DEPARTAMENT OF
ENVIRONMENTAL SCIENCES AND
ENGINEERING

HUMAN EXPOSURE TO FOOD CONTAMINANTS THROUGHOUT DIET: ASSESSMENT THROUGH TDS, BIOACCESSIBILITY AND CYTOTOXICITY ASSAYS *IN VITRO*

MARTA DO ROSÁRIO CRISTINO SILVA VENTURA

Master in Biomedical Sciences

DOCTORATE IN ENVIRONMENT AND SUSTAINABILITY

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To my lovely sons Tomás and Vasco, and my husband Marco

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“The world we have created is a proud of our thinking; it cannot be changed without changing our thinking.” *Albert Einstein*

ABSTRACT

Human health and environmental sustainability are interlinked through diet since food ingestion is one of the main routes of exposure to several contaminants in humans. In this sense, the transformation of healthy diets from sustainable food systems is crucial to achieving the Sustainable Development Goals defined by the United Nations, namely goals 2 and 3, related to food security and ensuring healthy lives, respectively. The present thesis aimed at i) characterising the presence of chemical contaminants in essential food items of the Portuguese diet; ii) evaluating the geographical influence and the importance of diffuse sources on trace elements levels in food; iii) assessing the potential risk of halogens, metals and metalloids to humans through food, and iv) estimating the bioaccessibility of food contaminants and their potential toxicity in intestinal cells. The results revealed that regarding the consumption of relevant food groups in the Portuguese population, the seafood group presented the highest As, Br, Cd and Pb levels compared to other analysed food groups, with an increased relative risk for total As and a clear progression of Cd relative risk with increasing consumption frequency of seafood. The results also demonstrated that the trace elements in areas affected by wildfires identified a hazard, especially for As, and other trace elements such as Cd, Br, Cr and Sr. Moreover, the bioaccessible fractions of As obtained after *in vitro* digestion of seafood were higher than the EC_{50} obtained for human intestine cells. This thesis contributed to increasing the knowledge about chemical contamination in food and their risk to the human population, as well as to the importance of using different approaches, including TDS methodology, risk assessment strategies, and *in vitro* assays, as essential tools for developing new monitoring methodologies in sustainable food systems and SDGs achievement.

Keywords: Food contaminants, TDS, bioaccessibility, toxicity, sustainability

RESUMO

A saúde humana e a sustentabilidade ambiental estão interligadas através da dieta, sendo a ingestão uma das principais vias de exposição a contaminantes nos seres humanos. Neste sentido, a transformação de dietas saudáveis a partir de sistemas alimentares sustentáveis é crucial para alcançar os Objetivos de Desenvolvimento Sustentável definidos pelas Nações Unidas, nomeadamente os objetivos 2 e 3, relacionados com a segurança alimentar e a garantia de vidas saudáveis, respetivamente. A presente tese visa i) caracterizar a presença de contaminantes químicos em alimentos essenciais da dieta portuguesa; ii) avaliar a influência geográfica e a importância de fontes difusas nos níveis de elementos vestigiais nos alimentos; iii) avaliar o potencial risco de halogéneos, metais e metalóides nos seres humanos através de alimentos, iv) estimar a bioacessibilidade de contaminantes alimentares e a sua potencial toxicidade em células intestinais. Os resultados revelaram que em relação ao consumo de grupos alimentares relevantes na população portuguesa, o grupo de produtos do mar apresentou os níveis mais elevados de As, Br, Cd e Pb comparativamente aos outros grupos analisados, um aumento do risco relativo de As total e uma progressão do risco relativo de Cd aumentando com a frequência de consumo de produtos do mar. Os resultados também demonstraram que os elementos vestigiais nas áreas afetadas pelos incêndios identificaram um risco, especialmente para As, Cd, Br, Cr e Sr. Evidenciaram que as frações bioaccessíveis de As após digestão *in vitro* dos produtos do mar foram superiores ao EC_{50} obtido nas células intestinais humanas. Esta tese contribuiu para aumentar o conhecimento sobre a contaminação química nos alimentos e o seu risco para a população humana, bem como para a importância de utilizar diferentes abordagens, incluindo a metodologia TDS, estratégias de avaliação de risco, e ensaios *in vitro*, para o desenvolvimento de novas metodologias de monitorização em sistemas alimentares sustentáveis e o alcance de ODS.

Palavras chave: Contaminantes alimentares, TDS, bioacessibilidade, toxicidade, sustentabilidade

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ACRONYMS

AAS	Atomic absorption spectrometry
ADI	Acceptable Daily Intake
AI	Adequate intake
ADME	Absorption, distribution, metabolism and excretion
ANOVA	Analysis of Variance
As	Arsenic
AsB	Arsenobetaine
ATSDR	Agency for Toxic Substances and Disease Registry of substances
BFRs	Brominated Flame Retardants
Br	Bromine
Br^-	Bromide
Br_2	Elemental bromine
bw	Body weight
Ca	Calcium
CA	Cluster Analysis
Cd	Cadmium
Co	Cobalt
Cr	Chromium
CRMs	Certified Reference Materials
DALYs	Disability-adjusted life years

DMA	Dimethylarsinic acid
EC	European Commission
EC_{50}	Median effective concentration to 50% of a population
EEE	Electric and electronic equipment
EFSA	European Food Safety Authority
EU	European Union
EVT	Extreme Value Theory
FA	Factor Analysis
FAO	Food and Agriculture Organization of the United Nations
FBD	Foodborne diseases
Fe	Iron
Ge	Germanium
GEMS	Global Environment Monitoring System
GIT	Gastrointestinal tract
HCA	Hierarchical Cluster Analysis
Hg	Mercury
IARC	International Agency for Research on Cancer
iAs	Inorganic arsenic
ICP-OES	Inductively coupled plasma/optical emission spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
In	Indium
INSA	National Institute of Health Doutor Ricardo Jorge
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LD_{50}	Lethal dose
LOD	Limit of detection
LOQ	Limit of quantification

LSD	Least Significant Difference
MeHg	Methylmercury
ML	Maximum level
MMA	Monomethylarsonic acid
MTT	3-[4,5dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide
MRL	Maximum residue limit
NCD	Noncommunicable diseases
NUTS	Nomenclature of Territorial Units for Statistics
Pb	Lead
PBDEs	Polybrominated Diphenyl Ethers
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PPCPs	Pharmaceuticals and personal care products
PI	Plug-in
PTWI	Provisional tolerable weekly intake
QALYs	Quality-adjusted life years
QC	Quality Control
Rh	Rhodium
ROS	Reactive oxygen species
RSD	Relative Standard Deviation
Se	Selenium
SeP	Selenoproteins
SD	Standard Deviation
SDGs	Sustainable Development Goals
Sr	Strontium
SRMs	Standard Reference Materials
tAs	Total arsenic

tBr	Total bromine
TDI	Tolerable daily intake
TDS	Total Diet Studies
Te	Tellurium
TE	Tail Estimation
TEs	Trace elements
tHg	Total mercury
TMAH	Tetramethylammonium hydroxide
THMs	Trihalomethanes
TWI	Tolerable weekly intake
UN	United Nations
UNESCO	United Nations Educational, Scientific and Cultural Organization
WHO	World Health Organization
Y	Yttrium
Zn	Zinc

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GENERAL INTRODUCTION

1.1 An overview of diet and nutrition importance for a healthy human life

Diet and nutrition are essential pillars for a healthy human life and are considered fundamental to preventing noncommunicable diseases (NCD), also known as chronic diseases; diet provides crucial nutrients for healthy human development and longevity. Adequate nutrition is essential through all the stages of life, from fetal development until old age, and is extremely important to guarantee physical growth, mental development, performance, productivity, health and well-being. Nutritional balance depends on several factors; the most important are: food and nutrition security, healthy literacy, caring for vulnerable people, good health, and a safe environment (FAO/WHO, 2021; WHO, 2000).

Diets are considered an essential link between human health and environmental sustainability. According to Food and Agriculture of the United Nations (FAO), sustainable diets are nutritionally adequate, safe, healthy, accessible, with low environmental impact, culturally acceptable, and protective of biodiversity and ecosystems; these premises are embedded on the three pillars of sustainability (environment, economy and society) (Fischer & Garnett, 2016).

Healthy diets and sustainable food systems should be integrated into a common framework to balance these global pillars. Unhealthy and unsustainable food production can be a risk for people and the planet since it is responsible for around 30% of global greenhouse gas emissions. The transformation of healthy diets from sustainable food systems is fundamental to achieving the Sustainable Development Goals (SDGs) defined by United Nations (UN) (Willett et al., 2019).

The SDGs aim to end extreme poverty, reduce inequality, and protect the planet until 2030 through the 17 SDGs. These universal goals for all human beings and adopted by the UN Member States require the adoption of scientific targets and the work at global scales of individuals and organisations (United Nations, 2015, 2021).

The link between nutrition and human health occurs since diets play a vital role as a risk factor for NCD; many NCD (obesity, diabetes, cancer, cardiovascular and dental diseases, among others) are preventable by combining an adequate diet with physical activity. On the other hand, World Health Organization (WHO) also prioritises the concern with inadequate diets, such as diets rich in salt, sugar, and saturated fatty acids and low in fruit, vegetables, and unrefined carbohydrates. These products' consumption has increased in the last decades and significantly compromised the population's worldwide health and nutritional status. Safe and varied food prevent malnutrition and reduce the risk of NCD (FAO/WHO, 2003a).

Unhealthy diets are estimated to be the second cause of death in the following decades in European countries, including Portugal (European Commission, 2020).

Mediterranean diet is considered one of the most worldwide healthy diets, which promotes a high intake of fresh vegetables and fruits, wholegrain cereals, moderate consumption of dairy, poultry and fish, and a low intake of red meat and sweets. Olive oil as fat is also recommended for cooking. Another advantage of this diet pattern is the sustainable way it is produced, avoiding impact on the environment (Maggi et al., 2021).

United Nations Educational, Scientific and Cultural Organization (UNESCO) considered the Mediterranean diet as an intangible cultural heritage in 2010. Initially, four Mediterranean countries were mentioned, and in 2013 the list was expanded to include other countries, among them Portugal. This diet pattern also contributes to a balanced nutritional diet (Trichopoulou, 2021).

Agriculture contributes to greenhouse gas emissions (Vermeulen et al., 2012); however, some strategies can reduce this effect by consuming local and seasonal products. A public health approach focused on plant-based foods could reduce environmental pressures (Springmann et al., 2018). The environmental impact of food production and reducing the overall carbon footprint should be a priority of different food chain players, such as the consumers, the producers, and the governments worldwide (Maggi et al., 2021).

The interaction between the ecosystems, animals and people can be assured through the One Health approach, a collaborative, multisectoral, transdisciplinary approach, which works at the local, regional, national, and global levels. This approach aims to achieve optimal health outcomes by recognizing the interconnection between people, animals, plants, and their shared environment (FAO/OIE/WHO, 2021). One Health is not a new concept, but the recent relevance assigned by FAO and WHO due to globalization and the increased movement of people, products and animals evidenced the importance of this approach. The 17 SDGs demonstrate a sustainable and healthy environment for all living beings, which are highly interconnected with the One Health approach. The areas of work in which the One Health approach is particularly relevant is food safety, the control of zoonoses and combatting antibiotic resistance (Naddeo, 2021). Biodiversity has an essential role in human nutrition, once ensuring the conditions of the soil and oceans to maintain crops, livestock, and marine species harvested for food. Environmental impacts and declines in biodiversity have been related to the increase of NCD. The use of fertilizers and pesticides to improve plant food production and the introduction of new crop varieties and cropping patterns can affect biodiversity and, consequently, the human nutrition status. (Garg & Banerjee, 2021).

WHO developed scientific risk assessments to define safe exposure levels, which form the basis for developing national and international food safety standards to protect consumers' health and ensure fair trade practices (WHO, 2015).

Food production has many critical points; food can be exposed to hazardous substances from production until consumption along the food supply chain; it is difficult to guarantee that food is entirely safe (Gallo et al., 2020).

1.2 Food safety and food contaminants

Food is considered one of the human beings pleasure's, although the presence of contaminants in food can be a risk for human health (WHO, 2021b). Food is exposed to different hazards at many stages of the food chain; the slogan "*farm to fork*" includes protecting human and animal health. A hazard is considered a biological, chemical or physical agent in, or condition of, food or feed with the potential to cause an adverse health effect on humans and animals (Silano & Silano, 2017).

Biological hazards are pathogenic microorganisms present in foodstuffs, including parasites, viruses, or bacteria, that cause disease when ingested. Chemical contaminants are a broad and significant group of distinct substances that occur throughout food production (Garvey, 2019). Physical hazards are objects in food that may cause injury if eaten and usually occur due to unsafe food handling practices or accidental contamination (FAO/WHO, 2019).

Environmental contaminants occur in food from diverse sources such as agriculture (soils, air, irrigation water, underground water, pesticides, fertilisers, greenhouse gases and mineral salts), forestry, atmospheric contamination, mining and mineral processing, fossil fuel combustion, industrial production and consumerism. These contaminants enter into the environment accidentally or deliberately, often, but not always, due to human activities. Some of these contaminants were produced for industrial use due to their stability (does not readily decompose); however, released into the environment, they can enter into the food chain at higher levels than would otherwise occur. Environmental contaminants can pollute soil, surface water, or aquatic sediments; these compounds can also enter groundwater and impact drinking water supplies (Wexler, 2014).

The food chain is a complex and multistep system in which food can be exposed to various contaminants at many stages on-route. The hazardous substances can contaminate the food through raw materials, transportation, packaging, storage, and cooking processing. Identifying all contaminants during food processing is a challenge for the scientific community (Li et al., 2021).

According to their growth environment, the foodstuffs can be contaminated from direct or diffuse sources and impact the food safety from production until consumption. Direct contamination occurs by point sources, and diffuse pollution is associated with atmospheric contamination, agricultural practices, and waste and wastewater treatment. In the case of vegetables and cereals, the contaminant sources are soil, irrigation water, or atmospheric contamination. The contaminants of meat and meat products are mainly metals introduced in feed, mineral supplements or undercooked meat. Fish and other seafood are exposed to pollutants from the aquatic environment, like metals, pesticides, biocides, pharmaceuticals and personal care products (PPCPs), and other industrial chemicals throughout the water column and sediment. Cooking processes like frying, grilling, boiling, and pre-cooking techniques such as peeling vegetables and fruits can also influence contaminants levels (Thakali & MacRae, 2021).

The WHO considers food safety an international public health priority and a fundamental human right. Access to sufficient amounts of safe and nutritious food is essential for human health (WHO, 2021a). Since the beginning of humanity, foodborne diseases (FBD) have been recognized as a significant public health concern; these diseases can be acute or chronic poisoning and affect people's health and well-being beyond the individual level, and are also a burden to the healthcare systems, reducing society productivity and influencing the social economy, and are also are responsible for many deaths worldwide (WHO, 2015). Due to the global scale of production, distribution, and processing that has become a food safety challenge, the concern about microbial pathogens and potentially hazardous chemicals in food has increased; however, the data caused by chemical contaminants has many knowledge gaps (WHO, 2021a).

The European Food Safety Authority (EFSA) is a keystone of the European Union (EU) on risk assessment for food and feed safety, ensuring safe, healthy food for consumers through the continued cooperation of the Member States and the EU's institutions and independent agencies. This organism provides independent scientific advice and clear communication on risk assessment, informs policymakers about food-related risks to ensure the safety of consumers and the environment. EFSA has an essential role in collecting and analysing data to estimate dietary exposure to food contaminants, resulting from combined data on food consumption and chemical occurrence (Ioannidou et al., 2021).

WHO and FAO elaborated food standards in a food code to protect consumers' health and ensure fair food trade practices (Codex Alimentarius) (FAO/WHO, 2019).

Food contaminants, which can be allergenic, microbial/biological, physical, or chemical agents that can cause adverse health effects, occur naturally or are added during food production or handling. These agents can contaminate the food in different stages of the food chain from production to storage, transport, distribution, packaging, cooking process, consumption, or as a result of environmental contaminants (FAO/WHO, 2019). Chemical hazards include environmental contaminants, pesticides, veterinary drugs used in the food chain, food additives, disinfectants, chemicals used directly to produce, package, and transport the food products and toxic metals such as arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) that can cause illness and specific toxicities. According to the International Agency for Research on Cancer (IARC), many of these contaminants are probably, possible or human carcinogenic (Borchers et al., 2010).

Implementing scientific and practical measures is essential to prevent and reduce food contaminants during production. The diet patterns influence the exposure to food contaminants. Specific diets should be considered once the consumed food belongs to the same food group, where the prevalence of food contaminants are similar, as is the case of populations whose protein intake comes exclusively from fish or plant-based foods (FAO/WHO, 2003a).

It is still important to emphasize that food safety is constantly being threatened by different changes occurring in the environment and through the food chain route, such as climate change's impact on food production, distribution and consumption, new technologies, emerging biological and environmental contamination, emerging pathogens and antimicrobial resistance (FAO, 2006).

1.3 Total Diet Studies

Total Diet Studies (TDS) are a public health tool to assess dietary exposure associated with contaminants and beneficial substances in food. WHO recommends TDS as the most cost-effective approach to estimating population risk assessment of food contaminants (Moy, 2013).

The TDS methodology consists of different phases: selecting a representative market basket according to a national food consumption survey, collecting foods purchased at the retail level, processing food for consumption, and pooling food into representative food groups. Pooling is essential in the TDS approach and consists of creating a unique food sample for analysis by combining various individual food items of the same type. TDS pooled samples (comprises twelve subsamples each) were homogenised and analysed for harmful and beneficial chemical substances. Foods were grouped by similarities and aggregated into the twenty FoodEx2 food groups defined by EFSA (EFSA/FAO/WHO, 2011).

FoodEx2 is a food classification and description system created by EFSA, aiming to cover the need to describe food in data collections across different domains, e.g. food consumption, chemical contaminants, etc. This system adopted by several European countries is a flexible combination of classifications and descriptions, including different hierarchies for different food safety domains. Also, it includes facets, which are collections of single descriptors from defined points of view applicable to specific food items. (EFSA, 2015).

Subsequently, the population's dietary exposure to the chemical substance is estimated by combining the analytical results obtained and the food consumption data. This approach estimates the population's dietary intake, it includes exposure through drinking water and the water used in cooking procedures. Chronic exposure is essential to evaluate the risk of toxic substances on human health. The essential principles of TDS are; representativeness of the whole diet, preparation and analysis of food as consumed by the targeted population, pooling the food, and combining the occurrence data with the consumption data to estimate the dietary exposure of the population (EFSA/FAO/WHO, 2011).

TDSs are adopted in different countries as a sound scientific basis to strengthen the linkage between food consumption and food analysis to assess the effect of harmful substances on health (EFSA, FAO, 2011). The application of TDS over several years provides essential information about the trends of toxic substances, contributes with information about food groups to dietary exposure, and indicates environmental contaminants. Metals such as Cd, Pb, and Hg, and metalloids as As are prioritized substances among TDS exposure chemical substances. The WHO's Global Environment Monitoring System/Food Contamination Monitoring and Assessment Programme (GEMS/Food) recognizes the value of TDS and recommends all countries to apply these studies in order to monitor environmental risk management (Moy, 2013).

The sampling plan (designed to collect the food items), food type, consumption, and cooking process were based on a food consumption survey collected at an individual level on the first harmonised Portuguese TDS. The TDS samples were representative of the Portuguese population diet (Vasco et al., 2021).

A TDS can be a valuable and cost-effective complementary approach to food surveillance and monitoring programs to assess the presence of chemical substances in the population diet and provide reliable data to perform risk assessments by estimating dietary exposure. TDSs have been performed in different countries worldwide. Sirot et al. emphasize that these studies should also be conducted in young children (under three years old) once they have specific nutritional needs due to immature development and their high food intake/body weight (bw) ratio (Sirot et al., 2018).

1.4 Trace elements occurrence in food

"*A knowledge of the chemical composition of foods is the first essential in the dietary treatment of disease or any quantitative study of human nutrition.*"

(McCance & Widdowson, 1940)

This statement is so true nowadays as when it was written in 1940. From a public health point of view, it is imperative to provide a diet that covers the recommended amount of nutrients. On the other hand, it ensures that the levels of toxic compounds do not exceed the maximum levels established in the legislation for those with legal values set (Greenfield H. & Southgate D.A.T., 2003).

Several trace elements (TEs) present in food can be divided into two main groups; non-essential elements as As, Cd, Hg, Pb, bromine (Br), chromium (Cr), and strontium (Sr); and essential elements such as selenium (Se) and zinc (Zn) (these two elements can contribute to the toxicity mitigation), and cobalt (Co) (Edelstein & Ben-Hur, 2018). Many of these chemical elements are metals, such as Cd, Hg, and Pb, and metalloids as As classified by IARC as human carcinogenic (IARC, 2021). These metals and metalloids occur naturally or from anthropogenic activities throughout the earth's crust, inducing environmental contamination and human exposure; they are toxic and nonessential elements, even at the lowest concentration, with accumulative tendencies in human tissues. (Tchounwou et al., 2012) and are also in the top ten of public health concern major chemicals defined by the Agency for Toxic Substances and Disease Registry of substances (ATSDR) (ATSDR, 2019). According to Grandjean & Herz (2015) and WHO, these trace elements can be retained for years in the human body and integrate the list of chemicals known to cause neurological effects as neurodevelopmental delay and neurological diseases (Grandjean & Herz, 2015; WHO, 2007).

Non-essential elements

Arsenic

As is a metalloid that occurs from natural phenomena or anthropogenic activities in the environment. The harmful effects of As are well known; however, the As toxicity depends on the chemical form present. This contaminant can occur in the inorganic or organic forms; the first forms are more toxic than the second; regarding the arsenosugars and arsenolipids, which toxicity level is not well known. The inorganic forms are trivalent arsenic or arsenite As(III), and the pentavalent arsenic or arsenate As(V); and organic forms are arsenobetaine (AsB), methylated arsenic species (monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)), arsenosugars, arsenolipids and other organoarsenic species. Inorganic forms frequently occur in natural ground water and food of terrestrial origin, and organic forms are usually found in marine organisms (EFSA, 2021). Figure 1.1 shows the arsenic toxicity level between organic and inorganic forms.

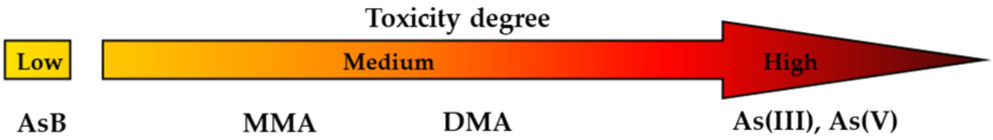

Figure 1.1. The arsenic toxicity degree in chemical forms (adapted from EFSA, 2009a).

IARC classifies inorganic compounds of arsenic in Group 1 (carcinogenic to humans); there is evidence that exposure to inorganic arsenic by inhalation or ingestion increases human cancer risk; over 80% of inorganic arsenic (iAs) is absorbed through the human gastrointestinal tract (GIT) (ATSDR, 2007). The most common cancers of human exposure to As chronically by the ingestion route are the skin, bladder and lung tumours. The cooking process can change the total arsenic content and the arsenic species, influencing dietary exposure to arsenic (EFSA, 2009a).

While the most occurrence data of As in food reported is total arsenic, the arsenic speciation is fundamental to estimate the risk assessment once the species demonstrates the real toxicity (EFSA, 2021). The accumulation of As in plants occurs mainly in the roots and accumulate to a lesser extent to different parts of the plant, causing plants damages (Pandey &

Gupta, 2018); this accumulation is affected by arsenic species; the organic arsenic species showed higher upward translocation than the inorganic (EFSA, 2009a).

The main route of human exposure to this contaminant is through drinking water and food. The regulatory limit for total arsenic in drinking water is 0.01 mg L^{-1}, considered by WHO as a provisional guideline (WHO, 2017), while Commission Directive 2003/40/EC established as a maximum level (ML) (EFSA, 2021).

The As accumulation in rice plants is a relevant global concern since the iAs in rice is higher than any other cereals due to its capacity to uptake and translocate iAs to the grain (EFSA, 2021). Rice is a semi-aquatic plant considered as the principal dietary source of iAs; the rice cultivation method could be the reason since it is flooded compared to non-flooded plants. Many factors influence the amount and forms of As in grains, such as the irrigation system, geographical location and environment (Awasthi et al., 2017; Rego A. et al., 2018). The European Commission (EC) established maximum levels of iAs in rice, rice products and rice intended for the production of food for infants and young children, once the principal forms of As present in rice are the inorganic and more toxic forms (European Commission, 2015).

Bromine

Br or dibromine (BR_2) is the elemental form of bromine, one of the principal halogens of the periodic table. In nature, it does not occur in the free state; it has different oxidation levels, and the most widespread is the anion (bromide). Exposure to high bromide levels has been associated with neurotoxicity (Wexler, 2014).

More than 50% of anthropogenic Br is associated with brominated flame retardants (BFRs). These compounds are added to various products to decrease their flammability; they can migrate and enter the environment during the materials' production, use, disposal, or recycling processes. BFRs are persistent and bioaccumulative in biota and the environment and are also one of the aquatic environment's principal hazards, especially in fish and other seafood. There are classes of BFRs considered contaminants of emerging concern acting as endocrine disruptors and mutagenic. BFRs are highly lipid-soluble, and fish oils are potential human exposure sources (Cruz et al., 2015). Br determination is considered a water quality indicator in the aquatic environment and the first screening to BFRs levels in biological and environmental materials. The interest in bromine compounds contamination has increased in the last decades (Winid, 2015). Due to the environmental hazardousness of BFRs, the European Union has established that plastic containing BFRs should be removed from any separately collected waste of electric and electronic equipment (European Union, 2012, 2018).

Br is also an oxidizing agent in the disinfection process of waters. The presence of bromide in the water has different results; it can originate bromate through the reaction of ozone with bromide or be involved in the reaction between chloride and organic matter that form brominated and mixed chloro-bromo by-products, such as trihalomethanes (THMs). Although chloroform is the most common THM, in the presence of bromide, the principal THMs formed are brominated THMs. IARC classifies bromate and chloroform as possible carcinogens to humans (category B2). WHO established a provisional guideline value for bromate in drinking water of 0.01 mg l^{-1}, and the acceptable daily intake (ADI) of inorganic bromide for humans is 0-0.4 mg kg^{-1} of the bw (WHO, 2017).

The EC established maximum pesticide residue levels, including bromide, on certain food products (European Commission, 2008).

Bromide has goitrogenic effects and, due to the chemical similarity to iodine, interferes with iodine metabolism in humans by decreasing iodide levels in the thyroid and enhancing iodide excretion by the kidneys. Bromide has various effects on iodide metabolism; in excess, bromide can replace iodide in the tissues inducing iodide deficiency and can result in thyroid goiters (Delgado et al., 2019; Sobolev et al., 2020).

Cadmium

Cd is a metal of toxicological concern found in the environment through a natural or anthropogenic occurrence. Cd can accumulate in aquatic organisms and agricultural crops; human exposure to this contaminant can come from several sources, including food, water, air or tobacco (tobacco leaves accumulate Cd from the soil), among others; however, in the non-smoking population, ingestion is the principal route of human exposure (ATSDR, 2012a). IARC classifies Cd as carcinogenic to humans (Group 1) (EFSA, 2012b); lung cancer is the more prevalent, and there is also limited evidence of prostate and kidney cancer (IARC, 2020).

The levels of Cd in foods can be influenced by many factors, such as growing conditions, agricultural and cultivation practices, meteorological conditions and anthropogenic contamination of soil or aquatic system (EFSA, 2012b). Several factors affect Cd absorption in human beings, including vitamin D, calcium (Ca) or iron (Fe) deficiency, metal-metal interactions with Fe, Pb or Cr, and metal-protein interactions or glutathione interaction (ATSDR, 2012a).

The legislation for Cd in different foodstuffs was reviewed, according to scientific opinion from EFSA reports concluded that the European population are close to or slightly exceeding the TWI of 2.5 µg kg^{-1} bw and establish the maximum levels in foodstuffs belonging to different food groups (EFSA, 2012b; European Commission, 2021b).

Chromium

Cr is a natural element present in the earth's crust, predominantly in rocks, soil, water and volcanic dust and is released to the environment from natural and anthropogenic sources.

This element occurs in nature in different oxidation states; the most common is trivalent form (Cr(III)), which is an essential nutrient present in diverse foods, and frequently used in dietary supplements; on the other hand, the hexavalent form (Cr(VI)) is considered a toxic industrial pollutant, classified by IARC as carcinogenic to humans (Group 1), (EFSA, 2014c), the prevalent cancers caused by Cr(VI) exposition are the lung and also cancer of the nose and nasal sinuses based on evidence from occupational studies (IARC, 2020).

Human beings are exposed to chromium by different sources, such as air, food, and water containing chromium. The primary route of nonoccupational workers is food ingestion; the amounts of Cr in foods depend on the food and the cooking process. EFSA estimates that the tolerable daily intake (TDI) of Cr(III) should be below of 300 µg Cr(III)/ kg bw *per* day (EFSA, 2014c).

The health effects associated with exposure to chromium depends on its oxidation state and the route of exposure, ranging from the low toxicity of the trivalent form to the high toxicity of the hexavalent form. Cr(III) plays an essential role in glucose, fat and protein metabolism. Once it can induce the action of insulin, its deficiency causes a decrease in glucose tolerance and an increase in the risk of cardiovascular disease. The Cr(VI) species usually occur by anthropogenic sources and are bio-available, mutagenic and carcinogenic (ATSDR, 2012b). Therefore, it is important to consider speciation analysis of Cr(III) and Cr(VI) in foodstuffs (Saraiva et al., 2021)

Lead

Pb is the most recognized environmental contaminant, widely present in nature, occurring naturally or via anthropogenic activities such as mining, battery manufacturing, smelting, etc. Since the late 1970s, lead exposure has decreased significantly in the environment in Europe as a result of multiple efforts, including unleaded fuel; however, the general population is exposed to this contaminant through food consumption, water, air, dust, and soil (ATSDR, 2020). The isotopic composition of Pb provides an essential tool to identify the sources of Pb contamination (Komárek et al., 2008).

The nervous system is the most vulnerable target of Pb poisoning, especially in children, since there is evidence that the developing brain is more vulnerable to the neurotoxicity of Pb, partly due to the immaturity of the blood-brain barrier than the mature brain and the gastrointestinal absorption of ingested Pb is higher in children compared to adults. In adults, Pb exposure can affect several organs, including the brain, cardiovascular, and renal systems; however, the Pb in the skeleton, which can be incorporated into Ca, represents the major body fraction. IARC classifies inorganic Pb as probably carcinogenic to humans (Group 2 A) (EFSA, 2010). Ingestion is a considerable route of Pb exposure, cereal products are the principal contributor to dietary Pb exposure, and dust and soil are critical non-dietary sources in children. There is a potential concern at current levels of exposure to Pb for effects on neurodevelopment, especially in children and pregnant women. (EFSA, 2010).

Due to the toxicity of this environmental contaminant, the European Commission established maximum levels of Pb in different foodstuffs by the regulation 2021/1317 (European Commission, 2021a), which amends the Regulation (EC) No 1881/2006 as regards maximum levels of Pb in certain foodstuffs (European Commission, 2006); the food products that overtake the legislated levels can not be marketed.

Mercury

Hg is a persistent toxic pollutant of worldwide concern that occurs in the environment from both natural and anthropogenic sources. The toxicity of this contaminant depends on its chemical form and can occur in three different forms; elemental (metallic), inorganic mercury, and organic mercury (mainly methylmercury (MeHg)). The most common and toxic is MeHg, which bioaccumulates in marine organisms and biomagnifies through the food chain (WHO, 2021c).

Environmental Hg poisoning was responsible for worldwide tragedies, such as the Minamata Bay tragedy in the late 1950s and onwards in Japan (UN environment, 2019).

Exposure to MeHg during pregnancy is a public health concern due to the capacity of this contaminant crossing the placental barrier, increasing the neurotoxicity of the fetus and consequently comprising the baby neurodevelopment. The fetal levels of exposure can be higher than maternal; MeHg can also cross the blood-brain barrier and enter the hair follicle. Children are more susceptible to central nervous system damage and the mercury's effect on neurodevelopment (Barbone et al., 2019).

Hg is considered one of the principal hazards of the aquatic environment and consequently a common contaminant in marine and freshwaters foods. The predatory fish that occupy the higher trophic levels have the highest concentrations of Hg in their muscle, over 90% of which may be MeHg. Cooking processes can not eliminate mercury from fish muscle (WHO, 2021c). Although marine organisms are a high source of Hg, they also are an essential contributor to the selenium dietary intake, which has been reported as a Hg antagonist (Ribeiro et al., 2022).

Human exposure to this contaminant is mainly through food consumption, especially seafood. EFSA established a TWI for MeHg of 1.3 μg/kg bw expressed as Hg, reducing the previously PTWI 1.6 μg kg^{-1} bw estimated by Joint FAO/WHO Expert Committee on Food Additives (JECFA), and a TWI for inorganic mercury of 4 μg/kg bw (EFSA, 2012a).

Strontium

Sr is a chemical element found in rocks, soil, dust, coal, oil, surface and underground water, air, plants, and animals (ATSDR, 2004b). It is regarded as a non-essential trace element for humans. This element has four stable isotopes that occur naturally and twenty-nine unstable isotopes, of which radioactive isotopes have higher toxicity. The radioactive isotope ^{90}Sr is considered toxic to human beings due to its relatively long half-life (Coelho et al., 2017).

Chemically Sr presents chemical similarities to Ca; Sr can replace Ca, causing a lack of Ca in the organisms and interfering with bone mineralization in the developing skeleton. Stable Sr isotopes are not considered toxic to humans; however, exposure to radioactive isotopes can be accumulated at bones and increase cancer risk. IARC has determined that radioactive Sr is a human carcinogen (Group 1) (ATSDR, 2004b).

The principal pathways of Sr exposure in humans are aerosol inhalation and ingestion of water and food. The food plants can absorb Sr from the soil and distribute it to the parts of the plant, such as leaves and fruits, the uptake in leaves is usually higher than in fruits; only soluble Sr can be absorbed by plant leaves and roots from soil, air and water. The amounts of Sr in the diet are influenced by geographical location and the type of foodstuffs (Burger & Lichtscheidl, 2019); the accumulation of Sr in soils is predominantly in acidic and Ca-poor soils (WHO, 2010).

Essential elements (micronutrients)

Selenium

Se is an essential micronutrient to human health, plays a vital role in oxidative stress, protecting human beings against oxidative damage. The absorption of Se compounds varies according to their chemical form. In the typical diet, Se is present in different chemical forms; organic components such as L-selenomethionine and L-selenocysteine and smaller amounts in inorganic compounds in the form of selenate and selenite. The principal sources of Se in the diet are Brazil nuts, viscera, fish, and eggs; on the other hand, grains and vegetables' contents generally depend on the soil selenium content (EFSA, 2014a).

The nutritional function of selenium is achieved by selenocysteine containing 25 selenoproteins (SeP) with various functions. Perhaps the most important is antioxidant, by protecting the body against oxidative damage. Deficiency of Se can lead to the appearance of cancer risk, male infertility, diabetes, and Keshan's and Kashin-Beck diseases, and affects the expression and function of SeP; the Se deficiency can be avoided through supplementation (Santhosh Kumar & Priyadarsini, 2014).

Se is recognised as having a protective factor against the toxicity of various contaminants. Se supplementation in rice influences nutrient regulation and reduces As accumulation and, consequently, As toxicity (Pandey & Gupta, 2018). Another antagonist effect of Se is decreasing Cd toxicity; this effect increases when used simultaneously with Zn; these two micronutrients act synergistically against oxidative stress (Jihen et al., 2009). Se is also a fundamental antagonist of Hg toxicity in marine organisms (Gochfeld & Burger, 2021; Ribeiro et al., 2022).

EFSA established an adequate intake (AI) of Se in human beings, ranging from 15 µg/day for children aged one to three years to 70 µg/day for adults and adolescents (EFSA, 2014a).

Zinc

Zn is an essential micronutrient that plays a role in the physiological functions of many enzymes, such as catalytic, structural and regulatory functions, as well as in the metabolism of proteins and nucleic acids (WHO, 2001). Zn is also a regulator of intracellular signalling, with a crucial role in the immune system (EFSA, 2014b). This micronutrient has relevant antiviral properties (Read et al., 2019), acting as an anti-inflammatory compound and reducing inflammatory risk. On the other hand, Zn deficiency is related to immune dysfunction and the consequent susceptibility to infectious diseases (Galmés et al., 2020).

This trace element occurs in air, water, and soil due to natural processes or human activities. The principal pathways of Zn exposure are ingestion and inhalation; this element is present in diverse foodstuffs but can be found mainly in grains, meats, vegetables and seafood, and can also be found in nutritional supplements (EFSA, 2014b).

Zn is a potent antioxidant agent to combat the toxicity of some contaminants; it has an effect against oxidative stress induced by Cd and has an important role in avoiding the impact of the redox status of the cell due to As toxicity (Ganger et al., 2016; Jihen et al., 2009).

According to EFSA, Zn's average requirements range from 6.2 to 10.2 mg/day for women and from 7.5 to 12.7 mg/day for men. Many factors influence Zn absorption, including the source of dietary protein (EFSA, 2014b).

Cobalt

Co is a metallic element produced primarily as a by-product of mining other metals that occurs in the environment from natural (rocks, soil, water, plants, and animals, forest fires) and anthropogenic sources (combustion of fossil fuels, metal smelting or sewage sludge) (ATSDR, 2004a). Co(III) is the cobalamin (vitamin B12) central atom, with 4.3 % molecular weight. It plays an essential role in this vitamin metabolism; as a crucial component of vitamin B12, it is found in most body tissues (EFSA, 2009b). Co is also an essential element for plant and animal growth since it is a component of several enzymes and proteins that participate in their metabolism. This element plays an important role in nitrogen fixation in plants, which is an essential macronutrient for them (Hu et al., 2021). Co in excess can interfere with iron absorption and give rise to iron deficiency (López–Luna et al., 2018).

The exposure to cobalt generally found in the environment is not considered harmful; however, exposure to high levels of Co compounds could be toxic and may entail adverse health effects in human beings, such as cardiac and developmental effects on the thyroid, immune system, and erythropoiesis, following inhalation, oral, or dermal exposure (EFSA, 2009b). According to IARC, Co and Co compounds are possibly carcinogenic to humans (Group 2B) (IARC, 2021).

1.4.1 Analytical methods

The importance of reliable analytical methods for measuring chemical elements in food relating to diet and health was first mentioned by McCance and Widdowson (1940) (McCance & Widdowson, 1940). Choosing suitable and appropriate analytical methods under quality assurance schemes and training staff is crucial to guarantee reliable data in foodstuffs. There are some points to consider, as analytical methods that have been recommended or adopted by international organisations and methods apply to a wide range of food matrices (Greenfield H. & Southgate D.A.T., 2003).

In the selection of the analytical method, the parameters of the method performance taken into account were the following: reliability, applicability, the limit of detection and limit of quantification, selectivity, sensitivity, accuracy, trueness, precision, linearity of a calibration curve, the procedure to calibrate the equipment response and approaches to evaluate method specificity, among others considered relevant for analytical techniques (Castanheira et al., 2016).

The majority of methods for inorganic constituents requires a previously digestion to remove the organic matrix of the foods or extraction and concentration before the analysis of inorganic components to be applied. The destruction of the food matrix removes many potential sources of interference and provides the inorganic material in a concentrated form. The organic matrix was incinerated in the classic food analysis, usually occurring in a muffle furnace at a controlled temperature. Another way to destroy organic matrix is being heated in concentrated acids in a closed vessel microwave digestor; this procedure minimizes losses during the oxidation and avoids any reaction between the inorganic constituents and the vessel used for dry incinerations (Greenfield H. & Southgate D.A.T., 2003).

The analytical methods considered appropriated to determine the trace elements referred to in section 1.4 were grouped in three different categories: 1) Inductively coupled mass spectroscopy (ICP-MS) is a golden standard multielementar technique that permits the analysis of a wide range of elements and can handle many samples and analytes in food. This method has high sensitivity and accuracy and low detection and quantification limits. 2) Inductively coupled plasma/optical emission spectrometry (ICP-OES) is a powerful technique to measure minerals presented in higher quantities in foodstuffs. 3) Atomic absorption spectrometry (AAS) is a classical method to measure minerals with similar performance to ICP-OES but less expensive. Currently, ICP-OES replace AAS in many laboratories (Castanheira et al., 2016).

1.5 The translocation of trace elements in plants

The contamination of soils by TEs is considered a major environmental concern worldwide; these elements can enter the food chain through contaminated soil, water sources and atmospheric deposition, becoming an effective hazard to the environment and human health (Afonne & Ifediba, 2020). Vegetables are foodstuffs more susceptible to contaminants accumulation from natural occurrences or anthropogenic activities. Industrialization, aerosols created by automobiles via combustion of fuels, metal smelting, and excessive pesticides, micronutrient fertilizers and manures can promote this contamination. Soil is the principal responsible for the TEs contamination into plants. Roots are the first plant component to absorb TEs and then transport them into the other plant parts, including edible parts; this contamination can modify or compromise the plants' physiological, biochemical or metabolic activities (Gupta et al., 2019).

Many TEs can produce reactive oxygen species (ROS) and cause oxidative stress in plants. This contamination causes several adverse effects on plants metabolism affecting their growth (Gupta et al., 2019). The accumulation of TEs in vegetables and root uptake of metals are influenced and controlled by many factors, among which the following can be highlighted; soil pH, soil temperature, soil texture, cation exchange capacity, organic matter, the soluble content of TEs in the soil, plant growth stages, crop type, fertilisers, and source and form of TEs (Afonne & Ifediba, 2020; Gupta et al., 2019).

Usually, higher amounts of TEs accumulation are found in leafy vegetables than legume vegetables, root vegetables, stalk vegetables, or inclusively solanaceous vegetables (Edelstein & Ben-Hur, 2018; Gupta et al., 2019).

There are two principal pathways for metal ions to enter into the roots: apoplastic and symplastic. Heavy metal can cross the plasma membrane and enter the xylem stream via the root symplasm; membrane transport proteins mediate this process; these contaminants also accumulate in plant leaves. The plants' uptake of TEs is influenced by soils properties and environmental conditions (Edelstein & Ben-Hur, 2018).

The standard behaviour of some trace elements translocation in plants depends on several factors. As is accumulated mainly in the plants' roots, and a lower concentration is extended to the above-ground organs, this contamination can cause physiological changes and damage plant metabolism. Cd and its compounds can move through the soil matrix toward the root system. This exposure route depends upon several factors, such as soil pH and organic matter content, which vary with the local environment (Edelstein & Ben-Hur, 2018). The Sr uptake into plants usually occurs from soil and water by plant roots. The root uptake in different kinds of *Brassica oleracea* is significantly higher than foliar absorption; however, in rainfall periods, the uptake through leaves is more significant than root uptake (Burger & Lichtscheidl, 2019). The plants' uptake to Hg depends on several factors as pH, texture and organic matter of the soil (Obrist et al., 2018).

The levels of Br in plants can vary significantly depending on plant species, plant part and the type of soil where the plants grow, among other factors (Shtangeeva et al., 2017).

1.6 Bioaccessibility and Toxicity of trace elements in food

Food and water ingestion is considered the principal exposure route of nutrients and xenobiotics to human beings (Garvey, 2019). The GIT is the linkage between the environment and the human body, which performs the absorption of nutrients and promotes protection from hazardous substances. Consequently, the diet has a primordial role in the composition and activity of the gut microbiota, being the human cells of the intestinal epithelial the first to be exposed to xenobiotics (Verhoeckx et al., 2015). The gut microbiome plays a modulator role in the presence of xenobiotic agents, influencing their absorption and metabolism. The balance of the gut microbiota is affected by the presence of these substances (Silbergeld, 2017).

However, the toxic effect of a xenobiotic depends on several factors, such as the type of molecule of the hazardous substance, the food matrix, and the toxicokinetic processes; absorption, distribution, metabolism, and excretion (ADME) (Fernández-García et al., 2009).

The toxicokinetic processes that lead to a xenobiotic's formation or distribution at the target tissue are essential to estimate the dose at the toxicological site. Absorption is defined as the rate of xenobiotic from the site of application into the bloodstream; distribution is considered the rate and extends of a xenobiotic movement out of the blood into the tissue; metabolism is the chemical biotransformation into secondary metabolites that are suitable for excretion, this process can be divided into two phases; phase I includes reactions adding a functional group to make the xenobiotic suitable for further modification, and phase II where occurs a conjugation of the xenobiotic with polar molecules to create a water-soluble compound, the products of biotransformation are used as exposure biomarkers; excretion is the final stage of toxicant disposition and refers to the elimination of the xenobiotic from the body through the excretory organs (Gehring & van der Merwe, 2014).

The amounts of contaminants in a portion of food that is eaten do not always reflect the bioaccessible level by the human body; bioavailability will influence its absorption. Oral bioavailability is the fraction of the nutrient or bioactive compound ingested available for use in physiologic functions or stored. This complex concept includes two processes; bioaccessibility and bioactivity (Verhoeckx et al., 2015), and is influenced by dietary factors, as chemical form, interactions with other nutrients and food components, food processing, host-related intestinal and systemic factors (FAO/WHO, 2019). Bioaccessibility is the fraction of an ingested bio compound released from its food matrix in the gastrointestinal tract. It thus becomes available for absorption through the epithelial layer of the GIT. Bioactivity is defined as the phenomena that occur after a compound reaches the systemic circulation; the transport to target tissues, interaction with biomolecules, metabolism in these tissues, and all the physiological events it generates (Verhoeckx et al., 2015).

Static and dynamic *in vitro* digestion systems have been developed to assess the sequence of events during digestion in the human gastrointestinal tract and, therefore, the bioaccessibility of food components in a realistic way. These methodologies have principal advantages: the quickness of the results, efficiency, lower costs, allowing a high number of samples and avoiding ethical issues compared to human nutritional studies. On the other hand, the principal disadvantage is the difficulty of reaching the results between different methods, mainly due to the differences in enzymes, pH, salt concentrations, digestion time, among other factors. A standardised static *in vitro* digestion model for food was developed by the COST action INFOGEST to overcome this difficulty; this consensus aimed to consolidate the conditions for simulating the digestion of food (Brodkorb et al., 2019). This digestion harmonised method takes into account the requirements of *in vivo* digestion. It is divided into three steps: oral, gastric, and intestinal phases, as represented in Figure 1.2 (Brodkorb et al., 2019).

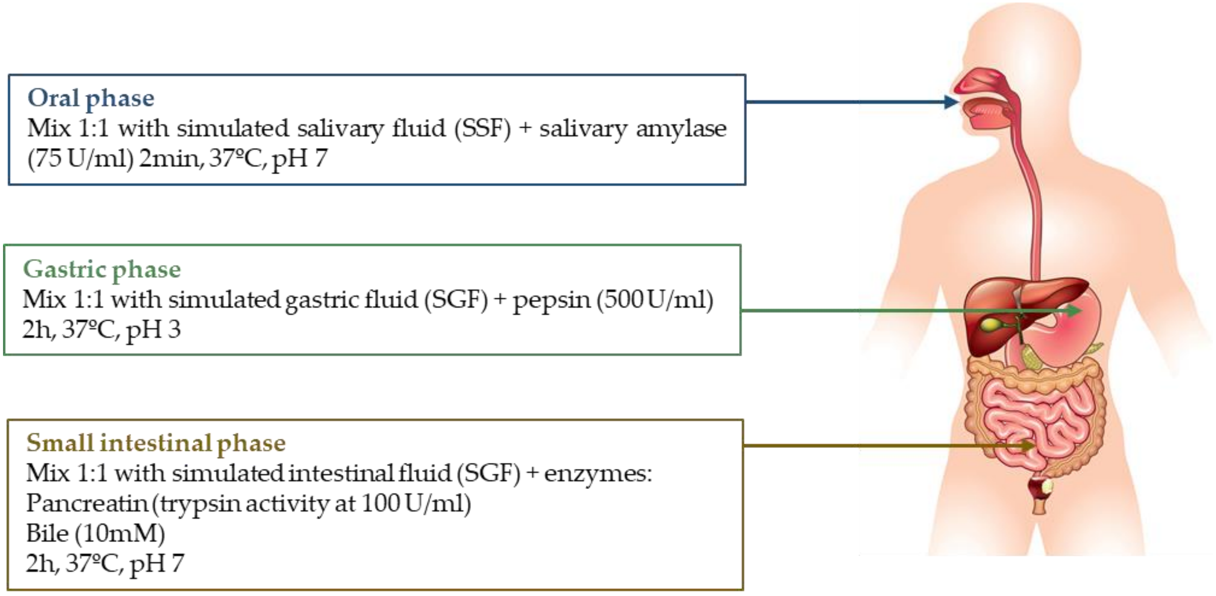

Figure 1.2. Overview of the INFOGEST diagram *in vitro* digestion model (adapted from Brodkorb et al., 2019).

The intestinal epithelium has a fundamental role in absorbing and transporting beneficial and harmful substances in human beings. Different intestinal cell culture models of human origin have been used in food and nutrition science to evaluate food components' absorption, transport, bioaccessibility, and bioavailability (Ponce de León-Rodríguez et al., 2018).

The principal intestinal cells lines are caco-2 cell line, frequently used for absorption, transport and bioavailability studies; T84 cell line, usually used to assess intestinal permeability; and HT-29 cell line derived from the human colon adenocarcinoma, which has target scientific interest, due to its capacity on expressing characteristics of mature cells, as enterocytes, representing the toxicity in the gut (Ponce de León-Rodríguez et al., 2018; Verhoeckx et al., 2015).

The toxicity of a substance can be classified into three different types; acute, subchronic or chronic. In acute toxicity, the durability of adverse effects induced by a xenobiotic persists over a finite period. In subchronic and chronic toxicity, these adverse effects hold on for long periods and denote repeated contact with the weird agent, with doses of exposure being lower than those that generate acute toxicity. Comparatively to the duration of the exposure period, subchronic and chronic toxicities are classified as being less than a lifetime or an entire lifetime, respectively (Wexler, 2014).

The toxicological effects are usually expressed as median lethal dose (LD_{50}) or effect concentrations (EC_{50}), the test concentration at which 50% of the organisms are affected or at which 50% effect is measured for a specifically defined endpoint (EFSA, 2019b).

Thesis research questions and objectives

Food safety has been considered a public health concern, being a topic of increased interest by the scientific community, and also interlinked with the food exposure to several contaminants through the food chain pathway. The exposure assessment to food contaminants in the Portuguese diet and its potential effects is aligned with this consideration.

Owing to this concern were formulated two main research questions:

1. Is the Portuguese population exposed to inorganic contaminants through food?
2. The food contaminants may become bioaccessible in order to cause toxicity to human intestine cells?

These research questions were answered throughout, the following objectives:

1. To characterize the presence of chemical contaminants in essential food items of the Portuguese diet;
2. To evaluate the geographical influence and the importance of diffuse sources on trace elements levels in food;
3. To determine the potential risk of halogens, metals, and metalloids to humans through food;
4. To estimate the bioaccessibility of food contaminants and their potential toxicity in intestinal cells.

These objectives were achieved through experimental work, which generated three manuscripts published in international journals with peer-review and one submitted.

The laboratory work resulted in several studies integrated into four chapters of this thesis. Chapter 2 characterised the chemical contaminants in essential food groups of the Portuguese diet and evaluated the influence of seasonality and proximate composition in the levels of elemental contents; Chapter 3 assessed the impact of forest fires in trace elements contents in food from areas affected by fires and consumed by the rural population; Chapter 4 evaluated the relative risk of inorganic contaminants associated with the consumption of relevant food groups; and lastly, Chapter 5 estimated the bioaccessibility of chemicals present in seafood and evaluated their potential toxicological effects. The principal results of these four chapters will be discussed and integrated throughout the general discussion (Chapter 6).

The thesis structure comprises seven interlinked chapters, including a general introduction, four chapters corresponding to the research achievements and composing four manuscripts (see Annex A.1), a chapter dedicated to a general discussion of the main findings and a final chapter with the conclusions and future perspectives. Figure 1.3 presents the thesis structure, including the four chapters of the laboratory work and research interlinked with research questions, aims and manuscripts.

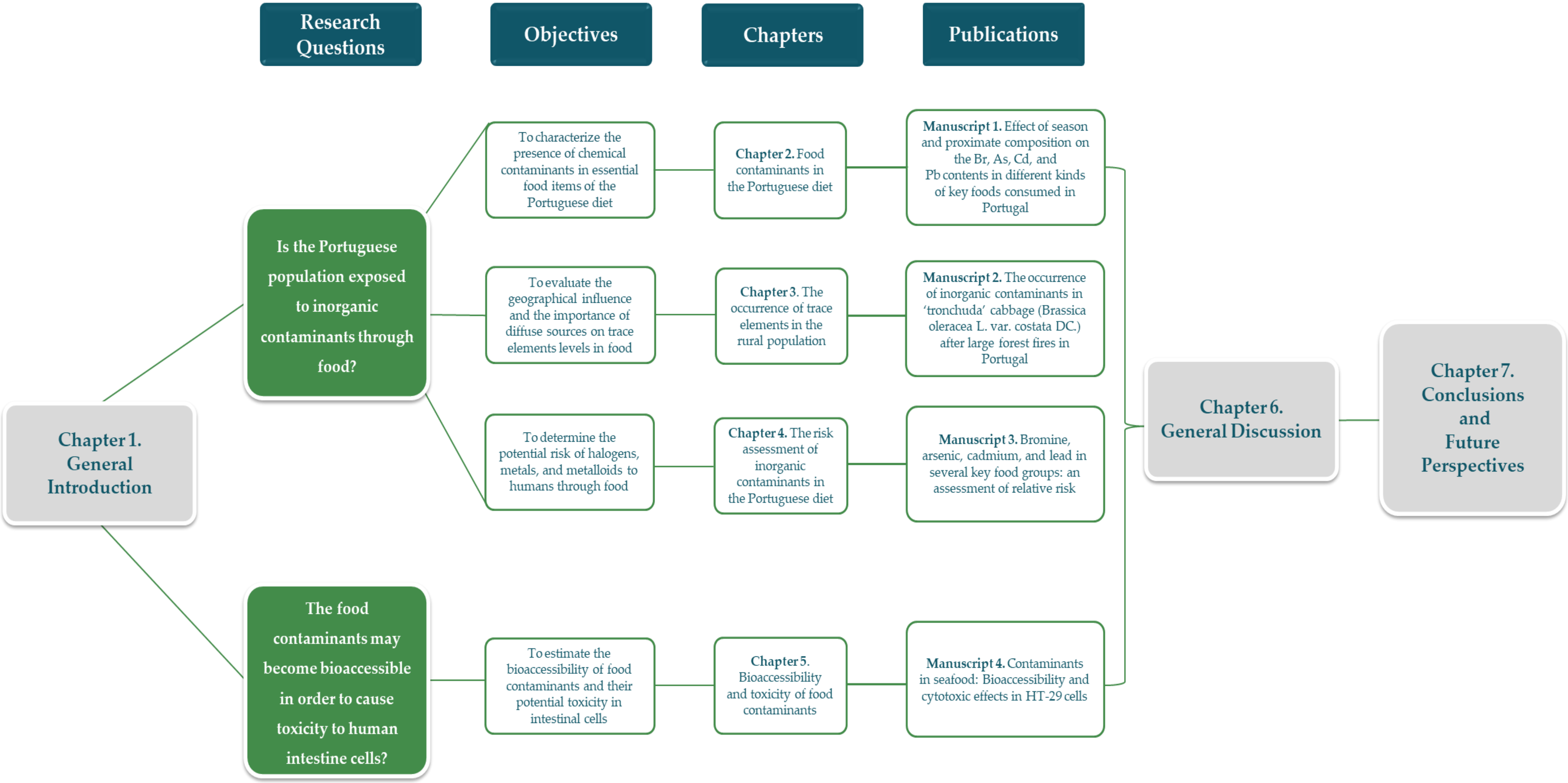

Figure 1.3. Diagram of thesis structure.

2

FOOD CONTAMINANTS IN THE PORTUGUESE DIET

MANUSCRIPT 1: Effect of season and proximate composition on the Br, As, Cd and Pb contents in different kinds of key foods consumed in Portugal

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Abstract

On the basis of a Total Diet Study, the Br, As, Cd and Pb contents in relevant food groups consumed in Portugal were determined. The studied groups were meat products, fatty fish, lean fish, shellfish, cephalopods, fresh and dried fruit, crucifers and other vegetables. It was observed that the concentrations of As, Br, Cd and Pb in fruit, vegetables and meat products were much lower than in seafood. Fresh fruit exhibited the lowest elemental contents. The concentrations of As, Br, Cd and Pb did not show any dependence on season. With respect to proximate composition, elemental content dependence on protein or ash was weak. PCA showed relevant associations: between Br and ash contents in fatty fish; between Cd, Pb and protein contents in crucifers; and between As, fat and protein contents in the other vegetables group. Therefore, the effect of the proximate composition on the studied elemental contents warrants investigation.

Keywords: Arsenic, bromine, cadmium, food, lead, proximate composition, season

2.1 Introduction

Portuguese diet, as other Western diets, is characterised by a large quantitative importance of fruit (fresh and dried), vegetables, meat and seafood (fatty and lean fish, shellfish and cephalopods) as key food groups (Gregório et al., 2017). However, this general knowledge has to be translated into a measurable reality, where nutrients, anti-nutrients, and contaminants mainly due to pollution (Zhou et al., 2016) - are determined. In particular, with the aim of assessing contaminants in foods, a Total Diet Study (TDS) has been established. A TDS is a complementary tool of a public health surveillance program, being characterised by the analysis of the positive and detrimental components of the diet. This particular study typology may be useful for the monitoring and control of dietary exposure to the various chemical compounds in food (EFSA/FAO/WHO, 2011). Precisely, the present study is based on a TDS project. A national list of prioritized components based on an European consensus was prepared (Vin et al., 2014). These components include As, a metalloid and the metals Cd and Pb as main hazardous elements in diet.

As, more specifically, inorganic As, is included in the Group I carcinogen list by the International Agency for Research on Cancer (ATSDR, 2007). Overexposure has been associated with physiological changes (Hojsak et al., 2015). With respect to Cd, it is linked with cancer, particularly, lung, kidney and prostate cancer (IARC, 2012a). Cd is also a possible initiator of atherosclerosis, being associated with coronary heart disease (Tellez-Plaza et al., 2013). Concerning Pb, alterations in the haematological, renal, nervous and reproductive systems have been claimed (Needleman, 2004; Patrick, 2006). Moreover, Pb is a competitor of calcium, causing disruption of neurotransmitter release and reduction of mineral density in the bone (Beier et al., 2013). Therefore, surveillance of As, Cd and Pb contents in relevant food groups is of critical importance.

On the other hand, bromine (Br) may only partially be viewed as a contaminant in that there is anthropogenic Br and more than 50 % of it is associated with brominated flame retardants (BFRs), which are deemed to be a main hazard to the aquatic environment (Winid, 2015). However, it should be remarked that the total Br content comprises compounds other than BFRs, such as polybrominated diphenyl ethers (PBDEs), wherefore Br hazards cannot be circumscribed to BFRs. Indeed, a low level of toxicity has been ascribed to inorganic Br (FAO/WHO, 1988; HCN, 2005). Long-term oral exposure to Br has been linked to skin alterations and to effects on conditioned reflexes and blood parameters (HCN, 2005). Nevertheless, there is an information deficit with respect to Br toxicology. The measurement of Br contents may also be considered a first approach to assess BFR contents in biological and environmental samples. Due to these reasons, research into Br contamination has grown in the last decade (Romarís-Hortas et al., 2009).

The As, Cd, Pb and Br contents in the foods belonging to the groups relevant to the Portuguese dietary patterns may depend on several factors, such as geographical origin, season or production system (for instance, wild fish *vs* farmed fish). In particular, seasonality is a prominent factor, which can exist ineither consumption and content data simultaneously, consumption data only or content data only (Elegbede et al., 2017). These authors used a TDS and showed significant differences between exposure levels in different seasons. It was also claimed that, perhaps, for most substances, the variable seasonal exposure is not mainly driven by consumption seasonality, but partly by content seasonality (Elegbede et al., 2017). The data presented in the current study should enable not only to test whether seasonality may play a role in the variation of some elemental contents, but also to test whether fat level or other proximate composition parameters display a relationship with such elemental contents.

Therefore, the main goal of the current study was to assess the extent of the influence of proximate composition parameters (moisture, ash, protein and lipid contents) and seasonality on the levels of Br, total As, Cd and Pb key food groups, targeted by the National Health Institute Doutor Ricardo Jorge (INSA) on the basis of its TDS (meat products, fatty fish, lean fish, shellfish, cephalopods, fresh fruit, dried fruit, crucifer vegetables and other vegetables).

2.2 Materials and methods

2.2.1 Sampling

The performed experimental activities belong to a TDS (Total Diet Study). The TDS is recognized by World Health Organization (WHO) and European Food Safety Authority (EFSA) as an important tool of public health for dietary exposure assessment to contaminants and to beneficial substances through food. This study consists of selecting, collecting and analysing foods representative of the diet, pooling the prepared food items into representative food groups and processing the food as for consumption. The selection and collection of food require a rational sampling plan supported by consumption data (EFSA/FAO/WHO, 2011). In order to obtain harmonised dietary exposure data between five European countries, a methodology was developed for establishing harmonised national TDS food lists and to test the feasibility of the proposed procedures in order to have comparable formats of food consumption data (Dofkova et al., 2016).

Sampling was conducted in accordance with a 2014-2015 Portuguese food consumption survey. All the food items were coded in FoodEx2 - a classification system that was created by EFSA and followed by several European countries and were grouped into composite samples in the twenty FoodEx2 food groups. Food groups were selected taking into account their potentially high Br levels and the absence of Br studies on them. Detailed taxonomical information is given in Table 2.1. It should also be noted that samples were selected in order to generate a representative collection (Dofkova et al., 2016) of the most frequently consumed foods in Portugal. Samples were purchased at representative supermarkets of the Lisbon metropolitan area.

Therefore, a total of nine main food groups were analysed: meat products (n=108), fatty fish (n=240), lean fish (n=60), shellfish (n=24), cephalopods (n=24), fresh fruit (n=252), dried fruit (n=60), crucifers (n=228) and other vegetables (n=216). This represented a total of 1212 samples. The edible part of each food product was used. In the case of fish, all bones and scales were separated after culinary treatment, shells were eliminated in the case of bivalves, and fruits were peeled or not as indicated by the results of the national food survey, being pits always discarded (Dofkova et al., 2016). Samples were cooked if this corresponded to the usual form of consumption in Portugal. Hence, culinary methods depended on the sample: for bivalves, boiling; for cephalopods, boiling and frying; and for fish, boiling, roasting, grilling and frying. In the case of fruits, samples were analysed raw, and for vegetables, samples were boiled.

After preparation, samples were mixed into pools of twelve samples of similar foods (Dofkova et al., 2016). It should be remarked that a pool could contain the same food prepared in various ways. Consequently, nine pools of meat products, twenty pools of fatty fish, five pools of lean fish, two pools of shellfish, two pools of cephalopods, twenty-one pools of fresh fruit, five pools of dried fruit, nineteen pools of crucifers and eighteen pools of other vegetables were attained (a total of 101 pools). These 101 pools representing 1212 samples (each sample corresponding to either a whole piece of meat, a whole fish/ shellfish/cephalopod, a whole fruit or a whole cabbage, etc.) were analysed in triplicate.

Table 2.1. Taxonomical information of the species considered in each food group.

Food Group	Taxonomical Information – Species List
Meat products	*Bos taurus, Gallus gallus domesticus, Meleagris gallopavo, Oryctolagus cuniculus & Sus domesticus*
Fatty fish	*Conger conger, Labroides sp., Lates niloticus, Lepidopus caudatus, Merluccius sp., Pagellus bogaraveo, Pagrus pagrus, Pangasius boucourti, Phycis phycis & Pleuronectes platessaSalmo salar, Sardina pilchardus, Scomber japonicus, Sparus aurata, Trachurus trachurus & Trisopterus luscus*
Lean fish	*Gadus morhua, Helicolenus dactylopterus, Molva molva, Thunnus sp.*
Shellfish	*Penaeus sp.Ruditapes sp. & Donax sp.*
Cephalopods	*Octopus vulgaris & Loligo vulgaris*
Fresh fruit	*Actinidia sp., Ananas comosus, Citrullus lanatus, Citrus sinensis, Cucumis sp., Fragaria ananassa, Malus pumila, Musa sp., Prunus persica, Pyrus Malus pumila, Musa sp., Prunus persica, Pyrus communis, & Vitis vinifera*
Dried fruit	*Ficus carica*
Crucifers	*Brassica oleracea, Brassica rapa, Brassica ruvo*
Other vegetables	*Allium cepa, Asparagus officinalis, Capsicum annuum, Daucus carota, Lactuca sativa, Solanum lycopersicum, & Vigna unguiculata*

Proximate composition

Moisture content

Moisture content was determined by gravimetric method using a dry air oven (Memmert, Schwabach, Germany) at 102 ± 2 °C during 2 h. A sample weight of 5 g was used, and dry weight was determined after reaching constant values as described in AOAC, 2000c (AOAC, 2000c).

Ash content

Total ash analysis was carried out in an M110 muffle furnace (Heraeus Instruments, Langenselbold, Germany) at 525 ± 25 °C for 20 h. A sample weight of 5 g was used, and ash weight was determined after reaching constant values as described in AOAC, 2000a (AOAC, 2000a).

Protein content

Each sample was analysed for total nitrogen by the Kjeldahl method, which includes three steps: digestion, distillation and titration. These steps were performed in the following equipments: digestion – Foss Tecator 2508 Digestor (Foss Analytical, Hillerød, Denmark), distillation – Foss 8400 Kjeltec Auto Distillation unit (Foss Analytical, Hillerød, Denmark) and titration-titrator system 808 (Teachware Metrohm, Herisau, Switzerland). The protein content was calculated by application of a conversion factor, according to the food matrice (AOAC, 2000d).

Lipid content

Total lipid determination was performed with an acid hydrolysis method followed by extraction using a Soxhlet apparatus (Soxtec™ 2050, Foss Analytical, Hillerød, Denmark) for 1 h 30 min with petroleum ether (40– 60 °C) as the extraction solvent. The attained residue was dried for 1 h 30 min at 102 ± 2 °C until constant weight (AOAC, 2000b).

Elemental composition

Inductively coupled plasma mass spectrometry (ICP-MS) was chosen for the quantification of trace elements in food.

Determination of elemental content

Br was determined by ICP-MS after alkaline extraction as described by Ventura et al. (2018) (Ventura et al., 2018). For the alkaline extraction, 0.5 g of sample was weighed and 8 mL of ultrapure water and 1 mL of tetramethylammonium hydroxide (TMAH) (Fluka Analytical, Buchs, Switzerland) 25%, v/v, were added, on a heating plate. After extraction, sample was diluted to 25-mL volumetric flasks with ultrapure water. The sample was then centrifuged at 22 °C using 15 550 g for 15 min, and the supernatant was filtered through a 0.45-µm syringe filter (Millipore) before ICP-MS (ICP-MS ThermoX Series II, Thermo Fisher Scientific, Bremen, Germany) measurements. Standard solution of bromine containing 1000 mg L^{-1} (Inorganic Ventures, Christiansburg, VA, USA) was used to prepare standard solutions. Standard solutions of tellurium and rhodium containing 1000 mg L^{-1} (Merck KGaA, Darmstadt, Germany) and 10 mg L^{-1} (Merck KGaA, Darmstadt, Germany), respectively, were used as internal standards. MRC Carrot NCS ZC73031 from China National Analysis Center, which had data for bromine, was used as SRM. Concentrations were expressed in wet weight.

Analysis of total As, Cd and Pb was carried out as described by Coelho et al. (2013). Concentrations were expressed in wet weight.

ICP-MS measurements were performed using Plasmalab database version 3.51. Further details of the instrumental settings are inscribed in Table 2.2.

Table 2.2. Experimental conditions for the analytical system of Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

ICP-MS Thermo X Series II	
Extraction	-106
Focus	10
Pole bias	-0.1
Hexapole bias	-3
Nebuliser flow rate (L s^{-1})	0.014
Forward power (W)	1400
Cool gas flow rate (L s^{-1})	0.217
Auxiliary gas flow rate (L s-1)	0.014
Sampling depth	95
Standard resolution	125
High resolution	125
Analogue detector	1902
PC detector	3100

Quality assurance

A quality assurance programme in accordance with the NP EN ISO/IEC 17025 (ISO/IEC, 2005) standard requirement was put in place for analysis of total As, Cd and Pb. This programme included appropriate qualification for involved operators and glassware calibration and traceability. Equipment was calibrated daily with new standard solutions. Acceptance criteria were established for repeatability and reproducibility. Concerning the Br analysis method, Certified Reference Material Carrot was used to test accuracy and precision. Recoveries between 80% and 120% were attained. The repeatability was always lower than 10%. Each sample was treated in triplicate, and one reagent blank was included in each extraction to check possible contamination. A quality control (QC) sample was included in each group of twelve test samples to validate the test run. Instrumental drift was controlled by difference between the results of first and last QC samples (<5%) (Table 2.3).

Limits of quantification (LOQ) were calculated by multiplying by three the standard deviation of the mean blank tests after correction by the sample weight and the appropriate dilution. The LOQ for the analysed samples varied within the following intervals: Br 0.03 (vegetables) - 0.21 (fish) mg kg^{-1}; total As 0.01 (fresh fruit) - 0.04 (fish) mg kg^{-1}; Cd 9 (fresh fruit) – 36 (fish) μgkg^{-1}; and Pb 11 (vegetables) – 71 (fish) μgkg^{-1}.

Correlation study

A correlation study involving, on the one hand, As, Br, Cd and Pb contents in the selected food groups and, on the other hand, ash, protein and lipid contents and seasonality (spring, summer, autumn and winter) was carried out. In particular, each of the four elements' contents was correlated with the other three elements' contents and with ash, protein and lipid contents, and season. These correlations were performed not only for all food groups taken together, but also for each main studied food grouping (meat products, fatty fish, lean fish, shellfish, cephalopods, fresh fruit, dried fruit, crucifers and other vegetables). For this purpose, it was used an algorithm that yielded the basic correlation factor (R^2) and several residuals. The application of the algorithm to the several sets of data was done with STATISTICA 6, 2003 version (StatSoft, Inc., Tulsa, OK, USA).

Principal component analysis

The principal component analysis (PCA) study also involved As, Br, Cd and Pb contents along with ash, protein and lipid levels. The PCA was always done without and with inclusion of the seasonal factor (a categorical variable). This enabled a better delineation of the effects of season on the analysis. Each PCA was done taking all samples of the nine food groupings without differentiating between them and for each one of the food groupings in separate. A PCA considering food groupings as a categorical variable was also done for an analysis of the relative relation of meat products, fatty fish, etc. Depending on the percentage of sum of squares explained by the extracted components, two or three principal components were considered relevant. The PCAs were done with STATISTICA 6, 2003 version (StatSoft, Inc., Tulsa, OK, USA).

2.3 Results and discussion

Raw data

The TDS results concerning, on the one hand, As, Br, Cd and Pb contents, and, on the other hand, moisture, ash, protein and lipid contents and season in the selected food groups consumed (combined in pools) in Portugal are presented in Table 2.4.

In general, the contents of the studied elements (As, Br, Cd and Pb) in fruit, vegetables and meat products were much lower than in seafood (encompassing fatty and lean fish and shellfish and cephalopods). However, there were differences in the elemental content ratios between seafood and the other groups; that is, the high seafood contents were particularly larger than the contents in other groups in the cases of As (a ratio >100 between the average As seafood content and the average As contents of the other groups) and Cd (ratio near 2 or higher, when lean fish is excluded from the seafood). On the other hand, this same ratio was ~3 for Br (after exclusion of dried fruits from the other groups) and ~2 for Pb. Outside the seafood groups, while dried fruits had the second highest average elemental contents in the cases of Br and As, closely followed by meat products, crucifers displayed the second highest average elemental concentrations for Cd and Pb.

Table 2.3. Quality assurance of the method Element.

Element	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Repeability (RSD %)	Intermediate precision (%)	Recovery (%)	Working range (µg kg^{-1})
As	0.06	0.21	7.9	8.6	95-115	0.25-2.5
Br	1.3	4.4	3.1	3.4	90 - 120	5.0-50
Cd	0.03	0.09	6.5	8.5	85-117	0.25-2.5
Pb	0.11	0.37	6.9	8.3	80-100	0.5-5.0

The fresh fruit group displayed the lowest average elemental contents in the comparison across the nine groups, that is, only 0.002 ± 0.001 mg kg^{-1}, 0.3 ± 0.4 mg kg^{-1}, 0.6 ± 0.8 µg kg^{-1}, and 3.6 ± 5.3 µg kg^{-1} in As, Br, Cd and Pb, respectively. Within seafood, shellfish and cephalopods exhibited higher Br, As and Cd levels than fatty and lean fish. The situation was only different in the case of Pb, whose lowest seafood contents were registered in the cephalopods group. Within vegetables, crucifers had higher Br, Cd and Pb concentrations than the other vegetables. This was very notorious for Cd. The standard deviations are high as a result of grouping a very wide gamut of foods in each one of the studied nine groups.

In fact, each one of the nine groups comprises very different foods with a wide diversity of taxonomical origins (Table 2.1).

The meat products range from beef to chicken and sausage. Nonetheless, the Br content did not differ much, being the highest and most different value observed for 'chouriço', which is a kind of Portuguese sausage with many ingredients. The As content also varied within a narrow range in the meat products. Regarding the Cd content, beef and pork (cutlet) displayed very low contents when compared with all other meat products. Finally, as with Br, 'chouriço' was distinguished from the other products by a very high Pb level.

With respect to seafood, Br concentration reached its highest value in the bivalve molluscs of the shellfish group, 40.3 ± 1.2 mg kg$^{-1.}$ Moreover, this seafood subgroup was also characterised by very high Cd and Pb concentrations, clearly much higher than the shellfish and seafood group averages. On the other hand, this did not happen with the bivalve As concentration. The highest As contents were found in the European conger and in octopus, which is a cephalopod. Very low As levels were observed in catfish and Nile perch, being the As level in sea bream, salmon, tuna (regardless of being canned) and squid also low, not exceeding 1 mg kg^{-1}. Within the cephalopod group, there was a striking contrast between octopus and squid, which are taxonomically relatively close to each other. Differently from As, squid exhibited a very high Cd content, 507 ± 36 µg kg^{-1}, when compared with the other seafood. Besides bivalve molluscs, relatively high Pb contents were determined in the cases of catfish, hake, scabbard fish, dried cod (a lean fish) and canned sardine.

Moisture level was very influential for the fruit group. Indeed, in the cases of Br, As and Cd levels, their highest values were observed in dried fruit (Figs). The lowest Br content was measured for apple, pear, grapes, peach and watermelon, not surpassing 0.1 mg kg^{-1}. Concerning Pb concentration, besides dried figs, relatively high values (in comparison with other fruits) were determined in the cases of the apple and fruit salad.

The comparison between crucifers and the other vegetables does not present very large elemental content contrasts. In the case of Br content, it varied between 0.4 (pepper, lower limit of its range) and 3.2 mg kg^{-1} (lettuce and turnip greens, upper limit of their range). Thus, no stark contrast between crucifers and the other vegetables was observed. Regarding As content, the mushroom pool was distinguished by a relatively very high value, 0.078 ± 0.002 mg kg^{-1}, which affected the average of the other vegetables group. Furthermore, broccoli, rapini and turnip greens had upper limits of their Cd content ranges that were among the highest values in the vegetables group, surpassing 15 µg kg^{-1}, this was influential in leading to higher Cd mean levels in the crucifers group. Broccoli had also a Pb content range whose upper limit reached relatively high values. The same occurred in the case of carrot. It should be remarked that these values in the 47–48 µg kg^{-1} level were only high in comparison with other vegetables - for instance, they were not so high if compared with some seafood samples, as mentioned above.

Regarding the nine main food groupings, variability as measured by the standard deviation was typically very pronounced across elements and groupings. In spite of such large variability, the observed contrasts are meaningful and point to the existence of clear patterns.

With respect to As and Pb, it is well known that some seafood species can accumulate these elements in their edible tissues (Anacleto et al., 2009; Storelli, 2008; Storelli et al., 2005). In particular, the high observed As concentrations in seafood are within values presented in the relevant literature (Afonso et al., 2013; Storelli et al., 2005).

Similarly, the low As levels in other food groups, such as vegetables, agree with literature concerning samples from not heavily polluted areas (Ciminelli et al., 2017; Upadhyay et al., 2019). In contrast, marine organisms are known to be very efficient accumulators of As, reaching levels in the 1–100 mg kg^{-1} range. It has been claimed that water near the sediments (with benthic seafood species) has more As than water close to the surface (Storelli & Marcotrigiano, 2000).

Table 2.4. Season, mean or range of moisture (%, w/w), ash (% w/w), protein (% w/w) and lipid (% w/w) contents, and mean or range of bromine (mg kg-1 w/w), arsenic (mg kg-1 w/w), cadmium (µg kg-1 w/w) and lead (µg kg-1 w/w) contents in the prepared pools of the selected food groups.

Food samples	**TDS Code**	**Season**	**Moisture (%)**	**Ash (%)**	**Protein (%)**	**Lipid (%)**	**Bromine (mg kg^{-1})**	**Total Arsenic (mg kg^{-1})**	**Cadmium (µg kg^{-1})**	**Lead (µg kg^{-1})**
Meat products	---	---	62.9 ± 7.3	2.4 ± 0.9	22.6 ± 7.8	10.2 ± 4.8	3.3 ± 1.2	0.02 ± 0.01	1.3 ± 1.3	11.6 ± 11.1
Beef	13-115	---	61.1	2.0	29.4	7.5	3.1 ± 0.2	0.020 ± 0.003	< 0.09	15 ± 2
Beef, cutlet	13-116	---	61.0	1.7	27.1	12.4	3.4 ± 0.0	0.013 ± 0.004	< 0.34	15 ± 1
Pork, cutlet	13-117	---	58.2	2.1	30.3	12.3	3.8 ± 0.0	0.017 ± 0.002	< 0.27	14 ± 3
Rabbit	13-119	---	65.9	1.6	13.5	7.3	2.5 ± 0.0	0.019 ± 0.003	3.0 ± 0.3	8 ± 2
Chicken	13-120	---	61.8	1.9	28.2	9.6	3.1 ± 0.1	0.018 ± 0.001	1.0 ± 0.1	3 ± 0
Turkey	13-121	---	62.3	2.2	29.2	7.5	3.2 ± 0.0	0.030 ± 0.003	2.0 ± 0.1	6 ± 2
Ham	13-123	---	74.2	3.6	15.6	2.8	2.7 ± 0.1	0.018 ± 0.003	1.0 ± 0.3	2 ± 1
‘Chouriço’	13-124	---	49.5	4.1	19.4	19.6	6.1 ± 0.1	0.030 ± 0.001	3.0 ± 0.1	38 ± 43
Sausage	13-125	---	71.9	2.2	10.5	12.8	1.7 ± 0.0	0.010 ± 0.003	2.0 ± 0.2	4 ± 0
Fatty fish	---	All 4	65.7 ± 5.3	2.2 ± 0.7	22.7 ± 4.2	8.4 ± 4.1	9.0 ±3.3	2.2 ± 2.9	17.8 ± 22.5	24.8 ± 25.0
Catfish	7-045	Autumn	69.4	2.6	14.8	8.2	4.9 ± 0.1	0.070 ± 0.003	2.0 ± 0.2	48 ± 5
Nile perch	7-046	---	68.3	1.7	24.8	6.3	11.4 ± 0.0	0.046 ± 0.003	2.0 ± 0.2	3 ± 0
Sea bream	7-048	---	68.1	1.9	24.6	6.9	6.8 ± 0.2	0.850 ± 0.027	2.0 ± 0.0	6 ± 1
Other coastal marine fish	7-049	Spring	70.0	2.0	20.0	6.6	7.8 ± 0.2	2.34 ± 0.04	8.0 ± 0.5	13 ± 2
Conger, European	7-050	Spring	72.6	1.2	19.0	7.3	5.2 ± 0.1	13.96 ± 0.93	5.0 ± 0.2	12 ± 5
Plaice, European	7-051	Summer	73.1	2.1	21.3	4.2	10.3 ± 0.1	2.72 ± 0.04	5.0 ± 0.2	1 ± 0
Hake	7-053	Winter	71.7	1.6	18.5	5.5	7.7 ± 0.4	1.73 ± 0.05	30.0 ± 1.0	44 ± 2
Horse mackerel	7-055	Summer	65.4	2.2	24.0	8.3	8.4 ± 0.2	1.78 ± 0.04	9.0 ± 0.7	5 ± 1
Scabbard fish	7-060	Spring	66.9	2.0	21.8	8.5	8.2 ± 0.0	1.27 ± 0.01	10.0 ± 1.2	56 ± 10
Salmon, Atlantic	7-047	Spring	55.8	1.6	23.1	18.7	6.0 ± 0.4	0.68 ± 0.03	1.0 ± 0.2	8 ± 1
Chub mackerel	7-56.1-4	All 4	63.0-70.8	1.7-2.1	22.8-26.7	3.9-9.9	5.8-9.2	0.9-1.5	9-20	3-46
Sardine, European	7-57.1-4	All 4	57.8-64.6	2.9-4.0	23.4-26.3	4.4-10.9	13.4-16.8	1.9-3.4	9-39	17-89

Food samples	TDS Code	Season	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Bromine (mg kg^{-1})	Total Arsenic (mg kg^{-1})	Cadmium (µg kg^{-1})	Lead (µg kg^{-1})
Canned tuna	7-067	---	56.3	1.6	22.8	18.9	6.4 ± 0.1	0.76 ± 0.01	23 ± 0	1 ± 3
Canned sardine	7-068	---	61.9	3.4	22.7	11.2	7.5 ± 0.2	2.07 ± 0.07	102 ± 2	54 ± 3
Lean fish	---	Exc Spr	70.2 ± 4.6	2.1 ± 0.5	26.5 ± 4.7	2.0 ± 0.5	8.3 ± 4.7	1.9 ± 1.2	10.0 ± 11.3	25.1 ± 29.1
Cod, Atlantic	7-052	Autumn	76.4	2.4	19.3	2.3	16.6 ± 0.3	3.54 ± 0.06	1.0 ± 0.0	14 ± 1
Ling	7-054	Summer	73.1	1.3	25.2	1.6	6.2 ± 0.1	1.45 ± 0.05	9.0 ± 0.6	5 ± 0
Other pelagic marine fish	7-059	Autumn	65.9	2.4	30.7	1.7	6.5 ± 0.1	2.65 ± 0.06	7.0 ± 0.4	18 ± 2
Cod, dried	7-065	Winter	69.8	1.9	26.4	2.7	5.8 ± 0.1	1.06 ± 0.02	3.0 ± 0.3	76 ± 2
Tuna	7-058	Summer	65.9	2.4	30.7	1.7	6.4 ± 0.1	0.81 ± 0.02	29 ± 31	12 ± 17
Shellfish	---	---	69.5 ± 2.5	2.8 ± 1.1	18.9 ± 8.8	6.7 ± 5.9	25.9 ± 20.4	3.9 ± 0.3	109 ± 23	146 ± 161
Marine shrimp/prawn	7-061	---	71.2	2.0	25.1	2.5	11.5 ± 0.6	4.10 ± 0.16	93 ± 0	32 ± 4
Bivalve molluscs	7-062	Winter	67.7	3.5	12.6	10.9	40.3 ± 1.2	3.67 ± 0.23	125 ± 1	260 ± 26
Cephalopods	---	---	64.9 ± 13.7	1.7 ± 0.1	17.1 ± 1.1	11.0 ± 13.2	11.5 ± 7.5	4.8 ± 6.1	311 ± 277	23.0 ± 17.0
Octopus, common	7-063	Winter	74.6	1.6	17.8	1.7	16.8 ± 0.1	9.14 ± 0.12	115 ± 3	35 ± 1
Squid, common	7-064	Spring	55.2	1.7	16.3	20.3	6.2 ± 0.1	0.54 ± 0.01	507 ± 36	11 ± 1
Fresh fruit	---	---	86.7 ± 3.4	0.5 ± 0.2	0.7 ± 0.3	0.1 ± 0.1	0.3 ± 0.4	0.002 ± 0.001	0.6 ± 0.8	3.6 ± 5.3
Orange, sweet	9-70.1-4	---	85.7-87.5	0.4-0.6	0.7-1.0	0.1-0.2	0.3-0.5	0.000-0.004	< 0.02	0-2
Apple	9-71.1-4	---	85.7-86.6	0.3	< 0.3	< 0.1	< 0.1	0.001-0.003	< 0.18	1-20
Pear	9-72.1-4	---	85.6-86.5	0.2-0.3	< 0.3	< 0.1	< 0.1	0.000-0.002	1-2	1-3
Grapes, table	9-73.1-4	---	80.8-84.4	0.4-0.5	0.5-0.8	< 0.1-0.2	0.1	0.001-0.003	< 0.04	1-2
Strawberry	9-074	---	92.2	0.4	0.6	0.2	0.9 ± 0.0	0.006 ± 0.001	2.0 ± 0.1	3 ± 1
Peach	9-075	---	87.1	0.7	0.8	< 0.1	0.1 ± 0.0	< 0.001	1.0 ± 0.0	1 ± 0
Kiwifruit	9-076	---	86.3	0.6	0.9	0.5	0.7 ± 0.0	0.001 ± 0.001	< 0.01	1 ± 0
Banana	9-077	---	74.3	1.1	1.4	< 0.1	1.1 ± 0.1	0.004 ± 0.002	< 0.03	< 0.1
Fruit salad	9-82.3-4	---	85.2-86.0	0.4-0.5	0.5-0.6	< 0.1	0.4	0.001-0.002	< 1.0	1-7

Food samples	TDS Code	Season	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Bromine (mg kg^{-1})	Total Arsenic (mg kg^{-1})	Cadmium (µg kg^{-1})	Lead (µg kg^{-1})
Melon	19-153.4	---	89.8	0.6	0.9	< 0.1	1.6 ± 0.0	0.001 ± 0.000	< 2.0	1 ± 0
Watermelon	19-154	---	91.4	0.3	0.6	< 0.1	0.1 ± 0.0	0.003 ± 0.001	< 2.0	2 ± 0
Dried fruit	---	---	24.9 ± 2.0	2.0 ± 0.1	2.8 ± 0.1	1.5 ± 0.3	9.9 ± 0.2	0.032 ± 0.001	9 ± 4	7 ± 1
Fig, dried	9-80.2-3	---	23.2-27.7	1.9-2.0	2.7-2.8	1.5-1.6	9.7-10.0	0.031-0.033	6-12	6-7
Vegetables, crucifers	---	All 4	93.5 ± 1.3	0.8 ± 0.1	2.1 ± 0.5	0.1 ± 0.2	1.7 ± 0.8	0.006 ± 0.003	9.5 ± 6.6	14.6 ± 12.5
Broccoli	19-143.1-3	Winter	90.8	0.8	2.9	0.3	0.6-1.3	0.002-0.004	3-25	7-48
Cauliflower	19-144.1	Winter	93.0	0.6	1.8	0.6	0.6 ± 0.0	0.003 ± 0.000	2.0 ± 0.0	4 ± 2
White cabbage	19-146	---	---	---	---	---	1.1 ± 0.0	0.007 ± 0.004	2.0 ± 0.2	5 ± 1
Portuguese cabbage	19-147	---	---	---	---	---	2.1 ± 0.0	0.013 ± 0.001	5.0 ± 1.0	23 ± 2
Rapini	19-156.1-4	All 4	---	---	---	---	1.4-3.0	0.005-0.009	8-17	6-10
Turnip greens	19-157.1-4	All 4	93.7-94.4	0.7-0.9	1.8-2.4	< 0.1	1.5-3.2	0.003-0.012	9-15	7-29
Vegetables, other	---	---	92.1 ± 4.2	0.7 ± 0.2	1.2 ± 0.8	0.8 ± 2.5	1.3 ± 0.8	0.011 ± 0.016	3.7 ± 2.7	8.7 ± 12.6
Onion	19-148	---	---	---	---	---	0.7 ± 0.0	0.003 ± 0.001	3.0 ± 0.6	1 ± 0
Asparagus	19-149	---	---	---	---	---	1.3 ± 0.0	0.004 ± 0.001	9.0 ± 0.1	5 ± 1
Tomato	19-150.1-4	All 4	94.0-94.1	0.6	0.7-0.9	< 0.1	0.6-1.6	0.001-0.005	1-2	1-2
Pepper	19-151.1-4	All 4	91.0-93.0	0.5-0.6	1.0	< 0.1 – 0.2	0.4-1.6	0.006-0.019	3-5	2-3
Lettuce	19-155.1-4	All 4	95.1-96.6	0.8-0.9	0.8-1.0	< 0.1	1.1-3.2	0.004-0.006	3-11	3-5
Green bean	19-158.1-4	All 4	93.9	0.5	1.3	0.3	0.6-0.8	0.005-0.006	1	6-8
Carrot	19-159.1-4	All 4	88.9-90.1	0.7	0.8-0.9	< 0.1	1.1-2.7	0.011-0.018	2-6	12-47
Mushroom	19-160	Spring	81.6	1.2	3.7	8.2	1.0 ± 0.1	0.078 ± 0.002	7.0 ± 0.1	7 ± 1

Most values are presented as average ± standard deviation.

The reported Pb levels in fish agree with the literature (Afonso et al., 2013), which stresses that Pb levels only become a serious concern in highly polluted waters. Bivalves may reach relatively very high Pb contents even in comparison with other seafood groups, just as seen in current study (Table 2.4), and fail to comply with upper Pb limits (Miedico et al., 2015). Low Pb contents in vegetables, similar to those of the current study, have been reported by other authors (Antoine et al., 2017).

For Br, research work focused on the total elemental content and not specifically directed to PBDEs is rare. Nonetheless, there are some studies that show similar levels of Br in marine fish (Fernandes et al., 2016; Wan et al., 2010) and, in particular, higher values in shellfish, 77.1 mg kg^{-1} (Fernandes et al., 2016). The tuna Br contents in this study were not dissimilar from those reported by Wan *et al.* (2010). As observed in the current study, meat products had lower Br contents than seafood. Regarding fruit and vegetables, Fernandes *et al.* (2016) are ambiguous, since they group these products with other food. Another study (Rose et al., 2001) also reported a low Br content in fruit, 0.7–1.4 mg kg^{-1}. However, green vegetables were claimed to have Br levels not much lower than in fish (Rose et al., 2001). Anyway, higher Br contents in foods and feeds of marine origin, fish, shellfish and fish feed have been observed and connected with the relatively high Br concentration in seawater (near 65 mg kg^{-1}) (Fernandes et al., 2016).

Concerning Cd, the relatively high values in cephalopods and fish are also reported by other authors (Galitsopoulou et al., 2009; BKKK Jinadasa et al., 2010). For instance, this study reported a mean Cd concentration of 20 µg kg^{-1} in yellowfin tuna – 23–29 µg kg^{-1} Cd in tuna products (Table 2.4) – and red snapper. Swordfish was reported to reach values as high as 360 µg kg^{-1} Cd (Jinadasa et al., 2010). Galitsopoulou *et al.* (2009) reported even higher values for squid, exceeding 1000 µg kg^{-1} Cd, which are not very dissimilar from the present study results. It should be noted that the EU establishes a maximum limit of 1000 µg kg^{-1} Cd for several seafood species, such as cephalopods (European Commission, 2006). On the other hand, according to literature (Norton et al., 2015), fruit and vegetables typically display lower Cd contents in the 1–10 µg kg^{-1} range. Higher values were observed in some open leaf vegetables, such as chard, lettuce and spinach, with mean Cd values in the 10–50 µg kg^{-1} range (Norton et al., 2015). This corroborates relatively higher Cd levels in the rapini and turnip greens analysed in the current study. Though it has been reported that cruciferous vegetables can accumulate high amounts of Cd and Pb (Kabata-Pendias & Pendias, 2001), the current study's results seem to indicate that being a crucifer may not be the most determining aspect in this case. It should also be mentioned that pollution and other environmental factors may lead to relatively higher Cd contents in vegetables (Antoine et al., 2017; Dziubanek et al., 2017).

Correlation with seasonality

The As, Br, Cd and Pb contents found in the studied food groupings may be correlated with seasonality. In order to test this possibility, all these elemental contents in the nine groups taken together and in each group *per se* (meat products, fatty fish, lean fish, shellfish, cephalopods, fresh fruit, dried fruit, crucifers and other vegetables) were correlated with the season information whenever this was available.

For the groups of meat products, fresh fruit and dried fruit, no statistically significant seasonal information was available; thus, no correlation was possible. For the four seafood groups, enough data were available, but correlation factors were low with a R^2 always below 0.32. Only if all seafood samples were analysed together, was this last value reached for the correlation between Pb content in seafood and season. There was an increasing Pb level with the progression from summer to spring, autumn and, finally, winter. The meaningfulness of this correlation is very doubtful, given the low correlation factor, the existence of many different species of four different groups and a not plentiful array of samples. Anyway, in some samples of fatty fish, particularly in chub mackerel and sardine (species with samples from all the four seasons), winter yielded the highest Pb concentrations.

For the two vegetable groups, correlation between elemental concentrations and season was even poorer than in seafood. Finally, taking together all samples regardless of their food group, correlations were extremely poor, R^2 not exceeding 0.10.

Regarding the Pb in seafood observation, it should be noted that there are seasonal variations of toxic metal contents in fish (Fallah et al., 2011; Mwashote, 2003; Zagh & Bachari, 2019). This seems to be more frequent in lakes, lagoons and estuaries, especially if the surrounding area has pollution sources. Mwashote (2003) reported elevated Pb concentrations in fish during the rainy season. Winter in Europe, especially in Portugal and Southwest Europe, is also a rainy season. Higher rainfall may cause Pb (and other toxic metals) to be transported to streams and the adjoining coastal area. Another explanation for the increase in metal levels in winter may lie in the production of reserves for spawning (Zagh & Bachari, 2019). These authors observed an increase of Pb content in sardine during winter, thus showing similarity to the current study.

Correlation with proximate composition

The concentrations of As, Br, Cd and Pb may also be correlated with proximate composition. The moisture, ash, protein and lipid contents were considered the most relevant, and data on these were plentiful - with exception of the vegetables group where some proximate composition parameters were missing. As with seasonality, the elemental contents in the nine groups taken together and in each group *per se* (meat products, fatty fish, lean fish, shellfish, cephalopods, fresh and dried fruit, crucifers and other vegetables) were correlated with either moisture, ash protein or lipid contents.

Most correlation factors were low ($R^2 < 0.80$) and not worth discussion. There was only one strong correlation for the other vegetables group, being relative to As (Figure 2.1). Indeed, As exhibited significant correlation with protein (R^2 - 0.89).

To the best of the authors' knowledge, there is no previous study (either in Portugal or in other countries) on Br correlating its content with proximate composition parameters in the studied food groups. Whereas As (and, possibly, Br) contents may correlate with dry matter components and be the subject of nutrient accumulation, Cd and Pb contents are related to the specific environmental pollutant exposure of each food sample. However, in the case of As, such supposition is controversial, given the association of As levels in fruit and vegetables with soil and water pollution (Ahiakpa et al., 2014; Punshon et al., 2017).

On the other hand, it was considered that it was worth investigating to which degree the elemental contents correlate with each other. This investigation was conducted for all food groups taken together and for each one of the nine groups. Results showed only poor correlations. Indeed, there was only a significant correlation (R^2 - 0.82) between Br and Pb levels in the meat products (Figure 2.1).

In the literature, there are no studies corroborating such correlations between Br and Pb or other elements in these specific food groups. In fact, the few studies on this subject oppose the existence of such correlations. For instance, in okra, the correlation between Br and As levels was weak, $R^2 < 0.5$ (Ahiakpa et al., 2014). Therefore, this correlation between Br and Pb and that involving As and protein warrant further research.

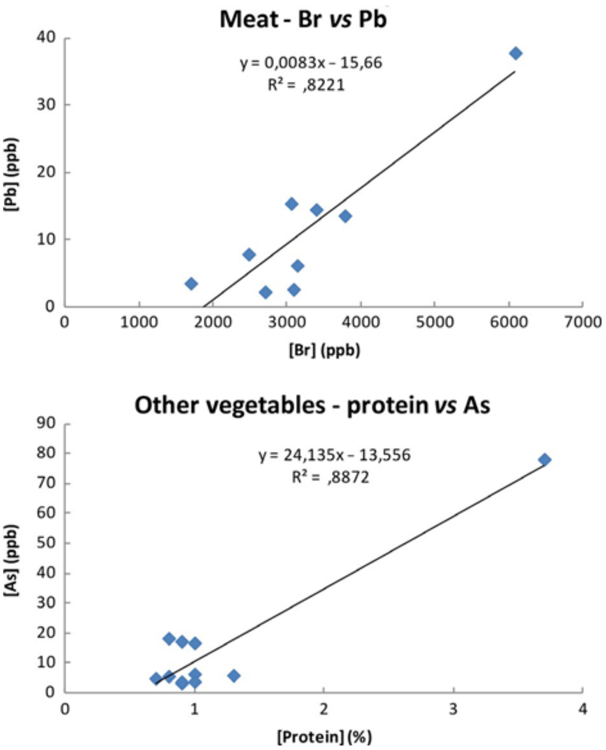

Figure 2.1. Correlations between Br vs Pb in meat products and between protein and As in the group of other vegetables.

Principal component analysis

A PCA study with the elemental contents in the nine groups and taking into account the seasonality and proximate composition aspects was performed with the purpose to shed light onto the associations underlying the attained data. The most meaningful PCA plots are presented in Figures 2.2 and 2.3.

In the case of the PCA encompassing all samples (101 pools), the studied variables form two distinct groups (Figure 2.2a), one composed by the elemental contents (As, Br, Cd and Pb) with loadings between 0.4 and 0.9 in the first principal component (p1) and another made up with the dry matter composition variables (ash, protein and lipid concentrations), which is located in the second quadrant (positive p1 and negative p2) and presents loadings between 0.7 and 0.9 in the first principal component (p1). Moisture was in the fourth quadrant (negative p1 and positive p2) with a p1 of almost -0.8 and in a position opposite to fat and protein contents. This is expected, since moisture typically correlates negatively with dry matter parameters. When seasonality is included in the PCA of all samples (Figure 2.2b), the relative positions of the proximate composition and elemental are largely conserved.

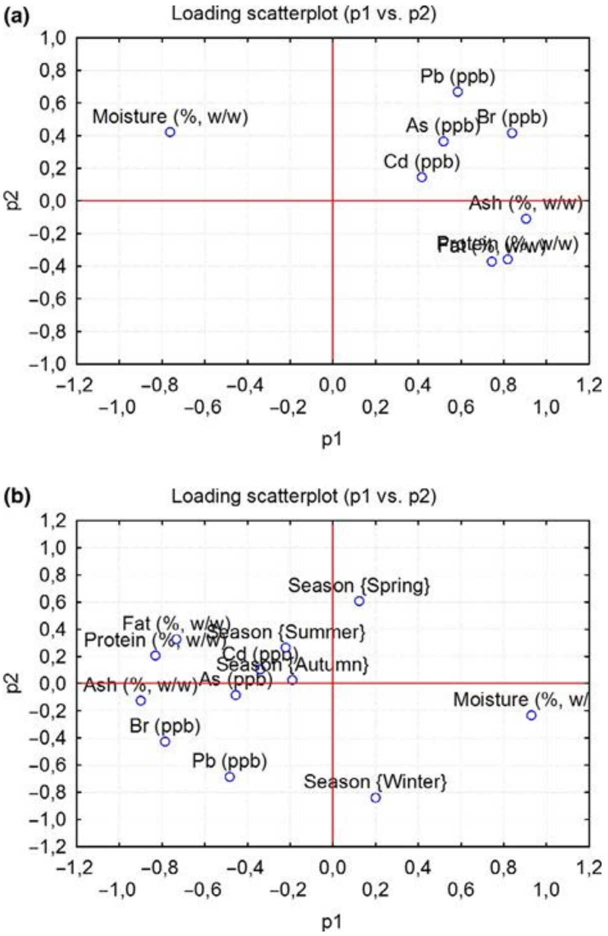

Figure 2.2. Significant principal component analysis plots generated on the basis of the elemental contents, seasonality and proximate composition data (a - all food groups; b - all food groups with inclusion of seasonality).

There is no clear association between any season and the variables, being winter more distant from the other seasons and parameters. It should be noted that such a broad statistical analysis encompassing all samples would hardly render clear seasonal patterns, since these would exist mostly within particular food groups.

When the PCA is done for all samples, but with differentiation between the nine food groups (Figure 2.3a), the same two variable groups are shown albeit in different position. Moisture again is opposite to the dry matter parameters. The novelty lies in the positioning of the food groups. In particular, the shellfish and cephalopods groups position themselves together with the elemental contents, being very near the Cd and Pb contents with p1 loadings between 0.2 and 0.4 and p2 loadings between -0.6 and -0.4. This observation may be related to the high levels of Cd and Pb in these particular food groups (Table 2.4). Moreover, whereas the meat products group is relatively isolated, the fatty fish group is near the dry matter composition variables and correlates strongly with the fat content. As expected, the lean fish group correlates much less with this parameter. With the exception of dried fruit, the fruit and vegetables groups are relatively near to each other in the third quadrant (both p1 and p2 negative). Hence, this global PCA only reinforced the insights of the previous sections.

A more detailed analysis within each food group is warranted and was enriched, whenever available, with seasonality data. For the meat products (Figure 3b), with exception of the Cd content - which was positioned opposite to fat content, thereby suggesting an inverse relationship - the elemental contents form a separate group in the second quadrant and with loadings near 0.8 for p1. Br and Pb contents were very near each other, which may further corroborate correlation levels seen in Figure 2.1.

In the case of fatty fish (Figure 2.3c), seasonality data were available and enabled a more complex PCA. Both proximate composition variables and elemental contents and the seasons are dispersed in the various quadrants of the PCA plot. The Br content is very near the ash content, which may support the presence of Br as halide salt in fatty fish (the data for the other seafood groups were insufficient for performing equivalent PCAs). Moreover, As and Pb contents distanced substantially from the Br and ash contents. Cd content had an intermediate PCA position between Pb and Br contents. Indeed, As in fish seems to be mostly in organic form, such as arsenobetaine and dimethylarsinic acid (Juncos et al., 2019), which may explain a lower correlation with the inorganic fraction. In addition, it has been reported that high Pb content in fish is associated with high content of organic matter (Ramos-Miras et al., 2019). It is also worth noting that fat content and summer were positioned very near each other. Winter was quite distant from these in the PCA plot. This suggests an accumulation of fat by fatty fish that reaches its maximum in summer, being its minimum in winter. Precisely, for pelagic fish – which are fatty fish species and are well represented in the corresponding group – there is literature (Bandarra et al., 1997, 2001) supporting a strong seasonal variation of the fat content with maxima and minima fitting the observed summer/winter opposition pattern.

The PCA for fresh fruit (Figure 2.3d) yielded a very specific plot with a large dispersion and did not provide any useful insight.

The crucifers group PCA (Figure 2.3e) also included the seasonality information, but no clear pattern is visible in the plot, except a distribution of all seasons along the p1 axis. This may suggest a seasonal character of p1. The Cd, Pb and protein contents form a group in the fourth quadrant, given their relative proximity in the plot. Their position is also characterised by very high p2 loadings in the 0.8–1.0 range. On the basis of the literature, this relationship is questionable, since these toxic metals are deemed to have a depressive effect on nitrogen assimilation and protein synthesis, at least, at high concentrations (Xiong et al., 2006). Finally, it is worth mentioning that the Br content is located almost on the p1 axis with a high p1 loading (0.8) and it is not distant from the ash and As contents.

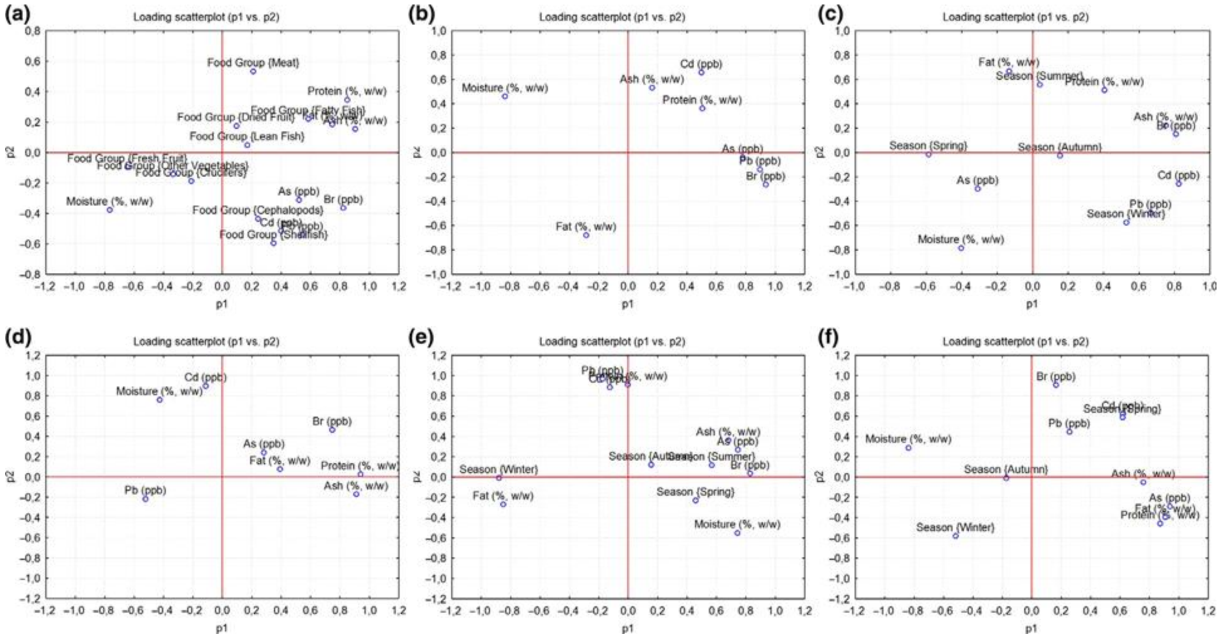

Figure 2.3. Significant principal component analysis plots generated on the basis of the elemental contents, seasonality and proximate composition data taking into account the studied food groups (a-all food groups with differentiation between these; b-meat products; c-fatty fish with inclusion of seasonality; d-fresh fruit; e-crucifers with inclusion of seasonality; f-other vegetables with inclusion of seasonality).

As last group, all other vegetables (with seasonal data) generated a PCA plot (Figure 2.3f) very distinct from that of the crucifers. There is no proximity between Cd/Pb contents and protein content or between Br and ash contents. A clear group is formed by As, fat and protein contents. This seems to reinforce the significant *R*^{2} determined for the As content–protein content association (Figure 2.1). Furthermore, the ash content positions itself not very far away from the As content. This may be related to a high share of inorganic As in several vegetables of this group, such as carrot, lettuce and tomato (Muñoz et al., 2002). Regarding the PCA plot, it can also be stated that there is a clear opposition between moisture and dry matter contents. Seasons do not yield a straightforward pattern.

This whole PCA highlights some possible connections between factors, such as protein and ash contents, and the studied elemental concentrations. However, the performed analysis leaves several unanswered questions, being necessary additional food samples and further elemental analyses, such as of Na, Mg, K, Ca, Mn, Fe, Cu and Zn, among others (Morais et al., 2017), for a deeper understanding of the possible associations between elements and food groups.

2.4 Conclusions

In general, the concentrations of As, Br, Cd and Pb in fruit, vegetables and meat products were much lower than in the seafood groups. The fresh fruit group exhibited the lowest average elemental contents. Regarding seasonality, its predictive value for the concentrations of As, Br, Cd and Pb was low even within very particular food groups. There seemed to exist a correlation between fat level in fatty fish and season. With respect to proximate composition, there was only a strong correlation in the other vegetables group. As correlated with the protein content. Furthermore, there was a significant correlation between the elemental concentrations themselves. In particular, Br and Pb levels correlated in the meat products. The overarching PCA revealed that the studied variables form two distinct groups, one composed by the elemental contents (As, Br, Cd and Pb) and another made up with the dry matter composition variables (ash, protein and lipid concentrations). Moreover, when the PCA was done for each of the nine food groups, some relevant associations seemed to exist: between Br and ash contents in fatty fish, thereby supporting the presence of Br as halide salt in this food group; between Cd, Pb and protein contents in crucifers; and between As, fat and protein contents in the other vegetables group. The effect of the proximate composition on the studied elemental contents is low, but still worth investigation.

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Author Contributions:

Marta Ventura participated in the conception and design of the study, carried out the literature review, execution of the laboratory analyses of the As, Br, Cd and Pb by ICP-MS, including interpretation of the data and writing of the manuscript. Carlos Cardoso participated in the study's design, performed the statistical analysis, and assisted in elaborating the manuscript. Narcisa Maria Bandarra conducted a critical review of the manuscript. Inês Delgado, Inês Coelho, and Sandra Gueifão participated in the laboratory analyses of the As, Br, Cd and Pb by ICP-MS. Marta Martins participated in the conception and design of the study and performed a critical review of the manuscript. Maria Helena Costa participated in the conception and design of the study and conducted a critical review of the manuscript. Isabel Castanheira participated in the conception and design of the study and performed a critical review of the manuscript. All authors read and approved the final manuscript.

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3

THE OCCURRENCE OF TRACE ELEMENTS IN THE RURAL POPULATION

MANUSCRIPT 2: The occurrence of inorganic contaminants in 'tronchuda' cabbage (*Brassica oleracea* L. var. costata DC.) after large forest fires in Portugal

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Abstract

European Mediterranean countries have been affected by unhealthy pollutants released by wildfire smoke. This work aims to determine the inorganic elements, present in the vegetable widely consumed by the rural population, provided by burned areas. Two groups composed the sampling plan; group one before forest fires and group two after forest fires under three campaigns: 1) immediately after forest fires; 2) after rainfall; 3) during springtime. ICP-MS ana- lysed all the samples to evaluate the contents of As, Br, Cd, Co, Cr, Pb, Se, Sr, and Zn. The amounts of trace elements founded were the following Pb < Cd < Co < As < Cr < Se < Zn < Br < Sr. Hierarchical Cluster Analysis evidenced 4 clusters; the first with the highest con- tents of Cr and Pb, the second cluster was marked by the highest amounts of As, and Br, higher values of Se characterised the third cluster, and the fourth cluster presented the highest values of Zn and Cd. The contents of contaminants in group one is not a matter of concern. The results obtained in group two identified a hazard, decreasing one year after the fires and maintaining these patterns afterwards. Occurrence data from local foods is a pioneer to study the impact of forest fires on human health through food consumption.

Keywords: Trace elements; cabbage; forest fires; public health; data quality; multivariate statistical analysis

3.1 Introduction

Forest fires are a source of contamination for the environment and human health. These environmental catastrophes influenced in the last years by climate changes have an impact on the health, the economy, and social life of the populations affected (Çolak & Sunar, 2020). Between 2010 and 2016 more than 85% of total burned areas in Europe were reported in Southern Europe in the five largest countries Portugal, Greece, Spain, France, Italy. In the majority of the countries a decrease in last years has been observed. However, in Portugal the forest fires had increased in the last decades and represent a leading concern for the European Union. In 2017 one of the biggest European catastrophes occurred in Portugal, raising a relevant Public Health concern due to the magnitude of fires (Rego F. et al., 2018).

The studies about wildfires usually focuses on assessing contaminants through soils and sediments in burned areas (Campo et al., 2017; Campos et al., 2016; Memoli et al., 2020). Moreover, there are different routes of exposure to the contaminants provided by forest fires, inhalation is the principal route studied by the scientific community, and there is a lack of information about ingestion, as a relevant route of exposure. Once there are burned areas affecting local farming products consumed by the rural population to maintain a balanced diet (Oliveira et al., 2020; Rogula-Kozłowska et al., 2020; Wan et al., 2021).

The principal pathways of human exposure are soils, air and water, this contamination occurs through different routes; dermic, inhalation and ingestion (Maul et al., 2017). Ingestion exposure occurs from local products (mainly vegetables) growing in contaminants soils. The contamination from the soil to the leafy vegetables occurs, mainly through the roots, xylem and plant surfaces. However, the root pathway is the largest way of contamination. Furthermore, the contaminants from the air can deposit in the soil or directly in vegetables (Pullagurala et al., 2018; Yang et al., 2016).

Tronchuda cabbage (*Brassica oleracea* L. var. costata DC.) is a variety of species *Brassica oleracea* L. (genus *Brassica*, family Brassicaceae, according to USDA plants database) (USDA, 2019). This family represents a group of vegetables highly produced and consumed around the world from ancestral times that includes different *Brassica* species (Ferreres et al., 2007). Tronchuda cabbage is a vegetal traditionally cultivated and widely consumed in rural communities, such as the Portuguese population and is part of the Mediterranean diet (Batista et al., 2011).

Leafy vegetables are the principal accumulators of chemical pollutants released to the atmosphere during forest fires which can cause adverse health effects and pose severe risks to the rural population with an average consumption higher than 100 g/day (Lopes et al., 2017).

Vegetables as *Brassica oleracea* accumulate toxic elements as arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb).

Additionally, these vegetables can also be indicators of environmental contaminants such as bromine (Br), depending on the type of soil and the environment where they grow (Shtangeeva et al., 2017; Vainikka & Hupa, 2012). The measurement of Br is an indicator of contaminants of emerging concern as brominated flame retardants, which are extremely dangerous for the environment and can reach the food chain through contaminated soils and waters (Romarís-Hortas et al., 2009).

Inductively coupled plasma mass spectrometry (ICP-MS) is a golden method for multielemental analysis due to its high sensitivity and precision and lower quantification and detection limits when compared with other methods (Castanheira et al., 2016). Chemometric tools help in data interpretation and give sustainable knowledge about the chemical system under study. Univariate statistical analysis such as analysis of variance (ANOVA) was applied in several studies, and One-way ANOVA was used to identify the existence of significant differences between two different *Brassica* species (rape and tronchuda cabbage), considering the nutritional and nutraceutical inflorescences (Batista et al., 2011), and different sprouting conditions were studied over the nutritional composition of four varieties of *Brassica oleracea* (Vale et al., 2015). However, a multivariate statistical analysis such as principal component analysis (PCA), factor analysis (FA) and hierarchical cluster analysis (HCA) permits us to reach a better interpretation of data by identifying important components or factors that allow explaining most of the variances of a system.

However, as far as we know, the occurrence of environmental inorganic pollutants originated by forest fires and accumulated in foods consumed by the population has not been reported.

This work aims to assess the accumulation of inorganic contaminants in food from areas affected by fires and consumed by the rural population, which eventually constitute a public health problem. The effect of exposure through the food of inorganic contaminants after forest fires will be achieved in three main steps; 1) to analyse trace elements contents (As, Br, Cd, Co, Cr, Pb, Se, Sr, and Zn) in Portuguese cabbage cultivated in all districts of Portugal and consumed as local farming products by the rural population, as a baseline; 2) to study the behaviour of trace elements profiles considering different geographical regions was studied by combining chemical analysis with chemometrics techniques; 3) The impact of forest fires in trace elements contents was evaluated by comparing baseline results with data obtained after the catastrophic forest fire occurred in the central region of the country.

3.2 Experimental

3.2.1 Sampling plan

Figure 3.1 presents a diagram of the sampling plan. To establish a representative control sample of cabbages consumed by the rural population, three samples from each of 33 rural localities (local farms cultivated by residents for subsistence use) were collected. Farms were selected based on the use of organic farming practices. Figure 3.2 illustrates the sampling sites. Collection of samples occurred between 2015 and 2018. Control samples were collected before forest fires in 2015. Large forest fires happened in June 2017 and subsequently, three sampling campaigns were promoted in the same sampling sites as the control samples. The 1st campaign was collected in August of 2017, the 2nd campaign was collected in December of 2017 after intense rainfall, and the samples analysed in 3rd campaign were collected in April of 2018, using the same approach (3 cabbages from each sample place). One hundred and seventy-one cabbages were analysed; where control region n = 99 and affected regions n = 72 (24 in each campaign).

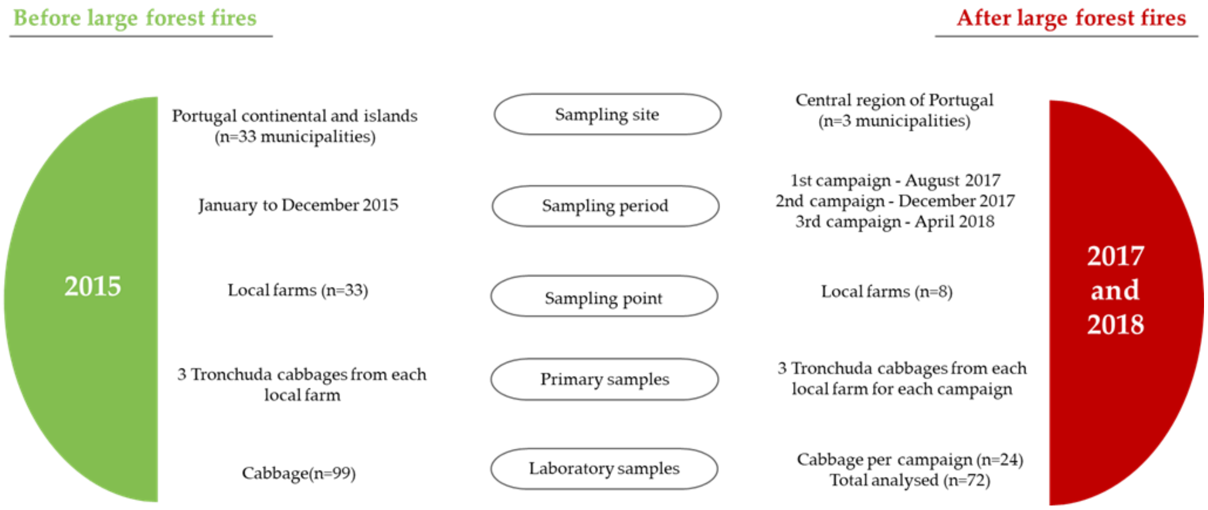

Figure 3.1. Diagram of sampling plan for all study.

Figure 3.2. Portugal map according to Nomenclature of territorial units for statistics (NUTS) II. The blue pins correspond to samples collected in 2015 before forest fires. The red pins correspond to the samples collected in 2017 and 2018 after large forest fires.

3.2.2 Sample preparation

The edible leaves of three cabbages from each place were washed thoroughly with tap water followed by MilliQ water, frozen at −20°C, freeze-dried (0.23 hPa, −50°C, 48 h), homogenised in a blender and analysed as a pooled sample.

3.3 Material and methods

3.3.1 Alkaline digestion (total Br)

Br was analysed by ICP-MS after alkaline digestion.

All the solutions were prepared using ultrapure water, resistivity 18 M cm, obtained from a Millipore purification system. For Br extraction, 0.5 g of sample was weighed and added 8 ml of ultrapure water and 1 ml of tetramethylammonium hydroxide (TMAH) (Fluka Analytical) 25%, v/v. The extraction procedure was done in a graphite digestion block for three hours at 90°C. The extract was transferred into a volumetric flask and made up to 25 ml with ultrapure water. Samples were centrifuged at 22°C at 15550 g for 15 min, and the supernatant was filtered through a 0.45 µm syringe filter (Millipore) before ICP-MS analysis (ICP-MS ThermoX Series II).

The Br calibration curve and quality control solution was prepared from standard solutions high purity ICP-MS stock standard solutions of Br containing 1000 mg L^{-1} (Inorganic Ventures). As internal standards were used standard solutions of tellurium (Te) and rhodium (Rh) containing 1000 mg L^{-1} (Merck) and 10 mg L^{-1} (Merck), respectively.

3.3.2 Acid digestion for total elements (As, Cd, Co, Cr, Se, Sr, Pb, Zn)

Analysis of total trace-elements was carried out as described in the literature (Coelho et al., 2013), with slight modifications in brief: samples weighing 0.5 g in a vessel microwave digestor (Ethos 1, Milestone), 3 ml of ultrapure water, 4 mL of concentrated HNO_3 and 1 mL of concentrated H_2O_2 were added to the vessel and closed. The digestion was made according to the following programme; in a first step, the temperature of 180°C was constant for 10 min, in a second step the temperature kept at 180°C during 5 min, in a third step the temperature increased to 210° C during 12 min, in a fourth step the temperature kept at 210°C during 5 min and in the last step the temperature decreases to 90°C during 6 min. One vessel was filled only with reagents, considered blank, to monitor the digestion process quality. After cooling down to room temperature, each vessel content was transferred into a volumetric flask and made up to 25 ml with ultrapure water. Then trace elements were analysed by ICP-MS.

Multi-element standard solutions and quality control solutions of these elements were prepared with high purity ICP stock standard solutions containing 100 mg L^{-1} of each element (Merck). As internal standards were used standard solutions of germanium (Ge) containing 1000 mg L^{-1} (inorganics ventures), yttrium (Y) and indium (In) containing 1000 mg L^{-1} (Merck).

3.3.3 ICP-MS analysis

ICP-MS measurements were performed using a quadrupole inductively coupled plasma mass spectrometry (ICP-MS) Thermo X series II (Thermo Fisher Scientific, Germany) with the software Plasmalab database version 3.51. Further details of the instrumental settings are given in Table 3.1. A quality control (QC) sample was included in each group of twelve test samples to validate the test run. Instrumental drift was monitored by the difference between the results of the first and last QC samples.

3.3.4 Quality assurance

A rigorous quality assurance programme following the NP EN ISO/IEC 17025 standard (ISO/IEC 17025, 2005) requirements was implemented for trace element analysis. Certified Reference Material GBW 10014 Cabbage was used to test the accuracy and precision of the method of analysis for acid digestion elements. Due to the lack of CRMs, Br method performance was assessed using spiked solutions. The repeatability was always below 10%. The limits of detection (LOD) and limits of quantification (LOQ) were estimated as described in the EFSA document (EFSA/FAO/WHO, 2011). The uncertainty of the studied elements ranged from 12% (Zn) to 26% (Se). The figures of merit of this work are presented in Table 3.2. Analytical procedures were carried out under rigorous metrological control and expressed by the average of three replicates. To control possible contaminations, in each acid digestion and alkaline extraction, one reagent blank was performed.

Table 3.1. ICP-MS operation conditions.

ICP-MS Thermo X Series II	
Extraction	-106
Focus	10
Pole bias	-0.1
Hexapole bias	-3
Nebuliser flow rate (L min^{-1})	0.83
Forward power (W)	1400
Cool gas flow rate (L min^{-1})	13
Auxiliary gas flow rate (L min-1)	0.84
Sampling depth	95
Standard resolution	125
High resolution	125
Analogue detector	1902
PC detector	3100

Table 3.2. Results of method validation parameters in acid or alkaline* digestion.

Element	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Repeability (RSD %)	Intermediate precision (%)	Recovery (%)	Working range ($\mu g\ kg^{-1}$)
As	0.06	0.21	7.9	8.6	107-111	0.25-2.5
Br*	1.3	4.4	3.1	3.4	91 - 120	5.0-50
Cd	0.03	0.09	6.5	8.5	82-117	0.25-2.5
Co	0.06	0.19	4.9	8.0	98-117	0.5-5.0
Cr	0.13	0.42	7.3	9.4	105-119	0.5-5.0
Pb	0.11	0.37	6.9	8.3	80-94	0.5-5.0
Se	0.13	0.44	5.2	7.5	99-105	0.5-5.0
Sr	0.12	0.41	0.44	0.66	101-118	0.5-5.0
Zn	1.1	3.7	7.5	9.8	82-119	5.0-50

3.3.5 Statistical analysis

All measurements, presented on a fresh weight basis, were done in triplicates and expressed as mean and standard deviation. A correlation study was performed, using the non-parametric *Spearman* R coefficient, to study possible relations between the nine trace elements. To obtain a better understanding of all data, considering the 33 geographical regions (samples) and the nine trace elements (variables), a multivariate analysis was conducted, using unsupervised techniques, such as Principal Component Analysis (PCA), Factor Analysis (FA) and Hierarchical Cluster Analysis (HCA). The first one, PCA, was performed to establish which variables were more correlated to each geographical region and if those variables allow reaching a clear distinction between the 33 geographic regions. Once obtained the principal components (PC) that would enable to capture the main variability on data, a Factorial Analysis (FA) was applied using an orthogonal varimax rotation, allowing a better interpretation of data. HCA was also involved since this technique is considered very effective in combining interpretation and grouping clarity. HCA was carried out on the nine trace elements using Ward's method as the amalgamation rule and the Euclidean distance as a measure similarity, and the graphical results were reported in a dendrogram. Results obtained in four different periods (one before and three after forest fires), collected from eight localities from the central region of Portugal, were analysed using one-way analysis of variance (ANOVA) followed by Least Significant Difference test (LSD) with $\alpha = 0.05$.

Before applying multivariate analysis (PCA, FA and HCA) all data were standardised to zero mean and unit standard deviation due to vast differences in data dimensionality. All statistical analyses (correlations, PCA, FA, HCA, ANOVA and LSD test) were conducted by Statistica v. 8 software (Statsoft Ibérica, Lisboa, Portugal). Significant Spearman correlations were considered at $p < 0.05$.

3.4 Results and discussion

3.4.1 Trace elements characterisation

The sampling plan was designed to guarantee the collection of cabbage cultivated in rural regions. The sampling plan before large fires was designed taking into account the areas affected by fires before 2015 and areas never affected. The samples collected in 2017 and 2018 were from areas dramatically affected by large forest fires in the country.

In Table 3.3 are presented the concentrations of trace elements in Portuguese cabbage. Among the nine elements analysed, Br and Sr were found in the highest concentration in all samples. However, a large variability was observed for almost all elements, probably due to distinct environmental conditions.

In this work, we studied three categories of elements: 1) toxic elements (As, Cd, Cr, Pb, Br); 2) micronutrients with effects on mitigation of toxic inorganic elements (Se, Zn) and 3) elements for geological identification (Co, Sr).

Se and Zn are essential elements and their effect as antioxidants are well recognised, in the last decades, they are described as antagonists of some heavy metals (Jihen et al., 2009). Due to their protective effects, these two micronutrients can reduce the hepatotoxicity of some heavy metals; beyond the liver they can protect other organs of oxidative stress. The action of Se and Zn simultaneously is more powerful as an antagonist of Cd adsorption and their deficiency constitutes a hazard for human health (Jihen et al., 2009). Some studies recommended the supplementation of Se and Zn in vegetables (He et al., 2004). Zn has chemical and physical similarities to Cd and Pb, leading to increased exposure to these two heavy metals in case of Zn deficiency (Yang et al., 2020). The antagonist effect of Se against As toxicity is recognised, and the genetic information has been already reported in the literature (Pandey & Gupta, 2018).

Table 3.3. Concentration of trace elements (fresh weight) determined in Portuguese cabbage cultivated in different regions of the country during 2015, before large forest fires (n=3). Expressed in mean ± SD.

Sample code	Location	As	Cd	Co	Cr	Pb	Se	Zn	Br	Sr
		μg kg-1						mg kg-1		
1	Açores	4.1 ± 0.3	15.7 ± 0.3	6.5 ± 0.1	32 ± 2	NQ	26 ± 2	2.3 ± 0.1	2.9 ± 0.1	6.5 ± 0.1
2	Alandroal	3.30 ± 0.01	2.28 ± 0.04	5.6 ± 0.1	30 ± 1	NQ	30 ± 2	1.24 ± 0.01	1.07 ± 0.05	4.91 ± 0.04
3	Albufeira	23 ± 1	9.3 ± 0.1	23 ± 1	30 ± 1	NQ	9.3 ± 0.2	2.01 ± 0.02	9.2 ± 0.4	18.9 ± 0.3
4	Almada	NQ	NQ	6 ± 1	10 ± 1	NQ	5.79 ± 0.06	1.1 ± 0.1	1.75 ± 0.04	4.5 ± 0.2
5	Aveiro	6.6 ± 0.3	20.2 ± 0.5	11 ± 1	26 ± 1	6 ± 1	9.8 ± 0.4	6.1 ± 0.2	3.3 ± 0.1	6.8 ± 0.1
6	Braga	15 ± 1	5.1 ± 0.4	3.7 ± 0.1	11.0 ± 0.5	NQ	40 ± 3	1.0 ± 0.1	5.4 ± 0.3	10.1 ± 0.1
7	Castelo Branco	9.3 ± 0.5	15.4 ± 0.4	20 ± 1	13 ± 1	NQ	10.9 ± 0.1	2.39 ± 0.05	1.76 ± 0.01	9.3 ± 0.2
8	Coimbra	2.9 ± 0.3	3.31 ± 0.04	3.1 ± 0.2	19 ± 2	NQ	39 ± 2	1.77 ± 0.02	1.19 ± 0.02	6.6 ± 0.1
9	Costa da Caparica	11 ± 1	4.6 ± 0.05	20.59 ± 0.04	72 ± 3	6.6 ± 0.1	13 ± 1	2.15 ± 0.01	8.8 ± 0.4	10.4 ± 0.1
10	Évora	7.7 ± 0.5	3.11 ± 0.03	6.0 ± 0.2	23 ± 2	4.3 ± 0.1	17 ± 1	1.29 ± 0.02	1.5 ± 0.1	4.7 ± 0.3
11	Figueiró dos vinhos	NQ	5.3 ± 0.1	6.4 ± 0.4	23 ± 2	NQ	6.7 ± 0.1	2.9 ± 0.1	0.51 ± 0.02	3.24 ± 0.05
12	Guarda	55 ± 2	4.1 ± 0.1	10.08 ± 0.05	17 ± 2	NQ	8.6 ± 0.5	2.9 ± 0.1	0.80 ± 0.03	6.7 ± 0.1
13	Lamego	19.1 ± 0.5	4.0 ± 0.3	10 ± 1	20 ± 1	NQ	26 ± 1	3.8 ± 0.1	0.57 ± 0.01	10.6 ± 0.1
14	Lisboa	17 ± 1	5.58 ± 0.07	18.7 ± 0.4	16 ± 1	NQ	43 ± 4	2.12 ± 0.02	2.10 ± 0.05	13.0 ± 0.3
15	Madeira	4.2 ± 0.4	7.9 ± 0.5	31 ± 3	15 ± 1	NQ	27 ± 2	1.2 ± 0.1	4.1 ± 0.2	13.2 ± 0.4
16	Minas S. Domingos	NQ	NQ	8.7 ± 0.2	15 ± 2	NQ	10.47 ± 0.02	1.71 ± 0.03	1.1 ± 0.1	2.93 ± 0.01
17	Montemor o Novo	4.3 ± 0.4	NQ	5.8 ± 0.4	28 ± 1	6.47 ± 0.01	11 ± 1	2.53 ± 0.01	2.5 ± 0.1	2.15 ± 0.01

Sample code	Location	As	Cd	Co	Cr	Pb	Se	Zn	Br	Sr
		µg kg-1						mg kg-1		
18	Mora	10.2 ± 0.2	3.5 ± 0.1	5.8 ± 0.2	15.7 ± 0.2	NQ	18 ± 1	2.4 ± 0.2	5.36 ± 0.02	7.1 ± 0.4
19	Pinhel	38 ± 3	5.9 ± 0.1	8.6 ± 0.4	18 ± 1	NQ	101 ± 4	2.24 ± 0.04	5.9 ± 0.2	13.3 ± 0.2
20	Ponte Lima	36 ± 2	17.0 ± 0.1	3.9 ± 0.3	13 ± 1	8.0 ± 0.3	47 ± 1	1.58 ± 0.02	14.5 ± 0.4	12.4 ± 0.2
21	Portalegre	8.3 ± 0.3	4.2 ± 0.1	NQ	24 ± 1	NQ	19 ± 1	1.86 ± 0.02	1.01 ± 0.03	4.30 ± 0.02
22	Portel	NQ	3.2 ± 0.1	10 ± 1	12 ± 1	NQ	13.9 ± 0.4	1.6 ± 0.1	0.56 ± 0.02	4.6 ± 0.1
23	Porto	11.7 ± 0.5	5.1 ± 0.1	5.4 ± 0.4	41 ± 1	NQ	22.8 ± 0.4	1.57 ± 0.04	6.9 ± 0.5	3.29 ± 0.03
24	Porto Santo	8.1 ± 0.4	9.3 ± 0.4	22 ± 1	37 ± 2	NQ	54 ± 2	0.7 ± 0.1	8.8 ± 0.3	29.6 ± 1.6
25	Santarém	3.4 ± 0.3	5.3 ± 0.2	4.3 ± 0.4	17 ± 1	11.4 ± 0.2	12 ± 1	1.4 ± 0.1	1.13 ± 0.04	4.61 ± 0.02
26	Setúbal	22 ± 1	2.5 ± 0.1	8.1 ± 0.3	30 ± 3	10 ± 1	38 ± 1	1.55 ± 0.04	3.3 ± 0.1	4.6 ± 0.1
27	Silves	8 ± 1	9.73 ± 0.04	28.7 ± 0.3	36 ± 1	NQ	25 ± 3	2.16 ± 0.02	4.8 ± 0.2	2.7 ± 0.1
28	Sintra	21 ± 2	NQ	11 ± 1	29.3 ± 0.5	6.8 ± 0.4	16 ± 1	2.1 ± 0.1	5.5 ± 0.2	5.0 ± 0.2
29	Trás os Montes	3.2 ± 0.3	NQ	24 ± 1	48 ± 5	NQ	83 ± 8	1.31 ± 0.01	0.61 ± 0.01	6.0 ± 0.1
32	Tomar	6.2 ± 0.4	3.86 ± 0.03	35 ± 2	28 ± 1	NQ	18 ± 2	2.71 ± 0.05	1.35 ± 0.02	3.3 ± 0.1
30	Vila Flor	8.2 ± 0.1	NQ	12.1 ± 0.3	44 ± 1	NQ	52 ± 1	1.51 ± 0.01	2.1 ± 0.1	13.3 ± 0.2
31	Vila Real	4.13 ± 0.01	5.7 ± 0.2	13 ± 1	7.3 ± 0.4	NQ	3.4 ± 0.2	2.5 ± 0.1	1.23 ± 0.04	2.6 ± 0.1
33	Viseu	10 ± 1	5.6 ± 0.2	9 ± 1	10.8 ± 0.2	NQ	7 ± 1	5.0 ± 0.1	1.891 ± 0.003	2.6 ± 0.1

NQ: not quantified

As is a metalloid present in nature that can occur from natural or anthropogenic sources, its toxicity depends on the chemical form. The inorganic forms are more toxic and more dangerous for human health and are classified by the International Agency for Research on Cancer (IARC) as carcinogenic in groups 1 for humans. On the other hand, organic forms have no toxicity (EFSA, 2009a). The highest amount of As was found in Guarda (55 ± 2 μg kg^{-1}). In five localities, the amount of As ranged from 38 μg kg−1 to 20 μg kg^{-1}. Cabbages from seven places exhibit amounts between 20 μg kg^{-1} and 10 μg kg^{-1}; however, 48% of the regions revealed values below 10 μg kg^{-1}. As was not quantified in four localities (Almada, Figueiró, Minas de São Domingos and Portel). Similar concentrations for As in vegetables were observed in the 2nd French total diet study (Millour et al., 2011).

Cd is a heavy metal of toxicological concern that can be found in the environment through natural or anthropogenic occurrence. It's a contaminant classified by IARC as carcinogenic to humans (group 1) (EFSA, 2012b). Food is one of the main sources of human exposure to Cd. The maximum amount of Cd in cabbage was found in Aveiro, with a concentration of 20.2 ± 0.5 μg kg^{-1}, whereas six localities revealed values below the quantification limits, namely Almada, Minas de S. Domingos, Montemor o Novo, Sintra, Trás os Montes and Vila Flor. In three localities (Açores, Castelo Branco and Ponte Lima), the amount ranged from 10 μg kg^{-1} to 20 μg kg^{-1}, although 90% of the collected cabbages showed values below 10 μg kg^{-1}. These results are expressly lower than those established for Cd in Brassicas (European Commission, 2014). The results of Cd presented in the literature were higher than the results observed in the present work (Millour et al., 2011).

Co is a transition metal that plays an important role in vitamin B12 metabolism as Co (III) in humans and animals (EFSA, 2009b). In this work, eight studied localities contained concentrations above 20 μg kg^{-1}, five localities showed concentrations between 11 and 20 μg kg^{-1}, nineteen localities presented values between LOQ and 10 μg kg^{-1} and only one locality presented values below the LOQ. The highest value was in Tomar (35 ± 2 μg kg^{-1}), and the lowest in Coimbra (3.1 ± 0.2 μg kg^{-1}). These results are in agreement with similar studies (Noël et al., 2011).

Cr is an element present in the earthy crust with different oxidation states, the trivalent form [Cr (III)] that is an essential nutrient with an important role in glucose mechanism in humans and the hexavalent form [Cr (VI)] that is a dangerous industrial contaminant considered as human carcinogenic by IARC (EFSA, 2014c; Tchounwou et al., 2012). All the results of Cr observed in this work were above the LOQ, sixteen localities presented values between the LOQ and 20 μg kg^{-1}, thirteen places contained concentrations between 21 and 40 μg kg$^{-1,}$ and only four localities presented values above 40 µg kg^{-1}. The highest value was presented in Costa de Caparica (72 ± 3 µg kg^{-1}) and the lowest value in Vila Real (7.3 ± 0.4 µg kg^{-1}). The results of Cr observed in this work were lower than other results in a similar group of food presented in the literature (Noël et al., 2011).

Pb is recognised as an environmental pollutant. The human being can be exposed to this contaminant through food, water, air, dust, and soil. This contaminant occurs naturally or via anthropogenic activities as mining, battery manufacturing, smelting, and others; because of unleaded fuel, the occurrence in the environment decreased in Europe. However, food can be a source of Pb exposure. Pb is toxic for the central nervous system of adults but especially in children. A systematic study based on epidemiological data links the concentration of Pb in foods with neurological effects in humans (EFSA, 2010). The highest amount was found in Santarém (11.4 ± 0.2 µg kg^{-1}), and the lowest value in Évora (4.3 ± 0.1 µg kg^{-1}). In the total geographical regions, we have detected Pb in seven localities, however, clearly lower than the maximum concentration established for Brassicas (Millour et al., 2011). The levels of lead founded in our work are similar with those published in the literature (Arnich et al., 2012; Vale et al., 2015).

Se is an essential micronutrient to human health with an important role in biological functions. The bioavailability of this micronutrient for humans can be related to Se contents in soils and this amount changes with the geographic location. Once the diet is the main source of Se for human intake, soil fortification has increased; however, the success of fertilisation depends on the chemical form of Se, and the properties of the soil; as soil pH, oxidation-reduction conditions and degree of aeration (Li et al., 2017). Within the obtained values for Se, ranging from 5.79 ± 0.06 µg kg^{-1} (Almada) to 101 ± 4 µg kg^{-1} (Pinhel), four localities presented values above 50 µg kg^{-1}, eleven regions between 21 and 50 µg kg^{-1} and half of the regions analysed presented values below 20 µg kg^{-1}. The recommended value for Se intake established by EFSA is 70 µg/day. Considering that the majority of these rural populations eat 200 g of cabbage per day, in some regions, this cabbage would make a significant contribution to the Se intake. The results of Se observed in this work were lower than similar studies in the same food group (Noël et al., 2011); the principal reason could be soil contents. However, the results presented in this work are in the same range of values in cabbage published for this region (Ventura et al., 2009).

Zn is an essential micronutrient very abundant in the Earth's Crust, with an important role in biological functions in humans (EFSA, 2014b). In this work, the mean value observed was 2.1 mg kg^{-1}. The highest value was in Aveiro (6.1 ± 0.2 mg kg^{-1}), and the cabbages with the lowest content of Zn were in Porto Santo (0.7 ± 0.1 mg kg^{-1}). Thirty-one out of thirty-three regions in the study contain a concentration up to 5 mg kg^{-1}. The recommended daily intake of Zn is 12 mg/day (EFSA, 2014b). Considering the low Zn contents determined in all samples, even if assuming that the amount consumed by this population is 200 g/day, cabbage represents a small contribution for Zn intake. These values are in range to those reported in the literature for zinc in vegetables (Noël et al., 2011).

Br is a contaminant which has an essential role as an indicator of contaminants of emerging concern as brominated flame retardants (BFRs), present in the environment and in the food chain (Pierre Hennebert; Montserrat Filella, 2018). In the analysed cabbage, the majority of the samples (25 farms) presented values between LoQ (0.04 mg kg^{-1}) and 5 mg kg^{-1}, seven localities contain concentration ranging from 6 to 10 mg kg^{-1} and only one place presented concentration above 10 mg kg^{-1}. The highest value was observed in Ponte Lima (14.5 ± 0.4 mg kg^{-1}) and the lowest was observed in Figueiró dos Vinhos (0.51 ± 0.02 mg kg^{-1}). These results were lower compared with others reported in the literature (Nguyen & Ludwig, 2014).

Sr is an alkaline earth metal widespread in nature, that can be found in soils and rocks; the principal route for humans is the food chain and water. Almost half of the total localities (16) presented values between LOQ (0.005 mg kg^{-1}) and 5 mg kg^{-1}, seven localities had values between 6 and 10 mg kg−1 and ten localities contain concentrations above 10 mg kg^{-1}. The highest result was in Porto Santo (29.6 ± 1.6 mg kg^{-1}), and the lowest in Montemor Novo (2.15 ± 0.01 mg kg^{-1}). The results of Sr present in the Portuguese cabbage were very high compared to similar studies in vegetables (Millour et al., 2012).

3.4.2 Data assessment through chemometrics analysis (PCA, FA and HCA)

For the chemometric study, the 33 regions were considered. The first approach was the Correlation Analysis so that a baseline of the relationships between the nine minerals (variables) could be established for further interpretation and justification of results. Once the data set dimension was not appropriate for an accurate distribution fitting or Normality assumption, the *Spearman* coefficient was chosen as the adequate metric. For a *p-value* < 0.05, the analysis showed the following significant correlations, presented on the format *Mineral1 – Mineral2* (*Spearman coefficient value*) for an easier interpretation: Cr – Se (0.354), Co (no correlations), Zn – Se (−0.464), As – Sr (0.471), As – Cd (0.351), As – Br (0.556), Se – Sr (0.530), Sr – Br (0.426), Cd

– Br (0.506), Pb – Br (0.489). In this work were observed positive correlations with Se and inorganic contaminants which could be explained by the heterogeneity of the soils, the same trends were described in literature (Ventura et al., 2009), they explain these differences due to diversity of the soils in Portugal. In opposite, a significant negative correlation was exhibited between Zn and Se. A possible explanation could be due to the competition of both elements for the same transporter or binding site (Vareda et al., 2019). Longchamp (Longchamp et al., 2016) observed that the contents of Zn translocation to the roots are lower in the presence of selenite, indicating that Se speciation is necessary to clarify this observation (Longchamp et al., 2016).

A positive correlation was observed between inorganic contaminants indicating that there is no competition during the translocation of heavy metals from roots to leaves; however, this behaviour can potentiate the accumulation of the toxic elements in humans through leaves intake (Gupta et al., 2019).

Anthropogenic factors, climatic conditions or geographical origin and geological characteristics could explain the variability observed in these results (Bong et al., 2013).

The Principal Components Analysis and the Factor Analysis were carried out to provide a comparative interpretation between regions. The factor analysis is a more powerful and agile method to study the relationship between the variables, was the predominant technique applied, being the *Principal Components Analysis* used on a first step to discovering the appropriate number of factors that should be studied in the *Factor Analysis*. Four factors were extracted, considering an eigenvalue higher than the unit, which correctly translates the core information of the data set. With a Cumulative Explained Variance of 74.25%, the 4 extracted factors in the PCA presented *eigenvalues* of: PC1 = 2.47, PC2 = 1.63, PC3 = 1.35, PC4 = 1.22. This initial estimate was proven accurate in the *Factor Analysis* also, and further improved once performed the *Varimax Axis Rotation*, with the 4 factors explaining the same amount of data variance but with a more even distribution among the factors. The Factor Loadings and *eigenvalues* are presented in Table 3.4, with highlighted Loadings > 0.6.

Considering that the variables (minerals) are included in a particular *Factor* for a *Loading* upper then 0.6 (except for Se (−0.542)) then PC1 is loaded by As, Br and Sr with an explained variance of 23%; PC2 is loaded by Cr, Pb and Sn and explains 21% of the data variance; Cd, Zn and Se (negatively correlated with the factor) load PC3, which contributes with 16% explained variance; PC4 is loaded by Co and explains approximately 14% of the variance. Similar behaviour was achieved using correlation analysis, as can be seen in PC1 between As, Br and Sr.

Once defined the 4 Principal Components, the PCA was carried out to approach the sampling locations. For this step, the Scores were examined under the criteria that a | Score|

> 1 indicates a strong association between a location and a certain PC. Compared to the average, high concentrations on a location, of the loaded minerals of a certain PC is translated by a Score > 1, and low concentrations by a Score < – 1.

Table 3.4. Varimax rotated Loadings and Eigenvalues for Portuguese cab- bage samples (n = 30, highlighted Loadings > 0.6).

Minerals	**Factors**			
	1	2	3	4
Cr	0.062	-0.189	0.384	**0.720**
Co	0.178	0.147	-0.294	**0.794**
Zn	-0.236	**0.788**	-0.189	-0.099
As	**0.659**	0.175	0.076	-0.419
Se	**0.666**	-0.441	-0.122	0.072
Sr	**0.808**	-0.004	-0.056	0.293
Cd	0.321	**0.794**	0.194	0.095
Pb	0.008	0.002	**0.920**	-0.067
Br	**0.692**	0.197	0.515	0.156
Eigenvalue	2.47	1.63	1.35	1.22

Figure 3.3 shows the loadings and the scores of the first three orthogonal vectors (PC1 x PC2; PC1 x PC3).

In Figure 3.3b), are presented four localities (Albufeira, Pinhel, Ponte de Lima and Porto Santo) with the highest amounts of As, Br and Sr, according to PC1. The maximum concentrations of Cr, Pb and Sn, evidenced in PC2, are showed in four localities (Aveiro, Costa de Caparica, Porto and Setúbal).

This allowed to translate the more relevant characteristics of a location in terms of Factors and then, if in major accordance with the rest of the *Cluster*, classify each *Cluster* for its mineral profile.

The *Cluster Analysis* was executed to group the 33 locations based on the similarity of the values presented for the 9 variables. In Figure 3.4 is presented the tree diagram or dendrogram that resumes the clustering process where 4 well-defined *Clusters* are visible, in figures a) and b).

In Figure (3.4b), where the 33 locations were organised by similarity, we can observe 4 groups of Clusters. The first one was characterised by the highest contents of Cr and Pb; these results are according to the pollution sources of Portuguese regions measured by the Portuguese Agency of Environment published in 2015, the same year of sampling of this work (Silva et al., 2015); the second cluster was marked by the highest amounts of As and Br mainly in North of Portugal, high values of As in the same regions, near to chemical industry was demonstrated in North of Portugal in groundwater sources (Farinha et al., 2009; Figueira et al., 2007).

The third cluster was characterised by higher values of Se in the South of Portugal, especially in Alentejo and in Coimbra (Central of Portugal); these results are according to the literature, demonstrated high contents of Se in cabbage in the same regions (Ventura et al., 2009). In the fourth cluster were presented the highest values of Zn and Cd in different regions of the country; no specific region was highlighted in this cluster.

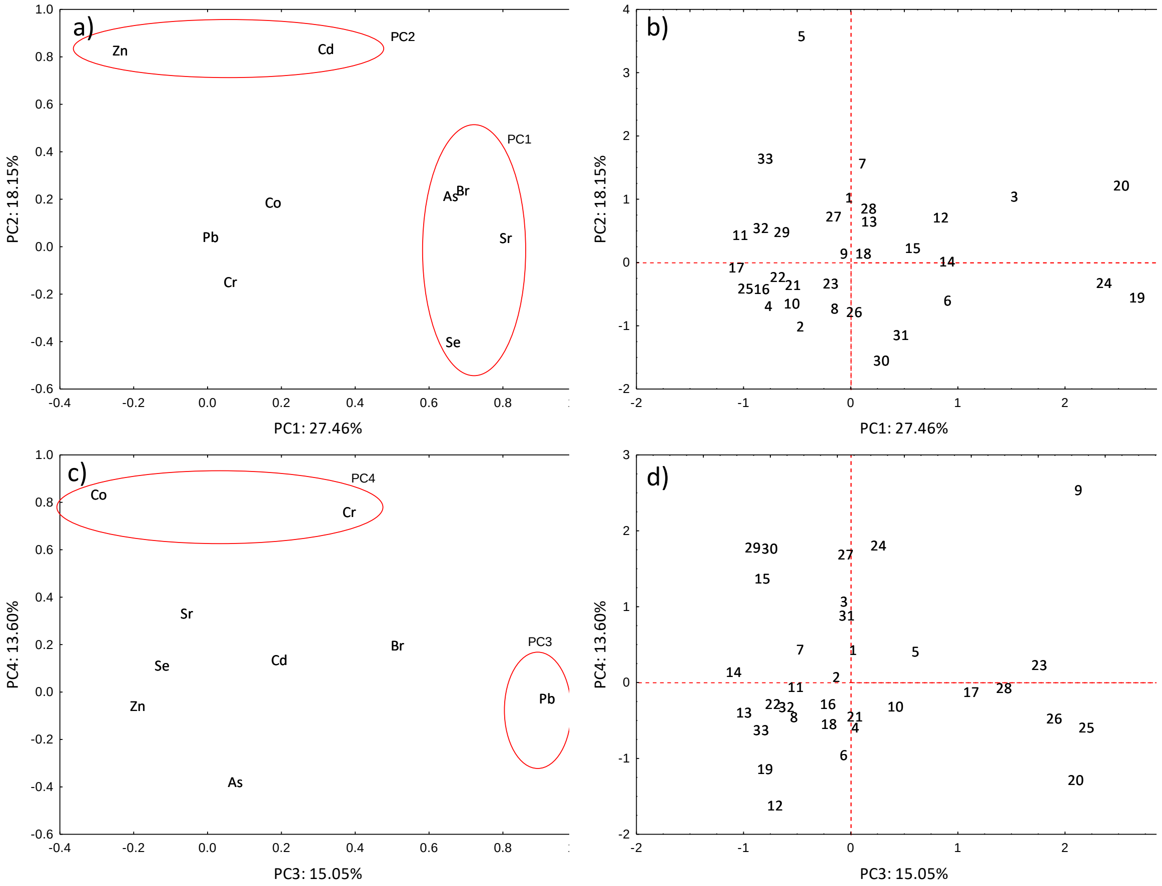

Figure 3.3. Loading (a and c) and Score (b and d) plots for Portuguese cabbage samples.

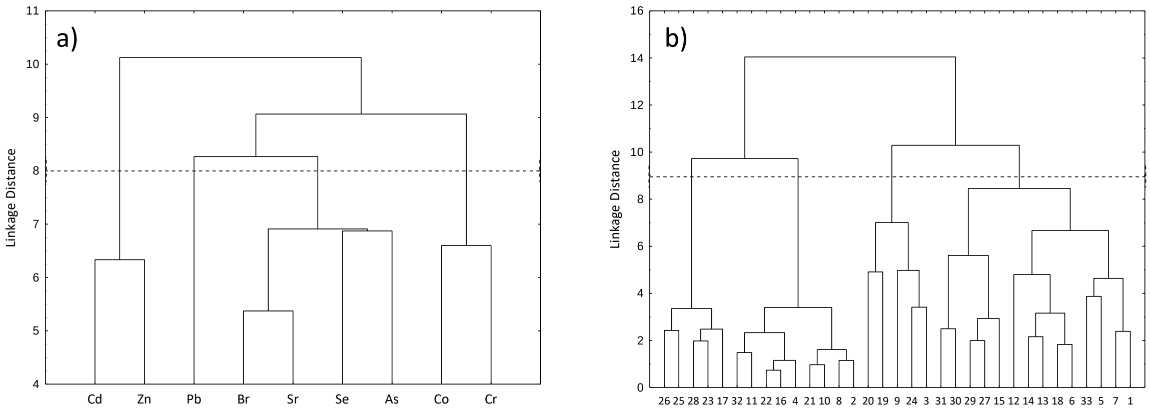

Figure 3.4. Dendrogram obtained by HCA considering (a) 9 trace elements and (b) 33 localities.

3.4.3 Comparative study between two periods of time – central region before and after forest fires

Forest fires can be one of the most relevant sources of environmental contamination. Due to climate change, forest fires have been increasing over the years. The contamination of soils after fires is a concern once the presence of contaminants increases in foods cultivated on burnt soils (Carvalho et al., 2019). In the present work, eight localities from the central region of Portugal were analysed at four different periods of time, one before (control group-CG) and three after the forest fires (three campaigns) (Table 3.5).

As observed in Table 3.5, the concentrations of all trace elements, with the exception of Cd and Pb, increased significantly between the control group and the first campaign. The comparison between the first and second campaigns only evidence significant differences for As, Cr and Sr.

The samples collected on the third campaign present the highest amounts of Cr and Pb when compared with the CG. No significant differences were observed for the remaining trace elements analysed in this study, notwithstanding presented lower averages regarding the CG.

In general, the concentration of trace elements studied for the eight localities increased in the second period of time. Climacteric conditions and soil changes could explain the concentration of this increase during cabbage growth.

In order to obtain greater robustness to this analysis, a test of Hypotheses was used to the average of the differences of the eight localities analysed, and that showed a statistically significant increase for Co, Zn, Cd and Cr.

The amounts of Br, Cd and Pb in the analysed samples before and after forest fires did not exceed the legislation values. Br values were compared to the MRL (maximum residue limit) for bromide ion contents in foods of the Brassica species (30 mg kg^{-1}) (European Commission, 2008), for Cd, the legislated value for brassicas is 0.050 mg kg^{-1} (European Commission, 2014), and for Pb, the legislated value is 0.30 mg kg^{-1} (European Commission, 2006).

Table 3.5. Concentration of trace elements (fresh weight) in Portuguese cabbage before (CG–Control Group) and after (1st, 2nd and 3rd campaigns) forest fires (mean and standard deviation) (n = 8).

Campaigns	As	Cd	Co	Cr	Se	Pb	Br	Sr	Zn
	μg kg^{-1}						mg kg^{-1}		
CG	6 ± 3^{c}	8 ± 6^{ab}	12 ± 10^{b}	19 ± 6^{b}	24 ± 31^{b}	3.0 ± 3.2^{b}	2.0 ± 1.7^{b}	6.0 ± 3.4^{bc}	3.0 ± 1.4^{b}
1^{a} campaign	31 ± 12^{b}	13 ± 8^{a}	27 ± 15^{a}	38 ± 15^{a}	79 ± 27^{a}	4.0 ± 1.3^{b}	5.0 ± 3.5^{a}	12 ± 4^{a}	5.0 ± 1.5^{a}
2^{a} campaign	53 ± 17^{a}	14 ± 14^{ab}	19 ± 6^{a}	27 ± 3^{b}	83 ± 59^{a}	4.0 ± 3.9^{b}	4.0 ± 1.7^{ac}	8.0 ± 1.6^{b}	4.0 ± 1.2^{a}
3^{a} campaign	8 ± 2^{c}	4 ± 2^{b}	8 ± 3^{b}	39 ± 7^{a}	16 ± 5^{b}	9.0 ± 4.5^{a}	2.0 ± 1.6^{bc}	3.0 ± 1.2^{c}	3.0 ± 1.0^{b}

3.5 Conclusions

In this study, we determined analytical data under rigorous metrological conditions providing reliable results to be used by risk assessors and public health authorities.

These results are part of a large project (CONFFIAR) *Impact of Inorganic contaminants from forest fires in local foods* – to estimate the real exposition to toxic elements by rural population after forest fires.

Occurrence Data from local foods is a pioneer to study the impact of forest fires to assess human health through food consumption in areas affected by forest fires and consumed daily by rural populations.

The results obtained in 2015 along the country are not affected by fires do not show any public health concern. Although data from samples collected in areas devasted by massive forest fires in 2017 identified a hazard, which decreases one year after the fires and maintains these patterns afterwards. The results show concern on As content estimated as inorganic As and should be a matter of monitorization in future. A trend was observed for inorganic toxicants like Br and needs to be followed.

This study gives a novel insight into the occurrence in local food plants of heavy metals and metalloids after forest fires and provides a reference for the prevention of toxicity. Complementary studies in the local population are conducted to biomonitor the presence of these inorganic elements, helping to target those elements on which public health measures should be a focus.

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Author Contributions:

M Ventura participated in the conception and design of the study, carried out the literature review, execution of the laboratory analyses of trace elements by ICP-MS, including interpretation of the data and writing the manuscript. H Cavaco collaborated in the statistical analysis. I Delgado, I Coelho, and S Gueifão participated in the laboratory analyses of trace elements by ICP-MS. M Martins participated in the conception and design of the study and performed a critical review of the manuscript. M. H Costa participated in the conception and design of the study and conducted a critical review of the manuscript. A Matos selected the statistical methods, carried out the statistical analyses and helped to write the manuscript. I Castanheira participated in the conception and design of the study and conducted a critical review of the manuscript. All authors read and approved the final manuscript.

4

THE RISK ASSESSMENT OF INORGANIC CONTAMINANTS IN THE PORTUGUESE DIET

MANUSCRIPT 3: Bromine, arsenic, cadmium, and lead in several key food groups: an assessment of relative risk

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Abstract

This study endeavoured to provide an overview of the bromine, Br, arsenic (As, a metalloid) and metal (cadmium, Cd, and lead, Pb) relative risk associated with the consumption of relevant food groups (lean fish, fatty fish, bivalves, cephalopods, fresh fruit, dried fruit, and green vegetables) in Portugal. Though As, Cd, and Pb have been frequently studied, a comprehensive overview applying the same state-of-the-art methodology to a wide array of foods is missing. Besides, there is a large deficit of content information and risk assessment studies on Br. Thus, it is important to apply a mathematically realistic and innovative methodology (extreme value theory) to recent and accurate databases for the aforementioned food groups and elements.

Best fits to Br, As (total), Cd, and Pb concentrations were attained through different functions, ranging from the normal distribution to the Weibull distribution. A semi-probabilistic risk assessment approach on the basis of four alternative scenarios (one monthly meal; one weekly meal; three weekly meals; one daily meal) showed low relative risk regarding Br and Pb in the selected food groups, including bivalves, which contained the highest Br and Pb contents. With respect to total As, high relative risk values were calculated for seafood, but it should be emphasized that this As is overwhelmingly organic, thus presenting low risk. For Cd, substantial relative risk associated with bivalves' and cephalopods' consumption, particularly with one or more weekly meal(s), was found. However, the current study enables us to compare between the relative risk of the studied food groups and scenarios, and other studies encompassing the whole diet and following long-term dietary patterns are needed for the assessment of absolute risk.

Keywords: Relative risk assessment; bromine; arsenic; metallic elements; seafood; fruit and vegetables

4.1 Introduction

Fruit, vegetables, and seafood are among the most important food groups with a strong presence in many diets, such as the Western, and more specifically, the Portuguese (Gregório et al., 2017). In the specific case of fish, the Food and Agriculture Organization/World Health Organization (FAO/WHO) has advocated the nutritional benefits of a diet with a strong fish component (FAO/WHO, 2011), thus propounding regular fish consumption. Dietary recommendations advising a weekly consumption of one to two portions of fatty fish have been issued (ISSFAL, 2004). Regarding fruit and vegetables, despite extensive promotion, worldwide per capita consumption is estimated to be 20 to 50 *per cent* short of the minimum daily recommended level of 400 g, or five 80 g portions (FAO/WHO, 2003a).

Even though the health benefits of seafood consumption have been widely recognized, it may have toxic effects due to the presence of some elements (EFSA, 2012a). Pollution may also lead to metal contamination of fruit and vegetables (Zhou et al., 2016). In order to assess contamination in foods, a Total Diet Study (TDS) has been set up. A TDS is considered a complementary tool of a public health surveillance program, consisting of the analysis of the beneficial and harmful chemical substances in the diet. This kind of studies can be an important approach to monitoring and surveillance of dietary exposure to chemical substances (EFSA/FAO/WHO, 2011). The current study belongs to a TDS exposure project. A national list of prioritized substances was formulated on the basis of European consensus (Vin et al., 2014). These substances include arsenic (As), a metalloid, and the metals cadmium (Cd) and lead (Pb) as main contributors for hazardous dietary exposure.

In particular, inorganic As, is listed as a Group I carcinogen by the International Agency for Research on Cancer (ATSDR, 2007). Besides some types of cancer, overexposure has been linked to alterations in gastrointestinal, cardiovascular, hematogical, pulmonary, neurological, immunological, and reproductive/developmental physiology (Hojsak et al., 2015). Regarding Cd, it has also been associated with cancer, namely, lung, kidney, and prostate cancer (IARC, 2012b). Moreover, Cd is able to initiate the atherosclerosis process and is linked to cardiovascular disease, especially coronary heart disease (Tellez-Plaza et al., 2013). Pb can result in various health effects, such as alterations in the hematological, nervous, renal, and reproductive systems (Needleman, 2004; Patrick, 2006). Furthermore, Pb competes with calcium in the organism, thereby disrupting neurotransmitter release and reducing bone mineral density (Beier et al., 2013). In this context, monitoring of As, Cd, and Pb levels in seafood, fruits, and vegetables is justified.

Regarding total bromine (Br) – an element that has not been very thoroughly studied, thereby generating a large data deficit –, more than 50% of anthropogenic Br is associated with brominated flame retardants (BFRs) and these compounds are considered nowadays as a main hazard to the aquatic environment (Winid, 2015). Nonetheless, total Br encompasses many more compounds than BFRs – such as polybrominated diphenylethers (PBDEs). Therefore, Br hazards cannot be reduced to BFRs. In particular, inorganic bromine, such as bromide ion, is deemed to have a low degree of toxicity (FAO/WHO, 1988; HCN, 2005). Chronic oral exposure to Br has been associated with alterations in the skin as well as changes in conditioned reflexes and blood indexes (HCN, 2005). Moreover, there is a lack of information regarding Br toxicology and Br toxicological limits. Since no thorough and systematic speciation of Br in foods has been conducted until now, an assessment of total Br hazard represents a first approach to the assessment of the Br exposure risks using the available information. Furthermore, Br determination is used as the first screening to measure BFRs levels in biological and environmental materials given that interest in bromine compounds contamination has increased in the last years (Romarís-Hortas et al., 2009).

The quantification of Br, total As, Cd, and Pb intake can be done by combining food consumption data with contaminant contents, following different approaches: deterministic or probabilistic. In the deterministic, it is based on a single 'worst case' point estimate of each input parameter (consumption and content). In the probabilistic, the variability of all input parameters can be taken into account by applying adequate probability distributions (Sioen, De Henauw, et al., 2007; Sioen, Van Camp, et al., 2007). Whenever no consumption frequency data are available, consumption scenarios represent an alternative, thus only requiring the application of probability distributions to the content data. This is the so-called semi-probabilistic approach. The outcome of a (semi-) probabilistic approach is a probability distribution yielding some insight into the variability of the intake under assessment in a given population. Accordingly, it is possible to calculate the probability that the individual exposure to a specific component surpasses the Adequate Daily Intake/Provisional Tolerable Weekly Intake/Tolerable Weekly Intake (ADI/PTWI/TWI) and to calculate relative risks on the basis of these probabilities for a comparison across different food groups and scenarios.

A realistic and reliable evaluation of relative risk requires statistical models and tools to study the tail of the intake distribution. A relatively recent and advanced statistical tool for this is the extreme value theory (EVT) (Tressou et al., 2004). The main novelty of EVT is to take full account of the very high (or very low) observed values, yielding more accurate calculations of low probability levels. The principle of EVT is to model the tail of an exposure distribution by a Pareto type distribution, described by a Pareto index, which can be viewed as a risk index (Tressou et al., 2004). This index is calculated by the Hill estimator, which shows a large instability, producing the so-called 'Hill-horror plot' (Emberchts et al., 1997). This instability may be greatly reduced by using bias correction methodologies (Beirlant et al., 1999; Feuerverger & Hall, 1999).

Regarding dietary risk for Portuguese consumers, there have been some previous studies mainly on methylmercury (Afonso et al., 2015; Afonso et al., 2013; Cardoso et al., 2012; Cardoso, Bandarra, et al., 2010; Cardoso, Farias, et al., 2010; Maulvault et al., 2013; Paiva et al., 2012) as well as As (Anacleto et al., 2009), Cd, and Pb (Afonso et al., 2013). These studies have highlighted the risk associated with the consumption of some fish species, such as black scabbardfish or swordfish. Nevertheless, there have been few studies, which are generally too specific, sometimes focusing on a single product. This does not enable a complete and balanced portrait of the dietary risk associated with specific element intake in Portugal. Hence, more comprehensive studies on As, Br, Cd, and Pb encompassing whole food groups are warranted.

This work aims to evaluate the Br, total As, Cd, and Pb relative risks associated with the consumption of relevant food groups, which were studied by the National Health Institute Doutor Ricardo Jorge (INSA) on the basis of its TDS (lean fish, fatty fish, bivalves, cephalopods, fresh fruit, dried fruit, and green vegetables). This involved an advanced mathematical-statistical model assuming four alternative scenarios (one monthly meal; one weekly meal; three weekly meals; one daily meal).

4.2 Experimental

4.2.1 Sampling

This study is part of a TDS (Total Diet Study). The TDS is recognised by World Health Organization (WHO) and European Food Safety Authority (EFSA) as an important tool of Public Health for dietary exposure assessment to contaminants and to beneficial substances through food. This study consists of selecting, collecting and analysing foods representative of the diet, pooling the prepared food items into representative food groups and processing the food as for consumption. The selection and collection of food require a rational sampling plan supported by consumption data (EFSA/FAO/WHO, 2011). In order to obtain harmonised dietary exposure data between five European countries, a methodology was developed for establishing harmonised national TDS food lists and to test the feasibility of the proposed procedures in order to have comparable formats of food consumption data (Dofkova et al., 2016).

Samples were collected according to Portuguese food consumption survey during 2014 and 2015. The food items were coded in FoodEx2 and were aggregated into composite samples in the twenty FoodEx2 food groups. It should be noted that the FoodEx2 is a classification system created by EFSA and adopted by several European countries. It is a hierarchical multifaceted system allowing classification and description of food in a harmonised way. FoodEX2 was conceived to help users of food exposure data to access data from different perspectives when making national and international comparisons for risk benefit analysis of contaminants and nutrients. This system is now a pan European system for electronic transmission of food data to EFSA (Akhandaf et al., 2015). Detailed food groups and different types of terms in the basic food list (hierarchy terms, core terms and extended terms) are the basis of the Food Ex2 system. In the current study, the analysed groups were chosen on the basis of their potentially high Br levels and the absence of Br studies on them. More detailed taxonomical information is given in Table 4.1. It should also be noted that analysed samples were chosen in order to ensure a representative collection (Dofkova et al., 2016) of the most consumed foods by the Portuguese population. Samples were bought at the main supermarkets of the Lisbon metropolitan area.

Accordingly, a total of seven food groups were analysed: lean fish (n = 39), fatty fish (n = 30), bivalves (n = 7), cephalopods (n = 8), fresh fruit (n = 102), dried fruit (n = 15), and green vegetables (n = 93). The distinction between lean and fatty fish used a fat content threshold of 5% w/w. The edible part of each food product was used. In the case of fish, all bones and scales were removed after cooking (see below), shells were discarded in the case of bivalves, and, in the case of fruits, they were peeled or not according to the results of the national food survey and pits were always removed (Dofkova et al., 2016). Samples were cooked whenever this corresponded to the usual consumption pattern according to the performed survey. In particular, cooking methods depended on the sample: for bivalves, boiling; for cephalopods, boiling and frying; for fish, boiling, roasting, grilling, and frying were used; for fruits, all samples were analysed raw; and for vegetables, all samples were boiled.

After preparation, samples were grouped into pools of 12 samples composed of similar foods (Dofkova et al., 2016). Note that a pool could contain the same food prepared in different ways; for example the pool of squid contained three different squid samples, prepared by different recipes (fried, grilled, and curry) as usual in the Portuguese diet.

Table 4.1. Taxonomical information of the species considered in each food group.

Food Group	Taxonomical Information – Species List
Lean Fish	*Conger conger, Gadus morhua, Lates niloticus, Merluccius sp., Molva molva, Pagellus bogaraveo, Pangasius boucourti,& Pleuronectes platessa*
Fatty Fish	*Salmo salar, Sardina pilchardus, Scomber japonicus, Sparus aurata,& Thunnus sp.*
Bivalves	*Meretrix lyrata, Mytilus sp., & Spisula solida*
Cephalopods	*Octopus vulgaris & Loligo vulgaris*
Fresh fruit	*Actinidia sp., Ananas comosus, Citrullus lanatus, Citrus sinensis, Cucumis sp., Fragaria ananassa, Malus pumila, Musa sp., Prunus persica, Pyrus communis,& Vitis vinifera*
Dried fruit	*Ficus carica*
Green vegetables	*Allium cepa, Asparagus officinalis, Brassica oleracea, Brassica rapa, Brassica ruvo, Capsicum annuum, Daucus carota, Lactuca sativa, Solanum lycopersicum,& Vigna unguiculata*

4.2.2 Analytical methodology

Inductively coupled plasma mass spectrometry (ICP-MS) was selected for determination of trace elements in foodstuffs. Recent metrological advances, such as method performance, reference materials, and proficiency testing schemes, have been important. As a consequence, analysis of trace elements in foods has become more robust, in spite of the shortage of Certified Reference Materials (CRMs) and Standard Reference Materials (SRMs) (Castanheira et al., 2016).

Determination of elemental content

Bromine was determined by ICP-MS after alkaline extraction. For the alkaline extraction, 0.5 g of sample was weighed and 8 ml of ultrapure water and 1 ml of tetramethylammonium hydroxide (TMAH) (Fluka Analytical) 25%, v/v, were added, on a heating plate. After extraction, sample was diluted to 25 ml volumetric flasks with ultrapure water. The sample was then centrifuged at 22°C using 15,550 × g for 15 min and the supernatant was filtered through a 0.45 µm syringe filter (Millipore) before ICP-MS (ICP-MS ThermoX Series II) measurements. Standard solution of bromine containing 1000 mg/l (Inorganic Ventures) was used to prepare standard solutions. Standard solutions of tellurium and rhodium containing 1000 mg/l (Merck) and 10 mg/l (Merck), respectively, were used as internal standards. MRC Carrot NCS ZC73031 from China National Analysis Center, which had data for bromine, was used as SRM. Concentrations were expressed in wet weight.

Analysis of total As, Cd, and Pb was carried out as described by Coelho et al. (2013) (Coelho et al., 2013). Briefly, an acid digestion was carried out in a closed vessel microwave digestor (Ethos 1, Milestone), with 4 ml of nitric acid, 1 ml hydrogen peroxide, and 3 ml of ultrapure water. Standard multielement solutions of Standard IX and Standard XVI containing 100 mg/l (Merck) were used to prepare standard solutions. Standard solutions of germanium, yttrium, and indium containing 1000 mg/l (Merck) were used as internal standards. Concentrations were expressed in wet weight.

Quality assurance

A rigorous quality assurance program following the NP EN ISO/IEC 17025 standard (ISO/IEC, 2005) requirement was implemented for analysis of total As, Cd, and Pb. This program included appropriate qualification for involved operators and glassware calibration and traceability. Equipment was calibrated every day with new standard solutions. Acceptance criteria were defined for repeatability – defined as an assessment of the results generated by the repeated performance of the same measure or test procedure, the intervention of the same operator, the utilization of the same equipment under the same conditions, in the same location and within a short period of time – and reproducibility. Certified Reference Material Carrot was used to test the accuracy and precision of the Br analysis method. Recoveries between 90% and 115% were obtained. The repeatability was always below 10%. Each sample was treated in triplicate and one reagent blank was included in each extraction to control possible contamination. A Quality Control (QC) sample was included in each group of twelve test samples to validate the test run. Instrumental drift was monitored by difference between the results of first and last QC samples (<5%).

Limits of Quantification (LOQ) were estimated as equal to three times the standard deviation of the mean blank tests after correction by the sample weight and the appropriate dilution. The LOQ ranged in the following intervals: Br 0.03 (vegetables) -0.4 (bivalves) mg/kg; total As 0.002 (molluscs) -0.04 (fish) mg/kg; Cd 2 (molluscs) – 36 (fish) µg/kg; and for Pb 5 (bivalves) -71 (fish) µg/kg.

Relative risk assessment

The relative risk assessment involved a determination of the risk levels for the seven food groups and each studied element. These risk levels were assessed through a mathematical model run with the software @ RISK® – Advanced Risk Analysis for Spreadsheets, from Palisade Corporation (Ithaca, NY, USA), version 4.5, 2005. A semiprobabilistic approach was applied. This combines variability in the concentration data with fixed consumption scenarios (ranging from one monthly meal to one daily meal for all food groups). Probability distribution fitting, intake level calculation and risk assessment determination were performed according to Cardoso et al. (2010a, 2010b) (Carlos Cardoso, Bandarra, et al., 2010; Carlos Cardoso, Farias, et al., 2010). In particular, the best probability distributions from an extensive library of 90 distributions were chosen according to χ^2 tests for goodness of fit. The risks were quantified based on the probability of exceeding threshold values.

Regarding these threshold values, it is worth noting that the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/ WHO) Expert Committee on Food Additives (JECFA) set a provisional tolerable weekly intake (PTWI) for Pb of 25 µg/kg body weight (FAO/WHO, 2010). For Cd, EFSA's Panel on Contaminants in the Food Chain (CONTAM Panel) established a tolerable weekly intake (TWI) of 2.5 µg/kg body weight (EFSA, 2011). For total As, a PTWI of 15 µg/kg body weight was established by the WHO in 1989 (FAO/WHO, 1989). In 2010 the JECFA withdrew the total As PTWI and the Pb PTWI has also been withdrawn. However, these threshold values were used in the current study, given the requirement of an intake limit for the application of the mathematical model. Inorganic bromine was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1966, which recommended an acceptable daily intake (ADI) for humans of up to 1.0 mg/kg bw (FAO/WHO, 1988).

There are two alternatives for estimating the risk: the plug-in (PI) and the tail estimation (TE) based estimators. Both were used, the PI estimator for large probabilities and the TE estimator for the other situations (especially, for very low probabilities). The statistical methodology based on the EVT was applied to the Br, total As, Cd, and Pb data of the studied food groups as described by Cardoso et al. (2010a, b) (Carlos Cardoso, Bandarra, et al., 2010; Carlos Cardoso, Farias, et al., 2010). For all the calculations, a meal of 160 g and an average human body weight of 60 kg (FAO/WHO, 2003b) were used.

For food with a concentration lower than LOQ, the methodology described by Verbovšek (2011) (Verbovsek, 2011) was adopted. Concentrations that ranged from Limit of Detection (LOD) to LOQ were treated as the values above the LOQ. This encompassed all Cd content values in lean fish. $\mathrm{LOD}/\sqrt{2}$ was used when the analytical values were below LOD. Finally, on the basis of the probability of exceeding the threshold values calculated for each food group, consumption scenario, and element, the relative risk was determined by using the 1:1 × 10^{10} probability (1 × 10^{-8} %) as the unit. Accordingly, for each element, a relative risk value enabled a comparison between food groups and consumption scenarios. This enabled assessment of how much risk was enhanced or reduced with the different scenarios or by switching between food groups.

4.3 Results and discussion

Raw data

A comparison of the values shows a wide variability of concentrations between food groups (Table 2). For total As and Pb, fruits and green vegetables formed a large group of low content foods and seafood (lean fish, fatty fish, bivalves, and cephalopods) exhibited high contents. This separation was not possible for Br, since dried fruit presented contents similar to those of lean fish. While dried fruit had higher Br, total As, and Cd concentrations than other vegetables, lean fish presented lower Br and Cd levels than other seafood groups. Regarding these two elements, bivalves and cephalopods groups exhibited the highest levels, reaching >20 mg/kg and >90 µg/kg in Br and Cd, respectively. For total As, cephalopods showed the highest concentration, 16.70 ± 8.53 mg/kg. For all other elements, bivalves contained the highest contents, reaching 87.15 ± 48.73 mg/kg in Br, 308.10 ± 348.20 µg/kg in Cd, and 192.18 ± 113.74 µg/kg in Pb. These were high values, doubling or more the other groups' values.

Variability as measured by the standard deviation was typically very pronounced across elements and food groups. This is a consequence of the wide array of different food products assembled together in each group. Notwithstanding such large variability, the observed contrasts are meaningful and point to the existence of clear patterns. Namely, it is known that mollusks – comprising bivalves and cephalopods – very often accumulate Cd in their digestive glands (Bustamante et al., 1998) and that Cd is also found in other body parts (Miramand & Bentley, 1992). This is corroborated by various authors performing analytical studies, such as Lourenço et al. (2009) (Lourenço et al., 2009), which have reported that Cd content in octopus sometimes exceeded the recommended European Union limit of 1000 µg/kg. Very high Cd values have also been reported for bivalves, such as oyster or clams (Ju et al., 2012).

Regarding total As and Pb, it is well known that some fish species can accumulate these metalloids and metals in their edible tissues (Anacleto et al., 2009; Storelli, 2008; Storelli et al., 2005). The high observed total As concentrations in seafood that were used afterwards in the risk assessment are within reported ranges (Afonso et al., 2013; Storelli et al., 2005). Marine organisms are known to accumulate As in the 1–100 mg/kg range. It has been recognized that the waters near the sediments (inhabited by benthic species) contain more As than those near the surface (Storelli & Marcotrigiano, 2000). Cephalopods may present relatively high total As contents as in the current study's, for instance, Nho et al. (2016) (Nho et al., 2016) reported 14.6 mg/kg for octopus. The observed Pb levels in fish agree with the available literature (Cláudia Afonso et al., 2013), which mentions that Pb levels are only of concern in highly polluted fishing grounds. Bivalves may display higher Pb contents than other seafood groups and fail to comply with upper Pb limits (Miedico et al., 2015).

Table 4.2. Average bromine (mg/kg w/w), total arsenic (mg/kg w/w), cadmium (µg/kg w/w), and lead (µg/kg w/w) contents in the selected food groups.

Food group	Bromine (mg/kg)	Total Arsenic (mg/kg)	Cadmium (µg/kg)	Lead (µg/kg)
Lean fish	8.14 ± 3.08	2.58 ± 3.48	< LOQ	76.40 ± 2.23
Fatty fish	10.24 ± 3.89	1.71 ± 0.87	45.90 ± 13.20	89.09 ± 10.07
Bivalves	87.15 ± 48.73	2.44 ± 1.75	308.10 ± 348.20	192.18 ± 113.74
Cephalopods	20.84 ± 6.91	16.70 ± 8.53	90.75 ± 172.13	39.07 ± 21.93
Fresh fruit	0.58 ± 0.76	0.003 ± 0.002	1.02 ± 1.04	3.71 ± 4.82
Dried fruit	7.54 ± 4.18	0.029 ± 0.008	7.86 ± 4.71	8.18 ± 2.19
Green vegetables	1.46 ± 0.87	0.007 ± 0.004	6.13 ± 5.65	13.19 ± 13.11

Values are presented as average ± standard deviation. Averages were calculated from all values above the respective elemental LOQ.

For Br, studies centered on the total elemental content and not only on PBDEs are scarce. That said, the high Br levels in the bivalves of the current study – that served as input for the risk assessment – are higher than in other studies on this element (Kumblad & Bradshaw, 2008; Pavlov et al., 2015). For other food groups, there seems to be no major disagreement with the available literature (Elson et al., 1983).

Risk assessment through scenarios

The assessment of the relative risk of Br, total As, Cd, and Pb exposure through seafood, fruit, and vegetables consumption requires a statistical analysis that takes into consideration uncertainty associated with data and assumptions. In this context, a semi-probabilistic approach with different consumption scenarios may be very useful. For the variability associated with the elements' concentrations, the best fit distributions for Br, total As, Cd, and Pb contents

(Table 3) were used and the probabilities of exceeding the Br ADI, P(Xi>ADI), the total As PTWI, P(Xi>PTWI), the Cd TWI, P(Xi>TWI), and the Pb PTWI, P(Xi>PTWI) were estimated (Table 4).

Table 4.3. Best fit distributions for bromine, total arsenic, cadmium, and lead contents in the selected food groups.

Food group	Bromine	Total Arsenic	Cadmium	Lead
Lean fish	Expon (3270)	Ext-value (1430;1560)	Ext-value (4.50;3.93)	Inv-Gauss (24.7;14.3)
Fatty fish	Expon (4250)	Inv-Gauss (1350;2170)	Inv-Gauss (28.0;121)	Beta-general (0.593;1.92;0.435;114)
Bivalves	Expon (46,800)	Expon (1360)	Ext-value (176;193)	Beta-general (0.258;0.271;46.1;343)
Cephalopods	Logistic (21.7;3410)	Logistic (17,500;4470)	Inv-Gauss (81.6;0.951)	Beta-general (0.293;0.332;11.0;79.6)
Fresh fruit	Beta-general (0.508;35.0;34.2;38,300)	Log-logistic (−0.927;3.19;2.63)	Expon (1.02)	Inv-Gauss (4.23;3.62)
Dried fruit	Expon (7100)	Ext-value (25.7;6.00)	Normal (7.86;4.71)	Expon (2.23)
Green vegetables	Expon (1050)	Weibull (1.34;6.38)	Expon (5.40)	Inv-Gauss (12.1;7.08)

For the Expon (exponential distribution), the rate parameter is the only numerical parameter; for the Logistic, there are two numerical parameters, μ relating to location and s relating to scale; for the Beta-general, there are four shape parameters, y, b, p, and q; for the Ext-value (extreme value distribution), there are two numerical parameters, μ relating to location and σ relating to scale; for the Inv-Gauss (inverse Gaussian distribution), there are two numerical parameters, λ and μ; for the Log-logistic, there are two numerical parameters, α relating to scale and β relating to shape; for the Weibull, there are two numerical parameters, λ relating to scale and k relating to shape; for the Normal, there are two numerical parameters, μ or mean and relating to location and σ 2 or variance and relating to scale.

The statistical distributions with the best fit to data —according to $\chi 2$ tests for goodness of fit— ranged from the normal distribution to the Weibull distribution. Some distribution function types were better at providing good fits to data, such as beta general or exponential. For instance, for Br, five of the seven adjusted distributions were exponential functions. These were defined by a single parameter, while beta general functions were defined by four parameters (see footnote of Table 4.3). Previous studies using EVT methodology have applied some similar distribution functions (Cardoso, Bandarra, et al., 2010).

Regarding Br, the probability of exceeding its ADI, 1 mg/(kg b.w. × day), was low across the various studied food groups, with the highest relative risk for a daily meal of bivalves, 6.1 × 10^6, which means that Br in bivalves is worth more attention than in fish or fruits and vegetables. For lower consumption levels, relative risk sank to very low levels. It should be remarked that whereas cephalopods as a group presented the second highest Br contents (Table 4.2), relative risk for a daily meal of dried fruit was higher than for cephalopods. In cephalopods, the relative risk was lower than one (1:1 × 10^{10} probability). Such low values were much better estimated through TE estimates using the EVT. TE estimation depends on the shape of the fitted distribution curve, sometimes resulting in a more serious concern for a wider curve with a lower average than a narrower curve with a higher average, thereby explaining some divergence between average contents and calculated probability. Therefore, total Br in bivalves may warrant further research. It should also be noted that the used threshold value, ADI of up to 1.0 mg/kg bw (FAO/WHO, 1988), incorporates a large safety factor, being quite below the level causing no toxicological effect on humans, 9.0 mg/kg bw (Sangster et al., 1983). Furthermore, in recent years, public health concerns have targeted PBDEs (Fernandes et al., 2016).

For total As, a much different risk portrait emerged, where higher relative risk values were calculated for all seafood groups. In the case of a daily meal, the highest relative risks were calculated for fish, bivalves, and cephalopods. For a sparse consumption frequency of a monthly meal of lean fish, fatty fish or bivalves the relative risk was not so high. However, for cephalopods with their high total As contents, an estimated relative risk of 2.1 × 10^9 was attained even in such a moderate consumption scenario. The relative risk for fresh fruit, dried fruit, and green vegetables was negligible.

These relative risk values concerning total As must be adequately interpreted. Indeed, it is known that the As toxicity depends on its oxidation state and molecular form. For instance, As (III) is more toxic than As (V), whereas arsenobetaine and arsenocholine, arseno-organic compounds, are relatively non-toxic (ATSDR, 2007; Peraza et al., 1998; Storelli & Marcotrigiano, 2000). Namely, high intakes of inorganic As are associated with several toxic effects including enhanced risk of cancer (ATSDR, 2007). However, the majority of this element in marine organisms is present as organoarsenic compounds (Anacleto et al., 2009; ATSDR, 2007), such as arsenobetaine (typically more than 90% of the total As) that is rapidly methylated and excreted via urine (ATSDR, 2007). Nonetheless, some authors (Taylor et al., 2017) have emphasized that the high concentrations of organic As in seafood and complex As speciation make the assessment of As exposure from diet difficult. For instance, it is known that arsenolipids can be a significant fraction in some oily fish (Lischka et al., 2013). This complexity warrants further investigation on organic As.

With respect to Cd, there was a clear progression of relative risk with increasing consumption frequency in the cases of the bivalves and cephalopods. Indeed, while a monthly meal of these foods was associated with a relative risk of less than 2×10^4, a weekly meal led to a relative risk in the 1.7–2.4×10^8 range, and a daily meal generated relative risk values of more than 6×10^8. For this consumption frequency, the bivalves exhibited the highest Cd relative risk, thus matching their high average Cd content (Table 4.2). For all other food groups, relative risk remained below 5.8×10^5, which was the value found for a daily meal of fatty fish.

Table 4.4. Relative risk associated with contaminants (Br, total As, Cd, and Pb) present in different food groups consumed in Portugal (based on the calculation of the probability in % of exceeding the respective threshold values*).

Food group	Consumption scenario	Bromine	Total Arsenic	Cadmium	Lead
Lean fish	1 monthly meal	n.s.r.	2.0×10^5	n.s.r.	n.s.r.
	1 weekly meal	n.s.r.	**7.9×10^8**	n.s.r.	n.s.r.
	3 weekly meals	n.s.r.	**5.9×10^9**	n.s.r.	27
	1 daily meal	n.s.r.	**8.5×10^9**	3.4×10^2	1.8×10^5
Fatty fish	1 monthly meal	n.s.r.	9.25×10^4	n.s.r.	n.s.r.
	1 weekly meal	n.s.r.	1.1×10^8	n.s.r.	n.s.r.
	3 weekly meals	n.s.r.	**3.2×10^9**	1.8×10^2	n.s.r.
	1 daily meal	n.s.r.	**9.1×10^9**	5.8×10^5	n.s.r.
Bivalves	1 monthly meal	n.s.r.	5.5×10^4	1.9×10^4	n.s.r.
	1 weekly meal	76	**3.7×10^8**	2.4×10^8	n.s.r.
	3 weekly meals	1.3×10^5	**5.0×10^9**	**4.3×10^9**	4.4×10^3
	1 daily meal	6.1×10^6	**9.9×10^9**	**7.8×10^9**	4.9×10^5
Cephalopods	1 monthly meal	n.s.r.	**2.1×10^9**	5.9×10^7	n.s.r.
	1 weekly meal	n.s.r.	**9.6×10^9**	1.7×10^8	n.s.r.
	3 weekly meals	n.s.r.	**9.9×10^9**	**3.9×10^8**	n.s.r.
	1 daily meal	n.s.r.	**9.9×10^9**	**6.2×10^8**	n.s.r.
Fresh fruit	1 monthly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	1 weekly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	3 weekly meals	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	1 daily meal	n.s.r.	1.4×10^4	4.2	n.s.r.
Dried fruit	1 monthly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	1 weekly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	3 weekly meals	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	1 daily meal	15	n.s.r.	n.s.r.	n.s.r.
Green vegetables	1 monthly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	1 weekly meal	n.s.r.	n.s.r.	n.s.r.	n.s.r.
	3 weekly meals	n.s.r.	n.s.r.	8.1×10^2	n.s.r.
	1 daily meal	n.s.r.	n.s.r.	6.2×10^3	2.9×10^3

*Threshold values: Br, ADI – 1 mg/(kg b.w. × day); total As, PTWI – 15 µg/(kg b.w. × week); Cd, TWI – 2.5 µg/(kg b.w. × week); Pb, PTWI – 25 µg/(kg b.w. × week). n.s.r. – No significant risk (< 1: 1 × 1010). Values in bold correspond to probabilities calculated through the 'plug-in' (PI) estimator.

In fact, there is an increasing concern about bivalve safety due to Cd accumulation in these marine organisms (Shi et al., 2016). A probabilistic risk assessment applied to the estimation of renal damage and prevalence of peripheral arterial disease from seafood consumption has cautioned against excessive consumption on the basis of health risk posed by long-term exposure to low level Cd in the general population (Ju et al., 2012). In addition, surveys conducted in the Pearl River Delta, south China, presented Cd contamination in edible marine bivalves as a potential serious hazard to public health (Fang et al., 2001). Another study (Li & Gao, 2014) has found that daily consumption of 9 g of the bivalve species *Chlamys farreri* is sufficient for Cd to reach the reference threshold. For the studied elements, Cd daily intake was considered to be the limiting factor for bivalve consumption as a food for a large number of consumers in some Chinese cities. In particular, authors (Li & Gao, 2014) warned that the situation demands attention, taking into account that a significant segment of the population regularly consumes large quantities of bivalves. This issue remains an important concern and may be worsened by ocean acidification, since it has been claimed that this process induces Cd accumulation in marine bivalves (Shi et al., 2016). What is more, it has been reported that Cd shows the highest capacity to accumulate in bivalves among all metals (Liu et al., 2017).

Concerning the relative risk for Pb, values lower than one were observed for most food groups and encompassing all studied scenarios. Otherwise, relative risk remained low, never going beyond 4.9×10^{5}, calculated for a daily meal of bivalves. This mirrored the relatively high average Pb content in the bivalves (Table 4.2). A daily meal of lean fish brought about a similar relative risk, 1.8×10^{5}. This is noteworthy, given the much lower average Pb content in lean fish in comparison to the average Pb content in the bivalves group, an approximately twofold ratio. As explained above, the shape of the fitted distribution – chosen on the basis of χ^2 tests for goodness of fit – may determine the behaviour of the tail of the curve and the calculation of relative risk.

Other studies have already shown that Pb is not the main health concern regarding metal toxicity in fish (Afonso et al., 2013) and other seafood (Cirillo et al., 2010). In fruits and vegetables, Pb health risks may be associated with the utilization of fertilizers (Yusuf et al., 2003), but such risks have been considered to be low in most cases (Ji-yun et al., 2016; Pan et al., 2016).

It should be stressed that for higher relative risk values PI estimates were used. Otherwise, TE estimates were always preferred as more accurate for low and, particularly, very low relative risk values. The PI estimator presents some drawbacks, since, when the TWI (or ADI/PTWI) is larger than the highest observed (estimated) intake value, P(Xi>TWI) (or

P(Xi>ADI/PTWI)) becomes zero, which is unacceptable, since a null risk/benefit never exists. TE estimates, albeit more accurate than PI ones, are not without error. It has been reported that TE may not ensure an accurate estimation of the probability of surpassing the TWI (or ADI/PTWI) whenever this probability is high (Tressou et al., 2004).

The performed assessment of relative risk is based on the assumption that the threshold (ADI/PTWI/TWI) is a binary outcome (hazard vs no hazard) parameter for the effect and that the severity of all effects is the same. The binary outcome approach is frequently used in risk assessment where safety is the only concern, but when comparing health effects across different food components it presents serious drawbacks. For the formulation of dietary recommendations, studies encompassing the whole diet and following dietary patterns long-term would be essential. Moreover, these studies would have to involve the calibration of the public health impacts of exceeding the Br ADI, the total As PTWI, the Cd TWI or the Pb PTWI. For this purpose, dose-response relationships concerning relevant health endpoints would have to be known. However, dose-response functions (Hoekstra et al., 2008) are difficult to achieve and use, given the various potential health endpoints, the poor evidence regarding some of these endpoints, and the existence of contradictions among several interventions and epidemiological studies (Hoekstra et al., 2013; Mozaffarian & Rimm, 2006). The used approach mainly focused on the threshold values for the calculation of relative risk, does not take into account these aspects. Besides, any quantified health outcome must be expressed in a common measure in order to be compared and to achieve a net health effect as a result of alternative scenarios. The disability-adjusted life years (DALYs) or the quality-adjusted life years (QALYs) may provide such a common currency (Hoekstra et al., 2008, 2013). There has been progress in this area leading to relevant studies (GBD 2016 DALYs and HALE Collaborators, 2017; Hoekstra et al., 2008, 2013), such as the Global Burden of Disease Study (GBD 2016 DALYs and HALE Collaborators, 2017), and future work on risk assessment should explore further this research area.

All the above discussion illustrates the importance of the various assumptions made in these studies. The relative risk values are calculated from probability results, which are based on assumptions that are not beyond doubt and may receive further improvement in future studies, especially if more information is rendered available. First, the utilization of the Br ADI, the total As PTWI, the Cd TWI or the Pb PTWI as sole references is disputable since these threshold values contain some uncertainty. Namely, it is difficult to set a reference for a whole population, which has a wide variability of health conditions and genetic features. Whenever a reference is established, safety factors are incorporated, which, for instance, lower the tolerable intake level for Cd TWI or underlie the admitted Br ADI. Nonetheless, these are the values generally recognized as the references – at least, in Europe – or, in the absence of currently accepted references, are required for the operation of the mathematical modeling. For instance, JECFA has withdrawn the Pb PTWI and the total As PTWI, leaving no alternative references. Another problem arises from the scarcity of detailed consumption surveys, which are crucial for achieving further progress in these matters, leading to somewhat arbitrary and uninformed consumption frequency scenarios. For instance, a daily consumption of a bivalve meal may be unrealistic. Additionally, the considered average body weight of 60 kg (FAO/WHO, 2003b) and the presumed meal size of 160 g are further assumptions. Finally, the statistical models used also involve assumptions, mainly regarding the application of the EVT (Tressou et al., 2004).

4.4 Conclusions

The applied semi-probabilistic risk assessment approach on the basis of four possible scenarios (one monthly meal; one weekly meal; three weekly meals; one daily meal) showed low relative risk regarding Br and Pb in the selected food groups, including bivalves, which contained the highest Br and Pb contents. For total As, high relative risk values were calculated for seafood, but it should be stressed that this As is overwhelmingly organic, thus presenting low risk. For Cd, substantial relative risk associated with bivalves' and cephalopods' consumption, particularly with one or more weekly meal(s), was calculated. There was a clear progression of Cd relative risk with increasing consumption frequency in the cases of the bivalves and cephalopods. For this reason, further study is warranted.

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Author Contributions:

Marta Ventura participated in the conception and design of the study, carried out the literature review, performed the laboratory analyses of the As, Br, Cd and Pb by ICP-MS, including interpretation of the data and writing the manuscript. Carlos Cardoso participated in the study's design, selected the statistical analysis, and helped to write the manuscript. Narcisa Maria Bandarra conducted a critical review of the manuscript. Inês Delgado, Inês Coelho, Sandra Gueifão and Mariana Ribeiro participated in the laboratory analyses of the As, Br, Cd and Pb by ICP-MS. Maria Helena Costa participated in the conception and design of the study and conducted a critical review of the manuscript. Isabel Castanheira participated in the conception and design of the study and performed a critical review of the manuscript. All authors read and approved the final manuscript.

5

BIOACCESSIBILITY AND TOXICITY OF FOOD CONTAMINANTS

MANUSCRIPT 4: Environmental contaminants in seafood: Bioaccessibility and cytotoxic effects in HT 29 cells

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In preparation

Abstract

Seafood, such as fish, shellfish and seaweed, constitute an essential source of essential nutrients. However, they can also be a source of harmful contaminants from the aquatic environment. Metals represent a public health concern, among the innumerable contaminants that can cause adverse effects in seafood due to their persistence and toxicological effects. Ingestion is considered the principal human exposure route of nutrients and xenobiotics absorbed by the gastrointestinal tract, which links the human body and the environment. This study aims to assess the bioaccessibility of chemical contaminants in contaminated seafood by applying the standardised *in vitro* digestion method and evaluating their potential cytotoxicity using the HT-29 (human colon adenocarcinoma) cell line model.

Concerning bioaccessibility values, in the analysed seafood samples, As and Br showed the higher values; on the other hand, Hg exhibited the lowest values.

Regarding the EC_{50} obtained by *in vitro* cytotoxicity tests with HT-29 cells, the results indicate that the bioavailable concentrations of As for some seafood items may induce cytotoxic effects at 72 h of exposure. The EC_{50} values obtained for tAs at 24, 48 and 72 h of exposure were estimated as 23.1, 7.2 and 3.0 mg L^{-1}, respectively.

The present research highlights the need for further studies on the cytotoxicological effects of seafood digestive fractions to improve the regulatory frameworks or review the existing ones and contribute to the risk assessment strategies. Furthermore, chemical speciation analysis of these contaminants and their mixtures' toxicity will clarify the impact of this exposure on human health.

Keywords: Seafood, bioaccessibility, cytotoxicity, intestinal cells, arsenic, bromine, mercury, TDS

5.1 Introduction

Seafood, such as fish, shellfish and seaweed, constitute an important source of essential nutrients for humans. This food group is rich in omega-3 long-chain polyunsaturated fatty acids, vitamins, micronutrients (e.g. iodine and selenium), easily digestible proteins rich in essential amino acids, and a lower caloric density. Although, the production and processing of seafood influence their nutritional value (Tilami & Sampels, 2018). Seafood is part of several healthy dietary patterns, including the Mediterranean diet, which has well-established the nutritional benefits of seafood consumption (Hidalgo-Mora et al., 2020). Seaweeds have been considered a novel food in European countries due to their beneficial effects (Cherry et al., 2019; Tilami & Sampels, 2018). Edible seaweeds as wakame (*Undaria pinnatifida*) contain essential protein, vitamins, and bioactive compounds as antioxidants with potential health-beneficial properties (Cofrades et al., 2008).

However, marine organisms as seafood can also be a source of contaminants from the aquatic environment, such as natural toxins, pathogenic microorganisms, chemical contaminants, or contaminants of emerging concern (EFSA, 2019a; Hellberg et al., 2012); these contaminants can be bioamplified in the trophic chain and, consequently, reach the top consumers such as humans. The predatory fish that occupy the higher trophic levels tend to accumulate more aquatic contaminants, while bivalves, filter organisms, accrue contaminants from the aquatic sediment. Therefore seafood is also considered an indicator of the quality of the marine environment, reflecting the water quality (Maulvault et al., 2015).

Metals and metalloids represent a public health concern among the innumerable contaminants that can cause adverse effects in seafood, due to their toxicological effects and persistence in the environment and the food chain (Næss et al., 2020; Varol & Sünbül, 2019).

The toxicity of metalloids as As, metals, such as mercury (Hg), and environmental contaminants as bromine (Br) depends on their chemical form.. In the case of As, the inorganic forms (arsenite (AsIII) and arsenate (AsV)) present higher toxicity (Fu et al., 2021); regarding Hg is the organic form (methylmercury (MeHg)) that has higher toxicity (Okpala et al., 2018); concerning bromine, the bromate formed in water during ozonolysis in the presence of the bromide ion (Br^-), is toxic and increases the risk of human carcinogenicity (Chen & Liu, 2021).

As and Hg have been documented as neurotoxic and carcinogenic to humans at a low level (Grandjean & Herz, 2015); these contaminants are also in the top ten of the major chemicals of public health concern defined by the Agency for Toxic Substances and Disease Registry of substances (ATSDR) (ATSDR, 2019). Indeed the International Agency for Research on Cancer (IARC) classifies many chemical substances such as As and Hg as carcinogenic and bromate as possibly carcinogenic to humans (IARC, 2021)(IARC, 2020).

Total diet studies (TDS) are recognised by the World Health Organization (WHO) and the European Food Safety Authority (EFSA) as an important Public Health tool for the assessment of dietary exposure to beneficial and harmful substances through food (EFSA/FAO/WHO, 2011). These studies are based on a food consumption data survey and consist of selecting, collecting at the retail level and analysing commonly consumed foods representative of the overall diet. The samples are pooled into representative foods grouped by similarities and aggregated into the twenty FoodEx2 food groups defined by EFSA (EFSA/FAO/WHO, 2011). FoodEx2 is a food classification and description system created by EFSA, aiming to cover the need to describe food in data collections across different domains, e.g. food consumption, chemical contaminants, etc. This system adopted by several European countries is a flexible combination of classifications and descriptions, including different hierarchies for different food safety domains. Also, it includes facets, which are collections of single descriptors from defined points of view applicable to specific food items (EFSA, 2015). The TDS data is important for estimating the dietary exposure by combining the occurrence data with consumption datasets (EFSA/FAO/WHO, 2011). A new methodology was developed between five European countries to establish harmonised national TDS food lists and test the feasibility of the proposed procedures to have comparable food consumption data formats and overcome different dietary exposure data (Dofkova et al., 2016). Seafood constituted more than one-third of the overall TDS samples in the first Portuguese harmonised TDS (Vasco et al., 2021).

Ingestion of food and water is considered the principal human exposure route of nutrients and xenobiotics absorbed by the gastrointestinal tract (GIT) (Garvey, 2019). GIT is the linkage between the environment and the human body, which accomplish the absorption of nutrients and the protection from hazardous substances. Thus, the diet has an essential role in gut function, and the intestinal cells become the first cells exposed to these substances (Verhoeckx et al., 2015). However, the toxic effects of a hazardous substance depend on several parameters: the type of molecule, the food matrix absorption, and the toxicokinetic process (absorption, distribution, metabolism, and excretion) (Fernández-García et al., 2009).

The amounts of the substances present in food may differ from the level available to exert their biological functions in the human body. Bioavailability is considered the fraction of the compound ingested and available for use in physiologic functions, stored or to exert its toxic action. This complex concept includes two processes; bioaccessibility and bioactivity. Bioaccessibility is the fraction of an ingested bio compound released from its food matrix in the gastrointestinal tract. It thus becomes available for absorption through the GIT. Bioactivity occur after a compound reaches the systemic circulation; the transport to target tissues, interaction with biomolecules, metabolism in these tissues, and all the physiological events it generates (Verhoeckx et al., 2015).

Different *in vitro* digestion methods are commonly used to simulate the human digestion process. These methodologies have as the principal advantages the quickness of the results, efficiency, lower costs, allowing a high number of samples and avoiding ethical issues compared to human nutritional studies. On the other hand, the principal disadvantage is the difficulty of comparing the results between different methods, mainly due to the differences in enzymes, pH, salt concentrations, digestion time, among other factors. A standardised static *in vitro* digestion model for food was developed by the COST action INFOGEST to get over this difficulty; this consensus aimed to consolidate the conditions for simulating the digestion of food (Brodkorb et al., 2019). The harmonised *in vitro* digestion (IVD) method takes into account the conditions of in *vivo* digestion. It is divided into three main steps: oral, gastric, and intestinal phases (Brodkorb et al., 2019).

Once the food components are released from the food matrix, the intestinal cells are the first biological barrier. Different cell lines are usually used to evaluate the cytotoxic effects of bioaccessible substances in the GIT. The human colon adenocarcinoma cell line HT29 is an *in vitro* model of the small intestine used in food digestion and bioavailability studies due to their capacity to express the characteristics of mature intestinal cells (Ponce de León-Rodríguez et al., 2018).

The present study aims 1) to assess the bioaccessibility of chemical contaminants present in contaminated seafood applying the standardised IVD method, and 2) to evaluate the potential contaminants cytotoxicity using the HT-29 (human colon adenocarcinoma) cell line model.

5.2 Materials and Methods

5.2.1 Sampling

The sampling strategy of the present study was established according to the TDS study for the Portuguese food consumption survey collected in the Portuguese market (Vasco et al., 2021). Based on the results obtained from the TDS study (Ventura et al., 2020) in the seafood group, five seafood samples were selected for the present study: cod Atlantic fresh, swordfish, European sardine, fresh tuna, bivalves (clams and wedge clams). The chemical analyses revealed that these seafood items presented the highest As, Br, and Hg levels in the seafood group. Seaweed wakame (*Undaria pinnatifida*) was also included.

Briefly, the analysed samples, n=61, were collected in selected supermarkets representing the major food chains in Portugal and cooked according to a Portuguese food consumption survey integrated into a Portuguese TDS pilot study. The seafood samples were analysed in five pools of twelve subsamples each, except edible seaweed wakame (*Undaria pinnatifida*), analysed as a single sample and subjected only to hydration before analysis.

5.2.2 *In vitro* bioaccessibility assay

The bioaccessibility assay was performed on food items (cod Atlantic fresh, swordfish, European sardine, fresh tuna, bivalves (clams and wedge clams), and seaweed wakame (*Undaria pinnatifida*)) consumed by the Portuguese population, and taking into account their content in hazard contaminants (As, Br, and Hg) to human health, i.e. considerable levels of contamination. To perform bioaccessibility was considered 1g of each sample. The procedure followed the harmonised static IVD model developed under INFOGEST COST Action and recently revised by Brodkorb et al. (2019) (Brodkorb et al., 2019). This model resulted from an international consensus concerning several aspects such as fluid composition, enzymatic activities and sample and fluid amounts. Briefly, IVD method consists of three sequential phases: oral phase, considering the simulated saliva fluid with amylase - 75 U/mL at the pH 7; gastric phase, considering the simulated gastric fluid with pepsin - 2000 U/mL at the pH 3; and intestinal phase, considering the simulated intestinal fluid containing a pancreatin-bile mixture - 100 U/mL and 10 mM, respectively, at the pH 7. A mechanical shaker was used to incubate the tubes at 37 ˚ C for two minutes (oral phase) and 120 minutes (gastric and intestinal phases). After intestinal phase incubation, the reaction was stopped using 1 mM of Pefabloc® (Sigma-Aldrich, St. Louis, MO, USA).

Additionally, digests were immediately immersed in liquid nitrogen, and after that, samples were kept at -80 °C until further determinations. All experiments were conducted in triplicate. A reagent blank was performed in every batch of samples, which was considered for all the bioaccessibility calculations. Bioaccessibility values were determined according to equation 1.

$$Bioaccessibility\ (\%) =$$

$$\frac{[Contaminants]\ in\ the\ digests}{}\,[Contaminants]\ in\ the\ undigested\ sample\text{x}100\ \text{(eq. 1)}$$

5.2.3 Chemical analyses of seafood digests

Determination of tAs content

The seafood samples digested previously, and the blank tests were diluted with HNO_3 2% (v/v) before analysing tAs by ICP-MS (ICP-MS ThermoX Series II, Thermo Fisher Scientific, Bremen, Germany). The nitric acid (HNO_3) (65%) was distilled to ultrapure grade in an acid distillation system (Milestone SubPUR). Calibration curve and quality control solutions of tAs were prepared with high purity ICP-MS stock standard solutions containing 1000 mg L^{-1} of tAs (Merck). As internal standards were used, standard solutions of germanium (Ge) containing 1000 mg L^{-1} (inorganics ventures), yttrium (Y) and indium (In) containing 1000 mg L^{-1} (Merck)

Determination of tBr content

The seafood samples digested previously, and blank tests were diluted with tetramethylammonium hydroxide (TMAH) 0.5 (v/v) (25%, Fluka, St. Gallen, Switzerland) before tBr ICP-MS analysis (ICP-MS ThermoX Series II, Thermo Fisher Scientific, Bremen, Germany). The Br calibration curve and quality control solutions were prepared from standard solutions with high purity ICP-MS stock standard solutions of Br containing 1000 mg L^{-1} (Inorganic Ventures). As internal standards were used, standard solutions of tellurium (Te) and rhodium (Rh) containing 1000 mg L^{-1} (Merck) and 10 mg L^{-1} (Merck), respectively.

Determination of tHg content

The total mercury (tHg) content was determined by atomic absorption spectrophotometry with thermal decomposition and amalgamation (TDA/AAS) using the Direct Mercury Analyzer DMA-80 with an automatic sampler, coupled to a terminal with easyDOC 2 software version 3.03 (Milestone Srl, Sorisole, BG). Briefly, the samples (0.04 - 0.3 g) were placed in nickel boats in the automatic sampler and then entered into the combustion tube. The samples were dried, thermally, and chemically decomposed within the decomposition furnace 650 ºC. The mercury is selectively trapped in a gold amalgamator, carried by flowing oxygen through absorbance cells, and quantified by atomic absorption spectrophotometry at 253.7 nm (EPA, 2007). The method was already accredited by NP ISO / IEC 17025 for fish.

Quality assurance

A rigorous quality assurance program following the NP EN ISO/IEC 17025 standard requirement was implemented to analyse total As, Br and Hg. Analytical procedures were carried out under rigorous metrological control and expressed by the average of three replicates. To control possible contaminations, one reagent blank was performed in each acid digestion and alkaline extraction. All standards and reagents were of high purity (over 99.5%). To quantify elements by ICP-MS (As and Br), every day is made a new calibration curve with no less than five standards in different concentrations. Recoveries between 87% and 108%; 103% and 113% were obtained for As and Br, respectively. The repeatability was consistently below 10%. A Quality Control (QC) sample was included in each group of twelve test samples to validate the test run. Instrumental drift was monitored by the difference between the first and last QC samples (<5%).

Regarding quality control in total mercury quantification, samples were analysed at least in duplicate and in each assay, blanks and quality control samples were analysed to guarantee the quality of the results.

Limits of Quantification (LOQ) were estimated as equal to ten times the standard deviation of the mean blank tests after correction by the sample weight and the appropriate dilution. The LOQ ranged in the following intervals: As (0.02 mg kg^{-1}); Br (0.2 mg kg^{-1}); Hg (0.01 mg kg^{-1}).

5.2.4 Cell viability assay

The cell viability assessment was performed using the HT-29 cell line as *in vitro* model; these cells were exposed to the same chemicals analysed in the bioaccessibility assay.

The cell viability was assessed by the 3-[4,5dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide (MTT) assay as described by (Branco et al., 2021). The principle of this assay is that in living cells, mitochondrial dehydrogenases convert MTT into formazan crystals. The absorbance at 550 nm is an indicator of living cells. Briefly, cells (5 × 10^3 cells/well) were cultured in 96-well plates and allowed to attach for 24 h before adding contaminants. To assess toxicity of the studied contaminants, were added the following concentrations: As (0.05; 0.1; 0.2; 0.39; 0.78; 1.56; 3.13; 6.25; 12.5; 25; 50; 100; 200 and 400 mg kg^{-1}), Br (1; 5; 10; 25; 50; 100; 200; 400; 600; 800; 1000; 2000; 4000; 6000; 8000; 10000 mg kg^{-1}) and Hg (0.1; 0.25; 0.5; 1; 2; 5; 10; 15; 20; 30 mg kg^{-1}). After 24, 48 and 72 h of exposure, 50 µl of MTT (400 µg/ml per well) was added to each well followed by incubation at 37 ºC for 2 h. After that, supernatants were discarded, and the formazan crystals dissolved with 4:1 dimethyl sulfoxide/glycine buffer (pH 10.5). The 96-well plate was placed on a shaker platform, kept in the dark to avoid light, and shaken lightly for 20 minutes to dissolve formazan crystals. The absorbance was measured at 570 nm on a microplate reader, and the EC_{50} was calculated as the compound concentration causing a 50% decrease in MTT reduction relative to the control group.

5.3 Results and discussion

Table 5.1 presents the tAs, tBr, and tHg concentrations in the seafood samples as mean ± S.D. of three independent replicates (n=3) and the correspondent bioaccessibility (%) after *in vitro* digestion of seafood items.

5.3.1 Levels of contaminants in seafood

The amounts of As and Br in seafood samples except for seaweed were published in the literature by Ventura et al. (2020) (Ventura et al., 2020).

Seaweed presented the highest levels of tAs (5.54 ± 0.03 mg kg^{-1}), and tBr (82 ± 4 mg kg^{-1}) and the lowest amounts of tHg (0.013 ± 0.003 mg kg^{-1}) in the analysed seafood samples.

The results of tAs, tBr and tHg in seaweed are usually reported in the literature in dry weight; however, the seaweed was analysed as wet weight in the present study. The comparison between the results in the present study and the literature demonstrates higher results for tAs and tHg in this study than the literature (EFSA, 2019a; Salomone & Riera, 2020; Taylor et al., 2017), and lower tBr levels, in this study, compared to published results (Romarís-Hortas et al., 2012); these comparisons took into account average moisture values of about 80% for seaweed (Madden et al., 2012).

Relatively to the highest value of tHg, it was presented in swordfish (0.57 ± 0.01 mg kg^{-1}); this result is in line with similar studies published in the literature by Millour et al. (2011) (Millour et al., 2011). Hg has been reported in different fish samples, and it is important to mention that items such as tuna, swordfish, and cod are the main contributors to methylmercury dietary exposure in the adult age groups (EFSA, 2012a); in this work, these items presented the occurrence of higher levels of total mercury. Swordfish is considered an aquatic reference fish to evaluate Hg contamination in the marine environment (Okpala et al., 2018). The maximum levels defined by EC for tHg in fish species such as cod, tuna and swordfish is 1.0 mg kg $^{-1}$, the levels of tHg in these fish species were lower than legislation (European Commission, 2006). The levels of tHg in the recommendation (EU) 2018/464 of 19 March 2018 for seaweed is 0.01 mg kg $^{-1}$; in the present study, the levels of tHg in this seafood sample was 0.013 ± 0.003 mg kg^{-1}.

5.3.2 Seafood bioaccessibility

Bioaccessibility was expressed as the percentage of the element content extracted from the food matrix at the end of the IVD assay.

The higher bioaccessibility values were observed for tAs (46-127%) and tBr (64-333%); on the other hand, the lowest bioaccessibility values were exhibited for tHg (5-24%); these results are demonstrated in Table 5.1. The tAs bioaccessibility was around 100% in almost analysed samples and higher than 100% for tBr, underlining the fresh tuna that presented 333% of tBr bioaccessibility. The seaweed bioaccessibility for tAs (46%) and tBr (64%) exhibited less than half of than other analysed seafood; similar results were reported in the literature for seaweed bioaccessibility (Afonso et al., 2021). The values of tAs bioaccessibility observed in the seafood samples agree with other studies reported in the literature (Fu et al., 2021; Moreda-Piñeiro et al., 2012). Regarding Br bioaccessibility in seafood, it should be noted that published results are scarce. It was not possible to compare the results of this study to those from the literature; however, it was reported by Alves et al. (2017) a study about bioaccessibility of contaminants of emerging concern, including BFRs in seafood, in which the bioaccessibility values ranged between 13.80-89.63% for the analysed BFRs compounds (PBDEs (BDE47, BDE100) and α-HBCD) (Alves et al., 2017). The high values of tBr bioaccessibility may be explained by the carbon levels and the organic matter present in the seafood digests.

Considering tHg, this contaminant presented the lowest bioaccessibility values (below 25%) in the analysed samples, as can be observed in Table 5.1, including for the swordfish, which presented the highest level of tHg. The tHg occurrence levels observed in swordfish are in the same range as those published in the literature (Okpala et al., 2018; Olmedo et al., 2013); however, the tHg bioaccessibility in these samples is lower than other results reported in the literature (Jadán-Piedra et al., 2017). The effect of cooking could explain the lower tHg bioaccessibility results. The cooking process can explain the low bioaccessibility values of Hg once the different forms of Hg bind to cysteine groups of proteins, which can be denatured during the culinary process, contributing to the loss of Hg (Mieiro et al., 2016; Torres-Escribano et al., 2011).

Table 5.1. Bioaccessibility (%) and occurrence (mean ± SD) of As, Br and Hg in samples (wet weight, n=3).

Samples	As		Br		Hg	
	x ± s (mg kg^{-1})	Bioaccessibility (%)	x ± s (mg kg^{-1})	Bioaccessibility (%)	x ± s (mg kg^{-1})	Bioaccessibility (%)
Cod Atlantic fresh	3.54 ± 0.06	114	16.6 ± 0.3	175	0.098 ± 0.003	8
Swordfish	1.27 ± 0.01	105	8.16 ± 0.03	239	0.57 ± 0.01	5
European Sardine	2.69 ± 0.06	91	16.8 ± 0.4	165	0.036 ± 0.001	14
Fresh tuna	0.81 ± 0.02	127	6.4 ± 0.1	333	0.24 ± 0.04	24
Bivalves	3.7 ± 0.2	95	40 ± 1	123	0.024 ± 0.001	8
Seaweed	5.54 ± 0.03	46	82 ± 4	64	0.013 ± 0.003	16

5.3.3 Cell viability and cytotoxicity

The HT-29 cells cell viability using the MTT assay was carried out to evaluate adverse effects after treatment with As, Br and Hg. The effective concentration at 50% (EC_{50}) for each contaminant and the respective exposure time was estimated, as can be observed in Figure 5.1. As expected, the cell viability decreased with increasing chemical concentration and time of exposure.

The EC_{50} obtained for HT-29 cells after tAs treatment for 24, 48 and 72 h of exposure were estimated as 23.1, 7.2 and 3.0 mg L^{-1}, respectively (Figure 5.1a). The EC_{50} of HT-29 cells after tBr treatment for 24, 48 and 72 h of exposure were estimated as 2009, 693 and 476 mg L^{-1}, respectively (Figure 5.1b). The EC_{50} of HT-29 cells after tHg treatment for 24, 48 and 72 h of exposure were estimated as 7.2, 4.6 and 5.6 mg L^{-1}, respectively (Figure 5.1c).

Hg, and As induce hepatotoxicity mainly through oxidative stress mechanisms in liver cells, producing reactive oxygen species (Renu et al., 2021). Cytotoxicity data in human cells for As and Hg is scarce; however, studies of metalloids and metals in hepatic cells were reported. There was reported in the literature by Cordier et al., 2021 a study of cytotoxicity in hepatic cells (HepG2) exposed to As and Hg that demonstrated IC50 of 6.71 mg L-1 for As and 26.23 mg L-1 for Hg at 24 h (Cordier et al., 2021), in the case of As was lower, whereas, in the case of Hg was higher than the present study. The EC_{50} observed in tHT-29 after treatment with Br was lower than other studies reported in the literature (Wexler, 2014; Winid, 2015).

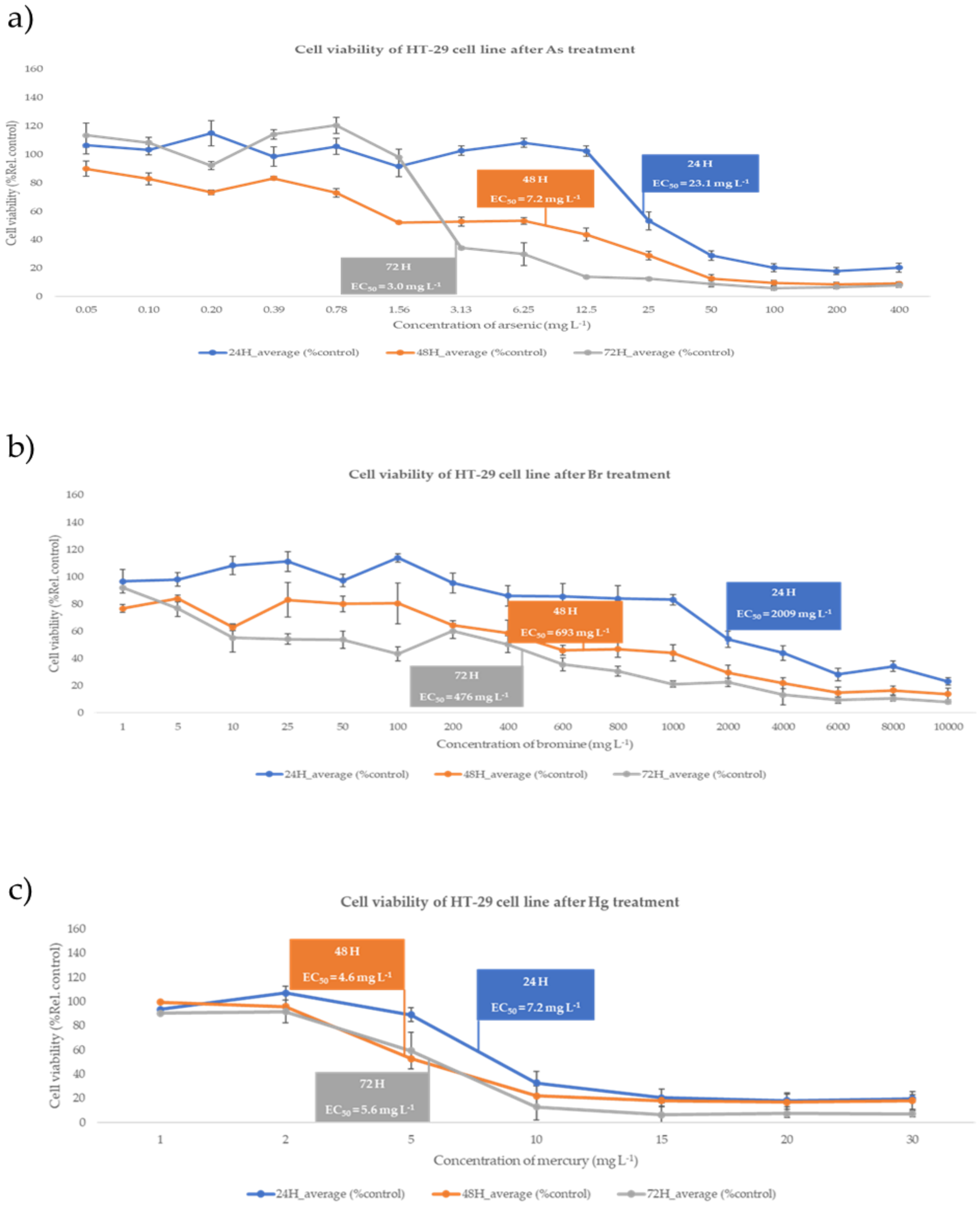

Figure 5.1. Cell viability of HT-29 cells based upon the MTT assay, EC_{50} for each contaminant and the respective exposure time. a) Effect of As treatment after 24, 48 and 72 h of exposure. b) Effect of Br treatment after 24, 48 and 72 h of exposure. c) Effect of Hg treatment after 24, 48 and 72 h of exposure. Each value represents three independent experiments (n=3).

Comparing the concentrations of tAs, tBr and tHg obtained in bioaccessible fractions after *in vitro* digestion for each seafood sample, with the EC_{50} obtained for these compounds taking HT-29 cells as a model (Table 5.2), we can observe that for some cases, the concentrations were above EC_{50}. The tAs in cod Atlantic fresh and bivalves samples presented higher levels than EC_{50} after 72h of exposure time. These results indicate that seafood digestive fractions can contain As concentrations bioaccessible to cause cytotoxic effects in HT-29 cells.

However, it is relevant to consider that the toxicity of As depends on its chemical form. The majority of As present in marine organisms, including seafood, are organic forms with low toxicity (Fu et al., 2021).

In the case of Br and Hg, the results obtained in bioaccessible fractions for all seafood samples are considerably lower than EC_{50} at 24, 48 or 72h of HT-29 cells exposure. These results suggest that these seafood samples' consumption will not increase the Br and Hg exposure.

Table 5.2. The concentration of As, Br and Hg in bioaccessible fraction after in *vitro* digestion (mean ± SD) for each sample and their EC_{50} values after different exposure times in HT-29 cells. All the results are expressed in mg kg^{-1}.

Samples	tAs				tBr				tHg			
	Bioaccessible fraction	EC_{50} 24h	EC_{50} 48h	EC_{50} 72h	Bioaccessible fraction	EC_{50} 24h	EC_{50} 48h	EC_{50} 72h	Bioaccessible fraction	EC_{50} 24h	EC_{50} 48h	EC_{50} 72h
Cod Atlantic fresh	4.0 ± 0.1	23.1	7.2	3.0	29.2 ± 0.9	2009	693	476	0.008 ± 0.001	7.2	4.6	5.6
Swordfish	1.3 ± 0.1				19.5 ± 0.3				0.027 ± 0.001			
European Sardine	2.45 ± 0.05				27.8 ± 0.7				0.005 ± 0.001			
Fresh tuna	1.03 ± 0.02				21.4 ± 0.3				0.06 ± 0.02			
Bivalves	3.5 ± 0.1				49.4 ± 0.8				0.002 ± 0.001			
Seaweed	2.6 ± 0.1				52.3 ± 0.3				0.01 ± 0.01			

5.4 Conclusions

This study assessed the bioaccessibility of As, Br and Hg present in contaminated seafood and their cytotoxicity potential. The total amount of As and Br bioaccessibility was higher than Hg bioaccessibility, and higher levels of these contaminants are expected to be bioaccessible after digestion. The low bioaccessibility of Hg can be explained by cooking processes.

The culture of the HT-29 cell line is an important model for investigating transport over intestinal epithelium and evaluating the toxicity of contaminants through diet. This study demonstrates that As can induce cytotoxicity in HT-29 cells and are dose and time-dependent.

The knowledge gap concerning toxicity and bioaccessibility of Br effects creates a lack of regulation on foodstuffs as seafood. This study was conducted under representative conditions and in compliance with the recommendations of international organisations; it can be used to understand better the toxicity of these contaminants and support policy makers' decisions. Moreover, this study is an important contribution to the risk assessment of these contaminants. Further studies on the toxicology of these contaminants will be required to clarify the regulatory authorities about their relevance and to elaborate adequate legislation or review the existing one.

Furthermore, the chemical speciation analysis of these contaminants and their mixtures' analysis will complement their toxicity and bioaccessibility in human health.

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Author Contributions:

Marta Ventura participated in the conception and design of the study, performed the literature review, carried out the *in vitro* bioaccessibility analyses, the chemical analyses of seafood samples and digests for As and Br, including interpretation of the data and writing the manuscript. Ricardo Assunção participated in the study's design, conducted the *in vitro* bioaccessibility analyses and participated in the writing of the manuscript. Inês Coelho participated in the laboratory analyses of the chemical analyses of seafood digests. Beatriz Matos and Joana Antunes participated in the cell viability assay. Susana Santiago performed the mercury analyses of the seafood samples and collaborated in writing the manuscript concerning these analyses. M. Helena Costa conducted a critical review of the manuscript. Isabel Castanheira participated in the conception and design of the study and performed a critical review of the manuscript. Marta Martins participated in the conception and design of the study, performed the mercury analyses of the seafood digests, participated in the *in vitro* bioaccessibility analyses and performed a critical review of the manuscript. All authors read and approved the final manuscript.

6

GENERAL DISCUSSION

The assessment of population dietary exposure to toxic chemicals in food consumed through the real diet is considered one of the main drivers of environmental impact. It is also essential to complement other areas that deal with the risk benefits of food, such as nutrition or food insecurity (EFSA/FAO/WHO, 2011). Therefore, procedures to estimate the contaminant levels are harmonised since the evaluation of dietary exposure is critical for the interpretation of data. Analytical results can contain putative artificial variations in which potential sources of errors need to be identified (Castanheira et al., 2016; Pereira et al., 2008; Pité et al., 2018).

This thesis addresses the procedures from sampling plan to biological methods in a feasible framework. Rigorous quality assurance procedures validated all determined results to guarantee a consistent linkage between the food ecosystem and the biological pathway. Therefore the present thesis features that should assist researchers, industry, and other key stakeholders who wish to have an overview of the availability and comparability of data at the international and national level overtime to reach a common vision on environmental health policies. It contributes to the policymakers warranty on food safety (FAO/WHO, 2021).

Dietary health is interlinked with environmental sustainability, and the transformation of healthy diets from sustainable food systems is fundamental to ensure population health and achieve the UN SDGs (Willett et al., 2019). The SDGs defined by UN 2030 Agenda for Sustainable Development and adopted by all the UN Member States provide a new global model to end poverty, promote prosperity and well-being, protect the environment, and combat climate change. Food security is part of goal 2 (end hunger, achieve food security, improve nutrition, and promote sustainable agriculture) integrated into the SDGs. (United Nations, 2021).

The main objectives of this thesis were achieved by contributing to attaining goals 2 and 3 of the UN SDGs. The work developed in this thesis included the assessment of food contaminants in relevant foodstuffs consumed by the Portuguese population and their potential toxic effects in intestinal cells, as well as the mapping of trace elements from different geographical regions across Portugal through a vegetable widely consumed by the rural population, contributed to food safety is aligned with goal 2 of the SDGs. This thesis is also in line with goal 3 of the UN SDGs, which ensure healthy lives and promote well-being for all ages, considering that good health is essential to sustainable development included in the 2030 Agenda.

This thesis highlighted the importance of providing credible and reliable data to estimate population risk assessment to the chemical contaminants through diet. The data generated during this thesis were reported to International Agencies as EFSA, contributing to the data update to dietary exposure of contaminants and dietary reference values in the case of micronutrients, and may influence new monitoring methodologies. The produced data concerning micronutrients will also integrate the national food composition table.

A significant contribution of this thesis was also the comparability of the data at the National and European levels to influence and clarify the risk managers, assessors, and policymakers in different countries to enable new regulations or update existing legislations.

The present work was also relevant to estimate toxicity effects of contaminants of public health concern in humans.

The impact of this thesis goes beyond the reported results boosting other studies published, highlighting the relevance of knowledge contaminants in the environment and food (P. Figueira et al., 2016; Henriques et al., 2017; Marinho Reis et al., 2016; Mieiro et al., 2016). Therefore the Interdisciplinary and Interconnected collaboration is a sustainable perspective to inspire policymakers for maximizing the relationship between food ecosystems and health and a redefine baseline for setting new scenarios with detailed targets of human health.

Food contaminants are a public health concern well established by International Agencies. Evaluating harmful chemical substances in food is essential for guaranteeing food safety once ingestion is one of the principal routes of exposure to several organic and inorganic contaminants. One of the recognised approaches to evaluate dietary exposure and risk assessment of harmful chemical substances in the population is TDS; these studies are a complementary public health tool, carried out based on national food consumption surveys, providing representative data on contamination of foodstuffs. TDS methodology is representative of the whole diet and measures the amount of each chemical substance of interest ingested, assuring that people are not exposed to unsafe levels of toxic chemicals through food; chemical substances are a priority in the framework of this methodology (EFSA/FAO/WHO, 2011).

According to the food wheel, seafood is one of the essential food groups as a protein source. It is an integral part of a healthy diet, for being a source of energy, vitamins, minerals, essential micronutrients such as selenium and iodine, and long-chain polyunsaturated fatty acids (EFSA, 2014d). However, seafood can also be a source of toxic contaminants for human beings, such as arsenic, cadmium, lead, and mercury (Jinadasa et al., 2021; Næss et al., 2020).

The seafood group showed the highest amounts of As, Br, Cd, and Pb in the present thesis compared to the other analysed samples. However, the results obtained for Cd and Pb in this food group are lower than the maximum level legislated by the European Commission for these two contaminants (European Commission, 2021b, 2021a). Concerning As and Br, there are no legislation values for the seafood, although the comparison with literature in this food group is discussed below (Chapters 2 and 4).

Shellfish and cephalopods exhibited higher levels of As, Br, and Cd than fatty fish and lean fish; in the case of Pb, cephalopods presented lower levels than other seafood groups. These results are evident through the principal component analysis (PCA) for all samples that demonstrated a strong association between the shellfish and cephalopods groups with the elemental contents (Cd and Pb), which can be related to the benthic habitat of these marine organisms. Regarding the levels of As, Cd and Pb, it's well known that several seafood species accumulate these elements in edible tissues; therefore, these results are in line with similar results reported in the literature (Afonso et al., 2013; Anacleto et al., 2009; Ramon et al., 2021) (Chapter 2). Marine organisms are usually accumulators of As, resulting in the contamination of aquatic biota and, consequently, represents a high risk to seafood consumers (Baeyens et al., 2019); due to his low solubility As can be retained in the water-sediment interface; this way As levels in water near sediments (benthic organisms) are higher than surface water (Li et al., 2018; Snelgrove, 2001), these facts can explain the high levels of As in seafood; although, the result of PCA between the proximate composition, the seasonality and the levels of As, Br, Cd and Pb in the fatty fish group (Figure.2.3c, chapter 2), demonstrated a low correlation between As and the inorganic fraction, which can be explained by the presence of mainly organic forms (lower toxicity) in seafood (Juncos et al., 2019).

Regarding Br levels, most seafood samples are in the same range as reported by Sobolev et al. (2020). In particular, the Atlantic cod species (*Gadus morhua*) presented higher amounts of Br (16.6 ± 0.3 mg kg^{-1}) in the current study than those referenced (7.45 mg kg^{-1}) in the literature (Sobolev et al., 2020); as well as overall seafood, exhibited similar results compared to those reported by Fernandes et al. (2016), particularly shellfish in this study presented lower levels (25.9 ± 20.4 mg kg^{-1}) than those reported in the literature (77.1 mg kg^{-1}) (Fernandes et al., 2016).

Bivalves presented higher levels of Br (40.3 ± 1.2 mg kg^{-1}) and Pb (260 ± 26 µg kg^{-1}) as can be observed in Table 2.4 (Chapter 2), and higher levels of Br (87.15 ± 48.73 mg kg^{-1}), Cd (308.10 ± 348.20 µg kg^{-1}) and Pb (192.18 ± 113.74 µg kg^{-1}) demonstrated in Table 4.2. The cephalopods group presented the highest amounts of Cd (311 ± 277 µg kg^{-1}) in chapter 2. These results evidenced that bivalves and cephalopods showed higher Br, Cd, and Pb levels; the results of Pb in seafood are discussed below. Various authors have reported higher contents of Cd in bivalves and cephalopods in the literature compared to other seafood groups (Miedico et al., 2015; Ramon et al., 2021; Storelli, 2008); this finding can be explained since metals accumulate preferentially in the aquatic sedimentary environment, where most marine molluscs species live (França et al., 2005).

The standard deviations are frequently very pronounced across elements and food groups due to grouping different food products in each studied group. They may also be due to the sampling dimension.

Concerning vegetables and meat groups, for As, both groups presented lower levels than those reported by Xue et al. (Xue et al., 2020); on the other hand, they are in line with the results reported in the 2nd TDS French, as well as Cd and Pb in such groups (Millour et al., 2011). The As levels in vegetables are influenced by soil and water pollution and the type of vegetable; once leafy and high surface area vegetables, can accumulate higher amounts of As than other vegetables (Punshon et al., 2017). As demonstrated a significant correlation with protein in other vegetables group, as can be noted in Figure 2.1; this correlation was not in evidence in the literature, although a study was reported that demonstrated a correlation between nitrogen and As in rice (Srivastava et al., 2019). The meat group showed a significant correlation between Br and Pb levels, which is corroborated by the result of PCA for the meat group, as can be observed in Figures 2.1, and 2.3b. There is no evidence of Br and Pb correlation in this food group in the literature. In the case of Br, research studies about total bromine are scarce, especially in other food groups besides the seafood group. However, Rose et al. (2001) reported results from a TDS, which were higher than those observed in this thesis for vegetables, meat, and fruits groups (Rose et al., 2001) (Chapter 2).

In the present work, was applied a semi-probabilistic relative risk assessment approach, which combines the variability of the concentration data of the total As, Br, Cd and Pb in relevant food groups with four possible consumption scenarios (one monthly meal; one weekly meal; three weekly meals; one daily meal), the relative risk was quantified based on the probability in exceeding the threshold values; considering for total As, a PTWI of 15 μg kg^{-1} bw (FAO/WHO, 1989), for Br an ADI of up to 1.0 mg kg^{-1} bw (FAO/WHO, 1988), for Cd, the TWI of 2.5 μg kg^{-1} bw (EFSA, 2011), and Pb the PTWI of 25 μg kg^{-1} in (FAO/WHO, 2010).

Accordingly, a relative risk value enabled a comparison between food groups and consumption scenarios for each element; these results can be observed in Table 4.4. In the case of total As, the highest relative risk was found in the seafood group; for a low consumption frequency of a monthly meal of lean fish, fatty fish or bivalves, the relative risk was not so high. However, for a moderate consumption scenario (1 monthly meal) of cephalopods, a relative risk of 21% was estimated; the total As levels corroborate these results. The interpretation of these results should consider that the toxicity of As depends on its chemical form. It has been reported that the principal forms of As present in marine organisms are the organic forms that are relatively non-toxic (ATSDR, 2007); the PCA result in fatty fish (Figure 2.3c) discussed above is in line with this scientific evidence.

The relative risk estimated for Br was low for all the food groups analysed, even for the bivalves in which Br presented the highest contents, compared to other analysed groups (Table 4.2), with a scenario of a daily meal (0.061%) for bivalves group. Regarding Cd, the relative risk was higher in bivalves and cephalopods groups, which is in evidence the increasing of consumption frequency with relative risk, for bivalves in a scenario of a daily meal, the relative risk estimate was 78%, and for cephalopods, in the same scenario, the relative risk was 6,2%. The concern of high levels of Cd in bivalves has been studied by different authors and reported in the literature (Li & Gao, 2014; Shi et al., 2016). Concerning Pb, the relative risk was lower for all food groups analysed, never going beyond 0.0049% in bivalves within a daily meal scenario. Other studies reported in the literature demonstrated that Pb is not a health concern in fish and other seafood, except in polluted areas (Afonso et al., 2013; Cirillo et al., 2010) (Chapter 4). Except for Cd in the bivalves group, the relative risk estimated for the other contaminants studied does not exceed the threshold values.

Seasonality has been documented as an important factor in dietary exposure to chemical contaminants; once seasons can influence the levels of these contaminants on foodstuffs, increasing their levels in the biological fluids of human beings (Levin et al., 2020). Seasonality is also considered an essential issue of TDS methodology. The exposure contents are achieved from the result of the consumption data and concentration data in corresponding foods; thereby, the effect of seasonality should be taken into account (Elegbede et al., 2017).

The seasonal variations of toxic metals contents in fish have been reported in the literature. Some authors mention the influence of rainfall, explaining the increased levels of Pb in seasons with high levels of rainfall (Fallah et al., 2011; Mwashote, 2003; Zagh & Bachari, 2019). In Chapter 2 was studied the effect of seasonality on the levels of contaminants in essential food groups of the Portuguese population. Although no correlations were detected between seasonality and the food groups individually, the seafood group (lean fish, fatty fish, shellfish and cephalopods), analysed together instead separately in 4 groups, demonstrated a progressive increase of Pb through seasons, summer < spring < autumn < winter; this fact is also in evidence in Figure 2.3c, where can be observed the proximity between winter and Pb in the PCA plot generated in the fatty fish group and seasonality.

The correlation of the Pb increase in fish and seasons after the rainy season, found in Chapter 2 according to the results of Pb in Portuguese cabbage after forest fires in Chapter 3, where the highest amount of Pb was detected in 3rd campaign (collected in April) after rainfall, as can be observed in Table 3.5. Portugal is an European country characterised by a rainy winter and sometimes spring. Although this is the norm for this country; December of 2017 (2nd campaign of Portuguese cabbage collection) was an exception for being a very dry month, with low rainfall, while the spring of 2018, particularly April of 2018 (3rd campaign of Portuguese cabbage collection) was quite rainy, with higher levels of rainfall (IPMA, 2017a, 2017b, 2017c, 2018a, 2018b).

The increase of Pb after the forest fires over the three campaigns can also be influenced by rainfall. Figure 6.1 demonstrates the relation between rainfall and Pb levels in Portuguese cabbage after forest fires throughout the three campaigns. Metals, like Pb, can move into the subsoil under rainfall leaching conditions; the literature corroborates this fact (Cao et al., 2013).

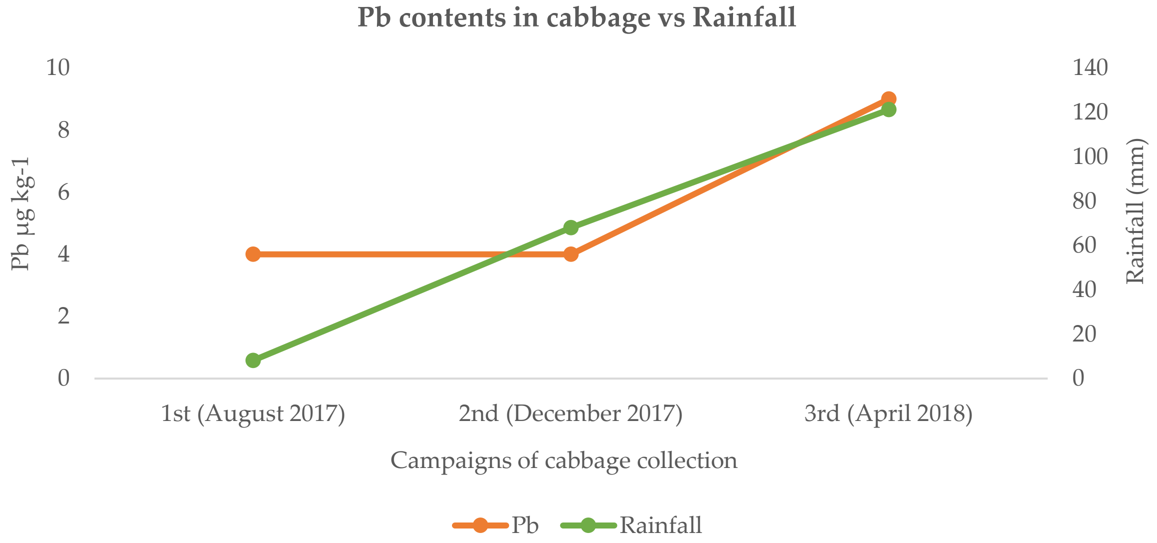

Figure 6.1. The relation of Pb contents in Portuguese cabbage after forest fires and rainfall.

The Portuguese cabbage is a cruciferous vegetable integrated on a healthy diet that has been classified as a superfood due to the antioxidant benefits, the levels in phenols and glucosinolates, which are associated with beneficial effects in cardiovascular diseases, and the protective effect in several cancers (Batista et al., 2011; Šamec et al., 2018), some studies demonstrated a relationship between the consumption of brassicas and the NCD decrease (Sanlier & Guler Saban, 2018; Veeranki et al., 2015). However, this vegetable can accumulate contaminants through the environment or soil, as ingestion is considered one of the principal exposure pathways to several contaminants; this way, human beings can be exposed (Gall et al., 2015). The components of this vegetable depend on several factors, among which processing and cooking methods since it will influence the levels of nutrients and chemical contaminants such as As (Martínez et al., 2020; Perello et al., 2008).

Portuguese cabbage was analysed after the cooking process, and the results obtained for As were (0.013 ± 0.001 mg kg^{-1}) (Chapter 2); on the other hand, all samples of Portuguese cabbage from different geographic regions of Portugal were analysed uncooked, and the results for As ranged from 0.0033 - 0.055 mg kg^{-1} (Chapter 3). Several studies demonstrate that cooking methods can influence the levels and chemical forms of As (Cheyns et al., 2017; Mwale et al., 2018); some authors reported a range of 0.019–2.334 mg kg^{-1} in cooked vegetables, although, in this study, the vegetable group does not only consist of cabbage (Upadhyay et al., 2019).

The Correlation Analysis as the first approach applied in the baseline samples of Portuguese cabbage, collected in all the studied geographic regions of Portugal (thirty-three regions) before forest fires revealed a positive correlation between Se and inorganic contaminants, which the heterogeneity of the soils can explain (Ventura et al., 2009); a negative correlation between Se and Zn; these two essential elements for humans, sometimes compete for the same binding site, similar results have been reported in the literature (Xu et al., 2019). Their availability in the soil depends on the mineral content and soil physicochemical properties, as soil pH, which in the case of Se, increases with the pH rise. At the same time, Zn decreases with the pH rise (Xu et al., 2019). A positive correlation between inorganic contaminants was also evidenced, suggesting no competition in the metals translocation from roots to leaves. On the other hand, it could increase the risk for the consumers of leafy vegetables (Gupta et al., 2019) (Chapter 3).

The FA results corresponded to the PCA illustrated in Figure 3.3a (PC1 with As, Br, Se and Sr, PC2 with Zn and Cd) and Figure 3.3c (PC3 composed by Pb, and PC4 constituted by Co and Cr). The obtained PCA agrees with the four *clusters* performed through HCA between the thirty-three localities and trace elements whereby evidenced in Figure 3.4a (trace elements).

In the *dendrogram* illustrated by Figure 3.4b (localities), can be observed the following *clusters*; highest levels of Cr and Pb mark the first *cluster*, these contents are in line with pollution sources reported by the Portuguese Agency of the environment in 2015 (the same year of sampling the samples included in this study) (Silva et al., 2015), higher levels of As characterise the second *cluster* and Br mainly in North of Portugal; increased amounts of As in groundwater and soils were reported in these regions (Farinha et al., 2009; R. Figueira et al., 2007), the third *cluster* presented higher levels of Se in South and Centre of Portugal, similar results have been reported in the literature (Ventura et al., 2009) and the fourth *cluster* was marked by the highest levels of Zn and Cd, where no specific region of the country was identified (Chapter 3).

Wildfires are increasing worldwide due to climate changes and global warming due to drier seasons, warmer temperatures, and precipitation decrease. These environmental catastrophes are also considered a source of environmental contaminants for human beings, affecting production, economics, and health of the exposed population to environmental risks such as fire, smoke, and other chemical products released from the wood combustion process in burned areas (Balmes, 2020; Isley & Taylor, 2020). This evidence aligns with goal 3 of the UN SDGs, which ensure healthy lives and promote well-being for all ages, including protecting chemicals from soil. Good health is essential to sustainable development and the 2030 Agenda (United Nations, 2015)

In the present work (Chapter 3), was analysed the occurrence of trace elements in Portuguese cabbage (*Brassica Oleracea* L. var. *costata* DC.) in eight localities from the central region of Portugal before (control group) and after forest fires (during three campaigns) (Table 3.5), once this vegetable is an indicator of soil quality.

The large wildfires occurred in a dry year with low levels of rainfall, as discussed above. The control group and 1st campaign results demonstrated a significant increase of all trace elements except for Cd and Pb; the comparison between 1st and 2nd campaigns showed an increase of As, Cr and Sr; and in the 3rd campaign, the highest levels were for Cr and Pb. Most of the analysed trace elements increased in Portuguese cabbage after forest fires; a possible explanation for this evidence could be the environmental alteration and soil change after wildfires influencing the growth of the vegetables in burned areas (Chapter 3).

Seafood is considered an essential food for humans for being a source of beneficial nutrients for human health, such as vitamins, n-3 polyunsaturated fatty acids, minerals, micro and macronutrients (Hosomi et al., 2012; Venugopal & Gopakumar, 2017). However, this food group is also a target of contaminants from the aquatic environment inducing adverse health effects; the evaluation of risk-benefit is crucial for the consumer's safety (Hellberg et al., 2012)

The seafood group demonstrated high levels of contaminants compared to all the analysed food groups. However, the levels of the substances present in food may be different from that will be available or accessible to exert their biological functions in the human body once the digestive process influences their absorption (Verhoeckx et al., 2015).

To assess the human digestive process in the GIT through bioaccessibility assays is fundamental to estimate risk assessment to contaminants. Different *in vitro* digestion methods simulate the human digestion process, avoiding ethical issues compared to human nutritional studies. However, the harmonised *in vitro* digestion method considers the conditions of *in vivo* digestion (Brodkorb et al., 2019).

The samples for the bioaccessibility assays were selected according to the consumption of the Portuguese population and the high levels of contaminants. Notwithstanding the high levels of Cd and Pb in shellfish and cephalopods groups, these contaminants were not included in bioaccessibility assays since their values in other seafood groups were lower.

Once the food components are released from the food matrix, the intestinal epithelium plays an important role in absorbing beneficial and harmful substances, being the intestinal cells the first biological barrier (Ponce de León-Rodríguez et al., 2018).

The present thesis evaluated the potential toxicological effects of contaminants (As, Br, Hg) and their bioaccessibility *in vitro*. The values of As and Br bioaccessibility were higher than Hg bioaccessibility; these results are corroborated by the other studies reported in the literature for As bioaccessibility (Afonso et al., 2018; Fu et al., 2021; Maulvault et al., 2011), although studies in seafood for Br bioaccessibility are very limited. However, Hg bioaccessibility presented lower values. The cooking process can explain the low percentages of Hg bioaccessibility once the different forms of Hg bind to cysteine groups of proteins, which can be denatured during the culinary process, contributing to the loss of Hg (Mieiro et al., 2016; Torres-Escribano et al., 2011) (Chapter 5).

Overall the cell viability decreased with increasing chemical concentration and time of exposure, taking into account the concentrations in bioaccessible fractions and the EC_{50} calculated using HT-29 cell line. According to As data for bioaccessible fractions and $EC_{50,}$ there should be an alert for consumers of bivalves. These data also contribute to strengthening the risk assessment of bivalves consumption and an effort to reduce the contamination in these seafood samples. In the case of Br and Hg, the results obtained in bioaccessible fractions for all seafood samples are considerably lower than EC_{50} at 24, 48 or 72h of HT-29 cells exposure. These results suggest that these seafood samples' consumption will not increase the Br and Hg exposure (Chapter 5).

The cytotoxic effects of As may occur after digestion of some marine species with the time of exposure (Chapter 5). However, it is relevant that the toxicity of As depends on its chemical form, and the main As forms present in marine organisms, including seafood, are organic forms with low toxicity (EFSA, 2021).

Although the present findings, further studies should be conducted to assess the cytotoxicity on other intestine cell models and consider chemical speciation to increase the accuracy and the toxic mechanisms associated with the human digestion processes and the potential toxicity and risk assessment.

7

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis intended to answer the research questions previously defined:

1. Is the Portuguese population exposed to inorganic contaminants through food?

Regarding the consumption of relevant food groups in the Portuguese population, the seafood group presented the highest As, Br, Cd and Pb levels compared to other analysed food groups (meat, vegetables and fruits). In contrast, the fresh fruits exhibited the lowest levels of these contaminants. A correlation between fat levels in fatty fish and seasons of the year was evidenced, increasing in summer compared to winter. The correlation between Br and Pb and the meat group was relevant. Another relevant correlation was the protein content and the As levels in other vegetables (Chapter 2).

The semi-probabilistic risk assessment approach showed a clear progression of Cd relative risk with increasing consumption frequency in the cases of bivalves and cephalopods. On the other hand, a low relative risk concerning Br and Pb was evident in the analysed food groups (seafood, fruits and vegetables), including bivalves, which contained higher contents of Br and Pb. For total As, high relative risk values were observed in seafood, but it should be regarded that As toxicity depends on chemical form and in seafood is overwhelmingly organic, thus presenting low risk (Chapter 4).

Considering trace elements assessment in a vegetable widely consumed by the Portuguese population, the results obtained across the country in different geographical regions before forest fires do not show any public health concern. Although the data from affected areas by dangerous forest fires in 2017 identified a hazard, especially for As content estimated as inorganic As, and other trace elements as Cd, Br, Cr and Sr, which increased in the two campaigns after the wildfires (Chapter 3).

2. The food contaminants may become bioaccessible in order to cause toxicity to human intestine cells?

The potential toxicological effects of food contaminants were evaluated in the intestinal cell lines, and their bioaccessibility in seafood samples was performed using a standardised *in vitro* digestion model.

The present thesis demonstrated that As and Br bioaccessibility values were higher than Hg bioaccessibility, and higher levels of these contaminants are expected to be bioaccessible after digestion. The present thesis demonstrates that As can induce cytotoxicity in HT-29 cells and are dose and time-dependent. However, it is important to consider that As toxicity depends on its chemical forms; the main As forms present in marine organisms, including shellfish, are organic forms with low toxicity (Chapter 5).

This thesis is aligned with two challenges of SDGs defined in the 2030 Agenda for Sustainable Development by the United Nations, more specifically with goals 2 and 3. Within goal 2, this thesis contributed to food safety by evaluating the impact of inorganic contaminants in relevant food groups consumed by the Portuguese population. Certain analysed contaminants are included in the top ten chemicals of public health concern, defined by International Agencies. Another contribution framed with food security aligned with this SDG was evaluating trace elements in a vegetable widely consumed by the rural population before and after forest fires. The present thesis analysed food contaminants in a vegetable indicator of soil quality and widely consumed by the rural population, aligning with goal 3 by promoting health and well-being, essential for sustainable development.

The results obtained during this work will contribute to the mentioned SDGs and, consequently, sustainable management.

This thesis contributed to increasing the knowledge about chemical contaminants in representative food consumed by the Portuguese population through several approaches; including TDS methodology, which emphasizes food as consumed, the assessment of the relative risk in the consumption of relevant food groups, and a mapping of several trace elements across the different geographical regions of the country throughout a vegetable of widely consumed by the rural population. The present work gave a novel insight into the trace elements occurrence in burned areas after wildfires, taking into account the human health risk of the population surrounding.

The present work was conducted under representative conditions and following international organisations' recommendations and highlights the behaviour of contaminants of public health concern through bioaccessibility assays and cytotoxicity in intestinal cells. It is essential to better understand the toxicity of chemical contaminants and support decisions by policymakers.

The following topics should be addressed in the future perspectives:

Furthermore, it will be essential to perform studies to evaluate the effects of proximate composition on the levels of contaminants and consider increasing seasonal sampling.

It will be important to perform additional studies regarding the relative risk of Cd in bivalves and cephalopods observed in the present thesis.

Further studies on the toxicology of chemical contaminants will be required to clarify the regulatory authorities about their relevance and elaborate adequate legislation or review the existing one. However, it will also be relevant to analyse the mixtures of food contaminants once the exposure through the diet combines several contaminants and not individually.

Further studies should be conducted to assess the cytotoxicity on other intestine cell models, as Caco-2 cells, which are an essential tool for studying properties of the intestinal epithelium. It is also important to consider performing additional studies in individual and mixtures of contaminants through hepatic and renal cells to complement the results of cytotoxicity obtained in intestinal cells.

The chemical form of contaminants such as As, Br and Hg influences their bioaccessibility and bioavailability. It is important to consider chemical speciation to increase the accuracy and the toxic mechanisms associated with the human digestion processes and the potential toxicity and risk assessment.

Considering the evaluation of trace elements across different geographical regions of Portugal after wildfires, it will be relevant to regard the study of trace elements in the respective soils in further studies. As well as perform biomonitoring studies from a public health point of view in the local population in order to evaluate the exposure to the inorganic elements.

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A

AN APPENDIX

A.1 Outputs of Thesis

This thesis originated the following papers:

International publications

Ventura, M., Cardoso, C., Bandarra, N. M., Delgado, I., Coelho, I., Gueifão, S., Martins, M., Costa, M. H., & Castanheira, I. (2020). Effect of season and proximate composition on the Br, As, Cd and Pb contents in different kinds of key foods consumed in Portugal. International Journal of Food Science and Technology, 55 (5), 2219–2231. https://doi.org/10.1111/ijfs.14475. (**Chapter 2**)

Ventura, M., Cavaco, H., Delgado, I., Coelho, I., Gueifão, S., Martins, M., Costa, M. H., Matos, A., & Castanheira, I. (2021). The occurrence of inorganic contaminants in 'tronchuda' cabbage (*Brassica oleracea* L. var. *costata* DC.) after large forest fires in Portugal. International Journal of Environmental Analytical Chemistry, 1–17. https://doi.org/10.1080/03067319.2021.1893709. (**Chapter 3**)

Ventura, M., Cardoso, C., Bandarra, N. M., Delgado, I., Coelho, I., Gueifão, S., Ribeiro, M., Costa, M. H., & Castanheira, I. (2018). Bromine, arsenic, cadmium, and lead in several key food groups: an assessment of relative risk. International Journal of Environmental Analytical Chemistry, 98 (15), 1398–1412. https://doi.org/10.1080/03067319.2018.1559307. (**Chapter 4**)

Marta Ventura, Ricardo Assunção, Inês Coelho, Inês Delgado, Sandra Gueifão, Beatriz Matos, Joana Antunes, Susana Santiago, M. Helena Costa, Isabel Castanheira, & Marta Martins. Environmental contaminants in seafood: Bioaccessibility and cytotoxic effects in HT-29 cells. In preparation. (**Chapter 5**)

National publications

Ventura, M., Ribeiro, M., Ferreira, M., Costa, M. H., & Castanheira, I. (2018). Bromo, um contaminante de preocupação emergente em couve portuguesa. INSA - Boletim Epidemiológico de Observações, 7 (23), 49–52.

A.2 Work presented in scientific meetings

Marta Ventura, Inês Coelho, Sandra Gueifão, Inês Delgado, Helena Costa and Isabel Castanheira. Emerging and persistent inorganic contaminants in prioritized foods: results from Portuguese Total Diet Pilot Study. International Conference on Pollutant Toxic Ions and Molecules, PTIM 2017, 6-9th of November 2017, Costa da Caparica, Portugal. **Oral communication**.

Marta Ventura, Inês Delgado, Inês Coelho, Mariana Ribeiro, Marta Ferreira, Isabel Castanheira. Avaliação dos teores de Bromo em alimentos consumidos pela população portuguesa. Encontros DAN, 25 maio, 2018, Lisboa; Portugal. **Oral communication**.

M. Ventura, I. Delgado, I. Coelho, M. Ribeiro, T. Moreira, M. Costa, I. Castanheira. Bromine occurrence in seafood products consumed in Portugal. Environmental & Food Monitoring Conference ISEAC 40, 19th-22nd of June 2018, Santiago de Compostela, Spain. **Oral communication**.

M. Ventura, I. Coelho, S. Gueifão, I. Delgado, M. Ribeiro, T. Moreira, I. Castanheira. 40th International Conference on Environmental & Food Monitoring, June 19 -22th 2018, Santiago de Compostela, Spain. **Painel communication.**

M. Ventura, I. Coelho, S. Gueifão, I. Delgado, M. Ribeiro, T. Moreira, I. Castanheira. Impact of Forest fires on the Content of Inorganic elements in cabbage. 40th International Conference on Environmental & Food Monitoring, June 19 -22th 2018, Santiago Compostela, Spain. **Painel communication.**

I. Delgado, **M. Ventura**, S. Gueifão, I. Coelho, M. Ribeiro, T. Moreira, I. Castanheira. Inorganic contaminants in foods most consumed by the Portuguese population. 40th International Conference on Environmental & Food Monitoring, June 19 -22th 2018, Santiago Compostela, Spain. **Painel communication.**

Marta Ventura, Inês Delgado, Inês Coelho, Mariana Ribeiro, Marta Ferreira, M. Helena Costa, Isabel Castanheira. Bromine occurrence in foods consumed by Portuguese population

Encontro com Ciência, julho 2018, Lisboa, Portugal. **Painel communication.**

Marta Ventura, Inês Delgado, Inês Coelho, Sandra Gueifão, Ana Sofia Matos, Marta Martins, Maria Helena Costa, Isabel Castanheira. Bromine occurrence in Portuguese cabbage from the center region of Portugal before and after forest fires. International Conference on Pollutant Toxic Ions and Molecules, PTIM 2019, 4 -7th of November 2019, Costa da Caparica, Portugal. **Oral communication**.

Marta Ventura, Carlos Cardoso, Narcisa Maria Bandarra, Inês Delgado, Inês Coelho, Sandra Gueifão, Marta Martins, Maria Helena Costa, Ana Sofia Matos, Isabel Castanheira Characterisation of Bromine, Arsenic, Cadmium and Lead contents in different key food items consumed by Portuguese population. II Conferência NOVAsaúde Nutrition "POLUENTES AMBIENTAIS, ALIMENTOS E IMPACTO NA SAÚDE - LIÇÕES PARA O FUTURO". 20th of January, 2021, online. **Oral communication**.

Marta Ventura, Susana Jesus, Inês Coelho, Sandra Gueifão, Inês Delgado, Isabel Castanheira. Segurança Alimentar. Webinar projeto AHFES - Identificação de aspectos legais no desenvolvimento de um produto. 21st of May, 2021, online. **Oral communication**.

B

ANOTHER APPENDIX

B.1 Bromo, um contaminante de preocupação emergente em couve portuguesa

Bromine, a contaminant of emerging concern in Portuguese cabbage

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Resumo

O bromo é um dos principais halogéneos da tabela periódica e tem vindo a ser classificado como contaminante de preocupação emergente, porque devido à sua bio-magnificação pode constituir um risco para a saúde humana. Os compostos bromados são contaminantes não biodegradáveis que se acumulam nos diferentes níveis tróficos da cadeia alimentar. O objetivo deste trabalho foi avaliar os teores de bromo em amostras de couve portuguesa (*Brassica Oleracea* var. *costata* DC), uma hortícola do género brássica bastante consumida pela população portuguesa. Foram recolhidas cento e dezassete amostras durante o período 2015-2017 em diferentes locais representativos de cada distrito de Portugal, em dois períodos de colheita. O teor de bromo foi determinado por espectrometria de massa com plasma indutivo acoplado (ICP-MS), precedido por uma extração alcalina com Tetrametilhidróxido de amónia (TMAH). Os valores de bromo encontrados na primeira colheita estavam compreendidos entre 0,5 e 14,5 mg/kg e na segunda colheita os valores observados estavam compreendidos entre 1,0 e 7,8 mg/kg. As amostras foram organizadas de acordo com a Nomenclatura de Unidades Territoriais NUTS II para Portugal e constatou-se que as regiões com valores mais elevados de bromo foram a Região do Algarve e a Região Autónoma da Madeira. Em comparação com o LMR (limite máximo de resíduos) para os teores do ião brometo em alimentos da espécie brássica (30 mg/kg) verificou-se que nenhuma das amostras analisadas ultrapassou esse valor estipulado. No entanto, serão necessários estudos adicionais sobre a avaliação da exposição, bem como sobre a toxicologia deste contaminante, para que entidades reguladoras possam avaliar a pertinência da revisão da legislação em vigor.

Abstract

Bromine is one of the principal halogens of the periodic table and has been classified as a contaminant of emerging concern because due to its biomagnification, it may pose a risk to human health. Brominated compounds are non-biodegradable contaminants that accumulate at different trophic levels in the food chain. The main goal of this work was to evaluate the bromine contents in samples of Portuguese cabbage (*Brassica oleracea* var. *costata* DC), a vegetal of the *Brassica* genus that is widely consumed by the Portuguese population. One hundred and seventeen samples were collected between 2015-2017 in different representative locations of each district of Portugal. This was done in two harvest periods. The Bromine contents were analysed by inductively coupled plasma mass spectrometry (ICP-MS), preceded by an alkaline extraction with 25% ammonium tetramethylhydroxide (TMAH). The Bromine amounts found in the first crop were between 0.5 and 14.5 mg/kg and in the second crop between 1.0 and 7.8 mg/kg. The samples were organized according to the Nomenclature of NUTS II Territorial Units for Portugal, and it was found that the regions with the highest bromine contents were the Algarve Region and the Autonomous Region of Madeira. Compared to the MRL (maximum residue limit) for bromide ion contents in foods of the Brassica species (30 mg/kg), none of the analysed samples exceeded this legislated value. However, further studies on the exposure assessment as well as the toxicology of this contaminant will be required so that regulatory authorities can assess the relevance of the review of existing legislation.

Introdução

O Bromo (Br) é o 35º elemento e é um dos principais halogéneos da tabela periódica, à temperatura ambiente forma um líquido vermelho-acastanhado denso que é extremamente tóxico para a saúde humana (1). Este elemento químico tem apenas dois isótopos estáveis na natureza; 79Br (50.57%) e 81Br (49,43%), no entanto no meio ambiente pode encontrar-se em várias formas químicas, mas é sobretudo na sua forma inorgânica, o ião brometo, que integra a maioria dos compostos (2). Encontra-se na crosta terrestre em águas do mar, massas de água em geral e ainda pode ser encontrado em solos, plantas e animais (3).

A presença, e respetivas concentrações, de Br no ambiente são essencialmente atribuídas à libertação antropogénica do elemento, através da utilização de pesticidas, fertilizantes e fumigantes na agricultura, emissões industriais, mineração, uso de desinfetantes e retardadores de chama bromados (*Brominated flame retardants* - BFRs), estes últimos são compostos aplicados na indústria têxtil, equipamentos elétricos, eletrónicos, plásticos, materiais de construção e mobiliário de forma a aumentar a resistência a incêndios, evitando a combustão (4). Estes compostos têm a capacidade de ficar retidos no meio ambiente de forma persistente e duradoura, com posterior efeito bioacumulador na cadeia alimentar, como é o caso de produtos de origem marinha (contaminação através do ambiente aquático) e vegetais (contaminação através dos solos e águas de rega) que serão posteriormente consumidos pela população (5).

A maior classe dos compostos BFRs são os difenil éter polibromados (*Polybrominated diphenyl ethers* - PBDEs) que são considerados contaminantes de preocupação emergente e disruptores endócrinos pois podem influenciar o metabolismo do iodo, ao nível da produção das hormonas tiroideias, no sentido de provocar uma diminuição de iodo na tiroide ou aumentar a excreção renal de iodo (6). A ingestão foi reconhecida como a principal via de exposição a PBDEs no ser humano (7).

A Organização Mundial da Saúde (OMS) recomenda um consumo diário de hortícolas entre 3 e 5 porções diárias. Para além dos benefícios nutricionais as hortícolas de folha, como é o caso das couves (*Brassica oleracea* L.), são alimentos acumuladores de metais pesados e sinalizadores de contaminantes ambientais, como é o caso do bromo. Uma das espécies de brássica mais consumida pela população portuguesa é a couve tronchuda ou, como é mais conhecida, a couve portuguesa (*Brassica oleracea* var. *costata* DC) (8-10). A avaliação do teor de bromo neste tipo de alimentos é de extrema relevância uma vez que é considerado um indicador do teor de compostos bromados no ambiente e na cadeia alimentar. A avaliação do bromo em alimentos tem despertado o interesse da comunidade científica pois continua a existir algum desconhecimento sobre a toxicidade, e o seu comportamento, a nível ambiental e humano.

Objetivo

Este trabalho teve como objetivo a determinação dos teores de bromo em amostras de folha de couve portuguesa representativas de todos os distritos de Portugal, uma hortícola bastante consumida pela população em Portugal, através do método de ICP-MS.

Materiais e métodos

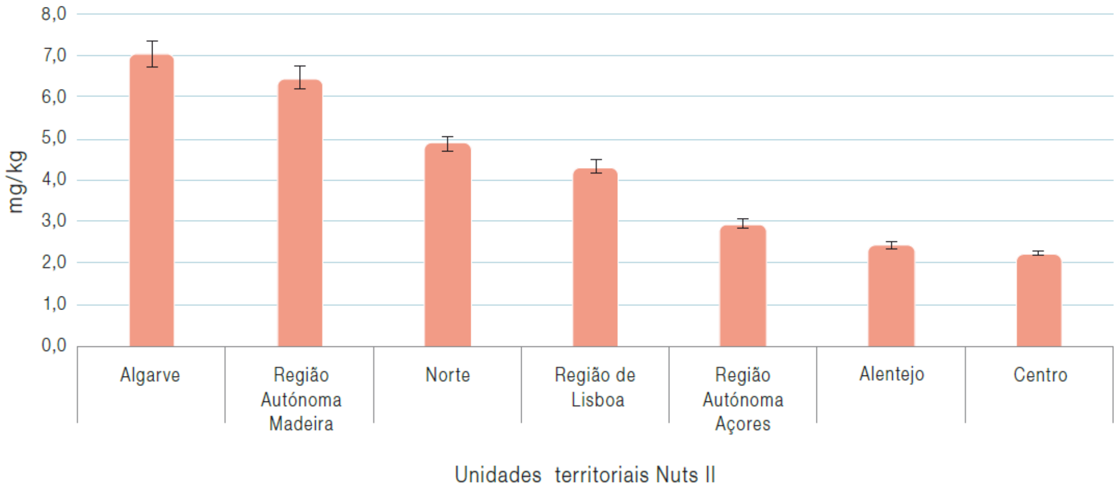
Foram recolhidas um total de cento e dezassete amostras em diferentes locais representativos de cada distrito de Portugal, em dois períodos distintos, no primeiro período (ano 2015) foram recolhidas 99 amostras em todas as regiões de Portugal e no segundo período (ano 2017) foram recolhidas 18 amostras apenas da região centro do país. As amostras foram agrupadas em 39 pools, cada uma constituída por 3 folhas exteriores de 3 couves diferentes. O teor de bromo foi determinado por espectrometria de massa com plasma indutivo acoplado (ICP-MS), precedido por uma extração alcalina com Tetrametilhidróxido de amónia (TMAH) 25%. Os resultados foram obtidos através de procedimentos analíticos que refletiram os requisitos de garantia da qualidade, descritos na norma ISO/IEC 17025:2005(11). A concentração foi expressa, em mg de bromo/kg de couve pela média de três réplicas.

Resultados e discussão

As amostras recolhidas no primeiro período de colheita apresentaram valores de bromo compreendidos entre 0,5 e 14,5 mg/kg. No segundo período de colheita os valores de bromo obtidos para as amostras compreenderam valores entre 1,0 e 7,8 mg/kg. De acordo com o Regulamento (UE) nº 839/2008 (12), os limites máximos de resíduos (LMR) para os teores do ião brometo em brássicas, como é o caso da couve portuguesa, correspondem a 30 mg/Kg. Os resultados obtidos neste trabalho não ultrapassaram este valor legislado. Constatou-se que 59% das amostras analisadas apresentaram valores entre 0 e 2,9 mg de bromo por kg de amostra. As 39 amostras analisadas em forma de pools, foram organizadas de acordo com a Nomenclatura de Unidades Territoriais NUTS II para Portugal presente no Regulamento (UE) n.° 868/2014 (13). Como se pode observar no gráfico 1, a Região do Algarve (7,0 mg/kg) e a Região Autónoma da Madeira (6,5 mg/kg) apresentaram os valores de bromo mais elevados. O Centro é a região onde foi observado o teor mais baixo de bromo (1,8 mg/kg).

Valores semelhantes aos deste estudo são reportados por Yuita e colaboradores, que concluíram que os valores de bromo presentes em solos e em plantas aumentariam com a proximidade da área costeira (14). No gráfico 2 pode-se observar a diferença do teor de bromo entre os dois períodos de recolha nos locais da região centro do país, e constatou-se que em todas as amostras os valores mais elevados foram encontrados no 2º período, exceto no caso das amostras com os números 34 e 39, em que se verificou um decréscimo no 2º período.

Gráfico 1: Avaliação do teor bromo de acordo com a classificação das Unidades Territoriais-NUTS II.



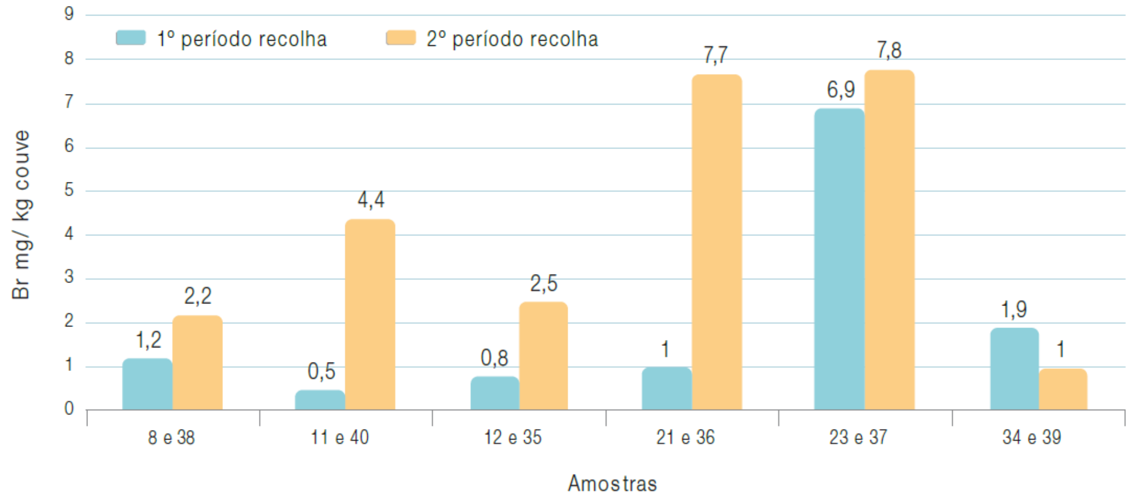


Gráfico 2: ⬇ Comparação dos teores de bromo entre os dois períodos de recolha de couve portuguesa na região centro de Portugal.

Conclusões

Em comparação com os LMR estipulados no Regulamento (UE) nº 839/2008 para os teores do ião brometo em alimentos da espécie brássica (30 mg/kg) verificou-se que nenhuma das amostras analisadas ultrapassou esse valor estipulado, não representando um perigo para o consumidor. Até ao momento, em Portugal, estão publicados poucos dados sobre o bromo em alimentos representativos da dieta nacional. O plano de recolha e de análise das amostras de couve portuguesa seguiu uma abordagem de forma a garantir a representatividade das amostras nacionais, o que permitiu a integração dos dados em bases de dados de âmbito europeu como as da Autoridade Europeia para a Segurança dos Alimentos (EFSA, na siga em inglês). Este trabalho permitiu então constatar que os teores de bromo nas amostras de couve portuguesa representativas do consumo da população, não constituem risco para a saúde humana. No entanto, os resultados aconselham estudos adicionais sobre a avaliação da exposição a este contaminante. Os dados analíticos obtidos quando combinados com os dados de consumo podem ser um ponto de partida fiável que permitirá às entidades reguladoras avaliar a pertinência da revisão da legislação em vigor.

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Human exposure to food contaminants throughout diet: Assessment through TDS, bioaccessibility and cytotoxicity assays *in vitro*

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