Universidade de Aveiro Departamento de Química
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NICOLE VIANA FERREIRA

VALIDATION OF A METHODOLOGY TO QUANTIFY MACRO, MICRO, AND POTENTIALLY TOXIC ELEMENTS IN FOOD MATRICES

VALIDAÇÃO DE UMA METODOLOGIA PARA QUANTIFICAÇÃO DE ELEMENTOS MACRO, MICRO E POTENCIALMENTE TÓXICOS EM MATRIZES ALIMENTARES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química, realizada sob a orientação científica da Doutora Maria Eduarda da Cunha Pereira, Professora Associada do Departamento de Química da Universidade de Aveiro e co-orientação do Doutor Bruno Manuel Galinho Henriques, Investigador do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, Daniela e Benedito, e à minha irmã, Thainara, pelo incansável apoio, pela orientação e pela força transmitida durante todo o meu percurso académico.

o júri

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"We must be willing to get rid of
the life we've planned, so as to have
the life that is waiting for us.

The old skin has to be shed
before the new one can come."

— Joseph Campbell

palavras-chave

Validação de método, alimentos, elementos potencialmente tóxicos, macro/microelementos, digestão ácida por microondas

resumo

Uma alimentação saudável fornece os nutrientes e a energia necessários ao desenvolvimento e crescimento do ser humano, e a um estilo de vida ativo e saudável. Embora alguns elementos sejam essenciais para o funcionamento metabólico, eles também podem trazer riscos para a saúde, dependendo da sua concentração no organismo.

Para regular a concentração de diversos elementos nos alimentos, e minimizar os efeitos prejudiciais na saúde humana, alguns países desenvolveram legislação que estipula concentrações máximas aceitáveis nos alimentos. De modo a garantir a qualidade exigida por lei, é necessário que as análises químicas aos alimentos sejam feitas em laboratórios credenciados, e utilizar métodos validados para a análise das amostras. O intuito de uma validação é garantir que os resultados obtidos estão próximos dos valores verdadeiros, e confirmar que as características dos métodos satisfazem as especificações exigidas.

O principal objetivo deste trabalho foi validar e implementar uma metodologia de quantificação de elementos macro, micro e potencialmente tóxicos em matrizes alimentares por meio de digestão ácida assistida por micro-ondas, e sua quantificação por atomização de plasma induzido associada à deteção por espectrometria de massa. Adicionalmente, foi ainda avaliada a influência que diferentes tipos de tratamento comuns (e.g. grelhar, cozer) podem ter na concentração dos elementos nos alimentos.

Neste trabalho foi validada uma metodologia de quantificação para nove elementos, nomeadamente, Na, Mg, Ca, Fe, Mn, Cu, Zn, As e Cd. Os parâmetros validados incluíram a calibração, os limites de quantificação e de deteção, a seletividade, a justeza e a precisão. Foi também avaliada a estimativa da incerteza para o método. Todos os parâmetros de validação estudados neste trabalho cumpriram os requisitos estipulados, de acordo com a associação de laboratórios acreditados de Portugal (RELACRE), resultando na correspondente ficha de validação do método.

A comparação elementar entre matrizes alimentares, após estas terem sido cozidas ou grelhadas, não indicou grandes alterações na concentração dos elementos nos alimentos. Exceção para o conteúdo de Na no peixe, que foi bastante reduzido (em 5 ordens de grandeza), após o seu processamento (cozedura ou grelhado). Os níveis dos elementos potencialmente tóxicos nas matrizes estudadas cumpriram com os limites máximos exigidos, com exceção do As no arroz, cuja concentração (0.30 µg/g) foi cerca de 1.5 superior ao valor legislado (0.20 µg/g).

keywords

Method validation, food, potentially toxic elements, macro/micro elements, microwave acid digestion

abstract

A healthy diet provides the nutrients and energy necessary for human development and growth, and for an active and healthy lifestyle. Although some elements are essential for metabolic functioning, they can also pose health risks, depending on their concentration in the body.

To regulate the concentration of various elements in foods, and minimize the harmful effects on human health, several countries have developed laws that stipulate maximum acceptable concentrations of these elements in food. To ensure the quality required by law, it is necessary that the chemical analyzes of foods are carried out in accredited laboratories, using validated methods for the analysis of samples. The intent of a validation is to guarantee that the obtained results are close to the true values, and to confirm that the method characteristics satisfy the required specifications.

The main objective of this work was to validate and implement a methodology for the quantification of macro, micro, and potentially toxic elements in food matrices by means of microwave-assisted acid digestion, and their quantification by induced plasma atomization associated with detection by mass spectrometry. Additionally, the influence that different types of common treatment (e.g. grilled, boiled) can have on the concentration of elements in food was also evaluated.

In this work, a methodology to quantify nine elements was validated, namely, Na, Mg, Ca, Fe, Mn, Cu, Zn, As and Cd. The validated parameters included calibration, limits of quantification and detection, selectivity, trueness, and precision. The measurement uncertainty for the method was also evaluated. All validation parameters studied in this work fulfilled the stipulated requirements, which are in accordance with the association of accredited laboratories in Portugal (RELACRE), resulting in the corresponding declaration on the suitability of the method.

The comparison between food matrices, after they have been cooked or grilled, did not indicate major changes in the concentration of the studied elements in the food. Exception for the Na content in fish, which was greatly reduced (by 5 orders of magnitude) after processing (boiled or grilled). The levels of the potentially toxic elements in the studied matrices complied with the maximum required limits, with the exception of As in rice, whose concentration (0.30 µg/g) was about 1.5 higher than the legislated value (0.20 µg/g).

Abbreviations

CAC	Codex Alimentarius Commission
CRM	Certified Reference Material
EA	European cooperation for Accreditation
EAM	Elemental Analysis Manual for Food and Related Products
EM	Electron Multiplier
EPA	United States Environmental Protection Agency
ETA	Electrothermal Atomization
FAAS	Flame Atomic Absorption Spectroscopy
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GFAAS	Graphite Furnace Atomic Absorption Spectroscopy
IAEA	International Atomic Energy Agency
IAF	International Accreditation Forum
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
ICP-MS	Inductively coupled plasma atomic mass spectrometry
ICT	Interlaboratory Comparison Test
IEC	International Electrotechnical Commission
IFT	Institute of Food Technologists
ILAC	International Laboratory Accreditation Cooperation
IPAC	Portuguese Accreditation Institute
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LCA	Central Laboratory of Analysis
LOD	Limit of detection
LOQ	Limit of quantification
MU	Measurement uncertainty
NIST	National Institute of Standards and Technology
NRC	National Research Council Canada
PTE	Potentially Toxic Elements
QMS	Quality Management Systems
RDA	Recommended Dietary Allowance

RF	Radio frequency
UL	Tolerable Upper Intake Level
USDA	U.S. Department of Agriculture
WHO	World Health Organization

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1. Introduction

1.1 Quality assurance in chemical laboratories

1.1.1 Quality management system

The concept of Quality was and will always be intrinsic to human nature since it persists from the most primordial civilizations to the present. The concern that man has in effectively carrying out his tasks, as good materials for building tools, is verified through historical records of past civilizations (Pereira and Requeijo, 2012)

Nowadays there are more and more demands being made on companies to export and import products, in addition to the requirement to comply with laws that guarantee consumer safety. Therefore, it is necessary to develop products with high quality so that customers' requirements are met. Quality in the production of products and in the process is a crucial demand that has been used in the social, technological, economic and scientific areas (Valcárcel and Ríos, 1994). Quality assessment manifests an increasing importance as a valid strategy to achieve an advantageous competitive position (Fornell, 1992). The differentiation of products and services by quality is an essential factor of competitiveness, so quality becomes one of the top priorities of companies. In this way, the quality of goods and services has become an essential factor for the continuity and survival of organizations in the long term (Margato, 2014).

Quality is defined by the International Organization for Standardization (ISO) as "the set of attributes and characteristics of a product, system or service that influences its ability to meet regulated or implied needs" (International Organization for Standardization, 2015). Quality, in general, integrates two approaches, the comparative approach and the performance/skill approach, which are complementary. The quality of a "thing" is reflected in the quality of a product (e.g. cheese), a system (e.g. a dairy factory) or a service (e.g. an analytical control laboratory). Total quality is the consequence of integrating these different types of quality and encompasses their obvious mutual relationships (Valcárcel and Ríos, 1994).

The phenomenon of globalization associated with the permanent evolution of the market has led companies to new challenges, conditioning them in the search for management tools that add value to the organization, improve the performance of processes, control operating costs and value existing resources (Martins, 2016). Quality has thus changed from a characteristic of the product/service to a characteristic of the entire organization, including the workers involved in the activities (Costa, 2016). The

Quality Management Systems (QMS) emerge in an attempt to organize the company, making decisions and continuously improving processes, thus promoting interest in external recognition (Martins, 2016). The QMS is a dynamic company management system to control and guarantee quality, the necessary resources, operational procedures and responsibilities established in order to achieve customer satisfaction, governed by a series of standards, which are intended to prevent deviations from occurring, at any stage of production of the product or service, from planning to after-sales service (Borba et al., 2016; Sousa, 2012).

Quality assurance (QA) is a system of activities whose purpose is to provide to the producer or user of a product or a service the assurance that it meets defined standards of quality with a stated level of confidence (Taylor, 1987), through the utilization of requirements, procedures, and assessment to ensure that the goal of the program is achieved. Specific goals that are expected from the quality assurance activities should be documented (Klesta and Bartz, 1996). Quality assurance is very important when it comes to the validation of a method, since it is known that not always the analyses performed, and the results obtained will be valid. Therefore, it should be taken into account that in a routine analysis all the procedures must be based on a quality control scheme, that will allow to determine deviations produced by equipment, analysts or by the experimental procedure itself, and to ensure that all results and conclusions are reliable (Bettencourt et al., 1999).

The quality of every analytical process is composed of two mainly components: 1) The quality of work done inside or outside the laboratory; 2) The quality of the materials, apparatus, instruments, and software used.

1.1.2 Laboratories accreditation systems

Accreditation is based on a set of rules and procedures used to recognize the technical competence of entities to carry out specified conformity assessment activities (e.g. tests, calibrations, certifications and inspections), and is governed by international standards called ISO standards (Instituto Português da Acreditação, 2019). To verify compliance with the requirements of the accreditation process, periodic evaluations are carried out at accredited laboratories. For an exemplary implementation of the requirements of good laboratory practices, which are reflected in good results, it is necessary that an accredited laboratory has adequate human resources, facilities, equipment, methods and procedures, and that follows all the requirements set in the

international standard ISO/IEC 17025:2018 (International Organization for Standardization, 2018).

Therefore, accreditation is a formal assessment that is given by a single accreditation body, where each EU Member State has designated a single national accreditation body. In Portugal, this accreditation body is the Portuguese Accreditation Institute (IPAC) (as provided for in Decree-Law Nº 23/2011, of 11 February, which responds to Regulation (EC) Nº 765/2008) (Instituto Português da Acreditação, 2020a). Therefore, only IPAC can guarantee the laboratory's competence to work according to specified norms or standards, in accordance with the international standard (International Organization for Standardization) ISO/IEC 17025:2018. Therefore, accreditation is used to determine whether laboratories have an adequate quality management system and whether they can properly perform analysis methods and/or perform calibrations.

With the need to develop international standards that guarantee the quality of the services of testing and calibration laboratories worldwide, the ISO/IEC 17025 - General Requirements for the Competence of Testing and Calibration Laboratories, presents the technical and management requirements to be followed to guarantee the quality of the services provided and demonstrate their technical competence (Martins, 2016).

It is possible to participate in Mutual Recognition Agreements between accreditation bodies as European cooperation for Accreditation (EA), International Laboratory Accreditation Cooperation (ILAC) and International Accreditation Forum (IAF) (Instituto Português da Acreditação, 2020b), due to global harmonization of standards, which ensures the free movement of goods and services that are included in the accreditation. As a result, IPAC is subject to the EA, peer review system and publishes the results of those assessments publicly (Martins, 2016).

The accreditation process consists of several stages, which are set out in the General Regulation on Accreditation (DRC001): application, evaluation and decision; based on the ISO/IEC 17025:2018 standard (Instituto Português da Acreditação, 2019). The laboratory that wants to be accredited must submit a complete application and send the corresponding forms to IPAC about the technical area that it intends to perform. In the assessment phase, a documentary analysis, or a face-to-face assessment, also called an audit, that is usually carried out to verify non-conformities, which can be resolved through the implementation of corrections or appropriate corrective actions. After the effective completion of the application phase, IPAC assigns the evaluation team, which

will study the documentation and proceed with the evaluation of the laboratory, informing the members of the laboratory and the audit team about the date of the audit.

The decision made by IPAC is made based on the analysis of the reports previously delivered, the opinion of the audit team and the analysis of corrective actions taken by the audit team during the audition. Subsequently, a report is written, in which the non-conformities to be corrected are mentioned, showing compliance with the accreditation standards, to which the entity will have to respond.

After the audit team carries out its study, a document is issued, which is followed by a procedural analysis by IPAC. Finally, the decision is taken by IPAC, which is valid as long as the laboratory shows compliance with the accreditation regulations and obligations. When the accreditation decision is favourable, IPAC issues a certificate of accreditation, after the audited entity has made the payment related to the process, in which the scope of accreditation is described (Instituto Português da Acreditação, 2020a). The draft of accreditation process is described in the document DRC004 (Instituto Português da Acreditação, 2017). **Figure 1** shows the schematic accreditation process.

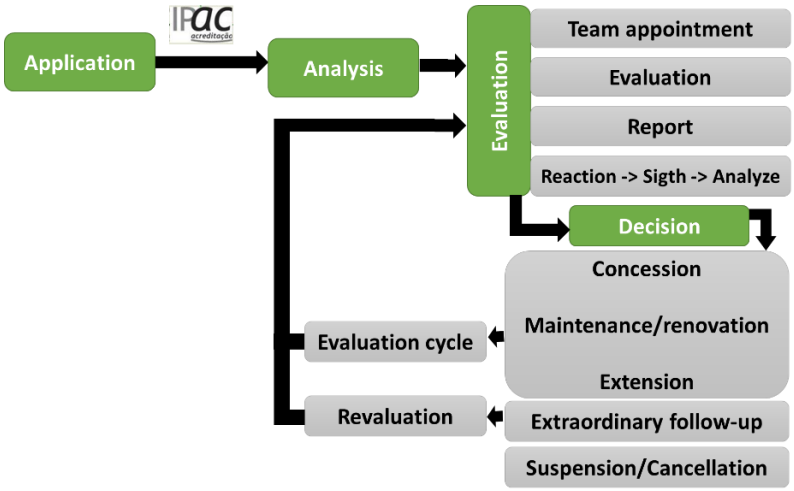

Figure 1: *Accreditation process (adapted from* Instituto Português da Acreditação (2020a)*).*

To ensure the quality and reliability of the results and conclusions acquired, it is necessary that the quality will be ensured by the accreditation of the laboratory in question.

1.1.3 Method validation

Method validation is not a new concept in laboratories and scientists have long validated their methods before using them routinely (Martins, 2015). The validation of an analytical method aims to ensure that the results obtained are close enough to the

unknown true value of the analyte in the analysed samples (González and Herrador, 2007). Also validation is the process of establishing the performance characteristics and limitations of a method, as well as identifying the influences that may change these characteristics (Feinberg et al., 2004).

The international standard ISO/IEC 17025:2018 is divided into two groups of requirements: management requirements and technical requirements and in accordance with requirement 7.2.2 - Method validation *"The laboratory must validate non-standard methods, methods developed by the laboratory itself and standard methods used outside the intended scope of use, or otherwise modified. The validation must be as extensive as necessary, in order to meet the needs of a given application, or field of application"* (International Organization for Standardization, 2018).

According to the Association of Accredited Laboratories of Portugal (RELACRE), the validation of an internal test method serves to demonstrate that the test performed, under the conditions in which it is practiced, has the necessary characteristics to obtain results with the required quality. The validation of an analysis method, when performed correctly, improves the reliability, consistency and precision of the analytical data obtained, being necessary whenever the method is not standardized or when it is an adaptation/modification of a standardized method (Martins, 2015).

In the practice of chemical analysis, the validation of an analytical method is very important, as it ensures that the analytical methodology studied is reproducible and accurate. This assessment also guarantees compliance with the requirements and the proposal defined by the analytical method (RELACRE, 1996). The validation of an analytic method performed in a laboratory is done after a selection, development, and optimization of the method (**Figure 2**). The development characteristics of the method, also called validation parameters, must be referred on a procedure (RELACRE, 2000).

The main objective of a validation is to confirm that the method characteristics satisfy the required specifications for the analytic results, as well as establish control limits to apply on the daily work (Coelho, 2010). It is of total importance that the determinations of the validation parameters are done on properly calibrated equipment, respecting the specifications of the international standard, and is also essential that all the method validation is described in a laboratorial procedure. Furthermore, all analysts and technicians must have qualifications and be properly trained (INMETRO, 2016; International Organization for Standardization, 2018).

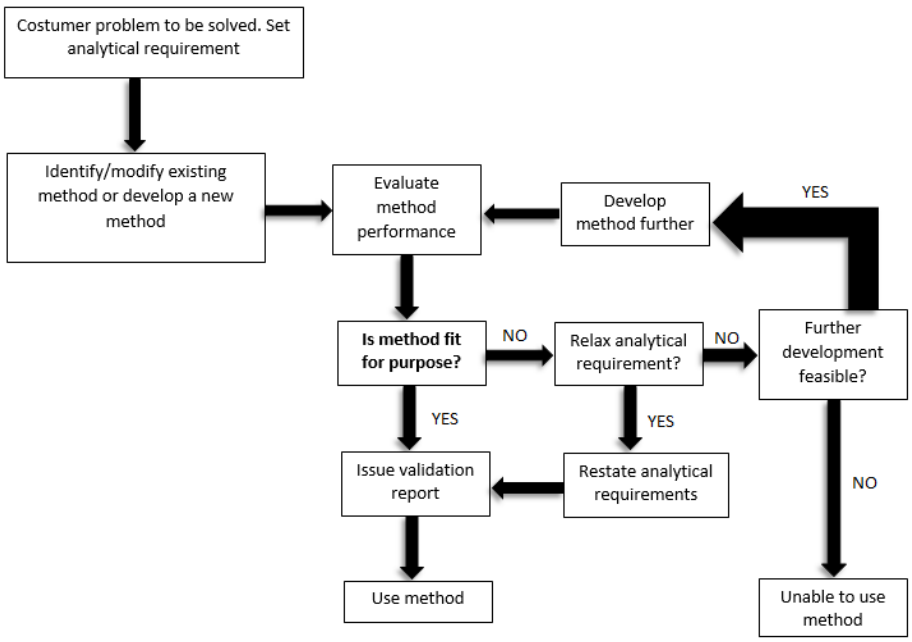

Figure 2: *Selection, development, and optimization of a validation process*

Recent directives from the International Union of Pure and Applied Chemistry (IUPAC) and also the International Conference on Harmonization report that for an analytical method to be fully validated, nine parameters must be evaluated, namely: identification, specificity and selectivity, sensitivity, analytical thresholds, precision, accuracy, linearity and analytical range, robustness and coherence (Thompson et al., 2002).

The validation of a method is effectuated by the utilization of direct evaluation tools, which intend to know the accuracy of assay methods, that means analyse the agreement between the result given by the method and the reference value that is taken for true value, and of indirect evaluation, which corresponds to the determination and evidence of the characteristics parameters of the method (**Table 1**) (de Carvalho, 2007; RELACRE, 2000). It is necessary to adapt the method parameters to each type of method. One example is that it is not useful to determine the limit of quantification, the precision and linearity of the work for a method of qualitative analysis, however these are relevant parameters for the quantitative analysis, as well as the limit of detection and its selectivity (RELACRE, 2000). At the end of the validation process, a declaration on the validity of the method must be issued, detailing its suitability for the intended use.

Table 1: *Tools for direct and indirect evaluation for validation of a method*

DIRECT EVALUATION	INDIRECT EVALUATION
Certified Reference Material (CRM)	**Specificity/Selectivity**
Interlaboratory Comparison Test (ICT)	**Quantification**
Comparative Tests	**Precision**
-	**Accuracy**
-	**Robustness**

The validation is an important process that encompasses different areas, and it continues to be developed in research at universities, in companies and industry. Method validation can be used in different matrices, as cattle bones (Zhang et al., 2021), human nails (Fleming, 2021), drinking water (Boselli et al., 2021), human urine (Jones et al., 2021; Laur et al., 2021), crude oil (Gab-Allah and Shehata, 2021), drug substances (Merusomayajula et al., 2021), vinegar (Paktsevanidou et al., 2021), food (Hwang et al., 2020), alcoholic beverages (Oliveira et al., 2021), blood and plasma (Tanvir et al., 2021), sediments (Carvalho et al., 2020), and dark chocolate (Mrmošanin et al., 2018), and for the analysis of different contaminants, such as rare earth elements (Baghaliannejad et al., 2021), trace elements (Laur et al., 2021; Paktsevanidou et al., 2021; Tanvir et al., 2021), iodine (Hwang et al., 2020), and macro elements (Carvalho et al., 2020; Hong et al., 2019; Mrmošanin et al., 2018).

1.2 Evaluation of food quality

After air and water, food is the third most basic need in life, which demonstrates its importance for living. Since human are born, food is needed to provide strength, to nourish, to supply the necessary nutrients to help in the development and growth of their bodies.

Currently, all food products that reach the market must first have gone through quality control and an analysis that requires prior validation. It is necessary to ensure the quality and comparability of analytical results generated by laboratories for compliance purposes, as food production faces frequent challenges such as large-scale production and possible contamination (Bratinova et al., 2009).

Throughout its evolutionary history, human being went through several stages: hunter, sower, farmer, until he reached the stage of industrialization, where he became a supplier of goods and services. It arose mainly from the need to answer social issues, such

as the need for food conservation, which would posteriorly set people free from the daily search for food. The need for adequate and consistent nutrition through food supply has also led to the development of the food industry, contributing in a major way to the foundation of a healthier human civilization, thereby helping society to prosper and evolve (Lund, 1989).

The Codex Alimentarius Commission (CAC) defines Food Safety as the guarantee that food will not cause harm to the consumer when it is prepared or eaten according to its intended use (Codex Alimentarius Commission, 2003). The request for food shaped the society development since the early ages, so food safety is as old as human history. Griffith (2006) suggests that the concept of food safety may have started with the recognition and avoidance of foods that were naturally toxic, and probably also by trial and error in the development of basic forms of food preservation that started to contribute to food safety (e.g. fermentation) (Selamat and Iqbal, 2016).

Over the years, food security, due to scandals, incidents and fears inherent in food consumption, has become an increasing global concern by government entities and the entire food sector, from producers to consumers (Muse, 2017). On the nineteenth century the biggest changes in food production occurred (Lásztity et al., 2004) and, due to a fast urbanization and poor hygiene, the creation of food laws as Codex Alimentarius Austriacus (Randell, 1995) and the Act of 1860 for "Preventing the Adulteration of Food and Drink" was essential to food safety, even though the importance of hygiene and the dangers of adulterations were neglected at that time.

During harvesting, transformation, storage, transportation, distribution and sale of a food, contamination can occur (World Health Organization, 2020), which can be biological, chemical and/or physical (**Figure 3**). The most significant biological contamination is the microbiological contamination, representing a significant portion of the hazards (Baptista and Venâncio, 2003).

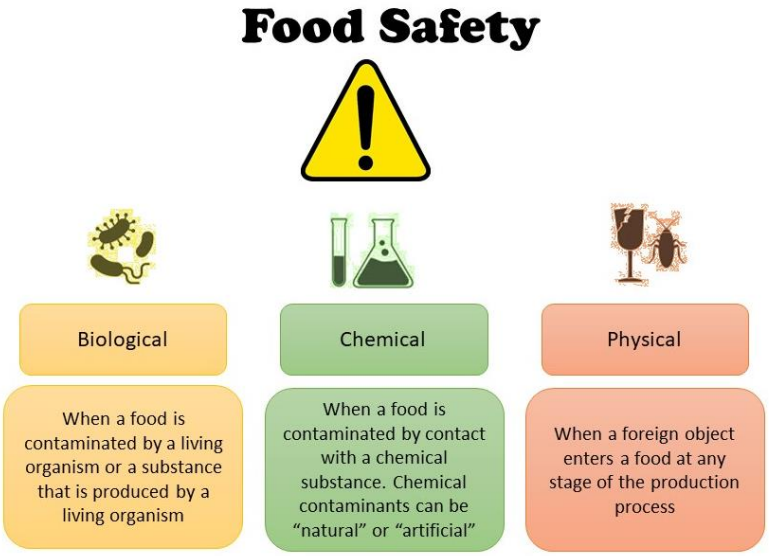

Figure 3: *Types of contamination that must be checked and eliminated to promote food safety.*

1.2.1 Toxicity associated with food intake

Food, by providing essential nutrients (macro and micronutrient), are beneficial for human health. However, it can also be considered a source of contamination, as food may be exposed to toxic elements, known as potentially toxic elements (PTEs).

Most natural elements and compounds are present in different forms all over the planet, with many of them available in food. The classification of chemical elements can be done according to their needs in the human organism, being divided in essential and non-essential (Torres et al., 2008). Metals and metalloids as lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) are classified as non-essential chemicals, since they do not present metabolic or biological functions, but present hazardous effects, even at low concentrations, on living organisms and ecosystems health (Gall et al., 2015). Due to their dangerousness and their harmful effects, these compounds were included in the top 20 list of dangerous substances by the United States Environmental Protection Agency (EPA) and the Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR, 2007; Khalid et al., 2017). Unlike persistent organic pollutants, metals are not created or destroyed, i.e. they are not biodegradable (El-Kady and Abdel-Wahhab, 2018). That characteristic leads to a very high tendency of its accumulation in the environment, such as soil and water (Gilbert and Weiss, 2006).

Some metals as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) are components needed in a vast group of metabolic processes as a part of the active center of enzymes and cytochromes (Marschner, 2011). Even though these metals are essential

to metabolic functioning of biota, they can also cause health risks depending on their concentration in the organism (e.g. nickel is one integral component of urease, but in excessive levels can cause health problems) (Rai et al., 2019). The importance of dosing essential and non-essential elements is highlighted in **Figure 4**, that shows how element dosage determines the response within the organism. In this context, the levels of essential and potentially toxic elements should be routinely determined in food products.

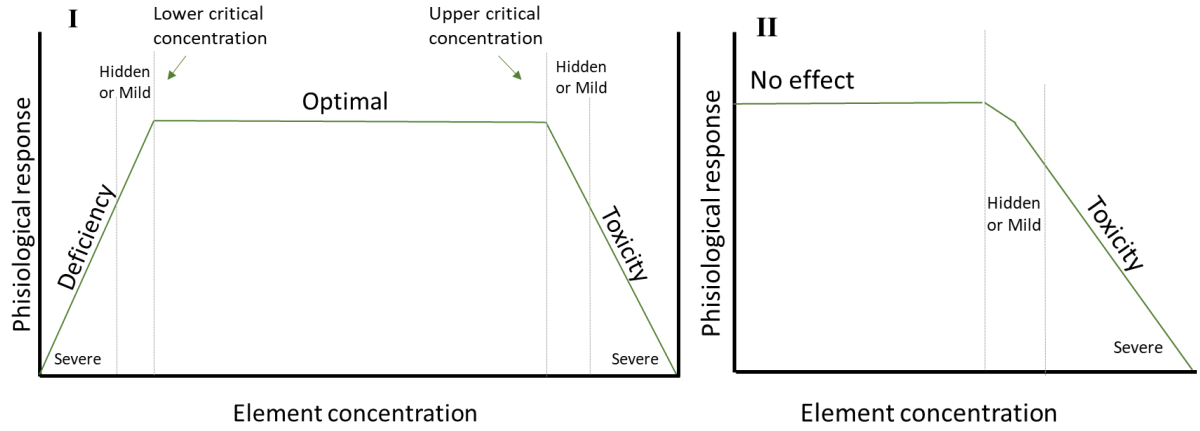

Figure 4: *Typical dose-response curves for essential (I) and non-essential (II) elements in physiological responses (adapted from* Alloway (2013)*)*

Contamination of agriculture food comes mainly from contamination in the soil, which primary sources are irrigation with wastewater or contaminated water, atmospheric deposition, pesticides and herbicides, phosphate-based fertilizers and sewage sludge-based amendments (El-Kady and Abdel-Wahhab, 2018; Hajeb et al., 2014; Nasreddine and Parent-Massin, 2002). The contamination of the environment mostly comes from human activity (Rai et al., 2019), and since the industrial revolution and economic globalization, the diversity of environmental contaminants has increased exponentially. Although metal concentration increased due to anthropogenic contamination, metals as Fe, Al and Mg occur naturally on the environment constituting 7.4, 4.7 and 2.1 % of global crust, respectively (Manahan, 1994). Continental weathering, forest fires and volcanic activities are also considered natural sources of trace metal.

Bioaccumulation of metals in food crops is a major concern worldwide due to its effects on human health. A global scenario for metal contamination of food crops in relation to their broad anthropogenic sources is represented in **Table 2**.

Studied cases shows variations according to geographic and socio-economic scenarios that account for remarkably different sources and extents of metal

contamination in soil–crop systems. Human health hazards are closely linked to the intake of metal-contaminated food crops and the ingestion of vegetables contaminated with metals can causes serious issues, such as gastrointestinal cancer, fragile immunological mechanisms, mental growth retardation, and malnutrition (Dickin et al., 2016; El-Kady and Abdel-Wahhab, 2018; Hu et al., 2013; Iyengar and Nair, 2000).

Table 2: *Metal contamination from diverse sources in global food crops (Adapted from* Rai et al., (2019)*)*

Food crops	***Country investigated***	***Sources of contamination***	***Metal concentrations***	***References***
Rice, wheat, soybean, corn, potato	Brazil	Industrial, intensive urban agriculture	Below the standard hazard limits to human health	(Corguinha et al., 2015)
Brassica sp., food grains, and leafy vegetables	China	Both sewage and industrial waste drained into river water used for irrigation	Cr 0.01-0.19 mg/kg, Pb 0.12-0.23 mg/kg, Cu 0.15–0.86 mg/kg, Zn 0.42-0.95 mg/kg	(Liu et al., 2005)
Industrially processed food stuffs and pharmaceuticals	United States of America, Spain, Portugal, Belgium, England, and Chile	Industries processing, modern pesticides-based agriculture	Cr (0.10-17.7 mg/kg), Ni (0.01-7.01 mg/kg), Cu (0.01-6.44 mg/kg), Zn (0.01-6.44 mg/kg), Pb (0.03-7.21 mg/kg)	(González-Martín et al., 2018)
Potato	China	Inadequately treated urban wastewater	Cu 1.03 mg/kg, Cr 0.03 mg/kg, Pb 0.067 mg/kg, Cd 0.015 mg/kg, Zn 3.77 mg/kg, Ni 0.054 mg/kg	(Song et al., 2009)
Potato/other foodstuffs	Egypt	Inadequately treated wastewater	Cu 0.83 mg/kg, Pb 0.08 mg/kg, Cd 0.02 mg/kg, Zn 7.16 mg/kg	(El-Kady and Abdel-Wahhab, 2018; Radwan and Salama, 2006)

One important criterion to assess global human health is the soil-plant transfer factor (TF) of the metals and metalloids (Rattan et al., 2005; Rothenberg et al., 2007; Woldetsadik et al., 2017), since metals can accumulate into the organisms, more specifically in fatty tissues and human bones through dietary intake, which leads to weakened immune defenses and depletion of essential nutrients (Rai et al., 2019). Metals

as Pb, Cd, Al and Mn are suspected to cause also intrauterine growth retardation (Iyengar and Nair, 2000; Rai et al., 2019).

To regulate and control the concentration of these elements in foods, minimizing their consumption and their harmful effects on human health, countries have developed and applied laws towards a healthier diet. Failure to comply with these laws leads to the closure of the food company.

1.2.2 International, European, and National food legislation

As technology evolves, and new diseases and food hazards are discovered every day, food safety legislation is constantly changing, as it is a very complex subject. Current food laws are designed to protect consumers from food fraud, adulteration and contamination, as well as protecting producers and traders from unfair competition (Lawley et al., 2012). The main participants in the history of food safety and quality regulation were the International Organization for Standardization (ISO), World Health Organization (WHO) and Food and Agricultural Organization (FAO) agencies (Schmidt and Rodrick, 2003; Selamat and Iqbal, 2016).

In the late 1990s, a series of incidents involving food draw attention to the necessity to establish requirements regarding food and feed law at a major level. As a result, the European Commission has developed one integrated approach to food security '*from farm to fork*', presented mainly in its *White Paper on Food Safety*. This approach covers all sectors of the food chain, which includes feed production, primary production, storage, transport and sale (European Commission, 2007a). The main forms of legislation, including food legislation, are EU Directives and EU Regulations (Schmidt and Rodrick, 2003). The European Parliament and the Council adopted, in 2002, the Regulation (EC) Nº 178/2002 that describes the general principles and requirements of food law (General Food Law Regulation) (European Commission, 2007a). Before the creation of EU Regulations, in 1963 the Codex Alimentarius Commission was created by FAO and WHO, at a conference on standards for food, being constituted by 187 countries members and by European Union (Machado, 2015; Selamat and Iqbal, 2016). Its main purposes are protect the health of consumers and ensure fair practices in the food trade, in addition to promoting coordination of all standards developed by international governmental and non-governmental organizations (Tavares, 2018). The creation of CAC allowed the orientation and promotion of the necessary requirements for semi-processed, processed or raw food, in order to facilitated and promote safety at international commercialization

(Machado, 2015; World Health Organization & Food and Agricultural Organization, 2010).

As an integral part of the program included in the White Paper for Food Safety, the European Food Safety Authority (EFSA) was founded, also in 2002, as an agency independent of European legislative and executive institutions, with the purpose of functioning as a source of scientific advice on the risks associated with the food chain for the bodies regulating food security in Europe. European Food Safety Authority operates in close collaboration with partners across Europe and the rest of the world (Commission of the European Communities, 2000; Costa, 2014; Rodriguez, 2012).

According to Lawley et al. 2012, three new EU regulations came into force on 1st March 2007 to deal with a great variety of chemical contaminants in food. From these three regulations the most important, taking into account food industry point of view, is the EC Regulation Nº 1881/2006 (Commission of the European Communities, 2006) that replaces Nº 466/2001. This regulation sets maximum permitted levels for a range of contaminants in foodstuffs, covering contaminants as heavy metals, PAH, mycotoxins, dioxins, and PCBs. From EC Regulation Nº 1881/2006 some set maximum permitted levels have been modified through application of 'amending' Acts. One example of such modification is the EU Regulation Nº 165/2010 (European Commission, 2010) that sets revised maximum levels for aflatoxins on a range of food commodities. Food industries and food businesses should always guarantee that they know what changes have been made to food safety regulations by such amending Acts (Lawley et al., 2012).

Regarding heavy metals the EC Regulation Nº 1881/2006 has also undergone by several changes as follows: Commission Regulation (EC) Nº 629/2008 of 2 July 2008 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants present in foodstuffs (Commission of the European Communities, 2008); Commission Regulation (EU) Nº 420/2011 of 29 April 2011 amending Regulation (EC) Nº 1881/2006 fixing the maximum levels of certain contaminants present in foodstuffs (European Commission, 2011); Commission Regulation (EU) Nº 488/2014 of 12 May 2014 amending Regulation (EC) Nº 1881/2006 with regard to maximum levels of cadmium in foodstuffs (The European Commission, 2014); Commission Regulation (EU) 2015/1005 of 25 June 2015 amending Regulation (EC) Nº 1881/2006 as regards maximum levels of lead in certain foodstuffs (European Commission, 2015a) and Regulation (EU) 2015/1006 of the commission of 25 June 2015, amending Regulation

(EC) Nº 1881/2006 with regard to the maximum levels of arsenic in inorganic form in foodstuffs (European Commission, 2015b).

According to Regulation (EC) Nº 178/2002 (The European Parliament and The Council of the European Union, 2002), the general principles and requirements of food law, establishing the European Food Safety Authority, and procedures in matters of food safety, it is a general principle of food law to provide a basis for consumers to make informed choices in relation to food they consume and to prevent any practices that may mislead the consumer. To achieve a high level of health protection for consumers, and to guarantee their right to information, the amending Act EU Nº 1169/2011 (The European Parliament and The Council of the European Union, 2011) ensures that consumers are appropriately informed as regards the food they consume.

Maximum levels of arsenic, cadmium, lead and mercury in certain food matrices as vegetable, animal and mineral origin, additives, pre-mixtures, complete and complementary feeding stuffs were established on EU Directive 2002/32/EC of 7 May 2002 on undesirable substances in animal feed (European Parliament and the Council of the EU, 2002). Criteria for sampling, sample treatment and methods of analysis for the official control of the maximum levels of these metals are laid down by Commission Regulation (EC) Nº 333/2007 of 28 March 2007 (European Commission, 2007b). Surveillance for residues of chemical elements in foods of animal origin is specified in Council Directive 96/23/EC (European Commission, 1996; López-Alonso, 2012).

To ensure that the food that reaches the consumer is of quality and is within the standards required by law, that is, that the levels of contamination are below the value indicated in European Union regulations, there must be a commitment to quality. To achieve this quality, it is necessary to use accredited laboratories and validated methods to analyze samples and ensure that the values found are true.

1.3 Preparation of food product for analysis

In the global food context, some foods are consumed more daily than others, however, when it comes to accreditation, laboratories should not only consider the global and social context, especially when they want to validate a method. For this, food matrices that can be accredited by IPAC must be considered, such as: meat and meat products, cereals and legumes, bivalve molluscs and fishery, and aquaculture products. Those matrices were observed on IPAC website as follows: accredited entities → testing laboratories → food and agri-food (Instituto Português da Acreditação, 2020c). Entities

as Silliker, IPMA, SGS Portugal, Instituto Superior Técnico, Autoridade de Segurança Alimentar Económica, CALSEG, AQUIMISA, among others, are accredited for the analysis on such food matrices.

There are currently 67 testing laboratories accredited by IPAC for food and agri-food analysis. Most of these laboratories carry out analyses such as: Determination of moisture content, Determination of crude protein content, Determination of total fat, Determination of Total aflatoxin, Determination of pH, Determination of nitrogen content, Research of Salmonella spp., Counting of Enterobacteriaceae, Count of Campylobacter spp. among other types of biochemical analysis. Among these 67 laboratories, only 10 carry out metal determination analyses. Of these 10 laboratories, 8 perform the analysis by atomic absorption spectroscopy (flame or graphite chamber). Only 2 laboratories are accredited to perform the analysis by inductively coupled plasma mass spectrometry (ICP-MS). To perform analysis of food matrices by ICP, it is necessary that the sample is in a liquid form. Methodologies for performing sample preparation include digestion methods that use concentrated acids or mixtures of acids (Parente, 2020).

This digestion can be carried out in an open vessel with heating, for example on a hotplate, or in a closed vessel with the aid of a microwave system. Microwave-assisted digestion is an appealing method, especially for small amounts of sample, as heating in this way has advantages over plate heating, since energy is generated in the digestion mixture and not transferred by conduction. The main advantages of microwave-assisted digestion are the digestion times, which are shorter when compared to digestion carried out on a hot plate, and the lower quantity of reagents needed to obtain a complete digestion (Duarte et al., 2014). One of the limitations of the microwave digestion process is the cooling time required before vessels can be opened, due to possible loss of analytes by volatilization.

In general, the reagents that can be used for sample decomposition in this process are HNO_3, HCl, H_2SO_4, H_3PO_4, $HClO_4$, HF and H_2O_2 (Bizzi et al., 2011; Gholami et al., 2016; Smita et al., 2013), with HNO_3 (compatible with most techniques of detection since it does not form insoluble products) and H_2O_2 (used to intensify the oxidant power of the medium) being the most used specially regarding biological samples (Arruda, 2007). Concentrated HNO_3 is the most favourable oxidant for the destruction of organic matter, but, due to its low oxidation potential, it can lead to incomplete digestion of some types

of samples (Muller et al., 2016; Smita et al., 2013). As such, mixtures of reagents are sometimes used to aid and make the digestion process more efficient (Bizzi et al., 2014).

To perform an acid digestion, it is necessary to identify which digestion method will be applied, considering mainly the sample to be analysed, and the reference materials that will be used for the analytical control of the method. In the literature there are scientific articles that portray different digestion methods for different CRMs and/or real matrices, most of them are done in real food samples at their raw state to assure that can be put in the market. Based on a literature review, a table containing different information on digestion methods for the previously mentioned food matrices was created (**Table 3**).

Table 3: *Methods for acid digestion of different food matrices as rice, chicken, mussels, and meat focusing on the amount of sample, reagents, and microwave procedure*

		Reagents					Experimental procedure		Reference
Matrix	Amount of sample	HNO_3	H_2O_2	HCl	HF	Deionized water	Microwave program (1st step and 2nd step)	Final Volume	
Rice	250 mg	7 mL of 4.5 mol/L	1 mL	-	-	-	4 min ramp up to 90 °C, then hold at 90 °C for 2 min. The next step was a 10 min ramp up to 200 °C.	15 mL	(Tarantino et al., 2017)
	400 mg	4 mL of 2 mol/L	3 mL	-	-	-	3 min at 30–85 ºC; 9 min at 85–145 º C, 4 min at 145–160 ºC; Variable: 15, 20, 25 min at 160 ºC; 5 min at 160–30 ºC	50 mL	(da Silva et al., 2018)
	400 mg	4 mL of 8 mol/L	2.5 mL	-	-	-	3 min at 30–85 ºC; 9 min at 85–145 º C, 4 min at 145–160 ºC; Variable: 15, 20, 25 min at 160 ºC; 5 min at 160–30 ºC	50 mL	(da Silva et al., 2018)
	500 mg	5 mL of 1 mol/L	2.5 mL	-	-	-	400 W for 4 min, 800 W for varying time intervals (4, 7, and 10 min)	-	(da Silva et al., 2020)
	100 mg	2 mL	1 mL	-	-	-	2 min at 167 W, ramp up to 333 W for 2 more minutes; then decreased to 0 W for one min. Ramp up to 333 W for 2 min; then decreased to 0 W for one min. This trend was repeated five times	-	(Gholami et al., 2016)
	500 mg	5 mL	1 mL	-	-	1 mL	2 min at 250 W; 2 min at 0 W, 5 min at 250 W; 5 min at 400 W and 5 min at 600 W	25 mL	(D'Ilio et al., 2002)
Corn Bran	200 mg	1.4 mL	1.7 mL	-	-	6.9 mL	5 min ramp up to 180 °C, hold for 15 min - 400 W power increased to 1200 W over 5 min and held for 5 min	10 mL	(Correia et al., 2017)
	0.200 mg	2 mL	1 mL	-	-	1 mL	-	15 mL	(Motta et al., 2020)
	1 g	10 mL	-	-	-	-	90 W for 9 min at 97 °C	-	(Khajeh, 2009)
	200 mg	2 mL	2 mL	-	-	-	33 % of heating power for 3 min, 55 % power for 5 min, 100 % power (700 W) for 3 min, and 77 % power for 3 min, 55 % power for 5 min, 100 % power (700 W) for 3 min	-	(Ke et al., 2006)

Table 3: *Continuation*

Chicken	300 mg	5 mL	2 mL	-	-	-	10 °C/min up to 120 °C - hold for 5 min, 10 °C/min up to 150 °C - hold for 5 min, 10 °C/min up to 180 °C - 5 min hold, 10 °C/min up to 200 °C - hold for 10 min.	-	(Bou et al., 2004)
	250 mg	7 mL (14.7/3.5 mol/L)	1 mL	-	-	-	10 min until reaching 180 °C, 15 min at 180 ºC with a maximum power of 1000 W	15 mL	(Souza et al., 2013)
	250 mg	4 ml of 7 mol/L	2 ml	-	-	-	2 min ramp up to 80 °C, 3 min hold, 4 min ramp up to 120 °C, 5 min ramp up to 180 °C, 5 min ramp up to 210 °C; cool down (15 min)	-	(Menezes et al., 2018)
	200 mg	2 mL	1 mL	-	-	-	20 °C (room) to 220 °C with ramp of 30 min; 15 min at 220 °C, 30 min cooling (from 220 °C to 60 °C)	20 mL	(Vieira et al., 2018)
Mussels	500 mg	6 mL	-	-	-	-	Heating for 20 min at 180 psi	25 mL	(Saavedra et al., 2004)
	500 mg	8 mL	-	-	-	-	90 ºC for 2.5 min at 1000 W; 140 ºC for 6.0 min at 1000 W, 200 ºC for 15 min at 1000 W	25 mL	(Seco-Gesto et al., 2007)
	250 mg	5 mL	1 mL	-	-	-	600 W for 5 min (5 min of ramp), 1400 W for 5 min (5 min of ramp) and 0 W for 20 min	30 mL	(Pereira et al., 2012)
Fish	100 mg	4 mL	2 mL	-	-	-	510 W for 60 s. After H_2O_2 addition 510 W for 120 s	10 mL	(Bugallo et al., 2007)
	250 mg	2.5 mL	-	0.5 mL	-	7 mL	Ramp up to 185°C in 10.5 min, hold for 14.5 min with 1600 W (50 %) of microwave power	-	(Low et al., 2012)
	200 mg	3 mL	2 mL	-	-	3 mL	Ramp up to 180 ºC at 580 W for 5 min, hold at 180 ºC at 470 W for 10 min; cooling at 0 W for 30 min	-	(Ashoka et al., 2009)
	200 mg	3.5 mL	2 mL	-	0.1 mL	2.5 mL	Ramp up to 180 ºC at 580 W for 5 min, hold at 180 ºC at 470 W for 10 min; cooling at 0 W for 30 min	-	(Ashoka et al., 2009)
Seaweed	500 mg	5 mL	-	-	-	-	Predigest for 20 min. Then 20 min ramp up to 200 °C and hold at 200 °C for 15 min	50 mL	(Yoganandham et al., 2019)
	500 mg	6 mL	-	-	-	-	-	25 mL	(Picoloto et al., 2017)
	300 mg	6 mL	-	-	-	-	15 min ramp up at 1400 W. Hold at 1400 W for 10 min, 0 W for 20 min (cooling step)	30 mL	(Guilherme et al., 2020)
	200 mg	7 mL	1 mL	-	-	-	5 min ramp up to 120 °C, then hold in 120 °C for 3 min. 10 min ramp up to 210 °C, hold at 210 °C for 15 min.	-	(Brito et al., 2012)

Depending on the food matrix under study, it may be necessary to use more or less aggressive digestion methods. It is possible to vary digestion parameters such as reagents used, temperatures and digestion time. Interferences in the detection of a given analyte also vary depending on the food matrix under study since the foods have different compositions, and as such, it may be necessary to vary the techniques used for their detection.

Important information can be seen in **Table 3**, namely the main reagents used to decompose food matrices. Nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) are the two main reagents used in literature to digest food matrices. There is no great agreement in the bibliography regarding the volume and concentration of acid used, with some authors using diluted acids while others (the great part) preferring concentrated acids (i.e., HNO_3 65 % and H_2O_2 30 %). The range of volume added was 1.4 to 10 mL of HNO_3, and 1 to 3 mL of H_2O_2.

The U.S. Food and Drug Administration's (FDA) has a mission that consists to maintain a safe food supply by monitoring food and related products for toxic and nutritional elements (Food and Drug Administration 2020). FDA laboratories perform sample analyses using sound analytical practices, and methodology which are documented in the Elemental Analysis Manual for Food and Related Products (EAM). This document act as a reference not only for FDA analysts, but also for analysts around the world providing detailed laboratory methods, procedures, and general analytical information.

Section 4 of the EAM document includes validated methodologies for different methods of analysis as Flame Atomic Absorption Spectrometric (section 4.1), Graphite Furnace Atomic Absorption Spectrometric (section 4.2 and 4.3), Inductively Coupled Plasma-Atomic Emission Spectrometric (section 4.4 and 4.6), Cold Vapor Atomic Absorption Spectrometric (section 4.5), Inductively Coupled Plasma-Mass Spectrometric (section 4.7), and High Pressure Liquid Chromatographic-Inductively Coupled Plasma-Mass Spectrometric (section 4.8 and 4.10).

An analysis technique rarely used in accredited laboratories for the analysis of metals in food matrices is the inductively coupled plasma. This technique has the great advantage of performing a multi-element analysis.

1.3.1 Element quantification in food samples

The quantification of essential elements and potentially toxic elements in several samples can be performed by different instrumental techniques, such as flame atomic absorption spectroscopy (FAAS), graphite furnace atomic absorption spectroscopy (GFAAS), electrothermal atomization (ETA), inductively coupled plasma mass spectrometry (ICP-MS), and inductively coupled plasma optical emission spectroscopy (ICP-OES) (Wilschefski and Baxter, 2019). The analysis techniques that use inductively coupled plasma as an atomization method have some advantages over those that have flame atomization or electrothermal, since they can perform multi-elemental analysis, in short analysis time with low sample use, giving a possibility of wider ranges of work.

Atomic Absorption (AA) occurs when a ground state atom absorbs energy in the form of light of a specific wavelength and is elevated to an excited state (Perkin Elmer, 2011). In atomic flame absorption (FAAS), a flame composed by air/acetylene or nitrous oxide/acetylene can be used to dissociate the sample into its corresponding atoms. When the light from a hollow cathode lamp (selection based on the element to be analysed) passes through the cloud of atoms, the atoms of interest absorb the radiation from the lamp. This is measured by a detector and used to calculate the concentration of that element in the original sample (Thermo Elemental, 2001). The relationship between the amount of light absorbed and the concentration of analytes present in known standards can be used to determine unknown sample concentrations by measuring the amount of light they absorb (Perkin Elmer, 2011).

The use of a flame limits the temperature to a maximum of about 2600 °C (N_2O/acetylene flame). For Mn, Ni, Cd, Pb and alkali metals that are efficiently atomized in F-AAS and can be quantified even at concentrations in the range of mg/L this is not a problem. However, there are several refractory elements, such as V, Zr, Mo and B, which do not perform well with flame atomization, because the maximum temperature reached is insufficient to decompose the compounds of these elements. As a result, the sensitivity of FAAS to these elements is not as satisfactory as in other techniques of elementary analysis (Thermo Elemental, 2001).

The atomic absorption technique with atomization in a graphite oven (GFAAS) is like that of the flame, but the flame is replaced by a small graphite tube, where the sample is directly introduced, GFAAS can reach temperatures up to 3000 ºC, to generate a cloud of atoms (Perkin Elmer, 2011). The higher atomic density and the longer residence time of the sample in the graphite chamber, relative to what happens in the flame, makes the

sensitivity of GFAAS higher than that of FAAS, which allows quantifying some elements with concentrations in the order of μg/L. However, due to the maximum temperature that is possible in the oven and the use of a graphite material, the problems associated with the formation of refractory compounds also occur in this type of technique (Thermo Elemental, 2001).

Inductively coupled plasma (ICP) techniques have plasma as their source of power, which is a highly ionized gas consisting of energetic electrons, ions, and atoms. Despite the high population of ions and electrons, the plasma is macroscopically neutral. The plasma is feed by a continuous supply of energy through electromagnetic induction, or inductive coupling, to a flowing gas. Usually the gas used for the plasma generation is argon, which gives ICP its unique characteristics of having lower interference on samples analysis (De Silva & Gregoire, 1998). This technique can perform multi-elemental analysis, in short analysis time and low sample use, giving a possibility of wider ranges of work, when compared to techniques such as atomic absorption spectrometry or atomic emission spectroscopy.

In an ICP temperatures reached are higher than those of FAAS and GFAAS (as high as 10000 K) and most of the refractory compounds are atomized efficiently. This allows the quantification of some elements with concentrations several orders of magnitude lower than the minimum concentrations quantifiable by FAAS. Not only ICP-OES but also ICP-MS allows the quantification of several elements per minute, being a technique faster than the previous ones. ICP-MS is the technique that offers the best detection limits for most elements, usually in range concentrations of ng/L (Thermo Elemental, 2001). Unlike the previously described techniques in which it is only possible to analyse one element at a time (mono-elemental), the ICP can analyse several elements in a few minutes (multi-elemental), with this characteristic and the detection limits being major factors that highlight this analytical technique. **Table 4** compares some of the characteristics of the mentioned analysis techniques.

***Table 4**: Comparison of some characteristics of elementary analysis techniques (adapted from* (Thermo Elemental, 2001; Tyler, 1994)*)*

	FAAS	**GFAAS**	**ICP-OES**	**ICP-MS**
Limits of detection	Very good for some elements	Excellent for some elements	Very good for most elements	Excellent for most elements
Analysis time	10-15 seconds per element	3-4 minutes per element	1-60 elements per minute	All elements in less than 1 minute
Applicable elements	68	50	73	82

Despite the advantages inherent to ICP techniques, they also have disadvantages such as higher costs for equipment and associated with its maintenance. However, when you want to analyse many elements in a short time and when you have many samples for analysis, the costs per analysis of an element are lower and the ICP compensates in terms of costs when comparing to other techniques (Thermo Elemental, 2001).

2. Objectives

The main objective of this work is to validate a methodology for quantifying macro, micro, and potentially toxic elements in food matrices through microwave-assisted acid digestion followed by quantification using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

Considering the main objective, some specific objectives to be achieved with this work are:

- Apply a methodology of treatment of food samples to selected matrices (chicken, mussels, fish, rice, and seaweed) and to certified reference materials of a similar matrix to the samples for quality control purposes;
- Evaluate quality control parameters associated with the treatment and analysis of food samples to validate the methodology and issue the corresponding declaration on the suitability of the method.

Another objective of this work, was to apply the validated methodology to different real food samples and to evaluate the influence of the processing (grilled, and boiled) on their concentrations of macro, micro, and potentially toxic elements.

3. Material and methods

3.1 Method validation: parameters evaluated

3.1.1 Indirect evaluation

The indirect analysis corresponds to the determination and evidence of the characteristic parameters of the method, which included the sensibility, selectivity, specificity and precision, among others. In this work the following parameters were evaluated.

- **Quantification**

The quantification of the method was determined by studying the calibration curves, analytical thresholds of the test method, and sensitivity.

Calibration curves

In quantitative analysis, calibration indicates a process by which the response of a measurement system relates to a known concentration or amount of substance (RELACRE, 2000). In the present work, standards with different concentrations were prepared through the dilution of standard stock solutions (IV-ICPMS-71A and IV-ICPMS-71B obtained from Inorganic Ventures^{TM}). To obtain the confidence interval on the calibration curve, 3 series of standards were prepared, and read independently (de Carvalho, 2007).

The number of standards used was never less than five, being evenly spaced within the working range, and for every 10 samples read, a reading of the highest and lowest standard concentration was made (Martins, 2015). The operating conditions of the analytical equipment was defined, in order to guarantee that both the standard solutions and the samples are analysed under the same operating conditions (RELACRE, 2000).

The calibration function of most analytical techniques is performed through linear calibration, which is obtained by adjusting a simple mathematical model through experimental data regarding the determination of the response of the analytical system to a given number of standards. Since the experimental values hardly fit the mathematical model, the methodology of minimising the sum of squares (SS) of the deviations between the data and the assumed model should yield the best estimate of the model parameters

(Almeida et al., 2002). This is called the "method of least squares" and the expression to be minimised is presented by **Equation 1**:

$$SS = \sum (y_{observed,i} - y_{predicted,i})^2$$

Equation 1

When using this method, and when the graphical representation is a line, the errors have a normal distribution and along the line there is homogeneity of variances (RELACRE, 2000). The equation of a calibration line is given by **Equation 2**:

$$y = a + bx$$

Equation 2

where *a* corresponds to the ordinate at the origin and *b* to the slope of the line. To be acceptable, the calibration curve must meet the following criteria: the correlation coefficient (ρ) must have at least two nines followed by a five; the graphical representation must be a straight line, with either a positive slope (ρ = +1) or a negative slope (ρ = -1) (Martins, 2015; RELACRE, 2000), that is represented on **Figure 5**.

In the least squares method, the horizontal axis (x-axis) always represents the concentrations of the standards and the vertical axis (y-axis) always represents the instrumental response of the equipment (signal), since it is assumed that the errors associated with the values of x are negligible compared to those of y.

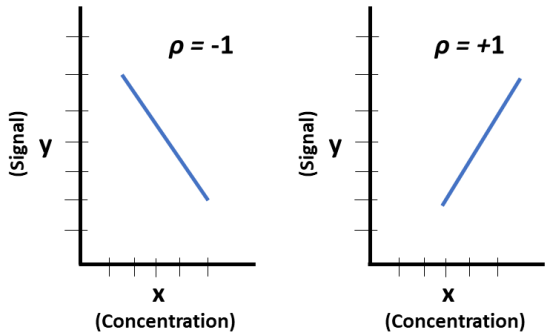

Figure 5: *Representation of positive slope and negative slope on a signal-concentration calibration curve*

The simple least squares method considers that, for each value of *x*, there is a subpopulation of y-values normally distributed, that the means of all the subpopulations of *y* lies on the same straight line and that all the subpopulations of y-values have equal variances (Almeida et al., 2002) (homoscedasticity). In statistics, homoscedasticity occurs when the variance in scores of one variable is somewhat similar at all the values of the other variable and in regression analysis, the assumption of homoscedasticity

occurs when at each level of the predictor variable, the residuals have similar variances (Issa and Nadal, 2011).

The homoscedasticity assumption was tested in the linear regression analysis. It was performed by plotting residuals versus concentration (Analytical Methods Committee, 1994) and by applying an F-test in accordance with **Equation 3**.

$$F_{exp} = \frac{s_2^2}{s_1^2}$$

Equation 3

where F_{exp} is the experimental value expressed as the ratio between the variances obtained at the lowest ($s\,_1^2$) and at the highest ($s\,_2^2$) concentration level of the working range. The tabled F-value is obtained from the F-table at a confidence level of 95 % for $f_1 = f_2 = (n-1)$ degrees of freedom (Almeida et al., 2002; Miller and Miller, 2010).

In ICP equipment's used in the present work, the calibration was performed using the weighted linear model. The use of this model was needed because there is no homogeneity of variances (homoscedasticity) in the instrumental signal. There are then differences in the variances of the lowest concentration and the highest concentration pattern (heteroscedasticity), and the use of linear regression using the least squares method is not appropriate in this situation. The regression line in weighted linear regression was calculated to give additional weight to those points where the error bars are smallest, as it is more important for the calculated line to pass close to such points than to pass close to the points representing higher concentrations with the largest errors (Miller and Miller, 2010).

The calculation formulas associated with the weighted model are like those of the unweighted linear model, however a weight factor, w_i, is introduced. The inverse of the variance (s_i^{-2}) associated with reading the signal of the calibration standards, will establish the weight that each of these standards will have in obtaining the calibration function. If the individual points (referring to each calibration standard) are represented by (x_1, y_1), (x_2, y_2), etc., as usual, and the corresponding standard deviations by s_1, s_2, etc., then the weights individual, w_1, w_2, etc., will be calculated using **Equation 4** (Miller and Miller, 2010).

$$w_i = \frac{s_i^{-2}}{\frac{\sum s_i^{-2}}{n}}$$

Equation 4

By using the n divisor in the denominator of the equation the weights have been scaled so that their sum is equal to the number of points on the graph: this simplifies the subsequent calculations (Miller and Miller, 2010). Since the weighted linear function is represented by $y = a_w + b_w x$ the slope (b_w) and the intercept (a_w) can be determined by **Equation 5** and **Equation 6**.

$$b_w = \frac{\sum_i w_i x_i y_i - n\bar{x}_w \bar{y}_w}{\sum_i w_i x_i^2 - n\bar{x}_w^2}$$

Equation 5

$$a_w = \bar{y}_w - b_w \bar{x}_w$$

Equation 6

where x_i is the individual concentration values, y_i is the individual signal values, $\bar{y}_w$ is the average values of weighted signal and $\bar{x}_w$ is the average values of weighted concentration.

Linearity

The ability of an analytical method to produce results that are directly proportional to the concentration of the analyte in the sample, in a given analytical range, is taken as the linearity of the method, which can be observed through a graph of the signal as a function of the concentration, and it was also determined through the calculation and analysis of the correlation coefficient. This last linearity test must be well interpreted, as the correlation coefficients are good indicators of correlation, but not necessarily of the linearity (RELACRE, 2000). According to the international standard ISO 8466-1, it can be evaluated by a statistical model, where a linear calibration function (ISO 8466-1) and the non-linear calibration function (ISO 8466-2) are calculated from a set of ordered pairs, as well as the respective residual standard deviations, $S_{y/x}$ and S_{y2} (RELACRE, 2000). If the linear regression line contains the origin, the method can be considered unbiased. Linearity deviations was verified by determining the residuals between the measured and calculated values, based on the regression line equation (Eurachem, 2012).

Work range

In any quantitative method there is a range of analyte concentrations within which the method can be applied, which is called the working range. The working range is the range in which the method provides results with a certain uncertainty, that was estimated,

and complied with the quality criteria accepted by the association of accredited laboratories in Portugal (RELACRE). The working range corresponds to the linear range of the calibration function and usually show homogeneity of variances in the dependent variable (Martins, 2016). The lower end of the working range was determined by the limit of quantification value and the upper end by the concentration at which the equipment fails to provide a linear response. During validation, it was necessary to confirm that the test method can be used in the working range defined, that is, the working range defined shows linearity (Magnusson and Örnemark, 2014).

The working range was determined by constructing a graph of the measured concentration as a function of the actual concentration of analyte in the analysed samples (calibration curve), then was necessary to resort to samples whose analyte concentration was strictly known (multi-element stock solutions). With this strategy, the working range of the method was that in which the determined concentration was not significantly different from the known actual concentration.

According to ISO 8466-1, 10 calibration points are recommended. In this work, at least five points were always considered, being distributed equally in the concentration range. The first and last standards were, most of the times, analysed in 10 independent replicates (RELACRE, 2000). The working range of each element was established for all matrices within the starting range of 0.15 µg/L – 110 mg/L. The method used was the same for all different food matrices, and even though the interferences and the method's ability to extract/recover the analyte may vary with the sample matrix, the working range was the same (Magnusson and Örnemark, 2014).

Here, the working range of the method was defined not only in µg/L, but also in µg/g (dry weight). Since the analyses carried out by ICP obtain concentrations in µg/L, it was necessary to convert these concentrations to mass/mass units. **Equation 7** show how this calculation to convert concentration is made for LOQ as an example. Considering that this method was carried out with digestions containing approximately 200 mg of sample (dry weight) for a final volume of approximately 25 mL, the limits of the method in mass/mass units were calculated according to the expression:

$$LOQ\left({}^{\mu g}/_{g}\right)(DW) = \frac{LOQ_{instrumental}\ (\mu g/L) \times V_{dilution}(L)}{m\ (g)}$$

Equation 7

where $V_{dilution} \cong 0.025\ L$ and $m \cong 0{,}2\ g$.

❖ Analytical thresholds

In Analytical Chemistry, the concepts of limit of detection (LOD) and limit of quantification (LOQ) are extremely important, since they allow the analyst to trace the analytical thresholds possible to achieve with the method to be used (Instituto Português da Acreditação, 2011; Martins, 2016, 2015; RELACRE, 2000). The analytical thresholds can be determined through the use of replicates of the blank, uncertainty of the parameters of the calibration curve, and through the uncertainty in the dispersion of the values around the calibration curve (Figueiredo, 2012).

In the interval between the limit of detection and the limit of quantification, numerical values should not be reported, as it is a semi-quantitative and non-quantitative detection zone. The laboratories must always consider the dilutions made on the samples, since the LOD and LOQ refer to the measured variable. In addition, these limits depend on factors that vary over time such as type of sample, contamination, operator, equipment used, among others. These limits should then be re-evaluated whenever there are variations in conditions (Instituto Português da Acreditação, 2011).

Limit of quantification

The limit of quantification (LOQ) corresponds to the lowest measured concentration at which it is possible to quantify the analyte with acceptable precision and accuracy. It can be determined by the measured content of blank analysis, by the calibration curve, or by defining that the LOQ corresponds to the calibration standard of the lowest concentration (excluding blank) (Martins, 2015; RELACRE, 2000). This last approach was used in the present work. After this threshold has been defined, it is necessary to ascertain whether the accuracy and precision found are satisfactory. It was ascertained with internal standards, under intermediate precision conditions, whose concentration is similar or equal to the limit of quantification. IUPAC recommends that the coefficient of variation ($CV = s/\bar{x}$) should not exceed 10 % (RELACRE, 2000).

Limit of detection

The limit of detection (LOD) is characterized by the minimum measured content, from which it is possible to detect the presence of the analyte with reasonable statistical certainty. This analytical threshold corresponds to the smallest amount of analyte that can

be detected in a sample, but not necessarily quantified as an exact value (RELACRE, 2000).

A reading below the LOD does not mean the absence of the analyte to be measured. It is only possible, through this reading, to state that, with a defined probability (normally 95 %) (Instituto Português da Acreditação, 2011), the concentration of the component in question will be below a certain value (RELACRE, 2000). For a more correct definition of the LOD, it is necessary to know two statistical concepts: type I and type II errors (Miller and Miller, 2010).

- type I error (risk **α**) – Is the probability of confirming the presence of the analyte under study in a sample, when, in fact, it is absent.
- type II error (risk **β**) – Is the probability of confirming the absence of the analyte under study in a sample, when, in fact, it is present.

For a more correct analysis of the analytical thresholds, these two types of errors should be minimized, preferably choosing to use the IUPAC recommendations ($\alpha = \beta =$ 5 %) (RELACRE, 2000)

The LOD can be calculated by different ways. The general case is where the LOD is calculated through blank replicates, since the reading of the solution blanks allows to evaluate the instrumental noise and to determine the concentration that gives an instrumental signal significantly different from the "background" signal of the equipment, whereas the reading of the digestion blanks allows to determine the concentration that gives an instrumental signal significantly different from the expected signal that may be caused by the contribution of digestion reagents and/or possible contamination that occurred during the stage of digestion or sample preparation. But LOD can also be calculated using a calibration curve, or through the lowest concentration standard of the calibration function. This last case was used in this work, where the LOD was 1/3 of the limit of quantification.

❖ Sensitivity

Sensitivity evaluates the method's ability to distinguish small differences in the concentration of the analyte, which can also be defined as the first order derivative of the calibration curve. This concept is often confused with the LOD. In the present work, the sensitivity was defined as the quotient of the value added to the read value (ΔL), and the variation in concentration (ΔC) to which the increase corresponds (**Equation 8**):

$$Sensitivity = \frac{\Delta L}{\Delta C}$$

Equation 8

When a linear model is used, as was the case of the present work, it is said that the sensitivity is constant over the working range and equal to the slope of the calibration curve (Martins, 2015; RELACRE, 2000). If the calibration function is not linear, the sensitivity will depend on the concentration of the analyte under study and will not have a constant value (Skoog et al., 2007).

The greater the variation in the value of the analytical signal measured with the addition of small concentrations of the analyte under study, the greater the sensitivity of the test method (Magnusson and Örnemark, 2014).

❖ Specificity/Selectivity

The determination of specificity and selectivity focuses on the interest in perceiving whether there are interferences in the method and, if so, its influence on the results, since the sample matrix may contain components that interfere with the measurement performance and interferences can affect the response (signal) (de Carvalho, 2007). Reagents, sample matrix, or other components can change the sensitivity of the detector, thus changing the signal (RELACRE, 2000) and so the matrices can directly affect and alter the sample signal (de Carvalho, 2007).

The ability of a method to distinguish and identify a particular analyte within a complex mixture, without interference from other components, is called selectivity (Harvey, 2000; RELACRE, 2000). The absolute absence of interference effects is considered to be the specificity of a method, therefore the specificity = 100 % selectivity (González and Herrador, 2007). As it is not feasible to consider all the potential interfering factors, in order to determine the selectivity, the study of the most probable cases in the present work was carried out using multi-element commercial standard solutions (González and Herrador, 2007; RELACRE, 2000).

Normally in food matrices, the quantification process is preceded by a sample digestion process, which is suitable for the matrix under study, which allows eliminating some types of matrix interferences, namely interferences of an organic nature. Most of the matrix interference effects are proportional to the obtained signal (proportional or rotational interferences) and cause a change in the slope of the calibration function (sensitivity). Fixed or translational interferences are independent of analyte concentration, influencing only the ordinate at the origin of the calibration function and

is often referred to as background or baseline interference (Magnusson and Örnemark, 2014).

One way to assess the presence of interference is through recovery tests, where samples are used, with the same matrix, whose concentration of the analyte varies in known proportions throughout the work range (performed with duplicates and under repeatability conditions) (RELACRE, 2000). The method is considered applicable (selective and specific) when a recovery rate close to 100 % is verified in the recovery tests. The range of the recovery rate depends on the applied methodology, where in some cases longer intervals are allowed, due to the studied characteristics of the method, while for others this admission cannot be tolerated (RELACRE, 2000).

In this work, selectivity was assessed through the study of matrix interference by performing recovery tests. Its concentration was previously determined and to these concentrations' volumes of solutions with known concentrations were added. The percentage of recovery was determined according to **Equation 9**:

$$Recovery\ (\%) = \frac{(C_f \times V_f) - (C_a \times V_a)}{(C_{add} \times V_{add})} \times 100$$

Equation 9

where C_f is the concentration of the analyte in the fortified sample, V_f is the volume of fortified sample, $C_a$is the concentration of the analyte in the sample, $V_a$is the sample volume, C_{add} is the concentration of the analyte in the fortifying solution and the V_{add} is the volume of added reinforcement solution.

Recovery tests were performed on different days using two commercial standards commercialized by Inorganic ventures: IV – STOCK - 2; 10 000 mg/L each of Ca; Mg; K; Na in HNO_3 2 %, and LCA-1; 250 mg/L of Fe; 100 mg/L each of Al, As, B, Ba, Be, Cd, Co, Cr(III), Cu, Li, Mn, Ni, Pb, Se, Sr, V, Zn in HNO_3 5 %. These multi-element standards were used to prepare the fortifications: A) 2 mL IV – STOCK – 2 + 1 mL LCA-1 + 7 mL HNO_3 1 %; and B) 1 mL IV – STOCK – 2 + 1 mL LCA-1. Different concentrations, which ensured results in the working range of the element of interest, were evaluated. Different fortifications were performed based on the fortification standards, where to 5 mL of each digested matrix, it was added 0.25 mL of standard A, 0.05 mL of standard A, or 0.1 mL of standard B.

❖ Precision

Precision is the parameter of the method that evaluates and determine the dispersion of the results obtained between independent tests, which are performed on the same sample, similar samples or on standards under pre-established conditions. The precision of the method may change over the range of concentrations (Martins, 2015). It is important to emphasize that it was more realistic to study the precision on samples, to minimize matrix effects (RELACRE, 2000).

Precision can be assessed using two extreme measures, repeatability, and reproducibility. Among these two extreme measures there is an intermediate situation known as intermediate precision or intra-laboratory variability (RELACRE, 2000). Precision normally variates with the work range, since it depends on analyte concentration. In this work, the precision was evaluated through repeatability and intermediate precision studies.

Repeatability

Repeatability refers to precision when the assay is performed under identical conditions, then in tests carried out on the same or identical samples, in the same laboratory by the same operator using the same equipment and the same reagents within a short period of time (RELACRE, 2000). Repeatability can be evaluated in the laboratory itself through tests, or it can be evaluated through an interlaboratory test. The determination of repeatability demands, at least, 10 tests when evaluated in the laboratory, which were performed in this work for each food matrix under study. Matrices samples were weighted (10 replicates) and digested at the same day and digestion cycle.

The repeatability limit ($\boldsymbol{r}$) is the maximum allowable value for the absolute difference between two tests, under repeatability conditions, determined for the 95 % confidence level, through **Equation 10**:

$$r = t \times \sqrt{2} \times S_{ri} = 1.96 \times \sqrt{2} \times S_{ri}$$

Equation 10

where S_{ri} are the standard deviation of repeatability associated with the results considered. It was important to consider the repeatability variation coefficient, $\boldsymbol{CV_r}$, which is numerically equal to the ratio between the repeatability standard deviation ($\boldsymbol{S_{ri}}$) and the average of the results obtained, $\boldsymbol{\bar{x}}$, according to the **Equation 11**:

$$CV_r = \frac{s_{ri}}{\bar{x}} \times 100$$

Equation 11

Intermediate precision

Intermediate precision refers to the precision assessed when the test is performed on the same sample, identical samples, or standards, following the same method in the same laboratory or in different laboratories, but it is necessary to define the conditions to vary, if the operator, equipment, or the time when the test is performed. This form of precision evaluation is characterized as the most representative of the variability of the results, being therefore the most recommended to be used (RELACRE, 2000).

A widely used resource for determining intermediate precision is the amplitude control charts (RELACRE, 1998), since there is a considerable random variation of experimental parameters when tests are carried out on different days, which can affect the performance of the method (RELACRE, 2000). In most cases, the intermediate precision value is a function of the concentration level of the assay, and its calculation is done, preferably, from the results obtained, after eliminating the discrepant results (INMETRO, 2016).

To determine the intermediate precision in the present work, results from duplicates of the different matrices analysed at different days were considered (November 2020 and April 2021). It was important to consider the intermediate precision variation coefficient, $\boldsymbol{CV_{SI}}$, which is numerically equal to the ratio between the intermediate precision standard deviation ($\boldsymbol{S_I}$) and the average of the results obtained, $\boldsymbol{\bar{x}}$, according to **Equation 12**:

$$CV_{SI} = \frac{s_I}{\bar{x}} \times 100$$

Equation 12

3.1.2 Direct evaluation

The direct evaluation aims to know the accuracy of the test method. Accuracy is defined as the agreement between a measured value and the reference value conventionally accepted as true (Instituto Português da Qualidade, 2012). The term accuracy, when applied to a series of test results, implies a combination of components of random errors, and components of systematic errors. The processes normally used to

assess the accuracy of a method are, among others, the following: certified reference material (CRM), interlaboratory comparison test (ICT) and comparative tests (RELACRE, 2000).

❖ Certified reference material

Certified reference materials (CRMs) are an important tool in the external quality control of a chemical analysis and should be used whenever possible in the method validation process (INMETRO, 2016), as they serve to verify quality and traceability both at the metrology level and at the validation level (RELACRE, 2000). The CRMs are accompanied by a document issued by a recognized entity, which provides one or more values of concentration (or other magnitude) specified for each parameter with respective associated uncertainties and traceability, using valid procedures (Instituto Português da Qualidade, 2012; RELACRE, 2000).

When talking about a multi-component CRM it may happen that not all parameters have uncertainty attributed. The acquisition of an CRM will have to be made from a recognized and credible supplier body such as: National Institute of Standards and Technology (NIST), US Environmental Protection Agency (EPA), Institute for Reference Materials and Measurements (IRMM), National Research Council Canada (NRC), International Atomic Energy Agency (IAEA), among others (RELACRE, 2000, 1996). The correct use of the CRM consists of its analysis to assess the performance of the Laboratory. The value obtained in the analysis of an CRM was compared with the certified value (Sutarno, 1985; Valcárcel and Ríos, 1995). When the value obtained is not within the range of uncertainty indicated for the certified value, the causes of this deviation must be sought and eliminated or accepted (RELACRE, 2000). The CRMs used in this work were NCS ZC73016 (chicken), ERM® - CE278k (mussels tissue), ERM® - BB422 (fish muscle), DORM-4 (fish protein), NCS ZC73028 (rice), NIST SRM 8433 (corn bran), NIST SRM 1547 (peach leaves), and BCR 060 (aquatic plant - *Lagarosiphon major*). Each CRM was evaluated at least 3 times.

There are several ways to evaluate the results generated in the analysis of certified reference material, among them: hypothesis test (t test), relative error, performance factor Z (Z score), and normalized error (RELACRE, 2000). In this work it was used the relative error to evaluate the CRM results.

Relative error

The calculation of the relative error is a way of evaluating the accuracy of a method (E_r), which is expressed as a percentage (%). The relative error was calculated by **Equation 13** (Bennett and Briggs, 2011; RELACRE, 2000):

$$E_r = \frac{(X_{lab} - X_v)}{X_v} \times 100$$

Equation 13

where Xl_{ab} is the value obtained experimentally, that is, the arithmetic average of values obtained and X_v is the value accepted as true, that is, the certified value of the CRM. The component of systematic errors is expressed through the relative errors, and each Laboratory must define its degree of demand in terms of the accuracy of the method under study (Miller and Miller, 2010; RELACRE, 2000).

❖ Measurement uncertainty

Measurement uncertainty (MU) is a non-negative parameter that characterizes the dispersion of the values assigned to a measurand, based on the available information used (Instituto Português da Qualidade, 2012). The measurement uncertainty is not a characteristic of the method, actually is the property of a measurement result. But if a method is under sufficient statistical control, some indicative estimates of MU of typical measurement results can be done (NATA, 2018).

A measurement uncertainty estimative should consider all the effects that operates on the final result. Measurements uncertainties associated with each effect are combined according to well-established procedures (Magnusson and Örnemark, 2014). The standard uncertainty is considered equal to the standard deviation of the series of measurements of the analyte, while the expanded uncertainty is considered as two times the standard uncertainty (coverage factor) (Magnusson and Örnemark, 2014). The expanded uncertainty is expected to encompass about 95 % of similar future measurements (AOAC, 2002).

Generally the most useful sources of information about measurement uncertainty are: a) statistics from collaborative trials, that may not be available in many situations of single-laboratory method validation; b) statistics from proficiency tests; c) results from the analysis of certified reference materials (Thompson et al., 2002).

For method validation if appropriate certified reference material are available, single-laboratory test can be done, allowing a laboratory to evaluate both, the laboratory bias and method bias, through analysis of the CRM *n* times (Thompson et al., 2002).

The measurement uncertainty can be calculated by several ways, where two main approaches are distinguished: bottom-up and top-down. Bottom-up approach consists of identifying all the characteristics of the analytical method that can contribute to the measurement uncertainty of the result, that way estimating the contributions of each one, assigning a numerical value, and combining them to obtain a final value. Top-down approach aims to simplify the measurement uncertainty estimate, that way reducing the total calculations, by grouping some measurement uncertainty terms into a single term, *e.g.* a measurement uncertainty term associated with precision that corresponds to the contribution of several sources of uncertainty. Consequently, the total number of terms to be considered in the estimation of global measurement uncertainty in the top-down approach becomes smaller. Each strategy has its advantages and disadvantages. The choice of the most appropriate depends on the characteristics of the analytical method (IPAC, 2007; Magnusson and Örnemark, 2014).

The methodology adopted in the present work to estimate the uncertainty component associated with the quantifications in food was the analysis of the certified reference material according to ISO 11352: 2012 (IPAC, 2007).

Measurement uncertainty was evaluated by both the component of systematic effect and the component of random effect. The component associated with the systematic effects was estimated based on the trueness of the method, whereas the component associated with random effects was estimated based on precision. At the end both components were combined to calculate and estimate the expanded uncertainty.

The component of the uncertainty associated with precision (u_r), that is expressed in the form of relative standard uncertainty was calculated using the determination of the variation coefficient of the measurements made in the CRM under repeatability conditions as represented by **Equation 14**:

$$u_r = \frac{s_r}{\bar{x}}$$

Equation 14

where s_r is the standard deviation of the results obtained in the CRM analysis and $\bar{x}$ is the mean concentration obtained at the CRM analysis.

The component of the uncertainty associated with trueness (u_b), expressed in the form of relative standard uncertainty, was calculated considering three components according to **Equation 15**, **Equation 16** and **Equation 17**:

1) The relative standard uncertainty of the CRM reference value ($u_{Cref,rel}$):

$$u_{Cref,rel} = \frac{U_{CRM}/k}{C_{ref}}$$

Equation 15

2) The relative error associated with the CRM readings (b_{rel}):

$$b_{rel} = \frac{\bar{x} - C_{ref}}{C_{ref}}$$

Equation 16

3) The variation coefficient of the CRM values measured (CV_b) that is equal to the precision component:

$$CV_b = u_r$$

Equation 17

After obtaining these data, it was possible to calculate the uncertainty component associated with trueness (u_b), which is expressed in the form of standard uncertainty, calculated according to **Equation 18**:

$$u_b = \sqrt{b_{rel}^2 + \left(\frac{CV_b}{\sqrt{n_M}}\right)^2 + u_{Cref,rel}^2}$$

Equation 18

where U_{CRM} is the expanded uncertainty associated with each analyte in the CRM, k is the expansion factor referred in the CRM analysis certificate, C_{ref} is the concentration indicated in the CRM certificate and n_M is the CRM reading number.

After calculating the relative uncertainty for both components, it was possible to calculate the combined standard uncertainty (u_c), which is expressed in the form of relative standard uncertainty and is given by the contribution of random effects and systematic effects. The combined standard uncertainty was determined according to **Equation 19**:

$$u_c = \sqrt{u_r^2 + u_b^2}$$

Equation 19

The expanded uncertainty (U) (**Equation 20**), that is expressed in the form of relative standard uncertainty, was obtained by multiplying the standard uncertainty by an expansion factor k = 2, to obtain a confidence level of about 95 %.

$$U = k \times u_c$$

Equation 20

At the end of the validation process, a declaration on the validity of the method must be issued, for every element and every technique used for the method validation, detailing the respective requirement values and values obtained for each validated parameter. This declaration is the main document that confers the suitability of the method to its intended use (International Organization for Standardization, 2018). The example of the declaration of validation used in this work is shown in **Table 5**.

Table 5: Declaration used in this work that is according to ISO/IEC 17025 and confers the suitability of the validated method to its intended use

Declaration on the suitability of the method on the intended use

Matrix: XXX

Assay: XXX

Requirements for assay method					
Characteristic parameters			**Requirement**	**Obtained value**	**Observations**
Specificity / selectivity		Matrix interference (recoveries)	80-120 %		Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix		n.a.
Quantification		Correlation coefficient (r)	> 0.995		Minimum value obtained
		Sensitivity / Slope	n.a.		n.a.
		Work range	n.a.		n.a.
		Limit of quantification	Depends on the element		*
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.		n.a.
		Coef. of variation of repeatability	≤ 10 %		*
		Duplicate evaluation (solutions)	n.a.		n.a.
		Intermediate precision	≤ 10 %		Maximum value obtained for a sample
	Trueness	Recovery trials	80-120 %		Minimum and maximum value obtained.
		Interlaboratory Comparison Test	< 2		Maximum value obtained in the interlaboratory comparison test
Uncertainty			n.a		ISO 11352

* Values obtained must be in accordance with the legislation

End of validation: DD/MM/YY

<u>Declaration on the suitability of the method on the intended use</u>

The results obtained in the validation of the test method "XXX" demonstrated that the method is suitable for the intended use.

The person in charge of the Laboratory

3.2 Material washing

As the ICP techniques have high sensitivity and some chemical elements are found in trace amounts in the food, for its determination it is essential to avoid contamination of the samples and possible losses of elements to be analysed, due to adsorption phenomena. It is necessary and important that all the material used during the execution of the method is properly cleaned, since dust in the laboratory environment, impurities in the reagents used and impurities that remained in the laboratory material after use are potential sources of contamination. The washing procedures adopted in the scope of this work for the *teflon* microwave vessels (polytetrafluoroethylene - PFA) was carried out according to the following procedure: washing of digestion vessels with 3 % (v/v) Decon detergent solution and tap water; after dried, addition of 10 to 20 mL of 50 % (v/v) aqueous HNO_3 solution to each microwave vessel and execution of the suitable microwave heating program for washing (the program depends on the type of microwave vessel); subsequent washing with ultrapure water at least 5 times (resistivity of 18 MΩ/cm); dry the vessels with the container openings facing downwards at room temperature on a plastic tray lined with absorbent paper.

Plastic and other materials used were previously washed according to the following procedure: initial washing with 3 % (v/v) Decon detergent solution and subsequent washing with tap water; following the immersion in the same 3 % (v/v) Decon detergent solution for at least 24 h, and subsequent washing with tap water; immersion in 50 % (v/v) HNO_3 solution for at least 24 h; final washing with ultrapure water; dry with the container openings facing downwards, at room temperature, in a plastic tray lined with absorbent paper.

3.3 Equipments

3.3.1 Homogenization system

Samples were homogenized through the use of an agate mill (mortar grinder) and through a coffee grinder. The RM200 can mix and homogenize powders, suspension, and pastes, even for samples with high viscosity, milling through pressure and friction. This combination of two loading mechanisms enables both soft and hard, brittle material to be crushed, milled, and mixed in this machine. Both dry and wet milling operations can be carried out in the RM200 (Retsch GmbH, 2020). Samples that could not be homogenized by RM200 were homogenized in a coffee grinder (SCM 2930) (**Figure 6**).

Figure 6: *Retsch RM 200 mortar grinder (left picture) and SCM 2930 coffee grinder (right picture) used to homogenize food samples*

3.3.2 Quantification

The quantifications performed in the scope of this work were made by inductively coupled plasma mass spectrometry technique (ICP-MS). The ICP-MS equipment used was a Quadrupole Thermo Scientific X Series (**Figure 7**), equipped with a Peltier Nebulizing Camera, a Burgener Nebulizer, and nickel cones. The detector is an electron multiplier. The operation of the ICP-MS equipment is a complex process that requires a qualified technician, so the analysis of the samples in the scope of this work were carried out by a specialist technician. The Argon flow was 13 L/min, nebulizer flow was 0.95 L/min, and the auxiliary gas flow was 0.90 L/min, working at 1400 W. The sample flow was 1 mL/min.

Figure 7: *ICP-MS Thermo X Series (Thermo Scientific)*

❖ Inductively Coupled Plasma Mass Spectrometry

The inductively coupled plasma (ICP) is an important instrument for elemental analysis, not only as a radiation source for optical emission spectrometry (OES), but also as ion source for mass spectrometry (MS) (Montaser and Golightly, 1992). Sensitivity and selectivity are characteristics that make techniques using coupled plasma stand out, especially when it comes to trace and ultra-trace elements (Montaser and Golightly, 1987; Mora et al., 2003). Sources from ICP allows the atomization and almost the complete ionization of all elements in a wide range of samples (Ammann, 2007). The ICP techniques is considered a 'hard' ionisation technique, since it completely atomises most molecules in the sample (Pitt, 2009; Wilschefski and Baxter, 2019).

Inductively coupled plasma is a highly ionized gas that consists of energetic electrons, ions and atoms, which is sustained by a continuous supply of energy to a flowing gas, but despite the high population of ions and electrons, the plasma is macroscopically neutral (De Silva and Gregoire, 1998). Although helium plasmas have been reported (Chan et al., 1986; Jorabchi et al., 2006; Montaser et al., 1987), argon is preferred as the cost of helium is prohibitive (Wilschefski and Baxter, 2019). The argon plasma induced by commercial ICP instruments has a gas kinetic temperature of 6000 to 10000 K, what makes it possible the fast desolvation, vaporization, atomization, and ionization of analytes (Templeton, 1994).

The plasma is formed in the end of a set of three concentric quartz tubes, referred as the torch (**Figure 8**) to where argon flows through. A tangential flow of argon, concentric to the injector, is called the auxiliary gas, which forms the plasma. The far end of the torch is surrounded by a copper induction coil or also known as 'load coil', which is connected to a radio frequency (RF) generator. The RF generator supplies power to the load coil, which creates a high-frequency alternating current that induces a time-varying electromagnetic field in the torch (Wilschefski and Baxter, 2019).

With argon gas flowing through the torch, a high-voltage discharge is applied, which ionises a fraction of the argon atoms generating in that way ions and electrons that collide with argon atoms. If sufficient energy is generated by these collisions, additional atoms are ionised creating electrons and ions, which propagates the cascade. The movement of electrons and ions in the torch generates a tremendous amount of heat resulting in temperatures up to 10000 K (Templeton, 1994).

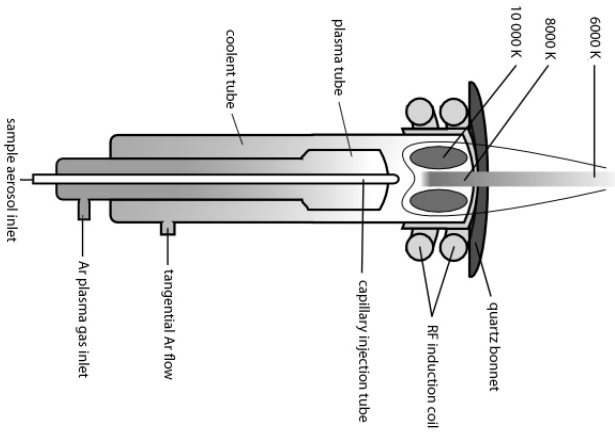

***Figure 8**: Inductively coupled plasma torch (retrieved from Chemistry LibreTexts, (2019))*

After the sample is nebulised, the tertiary aerosol exiting the spray chamber is swept in a stream of argon gas along the injector and into the plasma. After reaching the high-temperature plasma, the sample is desolvated, vaporised, atomized, and ionised. Due to the high temperature achieved by plasma most elements in periodic table can be almost completely ionised with a first ionization potential up to 10 eV (Pupyshev et al., 1999). Most elements form singly charged positive ions, however some elements may also form a small fraction of double charged ions (Pupyshev and Semenova, 2001).

Co-axial nickel cones separate the plasma from the mass spectrometer vacuum chamber (**Figure 9**). Ions, photons and neutral atoms or molecules are extracted from the plasma into the interface region via a small orifice, approximately 1 mm diameter, at the tip of the sample cone (Wilschefski and Baxter, 2019).

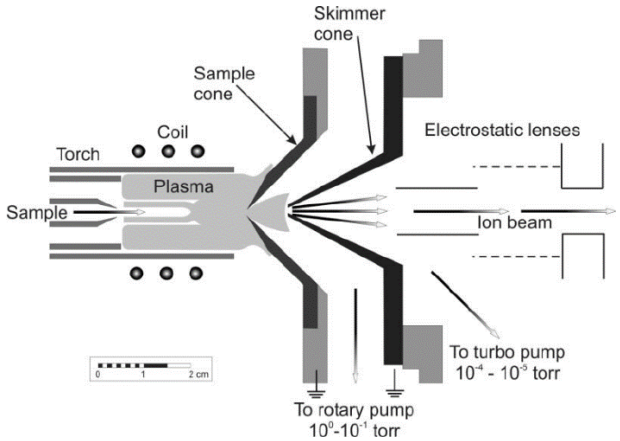

***Figure 9**: Cross section schematic of the interface region (retrieved from Košler and Sylvester (2003))*

As ions enter this interface region, a severe reduction in pressure causes an expansion of the ions, which generates a free jet (Farnsworth and Spencer, 2017). Extraction of ions are made through an even smaller orifice, approximately 0.45 mm

diameter, in the skimmer cone, and into the main vacuum chamber (Wilschefski and Baxter, 2019). At this pressure, ions can be guided effectively by charged surfaces called electrostatic lenses (ion optic system). The main role of the ion optic system is to guide the ion beam toward the mass analyser, preventing photons and other neutral species from reaching the detector. (Thomas, 2004).

After passing through the ion optics system, ions arrive at the mass analyser that in most ICP-MS equipment quadrupole is the mass analyser that separate the ions (Skoog et al., 2007; Templeton, 1994; Wilschefski and Baxter, 2019).

A quadrupole is a mass filter that separate ions based on their mass/charge (m/z) ratio (**Figure 10**) (Thomas, 2004). The operation of a quadrupole is made by placing an alternating current (AC) and direct current (DC) radio frequency potentials on opposite pairs of rods, which creates a time-varying electric field in the centre through which the selected ion pass to the detector (Thomas, 2004; Wilschefski and Baxter, 2019). Ions that have unstable trajectories collide with the rods not reaching the detector.

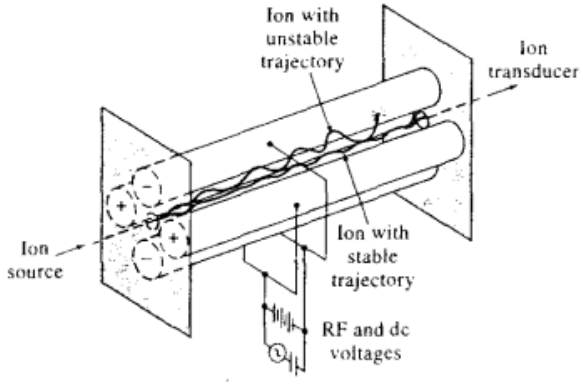

Figure 10: *A quadrupole mass analyser (retrieved from Skoog et al.* (2007)*)*

Ions are converted into electrical pulses by the detector which are then counted using its integrated measurement circuitry (Thomas, 2004). The magnitude of the electrical pulses corresponds to the number of analyte ions present in the sample, which is then used for element quantification by comparing the ion signal with known calibration or reference standards (Thomas, 2013). The most common detector used for ICP-MS is an electron multiplier (EM) (Wilschefski and Baxter, 2019). The high negative potential of the cone attracts the ions, when they emerge from the quadrupole mass analyser, (Thomas, 2013). The ion impact on the detector causes the emission of electrons from the surface, which, in turn, strike the next dynode releasing more electrons. Therefore, EM can generate a measurable signal pulse from the impact of a single ion on the detector, conferring very high analytical sensitivity (Wilschefski and Baxter, 2019).

Detection limits in ICP-MS are greater than flame atomic absorption and are comparable or even superior to graphite furnace atomic absorption (Thomas, 2004; Tyler, 1994; Wilschefski and Baxter, 2019). Typical limits of detection in ICP-MS are in the sub parts per trillion (ppt) range, but also enables quantitation at the high parts per million (ppm) level (Thomas, 2004). ICP-MS can be considered as one of the leading technologies in elemental analysis, which focus on the determination of ultra-trace levels of metals and metalloids in various sample types (Balcaen et al., 2015; Jin et al., 2020).

Interference

There are two classifications of interferences in ICP-MS, namely spectroscopic interference, and non-spectroscopic interference. When non-analyte ions have the same m/z ratio as the analyte there are spectroscopic interference (Templeton, 1994; Wilschefski and Baxter, 2019). Non-spectroscopic interference are the effects related with instrument drift and/or related to sample matrix (Wilschefski and Baxter, 2019). Spectroscopic interferences are divided into four groups: isobaric elements, double charged ions, polyatomic ions and tailing interference.

Elemental isobaric overlaps arise when two isotopes of different elements have the same m/z ratio to within the resolution of the mass spectrometer (*i.e.* $^{204}Hg/^{204}Pb$, $^{115}Sn/^{115}In$, $^{114}Sn/^{114}Cd$, $^{87}Rb/^{87}Sr$, $^{48}Ca/^{48}Ti$ and $^{40}Ca/^{40}Ar$) (Rowley, 2000; Wilschefski and Baxter, 2019).

Most elements form singly charged ions in the ICP, however elements with a second ionisation potential lower than the first ionisation potential commonly form double charged ions (Rowley, 2000; Wilschefski and Baxter, 2019). Elements most likely to form M^{2+} species are the alkaline earth elements, the rare earth elements, and elements such as U and Th (Pupyshev and Semenova, 2001). The formation of a doubly charged ion results in a loss of sensitivity for the singly charged species and generates an isotopic overlap (Rowley, 2000).

The most problematic type of spectroscopic interference is from polyatomic ions (Rowley, 2000; Wilschefski and Baxter, 2019). Polyatomic ions form in the high-temperature plasma, either due to incomplete atomisation or from recombination reactions during the extraction of ions into the mass spectrometer and comes typically from precursors in argon (*i.e.* Ar, O, H), atmospheric gases (*i.e.* N, O) and acids that were used during sample preparation (*i.e.* Cl, P, S, N) (Rowley, 2000). A way to monitor polyatomic ion formation in ICP-MS is measuring the degree of oxide formation, where

a tuning solution that contains cerium is normally used, since the oxide bond formation is strong and has a high oxide rate formation (Wilschefski and Baxter, 2019).

As previously referred non-spectroscopic interference are divided into matrix effects and instrument drift. Matrix effects can be defined as an enhancement or, more commonly, suppression of an analyte signal due to properties or constituents of the sample matrix (Karandashev et al., 2016). These effects are thought to arise from a complicated interplay of various mechanisms which occur in nearly all components of the instrument (Kim et al., 1990).

Instrumental drifts can occur over time, where dissolved solids in samples can deposit in the nebuliser and/or interface cones reducing ion transmission into the mass spectrometer by occluding the orifice, leading to signal suppression (Wilschefski and Baxter, 2019). By the time pass the degree of occlusion will increase causing a downward drift in signal, which will cause low results if not corrected (Douglas and Kerr, 1988; Wilschefski and Baxter, 2019). Instrumental drifts can also occur due to changes in room temperature, that could affect the spray chamber temperature leading to instrument drift. A well-controlled air-conditioning in the laboratory and periodic maintenance of ICP-MS equipment is essential to reduce instrumental drift (Wilschefski and Baxter, 2019).

❖ Element quantification by ICP

In this validation study, the isotopes used for the quantification of macro, micro, and potentially toxic elements were ^{7}Li, ^{9}Be, ^{11}B, ^{23}Na, ^{25}Mg, ^{27}Al, ^{28}Si, ^{31}P, ^{39}K, ^{44}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{85}Rb, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{118}Sn, ^{123}Sb, ^{137}Ba, ^{208}Pb, and ^{238}U and for the quantification of REE ^{139}La, ^{140}Ce, and ^{146}Nd. Polyatomic and isobaric interferences were minimized by setting the ratios $^{137}Ba^{++}/^{137}Ba$ and $^{140}Ce^{16}O/^{140}Ce$ to 0.010 under routine operating conditions (Carvalho et al., 2020). Interferences of polyatomic ions formed in the ICP-MS source were corrected mathematically when values exceeded the LOD of the analyte, considering an interference concentration that doubled the upper values found in the analysed samples.

The optimization aimed to choose the ideal analysis conditions for the day. The analytical calibration was made by reading the blank and the calibration standards previously prepared. The calibration function was defined based on standards that showed a relative error less than 10 %. The analysis of digestion samples was made always with control solutions between samples to guarantee the validation of results.

To proceed with the quantification of the samples in the ICP-MS, the mass/charge that corresponded to the isotope of the analytes of interest were selected. Then, the

calibration was carried out by reading the blank and the calibration standards. The reading of the samples interspersed with solutions was performed to control the quality of the results obtained.

3.4 Reagents and solutions

The water used to wash the material, make the dilutions, and prepare the standards solutions was of class 1, where the ultra-purification system, Helix coupled to a Milli-Q Element (Millipore), guarantees resistivity of less than 18.2 MΩ/cm and total organic carbon ≈ 1 µg/L. All reagents used in this work were of analytical grade obtained from certified sources with p.a. purity grade. Nitric acid 68 % (m/m) and hydrogen peroxide 30 % (m/m) were purchased from VWR Chemicals® and were used in the digestion procedure.

To assess matrix interferences in ICP-MS two multi-element standards were used to prepare two fortification standards. Both multi-element standards were purchased from Inorganic Ventures™. For macro elements IV-STOCK-2 (10 000 mg/L) was used and for micro and potentially toxic elements the standard used was LCA-1 (250 mg/L of Fe; 100 mg/L for all other elements) (**Figure 11**). In order to prepare the calibration standards, the multi-element commercial standards IV-ICPMS-71A and IV-ICPMS-71B from Inorganic Ventures™ were used.

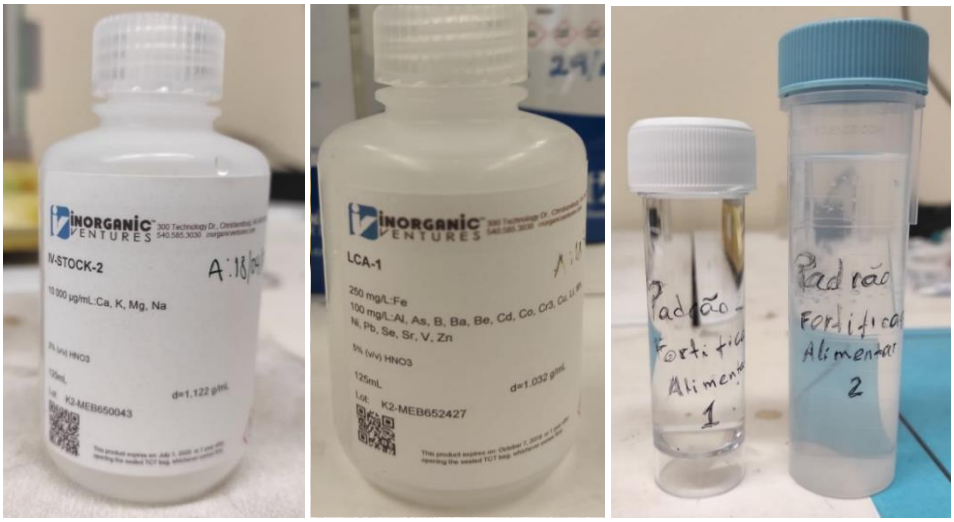

Figure 11: *Multi-elemental standards (left and middle picture) used to prepare the fortification standard (right picture)*

A 10 µg/L solution of Ba, Be, Ce, Co, In, Li, Pb, and U from Thermo Scientific was used as tuning solution to adjust the optic system and the nebulization conditions for ICP-MS (Carvalho et al., 2020). A 10 µg/L solution of ^{115}In was used as internal standard.

The solution prepared for the determination of instrumental noise (blank), and for preparing the calibration standards, was 1 % (v/v) nitric acid solution, resulted by a dilution of 65 % HNO_3 dilution, in ultrapure water. The washing solution, used between different sample readings, was a 2 % (v/v) nitric acid solution. All calibration standards used in ICP-MS were prepared by diluting a stock solution from a commercial multi-element solution.

3.5 Food samples preparation method

In this work, two types of food samples were considered: certified reference materials (CRM) and real food samples (chicken, mussels, fish, rice, and seaweed), which enabled the evaluation of the different performance characteristics of the method under study.

Different reference materials were used in the scope of this work to evaluate the several food matrices under study and the development of the method for each matrix. Eight CRMs were used taking into account the food matrix to be studied, these being: NCS ZC73016 (chicken), ERM® - CE278k (mussels tissue), ERM® - BB422 (fish muscle), DORM-4 (fish protein), NCS ZC73028 (rice), NIST SRM 8433 (corn bran), NIST SRM 1547 (peach leaves), and BCR 060 (aquatic plant - *Lagarosiphon major*) (China National Analysis Center for Iron and Steel, 2010, 2006; Institute for Reference Materials and Measurements, 2013a, 2013b; National Institute of Standards and Technology, 2019, 2008) represented in **Table 6**.

Samples of chicken, mussels, fish, rice, and seaweed were purchased from a supermarket in its raw state, where some amount of the chicken, mussels and fish were frozen at -20 ºC, for later lyophilization. The rice and the seaweed were directly homogenized in an agate mill and a coffee grinder until it became a dry powder. The chicken, mussel and fish after freeze drying were homogenized in a shredder until it was as close as possible to a powder, however these materials showed a degree of homogeneity less than the CRM because their granulometry was slightly higher.

Table 6: Food matrices and certified reference materials studied on this work

Food matrices	Certified Reference Material
Chicken	NCS ZC73016
Mussel	ERM-CE278k
Fish	ERM-BB422
	Dorm-4
Rice	NCS ZC73028
	NIST SRM 8433
Seaweed	NIST SRM 1547
	BCR 060

Samples of chicken, mussels, fish, and rice previously acquired were separated for processing. Chicken, and fish were boiled and grilled, rice and mussels were boiled. After processing samples were stored at -80 ºC. Tissue samples from all matrices were lyophilized and then ground to be homogenized.

As seen in **Table 3**, there are several methodologies for the digestion of food samples. In this work, the selected methodology consisted of using the smallest volume of concentrated reagents, being in accordance with Gholami et al. (2016), Motta et al. (2020), and Vieira et al. (2018). The mass used was the maximum allowed by the digestion vessel.

Before initiating the weighing process the CRM and the food samples had to be stirred for a few minutes to homogenize the material and ensure that the sampling performed was representative. About 200 mg of food sample and CRM were weighed directly into *teflon* vessels, that were coated with aluminium foil to minimize static electricity that can cause dispersion of the material, which could be then attached to vessel walls. The microwave digestion vessel used was MarsXpress™ digestion vessel (**Figure 12**). MarsXpress™ vessel is designed for microwave digestion in a Mars 5 or Mars 6 with iWave® or IR temperature control, being a vessel well suited for digestion of samples as: environmental samples, plant and animal tissue, mixed food, industrial hygiene, and consumer products (CEM Corporation, 2019a).

Figure 12: *MarsXpress™ vessel that is composed by a liner (1), a vessel plug (2), a cap (3), and a sleeve (4)*

To maintain quality control, in each digestion cycle a blank were also run following the same digestion procedure. After sample weighing, 2 mL of concentrated HNO_3 and 1 mL of concentrated H_2O_2 were added to each vessel. Subsequently, the vessels were properly closed and accommodated in the turntable (**Figure 13**) that posteriorly was placed in the microwave oven (CEM Mars 5). The microwave accelerated reaction system, CEM Mars 5, is designed for digesting, dissolving, extracting, and hydrolysing a wide variety of materials in a laboratory setting (Traces Centre, 2020). The system uses microwave energy to heat samples rapidly and at elevated pressures, which leads the sample to digest or dissolve in a short time (CEM Corporation, 2009). Microwave digestion greatly enhances the destruction of chemical bonds thereby achieving a faster digest (CEM Corporation, 2015). Its main purpose is for preparing samples for analysis by atomic absorption (AA), inductively coupled plasma emission spectroscopy (ICP), gas or liquid chromatography (CEM Corporation, 2009). The microwave oven used for the digestion of the samples was a CEM Mars 5 (**Figure 14**), equipped with 24 pressurized vessels.

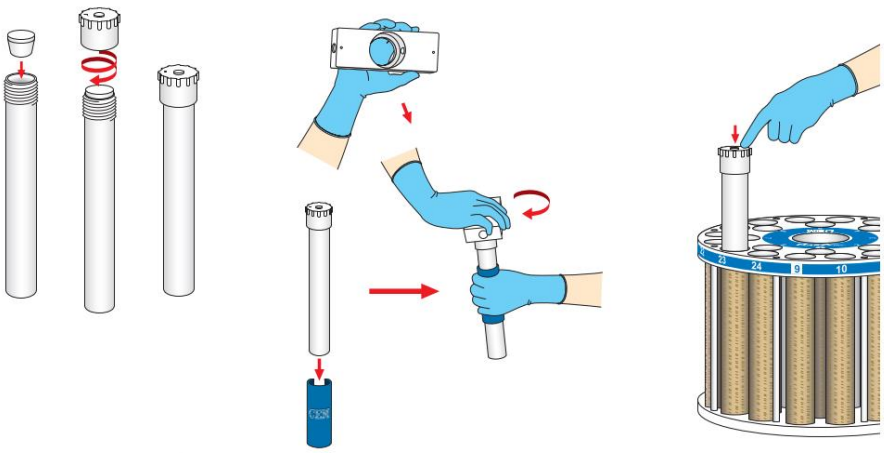

Figure 13: *Assembly of MARS Xpress Vessels (retrieved from CEM Corporation* (2015)*)*

Figure 14: *Microwave oven, model CEM Mars 5 240/50, used for the digestion of samples*

The vessels were then subjected to a digestion program consisting of two stages: the first one with the temperature ramping to reach 170 ºC ± 5 for 15 minutes and the second was holding this temperature for 10 minutes, at the maximum power of the microwave (1600 W), since more than 7 vessels were used in each digestion cycle (CEM Corporation, 2019b). At the end of the digestion cycle, the turntable was placed in the hood until the vessels cooled down, so that they could be opened. The digestion solutions were collected and diluted with ultrapure water to the final volume of 25 mL, obtaining a final acid percentage of approximately 2 % (v/v).

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4. Results and discussion

To ensure food quality, validated methods of analysis are crucial to quantify macro, micro, and potentially toxic elements, to assure that the values found are true. The matrices used in this work were selected based on food diversity and nutrition levels, as well as on the research on food matrices that can be accredited by IPAC.

The method used to analyse the chosen food matrices, like chicken, mussels, fish, rice, and seaweed, allowed to quantify 32 elements, but only As, Ca, Cd, Cu, Fe, Mg, Mn, Na and Zn were here validated using ICP-MS. Validation of the proposed method was done by imposing criteria accordingly to the association of accredited laboratories in Portugal (RELACRE). It will be presented the example of the work developed to validate the quantification of copper by ICP-MS. The remaining elements validated followed an identical process of analysis and treatment of the results and their main results are shown in the Annexes.

4.1 Method validation for food matrices by ICP-MS

4.1.1 Quantification

The weighted linear calibration model was used to perform the analytical calibration of the ICP-MS equipment, due to the lack of homoscedasticity of the instrumental response. The variance associated with the three replicates of the reading signal normally increases as the concentration increases. The homogeneity test of variances, applied to the lowest and highest concentration standards, with a degree of confidence of 95 %, indicated that the variances are statically different, suggesting that there was heteroscedasticity, which justified the use of a weighted calibration.

Prior to each analysis, the calibration functions were established by the ICP-MS *software*. Although the *software* of the equipment performs calibration calculations, during the validation of a method it was essential to check them using other *software*. In this work, that verification was performed using *Microsoft Excel*. Below is an example of one calibration function obtained during the validation process. The calculations were performed according to **Equation 5** and **Equation 6**. The calculated parameters for the construction of the Cu weighted linear calibration function, performed on April 26, 2021, are presented in **Table 7**, **Table 8**, and **Table 9**. This calibration model was followed for all the other elements under study. **Figure 15** shows the function obtained that confirms the results calculated by the ICP software and by *Excel*, validating these calculations.

Table 7: *Values for the determination of the inverse of the variation for the weighted linear calibration function of Cu in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si^2	$1/si^2$
P0	0.0	5.14E+02	2.10E+01	4.42E+02	2.26E-03
P1	0.15	5.89E+02	5.55E+01	3.08E+03	3.24E-04
P2	1.5	1.52E+03	4.30E+01	1.85E+03	5.41E-04
P3	7.5	6.25E+03	2.02E+02	4.09E+04	2.45E-05
P4	15	1.23E+04	1.18E+02	1.39E+04	7.19E-05
P5	75	5.51E+04	3.28E+02	1.07E+05	9.30E-06
P6	150	1.06E+05	1.40E+01	1.96E+02	5.09E-03
Sum	249	1.82E+05	7.82E+02	1.68E+05	8.32E-03
Mean	36	2.61E+04	1.12E+02	2.40E+04	1.19E-03

Table 8: *Values for the definition of the weighted linear calibration function of Cu in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	$wi*xi^2$
P0	0.0	1.90E+00	0.00E+00	9.77E+02	0.00E+00	0.00E+00
P1	0.15	2.73E-01	4.09E-02	1.61E+02	2.41E+01	6.14E-03
P2	1.5	4.55E-01	6.82E-01	6.89E+02	1.03E+03	1.02E+00
P3	7.5	2.06E-02	1.54E-01	1.29E+02	9.64E+02	1.16E+00
P4	15	6.04E-02	9.06E-01	7.41E+02	1.11E+04	1.36E+01
P5	75	7.82E-03	5.87E-01	4.31E+02	3.24E+04	4.40E+01
P6	150	4.28E+00	6.42E+02	4.54E+05	6.82E+07	9.64E+04
Sum	249	7.00E+00	6.45E+02	4.57E+05	6.82E+07	9.64E+04
Mean	36	1.00E+00	9.21E+01	6.54E+04	9.74E+06	1.38E+04

Table 9: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Cu in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Cu] calculated	|Relative error| (%)
P0	5.32E+02	-1.77E+01	-3.44E+00	-2.52E-02	-
P1	6.37E+02	-4.83E+01	-8.19E+00	8.14E-02	4.57E+01
P2	1.59E+03	-7.23E+01	-4.77E+00	1.40E+00	6.85E+00
P3	5.81E+03	4.42E+02	7.07E+00	8.13E+00	8.38E+00
P4	1.11E+04	1.17E+03	9.53E+00	1.67E+01	1.11E+01
P5	5.33E+04	1.84E+03	3.33E+00	7.76E+01	3.48E+00
P6	1.06E+05	-3.35E+00	-3.16E-03	1.50E+02	3.18E-03

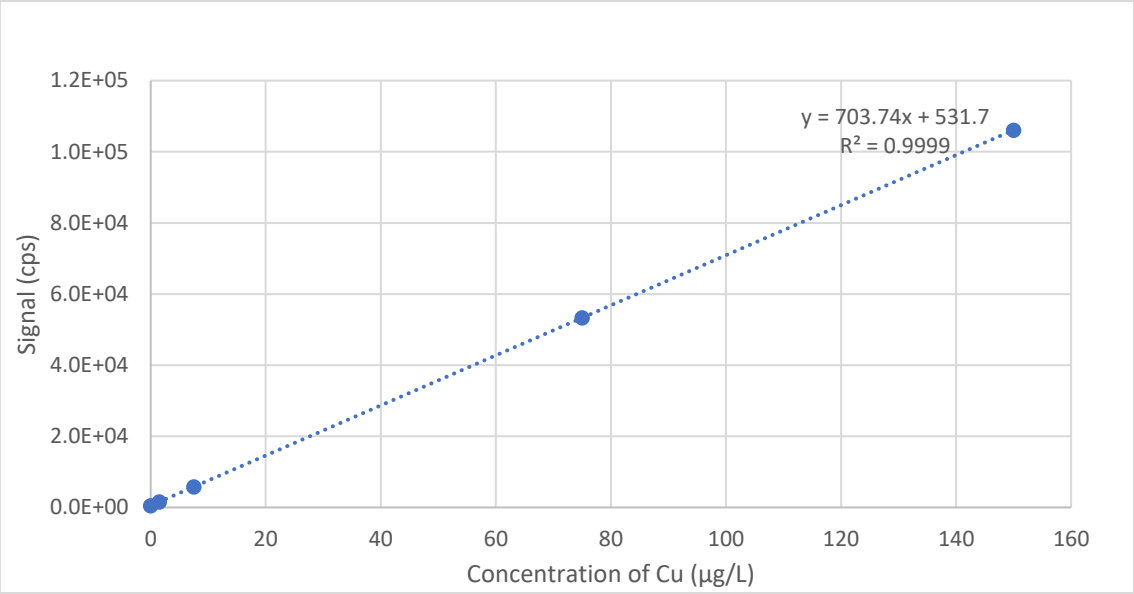

Figure 15: *Calibration curve for Cu in relation to the instrumental signal on 26/04/2021*

In the context of this work, valid calibration functions were obtained for the quantification of Cu by ICP-MS. The parameters of one of those calibration functions, *i.e.* the slope (703.74), the ordinate at the origin (531.7) and the coefficient of determination (> 0.999) are shown in **Figure 15**. Each calibration function was built with at least 5 calibration standards over the working range, and it was found that the correlation coefficient was greater than 0.995, value generally accepted in laboratories, as described in the RELACRE guide for method validation (RELACRE, 2000) and the criteria established for this work.

The instrumental work range for the quantification of Cu in food matrices by ICP-MS, was stipulated between 1.5 and 150 µg/L. These limits corresponded to the LOQ and to the highest concentration standard of the calibration curve, respectively. The working range of the method expressed in mass was obtained by converting these values into dry mass of sample, according to **Equation 7**. The working range of the method obtained for the quantification of Cu was: 0.18 to 18.47 µg/g (dry weight – DW).

The calculation of the correlation coefficient (r), and of the analysis of the residual error graph were performed to verify the linearity of the relationship between the instrumental signal and the concentration of the analyte. **Figure 16** shows the residual errors associated with the different calibration standards used in calculating Cu calibration function while **Figure 17** presents the relative error also associated with the different calibration standards used in the calculation of the calibration function for Cu on 26/04/2021.

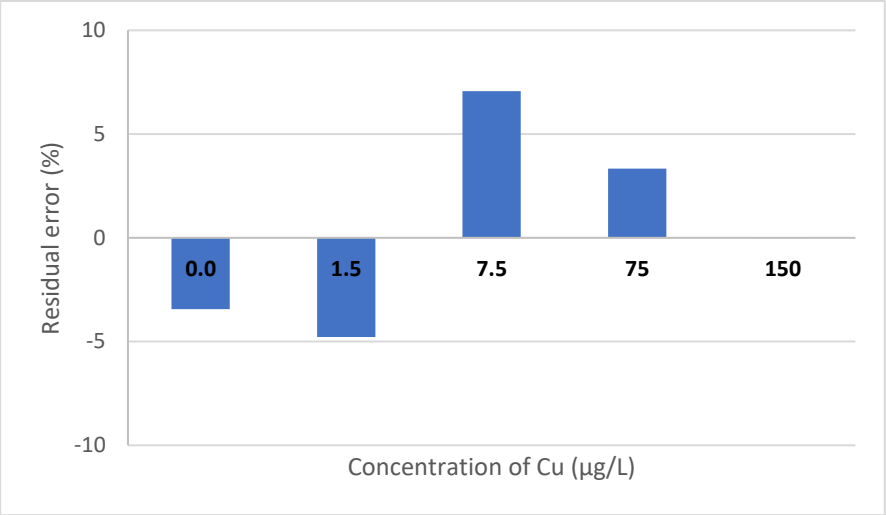

Figure 16: *Residual error (%) associated with different calibration standards for Cu on 26/04/2021*

Figure 16 and **Table 9** show the residual error calculated for Cu, and it is possible to notice that the error is below 10 %, a criteria stipulated for this work and also described in the literature (Carvalho et al., 2020). It is also possible to verify that there are no more than three consecutive positive or negative residues, which leads to the conclusion that the residual error does not present trends (Miller and Miller, 2010), proving the adequacy of the calculated calibration function to the results.

Based on the correlation coefficient (r) and on the residual error analysis, it is concluded that the weighted linear model adequately describes the instrumental response of the ICP-MS as a function of the Cu concentration.

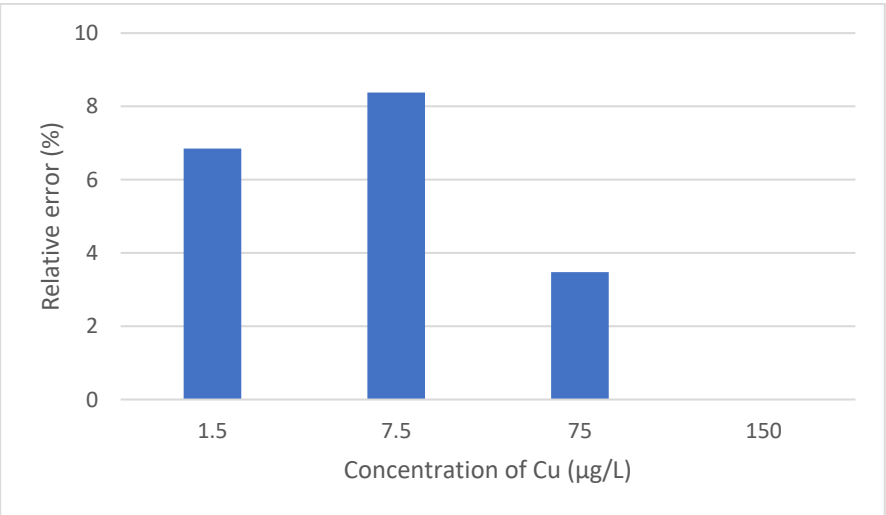

Figure 17: *Relative error (%) associated with different calibration standards for Cu on 26/04/2021*

The calibration curve for Cu quantification was subjected to calculation of the relative error (**Equation 13**). It was found that the values of 2 relative errors results were higher than 10 %, a criterion established by RELACRE and followed in this work. Those that were above the established value were marked in red (**Table 9**) and removed from the calibration curve. It was not necessary to redo the calibration curve since a total of 7 standards were used, of which two were removed, leaving the calibration curve with the minimum number of five standards. The other relative errors were equal to or less than 10 % (**Figure 17**).

The validation of the equipment's *software* for the remaining elements selected for this work was also performed, and the parameters of the respective calibration functions can be found in the Annex I. For those elements, the conclusions were the same of the ones for Cu. For Na, Ca, and Mn, all the standards showed relative errors less than or equal to 10 %, and there was no trend in the residual error. For Mg, Fe, and As, a standard showed a residual error greater than 10 %, which was removed from the calibration curve, and after that no trend was found. For Zn, 2 standards presented a relative error greater than 10 % (like for Cu), being removed from the calibration curve.

The instrumental working ranges were validated as follows: 0.11 to 110 mg/L for Na, Mg, and Ca; 0.15 to 150 µg/L for Mn; 1.5 to 150 µg/L for Zn, As; and 3 to 300 µg/L for Fe. The working ranges of the method that were validated are: 0.01 to 13.6 mg/g for Na, Mg, and Ca; 0.02 to 18.5 µg/g for Mn, Zn, As, and Cd; and 0.04 to 37.0 µg/g for Fe.

4.1.2 Limit of detection and limit of quantification

There are several approaches described in the literature to estimate the limits of detection and quantification. These estimates can be made using blanks, the parameters obtained for the calibration function, or by assuming that the limit of quantification corresponds to the standard of lower concentration of the calibration function, while the limit of detection corresponds to 1/3 of that value (Magnusson and Örnemark, 2014).

In this work, the LOQ followed the criteria of being the lowest standard of the calibration curve and 1/3 of the LOQ being the LOD.

The values of the concentration of Cu obtained during analysis of the digestion blanks are shown in **Figure 18**, where the LOQ (1.5 µg/L) and the LOD (0.45 µg/L) are in red line and yellow line, respectively.

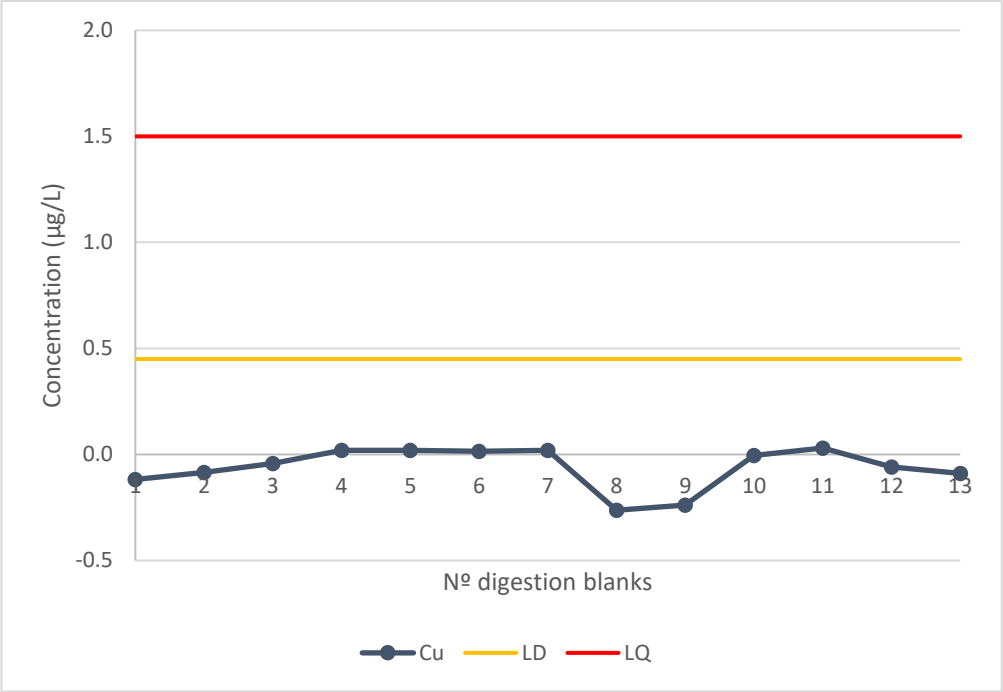

Figure 18: *Control verification chart of digestion blanks according to Cu concentration*

Through **Figure 18** it is possible to verify that the values of the blanks were always below the LOQ, which means that there was no contamination from the digestion step, and no correction on the concentration values of the samples was needed.

The verification charts of the values obtained by the digestion blanks, for the quantification of the other macro, micro and potentially toxic elements studied in this work can be found in Annex I. For all analytes it is possible to observe that the blanks were below the LOQ. The analytical thresholds of these elements are shown in **Table 10**.

Table 10: *Limit of quantification (LOQ) and limit of detection (LOD) obtained for macro, micro, and potentially toxic elements*

	Na	Mg	Ca	Mn	Fe	Zn	As	Cd
	(mg/L)			(µg/L)				
LOQ	0.1	0.1	0.1	0.15	3.0	1.5	1.5	0.15
LOD	0.03	0.03	0.03	0.05	0.9	0.45	0.45	0.05

4.1.3 Selectivity

- **Recovery tests – Matrix interference evaluation**

Control charts allow the detection of possible abnormal situations that may occur during the execution of the test methods. These charts are extremely useful if they are designed with a specific objective, like the control of the operations inherent to the

realization of the test method, such as recovery tests (RELACRE, 1998). Before the elaboration of the control chart, the acceptance limits for the recovery tests must be defined and for this study, the range of 80 to 120 % was defined as a criterion.

The control chart set as an example for Cu is showed in **Figure 19**, and it was obtained through the analysis of fortified solutions for each food matrix under study (chicken, mussel, fish, rice, and seaweed). This control chart refers to the percentages of recovery of Cu, where the warning lines, which are determined through the mean of readings ± 2 times the standard deviation ($\bar{x} \pm 2 \times s$), are represented in yellow color and the rejection lines, which are determined through the mean of readings ± 3 times the standard deviation ($\bar{x} \pm 3 \times s$), are shown in red color. The control chart shows that, although readings were made on different dates and at different fortification levels of the analyte, the process was always under control since the results obtained through the recovery test were always within the warning limits.

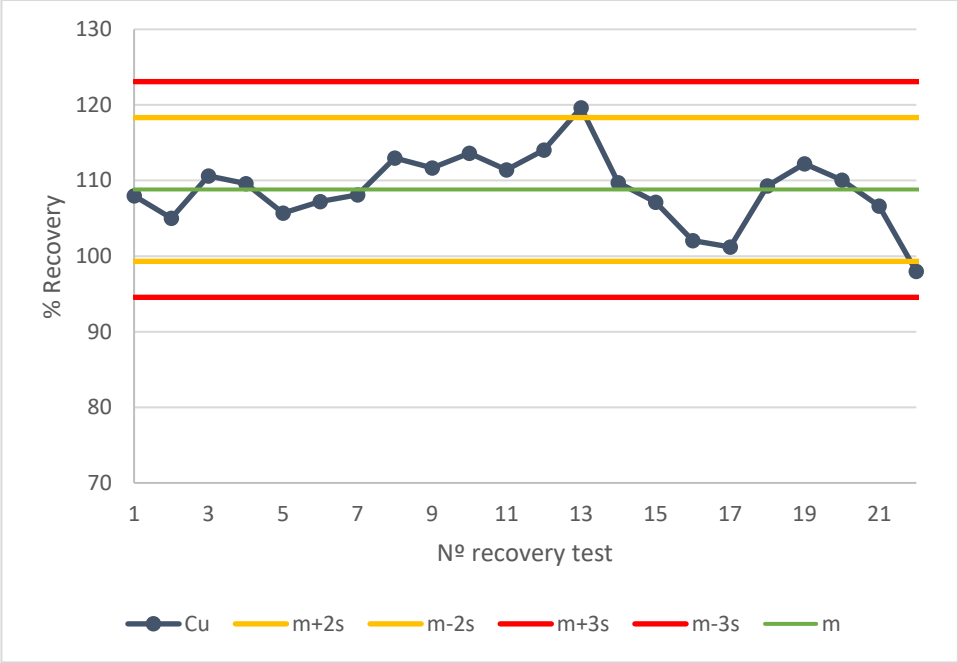

Figure 19: *Control chart for Cu recovery (%) by number of recovery readings*

For the adequate analysis of a control chart, it is necessary to understand the rules of routine analysis and corrective actions to be taken if necessary. According to RELACRE guide 9 (some examples of control charts in chemical analysis laboratories) the rules to be followed are (RELACRE, 1998):

- **Rejection limits (RL)**

If 1 point exceeds the rejection limits, the analysis must be repeated:

- if during this repetition it is found that the new point is within the rejection limits, continue and accept the results of the analysis;
- if during this repetition it is found that the new point is outside the rejection limits, interrupt the analysis and correct the problem.

- **Warning limits (WL)**

If 2 in 3 consecutive points exceed the warning limits, another point should be analysed:

- if during this repetition it is found that the new point is within the warning limits, continue and accept the results of the analysis;
- if during this repetition it is found that the new point is outside the warning limits, interrupt the analysis and try to correct the problem.

- **Central line**

When 6 consecutive points are located all above or all below the central line, care must be taken as to the location of the following point:

- if that point is located on the other side of the centre line, continue and accept the results of the analysis;
- if that point is still located on the same side of the centre line, stop the analysis, and correct the problem.

The mean recovery of Cu in fortified solutions was 109 % (**Figure 19**). In the 22 recovery trials performed, the recoveries obtained were in the range of 98 to 120 %, and therefore within the acceptance criteria established initially for percentages obtained in the recovery tests (80 and 120%).

Different levels of fortification were made on different days, and different recovery percentages were observed for those levels. According to RELACRE guide 9 (some examples of control charts in chemical analysis laboratories) (RELACRE, 1998) the control chart of Cu does not reveal any tendency.

This study was done for Cu 65 m/z. Based on the results it can be concluded that the quantification of Cu by ICP-MS in several food matrices does not present significant matrix interference at the studied mass/charge ratio.

The control charts for the validation of the other macro, micro and potentially toxic elements studied in this work can be found in Annex I. For all the elements, the control charts showed a distribution of results identical to that obtained for Cu, showing no tendency, and fulfilling the acceptance criteria. Recovery percentages (**Table 11**) varied between 96 and 115 % for Na, 99 and 109 % for Mg, 100 and 112 % for Ca, 101

and 113 % for Mn, 97 and 109 % for Fe, 90 and 117 % for Zn, 86 and 113 % for As, and 84 and 98 % for Cd.

Table 11: *Recovery (%) obtained for macro, micro, and potentially toxic elements from different food matrices*

Recovery (%)									
Matrix	Na	Mg	Ca	Mn	Fe	Cu	Zn	As	Cd
Chicken	98-107	100-106	104-108	104-106	102-109	105-114	100-105	96-106	87-97
Mussel	108-115	105-109	107-112	105-109	103-108	110-120	120-122	97-113	84-98
Fish	98-109	102-107	106-110	101-109	98-103	101-107	90-114	92-113	85-95
Rice	103-109	105-107	100-108	104-106	102-105	108-113	93-115	87-100	86-94
Seaweed	96-107	99-105	105-110	101-113	97-105	98-107	92-117	86-92	87-90

4.1.4 Accuracy

As previously stated, accuracy is the degree of agreement between a value measured by the laboratory and a true value of a quantity/measure (Instituto Português da Qualidade, 2012; RELACRE, 2000). A measurement is said to be more accurate when it provides a smaller measurement error (Instituto Português da Qualidade, 2012). Accuracy is influenced not only by random, but also by systematic errors. As accuracy describes how close the result is to its true value, it has to include the effect of both precision and trueness, being expressed as bias (NATA, 2018). **Figure 20** shows how errors are related to effects and terms.

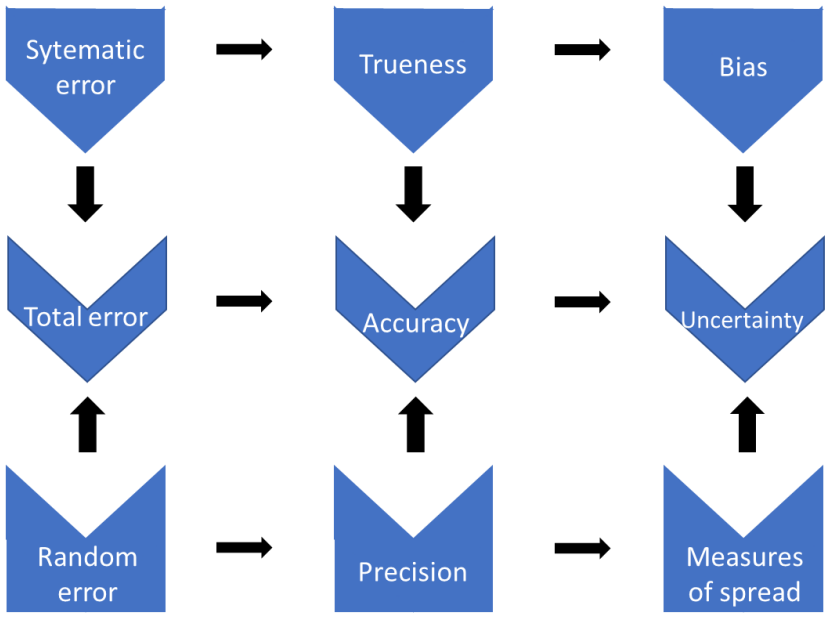

Figure 20: *Relationship between types of errors, performance characteristics and their quantitative expression. (adapted from Magnusson and Örnemark* (2014)*)*

4.1.4.1Trueness

Trueness is defined as the degree of agreement between the mean of an infinite number of repeated measurements and an accurate reference value, being inversely related to the systematic error (Instituto Português da Qualidade, 2012). Usually, trueness cannot be measured since it is not possible to carry out an infinite number of measurements. Nevertheless, a practical assessment of trueness can be made and this is usually expressed quantitatively in terms of "bias" (Magnusson and Örnemark, 2014). As trueness increases, bias decreases (NATA, 2018).

Trueness is one of the components that are part of the study of accuracy, being this one associated with systematic errors. In this work, the trueness associated with the method was evaluated, using several CRMs mimicking the different food samples under study. The trueness error, known as bias, associated with the quantification in these materials, performed on different days, were calculated, and this error was monitored using a control chart (**Figure 21**). The bias was obtained by calculating the relative error, according to **Equation 13**.

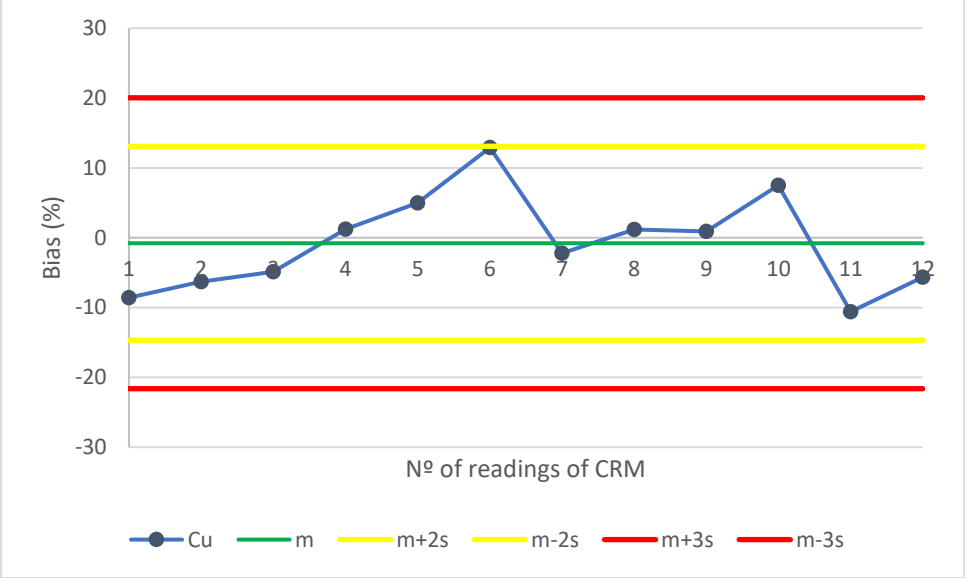

Figure 21: *Control chart of bias (%) for Cu by the number of CRM readings*

The bias average for Cu was close to zero. Bias was always within acceptable limits for different types of food matrices under study (< 20 %). It should be noted that the control chart presented has the bias associated with different CRMs, with different concentrations and that, on each day of analysis, a different number of solutions were read. The errors showed an absolute value for the bias of less than 20 %, being possible to conclude that the requirements for the bias of the method, stipulated by RELACRE and

followed in this work, were adequate for the quantification of Cu in food samples by ICP-MS. The maximum absolute bias value obtained was 13 %.

The control charts of the bias associated with the method (using CRMs) for Na, Mg, Ca, Mn, Fe, Zn, As, and Cd can be found in Annex I at **Figure 29**, **Figure 37**, **Figure 45**, **Figure 53**, **Figure 61**, **Figure 69**, **Figure 77**, **Figure 85**, respectively. Bias obtained were in the intervals: -17 to 2 % for Na, -17 to 9 % for Mg, -15 to 17 % for Ca, -11 to 11 % for Mn, -17 to 18 % for Fe, -19 to -10 % for Zn, -2 to 4 % for As, and -18 to -2 % for Cd.

4.1.4.2Precision

Precision is the parameter of the method that evaluates the dispersion of the results obtained between independent tests, which are performed on the same sample, similar samples, or on standards under pre-established conditions, and it depends only on the distribution of random errors (NATA, 2018). The precision of a measurement can be determined through measurement of the repeatability, intermediate precision and/or reproducibility (Magnusson and Örnemark, 2014; RELACRE, 2000). Precision is generally expressed numerically by characteristics such as dispersion, standard deviation, variance, or coefficient of variation of the replicates results, under specified measurement conditions (Instituto Português da Qualidade, 2012).

- **Relative standard deviation associated with instrumental reading**

The signal acquired by ICP-MS in the analysis of each solution corresponds to an average of the signals of three reading replicas of the same solution. With these three readings it is possible to obtain a relative standard deviation (RSD), which consists of a coefficient of variation of the repeatability of the instrumental reading. This parameter allows to evaluate possible drifts that may occur during the equipment quantification step, which is an important tool for the quality control of the results.

Relative standard deviation (RSD) helps to know the minimum concentration from which it is possible to quantify an analyte with satisfactory precision, as this deviation tends to increase with decreasing concentration. Therefore, the deviation tends to be greater when making measurements close to the LOQ of the equipment.

Figure 22 shows the relative standard deviations (%), obtained from the reading of various solutions analysed during the validation of the Cu quantification.

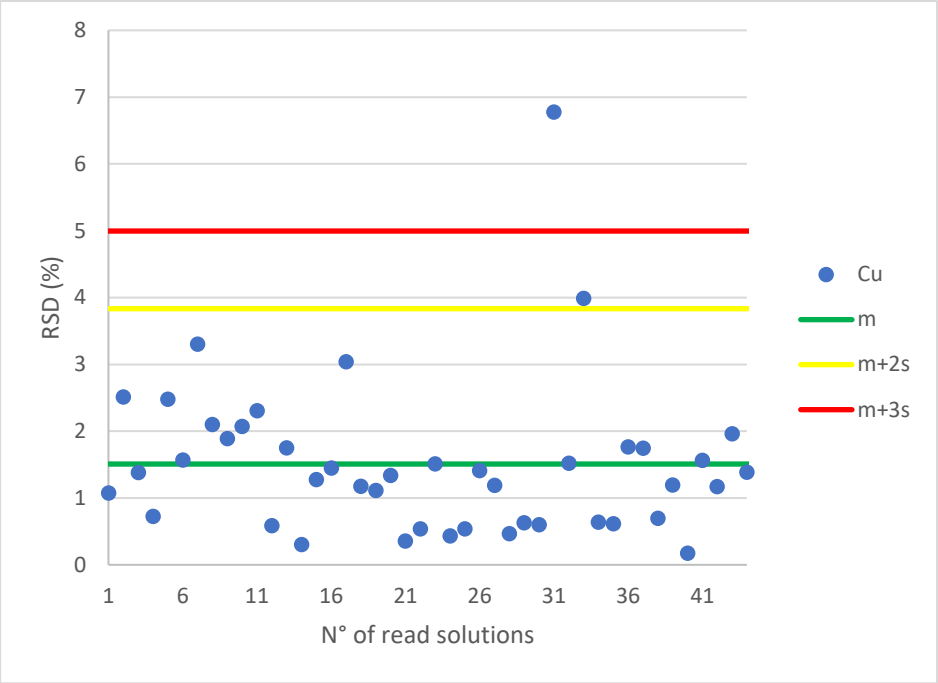

Figure 22: *Control chart of the relative standard deviations (RSD, %) for Cu by ICP-MS*

The acceptance criterion for the RSD is 10 % (established by RELACRE), and the average value obtained for the RSD in the case of Cu was approximately 1.5 %, with no value close to 10 % being observed. Bearing in mind the results obtained for RSD in the quantification of Cu in food matrices, it was possible to redefine the RSD acceptance criteria to 7 %, since no values higher than this were observed.

The control charts for the relative standard deviations obtained in the quantification of Na, Mg, Ca, Mn, Fe, Zn, As, Cd in foods are shown in Annex I. The maximum relative standard deviations were: 4 % for Na, 5 % for Mg, 5 % for Ca, 8 % for Mn, 7 % for Fe, 7 % for Zn, 10 % for As, and 10 % for Cd. With these results, it was possible to redefine/decrease the acceptance criteria of the RSD to 7 % for most elements. For As and Cd, the 10 % criterion remained the same.

- **Coefficient of variation of repeatability**

Repeatability is a measure of variability in results, when the measurements are performed in a laboratory, in identical samples, by a single analyst, using the same equipment, under conditions as constant as possible, in a short period of time (Magnusson and Örnemark, 2014; NATA, 2018). In a simplified way is the measurement of the minimum dispersion of results. The repeatability limit allows to decide whether the difference between duplicates of a sample under repeatability conditions is significant (Laboratório Central de Análises, 2018).

The coefficient of variation of repeatability (CVr) was used to evaluate the repeatability of the method. By calculating the standard deviation of repeatability, it was possible to obtain the coefficient according to **Equation 11**. **Table 12** shows the values of the coefficient of variation of repeatability estimated for the analysis of Cu in food samples.

Table 12: *Estimated CVr (%) for Cu*

Sample	CV_r (%)	Nº of reading samples
Chicken	6	10
Mussel	2	10
Fish	5	10
Rice	5	8
ZC 73016	2	2
ERM-CE278k	4	2
ERM-BB422	5	2
Dorm-4	2	2
ZC 73028	4	2
SRM 8433	4	2

Table 12 shows that all samples have a CVr less than 10 %, the criterion stablished by RELACRE. It is possible to notice that the different matrices under study are quite complex but also quite homogeneous. The CRMs analysed were highly homogeneous, being the variability presented only due to the quantification step.

The tables presenting the CVr's values for Na, Mg, Ca, Mn, Fe, As, and Zn may be found in the Annex I. The conclusions obtained for these elements are identical to those obtained for Cu, since for all analytes under study, the values of CVr were less than 10 %, thus considering that the repeatability of the method was satisfactory. Since the concentrations of As and Cd were below the LOQ for most of the food matrices, it was not possible to calculate the coefficient of variation in these matrices.

- **Duplicate evaluation**

Duplicate analysis was also used to assess the repeatability, as this is the characteristic number of replicates when the method is applied routinely. Control charts were represented for the relative difference between independent solutions, corresponding to digestion replicates. The calculation of the relative difference (Bennett and Briggs, 2011) between the duplicate analysis was carried out according to **Equation 21**.

$$Relative\ difference\ (\%) = \frac{|C_1 - C_2|}{\bar{C}} \times 100$$

Equation 21

where C_1 and C_2 correspond to the concentrations obtained in the reading of the sample and the duplicate, respectively, and $\bar{C}$ corresponds to the average of the concentrations obtained in the reading of the duplicates.

The control charts for the values of the relative differences (%), obtained by **Equation 21**, for the digestion of duplicates are shown in **Figure 23** (a digestion duplicate consisted of a sample digested in two different digestion vessels, but in the same digestion cycle and analysed by ICP one time).

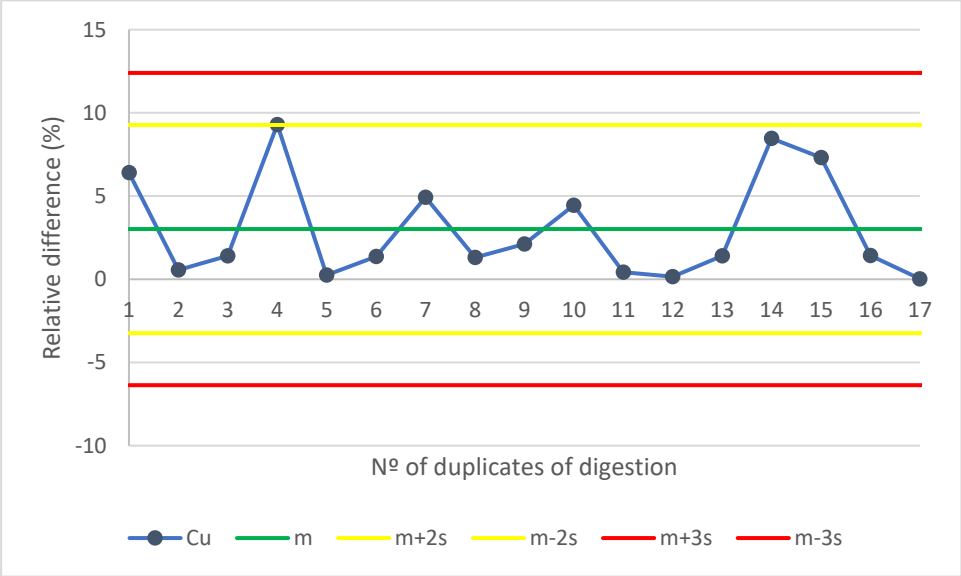

Figure 23: *Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Cu*

The relative differences obtained for the digestion of duplicates were affected by two types of repeatability, which contributed to the variability of the results. One is the repeatability associated with the digestion/preparation step of the sample, and the other one is the repeatability associated with instrumental reading. The module of the relative difference for digestion duplicates were always less than 10 %, criterion stipulated by the RELACRE.

The control charts for the values of the relative differences between the digestion duplicates for Na, Mg, Ca, Mn, Fe, Zn, As, and Cd are presented in Annex I. The conclusions drawn from these control charts are similar to those presented here for Cu.

❖ **Intermediate precision**

Table 13 shows the values obtained for the coefficient of variation of the intermediate precision (CV_{SI} (%)), as well as the number of readings obtained under repeatability conditions, in each food matrix, and the number of evaluated groups used to obtain these values.

Table 13: *Estimated CV_{SI} (%) for Cu*

Sample	CV_{Si} (%)	Nº of reading samples	Nº of groups
Chicken	7	20	2
Rice	8	20	2
Seaweed	7	10	2

It is possible to observe that the values of the coefficient of variation for the samples were close to the limit value stipulated (10 %) according to RELACRE (RELACRE, 2000), but none was out of the stipulated limits.

The coefficients of variation associated with the intermediate precision for the elements Na, Mg, Ca, Mn, Fe, Zn, As, and Cd are shown in **Table 13**, **Table 30**, **Table 36**, **Table 42**, **Table 48**, **Table 54**, **Table 60**, **Table 66**, and **Table 72**, respectively. The conclusions drawn from these tables were the same as those obtained for Cu. For Zn, low coefficients of variation (3 %) were obtained for chicken and rice, may be due to the high concentration of this element in these matrices. The concentrations for As and Cd were below the quantification limit for most of the food matrices, so it was not possible to calculate the coefficient of variation of these elements in these matrices, reason why their values are absent in the respective tables.

4.1.5 Estimation of the uncertainty of the results

Table 14 shows the value obtained for the estimation of the uncertainty associated with the quantification of Cu in food by ICP-MS, as well as the component associated with precision (random effects) and trueness (systematic effects). These values were obtained according to the expressions presented in **Equation 14** to **Equation 20** (values in percentage) and considering the CRM readings made during the method validation process.

Table 14: *Estimation of the uncertainty (%) for Cu by ICP-MS*

u_r	5.5 %
u_b	6.6 %
u_c	8.6 %
U	17.3 %

In this work, the uncertainty was estimated using the different certified reference materials, according to the food matrix, and due to this, the uncertainty may be underestimated. In future work, it is important to re-estimate uncertainty, if possible, based on interlaboratory comparison tests.

The estimative of the measurement uncertainty was obtained through a minimum number of CRM readings, under repeatability and reproducibility conditions, and the components associated with trueness and precision were representative, within the CRM concentration level. The expanded uncertainty (U), expressed in the form of estimated standard relative uncertainty through **Equation 20**, for the quantification of Cu in food by ICP-MS was 17 %.

The uncertainties associated with the quantification steps, as well as the maximum measurement uncertainties for Na, Mg, Ca, Mn, Fe, Zn, As and Cd in food by ICP-MS are presented in Annex I. The expanded uncertainties (U), expressed in the form of relative standard uncertainties estimated for the quantification of Na, Mg, Ca, Mn, Fe, Zn, As and Cd in food by ICP-MS were respectively: 12, 24, 14, 11, 17, 6, 11, and 10 %.

4.1.6 Method validation summary

Within the scope of this work, it was possible to validate the methodology for the quantification of Na, Mg, Ca, Mn, Fe, Cu, Zn, As and Cd by ICP-MS in food matrices. The data used for the validation of these methods can be consulted throughout the document for Cu and in Annex I, subsections 6.1 to 6.8, for Na, Mg, Ca, Mn, Fe, Zn, As and Cd. **Table 15** to **Table 23** present the performance parameters selected for the method, as well as the results obtained during the method validation process.

Regarding the evaluation of matrix interferences, it was found that the studied elements presented recovery percentages in accordance with the requirements established by RELACRE, with recovery percentage for all studied elements ranging between 84 and 120 %, indicating that there were no relevant matrix interferences.

For Na, Ca, Mn, and Cd, all standards showed relative errors within the stipulated criterion, while for Mg, Fe, and As, one standard presented a relative error above the stipulated criterion. For Cu and Zn, two standards presented values above the stipulated criterion. For all the elements, the correlation coefficient was always above 0.995 and there was no trend in the residual error, thus validating all the calibrations carried out in the scope of this work.

The trueness of the method was evaluated using CRMs and the method quantification of Cu and Mn by ICP-MS were the ones with the smallest trueness error.

As 8 different CRMs were used, the trueness could be well evaluated in different concentration ranges for different matrices.

The precision of the method was evaluated using CRMs and food samples (chicken, mussels, fish, rice, and seaweed). What was found for the assessment of precision using food matrices was that they had low coefficients of variation (repeatability), and within the established criteria, which demonstrates the homogeneity of the real food matrices studied in this work. The evaluation of precision using CRMs also resulted in coefficients of variation within the established criteria (the certified reference materials are very homogeneous, with well evaluated concentrations determined by competent entities).

For the methodology validated in ICP-MS (Na, Mg, Ca, Mn, Fe, Cu, Zn, As, and Cd) the uncertainty was estimated through readings of the different CRMs. The methodology that presented the least uncertainty was the quantification of Zn (6 %) by ICP-MS. Magnesium and iron were the elements that presented the greatest uncertainty (24 and 17 %, respectively), due not only to a greater trueness error but also to a lower precision.

The validated method has performance characteristics that allow the quantification of these elements in foods by ICP-MS. The requirements may later be readjusted as more results are obtained, since current data history is reduced due to having been carried out only one campaign and not having carried out interlaboratory comparison tests.

Table 15: *Performance parameters for Cu in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Cu in food by ICP-MS Isotope of 65 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	98-120 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	703.7	n.a.
		Work range	n.a.	1.5-150 µg/L 0.18-18.5 µg/g	n.a.
		Limit of quantification	Depends on the element	1.5 µg/L 0.02 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 7 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 6 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 9 %	n.a.
		Intermediate precision	≤ 10 %	≤ 8 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 13 %	Maximum value obtained
Uncertainty			n.a	17 %	ISO 11352

Table 16: *Performance parameters for Na in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Na in food by ICP-MS Isotope of 23 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	96-115 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	3973	n.a.
		Work range	n.a.	0.11-110 mg/L 0.01-13.6 mg/g	n.a.
		Limit of quantification	Depends on the element	0.11 mg/L 0.01 mg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 4 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 10 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 7 %	n.a.
		Intermediate precision	≤ 10 %	≤ 9 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 18 %	Maximum value obtained
Uncertainty			n.a	12 %	ISO 11352

Table 17: *Performance parameters for Mg in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Mg in food by ICP-MS Isotope of 25 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	99-109 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	321.1	n.a.
		Work range	n.a.	0.11-110 mg/L 0.01-13.6 mg/g	n.a.
		Limit of quantification	Depends on the element	0.11 mg/L 0.01 mg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 5 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 10 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 6 %	n.a.
		Intermediate precision	≤ 10 %	≤ 10 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 17 %	Maximum value obtained
Uncertainty			n.a	24 %	ISO 11352

Table 18: *Performance parameters for Ca in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Ca in food by ICP-MS Isotope of 44 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	100-112 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	113.5	n.a.
		Work range	n.a.	0.11-110 mg/L 0.01-13.6 mg/g	n.a.
		Limit of quantification	Depends on the element	0.11 mg/L 0.01 mg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 5 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 10 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 8 %	n.a.
		Intermediate precision	≤ 10 %	≤ 6 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 17 %	Maximum value obtained
Uncertainty			n.a	14 %	ISO 11352

Table 19: *Performance parameters for Mn in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Mn in food by ICP-MS Isotope of 55 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	101-113 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	5407	n.a.
		Work range	n.a.	0.15-150 µg/L 0.02-18.5 µg/g	n.a.
		Limit of quantification	Depends on the element	0.15 µg/L 0.02 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 8 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 5 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 4 %	n.a.
		Intermediate precision	≤ 10 %	≤ 9 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 14 %	Maximum value obtained
Uncertainty			n.a	11 %	ISO 11352

Table 20: *Performance parameters for Fe in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Fe in food by ICP-MS Isotope of 56 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	97-109 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	4399	n.a.
		Work range	n.a.	3.0-300 µg/L 0.04-36.9 µg/g	n.a.
		Limit of quantification	Depends on the element	3.0 µg/L 0.04 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 7 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 10 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 9 %	n.a.
		Intermediate precision	≤ 10 %	≤ 10 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 18 %	Maximum value obtained
Uncertainty			n.a	17 %	ISO 11352

Table 21: *Performance parameters for Zn in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Zn in food by ICP-MS Isotope of 64 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	90-117 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.998	Minimum value obtained
		Sensitivity / Slope	n.a.	1006	n.a.
		Work range	n.a.	1.5-150 µg/L 0.18-18.5 µg/g	n.a.
		Limit of quantification	Depends on the element	1.5 µg/L 0.18 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 7 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 10 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 5 %	n.a.
		Intermediate precision	≤ 10 %	≤ 7 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 20 %	Maximum value obtained
Uncertainty			n.a	6 %	ISO 11352

Table 22: *Performance parameters for As in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**As in food by ICP-MS Isotope of 75 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	86-113 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.999	Minimum value obtained
		Sensitivity / Slope	n.a.	452.7	n.a.
		Work range	n.a.	1.5-150 µg/L 0.18-18.5 µg/g	n.a.
		Limit of quantification	Depends on the element	1.5 µg/L 0.18 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 10 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 9 %	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 6 %	n.a.
		Intermediate precision	≤ 10 %	≤ 10 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 4 %	Maximum value obtained
Uncertainty			n.a	11 %	ISO 11352

Table 23: *Performance parameters for Cd in food by ICP-MS*

Requirements for assay method					
Characteristic parameters			**Cd in food by ICP-MS Isotope of 111 a.m.u.**		**Observations**
			Requirement	**Obtained value**	
Specificity / selectivity		Matrix interference (recoveries)	80-120 %	84-98 %	Minimum and maximum value obtained.
		Spectral interferences	Depends on the matrix	n.a.	n.a.
Quantification		Correlation coefficient (r)	> 0.995	0.998	Minimum value obtained
		Sensitivity / Slope	n.a.	711.3	n.a.
		Work range	n.a.	0.15-150 µg/L 0.02-18.5 µg/g	n.a.
		Limit of quantification	Depends on the element	0.15 µg/L 0.02 µg/g	n.a.
Accuracy	Precision	Coef. of variation of the samples (standard deviation)	n.a.	≤ 10 %	n.a.
		Coef. of variation of repeatability	≤ 10 %	≤ 8%	n.a.
		Duplicate evaluation (solutions)	n.a.	≤ 4 %	n.a.
		Intermediate precision	≤ 10 %	≤ 8 %	Maximum value obtained for a sample
	Trueness	Trueness error	≤ 20 %	≤ 18 %	Maximum value obtained
Uncertainty			n.a	10 %	ISO 11352

4.2 Evaluation of element concentrations in analysed food matrices

4.2.1 Element concentrations in unprocessed vs. processed food matrices

It is known that food is a source of macro and micronutrients necessary for the metabolic functioning of organisms in obtaining the necessary energy to carry out daily activities (Herreros-Chavez et al., 2019). Each food has a different composition and levels of nutrients, such as shellfish, which according to the Nutrient Database for Standard Reference published by the U.S. Department of Agriculture (USDA), are an excellent source of vitamin B12, omega-3 fatty acids, choline, iron, selenium and zinc (Wright et al., 2018). However, due to natural processes and human activities, such as industrial development, domestic life, and agricultural production, large amounts of potentially toxic elements (Gall et al., 2015) like Hg (Zhang et al., 2010) or As (Huang et al., 2015) can be released into the environment, and thus be incorporated into food (Liao et al., 2019).

After the validation of the method, where raw food matrices were used, a study was carried out to assess whether the processing commonly used daily for consumption affects the frequency and concentration of elements in the matrices. The digestion of processed food matrices was the same performed for the validation of the method, allowing the comparison among results.

Samples of raw food (unprocessed) and cooked food (boiled, grilled) were analysed to quantify the elements Na, Mg, Ca, Mn, Fe, Cu, Zn, As, Cd, Pb and Se. Results are shown in **Table 24**. Although the quantification method was not validated for all these elements (**Table 24**) it was still considered possible to include them in the study, once the recovery test and the CRM analysis gave good results for the validated elements in all tested food matrices.

Table 24: *Mean (m) and standard deviation (sd) of the concentration of macro, micro, and potentially toxic elements in unprocessed matrices vs. processed matrices*

Food matrices	Na (mg/g)		Mg (mg/g)		Ca (mg/g)		Mn (µg/g)		Fe (µg/g)		Cu (µg/g)		Zn (µg/g)		As (µg/g)		Cd (µg/g)		Pb (µg/g)		Se (µg/g)	
	m	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*std*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*	*m*	*sd*
Chicken	2.4	0.1	1.1	0.1	0.21	0.01	0.55	0.01	14.7	0.9	1.2	0.07	22.7	0.4	<LOQ	-	<LOQ	-	0.02	0.00	0.76	0.1
Grilled chicken	35.3	0.1	0.02	0.00	0.58	0.02	0.79	0.01	19.8	1.7	0.94	0.07	11.4	0.1	<LOQ	-	<LOQ	-	0.04	0.01	0.67	0.04
Boiled chicken	35.7	0.1	0.65	0.02	0.72	0.03	1.4	0.2	16.5	5.6	1.4	0.1	11.9	0.03	<LOQ	-	<LOQ	-	0.07	0.03	0.75	0.1
Mussel	15.2	0.6	3.4	0.1	1.9	0.1	6.5	0.1	135	2	5.5	0.1	135	2	12.9	0.4	3.15	0.04	0.06	0.00	5.9	0.4
Boiled mussel	6.9	0.01	2.3	0.01	1.8	0.1	7.6	0.1	114	0.1	5.7	0.1	144	2	10.2	0.4	4.45	0.05	0.07	0.00	3.8	0.1
Fish	53.6	2.6	0.88	0.05	0.58	0.01	0.69	0.02	11.8	0.5	0.76	0.04	10.9	0.1	<LOQ	-	<LOQ	-	0.02	0.00	0.94	0.1
Grilled fish	0.003	0.001	48.5	2.9	0.59	0.06	0.76	0.04	18.6	3.9	0.98	0.03	11.7	1.0	<LOQ	-	<LOQ	-	0.03	0.000	0.86	0.1
Boiled fish	0.003	0.0003	42.6	0.1	0.56	0.00	0.67	0.00	9.2	0.5	1.1	0.15	11.6	0.0	<LOQ	-	<LOQ	-	0.02	0.00	0.89	0.02
Rice	0.062	0.006	0.27	0.03	0.049	0.003	5.4	0.2	2.8	0.6	1.3	0.07	8.9	0.2	0.30	0.04	<LOQ	-	0.02	0.01	0.32	0.1
Boiled rice	0.095	0.002	0.24	0.01	0.13	0.01	4.7	0.2	28.0	0.4	1.8	0.09	9.4	0.03	<LOQ	-	<LOQ	-	0.02	0.01	0.24	-

It is possible to observe in **Table 24** that for most matrices the type of food processing does not influence the concentration of the elements in the different food matrices, once concentration was similar in raw and processed food.

For all food matrices, the macro elements such as Na, Mg and Ca had different responses regarding its concentration, when the sample is subjected to different types of processing. In general, Ca concentrations did not show a great difference in the order of magnitude of its concentration between the studied food treatments. Exception for rice, where Ca content increased after boiling, which may be associated to the hardness of the water, since in Aveiro, the tap water is categorized as of medium hardness, with a $CaCO_3$ concentration of 62 mg/L (AdRA, 2019), this is probably due to the greater capacity of rice to absorb water during cooking compared to other foods, thus incorporating this element into its composition. For Mg, it was not verified a great difference in the order of magnitude of its concentration, between the studied treatments for most matrices. Exception for chicken, where a reduction in Mg concentration was observed mostly for grilled samples, and for fish, where an increase in Mg concentration was observed in samples from both processing treatments. Sodium quantification revealed a different behavior for each matrix. In processed samples of chicken an increase in Na concentration was observed, while in mussels, and fish the opposite behavior was verified (decrease in Na concentration post processing treatment). This different behavior may be attributed to the ways each element is present in the studied food matrices, once it can be in free water inside the food, or in water that belongs to structure of the food or incorporated in the food tissues by association with several ligands present in food.

Micro elements, such as Mn, Fe, Cu, Zn, and Se were evaluated. Manganese concentration did not show a great difference in the order of magnitude of its concentration between the studied treatments. Iron concentration revealed an increase from 2.8 µg/g to 28.0 µg/g when the boiled treatment was applied to the rice matrix. This may be due to presence of high quantity of Fe in water, since the maximum allowable concentration of this element in drinking water is 200 µg/L (AdRA, 2021), and the greater capacity of rice to absorb water, consequently incorporating this element. Copper, zinc, and selenium did not show any relevant change in their concentration after the processing treatment in any matrix.

Potentially toxic elements such as As, Cd, and Pb were assessed to verify possible contamination. Arsenic and Cd concentrations were below the limit of quantification of

ICP-MS for most of the matrices under study, except for mussels, although no relevant change in their concentration was verified among processed and unprocessed samples. Marine organisms as shellfish can bioaccumulate metals from their surroundings since they feed by filtering particles from water (Houlbrèque et al., 2011). They are known to accumulate As from the environment and food sources in a range of 1 - 100 mg/g (Anacleto et al., 2009; Klarić et al., 2004). Cadmium concentration in molluscs species evaluated by Tapia et al., (2010) in Chile had values reaching 4.32 ± 0.12 mg/kg, in dry weight, for the *Ameghinomya antiqua* species. Lyophilised bivalves analysed in this work had values in line with those reported by Bruhn et al., (2002), 4.4 ± 0.3 mg/kg dry weight, which can be considered common for bivalves.

In general, we can say that for most of the elements quantified in this work, the food subjected to treatments like boiled and grilled had similar concentrations to the ones found in raw food. This way when comparing food concentration with the ones of the legislation or when comparing food items that are used in a meal, it is not necessary to consider the food treatment.

4.2.2 Levels of macro, micro, and potentially toxic elements in the analysed food matrices

As most studied food matrices are daily consumed a comparison between macro, micro, and potentially toxic elements among matrices was performed. The concentrations used to perform this comparison was the highest ones measured for each food item.

For macro elements, Na can be mostly found in chicken (highest value of 35.7 mg/g), while Mg is mostly found in fish (48.5 mg/g). Calcium has similar concentrations among all matrices with a slightly higher concentration in mussels (1.8 mg/g).

To classify the levels of micronutrient intake, the Recommended Dietary Allowance (RDA), and the Tolerable Upper Intake Level (UL) were here considered. If the population's average intake is below the RDA, or above the UL, it means that there is a deficiency or excess of the mineral, respectively (World Health Organization, 1996). Based on the RDA and the UL, the macro elements concentration in the daily diet should be of 900 – 1200 mg/day (RDA), and 2500 mg/day (UL) for Ca in adults (EFSA, 2006). When comparing with the studied food matrices, it is possible to verify that it would be necessary to eat 1 kg of mussels per day (matrix with the highest concentration of the element), to reach the daily recommended doses. Since it is impractical to eat 1 kg of mussel per day, other Ca sources should be consumed, such as milk and its derivates,

which are known to be rich in Ca. Curiously, it should be noted that the mussels analyzed in the present study had a higher content of Ca (1.8 mg/g) than milk and its derivatives (0.15 mg/g).

For Na, the RDA is 1200 – 1500 mg/day and the UL is 2300 mg/day. Based on the results obtained in this work, it appears that eating a 100 g chicken steak (white meat associated with healthy habits) leads to the intake of 3530 mg of Na. This value is about 1.5 times higher than the UL. It was also interesting to verify that the fish processing (grilled, and boiled) led to a reduction in the contents of this element, which allows to ingest this food abundantly without having concerns associated with this mineral. By contrary, the intake of 100 g of raw fish, which is a common habit in the orient (sushi), would lead to the ingestion of 2.3 times higher Na than the corresponding UL.

According to EFSA (2006) the RDA is 310 – 420 mg/day for Mg, and the UL is of 350 mg/day for supplements or fortified foods as there is no upper limit for Mg in food and water. Which means that an adult who eats a grilled fish steak (100 g) with boiled rice, can eat about 11 times the recommended amount.

For micro elements, Mn can be mostly found in mussels and rice (7.6 and 4.7 µg/g, respectively). Iron, copper, zinc, and selenium have higher concentrations in mussels 114, 5.7, 144, 3.8 µg/g, respectively, which may be related with their capacity to bioaccumulate metals from their surroundings by filtering particles from water (Houlbrèque et al., 2011). Iron deficiency is the most common in the world, causing anaemia (concentration of iron below the recommended medical threshold). By eating about 90 g of mussels, or about 250 g of grilled chicken accompanied by 200 g of rice, it possible to reach the RDA value (8 – 10 mg/day). The UL for Fe is 50 – 60 mg/day (EFSA, 2006).

Copper deficiency in humans is rare and symptoms of severe copper deficiency includes anaemia, neutropaenia, and bone abnormalities (EFSA, 2006). These symptoms can be avoided, according to the EFSA, by ingesting 0.9 to 2.3 mg/day of Cu (RDA), which would correspond to eat about 250 g of mussels per day. To exceed the maximum recommended value (UL; 10 mg/day), it would be necessary to eat 1.75 kg of mussels.

Zinc deficiency in humans is also rare, with symptoms of mild/marginal zinc deficiency including delayed wound healing and impaired resistance to infection and reduced growth rate. According to RDA and UL values, 8 - 11 mg/day and 40 - 50 mg/day (EFSA, 2006), respectively, an adult whose daily meals are 250 g of grilled chicken (2.85 mg Zn) with 100 g of rice (0.95 mg Zn), and 250 g of grilled fish (2.92 mg Zn) with 100

g of rice (0.95 mg Zn) ingests about 7.7 mg of Zn (70 % of the RDA). Instead, ingesting 80 g of mussels allows to reach the RDA. This food of marine origin is also very rich in Se, and an adult that ingests about 15 g/day can reach the recommended dose of 55 ug Se/day (RDA) (EFSA, 2006), avoiding possible adverse health effects associated with its deficiency (e.g. congestive heart failure).

Potentially toxic elements concentrations were only quantifiable in mussels (10.2 µg/g for As, 4.5 µg/g for Cd, and 0.07 µg/g for Pb) and rice matrices (0.02 µg/g for Pb).

It is noteworthy that the nutritional analysis was made only based on the total content of the elements determined in the analyzed foods. However, a correct evaluation must consider the bioavailable and/or bioaccessible fraction of the elements (e.g. by bioaccessibility/bioavailability tests as *in vitro* digestive model and Caco-2 cell cultures), which are better indicators of the absorption of the elements by the organism.

4.2.3 Legislated values and obtained food concentrations

The percentage of water in food samples (**Table 25**) was evaluated to allow to calculate the concentration (µg/g) in fresh weight (FW), and thus make it possible to compare the concentrations obtained in the scope of this work with those established by the legislation. Regarding the legal criteria only the potentially toxic elements will be evaluated. As mentioned in the introduction the most important regulation regarding food industry is EC Regulation Nº 1881/2006 that sets maximum permitted levels for a range of contaminants in foodstuffs. For the evaluation of As it was considered the amending Regulation (EU) 2015/1006.

Table 25: *Water content (%) in food matrices (mean and standard deviation (sd))*

Chicken	Water (%)	Fish	Water (%)	Mussels	Water (%)
Sample 1	73.5	Sample 1	94.7	Sample 1	89.5
Sample 2	86.5	Sample 2	95.4	Sample 2	89.8
Sample 3	90.6	Sample 3	95.1	Sample 3	90.1
Sample 4	90.1	Sample 4	95.6	Sample 4	91.7
Sample 5	90.2	Sample 5	95.4	Sample 5	88.6
Mean	86.2	Mean	95.2	Mean	89.9
sd	7.3	sd	0.36	sd	1.1

In chicken, the concentration of non-essential elements such as Cd and As were below the limit of quantification, which demonstrates that the levels of these potentially toxic elements are low enough to comply with the decree-law Nº 1881/2006 (0.05 µg/g) (Commission of the European Communities, 2006). The Pb levels were quantifiable,

however the concentrations found (0.002 – 0.004 µg/g) were 25 times lower than the maximum limit stipulated by the decree-law Nº 1881/2006 (0.10 µg/g), thus being in accordance with the legislation.

For mussels, the potentially toxic elements As, Cd and Pb showed concentration values above the quantification limit of the equipment. There is presently no Europe-wide regulation for arsenic in food (FSA, 2005) and few European countries have published legislation regulating the maximum concentration of this element in seafood products (Anacleto et al., 2009). The Food Regulation in the UK (SI 1959 nº. 831) for the concentration of As stablish a limit of 1 mg/kg wet weight (FSA, 2005). The FDA suggests a limit of 76 mg/kg for crustacea (Center for Food Safety and Applied Nutrition, 1993). Levels of Cd obtained on raw (0.64 ± 0.02 µg/g FW) and boiled matrices (0.51 ± 0.02 µg/g FW) were 1.5 - 2 times lower than the maximum concentration allowed by European Commission legislation (1 mg/kg) (Commission of the European Communities, 2006, 2001) being in accordance with the legislation. Values obtained for Pb (0.003 µg/g FW) were 500 times lower than the values allowed by decree-law Nº 1881/2006 (1.5 µg/g FW).

In fish, As and Cd presented values below the equipment quantification limit (ICP-MS) indicating that the concentrations present in raw and processed samples are very low, being in accordance with the legislation, 0.05 mg Cd /kg FW (Commission of the European Communities, 2006). For As in fish there is an absence of regarding maximum allowable levels (EFSA, 2009). Values for Pb were possible to quantify, but the results showed a concentration of 0.002 ± 0.0004 µg/g in FW for raw, grilled, and boiled samples, being in accordance with decree-law Nº 1881/2006 (0.30 µg/g FW) as it shows a value 150 times lower than the stablished value.

In rice, it was verified that all values were in accordance with the legislation, except for As in raw state (0.30 µg/g), that presented a concentration 1.5 times higher than that established in the amending regulation 2015/1006 (0.20 µg/g) (European Commission, 2015b). Similar values were found in the studies of Lamont (2003), where maximum value obtained was 0.27 µg/g, and of Liang et al. (2010), where maximum value obtained was 0.28 µg/g. However after the boiled treatment, the concentration of this element was below the quantification limit of the equipment, which highlights the importance of washing the rice before cooking it (Atiaga et al., 2020).

5. Conclusions

This work aimed to validate a methodology for the quantification of macro, micro, and potentially toxic elements in food matrices such as chicken, mussels, fish, rice, and seaweed by inductively coupled plasma techniques after microwave assisted acid digestion. This validation was developed at the Central Laboratory of Analysis of the University of Aveiro, and it was possible to achieve the validation for 9 elements (Na, Mg, Ca, Mn, Fe, Cu, Zn, As, and Cd). The elements were chosen due to their importance in nutrition and their role in human metabolism (macro and microelements), as well their dangerousness to human health (potentially toxic elements, As and Cd) to guarantee that their concentrations agreed with legislation. As a future perspective it would be necessary to participate in an interlaboratory test for the analysis of these elements in food to obtain an accreditation certificate for the analysis of these matrices, since this is a mandatory requirement for accreditation of a testing laboratory. It would also be important to evaluate the bioavailability and bioaccessibility of these elements in feed to understand how nutrition can be improved.

Several characteristics of the method were evaluated to achieve a full validation process aiming a future accreditation for the analysis of these matrices. The characteristics as matrix interferences, limit of detection, limit of quantification, working range, precision, accuracy, and uncertainty were studied and evaluated. All the studied parameters were in accordance with the requirements established by RELACRE and for this work, allowing the validation of the quantification of several elements by ICP-MS in food.

Another important approach was the comparison of the elements concentration when the food sample is submitted to different cooking treatments, *i.e.,* boiled, grilled, and fried. The raw sample (unprocessed) used in the validation process was used as reference value for the analysis of the processed samples. Results showed that for most of the elements there were no relevant alterations in the concentration when the sample is submitted to cooking treatment. An important achievement of this work was the guarantee that potentially toxic elements as As, Cd, and Pb did not present any results that disagreed with the legislation (decree-law Nº 1881/2006).

This work allowed to gain experience in quality assurance area, quality control, and validation of analytical methods, as well as developing laboratory skills and skills related to instrumental techniques of analysis by inductively coupled plasma.

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Annex I

AI.1 Validation of the method for Na in food by ICP-MS

❖ Quantification

Table 26: *Values for the determination of the inverse of the variance for the weighted linear calibration function of Na in 26/04/2021*

Standard	Concentration (mg/L)	Signal mean	si	si²	1/si²
P0	0.0	1.92E+02	1.72E+00	2.96E+00	3.38E-01
P1	0.1	6.17E+02	8.56E+00	7.32E+01	1.37E-02
P2	1.1	4.54E+03	1.06E+02	1.12E+04	8.93E-05
P3	5.5	2.27E+04	5.03E+02	2.53E+05	3.95E-06
P4	11	4.74E+04	1.03E+03	1.05E+06	9.50E-07
P5	55	2.18E+05	5.12E+03	2.62E+07	3.81E-08
P6	110	4.20E+05	8.27E+03	6.85E+07	1.46E-08
Sum	183	7.13E+05	1.50E+04	9.60E+07	3.52E-01
Mean	26	1.02E+05	2.15E+03	1.37E+07	5.03E-02

Table 27*: Values for the definition of the weighted linear calibration function of Na in 26/04/2021*

Standard	Concentration (mg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	6.73E+00	0.00E+00	1.29E+03	0.00E+00	0.00E+00
P1	0.1	2.72E-01	2.99E-02	1.68E+02	1.85E+01	3.29E-03
P2	1.1	1.78E-03	1.95E-03	8.06E+00	8.87E+00	2.15E-03
P3	5.5	7.85E-05	4.32E-04	1.78E+00	9.81E+00	2.38E-03
P4	11	1.89E-05	2.08E-04	8.94E-01	9.85E+00	2.29E-03
P5	55	7.58E-07	4.17E-05	1.65E-01	9.09E+00	2.30E-03
P6	110	2.91E-07	3.20E-05	1.22E-01	1.34E+01	3.52E-03
Sum	183	7.00E+00	3.26E-02	1.47E+03	6.95E+01	1.59E-02
Mean	26	1.00E+00	4.65E-03	2.10E+02	9.93E+00	2.28E-03

Table 28: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Na in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Na] calculated	|Relative error| (%)
P0	1.92E+02	4.59E-01	2.39E-01	1.16E-04	-
P1	6.29E+02	-1.16E+01	-1.88E+00	1.07E-01	2.65E+00
P2	4.57E+03	-2.55E+01	-5.63E-01	1.09E+00	5.84E-01
P3	2.21E+04	6.59E+02	2.90E+00	5.67E+00	3.02E+00
P4	4.39E+04	3.42E+03	7.22E+00	1.19E+01	7.82E+00
P5	2.19E+05	-8.38E+02	-3.84E-01	5.48E+01	3.83E-01
P6	4.38E+05	-1.76E+04	-4.20E+00	1.06E+02	4.03E+00

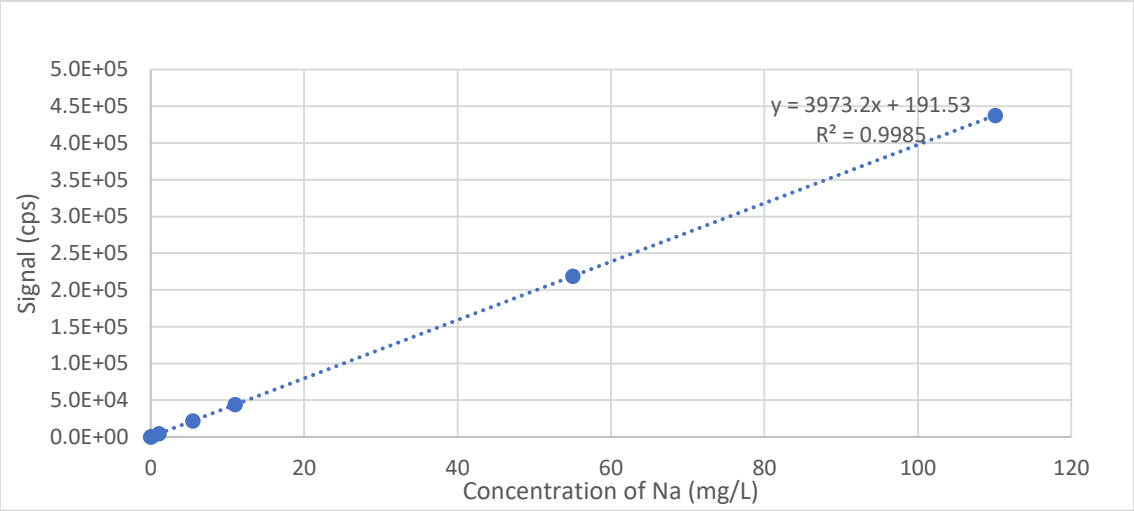

Figure 24: *Calibration curve for Na in relation to the instrumental signal on 26/04/2021*

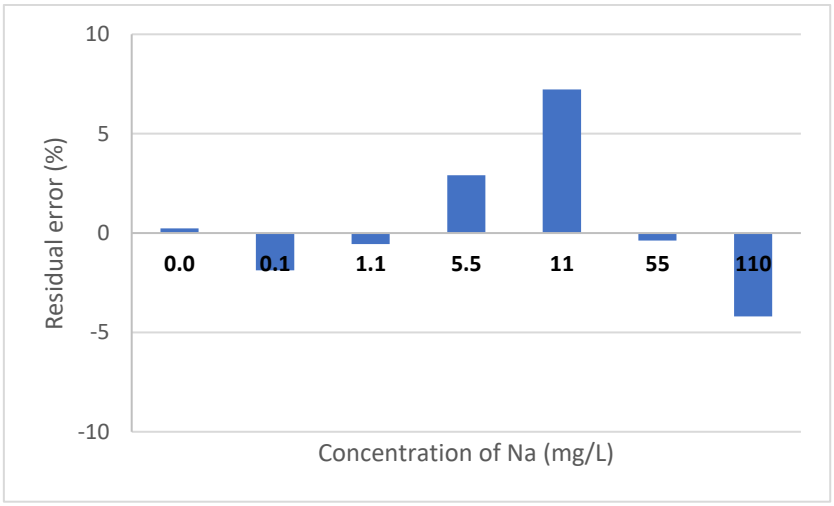

Figure 25: *Residual error (%) associated with different calibration standards for Na on 26/04/2021*

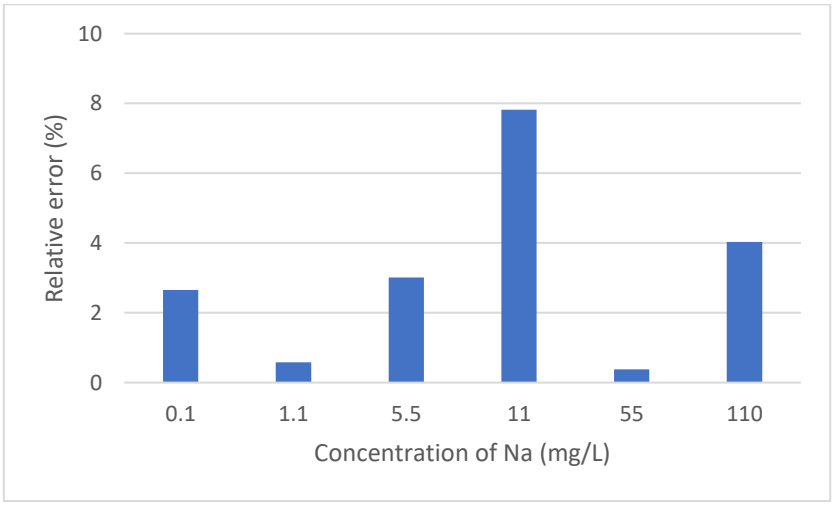

Figure 26: *Relative error (%) associated with different calibration standards for Na on 26/04/2021*

❖ **Limit of detection and limit of quantification**

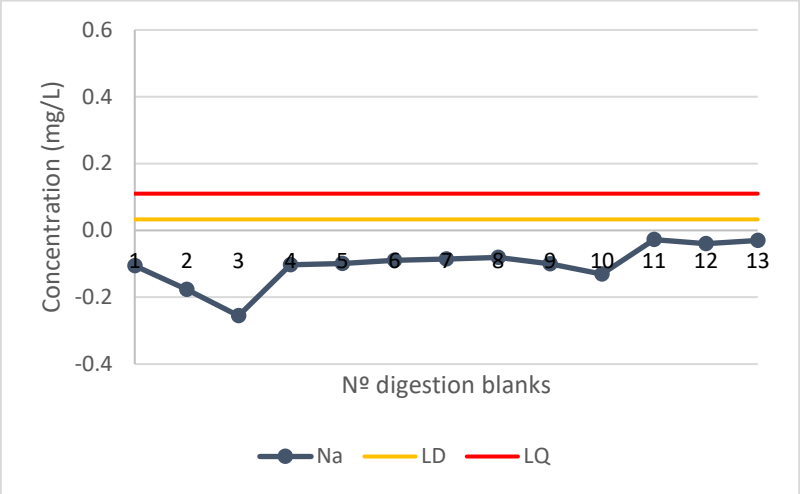

Figure 27: *Control verification chart of digestion blanks according to Na concentration*

❖ **Selectivity**

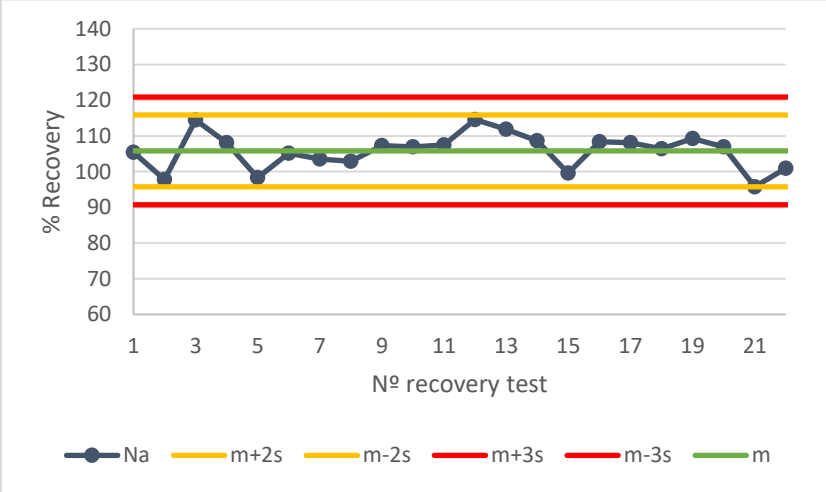

Figure 28: *Control chart for Na recovery (%) by number of recovery readings*

❖ **Trueness**

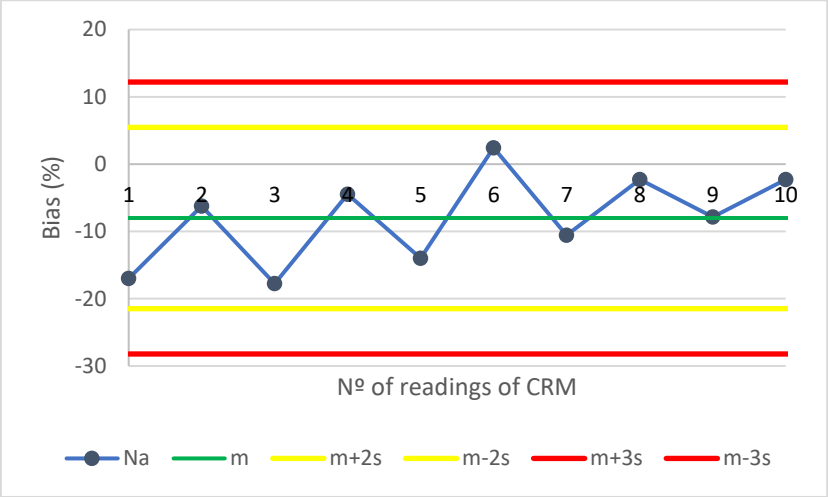

Figure 29: *Control chart of bias (%) for Na by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

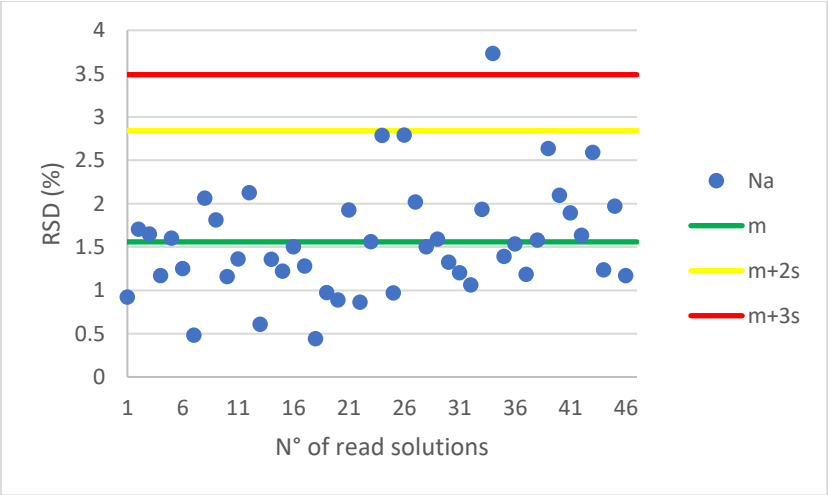

Figure 30: *Control chart of the relative standard deviations (RSD, %) for Na by ICP-MS*

- **Coefficient of variation of repeatability**

Table 29: Estimated CVr (%) for Na

Sample	CV_r (%)	Nº of reading samples
Chicken	6	10
Mussel	4	10
Fish	5	10
Rice	9	8
ZC 73016	9	2
ERM-CE278k	10	2
ERM-BB422	10	2
Dorm-4	6	2
ZC 73028	-	2
SRM 8433	4	2

- **Duplicate evaluation**

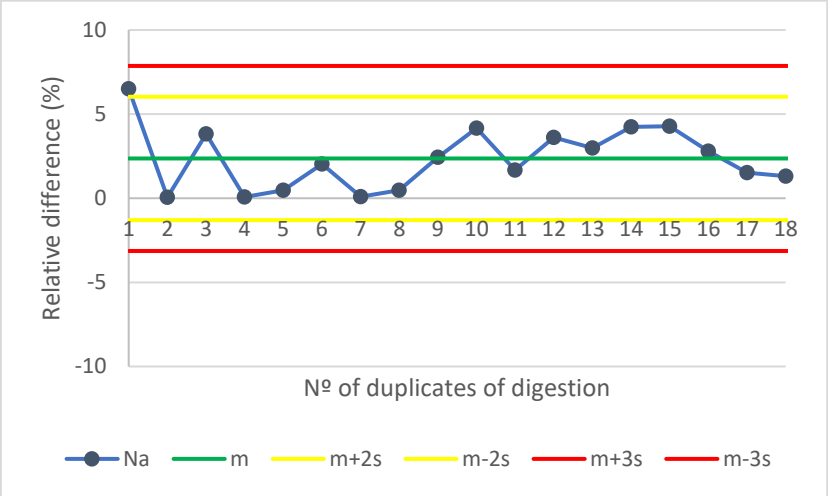

Figure 31: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Na

- **Intermediate precision**

Table 30: Estimated CV_{SI} (%) for Na

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	9	11	2
Rice	9	18	2
Seaweed	6	10	2

❖ **Uncertainty**

Table 31: Estimation of the uncertainty (%) for Na by ICP-MS

u_r	4.1 %
u_b	4.4 %
u_c	6.0 %
U	12.0 %

AI.2 Validation of the method for Mg in food by ICP-MS

❖ Quantification

Table 32: *Values for the determination of the inverse of the variance for the weighted linear calibration function of Mg in 26/04/2021*

Standard	Concentration (mg/L)	Signal mean	si	si^2	$1/si^2$
P0	0.0	2.43E-01	2.70E-02	7.26E-04	1.38E+03
P1	0.1	3.50E+01	3.58E-01	1.28E-01	7.79E+00
P2	1.1	3.41E+02	6.39E+00	4.08E+01	2.45E-02
P3	5.5	1.82E+03	2.85E+01	8.13E+02	1.23E-03
P4	11	3.93E+03	7.86E+01	6.18E+03	1.62E-04
P5	55	1.79E+04	3.17E+02	1.00E+05	9.96E-06
P6	110	3.42E+04	6.06E+02	3.68E+05	2.72E-06
Sum	183	5.82E+04	1.04E+03	4.75E+05	1.38E+03
Mean	26	8.32E+03	1.48E+02	6.79E+04	1.98E+02

Table 33: *Values for the definition of the weighted linear calibration function of Mg in 26/04/2021*

Standard	Concentration (mg/L)	wi	wi*xi	wi*yi	wi*xi*yi	$wi*xi^2$
P0	0.0	6.96E+00	0.00E+00	1.69E+00	0.00E+00	0.00E+00
P1	0.1	3.94E-02	4.33E-03	1.38E+00	1.52E-01	4.77E-04
P2	1.1	1.24E-04	1.36E-04	4.22E-02	4.65E-02	1.50E-04
P3	5.5	6.22E-06	3.42E-05	1.13E-02	6.24E-02	1.88E-04
P4	11	8.18E-07	9.00E-06	3.22E-03	3.54E-02	9.91E-05
P5	55	5.04E-08	2.77E-06	9.01E-04	4.96E-02	1.53E-04
P6	110	1.38E-08	1.51E-06	4.70E-04	5.18E-02	1.67E-04
Sum	183	7.00E+00	4.52E-03	3.13E+00	3.97E-01	1.23E-03
Mean	26	1.00E+00	6.45E-04	4.47E-01	5.68E-02	1.76E-04

Table 34: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Mg in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Mg] calculated	|Relative error| (%)
P0	2.39E-01	3.55E-03	1.46E+00	1.10E-05	-
P1	3.56E+01	-6.05E-01	-1.73E+00	1.08E-01	1.71E+00
P2	3.54E+02	-1.25E+01	-3.67E+00	1.06E+00	3.54E+00
P3	1.77E+03	5.64E+01	3.09E+00	5.68E+00	3.19E+00
P4	3.54E+03	3.97E+02	1.01E+01	1.22E+01	1.12E+01
P5	1.77E+04	2.11E+02	1.18E+00	5.57E+01	1.19E+00
P6	3.54E+04	-1.16E+03	-3.39E+00	1.06E+02	3.28E+00

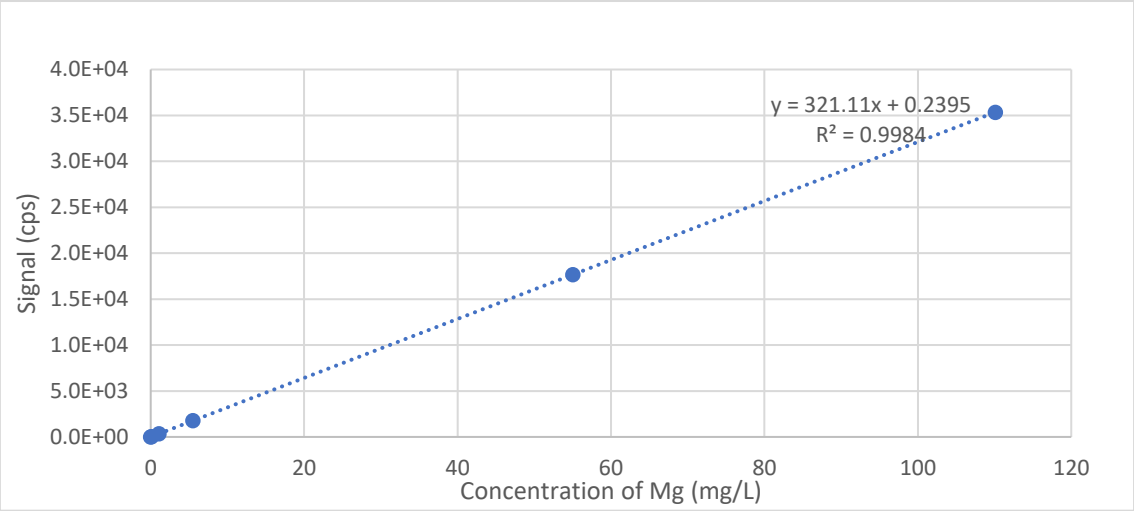

Figure 32: *Calibration curve for Mg in relation to the instrumental signal on 26/04/2021*

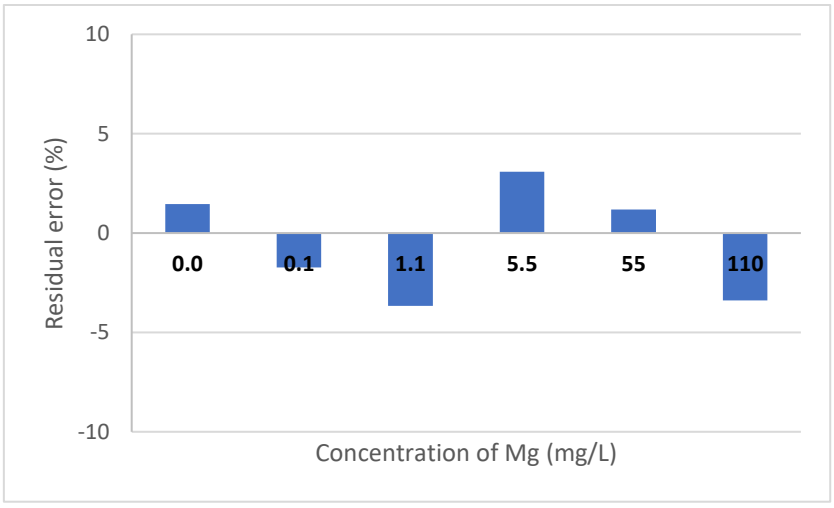

Figure 33: *Residual error (%) associated with different calibration standards for Mg on 26/04/2021*

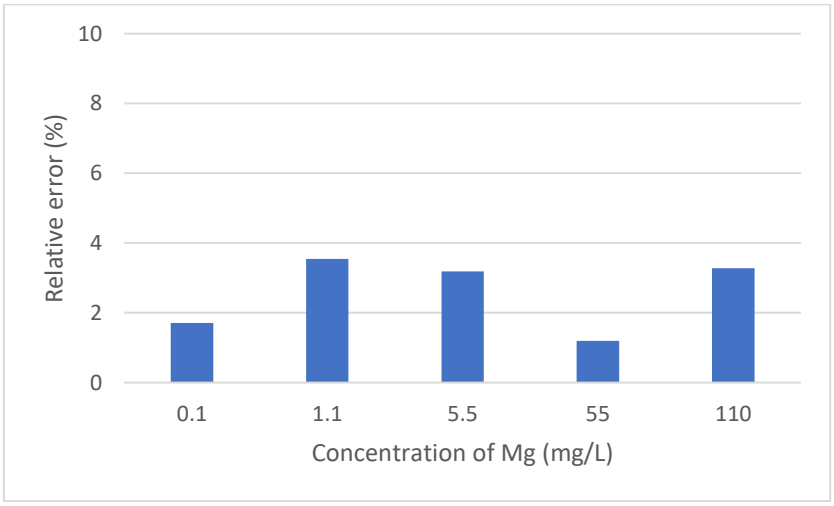

Figure 34: *Relative error (%) associated with different calibration standards for Mg on 26/04/2021*

❖ **Limit of detection and limit of quantification**

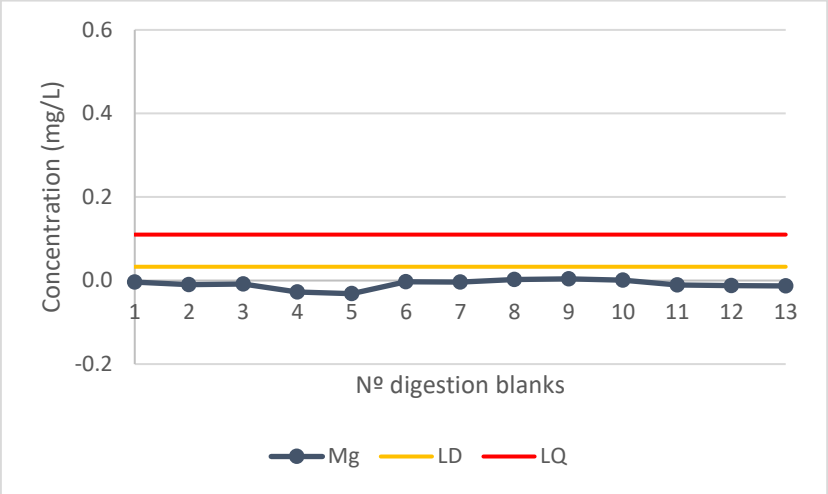

***Figure 35**: Control verification chart of digestion blanks according to Mg concentration*

❖ **Selectivity**

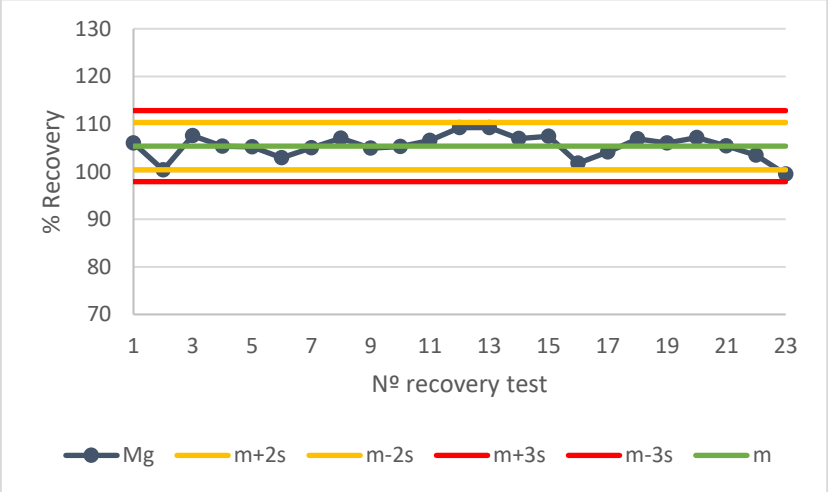

***Figure 36:** Control chart for Mg recovery (%) by number of recovery readings*

❖ **Trueness**

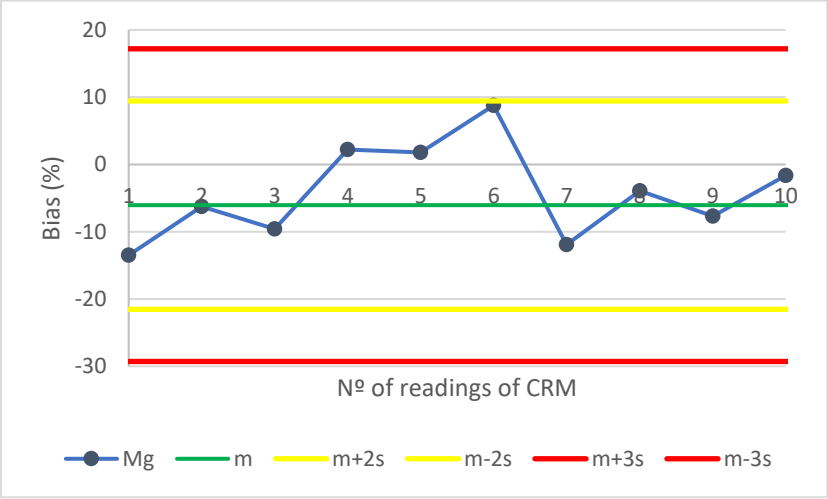

Figure 37: *Control chart of bias (%) for Mg by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

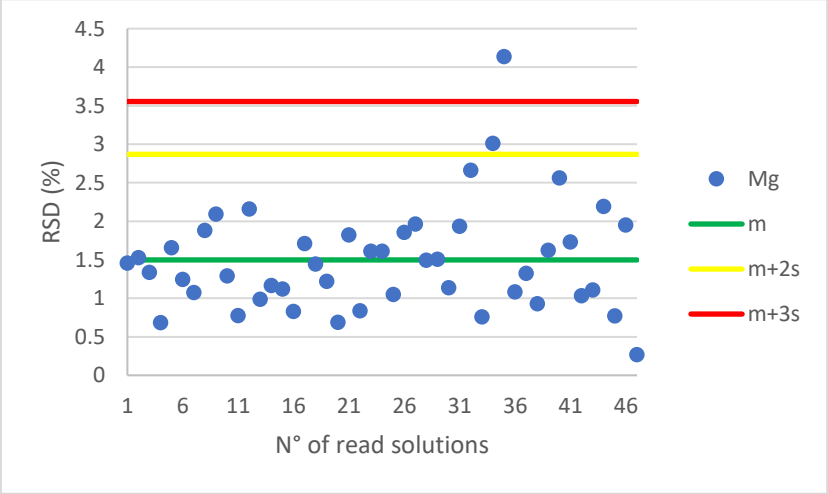

Figure 38: *Control chart of the relative standard deviations (RSD, %) for Mg by ICP-MS*

- **Coefficient of variation of repeatability**

Table 35: Estimated CVr (%) for Mg

Sample	CV_r (%)	Nº of reading samples
Chicken	6	10
Mussel	4	10
Fish	5	10
Rice	10	8
ZC 73016	6	2
ERM-CE278k	9	2
ERM-BB422	5	2
Dorm-4	6	2
ZC 73028	2	2
SRM 8433	4	2

- **Duplicate evaluation**

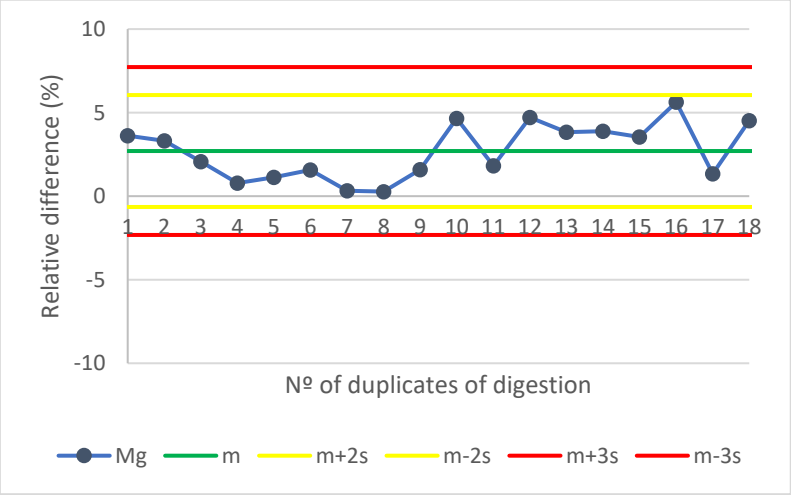

Figure 39: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Mg

- **Intermediate precision**

Table 36: Estimated CV_{SI} (%) for Mg

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	10	20	2
Rice	9	20	2
Seaweed	4	10	2

❖ **Uncertainty**

Table 37: Estimation of the uncertainty (%) for Mg by ICP-MS

u_r	6.1 %
u_b	10.0 %
u_c	11.8 %
U	23.5 %

AI.3 Validation of the method for Ca in food by ICP-MS

❖ Quantification

Table 38: *Values for the determination of the inverse of the variance for the weighted linear calibration function of Ca in 26/04/2021*

Standard	Concentration (mg/L)	Signal mean	si	si^2	$1/si^2$
P0	0.0	3.01E+01	6.87E-01	4.72E-01	2.12E+00
P1	0.1	4.31E+01	6.71E-01	4.50E-01	2.22E+00
P2	1.1	1.47E+02	2.89E+00	8.33E+00	1.20E-01
P3	5.5	6.47E+02	4.16E+00	1.73E+01	5.78E-02
P4	11	1.33E+03	2.35E+01	5.53E+02	1.81E-03
P5	55	6.47E+03	1.20E+02	1.45E+04	6.91E-05
P6	110	1.28E+04	1.37E+02	1.87E+04	5.35E-05
Sum	183	2.14E+04	2.89E+02	3.37E+04	4.52E+00
Mean	26	3.06E+03	4.13E+01	4.82E+03	6.46E-01

Table 39: *Values for the definition of the weighted linear calibration function of Ca in 26/04/2021*

Standard	Concentration (mg/L)	wi	wi*xi	wi*yi	wi*xi*yi	$wi*xi^2$
P0	0.0	3.28E+00	0.00E+00	9.86E+01	0.00E+00	0.00E+00
P1	0.1	3.44E+00	3.79E-01	1.48E+02	1.63E+01	4.17E-02
P2	1.1	1.86E-01	2.05E-01	2.72E+01	3.00E+01	2.25E-01
P3	5.5	8.95E-02	4.93E-01	5.80E+01	3.19E+02	2.71E+00
P4	11	2.80E-03	3.08E-02	3.72E+00	4.10E+01	3.39E-01
P5	55	1.07E-04	5.89E-03	6.93E-01	3.81E+01	3.24E-01
P6	110	8.29E-05	9.13E-03	1.06E+00	1.17E+02	1.00E+00
Sum	183	7.00E+00	1.12E+00	3.38E+02	5.61E+02	4.65E+00
Mean	26	1.00E+00	1.60E-01	4.82E+01	8.01E+01	6.64E-01

Table 40: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Ca in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Ca] calculated	|Relative error| (%)
P0	3.00E+01	8.70E-03	2.90E-02	7.67E-05	-
P1	4.25E+01	5.76E-01	1.34E+00	1.15E-01	4.61E+00
P2	1.55E+02	-8.41E+00	-5.74E+00	1.03E+00	6.73E+00
P3	6.54E+02	-7.07E+00	-1.09E+00	5.44E+00	1.13E+00
P4	1.28E+03	5.15E+01	3.87E+00	1.15E+01	4.12E+00
P5	6.27E+03	1.98E+02	3.06E+00	5.68E+01	3.17E+00
P6	1.25E+04	2.45E+02	1.92E+00	1.12E+02	1.96E+00

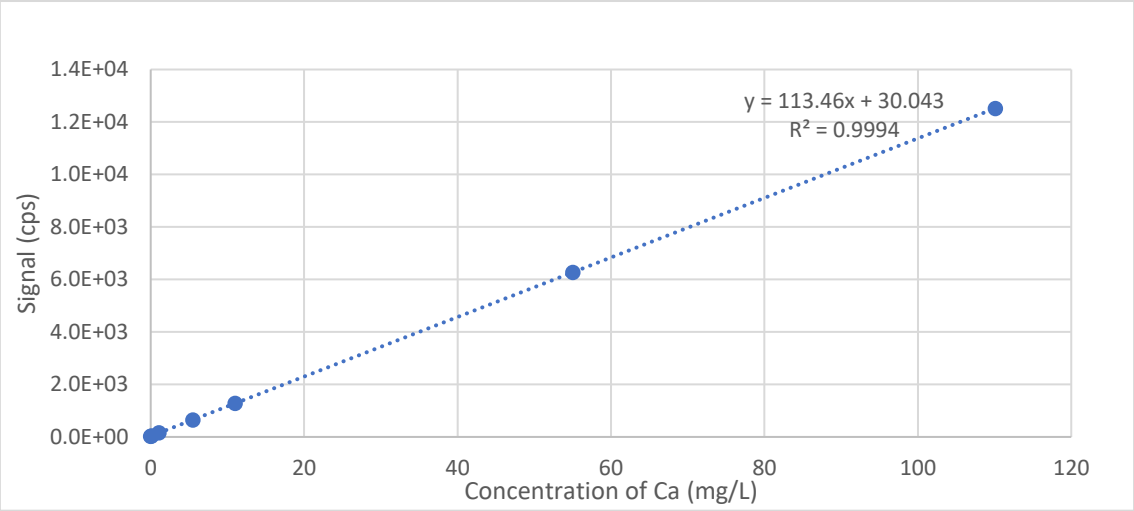

Figure 40: *Calibration curve for Ca in relation to the instrumental signal on 26/04/2021*

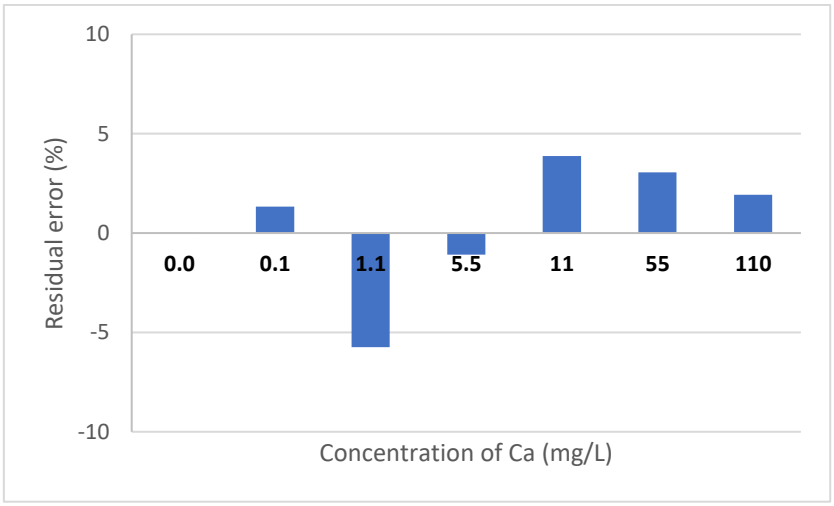

Figure 41: *Residual error (%) associated with different calibration standards for Ca on 26/04/2021*

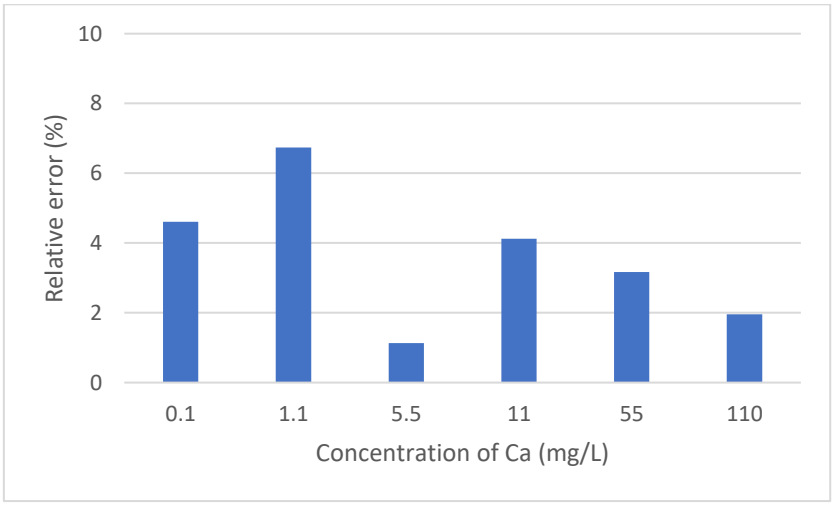

Figure 42: *Relative error (%) associated with different calibration standards for Ca on 26/04/2021*

❖ **Limit of detection and limit of quantification**

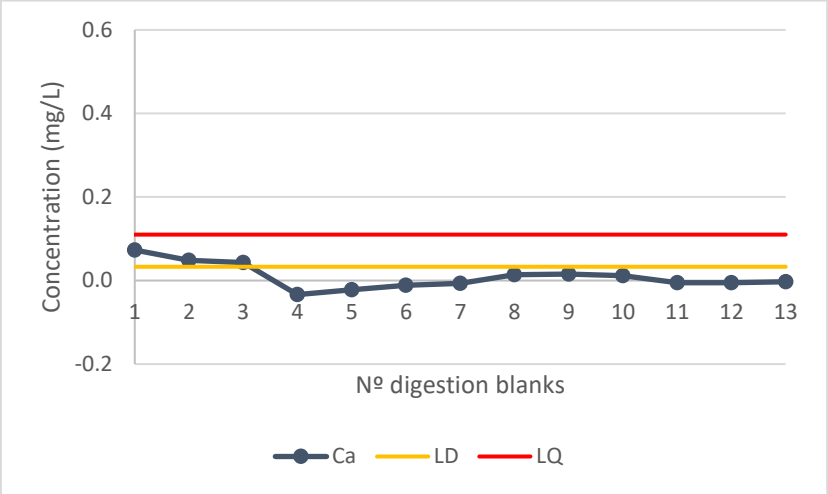

***Figure 43**: Control verification chart of digestion blanks according to Ca concentration*

❖ **Selectivity**

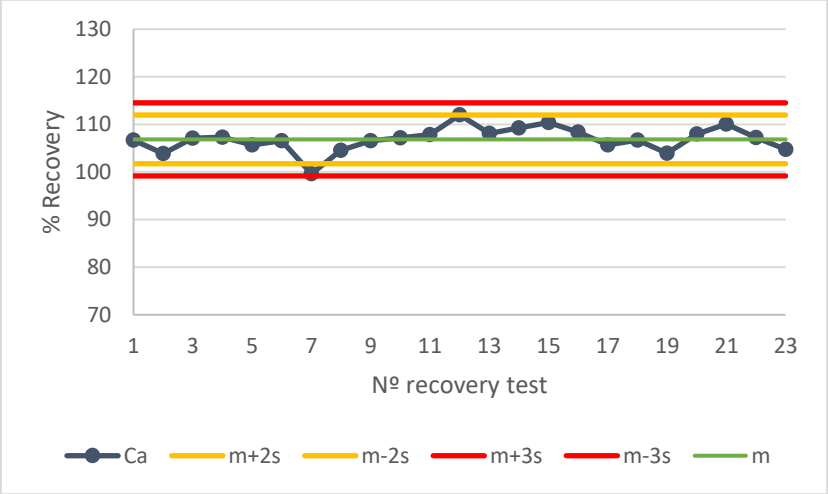

***Figure 44:** Control chart for Ca recovery (%) by number of recovery readings*

❖ **Trueness**

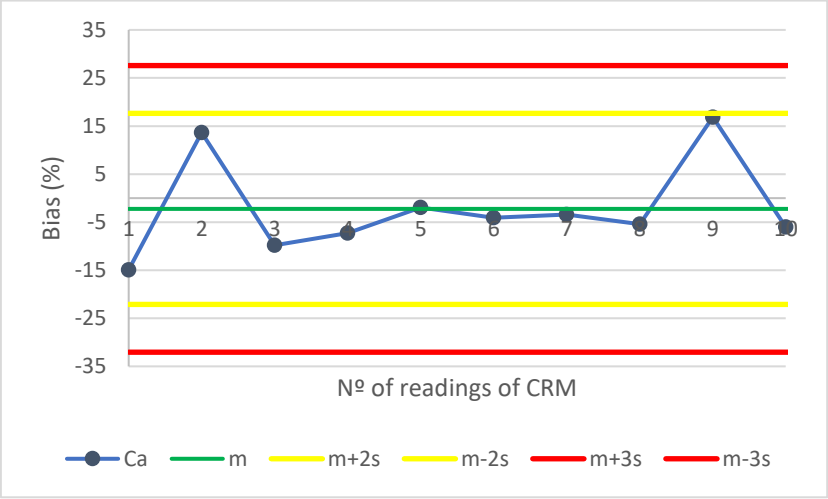

Figure 45: *Control chart of bias (%) for Ca by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

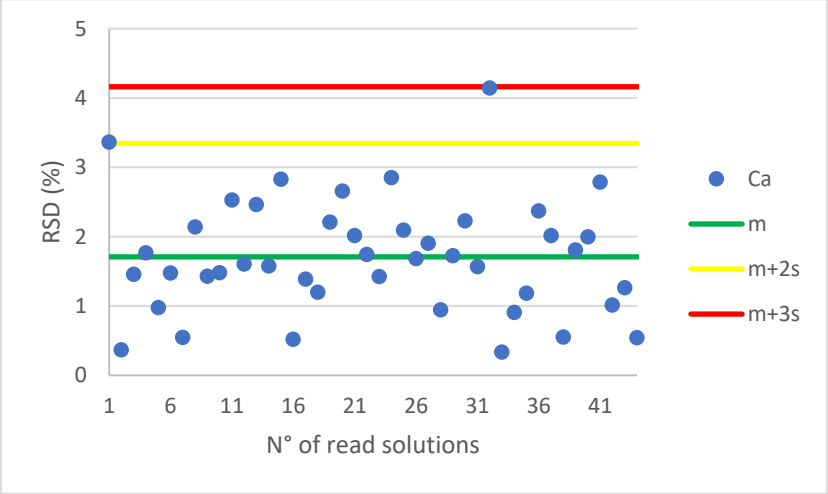

Figure 46: *Control chart of the relative standard deviations (RSD, %) for Ca by ICP-MS*

- **Coefficient of variation of repeatability**

Table 41: Estimated CVr (%) for Ca

Sample	CV_r (%)	Nº of reading samples
Chicken	4	10
Mussel	7	10
Fish	2	10
Rice	6	8
ZC 73016	10	2
ERM-CE278k	2	2
ERM-BB422	10	2
Dorm-4	1	2
ZC 73028	10	2
SRM 8433	10	2

- **Duplicate evaluation**

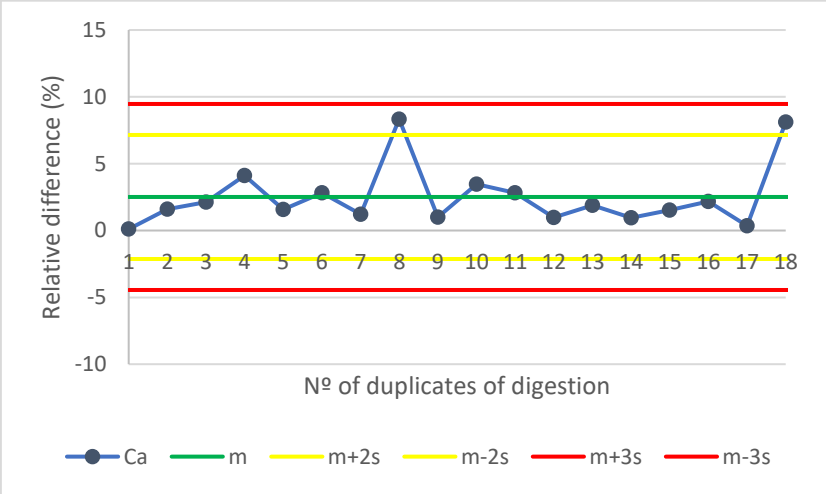

Figure 47: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Ca

- **Intermediate precision**

Table 42: Estimated CV_{SI} (%) for Ca

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	4	10	2
Rice	6	10	2
Seaweed	5	9	2

❖ **Uncertainty**

Table 43: Estimation of the uncertainty (%) for Ca by ICP-MS

u_r	1.5 %
u_b	6.7 %
u_c	6.9 %
U	13.8 %

AI.4 Validation of the method for Mn in food by ICP-MS

❖ Quantification

Table 44: *Values for the determination of the inverse of the variance for the weighted linear calibration function of Mn in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si²	1/si²
P0	0.0	1.81E+03	4.16E+01	1.73E+03	5.78E-04
P1	0.15	2.65E+03	3.69E+01	1.36E+03	7.36E-04
P2	1.5	9.62E+03	1.79E+02	3.20E+04	3.13E-05
P3	7.5	4.23E+04	5.19E+02	2.69E+05	3.72E-06
P4	15	8.57E+04	1.18E+03	1.38E+06	7.23E-07
P5	75	4.06E+05	3.16E+03	9.96E+06	1.00E-07
P6	150	8.12E+05	3.19E+03	1.02E+07	9.84E-08
Sum	249	1.36E+06	8.29E+03	2.18E+07	1.35E-03
Mean	36	1.94E+05	1.18E+03	3.11E+06	1.93E-04

Table 45: *Values for the definition of the weighted linear calibration function of Mn in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	3.00E+00	0.00E+00	5.42E+03	0.00E+00	0.00E+00
P1	0.15	3.82E+00	5.72E-01	1.01E+04	1.52E+03	8.58E-02
P2	1.5	1.62E-01	2.43E-01	1.56E+03	2.34E+03	3.65E-01
P3	7.5	1.93E-02	1.45E-01	8.16E+02	6.12E+03	1.08E+00
P4	15	3.75E-03	5.62E-02	3.21E+02	4.82E+03	8.44E-01
P5	75	5.21E-04	3.91E-02	2.12E+02	1.59E+04	2.93E+00
P6	150	5.10E-04	7.66E-02	4.15E+02	6.22E+04	1.15E+01
Sum	249	7.00E+00	1.13E+00	1.89E+04	9.29E+04	1.68E+01
Mean	36	1.00E+00	1.62E-01	2.69E+03	1.33E+04	2.40E+00

Table 46: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Mn in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Mn] calculated	|Relative error| (%)
P0	1.82E+03	-1.06E+01	-5.84E-01	-1.95E-03	
P1	2.63E+03	1.93E+01	7.29E-01	1.54E-01	2.38E+00
P2	9.93E+03	-3.13E+02	-3.25E+00	1.44E+00	3.86E+00
P3	4.24E+04	-4.00E+01	-9.45E-02	7.49E+00	9.86E-02
P4	8.29E+04	2.77E+03	3.23E+00	1.55E+01	3.41E+00
P5	4.07E+05	-1.13E+03	-2.79E-01	7.48E+01	2.80E-01
P6	8.13E+05	-5.27E+02	-6.49E-02	1.50E+02	6.50E-02

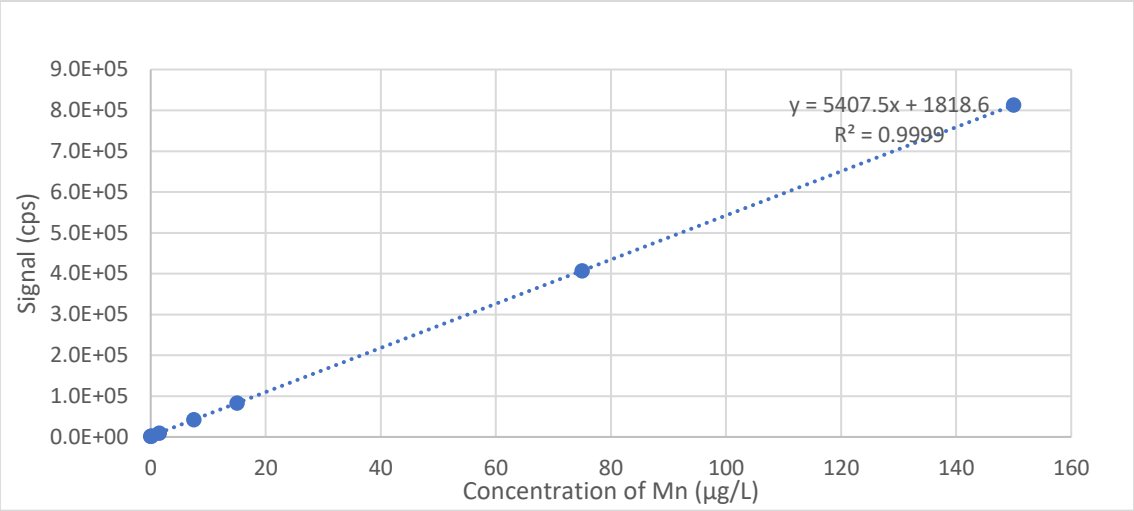

***Figure 48**: Calibration curve for Mn in relation to the instrumental signal on 26/04/2021*

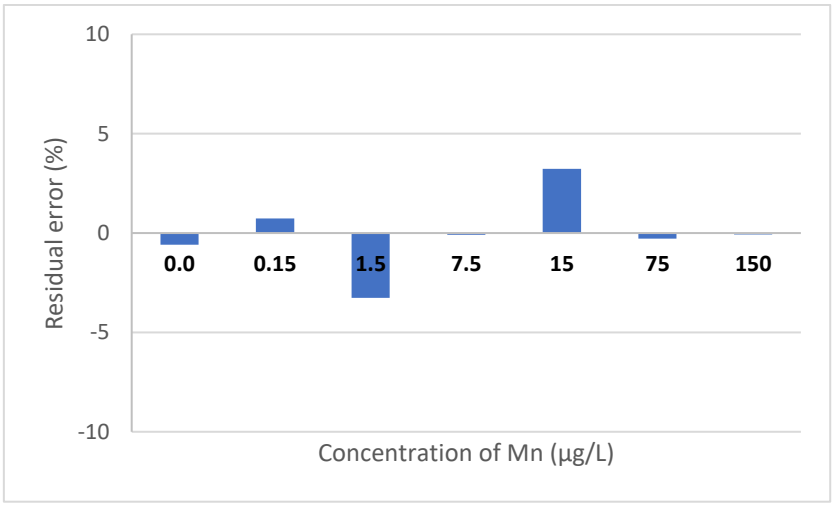

***Figure 49:** Residual error (%) associated with different calibration standards for Mn on 26/04/2021*

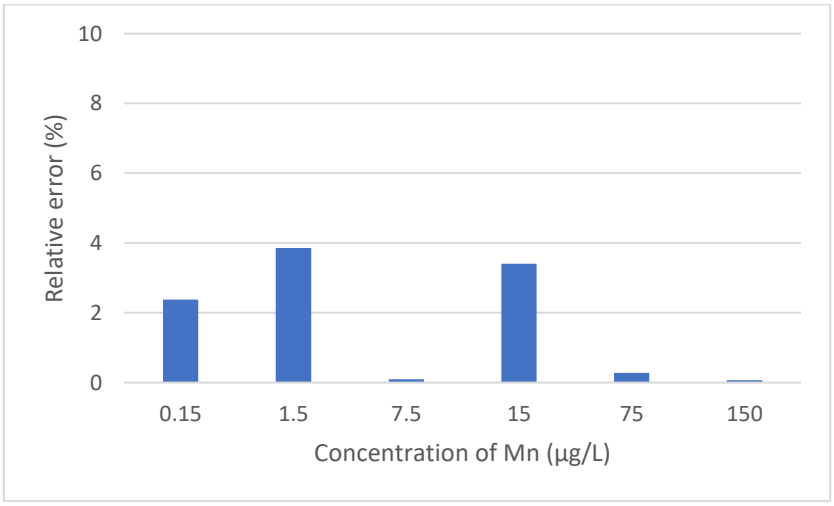

***Figure 50:** Relative error (%) associated with different calibration standards for Mn on 26/04/2021*

❖ **Limit of detection and limit of quantification**

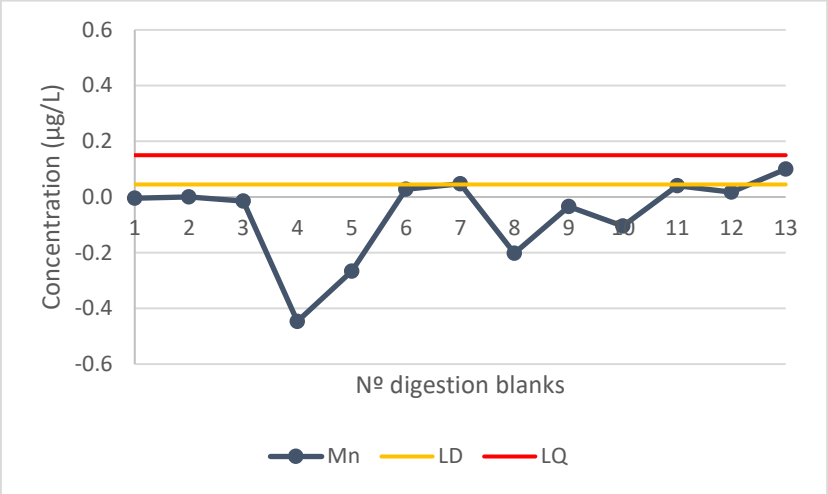

Figure 51: *Control verification chart of digestion blanks according to Mn concentration*

❖ **Selectivity**

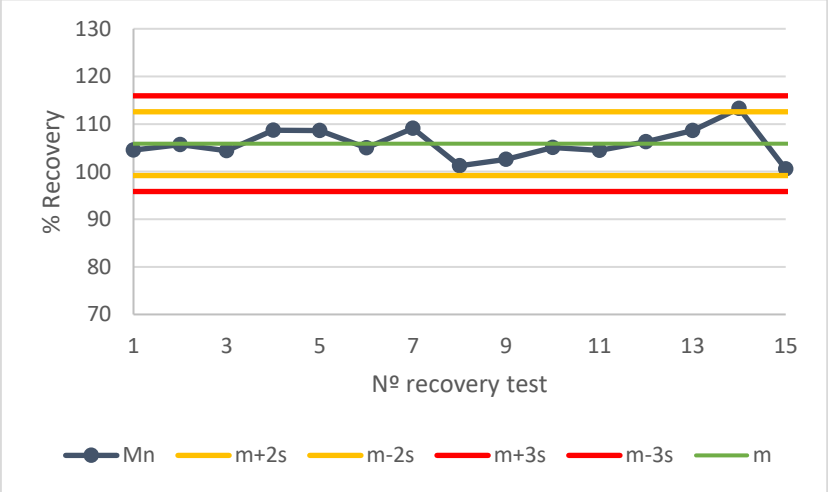

Figure 52: *Control chart for Mn recovery (%) by number of recovery readings*

❖ **Trueness**

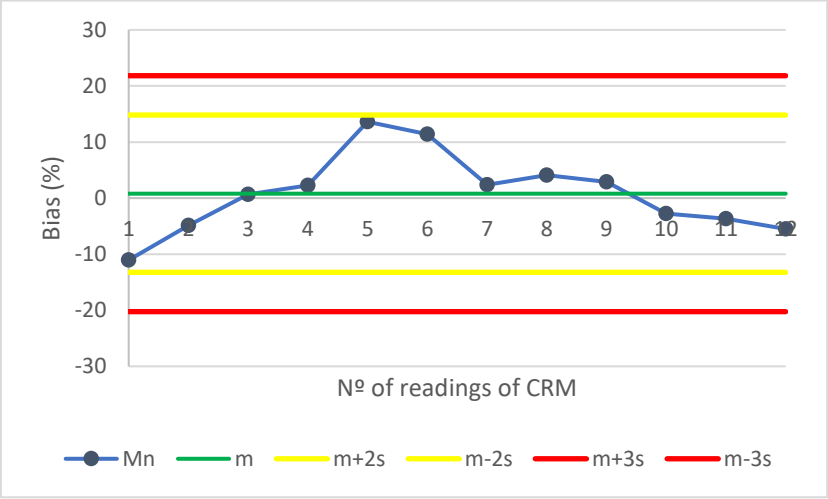

Figure 53: *Control chart of bias (%) for Mn by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

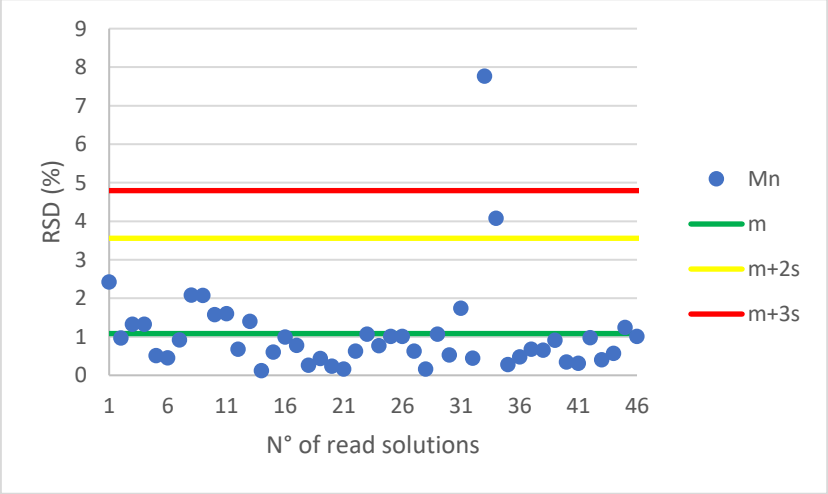

Figure 54: *Control chart of the relative standard deviations (RSD, %) for Mn by ICP-MS*

- **Coefficient of variation of repeatability**

Table 47: Estimated CVr (%) for Mn

Sample	CV_r (%)	Nº of reading samples
Chicken	3	10
Mussel	1	10
Fish	3	10
Rice	3	8
ZC 73016	5	2
ERM-CE278k	1	2
ERM-BB422	1	2
Dorm-4	1	2
ZC 73028	1	2
SRM 8433	4	2

- **Duplicate evaluation**

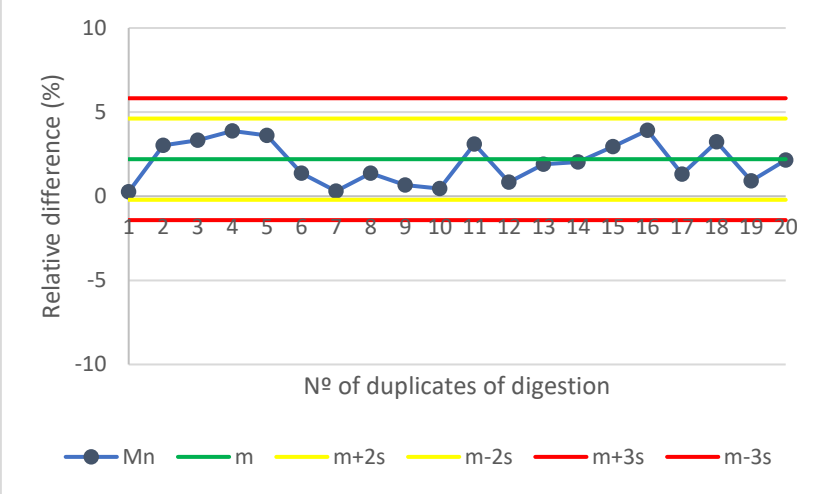

Figure 55: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Mn

- **Intermediate precision**

Table 48: Estimated CV_{SI} (%) for Mn

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	4	20	2
Rice	4	20	2
Seaweed	9	8	2

❖ **Uncertainty**

Table 49: Estimation of the uncertainty (%) for Mn by ICP-MS

u_r	1.2 %
u_b	5.3 %
u_c	5.4 %
U	10.8 %

AI.5 Validation of the method for Fe in food by ICP-MS

❖ Quantification

Table 50: *Values for the determination of the inverse of the variance of the weighted linear calibration function of Fe in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si²	1/si²
P0	0.0	2.69E+05	7.91E+02	6.26E+05	1.60E-06
P1	0.3	2.76E+05	6.00E+03	3.60E+07	2.78E-08
P2	3	2.82E+05	2.79E+03	7.76E+06	1.29E-07
P3	15	3.35E+05	3.85E+03	1.48E+07	6.74E-08
P4	30	3.92E+05	4.88E+03	2.38E+07	4.19E-08
P5	150	9.21E+05	1.54E+04	2.36E+08	4.24E-09
P6	300	1.59E+06	9.88E+03	9.77E+07	1.02E-08
Sum	498	4.07E+06	4.36E+04	4.17E+08	1.88E-06
Mean	71	5.81E+05	6.22E+03	5.95E+07	2.68E-07

Table 51: *Values for the definition of the weighted linear calibration function of Fe in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	5.95E+00	0.00E+00	1.60E+06	0.00E+00	0.00E+00
P1	0.3	1.04E-01	3.11E-02	2.86E+04	8.58E+03	9.32E-03
P2	3	4.81E-01	1.44E+00	1.35E+05	4.06E+05	4.32E+00
P3	15	2.51E-01	3.77E+00	8.41E+04	1.26E+06	5.65E+01
P4	30	1.56E-01	4.69E+00	6.13E+04	1.84E+06	1.41E+02
P5	150	1.58E-02	2.37E+00	1.45E+04	2.18E+06	3.55E+02
P6	300	3.82E-02	1.14E+01	6.08E+04	1.82E+07	3.43E+03
Sum	498	7.00E+00	2.37E+01	1.99E+06	2.39E+07	3.99E+03
Mean	71	1.00E+00	3.39E+00	2.84E+05	3.42E+06	5.70E+02

Table 52: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Fe in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Fe] calculated	|Relative error| (%)
P0	2.69E+05	1.39E+02	5.15E-02	3.15E-02	-
P1	2.70E+05	6.20E+03	2.24E+00	1.71E+00	4.70E+02
P2	2.82E+05	-3.61E+02	-1.28E-01	2.92E+00	2.73E+00
P3	3.35E+05	9.01E+01	2.69E-02	1.50E+01	1.37E-01
P4	4.01E+05	-8.90E+03	-2.27E+00	2.80E+01	6.74E+00
P5	9.29E+05	-8.17E+03	-8.87E-01	1.48E+02	1.24E+00
P6	1.59E+06	5.34E+03	3.35E-01	3.01E+02	4.04E-01

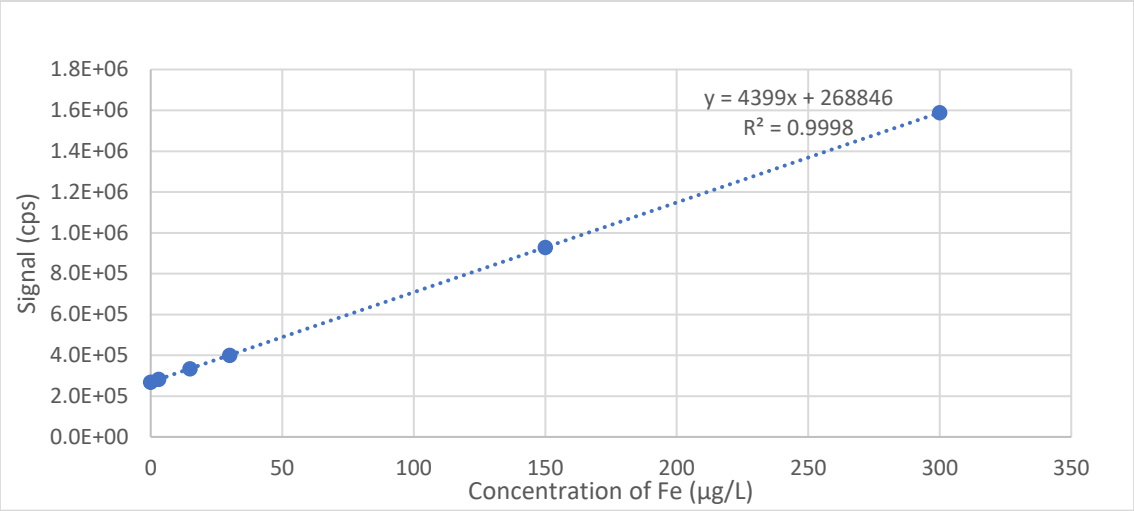

Figure 56: *Calibration curve for Fe in relation to the instrumental signal on 26/04/2021*

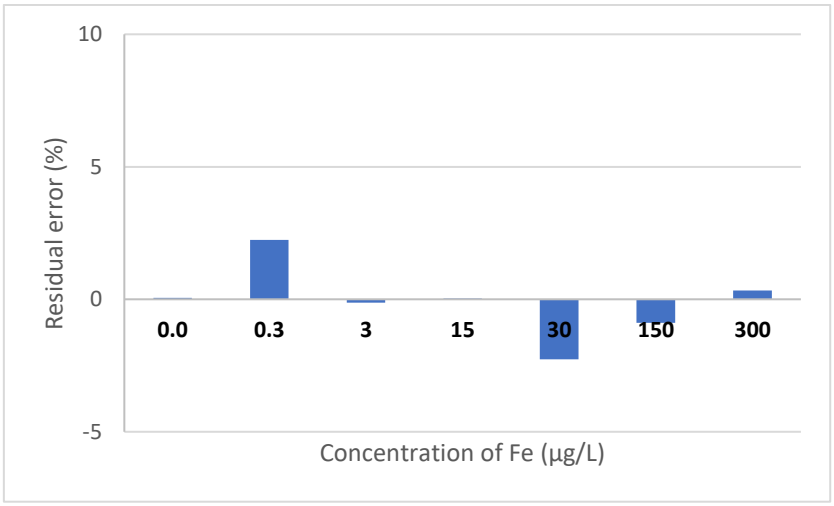

Figure 57: *Residual error (%) associated with different calibration standards for Fe on 26/04/2021*

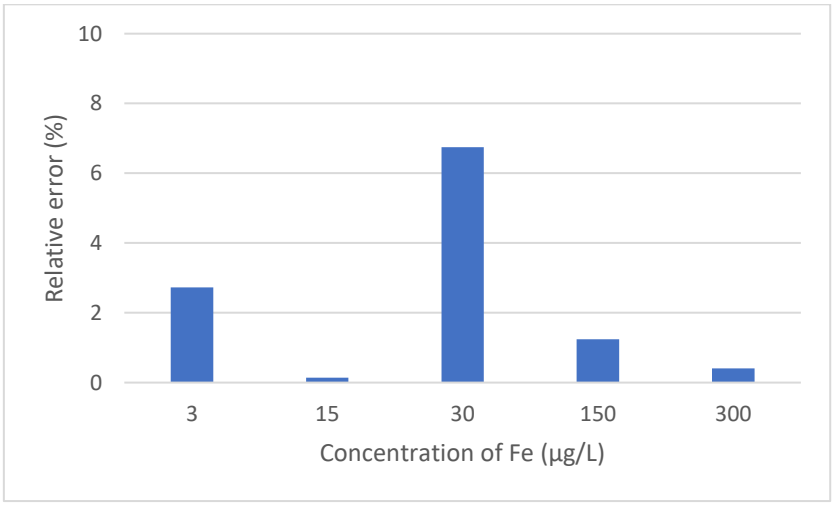

Figure 58: *Relative error (%) associated with different calibration standards for Fe on 26/04/2021*

❖ **Limit of detection and limit of quantification**

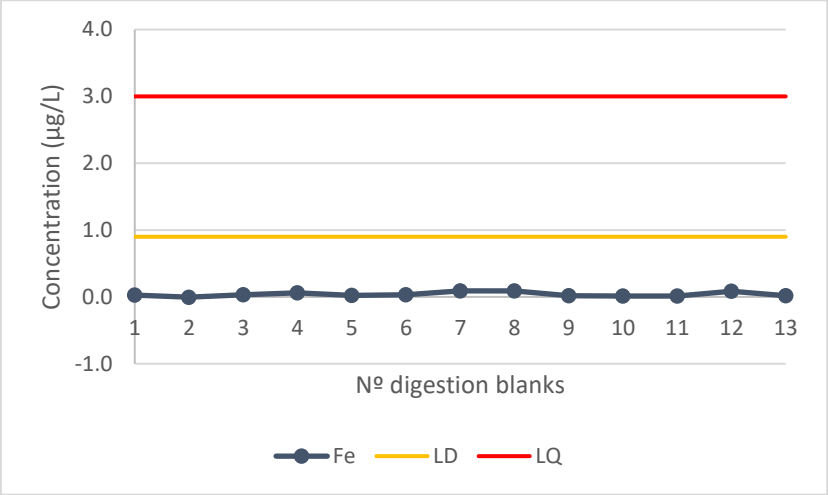

Figure 59: *Control verification chart of digestion blanks according to Fe concentration*

❖ **Selectivity**

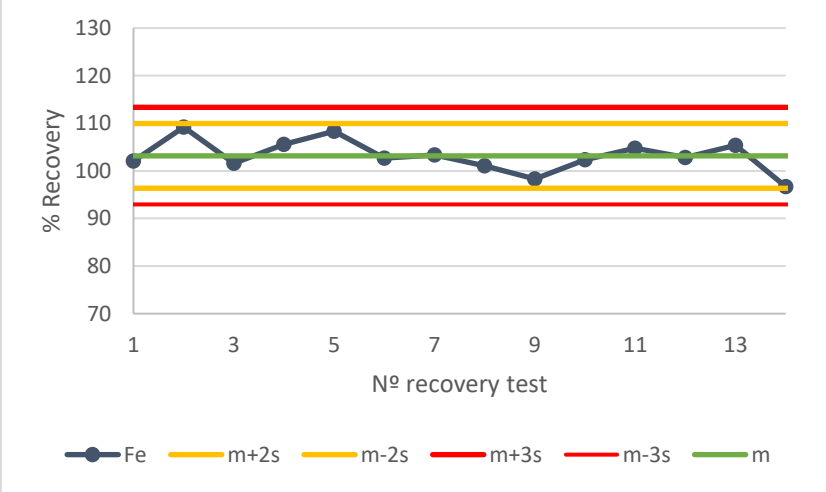

Figure 60: *Control chart for Fe recovery (%) by number of recovery readings*

❖ **Trueness**

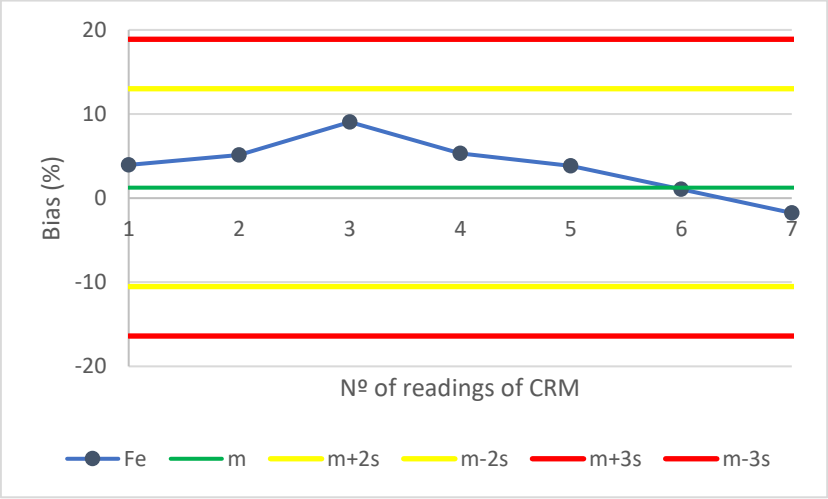

Figure 61: *Control chart of bias (%) for Fe by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

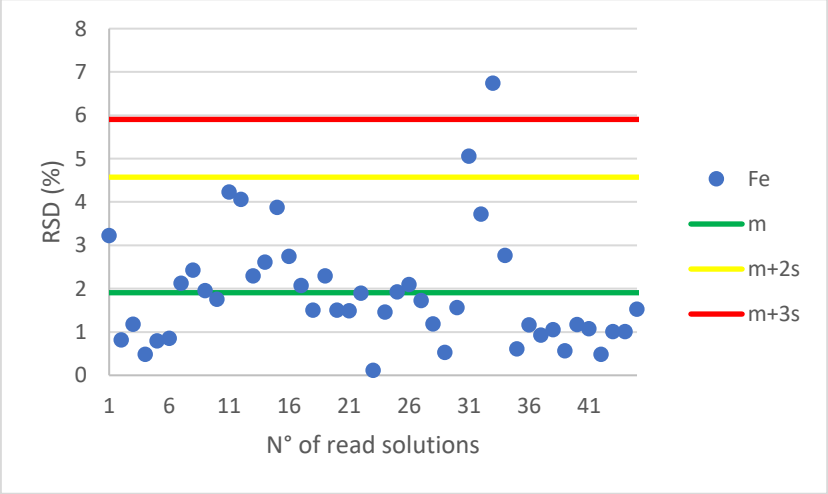

Figure 62: *Control chart of the relative standard deviations (RSD, %) for Fe by ICP-MS*

- **Coefficient of variation of repeatability**

Table 53: Estimated CVr (%) for Fe

Sample	CV_r (%)	Nº of reading samples
Chicken	6	10
Mussel	2	10
Fish	4	10
Rice	5	8
ZC 73016	10	2
ERM-CE278k	10	2
ERM-BB422	2	2
Dorm-4	9	2
ZC 73028	6	2
SRM 8433	2	2

- **Duplicate evaluation**

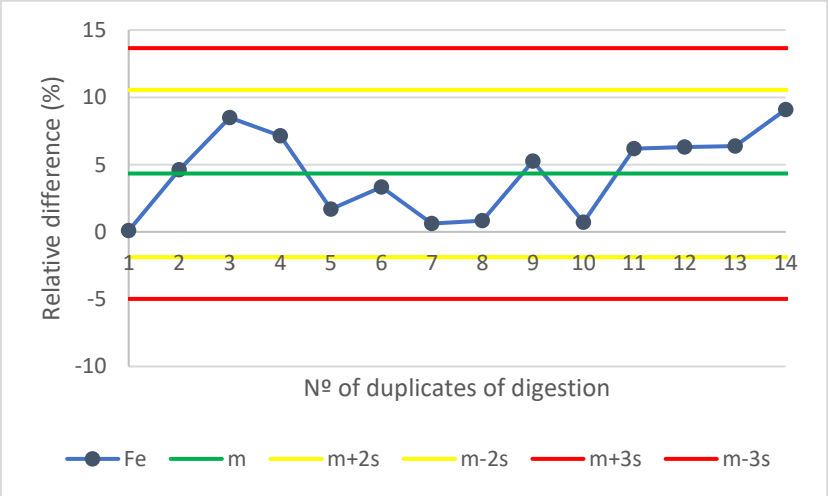

Figure 63: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Fe

- **Intermediate precision**

Table 54: Estimated CV_{SI} (%) for Fe

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	9	20	2
Rice	10	11	2
Seaweed	7	9	2

❖ **Uncertainty**

Table 55: Estimation of the uncertainty (%) for Fe by ICP-MS

u_r	4.5 %
u_b	7.0 %
u_c	8.3 %
U	16.6 %

AI.6 Validation of the method for Zn in food by ICP-MS

❖ Quantification

Table 56: *Values for the determination of the inverse of the variance for the weighted linear calibration function of Zn in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si²	1/si²
P0	0.0	9.08E+02	1.95E+02	3.78E+04	2.64E-05
P1	0.15	1.06E+03	1.08E+02	1.18E+04	8.51E-05
P2	1.5	2.48E+03	5.30E+01	2.81E+03	3.56E-04
P3	7.5	9.38E+03	1.02E+02	1.05E+04	9.57E-05
P4	15	1.84E+04	3.48E+02	1.21E+05	8.27E-06
P5	75	7.91E+04	9.90E+02	9.81E+05	1.02E-06
P6	150	1.48E+05	7.85E+02	6.16E+05	1.62E-06
Sum	249	2.60E+05	2.58E+03	1.78E+06	5.74E-04
Mean	36	3.71E+04	3.69E+02	2.54E+05	8.20E-05

Table 57: *Values for the definition of the weighted linear calibration function of Zn in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	3.22E-01	0.00E+00	2.93E+02	0.00E+00	0.00E+00
P1	0.15	1.04E+00	1.56E-01	1.09E+03	1.64E+02	2.33E-02
P2	1.5	4.34E+00	6.51E+00	1.07E+04	1.61E+04	9.77E+00
P3	7.5	1.17E+00	8.75E+00	1.09E+04	8.20E+04	6.56E+01
P4	15	1.01E-01	1.51E+00	1.85E+03	2.78E+04	2.27E+01
P5	75	1.24E-02	9.33E-01	9.84E+02	7.38E+04	7.00E+01
P6	150	1.98E-02	2.97E+00	2.94E+03	4.41E+05	4.45E+02
Sum	249	7.00E+00	2.08E+01	2.88E+04	6.41E+05	6.13E+02
Mean	36	1.00E+00	2.98E+00	4.12E+03	9.15E+04	8.76E+01

Table 58: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Zn in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[Zn] calculated	|Relative error| (%)
P0	1.13E+03	-2.18E+02	-2.41E+01	-2.17E-01	-
P1	1.28E+03	-2.22E+02	-2.11E+01	-7.10E-02	1.47E+02
P2	2.64E+03	-1.60E+02	-6.48E+00	1.34E+00	1.06E+01
P3	8.67E+03	7.03E+02	7.50E+00	8.20E+00	9.32E+00
P4	1.62E+04	2.15E+03	1.17E+01	1.71E+01	1.43E+01
P5	7.66E+04	2.50E+03	3.16E+00	7.75E+01	3.31E+00
P6	1.52E+05	-3.59E+03	-2.42E+00	1.46E+02	2.38E+00

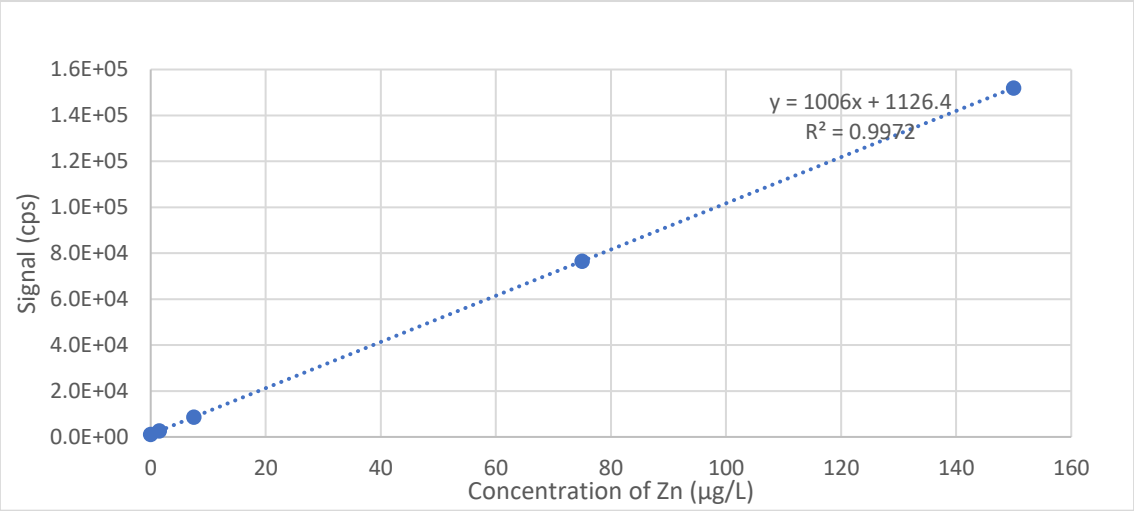

Figure 64: *Calibration curve for Zn in relation to the instrumental signal on 26/04/2021*

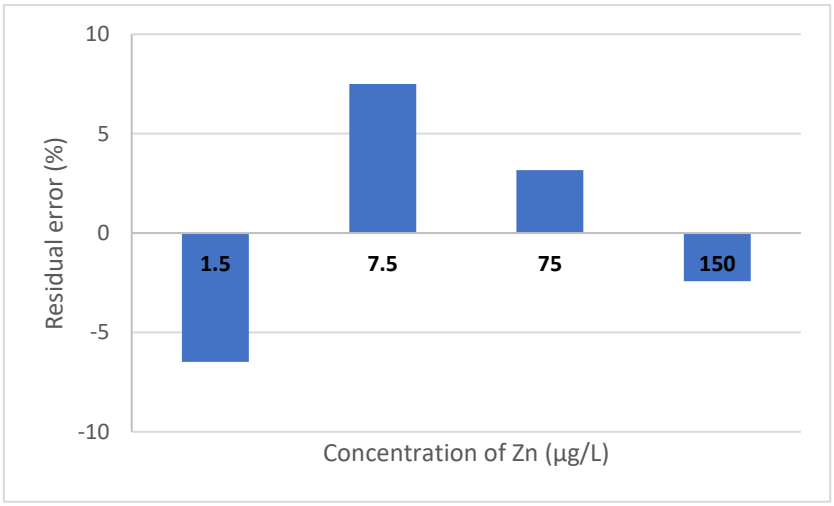

Figure 65: *Residual error (%) associated with different calibration standards for Zn on 26/04/2021*

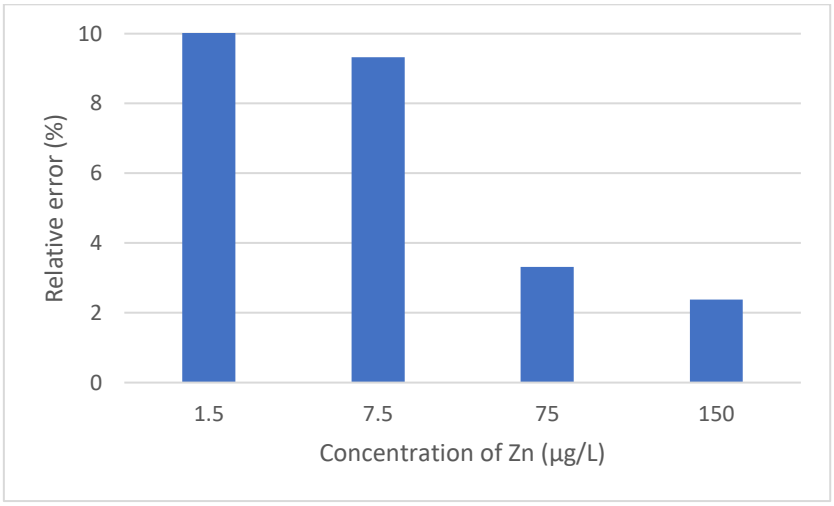

Figure 66: *Relative error (%) associated with different calibration standards for Zn on 26/04/2021*

❖ **Limit of detection and limit of quantification**

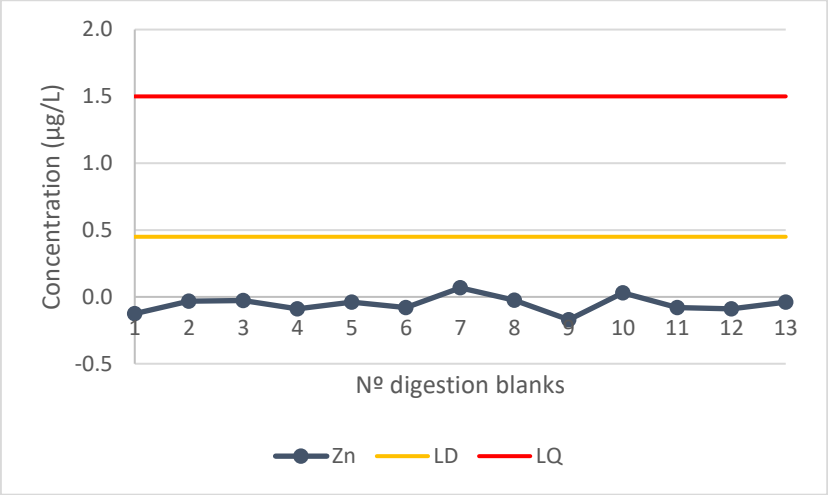

Figure 67: *Control verification chart of digestion blanks according to Zn concentration*

❖ **Selectivity**

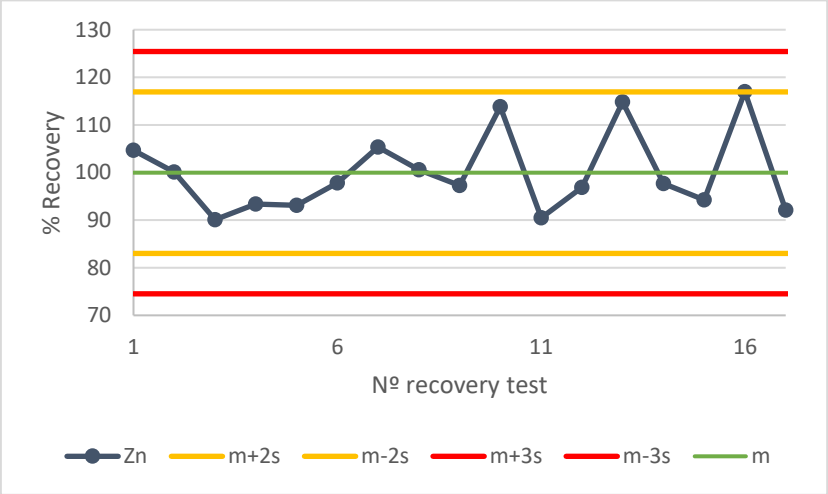

Figure 68: *Control chart for Zn recovery (%) by number of recovery readings*

❖ **Trueness**

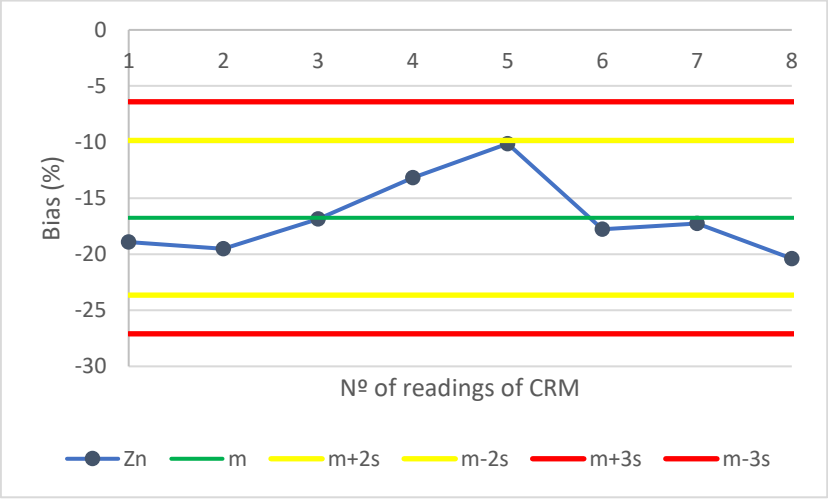

Figure 69: Control chart of bias (%) for Zn by the number of CRM readings

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

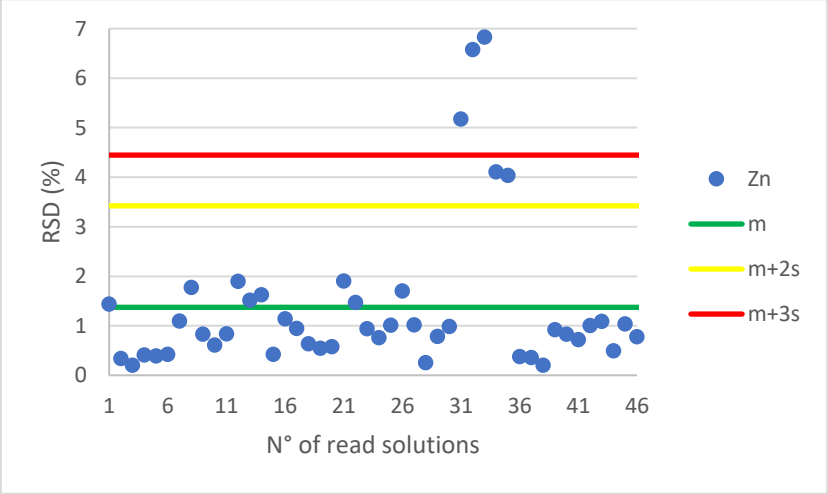

Figure 70: Control chart of the relative standard deviations (RSD, %) for Zn by ICP-MS

- **Coefficient of variation of repeatability**

Table 59: Estimated CVr (%) for Zn

Sample	CV_r (%)	Nº of reading samples
Chicken	2	10
Mussel	1	10
Fish	1	10
Rice	2	8
ZC 73016	10	2
ERM-CE278k	2	2
ERM-BB422	2	2
Dorm-4	0	2
ZC 73028	3	2
SRM 8433	0	2

- **Duplicate evaluation**

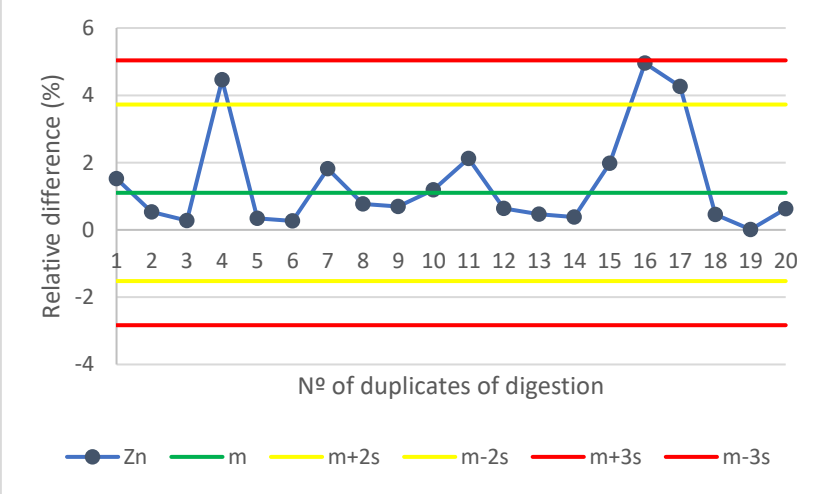

Figure 71: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Zn

- **Intermediate precision**

Table 60: Estimated CV_{SI} (%) for Zn

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	3	20	2
Rice	3	20	2
Seaweed	7	9	2

❖ **Uncertainty**

Table 61: Estimation of the uncertainty (%) for Zn by ICP-MS

u_r	1.7 %
u_b	2.3 %
u_c	2.9 %
U	5.8 %

AI.7 Validation of the method for As in food by ICP-MS

❖ Quantification

Table 62: *Values for the determination of the inverse of the variance for the weighted linear calibration function of As in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si²	1/si²
P0	0.0	-1.41E+02	5.10E+01	2.60E+03	3.85E-04
P1	0.15	-1.97E+02	3.18E+01	1.01E+03	9.88E-04
P2	1.5	4.29E+02	1.15E+02	1.32E+04	7.57E-05
P3	7.5	3.34E+03	8.00E+01	6.40E+03	1.56E-04
P4	15	7.17E+03	2.79E+02	7.77E+04	1.29E-05
P5	75	3.38E+04	4.68E+02	2.19E+05	4.56E-06
P6	150	6.62E+04	8.43E+02	7.11E+05	1.41E-06
Sum	249	1.11E+05	1.87E+03	1.03E+06	1.62E-03
Mean	36	1.58E+04	2.67E+02	1.47E+05	2.32E-04

Table 63: *Values for the definition of the weighted linear calibration function of As in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	1.66E+00	0.00E+00	-2.34E+02	0.00E+00	0.00E+00
P1	0.15	4.26E+00	6.39E-01	-8.39E+02	-1.26E+02	9.58E-02
P2	1.5	3.27E-01	4.90E-01	1.40E+02	2.10E+02	7.35E-01
P3	7.5	6.74E-01	5.05E+00	2.25E+03	1.69E+04	3.79E+01
P4	15	5.55E-02	8.33E-01	3.98E+02	5.97E+03	1.25E+01
P5	75	1.97E-02	1.47E+00	6.65E+02	4.99E+04	1.11E+02
P6	150	6.07E-03	9.10E-01	4.02E+02	6.03E+04	1.36E+02
Sum	249	7.00E+00	9.40E+00	2.78E+03	1.33E+05	2.98E+02
Mean	36	1.00E+00	1.34E+00	3.98E+02	1.90E+04	4.26E+01

Table 64: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of As in 26/04/2021*

Standard	Calculated signal	Residue	Residual error (%)	[As] calculated	|Relative error| (%)
P0	-2.10E+02	6.93E+01	-4.91E+01	1.53E-01	-
P1	-1.42E+02	-5.46E+01	2.77E+01	2.93E-02	8.05E+01
P2	4.69E+02	-3.97E+01	-9.26E+00	1.41E+00	5.85E+00
P3	3.18E+03	1.56E+02	4.68E+00	7.85E+00	4.60E+00
P4	6.58E+03	5.92E+02	8.26E+00	1.63E+01	8.72E+00
P5	3.37E+04	6.87E+01	2.03E-01	7.52E+01	2.02E-01
P6	6.77E+04	-1.46E+03	-2.21E+00	1.47E+02	2.15E+00

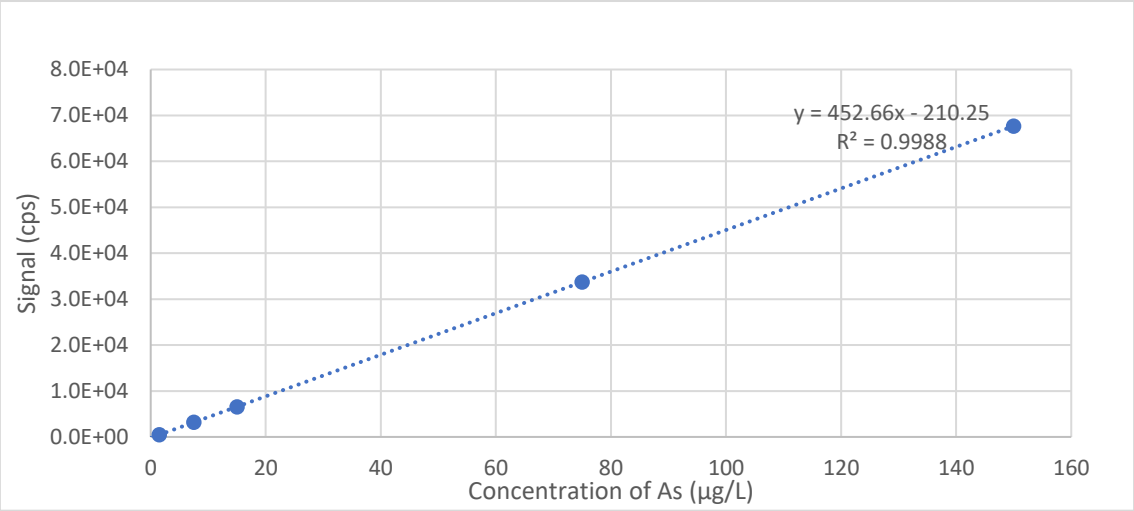

Figure 72: *Calibration curve for As in relation to the instrumental signal on 26/04/2021*

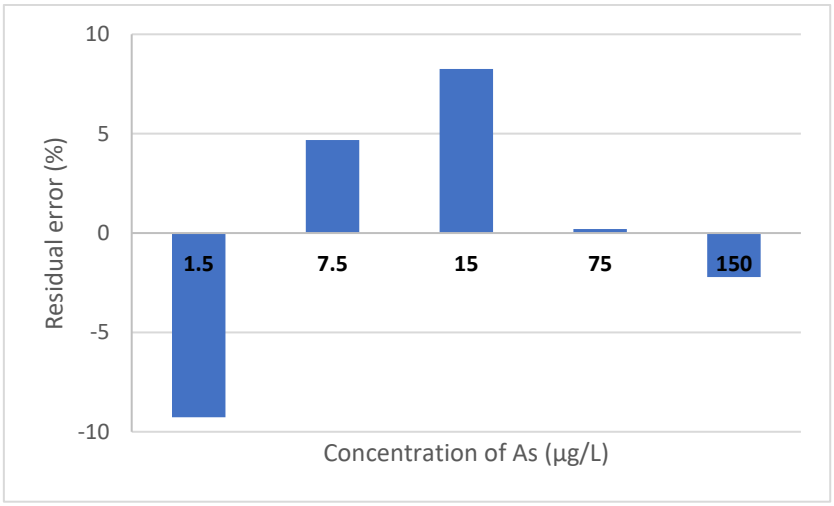

Figure 73: *Residual error (%) associated with different calibration standards for As on 26/04/2021*

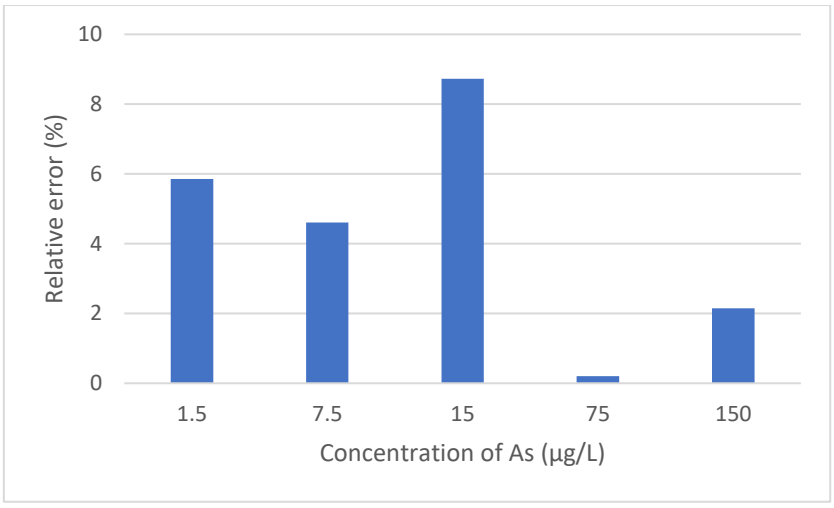

Figure 74: *Relative error (%) associated with different calibration standards for As on 26/04/2021*

❖ **Limit of detection and limit of quantification**

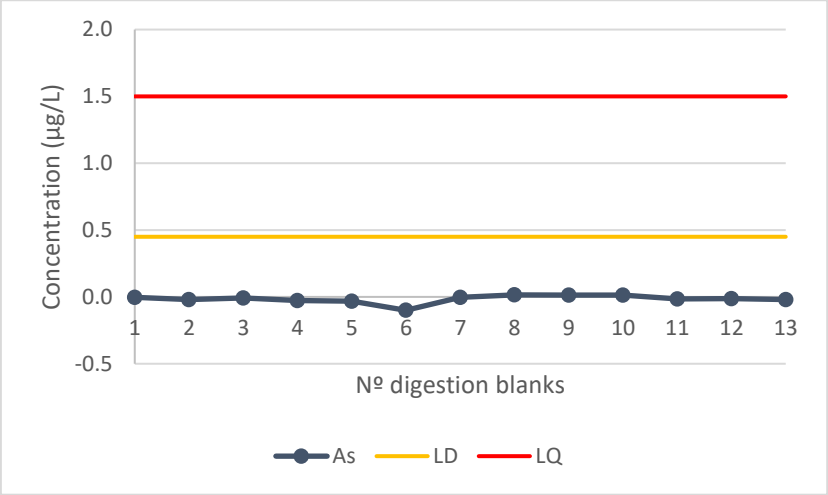

***Figure 75**: Control verification chart of digestion blanks according to As concentration*

❖ **Selectivity**

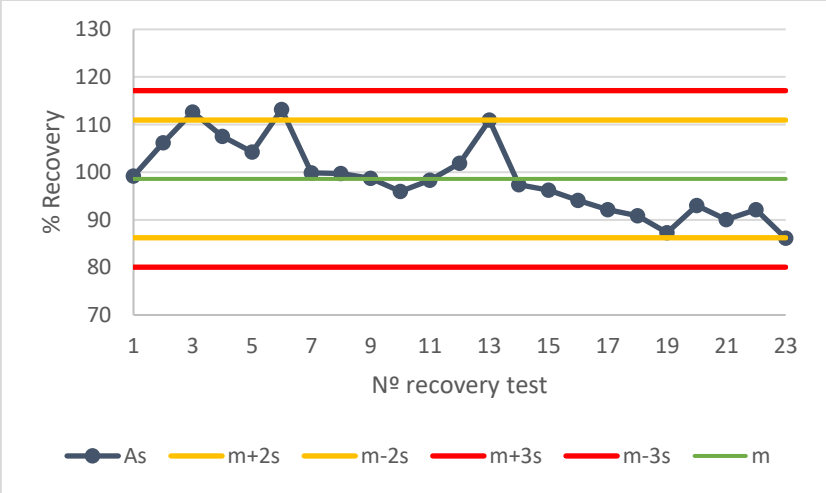

***Figure 76:** Control chart for As recovery (%) by number of recovery readings*

❖ **Trueness**

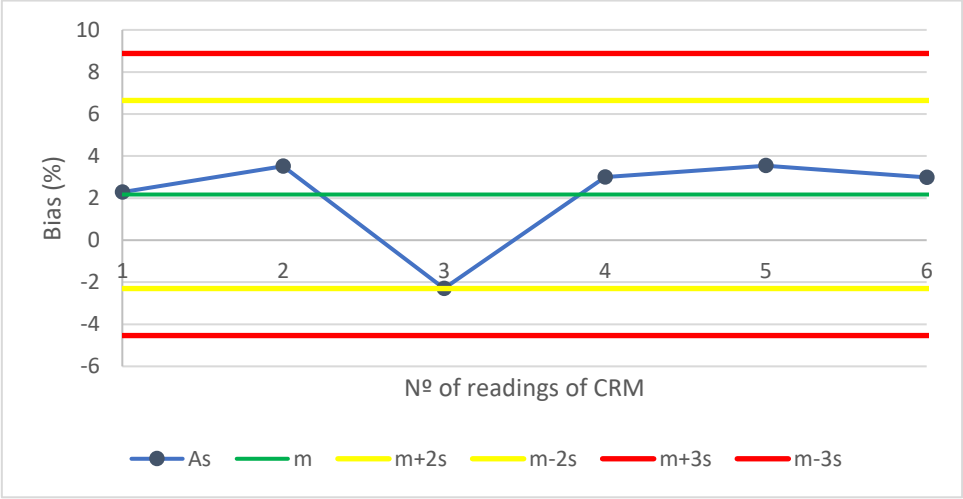

Figure 77: *Control chart of bias (%) for As by the number of CRM readings*

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

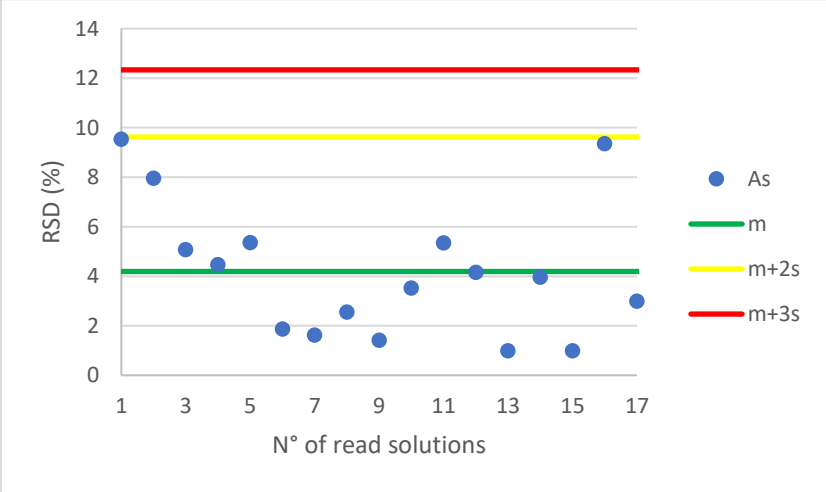

Figure 78: *Control chart of the relative standard deviations (RSD, %) for As by ICP-MS*

- **Coefficient of variation of repeatability**

Table 65: Estimated CVr (%) for As

Sample	CV_r (%)	Nº of reading samples
Chicken	-	10
Mussel	3	10
Fish	-	10
Rice	9	8
ZC 73016	-	2
ERM-CE278k	1	2
ERM-BB422	4	2
Dorm-4	0	2
ZC 73028	-	2
SRM 8433	-	2

- **Duplicate evaluation**

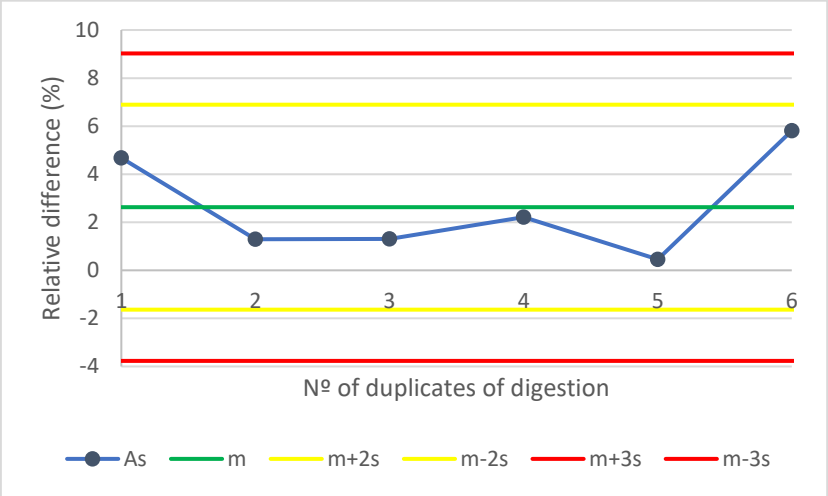

Figure 79: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for As

- **Intermediate precision**

Table 66: Estimated CV_{SI} (%) for As

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	-	20	2
Rice	10	20	2
Seaweed	9	9	2

❖ **Uncertainty**

Table 67: Estimation of the uncertainty (%) for As by ICP-MS

u_r	3.7 %
u_b	3.8 %
u_c	5.3 %
U	10.7 %

AI.8 Validation of the method for Cd in food by ICP-MS

❖ Quantification

Table 68: *Values for the determination of the inverse of the variation for the weighted linear calibration function of Cd in 26/04/2021*

Standard	Concentration (µg/L)	Signal mean	si	si²	1/si²
P0	0.0	3.00E+00	1.15E+00	1.33E+00	7.50E-01
P1	0.15	1.17E+02	6.51E+00	4.23E+01	2.36E-02
P2	1.5	1.07E+03	5.95E+01	3.54E+03	2.82E-04
P3	7.5	5.61E+03	9.78E+01	9.56E+03	1.05E-04
P4	15	1.16E+04	9.55E+01	9.13E+03	1.10E-04
P5	75	5.38E+04	3.65E+02	1.33E+05	7.51E-06
P6	150	1.02E+05	5.70E+02	3.25E+05	3.08E-06
Sum	249	1.74E+05	1.20E+03	4.80E+05	7.74E-01
Mean	36	2.49E+04	1.71E+02	6.86E+04	1.11E-01

Table 69: *Values for the definition of the weighted linear calibration function of Cd in 26/04/2021*

Standard	Concentration (µg/L)	wi	wi*xi	wi*yi	wi*xi*yi	wi*xi²
P0	0.0	6.78E+00	0.00E+00	2.03E+01	0.00E+00	0.00E+00
P1	0.15	2.14E-01	3.20E-02	2.50E+01	3.75E+00	4.81E-03
P2	1.5	2.55E-03	3.83E-03	2.73E+00	4.09E+00	5.75E-03
P3	7.5	9.46E-04	7.09E-03	5.31E+00	3.98E+01	5.32E-02
P4	15	9.91E-04	1.49E-02	1.15E+01	1.73E+02	2.23E-01
P5	75	6.79E-05	5.10E-03	3.66E+00	2.74E+02	3.82E-01
P6	150	2.78E-05	4.17E-03	2.84E+00	4.26E+02	6.26E-01
Sum	249	7.00E+00	6.71E-02	7.14E+01	9.21E+02	1.29E+00
Mean	36	1.00E+00	9.59E-03	1.02E+01	1.32E+02	1.85E-01

Table 70: *Calculated values for the signal, concentration, residual error, and relative error of the weighted linear calibration function of Cd in 26/04/2021*

Standard	Concentration (µg/L)	Residue	Residual error (%)	[Cd] calculated	|Relative error| (%)
P0	0.0	-3.83E-01	-1.28E+01	-5.38E-04	
P1	0.15	6.92E+00	5.92E+00	1.60E-01	6.49E+00
P2	1.5	-3.33E+00	-3.12E-01	1.50E+00	3.12E-01
P3	7.5	2.74E+02	4.88E+00	7.89E+00	5.13E+00
P4	15	9.71E+02	8.34E+00	1.64E+01	9.10E+00
P5	75	4.58E+02	8.52E-01	7.56E+01	8.59E-01
P6	150	-4.53E+03	-4.44E+00	1.44E+02	4.25E+00

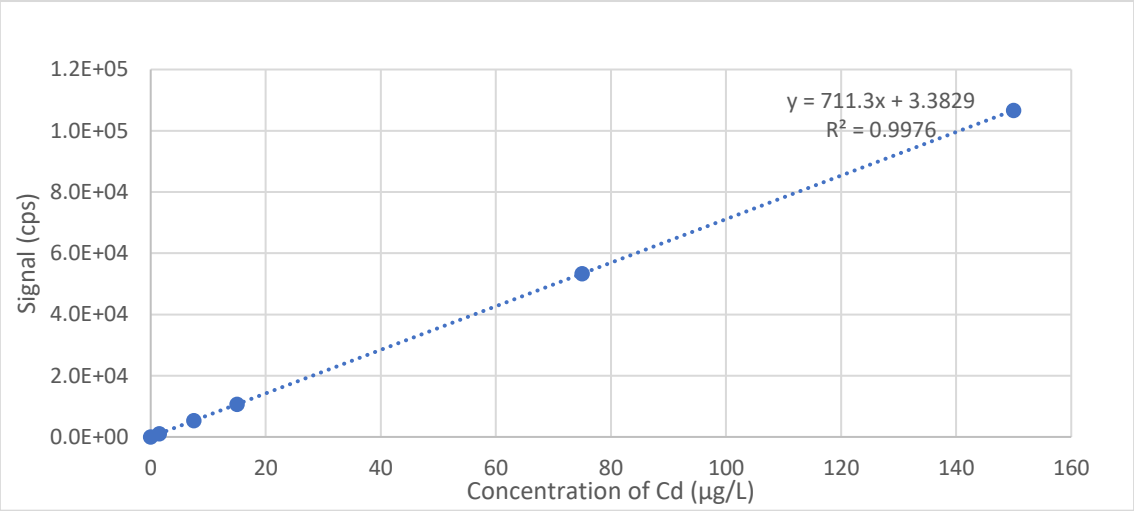

***Figure 80**: Calibration curve for Cd in relation to the instrumental signal on 26/04/2021*

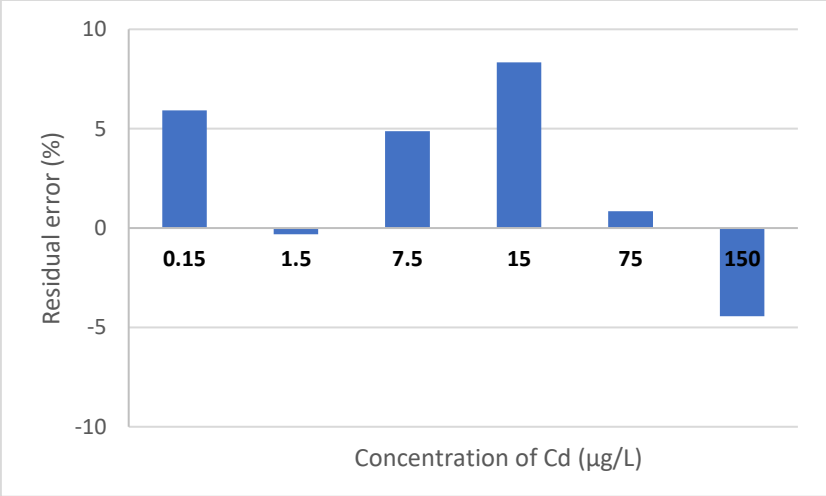

***Figure 81:** Residual error (%) associated with different calibration standards for Cd on 26/04/2021*

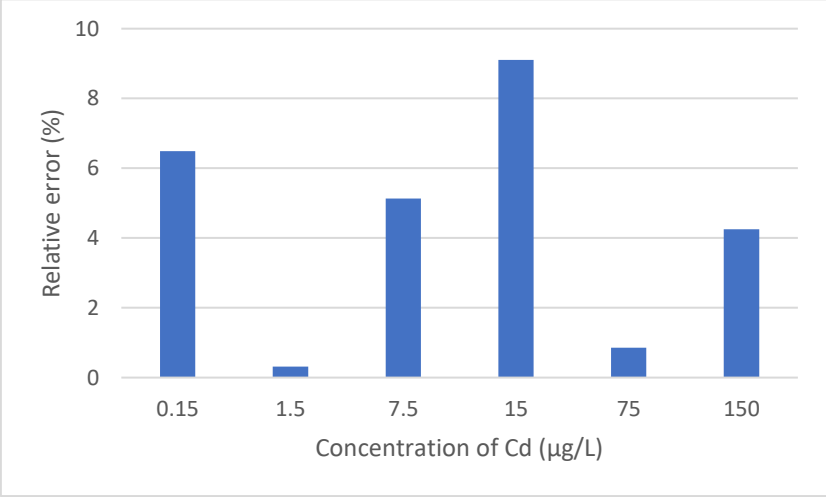

***Figure 82:** Relative error (%) associated with different calibration standards for Cd on 26/04/2021*

❖ **Limit of detection and limit of quantification**

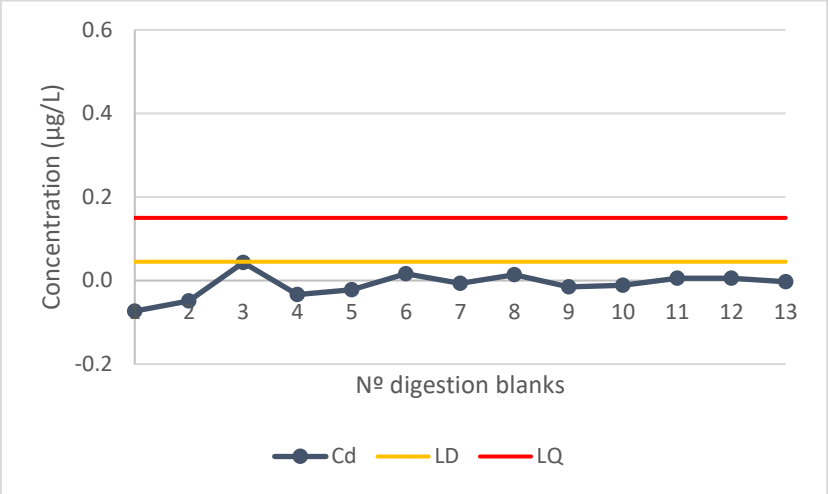

Figure 83: *Control verification chart of digestion blanks according to Cd concentration*

❖ **Selectivity**

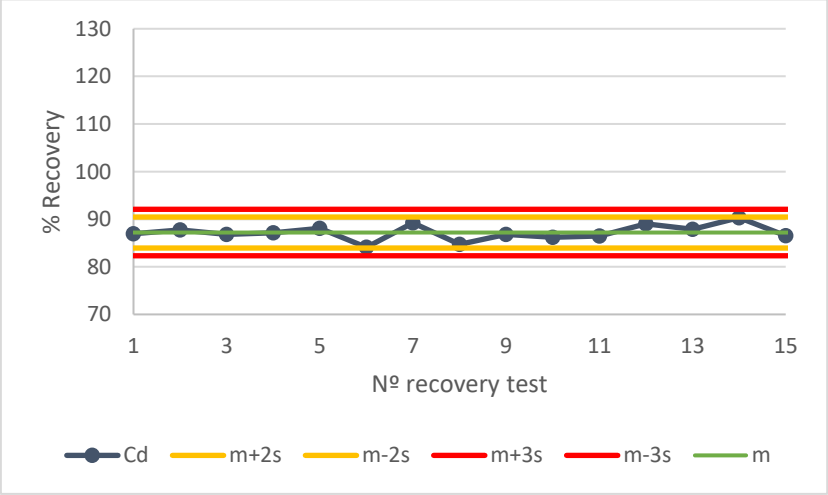

Figure 84: *Control chart for Cd recovery (%) by number of recovery readings*

❖ **Trueness**

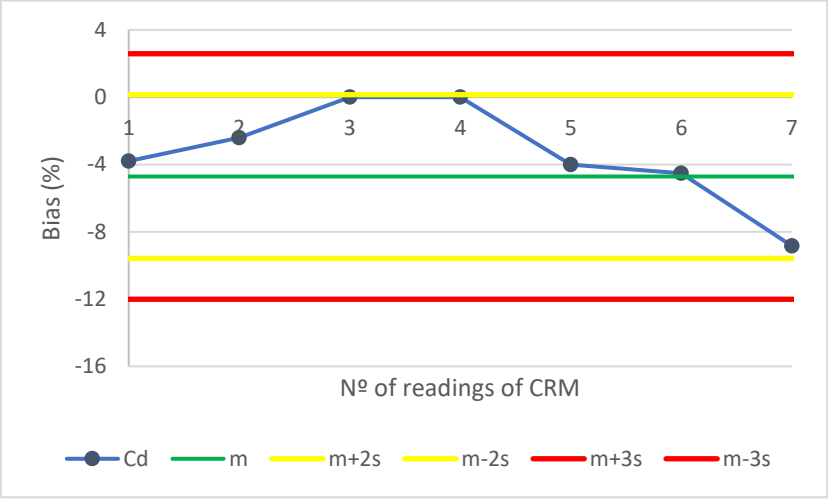

Figure 85: Control chart of bias (%) for Cd by the number of CRM readings

❖ **Precision**

- **Relative standard deviation associated with instrumental reading**

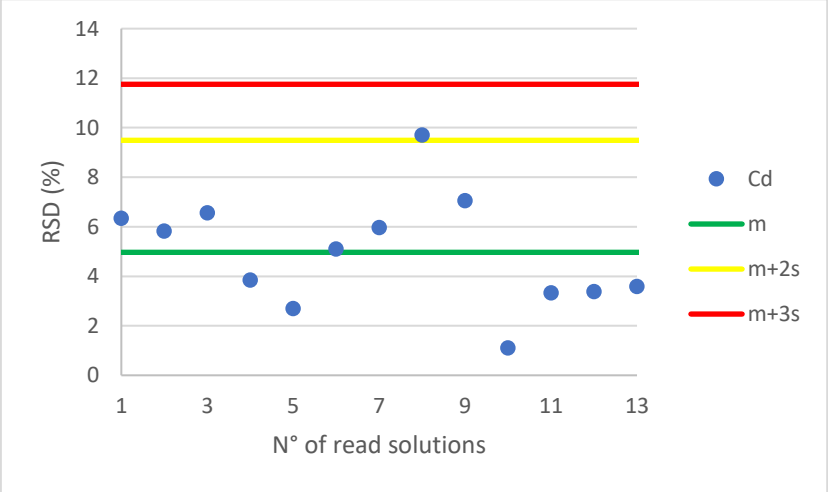

Figure 86: Control chart of the relative standard deviations (RSD, %) for Cd by ICP-MS

- **Coefficient of variation of repeatability**

Table 71: Estimated CVr (%) for Cd

Sample	CV_r (%)	Nº of reading samples
Chicken	-	10
Mussel	1	10
Fish	-	10
Rice	-	8
ZC 73016	-	2
ERM-CE278k	1	2
ERM-BB422	-	2
Dorm-4	0	2
ZC 73028	8	2
SRM 8433	-	2

- **Duplicate evaluation**

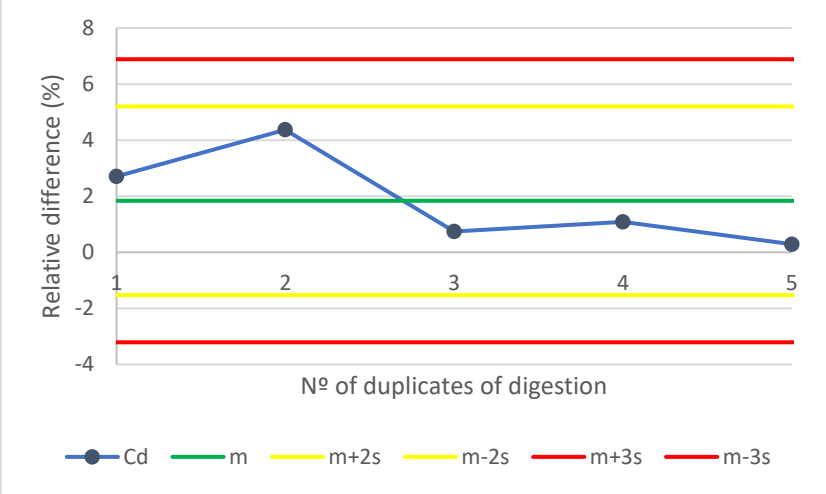

Figure 87: Control chart for the relative difference (%) of the duplicate values of digestion of food samples for Cd

- **Intermediate precision**

Table 72: Estimated CV_{SI} (%) for Cd

Sample	CV_{SI} (%)	Nº of reading samples	Nº of groups
Chicken	-	20	2
Rice	-	20	2
Seaweed	8	10	2

❖ **Uncertainty**

Table 73: Estimation of the uncertainty (%) for Cd by ICP-MS

u_r	1.0 %
u_b	5.1 %
u_c	5.2 %
U	10.4 %