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Validation of the Method Microwave Assisted Extraction to Total Fat Extraction in Fish

Relatório de Estágio para a obtenção do grau de Mestre em Engenharia Química e Biológica

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ABSTRACT

Although fatty acids are distinct substances with a well-known chemical composition, they can be found in a wide range of molecules with various chemical and physical properties. Analysis of the fatty acid profile is essential for nutritional labelling and aids in comprehension of the diversity of fatty acids present in various food commodities. Therefore, quantitative fatty acid extraction is a challenge.

This work, in partnership with AEMITEQ, had as main goal the validation of the method Microwave Assisted Extraction (MAE) for total fat measurement in the matrix Fish, ensuring the optimization conditions. Another objective of this work was the comparison between methods. This was done between the MAE method and the Soxhlet method, used by the Portuguese Institute of Sea and Atmosphere (IPMA), in order to understand which is the most advantageous.

An apparatus with 12 vessels, which can be used simultaneously, was used allowing increasing the extraction performance, being one of the key advantages of the method. An extraction commercial apparatus was used, the ETHOS X from Milestone. The extraction of total fat was performed with sulfuric acid (10 mL), using cyclohexane (25 mL) as the extraction solvent. The extraction time was set to 90 minutes, after which, the evaporation of the organic phase to obtain the total fat took 20 minutes.

For the validation of the method, it was studied experimentally, the limit of quantification, limit of detection, control standards, duplicates and replicates of samples, recovery tests and finally, reference materials. To validate the method, the expanded uncertainty (***U***), the precision and the trueness of the method were calculated.

The U value was calculated in four ways for different working ranges: 0.1-1 g/100g, 1-5 g/100g, 5-10 g/100g and >10 g/100g. From control standards, duplicates and reference material the following U values were obtained: 20%, 22%, 15% and 12%, respectively. Taking into account control standards, duplicates and recovery tests obtained values of 29%, 30%, 25% and 24% for each working range.

For the calculation of U per samples, the working ranges studied were only: 1-5 g/100g, 5-10 g/100g and >10 g/100g. The values obtained from samples and reference material were 20%, 11% and 12%, and for samples and recovery tests 28%, 23% and 24%, respectively. With this, the chosen value was 22% obtained by control standards and reference material, once it is the lowest value that covers all working ranges.

With the study performed, it can be confirmed that the method is very advantageous when compared to Soxhlet, since it presents a shorter extraction time, the amount of solvent to be used is lower, and it is also possible to analyze 12 samples at the same time. It is also concluded that the method was successfully validated, but that there is still room for improvement.

RESUMO

Os ácidos gordos podem ser encontrados numa ampla gama de moléculas com diversas propriedades químicas e físicas. A análise destes é essencial para a rotulagem nutricional, auxiliando na compreensão da diversidade de ácidos gordos presentes em diversos produtos alimentares, o que torna a sua extração um desafio.

Este trabalho, em parceria com a AEMITEQ, teve como principal objetivo a validação do método de extração assistida por micro-ondas (MAE) para avaliação da gordura total na matriz peixe, garantindo as condições de otimização. Outro objetivo deste trabalho foi a comparação entre métodos. Esta foi feita entre o método MAE e o método de Soxhlet, utilizado pelo Instituto Português do Mar e da Atmosfera (IPMA), de modo a perceber qual o mais vantajoso.

Para a realização deste trabalho foi utilizado o equipamento ETHOS X, da Milestone. Este equipamento permite a utilização de 12 vasos em simultâneo, permitindo aumentar o desempenho de extração e sendo assim uma grande vantagem do método quando comparado com outros. Para a extração da gordura total são utilizados dois solventes: ácido sulfúrico (10 mL) que origina a hidrólise, e ciclohexano (25 mL), que forma duas fases distintas. O tempo de extração é de cerca 90 minutos, no qual se segue 20 minutos de evaporação da fase orgânica para obter a gordura total.

Para a validação do método, os parâmetros estudados foram o limite de quantificação e deteção, padrões de controlo, duplicados e replicados de amostra, testes de recuperação, e finalmente, materiais de referência. Para validar o método foram calculadas a incerteza expandida (***U***), a precisão e a veracidade.

O valor de ***U*** foi calculado de quatro maneiras para diferentes gamas de trabalho: 0,1-1 g/100g, 1-5 g/100g, 5-10 g/100g e >10 g/100g. A partir de padrões de controlo + duplicados + material de referência obtiveram-se os seguintes valores de ***U***: 20%, 22%, 15% e 12%, respetivamente. Tendo em conta padrões de controlo + duplicados + testes de recuperação obtiveram-se valores de 29%, 30%, 25% e 24% para cada gama de trabalho.

Para o cálculo da ***U*** por amostras, as gamas de trabalho estudadas foram apenas de: 1-5 g/100g, 5-10 g/100g e >10 g/100g. Os valores obtidos a partir de amostras + material de referência foram de 20%, 11% e 12%, e para amostras + testes de recuperação obteve-se 28%, 23% e 24%, respetivamente. Com isto, o valor de ***U*** escolhido foi 22% obtido pelos padrões de controlo + material de referência, uma vez que é o menor valor que abrange todas as gamas de trabalho estudadas.

Com o estudo realizado, pode-se confirmar que o método é bastante vantajoso quando comparado com o Soxhlet, uma vez que apresenta um menor tempo de extração, a quantidade de solvente a utilizar é inferior, e ainda, é possível analisar 12 amostras ao mesmo tempo. Conclui-se também que o método foi validado com sucesso, podendo ainda haver espaço para melhorias.

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ACRONYMUS

ACIC	Associação Comercial e Industrial de Coimbra (Commercial and Industrial Association of Coimbra)
AEMITEQ	Associação para a inovação tecnológica e qualidade (Association to Technology and Quality Innovation)
AOAC	Association of Official Analytical Chemists
AOCS	Oil Chemists Society
DHA	Docosahexaeonic acid
EPA	Eicosapentaeonic acid
FA	Fatty Acid
FAO	Food and Agriculture Organization
FAME	Fatty Acid Methyl Ester
GL	Glycerolipid
GP	Glycerophospholipid
ILCNC	International Lipid Classification and Nomenclature Committee
INETI	Instituto de Engenharia e Tecnologia Industrial (Institute of Engineering and Industrial Technology)
IPAC	Instituto Português de Acreditação (Portuguese Accreditation Institute)
IPMA	Instituto Português do Mar e da Atmosfera (Portuguese Institute of Sea and Atmosphere)
ISO	International Organization for Standardization
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
MIR	Mid-infrared Spectroscopy
MUFA	Monounsaturated Fatty Acid
NIR	Near-infrared Spectroscopy
NOVOTECNA	Associação para o Desenvolvimento Tecnológico (Association for Technological Development)
PK	Polyketides
PL	Phospholipid
PR	Prenol
PTFE	Polytetrafluorethylene
PUFA	Polyunsaturated Fatty Acid
SFE	Supercritical Fluid Extraction
SL	Saccharolipid
SP	Sphingolipid
ST	Sterol
TAG	Triacylglycerol

TV	Test Value
UAE	Ultrasound Assisted Extraction
UC	Universidade de Coimbra (University of Coimbra)

SYMBOLS

S	Variances
i	Number of the standard
j	Number of repetitions
K	Constant
s	Standard deviation
b	Slope
CV	Coefficient of variation
$\overline{x}$	Average of results
r	Limit of Repeatability
S_r	Repeatability variance associated
S_L	Interlaboratory variance
S_R	Reproducibility variance
Er	Relative error
n	Number of samples
Z	Z-score
U	Expanded Uncertainty
uc	Combined Uncertainty
x	Obtained Value
$\mathbf{R}$	Recoveries
C	Concentration
$\overline{R}$	Average of Recoveries
b	Bias

1 INTRODUCTION

1.1 Framework And Motivation

The fat content of foods is of increasingly importance for both consumers and professionals in the food industry. For the consumers, the interest results from some reasons, being the most important, health. Manufacturers test food extracts in order to guarantee the quality of their products, being an essential part of food formulation (Jiang et al., 2014).

In the 20st century, the nutrition science focused on identifying the necessary nutrients, researching the effects of inadequate intakes, and figuring out the dosages required to prevent deficient conditions. Since then, it has progressively come to be understood that providing sufficient amounts of all the nutrients, does not constitute a good nutrition. However, nutritionists recognize the difficulty in changing people´s food intake behaviors, since diets are influenced by many factors besides the need to eat and the desire for well-being (Barasi, 2003).

The study of nutrition examines how food and drink affect our bodies with a focus on the vital nutrients required to support human health. Good nutrition is related to an adequate, well-balanced diet, with physical activity, which is the keystone for health. Good nutrition may help people to live longer, keeps teeth, skin and eyes healthy, boosts immunity, supports muscles and other. On the other hand, poor nutrition can impair physical and mental development, increase susceptibility to disease and reduce productivity. With this, efforts have been made to promote optimal health and thereby reduce the risk of chronic diseases such as cardiovascular disease and cancer (Mohanty et al., 2017).

There are seven major classes of nutrients, which can be split into macronutrients or micronutrients. The macronutrients are water, carbohydrates, proteins, fats, and fibers. Vitamins and minerals make up the micronutrients. Macrominerals such as potassium (K), sodium (Na), magnesium (Mg), calcium (Ca) and phosphorous (P), and microminerals like iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and selenium (Se) are among the important micronutrients. Micronutrients also include vitamins such as the water-soluble vitamins B complex, vitamin C, and the fat-soluble vitamins A, D, E, and K. Several variables, including each person's unique needs, age, lifestyle, level of physical activity, sex, and eating habits, affect how diverse, balanced, and healthful a diet is (Mohanty et al., 2019).

The MyPlateis an image or graphical representation that helps choose and combine the foods that should be part of the daily diet. It's a circle-shaped symbol which is divided into 7 segments of different sizes that bring together foods with similar nutritional properties. The 7 food groups and the proportion of weight each of them must be present in the daily diet are shown in **Figure 1.1.** Water, despite not having his own group, is also represented in the wheel, as it is part of the constitution of almost all foods. The need for water can vary between 1.5 and 3 liters per day (FCNAUP, 2004).

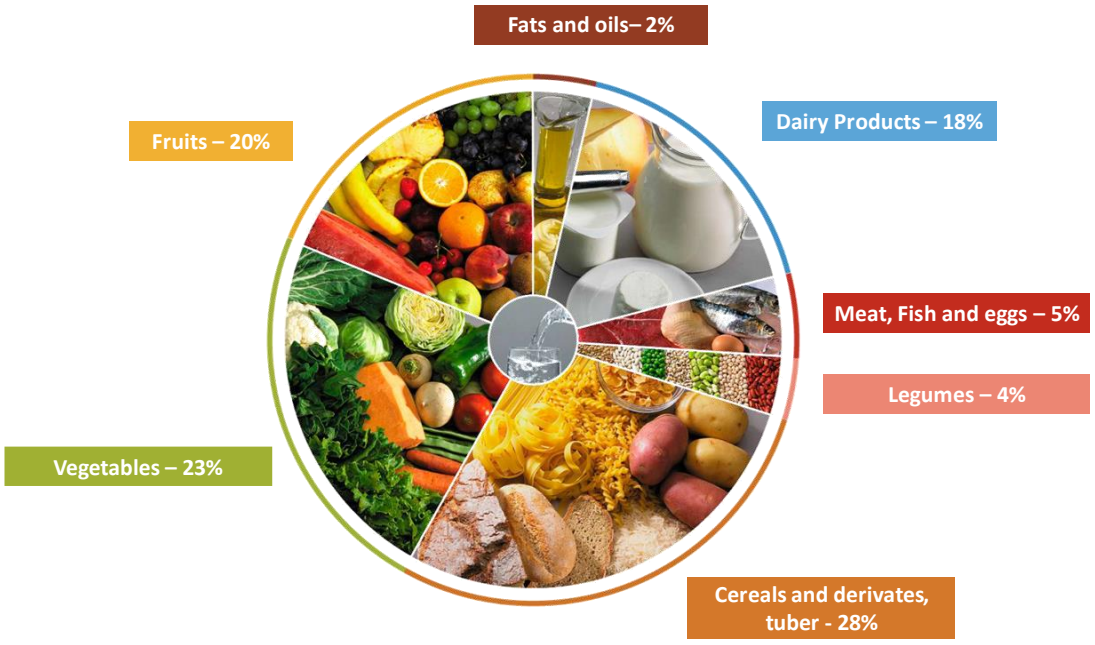


Figure 1.1 – Percentages to be consumed each day, for balanced healthy nutrition (Adapted from: (FCNAUP - Nova Roda Dos Alimentos, n.d.))

The purpose of a nutritional label is to inform consumers about their options and encourage the development and consumption of healthy products. Food scientists examine foods to understand more about their composition, flavor, texture, appearance, shelf life, etc., as well as to ensure the product´s quality. To ensure higher standards of safety and nutritional quality for food, various analytical methods have been required to obtain the best results, such as electrophoresis, spectroscopy, chromatography, sensory evaluation and rheological procedures. In general, the choice of an analytical method depends on the purpose of the analysis (Jain & Gupta, 2004; Azman & Sahak, 2014; Corporation, 2021).

The first device to extract lipids was developed by Franz von Soxhlet in 1879, who highlighted the importance of the degree of sample grinding in terms of the duration and effectiveness of the process. Soxhlet extraction has been a widely used technique for more than a century, and the techniques built on it continue to be the major standards against which the effectiveness of new leaching techniques is evaluated (Brum et al., 2009).

Some advantages that the Soxhlet method presents are, among others, that the sample is always in contact with the solvent, which is constantly being replaced, the system temperature stays relatively high because the heat applied to the evaporation process is constant. This procedure consists on the successive and intermittent treatment of the sample immersed in a pure solvent (petroleum ether, diethyl ether or n-hexane. However, procedures with solvent reflux for many hours, should be avoided, once they favor peroxidation and hydrolysis reactions and can compromise later analytical results, such as the quantification of certain lipid components (Brum et al., 2009).

One of the most versatile and effective extraction procedures, which overcomes the difficulties mentioned above, is the Bligh & Dyer methodology, a simplified version of the classical procedure, which used chloroform-methanol as the extraction solvent, as proposed by Folch et al. (1956). Another advantage presented by methods based on the binary mixture of chloroform and methanol is the capability to extract efficiently both neutral and polar lipids (Folch et al., 1956; Brum et al., 2009).

As these techniques are usually time-consuming and require relatively large amounts of non-green solvents, other methods have been developed, such as supercritical fluid extraction (SFE), microwave assisted extraction (MAE) and pressurized solvent extraction (PSE), being faster and more efficient when analytes are extracted from solid matrices (Kaufmann & Christen, 2002).

SFE is a rapid method that requires no organic solvents, but it is very expensive and complex. MAE has an operating short time and its more efficient than conventional heating. However, the energy demand is high as well as the extraction temperature (Saini et al., 2021).

Lipids analysis is a challenging task due to the wide variety of sample matrices, diversity of fatty acids and the broad range of total fat contents (Jiang et al., 2014).

The internship work described in this report results from the known difficulties of the previously mentioned methods. The idea of this work was to validate the same extraction method for as many matrices as possible. The Portuguese Institute of the Sea and the Atmosphere (IPMA) suggests the fat extraction by Soxhlet, although the limitations of this method as referred previously. Since the work with MAE has been applied to other matrices, IPMA saw an opportunity to compare the two methods and to establish the possible advantages of MAE, in which concerns the fat extraction step.

1.2 AEMITEQ

The Association to Technology and Quality Innovation (AEMITEQ) was found in 1990 by initiative of the University of Coimbra (UC), Institute of Engineering and Industrial Technology (INETI), Portuguese PHILIPS, Commercial and Industrial Association of Coimbra (ACIC) and Association for Technological Development (NOVOTECNA). AEMITEQ is located in Coimbra and exercises its activity since 1994.

AEMITEQ integrates the Sectorial Commission of Food, Agri-food and Water Laboratories from RELACRE, which is the committee responsible for the analysis and elaboration of technical documents and normative guides to be published by the Portuguese Institute of Accreditation (IPAC), in order to regulate the activity.

In the laboratories of AEMITEQ are developed studies based in analytical chemistry, such as: Chemical control of raw material and products; Composition of natural products; Quality control of waters; Analysis of industrial and urban waste; Analytical determination in biological materials; Development of analytical methods and fine chemistry.

AEMITEQ has a laboratory of rehearsals with IPAC accreditation as described in the accreditation certificate nº L0271, and the respective annex for accreditation for the analysis of fresh waters (except rainwater), for consumption, process and waste (fixed scope and scope flexible). It also have a calibration laboratory with accreditation certificate nº M0073, and the respective technical accreditation annex for the calibration of visible-ultraviolet spectrophotometers. **Figure 1.2** shows the laboratories of this institution.

Figure 1.2 – a) Chemistry laboratory; b) Chromatography laboratory; c) Atomic absorption and emission laboratory; d) Clean room support laboratory

1.3 Internship Goals

Traditional gravimetric analytical methods, such as Soxhlet extraction, or Bligh & Dyer extraction method, are typically used to determine total or crude fat in food matrices. Given the variation in sample fat content, the approaches utilized for fish matrix, result in limited reproducibility, making it challenging to assess.

This work proposes an innovative methodology for the determination of total fat that involves in a single step the hydrolysis and extraction of the same, in a short period of time.

In partnership with AEMITEQ, this work had as main goal the validation of the method Microwave Assisted Extraction (MAE) for total fat assessment in the Fish matrix , ensuring the optimization conditions.

In the beginning of the internship, once AEMITEQ has been in constructions, it was not possible to start immediately the theme that was propose. During this time, other works were done, such as: quantitative analysis of anions by ion chromatography and determination of pH in waters (electrometry).

1.4 Report Organization

The internship report is organized in 8 chapters where the work developed in AEMITEQ is described. In this chapter the context and the goals of the internship are introduced.

In chapter 2 the definition of lipids is provided and the difference between fats and oils is discussed.

In chapter 3 the methods usually applied for total fat extraction are described and a brief comparison between them is provided.

In chapter 4 the matrix fish is overviewed, specifically, how the fat content depends on several factors, being referred the commonly used methods for extracting fat from fish samples.

In chapter 5, the key steps for validating an analytical method are described.

Chapters 6 and 7 present the experimental procedure and the results as well as a discussion of the results, respectively.

Finally, the general conclusions drawn from this study, as well suggestions for future work are presented in chapter 8.

2 LIPIDS

Foods are highly complex matrices, and they are constituted by essential nutrients, like water, lipids, proteins, carbohydrates, vitamins, minerals and dietary fibers. Depending on the type of food, lipids are one of their main constituents, contributing to the organoleptic, physicochemical, and nutritional aspects, which are important in a regular diet. Lipids are composed of carbon, hydrogen and oxygen atoms, and in some cases phosphorus, nitrogen, sulfur, among others. Lipids are soluble in organic solvents (such as ether, hexane, or chloroform), but are insoluble in water. The insolubility in water is the analytical property used as the basis for its easy separation of proteins and carbohydrates. The possibility of lipids bonding with other molecules and the capacity to solubilize them in different mixtures of organic solvents, led to the concept of "total fat extraction" and "extractable lipids" (Cunha, 2011; Jiang et al., 2014; Srigley & Mossoba, 2017; Montesano et al., 2018). In this chapter, and taking into account the aim of the study, lipids will be highlighted.

The International Lipid Classification and Nomenclature Committee (ILCNC) classified lipids into eight categories (**Table 2.1**). Among these, the most common type of fat found in plants and animals are glycerophospholipids, also known as phospholipids (PLs), triacylglycerols (TAGs), and sterols (Saini et al., 2021).

Table 2.1 – Lipid category according to ILCNC (adapted from Saini et al. (2021))

Category	Abbreviation
Fatty Acids	FA
Glycerolipids	GL
Glycerophospholipids	GPL
Sphingolipids	SP
Sterol Lipids	ST
Prenol Lipids	PR
Saccharolipids	SL
Polyketides	PK

Lipids are particularly important in the growth and development of the organism, where mainly fatty acids will change with the age and physiological state of each individual. Furthermore, lipids can both prevent or be the cause of many diseases, especially chronic noncommunicable diseases, affecting the lipid requirements in humans (Valenzuela & Valenzuela, 2013).

2.1 Fats And Oils

Food lipids are divided in categories of fats and oils considering the origin of the lipid substance and its physical state at room temperature (20°C). Fats can remain in the solid state at room temperature due to a higher concentration of saturated fatty acids. On the other hand, vegetable and seed oils are mostly found in liquid state at room temperature once they present higher concentrations of monounsaturated and polyunsaturated fatty acids. However, it is also possible to find "solid" oils, such as coconut oil, once this contain high concentration of saturated and/or trans fatty acids (Srigley & Mossoba, 2017).

Fats and oils are tryacylglyceride mixtures (over 90%), i.e., structures formed by the bond of three different or similar fatty acids to the tri-alcohol glycerol. In addition, these substances frequently contain monoacylglycerides, diacylglycerides, phospholipids, terpenes, sterols, fatty alcohols, fat soluble vitamins, carotenoids and many others (Valenzuela & Valenzuela, 2013).

One molecule of fat consists in two types of components: a glycerol skeleton and three fatty acid tails. Glycerol (**Figure 2.1**) is a small organic molecule with three hydroxyl groups (OH), while fatty acid is a long chain of hydrocarbons bonded to a carboxyl group. To make a fat molecule, the hydroxyl groups in the glycerol skeleton must react with the carboxyl groups of fatty acids by dehydration synthesis reaction. This results in a molecule of fat with three tails of fatty acids linked to the glycerol skeleton by ester bonds (C=O) (Clark et al., 2012).

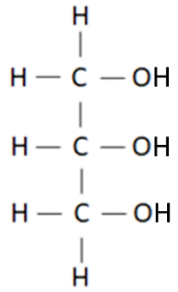


Figure 2.1 – Glycerol structure (adapted from Clark et al. (2012))

The reaction of a hydroxyl group, at any of glycerol´s three groups, with a fatty acid, origins a monoacylglyceride. The bond with a second fatty acid (similar or different from the initial one) gives a diacylglyceride. If all three hydroxyl groups of glycerol are linked with fatty acids, this will be a tryacylglyceride (**Figure 2.2**) (Valenzuela & Valenzuela, 2013).

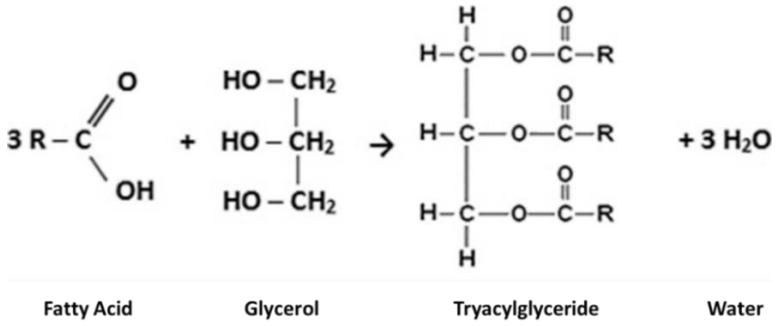


Figure 2.2 – Fatty acid and glycerol reaction to form a tryacylglyceride (adapted from Kalita & Netravali (2017))

Fatty acid chains can differ in length and in its degree of unsaturation. The melting point of the fatty acid varies with the number of carbon atoms. The higher the number of carbon atoms, the higher will be the melting point. Fatty acids that have between four and ten atoms of carbon, are referred as short-chain fatty acids, those having twelve to fourteen as medium, sixteen to eighteen as long, and those having twenty or more as very long-chain (Valenzuela & Valenzuela, 2013).

Fatty acids may be saturated or unsaturated (**Figure 2.3**). The fatty acid is saturated when, in a fatty acid chain, there are single bonds between carbons in the hydrocarbon chain. In saturated fatty acids, the number of hydrogen atoms bonded to the carbon skeleton, is maximized. When the hydrocarbon chain has a double bond, the fatty acid is unsaturated, having less hydrogen atoms. If there is only one double bound in the molecule, the fatty acid is monounsaturated (MUFA). On the contrary, if there is more than one double bond, the fatty acid is polyunsaturated (PUFA) (Clark et al., 2012).

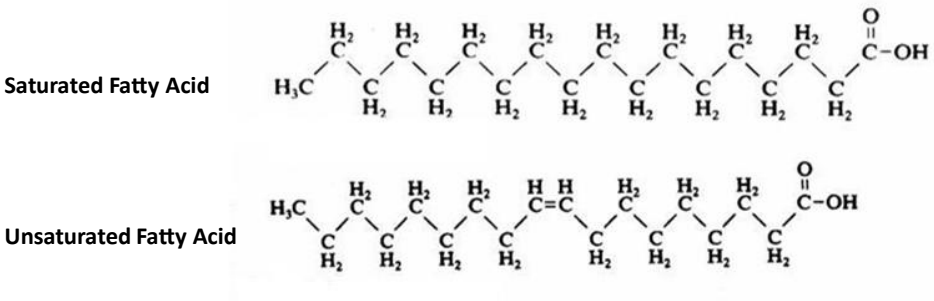


Figure 2.3 – Saturated (stearic acid) and unsaturated (oleate acid) fatty acids

In unsaturated fatty acids double bonds can be arranged in two ways: cis- or trans- configurations (**Figure 2.4**). In cis- configuration, the two hydrogens associated to the bond, are on the same side. In the trans- configuration, both hydrogens are in opposite sides. In food, double bonds are predominantly found in the cis- form. Trans-fatty acids are less common in nature but can be found in small amounts in milk and in the meat fat of ruminants. The most important fatty acids present in the diet can be seen in **Table 2.2.**

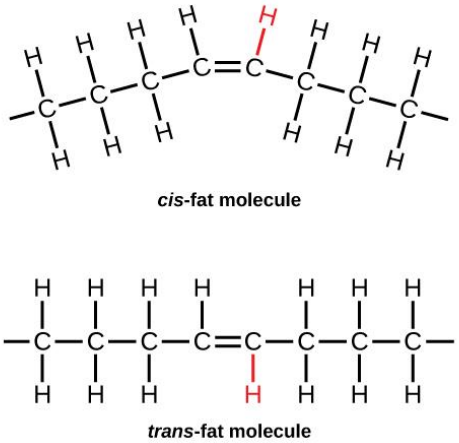


Figure 2.4 – Cis and Trans-fat Molecule (Adapted from: (Clark et al., 2012))

Table 2.2 - Major fatty acids from plant and animal origins (adapted from Sanders & Emery (2003))

	Trivial Name	Systematic Name	Rich Dietary Sources
Saturated fatty acids	Acetic (C2:0)	ethanoic	Butter
	Butyric (C4:0)	butanoic	Butter
	Caproic (C6:0)	hexanoic	Butter
	Caprylic (C8:0)	octanoic	Goat's Milk
	Capric (C10:0)	decanoic	Goat's Milk
	Lauric (C12:0)	dodecanoic	Coconut Oil
	Myristic (C14:0)	tetradecanoic	Coconut Oil, Animal Fats
	Palmitic (C16:0)	hexadecanoic	Palm Oil, Animal Fats
	Stearic (C18:0)	octadecanoic	Cocoabutter, Hydrogenated Fats
	Arachidic (C20:0)	eicosanoic	Peanuts

Table 2.2 – Major fatty acids from plant and animal origins (Continued) (adapted from Sanders & Emery (2003))

	Trivial Name	Systematic Name	Rich Dietary Sources
Monounsaturated fatty acids	Palmitoleic (C16:1n-7)	cis-9-hexadecenoic	Fish oil
	Oleic (C18:1n-9)	cis-9-octadecenoic	Olive oil, rapeseed oil, palm oil
	Elaidic (C18:1n-9 trans)	trans-9-octadecenoic	Partially hydrogenated fat
	trans-Vaccenic (C18:1n-7 trans)	trans-11-octadecenoic	Ruminant fats
	cis-Vaccenic (C18:1n-7)	cis-11-octadecenoic	Ruminant fats
	Erucic (C22:1n-9)	cis-13-docosenoic	Mustard seed oil
Polyunsaturated fatty acids	Linoleic (C18:2n-6)	9,12-octadecadienoic	Safflower oil, sunflower oil, corn oil
	Gamma (γ)-linolenic (C18:3n-6)	6,9,12-octadecatrienoic	Evening primrose oil
	Arachidonic (C20:4n-6)	5,9,11,14-eicosatrienoic	Ruminant meats (low levels)
	Alpha (α)-linolenic (C18:3n-3)	9,12,15-octadecatrienoic	Flaxseed oil
	Eicosapentaenoic (C20:5n-3)	5, 8,11,14,17-eicosapentaenoic	Fish oil
	Docosahexaenoic (C22:6n-3)	4,7,10,13,16,19-docosahexaenoic	Fish oil

3 EXTRACTION METHODS FOR TOTAL FAT ANALYSIS

A qualitative and quantitative analysis of food must be accurate and precise which is important for guarantee that the product fulfills the manufacturing specifications and for accurate nutritional labeling (Ellefson, 2017).

Due to a large range of total fat contents, wide variety of sample matrices and complex compositions of fatty acids, the analysis of lipids are a challenging task. Thus, there are a wide range of analytical techniques for the analysis of total fat and fatty acids in foods. The extraction methods are referred as official methods of analysis, and are evaluated for the method performance and validated according to national or international guidelines established by recognized organizations, such as the Association of Official Analytical Chemists (AOAC International), American Oil Chemists' Society (AOCS) and International Organization for Standardization (ISO) (Srigley & Mossoba, 2017).

Depending on the properties of the material, several methods are used for lipids' extraction. A compound or set of compounds are preferentially transported from a matrix into a separate phase during the extraction step of the analytical process. The final goal of this step is making the sample available for analysis using analytical instruments, ensuring that the target analytes are in an appropriate phase (e.g., liquid phase) and at appropriate concentrations for being analyzed, for example, in chromatographic systems (Rostagno & Prado, 2013).

The analysis of fatty acids in food products, requires 4 steps: extraction by acidic, basic or enzymatic hydrolysis, followed by extraction with organic solvents, methylation of fatty acids and, finally, qualitative and/or quantitative analysis. To ensure the total extraction of lipids, organic solvents are used, once triglycerides are very soluble in solvents like hexane or toluene, and in more polar solvents, such as chloroform and ethers (Teixeira, 2018).

Crude fat is the term used to describe the raw mixture of fat-soluble material in a sample. Lipidic material may include monoglycerides, diglycerides, triglycerides, phospholipids, free fatty acids, steroids, etc. Total fat refers to the sum of all types of fat found in a food (Shin et al., 2013; Hooper et al., 2015).

In **Figure 3.1**, it is presented an overview of the conventional analytical techniques for the quantification of total fat and fatty acids in foods.

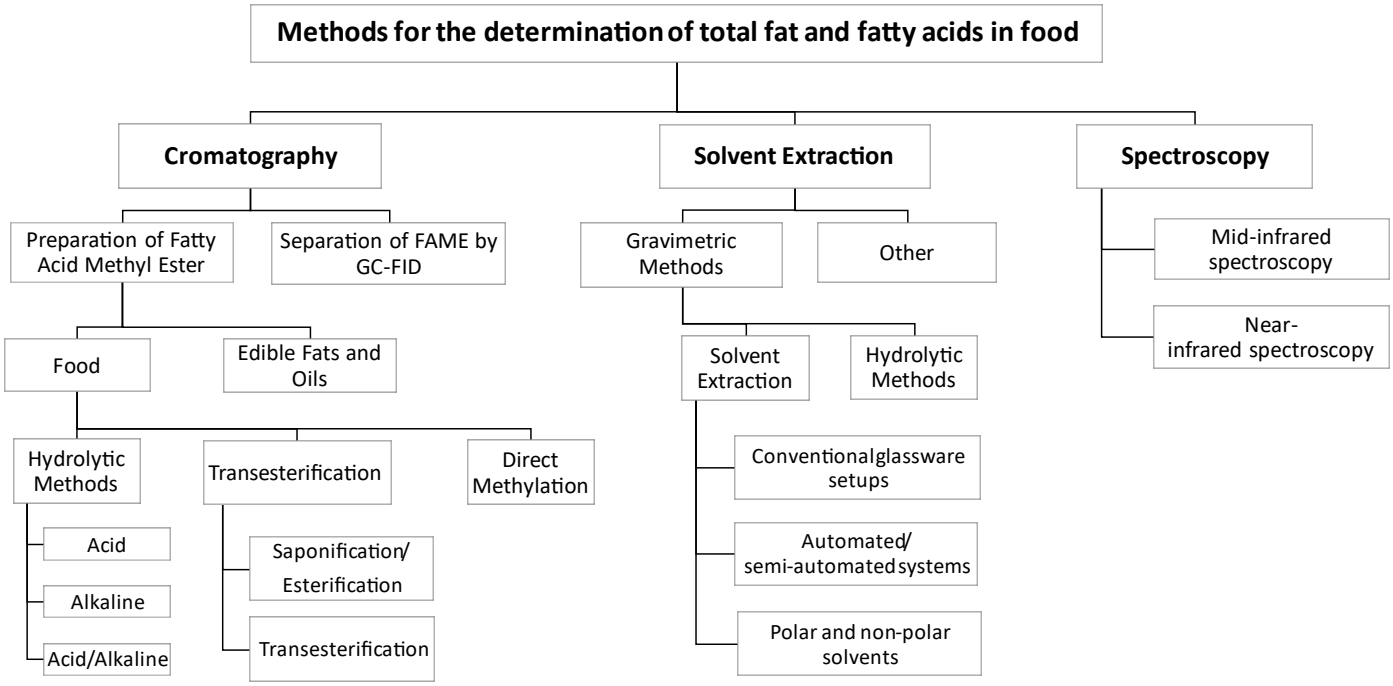


Figure 3.1 - Conventional analytical techniques for the quantification of total fat and fatty acids in foods (Adapted from: (Srigley & Mossoba, 2017))

The most common methods for fish lipid extraction are Soxhlet and Bligh & Dyer. When applying a solvent extraction, the total fat represents the content of crude fat, which may contain non-fat material. These techniques have been used during many years, but involve many steps, leading to a time-consuming procedure and demand a high consumption of organic solvents. Therefore, new extraction methods, such as Supercritical Fluid Extraction, Pressurized Liquid Extraction and Microwave Assisted Extraction, were proposed as possible sustainable alternatives for lipid extraction (Xiao, 2010; Pombal et al., 2017).

3.1 Soxhlet Method

Due to the complexity of some sample matrices and the need for several samples' pretreatments, prior to analysis and content assessment, the analysis of solid samples is a hard task. For the extraction and recovery of analytes from various solid matrices, Soxhlet extraction has been the method of choice (Zygler et al., 2012).

The Soxhlet technique was first developed to determine the fat content in milk. Later, this technique was generalized for agricultural and chemical extractions before becoming the most used tool for solid–liquid extraction in many fields like environment, food and pharmaceutical areas. Nowadays, the Soxhlet appliance is still common in laboratories and has been the standard and

reference method for solid–liquid extraction in most cases (Jensen, 2007; Virot et al., 2007).

However, the long extraction time (16-24h) and the high temperatures needed in this method, can result in changes in the composition of the extract. These alterations can influence the results of some analysis, as in the case of trans fatty acids determination (Shin et al., 2013).

To achieve an appropriate recovery of analytes, the matrices require a pre-treatment before extraction, being this the most challenging step. The efficiency of extraction is mostly related to three interrelated factors: solubility, mass transfer and matrix characteristics. So, it must be taken into account the questions related to the extraction technique, such as solvent choice, liquid-solid ratio, matrix characteristics, temperature, pressure and time of extraction and evaporation (Zygler et al., 2012).

To ensure the maximum efficiency of extraction of non-polar, non/semi volatile organic compounds, it is advised that the solid matrices containing water, such as soils, foods, vegetable tissues, etc., are dried before the extraction. In case of samples with fat or other non-polar analytes bonded to proteins, carbohydrates and/or minerals, an hydrolysis must be performed to break the bonds, which make the analytes unavailable for solubilization (Zygler et al., 2012).

The choice of the solvent significantly affects the extraction efficiency, and it should be selected considering the solubility power towards the target analyte without affecting the matrix. Another important aspect is the polarity of the solvent since this must be similar to that of the analytes. This condition provides enough contact with analytes, which controls the extraction recovery (Zygler et al., 2012).

The typical Soxhlet apparatus is shown in **Figure 3.2.** The thimble is positioned in a glass extraction chamber beneath the condenser and above a flask containing the extracting solvent. As the solvent boils, fresh solvent from the distillation flask slowly fills the extraction chamber. The extracted solutes are carried into the solvent reservoir below by a siphon as the condensed solvent is washed back into the distillation flask once it has filled the extraction chamber to its maximum capacity. The extraction thimble is currently devoid of any solvent. Typically, the cycle is repeated every 10–15 minutes. The target components must be less volatile than the solvent in the solvent flask because the solute is separated from the solvent by distillation. As a result, the fresh solvent evaporates and returns to the solid matrix while the solute remains in the flask. It must be recognized that each cycle includes an equilibrium step. In contrast to traditional soaking extraction, which results in solvent saturation, new solvent reaches the sample during each cycle, and this prevents even the use of hot solvents from causing solvent saturation. The extract is concentrated by

evaporation of the solvent following several hours of reflux (Luque de Castro & Priego-Capote, 2010).

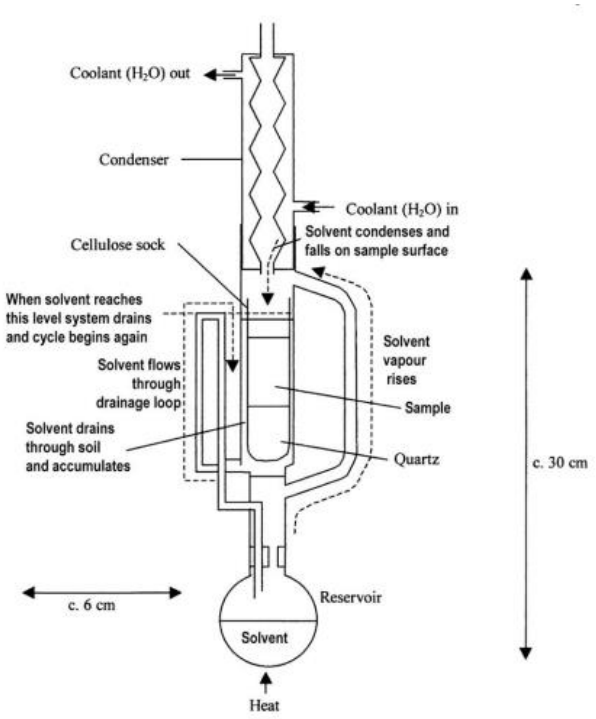


Figure 3.2 - Conventional Soxhlet extractor (adapted from Rostagno & Prado (2013))

The main advantages of conventional Soxhlet extraction are: the sample is repeatedly brought into contact with fresh portions of the extraction solvent, assisting in the displacement of the mass transfer equilibrium; the system temperature remains relatively high until reaching the extraction cavity; there is no need for filtration after the leaching step; the transfer rate of a sample could be increased by simultaneous parallel extractions since the equipment is inexpensive; the methodology is simple; compared to other approaches, a higher sample mass can be employed; a variety of compounds can be extracted from different solid matrices and there are many current official analytical methods that are based on this technique (Zygler et al., 2012; López-Bascón-Bascon & Luque de Castro, 2019).

However, there are some disadvantages, which include the large amounts of solvents required (waste disposal); the potential emissions of toxic compounds during extraction; the use of costly and pure solvents; the need of an evaporation/concentration step after extraction; and, the long time required (up to 48h) (Zygler et al., 2012; Hewavitharana et al., 2020).

3.2 Bligh & Dyer And Folch Methods

The Bligh & Dyer method has been known as an efficient method for the determination of lipids in microorganisms' matrices and fish. This method is an adaptation of the Folch method, using polar and non-polar solvents in specific ratios for lipid extraction. The solvents are needed to infiltrate into the fat cells and remove the lipid from the muscles including the phospholipids materials (Jensen, 2008; Adeoti & Hawboldt, 2014; Sundermann et al., 2016).

The main differences between the Folch and Bligh & Dyer methods are the ratios of the solvents in the mixture (chloroform/methanol/water), the volume of the solvents, and the presence or absence of salts in the added water fraction. While Folch method uses twenty times the sample's volume of a 2:1 (v/v) chloroform-methanol solution (assuming that the tissue had the specific gravity of water), the Bligh & Dyer method uses a chloroform-methanol stepwise extraction of 1:2 and 1:1 (v/v) amounting to a final volume of only four times the equivalent sample amount (Axelsson & Gentili, 2014).

Both methods are equally efficient in lipid extraction from marine tissues with 2% of lipids. However, Folch method extracts a higher amount of lipids due to the higher proportions of solvents used, compared to the Bligh & Dyer method (Axelsson & Gentili, 2014).

Both methods are widely used, as originally proposed or modified. These procedures have significant safety drawbacks, particularly when using chloroform, which is unsuitable for widespread use due to its high toxicity and carcinogenicity. Numerous researchers changed the Folch and Bligh & Dyer methods by substituting risky solvents in an attempt to turning them safer. Hexane-isopropanol combinations originally replaced the chloroform-methanol system (Hara & Radin, 1978). Despite this initial success, the outcomes indicated a reduced efficiency (Breil et al., 2017).

Several teams (e.g., Grima et al., 1994; Lee et al., 1998; Sheng et al., 2011; Caprioli et al., 2016) have conducted additional studies that demonstrated the efficacy of numerous potential solvents to replace the chloroform-methanol mixture. Nowadays, finding an effective extraction process with minimal energy use and greener solvents is a challenge for scientific and industrial researchers (Breil et al., 2017).

3.3 Microwave Assisted Extraction

The fundamentals of Microwave Assisted Extraction (MAE) are distinct from the conventional approaches since the extraction occurs as a result of changes in the cellular structure caused by electromagnetic waves. In MAE, a combination of two transport phenomena - heat and mass gradients acting simultaneously – can lead to an acceleration of the process and to a high

extraction yield. Contrary to the traditional extractions, in which the heat is transferred from within to outside, in MAE, the heat is dissipated volumetrically inside the irradiation medium, which transfers heat from the heating medium to the sample's interior (Chemat & Cravotto, 2013).

The use of microwave radiation for heating is based on the effect that microwaves have on molecules through ionic conduction and dipole rotation. Both processes operate simultaneously in many situations. Ions move electrophoretically when an electromagnetic field is applied, which is known as ionic conduction. Friction and heat generation are caused by the solution's resistance to the flow. The alignment of the dipoles with the applied field is known as dipole rotation (Eskilsson & Bjorklund, 2000).

The performance in microwave based processes, depends on some variables, such as the power of microwaves, time of exposure, pressure, viscosity and size of the sample and the solvent. The microwave power and the exposure time to the radiation have opposite effects: for a certain process, the use of a higher power gives a smaller time of extraction; on the other hand, the use of lower power requires a longer irradiation of the sample to apply the same amount of the energy (Lebovka et al., 2012).

Temperature also plays a key role in most analytical processes. In microwave, the temperature affects, for example, the speed of some reactions, the degradation of the thermolabile species, and the solubilization of some substances. Therefore, some microwave devices allow to monitor or control the temperature via a temperature probe (Lebovka et al., 2012).

Pressure is a highly influential factor in closed systems. The development of recipients capable of supporting pressures above 50 bar, allowed the digestion with pure liquid acids or mixtures at very high temperatures, increasing the efficiency of digestion and decreasing the time of exposure. Thus, some devices allow the control of pressure (Lebovka et al., 2012).

The input microwave frequency affects the depth of penetration of the energy. However, the bigger is the factor of dissipation of the sample, the less it will be penetrated by the microwave energy. In big samples with high factors of dissipation, the heating behind the depth of the energy of penetration is due to the thermal conductance trough molecular collisions. The use of small samples presents several disadvantages namely, the amount of energy absorbed decrease with the decrease of the sample size, being the energy reflected, which can damage the magnetron. Thus, it is advisable to employ microwave systems with magnetron protection (Lebovka et al., 2012).

The choice of the extraction solvent represents an important step to be optimized. When choosing the solvent, it is important to consider its capability to absorb microwaves, disrupt interaction with the matrix, solubilize the analyte and also its selectivity in relation to the analyte of interest. In solid-liquid extraction (leaching or lixiviation), the solvent must have a high selectivity in

relation to the target compounds and exclude unwanted matrix components. In closed vessels, the solvent can be heated above its normal boiling point, which enhances the extraction efficiency and increases the extraction speed. **Table 3.1** lists some of solvents that are used in 90% of the applications, including its boiling point and closed-vessel temperature (Eskilsson & Bjorklund, 2000; Lebovka et al., 2012).

Table 3.1 – Some solvents used in MAE (adapted from Eskilsson & Bjorklund (2000))

Solvent	Boiling Point (°C)	Closed-vessel temperature (°C)
Acetone	56	164
Acetonitrile	82	194
Ethanol	78	164
Hexane	69	[1]
Methanol	65	151
2-Propanol	82	145
Water	100	
Hexane-acetone (1:1)	52	156

[1] Indicates no microwave heating

There are two types of microwaves: closed and open vessels While an alternative method uses an open extractor container at atmospheric pressure, the most typical operation includes extraction in a closed vessel under controlled pressure and temperature. In this work a Milestone Ethos X microwave device was used, which comprises closed vessels, as shown in **Figure 3.3.**

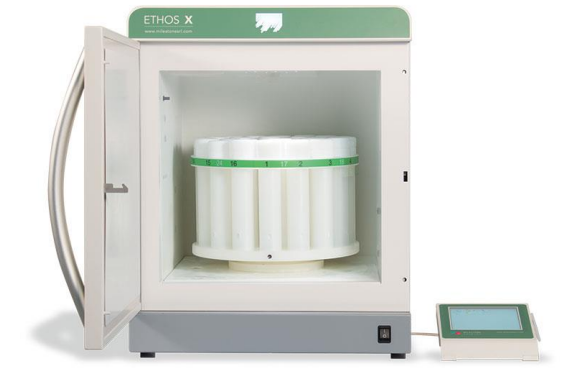


Figure 3.3 – Ethos X: Microwave Fat Determination in Food and Feed

This system allows the simultaneous irradiation of up to 12 extraction vessels. One of the vessels is a reference vessel (**Figure 3.4**) that regulates pressure and heat. Surface tension and solvent viscosity decrease as temperature rises, improving sample wetting and matrix penetration while increasing the solvent's ability to dissolve the target compounds. The effect of temperature on the extraction in a closed vessel demonstrates that increasing the solvent´s temperature from 60°C to 120°C greatly improves the extraction efficiency. This is due to the fact that increasing temperature causes a decrease of the intermolecular interactions within the solvent, resulting in a higher molecular mobility, which raises the solubility (Eskilsson & Bjorklund, 2000; Hemwimon et al., 2007; Moret et al., 2019).

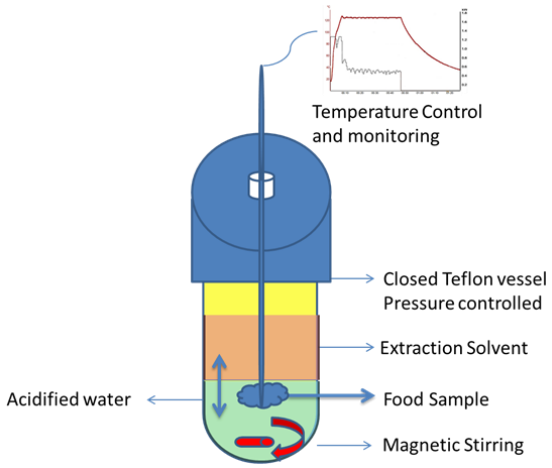


Figure 3.4 - Control Vessel Interior (adapted from Pombal et al. (2017))

Some advantages of the closed vessels can be resumed as follows (Lebovka et al., 2012):

- ✓ They can reach higher temperatures when compared to the open vessels, since higher solvents' boiling points are achieved due to the increase of pressure inside the vessel.
- ✓ The higher temperatures in the vessel, decrease the time of extraction.
- ✓ The loss of volatile compounds during the irradiation with the microwaves, are almost completely avoided by the absence of vapor losses.
- ✓ Less solvent is required. Since no evaporation occurs, there are no need to add solvent to maintain the volume. Also, the risk of contamination is avoided.
- ✓ The vapors that are produced during the treatment with acid microwaves, are contained inside the vessel.

On the other hand, the use of closed vessels have some disadvantages, such as (Lebovka et al., 2012):

- ✓ The high pressures used may represent a risk of security (explosion) derived from the production of hydrogen in acid treatments of metals and alloys.
- ✓ The quantity of sample that can be processed is limited (< 100 g).
- ✓ The material of the recipients is usual polytetrafluoroethylene (PTFE) and cannot support high temperatures.
- ✓ The recipient must be cooled down before it is open.

Digestion and extraction (leaching), both employed in food analysis, are the two main treatments provided by microwaves to food analysis. The features of the matrices and analytes, as well as the subsequent steps of the analytical process, determine the optimal conditions in each situation. Leaching must be chosen whenever possible since it enables the total removal of the target analytes, is less complex and is less prone to interferences. Leaching, however, only becomes more selective than digestion after maintaining the majority of sample interferences in the solid state. So, it is common the use of an extra cleaning step after leaching to remove any specie that behaves as the target analytes in the leaching step. The dissolution of the analytes must be assured for a precise quantification (Lebovka et al., 2012).

Despite this method has been successfully used for obtaining a variety of analytes, there are some constraints that need attention. There is a safety concern that must be considered when the microwaves are used, because the high temperatures and the conjunction of solvents used, can be a high risk to the operator. Despite the open vessels system being more safer than the closed vessels, the conditions of extraction are less reproductive and many of the samples cannot be processed simultaneously, which requires longer time of extraction to reach similar efficiencies to the closed vessels system (Kapoore et al., 2018).

Previous works (Batista et al., 2001; Virot et al., 2007; Virot et al., 2008a; Virot et al., 2008b) concluded that MAE is a well-suited alternative to conventional Bligh & Dyer and Soxhlet methods for the extraction of lipids followed by the determination of the fatty acids profile. Results showed that total fats and oils extracted by MAE were qualitatively similar to those obtained by conventional methods. No significant difference was obtained between the fatty acids composition of each extract, concluding that the technique is effective and valuable for the extraction of fats and oils from food products (Ramalhosa et al., 2012).

In **Table 3.2**, a brief comparison of the extraction methods previously mentioned, is given.

Table 3.2 - Comparison between different extraction methods: Soxhlet, Bligh & Dyer and MAE (Adapted from: (Adeoti & Hawboldt, 2014))

Extraction Method	Solvents	Extraction Time	Advantages	Disadvantages	Investment	References
Soxhlet	Organic solvents (hexane, toluene, acetone, petroleum ether, cyclohexane)	6-8h	It is simple Low labor intensive Can be operated with non-chlorinated solvents	Extractable lipids are determined not total lipids Large solvent volumes Special equipment required Results are very much operationally dependent Repeated extractions may be required	Low	Eskilsson & Bjorklund (2000); Adeoti & Hawboldt (2014); Suwal & Marciniak (2019)
Bligh & Dyer	Methanol, chloroform and water	5h	Simple and standard method Determines total lipids Direct analysis of samples with no pre-drying	Adverse effects of chloroform on the environment (EU regulation controlling chlorinated solvents) Laborious (requires filtration etc.)	Moderate	(Adeoti & Hawboldt, 2014; Kumar et al., 2015; Suwal & Marciniak, 2019)
MAE	Cyclohexane	3h	Fast and Multiple Extractions Low Solvent Volumes Elevated Temperatures	Extraction solvent must be able to absorb microwaves Clean up step needed Waiting time for the vessels to cool down	Moderate	Eskilsson & Bjorklund (2000)

As shown by the analysis of the above table, the MAE method is a fast method to determine the total fat, and may also be useful in sample preparation, when determining the fatty acids profile by chromatography.

4 FISH

Fish is one of the sources of proteins for humans. It is rich, not only in proteins, but also in essential fatty acids, minerals, and micronutrients, owning high nutritional value. In 2013, the Food and Agriculture Organization (FAO) of the United Nations, recommended that the States started to manage fisheries in a biologically sustainable way, since the majority of the fish stocks were fully fished (58.1%) or overfished (31.4%). FAO also encouraged the use of fish stocks considering their nutritional, social and economic values, where the preservation of their quality was primordial (Sogn-grundvåg et al., 2021).

Fish with high quality provide to the consumers safe and healthy meals, with high nutritional value. However, fresh fish is highly perishable when compared to other foods. Thus, the management of fish during fishing, and the posterior conservation are crucial to preserve the quality and the nutritional attributes of fish, besides reducing the waste (Sogn-grundvåg et al., 2021).

Fish is mostly constituted by water (70-80%), which is tightly bound to the proteins. The relation between the water and lipid content in fish is inverse. During the different seasons, with the raise of fat content, the water content decrease. The moisture content generally decreases too with the age of the fish. The superior part of the fish's fillet contains more protein than lipids when compared to the inferior part (Gökoğlu & Yerlikaya, 2015).

Fish oils are a rich source of PUFAs, in particular the n-3 series cis-5, 8, 11, 14, 17-eicosapentaeonic acids (EPA) and cis-4, 7, 10, 13, 16, 19-docosahexaeonic acid (DHA). These ingestion of PUFAs contributes to prevent and treat some coronary heart diseases, blood platelet aggregation, arthritis, hypertension, mental diseases, normal levels of cholesterol and autoimmune disorders. Fish oils are also a rich source of vitamins, including vitamin A, D, E and K (Smet, 2012; Adeoti & Hawboldt, 2014).

Lipid content and fatty acid composition are extremely variable in fishes from specie to specie. These differences depend on many factors, such as season, temperature of water, pH, salinity, life stage, species habitat and type and quantity of available food, specially, whether species are herbivorous, omnivorous or carnivorous. For example, depending on the season, the fat content in mackerel can vary from 4% to more than 30% (Moradi et al., 2011).

The lipid content in farmed fishes can also vary from wild fishes, due to the type of diet. Normally, the wild fishes have a diet richer in ω-3 highly unsaturated fatty acids. Consequently, PUFA ω-3 of long chain exists in higher concentration in the muscle of wild fishes. Most of the farmed fishes have diets rich in lipids to maximize their growth, and, because of that, they can present twice the lipid content when compared with the wild fishes (Moradi et al., 2011).

Lipids are mostly found in the subcutaneous tissue, belly flap, muscle tissue, liver, mesenteric tissue and head. The quantity of lipids decreases from the head to tail. Muscle tissue can be categorized in three principal types of muscle: principal white muscle, superficial red muscle and intermediate pink muscle. The white muscle is responsible for fast activity explosions, such as sudden fast swimming, and its covered by a thin layer of red muscle fibers. The red muscle is used to slow and continuous swimming. For this reason the flesh of migratory species, such as tuna, herring, mackerel, and sardine, have more tissues of red muscle and more fat than species that are more slow, like cod (Moradi et al., 2011; Ryu et al., 2021).

In **Figure 4.1** it is possible to visualize a fillet of fish where it is shown the white, red and pink muscles.

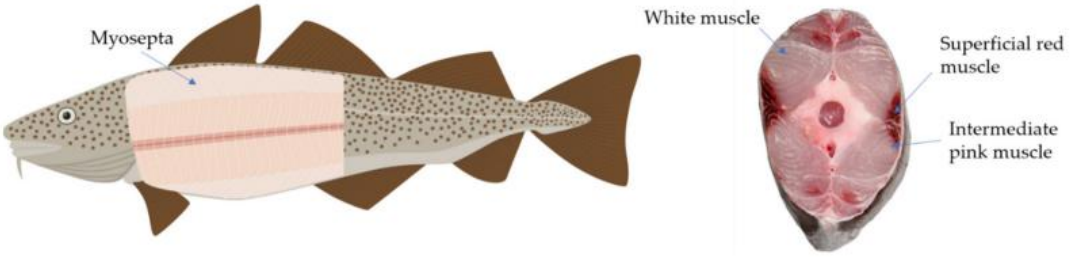


Figure 4.1 – Whole fish and sectional slide with myosepta, major white muscle, superficial red muscle and intermediate pink muscle (adapted from Ryu et al. (2021))

Fish can be classified as "lean," "extra lean," "fatty" and "extra fatty" based on the total fat content (**Table 4.1**). The distribution of lipids in muscle is heterogeneous, especially in fatty fishes. The lean fishes store lipids just in a limited extension of the liver, and the fatty fishes store lipids in fat cells distributed in other body tissues, such as subcutaneous tissue, belly flap muscle and muscles that move the fins and tail (Gökoğlu & Yerlikaya, 2015; Taşbozan & Gökçe, 2017).

Table 4.1 - Fish grouped by their fat contents (g/100 g, i.e. %), raw fillet or edible portion (adapted from Taşbozan & Gökç (2017) and Liu & Ralston (2021))

Very Low Fat(<2%)	Cod, Haddock, Flounder/Sole, Red Hake and Tuna
Low Fat (2-5%)	Tilapia, Atlantic Croaker, Ocean Perch and Salmon (Chum and Pink)
Medium Fat (5-10%)	Bluefish, Catfish, Rainbow Trout, Sword Fish and Salmon (Atlantic, Coho, Sockeye)
High Fat (>10%)	Atlantic Mackerel, Sardine, Herring and Salmon (Chinook/King)

When fish fatty acids (FAs) are analyzed, the results are mainly reported in relative abundance, i.e., in percentage (profile analysis) are mainly reported. However, the results are much more useful express in g/100g (absolute analysis) (Nevigato et al., 2012).

4.1 Methods For Lipid Extraction

For fish oil extraction, traditional methods such as, hydraulic pressing, heat extraction and solvent extraction can be used. However, there are some relatively new and environmentally friendly methods that can also be used: supercritical fluid extraction (SFE), enzyme extraction, microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE)(Ivanovs & Blumberga, 2017).

Microwave extraction, as referred previously, is considered a more advantageous method, when compared with the traditional methods, due to the higher extraction rates, lower temperatures, automatization and the possibility to study simultaneously different samples. However, this method present two major drawbacks: heat generation can lead to oxidation of unsaturated fatty acids and can present low efficiency when volatile solvents are used (Ivanovs & Blumberga, 2017).

Despite few, there are some papers that discussed oil extraction from fish using MAE. With these studies, it was revealed that extraction with microwaves, gives a similar or even greater yield than traditional extraction methods. For example, Ramalhosa et al. (2012) used a CEM MARS-X 1500W extraction unit to extract oil from mackerel, sardine and horse mackerel. In that study it was used petroleum ether:acetone (2:1, v/v) as solvent. It is important to emphasize that the lipid content of fishes varies with their life cycle, feeding growth cycles and other factors. The fat content determined by those researchers, for mackerel varied between 0.82 and 8.99 g/100g, in accordance with those reported by Karakoltsidis et al. (1995) and Stamatis & Arkoudelos (2007). Sardine fat content varied between 0.62 and 4.42 g/100g. According to some authors, sardine fat content significantly varied throughout the year. Bandarra et al. (1997) found values between 1.2 and 18.4 g/100g in sardines that were fished in the Portuguese coast, from Northeast Atlantic Ocean, monthly during a year. To horse mackerel, it was obtained a range from 3.22 to 5.01 g/100g. However, Aubourg & Ugliano (2002) reported total fat contents between 1.5 and 3.0 g/100g in horse mackerel from the Northeast Atlantic Ocean. These differences may be tentatively associated with factors like nutrition, living area, and catching season (Ramalhosa et al., 2012).

5 ANALYTICAL METHOD VALIDATION

One of the main requirements in any process that involves chemical analysis is to obtain quality data that meet the proposed goal. Thus, the validation of analytical methods aims to demonstrate, through laboratorial studies, that the method is adequate to the requirements demanded by the intended analytical application. In other words, the validation process intends to demonstrate that the method is suitable for the quantification of the target analyte in a matrix, at a certain concentration level, with satisfactory precision and trueness. (Almeida, 2012)

It is essential that laboratories demonstrate, trough the validation process, that the internal methods they use lead to credible and adequate quality results. This is why, methods used in laboratory must be validated, i.e., the methods must be tested and evaluated to ensure valid and adequate results for the intended purpose (Anderson et al., 2009).

According to the ISO/IEC 17025, to perform the method validation it may be necessary and convenient to perform some (or all) of the studies listed below. There are two types of studies, indirect evaluation and direct evaluation (Instituto Português de Acreditação, 2018). The indirect evaluation includes:

- ✓ The study of the representativeness of the method, i.e., that the determined characteristics correspond to the objective of the test.
- ✓ The study of the theoretical principles (fundaments) of the method to evidence the scientific base of the method.
- ✓ The study of the interference and error sources to outline its applicability and dominate its application.
- ✓ The study the performance characteristics of the method in order to know the quality of results.

Direct evaluation evolves:

- ✓ The comparison between standard or referenced methods.
- ✓ The comparison with patterns or reference material certificates.
- ✓ Interlaboratory comparisons.

The parameters to be checked for method validation are presented in the following scheme (**Figure 5.1**).

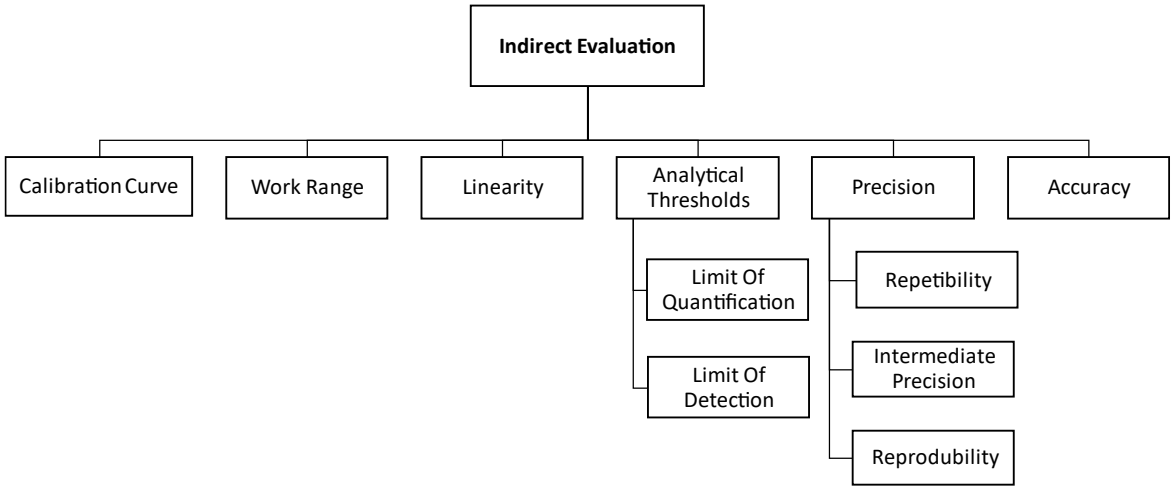


Figure 5.1 – Parameters for method validation (adapted from Augusto et al. (2000))

The process of validation starts with de definition of the application and the goal of the method, following the steps in **Figure 5.2.**

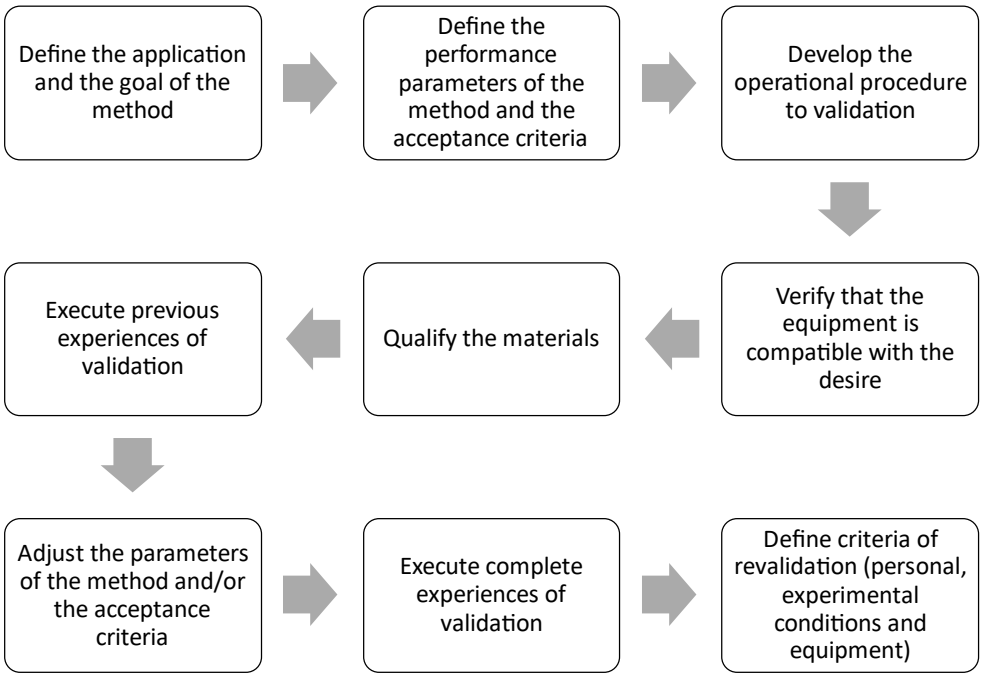


Figure 5.2 – General procedure for validation (adapted from Teixeira (2018))

5.1 Working Range

The working range corresponds to the concentration interval in which the analyte can be determined with good linearity, precision and trueness. For this to be defined, it is necessary that the instrumental answer be, preferably, linear and that there is homogeneity of variances in the dependent variable. With this, it is recommended the use of the regulation ISO 8466-1 to linear models and the regulation ISO 8466-2 to polynomials models of 2º degree (Augusto et al., 2000; Filipa & Martins, 2016)

Usually, the analysts start by selecting the working interval (based on the expected concentration level of the analyte in matrix under study) and determine if the relation signal *vs.* concentration is linear. By using a methodology that involves the representation of a calibration curve, the working range can be evaluated by the test of homogeneity of variances. For methods that not involve calibration curves, the working range must be defined previously and could be the function of some factors, such as the quantity of available sample (Thompson et al., 2002; Truta, 2012)

According to the regulation ISO 8566-1, it is recommended to use ten points for calibration. In case these are not carried out or that exists a rejection of some value, the calibration line must have a minimum of five points, distributed evenly along the working range (Filipa & Martins, 2016).

The first and last standards must be analyzed through ten independent replicas, performing the test of homogeneity of variances as follows:

- ✓ Determination of variances associated to the first and last standard ($\boldsymbol{S_1}, \boldsymbol{S_n}$), according to:

$$S_i^2 = \frac{\sum_{j=1}^{10}(y_{i,j} - \overline{y}_i)^2}{n_i - 1} \quad (1)$$

being,

$$\overline{y}_i = \frac{\sum_{j=1}^{10} y_{i,j}}{n_i} \quad (2)$$

- ✓ from $\boldsymbol{i = 1}$ to $\boldsymbol{i = 10}$, where $\boldsymbol{i}$ represents the number of the standard and $\boldsymbol{j}$ the number of repetitions performed to each standard.

The variances are tested to verify if there is a significant difference between them, in the limits of the working range, calculating the test value (TV):

$$TV = \frac{S_N^2}{S_1^2}, \text{ when } S_N^2 > S_1^2 \quad (3)$$

$$TV = \frac{S_1^2}{S_N^2}, \text{ when } S_N^2 < S_1^2 \quad (4)$$

Comparing the test value and the tabulated value of *F*-distribution, to n-1 degrees of freedom:

- ✓ If $\boldsymbol{TV \leq F_{crit}}$: the difference of the variances is not significant, and the working range is well adjusted;
- ✓ If $\boldsymbol{TV > F_{crit}}$: the difference of the variances is significant, and the working range must be reduced until the differences between the variances allow to get $\boldsymbol{TV \leq F_{crit}}$.

5.2 Linearity

The linearity of an analytical method is its ability to proportionally respond to the concentration of the analytes within a specified concentration range or with the aid of well described mathematical adjustments. By injecting a series of standards of stock solutions or diluted stock solutions, using the solvent or mobile phase, at least five distinct concentrations between 50 and 150% of the anticipated operating range are required to evaluate linearity. The Concentration *vs.* Peak Area Response (linearity) graph can be drawn using Microsoft Excel and it will be added to the respective study files (Stauffer, 2018).

5.3 Analytical Thresholds

It is important to know what is the lowest concentration value of the analyte that can be detected and quantified by the method, when the analysis is performed in samples with low levels of the analyte. The inferior limits of the calibration curve are concentrations that indicates the capacity to detect or quantify. These are estimated based in the uncertainty of the quantification of the analyte and can be obtained through: white replicas, uncertainty of the parameters from the calibration curve and uncertainty in the dispersion of the values around the calibration curve (Almeida, 2012).

The actualization of the Limit of Detection (LOD) and the Limit of Quantification (LOQ) must be performed when (Augusto et al., 2000):

- ✓ Changes in influencing factors, such as, analyst, chemicals, equipment, or environment occur;
- ✓ Whenever a new calibration curve is made, a study can be made over time and adopted as analytical thresholds the LOD and LOQ from a

significant series of calibration curves, since the values of the obtained thresholds are stable.

5.3.1 Limit Of Detection

The LOD of an individual analytical procedure, is defined as the lowest quantity of a component that can be detected, but not necessarily quantified under conditions established to the assay (Pum, 2019).

For a definition more correct of the LOD, it is necessary to known two concepts of statistic: type I error and type II error. Type I error (risk α) is the probability to conclude, due the presence of the component under analysis, when in fact that component does not exist in the sample. Type II error is the probability to conclude, due to the absence of the component under analysis, when in fact it does exist. In a way to do a correct analysis of analytical thresholds, these two types of errors should be minimized, opting to use IUPAC recommendations (α = β = 5%) (Augusto et al., 2000; Pum, 2019).

In quantitative terms, LOD can be obtained by:

$$LOD = \bar{x} + K\,\mathrm{s} \tag{5}$$

where, $\bar{\boldsymbol{x}}$ is the arithmetic average of the measured content of a series of whites or vestige patterns (between 10 and 20 rehearsals), prepared independently and read along several days of work, this is, reproducing the routine situation, as much as possible. $\mathbf{s}$ represents the standard deviation associated to $\boldsymbol{X_0}$ (Pum, 2019; Filipa & Martins, 2016).

If the probability law of $\boldsymbol{X_0}$ is sufficiently known and assuming it is Gaussian (normal distribution of errors), then $\boldsymbol{K} \cong 3.3$ to a level of confidence approx. 99.7% (Augusto et al., 2000; Francsico, 2008; Filipa & Martins, 2016).

If the method involves the use of a linear calibration:

$$LOD = \frac{3.3\,S_{y/x}}{b} \tag{6}$$

where, $\boldsymbol{S_{y/x}}$ is the residual standard deviation of the calibration curve (see minimum squares method) and $\boldsymbol{b}$ is the slope of the calibration curve (Francsico, 2008; Filipa & Martins, 2016).

5.3.2 Limit Of Quantification

LOQ is defined as the lowest measured concentration of an analyte, which is possible to be quantified, with a certain trueness and precision. In practice, it corresponds to the lower concentration of the calibration pattern (excluding the white). After the determination of this parameter, it must be tested to ascertain

if the trueness and precision achieved, are satisfactory. According to the recommendations of International Union of Pure and Applied Chemistry (IUPAC), the coefficient of variation (standard deviation / average of the values found) to this pattern should not exceed 10% (Augusto et al., 2000; Filipa & Martins, 2016).

Quantitatively, LOQ is determined by:

$$LOQ = \bar{x} + 10\ \mathrm{s} \tag{7}$$

where, $\bar{x}$ is the arithmetic average of the measured content of a series of whites or vestige patterns (between 10 and 20 rehearsals), prepared independently and read along several days of work, this is, reproducing the routine situation, as much as possible. **s** represents the standard deviation associated to $\bar{x}$ (Augusto et al., 2000).

LOQ can also be estimated by a set of trace patterns or fortified whites, independents, tested under conditions of intermediate precision, and it will be performed in studies of trueness and precision (Augusto et al., 2000).

If the method involves the use of a linear calibration:

$$LOQ = \frac{10\ S_{y/x}}{b} \tag{8}$$

where, $S_{y/x}$ is the residual standard deviation of the calibration curve (see minimum squares method) and b is the incline of the same (Francsico, 2008; Filipa & Martins, 2016).

5.4 Precision

The precision of an analytical method expresses the closeness of agreement between independent test results obtained under stipulated conditions. There are three levels of precision: repeatability, intermediate precision and reproducibility (Ribani et al., 2004).

Usually, precision is express as the standard deviation (s):

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \tag{9}$$

or as the coefficient of variation (CV):

$$\%\ CV = \frac{s}{\bar{x}} \times 100 \tag{10}$$

where $\boldsymbol{s}$ corresponds to standard deviation and $\boldsymbol{\bar{x}}$ to the average from the obtained results.

Repeatability and reproducibility refer to the agreement between successive measurements of the same analyte, performed under the same conditions. Both comprise different aspects of precision of a given method (Olivieri & Faber, 2009).

5.4.1 Repeatability

Repeatability represents the agreement between the results of successive measurements of the same method, made under the same measurement conditions, called conditions of repeatability: same procedure, same analyst, same instrument used under equal conditions, same local, repetitions in a short period of time (Ribani et al., 2004; Olivieri & Faber, 2009)

This parameter is determined through the study of n repetitions using the same matrix, covering all the levels of concentration and domains of the method application. First, it is necessary to examine if in the results obtained, there are values far from others, or if they present abnormal values (outliers), applying the Grubbs Test.

Repeatability is presented as the standard deviation associated to the average of results obtained. The limit of repeatability ($\boldsymbol{r}$) is the maximum value permitted to the difference between two rehearsals obtained in repeatability conditions, calculated for a confidence level of 95% through the following equation:

$$r = 2.8\sqrt{S_{ri}^2} \tag{11}$$

where 2.8 is the critical factor and $\boldsymbol{S_{ri}}$ is the repeatability variance associated of the consider results (Calçada, 2017; Teixeira, 2018).

Duplicates of determinations performed under conditions of repeatability are only accepted if the difference between the two values is lower than $\boldsymbol{r}$:

$$|x_{i1} - x_{i2}| \leq r \tag{12}$$

5.4.2 Intermediate Precision

Intermediate precision consists in the evaluation of the results obtained in the same laboratory, but in different days, with different analysts and/or equipment. To study this parameter, replicated measurements are performed under pre-defined conditions of repeatability (Teixeira, 2018; Calçada, 2017).

For replicates, the standard deviation of intermediate precision is calculated according to the following equation:

$$s = \sqrt{\frac{1}{2n}\sum_{i=1}^{n}(y_{i1} - y_{i2})^2} \quad (13)$$

5.4.3 Reproducibility

Reproducibility is the estimated precision obtained with the same method and identical tests, but in different laboratories, with different analysts and different apparatus. This parameter reflects the amplitude of random errors, and it can only be estimated trough interlaboratory studies (National Association of Testing Authorities, 2012; Olivieri & Faber, 2009).

The value of variance associated to the reproducibility is calculated by:

$$S_{Ri}^2 = S_{Li}^2 + S_{ri}^2 \quad (14)$$

being $\boldsymbol{S_{Ri}^2}$ the reproducibility variance, $\boldsymbol{S_{Li}^2}$ the interlaboratory variance and $\boldsymbol{S_{ri}^2}$ the repeatability variance (Augusto et al., 2000).

The limit of reproducibility is calculated similarly to that of repeatability (Equation (11)).

5.5 Trueness

Trueness measures the agreement between the test results and the reference value accepted as the true value. The term trueness, when applied to a set of test results, implies a combination of components of random errors and systematic errors. This can be evaluated by certified reference materials (CRM) and interlaboratory rehearsals (Olivieri & Faber, 2009; Ribani et al., 2004; Augusto et al., 2000).

Whenever possible, CRMs must be used in the validation process of a test method once they constitute an excellent tool in the external quality control of a chemical analysis. These are accompanied by a certificate that has the concentration value of a certain substance, or another characteristic for each parameter and one uncertainty associated. CRMs are provided from recognized and trustable organisms, like NIST (National Institute of Standards and Technology), LGC (Laboratory of the Government Chemist), and FAPAS (Food Analysis Performance Assessment Scheme) (Augusto et al., 2000; Ribani et al., 2004).

The correct use of CRM consists of its analysis to evaluate the laboratory performance. The value obtained from the analysis of the CRM must be compared with the certified value, determining the error and trueness of

analysis. When the obtained value falls the uncertainty range for the certified value, the laboratory must search the causes of that deviation and try to eliminate or accept them. The laboratory can also adopt different criteria for the acceptance of CRM results, according to the defined rigor for its results. Results evaluation obtained from the analysis of a CRM can be made using supplementary processes such as: relative error, statistical hypothesis testing (t-test), performance factor Z (z-score) and normalized error (Augusto et al., 2000).

5.5.1 Relative Error

The relative error (Er) expresses the component of systematic errors. The laboratory must define its degree of demand in terms of trueness for the method under study. This error is calculated by:

$$Er = \frac{(Xlab - Xv)}{Xv} \times 100 \quad (15)$$

where $\boldsymbol{Xlab}$ is the value obtained experimentally and $\boldsymbol{Xv}$ is the value accepted as real, i.e., the CRM value (Augusto et al., 2000).

5.5.2 Statistical Hypothesis Testing (t-test)

Hypothesis testing help the researcher to accept or reject a statistical hypothesis, based on the data (results) obtained for the samples (experimental) evaluated. With this, the laboratory can also ascertain the existence of systematic errors associated to the methodology applied, through a hypothesis test (Augusto et al., 2000; Assis et al., 2020):

$$t = \frac{(Xlab - Xv) \cdot \sqrt{n}}{sx_{lab}} \quad (16)$$

being $\boldsymbol{Xlab}$ the average of experimental values obtained by the laboratory in CRM analysis, $\boldsymbol{n}$ the number of samples tested and $\boldsymbol{sx_{lab}}$ the standard deviation associated to the average of the laboratory values.

Next, the $\boldsymbol{t}$ value (in module) is compared with the critical value $\boldsymbol{t_{tab}}$ (tabulated for a certain degree of confidence and $\boldsymbol{n - 1}$ degrees of freedom), taking as an acceptance criterion:

- ✓ If $\boldsymbol{|t| \leq t_{tab}}$**, there is no** statistically evidence regarding the existence of systematic errors and, therefore, the test is satisfactory;
- ✓ If $\boldsymbol{|t| > t_{tab}}$, **there is** statistically evidence regarding the existence of systematic errors and, therefore, the test is not satisfactory for fatty acid determination, for example.

5.5.3 Performance Factor Z (z-score)

Another way to evaluate the laboratory performance in the analysis of the CRM is to calculate the performance factor Z:

$$\mathrm{Z} = \frac{(Xlab - Xv)}{s} \tag{17}$$

where $\boldsymbol{Xlab}$ is the value obtained by the laboratory, $\boldsymbol{Xv}$ is the CRM value and $\boldsymbol{s}$ is the unity of deviation, that can be the uncertainty of CRM (Augusto et al., 2000).

The evaluation can be done according to the following score scale:

- ✓ $|z| \leq 2$: Satisfactory
- ✓ $2 < |z| \leq 3$: Questionable
- ✓ $|z| > 3$: Incorrect

5.5.4 Normalized Error

In case the laboratory calculates the uncertainty of its result ($\boldsymbol{U_{lab}}$), the real value ($\boldsymbol{X_v}$) must be inside of the uncertainty interval of $\boldsymbol{X_{lab}}$. When this does not happen, this interval can be underrated. In this case, the concept of normalized error is generally applied to assess the performance:

$$\mathrm{En} = \frac{(Xlab - Xv)}{\sqrt{U_{lab}^2 + U_{ref}^2}} \tag{18}$$

where $\boldsymbol{U_{ref}}$ is the uncertainty associated to the real value. If $\boldsymbol{|En|} \leq 1$, then $\boldsymbol{U_{lab}}$ is well valued (Augusto et al., 2000).

Whenever the evaluation processes used do not show satisfactory conditions, it must be elaborated a plan of actions for identifying the causes of the succeed, correct them and reevaluate the rehearsal (Augusto et al., 2000).

6 EXPERIMENTAL PROCEDURE

The present study aimed to validate a MAE method for the extraction of total fat from fish samples, and further assessment, in conditions previously defined by Milestone factory parameter settings. The extraction phase is done in the microwave at 1200 MW until a maximum temperature of 120°C is reached. The evaporation phase is performed at a power of 800 MW and a temperature of 110ºC.

The solvents used for the validation of the method are shown in **Table 6.1.**

Table 6.1 – Fixed conditions defined by UNICAM

	Trademark	**Analytical reagent grade (%)**	**Volume (mL)**
Volume H_2SO_4	CHEM-LAB	95-97	10 mL
Volume C_6H_{12}	CHEM-LAB	99,5	25 mL

The apparatus used in this study was the Ethos X, which enable the simultaneously study of 12 samples. In the 12 tubes were placed 11 samples and 1 blank. The fish was milled and weighed (≈1 g) in the tube, tared, and then 10 mL of H_2SO_4 (25% solution) were added. After tared again the tube, 25 mL of cyclohexane were added right after, registered the value of the solvent mass. Then a magnetic stirrer was placed inside each tube, which were then closed and positioned in the rotor (**Figure 6.1a**). Thus, the program "Fat (Hydrolysis + Extraction)" was initiated and took one hour and a half. When the program ends, the aluminum cups are weighed with and without a 10 mL aliquot with cyclohexane and fat (organic phase). To start the program "Solvent Evaporation" (**Figure 6.1b**), the refrigeration system was turned on, and after 30 minutes, the program ended. Finally, when the aluminum cups were cooled, they were weighed. This process is shown in **Figure 6.2.**

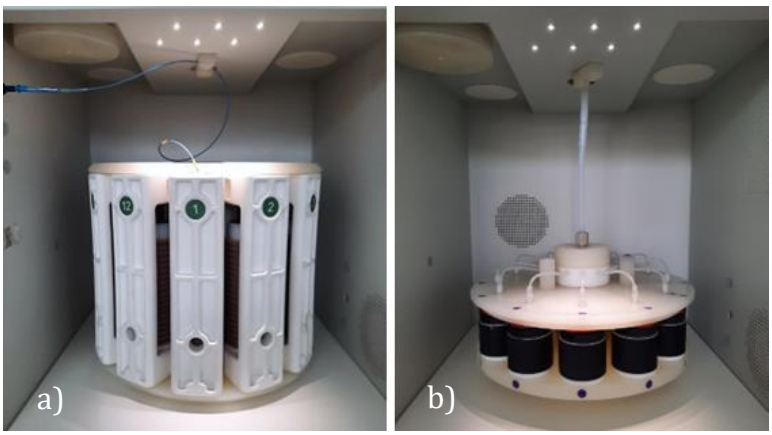


Figure 6.1 – a) Fat extraction rotor; b) Evaporation rotor

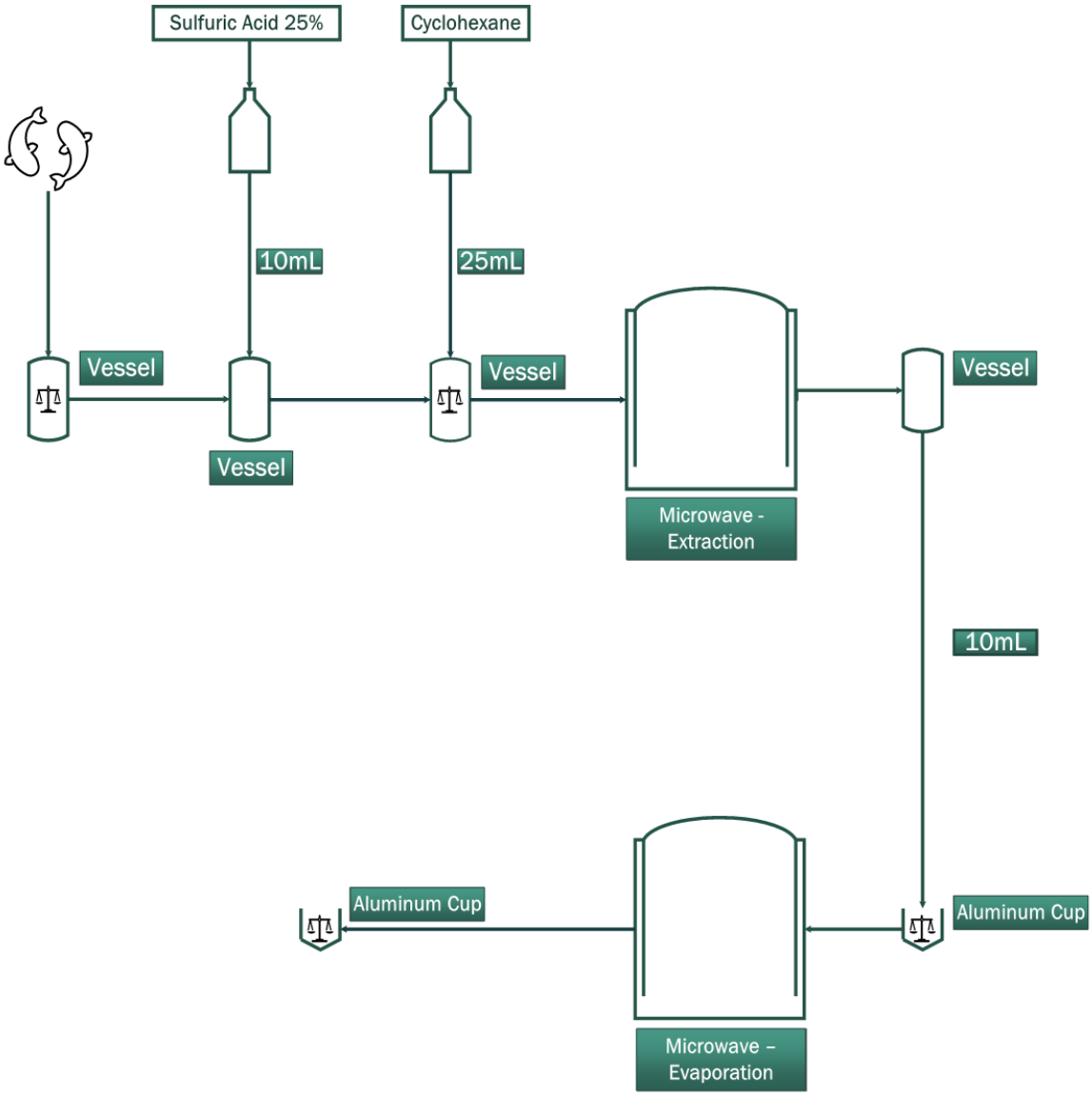


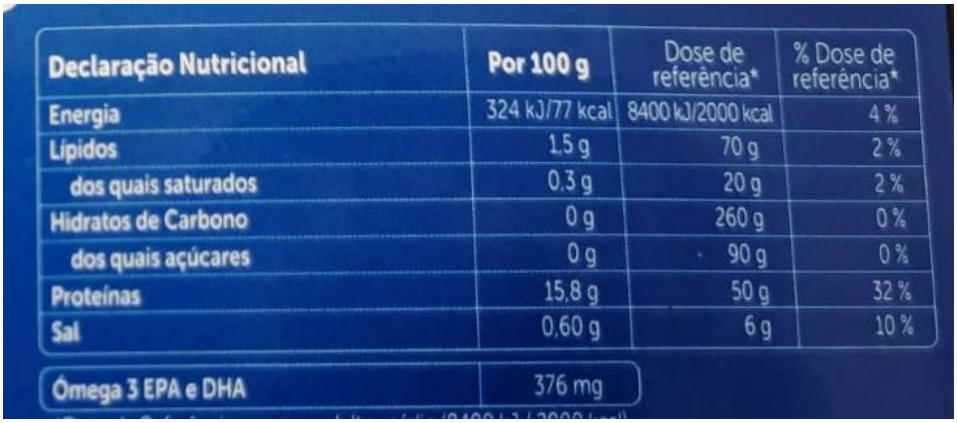
Figure 6.2 - Process of total fat extraction by MAE method

Fish samples provided by IPMA were used for the method validation. These matrices are listed in the **Table 6.2** according to the information provided. The values available in g/100g of fat, also presented in the table, were obtained by Soxhlet extraction, being only one value from each matrix. The samples were identified with letters A, B or C, being A for high fat, B for low fat and C for very low fat.

Table 6.2 – Information and samples obtained from IPMA

Sample	Fatty fish	Total Fat (g/100g)	Sample	Lean fish	Total Fat (g/100g)	Sample	Extra lean fish	Total Fat (g/100g)
A001	Blue Jack Mackerel	1.75	**B001**	Turbot	1.06	**C001.1**	Cod A	0.38
A002.1	Mackerel A	14.49	**B002.1**	Trout A	4.58	**C001.2**	Cod B	0.05
A002.2	Mackerel B	-	**B002.2**	Trout B	11.07	**C002.1**	Hake A	7.78
A003	Tuna Hamburger	9.42	**B002.3**	Trout C	2.44	**C002.2**	Hake B	2.66
A004	Canned Sardine	12.69	**B003**	Golden Bream	2.35	**C003**	Black Sword Fish	0.47
A005	Canned Mackerel	6.71	**B004**	Cooked Tuna	0.97	**C004**	Grouper	0.08
A006	Canned Tuna	7.02	**B005.1**	Skipjack A	1.30	**C005**	Ling	0.14
			B005.2	Skipjack B	1.22	**C006**	Monkfish	0.41
			B006	Swordfish	1.62	**C007**	Shrimp	0.29
			B007	Horse Mackerel	1.71	**C008**	Octopus	0.53
						C009	Cuttlefish	0.34
						C010	Squid Fish	0.39

In addition to these samples, it was also studied a commercial fish, more specifically, frozen hake burgers. All the nutritional information is found in **Figure 6.3.**



Declaração Nutricional	Por 100 g	Dose de referência*	% Dose de referência*
Energia	324 kJ/77 kcal	8400 kJ/2000 kcal	4 %
Lípidos	1,5 g	70 g	2 %
dos quais saturados	0,3 g	20 g	2 %
Hidratos de Carbono	0 g	260 g	0 %
dos quais açúcares	0 g	90 g	0 %
Proteínas	15,8 g	50 g	32 %
Sal	0,60 g	6 g	10 %
Ómega 3 EPA e DHA	376 mg		

Figure 6.3 – Nutritional table from commercial hake burgers

The hake presents a total fat value of 1.5 g/100g. With this, we can add this sample to the lean fishes, given it the name of B008.

7 RESULTS AND DISCUSSION

In order to implement the method, it was studied experimentally, the LOQ and LOD, control standards (olive oil), duplicates and replicates of samples, recovery tests and reference materials.

During the implementation of the method, the acceptance criteria (A.C) was established by us, from previously performed tests, and was set to <15% for all parameters.

During the validation of the method, the A.C used in the implementation, was compared to the expanded uncertainty obtained (***U***), and the precision and trueness of the method (coefficient of variation) is compared with the combined uncertainty (***uc***).

Whenever the standard uncertainties associated with the precision and trueness of measurement were calculated using a large number of experimental tests, the expanded uncertainty was related with the combined uncertainty trough the following equation:

$$U = uc \times \mathrm{k} \tag{19}$$

being $\boldsymbol{k} = 2$ for a confidence level of 95%.

7.1 Implementation Of The Method

7.1.1 Determination Of Theorical And Experimental LOQ

Initially, the LOQ and LOD were determined. The theoretical determination of these parameters was made following the previous discussed equations:

As mentioned before, the $\boldsymbol{K} \cong 3.3$ is used for a level of confidence approx. 99.7%. However, to other confidence intervals, the hypothesis test was used to estimate the LOD by the following equation:

$$LOD = \overline{\mathrm{X}} + (t_{n-1,1-a} \times s) \tag{20}$$

in which $\overline{\mathbf{X}}$ is the average of the blank samples, $\boldsymbol{t}$ is the abscissa of the student distribution, depending on the sample size and the degree of confidence, and $\boldsymbol{s}$ is the standard deviation of the blank samples (Perez, 2010).

For a level of confidence of 95%, the value of $\boldsymbol{K}$, according to the equation (20), is 2.16. With this, the LOQ and LOD obtained were 0.0031 g and 0.0009 g, respectively.

LOQ and LOD can also be presented in g/100g. For this, it was used the following equation:

$$\text{LOQ (g/100g)} = \frac{\frac{LOQ \times Solvent\ Mass}{Aliquot\ Mass}}{Sample\ Mass} \times 100 \quad (21)$$

$$\text{LOD (g/100g)} = \frac{\frac{LOD \times Solvent\ Mass}{Aliquot\ Mass}}{Sample\ Mass} \times 100 \quad (22)$$

Considering the solvent mass as 20 g, the aliquot mass as 10 g and the sample mass as 10 g, it was obtained a LOD of 0.018 g/100g and a LOQ of 0.061 g/100g.

To determine the experimental LOQ, some tests were realized. The resume of this determination is presented in **Figure 7.1**. For these tests it was used olive oil, which was considered as a solution with 100% of fat. To start the tests, different masses were weighted, in order to find the minimum mass that can be weighted and quantified. For this, the following masses were used: 0.4 g, 0.2 g, 0.1 g, 0.05 g, 0.02 g and 0.01 g.

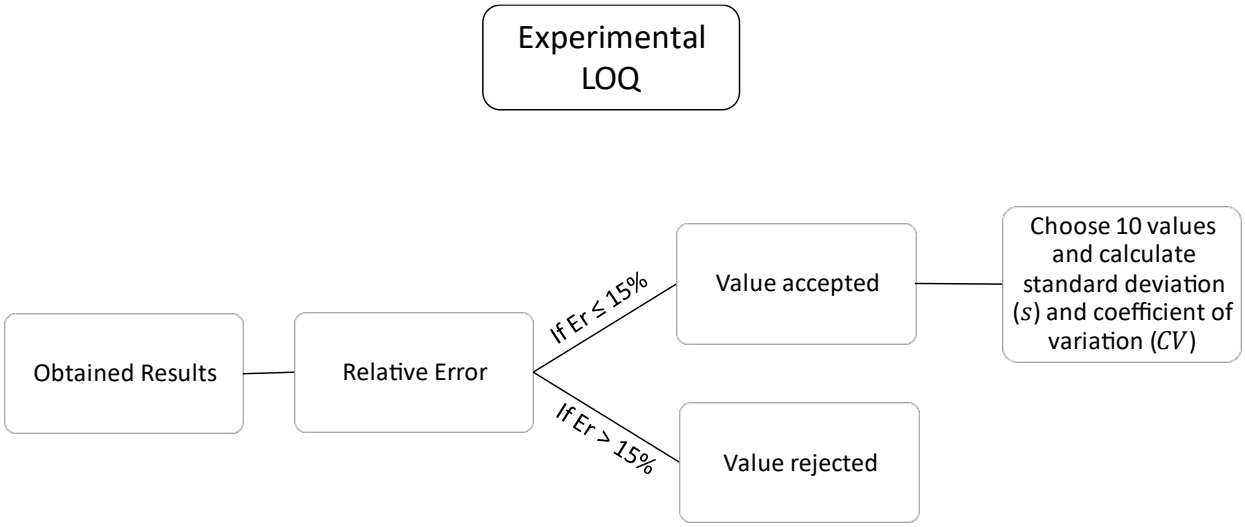


Figure 7.1 – Resume of determination of experimental LOQ

The chosen mass to determine the LOQ of the method was 0.01 g once it was the most comfortable mass to weight, from the operator´s point of view. For this mass, it was calculated the experimental fat (g/100g), that is the value that should be obtained to the maximum mass of the vessel (10 g), and the real fat (g/100g) that is the value of fat obtained through the mass weighted (≈0.01 g). The following equations were used to determine the experimental and real fat values:

$$\text{Experimental Fat (g/100g)} = \frac{\frac{Residue\ Mass\ \times Solvent\ Mass}{Aliquot\ Mass}}{Maximum\ Mass} \times 100 \quad (23)$$

$$\text{Real Fat (g/100g)} = \frac{Sample\ Mass}{Maximum\ Mass} \times 100 \quad (24)$$

The relative error between the experimental fat and the real fat was calculated by Equation (15). To accept the obtained values, it is considered as the acceptance criteria a value of 15%. In **Table 7.1** it is presented the values obtained and accepted for experimental fat, real fat and the correspondent mass weight.

Table 7.1 – Values obtained for Experimental and Real fat to a mass weight of 0.01 g

Mass Weight (g)	Exp. Fat (g/100g)	Real Fat (g/100g)
0.0110	0.099	0.110
0.0106	0.110	0.106
0.0120	0.118	0.120
0.0101	0.111	0.101
0.0114	0.115	0.114
0.0105	0.104	0.105
0.0121	0.120	0.121
0.0100	0.112	0.100
0.0102	0.103	0.102
0.0107	0.111	0.107

With this, the trueness and precision (repeatability) of the determination of the LOQ were studied. For this it was calculated the average and the standard deviation of the values obtained for the experimental fat. With the acceptance criteria presented in page 41 (≤ 10%), it was calculated the coefficient of variation and the relative error, being presented in **Table 7.2.**

Table 7.2 – Study of the trueness and precision of LOQ (0.01 g)

Exp. Fat Average (g/100g)	Standard Deviation (g/100g)	Number of Samples	Relative Error (%)	CV (%)
0.110	0.0067	10	-10.28	6.05

The determination of LOD can be made in two ways. It is possible to determine a theoretical LOD from the experimental LOQ, and an experimental LOD from the following equation:

$$LOD = t_{n-1,1-a} \times s \quad (25)$$

With this, it was obtained a theoretical LOD of 0.037 g/100g and an experimental LOD of 0.015 g/100g.

It is normal to obtain a higher value of the experimental LOQ than that of the theoretical LOQ. The obtained results confirmed this, being obtained an experimental LOQ of 0.110 g/100g and a theoretical LOQ of 0.061 g/100g.

The other masses, refered previously, were used as control standards, to assess the performance of the method. In **Figure 7.2** is possible to see the resume of the tests made to the standard controls. **Table 7.3** presents the average of the other weighted masses that were accepted, such as the average of fat obtained, standard deviation, number of rehearsals, relative error, and coefficient of variation.

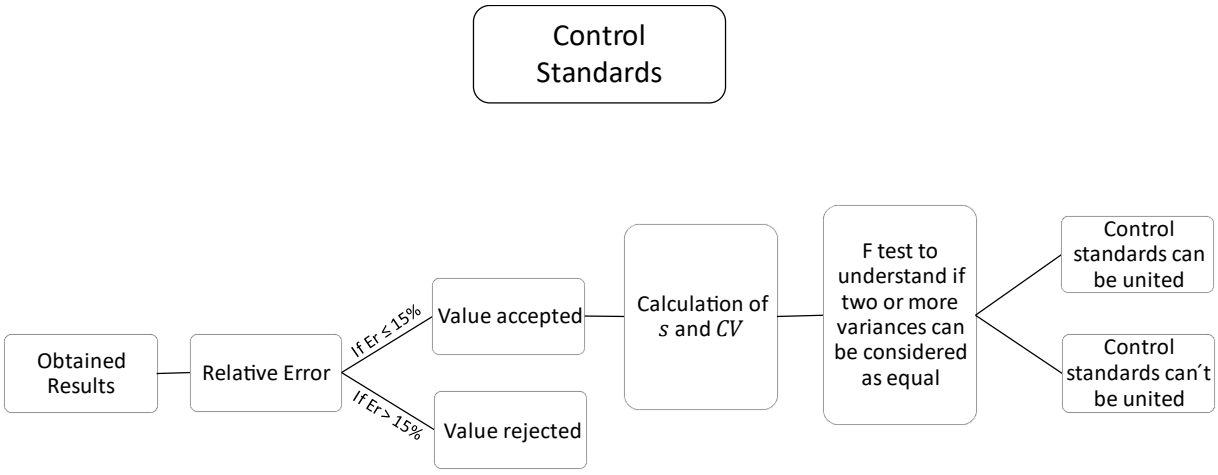


Figure 7.2 –Tests realized to standard controls

Table 7.3 - Study of the trueness and precision of the control standards

Average Mass (g)	Fat Average (g/100g)	Standard Deviation (g/100g)	Sample Number	Relative Error (%)	CV (%)
0.4140	20.074	0.848	5	-0.37	4.23
0.2080	10.270	0.172	11	-2.70	1.67
0.1077	5.241	0.316	11	-4.83	6.02
0.0544	2.704	0.184	4	9.86	6.79
0.0222	1.109	0.073	5	-10.89	6.55

The work ranges were defined by calculating the weighted coefficient of variation using standards coefficients of variation. To understand what standards can be considered equals, it is used the F test. This is a hypothesis test used to evaluate if two population variances can be considered as equal, or not, based in the obtained results of those samples. The variances that are compared are always the highest and the lowest. For this test it was used the "Data Analysis" from Excel. If F<Fcrit, the samples can be united, and it is possible to obtain a weighted coefficient of variation.

In the following tables (**Table 7.4** and **Table 7.5**) it is possible to verify that standards 1 and 3 g/100g and 5 and 10 g/100g, can be combined .

Table 7.4 – F test to standards 1 and 3 g/100g

	3 g/100 g	**1 g/100 g**
Average	2.704	1.109
Variance	0.034	0.005
n	4	5
(n-1)	3	4
F	6.394	
Fcrit	6.591	

Table 7.5 – F test to standards 5 and 10 g/100g

	5 g/100 g	**10 g/100 g**
Average	5.241	10.270
Variance	0.100	0.030
n	8	9
(n-1)	7	8
F	3.375	
Fcrit	3.500	

For both tables, since F<Fcrit, it is clear that there is no statistical difference between the variances, so it is possible to estimate a weighted coefficient of variation (absolute or relative). This parameter can be calculated by the following equation:

$$Weighted\ CV\ (\%) = \sqrt{\frac{\sum_{i=1}^{n}(n-1)\times cv^2}{\sum(n-1)}} \quad (26)$$

Based on the weighted CV, four working ranges were established. The weighted coefficients of variation, calculated using the standards, are shown in **Table 7.6.**

Table 7.6 – Weighted coefficients of variation to each work range

	0.1-1	**1-5**	**5-10**	**>10**
Weighted CV (%)	6.05	6.65	4.29	4.23

7.1.2 Study Of Precision

The precision of the method was assessed by studying the repeatability and the intermediate precision, being the process described in **Figure 7.3.** Reproducibility was not evaluated since the performance of the method has been determined and controlled in an intra-laboratory environment.

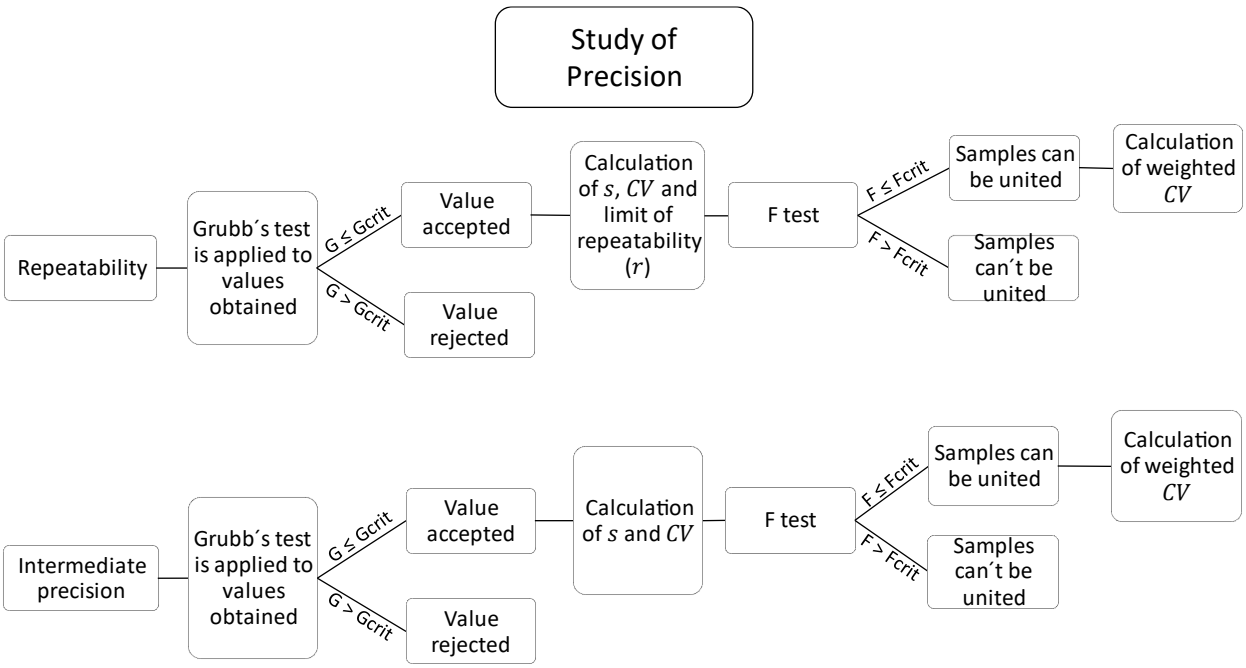


Figure 7.3 – Steps carried out to study the method's precision

The samples used to study the repeatability were A003, A004, A005, A006 and B008 being tuna hamburger, canned sardine, canned mackerel, canned tuna and hake burgers. The study of repeatability was done in stable conditions such as: same laboratory, same analyst, same equipment, same reagents and in a short period of time. For this, in the same day, 10 rehearsals of the same sample were studied. The percentage of total fat obtained was calculated by Equation (22).

To verify the acceptability of the values obtained, the Grubb´s Test was used. This test is used to detect outliers in a univariate data set. First, the experimental G is determined by Equation (27):

$$\text{Gexp} = \frac{[x_i - \bar{x}]}{s} \times 100 \tag{27}$$

being $\boldsymbol{x_i}$ the value obtained, $\boldsymbol{\bar{x}}$ the average of the values and $\boldsymbol{s}$ the standard deviation. After this, it is found the value of critical G in tables that already exist. If $G_{crit} > G_{exp}$, the value is accepted. Otherwise, the value is not accepted. In **Table 7.7**, **Table 7.8**, **Table 7.9**, **Table 7.10** and **Table 7.11** are shown all the values obtained from the different samples, and the evaluation of results according to the Grubb´s test.

Table 7.7 – Values obtained to study repeatability in sample A003

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0583	8.52	3.55	9	2.110	Rejected
1.0079	9.00	1.65			Accepted
1.0755	9.12	1.20			Accepted
1.0328	9.44	0.07			Accepted
1.0446	9.23	0.75			Accepted
1.0542	9.69	1.02			Accepted
1.0467	9.76	1.29			Accepted
1.0237	9.56	0.53			Accepted
1.0652	9.54	0.45			Accepted
1.0141	9.49	0.26			Accepted

Table 7.8 – Values obtained to study repeatability in sample A004

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0109	12.17	0.18	10	2.176	Accepted
1.0016	11.71	1.55			Accepted
1.0353	11.69	1.60			Accepted
1.0521	12.32	0.28			Accepted
1.0227	12.01	0.64			Accepted
1.0080	12.44	0.64			Accepted
1.0160	12.47	0.72			Accepted
1.0011	12.19	0.11			Accepted
1.0267	12.82	1.78			Accepted
1.0590	12.44	0.65			Accepted

Table 7.9 – Values obtained to study repeatability in sample A005

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0096	6.98	0.56	10	2.176	Accepted
1.0093	6.85	0.54			Accepted
1.0352	7.04	1.02			Accepted
1.0290	6.74	1.48			Accepted
1.0441	6.67	2.11			Accepted
1.0096	6.96	0.32			Accepted
1.0545	6.94	0.17			Accepted
1.0174	6.96	0.38			Accepted
1.0122	7.02	0.84			Accepted
1.0122	7.02	0.84			Accepted

Table 7.10 – Values obtained to study repeatability in sample A006

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0673	7.31	1.21	10	2.176	Accepted
1.0264	6.90	1.59			Accepted
1.0058	7.01	0.87			Accepted
1.0081	7.03	0.72			Accepted
1.0194	7.08	0.36			Accepted
1.1059	7.11	0.16			Accepted
1.0455	7.19	0.39			Accepted
1.0560	7.23	0.61			Accepted
1.0553	7.39	1.73			Accepted
1.0685	7.10	0.23			Accepted

Table 7.11 – Values obtained to study repeatability in sample B008

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
2.0526	1.18	1.52	10	2.176	Accepted
2.0628	0.99	0.95			Accepted
2.0093	1.11	0.60			Accepted
2.0374	1.16	1.18			Accepted
2.0774	1.04	0.29			Accepted
2.0836	1.03	0.50			Accepted
2.0619	1.07	0.01			Accepted
2.0508	1.10	0.46			Accepted
2.0229	0.92	1.85			Accepted
2.0166	1.05	0.20			Accepted

Using these values, the average, standard deviation, coefficient of variation, limit of repeatability and the relative limit of repeatability were calculated. **Table 7.12** lists the values obtained for each sample.

Table 7.12 – Values of average, standard deviation, coefficient of variation, limit of repeatability and relative limit of repeatability to each sample

	A003	A004	A005	A006	B008
Average	9.43	12.23	6.92	7.14	1.06
n	9	10	10	10	10
SD	0.26	0.35	0.13	0.15	0.08
CV (%)	2.71	2.88	1.81	2.06	7.31
r	0.72	0.99	0.35	0.41	0.22
Relative r (%)	7.60	8.08	5.07	5.76	20.47

From the F test it is possible to confirm that sample A003 can be combined with sample A004, and sample A005 with A006. From these, it was obtained a relative weighted CV of 7.85% and 5.43%, respectively. For sample B008 the CV is the same, being 7.31%. In **Table 7.13** and **Table 7.14** is possible to see the F test for these samples.

Table 7.13 – F test to samples A003 and A004

	A004	**A003**
Average	12.228	9.427
Variance	0.124	0.065
n	10	9
(n-1)	9	8
F	1.900	
Fcrit	3.388	

Table 7.14 – F test to samples A005 and A006

	A006	**A005**
Average	7.137	6.918
Variance	0.022	0.016
n	10	10
(n-1)	9	9
F	1.373	
Fcrit	3.179	

Precision, as mentioned before, is also calculated using the intermediate precision. Intermediate precision allows a more significant representation of the variability of the results, and for its determination, n measurements are performed in replicate or duplicate in a single assay.

Due to the amount of results, the tables with the values obtained are presented in Atpendix A1: Study of Intermediate Precision from Reproducibility. With these values, the standard deviation and coefficient of variation, to each sample were calculated. In **Table 7.15** it is possible to see the values obtained.

Table 7.15 – Values of average, standard deviation and coefficient of variation to each sample

	A003	**A004**	**A005**	**A006**	**B008**
Average	9.85	12.87	6.77	7.25	1.11
n	21	22	19	26	18
SD	0.46	0.35	0.20	0.23	0.06
CV (%)	4.64	2.70	2.92	3.11	5.57

The F test was applied, being obtained a relative weighted CV of 3.77% for samples A003 and A004, and 3.00% for samples A005 and A006. For sample B008 the CV was the same, being 5.57%. In **Table 7.16** and **Table 7.17** it is possible to see the F test for these samples.

Table 7.16 – F test to samples A003 and A004

	A004	**A003**
Average	9.852	12.872
Variance	0.209	0.121
N	21	22
(n-1)	20	21
F	1.733	
Fcrit	2.096	

Table 7.17 – F test to samples A005 and A006

	A006	**A005**
Average	7.250	6.768
Variance	0.051	0.039
N	26	19
(n-1)	25	18
F	1.300	
Fcrit	2.141	

With this study the ranges 1-5, 5-10 and >10 were covered. In **Table 7.18** it is possible to see the weighted coefficients of variation obtained for the repeatability and reproducibility.

Table 7.18 – Resume of the values obtained to weighted CV (%) to the study of repeatability and reproducibility

		1-5	**5-10**	**>10**
Repeatability	Weighted CV (%)	7.31	1.94	2.81
Reproducibility	Weighted CV (%)	5.57	3.03	3.77

7.1.3 Trueness

The trueness of the method was studied trough the recovery tests. In **Figure 7.4** the study of trueness based on the recovery tests is presented. These tests are very important in the qualitative analysis and must be carried out regularly and throughout the entire working range. The concept of "recovery" reflects the relationship between the amount of analyte recovered in the process *vs* the actual mount present in the sample.

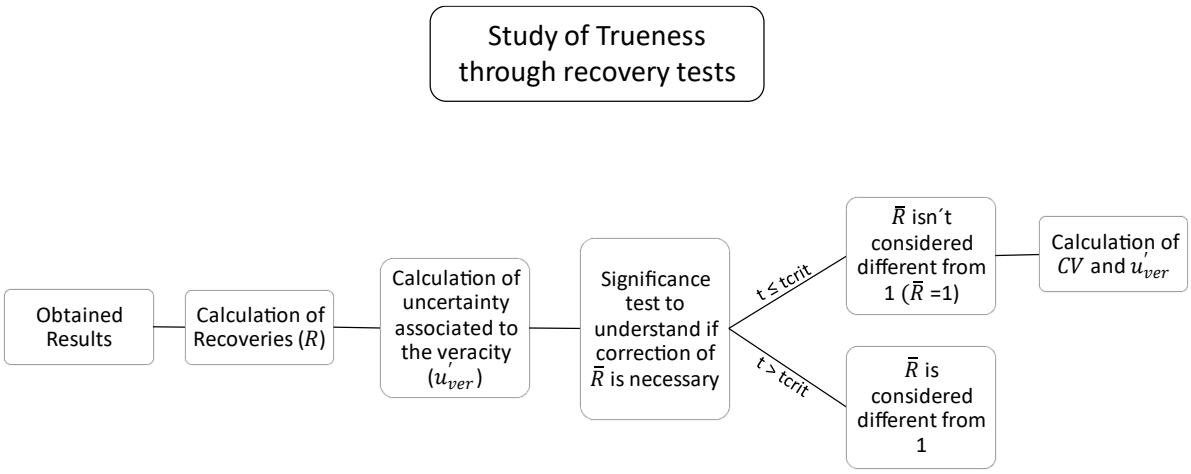


Figure 7.4 – Study of trueness through recovery tests

Recovery tests consist of adding a known amount of analyte to the sample. The analyte used was olive oil, considering to be 100% fat. For this study, duplicates were performed, one of them being a recovery, being later determined the percentage of recovery (Equation (28)).

$$\mathrm{R} = \frac{C_{AF} - C_A}{C_F} \times 100 \qquad (28)$$

$\boldsymbol{C_{AF}}$ is the concentration of analyte in the fortified sample, $\boldsymbol{C_A}$ is the concentration of analyte in the sample and $\boldsymbol{C_F}$ is the concentration of the fortification.

The samples used in this test were A003, A004, A005, B003, C006, A002.1 and B008, being tuna hamburger, canned sardine, canned mackerel, golden bream, monkfish, mackerel A and hake burgers, respectively. The idea of using different samples was to include all the working ranges considered in this study.

In **Table 7.19** there are shown the results obtained for this test. The acceptance criteria are between 80% and 120%. However, the laboratory may define another criterion, depending on the performance and the complexity of the method.

Table 7.19 – Results obtained to recovery tests for samples A003, A004, A005, B003, C006, A002.1 and B008

	Total Mass (g)	Sample Mass (g)	Olive Oil Mass (g)	Fat (g/100g)	Fortification	$\bar{R}$ (%)
A003	1.0795	1.0786	0.0232	13.27	2.11	87.1
A003 F	1.1018			15.10		
A004	1.0361	1.0357	0.0209	7.11	1.98	84.2
A004 F	1.0566			8.78		
A005	1.0177	1.0173	0.0229	7.34	2.20	88.3
A005 F	1.0402			9.28		
B003	2.2055	2.0723	0.0349	2.48	1.66	87.7
B003 F	2.1072			3.93		
C006	2.3133	2.192	0.0412	0.43	1.84	101.9
C006 F	2.2332			2.31		
A002.1	2.0389	2.0655	0.0203	14.45	0.97	57.8
A002.1 F	2.0858			15.01		
B008	2.0729	2.1192	0.0338	0.88	1.57	100
B008 F	2.1530			2.45		

With this, it is possible to infer that sample A002.1 cannot be accepted, since it has a recovery that does not fall in the range of the acceptance criterion.

After this, it was calculated the uncertainty associated to the trueness ($\boldsymbol{u'_{ver}}$), based on the evaluation of the recovery tests (Relacre, 2006):

$$u'_{ver} = \sqrt{b_{RMS}^2 + u'^2_{add}} \tag{29}$$

$$b_{RMS} = \sqrt{\frac{\sum_{i=1}^{n}(b'_i)^2}{n}} \tag{30}$$

$$b'_i = \frac{n_i - n_m}{n_m} \tag{31}$$

where, $\boldsymbol{b'_i}$ is the relative difference of the recuperation, $\boldsymbol{b_{RMS}}$ is the average quadratic value from the relative differences ($\boldsymbol{b'_i}$) calculated, $\boldsymbol{n}$ is the number of considered recoveries, $\boldsymbol{n_i}$ is the value of each recuperation, $\boldsymbol{n_m}$ is the average value of the recoveries, $\boldsymbol{u'_v}$ in the uncertainty of the additional volume and $\boldsymbol{u'_{conc}}$ is the relative uncertainty for the concentration of the fortification solution, that can be obtained from the RM. In **Table 7.20** are presented the parameters used for the calculation of $\boldsymbol{u'_{ver}}$.

Table 7.20 – Values obtained to calculate the uncertainty associated to the trueness

	R (%)	$\bar{R}$ (%)	b_i'	$(b_i')^2$	$\sum_{i=1}^{n}(b_i')^2$	n	b_{RMS}	b_{RMS} (%)	CV (%)
A003	87.1	91.5	0.0483	0.0023	0.0332	6	0.0744	7.44	6.86
A003 F									
A004	84.2		0.0805	0.0065					
A004 F									
A005	88.3		0.0350	0.0012					
A005 F									
B003	87.7		0.0419	0.0018					
B003 F									
C006	101.9		0.1135	0.0129					
C006 F									
B008	100.0		0.0922	0.0085					
B008 F									

To calculate the relative uncertainty of the added analyte concentration, it was study the repeatability of the measuring vessel. The uncertainty associated to a unit weighing (u_{add}) can be calculated trough the following equation.

$$u_{add} = \sqrt{2\,(u_{cal.bal})^2 + 2\,(u_{rep.bal})^2} \qquad (32)$$

being $u_{cal.bal}^2$ the uncertainty associated to the calibration/maximum permissible error of the balance, and $u_{rep.bal}^2$ the uncertainty associated to the repeatability of the weight.

According to an internal laboratory procedure (PTG 03 – Measurement Uncertainty), which describes the methodologies/approaches relating to the calculation of the uncertainty measurement, after the balance is tared:

$$u_{cal.bal} = \frac{U_m}{k} \qquad (33)$$

where U_m is the expanded uncertainty of the certificated balance calibration, for a calibration point with the closest tare and mass value, to the tare taken and the mass weighted in the test. k is the expansion factor.

The values of U_m and k, were taken from the **Figure 10.2** from Apendix A2: PTG 03 – Measurement Uncertainty, and are presented in **Table 7.21**.

Table 7.21 – Values for calculating the uncertainty associated to the calibration/maximum permissible error of the balance

U_m (mg)	k	$u_{cal.bal}$ (g)	Weight (g)	$u_{cal.bal}$ relative (%)
0.31	2.01	0.15	99.999792	0.00015

In **Table 7.22** it is possible to see the values obtained for the repeatability test of the balance, and the uncertainty associated to a unit weighing.

Table 7.22 – Repeatability test of the balance to the measuring vessel

n	Mass (g)	Average (g)	SD (g)	CV (%)	u_{add} (%)
1	100.2387	100.24	0.0023	0.0023	0.0033
2	100.2375				
3	100.2373				
4	100.2371				
5	100.2363				
6	100.2362				
7	100.2337				
8	100.2332				
9	100.2327				
10	100.2323				

With these results it is possible to calculate the uncertainty associated to the trueness ($\boldsymbol{u'_{ver}}$) that has the value of 7.44%.

Once the $\bar{\boldsymbol{R}}$ and the $\boldsymbol{u'_{ver}}$ are calculated, under certain condition of matrix and analyte, it is necessary to evaluate how different $\bar{R}$ is from the ideal value ($\bar{\boldsymbol{R}} = \boldsymbol{1}$), which can be made through a t-test. The first step of the significance test is the comparison of $\boldsymbol{t}$, defined by:

$$t = \frac{|1 - \bar{R}|}{u_{ver}} \tag{34}$$

with the $\boldsymbol{t_{crit}}$ value, to a level of significance of 95%. When $\boldsymbol{t \leq t_{crit}}$, $\bar{R}$ is not considered different from 1. When $\boldsymbol{t \geq t_{crit}}$, then $\bar{\boldsymbol{R}}$ is considered significantly different from 1.

In **Table 7.23** are presented the values of $\boldsymbol{t}$ and $\boldsymbol{t_{crit}}$, and the correspondent values used to the calculus.

Table 7.23 – Values of t and t_{crit}

$\bar{R}$	u'_{ver}	t	n	α	t_{crit}
0.915	0.074	1.14	6	0.05	2.57

As $t \leq t_{crit}$, $\bar{R}$ is not considered different from 1, so it is not necessary to make the correction of the recoveries. With this, we can calculate a new value of b_{RMS} from $\bar{R} = 1$ (**Table 7.24**).

Table 7.24 – Values obtained to calculate the uncertainty associated to the trueness to $\bar{R}$=1

	R (%)	$\bar{R}$ (%)	b'_i	$(b'_i)^2$	$\sum_{i=1}^{n}(b'_i)^2$	n	b_{RMS}	b_{RMS} (%)	CV (%)
A003	87.1	100	0.1289	0.0166	0.0708	6	0.1086	10.86	9.11
A003 F									
A004	84.2		0.1584	0.0251					
A004 F									
A005	88.3		0.1167	0.0136					
A005 F									
B003	87.7		0.1230	0.0151					
B003 F									
C006	101.9		0.0192	0.0004					
C006 F									
B008	100.0		0.0003	0.0000					
B008 F									

This study allows to check if the method can be selective or not. Selectivity is the capacity of the method to identify and distinguished a particular analyte in a mixture without interferents. Before the t-test, once the recoveries presented a **CV** and a b_{RMS} inferior to $U/2$, the selectivity of the method can be confirmed.

In order to establish the laboratory's own acceptance criteria, it is needed to calculate the range for the proposed method, through a graphic representation. This graph (control graph) includes a set of lines that are used to known if the process is controlled or not. The central limit (CL) specifies the most likely value (average of the results obtained). The superior and inferior control limits (SCL and ICL) are called action limits, that correspond to the limits established to an interval of confidence of 99%. The superior and inferior warning limits (SWL and IWL) are established to an interval of confidence of 95% and warn when we enter in a danger zone (Augusto et al., 2000).

The lines are calculated by the following equations:

$$\mathrm{CL} = \bar{X} \quad (35)$$

$$SCL = \bar{X} + 3s \quad (36)$$

$$ICL = \bar{X} - 3s \quad (37)$$

$$SWL = \bar{X} + 2s \quad (38)$$

$$IWL = \bar{X} - 2s \quad (39)$$

where $\bar{X}$ is the average of the obtained results and s is the standard deviation of the quantity to be controlled.

In **Table 7.25** are presented the values obtained for the limits of the graphic, such as the average of the obtained results and standard deviation. These limits can also be seen in **Figure 7.5**, as also the recoveries of each sample.

Table 7.25 – Values to build the graphic representation

Average (%)	s	SCL	ICL	CL	SWL	IWL
91.5	7.45	113.9	69.2	91.5	106.4	76.6

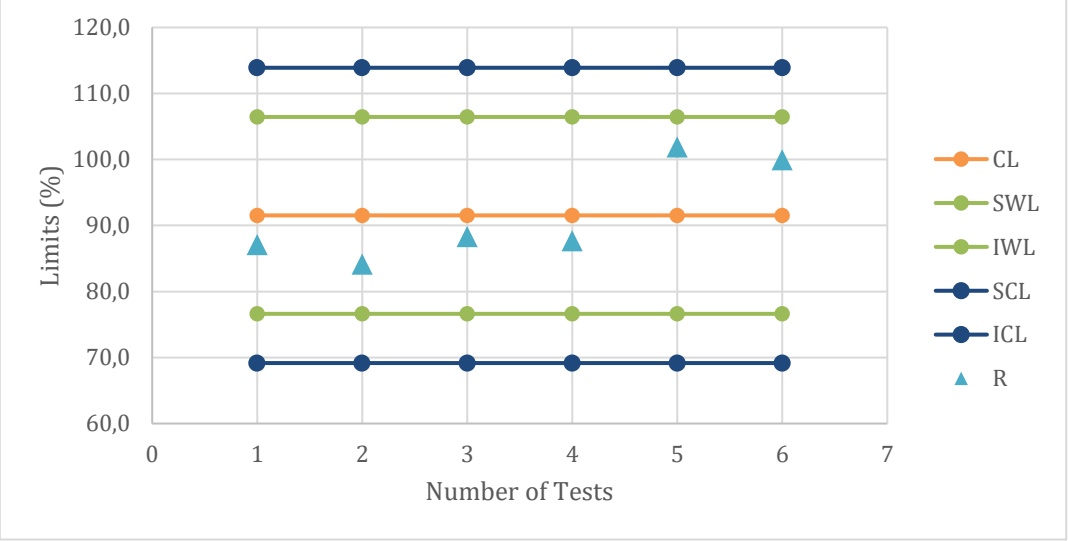


Figure 7.5 – Graphic representation of the limits and the recoveries of each sample

Through the graphic representation, the limits of 80% and 110% can be established, instead of 76.6% and 106.4%. However, to understand the evolution of recoveries, more trials would be needed.

Another way to control the trueness of the method is with certified reference materials (CRM). In this work it was used a reference material (RM) from FAPAS (Fish Paste - T25212QC), more specifically, fish paste. The value of total fat from this RM was 13.5 g/100g. This RM present a range from for $|z| \leq 2$, being the acceptance interval from 12.6 to 14.5 g/100g.

In **Figure 7.6** it can be observed a resume for the study of trueness through RM.

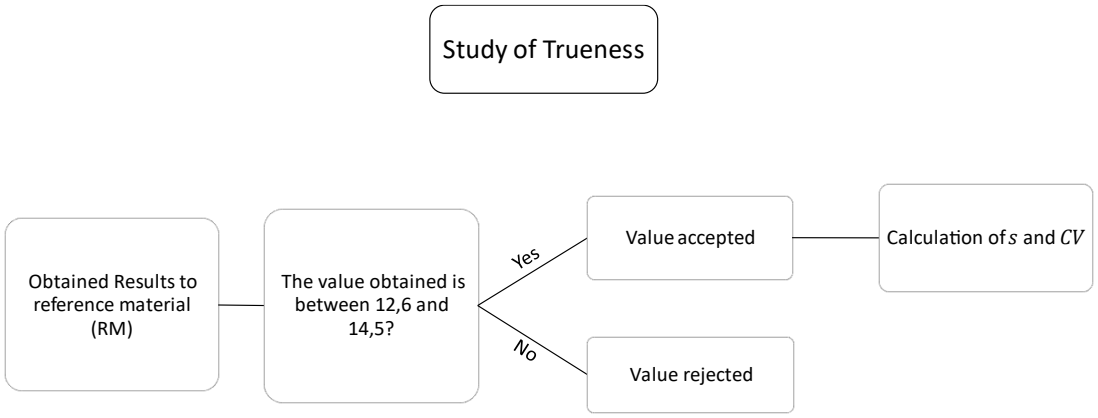


Figure 7.6 – Study of trueness through RM

In each rehearsal, the RM value was determined, being the results presented in **Table 7.26**. If the obtained value is contained in the range specified above, the value is accepted. For these values was calculated the average, standard deviation, and coefficient of variation, also presented on the table.

Table 7.26 – Evaluation of reference material

Mass (g)	Total fat (g/100g)	Evaluation	$\overline{RM}$ (g/100g)	s	CV (%)
1.2028	12.85	Accepted			
1.0947	13.40	Accepted			
1.0444	12.17	Rejected			
1.2543	13.13	Accepted			
1.0300	13.27	Accepted			
1.0325	13.32	Accepted			
1.0842	13.35	Accepted	13.21	0.16	1.21
1.1075	13.35	Accepted			
1.2586	13.07	Accepted			
1.0628	13.31	Accepted			
1.3114	13.24	Accepted			
1.0952	13.15	Accepted			
1.2191	13.06	Accepted			

As referred in the Trueness section, the evaluation of RM results can be made using the relative error, statistical hypothesis testing (t-test), performance factor Z (z-score) and normalized error. However, to determine these parameters, it is needed to know the value of the expanded uncertainty of the method, calculated through the validation of the method.

7.2 Validation Of The Method

7.2.1 Uncertainty Of The Method

The uncertainty of the measurement is related to the individual measurement results. The estimation of the uncertainty for each individual measurement result, is usually not necessary, if the measurement result comes from a regulated measuring method. The set of measurement results acquired using a particular analytical method is typically obtained under controlled conditions. As long as the measurement is done in accordance with a quality assurance program, the estimation of the measurement uncertainty is applicable to all of the measurement results in the set, regardless of the sample matrix or analyst (ISO 11352, 2012).

The procedure for the estimation of the measurement uncertainty consists of the steps shown in **Figure 7.7.**

In general, using appropriate data from the technique validation and the analytical control results, the method and laboratory bias (systematic error) and the within-laboratory reproducibility (random error) can be independently determined.

In this study the combined standard uncertainty ($\boldsymbol{u_c}$) was calculated considering the following cases:

- ✓ Through control standards, duplicates and reference material or recovery tests.
- ✓ Through the reproducibility and repeatability of samples and reference material or recovery tests.

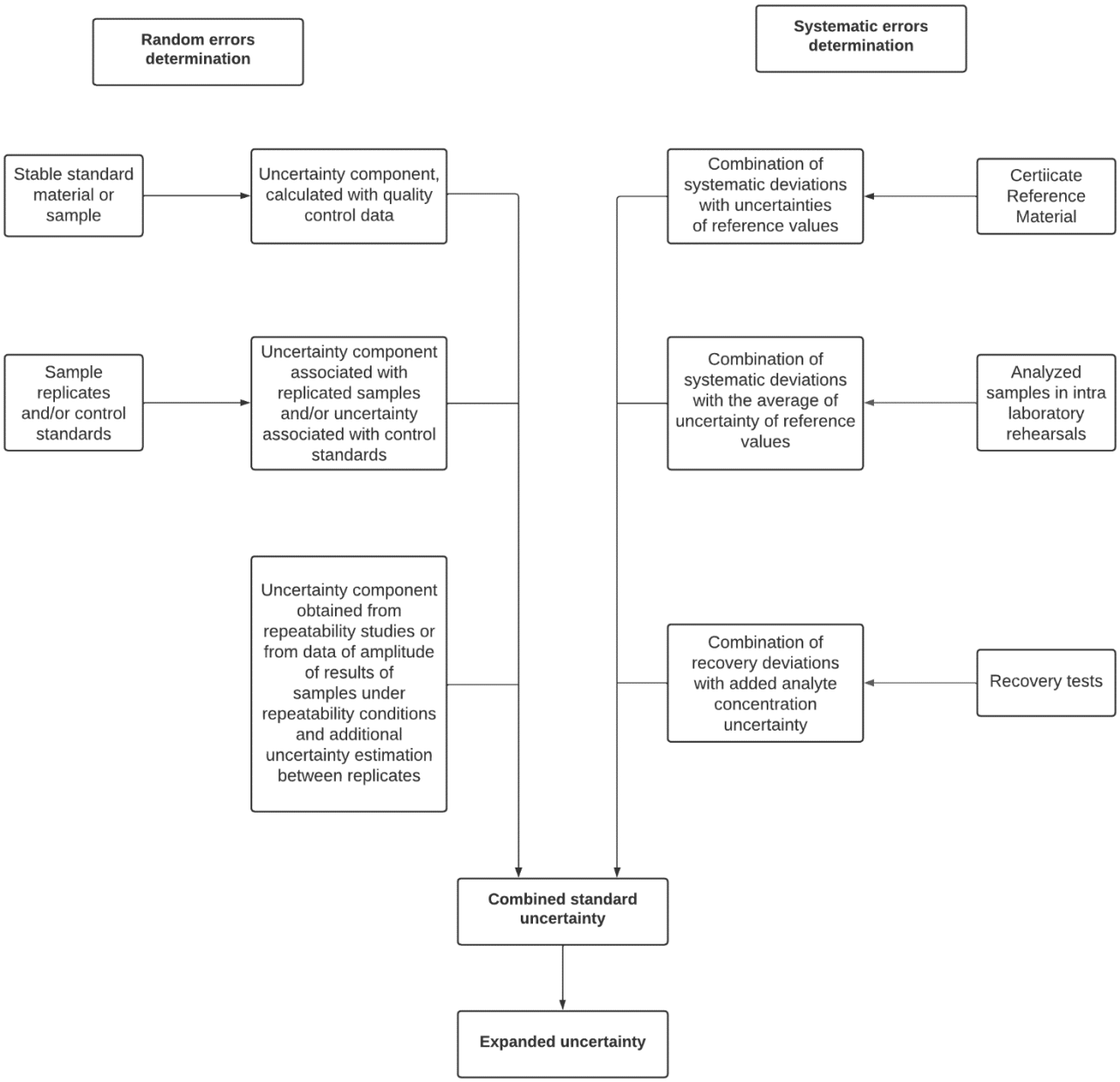


Figure 7.7 – Resume of the main methodologies for calculate standard uncertainties based on the overall performance of the test method (adapted from ISO 11352 (2012))

7.2.2 Uncertainty Trough Standards

The intra-laboratory uncertainty, related to the reproducibility for all the working ranges, is calculated as follows:

$$u_{Rw} = \sqrt{u_{Rw,stand}^2 + u_{r,range}^2} \tag{40}$$

where $\boldsymbol{u_{Rw,stand}}$ is the uncertainty component of the results from the standard solution which is used as quality control sample; $\boldsymbol{u_{r,range}}$ is the uncertainty component from the range control chart.

The standard uncertainty can be calculated from the mean range of a range control chart using the equation below:

$$u_{r,range} = \frac{\bar{R}}{d_2} \tag{41}$$

where $\boldsymbol{\bar{R}}$ is the mean range and $\boldsymbol{d_2}$ is taken from **Table 7.27**.

Table 7.27 – Factors to determine the standard deviation from the mean range (adapted from ISO 11352 (2012))

Number of replicates	Factor $\boldsymbol{d_2}$
2	1.128
3	1.693
4	2.059
5	2.326

In **Table 7.28Table 7.28** the results of $\boldsymbol{u_{Rw}}$ for all the working ranges studied, calculated trough the control standards and sample duplicates, are listed.

Table 7.28 – Values obtained to the intra laboratory reproducibility uncertainty for all the work ranges

		0.1 – 1	**1 – 5**	**5 – 10**	**>10**
Control Standards	$\boldsymbol{u_{Rw.stand}}$ (%)	6.31	6.65	4.29	4.23
Sample Duplicates	$\boldsymbol{u_{r.range}}$ (%)	6.99	7.51	4.60	2.19
	$\boldsymbol{u_{Rw}}$ (%)	9.42	10.03	6.29	4.76

The bias uncertainty component of the method and laboratory, based on the reference material, is calculated by:

$$u'_{ver} = \sqrt{(bias')^2 + \left(\frac{s'_{bias}}{\sqrt{n}}\right)^2 + \left(u'_{Cref}\right)^2} \tag{42}$$

being $\boldsymbol{bias'}$ the relative difference between the average of the experimental values and the reference value; $\boldsymbol{s'_{bias}}$ is the standard deviation associated to the results of RM; $\boldsymbol{n}$ is the number of tests performed on the RM; and $\boldsymbol{u'_{Cref}}$ is the relative standard uncertainty associated with the RM value.

For the calculation of $\boldsymbol{u'_{Cref}}$, it is needed the expanded uncertainty associated with the RM value. As referred previously, the acceptance interval for the value of RM (13.5 g/100g) is from 12.6 to 14.5. Since two values of uncertainty were determined (13.5 ± 0.9 or 13.5 ± 1.0), it was decided to use the lower uncertainty. From Equation (19), the combined uncertainty associated to the RM value is 0.45 g/100g. **Table 7.29** presents the parameters described above, and the respective values used to calculate them.

Table 7.29 – Values obtained to bias uncertainty component of the method and laboratory

$\overline{RM}$ (g/100g)	Real RM (g/100g)	$bias'$ (g/100g)	$bias'$ (%)	s'_{bias} (g/100g)	s'_{bias} (%)	u'_{Cref} (g/100g)	u'_{Cref} (%)	u'_{ver} (g/100g)	u'_{ver} (%)
13.21	13.5	0.29	0.022	0.160	0.012	0.45	0.033	0.040	3.99

Using the intra-laboratory reproducibility uncertainty and bias uncertainty, it is possible to calculate the combined relative uncertainty component:

$$u_c = \sqrt{{u_{Rw}}^2 + {u'_{ver}}^2} \qquad (43)$$

Table 7.30 shows all the values obtained regarding the combined standard uncertainty and the expanded uncertainty of the method.

Table 7.30 – Expanded uncertainty of method obtained through control standards and reference material

		0.1 – 1	**1 – 5**	**5 – 10**	**>10**
Precision	u_{Rw} **(%)**	9.42	10.03	6.29	4.76
Trueness	u'_{ver} **(%)**		3.99		
Combined Standard Uncertainty	u_c **(%)**	10.23	10.80	7.45	6.21
Expanded Uncertainty	U **(%)**	20	22	15	12

The $\boldsymbol{U}$ of the method must be higher than the A.C. Since the A.C was set equal to 15%, only the values of $\boldsymbol{U}$ obtained for the ranges 0.1-1 and 1-5 can be used.

7.2.3 Uncertainty From Recovery Tests

Bias can also be evaluated using recovery tests, which calculate the recovery of a known amount of analyte added to a sample that has already been subjected to analysis. The uncertainty associated with the method and laboratory bias ($\boldsymbol{u_b}$), consists of two components: the difference between observed recoveries of the analyte ($\boldsymbol{b_{rms}}$), and the uncertainty in the concentration of the analyte added ($\boldsymbol{u_{add}}$). The standard uncertainty ($\boldsymbol{u_b}$) estimated from the recovery experiments is given by:

$$u_b = \sqrt{{b_{rms}}^2 + {u_{add}}^2} \tag{44}$$

The root mean square of the deviations from the recovery experiments, $\boldsymbol{b_{rms}}$, is obtained from:

$$b_{rms} = \sqrt{\frac{\sum b_i^2}{n}} \tag{45}$$

where $\boldsymbol{b_i}$ is the deviation from the complete recovery (100%) from the mean recovery, and $\boldsymbol{n}$ is the number of rehearsals.

In **Table 7.31** are presented the values obtained to the parameters used for calculation of uncertainty associated with the method and laboratory bias.

Table 7.31 – Values obtained to calculate uncertainty of the method and laboratory bias

$\overline{\boldsymbol{R}}$ (%)	$\boldsymbol{b_{rms}}$ (%)	$\boldsymbol{u_{add}}$ (%)	$\boldsymbol{u_b}$ (%)
100	10.86	0.0033	10.86

With this, it was calculated the combined standard uncertainty and the expanded uncertainty of the method, being presented in **Table 7.32.**

Table 7.32 – Expanded uncertainty of method obtained through control standards and recovery tests

		0.1 – 1	1 – 5	5 – 10	>10
Precision	u_{Rw} **(%)**	9.42	10.03	6.29	4.76
Trueness	u'_{ver} **(%)**		10.86		
Combined Standard Uncertainty	u_c **(%)**	14.38	14.79	12.55	11.86
Expanded Uncertainty	U **(%)**	29	30	25	24

Since A.C was 15%, we can choose all the values of U obtained.

7.2.4 Uncertainty From Samples

The expanded uncertainty of samples can also be calculated trough the results obtained from the studies of reproducibility and repeatability of samples, shown in **Table 7.18.** With this, in **Table 7.33,** it is possible to observe the expanded uncertainty obtained from the study of the samples' precision, and from the trueness study by means of the RM and recovery tests.

Table 7.33 – Expanded uncertainty of method obtained through samples and RM/recovery tests

			1 – 5	5 – 10	>10
Samples + Reference Material	**Precision**	u_{Rw} **(%)**	9.19	3.60	4.70
	Trueness	u'_{ver} **(%)**		3.99	
	Combined Standard Uncertainty	u_c **(%)**	10.02	5.37	6.16
	Expanded Uncertainty	U **(%)**	20	11	12
Samples + Recovery tests	**Precision**	u_{Rw} **(%)**	9.19	3.60	4.70
	Trueness	u'_{ver} **(%)**		10.86	
	Combined Standard Uncertainty	u_c **(%)**	14.23	11.45	11.84
	Expanded Uncertainty	U **(%)**	28	23	24

All the accepted values of expanded uncertainty obtained, are presented in **Table 7.34**, allowing their comparison and to choose the $\boldsymbol{U}$ of the method.

Table 7.34 – All values obtained to expanded uncertainty of the method

		0.1 – 1	**1 – 5**	**5 – 10**	**>10**
Control standards + RM	$\boldsymbol{U}$ (%)	20	22	-	-
Control standards + Recovery tests		29	30	25	24
Samples + RM		-	20	-	-
Samples + Recovery tests		-	28	23	24

From the results presented in the table above, it can be decided what should be the uncertainty of the method. It is possible to observe that $\boldsymbol{U}$ increases with when the calculation is based on the recovery tests. So, it was decided to use the evaluation of control standard and RM to calculate the $\boldsymbol{U}$, being chosen the value of 22%, since it was the highest value.

With this value of $\boldsymbol{U}$, it was possible to evaluate the results of RM, trough the missing parameters referred in Trueness section. These parameters were calculated using Equations (15), (17) and (18). Remembering that $\boldsymbol{U_{lab}}$ is the expanded uncertainty associated to the estimated value by the laboratory and $\boldsymbol{U_{ref}}$ is the expanded uncertainty associated to the value of reference, $\boldsymbol{U_{lab}}$ can be calculated by the following equation:

$$U_{lab} = \frac{\mathrm{U} \times \overline{RM}}{100} \tag{46}$$

This calculation enables the evaluation of RM, which results are given in **Table 7.35**.

Table 7.35 – Values obtained to relative error, z-score and normalized error to evaluate the RM

Real value (g/100)	$\boldsymbol{\overline{RM}}$ **(g/100g)**	$\boldsymbol{Er}$ **(%)**	**Z**	$\boldsymbol{U_{lab}}$ **(g/100g)**	$\boldsymbol{U_{ref}}$ **(g/100g)**	$\boldsymbol{En}$
13.50	13.21	-2.16	-0.65	2.85	0.90	-0.10

As it can be inferred, since $|Z| \leq 2$, which means that the rehearsal is satisfactory, and, once $|En| \leq 1$, it is possible to confirm that U_{lab} is well estimated. The laboratory can also verify the existence of systematic errors through Equation (16), being the value of $\boldsymbol{t}$ presented in **Table 7.36.** $\boldsymbol{t_{crit}}$ was calculated through the operation "INVT" in Excel, considering a confidence level of 95%.

Table 7.36 – Evaluation of systematic errors

Real value (g/100)	$\overline{\boldsymbol{RM}}$ **(g/100g)**	**n**	**s (g/100g)**	**t**	$\boldsymbol{t_{crit}}$
13.50	13.21	12	0.16	6.30	2.20

Once $|t| \geq t_{crit}$, the existence of a systematic error was statistically evidenced.

8 CONCLUSION

The main goal of this work was to validate the MAE method, for the extraction of total fat in fish. This study demonstrated the advantages that make MAE an excellent alternative to traditional methods such as Soxhlet and Bligh & Dyer extraction, as well as other environmentally friendly technologies. In MAE, 12 samples can be extracted simultaneously in few minutes, resulting in higher sample throughput compared to Soxhlet extraction.

The validation process includes some characteristics parameters that, when combined with statistical techniques, makes the method acceptable. In this work, the method was first implemented and finally validated. The parameters calculated for the implementation of the method were LOQ, LOD, control standards, duplicates and replicates of samples, recovery tests and reference materials. For the validation of the method the expanded uncertainty (***U***), and the precision and trueness of the method (coefficient of variation) is compared with the combined uncertainty (***uc***).

The lowest amount of the analyte that can be quantitatively determined with defined precision under the stated experimental conditions (LOQ), was determined by a set of data with a known concentration, obtaining a value of 0,11 g/100g. The value of LOD were obtained from the LOQ obtaining a value of 0,015 g/100g.

The precision was studied by repeatability and intermediate precision, being calculated the weighted CV (%): for range 1-5 g/100g it was obtained a value of 7.31% and 5.57%; for range 5-10 g/100g a value of 1.94% and 3.03%; for range >10 g/100g a value of 2.81% and 3.77%, respectively.

The trueness of the method was calculated trough recovery tests or reference materials. The CV (%) obtained through recovery tests is 9.11 and trough reference materials is 1.21. Finally, the value of ***U*** was calculated by four different ways for the different ranges: control standards + reference material, control standards + recovery tests, samples + reference material and finally, samples + recovery tests. With this, the chosen value was 22% obtained by control standards + reference material, once it is the lowest value that covers all working ranges.

The validation of the method could be more robust if: certified reference materials were available for each range of work, which can also be considered control standards; further recovery tests had been performed; the fish matrix could have been seen by ranges instead as a whole; and the fish could have been homogenized in the laboratory, before reading. With the study performed, it is concluded that the method was successfully validated, but that there is still room for improvement.

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APENDIX

The attachments will present results or documents used to validate the method.

Atpendix A1: Study of Intermediate Precision from Reproducibility

In this attachment are presented all the results to determine the intermediate precision of reproducibility, obtained in different days.

Table 10.1 – Sample A003

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0329	9.73	0.27			Accepted
1.0262	9.45	0.88			Accepted
1.0136	9.77	0.17			Accepted
1.0347	9.70	0.33			Accepted
1.0147	9.76	0.20			Accepted
1.0971	10.49	1.41			Accepted
1.0198	9.70	0.32			Accepted
1.0534	9.86	0.01			Accepted
1.0756	8.88	2.14			Accepted
1.0731	8.97	1.93			Accepted
1.0360	9.43	0.93	21	2.580	Accepted
1.0128	10.13	0.61			Accepted
1.0373	10.12	0.59			Accepted
1.0320	10.15	0.65			Accepted
1.0492	10.37	1.12			Accepted
1.0780	9.73	0.27			Accepted
1.0171	10.69	1.83			Accepted
1.0425	10.48	1.37			Accepted
1.0287	9.85	0.00			Accepted
1.0315	9.87	0.04			Accepted
1.0350	9.77	0.18			Accepted

Table 10.2 – Sample A004

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0265	12.91	0.12	22	2.603	Accepted
1.0631	12.42	1.29			Accepted
1.0262	13.42	1.58			Accepted
1.0329	12.79	0.24			Accepted
1.0032	12.48	1.12			Accepted
1.0560	12.75	0.35			Accepted
1.0096	12.52	1.02			Accepted
1.0098	12.50	1.07			Accepted
1.0433	12.56	0.90			Accepted
1.0859	12.77	0.30			Accepted
1.0421	12.93	0.16			Accepted
1.0486	12.60	0.78			Accepted
1.0092	12.61	0.77			Accepted
1.0606	12.76	0.33			Accepted
1.0106	12.89	0.06			Accepted
1.0228	13.38	1.46			Accepted
1.0516	13.67	2.30			Accepted
1.1105	12.82	0.14			Accepted
1.0080	12.92	0.13			Accepted
1.0565	13.39	1.48			Accepted
1.1287	13.26	1.13			Accepted
1.0358	12.83	0.11			Accepted

Table 10.3 – Sample A005

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.1431	6.79	0.10			Accepted
1.1075	6.57	1.02			Accepted
1.0560	6.36	2.05			Accepted
1.1205	6.82	0.28			Accepted
1.0071	6.75	0.08			Accepted
1.0425	6.61	0.78			Accepted
1.1158	6.86	0.46			Accepted
1.3017	6.68	0.44			Accepted
1.0481	6.70	0.36			Accepted
1.0644	6.89	0.63	19	2.532	Accepted
1.1137	6.75	0.11			Accepted
1.0495	6.80	0.16			Accepted
1.0291	7.06	1.47			Accepted
1.0160	6.47	1.49			Accepted
1.0074	6.82	0.28			Accepted
1.0184	7.13	1.81			Accepted
1.0387	6.73	0.20			Accepted
1.0569	7.12	1.77			Accepted
1.0349	6.68	0.44			Accepted

Table 10.4 – Sample A006

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
1.0621	7.01	0.68			Accepted
1.0440	6.88	1.11			Accepted
1.0109	6.71	1.70			Accepted
1.0799	7.21	0.01			Accepted
1.0885	7.07	0.46			Accepted
1.0692	7.27	0.18			Accepted
1.0772	6.22	3.33			Rejected
1.0773	7.06	0.52			Accepted
1.0389	7.00	0.71			Accepted
1.0122	7.27	0.19			Accepted
1.0138	7.21	0.01			Accepted
1.0816	7.28	0.23			Accepted
1.0752	7.33	0.41			Accepted
1.0066	6.90	1.05	27	2.698	Accepted
1.0650	7.48	0.90			Accepted
1.0997	7.15	0.19			Accepted
1.0858	7.57	1.20			Accepted
1.0207	7.35	0.47			Accepted
1.0514	7.33	0.39			Accepted
1.0120	7.49	0.93			Accepted
1.0505	7.44	0.78			Accepted
1.0304	7.42	0.69			Accepted
1.0178	7.19	0.09			Accepted
1.0530	7.35	0.48			Accepted
1.0408	7.53	1.06			Accepted
1.0554	7.55	1.13			Accepted
1.0367	7.45	0.81			Accepted

Table 10.5 - Sample B008

Sample Mass (g)	Total Fat (g/100g)	Gexp	n	Gcrit	Evaluation
2.0645	1.17	0.91			Accepted
2.0570	1.10	0.24			Accepted
2.0211	1.07	0.66			Accepted
2.0025	1.06	0.86			Accepted
2.0423	1.14	0.42			Accepted
2.0496	1.27	2.48			Accepted
2.1112	1.11	0.04			Accepted
2.0676	1.10	0.19			Accepted
2.0548	1.23	1.81			Accepted
2.0385	1.10	0.27	19	2.532	Accepted
2.0781	1.10	0.27			Accepted
2.0330	1.10	0.19			Accepted
2.0431	1.06	0.88			Accepted
2.0628	1.11	0.04			Accepted
2.0407	1.12	0.18			Accepted
2.0685	1.00	1.76			Accepted
2.0325	0.94	2.83			Rejected
2.0543	1.05	0.96			Accepted
2.0785	1.15	0.56			Accepted

Apendix A2: PTG 03 – Measurement Uncertainty

CERTIFICADO DE CALIBRAÇÃO

Laboratório de Metrologia

IPAC acreditação M0039 Calibração Disponível em www.ipac.pt

ABIMOTA LEA

Nº CCLD 0936820 DATA DE EDIÇÃO: 2020 / 11 / 24

CLIENTE: AEMITEQ - Associação para a Inovação Tecnológica e Qualidade
Rua Coronel Júlio Veiga Simão, Loreto
3020-053 COIMBRA

EQUIPAMENTO: Designação: Instrumento de pesagem de indicação digital, funcionamento não automático e equilíbrio automático
Marca: RADWAG Modelo: AS 220.X2 Resolução: 0,0001g
Nº de Série: 4685653 Nº de Identificação: EQ2018001 Alcance: 0 - 220g

RASTREABILIDADE: Os resultados apresentados neste certificado estão rastreados a padrões nacionais ou internacionais que realizam as unidades de medição de acordo com o Sistema Internacional de Unidades (SI).

CONDIÇÕES AMBIENTAIS:
Temperatura = (17,2 - 17,1) ºC
Humidade relativa = (62 - 62) %

DOCUMENTO DE REFERÊNCIA:
PCLD35: 2019-01-13

RESULTADOS

Os resultados da calibração encontram-se na página seguinte.

INCERTEZA EXPANDIDA:

"A incerteza expandida apresentada, está expressa pela incerteza-padrão multiplicada pelo factor de expansão k = ki , o qual para uma distribuição t com νef = νeff graus de liberdade efectivos corresponde a uma probabilidade de cobertura de, aproximadamente, 95%. A incerteza foi calculada de acordo com o documento EA-4/02". A estabilidade a longo prazo do equipamento não foi considerada.

DATA DA CALIBRAÇÃO: 2020 / 11 / 24 PÁGINA: 1 / 2

EXECUÇÃO

Telmo Coelho de Sá

RESPONSÁVEL TÉCNICO

Assinado por : **César Valentino Ribeiro Coutinho**
Num. de Identificação: BI13020277
Data: 2020.12.02 11:29:39+00'00'

ASSOCIAÇÃO NACIONAL DAS INDÚSTRIAS DE DUAS RODAS, FERRAGENS, MOBILIÁRIO E AFINS
Rua Ramiro Soares de Miranda, 133 | 3750-866 Borralha - Águeda, Portugal | T. (351) 234 612 640 | www.abimota.org | E. lea@abimota.pt

Os resultados expressos neste relatório referem-se às amostras ensaiadas. Reprodução parcial proibida. O IPAC é signatário do Acordo de Reconhecimento Mútuo da EA e do ILAC para ensaios, calibrações e inspecções

IMDL15-9

Figure 10.1 – Page 1 from PTG 03

ABIMOTA LEA

CERTIFICADO DE CALIBRAÇÃO

Laboratório de Metrologia

Nº CCLD 0936820

DATA DE EDIÇÃO: 2020 / 11 / 24

ENSAIOS PRÉVIOS

Carga [g]	Indicação [g]	Erro de indicação [g]
49,999882	49,9997	-0,0002

Foi efectuado um ensaio prévio à carga indicada na tabela, tendo em seguida sido efectuada a regulação do IP segundo as instruções do fabricante.

ENSAIO DE EXACTIDÃO

Carga [g]	Indicação (Valor Médio) [g]	Erro de Indicação [g]	Incerteza Expandida U [mg]	k_i	v_{ef_i}
0,0100022	0,0100	0,0000	± 0,092	2,02	106
0,1000002	0,1000	0,0000	± 0,082	2,00	> 500
0,999983	1,0000	0,0000	± 0,083	2,00	> 500
9,999977	10,0000	0,0000	± 0,089	2,00	> 500
19,999973	20,0000	0,0000	± 0,10	2,00	> 500
49,999882	49,9999	0,0000	± 0,18	2,01	229
99,999792	99,9999	0,0001	± 0,31	2,01	130
219,99967	219,9999	0,0002	± 0,61	2,02	91

Ensaio com Tara

Tara = 25 g

Carga [g]	Indicação (Valor Médio) [g]	Erro de Indicação [g]	Incerteza Expandida U [mg]	k_i	v_{ef_i}
0,2499853	0,2499	-0,0001	± 0,094	2,00	> 500

Tara = 50 g

Carga [g]	Indicação (Valor Médio) [g]	Erro de Indicação [g]	Incerteza Expandida U [mg]	k_i	v_{ef_i}
0,999983	1,0000	0,0000	± 0,11	2,00	309

ENSAIO DE EXCENTRICIDADE

O valor de excentricidade máxima, calculado como a maior diferença de erros de indicação entre os cantos e o centro por aplicação excêntrica de uma carga de 50 g foi de 0,0001 g.

LOCAL DE REALIZAÇÃO DOS ENSAIOS

Instalações do cliente, na sala de balanças.

ASSOCIAÇÃO NACIONAL DAS INDÚSTRIAS DE DUAS RODAS, FERRAGENS, MOBILIÁRIO E AFINS
Rua Ramiro Soares de Miranda, 133 | 3750-866 Borralha - Águeda, Portugal | T. (351) 234 612 640 | www.abimota.org | E. lea@abimota.pt

Figure 10.2 – Page 2 from PTG 03

ApendixB: Beyond the Internship

In the beginning of the internship, once AEMITEQ has been in constructions, it was not possible to start immediately the theme that was propose for me. With that, I was introduced into quality control techniques, more specifically, ion chromatography, pH and migration tests.

Apendix B1: Quantitative analysis of anions by ion chromatography

Ion chromatography (IC) technique is used in implementation and validation of test method to determination of anions in Water and Liquid Effluents. The anions studied are Fluoride, Chloride, Nitrate and Sulfate. To each anion the best analysis conditions must be found e validated. After these studies, a specific procedure is defined to analyze these anions in a routine sample.

Fluoride in potable water in certain concentrations causes adverse effects including tooth decay (<0.50 mg/L), fluorosis (1.50-5 mg/L), and skeletal fluorosis (5-40 mg/L). The presence of Nitrate (NO_3^-) in potable water is harmful to animal and human health. The presence of excessive-high concentrations of NO_3^- in drinking water may create various diseases, including spontaneous abortions, thyroid disorders, cancer, teratogenesis, mutagenesis, and methemoglobinemia (blue baby syndrome). Other common anions, such as chloride and sulfate, are considered secondary contaminants. High levels of chloride can corrode and weaken metallic piping and fixtures, give a "salty" taste to the drinking water (400 mg/L), damage household appliances, boilers, and, if the water is being used for irrigation, it may inhibit the growth of vegetation. Sulfate in drinking water currently has a secondary maximum contamination level of 250 mg/L, based on aesthetic effects. Sulfate may have a laxative effect that can lead to dehydration and is of special concern for infants. With time, people become acclimated to the sulfate and the symptoms disappear (Varner et al., 2010; Duvva et al., 2021).

The laboratory has accredited the analytes to drinking and wastewater matrices. In these matrices, exists an obligation to comply with legal requirements established in legislation, due to the consequences described above. The following legal requirements are: decree law nº236 from 1998 to drinking water and decree law nº152 from 2017 to wastewater.

Apendix B2: Determination of pH in water (electrometry)

pH is one of the principles parameters to be controlled in wastewater treatment once wastewaters release without treatment in aquatic environmental bring deeply environmental damages (Santos et al., 2017).

In AEMITEQ, the pH determination is made in natural waters, drinking water, process water and wastewater, being the work range 4,01 - 10,01, and it follows the PTE 11.

The control of pH in industrial effluents, for discharge, is establish for state and federal laws. Due to not knowing the trueness of its measures and specially not knowing its traceability to national and/or international standards, it may occur the non-conformity when the discharge of this effluents its allowed (Ferreira Filho & Chui, 2006).

The pH of a sample could be determined in the local with resource of a portable potentiometer. This equipment normally requires a periodic analytical calibration using patterns defined by the manufacturer. The calibration can be evaluated by the analysis of asymmetry pH (zero pH) and the slope (sensitivity), being the acceptance criterion indicated by the manufacturer (RELACRE, 2017).

In order to carry out quality control, it is necessary:

- ✓ A buffer solution with inferior and superior values of expected pH values to samples in the beginning of the work session;
- ✓ One duplicate per session of work;
- ✓ A CRM (if exists) with periodicity appropriate to laboratory routine;
- ✓ Participation in interlaboratory field tests.

The patterns of verification must be analyzed in the local. The buffer solution utilized can be commercial or prepared in the laboratory and in the analytical calibration must be from a different origin than those used in the quality control (RELACRE, 2017).