INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior de Tecnologia e Gestão

# MECHANICAL, CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF OLIVE MILL WASTEWATERS

**Inês de Maria Santos Afonso**

*Dissertation presented to the*
***School of Technology and Management of the***
***Polytechnic Institute of Bragança***
*to obtain a Master of Science Degree in*
***Industrial Engineering (branch of Mechanical Engineering)***

**Bragança**

**2022**

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Supervised by

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*Para a minha mãe*

*“Nolite te Bastardes Carborundorum”*
*Margaret Atwood*

# Resumo

A indústria extrativa de azeite é uma atividade económica relevante nos países mediterrânicos. Contudo, do processo de extração resultam caudais volumétricos consideráveis de um efluente residual líquido, designado por águas-ruças (AG) que, se não for devidamente tratado, representa um grande problema de poluição ambiental. A composição físico-química das AG é muito variável, podendo ser caracterizada por uma alta carga orgânica, pH ácido, cheiro intenso a azeite e uma coloração castanho-escura. A alta complexidade orgânica contribui para a sua resistência à biodegradabilidade, causando efeitos ambientais nocivos. Não existe um tratamento padronizado para este resíduo, principalmente por limitações técnicas e económicas, mas as potencialidades para as AG em diversas áreas têm comprovado que podemos, hipoteticamente, transformar este efluente de um problema ambiental numa possível solução para muitos problemas industriais.

O principal objetivo deste trabalho consiste na caracterização mecânica, química e das propriedades bioativas das águas-ruças geradas durante a produção de azeite em lagares que utilizam métodos de extração tradicionais e contínuos, determinando as principais diferenças nas características das águas-ruças de diferentes lagares e perceber quais as potencialidades que este fluído pode ter na indústria mecânica.

Na caracterização mecânica estudaram-se as propriedades da viscosidade, condutividade térmica, calor específico e molhabilidade. A caracterização química incluiu o teor de água, densidade, composição em ésteres metílicos de ácidos gordos, ácidos gordos livres, índice de acidez, valor de pH, condutividade elétrica, sólidos totais, sólidos suspensos totais, carência química de oxigénio, carência bioquímica de oxigénio e a biodegradabilidade. Os parâmetros de bioatividade analisados foram as propriedades antioxidantes, com os fenóis totais e a captura de radicais DPPH, e as propriedades antimicrobianas foram determinadas pelo método de microdiluição em caldo face a diferentes bactérias Gram-positivo e Gram-negativo.

De um modo geral, a caracterização mecânica demonstrou que as águas-ruças são um fluido não-newtoniano, com uma boa molhabilidade com os materiais testados (alumínio, latão, nylon e aço carbónico). As análises químicas mostram que este fluido tem um alto teor de água, com uma densidade semelhante à mesma, uma baixa taxa de biodegradabilidade, um pH ácido em torno de 4,7 e uma acidez que varia, conforme as amostras analisadas, entre 3,2 a 10,6 %. Os resultados biológicos demonstram que as águas ruças possuem um bom poder redutor, e um bom poder inibitório, especialmente contra os micro-organismos gram-negativos *Klebsiella pneumoniae* e *Proteus mirabilis* e contra os gram-positivos *Enterococcus faecalis e Staphylococcus aureus*.

**Palavras-chave:** águas-ruças; propriedades mecânicas; propriedades físico-químicas, propriedades biológicas.

# Abstract

The olive oil production industry is a relevant economic activity in Mediterranean countries. However, in the process of extraction it is produced a large amount of an effluent called olive mil wastewaters (OMWW), which represents a large environmental problem. The physicochemical composition of the OMWW is very variable, and can be characterized by a high organic load, acidic pH, intense oily smell, and a dark brown colour. The high-level organic complexity contributes to their biodegradability resistance, causing negative environmental effects. There is not a globally standardised treatment for this residue, mainly due to technical and economically limitations, but the potentialities for the OMWW in various areas have proven that we can hypothetically turn this effluent from an environmental problem into a possible solution to many problems.

The main objective of this study is the characterization of the mechanical, chemical, and of the bioactive properties of the wastewaters generated during the olive oil extraction in traditional and 3-phased continuous olive oil mills, analysing the main differences in the characterization of the OMWW from the different mills, and to understand some of the potential applications this fluid may offer in the mechanical industry.

On the mechanical characterization were examined the properties of viscosity, thermal conductivity, specific heat, and wettability. The chemical characterization included water content, density, the composition of free fatty acid methyl esters, free fatty acids, acidity, pH value, electric conductivity, total solids, total suspended solids, chemical oxygen demand, biochemical oxygen demand, and biodegradability. The biological parameters analysed were the antioxidant properties, with total phenols and DPPH radicals' capture, and the antimicrobial properties were determined by the broth microdilution method with Gram-positive and Gram-negative bacteria.

In general, the mechanical characterization showed that the olive mill wastewaters have an hydrophilic behavior with the tested materials (aluminum, brass, nylon, and carbon steel). The chemical analysis concluded that this fluid has a high-water content, with a density similar to the water, a low rate of biodegradability, around 29 %, an acid pH of around 4.7, and an acidity that varies, according to the analyzed samples, between 3, 18 to 10.57%. The biological results obtained were satisfactory, with the OMWW showing good reducing power, and good inhibitory power, especially against the gram-negative microorganisms *Klebsiella pneumoniae* and *Proteus mirabilis*, and against the gram-positive *Enterococcus faecalis e Staphylococcus aureus*.

**Keywords:** olive mill wastewaters; mechanical properties; chemical properties, biological properties.

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# Nomenclature

| | | |
|---|---|---|
| **ATR** | Autothermal Reforming | |
| **ATCC** | American Type Culture Collection | |
| **BOD** | Biological Oxygen Demand | (g of $O_2$/L) |
| **CFU** | Colony forming unit | |
| **COD** | Chemical Oxygen Demand | (g of $O_2$/L) |
| **EC** | Electrical Conductivity | (mS/cm) |
| **FAME** | Fatty Acid Methyl Ester | |
| **FC** | Filtered Continuous | |
| **FFA** | Free Fatty Acids | |
| **FT** | Filtered Traditional | |
| **GC** | Gas Chromatography | |
| **GC-FID** | Gas Chromatography with Flame Ionization Detection | |
| **HTC** | Hydrothermal Carbonization | |
| **$IC_{50}$** | Extract concentration corresponding to 0.5 absorbance | (mg/mL) |
| **KF** | Karl Fischer | |
| **LLE** | Liquid-liquid extraction | |
| **MBC** | Minimal Bactericidal Concentration | (mg/mL) |
| **MF** | Microfiltration | |
| **MFU** | McFarland unit | |
| **MHB** | Mueller Hinton Broth | |
| **MIC** | Minimum Inhibitory Concentration | (mg/mL) |
| **NFC** | Non-filtered Continuous | |
| **NFT** | Non-filtered Traditional | |
| **OMWW** | Olive Mill Wastewaters | |
| **OP** | Olive Pomace | |
| **ROP** | Olive Raw Pomace | |
| **SE-ATR** | Sorption-enhanced Autothermal Reforming | |
| **TS** | Total Solids | |
| **TSS** | Total Suspended Solids | |
| **UF** | Ultrafiltration | |
| **VSS** | Volatile Suspended Solids | |

## Greek Letters

| | | |
|---|---|---|
| $A$ | Area | ($m^2$) |
| $f$ | Frequency | (Hz) |
| $G$ | Conductance | (S) |
| $L$ | Length | (m) |
| $\eta$ | Viscosity | (Pa.s) |
| $\eta_{min}$ | Lower boundary of the shear viscosity | |
| $\eta_{secf}$ | Upper limit of the measurable shear viscosity | |
| $\theta$ | Angle (degrees) | ($^o$) |
| $\sigma$ | Electrical Conductivity | (mS/cm) |
| $\rho$ | Density | ($kg/m^3$) |
| R | Geometry radius | (m) |
| $R$ | Resistance | ($\Omega$) |
| T | Temperature (Celsius) | ($^oC$) |
| $\tau$ | Extra stress tensor | ($N/m^2$) |
| $\tau_{xy}$ | Shear stress | (Pa) |
| $\mathfrak{T}$ | Torque | (N.m) |
| $\mathfrak{T}_{min}$ | Rheometer minimum torque resolution | |
| $y_{sl}$ | Free energy per unit of solid-liquid interfaces | |
| $y_{1g}$ | Free energy per unit of liquid-gas | |
| $y_{sg}$ | Free energy per unit of solid-gas | |
| $\dot{y}$ | Shear rate | (1/s) |
| $\omega$ | Angular velocity | (rad/s) |

# Chapter 1. Introduction

## 1.1. State of the Art

In agriculture in general, during the processing of food products, there are several by-products inherent to this process, which often have no useful use and can even become an environmental problem. As the Mediterranean area is one of the world's largest producers of olive oil, the wastewaters produced are becoming an increasing environmental problem with the growth of worldwide olive oil production, which from 2006 to 2019 has grown from about 2.6 to 2.97 million tons [1,2]. According to the FAO (Food and Agriculture Organization, 2017), 16.8 million ha of harvested area in the Mediterranean basin generated about 95% of the worldwide olive production [2]. In 2021, the main olive oil producing countries were Spain, Italy, Greece, Tunisia, and Portugal, with an annual production of around 30 million $m^3$ of olive mill wastewaters (OMWW) [3]. In Portugal, it is estimated that this value is between 100-350 thousand $m^3$/year [4,5,6].

The quantity of olive oil mill vegetation and washing effluents, or OMWW, generated, and consequently the environmental impact, depends on the method of olive oil extraction used. The traditional cold press method typically generates about 50% of OMWW relative to the initial weight of the olives, while the continuous centrifugation process generates 80–110% of OMWW due to the continuous washing of the olive paste with warm water prior to oil separation from the paste [6]. As mentioned before, the production of olive oil leads to the production of solid and liquid wastes at large amounts [8,9]. The olive raw pomace (ROP), which is the solid fraction, is widely employed in the amendment of soils, animals feeding and bio-energy production [9,10], but the liquid fraction, the OMWW, is poorly exploited, since it is considered economically and environmentally problematic. Due to the presence of phenolic compounds, OMWW have a high organic content and it is difficult to biodegrade. Since these compounds have antioxidant properties, responsible for toxic effects against microorganisms and plants, these waters contain a great power of contamination [4,5,6]. Nowadays, the standard procedure for this fluids is being left in outdoor storage/evaporation lagoons, but during the periods of high precipitations, they could reach water bodies and cause serious modifications to the underground water properties [8]. In addition, they also have acidic pH, high electrical conductivity and the total nitrogen, exchangeable phosphorus and potassium contents figure as “double role” factors, because although these nutrients are used for centuries to increase the agricultural soils fertility, their excessive presence in porous media could modify its overall ionic equilibrium [9,11].

The OMWW characterization isn’t always easy, as it depends on a variety of factors that cannot be controlled, such as climatic conditions, olive cultivars, degree of fruit maturation,

storage time and conditions, and oil extraction procedure [11]. Several studies regarding OMWW's physicochemical characterization are available, but their results are very variable. On the other hand, few information is available about the mechanical characterization of this fluid. About the physicochemical characterization, in 2001, Aktas *et al*. [12] conducted a study on the characterization of olive mill wastewaters and its treatment with lime, having characterized seventeen OMWW's samples. A recent study evaluated the physicochemical quality of three different types of mills (traditional mills, semi-modern, and modern units) in the province of Al-Hoceima, Morocco [13]. In 2008, Amaral *et al*. [11], published a study concerning the microbiological and physicochemical characterization of olive mill wastewaters from a continuous olive mill in north-east region of Portugal. In 2007, Aires *et al*. [14] conducted a study concerning the chemical characterization of OMWW, in which the results were included from the analysis of eight OMWW from mills with a press system and eleven from three phases ones, and then compiling the results obtained with those from other authors. The main characteristics of OMWW obtained by the previous references can be seen in Table 1.

**Table 1.** Main characteristics of traditional and continuous OMWW from different references.

| Ref. | Type of Mill | pH | TS[a] [g/L] | TSS[b] [g/L] | Oil [g/L] | TP[c] [g/L] | EC[d] [mS/cm] | COD[e] (g $O_2$/L) | $BOD_5$[f] (g $O_2$/L) |
|---|---|---|---|---|---|---|---|---|---|
| [12] | Traditional | 4.5±0.3 | 44.4±13.8 | 2.7±1.1 | 6.3±10.1 | 2.5±0.7 | - | 65.7±27.1 | - |
| | Continuous | 4.8±0.3 | 78.2±13.6 | 27.6±5.1 | 12.2±13.3 | 3.8±1.5 | - | 103.4±19.5 | - |
| [13] | Traditional | 4.9 | - | 6.82 | 1.15 | 4.4 | 18.25 | 213.44 | 93 |
| | Continuous | 4.5 | - | 5.94 | 0.38 | 4.2 | 16.22 | 93.6 | 44.3 |
| [11] | Traditional | 5.3 | 9.98 | - | 2.47 | 2.7 | - | 181.38 | 15 |
| | Continuous | 5.7 | 7.11 | - | 9 | 1.47 | - | 178.88 | 10 |
| [15] | Traditional | 4.7-5.7 | 12.90% | - | 0.20% | 1.4-14.3 | - | 42-390 | - |
| | Continuous | 4.6-5.9 | 6.10% | - | 0.60% | 0.4-7.1 | - | 15-189 | - |
| [14] | Traditional | 4.3-4.8 | 0.6-3.8% | - | - | - | 65-128 | - | - |
| | Continuous | 4.6-5.3 | 2.1-7.3% | - | - | 0.04-1.4 | 13-118 | 18-55 | 5.2-13.5 |

[a] Total Solids; [b] Total Suspended Solids; [c] Total Phenols; [d] Electric Conductivity; [e] Chemical Oxygen Demand; [f] Biochemical Oxygen Demand

There have been several studies on the potentials uses for the olive oil mill wastewaters and their treatment, which include biological and thermal processes, advanced oxidation, physicochemical methods or on membrane processes, and usually aim to recover water from OMWW. Concerning the conventional methods, membrane processes allow to separate different species without the use of chemicals or heat and can efficiently produce a polyphenols-rich concentrate. The recovery of polyphenols is very interesting, since they are valuable compounds that can be supplied to cosmetic and pharmaceutical industries [16]. Physicochemical methods include ozonation, coagulation, ultrafiltration, electrochemical oxidation, and biological methods include aerobic or anaerobic processes [7,18,19]. For example, aerobic pre-treatment on anaerobic digestion of olive mill wastewater studies were conducted, concluding that anaerobic digestion of OMWW will only be economically feasible if the waste is pre-treated by aerobic digestion, only if the period of return of investment obtained under these conditions does not exceed 6 years [18]. Another study evaluated the OMWW's suspended solids destabilization to improve membrane process performance, and was able to demonstrate that this pre-treatment proved to be relevant for achieving the complete

elimination of all the suspended solids making the OMWW more suitable for the following microfiltration (MF) and ultrafiltration (UF) processes [19].

As for the potentials, the majority of the studies that have been conducted, had shown a great potential to turn OMWW from a pollutant to an agricultural water source, a bio-fertilizer or even a green fuel. For instance, in 2022, *Tsigkou and Kornaros* [20] studied the development of an high-rate anaerobic thermophilic up-flow packed bed reactor for efficient bioconversion of diluted three-phase olive mill wastewater into methane. Their work consisted of a high-rate thermophilic anaerobic digester development, able to remove the high organic load amounts present on the OMWW, by converting it to biogas. Studies have also been conducted to evaluate the production of green hydrogen through OMWW [21], by a thermodynamic study and an energy analysis, performed on the autothermal reforming (ATR) and sorption-enhanced autothermal reforming (SE-ATR) of olive mill wastewater to produce green hydrogen. Another study had shown the potential to turn OMWW into a biofuel, dealing with the bio-oil production and characterization during the pyrolysis (thermal degradation of an organic substance in the absence of air to produce char, pyrolysis oil, and synthesis gas) of OMWW impregnated on olive pomace (OP) in a pyrolizer pilot unit [22]. The conversion into carbon rich materials for various energetic, environmental, and agricultural applications of wastes like the OMWW by hydrothermal carbonization (HTC) were also investigated, where the assessment of the physicochemical properties of the generated HTC by-products suggested the possible application of the hydrochars for energetic insights while the liquid fraction could be practical for the agricultural field [8]. Another research successfully synthesized carbon-based nanomaterials from olive mill wastewaters, using expedite and simple environmental-friendly procedures. The investigation showed that the resultant as-synthetized aqueous dispersions of carbon nanoparticles exhibit outstanding fluorescence emission properties, which encompass an astonishing quantum yield, relevant features to be used in several current and emerging technological applications, namely in bioimaging and nanomedicine, sensorial analysis, (photo)catalysis and optoelectronics [23].

Future uses, that aim not only the treatment of the OMWW but exploiting the many capabilities that it could and have shown to have, are vast and new studies are always turning up. As their pollutive ability is well known [26,27], environmentally friendly demands are higher than ever, for a less pollutive side but also for a lesser water usage [28,29]. On a mechanical engineering perspective, the industry has the same demands for their used fluids, so it would be an appealing perspective to try to reuse OMWW on an industrial level, turning a “burden” into something cheap, natural and with great potential.

## 1.2. Objectives

This work has as main objective the analysis of the mechanical, physicochemical, and biological properties, considered most relevant for the characterization of the olive mill wastewaters.

It is also intended to understand the differences between olive oil wastewaters derived from a traditional milling and from a three-stages olive oil extraction process. Among these variables, it is also intended to understand if there is any differences, on the various properties studied, considering the degree of filtration of the olive mill wastewaters.

## 1.3. Work's structure

The present dissertation is divided in 5 chapters, as follow:

In the first chapter (**Introduction**), the state of the art is presented, to frame what will be studied and to define the objectives of the work.

In the second chapter (**Theoretical Concepts**), a research and literature review are carried out where the fundamental theoretical concepts related to the work developed in this dissertation are described. The first sub-theme is about Olive Oil and Olive Mill Wastewaters, providing some background on the origin of olive oil in the Trás-os-Monte's region, the main varieties of olive trees in this region and the processes of olive oil extraction, that will provide OMWW with different properties, and that are an object of study in this work. On the second sub-theme, the main theorical concepts relative to the work done during this dissertation are presented, beginning for the mechanical properties, followed by the physical, chemical and biological properties.

In the third chapter (**Materials and Methods**), there is a description of the materials employed and the methods that were used for the preparation and elaboration of the experimental tests.

In the fourth chapter (**Results and Discussion**), all the data obtained from the experimental essays are shown, analysed, and compared.

In the fifth chapter (**Conclusion and Future Work's Proposals**), the main conclusions of this work are presented, as well as some study proposals for future work that may be carried out.

# Chapter 2. Theoretical Concepts

## 2.1. Olive Oil and Olive Mill Wastewaters

In this section a short review on Trás-os-Montes olive oil production methods history is presented, since they are strongly related with the quality and characterization of the olive oil wastewaters studied in this work.

### *2.1.1. Olive Tree*

#### 2.1.1.1. Olive Oil's Origins in Trás-os-Montes

The olive tree belongs to the Oleaceae family and the *Olea* genus, which comprises more than 30 species and subspecies. It is thought that its origins are from Minor Asia, where the oldest traces of its culture can be found, and it was already cultivated in Egypt, for over four thousand years [26].

This tree has been used for the most varied functions, from the honours of a funerary tomb in Sparta, where corpses were covered with olive branches, to the production of oil, which led to commercial exchanges early on; King Solomon, for example, sent oil to Hiram I in exchange for materials to build Temples [27].

Also in Christian culture, this tree has a special meaning, since it announces divine mercy when, in the Bible, the dove returns to Noah's ark with an olive branch in its beak, which, in addition to all the symbolism of peace associated, would also be guarantee of divine blessing, medicine and food.

It was by the hand of the Romans that the olive tree reached the entire Mediterranean basin, whose spiritual and religious components helped its expansion in Galicia and, later, in Minho, where its name was already engraved in Galician toponymy in the VII century and in the XI century, south of the Minho River (Oliveira do Douro). However, the olive tree still did not play a preponderant role in the rural economy at this time [27].

It is, once again, the religious component that will be concerned with the olive tree, and this is because the wax brought by the pilgrims to Santiago de Compostela was not enough to illuminate the Apostle in winter, leading to the Archbishop of Compostela, in 1190, obtaining from D. Afonso VII the donation of a property in order to have oil for the winter months, so that it would be able to illuminate the Apostle [27].

The introduction of the olive grove in Trás-os-Montes was also probably due to religion, since one of the three connections to the "Caminhos de Santiago" passed through High Trás-os--Montes. In the early days of the Portuguese nation, after the expulsion of the Moors, the repopulation of the devastated lands coincided with a period of great intensity on the pilgrimage to Santiago, leading the olive tree to settle permanently along the Douro River (Lamego/Régua). These routes were associated with a set of religious temples, places of

meditation, fortified villages and lodging places that, in addition to all the religious burden that the olive tree held, needed lighting, and saw in the olive tree a culture with an easy adaptation to the climatic and soil conditions of the region, and way of providing commercial development in the area, forming an agricultural triad of wheat-wine-olive oil, leading to an increased spread of olive cultivation around the Caminhos de Santiago [27].

Nowadays, the main olive-growing area of Trás-os-Montes and Alto Douro is in the Terra Quente Transmontana, which includes the councils of Alfândega da Fé, Carrazeda de Ansiães, Macedo de Cavaleiros, Mirandela and Vila Flor, but olive cultivation only happened in this region from the XVI century, spreading irreversibly through the XVII and XVIII centuries [27].

#### 2.1.1.2. Olive Tree

As previously mentioned, the olive tree (*Olea europaea L.)* belongs to the botanical family *Oleaceae*, with 35 species of the genus *Olea*, and it is the only species in the Oleaceae family that has the edible fruit [26].

*O. europaea* is a tree with an annual cycle, where the fruit's germination, the olive, takes place between the months of March and April. After the pollination of the flower, the fruit is fertilized and set, and the hardening of the stone takes place between July and August, ending with the development of the fruit in October. From this month onwards, being the exact time related to each variety, it ripens, and the fruit is ready to be picked. After the harvest, and during the winter state, the olive tree begins a vegetative rest phase [32-35].

In Figure 1, it is possible to observe the explanatory model of the development of the olive tree during the year.

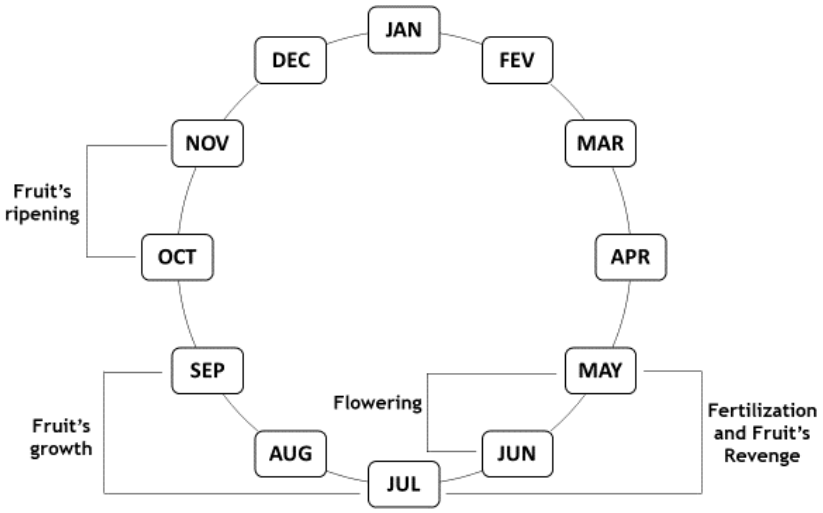



**Figure 1.** Explanatory model of the development of the olive tree during the year.

(Source: adapted from Jorge, R., 2007,[29])

#### 2.1.1.3. Portuguese varieties of olive trees in Trás-os-Montes

The varieties that complete the olive growing universe of Trás-os-Montes are, for the most part, those introduced in the XVI and XVIII centuries, and many adopted regional names, such as *Santulhana*, which owes its name to the parish of *Santulhão*, in the municipality of Vimioso, *Coimbreira*, *Cornalhuda*, *Zambulha*, *Azeitona-Rei*, among others. Other varieties introduced had a more recent origin, such as *Picual* or *Manzanilhas*, and others, the origin of which is unknown, *Maçanal* or *Casta Grande* [27].

In the municipality of Macedo de Cavaleiros, the most grown varieties are: *Cobrançosa*, *Madural* and *Verdeal Transmontana*, and they were also the main varieties used for the olive oils and the olive mill wastewaters which samples were used in this work. Their main characteristics are:

- *Cobrançosa* ("*Quebrançosa*" or "*Salgueira*") – small or medium sized tree with high productivity and medium olive oil yields. It produces sweet and mild oils, with sensory attributes of green apple and almond grass. Very low in oleic acid but average linoleic acid wealth [30].
- *Verdeal Transmontana* – medium-sized tree with regular production, medium-high yields and only used in the production of olive oil. In terms of its sensory attributes, it has a persistent fruity, leafy green and very sharp bitter and spicy flavor. Low in linoleic acid [29], [30];
- *Madural* ("*Negral*") - medium-sized tree with regular production and medium-high yields and only used in the production of olive oil. Very rare and produced only in Trás-os-Montes. Very rich in linoleic acid [30]. In **Figure *2***, it can be seen a photograph of one of the *Madural* olive trees that provided the samples used in these dissertation.

**Figure 2.** *Madural* olive tree in the village of Agrochão.

### *2.1.2. Olive Oil Extraction*

From olive harvesting to olive oil, olives go through a long process. Figure 3 represents schematically the various steps of the olive oil extraction.

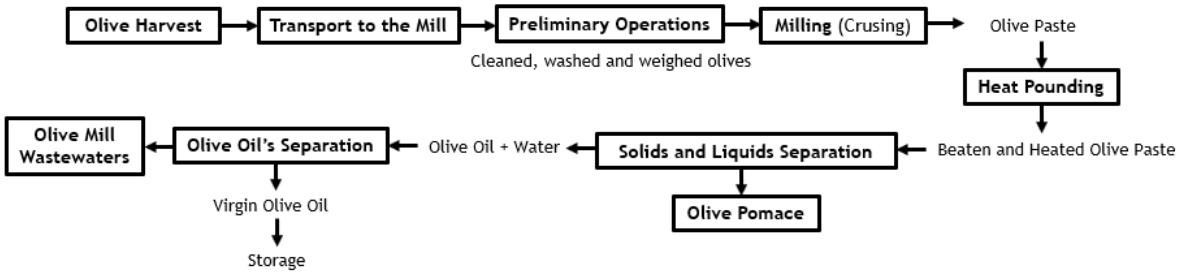



**Figure 3.** Olive oil's process of extracting diagram.

(Source: Adapted from Casa do Azeite, 2021, [31])

#### 2.1.2.1. Olive's Harvest Process

In my grandparents' time, harvesting the olives before January was unthinkable. There are, in fact, several popular sayings that prove this, such as "Whoever harvests oil before Christmas leaves olive oil in the olive grove" or "Whoever harvests the olive before Saint Andrew has the oil on his foot and, before January, it remains the oil on the logger", and Saint Andrew's Day is celebrated on November 30$^{th}$.

Nowadays, given the current market trend, the olive harvest is early, with farmers starting the campaign in November. According to the best standards, the quality of the oil is obtained when part of the fruits is not yet fully ripe. The result is a fruitier oil, with a fresh olive flavour, with a bitter and spicy flavour typical of the cultivars that gave rise to it and without negative odours that penalize it [30,32].

Figure 4 demonstrates the harvesting process of an olive tree, with an olive picking machine, and the green nets around the trees to catch the olives.

**Figure 4.** Harvesting process of a *Cobrançosa* olive tree.

#### 2.1.2.2. Preliminary Operations

To prepare the olives for the procedures that will take place, a set of processes that include sorting, peeling, washing, and weighing is necessary. The first step is to sort the olives, right after the arrival at the mill, according to their degree of maturation, with a selection also being carried out between the olives suitable or unsuitable for olive oil, for having been picked up from the ground or damaged by pests and diseases. Then, defoliation occurs, where leaves and branches are eliminated, as it is illustrated in Figure 5. After this, the washing process occurs, where the fruits go through a flow of water, which causes a separation, eliminating impurities, such as earth and stones, through the floating technique that separates the materials by their density. Finally, the olives are weighed [30,32].

**Figure 5.** Defoliation Process.

#### 2.1.2.3. Milling

The milling process is the crushing of the olives, forming a paste, to facilitate the extraction of the oil. The formed paste becomes thick because it is composed of the elements of the olive, the lump (endocarp) and the pulp (mesocarp), which are crushed through mills of metal or stone structures. An example of a stone crushing milling structure is presented in Figure 6. In this stage, the plant tissues are destroyed, so that the oil globules, coming mostly from the olive pulp, are released, facilitating their agglomeration. The type of mill chosen can interfere with the quality of the product and shows some differences in the composition of the olive oils [32,34,38-40].

**Figure 6.** Stone crushing milling structure.

#### 2.1.2.4. Heat Pounding

The heat pounding, or kneading process, consists of kneading the olive paste so that the oil droplets, which can diffuse into larger drops until forming "bags", can break the oil-water emulsion, a phenomenon called coalescence. This process is essential to increase the oil extraction yield, that is, to facilitate the subsequent step where the oil is separated from the water [29, 33,34].

#### 2.1.2.5. Separation of the Solid and Liquid Phases

When all the olive paste is well crushed, beaten, and heated, the extraction phase follows, which consists of separating olive pomace (solid phase) from the liquid phases (olive oil and vegetation water). The extraction may be carried out by pressing, which is the **Traditional System**, or by centrifugation, which is the **Continuous System** [35], which can have two or three phases. Figure 5 shows a scheme of the different olive oil extraction processes.

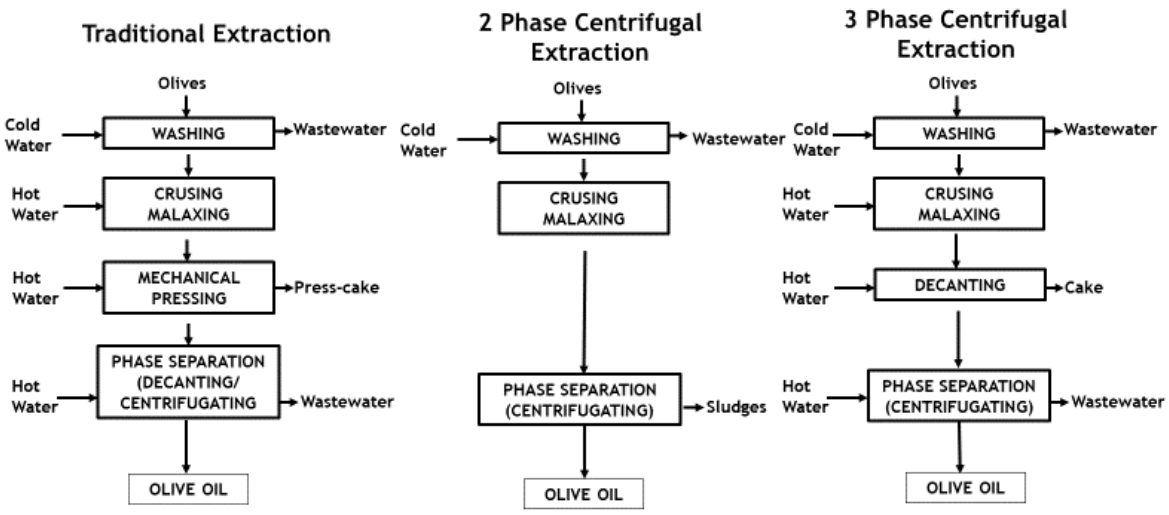



**Figure 7.** Traditional, 2 Phase and 3 Phase olive oil extraction processes.

(Source: Adapted from Zbakh, H. et. Al., [36])

- **Traditional System**

The Traditional System, or Discontinuous System, is considered the oldest and most artisanal procedure for obtaining olive oil. In it, the olive paste previously obtained by milling is deposited on pressing mats (Figure 8) placed on top of the other in the press and then squeezed, forming small and thin layers, so that the pressure exerted on the olives arranged in the pressing mats causes the release of the oily wort (olive oil and water) of the solid phase, which is called the olive pomace, OP [35,37].

**Figure 8.** Pressing by the Traditional System with the use of pressing mats.

- **Continuous System**

The Continuous System of olive oil extraction uses centrifugation for phase separation and are so called continuous because there is no need to stop the separating machine when the crushed olive mass is introduced, and the phases are separated. This system can, in a faster and more efficient way than the Traditional System, proceed the separation of solid and liquid phases, which is done using horizontal centrifuges or "*decanters*" [38].

The Continuous System can have 2 or 3 phases. It is considered a 3-phased system when, at the end of the process, three distinct phases are found, two liquid (one of olive oil and the other of OMWW) and a solid one. The system is considered continuous with 2 phases when, in the end, two phases are obtained, one liquid (olive oil) and the other solid. In this type of system, the wastewaters, OMWW, will be separated from the oil because they are incorporated in the solid phase that corresponds to the wet OP [35,39].

The olive pastes that result from the milling process are not a homogeneous mass, being composed of a mixture of three main components: olive oil (lowest density), OP (the densest component), and raw water (intermediate density). Is this difference in density that allows the three components of the olive mass to be separated by centrifugation, because after the olive paste is introduced into the horizontal type of centrifuge, which is practically a cylindrical or conical shaped drum, different separation forces are produced as the different rotation speed of the centrifuge drum. It is these differences in speed that allow the three components of the crushed olive mass to be separated. The force of lesser intensity will act in the separation of the oil and the one that generates greater intensity allows the separation of the OP, which is heavier. Due to the difference in densities of the components to be separated, and to the different speed inside the centrifuge, the OP will be located closer to the wall. The intermediate layer that will form in this phase of rotation is constituted by OMWW and finally a less dense phase will form, closer to the axis of rotation, which is the olive oil [35[40].

#### 2.1.2.6. Separation of the Liquid Phases

All the liquid obtained in the previous extraction process are a mixture of olive oil with water that the olive has and with water that is added to the process of separating the solid and liquid phases and may also contain suspended solids. It is then necessary to proceed with the separation of the liquid phases, to obtain olive oil, which can be done in two ways: Decanting and Vertical Centrifugation [37,41].

In decanting, which is the traditional way of separating the olive oil from the OMWW, the oily wort is treated and thrown into a tank where the two liquid phases are first separated. What happens is that the oil has a lower density than the OMWW and therefore tends to float, with the OMWW remaining denser at the bottom of the tank. Thus, and because the oil is less dense than the raw water, it leaves through the upper part of the tank, from where it is removed, and goes to another set of tanks where it is left to rest again, so that it can be clarified. The OMWW remains at the bottom of the ponds, being removed from there latter. [35,42]. Figure 9 represents the decantation tanks used for the traditional samples

With the same objective as the decantation tanks, there are the vertical centrifuges, that separates the liquid phases that constitute the oily most, resulted from pressing or centrifugation. This separation is done between 6000-8000 rpm to purify the olive oil. On the 3-Phased system, two vertical centrifuges are used, the first to retrieve some water and raw

particles and the second to recover some olive oil that have stayed on the liquid effluents, the OMWW [29] .

**Figure 9.** Decantation tanks used in the traditional mill.

### *2.1.3. OMMW – Olive Mill Wastewaters*

According to the International Olive Oil Council (*IOC, 1984*), OMWW are the residual aqueous liquid that is obtained from the olive oil processing process and that includes the water present in the olives, the addition and washing water and a variable percentage of solid elements. It is estimated that for every 100 kg of treated olives, 35 kg of solid waste (olive cake) and 55-200 L of liquid waste are produced, depending on the olive oil extraction process. In the Mediterranean countries, the effluents resulting from the olive oil extraction process are around 30 million $m^3$/year, and in Portugal the volume of these same OMWW is around 100.000 to 350.000 $m^3$ [14,42,43].

In aesthetics terms, OMWW show generally dark, reddish-brown coloration, and have an odor very similar to olive oil, but they can also have a very unpleasant odor. This odor difference may be due to a recent extraction or degradation, which comes to present a fetid smell. Figure 10 shows two OMWW samples, one from a Continuous 3-Phased Olive Mill, on the right, with a reddish-brow coloration, and the other from a Traditional one, on the left, with a darker coloration, both submitted to a coffee filter and vacuum filtration, and a sample of olive oil in the middle.

***Figure 10**. Traditional OMWW (left), Olive Oil (middle) and Continuous OMWW (right).*

#### 2.1.3.1. Composition of the Olive Mill Wastewater

The composition of the OMWW is not constant and may vary according to the composition of the vegetation water, that is, the water present in the olives and the one used during the process, the olive oil extraction process or the duration and characteristics of storage. For instance, the composition of vegetation water differs with the variety of olive trees, the ripening stage of the olives, the water content of the fruits, the characteristics of the soil where it is installed the olive grove, the time of harvesting the fruits, the presence of pesticides and the use of fertilizers, the production obtained, and the environmental conditions. Regarding the process of extraction, it is known that the amount of water used in the mill is very variable, not only due to the type of extraction equipment (the centrifugal mill consumes larger amounts of water, about 1 L per kg of olive), but also to the different operational techniques followed by the mill technicians. In other words, the greater the amount of water used in the mill, the lower the concentration of the various components present in the olive mill wastewaters. The composition of the OMWW may also vary on the storage characteristics, which can cause changes in the composition of the OMWW due to aerobic or anaerobic fermentations of various organic compounds, with the consequent emission of volatile substances, increase in acidity or precipitation of suspended solids [14, 42,44].

It can be stated that the most important factor that affects the chemical composition of OMWW is the technology used in the olive oil extraction process, and this happens mostly because in discontinuous processes, the amount of washing water is very restricted or practically nil, while in continuous processes, a little more than 1 L of water is used for each kg of olives. Of all the factors that contribute to the chemical composition of OMWW, the chemical component that appears in greater proportion is water, with organic substances in suspension and/or emulsion and small amounts of inorganic substances. From this, it can be deduced, from the characteristics presented in Tables 1 and 2, that the OMWW will present different physical, chemical, and biological properties, depending on the presence of organic

and inorganic chemical components and the relative concentration of each of the components. Some chemical compounds that can be found in OMWW are presented in Table 2.

**Table 2.** Chemical Components present in OMWW.

(Source: Adapted from Aires, 2007 [14])

| References | [15] | | [45] | [46] | | [14] | |
|---|---|---|---|---|---|---|---|
| Mill Method | Traditional Mill | Continuous Mill | Continuous Mill | Traditional Mill | Continuous Mill | Traditional Mill | Continuous Mill |
| **Properties** | | | | | | | |
| **O.M. [%]** | - | - | 4.7 | 10.5 | 5.5 | 1.4-4.4 | 0.6-2.1 |
| **P [g/L]** | 0.49 | 0.19 | 0.14 | 0.5 | 0.1 | 0.17-0.44 | 0.02-0.22 |
| **K [g/L]** | 2.47 | 0.95 | 3.8 | 3.6 | 1.2 | 1.99-4.98 | 0.08-3.32 |
| **Ca [g/L]** | 0.16 | 0.069 | 0.3 | 0.35 | 0.12 | 0.14-0.22 | 0.02-0.5 |
| **Mg [g/L]** | 0.19 | 0.09 | 0.13 | 0.2 | 0.05 | 0.06-0.12 | 0.02-0.06 |
| **Na [g/L]** | 0.11 | 0.04 | - | 0.15 | 0.05 | - | 0.1-0.3 |
| **Fe [mg/L]** | 33 | 14 | 68 | 50 | 16 | - | - |
| **Mn [mg/L]** | 5.3 | 1.6 | 1.1 | - | - | - | - |
| **Zn [mg/L]** | 3.6 | 2.1 | 4.1 | - | - | - | 0.7-6.2 |
| **Cu [mg/L]** | 3.1 | 1.6 | 1.5 | - | - | - | 0.3-2.0 |
| **Cr [mg/L]** | - | - | - | - | - | - | 0.04-0.3 |
| **Ni [mg/L]** | 0.78 | 0.57 | - | - | - | - | 0.18-2.1 |
| **Cd [mg/L]** | - | - | - | - | - | - | 0.02-0.1 |
| **Pb [mg/L]** | 1.05 | 0.42 | - | - | - | - | 0.3-3.2 |

O.M. - Organic Matter; P - Phosphorus; K – Potassium; Ca – Calcium; Mg – Magnesium; Na – Sodium; Fe – Iron; Mn – Manganese; Zn – Zinc; Cu – Cooper; Cr - Chromium; Ni – Nickel; Cd - Cadmium; Pb – Lead.

By the analysis of the data compiled on Tables 1 and 2, it is confirmed that the OMWW have very heterogeneous compositions, varying on the different references and on the type of mill. Nevertheless, olive mill wastewaters are generally acid, with a dark/reddish brown color, with different dissolved substances and in suspension, with a composition of 83 to 96 % water, 3.5 to 15 % organic matter (fats, sugars, nitrogenous substances, organic acids, polyalcohols, pectens, mucilages, tannins and polyphenols) and 0.2 to 2.0 % of salts (essentially consisting of potassium, sodium, carbonates and phosphates), and have a high contaminating power (BOD 35-110 g of $O_2$/L, COD 40-220 g of $O_2$/L; EC 8-22 dS/m) [14,42].

Of all the chemical components present in the OMWW, the phenolic compounds assume particular importance, since they are very resistant to degradation and have an inhibitory effect, hindering the biological processes of action of microorganisms. The presence of phenolic compounds, of which more than 50 have already been identified, is the responsible for the brownish color of the OMWW and for two of its most important properties: the bactericidal and phytotoxic character [14,43,44].

The phenolic compounds present in the OMWW come from the phenolic glucosides that exist in the pulp and stone of the olive. During the oil extraction process, the crushed olive paste changes color from an initial violet to a final brown, due to the disappearance of all anthocyanins and the formation of a polymer with a high molar mass. The chemical composition of raw water, in phenols, also varies and depends on: **i)** the state of ripeness of the olives (the

more mature the olives, the lower the concentration of phenols); **ii)** the time when the olives are harvested; **iii)** the methods used to extract the oil. Usually, this same chemical composition in phenols varies between 6000 to 17 500 mg/L [14,42].

OMWW hold a high concentration of various organic components. Although their use for fertilizers is well known, the immediate use of OMWW as fertilizer would not be adequate, due to its enormous phytotoxicity, in light of the presence of phenolic acids. The phenolic acids present in the raw water will precisely hinder the growth of crops, reducing or even inhibiting this same growth. Therefore, its treatment or its profitability becomes urgent. Currently, the direct discharge of liquid effluents from mills (water mills), in water courses are not allowed, and some measures must be taken before depositing these waters in the environment [14,43,44].

#### 2.1.3.2. Possible treatments for the OMWW

Effluents from olive oil mills, like the OMWW, generate massive environmental impacts, such as the coloring of natural waters, threat to aquatic life, pollution of the surface and groundwater, changes in soil quality, phytotoxicity and bad odors, turning the need to treat these effluents crucial [39,47,48]. The main characteristics that make the treatment of the OMWW particularly difficult are [49,50]:

- The high organic load, measured by the values of COD and BOD, which is much higher than that observed in most effluents from other agricultural and food industries.
- The seasonal nature of this industry, where the mills usually only work from November to February, no longer than 90 days.
- The number of olive oil mills geographically dispersed most of them small and very irregular in operation.
- The presence of organic compounds that are difficult to degrade by microbial action (phenolic compounds and long-chain fatty acids).
- The high percentage of dissolved salts and suspended solids.

Nowadays the method that is most used for the treatment of OMWW is their storage in ponds, followed by evaporation, where the water present in the liquid phase evaporates during the summer months. Although this is a simple process, some problems are associated, namely due to the inherent risks of infiltration if there is no effective isolation, and the formation of sludge that hinders the elimination and favors the separation/dehydration and not so much the degradation of organic matter, as would be expected [39,51]. The generation of odors, due to anaerobic activity, is other great issue, and the potential risk to the aquifers of the affected area make this method highly inadvisable [39,52]. In addition to the mentioned treatment, other processes have been tested, as mentioned in Table 3. However, all have significant drawbacks, which do not allow the choice of any technology as an autonomous treatment option for OMWW [39].

**Table 3.** Processes for the treatment of olive oil's effluent.

(Source: Adapted from Catalão, 2012, [50])

| Physical Processes | Thermal Processes | Physicochemical Processes | Biological Processes |
|---|---|---|---|
| Dilution | Forced Evaporation | Neutralization | Aerobic Biodegradation |
| Sedimentation | Drying | Flocculation or Precipitation | Anaerobic Biodegradation |
| Flotation | Combustion or Incineration | Adsorption | Aerobic/Anaerobic Biodegradation |
| Centrifugation | Lagoon Evaporation | Chemical Oxidation | Compost |
| Filtration | | Electro-chemical Oxidation | Phytoremediation |
| Reverse Osmosis | | Ion exchange | |
| Micro, Ultra and Nanofiltration | | | |

#### 2.1.3.3. Legal Framework

There is legislation available for industrial effluents in most of the industrialized countries, due to the destructive impact they could cause. In Portugal, the main laws that can be reached are the Law of Water (58/2005, from the 29th of December), Dispatch 118/2000 (from the 3rd of February) and Dispatch 626/2000 (from the 6th of June ), and they basically state the standards for the use of OMMW in the watering of agricultural soils, and the guides that promote the protection of the water resources. There is also legislation available for the environmental counteractions, namely Law n.° 50/2006, from the 29th of August and Dispatch 174/2008. [53].

On the Decreto-Lei n.º 226-A/2007, from the 31 of May, that has is 9th version, its most recent, from 2018, on the Decreto-Lei n.º 97/2018, the olive mill wastewaters are framed with uses of water resources, established the bases for sustainable water management and the institutional framework for the respective sector, based on the principle of the hydrographic region as the main planning and management unit

## 2.2. Mechanical Properties

### *2.2.1. Viscosity*

The viscosity analysis is typically performed on the rotational rheometer, where the fluid sample is confined between a rotating upper cone or plate geometry and a fixed lower plate, and the rotational motion of the upper geometry achieves a simple shear flow. In general, rotational rheometers work under controlled strain or under controlled stress. The most common measurement geometries used are the parallel plates (Figure 11 (b)), the cone-plate (Figure 11 (a)) and the concentric cylinders. In this dissertation, we will focus on cone-plate geometry systems [54].

The cone-plate geometry, schematically shown in the Figure 11 (a), is a common set-up used to characterize the viscosity of non-Newtonian fluids. The truncated apex of the cone is separated from the plate surface by a distance (gap) and the liquid to be tested fills the narrow gap formed by the cone and the plate. Small cone angles are important for accurate normal stress measurements and this arrangement ensures that the shear-rate remains constant everywhere in the sample. On the other hand, the smaller the cone angle, the greater the shear rate for a determined rotational speed. This geometry provides great precision and accuracy and is the most popular system to perform rheological measurements.

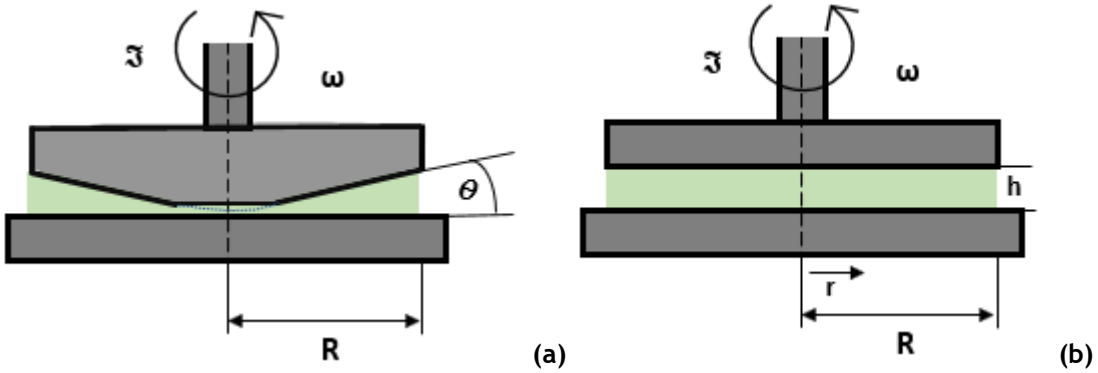



**Figure 11.** Schematic representation of the geometries cone-plate **(a)** and parallel plates **(b)**.

(Source: Adapted from Pinho, D., 2018 [54])

In the cone-plate geometry the shear-rate ($\dot{y}$) is constant throughout the sample and is given by:

$$\dot{y} = \frac{\omega}{\theta}, \tag{1}$$

where $\omega$ is the angular velocity of the rotating cone with angle $\theta$. The shear stress can be calculated from the torque necessary to apply on the cone ($\Im$) by:

$$\tau_{xy} = \eta(\dot{y})\dot{y} = \frac{3\tau}{2\pi R^{3'}} \tag{2}$$

where $\eta$ is the viscosity of the fluid being tested, $\tau$ is the extra stress tensor and $R$ is the geometry radius.

For this geometry system the lower boundary of the shear viscosity was determined using (3) and considering 20 times the minimum resolvable torque specifications of the shear rheometer:

$$\eta_{min} = \frac{3(20\Im_{min})}{2\pi R^3 \dot{y}} \tag{3}$$

where $\eta_{min}$ is the lower boundary of the shear viscosity and $\Im_{min}$ is the minimum torque resolution of the rheometer.

The secondary flow effects limit the upper measurable shear viscosity, where Taylor instabilities could appear due the inertial effects, and is given by

$$\eta_{secf} = \frac{\omega\theta^2\rho R^2}{6}\frac{3\tau}{2\pi R^{3'}} \tag{4}$$

where $\eta_{secf}$ is the upper limit of the measurable shear viscosity $\rho$ is the density of the studied fluid.

### *2.2.2. Wettability*

Wettability is the ability of a liquid to maintain contact with a solid surface, and it is controlled by the balance between the intermolecular interactions of adhesive type (liquid to surface) and cohesive type (liquid to liquid). This analysis is important to better understand the lubricant possible affinity of the OMWW, with working piece material surface, since it evaluates the capacity of a fluid to spread out, penetrate and cover the tool and workpiece [64,65].

Surfaces can be more or less repellent to fluids depending on each material properties. When a drop of a liquid is placed on a surface, tension forces will occur on the air-liquid and solid-liquid interfaces. If the forces between the solid and liquid are adhesives, the drop tend to spread over the surface but, if the forces are cohesive, the drop will acquire a spherical conformation, minimizing the contact with the surface. Thus, the wettability of a material can be classified depending on the angle that a liquid's drop forms when placed on its surface. Hydrophilic surfaces attract fluids, so the angle is below 90º while hydrophobic surfaces repel fluids, so the contact angle is over 90º. In cases where the angle is close to 0º or to 180º, the surfaces are called super hydrophilic or super hydrophobic, respectively. The contact angle ($\theta$) can also be defined mathematically according to Young's equation [66,67],

$$y_{1g}cos\theta = y_{sg} - y_{sl} \tag{5}$$

Where $y_{1g}$, $y_{sg}$ and $y_{sl}$ represent the free energy per unit area of the liquid-gas, solid-gas and solid-liquid interfaces respectively, as the surface free energy represents the amount of work to create a surface. For hydrophilic surfaces, $y_{sg}$ should be greater than $y_{sl}$ while in hydrophobic surfaces it should be smaller, as presented in Figure 12.

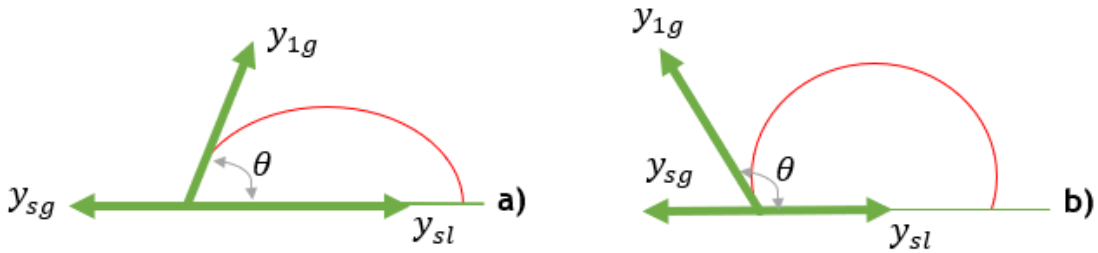



**Figure 12.** Representation of the free energy per unit area of the liquid-gas, solid-gas and solid-liquid interfaces in an **a)** hydrophilic and **b)** hydrophobic surface.

(Source: Adapted from I. Gonçalves, 2019, [57])

## 2.3. Physical Properties

### *2.3.1. Thermal Conductivity and Specific Heat*

Thermal techniques are typically classified under steady state and transient methods. The Hot Disk is an equipment based on Transient Plane Source theory, which was first developed by Gustafsson [58]. At a single measurement, the transient plane source technique has the advantage to simultaneously determine the thermal conductivity, thermal diffusivity, where the hot disk sensor itself, serves as both a heat source and a temperature sensor. The method employs a sensor of electrically conducting nickel, reinforced by layers of insulating Kapton. To carry out the measures, the sensor is placed between two identical samples and a current is applied to the sensor, which generates heat at the same time which the sensor monitors the temperature. Figure 13 represents the scheme of the TPS technique [59].

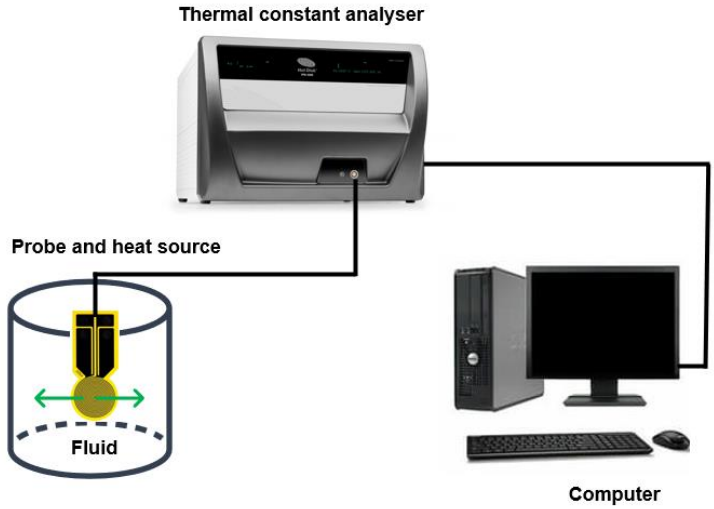



***Figure 13**. Illustration of the transient plane source (TPS) technique, where green arrows represent the direction of the heat flux.*

*(Source: Adapted Souza et. al, 2022* [59]*)*

Between the temperature responses versus the time, it is possible to determine the thermal conductivity of the material by Equations (12) and (13) using the inverse of thermal conductivity 1/k [59]

$$\Delta T(\emptyset) = \frac{Q}{\pi^{1.5} r k} D(\emptyset) \tag{6}$$

$$\emptyset = \sqrt{\frac{t\alpha}{r^2}} \tag{7}$$

.

Where, r is the sensor radius, D (∅) is as dimensionless theorical expression of the time dependent increase describes heat conduction of the sensor, t is the time, k is the thermal conductivity of the liquid (W/(m.K)) and $\alpha$ is the thermal diffusivity ($m^2.s^{-1}$).

### *2.3.2. Water Content*

Several methods are available to determine the water content level in fluids. A Karl Fischer (KF) coulometric titrator is one of the most accurate methods. During a volumetric titration, the content of a substance, such as water, is measured by adding a reactant of known concentration in carefully measured volume amounts until a chemical reaction between reactant and analyte is complete. There are two types of KF titrators: volumetric and coulometric titrators. The main difference between the two is that with the volumetric method, the titrant is added directly to the sample by a burette. Conversely, with the coulometric method, the titrant is generated electrochemically in the titration cell. The coulometric method can measure smaller water contents than the volumetric method [60].

The Karl Fischer titration uses a methanolic solution of iodine, sulphur dioxide and a base as buffer. Several reactions run in the titration of a water-containing sample and can be summarised by the following overall equation [61]:

$$H_2O\ (l) + I_2\ (aq) + [RNH]SO_3CH_3\ (aq) + 2\ RN\ (aq) \Leftrightarrow [RNH]SO_4CH_3\ (aq) + 2\ [RNH]I\ (aq) \qquad (8)$$

In the coulometric KF titration, the iodine needed is generated directly in the electrolyte by electrochemical means ("electronic burette") and the rigorously quantitative relationship between the electric charge and the amount of iodine generated is used for high-precision dispensing of the iodine, and no titer is need be determined. It is only necessary to ensure that the reaction which generates the iodine runs with 100% current efficiency, and that is achievable with the reagents. The titration end point is indicated voltametrically by applying an alternating current of constant strength to a double platinum electrode, which results in a voltage difference between the platinum wires of the indicator electrode which is drastically lowered in the presence of minimal quantities of free iodine. This fact is used to determine the end point of the titration [61].

### *2.3.3. Density*

Density, $\rho$, is a physical property of a substance that is dependent on temperature and pressure, being defined as mass per volume. Due to thermal expansion and compressibility, the density of a substance is influenced by the prevailing temperature and pressure, remaining the mass always constant, and these variables have a greater or smaller effect on the density, depending on whether the substance is a solid or a fluid, having larger influence for fluids than for solids. In order to obtain a precise density indication, the associated temperature and pressure must be known, especially with fluids. If the volume is reduced due to the influence of pressure and/or temperature, while the mass remains constant, the density will increase [62].

There are numerous devices and measuring methods with which the density of a substance can be determined, such as the areometer, pycnometer, buoyancy principle, MEMS chip or the resonator density measurement, which will be the main focus of this, being the technique used on the experiments [62].

With the resonator density measuring method, the density is measured indirectly by a frequency determination, where the liquid to be measured is filled into a tube (resonator) which is set into resonance vibration. The resulting oscillation frequency, which depends on the density of the liquid and the rigidity of the resonator, then provides information on the density. The properties of the resonator depend on temperature and pressure, which are determined based on calibration measurements and compensated for by the measuring instrument. The following equation illustrates the relationship between the density ρ of the liquid, the properties of the resonator (constants A and B) and the oscillation frequency $f$ [63]:

$$\rho = A + \frac{B}{f^2} \tag{9}$$

Basically, the working procedure that resonator measuring device follows starts with the resonator being firmly clamped at both ends, following the exciter which causes the tube to vibrate. Finally, the vibration sensors detect the vibration frequency [63].

### *2.3.1. pH value*

According to the Arrhenius Theory of Ionic Dissociation, a substance is considered acidic if, in an aqueous medium, it releases $H^+$ (or $H_3O^+$) as the only cation. The greater the amount of these ions in the medium, the greater the acidity of the solution. The Danish biochemist Peter Lauritz Sorensen (1868-1939) proposed using a logarithmic scale to work with the concentrations of the hydronium ion [$H_3O^+$ (aq.)] in solutions, which he called pH [64].

The pH is the acronym used for hydrogen potential because it refers to the activity of [$H^+$] (or $H_3O^+$) in a solution. Thus, the pH value serves to tell us if a solution is acidic, neutral or basic. Generally, the pH scale varies between 0 and 14 at a temperature of 25 °C. If the pH value is equal to 7 (water pH), the medium of the solution (or liquid) will be neutral. But if the pH is less than 7, it's acidic, and if it's greater than 7, basic [64].

To measure the pH of solutions in a precise way, an electronic pH meter can be used. The pH measurements can also be performed in a lesser accurate way by acid-base indicators, which are natural or synthetic substances that change color in the presence of acidic and basic solutions and in different pH ranges. Among the most used synthetic indicators is phenolphthalein, which is colorless in acidic medium and very pink in basic medium; litmus paper, which turns red in the presence of acids and blue in the presence of bases; and the universal indicator, which presents different colors for each pH value, being quite accurate. An example of a universal pH indicator used during this work is presented in Figure 14 [64].


***Figure 14** - Universal pH indicator.*

### *2.3.2. Electric Conductivity*

Electrical conductivity (EC), σ, expresses the ability of a substance or medium to conduct electricity. It ranges in value from $10^{-18}$ to $10^{7}$ S/m (Siemen per meter), depending on the material. The EC of normal whole milk is about 0.460 S/m [65], [66].

EC is most easily measured by applying a known DC voltage across a pair of parallel electrodes immersed in the sample, measuring the current produced, and calculating the resistance of the specimen (the volume bounded by the electrodes). Equation (7) shows that EC and electrical conductance (the reciprocal of resistance) are related via the dimensions of the specimen.

$$\sigma = \frac{1}{R}\frac{L}{A} = G\frac{L}{A}(S/m) \tag{10}$$

where *R* is the resistance (Ω), *G* the conductance (S), *L* the distance between the electrodes [m], and *A* the electrode area [$m^2$] [66].

Measurement of the electrical conductivity of various agri-food products is used to determine different parameters of foods, such as the moisture content and germinability of seeds or the resistance of fruits to frost. There have also been studies relating EC with the maturity index in several varieties of olives used for oil production in order to determine the optimal time for harvesting according to two key parameters, i.e., fat efficiency of the fruits and quality of the resulting oil [67].

## 2.4. Chemical Properties

### *2.4.1. Organic Phase Extraction*

In order to extract the organic phase of the samples, a liquid-liquid extraction (LLE), was used. This method, also known as solvent extraction and partitioning, is a common technique used in extracting and purifying analytes for further analysis. Although being an easy method, its main disadvantages are the large amounts of organic solvents used (such as dichloromethane, acetonitrile, or hexane, among others), and being a laborious process, involving longer extraction time [68].

This extraction approach is based on two immiscible solvents, the aqueous solvent, and the organic solvent. The solvent containing the analyte is placed in a funnel (Figure 15-1), and an immiscible solvent is added, forming two layers which are shaken together (Figure 15-2). The analyte then migrates from the initial solvent to the second solvent based on their relative solubility in the solvent (Figure 15-3) [68].

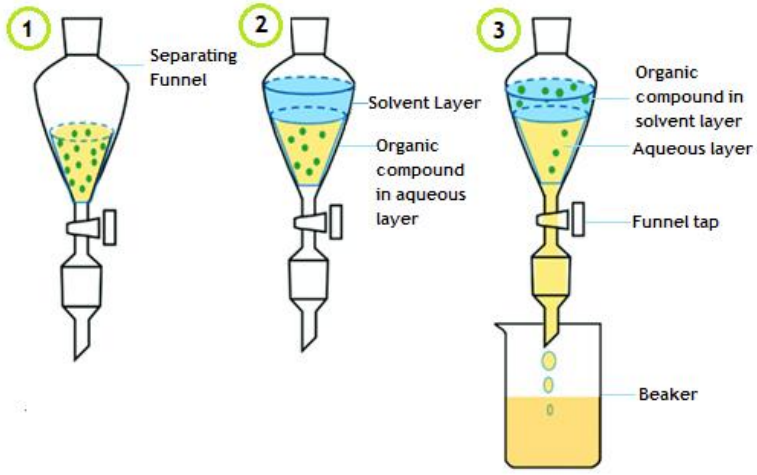



**Figure 15.** Illustration of liquid-liquid extraction.

(Source: Adapted from Targuma, 2021 [68])

When the liquid-liquid extraction is complete, it is then necessary to remove the solvent from the organic compound. In order to do so, it was used a rotary evaporator. A rotary evaporator is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation, and for the preparation of distillates and extracts [69].

### *2.4.2. Esterification of Fatty Acids with $BF_3$*

When it comes to the determination of a complete fatty acid composition of a lipid, the technique of gas chromatography (GC) can make it possible in a faster and more efficient way than no other. However, it is necessary that before the GC analysis, the fatty acid components of lipids are converted to the simplest convenient volatile derivative, usually methyl esters. The preparation of such esters has therefore become, by far, the most common type of chemical reaction for lipid analysts. Fatty acids can be found in nature in the free, unesterified, state, but they are mostly found as esters, linked to glycerol, cholesterol or long-chain aliphatic alcohols, and as amides in sphingolipids [70].

The derivatization procedure of methyl esters of fatty acids by boron trifluoride ($BF_3$) is one of the most commonly used methods for the derivatization of fatty acids. The Lewis acid, boron trifluoride, in the form of its coordination complex with methanol is a powerful acidic catalyst for the esterification of fatty acids. This process consists of the transformation of the triacylglycerols and fatty acids present in the samples into methyl esters, followed by the quantification of compounds between butyric acid methyl ester (C4) and lignoceric or nervonic acid methyl ester (C24) by gas chromatography [71,[72].

### *2.4.3. FAME content determination by gas chromatography*

Fatty acid methyl esters (FAME) are a type of fatty acid ester that are derived by transesterification of fats with methanol. Transesterification is a reversible reaction and is carried out by mixing the reactants, where a strong base or a strong acid can be used as a catalyst, usually sodium or potassium methanolate. In the transesterification process (Figure 16), a glyceride reacts with an alcohol in the presence of a catalyst, forming a mixture of fatty acids esters and an alcohol occurring the following reaction [73,74]:

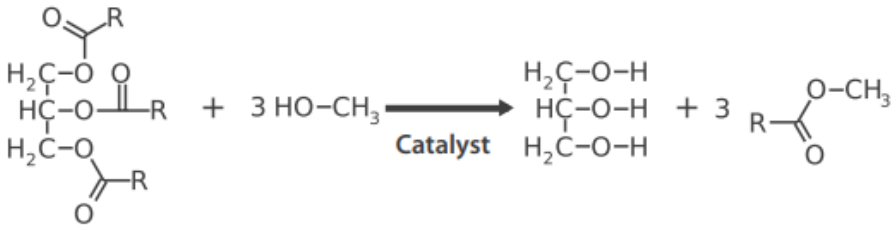



**Figure 16.** Transesterification reaction of FAME using methanol.

(Source: European Biofuels Technology Platform, 2011, [74])

### *2.4.4. Acidity*

Chemically, the components of olive oil can be divided into two fractions, one that is saponifiable (major fraction), which allows the formation of soaps (sodium and/or potassium salts) by hydrolysis in an alkaline medium, and the other, unsaponifiable (minor fraction). The saponifiable fraction constitutes about 97 to 99 % of the total weight of the oil, being mainly composed of triglycerides and a small fraction of di- and mono- glycerol's, phospholipids and free fatty acids, which are responsible for the acidity of olive oils [38].

The oil is naturally generated within the olive fruit forming triglycerides, and each triglyceride is a package made up of three fatty acids linked by a molecule called glycerol. The link between glycerol with the three fatty acids is weak, so before any aggressive or oxidizing atmosphere, it breaks and set free the three fatty acids, and consequently the olive oil starts degrading. Acidity measures how much of these free fatty acids are in the olive oil; therefore acidity is a general indicator of the quality of virgin olive oils. In other words, acidity measures the amount of free fatty oleic acid, the most abundant in the olive oils, and is given in the rate in weight of the free oleic acid over the total amount of olive oil [75].

In terms of acidity, olive oils can be classified as:

- **Extra virgin olive oil:** the acidity should be less than or equal to 0.8%.
- **Virgin olive oil:** the acidity should be less than or equal to 2%.
- "**Lampantes**": All virgin oils with acidity levels over 2%.

### *2.4.5. Determination of the solid content*

The definition of solids refers to suspended or dissolved matter present in samples, often used in the water treatment industry, and includes Total Solids, Total Suspended Solids

and Total Dissolved Solids. The designation of total solids corresponds to the residue resulting from the evaporation of free, occluded water and crystallization of salts, that of suspended solids corresponds to the portion of solids that is retained in a filter of defined porosity and the designation of volatile solids corresponds to the fraction lost by calcination [50,76].

**a) Total Solids**

Total solids (TS) are a measurement often used in the water treatment industry and that refers to matter suspended or dissolved in water or wastewater and is related to both specific conductance and turbidity. Total solids (also referred to as total residue) is the term used for material left in a container after evaporation and drying of a water sample. Total Solids includes both total suspended solids, the portion of total solids retained by a filter (usually with a pore size of 0.45 micrometres), and total dissolved solids, the portion that passes through a filter. This mixture includes any dissolved salts such as sodium chloride (NaCl) and solid particles such as silt and plankton salts such as sodium chloride (NaCl) and solid particles such as silt and plankton [76].

A high total solids level indicates that there is a high level of solid material in the liquid sample. Depending on the evaluation criteria, a high level of total solids could cause the sample to be considered contaminated [77].

**b) Total Suspended Solids**

Total Suspended Solids (TSS) are solids in water that can be trapped by a filter. TSS can include a wide variety of material, such as silt, decaying plant and animal matter, industrial wastes, and sewage. High concentrations of suspended solids can cause many problems for stream health and aquatic life. High TSS in a water body can often mean higher concentrations of bacteria, nutrients, pesticides, and metals in the water [78].

### *2.4.6. Chemical Oxygen Demand*

Chemical oxygen demand (COD) is used to quantify the amount of oxygen equivalent required to oxidize matter susceptible to being oxidized by a strong oxidant, in other words, COD is the amount of dissolved oxygen that must be present in water to oxidize chemical organic materials. COD is used to gauge the short-term impact wastewater effluents will have on the oxygen levels of receiving waters [79].

One of the most common methods for the determination of COD is Closed Reflux Method. This method of reflux with dichromat is preferable at others that use other oxidants because of its larger oxidation capacity applicability to a wide range of samples and ease of handling. This process is based on the fact that most organic compounds are oxidized by a boiling mixture of chromic and sulfuric acid. The sample is refluxed in a strongly acidic solution, with a known amount of potassium dichromate ($K_2Cr_2O_7$), in excess, in the presence of a catalyst ($Ag^+$). After digestion, reduced potassium dichromate is quantified by titration with iron and ammonia sulphate [76]

$$Cr_2O_7^{2-} + 6Fe^{2+} + 14H^+ \rightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \quad (11)$$

and related with the CQO value of the sample

$$2Cr_2O_7^{2-} + 16H^+ \rightarrow Cr^{3+} + 48H_2O + 3O_2 \quad (12)$$

### *2.4.7. Biochemical Oxygen Demand*

The Biochemical Oxygen Demand (BOD) determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters. The test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron. If the test is performed in an airtight bottle of the specified size and incubating it at the specified temperature for 5 days, it is called $BOD_5$ It also may measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5 [76].

### *2.4.8. Biodegradability*

Biodegradability is the capacity for biological degradation of organic materials by living organisms down to the base substances, such as water, carbon dioxide, methane, basic elements and biomass [80]. Evaluating the ability of the OMWW to biodegrade has a vital interest when choosing the method of treatment (biological or physicochemical) on these effluents.

When analysing the COD and BOD values, both pollution parameters, a good biodegradable approach can be reached, since COD represents the organic matter and BOD only the biodegradable fraction. If all the organic matter of OMWW were biodegradable, then their BOD should be equal to their COD value. However, many organic molecules present in the olive mill wastewaters are not biodegradable such as the polyphenolic compounds or have a very slow biodegradability rate or and, in this case, we observe that the BOD values are lesser than the COD values. $BOD_5$/COD allows getting a realistic idea of the biodegradability of an effluent such as OOMW. For industrial effluents, which may contain a significant fraction of non-biodegradable compounds can be considered as the $BOD_5$/ COD report that the ability to biodegradation is not conducive to biological treatment, the following rules are generally used [81]:

- $BOD_5$/ COD >0.5 - effluent easily biodegradable;
- 0.4 < $BOD_5$/ COD <0.5 - effluent average biodegradable;
- 0.2 < $BOD_5$/ COD <0.4 - effluent slowly biodegradable;

- $BOD_5$/ COD <0.2 - effluent non-biodegradable.

### *2.4.9. Phenolic Compounds*

Phenolic compounds can be chemically classified as simple or polyphenols, based on the number of phenol units present on the molecule, and they are substances widely distributed in nature, with more than 8000 already identified in plants. These compounds can be natural pigments, influencing the organoleptic characteristics of foods, or products synthesized by plant secondary metabolism, normally derived from plant defense reactions against environmental aggression and predators. Phenolic compounds are present in the majority of fruits and their identification and quantification provides information on the quality of foods and the potential benefits they can have on health [82,83].

Phenolic compounds can act as natural pesticides, antibiotic, markers for the establishment of symbiosis relationships with microorganisms, attraction of pollinating insects, protection against ultraviolet radiation, in addition to the structural function that gives stability to the plant. These components are considered primary antioxidants present in plants and it is considered to exist a linear relation between the total phenolic content and the antioxidant properties of a particular vegetal species [84,85].

For the total phenols' quantification, there are several methods available, being the colorimetric one of the most used. This method entails the phenols oxidation in basic medium by the yellow Folin-Ciocalteu reagent. From this oxidation reaction, a blue compost is obtained (molybdotungstophosphate), which maximum absorption that is read at 765 nm is directly proportional to the quantitative composition of the phenolic compounds from the analysed sample [86]. The Folin-Ciocalteu method application' requires a calibration curve in which different concentrations of gallic acid dissolved in water or hydroethanolic solvent are used. The results are presented in milligrams equivalent of gallic acid per gram of extract [87-89].

## 2.5. Biological Properties

### *2.5.1. Antioxidant Activity*

An antioxidant is a chemical substance that completely or partially inhibits the oxidation, which is a chemical reaction that can produce free radicals in a chain reaction that can damage the cell of organisms [90].

Antioxidants act at different levels in the protection of organisms. They prevent free radicals' formation by inhibiting chain reactions and chelate metals like iron and copper. They can also intercept/neutralize free radicals generated by cellular metabolism or by exogenous sources, preventing the attack on lipids, amino acids in proteins, the double bond of polyunsaturated fatty acids and DNA bases, preventing the formation of injury and loss of cellular integrity [86].

Nowadays, a growing interest in replacing synthetic antioxidant additives with those derived from natural sources has led researchers to screen plants and agricultural waste for the recovery of phytochemical substances, as they have high potency as a source of antioxidants which can be used for the development of new products [91,92].

Polyphenols are currently among the most important groups of natural antioxidants and frequently exist in plants, either as free molecules or esters/glycosides, and OMWW are a good source of phenolic antioxidants (1-1.8 % w/v) [93]. This happens because most of the phenols, around 90%, in olives are transferred to the water phase during the milling, being Oleuropein, hydroxytyrosol (HTyr), and tyrosol the most abundant phenolic compounds (PCs) in olive mill effluents, of which HTyr is the most bioactive [94],95].

#### 2.5.1.1. Assays to evaluate the Antioxidant Capacity

##### 2.5.1.1.1. Uptake of DPPH radicals

Cell damage of an organism can be due to the oxidative stress caused by increased release of oxygenated free radicals. Antioxidants donate electrons to free radicals to stabilize and neutralize them so it is important to perform antioxidant activity tests in order to detect those with high antioxidant capacity from natural products [96].

The DPPH (2,2-diphenyl-1-picrylhydrazyl) is a relatively simple and fast assay, being one of the most used assays to determine the antioxidant capacity. The DPPH radical (which has a strong violet color), in the presence of antioxidants that donate H atoms, such as phenols, or electrons, is reduced, forming a yellow product (Figure 17), with a consequent decrease in absorbance. When there are compounds in the solution that have antioxidant characteristics, this coloration loses intensity or disappears, so the DPPH radical is converted into pale yellow hydrazine, resulting in a decrease in absorbance between 515 and 528 nm, up to constant values. Its main disadvantage is that the assessment of antioxidant capacity by changes in DPPH absorbance should be carefully evaluated because the DPPH radical absorbance at 517 nm after

reaction with an antioxidant decreases with light, oxygen and the type of solvent used [86], [97,98].

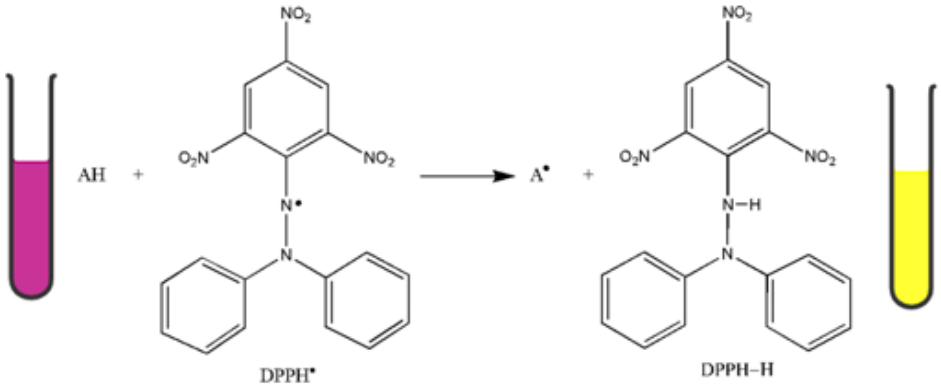



**Figure 17**. Representative scheme of the DPPH radical in the presence of a sample capable of capturing the radical.

(Source: Adapted from Madureira, L., 2020 [86]).

The percentage of antioxidant activity corresponds to the amount of DDPH consumed by the antioxidant, and the amount of antioxidant needed to decrease the initial concentration of DPPH by 50 % is called mean inhibitory concentration($IC_{50}$), that is, the greater the consumption of DPPH by a sample, the lower its $IC_{50}$ and the higher its antioxidant activity [86].

2.5.1.1.2. Reducing Power assay

The reducing power assay is based on the ability that antioxidants have to reduce the colourless ferric complex ($Fe^{3+}$) ($FeCl_3/K_3Fe(CN)_6$) to blue or green-coloured ferrous complex ($Fe^{2+}$) by the action of electron donating antioxidants at low pH, to maintain the solubility of the ferric complex [99].

It is an easy, fast, and reliable assay, that can be applied in a manually, automated, or semi-automated way, and can be measured spectrophotometrically at 690 nm. However, any substance with a redox potential lower than then the pair $Fe^{3+}/Fe^{2+}$ capable of donating electrons can influence the assay. On the other hand, an antioxidant capable of reducing pro-oxidants may be able to reduce $Fe^{3+}$, with compounds that scavenge radicals not being detected. Finally, compounds that absorb at the same wavelength can interfere with the determination, causing overestimation of results [86].

### *2.5.2. Antimicrobial Activity*

There have been several studies about the biological activity of OMWW, namely for its antiviral, antibacterial, antioxidant, anti-inflammatory, cancer-preventive, or even dermatologic properties. Phenolic compounds and their secoiridoid derivatives present in OMWW are proven to inhibit or delay the rate of growth of a wide range of bacteria. This activity was mainly related to the low polarity of some phenolic compounds, which facilitate their transport over the cell membrane, and also associated to the interaction with membrane

lipids, by a neutralisation of the membrane's electric potential following the penetration of the molecule state [100], [101].

To assess the susceptibility of microorganisms to the agent in question, several methods can be used. The agar diffusion method and the agar or liquid medium (broth) dilution methods are the simplest and most used. Dilution methods make it possible to determine the minimum inhibitory concentration (MIC), that is, the lowest concentration of an antimicrobial agent that prevents the growth of a microorganism. The agar diffusion method is the most commonly used for evaluating the antimicrobial resistance profile of isolated bacteria because of its ease of use, low cost, reproducibility, and flexibility in the type and number of samples that can be tested [86].

# Chapter 3. Materials and Methods

The OMWW fresh samples were obtained from mills located in Macedo de Cavaleiros, namely the mill from Lamalonga, for the traditional OMWW and the mill from Arcas, for the 3-phased ones. The samples were tote on carboys and stored in the dark for further analyses.

In the laboratory, the samples were filtrated firstly with coffee filter No. 4, giving their high density, and then through vacuum filtration, with Whatman No. 32 filter paper, with 0.45 mm of porosity, ending with their storage at 4°C. Most of the analysis were done considering these different types of filtrations, that is, samples with just the coffee filter filtration and samples with both the coffee filter and the Whatman filter paper filtration.

For better understanding during this chapter, the filtered samples with coffee filter will be considered single filtered, (1FT for the non-filtered traditional OMWW and 1FC for the NFC non-filtered continuous samples) and the samples filtered with both the coffee filter and the filter paper filtration will be considered double filtered (2FT for the filtered traditional samples and 2FC for the filtered continuous OMWW).

Figure 18 demonstrates the general methodology used for the analysis, since the OMWW were collected from the mills, stored and further filtered or used directly when it was required for some analysis.

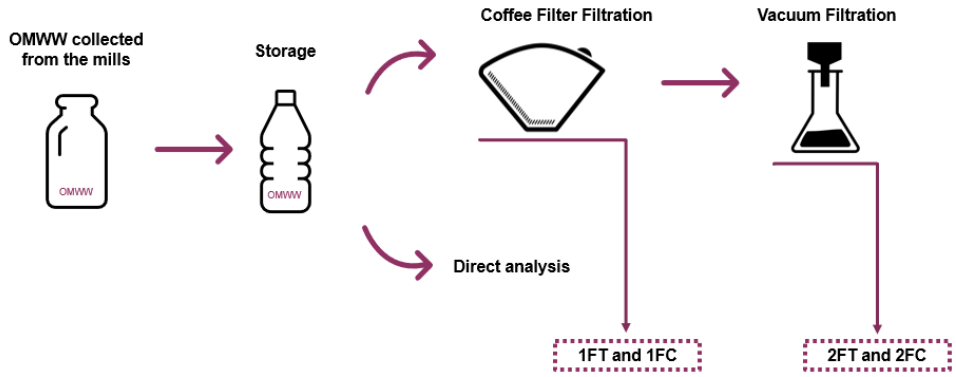



**Figure 18.** General methodology used for the analysis.

Figure 19 shows a summary of all the analysis performed during this dissertation, subdivided into mechanical, physical, chemical, and biological.

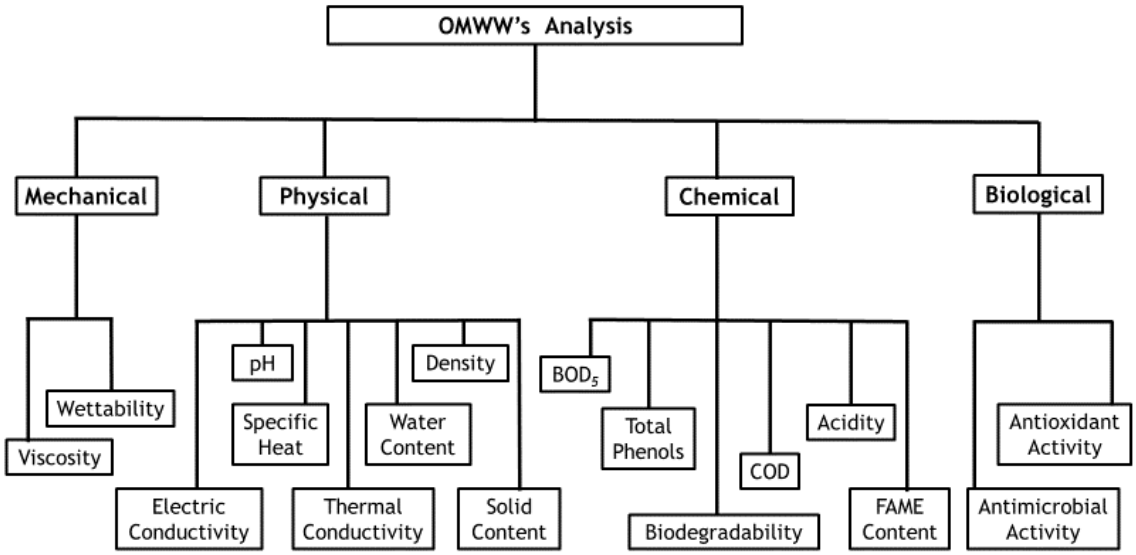



**Figure 19.** OMWW's sample analysis flowsheet.

The main standards and reagents used in this work are described in Table 4.

**Table 4.** Reagents used during the procedures.

| Parameter | Procedure | Reagent/Degree of Purity | Brand |
|---|---|---|---|
| **Physical** | Water Content | Methanol - 99.8% | Fisher |
| | Density | Methanol - 99.8% | Fisher |
| **Chemical** | Organic Phase Extraction | Methanol - 99.8% | Fisher |
| | | n-Hexane - 95% | Fisher |
| | | Anhydrous Sulphate Methanol - 99.6% | Carlo Erba |
| | Esterification of Fatty Acids and FAME | Methanol - 99.8% | Fisher |
| | | Potassium hydroxide - 99.995% | Panreac |
| | | Boron trifluoride - 99.5% | Sigma |
| | | n-Heptane - 99% | Carlo Erba |
| | | Sodium chloride - 0.85% | Panreac |
| | | Anhydrous sulphate - 99% | Carlo Erba |
| | Acidity | Ethanol - 96% | Fisher |
| | | Diethyl ether - 99.8% | HoneyWell |
| | | Potassium hydroxide - 99.995% | Panreac |
| | | Hydrochloric acid - 37% | HoneyWell |
| | | Phenolphthalein | Sigma |
| | Chemical Oxygen Demand | Potassium dichromate - 99.5% | Panreac |
| | | Sulfuric acid - 95% | HoneyWell |
| | | Silver sulphate - 99.8% | Riedel-de-Haen |
| | Biochemical Oxygen Demand | Glutamic Acid - 99% | Sigma |
| | | Glucose - 99.5% | Sigma |
| | | Magnesium sulphate - 99% | Pronolab |
| | | Calcium chloride - 97% | Sigma |
| | | Iron chloride - 97% | Sigma |
| | | Phosphate buffer solution | |
| | | Sodium hydroxide - 25% | Pronolab |
| | Total Phenols | Methanol - 99.8% | Fisher |
| | | Gallic Acid - 97.5% | Sigma |
| | | Folin-Ciocalteu | Sigma |
| | | Sodium Carbonate - 99.5% | HoneyWell |
| **Biological** | Capture of DPPH radicals | DPPH - 90% | Sigma |
| | Reducing Power | Potassium Ferricyanide - 99% | Sigma |
| | | Trichloroacetic Acid - 10% | Sigma |
| | | Iron (III) Chloride Hexahydrate - 0.1% | Sigma |
| | | Sodium phosphate - 0.2 mol/L | |
| | | Buffer solution - 0.2 mol/L | |
| | Antioxidant Analysis | Mueller Hinton Broth (MHB) | VWR |
| | | Iodonitrotetrazolium chloride | Sigma |
| | | Nutrient agar | Cultimed |

## 3.1. Mechanical Parameters

The mechanical parameters determined were viscosity and wettability. Wettability's analyses were performed on four different materials, nylon, aluminium alloy, carbon steel and brass. Figure 20 shows the equipment used for the analysis, as well as the different steps needed.

At least three replicates' measurements for each mechanical parameter were made to corroborate the reproducibility of the measurements.

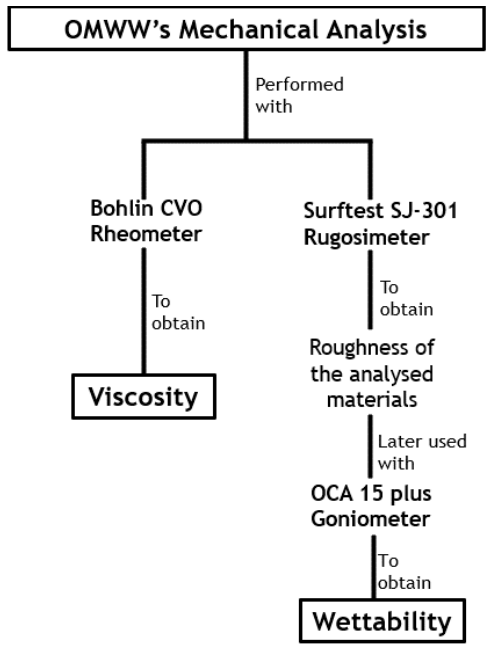



***Figure 20**. OMWW's mechanical analysis diagram.*

### *3.1.1. Viscosity*

The rheological measurements in steady-state and small amplitude oscillatory shear (SAOS) flows were carried by means of a stress-controlled rheometer (Bohlin CVO, Malvern, Worcestershire, UK) , with uncertainties of ± 0.00001 Pa.s, for both olive mill wastewaters, which has a minimum torque resolution of $5 \times 10^{-7}$ Nm. All the tests were performed with a controlled temperature performed by a Peltier heating system, at 20°, 30°, 40°, 50° and 60°C.

After initiating the Peltier heating system and the stress-controlled rheometer Bohlin CVO, a 60 mm diameter cone-plate (4°) geometry with a gap of 30 μm was used. Then, using a micropipette, 30 μm of the OMWW were inserted on the middle of the Peltier plate (Figure 17), initially at 20°C, and then going up until 60°C, providing the steady shear flow curves obtained in a range of shear rates $(\dot{y}/s^{-1})$.

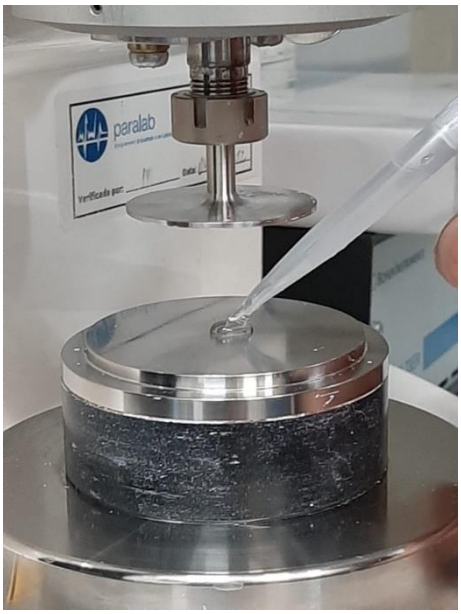



**Figure 21.** Stress-controlled rheometer Bohlin CVO with the samples.

### *3.1.2. Wettability*

Four materials commonly used in machining processes were chosen to better understand how the OMWW behaves in different surfaces, Nylon, Aluminium Alloy (AL6070-T6), Carbon Stell (Ck45) and Brass. Firstly, the materials roughness was measured, regarding the parameter Ra (arithmetic mean value between the peak and valley height values in the effective roughness profile) The values were measured according to the ISO 1997 standard using a portable surface roughness tester (Surftest SJ 301, Mitutoyo, Tokyo, Japan), with uncertainties of ± 0.001 µm. The equipment was used with a standard detector with 10µm of diameter and 83° of opening angle. The roughness values were recorded at five different areas on each material. The measurement length was 0.7 mm and cut off at 0.25 mm for 3 s. The materials surface used as well as the five different areas tested, identified in the red points, and the directions used, seen in the green arrows, is displayed in Figure 22.

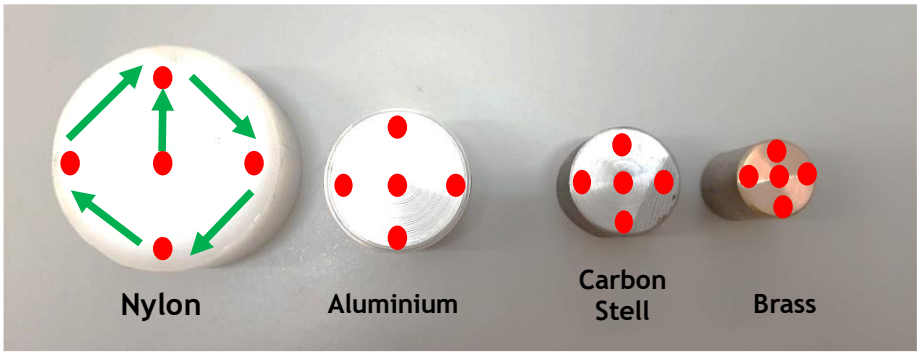



**Figure 22.** Materials surfaces used for wettability analysis.

Later, the wettability's analysis was performed in the Mechanical Engineering Department (MEtRICs) of the University of Minho, using the sessile drop method (2 µl fluid droplet) using a video-based drop shape analyser. The contact angles were measured using an optical tensiometer (OCA 15 plus, Dataphysics, Germany), with uncertainties of ± 0.01°. The droplet profile was analysed using the corresponding software (SCA 20, Dataphysics, Germany). The wetting behaviour was evaluated when the drop of OMWW was placed on the different materials and after 15 seconds, to better understand the differences in means of behaviour and equilibrium of the samples, on the tested material.

The measurements were carried out on each one of the two samples.

## 3.2. Physical Parameters

The physical parameters determined were thermal conductivity, specific heat, water content, density, pH and electric conductivity. Figure 23 demonstrates the equipment used for the analysis, as well as the different steps needed.

At least three replicates' measurements for each mechanical parameter were made to corroborate the reproducibility of the measurements.

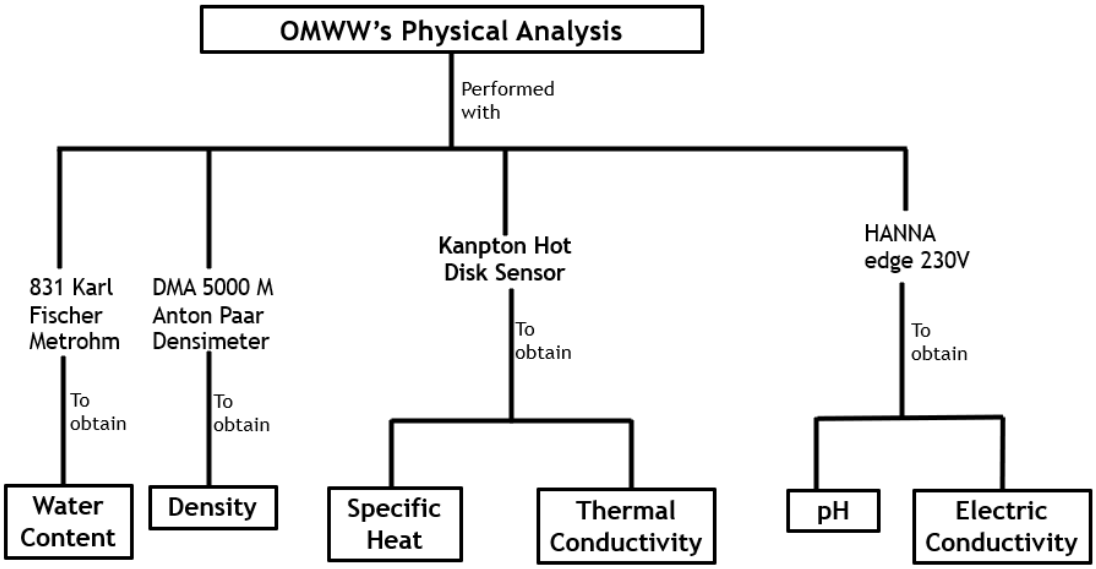



**Figure 23.** OMWW's physical analysis flowsheet.

### *3.2.1. Thermal Conductivity and Specific Heat*

As shown in the theoretical section above, the thermal properties of a material can be measured with this method using a hot disk sensor. For this analysis, it was used a Kanpton Hot Disk sensor C5465 (inserted on the beaker), with a 3.2 mm radius, recommended for liquids, and uncertainties of ± 0.0001 W/mK and ± 0.0001 MJ/m$^3$K. In the specifics introduced on the computer, it was used a 10 mm test piece, with an isotropic material, with an analysis for 3 seconds and a heating power of 25 mW. The analysis was repeated for 20°, 25°, 30°, 35°, 40° and 45 °C.

In Figure 24, it can be seen the apparatus for the measurement of the Thermal Conductivity and the Specific Heat, where it was also added extruded polystyrene and tape for better isolation.

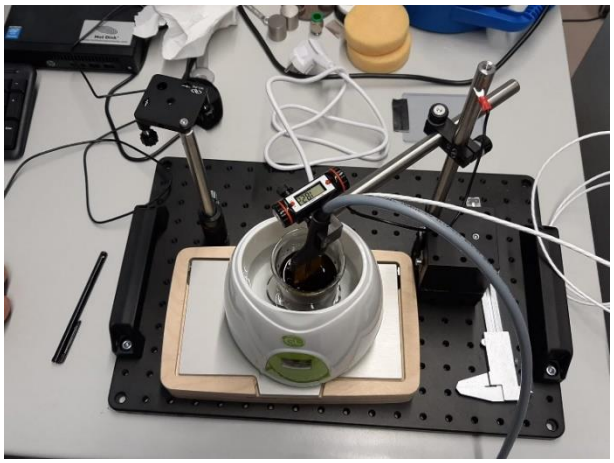


**Figure 24.** Apparatus for the measurement of the Thermal Conductivity and the Specific Heat.

### *3.2.1. Water Content*

The water content was determined by Karl-Fisher titration, which was taken into consideration in the preparation of the solutions and using a Metrohm 831 Karl Fischer coulometer (Figure 25) with Hydranal Coulomat AG, from Riedel-de Haen.

The dilutions were prepared using a 25 mL volumetric flask, adding 250 µL of the sample and prefilled with methanol as solvent. After the calibration of the scale, four drops of each sample were introduced on the septum of the coulometer, with uncertainties of ± 0.001 g/g.

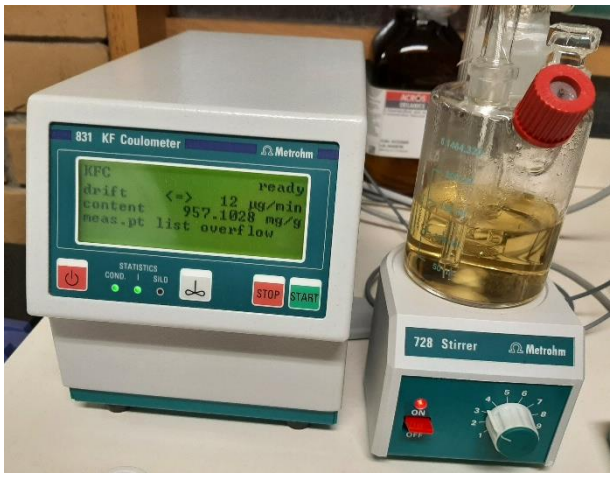



**Figure 25.** *Metrohm 831 Karl Fischer coulometer used in the water content analysis.*

### *3.2.2. Density Measurements*

The density measurements of the samples were carried out using a vibrating tube densimeter (DMA 5000 M, Anton Paar) coupled to a U-shaped tube (Figure 26), with uncertainties of $\pm 5 \times 10^{-5}$ g/cm$^{-3}$. The densities were measured from 293.15 K and 298.15 K at 0.1 MPa. All the measurements were preceded by a calibration step with ultra-pure water and air.

The solvent used to prepare the solutions was methanol.


**Figure 26.** Anton Paar densimeter used in the density analysis.

### *3.2.3. pH value*

The pH measurements of the aqueous solutions were carried out using a multiparameter meter (HANNA edge, 230V), with uncertainties of ± 0.01 (Figure 27). The pH values were measured between 23.7 °C and 24.4 °C. All the measurements were preceded by a calibration step with buffer solutions with pH values of 4.0, 7.0 and 10.0. After each measurement, the electrode was passed through deionized water for cleaning.


**Figure 27.** HANNA multiparameter meter used for the pH and electric conductivity analysis.

### *3.2.4. Electric Conductivity*

The electric conductivity measurements of the samples were carried out using a similar procedure and the same equipment used for pH value measurement (Figure 27). The multiparameter meter (HANNA edge, 230V) has uncertainties within ± 1 $mS/cm^2$ and the measurements were performed from 14.2 °C to 15.9 °C. All the measurements were also preceded by a calibration step with a buffer solution with a pH of 4.0, 7.0 and 10.0.

## 3.3. Chemical Parameters

The chemical parameters determined were fatty acids methyl esters (FAME) content, acidity, total solids (TS), total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD), biodegradability and total phenols. Figure 28 shows the equipment used for the analysis, as well as the different steps needed.

At least three replicates' measurements for each chemical parameter were made to corroborate the reproducibility of the measurements.

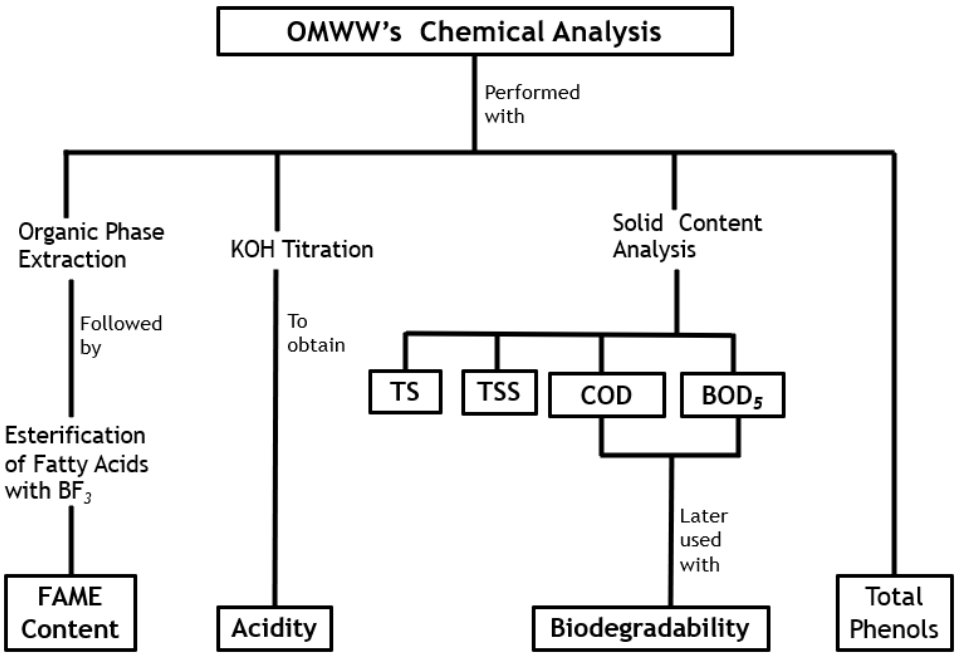



***Figure 28** - OMWW's chemical analysis diagram.*

### *3.3.1. Organic Phase Extraction*

The organic phase extraction of the OMWW samples was performed by a liquid-liquid extraction. Using a 250 mL separation funnel, 150 mL of sample was added to 50 mL of hexane. At the bottom of the separation funnel, a 50 mL flask was left for any residue that could leak out. Finally, the mixture was shaken and left for one week. After that, the stopper was carefully opened, and from the bottom of the funnel, the aqueous phase was removed. The upper (organic) phase was collected to a glass vial and weighted.

The removal of the remaining solvent from the sample, was done by evaporation, using a rotation evaporator, (Buchi R-114 Rotary Vap System) at 40 °C with a rotary speed of 40 rpm. The pressure of the rotary evaporator was controlled by a water circulation vacuum pump (SHZ-III, Shanghai Yarong Biochemical Instrument Factory, China), and a stable pressure was kept at 335 mbar. The dried sample was stored at 4 °C after the rotary evaporation process.

### *3.3.2. Esterification of fatty acids using $BF_3$*

The derivatization procedure of methyl esters of fatty acids using boron trifluoride ($BF_3$) was performed to study the distribution of the fatty acids present in the olive mill wastewaters samples, as well as the olive oil samples that originated them, and also to decrease the potential damage to the chromatographic column and/or instrument.

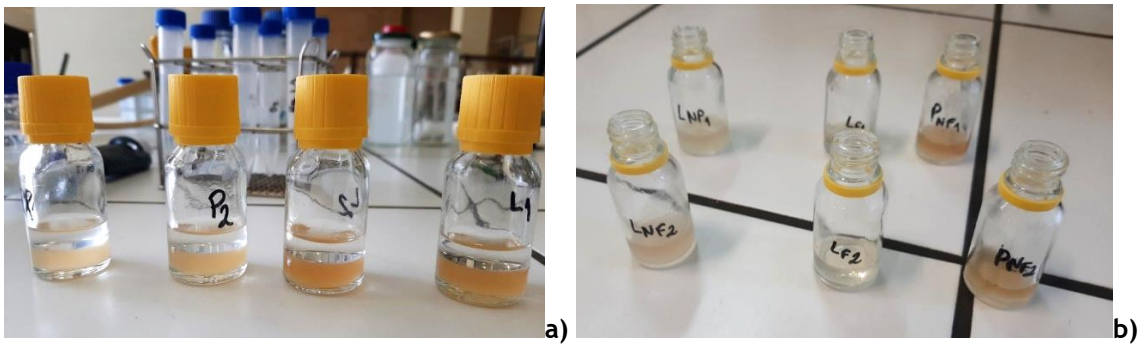
For the derivatization procedure, the first step was to prepare the necessary solutions of methanolic KOH and methyl heptadecanoate used as internal standard. For the methanolic KOH solution, KOH was added in methanol in order to achieve a 0.5 mol/L concentration.

In the second step, 25 mg of the samples of olive oils and olive mill wastewaters and 2.5 mL of the methanolic solution of KOH previously prepared were added to 20 mL glass flasks. Next, the flasks were submitted to a drying process in an oven, previously heated to 90 °C, for 10 min, and, thereafter, they were removed from the oven and waited to cool to room temperature, for approximately 5 min.

In the third step, 2 mL of $BF_3$ in methanol solution (10%, v/v) were added to the flasks, closed, and again taken to the oven at 90 °C, for more 30 min. Once again, when removed, the flasks were left to cool at room temperature, for about 5 min (Figure 29).

In the next step, 12 mL of methyl heptadecanoate solution were added to the solution and agitated, using a vortex apparatus. Next, 2 mL of saturated NaCl (sodium chloride) solution were added and taken to agitation using the vortex once again. The samples were then centrifuged at 3000 rpm for 5 min, to obtain the separation of the two phases.

Finally, 2 mL of the upper phase were transferred to 4 mL flasks and, using a micro spatula, a small amount of anhydrous sodium sulfate was added, in order to remove all moisture present, before gas chromatography analysis was performed.




**a)** **b)**

**Figure 29.** Olive Oil Samples **a)** and OMWW **b)** after the Esterification of Fatty Acids with $BF_3$.

### *3.3.3. FAME content by gas chromatography*

The FAME content of the olive oil and the olive mill wastewaters samples was characterized by gas chromatography (GC), according to the European Standard EN14103/2003 [102].

For the preparation of the olive oil samples, 25 mg of the samples prepared as described in section **3.2.2** and transferred to 15 mL flasks. Then, 3 mL of methyl heptadecanoate solution, prepared in heptane with a concentration of 10 mg/mL was added to samples and used as internal standard. A small amount of anhydrous sodium sulfate was added to remove any remaining moisture in the samples. Then, the solution was agitated and left to stand for at least 1 min, and a sample volume of 1 mL was transferred to a 2 mL glass vial to perform the GC analysis. The preparation of the OMWW samples were similar. Blank samples were processed through the above procedures along with samples [71].

The operating conditions used for the GC analysis were based on a helium flowrate of 1 mL/min, an oven temperature program which started with a temperature of 50 °C, maintained for 1 min and followed by an increase in temperature up to 200 °C at a rate of 25 °C/min. Then, it was once again increased to 230 °C with a heating rate of 3 °C/min for 3 min. The final temperature was maintained for 23 min, for a total running time of 40 min. The injector was operated at 250 °C. The injector was used in split mode, with a split ratio of 1:100, the detector temperature was 250 °C and the injected sample volume was 1 µL.

For the identification of each methyl ester present in the sample, a comparison was performed with the retention time of the FAME compound mixture analysis obtained in this work with the GC Shimadzu system under the operational conditions mentioned above. The chromatogram obtained from the 37 FAME compound mixture in this work is presented in Figure 30.

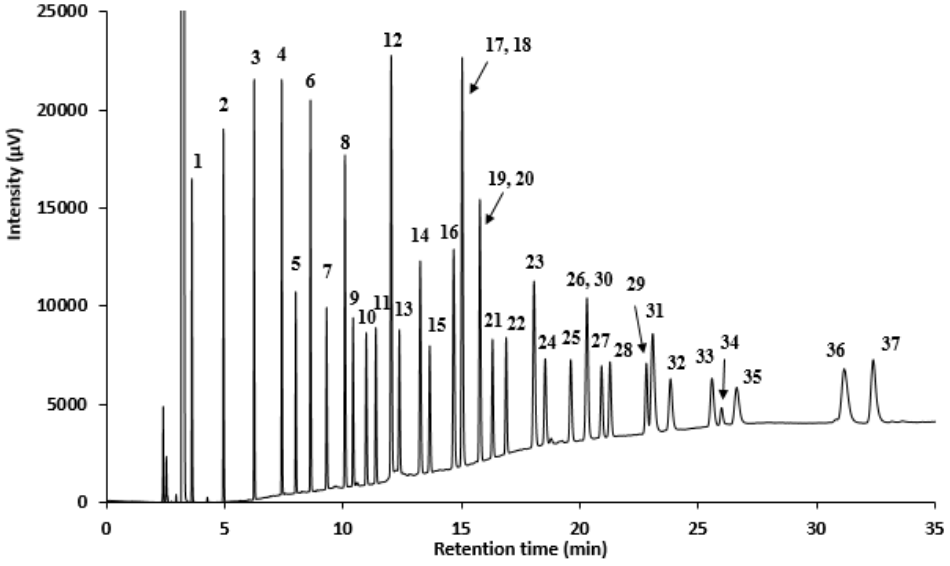



**Figure 30.** Chromatographic analysis obtained for the 37 compound FAME mix using the Shimadzu equipment.

(Source: A. Lima, 2020, [72])

Table 5 shows the elution order, compound name, compound ID, retention time and the obtained chromatographic area, for the analysis of the Supelco 37 compound FAME mix used in this work and presented in Figure 16. This table is used to identify each FAME peak in the analysed samples. These peaks are subsequently selected for the estimation of the individual FAME contents, and the total FAMEs content, in the olive oil and the OMWW's samples.

**Table 5.** Elution order, compound name, compound ID and retention time for the 37 FAME compounds.

| Elution Order | Compound Name | Compound ID | Retention time(min) | Area (μV) |
|---|---|---|---|---|
| **1** | Butyric acid methyl ester | C4:0 | 3.621 | 30583 |
| **2** | Caproic acid methyl ester | C6:0 | 4.953 | 37694 |
| **3** | Caprylic acid methyl ester | C8:0 | 6.250 | 42455 |
| **4** | Capric acid methyl ester | C10:0 | 7.416 | 45648 |
| **5** | Undecanoic acid methyl ester | C11:0 | 8.007 | 23521 |
| **6** | Lauric acid methyl ester | C12:0 | 8.629 | 48399 |
| **7** | Tridecanoic acid methyl ester | C13:0 | 9.310 | 24745 |
| **8** | Myristic acid methyl ester | C14:0 | 10.084 | 50129 |
| **9** | Myristoleic acd methyl ester | C14:1 | 10.427 | 24033 |
| **10** | Pentadecanoic acid methyl ester | C15:0 | 10.982 | 25267 |
| **11** | cis-10-Pentadecanoic acid methyl ester | C15:1 | 11.389 | 25247 |
| **12** | Palmitic acid methyl ester | C16:0 | 12.036 | 82633 |
| **13** | Palmitoleic acid methyl ester | C16:1 | 12.381 | 28309 |
| **14** | Heptadecanoic acid methyl ester | C17:0 | 13.263 | 45323 |
| **15** | cis-10-Heptadecanoic acid methyl ester | C17:1 | 13.662 | 25536 |
| **16** | Stearic acid methyl ester | C18:0 | 14.677 | 52918 |
| **17, 18** | Oleic acid methyl ester, Elaidic acid methyl ester | C18:1n9(c+t) | 15.033 | 92760 |
| **19, 20** | Linoleic acid methyl ester, Linolelaidic acid methyl ester | C18:2n6(c+t) | 15.784 | 59824 |
| **21** | gamma-Linolenic acid methyl ester | C18:3n6 | 16.315 | 25316 |
| **22** | alpha-Linolenic acid methyl ester | C18:3n3 | 16.891 | 25721 |
| **23** | Arachidic acid methyl ester | C20:0 | 18.068 | 52296 |
| **24** | cis-11-Eicosenoic acid methyl ester | C20:1n9 | 18.541 | 25933 |
| **25** | cis-11,14-Eicosadienoic acid methyl ester | C20:2 | 19.618 | 25832 |
| **26, 30** | cis-8,11,14-Eicosatrienoic acid methyl ester, Henicosanoic acid methyl ester | C20:3n6, C21:0 | 20.304 | 51710 |
| **27** | cis-11,14,17-Eicosatrienoic acid methyl ester | C20:3n3 | 20.920 | 22562 |
| **28** | Arachidonic acid methyl ester | C20:4n6 | 21.276 | 24669 |
| **29** | cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester | C20:5n3 | 22.811 | 24184 |
| **31** | Behenic acid methyl ester | C22:0 | 23.080 | 53019 |
| **32** | Erucic acid methyl ester | C22:1n9 | 23.832 | 25793 |
| **33** | cis-13,16-Docosadienoic acid methyl ester | C22:2 | 25.582 | 24786 |
| **34** | cis-4,7,10,13,16,19-Docosahexanoic acid methyl ester | C22:6n3 | 25.989 | 6549 |
| **35** | Tricosanoic acid methyl ester | C23:0 | 26.629 | 25197 |
| **36** | Lignoceric acid methyl ester | C24:0 | 31.164 | 49429 |
| **37** | Nervonic acid methyl ester | C24:1n9 | 32.393 | 47595 |

The percentage of FAME content was calculated using Equation 13.

$$C(\%) = \frac{\sum A_{FAMEs} - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m_{Olive\ Oil}} \times 100 \quad (13)$$

Where $\sum A_{FAMEs}$ is the total peak area of all methyl esters from C4:0 to C22:0, provided by the chromatograph, as shown in Table 5. $A_{IS}$ is the peak area corresponding to the methyl heptadecanoate, used as internal standard, $C_{IS}$ is the concentration, in milligrams per millilitre, of the methyl heptadecanoate solution, $V_{IS}$ is the volume, of the methyl heptadecanoate solution, and $m_{Olive\ Oil}$ is the mass, in milligrams, of the olive oil sample.

### *3.3.4. Acidity*

Based on the EN 14104 Standard [102], the acid values were determined to measure the degree of occurrence of FFA present in the olive oils.

The first step was to prepare a 1:1 (v/v) ethanol/diethyl solution to be used as solvent. Then, a 0.5 mol/L KOH was prepared in methanol and titrated with 0.09265 mol/L HCL standard solution. We then dilute the KOH solution to a concentration of 0.1 mol/L. The acidity of the samples was determined measuring 1 g of olive oil sample was measured using a micropipette and an analytical balance and placed into an Erlenmeyer.

The preceding step was the KOH standardization reaction, which at the equivalence point, as shown in Equation 14. The equation used (15) was:

$$\mathrm{HCl\ (aq) + KOH\ (aq) \rightarrow KCl\ (aq) + H_2O\ (liq)} \quad (14)$$

$$C_{KOH} = \frac{C_{HCl} \times V_{HCl}}{V_{KOH}} \quad (15)$$

The acid value (AV) is given in terms of mg of KOH / g sample by Equation 16:

$$AV = \frac{V_{KOH} \times C_{KOH} \times MW_{KOH}}{m_{sample}} \quad (16)$$

In this equation, $V_{KOH}$ is the volume, in mL of the KOH solution used in the titration, $C_{KOH}$ is the concentration of the KOH solution in mol/L , $MW_{KOH}$ is the KOH molecular weight, which is 56.1 g/mol, and $m_{sample}$ is the olive oil masses samples measured, in g.

In the acidity tests, for a concentration of HCl of 0.0927 mol/L, a concentration of KOH 0.0945 mol/L, with a degree of purity of 0.853, and a mass of olive oil of 1 g, a titration with KOH was performed, in order to obtain the acidity value. For the olive mill wastewaters, the process was similar but instead of 1 g of sample, 50 mL of each sample was taken and then the titration was done.

### *3.3.5. Determination of the Solid Content*

#### a) Total Solids

The TS measurements were done according to the 2540/-B procedure of the *Standard Methods for the Examination of Water and Wastewater, (APHA, 1999)*, where 20 mL of OMWW samples were taken and transferred to a crucible, previously weighted. Then, the crucibles with the samples were placed on top of a beaker, with about 600 mL of tap water, and on top of a heating plate and left to evaporate for about 4 h. The apparatus for the evaporation can be seen on Figure 31.

**Figure 31.** Apparatus for the evaporation step of Total Solids.

When the OMWW samples were dry, the crucibles were placed into an oven at 105 °C, for 24 h. Then, after cooling on a desiccator, the residue from the cooling was weighted, on a Kern ACJ 220-4M analytical scale. The content of the Total Solids, in g/L, is obtained from Equation 17:

$$TS = \frac{(m_2 - m_1) \times 1000}{V} \quad (17)$$

Where $m_2$ represents the weight of the crucible with the OMWW after the drying at 105 °C (g), $m_1$ the weight of the empty crucible (g) and V the volume of the sample (mL).

#### b) Total Suspended Solids

The TSS calculations were done according to the 2540/-D procedure of the *Standard Methods for the Examination of Water and Wastewater, (APHA, 1999)*. A volume of 10 mL of OMWW samples were subjected to vacuum filtration using a paper filter (ø 47 mm and 1.2 µm porosity). The residue retained in the filter was then put on a crucible and dried on stove at 105 °C, for 24 hours, and weighted after colling on a desiccator.

The content of the sample in TSS was obtained in a similar way of the one used in Equation 17, now considering $m_2$ the mass of the filter with the dryed residue (g) and $m_1$ the mass of the empty filter (g).

### *3.3.6. Chemical Oxygen Demand*

The COD calculations were done according to the 5220/-C procedure of the *Standard Methods for the Examination of Water and Wastewater, (APHA, 1999)*, Closed Reflux Method. The first step is to dilute the fresh samples, that are foreseeable to have higher COD, in order to reduce potential measurements errors, and also because the fresh samples are very dark, and hard to see using spectrophotometry. So, the samples from the traditional mill were diluted using a factor of 1:10 and 1:50 and the samples from the 3-phase mill were diluted 1:100 and 1:250 times, with distillated water.

The next step was to transfer 2.5 mL of the diluted samples to digestion tubes, with 16 mm x 15 mm and to extra tubes for the blank samples. Then, it was added 1.5 mL of the digestion solution, potassium dichromate, to each tube, except for the blank samples, that was added 2.5 mL of distilled water. After this, it was added 3.5 mL of sulfuric acid with silver sulphate. The tubes were then closed and inverted multiples time in order to mix completely, before going to the vortex. They were then placed on a digestion plate previously heated to 150 °C, for 2 h under reflux. After this time, the tubes were left to cool for about an hour.

Finally, the samples were analyzed by UV-vis spectrophotometry (Bio-Tek Synergy HT Microplate reader), with uncertainties of ± 0.010, and the results provided were then adjusted with a calibration curve (Figure 32).

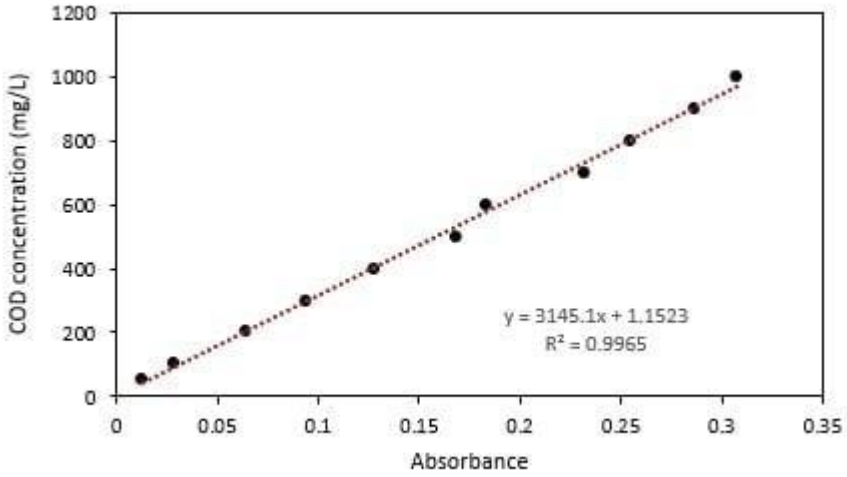



**Figure 32.** Calibration curve used for the COD analysis.

### *3.3.7. Biological Oxygen Demand*

The BOD determinations were done according to the 5210/-B. procedure of the *Standard Methods for the Examination of Water and Wastewater, (APHA, 1999)*. This procedure was used to measure the amount of dissolved oxygen (DO) that is consumed during biological aerobic oxidation activity of carbonated organic matter and inorganic ions for five days of incubation at 20 ± 1 °C, with agitation, in filled and stoppered bottles. Its value is calculated

from the difference between the initial oxygen and that present after the incubation period (if it is 5 days, it is called $BOD_5$), expressed in mg/L [50].

Before incubation, the olive mill wastewaters samples had to be neutralized and diluted using a dilution water rich in DO and in aerobes microorganisms, the inoculum. To each sample, a volume of 1 mL of inoculum was added. As control assay, it was used a solution of glutamic acid and dilution water. For the glutamic acid, it was added 150 mg of glutamic acid at 99 % and 150 mg of glucose on a 1000 mL Florence flask and prefilled with distilled water. For the dilution water, it was added distilled water, magnesium sulfate solution, calcium chloride solution, iron chloride solution and a phosphate buffer solution. The OxiTop® bottles containing the olive mill wastewarers as well as the blank sample and the glutamic acid are represented in Figure 33.

Then, 10 mL of each sample was diluted on a 100 mL Florence Flask, prefilled with the dilution water previously prepared. After this, 22.7 mL of the samples were transferred to OxiTop® bottles and was then added 1mL of the inoculum. There was also a white bottle, with 22.7 mL of dilution water and 1 mL of inoculum and the glutamic acid bottle, with 22.7 mL of the glutamic acid previously prepared and 1 mL of inoculum. Finally, two tablets of sodium hydroxide and stir bars were added to all the flasks and closed.

**Figure 33.** OxiTop bottles used in the $BOD_5$ analysis.

In the present work, the determination of $BOD_5$ was carried out using OxiTop® OC 100 (WTW), in which the evolution of the total pressure of a closed vessel is monitored over the days of the test. This equipment consists of [103]:

- Sample bottles, with a maximum capacity of 310 mL; in the gas phase of the vessel (in the cap) NaOH is placed which will fix the $CO_2$ that is being released during the test. Thus, the variation of the total pressure is only a consequence of the oxygen consumption by the biomass;
- Stirring plate, which keeps the samples in constant agitation;

- Controller, which allows reading the data from the OxiTop head;
- Pressure Sensors, which measure $BOD_5$ according to Equation 16.

Equation 18 demonstrates the progress of oxygen being consumed by converting the pressure given by the sensors, in mg/L.

$$BOD_5 = \frac{D_1 - D_2}{P} \quad (18)$$

Where:

$D_1$= DO of diluted sample immediately after preparation [mg/L]

$D_2$= DO of diluted sample after 5 days incubation at 20°C [mg/L]

$P$= decimal volumetric fraction of the used sample.

### *3.3.8. Biodegradability*

The biodegradability measurements considered that the $BOD_5$ values were smaller than the COD ones. The determination of the biodegradability ratio is made according to Equation 19.

$$Biodegradability = \frac{BOD_5}{COD} \quad (19)$$

### *3.3.9. Total Phenolic compounds*

The determination of the total phenolic compounds content of the extract obtained from the lyophilization of the samples was performed by spectrophotometry in a microplate reader (Epoch 2, Biotek), using the Folin–Ciocalteu reagent. To obtain the calibration curve, a methanolic gallic acid solution was used, a phenolic compound used as a standard, with concentrations of 0.005; 0.01; 0.05; 0.1 and 0.25 mg/mL. Different concentrations of extracts from the samples were prepared for analysis, using serial dilutions (1:2).

In a test tube, 250 µL of extract from each sample or standard was mixed with 1.25 mL of Folin-Ciocalteu reagent (1:10 *v*/*v*, in water) and 1 mL of sodium carbonate (75 g/L). The tubes were vortexed for 15 seconds and allowed to stand in the dark for 30 minutes at 40 °C for color development. Simultaneously, a blank containing 250 µL of water, 1.25 mL of Folin-Ciocalteu and 1 mL of sodium carbonate was prepared. At the end, if the solution was cloudy with suspended particles, the tubes were centrifuged at 7000 rpm for 3 minutes. Then, 300 µL of the samples from each test tube were transferred to a plate with the aid of a micropipette.

Finally, the absorbance was measured at 765 nm and the concentration in terms of total phenolic compounds was calculated using the standard curve obtained with gallic acid. Results were expressed as mg gallic acid equivalent per gram of extract (mg GA/g extract).

## 3.4. Biological Parameters

The biological parameters determined related with antioxidant properties were the capture of DPPH radicals and reducing power. For the antimicrobial properties, the microdilution method was used to obtain the MIC (Minimum Inhibitory Concentration) and the MBC (Minimal Bactericidal Concentration) against eight different microorganisms. Figure 34 shows the performed analysis, as well as the different steps needed.

At least three replicates' measurements for each biological parameter were made to corroborate the reproducibility of the measurements.

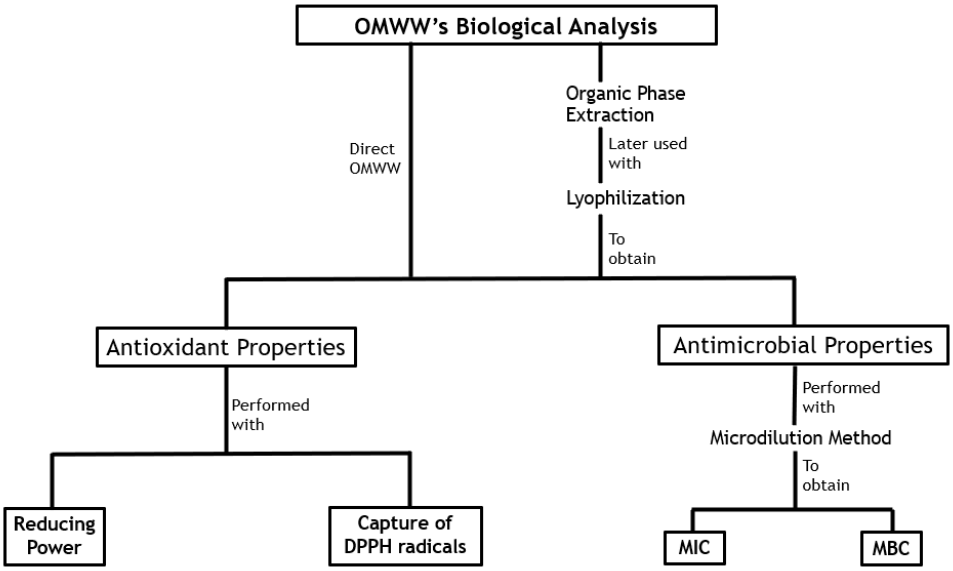



***Figure 34**. OMWW's biological analysis diagram.*

### *3.4.1. Extract preparation*

The organic phase extraction method was described in **2.4.1. Organic Phase Extraction,** with the use of the LLE method. For the biological properties, only the aqueous phase was reserved in Ambar flasks and later freeze dried and lyophilized. Lyophilization is a water removal process typically used to preserve perishable materials or make the material more convenient for transport. It works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the material to sublimate [104].

Before going to the lyophilization process, the samples were placed in plastic beakers, previously weighted, frozen, covered with stretch wrap containing small holes for better results at the lyophilization. After, they were weighted, homogenized, and maintained dried at a desiccator. The residue obtained was redissolved in the respective solvents for each of the following analysis and used after suitable dilution.

### *3.4.2. Antioxidant Properties*

#### 3.4.2.1. Capture of DPPH radicals

The determination of antioxidant activity of the extracts by the DPPH radical method was performed using a microplate reader (Epoch 2, Biotek). Different dilutions were prepared for the extract samples (0.016; 0.031; 0.063; 0.125; 0.25; 0.5 and 1 mg/mL). 30 µL of each of the different concentrations of extract was mixed with 270 µL of DPPH solution (6 x $10^{-5}$ mol/L prepared with distilled water), homogenized and the mixture obtained was placed in the dark for 1 hour. For the preparation of the blank reaction, a similar procedure was carried out using distilled water. The reduction of the DPPH radical was evaluated by measuring the absorbance at 517 nm. The free radical scavenging activity was calculated as a function of the percentage of DPPH discoloration using Equation 20:

$$\%\ \text{DPPH Radical Scavenging Capacity} = [(\text{Abs DPPH} - \text{Abs Sol})\ /\text{Abs DPPH}] \times 100 \quad (20)$$

Where Abs Sol represents the absorbance of the solution in the presence of extract in each concentration and Abs DPPH represents the absorbance of the DPPH solution (reaction blank). The minimum concentration of antioxidant necessary to reduce the initial concentration of radical scavenging activity by 50% ($IC_{50}$) was calculated by interpolation from the graph of DPPH Radical Scavenging Capacity percentage as a function of sample concentration. All extracts were analyzed in triplicate. Figure 35 shows an example of the DPPH assay.

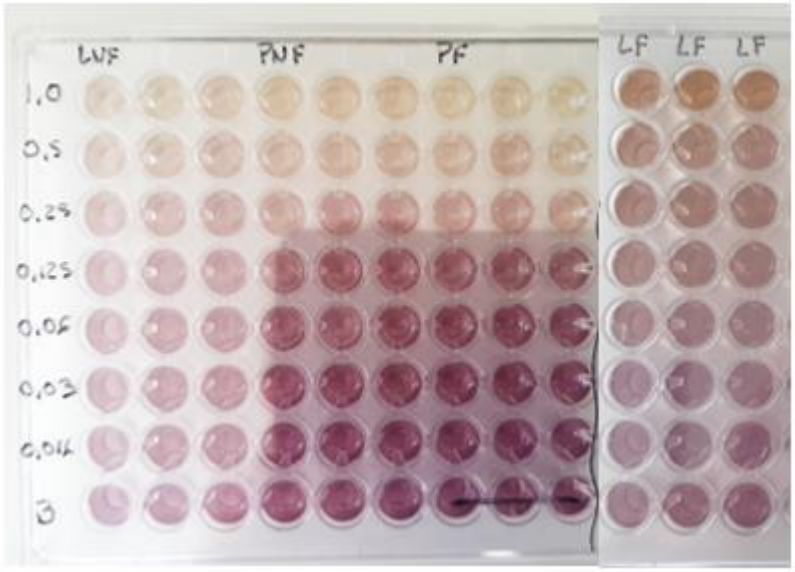



**Figure 35.** Apparatus for the determination of the percentage of DPPH scavenging capacity to the extracts.

#### 3.4.2.2. Reducing Power

The reducing power analysis were performed by spectrophotometry in a microplate reader (Epoch 2, Biotek). Different dilutions of the extracts were prepared, between 0.0156 mg/mL and 1 mg/mL. 500 µL of extract was mixed with 500 µL of sodium phosphate buffer solution (0.2 mol/L at pH=6.6) and 500 µL of potassium ferricyanide solution (1%, m/v). The mixture was homogenized and incubated at 50 °C for 20 minutes and, after cooling, 500 µl of trichloroacetic acid (10%, w/v) was added, then the mixture was homogenized and centrifuged at 12000 rpm for 2 minutes. 1 mL of the upper phase of the above mixture was withdrawn into a test tube and 1 mL of deionized water and 0.2 mL of $FeCl_3$ (0.1%, m/v) were added. For the preparation of the reaction blank, a similar procedure was carried out using distilled water. The absorbance of the mixture obtained was measured at 690 nm in a microplate reader. The extract concentration corresponding to $IC_{50}$ (extract concentration corresponding to 0.5 absorbance) was calculated from the graph of absorbance at 690 nm as a function of extract concentration. As a reference standard, a solution of Trolox in ethanol with a concentration of 2.5 mg/mL was used. Figure 36 shows the extracts with different dilutions before being read on the spectrophotometer (Bio-Tek Synergy HT Microplate reader).

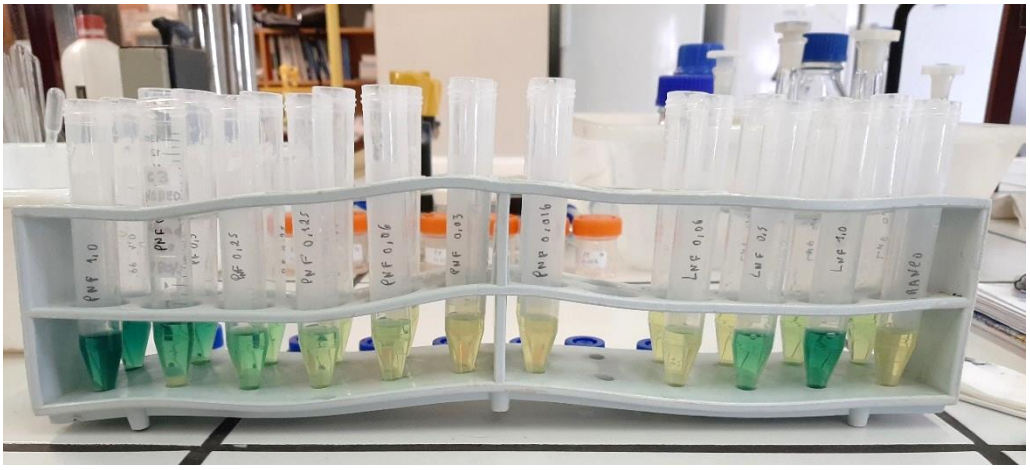


**Figure 36.** Determination of the reducing power of the OMWW.

### *3.4.3. Antimicrobial Properties*

The antimicrobial activity was evaluated by two different methodologies, the agar diffusion method and the broth microdilution method. The reference microorganisms used were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Klebsiella pneumoniae* (ATCC 13883), *Enterobacter aerogenes* (ATCC 13048), *Proteus mirabilis* (ATCC 14153), *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633) and *Enterococcus faecalis* (ATCC 33186) and *Pseudomonas aeruginosa* (ATCC 13048). The cultures were obtained by growing in nutrient broth for 24 hours at 37 °C.

#### 3.4.3.1. Microdilution Method

The method consists in determinate the minimal concentration of olive mill wastewaters capable of inhibit bacteria growth. For the study of the antimicrobial activity of the extracts, the broth microdilution method was used according to the Clinical & Laboratory Standards Institute (CLSI) method, with minor modifications, namely having adapted the volumes used. For each OMWW, five concentrations were tested (10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL and 0.6 mg/mL) in quadruplicate.

The first step is to prepare the OMWW's solutions in bacteria culture media, obtaining an aqueous solution with a known concentration of 20 mg/mL. Then use this solution to prepare a serial dilution (1/2) of each OMWW with 100 µL of Mueller Hinton Broth (MHB), as represented in Figure 37.

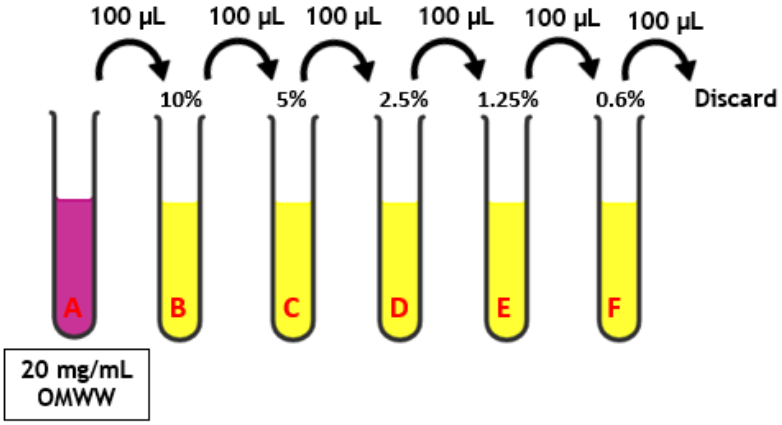



**Figure 37.** Serial dilution of OMWW inside the microplate.

To add bacteria inside the microplate wells is necessary to prepare an inoculum with adequate concentration and guarantee that each well will receive the same amount of bacteria. For this purpose, each bacteria was grown in an adequate agar media for 24h and then 5 isolated colonies transferred to nutrient broth, which was again incubated for 18-24h. A sample of this culture was then transferred to tubes containing Mueller Hinton broth (MHB) and adjusted to 0.5 McFarland unit (MFU). The inoculum was further diluted in MHB to reach a density of $10^6$ CFU/mL.

After, 100 µL were added in each microplate well from line A to H and columns 1 to 11, that were previously filled with OMWW dilutions or culture media only (column 11) (Figure 38). The columns 11 and 12 are used as controls, namely of bacteria positive growth (I) and culture media negative control (W) (to guaranty absence of media contamination). After the microplates were incubated with constant temperature of 37°C for 20 hours.

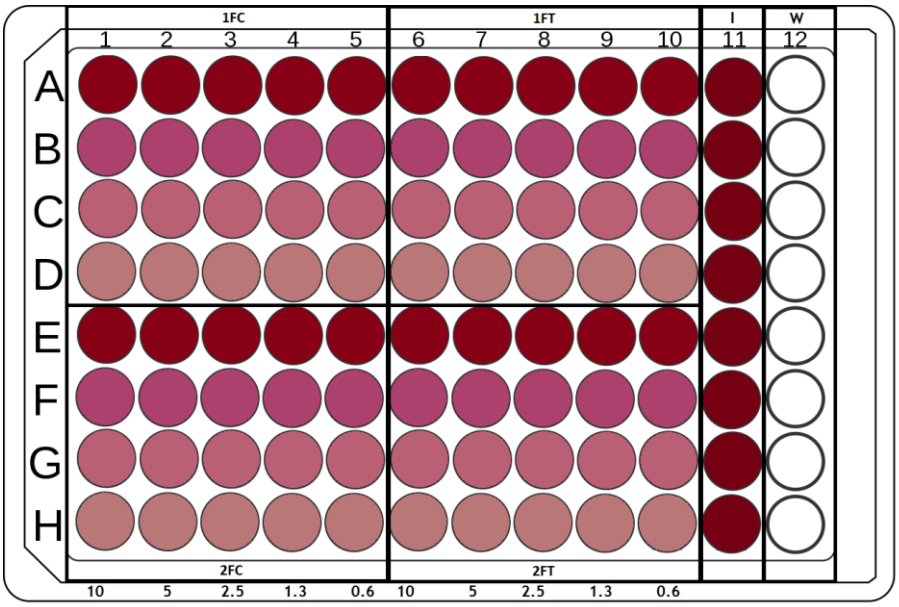



**Figure 38.** Final configuration of microplate before reserving it inside the kiln.

Subsequently, 30 µL of INT were added in each well, the plate was incubated for 30 min and the change of colour from yellow to pink was observed.

Moreover, a similar procedure was performed using the OMWW samples directly. In this case, the sample was seriously diluted with sterilized distilled water to a final volume of 100 µL and 100 µL of the inoculum prepared in double concentration MHB was added to each well.

To establish the minimal bactericidal concentration (MBC), 10 µL of the wells for which no visible growth was observed (wells equal or above the MIC concentration results) were subcultured into agar plates, and the presence of bacteria growth observed after incubation for 24 hours at 37 °C (Figure 39). The plating was made in duplicate.

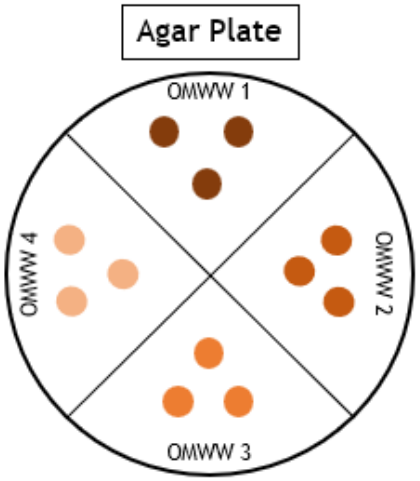



**Figure 39.** MBC test in agar plate.

# Chapter 4. Results and Discussion

## 4.1. Mechanical Analysis

### *4.1.1. Viscosity*

Rheologic tests are important to determine what type of fluid we are dealing with, a Newtonian fluid (equal viscosity for any flow velocity) or non-Newtonian fluids (viscosity varies with the shear rate).

On this work, the OMWW samples were rheologically characterized at different temperatures, namely for 20 °, 30 °, 40 °, 50 ° and 60 °C, and their results can be seen on Table 6, where 1FC is the continuous OMWW resulted from the first extraction with coffee filter filtration and 1FT is the traditional one.

***Table 6**. Viscosities of the OMWW samples.*

| OMWW Type | Temperature [°C] | Viscosity [Pa.s] | Standard Deviation [Pa.s] |
|---|---|---|---|
| 1FT | 20 | 0.00256 | 0.00011 |
| | 30 | 0.58120 | 0.06166 |
| | 40 | 0.10445 | 0.02617 |
| | 50 | 0.00154 | 0.00014 |
| | 60 | 0.00135 | 0.00002 |
| 1FC | 20 | 0.00221 | 0.00010 |
| | 30 | 0.00207 | 0.00022 |
| | 40 | 0.00178 | 0.00009 |
| | 50 | 0.00160 | 0.00014 |
| | 60 | 0.00152 | 0.00017 |

Through the analysis of Table 6, it is perceptible that the viscosity's values for both samples are very similar, showing a tendency for decreasing with higher temperatures. Nevertheless, the viscosity values for the traditional samples at 30 and 40 °C do not show a propensity to decrease, being in fact the highest levels, with 0.581 Pa.s and 0.104 Pa.s respectively for both temperatures. Considering the standard deviation of both this samples is superior to the others, and the fact that the samples were only filtrated through the coffee filter, and although the tests were done in triplicate, it is possible that this samples were badly filtrated and that some particles were in suspension, causing the rheometer to mislead the viscosity value. Nevertheless, it would be convenient to redo the analysis, to better understand the OMWW's behaviour at those temperatures, and with the samples that also passed through vacuum filtration.

Figure 40 shows the viscosity analysis of the Continuous OMWW, at different temperatures, and a reasonable agreement is observed to the tendency of increased viscosity

to all temperatures (that could signify a non-Newtonian behaviour), and confirming the values obtained in Table 6, where lower temperatures have higher viscosity values.

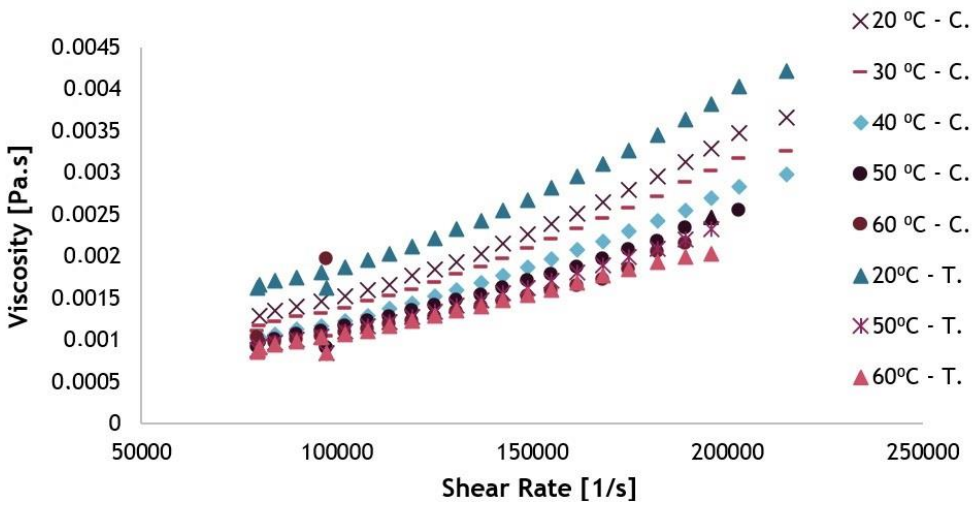



**Figure 40.** Viscosity curves for the Continuous and Traditional OMWW at different temperatures.

When comparing the viscosity curves obtained with water (Figure 43), we observe a similar behaviour with temperature, as both decrease their viscosity with the increase of temperature.

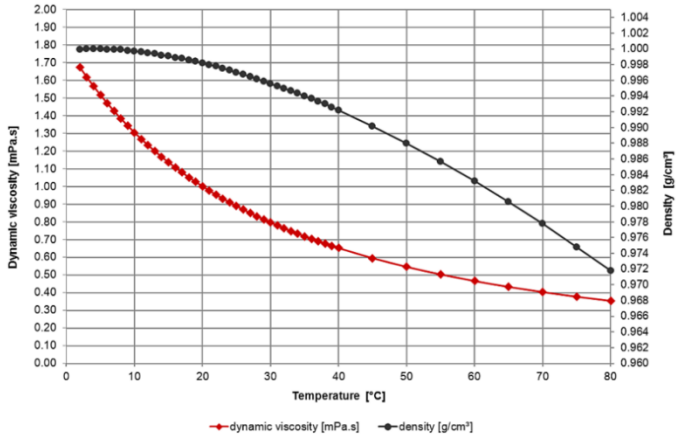



**Figure 41.** Dynamic Viscosity and Density of water over temperature.

(Source: Huber, M. et al, 2009, [105])

### *4.1.2. Wettability*

The mean roughness values of the measurements performed for the chosen materials are shown in Table 9. The nylon sample was the one with the higher roughness, with 8.738 µm, followed by aluminium alloy, with 4.489 µm, brass with 3.713 µm and carbon stell with 2.483 µm.

It is to be noticed that non of the materials were polished previously to the wettability analysis.

**Table 7.** Mean values of surface roughness measurements.

| Material | Roughness [µm] |
|---|---|
| Aluminium alloy | 4.489 ± 0.321 |
| Nylon | 8.738 ± 0.609 |
| Carbon Steel | 2.483 ± 0.187 |
| Brass | 3.713 ± 0.217 |

The wettability of the samples was measured by the sessile drop method, where the angle between the surface and the water drop was measured at two set points. The first measurement was performed at the moment that the drop contacted the surface, and the second measurement was recorded after 15 s, to better understand the equilibrium of the samples on the material, after the given time.

The mean values of the measured contact angle for both OMWW samples are shown in Figure 42. All the samples showed hydrophilic characteristics, as both the first and last measurements were lower than 90º. However, for the aluminium and carbon steel in both OMWW, the first angle values were lesser than the last, opposite to the brass and nylon. The mean values with the FC OMWW were 53.36º for aluminium alloy, 41.67º for brass, 54.79º for nylon and 38.47º for carbon steel. With the FT OMWW, the mean contact angle was 58.06º for aluminium, 74.86º for brass, 41.04º for nylon and 37.05º for carbon steel, being the aluminium alloy and carbon steel the values that showed the higher proximity with the different OMWW.

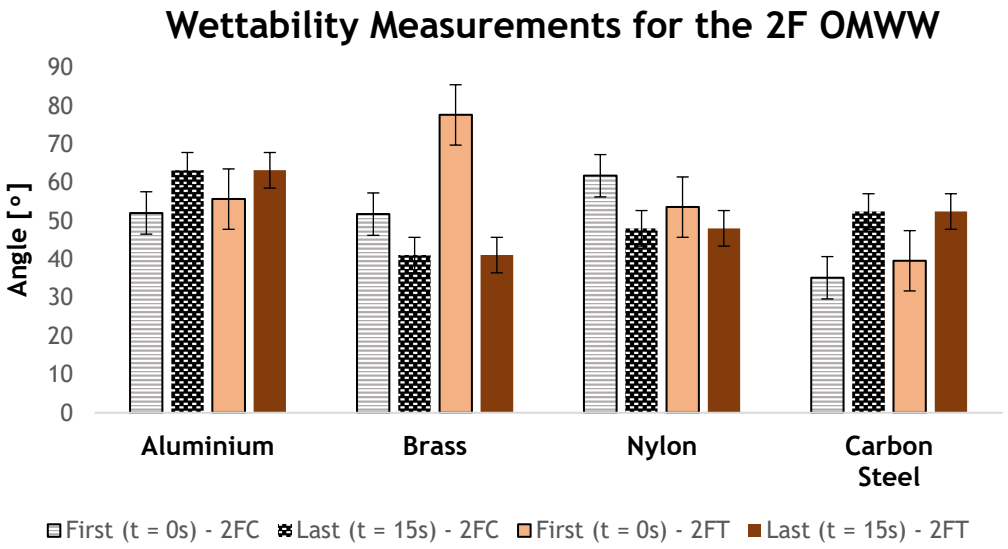



**Figure 42.** Mean values of water contact angle measurements for the OMWW.

## 4.2. Physical Analysis

### *4.2.1. Thermal Conductivity and Specific Heat*

The thermal conductivity and specific heat analysis were performed with the Hot Disk method, and the values obtained can be seen on Table 8, that shows a general increase for the thermal conductivity values with the increase of temperature, going from 0.6174 W/mK to 0.9097 W/mK in the continuous samples and from 0.6133 W/mK to 0.9172 W/mK in the tradicional ones. It also noticed that the increase is more significant between 35-40 °C, where its value boost from 0.7196 W/mK to 0.8931 W/mK (20% growth) in the continuous OMWW and in the 40-45 °C in the traditional OMWW, where the thermal conductivity values go from 0.7541 W/mK to 0.9172 W/mK (18%).

***Table 8**. Thermal Conductivity and Specific Heat values for the OMWW.*

| OMWW | 1FC | | 1FT | |
|---|---|---|---|---|
| Temperature [°C] | Thermal Conductivity [W/mK] | Specific Heat [MJ/m³K] | Thermal Conductivity [W/mK] | Specific Heat [MJ/m³K] |
| 20 | 0.6174 | 3.5833 | 0.6133 | 3.5144 |
| 25 | 0.6293 | 3.5505 | 0.6176 | 3.6184 |
| 30 | 0.6701 | 3.3326 | 0.6535 | 3.5491 |
| 35 | 0.7196 | 3.107 | 0.7374 | 2.9484 |
| 40 | 0.8931 | 2.2704 | 0.7541 | 2.9053 |
| 45 | 0.9097 | 2.6005 | 0.9172 | 2.2117 |

Figure 43 and Figure ***44*** show the thermal conductivity and specific heat analysis, respectively, for the OMWW. This analysis was also done on the olive oils that originated the olive mill wastewaters, to better understand their behaviour.

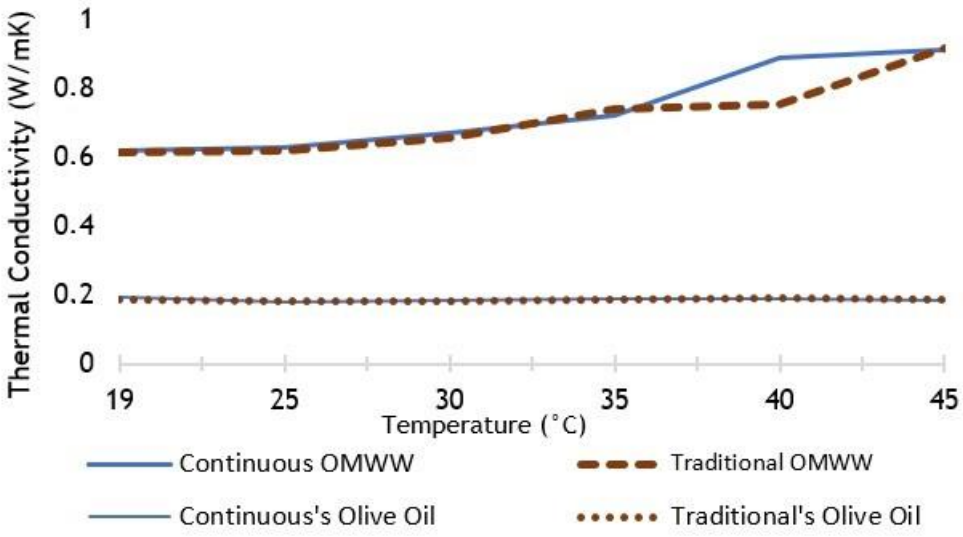



**Figure 43.** Thermal Conductivity of OMWW and their olive oils.

Through the analysis of Figure 43, it is noticeable that both olive oils have a similar behaviour, with a thermal conductivity practically constant at 0.2 W/mK. On the other and, the OMWW's conductivity shows a tendency to increase alongside the temperature, except for 35-45 °C interval, where the continuous sample has a more significative increase from 35-40°C, and from 40-45 °C in the traditional sample, which was already observed through Table 7.

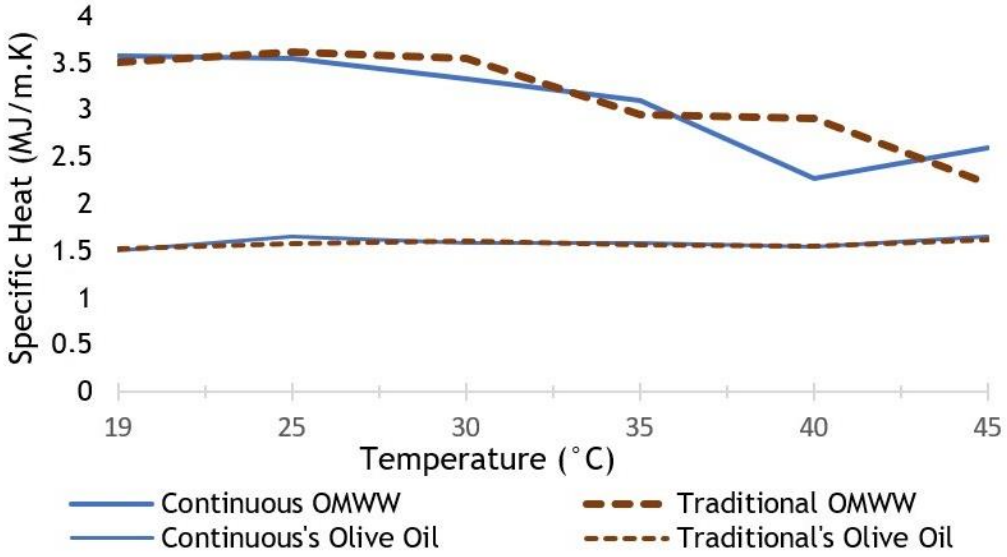



**Figure 44.** Specific Heat of OMWW and their olive oils.

As for the specific heat values, they do not show a particular trend but seems to generally decrease over temperature, varying from 3.5833 MJ/m$^3$.K at 20 °C to 2.6005 MJ/m$^3$.K at 45°C for the continuous OMWW samples and from 3.5144 MJ/m$^3$.K at 20°C to 2.2117 MJ/m$^3$.K at 45°C for the traditional ones. Although the apparent decrease, the major behaviour's difference is again between the 35-45°C interval, where the continuous sample has a larger decline until 40°C, achieving 2.2704 MJ/m$^3$.K, its lowest value, and then continuing to grow, unlike the traditional samples that, at 40°C, attain 2.9053 MJ/m$^3$.K, a value similar to the one obtained at 35°C (2.9484 MJ/m$^3$.K) and then as a more prominent decay. The olive oils' behaviour is once again very similar and constant.

The pure water behaviour in terms of its specific heat along temperature, and considering the range of temperatures between 5 and 95 °C, is practically constant, but has its lower range at 36 °C (1 bar). To directly compared the pure water to the OMWW behaviours, it would be necessary to redo the specific heat and the thermal conductivity analysis to temperatures values between 0 and 100 °C, but, for the given range of temperatures, between 20 and 45 °C, it seems like their similar, in particular for the continuous OMWW, that is the one with the higher water content.

### *4.2.2. Water content*

The water content was determined by coulometric Karl Fischer titration, and the mean of the obtained results are presented in Table 9.

**Table 9.** OMWW's water content.

| OMWW | Water Content [g/g] | Percentage [%] |
|---|---|---|
| **2FT** | 0.9483 | 94.83 ± 2.18 |
| **2FC** | 0.9837 | 98.37 ± 1.70 |

The results show a very high water content, with 94.83 % for the traditional OMWW and 98.37 % for the continuous OMWW, being higher from the ones found on the references, namely for [106] with an OMWW's water content of 80 % and [36] with 65-70 %.

These differences may due to the degree of maturation of the OMWW's samples and to the advance of the mill technique used, as the continuous OMWW's samples show a lesser water content, consistent to the 3-phased process, where the olive pomace is mixed with the olive mill wastewater and, therefore, more olive oil products are present, where in the traditional, the OMWW are the final waste, with most of the olive oil's products have already been removed.

On the total suspended solids, discussed on 4.2.3, the traditional OMWW were the one with the higher solid content, which agrees with the lower water content for the double filtered sample analysed for this analysis.

### *4.2.3. Density*

The density analysis was measured with the resonator density method, and the results are presented in Table 10.

**Table 10.** Density of the OMWW.

| OMWW | Temperature [°C] | Density [g/cm$^3$] | Average [g/cm$^3$] |
|---|---|---|---|
| **2FT** | 15<br>25 | 1.03145<br>1.02871 | 1.03007 ± 0.00137 |
| **2FC** | 15<br>25 | 1.02057<br>1.01804 | 1.01931 ± 0.00127 |

The density measurements were done using two different temperatures, 15 and 25 °C. The results for the traditional OMWW where 1.03145 g/cm$^3$ for 15 °C and 1.02871 g/cm$^3$ for 25 °C and 1.02057g/cm$^3$ for 15 °C and 1.01804 for 25 °C.

Although being very similar, the results show a tendency to slightly decrease with the increase of temperature, which is exactly what happens to liquid water at the same temperatures, which makes sense, as the OMWW samples have a high-water content, and their density is very similar to the liquid water itself. This can be because heating a substance causes

molecules to speed and get slightly apart from each other, occupying a larger volume that results in an decrease in density, as it happens with water, the larger substance present in the OMWW.

*Bouknana et al.*, in 2014, [81] conducted a study on the density of OMWW samples, obtained from 21 different mills at eastern Morocco, sorted by the type of mill (Figure 45. Density values of different OMWW of three extraction process of olive oil.), and their results where similar to the ones obtained, varying between 1.001 and 1.05g/cm$^3$. In their results, it also possible to observe that the higher density value is obtained in the 3-phase extraction, with 1.05 g/cm$^3$, but the values for this process of extraction are the ones with the superior amplitude, also obtaining values similar to the traditional extraction process as well, near 1.00 g/cm$^3$.

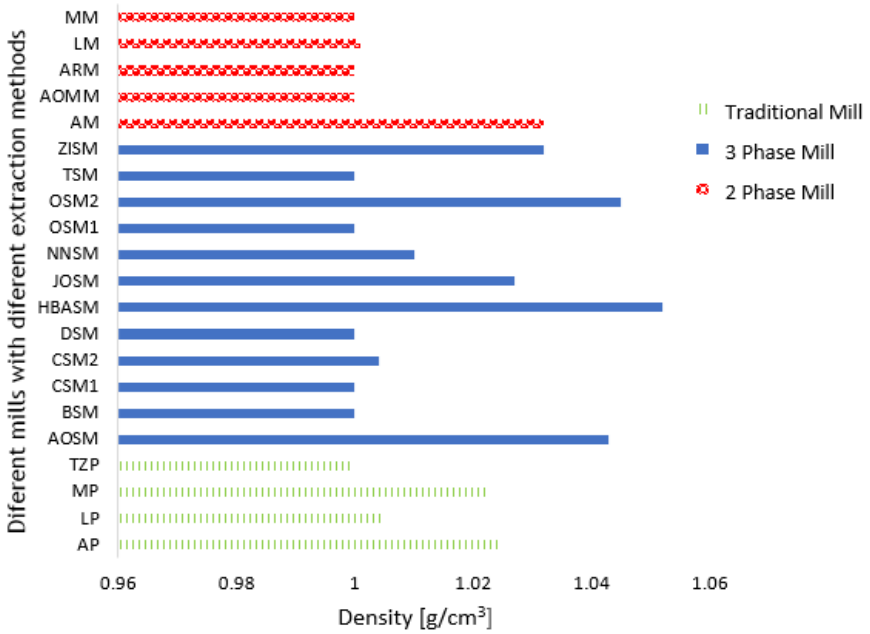



**Figure 45.** Density values of different OMWW of three extraction process of olive oil.

(Source: Adapted from Bouknana et. Al., 2014, [81])

### *4.2.1. pH value*

The pH values are presented in Table 11.

**Table 11.** pH values for the OMWW.

| OMWW | Temperature [°C] | pH |
|---|---|---|
| 2FT | 24.00 | 4.735 ± 0.025 |
| 1FT | 23.95 | 4.740 ± 0.000 |
| 2FC | 23.85 | 4.710 ± 0.020 |
| 1FC | 24.05 | 4.720 ± 0.010 |

The OMWW samples showed very consistent results for the pH measurements, as the pH for the double filtered Traditional Mill (2FT) were 4.735 ± 0.025 and for the single filtered ones (1FT) 4.740 ± 0.000. As for the 3-Phased samples, the 2FC showed a pH of 4.710 ± 0.020 and 1FC a pH of 4.720 ± 0.010. These results show that the pH measurements do not depend on the type of extraction used or the amount of filtration of the samples.

These results are also like the ones found on the references, in particular to Di *Giovacchino et. Al, 2005* (4.7-5.7 for the Traditional and 4.6-5.9 for the Continuous OMWW) and *Fiestas e Borja, 1991* (4.3-4.8 for the Traditional and 4.6-5.3 for the Continuous OMWW), which obtained similar values.

### *4.2.2. Electric Conductivity*

The Electric Conductivity results may be seen in Table 12.

**Table 12.** Electric Conductivity results for the OMWW samples.

| OMWW | Temperature [°C] | Electric Conductivity [mS/cm] |
|---|---|---|
| 2FT | 15.4 | 19.69 ± 0.02 |
| 2FC | 14.7 | 13.87 ± 0.05 |
| 1FT | 15.2 | 20.31 ± 0.05 |
| 1FC | 14.9 | 14.94 ± 0.01 |

The results show a similarity in the results for the double filtered and single filtered traditional samples, namely with 19.69 mS/cm and 20.31 mS/cm, and of 13.87 mS/cm and 14.94 mS/cm for the 2FC and 1FC OMWW, respectively, values in the same range of results presented in references [13] (18-25 mS/cm for the Traditional OMWW and 16-22 mS/cm for the Continuous OMWW), [14] (65-128 mS/cm for the Traditional OMWW and 13-118 mS/cm for the Continuous OMWW) and [81] (13-55 mS/cm for the Traditional OMWW and 23-41 mS/cm for the Continuous OMWW). This demonstrates that the type of extraction for the OMWW has a major role in the electric conductivity results, although the amount of filtration does not.

Besides these parameters, and according to reference [67], EC may also be related to the maturity index of the olives used for the olive oil production and, although the OMWW samples came from the same area, the places they were collected were different and so may be the maturity of the olives used. The conductivity measurement is also a good assessment of the degree of mineralization of olive oil mill wastewaters, where each ion is characterized by its concentration and specific conductivity, the electrical conductivity is strongly related to the concentration of dissolved substances and to their nature [81].

### *4.2.3. Solid Content*

The solid content analysis was done in total solids and the total suspended solids content. Total solids include any dissolved salts and solid particles, and a high level indicates a great amount of solid material in the liquid sample, as the total suspended solids represent all mineral and organic particles in the olive oil mill wastewaters and a large concentration can be regarded as a form of pollution. The results for the solid content can be seen in Table 13.

**Table 13.** Solid content results for the OMWW.

| OMWW | Total Solids [mg/L] | Total Suspended Solids [mg/L] |
|---|---|---|
| Traditional | 74.500 ± 1.880 | 33.260 ± 4.250 |
| Continuous | 43.750 ± 6.860 | 29.290 ± 10.312 |

The OMWW studied in this research are loaded with total solids and suspended solids; from the mean results obtained, the values of TS are 74.500 mg/L for the traditional process and 43.750 mg/L for the continuous process, that although being almost half of the value when comparing to the traditional samples, it is still very high, showing again the differences between the two processes. The TSS values are 33.260 mg/L for the traditional OMWW and 29.290 mg/L for the continuous ones, showing a lesser difference among both samples.

When compared to the references, the obtained results are higher, namely for [11] and [81]. Nevertheless, the majority of the references, except for [12] and [14], show that the traditional OMWW are higher than the continuous ones, many times almost the double value.

## 4.3. Chemical Analysis

### *4.3.1. FAME Content*

As mentioned in section **3.3.3**, olive mill wastewaters were analysed by gas chromatography to study the distribution of fatty acids. The characterization was done by identifying each fatty acid methyl ester by comparison with fatty acid methyl esters and the substances' retention time. For comparison purposes, the olive oils related to the OMWW samples were also analysed.

In Figure 44, it is presented the GC-FID chromatogram obtained after the derivatization of the double filtered continuous (2FC) OMWW. The GC analysis show that saturated stearic acid (C:18), unsaturated oleic acid (C18:1n9 (c+t)) and saturated palmitic acid (C16:0) were the major methyl esters in the filtered continuous olive mill wastewater.

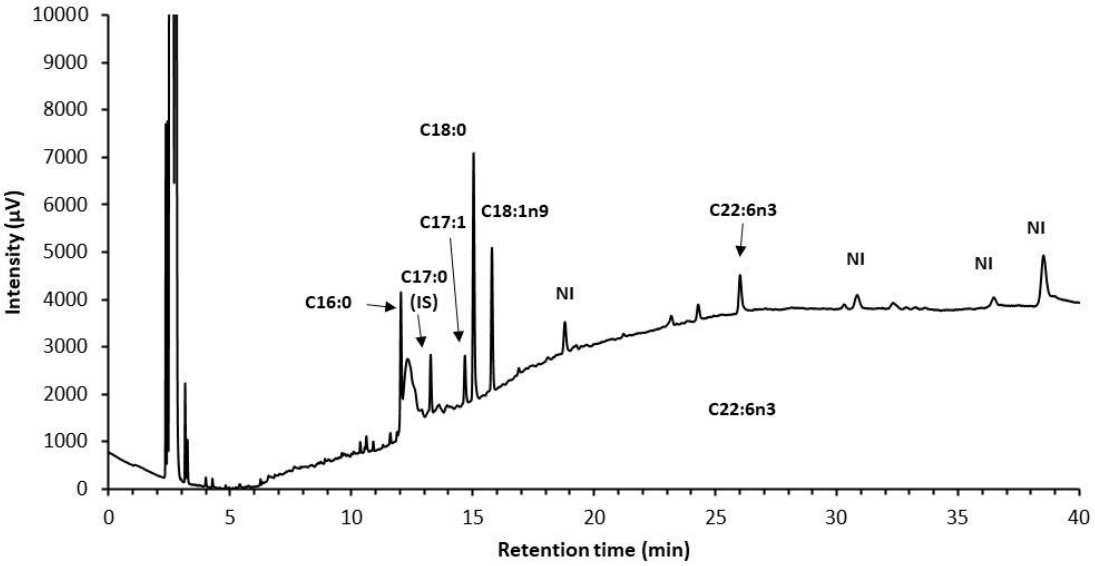



**Figure 46.** Chromatogram obtained after the derivatization of the 2FC OMWW.

The Continuous Mill FAME results distribution for the olive oil, the double filtered OMWW and the single filtered OMWW can be seen in Figures 47, 48 and 49, respectively.

The major components for the continuous olive oil were the where unsaturated oleic acid (C18:1n9 (c+t)) with 69 %, and the saturated palmitic acid (C16:0) with 15 %. As for the olive mill wastewaters, the main components for the double filtered OMWW were the saturated palmitoleic acid (C16:1) with 35 %, the unsaturated oleic acid (C18:1n9 (c+t)) with 25 %, the polyunsaturated linoleic acid (C18:2n9 (c+t)) with 14%, and for the single filtered OMWW, were, with the majority of FAME content, stearic acid (C18:0) with 68 %, the saturated palmitic acid (C16:0) with 14% and the unsaturated oleic acid (C18:1n9 (c+t)) with 11%.

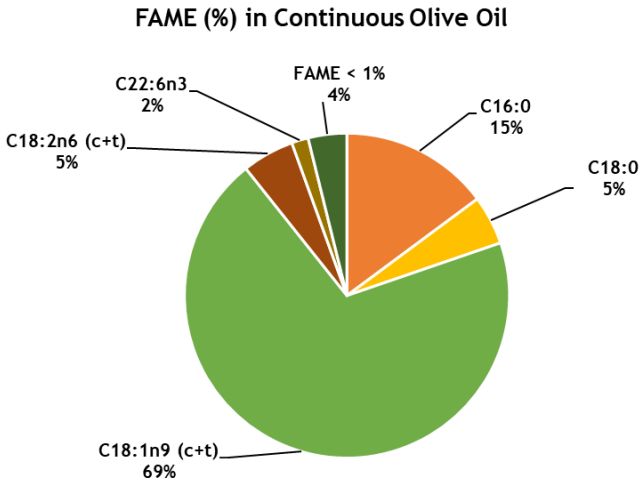



**Figure 47.** Percentage of fatty acids identified as methyl esters of triacylglycerols in Continuous Olive Oil.

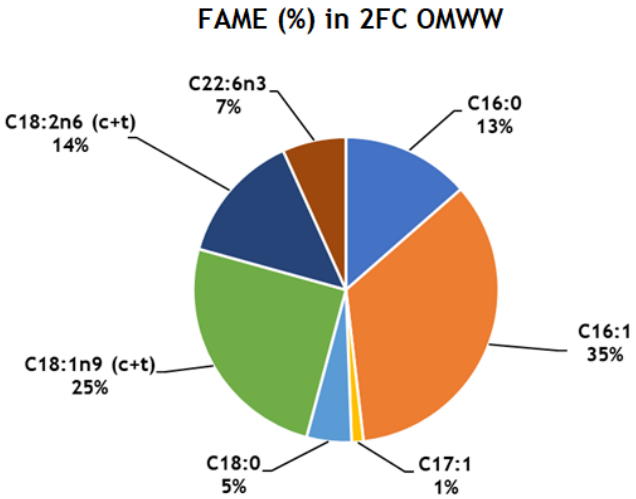



**Figure 48.** Percentage of fatty acids identified as methyl esters of triacylglycerols in the 2FC OMWW.

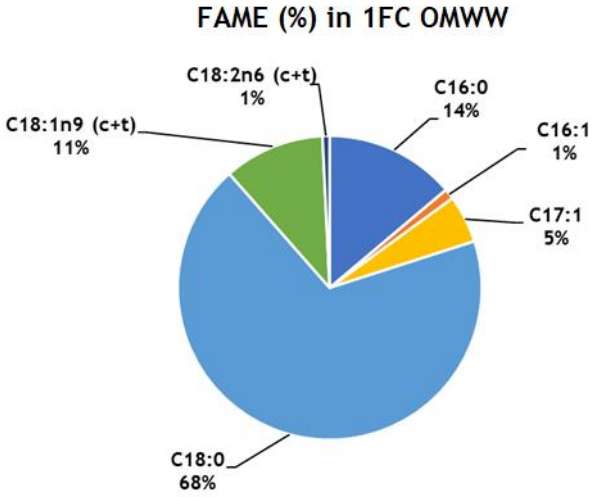



**Figure 49.** Percentage of fatty acids identified as methyl esters of triacylglycerols in the 1FC OMWW.

Figure 50 presents the GC-FID chromatogram obtained after the derivatization of the double filtered traditional OMWW (2FT), where the GC analysis showed that the unsaturated oleic acid (C18:1n9 (c+t)), the saturated palmitic acid (C16:0) and the polyunsaturated linoleic acid (C18:2n9 (c+t)), were the major methyl esters in the filtered continuous olive mill wastewater.

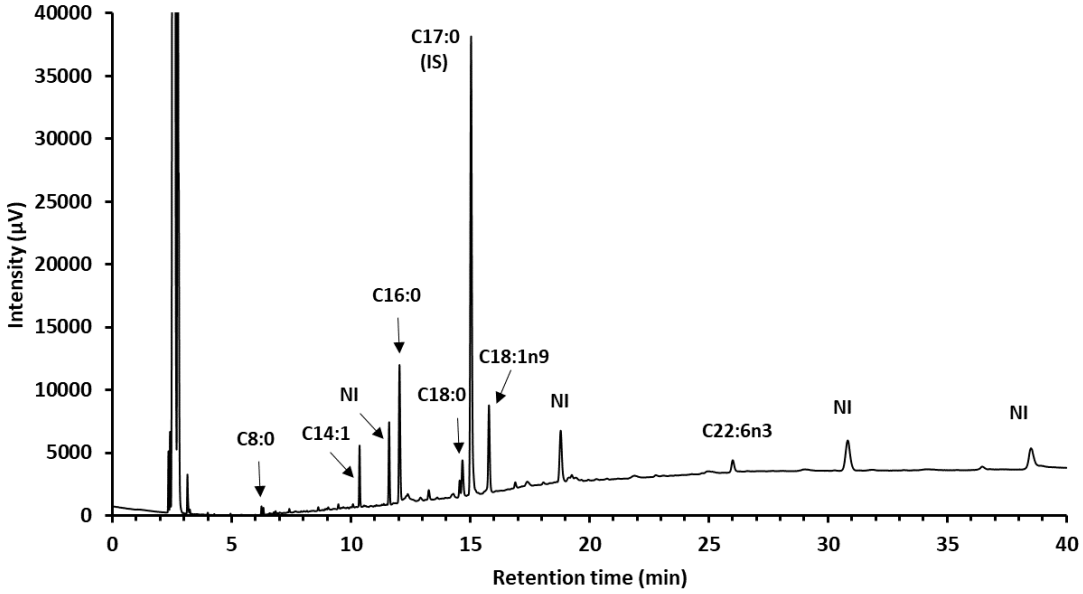



**Figure 50.** Chromatogram obtained after the derivatization of the 2FT OMWW.

The Traditional Mill graphic results for the olive oil, the double filtered OMWW and the single filtered OMWW samples can be seen in Figures 51, 52 and 53, respectively.

For the Traditional olive oil, the main components were the the unsaturated oleic acid (C18:1n9 (c+t)) with 66%, followed by the saturated palmitic acid (C16:0) with 10%. The traditional mill wastewaters also displayed the unsaturated oleic acid (C18:1n9 (c+t)) as their major methyl ester, with 58% in the double filtered sample and 67% on the single filtered one, followed by the saturated palmitic acid (C16:0), with 14% for the 2FT and 17% for the 1FT, and the polyunsaturated linoleic acid (C18:2n9 (c+t)), with 11% for the 2FT and 7% for the 1FT, being the main difference between the olive oil and the OMWW very much alike FAME's.

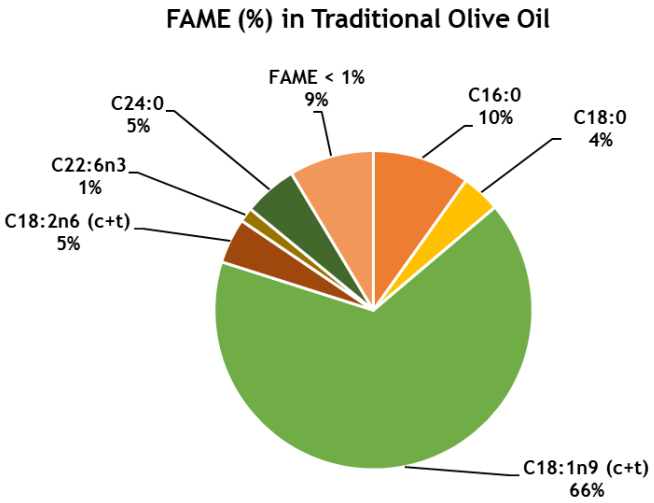



**Figure 51.** Percentage of fatty acids identified as methyl esters of triacylglycerols in Traditional Olive Oil.

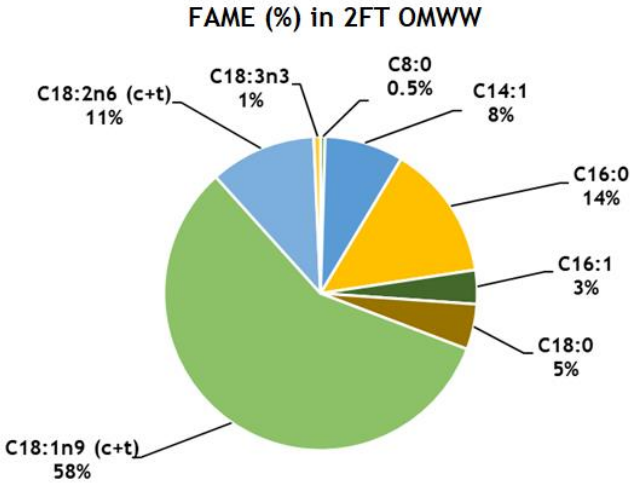



**Figure 52.** Percentage of fatty acids identified as methyl esters of triacylglycerols for the 2FT OMWW.

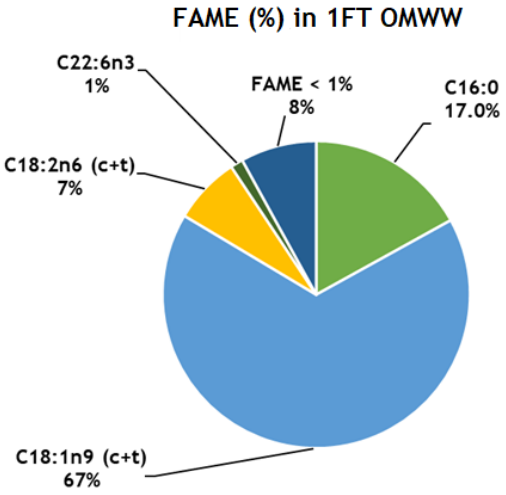



**Figure 53.** Percentage of fatty acids identified as methyl esters of triacylglycerols for the 1FT OMWW.

These results indicate a large similarity between the olive oils and the olive mill wastewaters, in particular with the traditional samples, where the two main components were the same, the unsaturated oleic acid (C18:1n9 (c+t)) and the saturated palmitic acid (C16:0). For the continuous samples, the unsaturated oleic acid (C18:1n9 (c+t)) was also the main methyl ester for the olive oil but only the second and third for the filtered and non-filtered OMWW, respectively. This can be due to the loss of doble bonds by the methyl esters that can be originated in the processing of the olive oil. Also, even factors such as light can lead to the breakage of these double bonds, oxygenation (lipid oxidation) and temperature causes a decrease in unsaturation. The change in colour, flavour, and aroma in the olive mill wastewaters but also in the olive oil itself has to do with compounds that are formed as a result of these reactions, leading to changes in the original characteristics of the fat/olive oil.

### *4.3.2. Acidity*

The acidity measurements were performed with the titration of KOH and can be obtained with Equation (16), and the results can be seen in Table 14.

**Table 14.** Acidity values for the OMWW and their Olive Oils.

| | Sample | AV [mg KOH/mg sample] |
|---|---|---|
| Olive Oil | Traditional | 1.043 ± 0.099 |
| | Continuous | 2.065 ± 0.619 |
| OMWW | 2FT | 6.864 ± 0.257 |
| | 1FT | 10.570 ± 1.360 |
| | 2FC | 3.121 ± 0.000 |
| | 1FT | 5.132 ± 0.936 |

Through the analysis of the results presented in Table 13, it can be observed that the acidity is very different for the studied samples. The traditional one has an acidity value of 1.043 mg KOH/mg olive oil and the continuous olive oil has almost the double, with 2.065 mg KOH/mg olive oil. Despite the traditional olive oil having the lesser AV value, the traditional OMWW have the higher acidity values, with 6.864 for the double filtered samples and 10.570 for the single filtered ones. As for the continuous OMWW, the filtered samples have an AV of 3.121 and the non-filtered one of 5.132, confirming that the single filtered samples are the ones with the higher AV.

These results are significantly higher than the values found in other studies, with values from 1.287 to 1.755 in [107] and 1.19 to 1.56 in [81], which are more similar to the olive oils' acid value. This difference can be explained by self-oxidation reactions and polymerization of phenolic compounds that took place during the storage olive oil mill wastewaters before the measurement of the acidity of the samples, and that can be seen by a change in the initial colour of olive oil mill wastewaters with a very dark black colour.

### *4.3.3. COD*

Due to the dark coloration of the olive mill wastewaters, the samples had to be diluted to be read in the spectrophotometer, but they showed different colour after the digestion step in the COD process. For example, the 10 times dilution was sufficient to the traditional samples to be read at the spectrophotometer, but for the continuous OMWW the dilution had to be at least 100 times so that the sample could be read. Figure 54 shows the samples after the dilution, namely the Continuous with 250 times dilution, Continuous with 100 times dilution, Traditional with 50 times dilution, Traditional with 10 times dilution and the blank sample in the back, in a left to right view.

**Figure 54.** OMWW after the digestion.

The COD's results may be seen on Table 15 and, once again, the values for the traditional OMWW are higher, with 93.361 g/L for the sample with a 10 times dilution and 90.627 g/L for the sample with a 50 times dilution. For the 100 times dilution of the continuous OMWW, the COD results were 63.500 g/L and 75.562 g/L.

**Table 15.** COD results for the OMWW.

| OMWW | Dilution | COD [g of $O_2$/L] |
|---|---|---|
| Traditional | 10 x | 93.361 ± 20.251 |
| Traditional | 50 x | 90.627 ± 7.697 |
| Continuous | 100 x | 63.500 ± 7.941 |
| Continuous | 250 x | 75.562 ± 15.293 |

When comparing the results to the ones found one the references, we observe that the COD values do not have a significant pattern, that is, the type of extraction does not seem to have a significant importance on the COD values. Considering that the chemical oxygen demand is the amount of dissolved oxygen that must be present in water to oxidize chemical organic

materials, the obtained results can be considered environmentally pollutive for the OMWW to be freely released into nature.

### *4.3.4. $BOD_5$*

The five-day BOD test's results, expressed on Table 16, show that the olive mill wastewaters are rich in organic matter, with 23 g of $O_2$/L for the traditional OMWW and 20.5 g of $O_2$/L for the continuous one. These results came along as the COD results, with the traditional samples having, once again, the higher amount of organic matter, but the difference was not as preeminent as the one found on the chemical oxygen demand.

**Table 16.** $BOD_5$ results for the samples.

| Sample | $BOD_5$ [g of $O_2$/L] |
|---|---|
| Glutamic Acid | 9 ± 0.0 |
| White | 2 ± 0.0 |
| Traditional | 23 ± 0.0 |
| Continuous | 20.5 ± 2.5 |

The BOD results found on the references were, in general, superior to the ones obtained, with the traditional samples having larger biochemical oxygen demand than the continuous OMWW, namely for [11,13], but with higher values, varying between 15-100 g of $O_2$/L for the traditional waters and 10-88 for the continuous ones.

### *4.3.5. Biodegradability*

Biodegradability is the BOD/COD ratio and measures the capacity for biological degradation of organic materials by living organisms down to the base substances such as water, carbon dioxide, methane, basic elements, and biomass. The biodegradability results can be seen on Table 17, that show that the BOD/COD ratio was approximately 25% for the continuous OMWW and 29% for the traditional ones, meaning that these waters, although not heavily loaded, were not totally degraded by biological means, probably due to the presence of some biological inhibitors for microbial aerobic growth. The results obtained in the references were similar for [11] but higher than the ones found on [81].

**Table 17.** Biodegradability results for the OMWW.

| OMWW | Biodegradability | BOD/COD [%] |
|---|---|---|
| Traditional | 0.29483 | 29 |
| Continuous | 0.25001 | 25 |

### *4.3.1. Total Phenols Content*

As mentioned, in this work the content of phenolic compounds was studied to better understand the antioxidant properties of OMWW, as it is considered to exist a correlation between the total phenolic content and the antioxidant properties of a particular vegetal species.

The total phenol content was determined by the Folin-Ciocalteu method, being expressed in mg equivalent of gallic acid. The determination was carried out using a plate reader. A calibration curve was prepared from the values obtained for the absorbance at 765 nm, using a standard gallic acid solution with defined concentration and respective dilutions, as it is shown in Figure 55. Calibration curve for the determination of total phenols obtained by reading the absorbance in a microplate reader.

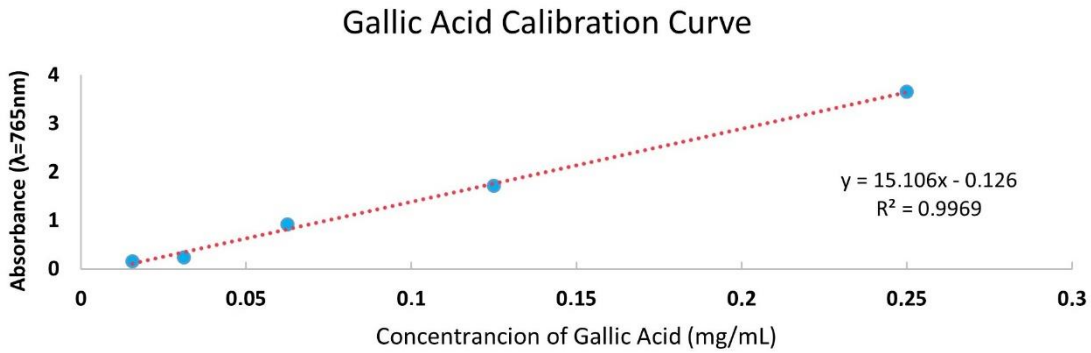



**Figure 55.** Calibration curve for the determination of total phenols obtained by reading the absorbance in a microplate reader.

The absorbance values obtained with gallic acid, used to construct the calibration curve are presented in Table 18.

**Table 18.** Absorbance results (λ= 765 nm) obtained in the analysis of total phenolic compounds for different dilutions of the same sample.

| Concentration [mg/mL] | Absorbance |
|---|---|
| 0.25 | 3.66 |
| 0.125 | 1.7130 |
| 0.0625 | 0.921 |
| 0.03125 | 0.24 |
| 0.015625 | 0.153 |

Table 19 presents the results obtained for the determination of total phenols of the OMWW's extracts. The results are presented as the mean of independent experiments, and associated standard deviation, having been carried out in triplicate.

**Table 19.** Determination of total phenolic compounds studied in the OMWW.

| Extracts | Solvent used for the extraction | Total Phenols (mg GAE/ g OMWW) |
|---|---|---|
| 1FC | $H_2O$ | 23.806 ± 0.373 |
| 1FT | | 29.811 ± 0.192 |
| 2FT | | 27.739 ± 0.084 |
| 2FC | | 24.071 ± 0.316 |

GAE: Gallic Acid Equivalents.

The results show concentrations of 23.806 mg GAE/ g OMWW for the single filtered 3-phased continuous OMWW (1FC), 24.071 mg GAE/ g OMWW for the double filtered ones (2FC), and 29.811 mg GAE/ g OMWW for the single filtered traditional discontinuous OMWW (1FT), and 27.739 mg GAE/ g OMWW for the double filtered ones (2FT). The values for the double and singled filtered samples are very similar, with slightly higher content being found for the traditional method. On the references [11–14], [81], [107], the reported content of total phenolic compounds ranged from 0.40 to 14.30 g/L, with values varying for the different mill extractions, values that are not directly compared to the ones found on this work.

It is known that the phenolic composition of OMWW depends not only on the variety, fruit maturity and climatic conditions, but also to the technological processes used to separate the aqueous phase (OMWW) of the oil phase. Therefore, the difference between the obtained values and those reported in the literature can be due to these differences.

## 4.4. Biological Analysis

### *4.4.1. Antioxidant Activity*

Antioxidant activity was evaluated using two different in vitro methods, the DPPH radical uptake assay and evaluation of reducing power. DPPH is a stable radical that loses its purple color when it accepts an electron from an antioxidant substance. The DPPH assay offers a convenient, accurate, and simple screening of the free radical scavenging activity of extracts from natural products. In the test to determine the reducing power, the presence of antioxidant compounds in the sample determines the reduction of $Fe^{3+}$ to the ferrous form. This reduction is highlighted by the spectrophotometric measurement of the intensity of the Prussian blue obtained, which depends on the reduction capacity of the extract.

Table 20 shows the obtained results by the application of the referred methods.

**Table 20.** Results for the Antioxidant Activity (values of IC50, mg/mL) determined by the different methods.

| Extracts | DPPH[a] | Reducing Power[b] |
|---|---|---|
| **1FC** | 0.6519 ± 0.006 | 0.477 ± 0.007 |
| **1FT** | 0.5486 ± 0.008 | 0.337 ± 0.002 |
| **2FC** | 0.4286 ± 0.006 | 0.359 ± 0.001 |
| **2FT** | 0.4657 ± 0.024 | 0.597 ± 0.012 |
| **TROLOX** | 0.040 ± 0.008 | 0.091 ± 0.001 |

Values expressed as mean ± standard deviation of three replicates; [a]$IC_{50}$ – Concentration for a DPPH radical uptake of 50%; [b]$IC_{50}$ – Effective concentration at which the absorbance is 0.5.

Being the antiradical activity $IC_{50}$ (mg/mL) defined as the concentration of extracts necessary to decrease the initial DPPH radical concentration by 50%, both samples demonstrated free radical's uptake activity in DPPH assay. Figure 56 presents the inhibition curve of the DPPH radical for all the extracts, where it is visible that as concentrations rise DPPH radical scavenging activity also increases, as expected. Except for the DPPH results, the antioxidant activity was superior for the single filtered traditional OMWW, which agrees with the higher phenolic content observed on these extracts (Table 20). Thus, the results obtained confirm to a certain extent the data presented by several studies, which demonstrate that the antioxidant capacity is dependent on the content of phenolic compounds present in the extracts of OMWW [108, 109].

The results obtained for the reducing power assay can be visualized in the graph of Figure 57, evidencing that the samples presented antioxidant activity also in this assay. Although the values were similar, the double filtered sample obtained by the traditional system (2FT) demonstrated a superior antioxidant activity since it obtained good results in both assays. Overall, the results were in good agreement, however, for the OMWW obtained by the continuous system, best results were obtained in the DPPH assay for the double filtered samples

while in the reducing power assay, the single filtered performed better. This can be due because the two assays are based on different chemical substances and principles as well to some loss of bioactive compounds during the filtering process. Therefore, more studies would be necessary to clarify this aspect.

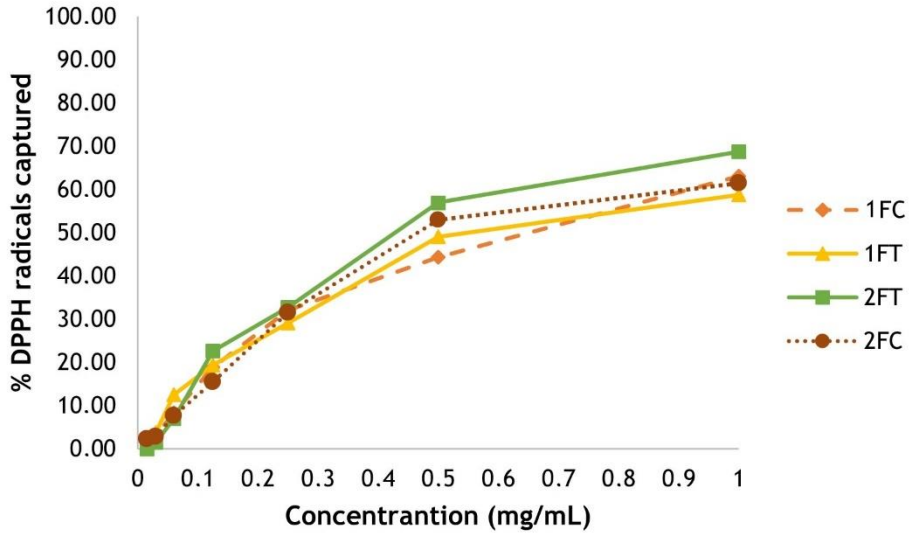



**Figure 56.** Percentage of DPPH radical inhibition of different extract concentrations of the OMWW extracts.

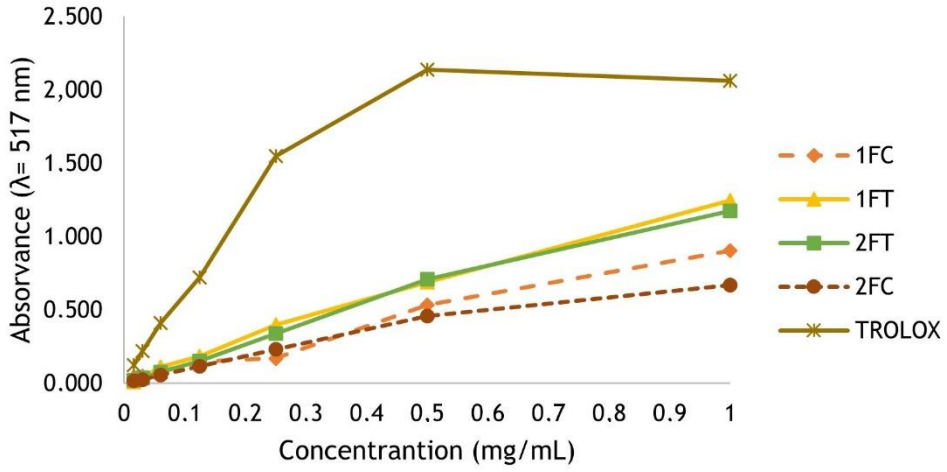



**Figure 57.** Result of the evaluation of the reducing power for different concentrations of the OMWW extracts.

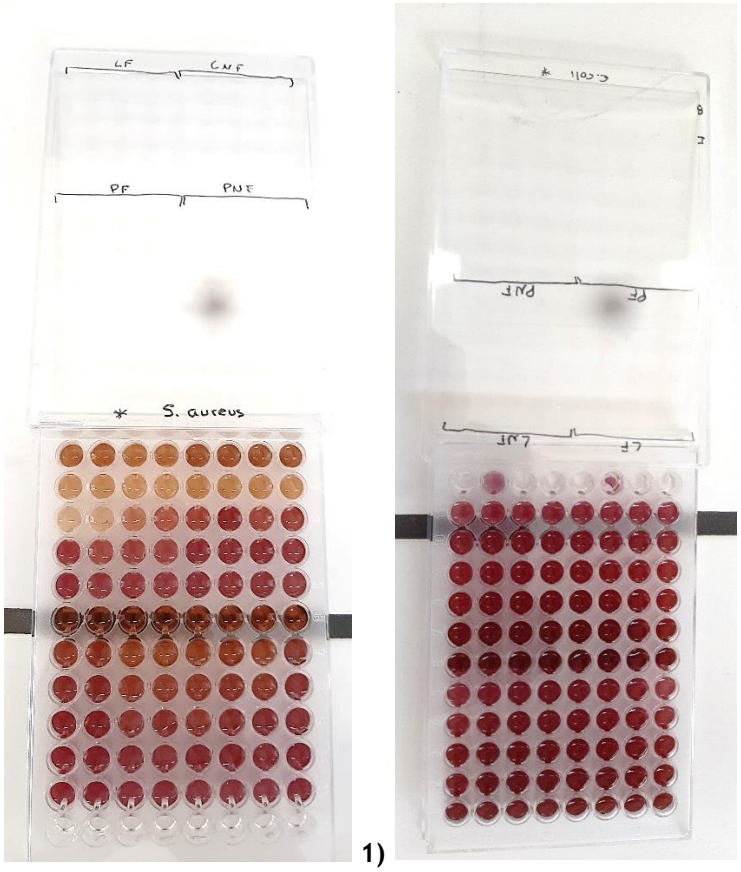
### *4.4.2. Antimicrobial Activity*

The assessment of antimicrobial activity is a complex determination and can vary due to multiple variables such as the culture medium used, the inoculum concentration, the methodology, the extraction method, among others.

The antimicrobial activity analysis was performed with the lyophilized OMWWs and also directly with the OMWW.

#### 4.4.2.1. Minimum Inhibition Concentration

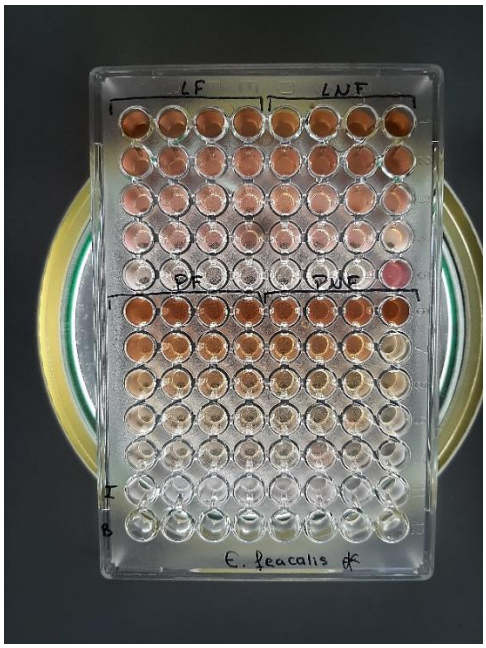
The purpose of this experiment was to determine the concentration of OMWW that inhibits bacteria growth. By analysing the results of turbidity, colour change, and colony formation, it is possible to determine which wells grew bacteria; for a concentration to be considered as MIC, the four wells (replicates) from each OMWW must be limpid after 24 hours. The photographic results can be seen in Figures 58-61.




**1)** **2)**

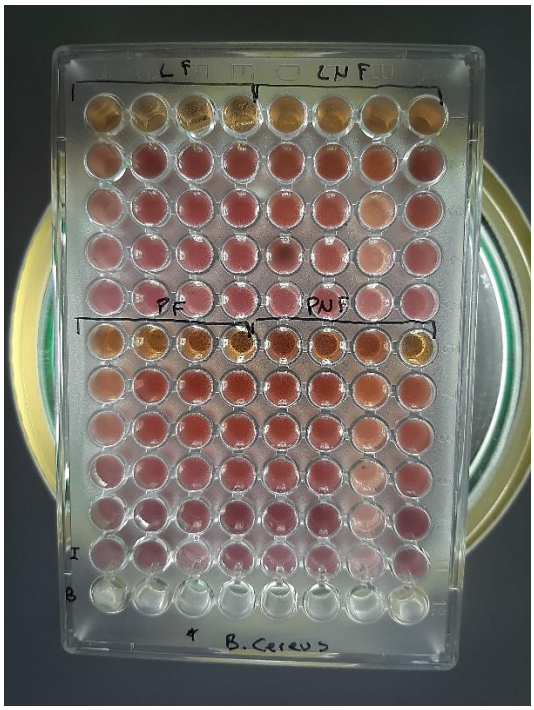
**Figure 58.** MIC for OMWW with **1)** *Staphylococcus aureus* and **2)** *Escherichia coli*.


3)

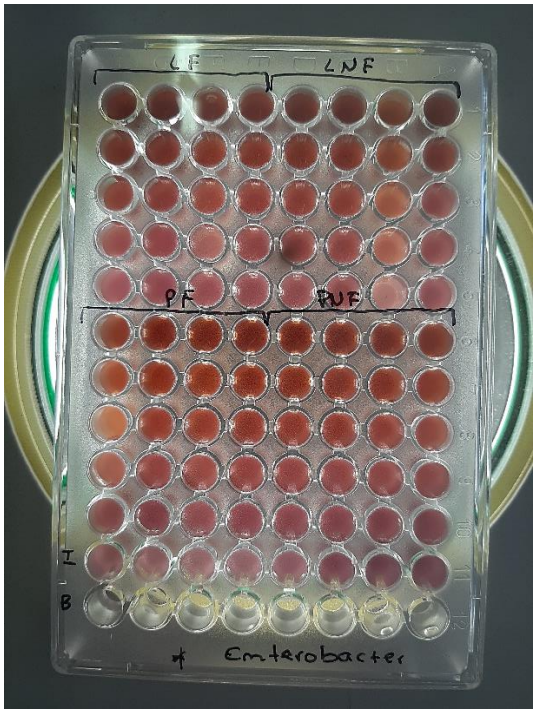



4)

**Figure 59.** MIC for OMWW with 3) *Klebsiella pneumoniae* and 4) *Enterococcus faecalis*.

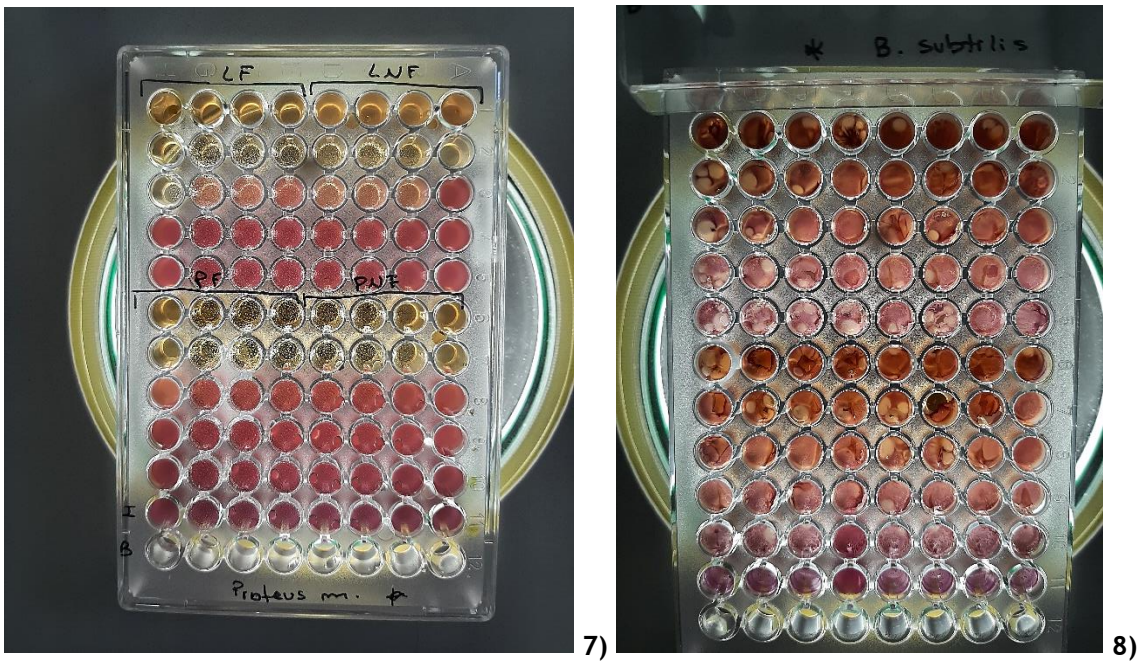



5)




6)

**Figure 60.** MIC for OMWW with **5)** *Bacillus cereus* and **6)** *Enterobacter*.

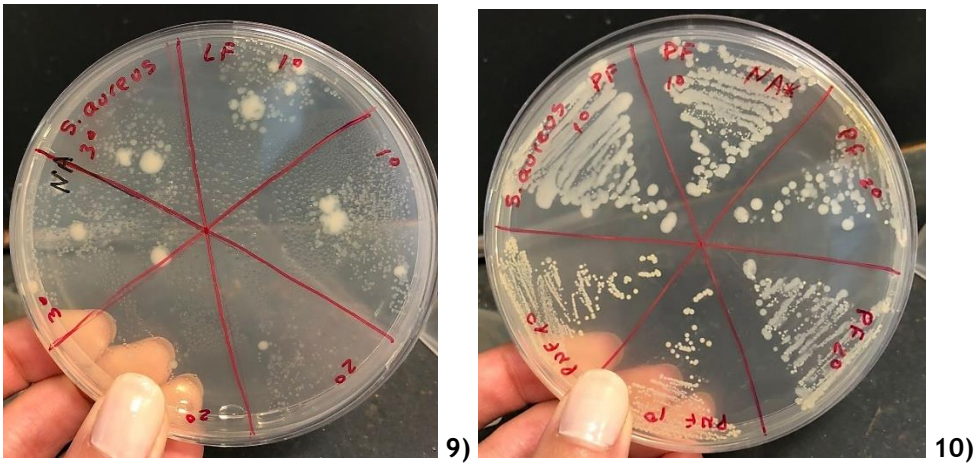



**7)** **8)**

**Figure 61.** MIC for OMWW samples with **7)** *Proteus mirabilis* and **8)** *Bacillus subtilis*.

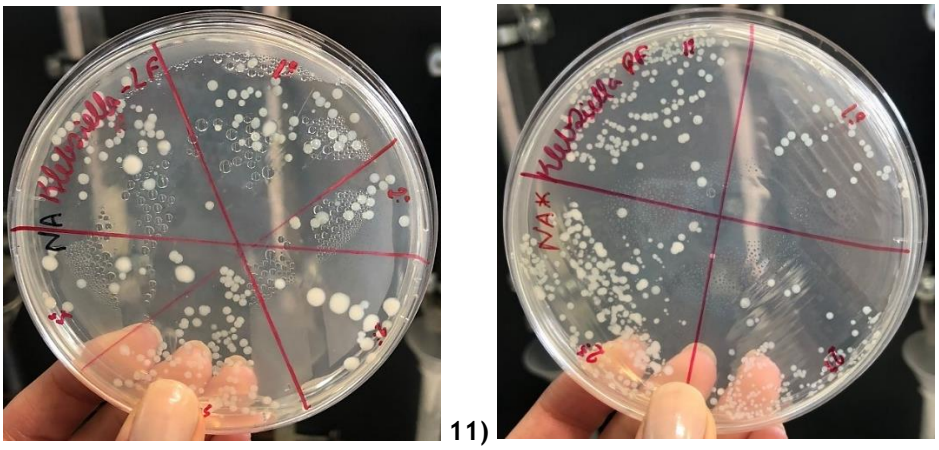
#### 4.4.2.2. Minimal Bactericidal Concentration

The MBC determines the minimum OMWW concentration capable of killing the bacteria. For establishing the MBC, the wells for which no growth was evidenced by turbidity and INT addition were plated to agar plates to estimate the bactericidal capacity of the lyophilized samples. The photographic results of the filtered OMWW can be seen in Figures 62-67.



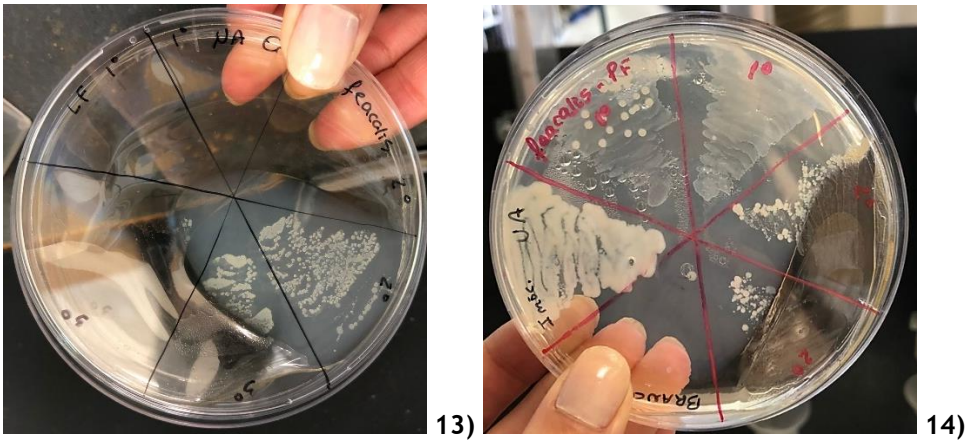

**9)** **10)**

**Figure 62.** MBC with *Staphylococcus aureus* for the **9)** Continuous OMWW and **10)** Traditional OMWW.



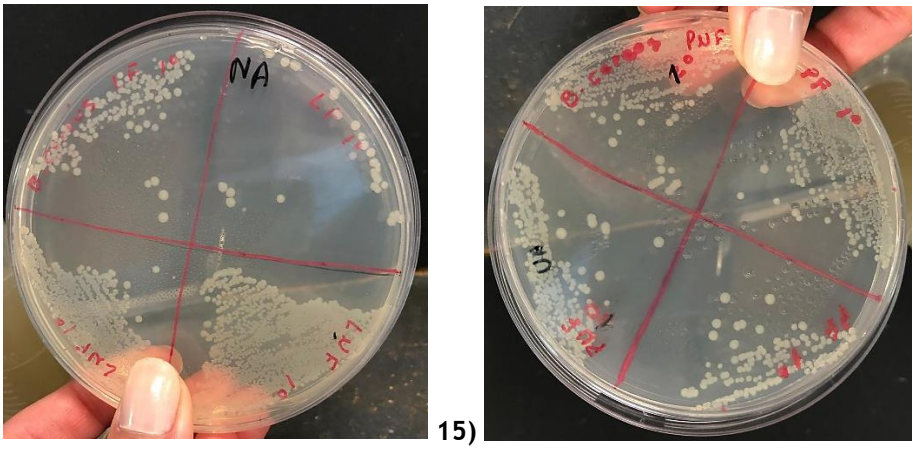

**11)** **12)**

**Figure 63.** MBC with *Klebsiella pneumoniae* for the **11)** Continuous OMWW and **12)** Traditional OMWW.




**13)** **14)**

**Figure 64.** MBC with *Enterococcus faecalis* for the **13)** Continuous OMWW and **14)** Traditional OMWW.




**15)** **16)**

**Figure 65.** MBC with *Bacillus cereus* for the **15)** Continuous OMWW and **16)** Traditional OMWW.

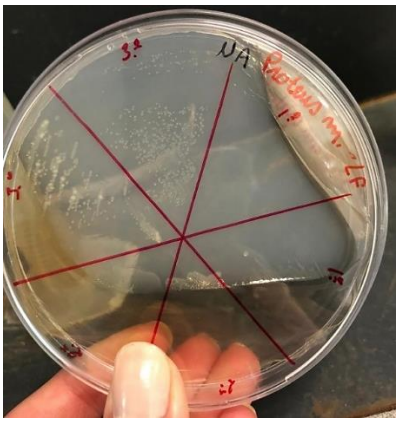
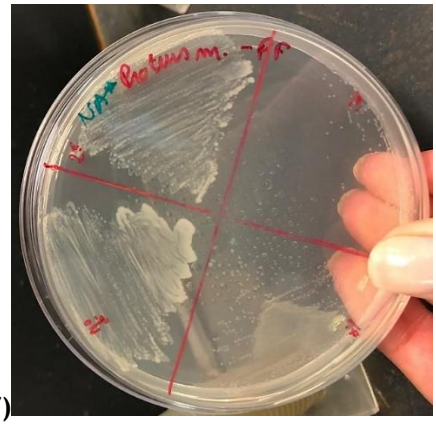

**17)**

**18)**

**Figure 66.** MBC with *Proteus mirabilis* for the **17)** Continuous OMWW and **18)** Traditional OMWW.


**19)**

**20)**

**Figure 67.** MBC with *Bacillus subtilis* for the **19)** Continuous OMWW and **20)** Traditional OMWW.

Table 21 provides a summary of the antimicrobial activity of the lyophilized OMWW against the tested microorganisms. The results showed that the lyophilized OMWW in the tested concentrations were ineffective against the Gram-negative *E. coli*, *E. aerogenes*, and Gram-positive *B. subtilis* and *B. cereus*. By the contrary, the samples presented good activity, both bacteriostatic and bactericidal, against *E. faecalis* and *P. mirabilis*. For *E. faecalis*, the samples obtained by the traditional method performed better since they presented lower MIC values than the continuous method samples while the opposite was observed for *P. mirabilis* for which the best results were obtained with the continuous method double filtered sample. Despite not presenting bactericidal activity, all lyophilized OMWW were able to inhibit the growth of *S. aureus*, *K. pneumoniae*, and *B. cereus*, with lower MIC values being generally obtained for the samples produced by the continuous method. The filtered sample obtained by this method presented the lowest MIC values (2.5 mg/mL) against *S. aureus, K. pneumonia* and *P. mirabilis*, which are relevant pathogenic bacteria that frequently present resistance to antibiotics.

**Table 21.** Results for the Antimicrobial Analysis for the OMWW extracts.

| Microorganisms | OMWW (mg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2FC | | 1FC | | 2FT | | 1FT | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| ***Staphylococcus aureus*** | 2.5 | >10 | 5 | >10 | 5 | >10 | 10 | >10 |
| ***Escherichia coli*** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| ***Klebsiella pneumoniae*** | 2.5 | >10 | 5 | >10 | 5 | >10 | 5 | >10 |
| ***Enterococcus faecalis*** | 5 | 10 | 5 | 10 | 2.5 | 10 | 2.5 | 10 |
| ***Bacillus cereus*** | 10 | >10 | 10 | >10 | 10 | >10 | 10 | >10 |
| ***Enterobacter aerogenes*** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| ***Proteus mirabilis*** | 2.5 | 10 | 5 | 10 | 5 | >10 | 5 | >10 |
| ***Bacillus subtilis*** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |

It is worth noticing that for some microorganisms, such as *S aureus*, it was perceptible the growth of more than one type of colony in the agar plates. This growth can possibly be attributed to microorganisms from the sample instead of the ones being tested in the antimicrobial activity assays. This hypothesis is supported by the observation of similar colonies growing in plates inoculated with some of samples and to the fact that the closed flasks containing samples of OMWW presented pressure when opened. To clarify this aspect, in future works the samples should be previously sterilized by filtration using a 0.2 μm membrane or the growth in agar being performed using selective media appropriated to each of the tested microorganisms. Moreover, it would be interesting to perform a genetic analysis to identify the microorganisms naturally present in the samples.

#### 4.4.2.3. Antimicrobial analysis with the direct OMWW

Since the lyophilizates showed good inhibitory activity against some microorganisms and considering also that OMWW can potentially be used directly as an ingredient in formulations for different aims, the OMWW as obtained from the mill was assayed for its antimicrobial activity. Since best results were obtained in the previous assay for the double filtered samples, only those two samples were evaluated. The sample was diluted with culture media, so it was tested from 50% to 3.125%, v/v. Table 22 presents the obtained MIC results.

**Table 22.** Results of antimicrobial activity for the OMWW as obtained from the mill.

| Antimicrobial Analysis | | | | |
|---|---|---|---|---|
| | 2FC [% v/v] | | 2FT [% v/v] | |
| **Microorganisms** | **MIC** | **MBC** | **MIC** | **MBC** |
| *Escherichia coli* | >50 | - | 25 | 50 |
| *Pseudomonas aeruginosa* | >50 | - | 25 | 50 |
| *Bacillus cereus* | >50 | - | 25 | >50 |
| *Klebsiella pneumoniae* | 25 | - | 25 | 50 |
| *Staphylococcus aureus* | 12.5 | - | 12.5 | 25 |
| *Proteus mirabilis* | 12.5 | - | 25 | >50 |
| *Enterococcus faecalis* | 12.5 | - | 3.125 | - |
| *Enterobacter aerogenes* | >50 | - | 25 | 50 |

In general, the results were in good agreement with results obtained for the lyophilized samples, since higher inhibition was also observed for *E. faecalis*, *S. aureus* and *P. mirabilis*. However, better results were observed particularly for the double filtered sample obtained by the traditional method that showed inhibitory activity against all 8 tested microorganisms. Nevertheless, the OMWW obtained by the continuous method also evidenced good results against *S. aureus*, *E. faecalis* and *P. mirabilis* (12.5%, v/v). The differences observed between results obtained with the liquid OMWW and lyophilized sample can possibly be related with an incomplete dissolution of the lyophilizates in the culture media, affecting the MIC values.

These results demonstrated that the direct use of the olive mill wastewaters, or its incorporation as ingredient in formulations, has potential to be used as an antimicrobial agent, especially for *Enterococcus faecalis*. Overall, this work demonstrates that these residues are, indeed, a good natural agent for microorganisms' inhibition.

# Chapter 5. Conclusions and Future Work

## 5.1. Main Conclusions

Olive oil wastewater are a major environmental problem worldwide. Every year, millions of tons of these toxic wastes are produced, and most of them cause significant environmental damage. Because they are generated seasonally, in small quantities and positioned mostly at scattered locations, the OMWW's matrix of pollutants is very hard to handle and treat at the desired level of new regulations for industrial wastewaters. Although several techniques are available to treat these residues and release them safely to the environment, the high organic content and phenolic contents present are still a problem, so the updated characterization is a good way to better understand the potentialities that these so-called residues might have in our world, a potentially turn an environmental problem into something industrially useful.

The samples of the olive oil mill wastewaters collected from the northeast of Portugal have shown a very complex and heterogeneous mechanical, physicochemical, and biological composition. They contain a variety of organic and inorganic compounds of varying natures and concentrations. The diversity of results can be due to different factors like the stage of the ripening olives, climatic conditions, variety of olive cultivation system, geographic location, time of storage before crushing the olives, techniques and storage space, nature conservation for the olives, and the process for extracting olive oil, which has proven the most significant factor for the heterogeneity of the OMWW samples.

A similarity has been found among the mechanical characteristics of OMWW and water because both wastewaters contain significant amounts of water. The mean viscosity for the continuous samples was 0.0018 Pa.s and for the traditional 0.1382 Pa.s. On the wettability analysis, the mean values with the 2FC OMWW were 53.36º for aluminium, 41.67º for brass, 54.79º for nylon and 38.47º for carbon steel, and with the 2FT OMWW, the mean contact angle was 58.06º for aluminium alloy, 74.86º for brass, 41.04º for nylon and 37.05º for carbon steel, where both samples demonstrated hydrophilic behaviour towards all four different materials tested.

The physicochemical characteristics exhibited a water content of 98.35 % for the continuous olive mill wastewater, with a density of 1.0193 g/cm$^3$, and 94.83 % for the traditional one, and a density of 1.0301 g/cm$^3$. The thermal conductivity was 0.7400 W/m.K for the continuous OMWW and 0.7155 W/m.K for the traditional sample and the specific heat was 3.1250 MJ/m3K for the continuous OMWW and 3.0741 MJ/m$^3$K for the traditional one. The samples displayed a similar behaviour until 40°C, when the traditional OMWW diverged from the continuous one. Since most microorganisms die at 37°C, this can be due to the biological activity of the samples. However, it would be convenient to repeat the tests, especially the viscosity parameter. The pH results were very similar, with 4.710 and 4.720 for the 2FC and 1FC OMWW, respectively, and 4.735 and 4.740 for the 2FT and 1FT. The acidity values shown a larger

discrepancy, with 3.1213 mg KOH/mg OMWW and 5.13 mg KOH/mg OMWW for the double and single filtered continuous OMWW, namely, and 6.86 mg KOH/mg OMWW and 10.57 mg KOH/mg OMWW for the 2FT and 1FT samples. These results are consistent with the values from the olive oils produced for each OMWW, as the continuous olive oil had an acidity of 2.07 mg KOH/mg olive oil and the traditional OMWW had an acidity of 1.04 mg KOH/mg olive oil, nearly half of value obtained for the continuous samples, a similar behaviour observed in their olive mill wastewaters also. The more interesting values are the single filtered ones, with almost double acidity than the one found for the filtered samples. This can be explained by self-oxidation reactions and polymerization of phenolic compounds throughout the storage of the OMWW samples, resulting in a change of colour of the waters, that could also explain the large similarity between the olive oils and the olive mill wastewaters FAME content, in particular with the traditional samples, where the two main components were the same, the unsaturated oleic acid (C18:1n9 (c+t)) and the the saturated palmitic acid (C16:0). For the continuous samples, the unsaturated oleic acid (C18:1n9 (c+t)) was also the main methyl ester for the olive oil but only the second and third for the filtered and non-filtered OMWW, respectively. Nevertheless, it is assumed that the single filtered samples have a higher phenolic content, resulting in a superior acidity level, which can occur as most of the phenols and other components capable of achieving a higher acidity content may not be hydrophilic, so they do not pass through the aqueous phase of the olive oil, during the extraction process, i.e., the OMWW.

The electric conductivity results shown a 13.87 mS/cm and 14.94 mS/cm for the continuous double filtered and single filtered OMWW, respectively, and 19.69 mS/cm and 20.31 mS/cm for the traditional samples. As the electric conductivity is strongly related to the concentration of dissolved substances and their nature, we can assume that the traditional samples have a higher rate of dissolved substances, which is proven by the total solids content, with 43.750 mg/L for the continuous OMWW and 74.500 mg/L for the traditional ones. It also interesting that, once again, is the single filtered samples that have a higher content, electric conductivity in this case. Nevertheless, when we analysed the total suspended solids, is the continuous samples who have the superior values, with 33.260 mg/L against 29.290 mg/L of the traditional OMWW, proven that the traditional OMWW have a higher dissolved content. The COD and BOD values were also higher for the traditional OMWW, with 91.994 g/mL in the COD and 23 g of $O_2$/mL in the BOD tests, versus 69.531 g/mL and 20.5 g of $O_2$/mL for the COD and BOD analysis on the continuous samples, respectively. Generally, the obtained results were higher than those found in the references, assuring environmental pollution. The biodegradability analysis revealed that only 29 % of the organic load could be biologically degraded by aerobic processes on the traditional samples and 25 % for the continuous OMWW. The mean values for polyphenols in continuous OMWW were 24.071 mg GAE/g and 23.806 mg GAE/g, for the filtered and non-filtered samples, and 27.739 mg GAE/g and 29.811 mg GAE/g, respectively for the filtered and

non-filtered traditional OMWW. In spite of the small difference, higher content of phenolic compounds was obtained for the OMWW obtained by the traditional system as compared to the continuous one. More samples produced under the different systems should be analysed to verify if the differences can be attributed to the different olive oil production methods.

The biological analysis of the antioxidant activity showed that, for the DPPH method, the concentration for a DPPH radical uptake of 50%, expressed in $IC_{50}$, where 0.4286 mg/mL and 0.6519 mg/mL for the double filtered and single filtered continuous OMWW and 0.4657 mg/mL and 0.5486 mg/mL for the 2FT and 1FT OMWW, respectively. These results showed a good scavenging activity, proving that the OMWW have a good antioxidant activity. For the reducing power method, the obtained results were 0.477 mg/mL and 0.337 mg/mL for the 2FC and 1FC OMWW, and 0.3559 mg/mL and 0.597 mg/mL for the double and single filtered traditional OMWW, respectively. The results from the two assays showed some differences, (mostly because they are different chemical processes, that tests different properties) however they demonstrate the potential of OMWW to be used as an ingredient due to its antioxidant capacity. The antimicrobial tests revealed that the OMWW has the capacity of inhibiting bacterial growth, especially against *Klebsiella pneumoniae, Staphylococcus aureus, Proteus mirabilis* and *Enterococcus faecalis*. The results are particularly interesting for *S. aureus,* which is a pathogenic bacterium frequently present in the skin and that can cause infection in cases of wounds/injuries of the skin. The results demonstrated that olive mill wastewaters have antibacterial activity even when used directly in the liquid form, and diluted to 50% or more, therefore suggesting that this residue can be a natural microorganism's inhibitory agent and can potentially be exploited for different purposes based on this activity.

## 5.2. Future Work Proposals

For future analyses, a detailed analysis of the biological activity of the olive mill wastewater could provide a better understanding of how the samples behaved in the different analyses. As most of the working fluids used in the mechanical industry have a high-water content, around 96%, it would be compelling to compare them with OMWW samples.

For possible better results, the mechanical performance could be improved by adding nanoparticles and essential oils. Thermal stability and wear tests may also be performed.

As the direct use of the OMWW showed a good inhibitory response and the possibly industrial use of these samples are being thought of, further tests on human skin for risk evaluation can also be staged.

Lastly, as it was mentioned along this work, some results raised doubts or would be convenient to repeat under different or during longer conditions or during, such as higher temperature ranges, to better understand the OMWW's behaviour under higher temperatures, or OMWW with an even lower pH, such as 2.0, so that the biological activity of the samples would not interfere with the results.

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