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# The application of the steam distillation on different organic matrices: optimization and innovation

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## List of publications

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Spadi, A., Angeloni, G., Guerrini, L., Corti, F., Parenti, A., Innocenti, M., Bellumori, M., Masella, P. (2021). Hydrodistillation of Coffee By-products to Recover of Bioactive Compounds: The Spent Coffee Ground and Coffee Silvers Skin Case-study. Chemical Engineering Transactions, 87, 313-318.

Spadi, A., Angeloni, G., Guerrini, L., Corti, F., Maioli, F., Calamai, L., Parenti, A., Masella, P. (2022). A conventional VOC-PID sensor for a rapid discrimination among aromatic plant varieties: classification models fitted to a rosemary case-study. Under review at Journal of Agricultural Engineering.

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## Participation at conferences

A. Spadi*, F. Maioli, G. Angeloni, L. Guerrini, A. Parenti, P. Masella. Un sensore VOC a fotoionizzazione (PID) per la stima rapida delle rese di estrazione di oli essenziali. VI Congresso Nazionale, Associazione

Scientifica S.I.R.O.E. (Società Italiana per la Ricerca sugli Oli Essenziali), Bologna, 18-19 ottobre 2019.

Agnese Spadi*, Virginie Xavier*, Piernicola Masella, Sandrina Heleno, Joana S. Amaral, Tânia C.S.P. Pires, Ricardo C. Calhelha, Isabel C.F.R. Ferreira, Lillian Barros. Chemical composition and bioactive properties of Eucalyptus globulus L. essential oil. 5th International Symposium on Phytochemicals in Medicine and Food. Nanchang, China, August 25-31, 2021. Carried out during a period abroad at CIMO - Centro de Investigação de Montanha Braganca Portogallo, Università Politecnica di Braganca.

## Abstract

In the following thesis steam distillation extraction process was studied and deepened. Steam distillation is a particular type of distillation, used for the recovery of volatile compounds with high boiling point, from inert and complex matrices, solid or liquid, using saturated or superheated steam as separation and energy agent. Steam distillation and its variants are the traditional methods used for the extraction of bioactive compounds from natural sources and it is unquestionably the most frequently used method for the extraction of essential oil from plants. In this contest, the overall aim of the research was to optimize and innovate the process, which is often considered too old, simple, expensive in terms of timing, energy consumed and raw material and thus, for these reasons not thorough by the scientific community. However, steam distillation is traditionally the most used method in the extraction of essential oils at industrial scale. Steam distillation has several considerable advantages such as ease of use, generates solvent-free products and by-products, no need for further separation of final products, extensive methodology know-how is available, and, as mentioned before, has a wide application on an industrial scale. On the other side, the main disadvantages are the long extraction times, the consumption of energy and sometimes of raw material. In particular for the case of essential oils, one of the critical points is the very low amount of essential oil extracted during the extraction process. Thus, in this view it is necessary to study and deepen several aspects of steam distillation. The following thesis was structured in four research with the overall aim to optimize and innovate the steam

distillation process. The first and the second study focused on the study of the extraction process, the third and fourth the possibility of to broaden the range of application of the process. Therefore, in the first study some of the operating factors, involved in the whole process, were studied. In the detail, it was proposed a novel statistical approach, a Plackett–Burman design to test the simultaneous effect of some factors in the essential oil extraction process and understand the relative importance of each factor on final yield, when plant material is distilled using steam and hydro distillation methods.

The second study allowed to develop the application of a photoionization detector (PID) for monitoring and controlling of different products in the phases of the distillation process. In detail, a general PID detector was applied to detect volatile organic compounds of the matrix entering the process, therefore distinguish different varieties of aromatic plants. In this case, two varieties of rosemary plants were studied. In a first step, PID detector was used to measure the volatile organic compounds of the two varieties. Hence, essential oils were extracted from both varieties and characterized by gas chromatography-mass spectrometry (GC-MS). Then, the well-established GC-MS method was used to characterize and differentiate the oil from each of the two varieties. The PID was able to capture different signals, and a sort of 'fingerprint' for each of the two varieties was obtained. The PID dataset was recorded via an electronic interface and transferred to a computer to be analysed by chemometric methods. Several statistical models were used to validate the dataset,

both unsupervised (principal component analysis and cluster analysis) and supervised (support vector machine and artificial neural network) methods. Finally, the third and fourth studies concerned a possible alternative application of the steam distillation process as a sustainable method for recovery. of bioactive compounds with high added value from the waste of some agri-food industries. In detail, the main waste from the coffee industry and the olive oil industry were considered. For this study, steam distillation was tested as a possible "green" extraction method to recover bioactive compounds and to enhance the agri-food waste. With the steam distillation plant, it was possible to recover two potentially different fractions. A richer fraction of the volatile component and a richer fraction of the less volatile component. Both the fractions have been suitably analysed and characterized by high-performance liquid chromatography with diode-array (HPLC) and gas chromatography–mass spectrometry (GC-MS) analysis to deepen the chemical profiles. Interesting compounds were found in the two fractions obtained, which could be potentially used in different industries.

Introduction part

# 1. Distillation

## 1.1. Distillation overview

Distillation is the oldest separation process and the most widely used unit operation in industry [1]. It has been an important process to humankind from the earliest civilizations. In fact, distilled products have determined and influenced cultural developments throughout the world [2]. Its origins date back to thousands of years ago. It is believed that art of distillation to have originated in Alexandria, Egypt, in the 1$^{st}$ century BC, when it was used for the extraction of essential oils from plants. In the following centuries it has been spread widely and, around the 11$^{th}$ century, has been used to produce alcoholic beverages, for the first time in northern Italy. Afterwards, in the 19$^{th}$ and 20$^{th}$ centuries, it developed rapidly prompted by the growing development of oil, petrochemical, chemical and pharmaceutical industry, becoming so the dominant separation process at industrial scale.

In ancient times this term was used to indicate all separation techniques indiscriminately. Later, it was used to indicate separation techniques based on through the succession of two different state changes: evaporation and condensation of a vapor. In fact, distillation is a well-defined separation unit consisting of the partial evaporation of a liquid mixture and successive condensation, with a composition that differs from that of evaporation [2]. The sample obtained after the condensation phase is called "distillate", richer in the volatile components, while the

remaining sample, not condensed, is called "residual" and it is richer in the less volatile components [3][4]. In fact, word “distillation” derives from the Latin verb “destillare o distillare”, meaning “to drop down” or to “trickle down”, referring to the dripping of the condensed vapor product from the condenser [5]. The equipment used for distillation flourished in Alexandria during the Roman Empire, and the apparatus did not change much until the sixteenth century. With the increased knowledge made possible by the invention of printing and with the larger demand for distilled products, various stills thrived. French scientists, English industrialists, and German craftsmen brought the equipment to the lab and fostered its industrial application. Until, the modern era with its development of high-tech information has made possible a much wider picture and large-scale global development [2].

Currently, there are several technologies used to conduct distillation, both for laboratory and industrial scale. However, most methods of distillation used are none other than variations of simple distillation.

## 1.2. Distillation theory

Distillation is a physical separation based on the vaporization of the different components of the mixture to be separated. Typically, a mixture is heated, vapours are produced, separated, and then condensed back into a liquid. As a result, each component can be separately recuperated in different fractions [6].

Distillation is based on the differences boiling points of the individual components and on the distributions of them between a liquid and gas phase in the mixture. Thus, the liquid mixture may have different boiling point characteristics depending on the concentrations of the components present in it.

The boiling point is related to the vapor pressure of liquid mixtures. The vapor pressure of a liquid at a particular temperature is the equilibrium pressure exerted by molecules leaving and entering the liquid surface. The liquid boils when the vapor pressure of mixture equals the surrounding pressure.

Furthermore, the ease with which a liquid boil depends on its volatility. Liquids with high vapor pressures will boil at lower temperatures. The greater the volatility of a liquid, the greater its tendency to pass from the liquid phase to the vapor phase and consequently the lower the boiling temperature will be. Distillation is accomplished because of the differences in the volatility of the components in a liquid mixture. The greater the difference, the better the separation. Furthermore, the vapor pressure and hence the boiling point of a liquid mixture depends on the relative amounts of the components in the mixture.

### 1.2.1 Vapour pressure

Some liquids will evaporate entirely over time if left in an open container at room temperature. However, this evaporation process can be significantly accelerated if the liquid is heated. As the liquid is heated, the molecules within it gain the energy to escape the liquid phase and transition into the gas phase in the form of bubbles. This phenomenon is called boiling. Consider a closed container of liquid. Initially, some of this liquid evaporates, but only until the rate of vaporization equals the rate of condensation. After this point is reached, there is no further change in the system, and the liquid and vapor are at equilibrium. Once this has been established, the pressure exerted by the vapor above the liquid is called the vapor pressure.

For a miscible mixture that forms a homogeneous solution, the vapor pressure of each component is dependent on the vapor pressure of the pure component and its mole fraction in the liquid mixture according to Raoult's law: $pA = pA^{*}xA$, where pA is the vapor pressure of one liquid component in a miscible liquid mixture, $pA^{*}$ is the vapor pressure of the pure liquid, and $xA$ is the mole fraction of that liquid in the mixture, which is equal to $nA/nt$. nA is the number of moles of the individual liquid in the mixture, and nt is the total number of moles of all the liquids in the mixture. The total vapor pressure above the miscible liquid mixture is equal to the sum of the partial vapor pressure of each component in it, which is known as Dalton's law. The vapor pressure of a liquid increases with temperature as more molecules gain kinetic energy to escape the

liquid phase to the gas phase. In a miscible mixture containing two liquids, the total pressure can be described as: $P = pA + pB$, where pA and pB are the vapor pressures of liquid A liquid B, respectively, above the mixture. $P$ is the total vapor pressure of the mixture. Combining the equations describes the relationship between the total vapor pressure of the solution and the mole fraction of the individual components: $P = pA^{*}xA + pB^{*}xB$.

On the other hand, in an immiscible mixture, where the components form a heterogeneous mixture, the vapor pressures of each component contribute independently to the total vapor pressure. Thus, the total vapor pressure is equal to the sum of the individual pure vapor pressures. In an immiscible mixture composed of two liquids, the total pressure is defined as the vapor pressure of the first liquid plus the vapor pressure of the second liquid: $P = pA^{*} + pB^{*}$. Furthermore, for this type of blends the steam distillation process is used.

### 1.2.2 Boiling point

The temperature at which a substance is changed from the liquid phase to the gas phase is known as the boiling point. In a mixture of miscible liquids, the solution boils when the total vapor pressure of the solution equals the atmospheric pressure. Thus, a mixture's boiling point occurs at a temperature between the boiling points of the two pure liquids. As the mixture is heated to its boiling point, some of the molecules escape the liquid phase and enter the gas phase. The temperature at which the first

bubbles start to form in a miscible solution that is being heated is the "bubble point" temperature. As result, the gas phase is rich with the molecules of the more volatile component, or the component with the higher vapor pressure and lower boiling point. The number of molecules that evaporate increases as more heat is applied. Thus, the liquid phase is rich with molecules of the less volatile component, or the component with the lower vapor pressure and higher boiling point. The temperature at which the first liquid drops begin to form during distillation is known as the "dew point".

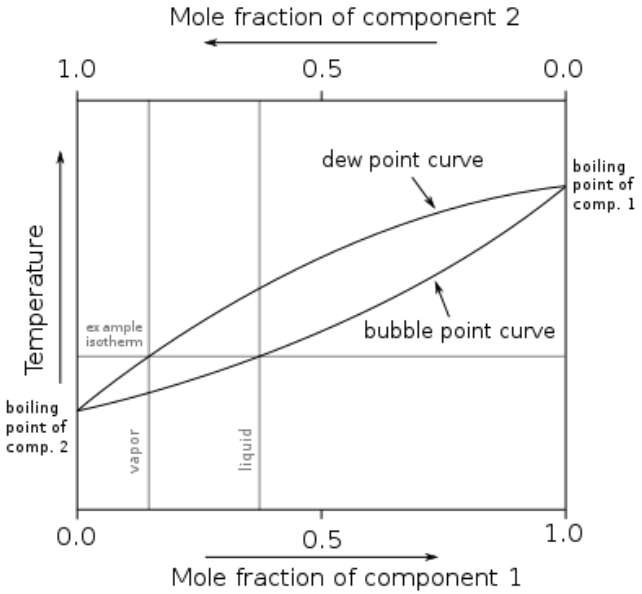


Figure 1: Vapor liquid equilibrium diagram.

### 1.2.3 Relative volatility

Relative volatility is a measure of the differences in volatility between two components, and hence their boiling points. It indicates how easy or difficult a particular separation will be. The relative volatility of component "$i$" with, respect to component "$j$" is defined by the following relationship: $a_{ij} = [x_i/y_i] / [x_j/y_j]$

where $y_i$ is the mole fraction of component "$i$" in the vapor, and $x_i$ is the mole fraction of component "$i$" in the liquid. We can conclude that if the relative volatility between two components is very close to one, it is an indication that they have very similar vapor pressure characteristics. This means that they have very similar boiling points and therefore, it will be relatively difficult to separate the two components by means of distillation.

## 1.3. Classification of distillation methods

Many distillation techniques exist, and the classification is based on the type of equipment used which, in turn, must be chosen based on the characteristics of the initial phase to be distillate. Base on the method, distillation can be carrying out on laboratory and industrial scale, with different types of equipment.

The most used techniques are:

- Simple distillation;
- Fractional distillation;
- Vacuum distillation;
- Steam distillation;
- Azeotropic distillation.

In simple distillation process, a volatile compound is evaporated and channelled through a distillation column into a condenser, where it is eventually captured. This technique can be used to separate mixtures containing non-volatile compounds such as particles and mixtures with differences of at least 70 °C in boiling points. Fractional distillation is used to separate mixtures with nearly equal relative volatility, and as small a difference in boiling points as 25 °C. Fractional distillation columns consist of an array of trays, in which the lowest and highest boiling liquids are collected at the top and bottom of the column, respectively. This process is commonly used in petroleum and food industries due to its better efficiency compared with simple distillation. Vacuum distillation

separates mixtures at a temperature much lower than their atmospheric boiling point, and thus it is mainly employed for high-boiling-point solvents such as dimethyl sulfoxide, benzyl alcohol, ethylene glycols, and glycerol. Steam distillation techniques is used to separate heat-sensitive compounds. Usually, steam is introduced to the mixture causing vaporization at lower temperature than the decomposition temperature of the heat-sensitive compound. This process is commonly used in the production of perfumes, essential oils, and waxes. At the end, azeotropic distillation is used in multipurpose solvent recovery systems for mixtures, whose separation is thermodynamically limited by the presence of azeotrope mixtures. Often, the addition of another component called an "entrainer" facilitates separation. An entrainer is a mass-separating agent that alters the relative volatility of mixtures and thus facilitates the separation process. For instance, benzene is often used as an entrainer to ease the separation of ethanol and water [7].

# 2. Steam distillation

Steam distillation is a particular type of distillation, used for the recovery of volatile compounds with high boiling point, from inert and complex matrices, solid or liquid, using saturated or superheated steam as separation and energy agent [8]. Steam distillation is unquestionably the most frequently used methods for the extraction of essential oil from plants [3].

## 2.1. Steam distillation process

The process uses steam as extraction agent to vaporize or liberate the volatile compounds from raw material. The compounds are volatilized by absorbing heat from steam and are transported to the steam where they are diffused. In case of essential oils, the release of volatile compounds present in the oil glands (cells) is due to the bursting of the oil cell walls caused by the increased pressure of the heat induced expansion of the oil cell contents. The steam flow acts as the carrier of the essential oil molecules. The resulting vapor phase is cooled and condensed prior to separating. The condensed distillate consists in a mixture of water and oil, the oil is separated from the water by means of a Florentine flask, which separates them based on their differing densities [9]. So, two products are obtained: oil and hydrosol. The volatile oil (essential oil) is in the upper phase and the hydrosol is in the bottom phase [3].

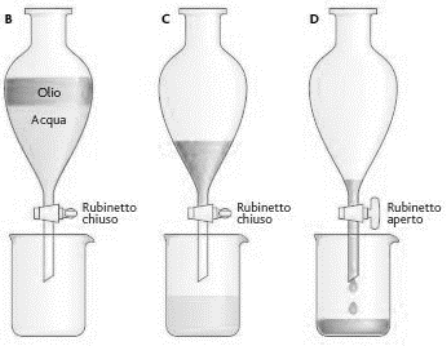


Figure 2: Separation of oil and water by Florentine flask, due to different densities.

Therefore, considering the manner of the contact between water and the original matrix is promoted, a terminology that distinguishes in two main different types of distillation has been proposed:

- Hydrodistillation,
- Steam distillation.

In the hydro distillation process, the plant material is completely immersed in boiling water. Sometimes, it is referred to as “indirect” steam distillation or “water distillation”. The characteristic feature of this process is that there is direct contact between water and the raw material. The plant material is soaked in water heated until it boils. It either by floating or by being completely immersed depending on its density. Agitation may be necessary to prevent agglutination. The

resulting steam from boiling water carries the volatile oils with it. Cooling and condensation subsequently separate the oil from the water.

Steam distillation is referred to a "direct" steam distillation. In this the matrix is supported on a perforated grid or screen inserted slightly above the bottom of the still. This scheme does not allow direct contact with water, whereas the boiler can be inside or outside the still. When it is outside, it is called “dry steam distillation” the steam is produced in a boiler and blown through a pipe into the bottom of the container. When the boiler is inside and steam is generated at the bottom of the container below the perforated tray, it can be called “water/steam distillation”, in this case both water and steam are utilized, also in this case the plant material is not in direct contact with water.

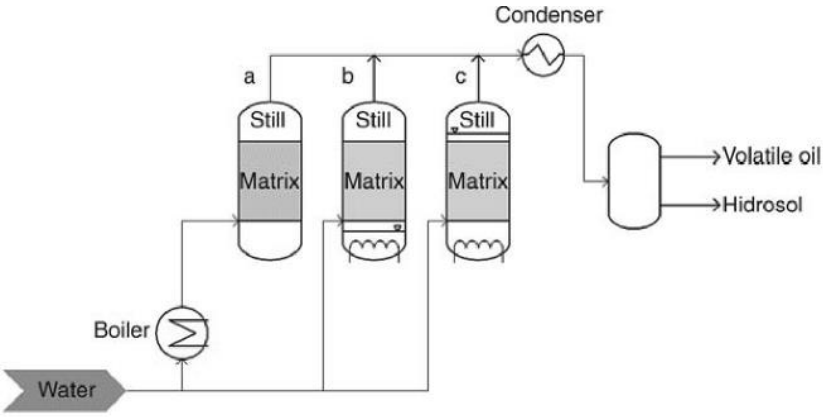


Figure 3: Generalized flowsheet of the different types of steam distillation (a: steam distillation or dry steam distillation; b: steam distillation or water/steam distillation; c: hydro distillation) [8].

## 2.2. Steam distillation theory

The theory of steam distillation is that two immiscible liquids (e.g., water and oil) A and B form two separate phases.

In this case, the vapor pressures of each component independently contribute to the total vapor pressure. Therefore, the total vapor pressure is equal to the sum of the individual pure vapor pressures:

$$p_{tot} = p_A + p_B$$

Consequently, the boiling temperature of the mixture is lower than the single boiling temperature of A and B. Since each individual component contributes independently, less heat is needed to raise the total vapor pressure to atmospheric pressure.

## 2.3. Steam distillation plant

Steam distillation plant of varying design abound all over the world. In some developing countries traditional and sometimes rather primitive methods are still being used. Industrialized countries employ technologically more evolved and complex equipment.

Steam distillation is the most commons used method at industrial scale [10]. A modern steam distillation plant consists of the biomass container (still pot), a cooling system (condenser), an oil separator, and a high-capacity steam generator.

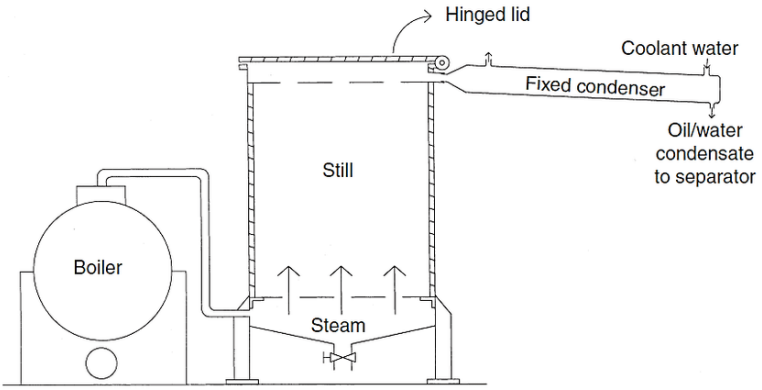


Figure 4: Steam distillation apparatus system at industrial scale [11] .

A high-capacity steam generator is usually used at industrial scale, while it doesn't use at laboratory scale. In this case, the steam is generated by a heat sources under the boiler, or most common is used the variant of steam distillation, the hydro distillation in a Clevenger type equipment.

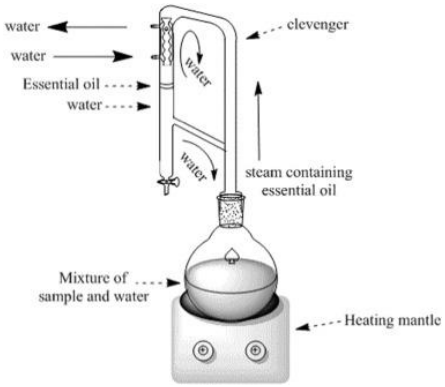


Figure 5: Hydrodistillation Clevenger apparatus system [12].

Perforated sieve-like plates are often used to separate the biomass and prevent compaction, thus allowing the steam unimpeded access to the raw material. The outlet for the oil-laden steam is usually incorporated into the design of the usually hemispherical, hinged still pot lid. The steam is then passed through the cooling system, either a plate heat exchanger or a surface heat exchanger, such as a cold-water condenser. The usually liquid condensate is separated into essential oil and distillation water in an appropriate oil separator such as a Florentine flask. The distillation water may, in some cases, be redistilled and any essential oil recovered dried and stored [3].

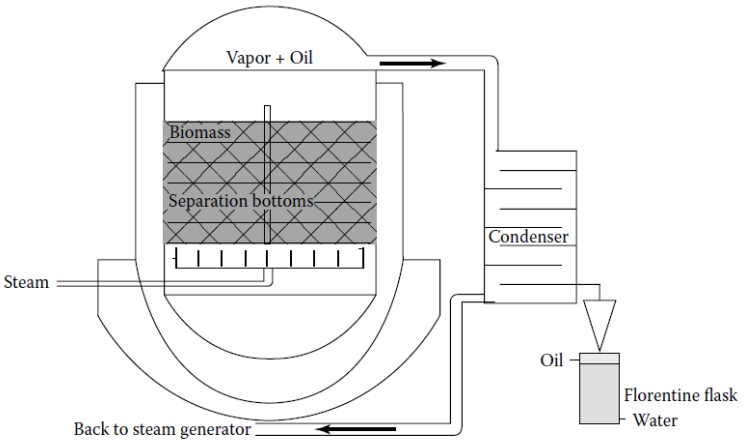


Figure 5: Cross section of hydro distillation apparatus system at industrial scale [3].

## 3. Use of steam distillation

Steam distillation and its variants are the traditional methods used for the extraction of bioactive compounds from natural sources [13]. It is the main conventional extraction technology for the recovery of essential oils from aromatic plants which is the main important product obtained from distillation process. However, five distinct products can be obtained during the aromatic plant distillation: essential oil, distilled biomass, ash from the distillation unit, residual water, and hydrosol. Essential oils and hydrosols are widely studied by research and used in several industries as the cosmetic, pharmaceutical and nutraceutical industries because their properties. However, some studies they highlighted and deepened the potential of the water residual inside the steam distillation plant too. In this sense, steam distillation could be apply as a method for the recovery value-added compounds from different organic matrices, for example food wastes [14]. Currently, in the contest of scientific research, steam distillation is considered an ancient method to use and often, it is only used to compare with other emerging extraction. However, steam distillation is worth developing for the advantages it has over other technologies such as, for example, the simplicity of execution and the relatively low cost of installation [15].

## 3.1. Bioactive compounds

Bioactive compounds can be defined as phytochemicals able to regulate metabolic functions leading to beneficial effects [16]. They are secondary metabolites found in small quantities in various plants and they are sourced naturally also from algae, foods, and by-products. It have been widely shown to impact human health and wellbeing positively [17], due to the many proprieties that they present like anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic antiallergenic, anti-inflammatory, and antimicrobial activities [18]. They have been used as ingredients to develop new functional food products with high rates of consumer interest and acceptance [19] and these could also be used as nutraceuticals in medicinal and pharmaceutical products [20]. For this reason, they are receiving much popularity in various commercial sectors such as food, chemical, and pharmaceutical industries [21]. All plant components, such as leaves, roots, barks, tubers, woods, gums or oleoresin, exudates, fruits, figs, flowers, rhizomes, berries, twigs, and the whole plant, produces active chemicals in smaller quantities and at variable concentrations [22]. Thus, the extraction process plays an important role in the recovery of bioactive compounds and the right choose of the extraction process is a crucial point to maximize the extract from the plants. However, there is a growing concern of the scientific community to develop environmentally sustainable methods for the extraction of bioactive compounds [33][23], since these methods reduce

or eliminate the use of organic solvents harmful to the environment, contributing to the safety, quality, and applicability of plant extracts [24].

The major class of bioactive compounds are:

- terpenes and terpenoids (approximately 25,000 types),
- alkaloids (about 12,000 types),
- phenolic compounds (about 8000 types) [25].

Bioactive compounds belong to one of the reported families, each of which has structural characteristics arising from the way in which they are built up in nature (biosynthesis). There are four major pathways for synthesis of secondary metabolites or bioactive compounds: Shikimic acid pathway, malonic acid pathway, mevalonic acid pathway and non-mevalonate (MEP) pathway [26].

In the detail, terpenes are characterized by a carbon skeleton of an isoprene unit, and terpenoid compounds are modified terpenes that may also contain other functional groups, commonly oxygen; examples of these compounds are limonene, carvone, squalene, humulene, lycopene, (α-, β-, γ-) carotene, and vitamin A [27].
Alkaloids are characterized by a nitrogen atom in a heterocyclic ring; in addition to carbon, hydrogen, and nitrogen, they may also contain oxygen, sulfur, and other elements; examples of alkaloid compounds are quinine, caffeine, piperine, nicotine, and theobromine [28].

At the end, the basic structural feature of phenolic compounds is one aromatic ring of hydroxyl groups; examples of phenolic compounds are phenolic acids, flavonoids, and tannins. These compounds have high antioxidant activity [29].

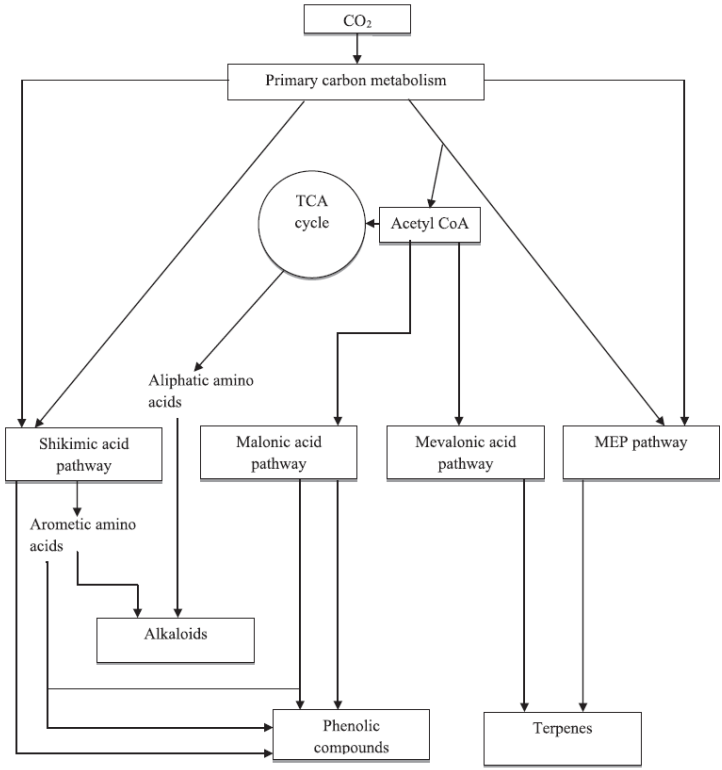


Figure 4: A simplified view of pathways for production of three major groups of plant bioactive compounds [26].

## 3.2. Terpenes and terpenoids

Terpenes and their oxygenated derivatives, terpenoids, are synthesized mainly by plants and are found in flowers, fruits, trees, and spices. They are one of the largest family of natural products synthesized as secondary metabolites [30]. Chemically, terpenes are grouped together because of their distinctive carbon skeleton. It consists of a basic five-carbon isoprene unit (2-methyl-1,3-butadiene). Terpenes generally are composed of two, three, four, or six isoprene units. These are called monoterpenes, sesquiterpenes, diterpenes, and triterpenes, respectively [31]. The terpenoids are the most important group of natural products as far as essential oils are concerned [3]. Chemically, the terpenes in the essential oils can be divided into two classes, the mono- and sesquiterpenoids, which differ in their boiling point range (monoterpenoids b.p. 140°-180°C, sesquiterpenoids b.p. .200°C) [32].

### 3.2.1. Essential oil

Essential oils can be given a simple definition as the predominantly volatile and odorous fraction isolated by some physical process from vegetable materials [33]. Pure essential oils are mixtures of more than 200 organic compounds classifiable in two groups: the volatile compounds, such as monoterpene and sesquiterpene hydrocarbons, and their oxygenated derivatives, which constituting of 90–95% of the oil in weight, and the non-volatile compounds such as hydrocarbons, fatty acids, sterols, carotenoids, waxes, and flavonoids, which constitute the 1-

10 % of the oil weight. Essential oils isolated and identified from the vast number of plant species amount to over 3000, and of these, several hundred are produced commercially. They are isolated from various plant components such as leaves, fruit, bark, root, wood, heartwood, gum, balsam, berries, seeds, flowers, twigs, and buds [33]. The amount of essential oil recovered in plants can vary from 0.01 to 10% of the total mass and several factors affect its availability such as agricultural factors, and the environment, climate, soil conditions, time of harvesting and postharvest handling prior to isolation. Furthermore, the extraction process plays a vital role in both the yield and quality of essential oils, especially because of their low amounts in plants [34]. Furthermore, is important to say that the conditions above mentioned can produce variations in the chemical profile of an essential oil of the same plant. Chemotypes of some plants vary substantially in the chemical composition of the essential oil. A prominent example of this is rosemary (Rosmarinus officinalis), of which there are three chemotypes. The borneol type, which contains a higher amount of camphor, is grown in Spain and the former Yugoslavia; the cineole type has a high cineole content and is grown in Africa; and the verbenone type is high in verbenone and is grown in France and Corsica [35].

Currently, the production and consumption of essential oils is rapidly growing due to consumer interest in their various biological effects, namely, antioxidant, antimicrobial, antiviral, and antitumor properties. For these reasons essential oils are of great interest in the food, cosmetic,

and pharmaceutical industries since their possible use as natural additives.

### 3.2.2. Hydrolate

Hydrosols are found in literature under various labels, including: hydrosol, hydroflorate, plant aromatic waste, aromatic water, floral water, essential aromatic water [36]. They are obtained during the process of essential oils extraction from aromatic plants. They are quite complex mixture containing traces of essential oils (usually less than 1 g/L) and several water-soluble components. They are constituted by the condensing water of the distillation process and by polar, oxygenated, odor imparting, hydrophilic, volatile oil components that form hydrogen bonds with water [37]. Contain numerous bioactive compounds, such as alkaloids, terpenes, and polyphenols.

For a long time, hydrosols have been defined as waste products of steam distillation process. Recently, they have attracted the attention of the scientific community because antioxidant, anti-inflammatory, antimicrobial properties. Several studies report the use of hydrosols in different fields of application such as cosmetic, pharmaceutical and food industries [38][39].

## 3.3 Phenolic compounds

Phenolic compounds are chemical metabolites derived from the secondary metabolism of plants characterized by their structures having at least one phenol unit. They are produced via the shikimic acid pathway in plants as secondary metabolites generally involved in plant adaptation to environmental stress conditions and they play different specific roles in the survival of plants as protection against UV rays, insects, and pathogens, among others [40][41].

Based on their chemical structures, phenolic compounds can be divided into different subgroups, such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids. They are widely distributed in the plant realm and are partly known for their numerous biological activities, mainly as antioxidants, but also as anti-inflammatory, anti-aging, cytotoxic and antitumor, antidepressants, and for these reasons, they are very attractive molecules in fields like pharmacy, cosmetics, agriculture, or agroindustry [42][43].

### 3.3.1. Phenolic compounds from agro-industrial by-products

Food industries produce a large amount of waste in all the phases of food life cycle, i.e., during agricultural production, industrial manufacturing, processing, and distribution. Industrial waste and by-products contain highly valuable components that can also be phytotoxic. For this, they

need specific processes to be disposed of properly that often require an economic expense or a highly skilled labor.

Over the past decade, advances in knowledge and the need to reduce environmental impacts have stimulated research into alternative ways to use these residues [44].

Some by-products result still rich in health promoting bioactive molecules, such as fiber, phenolic acids, flavonoids and anthocyanins [45]. Thus, the valorization of agro-industrial by-products is interesting from both environmental and economic aspects, since it contributes to the reduction of negative environmental impacts due to their disposal, and because of the recovery of high-added value compounds having many biotechnological applications [46].

Different agro-food industries generate different residues during their production. For example, coffee industries produce a large amount of residues, mainly coffee spent grounds and coffee silver skin [47]. Grape pomace represents the main by-products of the wine industry and it mainly consists of skin, pulp, and seeds [48]. Olive pomace, stone and olive leaves are the solid residues produced during the olive oil production [49].

These are some examples of residues produced by agro-food industries that each year produce millions of tons of organic residues. The current strategies to use of these residues in mainly three sectors biomass for food and feed, industrial bio-based products, and bioenergy. In the last

years, it is necessary to find alternatives application to valorize these types of residues and to permit to maintain the value of products and materials for as long as possible while minimizing resource use and waste generation. Several studies demonstrated these residues could contain interesting bioactive compounds that can be used in different fields of application. For example, spend coffee grounds contains high quantities of valuable compounds, such as caffeine and chlorogenic acids [44]. Caffeine is undoubtedly the most studied compound, given its widely known psychoactive effects and its exciting action on metabolism, on the other hand several studies report the strong antioxidant activity in vitro of chlorogenic acids, that are the main component in the phenolic fraction of green coffee seeds [50][51]. On the other hand, oleuropein, hydroxytyrosol, tyrosol and other compounds are contained in olive oil by-products and their numerous properties such as antioxidant, antimicrobial, anti-inflammatory are widely known and used such as dietary supplements, nutraceuticals, functional food ingredients or cosmeceuticals [52][53][54].

## 4. Pros and cons of steam distillation

Steam distillation is often considered to be a simple technique and generally, used only as a reference method to be compared with emerging extraction technologies. However, as report Galanakis et al., 2020 [14] this classical technology report several advantages, such as:

- the method generates organic solvent-free products,
- there is no need for subsequent separation steps,
- it has a large capacity for processing at the industrial scale,
- the equipment is inexpensive, and
- there is extensive know-how available for this technology.

On the other hand, the main disadvantages include:

- sensitive compounds could be thermally degraded and / or hydrolyzed,
- very long extraction times are required (1–5 h),
- high energy consumption.

## 5. Aim and structure of thesis

In the following thesis steam distillation process was studied and deepened. It was structured in four research with the overall aim to optimize and innovate the steam distillation process. Two main themes were addressed. The first concerned the optimization of the steam distillation process to minimize consumption and maximize the yield of the essential oil. The second, to innovate the process, understood as the possibility of expanding its field of application. In detail, the theme of optimization was addressed by two studies, focused on the investigation of the, of the factors affecting the yield of essential oil and, on the development the application of a photoionization detector for monitoring and controlling of the different products in several phases of the distillation process. The theme of "process innovation" was addressed with two further studies, on a possible alternative application of the process. In the detail, on the application of steam distillation as a sustainable method for the recovery of bioactive compounds with high added value from the waste of some agri-food industries.

Experimental part

# 6. Using a Plackett–Burman design to maximise yield of rosemary essential oil by distillation

Abbreviations

REO rosemary essential oil, ST steam distillation, HY hydro distillation, PBD Plackett–Burman design, ANOVA analysis of variance, GC-MS gas chromatography-mass spectroscopy.

## 6.1 Introduction

Rosemary *(Rosmarinus officinalis L. (Labiatae))* is a small, evergreen shrub that is found in the Mediterranean basin. Its health benefits have been recognised since antiquity, and it is part of many culinary traditions [55]. One popular use is essential oil extraction. Rosemary essential oil (REO) is widely used in the food, cosmetic and pharmaceutical industries, mainly due to monoterpene activity [56]. The current scientific literature reports antioxidant, antimicrobial, antiviral and antifungal properties [57][58][59][60][61][62]. These properties, and growing consumer demand for natural products with health benefits, mean that REO has become an important commercial product.

Rosemary essential oil represents about 1–2.5% of the total plant and, like other essential oils, both its quantity and quality are influenced by various intrinsic and extrinsic factors. In particular, its chemical composition differs according to the geographical area where the plant is grown, the climate, the part of the plant used, and the extraction method [56]. The extraction process plays a crucial role in the extraction of

bioactive compounds [17]. Among essential oils extraction methods, steam distillation and hydro distillation are the most used. In hydro distillation (HY), plant material is placed inside the distillation chamber and mixed with water. A heat source heats the mixture, and the steam that is created passes through a condenser that allows the recovery of essential oil. Steam distillation (ST) is carried out under the same conditions. The only difference is that, in this case, the plant is placed on a grid that is suspended above the water and steam passes through the plant material [63].

While such methods have been used since antiquity, few scientific studies have examined the operating conditions for an efficient and standard REO extraction. At the same time, several studies reported that current methods are very expensive in terms of energy and water consumption and have a negative environmental impact. Extraction conditions are therefore crucial in maximising REO recovery, and optimising parameters such as energy, time, raw materials and solvent [64].

Many factors contribute to HY and ST extraction processes. Distillation time is known to be important, and understanding its effect on yield and composition may increase production and decrease energy consumption [65][66]. Another relevant factor is the power of the heat source, as a more efficient system would decrease energy consumption and, consequently, lower extraction costs. The solid/ liquid ratio is another key factor. Optimising this ratio should maximise REO yield and reduce solvent use, improving efficiency both in economic and environmental

terms. Finally, one study [67] reports that pre-treatments, such as crushing raw material, can increase production. Furthermore, it is necessary to study the combined effect of these operational factors to understand the relations between them and final yield and maximise REO recovery. Currently, very few studies [68] have addressed the simultaneous effect of extraction factors during ST and HY.

In this context, we propose a novel approach. Specifically, we adopt a Plackett–Burman design (PBD) to test the simultaneous effect of several factors in the REO extraction process and understand the relative importance of each factor on final yield. The PBD belongs to the broad family of screening experiments, which are particularly important when there are too many factors to be able to test them all in depth. Screening experiments do not seek to develop an exhaustive model, but aim to identify the principal factors, which are generally set at only two levels. It should be noted that the approach removes any less-important factors and, thus, reduces data collection. At the same time, the PBD should be considered as a starting point for additional, full factorial experiments. We adopt this approach here. The aim of our study was, therefore, to identify the factors that have most influence on REO extraction yield when plant material is distilled using ST and HY extraction methods. Furthermore, we identify the optimal range for significant factors, with the aim of maximising REO yield and reducing energy, time and solvent consumption, and minimising waste.

## 6.2 Materials and methods

### Materials

Extractions were conducted using the leaves of the rosemary plant (Rosemary officinalis L. "Tuscan Blue") because, as reported Bousbia et al. (2009) [69], the essential oil secreted by glandular trichomes is mainly located in the plant's leaves. Fresh leaves were supplied daily by a local farm in Florence, Italy (Azienda Agricola Il Sorbo). Plants were cultivated in a mainly clay soil, managed with organomineral fertilisation. Average moisture content was 65.46% ± 5%. Trials were conducted in a commercial, stainless steel essential oil distiller (Spring 12 L, Albrigi Luigi Store, Italy) equipped with its own induction plate (Konig HA-INDUC-11). The internal solvent was commercial, deionised water, while tap water was used for the cooling circuit.

### Methods

The present study was divided into two parts. In the first part, a PBD was applied to identify the main factors influencing REO yield; hereinafter, this is referred to as experiment T1. This method was chosen as it is possible to simultaneously test a large number of factors at different levels, understand the importance of each factor compared with the others, and identify which ones to select to optimise the system [44]. We tested seven factors at two levels (−1 for the lower level and +1 for the higher level) for a total of eight extractions.

Experiment T1 tested seven factors at two levels (Table 1). Extraction method: steam distillation was designated the low level and hydro distillation as the high level; extraction time: 30 min and 120 min; cooling water flow rate: 1 L/m and 5 L/m; heating power: 600 W and 2000 W; and the ratio of rosemary leaves to deionised water: solid/ liquid ratio 1:2 and 1:6. Two leaf pre-treatments were considered: grinding and blast freezing. The former was performed by a screw extruder (Omega TE 22, Omega Foodtech) equipped with a 0.4 mm steel mesh (diameter 10 cm); here, the two levels were unground and ground leaves. In the blast freezing treatment, leaves were shock frozen using a blast freezer (Irinox Multifresh MF 25.1, Irinox S.p.a.) until a final temperature of about –18°C was reached, in a cycle lasting 20 minutes. According to the PBD these seven factors, at two levels, can be efficiently combined in eight runs, as reported in Table 2. Each run was performed in triplicate to estimate the factor's main effect, while no interactions could be estimated. In the second part of the study, hereinafter referred to as experiment T2, only the factors that had been found to have a significant effect on REO yield in T1 were tested and validated using a conventional, full factorial design. Specifically, T2 tested four extraction times (30, 60, 120 and 150 min) and two extraction methods (hydro distillation and steam distillation). Values for the solid/ liquid ratio, heat power and water flow were set by taking averages from T1, while neither pre-treatment was applied. Table 3 shows the full factorial design applied in T2. Three replicates were run of eight extractions, for a total of 24 extractions.

Sample preparation and extraction process

Extractions used one kilogram of fresh leaves. REO was recovered by decantation. In both T1 and T2, yield was calculated using the following equation:

$$R_{eo(\%)} = \frac{M_{eo}(g)}{M_d(g)} \times 100$$

where Reo = REO yield (% w/w), Meo = REO mass extracted (g) and Md = dry mass of rosemary leaves (g). REO samples from T2 were stored at −5°C until physical and chemical analyses were performed.

Physical analysis

Each REO sample was analysed. The following physical parameters were determined at 20°C [69]: specific gravity by weighing (BC310C, Orma s.r.l., Italy), refractive index using a benchtop refractometer (RMT, Optech Optical tech., Italy) and optical rotation using an optical polarimeter (PL1, Optech Optical tech., Italy).

GC-MS analysis

The GC-MS analysis used an Agilent 7820 gas chromatograph equipped with a 5975C mass selective detector operating in electron impact mode (Agilent Tech., Palo Alto, CA, USA). One µL of extract in solution was

injected into a split/ splitless injector operating in splitless mode. A Gerstel MPS2 XL liquid autosampler was used. Chromatographic settings were as follows: injector in splitless mode set at 260°C, J&W INNOWax column (30 m, 0.25 mm i.d., 0.5 µm df); oven temperature programme: initial temperature 40°C for 1 min, then 5°C min-1 until 200°C, then 10°C min-1 until 220°C, then 30°C min-1 until 260°C, held for 3 min. The mass spectrometer operated with electron ionisation of 70 eV, in scan mode in the m/z range 29–330, at three scans sec-1. Compounds were quantified using a calibration curve that was constructed by injecting known concentrations of authentic standards into the GC-MS. Deconvoluted peak spectra (obtained using the Agilent MassHunter software suite) were matched against the NIST 11 spectral library for initial identification. Kovats' retention indices were calculated for further confirmation, and compared with those reported in the literature for the chromatographic column used.

Statistical analyses

In T1, a multi-way analysis of variance (ANOVA) was performed for the seven factors, each at two levels, without interactions. The significance of main effects was checked with a conventional F-test ($p \leq 0.05$). Then, significant factors were compared and sorted in terms of their F-value. All statistical analyses were performed using R software.

In T2, a two-way ANOVA was used to test extraction time (four levels) and distillation method (two levels). In cases where the F-test was significant at the $p \leq 0.05$ level, multiple pairwise comparisons of group averages were checked for significance using the post hoc Tukey Honest Significance Difference test ($p \leq 0.05$).

## 6.3 Results and discussion

This study examined several factors involved in REO extraction. It considered the two, most-common extraction methods, hydro distillation and steam distillation. Two levels of extraction time, condenser water flow and heat power were examined, along with two leaf pre-treatments, namely grinding and blast freezing. The PBD identified the most important factors among the large number of factors involved in the extraction process. These factors have most influence on yield and should be optimised.

T1 - screening

Experiment T1 identified which factors were most important for REO yield. Table 4 reports yield for each run and shows that yield ranged from 0.26 to 0.90% (run 5 and run 1, respectively). The overall mean was 0.63%, with a standard error of 0.07% and coefficient of variation of about 32%. The magnitude of this variability reflects the importance of the tested factors, and our values are fully consistent with the recent literature [63][70][69][71]. Figure 1 compares extraction factors in terms

of their F-value after the multi-way ANOVA. This shows that yield was significantly affected by the grinding pre-treatment, extraction time and extraction method ($p \leq 0.05$). No significant effect was found for the other factors.

Figure 2 reports averages of the main effects of significant factors. Means for HY and ST are 0.55% ± 0.08% and 0.70% ± 0.04%, respectively. ST yield is significantly higher (by 0.15%) than HY. These results are consistent with the current literature. For example, Boutekedjiret et al. (2003) [63]reported REO yield of 0.44% by HY and 1.2% by ST. Similarly, Flamini et al. (2002)[72] reported REO yield of 0.91% for intermedia leaves and 1.44% for apical leaves by HY. On the other hand, a yield of 0.35% was reported by Bousbia et al. (2009)[69]; this difference is probably due to different plant cultivation conditions. Sartor et al. (2011)[71] reported a minimum yield of 0.231% and a maximum 0.605% by ST, and Conde-Hernández et al. (2017) [70] reported yield of between 0.35% and 2.35% using HY and ST methods.

We found a significant effect of extraction time on REO yield. The longer extraction time (120 min) corresponded to higher yield (0.74% ± 0.05%) compared with the shorter time (30 min) (0.51% ± 0.07%) (Figure 2). The literature confirms that REO yield increases with increasing extraction time (Rezzoug, 2005)[73]. In our HY condition, yield at 120 min was higher than the value reported in Bousbia et al. (2009)[69] and Okoh et al. (2010)[74] after 180 min (0.35% and 0.31%, respectively). Nevertheless,

several studies have reported higher yields for times ranging from 60 to 240 min [75][76][77] and our results are within these reported ranges.

Finally, our study identified that grinding had a statistical significant, but detrimental, effect on REO yield. Specifically, yield was higher when the treatment was not applied (0.74% ± 0.06%) than when it was (0.51% ± 0.06%). Conde-Hernández et al. (2017) [70] found similar results. The latter authors found that the use of whole rosemary leaves improved extraction compared with oil from ground leaves. The reduced yield obtained after grinding is presumably due to the temperature increase (from 15.4°C to 39.3°C) during grinding, which is likely to cause REO evaporation. In the light of these results, we tested the two factors that significantly affected REO yield (extraction time and extraction method) using a full factorial design. The remaining factors were set to the average level used in the PBD or were discarded because they were ineffective (blast freezing), or detrimental (grinding).

T2 - full factorial design

Based on the results of T1, the two extraction methods (HY and ST) and four distillation times (30, 60, 120, 150 min) were tested in order to determine the conditions that maximise REO yield. Overall, REO values were higher in T2 than T1 ($p \leq 0.05$). This was probably because 12 weeks elapsed between the two trials, and plants were at different stages of phenological growth [78]. In T2, the overall mean was 0.86%, with a

standard error of 0.05. The ANOVA highlighted a significant main effect of extraction time and extraction method, but their interaction was not significant. Figure 3 reports REO yields for the two extraction methods.

Yield was higher for ST (0.94% ± 0.07%) than HY (0.78% ± 0.06%), with an absolute increment of 0.16%, corresponding to a relative increment of 0.162. Our results can be compared with those of Boutekedjiretet al. (2003)[63] , who studied the influence of HY and ST methods on REO yield. The latter authors report values of 0.44% for HY and 1.2% for ST. Similarly, Conde-Hernández et al. (2017) [70] report lower yield for HY (0.35%) than ST (2.35%). It is likely that this variability is due to the different origin and agronomic conditions of plants.

Figure 4 reports REO yield as a function of extraction time. Yield was lowest (0.67% ± 0.09%) for the shortest extraction time (60 min), while highest yield was obtained at 150 min (1.00% ± 0.07%). However, there was no significant increase beyond 120 min (0.94% ± 0.11%).

Our results agree with the current literature. Boutekedjiret et al. (2003)[63] reported yields ranging from 0.42% to 1.2% at 120 min. Rasooli et al. (2008) [79] obtained REO yield of 1% at 90 min. Flamini et al. (2002) [72]recorded values between 0.91% and 1.44% at 120 min. Lowest yield (0.35%) was reported by Bousbia et al. (2009) [69] at 180 min.

However, studies concerning influence of extraction time on yield of rosemary essential oil are rather limited. Some authors have reported this aspect on essential oil yield but, the matrices they use differ from our

study. For example, Cannon et al. (2013) [66] studied the effect of extraction time on essential oil yield of peppermint *(Mentha×piperita L.)*, lemongrass *(Cymbopogon flexuosus Steud.),* and palmarosa *(Cymbopogon martinii (Roxb.))*. Zheljazkov et al. (2012; 2013) [72][73] examined lavender *(Lavandula angustifolia Mill.)* and oregano *(Origanum vulgare L.)*, and Sintim et al. (2015) [65] looked at dill (*Anethum graveolens L.)*. These studies report a significant effect of distillation time on essential oil yield for times ranging from 20 to 360 min, and our result is in the same range. More specifically, our study found optimal REO yield at 120 min.

Qualitative aspects

Physical analysis

Essential oil from T2 was subject to a physical analysis at 20°C. This focused on specific gravity, refractive index and optical rotation. Table 5 reports means and standard errors for main effects.

The results of the physical analysis were analysed with a two-way ANOVA (Table 5). No significant differences were found for specific gravity and optical rotation as a function of the extraction method (HY and ST) or the extraction time (60, 90, 120 and 150 min). A significant difference was found for the refractive index between the two extraction methods, but not extraction time. There are very few studies reporting the results of the physical analysis of REO. Bousbia et al. (2009) [69] reported REO

values for specific gravity, refractive index and optical rotation following hydro distillation. Our results were similar with respect to specific gravity and refractive index, but we found higher values for optical rotation.

GC-MS analysis

Table 6 reports the results of the two-way ANOVA on the chemical composition of our REO. Significant main effects were found, but no interactions. The main REO constituents were identified from the current literature. Our essential oils were characterised by high levels of borneol, 1,8-cineole and (-)borneol for all extraction times (60, 90, 120 and 150 min) and the two extraction methods. Levels were highest for borneol. Significant differences were observed between the two extraction methods for b-caryophyllene, ST values were higher (5.8% ± 0.4%) than HY (5.6% ± 0.4%).

## 6.4 Conclusions

In this study, PBD tested the simultaneous effect of several factors involved in REO extraction. The use of a PBD, certainly is among the most popular designs for industrial experimentations, was a novelty in the field of essential oils. The main scope was to maximize the yield of essential oil extracted and is an important novelty about this work, since the pertinent literature is mainly focused on the qualitative aspects. The results of this study showed that two factors had most effect on yield of REO: the extraction method and the extraction time. Specifically, yield was higher

for steam distillation than hydro distillation. With respect to extraction time, highest yield was obtained at 120 min, with no significant increase after this point. Physical and chemical analyses were conducted of the REO obtained and found significant differences in a few chemical components, and some physical aspects. In conclusion, the results obtained from the study are very interesting for the essential oil industry. These could allow to conduct more sustainable essential oil extractions, reducing waste and consumption, especially in terms of time, solvent, and raw material and, at the same time, to obtain the maximum extractable yield from the raw material of interest. This could allow the essential oils industry to reduce its environmental impact, and at the same time to reduce its economic impact, allowing them to be more competitive in essential oils extraction field.

Tables

Table 1: Factors tested using the Plackett–Burman Design (T1) at higher (+1) and lower (−1) levels.

| Factor | Level | |
|---|---|---|
| | −1 | +1 |
| Solid/liquid ratio | 1:2 | 1:6 |
| Extraction time | 30 min | 120 min |
| Heating power | 600 W | 2000 W |
| Grinding | unground | ground |
| Extraction method | steam distillation | hydro distillation |
| Blast freezing | not chilled | chilled |
| Cooling water flow | 1 L/min | 5 L/min |

Table 2: Factors tested in the PBD at higher and lower levels (T1).

| Extraction | Factor | | | | | | |
|---|---|---|---|---|---|---|---|
| | Solid/liquid ratio | Extraction time | Heating power | Grinding | Extraction method | Blast freezing | Cooling water flow |
| 1 | + | + | + | − | + | − | − |
| 2 | + | + | − | + | − | − | + |
| 3 | + | − | + | − | − | + | + |
| 4 | − | + | − | − | + | + | + |
| 5 | + | − | − | + | + | + | − |
| 6 | − | − | + | + | + | − | + |
| 7 | − | + | + | + | − | + | − |
| 8 | − | − | − | − | − | − | − |

Table 3: Factors tested in the full factorial design (T2).

| Extraction | Factor | | | | | | |
|---|---|---|---|---|---|---|---|
| | Solid/liquid ratio | Extraction time (min) | Heating power (W) | Grinding | Extraction method | Blast freezing | Cooling water flow (L/m) |
| 1 | 1:4 | 30 | 1400 | whole | HY | no | 3 |
| 2 | 1:4 | 60 | 1400 | whole | HY | no | 3 |
| 3 | 1:4 | 120 | 1400 | whole | HY | no | 3 |
| 4 | 1:4 | 150 | 1400 | whole | HY | no | 3 |
| 5 | 1:4 | 30 | 1400 | whole | ST | no | 3 |
| 6 | 1:4 | 60 | 1400 | whole | ST | no | 3 |
| 7 | 1:4 | 120 | 1400 | whole | ST | no | 3 |
| 8 | 1:4 | 150 | 1400 | whole | ST | no | 3 |

Table 4: Results of T1 (PBD). Means and standard errors are shown (n=3).

| Extraction | REO yield (%) | |
|---|---|---|
| 1 | 0.90 | ± 0.15 |
| 2 | 0.67 | ± 0.06 |
| 3 | 0.70 | ± 0.16 |
| 4 | 0.65 | ± 0.07 |
| 5 | 0.26 | ± 0.04 |
| 6 | 0.40 | ± 0.02 |
| 7 | 0.72 | ± 0.05 |
| 8 | 0.70 | ± 0.12 |

Table 5: Physical analysis (specific gravity, refractive index and optical rotation) for each extraction method (HY: hydro distillation and ST: steam distillation) and extraction time (60, 90, 120 and 150 min). Means and standard errors of main effects are shown (n = 3). Letters (a, b) indicate significance levels.

| Physical analysis | Extraction method | | Extraction time (min) | | | |
|---|---|---|---|---|---|---|
| | HY | ST | 60 | 90 | 120 | 150 |
| Specific gravity | 0.83 [a] ± 0.17 | 0.82 [a] ± 0.17 | 0.83 [a] ± 0.19 | 0.83 [a] ± 0.19 | 0.82 [a] ± 0.18 | 0.81 [a] ± 0.20 |
| Refractive index | 1.471 [a] ± 0.307 | 1.472 [b] ± 0.307 | 1.472 [a] ± 0.347 | 1.471 [a] ± 0.347 | 1.471 [a] ± 0.329 | 1.471 [a] ± 0.368 |
| Optical rotation | 5.20 [a] ± 0.31 | 4.49 [a] ± 0.31 | 5.78 [a] ± 0.35 | 5.09 [a] ± 0.35 | 4.10 [a] ± 0.34 | 4.41 [a] ± 0.39 |

Table 6: Chemical composition (≥ 1%) of rosemary essential oil as a function of the extraction method (HY: hydro distillation and ST: steam distillation) and extraction time (60, 90, 120 and 150 min). Means and standard errors of main effects are shown (n = 3). Letters (a, b) indicate significance levels.

| Compound | Extraction method (%) | | Extraction time (%) | | | |
|---|---|---|---|---|---|---|
| | HY | ST | 60 | 90 | 120 | 150 |
| (-)borneol | 24.7 [a] ± 0.9 | 25.0 [a] ± 0.9 | 24.3 [a] ± 1.2 | 22.0 [a] ± 1.8 | 25.0 [a] ± 4.4 | 24.8 [a] ± 0.9 |
| 1,8-Cineole | 18.7 [a] ± 0.2 | 18.7 [a] ± 0.2 | 19.0 [a] ± 0.4 | 18.5 [a] ± 0.4 | 18.7 [a] ± 1.0 | 18.7 [a] ± 0.2 |
| a-pinene | 10.4 [a] ± 1.0 | 10.3 [a] ± 1.0 | 10.1 [a] ± 1.3 | 14.4 [a] ± 2.1 | 10.4 [a] ± 4.7 | 10.3 [a] ± 1.0 |
| camphor | 7.9 [a] ± 0.3 | 7.8 [a] ± 0.3 | 8.9 [a] ± 0.6 | 7.0 [a] ± 0.6 | 7.9 [a] ± 1.6 | 7.9 [a] ± 0.3 |
| camphene | 5.6 [a] ± 0.1 | 5.7 [a] ± 0.1 | 5.6 [a] ± 0.1 | 5.6 [a] ± 0.1 | 5.7 [a] ± 0.6 | 5.7 [a] ± 0.1 |
| b-caryophillene | 5.6 [a] ± 0.4 | 5.8 [b] ± 0.4 | 5.7 [a] ± 0.6 | 5.2 [a] ± 0.8 | 5.7 [a] ± 1.8 | 5.7 [a] ± 0.4 |
| bornyl acetate | 4.6 [a] ± 0.4 | 4.6 [a] ± 0.4 | 4.2 [a] ± 0.6 | 4.5 [a] ± 0.7 | 4.4 [a] ± 1.8 | 4.6 [a] ± 0.4 |
| b-pinene | 4.0 [a] ± 0.2 | 4.0 [a] ± 0.1 | 4.0 [a] ± 0.2 | 4.4 [a] ± 0.3 | 4.1 [a] ± 0.6 | 4.0 [a] ± 0.1 |
| limonene | 3.0 [a] ± 0.1 | 3.0 [a] ± 0.1 | 3.2 [a] ± 0.1 | 2.9 [a] ± 0.1 | 3.1 [a] ± 0.4 | 3.0 [a] ± 0.1 |
| 3-Carene | 2.0 [a] ± 0.1 | 2.0 [a] ± 0.1 | 2.3 [a] ± 0.2 | 1.7 [a] ± 0.2 | 2.0 [a] ± 0.5 | 2.0 [a] ± 0.1 |
| linalool | 1.9 [a] ± 0.1 | 1.9 [a] ± 0.1 | 1.7 [a] ± 0.1 | 2.1 [a] ± 0.2 | 1.9 [a] ± 0.4 | 1.9 [a] ± 0.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| a-terpineol | 1.8 [a] ± 0.1 | 1.8 [a] ± 0.1 | 1.7 [a] ± 0.1 | 1.7 [a] ± 0.1 | 1.8 [a] ± 0.4 | 1.8 [a] ± 0.1 |
| terpinolene | 1.3 [a] ± 0.1 | 1.3 [a] ± 0.1 | 1.1 [a] ± 0.2 | 1.5 [a] ± 0.3 | 1.2 [a] ± 0.6 | 1.3 [a] ± 0.1 |
| myrcene | 1.2 [a] ± 0.0 | 1.2 [a] ± 0.0 | 1.3 [a] ± 0.1 | 1.2 [a] ± 0.0 | 1.3 [a] ± 0.2 | 1.2 [a] ± 0.0 |
| p-cymene | 1.2 [a] ± 0.1 | 1.2 [a] ± 0.1 | 1.3 [a] ± 0.1 | 1.0 [a] ± 0.2 | 1.2 [a] ± 0.4 | 1.2 [a] ± 0.1 |
| 4-ol-terpinen | 1.2 [a] ± 0.1 | 1.2 [a] ± 0.1 | 1.3 [a] ± 0.1 | 1.0 [a] ± 0.2 | 1.2 [a] ± 0.4 | 1.2 [a] ± 0.1 |

Figures

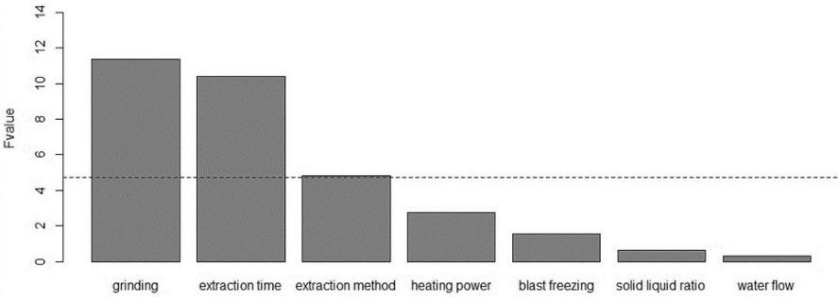


Figure 1: Bar chart of F-values for factors affecting REO yield in T1. The dashed horizontal line represents the F-value corresponding to a significance level of 95% ($p \leq 0.05$).

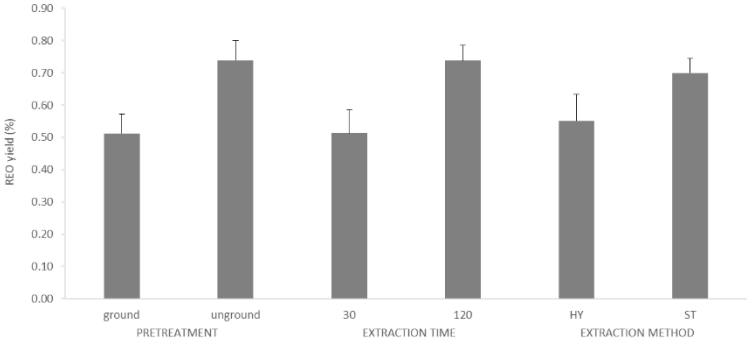


Figure 2: Bar chart of REO yield (%) as a function of the significant factors identified in T1. Pre-treatment (ground and unground), extraction time (30 and 120 min), extraction method (HY: hydro distillation and ST: steam distillation). Overall means and standard errors are reported.

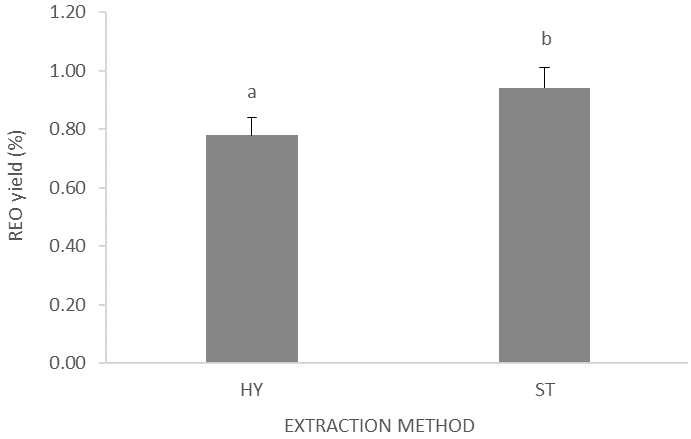


Figure 3: Bar chart of REO yield (%) for hydro distillation (HY) and steam distillation (ST) methods. Overall means and standard errors are reported. Letters (a and b) indicate the significance level.

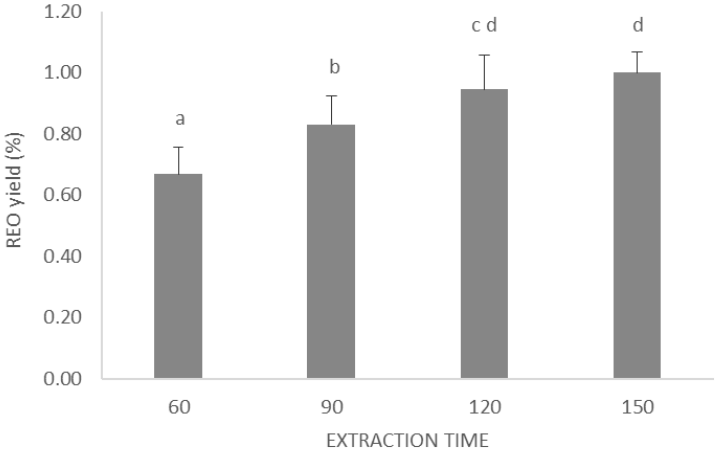


Figure 4: Bar chart of REO yield (%) as a function of extraction time (60, 90, 120, 150 min). Overall means and standard errors are reported. Letters (a, b, c, d) indicate significance levels.

# 7. A conventional VOC-PID sensor for a rapid discrimination among aromatic plant varieties: classification models fitted to a rosemary case-study

Abbreviations

PID photoionization detector, VOC volatile organic compound, PCA principal component analysis, CA cluster analysis, SVM support vector machine, ANN artificial neural network, GC-MS gas chromatography-mass spectrometry

## 7.1. Introduction

Aromatic plants are characterized by a peculiar aroma. Specialized secretory cells that are present in specific structures, called glandular trichomes [80][81], produce an amazing diversity of volatile organic compounds (VOCs). VOCs are characterized by low molecular mass (between 50 and 200 Da), a low boiling point and high vapor pressure. A consequence of their high volatility is a marked tendency to pass from the liquid to the vapor state, which allows them to disperse in the biosphere and act over long distances [82]. Plants that produce VOCs, in particular aromatic plants, are increasingly sought after, to the point that they have become an important economic resource worldwide, on a par with traditional food, forage, and fibre species. Among these plants, rosemary is widely cultivated, mainly due to its essential oil, which has high commercial value [56]. Rosemary leaf essential oil is dominated by monoterpenes (> 90%), hydrocarbons and oxygenated compounds. Among rosemary species, several chemotypes can be distinguished,

based on the predominant constituents of the essential oil. Examples include: 1,8-cineole; 1,8-cineole/ α-pinene/ camphor; myrcene; α-pinene/ verbenone/ bornyl acetate; and 1,8-cineole/ borneol/ p-cymene [83][84]. Furthermore, over 20 different types, varieties or cultivars can be distinguished as a function of morphological descriptors [83] such as the calyx, corolla, inflorescence and the presence of glandular trichomes [85], along with whether the plant prostrate, its leaf size and flower characteristics [86][87]. Given this diversity, the ability to easily discriminate between the great variety of rosemary plants would be a step forward, in order to process specific, high-quality products. Thus, in the following, we develop a simple method to measure VOCs from rosemary leaves, in order to classify these aromatic plants.

The main analytical method used to measure VOCs is gas chromatography-mass spectrometry (GC–MS). It is widely use because of its high sensitivity, accuracy, reproducibility and overall robustness. However, disadvantages include the cost of equipment, the complexity of the operation and the long analysis time [88]. Depending on the aim of the analysis, a possible alternative is the use of a standalone photoionization detector (PID). These sensors are simple to use, inexpensive and the analysis only takes a few seconds. However, in turn, they also have some disadvantages. For example, a PID sensor can only measure a VOC concentration, and no information is provided about the chemical composition. In general, PID technology is design to characterize

the overall profile of a VOC mixture into a digital fingerprint, rather than quantify individual compounds [89].

However, the growing need for a rapid method to analyse VOC concentrations has stimulated interest in more wieldy instruments in terms of time, use and cost. The current literature contains reports from many studies that test the use of rapid VOC measurement instruments, in particular electronic noses [90]. To the best of our knowledge, no studies have investigated the use of a standalone PID to classify plants based on their VOC emissions. Unlike electronic noses, which use a pattern recognition algorithm to analyse several signals from an array of semi-selective gas sensors, we adopt a different approach. Specifically, we explore the response of a single PID to a wide range of VOCs, and the use of temporal data acquisition. The underlying idea in our approach is that signal kinetics in the time domain may be a function of the morphological (shape, dimension, histological classification) and physiological (essential oil composition) characteristics of rosemary plants. Our hypothesis is that temporal kinetics of VOC emissions (emanation) from rosemary leaves has the potential to be a fingerprint for specific varieties. From this, it follows that the emission pattern could be used for classification purposes. Two varieties of rosemary plants were studied. In a first step, PID technique was used to measure VOCs from the two varieties. Then, essential oils were extracted from each of the two varieties and characterized by GC-MS. The purpose of this step was to establish a baseline for differences between the varieties under

examination. The analysis focused on whether the PID method would provide the same results as the GC-MS approach. PID data were analysed using advanced statistical models to assess and verify the sensor's classification capacity.

## 7.2. Materials and methods

### Materials

#### Rosemary samples

Two varieties of rosemary (Rosmarinus officinalis L. "Prostratus" and "Erectus") were bought at a local plant nursery in Florence, Italy (Società Cooperativa Agricola di Legnaia). Only fresh leaves were used and mean moisture content was 76.3%.

#### Essential oil extraction

Essential oil was extracted using petroleum ether (BAKER ANALYZED™ A.C.S. Reagent, J. T. Baker™). Samples were centrifuged and filtered to remove impurities and stored at 4°C until analysis.

#### The photoionization detector

A photoionization detector (PID) (PID-AH2, Alphasense Ltd, UK) was used to determine the range of VOCs in the headspace over fresh rosemary leaves. The sensor was equipped with a 10.6 eV krypton lamp, and was

sensitive to all target gases with photoionization potential less than or equal to this ionization threshold. The main features of the sensor, when used with isobutylene as the calibration gas, are as follows: minimum detection level 1 ppb; linear range (3% deviation) 40 ppm; overrange 40 ppm; sensitivity within the linear range > 25 mV / ppm; full stabilization time (minutes to 20 ppb) 5; warm up time (time to full operation) 5 s; offset voltage 46–60mV; and response time in diffusion mode <3 s. The main technical features are: power consumption 85 mW at 3.2 V (transient <300 mW for 200ms); supply voltage 3.2–3.6 V DC; output signal as voltage ranging from the offset (minimum 46 mV) to the supply voltage minus 0.150 V.

The sensor was driven by a dedicated electronic interface (LabQuest2, Vernier, USA), which supplied it with 3.6 V DC power and contextually recorded the output DC voltage signal at a set acquisition frequency of 1Hz (one reading per second). Output voltage signals were acquired as a function of time during each set of measurements, and stored locally by the software. Finally, all data were transferred to a computer and processed with Excel (Office 365, version 18.2008.12711.0).

## Methods

### Preparation of rosemary samples

Each plant was defoliated, and the leaves were homogenized to reduce internal variability. A total of 18 plants were studied, nine for each variety.

Three replicates were conducted per day, alternating the two varieties each day, over a total of six days.

Essential oil extraction

Essential oils were extracted from each plant. First, 5 g of fresh leaves was extracted using 45 ml of petroleum ether. For each sample, three washes were applied using 15 ml of solvent. Samples were then agitated using a vortex laboratory shaker. The extracted oils were stored in dark glass bottles at 4°C until analysed by GC–MS.

PID measurements

A bespoke measuring system was fitted to the PID sensor. The system consisted of a cylindrical thermostated (20 ± 1°C) reading chamber with nominal volume 100 ml, made from polypropylene (Sarstedt, Germany). The chamber was closed with a perforated screw cap, to allow a close connection with the sensor. Once the cap was screwed on to the chamber, the sensing side of the sensor was exposed to the air volume under measurement. The sensor was used in diffusion mode, above about 1 g of leaves. Before each measurement, it was ventilated for 5 minutes with ambient air using a laboratory fan at about 1600 L min−1, which established a steady baseline (it has humidity sensitivity that is near to 0). Then, the tested leaves were introduced into the measuring chamber and readings were recorded for 900 s (long enough for the

sensor response to become stable). At the end of each measurement, the sensor–cap assembly was unscrewed from the measuring chamber and reventilated before the next measurement. Figure 1 shows the experimental setup.

GC-MS analysis

The GC-MS analysis used an Agilent 7820 gas chromatograph equipped with a 5975C mass selective detector operating in electron impact mode (Agilent Tech., Palo Alto, CA, USA). One µL of extract in solution was injected into a split/ splitless injector operating in splitless mode. A Gerstel MPS2 XL liquid autosampler was used. Chromatographic settings were as follows: injector in splitless mode set at 260 °C, J&W INNOWax column (30 m, 0.25 mm i.d., 0.5 µm df); oven temperature programme: initial temperature 40 °C for 1 min, then 5 °C min−1 to 200 °C, then 10 °C min−1 to 220°C, then 30°C min−1 to 260°C, held for 3 min. The mass spectrometer operated at 70 eV in scan mode, in the m/z range 29–330, at 3 scans sec−1. Compounds were quantified using a calibration curve that was constructed by injecting known concentrations of authentic standard into the GC-MS. Deconvoluted peak spectra (obtained using the Agilent MassHunter software suite) were matched against the NIST 11 spectral library for initial identification. Kovats retention indices were calculated for further confirmation and compared with those reported in the literature for the chromatographic column used.

## Statistical analysis

### Essential oil characterization

A one-way analysis of variance (ANOVA) was performed for the data collected by GC-MS, in order to characterize qualitative aspects of the essential oils obtained from the two varieties. A conventional F-test tested for significant main effects ($p \leq 0.05$). The ANOVA was performed using R software (version 3.6.0 for Windows).

### PID measurements

Data (voltage versus time) collected by the PID were transferred to a computer. Each sample was tested in triplicate, and the resulting data were normalized as a function of the mass of leaves in the measuring chamber in each run. Therefore, signal responses were averaged over the three replicates (runs) corresponding to each read time. Then, for the purposes of drift compensation, the PID's base reading was removed. Specifically, at each read time, the voltage baseline was subtracted from the recorded response.

Then, the signal was regressed over time, in order to identify the best non-linear model, fit for the data. This was done for each data series using CurveExpert 1.40 software (D. Hyams, 1995–2009, Microsoft Corporation). The latter is a regression/ fitting tool that uses the Levenberg–Marquardt method to solve nonlinear regressions, making it possible to rank best-fit models on the basis of the coefficient of

determination. All the tested data series proved to be well-fitted by a three-parameter exponential model, with r2 ranging from 0.95 to 0.99:

$$signal = \alpha(\gamma - (\exp - \beta t))$$

where, $\alpha$, $\beta$, and $\gamma$ are the model parameters, and t is the acquisition time.

This model was then reapplied to each replicated data series and rechecked for goodness of fit using SigmaPlot 10.0 software (SystatSoftware Inc., USA). This software package adopts an iterative approach that is based on the Levenberg–Marquardt algorithm, and aims to minimize the sum of the squared differences between observed and predicted values of the dependent variable during regression testing. An example of the output of this analysis is reported in Table 2.

In addition to the exponential model, four other parameters were used to describe the signal versus time kinetic: i) the grand mean (GM) and, ii) the corresponding standard deviation (GMsd), both computed over the entire reading time (1–900 s); iii) the maximum recorded value (Max); and iv) the area under the curve (AUC). The latter was calculated following a numerical quadrature approach, as the definite integral of signal values between 1–1800 s reading time.

Following the application of this procedure, seven features of the sensor signal were identified and used in the subsequent multivariate pattern recognition techniques:

- $\alpha$, $\beta$, and $\gamma$ (the exponential model parameters)

- GM (the grand mean of readings computed over a data series acquisition time)
- GMsd (standard deviation of readings computed over a data series acquisition time)
- Max (the maximum signal reading)
- AUC (the area under the curve).

The complex set of data collected by the PID technique were then interpreted with several statistical models: principal component analysis (PCA), support vector machine (SVM), cluster analysis (CA), and artificial neural networks (ANN).

## Data analysis

### Principal component analysis

PCA is the most widely-used pattern recognition method applied to sensor data [91]. This linear, unsupervised technique is used to analyse, classify and reduce the dimensionality of numerical datasets in a multivariate problem [92]. PCA was performed using the XLSTAT Premium software package.

### Cluster analysis

CA is an exploratory multivariate technique that is used to explore the data structure. It seeks to identify natural groupings among data points, and present these groupings in the form of a hierarchical tree or

dendrogram [93]. It is based on the determination of the distance between objects (degree of similarity/ dissimilarity), and the application of an agglomerative (amalgamation) method to establish clusters of n-objects. The CA was performed using R software (version 3.6.0 for Windows).

Support vector machine

The SVM technique is one of the most widely-applied classification methods in electronic nose technologies. It was developed for the linear classification of separable data, but can be applied to nonlinear data with the use of kernel functions. The principal idea is that separating classes by a particular hyperplane maximises a quantity called the margin. The margin is the distance from the nearest point in the dataset to a hyperplane separating the classes. Four kernel types are available: linear, polynomial, radial basis function and sigmoid. Different kernel functions were tested to check the robustness of the classifier model (Sanaeifar, 2014). The SVM analysis was performed using XLSTAT Premium software.

Artificial neural networks

ANN are mathematical/ computational programs that are modelled on the central nervous system (neural) networks. These networks are composed of interconnecting nodes (neurons) that can recognize patterns and relationships in data [94]. In principle, an ANN is constituted of many artificial neurons, which are organised into layers, together forming a network. An artificial neuron is a simple processing element, which, like biological neurons takes signals from several inputs to produce

one output [95]. JustNN software (version 4.0) was used for the analysis. JustNN is a free software package; it is easy to use, frequently updated, and performs well. The model is based on three layers of nodes: input, hidden and output. Based on the inputs in the first layer, and the outputs from the third layer, the model develops a number of hidden nodes that comprise the middle layer [96].

## 7.3. Results

Our results can be divided into three, broad areas. The first was the GC-MS characterization of essential oil. Here, the aim was to differentiate between the two varieties of plants and establish a baseline for the rest of the study. Then, a generic PID was used to detect VOCs from the same batches of fresh leaves, in order to try to classify the two varieties based on their emissions. Finally, the two sets of measurements were compared. The complex dataset was processed with advanced statistical models (PCA, CA, SVM and ANN) to validate PID measurements.

Essential oil characterization

The ANOVA identified a significant main effect with respect to qualitative aspects of the two varieties. A total of 26 components, accounting for > 95% of the essential oil were identified. Table 1 reports the percentages of the main compounds (≥ 1%) found in the two essential oils. This highlights that “Erectus” is characterized by a high level (30.40 ± 1.71%) of bornyl acetate followed by alpha-pinene (13.17 ± 0.85%). “Prostratus”

is characterized by alpha-pinene (35.30 ± 1.50%) followed by bornyl acetate (13.31 ± 0.62%). Thus, alpha-pinene and bornyl acetate are the compounds with the highest differences between the two varieties. They are followed by camphor (8.28 ± 0.42% and 8.10 ± 0.35 %), and camphene (8.07 ± 0.30% and 10.02 ± 0.70%) for "Prostratus" and "Erectus", respectively. In general, significant differences were seen between the two varieties for all of the reported compounds. Both the identified compounds and their percentages are consistent with the results reported in the current literature [97][98].

PID measurements

Goodness of fit, representative of all of the analysed data series is reported in Table 2 (see Figure 2 for a graphical representation). Table 2 shows that the model converges after 87% of iterations, with an adjusted R2 of 0.9956 and a standard error of estimate of 0.0119. All three parameters describing the model were significantly different from zero at $p \leq 0.001$, and the ANOVA of the regression proved to be significant at the same level. As stated earlier, these results were representative of all of the tested data series; thus, the chosen model fits the PID data.

Data analysis

Principal component analysis

A PCA was run on the dataset. The first two components (F1, F2) explained nearly 82.59% of total variance, accounting for 60.59 and

22.00%, respectively. The scatter plot shown in Figure 3 reports the projection of variables on F1 and F2 axes. Here, the aim was to determine which variables influenced the distinction between the two varieties. A visual inspection shows the "Erectus" variety in the right quadrant, and "Prostratus" in the left; hence the two varieties can be clearly distinguished. Figure 3 highlights that the variables that have the greatest influence on the distinction between the two varieties are MAX, AUC, GM and GMsd (for F1), and $\alpha$,$\beta$, and $\gamma$ (for F2).

Cluster analysis

A hierarchical CA was run on the dataset, using the squared Euclidean distance as a measure of similarity and Ward's method as the amalgamation rule. The dendrogram is reported in Figure 4. This analysis subdivided the two rosemary varieties into two large groups. The first subgroup contains "Prostratus" plants, and the other "Erectus" plants. Thus, the two varieties were clearly classified by CA.

Support vector machine

The SVM technique was used to discriminate between rosemary varieties based on PID sensor data. Different kernel functions were tested to check the robustness of the classifier model, and a linear kernel was chosen. Cross-validation was applied to all data in the six test cycles. Classification was good, and accuracies of training and validation samples were found to be 100 and 83.33%, respectively. Results are reported in Tables 3 and 4.

Artificial neural networks

The software was trained using the backpropagation error algorithm with the following settings: learning rate 0.7; momentum 0.80. The target error was fixed when the average error was 0.01. One hundred cycles were run before the validation cycle, and 100 validation cycles were run. The learning process ended when all the validation samples were within 10 % of the validation error. The analysis was run with 12 training samples, and six samples were randomly chosen for validation. The process ended after 2000 cycles, with an average error rate of 0.000002. Validation accuracy was 83.33%. Figure 6 is a graphic visualization of the neural network in justNN. In Figure 6, the yellow circles represent the seven inputs: Max, GM, GMsd, AUC and A, B, C (corresponding to $\alpha$, $\beta$, and $\gamma$, respectively). The light blue circles are hidden nodes, and the purple node is the output, the rosemary variety. Lines between nodes represent relations between levels. The thickness of the line highlights the importance of the relation between variables. The thicker the line, the stronger the relationship. Red and green lines represent negative and positive relationships between inputs and outputs, respectively. Finally, the absolute and relative importance of each input column is reported in Figure 7. Importance is the sum of the absolute weights of connections from input nodes to all of the nodes in the first hidden layer. Inputs are shown in descending order of importance.

## 7.4. Discussion

The first encouraging result from our study was that the PID could measure VOC emissions from rosemary leaves. The second positive result is that it recorded different signals for the two varieties. This demonstrated that it was possible to obtain a sort of 'fingerprint' for each variety studied. As the two varieties were correctly identified, this confirmed that the PID can be used for classification purposes, which was the main aim of the present study. Furthermore, data analyses using different statistical models were satisfactory. Here, the aim was to interpret the dataset recorded by the PID. The two unsupervised methods (PCA and CA) were able to distinguish the two varieties. The PCA accurately differentiated the two varieties: 82.59 % of total variance was explained by F1 and F2 (60.59% and 22.00%, respectively). The variables that were most influential in distinguishing the two varieties were MAX, GM, AUC and GMsd (for F1), and $\alpha$, $\beta$, and $\gamma$, (for F2). The same result was achieved by CA. Here, two homogeneous groups were obtained: "Erectus" and "Prostatus" varieties.

Similar results were obtained using supervised methods. The ANN found a classification validation rate of 83%. Here, the variables that most influenced training were MAX, AUC, GM, GMsd, $\alpha$, $\beta$, and $\gamma$, in descending order of importance. A good classification rate was also obtained by SVM analysis, where validation accuracy was found to be 83.33 %. Hence, the two rosemary varieties were clearly separated by all of the models with a

high degree of accuracy. These findings verify the usefulness of the PID as a classification tool.

## 7.5 Conclusions

This study investigated whether a general-purpose, cheap, and easy-to-use PID could distinguish between two varieties of rosemary plant. The device was able to record two different VOC emission base signals, and provided a sort of 'fingerprint' for each variety. Advanced statistical models (PCA, CA, SVM and ANN) were applied to validate PID performance. Each model found high classification accuracy (> 80%). Both supervised and unsupervised methods were able to clearly distinguish the two varieties. The PID has multiple advantages: measurements are fast, it is easy to use, and the device is inexpensive. However, unlike other technologies, such as electronic noses, it does not provide additional information about chemical composition. Although there is clearly scope to improve the device, both in terms of performance and design, this study shows that it already functions very well, and further studies could focus on optimization.

Figure

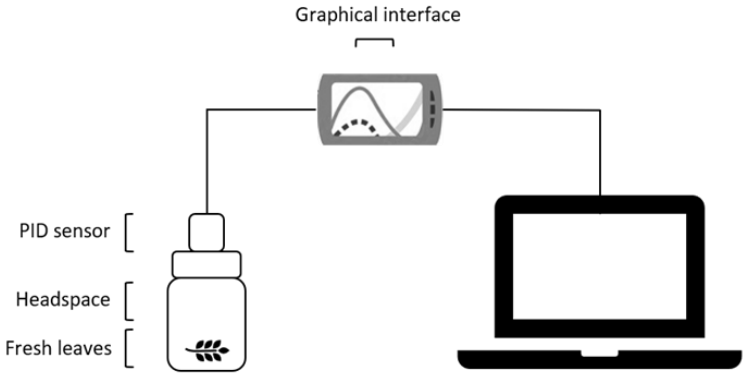


Figure 1: VOC measurement using PID sensor technology.

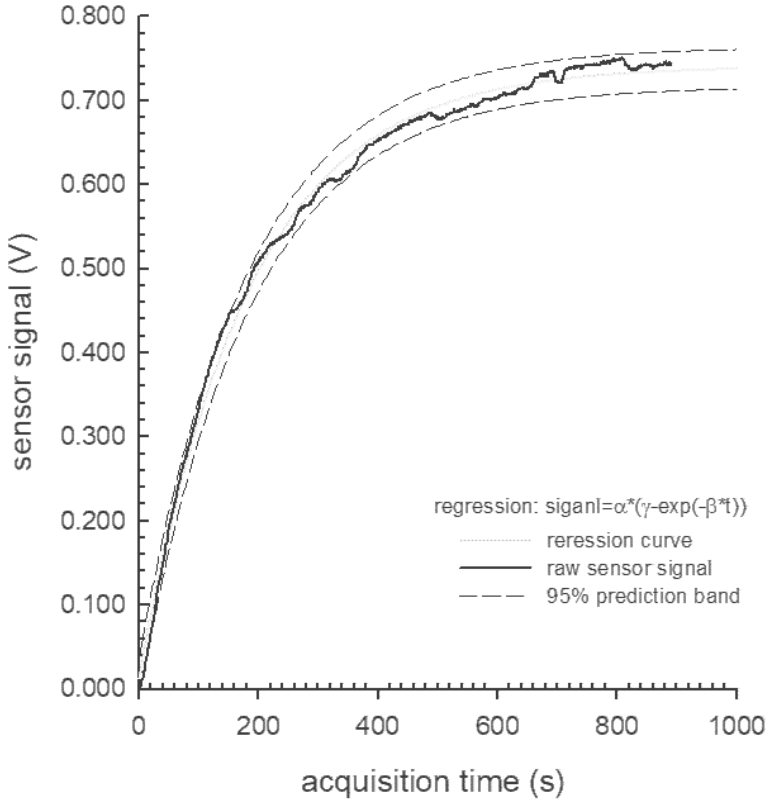


Figure 2: Statistical goodness of fit model.

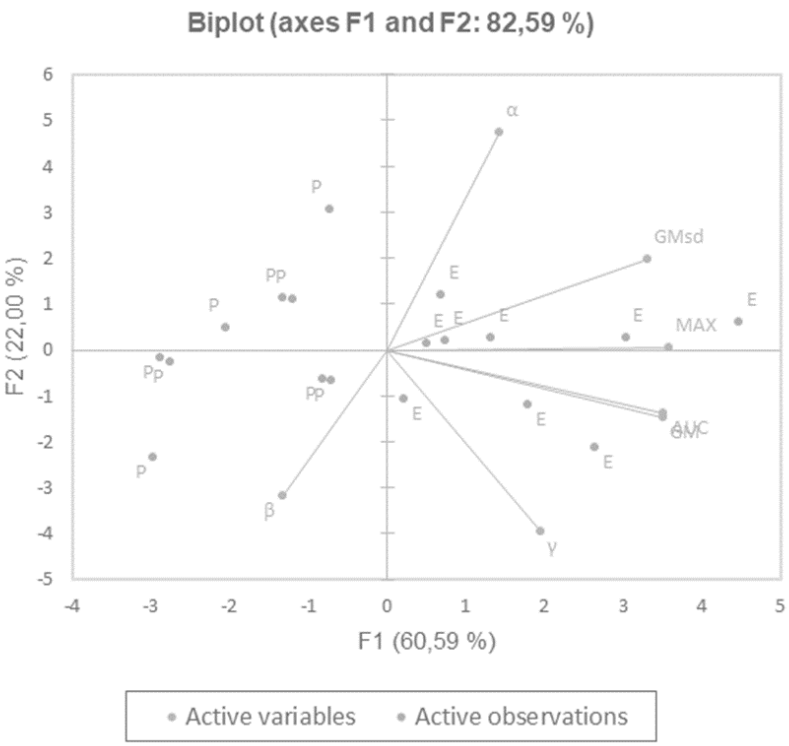


Figure 3: Biplot of the PCA of the two rosemary varieties. P = “Prostatus”, E = “Erectus”.

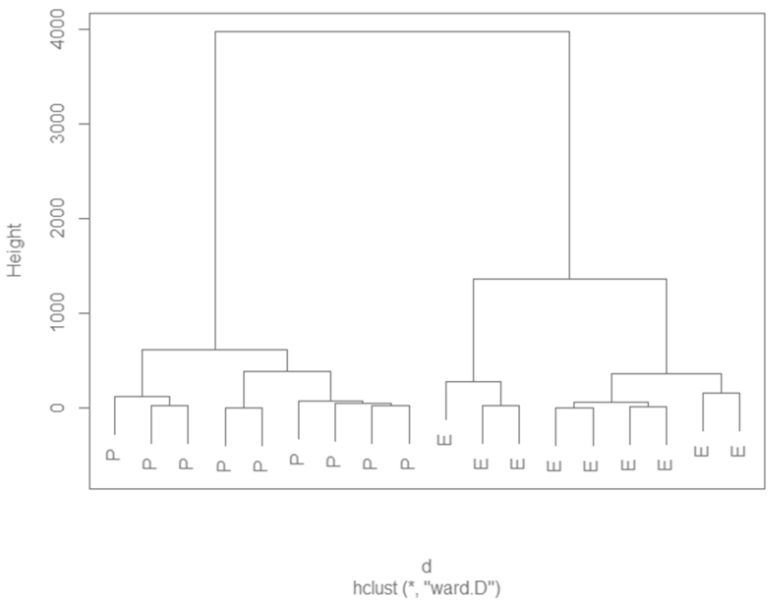


Figure 4: Dendrogram obtained by a cluster analysis of the two rosemary varieties. P = “Prostatus”, E = “Erectus”.

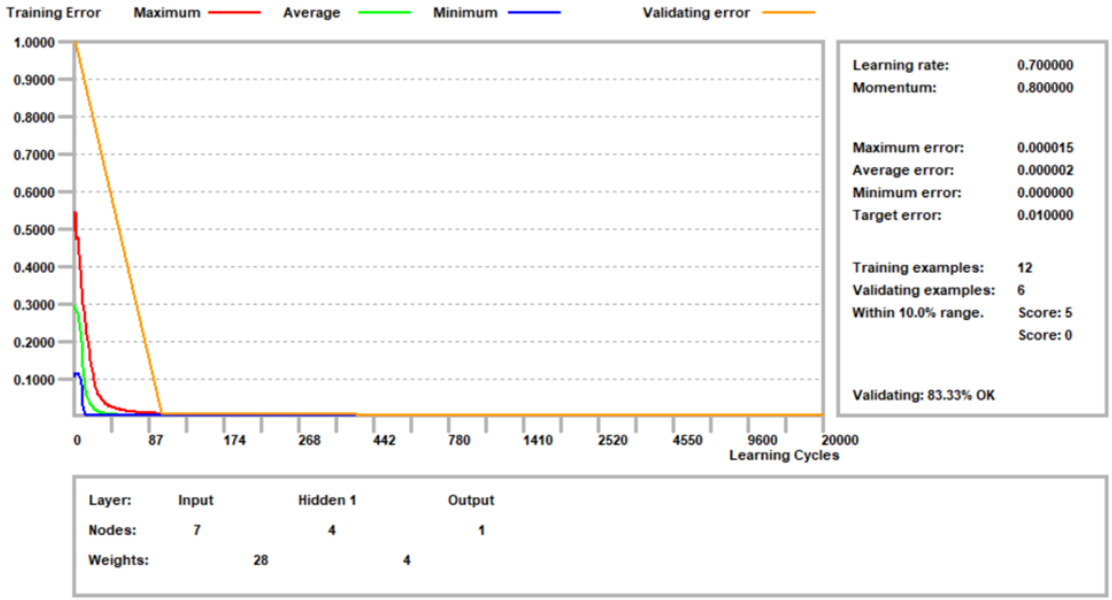


Figure 5: The ANN learning pr

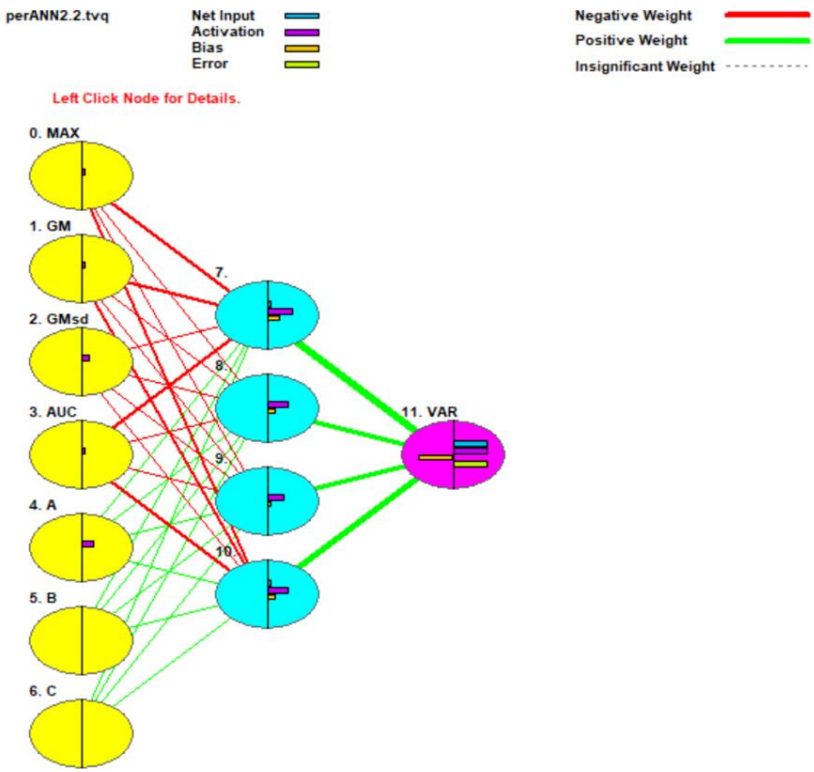


Figure 6: Graphic visualization of the neural network in ANN.

| Column | Input Name | Importance | Relative Importance |
|---|---|---|---|
| 1 | MAX | 11.7273 | |
| 2 | GM | 10.9167 | |
| 4 | AUC | 10.8775 | |
| 3 | GMsd | 7.9467 | |
| 7 | C | 5.9570 | |
| 5 | A | 0.9078 | |
| 6 | B | 0.8390 | |

Figure 7: ANN learning cycles parameters.

Tables

Table 1: Chemical composition of essential oils (≥ 1%) for the two rosemary varieties (mean ± standard deviation) (n = 3). Letters (a, b) indicate statistically significant differences using the Tukey HSD post-hoc test ($p < 0.05$).

| Compound | Variety (%) | |
|---|---|---|
| | "Prostratus" | "Erectus" |
| alpha-pinene | 35.30 [a] ± 1.50 | 13.17 [b] ± 0.85 |
| bornyl acetate | 13.31 [b] ± 0.62 | 30.40 [a] ± 1.71 |
| camphor | 8.28 [a] ± 0.42 | 8.10 [b] ± 0.35 |
| camphene | 8.07 [b] ± 0.30 | 10.02 [a] ± 0.70 |
| alpha-terpineol | 7.41 [a] ± 1.26 | 7.26 [a] ± 0.61 |
| p-cymene | 3.48 [a] ± 1.82 | 2.18 [a] ± 0.90 |
| endo-borneol | 2.65 [b] ± 0.71 | 8.78 [a] ± 0.52 |
| eucalyptol | 2.57 [a] ± 1.50 | 1.29 [a] ± 0.36 |
| 4-ol-terpinen | 2.31 [b] ± 0.27 | 4.40 [a] ± 1.10 |
| alpha-phellandrene | 1.98 [a] ± 0.40 | 0.42 [b] ± 0.07 |
| (+)-4-Carene | 1.78 [a] ± 0.11 | 0.70 [b] ± 0.05 |

| | | |
|---|---|---|
| limonene | 1.77 [a] ± 0.17 | 0.56 [b] ± 0.14 |
| cis-pinocamphone | 1.74 [b] ± 0.31 | 2.51 [a] ± 0.22 |
| eugenol | 1.52 [b] ± 0.66 | 3.99 [a] ± 0.86 |
| linalool | 1.49 [a] ± 0.38 | 0.11 [b] ± 0.15 |
| humulene | 1.42 [a] ± 0.21 | 1.29 [b] ± 0.15 |
| alpha-terpinene | 1.09 [a] ± 0.19 | 0.04 [b] ± 0.05 |
| compound identified (%) | 96.17 | 95.22 |

Table 2: Statistical output of the nonlinear regression (dynamic fitting).

| Dynamic Fit Options | |
|---|---|
| Total Number of Fits | 200 |
| Maximum Number of Iterations | 200 |

| Parameter Ranges for Initial Estimates | | |
|---|---|---|
| | Minimum | Maximum |
| $\alpha$ | −1.0000 | 3.0000 |
| $\beta$ | 0.0000 | 3.0000 |
| $\gamma$ | −1.0000 | 3.0000 |

| Summary of Fit Results | |
|---|---|
| Converged | 87.0% |
| Singular Solutions | 15.0% |
| Ill-Conditioned Solutions | 14.5% |
| Iterations Exceeding 200 | 13.0% |

Results for the Overall Best-Fit Solution

| R | R2 | Adj R2 | Standard Error of Estimate |
|---|---|---|---|
| 0.9978 | 0.9957 | 0.9956 | 0.0119 |

| | Coefficient | Std. Error | t | P |
|---|---|---|---|---|
| $\alpha$ | 0.7282 | 0.0018 | 413.2867 | <0.0001 |
| $\beta$ | 0.0055 | 2.9060E-5 | 188.1148 | <0.0001 |

| | | | | |
|---|---|---|---|---|
| γ | 1.0166 | 0.0026 | 387.0196 | <0.0001 |

Analysis of Variance

Uncorrected for the mean of the observations

| | DF | SS | MS |
|---|---|---|---|
| Regression | 3 | 341.3877 | 113.7959 |
| Residual | 889 | 0.1268 | 0.0001 |
| Total | 892 | 341.5145 | 0.3829 |

Corrected for the mean of the observations

| | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 2 | 29.0691 | 14.5345 | 101941.7917 | <0.0001 |
| Residual | 889 | 0.1268 | 0.0001 | | |
| Total | 891 | 29.1958 | 0.0328 | | |

Table 3: Confusion matrix for the training sample (VAR – E/ P)

| from \ to | E | P | Total | % correct |
|---|---|---|---|---|
| E | 6 | 0 | 6 | 100.00% |
| P | 0 | 6 | 6 | 100.00% |
| Total | 6 | 6 | 12 | 100.00% |

Table 4: Confusion matrix for the validation sample (VAR – E/ P).

| from \ to | E | P | Total | % correct |
|---|---|---|---|---|
| E | 3 | 0 | 3 | 100.00% |
| P | 1 | 2 | 3 | 66.67% |
| Total | 4 | 2 | 6 | 83.33% |

# 8. Hydrodistillation of coffee by-products to recover of bioactive compounds: the spent coffee ground and coffee silvers skin case-study

Abbreviations

SCG spent coffee ground, CSS coffee silver skin, CAF caffeine, CGAs chlorogenic acids, VOC volatile organic compound, HD hydrodistillation, HY hydrolate, PT phytocomplex, ORP oxidation-reduction potential, TDS total discolted solid, FC Folin–Ciocalteu, HPLC-DAD high-performance liquid chromatography with diode-array detector analysis

## 8.1 Introduction

Each year, coffee production results in millions of tons of residue. Spent coffee grounds (SCG) and silverskin (CSS) are produced in the largest quantities. SCG are the solid residue obtained during the processing of roasted coffee powder with hot water or steam to prepare instant coffee and other beverages [99]. CSS is a by-product of the coffee roasting process and consists of the innermost skin of the coffee bean. During roasting, a variety of chemical and structural changes occur which lead to the separation of this tegument from the beans [100]. Currently, these residues have no specific use, and are mostly considered as waste that is released into the environment. The toxic character of this organic matter, in particular SCG, makes it a significant source of pollution, and incorrect management can have negative effects [101]. In recent years, growing concern about the need to carefully manage these residues has

encouraged researchers to study possible reuses. Increasing demand from the pharmaceutical and food industries has led to the study of agro-food residues as a source of natural compounds. Recent studies have shown that both SCG [102] and CSS [103] are a natural source of bioactive compounds, and could be considered as new functional ingredients [104]. Caffeine (CAF) and chlorogenic acids (CGAs) have attracted particular interest due to their significant benefits. There are currently several techniques for recovering bioactive compounds from biomass. Of these, organic solvents are widely used for their high extraction capacity, however, they are highly polluting for the environment [105]. In recent years, several studies have investigated the use of water as an extraction solvent. For example, Bravo et al. (2013) [106] recovered bioactive compounds from SCG with good results in terms of efficiency and convenience. In another study, Ballasteros et al. (2017)[99] reported the use of an autohydrolysis technique to recover bioactive compounds from SCG with water as the extraction solvent. Similarly, an earlier study demonstrated that autohydrolysis, under mild reaction conditions, is a technology with great potential to recover phenolic compounds from SCG [105]. A recent study has evaluated various operative variables, and identified the optimal conditions for phytochemical recovery at mild temperatures (100–110°C), obtaining extracts with concentrations of phytochemicals comparable to those of other studies [107]. Costa et al. (2014) [103] reported that the use of a hydroalcoholic mixture (50% water: 50% ethanol) was the best compromise between the recovery of bioactive compounds and a sustainable CSS extraction process. In the

present study, we test hydrodistillation (HD) extraction as an environmentally friendly alternative method to recover valuable compounds from SCG and CSS. HD is a variant of steam distillation, in which the matrix is in direct contact with the solvent. This conventional technology is generally used for the extraction of secondary metabolites from plants. The process uses steam as an extraction agent to vaporize volatile compounds in the matrix. Subsequently, the mixture of steam and volatile compounds is collected and condensed again in the boiler in a recirculation circuit. When water is used as a solvent, the matrix being processed probably undergoes autohydrolysis under mild temperature conditions (about 100° C).

Thus, it can simultaneously obtain two, potentially different fractions: the condensate fraction, recovered in the condenser column, and the water-extract, i.e. a phytocomplex that is recovered inside the boiler. The aim of this study was to use HD extraction to recover bioactive compounds from SCG and CSS in order to obtain two fractions that could be characterized and differentiated. The applied technique can be considered as green, because it exploits resources and operations with a reduced environmental impact, First, it uses water as an alternative solvent; second, it produces co-products instead of waste [108] creating residues that can be used by other industries.

## 8.2 Materials and methods

Materials

Three varieties (Kaapi Royal AA, India; Santos, Brasile; Yirgacheffe gr.2, Ethiopia) of CSS and coffee powder were provided by a local company in Florence (Torrefazione Piansa, Firenze), while SCG were produced using a conventional bar machine (gs3, LaMarzocco, Italy). Coffee powder was studied as a benchmark. Before extraction, moisture was measured for each matrix with a conventional drying oven (Heraeus FunctionLine, Thermo Scientific Heraeus, USA) for 24h at 104°C (2.2 % Coffee Powder; 64.3 % SCG; 7.9 % CSS). Then, dry matrices were extracted using a stainless-steel essential oil distiller (Spring 12 l, Albrigi Luigi Store, Italy), equipped with its own induction plate (Konig HA-INDUC-11). The internal solvent was commercial, deionized water, while tap water was used for the cooling circuit.

Methods

The three matrices (CSS, SCG and Coffee Powder) were extracted by HD. Three replicates were conducted for each matrix for each variety, making a total of 27 extractions. Extractions were conducted at 1200 W for 30 min. The solid/ liquid ratio differed for the three matrices (1/3 for Coffee Powder; 1/15 for SCG and 1/50 for CSS) due to their different overall mass. After each extraction, two fractions were obtained: the condensate

fraction, recovered from the condenser column, and the water-extract, recovered from inside the boiler. We term the sample obtained from the condensate fraction the hydrolate (HY), while the water extract is termed the phytocomplex (PT). Then, all samples were filtered to separate solids and liquids. Finally, physical and chemical analysis were performed.

Physical and chemical analyses

Each sample obtained was analysed. TDS (total dissolved solids) were measured with a VST Lab Coffee III digital refractometer (VST, USA). The laser refractometer records the intensity of the light reflected by the solution under examination, directly returning a TDS% value [109]. Electrical conductivity was measured with a platinum cell conductivity probe sensor (Vernier, USA), and data were collected with LabQuest 2 software (Vernier, USA). Oxidation-reduction potential (ORP) was measured using an ORP sensor (Vernier, USA), here again, data were collected with LabQuest 2 software (Vernier, USA). Finally, a digital pH meter (GLP 21, Crison Instruments, Spain) was used to determine pH (Angeloni et al., 2019b) [109].

Measurement of caffeine with UV–vis spectrophotometry

Caffeine characterization was determined following the procedure given in Angeloni et al. (2019a) [107]. Absorbance was measured by UV–vis spectrophotometry at room temperature at a wavelength of 273 nm.

Once the calibration curve had been determined (five points) and the regression coefficient had been calculated (y = 16.82x + 3.35 and R2 =

0.97). For this, 0.1 mL of Coffee Powder extract was dissolved in 100 mL deionized water; while 0.1 ml of CSS and SCG extract were dissolved in 5 mL deionized water in order to obtain two dilutions: 1:1000 for Coffee Powder extract and 1:50 for CSS and SCG extract.

Total phenolic compounds by Folin–Ciocalteu (FC) assay

For all samples, Folin–Ciocalteu reducing capacity was evaluated following the method given in Bravo et al. (2013)[106]. In this study, only Coffee Powder PT samples were diluted before analysis (at 1:10 in demineralized water), while all other samples were analyzed without further processing. A volume of 500 µL Folin–Ciocalteu reagent was added to a mixture containing 100 µL extracted sample (as-is or diluted) and 7.9 mL demineralized water. After 2 min, 1.5 mL of a 7.5% sodium carbonate solution was added. Next, the sample was incubated in darkness at room temperature for 90 min. Absorbance was measured at 765 nm in a Lambda 25 UV–vis spectrophotometer (Perkin–Elmer Instruments). Gallic acid (GA) was used as the reference, and results were expressed as milligrams of GA equivalent per gram of SCG, CSS and Coffee Powder dry matter (mg GAE/g SCG dm).

High-performance liquid chromatography with diode-array detector analysis

Samples were centrifuged at 12000 rpm for 5 min and diluted 1:10 with water before high-performance liquid chromatography with diode-array detector (HPLC-DAD) analysis. HPLC was carried out using an Agilent HP

1100 system equipped with an auto sampler, column heater module and quaternary pump, coupled to a DAD all from Agilent Technologies (Palo Alto, CA, USA). An Infinity Lab 150 mm × 3 mm i.d., 2.7 m Poroshell 120, EC-C18 column (Agilent Technologies) was used, equipped with a pre-column of the same phase, and maintained at room temperature. Injection volume was 5 L. The elution method was performed at a flow rate of 0.4 mL/min using water at pH 3.2 by formic acid (solvent A) and acetonitrile (solvent B). All solvents were Chromasolv™ for HPLC grade (Sigma Aldrich S.R.L.). The multistep linear solvent gradient technique is described in detail in other work (Angeloni et al., 2019a) [107]. Starting at 95% A, and going up to 10% A, over 24 min (the total analysis time) UV–vis spectra were recorded in the range 220–600 nm. Chromatograms were registered at 330 nm for CGA, and 278 nm for caffeine. Caffeine and CGA were identified by comparing their retention times and UV–vis spectra to those of the respective standard, when possible, or with published data otherwise [107]. CGA was evaluated by HPLC-DAD using a five-point calibration curve (5-caffeoyl-quinic acid, purity 99%) (Extrasynthèse, Genay, France) at 330 nm (0–1.315 _g; R2= 0.9988), and caffeine content was determined by HPLC-DAD using a five-point calibration curve from Extrasynthèse (purity 95%) at 278 nm (0–0.34 _g; R2 = 0.9999).

Measurement of volatile organic compounds

As reported Angeloni, et al. (2020) [110], a photoionization sensor (PID) was used to detect volatile organic compounds (VOCs) of each sample. 10

gr of SCG and CSS and 1 gr of coffee powder were introduced in a container of 100 ml (Sarsted, Germany). Then, each sample was placed on a digital magnetic stirrer with heating plate (M2-D PRO ARGOlab, Italy) at 300 rpm and 35 ° C. Thus, VOCs were measured with PID sensor (Alphasense, United Kingdom) for a time of 1800 s. Then, all data were collected with a graphical interface LabQuest2 (Vernier, USA) and analysed.

Statistical analyses

Conventional analysis of variance (ANOVA) was used to compare means determined for each fraction obtained. The tested factors were considered significantly different at $p < 0.05$. All statistical analyses were performed using R software (version 3.6.0 for Windows). In cases where the F-test was significant at the $p \leq 0.05$ level, multiple pairwise comparisons of group means were checked for significance using the post hoc Tukey Honest Significance Difference test ($p < 0.05$).

## 8.3 Results and discussion

Physical and chemical analyses

Physical and chemical characteristics of all samples were analysed in order to characterize the two fractions (PT and HY) of the three extracted matrices (Coffee Powder, SCG, and CSS). Significant differences are reported. TDS, electrical conductivity, ORP, and pH results are reported in Table1. Regarding TDS, as expected, values were highest for both Coffee Powder fractions (PT and HY), with values of 6.95 and 3.14 %,

respectively, and a significant difference was found between them. No significant differences were found between the other samples. Regarding electrical conductivity, highest values were obtained for the Coffee Powder PT fraction (2590.38 µS/cm), followed by the respective HY fraction (1230.00 µS/cm). Lower values were obtained for SCG PT and HY fractions (488.97 and 204.84 µS/cm) and CSS (557.26 and 204.84 µS/cm). With respect to the ORP analysis, no significant differences were found for Coffee Powder PT and HY fractions. However, significant differences were observed for the two fractions of SCG and CSS. Specifically, higher values were found for SCG and CSS PT (343.87 and 366.09 mV), and lower values for the respective HY (278.78 and 306.78 mV). Finally, turning to pH, significant differences were found for Coffee Powder PT and HY fractions, while no significant differences were found for the two fractions of SCG and CSS.

Caffeine and total phenolic compounds

Concentrations of caffeine and total phenolic compounds are reported in Table 2. All samples were analysed and interactions between the two fractions (PT and HY), and the three matrices (Coffee Powder, SCG and CSS) are reported. In general, caffeine content in the PT fraction was higher than the HY fraction. In particular, caffeine content in the Coffee Powder PT fraction (3.42 mg/g) was significantly higher than the respective HY (3.19 mg/g), and the same trend was found for the other matrices studied. Highest content was obtained for SCG (3.21 mg/g) and CSS PT fractions (2.90 mg/g), while lowest yields were obtained for CSS

(2.49 mg/g) and SCG fractions (2.30 mg/g) HY, respectively. Regarding the PT fraction, our results agree with the current literature [111][106][107] who reported 3.59 mg/g, 4.52 mg/g and 3.10 ±1.98 mg/g for SCG, respectively. Panusa et al. (2017)[112] reported a maximum of 3.75 mg/g for CSS. Unsurprisingly, caffeine concentration in CSS was lower than in SCG. The high concentration of bioactive compounds in SCG is presumably due to the Espresso coffee technique that was used. Concerning phenolic compounds, significant differences were found between the two fractions (PT and HY) and the three matrices (Coffee Powder, SCG and CSS). In general, highest concentrations were obtained for PT with respect to HY. The highest value was obtained for SCG PT (21.47 mg/GAE g), while the lowest value was obtained for CSS PT (5.43 mg/GAE g). Yields were lowest for HY and, in this case, no significant differences were measured for SCG (1.96 mg/ GAE g) and CSS (2.11 mg/ GAE g). Concerning the PT fraction, Panusa et al. (2013) [113] reported a maximum yield of 17.43 mg/g of phenolic compounds for SCG using water as an extraction agent. Lower concentrations were reported by Ballesteros et al. (2014b) [114]using organic solvents for CSS, in a range between 5.26 and 13.53 mg GAE/g. However, Conde and Mussatto (2016) [105] used a hydrothermal pre-treatment, and measured 32.92 mg/g and 19.17 mg/g for SCG and CSS, respectively. The latter result was probably due to a short extraction process, but the method is less sustainable due to the greater number of operations.

HPLC-DAD analysis

Figure 1 shows HPLC-DAD chromatograms at 330 nm. Different chemical profiles were obtained for the two fractions of the three matrices. Here, we only report the chromatogram of six samples (two fractions of each matrix) for further characterization and differentiation. This analysis confirmed other differences found between the two fractions and the three matrices, and this aspect will be explored in more detail in subsequent studies.

Measurement of VOCs

Table 3 shows VOCs measured for each sample. As we expected, HY fraction has reported higher values than the PT fraction. In particular, Coffee Powder HY (1610.83 mV/g) was significantly higher than the respective PT fraction (30.31 mV/g). On the other hand, no significant different were found between the other matrices of the two fractions.

## 8.4 Conclusions

The two fractions showed interesting characteristics. Caffeine was detected for each fraction of Coffee Powder, SCG and CSS. High amounts of phenolic compounds were detected in the PT fraction of each of the matrices, while levels were lower in the HY fraction. TDS, electrical conductivity, ORP and pH results made it possible to characterize each of the fractions of each of the matrices. HPLC-DAD analysis revealed different chemical profiles, and this aspect will be investigated in more detail in future studies. Finally, measurements of VOCs shown different

characteristics, for Coffee Powder. Our green method was able to extract a considerable amount of bioactive compounds from industrial coffee waste, compared to conventional systems.

The efficiency of our method will be improved in future studies. In particular, we will examine the use of low impact pre-treatments to increase the amount of bioactive compounds that are extracted, reduce the amount of water needed, and shorten the extraction time.

Acknowledgments

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Tables

Table 1: Physical and chemical characteristics of the two fractions: phytocomplex (PT) and hydrolate (HY) and the three matrices: Coffee Powder, spent coffee grounds (SCG) and coffee silverskin (CSS). Means and standard deviation are reported. Letters indicate significant differences ($p \leq 0.05$).

| Fraction | Matrix | TDS (%) | Electrical conductivity (µS/cm) | ORP (mV) | pH |
|---|---|---|---|---|---|
| PT | Coffee powder | 6.95 [a] ± 5.12 | 2590.38 [a] ± 1826.82 | 254.12 [b] ± 40.05 | 4.31 [c] ± 0.85 |
| PT | SCG | 0.39 [c] ± 0.28 | 488.97 [c] ± 378.70 | 343.8 [a] ± 120.05 | 4.78 [b] ± 0.16 |
| PT | CSS | 0.25 [c] ± 0.17 | 557.26 [c] ± 497.34 | 366.09 [a] ± 91.42 | 5.42 [a] ± 0.84 |
| HY | Coffee powder | 3.14 [b] ± 4.60 | 1230.00 [b] ± 1645.48 | 246.08 [b] ± 47.49 | 3.66 [d] ± 0.86 |
| HY | SCG | 0.17 [c] ± 0.24 | 204.84 [e] ± 277.58 | 278.78 [b] ± 105.00 | 4.79 [b] ± 0.49 |
| HY | CSS | 0.15 [c] ± 0.22 | 285.74 [d] ± 426.99 | 306.78 [b] ± 82.87 | 5.93 [a] ± 0.96 |

Table 2: Recovered caffeine and phenolic compounds for the two fractions: phytocomplex (PT) and hydrolate (HY) and the three matrices: Coffee Powder, spent coffee grounds (SCG) and coffee silverskin (CSS). Means and standard deviation are reported. Letters indicate significant differences ($p \leq 0.05$).

| Fraction | Matrix | Caffeine (mg/g) | Phenolic compounds (mg/GAE g) |
|---|---|---|---|
| PT | Coffee powder | 3.42 [a] ± 0.10 | 17.67 [b] ± 2.34 |
| PT | SCG | 3.21 [b] ± 0.21 | 21.47 [a] ± 4.22 |
| PT | CSS | 2.90 [c] ± 0.18 | 5.43 [c] ± 0.60 |
| HY | Coffee powder | 3.19 [c] ± 0.23 | 0.20 [e] ± 0.07 |
| HY | SCG | 2.30 [e] ± 0.03 | 1.96 [d] ± 0.42 |
| HY | CSS | 2.49 [d] ± 0.70 | 2.11 [d] ± 0.25 |

Table 3: VOCs measured for the two fractions: phytocomplex (PT) and hydrolate (HY) and the three matrices: Coffee Powder, spent coffee ground (SCG) and coffee silverskin (CSS). Means and standard deviation are reported. Letters indicate significant differences ($p \leq 0.05$)

| Fraction | Matrix | VOC (mV/g) |
|---|---|---|
| PT | Coffee powder | 30.31 [b] ± 13.42 |
| PT | SCG | 2.79 [b] ± 0.40 |
| PT | CSS | 5.97 [b] ± 2.05 |
| HY | Coffee powder | 1610.83 [a] ± 528.37 |
| HY | SCG | 166.19 [b] ± 25.25 |
| HY | CSS | 81.91 [b] ± 10.48 |

Figures

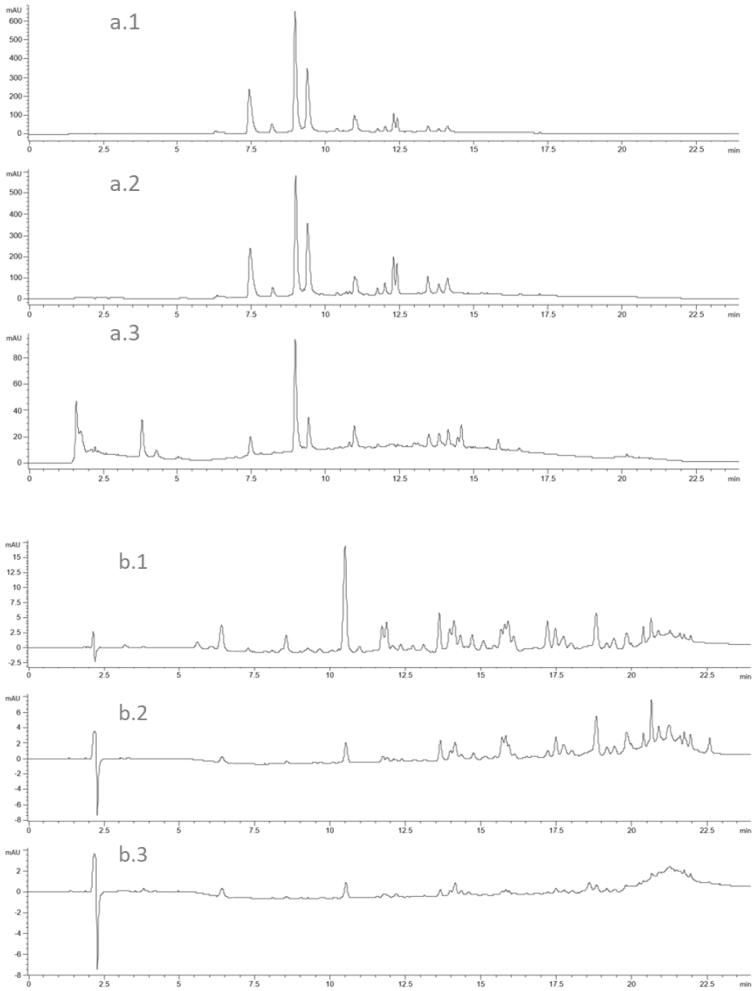


Figure 1: HPLC-DAD chromatograms at 330 nm for the two fractions (a: PT and b: HY) of the three matrices (1: Coffee Powder, 2: SCG and 3: CSS).

# 9. Preliminary study of the application of hydrodistillation to valorise olive oil by-products

Abbreviations

OL olive leave, OP olive pomace, OS olive stone, PT phytocomplex fraction, HY hydrolate, HD hydrodistillation

## 9.1 Introduction

Olive *(Olea europaea L.)* is one of most cultivate crops in the world, mainly for production of olive oil. During the whole process, a large amount of biomasses is produced by different phases as in the harvest, pruning and production phase [115]. These are mainly of olive leaves, olive pomace, olive stone and olive mill wastewater. Each year in fact, a great quantity of olive leaves (OL) and branches is cut during the pruning phase to improve the production and harvesting of olives in the next year. It is estimated about 25 kg of leaves per tree annually. Furthermore, another consistent amount of the leaves is collected also during the harvest phase. About 10% of the total weight of harvest consists in leaves and branches [116].

Generally, the leaves are separated from the olives harvested as soon as arrive in the mill, before to start the extraction process, by blowing air. Subsequently, the olives undergo a series of widely known processes as the crushing, kneading, centrifugal extraction, and filtration, in order to obtain olive oil. From the extraction phase the olive pomace (OP) another important biomass is obtained, in a quantity about 800–850 kg t-1 olives, depending on the two- or three-phase extraction system. It includes a

combination of liquid and solid wastes such as olive pulp, olive skin, stone and water [117]. Furthermore, OP can be further processed to separate the hard part, thus obtaining another important biomass, the olive stone (OS). Currently, these biomasses have a limited used and often represent only waste to be disposed of. They can be harmful for the environmental, and they must therefore be handled correctly. In general, require an important management both in term of manpower and in terms of storage. However, in the last years many researchers studied some possible use of these residues to valorise these biomasses and try to take advantage of their chemical composition.

OLs are generally used for direct combustion, animal feed, feedstock or pellet manufacturing. OS is used mainly for energy production and OP is further extracted to obtain olive oil pomace burned to energy scope [49]. The energetic use of olive biomasses is widely known and studied, however given the potential harmful character for the environment, it is necessary to focus on alternative uses. In this contest, is a priority to deepen the research on the olive biomasses as a source of bioactive compounds [118].

In the last few years, olive oil residues were studied as a source of valuable compounds especially phenols, thanks to the well-known antioxidant activity that present. In fact, these compounds are increasingly used for the antioxidant properties that present in pharmaceutical, cosmetic and food industry [119] [120]

Nowadays, there are many methods used for the recovery of bioactive compounds from agro-food industry. Generally, phenols compounds are typically extracted by conventional methods using a large quantity of organic solvents usually methanol or ethanol. On the other hand, other extraction techniques were developed in order to decrease the use of organic solvent and optimize the extraction conditions. Microwave, ultrasound, subcritical extraction, high hydrostatic pressure, pulsed electric field, high-voltage electrical discharge are the mainly studied by researchers [121][122][123].

However, the management of these technologies may be difficult, and may require the presence of specialized staff. This could translate into high costs for companies and in some cases may not be accessible by them. In this contest, it is necessary to develop a more sustainable technology, both in economic and environmental terms, which not require use of organic solvent or expensive equipment. In this sense, the water is the greenest solvent for its non-harmful character for both environment and human health. Water as a solvent is was already studied by several researchers as a extraction solvent to recover valuable compounds from different matrix showing interesting results [44].

Based on this, in the following work water is used as a green solvent and the hydrodistillation process (HD) is proposed as an environmentally friendly method to recover bioactive compounds from olive oil biomasses. HD is a relatively fast and easy-to-use system. It is a traditional method to extract secondary metabolites from plants in which the matrix

is in direct contact with the solvent inside a boiler. During the extraction process, the being processed matrix inside the boiler probably undergoes autohydrolysis under mild temperature conditions (about 100° C) allowing the extraction of valuable compounds.

At the same time, the generated steam by water vaporizes the volatile compounds in the matrix. So, the mixture of steam and volatile compounds is collected and condensed. HD allows to obtain simultaneously two different fractions: the condensate fraction, recovered in the condenser column, and the water-extract, i.e. a phytocomplex that is recovered inside the boiler [124].

The aim of this study was to use HD extraction and water as solvent to recover bioactive compounds from different biomasses from olive oil production process. Based on our knowledge, the application of this technology is new in the field of agri-food residues. Additionally, the possibility to obtain simultaneously two potentially different fractions is a novelty. Therefore, the objective was to ascertain the effectiveness or suitability of HD as a possible extraction technology from olive oil production residues. Hence, characterize and differentiate the two fractions obtained by the process. With a view to investigating further methodologies to valorize the different biomasses generated during the olive oil production process, HD could be an extraction method eco-friendly respect to the environment, sustainable in terms of costs and accessible to all interested industries. Furthermore, it could permit an

interesting and different use of olive oil residues as an alternative product to use in different application fields.

## 9.2 Materials and methods

Materials

Olive oil residues were provided by a local olive mill in Tuscany (Bucine, Ar, Italy) during the harvest campaign in 2020. Three different residues were considered, namely olive leaves (OLs), olive pomace (OP), and olive stone (OS). These residues in the following were called “matrix”.

OLs were separated from the mass of olives entering the olive mill by a defoliation apparatus (model DLE SUPER TD, MORI-TEM Srl, Italy) and then shredded by a mechanical shredder (MORI-TEM Srl, Italy), reducing them into small pieces of about 5 mm. The OP was recovered from the two-phases decanter (MORI-TEM Srl, Italy) and finally, the OS was obtained by a de-stoning machine (model DN/O, Clemente-industry, Italy). For each matrix, moisture was measured with a drying oven (Heraeus Function, Thermo Scientific Heraeus, USA) for 24h at 104°C (18.8 % OS, 47.5 % OL, 62.1 % OP). The dry matrices were extracted using a stainless-steel distiller (Spring 12 l, Albrigi Luigi Store, Italy) and an induction plate (Konig HA-INDUC-11) was used for the warm. Deionized water was used inside the boiler and tap water was used for the cooling circuit.

## Methods

### Extraction conditions

The extraction conditions were chosen based on careful preliminary tests [44][124]. The solid/ liquid ratio was 1/5 and 600 W was the power of the induction plate. Each extraction was stopped when the 50 % of the whole water solvent inside the boiler was recovered in the condenser column, for a total of 1 h. Before extraction, the moisture of each matric was appropriately measured (48 % OF; 62 % OS; 19 % OS).

Then, three replicates were conducted for each matrix (OF, OP, OS) by HD method for a total of 9 extractions. Two fractions were recovered for each extraction for a total of 18 extracts which were appropriately analyzed and characterized. A water-extract fraction, namely a phytocomplex, was recovered inside the boiler, while a condensate fraction was recovered in the condenser column. All samples were filtered to separate solids from the liquid phase. At the end, each sample was stored at −5 °C until physical and chemical analysis were performed.

### Chemical analysis

#### High-performance liquid chromatography with diode-array analysis

To determinate own chemical profile, samples of both fractions were centrifuged at 12000 rpm for 5 min and diluted 1:10 with water before high-performance liquid chromatography with diode-array detector (HPLC-DAD) analysis. HPLC was carried out using an Agilent HP 1100 system equipped with an auto sampler, column heater module and

quaternary pump, coupled to a DAD all from Agilent Technologies (Palo Alto, CA, USA). An Infinity Lab 150 mm × 3 mm i.d., 2.7 m Poroshell 120, EC-C18 column (Agilent Technologies) was used, equipped with a pre-column of the same phase, and maintained at room temperature. Injection volume was 5 L. The elution method was performed at a flow rate of 0.4 mL/min using water at pH 3.2 by formic acid (solvent A) and acetonitrile (solvent B). All solvents were Chromasolv™ for HPLC grade (Sigma Aldrich S.R.L.). The multistep linear solvent gradient technique is described in detail in other work [125]. Starting at 95% A, and going up to 10% A, over 24 min (the total analysis time) UV–vis spectra were recorded in the range 220–600 nm. Chromatograms were registered at 330 nm for CGA, and 278 nm for caffeine. Caffeine and CGA were identified by comparing their retention times and UV–vis spectra to those of the respective standard, when possible, or with published data otherwise [125]. CGA was evaluated by HPLC-DAD using a five-point calibration curve (5- caffeoyl-quinic acid, purity 99%) (Extrasynthèse, Genay, France) at 330 nm (0–1.315 _g; R2= 0.9988), and caffeine content was determined by HPLC-DAD using a five-point calibration curve from Extrasynthèse (purity 95%) at 278 nm (0–0.34 _g; R2 = 0.9999).

Gas chromatography–mass spectrometry analysis

The GC-MS analysis used an Agilent 7820 gas chromatograph equipped with a 5975C mass selective detector operating in electron impact mode (Agilent Tech., Palo Alto, CA, USA). One µL of extract in solution was injected into a split/ splitless injector operating in splitless mode. A

Gerstel MPS2 XL liquid autosampler was used. Chromatographic settings were as follows: injector in splitless mode set at 260°C, J&W INNOWax column (30 m, 0.25 mm i.d., 0.5 µm df); oven temperature programme: initial temperature 40°C for 1 min, then 5°C min-1 until 200°C, then 10°C min-1 until 220°C, then 30°C min-1 until 260°C, held for 3 min. The mass spectrometer operated with electron ionisation of 70 eV, in scan mode in the m/z range 29–330, at three scans sec-1. Compounds were quantified using a calibration curve that was constructed by injecting known concentrations of authentic standards into the GC-MS. Deconvoluted peak spectra (obtained using the Agilent MassHunter software suite) were matched against the NIST 11 spectral library for initial identification. Kovats' retention indices were calculated for further confirmation, and compared with those reported in the literature for the chromatographic column used.

Statistical analysis

One-way ANOVA was conducted to assess the effect of the different matrices in the recovery of phenolic compounds by HPLC analysis.

A principal component analysis (PCA) was applied based on the total area of the peaks of the volatile compounds identified as input variables. Subsequently, a multiway ANOVA was conducted to assess the effect of the matrix and fractions variables in the concentration of volatile compounds by GC-MS analysis. The significance of main effects and their interactions were tested. For both, the tested factors were considered

significantly different at $p < 0.05$. When the significance level was reached, a Tukey HSD post-hoc test was run. All statistical analyses were performed using R software (version 3.6.0 for Windows).

## 9.5 Results

The use of HD was able to obtain two fractions from each matrix (OL, OP and OS). The first one was nominated "phytocomplex" (PT), recovered inside the boiler, potentially rich of bioactive compounds and the second "hydrolate" (HY), recovered in the condenser column, potentially rich on volatile compounds. All the samples were analysed to characterize and differentiate the two fractions obtained. Thus, high performance liquid chromatography with diode-array detector analysis (HCLP-DAD) and gas chromatography–mass spectrometry (GC – MS) were used to determine the chemical profiles.

High-performance liquid chromatography with diode-array analysis

HPLC-DAD was conducted in an attempt to identify the mainly chemical compounds. The chromatograms of each fraction and each matrix at a wavelength of 280 nm where phenolic compounds adsorbed were reported in the Figures 1 and 2.

At this point of the study, it was decided to investigate only the PT fraction, based on the greater complexity of the chemical profile reported in the chromatograms (Figures 1 and 2) than HY fraction.

Based on the available equipment, the attempt to identify the main peaks of PT fraction of OL and OP was conducted. The main peak of OS matrix resulted small and not very resolute from the point of view of HPLC analysis. Therefore, it was decided to conduct further analyses for the missing samples which will be suitably integrated with current results for a complete and accurate characterization of the samples obtained. Currently, a total of 13 phenolic compounds were tentatively identified for OL and 11 for OP, in addition 4 and 3 peaks were respectively unknow for both the matrices. The compounds identified were summarize in Figures 4 and 5. According with current literature, for both the matrices the main families of phenolic compounds identified are phenolic alcohols, secoiridoids and flavonoids [126][127][128]. For OL, some peaks were unknow (1, 4, 6, 8). On the other hand, among simple phenols, peak 2, 3 and 5 were identified as hydroxytyrosol and its oxidized forms. Again, peak 7 was identified as caffeic acid. Instead, peak 9 was possible to affirm was an isomer of rutin, while peak 13 as rutin. Peak 10 was presumably cafselogoside. Between secoiridoids, peaks 11, 12 were oxidized of Oleuropein, while peak 17 as Oleuropein. At the end, peak 14, 15, 16 were identified as Luteolin-7-O-glucoside, Apigenin-7-O-glucoside and Luteolin-4-O'-glucoside, respectively.

For OP, also in this case some peaks were unknown (5, 6, 10). Among simple phenols peak 2,1 were identified as hydroxytyrosol and its oxidized, peak 3 as tyrosol glucoside and peak 4 as tyrosol. Peak 7, 8 as beta-OH acteroside 2, beta-OH acteroside 1. While, peak 9 as rutin. While, peak 11 as verbascoside and 12 as nuzhenide. Peak 13 as comselogoside. At the end, peak 14 was luteolina-7-O glucoside, confused with verbascoside.

Subsequently, the total of phenolic compounds was calculated, and the quantitative results are summarized in Table 1. Data were reported in mg per kg of dry weight of matrix under extraction. Significant difference ($p < 0.01$) was found among the total phenolic compounds identify in PT fraction for OL and OP. A higher amount of phenols compounds was extracted in OL (1767.35 ± 78.00 mg/kg) than OP (679.00 ± 73.75 mg/kg).

Chromatography–mass spectrometry analysis

GC-MS was conducted to analysis the volatile profile so the tentative to identify the main peaks was conducted for all the samples. In some cases, the commercial standard was used, for others the mass spectra of the peak was compared with the mass spectra in the NIST standard library database. All the chromatograms were reported in Figures 3 and 4.

In Table 2 were summarize the compounds identified. As reported, a great quantity of compounds associated to the typical positive flavours of olive oil were recovered for each matrix, while lower quantity to the off-

flavours [129][130][131]. In HY fraction a higher number of volatile compounds was detected respects PT fraction. So, for OL a total of 48 compounds are reported for HY fraction, while only 22 for PT. The same results are obtained for OP, 41 and 25 are reported for HY and PT, respectively. At the end also for OS a total of 44 compounds was found for HY fraction respect to 19 for PT and this demonstrates a greater complexity of the aromatic profile of HY fractions than PT.

Results of principal component analysis (PCA) summarized the differences related to the aromatic profile of all the sample. PCA was reported in Figure 7. The first two components (Dim1, Dim2) explained nearly 73.49% of total variance, accounting for 44.39 and 29.1%, respectively. The biplot shows the projection of all the samples on Dim1 and Dim2 axes. The PCA clearly separated the two fractions obtained (PT and HY) by HD. A visual inspection shows the samples of HY fraction in the right quadrant, and PT in the left. Hence, the fractions can be clearly distinguished.

Subsequently, for the detected compounds refer to olive oil, was possible to conduct a semi-quantification of peak area and for the semi-quantified compounds a multiway - ANOVA was conducted in terms of the volatile compound peak areas and the results are shown in Table 3.

The ANOVA highlighted a significant main effect of the two fractions (HY and PT) and the three matrices (OL, OP and OS). Significant differences were found between the two fractions. Higher values were reported for HY fraction than PT fraction. Furthermore, significant differences were

found between the three matrices. OL reported a higher value for both the HY and PT fractions, than OP and OS. Thus, it is possible to affirm, that the obtained result confirms that the HY fraction were found a greater content of volatile compound and so, a higher complexity of the aromatic profile.

## 9.6 Discussion

HD extraction has allowed to recover two fractions potentially different from different olive oil by-products. So, HPLC analysis and GC-MS analysis were conducted to characterize the chemical profiles.

By HPLC it was chosen to investigate the chemical profile of the PT fraction, inside the boiler, respect HY fraction, because the chromatogram of the peaks of the chromatograms of the latter fractions are small and not very resolute. A considerable number of valuable compounds were recovered for OL and OP, such as typical phenols compounds of olive oil and its by-products reported in literature [127][126]. Hydroxytyrosol, verbascoside, tyrosol are the main compounds recovered in OP, while oleuropein, hydroxytyrosol, luteolin-4-O'-glucoside, luteolin-7-O-glucoside are the main compounds identified in OL, in both cases the results agree with current literature [132][133][134]. A higher amount of total phenols compounds was recovered for OL (1767.35 ± 78.00 mg/kg), respect to OP (679.00 ± 73.75 mg/kg). For OP the concentration of phenolic compounds recovered with HD method is according with current literature [135] using water and

several emerging technologies as extraction method. On the other hand, the amount recovered for OL is lower respect the values reported in literature, could be due to several factors for example the cutting of the leaves, that could influence the recovery of these compounds, or the temperature of the extraction. At the end, regarding OS, in this step of the study, interesting compounds from the point of view of HPLC analysis were not recovered and therefore investigated. A further study will be conducted.

The aromatic profiles of all the samples were studied by GC-MS analysis. As expected, the HY fraction of all the matrices presented a more complex profile regarding volatile compounds. For all the three matrices of HY fraction were found compounds attributable to flavours and some off flavours of olive oil, according with current literature [129][130][132]. The highest number of detected compounds was for OL, follow by OP and OS. Results of ANOVA on peaks area also confirmed the previous values. Also in this case, a higher total area of peaks was recovered for HY fraction than PT.

Further studies will be conducted to deepen the fractions obtained. In light of the results obtained, it could be affirmed that the proposal method allowed to recover two different fractions from olive oil by-products. The PT fraction was resulted richer in valuable compounds as polyphenols and potentially used for the note antioxidant activity. On the other hand, the HY fraction was resulted richer in volatile compounds.

## 9.7 Conclusion

In conclusion, HD extraction is a novelty as method to recover valuable compounds from by-products. Certainly, further studies will be conducted in order to optimize the whole process and to deep all the matrices used. Furthermore, several aspects will be deepened to conduct an efficient extraction in both qualitative and quantitative terms. However, HD has the potential to be an easy, fast and relative sustainable method that use water as solvent in the field of agro-food by-products.

Figures

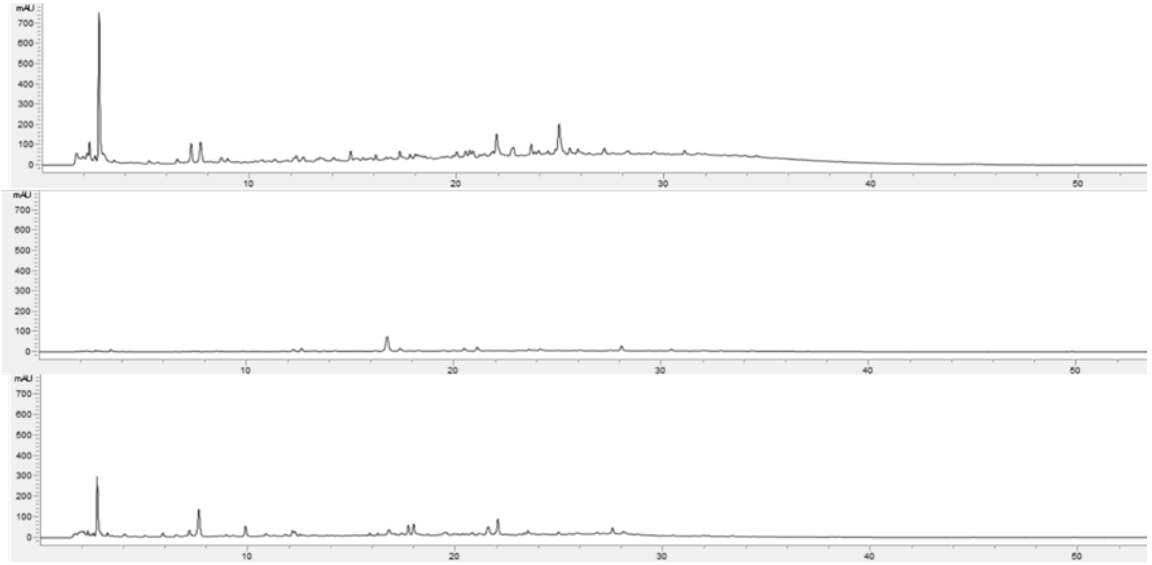


Fig. 1: Chromatograms of the PT fractions of the three matrices (in order OL, OS and OP) by HPLC – DAD at a wavelength of 280 nm. (PT = phytocomplex fraction, OL = olive leave, OP = olive pomace, OS = olive stone).

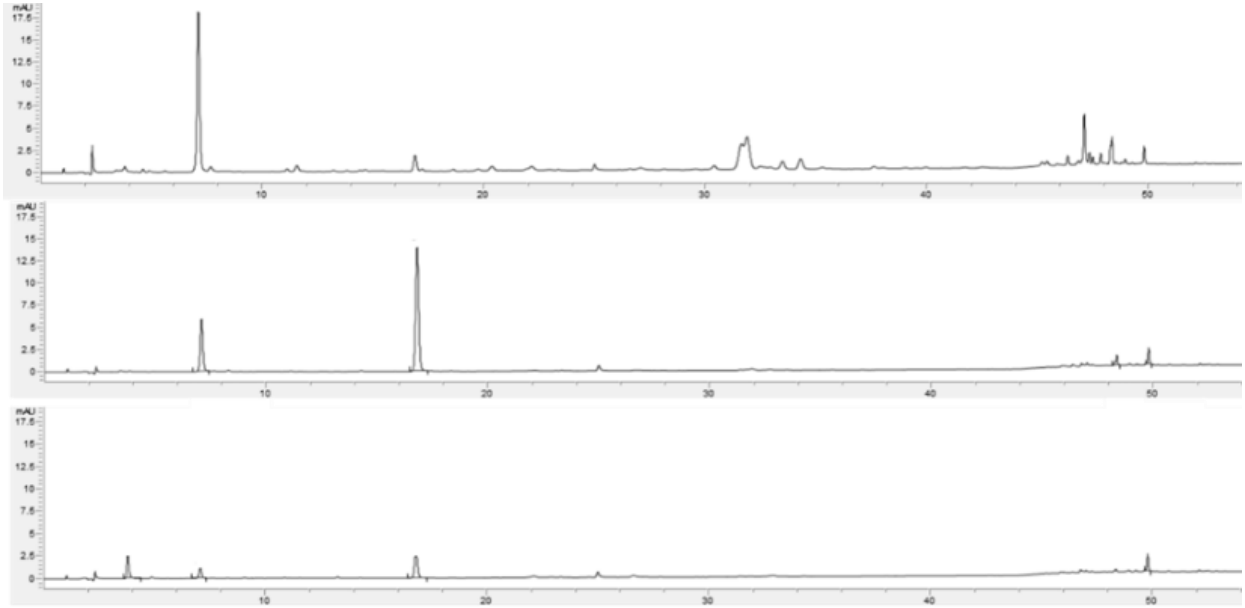


Fig. 2: Chromatograms of the HY fractions of the three matrices (in order OL, OS and OP) by HPLC – DAD at a wavelength of 280 nm. (HY = hydrolate fraction, OL = olive leave, OP = olive pomace, OS = olive stone).

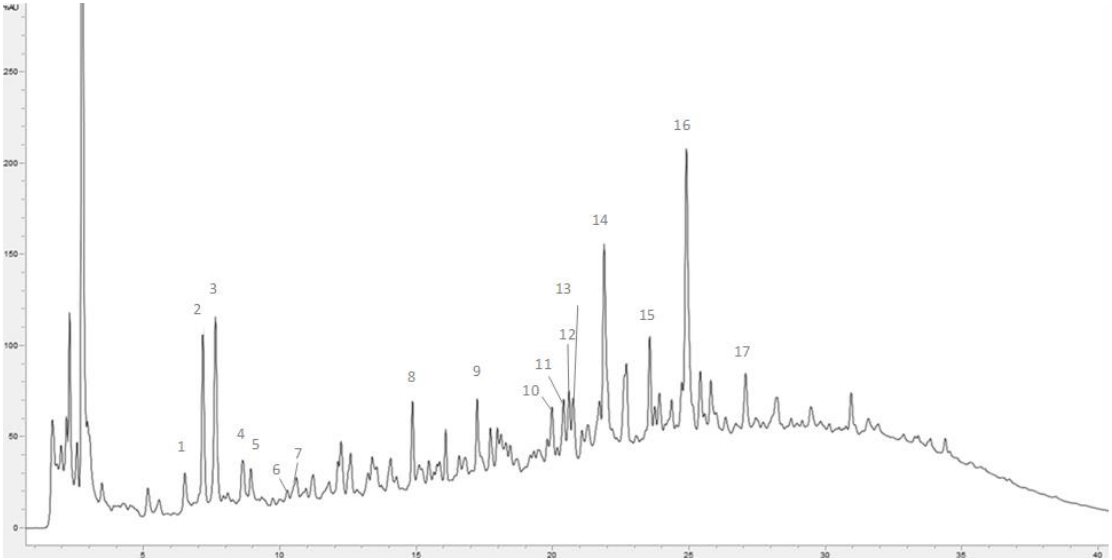


Fig. 3: Chromatograms of PT of OL by HPLC – DAD and main phenolic compounds identify. (PT = phytocomplex fraction, OL = olive leave).

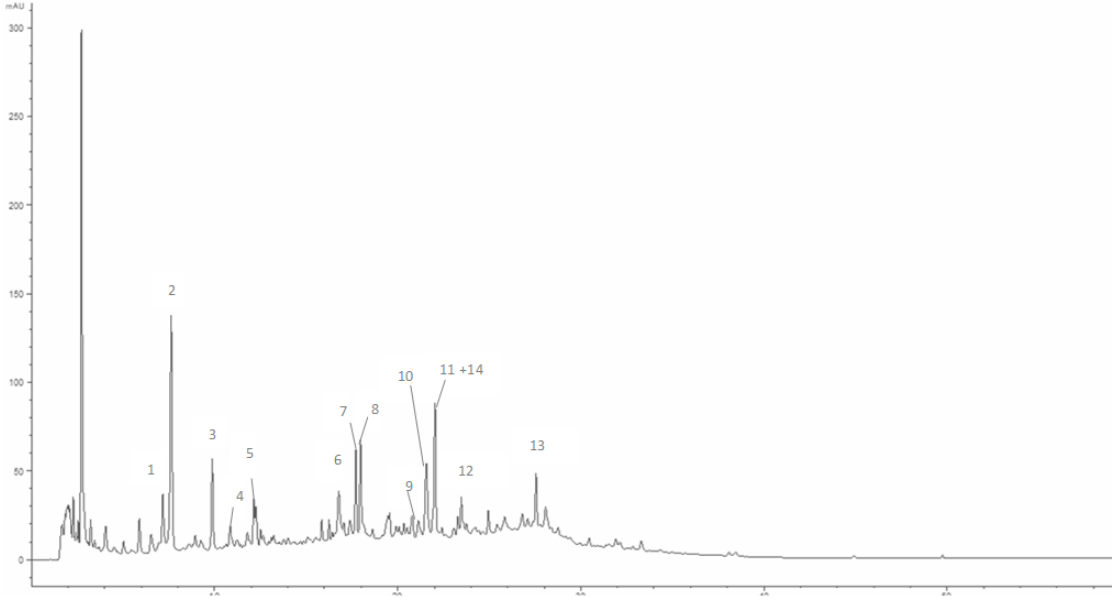


Fig. 4: Chromatograms of PT of OP by HPLC – DAD and main phenolic compounds identify. (PT = phytocomplex fraction, OP = olive pomace).

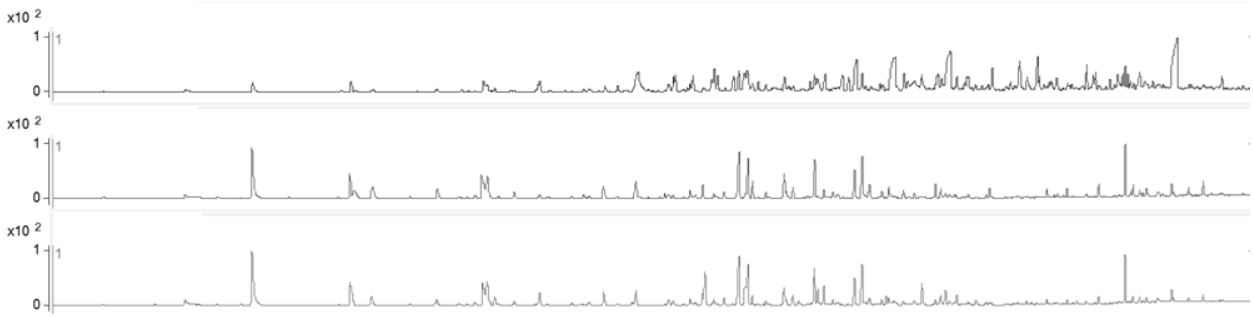


Fig. 5: Chromatograms of HY fraction of the three matrices (in order OL, OS and OP) (HY = hydrolate fraction, OL = olive leave, OS = olive stone, OP = olive pomace).

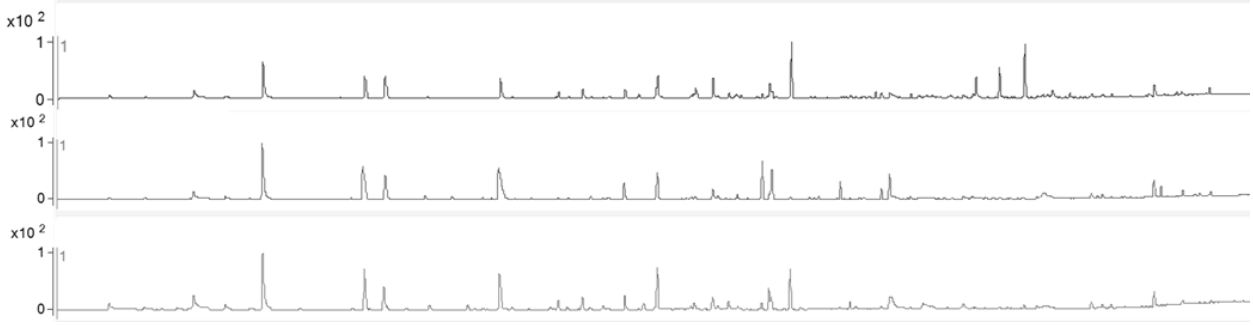


Fig. 6: Chromatograms of PT fraction of the three matrices (in order OL, OS and OP)(PT = phytocomplex fraction, OL = olive leave, OS = olive stone, OP = olive pomace).

Tables

Table 1: Phenolic compounds (mg/kg) in PT fraction of OL and OP detected by HPLC. Means and standard deviation are shown. The sig. columns report the ANOVA results (* = 0.05, ** = 0.01, *** = 0.001, ns = not significant, PT = phytocomplex fraction, OL = olive leave, OP = olive pomace).

| Fraction | Matrix | *Sig.* | Total phenolic compounds (mg/kg) |
|---|---|---|---|
| PT | OL | *** | 1767.35 ± 78.00 |
| PT | OP | *** | 679.00 ± 73.75 |

Table 2: Volatile compounds detected in PT and HY fraction of OL, OP and OS detected by GC- MS (PT = phytocomplex fraction, HY = hydrolate fraction, OL = olive leave, OP = olive pomace, OS = olive stone, D = detected, ND = not detected).

| Compounds | Rt | HY | | | PT | | | Scent |
|---|---|---|---|---|---|---|---|---|
| | | OL | OP | OS | OL | OP | OS | |
| hexanal | 13.3 | D | D | D | ND | ND | ND | green, apple |
| b-pinene | 15.5 | D | D | D | ND | ND | D | |
| a-pellandrene | 15.9 | D | ND | D | ND | ND | ND | |
| dodecane | 16.2 | ND | ND | ND | ND | ND | D | |
| octadecane | 16.4 | ND | ND | ND | ND | ND | D | |
| heptanal | 16.5 | D | ND | D | ND | ND | ND | fatty |
| limonene | 17.0 | ND | ND | ND | ND | ND | D | |
| eucaliptol | 17.4 | D | D | D | ND | ND | ND | |
| 2-hexenal | 17.7 | D | D | D | D | D | D | bitter, almonds, green |
| 3-carene | 18.4 | D | D | D | ND | ND | ND | |
| 3-octanone | 18.6 | ND | ND | D | ND | ND | ND | nut |
| o-cymene | 19.3 | D | D | D | ND | ND | ND | |
| 2-butenal | 19.3 | D | D | D | ND | ND | ND | |
| octanal | 19.7 | D | ND | ND | ND | ND | ND | fatty, soap, lemon, green |
| acido oleico | 20.0 | ND | ND | ND | ND | D | ND | |
| acido formico | 20.4 | ND | D | D | ND | ND | ND | |
| hexadecane | 20.5 | D | ND | ND | ND | ND | ND | |
| 2-heptenal | 20.9 | D | D | D | ND | D | ND | soap, fat, almond |
| 1-hexanol | 21.1 | D | D | D | ND | ND | ND | fruity, aromatic |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3-hexen-1-ol | 22.1 | D | D | D | D | D | D | fruity, pungent |
| tetradecano | 22.3 | ND | ND | ND | ND | D | ND | |
| 2-hexen-1-ol | 22.6 | D | ND | ND | ND | ND | ND | green, fruity |
| nonanale | 22.8 | D | ND | ND | ND | ND | ND | soapy, citrus-like |
| 1-heptanol | 22.7 | ND | D | D | ND | ND | ND | |
| 1-hexanol-3-metil | 22.8 | ND | ND | ND | D | D | D | |
| 1-octen-3-ol | 23.8 | ND | ND | D | ND | ND | ND | mushroom, moldy |
| benzene | 24.1 | D | D | D | ND | ND | ND | |
| p-mentatriene | 24.2 | D | ND | ND | ND | ND | ND | |
| tujone | 24.4 | ND | D | D | ND | ND | ND | |
| furfural | 24.7 | ND | ND | ND | D | D | D | |
| acido benzioico | 24.8 | D | D | D | ND | ND | ND | |
| 3 hexanol 2 ethyl | 24.9 | ND | ND | ND | D | D | D | |
| mentone | 25.1 | D | D | D | ND | ND | ND | |
| piridina | 25.2 | ND | ND | ND | D | ND | ND | |
| neptalene | 25.5 | D | ND | ND | ND | ND | ND | |
| 2,4-heptadienal | 25.7 | D | D | D | ND | ND | ND | fatty |
| p-mentone | 25.9 | ND | D | D | ND | ND | ND | |
| linalol | 26.4 | D | D | D | D | D | D | |
| etanone | 26.6 | D | ND | ND | ND | ND | ND | |
| isometol acetate | 27.4 | D | D | D | ND | ND | ND | |
| bornyl acetate | 28.0 | D | D | D | ND | ND | ND | |
| camphor | 26.7 | D | D | D | D | D | D | |
| 4-terpineol | 28.3 | D | D | D | ND | ND | D | |
| menthol | 29.1 | D | D | D | ND | D | D | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2-decenal | 29.5 | D | D | D | D | D | ND |
| pulegone | 29.9 | D | D | D | ND | ND | ND |
| cis.carveol | 30.4 | D | ND | ND | D | ND | ND |
| a-terpineol | 30.5 | ND | D | D | ND | D | D |
| tujopsene | 30.5 | ND | ND | ND | D | ND | ND |
| endoborneol | 30.8 | D | D | D | D | D | D |
| verbenone | 31.5 | D | D | D | D | D | D |
| geranil acetate | 31.8 | ND | D | D | ND | ND | ND |
| carvone | 32.0 | D | D | D | ND | ND | ND |
| 2,4 dedienal | 32.2 | ND | D | D | ND | ND | ND |
| drimenol | 32.7 | D | ND | ND | ND | ND | ND |
| endoborneol | 32.7 | ND | D | ND | ND | ND | ND |
| 2,4 decadiene | 33.4 | D | D | D | ND | ND | ND |
| geraniol | 33.6 | D | D | D | ND | ND | ND |
| Valeric acid | 33.7 | ND | ND | ND | D | ND | D |
| 2 buten 1 one | 33.8 | ND | ND | ND | D | ND | ND |
| demascnone | 33.9 | D | ND | ND | ND | ND | ND |
| 5.9 undecadien 2-one | 34.2 | D | ND | D | ND | ND | ND |
| 3 buten 2 one | 34.5 | D | ND | ND | ND | ND | ND |
| a-toluenol | 34.6 | ND | ND | ND | D | D | ND |
| 2 butanone | 34.9 | D | D | ND | ND | ND | ND |
| benzenethanol | 35.5 | D | ND | ND | D | D | D |
| 3-carene | 37.1 | D | ND | ND | D | ND | ND |
| falcarinol | 37.3 | D | D | D | ND | ND | ND |
| benzene | 37.4 | D | ND | ND | ND | ND | ND |
| nerodiol | 37.8 | D | ND | ND | ND | ND | ND |
| caryophillene ox | 37.8 | ND | ND | D | ND | ND | ND |
| 2-butanel | 38.1 | ND | D | D | ND | ND | ND |
| acetamide | 38.5 | ND | D | ND | ND | ND | ND |
| acido nonaico | 39.9 | D | ND | ND | D | D | D |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| thymol | 40.2 | D | D | D | ND | ND | D |
| eugenol | 40.3 | D | ND | ND | ND | ND | ND |
| cardinol | 40.5 | D | ND | ND | ND | ND | ND |
| juniper camphor | 41.9 | D | D | D | D | ND | ND |
| | | HY | | | PT | | |
| | Olive leaves | Olive pomace | Olive stone | Olive leaves | Olive pomace | Olive stone | Olive leaves |
| Tot. compounds identified | 48 | 41 | 44 | 19 | 25 | 22 | 48 |

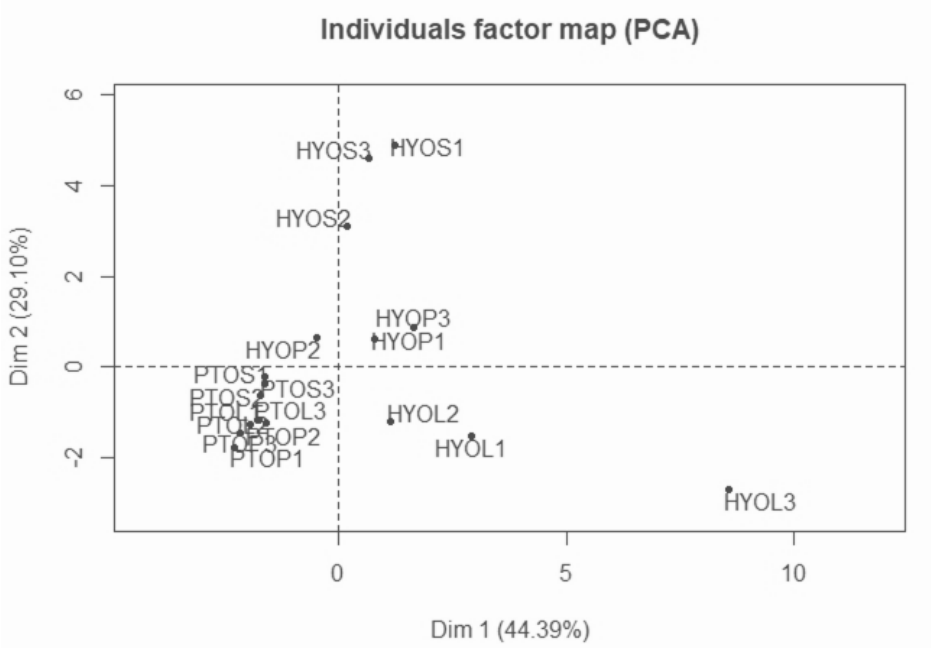


Fig. 7: Biplot of the extracted samples (PT = phytocomplex fraction, HY = hydrolate fraction, OL = olive leave, OP = olive pomace, OS = olive stone).

Table 3: Total area of the peaks of the volatile compounds identified. Mean and standard deviation are reported. The sig. columns report the ANOVA results (* = 0.05, ** = 0.01, *** = 0.001, ns = not significant), while lowercase letters represent the Tukey HSD post hoc test results. (PT = phytocomplex fraction, HY = hydrolate fraction, OL = olive leave, OP = olive pomace, OS = olive stone, D = detected, ND = not detected).

| Fraction | *Sig.* | Matrix | *Sig.* | Total peak area of identified compounds | |
|---|---|---|---|---|---|
| HY | * | OS | ** | 41628463.10 ± 5297044.94 | b |
| HY | * | OP | ** | 17743439.82 ± 3731139.99 | b |
| HY | * | OL | ** | 155044598.79 ± 80752072.57 | a |
| PT | * | OS | ** | 545249.07 ± 281341.00 | b |
| PT | * | OP | ** | 1868154.16 ± 1415872.82 | b |
| PT | * | OL | ** | 6747350.53 ± 232326.96 | b |

## 10. General conclusions

The topic of the PhD course was the study of steam distillation process. In the thesis, this was approached from an engineering point of view. The whole process was studied in the details. The operative factors involved were deepened in order to understand the relative importance of each factor in the essential oil yield and at the same time, to minimize the consumptions and waste. It was possible to establish the best operating conditions to extract the maximum yield of rosemary essential oil. That is, with the steam distillation method for an extraction duration of 120 min. Furthermore, it was possible to develop the application of a photoionization detector (PID) for monitoring and controlling of different products in the phases of the distillation process. In this contest, the matrices entering to the process was deepened. Two varieties of rosemary plant were studies. In the detail, PID detector was able to capture different signals, and a sort of 'fingerprint' for each of the two varieties was obtained. Thus, it was possible to distinguish different varieties of aromatic plants. Some preliminary research has also been conducted on the products coming out of the process and therefore hydrolats and essential oils. This topic will be further investigated in future studies. In this sense, the PID detector has the potential to be an easy, simple and fast method to monitor and control the whole process. Finally, steam distillation was applied as an alternative and sustainable method for the recovery of bioactive compounds with high added value from the waste of some agri-food industries. From coffee and olive oil industry by-products two fractions were obtained. One, richer in volatile

compounds and another less rich in volatile compound but rich in other compounds such as phenols. The studies allowed to obtain important information from the application of the distillation process in this sense. However, further studies will be necessary to optimize the process and ensure the maximum yield of the compounds sought.

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Appendix – Original papers