EFFECT OF AGRONOMIC AND TECHNOLOGICAL FACTORS ON THE FORMATION OF ETHYL ESTERS IN VIRGIN OLIVE OIL IN CATALONIA

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UNIVERSITAT ROVIRA i VIRGILI

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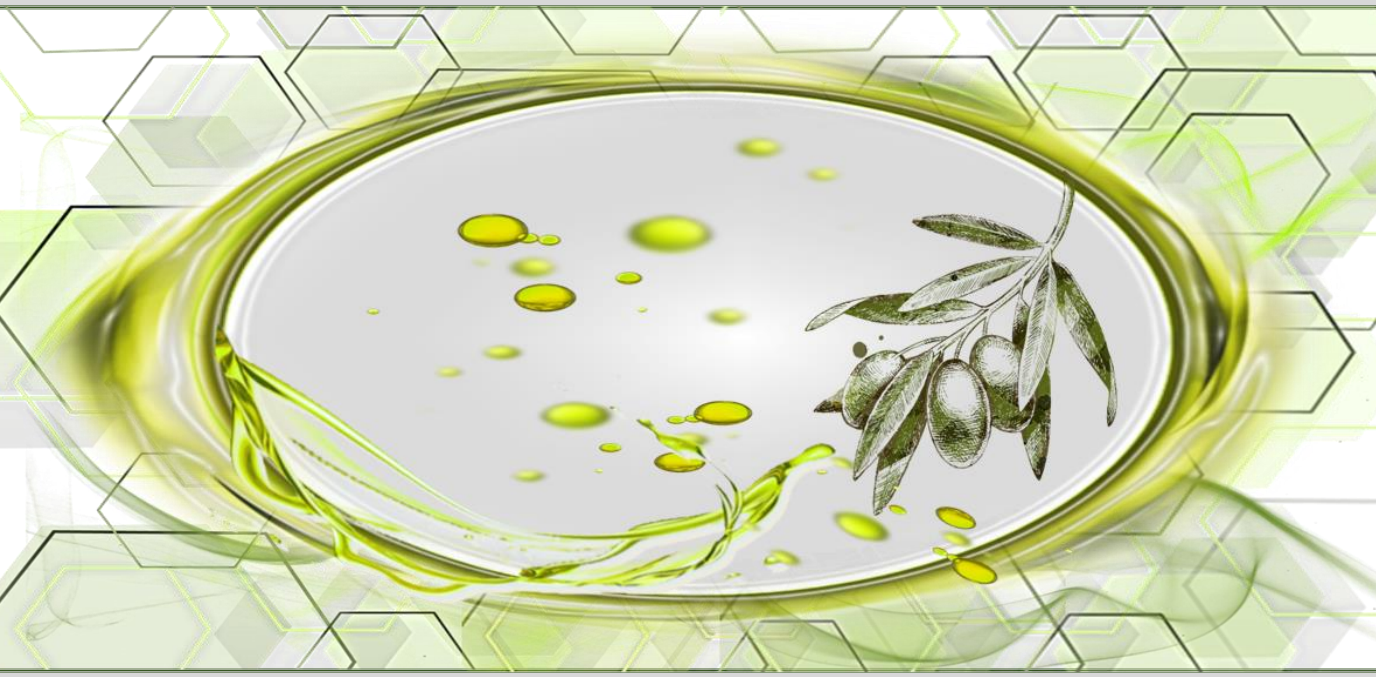
BOUDEBOUZ ABDELAZIZ



DOCTORAL THESIS

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Institut de Recerca i Tecnologia Agroalimentaria (IRTA)

EFFECT OF AGRONOMIC AND TECHNOLOGICAL FACTORS ON THE FORMATION OF ETHYL ESTERS IN VIRGIN OLIVE OIL IN CATALONIA

Doctoral Thesis presented by

Abdelaziz Boudebouz

To receive the degree of doctor by the Rovira I Virgili University

Tarragona, 2021

UNIVERSITAT ROVIRA I VIRGILI

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CERTIFY

That the Doctoral Thesis entitled "**Effect of Agronomic and Technological Factors on the Formation of Ethyl Esters in Virgin Olive Oil in Catalonia"**, submitted by **Abdelaziz Boudebouz** to receive the degree of Doctor by the Universitat Rovira i Virgili, has been carried out under our supervision, in the Department of Analytical Chemistry and Organic Chemistry of this university and the Institute of Agrifood Research and Technology, and all the results presented in this thesis were obtained in experiments conducted by the above mentioned student.

Tarragona, 10th June 2021

Dr. Ricard Boqué Martí Dra. Montserrat Mestres Solé Dr. Agustí Romero Aroca

This work constitutes a part of the research developed by the Instrumental Sensometry Group of the Rovira i Virgili University together with the Institute of Agrifood Research and Technology of the Government of Catalonia. All the results here presented have been obtained in the iSens laboratory at the Faculty of Oenology of Tarragona and the IRTA facilities at Mas Bové and Monells.

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The Cooperative was created in 1920 and is mainly dedicated to the production and marketing of extra virgin olive oil.

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A mi Hermano, Salah

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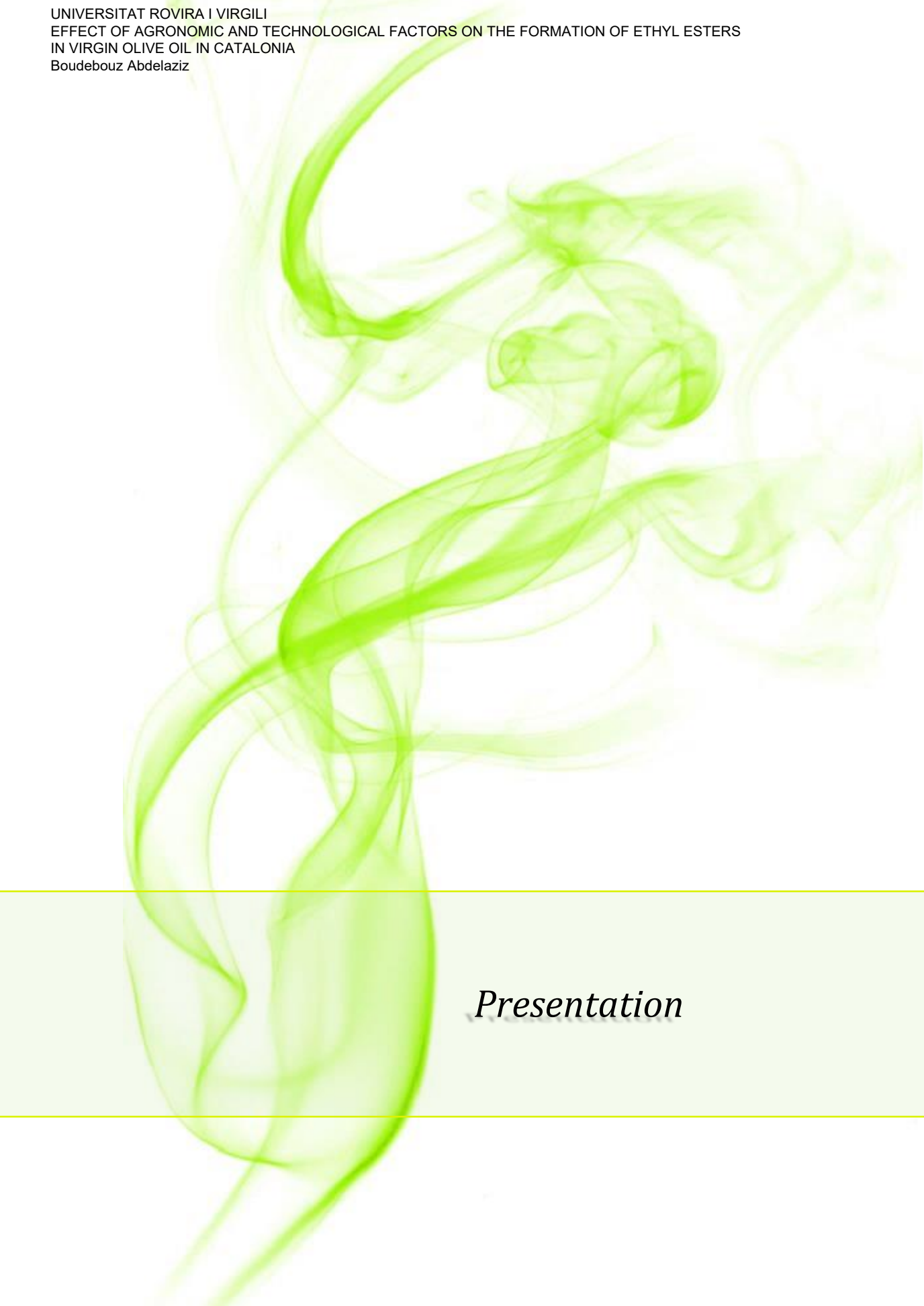

Presentation

Scope

THE VIRGIN OLIVE OIL sector is one of the most important economically and culturally in the entire Mediterranean basin, including Catalonia. The peculiarities of Catalonia, located in the northeast of the Iberian Peninsula, are the small size of the orchards and the variable size of the milling facilities, which implies strengths and weaknesses for the quality of oil. Although Catalonia represents only 6% of total Spanish production, its producers are very aware of the quality of olive oil, as 85% of the oils produced are of extra virgin category. Therefore, the Catalan olive oil sector is aware of the entry into force of any new quality index in the Official Regulations.

The quality parameter more recently included in the Official Standard is the content of ethyl esters of fatty acids. This parameter distinguishes extra virgin olive oil from other oil qualities by limiting the maximum amount to 35 mg/kg.

THE FATTY ACID ETHYL ESTERS are used as a marker for the freshness, quality and good handling of olives throughout the processing of olive oil. However, because these compounds result from a rapid esterification reaction between free fatty acids and ethanol, it is also necessary to prevent the formation of these precursors to avoid large amounts of ethyl esters. Thus, the factors that influence the formation of precursors must be controlled, both agronomic factors (variety and ripening stage) and technological ones (postharvest management, extraction process and storage conditions).

CATALONIA has different autochthonous olive varieties for the production of olive oil, with the 'Arbequina' variety being the most widespread. In addition, there are significant variations in the production conditions between the different olive growing areas, in particular those in southern Catalonia.

Currently, several studies reflect and describe the factors that increase the free acidity of olive oil; however, there is still little information on short chain alcohol synthesis, which through esterification with the free fatty acids deliver alkyl esters.

Taking into account the above, this thesis aims to study the key factors that can increase the content of alcohols (both in olives and in olive oil) because this increases the risk of formation of alkyl esters. It should be noted that in this thesis all postharvest and processing experiments were carried out in real conditions to obtain the most representative results possible.

Objectives

The main goals of this PhD thesis are: 1) to determine the actual prevalence of fatty acids ethyl esters in commercial Catalan virgin olive oil, and 2) which are the main factors, either agronomic or technological (including olive variety, fruits postharvest storage and oil extraction steps), which increase the risks of formation of fatty acid alkyl esters in Catalan production. The particular objectives are:

1. To estimate the current situation of the levels of ethyl esters in the virgin olive oil produced in Catalonia. This involves assessing the prevalence of the quality of virgin olive oils according to the new standard of the EU Commission (Regulation (EU) 2019/1604), which considers the content of ethyl esters as a quality parameter to distinguish extra virgin olive oils from other lower quality oils.

2. To assess the effect of olive variety on the endogenous amount of ethanol and methanol (precursors of alkyl esters), and acetaldehyde (precursor of ethanol) in healthy ripe olive fruits, including a wide range of the main Spanish and Catalan olive varieties.

3. To evaluate the effect of short-term storage of olives in a high-capacity hopper on the formation of alkyl alcohols in the fruits and its relationship with some organoleptic defects. This effect has been be evaluated using 'Arbequina' olives harvested at different ripening stages.

4. To study the effect of the oil separation on the balance of alkyl esters and their alcohol precursors during the processing of 'Arbequina' variety olives. Specifically, we have evaluated:

    4.1. The effect of the rate of paste injection into the decanter, as changing this rate is also an easy intervention that can be easily implemented at any time and in any type of decanter without stopping the process.

    4.2. The effect of the flow of water addition into the horizontal centrifuge (decanter), as this is a parameter that can be easily modified at any time and in any type of decanter without stopping the process.

    4.3. The influence of the "decanter-to-vertical centrifuge" step on the balance of these compounds, as it deals with the olive oil produced through the process.

Structure of the Thesis

The thesis consists of three main parts.

The first part presents the state of art, with a detailed bibliographic review. This is the starting point of this Doctoral Thesis, since the objectives were defined from the gaps detected in this research field. This part is divided into three Chapters:

- ***Chapter* 1.-** This chapter introduces definitions and the current situation of the virgin olive oil sector in the world and in Catalonia.
- ***Chapter* 2.-** In this chapter, the quality of virgin olive oil and the factors that influence it are described.
- ***Chapter* 3.-** This chapter focuses on the fatty acid alkyl esters in olive oil and their impact on the production of virgin olive oil in Catalonia.

The second part includes:

- ***Chapter* 4.-** that describes the sampling procedures and methodologies used to carry out the different experiments.
- ***Chapter 5.-*** that details the four experiments carried out to achieve the main objectives of this Thesis, presenting each one following the corresponding article structure and contents to make it easier to understand. Thus, the four articles to which this doctoral thesis has given rise are presented:

  Paper 1:- "Quantitation of endogenous amount of ethanol, methanol and acetaldehyde in ripe fruits of different Spanish olive varieties". This scientific article has been **published** in the *Journal of the Science of Food and Agriculture* and is particularly relevant because it describes the validated methodology developed to analyze alcohols in olives.

  Paper 2:- "Alcohols formation during short-term storage of 'Arbequina´ fruits and their relation with olive organoleptic quality losses". This article shows novel and consistent results and it has been **submitted** to the *Postharvest Biology and Technology* journal. This journal is devoted to the publication of articles on technological postharvest research, which includes the areas of postharvest storage and quality evaluation, which fits perfectly with our research.

Paper 3:- "Processing factors that affect the balance of alcohols and alkyl esters during 'Arbequina' olive oil production: separation and clarification steps". This paper has been **published** in the *LWT-Food Science and Technology* journal. This work is especially interesting because all the results have been obtained in a real oil mill, so the resulting conclusions and practical recommendations can be of great help to the producer.

Paper 4:- "Survey of the current situation of fatty acid ethyl esters and their prevalence in virgin olive oil in Catalonia". This study is already prepared for **submission**. It should be noted that the results were obtained from a very exhaustive sampling that took into account both the quality and the origin of the samples, that is, the results can be considered representative of the current situation of the Catalan olive sector.

The third part is also formed by two Chapters:

- ***Chapter 6.-*** presents a discussion of the most relevant results obtained in this Doctoral Thesis, but trying to provide a more general vision than that detailed in the articles. The most innovative and consistent results are highlighted.
- Finally, ***Chapter 7.-*** details the main conclusions derived from the results that match the main objectives of the thesis.

The bibliographic references are presented at the end of each section.

CHAPTER 1.

The Sector of Virgin Olive Oil

1.1. Origin and distribution of Olive Oil

The olive tree (*Olea Europea L.*) is a typical arboreal species that is part of the Mediterranean agricultural triad. Its origin dates back to 3000-4000 BC, on the eastern Mediterranean Coast and Asia Minor (in what is now Syria). Human's control over this tree led to its cultivation and gave rise to one of the first post-harvest activities of humanity, an activity that has been spreading since ancient times. The active dispersal of the olive fruit by birds (especially by the Common Starling) has contributed to his expansion, which has mainly allowed local propagation, although migrations have also allowed propagation over relatively long distances. However, it should be noted that human activity has been the main factor that has contributed to the intercontinental propagation of this species [1-3].

This is how the cultivation of olive trees expanded from the Asia Minor region to the Mediterranean basin. First, it was introduced in Greece thanks to the maritime expansion of the Phoenicians, in the 9th century BC and later its cultivation was spreading towards North Africa, Sicily and southern Italy and the Mediterranean coast, mainly southern of the Iberian Peninsula. However, the great expansion of this cultivar was promoted by the Roman Empire. Romans considered olive oil as an indispensable product, not only used for cooking but also as an ointment in the hot springs or as fuel for lamps. This involved a whole network of trade around olive oil that spread throughout the Roman domains.

Olives and olive oil became one of the most important elements of daily life in all the countries of the Mediterranean basin. For this reason, the peoples who have inhabited this area have always contributed all their knowledge to improve both the cultivation of the olive tree and the extraction of oil. In fact, the interest in this product was such that it became the heritage of the Mediterranean peoples.

Its expansion to the rest of the planet coincides with the adaptation of this crop in areas where there are conditions similar to those of this biotope. In fact, from the 15th century, with the discovery of the American continent, the trade of these products reached the new world, and this is why, in the 16th century, olive trees began to be cultivated in Mexico, Peru, Chile and Argentina. Later, already in the 18th century, it was also cultivated in areas of the southern United States such as California and Florida. The spread of cultivation did not stop here and, nowadays, the olive tree is grown in places as far away from its origin as Australia, China or Brazil.

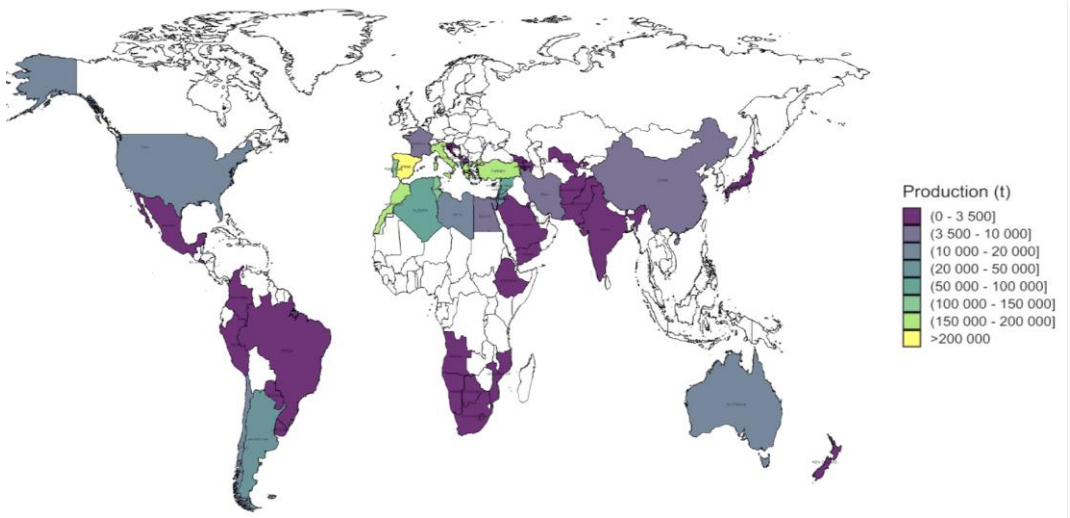

Figure 1.1. Distribution of the olive oil production in the world (Source: IOC, 2019)

In any case, the current reality is that, although the olive tree has spread across the five continents, the presence of this crop in areas other than the Mediterranean basin is merely testimonial. Thus, 98% of the olive trees planted in the world are distributed in the different areas of the Mediterranean and only the remaining 2% are distributed throughout the rest of the world, mainly in the southern of the American continent and Australia [4]. Focusing on the importance of the olive grove in Europe and Spain, in terms of cultivated area, the European Union represents 47% of the total area devoted to olive groves in the world, which implies approximately 4.5 million hectares. Spain is the country with the largest area dedicated to olive trees, representing 22% of the world area of this crop.

1.2. Olive oil (definition and characteristics)

According to the International Olive Council (T.15/NC No 3/ Rev. 11, July 2016) and the European Community (EC Reg. nº 1348/2013 that modify Reg. 2568/91), olive oil is the natural juice obtained from the fruits of olive tree (*Olea Europea L.*), with exclusion of those obtained using solvents or re-esterification.

Olive oil can be classified into different categories depending on both the process used to obtain it or its physicochemical characteristics. The so called virgin olive oil (VOO) is extracted

exclusively by physical-mechanical means and under thermal conditions that do not alter the oil, without any other treatment than washing, decanting, centrifuging and filtering [5, 6]. When the chemical composition is good enough and the organoleptic evaluation is excellent (without defects), it is classified as "extra virgin" olive oil (EVOO). Whereas, it is graded as "virgin" olive oil (VOO) if some leger sensorial or chemical problems appear. Finally, it is classified a "lampante" olive oil (LOO) if sensorial defects or chemical problems are very important and the oil cannot be bottled without additional refining processes. Besides, the IOC classifies the LOO as "ordinary", with medium intensity defects, and "lampante" with very high defects.

When the oil is obtained under more aggressive conditions or its organoleptic characteristics are not optimal, the IOC classifies these oils into other categories. So, if the oil is extracted from the olive pomace (by-product of the milling process) it is declared as "olive pomace oil" (OPO) and must be refined before consumption. The refining process can be either physical or chemical and produces the so called refined olive oil (ROO) and refined olive pomace oil (ROPO). In Europe, both refined oils must be mixed with some EVOO or VOO before bottling and retailing them as "olive oil" or "olive pomace oil".

Virgin olive oil is a valuable product both for its undeniable organoleptic qualities as well as for its nutritional and functional properties in comparison to other vegetable oils. These properties have made olive oil one of the pillars of the so-called Mediterranean diet, which is characterized by having one of the lowest incidences of chronic diseases in the world, while life expectancy is one of the longest.

The Phoenicians, Greeks and Romans already knew the antioxidant, anti-inflammatory and antimicrobial properties of olive oil so they used it to heal burns and sores, to strengthen skin and muscles, to relieve stomach aches or to treat infections. However, it has been during the last two centuries that these properties have been studied, documented and disseminated in such a way that there is an increasing evidence that the intake of this product has positive effects on oxidative and inflammatory diseases, such as cardiovascular diseases, cancer or neurodegenerative diseases, that are the most prevalent nowadays. Thus, now we know that the beneficial properties of the olive oil are due to the presence of different chemical compounds found at different concentrations but with a very suitable balance. The main ones are monounsaturated fatty acids (especially oleic acid) and other polyunsaturated acids such as omega 3 and 6 but the beneficial effects are also due to the action of other minor components such as polyphenols, tocopherols, chlorophylls or carotenoids [7, 8].

From a chemical point of view, olive oils are basically composed of two fractions which are called saponificable and unsaponificable. The first one represents about 98% of the total weight of the oil and is composed mainly of fatty acids esterified with glycerol, but also by other minor components such as free fatty acids, phospholipids, and waxes. The unsaponificable fraction represents about 2% of the total weight and includes more than 200 minor compounds not chemically related to fatty acids such as aliphatic and triterpenic alcohols, phenols, carotenoids, sterols, hydrocarbons, tocopherols and many other volatile compounds with very different chemical nature and that are responsible for the aroma [9-15]. Both fractions condition the physical-chemical properties of the oil and their exact composition depends on the olive variety, fruit characteristics, edapho-climatic conditions, extraction process and storage conditions [10, 16-18]. However, recent studies show that the compounds that actually differentiate the olive oils are the so-called minor compounds. In fact, these are used as fingerprint for the characterization, authenticity and even traceability of the geographic origin of olive oils [19-21].

1.3. Economic impact and benefits

Nowadays, consumers demand food products that, in addition to being organoleptically pleasing, also provide nutritional benefits. Since the virgin olive oil meets both characteristics, its demand and popularity has increased remarkably [22, 23]. The growing interest in this product has led to the distribution of olive cultivation in about 47 producing countries spread over the five continents. Many of these countries are members of the International Olive Council (IOC), the world's international intergovernmental organization in the field of olive oil and table olives. Thus, the IOC member countries represent 89% of the world's olive orchards, while the newest producing countries share 2.7% of the world olive crop area. As for the total area devoted to olive trees, it is approximately 11 million hectares, although not all having the same plantation density: 90% is planted with traditional spacing (80-200 trees/ha), 9% with intensive density spacing (200-600 trees/ha) and the remaining 1% is planted with super-high density spacing (more than 1200 trees/ha). It can be stated that the world olive oil sector generates a turnover between 6,500 and 11,000 million euros per crop year [24].

1.3.1. Worldwide production

The evolution of olive cultivar areas has been clearly positive over the last 20 years, with significant increases worldwide of almost 21%. This is mainly due to the increase that has

occurred in the southern hemisphere (especially in Argentine, Chile and Australia). However, for the EU countries, this trend has been very different as the planted area has been maintained or even reduced by 6 to 8% of its total production area (2015 data).

Regarding olive oil production, it varies from year to year depending on the natural alternate bearing cycle of the olive tree and the weather patterns that influence fruit set and crop load. The world production is estimated at almost 3.2 million tones and approximately 94% are produced by the IOC member countries. According to estimates of the International Olive Council, during the 2019/20 season, the contribution of EU countries was estimated at 60% of total production, but with a decrease of 15% compared to the average annual production. Spain leads the world ranking with a production of 1,125,300 tons (decrease of 37.1% compared to the previous season), followed by Italy with 366,000 tons (increase of 110.8%), Greece with 275,000 tons (increase of 48.6%) and Portugal with 140,500 tons (increase of 40.1%). The other current IOC member countries (of which: Tunisia, Turkey, Morocco, Algeria, Egypt, Argentina, Jordan, Palestine) produced about 1 million tons (increase of 20% compared to the previous season). The countries that do not belong to the IOC produced about 6% of total olive oil [24].

If we focus on the contribution of the UE countries, it can be stated that it is leaded by Spain with approximately 45% of the world olive oil production, followed by Italy, Greece and Portugal with 25%, 20% and 2%, respectively [25, http://faostat3.fao.org/home/E]. However, if we extend this study to the last 50 years, what we observe is that two countries have lost importance compared to two others. Thus, while Italy and Portugal have experienced a significant decrease in the percentage of EU production (from 40% to 25% for Italy and from 7.3% to 2% for Portugal, though in this country a lot is being planted), Greece and Spain have increased their contribution. Specifically, Greece increased from 15% to 20% of EU production and Spain stands out as the most dynamic country (especially after the 1990s), with an average contribution between 43% and 51% of the EU production.

Outside the EU, Tunisia surpasses the rest of the countries, although its share of production has decreased significantly in average over the last 20 years, with a 7% drop. A similar trend is observed for the whole group of the IOC member countries, as it has suffered a decrease of 10% on the world production, although it should be noted that, during the last decade, this decrease is attributed to the significant increase in production of non-IOC countries [16, 26-27].

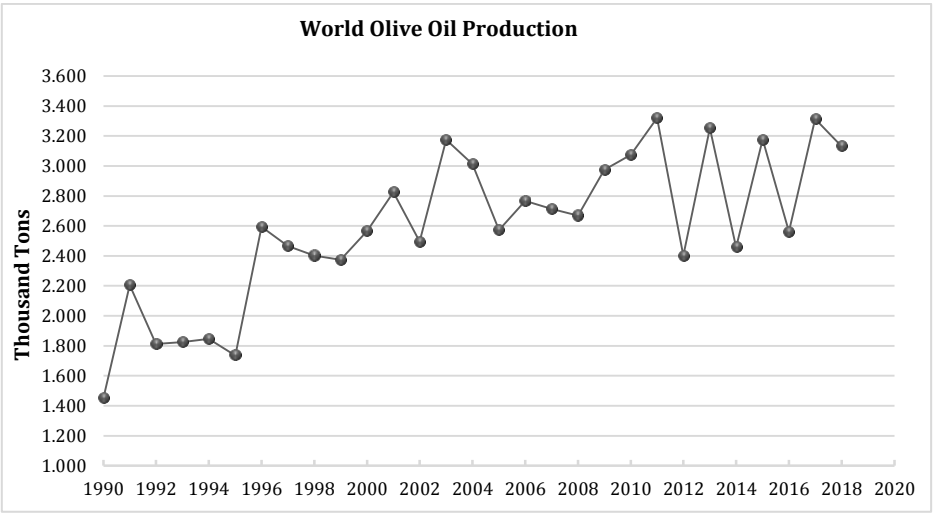

Figure 1.2. Evolution of world olive oil production between 1990 and 2019 (Source: www.statista.com)

1.3.2. Worldwide consumption

Consumers are increasingly concerned about the health benefits of olive oil. Related to this, the European Food Safety Agency (EFSA) accepts two claims about healthy:

- "*Replacing saturated fats in the diet with unsaturated fats contributes to the maintenance of normal blood cholesterol levels. The claim may be used only for food which is high in unsaturated fatty acids, as referred to in the claim HIGH UNSATURATED FAT as listed in the Annex to Regulation (EC) No 1924/2006*". Published in EFSA Journal 9(6):2203 (2011).
- "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil, containing at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil*." Published in EFSA Journal 9(4):2033 (2011)

Over the past 25 years, world consumption of olive oil has increased by more than 1 million tons: from 1.7 million tons at in the early 1990s to more than approximately 3 million tons in the 2020/21 season [28]. When analyzing global consumption over the last five years (2015 to 2018/19), it can be seen that the highest consumption occurs in the main IOC producer's countries (69.6%). Among the IOC member countries, Spain ranks first, with a consumption

share of more than 17.8% of the world consumption, followed by Italy with approximately 16%, Turkey with 5.5%, and Greece and Morocco with 4%. In America, the demand is mainly represented by the USA that consumes around 320,000 tons (10.7%), followed by Brazil with 68,000 tons (2.9%) and Canada with 44,000 tons (1.5%). In the rest of the countries, the consumption is basically situated in Japan that consumes around 58,000 tons (2.4%), followed by China and Australia with 45,000 tons each (1.7%), and Saudi Arabia with almost 31,000 tons (1.2%) [29].

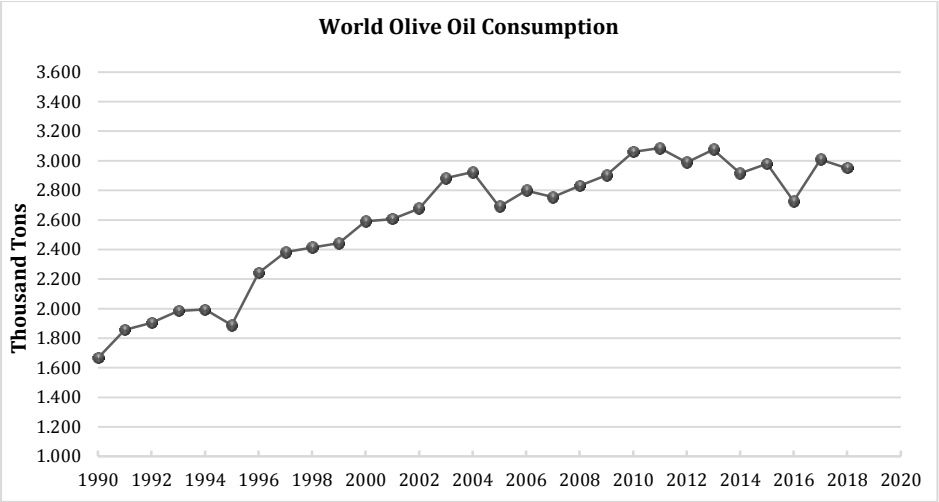

Figure 1.3. Evolution of world olive oil consumption between 1990 and 2019 (Source: www.statista.com)

The increasing demand for olive oil has generated a significant expansion of this market throughout the world. In fact, the imports and exports amount to more than 950,000 tons in the last years. However, even with this expansion it should be noted that the 3.1 million tons of olive oil produced and consumed per year in the world represent only the 2% of all edible vegetable and animal fats. Therefore, it is clear that the possibilities of this product have not yet been fully exploited, so that the world production of olive oil can be considered as a strategic economic sector and an influential player in the international arena. According to the IOC (2020), the US leads the world olive oil imports, accounting for around 36% of the world imports, followed by the UE (17%), Brazil (8%), Japan (7%), Canada (5%), China (4%) and, Australia and Saudi Arabia (3%) [30].

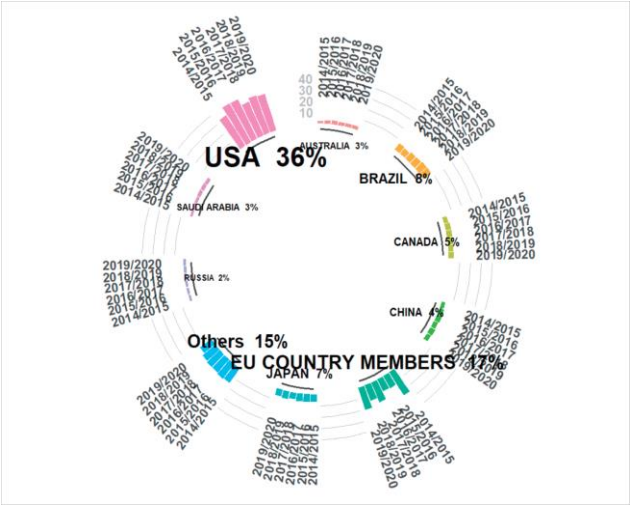

Figure 1.4. Worldwide olive oil imports in percentage (Source: IOC, 2021)

1.4. Current situation of the olive oil sector in Catalonia

Catalonia represents 4% of the Spanish olive-growing area, with a series of autochthonous varieties and an organization of production, elaboration systems, storing and marketing of the oils that is clearly different from the rest of Spain.

The main orientation of the olive sector in Catalonia is the production and commercialization of olive oil. That is why several public institutions collaborate with producers, to provide support to different aspects of the olive oil valorization chain (such as agronomic, technological, analytical quality, health, marketing and oleotourism aspects). The Generalitat de Catalunya (Catalan government) and the Institute of Agri-food Research and Technology (IRTA) develop different research programs oriented to detect, study and solve possible problems in the production chain that can compromise the quality of the oils and, therefore, their competitiveness.

In recent years (2013-2019), Catalonia produced an average of 29.2 tons of virgin olive oil, most of it of the extra virgin category. This production is distributed throughout the Catalan territory, although Tarragona and Lleida represent 95% of the production. In addition, Catalonia has five PDOs (Protected Designation of Origin) for extra virgin olive oil: Terra Alta, Baix Ebre-Montsià, Empordà, Siurana and Les Garrigues. Moreover, although the main cultivated variety is ‘Arbequina’, Catalonia has a great diversity of local and Spanish varieties such as ‘Empeltre’, ‘Morrut’, ‘Sevillenca’, ‘Farga’, ‘Argudell’, ‘Rojal’ and ‘Verdal’ (https://ruralcat.gencat.cat/web/guest/oficina-de-l-oli/olivicultura).

There are currently a total of 347 companies dedicated to the production of olive oil in Catalonia, and 30% of them are exporting companies, with destination mainly to France, Italy and the United States which, together, represent 43.4% of total olive oil exports (Figure.1.5.).

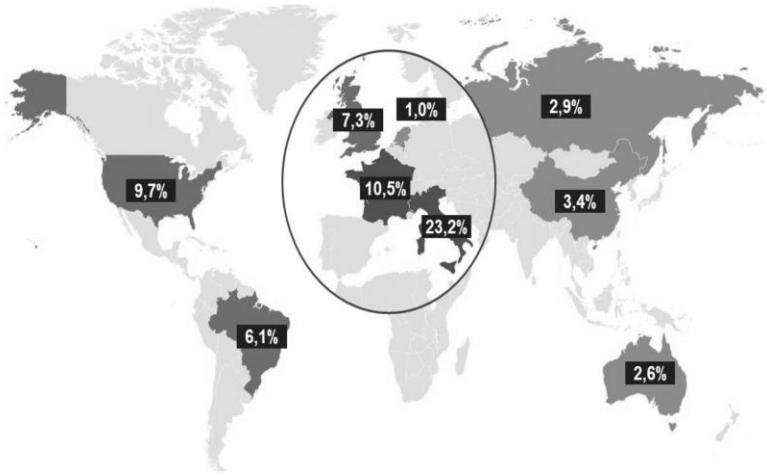

Figure 1.5. Main destinations for olive oil exports from Catalonia
(Source: Mapeig de l'oli d'oliva a Catalunya, Prodecca & ACCIÓ, November 2020)

Catalan trade companies manage more than 210,000 tons every year. However, only 15% of this amount is of local production. In addition, 63% (133,200 tons) of the olive oil sold is of extra virgin category, with a strong focus on the bulk market. The export of olive oil from Catalonia generates an annual average of 397 million euros, of which 80% come from EVOO.

The incorporation of alkyl esters into European and international standards for extra virgin olive oils, as a criterion of "freshness" and limiting their maximum content, has created great concern in the Catalan trade companies. In fact, there is no published information on the current values of these compounds in Catalan olive oils.

1.5. Production of olive oil

Traditionally, olive oil has been obtained by means of methods that resulted in a highly rectifiable product, since the treatment carried out on the olive was not the most appropriate. In fact, the available machinery implied that the pressing, extraction and separation processes were slow, so that the contact time of the water and oxygen with the sample was very long and this caused a significant deterioration of the oil obtained. If we add to this the lack of hygiene of the machinery used and the long storage times, the result is a poor-quality olive oil.

However, since the 90s, this situation has improved remarkably thanks to technological improvements and also to a greater knowledge about the composition and stability of the olive oil. Thus, the new production systems allow not only to reduce processing times, working with a continuous process, but also to obtain an efficient extraction while maintaining the highest quality of the oil.

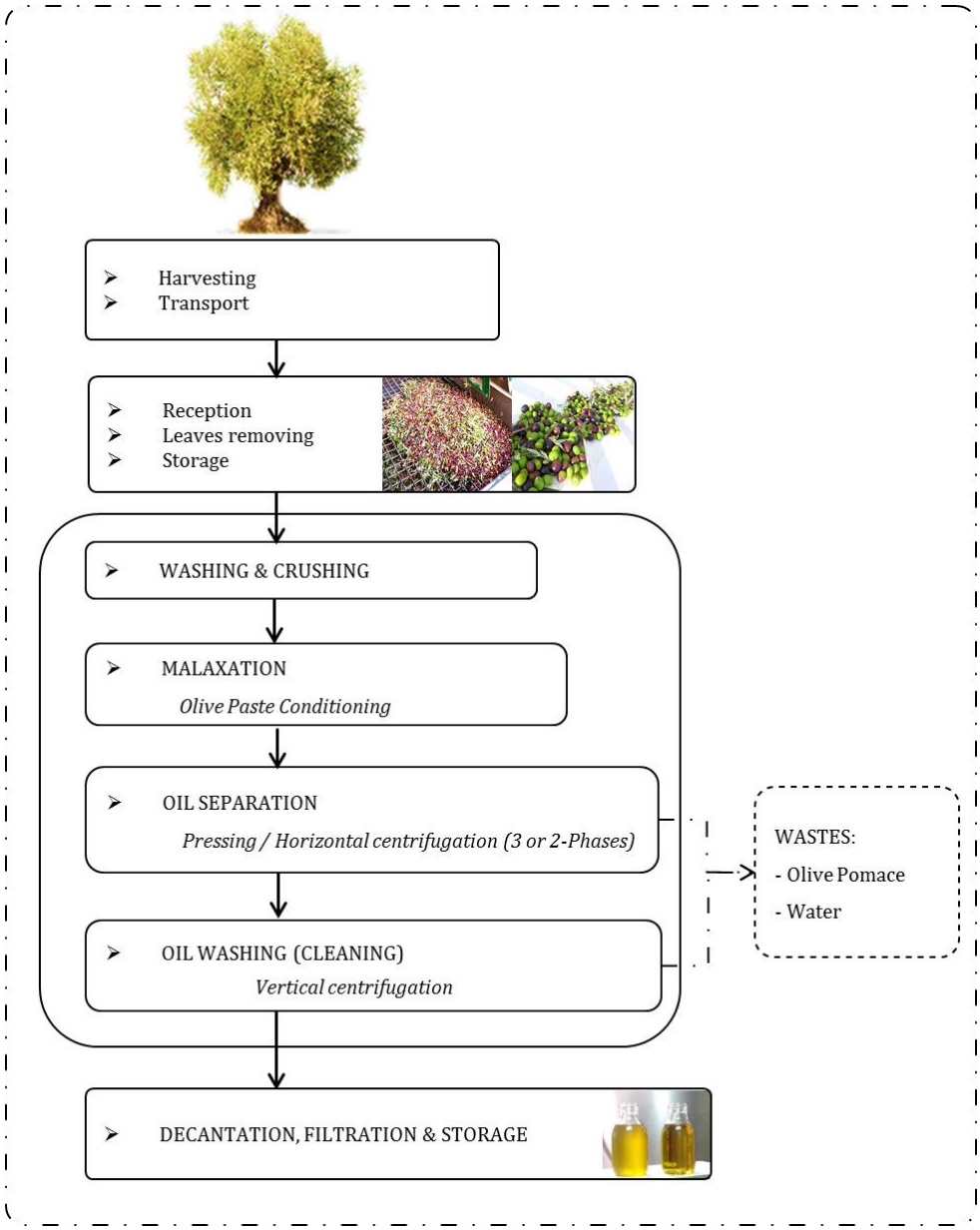

Figure 1.6. General scheme of virgin olive oil production processing

Currently, the most commonly used olive oil extraction process consists of the following steps (Figure1.6):

- **Harvest**

The quality of the oil largely depends on the health status of the olives. Therefore, to produce a high-quality oil, the olives must be harvested properly without altering the quality of the fruit and must be processed as quickly as possible to avoid undesirable evolutions of the fruit. The fruits must be separated according to their degree of quality, so that each grade can be processed separately [31-33].

Generally, as the fruit ripens, there is an increase in polyunsaturated fatty acids and a decrease in total content of polyphenols that implies an increase in oil instability [34-37]. These changes have a great commercial impact since they drastically influence the organoleptic characteristics of the oil as well as its shelf-life.

On the other hand, the early harvest consists of the harvest of immature or green fruits that give rise to an olive oil with high content of polyphenols that contribute to the bitterness and pungency. These oils are relatively more stable because of the antioxidant effect of the polyphenols [38]; however, early harvest results in a lower yield which implies a negative economic impact.

In conclusion, the harvest time is a very important parameter that the producer must consider, as it influences both the yield and the quality of the olive oil and will therefore directly affect the price at which the oil will be sold.

- **Reception and storage**

After being harvested, the olives are transferred to the mill where they must be processed within 12 to 24 hours after harvest. Once there, the leaves and other debris are removed. The most commonly used methods at this stage are vibration and ventilation. The clean olives are then weighed and stored until processed. In general, the olives are stored in large capacity hoppers (between 10,000 and 100,000 kilograms). During this step the olives may suffer different types of alterations due the high loading pressure of the hopper itself and, in some cases, also due to the long storage time. Under these circumstances, significant degradation and fermentation processes may occur that cause loss of quality of the oil obtained, both from a chemical and sensory point of view [39, 40]. For this reason, during the production of the highest quality

olive oil (the so-called extra virgin olive oil) it is recommended to minimize storage time and the use of small size hoppers.

- **Washing, crushing and malaxing**

Before being processed, the olives are washed to eliminate possible pesticide and other residues that may damage the quality of the oil. However, since this wash is done with water, care must be taken as too much water in the wash can cause problems. This is because the wash water left on the olives can reduce the efficiency of oil extraction due to the water/oil emulsions that can form. Moreover, oils made from washed olives are sometimes less desirable because this treatment can imply a reduction in bitterness and pungency and the development of sensorial defects if water is not refreshed [41-43].

Once clean, the olives are then crushed to break the cells and release the oil for extraction. The oldest methods consist of a stone base and vertical grinding wheels encased in a metal basin, often with scrapers and paddles to spread the fruit under the stones and to circulate and expel the paste. This method does not heat the paste because of the slow movement of the stone crushers, what results in less emulsification and makes easier the oil extraction. However, the bulky machinery and slowness, its high cost, and its inability to operate continuously are disadvantages of these old methods. This inefficiency is the reason why, over the past 30 years, most of the stone mills have been replaced by a continuous system with new technologies and higher capacities.

Thus, nowadays, the olives are crushed using hammer mills. This system consists of a metal body that rotates at high speed, hurling the olives against a metal screen (sieve). To optimize the extraction efficiency, the size of the sieve should be adjusted as the season progresses, so the sieve pore should be larger as the fruit becomes riper and softer to reduce the emulsions.

The main advantage of hammer mills is their speed and continuous operation, which implies high performance, compact size and low cost. However, although this method creates more emulsifications and favors higher temperatures, it should be noted that it produces oils usually with a stronger flavor because the pulp decomposes more [41, 44-45].

To reverse the emulsification that occurred during the crushing process and to improve the oil extraction yield, a step called malaxation is necessary, which is particularly important when the paste was produced in a hammer mill. It consists of mixing the olive paste in such a way that

the small oil droplets tend to coalesce, with the consequent reduction of the oil–water emulsion. To be exact, the paste is carefully and slightly heated and slowly stirred for 30 to 60 min to reduce the viscosity of the oil and to improve extractability, without degrading oil quality.

The optimal malaxation temperature to get good quality and extractability of olive oils is around 25-30ºC. Higher temperatures can cause organoleptic problems on the olive oil such as loss of fruit flavors or excess of bitterness and astringency.

However, regardless of the temperature or stirring time, the commonly used malaxation process may increase the oxidation of the paste. Consequently, one of the most recent trends in this step is to mix the paste avoiding oxygen, which can be done by covering the surface of the mixing tanks with nitrogen [46], or by using vacuum to exclude oxygen using special malaxation tanks [47]. Limiting oxygen exposure is believed to reduce enzyme activity that can break down polyphenols, which are major flavor compounds of olive oil [32, 44-45]. Another new trend to reduce the oxygen effect during this step is the so-called ultrasound assisted malaxation, which consists of the application of ultrasound emissions. The use of these waves significantly reduces the time spent on this step and therefore, also diminishes the oxidation process yield and, consequently, improves the quality of the oil [48, 49]. Alternatively, pulsed electric field technology is being studied as well [50]. However, the use of these methods is still not common and their inclusion into the olive oil extraction process remains a task for the machinery manufactures to convince the olive oil industries.

- **Separation step (pressing, horizontal centrifugation: 2 or 3-phases systems)**

Traditionally, the oil extraction process was carried out by using pressing methods. These methods consist of applying pressure to stacked filter mats, each covered with about 1.5 cm of olive paste, that alternate with metal disks to increase the pressure force. The liquid phase obtained is then accumulated in a deposit where the separation of the oil from the water takes place from their different density values (the oily phase floats on the top). Finally, a centrifugation process takes place to clean the oil from impurities or rests of water. As it can be seen, this method is very laborious and time-consuming as it is a discontinuous process. Moreover, it involves many sample manipulations, so that fermentation and/or oxidation processes can occur easily, introducing defects in the oil. Consequently, the use of this traditional method is obsolete [44].

The selective filtration method (percolation), though currently is rarely used, is an alternative separation method based on the principle of the adherence of the oil to the centrifuge blades when rotating into the olive paste. This adhered oil drips into a separated container, so that the solids and water are separated from the recovered oil. The extraction is stopped when vegetative–water begins to appear in the oil. This method produces a light "free-run" oil with a unique quality. However, like any other technique, it also has some drawbacks such as difficulty in cleaning the equipment (which is essential), the need for frequent maintenance, low oil extraction performance or the difficulty and high cost that implies the requirement of a constant heat source to keep the olive paste under constant and uniform temperature [33, 44].

At present, the so-called centrifugal decanters are the most frequently used system. These allow a continuous processing of the oil extraction. Specifically, there are two systems that are based on the same centrifugal principle and differ only in the number of separate phases: two or three phases. In both cases, the system consists of a horizontal centrifugation (decanter) of the olive paste that separate the oil from the solids and water very fast so, consequently, oxidative and/or fermentative conditions of the olive paste decrease.

The decanters are made up of two important parts: a drum that operates as a centrifuge and an auger that moves the olive paste inside the decanter. Both parts rotate in the same direction but with different rotational speed. Thus, the decanters operate at a spin rate between 2500 and 4000 rpm, depending on the decanter capacity and the manufacturer's recommendations.

After malaxing, the olive paste reaches the centrifugal decanter with the help of a pumping unit. Once into the decanter, the centrifugal force separates the heavier (solid) materials to the outside; a lighter water layer (either vegetative or added water) is formed in the middle and the oil, being lighter, remains inside. It is here, in the innermost part, where a regulated diaphragm is installed to let out the oily fraction.

In a three-phase system, water is added to the olive paste to get a fluid paste flow through the decanter, and the separation results in three phases; solid phase, oily phase and aqueous phase (containing water and small amounts of oil). The disadvantage of this system is that it consumes a lot of water, produces a huge amount of wastewater with environmental pollution concerns [51] and removes many polyphenols and pigments.

The two-phase system decanters were introduced in the early 1990s and are the most common throughout the world. This system consumes less water, managing to separate the oil from the solids and vegetative water. In fact, the latter come out together forming a fluid olive pomace

paste. One of the peculiarities of this system is that, although the olive paste contains enough water, it is always recommended to add small amounts into the decanter to facilitate the extractability of the oil. Furthermore, there are new injector devices that allow to deliver the water directly into the decanter without mixing it with the paste and overcoming the problem of polyphenol loss [52]. Compared to the three-phase system, the two-phase system produces insignificant waste-water but, on the contrary, with regard to solid waste management (wet olive pomace) it involves more difficulties [32, 41].

- **Oil washing (vertical centrifugation)**

The role of the vertical centrifuge is the clarification of the oily fraction (washing) by removing possible moisture and impurities coming from the separation stage. In general, the vertical centrifuges work at double decanter or horizontal centrifuged revolutions. In this case, the rotation occurs on a vertical axis and it provides up to four times the separation force of the decanter.

In this step, small amounts of water are usually added to clean (wash) the oil, which causes a greater separation and oils with less moisture and impurities (generally less than 2%). In the three-phase system, two centrifuges are used: one to clean the crude oil (oily fraction) coming from the decanter and a second to separate the oil from the aqueous phase also coming from the decanter [33, 44]. Oil clarification is an important step because oils with a high content of both moisture and impurities (mainly sugars from olive pulp) are more likely to have some sensory defects. Moreover, this is a delicate step as the use of water and high centrifugal forces cause a decrease in the amount of minor compounds in the oils obtained [53, 54].

- **Decantation and filtration**

After centrifugation, the oil has a cloudy appearance due to the remaining emulsions, the air bubbles and natural micro-particles of the olive pulp, which are still in suspension. The role of the decantation is to settle the oils and remove possible moisture and impurities by gravity.

In the usual way, the settling tanks are designed with a conical or inclined bottom, equipped with a tap to remove the deposited sediment. However, this is not an efficient practice to maintain the oil quality, as the longer time required and high moisture content and impurities lead to drastic losses in oil quality.

It is well known that the oil moisture is one of the main factors that cause deterioration in the oil quality. On the other hand, the micro-particles which are mainly olive sugars can enter a fermentative process causing negative sensory attributes in the oils produced, as well as the formation of ethyl ester [55, 56].

For this reason, oil filtration is an important procedure as a final step in the production process and it is recommended to carry it out as soon as possible to avoid loss of oil quality. The two objectives of the filtration step are: to remove the solid particles in suspension and to remove moisture. In this way, a shiny aspect and a significant increase in the shelf-life of the oil are achieved [41, 57].

The filtration is a mechanical process that is enhanced with the use of filter aids, which are normally organic or inorganic materials. However, it must be taken into account that this process can cause quantitative and qualitative changes on the minor compounds due to losses or contamination [55]. The most commonly used filter aids include diatomite in their composition, which is basically composed of silica (95-98%). The different permeability is given by the different particle sizes that this silica can acquire. However, the use of organic fibrous materials (cellulose fibers) as filter aids is becoming increasingly popular. Even, tandem filtration systems are being studied [58].

Finally, after filtration, the oils are stored under suitable conditions to avoid any interaction between the oil and the external factors (light, temperature, hygiene conditions, etc) that can damage their quality. Innovations at this stage concern both packaging materials and the use of inert gases [59, 60]. Thus, when the contact with oxygen is reduced, it is possible to limit the oxidative conditions and give a longer shelf-life to the oils.

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CHAPTER 2.

The Quality of Virgin Olive Oil

2.1. Introduction

The economic importance and health benefits of virgin olive oil are directly linked to it quality. Furthermore, the quality determines if a virgin oil can be bottled and under which category; in fact, the top-quality oils can be packaged as "extra virgin", while the medium grade oils can only be bottled as "virgin" and the lower quality oils can only be marketed in bulk (as "lampante" oils) and delivered to the refining industry. This is why a series of parameters and quality standards have been established for each oil category. In Europe, the classification of virgin olive oil is established by regulation standards adopted by the European Commission (EEC Regulation num. 2568/91, updated with the Regulation (EU) 2019/1604), which are very similar to those of the International Olive Oil Council (IOC). The limits established by current regulations are oriented towards the classification of olive oil within the corresponding commercial category according to its chemical characteristics and also to its organoleptic evaluation carried out by an accredited official tasting panel.

As described in the previous Chapter, virgin olive oil (VOO) is obtained only through physical-mechanical processes. The product obtained in this way can be classified into different categories depending on the values of different physico-chemical and sensory parameters, and according to current regulations [1, 2] (table 2.1).

The concept of quality can be defined as the totality of the characteristics of a product that condition its ability to satisfy the stated or implied needs and expectations of the consumer. In the virgin olive oil sector, the quality and purity evaluation are carried out by measuring a series of analytical parameters with the main goal of offering not only a safe food to the consumer but also ensuring the authenticity of the product. Traditionally, these controls are mainly aimed at classifying the oil into the different categories or detecting possible fraud, adulteration or degradation of the product.

2.2. Quality parameters in virgin olive oil and classification standards

2.2.1. Quality criteria for VOO

The quality criteria are basically oriented to the classification of olive oil into three different commercial categories ("extra virgin", "virgin" and "lampante"). The quality criteria include the evaluation of the following parameters:

- *Free acidity*, which is related to the amount of free fatty acids in the oil, expressed as percentage of oleic acid. The increase in the value of this parameter indicates an inadequate

handling during the processing or the presence of damaged fruits due to deficient harvest or storage. This increment in the content of free fatty acids is due to the hydrolysis of triacylglycerols when they come in contact with lipase in presence of water.

- *Oxidative parameters*, which include the peroxide value (PV) and the extinction coefficients K_{270} and K_{232}. The PV is used as an indicator of primary oxidation of the oil and it determines the content of lipidic hydroperoxides, expressed as milliequivalents of active oxygen per kilogram of oil. Moreover, the presence of peroxides and free radicals (that promote the formation of hydroperoxides) absorb at 232 nm, producing an increase in K_{232}. Finally, as the hydroperoxides decompose some of the final compounds absorb at 268-270 nm, increasing K_{270}.

- *The fatty acid ethyl esters (FAEEs)*, which come from the esterification of free fatty acids with ethanol. This parameter has been introduced in recent years as a quality parameter for the EVOO category. It is an indicator of improper handling/processing of the olive fruits but can also be used to detect the use of a soft-deodorization process in the VOO [1-3]. However, it should be noted that this parameter has changed since 2011, when it was adopted for the first time (CE 61/2011). In fact, the Commission Regulation of 24 January 2011 defined the official method of analysis and defined alternative limits: fatty acid alkyl esters (FAAE) $\leq$75 mg/kg or (75 mg/kg <FAAE $\leq$150 mg/kg and FAEE/FAME $\leq$1.5). These criteria have been updated several times until the current version that only considers FAEEs up to a limit of 35 mg/kg, though a limit of 30 mg/kg was proposed [4].

- *Sensorial evaluation*, which is based on the organoleptic evaluation of the aroma, flavours and smells by means of a tasting panel accredited and homologated by the authorities (COI/T.20/Doc. No 15/Rev. 7 and EEC Regulation No 640/2008) [5, 6]. The sensory evaluation consists on the assessment of both positive and negative attributes by trained tasters (8 to 12) who work under the standardized rules of the method. Each attribute is measured using a continuous scale of 10 cm. The final results are expressed as the median of the intensity measures for each single attribute. There are some statistical criteria to accept or refuse the final results.

In addition, quality criteria include the control of other parameters such as moisture and volatiles, insoluble impurities in ether and the presence of metallic traces (iron, copper) which should be taken into account for VOO commercialization. Other useful parameters to assess oil

quality are the polyphenol content, the pyropheophytin-A ratio, the 1,2-diacylglycerol percentage and the oxidative stability. The polyphenols are natural antioxidants, contribute to the stability of the oil and are related to their bitterness (a high polyphenol content raises the bitter taste). Pyropheophytins (PPP) are compounds derived from the decomposition of chlorophyll, promoted by light, temperature and time [7]. The 1,2-diacylglycerol (DAGs) content decrease and transformation into 1,3-DAGs on function of oil quality grade, time and storage temperature. Oxidative stability is related to oxidation resistance depending on the amount of natural antioxidants and composition of fatty acids.

Based all these considerations and taking into account the latest regulation described in the commercial standard applicable to olive oils and olive-pomace oils (CEE 2568/91 updated on October 20th, 2019) [8], the three commercial categories of the virgin olive oil are the following ones:

- *Extra virgin olive oil (EVOO):* it is the olive oil with the highest quality and both its chemical composition and organoleptic characteristics are excellent. Its free acidity, expressed as oleic acid, cannot exceed 0.8 g per 100 g (%), the peroxide value ≤20 meq O_2/kg, K_{232} ≤2.50, and K_{270} or K_{268} ≤0.22 and ΔK ≤0.01. Fatty acid ethyl esters (FAEE) must be lower than 35 mg/kg. Finally, the median of defects must be 0.0 and the median of the fruity attribute higher than zero.

- *Virgin olive oil (VOO):* it is very similar to EVOO but may present some imperfections. Its free acidity content cannot exceed 2 g per 100 g (%), the peroxide value ≤20 meq O_2/kg, K_{232} ≤2.60, K_{270} or K_{268} ≤0.25 and ΔK ≤0.01. Finally, the median of defects must be ≤3.5 and the median of the fruity attribute higher than zero. There is no limit for ethyl esters.

- *Lampante virgin olive oil (LVOO):* it is the lowest grade virgin olive oil, to such an extent that it is not suitable for direct human consumption. It is obtained from olives that have fallen to the ground due to meteorological causes or diseases, so it shows poor organoleptic qualities. It has a free acidity >2.0 and a median of sensory defects higher than 3. There is no limit for ethyl esters.

Table 2.1 summarizes the principal quality criteria for the categories of virgin olive oil according to the EC Regulation [8].

Table 2.1. Quality parameters and limits for each VOO category (Source: CEE 2568/91 updated on October 20th, 2019)

	Free acidity (%)	Peroxide value (mEq O_2/kg)	Fatty acids ethyl esters (mg/kg)	Sensorial evaluation*	UV Absorbance K_{232}	UV Absorbance K_{270}	UV Absorbance ΔK
EVOO	≤0.8	≤20	≤35	MD = 0.0, MF >0.0	≤2.5	≤0.22	≤0.01
VOO	≤2.0	≤20	--	MD ≤3.5, MF >0.0	≤2.6	≤0.25	≤0.01
LVOO	>2.0	--	--	MD >3.5	--	--	--

EVOO: Extra virgin olive oil
VOO: Virgin olive oil
LVOO: Lampante virgin olive oil
(*): Median of defect (MD) and median of fruity attribute (MF)

2.2.2. Parameters of purity applied to VOO

Due to its unique organoleptic qualities, its numerous nutritional properties and its health benefits, virgin olive oil is distinguished from other vegetable oils by its high market price. For this reason, on many occasions attempts have been made to adulterate it by mixing it with other low-cost oils, thus committing fraud and endangering public health. This is why different organizations such as the Food and Drug Administration (FDA), the EC, the IOC or the Codex commission have established a series of official methods for the control of the purity and authenticity of commercial olive oils, in order to detect possible fraud or adulteration. The current regulation of the European Union (Regulation (EU) 2019/1604) includes the limit values established for each one of the different purity parameters, such as the determination of the composition of fatty acids, total trans oleic isomers, total trans linoleic and trans linolenic isomers, stigmastadienes, difference between the determined and theoretical ECN 42,2-glyceryl monopalmitate, sterols composition, total sterols, erythrodiol and uvaol, and waxes.

2.3. Aroma of Virgin Olive Oil

The aroma of virgin olive oil is due to its odorous volatile compounds belonging to different chemical families (aldehydes, alcohols, ketones, acids, hydrocarbons, and esters). Most of them are rapidly formed during oil extraction through the lipoxygenase (LOX) pathway. The LOX pathway includes a set of endogenous enzyme activities that use lipids as substrates to stimulate a series of reactions and, therefore, to synthesize volatile compounds responsible for positive attributes in virgin olive oil. The positive attributes are strongly associated with the presence of C6 and C5 volatile compounds, mainly C6 aldehydes (hexanal, cis-hexenal and trans-hexenal).

The C6 and C5 volatile compounds represent most of the volatile components of virgin olive oils. Their synthesis derives from the polyunsaturated fatty acids (PUFA) through the LOX pathway, which consists on the formation of 9 and 13-hydroperoxides from linoleic and linolenic acids with subsequent cleavage by specific hydroperoxidelyases that lead to the production of C6 aldehydes. Later, these C6 aldehydes can be reduced by alcohol-dehydrogenase enzymes and give rise to their corresponding alcohols (hexanol and hexenol isomers). Then, alcohols may lead to the formation of acetate esters by means of the alcohol-acetyl-transferases. When using linolenic acid as substrate, 1,3-pentene radicals can dimerize to C10 hydrocarbons or react with hydroxyl-radicals to form C5 alcohols, which can be oxidized enzymatically and form C5 carbonyls. Since these C5 and C6 compounds are volatiles and many of them have specific aromas, they have a direct impact on the olive oil aroma providing fresh, green and fruity perceptions, which are usually very appreciated by consumers.

During olive oil processing, the LOX metabolic pathway starts while the olives are being crushed (cell wall rupture and oil droplet release) and continues during the paste preparation steps. Some of the volatile compounds formed pass into the oil and contribute to the aroma. Therefore, the enzymatic activities involved in this pathway will depend not only on the agronomic factors (variety, maturity stage) but also on the industrial processing conditions of virgin olive oil and will determine the olive oil aroma sensations and intensity [9-12]. The final aroma perception will depend on both the concentration and the specific aroma of each one of these volatiles [13, 14].

On the contrary, the defective aroma of VOO is associated to volatile compounds C7 to C11-monounsaturated-aldehydes, C6 to C10-dienals, C5-branched-aldehydes and alcohols or some C8-ketones, when they reach relatively high concentrations. In addition, there are some pathways, such as sugar fermentation, conversion of amino acids, enzymatic activities of molds, anaerobic microorganisms and oxidative processes that promote aromatic sensations described as ``winey´´, ``fusty´´, ``musty´´, ``muddy´´ and ``rancid´´, respectively, which are the most important negative attributes in virgin olive oils [15].

2.4. Factors that influence the quality of virgin olive oil

The good quality of the olive oil is never a coincidence, but it is the result of genius and efforts to maintain all the freshness and chemical composition of the olive juice, which distinguishes it from other vegetable oils, in view of its organoleptic and health-beneficial attributes. In fact, the

presence in virgin olive oils of the different volatile compounds, phenols, and monosaturated fatty acids found is influenced by several factors, both agronomic (pre-harvest) and technological (post-harvest).

2.4.1. The influence of agronomic factors

- ***Olive cultivars and geographic location***

The great varietal diversity existing in olive cultivars directly influences the phytochemical content and chemical composition of olive fruits and, consequently, the oils obtained from those fruits. The quality and composition of olive oils results from the complex interaction between several factors, among which are the genetic factors and the agro-climatic conditions. The genetic diversity (different varieties of olives) produces a significant variability in size, shape, oil content and biochemical characteristics of olive fruits, which allows the production of oils with different and specific composition (table 2.2). The relationship between cultivar genotype and differences on the profiles of fatty acids, triacylglycerols and phenolic compounds has already been reported [16, 17], although it should be noted that the environment has greater influence on the characteristics of the oil obtained from some varieties than others [18, 19]. Moreover, olive cultivars show different behaviour in different geographical areas, what implies differences in the chemical composition and organoleptic characteristics of the oils obtained, specifically, in the composition and contents of fatty acids, phenolic compounds, tocopherols, volatile compounds and oxidative stability [20-22]. It is in this context of variability, both for the variety of olives and for the geographical location, that the so-called mono-varietal oils and protected designations of origin (PDO) become important. Each PDO offers a characteristic and differentiated product mainly thanks to the differentiation made by a unique group of local varieties grown in a geographical area with specific climatic and soil conditions [23].

Table 2.2. Main chemical composition characteristics of the main Catalan cultivars and some very important olive varieties from other Spanish area [24]

Variety	Main chemical composition characteristics
Arbequina	- High content of palmitic acid, low content of oleic acid and mean to high content of linoleic acid - Low content of polyphenols - Low stability and bitterness
Argudell	- Low content of oleic acid and high content of linoleic and palmitic acids - Medium content of polyphenols - Medium stability and bitterness
Empeltre	- Mean content of oleic acid and mean to high content of palmitic acid - High content on tocopherols - Medium to high content of polyphenols - Medium to low stability and bitterness
Farga	- Medium to high content of linoleic and palmitic acids and medium content of oleic acid - Low content of polyphenols - Low stability and bitterness
Morrut	- High content of oleic acid and low content of palmitic and linoleic acids - Medium content of polyphenols - Medium stability and bitterness
Sevillenca	- Medium to high content of palmitic and linoleic acids, and low content of oleic acid - Low content of polyphenols - Low stability and bitterness
Cornicabra	- High content of oleic acid and low content of palmitic and linoleic acids - High content on polyphenols - Very high stability and bitterness
Hojiblanca	- High content of oleic acid and low content of palmitic and linoleic acids - Low levels on polyphenols (less than 300 mg/kg) - High content on tocopherols - Medium to low stability and bitterness
Picual	- Very high content of oleic acid and low content of palmitic and linoleic acids - Very high levels on polyphenols (more than 500 mg/kg) - Very high stability and bitterness

- ***Fruit maturity and metabolic process***

Both the quality and extraction efficiency of VOO are related to the quality and characteristics of olive fruits from which they are obtained. The agronomic factors such as cultivar, tree load and agricultural practices (irrigation, fertilization, planting system, pruning, pest and diseases management), and other ecological factors such as climate change, directly or indirectly affect the physiological characteristics of olive fruits, and therefore on their maturity index, size, oil content, texture and chemical composition which will ultimately affect the quality of the oil.

In fact, the metabolic processes that take place throughout the ripening of the fruits involve changes in the composition of the oils, such as the amount of fatty acids, tocopherols, phytosterols, volatile compounds and total phenols. In general, an early harvest results in a lower oil extraction yield, but with higher content of MUFAs and antioxidants and with a complex aromatic profile. This is because the green olives have a harder texture, are more acidic and their aromas are greener. However, a late harvest results in oils with a lower content in hydrophilic phenols and a poorer aromatic profile, due to reduction of LOX activity from overripe olives, and an increase of the free acidity [17, 25-27].

- ***Environmental and agricultural practice***

- *Edaphic-climatic factors:* Since the cultivation of the olive tree is distributed in different areas and regions, each olive grove will be exposed to the effect of various edaphoclimatic factors [28]. The altitude at which the olive trees are planted can have a positive effect on the characteristics of the oil obtained. Particularly, it can increase the oleic acid content and enhance the aromatic profile due to an increase in the phenolic content. On the other hand, the growth of the olive on the tree is also affected by the soil properties, especially by the availability of water and nutrients [12, 29]. Soils with a relatively intense or prolonged water deficit during the maturation period will favour an earlier fruit ripening. In addition, the fruits can be smaller, with low flesh/pit ratios and even wrinkled in soils with low clay or moisture content because of potassium deficiency. Regarding the acidic soils (low-pH), the lack of calcium can decrease the quality of fruits by favouring a higher incidence of the Anthracnose disease and, therefore, reducing the quality of oils [30-32].

As for the climatic conditions, the most important effect is caused by frost. It occurs when temperatures drop below -3ºC, which implies a fruit damage that negatively affects the quality of the oils with a reduction of the oxidative stability and a degradation of the phenolic

composition. Furthermore, oils obtained from frozen olives show a flatter sensory profile (slightly fruity, less pungent and bitter) with an unmistakable flavour of wet wood or wet straw, giving rise to the defect called "frostbitten" (frozen olives) [33]. In contrast, high temperatures during the ripening period, which occurs in warm cultivation areas, can lead to an overripe of the olive fruit. Since it affects the characteristics of the olive fruit, it will also negatively affect the quality of the VOO by increasing the content of palmitoleic and linoleic fatty acids (polyunsaturated), and decreasing the content of oleic acid [34-38]. However, it has to be highlighted that between these two extremes (too cold and too hot) it has been found that warmer springs, without water deficit during spring and autumn, improve the organoleptic quality of VOOs because these climatic conditions positively affect their volatile composition [39].

- *Agricultural practices:* The agronomic practices applied to the olive cultivar play an important role in the olive oil productivity in terms of quantity and quality. The production systems (traditional, intensive, hedgerow) have a regular impact on the fruit mesocarp characteristics and, therefore, on the oil composition [12, 31, 40-43]. In addition, an appropriate management of the tree canopy to control the vigour, fruit loading (which depends on the pruning, variety and biennial bearing of the cultivar) and the interception of solar radiation are factors that positively affect both the yield and quality of the olives and the oils [40, 44-47]. Thus, for example, the olive oils obtained from olives exposed to more sunlight tend to have a higher content of polyphenols and, therefore, more stability to oxidation and more bitterness in the tasting profile. These oils also show a reduction in the oleic acid and an increase in linoleic and palmitic acids [48, 49].

The application of other agricultural practices, such as irrigation and fertilization, reduces the deficiencies of water and nutrients necessary for the crop and, therefore, implies an important improvement in productivity but it has a certain effect on oil quality. Irrigation can reduce the oxidative stability of some varieties such as 'Arbequina' but also allows modulating the excessive bitterness in other varieties such as 'Cornicabra', 'Manzanilla de Sevilla' and 'Empeltre' [38, 50-51]. Moreover, irrigation may favour the synthesis of volatile compounds responsible for the attribute ``green-fruity´´ in VOO, such as (E)-hex-2-enal and (Z)-hex-2-enal, hexanal and hexan-1-ol, which favour VOOs from an organoleptic point of view [52-54]. However, when the olive grove is subjected to water stress during the summer period (application of regulated deficit irrigation), this stress has a positive effect on the quality of the

oil. It has been reported that this stress stimulates the synthesis of antioxidant and phenolic compounds and increases the bitterness and pungency of olive oil. In fact, different studies have shown that the application of a regulated deficit irrigation in summer is a good option to enhance EVOO quality by increasing the oxidative stability, the content of volatile compounds (mainly those related with the aroma), the content of total phenols and, therefore, the overall sensory quality [43, 50, 55-58].

The phytosanitary control is considered one of the most important practices to prevent the negative influence of pests and diseases that damage the olive fruits and, therefore, reduce the olive oil yield and quality. Among them, the olive fly is one of the most damaging pests. Olive fly causes the increase of free acidity and extinction coefficients (K_{232}, K_{270}), and a general reduction of the oil stability, green colour and flavours (appreciated by consumers). On the other hand, anthracnose is a very important disease in terms of quality losses. Oils from fruits damaged by anthracnose show a reddish colour together with a considerable increase in the values of free acidity, peroxide index, extinction coefficients and alkyl esters [31, 32].

2.4.2. The influence of technological factors

The good quality of olive fruits when harvesting is a decisive factor but it is also necessary to preserve this quality all along the oil production process to ensure the good quality of the final virgin olive oils. In other words, the quality is strongly influenced by the interaction of pre-harvest factors and post-harvest factors (in particular, harvesting and transportation systems, olive storage and technological processes such as olive crushing, malaxation, separation, decantation and filtration). In consequence, inappropriate management during the processing of olive fruits results in low VOO quality. Thus, the main objective during these operations is to keep the olives in good conditions from their harvest to their processing. Moreover, it is also sought to maximize its profitability, not only in terms of oil extractability but also in qualitative terms.

- ***Harvest and transport systems***

The first objective during harvesting is to maintain the physical integrity of the fruits by applying a suitable method that causes minimal damage to both the olive fruits and the olive tree. The injuries caused on the mesocarp and skin of the olives are the main sources of the quality deterioration since these wounds allow the penetration of microorganisms like molds, yeasts and bacteria and therefore cause considerable losses in the quality of the VOO [59]. As

an example, the most aggressive harvesting systems, such as hitting the olive branches with a long stick, generate losses of the antioxidant and flavour components of the virgin olive which seems to be associated to the physiological response of the olives [12, 60-63].

The transport of olives is another critical and very important operation in the process. It is well known that, if the olives are not transported quickly enough to the mill or the appropriate containers (both by material and by size) are not used, the olives can suffer physical damage. These injuries cause the deterioration of the olives and even fermentations that generate inappropriate enzymatic transformations in the fruits. This is the origin of some considerable organoleptic alterations in the oils such as the loss of ``fresh-fruitiness´´ and the presence of defects such as ``vinegar´´, ``fusty´´, ``earthy´´ and ``musty´´.

To guarantee a good quality of the oil obtained, fruits harvested from the canopy must keep separate from those picked from the ground. Then they must be transported in containers with limited load and upon arrival at the mill, they must be processed as soon as possible to avoid unwanted evolution of the fruits [13, 14].

- ***Storage and washing***

The storage of olive fruits, even during their transport, negatively affects the sensory quality of the oil. Storage in sacks, in large containers or in large capacity hoppers favours the proliferation and activity of diverse types of microorganisms, which results in the production of undesired metabolites such as branched aldehydes, alcohols and the corresponding carboxylic acids that contribute to negative sensory attributes such as the ``fusty´´ one. Moreover, it should be borne in mind that into these unsuitable containers the temperature of the olives increases, so that fermentation processes are favoured and the yeasts produce volatile phenols, ethanol and ethyl acetate, which contribute to the ``winey´´ defect. However, under low-temperature conditions, the proliferation of fungi and yeasts leads to the appearance of the ``musty-humid´´ defect [59, 64-65].

On the other hand, washing the olives can produce an excess of humidity that enhance the formation of emulsions and makes it difficult to process the olive paste. Furthermore, the excess of moisture in the olive paste can generate losses in the hydro-soluble components of virgin olive oil such as phenolic compounds.

In any case, the washing of the olives should always be the last operation before grinding and never be done before storage of olives. In this way, the acceleration of the yeasts activity is prevented [66].

- ***Extraction conditions***

Since the oil extraction process has a direct impact on the quality of the virgin olive oil obtained, the control of the extraction parameters becomes essential.

During the crushing of the olives, it is important to use a sieve of the right size, as it affects not only the efficiency of the oil extraction but also the extraction of polar and non-polar substances present in plant tissues, which increases both the oxidative stability and the content of volatile compounds in the oils obtained [67, 68]. In any case, it is obvious that the milling stage has an indisputable influence on the amount of total volatiles in the final oil, because when the olives are crushed, their aromas are released. However, it should be noted that some factors hinder this process. This is the case of the temperature increase due to the rubbing effect of the olive paste. This variation in the temperature of the sample negatively influences the activity of LOX, which implies a reduction in the formation of volatile compounds, mainly Z-3-hexen-1-ol, hexanal and E-2-hexenal [69].

The step that follows olive crushing is the so-called malaxation. During this operation, the main enzymatic activity responsible for the biosynthesis of olive oil aroma takes place thanks to lypoxygenase and also to hydroperoxide-lyase. The reactions depend on several factors related to the process conditions (temperature, time and sieve size) and on the variety and fruit characteristics (maturity and moisture). Thus, a high temperature during olive paste malaxation (even when improving the extractability yield) causes a decrease in the total amount of volatile compounds as well a decrease in the ratio C5/C6 volatiles, due to a decrease of LOX activity and prevalence of hydroperoxidolyase, which finally results in a reduction of the aroma intensity and fruitiness attribute values [69-71]. In addition, when increasing the malaxing temperature and time, a decrease in the content of phenolic compounds (such as secoiridoids) is found while the content of pigments in the oil decreases [70, 72]. Even though, the olive paste malaxation does not promote the oxidation of the oils due to the cellular respiration (which reduces the oxygen) and thanks to the large quantity of phenolic substances present in the olive paste [73, 74]. Furthermore, it has been reported that higher temperatures or prolonged malaxation times increase the concentration of some volatile compounds responsible for some sensory defects, such as 2-methyl and 3-methyl butanol [73].

Regarding the centrifugation processes (oil separation), these do not have an influence on the values of the parameters of VOO quality (free acidity, peroxide value, UV extinction coefficients). However, the effect that the changes or modulations in this process may have on the specific criteria for EVOO categories (mainly contents of FAEEs) is still not clear, making it difficult to make decisions about optimal operating conditions of the mill regarding both the extraction efficiency and oil quality. To make it even more complex, it is known that the amount of phenolic and volatile compounds decreases depending on the amount of water used during centrifugation. This is due to the fact that, as explained in the previous Chapter, the addition of water (mainly in the three-phase system) raises the fluidity of the olive paste, which promotes the separation of the phases (liquid-solid) and improves the oil extraction yield, but it affects negatively the volatile and phenolic contents in the oil and its oxidative stability. Moreover, the cleaning of the oily phase at the vertical centrifuge requires the addition of some amounts of water, which significantly decrease the concentration of the minor components of virgin olive oil [75-77].

In general, once obtained, the VOO is transferred and stored in large tanks for decantation and pending filtration and bottling. During these steps, the residual vegetative water and impurities, the light and the oxygen are the main parameters that influence the oil stability. Because of these factors, the VOO quality may suffer a progressive deterioration caused by the hydrolysis of triglycerides or the oxidation of fatty acids, which increases the free acidity. Moreover, after centrifugation the oil still contains a small amount of vegetative water in emulsified form and some impurities (mainly sugars and proteins from the olive pulp) that settle at the bottom of tanks. These residues can ferment forming substances that contribute to the appearance not only of some defective aromas (such as ``muddy-sediment´´) but also producing some undesirable chemical compounds such as alcohols (precursors of FAAEs) [78-80]. As for the solar light effect, it causes a degradation of the pigments and therefore reduces the freshness of the virgin olive oil aroma.

In order to avoid the deterioration of the VOO quality due to all these mentioned factors, once the production process is finished, the oils must be filtered as soon possible and stored under inert conditions. It is clear that there are many factors that contribute to the quality of virgin olive oil and must be controlled, but without forgetting that a good quality oil requires the maximum cleaning and hygiene of all materials used throughout the entire process.

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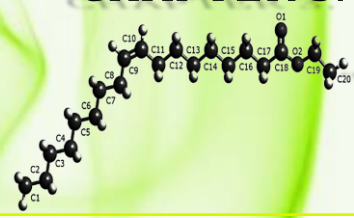
CHAPTER 3.



The Alkyl Esters of Fatty Acid in Virgin Olive Oil

3.1. Introduction

As mentioned in the previous Chapters, the quality of Virgin Olive Oil (VOO) is closely related not only to agricultural parameters, but also to all those variables on which the different stages of the oil production process depend. Thus, apart from edaphic-climatic conditions or growing factors, the variety, the health status and the maturity stage of the olives strongly influence the olive oil chemical composition, especially the phenolic and volatile compounds, which are the more influence the aroma and taste of the oil [1-5]. Together with these factors, the processing and storage conditions will determine the final quality and characteristics of the olive oil.

Extra virgin olive oil (EVOO) is the highest quality grade of olive oil. It must be obtained from fresh and healthy olives through an exclusively mechanic-physical process under not excessive temperature (it is advised processing below 35ºC in order to avoid peroxide induction). The EVOOs cannot be blended with other edible oils. However, due to the increasing demand for VOO (figure 3.1) and its economic relevance in the international market, the olive oil sector has become a common target for counterfeit. In fact, a great number of VOOs are fraudulently mixed with other cheaper olive oils, of lower quality and worse characteristics, seeking to take advantage of the virgin olive oil fame. Among these frauds, the addition of the so-called "soft-deodorized" olive oils in EVOO is one of the most difficult to detect. Thus, as explained in Chapter 2, the sectors of the olive oil production and marketing are nowadays subjected to a series of specific requirements and regulations to guarantee its genuineness and quality and therefore offer safety to the consumer.

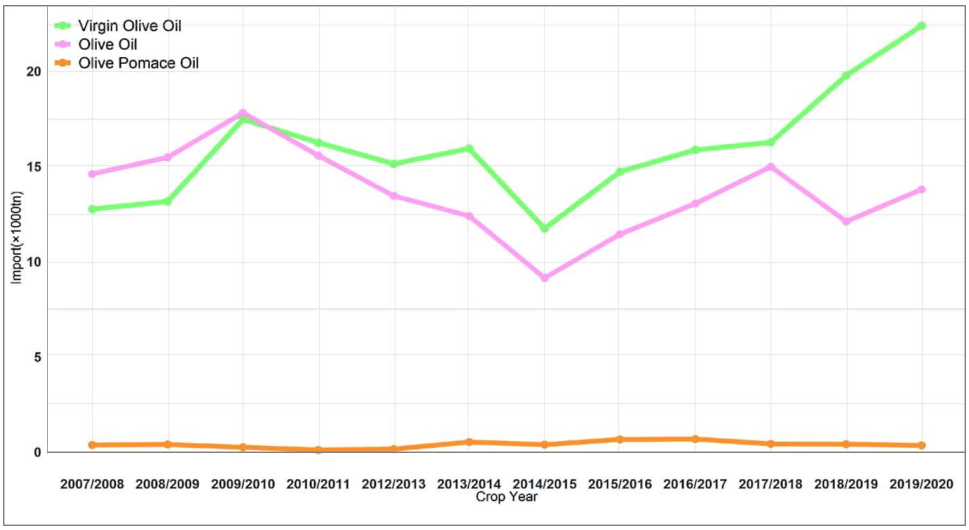


Figure 3.1. Evolution of olive oil imports from 2007 to 2020 (×1000 tn) (Source: IOC) [6]

The deodorization is actually a stripping process to remove off-flavours (defective aroma) present in edible oils. In the deodorization process, the oil is usually heated at temperatures above 200ºC and a stream of steam passes through it under reduced pressure. An example is the deodorization applied to the so-called "lampante" olive oil in order to transform this non-edible oil into edible to be commercialized. Standard deodorization highly affects some specific physico-chemical parameters, which makes deodorized oils easily distinguishable from EVOOs. In fact, several compounds were reported to be produced during deodorization, such as trans-fatty acids, saturated sn-2 fatty acids in triglycerides, dienes from sterols, 2-MCPD and 3-MCPD (monochloropropane-1,2-diol) esters or glycidyl fatty acid esters (GE). However, a soft deodorization without any other steps of the refining process is very difficult to detect due to the lack of chemical markers. Compared to the usual deodorization, this ´soft-deodorization´ is carried out by stripping at lower temperatures (120ºC), for a shorter time and under vacuum, or it can even be done by filtration. It is estimated that this type of adulteration is spreading dramatically and could explain why more and more EVOO brands can be found on supermarket shelves at low prices.

It is in this discouraging context where FAAEs acquire special relevance since there are some studies that show that the deodorization processes do not degrade the alkyl esters of fatty acids up to 150°C and that the presence of soft-deodorized oils in EVOOs is accompanied by a parallel increase in alkyl esters of fatty acids in treated oils. Based on this, some authors have suggested alkyl esters as quality markers, useful in detecting this kind of fraud but also to detect when a virgin olive oil has been obtained from poor quality olives [7-9]. In fact, the Regulation [EU] number 61/2011 establishes the use of the content of fatty acids ethyl esters as a parameter to ensure the use of good quality olives and their good management during the EVOO processing [10].

3.2. Sources and origins

The presence of Fatty Acid Alkyl Esters (FAAEs) in olive oils has been known since the late 1960s when they were identified among stripping products [11]. They are present in the waxy fraction of olive oils and constitute a natural neutral lipid family coming from the esterification of free fatty acids (FFAs) (mainly from C16 and C18 FAs) with low molecular alcohols (mainly ethanol and methanol), thus giving rise to fatty acid ethyl esters (FAEEs) and fatty acid methyl esters (FAMEs), respectively [9, 12]. An inappropriate handling of the olives in any of the stages from harvesting to storage, prior to olive oil extraction can cause the olive drupe to

break, allowing its contact with yeasts and also with lipolytic and pectolytic enzymes. The presence of these microorganisms and enzymes promotes the fermentation phenomena in the olive fruits with formation of alcohols that react with FFAs. Therefore, the presence of high amounts of FAAEs in virgin olive oil could be indicative of poor handling of the olives, inadequate management of VOO processing or inappropriate storage conditions [9, 13].

Regarding the formation of these compounds during the production of olive oil, some studies have highlighted that the content of FAAEs could be affected by the technological operations used during the extraction process of the virgin olive oil, while the oil storage effect is not totally clear [14-16]. In fact, big cooperatives that store oils in very big tanks are really concerned about the possible increase of FAEEs over time.

The esterification processes between the short alcohols (either methanol or ethanol) and free fatty acids present in the oil can easily occur in an acidic medium and catalyzed by the presence of certain enzymes (figure 3.2).

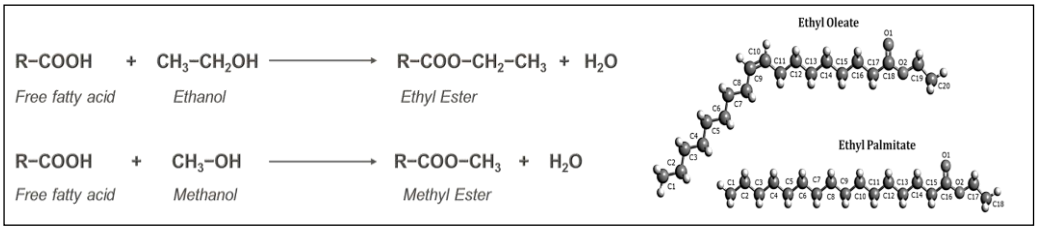


Figure 3.2. Esterification reaction of free fatty acids with short alcohols, and a 3D illustration structure of ethyl oleate and ethyl palmitate

However, there is another pathway that can generate these compounds: the transesterification of the triglycerides which implies the exchange of the organic group of an ester with the organic group of a short alcohol. Although the esterification reaction is faster than the transesterification, when the content of free alcohols and the esterified acids is high enough, the transesterification take place as a pathway for the formation of alkyl esters.

The consideration of FAAEs as a quality parameter was established for the first time with the Regulation [EU] 61/2011, which sets the total amount of alkyl esters (that is, the sum of FAEEs and FAMEs) at 75 mg/kg (or 120 mg/kg and FAMEs/FAEEs ratio <1.5) for the EVOO category [10]. However, the presence of some endogenous amounts of alcohols (mainly methanol), which have no relation with fermentative processes, has complicated the situation regarding the control of the amount of FAAEs that can be considered acceptable in the final oil. This is the reason why the later Regulation (Regulation [EU] 1348/2013) focused only on the

content of FAEE, the limit of which was set at ≤40 mg/kg in the 2013/14 season. Then, in 2014, it was reduced to ≤35 mg/kg and, during the 2015/2016 season, an attempt was made to set the FAEE values at 30 mg/kg [17]. However, this last adjustment did not prosper, so the current standard, which relies on the Regulation [EU] number 2019/1604, fixes the maximum content of FAEEs at 35 mg/kg for the EVOO category [18].

Regarding the effect of these compounds from the organoleptic point of view, the correlation between the presence of FAAEs and some fermentative sensory defects such as winey, moldy and fusty-muddy has been reported [19, 20].

3.2.1. Free fatty acids

The main components found in olive oils are triacylglycerols. These compounds result from the esterification between the trialcohol glycerol and different fatty acids (FAs) of which the most abundant is oleic acid (between 55 and 83%) [21]. Apart from this 18-carbon monounsaturated FA (C18:1), there are other important polyunsaturated FAs such as linoleic acid (C18:2) and linolenic acid (C18:3) and also some other saturated FAs such as palmitic acid (C16:0) and stearic acid (C18:0) [22, 23]. Table 3.1 shows the major fatty acids (C16 and C18) present in VOO from some Spanish and Catalan olive varieties.

Table 3.1. Principal fatty acids (%) in VOO from different varieties [24-26]

Fatty acids (%)	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)
Arbequina	15.2	2.1	1.7	68.0	11.5	0.7
Morrut	10.9	0.6	2.7	69.1	15.0	0.9
Fulla de Salze	14.3	2,2	1.4	66.5	17.6	1.0
Arbosana	13.6	2.0	1.9	73.1	8.1	0.9
Argudell	16.9	1.8	1.8	63.7	13.6	1.3
Marfil	12.2	1.3	1.4	71.9	11.2	1.2
Rojal	14.1	1.2	2.4	65.6	14.7	0.8
Menya	14.5	1.7	2.0	72.5	7.8	0.6
Sevillenca	12.5	0.7	2.0	68.4	14.6	0.7
Farga	10.5	0.8	1.9	75.6	9.8	0.7
Empeltre	13.9	1.5	1.4	68.6	13.0	0.8
Picual	12.3	1.1	2.6	79.8	3.0	0.6
Koroneiki	11.6	1.1	2.1	75.5	8.6	0.3
Leccino	13.7	1.3	1.9	75.6	5.7	0.2

The free fatty acids (FFAs) found in olive oil are formed from the lipolysis of triacylglycerols (TAG) carried out by lypolitic enzymes (lipase), which are present in the pulp and seed cells of the olives (figure 3.3). Taking into account that TAG is esterified with three fatty acids, the lipolysis of this molecule can give rise to diacylglycerol + one FA or to monoacylglycerol + 2FAs or to glycerol + 3FAs. In all cases, the lipolysis reaction is greatly enhanced by the presence of an aqueous phase, so when vegetative water is removed, as occurs after the oil extraction and filtration, the lipolysis reactions reduce and stop.

On the other hand, since the amount of FFAs depends on the TAG degradation, this is a parameter that is used to determine the degree of olive oil degradation and also to categorize the olive oils. Thus, for example, to be classified as EVOO, the free acidity value of the olive oil must not exceed 0.8% (expressed as oleic acid) (EEC 2568/91) [18]. It should be noted that the free acidity parameter is expressed as the percentage of oleic acid as it is the most abundant FA in olive oil.

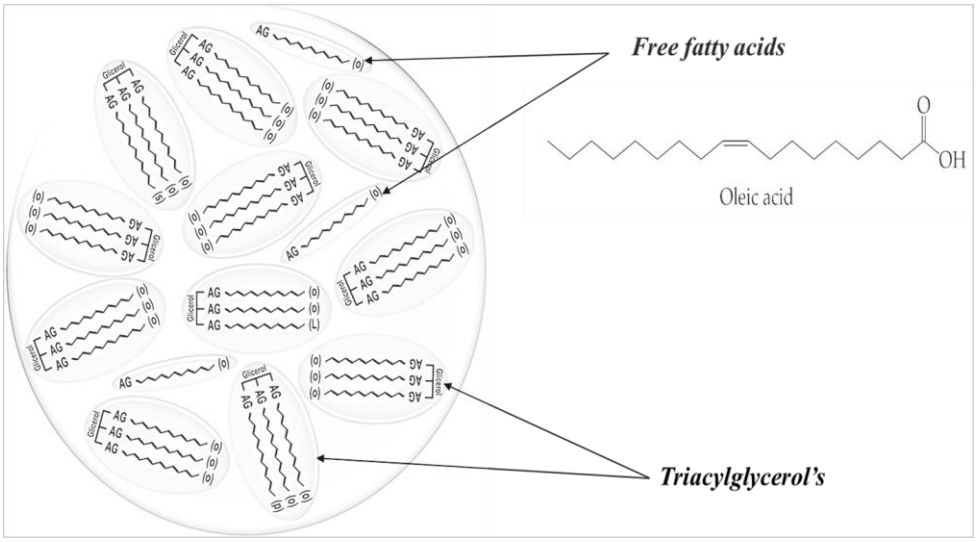


Figure 3.3. Representation of triacylglycerols and free fatty acids in oil

As explained in Chapter 2, the amount of FFA depends on different factors but, in a general and brief way, it tends to be increased by the use of damaged olives (due to fungal diseases, olive fly infestation and other pests), delays between harvesting and processing, prolonged contact between oil and vegetative water and careless extraction methods. In all cases, these factors increase the reaction of enzymes with triacylglycerol, causing FFAs release [27].

3.2.2. Short chain alcohols

3.2.2.1. Ethanol

The presence of endogenous ethanol in olive tissues is naturally generated as a result of fruit metabolism and, therefore, it can pass into the oily fraction during the processing of virgin olive oil [28-30]. Specifically, the endogenous ethanol in fruits comes from the consecutive action of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes on pyruvates (figure3.4). First, the action of PDC catalyzes the decarboxylation of pyruvate into its corresponding aldehyde (acetaldehyde) and then, the ADH activity catalyzes the reversible reduction of acetaldehyde to its corresponding alcohol (ethanol) using reduced pyridine nucleotides as cofactors [30].

It should be highlighted that, in olive fruits, the pyruvate metabolism follows a different pathway than that of other plant tissues (such as seeds), where pyruvate produces acetyl-CoA through the action of the pyruvate dehydrogenase (PDH) enzymatic complex. In olives, the synthesis of acetyl-CoA seems to be carried out from acetate through the so-called PDH bypass, which is promoted by the acetyl-CoA synthetase (ACS) activity [30, 31]. In this case, the pyruvate is catalytically transformed into acetate through the consecutive action of PDC and aldehyde dehydrogenase (AlDH). So, the ADH activity seems to play a security role: it protects cell tissues from excessive accumulation of cytotoxic acetaldehyde by reducing it to ethanol. Consequently, as claimed Beltran et al. [28], the ethanol generated during all these metabolic processes will accumulate in the olive throughout the fruit ripening process.

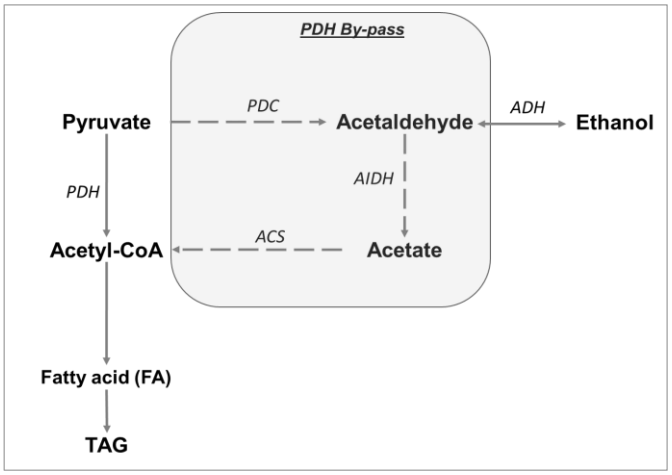


Figure 3.4. Metabolic pathway for endogenous ethanol in plants

This endogenous ethanol pathway in plants is not necessarily related to a reduction of oxygen in the tissues, as it can also occur under aerobic conditions. In addition, Tadege *et al*. [32] and Moyano *et al.* [33] have reported an increase of the ethanol content in plant tissues subjected to unfavorable environmental conditions such as cold, dryness or high concentrations of CO_2. This behavior is attributed to an activity induction of the PDC and ADH enzymes.

Besides these metabolic pathways, there are other sources of ethanol. One of the most important is the fermentative alteration of the olives. This unwanted process takes place due to inadequate handling during processing, which allows the yeasts to ferment the sugars naturally present in the olive pulp with the subsequent production of ethanol [34].

Another possible origin is the transport of olives when this is performed in unsuitable conditions. When transport is carried out in too large or non-ventilated containers or at high temperatures or pressures, the result is bruised fruit that offers optimal conditions for bacterial activity and yeast fermentation. This situation translates not only into the formation of ethanol, but also acetic acid and ethyl acetate, which leads to the release of some sensory defects such as ``winey´´ and ``vinegary´´.

Finally, it cannot be forgotten that each olive variety has a slightly different metabolism, so the huge diversity of olive cultivars may also play a key role in determining the content of endogenous ethanol in the olives and therefore in the oil produced.

3.2.2.2. Methanol

Unlike ethanol, methanol in plants is produced from the active growth of plant tissues and fruit ripening, so the presence of methanol in fruits, such as olives, is related to the physiological activity.

Particularly, methanol comes from the pectin degradation by the intervention of endogenous pectin-methyl-esterase (PME), an enzyme that exists in the olive drupe tissues. In fact, a recent study carried out with olives has shown a close correlation between PME activity and the amounts of methanol, which suggests that an important source of methanol in olives may be the pectin-methyl-esters that are hydrolyzed by the pectolitic enzyme to produce pectic substances (pectinic acids and pectic acids) and methanol [35]. Moreover, it has also been reported that methanol in fruits may be generated through the demethoxylation of wall cell pectins (cell wall disassembly) that mainly occurs at the veraison stage. Therefore, it can be concluded that

methanol is produced as a by-product of the olive's own metabolism and can accumulate during its ripening [35-37].

However, we cannot forget other less natural origins of methanol, such as inappropriate handling during harvesting, unsuitable transport and/or storage of olives or even infestation of fruits (mainly by olive fly). These poor conditions cause the rupture of the olive drupe and allow contact with the lipolytic and pectolytic enzymes, what implies methanol release. In addition, once the olives are crushed, the action of those enzymes can increase due to a prolonged time of the olive paste mixing process (before oil separation), thus causing the formation of larger amounts of methanol.

3.3. Impact of alkyl esters on virgin olive oil quality

As explained above, when we talk about alkyl esters, we refer to the sum of the methyl esters and the ethyl esters. However, although chemically they are very similar, it is necessary to differentiate between the two families since their contribution to the olive oil quality is very different. This differentiation is due to their origin. While the FAMEs are formed by the olive's own metabolism, the FAEEs mainly appear as a consequence of the low-quality of the olives or inadequate processing and/or unsuitable storage of the olives. This is why the European Union, in its Regulation 1348/2013, focuses mainly on setting a maximum content of ethyl esters, although it must be said that the FAEEs/FAMEs ratio could also be considered as another interesting parameter to detect possible fermentations during the production process [19].

The adoption of FAAEs as a parameter to detect possible frauds in virgin olive oil (mainly to avoid possible blends with other low-quality oils) has offered a margin of security not only for the consumer but also for producers, who suffer economic losses because of the competitive prices of counterfeit oils. However, since the formation of FAEEs in virgin olive oil does not exclusively depends on the incorrect practices or the use of low quality fruits but also on parameters such as the olive variety, the consideration of FAEEs as a standard for distinguishing EVOOs has also generated concern among producers.

In fact, the presence of endogenous amounts of FAAEs precursors makes a challenge for the producer when limiting their formation. This is due to the fact that FAs and alcohols can be released at different points in the oil production chain and, later, the esterification reaction between them is a fast reaction that easily occurs when these compounds are in the acidic medium of the olive oil and the temperature is around room temperature. Therefore, it is not enough to control the health and maturity of the olives to prevent fermentations that lead to the

formation of ethanol, but a control after oil extraction and mainly during oil decantation is also necessary. It should be noted that oil decantation is a particularly critical step, because the phase that settles at the bottom of the tanks is basically composed of water and organic matter (mainly sugars) from the olive drupe. Under these conditions, the presence of water causes both the hydrolysis of triglycerides (which results in an increase in the amounts of FFAs) and the fermentation of sugars (which results in the generation of ethanol). Therefore, at this stage both the precursors and the appropriate conditions for the generation of significant amounts of FAAEs are present.

Finally, a reference to the oil extraction yield should be made. As already mentioned in the previous Chapter, to increase yield and obtain a greater quantity of oil, it will be necessary to apply pressure and/or temperature conditions incompatible with obtaining extra quality oil, as these conditions favor the appearance of FAAEs and other compounds that lower the quality of the oil.

In Catalonia, the cultivation of the olive tree is deeply rooted and widespread throughout the territory (figure 3.5). However, given that the extent of most plantations is less than five hectares (see table 3.2), in general, the production of virgin olive oil is carried out in low-capacity oil mills. This allows a better control of the olive quality so, basically, these mills focus on the production and commercialization of good quality olive oils. This is why around 85% of the olive oils produced in Catalonia belong to the category Extra-Virgin (as it was mentioned in Chapter 1).

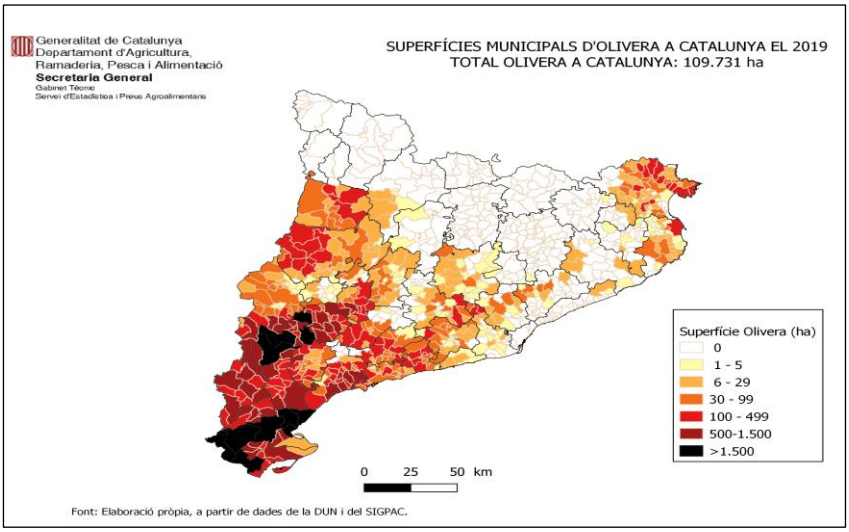


Figure 3.5. Distribution of the municipal's olive grove area in Catalonia in 2019 [38]

Tables 3.2. Dimensions and distribution of the exploitations on function of the olive grove area in Catalonia. Comparison for the years 2013 and 2016

Dimension	**2013**		**2016**	
	Exploitation	Area (ha)	Exploitation	Area (ha)
≤ 2 hectares	14,264	11,638	14,591	11,973
from 2 to 5 hectares	6,756	22,242	7,074	24,348
from 5 to ≤10 hectares	2,474	17,281	41,971	13,941
from 10 to ≤15 hectares	1,217	14,387	1,341	15,592
From 15 to ≤20 hectares	288	5,029	314	352
from 20 to ≤30 hectares	463	10,999	391	9,714
from 30 to ≤50 hectares	195	7,166	238	8,767
from 50 to ≤100 hectares	67	3,913	65	3,672
from 100 to ≤200 hectares	3	303	2	203
from 200 to ≤300 hectares	2	450	2	567
> 300 ha	0	0	0	0
TOTAL	**25,729**	**93,408**	**25,988**	**94,131**

Source : Institut d'estadistica de Catalunya (Idesca, 2018) [39]

Concerning the content of ethyl esters, there are currently no published data for the region of Catalonia. In this thesis, we are the first to make a global estimation for the levels of ethyl esters in virgin olive oils produced in Catalonia. We have found that some of these Catalan EVOOs show relatively high levels of FAEEs. Preliminary data indicate that, even being below the limit set by current regulation; there is a latent risk that these oils will lose this "extra" category if a minimum of care is not taken. Therefore, in these cases, good management of the oil during storage is essential to avoid its degradation, which would lower its category and involve significant economic losses. And it is becoming increasingly clear that controlling only the final product is not enough, but it is also necessary to control the whole production process.

From all these considerations, it is clear that it would be very interesting to study all those key points in the process that have a direct impact on the generation of FAEEs. However, from an industrial point of view, the control of all the stages of the production process is not always feasible. Therefore, what would be very useful for the olive oil sector would be to focus this

study on those points in the process that, in addition to being key points, are stages in the process that the producer can easily manipulate or modify.

This background is what has led to the completion of this Doctoral Thesis whose main objective is to determine the prevalence of FAEEs in Catalan virgin olive oil and which are the main factors that increase the risks of their formation. To achieve these objectives, the experimental research and studies, which are explained and largely discussed throughout the following sections, were carried out.

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CHAPTER 4.

Materials & Methods

4.1. General Approach

The experimental design planned for the development of this thesis has been divided into three main parts (figure 4.1):

(1) Survey of the current content of FAAEs in the commercial oils in Catalonia;
(2) Olive varieties and their natural content in alcohol precursors of FAAEs and,
(3) Milling process and the balance of FAAEs and alcohols along the processing line.

Although detailed experiments and methodologies are presented in each one of the papers, this Chapter summarizes them. Some methodologies are common for different experiments, as is the case of how to assess fruit characteristics, olive oil quality, or the method of analysis of FAAEs. Other methods are more specific for each experiment, due to the final goals or depending on the matrix to be analysed. When dealing with the usual parameters in olive oil, the analyses were performed following the methods described in the Implementing Regulation (EU) 2019/1604 (Ex. 2568/91) and, when new parameters or analytes had to be determined, specific methods were developed.

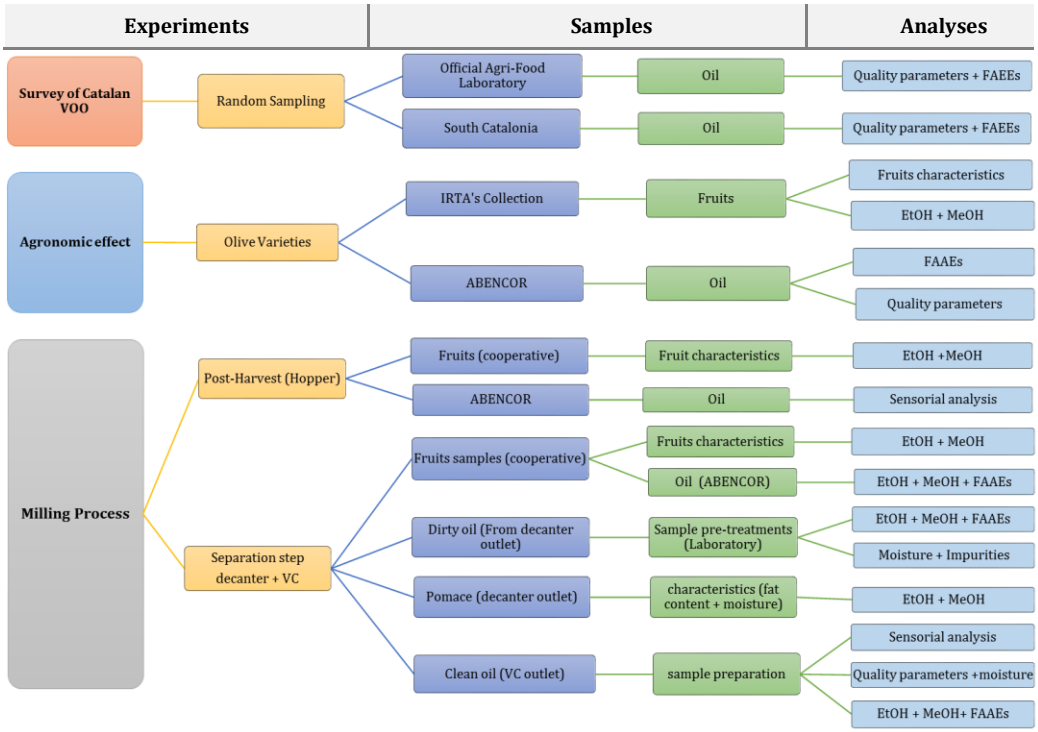

Figure 4.1. General approach of the experiments

4.2. Samples and Materials

For the development of this Thesis, different types of samples have been required to carry out the planned experiments, and it has been considered appropriate to include a brief summary of sampling in this section (figure 4.1). The detailed description of each sampling procedure can be found in the corresponding section of the Chapter 5.

4.2.1. Olive fruits

The olive samples used in this thesis basically came from two different origins depending on the experiment: (a) olive samples to study the varieties; (b) olive samples related to industrial experiments.

(a) To carry out the studies that required olives of different varieties and certain ripening stages, the olive samples were carefully handpicked from the Olive Germplasm Bank owned by Institute of Agrifood Research and Technology (IRTA) located at the Mas de Bover Center (Constantí, Tarragonès, Spain). The experiments that required these samples (figure 4.2) are the ones specified in Chapter 5.1 and 5.2.

Figure 4.2. Vegetal material (*Olea Europea L.*) used during the experiments:
A) Olive Germplasm Bank; B) Olive on the tree; C) olive samples

(b) The experiments planned to evaluate different parameters of the oil production process when working on a real scale were carried out at the Cooperative of La Granadella (Les Garrigues, Spain). In these experiments, each batch of olives was obtained from different farmers of the region (only of the 'Arbequina' variety). From each batch and when needed, a random sub-sample of fruits was taken to assess fruit characteristics (figure 4.3).

Figure 4.3. Preparation of batches of olive fruits at the industrial mill.
On the right, sub-sample of fruits taken to assess fruit characteristics

The description of the characteristics of the fruit consisted on:

- Fruit and pit average weight: weight a random sample of 50 olives using a precision balance. Use the same fruits to describe maturity index and healthy status, as described below. Take 10 fruits (randomly), remove the flesh by hand and weight the pits.

- Maturity index: It was determined based on the method described by Uceda and Frias (1975)[1] and consists on classifying 100 fruits in colour classes, from 0 (fully green skin) to 7 (fully black skin with 100% reddish flesh). Each colour class is weighed by its number of fruits ($n_{0\ldots7}$), using the following expression:

$$MI = \frac{0 \times n_0 + 1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4 + 5 \times n_5 + 6 \times n_6 + 7 \times n_7}{n_0 + n_1 + n_2 + n_3 + n_4 + n_5 + n_6 + n_7}$$

- Healthy status: classify 50 fruits according to its healthy aspect
  - No damage at all.
  - Some biting damages (scattered brownish dots due to biting olives during harvest or transport).
  - Affected by olive fly.
  - Mouldy.
  - Fermented (good quality fruits that became fermented during post-harvest).
  - Other visual problems.

- Fat and water contents: crush the remaining olives (apart from the 50 to assess former parameters) and measure fat and water in the grinded paste with a FOSS-OLIVESCAN NIR analyser (FOSS Iberia, Barcelona, Spain).

[1] Uceda M. and Frias L. Trend of the quality and quantitative composition of olive fruit oil during ripening. Proceedings of the International Meeting on Olive Oil. Cordoba, Spain. 25-46 (1975).

4.2.2. ABENCOR system to obtain virgin olive oil

Some of the experiments were performed at laboratory scale, using the ABENCOR system (Comercial ABENGOA, Seville, Spain) to extract the oil.

The collected olives were cleaned from leaves and twigs, rinsed with deionized cold water and crushed in the hammer mill of the ABENCOR System (figure 4.4). After that, the paste was malaxed for 30 min at <26ºC. A vertical centrifuge (3.000 rpm) was used to split the solid part (pomace). To clarify the oily fraction obtained in the previous step, a second high speed desktop centrifuge was used (KUBOTA Model, Japan). Finally, the oil was filtered to remove residual impurities.

The olive oil obtained was stored in a freezer (-20ºC) until analysis (quality parameters and FAAEs). The rest of the measurements (fat content and moisture from the olives) were carried out on the same pilot plant, as described above (FOSS-OlivScan NIR system).

Figure 4.4. ABENCOR system for olive oil extraction at laboratory scale

4.2.3. Samples from the industrial milling process

The industrial experiments to evaluate some processing factors were carried out at the mill of La Granadella Cooperative (La Granadella, Lleida). This mill process, approximately, 4,000 tons of olives per year. It has a single production line equipped with hoppers of medium capacity (20,000-25,000 kg each), a hammer mill for olive crushing (grinding) and a two-phase decanter DC-180 (TACSA, Técnicas Andaluzas de Centrifugación S.L.) that operates at ~2,410 RCF with a theoretical capacity of 5,000 kg/h, followed by an automatic vertical centrifuge (HAUS-Centrifuge technology) that operates at ~10,080 RCF. The variations of the evaluated process parameters are detailed in the corresponding section of Chapter 5.

The sampling strategy depended on each experiment. Figure 4.5 schematically shows the production process and the points from where the samples were taken for analysis.

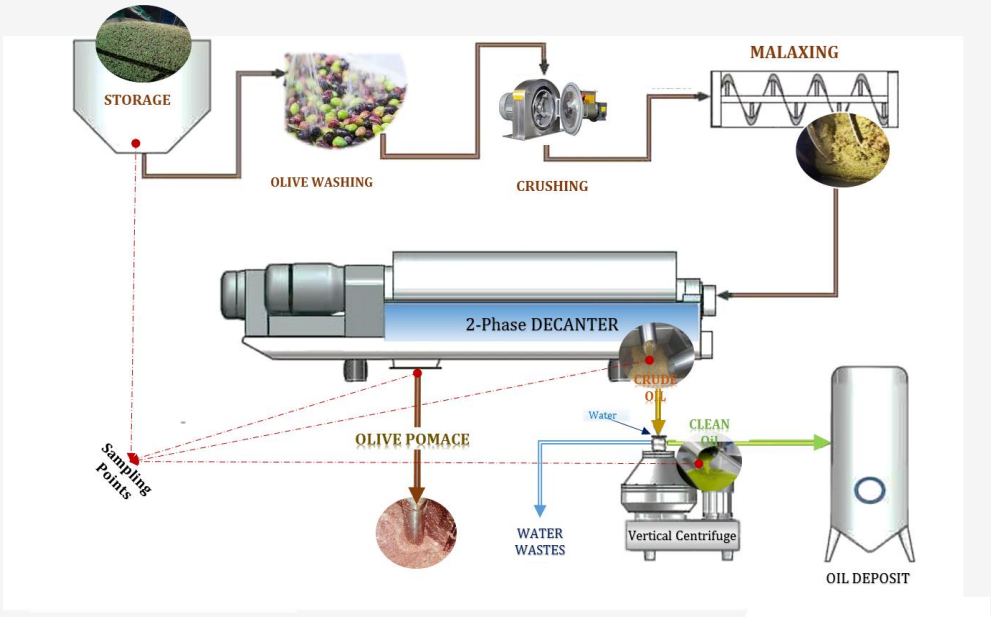

Figure 4.5. Main sampling points along the industrial olive oil extraction process

Olive oil and pomace samples were immediately placed in a freezer (-20ºC) and transported in dried ice to the analytical laboratories. Olive samples were sent to the IRTA laboratory the same day of the experiment, to be described for maturity, health status, fruit weight and stone weight.

4.3. Analytical methods

Due to the complexity of the samples used in this Thesis, a specific preparation of each type of sample was required before analyses. In each case, the most appropriate sample pre-treatment and analytical techniques were used, which are briefly explained below.

4.3.1. Sample preparation

- *Preparation of homogenate from olive and pomace paste*

For the analysis of alcohols (methanol, ethanol) and acetaldehyde in olive and olive pomace samples, it was necessary the preparation of a homogenate of olive paste (figure 4.6) was necessary. For that, the olives were triturated at room temperature with a small electric mill of laboratory use. It should be noted that the olives were kept in cold water (< 5ºC) to reduce their

temperature before grinding and thus avoid sample alterations. The paste obtained was mixed well (using a Vortex) with 50% milliQ water. Later, samples of these homogenates were taken and put in vials together with a saturated solution of calcium chloride ($CaCl_2$) in water (10%). This last solution was used as an inhibitor to stop the cellular metabolic activities during sample analysis. The prepared vials were hermetically sealed and finally stored in a freezer (-18ºC) until analysis.

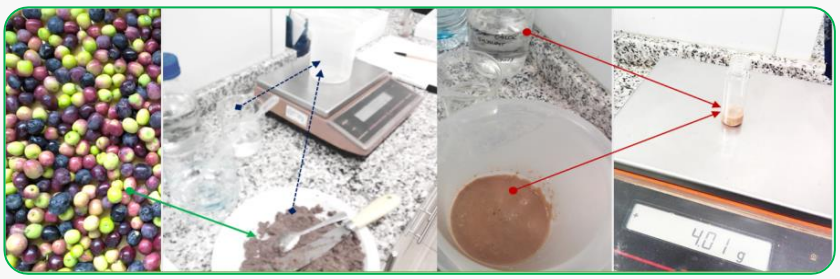

Figure 4.6. Preparation of the homogenates from the olive paste

❖ *Preparation of the oil samples*

Concerning oil samples, the analyses of the volatiles and alkyl esters were carried out on two different types of samples. The first were clean oil samples obtained after vertical centrifugation and filtration. In that case, no pre-treatment of the samples was required. The second were samples of crude oil from the decanter outlet (figure 4.5). These samples contained significant amounts of residual water and impurities, so to remove moisture and solids from the oil, the samples from the decanter outlet were centrifuged in the laboratory at 5ºC and 5,000 RCF during 3 min (figure 4.7). In addition, the moisture of each sample was measured and the reparation coefficient of alcohols between the oily and aqueous fractions was also determined, as explained in the Chapter 5.3.

Figure 4.7. Preparation of the oil samples

4.3.2. Analytical procedures

The determination of the standard physicochemical parameters in the olive oil samples was carried out using the official methods of the European Commission and the International Olive Council. Regarding the physicochemical characterization of the olives, it was evaluated following the methods described in Table 4.1. All these analyses were carried out in the laboratory of olive growing and oil technology at the Institute of Agrifood Research and Technology (IRTA) located in Mas de Bover. The determination of the physicochemical parameters of olive oils was carried out in the Agri-Food laboratory of Catalonia (Cabrils, Barcelona).

The other analytical methods used in each experiment are detailed in the corresponding sections of the next Chapter. In each case the most appropriate sample pre-treatment and analytical technique were used, and are briefly explained below.

Table 4.1. Analytical methods used for the standard physico-chemical determinations

	Determination	Method
Olive oil		
	Moisture and Volatiles matter	UNE-EN-ISO 662:2016 méthode B
	Impurities insoluble in ether	ISO 663:2017
	Acidity	R(CEE)2568/91 Annex II
	Peroxide value	R(CEE)2568/91 Annex III
	K_{232}	RCEE2568/91 DOUE L248 Annex IX
	K_{270}	RCEE2568/91 DOUE L248 Annex IX
	ΔK	RCEE2568/91 DOUE L248 Annex IX
Olive fruits		
	Maturity Index	Uceda & Frías (1975)
	Fruit and pit weight	Balance
	Fat and water content	NIR method using FOSS-OliveScan (FOSS IBERIA, S.A.U, Barcelona)
	Water content (oven method)	Norm UNE 55020

4.3.2.1. Short chain alcohols

a. Determination of alcohols in olive oil samples

❖ *Static Headspace sampling*

The analysis of short chain alcohols in olive samples was carried out using the static headspace technique (HS). Headspace sampling is a separation technique where the volatile compounds

may be extracted from the sample matrix. To achieve this separation, the sample is placed into a sealed vial and heated to a moderate temperature for a specific period of time. In this way, the more volatile compounds will tend to pass from the liquid or solid sample to the gas phase above the sample which is also called headspace. Therefore, if this headspace is analysed, only information on the volatile fraction of the sample will be obtained.

In each case, it is necessary to optimize the different parameters that influence the equilibrium between the sample and the headspace. In the present thesis, the conditions proposed by Gómez-Coca et al.[2] were checked and used. Specifically 3 g of sample were put into a 10 mL vial and heated at 80ºC during 50 min under medium orbital agitation. After that, 500 µl of the headspace of the sample were injected into the GC port through a transfer line at 110ºC as explained in Chapter 5.3.

- ***Instrumentation***

For these analyses a G1888 Automatic Static Headspace Sampler (Hewlett-Packard, USA) coupled to an HP-6890 gas chromatograph (HP, Palo Alto, CA, USA) equipped with an HP-5973 mass selective detector (HP, Palo Alto, CA, USA) were used (figure 4.8).

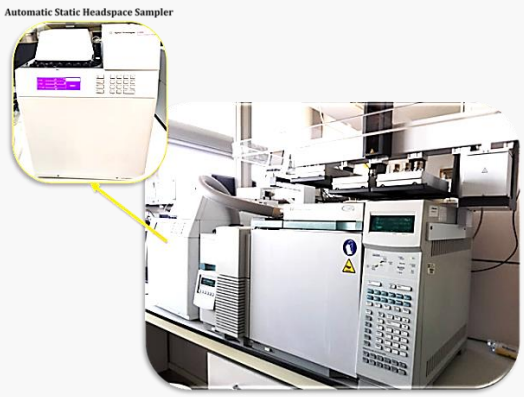

Figure 4.8. Automatic Static Headspace Sampler coupled to a GC-MS

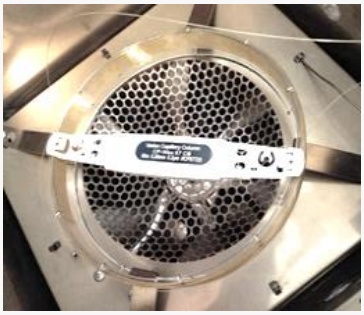

Figure 4.9. Capillary Column used for the analysis of methanol and ethanol

[2] Gómez-Coca, R.B., Cruz-Hidalgo, R., Fernandes, G.D., Pérez-Camino, M.D.C., Moreda, W. (2014). Analysis of methanol and ethanol in virgin olive oil. *MethodesX*. 1, e207-e211.

The chromatographic separations were carried out using a fused silica capillary column Chromapack (Varian, Middelburg, Netherlands) CP-WAX 57CB (50 m x 0.25 mm i.d., 0.2 µm film thickness) (figure 4.9). The chromatographic temperature program was: 40ºC (5 min), 5ºC/min to 100ºC and 10ºC/min to 215ºC (5 min). The carrier gas was helium (1.8 mL/min). Interface, ion source and mass quadrupole temperatures were 200ºC, 230ºC and 150ºC, respectively. To identify the compounds of interest, the spectra matching was performed using the Wiley library (Wiley NBS\library 275.L).

b. Determination of alcohols in the olive paste homogenates

❖ *Solid Phase Microextraction sampling*

The Solid Phase Microextraction (SPME) is a solvent-free sample preparation technique that is fast, clean and implies minimal sample manipulation. It uses a very thick silica fiber coated with a liquid (polymer), a solid (sorbent), or a combination of both to extract the compounds from the sample by absorption or adsorption. These extractions can be performed by introducing the fiber into the sample (if it is a liquid) or by exposing it to the headspace (HS-SPME). Once extracted, the analytes can be directly desorbed into the chromatographic injector inlet by means of thermal desorption (figure 4.10).

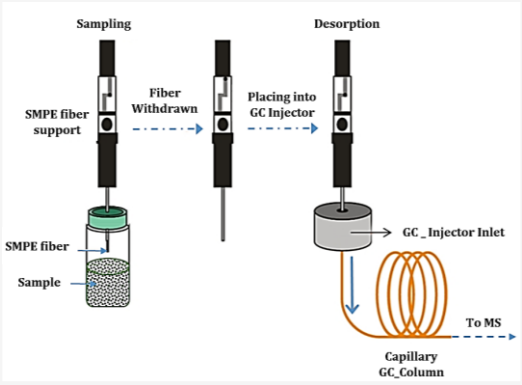

Figure 4.10. Solid Phase Microextraction technique

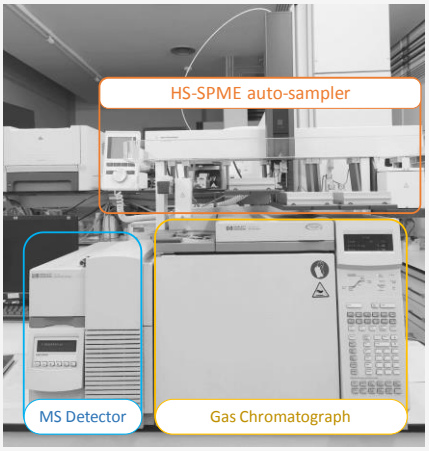

Figure 4.11. The SPME-GC-MS system

In this thesis, the contents of methanol and ethanol in the homogenates from olive paste were determined by using HS-SPME with a fiber coating of 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane. The optimal extraction conditions were: 3 g of sample into 20 mL vials;

15 min of pre-equilibration time at 50ºC; and HS-SPME during 50 min at 40ºC under medium orbital agitation. After that, the volatiles extracted were thermally desorbed into the GC injector port.

❖ ***Instrumentation***

For these analyses a HS-SPME CTC CombiPAL autosampler (CTC Analytics, Switzerland) coupled to an HP-6890 gas chromatograph (HP, Palo Alto, CA, USA) equipped with an HP-5973 mass selective detector (HP, Palo Alto, CA, USA) were used (figure 4.11).

4.3.2.2. Fatty acid alkyl esters

❖ *Fractionation by using column chromatography*

The determination of fatty acid methyl and ethyl esters (FAMEs and FAEEs) was carried out using the official method reported by the IOC (COI/T.20/Doc. No 31. 2012). This method is based on the fractionation of waxes and fatty acid alkyl esters by using column chromatography as a preparative technique (figure 4.12). About 100 mg of sample were exactly weighted and, after addition of a suitable amount of an internal standard (methyl heptadecanoate in heptane 0.02%), the sample was transferred to the chromatographic column filled with hydrated silica gel. Then, the solvent was added to start the chromatography and, after collecting the suitable fraction, first it was evaporated in a rotatory evaporator and later with a stream of nitrogen. Finally, the fraction containing the methyl and ethyl esters was diluted with n-heptane to obtain an extract, which was injected directly into the gas chromatograph.

Figure 4.12. Fractionation by using column chromatography

Figure 4.13. Gas chromatograph with automatic injector and PTV system

❖ ***Instrumentation***

For these analyses an Agilent 6890N gas chromatograph equipped with a programmable temperature vaporizing (PTV) inlet for on-column injection of the sample extracts was needed. Agilent G1530 flame ionization detector (FID) (Agilent Technologies, USA) was used (figure 4.13).

The chromatographic separations were performed using a fused silica capillary column, Zebron ZB-5MS, 30 m x 0.25 mm i.d. and 0.25 um d.f. from Phenomenex (Alcobendas, Spain).

The chromatographic program temperature was: 70ºC for 2 min, followed by three ramps of 10ºC/min until 180ºC, then 5ºC/min until 220ºC and finally 10ºC/min until 320ºC. The last temperature was kept constant for 16.5 min. The FID detector temperature was 350ºC, the hydrogen flow was 40 mL/min and the air (N_2) flow was set to 450 mL/min. The carrier gas was hydrogen at a constant flow of 1.5 mL/min and column head pressure of 23.9 psi.

4.3.2.3. Sensory Analysis

The sensory evaluation of the oil samples was carried out by the Official Olive Oil Tasting Panel of Catalonia (Reus, Spain), which has been recognized by the IOC since 1997 and by the Spanish Government since 2004. It relies under ISO 17025 standard since 2007. Each analytical session was conducted by eight trained tasters, who tasted 8-12 samples in groups of 4 samples, including a 10 min break between groups. Samples were coded using a random code that was unique for each single tasting glass. The assessors tasted the samples following the official rules of the EC Regulation 2568/1991. Each sample was described through its positive and negative attributes, using a continuous scale of 10 cm (figure 4.14). Finally, each attribute was scored as the median and robust standard deviation from all the tasters.

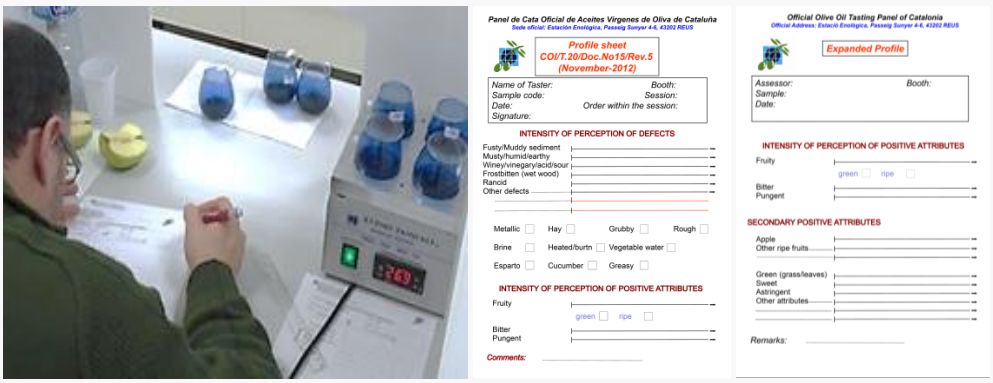

Figure 4.14. Tasting session of olive oil samples (on the right, example of a profile sheet used by the Official Taste Panel of Virgin Olive Oils in Catalonia, following the EU Regulation 640/2008

4.4. Statistical analysis

The statistical analysis is detailed in each paper. In summary, the statistical methods used were:

- Survey study of commercial virgin olive oils
  - **Distribution analysis and box-plot** to study the prevalence of alkyl esters in the Catalan olive oils.
  - **Correlation analysis** to explore the relationship between the content of alkyl esters and other quality criteria.
- Studies of varieties and ripening
  - **Analysis of Variance (ANOVA)** to explore differences between varieties and alcohol contents.
  - **Correlation analysis** to explore the relationship between the content of alkyl esters and other olive oil quality criteria, and fruit characteristics.
  - **Cluster analysis** to explore the relationships between olive varieties.
- Studies of the fruits storage in hopper
  - **ANOVA** to analyse the differences between time of downloading the hopper, including the harvest time factor and their interactions.
  - **Partial least Square analysis (PLS)** to identify the fruit characteristics and olive oil quality parameters that determine the final alcohol content in the olive oil, which are the precursors of alkyl esters.
- Studies along the extraction process
  - **ANOVA** to explore differences between the experimental factors (paste pumping rate and water addition).
  - **General Linear Model (GLM) analysis** to explore the linear or polynomic trend of the dose factor on the studied variable (alcohols or alkyl esters).

All the statistical analyses were carried out using the SAS software (SAS v 9.4, Cary, USA). Procedures UNIVARIATE, ANOVA, GLM, CORR and CLUSTER were used. More details can be found in the papers.

CHAPTER 5.

Results & Discussion

Chapter 5 – (PAPER 1)

Quantitation of endogenous amount of ethanol, methanol and acetaldehyde in ripe fruits of different Spanish olive varieties

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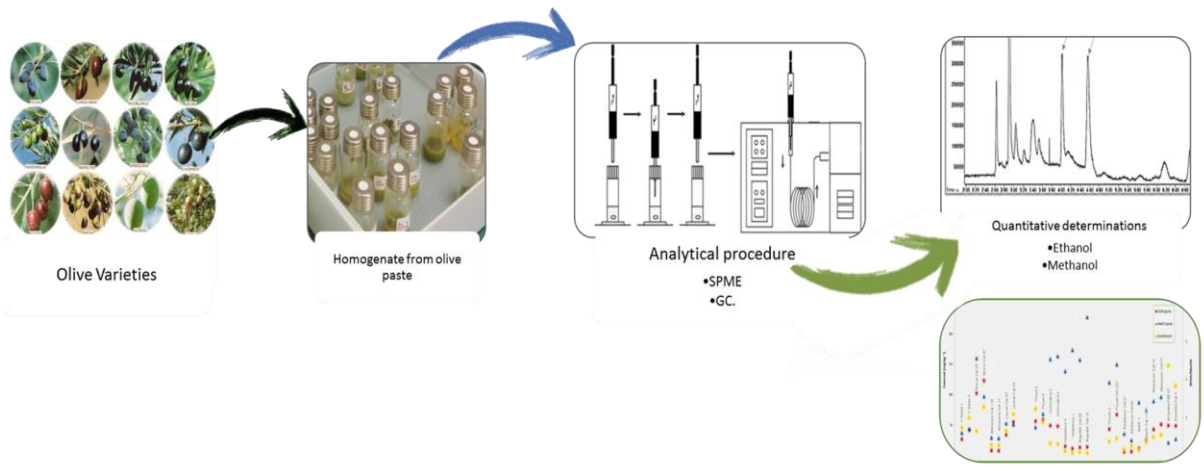
Olive Varieties
Homogenate from olive paste
Analytical procedure
•SPME
•GC.
Quantitative determinations
•Ethanol
•Methanol

ABSTRACT

BACKGROUND

The use of healthy olives and their good management along the production process are necessary to obtain the best quality virgin olive oils. One of the parameters related to the health of the olives is the content of fatty acid alkyl esters. Since these come from the esterification of C16 and C18 free fatty acids with short chain alcohols, the control of methanol, ethanol and acetaldehyde (precursor of ethanol) and their origin (endogenous or from fermentation) is essential.

This paper reports the endogenous amount of these compounds in some of the main Spanish olive varieties. For their analyses, headspace solid phase micro-extraction was applied and, to ensure the quantitation reliability, the matrix-matched technique was used to build the calibration lines.

RESULTS

For healthy and mature olives, the contents of ethanol and methanol are much higher and vary within a wider range than those corresponding to acetaldehyde. Since olives were not directly analyzed but previously homogenized, there was no correlation between the olive size parameters and the contents of the studied compounds. However, these contents are characteristic of each variety. When comparing healthy and unhealthy olives, significant differences were observed only for ethanol contents.

CONCLUSION

Higher contents of short alcohols are not always due to an unhealthy or poor state of the fruits, but to the variety. Therefore, as these alcohols are precursors of fatty acid alkyl esters, the maximum permissible content of the latter should not be set at a single value for all olive varieties.

Keywords: olive oil, olive variety, endogenous alcohols, fatty acid alkyl esters, solid-phase microextraction (SPME), reliable quantitation.

1. INTRODUCTION

Virgin olive oil is an important element in the Mediterranean diet; it is widely appreciated and consumed for its taste and aroma as well as for its nutritional and health benefits (1-3). Extra virgin olive oil (EVOO) is the highest quality oil, and comes from fresh and healthy olives and only by the application of mechanical extraction procedures. Because of its high added value the International Olive Oil Council (IOOC) and the European Commission have issued an international standard that regulates its quality by evaluating not only their organoleptic characteristics but also some physicochemical parameters.

In recent years, to ensure the use of healthy olives and their good management along the whole oil production process a new criterion has also been introduced: the content in Fatty Acid Ethyl Esters (FAEEs) (4). To classify an olive oil as EVOO the content of FAEEs has to be lower than 30 mg kg^{-1}, with a total amount of Fatty Acids Alkyl Esters (FAAEs) lower than 75 mg kg^{-1} (5- 7).

FAAEs derive from the esterification of C16 and C18 free fatty acids with low molecular weight alcohols such as ethanol and methanol, producing methyl and ethyl esters, respectively. This is a very fast reaction, so the FAAEs level in olive oil depends upon availability of precursors and enzymes (8-10). Therefore, to get a low FAAEs amount it is necessary to control the production of fatty acids and alcohols.

Fatty acids are specific to both the oil and the cell membrane of the pulp or can be produced after the lipolysis of the triacylglycerols. Short-chain alcohols come from many different sources such as pectin degradation, inadequate practices during olive harvest and post-harvest that cause the rupture of the drupe, fermentation of fruit sugars during the extraction and the storage process of olive oil or reduction of acetaldehyde to ethanol through the alcohol-dehydrogenase (ADH) activity during the glycolysis process. Therefore, it is obvious that the more damaged an olive is, the higher amounts of FAAEs the final oil will contain. However, Beltran et al., (11) claimed that part of the ethanol comes from the own metabolism of the olive and it is accumulated during fruit maturation on the olive tree, which makes the situation more complex. In fact, this implies that ripening stage and variety may have an effect on the amounts of ethanol and methanol, which may increase the content of FAAEs not related to the health status or quality of the olive, but will have legal implications on the limits of the alkyl esters.

Although there are many studies in the literature related to the origin of low molecular weight alcohols in plants and fruits, there are very few that quantify them in olive fruits and, so far,

only one has focused on the effect of the maturity of olive varieties over ethanol concentration (11). Therefore, a deeper knowledge on this topic is necessary for a better control of the natural content of FAAEs due to the lack of joint results about these compounds. This involves the analysis of more olive varieties and the determination not only of ethanol contents but also of methanol and acetaldehyde (as a precursor of ethanol). From these premises, the objective of the present study was to assess the effect of olive variety on the endogenous amount of ethanol, methanol and acetaldehyde in healthy ripe olive fruits, including a great range of the main Spanish varieties.

2. MATERIAL AND METHODS

2.1. Fruit sampling

The experiment was carried out by analysing 13 different Spanish varieties typical of different Spanish regions (specified in parentheses): 'Arbequina', 'Arbosana', 'Argudell', 'Fulla de Salze', 'Morrut' (Catalonia), 'Hojiblanca', 'Picual', 'Picudo' (Andalusia), 'Marfil', 'Llumet', 'Sevillenca' (Valencia), 'Cornicabra' (Castilla-La Mancha) and 'Empeltre' (Aragon). Olive samples were supplied by the Institute of Agrifood Research and Technology (IRTA) olive germplasm bank (12), located in an area of 0.6 ha at the Mas de Bover Center (Constantí, Tarragonès, Spain) with coordinates: 41º 10" north latitude, 1º 10" east longitude, 15 km from the Tarraconense coast, and 120 m above sea level. The climate is typically Mediterranean, with high environmental humidity (60-70%) and an average rainfall of 450 mm per year, irregularly distributed. The potential evapotranspiration (PET) is 965 mm per year. The soil is narrow (40-50 cm) and has a loamy texture, a basic pH (8.1) and a 4% content of active limestone. The collection keeps three trees by variety with a plantation framework of 7 x 5 m. All the olive trees grow in the same edaphoclimatic conditions and all of them have the same age (planted in 1992).

Fruit samples were collected by hand at the end of December 2017 so, taking into account the Mediterranean climate where the plantation is located, we could consider that all the olive varieties were mature and that there could be no further evolution since the cold would paralyze any type of plant metabolism. Between 3 kg and 5 kg of olive fruits were randomly taken around the olive canopy from each one of the two trees selected (the two ones with similar fruit load) of each variety and no distinction between healthy and unhealthy fruits was considered.

To determine the physical parameters of the olives, 100 olives were randomly taken from the sampling of each tree. Then, average values for fruit and pit weight, maturity index (M.I.) and

visual diagnostic of health status (undamaged olives, olives with knocking marks, olives damaged by fly, olives damaged by fungus, other defects) were measured.

The M.I. values were calculated by using the equation proposed by Uceda & Frias (13), based on the number of fruits of each maturity category (category 0 -when the skin of the fruit is deep green- and category 7 -when the colour of the skin and also of the pulp is purple-black) divided by the total number of fruits considered (100). Regarding health status, olive fruits were considered unhealthy when more than 10 olives (> 10%) present any defect, regardless of which one it was.

Finally, the healthy olives coming from the sampling of each tree were split into two groups and were processed separately. As far as the damaged fruits are concerned, these were separated from the rest of olive samples and were processed separately.

2.2. Preparation of the olive homogenates

The method followed to obtain the homogenates from each one of the two samples of each tree was based on the one proposed by Beltran *et al.* (11). Once the healthy olives (i.e. with a percentage of defects <10%) arrived at the laboratory, they were processed as soon as possible (15-20 min) to avoid any sample alteration. Each sample of olives (coming from the split into two from each tree) was rinsed with deionized cold water (4ºC) and totally dried with absorbent tissues for laboratory use. Immediately after, the olives were placed in a hammer mill (from the ABENCOR System) and were ground at room temperature obtaining a paste (sieve of 6 mm). 15 g of this olive paste were placed into a 50 mL tube and mixed together with 15 g of cold milli-Q water, homogenized and left to stand for 5 minutes in such a way that the particles corresponding to the pits were deposited in the lower part of the tube. Then, 1.00 g (± 0.01 g) of the homogenate was weighed in a 20 mL glass vial, together with 1.00 g (± 0.01 g) of saturated $CaCl_2$ solution and stirred to prevent any biological activity that could alter the sample before its analysis. This procedure was performed in duplicate for each pressing and the two vials were tightly sealed with a septum cap. Finally, the vials were kept at -18 ˚C until their analysis by solid phase microextraction and gas chromatography coupled to mass spectrometry (SPME-GC-MS).

2.3. Chemicals and standards

The standards of ethanol absolute (gradient HPLC grade) and methanol (supragradient HPLC grade) were purchased from Scharlab (Barcelona, Spain). Acetaldehyde (99% for synthesis) and

calcium chloride anhydrous (97%) were purchased from Panreac (Barcelona, Spain). Milli-Q quality water used was obtained from a purification system (Millipore, Bedford, USA).

2.4. Solid Phase Microextraction (SPME)

The SPME holder for manual sampling and the 75 µm/85 µm Carboxen/polydimethylsiloxane (Carboxen/PDMS), 100 µm polydimethylsiloxane (PDMS) and 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane (DVB/CAR/PDMS) fibres were purchased from SUPELCO (North Harrison Road -Bellefonte, PA - USA). All the fibres were conditioned before use and thermally cleaned between analyses by inserting them in the GC injector port at the temperature recommended by the manufacturer.

2.5. Analytical procedure: Headspace-Solid Phase Microextraction (HS-SPME)

Ethanol, methanol and acetaldehyde contents in the samples were determined using the solid phase microextraction technique applied to the headspace of the samples (HS-SPME) and followed by gas chromatography coupled to a quadrupole mass spectrometer (GC-MS). HS-SPME is a fast and clean technique that allows the extraction and the concentration of these compounds from the homogenate samples contained in the vials and with no extra sample handling.

The different parameters that affect the SPME yield were optimized: fiber coating, extraction time and temperature, and sample volume.

The best results were obtained by pouring 1.00 ± 0.01 g of sample homogenate with 1.00 ± 0.01 g of saturated $CaCl_2$ solution into a 20 mL glass vial and pre-equilibrating it in a thermostated water bath at 40 ºC for 5 minutes. Then, the SPME fiber DVB/CAR/ PDMS 50/30 µm, Stableflex, 2 cm fiber was inserted manually through the vial septum and exposed to the headspace over the sample for 50 min at 40 ºC under medium orbital agitation. Afterwards, the fiber was pulled into the sheath, removed from the vial and immediately introduced into the GC-MS injector port for thermal desorption at 270 ºC for 1 minute in the splitless mode.

2.6. Chromatographic conditions

Chromatographic analyses were performed with an HP-6890 gas chromatograph (HP, Palo Alto, CA, USA) equipped with an HP-5973 mass selective detector (HP, Palo Alto, CA, USA). Chromatographic separations were carried out by using a fused silica capillary column Chromapack (Varian, Middelburg, The Netherlands) CP-WAX 57CB (50 m x 0.25 mm i.d., 0.2 µm film thickness) and the best ratio “peak resolution/retention time” was achieved with the

following oven temperature program: 40 ºC (5 min), 5 ºC min-1 to 100 ºC, 10 ºC min-1 to 215 ºC (5 min). The carrier gas was helium (He), with a head pressure of 14.8 psi at a constant flow of 1.8 mL/min. The split-splitless injection port operated in the splitless mode at 270 ºC. The mass spectrometer operated in the electron impact ionization mode at 70 eV. Interface, ion source and mass quadrupole temperatures were 200 ºC, 230 ºC and 150 ºC, respectively. The mass-to-charge (m/z) ratio range used was 28-300 amu, and spectra matching were performed using the Wiley/NBS library.

2.7. Quantitation of the analytes

To get reliable calibration lines it was first necessary to evaluate the effect of the sample matrix over the SPME yield. Then, to avoid quantitation errors due to the matrix effect found, the calibration lines were built by using the matrix-matched calibration technique (14, 15). To obtain an olive fruit matrix without any of the studied compounds, a homogenate from unripe olive fruits was prepared. The absence of ethanol, methanol and acetaldehyde in the homogenate was checked by HS-SPME and GC-MS prior to its use.

Then, six different calibration standards were prepared by adding different known concentrations of each analyte to the homogenate of unripe olive fruits (not containing the analytes studied) by combining high, low and medium concentrations of the three analytes. In this way, it was possible to avoid measurements biases due to a different extraction yield when all the analyte concentrations were very low compared to when they were very high. Each calibration standard was prepared in triplicate and the calibration ranges were: ethanol (0-125 mg kg^{-1}), methanol (0-200 mg kg^{-1}) and acetaldehyde (0-15 mg kg^{-1}).

Finally, the concentration of ethanol, methanol and acetaldehyde in the olive fruits was calculated from the chromatographic peak area and taking into account the exact dilution of the sample performed when obtaining the homogenate by mixing water and also when adding $CaCl_2$ solution to the vial (already explained in "2.2. Preparation of the homogenates olive samples" section).

2.8. Statistical analysis

Statistical analysis was performed using the SAS-Stat Software (V9.3.Cary, SAS Institute Inc.). The variety factor was analysed by ANOVA (Analysis of Variance) using the Generalised Linear Model (GLM) procedure, and mean comparisons were performed by using the Duncan´s multiple range test ($\alpha < 0.05$).

The slopes and intercepts of the calibration lines – calculated by ordinary least-squares regression and evaluated by the coefficient of determination (r^2) and the standard errors of the slope and intercept coefficients – were obtained by using the ULC (Univariate Linear Calibration) software (16). This program was also used to carry out the comparison between the slopes of the calibration lines when it was required.

3. RESULTS AND DISCUSSION

3.1. HS-SPME optimization

Considering the low molecular weight and the high volatility of the compounds studied, the fiber coatings tested were 75 µm/85 µm Carboxen/polydimethylsiloxane, 100 µm polydimethylsiloxane and 50/30 µm divinylbenzene/Carboxen on polydimethylsiloxane. The results showed that the best overall extraction efficiency (20-40% higher) and the best reproducibility (5-10% better) was obtained when the last coating was used.

The sample weight (always maintaining the ratio 1:1 between the homogenate and the $CaCl_2$ saturated solution to ensure the biological inactivity) was optimized together with the vial volume. When using vials of 20 mL, more sample quantity resulted in more signal, until the maximum volume that prevented the immersion of part of the fiber into the sample was reached. The results showed that, when using vials of 40 mL, there was no significant signal increase.

Related to extraction time and temperature, ranges between 30-40 ºC (to avoid thermal sample alteration) and 30-60 minutes were essayed and the best results were obtained when extracting at 40 ºC for 50 min, values similar to the ones obtained in previous studies (17).

3.2. Performance parameters

The performance parameters evaluated were: matrix effect, limit of quantitation, repeatability and intermediate precision.

Due to the complexity of the olive paste composition and to the high extraction efficiency of the SPME fibers, it was necessary to check the effect that other olive compounds might have on the extraction of the analytes studied. Thus, known amounts of ethanol, methanol and acetaldehyde were added to Milli-Q water and to an olive paste (which did not contain any of the analytes). In each case 1.00 ± 0.01 g of spiked water or spiked paste were poured together with 1.00 ± 0.01 g of saturated $CaCl_2$ solution in a 20 mL vial. The chromatographic responses obtained after HS-SPME were compared and, as expected, when working with water as matrix, the response was 25-35% higher than when working with olive paste. However, it was still

necessary to determine the differences, if any, between the SPME responses provided by the different varieties. This effect was evaluated by studying four different olive pastes from four different varieties randomly selected: 'Arbequina', 'Picual', 'Panisello' and 'Hojiblanca'. Four different concentrations (in duplicate) of the compounds under study were added to each one of these olive matrices and they were analyzed following the procedure specified above. From the chromatographic peak areas obtained, four different calibration lines were built by plotting each response against the added concentration. Then, the slopes of the four lines were compared in order to detect a different behaviour related to the variety matrix effect. The results showed that the slope of the different matrices were comparable (significance level, $\alpha = 0.05$). This means that when working with the matrix-matched technique, the use of a paste of any olive variety as calibration matrix to build the calibration lines and quantify the analytes provides reliable results. Finally, the calibration lines were built using an 'Arbequina' olive paste as it provided the cleanest chromatographic signal.

The limits of quantitation (LOQ) were calculated from the amount of each compound required to give a signal/noise ratio 10:1 when working with olive paste. The LOQ values were 0.4 mg kg^{-1} for ethanol, 0.9 mg kg^{-1} for methanol and 0.2 mg kg^{-1} for acetaldehyde.

Within-day and between-day precision of the method were evaluated. In both cases, the results were calculated as a relative standard deviation (% rsd). Within-day precision was calculated by analyzing 5 times a matrix spiked with 25.0 mg kg-1 of ethanol, 50.0 mg kg^{-1} of methanol and 5.0 mg kg^{-1} of acetaldehyde. From their chromatographic response, the values obtained were rsd < 5.5%. The between-day precision was calculated from the results obtained when analyzing 10 times a matrix spiked with 5 mg kg^{-1} of each analyte, in alternate days. The precision values were rsd < 7.9 %.

Figure 1 shows the chromatogram obtained when analyzing a real sample, in this case from 'Arbequina' variety.

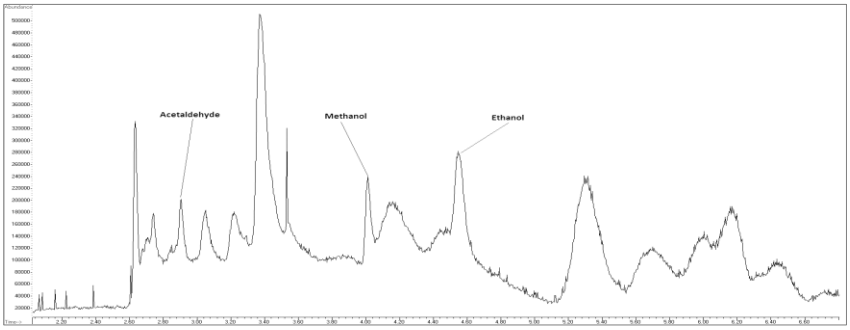

Figure 1. Chromatogram of an 'Arbequina' sample analysed with the proposed HS-SPME procedure

3.3. Physical analysis of samples.

Once samples were collected, 100 olives of each tree were randomly selected and their physical characteristics were immediately determined. Since all the olive samples were handpicked at the end of December and almost all the fruits looked black on the trees, the maturity index (M.I.) values were equal or greater than 4 for all samples. The most notable values were those corresponding to the 'Arbequina', 'Empeltre', 'Sevillenca' and 'Hojiblanca' varieties that, when studying the flesh colour in the lab, resulted in M.I. from 5 to 7. Table 1 shows the average M.I. values together with those corresponding to other parameters evaluated for each variety: fruit weight, pit weight and flesh/pit ratio. As expected and because of the great differences between the varieties studied, the statistical analysis showed significant differences in all cases (table 1). These values are in agreement with those previously reported for the same varieties (12, 18). Regarding the visual health status, no significant differences were found between varieties (data not shown). These results were the ones expected because all the trees under study were in good health and only 5-8% of the olives showed defects. It should be noted that these defective olives were studied separately, as explained below.

Table 1: Average values of fruit physical traits (fruit weight, pit weight, flesh/pit ratio and maturity index – M.I.)

						Reference values [12, 18]	
Variety		Fruit weight (g)	Pit weight (g)	Flesh/Pit ratio	M.I.	Fruit weight	Flesh/pit ratio
ARBEQUINA		1.6 ed	0.3 f	5.2 ± 0.0 cb	6.4 ± 0.5 ab	1.9	5.6
ARBOSANA		2.4 ced	0.4 fe	5.9 ± 0.7 cb	4.2 ± 0.9 ef	1.4	4.9
ARGUDELL		2.9 cbd	0.5 fde	6.2 ± 0.2 b	3.8 ± 0.3 f	2.2	5.6
CORNICABRA		4.0 b	0.8 b	4.9 ± 0.3 cbd	4.3 ± 0.9 def	3.5	4.1
EMPELTRE		3.4 cb	0.6 cde	5.8 ± 0.8 cb	6.9 ± 0.0 a	2.8	5.2
FULLA DE SALZE		1.5 e	0.4 f	3.9 ± 0.4 ed	4.5 ± 0.4 def	1.8	4.1
HOJIBLANCA		3.9 b	1.2 a	3.4 ± 0.1 e	5.1 ± 0.0 dc	4.5	3.7
LLUMET		2.5 ced	0.5 fde	5.5 ± 0.3 cb	4.9 ± 0.0 de	1.9	3.8
MARFIL		2.3 ced	0.4 fe	5.6 ± 0.2 cb	3.8 ± 2.7 f	2.1	4.3
MORRUT		3.4 cb	0.9 b	3.9 ± 1.2 ed	4.5 ± 1.4 def	3.9	5.7
PICUAL		2.9 cbd	0.6 cd	4.7 ± 0.3 cd	4.7 ± 0.5 de	3.6	3.5
PICUDO		5.9 a	0.8 cb	7.8 ± 0.9 a	4.6 ± 0.2 def	5.5	4.1
SEVILLENCA		3.5 cb	0.7 cb	4.9 ± 0.1 bd	5.8 ± 0.4 bc	3.4	5.3
Data analysis	*R-square*	*0.949*	*0.974*	*0.924*	*0.860*		
	F value	*11.52*	*23.14*	*8.11*	*15.69*		
	α <0.05	*0.0009*	*<0.0001*	*<0.0031*	*<0.0001*		

By column, means with the same latter are not significantly different according to Duncan's multiple range tests (p<0.05).

3.4. Determination of ethanol, methanol and acetaldehyde in samples

The chromatographic results after the HS-SPME application to the studied samples are summarized in Table 2. When considering the different varieties all together, we observed that the ethanol content varied over a very wide range (from around 6 mg kg^{-1} for 'Sevillenca' to around 111 mg kg^{-1} for 'Morrut'). These different content values could not be correlated to any of the physical parameters determined in the present study. This is because we did not analyze olives directly, instead we analyzed the homogenate prepared from each sample and all the olives were mature. In this way, we avoided the effect both of the olive size and the maturity status over the olive composition and, therefore, over ethanol concentration. However, each variety has a different average ethanol content with an uncertainty of less than 10% in all cases. This low data spread indicates that the ethanol content is characteristic of each variety. Thus, according to this content cultivars can be grouped in four main groups (Figure 2): 1) 'Morrut' and 'Hojiblanca', with values close to 100 mg kg^{-1}, 2) 'Picual', 'Llumet', 'Cornicabra', 'Picudo', 'Empeltre'and 'Arbequina', with values between 44 and 57 mg kg^{-1} of ethanol, 3) 'Fulla de Salze' and 'Marfil', with a quantity of ethanol between 25 and 35 mg kg^{-1}, and 4) 'Argudell', 'Sevillenca' and 'Arbosana', with less than 14 mg kg^{-1} of ethanol.

As it can be seen, the ethanol values found are noticeable higher than those presented by Beltrán et al. (11). This behaviour was the one expected because ethanol concentration increases with the fruit ripening and our samples presented higher M.I. in all cases.

Table 2: Average of the amount of ethanol, methanol and acetaldehyde contents for the studied varieties

Variety	Ethanol (mg kg^{-1})	Methanol (mg kg^{-1})	Acetaldehyde (mg kg^{-1})	EtOH/MeOH*	AA/EtOH*	∑EtOH+MeOH+AA (mg kg^{-1})*
ARBEQUINA	48.3 ± 2.0 bc	93.4 ± 1.6 bc	4.4 ± 0.1 a	0.52 ± 0.02 cd	0.10 ± 0.01 ef	146.2 ± 2.8 c
ARBOSANA	7.6 ± 0.2 e	28.5 ± 0.5 cd	4.1 ± 0.1 ab	0.27 ± 0.01 de	0.54 ± 0.01 a	40.2 ± 0.5 f
ARGUDELL	13.4 ± 0.4 e	180.6 ± 8.2 a	3.7 ± 0.1 bcd	0.07 ± 0.00 e	0.27 ± 0.02 d	197.7 ± 8.0 b
CORNICABRA	47.3 ± 0.8 bc	152.7 ± 5.4 ab	3.8 ± 0.1 bc	0.30 ± 0.01 de	0.08 ± 0.00 ef	203.8 ± 6.1 b
EMPELTRE	48.6 ± 0.5 bc	23.5 ± 0.9 d	3.2 ± 0.1 ecd	2.07 ± 0.08 a	0.07 ± 0.00 ef	75.3 ± 1.1 e
FULLA DE SALZE	34.4 ± 3.4 cd	39.7 ± 1.0 cd	3.5 ± 0.1 bcd	0.87 ± 0.06 bc	0.10 ± 0.01 ef	77.6 ± 4.4 e
HOJIBLANCA	98.1 ± 1.5 a	140.8 ± 9.9 ab	3.7 ± 0.1 bcd	0.70 ± 0.01 c	0.04 ± 0.00 c	257.5 ± 6.9 a
LLUMET	44.4 ± 3.2 cbd	51.5 ± 0.3 cd	2.6 ± 0.1 e	0.86 ± 0.06 bc	0.06 ± 0.01 ef	98.4 ± 2.9 de
MARFIL	25.3 ± 2.1 de	74.3 ± 2.3 cd	3.3 ± 0.1 cd	0.34 ± 0.03 de	0.20 ± 0.03 e	102.9 ± 2.0 de
MORRUT	111.2 ± 5.5 a	140.6 ± 9.2 ab	3.8 ± 0.2 bc	0.84 ± 0.09 bc	0.03 ± 0.00 f	255.5 ± 8.3 a
PICUAL	57.1 ± 3.2 b	53.3 ± 6.0 cd	3.1 ± 0.2 efd	1.13 ± 0.09 b	0.05 ± 0.01 ef	113.5 ± 8.7 d
PICUDO	48.7 ± 5.1 bc	137.2 ± 6.0 ab	3.3 ± 0.2 cd	0.36 ± 0.04 de	0.06 ± 0.01 ef	189.2 ±10.7 b
SEVILLENCA	5.9 ± 0.1 e	31.8 ± 2.1 dc	2.6 ± 0.1 ef	0.19 ± 0.02 de	0.43 ± 0.02 b	40.3 ± 2.0 f
Data analysis *R-square*	*0.9093*	*0.7869*	*0.7870*	*0.9193*	*0.9679*	*0.9589*
F value	*20.90*	*8.31*	*8.31*	*23.76*	*62.80*	*48.69*
α <0.05	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

By column, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05).
* EtOH: Ethanol; MeOH: Methanol; AA: Acetaldehyde.

Regarding the cultivars, our results agree and confirm that the 'Hojiblanca' variety is one of those with the highest content of endogenous ethanol. However, when comparing the results for 'Picual' and 'Arbequina', our results did not match those found by Beltrán et al. (11). As said before, in our study, these varieties showed a similar ethanol content but the results found by Beltrán et al. (11) showed a lower amount for 'Picual'. By carefully reviewing the results of these authors, it can be seen that, in the case of the 'Picual' variety, the amount of ethanol found at the last sampling point was 5 times higher than at the penultimate point while, for 'Arbequina', this increase was only of 3 times. Therefore, taking into account that the increase in ethanol is faster at the end of ripening, we can assume that if the MI of those samples were similar to ours, the ethanol values would tend to get closer as happened in our study.

Concerning methanol, its levels also varied significantly among cultivars, ranging from 23.5 mg kg^{-1} for 'Empeltre' to 180.6 mg kg^{-1} for 'Argudell' with a low dispersion of the results obtained for each cultivar (less than 7%). In this case, the application of hierarchical cluster analysis showed three groups (Figure 3). The first group contained the varieties with the highest methanol content, above 140 mg kg^{-1} ('Picudo', 'Morrut', 'Hojiblanca', 'Cornicabra' and 'Argudell'), the second group included the varieties having medium methanol content, between 74 and 94 mg kg^{-1} ('Marfil' and 'Arbequina'), and the third group contained the rest of cultivars, with methanol levels below 60 mg kg^{-1}.

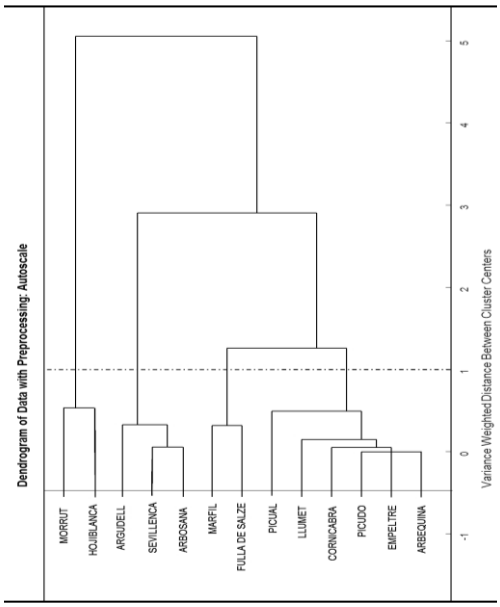

Figure 2. Hierarchical cluster for ethanol

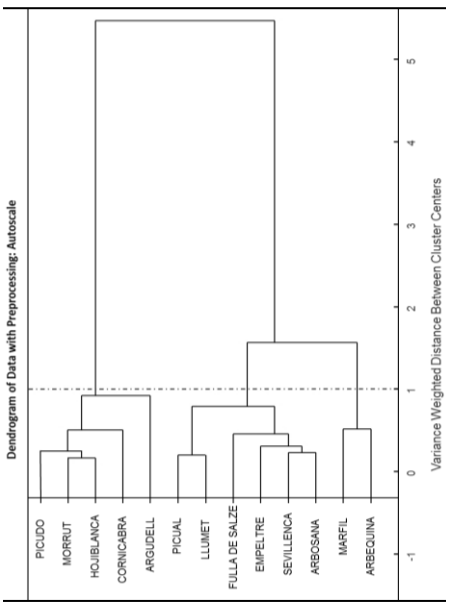

Figure 3. Hierarchical cluster for methanol

As it can be seen, in some cases such as 'Hojiblanca', 'Morrut', 'Picudo' or 'Cornicabra', a high amount of ethanol coincided with a high amount of methanol but, statistically speaking, there is no relationship between the concentrations of these two alcohols. The most obvious example of this lack of correspondence was observed in 'Argudell' behaviour since, for this variety, the higher methanol content corresponds to one of the lowest ethanol concentration values. In addition, as with ethanol, no correlation was found between methanol concentrations and physical parameters, so the different concentrations found are related to the variety.

Finally, for acetaldehyde, it can be seen that the amounts were much lower than the ones for ethanol and methanol. This finding is the expected one as acetaldehyde is reduced to ethanol by the enzyme ADH from 13 to 25 weeks after flowering and, considering the sampling data (December 2017), most of the acetaldehyde amount had been already consumed. No relationship between acetaldehyde and ethanol contents was observed, but the presence of low amounts of acetaldehyde in all samples suggests that the ethanol found might be derived from acetaldehyde by a natural physiological process from the healthy fruits. Depending on their acetaldehyde content, the olive varieties studied can be grouped in three different groups (Figure 4).

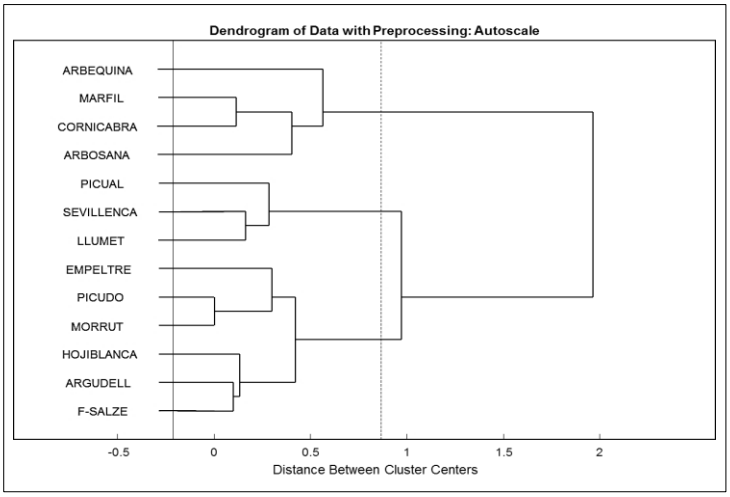

Figure 4. Hierarchical cluster for acetaldehyde

All these results were also reviewed by considering the two trees of each cultivar separately, as it can be seen in Figure 5, where ethanol and methanol for the two trees of each cultivar were graphically represented. It can be observed that results are very consistent. In fact, both trees of each cultivar have similar behaviour, except 'Morrut' variety which, moreover, shows the

highest ethanol concentrations. Acetaldehyde data are not shown in Figure 5, as the scale used is too large to see differences in such small concentrations.

Other values considered were the ratios between the analytes studied (table 2). Since ethanol and methanol are emitted by ripening fruit (19), the EtOH/MeOH ratio in fruits, and specifically in olives, can be considered a quality index because its value will mainly increase when the ethanol is above its natural concentration mainly due to fermentative processes. However, as shown in Table 2, this is a parameter highly depending on the olive variety. For example, the 'Empeltre' variety, even with healthy olives, will always show high EtOH/MeOH ratio values because it is a variety that has a very low MeOH content. The opposite case would be the 'Argudell' variety, with such high amounts of MeOH that, even when analysing damaged olives, the value of the EtOH/MeOH ratio will be very low.

Since in the present study the olives analysed were healthy and the ethanol origin could not be due to any spoilage, the EtOH/MeOH ratio was studied from another point of view. Concretely, we attempted to use this ratio as an index to predict whether the esters that will predominate in the final olive oil will be fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs). Thus, EtOH/MeOH >1 implies a probable greater proportion of FAEEs in the final oil and EtOH/MeOH <1 implies a probable greater proportion of FAMEs. By looking at the results, only 'Empeltre' is clearly over 1, whereas the rest of varieties resulted in ratios closer or lower than 1. Therefore, we could conclude that the majority of olive varieties will present a higher content of FAMEs than of FAEEs, which coincides with studies already published (8, 20).

In the other hand, the ratio acetaldehyde/ethanol (AA/EtOH) was proposed as an indicator of fruit anaerobic respiration occurring in ripen and over-ripen fruits, due to a reduction of the mitochondrial activity that implies a decrease of ADH activity. Table 2 shows the AA/EtOH ratios obtained; and it can be seen that this ratio is lower than 0.6 in all cases, value similar to the one reported by Beltran et al. (11) when dealing with ripe olives. For each individual variety we found that 'Arbosana', 'Argudell', 'Marfil' and 'Sevillenca' show a ratio higher than 0.2 (low metabolic activity) whereas the rest of varieties show a ratio lower than 0.2 (high metabolic activity).

In agreement with previous studies (21), from all these results it can be concluded that alcohols are clearly related to the olive cultivar when dealing with ripe healthy fruits. In fact, when looking at R-square values (%) (table 2), the cultivar accounts for 90% of the total variability for ethanol and 78% for methanol and acetaldehyde. Furthermore, the olive cultivar explains

more than 90% of the total variability for EtOH/MeOH ratio and total studied volatile compounds.

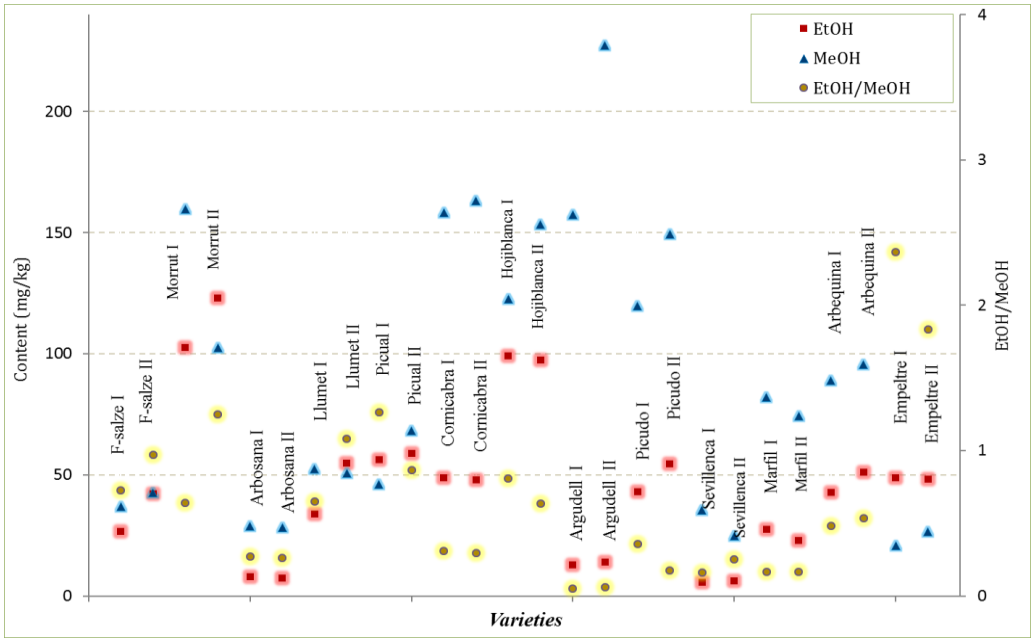

Figure 5. Representation of the individual results of the amounts of ethanol, methanol and the ratio ethanol/methanol (EtOH: Ethanol, MeOH: Methanol)

As discussed above, unhealthy fruits were processed separately and analyzed as described in the methodology to detect any possible abnormal behaviour. The chromatographic results were compared to those provided only by completely healthy olives, i.e. olives showing zero defects (the previously so - called “healthy olives” with defects <10%, were not included). In this way, we intended to assess that the differences, if any, were due solely to the health state of the olives. When comparing the alcohol content values obtained, no conclusive results were obtained. This was because the varieties that provided completely healthy olives did not match the varieties that gave damaged olives. For example, within the "unhealthy group” there were ‘Argudell’ olives that, naturally, have very low amounts of EtOH so, even when these olives were damaged (and their content in EtOH increases (22)), they still provided low values of EtOH. Contrary, within the “completely healthy group” there were ‘Hojiblanca’ olives that naturally present very high values of EtOH. Thus, to make a comparison possible, we evaluated the results obtained from varieties of which we had both completely healthy and unhealthy olives. Table 3 reports the average concentration values of ethanol, methanol and acetaldehyde

in 'Morrut' and 'Empeltre' varieties. It can be seen that both varieties show significant differences for ethanol: there is an increase of 43% (39 mg kg^{-1}) and 23% (10 mg kg^{-1}) of its content in damaged fruits compared to healthy fruits of 'Morrut' and 'Empeltre', respectively. However, there are not significant differences regarding both methanol and acetaldehyde contents of healthy and unhealthy olives. It should be noted that the average content for these two compounds (table 3) does not differ from the average values shown in Table 2 for these two varieties. Therefore, methanol and acetaldehyde contents in the so-called "healthy olives" (with less than 10% of defects) are at the same range level as the completely healthy and unhealthy ones.

Regarding the ratio EtOH/MeOH, it shows significant differences because of the significant differences of ethanol contents. As expected for individual varieties, there is a clear increase of the EtOH/MeOH value for the unhealthy fruits in both 'Morrut' and 'Empeltre', so in this case the ratio can be considered a good quality index.

Table 3: Comparative test on the mean values of ethanol, methanol and acetaldehyde contents for 100% healthy and unhealthy fruits from MORRUT and EMPELTRE varieties

Variety		Ethanol (mg kg^{-1})	Methanol (mg kg^{-1})	Acetaldehyde (mg kg^{-1})	EtOH/MeOH*
MORRUT	Completely healthy fruits	90.1 ± 5.7 a	124.6 ± 1.6 b	3.6 ± 0.4 a	0.72 ± 0.05 a
	unhealthy fruits	129.8 ± 3.7 b	132.6 ± 4.8 a	3.8 ± 0.3 a	0.98 ± 0.02 b
EMPELTRE	Completely healthy fruits	43.8 ± 0.4 A	25.4 ± 0.5 A	3.2 ± 0.1 A	1.86 ± 0.05 A
	unhealthy fruits	53.9 ± 1.8 B	22.6 ± 1.6 A	3.1 ± 0.1 A	2.37 ± 0.19 B

By column, means with the same letter are not significantly different according to Tukey Grouping (P<0.05).
** EtOH: Ethanol; MeOH: Methanol*

Finally, when analysing the overall data, it can be concluded that some healthy olive varieties provide an alcohol concentration (table 2) much higher than the values provided by unhealthy olives from other varieties (table 3). This behaviour proves that a high content of short alcohols is not always due to an unhealthy or poor state of the fruits. Hence, as these alcohols are the precursors of FAEEs, a unique value of FAEEs for all olive varieties should not be used. The olive variety has to be considered as it is closely related to this parameter.

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Chapter 5 – (PAPER 2)

Alcohol formation during short-term storage of 'Arbequina' fruits and their relationship with olive oil quality losses

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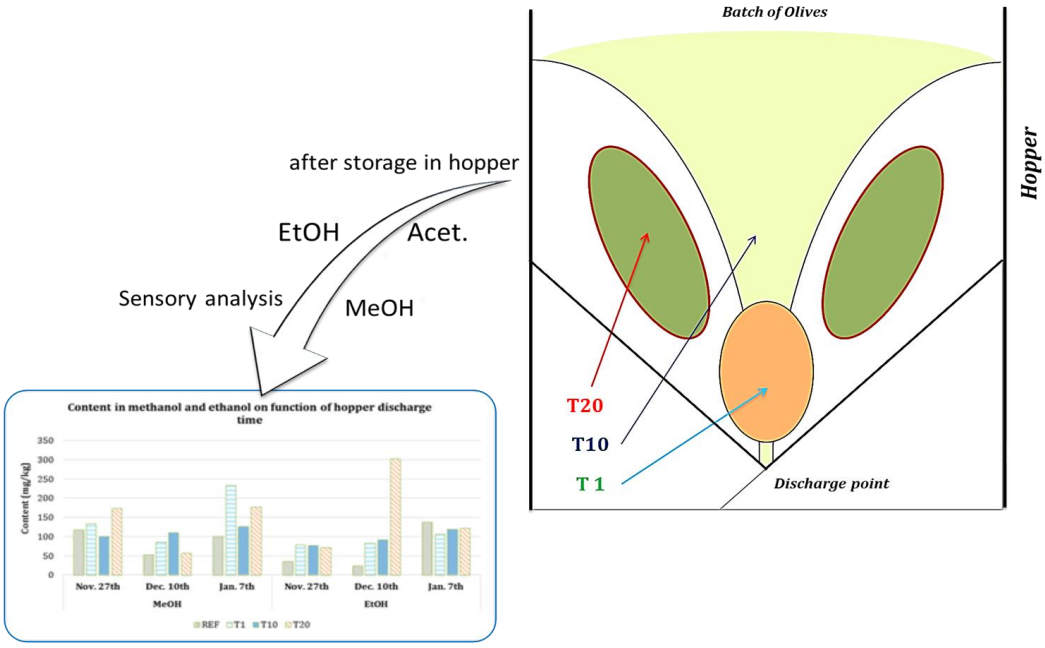
after storage in hopper
EtOH
Acet.
Sensory analysis
MeOH
Content in methanol and ethanol on function of hopper discharge time
Content (mg/kg)
350
300
250
200
150
100
50
0
Nov. 27th
Dec. 10th
Jan. 7th
Nov. 27th
Dec. 10th
Jan. 7th
MeOH
EtOH
REF
T1
T10
T20
Batch of Olives
Hopper
T20
T10
T 1
Discharge point

ABSTRACT

The quality of virgin olive oil (VOO) is strongly related to the quality of the processed olives, which implies a careful and good management of the olives from harvest to processing. However, large capacity hoppers are commonly used for the storage of olives, especially in large mills, due to the daily supply of a great quantity of olives. To ensure the use of good quality fruits during VOO processing a new quality parameter (content of fatty acid ethyl esters) has been recently proposed.

Therefore, the aim of this study has been to evaluate the effect that large hoppers have on the quality of olives but also on the organoleptic quality of the olive oil obtained.

Specifically, ethanol, methanol (precursors of alkyl esters) and acetaldehyde (precursor of ethanol) were measured in the flesh of the samples taken at different times throughout the hopper discharge, corresponding to T1 (1 min), T10 (10 min) and T20 (> 20 min). The organoleptic assessment was carried out on the oils obtained from each sample using the ABENCOR system. The experiment was repeated in three different harvest periods (Nov 27th, Dec 10th and Jan 07th).

Results showed that while the ethanol content dramatically increased during fruit storage, no significant differences were observed for the contents of methanol and acetaldehyde. Moreover, the organoleptic evaluation showed an increase in some negative descriptors such as ``winey´´ and ``fusty´´. Regarding the different positions into the hopper, as expected the fruits located at the bottom of the hopper (T1) suffered a greater effect. As for the hopper discharge, it was observed that it was done concentrically from the center towards the sides of the hopper wall, so the samples here located were the last fruits to be downloaded and showed the lower quality. The negative effect of the hopper was more evident when olives had a higher moisture content or a higher flesh to pit ratio, due to a greater sensitivity to mechanical impacts and microbiological deterioration.

Keywords: hopper, short-term storage, alcohols, quality, olives

1. Introduction

The olive oil quality directly depends on the fruit characteristics and their quality status before the milling process. Therefore, it is influenced by many agronomic, harvesting and processing factors (Mele et al., 2018). Control over these different steps is crucial, but because they are not independent of each other, it is also necessary to make sure that the transition from one step to the next is also controlled. To prevent the physic and biological deterioration of the olive fruits between harvest and processing, some strategies have been proposed, such as keeping the olives at low temperatures or in perforated boxes of less than 1000 kg capacity until processing (García et al., 2006; Kalua et al., 2007; Brkić et al., 2020). Currently, industrial practices are based on the reduction of the time elapsed between the harvest and the milling process, and the main recommendation is to increase the milling capacity to process the olives in a period of time not exceeding 24 hours (de Toro et al., 2002) or even below 12 h (Vichi et al., 2015). However, when this is not possible and the volume of fruit exceeds the capacity of the mill, the olives are stored for a longer period in large capacity hoppers, which can become a serious problem. During this storage period, the fruits may undergo fermentation, pressure and heat, which provide a medium for the growth of fungi and bacteria (Olias and Garcia, 1997; Vichi et al., 2009). Moreover, the deterioration of the olives during storage caused by both aerobic and anaerobic processes increases the acidity of the oil (Garcia et al., 1996) and promotes the production of alcohols and other compounds (Vichi et al., 2015), with the subsequent sensory impact on the final product.

The sensory quality is a very important parameter when dealing with olive oil as this attribute conditions the classification of the oil in different categories. Thus, extra virgin olive oil (EVOO), the highest quality olive oil, must have some positive sensory flavors and no sensory defects. Therefore, while it is important to enhance the formation of pleasant aromas, it is even more important to avoid the formation of unpleasant ones. Sensory olive oil defects like winey-vinegary, fusty, muddy and musty are not due to a single compound but to the combination of different volatile compounds (Procida et al., 2005; Angerosa et al., 2004; Morales et al., 2005; Aparicio et al., 2012). Therefore, the winey-vinegary defect is due to an aerobic fermentation process in the olives which leads to the formation of acetic acid, ethyl acetate, ethanol and, to a much lesser extent, methanol. The fusty perception is mainly due to the presence of ethanol together with octane, ethyl acetate and methanol. Regarding to the muddy defect, the greatest contribution is from ethanol, although the smaller contribution from butyl-formate, ethyl-acetate, 2-butanol, 1-propanol and ethyl-propionate must also be taken into account (Cayuela et

al., 2015). Finally, the musty defect is related with ethanol and hexanal followed by methanol, propanal, pentanal and propyl-propionate.

Although monitoring the generation of these bad flavors would be very interesting as a quality control of olive oil, the regulations do not set limits for these compounds. However, it does limit the amount of fatty acid alkyl esters (FAAEs), which originate as a result of the esterification of free fatty acids with short-chain alcohols (mainly ethanol and methanol) (Perez-Camino et al., 2008; IOC, 2015; Gómez-Coca et al., 2012). Therefore, to comply with the maximum content of FAAEs legislated, it is necessary to avoid the formation of these precursors throughout the entire production process, starting with the olive itself.

In Spain, most of the oil mills have a high capacity so, many times, olives are stored in hoppers of between 30 and 40 tons. Regarding medium to small mills, these also use big hoppers as this takes up less space in the building. In these conditions, the fruits are under pressure and, when the olives are very ripe, there is a risk of squeezing them (Di Giovacchino, 2013). It is assumed that only the olives at the bottom of the hopper can be damaged, while the rest of the fruit maintains a good quality. Nevertheless, no experimental results of any study under real conditions are available to confirm this statement. The only related study is from Vichi et al. (2009), who reported a significant increase in the winey and musty defects and volatile phenols during fruit storage in bags, and they concluded that the 'Arbequina' variety is more susceptible to postharvest damages than others like 'Arbosana' or 'Leccino'.

The fact that ethanol and methanol are involved in both sensory defects and FAAEs formation makes them good markers for checking the possible adverse effect of storing olives in large hoppers. The aim of the present research was to figure out how the short-term storage in a big hopper of 'Arbequina' olives, harvested at different ripening stages, affects alkyl alcohols formation in the fruits and produce sensory defects in the oil.

2. Materials and methods

2.1. Experiments

The experiments were carried out in a real mill (Cooperative of La Granadella, Lleida) and processing olives of the 'Arbequina' variety. In order to compare the results between different states of fruit maturity, the experiment was repeated on three different days (November 26th, December 12th and January 07th), corresponding to green, ripe and over-ripe fruits. Table 1 shows the mean values of the physico-chemical properties of the olives. On each date, the experiments were carried out using batches of 22,000 kg olives, harvested and processed the

same day and no later than three hours after delivery. The mill hopper had a capacity of 25,000 kg of fruit (with the dimensions 4 m x 4 m x 2.5 m); it was made of stainless steel and equipped with a vibrating system to download the olives from bottom of the hopper.

2.2. Sampling

Olive samples were taken during the downloading of the hopper (just as the olives fell from the bottom of the hopper) at three different times: 1 minute (T1), 10 minutes (T10) and 20 minutes (T20). At each time, a 1 kg olive sample was taken in small 3 kg perforated boxes. The downloading rate was 4000 kg h^{-1} and the sampling times are equivalent to the times that the olives remain in the hopper depending on the height at which they are inside the hopper (T1 at the base of the hopper, T10 in the middle of the hopper and T20 in the top of the hopper).

The samples were transferred directly to the laboratory and each one was split into three parts. With the first part, a visual diagnosis was made to determine the fruit characteristics (maturity index, health status, fruit weigh, flesh/pit ratio, moisture and oil content). The bruised, smashed, broken and fermented fruits were counted as damaged fruits. The second part was used to determine the ethanol and methanol content in olive fruit homogenates, following the method described in Boudebouz et al. (2020). Briefly, 100 g of olives were crushed and well homogenized, and then 15 g of the olive paste were weighed in a 50 mL falcon tube together with 15 g of MilliQ water and well mixed using a vortex stirrer. Two grams of this homogenate were weighed in a 20 ml vial together with two grams of a saturated solution of $CaCl_2$ in water (10 %) and were kept in a freezer (-18 ºC) until its analysis by headspace solid phase micro-extraction gas chromatography (HS-SPME GC-MS). Finally, third part of olives was processed using ABENCOR system to obtain representative oil from each sample. The olives were crushed and the olive paste was malaxed at 24 ºC during 30 minutes, and then centrifuged to obtain the oily fraction. In order to clarify the oil got from the previous step, a second high speed desktop centrifuge (KUBOTA Model, Japan) was used. The obtained oil samples were filtered to eliminate any residual impurities and sent to the tasting panel for their sensorial analysis.

To evaluate the hopper effect over the olive fruits, the results from each sampling were compared with those belonging to the so-called reference olive samples (REF). To obtain them, small quantities of olives were taken from each of the different batches as soon as arrived at the oil mill by using the scale's aspirator-sampler. These were stored under ambient conditions, next to the hopper and in small perforated boxes of 3 kg until the end of the experiment. This

sampling was carried out throughout the entire hopper loading process to ensure a representative reference sample of all partial inputs of olives to the hopper.

2.3. Chemicals and reagents

The standards of ethanol absolute (gradient HPLC grade) and methanol (supragradient HPLC grade) were purchased from Scharlab (Barcelona, Spain). Acetaldehyde standard (99 % for synthesis) and calcium chloride anhydrous (97 %) were purchased from Panreac (Barcelona, Spain). Milli-Q quality water used was obtained from a laboratory purification system (Millipore, Bedford, USA).

For the Headspace-Solid Phase Microextraction (HS-SPME) of the analytes, 2 cm length fibers 50/30 μm StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) purchased from SUPELCO (North Harrison Road -Bellefonte, PA - USA) were used.

2.4. Analytical procedure

The quantitative determination of the analytes (ethanol, methanol and acetaldehyde) in each sample was done in triplicate by using the method previously optimized (Boudebouz et al., 2020). It consists in applying the solid phase micro-extraction technique to the headspace of the samples (HS-SPME) with subsequent analysis of the extract by gas chromatography coupled to a quadrupole mass spectrometer (GC-MS).

Thus, the vials with the samples were preconditioned for 5 min at 40 ºC and, afterwards, the SPME fiber was inserted through the vial septum and exposed to the headspace above the sample for 50 min at 40 ºC with medium orbital agitation. Then, the fiber was removed from the vial and introduced directly into the injector port of the GC-MS for thermal desorption at 270 ºC for 1 min in the splitless mode.

The chromatographic analyses were performed with an HP-6890 gas chromatograph (HP, Palo Alto, CA, USA) equipped with an HP-5973 mass selective detector (HP, Palo Alto, CA, USA). The chromatographic separations were carried out using a fused silica capillary column Chromapack, CP-WAX 57CB (50 m x 0.25 mm i.d. and 0.2 μm film thickness) (Varian, Middelburg, The Netherlands) with an oven temperature program of: 40 ºC (5 min), 5 ºC min^{-1} to 100 ºC and 10 ºC min^{-1} to 215 ºC (5 min). The carrier gas was helium (He), with a head pressure of 14.8 psi at a constant flow of 1.8 mL min^{-1}. The mass spectrometer operated in the electron impact ionization mode at 70 eV. Interface, ion source and mass quadrupole temperatures were 200 ºC, 230 ºC and 150 ºC, respectively. The mass-to-charge (m/z) ratio range used was 28-300 amu. The compounds identification was done by spectrum matching with the Wiley/NBS library.

For the quantification of the studied analytes, the calibration lines were built by using the matrix-matched calibration technique. As explained in previous studies (Boudebouz et al., 2020), this technique makes it possible to avoid quantitation errors due to the matrix effect. The calibration standards ranged between 0–200 mg kg^{-1} for both ethanol and methanol and between 0–15 mg kg^{-1} for acetaldehyde. The regression lines showed determination coefficients R^2 > 0.96 in all cases.

2.5. Sensory evaluation

The sensory evaluation of the different olive oil samples was carried out by the Official Tasting Panel of Virgin Olive Oils of Catalonia (Reus, Spain), which has been recognized by the IOC since 1997 and by the Spanish Government since 2004. The panel follows the ISO 17025 standard since 2002. Join with the main profile sheet described by the official method, an expanded profile was used for this study, since the storing time was planned to be very short and only slight differences at sensorial level were expected (Romero et al., 1999; Guerero et al., 2001). Each sensorial attribute was measured by eight trained tasters using an open scale of 10 cm anchored on zero. The median of the eight tasters was used to describe the final intensity of each attribute. In addition, they were computed two new descriptors from the final results, according with Romero et al. (1999); complexity that is defined as the number of secondary aroma detected by more than 30 % of the assessors and global sensory score which is a value from 0 to 9 (0, very bad quality; 9, highest quality) using an algorithm developed by Romero et al. (1999). The global sensory score facilitates the comparison of the sensory quality of different samples. As a reference, global sensory scores for olive oils within the extra-virgin category should be at least 6.5 points.

2.6. Statistical analysis

Statistical analysis was carried out using the SAS-Stat Software (V9.4.Cary, SAS Institute Inc.) and the Unscrambler X software (v10.5.1, Camo Software AS). The effect of the hopper downloading time was analyzed by ANOVA (Analysis of Variance) using the Generalised Linear Model (GLM) procedure, and mean comparisons were performed by using the Duncan´s multiple range test ($\alpha < 0.05$). A Partial Least Squares (PLS) regression model was built between the predictor X variables (fruit characteristics and sensory attributes) and the predicted Y variables (EtOH, MeOH and acetaldehyde values) for the samples studied, to detect groupings and correlations between samples and variables. Prior to PLS regression, data were

autoscaled (column mean centered and standardized) to account for the scale differences between the variables.

3. Results and discussion

3.1. Fruit characteristics

The results of the visual diagnostic of olives at different hopper downloading times (Figure 1) show an important deterioration of the quality of fruits after passing through the hopper compared to the reference samples. Olives were damaged mainly due to the impacts that happen during the uploading and downloading of the hopper, in agreement with what was pointed out in other studies (Di Giovacchino, 2013; Mele et al., 2018).

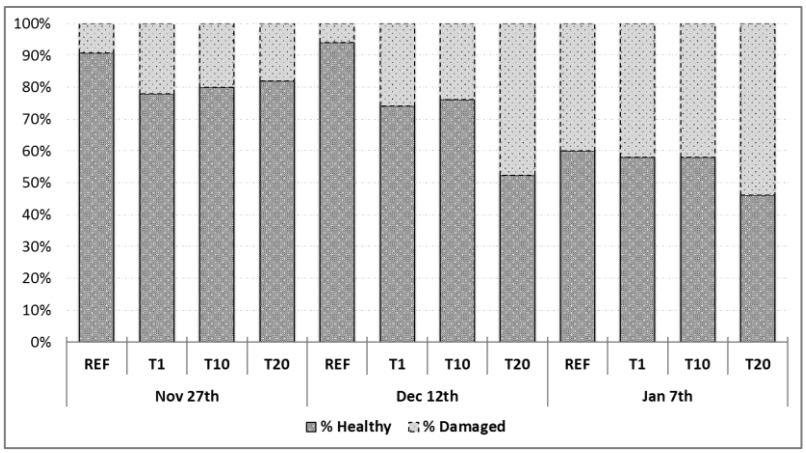

Figure 1. Representation of the visual diagnostic of olive fruits in different positions during hopper downloading

Olives entered in November 27th (first sampling date) showed 12 % of damaged fruits (table 1); the maturity index was 2.4 (yellow to reddish) and they were quite small (1.08 g), with a low flesh to pit ratio (4.1 g/g) and with high moisture and oil contents. After staying 3 hours in the hopper, visual damages increased from 12 to 18 % and so did the temperature that raised from 9 to 12 ºC meaning that some microbiological activity happened in the hopper.

On the other hand, olives delivered by growers on December 12th, (second sampling date) looked very healthy with only 6 % of damages (table 1); the maturity index (MI= 2.5) was very similar to the previous sampling, whereas fruits were larger (1.28 g) with higher flesh to pit ratio (5.1 g/g) and a similar moisture and fat contents. However, after staying 3 hours in the hopper, these fruits suffered more damages than in November, raising form 6 % to a maximum of 48 % with a temperature increase from 7 to 12 ºC. An explanation for such differences could

be the fact that, in this batch, fruits were larger with a higher flesh to pit ratio and a little more moisture content and the combination of all these factors could make the fruits more sensitive to the impacts that happen during post-harvest. In addition, the T20 sample, which corresponds to the olives located on the sides of the hopper, showed the worst fruit quality because these hopper parts are where the olives are exposed to higher bruise damage.

Finally, olives entered on January 7th (third sampling date) showed a very significant deterioration due to freezing issue in the orchards. This affectation implies the breakdown of the cell wall that facilitates the fermentation of the olive fruit with the loss of quality that this entails. In fact, up to 40 % of the fruits delivered by growers were damaged; they were quite small (1.03 g), with a maturity index from purple to black (MI= 3.5) and a very low flesh to pit ratio, related with a lower moisture content, consequently the fat content was very high. After staying 3 hours in the hopper, the proportion of damaged fruits increased from 40 to a maximum of 54 % while the temperature passed from 5 to 10 ºC.

Table 1. Ethanol and methanol contents (mg/kg) measured in olive samples on different positions (REF, T1, T10 and T20) during hopper downloading

Sample		% Healthy	% Damaged	Olives TºC	MI	Fruit weight (g)	Flesh/pit ratio	Moisture (%)	Fat content (%)
Nov 27th	**REF**	88	12	9	$2.4^{b} \pm 0.2$	1.08^{b}	$4.1^{b} \pm 0.3$	$53.1^{a} \pm 1.2$	$21.9^{a} \pm 1.6$
	T1	82	18	11					
	T10	82	18	12					
	T20	80	20	12					
Dec 12th	**REF**	94	6	7	$2.5^{b} \pm 0.2$	1.28^{a}	$5.1^{a} \pm 0.8$	$54.1^{a} \pm 2.3$	$21.5^{a} \pm 1.8$
	T1	76	24	10					
	T10	74	26	12					
	T20	52	48	12					
Jan 7th	**REF**	60	40	5	$3.5^{a} \pm 0.3$	1.03^{b}	$3.4^{b} \pm 0.4$	$49.4^{b} \pm 1.6$	$23.7^{b} \pm 1.1$
	T1	58	42	6					
	T10	58	42	7					
	T20	46	54	10					

By column, means with the same letters are not significantly different according to Duncan´s multiple range tests (P<0.05)

Thus, in the three harvest moments, fruits suffered microbiological damages in the hopper, even in the short-term of only three hours. Contrary to what it was expected, in the three cases the olives downloaded the last (T20) were of worst quality. This can be explained by the fact that such big hoppers download fruits from the central part faster than from laterals, were friction with fruits is higher. As a consequence, at the end of the downloading process they get out fruits

that have been in contact with the walls of the hopper and that suffered high pressure from upper layers and they are visually damaged.

3.2. Tasting panel results

As expected, the olive oil sensory quality was related to the quality of the olive fruits. In fact, in all cases, the oils obtained from the reference samples that did not pass through the hopper showed the best sensory profile. It must be pointed out that the first two experiments resulted in oils without any defect. Nevertheless, in the experiment of January 7th, the olives delivered by the growers were of very low quality and clearly damaged by freezing. In agreement with this, the tasters identified the frost defect in the oil extracted from these olives (table 2). Surprisingly, the frost defect resulted more intense after staying three hour in the hopper. This could be due to the fact that fruitiness decreased as well, and when this happens any aromatic defect is perceived as more intense because fruitiness use to overlaps other aroma in the oil.

When olives were stored in the hopper, even for a short time, suffered a degradative process that resulted in a loss of oil quality. This can be easily verified using the global score (table 2) that decreased from 7.2 to 5.9 in the first experiment (Nov 27th) and from 7.6 to 5.4 in the second experiment (Dec 12th). In the last experiment, very slight differences were observed because of the initial low quality of the fruits, though the trend is to decrease too.

Taking into account the distribution of the charge in the hopper, the higher pressure and load are exerted at positions T1 and T10. In fact, though the median of all the defects was equal to zero in the first experiment (Nov 27th), the solid standard deviation implies that some tasters detected some kind of fustiness, mustiness and winey smell at T1 and T10 positions, which are mainly due to a fermentation process (Cayuela et al., 2015). In the second experiment (Dec 12th), the worst condition seems to be the T20 position, with musty, fusty and winey defects higher than zero that matches with the higher level of damaged fruits (table 1). Winey was identified as well in T1 sample. Concerning the musty defect, it must be pointed out that this descriptor does not distinguish between molds and yeasts. In sample T20 from the second trial, it can be observed that bitterness, pungency and astringency significantly decreased, that could be due to yeasts that can depredate polyphenols delivering volatile phenols that are classified as musty by tasters (Vichi et al., 2009).

Table 2. Results of the tasting panel (ISO17025) of the oils obtained from each olive samples

	Attributes	NOV 27th				DEC 12th				JAN 7th			
		REF	T1	T10	T20	REF	T1	T10	T20	REF	T1	T10	T20
negative attributes	Fusty	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.8$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{b}\pm0.0$	$0.0^{b}\pm0.0$	$0.0^{b}\pm0.0$	$0.7^{a}\pm0.6$	$0.2^{a}\pm0.0$	$0.2^{a}\pm0.0$	$0.3^{a}\pm0.0$	$0.3^{a}\pm0.0$
	Musty	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.2$	$0.0^{a}\pm0.1$	$0.0^{a}\pm0.0$	$0.0^{b}\pm0.0$	$0.0^{b}\pm0.0$	$0.0^{b}\pm0.0$	$1.9^{a}\pm0.8$	$0.2^{a}\pm0.0$	$0.5^{a}\pm0.1$	$0.4^{a}\pm0.1$	$0.3^{a}\pm0.0$
	Winey	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.3$	$0.0^{a}\pm0.1$	$0.0^{a}\pm0.0$	$0.0^{b}\pm0.0$	$0.1^{ab}\pm0.3$	$0.0^{b}\pm0.0$	$0.5^{a}\pm0.4$	$0.0^{a}\pm0.0$	$0.5^{a}\pm0.2$	$0.0^{a}\pm0.0$	$0.3^{a}\pm0.0$
	Frost Olive	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$1.6^{a}\pm1.1$	$3.8^{a}\pm0.7$	$2.7^{a}\pm0.4$	$2.8^{a}\pm1.4$
	Rancid	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$
	Others	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.4$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.3$	$0.0^{a}\pm0.3$	$0.0^{a}\pm0.4$	$0.0^{a}\pm0.1$	$0.0^{a}\pm0.8$
positive attributes	Fruity	$4.8^{a}\pm0.2$	$3.7^{b}\pm0.4$	$3.6^{a}\pm0.4$	$4.3^{ab}\pm0.2$	$5.1^{a}\pm0.2$	$4.9^{a}\pm0.2$	$4.8^{a}\pm0.2$	$3.2^{b}\pm0.6$	$3.1^{a}\pm0.6$	$2.5^{a}\pm0.6$	$2.9^{a}\pm0.6$	$2.8^{a}\pm0.8$
	Bitter	$3.6^{a}\pm0.2$	$2.7^{b}\pm0.2$	$3.4^{ab}\pm0.4$	$3.6^{a}\pm0.2$	$4.2^{a}\pm0.3$	$4.2^{a}\pm0.2$	$4.3^{a}\pm0.3$	$2.1^{b}\pm0.4$	$2.1^{b}\pm0.4$	$3.2^{a}\pm0.5$	$2.7^{ab}\pm0.3$	$2.6^{ab}\pm0.5$
	Pungent	$4.4^{a}\pm0.2$	$4.2^{a}\pm0.3$	$4.5^{a}\pm0.2$	$4.5^{a}\pm0.2$	$4.7^{a}\pm0.2$	$4.8^{a}\pm0.2$	$4.6^{a}\pm0.3$	$3.7^{b}\pm0.2$	$3.2^{a}\pm0.4$	$3.6^{a}\pm0.3$	$3.4^{a}\pm0.4$	$3.5^{a}\pm0.4$
	Green	$2.7^{a}\pm0.4$	$2.0^{a}\pm0.9$	$2.3^{a}\pm0.2$	$2.4^{a}\pm0.1$	$3.7^{a}\pm0.1$	$3.7^{a}\pm0.4$	$3.2^{a}\pm0.3$	$1.2^{b}\pm0.6$	$1.2^{a}\pm0.5$	$1.1^{a}\pm0.7$	$1.1^{a}\pm0.7$	$1.3^{a}\pm0.7$
	Sweet	$4.3^{a}\pm0.1$	$4.6^{a}\pm0.1$	$4.0^{a}\pm0.3$	$4.4^{a}\pm0.2$	$4.3^{a}\pm0.2$	$4.2^{a}\pm0.3$	$4.1^{a}\pm0.6$	$4.7^{a}\pm0.3$	$5.1^{a}\pm0.2$	$4.6^{a}\pm0.1$	$5.1^{a}\pm0.1$	$4.8^{a}\pm0.2$
	Astringent	$2.2^{a}\pm0.1$	$1.1^{b}\pm0.6$	$1.8^{ab}\pm0.4$	$1.8^{ab}\pm0.3$	$2.1^{a}\pm0.4$	$2.1^{a}\pm0.4$	$1.9^{a}\pm0.4$	$0.2^{b}\pm0.2$	$0.4^{a}\pm0.1$	$1.1^{a}\pm0.8$	$0.9^{a}\pm0.4$	$0.6^{a}\pm0.4$
	Almond	$2.3^{a}\pm0.2$	$1.8^{a}\pm0.4$	$1.1^{a}\pm0.7$	$1.7^{a}\pm0.6$	$2.7^{a}\pm0.1$	$2.3^{a}\pm0.2$	$2.4^{a}\pm0.4$	$0.0^{b}\pm0.5$	$1.4^{a}\pm0.4$	$1.2^{a}\pm0.8$	$1.1^{a}\pm0.7$	$1.1^{a}\pm0.8$
	Wainut	$1.4^{a}\pm0.8$	$1.0^{a}\pm0.9$	$0.6^{a}\pm0.2$	$1.1^{a}\pm0.8$	$1.7^{a}\pm0.3$	$1.5^{a}\pm0.8$	$1.3^{a}\pm0.7$	$0.5^{a}\pm0.1$	$0.7^{a}\pm0.4$	$0.6^{a}\pm0.2$	$0.6^{a}\pm0.2$	$0.6^{a}\pm0.2$
	Other	$2.3^{a}\pm0.1$	$2.1^{a}\pm0.4$	$1.6^{a}\pm0.8$	$1.7^{a}\pm0.9$	$2.7^{a}\pm0.1$	$2.4^{a}\pm0.2$	$2.1^{ab}\pm0.2$	$1.4^{b}\pm0.6$	$1.6^{a}\pm0.5$	$1.3^{a}\pm0.7$	$1.7^{a}\pm0.6$	$1.6^{a}\pm0.4$
	Complexity	4.0	3.0	2.0	2.0	6.0	4.0	4.0	5.0	2.0	1.0	1.0	1.0
	Global score	7.2	5.9	6.3	6.4	7.6	6.7	7.2	5.4	5.6	5.3	5.5	5.4

By line & bloc, means with the same letters are not significantly different according to Duncan´s multiple range tests (P<0.05)

3.3. Content of alcohols

The results of the analysis of alcohols (ethanol and methanol) show an important increase of ethanol content in the fruits that stayed in the hopper, compared to the reference samples (table 3). In average of the three experiments, its content increased from 71.5 mg kg^{-1} at the reception point to 164.7 mg kg^{-1} at the worst point of the hopper. This increase in ethanol content seems related to a fermentation processes as corroborates the increase in olives temperature as result of this biological process (table 1). During the third experiment, no significant differences were observed due to the initial low quality of the fruits; the olives arrived with high amounts of ethanol (138 mg kg^{-1}), and specifically the fruits were mostly affected by frost

In the two first experiments, when fruits were not heavily affected by frost damage, it was pointed out a significant increase of acetaldehyde during the storage in the hopper. This could be due to a certain degree of physiological activity of the fruits, a part from the microbiological that can result in an extra increase of ethanol (Beltan et al., 2015).

Table 3. Ethanol and methanol contents (mg/kg) measured in olive samples on different positions (REF, T1, T10 and T20) during hopper downloading

	date	REF	T1	T10	T20	Average
Acet.	Nov. 27th	0.7^{a} ± 0.1	1.2^{ab} ± 0.4	1.3^{b} ± 0.2	1.9^{c} ± 0.2	**1.26 A**
	Dec. 10th	0.8^{a} ± 0.2	1.0^{ab} ± 0.1	1.2^{b} ± 0.2	1.9^{c} ± 0.1	**1.01 A**
	Jan. 7th	3.1^{a} ± 0.3	3.3^{a} ± 0.3	3.0^{a} ± 0.6	3.0^{a} ± 0.4	**3.10 B**
average		**1.76 A**	**1.76 A**	**1.78 A**	**1.94 A**	
EtOH	Nov. 27th	36.5^{a} ± 11.2	78.7^{b} ± 3.6	77.0^{b} ± 8.3	71.3^{b} ± 4.5	**65.9 A**
	Dec. 10th	23.7^{a} ± 9.5	83.6^{b} ± 6.3	91.8^{b} ± 4.5	301.3^{c} ± 19.1	**134.3 B**
	Jan. 7th	138.3^{a} ± 11.7	106.6^{a} ± 13.5	118.5^{a} ± 34.4	121.4^{a} ± 17.9	**122.6 B**
average		**71.5 A**	**87.5 B**	**95.8 B**	**164.7 C**	
MeOH	Nov. 27th	66.1^{a} ± 40.4	132.9^{a} ± 52.8	100.8^{a} ± 33.2	174.2^{a} ± 50.7	**123.3 AB**
	Dec. 10th	53.2^{a} ± 4.7	86.6^{a} ± 71.1	110.1^{a} ± 52.4	57.5^{a} ± 36.9	**79.0 A**
	Jan. 7th	100.7^{a} ± 29.8	232.7^{a} ± 135.0	126.9^{a} ± 68.6	176.3^{a} ± 41.5	**159.2 B**
average		**77.3 A**	**150.7 B**	**112.6 B**	**136.0 B**	

By line & bloc, means with the same letters are not significantly different according to Duncan´s multiple range tests (P<0.05)

Olives from T20 in the second experiment stand out for their very high ethanol content. This is in agreement with the highest level of damage observed in the olives (damaged fruits increased by eight times with respect to the delivered fruits by growers) and the sensory defects detected. These results corroborate those published in previous works (Aparicio et al., 2012; Cayuela et al., 2015; Beltran et al., 2020).

Regarding methanol, a certain increase between reference sample and those from the hopper was observed in all the experiments, though it was only statistically significant when the whole data are considered (table 3). It must be pointed out the high variation between replicates observed for methanol in all the samples from the hopper that were not observed either for ethanol or acetaldehyde. This makes difficult to get conclusions about methanol production during a short-term storage, in agreement with similar difficulties observed by other researchers (Boudebouz et al., 2020; Beltran et al., 2015).

Finally, a certain correlation between methanol and acetaldehyde was observed ($r = +0.50$ with $p=0.0025$). This behavior could be due to the fact that, as in other vegetables (Frenkel et al., 1998), methanol can act as an inhibitor of the enzymatic activity of alcohol dehydrogenase (ADH), which causes the reduction of acetaldehyde to ethanol to stop. This theory would explain that methanol and acetaldehyde show the same trend in all experiments (figure 2). Moreover, Frenkel et al. also reported that ethanol can act as an inhibitor of the enzymatic activity of pectin methylesterase (PME), and this could be the reason why, in some cases, the amount of methanol is very low when that of ethanol is high (such as in the case of T20 on Dec 12th). Therefore, the hypothesis that arises from our study is that the greatest increase in ethanol content is due to the fermentation of the olives after being stored in the hopper.

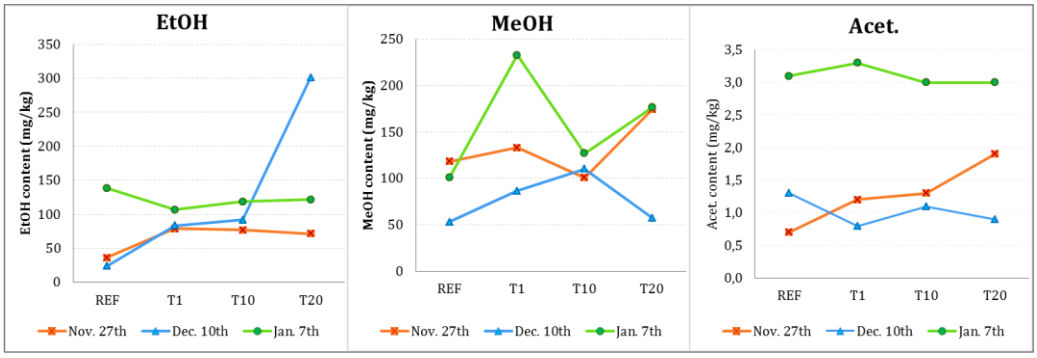

Figure 2. Variations of methanol, ethanol and acetaldehyde (mg/kg) on function of hopper downloading in each experiment

The PLS analysis permits a good explanation of the total variability using two factors (figure 3a). Factor 1 relates positively with fruit damages and oil defects, and negatively related with all the positive attributes detected in good quality olive oil. Factor 2 is related positively with green index, high pulp/pit ratio and ``musty´´ defect, and negatively with ripe index and pit weight (opposite to the pulp/pit ratio).

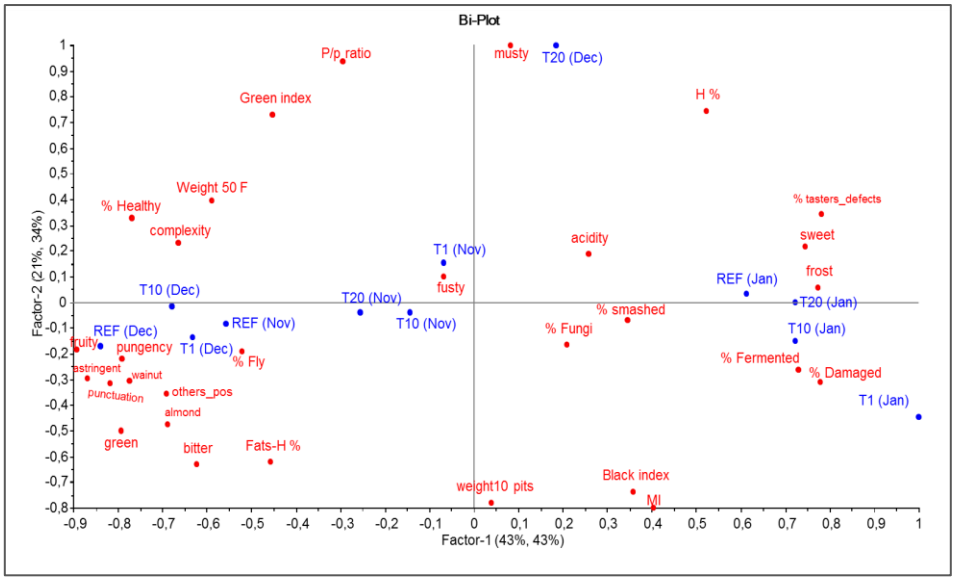

Figure 3a. Bi-plot of the scores and loadings of the PLS regression model using data from Tables 1-3

In addition, PLS highlights the difference between fruits harvested in November or December from those harvested in January. The first two consist of good quality fruits whereas those from January were of lower quality. Furthermore, in all cases reference samples that did not pass through the hopper had better quality.

On the other hand, the PLS correlations (figure 3b) show that ethanol is well correlated with Factor 2 (0.85) and medium with Factor 1 (0.4). In fact, the best predictor variables for ethanol are fruit moisture, sweet, musty defect and acidity on the positive side, and fruit fat content (inverse to fruit moisture) and bitterness. This makes sense because yeast that produce ethanol through sugar fermentation can also add certain ``musty´´ defect to the oil. Besides, yeasts can be more active in a medium with high moisture content. On the other hand, yeast can cause polyphenols degradation as well, with the consequent decrease of bitterness and increase of sweetness (Vichi et al, 2009).

Furthermore, methanol shows a positive correlation with Factor 1 (0.6) and negative with Factor 2 (-0.6). This behavior seems to be independent from the one of ethanol. The better predictors for methanol generation are fermented and damaged percentages, black index and frost defect. On the other hand, green index, and healthy fruits affect negatively on the methanol content. This behavior is in agreement with the hypothesis that methanol origins from fruit ripening process and cell wall degradation, which increases in the damaged fruits.

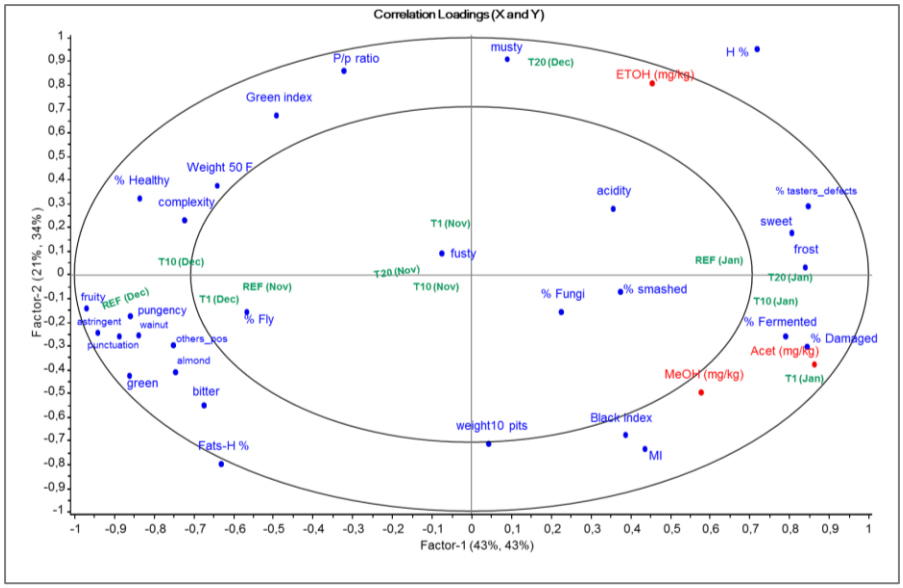

Figure 3b. Correlation loadings of the X-variables (fruit characteristics and sensory attributes) and the predicted Y variables (EtOH, MeOH and Acetaldehyde values)

Concerning acetaldehyde, this is very good correlated with Factor 1 (0.87) and medium correlated with Factor 2 (0.45) and it seems to be more related with methanol behavior than with ethanol. Acetaldehyde was positively correlated fruit damages, fermented fruit percentage, black index and sweet. Moreover, it was negatively correlated with healthy fruits, green index and oil complexity (a marker for high quality olive oil). To explain this behavior, there must be considered two processes. On the one hand, the acetaldehyde is generated from pyruvate pathway and may be, in this case, is more related with the fruit ripe index than with damages. In addition, it could happen that metabolism was more developed in fruits that staid in the orchard for longer time, as it was the case with the samples from January which are on the right of the vectorial space. On other hand, the methanol accumulation can reduce the chemical reduction of acetaldehyde into ethanol.

Furthermore, the positive sensory attributes detected in good quality oils (manly fruity, bitter and pungent) are negatively correlated with the levels of alcohols and acetaldehyde

4. Conclusions

The storage of olives in a big hopper is a widely used practice in olive mills that should be avoided during the processing of virgin olive oil to avoid loss of quality. Olives can deteriorate in quality due to the impacts in the hopper, the high pressure applied by the weight of the olives themselves and certain microbiological activity. This deterioration induces the generation of ethanol and the loss of sensory quality.

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Chapter 5 – (PAPER 3)

Processing factors that affect the balance of alcohols and alkyl esters during 'Arbequina' olive oil production: separation and clarification steps

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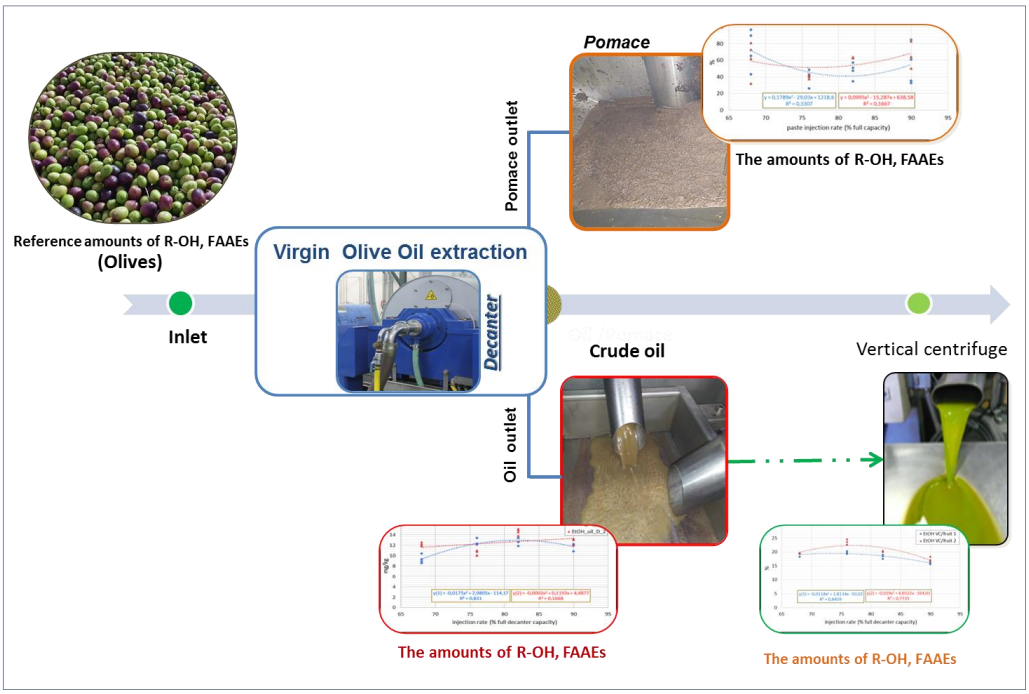
UNIVERSITAT ROVIRA I VIRGILI
EFFECT OF AGRONOMIC AND TECHNOLOGICAL FACTORS ON THE FORMATION OF ETHYL ESTERS IN VIRGIN OLIVE OIL IN CATALONIA
Boudebouz Abdelaziz



ABSTRACT

The aim of this study was to assess how different conditions used on the centrifugation step during olive oil extraction affect its quality by considering the balance of fatty acid alkyl esters (FAAEs) and their precursor alcohols. All the experiments were carried out under real working conditions in a two-phase decanter followed by a vertical centrifuge (VC) and different water injection doses and paste injection rates were tested. The fruits used were from 'Arbequina' variety at two different maturity stages and the balances of alcohols and FAAEs were measured at the outlets of both, decanter and VC, with respect to the system inlet.

Results show that the paste injection rate affects the content of alcohols and FAAEs in the final oil, which tend to increase when working closer to the maximum capacity of the decanter. Different behaviors have been detected when dealing with unripe or ripe fruits. Similarly, the water addition doses have different effects on the FAAEs and alcohols formation depending on the maturity status. Therefore, both the decanter and the step decanter to vertical centrifuge are key points that, when properly controlled, allow minimizing FAAEs formation, which is essential for obtaining quality oils.

Keywords: Virgin olive oil; Centrifugation processing factors; Alcohols; FAAEs; Quality control

1. Introduction

Virgin olive oil (VOO) is the juice of the olive fruits extracted only by physico-mechanical process and when its chemical composition is good enough and it organoleptic evaluation is excellent it is called extra virgin olive oil (EVOO). To maximize the product quality, the olives must be harvested in their optimal health and maturity state and the processing conditions must be controlled (Di Giovacchino, Sestili, & Di Vincenzo, 2002; Masella, Guerrini, Angeloni, Zanoni, & Parenti, 2019). Given that identifying reliable tools that preserves the quality of olive oil when maximizing extraction efficiency is still a challenge, there is growing number of studies related to EVOO processing (Clodoveo, 2012; Fregapane & Salvador, 2013; Jabeur, Zribi, Abdelhedi, & Bouaziz, 2015; Masella et al., 2019; Parenti, Spugnoli, Masella, & Calamai, 2007). Currently, the most commonly used mechanical process for extracting virgin olive oil is the so called "continuous"(Uceda, Jiménez, & Beltrán, 2006), although it is not completely continuous, as it consists of several steps (Fig_1): crusher, malaxer, horizontal centrifugation (decanter) and oil clarification (vertical centrifuge).

Among the different EVOO quality parameters, one of the most studied in recent years has been the content of fatty acids alkyl esters (FAAEs), which includes both ethyl esters (FAEEs) and methyl esters (FAMEs) (Alcalá et al., 2017; Beltrán et al., 2016; Biedermann, Bongartz, Mariani, & Grob, 2008; Di Serio et al., 2017; Gómez-Coca, Fernandes, Pérez-Camino, & Moreda, 2016; Lanza, Di Serio, & Di Giacinto, 2016). Their formation is due to the esterification and/or transesterification of free fatty acids with low molecular weight alcohols such as methanol or ethanol (Costa et al., 2017; Guilaume, Ravetti, Ruiz, & Zaparenkov, 2013; Pérez-Camino, Moreda, Mateos, & Cert, 2002). It is well known that alcohols are present in olive oil (García-Vico et al., 2018; Gómez-Coca, Cruz-Hidalgo, Fernandes, Pérez-Camino, & Moreda, 2014). When their origin is the natural pathway of fruit metabolism (what produces the so-called endogenous alcohols) their concentration depends on maturity, health status and olive cultivars (Beltrán, Bejaoui, Jimenez, & Sanchez-Ortiz, 2015; Boudebouz et al., 2020; García-Vico et al., 2018). However, when their presence is derived from the fermentation of olive sugars during the olive processing, the content of alcohols also depends on the manufacturing practices (Biedermann et al., 2008; Pérez-Camino, Cert, Romero-Segura, Cert-Trujillo, & Moreda, 2008). Thus, since alcohols and, consequently, the FAAEs values will increase when inappropriate practices are carried out during the processing, they have been used to assess the quality of olive fruits processed during EVOO extraction, to check the cleanliness of the material and to ensure a good management of the process (Pérez-Camino et al., 2008).

Both the International Olive Council (2013) and the European Commission (2013) have adopted the FAAEs standard to distinguish between EVOO and non-EVOO (Conte et al., 2019). However, the latest update of the standard, limiting the amounts of FAEEs to 30 mg/kg (IOC, 2013), has led to a worrying situation for the sector since, in some cases, it is very difficult not to exceed these limits, which would imply significant economic losses.

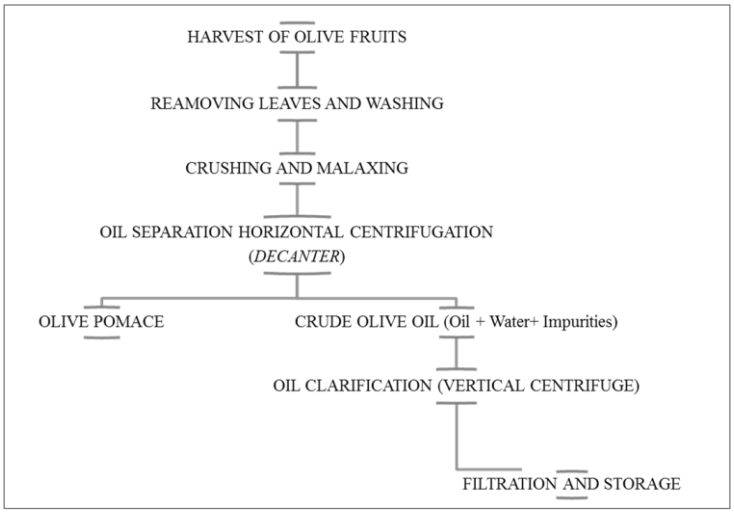


Figure 1: Olive oil processing scheme (Two-phase system)

To guarantee the limit values of FAEEs, the olive status control is not enough but it is also necessary to control the different stages during VOO production in order to intervene in those with major risk of increasing the content of alkyl esters. In the present work, we focused on how the separation steps of the process (decanter and the vertical centrifuge) affect the quality by evaluating the balance between FAAEs and short-chain alcohols (ethanol and methanol).

Specifically, the main objective was to study the effect of water addition flow and paste injection rate into the decanter, as these are two easy interventions that can be implemented at any time or type of decanter without stopping the process. The secondary goal of this work was to study the step decanter-to-vertical centrifuge and how is the oil at the end of the process. The experiments were carried out under optimal conditions for VOO production at a mill in operation (Cooperative La Granadella, Catalonia).

2. Material and methods

2.1. Experiments

The experiments were performed under the same extraction conditions on two different days (December 08th and 15th) using healthy ‘Arbequina’ olive fruits with a maturity index (MI) of

2.6 and 3.9, respectively (table 1). The MI was assessed using the method proposed by Uceda and Frias (1975).

Olive fruits were crushed using a hammer crusher operating at 3000 rpm, equipped with a 5mm sieve and with a capacity of 4500 kg per hour. Then, the olive paste was malaxed during 65 min at 27ºC. The separation of the oil was carried out using a two-phase decanter DC-180 (TACSA, Técnicas Andaluzas de Centrifugación S.L.), operating at ~2410 RCF and with a theoretical capacity of 5000 kg/h, followed by an automatic vertical centrifuge (HAUS-Centrifuge technology), operating at ~10080 RCF. While the vertical centrifuge (VC) operated under the same conditions for all experiments, different treatments of olive paste rates and water flow injected into the decanter were experimented.

The first group of experiments evaluated the effect of olive paste injection rate on the balance of alcohols and FAAEs between fruit, pomace and oil. This study was carried out by fixing at a constant flow of 150 L/h the water injected into the decanter and testing different rates of olive paste: 68% (3400 kg/h), 76% (3800 kg/h), 82% (4100 kg/h) and 90% (4500 kg/h) of theoretical decanter capacity. This range of working rates is within the recommended levels proposed by several authors for two-phase decanters (Di Giovacchino, 2013). The second group of experiments focused on the effect of small volumes of water injected into the decanter. Thus, the pumping of the olive paste was set at 76% of the decanter capacity and the water flow injection ranged between 0 L/h (0%), 100 L/h (3%), 200 L/h (5%) and 300 L/h (8%) respectively. In a two-phase system, in order to improve oil extraction, it is recommended to add small amounts of water into the decanter when working with difficult pastes (as is the case of 'Arbequina') as long as a limit of 10-15% water addition is not exceeded (Hermoso et al., 1996; Nieto et al., 2019).

Samples of pomace and oils were taken, in duplicate, at the decanter and VC outlets at approximately 10 min intervals. In all treatments, chemical characteristics of both pomace and oils samples were determined (table 2 and table 3).

2.2. Samplings

Sampling was carried out at different steps in order to study the balance of the compounds studied between the phases (oil, pomace) in each step. To get suitable conclusions, in all cases the results of the analysis were compared with those of the reference samples. In this way, it was possible to determine alcohols and FAAEs coming from olives, those formed during a specific production process or even alcohols lost by evaporation/transesterification (Masella et

al., 2019; Vidal, Alcalá, de Torres, Moya, & Espínola, 2019; Alcalá et al., 2017; Pérez-Camino et al., 2008).

2.2.1. Initial content in the olives

To check whether the compounds studied are generated throughout the process or if they enter the system coming from the fruits, initial amounts were measured when the olives arrived to the mill. These olives were called reference samples and to ensure that they were homogeneous and representative of the batch, small amounts were taken from the hopper every 10 minutes to get a final sample of ~5 kg of olives. Then reference samples were split into two parts. The first part was ground at room temperature and the homogenized paste obtained was used to quantify the alcohols. The second part was processed using the ABENCOR system to obtain olive oil and its content in ethanol (EtOH), methanol (MeOH) and FAAEs (FAEEs, FAMEs) was determined. The contents in the oil obtained by this controlled system were considered as reference values of these compounds at the inlet of the system (table 4).

2.2.2. Final content in pomace

Olive pomace samples were taken at the decanter outlet for each one of the tested water flow and paste injection rates. For each experiment two samples of 100 g pomace were taken, and these were analyzed to determine their moisture, oil content (table 2) and also their alcohol amounts.

2.2.3. Final content in the oil

Olive oil samples were taken after the two separation steps of the process: at the decanter outlet (crude oil) and at the VC outlet (clean oil). For each value of the tested parameters, two samples of 250 ml each were taken. Samples from the decanter were centrifuged in the laboratory at 5ºC and 5000 RCF during 3 minutes. In all cases, alcohols and FAAEs were quantified and moisture and impurities were measured (table 2) to evaluate the distribution of alcohols between the oil and wastes.

2.2.4. Sensory evaluation

The sensory evaluation of the oil samples was carried out by the Official Tasting Panel of Virgin Olive Oils of Catalonia (Reus, Spain), which has been recognized by the IOC since 1997 and by the Spanish Government since 2004. It relies under ISO 17025 standard since 2007. The final aroma evaluation represents the median from eight different trained tasters. Table 3 shows the results of the positive attributes of the sensorial analysis (fruitiness, bitterness and

pungency). The panel was unable to test the intermediate oil samples from the decanter, because the tasters are not trained for that purpose and because the oil contains high levels of moisture and impurities (table 2) that could interfere with taster's perception.

2.3. Analysis of alcohols

2.3.1. Sample preparation

To determine alcohols in olive homogenates and in pomace, 2 g of the homogenized paste were weighed into 20 mL vials together with 2g of saturated CaCl2 solution. The vials were tightly sealed with a septum cap and kept in the freezer (-18ºC) until their analysis. Regarding the quantitation of the alcohols in oil (either centrifuged crude oil or clean oil), the samples were prepared by pouring 3 g of oil together with 100 µL internal standard into a 10 mL vial. After hermetically sealed with a septum cap, it was kept in the freezer (-18ºC) until its analysis.

2.3.2. Materials and Reagents

All chemical reagents were of gradient HPLC grade. Ethanol and methanol were purchased from Scharlab (Barcelona, Spain). Calcium chloride ($CaCl_2$) and 1-propanol, used as internal standard, were provided by Sigma-Aldrich (St. Louis, USA). For the Headspace-Solid Phase Microextraction (HS-SPME) of the analytes, 2 cm length fibers 50/30 µm StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, USA) were used.

2.3.3. Analytical procedure

The quantification of alcohol contents in olive homogenates and pomace was carried out by using an HS-SPME CTC CombiPAL autosampler (CTC Analytics, Switzerland) and an HP-6890N gas chromatograph (GC) coupled to a mass detector (MSD) HP-5973 (Hewlett-Packard, USA). The optimal extraction conditions were: 15 min of pre-equilibration at 50ºC; HS-SPME during 50 min at 40ºC under medium agitation; thermal desorption at 270ºC for 1 min in the GC injector port in splitless mode.

Chromatographic separations were carried out using a fused silica capillary column, Chromapack CP-WAX 57CB, 50m x 0.25mm i.d. and 0.2 µm film thickness (Varian. Middelburg, Netherlands). The oven temperature program was: 40°C (5 min), 5°C.min^{-1} to 100ºC and 10ºC.min^{-1} to 215ºC (5 min). The carrier gas was helium (He) at a constant flow of 1.8 mL/min. Interface, ion source and mass quadrupole temperatures were 200°C, 230ºC and 150°C, respectively. The mass-to-charge (m/z) ratio range used was 28-300 amu, and spectra matching were performed using the Wiley/NBS library. To avoid quantification errors due to

the matrix effect, the calibration lines were built by using matrix-matched calibration technique as explained in a previous study (Boudebouz et al., 2020).

When dealing with oil samples, alcohols were determined by using an A G1888 Automatic Static Headspace Sampler (Hewlett-Packard, USA) coupled to a GC-MSD system. The optimal operating conditions were similar to the ones described by Gómez-Coca et al., (2014), so 3 g of sample into a 10 mL vial were heated at 80ºC during 50 minutes under medium agitation. Then, 500 μL of the headspace sample were injected into the GC port through a transfer line at 110ºC. The chromatographic conditions are the ones described above and the quantification of alcohols was carried out by means of the internal standard method by using 1-propanol for this purpose.

It should be noted that oil samples from the decanter were centrifuged to eliminate the water, therefore, part of alcohols were also eliminated due to their distribution between both phases. Since the different experiments carried out implied different oil: water ratios, a previous study to determine the repartition factor in each case was necessary. Thus, different oil/water mixtures were prepared ranging from 100:0 to 88:12 ratios. All the mixtures were spiked with the same amount of alcohols and then were agitated to facilitate partitioning of the analytes between both phases. Finally, the mixtures were centrifuged to separate the phases and the amounts of alcohols in each one were determined. In this way, we obtained the distribution factors that could be applied to the different samples to avoid quantification errors.

2.4. Analysis of fatty acids alkyl esters

2.4.1. Sample preparation

To determine the amount of FAAEs (FAMEs and FAEEs) in the different oil samples coming both from the decanter and from the VC, the IOC official method (COI/T.20/Doc. No31. 2012) was applied. Thus, a glass column for liquid chromatography was filled with 3 g of silica gel suspended in a hexane:ether mixture (98:2). This column was used to fractionate the sample (100±2 mg of the oil added with 25 μL of the internal standard (methyl heptadecanoate in heptane 0.02%)) and to get FAAEs fraction after evaporation of the solvent in a rotatory evaporator at 40ºC and subsequent dissolution of the residue in 1 mL of heptane. For each experiment, three extractions were performed and the extracts obtained were stored in the freezer, into 1.5mL vials hermetically closed until their analysis.

2.4.2. Materials and Reagents

The glass columns for liquid chromatography (10mm i.d, 40cm length) were provided by POBEL (Madrid, Spain). The solvents used were ethyl ether for HPLC, ≥99.00%

(CHROMASOLV), n-hexane for HPLC, ≥97.00% (CHROMASOLV) and n-heptane for GC, ≥97.00% (LICHROSOLV). The Silica gel used was Silica 60 from Merck-KGaA (Darmstadt, Germany).

The chemical standards for FAAEs identification (methyl palmitate, methyl linoleate, methyl oleate, methyl stearate, ethyl palmitate, ethyl linoleate, ethyloleate and ethyl stearate) and the internal standard (methyl heptadecanoate) were supplied by Sigma-Aldrich (Madrid, Spain) and their purity was ≥ 97% in all cases.

2.4.3. Analytical procedure

The GC analyses of FAAEs were carried out with an Agilent 6890N gas chromatograph equipped with an Agilent G1530 flame ionization detector (FID) (Agilent Technologies, USA) coupled to an automatic injector equipped with a programmable temperature vaporizing (PTV) inlet for on-column injection of the sample extracts. The chromatographic separations were done using a fused silica capillary column, Zebron ZB-5MS, 30m x 0.25mm i.d. and 0.25µm d.f. from Phenomenex (Alcobendas, Spain), which was protected with an empty pre-column of 30-40 cm. The oven temperature was programmed at 70ºC for 2 min, followed by a ramp of 10ºC.min-1 until 180ºC, then 5ºC.min^{-1} until 220ºC and 10ºC.min^{-1} until 320ºC, and held for 16.5 min. The detector temperature was 350ºC. Hydrogen was used as carrier gas at a constant flow of 1.5 mL/min. A sample volume of 1 µL was injected in on-column mode.

The identification of FAAEs was performed by injecting individual standards of C16 and C18 FAEEs and FAMEs. The quantification of each identified compound was performed based on the area ratio between the analyte and the IS by using the following mathyematical relationship (International Olive Council, 2012; Pérez-Camino et al., 2008; Gómez-Coca, Moreda, & Pérez-Camino, 2012):

$$\boldsymbol{FAAEs}\ (mg/kg) = \frac{(\boldsymbol{Ax} * \boldsymbol{ms}) * 1000}{(\boldsymbol{As} * \boldsymbol{m})}$$

***Ax**: area corresponding to the peak for the individual C16 and C18 esters*
***As**: area corresponding to the peak for the internal standard (methyl heptadecanoate)*
***ms**: mass of the internal standard added (in milligrams)*
***m**: mass of the oil sample taken for determination (in grams)*

2.5. Statistical analysis

Statistical analysis of the results was performed using the SAS-Stat Software (V9.4. SAS Institute Inc., Cary). The effects of water addition and paste injection rate were analyzed by one-way ANOVA (Analysis of Variance) using the Generalized Linear Model (GLM) procedure, exploring both linear and quadratic models. Comparison of means was performed by using the Duncan´s multiple range tests (α <0.05).

3. Results and discussion

As described above, each set of experiments was performed with olives at two different maturity stages. For each experiment, only olives of good visual quality were used, which implies that more than 94% of the fruits were healthy (data not shown).

To avoid any fermentation reaction from harvest to processing, the olive fruits were pressed no later than two hours after receiving them at the mill. This precaution is enough as the contents of alcohols in the reference samples averaged 9 mg kg^{-1} for ethanol and 120 mg kg^{-1} for methanol (data not shown), values very similar to those reported for healthy fruits in previous studies (Beltrán et al., 2015; Boudebouz et al., 2020; García-Vico et al., 2018).

To evaluate the effects of the decanter adjustments on the evolution of the content of alcohols and FAAEs when working under different conditions, the rest of the process steps (crushing, malaxing and vertical centrifugation) were kept constant. In this way the different experiments focused on the effect of the paste injection rate and the water injection flow into the decanter (keeping constant the spin and outlet diaphragm).

The values of the factors studied were chosen within the working ranges recommended by the decanter manufacturer. Specifically, the paste injection rates studied ranged between 3400 $kg.h^{-1}$ and 4500 $kg.h^{-1}$ and water addition between 0 and 300 $L.h^{-1}$, values within the interval recommended for a two-phase system (70-90% of decanter capacity and less than 10% water injection). It is well known that the use of water negatively affects minor compounds of VOOs, mainly volatiles and polyphenols, and eventually the VOOs shelf-life. However, when the contents of FAEEs are high, the concern is more related to the final commercial category than to the nutritional values. Furthermore, there are new injector devices that allow water to be delivered directly into the decanter without mixing it with the paste and thus overcome the problem of polyphenol loss (Hermoso, Boudebouz, Ninot, & Romero, 2021). In Catalonia, the prevalence of oils with high risk of being downgraded due to an excess of FAAEs is 15%. It

must also be stated that master millers need to balance between quantity and quality of the oil extracted, based on many reasons that have not been considered in this study, which aims to give them more criteria to take such decision.

Table 1. Olive characteristics according to the harvest date

date	Maturity Index (MI)	Flesh/pit ratio	Variety	Moisture (%)	Fat in wet basis (%)	Fat in dry basis (%)
December 8th	2.65	2.52	Arbequina	52.31	22.37	46.91
December 15th	3.90	2.61	Arbequina	48.69	22.64	44.13

The two batches of fruits used for the experiments were different (table 1). On December-8th the olives were turning from green to red color and were very rich in water, while on December-15th the olives were black and with less moisture. The oil yield of both batches was the expected for the 'Arbequina' cultivar in the Garrigues area in December. Regarding the characteristics of the pomace at the outlet of the decanter (table 2), the fat content was higher than expected for all experiments (12-14% dry basis, while the theoretical one is 8-12% db). This may be due to the fact that, to avoid the interference of too many variables, coadjutants were added to the malaxing step and mechanical adjustments of decanter were made (diaphragm, differential spin, distance of paste download in the decanter), apart from the two conditions studied.

The highest pomace fat content was observed at the minimum paste injection rate without water addition (table 2). This behavior may be because, under these conditions, a change of the decanter diaphragms was required. Therefore, the extractability of the oil was improved by increasing the paste injection rate or by adding a little water to the paste.

The oil moisture and impurity levels at the decanter outlet (table 2) were relatively high for all conditions and justified the use of the vertical centrifuge in order to clarify and stabilize the oil.

Finally, table 3 reports that all the processing conditions tested allowed to obtain oils of such good quality, as all quality criteria matched the category of extra virgin (free acidity, peroxide values, K_{232}, K_{270} and sensory evaluation).

Table 2. Olive pomace and oil characteristics at the outlet of horizontal centrifuge (decanter) (mean ± standard deviation)

Factor	date	Dose	Olive pomace (decanter exit)			Oil (decanter exit)	
			Moisture %	Fat in wet basis (%)	Fat in dry basis (%)	Moisture and volatiles (%)	Impurities (%)
Rhythm (kg/h)	Dec-8th	3400	63.39 **a** ± 0.32	5.32 **a** ± 0.01	14.55 **a** ± 0.10	5.73 **a** ± 1.15	0.50 **a** ± 0.25
		3800	63.10 **a** ± 0.58	4.44 **b** ± 0.01	12.04 **a** ± 0.18	3.90 **a** ± 1.15	0.39 **a** ± 0.20
		4100	61.13 **a** ± 0.99	4.75 **ab** ± 0.19	12.26 **a** ± 0.80	3.97 **a** ± 0.79	0.28 **a** ± 0.14
		4500	61.97 **a** ± 0.30	4.69 **ab** ± 0.12	12.35 **a** ± 0.41	4.30 **a** ± 0.86	0.50 **a** ± 0.25
	Dec-15th	3400	62.43 **b** ± 0.09	5.13 **a** ± 0.07	13.67 **a** ± 0.15	2.20 **a** ± 0.44	0.25 **a** ± 0.13
		3800	62.68 **b** ± 0.08	4.72 **a** ± 0.20	12.65 **a** ± 0.57	2.58 **a** ± 0.52	0.21 **a** ± 0.11
		4100	63.28 **a** ± 0.02	4.80 **a** ± 0.05	13.07 **a** ± 0.12	4.08 **ab** ± 0.82	0.23 **a** ± 0.11
		4500	63.54 **a** ± 0.02	4.94 **a** ± 0.10	13.56 **a** ± 0.26	5.75 **b** ± 1.15	0.18 **a** ± 0.09
Water (L/h)	Dec-8th	0	62.83 **b** ± 0.23	5.41 **a** ± 0.13	14.56 **a** ± 0.25	6.31 **a** ± 1.15	0.66 **a** ± 0.33
		100	63.66 **ab** ± 0.24	4.91 **b** ± 0.05	13.52 **b** ± 0.23	6.53 **a** ± 1.31	0.62 **a** ± 0.31
		200	64.44 **a** ± 0.09	4.79 **b** ± 0.01	13.46 **b** ± 0.06	6.66 **a** ± 1.33	0.23 **b** ± 0.11
		300	63.84 **a** ± 0.08	4.36 **c** ± 0.06	12.06 **c** ± 0.13	6.52 **a** ± 1.30	0.16 **b** ± 0.08
	Dec-15th	0	60.29 **a** ± 0.15	5.85 **a** ± 0.07	14.74 **a** ± 0.12	3.22 **a** ± 0.64	0.27 **a** ± 0.13
		100	61.15 **a** ± 0.24	5.02 **b** ± 0.05	12.91 **b** ± 0.04	2.32 **a** ± 0.46	0.35 **a** ± 0.18
		200	60.85 **a** ± 0.70	4.85 **b** ± 0.16	12.41 **b** ± 0.62	3.77 **a** ± 0.75	0.21 **a** ± 0.11
		300	62.73 **a** ± 0.41	4.78 **b** ± 0.03	12.84 **b** ± 0.07	5.01 **a** ± 1.00	0.42 **a** ± 0.21

By column and by group, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05)

3.1. The balance of alcohols and FAAEs

To better evaluate the effect of each processing factor studied, the balance of alcohols and FAAEs was made between the input and output of every studied step. Thus, the amount of each compound in every fraction (olive paste, pomace and oil) was calculated and expressed in grams per hour (g/h), taking into account the total amount of each fraction processed in one hour and the concentration of alcohols and FAAEs measured in aliquot samples of each fraction (table 4). Table 5 shows the balance taking into account inputs and outputs in each centrifugation step (decanter and VC) and the results are expressed as percentage relative to the inputs.

In most of the experiments, the samples showed lower contents of methanol both at the decanter and VC inlet than at the decanter and VC outlet, respectively. However, when looking at the ethanol contents, the values showed an opposite behavior as no generation of ethanol was observed in any experiment (table 4). This different trend seems to be related to the activity of pectin methyl-esterase and its hydrolytic processes that occur during the olive oil production

process, which implies methanol generation but has no effects on the ethanol contents. This corroborates the results found in the literature (Conte, et al., 2019).

Table 3. Olive oil characteristics at the vertical centrifuge outlet (mean ± standard deviation)

Factor	date	Dose	Moisture and volatiles content (%)	Acidity (% oleic acid)	Peroxide value (meq O2/kg)	K232	K270	Panel test			
								category	fruitiness	bitterness	pungent
Rhythm (kg/h)	Dec-8th	3400	0.21 a ± 0.04	0.14 a ± 0.01	7 a ± 1	1.60 a ± 0.16	0.14 a ± 0.04	Extra	4.7 a ±0.4	3.8 a ±0.5	4.3 a ±0.2
		3800	0.26 a ± 0.05	0.15 a ± 0.02	9 a ± 1	1.52 a ± 0.15	0.14 a ± 0.03	Extra	4.3 a ±0.2	3.1 a ±0.3	4.3 a ±0.4
		4100	0.24 a ± 0.05	0.15 a ± 0.02	7 a ± 1	1.54 a ± 0.15	0.17 a ± 0.04	Extra	4.8 a ±0.2	3.6 a ±0.2	4.4 a ±0.3
		4500	0.33 b ± 0.07	0.07 a ± 0.03	8 a ± 1	1.68 a ± 0.17	0.15 a ± 0.04	Extra	4.5 a ±0.3	4.4 a ±0.5	4.4 a ±0.4
	Dec-15th	3400	0.24 b ± 0.05	0.15 a ± 0.02	9 a ± 1	1.51 a ± 0.15	0.11 a ± 0.03	Extra	4.7 a ±0.2	3.9 a ±0.1	4.5 a ±0.2
		3800	0.27 a ± 0.05	0.14 a ± 0.01	9 a ± 1	1.64 a ± 0.16	0.13 a ± 0.03	Extra	4.7 a ±0.2	3.5 a ±0.2	4.7 a ±0.2
		4100	0.28 a ± 0.06	0.14 a ± 0.01	8 a ± 1	1.58 a ± 0.16	0.12 a ± 0.03	Extra	4.9 a ±0.2	3.6 a ±0.2	4.6 a ±0.2
		4500	0.29 a ± 0.06	0.12 a ± 0.01	8 a ± 1	1.76 a ± 0.18	0.12 a ± 0.03	Extra	4.6 a ±0.2	3.4 a ±0.2	4.4 a ±0.3
Water (L/h)	Dec-8th	0	0.28 a ± 0.06	0.11 a ± 0.01	6 a ± 1	1.50 a ± 0.15	0.09 a ± 0.02	Extra	5.1 a ±0.2	3.8 a ±0.2	4.7 a ±0.4
		100	0.27 a ± 0.05	0.11 a ± 0.01	6 a ± 1	1.49 a ± 0.15	0.09 a ± 0.02	Extra	5.0 a ±0.5	3.7 a ±0.1	4.4 a ±0.4
		200	0.27 a ± 0.05	0.11 a ± 0.01	6 a ± 1	1.49 a ± 0.15	0.11 a ± 0.03	Extra	4.7 a ±0.4	3.5 a ±0.2	4.4 a ±0.2
		300	0.30 a ± 0.06	0.12 a ± 0.01	7 a ± 1	1.51 a ± 0.15	0.11 a ± 0.03	Extra	5.0 a ±0.2	3.8 a ±0.2	4.6 a ±0.1
	Dec-15th	0	0.18 a ± 0.04	0.16 a ± 0.02	7 a ± 1	1.48 a ± 0.15	0.12 a ± 0.03	Extra	4.8 a ±0.3	4.4 a ±0.2	4.9 a ±0.4
		100	0.18 a ± 0.04	0.16 a ± 0.02	7 a ± 1	1.52 a ± 0.15	0.12 a ± 0.03	Extra	5.0 a ±0.5	4.1 a ±0.3	4.8 a ±0.3
		200	0.18 a ± 0.04	0.17 a ± 0.02	8 a ± 1	1.59 a ± 0.16	0.16 a ± 0.04	Extra	4.9 a ±0.3	4.0 a ±0.3	4.8 a ±0.4
		300	0.27 b ± 0.05	0.17 a ± 0.02	6 a ± 1	1.61 a ± 0.16	0.17 a ± 0.04	Extra	5.0 a ±0.1	4.3 a ±0.4	4.6 a ±0.2

By column and by group, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05)

Alcohols exist naturally in olives, so they can pass into the oil during the extraction process (Beltrán et al., 2015; Luna, Morales, & Aparicio, 2006; Boudebouz et al., 2020). However, as in previous studies (Biederman et al., 2008), the results showed that large amounts of ethanol and methanol are removed with water during processing although each alcohol has a different behavior. As shown in Table 5, while 90-95% of methanol is removed from the oil in the decanter, a significant amount of ethanol (15-25%) reaches the oily fraction. Therefore, special attention must be paid to ethanol and ethyl esters because, if the decanter does not work in the right conditions, these compounds can reach the oil.

According to the literature, the evaporation of a part of the alcohols can occur throughout the different process steps (Masella et al., 2019). However, our data do not support such fact but rather attribute some variations in the content of alcohols to their esterification into alkyl esters (Pérez-Camino et al., 2008). As shown in Tables 4 and 5, these esterification reactions showed a different yield depending on the olive ripeness status. Thus, when working with less mature fruits, the FAEE content at the decanter outlet can double the value found in the olive fruits.

However, these values do not reach the final oil since they are drastically reduced when the oil passes through the VC. Although centrifugation facilitates the elimination of a part of these compounds (Vidal et al., 2019), this separation process does not explain such a marked decrease. After carefully studying the results, it was concluded that at this point of the process, a certain hydrolysis of the alkyl esters can happen, which should be favored by the presence of high water content. Therefore, as less mature olives provided up to twice the water content in the oil obtained at the decanter outlet than more mature olives, the hydrolysis process in the latter should be much less. The results in Table 5 show that mature samples are not only unaffected by hydrolysis but even increase the value of the concentration of FAEE's. This behavior means that there must be an intermediate step between the decanter outlet and VC where ethyl esters are synthesized. This step can be related to the design of La Granadella mill, which implies that liquids can remain under the vibro-filter for a while and can facilitate the fermentation of sugars diluted in the vegetative water that is mixed with the oil in this step.

Regarding the contents of FAMEs, there is a similar trend for all the experiments and, as can be seen in Table 5, these values decrease in the decanter but increase again when passing through the VC. This opposite behavior to that observed for the FAEE's may be due to the high amount of methanol in the oil obtained at the decanter outlet. These great concentrations can promote the esterification reaction into the vertical centrifuge, with the consequent reduction of the methanol content in the final oil (due to both VC effect and esterification).

3.2. Effect of the paste injection rate

To easily visualize whether or not there was a relationship between the different paste injection rates and the concentration of the analytes studied in the final product, the plots shown in figures 2 and 3 were drawn up. These figures also show the balances between the input and output of the system from the ratio "analyte contents in VC / analyte contents in fruits" (VC/Fruit).

Concerning the relationship between the paste injection rate and EtOH content in the oil at VC outlet (that is, in the final product), a very weak quadratic trend was found, with a maximum between 76-82% of pumping rate and slightly higher values for the ripe olives (Fig. 2a). Regarding FAEE (Fig. 2b), a significant quadratic trend was pointed out for ripe olives. Maximum values of FAEEs were found when working at an injection rate between 76-82% of the whole decanter capacity. Therefore, about 15% to 25% of the EtOH that enters the system can reach the oil, either as ethanol or ethyl esters (Fig. 2c). When FAEE's in the oil are

compared to those in the olive fruits (Fig. 2d), it can be seen that during the process a significant FAEE synthesis occurs, which ranges between 100-150% of the values in the fruits. In the case of ripe olives processed at a very high rate (90%), significant losses of FAEEs are observed (balance below 100%) that possibly are carried along with the pomace due to a better separation as it happens with ethanol (Fig. 2g).

When only the decanter is considered, EtOH and mainly FAEE show a significant quadratic behavior related to the paste injection rates. As expected, ethanol and ethyl esters show inverse trends (Fig. 2e and 2f), that can be related to the ethanol conversion into FAEE since the esterification of free fatty acids with these alcohols is a fast reaction (Pérez-Camino et al., 2008). Thus, within the range 76% to 82% of working capacity, higher the injection rate lowers the time that the oil remains in the decanter and lower the EtOH transformed into EE. However, around the maximum capacity of the decanter (95-100%), the system deviates from the optimal working conditions and worsens the separation yield. Specifically, both the dry matter oil losses and oil moisture content increase (table 2), so this higher water content will drag more ethanol that could be esterified. This hypothesis is confirmed by observing the acidity of the final oil. As shown in Table 3, when working under these conditions, the acidity significantly decreases because some of the acids disappear when reacting with ethanol, giving rise to ethyl esters, which are the ones that increase their content.

In addition, and according to Guerrini, Pantani, & Parenti (2016), the effect of centrifugation together with the existence of a greater amount of vegetative water caused higher K232. These higher values were observed at the highest injection rates in all experiments, suggesting more oxidative conditions (table 2).

Table 4. Alcohols and FAAEs contents (mean ± standard deviation) in each step (loaded paste, pomace and oil), expressed in grams per hour (g/h) of processing.

Experiment		Decanter inlet		Decanter outlet				Vertical centrifuge outlet		
		Olive paste		Pomace	Oil			Oil		
Factor	dose	EtOH (g/h)	FAEE[z] (g/h)	EtOH (g/h)	EtOH (g/h)	FAEE (g/h)	EtOH_in_FAEE (g/h)[x]	EtOH (g/h)	FAEE (g/h)	EtOH_in_FAEE (g/h)
Rhythm (kg/h) Dec-8th	3400	33.29	7.72	24.49 ^{a} ± 2.72	5.85 ^{c} ± 0.17	16.92 ^{a} ± 0.66	2.63 ^{a} ± 0.10	5.13 ^{b} ± 0.08	9.33 ^{b} ± 0.04	1.45 ^{b} ± 0.01
	3800	37.20	8.63	14.63 ^{a} ± 1.19	8.99 ^{b} ± 0.14	18.85 ^{a} ± 0.83	2.93 ^{a} ± 0.13	5.62 ^{ab} ± 0.01	12.96 ^{a} ± 0.51	2.01 ^{a} ± 0.08
	4100	40.14	9.31	19.05 ^{a} ± 1.27	9.76 ^{ab} ± 0.19	10.87 ^{b} ± 0.56	1.69 ^{b} ± 0.09	6.12 ^{a} ± 0.16	9.79 ^{b} ± 0.52	1.52 ^{b} ± 0.08
	4500	44.06	10.22	23.48 ^{a} ± 3.57	9.85 ^{a} ± 0.19	18.28 ^{a} ± 0.56	2.84 ^{a} ± 0.09	5.80 ^{a} ± 0.09	10.19 ^{b} ± 0.04	1.58 ^{b} ± 0.01
Rhythm (kg/h) Dec-15th	3400	32.06	9.48	19.73 ^{bc} ± 2.29	7.45 ^{b} ± 0.07	12.65 ^{b} ± 0.53	1.97 ^{b} ± 0.08	5.23 ^{c} ± 0.07	8.54 ^{b} ± 0.52	1.33 ^{b} ± 0.08
	3800	35.83	10.60	19.18 ^{c} ± 3.29	7.30 ^{b} ± 0.12	12.86 ^{b} ± 0.28	2.00 ^{b} ± 0.04	6.71 ^{a} ± 0.08	14.04 ^{a} ± 0.36	2.18 ^{a} ± 0.06
	4100	38.66	11.44	24.30 ^{b} ± 0.06	11.06 ^{a} ± 0.15	10.65 ^{b} ± 0.38	1.66 ^{b} ± 0.06	5.89 ^{b} ± 0.06	14.94 ^{a} ± 0.4	2.32 ^{a} ± 0.06
	4500	42.44	12.55	32.82 ^{a} ± 3.83	10.78 ^{a} ± 0.15	18.76 ^{a} ± 0.11	2.92 ^{a} ± 0.02	6.65 ^{a} ± 0.10	6.82 ^{b} ± 0.37	1.06 ^{b} ± 0.06
Water (L/h) Dec-8th	0	37.20	8.63	10.58 ^{b} ± 2.71	8.46 ^{a} ± 0.17	12.28 ^{a} ± 0.73	1.91 ^{a} ± 0.11	6.70 ^{a} ± 0.14	7.83 ^{b} ± 0.33	1.22 ^{b} ± 0.05
	100	37.20	8.63	17.13 ^{ab} ± 1.33	8.57 ^{a} ± 0.25	9.91 ^{a} ± 0.81	1.54 ^{a} ± 0.13	7.25 ^{a} ± 0.04	12.11 ^{a} ± 0.48	1.88 ^{a} ± 0.07
	200	37.20	8.63	26.34 ^{a} ± 1.48	7.48 ^{b} ± 0.16	8.38 ^{a} ± 0.22	1.30 ^{a} ± 0.03	6.54 ^{a} ± 0.18	8.21 ^{b} ± 0.26	1.28 ^{b} ± 0.04
	300	37.20	8.63	19.66 ^{ab} ± 2.99	6.63 ^{b} ± 0.19	12.21 ^{a} ± 0.29	1.90 ^{a} ± 0.04	6.76 ^{a} ± 0.10	10.04 ^{ab} ± 0.42	1.56 ^{ab} ± 0.06
Water (L/h) Dec-15th	0	35.83	10.60	11.06 ^{b} ± 0.32	7.09 ^{b} ± 0.16	8.91 ^{a} ± 0.67	1.38 ^{a} ± 0.10	5.43 ^{a} ± 0.05	8.41 ^{b} ± 0.44	1.31 ^{b} ± 0.07
	100	35.83	10.60	29.09 ^{a} ± 1.82	6.89 ^{b} ± 0.13	7.68 ^{a} ± 0.74	1.19 ^{a} ± 0.12	5.19 ^{a} ± 0.04	11.48 ^{a} ± 0.11	1.78 ^{a} ± 0.02
	200	35.83	10.60	27.35 ^{a} ± 1.11	7.58 ^{b} ± 0.23	6.75 ^{a} ± 0.57	0.79 ^{a} ± 0.19	3.78 ^{c} ± 0.03	10.00 ^{ab} ± 0.29	1.55 ^{ab} ± 0.04
	300	35.83	10.60	24.65 ^{a} ± 2.26	10.53 ^{a} ± 0.20	8.95 ^{a} ± 0.17	1.39 ^{a} ± 0.03	4.61 ^{b} ± 0.09	9.82 ^{b} ± 0.14	1.53 ^{b} ± 0.02
Factor	dose	MeOH (g/h)	FAME[y] (g/h)	MeOH (g/h)	MeOH (g/h)	FAME (g/h)	MeOH_in_FAME(g/h)[t]	MeOH (g/h)	FAME (g/h)	MeOH_in_FAME (g/h)
Rhythm (kg/h) Dec-8th	3400	388.55	4.44	312.10 ^{b} ± 13.04	22.03 ^{d} ± 0.70	4.80 ^{ab} ± 0.04	0.55 ^{ab} ± 0.01	5.58 ^{c} ± 0.07	6.58 ^{a} ± 0.88	0.75 ^{a} ± 0.10
	3800	434.26	4.96	296.00 ^{b} ± 12.02	37.06 ^{c} ± 0.88	5.96 ^{a} ± 0.21	0.68 ^{a} ± 0.02	6.22 ^{c} ± 0.03	8.92 ^{a} ± 0.46	1.01 ^{a} ± 0.05
	4100	468.55	5.35	418.92 ^{ab} ± 13.90	43..83 ^{b} ± 0.91	4.30 ^{b} ± 0.23	0.49 ^{b} ± 0.03	7.32 ^{b} ± 0.24	5.62 ^{a} ± 0.68	0.64 ^{a} ± 0.08
	4500	514.26	5.87	531.30 ^{b} ± 18.18	50.68 ^{a} ± 1.81	3.71 ^{b} ± 0.12	0.42 ^{b} ± 0.01	10.85 ^{a} ± 0.21	6.97 ^{a} ± 0.42	0.79 ^{a} ± 0.05
Rhythm (kg/h) Dec-15th	3400	443.43	5.92	331.35 ^{b} ± 16.96	26.69 ^{c} ± 0.86	4.54 ^{ab} ± 0.68	0.52 ^{ab} ± 0.08	4.96 ^{c} ± 0.06	6.04 ^{ab} ± 1.14	0.69 ^{ab} ± 0.13
	3800	495.60	6.62	554.39 ^{a} ± 12.87	25.96 ^{c} ± 0.75	7.36 ^{a} ± 0.19	0.84 ^{a} ± 0.02	7.03 ^{b} ± 0.07	11.98 ^{a} ± 1.12	1.36 ^{a} ± 0.13
	4100	534.72	7.14	536.06 ^{a} ± 18.79	42.35 ^{b} ± 0.93	3.82 ^{b} ± 0.19	0.43 ^{b} ± 0.02	5.34 ^{c} ± 0.13	6.08 ^{ab} ± 0.82	0.69 ^{ab} ± 0.09
	4500	586.89	7.84	682.01 ^{a} ± 10.47	48.58 ^{a} ± 0.95	5.18 ^{ab} ± 0.42	0.59 ^{ab} ± 0.05	9.36 ^{a} ± 0.12	3.38 ^{b} ± 0.98	0.58 ^{b} ± 0.00
Water (L/h) Dec-8th	0	434.26	4.96	363.48 ^{a} ± 24.76	41.36 ^{a} ± 1.05	3.76 ^{b} ± 0.11	0.43 ^{b} ± 0.01	7.22 ^{b} ± 0.13	5.97 ^{a} ± 0.08	0.68 ^{a} ± 0.01
	100	434.26	4.96	374.99 ^{a} ± 10.59	33.37 ^{bc} ± 1.57	5.29 ^{ab} ± 1.37	0.60 ^{ab} ± 0.16	8.99 ^{a} ± 0.05	6.41 ^{a} ± 0.49	0.73 ^{a} ± 0.06
	200	434.26	4.96	465.64 ^{a} ± 21.93	37.23 ^{ab} ± 0.83	4.55 ^{ab} ± 0.60	0.52 ^{ab} ± 0.07	7.31 ^{b} ± 0.33	4.89 ^{a} ± 0.36	0.55 ^{a} ± 0.04
	300	434.26	4.96	455.18 ^{a} ± 13.80	30.86 ^{c} ± 0.72	9.36 ^{a} ± 0.93	1.06 ^{a} ± 0.11	8.94 ^{a} ± 0.14	5.24 ^{a} ± 0.08	0.60 ^{a} ± 0.01
Water (L/h) Dec-15th	0	495.60	6.62	425.96 ^{b} ± 23.09	27.05 ^{b} ± 0.75	2.82 ^{b} ± 0.47	0.32 ^{b} ± 0.05	7.02 ^{b} ± 0.11	7.86 ^{a} ± 0.11	0.89 ^{a} ± 0.01
	100	495.60	6.62	619.36 ^{a} ± 4.44	29.6 ^{ab} ± 0.68	3.68 ^{b} ± 0.33	0.42 ^{b} ± 0.04	5.98 ^{c} ± 0.17	6.11 ^{ab} ± 0.37	0.69 ^{ab} ± 0.04
	200	495.60	6.62	533.23 ^{ab} ± 13.14	34.23 ^{a} ± 1.04	5.31 ^{b} ± 0.75	0.60 ^{b} ± 0.08	9.05 ^{a} ± 0.16	6.07 ^{ab} ± 0.43	0.69 ^{ab} ± 0.05
	300	495.60	6.62	567.00 ^{ab} ± 14.33	32.05 ^{ab} ± 1.25	8.51 ^{a} ± 0.12	0.97 ^{a} ± 0.01	7.34 ^{b} ± 0.09	4.63 ^{b} ± 0.51	0.53 ^{b} ± 0.06

By column and by group, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05).

[z] EE for ethyl esters; [y] ME for methyl esters [t, x] The conversion (EtOH in FAEE and MeOH in FAME) explains the quantity of ethanol transformed into ethyl ester and methanol into methyl ester. It was calculated applying the equation:

EtOH in FAEE = (ethyl ester mass * ethanol molar mass)/(oleic acid molar mass) and the equivalent for MeOH in FAME

Table 5. Relative content (%) of alcohols and FAAEs at the decanter and vertical centrifuge outlets as a function of their initial content in the olive paste at the decanter inlet (mean ± standard deviation)

Experiment		Decanter outlet						Vertical centrifuge outlet						Balancez		
		Pomace			Oil			Oil								
date	dose	EtOH (%)			FAEE (%)			EtOH (%)			FAEE (%)			Decanter/CV (%)		
Rhythm (kg/h) Dec-8th	3400	73.57 **a**	±	8.18	219.24 **a**	±	8.55	17.57 **b**	±	0.50	15.41 **a**	±	0.28	120.90 **b**	±	0.59	77.86 **a**	±	1.15
	3800	39.32 **b**	±	3.21	218.49 **a**	±	9.67	24.15 **a**	±	0.39	15.09 **a**	±	0.04	150.18 **a**	±	5.88	63.69 **bc**	±	1.76
	4100	47.46 **ab**	±	3.17	116.72 **b**	±	6.05	24.31 **a**	±	0.47	15.23 **a**	±	0.40	105.19 **b**	±	5.63	67.51 **b**	±	0.90
	4500	53.29 **ab**	±	8.11	178.88 **a**	±	5.52	22.36 **a**	±	0.42	13.17 **b**	±	0.20	99.78 **b**	±	0.42	58.49 **c**	±	0.95
Rhythm (kg/h) Dec-15th	3400	61.54 **ab**	±	7.14	133.33 **ab**	±	5.62	23.22 **c**	±	0.22	16.30 **b**	±	0.22	90.03 **b**	±	5.44	69.33 **b**	±	0.40
	3800	39.80 **b**	±	9.17	121.27 **b**	±	2.68	20.37 **d**	±	0.32	18.73 **a**	±	0.22	132.45 **a**	±	3.36	96.80 **a**	±	1.51
	4100	62.84 **ab**	±	0.15	93.15 **c**	±	3.37	28.61 **a**	±	0.38	15.23 **b**	±	0.14	130.60 **a**	±	3.53	64.82 **b**	±	0.59
	4500	65.52 **a**	±	9.03	149.43 **a**	±	0.87	25.40 **b**	±	0.34	15.66 **b**	±	0.24	54.34 **c**	±	2.92	55.68 **c**	±	0.00
Water (L/h) Dec-8th	0	37.90 **b**	±	4.47	142.29 **a**	±	8.52	22.74 **a**	±	0.46	18.01 **a**	±	0.36	90.75 **b**	±	3.77	74.46 **b**	±	4.37
	100	46.05 **ab**	±	3.58	114.89 **a**	±	9.37	23.03 **a**	±	0.67	19.47 **a**	±	0.10	140.36 **a**	±	5.52	91.93 **ab**	±	0.88
	200	70.80 **a**	±	3.99	97.12 **a**	±	2.50	20.12 **b**	±	0.42	17.56 **a**	±	0.49	95.16 **b**	±	3.03	90.84 **ab**	±	3.32
	300	52.85 **ab**	±	8.05	141.56 **b**	±	3.35	17.82 **b**	±	0.50	18.16 **a**	±	0.26	116.34 **ab**	±	4.83	98.25 **a**	±	2.62
Water (L/h) Dec-15th	0	30.06 **b**	±	0.90	84.06 **a**	±	6.30	19.23 **b**	±	0.43	14.76 **a**	±	0.14	79.32 **b**	±	4.12	81.20 **a**	±	0.88
	100	78.18 **a**	±	5.09	72.43 **a**	±	7.00	18.52 **b**	±	0.37	13.93 **a**	±	0.11	108.30 **a**	±	1.02	81.89 **a**	±	1.43
	200	73.53 **a**	±	3.10	63.68 **a**	±	5.34	20.36 **b**	±	0.64	10.16 **c**	±	0.08	94.36 **ab**	±	2.72	62.88 **b**	±	2.53
	300	66.26 **a**	±	6.32	84.40 **a**	±	1.56	28.29 **a**	±	0.56	12.39 **b**	±	0.24	92.65 **b**	±	1.33	51.65 **c**	±	1.76
	date	MeOH (%)			FAME (%)			MeOH (%)			FAME (%)			Balance Decanter/VC		
Rhythm (kg/h) Dec-8th	3400	80.32 **a**	±	2.38	108.30 **a**	±	1.01	5.67 **c**	±	0.18	1.43 **b**	±	0.02	148.33 **a**	±	19.89	29.06 **a**	±	1.08
	3800	68.16 **a**	±	3.69	120.17 **a**	±	4.27	8.53 **b**	±	0.20	1.43 **b**	±	0.01	179.94 **a**	±	9.21	19.03 **b**	±	0.19
	4100	89.41 **a**	±	3.96	80.40 **b**	±	4.22	9.35 **ab**	±	0.19	1.56 **b**	±	0.05	105.05 **a**	±	12.76	18.66 **b**	±	0.82
	4500	103.31 **a**	±	4.71	63.24 **b**	±	2.06	9.85 **a**	±	0.35	2.11 **a**	±	0.04	118.72 **a**	±	7.13	24.75 **b**	±	1.08
Rhythm (kg/h) Dec-15th	3400	74.72 **b**	±	7.45	76.63 **ab**	±	11.51	6.02 **b**	±	0.19	1.12 **c**	±	0.01	102.0 **ab**	±	19.25	21.079 **b**	±	0.94
	3800	111.86 **ab**	±	8.38	111.14 **a**	±	2.86	5.24 **b**	±	0.15	1.42 **b**	±	0.01	180.98 **a**	±	16.91	34.50 **a**	±	0.98
	4100	100.25 **a**	±	5.47	53.51 **b**	±	2.66	7.92 **a**	±	0.17	1.00 **d**	±	0.02	85.15 **b**	±	11.48	14.80 **b**	±	0.55
	4500	116.21 **a**	±	3.14	66.08 **ab**	±	5.33	8.28 **a**	±	0.16	1.59 **a**	±	0.02	43.11 **b**	±	12.46	21.07 **b**	±	0.00
Water (L/h) Dec-8th	0	83.70 **a**	±	5.21	75.83 **b**	±	2.16	9.52 **a**	±	0.24	1.66 **b**	±	0.03	120.41 **a**	±	23.20	19.04 **b**	±	0.51
	100	86.35 **a**	±	3.95	106.80 **ab**	±	27.66	7.68 **ab**	±	0.36	2.07 **a**	±	0.01	129.24 **a**	±	9.94	28.25 **ab**	±	2.00
	200	107.23 **a**	±	5.77	91.79 **ab**	±	12.08	8.57 **bc**	±	0.19	1.68 **b**	±	0.07	98.61 **a**	±	7.29	21.43 **b**	±	1.17
	300	104.82 **a**	±	3.22	188.84 **a**	±	18.71	7.11 **c**	±	0.17	2.06 **a**	±	0.03	105.78 **a**	±	1.53	31.06 **a**	±	0.79
Water (L/h) Dec-15th	0	85.95 **b**	±	6.21	42.53 **b**	±	7.02	5.46 **b**	±	0.15	1.42 **b**	±	0.02	118.73 **a**	±	1.65	29.93 **a**	±	1.10
	100	124.97 **a**	±	1.20	55.56 **b**	±	4.91	5.97 **ab**	±	0.14	1.21 **c**	±	0.03	92.30 **ab**	±	5.64	21.04 **b**	±	0.73
	200	107.59 **ab**	±	3.54	80.17 **b**	±	11.27	6.91 **a**	±	0.21	1.83 **a**	±	0.03	91.69 **ab**	±	6.54	28.58 **ab**	±	1.48
	300	114.41 **ab**	±	9.44	128.5 **a**	±	1.83	6.47 **ab**	±	0.25	1.48 **b**	±	0.02	69.94 **b**	±	7.68	23.95 **ab**	±	1.20

By column and by group, means with the same letter are not significantly different according to Duncan's multiple range tests (P<0.05).
(1): The balance Decanter/CV explains the proportion of alcohol (either as alcohol or alkyl ester) between the vertical centrifuge outlet and the horizontal centrifuge outlet.

Regarding MeOH in the oil at the VC outlet, a significant data dispersion was found when it came from mature olives, probably due to its high inherent reactivity. However, for green olives a quadratic effect was observed with the paste injection rate (Fig. 3a). This is a trend opposite to ethanol's, as the recovery of methanol at the VC outlet increases with higher injection rates, especially with more than 90% of the total decanter capacity. The balance shows that between 1.5% and 2.5% of the methanol from the fruit reaches the final oil (Fig. 3c). Regarding FAME, no significant effect was observed (Fig. 3b).

Although most of the MeOH from the olive paste drags into the pomace at the decanter outlet, a certain amount reaches the oil following a quadratic trend, with higher concentrations of methanol in the oil at a higher injection rate (Fig. 3e), mainly when it comes to green olives. Regarding FAMEs, these showed an opposite behavior with lower concentrations at higher injection rates and, again, green fruits better fit the quadratic trend (Fig. 3f).

3.3. Effect of water addition

The relationship between the addition of water and the compounds studied (Fig. 4 and 5) shows different trends depending on the stage of the process. When looking at the amounts of ethanol that reaches the oil at outlet of the VC, no statistically significant effect was observed neither on green nor on ripe olives (regardless of the amount of water injected). However, the concentrations of ethanol in the oil were significantly lower for ripe olives in all experiments (Fig. 4a). The amounts of FAEE, which ranged between 10 and 20 mg kg^{-1}, also did not show any significant trend. Therefore, as it can be seen in figures 4c and 4d, whereas only between 12% and 25% of the total ethanol from the olives reaches the oil at the VC outlet, almost all the ethyl esters from the olives reach this oil, independently of the amount of water added.

When considering the decanter outlet, the results showed a significant quadratic relationship between the ethanol content in the oil and the water injection (Fig. 4e). In addition, a clear interaction with the state of maturity of the olives was identified, mainly in the greater addition of water (8%). Under these conditions, the ethanol concentration in the oil is reduced in green olives but increases in ripe olives.

At the decanter, water injection has an opposite behavior compared to the paste injection rate. In fact, the addition of water tends to drag ethanol with pomace following a quadratic trend with a maximum of 5% injection, which resulted in the highest oil extractability (Fig. 2g and 4g). However, the paste injection rate follows the opposite trend, with a minimum of ethanol dragged in the pomace when paste injection rate allows maximum oil extractability (Fig. 2g).

This suggests that water injection is a better regulation option when processing low quality fruits (i.e, with significant amounts of ethanol).

Regarding MeOH contents in the oil at the VC outlet, no significant relationship with the water injection flow was observed (Fig. 5a). This can be due to the fact that less than 2.5% of the total methanol coming from the fruits reaches the oil at the VC outlet, either as MeOH or as esterified in MEs (Fig. 5c). Methyl esters showed a slight but not significant trend to decrease with the addition of water (Fig. 5b). This trend is more evident in terms of ME balance, referred to the initial methyl esters in the olives, especially in ripe olives (Fig. 5d). Within the studied range (0 to 8% of water addition), the trend is almost linear.

At the decanter level, the results for MeOH in oil suggest an interaction between the water injection rate and the type of olives. For green olives, methanol decreases with the addition of water following a quadratic trend with a minimum outside of the range studied (and possibly over 10% of water addition). However, for ripe olives (with less moisture content), the trend was inverse, with a maximum greater than approximately 5% (Fig. 5e). This interaction is equivalent, though opposite in trend, to that observed for ethanol. Regarding ME, the addition of water tends to increase methyl esters in the oil at the decanter outlet, following a quadratic trend that does not depend on the type of olives (Fig. 5f). Finally, no significant relationship between methanol dragged into the pomace and water injection rate was observed (Fig. 5g).

4. Conclusions

In summary, during the EVOO extraction process, there is no generation of ethanol but a positive synthesis of methanol. However, decanter paste injection rate affects the content of alcohols and alkyl esters in the oil, which tend to increase when working closer to the maximum capacity of the decanter. Although most of alcohols are dragged within the aqueous phase, significant amounts of ethanol can reach the oil at the outlet of the vertical centrifuge (up to 25% of ethanol present in olives), which increases the risk of FAEEs formation during the decantation and storage of the oil. On the other hand, most of the alkyl esters are removed through by-products and few of them could be hydrolyzed according to the fruit moisture content and the total water available in the system. In fact, unripe and ripe fruits result in different FAAEs amounts depending on water injection and paste injection rate used during the EVOO extraction process.

Thus, it can be concluded that the decanter and the passage from the decanter outlet to the vertical centrifuge could be key points that must be controlled to avoid FAAEs formation, and that water injection flow is a good regulation option when low quality fruits are processed.

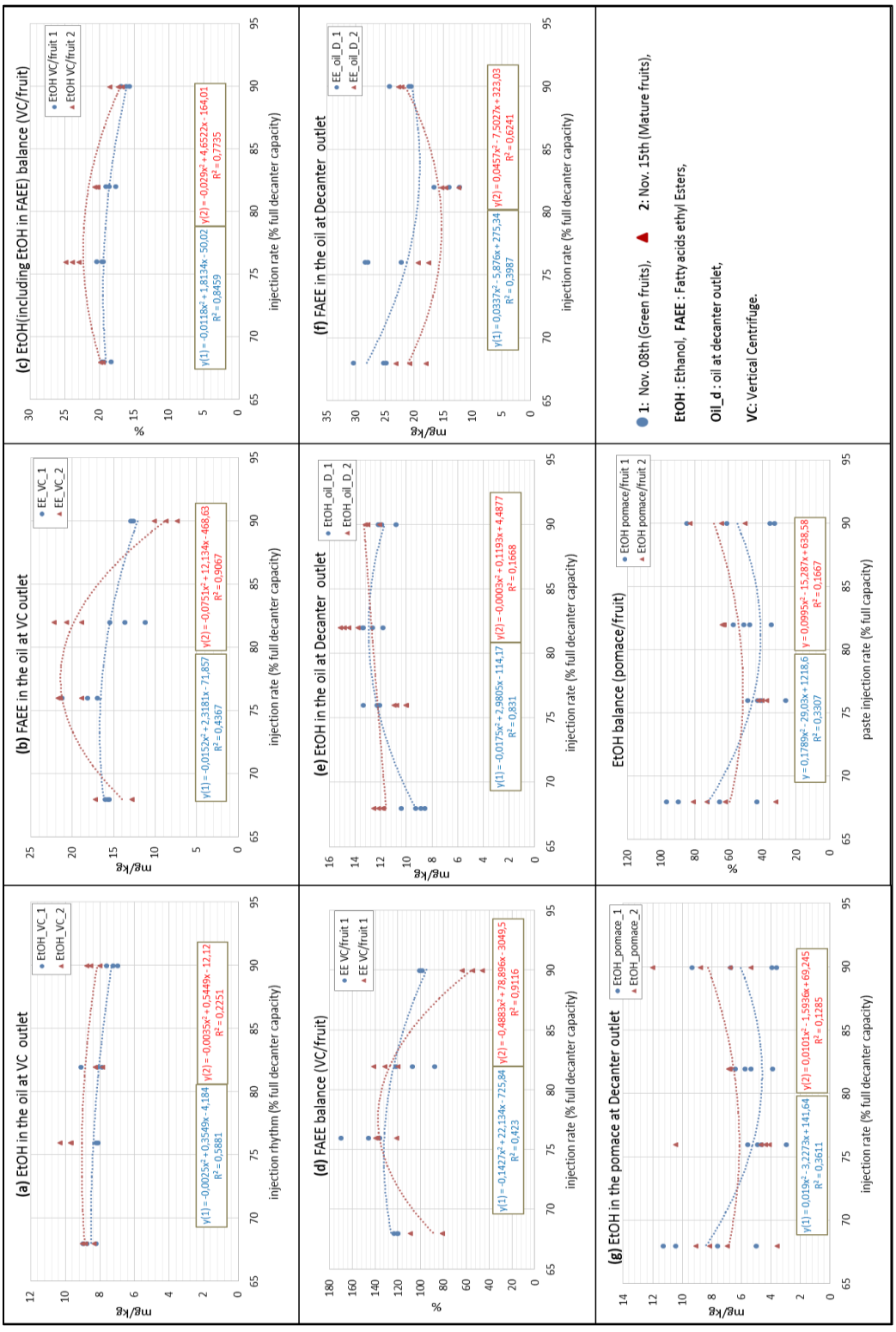


Figure 2. Paste injection rates effect on ethanol and ethyl esters in the decanter and the vertical centrifuge outlets.

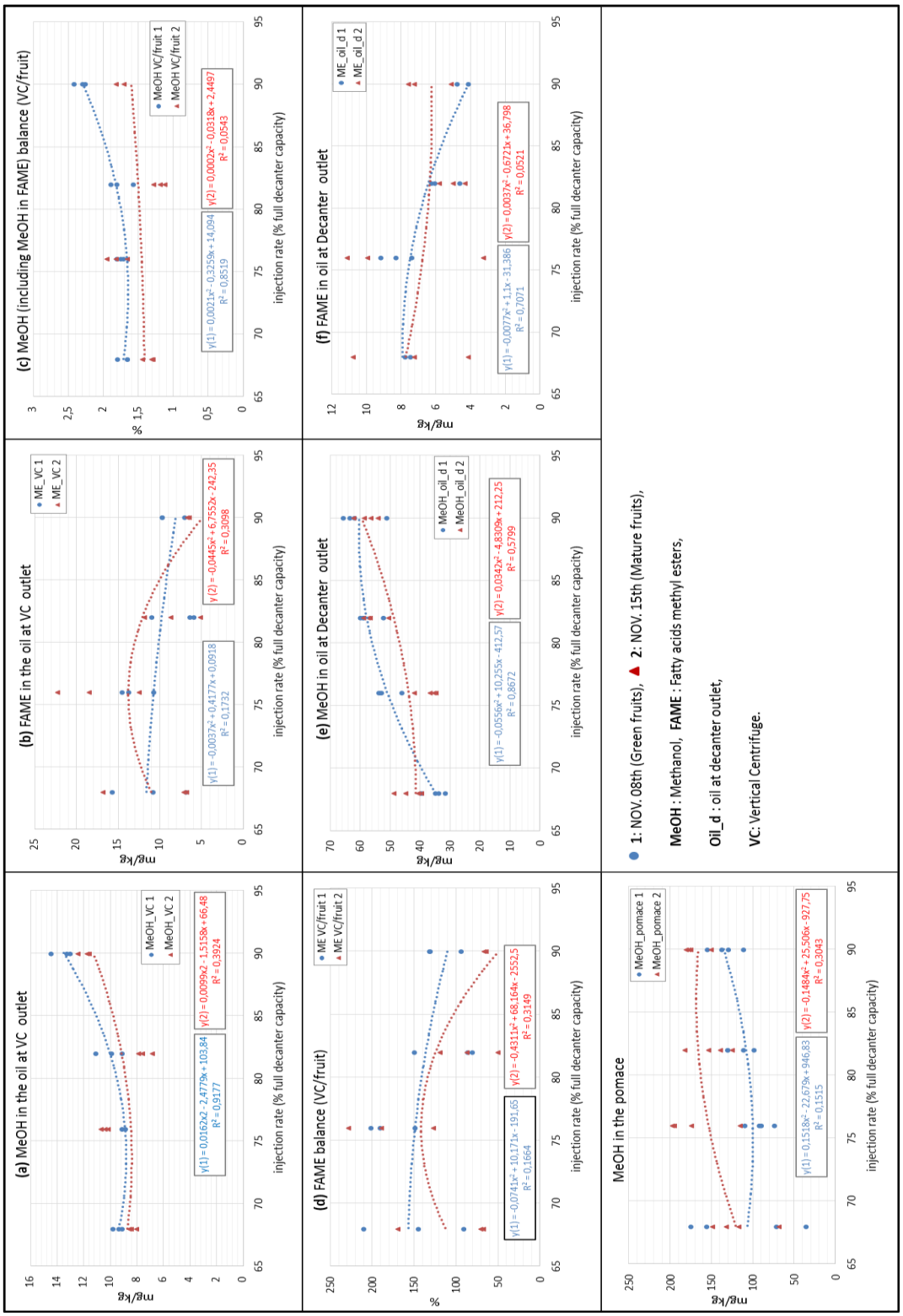


Figure 3. Paste injection rates effect on methanol and methyl esters in the decanter and the vertical centrifuge outlets

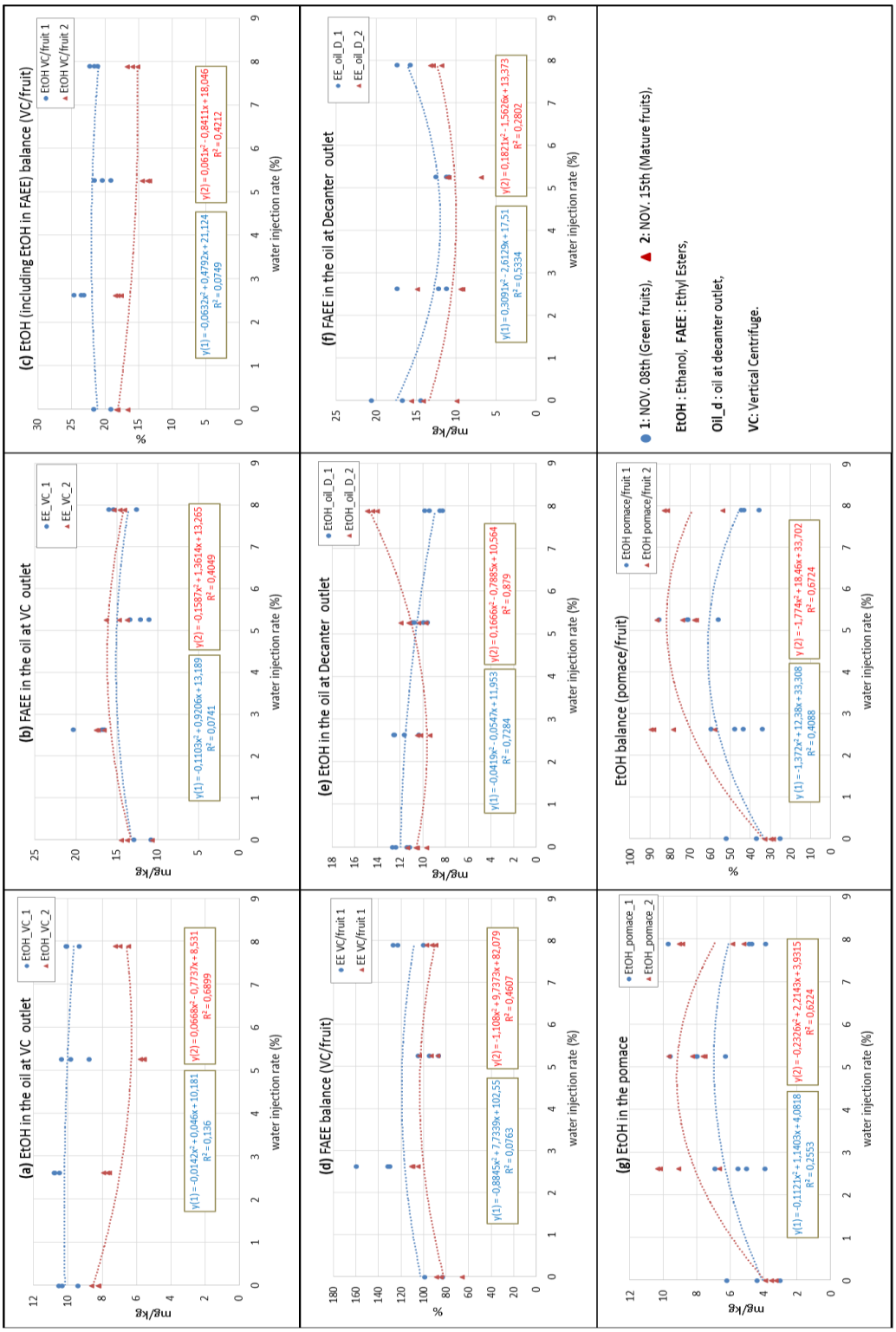


Figure 4. Water addition effect on ethanol (EtOH) and ethyl esters (FAEE) in the decanter and the vertical centrifuge outlets

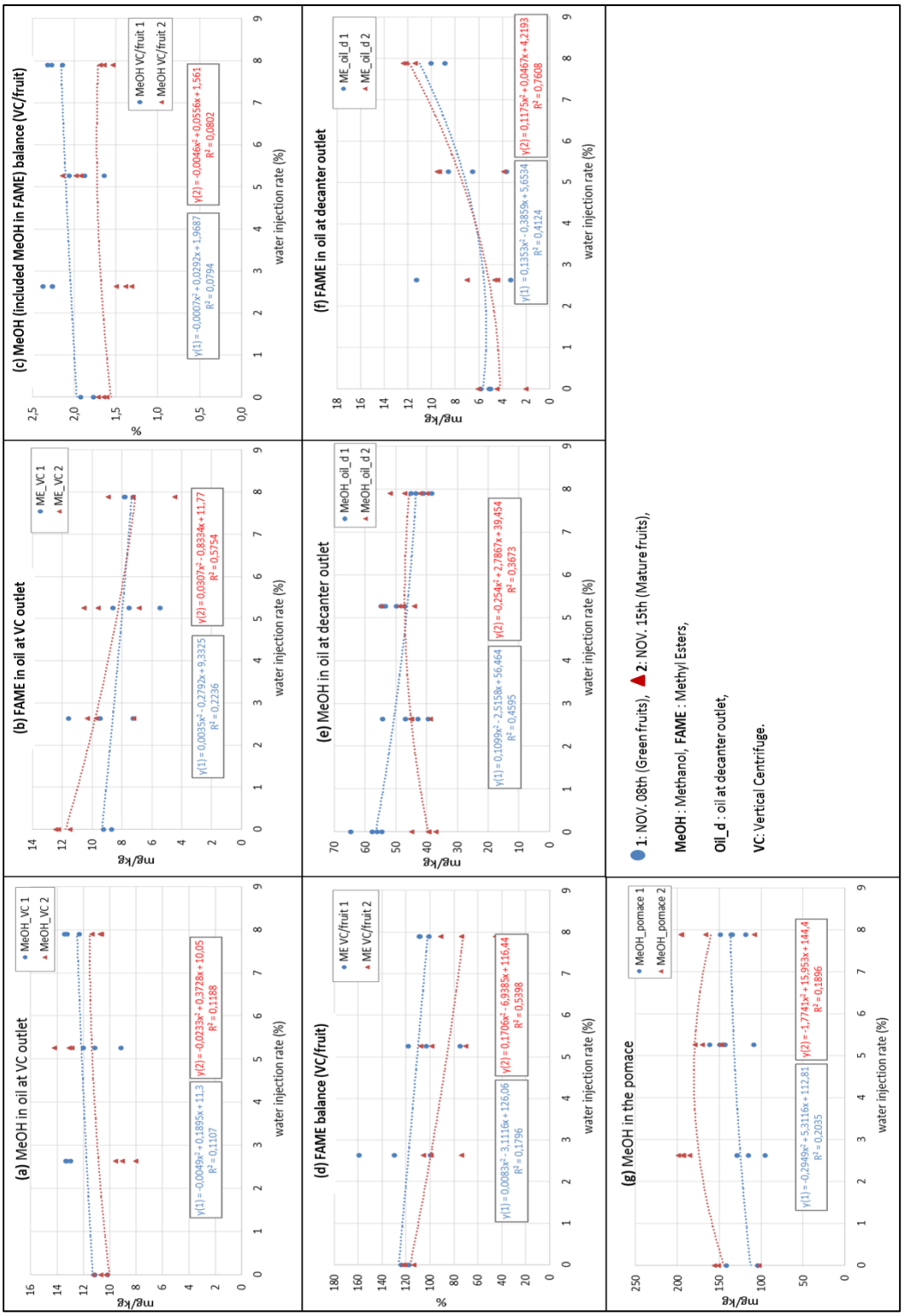


Figure 5. Water addition effect on methanol (MeOH) and methyl esters (FAME) in the decanter and the vertical centrifuge outlets

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Chapter 5 – (PAPER 4)

Survey of the current situation of fatty acid ethyl esters and their prevalence in virgin olive oil in Catalonia

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ABSTRACT

Virgin olive oil (VOO) is a highly valued commodity and is an essential element of the healthy Mediterranean diet. Although it represents only 6% of the Spanish production, Catalonia has focused on the elaboration of quality olive oils. The quality of VOO is controlled by an international standard, in which the content of ethyl esters of fatty acids (FAEEs) is used as a marker of the freshness, quality and good handling of the fruits throughout the processing of VOO.

The main goal of this study was to evaluate the contents of FAEEs in virgin oils from different productive areas in Catalonia, taking into account especially those areas considered at risk due the inadequate edaphoclimatic conditions. A total of 260 olive oil samples were analyzed over five harvests (2016-2020) and the possible interactions between FAEEs content and other quality parameters such as free acidity, peroxide value, UV-absorbancy, moisture, sterols, waxes and aliphatic alcohols were evaluated.

The results showed that most of the oils (>99%) bottled as extra virgin in Catalonia comply with the required FAEEs limits for this category (<35 mg/kg). However, it has been detected that around 6% of these oils are at risk of losing their category as they have a concentration of FAAEs between 25-35 mg/kg, close to the allowed limit. This behavior is not related to a specific area of Catalonia. Finally, a clear relationship was found between the increase in the content of ethyl esters and the increase in the values of free acidity, K_{270}, moisture, impurities and triterpene alcohols, which indicates that the presence of a high FAEEs content in Catalan VOOs is due to the use of poor-quality fruits or an incorrect storage of the oil.

Keywords: virgin olive oil, quality, ethyl esters.

1. INTRODUCTION

Extra virgin olive oil (EVOO) is one of the pillars of the Mediterranean diet known throughout the world for its large number of nutritional and healthy benefits. This is based on the traditional cuisine of the coastal countries of the Mediterranean Sea, so, Catalonia, located in the north-east of the Iberian Peninsula and bathed by more than 800 km of sea, is an area where this diet has a great tradition. In fact, so much so, that all the dishes of Catalan cuisine use, to a greater or lesser extent, olive oil for its preparation, which is why this valuable product is part of the Catalan culture.

The production of virgin olive oil (VOO) is distributed throughout all the Catalan territory, although the major production is situated in the areas of Tarragona and Lleida which represents 95% of the total production (GC, 2021). The main olive variety cultivated is 'Arbequina' but there is a large diversity of local varieties such as 'Empeltre', 'Morrut', 'Sevillenca', 'Farga', 'Fulla de Salze', 'Argudell', 'Rojal' or 'Verdiell' (Ninot, 2018, Tous et al., 1993). Thus, this diversity together with the different edaphological conditions justifies up to five Protected Designation of Origin (PDO) of olive oils throughout Catalonia, each with its own well-defined physical and organoleptic characteristics: ´´Oli de Terra Alta´´, ´´Oli del Baix Ebre-Montsia´´, ´´Oli d'Empordà´´, ´´Siurana´´ and ´´Les Garrigues´´ (Vichi et al., 2019).

The extension of most of the Catalan plantations is less than five hectares, so, generally, the production of virgin olive oil is carried out in small-capacity olive mills, which focus particularly on the production of high quality oils instead of massive production. In most areas, the harvest season begins in early November and finishes before January and only fruits directly harvested from the tree are processed. However, in some specific areas such as the southern Catalonia, the harvest lasts until February or March. At this time, many olive fruits have already fallen from the tree so these fruits are processed separately in the mills from those harvested from the tree. In any case, to get the best olive oil, the olive fruits processing begins in the shortest time possible from their collection which is usually less than 24h after harvest. In addition, most of the milling facilities belong to small cooperatives which allows a better control of both fruit management and the oil extraction process. The result of this good work is that 85% of the oils produced in Catalonia are of such quality that they can be marketed under the name of the best olive oil category: extra virgin olive oil (EVOO) (PRODECA & ACCIÓ, 2020).

The quality of VOO is directly linked to the quality of the fruits, as well as to their processing and storage conditions. In fact, several studies have reported that oils obtained from damaged

fruits (either by blows, diseases, insects, freezing, etc.) show not only a poor sensorial profile but also bad chemical characteristics (Rallo et al., 2018; Morales et al., 2014; Rufat et al., 2018, Vichi et al., 2009; Vichi et al., 2015). On the other hand, both inappropriate processing management during VOO extraction or unsuitable storage conditions negatively affect the olive oil quality (Vichi et al., 2015; Beltran et al., 2016; Morales et al., 2014).

There are several studies that have focused on the different chemical and biochemical reactions involved in these oil degradation processes. The different results have shown that, when dealing with damaged fruits and / or when working under incorrect conditions, mainly, the contact between oil, moisture and impurities favors the activity of both lipase and yeast with the consequent quality losses due to the oil acidity increase and fermentation processes, respectively (Di Giovacchino et al., 2002; Uceda et al., 2006; Vichi et al., 2011). Regarding this fermentation process, some studies relate it to an increase in the content of fatty acids ethyl esters (FAEEs). In addition, Gómez-Coca et al. (2016) have reported that this FAEEs content in olive oils increase dramatically after packaging, mainly in those oils stored under conditions that also cause an increase in acidity such as high values of temperature and moisture (Fortini et al., 2016).

This direct relationship between oil quality and FAEEs content has meant that the International Olive Council (IOC) and the European Commission have established FAEEs content as a quality parameter in the current trade standard applied to virgin olive oils. Thus, it is used to ensure the use of good quality fruits during VOO production and also to distinguish the EVOO category by limiting its content to 35 mg/kg (IOC, 2015; EC, 2019) although there is a proposal to reduce this content to 30 mg/kg.

Currently, there are no published data concerning the amounts of ethyl esters in VOO produced in Catalonia. However, it is assumed that southern Catalonia and some other areas with high prevalence of pests and diseases that damage the olives or cause them to fall to the ground, have a greater risk of presenting a high content of ethyl esters. This is why a large number of VOO produced in these areas are classified into the “Lampante” category.

Thus, the main goal of this work was to objectively estimate the actual situation of ethyl esters levels in the VOO produced in Catalonia. Therefore, the prevalence of the quality of virgin olive oils was evaluated according to the new standard of the European Union Commission (Regulation (EU) 2019/1604) considering the content of ethyl esters as a quality parameter to distinguish the extra virgin olive oils.

2. MATERIALS & METHODS

2.1. Olive oil samples

A total of 260 olive oil samples were analyzed over 5 years (2016-2020). The sampling strategy included two different groups. A first group consisted of 114 oils that arrived throughout the years 2017-2020 at the Official Food Analysis Laboratory of the Catalan Government (Laboratori Agroalimentari de la Generalitat de Catalunya, Cabrils, Barcelona) from all the olive growing areas of Catalonia. In fact, these samples were sent by many different producers and traders in order to check the quality of their oil batches. Therefore, this group can be considered as the control group as it was made up of random and representative samples from different origins and categories.

The second group consisted of 146 samples from 17 cooperatives and traders in southern Catalonia, where the risk of high FAAE content was considered to be greater. The sampling was carried out during the years 2016, 2017 and 2020 and it was organized and managed by the Institute of Agrifood Research and Technology (IRTA, Constantí, Spain).

2.2. Analytical determinations

The quality parameters that were determined for each of the analyzed oils were: FAEEs, free fatty acids or free acidity (as % oleic acid), peroxide value, ultra violet absorbency (or spectrophotometric constants K_{232} and K_{270}), moisture (as weight %), total amount of sterols, waxes content, quantity of aliphatic alcohols and % of oleic acid. The analyses were carried out at the Official Food Analysis Laboratory of the Catalan Government (Laboratori Agroalimentari de Cabrils, Generalitat de Catalunya) following the official methods proposed by the European Commission (Regulation (EC) 2568/91 and its updates).

2.3. Statistical analysis

Statistical analysis of the results was performed using the SAS-Stat Software (V9.4. SAS Institute Inc., Cary). A distribution analysis was carried out with ethyl esters contents. Average, median, percentiles 75% and 25% and inter-quartile range were calculated. Results were presented using boxplot graphics. The UNIVARIATE procedure from SAS software was used to do this. In addition, a correlation procedure was applied to find out possible interactions between ethyl esters content and other quality criteria. And, a linear regression between the FAEEs content and free acidity was performed.

3. RESULTS & DISCUSSION

When considering the traditional quality criteria, that is, regardless of the FAEEs content, the results obtained from the chemical analyses of the different samples showed two groups, depending on their potential market.

The first group included the oils suitable for direct consumption after bottling, that is, oils belonging to the "extra virgin" and "virgin" categories. The second group consisted of the oils which require a refining process before consumption i.e. the "lampante" category. According to this classification, among the 260 samples analyzed in this study, 123 samples were categorized as "extra virgin" or "virgin" and 137 samples as "lampante".

However, when analyzing the FAEEs contents, it can be observed that almost all the lampante samples exceeded the maximum value set by 35 mg/kg (Figure 1). In fact, only 5.8% of these samples had a lower FAEE content but, having acidity lower than 2% and a peroxide value higher than 20 meq O_2/kg, it could be concluded that these oils had degraded to the lampante category due to an oxidative problem.

Regarding to the 123 better quality samples, these showed different behaviors when considering the FAEEs values (Figure 1). Thus, 8.9% showed a content higher than the allowed 35 mg/kg, so these could no longer be considered suitable for bottling and marketing under the "extra virgin" category. On the other hand, 16.3% of the samples could be considered as the risky group as their FAEEs concentration ranged between 25 and 35 mg/kg and, therefore, could easily become non-consumable if alkyl esters increase during storage. Regarding the remaining 73.2% of the samples, these contained FAEEs concentrations lower than 25 mg/kg, so it could be considered a safety group. Finally, the hypothetical situation that would arise if the legal content of FAEE were reduced to the proposed 30 mg/kg was also considered. These circumstances would affect the samples with a concentration between 30 and 35 mg/kg, which represents 1.6% of the samples.

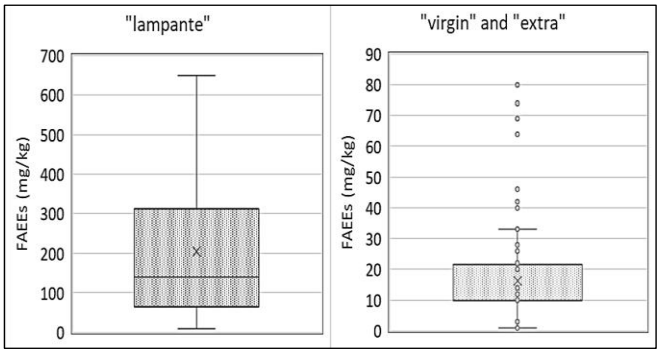

Figure 1. Boxplots for the distribution of fatty acid ethyl esters contents through the claimed categories

The box upper limit represents percentile 75%, the lower limit is for percentile 25%, the inner line is for percentile 50%, the inner cross is the average, edges of the vertical lines limit the maximum and minimum observations above which values can be considered as outliers.

Regarding the group of samples categorized as "extra virgin", only 0.8% of the samples exceeded 35 mg/kg while the samples considered as the risky group represented 5.7% of the total (Figure 2). These oils may overpass the limit established for FAEEs content during its storage even in the tanks (before bottling) or after their delivering to market, mainly when the conditions of storage provokes an increase in ethanol (fermentation) or free acidity (triglyceride lipolysis).

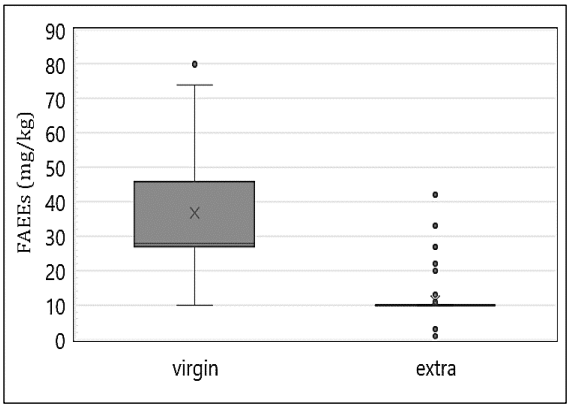

Figure 2. Boxplot of fatty acid ethyl esters (FAEEs) content in "virgin" and "extra virgin" categories

To study the effect of origin on the quality of the olive oil, the results obtained in the analysis of the 146 samples coming from southern Catalonia were evaluated separately. This evaluation corroborated what was already suspected about this area since, as already explained, its olive

fruits are prone to suffer more damage. In fact, when considering "extra virgin" and "virgin" groups together (representing 34.9% of the 146 samples), it was observed that up to 11.8% presented FAEEs contents greater than 35 mg/kg and 21.6% between 25-35 mg/kg. However, it has to be pointed out that, when evaluating only the "extra virgin" category the situation is equivalent to that previously described for the whole of Catalonia, since none of the samples exceeded the limit of 35 mg/kg, the 5.9% were between 25-35 mg/kg and 2% between 30-35 mg/kg.

❖ Relationship between FAEEs content and other quality parameters

To determine the possible relationship between the FAEEs content and the value of each of the quality parameters determined for the different samples analyzed, an attempt was made to establish the statistical correlations, if any, by using the Pearson's correlation coefficient. With this value it was possible to get information about the magnitude of the correlation, as well as the direction of the relationship. The results obtained are shown in Table 1. As can be observed, FAEEs content was mainly correlated with the oil acidity although there are also other important positive correlations with triterpenic alcohols, moisture, K_{270} absorbance, waxes and, to a lesser extent, with impurities and aliphatic alcohols. These results are consistent with the fact that ethyl esters increase when olives are damaged by fermentative microorganisms which also can favor the increase in acidity and triterpene alcohols. Moreover, moisture and impurities in the oil can also favor FAEEs formation as well.

Table 1. Pearson's correlation coefficients between FAEEs contents and other oil quality parameters

Parameter	Pearson's correlation coefficient	Probability	Number of observations [z]
Acidity (% oleic acid)	0.847	<0.0001	205
Peroxide value (meq O_2/kg)	-0.105	0.1333	204
K_{232}	0.068	0.4514	126
K_{270}	0.556	<0.0001	126
Moisture (%)	0.582	<0.0001	122
Impurities (%)	0.327	0.0014	93
Total sterols (mg/kg)	0.052	0.5495	136
Waxes (mg/kg)	0.484	<0.0001	136
Aliphatic alcohols (mg/kg)	0.318	0.0002	135
Erythrodiol + Uvaol (mg/kg)	0.643	<0.0001	132
Oleic acid (% C18:1)	-0.029	0.7386	134

(z) Not all the samples were analyzed for all the parameters

Taking into account the good correlation when dealing with oil acidity; this relationship was studied in more detail (Figure 3). In fact, the observations fit a linear model with a coefficient of determination (R^2) = 0.8715 although some atypical values were detected. Some of these outliers showed relatively high content of ethyl esters (600-700 mg/kg) with acidity values lower than 4.5% and other had relatively low concentration of ethyl esters (100-150 mg/kg) but with very high acidity values (more than 9.5%). A possible explanation for those atypical values could be related to the availability of FAEEs precursors, that is ethanol and free fatty acids. Thus, if an extremely high production of ethanol occurs during the oil production, this ethanol could esterify a large amount of free fatty acids resulting in a reduction in the acidity value. In contrast, if ethanol is not produced but a lipolysis process takes place, then a high acidity value could be reached without FAEEs formation. This should justify the convenience to consider both free acidity and FAEEs analysis in the quality parameters, in order to cover all the possibilities.

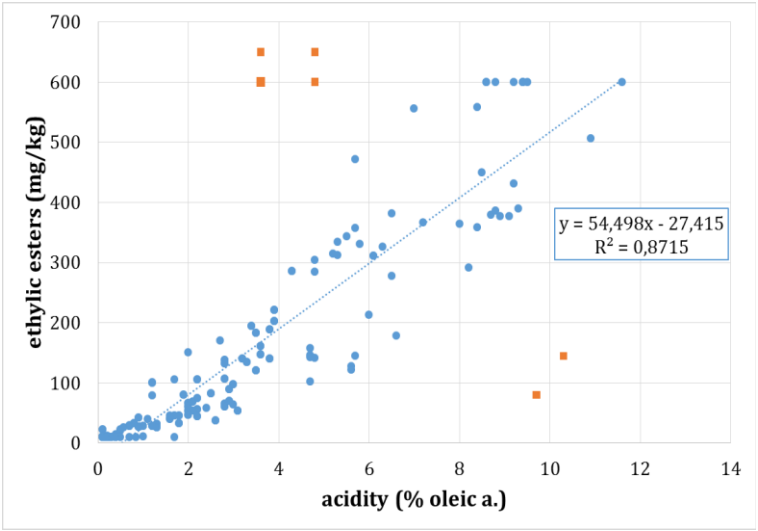

Figure 3. Linear model for FAEEs content as function of oil acidity. (Square dots highlights atypical values)

4. CONCLUSIONS

- Up to 99% of extra virgin oils in Catalonia present levels of fatty acid ethyl esters below the limits established by the standard.
- About 6% of Catalan extra virgin oils are at risk of losing their category due to their high content in ethyl esters (25-35 mg/kg).
- A clear correlation between the ethyl esters content and other quality parameters of olive oil quality deterioration was pointed out (i.e. acidity and moisture).

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CHAPTER 6.

General Discussion

6.1. Introduction

In recent years, fatty acid ethyl esters (FAEEs) have been incorporated into the European and International standards for olive oil trade, as a criterion of "freshness" of olive fruits and their good management during the production of virgin olive oil. Although the application of this normative is still under review (both in terms of maximum content allowed and the analytical method to be used), the current limit is 35 mg FAEEs/kg oil [1, 2]. Given the trend of recent years, it is becoming more and more evident that its incorporation into international standards and the possible requirement to display it on the packaging label will end up being a reality.

As it has been stated in the previous Chapters, Catalonia produces and commercializes high quality oils, although there are some risky areas where the olive fruits are prone to suffer damage, so the generation of high contents of FAEEs can be a handicap. This situation could be aggravated for olive varieties such as 'Arbequina', which tend to produce endogenous alcohols during the ripening process [3]. In addition, olive mills in Catalonia use big hoppers that favor fruit damaging during the storage and this fact could increase the production of ethanol [4, 5]. In all cases, the final problem is the same since the alcohols from the olive fruits can be transmitted to the oil during the production process or their storage, thus acting as precursors of FAEEs [6, 7]. However, to our knowledge there is no published information on current levels of FAEEs in commercial olive oils produced in Catalonia, so the need for a study on the current situation of FAEEs in commercial Catalan olive oils and what are the main factors that influence their content becomes evident.

It must be highlighted that during this study it was intended to carry out the experiments in the most realistic conditions possible for the production of VOO in Catalonia.

6.2. Prevalence of fatty acid ethyl esters in Catalan virgin olive oil

To assess the level of ethyl esters in virgin oils in Catalonia, the oil sampling was carried out in all production areas, with special attention to the most vulnerable areas where it was assumed that there was a greater risk of high content of FAEEs (southern area of Catalonia), due to different conditions such as climate, varieties and pests and diseases.

The prevalence of the contents of ethyl esters in different virgin olive oils analyzed in Catalonia shows that most of the oils (>99%) packaged as extra virgin comply with the required limits of FAEEs for this category. However, it has been detected that there are some olive oils that present the risk of ending up with a high content of FAEEs, as they have a concentration of these compounds between 25 and 35 mg/kg. These oils represent around 6% of the extra virgin olive oil in Catalonia. This group is not related to a specific area of Catalonia, but could be related to a late harvest of the olives. This delay in harvesting, results in a higher FAEE content not only from damage to the fruit but also from an advanced stage of ripeness. And in any case, it could also be related to some varieties of olives.

The physicochemical analyses of the oil samples indicate a clear relationship between the increase in the content of ethyl esters and the increase in the values of free acidity, K_{270}, moisture, impurities and triterpene alcohols. In addition, the relationships between these parameters, such as free acidity-moisture, impurities-K_{270} and moisture-impurities-aliphatic alcohols, indicate that the presence of high contents of FAEEs is due to poor quality fruits, an inaccurate milling process or inadequate storage conditions.

These results are consistent with those obtained in previous studies. In fact, the increase of free acidity has been well documented and referred to the low quality of olive fruits [8–11], but also correlating it with prolonged contact between the oil and moisture or even with incorrect storage conditions such as a too high temperature [12–14]. Moreover, there are also other studies that state that the fermentative process that occurs due to unsuitable olive storage conditions, together with fruit infestations (olive fly or anthracnose) and the presence of moisture and impurities in olive oil, are the main sources of ethanol [15, 16].

6.3. Effect of the cultivar on the endogenous amounts of ethanol, methanol and acetaldehyde

The use of healthy olives and their good management throughout the production process are necessary to obtain high quality virgin olive oils. It is already well known that the olive cultivar (olive variety) affects not only the fat content but also the chemical composition and sensory characteristics of the oils obtained [11, 17–19]. But even when using healthy olives, it is important to consider the content of FAEEs. This is because the fatty acid alkyl esters (FAAEs) come from a reaction between chemical compounds naturally present in healthy olives: the esterification of C16 and C18 free fatty acids with short chain alcohols. Therefore, having information on the amount of these precursors in the fruits is essential to control the content of FAAEs. Several authors have already reported factors that increase the content of free fatty acids, such as maturity, fruit quality and moisture [20–23]. However, it is also necessary to know the presence of alcohols (mainly methanol and ethanol) in olive fruits and their origin (endogenous or from fermentation).

Thus, a method for quantitation of low molecular alcohols in homogenates from olive fruits was developed, and the effect of the olive variety on the endogenous content of methanol and ethanol (FAAEs precursors) and acetaldehyde (Acet) (ethanol precursor) was assessed. Different olive varieties grown in Spain and, in particular, in Catalonia were used to carry out this study [24]. The results showed that ripen olive fruits from different varieties generate a significantly different content of endogenous alcohols (figure 6.1). Moreover, the results made it possible to group different varieties according to the amount of endogenous alcohols. In fact, some varieties such as ‘Hojiblanca’ and ’Morrut’ showed a very high level of endogenous ethanol (up to 100 mg/kg), which increases the risk of formation of ethyl esters during processing. On the contrary, there is group of varieties such as ‘Sevillenca’, ‘Arbosana’ and ‘Argudell’ with a very low ethanol content (lower than 15 mg/kg). These varieties also showed the highest Acet/EtOH ratio, which is related to a low physiologic activity of ADH enzymes [3]. Finally, a third group of varieties includes ‘Arbequina’ and ‘Picual’, with medium ethanol level (30 to 60 mg/kg). In this case, special care must be taken during processing to prevent ethanol from passing into the oil. Concerning methanol, significant differences in content were observed, mainly for ‘Argudell’ and ‘Cornicabra’ that showed concentrations higher than 150 mg/kg. The presence of methanol in olives is related to the degradation of the pectin cell wall by the PME enzyme activity, giving rise to the methyl esters in VOO [3, 25]. Nevertheless, so far, methyl esters are not limited in the official regulation.

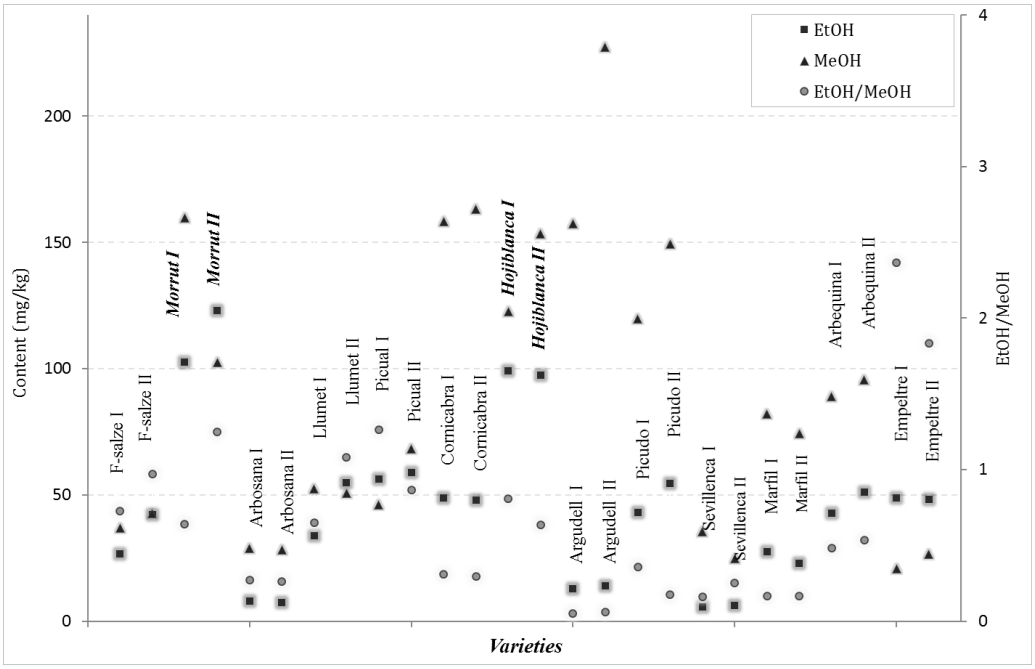

Figure 6.1. Ethanol and methanol levels in fruits from different olive varieties at their ripen stage of maturity (Boudebouz et al., 2020)

Regarding the state of the fruit, it was found that the healthy state of the olives significantly influences the amount of ethanol. Unhealthy fruits produce higher content of ethanol due to the fermentation of sugars present in the flesh. In addition, low quality fruits can produce olive oil with high free acidity, due to the action of the lipase enzyme, which could also result in an increase of ethyl esters [26–28]. A detailed study of ethanol levels in completely healthy or damaged fruits from varieties that produce different endogenous ethanol contents, concluded that a high content of short chain alcohols is not always due to an unhealthy or deficient state of the fruits. Hence, as these alcohols are the precursors of FAAEs, having the same value of FAEEs to assess the freshness of all the varieties seems to be meaningless. The olive variety has to be considered as it is closely related to this parameter [24].

Concerning methanol, the experimental results showed that its content is more related to the ripening stage than to the fruit quality, though it is known that fruits strongly affected by olive fly can release high contents of methanol [25].

6.4. Effect of short-term storage on the alkyl alcohol and their relationship with sensory quality losses

Once harvested, the fruits are transported to the mill and stored until processing. Fruit storage conditions play an important role in the quality of olive oil, as prolonged storage can cause damage to the fruit that can affect not only the chemical quality, such as an increase of free acidity, but also the sensory quality [21, 29]. This is why it is recommended to use medium-sized perforated boxes (maximum 1000 kg capacity) for the storage of olives, and proceed to their processing as soon as possible. However, it should be noted that the storage of olives in large hoppers is a common practice in the Catalan industry because of the large quantity of olives delivered every day during the harvest season.

The use of large hoppers for olive storage is a critical practice that results in important fruit damages, due to the high pressure exerted by the charge of the fruits. Moreover, the distribution of the charge inside the hopper is not homogenous and the impacts between fruits and the bruises from direct contact with the hopper walls cause irregular damage to the stored fruits. In fact, some of the experiments carried out in this thesis showed how the charge of the hoppers affects the fruit quality, the ethanol and methanol production and the final oil sensory characteristics (figure 6.2).

Specifically, the results indicate that the ethanol content increases dramatically during fruit storage, even in the short time used in the experiments (3 hours maximum), what is in agreement with Beltran et al. [30]. Furthermore, when evaluating the organoleptic characteristics of VOOs, an increase of some negative descriptors (``winey´´, ``musty´´ and ``fusty´´) were detected due to fruit damage and fermentations inside the hopper, which is in agreement with Di Serio et al. [31]. As expected, fruits located at the bottom of the hopper suffer a greater effect. Besides, it was observed that during hopper discharge, the fruits do not come out in layers from the bottom to the top, but concentrically from the center to the sides of the hopper. Therefore, as shown in figure 6.2, the fruits located at the position T1 are the first to be discharged, followed by those located at T10 and finally the ones located at the position T20. As a result, the last fruits to be downloaded are often of lower quality, since they were bruised by the walls of the hopper. The negative effect of the hopper is even stronger with olives with a higher moisture content and a high flesh to pit ratio because they are more sensitive to mechanical impacts and microbiological spoilage, especially to fermentations promoted by yeasts. This result is very important for the southwest area of Catalonia, where the experiments

were carried out, because new irrigation projects are being developed with the 'Arbequina' variety that will produce larger fruits, richer in moisture and with a higher flesh to pit ratio.

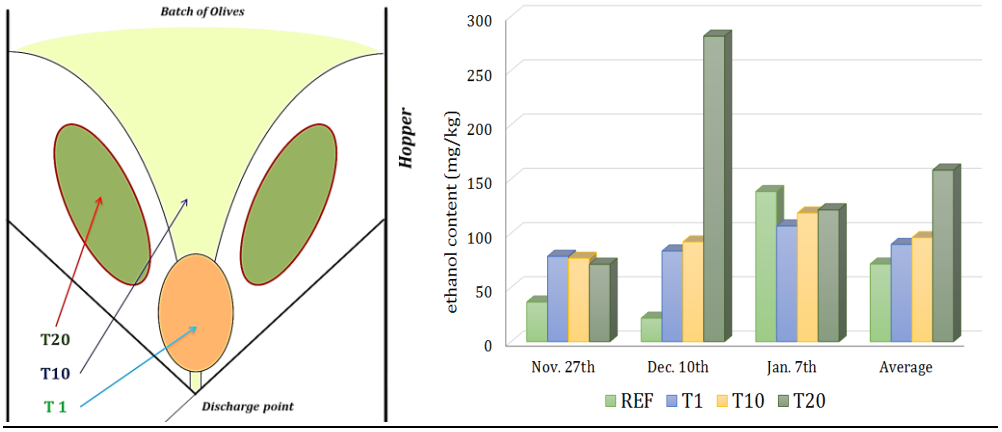

Figure 6.2. Variation of ethanol formation during olive storage in large capacity hoppers

6.5. Effect of processing factors on the balance of alcohols and alkyl esters: separation and clarification steps

Olive oil extraction using a two-phase system consists of several steps to separate the olive oil from solids and water. Since alcohols are polar, most of them are expected to be dragged through the pomace. On the other hand, FAAEs are non-polar and tend to move into the oil. However, these partitions need time and suitable physicochemical conditions. One of the elements in which the master miller can change the time and partition factors is the horizontal centrifuge (decanter). In fact, once the physical parameters (diaphragm position, feed pipe position and differential speed) have been set, the only two parameters that can be easily changed are the paste injection rate and the addition of water (below 10%). Thus, the study of the effect that these two options have on alcohols and FAAEs partition was one of the goals of this thesis.

Needless to say, the process is not completely continuous, as the connections between the different machines can favor that part of the paste remains for an uncertain time before moving on to the next machine. Fermentative reactions can take place at these discontinuous points with possible release of alcohols and FAAEs. One of these critical points could be the vibro-filter

unit located between the decanter and the vertical centrifuge, so the study of this step was another goal objective of this thesis.

The experiments were carried out with olives of the 'Arbequina' variety at two different ripening stages (green and ripe) and operating in a real two-phase processing unit (facility of La Granadella cooperative). Four water injection doses (0%, 3%, 5% and 8% referred to total paste) and four paste injection rates (68%, 76%, 82% and 90% of theoretical decanter capacity) were tested. In all cases, the balances of alcohols and FAAEs between the system inlet and the outlets of both the decanter and the vertical centrifuge were calculated.

Obviously, the use of water during VOO production is not the best option to obtain high quality oils. It has already been reported that the addition of water decreases the content of minor components of olive oil, mainly phenolic and volatile compounds [14, 32]. This decrease of phenolic compounds and antioxidants could be associated to a decrease of the oxidative stability and shelf life of the oils produced [33]. Therefore, in this thesis the addition of a maximum of 10% of water has been proposed, since several research studies have shown that the reduction of polyphenols within these values, and even more, is acceptable when working with a biphasic system [34, 35]. It should be taken into account that there are new innovative water injectors that supply water directly to the decanter without mixing it with the olive paste, which minimizes polyphenol losses [36]. When considering the industrial production, in many cases, the main objective of producers is to obtain VOO that meets the minimum requirements to be suitable for bottling as extra virgin, without any concern about healthy components such as polyphenols. Thus, the content of phenolic compounds does not seem to interest the producer, mainly in the period of full productive capacity where the reception of large quantities of olives implies an increase in storage time. In addition, the paste injection ranges chosen for this study were based on a previous work that reports that the best extraction for a two-phase decanter ranges between 60 and 90% of the theoretical machine capacity [37]. Finally, it should be noted that the study of these two adjustment options is particularly useful because they are universal for any type of decanter and can be changed at any time without stopping the machine and interrupting the process.

The results from the experiments carried out show that during the process there is no synthesis of ethanol, which is in line with the results reported by Masella et al. [38]. Concerning methanol, although its content may decrease due to evaporation, esterification or dilution in the aqueous phase, in this study we observed a positive synthesis that could be due to the increase

of pectin methyl esterase after the rupture of the cell wall, as reported by several authors [25, 39].

Regarding the paste injection rate and addition of water to the decanter, although a more detailed discussion can be found in the published article [40], the following results can be highlighted.

As for the paste injection rate, a significant quadratic trend was detected at the decanter level with respect to FAEEs and EtOH. Between 76% and 82% of work capacity, a shorter time oil remains in the system reduces the possibility of EtOH esterifying to FAEEs. Above 95% of work capacity, it seems that the higher moisture content in the oily phase dilutes more EtOH that can esterify free fatty acids, reducing the acidity and increasing the content of FAEEs. As for MeOH, higher concentrations are found at higher injection rates, inversely to what happens with fatty acid methyl esters (FAMEs).

As for the effects of water injection, the balance of alcohols and FAAEs shows an inverse relationship between the content of fatty acid ethyl esters and unreacted ethanol when adding water, although no differences were observed for methanol. However, this effect is not proportional to the amount of added water. Similarly to paste injection rates, higher concentrations of ethanol and FAEEs were observed at very high and very low injection rates, with a decrease when working at intermediate injection rates. In addition, the significant interaction observed between fruit ripening and processing factors suggests that the addition of regulated amounts of water during the separation step is a good option to reduce the risks of FAAEs formation. Especially when low quality fruits are processed, that is, those that arrive to the mill after the second half of December, usually overripe and sometimes damaged by the usual frosts in the center-west of Catalonia at that time of harvest.

The results obtained show that although the major part of alcohols are dragged within the aqueous phase, in agreement with Vidal et al. [14], almost all FAEEs from the olives reach the oil at the vertical centrifuge outlet, independently of the water addition, whereas FAMEs slightly decrease with water addition. Moreover, after studying carefully the oil path between the decanter outlet and the centrifuge inlet, it was confirmed that this is a critical step for the synthesis of ethyl esters. In fact, at this point an increase of the main precursors of FAEEs (ethanol and free acidity) was observed, which could be related to the high amount of moisture and impurities (mainly sugars form olive flesh). Therefore, its accumulation in the vibro-filter tank, together with the presence of favorable conditions of time and temperature, increase not

only the activity of yeasts (generation of ethanol), but also the lipolysis of triacylglycerides (TAGs), which results in an increase of free acidity. This result confirms the suspicion about the improper design of this step that concerns most of machine manufacturers.

From all these results, it is clear that in order to reduce the risk of presence of ethyl esters in VOO in Catalonia, selecting healthy olives is necessary but not sufficient. Earlier production is also recommended, especially when it comes with varieties with higher risks, such as 'Morrut', which produces greater amounts of endogenous alcohols. This risk increases mainly during fruit storage, due to the effect of the charge of olives when using large capacity hoppers (those commonly used in the Catalan mills). Moreover, the milling process must favor ethanol and methanol dilution in the waste phase, avoiding their concentration in the oily phase (especially when having olives of poor quality), as it has been shown in this thesis. Finally, pre- filtration of VOOs is recommended to remove moisture and organic impurities and thus prevent the formation of ethyl esters during storage.

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CHAPTER 7.

Conclusions

7. CONCLUSIONS

The main conclusions based on the initially defined objectives and derived from the results obtained throughout the development of this Doctoral Thesis are:

1. The great majority of extra virgin oils produced in Catalonia present levels of fatty acid ethyl esters below the limits established by the Official Regulation.
2. About 6% of these extra virgin oils are at risk of losing their category due to their content in ethyl esters that is very close to the allowed limit (25-35 mg/kg).
3. Catalan olive oils show a good linear correlation between the content in ethyl esters and the values of other quality parameters used to evaluate oil deterioration (i.e. acidity and moisture).
4. There are significant differences between varieties regarding their endogenous ethanol content. Particularly, the Catalan cultivars 'Morrut', 'Llumet', 'Empeltre' and 'Arbequina' produce high levels of ethanol, whereas 'Sevillenca', 'Argudell' and 'Arbosana' produce very low levels. Thus, a high content of short alcohols is not always due to an unhealthy or poor state of the fruits as the variety has also to be considered.
5. There are significant differences between varieties regarding their endogenous content of methanol and acetaldehyde. However, no significant differences were observed between healthy and unhealthy fruits.
6. A unique value of fatty acids ethyl esters should not be set for all the olive varieties, contrary to what establishes the Official Regulation.
7. The storage of olives in a large-capacity hopper, even for a short time, usually involves damages that induce certain microbiological activity with the subsequent generation of ethanol and sensory quality losses.
8. During the first step of the extraction process (crusher-malaxer-decanter), there is no generation of ethanol, but a synthesis of methanol occurs.
9. Significant amounts of ethanol can reach the oil at the outlet of the vertical centrifuge (up to 25% of ethanol present in olives), which increases the risk of formation of fatty acid ethyl esters during the decantation and storage of the oil.

10. During the olive oil processing some alkyl esters could be hydrolyzed by the water available in the system.

11. The rate of the paste injection into the decanter affects the content of alcohols and alkyl esters in the oil as these tend to increase when working closer to the maximum capacity of the decanter.

12. The flow of water injection into the decanter has an opposite effect to the paste injection rate because it tends to drag ethanol with pomace. The injection of a suitable amount of water into the decanter is a good regulation option when low quality fruits are processed.

13. Low amounts of ethyl esters can be synthesized when the oily fraction remains, during a short time, in the vibro-filter unit located in the connection between the decanter and the vertical centrifuge. The control of this connection step is a key point to avoid the formation of fatty acid alkyl esters.

APPENDIX

A1. Participation in Congress & Seminars

- Oral Communication. IRTA-PhD annual Seminar–2020. ´´Effect of agronomics and technological factors of the formation of alkyl esters in virgin olive oil ´´. *(December 2020). Online*
- Oral Communication. Meeting of the Technical Committee of the International Olive Council (IOC). ``Effect of agronomic and technological factors on the formation of fatty acids alkyl esters in virgin olive oil``. (*November 26th, 2019*). (Madrid, Spain)
- Oral Communication. IRTA-PhD annual Seminar – 2019. "Impact of agronomic and technological factors on the formation of ethyl esters in virgin olive oils". (*October 2019*). (Torre Marimon, Barcelona)
- Oral Communication. International Congress OLIVEBIOTEC´18. ''Parameters that increase the content of ethanol and methanol during post-harvest and processing of 'arbequina' ''. (*October* 2018). (Sevilla, Spain)

A2. Publications

- Boudebouz, A.; Romero, A.; Hermoso, J.F.; Boqué, R.; Mestres, M. (2021). Processing factors that affect the balance of alcohols and alkyl esters during 'Arbequina' olive oil production: centrifugation step. *LWT- Food science and Technology*, 149: 111842. DOI: https://doi.org/10.1016/j.lwt.2021.111842
- Boudebouz A., Agustí Romero A., Boqué R., Laura Aceña L., Busto O. and Mestres M. (2020). Quantitation of endogenous amount of ethanol, methanol and acetaldehyde in ripe fruits of different Spanish olive varieties. *Journal of the Science of Food and Agriculture, 100: 3173-3181*. DOI: https://doi.org/10.1002/jsfa.10352
- Boudebouz, A.; Romero, A.; Hermoso, J.F.; Boqué, R.; Mestres, M. Alcohol formation during short-term storage of 'Arbequina' fruits and their relationship with olive oil quality losses. *Postharvest Biology and Technology*. Submitted

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