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COORDINATORE Prof. Elisabetta Teodori

Extra virgin olive oil, olive milling by-products and "Virgin grape seed oil": chemical characterization, biological activity and sensorial studies for their nutraceutical valorization

Settore Scientifico Disciplinare CHIM/10

Dottorando
Dott. Lorenzo Cecchi

Tutore
Prof. Nadia Mulinacci

Coordinatore
Prof. Elisabetta Teodori

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Sommario

1. ABSTRACT

This thesis was focused on the nutraceutical valorization of extra virgin olive oil, “virgin” grape seed oil and olive milling by-products. This objective was pursued by a multidisciplinary approach involving chemical characterization and studies of biological activity and sensorial characteristic of the products.

In particular, the following specific objectives have been pursued:

1. to study the impact of several factors on olive oil quality and to develop suitable analytical methods to check this quality
2. to characterize composition and biological activities of phenolic fractions from “virgin” grape-seed oils
3. to develop suitable strategies for using by-products from virgin olive oil production for human consumption.

Regarding the first objective, a new methodological approach for evaluate the percentage of phenolic compounds transferred from olive fruits to olive oil during milling was developed and applied. Our results showed that up to 0.40% of phenols are transferred by the two-phase system and up to 0.19% with the three-phase system. These data are up to 25 times lower than those previously reported in the literature. At the same time, we studied the effect of olive paste moisture on content and profile of phenolic compounds in extractable oil at lab scale, by diluting freeze-dried olive pastes at a moisture range of 0-60%. Results showed that the % of extracted phenols reaches a maximum at a certain moisture content, that is lower than 40%, so that processing procedures able to control the olive fruit moisture may led to technological innovations.

In a further part of the thesis, we described, for the first time, the presence of new lignans, isobaric to the well known pinoresinol and 1-acetoxypinoresinol, in olive oil. Studying partially and fully-refined oils, it emerged that these isobaric lignans are formed during the bleaching step of the refining process, so that we were able to propose the detection of these molecules as markers of undeclared refining procedures in commercial virgin and extra virgin olive oils. Again, in order to check the quality of virgin olive oil, we developed and validated a new analytical method for the quantification of volatile organic compounds of virgin olive oil, with the aim to support the panel test in olive oil classification. This method, based on HS-SPME-GC-MS, uses up to 11 internal standard for area normalization and allows quantifying 70 VOCs in wide ranges of calibration.

In the second part, we investigated the phenolic composition of 17 monocultivar commercial cold-pressed grape seed oils, by HPLC-DAD-MS-TOF. Total phenolic content ranged between 1-15 mg/kg, and pinoresinol, ethyl caffeate and ethyl gallate were detected for the first time in this type of oil. Our studies also highlighted a good correlation between total phenolic content and inhibitory power of PTP-1B, an enzyme overexpressed in type-two diabetes, with pinoresinol, *p*-coumaric acid and quercetin that making the great contribution. Thid work contributed to clarify this matter because contradictory results were reported in the literature.

Finally, in the third part, we proposed different possible use of olive milling by-products, mainly for human nutrition. A first study was aimed to evaluate the quality and the stability of retentates obtained from olive mill wastewaters treated with a membrane filtration system. Results showed a reproducibility of the process over years, a high stability of hydroxytyrosol in the liquid retentates at 18-24°C over 24 months of storage and that retentates of reverse osmosis have the highest phenolic content. These retentates could be good sources of natural antioxidants and potassium and are suitable to formulate new food ingredient or food products.

At the same time, we characterized pâté, a new type of olive pomace, potentially suitable for human consumption. In the first part of the work, we determined the phenolic profile of pâté in fresh, dried and stored samples, and evaluated the antiaging effect in a cell senescence model. Results showed antiaging effects *in vitro*, comparable to those of pure hydroxytyrosol and that 1 g of paté provides a daily intake of total phenols comparable to that derived from 200 g of a typical virgin olive oil, demonstrating the potential value of this natural ingredient for human nutrition. However, before an ingredient could be successfully marketed, foods including this ingredient in their recipes must be perceived as pleasant by the consumer. For this reason, in the last part of my thesis, we tested the consumer acceptance of pasta, bread and granola bar fortified with suitable % of pâté, characterizing their sensory profile and evaluating the acceptance of naïve consumers. This part, performed at the University of California, Davis, allowed us highlighting that consumers accepted these products, confirming pâté as potential nutraceutical ingredient for improving the daily intake of phenolic compounds from *Olea europaea* L., also giving additional economic value to the olive oil production chain

2. PREAMBLE

This PhD work started about nine years after my degree in organic chemistry, in 2006. During these 9 years, I worked in the chemistry laboratory of the Chamber of Commerce of Florence. My activities were three-fold focused:

- To analyze olive oil samples for both certification and research purposes
- To develop applied research in the olive/olive oil sectors aimed to improve the quality of extra virgin olive oil (EVOO) and to transfer the acquired knowledges to the productive farms, in order to really improve the quality of the local EVOOs
- To combine chemical and sensorial data of the produced virgin olive oil samples as further approach to improve the quality of the EVOO

These activities were carried out under the supervision of Dr. Marzia Migliorini, which was the coordinator of the research activities of the laboratory.

Developing these activities allowed my acquiring specific skills in the olive oil field and starting a collaboration with the University of Florence mainly aimed to develop part of the researches. The main collaborations were with Professor Nadia Mulinacci (Department of NEUROFARBA), Professor Bruno Zanoni (Department of GESAAF), Professor Alessandro Parenti (Department of GESAAF) and Professor Luca Calamai (Department of DISPAA). These collaborations resulted in several publications before the beginning of my PhD. During the three years of my PhD several projects have been carried out, some of them started after the beginning of the PhD, some other continued from the previous period.

However, a common thread has been followed during the three years: it was the valorization of the whole olive oil production chain, with a special attention to develop analytical methods to protect the productive world and the consumers from frauds and to valorize the re-use of by-products. In this contest, in the last year of the PhD, part of the project was carried out at the University of California, Davis, thanks to a collaboration with Professor Jean-Xavier Guinard (Department of Food Science and Technology). In California it was also possible to visit and kept contact with a farm involved in the production of the so called “virgin grape seed oils”, a type of vegetable oil derived from the by-products of wine production and that is known to contain some specific compounds able to differentiate it from the vegetable oil, other than virgin olive oil and poorly investigated till now.

3. INTRODUCTION

In recent years, there have been strong evidences of an increasing attention towards a diet that could be preventive for the human health and not only a merely source of nutrients. In this sense, nutraceuticals are recognized as natural products able to provide extra health benefits, in addition to those provided by the well known macro and micro nutrients.

The first definition of nutraceuticals was given in 1989 by Defelice: "A food or parts of food that provide medical or health benefits, including the prevention and/or treatment of a disease". However, from the legislative point of view, nutraceuticals are not exactly defined by the European Union, and they stay in an area between Food and Drug (Gulati & Ottaway, 2006). A common point is that nutraceuticals are known to reduce disease risk factors, to enhance both physically and mentally performances and to cover health promotion and optimal nutrition.

In general, nutraceuticals *per se* consumed as tablets, capsules or liquid forms, are known as food supplements. A the same time, functional foods may be thought as food products in which nutraceuticals represents part of the components and fortified foods may be thought as products added with one or more nutraceuticals. In both these cases they maintain an appearance similar to the traditional food.

One of the main classes of natural compounds known for their nutraceutical properties are the phenolic compounds, produced by plants as secondary metabolites and used to communicate with the external environment. They are considered a widespread group of phytochemicals and plants use them to protect themselves from reactive oxygen species, photosynthetic stress and from predation by microorganism, insects, herbivores and other pathogens. Phenolic compounds are recognized to be able to exert a series of health effect on human health and they have been active components in several types of botanicals and herbal traditional medicines (Wollenweber, 1988). Thousands of phenolic compounds have been identified to date, the most of them recognized to possess several pharmacological properties (Beretz *et al*., 1977). These properties are not so strong, but when the intake of certain amounts of phenolic compounds are regularly maintained in the diet, they can exert positive long-term effects, substantially reducing the risk factor of several diseases.

Thanks to their numerous and well recognized biological activities (the most important of them being the antioxidant and anti-inflammatory properties), phenolic compounds represent a large group of nutraceuticals. The most of them belong to the class of anthocyanins, ellagitannins, flavonols, hydroxycinnamates, isoflavones, lignans, proanthocyanidins and stilbenes, even though, some specific families of plants are able to synthetize peculiar types of phenolic compounds. For example, the secoiridoids oleuropein, ligstroside, demethyloleuropein and nuzhenide, and their deglycosylated derivatives are the typical phenolic compounds from *Olea europaea* L. (Cecchi *et al*., 2013; Servili *et al*., 2004). These molecules, mainly present in their glycosilated form into the olive fruit, due to their hydrophilic nature are transferred in virgin olive oil only in a little percentage, and only after specific hydrolytic/enzymatic reactions that transform them in the corresponding aglycones (Hbaieb et al., 2016; Klen et al., 2012; Klen et al., 2015; Rodis et al., 2002).

However, in spite of the little amount of phenolic compounds recovered in olive oil, a cause and effect relationship between these compounds and protection of LDL from oxidative damage has been definitely established by several human studies (Covas et al., 2006; de la Torre Carbot et al., 2010). This evidence allowed the EFSA to approve a health claim for olive oil phenolic compounds that gives the possibility to insert "the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" in the oil label (EFSA, 2011). This health claim, representing a unique case in the field of vegetable oils, allows improving the commercial value of virgin olive oil.

Another type of vegetable oil for which the presence of phenolic compounds have been highlighted is the cold pressed grape-seed oil, also known as "virgin grape seed oils". The amount of phenolic compounds, even though lower than those of virgin olive oil, allows differentiating "virgin" grape-seed oils from the refined vegetable oils and can contribute to their healthy and sensory properties.

According to this introduction, the PhD thesis was three-fold focused:

- to study the impact of several factors on olive oil quality and to develop suitable analytical methods to check this quality
- to characterize composition and biological activities of phenolic fractions from "virgin" grape-seed oils

- to develop suitable strategies for using by-products from virgin olive oil production for human consumption.

3.1. Virgin olive oil

2.1.1. Overview on olive oil production and consumption

Statistical data from International Olive Council IOC indicates, in the last ten years, a world production of approx. 3 million of tons of olive oil. This is about 40% more of the productions until 1995/1996, confirming that the olive oil market is expanding over the years. In the last years, about 68% of this production is from European Union, for a total of approx. 2 million of tons, except for the harvesting year 2014/2015, strongly affected by olive oil fly attack (Cecchi et al., 2016). Spain is by far the main productive country in the EU and beyond, with approx. 1.3 million tons in the 2015/2016 crop season, which account for the 63% of the overall EU production, followed by Italy (0.351 million tons, 17%) and Greece (0.310 million tons, 15%) (UNAPROL, 2016).

Regarding consumption, data from 2015/2016 crop season highlighted a world consumption of about 3 million tons, 54% of which in European Union and 10% in the USA. Within the EU, consumption is mainly concentrated in Spain, Greece and Italy, while in not producing countries it is much lower, even though is growing in the last years (UNAPROL, 2016).

In Italy, the olive oil production chain is quite fragmented, with numerous small farms and the production that is decreasing in the last years, due to many types of challenges. One of these challenges is represented by the high costs of production, with the prize on the market not always able to cover these costs. Furthermore, confusion is often generated on the categorization of the product on the market and the consumer is not always able to choose knowingly. At the same time, the national demand is increasing and it is higher than the production, so that the difference is covered importing olive oil from abroad. This demand is mainly focused on EVOOs, also due to the growing attention of consumer to the quality and to the health property of the product.

2.1.2. Composition, health and sensory properties of Virgin Olive Oil

Virgin olive oil is unique within the vegetable oils, mainly due to its sensory and health properties, mainly linked to the peculiar composition of the olive oil, also in

comparison with other edible vegetable oils. Olive oil composition is briefly summarized in Figure 1.

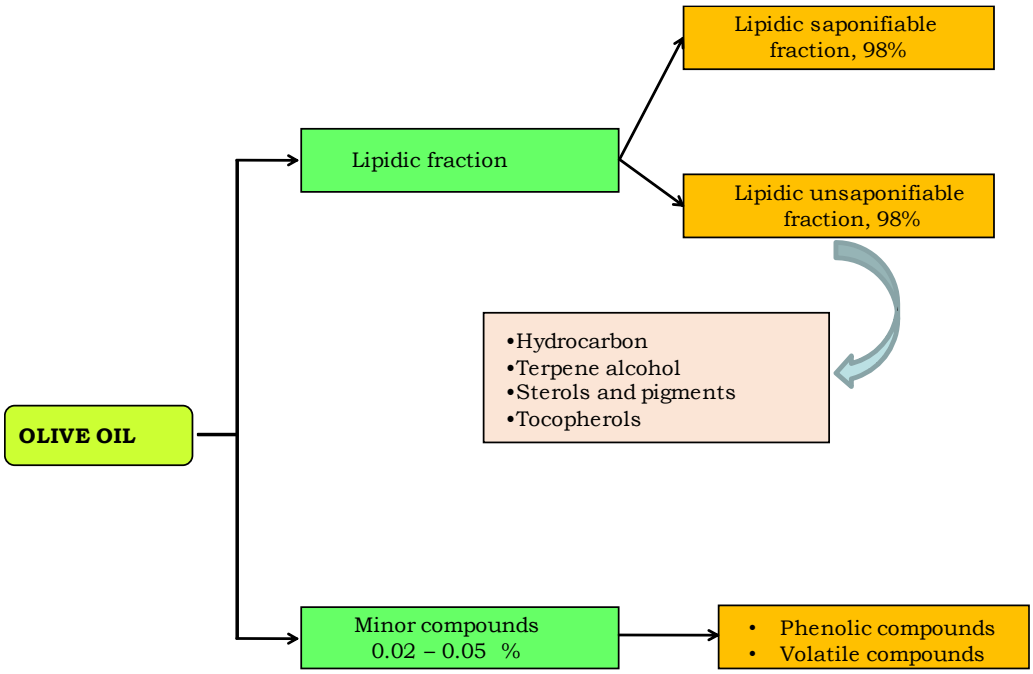

Figure 1. Chemical composition of olive oil

The health properties are in part due to the peculiar acidic composition of the triglyceride fraction, poor in saturated fatty acid, rich in monounsaturated fatty acids and also containing minor amounts of essential fatty acids, namely linoleic acid (ω6) and linolenic acid (ω3). The composition of this triglyceride fraction is affected by several factors, as cultivar, pedoclimatic conditions, irrigation and ripening stage (Ranalli, de Mattia, Ferrante & Giansante, 1997; Aparicio & Luna, 2002; Cortesi, Fiorino & Ponzetti, 2000; Aranda, Gomez-Alonso, Rivera del Alamo, Salvador & Fregapane, 2004).

However, what makes virgin olive oil really different from the other vegetable oils, is the presence of the so called minor compounds, mainly phenolic and volatile compounds. Phenolic compounds are recognized by the scientific community for the health properties of olive oils and for some sensations, as the taste of bitter and the pungency (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Gutierrez-Rosales, Rios, & Gomez-Rey, 2003). On the other side, volatile organic compounds (VOCs) are responsible for the peculiar sensory notes of virgin olive oil, mainly its characteristic smell (Campestre, Angelini, Gasbarri & Angerosa, 2017; Morales, Luna, Aparicio, 2005). Recently, it has been also pointed out a role of phenolic compounds in

the aroma release (Genovese, Yang, Linforth, Sacchi & Fisk, 2018), highlighting a sensorial interaction between these two classes of compounds.

Numerous are the papers focused on the health properties of phenolic compounds from *Olea europaea* L., as antiaging (Giovannelli, 2013; Casamenti & Stefani, 2017), antioxidant (Franco et al., 2014; Achat, Rakotomanomana, Madani, & Dangles, 2016) and anti-inflammatory (Beauchamp et al. 2005). Recently, the use of these molecules was patented thanks to their capability in improving the management of type two diabetes (De Bock, Hodgkinson, Curtfield, & Schlothauer, 2014). Finally, and likely most important, a dose effect relationship between these compounds and a protection of LDL from oxidative damage was established by several studies on humans (Covas et al., 2006; de la Torre Carbot et al., 2010), allowing the EFSA to approve a health claim for olive oil phenolic compounds, which contribute to the protection of blood lipids from oxidative stress (EFSA, 2011).

In order to have EVOOs with high sensory and health properties it is crucial to have high concentration of phenolic compounds, while, regarding volatile organic compounds, the main discriminating factor is the origin and the quality of the fruit. The pleasant fruity and green notes are given by C5 and C6 compounds, as aldehydes, alcohols and esters, originated from the lipoxygenase pathway (LOX), a well-known cascade of enzymatic transformations mainly involving linoleic and linolenic acids. On the contrary, most of the molecules responsible for the sensory defects, are mainly originated from oxidative and microbiological processes (Campestre et al., 2017). It is worth highlighting that the presence of molecules from the LOX contribute to partially covering the typical oil defects mainly when not so strong. At the same time, if the concentration of the molecules from LOX is too low, defects are always perceived also when their intensity is low.

Soil composition, irrigation, extraction parameters (crushing, malaxation time and temperature, kind of centrifugation, filtration), ripening time, storage time and condition of olive after harvesting, bottling and storage condition are the main factors that affect phenolic and volatile profile of virgin olive oils (Garcia-Gonzalez & Aparicio, 2010; Garcia-Gonzalez, Aparicio-Ruiz & Aparicio, 2009; Aparicio, 2000).

Regarding phenolic compounds, it is well known that only a small part is transferred from the fruit to olive oil. Literature reports percentage under 2% (Rodis et al., 2002), but these data seem to be not clear at all. However, this situation confirms how suitable

technological improvement for olive oil extraction would allow increasing the percentage of transferred phenols and, consequently, the health and sensory properties of virgin olive oils. Several attempts of increasing the capability of the olive mills in extracting higher amounts of phenols have been reported in the last years. In some of these attempts the use of ultrasounds and/or heat exchangers have been proposed (Clodoveo, Durante, La Notte, Punzi & Gambacorta, 2013; Veneziani et al., 2017), but, to date, only small increases of phenolic extraction were obtained.

Part of the activities discussed in this thesis (see Chapter 4), have been focused to better evaluating the real percentage of phenols transferred in EVOOs, investigating on the role of water content in the olive paste, but also to define indices of oxidative damage during malaxation.

2.1.3. Olive oil classification and frauds

Thanks to its high price and reputation, olive oil is one of the preferred targets for fraudsters in the world and one of the foods with the highest number of frauds in Europe, to date. For this reason, olive oil is strictly regulated by several established limits for analytical parameters aimed to guarantee the purity of the oil and the absence (or not) of adulterants. Usually, these limits are approved by the IOC and adopted by the EU regulatory body and the Codex Alimentarius (Garcia-Gonzalez & Aparicio, 2010). The final goal of this control is three-fold focused: i) ensure safety, ii) protect consumers and iii) protect the image of the olive oil. The purposes of these controls are to determine adulterations but also to confirm the authenticity of the oil with regards to geographical origin and cultivar. A continuous struggle is between analysts and fraudsters and the activity of the research has to be rapid enough to counteract fraudulent practices. The need to improving the effectiveness of the existing techniques and developing new ones to solve always new authenticity issues is a continuous challenge. In this context, during the first year of my PhD, a study aimed to set up a method for identifying illicit addition of refined oil to EVOO was developed and the results were published on Food Chemistry (See Chapter 4).

According to the IOC, "*virgin olive oils*" are the oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under

conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration. Within the category of *virgin olive oils*, the European legislation allows three categories:

- Extra virgin olive oil (EVOO): it is the *virgin olive oil* which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, median of the fruity higher than 0, median of defects = 0 and the other characteristics of which correspond to those fixed for this category in the IOC standard
- Virgin olive oil (VOO): it is the *virgin olive oil* which has a free acidity, expressed as oleic acid, of not more than 2.0 grams per 100 grams, median of the fruity higher than 0, median of defects not higher than 3.5 and the other characteristics of which correspond to those fixed for this category in the IOC standard
- Lampante virgin olive oil (LVOO): it is the *virgin olive oil* which has a free acidity, expressed as oleic acid, higher than 2.0 grams per 100 gram and/or median of the fruity = 0 and/or median of defects higher than 3.5 and other characteristics of which correspond to those fixed for this category in the IOC standard.

These three categories have very different commercial values, with EVOOs at the highest level, while LVOO is not intended for human consumption and it requires to be refined to obtain the refined oil that, mixed with virgin olive oils other than lampante virgin olive oil, is marketed and consumed as *olive oil* (OO). A correct classification of *virgin olive oil* has a very strong impact both for consumers and producers. It is based on chemical and sensorial analysis, this latter being carried out through sensory analysis by a panel of trained judges (the so called panel test). Unfortunately, this technique suffers of some drawbacks and a reliable and objective analytical method for supporting the panel test in *virgin olive oil* classification is still needed. For these reasons, in the first year of this PhD the development and validation of a HS-SPME-GC-MS method for the quantification of the VOO-VOCs was completed and published on Talanta (Chapter 4).

3.2. By-products from virgin olive oil production

Olive oil production is associated with generation of huge amounts of wastes: olive oil represents only a low percentage of the whole weight of the olives (approx. 15%). During milling, variable amounts of water are added, particularly when the three-phase decanter is used (Garcia-Gonzalez and Aparicio, 2010). The residual part of the olive (approx.. 85% of the whole fruit) and the added water contribute to form the final by-products from olive oil production. When olive oil is produced by a three-phase plant, the main by-products are olive-mill waste water (**OMWW**), also known as vegetation water or "alpechin" and pomace, a solid waste also called "orujo". Instead, when olive oil is produced by a two-phase plant, it produces a by-product called olive pomace or "alperujo", constituted by a combination of liquid and solid waste (Lozano-Sanchez et al., 2017).

The literature reported that approx. 3×10^7 m^3 of OMWW are produced annually only in the Mediterranean countries (Frankel, Bakhouche, Lozano-Sanchez, Segura-Carretero, & Fernandez-Gutierrez, 2013). Regarding olive pomace, the three-phase and the two-phase decanter produce approx. 500 kg and 800 kg per ton of olives, respectively (Frankel et al., 2013). The high amount of these by-products is a strong environmental problem, mainly in the Mediterranean area and also the several treatments proposed to date to counterbalance their negative effects on the environment are made very difficult by the high phenolic concentrations, being these molecules natural antimicrobials (Agalias et al., 2007; Pizzichini, 2005). However, thanks to the above mentioned health properties of phenolic compounds, these by-products can be seen as valuable source of these bioactive molecules, which can be then used for several applications (Lozano-Sanchez et al., 2017). The attempts to convert them in suitable source of bioactive compounds are numerous in the literature and continuously developing. Part of these studies are described in the introduction of the published results regarding both olive mill waste water (Chapter 6) and pâté (Chapter 6), a particular type of olive pomace, obtained by Leopard®. Leopard® is an innovative type of two-phase decanter able to pitting and partially dehydrating the olive pomace directly after the malaxation step, so reducing the possible oxidation of the paste (Leopard Series, Pieralisi Group S.p.A. Jesi, Italy).

A suitable recover of the phenolic compounds from these by-products could allow proposing nutraceutical ingredients for new types of foods focused towards a "protective

diet" for the consumer and not only a merely source of nutrients. Before the researches carried out in this PhD, only few studies were reported on the characterization of pâté and its possible use as ingredient for human nutrition. The large part of my PhD was focused to demonstrate this by-product as suitable for human nutrition. For developing this study we also kept into account that, before an ingredient could be successfully marketed, foods including this ingredient in their recipes must be perceived as pleasant by the consumer. In order to test the consumer acceptance of a new food or ingredient, it is necessary to characterize its sensory profile and to evaluate the acceptance of naïve consumers.

3.3. "Virgin grape seed oil"

The state of the art on this matter is reported in the introduction of the published results, reported in this thesis in Chapter 5. To my knowledges, no updates have been published after that publication.

4. STRUCTURE OF THE THESIS

According with the aims summarized at the end of paragraph 2, the next part of the thesis, concerning the obtained results, the most of them already published on international scientific journals, have been divided in three chapters. The first is focused towards the quality of extra virgin olive oil, the second towards characterizing the phenolic fraction of "virgin" grape seed oils and the third towards the valorization of by-products from olive oil production.

In each of these three parts, I reported all the published and unpublished results. Published results led to 7 scientific publications on journals with impact factor, while the unpublished results concern:

- the effect of the moisture content of rehydrated olive paste on the content and profile of phenolic compounds in extractable olive oil. This part has been submitted for publication to European Journal of Lipid Science and Technology
- the application of the HS-SPME-GC-MS analysis towards supporting the panel test, by using the method previously validated and published, as reported in the published results (Fortini et al., Talanta, 2017). This part has been presented at the 16th EuroFedLipid Congress: Science, Technology and Nutrition in a Changing world. September 16-19th 2018, Belfast, United Kingdom. Book of Abstract, 82.
- the use of pâté as ingredient for fortification of food products for human nutrition, and in particular, the characterization of the sensory profile of the fortified products and the evaluation of the acceptance by Californian consumers. This part will be submitted to Food Research International.

As mentioned above, some of the researches developed in this three years began before the beginning of the PhD. In particular:

- Studies focused on understanding what is the real percentage of phenolic compounds transferred from olive fruits to olive oils and how the moisture content of olive fruit affects the phenolic transfer have been developed in part in 2012 and in part during the PhD in 2016 and 2017. The first part of these studies is already published (Chapter 4 "An effective HPLC-based approach for the evaluation of the content of total phenolic compounds transferred from olives to virgin olive oil during the olive milling process"), while the second part has just been submitted

for publication (Chapter 4 “Effect of the moisture content of rehydrated olive paste on the content and profile of phenolic compounds in extractable olive oil”)

- Studies focused on recovering of olive mill by-products have been performed in the years 2013-2018 at the University of Florence – Department of NEUROFARBA. The activities of 2015-2018 have been part of my PhD. These studies, both already published, concerned the characterization of olive mill waste water as suitable sources of phenols for athletes and of pâté as suitable ingredient for human nutrition
- Studies focused at developing and validating analytical method based on HS-SPME-GC-MS for the quantification of volatile organic compounds of virgin olive oil were performed in the period 2014-2017. The first part was developed at the laboratory of the Chamber of Commerce of Florence, also in collaboration with professor Luca Calamai (University of Florence, Department o DISPAA). Then, the most of the work was developed during the first two years of my PhD, at the University of Florence, still in collaboration with professor Luca Calamai.

Finally, during the last year of the PhD, a collaboration with professor Jean-Xavier Guinard of University of California, Davis was activated with the aim of:

1. developing and fortifying with pâté food products very widespread in California and beyond
2. characterizing these products from a sensorial point of view
3. testing the acceptance of these products by Californian consumers

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6. RESULTS: THE QUALITY OF VIRGIN OLIVE OIL

An effective HPLC-based approach for the evaluation of the content of total phenolic compounds transferred from olives to virgin olive oil during the olive milling process

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Running title: A simple method to evaluate phenols transfer from olives into olive oils

Lorenzo Cecchi[1,] Marzia Migliorini[2], Bruno Zanoni[3], Carlotta Breschi[3] and Nadia Mulinacci[1]

[1] Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Firenze), and Multidisciplinary Center of Research on Food Sciences (M.C.R.F.S.-Ce.R.A), Italy

[2] Carapelli Firenze S.pA., Via Leonardo da Vinci 31, 50028 Tavarnelle Val di Pesa (Firenze), Italy

[3] Department of Agricultural, Food and Forestry System Management (GESAAF) and Food Science and Technology and Microbiology Section, Università degli Studi di Firenze, Via Donizetti 6, 50144, Florence, Italy

*Corresponding author: Nadia Mulinacci

Dipartimento di NEUROFARBA, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019, Firenze, Italy.

Tel.: +39 055 4573773

e-mail address: nadia.mulinacci@unifi.it

Abstract

Background: several studies demonstrate a strong interest in learning more about the phenolic transfer during oil extraction, with the main goal of increase the phenolic concentration in olive oils. We aimed to propose and apply a new methodological approach for evaluating phenolic transfer from olives into oil during milling, based on the quantification of phenolic content in whole lyophilized fruits and the corresponding oils and considering the oil extraction yields.

Results: we investigated the phenols transferred into the oil during olive milling in continuous extraction systems in Tuscany. In 2012, oils were extracted from cv Frantoio by a two-phase extraction system; in 2016, oils were extracted from cvs Leccio del Corno and Arbequina by a three-phase extraction system. Results highlighted very low percentages of extracted phenols: up to 0.40% by the two-phase system and up to 0.19% by the three-phase system (0.08% for cv Arbequina and 0.19% for cv Leccio del Corno).

Conclusion: The usefulness of a simple and effective methodological approach for evaluating the extracted phenols was highlighted. Values of extracted phenols were up to 25 times lower than previous literature data. The proposed approach is applicable in all types of milling processes.

Keywords: phenols extraction yield; secoiridoid; partition; HPLC-DAD-MS; oleuropein

1. Introduction

The interest for olive and olive oil phenols have been boosted in the last years, also thanks to their numerous health properties, such anti-inflammatory,[1] antioxidant, [2,3] anticancer,[4] anti-angiogenic[5] and anti-aterogenic[6] activities and no adverse effect known to date.[7] The use of olive phenols was even patented for its capability in improving the management of type 2 diabetes.[8] In light of *in vivo* tests on humans[9,10], the EFSA approved an important health claim for virgin olive oils rich in phenolic compounds giving the possibility to insert "the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" in the label.[11]

Olive fruits are very rich in phenolic compounds,[12] and the exact composition depends on different variables, the more relevant of which are cultivar, climatic conditions and degree of maturation.[13-16] Nevertheless, it is well known that only a minor part of this phenolic fraction passes in the olive oils during the extraction process, mainly depending on their predominant hydrophilic nature and the enzymatic activities.[17,18] Anyway, some technological conditions seem to be crucial to determine the percentage of phenols passed in the oil or lost in by-products as olive mill wastewater and solid pomace.[7,19,20] Nowadays, the oil production can be carried out by both traditional batch approach or two and three-phases continuous process.[21,22] However, the type of technology seems to influence only the quantitative aspects on the phenolic transfer during oil extraction, while the qualitative changes mainly depend upon the enzymatic activities, especially during the malaxation.[22-25]

Literature data reported that only up to 2% of the phenols available in the olive fruits are transferred to the oil due to the greater affinity of phenolic compounds towards the water phase.[7,26,27]

Klen & Vodopivec[22] evaluated the percentage of phenols transferred from olive to oil obtained both in a traditional press, and in two and three-phase centrifuge systems. It was stated that up to 1.5 % of olive phenols was transferred to the oil by the two phases centrifuge system, up to 1.2 % by the traditional press and up to 0.5 % by the three phases centrifuge system. No phenolic compounds content of oil was shown and the olive fruits phenolic content appeared to be very low with respect to the literature data.[12,28] In addition, the percentage of phenolic compounds was not normalized respect to the

relevant oil yield extraction of the above process systems; then, an its overestimation presumably occurred.

Goldsmith, et al.[29] investigated the phenolic compounds transfer during olive oil processing by a traditional press. The total phenolic content, measured by the Folin-Ciocalteu method, resulted in 250 mg kg^{-1} for olive oil and 18,470 mg kg^{-1} for olive; 1.4 % of olive phenolic compounds content was transferred to oil, but also in this case the above value was not normalized respect to the relevant oil yield extraction.

Klen et al.[20] evaluated that only 0.53 % of the phenolic amount of the olive fruit passed into the oil. The phenolic mass balance was evaluated only at a laboratory scale, without evaluations of the oil extraction in a real mill.

In our previous work,[12] the crucial effect of the freeze-drying of whole olives immediately after harvesting was demonstrated to preserve the "native" phenolic profile of olives. Measurement of phenolic compounds content on crushed or cut olives strongly modified olive phenolic profile due to enzymatic transformations. Therefore, a difference in calculating the yield of phenolic compounds transfer may occur if the "native" phenolic profiles of olives is lost.

Talhaoui et al.[30] studied the transfer of single phenolic compounds from olives to oil by working at laboratory scale for six different cultivars. The total phenol transfer rate varied markedly among cultivars with value between 0.38 % and 1.95 %. A very low amount of olive fruits phenolic content, characterized by only traces of oleuropein, appeared in disagreement with the literature data.[12,22]

All these attempts demonstrate a strong interest in learning more about the phenolic transfer during oil extraction, with the main goal of better known this process to increase the phenolic concentration in the olive oils. The aim of this work was to improve the methodological approach for evaluation of phenolic compounds transferred from olive to oil by (i) a measurement of the native phenolic compounds working on the whole freeze-dried olives, (ii) a determination of the phenolic compounds in virgin olive oils by the official IOC method and (iii) an evaluation of the oil yields after milling. The proposed approach was then applied to two different continuous milling systems, working on two different crop season in Tuscany.

2. Experimental

2.1 Chemicals

All chemicals for analysis were of analytical grade. Formic acid and hexane was from Sigma Aldrich (Steinheim, Germany) and phosphoric acid from Merck (Darmstadt, Germany). Methanol and acetonitrile of HPLC grade were from Baker (Phillipsburg, NJ) and Panreac (Barcelona, Spain) respectively. Deionized water was produced by the Milli-Q-system (Millipore SA, Molsheim, France). Syringic acid and tyrosol from Sigma Aldrich and oleuropein from Extrasynthese (Genay, France) were the standard compounds, stock solution of which were prepared in hydroalcoholic solution.

2.2 Samples

All the analyzed samples are summarized in Table 1.

Analyzed sample	Crop season	Cultivar	DAFB	Oil extraction system
Olive fruit and virgin olive oil	2012	Frantoio	150th	two-phase
Olive fruit and virgin olive oil	2012	Frantoio	164th	two-phase
Olive fruit and virgin olive oil	2016	Leccio del Corno	190th	three-phase
Olive fruit and virgin olive oil	2016	Arbequina	190th	three-phase

Table 1. List of all the analyzed samples; DAFB = Day After Full Blooming.

2.2.1 Olive fruits

During the 2012 crop season, 10 olive plants (*Olea europea* L.) of the cultivar Frantoio were selected from a farm located in Fiesole (Florence, Italy). Regular irrigation of orchard was applied and full blooming occurred by 15th June. Olive fly attacks were under 1%. Ripe olive fruits were sampled on the 150th and 164th Day After Full Blooming (DAFB) and the whole fruits were freeze-dried immediately after they arrived in the laboratory.

During the 2016 crop season, 10 olive plants (*Olea europea* L.) for each of the two cultivars, Leccio del Corno and Arbequina, were selected from farms located in province of Florence (Italy). No irrigation of orchard was applied and full blooming occurred by 28th April for the two cultivar. Olive fly attacks were under 1%. Ripe olive fruits were sampled on the 190th DAFB and were freeze-dried immediately after they arrived in the laboratory. Sampling was carried out by picking olives from all the selected plants along all their circumference at a height close to 170 cm.

Freeze-drying of olive samples was carried out as previously described by Cecchi et al.[12] Briefly, olives were deep-frozen in liquid nitrogen and then placed into the freeze-dryer at -20°C under 0.1 atm until reached a constant weight. Freeze-dried samples were stored at -20°C until analysis.

2.2.2 Virgin olive oil

During the 2012 crop season, three batches of olives at the 150th DAFB and four at the 164th DAFB were collected; the olive fruits were milled in a two-phase continuous extraction system within 24 h after harvest. Each batch of olives was about 600 kg, and was milled according to scheme in Figure 1A. Briefly, olives were de-foliated and de-branched, washed and drained by a vibrating table; then, a hammer mill was used to crush the fruits, and the obtained pastes were malaxed in two vertical tank kneaders equipped with a heating jacket, each of capacity of 300 kg; malaxation was carried out for 25 minutes at 27°C under vacuum. After malaxation, the virgin olive oil was extracted by a two-phase decanter (i.e. no water was added) and filtered by a filter press.

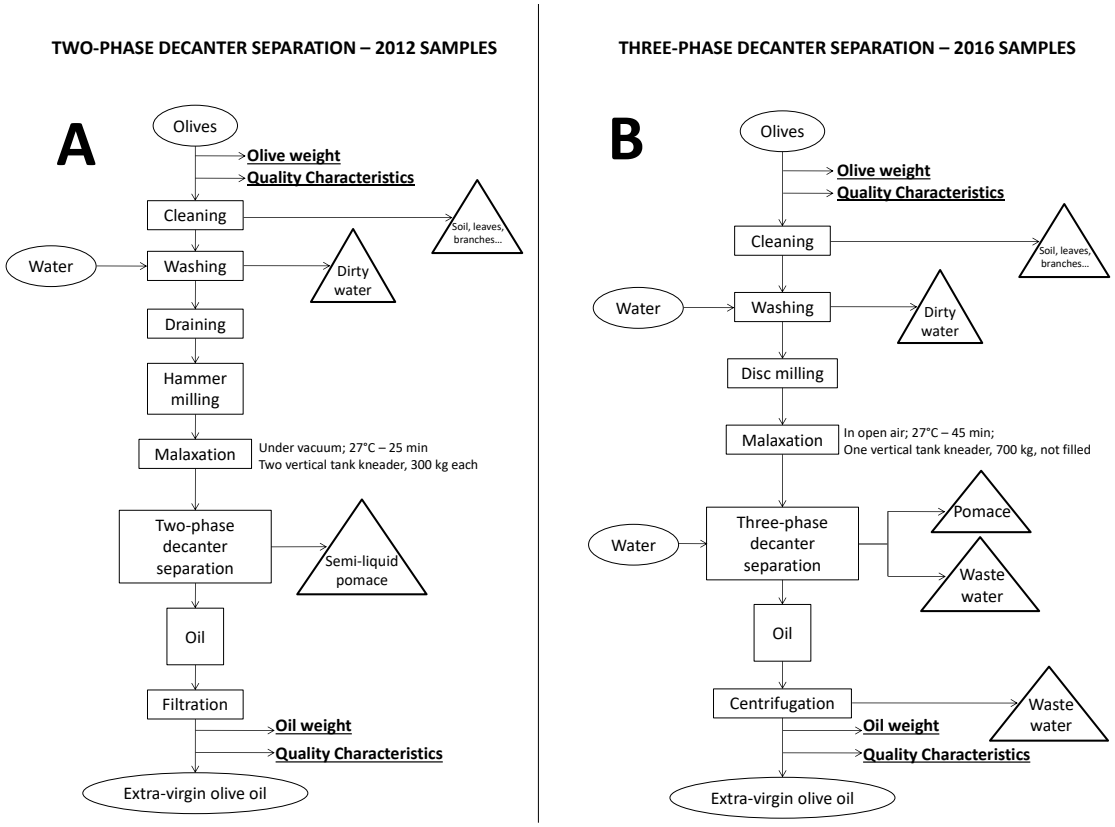

Figure 1. Comparison between the two- and three-phases continuous extraction system used for the virgin olive oil extraction.

During the 2016 crop season, three batches of olives of each cultivars (Leccio del Corno and Arbequina), harvested on the 190th DAFB, were milled in a three-phase continuous extraction system within 24 h after harvest. Each batch of olives was about 600 kg and was milled according to Figure 1B. The olives were washed, then a disc crusher was used to crush the olives, and the obtained pastes were malaxed in a horizontal tank kneader equipped with a heating jacket; malaxation was carried out in open air for 45 minutes at 27°C, with the kneader not completely filled. After malaxation, the virgin olive oil was extracted by a three-phase decanter (approx. 30 kg of water was added for 100 kg of olives) and were centrifuged at 7,000 rpm in a vertical centrifuge; no filtration was applied.

2.3 Measurements and determinations

2.3.1 Oil extraction yield

Oil extraction yields were determined during the olive milling processes by measurement of olive and oil weights as shown in Figure 1. The olives were weighted before washing, while the oils were weighted after filtration for the 2012 samples (Figure 1A) and after centrifugation for the 2016 samples (Figure 1B).

The yields were calculated both as actual yield (*OY*) obtained in the process and as *Extractability Index* (*EI*) or olive mill efficiency[31] as follows:

$$OY = \frac{OE_x}{Ol_m} \cdot 100 \qquad \mathbf{1}$$

$$EI = \frac{OE_x}{OC_{om}} \cdot 100 \qquad \mathbf{2}$$

where:

OE_x was the extracted olive oil (kg);

Ol_m was the milled olive fruits (kg);

OC_{om} was the oil content of milled olive fruits (kg).

2.3.2 Yield of phenolic compounds transfer from olive to oil

The yields of phenolic compounds transfer were determined during the olive milling processes by measurement of olive and oil phenolic compounds content as shown in Figure 1. The phenolic yields (*PY*) were calculated in percentage as normalized values respect to the above actual oil extraction yields (*OY*), as follows:

$$PY = \frac{PO_{Ex} \cdot OY}{POl_m} \qquad \textbf{3}$$

where:

PO_{Ex} was the total phenolic content of the extracted olive oil (mg kg^{-1});

POl_m was the total phenolic content of milled olive fruits (mg kg^{-1}).

2.3.3 Water, oil and sugar content of olive fruits

Moisture content (g kg^{-1}) was measured by gravimetry between the fresh and freeze-dried samples. The oil content (g kg^{-1}) was measured on freeze-dried olives by extraction with hexane in an automatic extractor (Randall mod.148, VELP Scientifica, Milan, Italy), following the method of Cherubini et al.[32] Sugar content (g kg^{-1}) was measured enzymatically, and expressed as sum of glucose and fructose, as previously reported by Trapani et al.[15]

2.3.4 European legal quality characteristics of virgin olive oil

Acidity (% oleic acid), peroxide value (meq O_2 kg^{-1}) and spectroscopic indices were measured according to EU official method.[33] Sensory evaluation of olive oil was performed by a panel test according to the EU official method.[34]

2.3.5 Phenolic compounds content

2.3.5.1 *Olive fruits*

Freeze-dried olives were crushed in a small laboratory crusher (Zeutec, Germany) so obtaining an olive cake as homogenous as possible, from which phenolic compounds were extracted as previously described.[28] Briefly, 4 grams of the olive cake were cold extracted twice with 30 mL of EtOH:H_2O 80:20 solution added with 0.5 mL of the internal standard (syringic acid 1.5 mg mL^{-1}). The obtained solution was concentrated, washed twice with hexane, centrifuged at 7.000 rpm and 10 °C and filtered in a 10 mL flask by a cellulose acetate membrane of 0.45 µm; final volume of 10 mL was reached adding MeOH:H_2O 50:50. The obtained solution was immediately used for the chromatographic analysis, which were carried out by an HP1100 liquid chromatograph equipped with DAD and MS detector with HP1100 MSD API-electrospray interface (all

by Agilent Technologies, California, USA). For the 2012 samples a Hypersil Gold QRP-18 (250 mm x 4.6 mm id, 3 µm particle size; Thermo Electron Corp., Austin, TX) column with a pre-column of the same phase was used. The oven temperature was 30°C. Elution was performed using H_2O (pH 3.2 by formic acid), acetonitrile and methanol at the condition previously described[12] and the chromatograms were acquired at the following wavelenghts: 240 nm, 280 nm and 330 nm. For the 2016 samples a new generation Poroshell 120, EC-C18 (150 mm x 3.0 mm id, 2.7 µm particle size; Agilent, USA) column with a pre-column of the same phase was used. The oven temperature was 26°C. Elution was performed using H_2O (pH 3.2 by formic acid) and acetonitrile with a flow rate of 0.4 mL min^{-1} with the following multistep linear gradient: the organic solvent, acetonitrile (A), changed from 5% at 0.1 min to 40% at 40 min, then remained at 40% until 45 min and changed to 100% at 50 min; after remaining at 100% until 53 min it returned to 5% at 55 min. The chromatograms were acquired at the following wavelenghts: 240 nm, 280 nm and 330 nm. Before using the new generation column for the 2016 samples, the two columns (Hypersil Gold QRP-18 and Poroshell 120, EC-C18) were preliminarily compared and the results were the same (data not shown).

Quantification of phenolic compounds was carried out by the internal standard method, according to our previous works.[12] Briefly, syringic acid was the internal standard, and the relative response factor (*RRF*) were evaluated with the following standards: oleuropein, verbascoside, tyrosol and luteolin-7-*O*-glucoside. Consequently, single phenolic compounds were expressed as follow: hydroxytyrosol, hydroxytyrosol glucoside and tyrosol glucoside as mg_{tyr} kg^{-1}; chlorogenic acid, caffeic acid, verbascoside and verbascoside isomers as mg_{verba} kg^{-1}; demethyloleuropein, nuzhenide, caffeoyl-6'-secologanoside, oleuropein aglycones, oleuropein, comselogoside and ligstroside as $mg_{oleurop}$ kg^{-1}; rutin and luteolin-7- *O*-glucoside as mg_{lut} kg^{-1}. Total phenolic content (*TPC*) was calculated by the integration of all the peaks present in the chromatogram at 280 nm and was expressed as $mg_{oleurop}$ kg^{-1}, taking into account that the main peaks in the phenolic profiles are oleuropein and similar secoiridoids.

2.3.5.2 Virgin olive oil

Phenolic compounds from olive oils were extracted and analyzed according to the IOC official method.[35] Briefly, phenolic compounds were extracted by a MeOH:H_2O

80:20 solution and immediately analyzed. Analysis were performed by an HP1200 liquid chromatograph, equipped with a HP 1200 auto-sampler and a HP1200 DAD detectors (all by Agilent Technologies, California, USA). A LiChrospher 100 endcapped RP-18, 5μm, 250 x 4.6 mm id column was used; elution was performed by using the acid H_2O (0.2% H_3PO_4)/acetonitrile/methanol gradient reported in the official method and by an injection volume of 20 μL; identification was carried out at 280 nm. Quantification was carried out by the internal standard method, for which syringic acid was used as internal standard and tyrosol as reference compound. As a consequence, *TPC* and the content of single secoiridoids, lignans, flavonoids and phenolic alcohols and acid were expressed as mg_{tyr} kg_{oil}^{-1}.

2.4 Data processing

The precision of the procedure for the quantitation of phenolic compounds of the olive fruits was previously reported by Cecchi et al.[12] Regarding the olive oil samples, standard deviation of total phenolic content was determined according to the official method.[35] To evaluate the precision of extraction and quantitation of each phenolic compound, one oil was selected and, starting from different aliquots of it, quantitation of phenols was repeated eight times and the obtained results were used to calculate the variation coefficient (CV%).

3. Results and discussion

Table 2 shows the quality characteristics of olives used for the oil extractions; Arbequina and Leccio del Corno cvs. had a significant higher moisture content (approx. 60%) than Frantoio cv in 2012; this moisture value could cause "difficult" olive pastes for oil extraction.[36] Phenolic content varied from 24,000 $mg_{oleurop}$ kg^{-1} for cv. Frantoio at the 164 DAFB to 31,000 $mg_{oleurop}$ kg^{-1} for cv. Arbequina.

Cultivar	Year	DAFB (die)	Sugar content ($g\ kg^{-1}$)	Oil content ($g\ kg^{-1}$)	Moisture ($g\ kg^{-1}$)	*TPC* ($mg_{oleurop}\ kg^{-1}$)
Frantoio	2012	150	$24.9^{a} \pm 1.7$	$175^{b} \pm 5$	$530^{b} \pm 20$	$28{,}643^{a} \pm 980$
Frantoio	2012	164	$25.6^{a} \pm 1.8$	$193^{a} \pm 6$	$500^{b} \pm 20$	$23{,}693^{c} \pm 810$
Arbequina	2016	190	$15.3^{b} \pm 1.1$	$123^{d} \pm 4$	$600^{a} \pm 20$	$30{,}633^{a} \pm 1{,}084$
Leccio del Corno	2016	190	$17.6^{b} \pm 1.2$	$133^{c} \pm 4$	$590^{a} \pm 20$	$26{,}302^{b} \pm 900$

Table 2. Olive quality characteristics; DAFB = Day After Full Blooming, *TPC* = Total Phenolic Content. Different small letters in the same column indicate significant differences (p = 0.01) for the different samples.

Quality characteristics of Frantoio cv. olives were congruent with the ripening degree expressed by the DAFB values: higher DAFB values reflected higher oil content values and lower total phenolic content values.[15]

Figure 2 shows two examples of chromatographic profiles at 280 nm of 2016 olive samples. All the main peaks were well resolved and they corresponded to the typical glycosilated phenols of the "native" fruits, e.g. oleuropein, demethyloleuropein, ligstroside, nuzhenide, verbascoside, rutin, luteolin-7-*O*-glucoside.[12,37,38] Minor or undetectable amounts of the degradation products, e.g. oleuropein aglycones, tyrosol, hydroxytyrosol, caffeic acid, were measured, confirming that our method preserved the olive "native" phenolic profile.

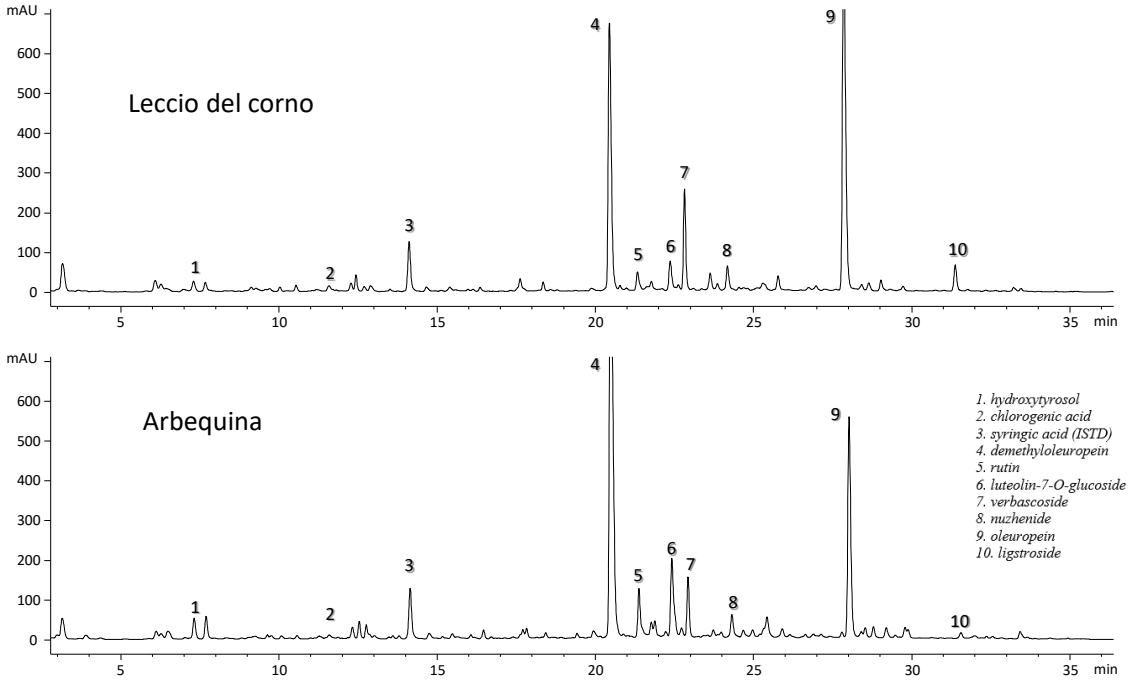

Figure 2. Examples of chromatographic profiles at 280 nm of 2016 olive samples with the main identified peaks

Table 3 shows the amounts of all the phenolic compounds, which were identified in the olive samples. Olive samples had higher values of total phenolic content than that reported in literature.[20,22,29,30] The glycosilated secoiridoids were the predominant phenolic compounds; they represented approx. 75% of the identified phenols and they were even 90.6 % in Leccio del Corno cv. olive sample in 2016. The high content of demethyloleuropein seemed to confirm that this phenolic compound was formed from endogenous esterase activity on oleuropein during olive ripening.[12]

Phenolic Compound	Frantoio 2012 DAFB 150	Frantoio 2012 DAFB 164	Arbequina 2016 DAFB 190	L. del Corno 2016 DAFB 190
Hydroxytyrosol	159.2	176.2	138.5	86.7
Hydroxytyrosol glucoside	n.d.	n.d.	138.5	86.7
Tyrosyl glucoside	n.d.	n.d.	147.9	66.6
Chlorogenic acid	62.5	38.5	19.2	31.5
Caffeic acid	n.d.	n.d.	14.2	nd
Demetyloleuropein	2939.2	4367.6	9992.5	5635.6
Rutin	115.4	74.3	160.6	67.4
Luteolin-7-O-glucoside	130.2	104.3	296.1	114.2
Verbascoside	630.2	637.6	211.4	389.9
Nuzhenide	577.0	458.8	383.8	438.6
Sum of isoverbascoside isomers	92.3	117.9	5.9	5.4
Caffeoyl-6'-secologanoside	274.3	299.3	64.0	9.8
Sum of oleuropein aglycone isomers	n.d.	692.4	83.0	96.3
Oleuropein	12286.5	6522.3	4115.6	7339.6
Comselogoside	324.6	391.4	175.9	nd
Ligstroside	626.1	321.4	110.8	509.9
Total phenolic compounds	**28643.2**	**23693.0**	**30633.1**	**26302.4**

Table 3. Amount of the phenolic compounds identified in the olive samples. Data are expressed as mg kg^{-1} on fresh fruit basis as explained in the Materials & Methods section; n.d. = not determined.

Arbequina cv. olive sample in 2016 had a particular phenolic profile, which was characterized by a higher percentage of demethyloleuropein content (approx. 33 %) and a lower percentage of oleoeuropein content (approx. 13 %) than the other tested olive samples, which were characterized by approx. 17 % of demethyloleuropein content and by approx. 33 % of oleuropein content.

All virgin olive oil samples extracted from olives were classified as extra virgin and their yields data and oil total phenolic contents are shown in Table 4.

Cultivar	Year	Oil extraction system	*OY* (%)	*EI* (%)	*TPC* (mg_{tyr} kg^{-1})	*PY* (%)
Frantoio	2012	Two-phases	15.8	90.3	$687^{a} \pm 78$	0.38
Frantoio	2012	Two-phases	16.1	83.4	$593^{a} \pm 64$	0.40
Arbequina	2016	Three-phases	9.8	79.7	$238^{c} \pm 32$	0.08
Leccio del Corno	2016	Three-phases	11.0	82.7	$445^{b} \pm 48$	0.19

Table 4. Yields data and oil total phenolic contents of extracted extra virgin olive oils; *OY* = oil actual yield; *EI* = oil extractability index; *TPC* = Total Phenolic Content (*TPC*), *PY* = yield of phenolic compounds transfer. Different small letters in the same column indicate significant differences (p = 0.01) for the different samples.

The total phenolic content was significant higher in oil samples extracted from Frantoio cv. olives in 2012 than in oil samples extracted from Arbequina cv. and Leccio del Corno cv. olives in 2016, although a similar difference was not occurred in their olive total phenolic content (Table 2). The application of a three-phase oil extraction system

may explain the above difference, related to the added water. The dilution of the aqueous phase of olive paste changed the partition equilibrium of phenolic compounds and most of phenolic compounds flushed away with the produced wastewater according to previous studied.[22,39]

Oil samples from Arbequina cv. olives in 2016 had also the lowest total phenolic content (238 mg_{tyr} kg^{-1}). The particular phenolic profile of Arbequina cv. olives, previously described in the text, could explain this behavior, since the phenolic compounds with high hydrophilic nature were predominant.[27]

The yields of phenolic compounds transfer (*PY*) from olive fruits to olive oils was calculated from data in Tables 2 and 4. In 2012 from 1 kg of Frantoio cv. olives at the 150th DAFB, a mean of 0.158 kg of olive oil were extracted with a total phenolic concentration of 687 mg kg^{-1}. This means that only approx. 109 mg of the potential 28,643 g of total phenolic content in the fruit were transferred and, then *PY* was 0.38 % (see also eq. **3**). Following the same approach, *PY* at the 164th DAFB was 0.40 %. These data were one order of magnitude lower than the 2 % value reported in literature,[7,26] and they were also lower than 0.53 % value determined at laboratory scale.[20] Furthermore, it should be taken into account that the oils from the 2012 crop season had a high phenolic content (i.e. 593 mg kg^{-1} and 687 mg kg^{-1}), higher even than mean phenolic concentration of high quality extra virgin olive oil which usually did not exceed 350 mg kg^{-1}.[40] These data suggested that for common extra virgin olive oils extracted by a two-phase system the yields of phenolic compounds transfer could be lower than 0.4 %.

In 2016, from 1 kg of Leccio del Corno cv. Olives, a mean of 0.110 kg of olive oil were extracted with a total phenolic content of 445 mg kg^{-1}, indicating that only approx. 49 mg of the potential 26,302 mg in olives were transferred to the oil. Then, a 0.19 % *PY* was determined (see also eq. **3**). Following the same approach, *PY* of Arbequina was lower and only of 0.08 %. The yields were up to 25 times lower than 2 % value reported in the literature and they were up to 5 times lower than the *PY* obtained during oil extraction from Frantoio cv. olives in 2012 by a two-phase system, confirming the effect of a three-phase extraction system on phenolic compounds content as previously reported in the text.

The above *PY* values were related to the oil actual yields (*OY*), which are also dependent to both the olive quality characteristics and the efficiency of processing steps

before the oil extraction, such as olive milling and olive paste malaxation. The highest *OY* values, and, consequently the *PY* values, were for Frantoio cv. in 2012 (Table 4). In our trials, the effect of the olive quality characteristics on *OY* values (that is, the more the oil content and the less the water content, the more oil yield value) seemed to prevail on the effect of processing steps.[22] Indeed, *OY* differences in Table 4 (about 15.9% for two-phase and 10.4% for three-phase) were very wider than *EI* differences.

Comparing the phenolic profiles of the extracted oils (Table 5), one of the main differences was the oleuropein content, which was higher in the oils from the two-phase system, also in terms of percentage on the total phenolic content. On the other side, the percentage of oleuropein derivatives was higher into the oils from the three-phase system. This behavior could be explained by both the higher amount of water in the olive fruits of 2016 and the added water in the three-phase system: each of these factors could promote a faster hydrolytic and enzymatic degradation of the secoiridoids.

Regarding lignans (i.e. pinoresinol and acetoxypinoresinol), which attracted many interest in the last years,[4,41] our data showed the higher percentage in the oils from Arbequina (15.3 %) then the other oils (Frantoio 2012 – 150th DAFB, 9.2%; Frantoio 2012 – 164th DAFB, 10.2%; Leccio del Corno 2016, 12.2%). These data are in agreement with previous results,[42] according with which lignans content in olive oils mainly depends on cultivar.

Phenolic compound (mg_{tyr} kg^{-1})	Frantoio 2012 - DAFB 150			Frantoio 2012 - DAFB 164			Arbequina 2016			Leccio del Corno 2016		
Hydroxytyrosol	3.2	±	0.2	1.4	±	0.1	1.8	±	0.1	3.3	±	0.2
Turosol	2.3	±	0.1	2.3	±	0.1	1.3	±	0.0	1.5	±	0.1
Caffeic + Vanillic acid	1.3	±	0.0	0.4	±	0.0	1.0	±	0.0	0.7	±	0.0
Vanillin	2.4	±	0.2	1.9	±	0.1	0.8	±	0.1	0.9	±	0.1
p-coumaric acid	0.9	±	0.1	0.0	±	0.0	0.8	±	0.1	0.6	±	0.1
Hydroxytyrosyl acetate	0.7	±	0.1	2.4	±	0.2	0.5	±	0.0	0.4	±	0.0
Ferulic Acid	0.2	±	0.0	0.7	±	0.1	1.5	±	0.3	0.6	±	0.1
o-coumaric acid	1.3	±	0.2	1.3	±	0.2	1.0	±	0.2	0.8	±	0.1
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	45.4	±	3.8	34.8	±	2.9	16.0	±	1.3	34.5	±	2.9
Dyaldehydic form of decarboxymethyloleuropein aglycon	116.8	±	1.3	101.2	±	1.1	56.4	±	0.6	95.7	±	1.0
Oleuropein	100.4	±	3.6	95.3	±	3.4	15.9	±	0.6	18.7	±	0.7
Dyaldehydic form of oleuropein aglycon	43.2	±	2.7	34.3	±	2.2	22.2	±	1.4	49.4	±	3.1
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	22.1	±	1.1	19.8	±	1.0	3.6	±	0.2	11.4	±	0.6
Dyaldehydic form of decarboxymethylligstroside aglycon	61.7	±	1.3	55.6	±	1.2	10.2	±	0.2	33.6	±	0.7
Pinoresinol + 1-acetoxypinoresinol	63.3	±	1.6	60.2	±	1.6	36.3	±	0.9	54.3	±	1.4
Cinnamic acid	14.5	±	2.2	11.4	±	1.7	3.6	±	0.5	9.3	±	1.4
Dyaldehydic form of ligstroside aglycon	5.2	±	0.6	7.4	±	0.8	7.8	±	0.9	11.1	±	1.2
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	33.9	±	1.5	26.3	±	1.2	13.3	±	0.6	26.5	±	1.2
Luteolin	24.4	±	3.5	19.3	±	2.8	7.4	±	1.1	10.2	±	1.5
Aldehydic and hydroxylic form of oleuropein aglycon	60.5	±	0.9	57.6	±	0.8	14.1	±	0.2	38.2	±	0.5
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	32.6	±	3.2	29.6	±	2.9	8.6	±	0.9	13.9	±	1.4
Apigenin	17.5	±	1.7	6.1	±	0.6	6.6	±	0.6	11.0	±	1.1
Methyl luteolin	17.8	±	1.4	6.9	±	0.6	5.6	±	0.4	11.0	±	0.9
Aldehydic and hydroxylic form of ligstroside aglycon	15.8	±	0.6	16.9	±	0.6	1.6	±	0.1	7.9	±	0.3
Total Phenolic Compounds	**687**	**±**	**78**	**593**	**±**	**64**	**238**	**±**	**32**	**445**	**±**	**48**
% lignans	9.2			10.2			15.3			12.2		
% oleuropein	14.6			16.1			6.7			4.2		
% oleuropein derivatives	43.6			42.8			51.2			54.9		

Table 5. Phenolic contents of extracted extra virgin olive oils. Data are expressed as mg_{tyr} kg^{-1}. The last three lines show the percentage of total lignans, oleuropein and oleuropein derivatives on the total phenolic content

4. Conclusions

Health claims related to phenolic compounds in olive oil are permitted in European Union. They guarantee that a cause and effect relationship has been established between the consumption of olive oil phenolic compounds and protection of LDL particles from oxidative damage. Therefore, both increase and control of yield of phenolic compounds transfer from olive to oil may be aims to an improved extra virgin olive oil processing.

In this study, a methodological approach for an improved measurement of the phenolic yields (*PY*) was set up. This approach is independent from the complexity of transformation and transfer phenomena of phenolic compounds during oil extraction.
The measurement of phenolic compounds content on whole lyophilized olive fruits allowed avoiding the enzymatic transformations of these molecules and the "native" phenolic profiles of olives was preserved. In this profile, secoiridoids, and in particular oleuropein, are by far the most abundant compounds, therefore we proposed to express the total phenolic content as mg of oleuropein per kg of fresh olives.

Regarding the phenolic yield (*PY*), more realistic values were determined by the proposed approach, which allows to express *PY* as percentage normalized values respect to the oil extraction yield (*OY*). By this approach, *PY* resulted approx. of one order of magnitude lower than previous data in the literature.

In agreement with previous literature, the three-phase extraction system appears less efficient than a two-phase continuous extraction system to allow high phenolic recovering. At the same time, the ripening degree of olive fruits confirmed to have a significant effect on *OY* and indirectly on *PY* values, and particularly a lower water content was associated to higher oil yield. The proposed approach is applicable in all types of milling processes.

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ABBREVIATIONS

EFSA = European Food Safety Authority
DAFB = Day After Full Blooming
cv = cultivar
OY = oil extraction yield
OE_x = extracted olive oil
Ol_m = milled olive fruits
OC_{om} = oil content of milled olive fruits
PO_{Ex} = total phenolic content of the extracted olive oil
POl_m = total phenolic content of milled olive fruits
PY = yield of phenols transferred from olive into olive oil
TPC = Total Phenolic Content
IOC = International Olive Council

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Indirect indices of oxidative damage to phenolic compounds for the implementation of olive paste malaxation optimization charts

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S. Trapani[a], C. Breschi[a], L. Cecchi[b], L. Guerrini[c], N. Mulinacci[b], A. Parenti[c], V. Canuti[a], M. Picchi[a], G. Caruso[d], R. Gucci[d], B. Zanoni[a, *]

[a]Department of Agricultural, Food and Forestry Systems Management (GESAAF) – Food Science and Technology and Microbiology Section, Università degli Studi di Firenze, Via Donizetti 6, 50144 Florence, ITALY

[b]Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), Università degli Studi di Firenze, Via Schiff 6, 50019 Sesto Fiorentino, ITALY

[c]Department of Agricultural, Food and Forestry Systems Management (GESAAF) – Agricultural, Forest and Biosystem Engineering Section, Università degli Studi di Firenze, Piazzale delle Cascine 15, 50144 Florence, ITALY

[d]Department of Agriculture, Food and Environment, Università degli Studi di Pisa, Via del Borghetto 80, 56124 Pisa, ITALY

*Corresponding author:

Tel.: +39 055 2755507;

Fax.: +39 055 2755500

Email address: bruno.zanoni@unifi.it

Running title: Malaxation oxidative damage indices

Abstract

An original kinetic study of the transformation phenomena of phenolic compounds in olive paste was carried out at different malaxation time-temperature conditions under exposure to air, using Abencor lab equipment to process olives (Frantoio cv) of a known degree of ripeness.

Empirical kinetic models and the relevant apparent kinetic constants were determined for the following significant indices: total phenolic compound content in waste water samples using the Folin-Ciocalteu method; verbascoside and β-OH-verbascoside contents in olive paste samples using HPLC; and 3,4-DHPEA-EDA contents in olive oil samples using HPLC. Two opposite phenolic compound transformation phenomena were proposed to explain the kinetic models: (i) enzymatic oxidative damage of phenolic compounds; (ii) physical and enzymatic release of phenolic compounds from cellular tissues. It was possible to propose a reference optimization chart to predict "selective" time-temperature conditions to maximize the apparent EVOO extraction yield while minimizing the degradation phenomena of phenolic compounds during malaxation.

Keywords:

Kinetics, Malaxation, Modelling, Olive oil, Phenolic compounds, Yield

1. Introduction

Oxidation is the most frequent degradation behaviour of food after microbial spoilage phenomena. Oxidative damage to food consists of oxidation reactions in lipids, proteins and minor compounds, causing a negative effect on food, particularly in terms of sensory and nutritional qualities. Oxidative reactions involve enzymatic or non-enzymatic phenomena and they are proportionally related to food temperature (Diplock et al., 1998; Parkin and Damodoran, 2003).

One of food technology's missions is to minimize oxidative damage in food processing where exposure to oxygen and, in general, operating conditions with high potentials of redox can occur. Therefore, it is necessary to select effective indices to both monitor and optimize operating conditions to control oxidative damage in food.

Extra virgin olive oil (EVOO) extraction processing can be an interesting example of how this approach can be applied in consideration of effects on the phenolic compounds in olive fruits. The phenolic profile has a critical role in the quality of EVOO. The amount of the different phenolic compounds is positively related to the preservation of oil quality from oxidation during shelf life, and it is responsible for EVOO's "bitter" and "pungent" sensory descriptors. Moreover, these compounds prevent ageing phenomena and several chronic diseases in humans (Clodoveo et al., 2014). Biochemical, chemical and physical phenomena that affect EVOO's phenolic profile, including enzymatic oxidative reactions, occur during the ripening of the olive fruits and the oil extraction process (Zanoni, 2014).

An impressive number of phenolic compounds (i.e. particularly oleoside compounds) are present in *Olea europaea* fruits. Secoiridoids, such as oleuropein, demethyloleuropein and ligstroside represent the predominant phenolic oleosides, whereas verbascoside is the main hydroxycinnamic derivative of olive oil fruits. Simpler phenolic compounds such as hydroxytyrosol and tyrosol are also present. The olive cultivar, geographical area of production, climatic conditions during the crop season, crop load and olive health conditions affect the phenolic profile of olive oil fruits (El Riachy et al., 2011).

However, the phenolic profile of olive oil fruits is not the same as the phenolic profile of extractable EVOO, since numerous transformation phenomena occur during the oil extraction process. Phenolic compounds are distributed greatly between the water and

oil phases of olive paste, obtained by crushing the olive fruits. The greater affinity of phenolic compounds towards the water phase means that only 0.3 % - 2 % of the phenols available in the olive fruits are transferred to the oil (Rodis et al., 2002). Secoiridoids are the compounds with the highest transfer rate from fruits to oil, followed by simple phenols; due to its structure, no verbascoside is found in EVOO (Klen and Vodopivec, 2012; Talhaoui et al., 2016). Moreover, rupturing of the olive cell tissues activates a series of enzymatic and non-enzymatic phenomena in the phenolic compounds. New phenolic compounds, which are hydrolytic forms of oleoeuropein and ligstroside, appear in the olive paste, whereas some fruit phenols disappear after crushing; therefore, the dialdehydic form of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) is often EVOO's most abundant phenolic compound (Zanoni, 2014; Klen et al., 2015a).

Three main steps in the oil extraction process affect the EVOO's phenolic profile: the crushing of the olive fruits, malaxation of the olive paste, and mechanical separation of the oil. The crushing step causes the initial physical partition of the phenolic compounds into the oil and water phases of the olive paste and activates the enzymatic (i.e. β-glucosidase activity) and non-enzymatic hydrolytic phenomena that transform oleoeuropein and ligstroside into their respective aglycones and decarboxymethylated forms (Clodoveo et al., 2014; Leone et al., 2015). The malaxation step consists of slow and continuous kneading of the olive paste to induce physical phenomena (i.e. oil droplet coalescence, rising of oil to the surface) that improve the oil process yield (Trapani et al., 2017); in general, malaxation is expected to continue the above hydrolytic phenomena without any enzymatic oxidative degradation (i.e. polyphenol oxidase and peroxidase activities) of the phenolic compounds (Clodoveo, 2012). Finally, the processing parameters during separation of the oil by centrifugation (i.e. use of a horizontal centrifuge with screw conveyor, namely "decanter") from the solid and water phases of olive paste have to be planned and controlled to maximize phenolic compound dissolution in the extractable EVOO (Altieri et al., 2013; Caponio et al., 2014). In view of the various possible combinations of operating conditions, such as time, temperature, oxygen exposure and kneading tools, several studies on the effect of malaxation on the phenolic profile of EVOO can be reported (Angerosa et al., 2001; Ranalli et al., 2001; Parenti and Spugnoli, 2002; Ranalli et al., 2003; Kalua et al., 2006; Migliorini et al., 2006; Artajo et al., 2007; Parenti et al., 2008; Servili et al., 2008; Boselli et al., 2009; Gomez-Rico et al.,

2009; Migliorini et al., 2009; Espinola et al., 2011; Migliorini et al., 2012; Catania et al., 2013; Taticchi et al., 2013; Tamborrino et al., 2014a; Klen et al., 2015a). The literature data shows that the malaxation behaves in a more complex way than the one described above. The secoiridoid profile depends on a combination of the following three kinds of opposite phenomena: (i) enzymatic oxidative degradation catalyzed by polyphenol oxidases (PPOs) and peroxidases (PODs), which cause a decrease in the phenolic compound content; (ii) enzymatic (i.e. β-glucosidase activity) and non-enzymatic hydrolytic phenomena that transform oleoeuropein and ligstroside into their respective aglycones and decarboxymethylated forms, especially the 3,4-DHPEA-EDA compound; (iii) physical and enzymatic (i.e. pectinase and cellulase activities) phenomena which promote the release of phenolic compounds from cellular tissues and then cause an increase in the phenolic compound content. Among the cinnamic acids, verbascoside content decreases, whereas its derivatives, such as the β-OH-verbascoside diastereoisomers, increase during malaxation. The literature data shows an incomplete and not uniform overview of the overall effect of the above phenomena on the phenolic profile of EVOO (relevant remarkable data are presented in Table 1). However, two common behaviours seemed to be observed: the content of the most representative phenolic compounds tends to decrease with malaxation time at a constant temperature, while it tends to increase with malaxation temperature at a constant time. These effects inversely depend on the oxygen exposure of the olive paste during malaxation: the higher the partial oxygen pressure, the greater the above decrease in phenolic compound content with time and the smaller the above increase in phenolic compound content with temperature.

No modelling based on pseudo *n*-order kinetics has been carried out on either the phenomena involved or the relationships of relevant rate constants with temperature. Therefore, the lack of quantitative time-temperature relationships makes it more difficult to apply the literature data to control olive paste malaxation. A kinetic approach to phenolic compound transformation phenomena may also link up to our previous time-temperature kinetic study to predict the potential effect of malaxation on extraction yield (Trapani et al., 2017), in order to strike a balance between oil yield and oil quality characteristics.

Analytical methodology		Malaxation operating conditions			Critical evaluation of results		Reference
Sample	**Phenolic compound measurement**	**Scale of trials**	**Time-temperature conditions**	**Level of oxygen exposure**	**In olive paste sample**	**In extracted oil sample**	
One batch of oil extracted by centrifugation of Dritta (23.1%[1]; 52.6%[2]), Caroleo (2i.6%[1]; 49.8%[2]) and Leccino (19.2%[1]; 59.8%[2]) olive paste	Total phenols by colorimetric Folin- Ciocalteu method as caffeic acid Single phenolic compounds by HRGC method as resorcinol	Industrial equipment (Rapanelli, Italy)	0, 15, 30, 45, 60 and 75 min at 30°C	Air exposure	-	Decrease in phenolic compound content with time	Ranalli et al. (2003)
One batch of oil extracted by centrifugation of Frantoio olive paste	Total phenols by colorimetric Folin- Ciocalteu method as gallic acid Single phenolic compounds by HPLC-DAD at 280 nm as tyrosol	Lab extraction unit	45 min at 21, 24, 27, 30, 33 and 36°C	Air exposure	-	Increase in total phenolic compounds and 3,4 DHPEA-EDA contents with temperature until 30°C referring to 45 min malaxation time	Parenti et al. (2008)
Two batches of oil extracted by centrifugation of Coratina and Ogliarola olive paste	Total and single phenolic compounds by HPLC-DAD at 278 nm as corresponding substances	Industrial equipment (Rapanelli, Italy)	40 min at 25°C	Air exposure/ N_2 without oxygen/High oxygen partial pressures (50 and 100 kPa)	Increase in degradation rate of total phenolic compounds and 3,4 DHPEA-EDA contents with time, on increasing the oxygen partial pressure; variation of verbascoside content	Decrease in 3,4 DHPEA-EDA content with oxygen partial pressure increase	Servili et al. (2008)
Two batches of oil extracted by centrifugation of Cornicabra (24.6-28.4%[1]; 39.8-37.5%[2]) olive paste	Total and single phenolic compounds by HPLC-DAD: - in olive paste at 280 nm (for secoiridoids) and 340 nm (for verbascoside) as corresponding substances or oleoeuropein; - in oil at 280 nm as corresponding substances	Industrial equipment (Pieralisi, Italy) Lab extraction unit (Abencor, Spain)	0, 17, 30, 43, 47, 60, 73, 77, 90 and 103 min at 20, 28 and 40°C 0, 15, 30, 45, 60 and 75 min at 20, 24, 28, 35 and 40°C	Air exposure	Decrease in 3,4 DHPEA-EDA content with time; no variation in verbascoside content	Increase in 3,4 DHPEA-EDA content with temperature referring to 20°C/60 min malaxation conditions	Gomez-Rico et al. (2009)
Two batches of oil extracted by centrifugation of Frantoio-Leccino and Coratina olive paste	Total and single phenolic compounds by HPLC-DAD at 280 and 350 nm as 3,4-dihydroxyphenylacetic acid and p-hydroxyphenylethanol	Industrial equipment (Pieralisi, Italy)	45 min at 25, 35 and 45°C	Air exposure	-	Increase in total phenolic compounds and 3,4 DHPEA-EDA contents with temperature referring to 45 min malaxation time	Boselli et al. (2009)
Four batches of oil extracted by centrifugation of Coratina, Ogliarola, Moraiolo and Peranzana olive paste	Total and single phenolic compounds by HPLC-DAD at 278 nm as corresponding substances	Industrial equipment (Rapanelli, Italy)	40 min at 20, 25 and 35°C	Air exposure/ High oxygen partial pressure (50 kPa)	Decrease in degradation rate of total phenolic compounds and 3,4 DHPEA-EDA contents with time, on increasing the temperature	Increase in total phenolic compounds and 3,4 DHPEA-EDA contents with temperature referring to 40 min malaxation time	Taticchi et al. (2013)

Table 1. Some remarkable literature data about the effects of malaxation operating conditions on phenolic compounds. [1]Olive fruit oil content; [2]Olive fruit moisture content.

The aim of this work is to apply a kinetic approach to phenolic compound transformation phenomena in order to select technological indices for the implementation of olive paste malaxation optimization charts.

2. Material and methods

2.1. Malaxation trials

The olive oil fruits (*Olea europea* L. Frantoio cv.) were supplied by the Pisa University experimental farm located in Venturina (Livorno, Italy) during the 2015 crop season. The ripe olive oil fruits were picked by hand at 08:00 a.m. at the end of October. Approximately 40 kg of olive oil fruits, which presented no infection or physical damage, were quickly transported to the laboratory.

The kinetic study was performed using Abencor lab equipment (Abencor analyser, MC2 Ingegneria Y Sistemas S.L., Seville, Spain) following Trapani et al. (2017). With respect to its usual use, the equipment was utilized both for the olive crushing and olive paste malaxation, but not for the olive paste centrifugation. The equipment consisted of an "MM-100" hammer mill (with 5.5 mm-diameter crusher holes) and a thermostated water bath (Thermo-mixer TB-100), with eight work sites; the work sites consisted of eight stainless steel mixing jars (speed of mixing blades: 50 rpm) under exposure to air, so that several olive paste malaxation treatments could be simulated in parallel. It was deliberately decided to perform the malaxation in this manner to make the oxidative degradation phenomena more evident.

The malaxation trials were carried out in triplicate at 22, 27, 32 and 37°C for 0, 20, 40, 60, 80 and 100 minutes; the water and paste temperatures were monitored using a type T thermocouple thermometer (Testo 926, Milan, Italy). Approximately 2.1 kg of olive paste, separated into six mixing jars each containing 350 g of olive paste, were used for each malaxation trial.

The olive paste samples were partly used to measure the phenolic compound content and partly to measure the apparent oil extraction yield, as reported below in the description of the analysis methods.

2.2. Analysis methods on olive oil fruits

The olive samples were analysed for weight, pulp/stone ratio and Maturity Index (Anonymous, 2011). The Maturity Index was based on the evaluation of the olive skin and pulp colours. The values ranged from 0 (deep green skin colour) to 7 (black skin colour with all the flesh purple to the stone).

A homogeneous batch of olives (i.e. approx. 300 g) were crushed in a laboratory crusher (Zeutec, Rendsburg, Germany), and the olive paste was used to make chemical analyses of the water and oil contents. The water content of the olive paste was measured by heating 60 g of the sample in an oven at 105°C until a constant weight was reached. The total oil content was determined on 5 g of dried olive paste (see the above oven method). Samples were extracted using hexane in an automatic extractor (Randall mod.148, VELP Scientifica, Milan, Italy), following the method of Cherubini et al. (2009). The characteristics of the processed olive oil fruits are given in Table 2.

Weight (g)	**Pulp/stone ratio**	**Maturity index**	**Water content (%)**	**Oil content (% dry basis)**	**Oil content (% fresh basis)**	**EY_{max} (%)**
1.9	3.1	3.2	45 ± 2	44 ± 3	24^a ± 2	22^a ± 2

Table 2. Mean values of *Frantoio* olive oil fruit characteristics for malaxation trials. Different small letters indicate significant differences (p = 0.01) for the different samples

2.3. Chemical analysis methods on the olive paste, olive oil and vegetation water

The phenolic compound content was extracted and determined on olive paste, vegetation water and olive oil samples. The olive oil and vegetation water samples were obtained by centrifugation (type 4239R, Alc Int. s.r.l, Milan, Italy); the olive paste samples, in 50 mL screw-cap tubes, were centrifuged at 4000 rpm (1800 G) for 15 min followed by a second centrifugation at 7000 rpm (5400 G) for 10 min. The oil and water phases were collected separately using a Pasteur pipette and then put into 15 mL test tubes for the following chemical analyses.

Phenolic compound content by Folin-Ciocalteu (Singleton and Rossi, 1965)

Olive paste. A 4.0 g olive paste sample was weighed in a 100 mL screw-cap tube and 80 mL of MeOH/H_2O solution (60/40, v/v) was added. The tube was shaken for 30 min and then was centrifuged at 4000 rpm for 15 min; the MeOH/H_2O phase was collected. The above extraction method was repeated and the collected MeOH/H_2O phases were brought to volume with MeOH/H_2O solution (60/40, v/v) in a 200 mL flask, which was stored in a freezer at least for 2 hours; then, the above solution was filtered

(FN 7 Munktell, Ahlstrom Falun AB, Falun, Sweden). 1.0 mL of the filtered phenolic extract was added to 5 mL of Folin-Ciocalteu reagent and 20 mL of Na_2CO_3 solution (20% w/v); the solution was brought to volume with purified water in a 100 mL flask and was stored for 1 hour at room temperature. The total phenolic compound content was detected at 765 nm (Lambda 35 UV/Vis Spectrometer, Perkin Elmer, Waltham, MA) and quantified using a gallic acid calibration curve ($r^2 = 0.997$) as mg gallic acid kg^{-1} of olive paste.

Olive oil. A 5.0 g olive oil sample was weighed in a 100 mL screw-cap tube and 10 mL of MeOH/H_2O solution (80/20, v/v) was added. The tube was shaken for 30 min and then was centrifuged at 4000 rpm for 10 min; the MeOH/H_2O phase was collected. The above extraction method was repeated and the collected MeOH/H_2O phases were brought to volume with MeOH/H_2O solution (80/20, v/v) in a 25 mL flask, which was stored in a freezer at least for 5 hours; then, the above solution was filtered. 1.0 mL of the filtered phenolic extract was added to 10 mL of Folin-Ciocalteu reagent (1/10 diluted) and the solution was brought to volume with Na_2CO_3 solution (7.5% w/v) in a 20 mL flask; it was stored for 2 hours at room temperature. The total phenolic compound content was detected at 765 nm and quantified using a gallic acid calibration curve ($r^2 = 0.997$) as mg gallic acid kg^{-1} of olive oil.

Vegetation water. The vegetation water sample was filtered and 1.0 g of the filtered vegetation water sample was weighed and then it was brought to volume with purified water in a 20 mL flask. 1.0 mL of the phenolic extract was added to 50 ml of purified water, 5 mL of Folin-Ciocalteu reagent and 20 mL of Na_2CO_3 solution (20% w/v); the solution was brought to volume with purified water in a 100 mL flask and was stored for 1 hour at room temperature. The total phenolic compound content was detected at 765 nm and quantified using a gallic acid calibration curve ($r^2 = 0.997$) as mg gallic acid kg^{-1} of vegetation water.

Phenolic compound content by HPLC

Olive paste. The phenolic compounds were extracted from the olive paste using the Cecchi *et al.* (2013) method. An 8.0 g olive paste sample was added to a test tube together with 0.500 mL of an internal standard (i.e. syringic acid, 1.5 mg mL^{-1} in a MeOH/H_2O 80/20, v/v solution) and 30 mL of EtOH/H_2O solution (80/20, v/v). The mixture was homogenized with ULTRA-TURRAX at 11,000 rpm in an ice bath for 3 min

and centrifuged (type PK121R, Alc Int. s.r.l, Milan, Italy) at 4,000 rpm (2000 G) at 0°C for 10 min. Then the supernatant was added to a 100 mL flask and it was stored in a freezer. The extraction procedure was repeated with 30 mL of EtOH/H_2O solution (80/20, v/v), and the obtained supernatant was added to the flask.

The obtained solution was concentrated in a vacuum at approx. 35°C, added to 2.5 mL of Milli-Q-Water (Millipore SA, Molsheim, France), washed twice with 25 mL of hexane in a separating funnel to remove lipid component, centrifuged at 14,000 rpm (24540 G) at 0°C for 5 min, and poured into a 10 mL flask. Five mL of methanol was added to the solution, which was brought to volume with Milli-Q-Water. The MeOH/H_2O solution of the phenolic extract was immediately used for the chromatographic analysis.

Chromatographic analyses were carried out using an HP1100L Liquid Chromatograph (Agilent Technologies, Palo Alto, CA), equipped with an autosampler, a column heater module, a quaternary pump, and coupled with DAD and MS/TOF detectors.

A Poroshell 120 EC-C18 column (3.0 mm, internal diameter; 150 mm, length; 2.7 µm, particle size) (Agilent Technologies, Palo Alto, CA) was used. It was equipped with a pre-column of the same phase. Elution was performed at a flow rate of 0.4 mL min^{-1} with a multistep linear gradient, using H_2O brought to pH 3.2 by formic acid (solvent A) and acetonitrile (solvent B). The three-step linear gradient of both solvents A and B changed as follows: from 95% A/5% B to 60% A/40% B in 40 min, with isocratic elution for 5 min, to 0% A/100% B in 5 min, with isocratic elution for 3 min, then to 95% A/5% B in 2 min. The total time of analysis was 55 min. All the solvents used were of HPLC grade. Syringic acid was chosen as the internal standard. The phenolic compounds were quantified at 280 nm; syringic acid and tyrosol were chosen as external calibration standards to evaluate the relative response factor (i.e. RRF = 4.74) and phenolic compound content values were expressed as $mg_{tyr}\ kg^{-1}$ of olive paste. Verbascoside and β-OH-verbascoside diastereoisomers were also quantified at 330 nm; syringic acid and verbascoside were chosen as external calibration standards to evaluate the relative response factor (i.e. RRF = 3.04) and verbascoside and β-OH-verbascoside diastereoisomers content values were expressed as $mg_{verb}\ kg^{-1}$ of olive paste.

Olive oil. The extraction, identification and determination of phenolic compounds were performed on the olive oil samples in agreement with the official IOC method

(Anonymous, 2009). The hydrophilic phenolic compound was extracted from the oil using a MeOH/H_2O (80/20, v/v) solution. The phenolic compounds in the mixture were separated and determined by an HPLC series 200 LC (Perkin Elmer Inc., Waltham, MA) consisting of a Perkin Elmer series 200 autosampler and a quaternary pump, coupled with a 9050 UV-Vis detector (Varian Inc, Palo Alto, CA). The analytical conditions were: pre-column: LiChroCART® 4-4 Purospher® STAR RP-18E, 5 µm (Merck KGaA, Darmstadt, Germany); HPLC column: LiChroCART® 250-4.6 Purospher® STAR RP-18E, 5 µm (Merck KGaA, Darmstadt, Germany); injection volume: 20 µl; solvent: acid H_2O (0.2% H_3PO_4)/acetonitrile/methanol gradient as described in the official method; wavelength: 280 nm.

Syringic acid was used as the internal standard; syringic acid and tyrosol were chosen as the external calibration standards to evaluate the relative response factor (i.e. RRF = 5.40). The phenolic compound content values were expressed as $mg_{tyr}\,kg^{-1}$ of olive oil.

2.4. Physical analysis methods on the olive paste, olive oil and vegetation water

Partition coefficient

Partition coefficients (*P*) were determined in order to compare the difference in solubility of the phenolic compound content in the different phases during malaxation. The ratio between the total phenolic compound content in olive oil and vegetation water ($P_{o/w}$) and the ratio between the total phenolic compound content in olive oil and olive paste ($P_{o/p}$) were determined using analytical data from the Folin-Ciocalteu and HPLC-DAD methods, respectively.

Apparent oil extraction yield

An apparent Extractability Index (EI_{app}) of oil during malaxation was measured following Trapani et al. (2017). This method permitted a quick measurement of the potential extraction performance by centrifugation of an olive paste malaxation treatment; hence, at increasing values this index would increase the effect of the malaxation on the olive paste, thus making the oil easier to extract industrially by way of centrifugation using a "decanter".

The apparent Extractability Index (EI_{app}) was calculated using the following ratio:

$$EI_{app}(\%) = \frac{EY\ (\%)}{EY_{max}\ (\%)} \cdot 100 \qquad [1]$$

where the extraction yields are expressed as percentage ratios of the mass of extracted oil and the mass of centrifuged olive paste; *EY (%)* is the percentage extraction yield and EY_{max} (%) is the percentage maximum oil extraction yield (Table 2).

2.5. Data processing

The analytical data were statistically processed according to a multifactor ANOVA using Statgraphics Centurion software (ver. XV, Statpoint Technologies, Warrenton, VA). Type III sums of squares were chosen and the contribution of each factor (i.e. time, temperature and replication) was measured after removing the effects of all of the other factors. The P-value test measured the statistical significance of each of the factors.

Time-temperature models were set up following the common kinetic approach to express the relationships between data and time as pseudo-chemical kinetics and then to correlate the relevant rate constant of the reactions with temperature. The kinetic data were processed using Table Curve 2D Version 4 software (Systos Software Inc., Richmond, CA).

3. Results and discussion

In our study the choice of which phenolic compounds to measure was based on criteria of both analytical effort and the relevance of the compounds in the literature in order to study the effect of malaxation on EVOO quality (Klen et al., 2015a). Therefore, measurements using the Folin-Ciocalteu method were carried out on olive paste, olive oil and vegetation water samples to determine the total phenolic compound content in a simple way; measurements using HPLC methods were carried out both to determine the total phenolic compound content, as well as the verbascoside and β-OH-verbascoside diastereoisomer contents in the olive paste samples, and to determine the total contents of phenolic compounds and oleuropein and derivatives in the olive oil samples.

In order to determine the kinetic models a prior assessment was performed of the statistic significance of the time-temperature variations of the measured indices (Table 3).

INDICES	TIME EFFECT		TEMPERATURE EFFECT	
	F-ratio	*p-value*	*F-ratio*	*p-value*
In olive paste samples:				
Total phenolic compounds by Folin-Ciocalteu	2.73	*	0.80	n.s.
Total phenolic compounds by HPLC	1.26	n.s.	0.75	n.s.
Verbascoside	11.84	***	5.01	**
β-OH-1 verbascoside diastereoisomer	9.92	***	3.91	*
β-OH-2 verbascoside diastereoisomer	10.24	***	4.73	**
In olive oil samples:				
Total phenolic compounds by Folin-Ciocalteu	9.41	***	0.07	n.s.
Total phenolic compounds by HPLC	4.37	**	0.79	n.s.
Oleuropein	3.60	*	1.20	n.s.
3,4-DHPEA-EA	2.04	n.s.	1.40	n.s.
3,4-DHPEA-EDA	16.70	***	6.28	*
Hydroxytyrosol	2.07	n.s.	3.70	*
	12.95	***	5.63	**
In vegetation water samples:				
Total phenolic compounds by Folin-Ciocalteu				
Apparent Extractability Index - EI_{app}	343.62	***	3.81	*
Partition coefficient - $P_{o/w}$	6.13	*	0.09	n.s.
Partition coefficient - $P_{o/p}$	1.22	n.s.	1.38	n.s.

Table 3. Effect of tested malaxation time-temperature operating conditions on changing of the measured chemical and physical indices (***: *p-value* ≤ 0.001; **: *p-value* ≤ 0.005; *: *p-value* ≤ 0.05; n.s.: not significant)

Significant chemical indices were highlighted for every type of sample; among these were indices of known importance (i.e. total phenolic compounds by Folin-Ciocalteu and 3,4-DHPEA-EDA) and indices about which less is known (i.e. verbascoside and β-OH-verbascoside diastereoisomers). Of the physical indices, the apparent Extractability Index proved to be significant, confirming what was reported by Trapani et al. (2017). Instead, the partition coefficients did not prove to be significant. These indices assumed values on average between 4 and 5%, similarly to the studies by Artajo et al. (2007). The fact that there were no variations suggests that the transformations of the phenolic compounds during malaxation did not display significant mass transfer phenomena between the water and oil phases of olive paste. The mean values of all the above significant indices are presented as supplementary material in Table 4.

Temperature (°C)	Time (min)	Verbascoside (mg_{verb} kg^{-1})	β-OH-1 (mg_{verb}/kg)	β-OH-2 (mg_{verb}/kg)	Sum of β-OH-1 and β-OH-2 (mg_{verb}/kg)	3,4-DHPEA-EDA (mg_{verb}/kg)	Total phenolic compounds ($mg_{gallic\ acid}$ /kg)	Apparent Extractability Index - EI_{app} (%)
22	**0**	2275	129	152	281	126	12981	0.0
	20	2397	178	207	385	144	12247	56.9
	40	2472	215	246	461	147	12267	79.2
	60	1901	223	249	472	118	11384	76.1
	80	1916	n.d.	n.d.	n.d.	94	11443	93.7
	100	497	298	334	632	46	10031	99.6
27	**0**	2717	166	189	355	265	13490	0.0
	20	1827	256	284	540	165	13123	68.9
	40	1097	294	332	626	93	11746	82.8
	60	757	337	381	718	82	11493	90.9
	80	694	360	440	800	68	10288	88.0
	100	201	n.d.	n.d	n.d.	n.d.	8457	100.0
32	**0**	2133	108	126	234	187	14883	0.0
	20	2248	175	204	379	181	13117	65.2
	40	1454	229	261	490	149	12311	88.4
	60	1075	248	280	528	n.d.	11895	96.4
	80	714	248	279	527	89	10367	98.8
	100	815	401	430	831	52	11201	99.6
37	**0**	2347	78	90	168	156	14287	0.0
	20	2141	136	148	284	155	13167	77.3
	40	2268	146	167	313	158	13601	87.3
	60	2365	196	223	419	146	13279	90.3
	80	2029	218	238	456	127	12002	98.3
	100	572	309	341	650	61	10903	97.3

Table 4. Mean values of the significant measured indices; n.d., not determined.

3.1 Kinetic models of phenolic compound transformation phenomena

Table 5 shows the kinetic models of the phenolic compound transformation phenomena, which were produced by normalizing the data in Table 4, that is, by processing the data to determine their relative variation in relation to the data measured at time $t = 0$ ($\triangle_{rel}$). In the case of the β-OH-verbascoside diastereoisomers it was preferred to determine the kinetic model relating to the sum of their contents. As they are complex phenomena all the kinetic models are empirical and the kinetic constants are apparent.

Indices	*Variation kinetics*	*Apparent kinetic constants*	*Equations of kinetic constants as a function of temperature*	*Constants of temperature equations*
In vegetation water samples: Total phenolic compounds by Folin-Ciocalteu	$\Delta_{rel} = 1 - K_{f(\vartheta)}t$	$K_{22°C}$ = 1.95 10^{-3} min^{-1} (R^2 = 0.89) $K_{27°C}$ = 3.20 10^{-3} min^{-1} (R^2 = 0.93) $K_{32°C}$ = 3.10 10^{-3} min^{-1} (R^2 = 0.75) $K_{37°C}$ = 2.00 10^{-3} min^{-1} (R^2 = 0.83)	$K_{f(\vartheta)} = a + \frac{b}{\vartheta} + \frac{c}{\vartheta^2}$ (R^2 = 0.98)	a = -1.68 10^{-2} min^{-1} b = 1.119 °C min^{-1} c = -15.53 $°C^2$ min^{-1}
In olive paste samples: Verbascoside	$\Delta_{rel} = \frac{\left(1 + exp\left(-K_{f(\vartheta)}t_{lag,f(\vartheta)}\right)\right)}{\left(1 + exp\left(K_{f(\vartheta)}\left(t - t_{lag,f(\vartheta)}\right)\right)\right)}$	$K_{22°C}$ = 0.104 min^{-1}; t_{lag} = 93.3 min (R^2 = 0.93) $K_{27°C}$ = 0.031 min^{-1}; t_{lag} = 0 min (R^2 = 0.97) $K_{32°C}$ = 0.026 min^{-1}; t_{lag} = 50.8 min (R^2 = 0.89) $K_{37°C}$ = 0.149 min^{-1}; t_{lag} = 92.3 min (R^2 = 0.98)	$K_{f(\vartheta)} = a + b\vartheta + c\vartheta^2$ (R^2 = 0.98) $t_{lag\,f(\vartheta)} = a + \frac{b}{\vartheta} + \frac{c}{\vartheta^2}$ (R^2 = 0.96)	a = 1.645 min^{-1} b =- 0.113 $°C^{-1}$ min^{-1} c = 1.96 10^{-3} $°C^{-2}$ min^{-1} a = 1454 min b = -79532 °C min c = 1089411 $°C^2$ min
Sum of β-OH-1 and β-OH-2 verbascoside diastereoisomers	$\Delta_{rel} = 1 + K_{f(\vartheta)}t$	$K_{22°C}$ = 1.28 10^{-2} min^{-1} (R^2 = 0.96) $K_{27°C}$ = 1.68 10^{-2} min^{-1} (R^2 = 0.95) $K_{32°C}$ = 1.79 10^{-2} min^{-1} (R^2 = 0.82) $K_{37°C}$ = 2.54 10^{-2} min^{-1} (R^2 = 0.95)	$K_{f(\vartheta)} = K_0 exp\left(-\frac{E_a}{RT}\right)$ (R^2 = 0.94)	K_0 =6413 min^{-1} E_a =32184 J mol^{-1}
In olive oil samples: 3,4 DHPEA-EDA	$\Delta_{rel} = \frac{\left(1 + exp\left(-K_{f(\vartheta)}t_{lag,f(\vartheta)}\right)\right)}{\left(1 + exp\left(K_{f(\vartheta)}\left(t - t_{lag,f(\vartheta)}\right)\right)\right)}$	$K_{22°C}$ = 0.088 min^{-1}; t_{lag} = 92.9 min (R^2 = 0.90) $K_{27°C}$ = 0.032 min^{-1}; t_{lag} = 0 min (R^2 = 0.92) $K_{32°C}$ = 0.037 min^{-1}; t_{lag} = 73.3 min (R^2 = 0.99) $K_{37°C}$ = 0.092 min^{-1}; t_{lag} = 95.2 min (R^2 = 0.99)	$K_{f(\vartheta)} = a + b\vartheta + c\vartheta^2$ (R^2 = 0.99) $t_{lag,f(\vartheta)} = a + \frac{b}{\vartheta} + \frac{c}{\vartheta^2}$ (R^2 = 0.87)	a = 0.987 min^{-1} b =- 0.065 $°C^{-1}$ min^{-1} c = 1.11 10^{-3} $°C^{-2}$ min^{-1} a = 1470 min b = -79482 °C min c = 1079542 $°C^2$ min
Apparent Extractability Index - EI_{app} (%)	$EI_{app} = EI_{app,max} \cdot \left(1 - exp\left(-K_{f(\vartheta)}t\right)\right)$	$K_{22°C}$ = 3.59 10^{-2} min^{-1} (R^2 = 0.99) $K_{27°C}$ = 4.94 10^{-2} min^{-1} (R^2 = 0.99) $K_{32°C}$ = 5.32 10^{-2} min^{-1} (R^2 = 0.99) $K_{37°C}$ = 6.47 10^{-2} min^{-1} (R^2 = 0.99)	$K_{f(\vartheta)} = K_0 exp\left(-\frac{E_a}{RT}\right)$ (R^2 = 0.95)	K_0 =3500 min^{-1} E_a =28064 J mol^{-1}

Table 5. Kinetic models of phenolic compound transformation phenomena; $\triangle_{rel}$ = relative variation with time (t) at different temperatures (ϑ).

In the vegetation water samples the normalized total phenolic compound content by Folin-Ciocalteu decreased linearly with time at the different tested temperatures (Figure 1); a maximum decrease of approx. 40% occurred at 27°C after 100 min of malaxation. The apparent decreasing rates ($K_{f(\vartheta)}$) showed an irregular trend with temperature: they increased from 22 to 27°C, then they decreased, at 37°C reaching a similar value to what was seen at 22°C. A polynomial model with a maximum point was suitable to describe this relationship (Table 5). Figure 1 shows an agreement between the experimental and predicted data.

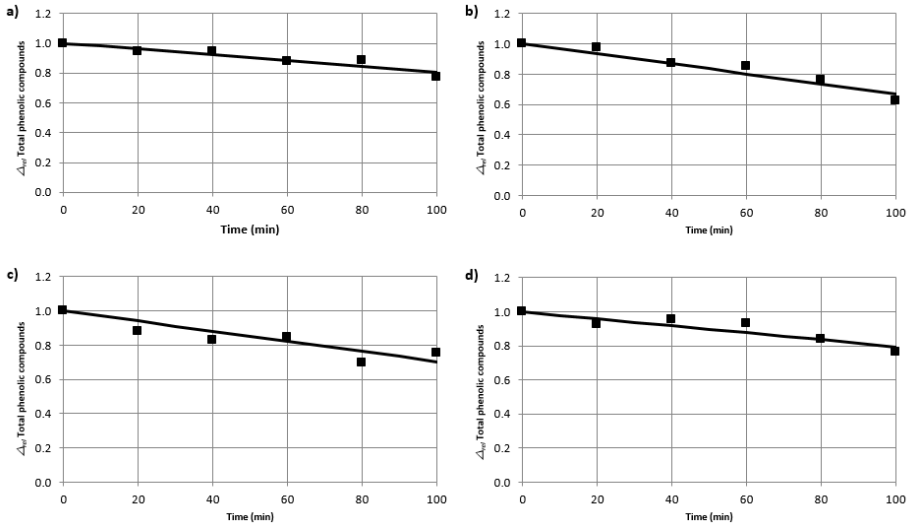

Figure 1. Kinetics of normalized total phenolic compound content using the Folin-Ciocalteu method in vegetation water samples at 22°C (**a**), 27°C (**b**), 32°C (**c**) and 37°C (**d**). The symbols ■ and ━━ are for experimental and predicted data, respectively.

The normalized verbascoside content in the olive paste strongly decreased with time; verbascoside disappeared almost completely at 27°C after 100 min of malax. (Fig. 2).

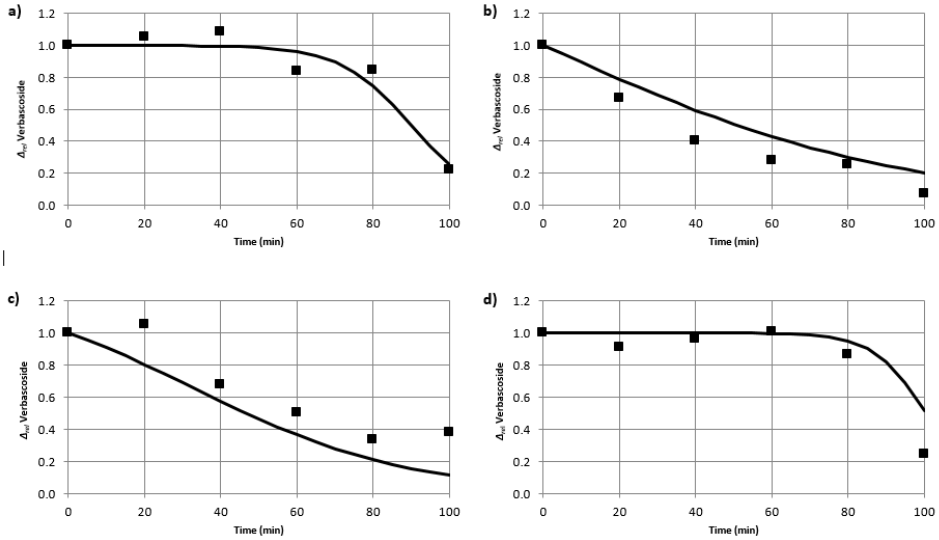

Figure 2. Kinetics of normalized verbascoside content in olive paste samples at 22°C (**a**), 27°C (**b**), 32°C (**c**) and 37°C (**d**). The symbols ■ and ━━ are for experimental and predicted data, respectively

Nevertheless, this decrease assumed a different trend as a function of temperature, with a clear concave curve at 22 and 37°C, but an exponential curve at 27°C. The general trend modelled by kinetics combined an apparent lag phase of verbascoside decrease with an apparent decreasing exponential phase (Table 5). The relationships of the apparent kinetic constants of the above phases ($t_{lag\ f(\vartheta)}$, $K_{f(\vartheta)}$) with temperature were described by polynomial models with a minimum point (Table 5). Figure 2 shows an agreement between the experimental and predicted data.

A similar behaviour compared with verbascoside content was evidenced for normalized 3,4-DHPEA-EDA content in the olive oil samples (Figure 3).

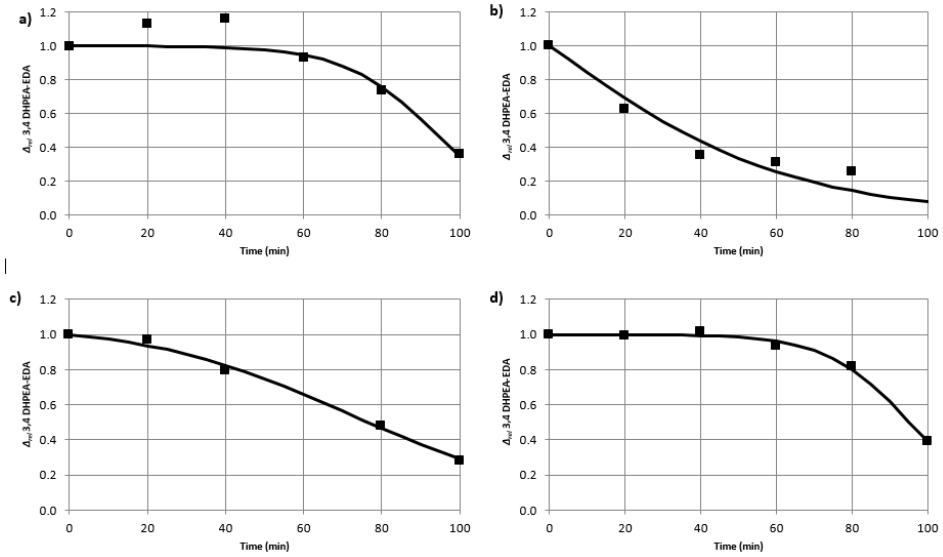

Figure 3. Kinetics of normalized 3,4 DHPEA-EDA content in olive oil samples at 22°C (**a**), 27°C (**b**), 32°C (**c**) and 37°C (**d**). The symbols ■ and ▬ are for experimental and predicted data, respectively.

As a result, the relevant kinetics were comparably modelled (Table 5). Figure 3 shows an agreement between the experimental and predicted data.

The normalized sum of the β-OH-verbascoside diastereoisomer content increased linearly with time at the different tested temperatures (Figure 4); an increase of four times occurred at 37°C after 100 min of malaxation. It was possible to significantly describe the experimental data using a pseudo zero-order kinetics with a rate constant ($K_{f(\vartheta)}$) that was temperature dependent through the Arrhenius equation (Table 5). Figure 4 shows an agreement between the experimental and predicted data.

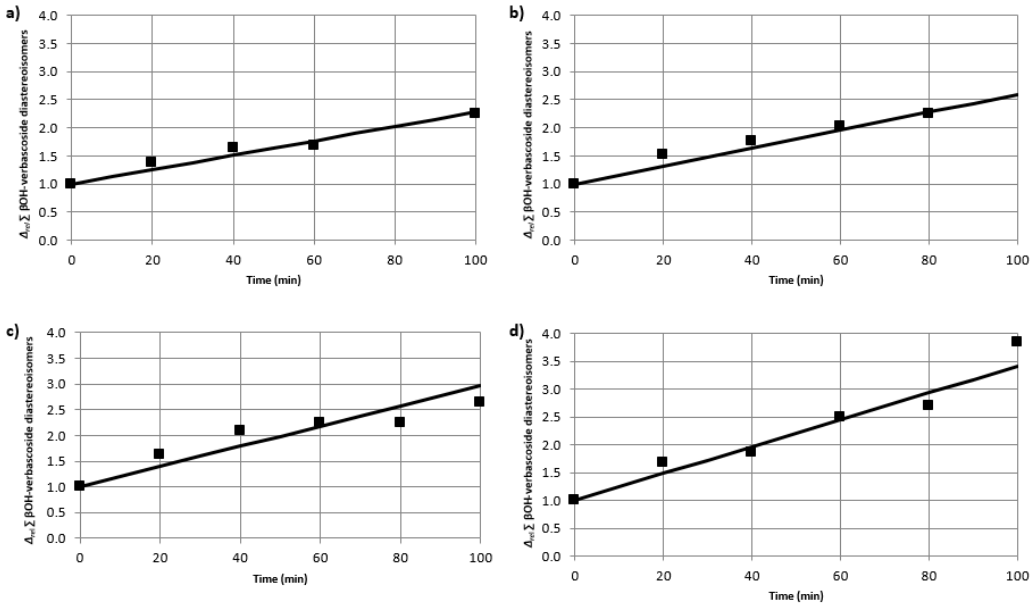

Figure 4. Kinetics of the normalized sum of β-OH-verbascoside diastereoisomer content in olive paste samples at 22°C (**a**), 27°C (**b**), 32°C (**c**) and 37°C (**d**). The symbols ■ and ━━━ are for experimental and predicted data, respectively

The overall vision of the above kinetics can coherently suggest that the phenolic compound transformation phenomena were caused by two opposing phenomena during olive paste malaxation, in line with the literature data (Boselli et al., 2009; Clodoveo, 2012; Taticchi et al., 2013; Clodoveo et al., 2014; Klen et al., 2015a): (i) a decreasing phenomenon probably due to enzymatic oxidative damage of the phenolic compounds; (ii) an increasing phenomenon probably due to a physical and enzymatic release of phenolic compounds from the cellular tissues. The effects of the above combination of phenomena were time-temperature dependent. In relation to the decreasing phenomenon it can be assumed that, after an activation phase, the speed increases as the temperature increases. In relation to the increasing phenomenon it can be assumed that it was absent or limited to 22 and 27°C, to then become present, at an increasing speed, at 32 and 37°C, so much so that at 37°C it cancelled out the effects of the decreasing phenomenon; this phenomenon tends to die out in time, seeing as at 37°C, even after a long period of malaxation, the effects of the decreasing phenomenon were seen. Hence, the irregular variation of the apparent decreasing rates with the temperature of the total phenolic compounds by Folin-Ciocalteu reflected the combination of the different speeds of the two aforesaid phenomena (Fig. 1). Similarly, the apparent lag phases of verbascoside and 3,4 DHPEA-EDA kinetics reflected either a slow decreasing phenomenon or an increasing phenomenon which disguised the effects of the decreasing phenomenon (Figures 2 and 3). The upshot is also that the different kinetics between the verbascoside

and its β-OH diastereoisomers must be related to the aforesaid transformation phenomena resulting from the verbascoside (Figures 2 and 4). The linear and exponentially temperature-dependent increase in β-OH verbascoside diastereoisomers could just be the expression of the decreasing phenomenon; that is, these diastereoisomers could be considered products of the verbascoside degradation due to a hydroxylation reaction, probably of an enzymatic nature. This consideration can be added to what was reported by Klen et al. (2015b).

3.2 Apparent oil extraction yield kinetic models

According to Trapani et al. (2017), the modelling of the evolution of the oil extraction yield, expressed as an apparent Extractability Index (EI_{app}), by pseudo first-order kinetics was statistically significant at every malaxation temperature (Table 5). It was reasonably assumed that for t = 0, EI_{app} = 0 and that EI_{app} tends in time to asymptotically reach a maximum value of 100% ($EI_{app,max}$). The rate constant ($K_{f(\vartheta)}$) was also significantly temperature dependent through the Arrhenius equation (Table 5). Figure 5 shows an agreement between the experimental and predicted data.

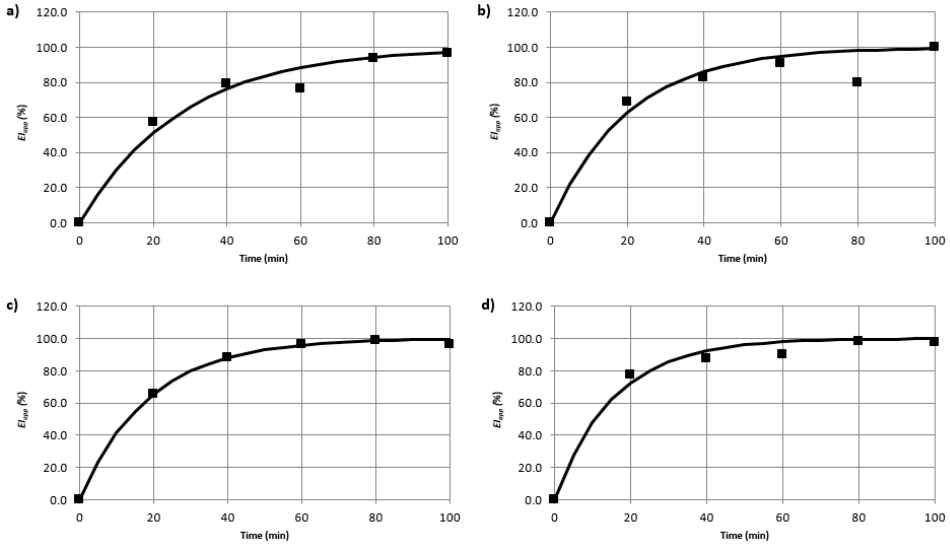

Figure 5. Kinetics of the apparent Extractability Index (EI_{app}) at 22°C (**a**), 27°C (**b**), 32°C (**c**) and 37°C (**d**).
The symbols ■ and ▬▬ are for experimental and predicted data, respectively.

Compared to the data of Trapani et al. (2017), the kinetic models were characterized by lower values of Arrhenius constants: K_0 = 3500 min^{-1} vs. K_0 = 7.50 10^7 min^{-1} and Ea = 28064 J mol^{-1} vs. Ea = 54512 J mol^{-1}. As a result, there was a faster increase in the apparent extraction yield during malaxation. It is thought that this was possible thanks to the greater oil content (24%) and the greater Maturation Index (3.2) of

the olive oil fruits (Table 2) compared to those referring (i.e. oil content = 20%; Maturation Index = 1.1) to the olives used in the experiment by Trapani et al. (2017). The data of Espinola et al. (2011) tend to confirm this hypothesis.

3.3. A malaxation time-temperature optimization chart

The direct application of the above kinetics enabled the construction of a synoptic chart to predict the potential effect of malaxation on phenolic compound content in isothermal conditions.

The chart was outlined with a logarithmic scale on the y-axis showing the malaxation time, and a linear scale on the x-axis showing the malaxation temperature (Figure 6).

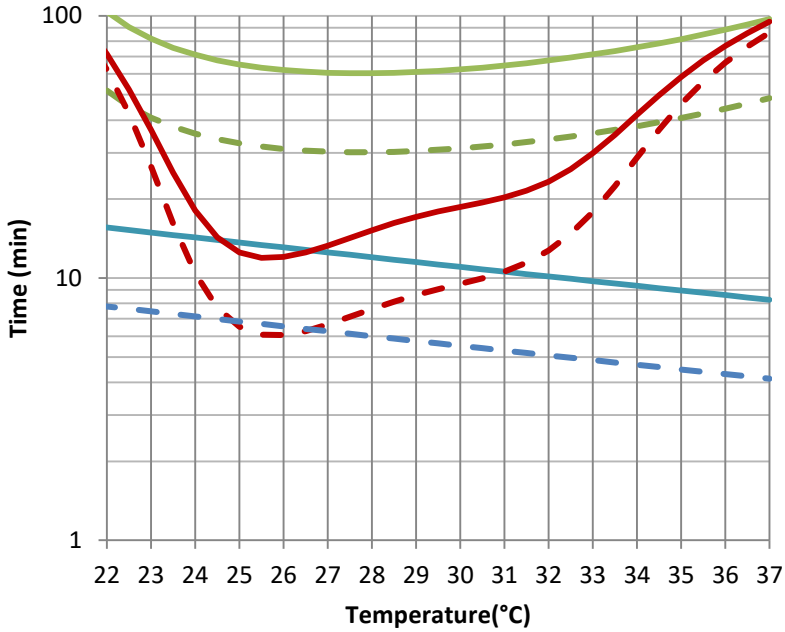

Figure 6. Time-temperature synoptic chart of olive past malaxation in relation to phenolic compounds: in green the curves referring to the total phenolic compounds, in red the curves referring to 3,4 DHPEA-EDA, in blue the curves referring to the sum of β-OH verbascoside diasteroisomers. The unbroken curves show a variation of 20%, and the dashed curves 10%.

On the chart it was possible to plot different relationships between the times and temperatures of malaxation, corresponding to defined quantitative levels of apparent phenolic compound oxidative damage, represented by the above selected indices. As the objective was to choose just one representative index for the vegetation water, the olive paste and the olive oil samples, it was opted to show the following defined medium-low levels of apparent oxidative damage by way of example in the synoptic chart: 10% and 20% apparent decrease in the total phenolic compound content using the Folin-Ciocalteu

method in the vegetation water, 10% and 20% apparent increase in the sum of β-OH verbascoside diastereoisomers in the olive paste and 10%, and 20% apparent decrease in 3,4 DHPEA-EDA in the olive oil.

The malaxation time (t) to reach the above set of apparent oxidative damage levels as a function of the olive paste malaxation temperature (ϑ) was calculated according to the relevant kinetics models in Table 5 as follows:

- for the total phenolic compound content using the Folin-Ciocalteu method:

$$t = \left(1 - \Delta_{rel,ref}\right) \cdot \frac{1}{K_{f(\vartheta)}} \quad [2]$$

where $\Delta_{rel,ref}$ is the chosen normalized value of reference (i.e. in our case 0.9 or 0.8 corresponding respectively to a 10% and 20% apparent decrease value);

- for the sum of β-OH verbascoside diastereoisomer content:

$$t = \left(\Delta_{rel,ref} - 1\right) \cdot \frac{1}{K_{f(\vartheta)}} \quad [3]$$

where $\Delta_{rel,ref}$ is the chosen normalized value of reference (i.e. in our case 1.1 or 1.2 corresponding respectively to a 10% and 20% apparent increase value);

- for 3,4 DHPEA-EDA content:

$$t = \frac{ln\left(\frac{1+\exp\left(-K_{f(\vartheta)} \cdot t_{lag,f(\vartheta)}\right)-\Delta_{rel,ref}}{\Delta_{rel,ref}}\right)+K_{f(\vartheta)} \cdot t_{lag,f(\vartheta)}}{K_{f(\vartheta)}} \quad [4]$$

where $\Delta_{rel,ref}$ is the chosen normalized value of reference (i.e. in our case 0.9 or 0.8 corresponding respectively to a 10% and 20% apparent decrease value).

The synoptic chart (Figure 6) shows how together the three chosen indices give an overall vision of the effects of the malaxation time-temperature conditions on the transformation phenomena of phenolic compounds.

The sum of β-OH verbascoside diastereoisomer content proved to be the most sensitive index among those chosen at the malaxation time-temperature conditions (i.e. levels of damage reached in lower malaxation times at the same temperature). Forming a straight line, it can be considered the index that expresses the substantially oxidative damage of the phenolic compounds only.

The total phenolic compound content using the Folin-Ciocalteu method proved to be the least sensitive index among those chosen to the malaxation time-temperature

conditions (i.e. levels of damage reached in longer malaxation times at the same temperature). Forming a convex curve, it can be considered the overall and simple measurement index that expresses the combination of phenolic compound damage and release phenomena.

The trends of time as a function of malaxation temperature to reach set levels of apparent degradation of 3,4 DPHEA-EDA took on the appearance of highly convex curves; this is due to the kinetics dealt with in the previous paragraph which, thanks to measuring a specific compound (and not a set of compounds like in the case of total phenolic compound content by Folin-Ciocalteu) made it easier to separate the phenolic compound degradation and release phenomena. As such, the 3,4 DPHEA-EDA content proved to be the index that best represents the effect of the malaxation time-temperature conditions on the phenolic compounds in the extractable oil.

The synoptic chart can also be used for optimization purposes, for example, if it plots, using straight lines, the different relationships between the times and temperatures of malaxation, corresponding to values of 60% and 80% (i.e. expression of insufficient and satisfactory oil process yields, respectively) of the apparent Extractability Index (Figure 7).

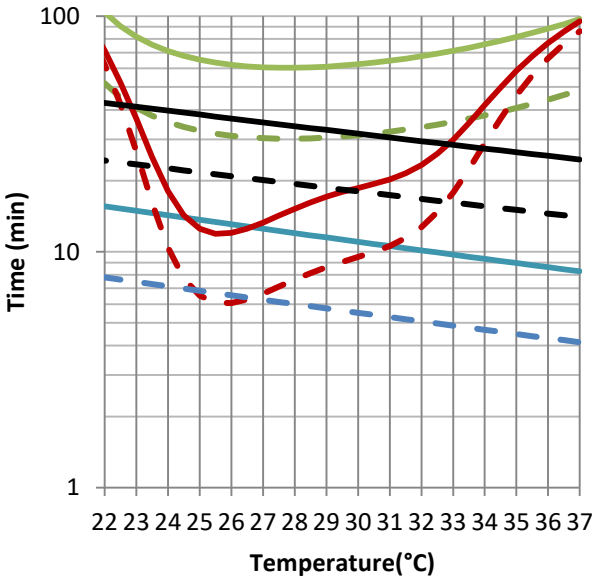

Figure 7. Olive paste malaxation time-temperature optimization chart obtained by overlapping the synoptic chart shown in Figure 6 with the black straight lines of apparent yield, unbroken to indicate an 80% yield and dashed for a 60% yield.

Please see Trapani et al. (2017) for the equation that expresses the malaxation time to reach the above set of apparent extraction levels as a function of the olive paste malaxation temperature, according to the relevant kinetic model in Table 5.

It is evident how in the adopted strong oxidative impact experimental conditions an acceptable apparent yield is not compatible with a lower degradation of the sum of β-OH verbascoside diastereoisomer content; from the synoptic chart it can be deduced that a lower degradation is compatible with an apparent yield of less than 50% or that an 80% apparent yield determines degradation of around 50% of the sum of β-OH verbascoside diastereoisomer content.

However, should the combination between phenolic compound degradation and release phenomena be considered, an apparent acceptable yield appears compatible with lower apparent degradation of the total phenolic compound content by Folin-Ciocalteu. Instead, an acceptable apparent yield only seems compatible with a lower apparent degradation of the 3,4-DHPEA-EDA content for some time-temperature combinations. For example, by moving along the straight line corresponding to 80% of the apparent yield, three zones can be seen with reference to the adopted experimental conditions: (i) a zone with an approximate temperature of < 23°C for time = 40 min compatible with a reduced apparent degradation of 3,4 DHPEA-EDA; (ii) a zone with an approximate temperature of > 23°C for times between 40 and 30 min responsible for a high apparent degradation of 3,4 DHPEA-EDA; (iii) a zone with an approximate temperature of > 33°C for time < 30 min compatible with a lower apparent degradation of 3,4 DHPEA-EDA.

4. Conclusions

This research is based on an original kinetic approach which enabled the prediction of the effects of time-temperature conditions of malaxation treatment under exposure to air on the transformation phenomena of phenolic compounds in olive paste.

It was possible to identify and quantify two contrasting phenolic compound transformation phenomena, which were measured on samples both of olive paste and its vegetation water and oil components: (i) a decreasing phenomenon probably due to enzymatic oxidative damage of phenolic compounds; (ii) an increasing phenomenon probably due to a physical and enzymatic release of phenolic compounds from the cellular tissues. These phenomena could be significantly monitored by three different but complementary technological indices. The sum of β-OH verbascoside diastereoisomer content in the olive paste samples proved to be a very sensitive index in expressing the degradation phenomena. The total phenolic compound content by Folin-Ciocalteu and the

3,4 DHPEA-EDA proved to express the combination of the two aforesaid transformation phenomena in the vegetation water and oil samples, respectively; of the two, the second appeared of particular interest as it specifically refers to the most important phenolic component present in EVOO.

With regard to the experimental conditions adopted in this work, it was possible to propose a reference optimization chart in order to predict “selective” time-temperature conditions to maximize the apparent EVOO extraction yield while minimizing the degradation phenomena of phenolic compounds during malaxation treatment when the olive paste is exposed to oxygen. The chart shows how an acceptable apparent yield is not compatible with a lower degradation of the phenolic compounds. Nevertheless, in consideration of the presence of phenolic compound release phenomena too, time-temperature combinations can be seen that are compatible for example with a minimization of the apparent degradation of the 3,4 DHPEA-EDA content.

Our kinetic approach could be also a useful reference to understand and quantify the potential efficacy on the optimization of malaxation treatment of several production elements. The effects of cultivar and the degree of ripeness of the olive oil fruits (Espinola et al., 2011, Caruso et al., 2013, Caruso et al., 2014), of technological innovations in the pre-treatment of olive paste prior to malaxation based on ultrasound or microwave techniques (Clodoveo et al., 2013; Tamborrino et al., 2014) and of malaxation treatment under no or controlled exposure of the olive paste to oxygen (Servili et al., 2008; Leone et al., 2014; Catania et al., 2016) could be compared with the kinetic model devised in this work.

Lastly, the proposed approach to quickly heat the olive paste to a particular temperature using a tubular heat exchanger, leave the paste in the malaxer for the desired time and then send it for extraction in a “decanter” seems a good idea in order to best exploit what is shown in this work (Veneziani et al., 2015; Leone et al., 2016). Such an approach would pave the way towards real control of malaxation treatments in order to achieve the desired phenolic profiles in EVOO.

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Nomenclature

a, b, c	regression coefficients (min^{-1} or min)
Ea	activation energy ($J\ mol^{-1}$)
EI_{app}	apparent Extractability Index (%)
$EI_{app,\ max}$	maximum apparent Extractability Index (%)
EY	extraction yield (%)
EY_{max}	maximum extraction yield (%)
$K_{f(\vartheta)}$	apparent kinetic constants as a function of malaxation temperature (min^{-1})
k_0	frequency factor (min^{-1})
R	gas constant ($J\ mol^{-1}\ K^{-1}$)
T	malaxation absolute temperature (K)
t	malaxation time (min)
t_{lag}	apparent lag phase (min)
$\triangle_{rel}$	relative variation with time at different temperatures
$\triangle_{rel,ref}$	relative variation chosen as reference
ϑ	malaxation temperature (°C)

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Effect of the moisture content of rehydrated olive paste on the content and profile of phenolic compounds in extractable olive oil

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Lorenzo Cecchi[1*], Carlotta Breschi[2], Marzia Migliorini[3], Valentina Canuti[2], Giovanna Fia[2], Nadia Mulinacci[1], and Bruno Zanoni[2]

[1]NEUROFARBA Department, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Florence), ITALY

[2]Department of Agricultural, Food and Forestry Systems Management (GESAAF) – Food Science and Technology and Microbiology Section, Università degli Studi di Firenze, Via Donizetti 6, 50144 Florence, ITALY

[3]Carapelli Firenze S.p.A., Via Leonardo da Vinci 31, 50028 *Tavarnelle* Val di Pesa, Florence, ITALY

*Corresponding author: Lorenzo Cecchi

Department of NEUROFARBA, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, Florence, Italy.

Tel.: +39 0554573707

E-mail address: lo.cecchi@unifi.it

Abstract

The effect of olive paste moisture on content and profile of phenolic compounds in extractable olive oil was studied at lab-scale. The experimental trials were carried out through the gradual dilution of freeze-dried olive paste, at a moisture range of 0-60%. The olive paste moisture produced a significant effect both on oil extraction and on phenolic transfer yields. Phenolic compounds in extractable oil reached a maximum (about 1200 mg/kg) at moisture contents of approx. 20-40% and then decreased. The measurement of olive paste moisture resulted essential to control EVOO processing equally to other operating conditions (time, temperature, air exposure), also playing a crucial role on the enzymatic phenomena that promote the release of phenolic compounds from the cellular tissues. Processing procedures able to control the olive fruit moisture may led to technological innovations and could be used to produce express olive oils using little mills recently proposed in the market.

Keywords: enzymatic phenomena, extra virgin olive oil, lignans, secoiridoids, water activity

1 Introduction

In addition to the many well-known health properties associated to olive oil phenolic compounds, an important health claim has been approved by the EFSA for olive oils rich in these bioactive molecules. This claim, based on *in vivo* tests in humans, guarantees that the consumption of olive oil phenols contributes to protect LDL from oxidative damage. Consequently, improving the ability to increase and control the amount of phenolic compounds transferred from olives to olive oils is one of the main goals in the production chain of extra virgin olive oil (Amirante, Clodoveo, Tamborrino & Leone, 2012; Lukic et al., 2017; Kiritsakis et al., 2017).

Biochemical, chemical and physical phenomena during olive fruit ripening, the olive oil extraction process and EVOO shelf life affect the phenolic compound content and profile of EVOO (Amirante, Clodoveo, Tamborrino, Leone & Paice, 2010; Gutierrez-Rosales, Romero, Casanovas, Motilva, & Minguez-Mosquera, 2012; Cecchi et al., 2013; Zanoni, 2014; Klen, Wondra, Vrhovsek, Silviotti & Vodopivec, 2015).

Water availability along the olive oil processing chain may have a critical role in the above phenomena. Water supplies in the olive orchard, water use during oil extraction in the olive mill and the moisture content of the different semi-finished products (i.e. olive fruits, olive paste, veiled oil) are operating conditions which can influence the phenolic compound content of EVOO. Several studies have upheld that the content of phenolic compounds in olive fruits is lower in fully irrigated trees than in trees under deficit irrigation or those that only receive complementary irrigation (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Caruso et al., 2014). Olive fruit moisture seems to negatively affect the phenolic content of EVOO; those cultivars with lower rates of phenolic transfer to the oil presented a high percentage of moisture in the olive fruits (Talhaoui et al., 2016). A moisture content greater than 50% was considered to cause "difficult" olive paste for oil extraction (Di Giovacchino, 1991). It is assumed to interfere with oil coalescence during malaxation, since the oil is bound in an emulsion with the vegetable water (Moya et al., 2010; Koprivnjak, Bubola, & Kosic, 2016). Therefore, a decrease could occur in the extraction yield (Aguilera, Beltran, Sanchez-Villasclaras, Uceda, & Jimenez, 2010, Zanoni et al., 2018) and, consequently, in the EVOO phenolic content (Cecchi, Migliorini, Zanoni, Breschi, & Mulinacci, 2018).

The content and profile of the EVOO phenolic compounds are not the same as those of the olive oil fruits, since numerous transformation phenomena occur during the oil extraction process. Enzymatic and non-enzymatic phenomena transform the phenolic compounds during crushing and malaxation (Migliorini et al., 2012; Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015; Trapani et al., 2017a). The presence of water is necessary for the above reactions, but the rate reaction constants depend on the water activity level (A_w) following a non-proportional behaviour, as shown by the "Food Stability Map" (Roos, 2003). As the water activity decreases, the rate of several reactions decreases, since water cannot enhance the diffusion of substrates to enzyme molecules. However, high values of water activity can cause a decrease in reaction rate under certain conditions, due to the dilution of reacting species above a certain water activity level (Labuza, 1980).

Phenolic compounds are mainly distributed between the water and the oil phases of olive paste. The greater affinity of phenolic compounds towards the water phase means that only 0.05%-1% of the phenols available in the olive fruits are transferred to the oil (Cecchi et al., 2018). A limitation of the water supply during oil extraction could be recommended in order to increase the phenolic compound content of EVOO. The three-phase decanter appeared less efficient than a two-phase decanter in allowing a high phenolic recovery in the EVOO. The added water in the three-phase decanter changes the partition equilibrium of the phenolic compounds and most of the phenolic compounds flush away with the wastewater that is produced (Klen and Vodopivec, 2012; Cecchi et al., 2018).

The knowledge of how the rheological behaviour of olive paste depends on moisture content was also useful for optimizing the decanter's oil extraction performances, particularly for the two-phase decanter where no extra water can be added (Guerrini, Masella, Angeloni, Migliorini, & Parenti, 2017). The olive paste showed a time-dependent behaviour, typical of a non-Newtonian pseudoplastic fluid, which was modelled by the Herschel-Bulkley equation (Di Renzo and Colelli, 1997). The relevant consistency coefficients decreased as the olive paste moisture increased and consequently an increase in oil extractability occurred for olive paste with a high moisture content (Masella, Parenti, & Spugnoli, 2008; Boncinelli, Daou, Cini, & Catalano, 2009).

Finally, EVOO filtration is a common procedure in oil clarification and oil dehydration; the EVOO moisture should not exceed 0.2 g/100 g in order to avoid enzymatic activities which could affect the EVOO quality during its shelf life (Fregapane, Lavelli, León, Kapuralin, & Salvador, 2006; Brkic Bubola, Lukic, Mofardin, Butumovic, & Koprivnjak, 2017).

A systematic approach to studying the thus evidenced critical role of water in the olive oil processing chain appears lacking in the literature, due to the complex phenomena involved. The aim of this work was to study the effect of olive paste moisture on the content and profile of phenolic compounds in the extractable olive oil. The experimental trials were carried out at lab scale by stressing physical and biochemical phenomena through the gradual dilution of freeze-dried olive paste at a wide range of moisture content.

2. Materials and methods

2.1 Chemicals

Methanol from Baker (Phillipsburg, NJ) and acetonitrile from Panreac (Barcelona, Spain) were of HPLC grade. All the other chemicals were of analytical grade. A Milli-Q-system (Millipore SA, Molsheim, France) was used to produce the deionized water. Formic acid, hexane, syringic acid and tyrosol were from Sigma Aldrich (Steinheim, Germany), oleuropein from Extrasynthese (Genay, France) and phosphoric acid from Merck (Darmstadt, Germany). Stock solutions of the standards (oleuropein, syringic acid and tyrosol) were prepared in a hydroalcoholic solution.

2.2 Samples

Olive oil fruits

Samples of ripe olive fruits of Frantoio cultivar were collected from 10 selected olive plants (*Olea europea* L.) during the 2012 (164st Day After Full Blooming, DAFB) and 2017 (161th DAFB) crop season. Samples of ripe olive fruits of Leccio del Corno cultivar were collected from 10 selected olive plants (*Olea europea* L.) during the 2016 (190th DAFB) and 2017 (169th DAFB) crop season. All the samples were collected in province of Florence.

Olives were picked along the whole circumference of all the selected plants, at a height of approx. 170 cm. For all the collected samples, the whole fruits were freeze-dried within four hours of collection as described in Cecchi et al. (2015) and stored at -20°C.

Olive paste and olive oil

For each freeze-dried sample, an aliquot of olives were crushed in a laboratory crusher (Zeutec, Rendsburg, Germany) obtaining dried olive pastes as homogeneous as possible. The batches of dried olive paste were rehydrated by mixing with different amounts of water (i.e. 0%, 5%, 10%, 20%, 40%, 50% and 60% w/w), in order to obtain homogeneous reconstituted olive pastes with different moisture contents. Olive oil was immediately extracted from the above olive pastes by centrifugation at 14,000 rpm for 20 min at 22°C. The olive paste and olive oil samples were coded with a letter (F or L) to identify the cultivar, a digit (17, 16 or 12) to identity the crop season, letters (OP or OO) to identify the material and a figure (+0%, +5%, +10%, etc.) to identify the rehydration level.

2.3 Measurements and determinations

Olive oil fruits

A batch of olives were crushed and the moisture content (%) of olive paste was measured by gravimetric analysis at 105°C. Sugar content (% of glucose + fructose) was measured following the enzymatic method described by Trapani et al. (2016) and oil content was measured as reported by Migliorini et al. (2011), working on freeze-dried olives.

The phenolic compounds were extracted from the freeze-dried olives in presence of an internal standard (syringic acid) as described by Cecchi et al. (2013). Immediately after the extraction, the chromatographic analysis of phenolic extracts was performed with an HP1100 liquid chromatographer coupled with a DAD and a MS detector with HP1100 MSD API-electrospray interface (Agilent Technologies, California, USA). The column used were a Hypersil Gold QRP-18 (250 mm x 4.6 mm id, 3 µm particle size; Thermo Electron Corp., Austin, TX) for the 2012 samples and a Poroshell 120, EC-C18 (150 mm x 3.0 mm id, 2.7 µm particle size; Agilent, USA) for the 2016 and 2017 samples; these two columns gave the same results during some analysis carried out for comparing them before using the Poroshell for the 2016 and 2017 samples (data not shown). The

adopted chromatographic conditions are described in Cecchi et al., 2018. All the chromatograms were registered at the following λ: 240 nm, 280 nm and 330 nm.

Phenolic compounds were quantified using the internal standard method, with syringic acid as the internal standard. Luteolin-7-*O*-glucoside, tyrosol, oleuropein and verbascoside were used for evaluating the RRFs and the single phenols were then expressed as reported by Cecchi et al. (2018). The total phenolic compounds content (TPC) was expressed as $mg_{oleuropein}/kg$.

Olive paste and olive oil

The moisture content (%) of both the dried and rehydrated olive pastes was measured by gravimetric analysis at 105°C. The water activity of both the dried and rehydrated olive pastes was measured at 25°C using a hygrometer (Hygroscop DT, Rotronic, Zurich, Switzerland).

The olive oil phenolic compounds were analysed according to the IOC official method, as already reported by Cecchi et al., (2018). The chromatographic conditions were the same already reported by Cecchi et al. (2018). Phenolic compounds were quantified using the internal standard method (internal standard, syringic acid; reference compound, tyrosol) and the content of the single phenols and of the Total Phenolic Compounds Content (TPC) were expressed as $mg_{tyrosol}/kg_{oil}$.

Oil extraction and phenolic compound transfer yields

The same formulas used in an our previous work (Cecchi et al., 2018) were used to calculate the oil extraction yield as actual yield (*OY*) and as an Extractability Index (*EI*):

$$OY = \frac{OE_x}{Ol_m} \cdot 100 \quad [1]$$

$$EI = \frac{OE_x}{OC_{om}} \cdot 100 \quad [2]$$

OE_x was the measured extracted olive oil (g);

Ol_m was the measured olive paste in dried or rehydrated forms (g);

OC_{om} was the oil content of the olive pastes in dried or rehydrated forms (g), which was determined from the oil content of the olive fruits (% dm) and the moisture content of the dried or rehydrated olive paste (%).

The phenolic compound olive-to-oil transfer yield (*PY*) was calculated as a percentage of the normalized values with respect to the above actual oil extraction yields (*OY*):

$$PY = \frac{PO_{Ex} \cdot OY}{POl_m} \quad [3]$$

PO_{Ex} was the measured *TPC* of the extracted olive oil (mg/kg);

POl_m was the *TPC* of the olive pastes in dried or rehydrated forms (mg/kg), which was determined from the *TPC* of the olive fruits (mg/kg dm) and the moisture content of the dried or rehydrated olive paste (%).

The relevant measured (i.e. in italic) and calculated (i.e. in roman) data are shown in Table 1.

2.4 Data analysis

The standard deviation of total phenolic compounds content in olive oil samples was calculated according to the IOC official method. The precision of the quantitation of each single phenol in olive oil and in olive fruit samples was evaluated as reported in previous works (Cecchi et al., 2018 and Cecchi et al., 2015, respectively).

The water adsorption data were processed using Table Curve 2D Version 4 software (Systos Software Inc., Richmond, CA).

3. Results and discussion

The quality characteristics of the batches of olives which were used to prepare the olive paste subject to this study are shown in Table 2. The Frantoio cultivar olive batches from 2012 (F12) and 2017 (F17) had a similar oil content, which was higher than the Leccio del Corno cultivar from 2016 (L16) and 2017 (L17).

The phenolic compound content was different for all the olive batches: it ranged between 23,693 $mg_{oleuropein}$/kg for F12 and 46,478 $mg_{oleuropein}$/kg for F17. The content of phenolic compounds identified in the olive fruit samples is presented as supplementary material in Table 3.

FRANTOIO	F17OP+0%	F17OP+5%	F17OP+10%	F17OP+20%	F17OP+40%	F17OP+50%	F17OP+60%
Moisture content of olive paste (%)	3.30 ± 0.04	8.1 ± 0.1	13.0 ± 0.2	22.6 ± 0.3	42.0 ± 0.5	51.7 ± 0.7	61.3 ± 0.8
Moisture content of olive paste - n_s (kg_{water}/kg dm)	0.0341 ± 0.0008	0.088 ± 0.002	0.149 ± 0.005	0.292 ± 0.008	0.72 ± 0.02	1.07 ± 0.03	1.58± 0.04
Water activity of olive paste - A_w	0.303 ± 0.001	0.647 ± 0.003	0.841 ± 0.004	0.939 ± 0.004	0.951 ± 0.004	0.955 ± 0.004	0.966 ± 0.004
Oil content of olive fruits (% dm)	40 ± 1	40 ± 1	40 ± 1	40 ± 1	40 ± 1	40 ± 1	40 ± 1
Oil content in 40 g of olive paste (g)*	30.9 ± 0.8	14.7 ± 0.4	13.9 ± 0.4	12.4 ± 0.4	9.3 ± 0.4	7.7 ± 0.4	6.2 ± 0.3
Oil content of olive paste (%)	39 ± 1	37 ± 1	35 ± 1	31 ± 1	23 ± 1	19 ± 1	15.5 ± 0.8
Water-oil ratio of olive paste	0.085 ± 0.003	0.220 ± 0.009	0.37 ± 0.02	0.73 ± 0.04	1.8 ± 0.1	2.7 ± 0.2	4.0 ± 0.3
Extracted oil (g)	2.4 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	5.9 ± 0.1	4.0 ± 0.1	3.1 ± 0.1	2.5 ± 0.1
Oil extraction yield - *EI* (%)	7.8 ± 0.5	42 ± 2	44 ± 2	48 ± 2	43 ± 3	40 ± 3	40 ± 4
Oil extraction yield – *OY* (%)	3.0 ± 0.1	15.5 ± 0.3	15.5 ± 0.3	14.8 ± 0.3	10.0 ± 0.3	7.8 ± 0.3	6.3 ± 0.3
TPC of olive fruits (mg/kg dm)	82262 ± 7859	82262 ± 7859	82262 ± 7859	82262 ± 7859	82262 ± 7859	82262 ± 7859	82262 ± 7859
TPC in 40 g of olive paste (mg)*	6364 ± 619	3024 ± 301	2863 ± 289	2547 ± 263	1908 ± 210	1589 ± 187	1273 ± 161
TPC of olive paste (mg/kg)	79550 ± 7837	75600 ± 7714	71575 ± 7403	63675 ± 6734	47700 ± 5369	39725 ± 4774	31825 ± 4105
TPC of extracted oil (mg/kg)	95 ± 24	230 ± 31	1144 ± 193	1021 ± 151	1158 ± 198	945 ± 130	772 ± 92
Phenolic transfer yield - *PY* (%)	0.004 ± 0.001	0.05 ± 0.01	0.25 ± 0.07	0.24 ± 0.07	0.24 ± 0.08	0.19 ± 0.05	0.15 ± 0.05

LECCIO DEL CORNO	L17OP+0%	L17OP+5%	L17OP+10%	L17OP+20%	L17OP+40%	L17OP+50%	L17OP+60%
Moisture content of olive paste (%)	5.90 ± 0.08	10.6 ± 0.1	15.3 ± 0.2	24.7 ± 0.3	43.5 ± 0.6	53.0 ± 0.7	62.4 ± 0.8
Moisture content of olive paste - n_s (kg_{water}/kg dm)	0.063 ± 0.002	0.119 ± 0.002	0.181 ± 0.005	0.328 ± 0.008	0.77 ± 0.02	1.13 ± 0.03	1.66 ± 0.04
Water activity of olive paste - A_w	0.402 ± 0.001	0.690 ± 0.003	0.837 ± 0.004	0.934 ± 0.004	0.948 ± 0.004	0.959 ± 0.004	0.971 ± 0.004
Oil content of olive fruits (% dm)	25 ± 1	25 ± 1	25 ± 1	25 ± 1	25 ± 1	25 ± 1	25 ± 1
Oil content in 40 g of olive paste (g)*	18.8 ± 0.8	8.9 ± 0.4	8.5 ± 0.4	7.5 ± 0.4	5.7 ± 0.3	4.7 ± 0.3	3.8 ± 0.3
Oil content of olive paste (%)	24 ± 1	22 ± 1	21 ± 1	19 ± 1	14.3 ± 0.8	11.8 ± 0.8	9.5 ± 0.8
Water-oil ratio of olive paste	0.25 ± 0.01	0.48 ± 0.03	0.72 ± 0.05	1.32 ± 0.09	3.1 ± 0.2	4.5 ± 0.4	6.6 ± 0.6
Extracted oil (g)	1.1 ± 0.1	4.5 ± 0.1	5.2 ± 0.1	4.8 ± 0.1	3.3 ± 0.1	2.5 ± 0.1	2.0 ± 0.1
Oil extraction yield - *EI* (%)	5.9 ± 0.8	51 ± 3	61 ± 4	64 ± 5	58 ± 6	53 ± 6	53 ± 7
Oil extraction yield – *OY* (%)	1.4 ± 0.1	11.3 ± 0.3	13.0 ± 0.3	12.0 ± 0.3	8.3 ± 0.3	6.3 ± 0.3	5.0 ± 0.3
TPC of olive fruits (mg/kg dm)	68690 ± 6385	68690 ± 6385	68690 ± 6385	68690 ± 6385	68690 ± 6385	68690 ± 6385	68690 ± 6385
TPC in 40 g of olive paste (mg)*	5171 ± 492	2456 ± 239	2327 ± 230	2069 ± 209	1552 ± 171	1291 ± 150	1033 ± 129
TPC of olive paste (mg/kg)	64638 ± 6231	61400 ± 6129	58175 ± 5895	51725 ± 5354	38800 ± 4372	32275 ± 3831	25825 ± 3290
TPC of extracted oil (mg/kg)	111 ± 24	374 ± 41	368 ± 41	487 ± 52	569 ± 61	552 ± 59	408 ± 44
Phenolic transfer yield - *PY* (%)	0.002 ± 0.001	0.07 ± 0.02	0.08 ± 0.02	0.11 ± 0.03	0.12 ± 0.03	0.11 ± 0.03	0.08 ± 0.02

Table 1. Mean value and standard deviations of experimental (in italic) and calculated data (in roman) of olive oil fruits, olive paste at different moisture content and extracted olive oil of Frantoio 2017 (F17) and Leccio del Corno 2017 (L17) samples; *TPC* = total phenolic content.
* 80 g of olive paste was prepared for the trials with freeze-dried samples.

Cultivar	Year	DAFB (die)	Moisture (g/kg)	Sugar (g/kg)	Sugar (g/kg dm)	Oil (g/kg)	Oil (g/kg dm)	*TPC* ($mg_{oleoeuropein}$/kg)	*TPC* ($mg_{oleoeuropein}$/kg dm)
Frantoio	2012	164	$500^{b} \pm 7$	$26^{a} \pm 2$	$52^{a} \pm 9$	$193^{b} \pm 6$	$386^{a} \pm 13$	$23{,}693^{d} \pm 810$	$47{,}386^{c} \pm 7{,}810$
Leccio del Corno	2016	190	$590^{a} \pm 8$	$18^{b} \pm 1$	$44^{a,b} \pm 9$	$133^{d} \pm 4$	$324^{b} \pm 12$	$26{,}302^{c} \pm 900$	$64{,}151^{b} \pm 8{,}900$
Frantoio	2017	161	$435^{c} \pm 6$	$19^{b} \pm 1$	$34^{b} \pm 7$	$228^{a} \pm 7$	$404^{a} \pm 13$	$46{,}478^{a} \pm 1{,}589$	$82{,}262^{a} \pm 7{,}859$
Leccio del Corno	2017	169	$410^{d} \pm 5$	$26^{a} \pm 1$	$44^{a,b} \pm 6$	$149^{c} \pm 5$	$253^{c} \pm 10$	$40{,}527^{b} \pm 1{,}385$	$68{,}690^{b} \pm 6{,}385$

Table 2. Olive fruit quality characteristics. DAFB = Day After Full Blooming; *TPC* = Total Phenolic Content; dm = dry matter. Different small letters in the same column indicate significant differences ($p = 0.01$) for the different samples

PHENOLIC COMPOUND CONTENT	Frantoio cv. 2012	Leccio del Corno cv. 2016	Frantoio cv. 2017	Leccio del Corno cv. 2017
Hydroxytyrosol	351	199	228	213
Hydroxytyrosol glucoside	nd	153	nd	nd
Tyrosol glucoside	nd	68	322	476
Chlorogenic acid	76	72	138	121
Caffeic acid	nd	nd	10	48
Demetyloleuropein	8700	12985	4576	2159
Rutin	148	155	400	255
Luteolin-7-*O*-glucoside	207	263	420	301
Verbascoside	1270	898	2703	1178
Nuzhenide	914	1010	827	1294
Sum of isoverbascoside isomers	234	12	139	46
Caffeoyl-6'-secologanoside	596	22	913	678
Sum of oleuropein aglycone isomers	1379	221	339	652
Oleuropein	12992	16911	34353	28818
Comselogoside	779	nd	1412	789
Ligstroside	640	1174	2683	1847
Total phenolic compounds	**47386**	**64151**	**68690**	**82262**

Table 3. Content of the phenolic compounds identified in the olive fruits samples. Data are expressed as $mg_{oleoeuropein}$/kg on dry basis; nd, not determined

3.1 Adsorption isotherm of olive paste

Table 1 shows the moisture contents and corresponding water activity values for the freeze-dried and rehydrated olive pastes that were obtained from the F17 and L17 olive batches. The relationship between the moisture content on dry basis (n_s) and the water activity (A_w) was similar for both olive batches (Figure 1).

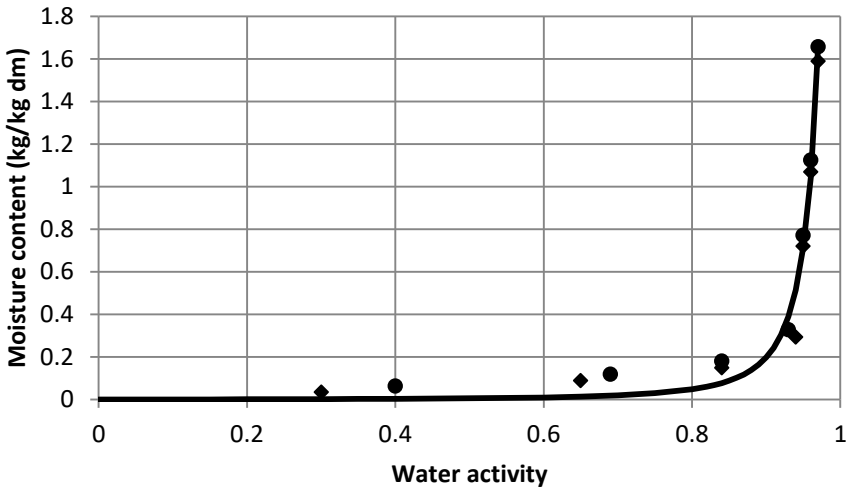

Figure 1. Olive paste adsorption isotherm at 25°C; experimental data for the F17 (◆) and L17 (●) samples

Hence, it was possible to build the adsorption isotherm of the olive paste at 25°C by applying the G.A.B. model (Giovanelli, Zanoni, Lavelli, & Nani, 2002):

$$n_s = \frac{n_{sm} \cdot C \cdot k \cdot A_w}{(1 - k \cdot A_w) \cdot (1 - k \cdot A_w + C \cdot k \cdot A_w)} \qquad r^2 = 0.98 \qquad [4]$$

where:

n_{sm} is the moisture content in the monolayer = 0.236 kg_{water}/kg dm; C is the adimensional constant related to the heat of adsorption in the monolayer = 0.011; k is the adimensional constant related to the heat of adsorption in the multilayer = 0.996.

The olive pastes displayed a hygroscopic behaviour similar to the combination of an adsorption isotherm of a lipid with that of an aqueous solution of salts and simple sugars (Iglesias & Chirife, 1982). The olive pastes displayed little hygroscopic behaviour up to $A_w < 0.90$; then, exponentially, they were very hygroscopic at $A_w \geq 0.90$ (Fig. 1). As a consequence, the susceptibility of the olive pastes to enzymatic and non-enzymatic reactions were potentially already present at a moisture content > 10 % (i.e. $n_s > 0.11$ kg_{water}/kg dm and $A_w \approx 0.70$). Values of $A_w > 0.90$, a condition indicating a potentially very reactive system, were already present at a moisture content > 20 % (i.e. $n_s > 0.25$ kg_{water}/kg dm).

3.2 Effect of olive paste moisture on oil extraction yield

Table 1 shows the experimental data used to determine, at laboratory scale, the effect of olive paste moisture on oil extraction yield, for the freeze-fried and rehydrated olive pastes that were obtained from the F17 and L17 olive batches, respectively.

An effect of olive paste moisture on oil extraction yield occurred for both cultivars. In Figure 2 (graphs 2a and 2b) two zones can be identified.

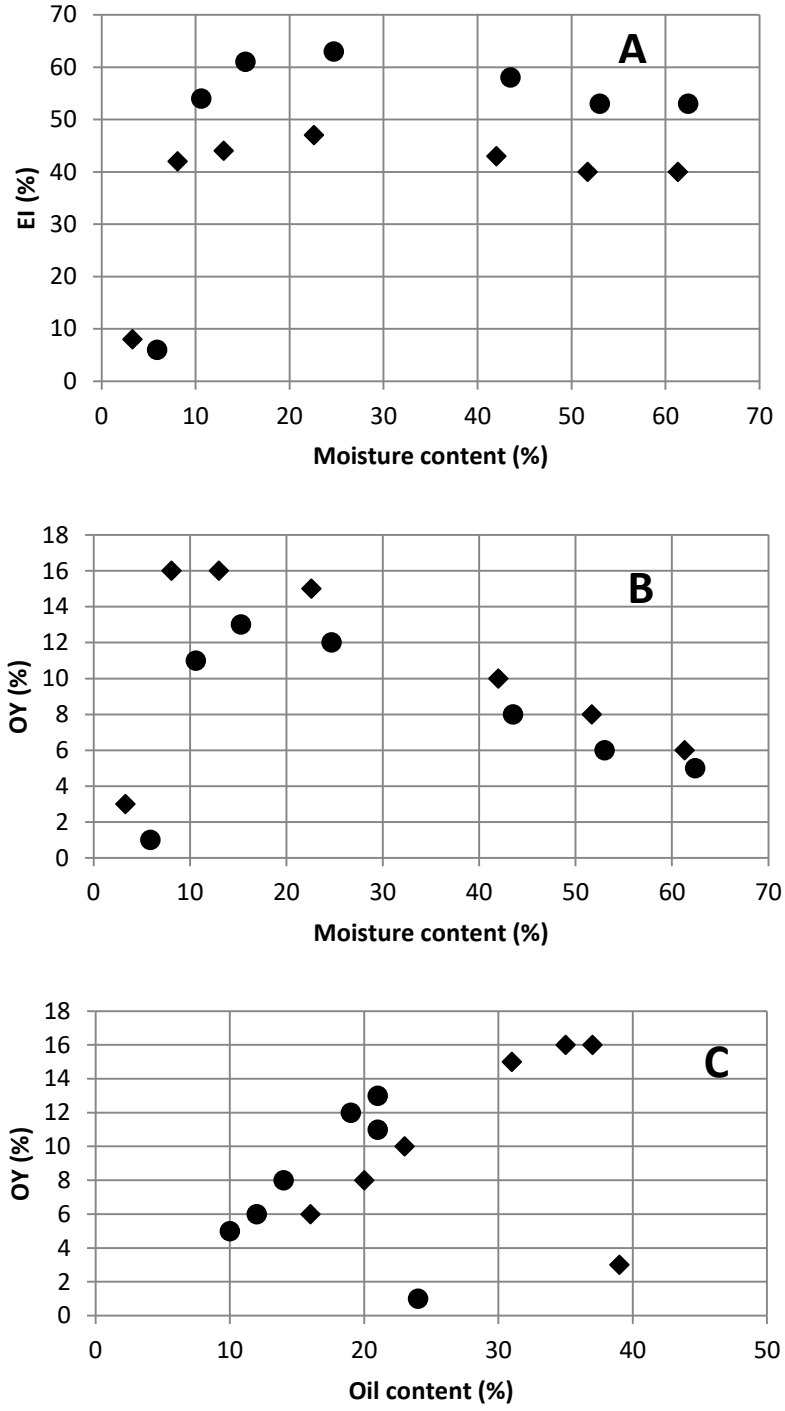

Figure 2. Effect of olive paste moisture on oil extraction yield: 2a) *EI* (%) as a function of moisture content (%); 2b) *OY* (%) as a function of moisture content (%); 2c) *OY* (%) as a function of oil content (%). The experimental data for the L17 and F17 samples are shown with the symbols ● and ◆, respectively

The first zone is characterized by the tendency for the *EI* and *OY* percentage values to increase as the moisture content increases. This behaviour can be deemed consistent with the literature (Masella et al., 2008; Boncinelli et al., 2009) on the positive effect of an increase in olive paste moisture on the extraction yield, due to the decrease in the consistency of the paste. The second zone is instead characterized by a tendency for the oil extraction yield to decrease (very evident in the *OY %* value trends) above a threshold moisture content, which in our study was approximately 30%. This behaviour can in turn be deemed consistent with the literature (Di Giovacchino, 1991; Aguilera et al., 2010; Moya et al., 2010; Koprivnjak et al., 2016; Zanoni et al., 2018) on the effect that increasing values of moisture content have in causing "difficult" olive paste for oil extraction.

Figure 2 (graphs 2a and 2b) also shows an effect of the cultivar on the ease of oil extraction. The olive pastes obtained from the L17 olive batches reached *EI* values that were always greater than the F17 batches (Fig. 2, graph 2a). An inverse effect among the cultivars on the extraction yield instead seems to be present if the yield is expressed as *OY* and the extracted oil is then related to the mass of paste used (Fig. 2, graph 2b). This behaviour can be considered to be only apparent and caused by the different oil content of the pastes from the two cultivars, which was greater for the F17 samples compared to the L17 samples; the relationship between *OY* values and oil content (Fig. 2, graph 2c) indeed shows how, at the same oil content in the paste, the yield expressed as *OY* also results greater for the L17 samples.

3.3 Effect of olive paste moisture on phenolic transfer yield

Table 1 shows the experimental data used to determine, at laboratory scale, the effect of olive paste moisture on the olive-to-oil phenolic compound transfer yield, for the freeze-dried and rehydrated olive pastes that were obtained from the F17 and L17 olive batches, respectively. The phenolic compound content of the extracted oils varied greatly as the olive paste moisture varied; furthermore, in some cases, very high phenolic compound content values were reached, of approx. 1200 mg_{tyr}/kg and 600 mg_{tyr}/kg for F17 and L17, respectively.

An effect of olive paste moisture on the phenolic transfer yield occurred for both cultivars. In Figure 3 (graph 3a) it is highlighted the tendency of the *PY* percentage values

to increase along with the moisture content, and then to decrease above a certain moisture content threshold, which in our study was approximately 40%. The olive pastes obtained from the batches of F17 olives always reached higher *PY* values than the L17 batches.

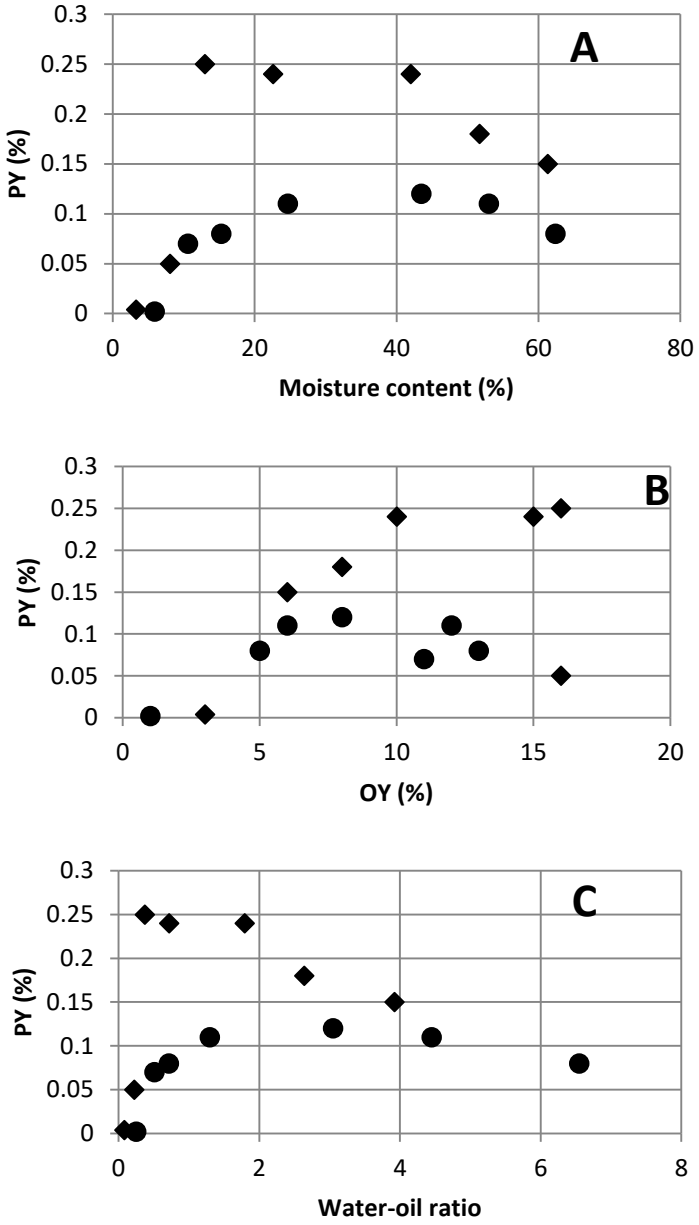

Figure 3. Effect of olive paste moisture on phenolic transfer yield: figure 3a) *PY* (%) as a function of moisture content (%); 3b) *PY* (%) as a function of OY (%); 3c) *PY* (%) as a function of the water-oil ratio. The experimental data for the L17 and F17 samples are shown with the symbols ● and ◆, respectively

Since for the calculation of *PY* (see equation [3]) the term oil extraction yield (i.e. *OY*) is present, the variation of *PY* could depend on the increase and then the decrease in *OY* depending on moisture content, as described in the previous paragraph (Fig. 2, graph

2b). Figure 3 (graph 3b) shows how this cause is not to be considered very important, since different *PY* values were calculated at the same *OY* value.

In line with the literature (Cecchi et al., 2018), it is instead possible to hypothesize that the variation in *PY* was due to the greater affinity of phenolic compounds towards the water phase. Figure 3 (graph 3c) shows how the variation in *PY* as the water-oil ratio in olive paste varies (Tab. 1) has a similar trend to the variation in *PY* with the moisture content (Fig. 3, graph 3a). The oil present in the olive pastes would seem to compete with the water content in the phenolic compound transfer from the olive pastes to the extracted oil. In our study, higher *PY* values corresponded to water-oil ratio values of between 1 and 2, while the *PY* decreased at water-oil ratio values of > 3.

However, what has been said above does not appear to be sufficient to fully explain the reasons for the variation of *PY* with the moisture content. In particular, it would not explain the large different in *PY* trends between the olive pastes obtained from the F17 olive batches compared to the L17 batches, whose pastes had greater water-oil ratios than the F17 batches (Table 1). Indeed, if the phenomenon only depended on the different affinity of the phenolic compounds between water and oil, at the same water-oil ratios the olive pastes from the two cultivars should have similar *PY* values, but this does not appear from the trends shown in Figure 3 (graph 3c).

Hence, it can be surmised that the enzymatic and non-enzymatic phenomena, which transform the olive paste phenolic compounds during the olive fruit processing, are also involved. The literature data (Tamborrino et al., 2014; Klen et al., 2015; Trapani et al, 2017b) show that the phenolic compound profile of EVOO depends on a combination of the following three kinds of phenomena occurring in the olive paste: (i) enzymatic oxidative degradation catalysed by polyphenol oxidases (PPOs) and peroxidases (PODs), which cause a decrease in the phenolic compound content; (ii) enzymatic (i.e. β-glucosidase activity) and non-enzymatic hydrolytic phenomena that transform oleuropein and ligstroside into their respective aglycone and decarboxymethylated forms, especially the dialdehydic form of decarboxymethyl oleuropein aglycone (i.e. 3,4 DHPEA-EDA compound); (iii) physical and enzymatic (i.e. pectinase and cellulase activities) phenomena which promote the release of phenolic compounds from cellular tissues and then cause an increase in the phenolic compound content.

cv Leccio del Corno 2017 - Phenolic compound (mg_{tyr}/kg)	No water	5 % water	10 % water	20 % water	40 % water	50 % water	60 % water
Hydroxytyrosol	0.9 ± 0.1	2.9 ± 0.2	1.9 ± 0.1	2.1 ± 0.1	1.4 ± 0.1	2.2 ± 0.2	1.6 ± 0.1
Turosol	0.7 ± 0.0	3.8 ± 0.1	4.0 ± 0.1	2.6 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	1.3 ± 0.0
Caffeic + Vanillic acid	1.0 ± 0.0	1.7 ± 0.1	1.6 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	0.9 ± 0.0	0.7 ± 0.0
Vanillin	0.8 ± 0.1	0.7 ± 0.0	1.2 ± 0.1	0.7 ± 0.0	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.1
p -coumaric acid	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.5 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.0
Hydroxytyrosyl acetate	2.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Ferulic Acid	3.8 ± 0.7	8.7 ± 1.7	1.1 ± 0.2	3.2 ± 0.6	2.7 ± 0.5	1.9 ± 0.4	1.8 ± 0.4
o -coumaric acid	1.2 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	1.2 ± 0.2	0.5 ± 0.1
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	2.9 ± 0.2	23.9 ± 2.0	16.4 ± 1.4	36.5 ± 3.1	30.7 ± 2.6	29.2 ± 2.5	24.4 ± 2.1
Dyaldehydic form of decarboxymethyloleuropein aglycon	22.7 ± 0.2	28.3 ± 0.3	37.6 ± 0.4	42.9 ± 0.5	62.8 ± 0.7	58.6 ± 0.6	33.0 ± 0.4
Oleuropein	2.3 ± 0.1	38.3 ± 1.4	50.4 ± 1.8	85.9 ± 3.1	94.9 ± 3.4	87.3 ± 3.2	69.6 ± 2.5
Dyaldehydic form of oleuropein aglycon	3.3 ± 0.2	24.3 ± 1.5	36.0 ± 2.3	56.5 ± 3.6	64.5 ± 4.1	66.0 ± 4.2	44.0 ± 2.8
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	1.9 ± 0.1	7.0 ± 0.3	7.5 ± 0.4	11.9 ± 0.6	17.0 ± 0.8	18.4 ± 0.9	13.9 ± 0.7
Dyaldehydic form of decarboxymethylligstroside aglycon	2.7 ± 0.1	4.5 ± 0.1	8.2 ± 0.2	9.4 ± 0.2	17.1 ± 0.4	17.9 ± 0.4	9.7 ± 0.2
Pinoresinol + 1-acetoxypinoresinol	3.6 ± 0.1	20.1 ± 0.5	24.0 ± 0.6	34.9 ± 0.9	40.6 ± 1.1	35.6 ± 0.9	31.4 ± 0.8
Cinnamic acid	2.4 ± 0.4	4.2 ± 0.6	5.4 ± 0.8	8.4 ± 1.3	9.6 ± 1.4	7.7 ± 1.2	6.7 ± 1.0
Dyaldehydic form of ligstroside aglycon	0.7 ± 0.1	12.8 ± 1.4	18.2 ± 2.0	31.9 ± 3.5	35.5 ± 4.0	37.1 ± 4.1	29.3 ± 3.3
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	3.2 ± 0.1	5.9 ± 0.3	4.8 ± 0.2	5.4 ± 0.2	4.7 ± 0.2	5.7 ± 0.3	4.1 ± 0.2
Luteolin	1.5 ± 0.2	4.4 ± 0.6	2.4 ± 0.4	3.1 ± 0.4	4.3 ± 0.6	6.2 ± 0.9	3.6 ± 0.5
Aldehydic and hydroxylic form of oleuropein aglycon	23.6 ± 0.3	144.3 ± 2.1	111.6 ± 1.6	112.8 ± 1.6	135.0 ± 1.9	130.2 ± 1.9	97.9 ± 1.4
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	1.2 ± 0.1	5.1 ± 0.5	6.3 ± 0.6	9.2 ± 0.9	9.9 ± 1.0	10.5 ± 1.0	8.0 ± 0.8
Apigenin	2.3 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Methyl luteolin	1.2 ± 0.1	2.4 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	2.0 ± 0.2
Aldehydic and hydroxylic form of ligstroside aglycon	23.4 ± 0.8	27.4 ± 1.0	24.3 ± 0.9	23.6 ± 0.8	28.1 ± 1.0	27.3 ± 1.0	21.7 ± 0.8
Total Phenolic Compounds	**111 ± 24**	**374 ± 41**	**368 ± 41**	**487 ± 52**	**569 ± 61**	**552 ± 59**	**408 ± 44**

Table 4. Phenolic compounds profile of the olive oil extracted from the freeze-dried and rehydrated olive pastes from the L17 olive batch. Data are expressed as mg_{tyr}/kg.

cv Frantoio 2017 - Phenolic compound (mg_{tyr}/kg)	No water	5 % water	10 % water	20 % water	40 % water	50 % water	60 % water
Hydroxytyrosol	1.1 ± 0.1	1.4 ± 0.1	2.6 ± 0.2	1.8 ± 0.1	2.0 ± 0.1	1.4 ± 0.1	1.7 ± 0.1
Turosol	0.8 ± 0.0	3.2 ± 0.1	4.6 ± 0.2	3.4 ± 0.1	3.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
Caffeic + Vanillic acid	0.7 ± 0.0	1.4 ± 0.0	2.5 ± 0.1	1.9 ± 0.1	2.2 ± 0.1	1.5 ± 0.0	1.3 ± 0.0
Vanillin	0.5 ± 0.0	0.8 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	2.9 ± 0.2	2.2 ± 0.1	1.7 ± 0.1
p -coumaric acid	0.9 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Hydroxytyrosyl acetate	4.1 ± 0.4	0.8 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Ferulic Acid	9.5 ± 1.9	0.9 ± 0.2	1.1 ± 0.2	0.7 ± 0.1	1.4 ± 0.3	0.6 ± 0.1	0.7 ± 0.1
o -coumaric acid	1.0 ± 0.2	0.6 ± 0.1	1.5 ± 0.3	2.2 ± 0.4	2.1 ± 0.4	1.5 ± 0.3	1.1 ± 0.2
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	9.2 ± 0.8	19.5 ± 1.6	122.9 ± 10.3	106.1 ± 8.9	90.2 ± 7.6	59.3 ± 5.0	60.7 ± 5.1
Dyaldehydic form of decarboxymethyloleuropein aglycon	2.7 ± 0.0	20.4 ± 0.2	111.6 ± 1.2	108.1 ± 1.2	127.7 ± 1.4	90.7 ± 1.0	65.8 ± 0.7
Oleuropein	7.0 ± 0.3	53.6 ± 1.9	310.8 ± 11.2	254.8 ± 9.2	259.4 ± 9.4	219.8 ± 8.0	148.2 ± 5.4
Dyaldehydic form of oleuropein aglycon	29.9 ± 1.9	22.8 ± 1.4	155.0 ± 9.8	145.3 ± 9.2	139.7 ± 8.8	121.9 ± 7.7	91.5 ± 5.8
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	2.0 ± 0.1	10.5 ± 0.5	38.4 ± 1.9	41.7 ± 2.1	38.1 ± 1.9	37.6 ± 1.9	33.5 ± 1.7
Dyaldehydic form of decarboxymethylligstroside aglycon	1.8 ± 0.0	7.7 ± 0.2	35.3 ± 0.8	36.5 ± 0.8	56.4 ± 1.2	40.6 ± 0.9	35.3 ± 0.8
Pinoresinol + 1-acetoxypinoresinol	3.7 ± 0.1	18.6 ± 0.5	112.7 ± 2.9	96.1 ± 2.5	142.4 ± 3.7	120.3 ± 3.1	108.1 ± 2.8
Cinnamic acid	0.9 ± 0.1	4.6 ± 0.7	28.7 ± 4.3	24.0 ± 3.6	20.8 ± 3.1	16.9 ± 2.6	12.8 ± 1.9
Dyaldehydic form of ligstroside aglycon	2.5 ± 0.3	16.8 ± 1.9	89.7 ± 10.0	85.9 ± 9.5	86.8 ± 9.7	78.7 ± 8.7	63.0 ± 7.0
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	2.6 ± 0.1	3.3 ± 0.2	8.6 ± 0.4	8.4 ± 0.4	29.9 ± 1.3	29.5 ± 1.3	33.3 ± 1.5
Luteolin	1.1 ± 0.2	0.9 ± 0.1	7.2 ± 1.0	8.4 ± 1.2	6.6 ± 0.9	6.1 ± 0.9	6.2 ± 0.9
Aldehydic and hydroxylic form of oleuropein aglycon	0.7 ± 0.0	26.1 ± 0.4	58.8 ± 0.8	47.9 ± 0.7	75.4 ± 1.1	56.6 ± 0.8	51.1 ± 0.7
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	5.1 ± 0.5	6.5 ± 0.6	29.4 ± 2.9	25.5 ± 2.5	31.0 ± 3.1	26.1 ± 2.6	20.7 ± 2.1
Apigenin	5.5 ± 0.5	0.5 ± 0.1	1.0 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	1.7 ± 0.2	1.9 ± 0.2
Methyl luteolin	0.9 ± 0.1	0.9 ± 0.1	3.2 ± 0.3	3.8 ± 0.3	10.8 ± 0.9	9.1 ± 0.7	10.4 ± 0.8
Aldehydic and hydroxylic form of ligstroside aglycon	1.2 ± 0.0	6.9 ± 0.2	13.6 ± 0.5	12.5 ± 0.4	23.5 ± 0.8	18.9 ± 0.7	19.0 ± 0.7
Total Phenolic Compounds	**95 ± 24**	**230 ± 31**	**1144 ± 193**	**1021 ± 151**	**1158 ± 198**	**945 ± 130**	**772 ± 92**

Table 5. Phenolic compounds profile of the olive oil extracted from the freeze-dried and rehydrated olive pastes from the F17 olive batch. Data are expressed as mg_{tyr}/kg.

cv Leccio del Corno 2016 - Phenolic compound (mg_{tyr}/kg)	No water	20 % water	40 % water
Hydroxytyrosol	2.6 ± 0.2	3.7 ± 0.3	4.8 ± 0.3
Turosol	3.3 ± 0.1	5.4 ± 0.2	4.5 ± 0.2
Caffeic + Vanillic acid	0.5 ± 0.0	1.0 ± 0.0	0.9 ± 0.0
Vanillin	0.4 ± 0.0	1.7 ± 0.1	1.7 ± 0.1
p -coumaric acid	0.4 ± 0.0	1.1 ± 0.1	1.0 ± 0.1
Hydroxytyrosyl acetate	0.6 ± 0.1	4.0 ± 0.4	4.1 ± 0.4
Ferulic Acid	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
o -coumaric acid	0.3 ± 0.1	2.6 ± 0.5	4.3 ± 0.8
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	14.5 ± 1.2	110.9 ± 9.3	80.8 ± 6.8
Dyaldehydic form of decarboxymethyloleuropein aglycon	36.9 ± 0.4	188.4 ± 2.0	154.2 ± 1.7
Oleuropein	16.5 ± 0.6	53.1 ± 1.9	39.9 ± 1.5
Dyaldehydic form of oleuropein aglycon	20.5 ± 1.3	50.2 ± 3.2	43.4 ± 2.8
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	2.4 ± 0.1	10.0 ± 0.5	12.8 ± 0.6
Dyaldehydic form of decarboxymethylligstroside aglycon	9.2 ± 0.2	62.3 ± 1.4	53.8 ± 1.2
Pinoresinol + 1-acetoxypinoresinol	4.0 ± 0.1	59.3 ± 1.5	83.3 ± 2.2
Cinnamic acid	10.0 ± 1.5	16.7 ± 2.5	11.3 ± 1.7
Dyaldehydic form of ligstroside aglycon	2.4 ± 0.3	18.5 ± 2.1	11.9 ± 1.3
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	7.0 ± 0.3	35.1 ± 1.6	38.9 ± 1.8
Luteolin	2.4 ± 0.3	9.5 ± 1.4	7.0 ± 1.0
Aldehydic and hydroxylic form of oleuropein aglycon	22.7 ± 0.3	39.0 ± 0.6	37.2 ± 0.5
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	3.9 ± 0.4	15.9 ± 1.6	18.2 ± 1.8
Apigenin	2.0 ± 0.2	8.1 ± 0.8	6.6 ± 0.6
Methyl luteolin	0.9 ± 0.1	4.6 ± 0.4	6.2 ± 0.5
Aldehydic and hydroxylic form of ligstroside aglycon	2.7 ± 0.1	10.3 ± 0.4	10.3 ± 0.4
Total Phenolic Compounds	**166 ± 27**	**712 ± 81**	**637 ± 70**

Table 6. Phenolic compounds profile of the olive oil extracted from the freeze-dried and rehydrated olive pastes from the L16 olive batch. Data are expressed as mg_{tyr}/kg.

cv Frantoio 2012 - Phenolic compound (mg_{tyr}/kg)	no water	5% water	10% water	20% water	40% water	50% water
Hydroxytyrosol	5.3 ± 0.4	4.3 ± 0.3	4.1 ± 0.3	4.2 ± 0.3	3.1 ± 0.2	3.2 ± 0.2
Turosol	3.1 ± 0.1	3.5 ± 0.1	5.0 ± 0.2	4.4 ± 0.2	3.1 ± 0.1	2.9 ± 0.1
Caffeic + Vanillic acid	2.0 ± 0.1	2.0 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	2.5 ± 0.1	1.7 ± 0.1
Vanillin	1.0 ± 0.1	1.2 ± 0.1	1.9 ± 0.1	2.5 ± 0.2	3.0 ± 0.2	1.9 ± 0.1
p -coumaric acid	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	0.9 ± 0.2
Hydroxytyrosyl acetate	2.8 ± 0.3	0.7 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.3 ± 0.1
Ferulic Acid	0.2 ± 0.05	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.05	0.2 ± 0.05	0.3 ± 0.1
o -coumaric acid	0.2 ± 0.1	0.8 ± 0.2	1.7 ± 0.3	2.4 ± 0.5	1.2 ± 0.2	1.4 ± 0.3
Dyaldehydic form of decarboxymethyloleuropein aglycon oxidized	23.0 ± 1.9	32.6 ± 2.7	64.4 ± 5.4	61.0 ± 5.1	58.1 ± 4.9	53.0 ± 4.5
Dyaldehydic form of decarboxymethyloleuropein aglycon	67.9 ± 0.7	55.9 ± 0.6	120.3 ± 1.3	171.6 ± 1.9	165.4 ± 1.8	121.0 ± 1.3
Oleuropein	38.0 ± 1.4	31.8 ± 1.1	184.2 ± 6.7	243.1 ± 8.8	165.8 ± 6.0	111.8 ± 4.0
Dyaldehydic form of oleuropein aglycon	15.4 ± 1.0	22.5 ± 1.4	102.3 ± 6.5	106.0 ± 6.7	88.2 ± 5.6	52.0 ± 3.3
Dyaldehydic form of decarboxymethylligstroside aglycon oxidized	4.3 ± 0.2	4.9 ± 0.2	9.7 ± 0.5	14.4 ± 0.7	14.2 ± 0.7	24.4 ± 1.2
Dyaldehydic form of decarboxymethylligstroside aglycon	12.4 ± 0.3	9.6 ± 0.2	22.6 ± 0.5	32.6 ± 0.7	32.5 ± 0.7	64.6 ± 1.4
Pinoresinol + 1-acetoxypinoresinol	3.1 ± 0.1	18.3 ± 0.5	52.0 ± 1.4	66.1 ± 1.7	65.8 ± 1.7	63.2 ± 1.6
Cinnamic acid	12.9 ± 1.9	6.9 ± 1.0	19.4 ± 2.9	28.9 ± 4.4	25.3 ± 3.8	18.6 ± 2.8
Dyaldehydic form of ligstroside aglycon	3.9 ± 0.4	6.0 ± 0.7	26.2 ± 2.9	28.6 ± 3.2	26.2 ± 2.9	6.5 ± 0.7
Aldehydic and hydroxylic form of oleuropein aglycon oxidized	29.7 ± 1.3	33.7 ± 1.5	67.1 ± 3.0	72.1 ± 3.20	60.6 ± 2.7	31.4 ± 1.4
Luteolin	5.1 ± 0.7	2.7 ± 0.4	5.5 ± 0.8	8.3 ± 1.2	15.3 ± 2.2	22.8 ± 3.3
Aldehydic and hydroxylic form of oleuropein aglycon	93.8 ± 1.3	99.5 ± 1.4	124.3 ± 1.8	141.8 ± 2.0	161.1 ± 2.3	46.2 ± 0.7
Aldehydic and hydroxylic form of ligstroside aglycon oxidized	10.4 ± 1.0	13.6 ± 1.4	25.9 ± 2.6	33.8 ± 3.4	29.0 ± 2.9	36.2 ± 3.6
Apigenin	1.8 ± 0.2	2.0 ± 0.2	4.1 ± 0.4	5.1 ± 0.5	4.1 ± 0.4	16.3 ± 1.6
Methyl luteolin	13.4 ± 1.1	14.4 ± 1.2	19.8 ± 1.6	16.2 ± 1.3	23.8 ± 1.9	18.4 ± 1.5
Aldehydic and hydroxylic form of ligstroside aglycon	3.9 ± 0.1	4.0 ± 0.1	4.8 ± 0.2	5.3 ± 0.2	5.5 ± 0.2	12.6 ± 0.5
Total Phenolic Compounds	**354 ± 40**	**371 ± 41**	**871 ± 112**	**1054 ± 161**	**956 ± 133**	**713 ± 81**

Table 7. Phenolic compounds profile of the olive oil extracted from the freeze-dried and rehydrated olive pastes from the F12 olive batch. Data are expressed as mg_{tyr}/kg.

The complete phenolic compound profiles of the oil extracted from the freeze-dried and rehydrated olive pastes are reported tables 4, 5, 6 and 7. The phenolic compounds considered most representative of the overall trend are shown in Table 2.

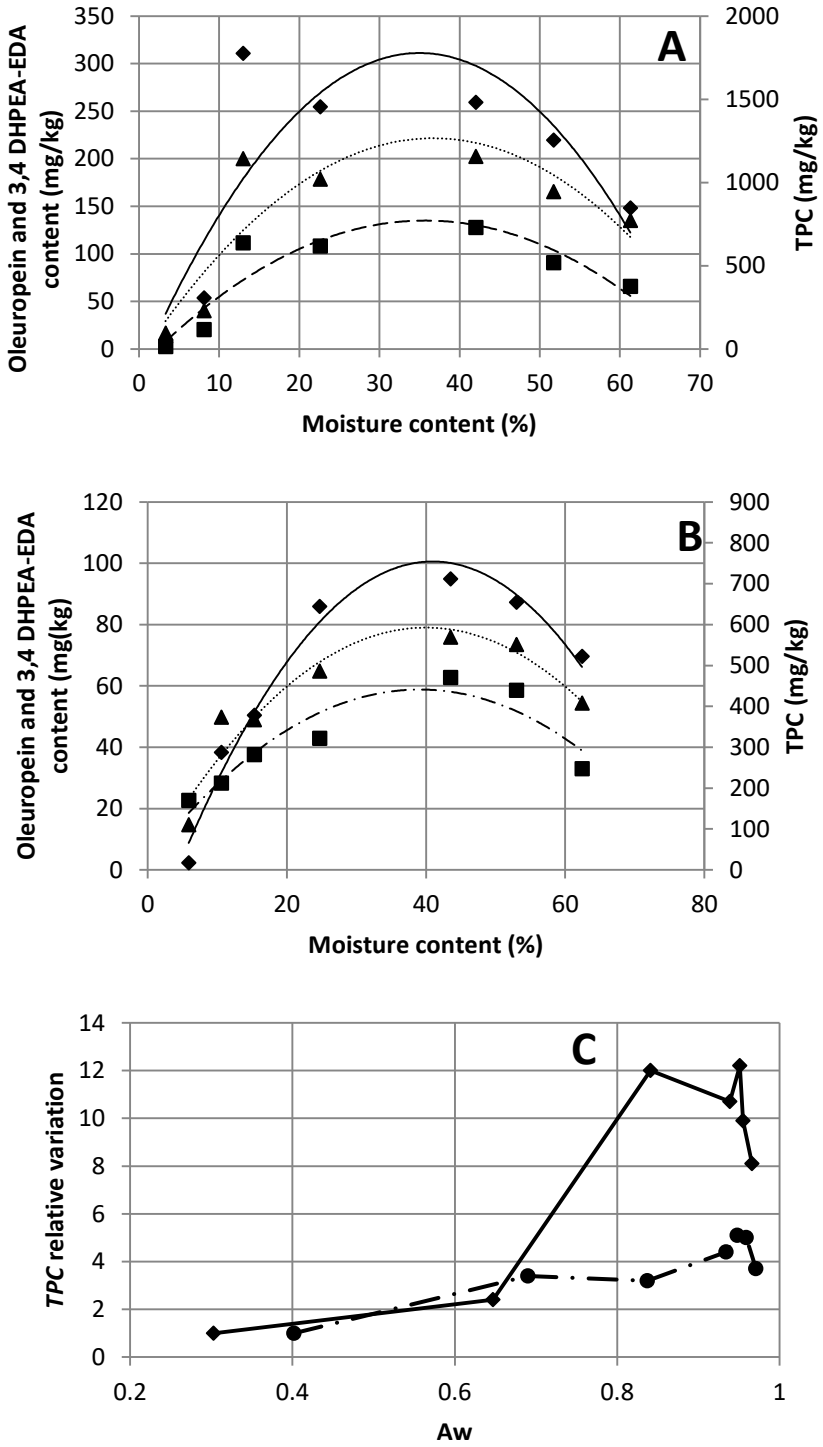

Figure 4. Effect of olive paste moisture on oil phenolic profile: figures 4a) and 4b) show the trend of the main phenolic compounds and total phenolic contents as a function of the moisture content of the oils extracted from the F17 and L17 olive paste samples, respectively; experimental data for oleuropein (◆), 3,4 DHPEA-EDA (■), total phenolic content (▲), with the relevant tendency curves. Figure 4c) shows the relative variation in total phenolic content as a function of the water activity (A_w) of the olive oil extracted from the L17 (●) and F17 (◆) olive paste samples.

There is an evident great change in the phenolic profile of the extracted oils compared to the olive fruit samples (Table 3), due to the enzymatic and non-enzymatic transformation phenomena in the phenolic compounds. This change is notably influenced by the olive paste moisture content for all the oils extracted from the olive pastes obtained both from the F17 and L17 olive batches (Fig. 4, graphs 4a and 4b). The increase in phenolic compounds, due to an increase in the olive paste moisture content, continues until a threshold value is reached, corresponding to a 30-40% moisture interval, followed by a progressive decrease in the phenolic content as the paste moisture increases. A similar behaviour was also noted for the phenolic profile of the oils extracted from the olive pastes both from the F12 and F16 olive batches (table 6 and 7).

If the variation in phenolic profile with the moisture content had only depended on the greater affinity of phenolic compounds towards the water phase, differences should have been seen between the behaviour of the oleuropein and the 3,4 DHPEA-EDA. Indeed, the 3,4 DHPEA-EDA has greater solubility in the oil phase of the oleuropein (Rodis et al., 2002). Instead, it is thought that the effect of the water content is above all due to the variation in the speed of the enzymatic and non-enzymatic transformation phenomena in the phenolic compounds owing to the olive paste water activity. Earlier in the text it was said how the reactivity of the olive pastes was potentially already present at a moisture content > 10 % and potentially continued to increase, with values of A_w > 0.90; however, the literature also reports that high values of water activity can cause a decrease in reaction rate (Labuza, 1980). While taking the increase, by way of example, in the total phenolic compound content in the rehydrated pastes compared to the corresponding value in the freeze-dried pastes as the indicator of the reaction speed of the phenolic compounds as a function of water activity, a progressive increase in the phenolic content reaction rate was effectively noted for both cultivars. At values of A_w > 0.95 this was followed by a sharp decrease in the speed of the reactions themselves (Fig. 4, graph 4c). Considering the paste sample preparation methods (i.e. crushing of freeze-dried olive oil fruits, olive paste rehydration and immediate olive oil extraction), it can be hypothesized that the variation in phenolic content is above all linked to the enzymatic (i.e. pectinase and cellulase activities) phenomena which promote the release of phenolic compounds from cellular tissues (Trapani et al., 2017b). From this point of view, the

Frantoio cultivar proved to have more reactive enzymes than the Leccio del Corno cultivar.

3.4 Lignan content of extracted oil

Table 8 shows the lignan content of the oil extracted from the freeze-dried and rehydrated olive pastes. Lignans were identified in the phenolic profile of EVOO only ten years after secoiridoids and nowadays they are recognized as the second most abundant class of hydrophilic phenolic compounds in olive oil after secoiridoids (Ballus et al., 2015). However, to date, they have not been identified in olive fruit (Oliveras-Lopez et al., 2008; Cecchi et al., 2017; Trapani et al., 2017a) and this fact could probably be explained by supposing that free lignans are initially absent in olive fruit because they are formed by enzymatic activity during the oil production (Lopez-Biedma et al., 2016). From the data in Table 8, it immediately appears evident that, for all four samples, lignans were almost totally absent in the oils extracted from the not reconstituted paste, while their content increased greatly until the water content was approx. 30-40%. All these results are in agreement with the hypothesis that lignans are absent or perhaps linked to other molecules in olive fruits and that they are released or biosynthesized during oil extraction thanks to specific enzymes and the water present in the olive fruits.

	Cultivar Frantoio 2017						
	F17OO+0%	**F17OO+5%**	**F17OO+10%**	**F17OO+20%**	**F17OO+40%**	**F17OO+50%**	**F17OO+60%**
Moisture content (%)	3.30 ± 0.04	8.1 ± 0.1	13.0 ± 0.2	22.6 ± 0.3	42.0 ± 0.5	51.7 ± 0.7	61.3 ± 0.8
Oleuropein	7 ± 1	54 ± 2	311 ± 11	255 ± 9	259 ± 9	220 ± 8	148 ± 5
3,4-DHPEA-EDA	3 ± 1	20 ± 1	112 ± 1	108 ± 1	128 ± 1	91 ± 1	66 ± 1
Lignans	3 ± 1	19 ± 1	113 ± 3	96 ± 3	142 ± 4	120 ± 3	108 ± 3
TPC	95 ± 24	230 ± 31	1144 ± 193	1021 ± 151	1158 ± 198	945 ± 130	772 ± 92
	Cultivar Frantoio 2012						
	F12OO+0%	**F12OO+5%**	**F12OO+10%**	**F12OO+20%**	**F12OO+40%**	**F12OO+50%**	**F12OO+60%**
Moisture content (%)	5.24 ± 0.07	10.0 ± 0.1	14.7 ± 0.2	24.2 ± 0.3	43.1 ± 0.6	52.6 ± 0.7	n.d.
Oleuropein	38 ± 1	32 ± 1	184 ± 7	243 ± 9	166 ± 6	112 ± 4	n.d.
3,4-DHPEA-EDA	68 ± 1	56 ± 1	120 ± 1	172 ± 2	165 ± 2	121 ± 1	n.d.
Lignans	3 ± 1	18 ± 1	52 ± 1	66 ± 2	66 ± 2	63 ± 2	n.d.
TPC	354 ± 40	371 ± 41	871 ± 112	1054 ± 161	956 ± 133	713 ± 81	n.d.
	Cultivar Leccio del Corno 2017						
	L17OO+0%	**L17OO+5%**	**L17OO+10%**	**L17OO+20%**	**L17OO+40%**	**L17OO+50%**	**L17OO+60%**
Moisture content (%)	5.90 ± 0.08	10.6 ± 0.1	15.3 ± 0.2	24.7 ± 0.3	43.5 ± 0.6	53.0 ± 0.7	62.4 ± 0.8
Oleuropein	2.3 ± 0.1	38 ± 1	50 ± 2	86 ± 3	95 ± 3	87 ± 3	70 ± 3
3,4-DHPEA-EDA	23 ± 1	28 ± 1	38 ± 1	43 ± 1	63 ± 1	59 ± 1	33 ± 1
Lignans	4 ± 1	20 ± 1	24 ± 1	35 ± 1	41 ± 1	36 ± 1	31 ± 1
TPC	111± 24	374 ± 41	368 ± 41	487 ± 52	569 ± 61	552 ± 59	408 ± 44
	Cultivar Leccio del Corno 2016						
	L16OO+0%	**L16OO+5%**	**L16OO+10%**	**L16OO+20%**	**L16OO+40%**	**L16OO+50%**	**L16OO+60%**
Moisture content (%)	5.24 ± 0.07	n.d.	n.d.	24.2 ± 0.3	43.1 ± 0.6	n.d.	n.d.
Oleuropein	17 ± 1	n.d.	n.d.	53 ± 2	40 ± 2	n.d.	n.d.
3,4-DHPEA-EDA	37 ± 1	n.d.	n.d.	188 ± 2	154 ± 2	n.d.	n.d.
Lignans	4 ± 1	n.d.	n.d.	59 ± 2	83 ± 2	n.d.	n.d.
TPC	166 ± 27	n.d.	n.d.	712 ± 81	637 ± 70	n.d.	n.d.

Table 8. Mean values and standard deviations of the olive oil main phenolic compounds at different moisture content of Frantoio 2017 (F17), Frantoio 2012 (F12), Leccio del Corno 2017 (L17) and Leccio del Corno 2016 (L16) olive paste samples. Data are expressed as mg_{tyr}/kg

n.d. not determined

4. Conclusions

This work showed a significant effect of olive paste moisture on oil extraction yield, phenolic transfer yield and profile of the phenolic compounds in the extractable oil at lab-scale. These parameters exhibited an increase up to a maximum point, and then a decrease, with the maximum values corresponding to an olive paste moisture content of approx. 40%.

High values of total phenolic content (up to 1200 mg_{tyr}/kg) were measured in some of the extracted oil, and in particular for the oils extracted from the olive paste of Frantoio cultivar, rehydrated with percentages of water in the range 10-40%. These values are very unusual in olive oil processed in a conventional way.

Our data indicated that the oil extraction yield was positively influenced by a decrease in olive paste consistency and negatively influenced by the effect of the high olive paste moisture content on oil coalescence (i.e. the "difficult" olive paste). Our findings suggested that the content and profile of the phenolic compounds in the extractable oil are strongly dependent on the greater affinity of these molecules towards the water phase: it was highlighted that a water-oil ratio > 3 caused a decrease in the phenolic transfer yield. However, the enzymatic phenomena that promote the release of phenolic compounds from the cellular tissues of olive fruits resulted to play a determinant role in increasing the phenolic compound content. The olive paste water activity (A_w) was related to these enzymatic phenomena, which were increased when $A_w > 0.70$ and then decreased when $A_w > 0.95$.

This study allowed to confirm that lignans, absent or linked to other molecules into the fruits, are released or biosynthesized during oil extraction thanks to the co-presence of specific enzymes and water.

In agreement with our results, the olive paste moisture content appeared to be as essential in controlling EVOO processing as the operating conditions of time, temperature and air exposure. Processing procedures along the olive oil chain that are able to reduce the moisture of olive oil fruits, even by a small amount, may complement the numerous proposed technological innovations for improving oil extraction and phenolic transfer yields. Suitable blends of olive batches with different moisture content can be used to control the olive paste moisture and, consequently, the extra virgin olive oil quality.

Little mills able to produce express olive oil from olive paste waffle were recently proposed in the market: results of this study also suggest the possibility to dry olives for obtaining dried olive paste waffles suitable to produce small amounts of express olive oils with the selected qualitative characteristics after rehydrating them at the suitable moisture content.

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New isobaric lignans from refined olive oils as quality markers for virgin olive oils

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Lorenzo Cecchi[1], Marzia Innocenti[1], Fabrizio Melani[1], Marzia Migliorini[2], Lanfranco Conte[3], and Nadia Mulinacci[1*]

[1] Dipartimento di NEUROFARBA, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Firenze), and Multidisciplinary Center of Research on Food Sciences (M.C.R.F.S.- Ce.R.A), Italy.

[2] PromoFirenze, Azienda Speciale della CCIAA di Firenze, Divisione Laboratorio Chimico, via Orcagna 70, 50121 Firenze, Italy.

[3]Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, via Sondrio 2/a, 33100 Udine, Italy.

*Corresponding author: Nadia Mulinacci

Dipartimento di NEUROFARBA, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Firenze, Italy.

Tel.: 0039-055-4573773

e-mail: nadia.mulinacci@unifi.it

Abstract

Herein we describe the influence of olive oil refining processes on the lignan profile. The detection of new isobaric lignans is suggested to reveal frauds in commercial extra-virgin olive oils. We analyzed five commercial olive oils by HPLC-DAD-TOF/MS to evaluate their lignan content and detected, for the first time, some isobaric forms of natural (+)-pinoresinol and (+)-1-acetoxypinoresinol. Then we analyzed partially and fully-refined oils from Italy, Tunisia and Spain. The isobaric forms occur only during the bleaching step of the refining process and remain unaltered after the final deodorizing step. Molecular dynamic simulation helped to identify the most probable chemical structures corresponding to these new isobars with data in agreement with the chromatographic findings. The total lignan amounts in commercial olive oils was close to 2 mg/L. Detection of these new lignans can be used as marker of undeclared refining procedures in commercial extra-virgin and/or virgin olive oils.

Keywords: (+)-pinoresinol; (+)-1-acetoxypinoresinol; lampante olive oil; HPLC-TOF; bleaching; olive oil frauds.

1. Introduction

All over the world, the Mediterranean diet is recognized for its health benefits.. Olive oil is one of the most important components of this diet (Buckland & Gonzalez, 2015) with special combination of fatty acids and bioactive minor constituents, which are particularly abundant in the highest quality extra-virgin olive oils (Frankel, 2011; Beauchamp et al., 2005; Salvini et al., 2006; Cecchi et al., 2013; Grossi et al., 2013; Cecchi, Migliorini, Cherubini, Innocenti & Mulinacci, 2015; Migliorini, Cherubini, Cecchi & Zanoni, 2013; Migliorini, Cecchi, Cherubini, Trapani Cini, & Zanoni, 2012).

According to the International Olive oil Council (IOC)), Virgin olive oils are obtained from olive fruit solely by mechanical or physical means under conditions that do not lead to alterations in the oil. Depending on their chemical and organoleptic properties, virgin olive oils are classified as Extra Virgin Olive Oil (EVOO), Virgin Olive Oil (VOO), Ordinary Virgin Olive Oil (OVOO) or Lampante Virgin Olive Oil (LVOO). The oils belonging to this latter category need to be refined to make it edible.

VOOs and, above all EVOOs, are widely appreciated for their health benefits and sensorial properties. The health benefits are mainly related to a high presence of monounsaturated fatty acids, mainly oleic acid (Cohen, Epstein, Pittman & Rivenson, 2000), and phenolic compounds (Covas et al., 2006; Coccia et al., 2014). The presence of these latter compounds has allowed the EFSA to approve the health claim, “the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” (EFSA Panel on Dietetic Products, 2011). The sensory properties are also influenced by this fraction together with the volatile compounds mainly derived from the lipoxygenase pathway (Bendini et al., 2007; Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Gutièrrez-Rosales, Rios, & Gomèz-Rey, 2003). All these properties justify the higher price of the EVOOs and VOOs when compared with other edible oils (Tena, Wang, Aparicio-Ruiz, Garcia-Gonzalez & Aparicio, 2015).

Oils of lower commercial value include Olive Oil (OO), which, according to the IOC, consists of a blend of Refined Olive Oil (ROO) and virgin olive oils (IOC). The country of retail sale may require a more specific designation; regarding Italy, virgin olive oil used to prepare OOs has to be different from LVOO, according to European Regulation 1308/13 (2013).

Oil refining is a physical-chemical multi-step process applied to LVOOs and other common seed oils to make them edible. This process requires, at least, a deacidification treatment and a deodorization as last step. It has been reported that phenolic compounds disappear in olive oil after the refining process, with the exception of lignans, (+)-pinoresinol and (+)-1-acetoxypinoresinol (Garcia, Ruiz-Mendez, Romero & Brenes, 2006). Nevertheless, the amount of these lignans in VOOs or crude LVOOs is consistently higher than in the refined oils (Garcia et al, 2006; Owen, Haubner, Wurtele, Hull, Spiegelhalder, & Bartsch, 2004).

Due to their high price, EVOOs, VOOs and OOs are very attractive targets for fraudsters. The most common frauds are: i) adulteration consisting of mixture of different categories of olive oils; ii) adulteration consisting of mixture with other vegetable oils. Consequently, there is a continuous search for new markers to detect adulterations and to guarantee the quality and safety of EVOO (Tena et al., 2015).

The lignans are a group of phytochemicals widespread in plants; they belong to the class of phytoestrogens and are beneficial for human health (Fini et al., 2008). Some of them are typical of *Olea europaea* L. and, although their quantity in olive oils is comparable to other classes of phenolic compounds, they were discovered 10 years after the first studies appeared on the oleuropein derivatives (Brenes et al., 2000). Lignans are the most abundant phenolic compounds after secoiridoids in the virgin olive oils (Bonoli, Bendini, Cerretani & Lercker, 2004). Their concentration mainly depends on the cultivar while the milling process does not affect their amount in a significant manner (Servili et al., 2014). To date, it is not yet clear how they are transferred from olives into oils (Oliveras-Lopéz, Innocenti, Ieri, Giaccherini, Romani, & Mulinacci, 2008) and how their amount changes after the chemical/physical treatment of oil.

The principal lignan in almost all virgin olive oils from different cultivars is (+)-1-acetoxypinoresinol with minor amounts of (+)-pinoresinol (Owen et al., 2004), hydroxypinoresinol and syringaresinol (Ballus et al., 2015). On the other hand, (+)-1-acetoxypinoresinol is the minor lignan compared to (+)-pinoresinol in the Picual cultivar oils, which represent approximately 25% of the world's production of olive oil.

(+)-pinoresinol has also been proposed as a marker to authenticate from Picual (Brenes, Garcia, Rios, Garcia & Garrido, 2002).

The main goals of this study were to investigate the influence of the olive oil refining process on the lignan profile and to propose the detection of new isobaric lignans as chemical markers of undeclared refining procedures in commercial EVOOs. We analyzed five Italian commercial OOs and three series of partially and fully-refined oils from Italian, Tunisian and Spanish industrial production. A mechanism for the formation of new isobaric lignans during the bleaching step is proposed by a comparison between the chromatographic findings and data from a dynamic molecular modeling study.

2. Materials and methods

2.1 Chemicals

All chemicals for the analyses were of analytical reagent grade: deionized water was produced by the Milli-Q-system (Millipore SA, Molsheim, France). Ethanol and n-hexane of analytical reagent grade and formic acid and acetonitrile of LC-MS grade were from J.T. Baker (Phillipsburg, New Jersey, USA). (+)-Pinoresinol from Sigma-Aldrich (Steinheim, Germany) was used as a standard compound. All stock solutions containing the standard (+)-pinoresinol were prepared in ethanol.

2.2 Samples

We sampled five Italian commercial OOs and three series of samples including oils collected at different stages of the refining process, as summarized in Table 1.

Provenience	Sample type	Code	Treatment
Italy	Crude Lampante Olive Oil	L_{ITA}	Crude Lampante Olive Oil; acidity ≈ 8.8%
	Neutral Oil	N_{ITA}	Chemical deacidification at room temperature and then at 90 °C; acidity ≈ 1.0%
	Bleached Oil	D_{ITA}	Bleaching with 1.5% of active earth at 90–100 °C and 40–50 mmHg
	Refined Oil	R_{ITA}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg
Spain	Crude Lampante Olive Oil	L_{SPA}	Crude Lampante Olive Oil; acidity ≈ 9.0%
	Neutral Oil	N_{SPA}	Physical treatment: degumming
	Bleached Oil	D_{SPA}	Bleaching with 0.8% of active earth
	Refined Oil	R_{SPA}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg
Tunisia	Crude Lampante Olive Oil	L_{TUN}	Crude Lampante Olive Oil; acidity ≈ 9.0%
	Neutral Oil	N_{TUN}	Chemical deacidification at 90 °C; acidity ≈ 1.0%
	Bleached Oil	D_{TUN}	Bleaching with 0.8% of active earth
	Refined Oil	R_{TUN}	Deodorization for 2.5 h at 230 °C and 1.5 mmHg

Table 1. List of the analyzed samples: in the different columns are indicated the provenience, the sample derived by a specific refining step, the codifying and a short description of the treatment applied to each refining step

The oils codified as “Italy”, “Spain” and “Tunisia” are samples purchased from Italy, Spain and Tunisia respectively and kindly obtained by an Italian factory of commercial olive oils. Each sample was representative of an industrial batch and was kept in the dark and stored at room temperature until the time of analysis. All the analyzed samples were

purchased in 2014-2015 and identified as follow: L for Crude Lampante Olive Oil; N for Neutral Oil; D for Bleached Oil and R for Refined Oil (Table 1).

2.3 Refining process

The Italian lampante olive oil (L_{ITA}) was subjected to a first chemical deacidification at room temperature, to obtain an oil with an acidity of approximately 7.8% and to a second step of deacidification at 90°C, giving an oil with an acidity of approximately 1.0% (N_{ITA}). In the following step the oil was bleached by using 1.5% of active earth at 90-100°C and 40-50 mmHg (D_{ITA}). Finally, the oil was deodorized for 2.5 hours at 230°C and 1.5 mmHg (R_{ITA}).

The Spanish lampante olive oil (L_{SPA}) was subjected to a physical treatment consisting of a degumming process (N_{SPA}). The obtained oil was bleached with 0.8% of active earth (D_{SPA}) and then deodorized for 2.5 hours at 230°C and 1.5 mmHg (R_{SPA}).

The Tunisian lampante olive oil (L_{TUN}) was subjected to a chemical deacidification at 90°C, to obtain an oil with an acidity of approximately 1.0% (N_{TUN}). The obtained oil was bleached with 0.8% of active earth (D_{TUN}) and then deodorized for 2.5 hours at 230°C and 1.5 mmHg (R_{TUN}). The initial acidity values of all these oils are reported in Table 1.

The active earths used for the bleaching step are bleaching earths “CLINOLIP CS 1060” mixed with 10% powder active carbon “FILTRACARB SK1-P75.

2.4 Extraction of phenolic compounds from the oils

The extraction conditions to recover the phenolic fraction were the same of our previous works (Oliveras-Lopez, Innocenti, Giaccherini, Ieri, Romani & Mulinacci, 2007). Briefly, approximately 20 grams of oil were extracted in 60 mL of the $EtOH/H_2O^+$ 70:30 solution by stirring for 30 minutes. The hydroalcoholic solution was brought to a pH=3.2 with formic acid. The mixture was then defatted three times with 20 mL of hexane. During the defatting of samples L_{SPA}, N_{SPA}, L_{TUN}, N_{TUN}, the separation between the hydroalcoholic and hexane phase was incomplete so that it was necessary to use 60 mL of hexane for the first defatting step. The hydroalcoholic solution was evaporated under reduced pressure at approximately 35°C and the residue was redissolved with 1.5

mL EtOH/H_2O^+ 70:30 solution. The sample was then centrifuged at 14,000 rpm and 10°C, and the supernatant immediately used for chromatographic analysis.

To evaluate the efficiency of the extractive procedure on the recovery of lignans, a spiking test was also used. Two amounts of (+)-pinoresinol, 0.36 and 0.73 mg_{PIN}/kg_{OIL}, were added to D_{TUN} oil and the percentage of recovery was evaluated by TOF Mass Spectrometer.

2.5 HPLC/DAD/TOF-MS analysis of lignans

The analyses were performed using an HP 1100L Liquid Chromatograph. The detector was a DAD coupled to a TOF Mass Spectrometer equipped with an electrospray (ESI) interface (all from Agilent Technologies, Palo Alto, CA, USA). The analysis parameters were set using a negative ion mode with spectra acquired over a mass range of 100-800 m/z. The conditions of the ESI source were as follows: drying gas (N_2) temperature, 350°C; drying gas flow-rate, 6 L/min; nebulizer, 20 psi; capillary voltage, 4000 V; fragmentation, 150 V; skimmer, 60 V. A 150 mm × 3 mm i.d., 2.7 μm Poroshell 120, EC-C18 column (Agilent, USA) equipped with a precolumn of the same phase was used; oven temperature 26°C. The acquisition and data analysis were controlled using Agilent LC-MS TOF Software (Agilent, USA).

The solvents for the mobile phase were (A) 0.1% formic acid/water and (B) CH_3CN; the multi-step linear solvent gradient used was: 0–5 min 10–15% B; 5–15 min 15–30% B; 15–20 min 30-35% B; 20–23 min 35–40% B; 23-26 min 40-45% B; 26-32 min 45-100% B; 32-37 min 100% B; 37-42 min 100-10% B; equilibration time 10 min; flow rate 0.4 mL min^{-1}; injection volume 2 μL. The following wavelengths were simultaneously selected: 240 nm, 280 nm, 330 nm, 350 nm and 540 nm.

The detection of lignans was carried out at 280 nm by comparing their UV-vis and mass spectra and with the pure standard, (+)-pinoresinol. The TOF mass spectrometer was calibrated immediately before the analyses. No internal reference was used during the analyses. The accurate mass of the molecules was measured; the mass accuracy was checked by analyzing the (+)-pinoresinol standard in the same conditions used for the samples. To calculate the elemental compositions, a maximum difference of 10 ppm between calculated and measured was considered. A mass difference always less than 6

ppm was observed for (+)-pinoresinol and its possible isomers and for (+)-1-acetoxypinoresinol and its isobaric species calculated on the deprotonated molecular ion.

The lignans were quantified only in the fully refined oils (ROOs) because this category of oil is used to prepare the commercial OOs. The total amount was the sum of (+)-pinoresinol, (+)-1-acetoxypinoresinol and their isobaric forms; the data were expressed as mg_{PIN}/kg_{oil}. This molecule was used as external standard to build a five-point calibration curve at 280 nm, linearity range 0-1.21 μg and R^2 0.9999.

2.6 Dynamic molecular modeling

The chemical structures of the new isobaric forms of lignans were created starting from the structures of the natural (+)-pinoresinol and (+)-1-acetoxypinoresinol (Brenes et al, 2000; Owen, Mier, Giacosa, Hull, Spiegelhalder, & Bartsch, 2000) by using the Discovery Studio 3.5 Visualizer free program (Accelrys, San Diego, USA). The partial atomic charges were derived using the AM1-BCC method implemented in the ANTECHAMBER suite (Wang, Wang, Kollman & Case, 2006). The energy minimizations and MD (Molecular Dynamic) were carried out using the SANDER module of AMBER 9 (Case et al., 2006) with the GAFF (Wang, Wolf, Caldwell, Kollman, & Case 2004) force field. Molecular dynamic simulations were performed in implicit solvent using the Generalized Born Surface Area (GBSA) at constant pH. The constant pH molecular dynamics method has been implemented in SANDER (Mongan, Case & McCammon, 2004). Before the dynamic simulation, 100 steps of steepest-descent and 900 steps of conjugate-gradient minimization on the entire complex were performed with a modified GB model with igb = 2 (Onufriev, Bashford & Case, 2004), the surface area was computed and included in the solvation term, and a cutoff of 30 A˚ for non-bonded interactions was used. The system was then heated from -273.15 °C to 90 °C in 10 ps by holding the complex fixed with a harmonic constraint of a strength of 0.05 kcal/(mol $Å^2$). After the minimization and heating, to equilibrate the system, 0.1 ns of dynamic simulations, with the molecular constraint (strength of 0.01 kcal/(mol $Å^2$), were performed at constant temperatures of 27 and 90 °C. Finally, 3 ns dynamic simulations, with the complex constraint (strength of 0.01 kcal/(mol $Å^2$), were performed at a constant temperature of 27 and 90 °C with SHAKE turned on for bonds involving hydrogens, allowing a time-step of 2.0 fs. 300 conformations were collected during the simulation

(one conformation every 10 ps). The stability of the different molecules was reported as the mean of 300 measurements and expressed in kcal/mol.

Data were subjected to analysis of variance using Microsoft Excel statistical software and F-Test ($P < 0.05$) was performed for statistical significance. The means were then compared by Fisher's LSD test by using the software DSAASTAT v. 1.1 (Onofri, 2007).

3. Results and discussion

3.1 Phenolic profiling of commercial olive oils

Five commercial OOs were purchased and analyzed with the aim of evaluating their phenolic content, and especially their lignan content. As required by law, these oils are a mix of ROOs and virgin olive oils and, due to the presence of these latter oils, it was expected to find several phenolic compounds. This hypothesis was confirmed only for three out of five samples highlighting at 280 nm the presence of several minor phenols. Surprisingly, the chromatographic profile at 280 nm of one sample was empty, while another oil showed four peaks in the typical chromatographic range of the phenolic compounds (Fig. 1A, rt 20-26 min). The UV and mass-TOF spectra of these analytes allowed us to identify (+)-pinoresinol and (+)-1-acetoxypinoresinol, together with their isobaric forms as clearly shown by the Extract Ion profiles (EI) at m/z 357.13 and m/z 415.14 (Figure 1B and 1C).

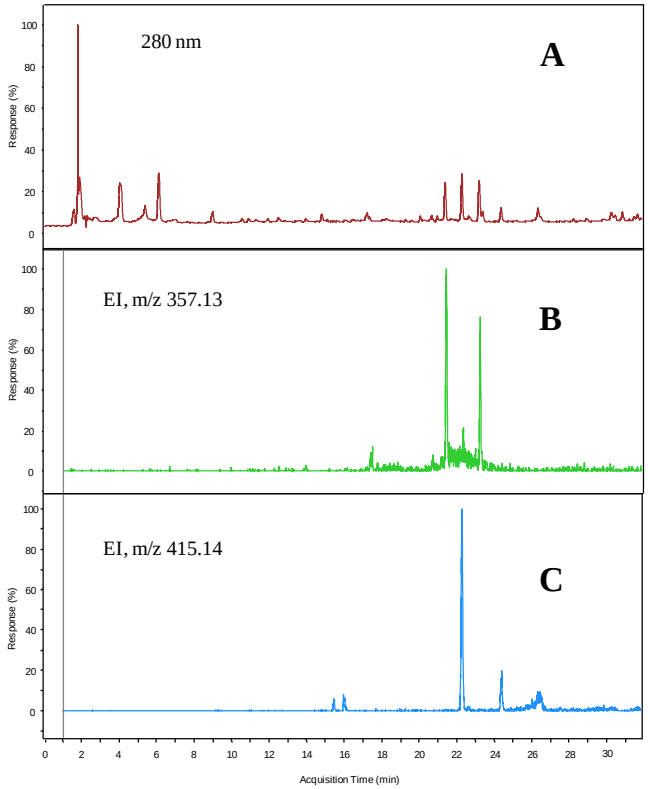


Figure 1. Chromatographic profiles at 280 nm of an Italian commercial Olive Oil: (A), Extract Ions at m/z 357.13 for (+)-pinoresinol (B) and at 415.14 for (+)-1-acetoxypinoresinol (C)

To the best of our knowledge, the presence of isobaric forms of lignans has never been described in VOOs and EVOOs derived only by mechanical means. Consequently it was hypothesized that their presence was induced by the refining process applied to LVOO. In light of this new evidence we decided to further investigate the origin of these new lignans by analyzing oil samples obtained after the different steps of the industrial refining process.

3.2 Phenolic profiles of partially and fully refined olive oils

Aiming to work on a pool of representative samples obtained after industrial refining, three series of oils derived from different LVOO (Table 1) were extracted and analyzed by HPLC-DAD-MS-TOF. To date, only one work (Garcia et al., 2006) was focused on phenolic determination in partially and fully refined LVOO, with a minor attention to lignan fraction.

The use of TOF allowed us to detect the accurate mass of lignans, and confirm the presence of the new isobars, identifying the step responsible for this formation. Figures 2A, 2B, 2C and 2D compare the profiles at 280 nm and the corresponding Extract Ions (m/z 357.13 and m/z 415.14) for L_{TUN}, N_{TUN}, D_{TUN} and R_{TUN}. It immediately appears that

the number and intensity of all the peaks detected at 280 nm strongly decreases after every step of the refining process with a total reduction of approximately 90% during the whole process from L_{TUN} to R_{TUN}.

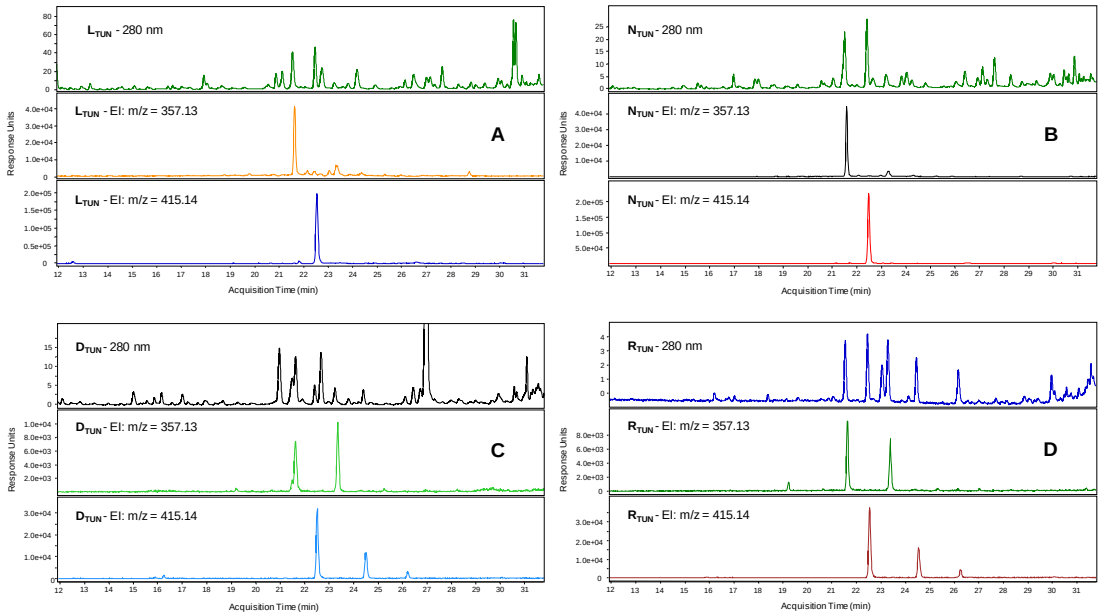


Figure 2. Comparison between chromatograms at 280 nm, EI at m/z 357.13 and at m/z 415.14 for the Tunisian samples: L_{TUN} (A), N_{TUN} (B), D_{TUN} (C) and R_{TUN} (D)

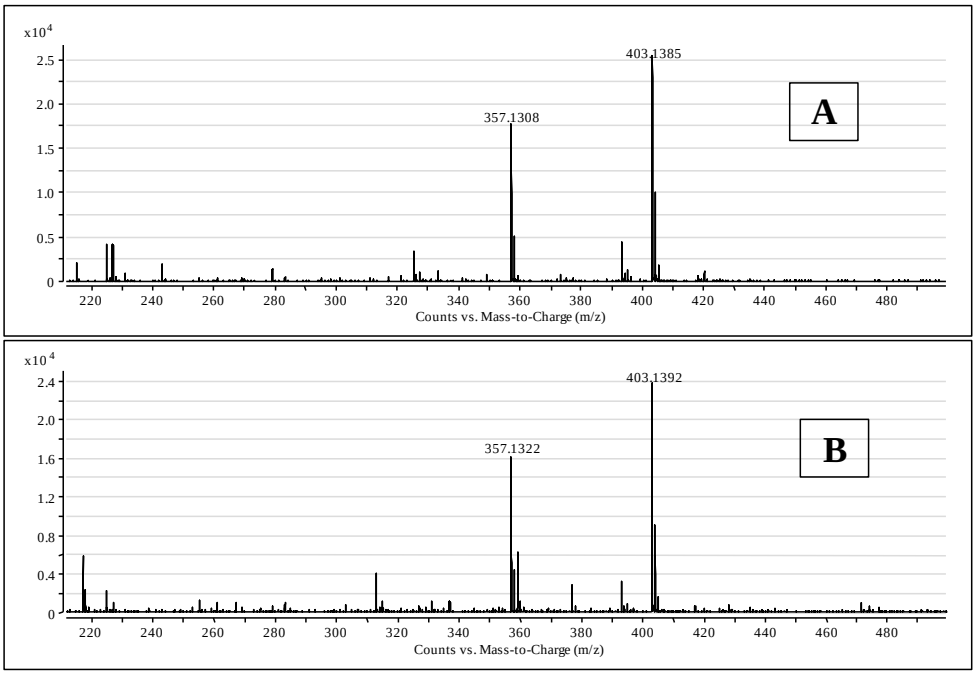
The very intense peak in chromatogram of D_{TUN} oil, (rt close to 27 min, Figure 2C), absent in lampante oil, was probably formed during the bleaching step but it disappeared after deodorization presumably because of its volatility and/or low thermal stability. Analogous behavior was observed in the corresponding Italian oils. Regarding EI profiles at m/z 357.13 of L_{TUN} and N_{TUN}, the UV and mass spectra of the smaller peaks at rt 22-23.5 min did not fit with those of known phenols. Their further characterization was not carried out since was not part of the scope of this study.

The chromatograms at 280 nm for L_{TUN} (Figure 2A) and N_{TUN} (Figure 2B) show no qualitative differences but only a general decrease in the intensity of all the peaks. The corresponding EI chromatograms of Tunisian crude (2A) and neutral (2B) oils almost overlap, and only after the bleaching step (2C) do the isobaric analogues of (+)-pinoresinol and (+)-1-acetoxypinoresinol appear. According to previous data (Garcia et al., 2006), and confirmed by the lower intensity of N_{TUN} (Fig 2C) *versus* D_{TUN} (Fig 2B) in the EI profiles, refining consistently reduces the amount of all the phenolic compounds. Nevertheless, the bleaching step also induces a chemical modification of the remaining lignans. To the best of our knowledge, this effect has never been reported before. Finally,

it should pointed out that the last step of the refining process, namely deodorization, does not induce further significant changes in the EI profiles.

Regarding the new isobaric lignans detected in D_{TUN}, the EI at m/z 315.13 (Figure 2C) shows two peaks with comparable intensity, UV and mass spectra (Figure 3, A-B) in agreement with those of (+)-pinoresinol. Two diagnostic ions, $[M–H]^-$ and its adduct with formic acid $[M+HCOOH]^-$, were detected for both the (+)-pinoresinol and its isobar at rt 23.3 min. Similarly, the EI profiles at m/z 415.14 (Figure 3, C-D-E) pointed out three peaks with the same UV and mass spectra of (+)-1-acetoxypinoresinol. The diagnostic ions were again the $[M–H]^-$ and the corresponding adducts with formic acid. Furthermore, two new isobars at rt 24.5 min and rt 26.2 min were detected.

The mass spectra in negative ionization mode of the natural lignans and of the three new isobaric forms are reported in supplementary material. None of these spectra show the presence of fragment ions and the relative intensity of the molecular ion and its adduct with formic acid are very similar. Our findings in Tunisian oils highlight that the new isobaric forms of (+)-pinoresinol and (+)-1-acetoxypinoresinol are formed during the bleaching step of the refining process.



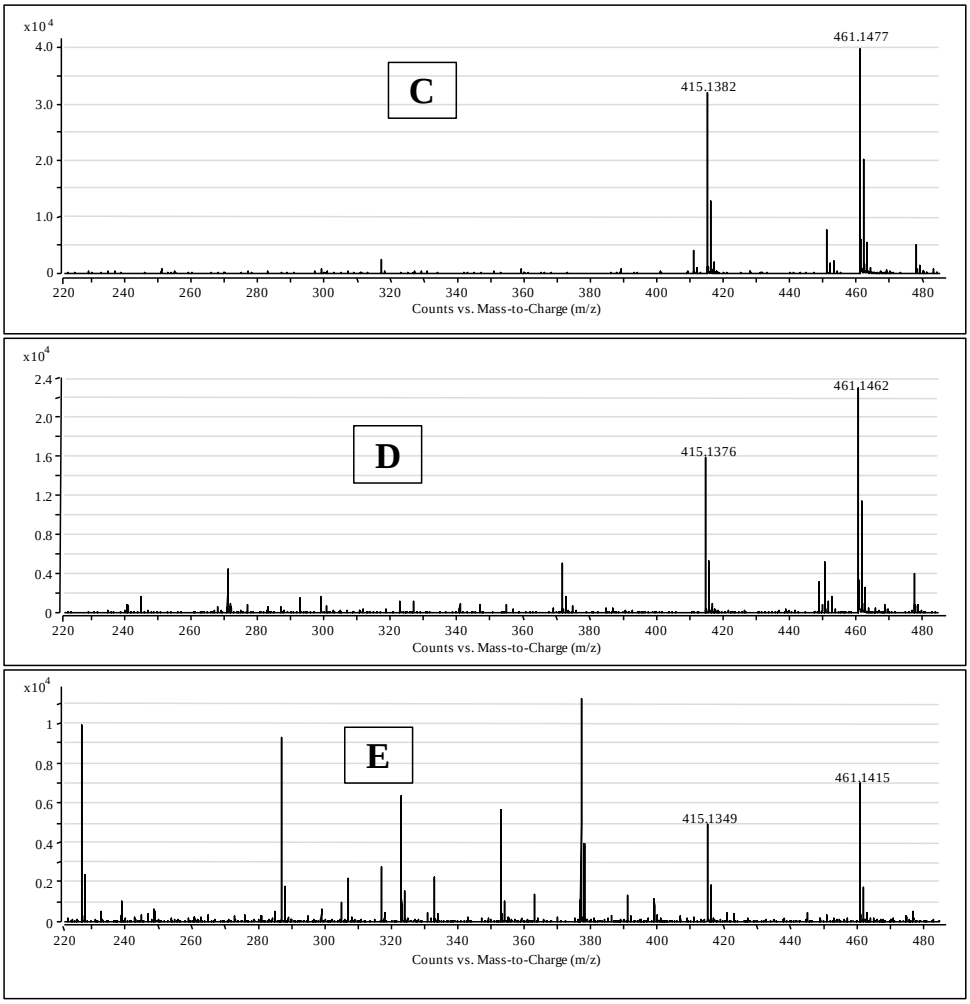


Figure 3. Mass spectra of the detected lignans in D_{TUN} oil obtained in negative ionization mode: (+)-pinoresinol (A) and its isobaric form (B), (+)-1-acetoxypinoresinol (C) and its isobaric forms (D-E)

The third series of samples were from the refining process applied of a Spanish oil. The profile at 280 nm of the lampante oil (L_{SPA}) presents several peaks: the most intense one was (+)-pinoresinol while the peak of (+)-1-acetoxypinoresinol appeared consistently lower than the lampante Tunisian and Italian oils. This result suggests the presence of Picual, a typical Spanish cultivar in which the relative concentration of pinoresinol/acetoxypinoresinol is inverted compared to the majority of virgin olive oils (Brenes et al., 2000) obtained from other cultivars in which the dominant lignan is always (+)-1-acetoxypinoresinol (Ballus et al., 2015).

The chromatograms for neutral and bleached oils show the same variations already described for the Tunisian and Italian series: the intensity of the peaks relating to (+)-1-acetoxypinoresinol and its isobaric forms is very low compared with (+)-

pinoresinol, and the formation of isobaric forms of lignans occurred again during the bleaching step.

The next step focused on elucidating the structure of these new isobars by using a molecular dynamic simulation, whose rationale entails the following considerations. The mass spectra obtained by TOF are accurate enough to confirm the presence of the previously cited isobars, because ions at 315.13 m/z and 415.14 m/z in olive oil can be associated only with (+)-pinoresinol and (+)-1-acetoxypinoresinol, respectively. The extraction and purification of these isobars requires the treatment of several liters of bleached oil because their final content is very low (less than 2 mg/Kg_{oil} as sum – see also next paragraph), their molecular weights are relatively high and the extractive yields are far away from 100%. Consequently, the dynamic molecular modeling at 27 and 90 °C was selected as a possible approach to acquire more information on the chemical structure of these new lignans. The chemical rearrangement behind this isomerization is discussed in the next paragraph.

3.3 Chemical rearrangement of lignans during the bleaching step

The UV spectra of new lignans, (+)-pinoresinol and (+)-1-acetoxypinoresinol, are identical indicating the presence of the same chromophore. MS-TOF analyses of isobaric forms showed exactly the molecular weight of the two natural precursors. In light of these findings no losses of groups of atoms and no definitive ring opening take place during this chemical rearrangement. To explain these isomerizations, it was hypothesized a ring opening/closing mechanism involving one of the C-O bond of the tetrahydrofuranic rings. If this rearrangement involves the achiral carbons (C4 and C8, Figure 4) the result is the re-formation of the natural lignan.

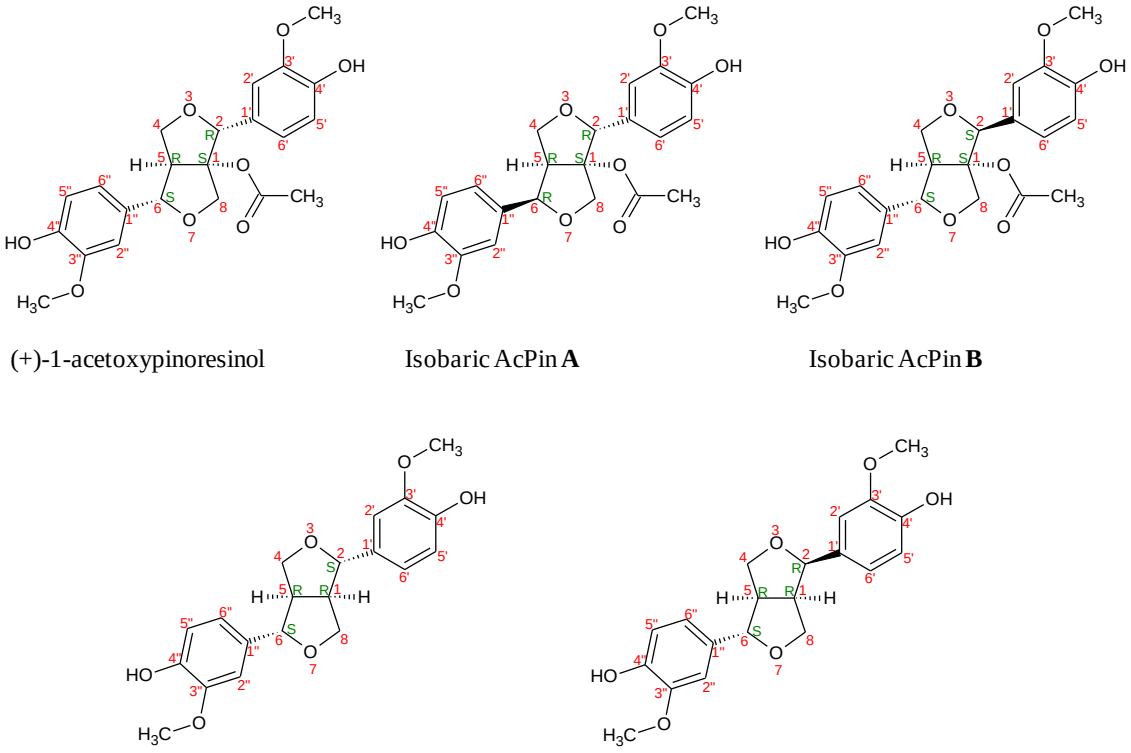


Figure 4. Chemical structures of natural (+)-pinoresinol, (+)-1-acetoxypinoresinol and their new isobaric forms

On the other hand, if the rearrangement involves one chiral carbon (C2 or C6, Figure 4), the re-formation of diastereomeric products is also possible. Taking into account the proposed chemical rearrangement and due to the presence of a C_2 axis perpendicular to the plane, we expect only one new isobar from (+)-pinoresinol. The chromatographic analyses (EI at m/z = 357.13 in Figure 2C), agree with our hypothesis pointing out only one isobaric form. In the case of (+)-1-acetoxypinoresinol, no axis of symmetry is present in the molecule, so we can have really two new diastereoisomers from this lignan. Again, the chromatographic behavior agrees with the previous hypothesis (Figure 2C, EI at m/z = 415.14) showing two isobaric forms.

The potential energies of all the possible diastereoisomers derived by the natural (+)-pinoresinol and (+)-1-acetoxypinoresinol, were calculated by applying dynamic molecular simulation. For (+)-1-acetoxypinoresinol with 4 chiral carbons and no symmetry axis, 16 (2^4) different diastereoisomers are possible, corresponding to 8 couples of enantiomers. The enantiomers are not distinguishable by chromatographic systems without chiral components and their potential energies are the same, consequently the structures of only eight diastereoisomers were considered for the calculations. The means

of potential energies after dynamic simulations of the eight diastereoisomers are shown in Figure 5.

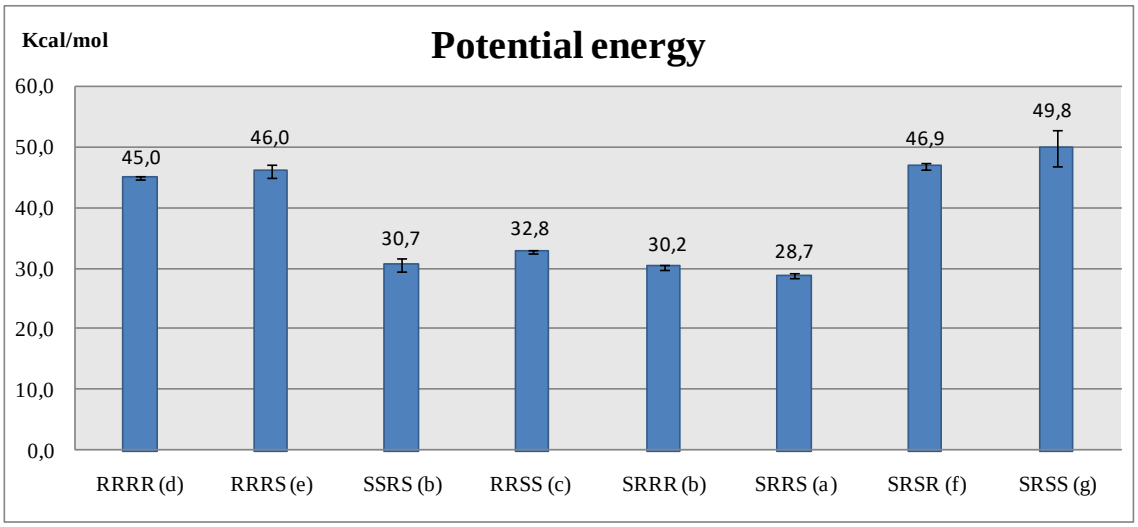


Figure 5. Potential energy of all possible different diastereoisomers of (+)-1-acetoxypinoresinol from dynamic molecular modeling at 90 °C. Each isomer is identified by the configuration of C1, C2, C5 and C6 according to Figure 3. Different letters (in brackets) point out significant differences by Fisher's LSD test

The more probable diastereoisomers were created starting from the natural (+)-1-acetoxypinoresinol (1*S*, 2*R*, 5*R*, 8*S*) by the inversion of C2 or C6 configuration, so obtaining two structures, called isobaricAcPinA and isobaricAcPinB (Figure 4). The histogram highlights these two isobars as the most stable isomers with energy values comparable with that of the natural (+)-1-acetoxypinoresinol. These findings agree with the chromatographic results in EI profiles at m/z = 415.14 (Figures 2C and 2D), where the natural form is the most abundant, followed by different amounts of only other two isobars. Similarly, for (+) pinoresinol the potential energy values (data no shown) are in agreement with the chromatographic results.

3.4 Determinations of lignans amount in refined oils (ROOs)

After the preliminary investigation on partially and fully refined LVOOs, we selected a pool of ROOs. Indeed, only this latter type of refined oils are used, in blends with virgin olive oils, to obtain the commercial OOs destined for human consumption. We confirmed the possibility of carrying out this determination using an HPLC-DAD method at 280 nm without the need of more expensive and often unavailable mass detectors.

To evaluate the efficiency of the extractive procedure on lignan recovery, spiking tests were carried out using the D_{TUN} oil and known amounts of (+)-pinoresinol. The

results of these experiments, evaluated by the integration of the peak areas in the lignan EI profiles, highlighted a recovery of 95% when the spiking was 0.36 mg_{PIN}/kg_{OIL} and 98% when the spiking was 0.73 mg_{PIN}/kg_{OIL}. These results indicated that the extractive method guarantees almost the full recovery of lignans.

We selected the HPLC-DAD at 280 nm using (+)-pinoresinol as external standard with the aim of providing a simple analytical tool to estimate the lignan content in ROOs but also to detect frauds in the commercial OOs. Overall, the total amount of lignans in these oils was very low (Figure 5) and always below 2 mg/kg_{OIL}; in R_{TUN} it was slightly higher than R_{SPA} and much higher than R_{ITA}; the (+)-pinoresinol content was higher than (+)-1-acetoxypinoresinol only in R_{SPA} oil in agreement with the hypothesis of a large presence of Picual cv.

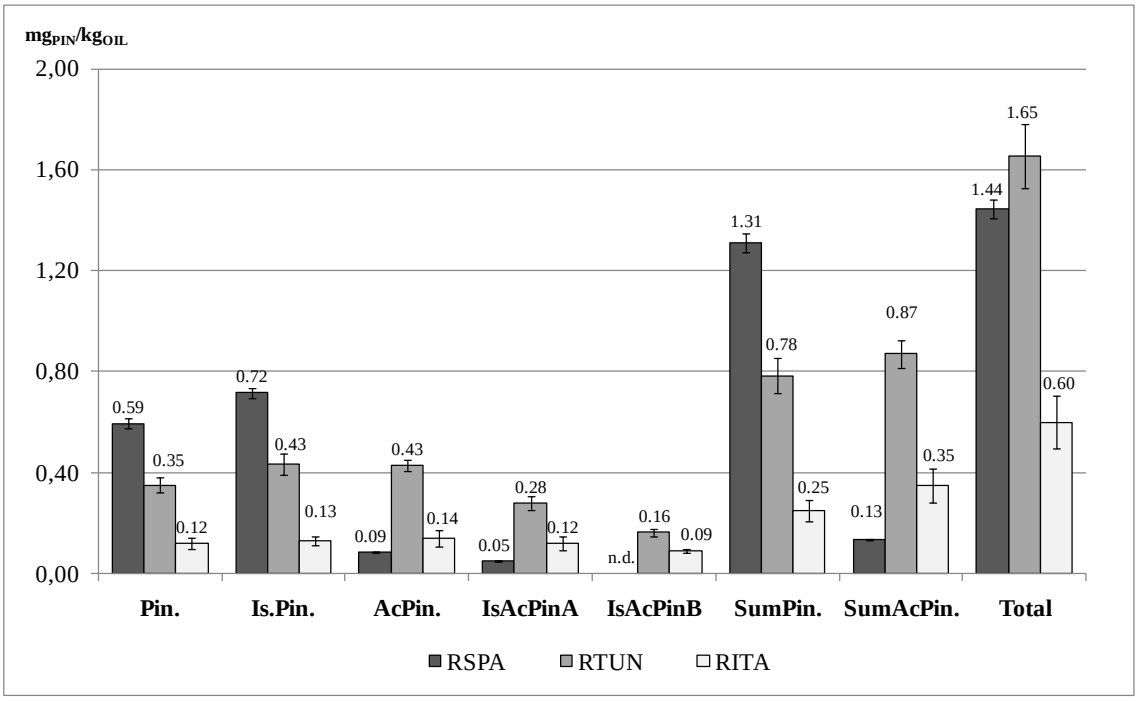


Figure 6. Lignan content of refined oils; *Pin*, (+)-pinoresinol; *AcPin*, (+)-1-acetoxypinoresinol; *Is.Pin*, Isobar of pinoresinol; *Is.AcPinA*, Isobar A of acetoxypinoresinol; *Is.AcPinB*, Isobar B of acetoxypinoresinol; *SumPin*, sum of pinoresinol and its isobar; *SumAcPin*, sum of acetoxypinoresinol and its isobars; *Total*, sum of all lignans

4. Conclusions

To the best of our knowledge, isobaric forms of lignans have never been described in edible olive oils before this report. We have confirmed their presence in three series of olive oils of different origin and derived by an industrial refining process. We have also demonstrated how the bleaching step induces this isomerization. The proposed

mechanism of rearrangement was confirmed by a dynamic molecular simulation, which provided results in agreement with our analytical findings.

The isobaric forms of (+)-pinoresinol and (+)-1-acetoxypinoresinol, never detected before, can also be evidenced by the use of HPLC-DAD systems, although mass spectrometric analysis is recommended to definitively confirm their presence.

Further investigations on a wider number of commercial olive oils are required for evaluate the minimum amount of refined oil, illegally added to VOOs, that this method is able to detect. The validation of the analytical method is the next step to improve the study.

These new isobaric lignans are proposed, to the producers and the analysts responsible for the oil quality control, as chemical markers in detecting frauds regarding the application of undeclared refining procedures in extra-virgin or virgin olive oils. The detection of these markers is possible by HPLC-DAD and without the need of more expensive mass spectrometric detectors. This aspect strongly facilitates the application of the method as a routine control for the oil quality in the next future

Acknowledgements

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Abbreviations.

EVOO: Extra Virgin Olive Oil
VOO: Virgin Olive Oil, as sub category of virgin olive oil
OVOO: Ordinary Virgin Olive Oil
LVOO: Lampante Virgin Olive Oil
OO: Olive Oil
ROO: Refined Olive Oil
cv: cultivar
EI: Extract ion
IOC: International Olive oil Council
EFSA: European Food Safety Authority

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Multiple internal standard normalization for improving HS-SPME-GC-MS quantitation in Virgin Olive Oil Volatile Organic Compounds (VOO-VOCs) profile

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Martina Fortini[1], Marzia Migliorini[1], Chiara Cherubini[1], Lorenzo Cecchi[2*], and Luca Calamai[3]

[1] PromoFirenze, Azienda Speciale della CCIAA di Firenze, Divisione Laboratorio Chimico, via Orcagna 70, 50121 Firenze, Italia

[2] Dipartimento di NEUROFARBA, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Firenze), Italia and Multidisciplinary Centre of Research on Food Sciences (M.C.R.F.S.- Ce.R.A).

[3] DISPAA, Università degli Studi di Firenze, Piazzale Cascine 28 50144 Firenze, Italy

*Corresponding author: Lorenzo Cecchi

Tel.: 0039-0554573707

e-mail: lo.cecchi@unifi.it

Abstract

The commercial value of virgin olive oils (VOOs) strongly depends on their classification, also based on the aroma of the oils, usually evaluated by a panel test. Nowadays, a reliable analytical method is still needed to evaluate the volatile organic compounds (VOCs) and support the standard panel test method. To date, the use of HS-SPME sampling coupled to GC-MS is generally accepted for the analysis of VOCs in VOOs. However, VOO is a challenging matrix due to the simultaneous presence of: i) compounds at ppm and ppb concentrations; ii) molecules belonging to different chemical classes and iii) analytes with a wide range of molecular mass. Therefore, HS-SPME-GC-MS quantitation based upon the use of external standard method or of only a single internal standard (ISTD) for data normalization in an internal standard method, may be troublesome. In this work a multiple internal standard normalization is proposed to overcome these problems and improving quantitation of VOO-VOCs. As many as 11 ISTDs were used for quantitation of 71 VOCs. For each of them the most suitable ISTD was selected and a good linearity in a wide range of calibration was obtained. Except for *E*-2-hexenal, without ISTD or with an unsuitable ISTD, the linear range of calibration was narrower with respect to that obtained by a suitable ISTD, confirming the usefulness of multiple internal standard normalization for the correct quantitation of VOCs profile in VOOs. The method was validated for 71 VOCs, and then applied to a series of lampante virgin olive oils and extra virgin olive oils. In light of our results, we propose the application of this analytical approach for routine quantitative analyses and to support sensorial analysis for the evaluation of positive and negative VOOs attributes.

Keywords: Virgin olive oil; Aroma; HS-SPME-GC-MS; Validation

1. Introduction

According to the IOC and UE [1], virgin olive oil (VOO) is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil. The green and fruity sensations of VOO are mainly due to volatile organic compounds (VOCs) derived from degradation of polyunsaturated fatty acids through a biosynthetic lipoxygenase (LOX) cascade pathway [2,3,4], occurring mainly during the crushing and malaxation steps within the extraction process [5,6,7,8].

The composition of VOCs in VOO is mainly affected by agronomical parameters, such as cultivar, pedoclimatic conditions and ripening stage [9,10,11,12,13] as well as by technological parameters, namely the drupes harvesting and storage conditions before transformation, the operations during the olives transformation and, later, the storage conditions of the obtained oils [3,4,14,15]. These processes can generate unpleasant sensory notes, also known as sensory defects [16], and modify the initial VOCs profile.

Based on sensory parameters and/or some legal limits for chemical analysis [17], VOO is classified as: extra virgin olive oil (EVOO), virgin olive oil (VOO) or lampante virgin olive oil (LVOO). These types of VOOs have very different commercial value, thus emphasizing the importance of their classification. In addition, within the EVOO class, VOCs profile contributes to the quality evaluation along with phenolic profile.

To date, the assessment of VOO defectness degree must be conducted by sensory analysis, through a panel of trained experts [18], even though the analytical identification and quantification of volatile compounds responsible for sensory defects is gaining increasing attention as an useful tool to support the panel test, this latter test being affected by some problems [19,20,21,22,23,24]. To this aim, it is necessary to develop robust and reliable instrumental analytical methods to at least screen out samples before the panel test. Different methods have been employed in recent years for VOO-VOCs sampling (e.g. dynamic head space, SPME) and detection [16-19-20-21-25]. Analytically, VOO represent a challenging matrix, since compounds at ppm (e.g. *E*-2-hexenal) and ppb (e.g. 1-octen-3-ol, 1-octen-3-one) concentrations are simultaneously present, and their quantification in a single analytical run may be troublesome.

Solid phase micro extraction from head space (HS-SPME) is a widespread and convenient sampling tool for VOCs, and its use in food analysis coupled with GC-MS

analysis is now increasingly accepted. However, when quantification is the goal, several devices must be used to ensure an unbiased quantification. Being HS-SPME a sampling technique at equilibrium, suitable internal standards (ISTDs) should be used for peak area normalization, to account for the differences arising from either absorption capacity of different fibers, fiber wearing upon usage, changes in sorption temperature, changes due to the competition of different molecules at different affinities for the sorbing material, among different samples [26]. The use of the appropriate ISTD generally allows to correct departures from linearity and, in some cases a single ISTD was employed for data normalization during quantification [14,27].

Recently, a SPME-GC-MS method has been published on the determination of off-odours in VOO [28]. Albeit a method validation was reported in such paper, the linear working range was so narrow as to render such method worthless for VOOs of different characteristics and provenance. When the evaluation of an overall VOCs profile is required, e.g. for the assessment of VOO quality, a quantitative method featuring a wide linear range should be developed such as to include pleasant VOCs along with off-odours [28].

The aim of this work is to propose and validate an analytical method based on multiple internal standard normalization for improving HS-SPME-GC-MS quantitation of VOO-VOCs profile. To this end it is necessary: i) to identify a suitable ISTD for each identified volatile molecule; ii) to expand the linear working range of all the identified volatile compounds, both those with low and high concentration.

2. Materials and methods

2.1 Chemicals

All chemicals and standards of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany) and were prepared in refined oil. Inert gasses (He and N_2 99.99999 % purity) were supplied by SOL gas company.

2.2 Samples and standards preparation

A stock standard solution mix of 71 analytes was prepared by weighting the standard compounds in a refined olive oil after verifying the absence of any analyte or ISTD by HS-SPME-GC-MS. Six levels of calibration scales were obtained by diluting

the stock standard solution mix in the refined olive oil. The external standards and their ranges of concentration were chosen according to data of previous works, in which Italian VOOs (with a prevalence from Tuscany) were analyzed [29]. The solutions of external standards were stored in the dark at -20°C until the chromatographic analysis.

Because the response of SPME fiber varies, depending upon the sample complexity, volatility of compounds, fiber wearing and composition of the VOCs present in the head space, an internal standard mixture (ISTD MIX) was prepared and added to samples and scales, to be used for quantitation. The ISTD MIX consisted of 11 compounds either labeled with at least 3 deuterium atoms or absent in the VOOs specimens and with no interferences with their volatile profiles, as established in preliminary analysis (Figure 1), i.e. 3,4-dimethylphenol, 4-methyl-2-pentanol, hexanoic acid-d_{11}, 1-butanol-d_{10}, ethyl acetate-d_8, toluene-d_8, ethyl hexanoate-d_{11}, acetic acid-2,2,2-d_3, 6-chloro-2-hexanone, 3-octanone, trimethylacetaldehyde.

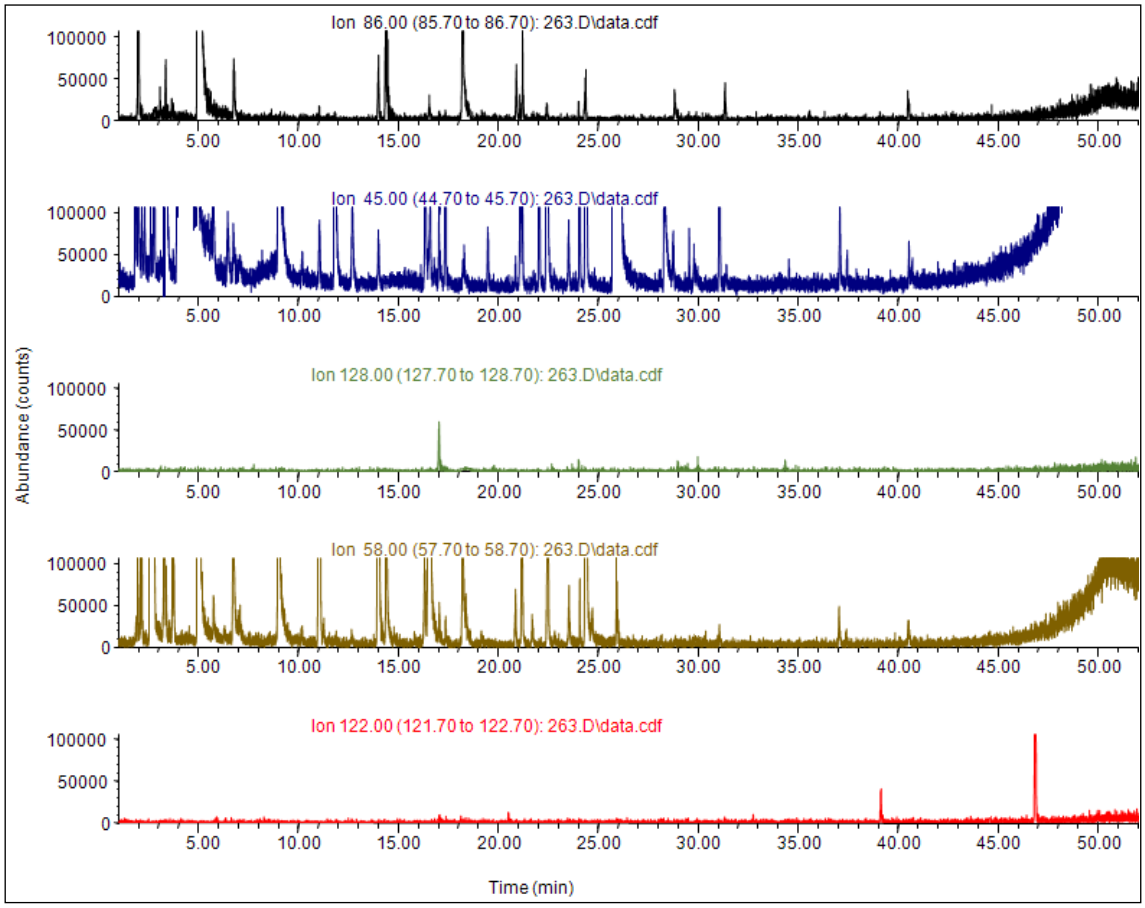

Figure 1. Extracted ion chromatograms for the quant-ion relative to the non-deuterated ISTDs for three commercial EVOOs

They were chosen in order to provide both low and high molecular mass ISTD compounds for several different compound classes, e.g. esters, alcohols, aldehydes,

ketones, carboxylic acids and aromatic hydrocarbons. The characteristics of each ISTD are summarized in Table 1.

Internal Standard	rt (min)	nominal mw (g/mol)	Chemical characteristic	Quantifier ions (Th)	Qualifier ions (Th)
trimethylacetaldehyde	2.63	86	aldehyde	86	57
ethyl acetate-d_8	3.30	96	ester	96	66/76
toluene-d_8	6.76	100	aromatic hydrocarbons	98	100
1-butanol-d_{10}	12.60	84	alcohol	64	46
4-methyl-2-pentanol	14.40	102	alcohol	45	69
ethyl hexanoate-d_{11}	17.11	155	ester	110	91/105
3-octanone	18.27	128	ketone	128	99
acetic acid-2,2,2-d_3	25.77	63	carboxylic acid	63	46
6-chloro-2-hexanone*	30.00	133	ketone	58	98
hexanoic-d_{11} acid	37.08	127	carboxylic acid	63	77
3,4-dimethylphenol	44.58	122	phenol	122	107

Table 1. List of the selected internal standards: i) rt, retention time; ii) nominal molecular weight; iii) chemical characteristic; iv) Identification ions – quantifier and v) Identification ions - qualifier. *Only monoisotopic mass for ^{35}Cl was considered

A bulk of refined oil solution of these ISTD compounds mix was prepared at the beginning of the experiment, and stored at −20°C until used. The peak areas of these compounds were used for area normalization in constructing the calibration lines of the target compounds, according to their chemical properties and elution order as reported in Table 1. The final concentration of each ISTD into the ISTD MIX was 75 mg/kg.

Samples analyzed were: 2 LVOOs supplied by the International Olive Council (IOC) and qualified with rancid (median intensity 9.5) and musty (4.7) sensory defects; 9 commercial EVOOs. To assure the wider variability of VOCs composition, the commercial EVOOs were of different provenance, and in particular from north Italy (1 sample), central Italy (1), south Italy (2), France (1), Spain (1), South Africa (1), Portugal (1) and Tunisia (1). All the samples were analyzed in triplicate.

2.3 HS-SPME-GC-MS analysis

HS-SPME-GC-MS analysis was carried out by weighing 4.3 g of sample or standard solution and 0.1 g of ISTD MIX into 20 ml screw cap vials fitted with a PTFE/silicone septa. Only for LVOO, 0.13 grams of oil were added to 4.03 grams of refined oil and 0.1 g of ISTD MIX (32-fold diluition) for taking into account their

recognized high contents of VOCs [16]. The final concentration of each ISTD into the samples was 1.74 mg/kg. As the same amount of ISTD MIX was added to samples and calibration scales, the ISTD concentration was arbitrarily set to 1 for convenience. In this way the ISTD amount ratio on the x axis in the calibration lines corresponded to the actual concentration of the analytes. Therefore the slopes of the calibration lines relative to the quantifier ion must be multiplied by 1.74 to obtain the sensitivity.

After 5 min equilibration at 60°C, SPME fiber (50/30 μm DVB/CAR/PDMS by Supelco) was exposed for 20 min in the vial headspace under orbital shaking (500 rpm). Then, the fiber was immediately desorbed for 2 min in a gas chromatograph injection port operating in splitless mode at 260°C. After each desorption a 15 min fiber backout at 260 °C was carried out in a backout unit such to avoid carryover phenomena among subsequent specimens.

2.4 Identification of volatile organic compounds

The identification of volatile compounds was performed by gas chromatography coupled to quadrupole mass spectrometry using a Trace CG-MS Thermo Fisher Scientific, equipped with a ZB-FFAP capillary column (Zebron) 30 m × 0.25 mm ID, 0.25 μm DF. Initial column temperature was held at 36°C for 10 min, then increased to 156°C at 4°C/min, then to 260°C at 10°C/min, and finally to 250°C at 10°C/min, with hold time of 2 min. Helium was used as the carrier gas at 0.8 ml/min constant flow. The temperature of both ion source and transfer line was 250°C. The mass detector was operated in scan mode within a 30 – 330 Th mass range at 1500 Th/s, with an IE energy of 70 eV.

Compounds were identified by comparison of their mass spectra and retention times with those of injected authentic standards.

2.5 Volatile organic compound quantification

Quantification was based on six point linear least squares calibration of analyte peak area over the relative ISTD peak (area ratio) plotted versus the analyte concentrations ratio (amount ratio). In order to verify the repeatability of the instrumental response on different days, for each standard compound, six replicates of a six level matrix-matched calibration curve were prepared and analyzed within 2 months. Finally,

to avoid mistakes in quantitation of VOCs due to variations of instrumental response in different days, a calibration curve was run for each analytical sequence by using the stored standard solution.

2.6 Statistical analysis

Microsoft Excel 2010 was used to building the calibration curves of 71 VOCs and to evaluate their linearity. All calibration curves were fitted to simple linear regression. Precision and accuracy of the calibration were estimated by building six different calibration curves in six different days.

2.7 Parameters for method validation

For each of the 71 analytes, limit of quantification (LOQ) was the lowest concentration level of the calibration line with precision up CV% ≤ 20% and accuracy in the 80-120% recovery range, according with literature [30]. Limit of detection (LOD) was calculated as one third of the LOQ. The upper end of the calibration was selected as the higher point with accuracy in the 80-120% recovery range and precision up CV% ≤ 20%, both calculated on the six replicates of the respective calibration levels. The linearity of the calibration was confirmed by evaluating the squared adjusted regression coefficient.

Accuracy was evaluated in terms of trueness and precision as defined in official method [31]. Both parameters were assessed by six replicates of two level spiked samples: the low concentration level was at 1.15*LOQ and the high concentration was at the penultimate point of the calibration curve. For each VOCs, mean (C_m) and standard deviation (s) from the six replicates were calculated, and C_m was compared with the reference values (C_{ref}). Precision was evaluated in terms of CV%, calculated as CV% = $s*100/C_m$, and trueness in terms of apparent recovery R, calculated as R = $(C_m*100)/C_{ref}$.

Sensitivity was assumed as the slope of the calibration straight-line multiplied by 1.74. Finally, selectivity was assured by the use of the most suitable ions for identification and quantitation of each analyte.

3. Results and discussion

3.1 Multiple internal standard normalization of VOCs quantitation

The HS-SPME-GC-MS Total Ion Current (TIC) chromatograms of the external standards solution supplemented with ISTD MIX (Figure 2A) and ISTD MIX (Figure 2B) show that the selected ISTDs are distributed all along the chromatogram at the conditions employed in this work and a complete separation of almost all the analytes was achieved for external standards and ISTDs (Figure 2A).

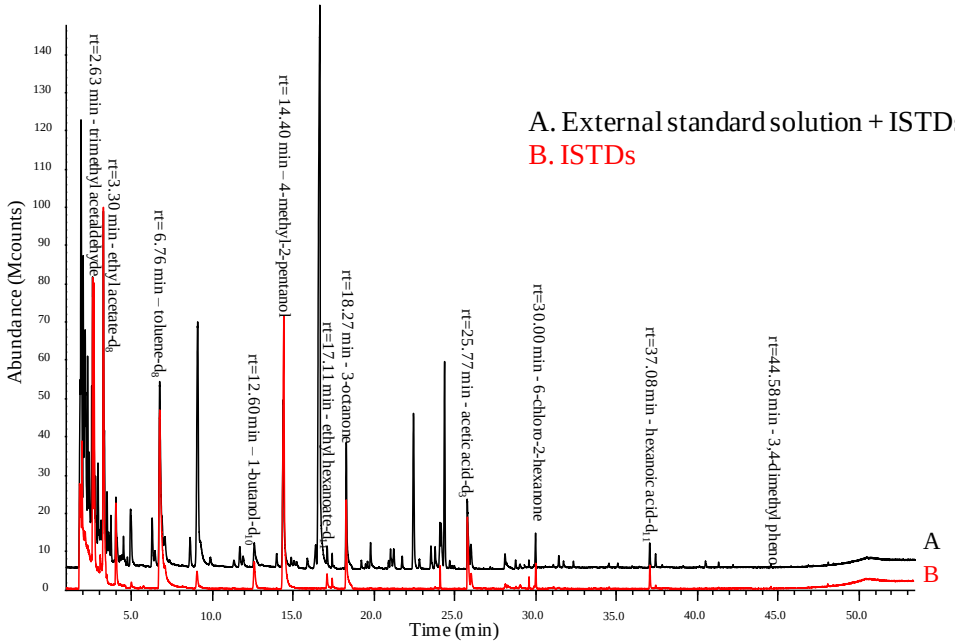

Fig 2. HS-SPME-GC-MS chromatograms of the deodorized oil supplemented (A) with ISTDs mix + STDs and (B) with ISTDs mix

When partial co-elution occurred, quantification on different target ions for the respective co-eluting compounds ensured the separation of the analytes peak without interferences. In addition, no interferences on the analytes of interest were found in the refined oil used for preparing the standard solutions in control experiments, as shown in Figure 2. All the chromatographic and mass spectrometry information concerning the quantified analytes (e.g. the selected quantifier and qualifier ions, molecular weight, chemical characteristics, retention time, the most suitable ISTD) are reported in Table 2.

Each analyte was calibrated by selecting the most appropriate ISTD. When an isotope analogue of an analyte was present among the ISTD list, it was obviously selected as the ideal ISTD. For other analytes, a trial-and-error approach was carried out with all the available ISTDs, until the best line fitting was obtained in the calibration. In this process, compounds similar by chemical classes and chromatographic retention times were initially selected. In some cases, the best linearity was obtained with apparently unsuitable ISTDs, on the basis of the previously discussed criteria.

Volatile compound	rt (min)	Internal Standard to VOC is referred for quantitation	quantifier/ qualifier ions	Range of linear calibration (mg/kg)	Slope	Intercept	R^2_{adj}	LOD	LOQ	Low Concentration Trueness (R%)	Low Concentration Precision (CV%)	High Concentration Trueness (R%)	High Concentration Precision (CV%)	Nominal mw	Chemical characteristic
heptane	2.17	1-butanol-d_{10}	100/71	0.060–0.931	1.641	0.0211	0.9985	0.018	0.060	83.7	28.1**	100.2	8.3	100	hydrocarbon
octane	2.57	toluene-d_8	85/71-114	0.097–1.519	0.1614	−0.0004	0.9975	0.029	0.097	90.1	16.8	115.2	3.8	114	hydrocarbon
methyl acetate	2.80	ethyl acetate-d_8	74/59	0.097–0.786	1.562	−0.0214	0.9937	0.029	0.097	110.3	3.2	100.4	1.8	74	ester
2-butanone	3.35	trimethylacetaldehyde	72/57	0.074–1.146	2.324	−0.1252	0.9920	0.022	0.074	82.3	5.6	70.9*	3.7	72	ketone
ethyl acetate	3.35	ethyl acetate-d_8	70/61	0.064–0.998	1.128	0.0042	0.9996	0.019	0.064	110.2	18.1	103.3	2.6	88	ester
methyl propanoate	3.62	ethyl acetate-d_8	59/88	0.023–0.359	1.563	0.0091	0.9979	0.007	0.023	100.8	8.0	101.0	1.1	88	ester
2-methyl butanal	3.74	trimethylacetaldehyde	41/57-58	0.052–0.420	0.5116	−0.0075	0.9970	0.016	0.052	91.2	11.9	78.0*	2.5	86	aldehyde
isovaleraldehyde	3.75	trimethylacetaldehyde	44/71	0.069–1.078	1.099	−0.0273	0.9963	0.021	0.069	102.9	11.8	84.2	3.2	86	aldehyde
ethyl propanoate	4.52	ethyl acetate-d_8	102/75	0.042–0.653	0.5078	−0.0035	0.9994	0.013	0.042	110.1	7.6	101.2	3.2	102	ester
3-pentanone	4.96	ethyl acetate-d_8	57/86	0.115–1.786	6.630	0.0490	0.9980	0.035	0.115	87.6	8.1	107.9	3.1	86	ketone
valeraldehyde	5.02	ethyl acetate-d_8	44/58	0.060–0.943	1.859	0.1313	0.9905	0.018	0.060	116.9	6.7	106.0	6.5	86	aldehyde
ethyl vinyl ketone	6.30	trimethylacetaldehyde	55/84	0.018–0.909	5.273	−0.2905	0.9881	0.005	0.018	69.8*	4.5	84.1	2.0	84	ketone
2-butanol	6.45	1-butanol-d_{10}	45/59	0.047–0.739	4.950	0.0771	0.9922	0.014	0.047	103.6	4.8	105.2	2.7	74	alcohol
ethyl butanoate	6.91	1-butanol-d_{10}	88/60	0.044–0.355	1.841	−0.0091	0.9987	0.013	0.044	116.2	7.3	104.8	4.9	116	ester
propanol	7.04	1-butanol-d_{10}	31/59	0.045–0.701	2.485	0.1004	0.9907	0.014	0.045	116.4	2.2	97.9	3.5	60	alcohol
buthyl acetate	8.64	1-butanol-d_{10}	43/56	0.108–0.873	4.094	−0.0564	0.9997	0.032	0.108	114.1	7.1	105.8	9.2	116	ester
hexanal	9.03	toluene-d_8	44/41	0.962–14.996	0.1016	0.0417	0.9908	0.289	0.962	123.0*	4.6	112.0	6.5	100	aldehyde
isobutanol	9.90	1-butanol-d_{10}	41/43	0.043–0.670	1.416	0.0290	0.9884	0.013	0.043	109.6	5.3	103.0	4.3	74	alcohol
2-pentanol	11.71	1-butanol-d_{10}	45/73	0.067–1.043	5.870	−0.0257	0.9993	0.020	0.067	114.3	8.0	103.1	4.1	88	alcohol
E-2-pentenal	11.88	4-methyl-2-pentanol	84/55	0.045–0.269	0.1404	−0.0014	0.9955	0.014	0.045	114.1	2.0	108.6	2.4	84	aldehyde
Z-3-hexenal	12.75	4-methyl-2-pentanol	69/98	0.087–0.514	0.0917	−0.0020	0.9945	0.026	0.087	99.6	5.5	108.5	4.0	98	aldehyde
1-penten-3-ol	14.00	1-butanol-d_{10}	57/27	0.047– 0.728	6.057	−0.0111	0.9992	0.014	0.047	102.2	6.3	111.5	4.5	86	alcohol
2-heptanone	14.84	3-octanone	58/71	0.048–0.388	14.40	0.0727	0.9971	0.014	0.048	106.5	6.6	107.7	1.1	114	ketone
heptanal	15.00	3-octanone	70/81	0.053–0.430	3.247	0.0836	0.9941	0.016	0.053	129.6*	25.7**	120.0	4.2	114	aldehyde
limonene	15.15	3-octanone	93/136-68	0.079–1.236	2.409	0.0089	0.9994	0.024	0.079	105.5	16.9	111.0	3.3	136	hydrocarbon
2-methyl-1-butanol + 3-methyl-1-butanol	16.33	1-butanol-d_{10}	57/70	0.127–1.018	0.7693	0.0187	0.9948	0.038	0.127	106.5	7.6	113.3	5.7	88	alcohol
E-2-hexenal	16.59	4-methyl-2-pentanol	83/69	0.567–56.064	9.170	0.0145	0.9992	0.170	0.567	117.6	9.9	102.9	2.8	98	aldehyde
ocimene	17.45	ethyl hexanoate-d_{11}	93/79	0.048–0.755	0.8170	0.0119	0.9935	0.014	0.048	104.9	6.5	106.0	5.3	136	hydrocarbon
pentanol	18.26	4-methyl-2-pentanol	70/55	0.049–0.433	0.1349	0.0057	0.9913	0.015	0.049	115.7	4.5	106.7	2.4	88	alcohol
hexyl acetate	19.18	4-methyl-2-pentanol	84/56	0.087–0.514	0.0156	−0.0003	0.9938	0.026	0.087	109.7	6.8	110.2	5.3	144	ester
2-octanone	19.57	3-octanone	58/71	0.067–1.039	5.899	0.1336	0.9969	0.020	0.067	110.8	7.0	112.4	2.5	128	ketone
octanal	19.76	3-octanone	84/43	0.345–5.385	1.152	0.0689	0.9962	0.104	0.345	117.8	9.5	111.2	8.1	128	aldehyde
1-octen-3-one	20.29	3-octanone	70/55	0.056 – 0.878	1.348	0.0230	0.9998	0.017	0.056	107.0	11.3	103.6	3.7	126	ketone
E-2-pentenol	20.89	4-methyl-2-pentanol	57/86	0.043–0.251	0.2471	−0.0032	0.9926	0.013	0.043	104.7	7.2	113.4	4.6	86	alcohol
Z-3-hexenyl-acetate	21.00	4-methyl-2-pentanol	82/67	0.187–1.106	0.0524	−0.0025	0.9923	0.056	0.187	116.6	6.1	97.9	5.4	141	ester
E-2-heptenal	21.06	3-octanone	83/70	0.083–1.288	1.869	0.0453	0.9978	0.025	0.083	112.1	6.4	106.8	3.7	112	aldehyde
2-heptanol	21.17	4-methyl-2-pentanol	57/68	0.061–0.362	0.1932	−0.0022	0.9960	0.018	0.061	111.3	6.2	112.9	4.6	116	alcohol
Z-2-pentenol	21.19	4-methyl-2-pentanol	45/55	0.067–0.393	0.2347	−0.004	0.9969	0.020	0.067	101.4	4.5	111.3	6.6	86	alcohol
E-2-hexenyl-acetate	21.72	4-methyl-2-pentanol	100/82	0.072–0.427	0.0101	−0.000233	0.9902	0.022	0.072	102.3	9.6	112.7	4.6	141	ester
6-methyl-5-hepten-2-one	21.73	3-octanone	108/69	0.081–1.270	1.897	0.0472	0.9944	0.024	0.081	116.0	13.0	102.7	2.8	126	ketone
hexanol	22.42	4-methyl-2-pentanol	56/69	0.633–3.739	0.2765	−0.0391	0.9960	0.190	0.633	111.9	7.2	103.1	2.8	102	alcohol
E-3-hexenol	22.77	4-methyl-2-pentanol	67/82	0.050–0.293	0.1019	−0.0014	0.9934	0.015	0.050	109.8	5.9	109.7	5.1	100	alcohol
Z-3-hexenol	23.49	4-methyl-2-pentanol	67/82	0.148–0.876	0.1047	−0.0048	0.9943	0.044	0.148	103.1	6.2	112.2	4.6	100	alcohol
2-nonanone	23.58	3-octanone	58/43	0.074–1.155	2.574	−0.0099	0.9995	0.022	0.074	108.8	5.7	99.8	1.2	142	ketone
nonanal	23.76	3-octanone	57/98	0.835–13.010	0.7212	0.2148	0.9973	0.251	0.835	117.2	16.1	105.7	3.3	142	aldehyde
2,4-hexadienal	24.14	3-octanone	81/69	0.109–0.877	33.05	0.9345	0.9948	0.033	0.109	104.9	13.4	99.1	1.5	96	aldehyde
E-2-hexenol	24.18	4-methyl-2-pentanol	57/82	0.956–5.644	0.2327	−0.0597	0.9960	0.287	0.956	110.6	6.9	106.9	4.3	100	alcohol
Z-2-hexenol	24.32	4-methyl-2-pentanol	57/82	0.048–0.285	0.1151	−0.0013	0.9927	0.014	0.048	99.7	9.1	108.2	5.9	100	alcohol
2-octanol	24.33	4-methyl-2-pentanol	45/55	0.047–0.275	0.0635	−0.0006	0.9922	0.014	0.047	101.3	6.9	112.0	5.8	130	alcohol
E-2-octenal	25.00	3-octanone	70/83	0.080–1.240	0.7091	0.0065	0.9997	0.024	0.080	125.3*	19.3	112.0	3.2	126	aldehyde
1-octen-3-ol	25.86	4-methyl-2-pentanol	57/72	0.054–0.320	0.1289	−0.0017	0.9938	0.016	0.054	105.6	7.9	119.6	6.6	128	alcohol
heptanol	26.00	4-methyl-2-pentanol	70/56	0.048–0.283	0.0378	−0.0003	0.9911	0.014	0.048	113.4	6.6	113.7	5.1	116	alcohol
2,4-heptadienal	27.18	3-octanone	81/110	0.070–1.096	1.791	0.0061	0.9991	0.021	0.070	108.6	8.0	103.5	7.3	110	aldehyde
decanal	27.32	3-octanone	82/70	0.454–2.243	0.0387	0.0065	0.9688	0.136	0.454	111.6	13.6	102.1	4.8	156	aldehyde
benzaldehyde	28.11	3-octanone	105/77	0.074–1.155	4.398	0.1770	0.9974	0.022	0.074	101.6	9.3	109.8	2.6	106	aldehyde
E-2-nonenal	28.43	3-octanone	70/83	0.053–0.828	0.1882	0.0060	0.9850	0.016	0.053	113.8	9.0	102.2	4.2	140	aldehyde
propanoic acid	28.76	4-methyl-2-pentanol	74/57	0.085–0.745	0.0861	−0.00006	0.9909	0.026	0.085	95.2	12.1	102.7	2.5	74	carboxylic acid
octanol	29.30	4-methyl-2-pentanol	70/56	0.047–0.280	0.0074	−0.00002	0.9979	0.014	0.047	100.7	3.3	101.0	3.4	130	alcohol
butanoic acid	31.42	acetic acid-2,2,2-d_3	60/73	0.097–1.519	0.4753	0.0153	0.9987	0.029	0.097	100.1	6.5	106.3	3.5	88	carboxylic acid
E-2-decenal	31.73	3-octanone	70/83	0.659–10.280	0.1331	−0.0231	0.9978	0.198	0.659	99.2	6.7	101.6	6.5	154	aldehyde
nonanol	32.33	4-methyl-2-pentanol	56/98	0.295–1.743	0.0074	−0.0007	0.9869	0.089	0.295	99.7	5.8	110.5	8.7	144	alcohol
2,4-nonadienal	33.43	3-octanone	81/138	0.080–1.250	0.2328	−0.00002	0.9958	0.024	0.080	115.2	15.4	101.0	4.1	138	aldehyde
pentanoic acid	34.54	hexanoic-d_{11} acid	60/73	0.087–0.223	1.145	0.0249	0.9943	0.026	0.087	141.7*	38.7**	81.3	28.1**	102	carboxylic acid
2,4-decadienal	36.42	6-chloro-2-hexanone	81/152	0.430–6.698	0.0781	−0.0098	0.9983	0.129	0.430	99.5	11.3	108.2	8.2	152	aldehyde
hexanoic acid	37.43	hexanoic-d_{11} acid	60/73	0.117–0.944	0.8508	0.0828	0.9799	0.035	0.117	153.2*	48.9**	97.5	17.5	116	carboxylic acid
guaiacol	37.83	3,4-dimethylphenol	109/124	0.112–1.746	4.2283	−0.0132	0.9992	0.034	0.112	109.3	9.2	104.7	9.1	124	phenol
phenyl ethanol	39.14	3,4-dimethylphenol	91/122	0.111–1.735	4.333	0.0410	0.9980	0.033	0.111	112.7	9.0	105.6	7.1	122	Pieno
phenol	41.33	3,4-dimethylphenol	94/66	0.107–0.861	7.646	−0.7933	0.9976	0.032	0.107	126.0*	16.9	106.2	8.7	94	Pieno
4-ethylguaiacol	42.40	3,4-dimethylphenol	137/152	0.316–1.562	0.7385	−0.0474	0.9960	0.095	0.316	99.5	10.0	99.8	3.0	152	Pieno
4-ethyl-phenol	43.97	3,4-dimethylphenol	107/122	0.095–1.509	1.057	0.0128	0.9965	0.029	0.095	108.6	12.2	102.3	7.5	122	Pieno

rt, retention time; R^2_{adj}, squared adjusted regression coefficient; mw, molecular weight. Sensitivity is the slope multiplied by 1.74.
* Values of accuracy out of the range 80–120%
** Values of precision over to 20%.

Table 2. Quality parameters for method validation

The effect of the selection of a different ISTD or no ISTD for calibration is reported, for example, in Figure 3 and in Figure 4 for *E*-2-hexenyl acetate and *E*-2-hexenal, respectively. In the case of *E*-2-hexenyl acetate, when the scale at the highest concentration was excluded from the regression, the calibrations were linear in almost all cases, even in the case of external standard calibration (Figure 3, last pane, bottom-right, NO ISTD). On the other hand, when the highest concentration was considered, a linear calibration was obtained only in the case of a normalization with 3,4-dimethylphenol as

ISTD. This behavior was evidently the results of a partial saturation of the SPME fiber, and/or from analyte–to–analyte interactions. In this respect, a normalization with an ISTD whose behavior was almost identical to the analyte of interest, was capable of correcting these departures from linearity, as in the case of 3,4-dimethylphenol for *E*-2-hexenyl acetate (Figure 3). However, the range of concentration of this analyte in the VOOs never exceeds the amount of the penultimate point of the calibration curves. For this reason, and taking into account that, including the last point in the calibration, no ISTD gave an R^2 of at least 0.99, the choice of the ISTD for this analyte was 4-methyl-2-pentanol: in fact, by excluding the last point, its R^2 was highest to 0.99, its rt was near those of the analyte and its intercept was almost zero.

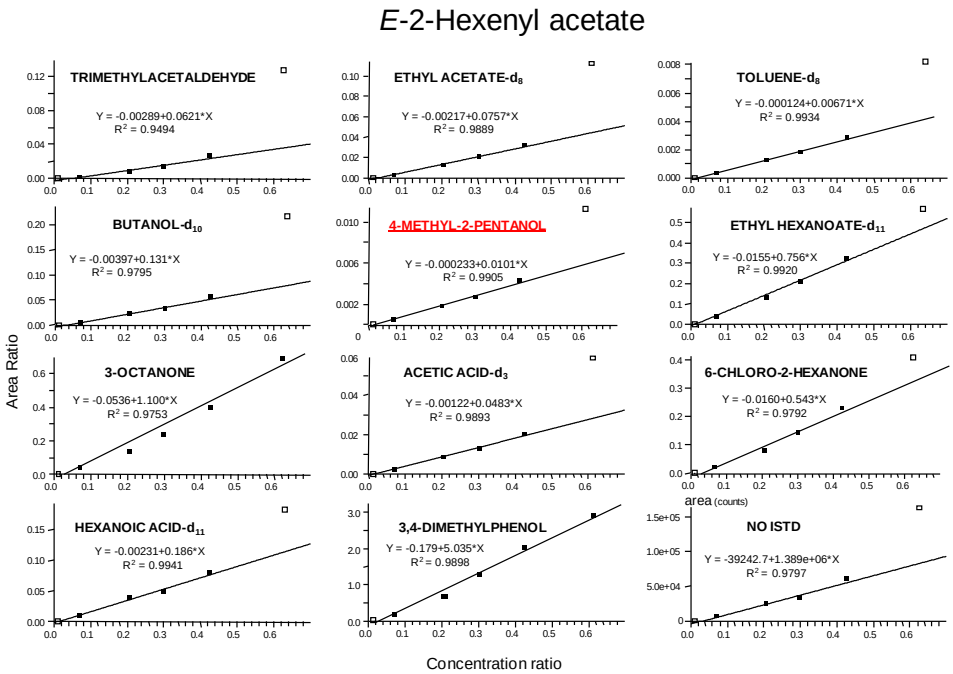

Figure 3. Calibration curves for *E*-2-hexenyl acetate with each of the ISTDs and without ISTD. For each ISTD, the last point of calibration was excluded if its exclusion gave the highest R^2; the excluded points are indicated by the unfilled square. The chosen ISTD is underlined

In the case of *E*-2-hexenal, a linear calibration over the entire calibration range was obtained, in addition to the normalization with 4-methyl-2-pentanol, toluene-d_8 or hexanoic acid-d_{11}, also without ISTD (Figure 4, last pane, bottom-right, NO ISTD). This probably occurred since *E*-2-hexenal is by far the major VOC of VOOs and it was only minimally affected by the competition of other VOCs for sorption over the fiber. On the other hand, the normalization with other ISTDs resulted in worst line fitting also in comparison with the external standard calibration (figure 4, NO ISTD). In Figure 4, the last point was excluded if, without it, a better calibration curve was obtained, and in this

case the excluded point was indicated by an empty symbol. It is evident that the choice of a correct ISTD for data normalization seems to be critical for a correct quantitation in a wide working range, and therefore the choice of a wrong ISTD could cause an inexact quantification of the VOCs in VOOs. The most suitable ISTD for *E*-2-hexenal was 4-methyl-2-pentanol, in fact its R^2 was the highest, rt was near that of the analyte and its intercept was close to zero.

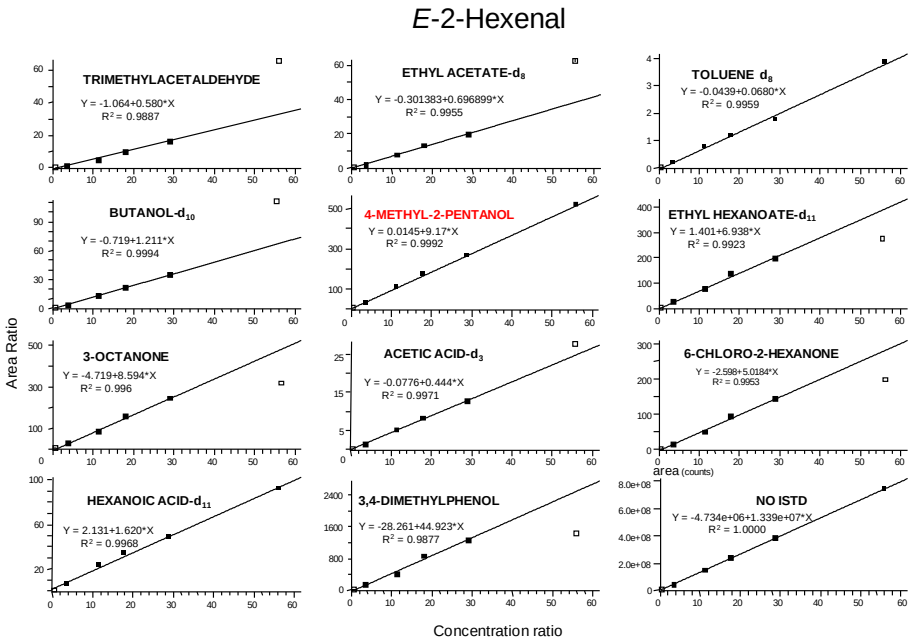

Figure 4. Calibration curves for *E*-2-hexenal with each of the ISTDs and without ISTD. For each ISTD, the last point of calibration was excluded if its exclusion gave the highest R^2; the excluded points are indicated by the unfilled square. The chosen ISTD is underlined

The effect of normalization on a single ISTD (i.e. 4-methyl-2-pentanol) for 12 VOCs, selected on the basis of the chemical classes present in the volatile profile of the VOOs, is reported in Figure 5. It is evident that a successful data normalization was analyte-dependent. In fact, the calibration was linear in all cases only for the lower concentrations, while the scale at the highest concentration was linearized only for some of the selected compounds (e.g. hexanoic acid, butyl acetate, 2-butanone, *E*-2-hexenal). Moreover, for some analytes, the intercept was far from zero, resulting in significant errors in quantifying the lower concentrations. These evidences indicate that a proper ISTD must be selected depending on the analytes (on the basis of their R^2, and intercept near to zero) and that the use of a single ISTD for the normalization of all analytes in a HS-SPME-GC-MS quantification suffers from serious limitations. These evidences suggest the need for a multiple internal standard normalization within the VOOs profile.

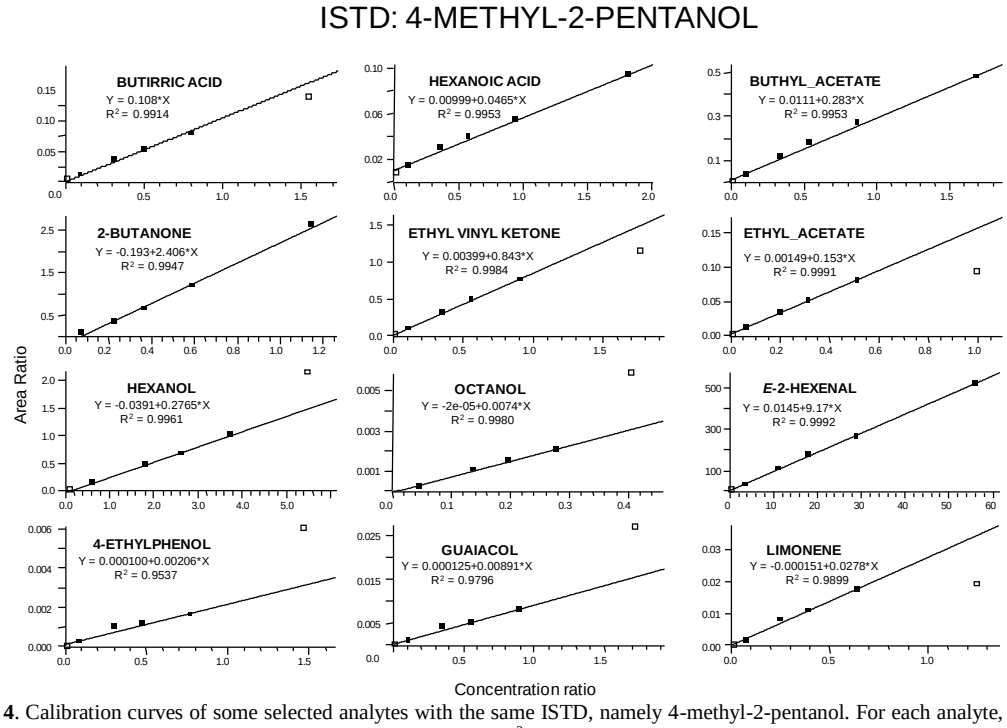

Figure 4. Calibration curves of some selected analytes with the same ISTD, namely 4-methyl-2-pentanol. For each analyte, the last point of calibration was excluded if its exclusion gave the highest R^2; the excluded points are indicated by the unfilled square.

On the other hand, the comparison between internal standards vs external standard approaches is shown in Figure 6. Calibration of a 12 case-study VOO-VOCs selected from different compound classes after the normalization over the most appropriate ISTD was compared to the calibration following the external standard approach. Most of the selected compounds showed a larger linear calibration range when normalized on the proper ISTD, in comparison with the external standard calibration.

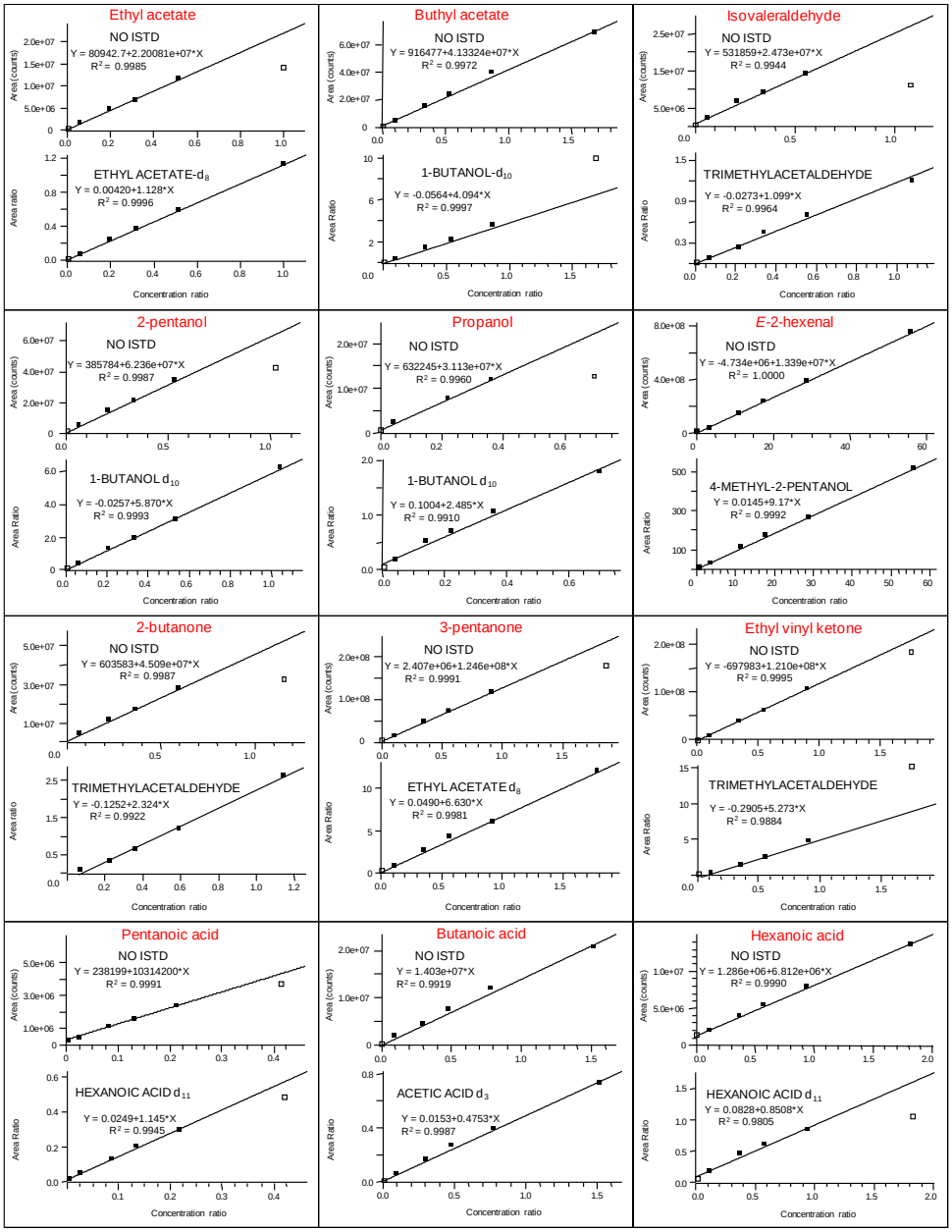

Figure 5. Comparison of the calibration curves obtained without ISTD or with the most suitable ISTD for some selected analytes. The last point of calibration was excluded if its exclusion gave the highest R^2; the excluded points are indicated by the unfilled square.

This clearly indicates that at the highest concentration the SPME fiber was sorbing the analytes in saturated to oversaturated conditions, resulting in a breakdown of the linearity of responses. However, the normalization with ISTD exhibiting the same behavior was capable of compensating these biases.

For the most represented VOCs in Tuscan VOOs, e.g. *E*-2-hexenal, a quantification without the aid of ISTD gave satisfactory results, as reported in Figure 3. This probably occurred because the sorption of this compound onto the SPME fiber suffers of the competition of the others only minimally, since its concentration in the

calibration scales was about 33% of the total compound amount (see also Table 2). However, this was not the case for most of the other VOCs, whose concentration was at lower levels; in these cases the use of a normalization over a suitable ISTD, is mandatory for a correct quantitation. These evidences again indicate that the selection of the most suitable ISTD is a key step in obtaining linear calibration lines over a wide working range.

3.2 Method validation

The linearity of the calibration range, the LOD and LOQ, the accuracy at low and high concentration levels, estimated as trueness and precision, the sensitivity and selectivity of the method were assessed as described in the previous paragraph 2.7. All the calibration parameters obtained for all analytes are summarized in Table 2.

3.2.1 Linearity of the working range, LOD and LOQ

Linear ranges of calibration as wide as 10 - 100 fold with respect to the LOQ were obtained for almost all the considered analytes, albeit, narrower ranges were obtained for some of them (e.g pentanoic acid, decanal, 4-ethylguaiacol). The linearity of the working range, defined based on the criteria described in paragraph 2.7, was confirmed by the squared adjusted regression coefficient, which was higher than 0.95 for all the 71 analytes. Regarding the LOQ, the highest values were obtained for aldehydes; however, values minor to 1 mg/kg were obtained even for the analytes present at the higher concentrations.

3.2.2 Accuracy

Table 2 shows trueness and precision at low and high concentration levels for all the 71 analytes, estimated as described in paragraph 2.7. At high concentration, trueness was assessed for 69 analytes, with R% within the range 80-120%, and precision was assessed with CV% < 20% for all the analytes, the only exception being pentanoic acid. At low concentration, trueness was assessed for 64 analytes, which R% within the range 80-120%; values within the range 70-130% were obtained for phenol, ethyl-vinyl-ketone and three aldehydes (hexanal, heptanal and *E*-2-octenal), while values out of this range were obtained only for pentanoic and hexanoic acid. Regarding precision, CV% was < 20% for 67 analytes; the highest values (CV% > 30%) was obtained again for pentanoic and hexanoic acid. This behavior was probably related to both the poor volatility and the affinity for the oily matrix of these carboxylic acids, owing to the length of their

hydrophobic tail, since no problems were observed for the carboxylic acids with a shorter chain.

3.2.3 Sensitivity and selectivity

Sensitivity is defined as "the change in the response of a measuring instrument divided by the corresponding change in the amount of the measurand" [32]. The highest values of sensitivity, in terms of slope of the calibration straight-lines multiplied by 1.74, were obtained for carbonyl compounds as 2,4-hexadienal, 2-heptanone and *E*-2-hexenal (one of the main volatiles of the high quality EVOO), while the lowest values were obtained for octanol and nonanol.

Selectivity is defined as "the ability of the method to accurately and specifically determine an analyte of interest in the presence of other components in a sample matrix under the stated condition of the text" [32]. This method allows to analyze up to 71 VOCs; as a consequence, a good selectivity is necessary for a correct identification and quantitation of all the analytes. The identification and quantitation of each VOCs were based on different target ions for each compounds (Table 2). This approach allowed the unique identification of all the analytes, even if the retention times were very similar, as clearly shown in Supplementary Figure B. The only exception was for 2-methyl-1-butanol and 3-methyl-1-butanol, due to their very similar chemical nature and mass spectra. As a consequence, these two analytes were quantified as sum of their amounts.

3.3 Analysis of different EVOOs and defected LVOOs

Several commercial EVOOs of different provenance (nine samples) and two defected LVOOs (qualified with rancid and musty defects, respectively) were analyzed with the proposed method. All the analytes were present in at least one of the analyzed samples (Table 3), with the only exception for ethyl propanoate, 2-butanol and 3-butanol, confirming the complexity of the volatile fraction of VOOs.

As expected, the volatile profiles of the analyzed LVOO samples were very different from EVOOs [16,28]. In general, the total VOCs concentrations of LVOOs (41.69 and 22.64 mg/kg for the rancid and musty samples, respectively) were much higher than EVOOs (average values of 21.91 mg/kg), also in spite of the LVOOs dilution during sample preparation (see also paragraph 2.3).

VOC	Lampante virgin olive oils		Commercial extra virgin olive oils								
	Rancid	Musty	Northern Italy	Central Italy	Southern Italy	Southern Italy	France	Spain	South Africa	Portugal	Tunisia
heptane	0.93 ± 0.08	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
octane	1.50 ± 0.09	0.14 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd	0.18 ± 0.03
methyl acetate	0.04 ± 0.002	0.31 ± 0.01	0.06 ± 0.001	0.03 ± 0.001	0.04 ± 0.002	0.03 ± 0.001	0.03 ± 0.002	0.05 ± 0.004	0.31 ± 0.02	0.11 ± 0.01	0.18 ± 0.01
2-butanone	nd	0.03 ± 0.001	0.06 ± 0.002	0.06 ± 0.01	0.06 ± 0.002	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
ethyl acetate	nd	0.26 ± 0.01	nd	0.03 ± 0.01	0.04 ± 0.001	nd	nd	0.03 ± 0.004	0.03 ± 0.002	0.41 ± 0.04	0.57 ± 0.04
methyl propanoate	0.01 ± 0.001	0.01 ± 0.001	0.013 ± 0.001	0.014 ± 0.001	0.015 ± 0.001	0.017 ± 0.001	0.019 ± 0.001	0.011 ± 0.001	0.011 ± 0.001	0.024 ± 0.002	0.021 ± 0.001
2-methyl butanal	nd	nd	0.03 ± 0.01	0.14 ± 0.02	0.04 ± 0.002	0.03 ± 0.001	0.09 ± 0.01	0.07 ± 0.01	0.08 ± 0.04	0.06 ± 0.01	0.13 ± 0.02
isovaleraldehyde	nd	nd	nd	nd	nd	nd	nd	nd	0.03 ± 0.002	nd	0.07 ± 0.01
ethyl propanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3-pentanone	nd	0.12 ± 0.01	0.09 ± 0.01	0.28 ± 0.03	0.16 ± 0.001	0.11 ± 0.02	0.18 ± 0.01	0.20 ± 0.03	0.14 ± 0.02	0.42 ± 0.04	0.27 ± 0.04
valeraldehyde	*1.10 ± 0.08*	*1.56 ± 0.07*	0.16 ± 0.01	0.17 ± 0.04	0.22 ± 0.04	0.20 ± 0.01	0.20 ± 0.01	0.14 ± 0.02	0.13 ± 0.02	0.26 ± 0.03	0.30 ± 0.01
ethyl vinyl ketone	0.01 ± 0.001	0.01 ± 0.001	0.56 ± 0.06	0.46 ± 0.05	0.68 ± 0.02	0.50 ± 0.08	0.34 ± 0.03	0.53 ± 0.05	0.57 ± 0.04	0.23 ± 0.02	0.13 ± 0.01
2-butanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ethyl butanoate	0.02 ± 0.001	0.02 ± 0.001	0.05 ± 0.01	0.05 ± 0.001	0.06 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.06 ± 0.001	0.04 ± 0.001
propanol	nd	0.03 ± 0.001	nd	nd	nd	nd	nd	nd	nd	0.04 ± 0.002	0.03 ± 0.003
buthyl acetate	0.07 ± 0.01	0.07 ± 0.01	0.16 ± 0.02	0.21 ± 0.01	0.23 ± 0.02	0.25 ± 0.01	0.16 ± 0.02	0.18 ± 0.01	0.24 ± 0.02	0.13 ± 0.01	0.26 ± 0.01
hexanal	5.16 ± 0.31	5.06 ± 0.20	nd	nd	0.31 ± 0.10	0.73 ± 0.10	nd	nd	0.69 ± 0.23	nd	1.01 ± 0.19
isobutanol	0.04 ± 0.003	0.03 ± 0.004	nd	nd	nd	nd	nd	nd	nd	nd	0.12 ± 0.01
2-pentanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
***E*-2-pentenal**	0.05 ± 0.002	0.13 ± 0.01	0.16 ± 0.03	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.02	0.14 ± 0.01	0.09 ± 0.01	0.07 ± 0.01
***Z*-3-hexenal**	nd	0.03 ± 0.001	0.50 ± 0.05	0.39 ± 0.02	0.55 ± 0.12	0.47 ± 0.04	0.21 ± 0.01	0.32 ± 0.02	0.53 ± 0.04	0.27 ± 0.02	0.16 ± 0.01
1-penten-3-ol	0.02 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.55 ± 0.04	0.67 ± 0.03	0.62 ± 0.04	0.34 ± 0.04	0.50 ± 0.06	0.50 ± 0.05	0.27 ± 0.03	0.25 ± 0.02
2-heptanone	*1.49 ± 0.12*	0.01 ± 0.004	nd	nd	nd	nd	nd	nd	nd	nd	nd
heptanal	*2.06 ± 0.16*	0.28 ± 0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd
limonene	nd	0.03 ± 0.004	nd	nd	nd	nd	0.03 ± 0.01	nd	0.11 ± 0.01	nd	nd
2-methyl-1-butanol+ 3-methyl-1-butanol	nd	nd	0.04 ± 0.001	0.05 ± 0.01	0.05 ± 0.001	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.13 ± 0.01
***E*-2-hexenal**	nd	0.17 ± 0.03	11.34 ± 1.75	8.61 ± 0.74	16.43 ± 0.63	11.00 ± 0.83	4.46 ± 0.42	3.07 ± 0.41	2.17 ± 0.13	5.62 ± 0.44	3.47 ± 0.15
ocimene	nd	0.02 ± 0.001	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.003	0.03 ± 0.01	0.03 ± 0.01	nd	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
pentanol	0.16 ± 0.01	0.05 ± 0.002	nd	0.02 ± 0.001	0.02 ± 0.001	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
hexyl acetate	0.04 ± 0.002	0.07 ± 0.003	0.13 ± 0.01	0.28 ± 0.02	0.11 ± 0.003	0.10 ± 0.01	0.41 ± 0.03	0.14 ± 0.01	0.09 ± 0.01	0.15 ± 0.01	0.11 ± 0.01
2-octanone	0.46 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
octanal	3.20 ± 0.15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-octen-3-one	0.03 ± 0.003	0.06 ± 0.004	0.11 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.10 ± 0.05	0.04 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
***E*-2-pentenol**	nd	nd	0.19 ± 0.02	0.25 ± 0.02	*0.27 ± 0.01*	*0.33 ± 0.02*	0.16 ± 0.01	*0.31 ± 0.03*	0.24 ± 0.02	0.14 ± 0.01	0.09 ± 0.01
***Z*-3-hexenyl-acetate**	0.11 ± 0.01	0.19 ± 0.01	0.35 ± 0.04	0.86 ± 0.08	0.29 ± 0.01	0.51 ± 0.01	*2.17 ± 0.20*	1.09 ± 0.07	0.28 ± 0.02	0.77 ± 0.06	0.29 ± 0.01
***E*-2-heptenal**	0.41 ± 0.04	0.45 ± 0.05	nd	0.03 ± 0.01	0.06 ± 0.01	nd	nd	nd	nd	nd	0.34 ± 0.02
2-heptanol	0.03 ± 0.001	0.02 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.01
***Z*-2-pentenol**	nd	nd	0.08 ± 0.004	0.12 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
***E*-2-hexenyl-acetate**	nd	0.02 ± 0.001	0.07 ± 0.01	0.09 ± 0.01	0.07 ± 0.001	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
6-methyl-5-hepten-2-one	nd	*2.76 ± 0.14*	nd	nd	nd	nd	nd	nd	nd	nd	nd
hexanol	0.17 ± 0.02	nd	0.45 ± 0.02	0.64 ± 0.02	0.48 ± 0.01	0.49 ± 0.01	0.45 ± 0.01	0.51 ± 0.02	0.36 ± 0.01	0.74 ± 0.04	0.53 ± 0.02
***E*-3-hexenol**	nd	nd	0.04 ± 0.001	0.05 ± 0.01	0.04 ± 0.001	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	0.04 ± 0.01
***Z*-3-hexenol**	nd	0.05 ± 0.001	0.50 ± 0.05	*0.98 ± 0.05*	0.47 ± 0.02	0.69 ± 0.03	0.84 ± 0.07	*2.24 ± 0.18*	0.44 ± 0.04	*1.93 ± 0.16*	0.32 ± 0.03
2-nonanone	1.09 ± 0.10	nd	0.07 ± 0.04	0.23 ± 0.02	0.14 ± 0.01	0.11 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	nd	0.36 ± 0.01	0.06 ± 0.04
nonanal	2.65 ± 0.22	2.43 ± 0.35	4.22 ± 0.52	11.15 ± 0.92	6.68 ± 0.19	4.94 ± 0.12	2.22 ± 0.20	1.66 ± 0.24	0.95 ± 0.09	*17.50 ± 0.63*	5.66 ± 0.46
2,4-hexadienal	nd	nd	0.48 ± 0.06	0.41 ± 0.05	0.77 ± 0.05	0.49 ± 0.01	0.16 ± 0.03	0.28 ± 0.06	0.37 ± 0.06	0.23 ± 0.02	0.06 ± 0.01
***E*-2-hexenol**	0.26 ± 0.01	0.29 ± 0.01	0.65 ± 0.02	0.97 ± 0.02	0.72 ± 0.01	0.70 ± 0.01	0.57 ± 0.01	0.55 ± 0.01	0.53 ± 0.01	1.22 ± 0.03	0.71 ± 0.02
***Z*-2-hexenol**	nd	nd	0.03 ± 0.001	0.05 ± 0.01	0.04 ± 0.001	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
2-octanol	0.02 ± 0.001	nd	0.04 ± 0.002	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
***E*-2-octenal**	0.92 ± 0.13	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-octen-3-ol	0.08 ± 0.01	nd	0.04 ± 0.001	0.05 ± 0.01	0.04 ± 0.001	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
heptanol	0.17 ± 0.01	0.02 ± 0.003	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.001	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
2,4-heptadienal	0.71 ± 0.06	0.24 ± 0.01	nd	nd	nd	0.06 ± 0.01	0.18 ± 0.03	nd	0.07 ± 0.01	nd	nd
decanal	*3.32 ± 0.44*	0.62 ± 0.08	nd	nd	nd	0.31 ± 0.07	0.74 ± 0.06	nd	0.27 ± 0.06	nd	nd
benzaldehyde	nd	0.08 ± 0.01	0.03 ± 0.002	0.04 ± 0.01	0.06 ± 0.01	nd	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
***E*-2-nonenal**	0.66 ± 0.12	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	nd	nd	0.03 ± 0.01	0.15 ± 0.02	nd	0.06 ± 0.01
propanoic acid	*0.86 ± 0.07*	*0.82 ± 0.07*	nd	0.04 ± 0.03	nd	nd	nd	nd	nd	nd	nd
octanol	0.15 ± 0.02	0.03 ± 0.003	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.002	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
butanoic acid	1.31 ± 0.15	0.18 ± 0.01	nd	0.03 ± 0.01	0.03 ± 0.002	nd	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
***E*-2-decenal**	1.02 ± 0.26	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
nonanol	0.35 ± 0.01	0.16 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.25 ± 0.02	0.25 ± 0.05	0.26 ± 0.04	0.25 ± 0.01	0.26 ± 0.03	0.28 ± 0.02
2,4-nonadienal	0.17 ± 0.03	0.12 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.11 ± 0.01	0.03 ± 0.01	0.07 ± 0.01
pentanoic acid	*2.65 ± 0.45*	*0.48 ± 0.03*	nd	nd	nd	nd	nd	nd	nd	nd	nd
2,4-decadienal	0.54 ± 0.22	nd	0.12 ± 0.01	0.14 ± 0.03	nd	nd	0.13 ± 0.01	nd	0.16 ± 0.03	nd	nd
hexanoic acid	*7.47 ± 1.13*	*1.16 ± 0.23*	nd	nd	nd	nd	nd	nd	nd	nd	0.04 ± 0.02
guaiacol	nd	0.54 ± 0.03	nd	nd	nd	nd	nd	nd	nd	nd	nd
phenyl ethanol	nd	0.31 ± 0.02	0.14 ± 0.02	0.31 ± 0.02	0.14 ± 0.02	0.16 ± 0.01	0.28 ± 0.07	0.12 ± 0.02	0.11 ± 0.01	0.14 ± 0.02	0.22 ± 0.02
phenol	nd	0.22 ± 0.01	0.03 ± 0.03	0.10 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.06 ± 0.01
4-ethylguaiacol	nd	0.35 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd
4-ethyl-phenol	nd	*2.14 ± 0.16*	nd	nd	nd	nd	nd	nd	nd	nd	0.07 ± 0.02
Total VOCs	41.69 ± 4.560	22.64 ± 1.70	21.83 ± 2.92	28.94 ± 2.45	31.22 ± 1.43	24.16 ± 1.64	15.70 ± 1.53	13.96 ± 1.95	11.00 ± 1.21	32.69 ± *1.87*	17.70 ± 1.48

Table 2. Concentration of the 71 volatiles quantified in the 11 analyzed samples, expressed as mean ± standard deviation of triplicates. Values higher than the upper end of the working range are in italic.

The most abundant volatiles in the mustiness LVOO were hexanal and 6-methyl-5-hepten-2-one, while the LVOO qualified as rancid differed from the EVOO samples for the high content of octane, carboxylic acids (mainly hexanoic acid) and carbonyl compounds, such as saturated C6-C10 aldehydes, in agreement with the data reported in literature [16]. For these defected LVOOs, the 32-fold dilution prevented the detection of some of the least concentrated analytes, which can, of course, be quantitated by simply repeating the analysis at lower dilution.

The analyzed EVOO samples differed from the LVOO samples for the higher concentration of C5-C6 LOX-pathway related compounds: *E*-2-hexenal, which is associated to green aroma [28], was absent in the rancid specimen, while in EVOOs concentrations of 2.17-16.42 (average of 7.35) mg/kg were retrieved. For ethyl-vinyl-

ketone, which is responsible for green/pungent aroma [4], a range of 0.13-0.68 mg/kg was measured in the EVOO samples, while only trace amounts were present in LVOO specimens.

These data confirmed again the need for wide working ranges for VOCs profile quantitation and highlighted the ability of this method to discriminate among VOOs of different characteristics.

4. Conclusions

The proposed multiple internal standard normalization for improving HS-SPME-GC-MS quantitation of VOO-VOCs allows to investigate a large number of the main volatile compounds responsible for positive and negative attributes in VOOs. The method requests the initial preparation of the standard solutions (which can be frozen and easily stored over time at -20°C) and the optimization of the quantitation method; after these steps, it allows a reliable quantitation of VOCs profile in VOOs of different characteristic in routine analysis.

Calibrations with good linearity and wide concentration range were obtained for each analyte after data normalization with a suitable ISTD. Linear ranges as wide as 10-100 fold of the LOQ were obtained, such as to allow an easy quantitation of the volatile compounds in most of the commercially available VOOs. Good linearity of the quantitation without the use of ISTD was found only for *E*-2 hexenal, i.e. the most represented VOC in VOOs. Linearity of the calibration in narrower ranges was achieved without the use of ISTD, confirming the need to use ISTD for an exact quantitation of a larger number of analytes. The approach proposed in this paper gives the possibility of recalibration the calibration curve with the suitable ISTD for each analyte, thus improving the versatility of the analytical method.

The proposed method was validated for 71 VOCs responsible for positive or negative sensory perceptions, and then applied to a series of LVOOs and EVOOs. The results showed a wide variability of VOCs profile from both qualitative and quantitative standpoint, confirming the usefulness of wide linear working ranges of quantitation in the analytical methods. Finally, the method was able to quantify the main VOCs responsible for the defectiveness in LVOO and for green attributes of EVOO.

In this way, this method can be used to support sensorial analysis for the evaluation of positive and negative attributes of VOOs, aimed at the creation of a objective tool for the classification of the VOOs.

Future developments are possible for this methodology with the use of larger number on isotopologues and with the use of SPME fibers of larger capacity.

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HS-SPME-GC-MS analysis towards supporting the panel test: quantitation of VOCs in Virgin Olive Oils

Cecchi, L.[a], Guariglia, C.,[a] Migliorini, M.[b], Giambanelli, E.,[b] Rossetti, A.[b], Cane, A.[b], Calamai, L.[c], Mulinacci, N.[a]

a. Dipartimento di NEUROFARBA, Università degli Studi di Firenze
b. CARAPELLI Firenze S.p.A.
c. Dipartimento di Gestione dei Sistemi Agrari, Alimentari e Forestali, Università degli Studi di Firenze

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In the next pages there is a summary of the results presented as oral presentation at the 16th EuroFedLipid Congress: Science, Technology and Nutrition in a Changing world. September 16-19th 2018, Belfast, United Kingdom. Book of Abstract, 82

1. INTRODUCTION

The importance of virgin olive oil classification is well known: it can be classified as extra virgin, virgin, ordinary or lampante virgin olive oil. These categories have very different commercial value, in that, for example, extravirgin olive oil has the highest commercial value, while lampante virgin olive oil is not edible as it is, but need to be refined to render it edible.

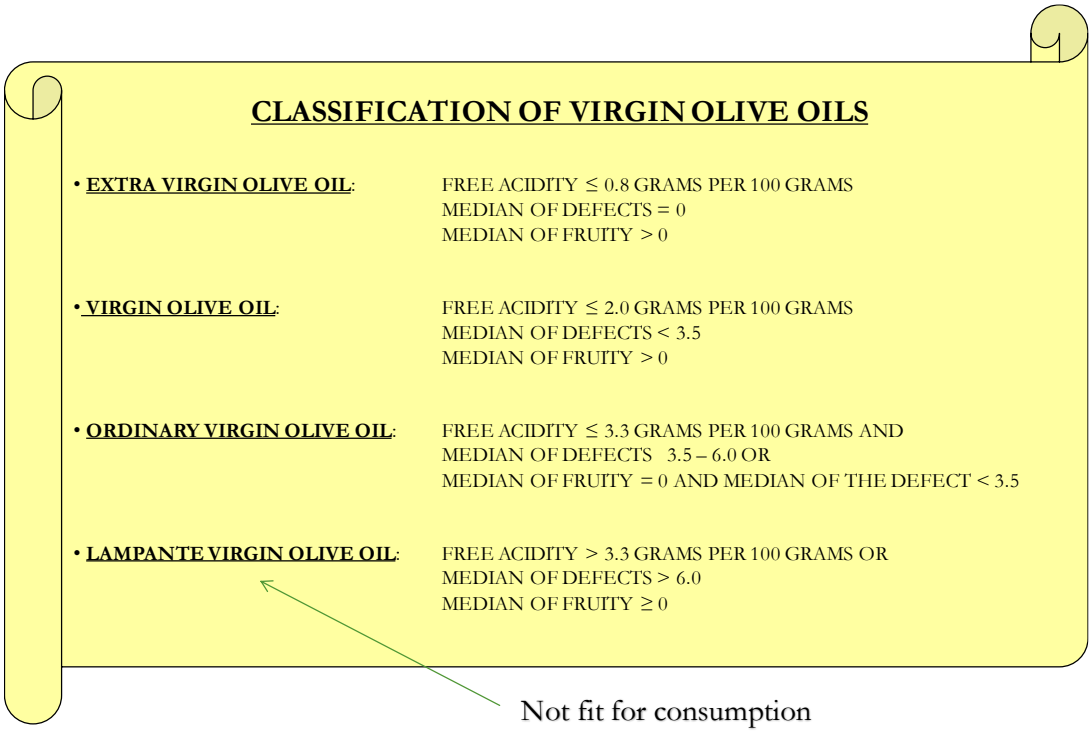

Figure 1. Classification of Virgin Olive Oil according to IOC

This classification is based on chemical and sensorial characteristic of the oils, as briefly summarized in figure 1. To date, the evaluation of sensory defects has to be carried out through the sensorial analysis by a panel of trained experts (panel test).

Panel test is a very useful tool and, over the last decades contributed to the overall increasing of the quality of extra virgin olive oil. However, it also suffers of some drawbacks:

- it is affected by subjectivity and emotionality
- it is slow and time consuming due to the limited number of samples for each session needed to avoid tasters sensory fatigue and the consequent inconsistent judgment, this slowness resulting in difficult to perform the numerous daily tests
- it can be affected by low reproducibility resulting in legal uncertainty

- it is also expensive, due to the need of at least eight tasters, in addition to the head-panelist, which are formed in many years of training and must be kept constantly trained.

In our opinion, for these reasons but as also confirmed by important projects founded by the European Union still ongoing on this matter, it is time for having available a reliable and robust analytical method for supporting the panel test. Several approaches have been proposed in the last years to this goal, mainly based on chemical-analytical tools and statistical tools.

To developing this work, we used an our method previously validated (Fortini et al., 2017) and based on the use of several internal standards and not only one reference molecule. The availability of several internal standards belonging to different chemical classes and having different molecular weights, allows selecting the suitable internal standard for each of the quantified volatile organic compounds, so overcoming some issues that usually limit this techinque, as:

1. different absorption capacity of different fibers
2. fiber wearing
3. competition of molecules at different concentration in different samples
4. different affinity of different molecules for the coating material of the fiber

resulting in more reliability of volatile organic compounds quantitation in wider ranges of calibration.

2. AIM

This study stems with the aim to satisfy the needs of producers and companies working in the olive oil field. These needs required:

- the evaluation of the quality of the product, which results in several practical issues (selection of raw material, the evaluation of the evolution of volatile fraction of virgin olive oils over time, the standardization of blends and products and the detection of virgin olive oils with poor quality by only a fast chemical analysis).
- the need to satisfy legislative requirements, which is supporting the panel test for the olive oil classification, according to reg. CE 2568/1991, with the main goal to protect the productive world but, first of all, consumers from frauds.

In this context, this work is aimed to propose a new chemical approach for the classification of virgin olive oil, limiting the activity of the panel to those cases for which the proposed approach is not able to classify the oil

3. DISCUSSION

To reach this aim, the work was performed through the steps reported following.

1. Method set up and validation were mainly aimed to the routine use in the laboratory of Carapelli. The method was slightly modified with respect to that previously validated and published. These changes were the addition of some molecules involved in some defects, as methanol, ethanol and acetic acid within the quantified VOCs, and the enlargement of the range of linear calibration for those molecules that are present in oil samples in concentrations higher than the upper end of calibration of the previously validated method. Parameters for validation were the same already used previously. Briefly, the LOQ and the upper end of the calibration were, respectively, the lower and the higher point of the calibration with accuracy and precision within the selected limits, while the linearity of calibration was assured by calculating the squared adjusted regression coefficient, which was higher than 0.95 for all the analytes. Except for three molecules (1-penten-3-ol, decanal and *Z*-2-hexenol), the obtained linear ranges of calibration were between 10-200 folds the LOQ. Accuracy was evaluated in terms of trueness and precision at low and high concentration. For each VOC, sensitivity was the slope of the calibration line multiplied by the concentration of the internal standard selected for that VOC in samples. Finally, the selectivity was assured by the use of the suitable ion for identification and quantification of each analyte.
2. The selection of possible markers of unpleasant notes in virgin olive oils was done bearing in mind the knowledges about the processes behind development of both oxidative and microbiological defects and information from the literature, but also the information gathered by the analysis of the standards provided by the international olive council for the defects. Regarding fruity and green notes, markers were the molecules originated from the lipoxygenase pathway (Figure 2), a well-known cascade of enzymatic transformations mainly involving unsaturated fatty acids as linoleic and linolenic acids, that lead to C5 and C6 VOCs as

aldehydes, alcohols and esters, responsible for green and fruity notes of extra virgin olive oils. It is worth highlighting that the presence of medium to high amounts of these molecules can contribute to cover the typical oil defects, mainly when these defects are not so strong. At the same time, if the level of the molecules from LOX is low, defects are also perceived when their intensity is low.

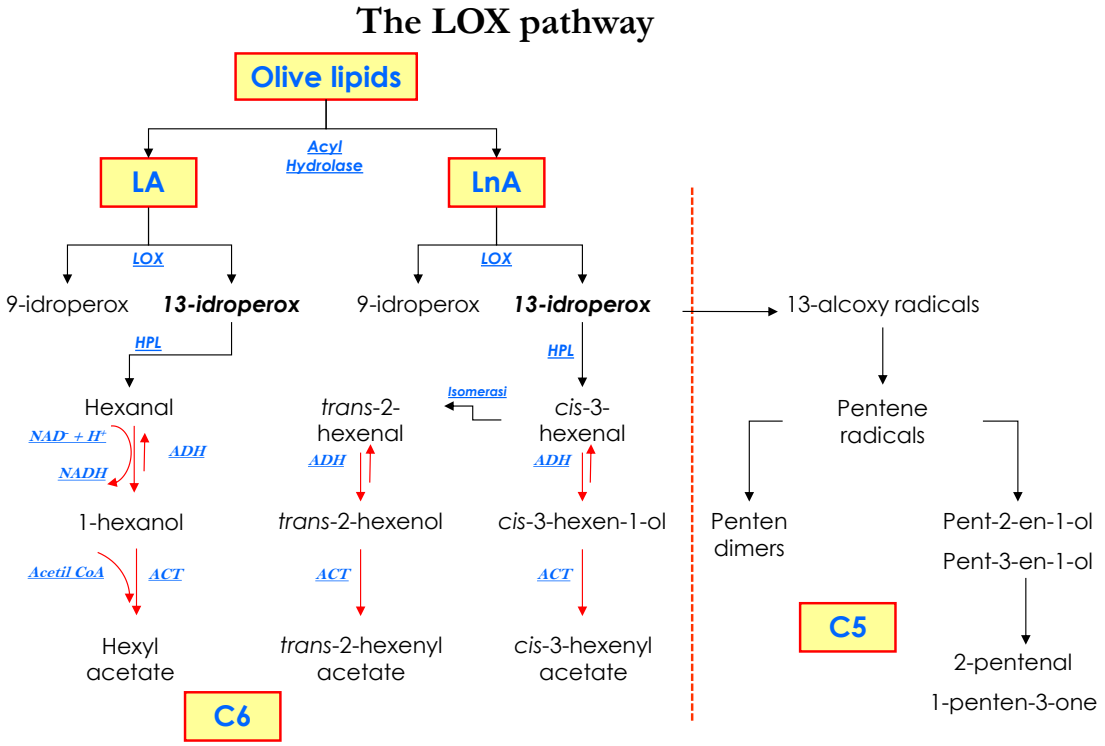

Figure 2. The LOX pathway

3. In the next step, the panel of the Carapelli company, acknowledged by the Italian ministry of agricultural policies, tasted day by day more than 1000 virgin olive oil samples, potentially belonging to the three categories, namely extra virgin, virgin olive oil and lampante virgin olive oil. These oils were mainly from the Mediterranean area and were classifiable as EU or non-EU virgin olive oils. Spain, Italy and Greece were the country with a higher number of samples, followed by Portugal and Tunisia. Samples were from 26 cultivars, and from two different crop seasons. After panel test, samples were classified as extra virgin olive oils (406), virgin olive oils (529) defected for different types of defects, and only 22 lampante virgin olive oils. It is worth note that these oils, selected for the training set, were

almost all border line, that is virgin olive oils with low intensity defects or extra virgin olive oils with not so high level of quality. This choice was done in order to create a model suitable for those samples for which an objective and unique classification by the panel is difficult.

4. This group of samples was then analyzed by the HS-SPME-GC-MS validated method and data from both chemical and sensory analysis constituted the training set for our model.
5. Starting from these data and from the markers previously defined, some indexes for positive attributes and defects were generated (Figure 3).

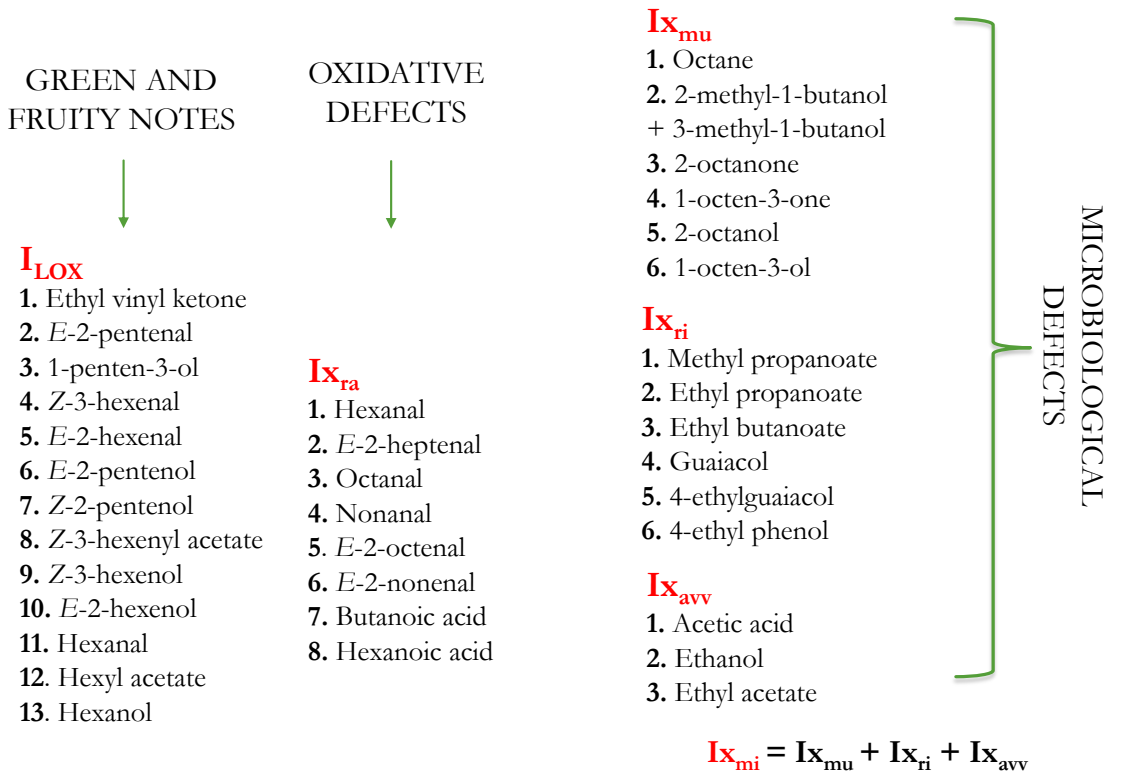

Figure 3. The generated indexes

They were:

- **I_{LOX}**: sum of the amounts of volatiles generated by the LOX pathway
- **Ix_{RA}**: an index for the rancid defects given by the sum of the VOCs mainly related to the oxidative defects
- **Ix_{MI}**: an index for microbiological defects, given by the sum of other three indexes: **Ix_{MU}**, **Ix_{RI}**, and **Ix_{AVV}**. These three indexes are, respectively, indexes related to the defects of musty (MU), fusty (RI) and winegar (AVV).

Regarding this last index, we decided to merge the microbiological defects together for a reason and an aim:

- the reason was that, in our experience, the presence of one of these defects usually also involves the other microbiological defects, even though at different levels;
- the aim was to create an easy model able to discriminate between defected and not defected oils and between defects generated by oxidative or microbiological activities.

6. Starting from these indexes and from their values in the analyzed olive oils, we defined the criteria for classification of the oils.

	EVOO		Rancid		Other defects		Total	
Values	**n° oil**	**% oil**	**n° oil**	**% oil**	**n° oil**	**% oil**	**n° oil**	**% oil**
$Ix_{ra} > 3$	3	1%	25	26%	21	8%	49	6
$1 < Ix_{ra} < 3$	224	56%	54	57%	125	48%	403	53
$Ix_{ra} < 1$	176	43%	16	17%	114	44%	306	40
Total n° of oils	403	100.0%	95	100.0%	260	100.0%	758	100.0%

7. **Table 1.** Values of Ix_{RA} for the oils of the training set

	EVOO		Rancid		Microbiological defects		Total	
Values	**n° oil**	**% oil**	**n° oil**	**% oil**	**n° oil**	**% oil**	**n° oil**	**% oil**
$Ix_{mi} > 15$	5	1%	9	9%	38	15%	52	7
$5 < Ix_{mi} < 15$	133	33%	51	54%	164	63%	348	46
$Ix_{mi} < 5$	264	66%	35	37%	58	22%	357	47
Total n° of oils	402	100%	95	100%	260	100%	758	100%

8. **Table 2.** Values of Ix_{MI} for the oils of the training set

Regarding oxidative defects (Table 1), all samples were characterized by index for rancid defect minor than 3.5 ppm, and 99 % of the extra virgin olive oils had this index below 3.0 ppm, while 83 % of rancid oils over 1 ppm.

Summarizing:

- If the Ix_{RA} is over 3 ppm, 94% of oils are defected, with about 50% of rancid oils,
- If the Ix_{RA} is between 1 and 3 ppm, 44% of oils are defected, but if the I_{LOX} is below 10 ppm, 80% of samples resulted defected
- Finally, if Ix_{RA} is below 1 ppm, only 5% of oils are rancid.

The same approach was repeated using the index of microbiological defects (Table 2). This index was below 18 ppm for all the samples and was below 15 ppm for 99% of the extra virgin olive oils, while 78% of oils with microbiological defects had values over 5 ppm. In this case, we can say that:

- If the Ix_{MI} is below 5 ppm, 74% of samples are extra virgin olive oils.
- If the Ix_{MI} is over 15 ppm, 73% of samples resulted with microbiological defects and 90% of samples resulted defected
- If the Ix_{MI} is between 5 and 15 ppm, about 47% of the oils resulted with microbiological defects, but if the I_{LOX} is below 15 ppm, 71% of oils are defected.

Ix_{MI} (mg/kg)	Ix_{RA} (mg/kg)	I_{LOX} (mg/kg)	Classification
> 15	> 3	-	VOO (Micro + Rancid)
> 15	$1<Ix_{RA}<3$, $I_{LOX}<10$	$I_{LOX}<10$	VOO (Micro + Rancid)
> 15	$1<Ix_{RA}<3$, $I_{LOX}>10$	$I_{LOX}<10$	VOO (Micro)
> 15	< 1	-	VOO (Micro)
$5<Ix_{MI}<15$ ($I_{LOX}<15$)	> 3	$I_{LOX}>15$	VOO (Micro + Rancid)
$5<Ix_{MI}<15$ ($I_{LOX}<15$)	$1<Ix_{RA}<3$, $I_{LOX}<10$	$I_{LOX}>15$	VOO (Micro + Rancid)
$5<Ix_{MI}<15$ ($I_{LOX}<15$)	$1<Ix_{RA}<3$, $I_{LOX}>10$	$I_{LOX}>15$	VOO (Micro)
$5<Ix_{MI}<15$, $I_{LOX}<15$	< 1	$I_{LOX}>15$	VOO (Micro)
$5<Ix_{MI}<15$, $I_{LOX}>15$	> 3	$I_{LOX}>15$	VOO (Rancid)
$5<Ix_{MI}<15$, $I_{LOX}>15$	$1<Ix_{RA}<3$, $I_{LOX}<10$	$I_{LOX}>15$	VOO (Rancid)
$5<Ix_{MI}<15$, $I_{LOX}>15$	$1<Ix_{RA}<3$, $I_{LOX}>10$	$I_{LOX}>15$	Not classified
$5<Ix_{MI}<15$, $I_{LOX}>15$	< 1	$I_{LOX}>15$	EVOO
< 5	> 3	-	VOO (Rancid)
< 5	$1<Ix_{RA}<3$, $I_{LOX}<10$	$I_{LOX}<10$	VOO (Rancid)
< 5	$1<Ix_{RA}<3$, $I_{LOX}>10$	$I_{LOX}<10$	EVOO
< 5	< 1	-	EVOO

Table 3. Values of Ix_{MI} for the oils of the training set

7. These observations allowed us building a decision table based on some criteria (Table 3). For each sample, the inputs were I_{LOX}, Ix_{MI} and Ix_{RA}, while the outputs were if the sample is EVOO or not and, in case of not, if it is defected for oxidative or microbiological defects. Only in the case when both Ix_{MI} and Ix_{RA} have medium values and I_{LOX} is over 15 ppm, we established that this approach is not able to classify the olive oil sample.
8. In order to validate the proposed approach, a further set of samples with 91 virgin olive oils of different categories was used as validation set. All these samples were analyzed by HS-SPME-GC-MS and I_{LOX}, Ix_{MI} and Ix_{RA} were calculated. Starting from the calculated values, each sample was classified based on the decision table, and the results were compared with classification by panel test.

Result	n°	%	%
NOT CLASSIFIED	9	-	
OK	64	78.0	84.1
OK BUT OTHER DEFECT	5	6.1	
NO	13	15.9	15.9

Table 3. Values of Ix_{MI} for the oils of the training set

The results of this comparison are summarized in table 4, in which we indicated **not classified** if our approach was not able to classify that sample, **ok** if the results of panel test and our approach were the same, **ok but other defect** if the sample resulted defected by panel test and our approach but for different defects, and **no** if our results are in disagrement with the panel test**.**

Nine samples were not classified according to the decision table. For the other oils, 84% of samples were successfully classified by our approach according with the results of the panel, with 78% in agreement also for the type of defect when the oil was defected.

Only 15.9% of samples were classified differently by our approach and panel test. However, it is worth to remember that almost all the samples used for both training set and validation set were oils with weak defects or, if extra virgin, with

not so high quality. Consequently, the discrepancy between sample classification by our approach and panel test can be considered more than acceptable.

Conclusion

In conclusion, the quantification of 73 VOCs by using multiple internal standard area normalization and HS-SPME-GC-MS was validated in the laboratory of Carapelli SpA. Using this method and the company panel, more than 1000 virgin olive oil samples were analyzed from both chemical and sensory point of view, allowed defining indexes for oxidative and microbiological defects and for positive attributes. These indexes were then used for developing and easy approach for classification of virgin olive oils, based on only chemical analysis. Finally, the approach was validated using a validation set of 91 virgin olive oils with different category: it allowed classifying 90% of samples, 84% of which in agreement with panel test.

The next step of the work are i) the evaluation of the reproducibility of quantification of VOO-VOCs by the validated HS-SPME-GC-MS method by interlaboratory tests and ii) the extension of our approach involving several panels. By this way, we will make the proposed indexes more robust and suitable for routine use by the olive oil companies and analytical laboratories.

References

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7. RESULTS: VIRGIN GRAPE SEED OIL

In depth study of phenolic profile and PTP-1B inhibitory power of cold-pressed grape seed oils of different varieties

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Lorenzo Cecchi[1], Marzia Innocenti[1], Silvia Urciuoli[1], Marco Arlorio[2], Paolo Paoli[3], Nadia Mulinacci[1*]

[1] Dipartimento di NEUROFARBA, and Multidisciplinary Centre of Research on Food Sciences (M.C.R.F.S.- Ce.R.A). Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Firenze), Italy

[2] Dipartimento di Scienze del Farmaco and DFB Center, Università degli Studi del Piemonte Orientale "A. Avogadro", Largo Donegiani 2, Novara, Italy

[3] Dipartimento di Scienze Biomediche, Sperimentali e Cliniche "Mario Serio", Università degli Studi di Firenze, Via Viale Morgagni 50, Firenze, Italy.

*Corresponding author

Tel.: +39 055 4573773; fax: +39 055 4573737

e-mail: nadia.mulinacci@unifi.it

Abstract

This paper investigates the phenolic composition of 17 monocultivar commercial cold-pressed grape seed oils. Chromatographic profiles showed the presence of more than 28 molecules, 11 of which were successfully identified by HPLC-DAD-MS-TOF and HPLC-FLD analysis. Pinoresinol, ethyl caffeate and ethyl gallate were detected for the first time in these oils. The total phenolic content ranged between 0.83 mg/kg for Viognier sample to 15.16 mg/kg for Merlot org sample. The detected ethyl esters can be suggested as markers to evaluate the intensity of fermentation in grape seeds before oil extraction, and to control the sensorial quality of the produced oils. In addition, the inhibitory power of these phenolic extracts against Protein Tyrosine Phosphatase 1B enzyme (PTP-1B), overexpressed in type-two diabetes, was investigated for the first time. Data highlighted a good correlation between total phenolic content and inhibitory power, with pinoresinol, *p*-coumaric acid and quercetin making the greater contributions.

Keywords: phenolic compounds, lignans, pinoresinol, virgin grape seed oils

1. Introduction

Grapes are primarily used in winemaking, with several European countries being the main producers together with Argentine and Chile in South America and California in the U.S.A. Grape pomace and grape seeds are the main by-products of the winery industry (Chemat, Li, Tomao, Ginies & Cravotto, 2014). In 2008, Matthäus estimated worldwide grape production at 60 million tons/year, with grape seeds close to 20% of fresh fruit and 40-60% of dried matter. Regarding the oil content, several studies reported values from 13% to a maximum of 19%, but also underlined that the oil is only partially recovered in cold-pressed extraction (Matthäus, 2008; Özcan, Al Juhaimi, Gülcü, Uslu & Geçgel, 2017a; Özcan, Endes & Er, 2010a; Özcan et al., 2017b). Other authors (Venkitasamy, The, Atungulu, McHugh & Pan, 2014) reported that 20% of grape production is typically formed by by-products (grape pomace), 47% of which are seeds. Data from the USDA (2013) reported for 2012 an estimated production of 150,000 tons/year of dried seeds, derived from 5.8 million tons of processed grapes. In this case, the reported range of estimated oil production is 10-22% of dry seeds, with values similar to those previously reported (Matthäus, 2008). A not negligible amount of grape seeds as by-products derived also from juice extraction: in Brazil, approximately 42 % of total grape production was marketed as fresh grape fruit (Shinagawa, De Santana, Torres & Mancini-Filho, 2015).

Grape seeds have different morphological aspects, variable content of lipids and minerals (Özcan, 2010b), and present a certain difficulty to manage after wine production, mainly due to the risk of fermentation. A large part of these by-products are utilized for oil recovery by solvent extraction; a few preliminary studies were conducted to evaluate the effect of microwave on oil extraction yield (Özcan & Juhaimi, 2017c), but some aliquots were used for oil extraction by mechanical process only. These latter products are commonly defined as cold-pressed grape seed oils or, in some cases, also virgin grape seed oils. The most critical aspects during production of cold-pressed grape seed oils are linked to seed size and to the drying process applied to strictly control the residual moisture content of the matrix after wine production. At the same time, technological aspects, such as correct screw-press parameters, are recognized as crucial to obtain a good oil with acceptable yields (Venkitasamy et al., 2014; Rombaut et al 2015). ‘Virgin’ grape seed oils are characterized by high levels of poly-unsaturated fatty acids and vitamin E

(Bertrand & Özcan, 2011) and a light flavor with fruity notes, even though their organoleptic characteristics are strongly affected by the quality of by-products (Zhao, Yagiz, Xu, Fang & Marshall, 2017; Al Juhaimi, Geçgel, Gülcü, Hamurcu & Özcan, 2017; Garavaglia, Markoski, Oliveira & Marcadenti 2016; Shinagawa et al., 2015).

What has been proven for extra virgin olive oil and its properties for health has induced a renewed interest of the market toward cold-pressed grape seed oils. Grape seed oils are often cited for their potential benefits (Shinagawa et al., 2015; Garavaglia et al., 2016) but often it is not specified if the effects have been observed with refined or virgin oils.

Several works are available in literature on phenols in grape seeds extracts, obtained from fresh or dried seeds or from the residual cake after oil extraction (Rustioni & Failla, 2016; Maier, Schieber, Kammerer & Carle, 2009). On the contrary, findings regarding the phenolic profiles of cold-pressed grape seed oils are scarce. Almost all the available works since 2007 (Rombaut et al., 2014; Rombaut et al., 2015; Lutterodt, Slavin, Whent, Turner & Yu, 2011; Matthäus, 2008; Bail, Steuebiger, Krist, Unterweger & Buchbauer, 2008; Baydar, Özcan & Çetin, 2007) report only the evaluation of the total phenolic content, often applying the non-specific Folin Ciocalteau method (Baydar et al 2007; Bail et al 2008; Lutterodt et al., 2011), without identifying the chemical structure of at least the main constituents. Few groups have applied chromatographic analyses to study the composition of this fraction, reporting only the presence of catechin (Assumpçåo et al., 2014), vanilline and vanillic acid (Rombaut et al, 2014) and, recently, quercetin and rutin together with gallic and chlorogenic acids (Al Juhaimi & Özcan, 2017). Other authors detected epicatechin, epicatechin gallate and pentagalloylglucose in cold-pressed oils obtained from the Muscadine variety (Zhao et al, 2017). Nevertheless, these last works did not report mass spectra or a comparison with pure standards, which are necessary to definitively confirm the structure identification of the detected compounds. Consequently, some doubts remain regarding the presence of very polar phenolic compounds such as glycosylated and galloylated derivatives, which are known to have poor solubility in oil.

The aim of this work was to investigate the phenolic composition of cold-pressed grape seed oils from both qualitative and quantitative points of view. Furthermore, until now no data have been available regarding cold-pressed grape seed oils and the

interaction with the enzyme Protein Tyrosine Phosphatase 1B (PTP-1B), a typical target of investigations on type-two diabetes. A recent review discussed the numerous approaches applied to find selective inhibitors of PTP-1B enzyme (Verma, Ji Gupta, Chaudhary & Garg, 2017), which acts as a negative regulator of insulin and leptin receptor signaling pathways. Pharmacological inhibition of PTP-1B enhances insulin sensitivity, improves glycemic control, and favors loss of body weight (Qian, Zhang, He, Wang & Liu, 2016).

Our investigation focuses on studying, by HPLC-DAD-MS-TOF and HPLC-FLD, the phenolic profile of several cold-pressed grape seed oils from qualitative and quantitative points of view, and to examine the *in vitro* power of their phenolic fractions to inhibit the PTP-1B enzyme, overexpressed in type-two diabetes. It is worth noting that the study is based on 17 commercial oils, most of them monocultivar, obtained not at laboratory scale but at industrial scale and derived from the same producer.

2. Material and methods

2.1 Chemicals

Ethanol, hexane and formic acid of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany). Acetonitrile of HPLC and HPLC-MS grade were purchased from Panreac (Barcelona, Spain). The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce deionized water. Pinoresinol (≥ 95%) and *p*-coumaric acid (≥ 98%) from Sigma-Aldrich (Steinheim, Germany) were used as standard compounds. Other standards as vanillic, ferulic and syringic acids, *E*-resveratrol, tyrosol, and kaempferol were purchased from Extrasynthese (France).

Human recombinant PTP-1B was expressed in Escherichia coli TB1 strain, and purified as previously described (Paoli et al., 2013). 4-nitrophenyl phosphate disodium salt hexahydrate were from Santa Cruz Biotechnology.

2.2 Samples

'Virgin' grape seed oils. All the selected mono-varietal cold pressed grape seed oils are listed in Table 1, with the best-before-date and the corresponding grape variety. The 15 Californian samples, three of which being organic samples, were from SaluteSantè, Napa, California, USA, while the further two organic samples of unknown

variety were purchased from Italian market. All samples from SaluteSantè were from sun-dried grape seeds, then cold pressed with a screw press applying a pressure of 35-55 MPa. One ton of grape seeds was pressed in each extraction cycle; the oil yields were about 10% w/w. No filtration was applied, but the oils were clarified by sedimentation.

n°	Sample name	Best before date
1	Viognier	Jan-16
2	Sangiovese	Jan-16
3	Cabernet Sauvignon	Oct-16
4	French Colombard	Oct-16
5	Sauvignon blanc	Oct-16
6	Riesling	Oct-16
7	Chenin blanc	Nov-16
8	Pinot noir	Dec-16
9	Merlot	Dec-16
10	Petite Sirah organic	Mar-17
11	Merlot organic	Mar-17
12	Cabernet Sauvignon organic	Mar-17
13	Zinfandel	Mar-17
14	Chardonnay	Sep-17
15	Sirah	Nov-17
16	Sample A organic *	Sep-16
17	Sample B organic *	Sep-16

Table 1. List of the analyzed grape seed oil samples ***** *From italian market*

Grape seeds. A sample of sun-dried grape seeds of Sangiovese variety was purchased from a Tuscan farm, then milled at laboratory scale in order to obtain a homogenous powder which was used for the successive extraction with the sunflower oil.

2.3. Extraction of phenolic compounds

Extraction of oil samples. The extraction conditions to recover the phenolic fraction were the same already applied to olive oil samples (Cecchi et al., 2017). Briefly, 20 g of sample were added to a flask, extracted in 60 mL of ethanol/acidic water (pH 3.2 by formic acid) 7:3 v/v and stirred for 30 min. The obtained mixture was defatted three times with n-hexane (20 mL each time); when the separation of the two phases was incomplete due to formation of emulsion, few mL of ethanol were added to broke this emulsion. The hydroalcoholic phase was recovered, evaporated under vacuum at room temperature, and the residue redissolved in 1.5 mL of ethanol/acidic water (pH 3.2 by

formic acid) 7:3 v/v. The obtained solution was centrifuged at 14,000 rpm and the supernatant was used for the chromatographic analysis.

Extraction of grape seed sample. The seeds from Sangiovese were extracted in commercial sunflower oil; the oil was previously analyzed to rule out the presence of native phenols. 10 g of powdered grape seeds were added to a flask together with 100 g of sunflower oil and extracted for 25 min in an ultrasound bath at 30°C, and then stirred for 60 min at room temperature. The mixture was filtered and the phenolic fraction was extracted from the obtained oil as previously described for the cold pressed oils.

2.4. HPLC-DAD-MS-TOF and HPLC-DAD-FLD analysis of phenolic extracts

The analyses of phenolic compounds were carried out with an HP 1100 Liquid Chromatograph coupled with DAD and TOF Mass Spectrometer detector equipped with electrospray interface (ESI), all from Agilent Technologies (Palo Alto, CA, USA). The column was a Poroshell 120, EC-C18 (150 mm x 3 mm i.d., 2.7 μm) equipped with a precolumn of the same phase (Agilent Technologies); oven temperature, 26°C. Solvents for elution were (A) 0.1% formic acid/water and (B) acetonitrile. The multi-step linear solvent gradient varied as follow: 0-5 min 10-15% B; 5-15 min 15-30% B; 15-20 min 30-35% B; 20-23 min 35-40% B; 23-26 min 40-45% B; 26-32 min 45-100% B; 32-37 min 100% B; 37-42 min 100-10% B; equilibration time 10 min; flow rate 0.4 mL/min. We acquired chromatograms at 240 nm, 280 nm, 330 nm, 350 nm and 540 nm, and UV spectra in the wavelength range of 200-600 nm. Mass spectra were acquired in negative ion mode in a mass range of 80-1200 m/z. The ESI source was set as follow: drying gas (N_2), temperature 350°C, drying gas flow rate 6 L/min, nebulizer 20 psi, capillary voltage 3800 V, fragmentation 150 V, skimmer 60 V. The acquisition data was done by the Agilent MassHunter Qualitative Analysis Software, version B.06.00 (Agilent Technologies). The TOF mass spectrometer was calibrated immediately before the analyses and no internal reference was used. The accurate mass of the molecules related to the main peaks was measured and the elemental compositions were calculated, considering a maximum difference of 10 ppm between the mass of the calculated and measured formulas.

To better investigate the lignan content, some analysis were repeated using the same chromatographic conditions and the same apparatus, but equipped with a

fluorimetric detector (FLD). Regarding the FLD, the excitation wavelength was 280 nm, and the emission wavelength was set at 339 nm, according to Servili et al. (2007).

Quantification of phenolic compounds was carried out by the external standard method, using the following standards: *p*-coumaric acid was used to build a five-point calibration curve at 280 nm; vanillic acid, *p*-hydroxybenzoic acid, syringic acid, *p*-coumaric acid, ferulic acid, ethyl gallate, ethyl caffeate, *E*-resveratrol, quercetin and kaempferol were expressed as mg of *p*-coumaric acid per kg of oil ($mg_{p\text{-}cum}/kg$). Total phenolic content (TPC) was evaluated on the total area of peaks in the range 4-33 minutes of the chromatograms at 280 nm and was expressed as $mg_{p\text{-}cum}/kg$. Pinoresinol was used to build a five-point calibration curve at 280 nm; pinoresinol was expressed as mg of pinoresinol per kg of oil (mg_{pin}/kg). All the analytes in Table 2 were evaluated at 280 nm with the only exception of *p*-coumaric acid, for which a calibration curve at 330 nm was built because this molecule was partially co-eluted with ethyl gallate, which does not shown absorbtion at 330 nm.

Limit of quantifications (LOQ) were estimated according to the Eurachem Guide (Magnusson & Ornemark, 2014) using the standards pinoresinol (LOQ, 0.053 mg/kg) and *p*-coumaric acid (LOQ, 0.015 mg/kg).

2.5. Inhibition's assays of enzyme PTP-1B by the phenolic grape seed oil extracts

Inhibition assays of the enzyme PTP-1B was carried out using *p*-nitrophenylphosphate (*p*NPP) 2.5 mM as substrate. The assay buffer contained sodium *β*,*β*-dimethylglutarate buffer (75 mM, pH 7.0), EDTA (1 mM), and dithiothreitol (1mM) in addition to *p*NPP. Solutions of grape seed oil extracts (0.66 g_{oil}/mL) were used as putative inhibitor of PBP-1B.

Inhibitory assays were carried out at 37°C on a solution of inhibitor (10 μL) and substrate (990 μL). Reactions started by addition of aliquots of the enzyme preparation (Paoli et al., 2013) and stopped with KOH 0.2 M (2 mL). The released *p*-nitrophenolate was quantified by reading the absorbance of the final solution at 400 nm (ε = 18,000 M^{-1} cm^{-1}). Percentage of inhibition of each extract was calculated by comparing the absorbance of the assays with that of a control test, carried out in the same condition but in absence of the inhibitor solutions. The results of all the assays were reported as a mean of three experiments.

IC_{50} values for some inhibitors were calculated. To this aim, 12 different dilutions of the phenolic extracts (range of concentration 0.001333-13.33 g_{oil}/mL) obtained from grape seed oils as described in paragraph 2.3, were used for the inhibitory assays, carried out as described above. The IC_{50} values for the PTP-1B inhibitors were determined by fitting experimental data using the following equation (Paoli et al., 2013):

$$y = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{IC_{50}}\right)^{\text{slope}}} + \text{Min}$$

where $y=v_i/v_o$, is the ratio between the activity measured in the presence of the inhibitor (v_i) and the activity of the control without the inhibitor (v_o). The parameter "x" is the inhibitor concentration.

2.6 Precision parameters

To evaluate the precision of the procedure for the quantitation of each phenolic compound, we prepared a blend of all the oil samples, weighting and mixing aliquots of each of them until reaching a homogeneous oil solution. This solution was used as reference sample. The extraction and analysis of the phenolic compounds were repeated six times starting from different aliquots of the reference sample and the obtained results expressed in terms of CV% (Table 3).

Compound name	Retention time (min)	mw	Average (µg/kg)	SD	CV%	λ_{max} (nm)	Major ions
***p*-hydroxybenzoic acid**	6.7	138	42.1	3.3	7.8	256	137, 93
vanillic acid	8.1	168	267.6	15.1	5.6	260, 293	167, 123
syringic acid	8.8	198	243.3	12.4	5.1	274	197, 131
***p*-coumaric acid**	12.0	164	775.0	40.5	5.2	309	163, 119
ethyl gallate	12.0	198	68.1	5.9	8.7	274	197, 395
ferulic acid	13.4	194	62.7	5.7	9.1	322	193
***E*-resveratrol**	18.7	228	68.2	7.1	10.4	307	227, 273, 171
quercetin	21.2	302	63.3	14.0	22.1	256, 372	301, 603
pinoresinol	21.7	358	2974.1	168.0	5.6	280	357, 403
ethyl caffeate	21.9	208	43.3	3.8	8.8	295, 317	207, 415
kaempferol	25.1	286	83.4	13.3	16.0	266, 367	285, 571
Total phenols (280 nm)			8726.8	1070.0	12.2		

Table 3. List of the identified compounds in cold pressed grape seed oils; the analyzed sample was a blend obtained mixing equal amounts of all the analyzed oils. Data were the mean of six determinations, each obtained from the extraction of six different aliquots of the blend.

2.7 Statistical analysis

All computations related to the Pearson Correlation Coefficient reported in Figure 4 were carried out by EXCEL software (version 2013) in-house routines

3. Results and discussion

3.1 Phenolic characterization

Our aim was to investigate the phenolic composition of 'virgin' grape seed oils. To this aim, we selected and analyzed 17 oils by HPLC-DAD-MS and HPLC-DAD-FLD, in order to compare their phenolic profiles and to estimate the total phenolic content using suitable external standards. The applied liquid/liquid extraction was similar to that previously used to recover the minor polar compounds of olive oil (Cecchi et al., 2017) and the separation was obtained without the need to add Tween 20, as previously suggested for these oils (Maier, Schieber, Kammerer & Carle, 2009). Figure 1 shows the chromatographic profiles at 280 nm for the Pinot Noir sample: 11 compounds were successfully assigned to specific phenols on the basis of their retention time, UV-Vis and mass spectral data, and the comparison with a pool of pure standards (Table 3).

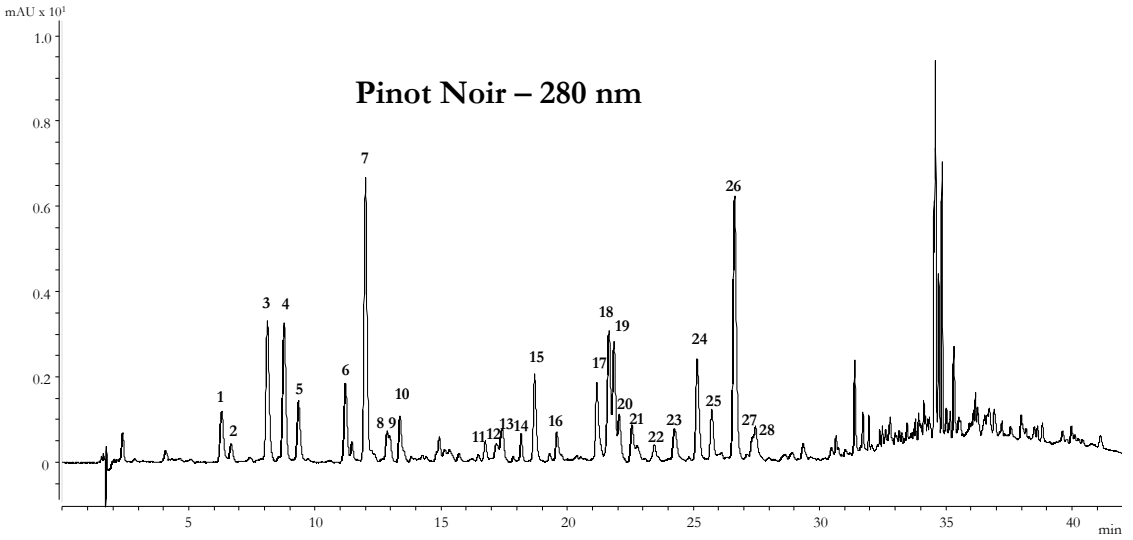

Figure 1. Chromatographic profile at 280 nm for the Pinot Noir sample. The identified molecules: **2**, *p*-hydroxybenzoic acid; **3**, vanillic acid; **4**, syringic acid; **7**, ethyl gallate + *p*-coumaric acid; **10**, ferulic acid; **15**, *E*-resveratrol; **17**, quercetin; **18**, pinoresinol; **19**, ethyl caffeate; **24**, kaempferol. (Spectral data of both identified and unidentified compounds are reported in Supplementary material, Tables 1S and 2S).

Other peaks were not identified despite the acquisition of their spectral data (Table 4) and the consultation of a specific data bank for phenolic compounds (http://phenol-explorer.eu/). Further analytical efforts will be required for their structural identification.

Compound	Retention time (min)	λ_{max} (nm)	Major ions
1	6.3	275, 313	137, 275, 279, 325
5	9.4	284	121, 131,
6	11.2	280, 311	151, 135, 165
8	12.8	274	159, 175, 197
9	13.0	293	163, 119, 197
11	16.8	280	187, 169, 375
12	17.2	265	181, 138, 243, 363
13	17.4	265	211, 181, 287, 407
14	18.2	281	405, 171, 191, 531
16	19.6	265	263, 309, 325, 527
20	22.1	274	409, 165, 207, 225
21	22.6	261, 294	409, 199, 279
22	23.4	280	157, 227, 301, 410
23	24.3	286	271, 357, 403, 543
25	25.7	256, 372	315, 409, 631
26	26.6	311	191, 329, 659
27	27.3	295, 321	241, 183, 467
28	27.5	295, 323	187, 221, 467

Table 4. Spectral data of the unidentified phenols (numbered according to the chromatographic profile of Figure 1).

With the help of pure standards and use of the extract ion technique, it was possible to exclude the presence of detectable amounts of epicatechin and catechin, previously cited as main components of the phenolic fraction of these oils (Assumpção et al., 2014; Zhao et al., 2017), and of epicatechin gallate and pentagalloyl glucose recently reported in oils from Muscadine variety (Zhao et al., 2017). Analogously, gallic and chlorogenic acids, previously found in 'virgin' grape seed oils from Muscadine variety, were not detected in our samples (Zhao et al., 2017). Furthermore, the total amounts reported by these latter authors appears really too high, with values up to 697 mg/kg; these values are largely higher even than the amounts detected in high quality extra virgin olive oils. The amount of identified phenolic compounds in each of the analyzed grape seed oil samples is summarized in Table 2.

Sample	*p*-hydroxybenzoic acid	vanillic acid	syringic acid	*p*-coumaric acid	ethyl gallate	ferulic acid	*E*-resveratrol	quercetin	ethyl caffeate	kaempferol	TPC
Viognier	< LOQ	< LOQ	0.079 ± 0.004	0.161 ± 0.008	< LOQ	< LOQ	< LOQ	0.082 ± 0.018	< LOQ	< LOQ	**0.827 ± 0.099**
Sangiovese	< LOQ	0.095 ± 0.005	0.104 ± 0.005	0.381 ± 0.020	0.017 ± 0.002	0.029 ± 0.003	< LOQ	< LOQ	< LOQ	< LOQ	**2.713 ± 0.326**
Cabernet Sauvignon	0.043 ± 0.003	0.127 ± 0.007	0.260 ± 0.013	0.191 ± 0.010	< LOQ	0.038 ± 0.004	0.096 ± 0.010	0.207 ± 0.046	< LOQ	0.043 ± 0.007	**4.629 ± 0.556**
French Colombard	0.036 ± 0.003	0.122 ± 0.007	< LOQ	0.573 ± 0.030	< LOQ	0.048 ± 0.004	< LOQ	0.343 ± 0.076	< LOQ	0.031 ± 0.005	**4.973 ± 0.597**
Sauvignon Blanc	0.032 ± 0.003	0.124 ± 0.007	< LOQ	0.712 ± 0.037	< LOQ	0.051 ± 0.005	< LOQ	0.379 ± 0.084	< LOQ	0.025 ± 0.004	**5.563 ± 0.668**
Riesling	0.040 ± 0.003	0.130 ± 0.007	< LOQ	0.891 ± 0.047	< LOQ	0.068 ± 0.006	< LOQ	< LOQ	< LOQ	0.029 ± 0.005	**6.776 ± 0.813**
Chenin Blanc	0.036 ± 0.003	< LOQ	< LOQ	0.717 ± 0.038	< LOQ	0.052 ± 0.005	< LOQ	0.493 ± 0.109	< LOQ	0.025 ± 0.004	**5.896 ± 0.708**
Pinot Noir	0.060 ± 0.005	0.437 ± 0.025	0.398 ± 0.020	0.257 ± 0.014	0.590 ± 0.051	0.131 ± 0.012	0.252 ± 0.026	0.241 ± 0.053	0.341 ± 0.027	0.349 ± 0.057	**9.172 ± 1.101**
Merlot	0.025 ± 0.002	0.101 ± 0.006	0.114 ± 0.006	0.552 ± 0.029	< LOQ	0.031 ± 0.003	0.030 ± 0.003	0.129 ± 0.029	0.062 ± 0.005	0.036 ± 0.006	**2.944 ± 0.353**
Petit Syrah Org.	0.023 ± 0.002	0.190 ± 0.011	0.420 ± 0.021	0.249 ± 0.013	0.061 ± 0.005	0.064 ± 0.006	0.055 ± 0.006	0.458 ± 0.101	0.048 ± 0.004	0.131 ± 0.022	**6.253 ± 0.750**
Merlot Org.	0.080 ± 0.006	0.537 ± 0.030	0.874 ± 0.044	0.528 ± 0.028	0.170 ± 0.015	0.092 ± 0.008	0.094 ± 0.010	1.094 ± 0.242	< LOQ	0.218 ± 0.036	**15.158 ± 1.819**
Cabernet Sauvignon Org.	0.049 ± 0.004	0.317 ± 0.018	0.831 ± 0.042	0.459 ± 0.024	0.085 ± 0.007	0.061 ± 0.006	0.060 ± 0.006	< LOQ	0.100 ± 0.008	0.227 ± 0.037	**9.962 ± 1.196**
Zinfandel	< LOQ	0.066 ± 0.004	< LOQ	0.500 ± 0.026	< LOQ	0.031 ± 0.003	< LOQ	< LOQ	< LOQ	0.016 ± 0.003	**2.553 ± 0.306**
Chardonnay	0.045 ± 0.004	< LOQ	< LOQ	0.218 ± 0.011	0.072 ± 0.006	0.042 ± 0.004	0.041 ± 0.004	0.132 ± 0.029	< LOQ	0.051 ± 0.008	**3.835 ± 0.460**
Syrah	0.050 ± 0.004	0.289 ± 0.016	0.518 ± 0.026	0.615 ± 0.032	0.083 ± 0.007	0.089 ± 0.008	< LOQ	< LOQ	< LOQ	0.036 ± 0.006	**8.173 ± 0.981**
Italan SampleA Org.	0.037 ± 0.003	0.314 ± 0.018	0.579 ± 0.030	0.417 ± 0.022	0.078 ± 0.007	0.068 ± 0.006	0.048 ± 0.005	< LOQ	0.040 ± 0.003	0.148 ± 0.024	**7.861 ± 0.943**
Italian SampleB Org.	0.047 ± 0.004	0.328 ± 0.018	0.629 ± 0.032	0.434 ± 0.023	0.091 ± 0.008	0.080 ± 0.007	0.049 ± 0.005	< LOQ	0.049 ± 0.004	0.179 ± 0.029	**9.202 ± 1.104**

Table 2. Phenolic compounds identified in the cold pressed oils. Results are expressed as $mg_{p\text{-coum}}/kg$ (mean ± SD); LOQ, 0.015 mg/kg

It is worth pointing out that none of the previous works mentioned the presence of lignans in the phenolic fraction of cold-pressed grape seed oils. In the present work, the presence of these molecules was confirmed by HPLC-MS-TOF analyses in negative ionization mode, the extract ion chromatogram at 357.13 Th, and the comparison with pinoresinol standard (retention time, UV and mass spectra). A second peak with the same molecular ion of pinoresinol (confirmed by the adduct with formic acid) and retention time higher than pinoresinol was detected in a few samples. In order to better investigate the structure of this molecule, the same chromatographic analyses were repeated using a fluorimetric detector (FLD). This detector, selective for the lignans pinoresinol and 1-acetoxypinoresinol in olive oil (Servili et al., 2007), allowed exclusion of this molecule as an isobaric derivative of pinoresinol. As shown in Figure 2, the presence of pinoresinol was confirmed in all the analyzed samples, with values in the range between 0.513 mg/kg (Viognier sample) and 6.468 mg/kg (Merlot Org sample), with a mean concentration close to 2-3 mg/kg in the other oils.

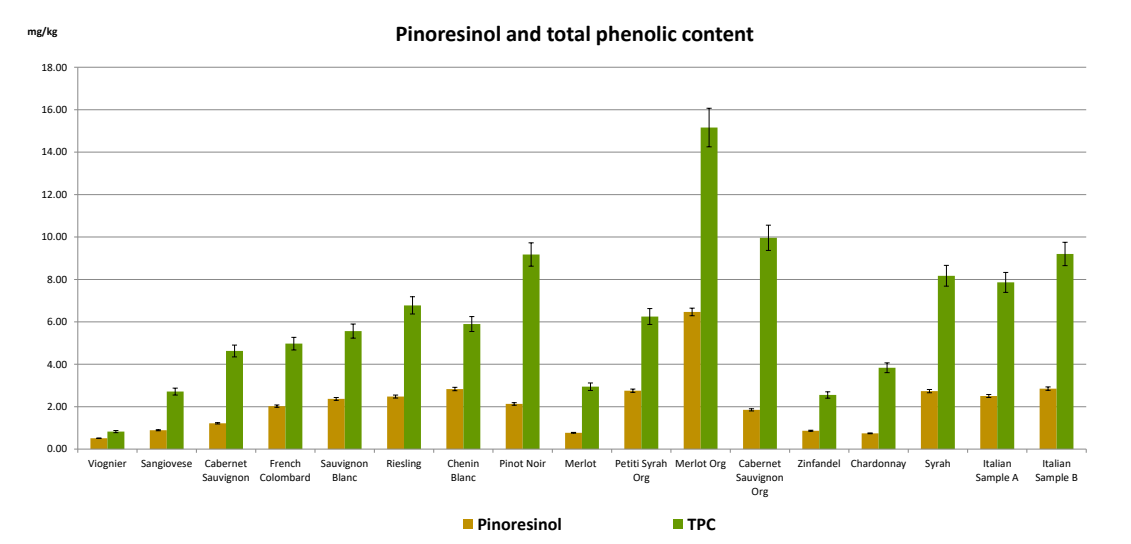

Figure 2. Pinoresinol and total phenolic content (TPC) of the 17 oil samples. Pinoresinol content is expressed as mg_{pin}/kg; total phenolic content is expressed as mg_{coum}/kg.

The presence of the ethyl esters of caffeic and gallic acids in the phenolic fraction of several oils was revealed; ethyl gallate was detected in nine of the 17 samples in concentrations up to 0.590 mg/kg, and ethyl caffeate was detected in seven oils in concentrations up to 0.341 mg/kg. The highest content of the two esters was detected in the Pinot Noir sample (Table 2), suggesting these grape seeds had undergone to a stronger fermentation of the residual sugars, presumably developed during the drying process (Ovcharova, Zlatanov & Dimitrova, 2016). It is well known that sugar fermentation leads

to ethanol formation (Angerosa, Lanza & Marsilio, 1996) which is needed for the synthesis of ethyl esters. According to the literature, these esters can be suggested as possible markers to evaluate the intensity of the fermentation process in grape seeds before oil extraction, but also to control the sensorial quality of the final pressed oil (Di Serio et al., 2017).

Overall, the quantitative data for the total phenolic compounds content are in agreement with those obtained with a similar analytical approach by Maier et al. (2009), who reported 2.9 mg/kg as maximum amount. At the same time, our results strongly disagree with other authors who recently reported concentrations of total phenols over 600 mg/kg (Zhao et al., 2017); a clearly described quantitative procedure was not applied in this work. However, bearing in mind that the total phenolic compounds content in extra virgin olive oils exceeds only in a few cases 500-600 mg/kg, the values indicated for the grape seeds oils by these latter authors seem to be largely overestimated.

3.2 Research of pinoresinol in grape seeds

To the best of our knowledge, this is the first time the presence of pinoresinol is reported in cold-pressed grape seed oils; we presume it is also present in grape seeds. In another oleaginous matrix, namely olives (*Olea europaea* L.), the presence of lignans (pinoresinol and 1-acetoxypinoresinol) before the milling process has not yet been confirmed, despite their presence in the corresponding virgin olive oils (Cecchi et al., 2013; Cecchi, Migliorini, Cherubini, Innocenti & Mulinacci, 2015).

In this study, we analyzed a set of oils purchased from a production plant that works with about one ton of seeds per time and, consequently, it is not possible to completely exclude the co-presence of vine shoots as a possible source of lignans. To clarify this, we investigated the presence of the lignan pinoresinol at laboratory scale in fresh grape seeds of a widespread wine variety, namely Sangiovese. In order to simulate the seed contact with the extracted oil during the productive process, seeds were ground and extracted with a commercial refined sunflower oil, previously analyzed to exclude the presence of detectable amounts of phenolic compounds.

The histogram in Figure 3 clearly shows that the typical components (catechins, procyanidins, gallocatechins) of either aqueous or hydroalcoholic extracts of grape seeds are absent in the oil extract. On the other hand, the presence of pinoresinol as the principal

extractable compound from the seeds was confirmed. Even though the test was carried out on a raw material different from that used to produce the analyzed oils, it demonstrated that this lignan can be considered a component of grape seeds. To date, pinoresinol has never been detected in this matrix, presumably because the extraction procedures were not suitable to recover this lipophilic phenol and/or because the molecule is present at very low concentrations in seeds.

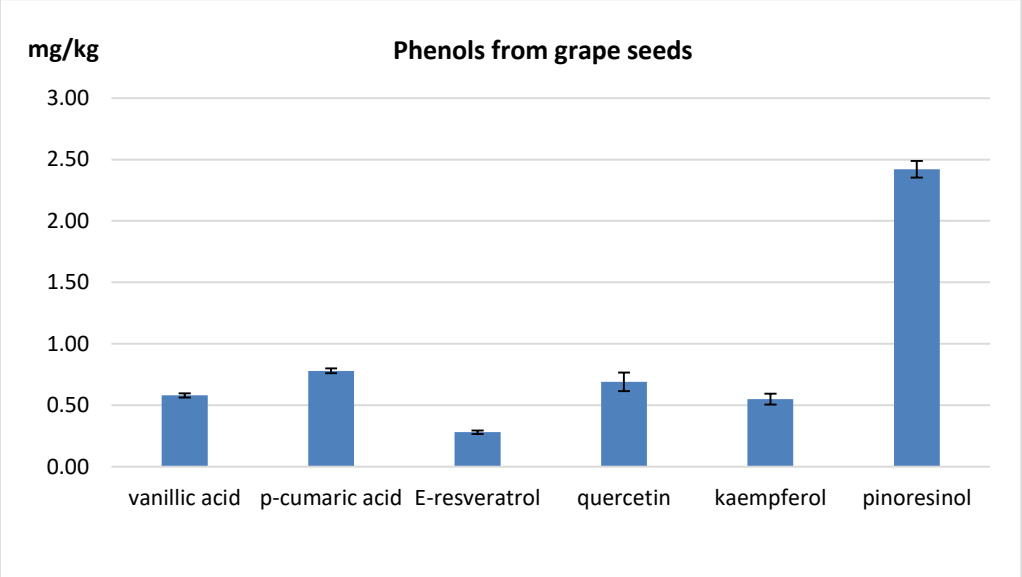

Figure 3. Phenols extracted by refined oil from dried powder of Sangiovese grape seeds. All phenolic compounds are expressed as $mg_{p\text{-}coum}$/kg, with the only exception for pinoresinol, which is in mg_{pin}/kg.

3.3 *In vitro* inhibition of PTP-1B enzyme

To evaluate the potential contribution of consuming cold-pressed grape seed oils to reduce the risk of type-two diabetes, a study on a specific enzymatic target, PTP-1B, was initially carried out working with the phenolic extracts of the selected oils.

After some preliminary tests (necessary to select the suitable concentration), the inhibitory power of the extracts was evaluated testing all the samples at a concentration of 6.67 mg_{oil}/mL. As summarized in Figure 4A, different potencies were found for the 17 samples. Maximum inhibition was close to 93-98 % for a group of five oils, while minimum inhibition power was shown by Caberbet Sauvignon (close to 40 %).

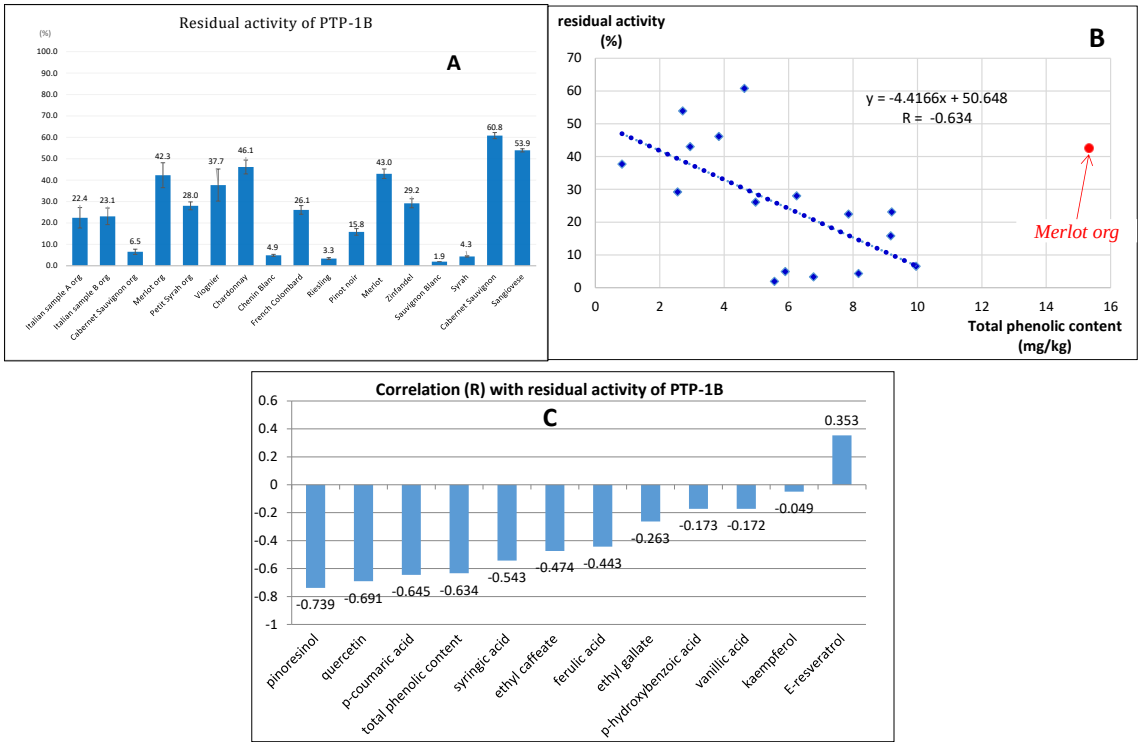

Figure 4. Residual activity of PTP-1B in presence of hydroalcoholic extracts of the analyzed samples evaluated at the same concentration (6.67 mg_{oil}/mL) (**A**). Correlation between residual activity of PTP-1B and total phenolic content of the analyzed extracts (**B**); the red point indicates the outlier. Correlations between the amount of each phenolic compound and residual activity of PTP-1B for the seventeen 'virgin' grape seed oils (**C**).

In order to verify a correlation between the inhibitory activity and the total phenolic content (TPC), we estimated the IC_{50} value of the samples at the lowest (Viognier) and highest (Merlot org) TPC. The curves in Figures 5A and 5B show a very similar power for both extracts (corresponding to 5.33 mg_{oil}/mL) in spite of the consistently different phenolic content (Figure 2), suggesting no correlation between TPC and inhibitory power. Nevertheless, Figure 4B shows a good correlation between TPC and the inhibitory power and points out that Merlot Org is clearly an outlier. This result can partially explain the similar IC_{50} values obtained for Viognier and Merlot Org samples.

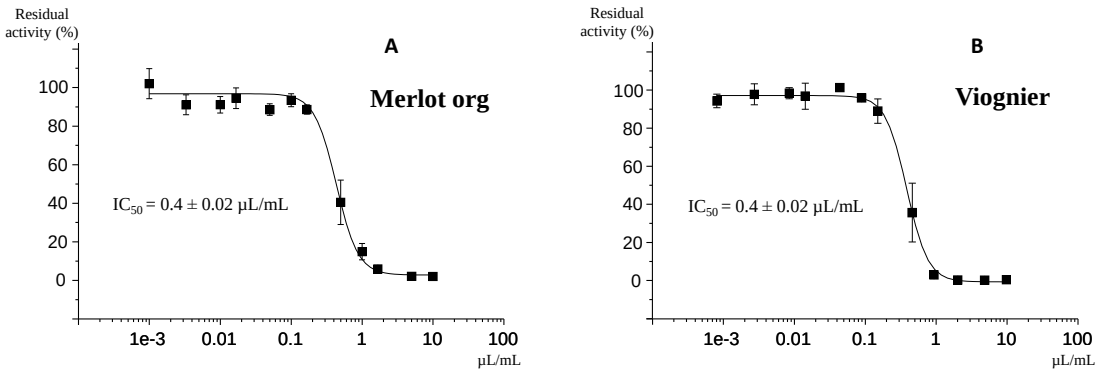

Figure 5. Evaluation of IC_{50} values for the "Merlot org" (**A**) and "Viognier" (**B**) extracts

Figure 4C reports the Pearson correlation coefficients between the residual activity of PTP-1B and the amount of each identified phenolic compound for the 17 'virgin' grape seed oils. Pinoresinol showed the highest negative correlation value among the identified phenols (R, -0.739); a slightly lower correlation was observed for *p*-coumaric acid and quercetin. The latter molecule is a flavonol already known as an inhibitor of PTP-1B, with an IC_{50} value of 0.98 µM as pure molecule (data not shown). All the other phenolic compounds identified in the extracts showed R values close to or below -0.5.

These preliminary results showed that the inhibitory power is partially correlated to the phenolic content of these 'virgin' grape seed oils and that pinoresinol and quercetin seem to give the highest contribution within this group of molecules. However, further studies are needed to complete identification of the other minor constituents of the seed oils and of their inhibition power of PTB-1B enzyme.

4. Conclusions

A detailed study on the phenolic content of a large pool of commercial cold-pressed grape seed oils determined by HPLC-DAD-MS-TOF and HPLC-FLD analysis is reported in this work. The presence of pinoresinol was confirmed for the first time in all these oils, together with some main flavonols such as quercetin and kaempferol, while catechin and its gallate forms were not detected, a result in disagreement with some previous works. Ethyl caffeate and ethyl gallate, detected in many of these oils, can be suggested as markers to evaluate the intensity of fermentation in grape seeds before oil extraction, but also to control the sensorial quality of the final oils.

Lastly, an inhibitory activity exerted by the phenolic fraction isolated from these oils against PTP-1B, an enzyme overexpressed in type-two diabetes, was demonstrated. This interesting data begs for further studies to confirm this action with other cold-pressed grape seed oils and to understand the mechanism behind this action.

Overall, these results highlight the greater health properties of the cold-pressed grape seed oils with respect to refined oils, which do not contain the pool of the phenolic molecules.

Acknowledgments

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8. RESULTS: STRATEGIES FOR USE BY-PRODUCTS FROM OLIVE OIL PRODUCTION

Recovery and stability over time of phenolic fractions by an industrial filtration system of olive mill wastewaters: a three years study

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Running title: Phenolic compounds in olive mill wastewaters obtained by an industrial filtration system

Maria Bellumori[a†], Lorenzo Cecchi[a†], Annalisa Romani[b], Nadia Mulinacci[a*], Marzia Innocenti[a]

[†]these authors contributed equally to this work

[a]Department of Neuroscience, Psychology, Drug and Child Health (NEUROFARBA), University of Florence, Via Ugo Schiff 6, 50019 Sesto F.no, Florence, Italy
[b]Department of Statistic, Informatics and Applications, "G. Parenti", University of Florence, Viale Morgagni 59, Florence, Italy

* **Corresponding author**: Tel +39-055-4573773; Fax +39-055-4573737
E-mail address: nadia.mulinacci@unifi.it

Abstract

Background. The recovery of phenolic compounds from olive milling is recognized as strategic for producers. The aim of this work was to evaluate the quality and stability of retentates obtained from olive mill wastewaters treated with a membrane filtration system constituted by a micro-, ultra- and nanofiltration followed by a final reverse osmosis, over three crop seasons. Efficiency was evaluated in terms of phenolic amount in the retentates and of organic load in the final discarded waters. Phenolic compounds were quantified using tyrosol as external standard.

Results. Our study highlighted a reproducibility of the process over years and a low organic load in permeates from reverse osmosis. Hydroxytyrosol was very stable in the liquid products at 18-28°C over 24 months of storage. The retentates from reverse osmosis showed the highest phenolic content (78.6 mg g^{-1} dm in 2015), associated with a potassium content of 22 g kg^{-1}.

Conclusions. The liquid concentrated retentates showed an unexpected stability over time of their bioactive phenolic compounds, particularly of hydroxytyrosol. These samples recovered from olive mill wastewaters can be good sources of natural antioxidants and potassium to guarantee the correct intake and to formulate new food ingredient or food products.

Keywords: Temperature effect; storage stability; hydroxytyrosol; verbascoside; olive mill wastewaters

1. Introduction

In the last decades the interest of the scientific community towards phenolic compounds of olive oil was strongly increased,[1–4] mainly thanks to the health benefits that they confer to the oils.[5–7] A series of *in vivo* human studies[2,8] allowed EFSA to approve an important health claim for virgin olive oils with enough amounts of phenolic compounds. It is possible to add in label "the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress", when at least 5 mg of total hydroxytyrosol are present in 20 mL of extra virgin olive oil.[9]

It is well known that whole lyophilized olives are very rich in phenolic compounds (e.g. oleuropein was up to 80 g kg^{-1} in Frantoio cv[10]), but at the same time only a minor part of this fraction passes into the oil during the milling process.[11,12] Due to their predominant hydrophilic nature, the huge amount of phenolic compounds is lost in olive mill wastewaters (OMWWs) and in solid pomace residue recovered after the separation of the oil in the decanter. The amount of OMWWs and the percentage of phenolic compounds in OMWWs and in solid pomace strongly depend on the applied processing: the widely used three-phase system requires addition of variable amounts of water.

The worldwide production of OMWWs (composed by a complex mixture of sugars, tannins, pectin, lipids, minerals and phenolic compounds) was estimated in more than 30 million m^3 per year.[12] The organic load of these wastewaters is responsible for the high BOD (from 15 to 135 g L^{-1}) and COD (from 37 to 318 g L^{-1}) values.[13] Due to their composition, OMWWs have a high environmental impact, are phytotoxic, and their disposal in fertile lands is strongly limited by law in terms of quantities h^{-1} and type of cultures.[14] To date, the disposal of this high pollutant waste is a problem for the oil mills and requires additional costs.[15,16]

In recent decades, the increasing worldwide demand of olive oil results in a strong growth of operating mills, hired people and amount of olive oil produced. One undesired side-effect of such a growth is the strong increase of milling wastes and, among these, of produced OMWWs.[14]

Among the several treatments proposed to reduce the organic load of OMWWs, most of them resulted not fully satisfactory and unable to resolve the problem. At the same time, the high content of phenolic compounds with strong antioxidant activity (mainly hydroxytyrosol, oleuropein derivatives and verbascoside) makes the OMWWs

an interesting source for these bioactive compounds potentially applied in nutraceutical market, in food and beverages formulations as well as in new cosmetic products.[12,17]

Among the different methods proposed and adopted to recover phenolic compounds from OMWWs, the use of membrane-systems seems to be the most promising and applied on large scale nowadays. Several works and experimental results confirmed that the future direction of the processes for the recovery of antioxidants from OMWWs is toward the utilization of membranes in a sequential design.[18–21] Conventional physicochemical technologies, like microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), are generally assumed as being safe and cheap since most of them have been widely applied in different food industry and potable water sectors.[19]

One key aspect is to select the suitable membranes to recover and concentrate the phenolic compounds from vegetation waters. Cassano et al. (2011)[22] proposed UF membranes with regenerated cellulose suitable to guarantee an enhancement of the phenolic concentration in the permeate stream in comparison with the feed solution. NF and RO processes have been proposed alternatively to concentrate specific phenolic classes,[23] whereas Garcia-Castello et al. (2010)[24] used a system including MF and NF, osmotic distillation and vacuum membrane distillation (VMD) to purify and concentrate phenolic fractions from OMWWs obtaining a recovery of 78% of the initial content of phenolic compounds in the permeate stream.

Nevertheless, a systematic study on industrial filtration-membrane systems associated to the phenolic composition and the evaluation of the stability over time of the produced retentates is not available to date. A detailed characterization of the final retentates, correlated to the applied filtration process, is a necessary step for the olive oil producers to valorize these milling by-products and to transform them in commercial products.

Aim of this work was to evaluate the reproducibility and the efficiency over three years (2013-2015) of an industrial membrane-system dedicated to recover the phenolic fractions from the treatment of OMWWs. The further goal was to evaluate the effects of concentration at different temperatures on the retentates in terms of phenolic profiles and stability after several months of storage. The retentates from ultrafiltration, nanofiltration and reverse osmosis were analyzed by HPLC, both as such and in liquid concentrated

forms obtained at various temperatures. To better characterize the retentates, the phenolic content was calculated both as mg L^{-1} and as mg g^{-1} dry sample.

2. Experimental

2.1 Chemicals

All chemicals for the analyses were of analytical reagent grade. The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce deionized water. Methanol of HPLC grade was purchased from J.T. Baker (Phillipsburg, New Jersey, USA); ethanol and acetonitrile of HPLC grade were purchased from Panreac (Barcelona, Spain). Tyrosol (98%), hydroxytyrosol (98%) and verbascoside (>99%) from Sigma-Aldrich (Steinheim, Germany) were used as standard compounds for the quantitative determination.

2.2 Samples

Samples of OMWWs (cultivar Carolea) from 2013, 2014 and 2015 crop seasons were treated with a sequence of filtration steps by a membrane system (flow rate 1000 L h^{-1} OMWWs) in Azienda Agricola Fangiano (Nocera Terinese, Catanzaro, Italy), as described in Figure 1.

Briefly, a pre-filtration step was carried out in a series of three cartridges with decreasing pore diameters of 100, 50 and 25 μm. Aiming to remove the suspended particles, a pre-clarification step, namely microfiltration (MF), was carried out operating in the particle size range 0.1-1 μm. Permeates from both pre-filtration and filtration steps were pushed towards the next membrane by a pressure pump. The first filtration step was ultrafiltration (UF), which operated in the particle size range of 0.01-0.1 μm and generated the corresponding retentate (RUF) and permeate (PUF); the next step was nanofiltration (NF) with particles size range of 1-10 nm, which generated the relative retentate (RNF) and permeate (PNF). The last filtration step was reverse osmosis, (particles size range 0.1-1 nm), which generated the final retentate (ROI) and the corresponding permeate (POI).

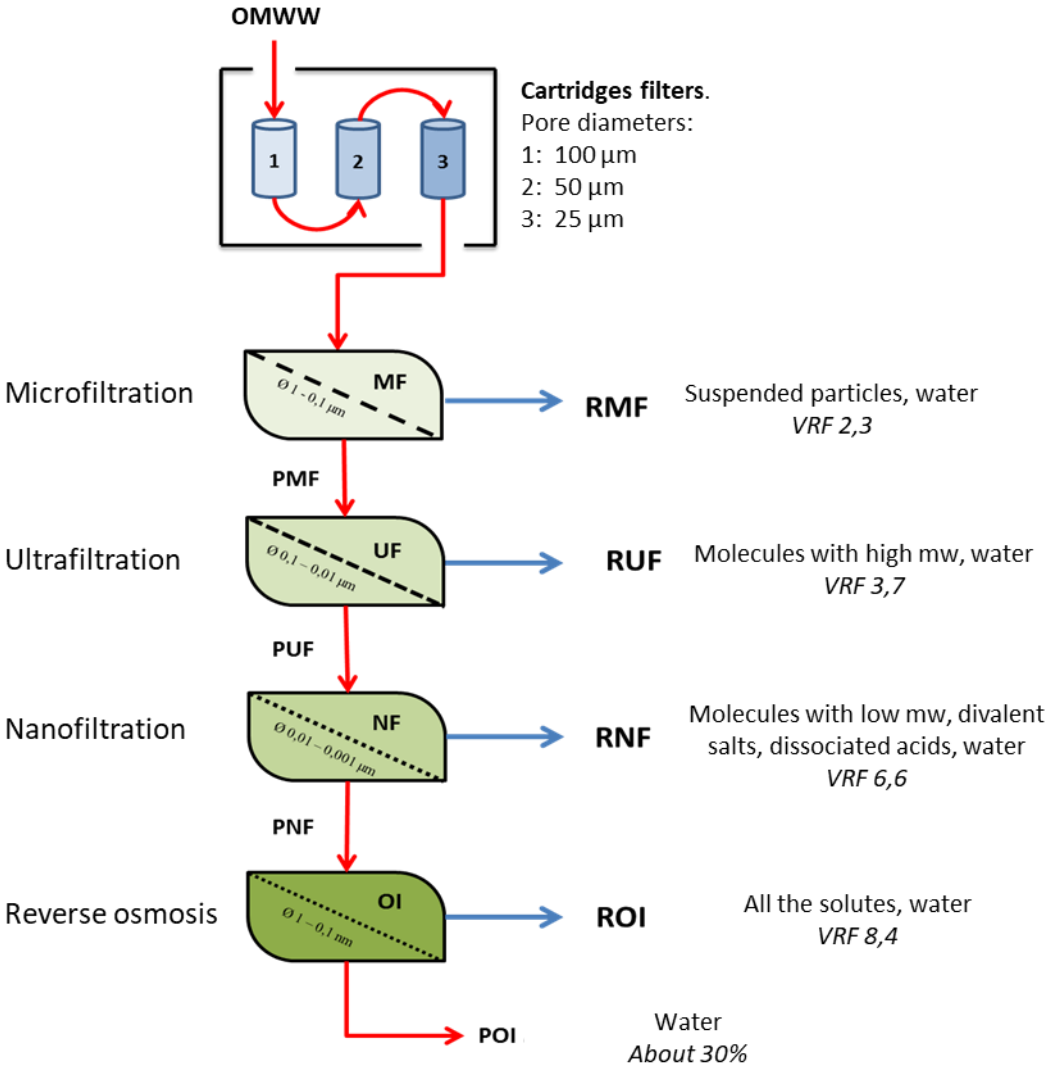

Figure 1. Scheme of the processing of olive mill waste water (OMWW). R, retentate; P, permeate; MF, microfiltration; UF, ultrafiltration, NF, nanofiltration; OI, reverse osmosis; VRF, volume reduction factor

As summarized in Table 1, aliquots of RUF, RNF and ROI were concentrated at different temperatures. Samples from 2013 and 2014 were concentrated by a shell and a tube vacuum evaporator, with a required time of 5 hours for a volume of 50 L. Retentates from 2015 were concentrated by a jacketed vacuum evaporator, with a concentration time of 4 hours for a volume of 70 L.

The OMWWs were produced in November; the name of each sample was built taking into account the type of retentate, the year of production, the applied temperature for concentration, and the theoretical concentration factor, calculated by the volume reduction.

Sample	Year	Applied Temp. (°C)
RUF1324	**2013**	-
RNF1324		-
ROI1324		-
RUF1330		30
RNF1330		30
ROI1330		30
RUF1355a		55
RNF1355a		55
ROI1355a		55
RUF1355b		55
RNF1355b		55
ROI1355b		55
RUF1424	**2014**	-
RNF1424		-
ROI1424		-
RUF1445		45
RNF1445		45
ROI1445		45
PUF1424		-
PNF1424		-
POI1424		-
RMF1524	**2015**	-
RUF1524		-
RNF1524		-
ROI1524		-
RUF1560		60
RNF1560		60
ROI1560		60
PMF1524		-
PUF1524		-
PNF1524		-
POI1524		-

Table 1. List of the analyzed samples. The first three letters define the type of retentate (R) or permeate (P), the successive two numbers indicate the year and the last two numbers indicate the temperature applied during the concentration ("24" indicate the non-concentrated samples). The letters "a" and "b" for 2013 retentates indicate two different concentrations of these samples. MF, microfiltration, UF, ultrafiltration; NF, nanofiltration; OI, reverse osmosis

The evaluation of the dry weights was performed in triplicate on 5 mL (concentrated samples) and 10 mL (non-concentrated samples), in oven at 105°C until constant weight.

2.3 HPLC-DAD-MS-TOF analysis of phenolic compounds

Each sample was centrifuged (14,000 xg, 20°C, 5 min) before the chromatographic analysis for fully removing the suspended particles. The concentrated

samples were diluted 1:10 with distilled water in order to analyze samples with similar concentration in phenolic compounds. Analysis of phenolic compounds were performed using a HP 1200L Liquid Chromatograph coupled in series with a DAD detector and a TOF Mass Spectrometer equipped with electrospray interface (ESI), all from Agilent Technologies, (Palo Alto, CA, USA). A 150 mm x 3 mm i.d., 2.7 μm Poroshell 120, EC-C18 column equipped with a precolumn of the same phase was used (Agilent Technologies). The analysis were carried out in negative ion mode, with spectra acquired in a mass range of 80-1000 Th. The conditions of ESI source were: drying gas (N_2), temperature 350°C, drying gas flow rate 6 L min^{-1}, nebulizer 20 psi, capillary voltage 4000 V, fragmentation 150 V, skimmer 60 V. Acquisition and data analysis were controlled using the Agilent MassHunter Qualitative Analysis Software version B.02.01 (Agilent Technologies).

Solvents for the mobile phase were (A) 0.1% formic acid/water and (B) CH_3CN; flow rate 0.4 mL min^{-1}; the multi-step linear solvent gradient used was: 0-40 min 5-40% B; 40-45 min 40% B; 45-50 min 40-100% B; 50-53 min 100% B; 53-55 min 100-5% B; equilibration time after each analysis was 10 min. The wavelengths simultaneously selected were: 240 nm, 254 nm, 280 nm, 330 nm and 350 nm. Tyrosol, hydroxytyrosol and verbascoside were identified by comparison with retention time, UV and mass spectra of the corresponding pure standards; tyrosil- and hydroxytyrosol-glucosides were identified by their UV and mass spectra and according to literature data.

2.4 Quantitative evaluation by HPLC-DAD

The standards, all at the same purity grade (98%), were used to prepare three five-point calibration curves: tyrosol with R^2 0.9999, hydroxytyrosol with R^2 1, and verbascoside with R^2 0.9993. All the standards were evaluated at 280 nm and their final concentration in the liquid samples were expressed as mg L^{-1}. The total phenolic content was expresses as sum of all the phenolic compounds, including minor derivatives of tyrosol and hydroxytyrosol.

2.5 Chemical analysis for the nutritional values

The moisture content (U, g kg^{-1}) was evaluated by gravimetric analysis, placing the suitable amount of sample in oven at 105°C until reaching constant weight, according

to Rapporti ISTISAN 1996/34.[25] Protein content (PC) was evaluated by Kjeldhal assay and applying: PC (g kg^{-1}) = N*6.25, where N is total nitrogen, according to ISS protocol.[25] Ashes content (AC) was evaluated by gravimetric assay, according to ISS protocol. The total fat content (TFC) was determined on the dried samples by Soxhlet extraction, as previously described.[10] Total Carbohydrates Content (TCC) was calculated according to USDA,[26] as TCC (g kg^{-1}) = 1000 – U - PC – AC – TFC. Regarding the carbohydrate composition, simple sugars (glucose, fructose and sucrose) were assayed by an enzymatic method, as described elsewhere,[27] while dietary fiber (both soluble and insoluble) were quantified according to official methods.[28] Sodium and potassium were assayed by using atomic absorption spectroscopy and five point calibration curves. Finally, energy values (EV) were calculated by using the second Atwater approximation: EV (Kcal kg^{-1}) = 4*(TCC+PC) + 9*TFC.[26]

2.6 Stability over time of the concentrated retentates

Changes in the phenolic profiles of the concentrated retentates were evaluated by repeating the HPLC-DAD-MS-TOF analysis over time. The analysis of six concentrated samples from 2013 (RUF1355a, RNF1355a, ROI1355a, RUF1355b, RNF1355b, ROI1355b) and three of 2014 (RUF1445, RNF1445, ROI1445) were repeated 18 months after their concentration. The analysis of the three concentrated samples from 2015 (RUF1560, RNF1560, ROI1560) were repeated 6 months after the concentration step. In order to simulate storage conditions that can be applied in the mill and to evaluate the temperature effects, samples were maintained in the dark, in a plastic bottle (100 mL), at temperatures ranging from 18 to 28 °C.

2.7 Precision parameters and statistical analysis

Several retentates (RUF1355a, RNF1355a, ROI1355a, RUF1355b, RNF1355b, ROI1355b, RUF1445, RNF1445, ROI1445, RUF1560, RNF1560 and ROI1560) were selected to evaluate the precision of the analytical procedure for the quantitative analysis of the principal phenolic compounds. These samples were blended (each in the same volume) to obtain a reference sample that was homogenized and analyzed six times. Data were expressed in terms of CV% (Table 2). Analysis of variance and F-Test ($P < 0.05$) were performed using Microsoft Excel statistical software to evaluate statistical

significance. Fisher's LSD test was then applied to compare the means by using the software DSAASTAT v. 1.1.

Compound name	Average (mg L^{-1})	SD	CV%
hydroxytyrosol	16166.6	155.6	1.0
tyrosol	2413.8	30.5	1.3
verbascoside	314.1	4.7	1.5
total phenolic compounds	21630.2	222.0	1.0

Table 2. Average content of the main detected compounds obtained analyzing a mix of nine retentates, aimed to the estimation of precision of the quantitative evaluation. The average values were calculated by six independent injections of the mixture carried out in the same day.

3. Results and discussion

This work, developed over three successive years, allowed to collect and analyze by HPLC-DAD-MS-TOF different retentates obtained using the same filtration system (Table 1). Figure 2 shows a representative profile at 280 nm of one of these retentates, the RNF1524, with the main identified phenolic compounds. It is well known that oleuropein is quickly deglycosilated after olive crushing and then stepwise degraded to different derivatives and finally hydroxytyrosol; according to this, this secoiridoid was not detected in any of our samples.

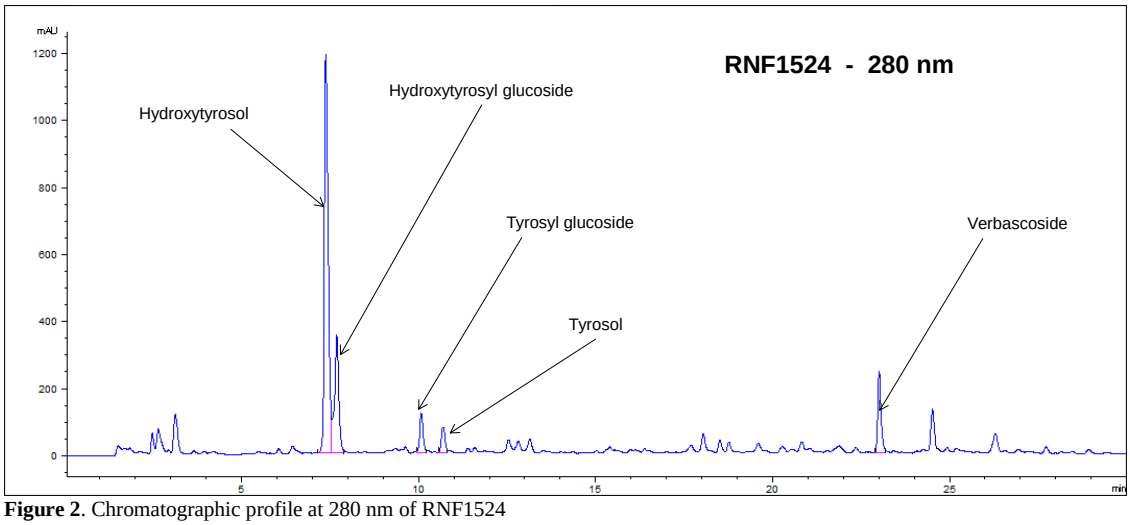

Figure 2. Chromatographic profile at 280 nm of RNF1524

3.1 Optimization of the quantitative evaluation

This step was carried out working on a representative sample built as a mixture of twelve different retentate. Table 2 shows the average values (six replicates of the same

blend sample), standard deviation and CV%, for the main detected compounds. As expected, CV% values were very low, according to the simple management of samples before the chromatographic analysis (only a dilution and a centrifugation) and to the good chromatographic resolution (Figure 2).

In order to express the amount of the single phenolic compounds and to define a rapid, not expensive and accurate method, two different approaches were applied and compared. Firstly, we measured tyrosol, hydroxytyrosol and verbascoside by the calibration curves of the corresponding pure standards; the minor derivatives of tyrosol and hydroxytyrosol were expressed as $mg_{tyr}\ L^{-1}$ and $mg_{OH\text{-}tyr}\ L^{-1}$, respectively. Nevertheless, we observed a rapid degradation of hydroxytyrosol in water media. This finding, and the high cost of this standard (25 times higher with respect to tyrosol), induced us to propose the use of tyrosol as reference standard to quantify all the identified molecules. Using the calibration curve of tyrosol at 280 nm, the content of hydroxytyrosol resulted overestimated of 35% and consequently the following formula has to be applied to obtain the accurate amount of hydroxytyrosol and its minor monoglycosides:

$$mg_{OH\text{-}tyr} = mg_{tyr} * 0.65$$

The same approach was used to express also the verbascoside amount applying the following formula:

$$mg_{verb} = mg_{tyr} * 0.64.$$

In conclusion, the use of tyrosol as unique external standard and the application of the suitable corrective factors to quantify the main phenolic compounds in the OMWWs, allowed to short and simplify the analytical method, to reduce the total cost of analysis and to maintain a good accuracy of the data.

3.2 Phenolic characterization

The main findings for all the retentates are summarized in Table 3, which reports the dry weights correlated to tyrosol, hydroxytyrosol and total phenolic content (TPC). It clearly appears how the dry weights of the not concentrated retentates from 2013 and

Sample	dry weight		Hydroxytyrosol				Tyrosol				Total Phenolic Compounds			
	g L^{-1}	Conc. factor	mg_{OHTYR} L^{-1}	Conc. factor	mg g^{-1} dm	% p/p	mg_{TYR} L^{-1}	Conc. factor	mg g^{-1} dm	% p/p	g L^{-1}	Conc. factor	mg g^{-1} dm	% p/p
RUF1324	40.8 ± 1.0 B	-	702.9 AB	-	17.2	1.7	274.6 B	-	6.7	0.7	1.87 B	-	45.7	4.6
RNF1324	82.3 ± 1.2 F	-	872.5 B	-	10.6	1.1	270.1 B	-	3.3	0.3	2.48 C	-	30.2	3.0
ROI1324	51.9 ± 0.4 C	-	2287.7 E	-	44.1	4.4	826.9 G	-	15.9	1.6	4.48 F	-	86.3	8.6
RUF1330	115 ± 0.8 G	2.8	3704.8 F	5.3	32.2	3.2	755.0 F	2.7	6.6	0.7	5.40 H	2.9	46.9	4.7
RNF1330	242.3 ± 2.3 K	2.9	6264.6 J	7.2	25.9	2.6	1435.8 J	5.3	5.9	0.6	9.49 L	3.8	39.2	3.9
ROI1330	155.1 ± 2.2 I	3.0	8940.9 L	3.9	57.6	5.8	1238.9 I	1.5	8.0	0.8	11.98 N	2.7	77.2	7.7
RUF1355a	145.6 ± 1.5 H	3.6	6256.3 J	8.9	43.0	4.3	1061.5 H	3.9	7.3	0.7	8.78 K	4.7	60.3	6.0
RNF1355a	277.7 ± 3.9 L	3.4	7613.1 K	8.7	27.4	2.7	1703.6 K	6.3	6.1	0.6	11.43 M	4.6	41.2	4.1
ROI1355a	229.6 ± 1.5 J	4.4	12658.1 M	5.5	55.1	5.5	1906.8 M	2.3	8.3	0.8	17.27 O	3.9	75.2	7.5
RUF1355b	383.7 ± 3.6 O	9.4	15010.2 N	21.4	39.1	3.9	2723.1 P	9.9	7.1	0.7	21.52 Q	11.5	56.1	5.6
RNF1355b	668.8 ± 4.6 R	8.1	18976.7 O	21.8	28.4	2.8	4062.1 S	15.0	6.1	0.6	28.78 R	11.6	43.0	4.3
ROI1355b	480.8 ± 1.9 P	9.3	24364.5 R	10.7	50.7	5.1	3874.9 R	4.7	8.1	0.8	33.66 T	7.5	70.0	7.0
RUF1424	36.4 ± 1.1 A	-	504.5 A	-	13.9	1.4	182.3 A	-	5.0	0.5	1.27 A	-	35.0	3.5
RNF1424	82.6 ± 1.5 F	-	575.8 A	-	7.0	0.7	180.7 A	-	2.2	0.2	1.84 B	-	22.3	2.2
ROI1424	51.6 ± 1.1 C	-	2007.0 D	-	38.9	3.9	752.1 F	-	14.6	1.5	3.97 E	-	76.9	7.7
RUF1445	279.2 ± 2.7 L	7.7	5354.5 H	10.6	19.2	1.9	632.4 E	3.5	2.3	0.2	7.78 I	6.1	27.9	2.8
RNF1445	352.3 ± 2.6 N	4.3	5614.3 I	9.8	15.9	1.6	616.3 E	3.4	1.7	0.2	8.28 J	4.5	23.5	2.4
ROI1445	333.6 ± 2.1 M	6.5	12521.0 M	6.2	37.5	3.8	1816.5 L	2.4	5.4	0.5	17.81 P	4.5	53.4	5.3
RMF1524	53.6 ± 0.5 CD	-	1634.7 C	-	30.5	3.0	282.7 B	-	5.3	0.5	2.02 B	-	37.7	3.8
RUF1524	56.8 ± 1.1 DE	-	1792.5 C	-	31.6	3.2	329.5 C	-	5.8	0.6	2.42 C	-	42.6	4.3
RNF1524	59.2 ± 1.7 E	-	2008.9 D	-	33.9	3.4	203.3 A	-	3.4	0.3	3.47 D	-	58.6	5.9
ROI1524	39.1 ± 0.8 AB	-	4109.3 G	-	105.1	10.5	378.9 D	-	9.7	1.0	4.88 G	-	124.9	12.5
RUF1560	786 ± 3.9 S	13.8	22498.9 Q	12.6	28.6	2.9	2556.7 O	7.8	3.3	0.3	31.42 S	13.0	40.0	4.0
RNF1560	908 ± 4.5 T	15.3	21875.6 P	10.9	24.1	2.4	2001.9 N	9.8	2.2	0.2	35.38 U	10.2	39.0	3.9
ROI1560	651 ± 3.2 Q	16.6	39000.5 S	9.5	59.9	6.0	3322.2 Q	8.8	5.1	0.5	51.15 V	10.5	78.6	7.9

Table 3. Dry weight and phenolic content of retentates from 2013, 2014 and 2015 crop seasons. The concentration factors were calculated as dry weight ratio between fresh and corresponding concentrated retentates. The concentration factors for the Total Phenolic Compounds (TPC) were calculated as the phenolic content ratio between fresh and correlated concentrated retentate. Dry weight data (g L^{-1}) are the mean of three determinations; variability of data for phenolic compounds (expressed as mg L^{-1} for single phenols and g L^{-1} for TPC on dried matter) are expressed as CV% evaluated on six replicates (see also table 2). In each column, different letters indicate significant differences at $P < 0.05$.

2014 crop season were very similar (RUF, 40.8 g L^{-1} vs 36.4 g L^{-1}; RNF, 82.3 g L^{-1} vs 82.6 g L^{-1}; ROI, 51.9 g L^{-1} vs 51.6 g L^{-1}) and no significant differences were highlighted for RNF and ROI. Conversely, values from 2015 were quite different.

The differences among the samples of 2013/2014 and those of 2015 can be attributable to the variability of OMWWs composition, to the applied milling process, but also to the different concentration process. Taking into account the concentration factors (calculated on the dry weight ratios of each couple of fresh and concentrated retentates), samples of 2015 crop season resulted those subjected to the highest level of concentration. These findings were in agreement with the physical characteristics of the samples, which showed a higher density than the corresponding ones of 2013 and 2014.

As expected, and according to the literature,[29,30] hydroxytyrosol was the major component of all the retentates in fresh and concentrated samples; its concentration increased from RUF to RNF, and reached the maximum values in ROI. Particularly, hydroxytyrosol was 30-40% of TPC in RUF and RNF of 2013 and 2014, reaching 55-70% in RUF and RNF of 2015. Analogously, hydroxytyrosol concentration was close to 50% of TPC in ROI of 2013 and 2014, and strongly increased (up to 84% of TPC) in ROI of 2015.

It is worth to underline the increased amount of hydroxytyrosol in the concentrated RUF and RNF of 2013 and 2014, with values higher than those simply calculated applying the concentration factor based on their dry weights ratios. On the opposite, in ROI samples the hydroxytyrosol amounts were approximately the same of those calculated, because the potential precursors of this phenol were absent in this retentate. ROI was obtained at the end of the filtration process, and the molecules with high molecular size (glycosil derivatives of tyrosol, hydroxytyrosol and secoiridoids) were not concentrate in this kind of retentate, but remained in RUF and RNF.

Comparing the quantitative data in Table 3, it emerges that the temperatures applied during the concentration step, induced in RUF and RNF a partial hydrolysis of the glycosidic forms, with a consequent increase of free hydroxytyrosol concentration. The only exception were RNF1560 and ROI1560, in which the amount of hydroxytyrosol decreased after the concentration step. Overall, the concentrated retentates of 2015 showed a consistently higher amount of hydroxytyrosol with respect to 2013 and 2014. The different behavior of the concentrated retentates from 2015 can be associated to the

composition of the fresh OMWWs richer in free hydroxytyrosol and with lower contents of its glycosides, but also to the higher temperature applied for their concentration.

Regarding tyrosol, its amount in retentates of 2013 and 2014 was about one-third than hydroxytyrosol, while in 2015 was from one-fifth to one-tenth inferior than hydroxytyrosol. The amounts in the non-concentrated RUF and RNF samples were similar for all the three years (from 180.7 to 329.5 mg_{tyr} kg^{-1}); higher concentrations were in ROI from 2013 and 2014 (826.9 mg_{tyr} kg^{-1} and 752.1 mg_{tyr} kg^{-1} respectively) and a lower amount was found in ROI of 2015 (378.9 mg_{tyr} kg^{-1}). In all the three years tyrosol did not increase from RUF to RNF, but only in ROI, confirming the good reproducibility of the filtration system and the ability to concentrate these small phenols during the latter filtration step. Overall, the tyrosol amounts in all the concentrated retentates were about one order of magnitude lower than those of hydroxytyrosol.

Due to its high polarity, verbascoside is abundant in olives but not into the oil and it is fully lost into the by-products. Although verbascoside is a minor component, is well recognized as a bioactive molecule.[31–33] Its chemical structure contains one moiety of caffeic acid and one of hydroxytyrosol (Figure 3) and the enzymatic hydrolysis processes during the milling can contribute to reduce its concentration in OMWWs.

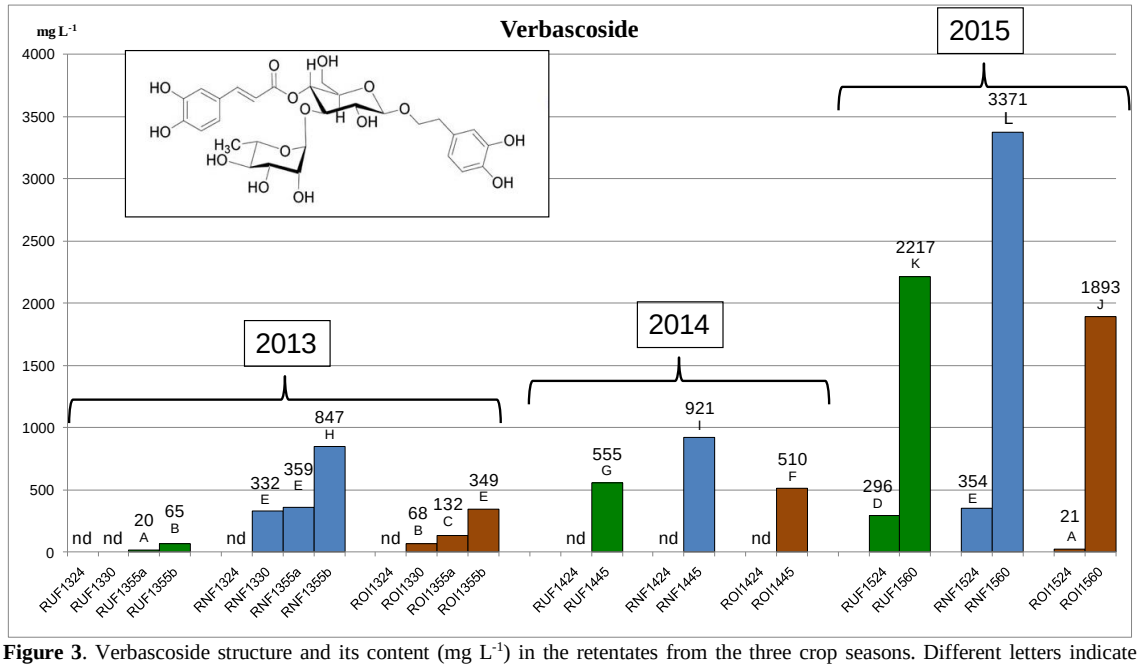

Figure 3. Verbascoside structure and its content (mg L^{-1}) in the retentates from the three crop seasons. Different letters indicate significant differences at $P < 0.05$. Variability of data (as CV%) was evaluated on six replicates.

Due to its molecular size and structural conformation, verbascoside was mainly retained by nanofiltration membrane, and consequently the richest retentates were RNFs (Figure 3).

The TPC in all the series of fresh samples increased from RUF to ROI (from 1865.3 mg kg^{-1} to 4479.4 mg kg^{-1} in 2013, from 1273.4 mg kg^{-1} to 3968.0 mg kg^{-1} in 2014, from 2417.0 mg kg^{-1} to 4884.0 mg kg^{-1} in 2015), highlighting the good efficiency and reproducibility of the filtration system used in this study. As expected, the same trend was confirmed in the concentrated retentates (Table 3).

3.3 Phenolic characterization of permeates

After the preliminary studies on the retentates from 2013 crop seasons, the permeates of the next two years were analyzed for their phenolic content to indirectly evaluate the efficiency of the membrane filtration system. As reported in Figure 4, dry weights of 2015 permeates were consistently lower than those of 2014.

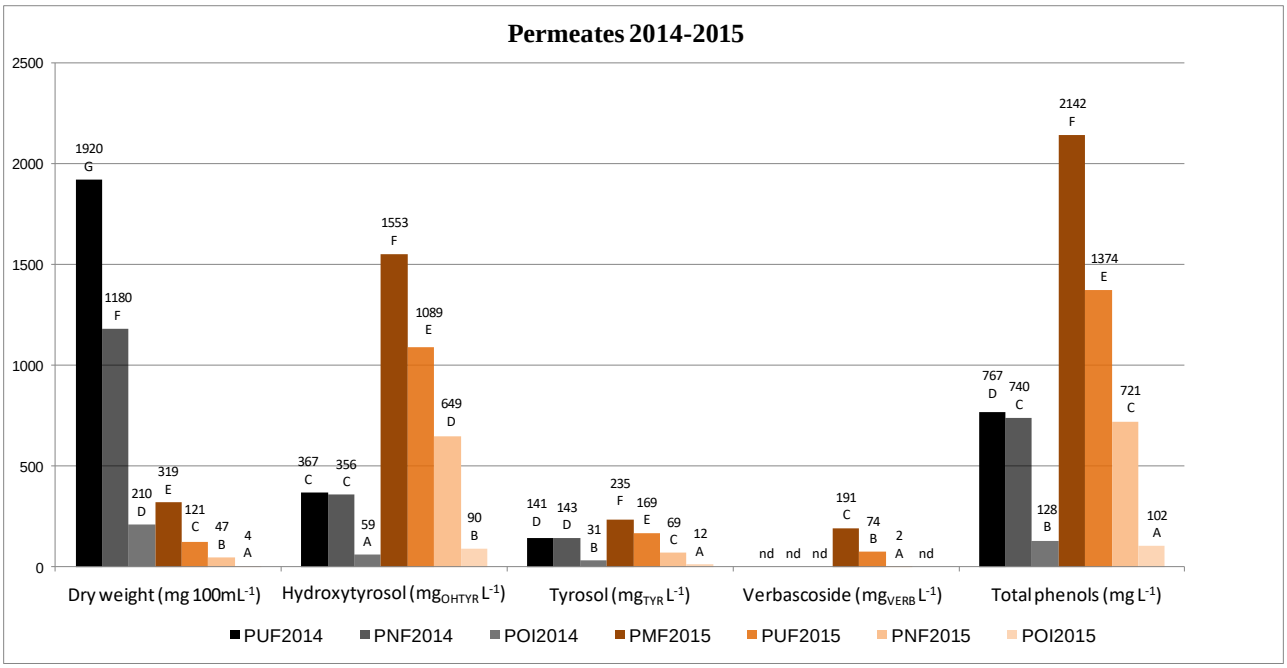

Figure 3. Dry weights and phenolic contents of the permeates from 2014 and 2015 crop seasons. For each parameters, different letters indicate significant differences at $P < 0.05$. Dry weight data are the mean of three determinations; variability of data for phenolic compounds (as CV%) was evaluated on six replicates.

These data pointed out the plant as an efficient system to recover almost all the phenolic compounds, particularly after the reverse osmosis step, producing final permeates (POI2014 and POI2015) with a very low content of solid residue (0.21 g L^{-1} for POI2014 and 0.04 g L^{-1} for POI2015) and consequently of organic load. Particularly,

hydroxytyrosol and total phenolic compounds were strongly reduced from PUF to POI of 2015 (hydroxytyrosol from 1088.5 mg L^{-1} to 90.0 mg L^{-1} and total phenolic compounds from 1373.7 mg L^{-1} to 101.9 mg L^{-1}), in agreement with the observed strong re-concentration step in the corresponding retentates (e.g. 4109.3 mg L^{-1} of hydroxytyrosol in ROI2015).

3.4 Stability over time of the concentrated retentates

Because these samples can undergo variable storage times and conditions before their use and/or commercialization (e.g. as natural source of antioxidant phenolic compounds), one aim of this work was evaluating the effects of long storage times on the phenolic composition of the concentrated retentates. The scope was to estimate if after several months (from 6 to 24) of storage in the dark, in closed plastic bottles, at temperature range of 18-28°C, the amounts of the main phenolic compounds drastically diminished. The purpose was to simulate a possible storage in a common shelf and in a room with temperature and relative humidity not fixed.

Data in Figure 5 on the concentrated samples of 2015, showed an unexpected stability for hydroxytyrosol (if compared with the pure standard in water) and for tyrosol after 6 months of storage, with only minor variations observed for verbascoside and TPC. The same behavior was observed for hydroxytyrosol, tyrosol, verbascoside and TPC for the concentrated samples of 2013 and 2014, submitted to a definitely longer storage time of 24 months.

It is worth noting that hydroxytyrosol, very unstable in pure form in water media, showed a very high stability in these aqueous concentrated samples after several months of storage, also in not fully controlled conditions. Indeed, it significantly diminished only in ROI1355a, RUF1355b and RNF1560, but in this latter sample the variation was very low (Figure 5A). Usually, to guarantee the stability of these samples, they are dried by adding maltodextrins to the aqueous extracts (from 40% to 50 % of the final weight) and then spray dried. Nevertheless, this practice is expensive, time consuming and unavoidably, and the dry sample with maltodextrin present a lower antioxidant content per g of product.

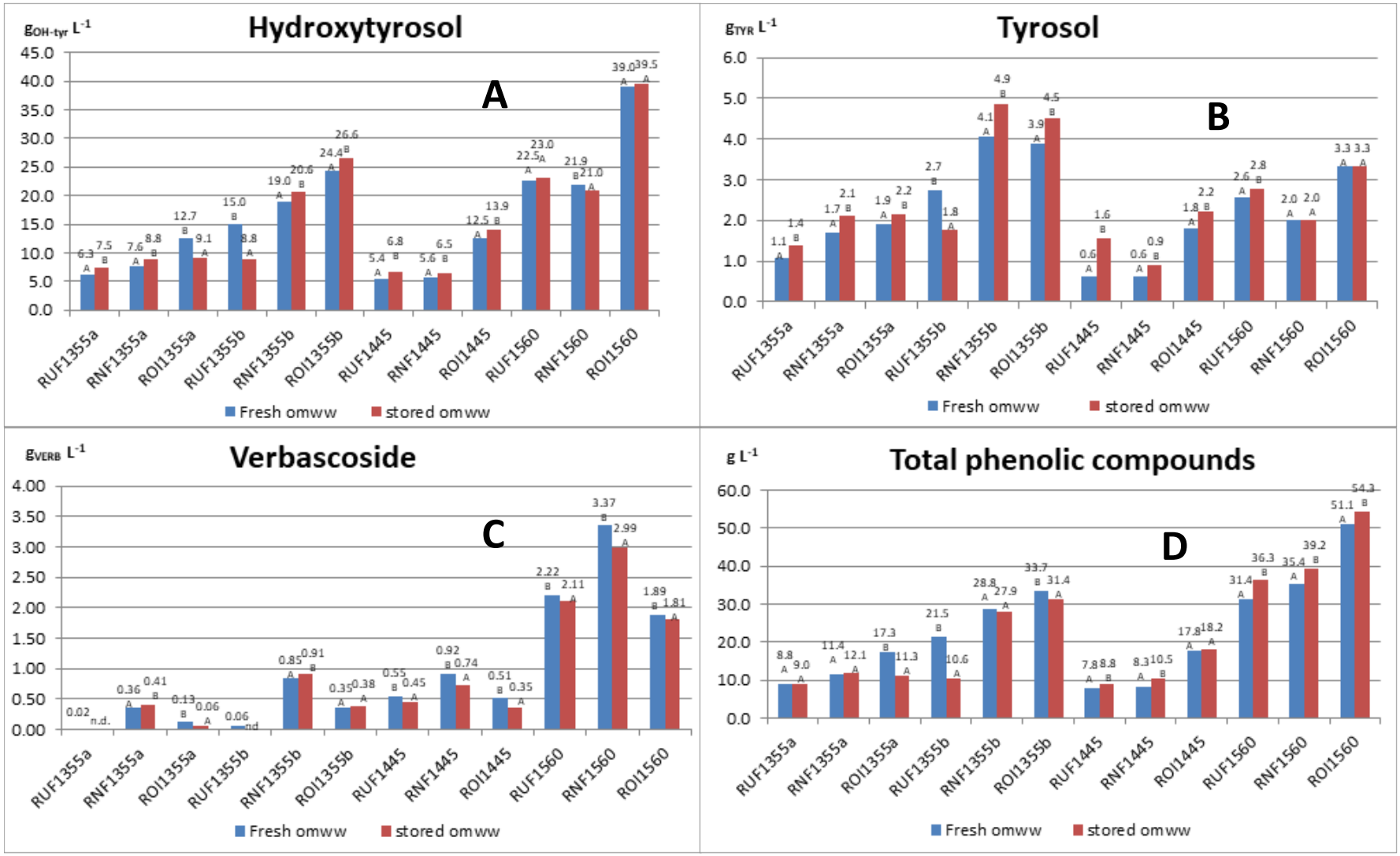

Figure 5. Effect of storage over time on the phenolic content of concentrated retentates: the analysis were repeated after 24 months on samples of 2013 and 2014, and after 6 months for samples of 2015. Different letters indicate significant differences at $P < 0.05$. Variability of data (as CV%) was evaluated on six replicates.

3.5 Nutritional evaluation of concentrated retentates of 2015

With the aim of a more complete characterization, the retentates of 2015 were analyzed for their nutritional profile. Results in Table 4 showed a negligible content of fats and proteins, a low sodium content and a high mineral amount (ashes from 106 to 125 g kg^{-1}), characterized by an high potassium content (up to 22 g kg^{-1} in ROI). It is recognized that athletes produce high levels of endogenous free radicals during their activity and, at the same time, they lose salts, particularly potassium. These concentrated retentates can be used to formulate food supplements for athletes, who often need the simultaneous supplementation not only of antioxidants but also of potassium in order to guarantee the recommended daily intake of this element estimated in 3g die^{-1}.

		RUF1560	RNF1560	ROI1560
Carbohydrates (g kg^{-1})		461 ± 12	540 ± 12	385 ± 12
Simple sugars (g kg^{-1})		3 ± 1	57 ± 4	15 ± 2
Fats (g kg^{-1})		< 2	< 2	< 2
Protein (g kg^{-1})		43 ± 1	30 ± 2	13 ± 2
Water content (g kg^{-1})		386 ± 2	313 ± 2	474 ± 2
Dietary fiber (g kg^{-1})	**Insoluble**	< 1	< 1	< 1
	Soluble	< 6	< 6	< 6
Ashes (g kg^{-1})		106 ± 5	114 ± 6	125 ± 6
Sodium (mg kg^{-1})		1046 ± 50	668 ± 30	727 ± 40
Potassium (mg kg^{-1})		20959 ± 1050	19557 ± 980	22365 ± 1110
Energy value (Kcal kg^{-1})		2030 ± 60	2290 ± 60	1600 ± 60
Energy value (KJ kg^{-1})		8610 ± 250	9720 ± 250	6810 ± 250

Table 4. Nutritional label of the concentrated retentates from 2015 crop season. Data are a mean of three determinations.

4. Conclusions

Samples obtained by a filtration system applied to treat OMWWs over three years were evaluated in terms of phenolic concentration. We proposed a simple analytical method, using tyrosol as unique standard and the application of suitable corrective factors to quantify all the main phenolic compounds in retentates. Due to the stability over time of their bioactive phenols, the concentrated liquid retentates can be proposed as possible commercial products, particularly as natural source of hydroxytyrosol. This simple

phenol, known as an unstable molecule when dissolved in water media, remained almost unaltered in these more complex samples for several months. Finally, these samples could be commercialized to formulate new foods or dietary supplements suitable to guarantee an intake of natural antioxidants from *Olea europaea* L. and of potassium, both recognized as important components, particularly in dietary supplements employed by the athletes.

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A two-phase olive mill by-product (pâté) as a convenient source of phenolic compounds: content, stability, and antiaging properties in cultured human fibroblasts

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Lorenzo Cecchi[1†], Maria Bellumori[1†], Caterina Cipriani[2], Alessandra Mocali[3], Marzia Innocenti[1,4], Nadia Mulinacci[1,4*], Lisa Giovannelli[2,4*]

†these authors contributed equally to this work

[1]Department of Neuroscience, Psychology, Drug and Child Health, University of Florence, Nutraceutical and Pharmaceutical section, via Ugo Schiff 6, Sesto Fiorentino, Florence, Italy

[2]Department of Neuroscience, Psychology, Drug and Child Health, University of Florence, Pharmacology and Toxicology section, viale Pieraccini 6, Florence, Italy

[3]Department of Biomedical, Experimental and Clinical Science Mario Serio, viale Morgagni 50, Florence Italy

[4]Multidisciplinary Centre of Research on Food Sciences (M.C.R.F.S.-Ce.R.A), University of Florence Italy

*Corresponding authors

Tel.: +39 055 4573773; fax: +39 055 4573737

e-mail: nadia.mulinacci@unifi.it

Tel.: +39 055 2758316

e-mail: lisa.giovannelli@unifi.it

Abstract

Pâté is a new olive mill by-product potentially suitable for human consumption. This work aimed to characterize the phenolic profile of pâté samples from four crop seasons (2013-2016) in fresh, dried and stored samples, applying HPLC-DAD-MS-TOF, and to evaluate the antiaging effect in a cell senescence model. The dried pâté contains high levels of hydroxytyrosol, oleuropein derivatives and other phenolic compounds and is stable for several months. A diluted hydroalcoholic extract showed antiaging effects *in vitro*, comparable to those of pure hydroxytyrosol. Pâté can thus be proposed as an additional economical and environment-friendly source of olive bioactive phenolic compounds, particularly hydroxytyrosol: 1 g of pâté provides a daily intake comparable to that derived from 200 g of a typical virgin olive oil. This work lays the basis for a possible use of this food by-product as a natural ingredient for innovative foods or food supplements, contributing to a healthier lifestyle.

Keywords: Olive mill by-product; phenolic compounds; hydroxytyrosol; antiaging effect

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2006), 2.7 million tons of olive oil are produced annually worldwide, 76% of which in Europe, with Spain (35.2%), Italy (23.1%) and Greece (16.1%) being the highest olive oil producers. The production of olive oil yields a considerable amount of olive mill waste (OMW), which have a negative impact on land and water environment, being phytotoxic and powerful pollutants.

Although the high phenol, lipid and organic acid content is responsible for phytotoxicity, these by-products also contain valuable resources such as a great amount of interesting phytochemicals that could be recovered (Roig, Cayuela, & Sánchez-Monedero, 2006). In fact, although the olive fruit is very rich in phenolic compounds (Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015), the largest portion of these compounds is lost in the olive mill by-products during the milling process. In particular, the phenolic fraction in olive oil is below 2% of the total phenolic content of the olive fruits, with the remaining 98% being lost in OMW (Ciriminna, Meneguzzo, Fidalgo, Ilharco, & Pagliaro, 2016; Rodis, Karathanos, & Mantzavinou, 2002).

Since the importance of natural products, particularly from olives, has been highlighted (Ahmad Farooqi et al., 2017; Waltenberger, Mocan, Šmejkal, Heiss, & Atanasov, 2016), these huge quantities of olive mill by-products are potential rich sources of phenolic compounds, endowed with a wide array of biological activities. The most extensively studied is the antioxidant action, but also antimicrobial and a diverse range of other bioactivities have been demonstrated both for OMW as such and for phenolic compounds, which have been reported to be present in this wastes (Obied et al., 2005). In 2011, EFSA stated that "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" (European Food Safety Authority, 2011). Among these activities, much attention has focused on the antiaging properties: studies in rodent models of normal and accelerated aging have shown improvement in age-related dysfunctions upon administration of olive oil phenolic compounds, which have been proposed as candidates to counteract age-associated neurodegeneration (Casamenti & Stefani, 2017). Mechanistic studies indicate that these compounds are able to act at different sites, modulating cellular pathways relevant to the aging process, interfering with protein function and gene expression modulation (Giovannelli, 2013). Hydroxytyrosol is the most

studied among olive phenolic compounds, and showed several superior biological activities, some of which demonstrated in humans (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010; Visioli & Bernardini, 2011).

The technology for olive oil extraction has progressed significantly since the beginning of the 1970s, when the three-phase centrifugation system proved to be more efficient than the traditional pressing used for many centuries. In the 1990s, the two-phase centrifugation system was introduced in Spain as a more ecological approach for olive oil production, drastically reducing the amount of added water and producing a semi-solid by-product named alperujo, or olive pomace.

Recently, Pieralisi S.p.A. developed an innovative two-phase decanter named Leopard. This decanter produces a dehydrated husk similar to the one obtained from a three-phase decanter, but it also separates the pulp (pâté) from the husk directly after the malaxation step (Leopard Series, Pieralisi Group S.p.A. Jesi, Italy), so reducing the possible oxidation processes. This by-product, named pâté, consists of a wet homogeneous pulp free from residuals of kernel, peculiarity making it a suitable ingredient for possible commercial applications after drying. It is potentially suitable for various uses, including animal feeding, but also for human consumption in the form of food supplement or food ingredient. The possibility to use the fresh pâté was evaluated in one study (Luciano et al., 2013), who demonstrated that the inclusion of olive cake into a concentrate-based diet for lambs could be proposed as a strategy to improve the nutritional quality of meat without compromising its oxidative stability. Indeed, the inclusion of this pâté in the animal diet increased the concentration of vitamin E in muscle and extended meat oxidative stability.

To the best of our knowledge, reports on the use of this particular pâté for food formulations to be used in the human diet are not available so far. Clearly, the possibility of turning a by-product into a valuable resource, particularly for human consumption, would represent an important benefit for the miller. Recently, one study focused on the qualitative and quantitative characterization of this pâté (Lozano-Sánchez et al., 2017); the authors analyzed one sample recovered in 2015 from a mill in the Marche region (Italy) and concluded that this particular by-product can be used as source of bioactive hydrophilic and lipophilic compounds. The authors highlighted the high oxidative

stability of the pâté, even if the high moisture content could be a serious technological processing problem for long-term storage of this by-product.

The aim of the present work was to characterize pâté samples obtained from different crop seasons from the Leopard decanter during the production of VOO. The samples collected over four years (2013-2016) from two productive mills were characterized in terms of phenolic compounds, and the proximate composition was determined for a subset of these. A further aim was to apply drying technologies to evaluate the shelf life of the pâté. The thermal effects of an industrial spray-drying process, as well as the changes of the phenolic profile of several lyophilized pâté samples during storage in different conditions have been evaluated by HPLC-DAD-MS-TOF. Finally, the antiaging effect of a phenolic extract from this pâté was carried out in cultured human fibroblasts, a well-known and widely applied model of cell senescence, and the results compared with those obtained with hydroxytyrosol used as reference compound.

2. Materials and methods

2.1 Chemicals

All chemicals for analysis were of analytical grade. Formic acid, methanol and hexane were from Sigma Aldrich (Steinheim, Germany). Acetonitrile of HPLC grade was from Panreac (Barcelona, Spain). The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce deionized water. Syringic acid from Sigma Aldrich (Steinheim, Germany) was used as internal standard; caffeic acid and tyrosol from Sigma Aldrich (Steinheim, Germany) and oleuropein from Extrasynthese (Genay, France) were used as external standard. Stock solution of all the standards were prepared in hydroalcoholic solution. Sulfuric acid (96%, Sigma Aldrich, Steinheim, Germany) was used to prepare 1 M sulphuric acid.

2.2 Samples and sample preparation

Pâté samples of typical Tuscan cultivars (Frantoio, Moraiolo and Leccino) from two olive-mills located in two different provinces of Italy (Ancona and Livorno) were collected during the four crop seasons 2013-2016. In particular, the 2013 and 2014 samples were collected from Monteschiavo (M13 and M14) olive-mill (Maiolati Spontini, Ancona, Latitude: 43.509164; Longitude: 13.166397) and obtained by

processing an olive batch (about 0.5 ton) of Leccino and Frantoio as prevalent cultivars. The 2015 and 2016 samples were collected from Terre dell'Etruria (TE 15 and TE16) olive-mill (Castagneto Carducci, Livorno, **Latitude:** 43.166798; **Longitude:** 10.580778) and obtained by processing olives from Frantoio and Moraiolo as prevalent cultivar (each close to 0.5 ton).

Samples were divided into several aliquots: one was immediately freeze-dried (FD), one was immediately frozen and then stored at -22°C for four months before the freeze-drying process (SFD), and other aliquots were stored for one, two or four months at room temperature, i.e. in non-fully controlled conditions, and then freeze-dried (1FD, 2FD, 4FD). For the pâté of the 2013 campaign, a further sample was immediately frozen, stored at -22°C for four months and then dried using a spray-drier (M13-SD). All the analyzed samples are summarized in Table 1.

Sample	Year	Origin	Storage treatment	Analysis conditions	Quantitation
M13-FD	2013	Monteschiavo (AN)	Freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-S4FD	2013	Monteschiavo (AN)	Stored at RT for 4 months + freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-SFD	2013	Monteschiavo (AN)	Stored at −22 °C for 4 months + freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-SD	2013	Monteschiavo (AN)	STORED at −22 °C for 4 months + spray-dried	LiChrosorb RP-18 (Merck)	ES method
M13-FD	2013	Monteschiavo (AN)	freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
M14-FD	2014	Monteschiavo (AN)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE15-FD	2015	Terre dell'Etruria (LI)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE15-S4FD	2015	Terre dell'Etruria (LI)	Stored at RT for 4 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-FD	2016	Terre dell'Etruria (LI)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S1FD	2016	Terre dell'Etruria (LI)	Stored at RT for 1 month + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S2FD	2016	Terre dell'Etruria (LI)	Stored at RT for 2 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S4FD	2016	Terre dell'Etruria (LI)	Stored at RT for 4 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method

ES, external standard; IS, internal standard; RT, room temperature
Table 1. List of the analyzed pâté samples

2.3 Phenolic compounds

2.3.1 Extraction

All the samples listed in Table 1 and quantified using the external standard (ES) or the internal standard (IS) methods were extracted in triplicate. The phenolic extracts from the 2013 pâté were obtained as follows: 1 g dried sample was extracted with 2 × 10 mL of EtOH:H_2O 8:2 v/v, under magnetic stirring for 2 h, and then filtered. The obtained extract was defatted with *n*-hexane, dried under vacuum (-0.1 MPa) at 40 °C for 1 h on a Rotavapor® R-100 (from Büchi), re-dissolved in an exact volume of the same extractive mixture, and then analyzed by HPLC-DAD-MS-TOF.

The following procedure was applied for the IS method: 3 g of dried sample were extracted twice with 35 mL of EtOH:H_2O 8:2 v/v, in presence of 0.5 mL of internal standard solution (syringic acid, 3.25 mg/mL); each extraction was carried out under

magnetic stirring for 1 h. The obtained mixture was centrifuged for 10 min at 10°C and 5000 rpm, then the supernatant was defatted twice with hexane (35 mL), dried under vacuum at 40 °C for 1 h and re-dissolved in 1.5 mL of the extractive solution. An aliquot of this solution was centrifuged for 4 min at room temperature (RT) and 14,000 rpm and immediately used for the chromatographic analysis.

2.3.2 Hydrolysis of the hydroalcoholic extracts

The hydroalcoholic extracts, obtained from the immediately freeze-drying pâté samples, were submitted to a hydrolytic process in acidic medium. This method was previously proposed to evaluate the total content of free and bound tyrosol and hydroxytyrosol in VOO (Mulinacci et al., 2006). Briefly, 300 μL of hydroalcoholic extract were treated with 300 μL of H_2SO_4 1M for 2 hours at 80°C in a vial for HPLC. Then, 400 μL of EtOH were added, the obtained sample was centrifuged at 14,000 rpm for 5 minutes and the solution was immediately used for the chromatographic analysis.

2.3.3 Identification and quantification

The analysis was carried out using a HP 1200 liquid chromatograph equipped with a DAD detector coupled to a TOF-MS with an ESI interface (all from Agilent Technologies), under the following conditions: gas temperature 300°C, nitrogen flow rate 12 L/min, nebulizer pressure 20 psi, capillary voltage 3800 V. The mass spectra were acquired in the m/z range 100-1000 Th in negative ion mode, setting the fragmentation energy between 80 and 180V. Data acquisition and evaluation were performed using MassHunter software: acquisition module (Acq) B.05.01, qualitative analysis module (Qual) B.06.00.

The column LiChrosorb RP-18 250 × 4.6 mm (5 μm) (Merck) was used to analyze the extracts obtained from the pâté 2013. The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B) and the analyses were carried out applying the following multistep gradient: from 100% A to 89% A in 23 min followed by a 10 min plateau; 8 min to 87% A followed by a 4 min plateau; 10 min to 80% A and a 13 min plateau; 2 min to 75% A and a 5 min plateau; 10 min to 65% A and a 3 min plateau; 8 min to 55% A and a 3 min plateau; to 100% B within 4 min, and a final plateau of 7 min. Total time of analysis 117 min; oven temperature 26°C; flow rate 0.8 mL min^{-1}.

A Poroshell 120 EC-C18 150 × 3 mm, 2.7µm (Agilent Technologies) column was used to analyze the samples listed in Table 1. A six-step linear solvent gradient was used, starting from 95% H_2O adjusted to pH 3.2 by formic acid (A) up to 100% acetonitrile (B), as follows: from 95% A to 60% A in 40 min followed by a 5 min plateau; to 100% B within 5 min and a final plateau of 3 min. Total time of analysis 53 min, flow rate 0.4 mL min^{-1}.

The quantitative evaluation of all the 2013 samples was performed through the use of three external standards, each with a five-point regression curve: tyrosol (Y = 1548.2*X+6.7; $R^2 \geq 0.999$), oleuropein (Y = 531.8*X+18.3; $R^2 \geq 0.999$) and caffeic acid (Y = 12514.0*X+33.3; $R^2 \geq 0.999$), according to our previous works (Oliveras-López et al., 2007; Romani et al., 2007). Tyrosol and hydroxytyrosol derivatives were evaluated at 280 nm using tyrosol as reference; secoiridoids at 280 nm with oleuropein as standard; verbascoside and the cinnamoyl derivatives at 330 nm with caffeic acid as surrogate standard.

The immediately freeze-dried 2013 sample, the samples from 2014, 2015 and 2016 crop season, and all the samples obtained with the acidic hydrolytic protocol (paragraph 2.4), were quantified applying the internal standard method and following the same approach used by the official International Olive Council method for the analysis of olive oil biophenols (COI/T.20/Doc No 29, 2009). Syringic acid was used as internal standard, and tyrosol as external standard; consequently, quantitative data were expressed as mg_{tyr}/kg of dried pâté.

2.4 Proximate composition and dietary fiber analyses

The proximate composition was evaluated for the two samples collected from Terre dell'Etruria in 2015 and 2016. The fat content was gravimetrically determined after Soxhlet extraction, according to the ISS protocol (ISS, 1996). Proteins were evaluated using the Kjeldhal method and applying the formula: (g/100g) = N*6.25, where N is total nitrogen. Dietary fiber content was determined according to the AOAC method 991.43 (AOAC, 1995).

2.5 Biological assays in human fibroblasts

For these tests, the hydroalcoholic extract obtained from the 2013 pâté (freeze-dried within 3 days, M13-FD) was used. This extract, analyzed by the external standard method (Table 1) contained 0.58 mg/mL hydroxytyrosol (corresponding to 3.8 mM) and 5.4 mg/mL total phenolic compounds.

2.5.1 Cell cultures and experimental conditions

Neonatal Human Dermal Fibroblasts are a primary cell line derived from neonatal human dermal tissue (NHDFs Clonetics, Lonza). They were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS (Gibco, NY), 100 units/mL of penicillin G, 0.1 mg/ml streptomycin and 2 mM glutamine (PAN-Biotech GmbH, Germany), at 37 °C in a humidified incubator containing 5% CO_2. Culture medium was changed every 2-3 days until cells reached 90–95% confluence. They were then sub-cultivated by trypsinization (Trypsin-EDTA 1x in PBS, Euroclone) and the attained population doubling level (PDL) was calculated according to the equation: $PDL = 3.32 \times \log N/N_o$ (where N and N_o are the recovered and seeded cell numbers, respectively). The experiments were conducted starting from pre-senescent (PDL = 24) to senescent fibroblasts (PDL= 35), as described previously (Menicacci, Cipriani, Margheri, Mocali, & Giovannelli, 2017).

For short-term viability assay, NHDFs cells were seeded into 96-well plates (3000 cells per well) and grown for 72 hours in the presence of M13-FD at three different dilutions: 1:100, 1:1000, 1:10000. Control cultures were treated with ethanol 0.8%, i.e. the concentration corresponding to the lower dilution, or DMEM only.

Cell viability was then assessed by means of MTS cytotoxicity assay (see below). Cytotoxicity was evaluated by measuring the absorbance in two independent experiments and data were expressed as percentage of control.

For long-term experiments, cell cultures were treated continuously with M13-FD 1:1000, 1:5000, 1:10000 or 1 μM hydroxytyrosol until senescence. The culture medium was replaced every 2 days to maintain the treatment concentration relatively constant over time. At each passage, the number of cells recovered after trypsinization for each treatment was measured in a Burker chamber to evaluate cell growth over time.

2.5.2 MTS viability assay

The Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega) was used. The assay is based on the bioreduction of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium:inner salt] into formazan by NADH and NADPH produced by dehydrogenase enzymes only in active and viable cells. The reagent was added to each well and 96-well plates were incubated at 37°C in humidified 5% CO_2 atmosphere until color development had occurred (1-2 hours). The formation of a purple formazan product was then measured spectrophotometrically at 490 nm. The measured absorbance value is a function of the amount of formazan produced and is proportional to the number of viable cells.

2.5.3 SA-β-galactosidase assay

The senescence-associated β-galactosidase (SA-β-gal) assay is designed to detect β-galactosidase activity at pH 6, typically expressed in senescent cells. Cells were washed twice in phosphate- buffered saline (PBS) and fixed for 5 minutes at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed twice in PBS and then incubated at 37°C, with fresh SA-β-gal staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-D-galactoside, 40 mmol/L citric acid/sodium phosphate dibasic at pH 6, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L $K_3[Fe(CN)_6]$ and 5 mmol/L $K_4[Fe(CN_6)]3H_2O$). Staining was evident in 2-4 hours and maximal in 12-16 hours. The next day hematoxylin was used to counter-stain the cells. Cells were finally manually counted to determine the percentage of SA-β-gal positive cells over the total.

2.5.4 LDH release

Cell damage was evaluated by the lactate dehydrogenase (LDH) release assay. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity was determined in an enzymatic test (Cytotoxicity Detection Kit, Roche), based on the reduction of NAD^+ to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate, and on the further diaphorase/$NADH/H^+$-mediated reduction of the tetrazolium salt INT to formazan.

NHDFs were seeded into 96-well plates (3000 cells per well). The next day, DMEM was replaced in each well with 200 µL of red phenol-free DMEM containing the

different treatments. After 24 h the medium was withdrawn, centrifuged at 250 RCF for 5 minutes, and the supernatant used for enzyme assay. To determine LDH activity in 100 µL of supernatant, 100 µL of freshly prepared Reaction Mixture were added to each well and incubated for up to 30 minutes at room temperature. Finally, the absorbance at 490-492 nm was measured spectrophotometrically.

2.5.5 Western Blot Analysis

After washing the wells with PBS, cellular proteins were extracted in RIPA buffer [50 mm Tris–Cl (pH 7.5), 150 mm NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS] containing 1% protease and phosphatase inhibitor Cocktail (Sigma–Aldrich Chemicals) with the aid of a cell scraper. Cell lysates were then sonicated (Microson XL 2000; Misonix, Farmingdale, NY, USA), clarified by centrifugation and supernatants collected and stored at -20°C. Protein content was measured by using the Bio-Rad DC protein assay kit (Bio-Rad).

Forty-fifty micrograms of proteins for each sample were subjected to 4-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation (Bis-Tris Plus BOLT, Invitrogen, Thermo Fisher Scientific, San Giuliano Milanese, Italy) and transferred to polyvinylidene fluoride membranes ([PVDF] Millipore, Billerica, MA, USA). Total amounts of p16 and GAPDH protein (the latter used as loading control) were determined by immunostaining with primary rabbit polyclonal antibodies: anti p16 (N-20), sc-467 (Santa Cruz Biotechnology); anti-GAPDH, 14C10 (Cell Signaling Technology) and suitable peroxidase-conjugated secondary antibodies (Sigma–Aldrich Chemicals Co.). Proteins were visualized using the enhanced chemiluminescence procedure with Immobilon Horseradish Peroxidase Substrate (Millipore) and immune-reactive bands were quantified by densitometric analysis using the Quantity-One software (Bio-Rad Laboratories). Each density measure was normalized by using the corresponding GAPDH level as an internal control.

2.6 Statistical Analysis

All the quantitative data were expressed as the mean ± standard error. Statistical analyses of data were performed by one-way ANOVA and F-Test ($P < 0.05$); Fisher's

LSD test was then applied to data on phenolic composition and Bonferroni's post-hoc test to data from biological assays (DSAASTAT software v. 1.1).

3. Results and Discussion

Differently from wastewaters or solid olive residue (pomace), pâté is a new olive by-product of particular interest in that it is recovered during and not after the milling process of an edible fruit by an exclusively mechanical treatment. To the best of our knowledge, only one recent publication is focused on the composition of this by-product (Lozano-Sánchez et al., 2017).

One main goal of the present work was the evaluation of content and stability of the phenolic fraction in pâté after drying treatments and after different storage times. The dried pâté appears homogeneous, devoid of woody parts and non-hygroscopic also after several months of storage (Figure 1).

Figure 1. Dried pâté from 2016 campaign: a) dried after 18 months of storage in non-controlled conditions; b) after a rapid and preliminary grinding, 15 days after drying; c) after a rapid and preliminary grinding and sifting, 15 days after drying

To date, we know that the phenolic fraction extracted from olive into olive oil never exceeds 2%, remaining almost completely in the milling by-products (Jerman Klen, Golc Wondra, Vrhovšek, Sivilotti, & Vodopivec, 2015). However, consumption of table olives does not guarantee a significant intake of these phenolic compounds, as they are almost completely degraded during the common industrial processes used to remove olive bitterness.

In this context, we aimed at evaluating the possibility of using the dried pâté as a natural ingredient for formulating innovative foods or food supplements to improve the daily intake of these bioactive natural compounds, thus adding value to this by-product. First, several samples were collected starting from 2013 campaign to evaluate the composition of the pâté. Successively, the phenolic content was determined in other pâté samples harvested over four years in two productive mills (Table 1).

The water content of all samples ranged between 78-80%, in agreement with recent data on a pâté recovered in Italy during the 2015 season (Lozano-Sánchez et al., 2017). As the pâté production is concentrated in a few months and in view of its high water content, we decided to investigate the effect of a room temperature (18-20°C) storage of the fresh pâté in plastic closed tanks for several months. This model was chosen to simulate a simple storage process, directly applicable in the mill, and aimed to maintain the fresh pâté before the drying process for several weeks. The freeze-drying procedure was chosen as an elective method to stabilize the fresh pâté samples over time, and all the phenolic extracts were derived from the treatment of these dried samples.

Sample	Hydroxytyrosol									Tyrosol								
	Free			Glucoside			Total			Free			Glucoside			Total		
M13-FD	12,074	(156)	B	1394	(62)	E	13,468	(218)	C	383	(10)	B	1336	(18)	F	1719	(28)	C
M14-FD	2883	(43)	A	371	(17)	A	3253	(60)	A	85	(8)	A	85	(8)	A	170	(16)	A
TE15-FD	4103	(131)	A	1532	(68)	E	5635	(199)	B	382	(5)	B	844	(24)	E	1226	(29)	B
TE15-S4FD	21,499	(2888)	D	1682	(203)	F	23,181	(3090)	E	3175	(373)	G	770	(68)	D	3945	(441)	F
TE16-FD	11,317	(415)	B	844	(36)	C	12,161	(451)	C	840	(23)	C	710	(21)	D	1550	(44)	C
TE16-S1FD	12,383	(154)	B	1056	(79)	D	13,438	(233)	C	1814	(42)	D	594	(64)	C	2408	(107)	D
TE16-S2FD	15,130	(414)	C	1011	(25)	D	16,141	(440)	D	2457	(58)	E	270	(9)	B	2727	(67)	E
TE16-S4FD	16,886	(319)	C	565	(12)	B	17,451	(331)	D	2847	(60)	F	62	(2)	A	2909	(63)	E

Sample	Verbascoside			Caffeic acid			β-OH acteoside 1			β-OH acteoside 2			Luteolin			Oleuropein derivatives			Total phenols		
M13-FD	3380	(15)	C	90	(3)	A	292	(12)	D	343	(8)	C	456	(8)	A	9200	(186)	D	49,550	(421)	BCD
M14-FD	118	(13)	A	nd	49	(7)	A	69	(11)	AB	1203	(43)	B	2767	(79)	A	27,695	(524)	A		
TE15-FD	6702	(168)	E	219	(30)	A	1272	(16)	F	1405	(43)	D	1020	(102)	B	13,232	(357)	E	52,969	(2019)	CD
TE15-S4FD	5006	(914)	D	4038	(502)	C	1616	(86)	G	1812	(134)	E	3234	(530)	D	3695	(571)	B	75,748	(6276)	E
TE16-FD	2509	(89)	B	3070	(75)	B	420	(4)	E	310	(6)	C	1186	(111)	B	5225	(214)	C	45,573	(1525)	B
TE16-S1FD	546	(22)	A	4982	(69)	D	202	(11)	C	151	(12)	B	1685	(49)	C	3514	(366)	B	48,507	(1154)	BC
TE16-S2FD	379	(17)	A	5409	(156)	E	122	(12)	B	97	(15)	AB	1650	(54)	C	3474	(87)	B	52,367	(923)	CD
TE16-S4FD	154	(10)	A	4907	(121)	D	44	(6)	A	33	(9)	A	1703	(40)	C	3550	(81)	B	51,123	(850)	CD

M, mill of Monteschiavo; TE, mill of Terre dell'Etruria; FD, freeze-dried; S1FD, S2FD, S4FD, freeze-dried after 1, 2, 4 months of storage in non-controlled conditions

Table 2. Phenolic content of pâté samples quantified by the internal standard method according to the IOC protocol for biophenols from olive oils; data are the mean (SD) of three independent measurements, expressed as mg_{tyr}/kg on dry matter basis. In each column, different letters indicate significant differences at $p < 5\%$.

Compound name	Retention time (min)	λ_{max} (nm)	mw	Major ions
hydroxytyrosol glucoside	7.4	202, 276	316	315, 153, 631
hydroxytyrosol	6.9	210, 280	154	153, 307, 123
tyrosol	10.3	221, 276	138	137
tyrosil glucoside	9.8	222, 276	300	299, 599
caffeic acid	13.3	218, 322	180	179, 135, 359
verbascoside	22.8	329	624	623, 461, 161
β-OH acteoside 1	18.2	286, 328	640	639, 621, 179, 161
β-OH acteoside 2	18.4	286, 329	640	639, 621, 179, 161
luteolin	31.9	347	286	285, 571
oleuropein derivatives	36-30.5	280	378	377

Table 3. Spectral information about the identified phenolic compounds

The phenolic compounds were identified by HPLC-DAD-MS-TOF studying the typical profiles at 280 nm and 330 nm. The detected molecules are those typical of olive oil and olive fruit and already known as derived by *Olea europaea* L. Two different quantitative approaches were applied as discussed below and summarized in Tables 1, Table 2 and Table 3.

3.1 Preliminary evaluation on pâté 2013

The 2013 pâté was used for a preliminary screening primarily focused on evaluating the suitability of the sample to be dried by an industrial spray drier, widely used for treating liquid and semi-liquid formulations for foods or pharmaceutical products. At the same time, we investigated the changes in the phenolic content and phenolic profile of the fresh pâté after a four months storage in plastic tank. The antiaging efficacy of the phenolic extract from this pâté was then evaluated choosing cultured human fibroblasts as a suitable cellular model to study the modulation of senescence.

Firstly, we used an analytical method previously applied by our research group to determine the phenolic compounds in EVOOs (Mulinacci et al., 2013; Oliveras-López et al., 2007; Oliveras Lopez et al., 2008; Romani et al., 2007). This method was recognized as particularly suitable to investigate the composition of the secoiridoid fraction, mainly constituted by oleuropein derivatives. These molecules are known to be sensitive to oxygen during the milling process and consequently the successive management of the fresh pâté can affect the phenolic concentration. Figure 2 shows the effect of the different storage treatments on the phenolic content of pâté 2013.

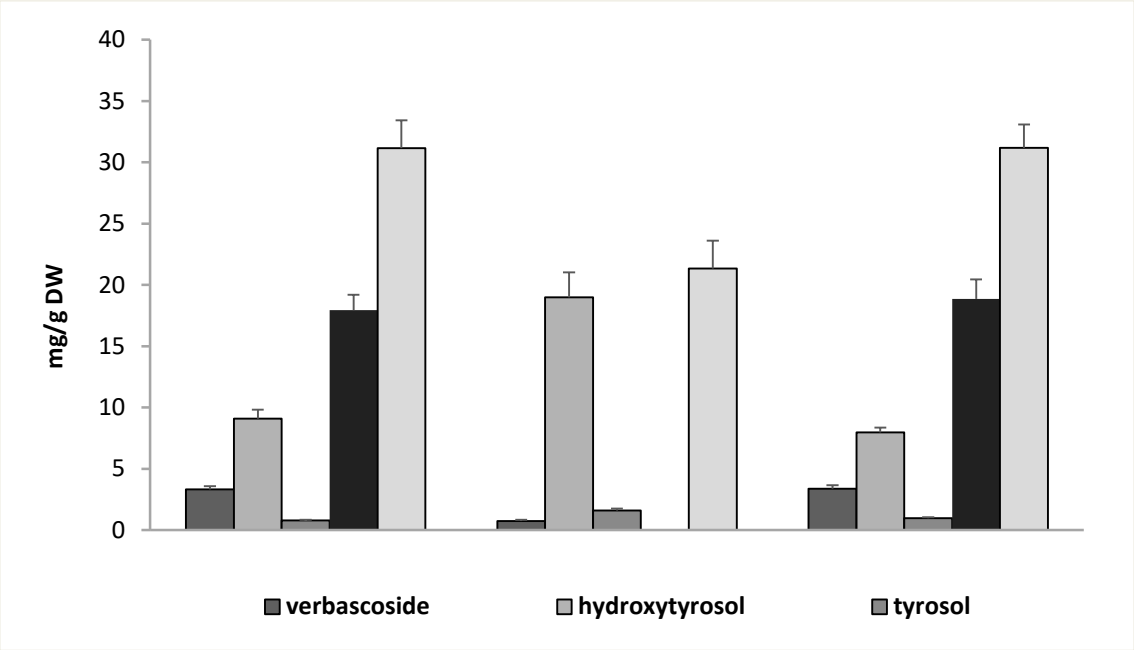

Figure 2. Effect of different storage treatments on the phenolic content of pâté 2013; data are expressed in mg/g dried weight (DW) as a mean of three independent analysis.
M13-FD freeze-dried within 3 days; **M13-S4FD**, freeze-dried after 4 months of storage in not controlled conditions; **M13-SFD**, freeze-dried after 4 months of storage at -22 °C

The principal components of this by-product were hydroxytyrosol, tyrosol, dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and verbascoside. As expected, the storage for four months at -22°C of the fresh pâté before the freeze-drying process (M13-SFD) did not modify the phenolic profiles. On the opposite, the storage in non-controlled conditions at room temperature (18-20°C) for the same amount of time induced an almost complete hydrolysis (presumably enzyme-mediated) of the oleuropein derivatives, with a corresponding increase of free tyrosol and hydroxytyrosol (M13-S4FD). Upon spray-drying of pâté (over 100°C for a few seconds) after addition of maltodextrins, the phenolic fraction was stable (data not shown); the final sample appeared as a fine and non-hygroscopic powder. Nevertheless, the high viscosity of the pâté resulted to be the limiting factor to apply the spray-dryer technology on a larger scale to dry the fresh pâté, because during the process the small holes of the spray-cone were partially or completely obstructed. Nevertheless, the test allowed verifying that the olive phenolic compounds are not too sensitive to thermal degradation up to around 100°C, and this information can be useful for further studies aiming to find sustainable and alternative drying processes to treat the fresh pâté.

3.2 Phenolic profile in pâté samples over years

After the first evaluation of pâté 2013, other samples derived from different cultivars and two different geographical areas were collected during the years 2014-2016. To carry out this comparative analysis of the phenolic profiles, a more efficient method, based on a next generation column (Poroshell 120 EC-C18 Agilent Technologies) was applied. This approach allowed to strongly reduce the required time for analysis and the costs, improving the efficiency of the chromatographic separation. All the extracts from the pâté samples harvested during the four years were quantified using the internal standard method, according to the IOC protocol for biophenols in olive oils, with the final data expressed as mg_{tyr}/kg dried pâté. Chromatographic profiles at 280 nm of the hydroalcoholic extracts of pâté 2015 are compared in Figure 3, while the quantitative results are summarized in Table 2.

HPLC-DAD-MS-TOF allowed to detect 21 phenolic compounds, some of which previously described in olive, olive oil and other olive oil waste (Kanakis et al., 2013; Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013). The analyses showed that pâté contains appreciable amounts of phenolic compounds as reported in other studies (Lozano-Sanchez et al., 2011; Lozano-Sánchez et al., 2017). As expected, the lowest value was reported for the extract from pâté 2014 (M14-FD sample) deriving from olives of the 2014 campaign, severely damaged by *Bactrocera oleae* infestation (Cecchi, Migliorini, Cherubini, Trapani, & Zanoni, 2016).

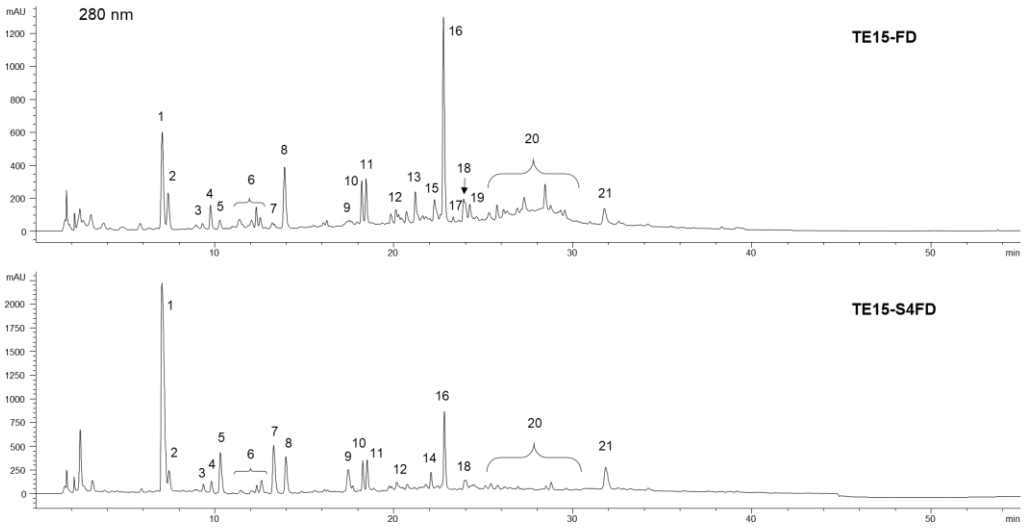

Figure 3. Comparison of the phenolic profile at 280 nm of the pâté 2015 (TE15-FD) and the corresponding sample maintained for 4 months at 18-20°C (TE15-S4FD). *1, hydroxytyrosol; 2, hydroxytyrosyl glucoside; 3, unknown 1; 4, tyrosyl glucoside; 5, tyrosol; 6, unknown 2; 7, caffeic acid; 8, syringic acid (internal standard); 9, p-coumaroyl derivative; 10, β-OH acteoside 1; 11, β-OH acteoside 2; 12, flavonoid 1; 13, flavonoid 2; 14, caffeoyl derivative 1; 15, flavonoid 3; 16, verbascoside; 17, caffeoyl derivative 2; 18, flavonoid 4; 19, caffeoyl derivative 3; 20, oleuropein derivatives; 21, luteolin*

Considering the samples freeze-dried within 3 days (FD), the total phenolic content ranged from 45,573 (TE16-FD sample) to 52,969 (TE15-FD sample) mg_{tyr}/kg dried matter basis (DM), with a comparable content in pâté from the same mill. Hydroxytyrosol was the most abundant phenol for the extracts from 2013, 2014 and 2016 campaigns while for the year 2015 the major compounds were the oleuropein derivatives.

The effect of four months storage in non-fully controlled conditions (10-20 °C in plastic tank) on the phenolic content was also evaluated for the 2015 sample. As showed in Table 2 and Figure 2, the phenolic profile varied, with hydroxytyrosol and tyrosol considerably increased. Regarding hydroxytyrosol (free + glucoside) the amount ranged from 5,635 to 23,181 mg_{tyr}/kg DM. At the same time, the verbascoside and the oleuropein derivatives showed a reduction of their content after the four months of storage.

Finally, for the samples from 2016, the study was carried out considering the effect of storage at 18-20°C also for shorter times (one and two months). As expected, the trend observed for the 2015 sample was confirmed: the amount of hydroxytyrosol and tyrosol increased gradually and proportionally to the storage time; verbascoside content again showed a drastic reduction, from 2,509 to 154 mg_{tyr}/kg DM; oleuropein derivatives content decreased from 5,225 to 3,550 mg_{tyr}/kg DM. The decrease of verbascoside and oleuropein derivatives is associated to increase in hydroxytyrosol, the main product of the spontaneous hydrolysis, taking place during the fresh pâté storage. During the four months of storage, no apparent fermentative process was noticed, and the smell of samples remained similar to that of fresh pâté. Nevertheless, further studies will need to further explore this aspect. To complete and simplify the analytical determination, an acid hydrolysis was applied on the phenolic extracts of the four pâté collected in this study (Table 4), aimed to evaluate the actual hydroxytyrosol and tyrosol content, including both the free forms and those linked to the secoiridoid nucleus.

Sample	Hydroxytyrosol (mg/Kg)			Tyrosol (mg/Kg)			Sum of tyr + OH-tyr (mg/Kg)		
M13-FD	29,145	(316)	D	4283	(66)	C	33,428	(382)	D
M14-FD	5920	(86)	A	569	(28)	A	6489	(114)	A
TE15-FD	23,667	(196)	C	5522	(81)	D	29,189	(277)	C
TE16-FD	18,867	(157)	B	4023	(73)	B	22,890	(230)	B

Table 4. Quantitation of total tyrosol and hydroxytyrosol (free and bound forms) after acid hydrolysis. Data are the mean (SD) of three independent measurements, expressed as mg_{tyr}/kg on dry matter basis. In each column, different letters indicate significant differences at $p < 5\%$

This approach, previously proposed for the determination of these phenolic compounds in EVOOs (Mulinacci et al., 2006), allows to simplify their determination

avoiding underestimations of the minor derivatives; only one peak for tyrosol and one for hydroxytyrosol were obtained after the hydrolysis. The total amount of both these phenolic compunds is considerably lower if determined at the start of the storage (Table 2, t_0) without applying the acid hydrolysis (Table 4). When comparing the samples of 2015 and 2016, similar results were obtained. The content of hydroxytyrosol after four months of storage at 18-20°C was almost the same obtained after the acidic hydrolysis of the phenolic fraction of 2015 pâté, showing values of 23,181 ± 3,090 mg_{tyr}/kg DM and 23,667 ± 196 mg_{tyr}/kg DM, respectively. Similarly, for the 2016 pâté the hydroxytyrosol amount in TE16-S4FD was 17,451 ± 331 mg_{tyr}/kg DM, close to the value in the hydrolyzed extract (TE16-FD): 18,827 ± 157 mg_{tyr}/kg DM.

Regarding the tyrosol content in pâté of 2015 and 2016, the amounts evaluated after the hydrolysis were consistently higher (+28%) of those measured in the samples after the four month-storage without hydrolysis. This discrepancy can be attributed to underestimation of tyrosol derivatives present in lower concentration in the extract with respect to hydroxytyrosol derivatives.

In view of a possible use of the pâté for human consumption, we carried out the proximate analysis on the pâté samples recovered from 2015 and 2016 in Tuscany: the amount of fat and proteins was similar (from 10 to 15%) and the dietary fiber content was close to 50% of the dry pâté, with a 6% of fermentable fiber, in both the 2015 and 2016 samples.

This dry pâté was stable over time, as confirmed by the analysis of TE15-FD after 12 months of storage in a dark closed bottle at room temperature (Table 5): the total phenolic content was the same than t_0. This finding confirms that the dried pâté can be proposed as an additional source of the olive bioactive phenolic compounds, particularly of free and bound hydroxytyrosol. It is worth noting that the total phenolic compounds in 1 g of dry 2015 pâté (evaluated by internal standard method, according to the IOC method for olive oils) can guarantee a daily intake close to 40 mg of total phenolic compounds, of which 25 mg of hydroxytyrosol, i.e. an amount comparable to that derived by the daily consumption of 200 g of a EVOO with a total phenolic content close to 200 mg/kg.

TE15-FD		
	t_0	**12 months**
Hydroxytyrosol	4103 ± 131	5947 ± 168
Hydroxytyrosyl glucoside	1532 ± 68	1575 ± 46
Tyrosyl glucoside	844 ± 24	1191 ± 38
Tyrosol	382 ± 5	473 ± 8
β-OH acteoside 1	1272 ± 16	1216 ± 19
β-OH acteoside 2	1405 ± 43	1347 ± 8
Verbascoside	6702 ± 168	6508 ± 16
Luteolin	1020 ± 102	1018 ± 35
Oleuropein derivatives	13232 ± 357	10274 ± 279
Caffeic acid	219 ± 30	75 ± 14
Total phenolic compounds	**52970 ± 2019**	**52410 ± 1563**

Table 5. Stability of the freeze-dried pâté, evaluated on the 2015 pâté sample analyzed at t_0 and after 12 months

3.3 Toxicity testing and anti-aging activity in human fibroblasts

3.3.1 Short term toxicity

To define the working dilutions of the M13-FD extract, we first carried out short-term toxicity experiments evaluating cell viability with the MTS method. The results showed that the 1:100 dilution reduced cell viability upon 72 h incubation and was discarded, whereas the 1:1000 and 1:5000 dilutions were not toxic (Figure 4). For the long term experiments, the 1:1000 and 1:5000 dilutions were used.

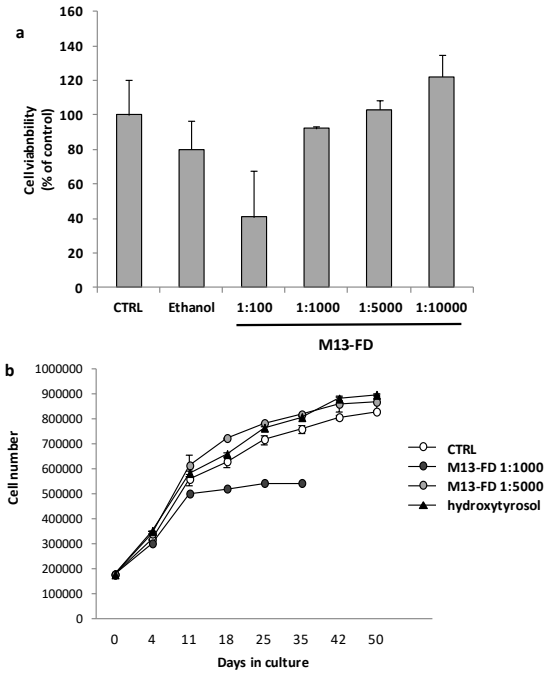

Figure 4. Panel a: NHDF viability upon short-term (72h) treatment with the indicated dilutions of the extract (M13-FD) and the vehicle (ethanol, 0.8%) measured with MTS. Data are expressed as the percentage absorbance value of the control, untreated fibroblasts (mean ± SE of three experiments). Panel b: Number of NHDFs recovered at each passage upon long-term treatment with the indicated dilutions of the extract (M13-FD) and hydroxytyrosol (1 μM). Data from one representative experiment (out of three experiments) are expressed as the mean ± SE of technical duplicates

3.3.2 Long term effects on cell proliferation, survival, and markers of cell senescence

When cells were continuously treated with either M13-FD or hydroxytyrosol, the 1:1000 dilution of the extract showed slowing effects on cell proliferation, starting at about 10 days of treatment. These cultures were terminated at 35 days. On the contrary, both 1 μM hydroxytyrosol and 1:5000 M13-FD were able to increase the number of cells recovered at each passage, and although the differences were small the effect was statistically significant (Figure 5).

This effect of hydroxytyrosol and M13-FD 1:5000 was paralleled by reduced LDH release measured at senescence, indicating that the two treatments strongly reduced cell death at this time point (Figure 5).

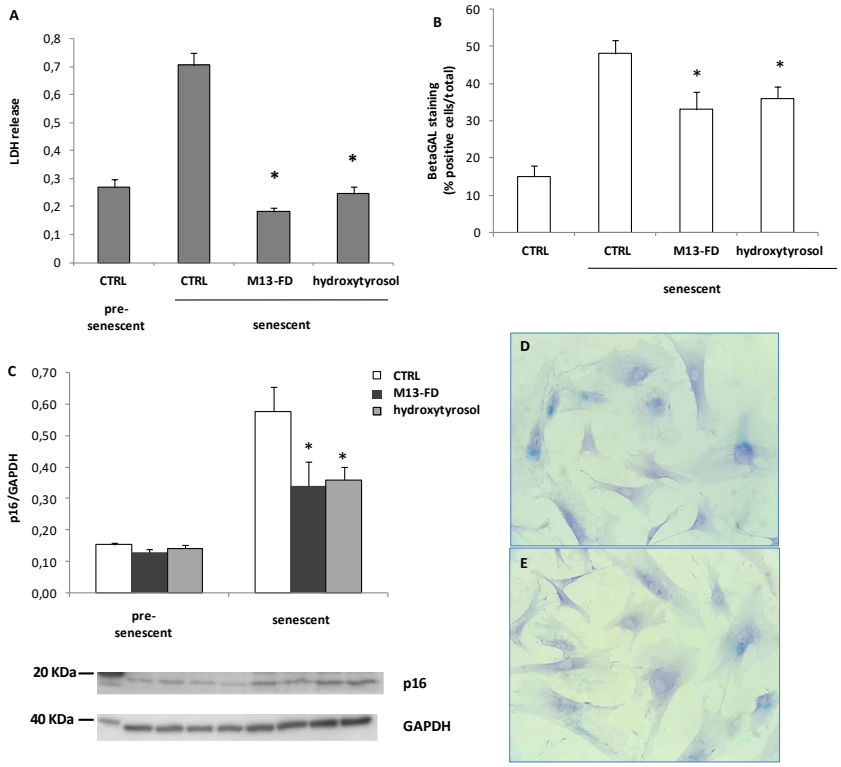

Figure 5. A: LDH release in the NHDF culture medium at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM). **B**: SA-β-gal staining in NHDFs at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM): cell counts. **C**: P16 protein expression evaluated by western blot at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM). Representative images of the blotted membranes are shown below the graph. Data are the mean ± SE of three experiments. *$p<0.05$ statistically significant difference from senescent control fibroblasts. **D** and **E**: representative microphotographs of senescent control (D) and M13-FD-treated (E) NHDFs

The expression of the senescence marker protein p16, involved in cell cycle arrest at senescence, was found increased at the end of the experiment as expected, and strongly reduced by the treatment with both hydroxytyrosol and M13-FD 1:5000 (Figure 5 C). Finally, the expression of the enzymatic activity of SA-β-gal was markedly

increased in senescent fibroblasts, and this effect was counteracted by the two treatments, which brought about a 30% reduction of the percentage of positive cells at the end of the treatment (Fig. 5 B, D, E).

Testing the antiaging activity of a compound *in vivo* involves time- and animal consuming complex experiments in which specific functions affected by age, such as cognitive, motor, autonomous or metabolic, are measured. The aging process *per se* can be investigated at the cellular level in a widely used simple *in vitro* model, that has repeatedly shown predictive value towards *in vivo* effects: the senescent primary cell culture. Cellular senescence is characterized by complex modifications in the protein expression profile of the cell, leading to replicative arrest and cell dysfunction. Senescent human fibroblasts, the most widely used cell type in this context, can be protected against replicative senescence upon long-term treatment with natural compounds such as resveratrol (Giovannelli et al., 2011) and epigallocatechingallate (Han et al., 2012) as well as different plant extracts (Ding et al., 2017); among the latter, an extract from *Olea europaea* delayed senescence in IMR90 and WI38 human fibroblasts (Katsiki, Chondrogianni, Chinou, Rivett, & Gonos, 2007). Hydroxytyrosol (1 μM) has been shown to lengthen the chronological lifespan of cultured human fibroblasts (Sarsour et al., 2012). Recently, we have shown that 1 μM hydroxytyrosol is able to counteract replicative senescence and the associated inflammatory phenotype in long-term treated cultured human fibroblasts, showing reduced beta-galactosidase activity and p16 expression, along with enhanced cell protection (Menicacci et al., 2017). In the present work, we have evaluated the antiaging activity of the FD extract by comparison with 1 μM hydroxytyrosol in the same model of replicative senescence.

The final concentration of hydroxytyrosol in the 1:5000 dilution of the hydroalcoholic extract, effective in the antiaging tests, was 0.76 μM. This dilution induced the same protective effects than pure hydroxytyrosol at 1 μM concentration, reasonably due to the presence of other phenolic compounds in the extract. Thus, these experiments indicate that the extract is as active as the pure reference compound, hydroxytyrosol, in delaying the senescence process upon a long-term exposure.

4. Conclusions

In conclusion, we studied a particular two-phase olive by-product (pâté), obtained directly after the malaxation step and characterized by a wet homogeneous pulp free from residuals of kernel. Our data show that pâté is a convenient source of phenolic compounds and that the main components of this by-product are hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA and verbascoside.

Regarding the storability of fresh pâté, storage in non-controlled conditions leads to a strong increase of hydroxytyrosol content over time. After freeze-drying, the dry pâté is a homogeneous powder, that resulted stable for several months.

Biological assays carried out on a simple hydroalcoholic extract of pâté proved to have antiaging activity in cultured human cells upon long-term exposure, with a potency comparable to that of pure hydroxytyrosol. Thus, pâté can be proposed as an environment-friendly source of olive bioactive phenolic compounds, particularly of free and bound hydroxytyrosol: 1 g of pâté is able to provide a daily intake comparable to that derived from 200 g of an EVOO with a phenolic content of 200 mg/kg.

As a whole, this work lays the basis for the possibility of using this food by-product as a natural ingredient for formulating innovative foods or food supplements contributing to a healthier lifestyle.

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Abbreviations

OMW, olive mill waste
VOO, virgin olive oil
EVOO, extra virgin olive oil
NHDFs, neonatal human dermal fibroblasts
DMEM, Dulbecco's modified Eagle's medium
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium:inner salt
SA-β-gal, Senescence-Associated β-galactosidase
PBS, phosphate-buffered saline

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Use of pâté, a by-product from virgin olive oil production, as functional ingredient for fortification of pasta, bread and granola bar: sensory profiling and acceptance by Californian consumers

Unpublished results

Lorenzo Cecchi[1], Noah Shuster[2], Rose Bechtel[2], Dan Flynn[3], Maria Bellumori[1], Marzia Innocenti[1], Nadia Mulinacci[1] and Jean-Xavier Guinard[2]

[1]NEUROFARBA Department, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto F.no (Florence), ITALY

[2]Department of Food Science and Technology, University of California-Davis, Davis, CA, United States

[3]Olive Center, University of California-Davis, Davis, CA, United States

Abstract

In this work we used pâté, a by-product from virgin olive oil production, for fortification of three type of food products very widespread in California and beyond, namely Pasta, Bread and Granola Bar. For each dish, we prepared Control (without pâté) and Sample (with pâté, 7% in pasta and 5% in Bread and Granola Bar). Firstly, after having figured out the recipes, we evaluated the percentage of phenolic compounds from pâté recovered in the Samples when they are ready-to-eat: the highest recover (76%) was for granola bar, followed by bread (49%) and pasta (10%). Then, we characterized the sensory profile of the six product using both Check-All-That-Apply analysis (for gaining insight into naïve consumers) and Descriptive Analysis (for a more detailed description and differentiation of Sample and Control by a panel of trained judges). In the next step, we tested the hypothesis that consumers still accept the fortified products, despite the presence of pâté, which, in our hypothesis, at least affects the appearance and the bitterness. In a Central Location Test, for each kind of dish, 175 Californian consumers evaluated Control and Sample in a fully-randomized design for overall liking and liking of appearance, texture and flavour/taste/smell on the 9 point hedonic scale. Adequacy of flavor intensity, saltiness (sweetness for granola bar), firmness and color was then evaluated on 5 point just-about-right (JAR) scale. Purchase intention, consumer preference and consumer willingness to pay more for the fortified products were also measured. Finally three focus group sessions were performed to gain further qualitative information about the products and the idea of re-using pâté. For each type of dish, the overall acceptance of the Sample was lower than Control, as well as the liking of appearance, flavour/taste/smell and texture. However, all products were overall accepted by consumer, with only the liking of appearance of pasta sample that had a mean score slightly under the "neither like nor dislike" option. Some consumers preferred the Sample and 50% declared to be available to pay more for the fortified products. This study demonstrated that pâté can be used for fortification of food products very widespread in California and beyond, so giving additional economic value to the virgin olive oil production chain and allowing a higher daily intake of phenols from *Olea europaea* L., whose health properties are well recognized as also stated by a health claim allowed in Europe by the EFSA.

Keywords: Descriptive analysis, Consumer test, Focus Group, Phenolic compounds, Check-All-That-Apply (CATA), Consumer preferences, Acceptability, Consumer attitutes

1. Introduction

Phenolic compounds from *Olea europaea* L. has been associated to a wide array of biological activities, including antiaging (Giovannelli, 2013; Casamenti & Stefani, 2017), antioxidant (Franco et al., 2014; Achat, Rakotomanomana, Madani, & Dangles, 2016) and anti-inflammatory (Beauchamp et al. 2005). Their use was patented thanks to their capability in improving the management of type two diabetes (De Bock, Hodgkinson, Curtfield, & Schlothauer, 2014). Furthermore, a cause and effect relationship between these compounds and protection of LDL from oxidative damage was established by some *in vivo* tests on humans (Covas et al., 2006; de la Torre Carbot et al., 2010), allowing the EFSA to approve a health claim for olive oil phenolic compounds; this health claim gives the possibility to insert "the olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" in the olive oil label (EFSA, 2011).

Olive fruits are very rich in phenolic compounds (Cecchi, Migliorini, Cherubini, Innocenti & Mulinacci, 2015), but only less than 1% of them are extracted in olive oils, as demonstrated in one of the published work of this thesis (Cecchi, Migliorini, Zanoni, Breschi & Mulinacci, 2018a). On the contrary, the most are lost in the olive waste, namely olive mill wastewater and olive pomace (Bellumori, Cecchi, Romani, Mulinacci & Innocenti, 2018; Cecchi et al., 2018b). Consequently, only daily consumers of olive oil can get the phenolic compounds' intake recommended by EFSA in order to obtain the health benefits. Not even consumption of table olives, whose phenolic compounds are almost fully removed in order to reduce olive bitterness, allows guaranteeing a significant intake. Consuming of olive oil is daily only in some Mediterranean countries, mainly Italy and Spain. In other countries, particularly in US, olive oil consumption has been strongly growing in the last 20 years (Delgado & Guinard, 2011), but to date olive oil is not consumed on a daily base by the most population. Furthermore, olive oil rich in phenolic compounds is usually characterized by high level of bitter and pungent notes, which appeared as negative drivers of liking by Italian, Spanish and Californian consumers even with consumers with high level of involvement with and subjective knowledge to extra virgin olive oil (Caporale, Policastro & Monteleone, 2004; Delgado & Guinard. 2011; Recchia, Monteleone & Tuorila, 2012; Delgado, Gòmez-Rico, & Guinard, 2013). Consequently, consumers tend to use those olive oils that are not very rich in phenolic compounds.

The other consequence of the high amount of phenolic compounds lost in the olive waste is that these latter become phytotoxic and powerful pollutants. However, the other side of the coin is that these residues from olive oil production can be seen as by-products very rich of phenolic compounds and used as convenient sources of these molecules (Lozano-Sanchez et al., 2017; Cecchi et al., 2018b).

There is a strong interest in recover and utilize olive mill by-products, namely olive mill waste water and olive pomace (Bellumori et al., 2018; Malapert, Reboul, Loonis, Dangles & Tomao, 2018; Parascanu, Sanchez, Soreanu, Valverde, & Sanchez-Silva, L., 2018a; Parascanu et al., 2018b). For this reason, an innovative two-phase decanter (Leopard Series, Pieralisi Group S.p.A. Jesi, Italy) was recently developed. It produces "pâté", that is an olive pomace pitted and partially dehydrated directly after the malaxation step so reducing the possible oxidation processes and preserving phenolic compounds (Cecchi et al., 2018b). These peculiarities make it a suitable ingredient for possible commercial applications after drying. Luciano et al. (2013) included the fresh pâté cake into a concentrate-based diet for lambs, so improving the nutritional quality of the meat (the concentration of vitamin E was increased in muscle) without compromising its oxidative stability (the meat oxidative stability was increased).

Pâté is also potentially suitable for human consumption in the form of food supplement or food ingredient. Fortification of widely spread food products with dried pâté might allow on the one hand to increase the daily intake of phenols from *Olea europaea* L. with the consequent health benefits for consumers, and on the other hand to give added economic value to the olive oil production chain.

To the authors' knowledge, no reports about the use of pâté as ingredient for human food are present in the literature to date. For the first time, we used the dried pâté for the fortification of the recipes of several dishes very widespread worldwide and in particular in the U.S.A., namely pasta, bread and granola bar.

Sensory characteristics of these new types of dish are completely unknown, but they are important for consumers' selection and acceptance (Saldana et al., 2018). Consequently, characterization of these products in terms of sensory profile is strongly demanding. Descriptive Analysis (DA) with expert panelists is the most commonly used methodology in this sense (Lawless & Heymann, 2010). At the same time, it is well known that, with the constant evolution of the food industry, in recent years a shift

towards rapid tools for gathering sensory profile data of food products was observed (Oliver, Cicerale, Pang & Keast, 2017). One of these rapid tools is Check-All-That-Apply (CATA), which in recent years was used for sensory profiling of food products by naïve consumers.

In this scenario, the main aims of the first part of this study were:

- to evaluate in which extent phenols are recovered in the food samples fortified with pâté, when they are ready-to-eat
- to characterize the sensory profile of samples using both DA by a panel of expert judges and Check-All-That-Apply analysis by naïve consumers
- the third objective was to study the impact of the presence of pâté on the sensory characteristic of pasta, bread and granola bar, based on data gathered with sensory profiling

The second part was focused on evaluating the consumer acceptance of the fortified samples, also in comparison with the not fortified ones. Indeed, it is well known that, although the success of a products in the marketplace is affected also by factors like price, market image, packaging, niche ecc, a new food product that does not score well in a consumer acceptance test will probably fail in the marketplace in spite of great marketing (Lawless & Heymann, 2010). We hypothesized that the acceptance of the fortified products is lower than the not fortified ones. However, the question we want to answer to in this part of the research was if consumers still accept the fortified dishes. Data obtained in these two parts were also used for identifying possible drivers of liking, also aimed to uncover possible strategies to improve the recipes of the tasted dishes.Finally, we aimed to gain further qualitative information about the idea to use pâté as ingredient for the tasted products and beyond and further possible applications, by small groups of naïve consumers.

To this aims, two versions of each of the three dishes, namely pasta, bread and granola bar, were developed and produced. They were Control, the dish without pâté, and Sample, the same but fortified with pâté. These six products were evaluated by 175 naïve consumers at University of California, Davis. Descriptive Analysis was then carried out with a panel of expert panelists, and finally three focus group sessions were performed with small groups of 10 naïve consumers.

2. Materials and methods

2.1 Samples

A sample of pâté from typical Tuscan cultivars (Frantoio and Moraiolo as prevalent cultivars) was used as ingredient for sample preparation. About 60 kg of pâté, obtained processing an olive batch of approx. 0.5 ton, were collected on October, 23th 2017 from Terre dell'Etruria olive-mill (Castagneto Carducci, Livorno), immediately after oil extraction process. Freeze-drying of the sample started within 3 hours after collection (Laboratorio Terapeutico M.R., Firenze, Italy), and finished 4 days later, after reaching constant weight (water content, 79% w/w). Freeze-dried sample was ground until obtaining a homogenous powder and stored under vacuum and at room temperature until using (Figure 1).

Figure 1. Dried and ground pâté

Before preparing the food products described in the following paragraph, an aliquot of sample was analyzed with the accredited analytical method (UNI EN 15662:2009, Analytical Group, Florence) and the presence of pesticides was excluded.

Pasta: pasta samples were purchased by Fiordimonte s.r.l. (Cerreto D'Esi, Ancona, Marche, Italy). They were: 1) Pasta Control (**PC**): typical Italian egg dry noodles (ingredients: durum wheat flour, fresh eggs and little amounts of water and salt) and 2) Pasta Sample (**PS**): the same of PC but fortified with 7 % w/w of freeze-dried pâté. Pasta was cooked immediately before testing, as follow: 600 g of pasta were cooked for 390 sec (PC) or 480 sec (PS) in 6 L of boiling salty water (38 g NaCl); immediately after cooking, 40 g of a typical Californian Extra Virgin Olive Oil was added, pasta was well homogenized and immediately served to the subjects.

Bread: bread (both the Bread Control, **BC**, and Bread Sample, **BS**) was baked in the "Carlos Alvarez Food Innovation Laboratory" of the Robert Mondavi Institute – Sensory Building (**FLI-RMI**) of UC Davis the day before each testing sessions. Both recipe and leavening/baking procedure were figured out after several attempts, in order to allow a good leavening of the BS and to have a balanced overall flavor in both BC and BS. Ingredients were: whole wheat flour, bread flour, kosher salt, active dry yeast, Davis municipal tap water, freeze-dried pâté (this latter only for BS). An aliquot of both BC and BS was freeze-dried for the following chemical analysis.

Detailed procedures for bread leavening/baking are following:

Procedure for 1 Loaf of Control Bread:
6.0g active dry yeast was whisked into 400g warm tap water (33 – 37°C) and left to sit for 10 minutes. 250g each of whole wheat flour and bread flour were added to the bowl of an electric mixer (Blakeslee mixer, Nevada Restaurant Supply, Sparks, NV), along with 10.0g kosher salt. The water and yeast mixture was added to the mixer bowl after 10 minutes, and the mixer was fitted with the dough hook attachment. The mixer was turned on to the first speed setting, and the dough was kneaded mechanically for 20 minutes. Then, the dough hook was removed, and the bowl was covered with plastic wrap. The dough was left to rise for 1 hour at room temperature (22 – 24°C).
After rising, the dough was turned out of the bowl onto a floured surface. The dough was punched down by flattening it out with one's knuckles and then folding it in thirds. This was performed twice such that the second round of folds were orthogonal to the first. The dough was shaped into a loaf and set into a pan that was greased with Pam Original No-Stick Cooking Spray (ConAgra, Chicago, IL) and lined with parchment paper. The dough was covered with plastic wrap and left to rise again for 40 minutes at room temperature.
The dough was then uncovered and put into a 232°C oven. Once the loaf was in the oven, the temperature was lowered to 204°C. The bread baked for 30 minutes, and an internal temperature of 99°C was reached. The dough was removed from the pan and left to cool completely on a cooling rack.
Procedure for 1 Loaf of Sample Bread:
6.0g active dry yeast was whisked into 400g warm tap water (33 – 37°C) and left to sit for 10 minutes. 250g each of whole wheat flour and bread flour were added to the bowl of an electric mixer (Blakeslee mixer, Nevada Restaurant Supply, Sparks, NV), along with 10.0g kosher salt. The water and yeast mixture was added to the mixer bowl after 10 minutes, and the mixer was fitted with the dough hook attachment. The mixer was turned on to the first speed setting, and the dough was kneaded mechanically for 20 minutes. Then, the dough hook was removed, and the bowl was covered with plastic wrap. The dough was left to rise for 1 hour at room temperature (22 – 24°C).
After rising, the dough was turned out of the bowl onto a floured surface. The dough was punched down by flattening it out with one's knuckles. Then the olive pomace was kneaded into the dough. The pâté was added in amounts such that it represented a fraction of the total weight of the flour in the dough. For example, 25.0g pâté was added to the breads labeled 5%. The pâté was kneaded into the dough by hand for about 5 minutes, or until the pâté had been hydrated and evenly distributed throughout. The dough was shaped into a loaf and set into a pan that was greased with Pam Original No-Stick Cooking Spray (ConAgra, Chicago, IL) and lined with parchment paper. The dough was covered with plastic wrap and left to rise again for 80 minutes at room temperature.
The dough was then uncovered and put into a 232°C oven. Once the loaf was in the oven, the temperature was lowered to 204°C. The bread baked for 35 minutes, and an internal temperature of 99°C was reached. The dough was removed from the pan and left to cool completely on a cooling rack.
Additional Notes
Because the loaves were made in large batches rather than one at a time, the dough had to be portioned after the first rise. The dough was sectioned into portions of 885 + 3g. This is less than the theoretical 941g that each loaf should weigh if all ingredients (except pomace) were totaled. The discrepancy is due to loss during the transfer of dough from mixer bowl to countertop, and it's also a result of the fermentative action of the yeast on the flour. The pâté amount to add in Bread Sample Loaves was recalculated due to this discrepancy.

Granola bar: granola bar (both the Granola Bar Control, **GBC**, and Sample, **GBS**) was baked in the **FLI-RMI** the day before each testing sessions. Ingredients needed to obtain 2 kg of granola bar are reported in Table 1; sourdough was prepared with starter (100 g), water (900 g) and whole wheat flour (900 g) 24 hours before using it as ingredient for granola bar. Ingredients were mixed all together, well blended and pat out on sheet pan until about 12 mm thick. Baking time (25 min) and temperature (153°C) were set in order to obtain chewy granola bar. An aliquot of both GBC and GBS was freeze-dried for the following chemical analysis.

Ingredient	Granola bar Control	Granola Bar Sample
Sourdough (g)	541	514
Rolled oats (g)	207	197
Raw sesame seeds (g)	257	244
EVOO (g)	144	137
Coconud flakened and sweetened (g)	286	272
Almond, roasted and unsalted (g)	149	142
Multiflower honey (g)	258	245
Chocolate chips (g)	144	137
Salt (g)	14	13
Pâté (g)	0	100
Totale (g)	**2000**	**2000**

Table 1. Recipe composition for Granola Bar

2.2. Analysis of phenolic compounds

Extraction of phenolic compounds was carried out as follow:

- dried pâté: the extraction procedure reported by Cecchi et al. (2018b), suitable for internal standard method, was applied with some modification. Briefly, 1 g of sample was extracted twice with 35 mL of EtOH:H_2O 8:2 v/v, in presence of 0.5 mL of internal standard solution (ISTD, syringic acid, 0.75 mg/mL). The obtained mixture was centrifuged for 10 min at 10 °C and 5000 rpm, defatted twice with hexane, dried under vacuum at 40 °C, re-dissolved in 6 mL of MeOH:H_2O 50:50 and centrifuged for 5 min at room temperature and 14,000 rpm.

- pasta, bread and granola bar: extraction of phenolic compounds from freeze-dried bread and granola bar Control and Sample was carried out with the same extraction procedure applied to the dried pâté, but starting from 3 g of sample. Pasta samples were analysed after cooking it as described in paragraph 2.1. Forty-five g of pasta sample were cooked, resulting in 97.5 g of cooked pasta. Thirty g of cooked pasta were extracted with 65 mL EtOH + 80 mL EtOH:H_2O 8:2 v/v in presence of 0.5 mL of ISTD. The obtained mixture was centrifuged for 10 min at 10 °C and 5000 rpm and the supernatant was collected in a 250 mL flask. The precipitate was extracted again with 160 EtOH:H_2O 8:2 v/v and after centrifugation the supernatant was added to the 250 mL flask. The obtained solution was defatted twice with hexane (150 mL), dried under vacuum at 40 °C, re-dissolved in 6 mL of MeOH:H_2O 50:50 and centrifuged for 4 min at room temperature and 14,000 rpm.

Hydrolysis of the hydroalcoholic extracts

All the obtained hydroalcoholic extracts were submitted to acidic hydrolysis according to Mulinacci et al., (2006) in order to evaluate the total content of free and bound tyrosol and hydroxytyrosol. Briefly, 300 µL of hydroalcoholic extract were treated with 300 µL of H_2SO_4 1M for 2 h at 80 °C in a vial for HPLC. Then, 400 µL of EtOH

were added, the obtained sample was centrifuged at 14,000 rpm for 5 min and the solution was immediately used for the chromatographic analysis.

Chromatographic analysis and phenolic identification and quantification of all the hydrolysed extracts were carried out according to the internal standard method reported by Cecchi et al. (2018).

All the analysis were carried out in triplicate.

2.3. Consumer test

A Central Location Test (CLT) was conducted in the Robert Mondavi Institute – Sensory Building on the University of California, Davis campus in two testing days between 11:00 am and 3:30 pm. Consumers were recruited at farmers markets and through internet sources as the E-mail directory of the University of California, Davis and Sona. Criteria for consumer' selection were that they had to be regular consumers of bread, pasta, and granola bars, 18 and older, not pregnant and had to have no food allergies or dietary restrictions. They were asked not to eat in the three hours before the test and received a $10 gift card for their participation in the study.

The six samples described in paragraph 2.1 were tested. Bread and granola bar were served in small plastic cups (about 20 g) the day after baking. Pasta (about 20 g) was served in biodegradable clamshell containers immediately after cooking. Samples were served with blind 3-digit codes and the only information given the subject was that products were made only with natural ingredients; consequently, the only biases would be from expectations mainly based on the color differences between control and samples (Figure 2).

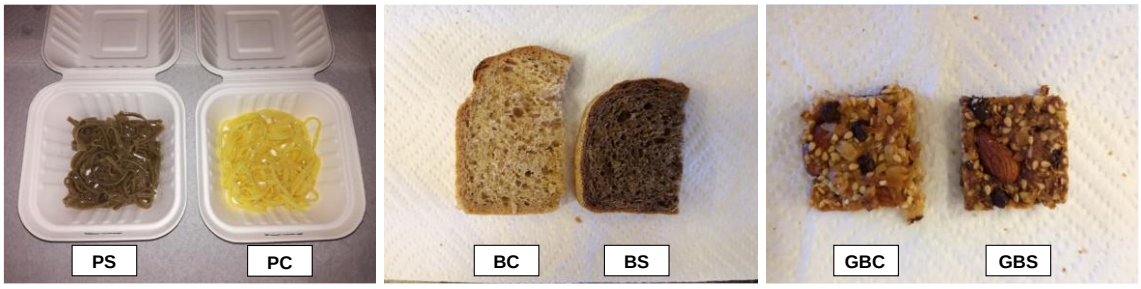


Figure 2. The six tasted products

The two pasta samples were evaluated first, then the two bread samples and finally the two granola bar samples, according to the eight different serving order reported in

Table 2. Each pair of samples was evaluated in counterbalanced two-sample serial monadic sequences.

Serving order n°	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6
1	PC	PS	BC	BS	GBC	GBS
2	PC	PS	BC	BS	GBS	GBC
3	PC	PS	BS	BC	GBC	GBS
4	PC	PS	BS	BC	GBS	GBC
5	PS	PC	BC	BS	GBC	GBS
6	PS	PC	BC	BS	GBS	GBC
7	PS	PC	BS	BC	GBC	GBS
8	PS	PC	BS	BC	GBS	GBC

Table 2. Serving order of the six samples during consumer test. PC = pasta control, PS = pasta sample, BC = bread control, BS = bread sample, GBC = granola bar control, GBS = granola bar sample. The same number of consumers (about 20-22) tasted the samples following the different serving orders.

2.3.1 Data collection

Qualtrics online survey software (Qualtrics, LLC; Seattle, WA, USA) was used for collecting data from CLT. All subjects completed each part of the questionnaire and data were uploaded automatically, so that there were no missing values. Subjects were asked how hungry they were, using a 5-point scale (Table 3). For all samples, four hedonic questions were answered using a 9-point hedonic scale, according to Peryam & Pilgrim (1957). These questions were used to determine the acceptability of the samples; hedonic attributes were liking of the overall appearance, overall liking, liking of flavor, taste and smell, and liking of texture/mouthfeel. Then, 4 Just-About-Right (JAR) five-point nominal scale questions were proposed to evaluate the adequacy of specific attribute for each type of sample: flavor intensity, saltiness, firmness and color for both bread and pasta, and flavor intensity, sweetness, firmness and color for granola bar. For each sample, a 5-point scale question was used to determine purchase intention.

After this, a Check-All-That-Apply (CATA) task was proposed. The proposed CATA descriptors were: al dente, astringent, bitter, bland, buttery, chewy, coarse, dark, elastic, eggy, fatty, firm, floury, fresh, gritty, mild, nutty, oily, olivey, salty, savory and thick for pasta; astringent, bitter, bland, burnt, chewy, cohesive, crumbly, dark brown, dense, dry, flavorful, fresh, gritty, light brown, moist, nutty, olivey, porous, salty, savory, sour, smooth, stale, sweet, toasted/toasty and yeasty for bread; bitter, bland, burnt, caramel, chewy, chocolatey, coconut, crunchy, fatty, fresh, honey, mild, nutty, oily, olivey, savory, sour, sweet, thick and toasted for granola bar. Furthermore, a free space labelled as “other” were in all CATA task. After CATA, three sections for comments

1. *5-point hunger rate scale*

□ Not at all hungry □ Slightly hungry □ Somewhat hungry □ Very Hungry □ Extremely hungry

2. *9-point hedonic scale*

□	□	□	□	□	□	□	□	□
Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

3. *5-point nominal Just About Right scale*

Attribute	Scale				
Flavor intensity	□ Much too weak	□ Somewhat too weak	□ Just About Right	□ Somewhat too strong	□ Much too strong
Saltiness	□ Not at all salty enough	□ Not quite salty enough	□ Just About Right	□ Somewhat too salty	□ Much too salty
Sweetness	□ Not at all sweet enough	□ Not quite sweet enough	□ Just About Right	□ Somewhat too sweet	□ Much too sweet
Firmness	□ Much too soft	□ Somewhat too soft	□ Just About Right	□ Somewhat too firm	□ Much too firm
Color	□ Much too light	□ Somewhat too light	□ Just About Right	□ Somewhat too dark	□ Much too dark

4. *5-point purchase intent rating scale*

□	□	□	□	□
Definitely would not purchase	Probably would not purchase	Might or might not purchase	Probably would purchase	Definitely would purchase

5. *% of price that consumers are willing to pay more for products fortified with antioxidants obtained from the olive oil extraction process*

□ 0% □ 10% or less □ 10% to 20% □ 20% to 30% □ 30% or more

6. *7-point agreement scale used to measure attitude on healthiness and Food Neophobia*

□ Strongly disagree □ Disagree □ Disagree slighlty □ Neither agree nor disagree □ Agree slightly □ Agree □ Strongly agree

Table 3. Scales used by consumers to evaluate each sample during the Central Location Test

were proposed for asking the subjects what else they liked, what else disliked and other comments. After tasting the two products of each type of dish, a preference question was proposed to assess which between Sample or Control was preferred by subjects, including also "no preference" as possible choice. After tasting, an exit survey was proposed to the consumers with question about demographics, psychographics and consumption attitudes. It was focused on the subjects' habits concerned bread, pasta and granola bar, on their availability to pay more for these dishes fortified with antioxidants obtained from the olive oil extraction process. Consumers were asked how frequently they consume pasta, bread and granola bar on a 3-point scale (1 = 1/2 times a week; 2 = 3/6 times a week; 3 = everyday). We also measured attitudes regarding healthiness of foods using the questionnaire from Spencer et al., (2018). The Food Neophobia Scale (FNS) was used to assess the consumers' neophobia and willingness to try unfamiliar foods (Pliner & Hobden, 1992).-All scales used by consumers are reported in Table 3

2.4. Descriptive Analysis

The intensity of the sensory attributes of the same samples tasted during the CLT was evaluated by a trained sensory panel using a modified version of Quantitative Descriptive Analysis (QDA®), in which standards were not provided and only verbal definitions were used (Stone Bleibaum & Thomas, 2012; Murray, Delahunty & Baxter, 2001).

The panel was made up of 10 expert panellists (2 male, 8 female) aged between 20 and 65 years from Davis, California (USA) population. Recruitment was carried out based on the fact that panellists had to be regular consumers of bread, pasta and granola bar and that they had already participating in several sessions of Descriptive Analysis (expert panellists). Panelists had also to be English native language. For each type of dish (pasta, bread and granola bar), descriptors were generated by consensus for the categories appearance, aroma, taste, flavor, texture/mouthfeel and aftertaste, after presenting the panellists a wide range of variations of products (our Control and Sample and a series of commercial products each other as different as possible). Descriptors generated and scored by the panellists are summarized in Table 4 for each type of dish.

After generating descriptors, the panellists were trained in several group sessions of practice in rating the attributes' intensity of each product. During these sessions, in

order to ensure concept alignment across the panel, panellists shared their ratings and, for those attributes for which major differences in panellists' ratings emerged, panellists were asked to taste the products again until reaching consensus (Stone et al., 2012).

	PASTA	BREAD	GRANOLA BAR
		APPEARANCE	
1	Color *(Yellow to Greenish Grey)*	Internal color *(Grey to Brown)*	Color *(Golden to Brown)*
2	Color intensity *(Light to Dark)*	Internal color intensity *(Light to Dark)*	Color intensity *(Light to Dark)*
3	Evenness of color *(Uneven to Even)*	External color *(Light brown to Dark brown)*	Evenness of color *(Uneven to Even)*
4	Shininess *(Matte to Shiny)*	Evenness of color *(Uneven to Even)*	Shininess *(Matte to Shiny)*
5	Surface roughness *(Smooth to bumpy)*	Porosity *(No bubbles to Lots of bubbles)*	Thickness *(Thin to Thick)*
6	Thickness *(Thin to Thick)*	Bubble size *(Small to Large)*	
7		Degree of leavening *(Not risen at all to Very well risen)*	
8		Crust thickness *(Thin to Thick)*	
		AROMA	
9	Eggy	Yeasty	Cardboard
10	Olive oil	Earthy	Rancid oils
11	Buttery	Rancid oil	Toasted *(Light to Burnt)*
12	Earthy	Toasted	Nutty
13	Wheat	Grain	Honey
14		Nutty	Coconut
15			Dried fruit
16			Chocolatey
17			Buttery
		TASTE	
18	Sweet	Sweet	Sweet
19	Salty	Salty	Salty
20	Bitter	Bitter	Bitter
21	Sour	Sour	Sour
22	Umami		
		FLAVOR	
23	Buttery	Olives	Toasted *(Light to Burnt)*
24	Eggy	Toasted	Nutty
25	Olive oil	Nutty	Oats
26	Wheat	Grain	Chocolatey
27	Cardboard	Yeasty	Caramel
28	Earthy	Earthy	Coconut
29			Dried figs
30			Vanilla
31			Buttery
		TEXTURE/MOUTHFEEL	
32	Dryness *(Moist to Dry)*	External dryness *(Moist to Dry)*	Dryness *(Moist to Dry)*
33	Firmness *(Soft to Firm)*	Internal dryness *(Moist to Dry)*	Firmness *(Soft to Firm)*
34	Chewyness	External firmness *(Soft to Firm)*	Crunchyness
35	Stickyness	Internal firmness *(Soft to Firm)*	Stickyness
36	Slippery	Crust chewyness	
37	Evenness of texture *(Uneven to Even)*	Crumb chewyness	
38	Cooking Level *(Undercooked to Overcooked)*	Astringent	
39	Astringent		
		AFTERTASTE	
40	Salty	Sweet	Sweet
41	Astringent	Salty	Salty
42	Eggy	Bitter	Bitter
43	Oily	Sour	Astringent
44		Astringent	Chocolatey
45		Olive oil/Olives	

Table 4. List of the descriptors generated by the trained panelists for Descriptive Analysis. Low and High were usually used at the anchored points of the unstructured line scale; for those attributes for which panelists chose different words, these are reported in the brackets

After training, each panellist evaluated both Sample and Control in triplicate (i.e. complete block design) in isolated booths. Samples were presented in a monadic way and in a fully randomized order, coded with 3-digit number, and in the same conditions of the CLT. Filtered tap water was used for mouth rinsing after each sample. The attributes were

evaluated following the order: appearance, aroma, taste, flavor, texture/mouthfeel and aftertaste.

RedJade Sensory Software (RedJade Software Solution, LLC; Palo Alto, CA, USA) was used for online collecting data. Panellists used a 10-cm unstructured graphic line scales to describe the intensity of rated attributes across the products. The scales were anchored at 0.8 cm and 9.2 cm with the words reported in Table 4.

All sessions of descriptive analysis were carried out in the Robert Mondavi Institute – Sensory Building on the University of California, Davis.

2.5. Focus Group

Three focus group sessions were conducted in the Robert Mondavi Institute – Sensory Building on the University of California, Davis campus on three different days between 1:30 am and 4:00 pm. Groups consisted of 6, 7 and 9 consumers. They were recruited based on the same criteria of the CLT. They were 59% female, with 27% ranging in age from 18 to 24, 27% from 25 to 34, 32% from 35 to 54, and 14% aged 55 and older. The participants were 72% White/Caucasian, 5% Hispanic/Latino(a), and 23% Asian. Information obtained during the sessions was reported by three observers per session. The sessions were also recorded and listened to again. The focus groups were conducted using the following script.

Focus group – pâté: a new food ingredient

Greeting. 5 minutes

Today we are interested in getting your honest opinion about a new food ingredient and how it could be incorporated into new products. This focus group will last about 1 hour and a half. Please feel free to speak openly about your feelings. Any comments, suggestions or other input will be greatly appreciated and please know that there are no right or wrong answers! You may find that others have a different opinion than you, please don't let that stop you from expressing your own opinion. In order to make sure that everyone has a chance to express their opinion, please try to talk one at a time and to not interrupt each other.

Your answers and identity will be kept confidential and will not be connected to you. This session will be videotaped as was explained to you. Videotapes will only be used to ensure that all notes collected during the sessions are complete. There also may be some researchers watching and listening to us on the other side of the one-way mirror behind me.

If you feel uncomfortable and would like to withdraw from this study, you may do so at any time.

Introduction 5 minutes

Let's find out a little more about each other by going around the table and introducing ourselves.

Tell me a little about you: What is your name? What do you do for living? Then, just for fun, tell us the first word that comes to your mind when you think about extra virgin olive oil

1. General question about olive oil. No more than 10 minutes

Now, I'd like to talk more specifically about olive oil.

1. How often do you consume olive oil?
2. What do you think about olive oil? Why is it important for you?
   (*If health benefits come up, probe and expand*)
3. Please, tell me something more about your opinion on the health benefits of olive oil. (*Ask this question only if health benefits don't come up while they answer the previous questions*).
4. Do you associate any of the sensory qualities of olive oil with its health properties?
   (*Suggest bitterness and pungency if they don't come up with them*)

2. Questions about the pomace No more than 10 minutes

Now, I'd like to talk about a new ingredient obtained from olive oil production process.

1. What do you think about recover food waste and use them in the food industry? Why do you think it is important?
   Health and sensory properties of olive oils mainly depend on polyphenols.

They are present in high amounts in olive fruits, but only a very low part is recovered in virgin olive oil during the extraction process, while the larger amount finishes in the residues, that today we will named pomace.
Today, thanks to new technologies, it is possible to recover this pomace immediately after olive oil extraction with no chemical or coadjutants, so getting a fully natural product, very rich in polyphenols. Consumption of very low amounts of this pomace after drying, allows to intake the amount of polyphenols recommended by many food authorities.
For these reasons we are trying to use the dried pomace as food ingredient.
Now I'm going to show you the dry pomace in the form to be used as food ingredient.

2. What do you think about it?
3. What do you feel about incorporating it in different foods?
4. What do you expect it will do to the sensory profile?
5. In general, what do you think about fortification of food with antioxidants/polyphenols?

3. Questions about the use of the pomace No more than 10 minutes

Now, I'm going to ask you your opinion about application of the pomace.

1. In which type of food would you put this ingredient?
2. Why is this food important for you? And why would you put the pomace in it?
3. In general, would you propose some other application for the pomace?
4. Why did you propose that application?

Break 5 minutes

We put the pomace in pasta, in bread and in granola bar. Now, I'll ask you to taste these product and to tell me about your opinion. Before, you have a 5-minutes break, during which the samples are prepared. Please wait in the room.

4. Tasting session No more than 30 minutes

PASTA: **10 minutes.** Now, please taste the pasta.

1. What do you think about it? Why do you like (dislike) it?
2. How would you consume it? Why?
3. Who would you serve this to? Children, guests..? Why? Why not?

BREAD: **10 minutes.** Now, please taste the bread.

1. What do you think about it? Why do you like (dislike) it?
2. How would you consume it? Why?
3. Who would you serve this to? Children, guests..? Why? Why not?

GRANOLA BAR: **10 minutes.** Now, please taste the granola bar.

1. What do you think about it? Why do you like (dislike) it?
2. How would you consume it? Why?
3. Who would you serve this to? Children, guests..? Why? Why not?

5. Purchase and consumption intentions. No more than 10 minutes

This is the last session, in which I'll ask you something about your purchase and consumption intentions.

1. Would you buy and consume something like that? Why yes/no? How often?
2. When and where would you consume it?
3. After tasting these products, what other application would you propose for the pomace? Why?

Wrap-Up Question 5 minutes

These were all the questions I had for you. Is there anything else you'd like to know about our project or other comments you would like to share with us in order to help us understand more about fortification of food products with pomace?
Thank you for your time. It was a pleasure to meet all of you. Your answers will be very helpful as we move forward with this project. We'll give you your gift certificates on your way out.

2.6 Data analysis

Consumer Test Data: For each category of sample, in order to compare the hedonic ratings for Control and Sample, t-test was run in XLSTAT-Sensory® (Addinsoft, New York City, NY, USA).

Data from JAR questions were collapsed into three categories before analyzing them: those above the just-right, the just-right and those below the just-right. For each dish, chi-squared tests based on the Stuart-Maxwell test were run for each Just-About-Right attribute for evaluating if they were able to differentiate the two kind of products. Finally, the effect of the scores of JAR attributes on the mean overall liking of each product was evaluated running penalty analysis in XLSTAT-Sensory® (Addinsoft; New York, NY, USA).

For each of the three tested dishes, CATA data were analyzed separately in XLSTAT-Sensory®. The independence of the samples from the attributes was tested by running a χ^2 test (α, 0.05) and the usefulness of each attribute in discriminating between the two products was tested using the Cochran's Q test with McNemar-Bonferroni mean comparison test. For each dish, a binary matrix was built with the CATA data and used to generate a contingency table. For each sample, we compared the effect of each of the significant CATA attribute on the consumers' acceptance of the samples; to this aim, the mean impact on the overall liking (measured on the 9-point hedonic scale) of those CATA attributes was measured as the difference between the mean overall liking of samples with the presence of that attribute and the mean overall liking of samples with the absence of the same attribute. Finally, in order to provide a map of the relationships between the attributes and the consumers' liking, a Principal Coordinate Analysis (PCA) was run for each of the three dishes and the first two component were plotted; the three PCAs were based on a correlation matrix generated from tetrachoric correlation coefficients between CATA attributes and biserial correlation coefficients between each CATA attribute and overall liking.

For the analysis of the preference data, which included also the "no preference" option, we applied a confidence interval analysis (Lawless & Heymann, 2010) suitable for a dataset with over than 100 samples and with the no preference option used less than 20% (Quesenberry & Hurst, 1964).

Descriptive Analysis Data: for each of the three dishes, the significance of overall differences between Sample and Control was initially evaluated performing a multivariate analysis of variance (MANOVA). Then, a three-factor, fixed effects ANOVA was run for each attribute, the three factors being the products, the judges and the replications. All one-way and two-way interactions were calculated. Since judges have been considered a fixed effect, a pseudomixed model ANOVA was applied for re-analyzing the "sample" *F*-ratio, using the "Sample*Judge" interaction as the error term. All MANOVAs and ANOVAs were run in RStudio 2016 (RStudio, Boston, MA, USA).

3. Results and discussion

3.1 Chemical Data

The first goal of this work was to evaluate the percentage of the phenols from pâté recovered in the fortified products, when they are ready-to-eat. To this aim, the phenolic fraction of dried pâté used as ingredient for the fortification, and the three fortified samples were analyzed following the same approach, as reported in the "materials and methods" section.

	Pâté	Pasta Sample		Bread Sample		Granola Bar Sample	
	(mg/kg)	(mg/kg)	%	(mg/kg)	%	(mg/kg)	%
Hydroxytyrosol (OHtyr)	9764 ± 413	63 ± 5	9	235 ± 4	48	364 ± 12	75
Tyrosol (tyr)	1511 ± 84	16 ± 2	15	43 ± 1	57	65 ± 2	86
Sum of tyr + OHtyr	11275 ± 497	79 ± 7	10	278 ± 5	49	429 ± 14	76

Table 5. Content of total tyrosol and hydroxytyrosol (free and bound forms) after acidic hydrolysis in dried pâté, in cooked Pasta Sample, Bread Sample and Granola Bar Sample quantified by the internal standard method, according to the IOC protocol for biophenols of olive oil. Data are expressed as mg_{tyr}/kg mean ± SD of three independent measurements. For each type of dish the percentage of recoveder tyrosol and hydroxytyrosol is also reported.

Table 5 shows the total content of tyrosol (tyr) and hydroxytyrsol (OHtyr) in their free and bound forms, after acidic hydrolysis, in dried pâté, in cooked Pasta Sample and in Bread and Granola Bar Samples and the recovered % of these molecules based on the amount of dried pâté in recipes. Analysis of Pasta, Bread and Granola Bar Controls evidenced no presence of these molecules (data not shown). Data show a higher recover of tyr and OHtyr in GBS (76%) and BS (49%) and a lower recover (10%) in PS. These differences could be due to both the baking conditions (i.e. higher temperature and baking time for bread than granola bar) and interaction between pâté phenols and other ingredients (e.g. gluten and egg proteins in pasta). Further studies, out of the aim of this work, are needed to better clarify this aspect. Anyway, these data highlighted that to reach the minimum intake of olive oil phenols (e.g. 5 mg of hydroxytyrosol and its derivatives, e.g. oleuropein complex and tyrosol) that the EFSA recommend consuming daily in order to benefit from the health effects stated by the aforementioned health claim, the following amount of our fortified dishes are enough: Pasta 63 g, Bread 18 g, Granola Bar 12 g.

3.2 Characterization of the sensory profile of the tasted products

The next step of the work was aimed to describe the sensory profile of the six products and to evaluate the effect of the presence of pâté in the sensory profile of the three kind of dishes. We pursued this aim following two different approaches:

- The first approach was the Check-All-That-Apply (CATA) analysis, carried out during the Consumer Test by 175 naïve consumers. Application of CATA to description of the sensory profile of food products is finding application mainly due to the growing request of fast description of food products by the food industry (Oliver et al., 2017)
- The second approach was the application of the typical methodology used by sensory scientist to describe the sensory profile of food products, namely Descriptive Analysis (DA), carried out by a panel of trained expert judges. DA is reported to be more suitable to describe smaller differences between samples (Oliver et al., 2017).

Following these two different approaches in an independent way, we aimed to gather information on the sensory profile of the tasted products by both naïve consumers and expert judges. In this sense, it should be noted that the attributes for the CATA analysis were crated before Descriptive Analysis was performed.

3.2.1 Characterization of products by Check-All-That-Apply attributes

CATA questions were proposed to consumers in order to obtain a first rapid characterization of samples from naïve consumers (Meyners, Castura and Carr, 2013). The application of CATA was considered suitable to a first rapid description of the sensory profile of these products since, for each type of dish, the sample set was relatively simple (two variants with only one different ingredient in the recipe) and the differences were suppose to be rather clear.

With the constant evolution of food industry, the impact of consumers on the products available on the market is becoming more and more important (Oliver et al., 2017). This evolution has had a further consequence in the shift towards the use of rapid tools for description of the sensory profile of the food products (Dehlholm, Brockhoff, Meinert, Aaslyng & Bredie, 2012). For these reasons, within the questionnaires developed for the consumer test, which was performed for gathering acceptance data by

naïve consumers, we decided to insert a Check-All-That-Apply task, with the aim of obtaining a description of the sensory profile of the products also by naïve consumers. It is worth nothing that performing CATA analysis didn't result in any additional cost of this work.

Descriptors for CATA were generated before that descriptive analysis was carried out. By this way, description of the sensory profiles through both CATA analysis by naïve consumers and Descriptive Analysis by trained expert judges led to independent results and a more detailed description of the sensory profiles was obtained.

The number of consumers that checked each attribute is presented as contingency tables for samples of pasta (Table 6a), bread (6b) and granola bar (6c). We considered able to differentiate between Sample and Control those attributes used by at least 20% of consumers for at least one among Sample and Control and for which the Cochran's Q test with McNemar-Bonferroni mean comparison gave significant differences at $p < 0.05$). Following this approach 11 of the 22 proposed attributes were able in differentiating between pasta samples (50%), 9 of 26 differentiated bread samples (35%) and 9 of 20 differentiated granola bar samples (45%).

Pasta: from data in Table 6a, the attributes able to differentiate between PC and PS were "buttery", "chewy", "dark", "eggy", "firm", "fresh", "gritty", "mild", "nutty", "olivey", "thick". It immediately appears that "bitter" and "astringent" were checked by a very few number of consumers for both PC (2 and 1 times, respectively) and PS (9 and 1). This could be partially unexpected for PS, since pâté is very rich of phenolic compounds from *Olea europaea* L., a class of chemical compounds well known for their bitterness (Gutiérrez-Rosales, Rios & Gòmez-Rey, 2003); however, results from chemical analysis showed that only a minor part of phenols are recovered in the PS when it is ready-to-eat (≈10%, table 5) and, although this fact doesn't compromise the capability of this pasta in providing good source of phenols for the daily diet, it reduces the impact of pâté on perception of bitter and astringent attributes.

Interestingly, "Al dente" was used by about half of consumers for both PC and PS, confirming that the different cooking times set at the beginning of the study were suitable to obtain similar cooking levels. The presence of pâté in PS seemed to negatively affect some attributes typical of egg dry noodles, as "buttery" (checked 94 for PC vs 48 for PS), "eggy" (88 vs 27) and "fresh" (57 vs 35), while, as expected, "dark" was the attribute that

mostly differentiated PS (checked 135 times) from PC (3). Also “mild” was checked by a lower number of consumers for PS than PC, likely indicating that the presence of pâté increase the overall complexity of PS. In this sense, attributes like “firm”, “gritty”, “nutty” and “olivey” had been positively affected by the presence of pâté.

a) Pasta		
Attributes\Products	**Control**	**Sample**
al dente	83 (a)	76 (a)
astringent	1 (a)	1 (a)
bitter	2 (a)	9 (b)
bland	65 (a)	62 (a)
buttery	94 (b)	48 (a)
chewy	84 (a)	100 (b)
coarse	8 (a)	27 (b)
dark	3 (a)	135 (b)
elastic	21 (a)	21 (a)
eggy	88 (b)	27 (a)
fatty	14 (a)	8 (a)
firm	79 (a)	109 (b)
floury	23 (a)	22 (a)
fresh	57 (b)	35 (a)
gritty	6 (a)	46 (b)
mild	78 (b)	54 (a)
nutty	9 (a)	40 (b)
oily	79 (a)	69 (a)
olivey	36 (a)	53 (b)
salty	33 (a)	28 (a)
savory	62 (a)	60 (a)
thick	47 (a)	74 (b)
other	6 (a)	5 (a)

b) Bread		
Attributes\Products	**Control**	**Sample**
astringent	2 (a)	7 (a)
bitter	5 (a)	63 (b)
bland	41 (b)	21 (a)
burnt	4 (a)	20 (b)
chewy	95 (a)	105 (a)
cohesive	25 (a)	21 (a)
crumbly	11 (b)	3 (a)
dark brown	14 (a)	134 (b)
dense	42 (a)	105 (b)
dry	56 (a)	47 (a)
flavourful	42 (a)	49 (a)
fresh	69 (b)	42 (a)
gritty	8 (a)	11 (a)
light brown	127 (b)	10 (a)
moist	73 (a)	65 (a)
nutty	36 (a)	55 (b)
olivey	14 (a)	23 (b)
porous	72 (b)	30 (a)
salty	18 (a)	21 (a)
savory	47 (a)	36 (a)
sour	17 (a)	28 (b)
smooth	32 (b)	16 (a)
stale	15 (a)	19 (a)
sweet	14 (a)	8 (a)
toasted/toasty	39 (b)	26 (a)
yeasty	28 (a)	28 (a)
other	16 (b)	5 (a)

c) Granola Bar		
Attributes\Products	**Control**	**Sample**
bitter	19 (a)	67 (b)
bland	4 (a)	7 (a)
burnt	18 (a)	62 (b)
caramel	35 (b)	20 (a)
chewy	142 (a)	137 (a)
chocolatey	93 (a)	77 (a)
coconut	101 (b)	64 (a)
crunchy	57 (a)	59 (a)
fatty	8 (a)	6 (a)
fresh	36 (b)	24 (a)
honey	51 (b)	28 (a)
mild	29 (b)	17 (a)
nutty	144 (a)	138 (a)
oily	12 (a)	14 (a)
olivey	2 (a)	9 (b)
savory	31 (a)	28 (a)
sour	4 (a)	13 (b)
sweet	107 (b)	66 (a)
thick	21 (a)	41 (b)
toasted	79 (a)	97 (b)
other	14 (a)	17 (a)

Table 6. Contingency tables for the CATA evaluation for the three tasted dishes. For each attribute, different letters in the brackets indicate the capability of that attribute in discriminating between the two products (Cochran’s Q test with McNemar-Bonferroni mean comparison test, $p < 0.05$).

Bread: data in table 6b show that “dark brown” was the attribute more used for BS (134 times, 77%) and “light brown” for BC (127 times, 73%). These two were also the attributes that differentiated between BC and BS the most. Unlike what we saw for pasta, “bitter” strongly differentiated BS (63) from BC (5), again in agreement with chemical data (recover of phenols for BS, ≈50%). Other attributes able to differentiate between BC and BS were “bland”, “dense”, “fresh”, “nutty” “porous” and “toasted/toasty”. The presence of pâté reduced the perceived freshness of BS but enhanced “nutty”, as already seen for Pasta Sample respect to Pasta Control. The presence of phenols from pâté makes leavening of the dough for BS more difficult, resulting in much higher density and in lower porosity.

Granola bar: table 6c shows that attributes able to differentiate between GBC and GBS were “bitter”, “burnt”, “caramel”, “coconut”, “fresh”, “honey”, “sweet”, “thick” and “toasted”. “Chewy” was checked by about 80% of consumers for both GBC and GBS, confirming that the baking process set up at the beginning of the research was suitable to obtain chewy granola bars, that was what we wanted. Like for bread and again in agreement with results from chemical analysis, “bitter” strongly differentiated GBS from GBC. Also the attributes “burnt”, “thick” and “toasted” were checked more times for GBS than for GBC, likely indicating that we could try to reduce the baking time for GBS in order to soften the increase of perceived “burnt”. For granola bar, the attribute “nutty” had not been affected by the presence of pâté in GBS, because of the presence of nuts in the recipes that made the “nutty” strongly perceived for both GBC and GBS. Finally, the attributes “caramel”, “coconut”, “fresh”, “honey” and “sweet” were negatively influenced by the presence of pâté in GBS, even though the number of consumers that perceived the attribute “fresh” was quite low and similar for both GBC and GBS

3.2.2 Characterization of products by Descriptive Analysis

To our knowledges, this work represent the first attempt at documenting the effect of incorporation of pâté in the recipes of human foods, and in particular, in these three types of dishes. Since for each type of dish, the panel had to evaluate only two different products (Control and Sample) and these samples were quite simple (no sauces or condiments and really basic recipes), the panel had need not too long training (a total of nine hours). However, in order to check the reliability of the obtained results, the following step were carried out:

- Application of MANOVA for evaluate the significance of the overall differences between Control and Sample for each type of dish;
- Evaluation of the performance of the panel, according to previously published papers (Saldana et al., 2015). In particular, the performance of the panel was evaluated through analysis of variance in terms of:
    1. Discrimination: when there was a significant effect of the sample ($p < 0.05$), the panel was considered able to discriminate between the two products (Sample and Control) with respect to the given attributes

2. Consensus: when there was a not significant effect of the interaction between sample and judges ($p > 0.05$), panel was considered able to percept similarly the given attribute across the products. It should be noted that, when there was a significant effect of this interaction, the sample *F*-ratio was recalculated, but this time using the "Sample*judge" interaction as the error term and the capability of the panel in discrimination between samples was re-evaluated, as at point 1.
3. Repeatability: when there was a not significant effect of the interaction between sample and replications ($p > 0.05$), panel was considered able to use similarly an attribute across the replication.

- Identification of those attributes that were able to discriminate between the samples, by Analysis of Variance

3.2.1.1. Descriptors' generation

For each type of dish, in the first session, judges tasted a set of products and wrote down all the words/attributes that in their opinion were able to describe and differentiate among the products. The products tasted in this session were our Sample and Control, but also other products available on the market and each other as different as possible. Words were generated for the categories appearance, aroma, taste, flavor, texture/mouthfeel and aftertaste. In this session, each panelist tasted the product and wrote down the words by itself and in silence. In the next session, panelists tastes a reduced set of products (our Sample and Control and a third product not so different from them) in order to uniform concepts and define the words at the anchored end of the scale to be used for training and products' evaluation (Table 4). For each categories of attribute, synonyms and antonyms were grouped so obtaining orthogonal descriptors (Lawless & Heymann, 2010). They were, 34 descriptors for pasta, 37 for bread and 36 for granola bar.

3.2.1.2. Pasta

For the descriptive analysis data of each of the three dishes, the application of a MANOVA indicated an overall significant difference between Control and Sample exhibited by the attributes (Pillai's trace: pasta 0.98280, bread 0.97766, granola bar 0.94597; $p < 0.001$).

Table 7 shows the *F*-ratios calculated for the different sources of variation from the ANOVAs performed for each attribute for Pasta (Sample and Control). Immediately, it appears that for only 1 of the 34 attributes there was a significant effect of the interaction Sample*Replication, so that we can conclude that the repeatability of the panel across the replication was very good. In order to identify the attributes able in differentiating among Sample and Control we applied the following approach: for each attribute, firstly, we kept into account the effect of the sample. If this effect was not significant, we conclude that this attribute didn't contribute to differentiate among the samples. On the contrary, if this effect was significant, we evaluated the consensus among assessors keeping into account the effect of the interaction judge*sample. A not significant interaction indicated consensus among judges that allowed us concluding that this attribute was significant in discriminating between the samples; a not significant interaction indicated a lack of consensus among judges. In this latter case, in order to evaluate if that attribute contributed in differentiating among the samples, we kept into account the effect of the sample again, but this time after recalculating it considering the effect judge*sample as the error term: if this effect was significant, that attribute was able to discriminate among the samples, and vice versa. Those attributes that, following this approach, showed significant difference among the samples are reported in italic and underlined in Table 7. Figure 3 shows Spider web plots for the six categories of attributes, with attributes with significant differences ($p < 0.05$) among the sample reported in red.

In the category of appearance, the attribute mainly affected by the presence of pâté was the color, which was yellow for the PC and greenish grey for PS, as expected (see also Figure 1). Also evenness of color was affected by the presence of pâté, while weaker differences for surface roughness and thickness and no significant differences for shininess and color intensity were found. The presence of pâté had a strong effect in softening the eggy and in increasing the earthy aromas, while significant but lower effects

	Attribute	Judge (J)	Sample (S)	Replication (R)	Judge*Sample	Judge*Replication	Sample*Replication	Sample (e = Judge*Sample)
APPEARANCE	*Color*	**3.83**	**1798.56**	1.06	**5.98**	0.86	0.85	***300.67***
	Color intensity	**4.24**	**25.32**	0.23	**5.98**	1.03	0.01	4.24
	Evenness of color	1.98	***106.9***	1.00	2.20	0.96	1.00	**48.66**
	Shininess	**17.05**	2.17	0.14	1.67	2.14	2.65	1.30
	Surface roughness	**12.26**	**77.85**	1.03	**2.51**	1.09	1.99	***30.97***
	Thickness	**6.97**	***55.92***	0.50	2.22	0.82	1.44	**25.19**
AROMA	*Eggy*	**9.56**	**75.13**	**6.11**	**3.51**	1.73	0.73	***21.37***
	Olive oil	2.28	***4.56***	1.27	1.37	1.40	2.62	3.34
	Buttery	**9.33**	***6.21***	1.99	0.61	0.52	0.07	**10.16**
	Earthy	**13.74**	**410.37**	1.41	**13.26**	**2.44**	0.05	***30.96***
	Wheat	**10.77**	***10.69***	0.63	1.42	0.70	0.21	**7.51**
TASTE	Sweet	**5.10**	1.54	0.59	2.34	1.25	2.01	0.66
	Salty	**8.32**	***7.29***	1.50	1.8	0.95	0.26	3.88
	Bitter	**5.24**	**6.63**	2.60	**3.13**	1.74	0.08	2.12
	Sour	**7.31**	***4.83***	0.34	1.85	0.56	0.72	2.61
	Umami	**12.80**	4.36	3.21	**4.28**	1.09	0.35	1.02
FLAVOR	Buttery	**22.46**	**14.87**	**9.31**	**3.89**	**2.24**	2.21	3.82
	Eggy	**8.12**	***11.64***	0.04	0.88	0.88	1.55	**13.21**
	Olive oil	**2.99**	***6.67***	**7.68**	1.28	**2.30**	1.620	**5.21**
	Wheat	**19.94**	***26.37***	**3.67**	1.90	1.51	0.88	**13.85**
	Cardboard	**14.26**	**23.28**	0.50	**8.26**	1.08	0.77	2.82
	Earthy	**11.10**	**200.39**	2.35	**8.86**	0.81	0.40	***22.61***
TEXTURE	*Dryness*	**7.59**	***37.96***	1.11	2.13	**2.38**	0.27	**17.80**
	Firmness	**12.84**	**25.46**	2.43	**4.52**	0.76	0.42	***5.63***
	Chewyness	**5.02**	***4.43***	1.28	1.29	0.77	1.03	3.44
	Stickyness	**15.01**	0.01	**5.35**	2.38	1.69	0.38	0.01
	Slippery	**3.75**	***20.71***	0.58	1.78	1.48	0.45	**11.61**
	Evenness of Texture	**6.12**	**45.55**	1.64	**2.50**	0.86	0.32	***18.21***
	Cooking level	1.70	***15.75***	0.74	0.86	0.85	0.23	**18.42**
	Astringent	**4.52**	***20.38***	1.41	0.81	0.88	0.26	**25.15**
AFTER TASTE	Salty	**12.90**	0.44	0.90	1.42	1.45	0.31	0.31
	Astringent	**18.34**	***23.41***	**12.40**	1.70	**2.36**	1.49	**13.73**
	Eggy	**8.44**	**8.96**	0.52	**3.33**	2.02	**5.68**	2.69
	Oily	**9.89**	4.20	0.09	1.11	0.95	0.18	3.76

Table 7. *F*-ratios of the different sources of variation from ANOVA for the 34 attributes rated by descriptive panel for the 2 pasta samples. *F*-ratios for which significance was at $P < 0.05$ are reported in bold text. The attributes that showed significant difference among the samples are in italic and underlined

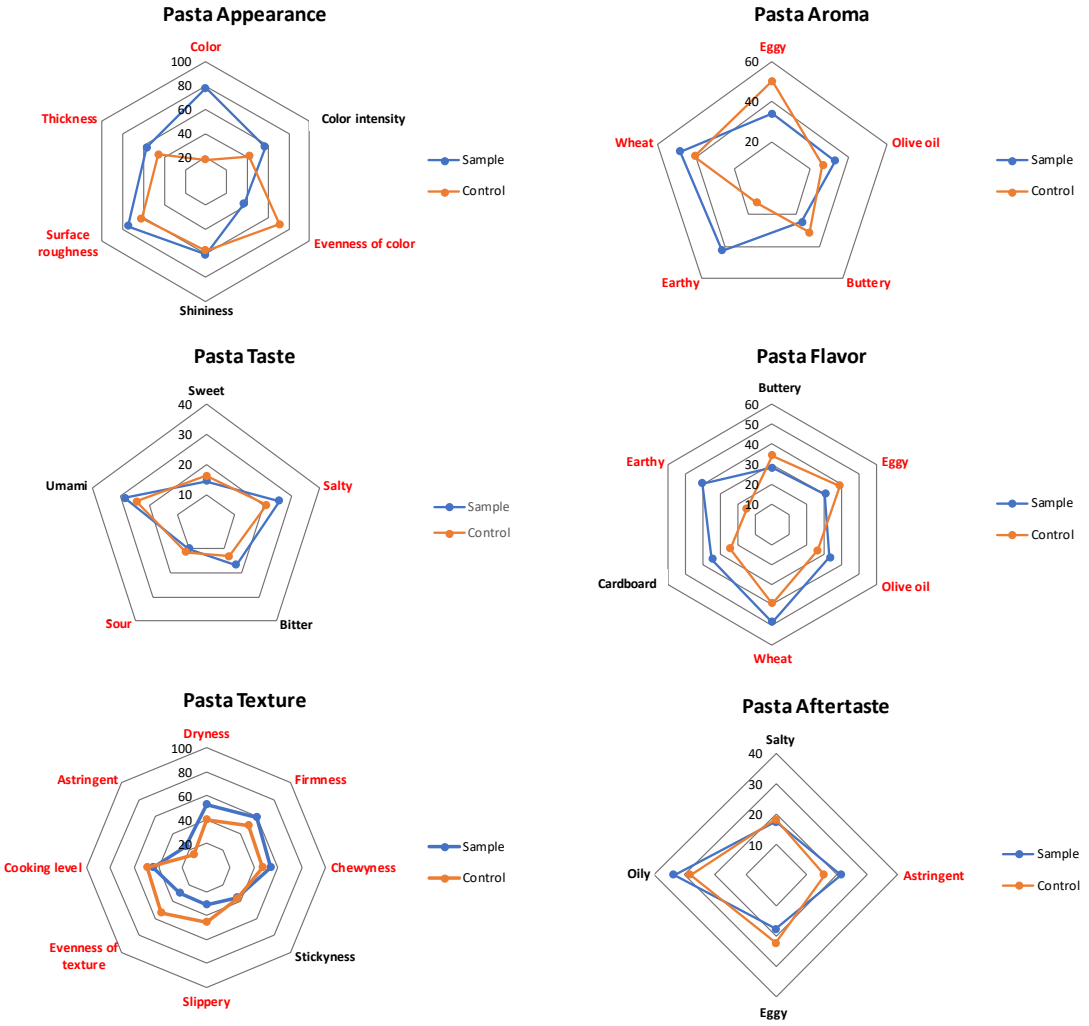


Figure 3. Spider web plot of pasta descriptive analysis for six categories of attributes (Appearance, Aroma, Taste, Flavor, Texture, Aftertaste). Attributes for which there was a significant difference ($p < 0.05$) in the panelists' rates are in red.

were found for the other aroma attributes. Interestingly, the presence of pâté had a very low impact on the typical attributes of taste and, quite surprisingly, the taste of bitter was not significantly affected. However, this latter evidence is not fully surprisingly, in light of the results from chemical analysis that showed that only about 10% of phenols from pâté were recovered in the ready-to-eat PS, the phenols being responsible for the bitterness of products from *Olea europaea* L. Within the category of flavour, earthy was strongly affected by the presence of pâté, as also already seen for the earthy aroma; significant but lower effects were highlighted for eggy (which was lowered by the presence of pâté) and for wheat and olive oil (which were increased) aromas, while buttery and cardboard were not affected by pâté. All the texture attributes, except stickiness, were affected by the presence of pâté, even though these effects were not so strong. Interestingly, the effect on the attribute cooking level was very small, and, even

though it resulted significant, the difference between Control and Sample could be considered negligible. This similarity in the perceived cooking level between PC and PS is the result of the different cooking time set up at the beginning of the experiment, as reported in the material and method section and confirms that this difference in cooking times is suitable to make level of cooking similar in spite of the effect of the presence of pâté. Moreover, the panel perceived the level of cooking at the half of a scale anchored with undercooked and overcooked (Table 4), confirming this cooking time as suitable to obtain the pasta close to the ideal cooking level. Finally, a very low effect of the presence of pâté has been highlighted for the attributes in the category of aftertaste.

3.2.1.3. Bread

As mentioned above, MANOVA indicated an overall significant difference between Control and Sample exhibited by the attributes for bread.

Table 8 shows the *F*-ratios calculated for the different sources of variation from the ANOVAs performed for each attribute for Bread (Sample and Control). Also in this case, the repeatability of the panel across the replication was very good. For identifying the attributes able in differentiating between Sample and Control, the same approach already described for pasta was used. Figure 4 shows Spider web plots of the six categories of attributes, with attributes with significant differences ($p < 0.05$) among the sample reported in red.

The presence of pâté had a strong impact on all the attributes in the category of appearance, with the only two exceptions of porosity and external color. It is worth noting that the presence of pâté in the recipe of BS strongly affected the degree of leavening, which, for BS, resulted lower than BC. This difference led to different size of the bubbles and crust thickness, while the number of bubbles were not significantly affected. In this sense, DA confirmed to be able to uncover and describe more detailed differences than CATA, which, in our experiments, described the different degree of leavening with only a more generic attribute, namely “porosity”. Within the category of aroma, the more affected attribute was earthy, as already seen also for pasta; the other attributes were

	Attribute	Judge (J)	Sample (S)	Replication (R)	Judge*Sample	Judge*Replication	Sample*Replication	Sample (e = Judge*Sample)
APPEARANCE	*Internal color*	0.96	**160.01**	0.28	**12.03**	1.10	0.62	***13.31***
	Internal color intensity	**9.08**	**274.14**	**4.06**	**4.02**	1.28	**6.42**	***68.28***
	External color	1.17	0.05	0.11	1.20	0.34	0.20	0.04
	Eveness of internal color	2.26	***88.41***	1.30	2.51	1.00	0.98	**35.17**
	Porosity	1.23	3.35	0.32	**3.28**	0.29	0.55	1.02
	Bubble size	**3.21**	**91.64**	0.54	**2.68**	0.60	0.06	***34.22***
	Degree of leavening	**3.10**	***225.76***	1.59	1.97	0.89	0.34	**114.75**
	Crust thichness	**5.60**	***26.02***	0.74	2.24	0.78	**3.96**	**11.63**
AROMA	Yeasty	**16.52**	0.88	**3.98**	**4.81**	1.43	0.78	0.18
	Earthy	**4.16**	**54.38**	1.98	**5.04**	1.48	0.81	***10.78***
	Rancid oils	**18.73**	1.89	0.32	0.57	0.46	0.74	3.33
	Toasted	**3.56**	***7.51***	0.17	0.93	2.00	0.62	**8.09**
	Grain	**3.97**	0.19	0.36	1.53	2.14	0.34	0.13
	Nutty	**2.71**	***11.21***	1.67	1.41	0.53	0.05	**7.96**
TASTE	*Sweet*	**7.47**	**40.13**	0.23	**3.11**	2.25	1.44	***12.91***
	Salty	**12.48**	**7.35**	1.72	**2.76**	0.93	0.88	2.66
	Bitter	**5.40**	***175.38***	2.49	1.34	1.09	0.02	**130.48**
	Sour	**6.77**	3.87	0.81	0.63	0.60	2.48	**6.15**
FLAVOR	*Olives*	**13.08**	**33.29**	0.22	**3.13**	1.88	1.11	***10.62***
	Toasted	**7.42**	**26.09**	0.15	**4.74**	0.35	0.62	***5.51***
	Nutty	**11.43**	***20.15***	1.30	2.32	1.99	1.77	**8.68**
	Grain	**3.12**	0.28	3.18	2.52	**2.48**	1.87	0.11
	Yeasty	1.39	1.45	**4.00**	1.86	0.54	1.23	0.24
	Earthy	**6.50**	**78.88**	1.37	**8.97**	1.76	0.49	***8.80***
TEXTURE	External dryness	**8.83**	1.02	1.38	1.56	1.52	**6.71**	0.65
	Internal dryness	**9.06**	2.65	2.26	2.39	**2.40**	3.59	1.11
	External firmness	**14.08**	**44.24**	0.12	**6.32**	**3.14**	**4.62**	***6.99***
	Internal firmness	**8.54**	***49.88***	0.69	0.96	2.04	0.57	**51.83**
	Crust chewyness	**12.47**	**39.50**	1.95	**4.72**	**3.75**	0.80	***8.37***
	Crumb chewyness	**9.54**	***56.46***	0.77	1.17	0.63	1.56	**48.13**
	Astringent	**13.20**	***10.20***	0.78	1.19	1.29	2.39	**8.55**
AFTERTASTE	*Sweet*	**4.69**	***6.74***	2.62	1.06	0.69	0.10	**6.33**
	Salty	**5.51**	1.11	1.13	1.08	1.19	0.52	1.03
	Bitter	**4.86**	***90.55***	1.25	2.01	0.63	0.70	**44.98**
	Sour	**6.29**	3.27	3.20	**3.38**	**2.89**	2.14	0.97
	Astringent	**4.87**	1.32	0.83	0.23	0.39	0.17	**5.72**
	Olive oil/Olives	**8.81**	***10.29***	0.47	2.08	0.67	1.48	4.95

Table 8. *F*-ratios of the different sources of variation from ANOVA for the 38 attributes rated by descriptive panel for the 2 bread samples. *F*-ratios for which significance was at $P < 0.05$ are reported in bold text. The attributes that showed significant difference among the samples are in italic and underlined

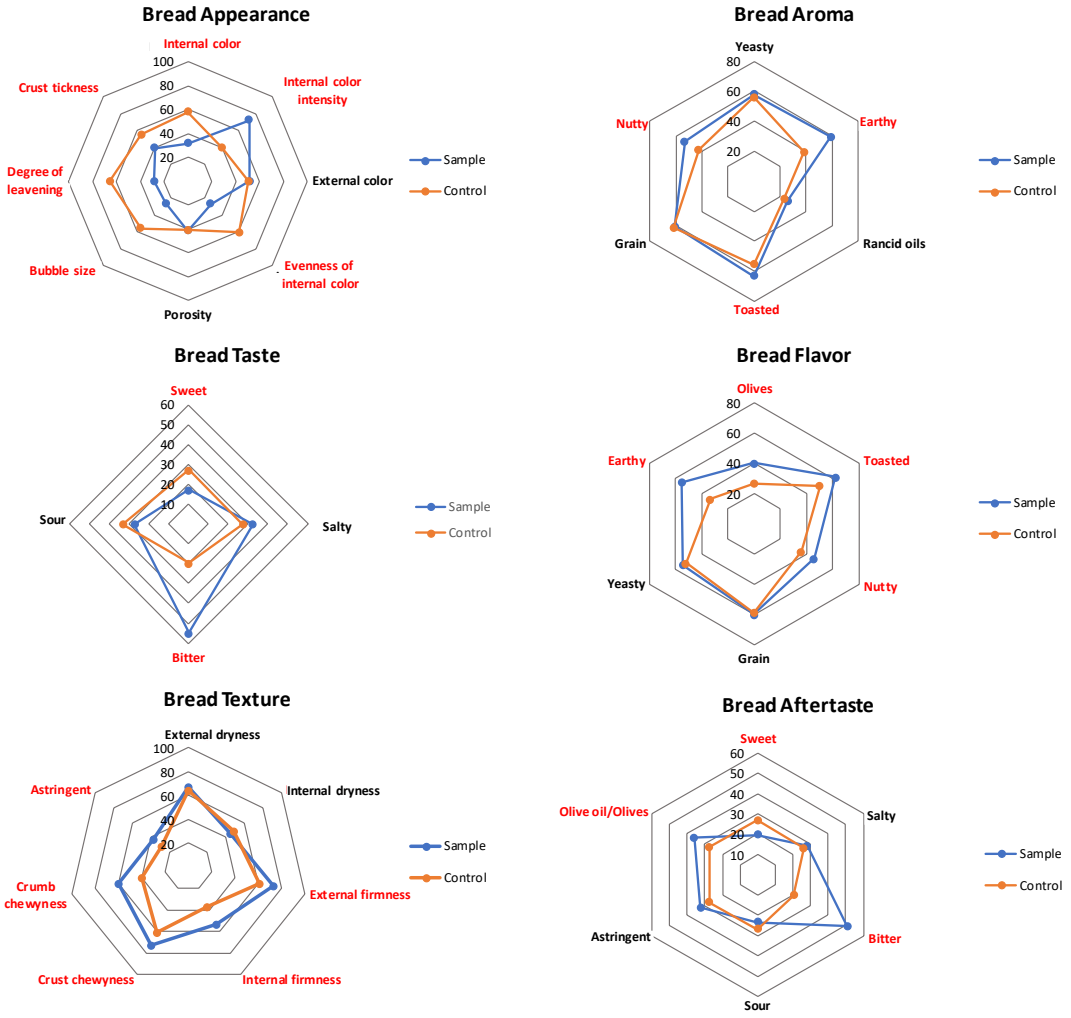


Figure 4. Spider web plot of bread descriptive analysis for six categories of attributes (Appearance, Aroma, Taste, Flavor, Texture, Aftertaste). Attributes for which there was a significant difference ($p < 0.05$) in the panelists rates are in red.

affected only slightly or in a not significant extent. The tastes of salty and sour didn't contribute in differentiating between BC and BS, while sweetness was lower in BS than in BC. These lower perception of the taste of sweet in BS was likely due to the strong increase of bitterness in the BS (Keast & Breslin, 2003), which was the consequence of the higher availability of phenols from pâté in BS (Table 5). All the flavour attributes except yeasty and grain were increased in the BS, with earthy that was again the attribute with the higher difference. Regarding texture attributes, dryness was not affected by the presence of pâté, while firmness (both external and internal) and chewiness (of both crumb and crust) were higher in BS than BC and also astringency was slightly increased by the presence of pâté. Finally, the observations for aftertaste confirmed what was observed for taste attributes, with a strong increase of bitter perception and a consequent decrease of sweet perception

3.2.1.4. Granola bar

Finally, also for granola bar MANOVA indicated an overall significant difference between Control and Sample exhibited by the attributes. Table 9 shows the *F*-ratios calculated for the different sources of variation from the ANOVAs performed for each attribute for Granola Bar (Sample and Control) and also in this case, the repeatability of the panel across the replication was very good. For identifying the attributes able in differentiating between Sample and Control, the same approach already described for pasta was used. Figure 5 shows Spider web plots of the six categories of attributes, with attributes with significant differences ($p < 0.05$) among the sample reported in red.

Looking at Table 9 and Figure 5, immediately appear that, for granola bar, the differences between Control and Sample are less than those highlighted for Pasta and Bread. Indeed, significant differences were observed for 23 of the 34 rated attributes (68%) for pasta and for 23 of the 37 rated attributes (62%) for bread, while for only 13 of the 36 rated attributes (36%) for granola bar. Moreover, looking at the effect of the samples on the attributes for which there was significant differences (Table 7, 8 and 9, columns "Sample (S)" and Sample (e = Judge*Sample), values in italic), mean *F*-ratios were higher for Bread (mean *F*-ratio 41.58) and Pasta (34.53) than granola bar (20.3), highlighting that the significance of the differences was higher for the first two types of dishes. Consequently, also the differences for the rated attributes between GBC and GBS showed in the spider web plots in figure 5 are less and at a lower extent with respect to those seen for Pasta and Bread (Figures 3 and 4). This fact could be explained by the higher complexity of color, aromas, taste and flavor of GBC that results in a lower impact in GBS due to the presence of pâté. The highest differences were seen for the appearance attributes color and color intensity. Aroma was very slightly affected by the presence of pâté with only a small increase of the attribute dried fruit and a small decrease in honey perception. Bitter was the only taste attribute affected by the presence of pâté, with a quite strong increase in the GBS. Flavor and texture attributes were very slightly affected, with only a certain increase of perception of toasted in GBS. In aftertaste, a certain increase of bitter perception and a consequent decrease of sweet perception were observed.

	Attribute	Judge (J)	Sample (S)	Replication (R)	Judge*Sample	Judge*Replication	Sample*Replication	Sample (e = Judge*Sample)
APPEARANCE	*Color*	**9.71**	**357.14**	0.63	**6.32**	1.37	1.54	***56.54***
	Color intensity	**8.43**	**352.48**	0.24	**5.86**	1.28	3.09	***60.15***
	Evenness of color	**7.00**	0.40	0.62	**5.58**	1.66	0.38	0.07
	Shininess	**13.79**	***20.25***	0.05	1.79	1.13	0.95	**11.33**
	Thichness	**4.01**	0.09	0.24	0.97	0.78	2.87	0.10
AROMA	Cardboard	**9.72**	0.58	0.38	**2.52**	1.12	0.67	0.23
	Rancid oils	**33.00**	**9.20**	0.35	**3.28**	0.79	0.24	2.80
	Toasted	**5.05**	**8.50**	0.96	**2.98**	1.43	0.39	2.85
	Nutty	**10.40**	0.29	1.14	**2.89**	1.96	0.88	0.10
	Honey	**8.45**	***7.27***	1.65	1.08	1.06	0.14	**6.71**
	Coconut	**16.24**	0.03	1.08	1.11	1.19	0.68	0.03
	Dried fruit	**36.44**	***9.75***	0.24	1.66	1.26	2.19	**5.88**
	Chocolatey	**8.26**	1.05	1.43	0.45	1.07	2.11	2.30
	Buttery	**45.41**	2.04	3.32	1.40	1.93	0.43	1.46
TASTE	Sweet	**12.40**	2.50	0.12	0.63	0.85	0.25	3.96
	Salty	**18.92**	1.32	1.58	0.96	**2.28**	0.31	1.38
	Bitter	**8.36**	**47.51**	1.40	**3.69**	0.88	1.74	***12.88***
	Sour	**6.07**	2.18	1.83	1.66	**2.27**	1.97	1.31
FLAVOR	*Toasted*	**4.10**	**50.29**	3.52	**3.16**	1.44	0.34	***15.91***
	Nutty	**7.72**	0.03	0.41	0.98	0.73	0.29	0.03
	Oats	**17.02**	2.31	2.89	1.11	1.69	3.16	2.08
	Chocolatey	**8.54**	***23.80***	1.45	1.94	**5.50**	**4.25**	**12.30**
	Caramel	**8.12**	1.82	0.72	**3.16**	0.99	0.04	0.57
	Coconut	**18.87**	***6.09***	3.34	0.97	1.73	0.20	**6.24**
	Dried figs	**20.18**	**11.89**	2.15	**3.79**	1.62	0.60	3.14
	Vanilla	**10.98**	***12.75***	< 0.01	2.08	1.28	2.76	**6.12**
	Buttery	**6.14**	3.98	0.22	1.11	0.50	0.99	3.59
TEXTURE	Dryness	**17.36**	**8.56**	1.05	**5.58**	**2.77**	**4.45**	1.53
	Firmness	**7.05**	***8.10***	0.49	1.37	1.13	0.85	**5.93**
	Crunchyness	**8.10**	0.05	1.37	1.67	0.89	3.17	0.03
	Stickyness	**18.59**	1.73	2.64	**2.70**	0.88	0.15	0.64
AFTERTASTE	*Sweet*	**5.41**	***9.27***	0.07	0.62	0.72	1.37	**14.99**
	Salty	**2.93**	0.88	1.03	1.29	1.31	0.57	0.68
	Bitter	**7.87**	***21.72***	0.14	1.57	0.82	1.03	**13.82**
	Astringent	**8.21**	0.55	0.59	0.37	0.86	1.84	1.49
	Chocolatey	**5.39**	4.25	0.43	0.75	2.14	1.46	**5.64**

Table 9. *F*-ratios of the different sources of variation from ANOVA for the 36 attributes rated by descriptive panel for the 2 granola bar samples. *F*-ratios for which significance was at $P < 0.05$ are reported in bold text. The attributes that showed significant difference among the samples are in italic and underlined

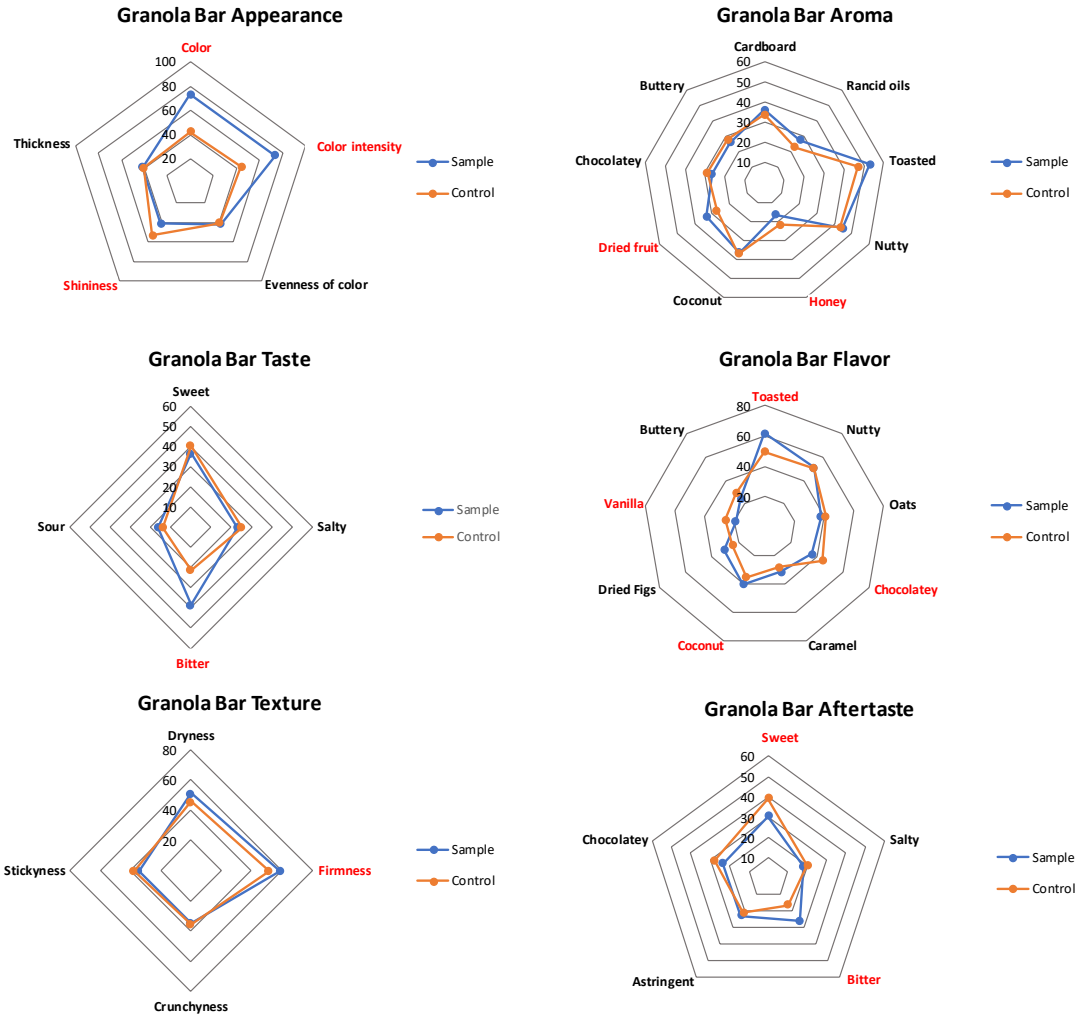


Figure 5. Spider web plot of granola bar descriptive analysis for six categories of attributes (Appearance, Aroma, Taste, Flavor, Texture, Aftertaste). Attributes for which there was a significant difference ($p < 0.05$) in the panelists rates are in red.

In conclusion, a first description of the sensory profile of pasta, bread and granola bar as they are and fortified with pâté were obtained using a fast technique (CATA) by naïve consumers. Applying CATA to products' sensory profiling, allowed us to gather insight into the consumers, and these information will help a further improvement of the recipes towards meeting the consumer demands (Oliver et al., 2017). Then, a more detailed description was obtained using Descriptive Analysis with a panel of expert judges. Findings obtained from DA e CATA will be used together with data from consumer test for identifying consumers' drivers of liking.

3.3 Consumer test

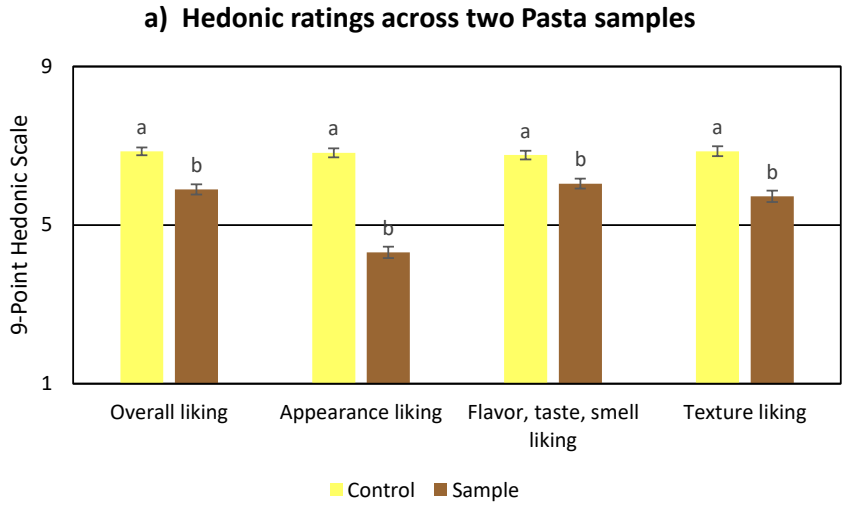
The first thing for a dish being successful in the market is that consumers perceive it tastes good (Predieri, Medoro, Magli, Gatti & Rotondi, 2013). For this reasons, in the

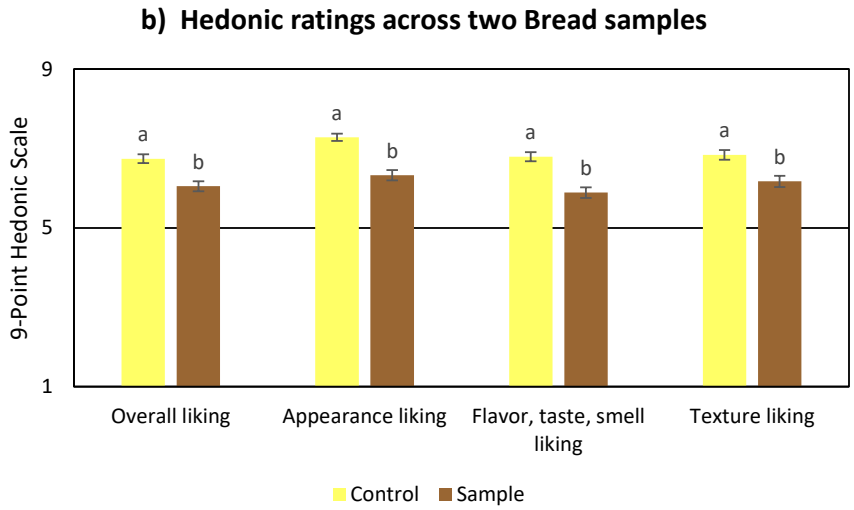
second part of the work, we aimed to evaluated the liking and acceptability of our products by Californian consumers. For each kind of dish, it was hypothesized that the acceptability of the Sample is inferior to the Control, mainly due to the dark color and the bitterness that pâté confers to the recipes, but also that some segments of consumers accept and even prefer the sample to the control.

A consumer population sample (n = 175) completed the consumer test. They were 58% female, with 41% ranging in age from 18 to 24, 21% from 25 to 34, 19% from 35 to 54 and 19% aged 55 and older. 50% were students. Their ethnicity was representative of likely target consumers of the proposed products: 45% White/Caucasian; 31% Asian; 17% Hispanic/Latino(a); 7% other or more than one. Educational level was distributed in the following categories: graduate or professional school (37%), bachelor's degree (27%), some college (31%), high school diploma (3%). Income level was distributed almost evenly in the categories "less than $25,000", "$25,000 to $50,000", "$50,000 to $100,000" and "more than $100,000". Most consumers (94%) declared not to have eaten before the test and only 2% declared to be not at all hungry.

3.3.1 Hedonic comparisons

Figure 6 shows the hedonic ratings across samples of Pasta (6a), Bread (6b) and Granola Bar (6c). It is immediately evident that, for all the three kind of dishes, the overall liking of the Controls was significantly higher ($p < 0.05$, Cohen's d effect size: Pasta 0.630, Bread 0.443, Granola Bar 0.383) than the Samples, but also that all the six products have been overall accepted by consumers with mean ratings always higher than 5.80 on the 9-point hedonic scale. The same trend was observed for flavor, taste and smell liking, which was rated significantly higher for the Controls than the Samples ($p < 0.05$, Cohen's d: Pasta 0.463, Bread 0.554, Granola Bar 0.397) with mean ratings always higher than 5.90. Regarding texture liking (mean ratings always higher than 5.70), no significant differences were observed between GBS and GBC ($p > 0.05$, Cohen's d 0.163), while for PC and BC, ratings were higher than PS and BS, respectively ($p < 0.05$, Cohen's d: Pasta 0.632, Bread 0.386).





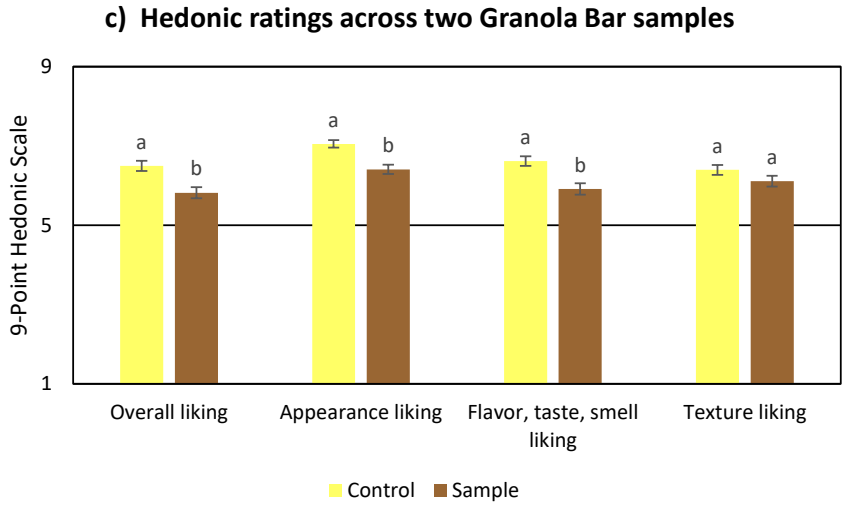


Figure 6. Mean ratings on the 9-point hedonic scale and standard error of the means (SEM) for a) Pasta Control and Pasta Sample b) Bread Control and Bread Sample and c) Granola Bar Control and Granola Bar Sample for four hedonic categories: overall liking, appearance liking, flavor, taste and smell liking, and texture liking. n=175 consumers for all the 6 samples. Within each hedonic category, means with different superscripts are significantly different at $p < 0.05$.

Finally, in liking of appearance, Controls were rated again higher than Samples ($p < 0.05$, Cohen's d: Pasta 1.480, Bread 0.638, Granola Bar 0.462), but in this case, the appearance of the PS hasn't been accepted by consumers (mean rating, 4.31±0.14). This fact could be explained bearing in mind that the appearance of PS was very unfamiliar for the Californian consumers, while the appearance of BS and GBS was more familiar, since in the Californian market a great variety of kinds of bread and granola bar are present, with very different colors. This lack of acceptability of the appearance of the PC results in needing to develop suitable marketing strategies, then, once it has been sold for the first time, our data suggest that the consumers will like it.

It has been reported that food neophobia, that is the fear of new foods, may affect the degree of liking of products but not how the products are ranked (Henriques, King & Meiselman, 2009). In agreement with Pliner & Hobden (1992), we divided the consumers in neophilics and neophobics, if the FNS is <25 or >35, respectively. As expected, neophobics were underrepresented in our CLT, which was a traditional CLT, this not being a problem since consumers tasted existing products with only a change (Henriques et al., 2009). Consumers had no information about the products before tasting them, consequently expectations were based only on their appearance. Table 10 shows the hedonic ratings for all the products for neophobics and neophilics.

		Overall liking		Overall appearance		Flavor, taste, smell liking		Texture/mouthfeel liking	
		Control	Sample	Control	Sample	Control	Sample	Control	Sample
PASTA	Neophilics (FNS < 25)	7.1 ± 0.1	6.2 ± 0.1	7.0 ± 0.1	4.5 ± 0.1	7.0 ± 0.1	6.4 ± 0.1	7.1 ± 0.1	6.0 ± 0.2
	25 < FNS < 35	6.6 ± 0.1	5.6 ± 0.1	6.6 ± 0.1	4.2 ± 0.1	6.4 ± 0.1	5.7 ± 0.1	6.5 ± 0.1	5.5 ± 0.2
	Neophobics (FNS > 35)	6.7 ± 0.1	5.5 ± 0.1	6.7 ± 0.1	4.0 ± 0.1	6.7 ± 0.1	5.5 ± 0.1	6.7 ± 0.1	5.3 ± 0.1
BREAD	Neophilics (FNS < 25)	6.9 ± 0.1	6.2 ± 0.1	7.3 ± 0.1	6.7 ± 0.1	7.0 ± 0.1	6.1 ± 0.1	6.9 ± 0.1	6.4 ± 0.1
	25 < FNS < 35	6.6 ± 0.1	5.8 ± 0.1	7.2 ± 0.1	5.9 ± 0.1	6.7 ± 0.1	5.6 ± 0.1	6.7 ± 0.1	5.8 ± 0.1
	Neophobics (FNS > 35)	6.5 ± 0.1	6.0 ± 0.1	7.3 ± 0.1	6.1 ± 0.1	6.5 ± 0.1	5.6 ± 0.1	6.8 ± 0.1	6.1 ± 0.2
GRANOLA BAR	Neophilics (FNS < 25)	6.4 ± 0.1	6.0 ± 0.1	7.1 ± 0.1	6.5 ± 0.1	6.6 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.2 ± 0.1
	25 < FNS < 35	6.4 ± 0.1	5.5 ± 0.2	6.9 ± 0.1	6.1 ± 0.1	6.5 ± 0.1	5.5 ± 0.2	6.4 ± 0.1	6.0 ± 0.1
	Neophobics (FNS > 35)	7.1 ± 0.1	5.7 ± 0.1	7.4 ± 0.1	6.7 ± 0.1	7.1 ± 0.1	5.9 ± 0.1	6.7 ± 0.1	6.3 ± 0.1

Table 10. Mean ratings on the 9-point hedonic scale and standard error of the means (SEM) by the segments of consumers based on the Food Neophobia Score (FNS) for a) Pasta, b) Bread and c) Granola Bar for four hedonic categories: overall liking, liking of appearance, flavor, taste and smell liking, and texture liking. Neophilics are consumers with FNS < 25. Neophobics are consumers with FNS > 35.

Data confirmed that the ranking order of the products didn't change between neophobics and neophilics, but the differences in the hedonic scores between Controls and Products were higher for neophobic than neophilics and were higher for Pasta than

Bread and Granola Bar, particularly for the liking of appearance. Regarding the differences within the same sample between neophilics' and neophobics' scores, mean ratings of all the four hedonic attributes lowered more for PS than PC. This trend was not confirmed for Bread (for some attributes, mean ratings lowered more for BC than BS) and Granola Bar (for some attributes, mean rating by neophobics were even higher than neophilics for both GBS than GBC), confirming that consumers perceived only PS as an unfamiliar food.

3.3.2 Product preference segments

Figure 7 shows that the Control was significantly (confidence interval, no overlapping) preferred to the Sample for each of the three kind of dishes, in agreement to the initial hypothesis. In spite of this, a not small percentage of consumers preferred the Sample: 23% for pasta, 27% for bread and 34% for granola bar. These percentages seems to be affected more by the differences in liking of appearance than in overall liking. The no preference option was used rarely (less than 10%) for all the kind of samples.

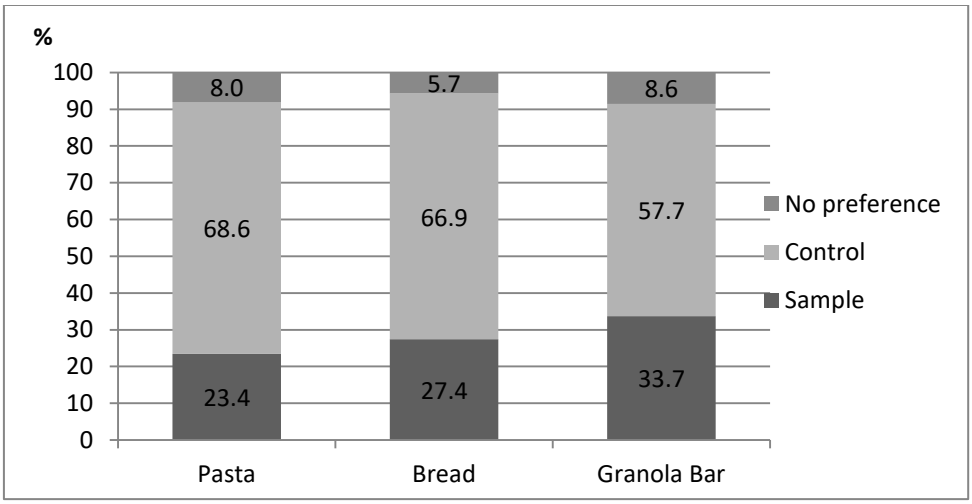


Figure 7. Percentage of consumers that preferred Sample or Control for each of the three dishes, including also the "no preference" choice.

Consumers were divided based on the abovementioned preferences, so that three segments were generated for each kind of dish. Tables 11 show the characteristics of these segments for Pasta (11A), Bread (11B) and Granola Bar (11C), while Tables 12 shows the hedonic ratings by the three segments across the samples of Pasta (12a), Bread (12b) and Granola Bar (12c). Furthermore, consumers of the three segments were divided based on ethnicity (White/Caucasians, Asians, Hispanic/Latinos and other) and the preferences

	A) Pasta			B) Bread			C) Granola bar		
	Pref Control	**Pref Sample**	**No pref**	**Pref Control**	**Pref Sample**	**No pref**	**Pref Control**	**Pref Sample**	**No pref**
% males	42.5%	41.5%	42.9%	40.2%	41.7%	70.0%	35.6%	49.2%	60.0%
% students	45.0%	61.0%	57.1%	53.8%	41.7%	40.0%	48.5%	50.8%	53.3%
Age (18-24)	35.8%	51.2%	57.1%	41.9%	39.6%	40.0%	44.6%	35.6%	40.0%
Age (25-34)	24.2%	14.6%	14.3%	25.6%	12.5%	10.0%	19.8%	23.7%	20.0%
Age (35-54)	21.7%	14.6%	7.1%	17.1%	22.9%	20.0%	18.8%	18.6%	20.0%
Age (55 or more)	18.3%	19.5%	21.4%	15.4%	25.0%	30.0%	16.8%	22.0%	20.0%
(Ethnicity) White/Caucasian	47.5%	36.6%	7.1%	41.9%	39.6%	50.0%	36.6%	47.5%	53.3%
(Ethnicity) Asian	29.2%	34.1%	42.9%	33.3%	25.0%	40.0%	33.7%	25.4%	40.0%
(Ethnicity) Hispanic/Latino(a)	14.2%	19.5%	21.4%	14.5%	20.8%	10.0%	17.8%	15.3%	6.7%
(Ethnicity) Other	9.1%	9.8%	28.6%	10.3%	14.6%	0.0%	11.9%	11.9%	0.0%
% neophobic	14.17%	4.88%	14.29%	13.68%	10.42%	0.00%	14.85%	6.78%	13.33%
% neophilic	50.83%	63.41%	50.00%	50.43%	54.17%	90.00%	51.49%	55.93%	60.00%
Q1. "I always follow a healthy and balanced diet"	4.57 ± 0.12	4.59 ± 0.10	4.71 ± 0.12	4.69 ± 0.11	4.38 ± 0.11	4.30 ± 0.13	4.58 ± 0.10	4.54 ± 0.11	4.73 ± 0.15
Q2. "It is important to me that my daily diet contains lots of vitamins and minerals"	5.36 ± 0.10	5.54 ± 0.10	5.14 ± 0.09	5.41 ± 0.10	5.23 ± 0.10	5.80 ± 0.07	5.40 ± 0.10	5.37 ± 0.10	5.33 ± 0.11
Q3. "The healthiness of food strongly impact my food choices"	5.07 ± 0.11	5.02 ± 0.11	5.50 ± 0.08	5.13 ± 0.11	5.00 ± 0.11	5.10 ± .013	5.06 ± 0.10	5.17 ± 0.12	5.00 ± 0.13
Q4. "I eat what I like and I do not worry about the healthiness of food"	4.52 ± 0.12	4.56 ± 0.13	4.07 ± 0.14	4.51 ± 0.13	4.50 ± 0.12	4.20 ± 0.12	4.51 ± 0.13	4.29 ± 0.13	5.13 ± 0.10
Q5. "I do not avoid any foods even if know they are considered unhealthy"	4.52 ± 0.13	3.71 ± 0.13	4.86 ± 0.11	4.45 ± 0.13	4.15 ± 0.14	4.20 ± 0.09	4.25 ± 0.13	4.44 ± 0.12	4.73 ± 0.12
Reading labeling often or always	71.7%	73.2%	78.6%	71.8%	70.8%	90.0%	73.3%	69.5%	80.0%
Willingness to pay up to 10% more	30.0%	34.1%	28.6%	33.3%	22.9%	30.0%	31.7%	25.4%	20.0%
Willingness to pay 10 to 20% more	20.0%	14.6%	28.6%	16.2%	16.7%	20.0%	15.8%	16.9%	13.3%
Willingness to pay 20 to 30% more	0.0%	4.9%	0.0%	2.6%	4.2%	0.0%	3.0%	0.0%	0.0%
Willingness to pay more than 30% more	0.8%	2.4%	7.1%	0.0%	4.2%	0.0%	0.0%	6.8%	0.0%
Willingness to pay more: total	50.83%	56.10%	64.29%	52.14%	47.92%	50.00%	50.50%	49.15%	33.33%

Table 11. Characteristics of segments of consumers that prefer Sample, Control or neither Sample nor Control for A) Pasta, B) Bread, and C) Granola Bar. For all questions except those related to the attitude regarding healthiness of foods, the values represent percentages of the sample population. For the questions on the attitude regarding healthiness of foods, the values are "mean ± SEM", based on values ranging between 1 (Strongly disagree) to 7 (Strongly agree) after reversing the scale for questions Q4 and Q5.

		Pref Control		Pref Sample		No preference	
		Control	Sample	Control	Sample	Control	Sample
a) pasta	Overall liking	7.01 ± 0.09	5.45 ± 0.12	6.51 ± 0.11	7.24 ± 0.10	6.57 ± 0.10	5.79 ± 0.09
	Overall appearance	6.89 ± 0.11	3.93 ± 0.13	6.68 ± 0.13	5.39 ± 0.16	6.64 ± 0.12	4.43 ± 0.13
	Flavor, taste, smell liking	6.99 ± 0.10	5.68 ± 0.13	6.29 ± 0.12	7.15 ± 0.10	6.21 ± 0.09	6.00 ± 0.08
	Texture/mouthfeel liking	6.95 ± 0.12	5.29 ± 0.14	6.83 ± 0.13	7.10 ± 0.11	6.21 ± 0.14	5.42 ± 0.12
		Control	Sample	Control	Sample	Control	Sample
b) bread	Overall liking	7.02 ± 0.11	5.65 ± 0.13	6.13 ± 0.11	7.04 ± 0.08	6.40 ± 0.08	6.00 ± 0.12
	Overall appearance	7.37 ± 0.09	6.06 ± 0.13	7.13 ± 0.09	6.92 ± 0.11	7.00 ± 0.06	6.70 ± 0.14
	Flavor, taste, smell liking	7.15 ± 0.10	5.43 ± 0.13	6.02 ± 0.12	6.98 ± 0.10	6.40 ± 0.10	6.00 ± 0.11
	Texture/mouthfeel liking	7.16 ± 0.11	5.81 ± 0.15	5.96 ± 0.13	6.94 ± 0.11	7.30 ± 0.06	6.70 ± 0.12
		Control	Sample	Control	Sample	Control	Sample
c) granola bar	Overall liking	6.74 ± 0.12	5.14 ± 0.15	6.03 ± 0.13	6.86 ± 0.09	6.67 ± 0.12	6.27 ± 0.13
	Overall appearance	7.05 ± 0.10	6.26 ± 0.12	7.03 ± 0.09	6.64 ± 0.11	7.13 ± 0.08	6.47 ± 0.11
	Flavor, taste, smell liking	6.92 ± 0.11	5.24 ± 0.15	6.10 ± 0.14	6.92 ± 0.10	6.60 ± 0.12	6.53 ± 0.13
	Texture/mouthfeel liking	6.52 ± 0.13	5.59 ± 0.15	6.14 ± 0.12	6.97 ± 0.10	6.53 ± 0.14	6.20 ± 0.13

Table 12. Mean ratings on the 9-point hedonic scale and standard error of the means (SEM) by the segments of consumers that prefer Control ("Pref Control"), Sample ("Pref Sample") or neither Sample nor Control ("No preference") for a) Pasta, b) Bread and c) Granola Bar for four hedonic categories: overall liking, appearance liking, flavor, taste and smell liking, and texture liking.

		White/Caucasian		Asian		Hispanic/Latino		Other	
		Control	Sample	Control	Sample	Control	Sample	Control	Sample
a) pasta	Overall liking	7.11	6.11	6.62	5.58	6.82	5.68	6.63	6.32
	Overall Appearance	7.03	4.51	6.51	4.02	6.79	3.93	7.00	4.95
	Flavor, taste, smell liking	7.00	6.19	6.40	5.87	7.00	6.00	6.58	6.05
	Texture/mouthfeel	7.19	6.04	6.65	5.36	6.68	5.79	6.47	5.47
		Control	Sample	Control	Sample	Control	Sample	Control	Sample
b) bread	Overall liking	6.90	5.99	6.53	5.96	7.04	6.29	6.32	6.21
	Overall Appearance	7.42	6.51	7.04	5.98	7.57	6.29	7.00	6.74
	Flavor, taste, smell liking	6.90	5.92	6.67	5.67	6.89	6.14	6.58	6.05
	Texture/mouthfeel	7.08	6.12	6.75	6.09	6.93	6.46	6.05	6.16
		Control	Sample	Control	Sample	Control	Sample	Control	Sample
c) granola bar	Overall liking	6.12	5.90	6.85	5.87	6.57	5.14	6.79	6.32
	Overall Appearance	6.89	6.33	7.09	6.38	7.29	6.46	7.21	6.68
	Flavor, taste, smell liking	6.29	5.89	7.04	5.89	6.61	5.50	6.68	6.68
	Texture/mouthfeel	6.18	5.96	6.40	6.18	6.61	6.29	6.89	6.21

Table 13. Mean ratings on the 9-point hedonic scale by consumers of different ethnicities for a) Pasta, b) Bread and c) Granola Bar for four hedonic categories: overall liking, appearance liking, flavor, taste and smell liking, and texture liking

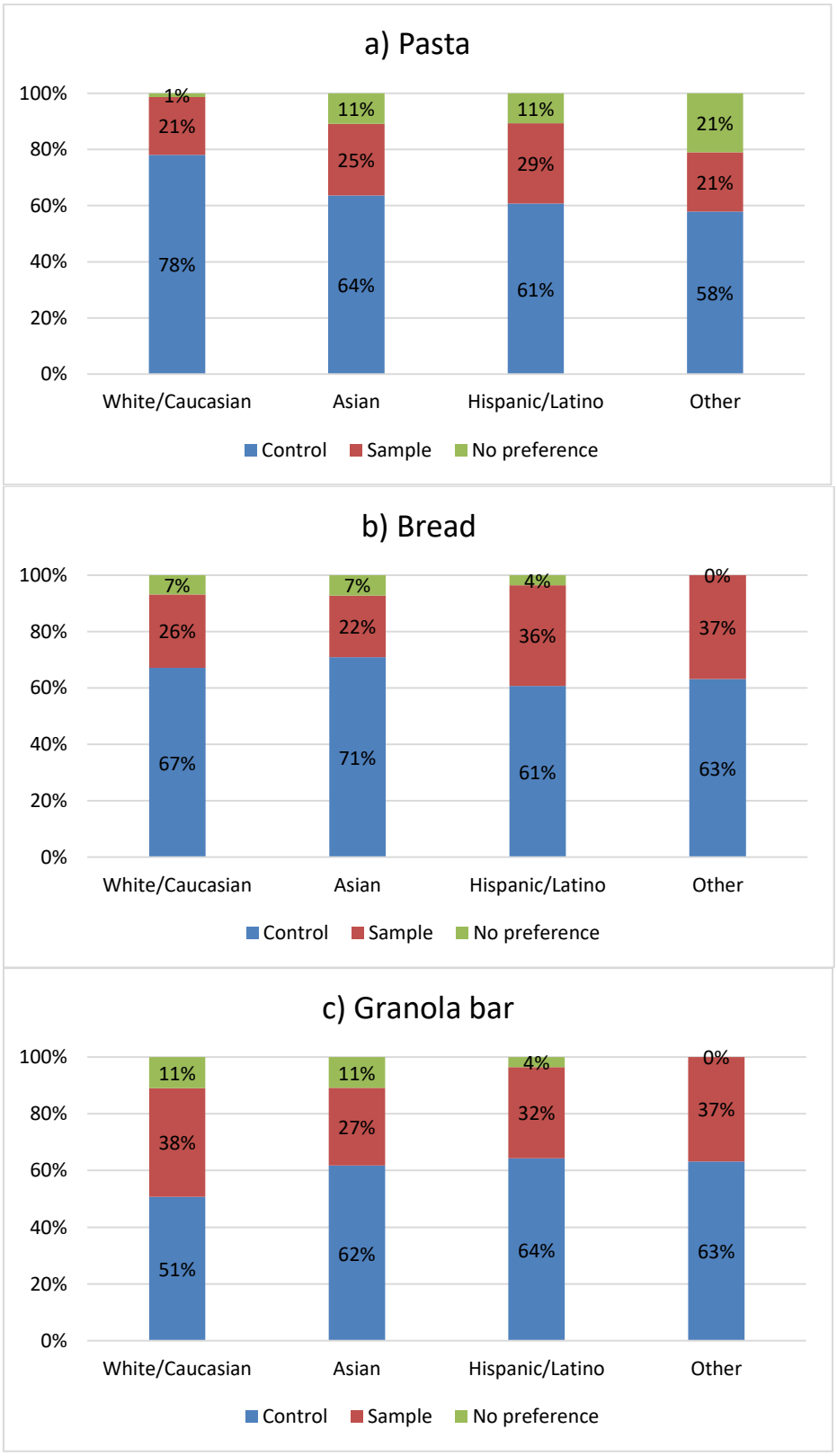


Figure 8. Percentage of consumers of different ethnicities that preferred Sample or Control for each of the three dishes (a, pasta; b, bread; c, granola bar), including also the "no preference" choice.

of each group are showed in Figure 8, while Table 13 shows the mean ratings by consumers of different ethnicities for the four hedonic categories for Pasta (13a), Bread (13b) and Granola Bar (13c).

Pasta: table 11a shows that consumers' gender had no impact in preferring PC or PS, while % of students that preferred PS was higher than those that preferred PC. This fact is consistent with the % of consumers aged 18-24 that preferred PS, which was higher than those that preferred PC. On the opposite, % of consumers aged 25-54 that preferred PS was lower than those that preferred PC, while percentages of consumers aged 55 or more that preferred PC or PS were similar. We can hypothesize that young consumers are more familiar with kinds of pasta different from the traditional ones, in that the former ones being spreading only in the last years particularly in countries like California. This fact makes young people more exposed to those kinds of pasta with a not traditional appearance, resulting in an increased liking of PC, in agreement to previous work, which reported that exposure drives liking (Spencer et al., 2018; Pliner, 1982). Surprisingly, % of White/Caucasian consumers that preferred PC was higher than those that preferred PS, while the opposite was observed for Asian and Hispanic/Latino consumers. When consumers were divided by ethnicity, it emerged that almost 78% of White/Caucasians preferred PC, while % of Asians and Hispanic/Latinos were slightly more than 60% (Figure 8). However, data reported in Table 13 show that mean ratings by White/Caucasian consumers were higher than those by Asian and Hispanic/Latinos consumers for all the hedonic attributes and for both PS and PC. These data confirm that, in general, White/Caucasian consumers like pasta more than other consumers, but also suggest that Asians and Hispanic/Latinos could be a segment of population that prefer PS. Consumers that preferred PC declared to avoid foods considered unhealthy more that those that preferred PS (Table 11a).

Bread: table 11b shows that consumers' gender had again no impact in preferring BC or BS and a high percentage of males selected the no preference option, while % of students that preferred BC was higher than those that preferred BS, unlike what happened for pasta. Percentage of consumers aged under 35 that preferred PC was higher than those that preferred PS, vice versa for consumers aged 35 or more. We can hypothesize that this observation is due to the fact that BS was perceived healthier by consumers, and it is well known that the older people are more health conscious; furthermore, older people accept

the bitterness more than young people, and BS was perceived much more bitter than BC (see also section 3.2). Hispanic/Latinos were the consumers that preferred BS in percentages (36%) higher than White/Caucasian (26%) and Asian (22%) (Figure 8). Asian were also the consumers that rated the samples of bread the lowest for all the hedonic attributes and for both BS and BC, even though the mean ratings were always higher than 6.5 for BC and higher than 5.7 for BS (Table 13). These data suggest that BC is slightly preferred by consumers and that BS could be bought by Californian consumers from the different ethnicities, allowing commercializing it through several strategies. Again, consumers that preferred BC declared to avoid foods considered unhealthy more that those that preferred BS (Table 11b).

Granola bar: table 11c shows that gender had a strong impact on granola bar preferences, with higher percentage of male that preferred GBS (49%) and that had no preferences (60%) than those preferring GBC (36%). Granola bar is expected to be sweet, and usually women like sweet dishes more than men, so the bitterness of the GBS could have had an impact on women more negative than on men. Students and non-students preferred GBC and GBS almost the same. People aged under 25 preferred GBC more than GBS, vice versa for people aged 55 or more. Again, we can hypothesize that older people accept the bitterness more than young people. Regarding ethnicity, only 51% of White/Caucasians preferred GBC, with 38% that preferred GBS and 11% that had no preference (Figure 8), this fact indicating that White/Caucasians are a really good target of Californian consumers for GBS. On the opposite, Asians were the consumers that preferred GBC the more, even though 28% of them still prefer GBS. However, data in Table 13 show that Asians rated GBS similar to White/Caucasians and better than Hispanic/Latinos, and that these latter consumers rated the overall liking of GBS 5.14, a mean rating very close to the "neither like nor dislike" option, suggesting that Hispanic/Latinos are not the best segment of consumers for GBS.

As expected, table 11 shows that % of neophobics that preferred the Sample was lower than those that preferred the Control for all the three dishes, and in particular for pasta, in agreement with the fact that PC was perceived as the most unfamiliar food.

3.3.3 Purchase intentions

Figure 9 shows the purchase intention expressed by 175 consumers after tasting each of the six products. Before calculating the reported percentages, the categories

"Definitely would not purchase" and "Probably would not purchase" were collapsed in "Would not purchase" and the categories "Definitely would purchase" and "Probably would purchase" were collapsed in "Would purchase".

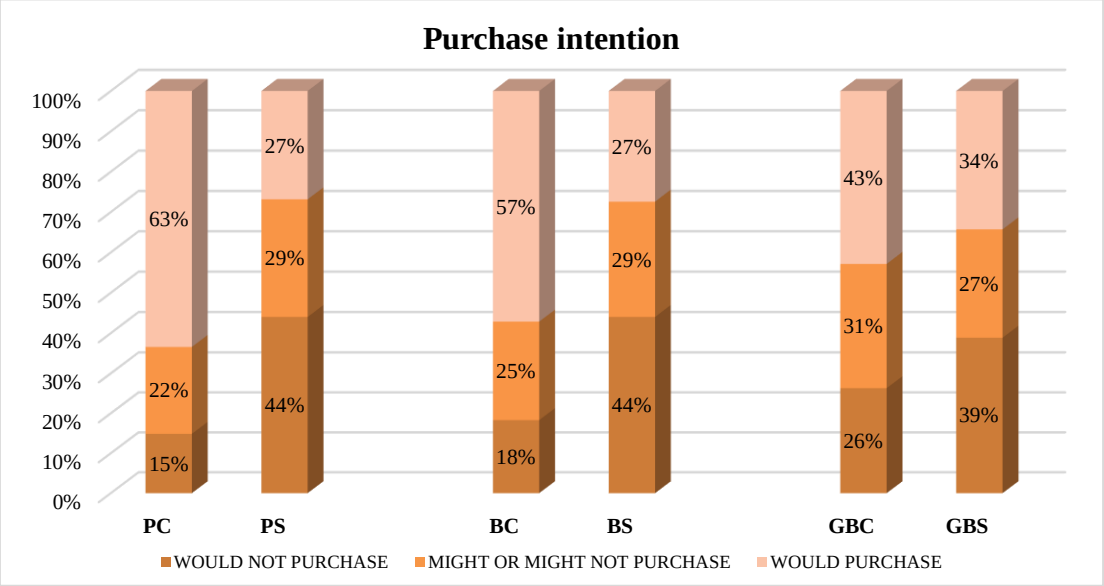


Figure 9. Percentages of consumers that declared to would purchase the tastes products. Categories of "definitely would purchase" and "probably would purchase" were collapsed in "would purchase"; categories of "definitely would not purchase" and "probably would not purchase" were collapsed in "would not purchase"

As expected, percentages of consumers declared they would purchase the Control were higher than those declared would purchase Sample, for all the three kind of dish. The differences in these percentages are higher for pasta than bread, both being much higher than for granola bar. Indeed, while for the three Samples, results were very similar (about 40% of consumers declared they would not purchase and about 30% declared they would purchase all the three Samples), for Controls were quite different, with 63% of consumers declared they would purchase PC, 57% BC and only 43% GBC. These findings for the Samples confirm that there is a quite high percentage of consumers available to purchase the fortified products and this is confirmed by the results obtained in the exit survey, regarding the willingness to pay more for the products fortified with antioxidants obtained from the olive oil extraction process: Figure 10 shows that up to 53% of consumers declared they are willing to pay more for the fortified pasta, this % being higher for consumers that preferred PS (56%, Table 11), and about 7% of consumers that preferred PS declared they are willing to pay even more than 20% more for the fortified pasta. Similarly, 51% of consumers declared they are willing to pay more for the fortified bread (Figure 10), this % being higher for consumers that preferred BC (52%, Table 11), but about 8% of consumers that preferred BS declared they are willing

to pay even more than 20% more for the fortified bread. Finally, 48% of consumers declared they are willing to pay more for fortified granola bar (Figure 10), with similar percentages for consumers that preferred GBC and GBS (Table 11). Noteworthy, almost 7% of consumers that preferred GBS declared they are willing to pay even 30% more for fortified granola bar.

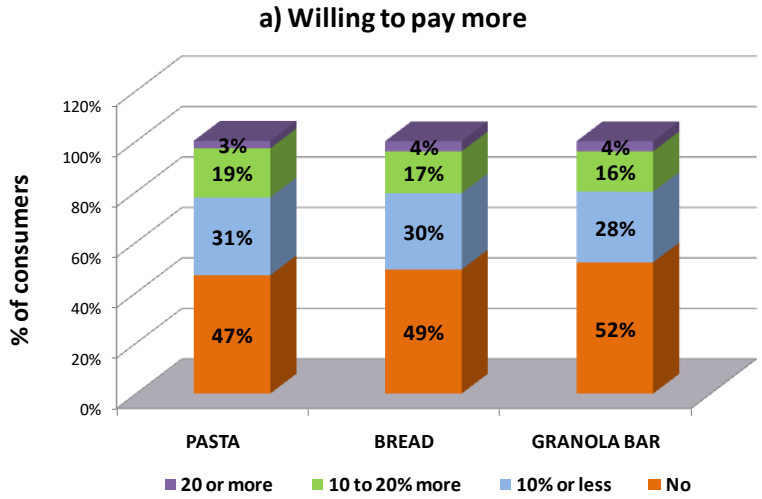


Figure 10. Percentages of consumers willing to pay more (or not) for products fortified with antioxidants obtained from the olive oil extraction process.

3.3.4 Just-About-Right frequencies and impact of the attributes on acceptability

Figure 11 shows the frequencies (%) of the three categories (too much, too little and just right) for the attributes rated in the JAR section for each of the tasted dish. Penalty analysis was then run to evaluate how the attributes rated with the JAR-scale impacted on the mean overall liking of each product and also in order to gain useful information for optimizing the recipes. This effect is reported in Figure 12, which shows the mean drop of each attribute on the mean overall liking of each product vs the % of too little and too much.

Pasta: the χ^2 values calculated by the Stuart-Maxwell test indicated that the attributes "Flavor intensity" and "Saltiness" didn't differentiate between PC and PS, while "Firmness" and, mostly, "Darkness" did it. Figures 11a and 11b show that both the samples were rated just right in flavor intensity and saltiness by 50% or more of the consumers, but more than 33% of them rated the samples too little in these two attributes. This was not surprising, since the pasta was served without any sauce or condiment, and the salt into the cooking water was limited to a minimum amount for avoiding the

predominance of saltiness. Regarding firmness, PC was evaluated just right by 68% and too much by 30% of consumers, while PS was just right for 48% and too much for 51% of consumers.

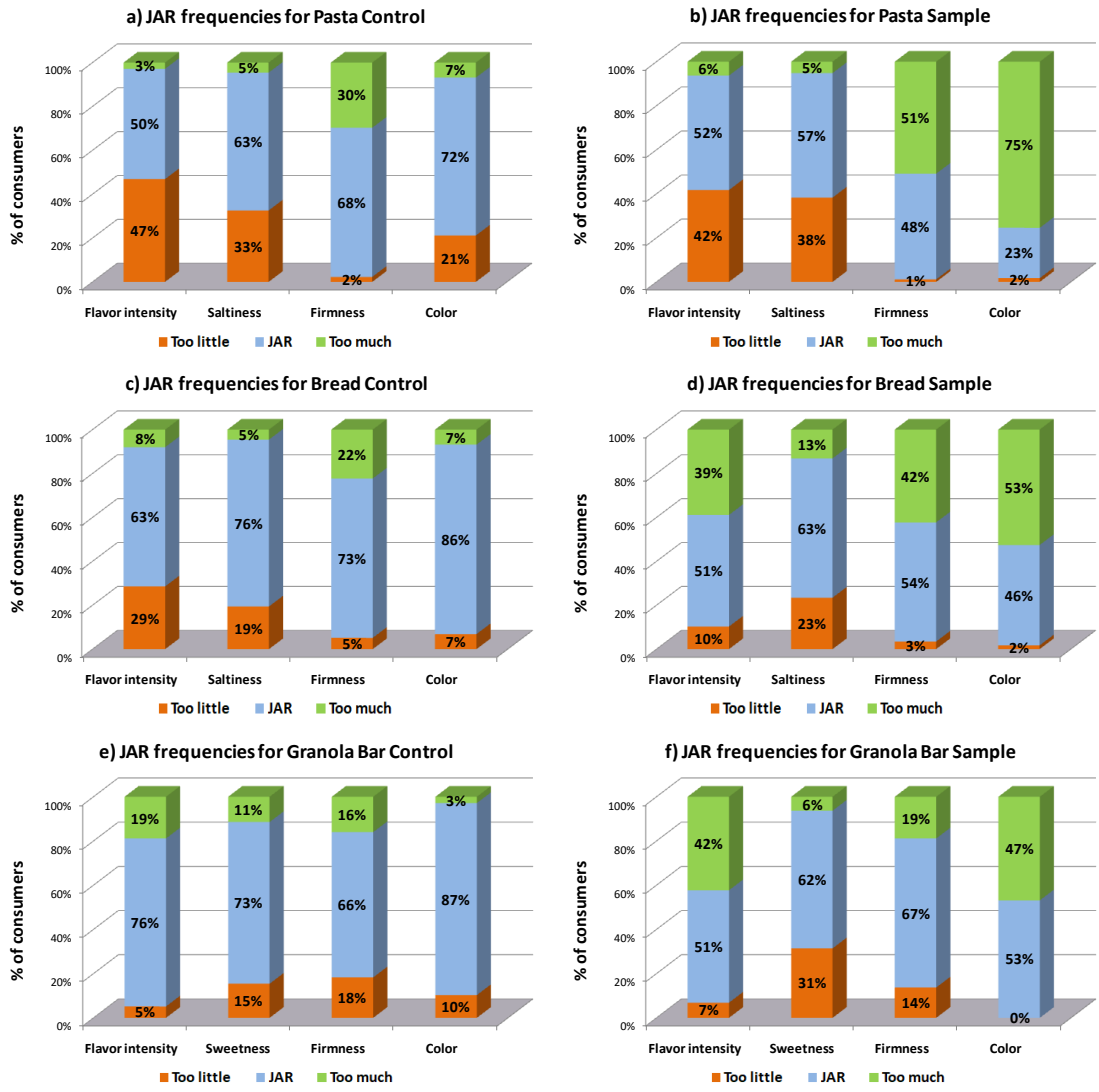


Figure 11. Just-About-Right (JAR) scores rated by consumers for the proposed attributes for each dish (Pasta, Bread and Granola Bar). For the Color attribute, "Too little" means "Too light" and "Too much" means "Too dark".

This data indicate that, despite the cooking time higher for PS than PC, the consistence of PS was still firmer. This finding confirm again the need of more cooking time for PS, as set up at the beginning of the research. Finally, as expected, the color was the attribute that differentiated PC and PS the most: PC was evaluated just right by 72% of consumers and too light by 21% of them, while PS was evaluated just right by only 23% of consumers and too dark by 75% of them. This observation was again in agreement with the fact that this latter kind of pasta is very unfamiliar for the Californian consumers.

Figure 12a shows that a too low flavor intensity caused the higher mean drop (approx. 1.3) in overall liking of PC, followed by too low saltiness (≈1) and too high firmness and too light color (≈0.6). Figure 12b, instead, shows that too low flavor intensity (mean drop approx. 1.3), to high firmness (≈1.3) and too dark color (≈1.2) had the highest negative impact on the overall liking of PS, followed by too low saltiness

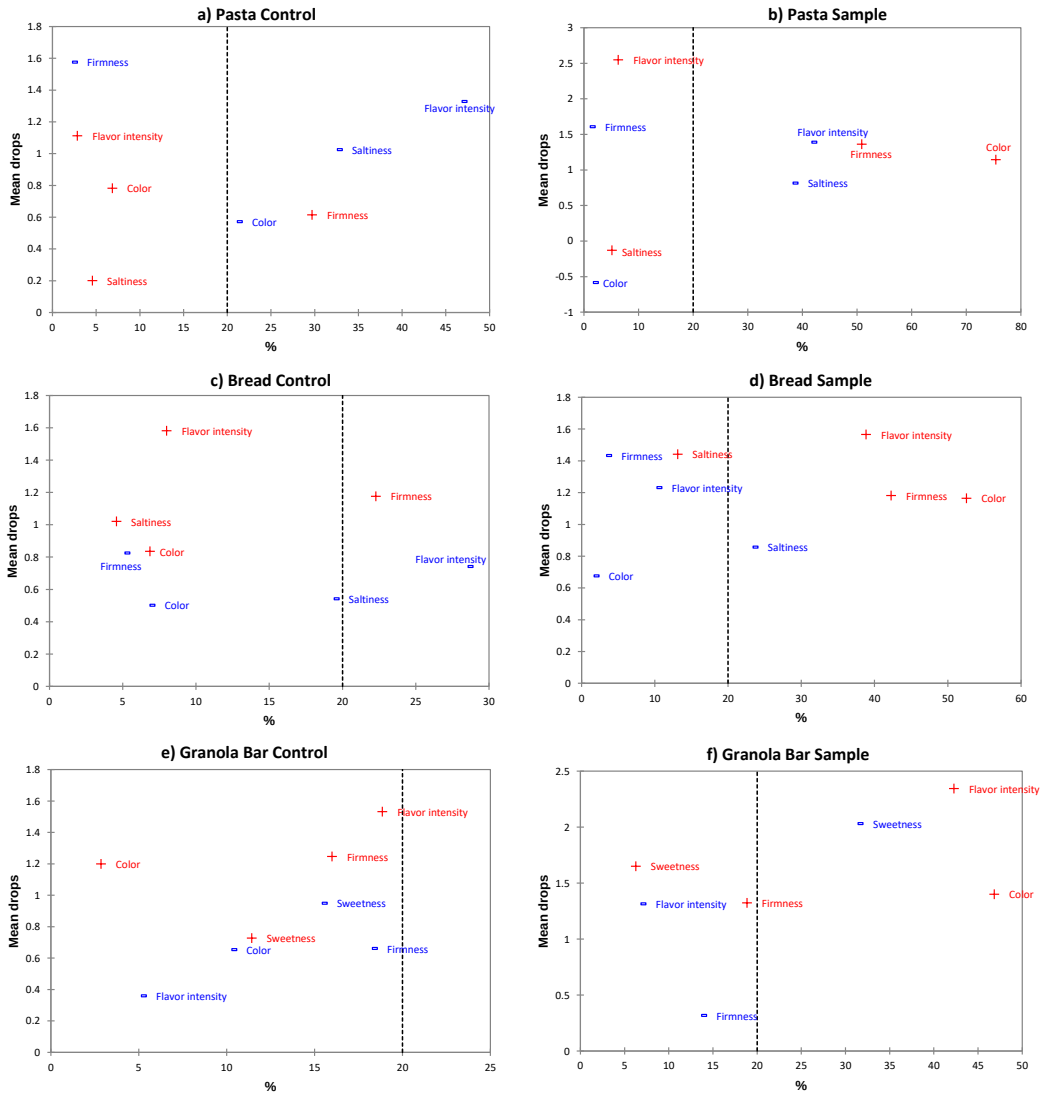


Figure 12. Mean Drops vs % of too little (- in light blue) and too much (+ in red) chart for each JAR attribute for all products. Mean Drops is the difference between the liking mean for the JAR level minus the liking mean for the "Too Little" or "Too Much" levels. The dotted vertical line indicates the threshold for population size (set to 20%) over which the results are considered significant. *For the Granola Bar Sample, no consumer indicated too little for the attribute "color" and this is not reported in the chart.*

(≈0.8). Low flavor intensity and saltiness seem to be not a problem, in that the presence of some sauces or condiment will allow to adjust these attributes. Also the firmness can became more suitable for Californian consumers by optimizing the cooking time. On the opposite, the problem of the too dark color of PS seems to be more difficult to overcame

in the phase of the pasta production, since the presence of pâté as ingredient makes the color very dark. Consequently, appropriate sales strategies are needed: for example, we propose to sell pasta in mixed packs with both PS and PC, to have something of similar to "paglia e fieno", which, with its variety of colors, could be more accepted by Californian consumers.

Bread: the χ^2 values indicated that all the rated attributes were able to differentiate between BS and BC (data not shown). Figure 11c shows that BC was rated just right by more than 63% of consumers for all the 4 attributes, while lower % rated just right BS (Figure 11d). About 29% of consumers rated too low BC and 39% of them rated too high BS for flavor intensity; BC was rated too high by 22% of consumers and BS by 42% of them for firmness; finally, BC was rated just right by 86% of consumers for color, while BS was rated too dark by 53% of them. These data suggest that a more high acceptability of BS could be obtained by reducing the % of pâté in the recipe of BS, for example to 3%. This reduction would not preclude the intake of olive oil phenols (30 g of BS would be still enough to benefit from the health effects stated by EFSA health claim) and would allow to reduce flavor intensity and firmness of BS, but also would allow simplifying the baking process and reducing the darkness of BS. These would result in a strong improving in acceptability of BS: in fact, as shown in Figure 12d, too high firmness and flavor intensity and too dark color had the higher negative impact on the overall liking of BS.

Granola bar: the $\chi 2$ values indicated that all the evaluated attributes, except firmness, were able to differentiate between GBS and GBC (data not shown). GBC was rated just right by at least 66% of consumers for all the attributes and none of the attributes were rated too low or too high by 20% consumers or more (Figure 11e), so that none of them was found to have a significant impact on overall acceptance of GBC (figure 11f). On the contrary, although GBS was rated just right by at least 51% of consumers for all the attributes, it was rated too high by 42% of consumers for flavor intensity, too low by 31% of consumers for sweetness and too dark by 47% of consumers. Figure 12f shows that the too high flavor intensity and the too low sweetness had the highest negative impact on the overall liking, with mean drops of approx. 2.3 and 2.0, respectively, while the too dark color (mean impact, $\approx$1.3) had a negative impact on overall liking similar to PS and BS (Figures 12b and 12d). Like for bread, these data suggest that a more high acceptability of GBS by Californian consumers could be obtained by reducing the % of

pâté in the recipe, for example to 3%, resulting in a partial reduction of flavor intensity and darkness but also of bitterness, with a consequent increase of the perceived sweetness. Also in this case, this reduction would not preclude the intake of olive oil phenols (20 g of GBS would be still enough to benefit from the health effects).

3.3.5. Impact of the Check-All-That-Apply attributes of products acceptability

Description of the products through CATA analysis was already reported (see paragraph 3.2.2). Results of Principal Coordinate Analysis, reported in two-dimensional maps in figure 13 for pasta (13a), bread (13b) and granola bar (13c), allow visualizing how the CATA attributes were associated with higher overall consumer acceptability. Finally, charts with mean impact vs % of responses for each CATA attribute significant in discriminating between Sample and Control are shown in figure 14 for pasta (14a), bread (14b) and granola bar (14c).

Pasta: "Al dente", "fresh", "buttery", "mild" and "savory" were the attributes more positively correlated to high overall liking scores (Figure 13a). Figure 14a shows that, among the attributes checked at least 20% of times, "fresh" (+ 1.2), "buttery" (+ 0.9), "eggy" (+ 0.6) and "mild" (+ 0.5) had the higher positive impact on overall liking scores, and, interestingly, all of them where checked a higher number of times for PC than for PS. On the opposite, "dark" (- 1.0) had the higher negative impact, and it was checked almost only for PS, by 77% of consumers. These findings indicate that the presence of pâté in the recipe of PS has a negative impact on the consumer acceptance of this kind of pasta, mainly due to its dark color and to lower perception of some attributes, most of which seeming to be intercorrelated ("buttery", "eggy", "fresh"). These findings are partially in agreement with results from descriptive analysis, from which the main negative drivers of liking of PS seemed to be "color", "eggy" aroma and flavor, "earthy" aroma and flavor and "evenness of texture".

Bread: data in table 5b confirmed our findings from JAR attributes, that "light brown" and "dark brown" were the attributes that differentiated between BC and BS the most. Figure 13b shows that bitter is one of the main negative drivers of liking, as also confirmed by figure 14b, which shows that "bitter" had a high negative impact (-1.3) on overall liking scores. This is not surprisingly, in that several studies reported bitterness as a negative driver of liking in several foods (Drewnowski & Gomez-Carneros, 2000;

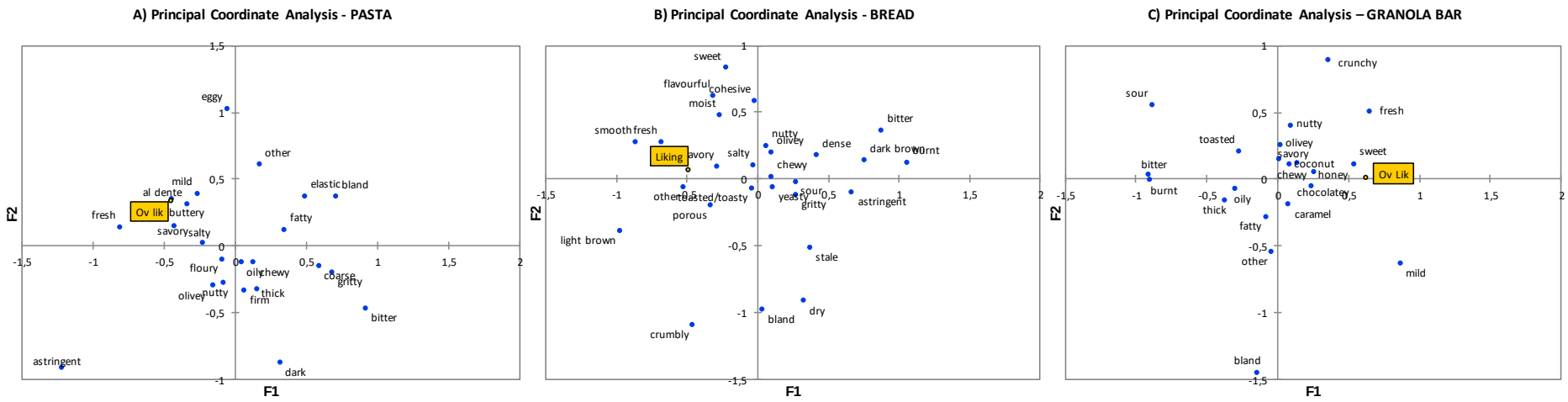


Fig 13. Principal Coordinate Analysis based on the correlation matrix of the CATA attributes with overall liking scores for Pasta (**A**), Bread (**B**) and Granola Bar (**C**) rated by all the 175 consumers.

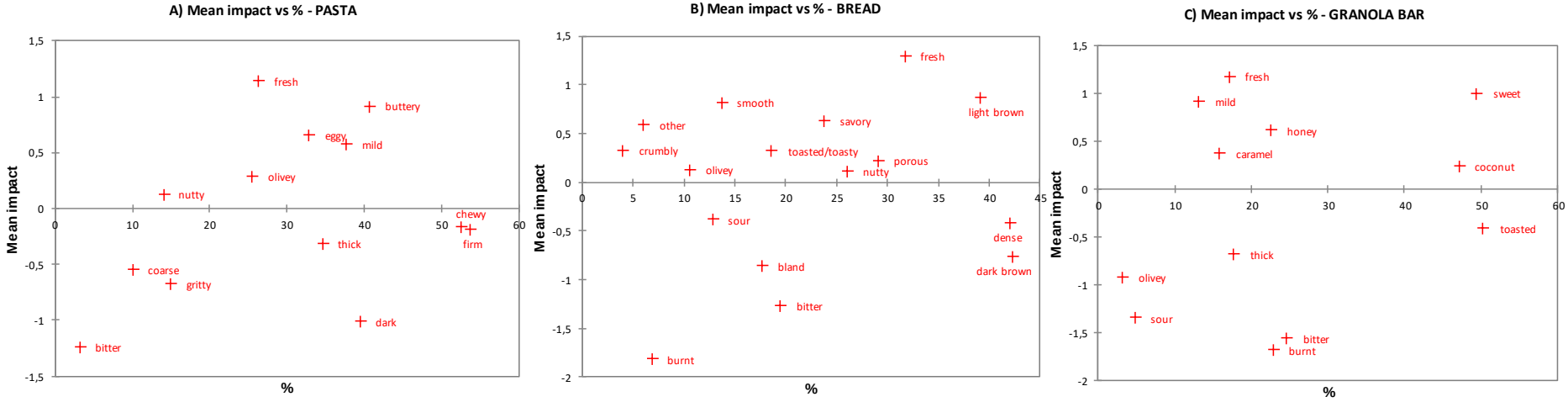


Figure 14. Mean Impact vs % of responses of those CATA attributes significant according to Table contingency for Pasta (**A**), Bread (**B**) and Granola Bar (**C**). The Mean Impact is the difference between the mean overall liking of samples with the presence of that attribute and the mean overall liking of samples with the absence of the same attribute

Guinard, et al., 1996), particularly in emerging markets like California, in which consumers were not exposed enough to bitterness of products from *Olea europaea* L., as high quality extra virgin olive oil (Delgado & Guinard, 2011). "Nutty" and "porous" had a low impact on overall liking scores. "Light brown" (+0.8) and "fresh" (+1.3), both checked more times for BC than for BS, had a positive impact and seemed to be positive drivers of liking. On the opposite, "dense" (-0.5) and "dark brown" (-0.8) together with "bitter" (all of them were checked more times for BS than for BC) had a negative impact. Bitter seemed also to be the main negative driver of liking from results of descriptive analysis, from which other drivers of liking are almost all the appearance attributes and "hearty" aroma and flavor. The above mentioned idea of reducing % of pâté in the recipe of BS would allow reducing the negative effect of the latter attributes on consumers acceptability. Indeed, if on one hand bitterness and darkness would be lowered, on the other hand a lower amount of pâté (and consequently of phenols) would render the leavening of loaves easier and also the "dense" attribute would be lowered

Granola bar: the attributes "toasted", "bitter" and "burnt" were checked more times for GBS than for GBC and had a negative impact on the overall liking scores (-0.4; -1.6 and -1.7, respectively), while "coconut", "honey" and "sweet" were checked more times for GBC than for GBS and had a positive impact on the overall liking scores (+0.2, +0.6 and +1.0, respectively). The negative impact of bitterness was not surprisingly, and was already evidenced by descriptive analysis, which also suggested "color" and "color intensity" as possible negative drivers of liking. Again, it appears evident that the negative impact of the three negative drivers of liking ("bitter", "burnt", "toasted") could be softened reducing the % of pâté in the recipe of GBS. However, in this case, we can also hypothesize that a partial decrease of cooking time and/or temperature could allow reducing "burnt" and "toasted" attributes. Figure 13c shows that "sweet" is the attribute more strongly associated to high overall liking scores, while "burnt" and "bitter" are confirmed to be the attributes more separated from high overall liking scores on the first component.

3.4 Focus Group

Focus group sessions were conducted to gain further qualitative information about the idea to use pâté as ingredient for the tasted products and beyond and further possible

applications, by small groups of naïve consumers. Focus group is usually used to communicate effectively with the consumers for determining what they want and what needs they have that are not already being met (Von Arx, 1986).

The focus groups associated olive oil with the Mediterranean as well as California. All were very familiar with olive oil, with most describing olive oil as a staple at home, used several times per week in cooking and salads for both perceived healthiness and taste. Someone perceives “extra virgin” like a marketing term rather than something real, while healthy properties of olive oil are mainly associated to the taste of bitter.

Participants responded positively in general to recovery of food waste, as a way to improve nutritional content of supplemented food products, increase sustainability of olive oil production, and potentially reduce prices of commercially available EVOO. Participants declared they want to know more about the product before eating and, in any case, to do not want eat it if it tastes bad. Noteworthy, several participants suggested the name “pomace” as not attractive.

Primary factors in purchasing habits of products supplemented with olive pomace were cost and hedonics. Participants suggested the pomace could be acceptable in products in all categories (with examples given of beer, baked products, soil, skincare products, spreads, products with low nutritional density, and “health” products), particularly if impacts to sensory characteristics are minimal or positive due to strong colors and flavours already present. Some expressed interest in availability of pomace as a consumer ingredient for addition to bread or as a spice.

Bread Sample, Granola Bar Sample, and Pasta Sample prepared as above described were presented to participants. The pasta was described as chewy and earthy, with no obvious difference in flavour from conventional pasta. The color of the pasta was described as potentially off-putting, but still remained an appropriate choice for special occasions and “adventurous eaters”. The bread was described as rustic, artisanal, and heavy, appropriate for fine dining restaurants, an accompaniment to cheeses and charcuterie, and other special occasions. The granola bar was also associated with the “health food” area of the product space, appropriate for health-conscious and athletic consumers rather than children, with some feeling it was not sweet enough. More than half of participants across all groups reported an interest in buying the three products. Overall, participants associated the darkened color of products with the appearance of

"healthy" foods, and few noted unexpected bitterness. All products were identified as being primarily suitable for special occasions, and likely not attractive to children.

4. Conclusions

In this work, three kind of food products (pasta, bread and granola bar) very widespread worldwide, and particularly in California, were selected and their recipes were slightly modified adding suitable % of a by-product from the olive oil production (pâté). The products had been characterized from a chemical and sensorial point of view: chemical analysis showed that different percentages of the phenols from pâté were recovered in the products ready-to-eat, so that we can state that about 63 g of fortified pasta, 18 g of fortified bread and 12 g of granola bar are enough to reach the minimum intake of olive oil phenols that the EFSA recommends consuming daily in order to benefit from the health effects stated the health claim.

The characterization of the sensory profile of both the fortified and not fortified products was performed using two different methodologies:

- CATA analysis, which allowed gaining insight into the consumers
- Descriptive analysis, which allowed a more detailed description of the differences between fortified and not fortified products

Results of the following consumer testing and focus groups, together with the characterization of the sensory profiles, confirmed that Californian consumers are receptive to these fortified products. The increasing exposure of consumers of emerging markets to bitter products as high quality extra virgin olive oils, will make easier to consumers accepting the bitterness of these type of products. Furthermore, we tested the products without any sauce or condiment, in order to only evaluate the impact of pâté: it would be reasonable to think that the acceptability of the products (mainly pasta and bread) will be increased by adding sauces or condiments. Recipes and products preparation could be slightly modified in order to softened the impact of some negative drivers of liking, the main of which seemed to be linked to the appearance and, to a lower extent, to the increase of bitterness; this is mainly true for bread and granola bar, since the bitterness seemed not to be significantly affected in the fortified pasta. Moreover, a proper labeling and marketing of the fortified products, mainly aimed to make more

familiar their appearance and to state their potential health benefits also partially linked to the bitterness, would allow improving the acceptance of the products.

In conclusion, it was demonstrated that pâté is suitable as ingredient for improving the daily intake of phenolic compounds from *Olea europaea* L., in order to meet the health benefits provided by these compounds, as also stated by the heath claim allowed by EFSA. The use of pâté as ingredient in products very widespread in several parts of the world would also give additional economic value to the olive oil production chain.

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9. LIST OF PUBLICATION

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