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"OLIVE CHAIN BY-PRODUCTS FOR THE FUNCTIONALIZATION OF FOODS"

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Abstract

The olive oil industry generates an important number of by-products, such as olive mill waste water, olive pomace and olive leaves. It has been demonstrated that these vegetable wastes are rich in almost all the phenolic compounds which are present in olive oil. Nevertheless, olive oil by-products have not yet been exploited at industrial scale, for example as sources of bioactive compounds. For this purpose, it is necessary to study how the processing conditions (raw material pre-treatment, extraction, etc.) affect their properties, as well as explore new applications in the food industry. Therefore, the main goal of this thesis was related to the possibility of using olive oil by-products for the formulation of new fortified foods. To this aim, it was necessary to consider that the potential incorporation of by-products into food formulation could alter the sensory properties, thus suggesting that careful selection of the type and the amount of these ingredients and proper technological options, should be adopted.

Specifically, in this research activity the effects of drying methods applied to the extracts from olive oil by-products on the polyphenol content and antioxidant capacity were investigated. Supercritical fluid extraction, pressurized liquid extraction and ultrasonic assisted extraction were adopted to choose the best extraction conditions to be applied to by-products. Fresh olive pomace and olive leaves were air dried at low temperatures to preserve bioactive compounds and then used to fortity food. Olive mill waste water was pretreated by membrane technology, with the dual aims of reducing the organic load waste water and recovering polyphenols. This technology, through the use of four membranes in cascade of microfiltration, ultrafiltration, nanofiltration and reverse osmosis is able to extract polyphenolic compounds. Innovative ceral-based and fish-based products were realized using these by-products rich in bioactive molecules. The enriched food products were characterized for the content of bioactive compounds, for sensory properties and then subjected to *in vitro* digestion with to purpose of evaluating the bioaccessibility of total polyphenols. The experimental results are very interesting and highlight that olive oil by-products could be valorized as promising ingredients to realize new products rich in phenolic compounds, also facing the problem of the environmental pollution.

Sommario

L'industria dell'olio d'oliva genera un numero importante di sottoprodotti, come le acque di scarico delle olive, la sansa di oliva e le foglie di ulivo. È stato dimostrato che questi scarti vegetali sono ricchi degli stessi composti fenolici presenti anche nell'olio d'oliva. Tuttavia, i sottoprodotti di olio d'oliva non sono ancora stati sfruttati su scala industriale, ad esempio come fonti di composti bioattivi. A tal fine, è necessario studiare a fondo in che modo le condizioni di lavorazione (pretrattamento della materia prima, estrazione, ecc.) influenzano il potenziale di bioattività, nonché esplorare nuove applicazioni nell'industria alimentare. Pertanto, l'obiettivo principale di questa tesi era legato alla possibilità di utilizzare i sottoprodotti oleari ricchi di composti polifenolici per la formulazione di nuovi alimenti funzionali. Per realizzare nuovi alimenti è necessario considerare che la potenziale incorporazione di sottoprodotti nella formulazione alimentare potrebbe alterare le proprietà sensoriali, suggerendo quindi che un'attenta selezione del tipo e la quantità di questi ingredienti e le opportune opzioni tecnologiche, dovrebbero essere adottate. In particolare, in questa attività di ricerca sono stati studiati gli effetti dei metodi di essiccazione applicati agli estratti dai sottoprodotti dell'olio di oliva sul contenuto di polifenoli e sulla capacità antiossidante. Estrazione di liquidi supercritici, estrazione liquida pressurizzata e estrazione assistita ad ultrasuoni sono stati adottati per scegliere le migliori condizioni di estrazione da applicare ai sottoprodotti. La sansa d'oliva fresca e le foglie di olivo sono state essiccate all'aria a basse temperature per preservare i composti bioattivi e poi utilizzate per alimentare la fortuna. Le acque reflue dei mulini sono state pretrattate dalla tecnologia a membrana, con il duplice scopo di ridurre l'acqua di scarico del carico organico e recuperare i polifenoli. Questa tecnologia, attraverso l'uso di quattro membrane in cascata di microfiltrazione, ultrafiltrazione, nanofiltrazione e osmosi inversa è in grado di estrarre composti polifenolici. Innovativi prodotti a base di cerali e di pesce sono stati realizzati utilizzando questi sottoprodotti ricchi di molecole bioattive. I prodotti alimentari arricchiti sono stati caratterizzati per il contenuto di composti bioattivi, per le proprietà sensoriali e quindi sottoposti a digestione in vitro allo scopo di valutare la bioaccessibilità dei polifenoli totali. I risultati sperimentali sono molto interessanti e sottolineano che i sottoprodotti dell'olio d'oliva potrebbero essere valorizzati come ingredienti promettenti per realizzare nuovi prodotti ricchi di composti fenolici, affrontando anche il problema dell'inquinamento ambientale.

1. INTRODUCTION

1.1 Olive oil industrial by-products

Olive (*Olea europaea L.*) is one of the most important crops in the Mediterranean countries. Around world more than eight million ha of olive trees are cultivated and about 98% of them is present the Mediterranean basin (Peralbo-Molina and Luque deCastro, 2013). Olive oil production is particularly important for Spain, Italy and Greece as the major producers in EU. Spain, with 2.47 million ha, has the largest area under cultivation, followed by Italy (1.16 million ha), Greece (0.81 million ha) and Portugal (0.38 million ha) (Inglezakis et al., 2012). Olives have attracted considerable attention during the past few years as sources of biophenols useful for the food and pharmaceutical industries. The technology for olive oil extraction has progressed significantly since the beginning of the seventies, when the three-phase centrifugation system appeared. By means of this system, the oil, vegetation water and solid phase of the olive can be separated in a continuous process, with the result that the traditional discontinuous press system is almost non-existent at present. The main inconvenience of the three-phase system is the generation during a short period of the year (November–February) of large quantities of olive mill waste water (OMWW), which is a very polluting liquid made of the olive vegetation water plus the water added in the different steps of oil production. In an attempt to reduce its environmental impact, different methods have been tried to make the best use of OMWW; these include storage in evaporation ponds and its direct application to agricultural soils as fertilizers. However, these methods have gradually become less viable for OMWW disposal, and so a new two-phase centrifugation system for oil extraction was developed during the early nineties. Although this is called the ecological system because it greatly reduces wastewater generation and its contaminant load, it still produces a solid and very humid by-product called ‘‘alperujo’’or olive pomace. Almost all of the olive mills in Spain use the two–phase centrifugation system for oil extraction to reduce wastewater generation and lower the contaminant load, compared with the three–phase centrifugation system (Dios–Palomares et al., 2005) which is corrently used by also other Mediterranean countries. The production of olive oil with this new centrifugation system, which saves both water and energy compared with the three-phase system, is estimated to represent about 75% of the total and the system is used roughly by 90% of olive-mills. As can be

seen in **Figure 1**, which schematically compares the three and two-phase centrifugation systems, approximately 800 kg of alperujo per ton of processed olives result from using the two-phase system, meaning that the yearly production of this by-product from the whole olive oil industry may approach four million tons. Recently, was developed an innovative two-phase decanter that 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, so reducing thepossible oxidation processes. This by-product, named pâté, consists of awet homogeneous pulp free from residuals of kernel, peculiarity making it a suitable ingredient for possible commercial applications after drying. The phenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remainder being lost in olive mill waste, in the form of a solid waste and an aqueous liquid. Several research demonstrates that these by-products from olive grove farming are a good source of antioxidant compounds with antibacterial properties, which have potential applications in the food and pharmaceutical industries.

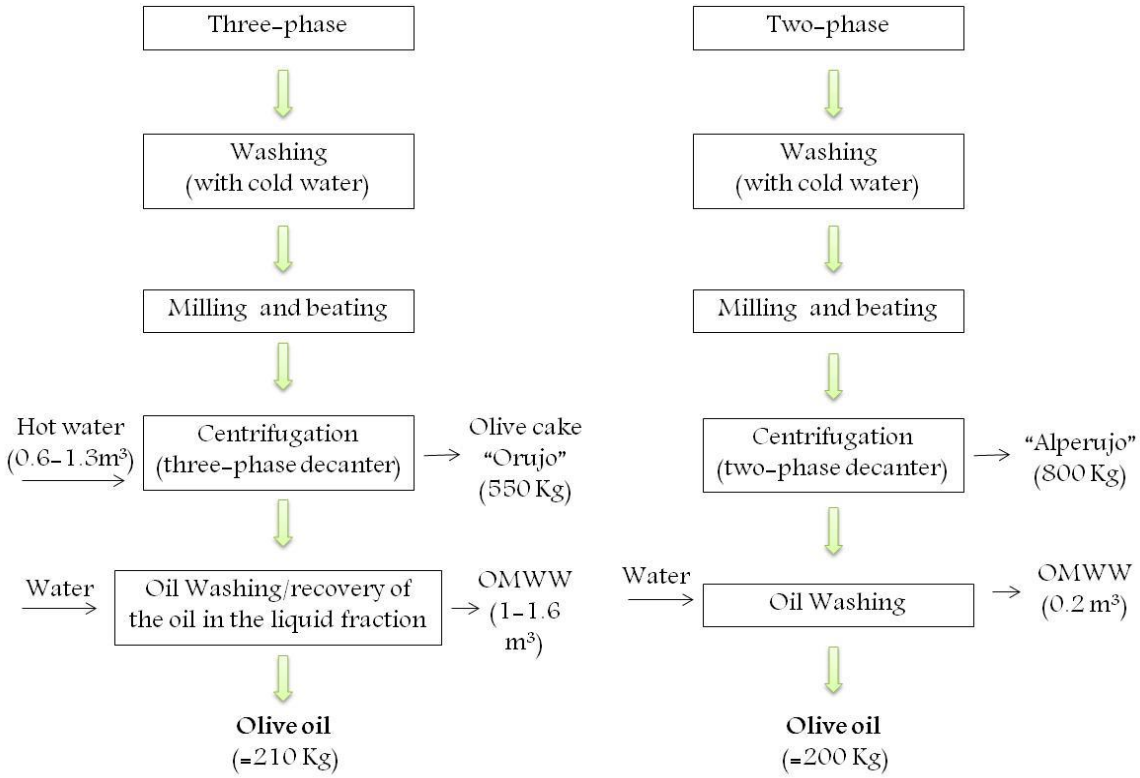


Figure 1 *Comparison of the three and two-phase centrifugation systems for olive oil extraction.*

1.1.1 Olive mill waste water

During olive oil production a considerable amount of water is employed, this water named "olive mill waste water" (OMWW) is discarded. Huge amounts (6-7 million tons/year) of OMWW, a complex medium containing polyphenols of different molecular masses, are produced in the Mediterranean countries cultivating the olive tree. In Italy, approximately 2 million cubic meters of wastewater are produced each year by the olive oil industry. This waste is claimed to be one of the most polluting effluents among those produced by the agro-food industries because of its high polluting load and high toxicity to plants, bacteria, and aquatic organisms, owing to its contents (14-15%) of organic substances and phenols (up to 10 g/L). These latter compounds, characterized by high specific chemical oxygen demand (COD) and resistance to biodegradation, are responsible for its black color, depending on their state of degradation and the olives they come from, and its phytotoxic and antibacterial properties (Capasso et al., 1992). For long time, OMWW has been regarded as a hazardous waste with negative impact on the environment and an economic burden on the olive oil industry. However, this view has changed to recognize OMWW as a potential low-cost starting material rich in bioactive compounds, particularly phenolics, that can be extracted and applied as natural antioxidants for the food and pharmaceutical industries. There is growing evidence that free radical mediated events are involved in several human diseases, because free radicals attack biomolecules such as lipids, protein, DNA, and bio-membranes, and play major roles in the oxidative degradation of food, animal feed, and cosmetics. Therefore, the extraction of biologically active phenolics from OMWW constitutes a viable alternative for valorizing this problematic agro-industrial waste (Capasso et al., 1992; Obied et al., 2008). The scientific interest in this material derives from the fact that its phenolic fraction possesses interesting biological activities. Several *in vitro* and *in vivo* studies have shown that phenols found in olives, olive oil, and OMWW exert potent biological activities including, but not limited to, antioxidant and free radical scavenging actions. They are potentially capable of preventing passive smoke-induced oxidative stress, reducing thromboxane B_2 production by whole blood, and ameliorating symptoms of inflammatory diseases such as osteoarthritis (Visioli et al., 2000; Visioli et al., 2009). The antioxidant efficiency of olive phenols has been assessed in various tests (Roche et al., 2005). It has been also observed that the administration of OMWW extract fractions as well as purified

hydroxytyrosol in diabetic rats caused a decrease in the glucose level in plasma (Hamden et al., 2009). A typical phenolic substance identified in olive fruit is oleuropein, a secoiridoid glucoside that is absent in OMWW due to enzymatic hydrolysis during olive oil extraction resulting in the formation of side products such as hydroxytyrosol and elenolic acid. Other phenolics identified in OMWW are verbascoside, tyrosol, catechol, 4-methylcatechol, p-hydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid (Visioli et al., 1999). Another potentially important compound present in OMWW is verbascoside and its isomer isoverbascoside (Wu and others, 2006). Verbascosides have demonstrated antioxidant protective effects on phospholipid membranes and have demonstrated the ability to modulate plasma antioxidant measures *in vivo* (Funes et al., 2009).

1.1.2 Olive pomace

The main by–product of the two–phase extraction system is olive pomace (OPO), which in Mediterranean areas is produced during a short period over the winter, from November to February (Moraetis et al., 2011), the amount generated varying between 7 and 30 million m^3 per year (López-Piñeiro et al., 2011). Typically, OPO is a semi–solid to semi–liquid by-product resulting from the mix of "alpechin", the main by–products resulting from the older three–phase extraction procedure, and "orujo". This by-product is made mainly with water, seed and pulp and is a potentially harmful by–product for the environment, because of the phytotoxic and antimicrobial properties, low pH, relatively high salinity and organic load, and the phenolic and lipid constituents (Piotrowska et al., 2011). Direct application to rivers or soil is not allowed under most of the national regulations of the producer countries. The main physico–chemical characterization of OPO can be found in many reports (Sierra et al., 2007; Paredes et al., 1999; Alburquerque et al., 2004). According to these studies, OPO is acidic, with a very high content of organic matter and carbon, rich in potassium (K), poor in phosphorus (P), with intermediate levels of nitrogen (N) and may also contain phenolic and lipid compounds. Although being lignocelluloses material, OPO contains a great amount of bioactive compounds, with a wide range of physiological properties, such as, anti-artherogenic, anti-allergenic, anti-cancer, anti-inflammatory, antioxidant, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Lozano-Sánchez et al., 2014). Among them, three glucosides, including salidroside (tyrosol–glucose),

nuezhenide (glucose–elenolic acid–glucose–tyrosol) and nuezhenide-oleoside, have been identified in OPO. Nuezhenide is found only in seeds as a predominant phenol, whereas verbascoside only appears in significant quantities in the seeds and pulp (Azmir et al., 2013). Thus, thanks to its low cost and large availability, several extraction techniques for OPO antioxidant components have been developed with the aim of re-valorizing the olive oil by-product and minimizing the environmental impact associated with its disposal.

1.1.3 Olive pâtè

The olive oil extraction technique 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 was introduced in Spain the two-phase centrifugation system 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 that can combine modern extraction technology without the addition of water. This decanter produces a dehydrated husk similar to the one obtained from a three-phase decanter, but it also separates the pulp 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 olive pâté (OP), 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 OP was evaluated in one study (Luciano et al., 2013), who demonstrated that the inclusion of OP 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. 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.

1.1.4 Olive leaves

More than 8 million ha of olive trees are cultivated worldwide. It has been estimated that an average of three tons of pruning biomass is obtained each year from one olive tree hectare, making these residues a huge, cheap, and unexploited source of energy or chemicals (Conde et al., 2009). Among them, residual biomass derived from olive trees includes two types of by-products: olive tree pruning and olive mill leaf biomass. The firsts is generated in olive groves when unproductive branches are eliminated, while the olive leaf biomass is obtained in olive mills by separating the leaves and thin branches fromthe olives. These olive residues do not have industrial applications and are usually burnt or just left in the field for fertilisation purposes; however, both practices have associated environmental risks (Ruiz et al., 2017). Olive leaves (OL) are also used for animal feed (Romero-Garcı´a et al., 2016), but their medicinal properties have aroused great attention. As the other parts of olive tree, leaves contain considerable amount of biophenols (Jilani et al., 2016). Olive leaves, which historically served as folk medicine in the Mediterranean region (Talhaoui et al., 2016), became even more valuable with reports of their extract effectively curing fever and malaria in 1854 (Altınyay and Altun, 2006). Polyphenols and flavonoids from olive leaves have demonstrated anti-carcinogenic, anti-inflammatory, antihypertension, and antimicrobial properties and thus are essential to the effects that gave olive leaves their significance. Biophenols in leaves are different from those of the flowers, fruits and the branch (Ozcan and Matthaus, 2017). Folklore reports about the therapeutic effects of tea made of olive leaves against sicknesses like coughing, sore throat, fever and cystitis in the Middle East culture for hundreds of years. Additionally, the leaf was used for dermatological diseases (Marsilio et al., 2001). Olive leaf was first used medicinally in ancient Egypt, and was the symbol of heavenly power. Several brands of medical supplements containing OL have been offered as liquid extracts or tablet forms against diabetes, high

blood pressure, cardiovascular diseases, common cold, urinary tract infections, chronic fatigue syndrome, and to support time of recovery, immune system (Ben Salem et al., 2015). Products containing olive leaf extract are used for anti-ageing activities in the cosmetics industry. Drugs made of OL extract are used not only for human health, but also for animal health due to their antibiotic and antiparasitic properties. In addition to the phenols commonly found in other plants, OL contain secoiridoid phenols, mainly oleuropein, which are exclusive to Oleaceae (Talhaoui et al., 2015). Since OL are inexpensive, they are a useful source of by-products with high phenolic value (Vergara-Barberan et al., 2015). Some research has been reported on the use of this by-products as raw materials in the biorefinery context to producemainly sugars and bioethanol through different thermochemical pretreatments (Negro et al., 2017). Such bioactivity drives the use of different olive oil by-products infunctional and food applications. In this sense, new applications have focused in enriching the food nutritional profile, replacing or improving the technological properties of food additives, and extendingthe food product shelf-life (Nunes et al., 2016).

1.2 Management of olive oil by-products

1.2.1 Waste or by-products?

The traditional waste management practices in the olive oil sector result in environmental problems as soil contamination, underground seepage, water-bodies pollution and foul odor emissions (Cabrera et al., 1996). Phenolic concentration deserves special attention for it influence on the antibacterial effects, phytotoxic effect and dark colour of OMWW. Discharging untreated or partially safe olive mill wastewater back into natural water systems can result in a rapid rise of microorganism numbers and this could quickly offset the equilibrium of an entire ecosystem (LIFE Focus, 2010). While phenolics are mainly held responsible for the olive oil wastewater strong antimicrobial and phytotoxic properties, non-phenolic-related toxicity attributed to long-chain fatty acids and volatile acids was also reported (Ouzounidou et al., 2010). Moreover, OMWW has been shown to affect the physical and chemical properties of soil and its microbial community, while several studies have evidenced its phytotoxic effects and antimicrobial activity. Olive oil waste can be toxic to anaerobic bacteria, which may inhibit conventional secondary and anaerobic treatments in municipal treatment plants (Karaouzas et al., 2011). Furthermore, the very high BOD_5 and COD,

which cannot be reduced in appropriate levels by anaerobic digestion poses another threat for the receivers (LIFE Focus, 2010). Landspreading and treatment in evaporation ponds could lead in groundwater pollution problems. The presence of organic matter as well as many inorganic compounds causes severe pollution when olive mill wastewater is disposed of into water bodies, but in soil it prevents erosion and can be beneficial to soil fertility (Kapellakis et al., 2008). Controlled land application of olive oil waste results in increase of soil organic matter and nutrient availability and thus, improves soil fertility and productivity by controlling the nutritional and biological equilibrium in the soil–plant interface. The use of olive oil waste in agriculture may also affect acidity, salinity, N immobilization, microbial response, leaching of nutrients and concentration of lipids, organic acids and phenolic.

One important question is "waste or by-product?". It is true that the characterization of a waste as by-product simplifies its further use. These materials are generated from industrial processes, which are considered, by the producers at least, to be by-products rather than wastes so that they can be recycled to land as soil improvers and fertilisers with minimum restriction (EC-DG, 2001). EC Directive 2008/98 specifies in the preamble under point 22, the need to avoid confusion with regards to the various aspects of the concept of waste and so make a distinction between waste and by-products (Taccogna, 2010). However, confusion still exists and in some cases the courts resolve these issues. Pursuant to article 5 of Directive 2008/98/EC, the by-product is a substance or object resulting from a production process the primary aim of which is not the production of that item. Such a substance or object can be regarded as not being waste if the following conditions are fulfilled:

(a) further use of the substance or object is certain;

(b) the substance or object can be used directly without any further processing other than normal industrial practice;

(c) the substance or object is produced as an integral part of a production process;

(d) further use is lawful; the substance or object fulfills all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts.

Concerning olive oil pomace and, together with above definitions, is also reported (Taccogna, 2010):

(a) the conditions for using, as a by-product, pomace from the pressing process in an olive mill, without applying the rules on waste, can exist with regard to its possible

uses: selling it to olive pomace refineries for chemical processing to extract olive pomace oil; spreading it as fertilizer (or mixed with backfill ground); as an additive in animal feed; for fuel (or similar), such as biomass, at energy production installations.
(b) olive oil pomace (OPO) deriving from mechanically pressing olives in the mill, for producing olive oil, conclusively meets the second condition where a by-product can be obtained only if the substance is produced as an integral part of the process (or not as the primary aim of production).
(c) it might be possible to consider that OPO can be used directly without needing any special processing other than normal industrial practice and so essentially in the same state as when it is pressed in the mill. Although it is true that further drying may sometimes be needed, it can still be considered as "common industrial practice" and so it complies with the legal rules provided for by-products.
(d) since OPO is a natural substance, and considering its properties it might be assumed that there is no adverse impact. In the EWC Decision 2002/532, olive oil waste falls under Category 2: "Wastes from agricultural, horticultural, hunting, fishing and aquacultural primary production, food preparation and processing". More specifically, the appropriate code is 02 03 "Wastes from fruit, vegetables, cereals, edible oils, cocoa, coffee and tobacco preparation and processing; conserve production". This kind of waste is not marked by asterisk and thus is not an "absolute" hazardous waste. However, in publication for oil olive waste of the European Union Network for the Implementation and Enforcement of Environmental Law (IMPEL) is stated that "special legislative provisions are needed for the solid residue, since, in principle, could be considered hazardous waste" (IMPEL, 2003). As is well known, olive oil waste contains polyphenols which render this waste as potentially "ecotoxic", under code H14: "'waste which presents or may present immediate or delayed risks for one or more sectors of the environment", in the meaning of in Annex III to Directive 91/689/EEC and to Directive 2008/98/EC.

1.2.2 EU legislation of olive by-products

The Legislative Decree n° 152 of 1999, transposition of the European Directives 91/271/CEE and 91/676/CEE, regulates the waters safeguard from pollution. The article 38 of this act makes reference to the Italian Law n° 574 of 1996 with regards to agronomic use of sewage sludge and other wastes. According to this law (n° 574), the

agronomic use of these by-products is allowed on the ground of their composition and the characteristics of soils. Such use has to be authorised each time by the competent public authority on the ground of simple documentation but subordinate to limitations, verifications and possible sanctions in order to avoid any fraudulent activity that can pollute water tables (More, 2009). This law allowed the direct application of the olive mill wastewater without previous treatment (Kapellakis et al., 2008). Technical aspects according to the Law 574/96 (More, 2009; Res-Hui, 2006):

- Maximum tolerance limit for soils: 50 m^3/ha/year for olive mill waste waters deriving from traditional mills (discontinuous extraction systems); 80 m^3/ha/year for vegetable water deriving from centrifugal extraction (continuous extraction systems);
- Uniform spreading in order to avoid surface runoff;
- Submission of the agronomic report to the Municipality at least 30 days before the spreading. The report has to be written by an expert technician and has to cover topics such as the characteristics of the soil, the time and means of spreading;
- Possibility for the Mayor of any municipality of modifying those limits or suspending fertirrigation in case of environmental risk;
- It is forbidden to spread vegetable water on:
  - soils which are at less than three hundreds meters from the preservation areas for water
  - soils which are less than two hundred meters from inhabited areas;
  - collection destined to the human consumption;
  - soils which are cultivated with vegetable crops;
  - soils where water tables are at less than ten meters depth;
  - soils which are frozen, covered by snow, awashed or saturated with water.
- Waste storage in the oil mill less than 30 days (limit protracted to 3 months – D Lgs 22/1997)

With the 6 July 2005 Decree, "*Criteria and technical rules regarding regional regulation of the agronomic use of olive mill waste waters and other mill wastes*" that makes reference to article n° 38 of Decree n° 152 of 11 May 1999, some more exclusions of lands are added (Res-Hui, 2006):

- Distance <10m from river banks

- Distance <10 m from sandy shore or lake water
- Lands with slope >15% and lacking of hydraulic and agricultural setting
- Woods
- Quarry
- Gardens and public areas

Furthermore, the same Decree prohibits the mixing of olive oil wastewater with other wastewater (e.g. animal slurry) or waste. Finally, oil mill water plus stone fragments and fibrous part of the fruit can be used in agriculture and are not subject to Fertiliser Law No 748. According to Law n° 574 of 1996 wet olive husks can be used as soil amendment notwithstanding to the indications given in the Italian Law n° 748 of 1984 on fertilizers and subsequent modifications such as legislative Decree n° 217 of 29 April 2006 "*Revision of regulations on fertilizer use*". According to the later, wet olive husks can be considered as a "*simple notcomposted plant amendment*" and therefore they can be applied to soil without any specific limitations if they comply with the thresholds set by the decree regarding some specific parameters, i.e. humidity, pH, organic carbon, organic nitrogen, total Cu referred to dry matter, total Zn referred to dry matter, total peat content and other heavy metals contents. With the D.M. 05/02/1998 and D. Lgs n. 22/97, by-products coming from olive oil pressing are allowed to be placed in the market. The same Decree specifies that olive husks (pomace) are non-dangerous waste. The same waste can be used in the energy sector, as defined in the DPCM 08/10/2004 (More, 2008; Res-Hui, 2006). Finally, The disposal of wastewaters (of any kind and, therefore, of OMWW too) in sewage systems or in superficial water bodies (rivers, lakes etc) is regulated by the Decree 152/2006. The specific thresholds set by the Decree for all the parameters to be taken in consideration for disposal are listed in Table 3 of Annex 5 of the Decree. The ELVs are set for national level (IPPB BREF, 2006b).

1.3 Re-use of olive oil by-products

Olive mill wastes require a complex management and disposal system due to their textural and organoleptic properties, as well as their negative effects on the environment (Roig et al., 2006). Nevertheless, have been studied as organic fertilizers (Fernandez-Hernandez et al., 2014; Magdich et al., 2012). Some evidences have shown that the high levels of phenolic compounds in olive by-products may be responsible for phytotocixity

and modifications in soil microflora (Kotsou et al., 2004). However, at a maximum spreading rate of 100 m^3 ha^{-1}, olive oil by-products did not affect olive production, despite the increasing levels of phenolic content when compared to the control soil (Magdich et al., 2012). Furthermore, phenolic compounds from olive oil by-products may act against specific plant pathogens, showing potential as natural pesticides (Cayuela et al., 2008). Olive by-products were also tested as a natural supplement in feeds of small ruminants (Yanez-Ruiz and Molina-Alcaide, 2007) and fish (Sicuro et al., 2010). However, the presence of complex polyphenols may have exhibited a negative impact on nutrient digestibility and intestine flora (Vasta et al., 2008). Olive pomace was included in sheep and goat feeds to study the effects of its phenolic content on digestibility, nitrogen losses and liver and kidney functions (Yanez- Ruiz and Molina-Alcaide, 2007). In this case, although being considered anti-nutritional factors, the phenolic compounds did not cause toxicity in the animals for the tested inclusion levels of olive pomace (122 and 128 g/kg of feed DM). Despite their controversial effects on plant growth and nutrient digestibility in animals, phenols from olive by-products are considered valuable compounds to be employed in other fields. For instance, their introduction in foodstuffs could decrease the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ). Due to their potential negative effects on health, these synthetic antioxidants are restricted to maximum concentrations in foodstuffs. In oils and fats, for example, those concentrations should not exceed 200 mg/kg of fat (individually or in combination with other added antioxidants) for BHA and TBHQ and 100 mg/kg for BHT (EC, 2011). These restrictions imposed by legislation, along with the health concerns related with the above mentioned synthetic antioxidants, are challenging matters for industries that need to find safer and costeffective alternatives. For instance, phenolic compounds recovered from vegetable by-products, such as olive oil by-products, can be applied in food matrices, improving their preservation and antioxidant properties. Bouaziz et al. (2010) observed that the introduction of a hydrolyzed phenolic extract of olive leaves in refined olive oil and olive oil pomace revealed a higher oxidation resistance when compared to the use of oleuropein and α-tocopherol. The authors related this antioxidant protection to the high levels of hydroxytyrosol and oleuropein aglycone in the hydrolyzed phenolic extract. Additionally, phenolic compounds of olive pomace were included in commercial sunflower oil (without added antioxidants) and compared to the use of other food

antioxidants by means of induction periods at 100 C (Lafka et al., 2011). Results from induction time measurement showed that phenolic extracts combined with ascorbyl palmitate contributed to a longer antioxidant protection than the one observed with the addition of ascorbyl palmitate, BHT and vitamin E. In another study, phenolic extracts from olive pomace were dissolved in ethanol/water and included in olive oil (Suarez et al., 2010). The results showed a significant improvement of the antioxidant capacity (up to 73%) of phenol-enriched olive oil when compared to the control group. In another research, De Leonardis et al. (2008) included hydroxytyrosol and oleuropein extracts from olive leaves in cod liver oil and solid fats, which led to an induction time increase with the increasing concentration of extract for all the tested matrices. Troise et al. (2014) included OMWW phenolic powder in raw milk that was then ultra-pasteurized and monitored the products derived from this thermal treatment. The author reported that the addition of this phenol-rich powder inhibited the formation of off-flavor compounds resulting from Maillard reactions during UHT treatment, improving the sensory quality of ultra-pasteurized milk. Hence, phenolic compounds from olive oil by-products may contribute to an improvement of nutritional and sensory quality in food products. The advantage of using olive oil by-products as feed supplements to reduce oxidative stress and improve meat antioxidant status, oxidative stability, and shelf life, has been demonstrated in different food producing animals, such as lambs (Luciano et al., 2013), chickens (Gerasopoulos et al., 2015; Tufarelli et al., 2016), rabbits (Dal Bosco et al., 2012), and beef cattle (Branciari et al., 2015). However, no reports are available on the use of olive pomace and the metabolic pathway of these compounds in poultry. Poultry meat is particularly prone to oxidative deterioration due to its high concentration of polyunsaturated fatty acids (Igene et al., 1979). There are many studies showing an improvement in the oxidative stability of chicken meat after feeding poultry with natural antioxidants as dietary additives (Gerasopoulos et al., 2015; Tufarelli et al., 2016; Botsoglou et al., 2002), causing an increase in the market value of the resulting products. Olive industry by-products could represent a different source of nutrients for animals and their inclusion in animal diets could be a convenient strategy to reduce oxidative deterioration in meat and increase olive oil production sustainability. Furthermore, when these compounds are present in small amounts in food, they are capable of preventing or retarding the oxidation of oils and fats, being a potential preservative of food quality (Tufarelli et al., 2016; Oroian et al., 2015). As example, one of the latest uses reported in the literature has been to include

thepurifiedphenol extract from OMWW to fresh sausage as a potential bioactive ingredient (Lucci et al., 2017). Currently, technology solutions are aimed at minimizing the amount of vegetable waste water effluent (Taticchi et al., 2017) andutilizing olive residues, either through promoting their overall sustainable management and conversion into an affordable source of bioactive compounds.

1.3.1 Extraction from olive mill waste water

From the oil extraction process regardless of the method used, extraction by pressure or centrifugation, olive mill waste water are formed in considerable amount. It has been estimated that for the process of the olives milling the OMWW produced are 1.1-1.5 times the weight of the minced olives. The annual production of vegetation waters in the Mediterranean countries is greater than $3x10^7$ m^3 and this is a disadvantage for the Italian crushers, because there is the problem to dispose large quantities concentrated in periods from October to January. Always the OMWW represent an environmental problem due to their high content of pollutants and their disposal add an additional cost to the oil producers. Several studies for their use as agricultural fertilizer are been found on the literature, but the centralization of milling processes in large mills that use advanced technologies hinders this traditional procedure. On the other hand, the OMWW are rich in phenolic compounds, potentially valuable if could be possible to develop an efficient recovery process and especially if there is the ability to formulate the final products that can be used as ingredients, food, nutraceutical or cosmetic. Recently, studies have used membrane technology, with the dual aims of reducing the organic load of OMWW and recovering polyphenols from OMWW. This technology, through the use of four membranes in cascade: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and Reverse Osmosis (RO), is able to recovered, continuously, high nutritional value able to be used in various fields of food and pharmaceutical industry and deionized water for possible agronomic applications.

This system, mainly applaied for the OMWW treatment has the dual purpose of recovering substances with high added value, to be used in various fields such as pharmaceutical, food and cosmetics, and obtaining at the same time the purification of water, with obvious advantages from the environmental point of view. The flow chart of the polyphenols extraction from the vegetation water is shown in **Figure 2** and described briefly below.

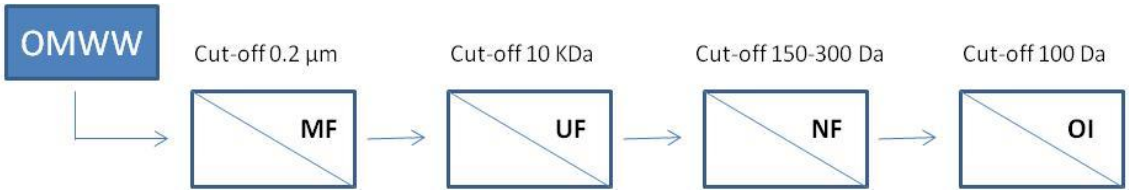


Figure 2 *Flows diagrams*

Microfiltration (MF) is made by tubular polymeric membranes with a 0.03 µm filter surface. From the storage tanks and the OMWW are fed into the circuit of the MF max 1200 l/h at a pressure of 5 bar pumped with an inverter for the flow regulation. Through the MF retentate side, is realized with a recycle flow rate of 110 m^3/h approximately with centrifugal pump appropriate, to maintain the speed inside the membrane of 4 m/s, that should avoid the solids accumulation on the membrane filter walls, preventing so the occlusion. The pressure, in the system, is maintained by a valve skimming, self-regulating pressure upstream, settable in the range 2 to 5 bar. Bleeding is conveyed in the system of collection and disposal drainages. Part of this water, that giving rise to the permeate through the MF membrane, has the pressure regulated by a valve skimmingwith self-regulating pressure upstream, settable in the range 1 ÷ 4 bar, whose set is fixed manually 0.5 ÷ 1 bar below the pressure set up in the circuit of the retentate, to maintain a trans-membrane pressure of about 1 bar. The overflow of permeate is conveyed to a suitable storage tank, from which it will be further used to feed the next section of Ultrafiltration (UF). The tank is equipped with the system of blanketing with nitrogen and with heating jacket. The MF retentate is conveyed to the waste for the untreated OMWW as it is poor in polyphenols and a few other impurities.

Figure 3 *Micro-filtration membrane system*

Ultrafiltration (UF) is carried out by a spiral wound membrane having a porosity of 5000 Da. The feeding is the permeate of the MF collected in a storage tank of 2.7 m^3 to max flow of 1200 l/h with a pressure of 10 bar with a manually operated pump with inverter for adjusting the flow rate. Through the UF retentate side, is realized with recycler with a flow rate of 10 m^3/h approximately by centrifugal pump. The pressure in the system is maintained by a settable valve. Part of the water through the membrane to give the UF permeate. The produced permeate is conveyed to a suitable tank, kept under nitrogen, from which will be picked up and fed to the next section of nanofiltration (NF). The UF retentate is stored in a storage tank of 2.7 m^3 equipped with nitrogen blanketing system.

The Nanofiltration (NF) is carried out by a spiral membrane having a pore size of 200 Da. Power is carried out with the UF permeate collected in a small tank with a flow rate of 1200 l/h at a pressure of 20 bar with a manually operated pump with inverter for the flow rate setting. Through the NF retentate side, is realized with a recycler with a flow rate of about 10 m^3/h by centrifugal pump. The pressure in the system is maintained by a settable valve. Part of the water through the membrane to NF give the UF permeate. The produced permeate is conveyed to a suitable tank, kept under nitrogen, from which will be picked up and fed to the Reverse osmosis.

Figure 4 *Ultra- and nano-filtration membrane system*

The Reverse Osmosis (RO) spiral membrane has a porosity of 100 Da. The NF permeate collected in its storage tank is drawn from a triplex pump, with flow rate of max 1200 l/h manually controlled by an inverter, feed the circuit of RO. The system can work in a range of pressures between 30 bar and 70 bar. For pressures around 30 ÷ 40 bar the pressure is maintained by a valve. Through the RO, the retentate side, is realized with a recycle flow rate of 10 m^3/h approximately always with a special triplex pump. For higher values, however, the pressure is adjusted manually by a needle valve. Part of the water through the membrane of RO give the permeate. The permeate is produced is partly used for the circuit Water Services while the excess is discharged as completely made up of purified water.

1.3.2 Extraction from olive pomace

The olive pomace (OPO) is an organic lignocellulosic material with a difficult commercial collocation due to its high moisture content (55–65%), which causes a noticeable increase in oil extraction costs, but it could be conveniently reused in agriculture as a valid soil amendment (L. 574/96). The maximum amount of wet pomace that farmers may apply over a 3 year period is 210 Mg ha^{-1}. In Italy, where the soil organic matter content is on average low and the Mediterranean climate contributes to its fast mineralization, the spreading of wet pomace on agricultural soil could increase its fertility; moreover, crops could also benefit from the dissolved water and

nutrients without toxic effects or yield reductions (Bonari and Ceccarini, 1993; Papini et al., 2000). Nevertheless, the OPO has some inconveniences when it is used in agriculture. The first is the difficulty to uniformly incorporate it into the soil. The second is its toxicity to plants due to the presence of high amounts of polyphenols (Marambe and Ando, 1992) which have, however, a fast decay (Camacho et al., 2000), and of oil residues that induce a slowing of the metabolism and of zymogene soil microflora multiplication, attenuated for some physiological groups after about 30 days, but continuing until 300 days for other groups such as autotrophic nitrifiers (Bedini et al., 1998). The spreading of olive-processing wet pomace on the soil seems, however, to improve the soil physical fertility. In particular, it induces an increase in the storage and transmission pores with beneficial effects on water retention, water flow in the soil and the structural stability, with the subsequent prevention of surface crusting (Bonari et al., 2001). The reductions of macro-porosity and water infiltration in the soil, just after treatments, are temporary unless applications are very high and/or take place in periods in which the biological activity is low, like in winter. It is known that the soil structural quality mostly depends on its interaction with organic matter, in the sense that the accumulation of the latter contributes to the formation and subsequent stabilization of aggregates against the dispersing action of water (Spaccini et al., 2001; Cavazza et al., 2002). An alternative for the disposal of large amount of OPO could be in evaporative ponds, but large areas would be required for this option which might also pose several potential environmental problems such as bad odor, leaching and insect proliferation (Cayuela et al., 2007). Another major preferred option would include the generation of renewable energy taking advantage of the relatively high calorific value of OPO. The process can be carried out in relatively inexpensive and simple reactor designs and operating procedures. Anaerobic digestion converts the carbonaceous matter into biogas leaving a stabilized slurry in a form suitable for reapplication to land as fertilizer. Common materials used for methane generation are often defined as waste materials, i.e. crop residues, animal and urban wastes. Manures and process waste waters have been extensively investigated as sources of biogas (Di Berardino et al., 2000).

In addition to these reuses, olive pomace can be used to enrich the human diet thanks to the many bioactive substances it contains. Although being lignocelluloses material, OPO contains a great amount of bioactive compounds, with a wide range of physiological properties, such as, anti-artherogenic, anti-allergenic, anti-cancer, anti-inflammatory, antioxidant, anti-microbial, anti-thrombotic, cardio-protective and

vasodilatory effects (Lozano-Sánchez et al., 2014). Thus, thanks to its low cost and large availability, several extraction techniques for OPO antioxidant components have been developed with the aim of re-valorizing the olive oil by-product and minimizing the environmental impact associated with its disposal. Generally, the conventional extraction process are mainly based on basic hydrolysis or saponification and on liquid–liquid or liquid–solid extraction with the use of toxic polar solvents (McCarthy et al., 2013). Nowadays, the use of environmental-friendly extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and ultrasonic assisted extraction (UAE) is increasingly required. In fact, these extraction techniques provide a lot of advantages, mainly connected to shorter extraction times, higher selectivity and lower consumption of organic solvents (Herrero et al., 2006, Azmir et al., 2013).

The supercritical fluid extraction (SFE) technology has advanced tremendously since its inception and is a method of choice in many food processing industries. The SFE technique is known as a green technology that minimizes damages to the environment while extracting high value-added compounds using a supercritical fluid, such as carbon dioxide (CO_2) (Veggi et al., 2014). The most recent advances of SFE applications in food science, natural products, by-product recovery, pharmaceutical and environmental sciences have been published in extensive reviews. Solvent extraction (SFE) is one of the old methods of separation known and certainly dates back to Paleolithic age. The science of solvent extraction has evolved over a long period of time and much progress has been made in the understanding of solvation and the properties of liquid mixtures used in extraction processes. Hannay and Hogarth's (1879) early observations of the dissolution of medium. However, it is only quite recently (around1960) that commercial process applications of supercritical fluid extraction have been extensively examined. Since the end of the 1970s, supercritical fluids have been used to isolate natural products; industrial applications of SFE have experienced a strong development since the early1990s in terms of patents. The main supercritical solvent used is carbon dioxide. Carbon dioxide (critical conditions tc = 31.3 °C and pc = 72.8 bar, dc = 0.467 gm/ml) is cheap, environmentally friendly and generally recognized as safe by FDA and EFSA (Dudai et al., 2006). The main downside of CO_2 is its low polarity, problem that can be overcome using co-solvents, capable of hydrogen-bonding, dipole-dipole and other polarity interactions with the analyte of interest. The solvents could be unsafe to handle and unacceptable as it is harmful to human health and the environment,

restricting its use in the food, cosmetic and pharmaceutical industries. Furthermore, the major drawback of the solvent extracted products is the high level of residues left in the final products that must be desolventizer before consumption. Therefore, for its high safety in the food field, only food ethanol was used to improve the efficiency of extraction from olive oil by-products.

Pressurized liquid extraction (PLE) is another green extraction technique for natural product extraction from food and botanical sample matrices. PLE is a widely considered advanced extraction technique which is able to efficiently extract interesting compounds from natural matrices using low volumes of organic solvents, if any, as well as producing high extraction yields in short extraction processes (Herrero et al., 2011). Using PLE, a relatively faster extraction rate is attained due to a combination of the following:

1. liquid solvent interaction with matrix molecules
2. elevated temperature and pressure for efficient extraction of targeted components.

Elevating the temperatures of employed solvents above their atmospheric boiling points allows increased solubility and mass transfer rates between the plant matrix and the solvent of choice. Eventually, enhanced diffusivity of the solvent and plant matrix causes more prominent extraction kinetics. The application of elevated temperature decreases extract viscosity, resulting in enhanced wetting of the plant matrix, and this leads to high solubility of the targeted molecules. It also causes breakage of bonding forces (dipole-dipole, van der Waals, and H_2-bonding) in order to facilitate diffusion of targeted phenolic compounds to the outer surfaces of solid matrices. Eventually, increased diffusion rates allow high extraction efficiencies with improved recovery rates (Carabias-Martınez et al., 2005; Wang and Weller, 2006). During the extraction process, the sample is placed into the extractor, followed by solvent pumping into the extraction vessel using an HPLC pump. The sample placed in the extraction cell is maintained at the desired temperature, using an electric heating jacket, until the required pressure is attained. After that the desired combination of temperature and pressure variables is reached, the extraction process starts. Extraction processes having more than 1 extraction cycles involve extraction solvent replenishment during each extraction cycle. Owing to back pressure, blocking valves are opened carefully after completion of the extraction cycle at an appropriate level in order to maintain the desired flow rate.

Once the extraction process is completed, the heating system and HPLC pump are shut down. Inert gases such as nitrogen may be utilized for purging the pressurized liquid extractor for the removal of residual solvent within the extractor. Also in PLE extraction food ethanol was used to improve the efficiency of extraction from olive oil by-products.

Ultrasound-assisted extraction (UAE) is a extraction process simple, inexpensive and green (Rombaut et al., 2014). UAE stands out as a sustainable alternative which requires a moderate investment of solvent and energy. Furthermore, it is easy to handle, safe, economical and reproducible due to the fact that this technology allows its development under conditions of atmospheric pressure and at an ambient temperature (Soria et al., 2010; Vieira et al., 2013). UAE is based on the principle of acoustic cavitation which is capable of damaging the cell walls of the plant matrix and thereby favoring the release of bioactive compounds (Tiwari et al., 2015). This technology can be applied to obtain different phytochemicals of which phenolic compounds stand out. These are appreciated by various fields of industry, particularly the food and pharmaceutical industries, thanks to their antimicrobial, anti-inflammatory, anticancer properties and mainly for their antioxidant capability (Parisi et al., 2014). It has been used to extract phenolic compounds from various plants, such as spruce wood (Ghitescu et al., 2015), haskap berries (Celli et al., 2015) and blueberry (He et al., 2016). Compared to conventional solvent extraction, UAE can significantly enhance the extraction yield of phenolic compounds (Mane et al., 2015). The optimization of extraction conditions with ethanol as solvent has been studied in order to obtain extra yield and better quality of the enriched product.

1.3.3 Extraction from olive pâtè

Olive oil extraction generates a variety of by-products, in different amounts depending on the production techniques used, which are all considered and treated as potential pollutants (Branciari et al., 2015). Among these, solid olive residues account for approximately 35% (w/w) of the processed olives according to the extraction method used. The particularity of the new centrifugal separator (Pieralisi Leopard DMF Technology) is the possibility to obtain a fraction of olive by-product called pâtè devoid of woody part. Indeed, this spreadable olive by-product is lignin free and consists of lipophilicandhydrophilic fractions, both characterized by technological and functional

properties. The absence of stone allows the use of the pâtè in the food field without further extraction. “Pâté” contains 75% moisture (both including conventional olive cake and olive mill waste waters). Moreover, it also contains a variable amount of olive oil (approximately 8–12%), depending on the water content), high levels of structural carbohydrates and sugars, and moderate concentrations of crude protein. Fatty acids are mainly represented by oleic acid and polyunsaturated fatty acids. Major phenolic compounds contained in olive cake are included in the following classes as already reported for the other by-products (Dal Basco et al., 2012; Cardinali et al., 2012; Servili et al., 2013):

(a) hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) (class of phenolic alcohols);

(b) dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA or p-HPEA 3,4-DHPEA-EDA or p-HPEA-EDA) (class of secoiridoids derivatives);

(c) verbascoside (a derivative of the hydroxicinnamic acid);

(d) caffeic acid, p-coumaric acid and vanillic acid (class of phenolic acids and derivatives);

(e) lutein (class of flavones);

(f) (+)-acetoxypinoresinol and (+)-pinoresinol (class of lignans).

This kind of compounds are well-known for their beneficial effects in the human health. So, these results suggest that the olive by-product, olive pate, could be used as functional ingredients, in the production of nutraceuticals for human and animal feeding. The particularity of the new centrifugal separator is the possibility to obtain a fraction of olive by-product called pâtè devoid of woody part. Indeed, this spreadable olive by-product is lignin free and consists of lipophilicandhydrophilic fractions, both characterized by technological and functional properties. The absence of stone allows the use of the pate in the food field without the need for extraction.

1.3.4 Extraction from olive leaves

Olive leaves contain many potentially bioactive compounds that may have antioxidant, anti-hypertensive, anti-inflammatory, hypoglycaemic and hypocholesterolemic properties and antimicrobial properties against some microorganisms such as bacteria, fungi, and mycoplasma. Due to these activities and valuable biophenol compounds, usage of whole olive leaf and olive leaf extract has increased rapidly in both the pharmaceutical and food industries as food additives and functional food materials. The

whole leaf extract is recommended to achieve health benefits due to the presence of additive and/or synergistic effects of their phytochemicals (Ghanbari et al., 2012). Several techniques have been used to recover phenolic compounds from olive by-products, including enzymatic preparation, solvent extraction, supercritical fluids (supercritical CO_2) membrane separation, centrifugation, and chromatographic procedures. Since ultrasound assisted extraction (UAE) seemed to be the most suitable technique for extraction of bioactive compounds from olive oil by-products (Jap_on-Luj_an et al., 2006), so this method was preferred to extract phenolic compounds from olive leaves.

2. OBJECTIVE

The possibility of turning olive oil by-product into a valuable resource, particularly for human consumption, would represent an important benefit for the miller. Reports on the use of these particular by-products for food formulations are not available so far. In this contest, my Ph-D project had the aim to realize innovative cereal-based and fish-based food enriched with by-products rich in bioactive molecules. In order to realize new foods it was necessary to consider that the potential incorporation of by-products into food formulations could alter the sensory properties, therefore, careful selection of the type and the amount of these ingredients and proper technological options were adopted.

To the aim of the research, different steps were carried out: (i) stabilization and characterization of by-products of the oil industry; (ii) addition of olive oil by-products to food formulation; (iii) sensory evaluation of new products; (iv) chemical and biological characterization of new food to evaluate macro-components, polyphenol content and antioxidant activity; (v) evaluation of bioaccessibility of fortified food by *in vitro* gastro intestinal digestion model.

3. MATERIALS AND METHODS

3.1 Fish burger with olive pâtè

3.1.1 Raw materials

The olive pâtè (OP) was obtained by a local olive mill (Bisceglie, Bari, Italy) from the organic *Coratina* cultivar milled using a Pieralisi Leopard with DMF technology (Multi Phases Decanter). The OP was dried at 35 °C in a dryer (SG600, Namad, Rome, Italy) for 72 h. The dried olive pâtè was reduced in a fine powder (< 500μm) by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) and then stored at 4 °C until further utilization. The fish, tuna trance, was purchased by Minaba srl (Manfredonia, Foggia, Italy).

3.1.2 Fish burger preparation

Fish burger (40 g) were manufactured using tuna trance, previously thawed and chopped until to a diameter of about 12 mm (28 g), mixed with potato flour (2 g), parsley (0.2 g), salt (0.2 g) and a whey protein based crumb soaked in extra-virgin oil, as reported by (Del Nobile et al., 2009). The mixture was added with 10% w/w of dry OP. OP was added to burger formulation either as it was or pre-treated. In particular, two different pre-treatments were adopted: hydration and hydration/extraction. In the first case OP was hydrated either with water or milk with a OP/liquid ratio of 1:1 for 1 h, it will be referred as OP-H. In the second case, OP was first hydrated with either water or milk with a OP/liquid ratio of 1:5 for 1 h (extraction stage). Afterwards, the excess liquid was drained (separation stage), it will be referred as OP-H/E. The control was prepared without the olive pâtè. The mixture was mixed for a few minutes to have a greater homogenization and burger were made with a diameter of 5 cm and a height of 1 cm. Finally, the burgers were cooked in an electric convention oven (H2810, Hugin, Milan, Italy) at 240 °C for 12 min.

3.1.3 Total phenols, flavonoids and antioxidant activity determination

To determine total phenols, flavonoids and antioxidant activity, the extraction was performed as described by Meneses et al. (2013) with slight modifications. Burger with and without olive pâtè, both raw and cooked, were dried at 35 °C with a ventilated stove

(BINDER GmbH, Tuttlingen, Germany), than milled to obtain a powder. For the extractions, 1 g of dried sample was mixed with 20 ml of equal mixture water: ethanol (v/v) (Sigma-Aldrich, Milan, Italy) in Erlenmeyer flasks, which were duly covered to avoid solvent loss, and maintained during 30 min in a water-bath (GRANT OLS200, Cambridge, England) with magnetic agitation at 60 °C. After this time, the extracts were filtered with Nylon 0.45 µm to obtain a clear supernatant. The volume of extract recovered after each extraction was quantified and used for calculation. Triplicate extractions were made for each sample.

Total phenolic compounds were determined by UV-vis spectrophotometry according to Folin-Ciocalteu method (Spinelli et al., 2015). In particular, OP was 1:20 diluted with water before analysis, while the extracts obtained from samples, previously described, were analyzed without any dilution. Specifically, 0.5 mL of dry OP or burger extract was mixed with 2.5 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, diluted 1:10 with water) and, after 5 minutes, 2 mL of 75 g/L Na_2CO_3 (Carlo Erba, Milan, Italy) was added. The sample was kept in darkness at room temperature for 2 h. The equal solution water: ethanol (v/v) was used as control sample. The absorbance was measured at 740 nm by an UV-vis spectrophotometer (UV1800, Shimadzu Italia s.r.l., Milan, Italy). Total phenolic compounds were quantified by a calibration curve previously built (3.12-100 mg/L; $R^2 = 0.9989$) using standard solution of gallic acid (Sigma-Aldrich), and the total phenolic content was expressed as mg gallic acid/g of dry weight (dw). All tests were carried out in triplicate.

Total flavonoids content both in OP and in all the burger's extracts was determined by aluminum chloride colorimetric method, according to (Kuo et al., 2015) with modifications, using quercetin (Sigma-Aldrich) as standard. Extracts (0.5 mL), prepared as previously described, were mixed with 2 mL of distilled water and 150 μL of a 5% sodium nitrite (Sigma-Aldrich) solution. After 6 minutes, 150 μL of a 10% aluminum chloride (Sigma-Aldrich) solution was added and the mixture was allowed to stand for 6 minutes. Finally, 1 mL of 1M sodium hydroxide (Sigma-Aldrich) was added until had volume was made up to 5 mL with distilled water. Then, the solutions were mixed and for each sample the absorbance was read in triplicate against blank at 415 nm. The standard curve was prepared using quercetin as standard in the range 12.5-200 mg/L ($R^2 = 0.9954$) and total amount of flavonoids was expressed in mg of quercetin/g of dry weight (dw).

The antioxidant activity was evaluated using ABTS assay according to the method of Arnàiz et al. (2016) with slight modifications. The ABTS assay is based on the ability of antioxidants to interact with the radical cation (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich) inhibiting its absorption at 728 nm. 7 mM ABTS stock solution and 140 mM potassium persulfate (Sigma-Aldrich) were utilized. The ABTS radical cation ($ABTS^{\cdot+}$) was obtained by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and leaving the mixture in the dark at room temperature for 12-16 h. The $ABTS^{\cdot+}$ solution was diluted to an absorbance 0.700±0.020 at 728 nm, with 5 mM phosphate buffered saline (pH 7.4). For the preparation of the phosphate buffered saline (PBS), the following salts were used: sodium phosphate monobasic monohydrate ($H_2NaO_4P \cdot H_2O$) and sodium phosphate dibasic heptahydrate ($HNa_2O_4P \cdot 7H_2O$) (Sigma-Aldrich). Then, 300 μL of sample extract was added to 2.2 mL of $ABTS^{\cdot+}$ diluted solution and after 6 minutes at room temperature the mixture was measured through a spectrophotometer (UV1800, Shimadzu Italia s.r.l., Milan, Italy) at 728 nm. A calibration curve was previously built using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich) as standard, at concentrations between 0.94 and 40 mg/L ($R^2 = 0.9995$) and the antioxidant activity was expressed as mg Trolox equivalents for gram of dry weight (dw). The analyses were carried out in triplicate. The free radical activity was defined by measuring the ability of the extracts to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich). The antioxidant activity was determined using a method described by (Meneses et al., 2013) with modification. For the reactions, 100 µl of each extract were added to 2.9 ml of DPPH solution (6×10^{-5} M in ethanol). The resulting solutions were vortexed and allowed to stand for 30 min in darkness at room temperature. Then the absorbance was measured at 517 nm in a spectrophotometer (UV1800, Shimadzu Italia s.r.l., Milan, Italy), using ethanol as blank. The control solution was constituted by ethanol instead of the sample. The radical scavenging activity was expressed as the inhibition percentage using the equation (1), where A_C and A_S are the absorbance of the control solution and the absorbance of the sample solution, respectively.

$$\% \text{ Inhibition of DPPH} = (1 - A_S/A_C) \times 100 \qquad (1)$$

3.1.4 Sensory analysis

Fish burger samples were submitted to a panel of 10 trained tasters in order to evaluate the sensory attributes. The panelists were selected on the basis of their sensory skills (ability to accurately determine and communicate the sensory attributes as appearance, odor, flavor and texture of a product). They were asked to indicate color, odor and texture of raw burger. Color, odor, taste, texture, juiciness and tenderness were evaluated on cooked fish burger. To the aim, a nine-point scale, where 1 corresponded to extremely unpleasant, 9 to extremely pleasant and 5 to the threshold acceptability, was used to quantify each attribute. On the basis of the aforementioned attributes, panelists were also asked to score the overall quality of both cooked and uncooked products using the same nine-point scale.

3.1.5 Statistical analysis

Experimental data were compared by a one-way analysis of variance (ANOVA). Duncan's multiple range test, with the option of homogeneous groups ($p<0.05$), was carried out to determine significant differences between spaghetti samples. STATISTICA 7.1 for Windows (Stat Soft, Inc., Tulsa, OK, USA) was used.

3.2 Bread with olive pâtè

3.2.1 Bread-making process

Bread dough mixing, processing and baking were carry out on laboratory-scale equipment as described by Saccotelli and colleagues (2017). The control sample (B-CTRL) was obtained with a pre-mixture of 1500 g of wheat flour and 900 g of water stirred at high speed for 10 min in a mixer (Conti, Bussolengo, Verona, Italy). Once a homogeneous mixture was obtained, 45 g of compressed fresh yeast, 15 g of sugar, and 30 g of salt were added and it was mixed at the average speed for 15 min. All ingredients were purchased from a local market (Foggia, Italy). After complete mixing, the dough rested in bulk in the incubator (Thermogel, Varese, Italy) at constant temperature (30 °C) and relative humidity (85%) for 60 min. Subsequently, dough portions of about 800 g were manually rounded and placed above a baking tray in the incubator at the same conditions reported above, for the final fermentation, lasting 30 min. After the fermentation process, the samples were baked in an electric oven (Europa

Forni, Vicenza, Italy) at 230 °C for 15 min, followed by 35 min at 200 °C. To enrich the bread, was used the pâtè (OP) that was obtained by a local olive mill (Lecce, Bari, Italy) from the organic *Cellina* cultivar milled using a Pieralisi Leopard with DMF technology (Multi Phases Decanter). The olive pâtè (OP) was dried at 35 °C in a dryer (SG600, Namad, Rome, Italy) for 72 hr and than reduced in a fine powder (< 500μm) by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy). The enriched experimental sample (B-OP) was prepared using the same procedure as for the B-CTRL sample, with dry OP (150 g), in partial substitution of wheat flour, and in addition to compressed fresh yeast sodium bicarbonate (18 g) and cream of tartar (36 g) were also added. The OP was pre-treated with milk as described previously for burger case.

3.2.2 Sensory analysis

Bread samples were submitted to a panel of 10 trained tasters in order to evaluate the bread sensory attributes. Before sensory analysis, samples were sliced with an electric slicing knife (thickness of 15 mm) (Atlantic; Calenzano, Firenze, Italy) without removing the crust. The bread samples were evaluated on a 9 points scale anchored where 1 corresponded to *extremely unpleasant*, 9 to *extremely pleasant* and 5 to the *threshold* acceptability. In particular, seven attributes were considered for the bread acceptance: color, odor, taste, crust and crumb firmness, presence of large bubbles and overall quality.

3.2.3 Total phenolic compounds, total flavonoids and trolox equivalent antioxidant activity

To determine total phenols, flavonoids and antioxidant activity, the extraction was performed as described by Biney and Beta (2014) with slight modifications. Bread samples, without crust, were dried with a ventilated stove (BINDER GmbH, Tuttlingen, Germany) at 35 °C and milled to obtain a powder. 1 g of each dried sample was mixed with 10 ml of acidified methanol (HCl/H_2O:MeOH, 20:80) and they were shaken at room temperature in darkness for 2 h at 300 rpm using orbital shaker (HS 260 BASIC, IKA, Staufen, Germany). Next, the samples were centrifuged at 5 °C for 15 minutes at 10000 rpm (5804R, Eppendorf, Milan, Italy) and supernatant was collected and filtered (PTFE 0.45 μm) prior to the analytical determinations. Triplicate extractions were made for each sample. Total phenolic compounds, total flavonoids content and antioxidant

activity, evaluated using the ABTS assay, were conducted in triplicate with the same methods described previously for burger case.

3.2.4 Glycemic index

Digestion was carried out as described by Chillo and others (2011) and Monro and others (2009) with slight modifications. Bread samples without crust (5 g) were chopped and tipped into a digestion vessel with 50 ml of distilled water and 5 ml maleate buffer (0.2 M pH 6.0, containing 0.1 g sodium azide and 0.15 g $CaCl_2$ per liter) in an block at 37 °C (GFL 1092; GFL Gesellschaft für Labortechnik, Burgwedel, Germany) and allowed to equilibrate for 15 min. Digestion was started by adding 1 ml of 2% pancreatin (P7545; Sigma Aldrich 2 g in 100 ml of maleate buffer) and 0.1 ml amyloglucosidase (A7095; Sigma Aldrich) and the vessels were stirred at 130 rpm. An amount of 0.5 ml of the digested samples was taken at 0, 20, 60 and 120 min for the released glucose analysis. These samples, taken during the different times of digestion, were added to 2.0 ml of ethanol and mixed. After 1 h, the ethanolic sub-samples were centrifuged at 2000 g for 2 min (Biofuge fresco; Heraeus, Hanau, Germany). Finally, the reducing sugar concentration was measured colorimetrically at 530 nm. Glucose standards of 10 mg/ml were used. Amyloglucosidase (0.25 ml) (EAMGDF, 1 ml per 100 ml in sodium acetate buffer 0.1 M, pH 5.2; Megazyme International 205 Ireland Ltd., Wicklow, Ireland) was adjunct to 0.05 ml of the supernatant and incubated for 10 min at 20 °C. Afterwards, 0.75 ml DNS solution (16% NaOH, 10% 3,5-dinitrosalicylic acid and 30% Na-K tartrate – Sigma Aldrich) was conjoint to the above solution, heated to 100 °C for 15 min and allowed to cool for 1 h at 15 °C. Finally, 4 ml of distilled water (15 °C) were added to the solution. The results were plotted as glucose release (mg) per g of sample vs. time. The starch digestibility was calculated as the area under the curve (0–120 min) for the tested products, and expressed as the percentage of the corresponding area for white bread (Chillo et al., 2011).

3.2.5 Bio-accessibility of enriched bread

Polyphenol bio-accessibility from enriched bread was determined using a three-stage simulated digestion including oral, gastric and small intestinal phase, as described by D'Antuono and others (2016) with slight modifications. The oral phase solution (6 ml), containing 5 % mucin (M2378; Sigma Aldrich), 3 % uric acid (U2625; Sigma Aldrich),

40 % urea (U5378; Sigma Aldrich) of sample and 10.6 g of α-amylase (A3176; Sigma Aldrich), was added to the previously chopped bread without crust (1 g). The reaction tube was mixed and incubated in a covered shaking water bath (37 °C, 85 rpm, 10 min). Samples were then diluted to 30 ml with saline solution (0.9 % NaCl). The gastric phase was initiated with addition of 2 ml porcine pepsin (P7000; Sigma Aldrich) solution (20 mg/ml in 0.1 M HCl) and adjustment of the pH to 3.0 ± 0.1 with 1.0 M HCl. After the addition of saline solution to 40 ml total volume, samples were incubated in a covered shaking water bath (37 °C, 85 rpm, 1 h). The small intestinal phase was initiated by the addition of 1.0 M $NaHCO_3$ to adjust the pH of gastric digesta to 6.5 ± 0.1. A cocktail of small intestinal enzyme, 2 ml of 15 mg/ml lipase (L3126; Sigma Aldrich) in 0.1 M $NaHCO_3$ and 30 mg/ml pancreatin (P7545; Sigma Aldrich), and porcine bile salts (B8631; Sigma Aldrich) (3 ml of 120 mg/mL bile extract in 0.1 M $NaHCO_3$) were added to the solution. Samples were then standardized to 50 ml with saline solution and incubated in a covered shaking water bath (37 °C, 85 rpm, 2 h). At the end of the small intestinal phase, an aliquot (3 ml) representing the crude digested (CD) was collected and the samples were centrifuged at 10000 × g for 1 h at 4 °C to separate the aqueous intestinal digested (AQ) from the residual solid. Aliquots of AQ, for both control and enriched bread, were collected, filtered using a 0.2 μm PTFE filter and analyzed with Folin-Ciocalteu spectrophotometric method. The bio-accessibility of phenols was expressed as the bio-accessibility percentage using the following expression:

$$\text{Bioaccessibiliy (\%)} = (CF / CI) \times 100 \qquad (2)$$

where CF is the final concentration of total polyphenols in the aqueous intestinal digesta and CI is the initial total polyphenols concentration in the undigested bread.

3.2.6 Statistical analysis

Experimental data were compared by a one-way analysis of variance (ANOVA). Duncan's multiple range test, with the option of homogeneous groups ($p<0.05$), was carried out to determine significant differences among bread samples. STATISTICA 7.1 for Windows (Stat Soft, Inc., Tulsa, OK, USA) was used.

3.3 Pasta with olive pâtè

3.3.1 Spaghetti preparation

The olive pâtè (OP) was obtained by a local olive mill (Bisceglie, Bari, Italy) from the organic *Coratina* cultivar milled using a Pieralisi Leopard with DMF technology (Multi Phases Decanter). The OP was dried at 35 °C in a dryer (SG600, Namad, Rome, Italy) for 72 h. The dried olive pâtè was reduced in a fine powder (< 500μm) by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) and then stored at 4 °C until further utilization. The OP was added to the dough at 10% and 15% (w/w). In the subsequent trial, to the formulation with 10% OP, 0.3 and 0.6% Transglutaminase Activa WM (TG) (Perrins Chemical, Triggiano, BARI, Italy) were also added, respectively. In order to ensure the solubility of the enzyme as powder, it was previously dissolved in water. Spaghetti without any addition were also manufactured and used as the reference sample (CTRL). In both steps, dough was extruded with a 60VR extruder (Namad) and dried in a dryer (SG600; Namad), as described by Padalino et al. (2013). Spaghetti samples of each batch were produced twice.

3.3.2 Sensory analysis

Dry spaghetti samples were examined by a panel of 10 trained tasters in order to evaluate the sensory attributes. The panelists had at least several years of experience in evaluation of pasta prior to this study; however, they were retrained for this study in a session of 2 h to be experienced in the products and terminology (ISO 11036/7304-2). Appropriate descriptive terms for sensory evaluation were decided during the retraining sessions. After retraining, experienced graders were able to evaluate color and resistance to break of uncooked spaghetti and elasticity, firmness, bulkiness, adhesiveness, color, odor and taste on cooked spaghetti. The elasticity is the measure of the degree of extension of the spaghetti before the break and it is evaluated on the single sample practicing a slight traction in two points distant 10 cm. The firmness is the resistance of cooked pasta to compression by the teeth, it is measured by compressing the spaghetti strand against the palate with the tongue. The bulkiness is the measure of the degree of jamming among the spaghetti strands and it is evaluated by placing two spaghetti strands together and determining the force required for detachment. The adhesiveness is related to the formation of a surface coating made of amylose and it is evaluated by placing the spaghetti in the mouth, pressing it against the palate and

determining the force required to remove it with the tongue. For the evaluation, a nine-point scale was adopted, where 1 and 9 represented the lowest and the highest intensity of a particular attribute, respectively. On the basis of the above-mentioned attributes, the panel evaluated overall acceptability of each pasta sample using a nine-point scale where 1 = dislike extremely, and 9 = like extremely. Pasta products with an overall acceptability mean score above 5 were considered as acceptable (Padalino et al., 2013). Sensory evaluation was repeated twice on two different batches of samples.

3.3.3 HPLC standards for chemical analyses

Tocopherol isoforms (α-, β-, and γ-tocopherol), triterpenic acids (maslinic and oleanoic acids), methyl tricosanoate, myristic, palmitic, pentadecanoic, stearic, arachidic, palmitoleic, heptadecanoic, oleic, linoleic and linolenic acids used as standard as well as all High Performance Liquid Chromatography (HPLC) grade solvents were all purchased from Sigma–Aldrich (Milan, Italy). Tocotrienol isoforms and carotenoid standards were purchased from Cayman chemicals (Ann Arbor, MI, USA) and CaroteNature (Lupsingen, Liestal, Switzerland), respectively. Phenolic standards used for the High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis were purchased from PhytoLab GmbH & Co. KG Dutendorfer Straße 5-7 91487 (Vestenbergsgreuth, Germany) and listed as follows: tyrosol, caffeic acid, vanillic acid, coumaric acid, vitexin, ferulic acid, oleuropein, quercetin, luteolin, apigenin.

3.3.4 Extraction and analysis of triterpenic acids

Triterpenic acids extraction was carried out according to Romero et al. (2010). Briefly, 1 g of each sample was extracted 6 times using 4 mL of methanol/ethanol (1:1, v/v), the extracts were combined and re-dissolved in 1 mL of methanol and filtered through a 0.45 μm syringe filter (Millipore Corporation, Billerica, MA, USA). Quali-quantitative analysis of triterpenic acids was carried out by the method of Lozano-Mena et al. (2012) slightly modified, using an Agilent 1100 Series HPLC system equipped with Phenomenex-luna 5 μm C18 (2) 100 Å column (250 x 4.6 mm). To record HPLC runs the Agilent ChemStation software was used. The mobile phases were: acetonitrile (A) and 1% (v/v) H_3PO_4 in water (B). The gradient elution was as follows: 0 min, 60% A and 40% B; 0–5 min, 50% A and 50% B; 5–10 min, 40% A and 60% B; 10–20 min,

30% A and 70% B; 20–25 min, 25% A and 75% B; 25–30 min, 20% A and 80% B; 30–35 min, 80% A and 20% B, and 40 min, 0% A and 100% B. The flow rate was 1.0 mL/min and the column temperature was maintained at 30 °C. Absorbance was registered at wavelengths of 210 nm. Triterpenic acids were identified and quantified by the retention time, spectra and response factors of the pure standards.

3.3.5 Tocochromanols and carotenoids extraction and analysis

Tocochromanols and carotenoids were extracted by mild saponification, as described by Panfili et al. (2003) slightly modified. Two grams of sample were saponified with 2 mL methanolic KOH (60%, w/v), 2 mL ethanol (20%, v/v), 1 mL NaCl (0.1% w/v) and 5 mL BHT (0.05%, w/v) in acetone. After a digestion time of 30 min at 60 °C the samples were cooled and 15 mL sodium chloride solution (1%, w/v) was added. The mixture was then extracted twice with 15 mL n-hexane/ethyl acetate (9/1, v/v). The upper layer was dried under nitrogen flux and was re-dissolved in 1 mL ethyl acetate, filtered through a 0.45 μm syringe filter. Quali-quantitative analyses of tocochromanols and carotenoids were carried out according to Fraser et al. (2000) with some modifications, using an Agilent 1100 Series HPLC system equipped with a reverse-phase C30 column (5 μm, 250 Å–4.6 mm) (YMC Inc. Wilmington, NC, USA) at 25 °C. The mobile phases were: methanol (A), 0.2% ammonium acetate aqueous solution/methanol (20/80 v/v) (B), and tert-methyl butyl ether (C). The gradient profile was as follows: 0 min, 95% A and 5% B; 0–12 min, 80% A, 5% B, and 15% C; 12–42 min, 30% A, 5% B, and 65% C; 42–60 min, 30% A, 5% B, and 65% C; 60–62 min, 95% A, and 5% B. The flow rate was 1.0 mL/min and the column temperature was maintained at 25 °C. Absorbance was registered by DAD at wavelengths of 290 nm and 475 nm for tocochromanols and carotenoids, respectively. Tocochromanols and carotenoids were identified and quantified by the retention time, spectra and response factors of the pure standards.

3.3.6 Extraction and fatty acids analysis

The lipids were extracted from olive pâtè and pasta using acid hydrolysis (AAAC, 2003). A volume of 2.5 mL HCl (25%, v/v) was added to 0.5 g of each sample. The mixture was incubated at 80 °C for 30 min in a water bath and then rapidly cooled in an ice bath before the addition of 1.5 mL diethyl ether and centrifuged for 7 min at 4000 rpm. The extraction procedure was repeated twice and the organic layers were collected.

One mL of extract was supplemented with 75 µL of methyl tricosanoate (1 mg/mL) as internal standard and dried under nitrogen flux. The residues were re-dissolved in 250 µL $CHCl_3$ and 250 µL acetyl-chloride in methanol (3% v/v) (Instant Methanolic HCl kit-Alltech, Deerfield, Illinois, USA) were added. The samples were heated at 60 °C in a water bath for 30 min. The fatty acid methyl esters (FAMEs) were dried under nitrogen at 40 °C and the residues re-dissolved in 500 µL of hexane and analyzed by GC-MS. The analysis of fatty acids was performed on an Agilent 5977E GC/MS system (Boblingen, Germany) as described by Durante et al. (2016).

3.3.7 Extraction and analysis of free and total phenolic compounds

The phenolic compounds were extracted from semolina, olive pâtè (OP), spaghetti CTRL and spaghetti enriched with 10% OP in two separated fractions: soluble free and bound, following the method reported by Mattilla (2005) without acid hydrolysis. The difference between total and free polyphenols represented the bound phenolic compounds. HPLC-DAD analysis was performed using the Agilent 1260 infinity system, equipped with a 1260 binary pump, 1260 HiP Degasser, 1260 TCC Thermostat, 1260 Diode Array Detector and Agilent Open Lab Chem Station Rev C.01.05 (35) software. The UV–visible absorption chromatogram was detected at 280 nm, 325 nm and 360 nm. The separation was performed by gradient elution on a 4.6 x 250 mm reversed phase Luna C-18 (5 µm) column (Phenomenex Torrance, California, USA). The elution was performed using methanol (eluent A) and water/acetic acid 95:5 (eluent B). The gradient profile was: 85–60% B (0–25 min), 60% B (25–30 min), 60–37% B (30–45 min), 37% B (45–47 min), 37–0% B (47–52 min). The flow rate was 1 mL/min and the injection volume was 25 µL. Phenolics were identified and quantified by the retention time, spectra and response factors of the pure standards.

3.3.8 Pasta characteristics determination

The optimal cooking time (OCT) of pasta and the cooking loss (the amount of solid substance lost into the cooking water), were both evaluated according to the American Association for Clinical Chemistry (AACC) approved method. The swelling index and the water absorption of cooked pasta (grams of water per gram of dry pasta) were determined according to the procedure described by Padalino (2013). Hardness and adhesiveness were determined by a Texture Analyzer (Zwick Roell Group, Ulm-

Germany; model Z010) equipped with a stainless steel cylinder probe (2 cm diameter). The hardness (mean maximum force, N) and the adhesiveness (mean negative area, Nmm) of cooked spaghetti were measured according to the procedure described by Padalino (2013). Six measurements for each sample were performed.

3.3.9 Polyphenols bio-accessibility of enriched pasta

Polyphenols bio-accessibility from enriched pasta was determined using a three-stage simulated digestion including oral, gastric and small intestinal phase, as described previously for bread samples. Aliquots of all supernatant for both control and enriched pasta, were recovered for HPLC analysis.

3.3.10 Statistical analysis

Analytical results were reported as the mean value ± standard deviation of three independent replicate experiments (n = 3). Statistical analysis was based on a one-way ANOVA test. Tukey's post hoc method was applied to establish significant differences between means ($p<0.05$). All statistical comparisons were performed using Sigma Stat version 11.0 software (Systat software Inc., Chicago, IL, USA). Experimental data on pasta characteristics were compared by one-way analysis of variance (ANOVA). A Duncan's multiple range test, with the option of homogeneous groups ($P < 0.05$), was carried out to determine significant differences between samples. STATISTICA 7.1 for Windows (Stat Soft, Inc., Tulsa, OK, USA) was used.

3.4 Bread and pasta with olive pâtè and olive mill waste water

3.4.1 Raw materials

The olive pâtè (OP) was obtained by a local olive mill (Bisceglie, Bari, Italy) from the organic *Cellina* cultivar milled using a Pieralisi Leopard with DMF technology (Multi Phases Decanter). The OP was dried at 35 °C in a dryer (SG600, Namad, Rome, Italy) for 72 h. The dried olive pâtè was reduced in a fine powder (< 500µm) by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) and then stored at 4 °C until further utilization. Olive mill waste water **(**OMWW) came from the *Coratina* cultivar. It is processed with a laboratory-scale system (Permeare s.r.l., Milano, Italy) situated in the

laboratory of ISPA-CNR of Bari (Italy). This system, that utilizes a continuous parallel flow, consists in a series of membranes at different porosity (from 0.1 to 0.005 μm) to give 3 types of permeated fractions: micro- (MF, above 5000 Da), ultra- (UF, from 5000 to 200 Da), and nano-filtrate (NF, below 200 Da) (D'Antuono et al., 2014). The UF fraction, that represents the sample with a better compromise between amount of polyphenols and degree of purification, was stored at 4 °C to be successively used in the food formulation.

3.4.2 Sensory analysis

The samples were submitted to a panel of 10 trained tasters from the packaging laboratory of the University of Foggia in order to evaluate the sensory attributes of examined samples. The bread samples were evaluated for acceptance of seven attributes i.e. color, odor, taste, crust and crumb firmness, presence of large bubbles and overall quality. Before sensory analysis, bread samples were sliced with an electric slicing knife (thickness of 15 mm) (Atlantic; Calenzano, Firenze, Italy) without removing the crust. In the spaghetti case the panelists were asked to indicate color, odor and overall quality of dry uncooked spaghetti. In addition, elasticity, firmness, bulkiness, adhesiveness, taste and overall quality were evaluated on cooked spaghetti. To the aim, a nine-point scale, where 1 corresponded to *extremely unpleasant*, 9 to *extremely pleasant*, and 5 to the *threshold acceptability*, was used to quantify each attribute.

3.4.3 Chemical analyses

To determine total phenols and antioxidant activity, the extraction from bread and spaghetti was performed as described by Biney and Beta (2014) as previously described.

Total phenolic compounds in spaghetti and bread samples were determined by UV-vis spectrophotometer, according to the Folin-Ciocalteu method. The methodology used and the experimental conditions were according to Spinelli et al. (2015). Total phenolic compounds were quantified by a calibration curve previously built (3.12-100 mg/L; R^2 = 0.9988) using standard solution of gallic acid, and the total phenolic content was expressed as mg gallic acid/g of dry weight (dw). Total phenolic compounds were also measured in OMWW and dry OP.

The antioxidant activity was assessed using ABTS assay according to the method of Re et al. (1999). A calibration curve was previously built using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard, at concentrations between 1.56 and 50 mg/L (R^2 = 0.999) and the antioxidant activity was expressed as mg Trolox equivalents for gram of dry weight (dw). The free radical activity was determined by measuring the ferric reducing ability of plasma (FRAP) using a method described by Mohd Salleh and Faraniza (2013) with slight modifications. An amount of 200 µl extracted samples were mixed with 3 mL FRAP reagent (Sigma-Aldrich) in test tubes and undergoes vortex. The samples were incubated in water bath for 30 min at 37 °C and the absorbance was determined against blank at 593 nm. A calibration curve was previously built using aqueous solution of $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) as standard, at concentrations between 12.5 and 600 µM (R^2 = 0.9999) and the antioxidant activity was expressed as µM of ferrous equivalent Fe (III) for gram of dry weight (dw). All tests were carried out in triplicate.

3.4.4 Whole quality index

In order to assess which cereal product, between bread and pasta, is the best food matrix to be fortified with by-products from oil industry, the following whole quality index (WQI) is proposed:

$$WQI = (|NQF - NQC|/NQC) \cdot ((SQF - SQmin) / (SQC - SQmin)) \quad (3)$$

where: NQF is the fortified sample nutritional quality expressed as total phenolic compounds; NQC is the nutritional quality of the control sample (food product without by-products), expressed as total phenolic compounds; SQF is the fortified sample overall quality, SQC is the sensory quality of the control sample (food product without by-products); SQ min is the sensory threshold for product acceptability = 5. The equation is given by the product of two terms: the former one takes into account the nutritional quality of fortified sample (it increases with by-products addition respect to the control sample), the latter one, instead, is related to the sensory quality of the sample (it decreases with the increase of by-products addition).

3.4.5 Statistical analysis

Experimental data were compared by a one-way analysis of variance (ANOVA). Duncan's multiple range test, with the option of homogeneous groups ($p<0.05$), was carried out to determine significant differences between the samples. STATISTICA 7.1 for Windows (Stat Soft, Inc., Tulsa, OK, USA) was used.

3.5 Extract from Olive Pomace

3.5.1 Raw materials

The olive pomace (OPO) of *Coratina* cultivar was milled using a three-phase oil extraction decanter (UVNX X20B-11G, Alfa Laval, Italy) at a local olive mill, Oleificio D'Aries s.r.l. (Lucera, Foggia, Italy). Then, olive pomace was dried in a dryer (SG600, Namad, Rome, Italy) at 35 °C for 48 hr. Finally, the dried reduced in a fine powder by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy).

3.5.2 Supercritical fluid extraction (SFE)

SFEs were carried out in triplicate by a speed SFE-2 supercritical fluid extractor (Applied Separation, Allentown, USA) using the extraction conditions identified by Lafka et al. (2013) for wild olive leaves. In particular, dried olive pomace was subjected to extraction process using carbon dioxide (4.5 purity degree; Sapio, Monza; Italy) as solvent (SC-CO_2) carry out at 40 °C, 35 MPa for 60 min of dynamic phase. The SC-CO_2 flow was fixed at rate of 2 L/min. Furthermore, to improve SFE effectiveness, ethanol was flowed at different concentrations (5, 10, 15 or 20%; v/v). The obtained extracts were filtered through a 0.45μm syringe filter (OlimPeak Filters with Nylon Membrane, Teknokroma Anlítica, SA. Sant Cugat del Vallés, Barcelona, Spain) before chemical analysis.

3.5.3 Pressurized liquid extraction (PLE)

PLEs were performed in triplicate by PLE-1 pressurized liquid extractor (LabService Analytica srl, Anzola Emilia, Bologna, Italy). Extractions were performed using a single cycle mode divided into the following steps: a) sample loading into cell (30 g); b) cell preparation (3 min); c) pressurization and heating (3 min, 10 Mpa and 40 °C); d)

constant pressure (10 min, 10 MPa and 40 °C); e) depressurization (0.1 min); f) flush volume (60%); and finally, g) N_2 purge (3 min). Also in this case, different concentrations of ethanol (0, 5, 10, 15, 20, 40, 60, 80 and 100%; v/v) have been tested. To remove any process carryover, a washing cycle was made between extractions. The resulting extracts were filtered through a 0.45 μm nylon syringe filter before chemical analysis.

3.5.4 Ultrasound assisted extraction (UAE)

UAEs were carry out in an ultrasonic bath, model CP104 (39 kHz, 200 W) produced by C.E.I.A. s.p.a. (Viciomaggio, Arezzo, Italy) using 5 g of OPO and 50 mL of the solvents at the same ethanol/water ratios used for PLE (0-100% of ethanol). Extractions were carried out at 40 °C for 15 min. The experiments were performed in triplicate and the resultant extracts were filtered through a 0.45 μm nylon syringe filter before chemical analysis.

3.5.5 Chemical characterization of the extracts

The total phenol content (TPC), determined with Folin-Ciocalteu assay, was expressed as milligrams of gallic acid equivalents (GAE) per gram of olive pomace (OPO), according to a gallic acid calibration curve (100-3.125 ppm; R^2=0.998). The total flavonoid content (TFC), based on the aluminium trichloride method, was expressed as milligrams of quercetin equivalents (QE) per gram of OPO, according to a quercetin calibration curve (400-6.25 ppm; R^2=0.999). For each sample, the phenol and flavonoid measurements were performed in triplicate according to the method described by Spinelli et al. (2017). The antioxidant activity has been determined in triplicate using two methods ABTS assay and FRAP methods following the methods previously described.

3.5.6 Statistical analysis

Statistica 7.1 for Windows (Stat Soft Inc., Tulsa, USA) was used to identify significant differences ($p<0.05$) among different extracts. In particular, a one-way ANOVA, followed by a post-hoc Fisher's test, was carried out.

3.6 Taralli with extract from olive leaves

3.6.1 Raw materials

The olive leaves (OL) were obtained from leaves of *Coratina* cultivar, washed with water and dried in a dryer (SG600, Namad, Rome, Italy) at low temperature (35 °C for 48 hr). Then, OL was reduced to a fine powder by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) and stored at 4 °C until further utilization. Ultrasound-assisted extraction from OL was conducted in the ultrasonic bath, model CP104 (39 kHz, 200 W) produced by C.E.I.A. s.p.a. (Viciomaggio, Arezzo, Italy) in the same condition used for olive pomace.

3.6.2 Taralli preparation

Taralli were prepared with unleavened dough of flour (250 g), salt (3.75 g), sugar (15 g), extra-virgin olive oil (75 g), instant yeast (4 g) and white wine (75 g), for T-CTRL-W sample. The enriched experimental sample (T-EXT) was prepared using the same ingredients as for the T-CTRL-W sample, with OL extract, in substitution of white wine. Also, a sample with water (T-CTRL-H_2O) in substitution of white wine was prepared. Finally, the taralli were cooked in an electric convention oven (H2810, Hugin, Milan, Italy) at 200 °C for 12 min.

3.6.3 Sensory and Chemical characterization of taralli

Sensory evaluation was designed to measure the degree of liking of samples according to a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely). A panel formed by 10 members of the laboratory performed sensory evaluation of taralli in terms of colour, odour, appearance, taste, friability and overall quality.

3.6.4 Bio-accessibility of enriched taralli

The bio-accessibility of bioactive compounds from enriched taralli was determined using a three-stage simulated digestion including oral, gastric and small intestinal phase, as previously described. Aliquots of intestinal digested, for both control and enriched taralli, were filtered using a 0.45 μm PTFE filter and the amount of polyphenols, flavonoids and antioxidant activity were evaluated.

3.6.5 Statistical analysis

Experimental data were compared by a one-way analysis of variance (ANOVA). Duncan's multiple range test, with the option of homogeneous groups ($p<0.05$), was carried out to determine significant differences among bread samples. STATISTICA 7.1 for Windows (Stat Soft, Inc., Tulsa, OK, USA) was used.

4. RESULTS AND DISCUSSION

4.1 Fish burger with olive pâtè

The work has been organized in three subsequent phases: first the dry olive pâtè has been produced and characterized for total polyphenols, flavonoids content and antioxidant capacity; afterward olive pâtè **(**OP) has been added to fish burger to enhance its quality characteristics; finally, the formulation of OP loaded fish burger has been optimize in order to improve its sensory quality.

4.1.1 Production and characterization of olive pâtè

In order to render olive pâtè shelf stable, it has been dried according to the drying cycle reported beforehand. A low temperature drying cycle was used to preserve olive pâtè quality characteristics. The total phenolic compounds (mg gallic acid/g dw), flavonoids (mg quercitin/g dw) and antioxidant activity (mg Trolox/g dw), measured by ABTS assay and as sequestrating activity on DPPH (Free radical scavenged %), of OP were shown in **Table 1.** The obtained results indicate that the dry olive pâtè has a high phenols content, equal to 31.2 mg acid gallic/g dw, not much lower than that reported by Chiung-Tsu et al. (2015), who studied the total phenolic compounds and antioxidant properties of Chinese olive fruit.

Additionally, flavonoids, which constitute the largest group of plant phenolic accounting for over half of the eight thousand naturally occurring phenolic compounds (Asfaw & Demissew, 1994), were present in high amounts in dry olive pâtè (61.3 mg quercetin/g dw).

Finally, the antioxidant potential of the OP extracts was determined by two methods based on different approaches, ABTS assay and DPPH radical scavenging method, which have been extensively used to determine the antioxidant potential of various plant extracts and natural products. According to Alonso et al. (2002) there is a positive correlation between the antioxidant activity and the total polyphenol content contained in the product. In fact, the dry olive pâtè present a high antioxidant activity (43.33 mg Trolox/g dw) and a sequestrating activity on DPPH (88.19 %) capable to improve the nutritional quality of the products in which it is added as an ingredient.

Table 1 Total phenols, total flavonoids and antioxidant activity of dry OP.

Sample	Total phenols (mg GAE/g dw) ± SD	Total flavonoids (mg QE/g dw) ± SD	Antioxidant activity (mg Trolox/g dw) ± SD	Antioxidant activity DPPH (%) ± SD
OP	31.2 ± 0.3[a]	61.3 ± 0.9[a]	43.33 ± 0.01[a]	88.19 ± 0.01[a]

GAE: gallic acid equivalents; QE: quercetin equivalent; DPPH: 2, 2-diphenyl-1-picrylhydrazyl. The different letters show significant difference between means of triplicate determinations ($P < 0.05$).

4.1.2 Nutritional quality of enriched burger

The total phenolic content, flavonoids and antioxidant activity of the fish burgers enriched with 10 % OP were determined and compared with those of the control (CTRL). The results on both the raw and cooked products are presented in **Table 2.**

The content of total phenols in experimental samples, which was expressed as gallic acid equivalents (GAEs), varied from 0.47 mg GAE/g dw for CTRL sample to 6.2 mg GAE/g dw in the case of DOPF enriched burger, which represent a 13.0 fold increase in the phenols content. It is worth noting that the cooking process slightly increased the phenols amounts (6.6 mg GAE/g dw). This is most probably due to the fact that in this step the bounded compounds become available as reported by Marinelli et al. (2015). In fact, according to Abdel-Aal and Rabalski (2013) the effect of cooking on polyphenols is not always in the same way, but it depends on the type of bioactive compound and type of product. Baking is reported to increase the phenols content slightly (Gelinas & McKinnon, 2006), whilst others have claimed that phenolic compounds are destroyed during baking (Leenhardt et al., 2006). Similarly to phenols, the flavonoids content of OP enriched burger, both raw and cooked, is much higher than the corresponding CTRL sample (10.1 to 0.03 mg QE/g dw). Flavonoids as all phenolics compounds, exhibit a variety of activities including anti-inflammatory, antioxidant, and antiallergenic, in addition, they also reduce the risk of cardiovascular disease and cancer (Srivastava & Gupta, 2007). According to obtained data, dry olive pâtè enrichment of burger leads to a significant increase in the burger antioxidant capacity. In fact, according to both analysis methods used in this investigation, the active sample showed higher antioxidant activity than the CTRL, 0.59 against 6.06 mg Trolox/g burger for ABTS method, and 1.12 against 84.87% for DPPH method. This antioxidant activity turned out to be in agreement with the total phenolic content previously reported. The DPPH method is based on the ability of DPPH radical to

react with hydrogen donor species, such as phenols and flavonoids, present in the extract material (Brand-Williams et al., 1995). According to Shabir et al. (2011), the correlation analyses between the DPPH results, obtained for extracts, and the total phenols and flavonoids contents in the extracts, was positive for both cases, indicating that the antioxidant activity increased with the increasing of the total phenols and flavonoids concentrations.

Table 2 Total phenols, total flavonoids and antioxidant activity of dry olive pâtè and burgers with and without olive pâtè.

Sample	Total phenols (mg GAE/g dw) ± SD	Total flavonoids (mg QE/g dw) ± SD	Antioxidant activity (mg Trolox/g dw) ± SD	Antioxidant activity DPPH (%) ± SD
OP	31.2 ± 0.3^{a}	61.3 ± 0.9^{a}	43.33 ± 0.01^{a}	88.19 ± 0.01^{a}
R-CTRL	0.47 ± 0.01^{b}	0.03 ± 0.03^{b}	0.59 ± 0.02^{b}	1.12 ± 0.01^{b}
C-CTRL	0.51 ± 0.01^{b}	0.05 ± 0.03^{b}	0.61 ± 0.01^{b}	1.37 ± 0.01^{c}
R-B-OP	6.2 ± 0.2^{c}	10.1 ± 0.5^{c}	6.06 ± 0.02^{c}	85.77 ± 0.02^{d}
C-B-OP	6.6 ± 0.2^{d}	10.5 ± 0.3^{c}	5.99 ± 0.01^{d}	84.87 ± 0.01^{e}

GAE: gallic acid equivalents; QE: quercetin equivalent; DPPH: 2, 2-diphenyl-1-picrylhydrazyl. The different letters show significant difference between means of triplicate determinations ($p<0.05$). R-CTRL: burger uncooked without olive pâtè; C-CTRL: burger cooked without olive pâtè; R-B-OP: burger uncooked with olive pâtè. C-B-OP: burger cooked with olive pâtè.

4.1.3 Sensory quality of enriched burger

The sensory properties of the investigated samples wre evaluated by means of a group of ten trained panelists. The results are listed in **Table 3 a, b** for the burgers with and without the addition of dry olive pâtè (uncooked and cooked). As far as the sensory quality of raw burgers is concerned, the addition of OP in fish burger has determined a worsening of the color (4.6) due to the color dark green of olive pâtè. The same trend was found for the texture, enriched burger had a score (5.1) lower than the control sample (7.6). This is probably because the addition of dry olive flour has contributed to make the burger more bulky and less juicy (Spinelli et al., 2015). So the overall quality of the OP enriched burger (4.8) is much lower respect the CTRL (7.5) thus receiving a global score for overall quality under the acceptability threshold. In the case of cooked burgers, the color and the texture (5.8 and 5.5) is decreased compared to the control (6.6 and 7.1). However, the odor and

taste of the enriched burgers (4.5 and 3.2) are the main responsible for product unacceptability. The enriched burger had very bitter and spicy taste, probably due of high content of polyphenols, in particular oleuropein, the characteristic compound of olive oil chain that is responsible for the characteristic bitter taste (Cardinali et al., 2010). The overall quality score of enriched burger was much lower than the acceptability threshold (3.3). Based on sensory data beforehand reported, the final phase was aimed to improve the sensory quality of the 10% OP enriched burger.

Table 3a Sensory characteristics of uncooked samples prepared in the second phase.

Sample	**Uncooked sample**			
	Color	**Odor**	**Texture**	**Overall Quality**
CTRL	7.6 ± 0.4^{b}	7.8 ± 0.3^{b}	7.6 ± 0.5^{b}	7.5 ± 0.3^{b}
BURGER OP	4.6 ± 0.5^{a}	6.6 ± 0.5^{a}	5.1 ± 0.4^{a}	4.8 ± 0.5^{a}

Table 3b Sensory characteristics of uncooked and cooked samples prepared in the second phase.

Sample	**Cooked sample**						
	Color	**Odor**	**Taste**	**Texture**	**Juiciness**	**Tenderness**	**Overall Quality**
CTRL	6.6 ± 0.4^{a}	7.5 ± 0.4^{b}	7.6 ± 0.2^{b}	7.1 ± 0.5^{b}	6.7 ± 0.6^{b}	7.4 ± 0.4^{b}	6.9 ± 0.4^{b}
BURGER OP	5.8 ± 0.5^{a}	4.5 ± 0.6^{a}	3.2 ± 0.5^{a}	5.5 ± 0.4^{a}	5.3 ± 0.6^{a}	6.3 ± 0.4^{a}	3.3 ± 0.6^{a}

^{a,b} Data in columns with different superscripts are significantly different ($P < 0.05$).

4.1.4 Optimization of burger enriched with olive pâtè

Bitter and spicy taste is one of the main problems related to the use of waste oil industry as ingredient in the food. As beforehand reported, to reduce the bitter component of enriched burgers DOPF were pre-treated with either water or milk before to obtain OP-H and OP-H/E. After pre-treatment, it was used as burger ingredient. The fish burger sensory properties are listed in **Table 4 a, b**. As can be seen, a simple hydration of OP with either water or milk, does not contribute to improve the fish burger sensory quality. In particular, the cooked OP-H-H_2O and OP-H-MILK samples have recorded low values of odor and taste, thus receiving a global quality score under the acceptability threshold (3.8 and 4.3). Conversely, the hydration/extraction with either water or milk significantly improved the

sensory quality. As can be inferred from the table, the overall quality of OP-H/E-H_2O (6.7) and OP-H/E-MILK (7.2) samples recorded a score very similar to the CTRL (7.5) sample, for both cooked and uncooked burger. In fact, the hydration/extraction with either water or milk leads to a decrease in the bitter taste related to OP. In particular, the fish burger bitterness attribute of cooked burger markedly decreases when OP-H/E-MILK is used to prepare the burger. This is most probably becouse drained milk retains part of bitter substances present in OP such as polyphenols. This is in agreement with Pripp et al. (2004), who have observed that the presence of milk proteins, sodium caseinate, in emulsion with the extra virgin olive oil attenuates the intensity of perception of bitterness. They indicated that the presence in the oil of an amount comprised between 1-4% of caseinate, results in a reduction of the bitter perception of about 60%. The formation of protein-phenols ties would prevent the interaction between the salivary proteins and the taste receptors to interact with the phenolic molecules, reducing the perception of bitterness (Pripp et al., 2004). These results are confirmed by the analysis made on the drained liquid, either water or milk (data not shown). According to analysis performed on water and milk used for the hydration/extraction process, the compounds responsible of the bitter taste pass in the extracting liquid and so reducing their concentration in the burger.

Table 4a Sensory characteristics of uncooked samples prepared in the finally phase.

Sample	**Uncooked sample**			
	Color	**Odor**	**Texture**	**Overall quality**
CTRL	7.6±0.4^{c}	7.8±0.3^{b}	7.6±0.5^{b}	7.5±0.3^{b}
OP H-H_2O	5.0±0.6^{a}	6.9±0.4^{a}	6.6±0.3^{a}	6.8±0.5^{a}
OP H-MILK	4.8±0.3^{a}	6.7±0.4^{a}	6.5±0.3^{a}	5.2±0.4^{a}
OP H/E-H_2O	6.6±0.4^{b}	7.3±0.4^{a,b}	7.3±0.3^{b}	6.9±0.4^{b}
OP H/E-MILK	6.7±0.5^{b}	7.3±0.5^{a,b}	7.3±0.3^{b}	6.8±0.5^{b}

Table 4b Sensory characteristics of cooked samples prepared in the finally phase.

Sample	Cooked sample						
	Color	**Odor**	**Taste**	**Texture**	**Juiciness**	**Tenderness**	**Overall quality**
CTRL	6.6±0.4^{a}	7.5±0.5^{a}	7.6±0.2^{c}	7.1±0.5^{a}	6.7±0.6^{a}	7.4±0.4^{a}	7.5±0.5^{a}
OP H-H_2O	6.8±0.5^{a}	5.4±0.5^{b}	4.0±0.7^{a}	5.8±0.5^{b}	5.7±0.5^{b}	6.8±0.8^{a}	3.8±0.7^{b}
OP H-MILK	6.9±0.4^{a}	6.0±0.5^{b,c}	4.3±0.3^{a}	6.1±0.2^{b}	5.9±0.2^{b}	7.1±0.4^{a}	4.3±0.3^{b}
OP H/E-H_2O	7.0±0.3^{a}	6.7±0.5^{a,c}	6.8±0.4^{b}	6.8±0.5^{a}	6.8±0.5^{a}	7.4±0.2^{a}	6.8±0.4^{a}
OP H/E-MILK	7.3±0.5^{a}	7.4±0.4^{a}	7.1±0.4^{b,c}	7.4±0.4^{a}	7.4±0.4^{a}	7.4±0.4^{a}	7.2±0.3^{a}

^{a,b} Data in columns with different superscripts are significantly different ($P < 0.05$). CTRL: burger without the addition of OP; OP H-H_2O: burger with OP hydration with water; OP H-MILK: burger with OP hydration with milk; OP H/E-H_2O: burger with OP hydration/extraction with water; OP H/E-MILK: burger with OP hydration/extraction with milk.

As one would expect, although the hydration/extraction of OP improved the sensory aspect of the investigated burger, a lower polyphenols amount and an antioxidant activity was recorded as compared to hydrated samples (**Table 5**). In particular, the hydration/extraction process with milk leads to a greater loss of bioactive compounds. The total phenols varied from 6.5 mg GAE/g dw to 1.9 mg GAE/g dw. It is worth noting that this latter value is 3.6 times higher than the control sample. The results beforehand mentioned are in agreement with the sensory analysis reported in Table 4. Similar results were found by Ye et al. (2013), who studied the interactions between polyphenols and milk proteins. They have reported that casein micelles are able to bind highly polymerized polyphenols. Instead, in the case of the hydration/extraction process with water, a lower loss of polyphenols was observed. The polyphenols content decreased from 6.1 mg GAE/g dw to 4.1 mg GAE/g dw. It is worth noting that this latter value is 8 times greater than the control. Regarding the antioxidant activity, OP-H/E samples have lower value than the OP-H samples, probably because the extraction process involves the pass of bioactive components in the solvent (Liu et al., 2015).

Table 5 Chemical data of burger samples prepared in the finally phase.

Sample	Total phenols (mg GAE/g dw) ± SD	Total flavonoids (mg QE/g dw) ± SD	Antioxidant activity (mg Trolox/g dw) ± SD	Antioxidant activity DPPH (%) ± SD
R-CTRL	0.47 ± 0.01^{a}	0.03 ± 0.03^{a}	0.59 ± 0.02^{b}	1.12 ± 0.01^{a}
C-CTRL	0.51 ± 0.01^{a}	0.05 ± 0.03^{a}	0.61 ± 0.01^{b}	1.37 ± 0.01^{b}
R-OP-H-H_2O	6.1 ± 0.1^{b}	8.9 ± 0.3^{c}	6.20 ± 0.01^{g}	85.53 ± 0.01^{i}
C-OP-H-H_2O	$6,1 \pm 0.2^{b}$	9.3 ± 0.5^{c}	5.77 ± 0.01^{a}	85.73 ± 0.01^{l}
R-OP-H-MILK	5.66 ± 0.08^{g}	8.3 ± 0.5^{f}	5.79 ± 0.01^{a}	82.45 ± 0.01^{h}
C-OP-H-MILK	6.47 ± 0.05^{h}	11.5 ± 0.4^{g}	5.78 ± 0.02^{a}	86.94 ± 0.01^{m}
R-OP-H/E-H_2O	3.7 ± 0.2^{e}	3.4 ± 0.2^{d}	3.39 ± 0.01^{e}	57.97 ± 0.01^{f}
C-OP-H/E-H_2O	4.1 ± 0.3^{f}	5.8 ± 0.5^{e}	3.50 ± 0.01^{f}	73.22 ± 0.02^{g}
R-OP-H/E-MILK	1.61 ± 0.07^{c}	1.07 ± 0.07^{b}	1.63 ± 0.01^{c}	32.46 ± 0.02^{c}
C-OP-H/E-MILK	1.86 ± 0.07^{d}	1.33 ± 0.04^{b}	2.26 ± 0.01^{d}	46.21 ± 0.02^{d}

[a,b] Data in columns with different superscripts are significantly different ($P < 0.05$). R-C-OP-H-H_2O: burger with OP hydration with water uncooked and cooked R-C-OP-H-MILK: burger with OP hydration with milk uncooked and cooked R-C-OP-H/E-H_2O: burger with OP hydrated/ extraction with water uncooked and cooked R-C-OP-H/E-MILK: burger with OP extraction with milk uncooked and cooked.

4.2 Bread with olive pâtè

The impact of olive oil by-products on both sensory and nutritional quality of bread was evaluated. Afterward the enriched bread was subjected to *in vitro* digestion to evaluate the glycemic response and then, the bio-accessibility of polyphenols available for intestinal absorption was also evaluated.

4.2.1 Sensory and nutritional characteristics of bread

The results of sensory test are listed in **Table 6** for bread with and without olive pâtè (OP). Samples of enriched bread were found generally acceptable as the control bread, with scores much higher than the sensory threshold. In particular, slight differences were found in terms of color, due to the purplish color of the olive oil by-products, and in terms of taste, due to the high content of polyphenols. As a fact, olive oil contains in particular tyrosol and oleuropein, phenols with very bitter and spicy taste (Cardinali et al., 2010). The

addition of OP also slightly interfered with the network formation, thus influencing the final bread bubbles that were considered more acceptable in the control samples than in the enriched bread. The literature also confirms that other ingredients added to the main raw materials for bread enrichment generally modify the network formation and destabilize the gas cells, thus causing a lower gas retention (Saccotelli et al., 2017). The addition of chemical leavening agents greatly improved the formation of gas in the dough. Odor, crust and crumb firmness remained similar in both control and fortified bread.

The total phenolic compounds (mg gallic acid/g dw), flavonoids (mg quercitin/g dw) and antioxidant activity (mg Trolox/g dw), measured by ABTS assay are shown in **Table 7**. The obtained results indicate that the dry olive pâtè has high phenols content, equal to 45.09 mg GAE/g dw, capable to improve the nutritional quality of enriched food, as also found in other applications of oil by-products (Cedola et al., 2017). Specifically, the content of total phenols in the experimental samples varied from 0.28 mg GAE/g dw in the CTRL sample to 1.96 mg GAE/g dw in the enriched bread, that means 7 fold increase.

Flavonoids, that constitute the largest group of plant phenolic compounds (Harborne et al., 1999), are present in high amounts in dry olive pâtè (36.11 mg QE/g dw). Therefore, the addition of OP to bread significantly increased also the flavonoid content, which varied from 0.06 mg quercetin/g dw in the CTRL sample to 0.85 mg QE/g dw in the enriched bread. As a consequence of the enrichment by bioactive compounds from oil by-products, a significant antioxidant capacity was also found in the fortified bread, compared to the control sample (1.12 against 0.02 mg Trolox/g bread). The total phenols are considered the main responsible for antioxidant activity; several studies have been carried out to correlate phenolic composition of by-products with their antioxidant properties (Gregoris and Stevanato, 2010; Gawlik-Dziki et al., 2015).

Table 6 Sensory characteristics of bread samples.

Sample	Color	Odor	Taste	Crust firmness	Crumb firmness	Large bubbles	Overall quality
B-CTRL	8.0±0.1^{a}	8.0±0.1^{a}	7.8±0.3^{a}	7.5±0.1^{a}	7.5±0.1^{a}	7.0±0.1^{a}	7.8±0.3^{a}
B-OP	6.6±0.3^{b}	7.8±0.1^{a}	6.8±0.3^{b}	7.5±0.1^{a}	7.5±0.1^{a}	6.7±0.2^{b}	7.0±0.3^{b}

^{a,b} Data in columns with different superscripts are significantly different ($P < 0.05$).
B-CTRL: bread without OP; B-OP: bread with OP.

Table 7 Total phenols, total flavonoids and antioxidant activity of dry olive pâtè (OP) and bread, with and without OP.

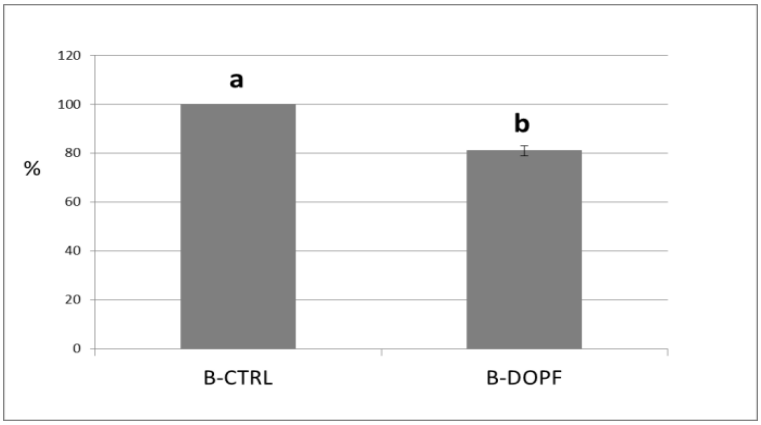
Sample	Total phenols (mg GAE/g dw) ± SD	Total flavonoids (mg QE/g dw) ± SD	Antioxidant activity (mg Trolox/g dw) ± SD
OP	45.09 ± 0.91[a]	36.11 ± 0.71[a]	21.64 ± 0.38[a]
B-CTRL	0.28 ± 0.01[b]	0.06 ± 0.01[b]	0.02 ± 0.01[b]
B-OP	1.96 ± 0.04[c]	0.85 ± 0.03[c]	1.12 ± 0.02[c]

GAE: gallic acid equivalents; QE: quercetin equivalent; [a,c] Data in columns with different superscripts are significantly different (P < 0.05). B-CTRL: bread without OP. B-OP: bread with OP.

4.2.2 Glycemic index

The glycemic index is a ranking parameter for carbohydrate-containing foods, varying from 0 to 100, based on the ratio of the area under the curve (0-180 min), compared to a reference (white wheat bread). Figure 5 highlights that bread sample manufactured with olive pâtè recorded a significant lower glycemic index value compared to the CTRL sample, most probably, because the olive pâtè enriched bread is rich in fibers. Numerous studies have focused on determining the dietary fiber content of olive fruits and their by-products, thus identifying pectins (arabinans, homogalacturonans and rhamnogalacturonans), cellulose, hemicelluloses and lignin as abundant fibers sources (Niaounakis, 2004). Scientific literature also confirms that addition of dietary fibers to food very rich in carbohydrates as bread and pasta can reduce the *in vitro* glycemic response (Vosloo, 2005). In addition, also the increased amount of polyphenols can contribute to the modulation of glucose absorption. In particular, in the OP enriched bread is present, with high probability, cyanidin-3-glucoside, anthocyanin which gives the characteristic purple color to the cultivar *Cellina di Nardò*. This compound has an important effect on the glucose absorption modulation as reported by some authors on the anthocyanic component of blueberry and pomegranate using a Caco-2TC7cells/biosensors telemetric device (Barberis et al., 2017). The results reported by these authors were in agreement with the *in vivo* hypoglycemic effect reported by McDougal and others (2005), thus confirming the inhibitory effect of anthocyanins on alpha-glucosidase activity. Moreover, other authors in diabetic mice, compared the hypoglycemic activity of an anthocyanin-enriched fraction respect to blueberry polyphenols extract, ascribing to the anthocyanins the hypoglycemic activity of blueberry (Grace et al., 2009).

Figure 5 Glycemic index of bread with and without dry olive pâtè addition.



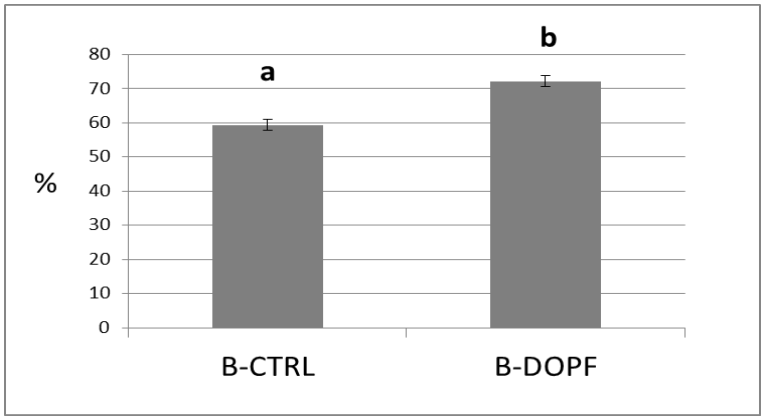
B-CTRL: bread without OP; B-DOPF: bread with OP. *Different letters show significant difference of triplicate determinations (P < 0.05).*

4.2.3 Polyphenol bio-accessibility

Figure 6 reports the percentage of bio-accessibility of total polyphenols after the *in vitro* digestion process. The bio-accessibility represents the polyphenolic fraction stable to the gastro-intestinal conditions and potentially available for the intestinal absorption. From the figure is noteworthy to underline that the total polyphenols of the enriched bread are stable under gastric and small-intestinal digestive conditions respect to the small phenolic components of the control, thus highlighting the bio-accessibility of olive oil by-products compounds. This high stability could be also confirmed by the presence of milk proteins used in the production of enriched bread as was discussed by Lamothe and others (2014) on green tea extract. These authors found that the addition of green tea extract to dairy matrices promoted the formation of polyphenol–protein complexes, which significantly improved the polyphenol stability in simulated gastro-intestinal environment and consequently enhanced the antioxidant activity. Several authors have also discussed the influence of the food matrix in the digestion process, highlighting as the food can control the release of bioactive compounds in the gastro-intestinal environment (D'Archivio et al., 2010). As a matter of fact, when verbascoside and isoverbascoside, both polyphenols present in olive mill wastewater, were digested as extracts, their bio-accessibility values were lower than when the matrix (table olive) was present (Cardinali et al., 2011; D'Antuono et al., 2016). The bio-accessibility of the main polyphenols present in olives was already investigated. A study performed on table olives *cv Bella di Cerignola* described the high stability of different identified polyphenol classes (D'Antuono et al.

2016). The authors showed a percentage of bio-accessibility ranging from 7% of the flavonoid luteolin to 99% of tyrosol. In addition, the phenolic characterization of the olive cultivar *Cellina di Nardò* was recently studied (D'Antuono et al., 2018; Durante et al., 2018). The authors reported that the cultivar, together with the main phenolic compounds characteristic of the olive matrix, such as hydroxytyrosol, tyrosol, verbascoside and luteolin, showed the peculiar presence of cyanidin-3-glucoside and -3-rutinoside, both compounds responsible for the red/purple color of the olive pulp. Besides, among the identified polyphenols, hydroxytyrosol and tyrosol were the most bio-accessible (~90% for both), instead cyanidin-3-glucoside showed a lower bio-accessibility level (almost 40%) (D'Antuono et al., 2018). To sum up, the great polyphenols stability recorded in the enriched samples studied in this paper permits to speculate about the possibility to increase the polyphenols intake through the consumption of supplemented bread. Studies are in progress in order to elucidate the polyphenolic composition of the enriched matrix to the aim to understand which specific compound present in the matrix could produce significant biological effects.

Figure 6 Bio-accessibility of polyphenols of bread with and without dry olive pâtè addition. B-CTRL: bread without OP; B-DOPF: bread with OP.



Data represents mean ± standard error of mean for n = 3 digestion experiments. The different letters show significant difference among means of triplicate determinations (P < 0.05).

4.3 Pasta with olive pâtè

The work was organized in three consecutive steps: first, the olive pâtè (OP) flour was produced and characterized; afterwards, it was added to durum wheat semolina dough in different concentrations; finally, transglutaminase was used to improve the sensory quality of OP enriched pasta. In the following paragraphs, the above-mentioned steps will be discussed separately.

4.3.1 Biochemical Composition of Semolina and olive pâtè

The biochemical composition of both OP and durum wheat semolina are reported in **Table 8a,b**. OP and durum semolina showed similar total carotenoids (5.36 and 5.66 μg/g dw, respectively) with lutein as the most abundant one. The quali-quantitative characterization of tocochromanols evidenced a remarkable difference between samples. Olive pâtè was characterized by the exclusive presence of μ-tocopherol (107.17 μg/g dw), the most biologically active form of vitamin E. On the contrary, semolina showed the highest μ-tocotrienol content (11.95 μg/g dw), in agreement with data reported by Laddomada et al. (2015). Olive fruits are rich in triterpenic acids, such as oleanolic and maslinic acids, present in the epicarp, in the endocarp, in the wood shell and in the seeds of olives (Bianchi, 2003). Total triterpenic acids represented one percent weight of olive pâtè analyzed, the concentration of maslinic acid (6.76 mg/g dw) was higher than the oleanolic acid (3.65 mg/g DW). **Table 8a** also reports the fatty acid profile (expressed as relative percentage) of OP and semolina. In the OP, oleic acid was the most abundant fatty acid, contributing for 57.20% to the total, followed by palmitic (21.40%) and linoleic (12.50%) acids. In the semolina, palmitic (43.93%) and linoleic acids (35.20%) were the main saturated and unsaturated fatty acids, respectively, in agreement with Belleggia et al. (2009). The polyphenols composition of OP and semolina is also shown in **Table 8b**. Phenolic acids and flavonoids are present in plant in both free and conjugated/bound forms; while the free polyphenols are extracted using a hydro-alcoholic solution at room temperature, for conjugated and bound compounds an alkaline or acidic hydrolysis treatment is necessary. The OP resulted very rich in free phenolic compounds identified by HPLC-DAD analysis, with a total amount of 2616 μg/g dw. It is interesting to note the presence of several flavonoids; among them, luteolin and quercetin aglycons were the most abundant (533.59 and 308.23 μg/g dw, respectively). Among the phenolic acids, caffeic, vanillic and coumaric acids were detected; these compounds although present at very low

concentrations, are noteworthy for their biological activity (Breinholt et al., 2009). In addition, tyrosol, characteristic compound of olive products, was the most abundant phenols recovered in the OP (936.12 μg/g dw), followed by oleuropein (371.42 μg/g dw). The presence of the latter could be related to the early ripeness degree of olives, as demonstrated by the absence of hydroxytyrosol, hydrolysis product of oleuropein, normally recovered in the ripe olives (Brenes et al., 1998). The fraction of OP not solubilized by the aqueous organic solvent (non-extractable polyphenols) showed that caffeic acid was the major compound produced by alkaline hydrolysis (576.70 μg/g dw) followed by coumaric and ferulic acids. The presence of low molecular weight phenolics after alkalin treatment are in agreement with results also obtained by Arranz et al. (2010), using different plant-derived foods, that demonstrated the bonds break between arabinoxylan and ferulic acid in cell wall plants, with the release of ferulic acid. Further, the total amount of phenol compounds (extractable and non-extractable) recovered was 3249.30 μg/g dw, underlining the high composition of bioactive compounds in the OP. The semolina sample used in this work was also analyzed for the phenolic composition (**Table 8b**). The main amount of polyphenols was recovered as bound form, with the ferulic acid as the most abundant (72.50 μg/g dw), followed by sinapic, coumaric, 4-hydroxybenzoic, syringic, vanillic acids and caffeic derivatives acid. In addition, in the soluble free fraction some flavonoids, such as apigenin derivative, vitexin, luteolin and apigenin, were recognized.

Table 8a Tocochromanols, carotenoids, fatty acids and phenolic composition of olive pâtè (OP) and semolina DryWeight (DW), not detectable.

	OP	Semolina
Tocochromanols (µg/g DW)		
β T3	ND	11.95±0.71
α T	107.17±1.78	ND
Carotenoids (µg/g DW)		
Lutein	4.03±0.27	5.59±0.26
Zeaxanthin	0.19±0.001	0.25±0.004
α-carotene	0.45±0.03	ND
β-carotene	0.69±0.06	0.037±0.001
Triterpenic acids (mg/g DW)		
Maslinic	6.76±0.45	ND
Eonolic	3.65±0.32	ND
Fatty acids (%)		
Myristic C14:0	ND	0.24±0.01
Palmitic C16:0	21.40±2.70	43.93±0.63
Palmitoleic C16:1	1.81±0.21	5.96±0.24
Stearic C18:0	3.25±0.47	5.04±0.15
Cis Oleic C18:1	57.20±0.24	7.01±0.16
Trans oleico C18:1	3.08±0.37	0.82±0.02
Linoleic C18:2	12.50±1.36	35.20±0.50
Linolenic C18:3	0.79±0.06	1.80±0.04

Table 8b Phenolic composition of olive pâtè (OP) and semolina DryWeight (DW), not detectable.

	OP			Semolina		
	Phenols (µg/g DW)					
	Free	NonExtractable	Total	Free	Conjugated+ bound	Total
Caffeic acid	52.01±2.54	576.70±2.83	628.71±5.37	ND	ND	ND
4-Hydroxybenzoic acid	ND	ND	ND	ND	0.94±0.17	0.90±0.20
Vanillic acid	156.13±7.83	ND	156.13±7.83	0.64±0.07	0.60±0.18	1.20±0.30
Syringic acid	ND	ND	ND	ND	0.71±0.24	0.70±0.20
Cumaric acid	117.74±7.09	39.57±2.33	157.31±9.42	0.25±0.01	1.80±0.34	2.00±0.35
Ferulic acid	ND	16.46±2.31	16.46±2.31	0.67±0.04	72.50±8.54	73.17±8.78
Sinapic acid	ND	ND	ND	0.21±0.01	8.50±1.30	8.71±1.31
Caffeic acid derivative	ND	ND	ND	ND	1.41±0.34	1.41±0.30
Apigenin derivate	ND	ND	ND	0.74±0.38	ND	0.74±0.38
Vitexin	ND	ND	ND	2.36±1.21	ND	2.36±1.21
Quercetin 3-O-glucoside	72.90±3.81	ND	72.90±3.81	ND	ND	ND
Glicosylated luteolin derivate	27.31±0.91	ND	27.13±0.91	ND	ND	ND
Quercetin derivate	308.23±34.36	ND	308.23±34.36	ND	ND	ND
Luteolin	532.59±53.91	ND	532.59±53.91	1.07±0.08	ND	1.07±0.08
Apigenin	30.06±3.02	ND	30.06±3.02	0.10±0.02	ND	0.10±0.02
Luteolin derivate	12.24±1.24	ND	12.24±1.24	ND	ND	ND
Tyrosol	932.12±42.13	ND	936.12±42.13	ND	ND	ND
Oleuropein	371.42±25.41	ND	371.42±25.41	ND	ND	ND
Total	**2616.57±182.25**	**632.73±7.47**	**3249.30±189.7**	**6.04±1.84**	**86.50±11.30**	**92.54±13.10**

4.3.2 Sensory quality of spaghetti enriched with olive pâtè

The sensory properties of dry spaghetti investigated in this work are showned in **Table 9a, b**. Data highlight that the overall quality of both uncooked and cooked control samples

(CTRL) was higher than pasta enriched with OP. In fact, the overall quality of these samples decreased as the olive pâtè increased. In particular, poor color (dark green) and break to resistance were found in the uncooked spaghetti with 15% OP. Pasta color is an important parameter for the assessment of pasta quality, and generally, consumers prefer pasta with a bright yellow color (Debbouz et al., 1995). Regarding the cooked samples, the pasta with 15% OP recorded low elasticity and firmness, thus receiving a global score for overall quality slightly under the acceptability threshold (4.42). Most probably, this is due to the inclusion of fibers from olive pâtè that promoted the formation of discontinuities or cracks in the pasta strand, thus weakening its structure (Lonˇcari´c et al., 2014). A weak or discontinuous protein matrix results in a protein network that is too loose and permits greater amount of leaching during starch granule gelatinization, causing increased adhesiveness and bulkiness (Padalino et al., 2013). In fact, the sample enriched with 15% OP recorded the smallest adhesiveness and bulkiness values. In addition, spaghetti enriched with 15% OP had very intense taste and odor that further compromized their quality. Based on the sensory evaluation, spaghetti sample enriched with 10% OP was selected for the subsequent optimization.

Table 9a Sensory characteristics of uncooked dry spaghetti samples.

Sample	Uncooked sample		
	Color	**Resistence to Break**	**Overall quality**
CTRL	7.2±0.3^{a}	7.3±0.3^{a}	7.2±0.3^{a}
10% OP	6.1±0.3^{a}	6.2±0.3^{b}	6.2±0.3^{b}
15% OP	5.4±0.4^{b}	5.2±0.3^{c}	5.3±0.4^{c}

a–c Mean in the same column followed by different superscript letters differ significantly ($p < 0.05$). OP: Olive Pàtè.

Table 9b Sensory characteristics of cooked dry spaghetti samples.

Sample	Cooked sample							
	Color	**Odor**	**Taste**	**Elasticity**	**Firmness**	**Bulkiness**	**Adhesiveness**	**Overall quality**
CTRL	7.6±0.4^{a}	7.23±0.3^{a}	7.0±0.4^{a}	7.1±0.3^{a}	7.2±0.3^{a}	6.1±0.3^{a}	6.2±0.3^{a}	7.2±0.3^{a}
10% OP	6.7±0.3^{b}	7.0±0.3^{a}	6.7±0.4^{a}	5.2±0.3^{b}	6.2±0.3^{b}	5.3±0.3^{b}	5.3±0.3^{b}	5.32±0.3^{b}
15% OP	5.36±0.5^{c}	6.2±0.3^{b}	5.8±0.3^{b}	4.2±0.3^{c}	5.8±0.3^{b}	4.7±0.4^{b}	4.95±0.3^{b}	4.4±0.4^{c}

4.3.3 Effect of transglutaminase on spaghetti enriched with olive pâtè

4.3.3.1 Sensory quality

Sensory properties of dry spaghetti with and without TG are listed in **Table 10a, b**. Results highlighted that the addition of TG significantly improved the sensory quality of pasta. In particular, **Table 10a** shows that the overall quality of 10% OP with 0.6% TG sample recorded an overall quality score verys imilar to the CTRL sample for uncooked spaghetti and **Table 10b** shows the results for uncooked spaghetti. As reported, the overall quality rose with the increase of TG amount, above all forelasticity, firmness, adhesiveness and bulkiness. These results are in agreement with Kang (2014), who also found that TG addition to noodles prevented texture deterioration after cooking, increased hardness and elasticity and decreased stickiness. It is expected that the cross-linking introduced by TG are heat-stable and reduced leaching of starchy materials (Wu et al., 2005). Regardin the color, odor and taste, no significant differences were found among samples. The cooking performances in terms of optimum cooking time, cooking loss, water absorption, swelling index, hardness and adhesiveness are given in **Table 11**. The OCT value of the spaghetti with OP was smaller than the other samples. It is conceivable that a physical disruption of the gluten matrix due to the presence of fibers may have facilitated the penetration of water into the pasta core (Padalino et al., 2013). The cooking loss results suggest that the incorporation of OP prevented the formation of the gluten network and therefore, negatively influenced pasta cooking quality. In fact, while the 10% OP sample recorded the highest cooking loss, the supplementation of spaghetti with TG resulted in a significant decrease of cooking loss, indicating improved spaghetti quality, above all when 0.6% TG was used. Literature data also demonstrated that cooking loss was reduced by TG treatment of noodle dough because starch is better held in the gluten network and reduced solid loss into the boiling water (Kuraisci et al., 2011). The significant decrease in cooking loss of the TG-supplemented spaghetti can be also explained in terms of formation of covalent cross-links catalyzed by TG that reduced the solids released during cooking. Cross-linked proteins might form a network around the starch granules and encapsulate them during cooking and restrict the diffusion of starch. Concerning the swelling index andthewaterabsorption, results showed that the 10% OP samples howed the highest values. Increase of water absorption and swelling index with OPF addition could be explained by the high fiber content addition. Loncˇaric´ (2014) also observed a rise in water absorption for pasta enriched with apple flour with respect to control pasta. The addition of TG caused

a decline of these two parameters. Most probably, the structural change of gluten, due to the TG cross-linkage, increased water-holding capacity and TG had a profound influence on the decrease of water absorption (Gerrard et al., 1998). Wu and Corke (2005) also speculated that the action of TG leads to gluten proteins by the hydrolysis of glutamine residues to glutamic acid. As result, the strong protein network prevents water diffusion into the starch granules, thus limiting the swelling ndex (Sozer et al., 2003). Infact, the 10% OPF sample with 0.6% TG had the lowest swelling index compared to the other samples. No differences among samples were recorded in terms of adhesiveness. From the **Table 11** it can be also inferred that spaghetti loaded with TG showed higher hardness than 10% OP sample, due to the stronger and tighter protein network, also responsible for limiting the excessive water uptake during cooking (Kovacs et al., 2004). The decrease in surface stickiness of spaghetti samples can be attributed to the protein network created throught hecross-linking of gluten protein scatalyzed by the TG that might be responsible for preventing leaching of starchy material to the surface of spaghetti strands, thus also decreasing the stickiness. Kuraishi (2001) also reported that starch granules in semolina dough are better held within the gluten network that is strengthened by the addition of TG and therefore, it would be responsible for the surface less sticky of noodles and for the reduction in bulkiness. These data are in agreement with sensory analysis because the panel test also assessed that OP spaghetti were less adhesive compared to the sample without TG.

Table 10a Sensory characteristics of uncooked dry spaghetti samples than improvement.

Sample	Uncooked sample		
	Color	**Resistence to Break**	**Overall quality**
CTRL	7.2±0.3^{a}	7.3±0.3^{a}	7.2±0.3^{a}
10% OP	6.1±0.3^{b}	6.2±0.3^{c}	6.2±0.3^{b}
10% OP+0.3 TG	6.0±0.3^{b}	6.4±0.4^{b,c}	6.6±0.4^{c}
10% OP+0.6 TG	6.1±0.3^{b}	6.9±0.3^{a,b}	6.8±0.3^{c}

^{a,c}Mean in the same column followed by different superscript letters differ significantly ($p < 0.05$). TG: Transglutaminase.

Table 10b Sensory characteristics of cooked dry spaghetti samples than improvement.

Sample	Cooked sample							
	Color	**Odor**	**Taste**	**Elasticity**	**Firmnes**	**Bulkines**	**Adhesiveness**	**Overall quality**
CTRL	7.6±0.4[a]	7.23±0.3[a]	7.0±0.4[a]	7.1±0.3[a]	7.2±0.3[a]	6.2±0.3[a]	6.1±0.3[a]	7.2±0.3[a]
10% OP	6.7±0.3[b]	7.0±0.3[a]	6.7±0.4[a]	5.2±0.3[b]	6.2±0.3[b]	5.3±0.3[b]	5.3±0.3[b]	5.32±0.3[b]
10% OP+0.3 TG	6.75±0.28[c]	7.0±0.3[b]	6.7±0.4[b]	5.3 ±0.4[c]	6.26±0.3[b]	5.6±0.4[b]	5.4±0.4[b]	5.7±0.4[c]
10% OP+0.6 TG	6.21±0.3[c]	7.0±0.3[b]	6.8±0.3[b]	5.7±0.2[c]	6.3±0.3[b]	6.6±0.3[b]	6.1±0.3[b]	6.6±0.4[c]

[a,c]*Mean in the same column followed by different superscript letters differ significantly ($p < 0.05$). TG: Transglutaminase.*

Table 11 Cooking quality of dry spaghetti studied.

Sample	OCT	Cooking Loss (%)	Swelling Index	Water Adsorption (%)	Adesiveness (Nmm)	Hardness
CTRL	10.30	5.05±0.28[c]	1.86±0.07[a]	141±3.64[a]	0.69±0.06[a]	6.69±0.32[c]
10% OP	9.00	6.20±0.12[a]	1.75±0.05[b]	138±8.78[a,b]	0.78±0.13[a]	7.96±0.23[b]
10% OP+0.3 TG	9.30	5.93±0.23[a,b]	1.62±0.04[c]	128±4.38[b,c]	0.68 ±0.12[a]	8.15±0.42[b]
10% OP+0.6 TG	10.00	5.65±0.14[b]	1.61±0.02[c]	126±2.36[c]	0.62±0.13[a]	9.55±0.71[a]

[a,c]*Mean in the same column followed by different superscript letters differ significantly ($p < 0.05$). TG: Transglutaminase.*

4.3.3.2 Biochemical composition

Considering that the OP spaghetti sample had a good sensory quality score, its biochemical composition, compared to the CTRL, was evaluated (**Table 12a,b**). Results showed an enrichment of α-tocopherol, α- and β-carotene, maslinic and oleanolic acids in OP spaghetti, respect to the CTRL. The ratio of polyunsaturated (PUFA) to saturated fatty acids (SFA) is commonly used to assess the nutritional quality of the lipid fraction of foods (Durante et al., 2016) and according to the current dietary guidance for healthy nutrition, PUFA/SFA ratio above 0.4–0.5 is considered optimal (FAO, 1997). In OP spaghetti the PUFA/SFA ratio resulted higher (1.16) than the CTRL (0.69). The total polyphenols content in dry spaghetti samples increased from 82.39 μg/g DW to 245.08 μg/g dw after 10% OP enrichment (**Table 12b**). Generally, the majority of studies on functional pasta were related to the whole-wheat flours or adding flours with different cereals typologies (barley, oat, rice) (Verardo et al., 2011). Instead, very few works are focused on pasta enriched by polyphenols. Sun-Waterhouse (2013) showed that the addition of elderberry juice in fibre-enriched pasta increased the total extractable polyphenols contents about 60 times. The amount of total free phenolics in the enriched samples, was almost 50 times higher respect to the CTRL (107.61 and 2.3 μg/g dw, respectively), with tyrosol and oleuropein as the most abundant. In addition, it is interesting to underline that the spaghetti preparation process preserved an aliquot of flavonoids, such as apigenin, luteolin and quercetin present in the OP, 15 times higher than CTRL. Further, the OP spaghetti showed the highest amount of phenolic acids, mainly present as conjugated and bound forms, followed by the free form. These results are in agreement with the study of Verardo (2011) on spaghetti enriched with barley coarse fraction. These authors demonstrated that the functional spaghetti had high fiber content and at the same time a high antioxidant activity for the presence of flavan-3-ols and phenolic acids.

Table 12a Tocochromanols, carotenoids and fatty acids of control spaghetti (CTRL) and spaghetti enriched with 10% olive p*àtè* (OP). Different letters indicate diferences between the CTRL and OPF ($p < 0.05$). ND: not detectable.

	Spaghetti CTRL	Spaghetti 10% OP
Tocochromanols (µg/g DW)		
α T	ND	8.63±0.04
Carotenoids (µg/g DW)		
Lutein	4.22±0.95[a]	4.72±0.09[a]
Zeaxanthin	0.18±0.002[a]	0.16±0.02[a]
α-carotene	ND	0.08±0.001
β-carotene	ND	0.27±0.003
Total	**4.40±0.95[a]**	**5.23±0.11[b]**
Triterpenic acids (mg/g DW)		
Maslinic	ND	1.32±0.04
Eonolic	ND	0.54±0.05
Total	**ND**	**1.86±0.07**
Fatty acids (%)		
Myristic C14:0	0.37±0.01[a]	0.12±0.01[b]
Palmitic C16:0	41.73±0.19[a]	23.20±0.32[b]
Palmitoleic C16:1	1.95±0.11[a]	2.31±0.09[b]
Stearic C18:0	7.50±0.44[a]	2.49±0.29[b]
Cis Oleic C18:1	12.60±2.22[a]	38.90±2.41[b]
Trans oleico C18:1	1.48±0.15[a]	2.96±0.20[b]
Linoleic C18:2	32.61±1.46[a]	28.68±2.75[a]
Linolenic C18:3	1.76±0.18[a]	1.34±0.64[a]

Table 12b Phenolic composition of control spaghetti (CTRL) and spaghetti enriched with 10% olive p*àtè* (OP). Different letters indicate differences between the CTRL and OPF ($p < 0.05$). ND: not detectable.

	Phenols (µg/g DW)					
	Free	**Conjugated+bound**	**Total**	**Free**	**Conjugated+bound**	**Total**
Caffeic acid	ND	ND	ND	1.32±0.14	2.25±0.31	3.57±0.45
4-Hydroxybenzoic acid	ND	1.28±0.07[a]	1.28±0.07[a]	ND	2.81±0.16[b]	2.81±0.16[b]
Vanillic acid	0.56±0.04[a]	0.78±0.01[a]	1.34±0.05[a]	7.28±0.46[b]	1.99±0.62[b]	9.27±1.08[b]
Syringic acid	ND	0.75±0.08	0.75±0.08	ND	ND	ND
Cumaric acid	0.17±0.01	1.75±0.01[a]	1.92±0.02[a]	1.22±0.13[b]	23.47±1.90[b]	24.69±2.03[b]
Ferulic acid	0.35±0.02	67.70±0.19[a]	68.05±0.21[a]	ND	60.93±4.28[a]	60.93±4.28[b]
Sinapic acid	ND	5.78±0.55[a]	5.78±0.55[a]	ND	9.79±0.73[b]	9.79±0.73[b]
Caffeic acid derivative	ND	2.05±0.12	2.05±0.12[a]	0.84±0.08	ND	0.84±0.08[b]
Apigenin derivate	0.27±0.02	ND	0.27±0.02	ND	ND	ND
Vitexin	0.78±0.12	ND	0.78±0.12	ND	ND	ND
Quercetin 3-O-glucoside	ND	ND	ND	ND	ND	ND
Quercetin	ND	ND	ND	5.49±0.89	ND	5.49±0.89
Luteolin	0.17±0.08[a]	ND	0.17±0.08[a]	11.46±2.16b	ND	11.46±2.16[b]
Apigenin	ND	ND	ND	1.18±0.19	ND	1.18±0.19
Tyrosol	ND	ND	ND	66.10±2.47	36.23±4.52	102.33±6.99
Oleuropein	ND	ND	ND	12.72±1.17	ND	12.72±1.17
Total	**2.30±0.29[a]**	**80.09±1.03[a]**	**82.39±1.32[a]**	**107.61±7.69[b]**	**137.47±12.52[b]**	**245.08±20.21[b]**

4.3.4 Polyphenols bioaccessibility of enriched pasta

Chemical characterization of cooked and uncooked pasta extracts, before and after in vitro digestion, was carried out by HPLC-DAD analysis. Polyphenols identification was performed using external standards. The same analysis were conducted both OP enriched pasta and CTRL pasta and the results are reported in **Table 13**. The quali-quantitative characterization of the main polyphenols present in the olive by-products evidenced a remarkable difference between CTRL samples. Olive patè enriched pasta (S-OP) was characterized by the exclusive presence of a high quantity of tyrosol (149.25 ug/g dw) and

luteolin (58.35 ug/g dw) in agreement with data reported by Arouri et al. (2009). They reported that the main phenolic alcohols in the studied Chétoui olive oils were hydroxytyrosol and tyrosol, which were derived from the hydrolysis of oleuropein aglycon and ligstroside aglycon, respectively. Even after the cooking process, the tyrosol and luteolin continue to be the compounds in greater quantities (65,65 ug/g dw and 42,34 ug/g dw, respectively). According Le Marchand (2002), luteolin is heat stable and losses due to cooking are relatively low. Also, Daskalaki et al. (2009) had study the contents of tyrosol derivatives and lignans during period of frying of olive oil. They had observed that these compounds seemed stable, after the same period of frying and no statistically significant differences were observed. The OP enriched and CTRL pasta were submitted to an in vitro gastrointestinal digestion model to evaluate the stability and potential bioaccesibility of the phenol components of the OP compared with the control. After gastric digestion, a reduction of about two times less than the starting quantities for most of the compounds analyzed was observed. Suarez et al. (2010) had observed that almost all of the compounds incorporated into the oil in their free form (monomers), was rather unstable during gastric digestion. Garcia-Villalba (2010) and coworkers reported that almost all phenols contained in an olive oil oral administration appeared in urine as metabolites (Ting Wu et al., 2017), possibly due to hepatic metabolism (Ambra et al., 2017), suggesting that they are not absorbed but could have local antioxidant action in the gastrointestinal tract, in accordance with the free radical scavenging capabilities that have been reported both in the fecal matrix and intestinal cells (Ting Wu et al., 2017). Of special interest was the increase in the amount of secoiridoid derivatives after gastric digestion of phenol such as Quercetin and Caffeic acid derivaties. The luteolin and apigenin, the main flavonoids of olive fruits (Bendini et al., 2007), during the digestion process were unstable, resulting not bioaccessible in pasta sample. The low flavonoids stability, as already reported by other authors (Hwang et al., 2011) is probably due to their poor resistance to gastrointestinal acidic conditions and their high hydrophobicity.

Table 13 Chemical profile of uncooked and cooked Ctrl and OP enriched pasta and bio-accessibility of samples.

CRTL PASTA				
	Extract (ug/g D.W.±dw)		Digested (ug/g D.W.±dw)	Bio-accessibility (%)
	Uncooked	**Cooked**		
Vanillic acid	0.59±0.12	0.38±0.14	-	-
Cumaric acid	1.05±1.24	0.73±0.10	0.89±0.11	122
Ferulic acid	1.56±0.37	1.03±0.13	1.04±0.13	101
Caffeic ac. der.	-	-	1.59±0.90	-
TOTAL	3.20±5.68	2.14±0.98	3.52±0.83	164

PASTA with olive pâtè				
	Extract (ug/g D.W.±dw)		Digested (ug/g D.W.±dw)	Bio-accessibility (%)
	Uncooked	**Cooked**		
Tyrosol	149.25±3.50	65.65±2.96	72.18±8.84	110
Caffeic acid	13.92±2.80	7.35±2.01	7.46±0.21	101
Vanillic acid	12.28±0.89	9.96±1.98	9.52±0.17	96
Cumaric acid	17.00±1.23	9.01±2.54	9.56±0.21	106
Ferulic acid	3.54±0.78	2.04±0.97	1.78±0.22	87
Quercetin der.	-	-	6.27±0.91	-
Caffeic ac. der.	-	-	2.19±0.46	-
Quercetin glyc.	12.52±0.54	8.06±1.26	11.70±3.24	145
Luteolin glyc.	3.43±0.23	2.34±0.89	1.28±0.13	55
Oleuropein	37.79±1.18	26.90±1.13	-	-
Quercetin	30.72±2.10	30.87±2.31	-	-
Luteolin	58.35±3.10	42.34±3.11	-	-
Apigenin	2.98±0.98	2.40±0.79	-	-
TOTAL	341.79±5.68	206.92±4.96	121.94±11.40	59

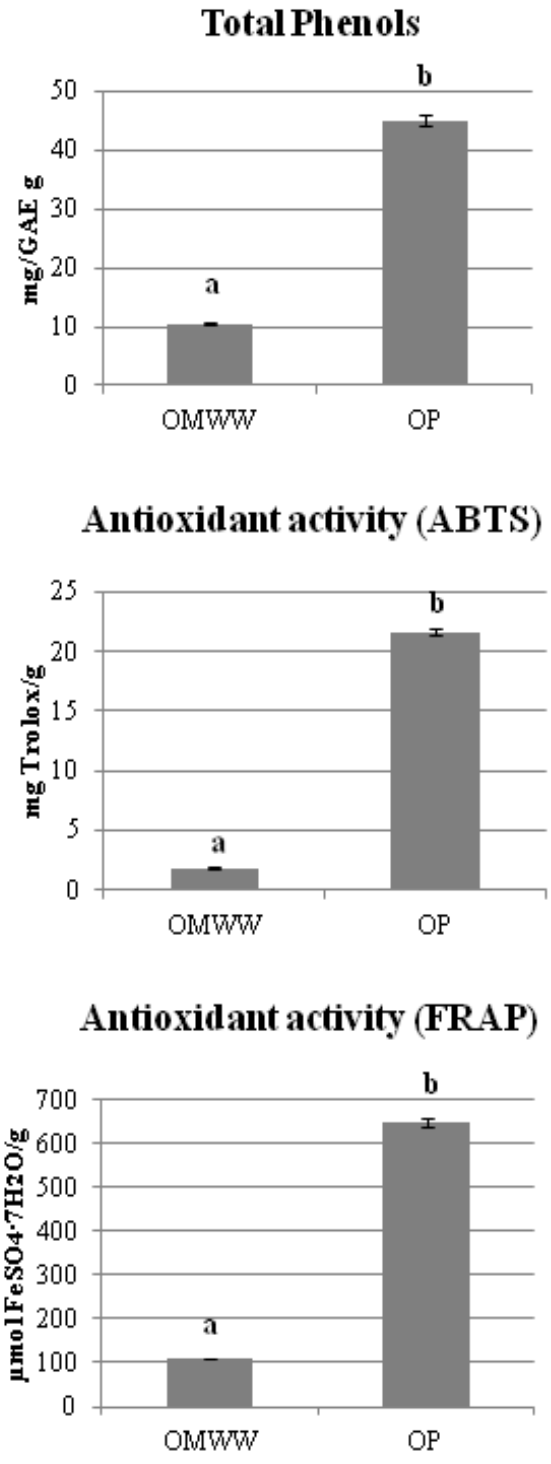
4.4 Bread and pasta with olive pâtè and olive mill waste water

The optimization of cereal-based products enriched with olive oil by-products is proposed as balance between chemical and sensory quality. In particular, olive mill waste water (OMWW) and olive pâtè (OP) were chosen to increase the polyphenols content of bread and pasta. Therefore, these two aspects of food quality will be separately discussed.

4.4.1 Chemical quality

The two by-products (OMWW and OP) used to fortified bread and pasta were found very rich in phenolic compounds (**Figure 7**). It is important to underline that OP phenolic compounds are more than 4 times higher than values of OMWW. Also for the antioxidant activity was observed a similar trend. The OP presented higher values of antioxidant activity respect to OMWW by-product both in terms of ABTS and FRAP methods.

Figure 7 Total phenols, ABTS antioxidant capacity and FRAP antioxidant capacity of OMWW and OP used to fortified bread and pasta.



OMWW: olive mill waste water; OP: olive pâtè.
[a,b] *Data with different superscripts are significantly different ($p < 0.05$).*

Table 14 (first column) shows the theoretical polyphenols content (ThPC) in all the fortified food, obtained from mass balance calculations. These values have to be intended as the polyphenols content that would expect just by mixing wheat flour and olive oil by-products, and they were calculated assuming that the contribute of wheat flour and olive oil by-products in the enriched products was additive and the temperature abuse during processing, was neglected. Furthermore, in case of spaghetti samples, the ThPC was calculated also supposing that the amount of polyphenols leached out during the cooking process was negligible. As one would expect, the ThPC of OMWW, for both bread (B-OMWW) and spaghetti (S-OMWW) was higher than that calculated for OP products (B-OP and S-OP), due to the high amount of OMWW compared to the small quantity of OP adopted in both foods. In fact, the ThPC of B-OMWW is 5.97, about 1.4 times higher than the B-OP (4.25). A similar trend was recorded for spaghetti samples where the ThPC of S-OMWW is about 2.82 times higher than that found in S-OP sample (3.16 for S-OMWW against 1.12 for S-OP). Looking at the second column of the **Table 14** it's worth noting that the real phenolic compounds recovered in the enriched samples (B-OMWW, B-OP, S-OMWW and S-OP) after processing and cooking were considerably lower than those expected (ThPC). They moved from values of 5.97 and 4.25 to 0.49 and 1.33 mg GAE/g in bread samples and from 3.16 and 1.12 to 0.24 and 0.76 mg GAE/g in spaghetti samples. This finding is in agreement with other studies that suggest that phenolic are unstable to heat and this is a reason why the detected polyphenol compounds are lower than predicted (Leenhardt et al., 2006; Visioli et al., 2009). Vogrinčič et al. (2010) also studied the impact of bread making and baking on rutin, quercetin and polyphenol concentrations as well as the antioxidant activity of tartary buckwheat bread, demonstrating a decrease in polyphenols concentrations as a result of baking process. Delgado-Andrade et al. (2010) showed that baking involves thermal and moisture conditions that facilitate the Maillard reaction and, at the same time, the destruction of labile antioxidant compounds. As regards spaghetti, the high temperature reached during drying and cooking process may be the responsible factor for phenolic compounds degradation (Hirawan et al., 2010). Verardo et al. (2011) also studied the effects of pasta-making process and boiling process, demonstrating a decrease by about 53% of total phenolic compounds in the cooked spaghetti, due to the solubility of phenolic compounds in the cooking water. The same behavior was also confirmed in bread and pasta enriched with both types of by-products with values of 1.75 and 0.98 mg GAE/g for B-OMWW-OP and S-OMWW-OP, respectively. Another interesting feature of the recorded results also highlighted in the

second column of the same **Table 14** is the reversed situation between OMWW and OP in the two cereal products. This means that samples with OMWW always present a concentration of phenolic compounds lower than samples enriched with OP, contrary to what measured using the theoretical approach. Abdel-Aal and Rabalski (2013) argued that the decrease in bioactive compounds depends on the type of product, on the recipe and processing conditions but mainly on the type of phenolic compounds. As a fact, the characterization of OMWW and OP underlined a different phenolic composition, being the OP rich in oleuropein and triterpenic acids (oleanolic and maslinic acids) present in the epicarp, endocarp, wood shell and seeds of olives (D'Antuono et al., 2014; Padalino et al., 2018). The antioxidant activity of the cereal products turned out to be in agreement with the total phenolic content (Gregoris and Stevanato, 2010; Swieca et al., 2014). Therefore, the enrichment with only OMWW for both bread and spaghetti did not provoke a significant increase in the antioxidant activity ($p \geq 0.05$), whereas, the OP enrichment considerably improved the antioxidant activity of the two cereal products.

Table 14 Theoretical polyphenols content (ThPC) of enriched products and chemical characteristics of bread and spaghetti samples and olive oil by-products.

Sample	ThPC (mg GAE/ g dm)	Total phenols (mg GAE/g dw) ± SD	Antioxidant activity (mg Trolox/g dw) ± SD	Antioxidant activity (µmol $FeSO_4{\cdot}7H_2O$/g dw) ± SD
B-CTRL	-	0.14 ± 0.01^{a}	$0.046\pm0.003^{a,b}$	1.8 ± 0.1^{a}
B-OMWW	5.97	0.49 ± 0.01^{c}	0.08 ± 0.01^{c}	5.6 ± 0.4^{d}
B-OP	4.25	1.33 ± 0.04^{f}	0.42 ± 0.02^{f}	17.7 ± 0.8^{e}
B-OMWW-OP	10.22	1.75 ± 0.06^{g}	0.67 ± 0.02^{g}	25.3 ± 0.4^{f}
S-CTRL	-	0.11 ± 0.01^{a}	0.043 ± 0.002^{a}	0.68 ± 0.03^{a}
S-OMWW	3.16	0.24 ± 0.02^{b}	$0.071\pm0.002^{b,c}$	2.2 ± 0.1^{c}
S-OP	1.12	0.76 ± 0.04^{d}	0.28 ± 0.01^{d}	13.8 ± 0.5^{b}
S-OMWW-OP	7.67	0.98 ± 0.07^{e}	0.30 ± 0.03^{e}	13.5 ± 0.2^{b}

B-CTRL: bread control; B-OMWW: bread with olive mill waste water; B-OP: bread with olive pâtè; B-OMWW-OP: bread with olive mill waste water and olive pâtè; S-CTRL: Spaghetti control; S-OMWW: spaghetti with olive mill waste water; S-OP: spaghetti with olive pâtè; S-OMWW-OP: spaghetti with olive mill waste water and olive pâtè. ^{a,g} *Data in columns with different superscripts are significantly different ($P < 0.05$).GAE: gallic acid equivalents.*

4.4.2 Sensory Quality

In **Table 15** can be seen that sensory quality of bread decreased with the addition of by-products; however, all the fortified bread samples remained acceptable above the sensory threshold. The enrichment of bread with alternative ingredients to dough generally decreases the sensory quality of bread because alter the network formation and destabilizes the gas cells, causing low gas retention (Hemdane et al., 2015; Saccotelli et al., 2017). This phenomenon was more evident when OMWW was added to the dough (B-OMWW), thus provoking a worsening of crumb firmness and large bubbles. When OP was included in the bread formulation (B-OP), the attributes that mainly affect sensory quality were color and taste, highly compromised by the bitter and spicy taste of OP phenols (Padalino et al., 2018). When both by-products were adopted (B-OMWW-OP) the texture defects (crumb firmness and large bubbles) were more accentuated. Anyhow, the overall quality of all types of fortified breads was perceived by the panelists without significant differences ($p<0.05$) among them. In **Table 16** the sensory characterization of spaghetti with and without by-products is reported. Data highlight that the overall quality of both uncooked and cooked control samples (S-CTRL) was higher than spaghetti supplemented with olive oil by-products. The attributes that mainly compromise spaghetti acceptability were linked to the strength of gluten network, such as elasticity, firmness and bulkiness, as well as those linked to the aesthetical aspect, such as adhesiveness and color. Attributes of odor and taste decreased mostly when both by-products were combined (S-OMWW-OP). In addition, elasticity and firmness were found to be very low in the cooked spaghetti with olive pâtè (S-OP and S-OMWW-OP). Most probably, this was due to the inclusion of fibers from oil by-products that promoted the formation of discontinuities or cracks in the pasta strand, thus weakening its structure (Lončarić et al., 2014). A weak or discontinuous protein matrix results in a protein network that is too loose and permits greater amount of leaching during starch granule gelatinization, causing an increased adhesiveness and bulkiness (Chillo et al., 2011). As a fact, samples enriched with olive pâtè (S-OP and S-OMWW-OP) had the smallest adhesiveness and bulkiness values. Pasta color is essential for assessing pasta quality. Generally, consumers prefer pasta with a bright yellow color (Debbouz et al., 1995). Therefore, the color of the spaghetti produced only with the olive mill waste water (S-OMWW), which is colorless, was not significantly altered, compared to the control sample (S-CTRL). On the contrary, the use of olive pâtè (S-OP and S-OMWW-OP) determined a worsening of color due to the OP dark green color. As

happened for bread, also the various fortified pasta samples were found similar among them, with score values ranged from 6 to 6.94.

Table 15 Sensory characteristics of bread samples.

Sample	Color	Odor	Taste	Crust firmness	Crumb firmness	Large bubbles	Overall quality
B-CTRL	8.13±0.23^{a}	8.19±0.26^{a}	7.94±0.32^{a}	7.88±0.23^{a}	7.75±0.27^{a}	7.81±0.26^{a}	7.81±0.26^{a}
B-OMWW	7.31±0.25^{b}	7.69±0.26^{b}	7.25±0.38^{a,b}	6.81±0.26^{b,c}	5.88±0.23^{c}	6.13±0.44^{b,c}	6.38±0.23^{b}
B-OP	6.50±0.38^{c}	7.75±0.26^{b}	6.75±0.37^{b,c}	7.19±0.26^{b}	6.69±0.26^{b}	6.63±0.23^{c}	6.75±0.27^{b}
B-OMWW-OP	6.75±0.27^{c}	7.33±0.23^{b}	6.92±0.53^{c}	6.38±0.44^{c}	5.63±0.44^{c}	5.75±0.27^{b}	6.31±0.26^{b}

B-CTRL: control bread; B-OMWW: bread with olive mill waste water; B-OP: bread with olive pâtè; B-OMWW-OP: bread with olive mill waste water and olive pâtè. ^{a,c}*Data in columns with different superscripts are significantly different (*$p < 0.05$*).*

Table 16a Sensory characteristics of uncooked spaghetti samples.

Sample	Uncooked sample		
	Color	**Odor**	**Overall quality**
S-CTRL	7.6±0.4[a]	7.1±0.2[a]	7.1±0.2[a]
S-OMWW	7.6±0.2[a]	7.0±0.2[a]	7.0±0.3[a]
S-OP	6.2±0.3[b]	6.8±0.3[a]	6.2±0.3[b]
S-OMWW-OP	6.3±0.3[b]	6.9±0.2[a]	6.3±0.3[b]

Table 16b Sensory characteristics of cooked spaghetti samples.

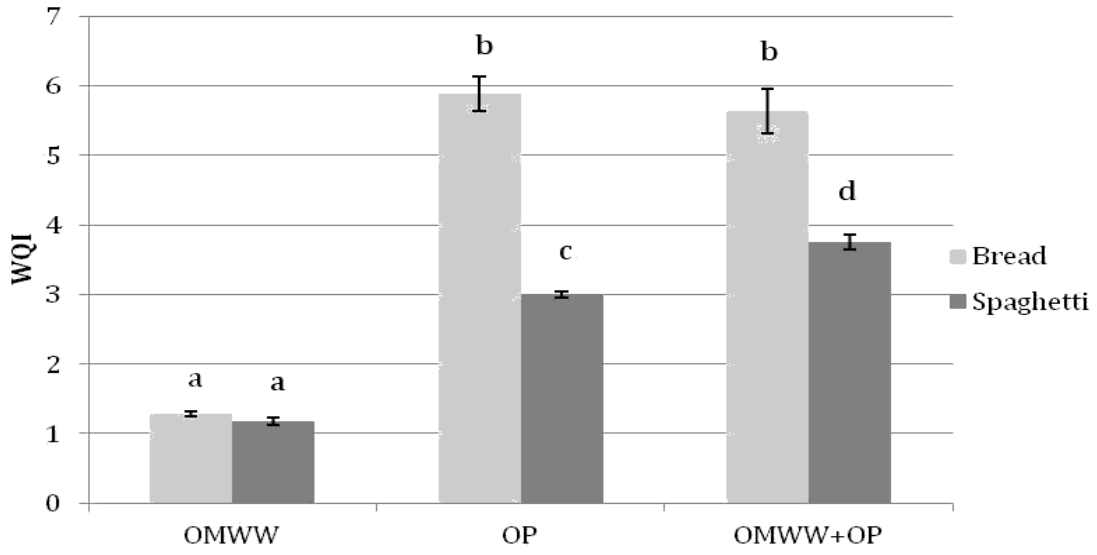
Sample	Cooked sample							
	Color	**Odor**	**Taste**	**Elasticity**	**Firmness**	**Bulkiness**	**Adhesiveness**	**Overall quality**
S-CTRL	7.6±0.3[a]	7.1±0.2[a]	7.1±0.2[a]	7.2±0.4[a]	7.3±0.5[a]	6.3±0.3[a]	6.6±0.2[a]	7.3±0.3[a]
S-OMWW	7.4±0.2[a]	7.1±0.2[a]	7.0±0.3[a]	7.1±0.2[a]	7.1±0.2[a]	6.3±0.3[a]	6.6±0.2[a]	6.9±0.2[a]
S-OP	6.8±0.3[b]	6.8±0.3[a]	6.3±0.3[b]	5.9±0.2[c]	6.2±0.3[b]	5.7±0.3[b]	5.8±0.3[a]	6.3±0.3[b]
S-OMWW-OP	6.6±0.3[b]	6.7±0.4[a]	6.2±0.3[b]	5.3±0.3[b]	6.1±0.2[b]	5.6±0.2[b]	5.6±0.2[a]	6.0±0.3[b]

S-CTRL: control spaghetti; S-OMWW: spaghetti with olive mill waste water; S-OP: spaghetti with olive pâtè; S-OMWW-OP: spaghetti with olive mill waste water and olive pâtè. [a,c]*Data in columns with different superscripts are significantly different* ($p < 0.05$)

4.4.3 Whole quality index

Figure 8 shows the whole quality index (WQI) of investigated bread and spaghetti, as a function of the different by-products used for their fortification, individually or combined. This index is given by the product of two terms: the former takes into account the nutritional quality of fortified sample (it increases with by-products addition respect to the control sample), the latter, is related to the sensory quality of the sample (it decreases with the increase of by-products addition). As can be seen in the figure 2, the WQI for both bread and spaghetti is much lower when OMWW was used to enrichment than the OP. During the spaghetti (mixing, extrusion, drying, cooking) and bread making process (mixing, fermentation, cooking), the most OMWW phenols were destructed because these substances are very labile to high cooking temperature (Abdel-Aal and Rabalski, 2013). In OP enrichment, instead, the WQI was lower for the spaghetti respect to the bread samples. Probably, the boiling process induced the leaching of most OP phenolic compounds in the cooking water, causing a significant loss of nutritional quality in pasta samples. When the two by-products were combined the best product continued to be the bread, with a final WQI not statistically different from the value recorded when the sole OP was used. Therefore, the WQI shows that OP is the best ingredient to enrich cereal products and among them, bread is better than pasta.

Figure 8 Whole Quality Index of Bread and Spaghetti enriched with OMWW and OP individually or combined.



WQI: Whole Quality Index; OMWW: olive mill waste water; OP: olive pâtè. [a,d] *Data with different superscripts are significantly different ($p < 0.05$).*

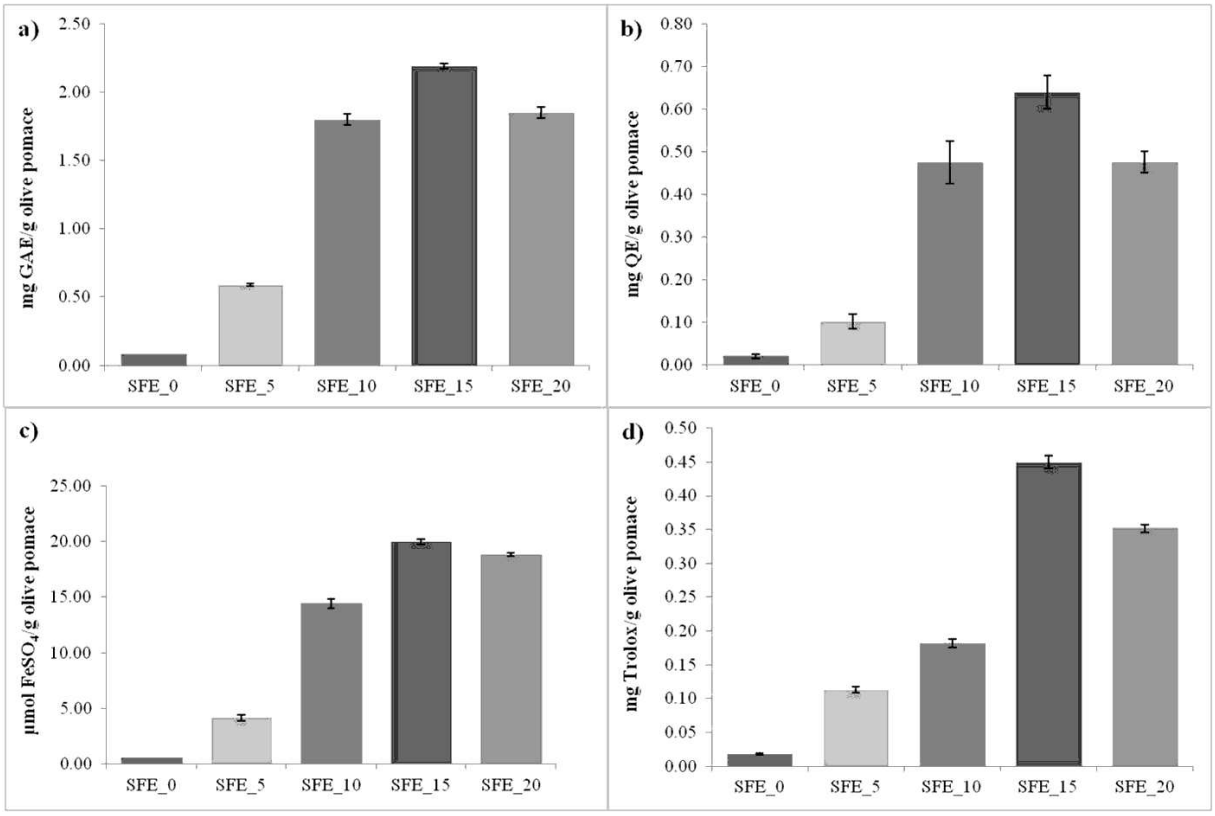
4.5 Olive pomace

To choose the best supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and ultrasonic assisted extraction (UAE) extraction conditions, in terms of solvent concentration, the effect of increased ethanol concentration on total phenolic content, total flavonoid content and antioxidant capacity of olive pomace (OPO) extract was assesed.

4.5.1 Supercritical fluid extraction

According to several studies reported in the literature, SFE optimal conditions to extract phenolic compounds from olive by-products are 40 °C and 35 MPa (Lafka et al., 2011, Lafka et al., 2013). As can be seen from **Figure 9**, TPC, TFC and antioxidant activity of SFE samples have shown the lowest values when only CO_2 was used for extraction. Most probably, this could be explained considering that the OP extracts are rich in polar high molecular weight substances, which appear to have a low solubility in CO_2 (Junior Maróstica *et al,*. 2010). In fact, pure CO_2 at moderate pressures is not capable to extract high molecular weight polar compounds (Singh et al., 2014). Kraujalis et al. (2017) have also observed that SFE-CO_2 of berry pomace with pure CO_2 was not effective for polyphenolic antioxidants extraction, but ethanol addition in SFE-CO_2 was required for achieving better extraction of polyphenols. In accordance to what reported in the literature, to enhance the extraction efficiency of supercritical CO_2, a polar co-solvent such as ethanol was used. Its concentration was increased stepwise until the maximum efficiency was achieved. According to Paes et al. (2014) and Grunovaite et al. (2016) the extraction capacity of ethanol was higher than those of other solvents. As can be seen from **Figure 9**, TPC, TFC and antioxidant activity increased with the addition of the co-solvent, until it reached a maximum value at ethanol concentration of 15% (SFE_15) (2.19 ± 0.02 mg GAE/g of OP, 0.64 ± 0.04 mg QE/g of OP, 19.97 ± 0.24 μmol $FeSO_4$/g of OP and 0.35 ± 0.01 mg Trolox/g OP). Benelli et al. (2010) suggested that adding ethanol provokes a strong effect, probably due to the break in solute/matrix interactions and consequent substitution with co-solvent molecules in solid active sites. Moreover, as argued by Veggi *et al,*. (2014), a solvent can swell the plant cells, which, in turn, favors both solvent penetration and solute counter diffusion out of solid matrix.

Figure 9 Total phenolic content (a), total flavonoid content (b), FRAP antioxidant capacity (c) and ABTS antioxidant capacity (d) using supercritical fluid extraction.



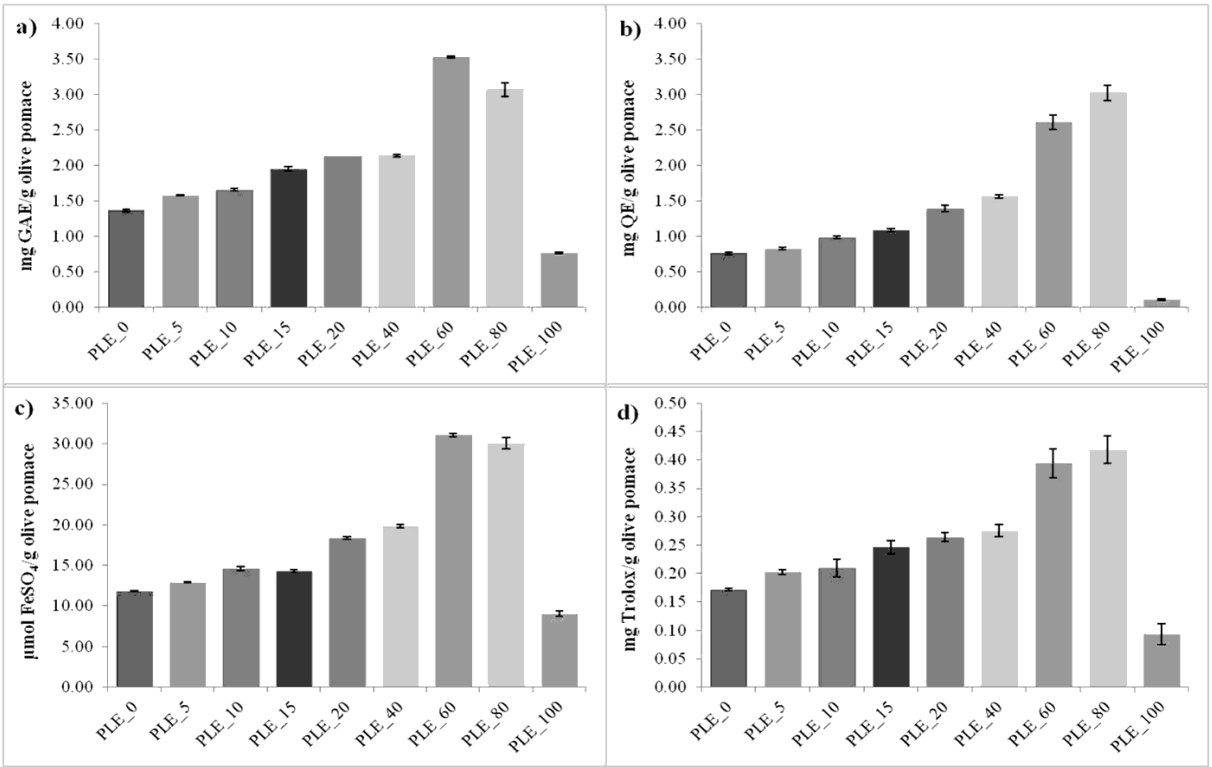
Note: SFE_X where X refers to ethanol concentration. GAE: gallic acid equivalent. QE: quercetin equivalent.

4.5.2 Pressurized liquid extraction

To study PLE efficacy in extracting bioactive compounds from OP, different percentages (0-100%; v/v) of water and ethanol solvents were tested. PLE extraction was carried out at 40 °C and 10 MPa. The data collected are summarized in **Figure 10**. As it can be observed, with the use of pure extraction solvents (100% H_2O=PLE_0 and 100% EtOH=PLE_100) the lowest results in terms of bioactive compound and antioxidant activity were obtained. In particular, the worst result was observed using pure ethanol (PLE_100) (0.76 ± 0.01 mg GAE/g of OP). The extract obtained by using water (PLE_0) showed a significant increase of total polyphenols (1.36 ± 0.02 mg GAE/g of OP) as compared to pure ethanol. Data shown in the figure also highlight that TPC increased as ethanol concentration increased, reaching its highest value at 60% ethanol (3.52 ± 0.02 mg GAE/g of OP). The use of dual mixtures determines an improvement of the extraction efficacy. In fact, as reported by other authors (Mustafa and Turner, 2011), extractions with water-based binary mixtures exploit the ability of water to break the hydrogen bonding between matrix and analytes,

while the organic solvent enhances the solubility of the extracted species. The maximum flavonoid amounts were obtained at an ethanol concentration of 80% (PLE_80) (**Figure 10b**), which is slightly higher than the optimal ethanol concentration reported beforehand. It has been suggest that the solubility of the flavonoids could be modified by changes in ethanol concentration, thus affecting the properties of the solvent (i.e. density, dielectric constant, etc.) (Cacace and Mazza, 2003). The antioxidant capacity of the extracts can be highly correlated to the presence of phenolic compounds (Herrero et al., 2010). As it can be observed in **Figure 10c** and Figure 2d, the extract antioxidant capacity followed the same trend as the amount of total phenols, with a maximum at an ethanol concentration equal to 60% (31.10 ± 0.19 μmol $FeSO_4$/g of OP and 0.39 ± 0.03 mg Trolox/g OP for Frap and $ABTS^+$ methods, respectively). In support of this thesis, also, Corrales et al. (2008) affirmed that the radical activity test $ABTS^+$ defines the scavenging activity of polyphenolic compounds and other substances with antioxidant capacity present in the extracts such as anthocyanin condensate products, aminoacids, vitamins, minerals and synergistic effects among them.

Figure 10 Total phenolic content (a), total flavonoid content (b), FRAP antioxidant capacity (c) and ABTS antioxidant capacity (d) using pressurized liquid extraction.

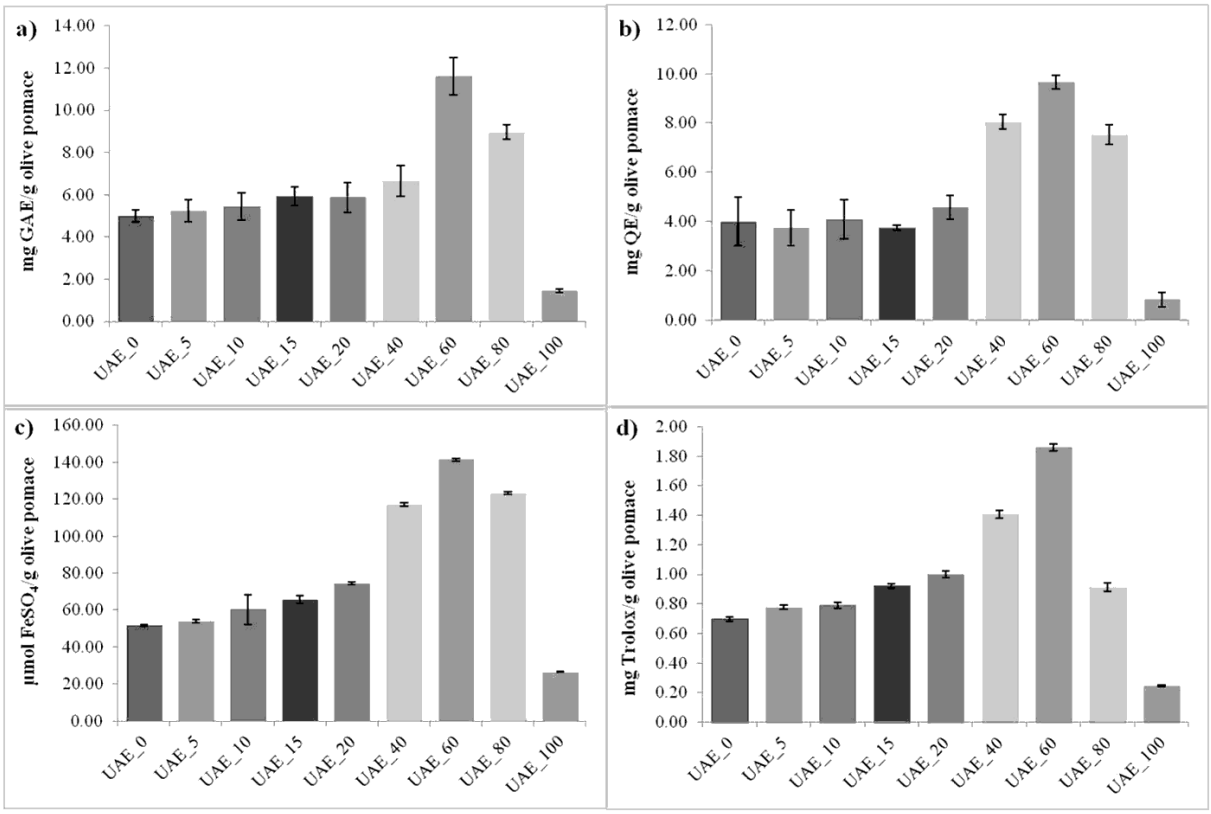


Note: PLE_X where X refers to ethanol concentration. GAE: gallic acid equivalent. QE: quercetin equivalent.

4.5.3 Ultrasonic assisted extraction

The effects of increased ethanol concentration (0-100%) on bioactive compound concentration in extract obtained from UAE was also studied, data are shown in **Figure 11**. The obtained results show a trend comparable to data obtained with the PLE. Specifically, the addition of ethanol to the water based solvent mixture led to an increase of TPC (**Figure 11a**); this was particularly evident when the concentration of ethanol increased from 40 to 60% (from 6.66 ± 0.71 to 11.62 ± 0.89 mg GAE/g of OP, respectively). These results are in accordance with those obtained by Paini et al. (2016), who observed a considerable increase in phenol compounds following an increase in ethanol fraction from 25% to 50% (v/v). This is probably due to the high ethanol concentration that led to a better phenolic compound solubility. A further increase in the ethanol fraction does not correspond to an increase in TPC, in fact elevated ethanol concentration caused a significant decrease in TPC value (8.97 ± 0.33 mg GAE/g of OP). Specifically, with absolute ethanol (UAE_100) the lowest TPC value was reached (1.44 ± 0,08 mg GAE/g of OP). This can be explained by the fact that the higher viscosity of ethanol, with respect to water, can decrease the force of the implosion of the cavitation bubbles (Hemwimol et al., 2006), leading to a less effective extraction process. TFC is closely related to ethanol concentration in the extracting mixture, it follows a trend similar to that of TPC (**Figure 11b**). Accordingly, the maximum value was obtained with 60% of ethanol (UAE_60), equal to 9.66 ± 0.29 mg QE/g of OP. Similarly to TPC results, a consistent decrease in TFC was detected (0.84 ± 0.29 mg QE/g of OP) when pure ethanol was used. Also for the antioxidant activity (**Figures 11c, d**), the addition of increasing amount of ethanol generated statistically significant differences in FRAP and ABTS activities. This findings confirmed that the maximum extraction of bioactive compounds was obtained with 60% of ethanol (UAE_60), with values equal to 141 μmol $FeSO_4$/g of OP and 1.86 mg Trolox/g OP. Antioxidant activity decreased to 26.45 μmol $FeSO_4$ and 0.25 mg Trolox in UAE_100 sample, confirming that a fraction of water is necessary to obtain an optimal phenolic compound extraction from this matrix.

Figure 11 Total phenolic content (a), total flavonoid content (b), FRAP antioxidant capacity (c) and ABTS antioxidant capacity (d) using ultrasonic assisted extraction.



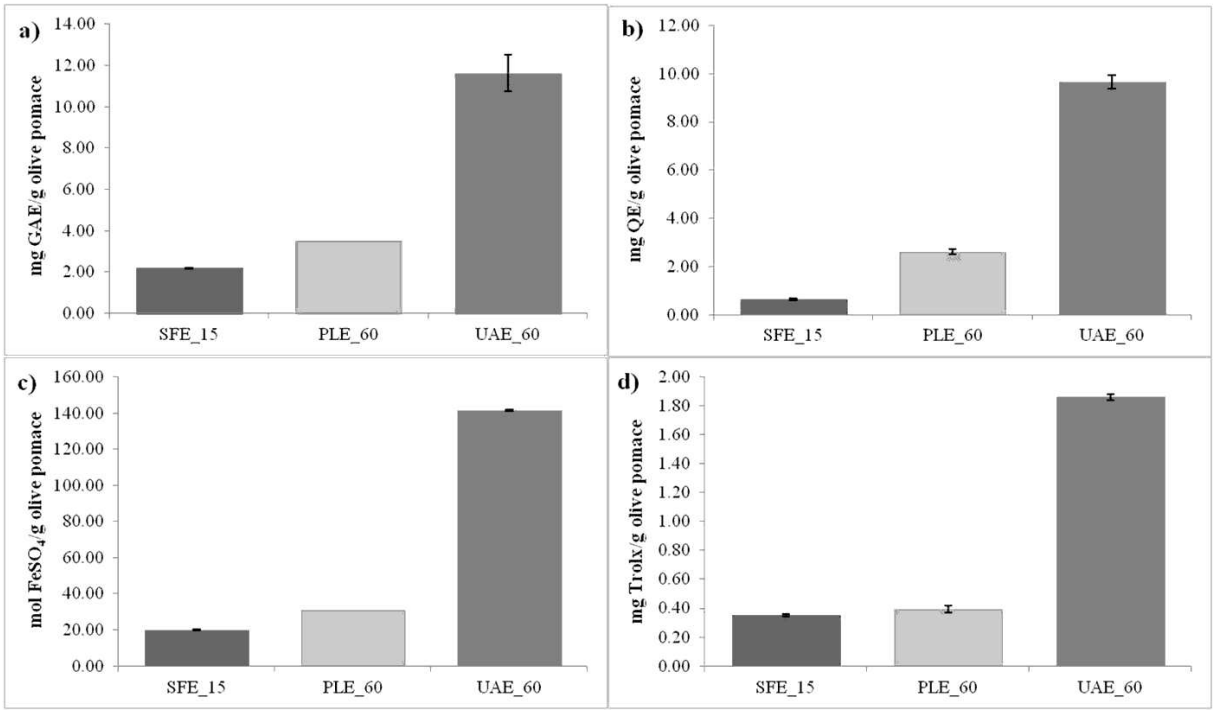
Note: UAE_X where X refers to ethanol concentration. GAE: gallic acid equivalent. QE: quercetin equivalent.

4.5.4 Comparison among SFE, PLE and UAE

Total phenolic, total flavonoid content and antioxidant activity of the extract obtained using the optimal extraction conditions reported beforehand (SFE_15, PLE_60 and UAE_60) were shown and compared in **Figure 12**. As one can observe, a one-way analysis of variance showed that the content of valuable compounds was statistically influenced by the method used for extraction ($p<0.05$). In particular, the results suggested that SFE was not suitable for the extraction of bioactive compounds from olive pomace, this is most probably due to the raw material composition, which could compromise the efficacy of supercritical extraction technology. In general, to improve the extraction power of supercritical CO_2, it is necessary to use polar co-solvents such as ethanol. However, da Costa et al. (1999) argued that sometimes this is still insufficient. Indeed, Esquivel-Hernández et al. (2017) reviewed that SFE is greatly recommended for the extraction of other compounds, such as fatty acids and carotenoids. An increase of 60.73% in TPC,

306.25% in TFC and 55.73% in Frap was observed in PLE_60 extract compared to SFE. Most probably, the application of elevated pressure promoted the penetration of the solvent within the matrix, by increasing mass transfer of the analyte from the sample to the solvent (Mustafa and Turner, 2011, Kadam et al., 2015). Moreover, PLE method was less time-consuming (from 60 to 20 minutes). Using UAE condition (UAE_60), an higher concentration of bioactive compounds was achieved if compared to both SFE and PLE methods. It can be supposed that UAE effectiveness mainly derived from the cavitation phenomenon, which promoting both breakage of cell membrane and increase of solvent-solute contact surface increased the release of intracellular compounds, in this way the extraction of bioactive compounds was improved (Chimsook and Wannalangka, 2015; Lazar et al., 2016). Actually, some authors have already demonstrated the positive conseguence of ultrasounds on the extraction of benefic compounds (Galvan d'Alessandro et al., 2012, Wang et al., 2013, Ghitescu et al., 2015, Cai et al., 2016). In addition, it is worth noting that the ultrasound assisted extraction needed shorter time (15 min) to perform the extraction process than the other extraction techniques investigated in this study. Hence, UAE appeared to be the most suitable technique for obtaining phenols and antioxidants from olive pomace.

Figure 12 Comparison among the best extraction conditions (SFE_15, PLE_60, UAE_60).



4.6 Taralli with extract from olive leaves

Since UAE seemed to be the most suitable technique for extraction of bioactive compounds from olive pomace, this method was used to extract phenolic compounds from olive leaves (OL). Olive leaf extract was added to taralli dough in place of the wine to enhance the nutritional quality of a snach larghely used in Italian country.

4.6.1 Sensory and Chemical Analysis of Enriched Taralli

According to the literature, the OL is a by-product that represents a good source of phenolic compounds (Difonzo et al., 2018). The UAE leaves extract showed a content of polyphenols, determined by Folin-Ciocalteu, equal to 24.08 mg g^{-1} gallic acid equivalents (GAE), total flavonoids content of 33.27 mg QE g^{-1} leaves, and an antioxidant activity, determined by FRAP, accounting for 518 μmol $FeSO_4$ g^{-1}. The recorded rich extract from leaves was added to taralli dough in place of the wine (T- EXT). Both traditional taralli with water (T- CTRL-H_2O) and with wine (T-CTRL-W) were also produced and compared to taralli with extract. As can be seen in the **Table 17**, the overall quality of T-CTRL-W was not statistically different from T-EXT sample (8.25 ± 0.27 respect to 7.88 ± 0.23). Slight differences in sensory parameters were found in terms of color and odor. Specifically, the color of T-EXT sample is rather brown due to the dark color of the extract, whereas, the odor of the T-EXT sample is less persistent than the T-CRTL-W sample.

Table 17 Sensory characteristics of taralli samples.

Sample	Color	Odor	Taste	Appearance	Friability	Overal Quality
T-CTRL-H_2O	8.1±0.2^a	7.3±0.3^a	8.0±0.3^a	7.3±0.3^a	7.3±0.3^a	6.8±0.3^a
T-CTRL-W	8.1±0.2^a	8.4±0.2^b	8.1±0.2^a	8.3±0.3^b	7.9±0.2^b	8.6±0.3^b
T-EXT	7.4±0.2^b	7.8±0.3^c	7.4±0.2^b	7.8±0.3^b	7.4±0.2^a	7.9±0.2^b

T-CTRL-H_2O: taralli control water; T-CTRL-W: taralli control wine; T-EXT: taralli extract.

4.6.2 Bioaccessibility of Enriched Taralli

The **Table 18** reports the TPC, TFC and FRAP antioxidant capacity of taralli samples before and after *in vitro* digestion process. The lowest TPC was determined for T-CTRL-H_2O sample whereas the highest one for taralli with extract addition (0.24 and 0.54 mg GAE/g, respectively). A similar trend was observed for TFC and antioxidant activity. Simulated gastrointestinal digestion caused a tested taralli. So, a significant ($P < 0.05$) increased in the total phenols was observed for all samples after the *in vitro* digestion process. An explanation to this was given by Gawlik-Dziki et al. (2015) who clearly showed that simulated gastrointestinal tract consists of an effective extractor of phenolic compounds from all samples about two times more effective than chemical extraction. This behaviour was different in the TFC case where a decrease of compounds was only observed for control samples. For antioxidant activity values, the digestion samples showed a significant higher antioxidant capacity than their undigested counterparts. Previously, similar trend was obtained in the study of Ahmad-Qqasem et al. (2014) indicating an increase in antioxidant capacity proportionally to the increase in TPC after digestion of gooseberry.

Table 18 Total phenolic content, total flavonoid content and antioxidant capacity of taralli samples before and after digestion.

	Before digestion			**After digestion**		
	Total Phenols (mg GAE/g)	**Total Flavonoids (mg QE/g)**	**FRAP(µmol $FeSO_4 \cdot 7H_2O$/g)**	**Total Phenols (mg GAE/ml digested)**	**Total Flavonoids(mg QE/ml digested)**	**FRAP (µmol $FeSO_4 \cdot 7H_2O$ /ml digested)**
T-CTRL-H2O	0.24±0.01[a]	0.06±0.02[a]	2.15±0.10[a]	1.75±0.12[a]	n.i.	8.81±0.31[a]
T-CTRL-W	0.39±0.02[b]	0.12±0.01[b]	3.48±0.05[b]	2.23±0.07[b]	n.i.	15.14±0.19[b]
T-EXT	0.54±0.04[c]	0.36±0.02[c]	4.86±0.04[c]	3.23±0.17[c]	0.88±0.04[a]	20.98±0.22[c]

T-CTRL-H_2O: taralli control water; T-CTRL-W: taralli control wine; T-EXT: taralli extract.

5. CONCLUSIONS

The impact of olive pâtè (OP) addition on both sensory and nutritional characteristics was assessed for the first time on fish burger. In the first step, OP was produced and characterized for total bioactive composition. The obtained results indicate that OP has a high content of phenols, flavonoids and consequently an high antioxidant activity, capable to improve the nutritional characteristics of the products in which it is added as an ingredient. Subsequently, OP has been added in fish burger at concentration of 10% to enhance its quality characteristics. The OP enrichment considerably improved the quality characteristics of the fish burger sample, but it negatively influenced its sensory quality. In particular, the enriched burger had very bitter and spicy taste; in fact, it was scored under the acceptability threshold. Finally, the formulation of OP loaded fish burger has been optimized in order to improve its sensory quality. To reduce the bitter component of enriched burgers, OP was pre-treated with either water or milk before it was added to burger's formulation. Between the two treatments, milk hydration/extraction, significantly improved the burger sensory quality by reducing the polyphenols concentrations and consequently the characteristic bitter taste and spicy note. Moreover, the polyphenolic enrichment of almost 8 times compared to the control permits to consider the fish burger suitable for a more balanced diet.

In the second study, the impact of OP on sensory and nutritional characteristics of bread was evaluated. In addition, the glycemic response and the bio-accessibility of polyphenols of bread samples were also estimated. The bread fortified with OP 10% recorded high content of phenols and flavonoids and, consequently exhibits a higher antioxidant activity than the control sample. The phenolic content of olive pâtè was found stable under digestive conditions, so the enriched bread reduced the glycemic index. The *in vitro* digestion also confirmed that the phenols are bio-accessible components. From the sensory point of view the fortified bread was acceptable. Therefore, the fortification of bread with OP considerably improved the nutritional quality, without compromising the product acceptability.

The effects of addition of OP on chemical composition, cooking and sensory quality of pasta were also evaluated. The best amount to enrich pasta with olive pâtè corresponds to 10% (w/w) but further technological options needed to be applied to make the product more acceptable. Therefore, 0.6% TG addition increased the overall quality of pasta in

terms of elasticity, firmness, adhesiveness and bulkiness. Additionally, the supplementation of spaghetti with 0.6% TG resulted in a significant decrease of cooking loss, swelling index and water absorption. From the biochemical point of view, the spaghetti enriched with 10% OP and 0.6% TG considerably improved the bioactive components of sample. In particular, the PUFA/SFA ratio resulted higher (1.16) than the control (0.69) and the total polyphenols content in dry spaghetti increased from 82.39 μg/g DW to 245.08 μg/g DW. In addition, it was interesting to underline that the spaghetti preparation process preserved an aliquot of flavonoids that are known to be important for their several biological effects. In particular, the results showed that levels of apigenin, luteolin and quercetin, observed in enriched spaghetti, was 15 times higher than the control. In conclusion, the proper addition of pâtè and TG for pasta enrichment could represent a means to valorize olive oil industrial by-products.

In order to assess which cereal product, between bread and pasta, is the best food matrix to be fortified with by-products from oil industry, in the successive study the Whole Quality Index (WQI) was proposed. In particular, the impact of olive oil by-products addition as olive mill waste water and olive pâtè on both nutritional and sensory characteristics of bread and pasta was evaluated. Results show that the enrichment of bread and pasta with olive mill waste water slightly improved the nutritional quality of samples whereas the olive pâtè enrichment considerably improved the cereal products nutritional quality. The olive pâtè in particular negatively influenced the sensory properties of pasta and bread due to a very bitter and spicy taste. The WQI is higher for bread respect to spaghetti when both by-products were used. The WQI also shows that the pâtè is the best olive oil by-product to be used for bread fortification.

Three different environmental-friendly extraction techniques methods (i.e. SFE, PLE and UAE) were used to obtain bioactive compounds from olive pomace. The investigated extraction methods were optimized in terms of ethanol content, to obtain the highest value of total phenolic and total flavonoid content and antioxidant capacity. Results show that the best values were found in UAE extracts obtained with 60% of ethanol.

Finally, food application of olive leaves extract from ultrasonic assistment extraction for taralli production suggests that the olive oil by-products constitute a valuable supplement also for the development of taralli with enhanced nutritional properties.

In overall terms, olive by-products can be considered a potential source of natural bioactive compounds. The raw materials and the process to extract the active molecules are of relevant importance for maintaining the nutritional properties. Proper food formulation is

necessary to assure the complete acceptance of the final food. The evidence of the stability of polyphenols and active compunds also after digestion confirms the possibility that olive oil by-products can be used in different way to valorize wastes and to realize high value food.

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