

# Enrichment of Bread with Olive Oil Industrial By-Product

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**Abstract:** The aim of the study was to investigate the effect of bread fortification with dry olive paste flour (DOPF), coming from Cellina di Nardò cultivar. Specifically, sensory and nutritional properties of enriched bread were investigated. In addition, the glycemic response and the bio-accessibility of polyphenols contained in the bread samples were also estimated. The addition of DOPF leads to an increase of the total phenolic compounds, total flavonoids and antioxidant activity, without compromising the final acceptability. The recovery of bio-accessible polyphenols from the simulated gastro-intestinal digestion was 59.3% for the control and 72.1% for the enriched bread. Also the glycemic response was improved in the enriched bread.

**Key words:** Antioxidant activity, bio-accessibility, dry olive paste flour, oil by-products, sustainable food.

## Abbreviations

DOPF: dry olive paste flour;  
ABTS: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt);  
PBS: phosphate buffer saline.

## 1. Introduction

In the olive plant fruits there are a high quantity of bioactive compounds that can be recovered and used as valuable substances. From the olive oil extraction process, regardless of the method used, extraction by pressure or centrifugation, three different by-products are formed in considerable amount: olive paste, olive pomace and olive mill waste water. During olive oil processing, most of substances of high benefic effect remain in the oil wastes. So, the bioconversion of these wastes to useful products is receiving increased attention. Nevertheless, the extraction system generates a liquid/solid waste rich in hydroxytyrosol, with a concentration 10-100 fold higher than olive oil [1]. Up to now the emphasis has been focused on detoxifying these wastes prior to disposal, feeding, fertilization/composting, because they are not easily degradable by natural processes, or even used in

combustion as fuel [2]. However, recovery of high value compounds or the utilization of these wastes as raw materials for new products is particularly attractive, being the recovery process of economic and practical interest. This aspect, added to the alternative proposals to diminish the environmental impact, will allow the placement of olive market in high competitive position [3]. At the epidemiological level, plant polyphenols have been suggested to reduce the risk of cardiovascular diseases, neurodegenerative disorders, cancer and diabetes [4]. Fortification with phenolics-rich ingredients is the most commonly used for improving the antioxidant potential of food and the resulting improvement of the consumer antioxidant status [5-9]. Several research groups have been worked on the alternative use of these organic residues and the recovery of valuable substances. Many of them are focused on solvent extraction with methanol, ethanol or ethyl acetate from olive pomace [10], but very scarce applications to food are recorded [11]. Among food, cereals-based products are considered the most interesting vehicle for functional supplements because in developed communities they provide more than 50% of the total energy intake [12].

Therefore, the main aim of this work was to develop a novel enriched bread with dried olive

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pomace, rich in phenolic compounds, to increase its nutritional quality and health promoting properties. Further, in order to give information about the possibility to increase the polyphenols intake through the consumption of enriched bread, the amount of total polyphenols was estimated and their total bio-accessibility, after a simulated gastro-intestinal digestion, was also assessed. Finally, in order to highlight the potential beneficial effects on health, the glycemic index modulation and the antioxidant capacity of the enriched bread were also evaluated.

## 2. Materials and Methods

### 2.1 Raw Materials

The olive paste from the Cellina di Nardò cultivar was obtained by a local olive mill (Lecce, Bari, Italy) using a Pieralisi Leopard with Multi Phases Decanter (DMP) Technology. Leopard is the two-phase decanter that can combine modern extraction technology without the addition of water. It produces a dehydrated husk similar to the one coming from a three-phase decanter; it also recovers a certain quantity of husk (olive paste) made up of wet pulp without any traces of kernel.

The olive paste was dried in a dryer (SG600, Namad, Rome, Italy) at 35 °C for 48 h to reach a moisture of  $7.18 \pm 0.61$  and then reduced to a fine powder by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) to obtain the olive paste flour ( $\leq 500 \mu\text{m}$ ). Wheat flour (WF) for bread production was supplied from Agostini mill (Montefiore dell'Aso, Ascoli Piceno, Italy). Fresh compressed yeast, cream of tartar, sodium bicarbonate, sugar and salt were purchased from a local market. All solvents and the enzymes used for the *in vitro* digestion were obtained from Sigma-Aldrich (Milan, Italy).

### 2.2 Bread-Making Process

Bread dough mixing, processing and baking were carried out with laboratory-scale equipment as described by Saccotelli *et al.* [13]. The DOPF was

added to the dough at 10% (w/w). The control sample (bread without DOPF, B-CTRL) was obtained with a pre-mixture of 1,500 g of WF 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. 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. The enriched experimental sample (bread with DOPF, B-DOPF) was prepared using the same procedure as for the B-CTRL sample, with dry olive paste (150 g), in partial substitution of WF, and in addition to compressed fresh yeast sodium bicarbonate (18 g) and cream of tartar (36 g) were also added. The DOPF was pre-treated with milk as described by Cedola *et al.* [11]. Bread samples were produced three times.

### 2.3 Sensory Analysis

Bread samples were submitted to a panel of six trained tasters (women, aged between 25 and 48) in order to evaluate the bread sensory attributes. The panelists had at least several years of experience in sensory evaluation prior to this study; however, they were retrained for this study in a session of 2 h to be experienced in the product and terminology. 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-point 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.

#### 2.4 Total Phenolic Compounds, Total Flavonoids and ABTS Antioxidant Activity

To determine total phenols, flavonoids and ABTS antioxidant activity, the extraction was performed as described by Biney *et al.* [14] 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. One gram (1 g) of dried sample was mixed with 10 mL of acidified methanol (HCl/H<sub>2</sub>O:MeOH, 20:80). The mixtures were included in 50 mL centrifuge tubes and 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 min at 10,000 rpm (5804R, Eppendorf, Milan, Italy) and supernatant was collected and filtered (polytetrafluoroethylene (PTFE) 0.45 µm) prior to the analytical determinations. Triplicate extractions were made for each sample.

Total phenolic compounds were determined at 740 nm by UV-vis spectrophotometry (UV1800, Shimadzu Italia s.r.l., Milan, Italy) according to Folin-Ciocalteu method [15]. Specifically, 0.5 mL of sample was mixed with 2.5 mL of Folin-Ciocalteu reagent and, after 5 min, 2 mL of NaCO<sub>3</sub> (75 g/L) was added. The sample was kept in darkness at room temperature for 2 h. The total phenols were expressed as milligram of gallic acid equivalents (GAE) per gram of dry weight (dw), according to a calibration curve (3.12-100 mg/L;  $R^2 = 0.9989$ ).

Total flavonoids content was determined by aluminum chloride colorimetric method, according to Chiung-Tsu *et al.* [16]. Samples (0.5 mL) were mixed with 2 mL of distilled water and 150 µL of a 5% sodium nitrite (NaNO<sub>2</sub>) solution. After 6 min, 150 µL

of a 10% aluminum chloride (AlCl<sub>3</sub>) solution was added and the mixture was allowed to stand for 6 min. Finally, 1 mL of 1 mol/L sodium hydroxide (NaOH) was added until the volume was made up to 5 mL with distilled water. The final mixture was filtered through a 0.45 µm nylon filter and measured at 415 nm with a spectrophotometer (UV1800; Shimadzu Italia s.r.l.). The total flavonoids were expressed in milligram of quercetin equivalent (QE) per gram of dw. Quercetin standard solutions were used for constructing the calibration curve (12.5-200 mg/L;  $R^2 = 0.9986$ ). The antioxidant activity was evaluated at 728 nm by UV-vis spectrophotometry (UV1800, Shimadzu Italia s.r.l., Milan, Italy) using the ABTS assay according to the method of Re *et al.* [17]. To the aim, ABTS stock solution (7 mmol/L) and potassium persulfate (140 mmol/L) were utilized. The ABTS radical cation (ABTS<sup>•+</sup>) was obtained by reacting ABTS stock solution with 2.45 mmol/L potassium persulfate and leaving the mixture in the dark at room temperature for 12-16 h. The ABTS<sup>•+</sup> solution was diluted to an absorbance of  $0.700 \pm 0.020$  at 728 nm, with 5 mmol/L phosphate buffered saline (pH 7.4). Then, 300 µL of sample extract was added to 2.2 mL of ABTS<sup>•+</sup> diluted solution and after 6 min at room temperature the mixture was measured. A calibration curve was built using Trolox as standard, at concentrations between 1.56 mg/L and 50 mg/L ( $R^2 = 0.9988$ ) and the antioxidant activity was expressed as milligram Trolox equivalents for gram of dw.

#### 2.5 Glycemic Index

Digestion was carried out as described by Padalino *et al.* [18] with some modifications. Briefly, 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<sub>2</sub> per liter) in an block at 37 °C (GFL 1092; GFL Gesellschaft für Labortechnik, Burgwedel, Germany) and allowed to balance for 15 min. The other phases were performed

following the above-reported method [18] without any modifications. The results were plotted as glucose release (mg) per g of sample vs. time. The glycemic index was calculated as the incremental area under the curve (iAUC) for the tested products divided by the iAUC of a reference food (white bread) [19].

### 2.6 Polyphenol Bio-accessibility by *in Vitro* Digestion

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 *et al.* [20] 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  $\alpha$ -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 at the temperature of 37 °C, the speed of 85 rpm and for a time of 10 min. Samples were then diluted to 30 mL with saline solution (0.9% NaCl). The last two phases (gastric and small intestinal) were performed following the above-reported method [20] without any modifications. 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 10,000× 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  $\mu$ m PTFE filter and analyzed with Folin-Ciocalteu spectrophotometric (UV1800, Shimadzu Italia s.r.l., Milan, Italy) method at 740 nm. The bio-accessibility of phenols was expressed as the bio-accessibility percentage using the following expression:

$$\text{Bio-accessibility (\%)} = (CF/CI) \times 100 \quad (1)$$

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.

### 2.7 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. Results and Discussion

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, then the bio-accessibility of polyphenols available for intestinal absorption was also evaluated.

### 3.1 Sensory and Chemical Characteristics

The results of sensory test are listed in Table 1 for bread with and without olive oil by-products. It is well known that olive oil contains tyrosol and oleuropein, phenols with very bitter and spicy taste [21]. For this reason the addition of olive oil by-products to food is expected to affect the sensory quality. Results of the study demonstrate that in general, the enriched bread recorded acceptable score, much higher than the sensory threshold. Some differences compared to the control were highlighted 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.

**Table 1** Sensory characteristics of bread samples.

Sample	Color	Odor	Taste	Crust firmness	Crumb firmness	Large bubbles	Overall quality
B-CTRL	8.00 ± 0.00 <sup>a</sup>	8.00 ± 0.00 <sup>a</sup>	7.75 ± 0.29 <sup>a</sup>	7.50 ± 0.00 <sup>a</sup>	7.50 ± 0.00 <sup>a</sup>	7.00 ± 0.00 <sup>a</sup>	7.75 ± 0.27 <sup>a</sup>
B-DOPF	6.55 ± 0.27 <sup>b</sup>	7.75 ± 0.27 <sup>a</sup>	6.75 ± 0.27 <sup>b</sup>	7.50 ± 0.00 <sup>a</sup>	7.50 ± 0.00 <sup>a</sup>	6.68 ± 0.24 <sup>b</sup>	7.00 ± 0.32 <sup>b</sup>

B-CTRL: bread without DOPF; B-DOPF: bread with DOPF.

<sup>a, b</sup> Data in columns with different superscripts are significantly different ( $p < 0.05$ ).

The addition of DOPF 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 [22] 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 low gas retention. Saccotelli *et al.* [23] also observed that adding bran to bread formulation compromised the retention of gas and the final loaf volume. However, in the current study, the addition of chemical leavening agents greatly improved the formation of gas in the dough, thus maintaining an acceptable quality. Odor, crust and crumb firmness remained similar in both control and fortified bread.

The total phenolic compounds (mg GAE/g dw), flavonoids (mg QE/g dw) and antioxidant activity (mg Trolox equivalent/g dw), measured by ABTS assay are shown in Table 2. The obtained results indicate that the dry olive paste 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 [11]. Specifically, the content of total phenols in the experimental samples, showed in Table 3, varied from 0.28 mg GAE/g dw in the B-CTRL sample to 1.96 mg GAE/g dw in the enriched bread, that means seven fold increase. Flavonoids, that constitute the largest group of plant phenolic compounds [24], are present in high amounts in dry olive paste (36.11 mg QE/g dw). Therefore, the addition of DOPF to bread significantly increased also the flavonoid content which varied from 0.06 mg QE/g dw in the B-CTRL sample to 0.85 mg QE/g dw in the enriched bread. As reported by

others authors [25] the olive paste is very rich in flavonoid compounds and among them, luteolin and quercetin aglycons are the most abundant. 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 equivalent/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 [26, 27].

### 3.2 Glycemic Index

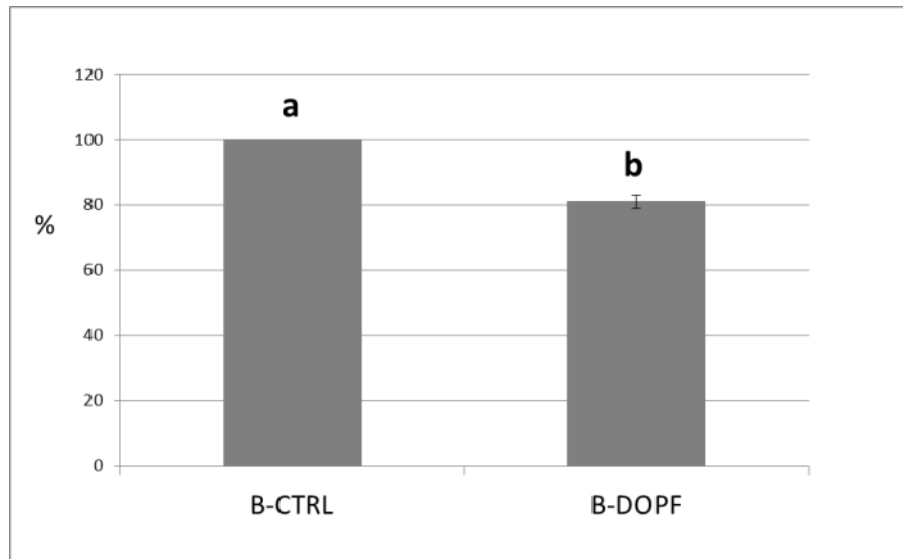
The glycemic index is a ranking parameter for carbohydrate-containing foods, varying from 0 to 100, based on the ratio of the area under the curve (0-180 min), compared to a reference (white wheat bread). Fig. 1 highlights that bread sample manufactured with olive paste recorded a significant lower glycemic index value compared to the B-CTRL sample, most probably because the olive paste 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. Scientific literature [28] also confirms that addition of dietary fibers to food very rich in carbohydrates as bread and pasta can reduce the *in vitro* glycemic response. In addition, also the increased amount of polyphenols can contribute to the modulation of glucose absorption. In particular, in the B-DOPF is present, with high probability, the cyaniding-3-glucoside, the anthocyanin which gives the

**Table 2 Total phenols, total flavonoids and antioxidant activity of wheat flour (WF) and DOPF.**

Sample	Total phenols (mg GAE/g dw) ± SD	Total flavonoids (mg QE/g dw) ± SD	Antioxidant activity (mg Trolox equivalent/g dw) ± SD
WF	0.29 ± 0.01 <sup>a</sup>	0.11 ± 0.02 <sup>a</sup>	0.24 ± 0.02 <sup>a</sup>
DOPF	45.09 ± 0.91 <sup>b</sup>	36.11 ± 0.71 <sup>b</sup>	21.64 ± 0.38 <sup>b</sup>

GAE: gallic acid equivalents; QE: quercetin equivalent.

<sup>a, b</sup> Data in columns with different superscripts are significantly different ( $p < 0.05$ ).



**Fig. 1** Glycemic index of bread with and without dry olive paste addition.

B-CTRL: bread without DOPF; B-DOPF: bread with DOPF.

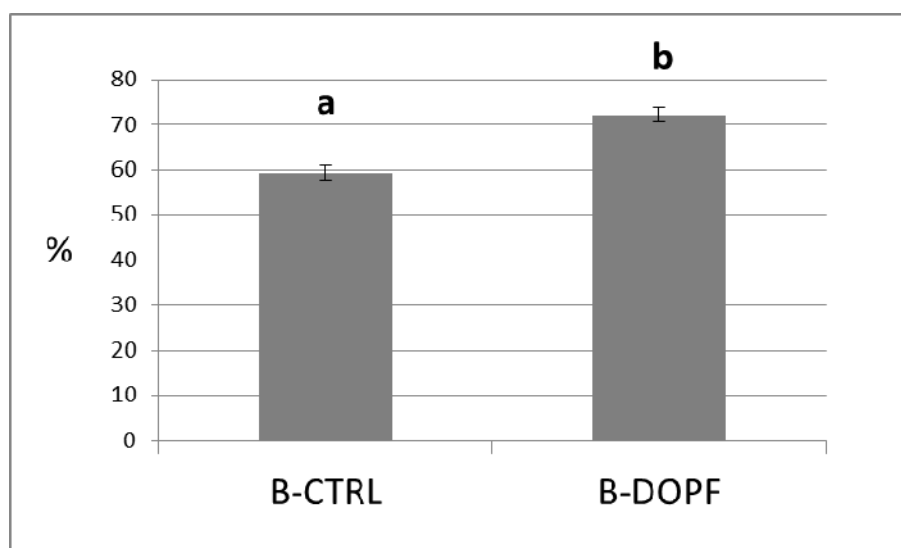
The different letters show significant difference among means of triplicate determinations ( $p < 0.05$ ).

characteristic purple color to the cultivar Cellina di Nardò [29]. 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 [30]. The results reported by these authors were in agreement with the *in vivo* hypoglycemic effect reported by McDougal and Stewart [31], thus confirming the inhibitory effect of anthocyanins on alpha-glucosidase activity. Moreover other authors compared the hypoglycemic activity of an anthocyanin-enriched fraction with respect to blueberry polyphenols extract, ascribing to the anthocyanins the hypoglycemic activity of blueberry [32].

### 3.3 Polyphenol Bio-accessibility

Fig. 2 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 Fig. 2, it is noteworthy to underline that the total polyphenols of the enriched bread are stable under

gastric and small-intestinal digestive conditions with 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 *et al.* [33] 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 [34]. 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 [20, 35]. 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



**Fig. 2 Bio-accessibility of polyphenols of bread with and without dry olive paste addition.**

B-CTRL: bread without DOPF; B-DOPF: bread with DOPF.

Data represent mean  $\pm$  standard error of mean for  $n = 3$  digestion experiments. The different letters show significant difference among means of triplicate determinations ( $p < 0.05$ ).

**Table 3 Total phenols, total flavonoids and antioxidant activity of bread with and without DOPF.**

Sample	Total phenols (mg GAE/g dw) $\pm$ SD	Total flavonoids (mg QE/g dw) $\pm$ SD	Antioxidant activity (mg Trolox equivalent/g dw) $\pm$ SD
B-CTRL	$0.28 \pm 0.01^a$	$0.06 \pm 0.01^a$	$0.02 \pm 0.01^a$
B-DOPF	$1.96 \pm 0.04^b$	$0.85 \pm 0.03^b$	$1.12 \pm 0.02^b$

B-CTRL: bread without DOPF; B-DOPF: bread with DOPF. GAE: gallic acid equivalents; QE: quercetin equivalent.

<sup>a, b</sup> Data in columns with different superscripts are significantly different ( $p < 0.05$ ).

identified polyphenol classes [20]. The authors showed a percentage of bio-accessibility ranging from 7% of the flavonoid luteolin to 99% of tyrosol. Also the phenolic characterization of the olive cultivar Cellina di Nardò was recently studied [36, 37]. 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 cyanidin-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%) [36].

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.

#### 4. Conclusions

In this study the impact of olive oil by-products on both 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 10% of olive paste recorded high content of phenols and flavonoids and, consequently exhibiting a higher antioxidant activity than the control sample. The phenolic content of olive paste was found stable under

digestive conditions, so the enriched bread reduced the glycemic index. The *in vitro* digestion model also confirmed that the phenols are bio-accessible components. From the sensory point of view the fortified bread was acceptable. Therefore, it is possible to conclude that the fortification of DOPF considerably improved the nutritional quality, without compromising the product acceptability. Slight differences between control and enriched bread were found in terms of taste and color but in general enriched bread remained acceptable.

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