Evaluation of enrichment with antioxidants from olive oil mill wastes in hydrophilic model system

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Abstract

Agro-food industry generates significant amounts of by-products, as olive oil mill waste waters, which could be valorise for their extraordinary content of bioactive compounds. The aim of this work was to evaluate the antioxidant stability of a hydrophilic model system with the possibility to create a functional beverage. The study was divided into different steps: extraction of phenolic compounds from olive oil waste waters; formulation of enriched water fortified with phenolic extract (50 and 100 mg tyrosol L^{-1}); evaluation of its physicochemical and antioxidant parameters during storage. Ultra-high performance liquid chromatography was used for the evaluation of single phenols present in the extract and the highest content was observed for tyrosol (12.9 g L^{-1}). The results of this study showed that antioxidant capacity measured by DPPH and ABTS assays is relatively stable during the storage in the samples enriched with lower concentration of phenolic extract (50 mg tyrosol L^{-1}).

Practical Applications

The olive oil industry generates huge quantities of waste, with shown significant amounts of by-products that are discarded and can be a serious environmental problem. This food by-products are an extraordinary source of bioactive compounds, which can be recovered in order to produce valuable metabolites via chemical and biotechnological processes. The study demonstrated that: the Olive Oil Mill Waste Waters have a high concentration of phenolic compounds and antioxidant activity. These antioxidant compounds can be used in the food industry for the production of beverages and/or enriched foods.

Keywords: Antioxidant activity, Enrichment, Olive Oil Mill Waste Water, Phenolic Compounds, Tyrosol

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1. Introduction

The olive oil extraction process involves the production of a considerable amount of Olive Oil Mill Waste Waters (OMWW) which are rich in organic compounds, mainly phenols. In particular, the olive oil contains only 2% of the total phenolic content of the olive fruits, while the residual part is lost in OMWW. These wastes are claimed to be one of the most polluting effluents among those produced by the agro-food industries, for their contents of organic substances (14–15%) and polyphenols (0.3-24 g L^{-1}) (Feki et al., 2006; Servili et al., 2011). Therefore, OMWW are potentially a rich source of phenols with a wide array of biological activities and antioxidant activity (Tafesh et al., 2011, Giuffrè et al., 2012).

The use of phenols extract obtained by OMWW as functional ingredients constitutes a viable alternative to transform an agro-industrial waste into a resource from the economic and environmental points of view. Over the last decade, demand for "healthy" foods and beverages has increased, in particular the market of functional drinks is rapidly growing. Many functional drinks have been developed to provide specific medical or health benefits to the human organism, indeed epidemiological studies have strongly suggested the existence of a correlation between intake of polyphenol-rich foods and low mortality due to coronary heart disease (Zbakh & El Abbassi, 2012). Tyrosol is a well-known phenolic compound that is mainly present in extra-virgin olive oil and has been reported to have scavenging effects, (the optimum intake of phenolic compounds should be 7-9 mg day, about 25-50 mL of olive oil per day (Segura-Carretero & Curiel, 2018; Morató et al., 2015). This phenolic compound exhibits antioxidant, antibacterial, anticancer, anti-depressant, anti-stress, cardio protective, anti-osteoporosis, anti-inflammatory and neural protective effects (Chandramohan et al., 2015; Tafesh et al., 2011). Functional drinks can be divided into different sectors: ready-to-drink essence-flavoured beverages; ready-to-drink beverages containing fruits or fruit juice; beverages ready-to-drink after dilution (Kregiel, 2015). The drinks intended for human consumption are regulated by codes and standards, in the EU, beverages are subject to legislation on microbiological criteria, food additives, and general hygiene requirements for the production, storage, and trade of food products (Ristovska et al., 2012).

The most frequently used approach to recovery phenol from olive fruit, olive tree leaves, olive pomace and OMWW is solvent liquid extraction (Caporaso, Formisano & Genovese, 2017; De Bruno et al., 2018). The presence of various compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent or organic solvent (Do et al., 2014). Among the different extractive solvents, diethyl ether, ethyl acetate and a mixture of chloroform with isopropyl alcohol have shown a high extraction efficiency, according to different studies (Araújo et al., 2015; De Marco et al., 2007). At the end of the extraction procedure, these solvent can be totally eliminated

or present in traces in the phenolic extract for the food enrichment according to the European regulation (EU, 2012).

This study aim to evaluate the antioxidant stability of an hydrophilic model system enriched with a phenolic extract obtained from OMWW. The obtained results can be useful for the knowledge of further operations and production linked to the recovery and valorisation of agricultural food industry wastes.

2. Materials and methods

2.1 Chemicals and substrates

The phenolic compounds, gallic acid (99%) vanillic acid (97%), vanillin (98%), tyrosol (97%), ferulic acid (99%), *p*-coumaric acid (98%) were purchased from Fluka (Germany). Caffeic acid (98%), Apigenin (99%), luteolin (99%) and oleuropein (99%) were acquired from Extrasynthèse (France). Hydroxytyrosol ((3,4-dihydroxyphenyl)ethanol) was acquired from TCI (Japan). The solvents used for chromatographic analysis (methanol, water and acetonitrile) were UHPLC-MS grade (Carlo Erba, Italy). ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin–Ciocalteu's phenol reagent and Trolox were purchased from Sigma Chemical Co. (USA). AAPH (2,2′-azobis (2-amidino-propane) dihydrochloride) and Fluorescein sodium were purchased by Acros Organics (USA) and Panreac (Spain) respectively. For microbiological analysis Plate Count Agar (Oxoid, Milan, Italy), MRS Agar (Oxoid Milan, Italy) and Sabouraud Glucose Agar with Chloramphenicol (VWR International) were used.

2.2 Sampling preparation

The OMWW were obtained during the crop seasons 2016 from Ottobratica olive cultivars and produced according to a 3-phase centrifugation process. The experimental procedure to obtain the phenolic extract and the following enrichment were illustrated in Figure 1.

2.3 Extraction of phenolic compounds from Olive Oil Waste Water

The phenolic extract (PE) was obtained following the method reported by De Marco (2007) with some modifications. Two litres of OMWW were acidified to pH 2 with HCl and washed three times with hexane (1:1, v:v) in order to remove the lipid fraction. The mixture was vigorously shaken and centrifuged under 3000 rpm for 3 minutes at 10 °C. The phenolic compounds were extracted by mean of ethyl acetate for three times in a separating funnel (1:4 v:v) and the combined extracts centrifuged for 5 minutes at 3000 rpm at temperature of 10°C. The organic phase was separated and filtered

through a sintered glass Buchner apparatus. Then the ethyl acetate was evaporated under vacuum using a rotary vacuum evaporator at 25 °C (headspace analysis has been performed). Finally, the dry residue was again dissolved in 100 mL of water, filtered using PTFE 0.45 μm (diameter 15 mm) syringe filter and stored at 4 °C until subsequent analyses.

2.4 Production of Enriched Water (EW)

Production of enriched water (EW) was performed in the laboratory of Food Technologies of the Mediterranean University of Reggio Calabria (Italy). PE containing 50 and 100 mg of Tyrosol L^{-1} were added and homogenized by vortex (Power Mix-Labinco L46, Italy) in mineral waters named respectively EW^{50} and EW^{100}. 50 g of fructose, 10 g of black cherry flavouring and red food colorant were added in enriched waters and then they are stored in aseptically sterile glass bottles (60 mL of capacity) at 4 ºC and at 25 °C. All samples were monitored for physicochemical, microbiological and sensory analyses at 0, 7, 15, 30, 60 days of storage.

2.5 Microbiological, physicochemical and antioxidant analyses of PE and EW

Viable populations of the principal groups of microorganisms were counted by plating in the following selective media: Plate Count Agar (PCA) for total mesophilic bacteria; MRS Agar (LAB) for lactic acid bacteria; Sabouraud Glucose Agar with Chloramphenicol for yeasts and moulds.

The analysis of the colour of PE was performed on 10 mL using a reflection colorimeter (Minolta CR 300, Japan) with reference to e CIE L*a*b* coordinates by using of a D65 illuminant.

pH and titratable acidity (TA) of EW were carried out by the routine methods: pH was measured with a pHmeter (Crison Basic 20, Spain), TA by titration with NaOH 0.01 N and expressed as g 100 mL^{-1} (w/v) citric acid.

The total phenol content was determined spectrophotometrically following the method described by De Bruno (2018) with some modifications. An aliquot portion (0.1 mL) of PE were placed in a 25 mL volumetric flask and mixed with 20 mL of deionized water and 0.625 mL of the Folin-Ciocalteau reagent. After 3 minutes, 2.5 mL of saturated solution of Na_2CO_3 (20%) were added. The content was mixed and diluted to volume with deionized water. Thereafter the mixture was incubated for 12 hours at room temperature and dark. The absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Agilent 8453 UV- Vis, Germany) and compared with a gallic acid calibration curve (concentration between 1 and 10 mg L^{-1} of gallic acid). The results were expressed as g of gallic acid equivalent L^{-1} of PE.

The determination of the total antioxidant activity by DPPH assay was performed using the Brand-Williams (1995) method, which is based on the reaction mechanism between the DPPH and the antioxidants in the samples. 10 μL of diluted PE (1:50) and 100 μL EWs were added to 6 10^{-5} mM of

DPPH solution to achieve a final volume of 3 mL and leaved in the dark for 30 minutes (till stabilization). The decrement of absorbance was determined at 515 nm against methanol using a spectrophotometer (Agilent 8453 UV-Vis, Germany) at 20°C to eliminate the risk of thermal degradation of the tested molecules (Bondet et al., 1997). The radical scavenging activity of the tested samples, reported as percentage of inhibition, was calculated by the following formula:

$$\% \text{ Inhibition} = 100 \cdot \frac{(At0 - Ate)}{At0}$$

Where A_{te} is the value of absorbance measured at the end of reaction while A_{t0} is the value of absorbance at the initial time of reaction.

The properly modified method of Re (1999) was adopted for the determination of antioxidant activity by ABTS assay. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulphate ($K_2S_2O_8$) solution. The mixture was placed at room temperature for 12 hours at dark in order to achieve a stable value of absorbance. The resulting $ABTS^{\cdot+}$ solution was diluted with ethanol to obtain a blue-green chromogen that showed an absorbance of 0.70 (±0.02) at 734 nm.

The reaction mixture was prepared by mixing 50 µL diluted solution (1:100) of PE and 50 µL EWs to achieve a final volume of 3 mL and the absorbance was measured after 6 minutes. The quenching of initial absorbance was plotted against the Trolox concentration (from 1.5 to 24 µM) and obtained results were expressed as TEAC values (mmol Trolox L^{-1} of PE).

The ORAC assay is based up on the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of AAPH. The reactive oxygen species (ROS) generated from this thermal decomposition quenches the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples was assayed according to Suarez (2010), with minor modifications. The ORAC assay was carried out on VICTOR™ X2 2030 Multilabel Plate Readers (PerkinElmer, USA) in 96-well black microplate (PerkinElmer, USA) using a fluorescence filter with an excitation wavelength of 485 nm and emission wavelength of 520 nm. The samples (20 µL of diluted 1:50(v/v) EWs, 20 µL of Trolox, or 20 µL of diluted 1:4000 (v/v) PE) were mixed with 130 µL of fluorescein and 50 µL of AAPH. The fluorescence was measured at 37 °C immediately after the addition of fluorescein (time 0) and measurements of fluorescence kinetic were taken every 1 minute for 30 times until the relative fluorescence intensity was less than 5% of the initial value. The ORAC values were expressed as mmol Trolox L^{-1} of PE.

2.6 Identification and quantification of single phenolic compounds (PC) by UHPLC of PE and EW

Identification and determination of the main bioactive phenolic compounds were performed by ultra-high performance liquid chromatography (UHPLC) following the method described by Becerra-Herrera (2014), with some modifications. The UHPLC system consisted of an UHPLC PLATINblue (Knauer, Germany) equipped with a binary pump system using a Knauer blue orchid column C18 (1.8 µm, 100 x 2mm) coupled with a PDA-1 (Photo Diode Array Detector) PLATINblue (Knauer, Germany). The used software was Clarity 6.2. The samples were filtered with a 0.22 µm nylon syringe filters (diameter 13mm) and then injected in the system with a volume of 5 µL. The mobile phases were: (A) water acidified with acetic acid (pH 3.10) and (B) acetonitrile; the gradient elution program consisted in: 0-3 min, 95% A and 5% B; 3-15 min, 95-60% A and 5-40% B; 15-15.5 min, 60-0% A and 40-100% B; finally, returning to the initial conditions was achieved during analysis keeping the column at 30°C and the injection volume 5 µL. External standards (concentration between 1 and 100 mg L^{-1}) were used for the quantification and the results were expressed as mg L^{-1}.

2.7 Statistical analysis

All experimental results in this study were expressed as mean values ± standard deviation (SD) of three measurements (n=3). The significant differences ($p<0.05$) among treatment means were determined by analysis of variance (Multivariate and ANOVA analysis) with Tukey's *post-hoc* test. SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) was used for data processing. In addition, Pearson's correlation coefficients (r) to determine the relation between two variables were analysed.

3. Results and discussion

3.1 Phenolic Extract (PE) characterization

Ethyl acetate was the solvent used for the extraction from OMWW of phenols: in order to apply extracts as functional ingredients in food matrixes, the solvent had to be evaporated at the end of the extraction and the solutes had to be recovered with water. European Regulation (2012) allows to use ethyl acetate as extraction solvent with a final content not exceeding 50 mg kg^{-1}. OMWW sample showed a pH value close to 5 (data not shown) that is a common characteristic in OMWW, as reported in literature (Chaari et al., 2015). Before the extraction, the OMWW sample was acidified at pH 2 in order to prevent oxidative reactions which can occur at higher pH values. In addition, acidification treatment allows the precipitation of proteins, the release of phenolic compounds bounded by either covalent or non-covalent bonds to polysaccharides and increases the solubility of phenolic compounds in the extraction solvent (Sellami et al., 2016). These effects are confirmed by high value

of TPC showed in table 1 (19.58 g L^{-1}). The obtained value was higher than the data reported in literature. This is most probably due to the different milling procedures from which the OMWW were collected. In our investigation OMWW samples were collected from three-phase system. In the three phase systems most of the phenols are flushed away with the wastewater, only 0.3-1.5% of phenols remained trapped in the oil and about 4-6 % is lost with the pomace (Klen & Vodopivec, 2012). Moreover, different values were found in literature studies for three phase olive wastewaters: De Marco (2007) reported a value of 3481 mg L^{-1} of extract, while other authors detected a range of values between 6110 and 9820 mg L^{-1} of extract for OMWW collected from semi-modern and modern three-phase processes (El-Abbassi et al., 2011). In addition, the differences in total phenolic content can be explained by the impact of geographic and climatic conditions (Piscopo et al., 2016), period of harvest (Piscopo et al., 2018) and olive variety (Dermeche et al., 2013; Aggoun et al., 2016,).

The phenol compounds identified in the PE were phenyl acids (vanillic acid, caffeic acid, *p*-cumaric acid, ferulic acid), phenyl alcohols (hydroxytyrosol and tyrosol), flavonoids (luteolin), verbascoside and oleuropein, but only the principal ones were showed (Fig. 2 and Table 2). The amount of hydroxytyrosol (1.2 g L^{-1}) appeared to be in agreement with the value reported by De Marco (2007) and Fki (2005). High concentrations of tyrosol (12.9 g L^{-1}) and oleuropein (2.1 g L^{-1}) were detected in the extract, according to Aggoun (2016). Several studies reported a lower concentration of oleuropein in extract from OMWW (Lafka et al., 2011; El-Abbassi et al., 2011). The obtained results can be explained considering the chemical structure of these compounds: probably a non- polar interaction occurred between aromatic group of tyrosol and aliphatic chain of solvent. Moreover, the higher concentration of oleuropein respect to the hydroxytyrosol amount indicates that the oleuropein molecule had more polar interactions with ethyl acetate probably due to its glucoside structure (Julio et al., 2018). In addition, oleuropein showed a high solubility in OMWW compared to oil phase and it explains its higher concentration in OMWW obtained by 3-phases centrifugal systems (Aggoun et al., 2016). At the same time, these compounds could be at different concentrations in OMWW, depending of several factors such as cultivar, maturity of the fruit, climatic conditions, storage time, malaxing time and process of milling (Jiménez et al., 2014).

Given the importance of multidimensional evaluation of antioxidant activity, the extract was subject to three antioxidant assays: ABTS, DPPH and ORAC. In accord with the total phenol content and chromatographic results, PE showed the best performance in ABTS test (Table 1) that could be related to high concentration of tyrosol and hydroxytyrosol. A lower value of antioxidant activity was showed by DPPH assay (37% corresponding to about 415 mM Trolox) while ORAC assay showed values of about 1576 mg L^{-1}. Different response for DPPH and ORAC assay was also obtained by Suarez (2009) who affirmed that DPPH assay is not strongly adequate for the determination of antioxidant

activity of complex matrix, because it uses stable radicals instead of peroxyl ones, as it is performed by the ORAC assay (Becker et al., 2004) Even though all three assays are based on the electron transfer from the deprotonated antioxidant to the probe, the different reaction mechanism, the structure of the antioxidant molecule, the type of solvent and the pH of the assay solution have a large influence on the reactivity of antioxidants and, consequently, the obtained results are not comparable (Abramovič et al., 2018).

3.2 Antioxidant Stability of EW during storage

Food safety and quality are important to consumers and they continue to be a basic requirement of any modern food system. For this, in order to evaluate the potential application of PE obtained by OMWW in the food industry, all samples were subjected chiefly to microbiological analysis. The samples did not show measurable mesophilic aerobic microorganism colonies, yeast and lactic bacteria (<1 cfu mL^{-1}, data not shown) over time regardless the storage conditions. Previous studies on bioactive compounds contained in OMWW extract showed that single phenolic compounds or their combination resulted in growth inhibition of different bacteria (Galanakis, 2017; Medina et al., 2013). Considering that the viability of main microorganisms depends on the pH, also the analysis of this parameter was performed. In Fig. 3 (a and b) the change in pH of EWs during storage at different temperature (4 and 25°C) is reported. The obtained data were included in the range of 2.5–4.0, as also reported by Azeredo and colleagues (2016) regarding to different kinds of soft drink available on the market. The sample EW^{50}, that was enriched with lower content of antioxidant extract showed higher value of pH (with statistical differences between the two concentrations, $p<0.05$), while the TA value decreased during the storage in all the treated samples. Overall, chemical preservatives are used in commercial soft drink to improve their microbiological stability. These results confirm that PE can acts as a natural preservative to avoid the microbiological growth.

Considering the important role of temperature on the stability of phenolic compounds, the samples were stored at two different temperatures (4°C and 25°C) monitoring the changes that occurred during the storage. TPC raised significantly in all the samples during the storage period at different temperatures (table 3 a): in particular the increasing after 60 days of storage in all the samples can be explained, by a possible interference of polymerized substances with the Folin reagent (Prior et al., 2005). The highest value of TPC (about 0.33 g L^{-1}) was quantified in EW^{100} after 60 days of storage at both temperatures.

Samples responded better to ABTS than the other assays for the antioxidant activity expressed as TEAC values. TEAC values of EW^{100} increased also at the end of storage at both temperatures, as

confirmed by TPC. $ABTS^{+}$ reacts not only with antioxidant compounds but also with any hydroxylated aromatic compounds independently of their antioxidant potential occurred in the sample. However, detected values with DPPH and ORAC assay were characterized by different trend for the same sample. A loss of 7% of percentage of DPPH inhibition was showed at the end of storage at 4°C in EW^{100}, while a slight variation was observed at higher storage temperature ($p>0.01$). Regarding the ORAC assay, a high variability in results was detected for both samples. Significant variations were observed also for EW^{50} samples but the higher temperature seemed to improve the stability of sample. However, the highest values were obtained in ORAC assays after 15 and 30 days regardless the storage conditions. No correlations were found between total phenol compounds and the results obtained from ORAC assay ($r\leq0.1$). Statistical analysis revealed lower or negative correlation between TPC and DPPH assay at both temperatures $r\leq0.530$ in EW^{100} $r \geq -0.500$ in EW^{50}). In contrast, a strong correlation ($r=0.849$ at 4°C and $r=0.878$ at 25°C) was found in EW^{100} between TEAC values and total phenol content.

The different relationships between the antioxidant activity and the total phenolic content can be due to many factors. The results of this study showed that antioxidant capacity is relatively stable when a low concentration of extract was added to hydrophilic matrix. As can be seen in Table 3 (a), the antioxidant values obtained by the different assays did not show significant variations ($p>0.05$) over time regardless the storage temperature (as evidenced by multivariate analysis in table 3 b. The phenolic compounds can act as pro-oxidants when their concentration is above a narrow range (Lafka et al., 2011). This could explain why the use of only 50 mg L^{-1} of tyrosol allows to obtain a product with a high stability compared to the product added with a more amount of phenol. The multivariate statistical analysis (Table 3 b) shows that different treatments, time and the combination of these factors significantly affected ($p<0.05$) the total phenol content, performed by Folin-Ciocalteau assay, and the antioxidant activity.

Quantification of major compounds present in EW^{100} and EW^{50} over time at different temperatures is given in Table 4. No significant variation of principal phenolic compounds (hydroxytyrosol and tyrosol) was observed in the storage time, as reported in table 4. The use of food flavouring ethanol-based could explain the stability of hydroxytyrosol during storage, as reported by Feki (2006). In contrast, the concentration of the other phenol compounds decreased during storage, in agreement with Romero (2004). It was demonstrated that in the olive juice the main phenol was hydroxytyrosol at the end of the storage period. These results could confirm that hydroxytyrosol and tyrosol are the major compounds responsible of antioxidant stability of enriched water.

4. Conclusions

The recovery of phenol compounds contributes to the sustainability of olive waste sector reducing their environmental impact and allowing to obtain an extract which can be an antioxidant ingredient to be used in food industry and other.

This work showed that the addition of low concentrations (50 mg of Tyrosol L^{-1}) of the phenolic extract to hydrophilic matrix allows to increase its chemical and microbiological stability. Based on these results, the future purpose will be the addition of the same extract to more complex foods.

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Figure Captions

Figure 1: Schematic overview of the experimental plan

Figure 2: UHPLC chromatogram of phenolic extract (OMWW). Identified peaks: 1) Hydroxytyrosol; 2) Tyrosol; 3) Vanillic acid; 4) Caffeic acid; 5) p-cumaric acid; 6)Ferulic acid; 7) Verbascoside; 8) Luteolin; 9) Apigenin; 10) Oleuropein

Figure 3 (a): Changes in the pH and TA values for the samples stored at 4°C

Figure 3(b): Changes in the pH and TA values for the samples stored at 25°C

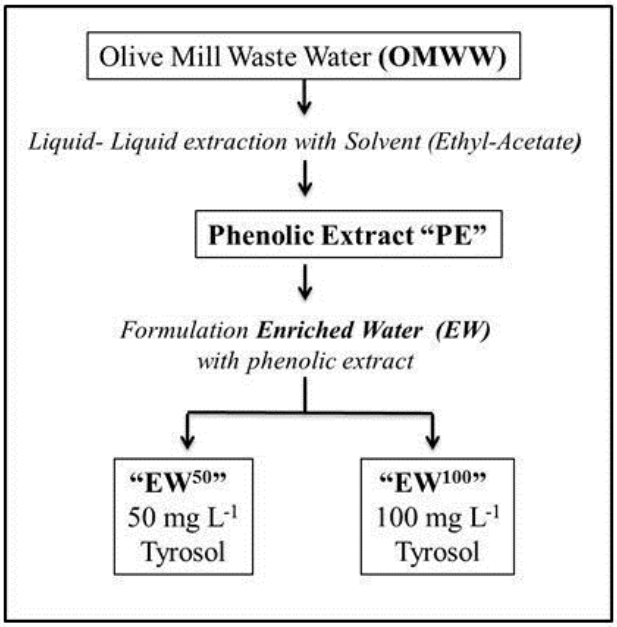

Figure 2

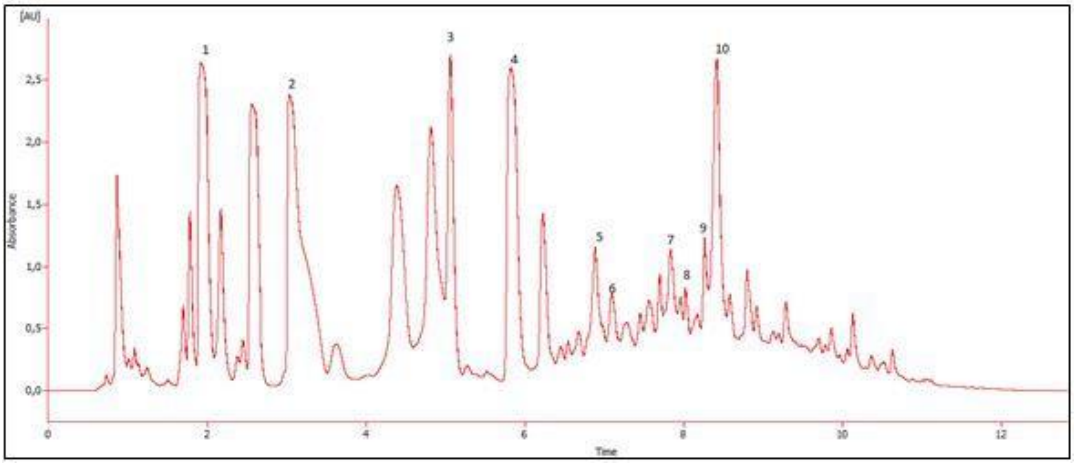

Figure 2

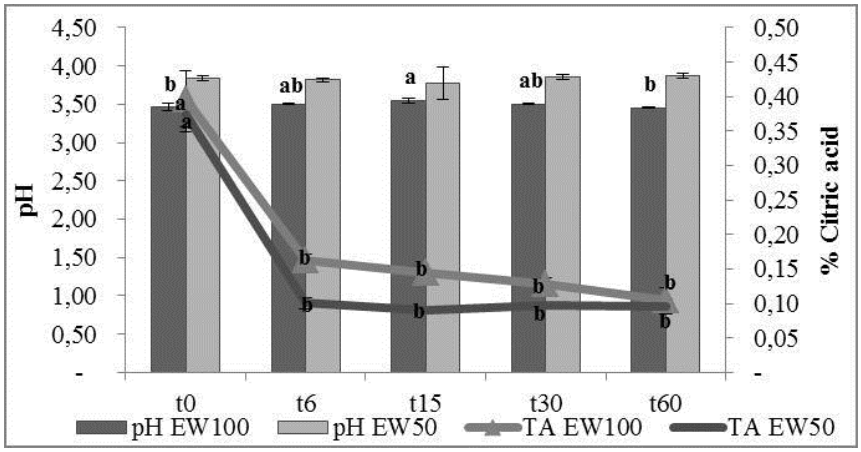

Figure 3

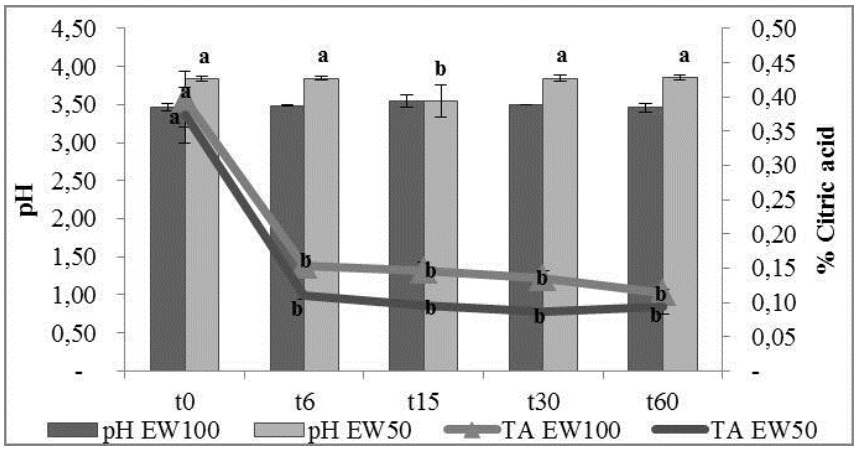

Figure 4

Table 1 Characterization of PE

Phenolic Extract (OMWW)	
L*	33.82±0.43
a*	0.45±0.06
b*	3.24±0.43
TPC (g L^{-1})	19.58±0.10
ABTS (mmol L^{-1})	3247±12
DPPH (%)	37.4±0.6
ORAC (mmol L^{-1})	1576±10

Table 2 Concentration of single phenolic compounds in the phenolic extract (OMWW) performed by UHPLC

Phenolic Compounds	g L^{-1}
Hydroxytyrosol	1.22±0.01
Tyrosol	12.9±0.05
Vanillic acid	1.4±0.02
Caffeic acid	1.3±0.01
p-cumaric acid	0.1±0.00
Ferulic acid	0.050±0.00
Verbascoside	0.8±0.00
Luteolin	0.3±0.00
Apigenin	0.09±0.00
Oleuropein	2.1±0.02

Table 3 TPC values and antioxidant capacity for the samples stored at both temperatures

Samples	Time (days)	TPC (g L^{-1})	ABTS (mmol L^{-1})	DPPH (%)	ORAC (mmol L^{-1})
EW^{100} 4°C	t0	0.26±0.00^{c}	8.6±0.6^{c}	74.9±2.6^{a}	2.9±0.2^{b}
	t6	0.27±0.00^{c}	11.4±0.3^{ab}	73.1±3.4^{ab}	2.4±0.2^{c}
	t15	0.28±0.00^{b}	12.8±0.1^{ab}	67.5±1.6^{b}	4.0±0.1^{a}
	t30	0.28±0.00^{b}	11.1±0.6^{b}	66.8±1.5^{b}	3.8±0.0^{a}
	t60	0.33±0.02^{a}	11.4±0.2^{a}	68.7±3.5^{ab}	2.8±0.2^{b}
	Sign.	**	**	*	**
EW^{100} 25°C	t0	0.26±0.00^{c}	8.6±0.6^{d}	74.8±2.6^{a}	2.9±0.2
	t6	0.27±0.00^{c}	10.4±0.2^{c}	73.3±2.1^{ab}	2.7±0.4
	t15	0.28±0.00^{b}	11.7±0,33^{b}	71.5±2.4^{ab}	3.9±0.1
	t30	0.28±0.00^{b}	10.7±0.5^{bc}	68.1±0.6^{b}	3.7±1.0
	t60	0.33±0.03^{a}	13.6±0.3^{a}	69.3±2.8^{ab}	3.1±0.1
	Sign.	**	**	*	**ns**
EW^{50} 4°C	t0	0.13±0.00^{b}	6.6±0.4	42.2±5.7	2.0±0.1^{a}
	t6	0.14±0.00^{b}	6.2±0.2	50.8±1.4	1.2±0.2^{b}
	t15	0.15±0.00^{ab}	6.3±0.4	49.1±3.3	2.8±0.5^{a}
	t30	0.14±0.00^{ab}	6.0±0.2	50.4±2.9	2.6±0.4^{a}
	t60	0.16±0.02^{a}	6.7±0.2	50.9±3.5	1.7±0.1^{b}
	Sign.	**	**ns**	**ns**	**
EW^{50} 25°C	t0	0.13±0.00^{d}	6.6±0.4	39.1±1,6	2.0±0.1^{bc}
	t6	0.14±0.00^{cd}	6.2±0.1	49.7±0.3	1.5±0.1^{c}
	t15	0.15±0.00^{bc}	6.2±0.2	50.1±0.6	2.7±0.2^{ab}
	t30	0.15±0.00^{b}	6.1±0.1	46.9±1.3	3.0 ±0.7^{a}
	t60	0.16±0.00^{a}	6.7±0.2	49.1±3.7	1.7±0.1^{bc}
	Sign.	**	**ns**	**ns**	*

The data are presented as means ± SDs. Means within a row with different letters are significantly different by Tukey's post hoc test. ** Significance at $P < 0.01$. * Significance at $P < 0.05$; n.s. not significant.

Table 4 Multivariate statistical analysis of EW

	TPC[a]	ABTS	DPPH	ORAC
TREATMENT	**	**	**	**
TEMPERATURE	n.s.	n.s	n.s	n.s
TIME	**	**	**	**
TREATMENT*TIME	*	**	*	*

Table 5 Changes in the amount of single phenolic compounds (mg L^{-1} of extract) in the samples during the storage at both temperatures

Phenolic compounds	1 day	30 days	60 days	Sig n.	1 day	30 days	60 days	Sign.
EW[100]		**4°C**				**25°C**		
Hydroxytyrosol	55±1	51±0	52±1	**ns**	55±1	53±0	53±1	**ns**
Tyrosol	57±1	40±14	46±17	**ns**	57±1	56±14	57±1	**ns**
Caffeic acid	13±1	15±0	15±0	**ns**	13±1^{ab}	13±0^{b}	15±0^{a}	**
Apigenin	4±0^{a}	3±0^{c}	5±0^{a}	**	4±0^{c}	6±0^{b}	5±0^{b}	**
Oleuropein	3±0^{a}	3±0^{a}	2±0^{b}	**	3±0^{a}	4±0^{a}	3±0^{b}	**
EW[50]		**4°C**				**25°C**		
Hydroxytyrosol	26±0	27±0	28±1	**ns**	26±0^{b}	28±0^{ab}	29±1^{a}	*
Tyrosol	29±0	29±0	30±1	**ns**	29±0^{b}	29±0^{ab}	31±1^{a}	*
Caffeic acid	15±1^{a}	13±1^{b}	14±0^{ab}	*	15±1^{a}	13±1^{b}	13±0^{b}	*
Apigenin	9±0^{a}	4±0^{b}	4±0^{b}	**	9±0^{a}	6±0^{b}	5±0^{c}	**
Oleuropein	2±0^{a}	2±0^{ab}	2±0^{b}	**	2±0^{a}	2±0^{a}	1±0^{b}	*

The data are presented as means ± SDs. Means within a row with different letters are significantly different by Tukey's post hoc test. ** Significance at $P < 0.01$. * Significance at $P < 0.05$; n.s. not significant.