J Am Oil Chem Soc (2017) 94:1077–1085
DOI 10.1007/s11746-017-3011-1

ORIGINAL PAPER

Enhancement of Bioactive Phenols and Quality Values of Olive Oil by Recycling Olive Mill Waste Water

Konstantinos Kiritsakis[1] · Eleni Melliou[2] · Prokopios Magiatis[2] · Dimitrios Gerasopoulos[1]

Received: 4 October 2016 / Revised: 27 May 2017 / Accepted: 14 June 2017 / Published online: 23 June 2017

Abstract Mature 'Chondrolia Chalkidikis' olives were processed in an industrial olive oil mill equipped with a three-phase decanter. Water was added to the decanter at a 1:2 water-to-paste ratio. Olive mill waste water (OMWW) was used to replace the added water at a rate of 50 or 100%. Following the final separation, the obtained oil was used for chemical analysis and sensory evaluation. All oils had similar acidity, peroxide and K values. OMWW-treated olive oils presented higher total phenolic content and higher antioxidant activity based on DPPH and oven tests, but lower chlorophyll and carotenoids content. However, there was no significant difference between the 50 and 100% replacement. The phenolic profile of the treated olive oils analyzed by quantitative ^{1}H NMR revealed more than twofold oleocanthal and oleacein as well as oleuropein and ligstroside aglycone contents than in the control. Sensory evaluation of treated oils also showed an enhancement of fruity, bitter and pungent attributes compared to the control.

Keywords Enriched olive oil · Waste water · Oleocanthal · Oleacein · Oleuropein · Bioactive phenols · Secoiridoids

✉ Dimitrios Gerasopoulos
dgerasop@agro.auth.gr

[1] Laboratory of Food Processing and Engineering, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, Panepistimiopolis, 54124 Thessaloníki, Greece

[2] Laboratory of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece

Introduction

Olive oil is the principle source of fat in the Mediterranean diet, consumed daily in salads and cooked food. Besides its high monounsaturated fatty acid content, olive oil is known to contain several phenolic compounds, contributing to its oxidative stability, functional activity and unique sensory properties. Phenolic compounds of olive oil include simple phenols (hydroxytyrosol, tyrosol), secoiridoid derivatives (oleocanthal, oleacein, oleuropein and ligstroside aglycones) and lignans [1–3].

The phenolic fraction of olive oil imparts many beneficial effects to human health and has been linked to protection from inflammatory degenerative joint diseases, cardiovascular disorders, certain types of cancer and other chronic diseases [4]. Among phenolic compounds, oleuropein aglycone, oleocanthal and oleacein have attracted researchers' attention to a greater extent.

Oleuropein aglycone has been found to be one of the substances of olive oil with antiatherogenic properties, exhibiting an ability to inhibit endothelial activation and having anti-breast cancer properties [5].

Oleocanthal is the dialdehydic form of decarboxymethyl ligstroside aglycone, responsible for the pungency associated with some extra virgin olive oils [6]. Beauchamp et al. [7] reported that oleocanthal mimics the antiinflammatory activities of ibuprofen in inhibiting cyclooxygenase activity. Oleocanthal has been reported to act on inflammatory markers associated with neurodegenerative disease, joint degenerative disease and cancer [4] as well as to display antimicrobial activity against *Helicobacter pylori* [8]. Nevertheless, the oleocanthal concentration does not remain steady during the olive maturation period. Cicerale et al. [9] reported a 15% decrease in total oleocanthal concentration for both olive oil and olive pomace across harvest time.

Oleacein is the dialdehydic form of decarboxymethyl oleuropein aglycone, which, similarly to oleocanthal, is also responsible for the pungency associated with some extra virgin olive oils [6] and has potent antioxidant activities, in addition to significant anti-breast cancer properties [1].

Olive oil retains only a small portion of the fruit polar phenols (2%) during extraction because of their low solubility in lipid matrices. Thus, current research is attempting to enrich olive oil with phenolic compounds. The use of olive plant by-products, such as olive leaves, olive cake or olive mill waste water (OMWW), for this purpose has gained attention lately as OMWW extracts have been reported to inhibit human LDL oxidation and to scavenge superoxide anions and hypochlorous acid in addition to inhibiting leukotriene production by human neutrophils [10].

Olive fruit processing systems to obtain olive oil are the two-phase centrifugals, which do not require addition of water, and the three-phase ones, which require about 40% water (on a processed olive paste basis) [11]. As a result, the two-phase systems produce wet olive pomace and a small amount of OMWW, while the three-phase ones produce more OMWW and dry olive pomace.

Concerning the OMWW by-product, Suarez et al. [12] reported the enrichment of olive oil with phenolic compounds extracted from the vegetation water. Moreover, Boudissa and Kadi [13] studied the transfer of phenolic compounds from OMWW to olive pomace oil, and, under optimal conditions, the increase in phenolic content in the olive pomace oil was from 0.04 ± 0.01 to 0.13 ± 0.02 g/L. Servili et al. [14] used a three-phase membrane system to produce a crude phenolic concentrate, which increased the phenolic content of the virgin olive oils without any alteration of their aroma profiles. Individual and combined phenolic compounds of different concentrations were added to refined olive oil as a lipid matrix, increasing the oils' antioxidant activity. Farag et al. [15] found that phenolic extracts obtained from the olive pomace by-product showed remarkable antioxidant activity in retarding sunflower oil oxidative rancidity.

Recent reports supporting the healthy properties of olive oil phenols and the interest in utilizing by-products from the oil extraction process make the development of phenol-enriched olive oils substantial. Considering the above, the present study aims at contributing to the ongoing efforts for future exploitation of OMWW in the olive oil industry using a simple process, which, not employing any additional processing, saves water and energy. More specifically, mature olives were selected instead of any other maturity stages for extracting olive oil, which are rather poor in phenolic components [16]. Furthermore, addition of different proportions of water and/or OMWW to the olive paste, before centrifugation in a three-phase decanter, was evaluated. A flow chart with the proposed mechanism of recycling OMWW during the olive oil production procedure, along with the quantities of the materials used and produced, is shown in Fig. 1. Quality characteristics of the enriched olive oils were examined with regard to the main bioactive constituents (oleuropein and ligstroside aglycones, oleocanthal and oleacein). A sensory evaluation test was carried out to grade the organoleptic characteristics of OMWW-treated olive oil.

Experimental Procedures

Plant Material

Fully ripe olive fruits (cv. Chondrolia Chalkidikis) were collected from the olive orchard of Aristotle University of Thessaloniki farm at the end of the harvesting period (end of November). Olives were harvested at a complete maturity stage (black color).

Reagents

For the analyses, solvents (HPLC grade) and all other common reagents were purchased from various suppliers. Sigma Chemical Co. (St. Louis, MO, USA) supplied caffeic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteu (F-C) reagent.

Extraction Procedure

A quantity of 3000 kg of olive fruit was divided into three equal parts (1000 kg) by weight; they were crushed and malaxed separately in individual malaxators (Alfa Laval) for 40 min at 27 °C. Each malaxator provided three batches of about 333 kg of malaxed olive paste in a three-phase decanter (318 model) and a vertical centrifugal separator (307 model). In each case, the first batch was centrifuged with the addition of about 130 kg clear tap water. The second batch was centrifuged with the addition of about 65 kg of tap water and 65 kg of OMWW from the vertical centrifuge (50% OMWW), while the third one with the addition of about 130 kg of OMWW (100% OMWW) (Fig. 1).

The OMWW used was obtained from the vertical centrifuge at the final separation stage. We decided to use it immediately after its generation, without any intermediate storage, filtration or processing of any kind, because we wanted the procedure to take place online in order not to give sufficient time for fermentation to take place. This OMWW was very low in solid matter, because the solid matter was removed from the three-phase decanter (horizontal centrifuge) along with the stream of olive pomace

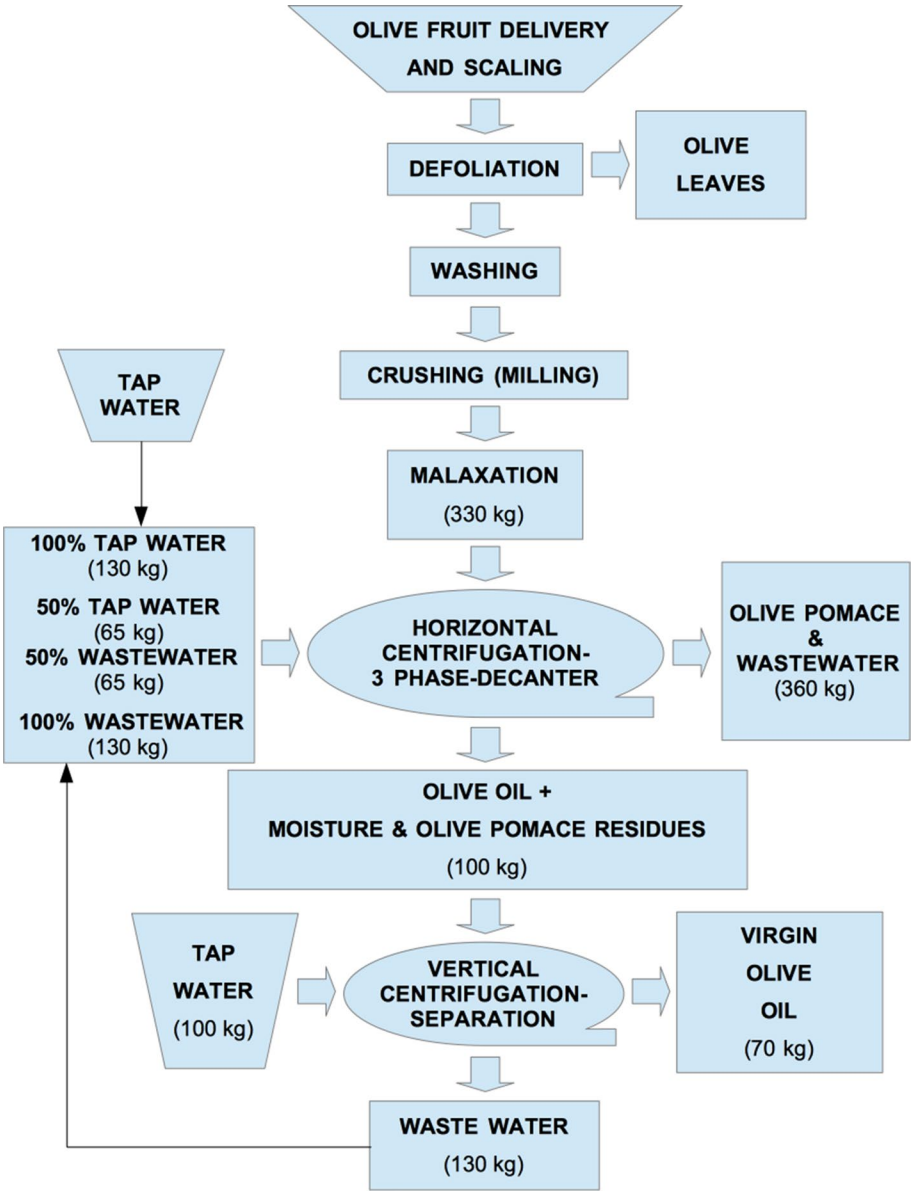

Fig. 1 Flow chart followed in the olive oil mill and material balance

and the stream of OMWW. The latter probably contained phenolics because during the centrifugation of the olive paste in the decanter, some of these compounds were removed from the oily phase and went to OMWW. Besides, it is known that OMWW always contains olive oil [17], which can be up to 4.5% [18]. Therefore, the OMWW, which was directly pumped into the decanter, along with the olive paste from the malaxator at a ratio of 0, 50 or 100%, contained some olive oil as well (Fig. 1).

Each batch gave about 65–70 kg of olive oil. As the oil started coming out of the vertical centrifuge minutes later, samples of about 0.5 kg each were collected (a total of 5 kg). Special attention was taken to avoid sample collection during the change from one treatment to the other. The above procedure was repeated for each one of the three treatments. The obtained samples were then transferred to 50-mL tubes, filled to the top, capped, covered with aluminum foil and stored in the freezer (−20 °C) until analysis.

Quality Attributes, Total Phenol Content, Radical Scavenging Capacity and Oxidative Stability

The free acidity (as % oleic acid), peroxide value (meqO_2/kg oil) and K values (K_{232}, K_{270} and ΔK) of the oil samples were measured according to the EU Regulation 2568 methods. Spectrophotometric analysis of the chlorophyll and carotenoid pigments was determined using the method of Moyano et al. [19]. The total chlorophyll and carotenoid content was expressed as mg of pheophytin a and mg of

lutein per kg of oil, respectively. A Minolta CR-310A colorimeter with a DP-301 data processor (Minolta Camera Co. Ltd., Osaka, Japan) was used to record the CIE-Lab color. L^* (light/dark), a^* (red/green) and b^* (yellow/blue) values for each sample were measured. The total phenol content was determined using oil samples of 2.5 g [20]. The radical scavenging activity (RSA) of the samples was determined using the DPPH* assay [21] and expressed as the % reduction in the concentration of DPPH* by the constituents of the oils. To estimate the differences in oxidative stability among enriched oil treatments, 50-mL oil samples were stored in the oven at 50 °C in 125-mL open amber glass bottles, i.d. 4.2 cm, with a surface area of 13.85 cm^2 exposed to the atmosphere. At scheduled times, oil samples were taken from the incubator for peroxide and K value analyses.

NMR Quantitation of Phenolic Compounds

Olive Oil Extraction and Sample Preparation for NMR Analysis

^{1}H NMR analysis of the phenolic compounds was performed according to Karkoula et al. [22]. More specifically, a quantity of 5.0 g olive oil was mixed with cyclohexane (20 mL) and acetonitrile (25 mL). The mixture was homogenized using a vortex mixer for 30 s and centrifuged at 4000 rpm for 5 min. A part of the acetonitrile phase (25 mL) was collected, mixed with 1.0 mL of a syringaldehyde solution (0.5 mg/mL) in acetonitrile and evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland).

NMR Spectral Analysis

The residue of the above procedure was dissolved in $CDCl_3$ (750 μL), and an accurately measured volume of the solution (550 μL) was transferred to a 5-mm NMR tube. ^{1}H NMR spectra were recorded at 600 MHz using an NMR spectrometer (Bruker Avance 600). Typically, 50 scans were collected into 32-K data points over a spectral width of 0–16 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s. Prior to Fourier transformation (FT), an exponential weighing factor corresponding to a line broadening of 0.3 Hz was applied. The spectra were phased, corrected and integrated automatically using TOPSPIN. When necessary, accurate integration was performed manually for the peaks of interest.

Organoleptic Test

Considering the bitter flavor of olive oils enriched with the main bioactive constituents, an organoleptic test was carried out to ensure that the organoleptic quality was maintained. Organoleptic assessment of three selected samples (control olive oil, olive oil with 50 and 100% OMWW) was carried out by the official evaluation panel of the Ministry of Economy and Tourism-Greece, according to the EU Regulation 2568 methods.

Statistical Analysis

All analyses were performed in triplicate. An ANOVA analysis was applied to the data using the SPSS statistical software for Windows (Release 10.0.1). Mean separation was conducted by LSDs at a 0.05 level of confidence.

Results and Discussion

Oil Quality Characteristics, Pigment Content, Color Attributes, Total Phenol Content, Radical Scavenging Activity and Oil Stability

To evaluate the results of the selected treatments, the standard quality parameters (acidity, peroxide value, K_{232}, K_{270} and ΔK) proposed by the EU Regulation 2568 methods were applied. Moreover, total chlorophyll and carotenoid contents were evaluated and compared with those obtained from the control samples. The values of the quality parameters in all of the olive oils were within the official range for extra virgin olive oil. The process had virtually no effect on the free fatty acids (% oleic acid), peroxides and K_{232}, while it caused a slight increase in the values of K_{270} and ΔK (Table 1).

A slight but significant decrease was observed in the total chlorophyll and carotenoid values of olive oils produced using OMWW compared to those of the control, while there was no significant difference between the samples produced using 50 and 100% OMWW (Table 1). For characterizing the oil color, chromatic coordinates L^*, $-a^*$ and b^* can be used. Related parameters, such as chroma (C^*) and the ratio of absolute values of a^* and b^*, are also evaluated [23]. Higher L^* values reveal high light transmittance through the sample. Moreover, the greater the absolute values of the a^* or b^* coordinates are, the higher the levels of pheophytins (green hues) and carotenoids (yellow hues), respectively. When C^* values are greater, darker colorations of the samples are expected to be observed, while higher a^*/b^* ratio values are related to samples with more green tonalities. Values of chromatic coordinates and related parameters, as well as total chlorophyll and carotenoid content values as an index of green and yellow pigment levels, respectively, for all samples are presented in Table 1. L^* values showed slight differences in transmission of light among

Table 1 Mean values of quality characteristics, total carotenoids and chlorophyll content as well as chromatic ordinates of olive oil enriched by replacing water in a three-phase decanter system with 0 (control), 50 or 100% OMWW

Attributes	Treatment		
	Water (control)	50% OMWW	100% OMWW
Free acidity (% oleic acid)	0.71[a]	0.70[a]	0.71[a]
Peroxide value (meq O_2/kg)	10.1[a]	9.95[a]	10.0[a]
K_{232}	1.739[a]	1.743[a]	1.733[a]
K_{270}	0.172[a]	0.143[a]	0.135[b]
ΔK	0.001[a]	0.001[a]	0.001[a]
Total chlorophyll (mg/kg pheophytin a)	2.07[a]	1.76[b]	1.74[b]
Total carotenoids (mg/kg lutein)	1.65[a]	1.44[b]	1.42[b]
L^*	92.51[a,b]	92.40[b]	92.56[a]
a^*	−12.31[b]	−10.85[a]	−10.72[a]
b^*	42.19[a]	37.79[b]	36.40[c]
C^*	43.95[a]	39.32[b]	37.94[c]
a^*/b^*	−0.291[b]	−0.287[a]	−0.294[c]

[a,b,c] Values within each row with the same lower case letter are not significantly different at 0.05

treatments. Similarly, enrichment with OMWW caused a small decrease in a^* and b^* values in comparison with the control. In every case, the values recorded for both a^* and b^* chromatic indices were in the average ranges reported in the literature for those of virgin olive oil from different degrees of ripeness. Chroma values (C^*) showed that increasing the OMWW levels results in less vivid color (darkening) of the treatments. The tonalities of both kinds of enriched oils were comparable according to the a^*/b^* values. All the above comments are in accordance with the calculated total chlorophyll values.

The total polar phenol content, radical scavenging capacity, oxidative stability based on the oven test and composition of phenols of the olive oil samples (control, 50 and 100% OMWW) were also examined in this study. Determination of total polar phenol contents (as measured by the Folin-Ciocalteau method) [20] showed that the use of 50 or 100% OMWW resulted in an ~10% increase in the total phenol content for both OMWW treatments. It is to this higher level of phenolic antioxidants that the improvement of the radical scavenging properties of the oils produced with OMWW treatment is attributed (Fig. 2).

Oxidative stability of oil gives a good estimation of its resistance to oxidative deterioration; according to Nenadis et al. [24], it is considered the most important quality parameter of oils and fats. Figure 3 shows the kinetic behavior of the hydroperoxides measured as the peroxide value (PV, Fig. 3a), the formation of the conjugated dienes (K_{232}, Fig. 3b) and the formation of the conjugated trienes (K_{270}, Fig. 3c) of the autoxidation reaction at 50 °C. For each sample, the oven test period showed a linear increase in PV, conjugated dienes and conjugated trienes. Our results agree with those of Cinquanta et al. [25].

The kinetics of oxidation parameters of olive oil as a function of temperature were described in the literature

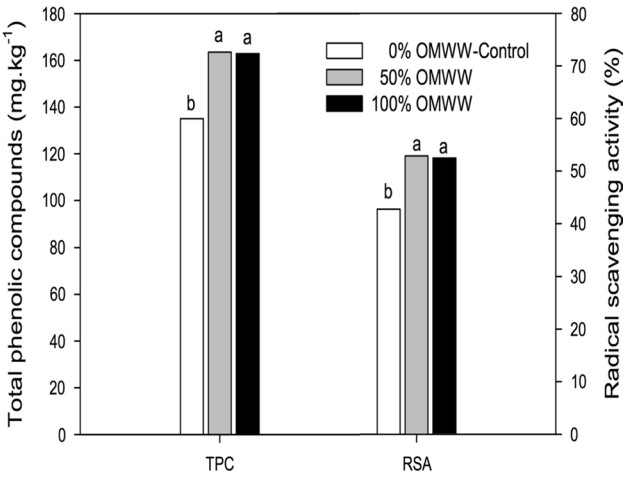

Fig. 2 Total polar phenol (TPC) content (based on the Folin-Ciocalteau assay) and radical scavenging activity (RSA) of olive oil enriched by replacing water in a three-phase decanter system with 0 (control), 50 or 100% OMWW. (Values within the same letter are not significantly different at 0.05)

long ago. In this article, our aim was to use a single temperature at which the differences between treatments regarding autoxidation parameters would be revealed. Since autoxidation parameters (PV, K_{232}, K_{270}) evolve as a function of time, we presented such data using a linear regression, and the rate constants (k) of control and treatments were finally compared to obtain and show possible differences.

The autoxidation reaction followed a pseudo-zero-order kinetic behavior, where the reaction rate is independent of the oxidizing substrate concentration (Eq. 1).

$$C = \mathrm{Co} + kT \quad (1)$$

where Co and C represent the concentration of hydroperoxides in the oil before and after time T of storage at 50 °C,

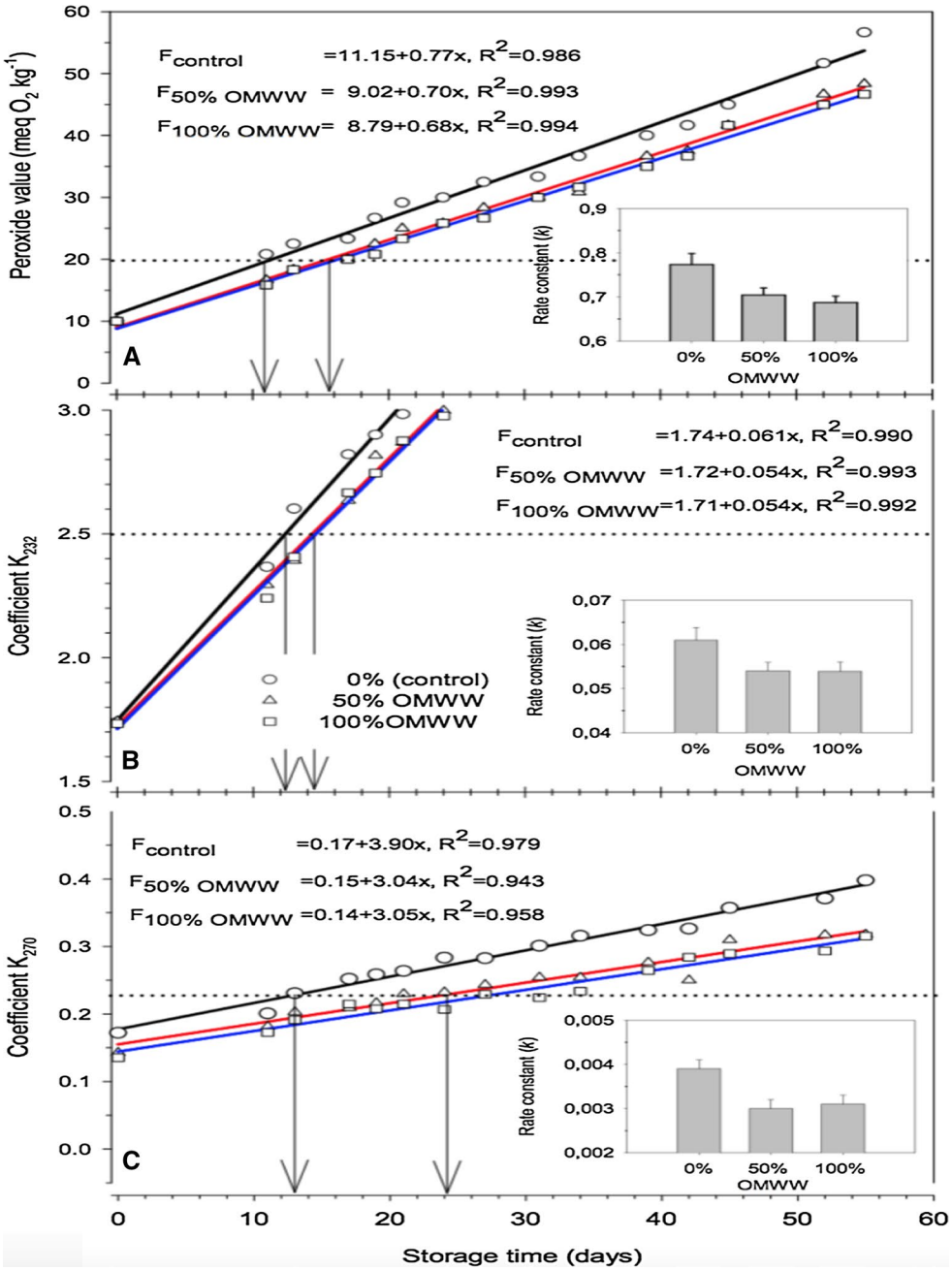

Fig. 3 Changes of quality parameters and linear regression analysis of the peroxide value (**a**), K_{232} (**b**) and K_{270} (**c**) of olive oil enriched by replacing water in a three-phase decanter system with 0 (control) (*circle*), 50 (*triangle*) or 100% (*square*) OMWW during storage at 50 °C. *Inserted figures* within each quality parameter figure represent respective rate constants (k) following linear regression; *vertical bars* represent standard errors

respectively, and k is the rate constant of the reaction. The behavior during the autoxidation reaction of conjugated dienes (K_{232}) and conjugated trienes (K_{270}) of oxidized oils was very similar to that observed for PV, as was also observed by Gomez-Alonso et al. [26]. Values of the oxidation rate constants k and regression coefficients for PV, K_{232} and K_{270} are also reported in Fig. 3. The regression coefficient (r^2) was always higher than 0.94 ($p > 0.5$) for PV, K_{232} and K_{270}, indicating a good linear relationship with storage time at 50 °C. The k values of all the parameters measured were significantly higher in the control oil than in the oils enriched using 50 or 100% OMWW, although there was no difference between the last two (inserted figures within Fig. 3). The PV, K_{232} and K_{270} values exceeded the virgin olive oil limits after 10.5, 12 and 13 days for the control and after 16, 14 and 24 days for the enriched oils, respectively, when stored at 50 °C. These results indicate that enriching olive oil with recycled OMWW had a positive effect on oxidative stability.

NMR Analysis of Phenolic Compounds

The enrichment process of olive oil usually leads to an increase in the concentration of olive oil phenolic

compounds. However, besides the total phenolic content, of special interest is the individual concentration of the secoiridoid derivatives, specifically oleocanthal, oleacein and oleuropein aglycone. Oleocanthal is found in quantities ranging from 0.2 to 711 mg/kg, whereas oleacein was reported to range from 0.2 to 588 mg/kg in extra-virgin olive oil [2]. This high variation depends on many factors, such as the olive cultivar and harvest time [27]. Thus, the quantitative enrichment of the individual phenolic compounds observed in the oils produced with OMWW is of great importance. The concentrations of oleocanthal and oleacein recorded in the control oil of the experiment were as low as 87 and 23 mg/kg, respectively. After the enrichment of oil using OMWW, the major phenols transferred to the oil matrix were oleocanthal at levels of 184 mg/kg and oleacein at 88 mg/kg of oil for samples produced with replacement of water with 50% OMWW. For 100% replacement, they were 193 mg/kg for oleocanthal and 74 mg/kg for oleacein.

The oleuropein and ligstroside aglycone (monoaldehydic forms) contents of the control were as low as 1 mg/kg and also increased to almost 25 mg/kg of oil for the samples produced using 100% OMWW (Fig. 4).

A significant difference is observed between the results obtained with the Folin-Ciocalteau method and the NMR analysis. This difference is attributed mainly to the fact that NMR evaluates and quantifies each compound separately, whereas the Folin-Ciocalteau assay quantifies the phenolic compounds as caffeic acid equivalents in a mechanism that is not yet definitely known [28]. Moreover, Mastralexi et al. [29] observed that, even when the same method (RP-HPLC analysis) was applied to analyze the phenolic profile, two- to seven-fold higher levels of specific phenolic compounds were observed among the samples because different protocols were applied for the extraction of phenols from the samples.

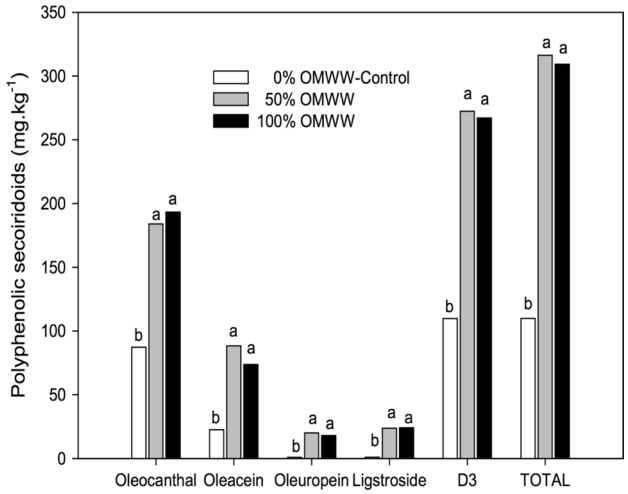

Fig. 4 Oleocanthal, oleacein, oleuropein aglycon, ligstroside aglycon, oleocanthal + oleacein (D3) and total phenolic secoiridoids of olive oil enriched by replacing water in a three-phase decanter system with 0 (control), 50 or 100% OMWW (values within the same letter are not significantly different at 0.05)

Karkoula et al. [22] suggested the need for a new type of classification based on the possible health claims of the bioactive polyphenolic secoiridoids of olive oil because of the variation of the concentration of these compounds observed in several samples of virgin olive oils. Recently, the European Food Safety Authority (EFSA) issued a health claim that consumption of olive oil containing at least 5 mg of polyphenols (hydroxytyrosol and its derivatives) per 20 g of olive oil (or 250 mg/kg of oil) contributes to the protection of blood lipids from oxidative stress.

Unlike the control sample, oils of both enrichment levels using OMWW passed the threshold of 5 mg/20 g (Fig. 4), having a final sum of oleocanthal, oleacein, oleuropein and ligstroside aglycones of 300–315 mg/kg. Figure 5 shows the NMR spectra of these compounds (400 MHz) comparing olive oil enriched by replacing water in a three-phase decanter system with 0% (control) (down) and 50% OMWW (up). It is obvious that oleocanthal, oleacein, oleuropein aglycon and ligstroside aglycon are present in higher values in the olive oil samples where 50% OMWW was used.

As these compounds are known to possess various biological properties (e.g., scavenging of reactive oxygen and nitrogen species, possible protection from cardiovascular diseases and certain type of cancers, etc.), consumption of olive oils enriched with these compounds may be a promising approach.

Organoleptic Test

Considering the bitterness of polyphenolic secoiridoids [6], enrichment of olive oil with oleuropein, as well as oleacein and oleocanthal, may affect the bitter and pungent organoleptic attributes and, as a result, the consumer's preference. To introduce such olive oils to the market and consequently attract buyers, both the appearance of the product and its organoleptic properties should satisfy potential consumers. For this reason, organoleptic evaluation was applied to detect the acceptability of the enhanced organoleptic characteristics of olive oil produced using OMWW at the 50 and 100% level. The control samples showed low mean values regarding fruity, bitter and pungent attributes (Fig. 6). Panelists judged that the control oil had a very week aroma, while the respective enriched samples had an enhanced average for fruity, bitter and pungent attributes, without any statistically significant differences between bitter and pungent when 50 or 100% OMWW was used ($p > 0.05$). A stronger pungency (a rough, burning or biting sensation

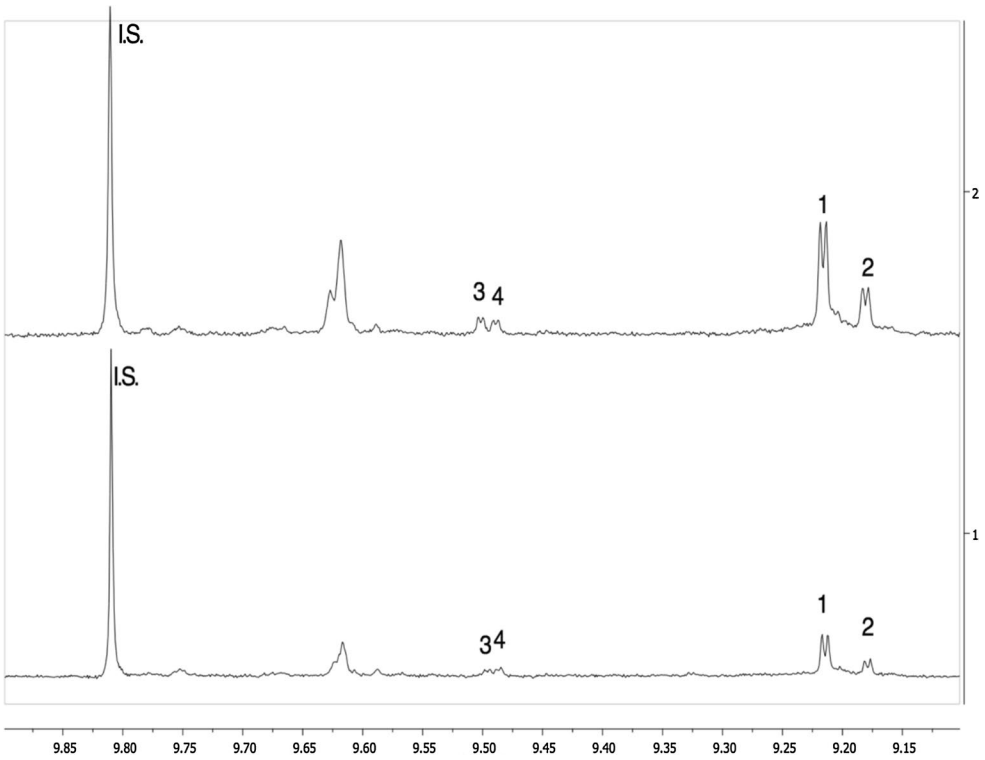

Fig. 5 Comparison of NMR spectra (400 MHz) between olive oil enriched by replacing water in a three-phase decanter system with 0 (control) (*down*) and 50% OMWW (*up*). *1* Oleocanthal, *2* oleacein, *3* oleuropein aglycon, *4* ligstroside aglycon *I.S.* internal standard. The ratio of the internal standard integration to the integration of peak of interest is used for quantitation

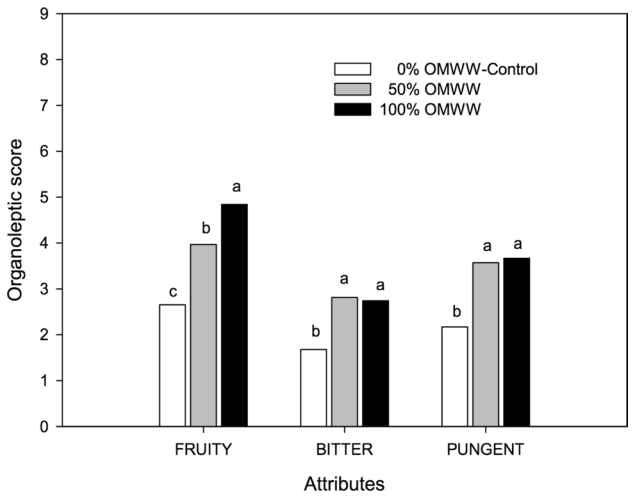

Fig. 6 Organoleptic attributes of olive oil enriched by replacing water in a three-phase decanter system with 0 (control), 50 or 100% OMWW (values within the *same letter* are not significantly different at 0.05)

in the throat) was also identified in samples with 50 or 100% OMWW. This was in accordance with the findings previously reported on NMR analysis of phenols and the possible presence of higher levels of oleocanthal and oleacein (Fig. 4).

Conclusions

Although the benefits of the two-phase decanters have been widely described in the literature, for several reasons, many olive mills have not yet replaced their systems. The authors suggest a way to benefit from the use of the three-phase systems in favor of olive oil quality, in terms of both its total phenolic content and its oxidative stability. More specifically, research has showed that enhancing olive oil by recycling OMWW can have a positive effect on the concentrations of the individual secoiridoid derivatives oleocanthal and oleacein as well as oleuropein and ligstroside aglycones. The produced olive oil even exceeded the threshold of 5 mg/20 g, which satisfies the EFSA health claim. Meanwhile, the environmental effects of the three-phase decanters can be reduced through this process by eliminating the use of tap water and at the same time reducing the amount of generated wastewater.

Acknowledgements Sincere appreciation is extended to the official panel team of the Greek Ministry of Economy and Tourism, General Secretariat for Commerce and Consumers, Section for Chemical Analysis and Organoleptic Assessment Affairs, for examining our samples. Also special thanks go to Mr. Kostas Sphondilas, food technologist, for using his commercial olive oil mill for the needs of the experiments. Panagiotis Diamantakos is acknowledged for technical assistance.

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