Polyphenolic profile and antioxidant activity of olive mill wastewater from two Sicilian olive cultivars: Cerasuola and Nocellara etnea

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Abstract

During the last years there has been an increasing interest in the valorization of the food processing side streams due to their potentially valuable phytochemical content. Olive mill wastewater (OMWW), the main by-product of olive oil extraction process, are rich in polyphenolic compounds, widely known for their health-promoting benefits. However, different parameters - such as the distinct olive cultivar, ripeness of the fruit, processing techniques, climate and storage conditions - play an important role in determining the specific quali-quantitative polyphenolic composition of OMWW. In this work, for the first time, we have characterized and compared the polyphenolic profile of different OMWW generated by centrifugal three-phase olive oil mills processing of two important Sicilian cultivars, Cerasuola and Nocellara etnea. Moreover, the correlated antioxidant activity and the stability of both OMWW samples stored at different conditions were evaluated. Our results show that even if OMWW are characterized by different and individual polyphenolic profile, both side streams have high levels of antioxidant activity. From a comparative point of view, we found that Cerasuola-OMWW showed higher values of total phenols (5.20±0.21 g/l gallic acid), total flavonoids (2.28±0.23 g/l catechin) and hydroxytyrosol content (821.86±0.01 mg/l) respect to the analogue parameters measured in Nocellara etnea-OMWW; these phytochemical values showed a significant stability in both OMWW samples stored at -20°C for 6 months. Conversely, a decrease in the level of these compounds was observed in samples maintained at 4°C or 25 °C for 45 days.

Keywords

Olive mill wastewater, polyphenolic profile, hydroxytyrosol, antioxidant activity, stability study.

1. Introduction

Food production and processing activities are the oldest and the most impacting human industry, responsible for more than 25 billion ton CO_2-eq. [1]. Agriculture itself is responsible for 70–85% of water footprint [2] and the foreseen increase of food production by 70% till 2050 [3] imposes additional precautions for the global sustainability and environmental security. One of the most important food supply chains refers to the olive oil, with a European Union (EU) production for the year 2014 of 2482.6 t, the majority of which in the Mediterranean region. Mediterranean countries alone produce 97% of the total olive oil, while EU countries produce 80-84%. The biggest olive oil-producing country is Spain (1781.5 t in 2014), then Italy (463.7 t) and Greece (132.0 t), followed by Turkey, Tunisia, Portugal, Morocco and Algeria [4]. Olive mill wastewater (OMWW) arising from olive (*Olea europaea* L.) processing (0.5–1.5 m^3 per 1000 kg of olives, depending on the process) is one of the strongest industrial effluents: with chemical oxygen demand (COD) values of up to 220 g l^{-1} and corresponding biochemical oxygen demand (BOD) values of up to 100 g l^{-1}, its disposal is considered a significant economical and environmental issue.

Olea europaea L. fruit is rich in antioxidant and other biological activities strictly related to the high polyphenolic content [5-6], but olive oil contains only 2% of the total phenols occurring in the olive fruit since most of them are lost, during the processing operations, in the olive mill wastewater [7-9] depending on their low partition (oil/water) coefficients [10]. As a consequence, OMWW, the principal olive oil by-product, are actually regarded as a potential and very valuable source of antioxidant compounds useful in food, cosmetic and pharmaceutical industries [11-13].

Polyphenols represent a wide group of secondary plant metabolites, arising from phenylalanine or shikimic acid, playing a crucial role in counteracting various type of stress (ultraviolet irradiation, aggression by pathogens, parasites and plant predators), other than contributing to organoleptic properties of plants and plant-derived food [14-15]. Potential beneficial effects of polyphenolic substances on human health are widely known [16], due to their antioxidant, cardioprotective, anticancer, anti-inflammatory and antimicrobial properties [17-21]. Furthermore, recent studies highlighted that polyphenols may also prevent neurodegenerative diseases and ageing [22-23].

Most of the polyphenols commonly identified in OMWW include phenyl alcohols, phenolic acids, secoiridoids and flavonoids [24-25].

A body of literature data evidenced that both the qualitative and quantitative phenolic profile of OMWW change depending on different parameters such as olive cultivar, ripeness of the fruit, processing techniques, climate and storage conditions [25-27]. For example, an increase in the malaxation temperature causes a general reduction of total phenols and antioxidant activity of OMWW, moreover the pH may be responsible of oxidations processes and the same effects can also arise from many metabolic pathways including enzymatic activities [27]. Analogously, it has been observed that side streams produced from traditional discontinuous press processes have a higher phenolic content than OMWW obtained from more modern 3-phases centrifugal systems [25]. Very recent studies have reinforced the concept that changes of OMWW phenolic composition are strictly related to olive variety and/or to the production process [28-29]. Although OMWW polyphenolic composition has been investigated in samples coming from Algerian (Azerraj, Sigoise, Chemlal), Australian (Barnea), Greek (Koroneiki, Lianolia and Asprolia) and Italian (Cellina and Coratina) olive cultivars [24, 28-29], there are no data about some of the most important Mediterranean productions, such as the Sicilian ones.

In this study we report the first investigation concerning Sicilian OMWW samples from local Cerasuola and Nocellara etnea cultivars, cultivated respectively in the western and eastern part of the island: in order of possible applications as raw material in the nutraceutical field we studied and compared, the specific polyphenolic profiles and the antioxidant activity of OMWW obtained by a centrifugal three-phase olive oil mill processing system, and we additionally evaluated the stability of both effluents stored at different conditions.

2. Materials and Methods

2.1 Materials

Folin-Ciocalteu reagent, 1-diphenyl-2-picrylhydrazyl (DPPH), trifluoroacetic acid (TFA) and all standards were purchased from Sigma-Aldrich. HPLC grade solvents were purchased from Carlo Erba (Italy). Cerasuola-OMWW and Nocellara etnea-OMWW were freshly collected from three-phase olive oil mill processing systems located respectively in Menfi (Agrigento, Italy) and in Mascalucia (Catania, Italy).

2.2 OMWW pretreatment and physicochemical analysis

OMWW were centrifuged at 4000 rpm for 20 minutes and the supernatant was filtered through filter paper under vacuum condition. Filtered OMWW were stored at -20°C before use. The pH values of OMWW samples were determined by using a Mettler Toledo SevenCompact pH meter. Chemical Oxygen Demand (COD), total nitrogen, total phosphorous and metals were determined according to EPA (U.S. Environmental Protection Agency) methods 410.3, 352.1, 365.3 and 200.8.

Total sugars were determined according to Dubois method [30]. The absorbance was measured at λ 490 nm and compared against a glucose calibration curve (R^2=0.999) (Cary UV Agilent Technology). Results were expressed as g/l of glucose.

2.3 Total phenolic content

The total phenolic content of OMWW samples was determined using the Folin-Ciocalteu assay [31-33]. Briefly, 10 µl of OMWW or gallic acid standard solution, appropriately diluted, were added to 90 µl of water. Folin-Ciocalteu reagent (10 µl) was added to the mixture and shaken. After 5 minutes, 100 µl of 7% (w/v) Na_2CO_3 were added and the obtained solution was diluted with water up to 250 µl, shaken and incubated for 90 minutes at room temperature. The absorbance was determined at λ 750 nm with a microplate spectrophotometer reader (Synergy HT multi-mode microplate reader, BioTek, Milano, Italy) and compared against a gallic acid calibration curve (y=0.002x+0.030, R^2=0.9997). The total phenolic content was expressed as g/l of gallic acid. The data are presented as means ± standard deviations for 3 experiments in triplicate.

2.4 Total flavonoid content

An aliquot (25 µl) of OMWW or gallic acid standard solution, appropriately diluted, was transferred to test tube containing 100 µl of water. At time zero, 7.5 µl of 5% (w/v) $NaNO_2$ were added; at 5 minutes 7.5 µl of 10% (w/v) $AlCl_3$ were added; finally, at 6 minutes 50 µl of 1 M NaOH were added. Each reaction mixture was diluted with water up to 250 µl and mixed [34]. The absorbance was measured at λ 510 nm with a microplate spectrophotometer reader (Synergy HT multi-mode microplate reader, BioTek, Milano, Italy) and compared against a catechin calibration curve (y= 0.0008x-0.0094, R^2 = 0,9968). The total flavonoid content was expressed as g/l of catechin. The data are presented as means ± standard deviations for 3 experiments in triplicate.

2.5 Oil residue content determination

10 ml of OMWW were extracted three times with 10 ml of hexane in a separator funnel. The organic phases were collected, dried over anhydrous Na_2SO_4, filtered off and evaporated under vacuum.

2.6 HPLC-DAD analysis

The chromatographic analysis of polyphenolic compounds was performed by HPLC-DAD (HITACHI) using a Kinetex C-18 (4.6x250mm, 5µm) column (Phenomenex) with a security guard cartridge (Phenomenex), thermostated at 30°C±1°C. The samples were eluted with water (A) and acetonitrile (B) both added with 0.1% trifluoroacetic acid (TFA) according to the following gradient: 100% A as initial condition, maintained for 5 minutes; 58% A in 25 minutes; 100% B in 15 minutes, maintained for 5 minutes. The flow rate was 0.8ml/min. The chromatograms were acquired at 280nm. The polyphenolic compounds were identified by comparison of retention times and UV spectra with the corresponding commercial standards: gallic acid, hydroxytyrosol, tyrosol, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid (PHPA), caffeic acid, vanillic acid, floretic acid, verbascoside, p-coumaric acid, trans-ferulic acid, oleuropein, catechol, 4-hydroxybenzoic acid, 4-methylcatechol, 3-hydroxyphenylpropionic acid, 3,4,5-trimethoxybenzoic acid and trans-cinnamic acid. A 5-points calibration curve of each standard was used for the quantification.

2.7 Antioxidant activity of OMWW

The antioxidant activity of OMWW was evaluated by DPPH assay [34-35]. The reaction mixture contained DPPH radical (86 µM) and different amounts of OMWW in 1 ml of ethanol to obtain concentrations of OMWW, expressed as µM of hydroxytyrosol, ranging from 1 to 100 µM. The samples were incubated for 10 minutes at room temperature, then the absorbance was measured at λ 517 nm with a microplate spectrophotometer reader (Synergy HT multi-mode microplate reader, BioTek, Milano, Italy). The results were expressed as percentage decrease in absorbance with respect to control. Hydroxytyrosol was used as standard. The data are presented as means ± standard deviations for 3 experiments in triplicate.

2.8 Stability studies

The stability of OMWW samples stored in the dark at different temperatures (-20°C, 4°C and 25°C) under aerobic conditions was evaluated by measuring total phenols, total flavonoids and hydroxytyrosol content as previously described. The data are presented as means ± standard deviations for 3 experiments in triplicate.

2.9 Statistical analysis

All technological treatments and analysis were done in triplicate. Analysis of variance (ANOVA) followed by Bonferroni's test was performed in order to estimate significant differences among samples. Data were reported as mean values ± standard deviations (SD). Differences between groups were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1 Pretreatment and physicochemical analysis

OMWW produced during the processing of Cerasuola and Nocellara etnea, two very important Sicilian olive cultivars, were sampled, during their formation, in the middle-late of the milling period; since they originated from a three phase continuous apparatus, samples contained solid residues which were eliminated by centrifugation and filtration. To reduce the formation of artifacts and to inhibit chemical modifications to the contained biophenols, samples were flash-freezed straight after collection and stored at -20 °C into airtight screw-capped tanks. Samples were treated in such a way as to prevent contacts with the ambient and analytical operations were carried out, when possible, under nitrogen or argon to avoid contacts with the atmospheric moisture. The pH of the OMWW were in the range 4.98-5.24, consistent with values (pH 3-6) reported by literature [36]. The fat content (mainly residual olive oil) was evaluated by extraction of a hexane soluble fraction and it was found to be, in both cases, less than 1% (based on the weight of the freeze-dried materials);

this value is strongly dependent on the olive oil production process which is used, on the preliminary operations that OMWW undergo (centrifugation and filtration of solid and floating materials) and on the solvent employed for the extraction (some authors report the use of petroleum ether). OMWW were preliminarily analyzed to estimate the polluting power: COD values were 73.6 g/l and 50.00 g/l respectively for Cerasuola-OMWW and Nocellara Etnea-OMWW, evidencing a high chemical load present in the waters. The main results arising from the physicochemical characterization are reported in Table 1. It is noteworthy that both OMWW contain important concentrations of potassium (in the range 4.73-7.38 g/l), suggesting this raw material as a potential source of food supplements in cases of low mineral salts intake.

TABLE 1

3.2 Polyphenolic characterization of OMWW

In general, OMWW are a complex matrix known for their very variable amounts of polyphenols ranging from 0.5 to 24 g/l [29]. Most of data literature report the polyphenolic composition of OMWW extracts obtained through membrane filtration systems, adsorption/desorption processes or solvent extraction [24, 28-29] but this approach could modify the original quali-quantitative profile; for this reason, we chose to perform a characterization of the untreated waters avoiding any process that could, even slightly, stress them. The total phenolic contents of the investigated OMWW samples was determined using the Folin-Ciocalteu assay and are reported in Table 2.

TABLE 2

These data indicate a coherence between the experimental values and those commonly found in literature and concerning different cultivars. It is worth noting that Cerasuola-OMWW present a total polyphenolic content (5.20 g/l) almost double compared to Nocellara etnea-OMWW (3.02 g/l). Analogous results have been obtained by comparing the total flavonoid content colorimetrically determined (Table 2).

Characterization of OMWW was carried out by reversed-phase (RP)-HPLC: among the several mobile phase gradients tested, the most suitable involved water and acetonitrile with 0.1% trifluoroacetic acid (TFA). Further decreasing the amount of TFA from 0.1:100 to 0.05:100 (with a slight increase of solvent pH) ended-up with a poor peak resolution and the retention time augmented for nearly all compounds by up to 1 min. The olive biophenols were identified using commercially available standards. The chromatographic retention times of standards and principal peaks detected in the matrix were highly reproducible (coefficient of variation, CV, smaller than 3.4% during the months this investigation occurred).

3-D diagrams of the diode array analyses (data not reported) and the chromatograms registered at specific wavelengths, depict how complicated are OMWW matrix and how troublesome is to report a large number of products at a few single wavelenghts. Recording spectra at 280 nm made possible to detect most of biophenols present in the matrix with acceptable intensity and signal to noise ratio (Figure 1).

FIGURE 1

The chromatograms highlighted a quantitative rather than a qualitative difference in the polyphenolic profiles of Cerasuola-OMWW and Nocellara etnea-OMWW. Moreover, a continuous low intensity band spreading along the chromatograms suggests the presence of a polyphenolic polymer as the origin of the OMWW dark pigmentation. The

quantitation of such complicated mixtures is a challenge; the concentration of the single species present in the samples was determined on the base of the corresponding peak area value related to a calibration curve obtained as depicted in the Materials and Methods section: hydroxytyrosol, tyrosol, caffeic acid, pyrocatechol, floretic acid, *p*-coumaric acid and *trans*-ferulic acid were identified and quantified as reported in Table 2.

Data show that hydroxytyrosol and tyrosol are the main phenolic constituents of both OMWW (Table 2), according to several previous reported investigations [29]. In particular, Cerasuola-OMWW showed the highest concentration of hydroxytyrosol (821.86 mg/l) and tyrosol (105.93 mg/l) in comparison to Nocellara etnea-OMWW. In contrast, caffeic acid, floretic acid and *p*-coumaric acid were most abundant in Nocellara etnea-OMWW. Verbascoside was identified in traces only in Cerasuola-OMWW.

However, the qualitative and quantitative profiles of the biophenolic content in OMWW from different sources change on a case by case basis. As an example: none of the principal biophenolic constituents found by Casa et al. [37] in an Italian OMWW (catechol, 4-hydroxybenzoic acid, 4-methylcatechol, 3-hydroxyphenylpropionic acid, 3,4,5-trimethoxybenzoic acid and trans-cinnamic acid) has been detected in the current analyzed samples. Furthermore, to underline the specificity of each biomass we can observe that oleuropein, (the ester of elenolic acid and hydroxytytrosol) already reported as the most abundant polyphenol in OMWW by some authors [6], is present only in small amounts (not quantifiable) in our samples; these differences in oleuropein concentrations are likely due to the different sampling period. In our case, olives were milled when mature and probably, at that time, most of oleuropein had already been metabolized into the corresponding phenolic and acidic precursors. As a support to this hypothesis, we found high amounts of hydroxythyrosol in both our OMWW. In addition, gallic acid and vanillic acid often found in other OMWW [24, 28], were not identified in any sample too.

3.3 Antioxidant activity of OMWW

The free radical scavenging activity of OMWW was evaluated measuring its ability to quench the stable DPPH radical. The results, expressed as percentage decrease in absorbance with respect to control, showed that both Cerasuola-OMWW and Nocellara etnea-OMWW were able to quench the DPPH radical in a dose dependent manner and had a higher radical scavenging activity than the positive standard hydroxytyrosol ($p < 0.05$), probably due to the synergistic effect of the other phenolic compounds (Figure 2).

FIGURE 2

More specifically, Cerasuola-OMWW showed a higher antioxidant activity with an IC_{50} value of 7.1 μM with respect to hydroxytyrosol and Nocellara etnea-OMWW, whose IC_{50} values were equal to 34.9 μM and 7.7 μM respectively; these results can be easily explained and reflect the differences in the total phenols and flavonoids content values previously reported.

3.4 Stability study

Polyphenols are highly unstable species which are involved in several biochemical and chemical reactions such as oxidation, condensation, polymerization and hydrolysis during food processing and storage [38-40]. In this study, we evaluated the effects of storage time and temperatures on the specific OMWW polyphenolic compositions by measuring the variations of total phenolic, flavonoid and hydroxytyrosol contents. In particular, OMWW samples stored at 4°C and 25°C were controlled for 45 days, whereas the monitoring of OMWW samples stored at -20°C was extended to 6 months.

No significant changes were observed with respect to total phenolic, flavonoid and hydroxytyrosol content in Cerasuola-OMWW and Nocellara etnea-OMWW samples stored at -20°C for 6 months (data not shown). On the other hand, the storage at 4°C and 25°C determined a decrease of total phenols and total flavonoids, especially in Nocellara etnea-OMWW, in a temperature- and time-dependent manner as shown in Table 3. Other authors evaluated the relative changes in the total phenols concentration in samples stored at different conditions, but they found only a very slight reduction ranging from 2% to 5% in samples stored at 4°C and 23°C under aerobic conditions [24]. Hydroxytyrosol concentration also decreased in Nocellara etnea-OMWW samples stored at 4°C and 25°C (Figure 3); in contrast, a slight increase was observed in Cerasuola-OMWW samples stored at 4°C, probably due to the hydrolysis of oleuropein and verbascoside. These results allowed us to establish the best storage conditions in order to minimize the loss of polyphenolic compounds, and imply the need of short storage periods in the olive mills collecting tanks (usually kept at room temperature) when a work-up of OMWW is expected.

TABLE 3

FIGURE 3

4. Conclusions

Nowadays, according to the modern paradigm "From waste to value", valorization is a widely accepted concept in the field of industrial residues management promoting the principle of sustainable development. One of the valorization objectives regarding food processing wastes is the recovery of fine chemicals and the production of precious pools of metabolites.

In this work, we have characterized for the first time OMWW from two local Sicilian olive cultivars (Cerasuola e Nocellara etnea) and highlighted that their polyphenolic profiles are suitable for a chemical exploitation aimed at (but not only) the production of nutraceuticals formulations. Our data indicate that OMWW could represent a potential source of molecular pools with antioxidant properties which could find application as nutraceuticals in the prevention and/or management of pathologies in which oxidative stress is involved. Moreover, in consideration of the reported value of hydroxytyrosol, the major phenolic component in our OMWW samples (1 g of pure substance is about $U.S. 1000-2000 for scientific/experimental purposes), a large scale extraction of this compound from easily available OMWW, would be an extremely valuable way to valorize this troublesome and polluting side stream. Indeed, after specific pretreatments with physical and biological agents followed by tailored recovery procedures, OMWW from Cerasuola and Nocellara etnea cultivars, might provide not just value-added natural antioxidants, but also sugars and mineral salts of great interest to the pharmaceutical, cosmetic and food industries.

Now, the main challenge is to efficiently integrate the biomass pretreatment and recovery of readily exploitable biogenic chemicals with successive bioconversion processes to fully exploit the matrix and obtain sequentially all the main classes of products, from fine and pharmaceutical/nutraceutical chemicals (which have higher market value) to bulk chemicals and eventually biofuels (which have lower market value).

Conflict of interest

The authors declare that they have no conflict of interest.

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	Unit	Cerasuola-OMWW	Nocellara etnea-OMWW
pH	-	4.98	5.24
COD	g/l	73.60	50.00
Total sugars	g/l	34.00	16.04
Total nitrogen	mg/l	350.00	116.00
Total phosphorous	mg/l	186.00	229.00
Metal:	mg/l		
▪ **Sb, Hg, As, Ag, Cd, Se, Bi, Be**		< 0.01	< 0.01
▪ **Mo**		0.01	0.02
▪ **Pb**		0.03	< 0.01
▪ **Al**		0.30	0.30
▪ **Cr**		0.02	0.01
▪ **Co**		0.02	0.01
▪ **Cu**		0.60	0.37
▪ **Zn**		3.00	2.28
▪ **Fe**		1.99	3.98
▪ **Mn**		2.17	1.24
▪ **Ni**		0.11	0.05
▪ **Na**		303.34	109.97
▪ **K**		7379.34	4732.58
▪ **Ca**		61.74	42.58
▪ **Mg**		240.56	161.41

2 **Table 1:** Physicochemical characterization of OMWW

1

	Unit	Cerasuola-OMWW	Nocellara etnea-OMWW
Total phenolic content	g/l gallic acid	5.20±0.21	3.02±0.18
Total flavonoid content	g/l catechin	2.28±0.23	0.95±0.17
Gallic acid	mg/l	not identified	not identified
Hydroxytyrosol	mg/l	821.86	267.17
Pyrocatechol	mg/l	6.59	traces
Tyrosol	mg/l	105.93	37.49
***p*-Hydroxybenzoic acid**	mg/l	not identified	not identified
4-Hydroxyphenylacetic acid (PHPA)	mg/l	traces	traces
Caffeic acid	mg/l	9.12	10.42
Floretic acid	mg/l	21.17	33.87
Verbascoside	mg/l	traces	not identified
***p*-Coumaric acid**	mg/l	4.27	14.44
***trans*-Ferulic acid**	mg/l	not identified	2.35
Oleuropein	mg/l	14.32	traces

2 **Table 2:** Total phenolic and flavonoid content of OMWW

	Days			
	0	**10**	**25**	**45**
Total phenols				
Cerasuola-OMWW (4°C)	100%	92.69%±0.79%[a]	79.16%±4.28%	76.84%±1.58%
Cerasuola-OMWW (25°C)	100%	81.09%±0.55%[a]	72.66%±3.15%	71.28%±5.96%
Nocellara etnea-OMWW (4°C)	100%	78.00%±1.17%[b]	68.85%±3.99%	67.44%±1.04%[b]
Nocellara etnea-OMWW (25°C)	100%	72.48%±1.44%[b]	65.65%±1.97%	64.52%±1.25%[b]
Total flavonoids				
Cerasuola-OMWW (4°C)	100%	81.57%±1.52%	78.36%±0.54%[c]	46.45%±3.30%
Cerasuola-OMWW (25°C)	100%	75.22%±2.33%	53.42%±1.78%[c]	41.59%±5.12%
Nocellara etnea-OMWW (4°C)	100%	59.30%±1.47%	56.34%±0.90%	56.34%±1.79%
Nocellara etnea-OMWW (25°C)	100%	58.88%±2.73%	58.88%±0.90%	57.61%±0.75%

Table 3: Relative changes in the concentration of total phenols and flavonoids determined in Cerasuola-OMWW and Nocellara etnea-OMWW during storage at 4°C and 25°C. Values with a common letter within a column are significantly different ($p < 0.05$).

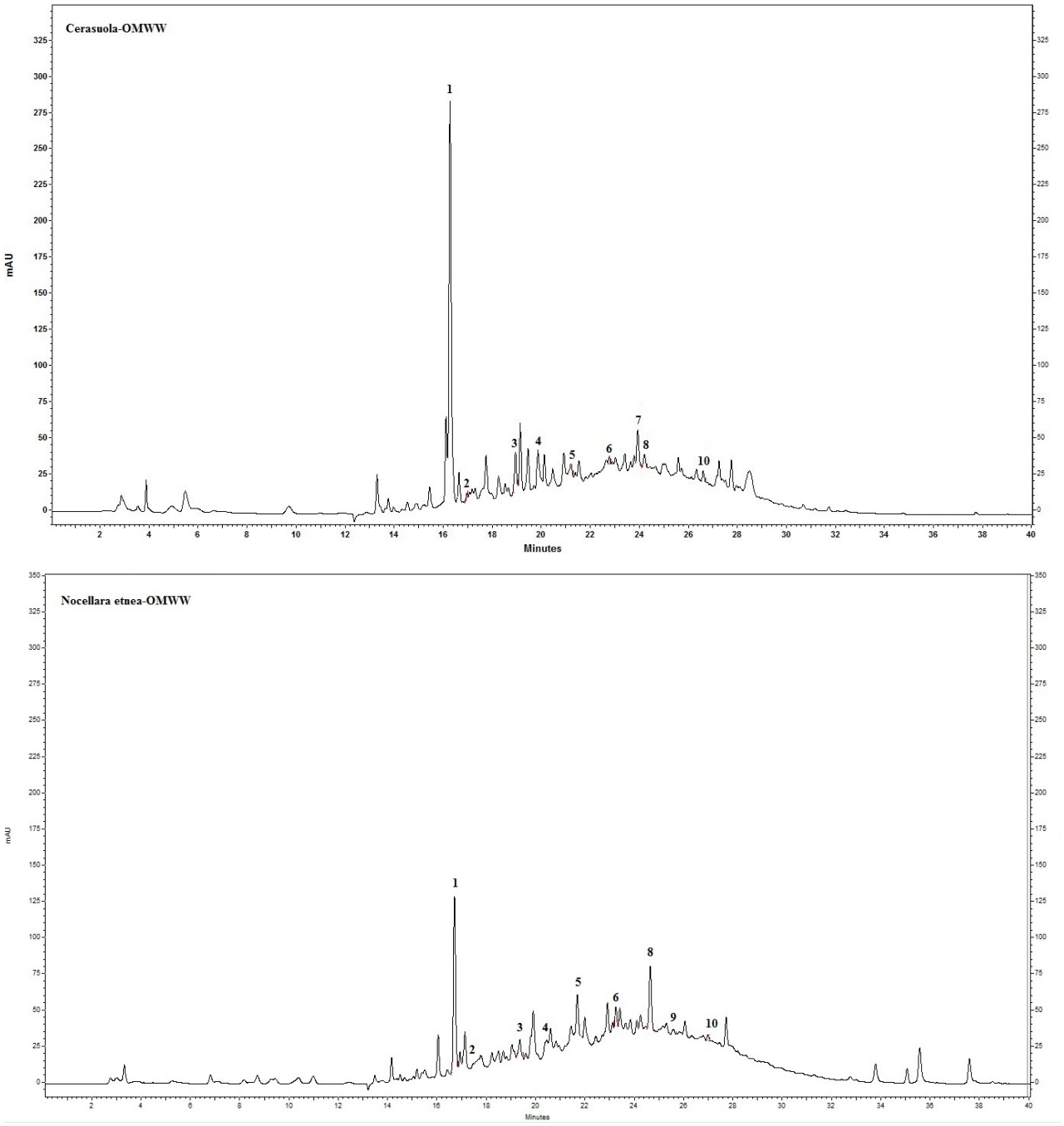

Figure 1: Chromatograms at 280 nm of Cerasuola-OMWW and Nocellara etnea-OMWW: 1) Hydroxytyrosol, 2) Pyrocatechol, 3) Tyrosol, 4) 4-Hydroxyphenylacetic acid (PHPA), 5) Caffeic acid, 6) Floretic acid, 7) Verbascoside, 8) *p*-Coumaric acid, 9) *trans*-Ferulic acid, 10) Oleuropein.

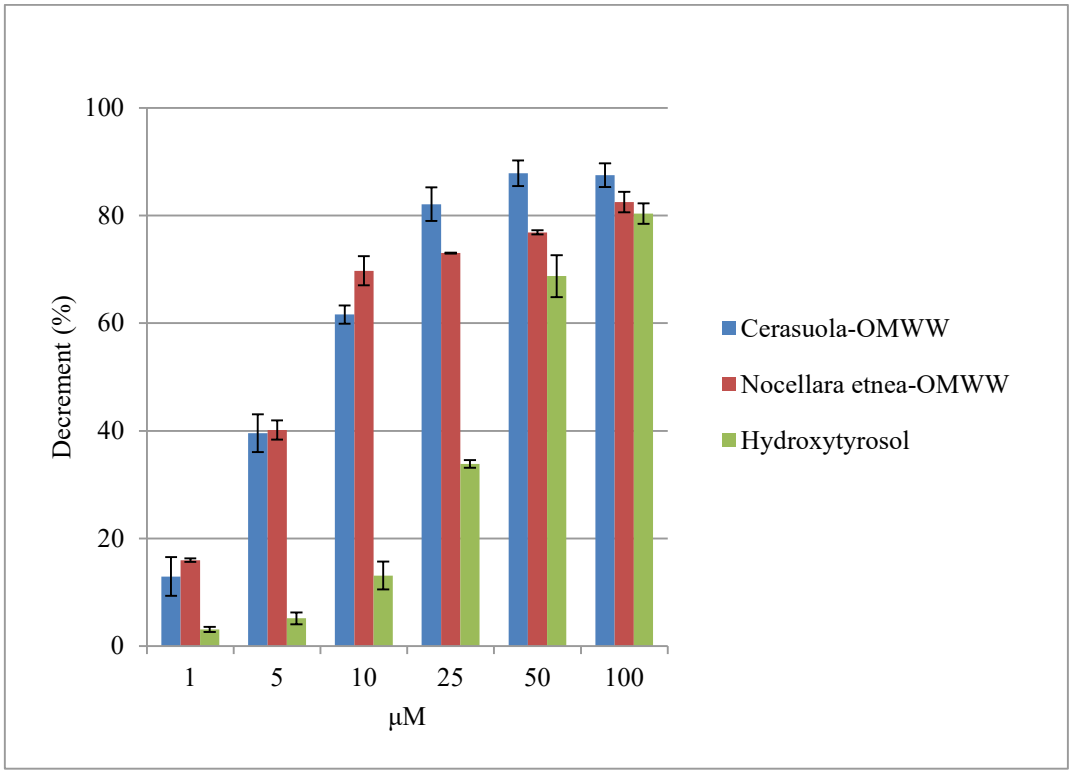

Figure 2: Antioxidant activity of Cerasuola-OMWW and Nocellara etnea-OMWW compared to hydroxytyrosol. The results are expressed as µM of hydroxytyrosol. Both Cerasuola-OMWW and Nocellara etnea-OMWW values in the range 1-50 µM are significantly different ($p < 0.05$) vs hydroxytyrosol.

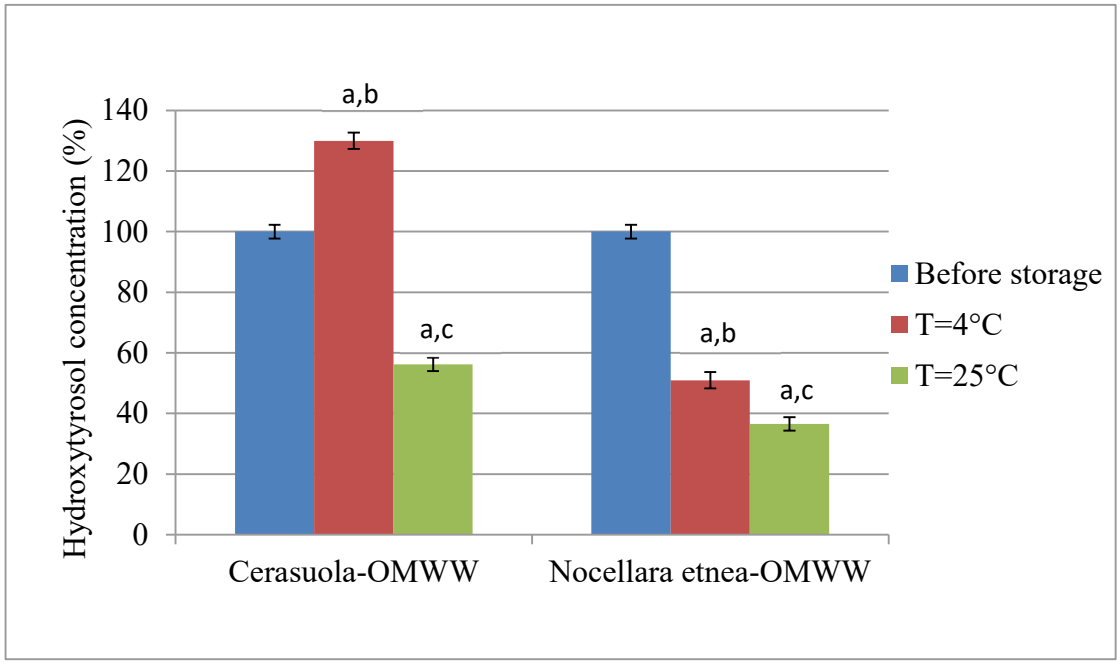

Figure 3: Relative changes in the hydroxytyrosol concentration determined in Cerasuola-OMWW and Nocellara etnea-OMWW after storage at 4°C and 25°C for 45 days. Values with letter "a" are significantly different ($p < 0.05$) vs hydroxytyrosol concentration before storage; values with other common letters ("b" or "c") are significantly different ($p < 0.05$).