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Effect of Processing Conditions, Prestorage Treatment, and Storage Conditions on the Phenol Content and Antioxidant Activity of Olive Mill Waste

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The impact of two- and three-phase processing systems and malaxation conditions on phenol content (both total and individual phenols) and antioxidant capacity of laboratory-generated olive mill waste (OMW) was assessed. Two-phase olive processing generated a waste with higher phenol content and antioxidant capacity. Using the two-phase system, both malaxation time and temperature affected the phenol content and antioxidant capacity. The effects of different prestorage drying treatments on phenol content and antioxidant capacity were also compared. Air drying and drying at 60 °C resulted in a substantial decrease in the phenol content and antioxidant capacity. Drying at 105 °C and freeze-drying produced less degradation. The phenol content and antioxidant capacity of OMW stored at 4 °C and of OMW preserved by 40% w/w ethanol and 1% w/w acetic acid and stored at 4 °C were monitored for 30 days and compared with those of OMW stored at room temperature. None of these storage conditions could prevent the rapid decrease in phenolic concentrations and antioxidant capacity, which happened within the first 24 h.

KEYWORDS: Phenol profile; total phenol; antioxidant activity; phenol recovery

INTRODUCTION

Olive mill waste (OMW) is the byproduct of processing olive fruits to produce virgin olive oil. The huge amounts of biodegradation-resistant OMW pose an economic and environmental burden on the olive oil industry (*1, 2*). This is further complicated by the seasonal nature and restricted discharge options of OMW. The noxious properties of OMW result essentially from its high phenol content, which ironically at the same time is reported to have myriad bioactivities, especially antioxidant activity. Up to now there is no widely adopted practice for managing this waste. Recently, production of bioactive compounds from OMW has attracted the interest of researchers and offered producers potential for a profit from a traditional economic burden (*3, 4*).

In our earlier research, we investigated various aspects of the recovery of phenols from Australian OMW and their stability during the extraction process and upon storage of OMW extracts (*5–7*). Other studies have emphasized different aspects of the recovery of phenols from OMW such as the stability of hydroxytyrosol (*8*) and recovery from the aqueous component of the waste (*9, 10*). To maximize the potential for future commercial-scale production of antioxidant phenols from Australian OMW, a number of issues must be addressed including olive oil processing conditions and preservation (including drying) and storage of the OMW prior to phenolic extraction, and these are the subject of this paper.

Olive mill wastes from two- and three-phase processing systems have been compared for their phenol content and antioxidant capacity (*11*), but there are no results available on the effect of malaxation conditions on OMW phenols. The high water content of OMW constitutes a challenge for any subsequent value-adding processes. Drying is often used for plant tissues with low moisture content, for example, leaves, and also as a preservation technique for fruit, although the chemical composition of dried plant material differs from that of the fresh material (*12*). Oven-drying and air-drying at ambient temperature are among the conventional sample pretreatments for phytomaterials. A stability study of phenols in their original sample matrix is a prerequisite for any decision taken toward adding value to OMW. Whether the value-adding facility is at the olive oil mill or off-site, for which transport would be required, the stability of phenols is crucial for determining storage and transfer conditions.

This study focuses on two-phase mill waste as this is the main waste-stream from Australian mills. Traditional three-phase processing was included for purposes of comparison. Laboratory-scale processing was employed as this facilitates control of variables and determination of potential recoveries under optimized conditions.

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MATERIALS AND METHODS

Samples. Olive fruits were harvested in June 2004/2005 from an olive grove at Cookathama near Darlington Point, NSW, Australia, and stored at 3 °C and processed within 48 h. The OMW samples were collected from a laboratory-scale olive oil mill (Abencor). OMW was instantaneously packed in airtight amber glass containers in triplicate, transferred on ice, and extracted within 1 h. The effects of malaxation conditions were examined in samples of Mission collected on June 9, 2004. Mission and Correggiola samples collected on June 6, 2005, were used to assess the impact of two- and three-phase processing systems. Samples of Correggiola collected on the same day were used to assess effects of prestorage drying and storage conditions.

Olive Oil Extraction Systems. Two methods were used for processing olive fruits in the current study using the same milling system (Abencor laboratory-scale olive mill). The main difference between the three-phase and two-phase methods was the amount of water added (vide infra).

Two-Phase Olive Oil Extraction. The method of Kalua et al. (*13*) was used without any modification. One kilogram of fruit was crushed by a hammer mill, and 100 mL of water was added during the malaxation to improve the rheology of the paste. A malaxation time of 45 min was ordinarily used unless otherwise specified (effect of malaxation time and temperature experiment) and resulted in two phases: the upper oil phase was decanted, and the lower paste-like pomace phase was analyzed. The pomace is described here as OMW or two-phase OMW (2P-OMW).

Three-Phase Olive Oil Extraction. The method of the Australian Oils Research Laboratory was used without any modification (*14*). Approximately 1 kg of olive fruit was ground to a paste using the hammer mill. Seven hundred grams of the paste was placed into a mixing jar and malaxed for 20 min at 25 °C in the thermomalaxer. Afterward, 300 mL of boiling water was added and remalaxed for a further 10 min. Centrifugation of the paste resulted in three phases: semisolid pomace, wastewater, and olive oil. The semisolid pomace only was analyzed in the current study and described as OMW or three-phase OMW (3P-OMW).

Effect of Malaxation Time and Temperature. The OMW from Mission fruits collected in June 2004 was processed under two-phase processing conditions at 15 °C for 30 min and also for 60 min and at 30 °C for 60 min.

Drying Treatments. *Air-Drying.* OMW samples were weighed in three preweighed large porcelain plates and spread over the surface to form a thin layer. The plates were stored in a clean dry place at ambient temperature (20 ± 2 °C) away from air drafts and direct sunlight. These plates were used to determine the change in moisture content of the OMW. Approximately 200 g of the OMW was spread over an aluminum tray and placed next to the plates. Three aliquots each of 10 g of OMW were taken at every sampling point (7, 15, and 30 days) and extracted as described below to obtain extracts from air-dried waste at ambient temperature.

Oven-Drying at 60 and 105 °C. Four aliquots each of 30 g of OMW were weighed in porcelain dishes and placed in a forced-convection oven adjusted to the required temperature. The weight was checked regularly until the change in dry weight between measurements was less than ±0.05%.

Freeze-Drying. OMW samples (200 g) were placed in a Dynavac FD12 freeze-dryer. Freeze-drying required 2 weeks to reach minimum moisture content.

Storage Treatments. *Room Temperature Storage.* OMW was stored in screw-capped amber glass containers. The containers were kept at ambient temperature on a laboratory shelf. Sampling of the stored material was performed by removing the upper layer with a spatula and weighing 10 g from the bulk of the sample.

Storage at 4 °C. OMW samples in clean and dry amber glass jars with screw-capped lids were stored in a refrigerator at 4 °C. Sampling was performed as above.

Preserved Sample. OMW (200 g) was weighed and mixed with 80 g of ethanol and 2 g of glacial acetic acid. The mixture was stored in screw-capped amber glass containers at 4 °C.

Extraction of Phenols. Ten grams of the fresh OMW or 5 g of dried OMW was extracted. OMW was extracted with methanol/water/HCl (80:20:1; 15 mL) for 30 min with stirring. After recovery of the extract, the process was repeated for 15 min with fresh solvent (10 mL). The combined extracts were filtered through Whatman no. 1 filter paper and defatted by *n*-hexane (30 mL × 2). The defatted extract was filtered through GF/F filter paper and then refiltered using 0.2 μm nylon nonsterile syringe filters (Phenomenex). All extractions were performed at room temperature (20 ± 2 °C). The crude extracts were stored at −18 °C until analyzed.

Dry Weight, Extractable Matter, and pH. These were determined as described earlier (*5*).

Folin−Ciocalteu Total Phenols. One milliliter of crude extract was volumetrically diluted to 10 mL with water. The diluted extract was used for determination of total phenols as described earlier (*5*). Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg of GAE/g of DW).

Phenolic Profiling and Recovery of Individual Phenols. HPLC-DAD was performed with a Varian 9021 solvent delivery system equipped with a Varian 9065 Polychrom UV diode array detector (190−367 nm). Separation was performed by gradient elution on a Luna C-18(2) column, 5 μm particle size (150 mm × 4.6 mm) (Phenomenex) attached to a SecurityGuard guard cartridge (Phenomenex). Analysis conditions were described previously (*5*). Individual phenols [hydroxytyrosol glucoside, hydroxytyrosol, verbascoside, *p*-coumaroyl-6′-secologanoside (comselogoside), and luteolin] were quantified using peak areas at 278 nm and standard curves of commercially available reference compounds.

Antioxidant Capacity. Antioxidant capacity was determined using DPPH radical scavenging assay as described previously (*6*). EC_{50} values were expressed as parts per million (μg/mL) of extractable matter, and then antioxidant capacity was calculated as $100/(EC_{50})$. The expression of results as antioxidant capacity facilitated graphical presentation and sample comparisons; hence, antioxidant capacity was directly proportional to activity unlike EC_{50}.

Data Treatment. Sampling was performed in triplicate, and at least duplicate samples were analyzed. Data were expressed as means ± standard deviations. Data analysis was performed by Microsoft Excel. One-way ANOVA was carried out to test for significant differences using SPSS 11.5 (SPSS Inc., Chicago, IL). Results were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Impact of Olive Oil Extraction Conditions. *Three-Phase versus Two-Phase Processing.* Technically, the difference between three-phase and two-phase processes resides in the amount of processing water required (*1*). For three-phase processing, the method of Mailer et al. (*14*) was applied (ca. 45% water). The method of Kalua et al. (*13*) was used for two-phase processing (10% water). The difference in phenol content between waste generated by two-phase and three-phase processes was found to be a quantitative rather than qualitative one (*11*).

In the current study (**Table 1**), the two-phase OMW (2P-OMW) had higher dry weight, extractable matter, total phenol (ca. 1.6-fold), and antioxidant capacity (ca. 1.2-fold). At the level of individual phenols, the recovery of all investigated phenols was higher from 2P-OMW (1.3−2.0-fold) than from 3P-OMW (**Table 1**). Lessage-Meessen et al. recorded a 1.4-fold increase in the total phenol content and a 2-fold increase in antioxidant capacity of the French 2P-OMW compared with 3P-OMW (*11*). The total phenol content was found to differ between 2P-OMW and 3P-OMW depending on the degree of maturation. Gimeno et al. reported a 1.7-fold increase in green maturation and a 1.9-fold increase in black maturation of total phenols (*15*). However, the discrepancy between total phenol content and antioxidant capacity in the present study and Lessage-Meesen's findings is most probably due to experimental

Table 1. Effect of Extraction System on Phenol Content and Antioxidant Activity of Olive Mill Waste Extracts

	CR-3P[h]	CR-2P	MS-3P	MS-2P
dry weight (DW) [a]	33.5 ± 0.9a	42.0 ± 2.0b	33.3 ± 2.0a	35.3 ± 2.7a
extractable matter[b]	195.5 ± 8.8a	264.0 ± 3.7b	231.2 ± 13.0c	335.5 ± 24.7d
Folin−Ciocalteu total phenols[c]	22.9 ± 0.5a	39.5 ± 2.0b	32.9 ± 2.0c	49.9 ± 3.5d
antioxidant capacity[d]	4.73 ± 0.07a	5.69 ± 0.12b	5.73 ± 0.02b	7.25 ± 0.12c
hydroxytyrosol glucoside[e]	0.62 ± 0.01a	0.97 ± 0.08b	2.75 ± 0.24c	4.05 ± 0.12d
hydroxytyrosol [f]	0.45 ± 0.06a	0.64 ± 0.11b	0.84 ± 0.10b	1.32 ± 0.11c
verbascoside[f]	0.92 ± 0.01a	1.23 ± 0.02b	1.57 ± 0.09c	3.19 ± 0.66d
oleuropein[f]	0.06 ± 0.01a	0.10 ± 0.01b	0.11 ± 0.02b	0.15 ± 0.01c
comselogoside[g]	0.06 ± 0.01a	0.08 ± 0.01b	0.31 ± 0.01c	0.46 ± 0.09d
luteolin[f]	0.23 ± 0.01a	0.24 ± 0.01a	0.19 ± 0.01a	0.28 ± 0.06b

[a] Percent w/w fresh weight. [b] Milligrams per gram of DW. [c] Milligrams of GAE per gram of DW. [d] Antioxidant capacity = 100 × 1/EC_{50} (ppm). [e] Milligrams of hydroxytyrosol equivalent/g of DW. [f] Milligrams per gram of DW. [g] Milligrams of *p*-coumaric acid equivalent/g of DW. [h] CR, Correggiola cultivar; MS, Mission cultivar; 3P, three-phase olive oil extraction conditions; 2P, two-phase olive oil extraction conditions; 2004, samples collected in June 2004 season; 2005, samples collected in June 2005; different letters in the same row indicate significantly different ($p < 0.05$) mean ± standard deviation of duplicates.

Table 2. Effect of Different Malaxation Temperatures and Times on OMW [Generated from Mission Cultivar Harvested in June 2004 (MS 2004)] Composition and Antioxidant Activity

	MS 2004[h]		
	15 °C and 30 min	15 °C and 60 min	30 °C and 60 min
dry weight (DW)[a]	35.4 ± 2.1a	34.7 ± 1.6a	36.0 ± 1.8a
extractable matter[b]	370.6 ± 25.0a	380.8 ± 22.8a	320.2 ± 22.4b
Folin−Ciocalteu total phenols[c]	44.9 ± 1.4a	49.5 ± 0.1b	39.7 ± 1.2c
antioxidant capacity[d]	3.54 ± 0.14a	4.30 ± 0.09b	3.04 ± 0.06c
hydroxytyrosol glucoside[e]	1.68 ± 0.07a	2.11 ± 0.01a	1.96 ± 0.20a
hydroxytyrosol[f]	0.52 ± 0.01a	0.49 ± 0.01a	*i*
verbascoside[f]	2.61 ± 0.28ab	3.22 ± 0.24a	2.20 ± 0.24b
comselogoside[g]	0.47 ± 0.08ab	0.35 ± 0.06a	0.54 ± 0.07b
luteolin[f]	0.51 ± 0.01a	0.83 ± 0.03b	0.22 ± 0.03c

[a] Percent w/w fresh weight. [b] Milligrams per gram of DW. [c] Milligrams of GAE per gram of DW. [d] ARE = 100 × 1/EC_{50} (ppm). [e] Milligrams of hydroxytyrosol equivalent per gram of DW. [f] Milligrams per gram of DW. [g] Milligrams of *p*-coumaric acid equivalent per gram of DW. [h] Different letters in the same row indicate significantly different ($p < 0.05$) mean ± standard deviation of duplicates. [i] Peak was not detected.

differences. The hydroalcoholic extraction solvent used here tends to extract polymeric compounds, which add to the total phenol content but have little effect on antioxidant capacity, or hydrophilic nonphenolic reducing compounds that interfere with Folin−Ciocalteu reagent (*5*).

Malaxation Conditions (Time and Temperature). Malaxation is the process of slow kneading of crushed olive paste in a special mixer (malaxer) for certain times (30−60 min) to allow coalescence of small oil droplets into larger ones and subsequent formation of a separable oil phase (*13*). Malaxation conditions (time and temperature) are known to affect the olive oil quality and yield (*13*). There are no universal optimum malaxation time and temperature for oil production; rather, the optimum temperature was found to be cultivar dependent (*16*).

OMW samples of Mission milled by the two-phase process at different malaxation conditions were collected. Three different treatments were assessed: malaxation at 15 °C for 30 min, malaxation at 15 °C for 60 min, and malaxation at 30 °C for 60 min. The dry weight did not change significantly among the three treatments (**Table 2**). The extractable matter decreased significantly when the malaxation temperature was increased (**Table 2**). This decrease can be attributed to the increased partitioning of the more lipophilic components into olive oil by increasing malaxation temperature, as under two-phase conditions the oil yield was enhanced by raising the malaxation temperature (*13*). The highest total phenol content and antioxidant capacity were found for malaxation at 15 °C for 60 min (**Table 2**). Nonetheless, at the level of individual phenols the picture is more complicated; the recovery depends on relative thermal, enzymatic, and autoxidative stability in addition to the physicochemical properties of the phenols (solubility and partitioning) (*17*). Hydroxytyrosol recovery changed insignificantly when the malaxation time was increased, whereas complete degradation was observed at higher temperature (30 °C). At the same time, hydroxytyrosol glucoside was highly stable and its concentration was not affected by the malaxation time or malaxation temperature (**Table 2**). Increasing malaxation time enhanced the recovery of luteolin at 15 °C, whereas increasing the temperature (30 °C) caused a reduction in recovery. The change in verbascoside and comselogoside recovery with malaxation time was statistically insignificant (**Table 2**). Increasing the malaxation temperature (at malaxation time = 60 min) significantly increased the recovery of comselogoside and significantly decreased the recovery of verbascoside. In summary, both malaxation time and temperature affected the phenolic content of OMW. However, the malaxation temperature effect was more significant.

Drying Treatments. Analysis of fresh plant materials is always the ideal situation for determination of the phenolic composition of plant samples, followed by freeze-drying or freezing (*18*). When storage is required for an extended period, as anticipated upon commercialization of OMW extraction, drying is needed to quickly halt or slow the degrading enzymes and also to facilitate the transport and storage of high moisture content OMW. Rapid drying at high temperature quickly inactivates enzymes, but it may lead to degradation of thermolabile phenols.

Air-drying at ambient temperature (21 ± 1 °C), oven-drying at 60 °C, oven-drying at 105 °C, and freeze-drying after flash-freezing (liquid nitrogen) were compared with the freshly analyzed OMW sample from Correggiola. The moisture content for different drying methods was monitored periodically, and drying was stopped upon reaching constant weight. Drying kinetics showed a biphasic behavior, a rapid sharp decrease in the moisture content followed by a slow elongated decrease to a stable level. Drying at 105 °C was stopped after 12 h (the plateau was reached after 8 h); drying at 60 °C was stopped after 36 h; air-drying was stopped after 30 days (the plateau was reached after 7 days); freeze-drying took 2 weeks. The reference dry weight used was that obtained from drying at 105 °C for 12 h (assuming complete removal of moisture). Other

Table 3. Effect of Different Prestorage Drying Processes on Phenol Content and Antioxidant Activity of Correggiola 2P-OMW

	fresh[h]	drying at 105 °C (12 h)	drying at 60 °C (36 h)	air-drying (1 week)	air-drying (1 month)	freeze-drying
dry weight (DW)[a]	42.0 ± 0.1a		44.0 ± 0.4b	49.7 ± 3.6c	49.1 ± 3.5c	46.0 ± 1.8d
extractable matter[b]	264.0 ± 3.7a	146.8 ± 10.5b	201.9 ± 5.4c	264.5 ± 19.3a	244.2 ± 5.5a	263.2 ± 9.6a
Folin−Ciocalteu total phenols[c]	39.9 ± 2.0a	14.7 ± 0.1b	12.1 ± 0.7c	9.3 ± 0.9d	5.7 ± 0.3e	29.0 ± 1.1f
antioxidant capacity[d]	5.69 ± 0.12a	3.91 ± 0.10b	1.83 ± 0.10c	0.92 ± 0.02d	0.72 ± 0.01e	4.72 ± 0.14f
hydroxytyrosol glucoside[e]	0.97 ± 0.08a	0.33 ± 0.05b	0.37 ± 0.03b	0.16 ± 0.02c	NA	0.34 ± 0.06b
hydroxytyrosol[f]	0.64 ± 0.11a	1.25 ± 0.06b	0.11 ± 0.01c	NA	NA	0.89 ± 0.06d
verbascoside[f]	1.23 ± 0.02b	0.61 ± 0.02b	0.16 ± 0.02c	0.038 ± 0.001d	NA	2.05 ± 0.06e
comselogoside[g]	0.080 ± 0.01a	0.044 ± 0.003b	0.048 ± 0.010b	0.017 ± 0.001c	NA	0.085 ± 0.010a
luteolin	0.239 ± 0.010a	0.015 ± 0.003b	0.096 ± 0.014c	0.077 ± 0.009c	NA	0.156 ± 0.036d

[a] Percent w/w fresh weight. [b] Milligrams per gram of DW. [c] Milligrams of GAE per gram of DW. [d] Antioxidant capacity = 100 × 1/EC_{50} (ppm). [e] Milligrams of hydroxytyrosol equivalent per gram of DW. [f] Milligrams per gram of DW. [g] Milligrams of *p*-coumaric acid equivalent per gram of DW. [h] Different letters in the same row indicate significantly different ($p < 0.05$) mean ± standard deviation of at least duplicates.

drying methods removed less water: 96% for drying at 60 °C; 91% for freeze-drying; and 87% for air-drying.

All of the applied drying methods, including freeze-drying, resulted in a significant degradation and loss of antioxidant capacity compared with the fresh sample (**Table 3**). The least decreases in total phenols and antioxidant capacity were reported for freeze-drying. Drying at 105 °C had significantly higher total phenols and antioxidant capacity than drying at 60 °C. The lowest recovery of phenols and the largest drop in antioxidant capacity were found for air-drying.

Drying at 105 °C for 12 h resulted in the lowest recovery of extractable matter, which may be due to degradation of thermolabile constituents, loss of volatile compounds by evaporation, or formation of insoluble oxidation and polymerization products. Total phenol content was decreased to 37% of the fresh OMW content and antioxidant capacity was 69% of the fresh OMW (**Table 3**). It may be inferred that thermal degradation of some large molecular weight phenols generated more active small molecular weight antioxidants that compensated for the sharp decrease in the total phenol content. Whereas a reduction in the recovery of hydroxytyrosol glucoside, verbascoside, and comselogoside was in accord with total phenol reduction (50−60%), luteolin was the most thermolabile of the studied phenols (93% reduction) (**Table 3**). In contrast, the recovery of hydroxytyrosol was nearly doubled (**Table 3**), most likely due to the hydrolysis of hydroxytyrosol-containing compounds. Although hydroxytyrosol was totally degraded when the malaxation temperature was increased to 30 °C for 1 h (vide supra), hydroxytyrosol had good stability at 105 °C.

Oven-drying at 60 °C for 36 h gave an amount of extractable matter intermediate between those for fresh and oven-drying at 105 °C (**Table 3**). Both total phenol content and antioxidant capacity had significantly decreased compared to the fresh OMW and OMW dried at 105 °C. Drying at 60 °C was expected to produce less thermal degradation than drying at 105 °C, but the extended drying time (36 h) allowed substantial aerial oxidation to take place, and enzymatic degradation could have occurred in the lag phase before enzyme inactivation (i.e., longer lag period before inactivation at 60 cf. 105 °C). Unlike oven-drying at 105 °C, the decrease in antioxidant capacity (32%) was paralleled by a decrease in the total phenol recovery (30%). All phenols experienced a sharp decrease in their recovery for samples dried at 60 °C compared with fresh OMW (40−87%). Compared to drying at 105 °C, more degradation of hydroxytyrosol and verbascoside was observed upon drying at 60 °C. On the contrary, the recovery of hydroxytyrosol glucoside and comselogoside was somewhat enhanced for samples dried at 60 °C (although not statistically significant). Only the recovery of luteolin was significantly increased for drying at 60 versus 105 °C.

Air-drying was the least efficient in decreasing the water content of OMW (86% after 1 week). Extractable matter content was not significantly different from that of fresh OMW even after 30 days. Air-drying allows slow withdrawal of moisture at ambient temperature, which maintains the action of degrading enzymes until the water content and a_w are critically low. After 1 week, the total phenol content was 25%, and antioxidant capacity was around 16%, of the starting value of fresh OMW. After 30 days, the total phenol content reached 14% and the antioxidant capacity reached 13%. Only a broad hump of polymeric substances appeared in the chromatograms at 280 nm (**Figure 1**) with peaks due to individual phenols completely absent.

Freeze-drying is considered as the first alternative, if analysis of the fresh sample is not possible (*18*), and would be expected to be the method most likely to preserve commercially relevant phenols prior to extraction. The present study revealed that a residual amount of moisture, ca. 9%, was not removed by freeze-drying compared with drying at 105 °C. Thus, the use of freeze-dried weight is expected to underestimate the amount of phenols when recoveries are expressed per freeze-dried weight instead of dry weight. Whereas the best recoveries after the fresh OMW could be achieved only by freeze-drying, a 27% reduction of total phenols and a 17% reduction of antioxidant capacity were found (**Table 3**). The recovery of hydroxytyrosol glucoside was not significantly improved compared with drying at 105 and 60 °C (**Table 3**). The increased recovery of hydroxytyrosol can be explained by hydrolysis of hydroxytyrosol-containing higher molecular weight phenols, whereas the increase in the recovery of verbascoside upon freeze-drying is most unlikely to result from degradation of higher molecular weight verbascoside derivatives (*7*). Possible explanations for this increase include the presence of considerable amounts of verbascoside in fruit compartments that were made more accessible for extraction solvent through freeze-drying or through enhanced solubility or increased surface area known for lyophilized powder. Above all, freeze-drying efficiently preserved verbascoside. Comselogoside was highly stable under freeze-drying conditions (**Table 3**). The recovery of luteolin was significantly increased compared to other drying methods.

Visual comparison of the chromatograms generated at 278 nm (**Figure 1**) reflects the quantitative changes discussed above. For oven-drying at 105 °C, two new peaks were observed that were not present in fresh OMW (**Figure 1A**): *p*-coumaric acid [**Figure 1B**(b)], a hydrolysis product of comselogoside; and an unidentified hydroxytyrosol derivative [**Figure 1 B**(c)], most probably resulting from the hydrolysis of oleuropein derivatives [3,4-dihydroxyphenylethyl alcohol−deacetoxy elenolic acid dialdehyde (3,4-DHPEA-DEDA) and hydroxytyrosol acyclodihydroelenolate (HT-ACDE), peak (a) in **Figure 1A**,**E**]. Broad

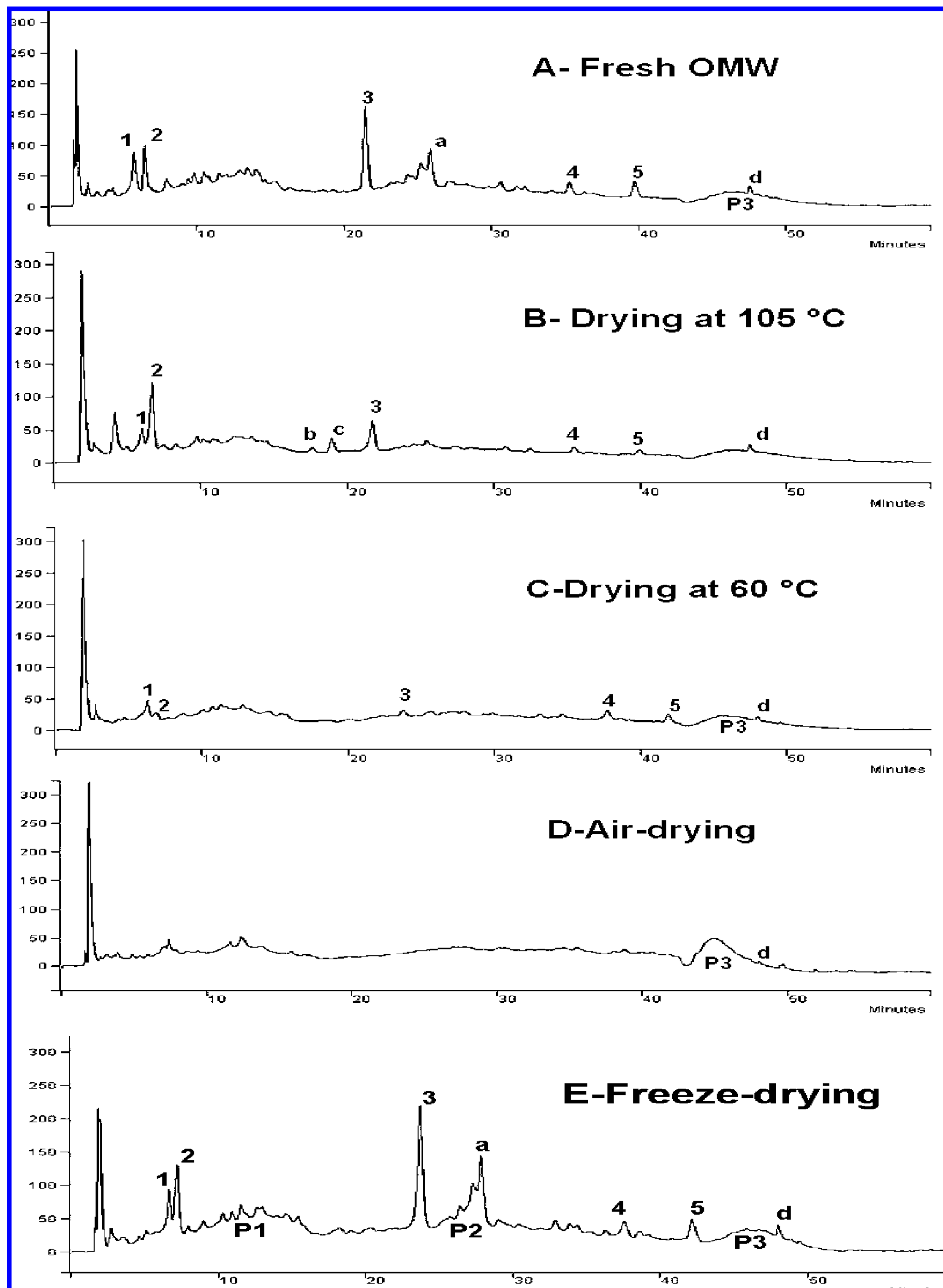

Figure 1. Effect of different drying conditions [105 °C for 12 h; 60 °C for 36 h; air-drying at room temperature (21 °C) for 30 days; freeze-drying for 2 weeks] on phenolic profiles of chromatograms at 278 nm: (1) hydroxytyrosol glucoside; (2) hydroxytyrosol; (3) verbascoside; (4) comselogoside; (5) luteolin; (a) coeluting peaks (oleuropein derivatives and rutin); (b) *p*-coumaric acid; (c) unidentified hydroxytyrosol derivative; (d) detergent artifact; (P1, P2, and P3) polymeric compounds.

peaks designated P1, P2, and P3 (**Figure 1D**,**E**) were previously reported in freeze-dried Frantoio OMW (*5*). The P3 hump increased greatly under air-drying (**Figure 1D**) conditions, suggesting aerial oxidation products. Although P3 was detected in the fresh OMW chromatogram (**Figure 1A**), P1 and P2 were significantly enhanced by freeze-drying, which suggests that they are artifacts (**Figure 1E**).

The less expensive drying options (air-drying or drying at 60 °C) resulted in a substantial decrease in the phenol content and antioxidant capacity. The more expensive drying techniques (drying at 105 °C and freeze-drying) suffered less degradation, but they may not be commercially viable.

Storage Treatments. Freezing is the most common practice to reduce or stop enzyme activity for plant samples (*18*). Due

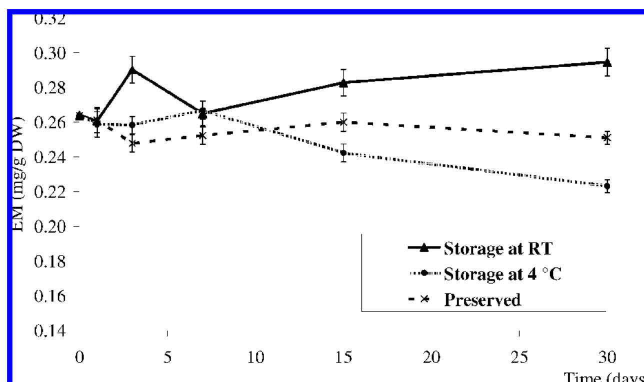

Figure 2. Change of extractable matter (EM) recovery from OMW stored under different storage conditions. DW, dry weight.

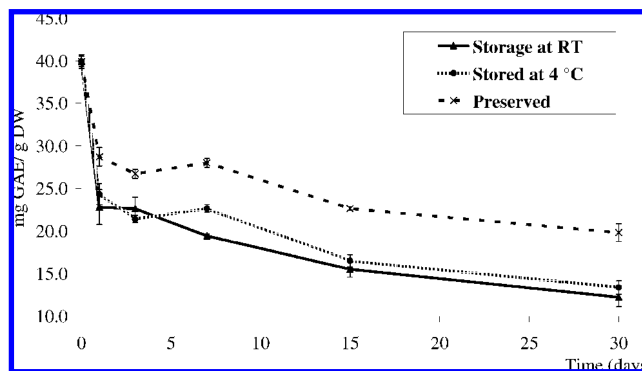

Figure 3. Change of total phenol content in OMW stored under different storage conditions. GAE, gallic acid equivalent; DW, dry weight.

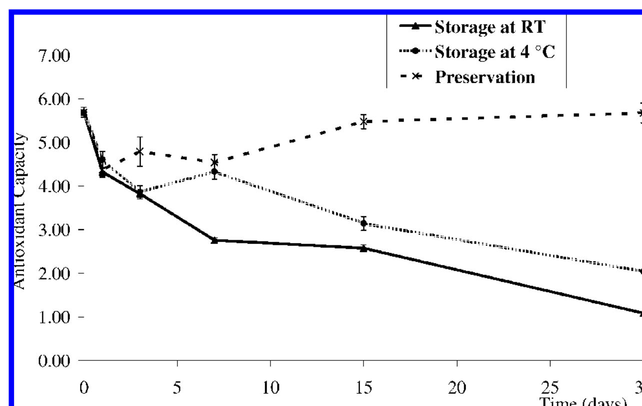

Figure 4. Change of antioxidant capacity in OMW stored under different storage conditions.

to higher costs associated with freezing and the subsequent need for thawing of frozen materials, cooling at 4 °C was chosen as a more convenient and economic solution in this study. Samples with high moisture content such as fruits may be preserved (pickled) using alcohol (*18*). Lessage-Meessen et al. used 30% v/v ethanol to stabilize OMW samples and claimed long-term stability of phenols at 4 °C in dark storage (*11*). More recently, hydroxytyrosol concentration has been stabilized in fresh olive mill wastewaters by the addition of 10% ethanol, although the concentrations of other phenols decreased during storage (*8*). On the other hand, hydroxytyrosol concentration increased during storage when 0 or 5% ethanol was added to the waste. Hydroxytyrosol is a major phenol in many OMW samples, and stability data are seemingly contradictory. However, data collected in such samples represent the hydroxytyrosol concentration at a particular point in time resulting from various hydrolytic, oxidative, and other reactions that potentially lead to both formation and degradation of hydroxytyrosol. Thus, they cannot be used to infer information on the stability of hydroxytyrosol. Indeed, many data describe hydroxytyrosol as chemically unstable unless preserved dried in the absence of air and in the dark (*19, 20*). It has also been reported that isolated pure hydroxytyrosol was stable for 5 days under ambient temperature, exposed to light, and in a direct continuous air current (*21*).

Extractable Matter. A significant change was observed in the amount of extractable matter recovered per dry weight of OMW after 30 days (**Figure 2**). Storage at room temperature for 30 days increased the recovery of extractable matter by 12%, whereas storage at 4 °C and preservation at 4 °C decreased the recovery of extractable matter by 15 and 5%, respectively (**Figure 2**).

The increase in the recovery of extractable matter for samples stored at room temperature can result from biotransformation, olive fruit enzymatic activity, or chemical transformations (aerial oxidation). The storage in airtight containers may limit the effect of autoxidation to the surface layer, a phenomenon that could be observed from the dark brownish black surface layer compared to the light brown color of the bulk. Because no preservative was added to the samples stored at room temperature, microbial growth was expected. Fungal growth could be observed after 2 week and covered the whole surface after 15 days in most containers, with a strong foul odor. Mycelia of three different colors (white, brown, and green) were observed on the surface layer after 2 weeks, and black spores were noticed after 30 days. Large numbers of fungi (*22*) and bacteria (*23*) have been separated from OMW. The surface layer was scratched, and sampling was always done from the core to minimize any potential effects of aerobic fungal growth on phenol content. However, the metabolic effect of bacteria and anaerobic fungi was unavoidable.

Total Phenols. A significant drop (30−40% decrease) in the total phenol content was observed after 24 h under all storage conditions tested (**Figure 3**). Storage at 4 °C did not offer any advantage over storage at room temperature, whereas the preserved sample showed higher recovery of total phenols. After 30 days, 70% of total phenols were lost for samples stored both at room temperature and at 4 °C, and 50% loss was found for the preserved sample.

Phenols are reactive chemical compounds that can undergo a vast array of reactions in an OMW matrix (*3*). Cooling and acidification are known to improve the stability of phenols. Neither of these practices, however, could stop the loss of total phenols under the current experimental conditions. On the other hand, acidification and preservation with alcohol significantly improved the recovery compared to cooling alone.

Antioxidant Capacity. Antioxidant capacity under the three storage conditions was sharply decreased to 80% of the fresh sample after 24 h (**Figure 4**). The decrease was again not parallel to the decrease in the total phenols (vide supra). All storage conditions resulted in a decrease in the antioxidant activity in the first week. The antioxidant capacity of the preserved samples increased gradually, reaching the same activity of the fresh sample. Samples stored at room temperature had only 20% of fresh sample activity after 30 days. Storage at 4 °C significantly preserved activity over storage at room temperature after 30 days (36%).

Recovery of Individual Phenols. Storage at room temperature for 30 days significantly decreased the recovery of all studied phenols. Storage of OMW at 4 °C significantly protected

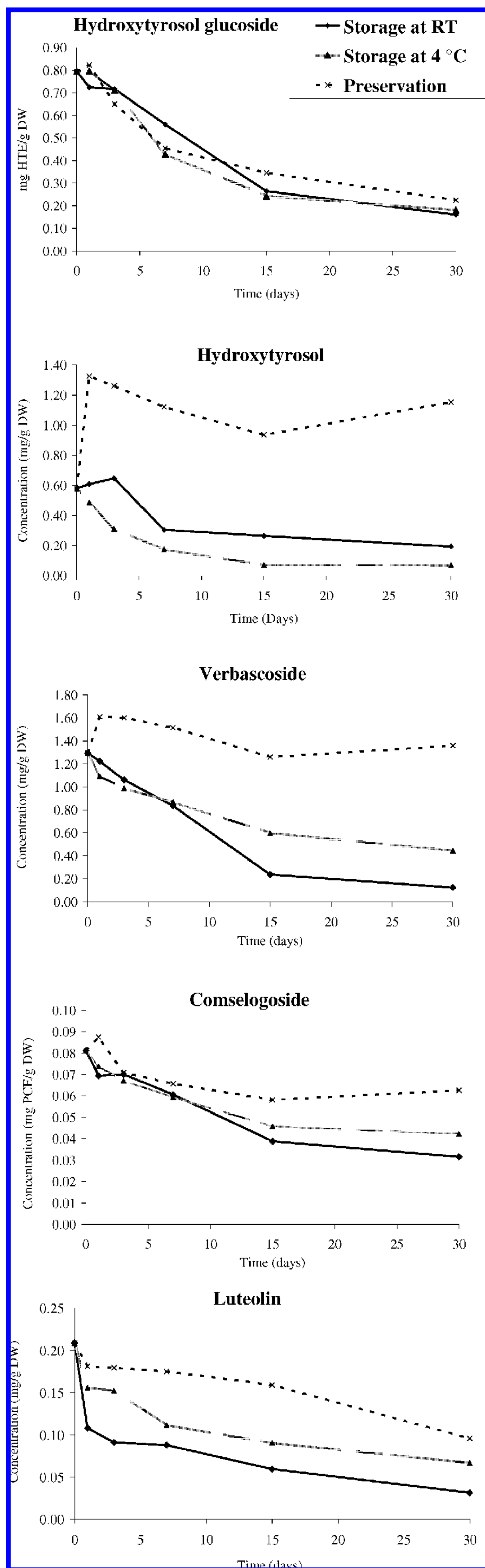

Figure 5. Changes of recovery of individual phenols from OMW stored under different conditions quantified by HPLC-DAD. RT, room temperature; HTE, hydroxytyrosol equivalent; PCE, *p*-coumaric acid equivalent; DW, dry weight; results are average of triplicate analyses [relative standard deviation (RSD) < 10%].

verbascoside, comselogoside, and luteolin relative to storage at room temperature. Preserved samples kept at 4 °C had a higher recovery of all studied phenols except for hydroxytyrosol glucoside.

Hydroxytyrosol glucoside underwent a gradual degradation under all of the tested storage conditions. Neither cooling at 4 °C nor preservation improved its recovery at all sampling points (**Figure 5**). Only 25% of the starting concentration could be recovered irrespective of the applied storage conditions after 30 days. Comselogoside degraded under the three storage conditions during the first week. After 15 days, higher recoveries were reported for preservation and storage at 4 °C compared to storage at room temperature (**Figure 5**). It was possible to recover 77% of the starting concentration of comselogoside after 30 days for preserved samples compared with 39% for room temperature stored samples.

Luteolin gradually degraded under all storage conditions, although preservation and storage at 4 °C resulted in higher recoveries than storage at room temperature at all sampling points (**Figure 5**). After 30 days, only 15% of the original fresh sample starting concentration of luteolin was recovered for samples stored at room temperature. This value was doubled for samples stored at 4 °C (32%) and tripled for preserved samples (46%).

Verbascoside had a good stability in the preserved samples. In fact, verbascoside recovery after 30 days was 105% of the original concentration. An initial increase (24%) was noticed after 24 h, although it was statistically insignificant. This may be due to improved extraction of verbascoside from the seed compartment under acidic conditions of the preserved samples. Ryan et al. (*24*) reported accumulation of verbascoside in seeds and its translocation to the pulp with maturation. Storage at 4 °C had significantly higher recovery than room temperature stored samples only after 15 days (**Figure 5**).

The recovery of hydroxytyrosol from samples stored at 4 °C was less than that from samples stored at room temperature. The concentration of hydroxytyrosol was doubled in the preserved samples (229%) after 24 h and then gradually decreased to 160% after 15 days and increased again to 199% after 30 days (**Figure 5**). All of these changes in hydroxytyrosol concentration could be explained by oxidation of hydroxytyrosol and hydrolysis of hydroxytyrosol-containing compounds. For samples stored at room temperature, both factors were working simultaneously, antagonizing each other. An overall decrease in the concentration of hydroxytyrosol was observed, but it was less than for those samples stored at 4 °C, as the activity of hydrolyzing enzymes was reduced by cooling. In the case of preserved samples, acid hydrolysis took place within 24 h, which accounts for the initial increase in the free hydroxytyrosol concentration.

Overall, preservation with acidified alcohol at 4 °C was superior to storage at room temperature and storage at 4 °C. Commercial feasibility is to be ascertained, but bulk storage at 4 °C is likely to be expensive.

Freeze-drying resulted in a substantial loss of phenols and antioxidant activity compared with the fresh sample, in addition to the generation of some artifacts. High-temperature treatments for a short time may be used as a cheaper and faster alternative to freeze-drying for thermostable phenols. However, this option needs to be optimized for the recovery of target compound(s).

None of the proposed storage or preservation treatments were successful enough to stop the fast degradation of phenols and the drop in antioxidant activity, which happened within 24 h of fresh sample collection. Extraction of fresh OMW is recom-

mended without delay (<24 h) until a more effective and more economical storage alternative is found. Once it is determined whether an individual phenol to be separated or an extract of OMW is to be used, the data from this study may assist in designing the most suitable handling techniques.

A two-phase extraction system results in higher phenol content and antioxidant activity of OMW relative to three-phase extraction. Increasing the malaxation time slightly improved total phenol content, antioxidant activity, and recovery of most phenols, whereas increasing the malaxation temperature had more detrimental effects.

OMW is a very viable resource for the production of bioactive molecules that can be used in the food or pharmacy sectors. Commercialization scenarios will depend on the intended use, whether individual compounds or a multicomponent mixture (phytoextract) is to be recovered.

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