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Fate of the phenolic compounds during olive oil production with the traditional press method

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Abstract

In the traditional press method for olive oil production, olives are crushed and malaxed into a paste, which is spread on mats. Pressure is applied to squeeze out the oil and wastewater, leaving a material on the mats called pomace. The oil and wastewater are then separated by gravity. The fate of the olive phenolic compounds, including oleuropein, and antioxidant activity was investigated at each stage of the process and the waste products (pomace and wastewater) were evaluated as potential sources of valuable phenolic compounds and antioxidant activity. The largest loss of phenolic compounds was seen at the crushing stage (60% of phenolic compounds, 70% of oleuropein) but only 21% of antioxidant activity was lost. Malaxation did not cause significant losses of phenolic compounds but the antioxidant activity was affected (43% loss). Pomace retained 26% of the phenolic compounds, 21% of the oleuropein and 33% of the antioxidant activity. When dried, the phenolic compounds and oleuropein were 3.5-fold concentrated in the wastewater and it exhibited a 2.7-fold increase in antioxidant activity compared to whole olives. The olive waste products from the traditional press method, pomace and wastewater, are good sources of valuable phenolic compounds and antioxidant activity.

Introduction

Adherence to a Mediterranean diet and consumption of olive oil has been associated with a number of health benefits including a reduced risk of morbidity and mortality (Cicerale *et al.*, 2009), particularly by reducing the risk of cardiovascular disease (de Lorgeril *et al*., 1999), atherosclerosis (Visioli *et al*., 2000) and certain types of cancer (Kapiszewska *et al*., 2005). The Mediterranean diet is characterized by a high consumption of fruits, vegetables, fish, legumes and whole grains. However, fat consumption is also high; it accounts for approximately 40% of caloric intake, the main source of which is olive oil (Stark and Madar, 2002).

Historically, the healthful properties of olive oil have been attributed to its high proportion of monounsaturated fatty acids (MUFAs), in particular oleic acid which represents 70-80% of the total fatty acids present in virgin olive oil (Cicerale *et al*., 2009). In addition to MUFAs, virgin olive oil contains a minor yet significant phenolic compound fraction, which has garnered much interest in relation to the health promoting properties of olive oil (Cicerale *et al*., 2009).

Olive oil extraction aims to separate the liquid oil phase from the other constituents of the fruit. Currently, commercial olive oil production is carried out using both continuous (centrifugation) and batch (traditional press) approaches. However, centrifugal systems face larger waste disposal issues and produce oils, which can be of lower quality especially in terms of phenolic compound content (Di Giovacchino *et al*., 2002; Issaoui *et al*., 2009; Torres and Maestri, 2005).

The traditional press method works by first grinding the olives in a hammer mill followed by malaxing the pulp into a paste, which is then spread on spherical mats before pressure is applied using a hydraulic piston press to squeeze the oil and the water from the paste and leaving a solid material on the mats referred to as pomace. The oil and water phases are then separated by gravity and collected by decantation. Therefore, the traditional press method produces three fractions, olive oil plus large amounts of two waste products – a relatively dry and solid pomace and wastewater (Jerman Klen and Mozetic Vodopivec, 2012).

The wastes, especially the pomace, possess high amounts of organic substances (14-15%) including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats (Lafka *et al*., 2011) and high concentrations of phenolic compounds (up to 10 g L^{-1}) (Ranalli *et al*., 2003). Therefore, disposal of these waste products has been a major environmental issue in a number of olive growing countries (Capasso

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et al., 1992).

Extraction of the phenolic compounds from olive pomace and wastewater has the potential to somewhat limit the environmental damage that can be caused by these waste fractions and may even provide another source of income for olive oil producers (Obied *et al*., 2005). For example, extraction of oleuropein, the most abundant phenolic compound in olives could add value to the olive oil production process. Although it is responsible for the characteristic bitterness of the olive fruit, a number of the beneficial effects of virgin olive oil have been attributed to oleuropein; the phenolic compound has been found to have anti-atherogenic (Covas, 2007), anti-inflammatory (de la Puerta *et al.*, 1999), anti-cancer (Menendez *et al*., 2007) and antimicrobial (Bisignano *et al.*, 1999) properties.

These valuable phenolic compounds, including oleuropein, are undoubtedly worth isolating from the waste products of olive oil production. Therefore, it is important to determine how the phenolic compounds partition and degrade throughout the olive oil production process in order to determine how the value of these waste products can be maximized in terms of being sources for these compounds. However, to date, there has been no comprehensive investigation into the partitioning behavior of the phenolic compounds and their degradation during all the steps of the traditional press method.

Therefore, the aims of this study were to investigate the partitioning and degradation patterns of the olive phenolic compounds, including oleuropein, during each stage of the olive oil production process using the traditional press system. The phenolic compounds were followed from the whole olives through to the crushing and malaxation steps to produce the olive paste, then through to the pressing step to produce the pomace and finally through to the separation and decanting of the liquids to produce the oil and the wastewater.

Materials and Method

Materials and reference compounds

Gallic acid, sodium carbonate, Folin Ciocalteu's phenol reagent, 1,1-diphenyl-2-dipicrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), oleuropein, 2-(4-hydroxyphenyl)ethanol (tyrosol), hydroxytyrosol, 3,5-dimethoxyphenol, syringic acid, vanillin and ferulic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Methanol (HPLC grade), chloroform, phosphoric acid and acetonitrile (HPLC grade) were from Lomb Scientific (Taren Point, NSW, Australia). Liquid nitrogen was from BOC (Gosford, NSW, Australia). Ultra-pure (type 1) de-ionized (DI) water was prepared by reverse osmosis and filtration using a Milli-Q Direct 16 system (Millipore Australia Pty Ltd, North Ryde, NSW, Australia).

Olives and olive oil extraction

Green olives (mature but still unripe) of the Frantoio cultivar were harvested at Houndsfield Estate (Hunter Valley, NSW, Australia) and processed on-site the next day using a semi-continuous Enorossi 150 traditional olive oil pressing system (Enoagricola Rossi, Calzolaro di Umbertide, Perugia, Italy) standardized to press a maximum of 150 kg of olives at a time. The room temperature was constant at 21^{o}C.

First, samples of the olives, referred to as 'whole olives' were taken before they were crushed into a paste using a hammermill; samples were taken at this stage and referred to as 'crushed olives'. The pulp was then malaxed for 25 min at 23^{o}C to produce a paste; samples were taken at this stage and referred to as 'malaxed olives'. The olive paste was then spread onto mats and pressed for a total of 60 min, first at 150 atm for 20 min, then at 200 atm for 10 min and finally at 400 atm for 30 min.

During the pressing stage, the liquids were forced out of the paste until they spilt over the sides of the mats and they were collected in a reservoir positioned below the mats. Some water was used to wash the remainder of the oily must down from the sides of the mats into the reservoir below. The liquids were then transferred to settling tanks and left to stand in order to separate the oil from the wastewater. Samples of the separated olive oil, referred to as the 'oil', the water, referred to as 'wastewater', and of the relatively dry solids left behind on the mats, referred to as the 'pomace', were also taken.

Altogether, samples were taken from each stage in the olive oil production process (whole olives, crushed olives, malaxed olives, pomace, wastewater and oil) from three separate runs on the day (triplicate runs). The samples were collected in opaque containers, which were immediately placed on ice and then stored at -20^{o}C until analysis.

Moisture analysis

Moisture analysis was conducted on all samples in order to express all results in terms of dry weight (dw). Each sample (2 g), including oil and wastewater, was placed in pre-dried and weighed crucibles. Sample + crucible weight was recorded and then were dried at 70^{o}C in a vacuum oven (Thermoline,

Wetherill Park, NSW, Australia) until constant weight was achieved (72 h). Weight loss was used to calculate total moisture and total solids.

Determination of biophenols

Sample preparation

The sample preparation was based on the standard method for the determination of biophenols in olive oils of the International Olive Council (2009) with a few modifications. For whole olives, 1 g of sample was added to 12 ml of methanol:water (80:20 v/v). For crushed, malaxed and pomace samples, 2 g of sample was added to 12 ml of methanol: water (80:20 v/v). For olive oil and wastewater, 5 g were added to 15 ml of methanol: water (80:20 v/v). Samples were vortexed for 2 min before extraction in an ultrasonic bath for 15 min and centrifuging at 3000× *g* for 25 min at 4°C in a JA-20 rotor on a Beckman J2-MC centrifuge (Beckman Coulter, Lane Cove, NSW Australia). An aliquot of the supernatant phase was then taken and filtered through a 0.45 µm Nylon PVF filter (Phenomonex Australia Pty Ltd, Lane Cove, NSW Australia) before further analysis.

Determination of total phenolic compounds

Folin Ciocalteu method

The Folin Ciocalteu method was used to determine total phenolic compounds in the methanol:water (80:20 v/v) extracts. This method was based on Cicco *et al.* (2009) with a few minor modifications. A standard curve was developed using Gallic acid, which was linear between 10-100 µg ml^{-1}.

To each of the standard samples, the appropriately diluted olive extract samples and a blank (methanol:water, 80:20 v/v) (all 300 µl), 300 µl of Folin Ciocalteu's reagent was added and left to equilibrate for 2 min. Then, 2.4 ml of 5% (w/v) sodium carbonate solution was added to each preparation and left to react in the dark at room temperature for 1hour. Absorbance was then read on a Carry 50 Spectrophotometer (Varian, Melbourne, VIC, Australia) at a wavelength of 760 nm. The values were determined using a gallic acid standard curve (prepared each day). Results were expressed as mg gallic acid equivalents (GAE) g^{-1} sample (dw).

HPLC

The methanol:water (80:20 v/v) extracts were analyzed using a Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6 mm Synergi 4 µm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with detection at 254 nm. The column was maintained at 30°C, the flow rate was 1 ml min^{-1} and three solvents were used for the mobile phase: solvent A was 1% acetonitrile in 0.2% H_3PO^4 (v/v), solvent B was 100% methanol and solvent C was 100% acetonitrile. A gradient elution schedule was used. The initial solvent system at the time of injection was 96% A, 2% B and 2% C. The eluting solvent was then changed, in a linear gradient manner, to 40% A, 30% B and 30% C by 30 min and held there for 10 min. From 40 to 42 min, the solvent was then returned to 96% A, 2% B and 2% C and maintained there for 10 min to re-equilibrate the column with the initial solvent system before the next injection. Syringic acid (3,5 dimethoxy 4-hydroxy benzoic acid) was used as an internal standard. A standard curve using tyrosol was prepared in methanol:water (80:20 v/v), which was linear between 0.06 and 1.2 mg ml^{-1}. Values for the total HPLC peaks were determined using the tyrosol standard curve and the results were expressed as mg Tyrosol Equivalents (TRE) g^{-1} sample (dw).

Oleuropein

The HPLC peak corresponding to oleuropein was identified and its content in the extracts was quantified using a standard curve of oleuropein prepared in methanol:water (80:20 v/v), which was linear between 0.05 and 0.925 mM. The results were expressed as mmol oleuropein g^{-1} sample (dw).

Determination of antioxidant activity using the DPPH method

The antioxidant activities of the methanol:water (80:20 v/v) extracts were determined using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical based on the procedure described by Thaipong *et al.* (2006). A standard curve using Trolox was prepared, which was linear between 20 - 200 µg ml^{-1}. Samples (150 µl) of olive sample extracts and standards at the appropriate dilutions were allowed to react with 0.1M DPPH (2.85 ml) for 24 h in the dark. The absorbance was then read at 515 nm on a Carry 50 spectrophotometer (Varian, Melbourne, VIC Australia). Values were determined using the trolox standard curve (prepared each day) and the results were expressed as mg Trolox Equivalents (TE) g^{-1} sample (dw).

Statistical analysis

All analyses were performed on samples from the three separate olive oil extraction runs on the day (triplicate runs, n = 3) and all results were expressed as means ± SD for the triplicate runs. The one-way ANOVA was then used to examine differences

between the mean values for total phenolic compounds (Folin and Ciocalteu assay), antioxidant activities (DPPH), total HPLC peaks and oleuropein levels for the different samples and the Tukey HSD post-hoc test was performed to determine where any differences lay. However, when the values of the four measurements for wastewater were included in the statistical analysis it was found that they were significantly different ($p < 0.05$) from all the other samples but the values were so highly variable that they caused other differences to appear insignificant. Therefore, the statistical analysis was also conducted without the values for wastewater to determine whether there were any differences between the values for the other samples and superscript letters were used to indicate differences between these values.

Linear regression analysis was also conducted to identify if any significant relationships existed between the four measurements. Statistical significance and linear regressions were evaluated using SPSS statistical software version 18 and p-values <0.05 were taken as indicating statistical significance.

Results

Total phenolic compounds

The total phenolic compounds, as measured using the Folin and Ciocalteu assay (2009) were found to decrease along the olive oil extraction process (Table 1). A significant difference was found between the total phenolic compounds of whole, crushed and malaxed olives and in pomace and oil samples ($p < 0.001$). Crushing caused a 23% loss of phenolic compounds, while after 30 min of malaxation, the total phenolic compound content dropped a further 30% leaving 46% of the phenolic compounds originally present in the whole olive samples to be measured in the malaxed samples (Table 1). The wastewater and oil were then removed from the malaxed paste during the hydraulic press step and the phenolic compounds partitioned into the different fractions. On a dry weight basis, there was a further 51% loss of phenolic compounds in the pomace compared to the malaxed olives. However, the pomace still retained over 26% of the phenolic compounds originally measured in the whole olives (Table 1).

In contrast, on a dry weight basis, the oil only exhibited 1.4% of the original content of phenolic compounds relative to the whole olives. The wastewater fraction was difficult to compare directly with the other samples because it contained a high amount of water and a low amount of solids. However, because of its low solids, the relative content of phenolic compounds was 3.5 times higher in this fraction than in the whole olives when expressed in terms of dry weight.

The total phenolic compound results for wastewater (Table 1) were highly variable (63.77 ± 27.01 mg GAE g^{-1}). When included in the statistical analysis it was significantly different ($p < 0.05$) from all other samples but caused other differences to appear insignificant due to its high variability. Therefore, for further analysis the statistical comparison was repeated without the wastewater results.

Table 1. Phenolic compounds in whole olives and in olive material sampled through the oil extraction process

Sample	Total Phenolic Compounds (GAE g^{-1})	Total HPLC Peaks (TRE g^{-1})
Whole olives	18.47 ± 0.62^{a}	56.89 ± 6.32^{a}
Crushed olives	14.21 ± 0.97^{b}	23.45 ± 2.76^{b}
Malaxed olives	9.99 ± 0.31^{c}	23.45 ± 2.11^{b}
Pomace	4.89 ± 0.39^{d}	18.92 ± 5.83^{b}
Oil	0.25 ± 0.0001^{e}	0.74 ± 0.27^{c}
Wastewater*	63.77 ± 27.01*	123.27 ± 27.81*

Values are means ± SD for triplicate runs and values in a column not sharing a superscript are significantly ($p < 0.5$) different from each other.
*When wastewater was included in the statistical analysis it was significantly different ($p < 0.05$) from all other samples but caused other differences to appear insignificant due to its high variability. Therefore, the statistical analysis was also conducted without the values for wastewater to determine whether there were any differences between the values for the other samples and superscript letters were used to indicate differences.

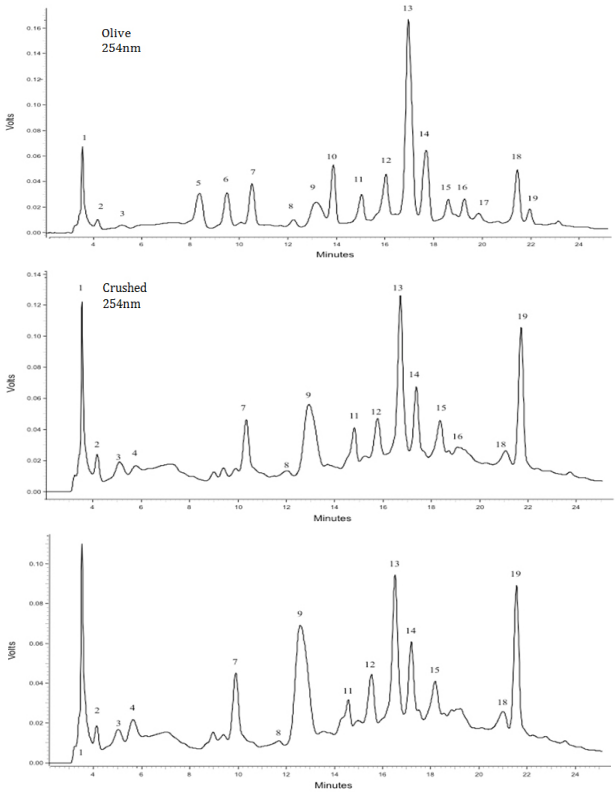

Figure 1. Typical HPLC chromatograms of olive and olive materials (whole, crushed and malaxed olive samples). Peaks which were identified are: (3) hydroxytyrosol, (4) tyrosol, (7) syringic acid (Internal standard), (14) oleuropein

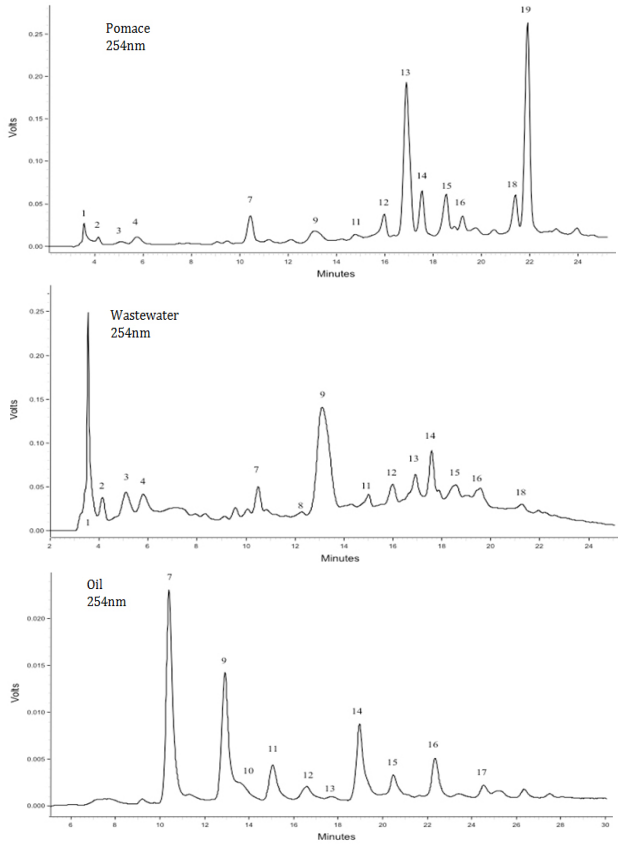

Figure 2. Typical HPLC chromatograms of olive pomace, wastewater and oil samples. Peaks which were identified are: (3) hydroxytyrosol, (4) tyrosol, (7) syringic acid (Internal standard), (14) oleuropein. Note that the scale for the olive oil sample is different from the scale for the other chromatograms in Figures 1 and 2

Total phenolic compounds (HPLC)

Examination of the methanol:water (80:20 v/v) extracts from the samples using HPLC resulted in the detection of 19 major peaks. Chromatograms showing the typical phenolic compound profile of samples from each stage of the process are pictured in Figures 1 and 2. This provides some basic insights into the phenolic compound partition trail from the whole olives to the crushed and malaxed olive samples (Figure 1) and finally to the oil and waste products (Figure 2).

The areas of the 19 detected peaks were pooled and expressed as mg tyrosol equivalents g^{-1} of sample (dw). As with the total phenolic compound assay, the values were found to generally decrease along the olive oil production process (Table 1). When wastewater was not included in the statistical analysis, the whole olives were found to be significantly different to all other samples ($p < 0.001$). There was a 59% loss of phenolic compounds at the crushing step. However, based on dry weight, there was no difference in the total HPLC peak values between crushed, malaxed and olive pomace samples and the pomace's relative content of these peaks was 33% compared to the whole olives. In contrast, the relative content in oil was only 1.3% for these compounds when compared to the whole olives ($p < 0.002$).

Table 2. Oleuropein levels in samples determined using HPLC

Sample	* Oleuropein (μmoles g^{-1})	**Oleuropein % total HPLC peaks
Whole olives	3.47 ± 0.83^{a}	11.99 ± 3.01^{a}
Crushed olives	1.13 ± 0.20^{b}	8.19 ± 1.91^{a}
Malaxed olives	0.89 ± 0.23^{b}	7.43 ± 2.46^{a}
Pomace	0.72 ± 0.17^{b}	8.26 ± 4.03^{a}
Oil	0.17 ± 0.02^{c}	22.86 ± 5.17^{b}
Wastewater#	$12.18 \pm 3.30^{\#}$	$9.60 \pm 1.58^{\#}$

Values are means ± SD and values in a column not sharing a superscript are significantly ($p < 0.05$) different from each other.

* HPLC analysis of oleuropein expressed as μmoles oleuropein/g of fresh sample (dw)

** Oleuropein expressed as a percentage (%) of the total HPLC peaks

When included in the statistical analysis, wastewater values were significantly different ($p < 0.05$) from all other samples but caused other differences to appear insignificant due to its high variability. Therefore the statistical analysis was also conducted without the values for wastewater to determine whether there were any differences between the values for the other samples and superscript letters were used to indicate differences.

Table 1 also shows that the HPLC peak values were higher (2x) in the wastewater than in the whole olives when expressed in terms of dry weight. Again, the values for wastewater were significantly higher from all of the samples when included in the statistical analysis but it caused the differences between the other samples to appear insignificant due to its high variability. Therefore, the statistical comparison was repeated without the wastewater results. Of the 19 peaks, only 3 were identified (3. hydroxytyrosol, 4. tyrosol and 14. oleuropein). Since hydroxytyrosol and tyrosol were only detected in very small amounts they were not individually quantifiable. However, a standard curve was used for oleuropein to enable its quantification.

Oleuropein was found to degrade quickly during olive oil processing, with the simple act of crushing causing a loss of more than 60% (Table 2). Interestingly, malaxation did not cause a significant reduction in oleuropein levels nor did pressing as no difference was found between the oleuropein levels in the crushed, malaxed and pomace samples when the values were expressed on a dry weight basis. There was also no difference between these three samples when oleuropein was expressed as a percentage of the total HPLC peak values (Table 2).

Only a low relative content, approximately 4.6% of the oleuropein content in whole olives was detected in the olive oil samples. However, oleuropein accounted for almost a 2 times higher percentage of the total HPLC peaks (Table 2) in the olive oil samples (23%) compared to the whole olives (12%). The wastewater samples were found to have a high content of oleuropein (Table 2). However, as a percentage of the total HPLC peaks the oleuropein

Table 3. Antioxidant activity in whole olive and olive material sample extracts

Sample	*Antioxidant Activity (TE g^{-1})	** Antioxidant Activity (% of whole olives)
Whole olives	29.53 ± 1.37^{a}	100 ± 0^{a}
Crushed olives	23.21 ± 1.05^{b}	78.6 ± 2.13^{b}
Malaxed olives	16.94 ± 0.73^{c}	57.4 ± 3.04^{c}
Pomace	9.65 ± 0.38^{d}	32.7 ± 2.71^{d}
Oil	0.44 ± 0.01^{e}	1.49 ± 0.1^{e}
Wastewater#	$80.43 \pm 37.33^{\#}$	$272.4 \pm 115.96^{\#}$

Values are means ± SD and values in a column not sharing a superscript are significantly ($p < 0.05$) different from each other.
* Antioxidant activity measured using the DPPH assay and expressed as mg trolox equivalents (TE)/g of sample (dw)
** Antioxidant activity expressed as a percentage of the activity in whole olives.
# When included in the statistical analysis the wastewater values were significantly different ($p < 0.05$) from all other samples but caused other differences to appear insignificant due to its high variability. Therefore the statistical analysis was also conducted without the values for wastewater to determine whether there were any differences between the values for the other samples and superscript letters were used to indicate differences.

content in wastewater was more similar the whole olives (10%) than to the oil.

In the chromatograms (Figures 1 and 2) it can generally be seen that as certain peaks decreased in area throughout the process (e.g. peak 14. oleuropein) other peaks increased such as peak 3. hydroxytyrosol, peak 9 and 19. It can also be noted that the more hydrophilic early-eluting compounds (peaks 1-9), which were present in the whole, crushed and malaxed olive samples, were also detected in the wastewater samples. However, these hydrophilic compounds were either not detected or only detected in small amounts in the pomace samples. In contrast, the more hydrophobic late-eluting compounds (peaks 12-19), were detected in the pomace but not in the wastewater. Interestingly, the phenolic compound profile of the oil was very different from the other samples ($p < 0.01$) and despite its hydrophobicity, the late-eluting compounds were only detected in very small amounts in the oil.

Antioxidant activity

The antioxidant activities of the different olive samples are displayed in Table 3. As with the total phenolic compounds (Table 1), the antioxidant activity of the extracts was found to decrease along the olive oil extraction process when the analysis was done without the wastewater values (Table 3). Crushing caused a significant decrease in antioxidant activity (21%) while malaxation caused the largest reduction (27%). After pressing and extraction of the oil and wastewater, there was a further 43% loss of activity in the extracts from the pomace compared to the malaxed olives on a dry weight basis. However, the pomace retained over 32% of the antioxidant activity exhibited by the whole olives. In contrast, the oil only retained 1.5% of the antioxidant activity of the whole olive extracts. The wastewater was a concentrated source of phenolic compounds when

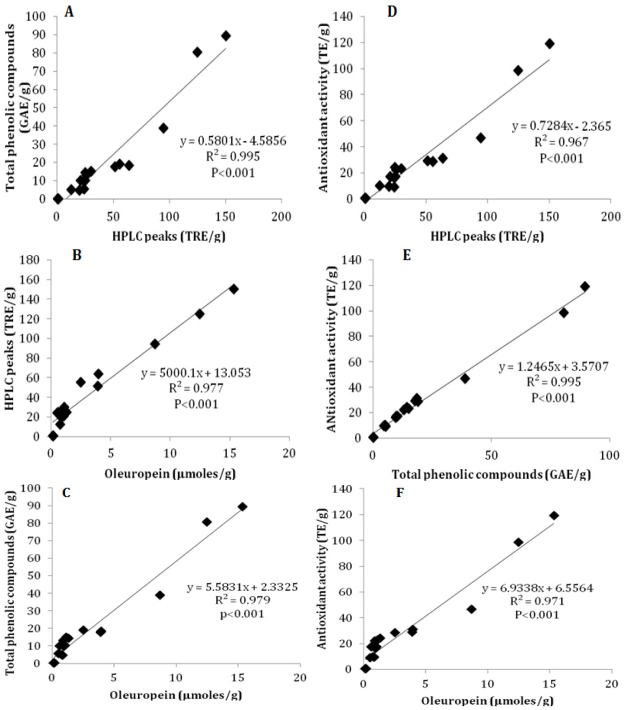

Figure 3. Comparison between the total phenolic compounds (GAE/g), HPLC peaks (mg TRE/g), antioxidant activity (TE/g) and oleuropein levels (µmoles/g) of all the olive samples The values for all three samples of whole olives, crushed, malaxed, pomace, oil and wastewater were included in the analyses and the total number of values was 18

expressed in terms of dry weight (Table 1). This fraction also exhibited a very high antioxidant activity (Table 3), which was significantly higher than in all other samples ($p < 0.001$).

Linear regression analyses

When the total phenolic compounds and HPLC peaks of all 18 samples (n = 3 for whole, crushed and malaxed olives and for pomace, wastewater and oil samples) were compared using linear regression (Figure 3A), they were highly correlated ($R^2 = 0.995$). Similarly, the HPLC peaks (Figure 3B) and the total phenolic compounds (Figure 3C) were both highly correlated with oleuropein levels ($R^2 = 0.977$ and 0.979, respectively).

The antioxidant activity of the extracts was also highly correlated with the HPLC peaks (Figure 3D, $R^2 = 0.967$) and the total phenolic compounds (Figure 3E, $R^2 = 0.995$). A strong positive correlation was also seen when the antioxidant activity was compared to the oleuropein levels (Figure 3F, $R^2 = 0.971$).

Discussion

This study showed that olive wastes from the traditional press oil extraction method are a good source of phenolic compounds. Between 26% (Folin Ciocalteu) and 33% (HPLC) of the total

phenolic compounds and 21% of the oleuropein were recovered in the pomace compared to whole olives. Furthermore, the pomace exhibited 32% of the antioxidant activity originally measured in the whole olives. Although dilute, the wastewater was also a good source of phenolic compounds. When expressed in terms of concentration per dry weight of material, the wastewater displayed 3 times the concentration of phenolic compounds (Folin Ciocalteu), twice the total HPLC peaks, twice the oleuropein levels and almost three times the antioxidant activity compared to the whole olives. In contrast, only 1.4% of the phenolic compounds, 1.3% of the total HPLC peaks, 4.9% of the oleuropein and 1.5% of the antioxidant activity originally present in the whole olives ended up in the oil. Therefore, the waste products of the traditional press olive oil extraction method, the pomace and wastewater, have been shown to be good sources of phenolic compounds that exhibit high antioxidant activity.

Crushing caused the biggest loss of phenolic compounds, including oleuropein, most likely due to the breaking of the cell walls and the liberation of enzymes able to degrade the phenolic compounds. Crushing alone was found to result in a 59% loss of total HPLC peaks (Table 1) and a 60% loss of oleuropein (Table 2), both determined via HPLC. However, there was only a 23% loss in total phenolic compounds as measured by the Folin Ciocalteu method (Table 1). This suggests that there may have been interfering compounds present in the samples that yield higher values due to false positive reactions of the Folin Ciocalteu reagent with substances such as sugars, pectins or polyalcohols (Obied *et al*., 2008). A similar result was observed in a previous study, which demonstrated that higher values were obtained for the total phenolic compounds determined via the Folin Ciocalteu total phenolic compound assay compared to analysis by HPLC (Jerman Klen and Mozetic Vodopivec, 2012). Nonetheless, despite the criticisms of the Folin Ciocalteu assay for its non-specificity (Obied *et al*., 2008), the total phenolic compounds were still highly correlated to total HPLC peak areas (R^2 = 0.995) across all of the samples in the present study (Figure 3). Furthermore, the Folin Ciocalteu total phenolic compounds were also highly correlated with antioxidant activity (R^2 = 0.995), as were the HPLC peaks (R^2 = 0.967).

Similarly, despite the loss of 59% of the phenolic compounds (HPLC peaks) (Table 1) and 60% of the oleuropein (Table 2) due to crushing, there was only a 23% loss of antioxidant activity (Table 3). There is no other available research conducted on the crushed olive paste from the modernised traditional press method. However, the differential effects on the HPLC phenolic compounds compared to the antioxidant activity could be explained by the degradation of the more complex olive phenolic compounds (i.e. oleuropein, demethyloleuropein and ligstroside), to more active DPPH scavengers of lower molecular weights, such as hydroxytyrosol, tyrosol, 3,4 DHPEA-DEDA and oleuropein aglycone as suggested by Jerman Klen and Mozetic Vodopivec (2012). However, hydroxytyrosol and tyrosol were not detected in high amounts in the present study.

Prior to the hydraulic pressing step in the production process, any loss of phenolic compounds or antioxidant activity had to be due to degradation. However, after pressing, settling and decantation, the olive paste was fractionated into pomace, wastewater and oil. The phenolic compounds therefore partitioned into each of these three different phases depending on their polarity and the different concentrations of hydrophilic or hydrophobic material in each phase.

The olive pomace retained between 26% (Folin Ciocalteu) and 33% (HPLC) of the total phenolic compounds and 21% of the oleuropein whilst having 32% of the antioxidant activity originally in the whole olives. This was much higher than previously reported by Jerman Klen and Mozetic Vodopivec (2012), who found that only 7.6% of the total phenolic compounds (Folin Ciocalteu) partitioned into the olive pomace from the traditional press method. They also found that the pomace only had around 17% of the initial antioxidant activity of the olives, also measured using the DPPH assay. A possible reason for these differences could be that, in the previous study (Jerman Klen and Mozetic Vodopivec, 2012), the pomace samples were freeze dried, a process which has been shown to have a detrimental effect on phenolic compounds(Michalczyk *et al*., 2009). Alternatively, because the authors also diluted the olive paste during the malaxation step (Jerman Klen and Mozetic Vodopivec, 2012), a higher percentage of the phenolic compounds may have partitioned into the wastewater fraction rather than into the pomace.

Although, there have not been many studies that have compared wastewater to the whole olives from which it came, some have compared the phenolic compound content of wastewater to the oil produced. Angelino *et al.* (2011) found that wastewater from the traditional press method had over 52 times the total phenolic compound content of oil. This was much less than in the present study where the wastewater was found to have 255 times the total phenolic compounds of the oil. Nonetheless, Angelino *et al.* (2011) found their wastewater to have 253 times the antioxidant activity of oil, which was higher than

in the present study where the wastewater had an antioxidant activity 167 times higher than the oil. Nonetheless, it is clear from both the previous 20 and the present study that wastewater has a higher level of total phenolic compounds and antioxidant activity than the oil.

The wastewater in the present study contained 12.2 μmoles g^{-1} of oleuropein (dw) equating to 6.6 mg g^{-1} (dw) and the pomace contained 0.7 μmoles g^{-1} oleuropein (dw), which equates to 0.4 mg g^{-1} (dw). Oleuropein (98% pure) currently retails for $177 AUD for 10 mg (Sigma-Aldrich). Therefore, the extraction of this compound alone from olive pomace or wastewater could prove to be a feasible venture for traditional press olive oil producers.

In summary, the results of the present study have shown that a substantial amount of phenolic compounds are present in the waste products from the modernised traditional press process. In fact, olive pomace and wastewater were shown to have over 20 and 255 times the total phenolic compounds (Folin Ciocalteu), 26 and 167 times the total HPLC peaks, 4 and 72 times the oleuropein levels and 22 and 183 times the antioxidant activity respectively, when based on dry weight, compared to the oil samples. Therefore, the waste products of the traditional press olive oil extraction method, the pomace and wastewater, have been shown to be good sources of phenolic compounds that exhibit high antioxidant activity.

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