COMPARISON OF CHEMICAL COMPOSITION, BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF THREE OLIVE-WASTE CAKES

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ABSTRACT

The objectives of this study were to determine the physicochemical composition, phenolic, tocopherol, flavonoid, flavanol, dietary fiber and β-carotene contents, as well as the antioxidant activity of three olive-waste cakes from varieties Picual, Frantoio and Arbequina. Frantoio presented the highest dietary fiber content and Arbequina showed the highest β-carotene content. Results indicated that total phenolic content followed the sequence (Frantoio > Picual > Arbequina). Picual presented the highest free phenolic and flavonoid contents (3499.11 mg gallic acid equivalent/100 g dry weight (DW) and 1331.45 mg CTE/100 g DW, respectively) showing the highest antioxidant capacity. The identified free phenolic compounds of the three varieties were gallic acid, 3-hydroxytyrosol, rutin hydrate, tyrosol and p-coumaric acids. The α-tocopherol was the predominant for the three varieties.

PRACTICAL APLICATIONS

Olive oil processing produces significant amount of waste that can be utilized for the production of high value-added ingredients for various industrial applications. Therefore, the information here reported may stimulate engineers to re-evaluate these wastes, with the possibility to recover significant amounts of bioactive compounds with high antioxidant capacity and dietary fiber in order to produce food additives in the scenario of functional foods.

INTRODUCTION

Along with mechanical olive oil extraction, the olive industry has to face an important issue with accumulation of solid residues. With increasing emphasis on cost reduction of industrial processes and value addition to agro-industrial residues, oil cakes are seen as an ideal source of nutrients (Uribe *et al.* 2014). This concern is providing a renewed incentive for generating value-added by-products. Moreover, there is a rising interest in antioxidative phytochemicals for their potential roles in human disease prevention and food quality improvement (Yu *et al.* 2002; Albishi *et al.* 2013). Phenolic compounds are of considerable interest because of their antioxidant properties (Duthie and Crozier 2000; Balasundram *et al.* 2006). The antioxidant compounds from waste products of food industry could be used for protecting against the oxidative damage in living systems by scavenging oxygen free radicals, and also for the stability increase of foods by preventing lipid peroxidation (Vulic *et al.* 2014).

Particularly, the olive-waste cake is an excellent natural source of phenolic compounds and can add up to 30% of olive oil manufacturing (Suarez *et al.* 2010; Lafka *et al.* 2011). The analysis of phenolic extracts from these

by-products has demonstrated their high antioxidant activity and suggested their potential use as additives for the food industry (Piperidou *et al.* 2000; Fiorentino *et al.* 2003; Allouche *et al.* 2004; Obied *et al.* 2007; Uribe *et al.* 2014).

Moreover, dietary fiber and other bioactive compounds such as flavonoids are widely used as functional food ingredients in processed foods because of their potential health benefits (Visioli *et al.* 2002; Rodriguez *et al.* 2006; Ajila and Prasada Rao 2013). Olive-waste cake contains pectic materials that could be transformed into a potential source of gelling material (Cardoso *et al.* 2003).

In addition, Chile has increased the olive oil production as well as enhanced olives varieties, time and system of harvest because of appropriate cultivation techniques and climatic conditions (ODEPA 2008). Among the varieties, Picual, Frantoio and Arbequina represent the most important ones. In particular, Picual represents 20% of the world total olive plantation and 50% of the planting in the Iberic peninsula. Frantoio produces oil with excellent organoleptic characteristics, fruity, green color and very stable because of its richness in polyphenols. Finally, Arbequina is the main variety of olive trees that are grown in Chile and represents 70% of the total national production (www.chileoliva.cl). The cultivated areas have increased from 16,000 ha in 2008 to 20,000 ha in 2009 representing an increase of 25%. Thus, the amount of olive-waste cake produced by the olive oil industry has highly increased.

The aims of this study were to determine the physicochemical characteristics as well as phenolic, tocopherol, flavonoids, flavanols, dietary fiber and β-carotene contents and antioxidant capacity of three olive-waste cakes from Picual, Frantoio and Arbequina olive varieties as potential sources of bioactive compounds. Identification of the main phenolic compounds in their free and bound forms was also presented.

MATERIALS AND METHODS

Raw Material

The olive-waste cakes were supplied by an agro-food company (Agronoble S.A) from Ovalle, Chile. The wastes resulted from a continuous cold process of the olive oil by means of the conventional three-phase extraction (Borja *et al.* 2006). The olives varieties used in this investigation were Picual, Frantoio and Arbequina, harvested at optimum ripeness and pressed without delay. The classic production of olive oil generates: olive oil (20%), solid waste (30%) and aqueous liquor (50%). The solid waste (olive oil cake or "orujo") is a combination of olive pulp and stones (Fernandez-Bolaños *et al.* 2006). The samples used for analysis were packed in polyethylene bags and kept in a

freezer at −20C. The olive cake samples were freeze-dried (Virtis Advantage Plus, Gardiner, NY) until processing.

Physicochemical Analysis

The crude protein content was determined by using the Kjeldahl method with a conversion factor of 6.25. The lipid content was obtained gravimetrically following Soxhlet extraction, using petroleum ether (Merck, p.a., Darmstadt, Germany) as a solvent according to the method described in AOAC method no. 920.39 (AOAC 1990). The crude fiber content was estimated by the Weende's method through an acid/alkaline hydrolysis of insoluble residues as described in AOAC method no. 962.09 (AOAC 1990). The crude ash content was estimated by incineration in a muffle furnace (Felisa, FE-341, Jalisco, Mexico) at 550C. All methodologies followed the recommendations from the Official Methods of Analysis (AOAC 1990). The available carbohydrate was estimated by difference. The moisture level was determined by means of AOAC method no. 934.06 (AOAC 1990). The pH value was measured directly on moist sample as described in AOAC method no. 945.10 (AOAC 1990) using a pH meter (Orion,Thermo Scientific, MA) and the level of titrimetric acidity at a final pH 8.2 was expressed as an oleic acid according to AOAC method no. 939.05 (AOAC 2000). All measurements were done in triplicate. The chemical compounds were expressed as g/100 g DW.

Determination of Dietary Fiber

Samples of waste olive cakes were analyzed for soluble and insoluble dietary fiber fractions according to a gravimetric-enzymatic method (AOAC n° 991.43) by using a Total Dietary Fiber Assay Kit (TDF100A; Sigma-Aldrich, St. Louis, MO) by an Enzymatic Digestion Unit and a Filtration System (VELP Scientifica, Usmate, Italy). Briefly, the samples were suspended in MES-TRIS buffer pH 8.2 and digested sequentially with heat stable α-amylase at 95–100C, protease at 60C, and amyloglucosidase at 60C. Enzyme digestates were filtered through tared fritted glass crucibles and collected in an Erlenmeyer flask. Crucibles containing insoluble dietary fiber were rinsed with an ethanol 95% solution followed by acetone, and dried overnight in a 105C oven. Each collected filtrate was mixed with four volume of 95% ethanol to precipitate materials that were soluble in the digestates. After allowing standing overnight, precipitates were filtered through tared fritted glass crucibles and washed. One of each set of duplicate insoluble fiber residues and soluble fiber residues was ashed in a muffle furnace at 525C for 5 h and another set of residues was used to determine protein as Kjeldahl nitrogen 6.25. Total dietary fiber was calculated as the sum of soluble and insoluble dietary fiber, and expressed as g/100 g DW.

β-Carotene Determination

The β-carotene extraction method was performed according to the method by Barba *et al.*, 2006, using a solvent mixture of hexane/acetone/ethanol (50/25/25 v/v/v).

Three grams of dehydrated sample was extracted with 25 mL of the solvent mixture using a orbital shaker (OS-20, Boeco, Hamburg, Germany) at 200 rpm for 30 min. The extracts were centrifuged to separate the supernatant, and these operations were repeated twice. Then, the filtrates were combined and placed in a round-bottomed flask and the extracts were evaporated to dryness. The dried extract was dissolved to a final volume of 10 mL with ethyl acetate and filtrated through 0.45 µm membrane filters and 10 µL were injected for high-performance liquid chromatography (HPLC) analysis.

The chromatographic conditions being used followed the methodology of Laur and Tian (2011). Analyses of carotenoid content were performed using an Agilent 1200 series HPLC system (Santa Clara, CA), including a quaternary pump (model G1311A), a autosampler (model G1329B), a column oven (model G1316A) and a diode array detector (DAD, model G1315D). The column used was a Kromasil 100-5C18, 250×4.6 mm, connected with a Kromasil guard column. The column temperature was controlled at 30C during the HPLC runs. Data were processed using the Agilent ChemStation software.

The flow rate was at 1 mL/min. The mobile phases were (1) acetonitrile : H_2O : triethylamine (900:99:1, v/v/v) adjusted to pH 8.5 with a solution of 1 M phosphoric acid, and ethyl acetate (2). The gradient elution program was 0–5 min, 100–75% A; 5–10 min, 75–30% A; 10–14 min, 30–0% A; 14–15 min, 0–100% A; 15–20 min, 100% A. The absorbance was read at 450 nm, β-carotene was identified based on the retention time and peak areas compared against authentic standards. The calibration curves were prepared between 20 and 80 µg of β-carotene/mL. Results were expressed as β-carotene/100 g DW.

Extraction of Free Phenolic Compounds

The olive-waste cake (2.5 g) was extracted using 250 mL n-hexane for 60 min in continuous extraction by means of a Soxhlet apparatus, for fat removal. The defatted extract was weighed and brought into phenolic compound extraction conditions.

Extraction was performed by adding of 20 mL of methanol to the deffated sample and agitated on an orbital shaker (OS-20, Boeco) at 250 rpm for 60 min, according to the methods by Lopez-Martinez *et al.* (2009) and Alu'datt *et al.* (2010) with some modifications. Afterwards, centrifugation at 5,000 rpm for 3 min was done, and then the supernatant was removed and extraction was repeated once

more in a similar way. The combined extracts were evaporated to 37C and redissolved in 10 mL methanol-formic acid (99:1). Aliquots of 10 µL were injected into the HPLC column. The phenolic compounds obtained from this technique were designated as FPC. The residue was kept for further extraction (for 24 h as maximum storage time).

Extraction of Bound Phenolic Compounds

Extraction procedures of bound phenolic compounds (BPC) followed the method described by Lopez-Martinez *et al.* (2009) with some modifications. The residue was hydrolyzed with 20 mL of 3 N NaOH and was agitated in a orbital shaker at 250 rpm for 88 min. The hydrolizate was acidified to pH 3 with concentrated HCl. The liberated BPC in the clear solution was extracted seven times with 10 mL ethyl acetate. The pooled ethyl acetate extracts were evaporated to dryness under vacuum in a rotary evaporator (Büchi R-210, Flawil, Switzerland) at 37C. The dried residue was dissolved in 10 mL methanol : formic acid (99:1). Aliquots of 10 µL were injected into the HPLC column.

HPLC Analysis

FPC and BPC analyses were performed by HPLC. A reverse phase- HPLC system, Agilent 1200, equipped with a high pressure pump; automatic inyector; a UV-Visible Diode Array Detector (UV-Visible-DAD); controlled by ChemStation software, was used. The analytical column was a Kromasil 100-5C18 (250×4.6 mm; Eka Chemicals, Alby, Sweden). The flow rate was 0.7 mL/min and the eluates were monitored at 280 and 310 nm at 25C. The mobile phase was composed of solvent A (0.1% formic acid pH 3) and B (100% acetonitrile). The elution gradient started with 87% A and 13% B; the solvent B to reach 55% at 18 min, 60% at 23 min, 13% at 25 min and then return to initial conditions for 2 min. The phenolic extracts and standard compounds were analyzed under the same analysis conditions.

Identification of some of the main phenolic compounds (gallic acid, 3-hydroxytyrosol, protocatechuic acid, caffeic acid, rutin hydrate and p-coumaric acid) in methanol/formic acid (99:1) was performed by comparison of retention times, spectra and peak areas at maximum absorption wavelength. The results of the main phenolic compounds were expressed as mg/100 g DW.

Determination of Total Phenolic Content

Total phenolic content (TPC) was determined colorimetrically by using a Folin–Ciocalteu (FC) reagent according to

Chuah *et al.* (2008) with some modifications. In brief, BPC and FPC extracts were diluted in methanol, and an aliquot of 0.5 mL of these diluted extracts was transferred to a 15 mL falcone tube. Then, 0.5 mL of FC reagent (Merck KGaA, Damstadt, Germany) was added after 5 min followed by a neutralization with 2 mL of Na_2CO_3 solution (200 mg/mL). The sample was then mixed on a vortex mixer (VELP SCIENTIFIC $2\chi^3$, Milan, Italy) and allowed to stand for 15 min at room temperature. 10 mL of ultra-pure water were then added and the precipitate being formed was removed by centrifugation at 5,000 rpm for 5 min. Finally, absorbance was measured at 725 nm in a spectrophotometer (Spectronic20, Genesys, Rochester, NY) and compared with a gallic acid (UCB, Brussels, Belgium) calibration curve (concentrations between 25 and 500 µg gallic acid/mL). Results were expressed as mg gallic acid equivalents (GAE)/100 g DW. All measurements were done in triplicate.

Determination of Total Flavonoid and Total Flavanol Contents

The total flavonoid content (TFC) from the olive-waste cake extracts was performed by a previously described protocol by Kim *et al.* 2003, slightly modified. BPC and FPC extracts were diluted in methanol and a 0.5 mL aliquot of methanolic extract was mixed with 2.0 mL deionized water in a 5 mL microcentrifuge tube, added 0.15 mL $NaNO_2$ (50 mg/mL) and allowed to react for 5 min. Following this, 0.15 mL $AlCl_3$ (100 mg/mL) was added and the mixture allowed standing for a further 6 min.

Finally, 1.0 mL 1 M NaOH and 1.2 mL desionized water were added to the reaction mixture and the absorbance at 510 nm was obtained against a blank that had been prepared in a similar manner, by replacing the extract with deionized water. Total flavonoid content was calculated from a calibration curve using catechin as a standard, and expressed as mg catechin equivalents (CTE)/100 g DW. All measurements were done in triplicate.

Total flavanol content (TF) was determined after derivatization with *p*-Dimethylaminocinnamaldehyde (p-DMACA), using an optimized methodology (Nigel and Glories 1991). A 0.2 mL aliquot of BPC and FPC extracts, respectively, was introduced into a 2 mL microcentrifuge tube. Then, 0.5 mL of HCl (0.24 N in methanol) and 0.5 mL p-DMACA solution (0.2% in methanol) were added. The mixture was allowed to react for 5 min at room temperature, and the absorbance was read at 640 nm in a spectrophotometer (Spectronic20 Genesys). A blank was prepared by replacing sample with methanol. Total flavanols were calculated from a calibration curve using catechin as a standard, and expressed as mg CTE/100 g DW. All measurements were done in triplicate.

In vitro Antioxidant Assays

Free Radical Scavenging Activity (DPPH). Different dilutions of the methanolic extracts were prepared in triplicate. Free radical scavenging activity of the samples was determined using the 2,2,-diphenyl-2-picrylhydrazyl (DPPH) method (Lafka *et al.* 2011) with some modifications. An aliquot of 3.9 mL of 0.51 mM DPPH (Calbiochem, Darmstadt, Germany) radical in methanol was added to a test tube with 100 µL of the sample extract (BPC and FPC, respectively) diluted in methanol. The reaction mixture was vortex-mixed for 30 s and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm, by using a spectrophotometer (Spectronic20, Genesys). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard for the calibration curve using concentration between 0.1~1.0 mM Trolox and the DPPH radical scavenging activities were expressed as mmol Trolox equivalents (TE)/g DW. Methanol was used as blank.

Ferric-Reducing Antioxidant Power Assay

The ferric-reducing antioxidant power (FRAP) assay was carried out according to Stratil *et al.* (2006) with slight modifications. The FRAP solution was freshly prepared on the day of use, by mixing acetate buffer (pH 3.6), ferric chloride solution (20 mM) and a 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution (10 mM TPTZ in mM HCl) in a proportion of 10:1:1, respectively. Following this, the FRAP solution was heated and protected from light, until it had reached a temperature of 37C. Extracts were prepared from appropriate dilutions in methanol BPC and FPC, respectively. 60 µL of the diluted sample extract (or for blank 60 µL methanol) and 180 µL of distilled water in glass tube until it reached a temperature of 37C and 1.8 mL FRAP solution were added into glass tubes. The tubes were vortexed and left at 37C for exactly 120 min, and the absorbance was measured at 593 nm. A Trolox standard curve was used (50–1,000 µM) to calculate the antioxidant activity of the samples in relation to Trolox and were expressed as mmol TE/100 g DW.

Tocopherol Content

Tocopherols were determined from the lipid extracts of the olive-waste cake by an HPLC analysis with fluorescence detection, following the standard method Ce 8–89 (AOCS 1993). Tocopherols were identified and quantified by using the external standards of the different molecules tested (α-, β- and γ-tocopherol; Merck). Results were calculated as mg/100 g of DW.

TABLE 1. PROXIMATE ANALYSIS OF THE THREE OLIVE-WASTE CAKES VARIETIES

Component/variety	Picual	Frantoio	Arbequina
Proximal analysis, g/100 g DM			
Moisture	4.65 ± 0.02^a	4.44 ± 0.06^b	3.86 ± 0.09^c
Ash	6.51 ± 0.34^a	6.73 ± 0.83^a	5.92 ± 0.45^a
Protein (N × 6.25)	8.44 ± 0.20^a	6.24 ± 0.56^b	8.62 ± 0.33^a
Fat	9.11 ± 0.34^a	4.59 ± 0.12^b	8.18 ± 0.32^c
Crude fiber	29.11 ± 2.64^a	37.27 ± 0.86^b	36.37 ± 0.59^b
Total carbohydrates	71.50 ± 0.26^a	78.19 ± 1.20^b	73.57 ± 0.34^c
a_w	0.137 ± 0.001^a	0.131 ± 0.004^b	0.116 ± 0.002^c
Dietary fiber, g/100 g DM			
TDF	52.12 ± 0.25^a	71.38 ± 0.32^b	63.64 ± 0.28^c
IDF	48.85 ± 0.50^a	69.35 ± 0.35^b	62.28 ± 0.64^c
SDF	3.27 ± 0.25^a	2.03 ± 0.04^b	1.36 ± 0.36^b
β-Carotene, mg 100 g/DM	18.84 ± 0.57^a	14.94 ± 0.98^b	21.47 ± 0.26^c

Data are expressed as the mean ± standard deviation of three replicates.
[a-c] Means in the same column followed by same letter type are not significantly different ($P < 0.05$).
a_w, water activity; IDF, insoluble dietary fiber; SDF, soluble dietary fiber; TDF, total dietary fiber.

Statistical Analysis

The effect of air-drying temperature on parameters was estimated using Statgraphics Plus 5.0 (Statistical Graphics Corp., Herndon, VA). One-way analysis of variance and three replicates for each olive cake were performed. Differences among the media were analyzed using the least significant difference test with a significance level of α = 0.05 and a confidence interval of 95%. In addition, the multiple range test included in the statistical program was used to demonstrate the existence of homogeneous groups within each of the parameters.

RESULTS AND DISCUSSION

Physicochemical Composition, Dietary Fiber and β-Carotene Contents

Table 1 shows the physicochemical composition analysis of the lyophilized olive-waste cakes from the varieties called Picual, Frantoio and Arbequina, respectively. The physicochemical composition of olive-waste cakes showed significant differences ($P < 0.05$) in each of their parameters except for ashes. These differences are related to genotypes, culture conditions, origin of the olives and extraction process. The major constituent of three olive cakes was the carbohydrates followed by the crude fiber. The high percentage of crude fiber is typical to olive cake, which is comparable with the results presented by Alhamad *et al.* (2012). In particular, Frantoio showed the highest content of carbohydrates (78.19 g/100 g DW; $P < 0.05$). These values are higher compared with another by-product of the olive oil industry (olive mill wastewater) and numerous other agricultural and fruit by-products, like cider waste and cucum-

ber pulp (Grigelmo-Miguel and Martín-Belloso 1998; Galanakis 2013).

Lyophilized samples presented an average water activity of 0.13. Regarding the dietary fiber, the total dietary fiber content in the olive-waste cakes being studied was found to be in the range of 52.12–71.38%. In particular, Frantoio showed the highest dietary fiber content (71.38 g/100 g DW), which was comparable with the reports by Llobera *et al.* (2007). The high content of dietary fiber was owing to the fact that olive cake is composed of skin, flesh and bone and lesser amounts of olive leaves. Regarding this aspect, this by-product is a good source for animal feeding (Chiofalo *et al.* 2004). The insoluble dietary fiber to soluble dietary fiber ratios were in the range of 15–46.

On the other hand, the β-carotene content of the studied varieties was also presented in Table 1. Arbequina variety presented the highest content of this chemical compound (21.47 mg/100 g DW), showing significant differences ($P < 0.05$) with the other two olive cake varieties. These results are in agreement with the reports of Azaizeh *et al.* (2012).

Total Phenolic Compounds: Free and Bound

Figure 1 shows the total phenolic content of the three varieties in the free and bound form. Results indicated that total amount of phenolics in olive-waste cake varies significantly among different varieties (Frantoio [5305.19 mg GAE/100 g DW] > Picual [4980.49 mg GAE/100 g DW] > Arbequina [3110.50 mg GAE/100 g DW]). These values are comparable with those corresponding to different fruit by-products of tropical fruits (Ribeiro da Silva *et al.* 2014) and higher than those reported by Terpinc *et al.* (2012) for oil cakes extracts from camelina, linseed, rapeseed and white mustard.
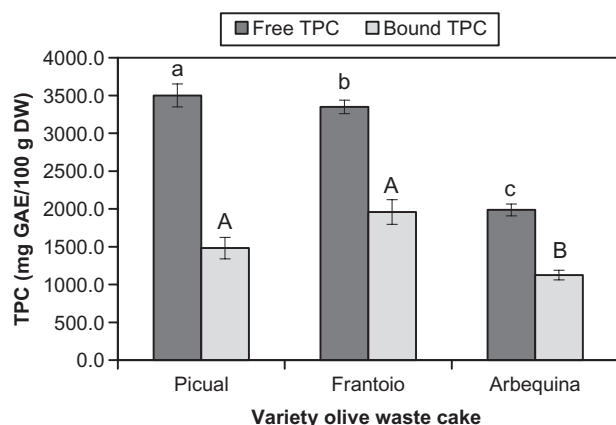
FIG. 1. FREE AND BOUND TOTAL PHENOLIC CONTENTS OF THREE OLIVE-WASTE CAKE VARIETIES

*Values having the same letter for each parameter are not significantly different at a confidence level of 95%.

The free-form percentage of total phenolic compound was 70% for Picual and 63% for Frantoio and Arbequina varieties. Particularly, Picual presented the highest free content (3499.11 mg GAE/100 g DW) and regarding the bound phenolic compounds, results indicated that Picual and Frantoio showed the highest content (1957.43 mg GAE/

100 g DW; $P < 0.05$). Comparable results were reported by previous investigations (Lesage-Meessen *et al.* 2001; Alu'datt *et al.* 2010). Significant differences among varieties might be attributed to genotypes, which influence the accumulation of phenolic compounds by synthesizing different quantities and/or types of phenolics (Albishi *et al.* 2013).

Table 2 shows the identified FPC of the three varieties of olive-waste cake. The detected phenolic compounds were gallic acid, 3-hydroxytyrosol, rutin hydrate and p-coumaric acid. Gallic acid and 3-hydroxytyrosol were detected in the three varieties ($P < 0.05$). The 3-hydroxytyrosol fraction in Frantoio variety presented the highest contents (49.83 mg/100 g DW). On the other hand, the highest gallic acid content was observed in the Picual variety (10.40 mg/100 g DW). Presence of gallic acid and 3-hydroxytyrosol has also been reported previously (Alu'datt *et al.* 2010; Suarez *et al.* 2010; Aliakbarian *et al.* 2011).

Table 3 shows the identified bound phenolic compound content of three varieties of olive-waste cake. The detected bound phenolic compounds were 3-hydroxytyrosol, protocatechuic, caffeic, tyrosol and p-coumaric acids. 3-hydroxytyrosol, caffeic and p-coumaric acids were detected in the three varieties ($P < 0.05$). The highest fraction was observed in 3-hydroxytyrosol (286.67 mg/100 g DW) for the Picual variety followed by caffeic (72. 87 mg/100 g DM) and tyrosol (63.76 mg/100 g DM) for Frantoio and p-coumaric (20.29 mg/100 g DM) for Arbequina. Based on results, 3-hydroxytyrosol (free and bound form) was the main phenolic compound detected in the olive-waste extracts. Hydroxytyrosol is one of major phenolic compounds present in olive fruit and it has been revealed to be the most interesting because of its remarkable pharmacological and antioxidant activity (Fernandez-Bolaños *et al.* 2006; Granados-Principal *et al.* 2010).

The presented results are in agreement with those reported in previous works: caffeic acid (Lesage-Meessen *et al.* 2001; Bianco *et al.* 2003); hydroxytyrosol (Fernandez-Bolaños *et al.* 2002) and p-coumaric acid and rutin (Romero *et al.* 2002).

TABLE 2. IDENTIFICATION OF FREE PHENOLIC COMPOUND OF THE THREE VARIETIES OF OLIVE-WASTE CAKE (MG/100 G DW)

Compound/variety	Picual	Frantoio	Arbequina
Gallic acid	10.40 ± 1.95[a]	4.36 ± 0.68[b]	7.47 ± 1.26[c]
3-Hydroxytyrosol	36.52 ± 4.02[a]	49.83 ± 5.02[b]	38.61 ± 3.89[a]
Protocatechuic acid	ND	ND	ND
Caffeic acid	ND	ND	NQ
Rutin hydrate	22.88 ± 1.39[a]	NQ	19.35 ± 1.33[b]
Tyrosol	ND	ND	ND
p- Coumaric acid	NQ	NQ	5.55 ± 0.32

Data are expressed as the mean ± standard deviation of three replicates.
[a–c] Means in the same column followed by same letter type are not significantly different ($P < 0.05$).
ND, not detected; NQ, not quantifiable; its retention time matches only (not its spectrum).

TABLE 3. BOUND PHENOLIC COMPOUND CONTENTS OF THREE OLIVE-WASTE CAKE VARIETIES (MG/100 G DW)

Compound/variety	Picual	Frantoio	Arbequina
Gallic acid	ND	ND	ND
3-Hydroxytyrosol*	286.67 ± 19.81[a]	132.14 ± 18.33[b]	104.08 ± 11.88[b]
Protocatechuic acid*	NQ	6.46 ± 1.36	NQ
Caffeic acid*	15.27 ± 1.11[a]	72.87 ± 7.56[b]	32.38 ± 3.48[c]
Rutin hydrate	ND	ND	ND
Tyrosol*	38.61 ± 2.20[a]	63.76 ± 1.51[b]	13.06 ± 0.09[c]
p- Coumaric acid*	14.79 ± 1.30[a]	16.23 ± 0.39[a]	20.29 ± 1.57[b]

* Data are expressed as the mean ± standard deviation of three replicates.
[a–c] Means in the same row followed by same letter are not significantly different ($P < 0.05$).
ND, not detected; NQ, not quantifiable; its retention time matches only (not its spectrum).
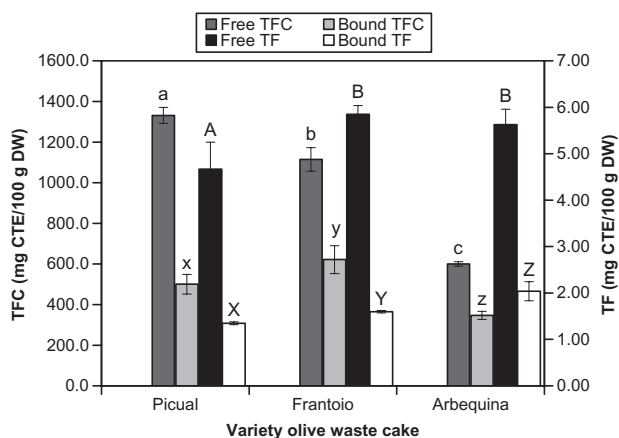
FIG. 2. FREE AND BOUND TOTAL FLAVONOID (TFC) AND FLAVANOL (TF) CONTENTS OF THREE OLIVE-WASTE CAKE VARIETIES
*Values having the same letter for each parameter are not significantly different at a confidence level of 95%.

Total Flavonoid and Total Flavanol Contents

Figure 2 shows that the total flavonoid as well as total flavanol contents of the olive cake extracts (free and bound) for the three varieties ($P < 0.05$). Regarding the flavonoids, Picual presented the highest free total flavonoid content (1331.45 mg CTE/100 g DW). Flavonoids are one of the major groups of phenolic compounds present in olive fruits and in their by-products (Vlahov 1992). However, Frantoio showed the highest bound flavonoid total content (621.62 mg CTE/100 g DW). These results are comparable with those reported in the investigation by Suarez *et al.* 2010. In addition, antioxidant capacity exerted by flavonoids was related to interaction of two flavonoids in terms of synergistic and antagonistic effects (Hidalgo *et al.* 2010).

Flavanol content ranged from 4.6 mg CTE/100 g DW to 5.9 100 mg CTE/100 g DW (free extracts) and 1.3 mg CTE/100 g DW to 2.1 mg CTE/100 g DW (bound extracts) for the three olive cakes variety. These results were comparable with those reported by several fruits by-products (Ribeiro da Silva *et al.* 2014) and apple peel and olive leaf extracts (Makris *et al.* 2007). Previous reports suggested that flavonoids and flavanols have a great potential and health benefits because of their antioxidant activity (Hidalgo *et al.* 2010).

In vitro Antioxidant Activity of Free and Bound Phenolic Extracts: DPPH and FRAP Assays

Figure 3 shows the free and bound phenolic compound antioxidant activities by means of DPPH and FRAP assays of three olive-waste cake varieties. The FRAP assay measures

the total level of redox-active compounds in a solution (Siriamornpun *et al.* 2012). DPPH radical scavenging activity is influenced by the polarity of the reaction medium, chemical structure of the radical scavenger and the pH of the reaction mixture (Sharma and Bhat 2009). Consequently, comparison against different analytical methods to determine total antioxidant capacity is a key factor to help investigators choose a method and understand the result obtained (Uribe *et al.* 2014).

The Picual variety showed the highest antioxidant capacity related to the free form of the phenolic compounds as compared with the other varieties in both DPPH (6.72 mmol TE/100 g DW) and FRAP (17.67 mmol TE/100 g DW) assays ($P < 0.05$). Bound phenolic compounds showed a lower antioxidant capacity compared with the free forms in both DPPH and FRAP assay. In particular, Picual and Frantoio presented the highest values for FRAP assay (7.52. mmol TE/100 g DW) showing no significant differences ($P < 0.05$). Similar results have been reported in previous investigations with different by-products (Albishi *et al.* 2013; Ajila *et al.* 2013). Based on results, Picual and Frantoio resulted in the varieties that showed high antioxidant capacity compared with Arbequina.

To explore the influence of phenolic compounds on the antioxidant capacity in olive-waste cake extracts, correlations between the antioxidant capacity and FPCs were determined. Results of the FRAP and DPPH assays were positive to FPCs ($r = 0.888$ and $r = 0.929$), respectively. These results indicated that the measured antiradical activity could be attributable to the phenolic compounds present in the wastes. In addition, a good correlation was found between the FRAP and DPPH ($r = 0.994$). This correlation suggested that the relatively simple antiradical activity
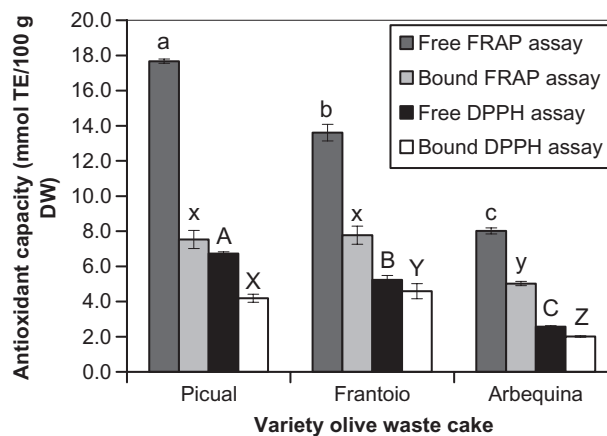

FIG. 3. ANTIOXIDANT CAPACITY OF FREE AND BOUND EXTRACTED OF THREE OLIVE-WASTE CAKE VARIETIES
*Values having the same letter for each parameter are not significantly different at a confidence level of 95%.

Compound/variety	Picual	Frantoio	Arbequina
α-Tocopherol	31.148 ± 0.195[a]	21.648 ± 0.065[b]	22.561 ± 0.138[c]
α-Tocotrienol	2.336 ± 0.065[a]	2.170 ± 0.032[b]	2.219 ± 0.037[b]
β-Tocopherol	0.741 ± 0.045[a]	0.379 ± 0.011[b]	0.352 ± 0.038[b]
γ-Tocopherol	2.133 ± 0.138[c]	0.585 ± 0.014[b]	0.561 ± 0.024[b]

TABLE 4. TOCOPHEROL CONTENTS OF THE THREE OLIVE-WASTE CAKE VARIETIES (MG/100 G DW)

Data are expressed as the mean ± standard deviation of three replicates.

[a–c] Means in the same column followed by same letter type are not significantly different ($P < 0.05$).

DPPH test under the adopted experimental conditions may be a good predictor of antioxidant activity in these wastes as measured by the FRAP assay.

Tocopherol Content

Vitamin E is a general term employed for the designation of tocopherols and tocotrienols, including α, β, γ and δ species. Primary antioxidants include phenolic compounds of the lipophilic group such as α-tocopherol. The importance of these compounds is supported by verifiable evidence as antioxidants at the cell membrane level, protecting the fatty acids of the membranes against damage caused by free radicals (Uribe *et al.* 2012).

Table 4 shows the α-tocopherol as the predominant tocopherol for the olive cake samples of the three varieties. The vitamin E contents (presented as α-tocopherol) were 31.148, 21.648, 22.561 mg/100 g DW for Picual, Frantoio and Arbequina, respectively ($P < 0.05$). These results are comparable with those reported by Escuderos *et al.* (2009) in virgin olive oils and but higher respect to those reported for other waste products (Kalogeropoulos *et al.* 2012).

α-Tocotrienol contents were in the range of 2.17–2.34 mg/100 g DW ($P < 0.05$). Besides the above mentioned health-promoting properties, several lines of evidence support the beneficial effect of tocotrienols on inhibition of tumor development (Miyazawa *et al.* 2009).

CONCLUSION

The results obtained in the present study have demonstrated that the three olive-waste cakes are excellent sources of bioactive compounds. Arbequina variety presented the highest β-carotene content (21.47 mg/100 g DW). Picual presented the highest free phenolic content (3499.11 mg GAE/100 g DW). Gallic acid, tyrosol and 3-hydroxytyrosol were detected in the free extracts of the three varieties. Regarding the flavonoids, Picual presented the highest free total flavonoid content (1331.45 mg CTE/100 g DW). Picual and Frantoio resulted in the varieties that showed high antioxidant capacity compared with Arbequina. In particular, Picual showed the highest antioxidant capacity related to the free form of the phenolic compounds as compared with

the other varieties in both DPPH (6.72 mmol TE/100 g DW) and FRAP (17.67 mmol TE/100 g DW) assays. The compound α-tocopherol was the predominant tocopherol for the three olive cake varieties (31.148 mg/100 g DW, Picual). Based on these results, future investigations are required to optimize the bioactive compounds extraction methods on industrial scale as well as to study the incorporation of these compounds extracts in real food matrices for innovative new functional foods.

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REFERENCES

AJILA, C.M. and PRASADA RAO, U.J.S. 2013. Mango peel dietary fibre: Composition and associated bound phenolics. J. Funct. Food *5*, 444–450.

ALBISHI, T., JOHN, J., AL-KHALIFA, A. and SHAHIDI, F. 2013. Phenolic content and antioxidant activities of selected potato varieties and their processing by-products. J. Funct. Food *5*, 590–600.

ALHAMAD, M., RABAHAD, T., ALU'DATT, M., EREIFEJ, K., ESOH, R., FENG, H. and YANG, W. 2012. The physicochemical properties, total phenolic, antioxidant activities, and phenolic profile of fermented olive cake. Arab. J. Chem. doi: 10.1016/j.arabkc.2012.01.002; in press.

ALIAKBARIAN, B., CASAZZA, A. and PEREGO, P. 2011. Valorization of olive oil solid waste using high pressure-high temperature reactor. Food Chem. *128*, 704–710.

ALLOUCHE, N., FKI, I. and SAYADI, S. 2004. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. J. Agric. Food Chem. *52*, 267–273.

ALU'DATT, M.H., INTEAZ, A., EREIFJ, K., ALHAMAD, M., AL-TAWAH, A. and RABABAH, T. 2010. Optimisation, characterization and quantification of phenolic compounds in olive cake. Food Chem. *123*, 117–122.

AOAC. 1990. *Official Method of Analysis no. 920.39/962.09 and 934.06*, 15th Ed., Washington, Arlington, MA.

AOAC. 2000. *Official Method of Analysis no. 939.05* (Determining titratable acidity), 15th Ed., Washington, Arlington, MA.

AOCS. 1993. *Official Methods and Recommended Practices of the American Oils Chemists' Society*, 4th Ed., pp. 1–2, AOCS Press, Champaign, IL.

AZAIZEH, H., HALAHLIH, F., NAJAMI, N., BRUNNER, D., FAULSTICH, M. and TAFESH, A. 2012. Antioxidant activity of phenolic fractions in olive mill wastewater. Food Chem. *13*, 2226–2234.

BALASUNDRAM, N., SUNDRAM, K. and SAMMAN, S. 2006. Phenolic compounds in plant and agri-industrial byproducts: antioxidant activity, occurrence, and potential uses. Food Chem. *99*, 191–203.

BARBA, O., CÁMARA HURTADO, A., SÁNCHEZ MATA, M., FERNÁNDEZ RUIZ, V. and LÓPEZ SÁENZ DE TEJADA, M. 2006. Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and b-carotene in vegetables. Food Chem. *95*, 328–336.

BIANCO, A., BUIARELLI, F., CARTONI, G., COCCIOLI, F., JASIONOWSKA, R. and MARGHERITA, P. 2003. Analysis by liquid chromatography-tandem mass spectrometry of biophenolic compounds in olives and vegetation waters, part I. J. Sep. Sci. *26*, 409–416.

BORJA, R., RAPOSO, F. and RINCÓN, B. 2006. Treatment technologies of liquid and solid wastes from two-phase olive oil mills. Grasas y Aceites *57*, 32–46.

CARDOSO, S.M., COIMBRA, M.A. and LOPEZ DA SILVA, J.A. 2003. Calcium-mediated gelation of an olive pomace extract. Carb. Pol. *52*, 125–133.

CHILEOLIVA. 2014. Chile productor. Estadisticas. Boletin informativo 2014. http://www.chileoliva.cl/index.php/en/productor/estudios-proyectos-y-estadisticas (accessed June 12, 2014).

CHIOFALO, B., LIOTTA, L., ZUMBO, A. and CHIOFALO, V. 2004. Administration of olive cake for ewe feeding: Effect on milk yield and composition. Small Rum. Res. *55*, 169–176.

CHUAH, A.M., LEE, Y.-C., YAMAGUCHI, T., TAKAMURA, H., YIN, L.-J. and MATOBA, T. 2008. Effect of cooking on the antioxidant properties of coloured peppers. Food Chem. *111*, 20–28.

DUTHIE, G. and CROZIER, A. 2000. Plant-derived phenolic antioxidants. Curr. Opin. Lipidol. *11*, 43–47.

ESCUDEROS, M.E., SAYAGO, A., MORALES, M.T. and APARICIO, R. 2009. Evaluation of α-tocopherol in virgin olive oil by a luminescent method. Grasas y Aceites *60*, 336–342.

FERNANDEZ-BOLAÑOS, J., RODRIGUEZ, G., RODRIGUEZ, R., HEREDIA, A., GUILLEN, R. and JIMENEZ, A. 2002. Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two phase olive oil processing or "Alperujo." J. Agric. Food Chem. *50*, 6804–6811.

FERNANDEZ-BOLAÑOS, J., RODRÍGUEZ, G., RODRÍGUEZ, R., GUILLÉN, R. and JIMÉNEZ, A. 2006. Potential use of olive by-products. Grasas y Aceites *57*, 95–106.

FIORENTINO, A., GENTILI, A., ISIDORI, M., MONACO, P., NARDELLI, A. and PARRELLA, A. 2003. Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular weight phenol components. J. Agric. Food Chem. *51*, 1005–1009.

GALANAKIS, C.M. 2013. Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges. Food Bioprod. Proc. *91*, 575–579.

GRANADOS-PRINCIPAL, S., QUILES, J.L., RAMIREZ-TORTOSA, C.L., SANCHEZ-ROVIRA, P. and RAMIREZ TORTOSA, M.C. 2010. Hydroxytyrosol: From laboratory investigations to future clinical trials. Nutr. Rev. *68*, 191–206.

GRIGELMO-MIGUEL, N. and MARTÍN-BELLOSO, O. 1998. Characterization of dietary fiber from orange juice extraction. Food Res. Intern. *31*, 355–361.

HIDALGO, M., SÁNCHEZ-MORENO, C. and DE PASCUAL-TERESA, S. 2010. Flavonoid–flavonoid interaction and its effect on their antioxidant activity. Food Chem. *121*, 691–696.

KALOGEROPOULOS, N., CHIOU, A., PYRIOCHOU, V., PERISTERAKI, A. and KARATHANOS, V.T. 2012. Bioactive phytochemicals in industrial tomatoes and their processing byproducts. Food Sci. Tech. *49*, 213–216.

KIM, D.O., CHUN, O.K., KIM, Y.J., MOON, H.Y. and LEE, C.Y. 2003. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J. Agric. Food Chem. *51*, 6509–6515.

LAFKA, T., LAZOU, A., SINANOGLOU, V. and LAZOS, E. 2011. Phenolic and antioxidant potential of olive oil mill wastes. Food Chem. *125*, 92–98.

LAUR, L.M. and TIAN, L. 2011. Provitamin A and vitamin C contents in selected California-grown cantaloupe and honeydew melons and imported melons. J. Food Comp. Anal. *24*, 194–201.

LESAGE-MEESSEN, L., NAVARRO, D., MAUNIER, S., SIGOILLOT, J.C., LORQUIN, J. and DELATTRE, M. 2001. Simple phenolic content in olive oil residues as a function of extraction systems. Food Chem. *75*, 501–507.

LLOBERA, A. and CAÑELLAS, J. 2007. Dietary fibre content and antioxidant activity of Manto Negro red grape (Vitis vinifera): pomace and stem. Food Chem. *101*, 659–666.

LOPEZ-MARTINEZ, L.X., OLIART-ROS, R.M., VALERIO-ALFARO, G., LEE, C.-H., PARKIN, K.L. and GARCIA, H.S. 2009. Antioxidant activity, phenolic compounds and anthocyanins content of eighteen strains of Mexican maize. Food Sci. Tech. *42*, 1187–1192.

MAKRIS, D.P., BOSKOU, G. and ANDRIKOPOULOS, N. 2007. Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts. J. Food Comp. Anal. *20*, 125–132.

MIYAZAWA, T., SHIBATA, A., SOOKWONG, P., KAWAKAMI, Y., EITSUKAA, T., ASAI, A., OIKAW, S. and NAKAGAWA, K. 2009. Antiangiogenic and anticancer potential of unsaturated vitamin E (tocotrienol). J. Nutr. Biochem. *20*, 79–86.

NIGEL, C.W. and GLORIES, Y. 1991. Use of a modified dimethyl aminocinnamaldehyde reagent for analysis of flavanols. Am. J. Enol. Vit. *42*, 364–366.

OBIED, H.K., BEDGOOD JR. D.R., PRENZLER, P.D. and ROBARDS, K. 2007. Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities. Food Chem. Toxicol. *45*(7), 1238–1248.

ODEPA, 2008. Oficina de Estudios y Políticas Agrarias. Consumo en Chile de aceitunas y aceite de oliva. http://www.odepa.gob.cl/odepaweb/jsp/odepad.jsp (accessed June 20, 2014).

PIPERIDOU, C.I., CHAIDOU, C.I., STALIKAS, C.D., SOULTI, K., PILIDIS, G.A. and BALIS, C. 2000. Bioremediation of olive oil mill wastewater: Chemical alterations induced by *Azotobacter vinelandii*. J. Agric. Food Chem. *48*, 1941–1948.

RIBEIRO DA SILVA, L., TEIXEIRA DE FIGUEIREDO, E., PONTES SILVA RICARDO, N., VIEIRA, N., WILANE DE FIGUEIREDO, R., MONTENEGRO BRASIL, I. and GOMES, C. 2014. Quantification of bioactive compounds in pulps and by-products of tropical fruits from Brazil. Food Chem. *143*, 398–404.

RODRIGUEZ, M.R., ROMERO PECES, R., CHACON VOZMEDIANO, J.L., MARTINEZ GASCUENA, J. and GARCIA ROMERO, E. 2006. Phenolic compounds in skins and seeds often grape Vitis yin ([era varieties grown in a warm climate. J. Food Comp. Anal. *19*, 687–693.

ROMERO, C., BRENES, M., GARCIA, P. and GARRIDO, A. 2002. Hydroxytyrosol 4-a-Dglucoside, an important phenolic compound in olive fruits and derived products. J. Agric. Food Chem. *50*, 3835–3839.

SHARMA, O.P. and BHAT, T.K. 2009. DPPH antioxidant assay revisited. Food Chem. *113*, 1202–1205.

SIRIAMORNPUN, S., KAISOON, O. and MEESO, N. 2012. Changes in colour, antioxidant activities and carotenoids (lycopene, b-carotene, lutein) of marigold flower (*Tagetes erecta* L.) resulting from different drying processes. J. Funct. Food *4*, 757–766.

STRATIL, P., KLEJDUS, B. and KUBAN, V. 2006. Determination of total content of phenolic compounds and their antioxidant activity in vegetables-evaluation of spectrophotometric methods. J. Agric. Food Chem. *54*, 607–616.

SUAREZ, M., ROMERO, M. and MOTILVA, M. 2010. Development of phenol enriched olive with phenolic compounds from olive cake. J. Agric. Food Chem. *58*, 1036–1043.

TERPINC, P., CEH, B., POKLAR ULRIH, N. and ABRAMOVIC, H. 2012. Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. Ind. Crop. Prod. *39*, 210–217.

URIBE, E., LEMUS-MONDACA, R., VEGA-GÁLVEZ, A., LÓPEZ, L., PEREIRA, K., LÓPEZ, J., AH-HEN, K. and DI SCALA, K. 2012. Quality characterization of waste olive cake during hot air drying: nutritional aspects and antioxidant activity. Food Bioprocess Tech. *6*, 1207–1217.

URIBE, E., LEMUS-MONDACA, R., VEGA-GÁLVEZ, A., ZAMORANO, M., QUISPE, I., PASTEN, A. and DI SCALA, K. 2014. Influence of process temperature on drying kinetics, physicochemical properties and antioxidant capacity of the olive-waste cake. Food Chem. *147*, 170–176.

VISIOLI, F., POLI, A. and GALLI, C. 2002. Antioxidant and other biological activities of phenols from olives and olive oil. Med. Care Res. Rev. *22*, 65–75.

VLAHOV, G. 1992. Flavonoids in three olive (*Olea europaea*) fruit varieties during maturation. J. Sci. Food Agric. *58*, 157–159.

VULIC, E., CEBOVIC, T., CANADANOVIC'-BRUNET, J., CETKOVIC, G., CANADANOVIC, V., DJILASA, S. and SAPONJAC, T. 2014. *In vivo* and *in vitro* antioxidant effects of beetroot pomace extracts. J. Funct. Food 6, 168–175.

YU, L., HALEY, S., PERRET, J., HARRIS, M., WILSON, J. and QIAN, M. 2002. Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. *50*, 1619–1624.