Phenolic Compounds: Their Role During Olive Oil Extraction and in Flaxseed – Transfer and Antioxidant Function

Luz Stella Artajo Medina
Food Engineer

A thesis submitted to the Faculty of Agromical Engineer of the University of Lleida in partial fulfillment of the requirements of the degree of Doctorate of Philosophy

Supervisors:
María José Motilva Casado, Ph.D
María Paz Romero Fabregat, Ph.D

University of Lleida
Agronomical, Forestal and Food Systems Doctorate Program
Food Technology Department
Lleida, Spain, 2006

2.5 ENHANCEMENT IN THE OXIDATIVE STABILITY OF EXTRA VIRGIN OLIVE OIL MATRICES BY THE ADDITION OF PHENOLIC COMPOUNDS

In Preparation

Objective 2.1

Objective 2.2

Objective 2.3

Paper in Preparation

ENHANCEMENT IN THE OXIDATIVE STABILITY OF EXTRA VIRGIN OLIVE OIL MATRICES BY THE ADDITION OF PHENOLIC COMPOUNDS

Luz S Artajo, M Paz Romero, and M José Motilva

Abstract:

The study of the antioxidant effects of biophenolic compounds is supported by the current interest in natural products and the on-going replacement of synthetic antioxidants by natural antioxidants from plant sources. This research was focused on the enrichment of olive oil matrices with individual compounds from the phenolic fraction of virgin olive oil, and the evaluation of its antioxidant capacity in order to discover possible functional applications in food. Two commercial extra virgin olive oil matrices (EVOO) from *Arbequina* and *Morrut* cultivars were used to perform the antioxidants assays through enrichment with different phenolics. They differed in their percentages of saturated, monounsaturated and polyunsaturated fatty acid content. Diverse concentrations of individual phenolic compounds (commercial standards and isolated from virgin olive oil by semipreparative HPLC) were added to the lipid matrices, and the antioxidant activity (AA), expressed as oxidative stability in hours, was determined. Regression analysis was carried out to evaluate the correlation between the induction time (IT) (hours) and the enrichment concentration of individual phenols. A positive linear trend was established between IT and the concentration of phenol with a good correlation coefficient ($r^2 \geq 0.90$) and higher slope for gallic acid and luteolin compounds.

Keywords: Phenolic Compounds, Antioxidant Activity, Oxidative Stability, Virgin Olive Oil

Introduction

Phenolic compounds, as a diverse range of secondary metabolites from the shikimate pathway and phenylpropanoid metabolism, play an important role on human health. Interest in phenolic compounds has been increased during last years since they have potential antioxidant activity and potential effects against degenerative illness. Polyphenols are significant in olive fruit because of their wide range of biochemical and pharmaceutical effects such as anticarcinogenic, antiatherogenic and antimicrobial [1-3]. Some authors have estimated their contribution to the increase of oil stability close to 30% in terms of induction time [4].

Free radicals are formed as natural by-products when the human body converts oxygen into energy. However, their overproduction as well as the exposure to various environmental factors such as pollution, smoke and pesticides causes damage to the body. This process, known as oxidation, causes harm to cells and can contribute to any number of debilitating diseases, including cancer and heart disease. Antioxidants prevent oxidation from happening and could be considered substances that, when present at low concentrations, compared with those of an oxidizable substrate, significantly retards oxidation of that substrate [5]

Fruits and vegetables are important sources of antioxidants such a polyphenols [6]. A polyphenol must delay, retard or prevent the autoxidation or free radical-mediated oxidation to be considered antioxidant. Therefore, the resulting radical formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation [7].

On the other hand, the study of antioxidant effects of biophenolic compounds was supported with the current interest in natural products and the continuously replacing of synthetic antioxidants by natural antioxidants from plant sources as well as the screening of raw materials to identify new antioxidants [8-10]. The effectiveness as antioxidants is demonstrated by structure–activity relationships [11]. Enrichment of processed food with polyphenols protects against oxidation and has better keeping quality because formation of toxic oxidation products, like cholesterol oxides, is being prevented such enrichment also benefits human health. Both of these benefits, however, hinge on the availability of the phenolic substances [12].

Olives and olive oil, in particular extra virgin olive oil, contain a variety of bioactive compounds considered to be potentially beneficial to health. There are consistent

evidences regarding the health-beneficial properties (protection against cardiovascular diseases and cancer) of the virgin olive oil. The responsible for such beneficial properties are both an adequate fatty acid profile and the presence of antioxidants such as the phenolic compounds. The most important phenolic compound is the antioxidant hydroxytyrosol which has been widely studied demonstrating its health-beneficial properties as well as its good bioavailability [13].

Studies have shown the biological antioxidant effects of the olive phenols through *in vitro*, rat and human studies. *In vitro*, the phenols inhibited LDL oxidation, leukocyte activation, superoxide anion production, and the enzyme glutathione-S-transferase. It has been suggested that water-soluble olive oil polyphenols might be a good candidate as a functional food ingredient in the future, potentially influencing the development of cardiovascular disease, cancer and inflammatory reactions. In that sense, functional foods are foods or dietary components that may provide a health benefit beyond basic nutrition [14].

In addition, the synergism effect between diverse natural antioxidants has been studied. Polyphenols and α-tocopherol influence the stability of some food systems, it can be described as an effect of regeneration of the more-active by the less-active antioxidant. The synergism between ascorbate and the tocopherols rather depends on the phase distribution of the two types of antioxidants. When a better understanding of the mechanism behind antioxidant synergism is available, protective systems based on such an understanding will probably be developed. The use of green tea extract in meat systems may present a breakthrough in exploitation of synergism between nutrient and non-nutrient antioxidants [15]. The use of plant material and plant extracts, as food ingredients will go beyond antioxidative effects. The "green revolution" for the food ingredients industry is to modify plants to produce molecules that combine emulsifying and/or thickening effects with antioxidative and antimicrobial effects.

In this study, the enrichment of Extra Virgin Olive Oil with different polyphenols was evaluated trough the antioxidant capacity measured by Rancimat. Individual and combined phenolic compounds- standards and isolated- were added to different lipid matrices. Thus, to develop a potential functional olive oil.

Materials and Methods

Lipid Matrices. Extra Virgin Olive Oil (EVOO) from both Arbequina and Morrut cultivars were used to perform the antioxidant assay. Samples were collected from olive oil mills established in the region "Les Garrigues" (Lleida-Catalonia, Spain). After sampling, olive oil was filtered through a paper filter Ahlstrom, d.150mm, dark bottling with nitrogen in head sace and finally stored at 5ºC.

Acidity, peroxide value (PV) and spectroscopic indices, K_{270}, K_{232}. These parameters were determined according to the UE official method [16].

K_{225} (Bitter index). A C18 column (Waters Sep-Pack cartridges) was activated with 6 mL of methanol (6 mL) and then washed with 6 mL of hexane. 1.00±0.01 g of oil dissolved in 4 mL of hexane was passed through the column. After elution 10 mL of hexane were passed to eliminate the fat and the retained compounds were eluted with 25 mL of methanol/water (1/1). The absorbance was measured at 225 nm against methanol/water (1/1) in a 1 cm cuvette.

Fatty acid composition analysis. The fatty acid composition of the oils was determined by gas chromatographic (GC) according to the European Union Commission Modified Regulation EEC 2568/91) [16].

α-Tocopherol determination. α-Tocopherol was evaluated by High-Performance Liquid-Chromatographic with direct injection of an oil dissolved in hexane. [17].

Phenolic and Pigment Characterization. Phenolic compounds from olive oil were extracted as reported by Artajo [18].

The chlorophyll and carotenoid compounds were evaluated following the HPLC method reported by Mínguez-Mosquera [19].

Reference Compounds. α-Tocopherol was used form Sigma-Aldrich Chemical Co. (USA). Phenolic standards were used without further purification: apigenin, apigenin 7-O-glucoside, *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin 7-O-glucoside, oleuropein, tyrosol, *p*-HPEA, and vanillin from Extrasynthese (Genay, France); caffeic acid, *o*-coumaric acid, ferulic acid, gallic acid, and vanillic acid from Fluka Co. (Buchs, Swiss), and 3,4-dyhydroxyphenilacetic acid (DOPAC), 4-hydroxybenzoic acid; homovanillic acid, *trans*-Cinnamic acid from Sigma-Aldrich Chemical Co. (USA). Chlorophylls a (No. C-6144 from algae), chlorophyll b (No. C-5878 from spinach) and β-carotene (No. C-4582) were supplied by Sigma (St. Louis, MO).

Isolated and Acquired Compounds. Secoiridoids derivatives 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC); 4-Hexenoic acid, 4-formyl-3-(2-oxoethyl)-2-(3,4 dihydroxyphenyl) ethyl ester (3,4-DHPEA-

EDA); 4-Hexenoic acid, 4-formyl-3-(2-oxoethyl) 2-(4-hydroxyphenyl) ethyl ester (p-HPEA-EDA); 2H- Pyran-4-acetic acid, 3-formyl -3,4-dihydro-5-(methoxycarbonyl) -2-methyl-2-(4-hydroxyphenyl) ethyl ester (3,4-DHPEA-EA); 2H-Pyran-4-acetic-, 3-formyl-3,4-dihydroxy-5-(methoxycarbonyl)-2-methyl-2-(4-hydroxyphenyl) ethyl ester (p-HPEA-EA) and methylated form of 3,4-DHPEA-EA were isolated from EVOO by using semipreparative method.

Pheophytins a and b were obtained from the respective solutions of chlorophylls by shaking the ether solution with 2 – 3 drops of 13% HCl until the green chlorophyll colour changed to the greyish pheophytin colour (Holden, 1976; Sievers & Hynninen, 1977). Pheophorbide a was formed by enzymatic de-esterification of pheophytin a (Hynninen, 1973). Chlorophyllides a and b were obtained from the respective solutions of chlorophylls by enzymatic de-esterification (Jones, Butler, Gibbs & White, 1972). The enzymatic extract of chlorophyllase was obtained from Ailanthus altissima leaves (Terpstra & Lambers, 1983). Lutein, antheraxanthin, violaxanthin and neoxanthin were obtained from a pigment extract of fresh spinach and separated by TLC on silica gel GF254 (0.2 mm) on 20 x 20 cm plates using petroleum ether (65 – 95° C)/acetone/diethylamine (10:4:1, v/v/v) [19].

High - Performance Liquid Chromatographic. HPLC analyses were performed using a Waters 600 E pump, a Waters column heater, a Waters 717 plus autosampler equipped with a 20 µL loop injector and a Waters 996 photodiode array detector. Separation was achieved on a 5 µm, 15 cm x 4.6 mm i.d., Inertsil ODS-3 column (GL Sciences Inc.) equipped with a 5 µm, 1 cm x 4.6 mm i.d., Spherisorb S5 ODS-2 precolumn (Technokroma, Barcelona, Spain). The mobile phases were degassed under vacuum using continuously sparged with high-purity helium during analysis. Water/acetic acid (100:02 v/v) was used as solvent A and methanol as solvent B.

Semipreparative Isolation of Compounds from Olive Oil. Phenolics compounds extracted from olive oil were isolated following the proceeding reported by Artajo *et al* [20].

Liquid Chromatography - Mass Spectrometry. Samples extracts corresponding to phenolic compounds in methanol/water (50:50 v/v) were analyzed using a Waters System (Alliance 2596 Separations Modul, and a Micromass Quattro II quadrupole mass spectrometer by electrospray ionization (ESI). A Inertsil ODS-3 (5 µm, 15 cm x 4.6 mm i.d., GL Sciences Inc.) column used for HPLC analyses was used in conjunction with the gradient program described for routine HPLC. A flow rate of 1 mL/min was used with a split ratio of 20:1 (PDA detector/MS). The photodiode array detector output was monitored at 280 nm. Masslynx Data System (Micromass) for alignment with the mass spectral data. The mass spectral data were acquired at four alternating scans from m/z 100 to 700 in 2 s using both positive and negative ion modes at cone voltages of 30 V and 70 V.

Evaluation of Antioxidant Activity (AA). The AA was evaluated according to Rancimat method (Metrohm, Ltda). A flow of air (20 L/h) was bubble through the heated oil at 120 °C and cold water and Induction Time (IT) were recorded. Standard and isolated compounds were prepared in Me-OH/H_2O (80:20 v/v) to the desired concentration in a range of 0.05 to 2.00 mmol/kg oil (µM) and added to a reaction vessel containing 2.5 g of the lipid matrix and then the mixtures were homogenized with vortex. Each analysis was done in duplicate.

Lipid matrices without any added compounds were analyzed as control samples and the antioxidant activity was expressed as the antioxidant activity index: AAI= ITs/ITc, where ITs is the induction time of a stabilized sample and ITc is the induction time of a control sample.

Statistical analysis. Regression analysis was carried out with the 6.12 version SAS System package (SAS Institute Inc. Cary, NC, USA) to evaluate the correlation between the induction time (hours) and the enrichment concentration of individual phenolic compounds.

Results and Discussion

Characterization of Lipid Matrices. Adding antioxidants to food matrices lead to evaluate the real effect of these compounds and their possible interaction with other components naturally occurring in product. Olive oil, primary source of fat in the Mediterranean diet is a good matrix to be studied for enrichment processes, not only as product of direct consumption but also as part of ingredient of new industrial foodstuffs. Thus, characterization of the olive oil matrices contributes to establish the basis of this study. **Table 1** shows the quality parameters of Extra Virgin Olive Oil (EVOO) matrices. The fatty acid compositions of the two extra virgin olive oil matrices are shown in **Table 2. Table 3** and **Table 4** show the phenolic and pigment profile of both EVOO.

Table 1. Physical - Chemical Profile and Pro-oxidant and Antioxidant Factors. Extra Virgin Olive Oil (EVOO). [Means Values]

	EVOO (Arbequina cultivar)	EVOO (Morrut cultivar)
QUALITY PARAMETERS		
Moisture (%)	0.06 ± 0.01	0.05 ± 0.01
Acidity (% oleic acid)	0.11 ± 0.02	0.40 ± 0.08
Peroxide Value ($meqO_2$/kg oil)	6.03 ± 0.95	15.92 ± 1.01
Absorbance en el UV		
K_{270}	0.07 ± 0.01	0.15 ± 0.02
K_{232}	1.60 ± 0.2	2.84 ± 0.1
COMPOSITION		
Tocopherols (mg/kg oil)	151.49 ± 1.2	149.96 ± 1.4
Total Phenol content (mg/kg oil)	129.37 ± 0.9	178.15 ± 1.1
Total Pigments content (mg/kg oil)		
Chlorophylls	5.40 ± 0.02	6.38 ± 0.02
Carotenoids	5.77 ± 0.3	11.9 ± 0.5
β-Carotenoid	0.93 ± 0.02	1.41 ± 0.02
OXIDATIVE STABILITY (h)	9.38 ± 0.8	5.72 ± 0.4
BITTER INDEX (K_{225})	0.160 ± 0.02	0.170 ± 0.03

Table 2. Fatty Acid Composition of Refined Olive Oil (ROO) and Extra Virgin Olive Oil (EVOO) [Means Values]

	EVOO (Arbequina cultivar)	EVOO (Morrut cultivar)
Fatty Acid (%)		
Palmitic Acid (C16:0)	12.07 ± 0.5	13.51 ± 0.4
Palmitoleic Acid (C16:1)	1.29 ± 0.06	1.11 ± 0.04
Margaroleico Acid (C17:1)	0.20 ± 0.03	0.20 ± 0.01
Stearic Acid (C18:0)	1.84 ± 0.01	2.52 ± 0.03
Oleic acid (C18:1)	74.44 ± 0.6	69.33 ± 0.5
Linoleic acid (C18:2)	8.88 ± 0.3	11.70 ± 0.2
Linolenic acid (C18:3)	0.45 ± 0.02	0.51 ± 0.01
Arachidic acid (C20:0)	0.40 ± 0.01	0.58 ± 0.01
Gadoleic acid (C20:1)	0.37 ± 0.02	0.41 ± 0.03
Behenic acid (C22:0)	0.11 ± 0.02	0.13 ± 0.01
Saturated	14.42 ± 0.8	16.73 ± 1.1
Mono-unsaturated	76.30 ± 1.5	71.04 ± 1.9
Poly-unsaturated	9.33 ± 0.9	12.21 ± 1.0

Table 3: Phenolic profile of Extra Virgin Olive Oil (EVOO) from Arbequina and Morrut cultivars Means Values] N.D= not detected

Phenolic compound (mg/kg oil)	EVOO (Arbequina cultivar)	EVOO (Morrut cultivar)
Phenolic acids and Derivatives		
Hydroxytyrosol	2.64 ± 0.3	10.36 ± 0.9
Tyrosol	2.90 ± 0.9	6.04 ± 0.4
Vanillic Acid	0.17 ± 0.03	1.44 ± 0.02
Vanillin	0.22 ± 0.02	0.53 ± 0.02
p- coumaric acid	ND	0.24 ± 0.03
Flavonoids		
Luteolin	2.40 ± 0.07	1.28 ± 0.03
Apigenin	1.34 ± 0.03	1.38 ± 0.02
Secoiridoid Derivatives		
3,4-DHPEA-AC	30.12 ± 1.9	2.66 ± 0.06
3,4-DHPEA-EDA	47.84 ± 2.1	43.65 ± 1.7
p-HPEA-EDA	19.33 ± 0.5	26.15 ± 0.8
p –HPEA-EA	3.03 ± 0.02	3.95 ± 0.03
3,4-DHPEA-EA	50.71 ± 1.9	75.73 ± 2.3
Methylated form of 3,4 -DHPEA-EA	22.63 ± 0.8	25.08 ± 1.1
Lignans		
Acetoxypinoresinol and pinoresinol	38.79 ± 1.3	46.44 ± 1.1

EVOO (Extra Virgin Olive Oil) matrices had a remarkable difference in peroxide value and K_{232} parameter as well as carotenoid content. Total phenol content gave an important dissimilarity between samples and in consequence the oxidative stability. The fatty acid profile showed a slight difference in stearic, oleic and linoleic acids. At the same time, the α- tocopherol content was similar in the two extra virgin olive oils.

In extra virgin olive the most abundant phenolic compounds were secoridoid derivatives. 3,4-DHPEA-EDA, *p*-HPEA-EDA, *p*-HPEA-EA, methylated form of 3,4-DHPEA-EA (maxim absorption at 210 and 280 nm) followed by lignans (maxim absorption at 230 nm and 280 nm) and 3,4-DHPEA-AC (maxim absorption at 205 nm and 280 nm) and simple phenols such as phenolic alcohols (hydroxytyrosol and tyrosol) and phenolic acids (caffeic, ferulic, *p*-coumaric, *o*-coumaric). Flavones aglycones such as luteolin and apigenin played also an important role. These compounds have been previously identified by spectrum characteristics. Although, methylated form of 3,4-DHPEA-EA was recently found as an important component present in olive oil [20].

The chemical properties of polyphenols, in terms of the availability of the phenolic hydrogens, as hydrogen-donating radical scavengers, could predict their antioxidant activity. Free radicals had an unpaired electron, which they tried to find a matched for by stealing an electron from another molecule. Antioxidants cleaned up as possible by stabilizing the free radicals before damage occurred by giving up one of their electrons.

Relation between Oxidative Stability and Antioxidant Compounds. A positive linear trend between IT and concentration of antioxidant **(Table 5)** with a good correlation coefficient ($r^2 \geq 0.90$) and higher slope could be established for some phenolic compounds evaluated in EVOO matrices at concentration range of evaluation. Gallic acid and luteolin showed the highest slopes in both matrices from Arbequina and Morrut cultivar, indicating a significant increase in induction time at different concentrations. However, 3,4-DHPEA-EDA did not show a good linear trend as it was observed on Refined Olive Oil in previous studies [20].

Table 4. Pigment profile of Extra Virgin Olive Oil (EVOO) from Arbequina and Morrut cultivars [Means Values± SD] Tr = trace amount N.D= not detected

	EVOO (Arbequina cultivar)	EVOO (Morrut cultivar)
Pigments (mg/kg oil)		
Feoforbide a	0.02 ± 0.001	Tr
Neoxanten	0.10 ± 0.02	0.03 ± 0.002
Violaxanten	0.12 ± 0.09	0.15 ± 0.02
Luteoxanten	0.09 ± 0.02	0.09 ± 0.01
Anteraxanten	0.11 ± 0.04	0.10 ± 0.03
Mutatoxanten	0.05 ± 0.01	ND
Lutein	1.84 ± 0.1	1.99 ± 0.3
Chlorophyll b	0.10 ± 0.02	ND
B-criptoxanten	0.07 ± 0.01	0.04 ± 0.01
Chlorophyll a	0.02 ± 0.001	ND
Mono-Estherified Violaxanten	0.02 ±	ND
Feofitin b	0.18 ± 0.03	0.08 ± 0.01
Estherified Neoxanten	ND	ND
Feofitin a	2.21 ± 0.3	4.89 ± 0.9
α-tocopherol	0.01 ± 0.001	0.01 ± 0.001

Effect of Individual Phenolic Compounds Addition on Olive Oil Matrices. The systematic study of the antioxidants on EVOO makes more understandable the individual behavior of phenolic compounds against oxidative process. During recent years the antioxidant or pro-oxidative effect of natural antioxidants has been widely studied and it has been highlighted that many factors influence their response on lipid matrices. In general, free radicals have an unpaired electron, which they try to find a match for by stealing an electron from something around it. Antioxidants clean up as many free radicals as possible by stabilizing the free radicals before damage occurs by giving up one of their electrons.

In spite of the fact that wide data on the importance of natural antioxidants in conferring stability towards or protection from oxidation have been reported [10, 22], the connection between antioxidant activity and chemical structure (the position and the degree of hydroxylation) is has not been entirely elucidated. Factors such a system of assay, temperature, pH, and concentration could be influenced founded results by researchers [7, 23].

Phenolic antioxidants are primary antioxidants which act as free-radical terminators. The position and the degree of hydroxylation are of primary importance in determining antioxidant activity and chemical structure could be criteria to evaluate the activity of polyphenols in enhancing the Rancimat stability of oils. The specific mode of inhibition of oxidation by the individual polyphenols is not clear but they may act by chelating copper ions via the ortho dihydroxy phenolic structure scavenging lipid alcoxyl and peroxyl radicals or by acting as chain breaking antioxidants, as hydrogen donors.

Table 5. Model to describe the relationship between oxidative stability (Induction Time) and phenolic concentration[a]

Correlation Parameters [b]

EVOO (Extra Virgin Olive Oil, Arbequina cultivar)				
Phenolics Acids and Derivatives	**a**	**B**	**c**	**r^2**
Caffeic Acid	10.01	5.57	---	0.985
Gallic Acid	11.33	11.20	---	0.966
Flavonoids				
Luteolin	9.42	2.67	---	0.991
Secoiridoids and Derivatives				
Oleuropein	9.97	1.65	---	0.809
3,4-DHPEA-EDA	10.04	-2.5×10^{-3}	3.00×10^{-5}	0.874
Methylated form of 3,4-DHPEA-EA	9.97	3.07	---	0.889
EVOO (Extra Virgin Olive Oil, Morrut cultivar)				
Phenolics Acids and Derivatives	**a**	**B**	**c**	**r^2**
Caffeic Acid	6.25	3.62	---	0.988
Gallic Acid	6.90	4.80	---	0.923
Flavonoids				
Luteolin	5.76	1.06	---	0.963
Secoiridoids and Derivatives				
Oleuropein	5.75	2.45	---	0.905
3,4-DHPEA-EDA	5.34	7.7×10^{-3}	-1.00×10^{-5}	0.711

[a] Values represent the mean of two replicates of two set of experiments
[b] Simple regression y = a + bx; Polynomial regression y = a +bx + cx^2

Phenolic Alcohols. The antioxidant activity expressed as AAI of hydroxytyrosol and tyrosol were evaluated at concentrations from 0.25 to 2.0 μM. Hydroxytyrosol do have a significant effect in the concentration range. The introduction of a second hydroxyl group in the ortho position increases antioxidant activity; this effect was corroborated with the AAI maximum values reached (1.76 and 2.36) in Arbequina and Morrut EVOO respectively. This is in agreement with the study performed by Baldioli *et al* [24,25], considering this phenolic compound the main ortho-diphenol occurring in olive fruit and olive oil. Conversely, tyrosol as a monophenol, was far less efficient than the polyphenols, which is reflected in a no significant effect on EVOO matrices. This agrees with the information reported by several authors [20,26,27].

The results obtained from phenolic acids were expected. Gallic acid exhibited the most antioxidant effect on refined olive oil matrix [20], while the increment in AAI was more important in EVOO from Arbequina cultivar. Caffeic acid was founded to have a very huge antioxidant activity compared to some of the other phenolic acids in EVOO matrices; it might be due to its *ortho* phenolic condition. In addition, derivates of cinnamic acid, such as ferulic and *p*-coumaric were more active than hydroxyl derivatives of benzoic such as vanillic acid and 4-dihydroxybenzoic acid, which exhibited only a weak antioxidant activity due to the presence of the CH=CH-COOH [21, 28].

Flavonoids, As can be observed in this study, luteolin and apigenin present a higher AAI related to their 7 position glycosilated forms, this agree with the information reported in several studies [29-31]. Although, there is, occasionally, unpredictable relationship between the structure of some flavonoids and their antioxidant activities.

Secoiridoid and Derivatives. 3,4-DHPEA-EA has been reported as strong antioxidant in several lipid systems including oil emulsions. However, our result showed slight antioxidant activity. 3,4-DHPEA-EDA is the compound of especial interest because of its presence as one of the major secoiridoid antioxidant compounds in virgin olive oil [32]. This compound had a positive result as an antioxidant having evidence that bear two hydroxyl groups linked to an aromatic ring on the *ortho* position. Ligstroside derivative (*p*-HPEA-EA), bearing one hydroxyl substituent, had a negative effect on antioxidant activity evaluated in EVOO matrices from Arbequina and Morrut cultivars, the antioxidant activity was lower than 1, having an evidence of its potency pro-oxidant action. These results are in agreement with those reported by Morelló *et al* [26].

The Antioxidant Activity Index expressed in terms of Induction Time is shown in **Table 6.** This measure could be considered a reliable evaluation of the susceptibility to oxidative degeneration and specifies the shelf life of an olive oil.

Table 6. Antioxidant Activity Index (AAI) of EVOO (Arbequina and Morrut cultivar). Induction Time of sample ITs / Induction Time of control ITs

	PHENOLIC COMPOUND CONCENTRATION (mmol/kg oil) (μM)					
	(EVOO Arbequina / EVOO Morrut)					
PHENOLIC COMPOUNDS						
Phenolic Alcohols	**0.00**	**0.25**	**0.50**	**1.00**	**1.50**	**2.00**
Hydroxytyrosol	1.00	1.11 / 1.23	1.32 / 1.55	1.49 / 1.76	1.70 / 2.11	1.76 / 2.36
Tyrosol	1.00	0.99 / 1.01	1.00 / 1.03	1.02 / 1.06	1.03 / 1.07	1.01/ 1.04
Phenolic Acids and Derivatives	**0.00**	**0.25**	**0.50**	**1.00**	**1.50**	**2.00**
Vanillic Acid	1.00	1.04 / 0.98	1.02 / 1.00	1.02 / 1.02	1.04 / 1.05	1.05 / 0.98
Homovanillic Acid	1.00	1.02 / 1.05	0.93 /1.00	1.00 / 1.05	0.93 / 1.05	0.93 / 1.05
Vanillin	1.00	1.00 / 0.94	1.06 / 0.96	0.99 / 0.4	1.01 / 0.91	0.97 / 0.92
Caffeic Acid	1.00	1.22 / 1.12	1.28 / 1.25	1.56 / 1.48	1.96 / 1.91	2.12 / 2.08
Ferulic Acid	1.00	1.06 / 0.97	1.01 / 0.97	1.04 / 1.08	1.08 / 1.08	1.04 / 1.08
Gallic Acid	1.00	1.47 / 1.34	1.88 / 161	2.65 / 2.21	2.92 / 2.53	3.42 / 2.55
o-coumaric Acid	1.00	0.99 / 1.00	0.98 / 0.96	1.06 / 1.04	1.04 / 1.05	0.98 / 1.06
p-coumaric Acid	1.00	1.00 / 0.98	1.00 / 1.00	1.02 / 1.01	1.03 / 0.98	0.98 / 1.01
DOPAC	1.00	1.05 / 0.99	1.02 / 1.02	1.00 / 1.00	1.01 / 1.05	1.02 / 1.06
4-hidroxybenzoic Acid	1.00	0.97 / 1.07	0.97 / 1.04	0.99 / 1.03	1.01 / 1.09	0.97 / 1.07
trans-cinnamic Acid	1.00	0.96 / 1.05	0.99 / 1.01	0.95 / 1.06	0.85 / 1.03	1.01 / 1.04
Hydrocinnamic Derivative	**0.00**	**0.10**	**0.20**	**0.30**	**0.40**	**0.50**
Verbascoside	1.00	1.03 / 1.04	1.00 / 1.04	1.02 / 1.10	1.00 / 1.13	1.03 / 1.13
Flavones	**0.00**	**0.15**	**0.30**	**0.60**	**0.90**	**1.20**
Luteolin	1.00	1.07 / 1.07	1.08 / 1.13	1.18 / 1.17	1.25 / 1.34	1.36 / 1.36
Apigenin	1.00	1.04 / 0.96	1.02 / 0.97	1.03 / 0.98	1.06 / 1.01	1.02 / 1.00
Luteolin 7-O-glucoside	1.00	1.04 / 1.06	0.97 / 1.05	0.97 / 1.07	1.02 / 1.11	0.96 / 1.05
Apigenin-7-O-glucoside	1.00	0.96 / 0.97	0.97 / 0.99	1.03 / 1.01	0.98 / 0.99	0.94 / 1.02
Secoiridoids and Derivatives	**0.00**	**0.10**	**0.15**	**0.30**	**0.45**	**0.60**
Oleuropein	1.00	1.02 / 1.05	1.07 / 1.17	1.06 / 1.20	1.11 / 1.20	1.10 / 1.30
	0.00	**0.20**	**0.40**	**0.80**	**1.20**	**1.60**
3,4-DHPEA-AC	1.00	1.00 / 1.01	1.01 / 1.11	1.10 / 0.98	1.00 / 1.00	0.93 / 0.99
	0.00	**0.10**	**0.20**	**0.40**	**0.60**	**0.80**
3,4-DHPEA-EDA	1.00	1.07 / 1.17	1.03 / 1.25	1.07 / 1.23	1.09/ 1.22	1.24 / 1.35
	0.00	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
p-HPEA-EDA	1.00	0.92 / 0.96	0.96 / 0.94	0.99 / 0.92	0.97 / 0.90	0.92 / 0.84
3,4-DHPEA-EA	1.00	0.96 / 1.03	0.98 / 1.08	0.99 / 1.08	0.98 / 1.11	0.97 / 1.08
Methylated form of 3,4-DHPEA-EA	1.00	0.96 / 1.08	1.02 / 1.08	1.06 / 1.10	1.09 / 1.07	1.12 / 1.10
Lignans	**0.00**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
Acetoypinoresinol + pinoresinol	1.00	1.05 / 1.00	1.06 / 1.00	1.07 / 1.08	1.11 / 1.10	1.13 / 1.10

CONCLUSIONS

The position and the degree of hydroxylation are of primary importance in determining antioxidant activity and chemical structure could be a criteria to evaluate the activity of polyphenols in enhancing the Rancimat stability of oils.

Gallic and caffeic acid had a good linear trend relationship ($r2 \geq 0.90$; high slope) in EVOO matrices. Luteolin showed a similar behavior. However, it appeared that the differences in the oxidative stability of extra virgin olive related to the addition of phenolic compounds might be affected by the fatty acid, α-tocopherol, phenolic and pigment composition.

In conclusion, a great antioxidant activity corresponded to 3,4-dihydroxy and 3,4,5-trihydroxy structures linked to an aromatic ring that conferred the moiety with a higher proton dislocation, thus facilitating the scavenging activity.

ACKNOLEGMENTS

This work was supported by grant AGL2002-00289 from the "Comisión Interministerial de Ciencia y Tecnología" (CICYT, Spain) and by the "Comissió Interdepartamental de Recerca i Innovació Tecnològica" (CIRIT, Catalonia Government, European Social Fund).

REFERENCES

1. Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in Olea europaea L. *Sci. Hort.* **2002,** *92,* 147-176.

2. Patumi, M.; D'Andria, R.; Marsilio, V.; Fontanazza, G.; Morelli, G.; Lanza, B. Olive and olive oil quality after intensive monocone olive growing (Olea europaea L., cv. Kalamata) in different irrigation regimes. *Food Chem.* **2002,** *77,* 27-34.

3. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998,** *56,* 317-333.

4. Morello, J.R.; Romero, M.P.; Motilva, M.J. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga, and Morrut cultivars. *J. Agric. Food Chem.* **2004,** *52,* 6002-6009.

5. Shahidi, F. and Naczk, M. *Food phenolics: sources, chemistry, effects and applications.* Technomic Publishing Company, Inc.: Lancaster, Pennsylvania, 1995; pp. 331.

6. Antolovich, M.; Bedgood Jr., D.R.; Bishop, A.G.; Jardine, D.; Prenzler, P.D.; Robards, K. LC-MS Investigation of Oxidation Products of Phenolic Antioxidants. *J. Agric. Food Chem.* **2004,** *52,* 962-971.

7. Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *FREE RADIC. BIOL. MED.* **1996,** *20,* 933-956.

8. Kæhkoœnen, M.P.; Hopia, A.I.; Vuorela, H.J.; Rauha, J.-.; Pihlaja, K.; Kujala, T.S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999,** *47,* 3954-3962.

9. Ryan, D.; Antolovich, M.; Herlt, T.; Prenzler, P.D.; Lavee, S.; Robards, K. Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. *Journal of Agricultural & Food Chemistry* **2002,** *50,* 6716-6724.

10. Briante, R.; Febbraio, F.; Nucci, R. Antioxidant Properties of Low Molecular Weight Phenols Present in the Mediterranean Diet. *J. Agric. Food Chem.* **2003,** *51,* 6975-6981.

11. Zhongkai Zhou, Robards, K., Helliwell, S.,Blanchard, C. The distribution of phenolic acids in rice. *Food Chem.* **2004,** *87,* 401-406.

12. Britt, C.; Gomaa, E.A.; Gray, J.I.; Booren, A.M. Influence of Cherry Tissue on Lipid Oxidation and Heterocyclic Aromatic Amine Formation in Ground Beef Patties. *J. Agric. Food Chem.* **1998,** *46,* 4891-4897.

13. Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F.F.; Galli, C. Antioxidant and other biological activities of olive mill waste waters. *J. Agric. Food Chem.* **1999,** *47,* 3397-3401.

14. Galli, C. and Visioli, F. Antioxidant and other activities of phenolics in olives olive oil, typical components of the Mediterranean diet. *Lipids* **1999,** *34,* S23-S26.

15. Hu, M. and Skibsted, L.H. Kinetics of reduction of ferrylmyoglobin by (-)-epigallocatechin gallate and green tea extract. *J. Agric. Food Chem.* **2002,** *50,* 2998-3003.

16. European Union Commission Regulation EEC 2568/91 on the characteristics of olive and olive pomace oils and their analytical methods. *Off. J. Eur. Communities* **1991,** *L248.*

17. Romero, M.P.; Tovar, M.J.; Ramo, T.; Motilva, M.J. Effect of crop season on the composition of virgin olive oil with protected designation of origin "Les Garrigues". *J. Am. Oil Chem. Soc.* **2003,** *80,* 423-430.

18. Artajo, L.S.; Romero, M.P.; Motilva, M.J. Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of the fruit. *J. Sc. Food Agric.* **2006,** *86,* 518-527.

19. Minguez-Mosquera, M.I.; Rejano-Navarro, L.; Gandul-Rojas, B.; Sanchez-Gomez, A.H.; Garrido-Fernandez, J. Color-pigment correlation in virgin olive oil. *JAOCS J Am Oil Chem Soc* **1991,** *68,* 332-336.

20. Artajo, L.-.; Romero, M.-.; Tovar, M.-.; Motilva, M.-. Effect of irrigation applied to olive trees (Olea europaea L.) on phenolic compound transfer during olive oil extraction. *Eur. J. Lipid Sci. Technol.* **2006,** *108,* 19-27.

21. Marinova, E.M. and Yanishlieva, N.V. Antioxidant activity and mechanism of action of some phenolic acids at ambient and high temperatures. *Food Chem.* **2003,** *81,* 189-197.

22. Moure, A.; Cruz, J.M.; Franco, D.; Manuel Domínguez, J.; Sineiro, J.; Domínguez, H.; Núñez, M .J.; Carlos Parajó, J. Natural antioxidants from residual sources. *Food Chem.* **2001,** *72,* 145-171.

23. Skerget, M.; Kotnik, P.; Hadolin, M.; Hras, A.R.; Simonic, M.; Knez, Z. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem.* **2005/2,** *89,* 191-198.

24. Servili, M. and Montedoro, G. Contribution of phenolic compounds to virgin olive oil quality. *Eur. J. Lipid Sci. Technol.* **2002,** *104,* 602-613.

25. Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **1992,** *40,* 1571-1576.

26. Morelló, J.; Romero, M .; Ramo, T.; Motilva, M. Evaluation of l-phenylalanine ammonia-lyase activity and phenolic profile in olive drupe (Olea europaea L.) from fruit setting period to harvesting time. *Plant Sci* **2005,** *168,* 65-72.

27. White, P.J. and Xing, Y. Antioxidants from cereals and legumes, In *Natural Antioxidants Chemistry, Health Effects, and Applications,* AOCS Press; Shahidi, F.: Champaign, Illinois, 1977; pp. 25-63.

28. Marinova, E.M. and Yanishlieva, N.V. Effect of lipid unsaturation on the antioxidative activity of some phenolic acids. *JAOCS, J Am Oil Chem. Soc.* **1994,** *71,* 427-434.

29. Das, N.P. and Pereira, T.A. Effects of flavonoids on thermal autoxidation of palm oil. Structure-activity relationships. *JAOCS J. Am. Oil Chem. Soc.* **1990,** *67,* 255-258.

30. Shahidi, F. and Wanasundara, P.K. Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr.* **1992,** *32,* 67-103.

31. Cuppet, S.; Schnepf, M.; Hall III, C. Natural antioxidants- Are they a reality? In *Natural Antioxidants Chemistry, Health Effects, and Applications,* AOCS Press; Shahidi, F.: Champaig, Illinois, 1997; pp. 12-24.

32. Paiva-Martins, F. and Gordon, M.H. Isolation and characterization of the antioxidant component 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from olive (Olea europaea) leaves. *J. Agric. Food Chem.* **2001,** *49,* 4214-4219.