GRASAS Y ACEITES, 61 (3),
JULIO-SEPTIEMBRE, 303-311, 2010,
ISSN: 0017-3495
DOI: 10.3989/gya.064809

Improvement of phenolic antioxidants and quality characteristics of virgin olive oil with the addition of enzymes and nitrogen during olive paste processing

By **D. Iconomou,* D. Arapoglou** and **C. Israilides**

National Agricultural Research Foundation (NAGREF), Institute of Technology of Agricultural Products, Laboratory of Biotechnology, 1, Sof. Venizelou Str, Lykovrissi GR-14123, Athens, Greece.
(* Corresponding author: diconomou@yahoo.gr)

RESUMEN

Mejora de las caracterísiticas de calidad y de los antioxidantes fenólicos de aceite de oliva virgen por la adición de enzimas y nitrógeno durante el procesado de la pasta de aceituna.

La evolución de los compuestos fenólicos y su contribución a las caracterísiticas de calidad de aceite de oliva virgen durante el procesado del fruto fue estudiado mediante la adición de una combinación de varias enzimas comerciales conteniendo pectinasas, poligalacturonasa, celulasa y β-glucanasa con y sin flujo de nitrógeno. Las aceitunas (*Olea europaea,* L.) de la variedad Megaritiki, con un estado de madurez correspondiente a una pigmentación semi-negra, fueron usadas en un experimento a escala industrial mediante un sistema de extracción de 3-fase. La adición de enzimas a la pasta de aceituna durante el procesado incremento, en el aceite de oliva, el contenido total de fenoles y orto-difenoles, así como algunos compuestos fenólicos sencillos (3,4-DHPEA, *p*-HPEA) y derivados secoiridoides (3,4-DHPEA-EDA and 3,4-DHPEA-EA) y además mejoró su actividad oxidativa. Más aún, el tratamiento con enzimas mejoró los parámetros de calidad del aceite de oliva producido (acidez y valor de peróxidos) y sus atributos sensoriales. El uso adicional de nitrógeno en el tratamiento enzimático no mejoró los parámetros de calidad del aceite de oliva en ningún caso. Sin embargo, no afectó a la concentración individual o total de esteroles así como a la mayoría de los ácidos grasos. Consecuentemente, el tratamiento de la pasta de aceitunas con enzimas no solo mejoró las características de calidad del aceite de oliva y la calidad organoléptica global, sino que también aumento el rendimiento de aceite de oliva.

PALABRAS CLAVE: Aceite de oliva virgen – Batido de la pasta de aceituna – Compuestos fenólicos – Enzimas – Parámetros de calidad.

SUMMARY

Improvement of phenolic antioxidants and quality characteristics of virgin olive oil with the addition of enzymes and nitrogen during olive paste processing.

The evolution of phenolic compounds and their contribution to the quality characteristics in virgin olive oil during fruit processing was studied with the addition of a combination of various commercial enzymes containing pectinases, polygalacturonases, cellulase and β-glucanase with or without nitrogen flush. Olive fruits (*Olea europaea,* L.) of the cultivar Megaritiki, at the semi black pigmentation stage of maturity, were used in a 3-phase extraction system in an experiment at industrial scale. The addition of enzymes in the olive paste during processing increased the total phenol and ortho-diphenol contents, as well as some simple phenolic compounds (3,4-DHPEA, *p*-HPEA) and the secoiridoid derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA) in olive oil and therefore improved its oxidative stability. Furthermore, enzyme treatment ameliorated the quality parameters of the produced olive oil (acidity and peroxide value) and their sensory attributes. The use of additional N_2 flush with the enzyme treatments did not improve the quality parameters of olive oil any further; however it did not affect the concentration of individual and total sterols or most of the fatty acid composition. Consequently, olive paste treatment with enzymes not only improved the quality characteristics of olive oil and enhanced the overall ogranoleptic quality, but also increased the olive oil yield.

KEY-WORDS: Enzymes – Olive paste malaxation – Phenolic compounds – Quality parameters – Virgin olive oil.

1. INTRODUCTION

The world production of olive oil amounts to approximately 2.800.000 t, with Greece ranking third in the world in terms of olive oil quantity (331.310 t) after Spain and Italy (FAOSTAT, 2007). The virgin olive oil, apart from the oily phase (fatty acids) contains precious substances of high biological value such as phenols, ortho-diphenols and various natural antioxidants, tocopherol (Vitamin E), flavones as well as a high content of oleic acid, alcohols, sterols, chlorophyll, volatile aromatic substances, *etc*. (Baldioli *et al*., 1996; Giovannini *et al*., 1999; Iconomou *et al*., 2005; De Faveri *et al*., 2008). This fact leads to health claims, particularly regarding children, concerning the prevention of cardiovascular and gastrointestinal diseases, the deceleration of ageing and cancer prevention.

During malaxation, centrifugation and separation, a small fraction of phenolic compounds (ca 10-15 %) is released into the oily phase. The remaining larger fraction is removed with the wastewater and pomace (Servili *et al*., 1999). The concentration of phenolic compounds in olive oil is affected by the extraction conditions during processing (Montedoro *et al*., 1992). However, the exact mechanism that

explains the quantitative modification of secoiridoids in the olive oil during malaxation is unknown. The phenolic compounds (natural antioxidants) of olive fruits are distributed among oil, vegetation waste water and solid phase and some may be linked to the colloid oil droplets, such as phenolic polymeric structures and pectins, hemicelluloses, proteins, *etc.* The relationship between phenolic compounds in virgin olive oil the organoleptic characteristics and its oxidative stability has been studied (Montedoro *et al*., 1992; Baldioli *et al*., 1996; Garcia *et al*., 2001). Olive fruit phenol concentration ranges from 1.0 – 3.0 % (w/w), on a raw fruit basis, and depends on various factors such as variety, cultivation environment and ripening stage during harvesting, storage conditions, processing methods *etc.* (Solinas *et al*., 1978; Montedoro *et al*., 1992; Cert *et al*., 1999; Iconomou *et al*., 2005; Garcia *et al*., 2001).

For the assessment of virgin olive oil quality, the phenolic compounds are considered to be of paramount importance as they affect its quality parameters. They are also related to resistance to oxidation (Roncero *et al*., 1975; Gutiérrez *et al.,* 1977; Roncero, 1978; Gutfinger, 1981; Tsimidou *et al*., 1992; Baldioli *et al*., 1996; Giovannini *et al*., 1999) and organoleptic properties, preventing the degradation of volatile compounds and finally they are responsible for the typical pungent and bitter taste in olive oil (Gutiérrez-Rosales *et al*., 1992; Montedoro *et al.,* 1992; Angerosa and Di Giacinto, 1995; Tous *et al*., 1997; Servili *et al*., 1999; Iconomou *et al*., 2005).

The most characteristic phenol of the olive fruit and leaves is oleuropein. Other principal phenolic compounds in olive oil are phenolic acids (such as vanillic acid, syringic acid, *p*-coumaric and *o*-coumaric acid, caffeic acid, protocatechuic and ferulic acid), phenolic alcohols {such as 3,4-dihydroxyphenyl-ethanol (OH-tyrosol or 3,4-DHPEA), *p*-hydroxyphenyl-ethanol (tyrosol or *p*-HPEA)} and secoiridoids {such as dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA), the dialdehydic form of decarboxymethyl elenolic acid linked to *p*-HPEA (*p*-HPEA-EDA) and oleuropein aglycon (3,4-DHPEA-EA)}.(Solinas *et al*., 1978; Montedoro *et al*., 1992; Tsimidou *et al*., 1992; Vierhuis *et al*., 2001). During crushing and malaxation, most of these phenols are produced from the hydrolysis of oleuropein glycoside and secoiridoid derivatives *via* the endogenous enzymes of the olive fruit (Angerosa and Di Giacinto, 1995; Servili *et al.,* 1999). It has been shown that there is a correlation among the activity of some endogenous enzymes in olives during ripening, the extraction treatment and the release of total phenols and antioxidants found in olive oil (Servili *et al*., 1999; Ranalli *et al*., 2003; Milan-Linares *et al*. 2006). Endogenous enzymes (pectinolytic, cellulolytic *etc.*), despite their minimal presence in the paste, break up the surrounding oil droplet membrane (Ranalli and Serraiocco, 1995 and 1996; De Faveri *et al*., 2008). Cellulase opens up the solid structure of olive flesh releasing oleuropein aglycon and 3,4-DHPEA**.**

It has been shown that the addition of exogenous enzymes to the olive paste increases the oil yield and the antioxidant content in virgin olive oil, depending on the olive variety and degree of ripening (Garcia *et al*., 2001; Chiaccheirini et *al*., 2007; Aliakbarian *et al.* 2008; Najafian *et al*., 2009). Exogenous enzymatic preparations *(i.e.* β-glucanase) aid the hydrolysis of secoiridoid glycosides. The enzymes degrade the polysaccharides and liberate phenolic antioxidants, which, after equilibrium, are distributed among three phases: oil, water and solid. Antioxidants are sensitive to oxidation during malaxation of the olive paste and the nitrogen atmosphere protects them against oxidative destruction (Gutiérrez *et al*., 1977; Giovannini *et al*., 1999). In general the native enzymes present in the olive fruit are deactivated during the oil extraction process or crushing step. It was shown that a few native enzymes (such as lipoxygenase: LOX and polyphenol oxidase: PPO), retain some detectable activity even in virgin olive oils (De Faveri *et al.* 2008). It has been suggested that LOX plays a role in the oxidation of unsaturated fatty acids and pigments of the olive fruits (Georgalaki M.D., *et al.* 1998). After a series of reactions, LOX, together with other enzymes, end up in the formation of volatile compounds responsible in the development of the most characteristic “green” and “fruity” aroma of olive oil (Kyritsakis and Markakis, 1987). Although the effect of the use of enzymes on the quality of olive oil has been studied, its action has not been fully described in combination with a nitrogen atmosphere in the malaxation process at industrial scale.

This paper deals with the effect of various enzyme combinations during the treatment of the olive paste of cv. Megaritiki, with or without nitrogen flush, on the release of phenolic antioxidants in virgin olive oil and on its qualitative and sensory characteristics using a three-phase extraction system at industrial scale.

2. MATERIALS AND METHODS

2.1. Olive variety

4080 kg of healthy olive fruits of *c.v.* Megaritiki at semi-black maturity index, (M.I. =2.75), were harvested from an olive orchard near Agios Konstantinos, Central Greece. (Garcia *et al*., 2001; Iconomou *et al*., 2005). The maturity index (M.I.) was calculated as a subjective evaluation of the skin color and flesh as developed in the Research Station of Venta del Llano (Jaen, Spain) and proposed by Uceda and Frias (1975). The following formula was applied to 100 olives that were randomly selected.

Maturity Index (M.I**.**) = (Ax0 + Bx1 + Cx2 + Dx3 + Ex4 + Fx5 + Gx6 + Hx7) /100.

A,B,C,D,E,F,G,H, are the number of fruits in the various types described below and 0,1,2,3,4,5,6,7, the grade of ripeness respectively. (i) type 0: intense green skin; (ii) type 1: yellowish green skin; (iii) type 2: green skin with red spots, in less than half of the fruits; (iv) type 3: reddish or purple skin

in more than half of the fruits; (v) type 4: black skin and white pulp; (vi) type 5: black skin and pulp purple; (vii) type 6: black skin in more than half of the pulp purple; (viii) type 7: black skin and totally purple pulp.

2.2. Enzyme preparations and industrial scale experiments

Olivex and Glucanex were chosen in industrial scale experiments and were kindly supplied by Novo Nordisk Ferment Ltd. (Dittingen, Switzerland). Olivex is an enzyme preparation (produced by the fungus *Aspergillus aculeatus)* rich in pectinolytic, hemicellulolytic and cellulolytic side activities. Olivex activity was 26.000 PGU (polygalacturonase units) per mL at pH 3.5. Glucanex is a β-glucanase preparation produced by a selected strain of *Trichoderma sp*. It contains all the enzymes needed for the complete hydrolysis of β-glucan to glucose. Glucanex activity was 300 BGU (β-glucanase units) per gram.

There were three treatments plus the control with three runs in each treatment as follows: *(i)* E_1: addition of 0.25 mL of Olivex and 0.03 g of Glucanex per kg of olive paste at the beginning of malaxation; (*ii)* E_2: addition of 0.5 mL of Olivex and 0.06 g of Glucanex per kg of olive paste; (*iii)* E_2+N_2: E_2 enzymes used under nitrogen flush (with 2 L / min in a covered malaxator), and (*iv*) control treatment without enzymes. 340. 0 kg of olive fruits were used in each run.

Fig. 1 shows the flow sheet of the extraction system used to obtain virgin olive oil. An Alfa-Laval 3phase olive oil extraction system with 2 parallel malaxators and maximum working capacity of 1.0 tonnes per hour was used. Firstly the leaves were removed from olive lots and then olive fruits were subjected to a milling of drupes by a hammer crusher operating at 2800 rpm with a sieve with 6 mm holes working at 100 rpm in the same direction as the crusher. The olive paste was malaxed for 30 min at 30±2°C with or without nitrogen flush. After malaxation, 40 L of water was added to 100 kg of olive paste before entering the 3-phase decanter. Finally, separation of the oily must into oil and vegetation water took place using a simple centrifugal oil separator operating at 1700 rpm (Angerosa and Di Giacinto, 1995; García *et al*., 2001; Iconomou *et al.,* 2005; De Faveri *et al*., 2008). The produced olive oil was stored in 1.0 L plastic bottles at room temperature, without nitrogen addition, for a period of 5 months, in the absence of light in order to test the effect of phenolic compounds on the shelf-life of olive oil.

2.3. Extraction and colorimetric determination of total phenols and ortho-diphenols

The extraction of phenolic compounds from olive oil was carried out according to Montedoro *et al.* (1992) and Servili *et al.* (1999). The concentration of total phenols in the methanolic extract was determined colorimetrically using the Folin-Ciocalteau reagent. The absorbance was measured at 725 nm (in the range 0.01-1.00 mg/mL) against a blank, using a UV-VIS spectrophotometer (GBS model 916). Results were expressed in mg/kg of gallic acid (Gutiérrez *et al*., 1977). Ortho-diphenol content in the methanolic extract was determined (in mg/kg of caffeic acid) according to the procedure described by Gutfinger (1981).

2.4. HPLC separation of phenolic compounds

The separation of phenolic compounds was performed according to Montedoro *et al.* (1992) and Servili *et al.* (1999). The HPLC system consisted of a Spectra System liquid chromatograph model 2000 (Thermo Separation Product, USA), equipped with a 250mm x 4.6mm C18 NovaPak column coupled with a UV detector. Individual phenolic compounds were detected at 278nm. The flow rate was 1 mL/min. The mobile phase used was 0.2 % (v/v) acetic acid in water (A) *vs.* methanol (B) for a total running time of 60 min and the gradient changed as follows: 95 % A / 5 % B for 2 min, 80 % A / 20 % B for 10 min, 70 % A / 30 % B for 10 min., 60 % A / 40 % B for 10 min, 40 % A / 60 % B for 10 min., 100 % A / 0 % B for 10 min until the end of running (Angerosa and Di Giacinto, 1995). Samples were dissolved in methanol; a sample loop of 20-μL capacity was used for the introduction of the sample.

Gallic, protocatechuic, *p*-hydroxybenzoic acid, vanillic, caffeic, syringic, *p*-coumaric, ferulic and

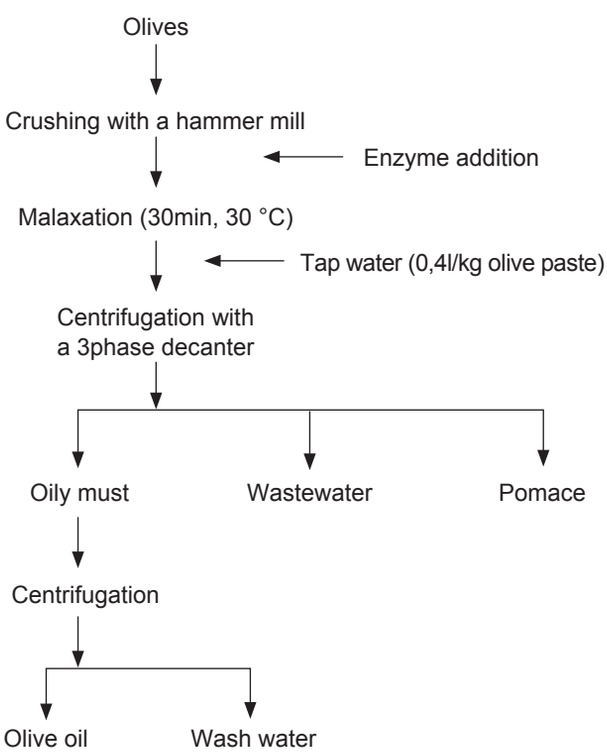

Figure 1.
Flow-sheet of the three-phase extraction system used to obtain virgin olive oil, from the olive paste of the Megaritiki variety at industrial scale.

o-coumaric acids were HPLC grade and purchased from Sigma Chemical Co (USA), tyrosol (98%) from Aldrich Chemie (Germany) and extra pure oleuropein from Extrasynthese Co. (Genay, France). The pure compounds 3,4-DHPEA, 3,4-DHPEA-EDA and the isomer 3,4-DHPEA-EA were prepared and kindly offered by Prof. Montedoro (Italy).

2.5. Quality characteristics

The analyses of free acidity (% oleic acid) and Peroxide value (meq O_2/kg) as well as sterols (mg/kg olive oil) and the fatty acid (%) composition of olive oil were carried out according to the official methods of the EC Regulation 2568/91. Indices K_{270} and K_{232} extinction coefficients (absorption of 1 % solution in isooctane at 270 and 232 nm, respectively, with 1 cm of passage length) were measured using a double beam UV/Visible spectrophotometer, model GBC-916 (Scientific Equipment Ltd, Victoria, Australia). Chlorophyll was determined (in mg/kg) according to AOCS (1978). Resistance to oxidation was determined using the Rancimat apparatus (Methrohm, Basel, Switzerland) at 120°C with an air flow of 20 L/h. Results were expressed as induction time in hours: Rancimat stability (Läubli and Bruttel, 1986: Kyritsakis and Markakis, 1987).

A sensory evaluation of the sample was performed by a panel of experts according to the official methods of Annex XII of the EC Regulation: 2568/1991. The descriptive analysis used a five-point intensity scale, ranging from 0 (no perception) to 5 (extreme). Overall grading used a nine-point scale, 9 for exceptional quality and 1 for the worst. Ten trained tasters were used (Tous *et al.*, 1997).

2.6. Statistical analysis

Samples were taken at random in triplicate runs. The results were calculated as the means of three separate runs. Average values were compared with the least significant difference (LSD) at p=0.05 and with the Students t-test where appropriate.

3. RESULTS AND DISCUSSION

3.1. Quality parameters and resistance to oxidation

The effects of the enzymatic preparations with or without nitrogen flush (E_1, E_2 and E_2+N_2) during malaxation of the olive oils are presented in Table 1. Regarding the quality parameters of the olive oils, there was a decreasing trend in olive oil acidity and peroxide value, with the addition of enzymes compared to control, which is in agreement with previous studies (García *et al.*, 2001; Iconomou *et al.,* 2005, Chiaccheirini et *al.*, 2007, De Faveri *et al.* 2008).

The use of nitrogen (E_2+N_2) resulted in a significant increase in peroxide value compared with the E_2 treatment (Table 1). The increase in peroxides was probably due to a reduction in the rate of their degradation in the presence of nitrogen, illustrating their relative increase (E. Stefanoudaki, Greece personal communication).

All enzymatic treatments resulted in the improvement of all parameters tested compared to the control, even after 5 months of storage in plastic bottles in the absence of light. By doubling the added enzymes (E_2 vs. E_1) there was a significant decrease in olive oil acidity and peroxide value and an increase in chlorophyll ($p<0.05$). The decrease in peroxide value (Table 1) is likely due to the total phenol increase by the use of enzymes, as shown in Table 2 (Ranalli and Serraiocco, 1996).

The quality parameters (acidity, peroxide value, K_{232}-K_{270} indices) of the obtained olive oil in all treatments classify it in the extra-virgin olive oil category according to the E.U. Regulation 2568/91.

The addition of enzymes in all treatments resulted in a significant increase ($p<0.05$) in olive

Table 1
Qualitative characteristics and yield of virgin olive oil of the cv. Megaritiki treated during malaxation with commercial enzyme preparation (E_1, E_2 and E_2+N_2)*

	Control	E_1	E_2	E_2 + N_2
Acidity (% oleic acid)	0.57±0.12[a]	0.46±0.14[b]	0.29±0.18[c]	0.32±0.04[c]
Ac. after 5 months storage	0.63±0.09[a]	0.47±0.11[b]	0.30±0.14[c]	0.34±0.03[c]
Peroxide value (meq O2/kg)	10.54±0.05[a]	8.32±0.08[b]	6.79±0.12[c]	8.31±0.04[b]
P.V. after 5 months storage	14.11±0.05[a]	12.28±0.08[b]	10.77±0.13[b]	12.19±0.02[b]
K232	2.30±0.13	1.97±0.27	1.98±0.17	2.03±0.12
K270	0.15±0.02	0.13±0.03	0.14±0.03	0.13±0.01
Chlorophyll ppm	4.91±0.18[a]	5.62±0.19[b]	6.15±0.24[b]	5.97±0.31[b]
Yield kg oil/100 Kg olive	13.28±0.10[a]	14.76±0.15[b]	15.31±1.40[b]	15.00±0.82[b]

*The results in the table represent the average values of the means of 3 runs ± S.D.. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment). E_2+N_2: Olivex+Glucanex+Nitrogen.
[a,b,c] Different superscripts are statistically significant, p=0.05.

oil yield of about 15.0% compared to the control. This corresponds to an oil yield increase of about 2.0 kg olive oil per 100Kg of olives. This was also reported by other researchers (Uceda and Frias, 1975; Ranalli and Serraiocco, 1995; García *et al.,* 2001; Vierhuis *et al*., 2001; Chiacchierini *et al.,* 2007). The action of enzymes in the olive paste is not fully understood. It could be stipulated that enzymes degrade the olive cell wall and therefore change the rheologic behavior of the paste. The disruption of cell walls by enzymes may explain the increase in olive oil yield (Iconomou *et al.,* 2005; De Faveri *et al.* 2008).

Table 2 shows resistance to oxidation, total phenols and the ortho-diphenol content of the obtained olive oil. The addition of enzymes E_1, E_2 and E_2+N_2 in pastes increased the resistance to oxidation - induction time in Rancimat- ($p<0.05$) and the content of total phenols compared to the control ($p<0.05$). There was no significant difference among enzyme treatments E_1, E_2 and E_2+N_2 oils in total phenol concentration. Ortho-diphenol content increased in E2 and E2 +N2 treatments ($p<0.05$) but not in E1. This is in agreement with other studies on olive oils obtained from treated olive pastes with other enzyme combinations (Garcia *et al*., 2001; Aliakbarian *et al*. 2008; Najafian *et al*., 2009).

3.2. Phenolic compounds in various treatments

Table 3 shows that the use of enzymes, resulted in a significant increase ($p<0.05$) in phenolic compound concentration (3,4-DHPEA, p-HPEA, syringic acid, 3,4-DHPEA-EA and 3,4-DHPEA-EDA) in all treatments compared to the control. The E_2 treatment showed the highest concentration in the above phenolic compounds, while there was no difference ($p>0.05$), between E_1 and E_2+N_2.

During malaxation, oleuropein degradation continues by endogenous enzymes which slowly formed the phenolic antioxidants (3, 4-DHPEA, *p*-HPEA) and other secoiridoid derivatives: 3, 4-DHPEA-EDA, 3, 4-DHPEA-EA (Roncero *et al*., 1975; Ranalli and Serraiocco, 1996; García *et al*., 2001; Vierhuis *et al*., 2001; Milan-Linares *et al.* 2006).

The antioxidants may be absorbed by polymers and/or dissolved into the water around the hydrophilic sites and trapped inside and among the poly-

Table 2
Colorimetric determination of total phenols, ortho-diphenols and resistance to oxidation (Rancimat stability) in virgin olive oil from the cv. Megaritiki during malaxation with commercial enzyme preparations E_1, E_2 and E_2+N_2 *

	Control	E_1	E_2	$E_2 + N_2$
Resistance to oxidation (Rancimat stability: h)	2.72±0.03[a]	3.26 ±0.14[b]	3.56±0.07[b]	3.47±0.09[b]
Total phenols (mg/kg gallic acid)	113.4±3.8[a]	180.8±5.8[b]	188.8±6.1[b]	179.2±4.2[b]
T.ph. after 5 months storage	87.8±5.6[a]	134.9±8.1[b]	174.3±6.4[c]	149.1±7.7[b]
ortho-diphenols (mg/kg caffeic acid)	17.9±0.8[a]	18.5±1.3[a]	23.2±0.9[b]	22.5±0.9[b]

*The results in the table represent the average values of the means of 3 runs ± S.D.. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment). E_2+N_2: Olivex+Glucanex+Nitrogen.
[a,b,c] Different superscripts are statistically significant, p=0.05.

Table 3
Phenolic compound concentration (mg/kg) in virgin olive oil (HPLC) of the cv. Megaritiki treated with commercial enzyme preparations (E_1, E_2 and E_2+N_2) during malaxation*

	Control	E_1	E_2	E_2+N_2
3,4-DHPEA	0.04±0.01[a]	0.12±0.03[b]	0.19±0.03[c]	0.14±0.01[b]
p-HPEA	1.57±0.10[a]	2.18±0.09[b]	2.06±0.06[b]	1.92±0.04[b]
Vanillic acid	0.13±0.09[a]	0.16±0.10[a]	0.18±0.09[a]	0.16±0.04[a]
Caffeic acid	0.29±0.08[a]	0.55±0.11[a]	0.59±0.12[a]	0.49±0.09[a]
Syringic acid	0.08±0.01[a]	0.20±0.04[b]	0.24±0.07[b]	0.19±0.03[b]
p-Coumaric acid	0.24±0.10[a]	0.25±0.08[a]	0.13±0.08[a]	0.22±0.05[a]
3,4-DHPEA EDA	23.29±1.71[a]	35.25±1.82[b]	47.50±2.11[c]	36.62±1.82[b]
3,4-DHPEA EA	81.37±1.10[a]	94.60±1.24[b]	100.35±1.12[c]	89.14±2.77[b]

*The results in the table represent the average values of the means of 3 runs ± S.D.. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment). E_2+N_2: Olivex+Glucanex+Nitrogen.
[a,b,c] Different superscripts are statistically significant, p=0.05.

mers (Roncero *et al.*, 1975; Solinas *et al.*, 1978; Montedoro *et al.*, 1992**).**

It has been shown that enzyme formulation degrades the walls of the oil-bearing cells. Also the enzymes breaks up the liquid/solid and the liquid/liquid emulsions mainly caused by crushing and centrifuging the paste and through its endo-polygalacturonase activity. These results in the release and merging of the oil droplets into larger ones forming a mass of free oil, which is more easily extracted mechanically (Chiacchierini *et al.,* 2007: De Faveri *et al.*, 2008). Additionally it was noted that exogenous enzymes free more antioxidants in olive oil. For example, β-glycosidase was observed to increase the oleuropein aglycon concentration (García *et al.*, 2001; Vierhuis *et al.*, 2001).

The concentrations of total phenols and 3, 4-DHPEA-EA were also correlated to the resistance in oxidation - Rancimat (Iconomou *et al.*, 2005; De Faveri *et al.*, 2008). The maximum correlation (r=0.897) was found between the total phenols and the resistance to oxidation, while the correlation between 3, 4-DHPEA-EA and resistance to oxidation was found to be lower (r=0.792).

3.3. Fatty acid and sterol content

Table 4 shows the effect of the olive paste treatments with the enzyme combination E1 and E_2 in the fatty acid composition of virgin olive oil. There was a significant increase in oleic acid in E_1 and E_2 treatments and a simultaneous decrease in linoleic acid compared to the control ($p<0.05$). During malaxation, the linoleic acid oxidizes quickly and probably the presence of enzymes accelerates its degradation.The reduction of the percent of linoleic acid might have changed the value of the other fatty acids making the increase in oleic acid significant. LOX may be involved in this procedure (Kyritsakis, A. and Markakis P., 1987: Ranalli *et al*. 2002: De Faveri *et al.*, 2008). The concentration of the rest of the fatty acids was not affected by the addition of the same enzymes E_1 and E_2 ($p>0.05$).

In addition, the addition of the enzymes (E_1, E_2) did not influence the sterol content of olive oil (Table 5). The composition of fatty acids and sterols in olive oil remained at levels which are in accordance with the limits mentioned in the EU Regulation 2568/91 for all treatments and the control (Iconomou *et al.*, 1998).

3.4. Sensory evaluation of olive oil

Table 6 shows the sensory evaluation of virgin olive oil obtained with enzymes (E_1 and E_2), by a panel of trained tasters. Treatments E_1 and E_2 resulted in a significant improvement ($p<0.05$) of most of the desirable attributes of the sensory characteristics of olive oil, especially the flavor of “olive fruity”, “green leafy” and “apple” compared to the control. The overall organoleptic grades from a taste panel for E_1 and E_2 were 7.0 and 6.50, respectively, while the corresponding value for the control was lower than the accepted limit (≥ 6.50) for extra virgin olive oil (E.U. Regulation 2568/91). Overall, E_1 and E_2 did not differ significantly ($p>0.05$) in every individual sensory characteristic and overall grading, although they scored higher compared to the control.

The use of enzymes improved olive oil quality by enrichment with natural antioxidants and increase in its protection to oxidation and the amelioration of its organoleptic characteristics.

Table 4
Effect of olive paste treatment with enzyme preparations E_1 and E_2 on the fatty acid (%) composition of virgin olive oil of the cv. Megaritiki*

Fatty acid %	Control	E_1	E_2
Palmitic (C16)	16.51±0.23[a]	16.30±0.15[a]	16.01±0.10[a]
Palmitoleic (C16:1)	1.86±0.05[a]	1.84±0.20[a]	1.51±0.14[a]
Decaheptanonic (C17)	0.02±0.01	Trace	Trace
Decaheptenoic (C17:1)	0.05±0.01	0.04±0.01	Trace
Stearic (C18:0)	1.84±0.15[a]	1.90±0.04[a]	2.02±0.11[a]
Oleic (C18:1)	62.96±0.21[a]	64.92±0.41[b]	66.86±0.17[c]
Linoleic (C18:2)	14.39±0.36[a]	12.95±0.25[b]	11.43±0.31[c]
Arachidic (C20)	0.30±0.05[a]	0.30±0.07[a]	0.31±0.02[a]
Linolenic (C18:3)	0.95±0.05[a]	0.86±0.11[a]	0.83±0.17[a]
Eicosenoic (C20:1)	0.08±0.02[a]	0.08±0.01[a]	0.70±0.10[b]
Vechenic (C20:1)	Trace	Trace	Trace
Lignoceric (C24)	0.05±0.01[a]	0.04±0.01[a]	0.04±0.01[a]

*The results in the table represent the average values of the means of 3 runs ± S.D.. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment). [a,b,c] Different superscripts are statistically significant, p=0.05.

Table 5
Total sterol (g/kg) and sterol fraction content (%) of virgin olive oil of the cv. Megaritiki treated during malaxation with commercial enzyme preparations (E_1, E_2)*

	Control	E_1	E_2
Cholesterol	0.23±0.05[a]	0.26±0.02[a]	0.23±0.04[a]
Brasicasterol	Trace	Trace	Trace
24-methylen-cholesterol	Trace	Trace	Trace
Campesterol	2.56±0.12[a]	2.60±0.08[a]	2.53±0.09[a]
Campestanol	0.31±0.71[a]	0.30±0.04[a]	0.35±0.04[a]
Stigmasterol	0.74±0.10[a]	0.76±0.07[a]	0.80±0.18[a]
Δ^7-Campesterol	Trace	Trace	Trace
$\Delta^{5,23}$-Stigmastadienol	Trace	Trace	Trace
Chlerosterol	0.95±0.03[a]	0.95±0.03[a]	1.00±0.02[a]
β-Sitosterol	90.00±0.25[a]	90.24±0.54[a]	90.20±0.86[a]
Sitostanol	0.08±0.01[a]	0.10±0.02[a]	0.10±0.01[a]
Δ^5-Avenasterol	4.39±0.10[a]	4.00±0.21[a]	4.30±0.15[a]
$\Delta^{5,24}$-Stigmastadienol	0.21±0.05[a]	0.20±0.01[a]	0.20±0.14[a]
Stigmastenol	0.18±0.02[a]	0.19±0.03[a]	0.18±0.02[a]
Δ^7-Avenasterol	0.36±0.07[a]	0.40±0.09[a]	0.11±0.08[b]
Erythrodiol+Uvaol	1.06±0.09	1.32±0.15	1.30±0.10
Σ β-Sitosterol**	95.63±0.98[a]	95.49±1.41[a]	95.80±1.01[a]
Total Sterols	1.51±0.22[a]	1.50±0.14[a]	1.51±0.33[a]

*The results in the table represent the average values of the means of 3 runs ± S.D.. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment).**Σ β-Sitosterol was the sum of $\Delta^{5,23}$-Stigmastadienol+Chlerosterol+β-Sitosterol+Sitostanol+Δ^5-Avenasterol+$\Delta^{5,24}$-Stigmastadienol. [a,b] Different superscripts are statistically significant, p=0.05.

Table 6
Sensory evaluation of virgin olive oil treated during malaxation with commercial enzyme preparations (E_1, E_2), performed by a panel of ten trained tasters*

Characteristic	Control	E_1	E_2
Olive fruity	0.65±0.28[a]	1.90±0.40[b]	2.56±0.38[b]
Apple	0.57±0.15[a]	1.25±0.12[b]	1.86±0.42[b]
Green leaves	0.71±0.12[a]	1.56±0.15[b]	1.14 ±0.10[b]
Bitter	0.00	0.25	0.00
Pungent	0.56±0.20[a]	1.00±0.25[a]	0.86±0.30[a]
Sweet	1.57±0.22[a]	2.00±0.25[a]	2.43±0.40[a]
Overall grade**	5.50±0.30[a]	7.00±0.40[b]	6.50±0.20[b]

*The results in the table represent the average values of the means of 3 runs ± S.D.. **Limit acceptance for extra virgin olive oil: Overall grade ≥ 6.50. E_1: Olivex+Glucanex. E_2: 2 x E_1 (Concentration of enzymes is double the concentration in the E_1 treatment). [a,b] Different superscripts are statistically significant, p=0.05.

E.U. Regulations (2568/91 and theirs amendments) prohibit the use of any external adjuvant (chemicals, enzymes) except the addition of water and/or the use of talk as co-adjuvant, for virgin olive oil extraction during paste malaxation. However there may be amendments to the above E.U. regulations in the future for olive oil production as other enzymes have already been approved and used in the agro-food and juice industry with technological and economic benefits (Milan-Linares *et al.* 2006; De Faveri *et al.*, 2008). Therefore the results of this study may have a potential practical application in the production of olive oil.

Previous reports on the simultaneous use of enzymes and nitrogen on the olive oil quality are scarce in the literature and do not show a definite trend in all cases (Vierhuis *et al.*, 2001; Garcia *et al.*, 2001and Chiacchierini *et al.,* 2007) and are mixed depending on the olive cultivar used, endogenous enzymes, etc.

According to our results the effect of both enzymes and nitrogen exhibited either no improvement or even

a slight decrease in the quality of olive oil compared to the use of one enzyme alone. This may be due to the different behavior of N_2 interference during malaxation on the effect of endogenous and exogenous enzymes in various olive cultivars on olive oil qualitative characteristics, as observed in the case of two cultivars Koroneiki *vs.* Megaritiki. (Garcia *et al*., 2001; Ranalli *et al.,* 2003; Iconomou *et al*., 2005 and Chiacchierini *et al.,* 2007). This adverse effect of nitrogen was reported with the use of some other enzymes, like tyrosinase (Zhang *et al*. 2001).

4. CONCLUSIONS

The addition of a mixture of exogenous enzymes during the olive paste malaxation of *c.v.* Megaritiki improves the quality characteristics of the obtained olive oil such as acidity, peroxide value and chlorophyll. Sterols and most of fatty acids were not affected by paste enzymatic treatment, except for an increase in oleic acid and a decrease in linolenic acid. The addition of enzymes increased the amount of total phenols and ortho-diphenols, as well as some simple phenolic compounds (3,4-DHPEA, *p*-HPEA),especially, the secoiridoid derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA) in olive oil. There was also an increase in olive oil yield and an improvement in the resistance to oxidation and shelf life in the produced virgin olive oil. The use of an N_2 flush with the enzyme treatments during paste processing did not improve the quality parameters of olive oil compared to enzyme treatments alone. The addition of enzymes considerably improved the olive oil yield and increased the release of antioxidants and total phenols into the virgin olive oil. It also contributed to the enhancement of its quality characteristics of resistance to oxidation, the improvement of the olive oil aroma and the overall organoleptic quality compared to the control.

ACKNOWLEDGEMENTS

This research work was supported by the European Union under Project AIR3-CT-94-1355. We would like to thank Dr. Evaggelia Stefanoudaki (Olive Trees & Plants Institute, NAGREF, Greece) for her scientific assistance and Dr. Yannis Mastroyannis (Nutria Co, Greece), Dr. Stathis Panagou and Theodora Pitsoli for their technical assistance.

REFERENCES

Aliakbarian B, De Faveri D, Converti A, Perego P. 2008. Optimisation of olive oil extraction by means of enzyme processing aids using response surface methodology. *Biochemical Engineering Journal* **42**, 34-40.

Angerosa F, Di Giacinto L. 1995. Crushing influence on the quality characteristics of virgin olive oil. Note II. *Rivista Italiana delle Sostanze Grasse* **72***,* 1-4.

Baldioli M, Servili M, Perretti G, Montedoro GF. 1996. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *Journal of the American Oil Chemists*' *Society* **73**, 1589-1593.

Chiacchierini E, Mele G,Restuccia D, Vinci G. 2007. Impact evaluation of innovative and sustainable extraction technologies on olive oil quality. *Trends in Food Science & Technology* **18**, 299-305.

Cert A, Alba J, Pérez-Camino MC, Ruiz-Gómez A, Hidalgo F, Moreda W, Moyano MJ, Martinez R, Olias JM. 1999. Influence of extraction methods on the characteristics and minor components of extra virgin olive oil. *Olivae* **79**, 41-50.

The Commission of the European Communities, Regulation No 2568/1991. On the characteristics of olive oil and olive residue oil and on the relevant methods of analysis, *Official Journal* of the *European Communities* L **248**, 1-83.

De Faveri D, Aliakbarian B, Avogardo M, Perego P, Converti, A. 2008. Improvement of olive oil phenolics content by means of enzyme formulation: Effect of different enzyme activities and levels. *Biochemical Engineering Journal* **41**, 149-156.

García A, Brenes M, Moyano MJ, Alba J, García P, Garrido A. 2001. Improvement of phenolic compound content in virgin olive oils by using enzymes during malaxation. *Journal of Food Engineering* **48**, 189-194.

Georgalaki MD, Sotiroudis T*G,* and Xenakis *A*. 1998. The presence of oxidizing enzyme activities in virgin olive oil. *JAOCS*, **75**, 155-159.

Giovannini C, Straface E, Modesti D, Coni E, Cantafora A, De Vincenzi W, Malorni W, Masella R. 1999. Tyrosol, the major olive oil biophenol protects against oxidized-LDL-induced injury in Caco-2 cells. *Journal of Nutrition* **129**, 1269-1277.

Gutfinger T. 1981. Polyphenols in Olive Oils. *Journal* of the *American Oil Chemists*' *Society* **58**, 966-968.

Gutiérrez Gonzales-Quijano R, Janer del Valle C, Janer del Valle ML, Gutiérrez Rosales F, Vazquez Roncero A. 1977. Relationship between polyphenol content and the quality and stability of virgin olive oil. *Grasas y Aceites* **28**, 101-106.

Gutiérrez-Rosales F, Garrido-Fernandez J, Gallardo-Guerrero L, Gandul Rojas B, Minguez-Mosuera MI. 1992. Action of chlorophylls on the stability of virgin olive oil. *Journal of the American Oil Chemists*' *Society* **69**, 866-871.

Iconomou D, Israilides C, Arapoglou D, Stefanoudaki E, Koutsaftakis A. 1998. Contribution on the extraction methods of endogenous enzymes of olive tree and their effect on the secretion of the total phenol of the olive oil. *Proceedings of Sixth Greek Conference of the Greek Company of Scientists-Food Technicians 34-46.* Thessaloniki, Greece,19-21 November 1998.

Iconomou D, Stefanoudaki E, Koutsaftakis A, Arapoglou D, Israilides K. 2005. Effect of endogenous enzymes during ripening on qualitative characteristic of Koroneiki and Megaritiki olive oil cv's. *Proceedings of the 1st International Symposium, on the Olive Tree and the Environment* 388-394. Chania, Greece, 1-3 October 2003.

Kyritsakis, A, Markakis P. 1987. Olive Oil: A Review, Adv. *Food Res*. **31**: 453-482.

Läubli MW, Bruttel PA. 1986. Determination of the oxidative stability of fats and oils: Comparison between the active oxygen method (AOCS Cd 12-57) and the Rancimat

method. *Journal* of the *American Oil Chemists' Society* **63**, 792-795.

Milan-Linares H, Alba Mendoza J, Moyano Pérez J, Oliver Jackfish B. 2006. Influences of using enzymatic complexes in the second centrifugation of olive paste. *Grasas y Aceites* **57**, 301-307.

Montedoro GF, Servili M, Baldioli M, Miniati E. 1992. Simple and hydrolysable phenolic compounds in virgin olive oil. 1. Their extraction, separation, quantitative and semi quantitative evaluation by HPLC. Journal of *Agricultural* and *Food Chemistry* **40,** 1571-1576.

Najafian L, Ghodsvali A, Khodaparast MH, Diosady LL. 2009. Aqueous extraction of virgin olive oil using industrial enzymes. *Food Research International* **42**, 171-175.

Official and Tentative Methods *of the American Oil Chemists' Society*, 3rd ed. 1978. AOCS, Champaign, Illinois, USA.

Ranalli A, Serraiocco A. 1995. Effect induced by a pectolytic adjuvant in olive oil extraction by the present technological systems. Pluriannual research results. *Rivista Italiana delle Sostanze Grasse* **72**, 355-364.

Ranalli A, Serraiocco A. 1996. Quantitative and qualitative effects of a pectolytic enzyme in olive oil production. *Grasas y Aceites* **47**, 227-236.

Ranalli A, Pollastri L, Contento S, Lucera L, Del Re P. 2003. Enhancing the quality of virgin olive oil by use of a new vegetable enzyme extract during processing. *European Food Research and Technology* **216**, 109-115.

Roncero Vazquez A, Janer del Valle C, Janer del Valle ML. 1975. Polyphenols content and stability of olive oils. *Grasas y Aceites* **26**, 14-18.

Roncero Vazquez A. 1978. The polyphenols of olive oil and their influence on the characteristics of oil. *Revue Francaise des Corps Gra,* **25**, 21-26 .

Servili M, Begliomini AL, Montedoro GF, Petruccioli M, Federici F. 1992. Utilization of a yeast pectinase in olive oil extraction and red wine making processes. *Journal of the Science of Food and Agriculture* **58**, 253-260.

Servili M, Baldioli M, Mariotti F, Montedoro GF. 1999. Phenolic composition of olive fruit and virgin olive oil: Distribution in the constitutive parts of fruit and evolution during the oil mechanical extraction process. *Acta Horticulturae* **474**, 609-613.

Solinas M, Di Giovacchino L, Mascalo A. 1978. The polyphenols of olives and olive oil. Note III: Influence of temperature and needing time on the oil polyphenol content. *Rivista Italiana delle Sostanze Grasse* **55**, 19-23.

Tsimidou M, Papadopoulos G, Boskou D. 1992. Phenolic compounds and stability of virgin olive oil, Part 1. *Food Chemistry* **45**, 141-144.

Tous J, Romero A, Plana J, Guerrero L, Díaz I, Hermoso JF. 1997. Chemical and sensory characteristics of Arbequina olive oil obtained in different growing areas of Spain. *Grasas y Aceites* **48**, 415-424.

Uceda M, Frias L. 1975. Trend of the quality and quantitative composition of olive fruit oil during ripening. *Proceedings of the International Meeting on Olive Oil* 25-46. Cordoba, Spain.

Viethuis E, Servili M, Baldioli M, Scholsm HA, Voragen AGJ, Montedoro GF. 2001. Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *Journal of Agricultural and Food Chemistry* **49**, 1218-1223.

Zhang D, Quantick P, Grigor J, Wiktorowicz R, Irven J. 2001. A comparative study of effects of nitrogen and argon on tyrosinase and malic dehydrogenase activities. *Food Chemistry* **72,** 45-49.

Recibido: 15/6/09
Aceptado: 22/1/10