GRASAS Y ACEITES 69 (2)
April-June 2018, e250
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1220172

Modeling of volatile and phenolic compounds and optimization of the process conditions for obtaining balanced extra virgin olive oils

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Submitted: 05 December 2017; Accepted: 12 February 2018

SUMMARY: The main objective of this paper is to obtain extra virgin olive oils (EVOOs) which are balanced in volatile and phenolic compounds. An experimental design was performed and response surface methodology was applied. The factors for malaxation were: temperature 20-40 °C, time 30-90 min, and hole diameter of hammer-crusher 4.5-6.5 mm. The results show that high temperatures and small hole diameter must be used in order to obtain a higher content in phenolic compounds, while for volatile compounds a low temperature and large hole diameter must be used. The models predict that the best and more balanced EVOO are obtained with the hole diameter of greater size and a medium-low temperature. Thus, for a hammer-crusher hole diameter of 6.5 mm 337 and 356 mg/kg total HPLC phenols were obtained for malaxation temperature of 20 and 25 °C, respectively and, likewise, 12.7 and 11.5 mg/kg total LOX volatiles.

KEYWORDS*: Balanced olive oils; Oil mill; Phenolic compounds; Response Surface Methodology; Volatile compounds*

RESUMEN: ***Modelado de compuestos volátiles y fenólicos y optimización de las condiciones de operación para obtener aceites de oliva virgen extra equilibrados*****.** El principal objetivo es obtener aceites de oliva vírgenes extra (AOVEs) equilibrados en compuestos volátiles y fenólicos. Se ha realizado un diseño experimental y aplicado metodología de superficie de respuesta. El rango de los factores de batido fue, temperatura 20-40 ºC y tiempo 30-90 min, y diámetro de orificio del molino de martillos 4,5-6,5 mm. Los resultados muestran que a altas temperaturas y pequeño diámetro de orificio se obtienen elevados contenidos en compuestos fenólicos, mientras que para volátiles se debe usar temperatura baja y orificio de gran diámetro. Los modelos predicen que el mejor y más equilibrado EVOO se obtiene con el orificio de mayor tamaño y temperatura media-baja. Así, para diámetro de orificio de 6,5 mm se obtienen 337 y 356 mg/kg de fenoles totales HPLC, para temperaturas de batido de 20 y 25 °C respectivamente y, asimismo, 12,7 y 11,5 mg/kg de volátiles totales LOX.

PALABRAS CLAVE*: Aceites de oliva equilibrados; Almazara; Compuestos fenólicos; Compuestos volátiles; Metodología de superficie de respuesta*

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Citation/Cómo citar este artículo: Vidal AM, Alcalá S, Ocaña MT, De Torres A, Espínola F, Moya M. 2018. Modeling of volatile and phenolic compounds and optimization of the process conditions for obtaining balanced extra virgin olive oils. *Grasas Aceites* **69** (2), e250. https://doi.org/10.3989/gya.1220172

1. INTRODUCTION

Volatile and phenolic compounds are different groups of compounds present in virgin olive oil (VOO). Most of the taste and aroma of olive oil depend on these compounds. The volatile compounds present in olive oil are related to sensory attributes, and play an important role in consumers' sensory perceptions. These compounds are originated during the olive oil production process, most of them through the actions of enzymes which are released during the olive milling process. Many pathways are involved in the production of volatile compounds. Unsaturated fatty acids, such as linolenic and linoleic acid, are transformed into compounds of five and six carbon atoms through the Lipoxygenase (LOX) pathway (Gómez-Rico *et al.*, 2006; Sanchez and Salas, 2003). These compounds contribute to the pleasant aromas of olive oil (Angerosa *et al.*, 2000).

Sensory characteristics are a key factor in the perception of a product's quality level on the part of consumers. Volatile compounds are mainly responsible for aroma, even though phenolic compounds are related to the sense of the taste of olive oil (Angerosa *et al.*, 2004). Several factors influence the composition of volatile compounds of olive oil, from the agronomic and climatic to the technological ones (Luna *et al.*, 2006). However, the activity of the enzymes involved in the LOX pathway is genetically determined (Clodoveo *et al.*, 2014).

On the other hand, the phenolic compounds of olive oil are a complex group of chemical compounds which contribute to the stability of olive oil (Franco *et al.*, 2014). The composition of these compounds is mainly determined by the elaboration process, production technology, the variety and maturation of olives and the agro-climatic parameters (Romero *et al.*, 2016; Servili *et al.*, 2004). An overripe harvest of olives involves a decrease in the concentration of phenolic compounds. Therefore, olives should be harvested at the early stage of ripeness to obtain the maximum amount of phenolic compounds.

The antioxidant capacity, the increase in the quantity of HDL and the decrease in the quantity of LDL, the inhibition of the proliferation of cancer cells, the prevention of many diseases and the decrease in oxidative stress are just some of the properties of these compounds (Tripoli *et al.*, 2005). Several research papers (Beauchamp *et al.*, 2005; Cicerale *et al.*, 2012) have stated that oleocanthal has anti-inflammatory properties similar to classic non-steroidal anti-inflammatory drugs (NSAID) such as Ibuprofen, suppressing the Cyclooxygenase enzyme (COX) involved in the prostaglandin synthesis pathway. Recently, oleocanthal has been unveiled as a powerful therapeutic molecule for several diseases. It can show pharmacological properties for various pathogenic processes, including inflammation, cancer and neurodegenerative diseases (Scotece *et al.*, 2015).

The hole diameter of the hammer-crusher and temperature and time in the malaxation stage are the main technological factors which influence the EVOO production process. These parameters can be modified to obtain olive oils of excellent quality. On the basis of the above, the main aim of this research is to determine the best conditions to obtain healthy and high quality EVOO, with a large quantity of phenolic compounds and a good profile of volatile compounds (balanced EVOO).

2. MATERIALS AND METHODS

2.1. Olives

Olive fruits, *Olea europaea* L.*,* were hand-picked from a traditional grove in Sierra Mágina (Jaén, Spain). The cultivated variety is Picual with a 4.9 maturity index, determined according to Uceda and Frias and described by Espínola *et al.* (2009); 486 g/kg moisture content, determined by drying milled paste at 105 ºC, and a 284 g/kg oil content, determined by the Soxhlet method. The olives were collected from unirrigated land.

2.2. Olive oil extraction

Oils were obtained under laboratory-scale conditions using an Abencor centrifugal system (Abencor analyzer, MC2, Ingeniería y Sistemas S.L., Seville, Spain) (Espínola *et al.*, 2011). The oils obtained were decanted into a graduated test tube for at least three hours, paper filtered and stored in amber glass bottles, under N_2 atmosphere, at -18 ºC until they were analyzed.

2.3. Analysis of phenolic compounds

The method proposed by the International Olive Council (COI/T.20/Doc No 29) was used to determine the phenolic compounds present in virgin olive oils via High Performance Liquid Chromatography (HPLC). The equipment used was a liquid chromatograph (Shimadzu Corp., Kyoto, Japan) with the essential components: an elution pump (model LC-20AD), solvent degasser (model DGU-20A5), a refrigerated automatic injector (model SIL-20ACHT), a column oven (model CTO-10AsvpC), a diode array detector (model SPD-M20) and LC LabSolutions V.5.42.SP3 software (Shimadzu Corp., Kyoto, Japan). The column used was BDS Hypersil C18 (Thermo Scientific, USA), the particle size was 5 μm, the column size was 25 cm and the internal diameter was 4.6 mm. The mobile phase was a

ternary gradient made up by orthophosphoric acid-water to 0.2% (A), Methanol (B) and Acetonitrile (C). The proportions of the phases are changed over time by means of a flow ramp. The initial proportion of phase A was 96%, B and C were 2%. At minute 40, the proportion of phase A was 50%, B and C were 25%. At minute 45, the proportion of phase A was 40%, B and C were 30%. At minute 60, the proportion of phase A was 0%, B and C were 50%. From minute 72 to 80 the proportions of the phases were identical to the initial ones; minute 80 was the end of the chromatogram. The elution flow was 1 mL/min. The oven temperature was set at 30 ºC and the injected volume of sample was 20 µL. The detector UV provided a signal at 280 nm. The phenolic compounds were quantified through the addition of syringic acid as internal standard and tyrosol as external standard. The results obtained were expressed as mg of tyrosol per kg of oil. The phenolic compounds were identified through comparison with the following analytical standards: Syringic acid, vanillin, luteolin, vanillic acid, oleuropein, *trans*-ferulic acid and *trans*-cinamic acid, all purchased from Fluka (Milan, Italy). Tyrosol, pinoresinol, caffeic acid, apigenin and p-coumaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). *o*-coumaric acid and hydroxytyrosol were purchased from Extrasynthese (Genay Cedex, France). 3,4-DHPEA-EDA (oleacein) and p-HPEA-EDA (oleocanthal) were identified by analytical standards supplied by the Department of Organic Chemistry from the University of Jaén. p-HPEA-EA and 3,4-DHPEA-EA were identified using the method proposed by COI, Determination of bio-phenols in olive oils by HPLC, (COI/T.20/Doc No 29).

The Folin-Ciocalteau reagent was used to determine the total quantity of phenolic compounds in the olive oil, with slight adjustments (Vázquez-Roncero *et al.*, 1973). The weight of the oil samples was 1 g. The sample was placed in a tube and dissolved in 5 mL of hexane. The liquid-liquid extraction was performed by repeating contact with a 2 mL methanol-water solution (60:40). This extraction was repeated three times. Each contact was shaken for 2 min with a Vortex type agitator. The methanolic extracts were collected in a test tube of 10 mL. To conclude, the methanol-water solution was added to complete 10 mL. The methanolic extracts were reacted with the Folin-Ciacolteau reagent. The absorbance of the solutions was measured at a wavelength of 725 nm. The equipment used was a UV spectrophotometer, model Shimadzu UV-Spectrophotometer 1800 (Kyoto, Japan). A standard curve was used to determine the quantity of phenolic compounds, using caffeic acid as external standard. The results were expressed as mg of caffeic acid per kg of olive oil.

The antioxidant potential was determined as DPPH free radical scavenging activity. A DPPH stock solution (0.1 mmol/L) was prepared in methanol, and further diluted to 1.0 AU at 515 nm prior to use. Aliquots of extract (20-2000 mL) were adequately diluted to a volume of 200 mL and added to 1.5 mL of DPPH solution cuvettes, shaken and kept in the dark for 60 min, and then the absorbance was measured at 515 nm using methanol as a blank. The percentage of scavenged DPPH radicals was calculated according to Equation 1:

$$\%DPPH_{rem} = \frac{A_0 - A_{sample}}{A_0} \times 100 \quad (1)$$

where A_0 and A_{sample} stand for the absorbance of the control and sample, respectively. The percentage of inhibition was converted into antioxidant activity by using Trolox as standard antioxidant.

2.4. Analysis of volatile compounds

Headspace solid-phase micro extraction (HS-SPME) and the gas chromatography-flame ionization detector (GC-FID) technique were used for the analysis of volatile compounds.

Two sample grams were placed in a 20 mL amber glass vial tightly capped with polytetrafluoroethylene (PTFE)/silicone septum and a magnetic cap. The vial was heated up to 40 ºC for 10 minutes to reach the equilibration of volatile compounds in the headspace. Afterwards, the SPME needle was inserted through the septum and the fiber was exposed for 40 min. The SPME fiber (2 cm length and 50/30 µm film thickness), purchased from Supelco (Bellefonte, PA, USA), was composed of Carboxen/DVB/polydimethylsiloxane (PDMS). Previously, the fiber had been conditioned following the instructions of the supplier.

The GC-FID analysis was performed using a gas chromatograph model 7890B (Agilent Technologies, Santa Clara, CA, USA). The gas chromatograph was equipped with a split/splitless injector and a flame ionization detector. The volatile compounds adsorbed in the fiber were desorbed into the injector port for 1 min in splitless mode. The DB-WAXetr polyethylene glycol capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm coating) (Agilent Technologies, USA) was used for the chromatographic separation. The carrier gas was helium at a flow rate of 1 mL/min. The injector temperature was 260 ºC and the detector temperature was 280 ºC. The oven temperature was initially 40 ºC for 10 min. Afterwards, the temperature was increased with a ramp of 3 ºC/min up to 160 ºC and immediately increased with a ramp of 15 ºC/min up to 200 ºC and held for 5 min to the end. The integrations were

performed with Agilent OpenLAB ChemStation C.01.06 Software (Agilent Technologies, CA, USA).

The chromatographic peaks were quantified by the Internal Standard Method. 4-Methyl-2-pentanol was the internal standard and each and every one of the compounds was used as external standard. The results obtained were expressed as mg of each standard compound per kg of oil.

For the analysis of volatile compounds, 39 analytical standards were used: acetic acid, *trans*-2-pentenal, 1-penten-3-one, pentanal, 1-penten-3-ol, pentan-1-ol, 2-methyl-1-butanol, hexanal, *cis*-3-hexen-1-ol, hexan-1-ol, heptanal, octane, octanal, 1-octen-3-ol, cis-3-hexenyl acetate, hexyl acetate, *trans,trans*-2,4-decadienal, propionic acid, 2-methylpropan-1-ol and butanoic acid, all supplied by Fluka (Buchs, Switzerland); 4-methylpentan-2-ol, *trans,trans*-2,4-heptadienal, *trans*-2-heptenal, heptan-2-ol, *trans*-2-penten-1-ol, 2-methylbutanal, 3-methylbutanal, 3-methyl butanoic acid, ethyl acetate, *cis*-3-hexenal, 2-methylpropanoic acid, ethyl butanoate, *trans,trans*-2,6-nonadienal, and *trans*-2-hexenyl acetate were supplied by Sigma-Aldrich (St. Louis, MO, USA); pentan-3-one and nonanal were supplied by Supelco (Bellefonte, PN, USA); cis-2-penten-1-ol and *trans*-2-hexen-1-ol were supplied by SAFC; *trans*-2-hexenal was supplied by Acrös Organics (Geel, Belgium).

2.5. Experimental design and statistical analysis

The Statistical Design of Experiments (SDE) and Response Surface Methodology (RSM) were used to plan and analyze the experiments. Both constitute a planning methodology and analysis based on statistical tools, where SDE selects the optimal experimental strategy to obtain the desired information with the minimum cost of analysis and RSM evaluates the experimental results ensuring maximum reliability in the conclusions (Box *et al.*, 2005).

In this process, a Box-Behnken design, with five repetitions of central points, for three factors was used: diameter of the holes of the hammer-crusher, temperature and malaxation time. The range of variation of these factors is 4.5 to 6.5 mm, 20 to 40 ºC and 30 to 90 minutes, respectively. Table 1 shows the different trials proposed by the design and their order of execution. Likewise, Table 1 also shows the actual and coded values of the factors used in each trial. The design tests allow to determine the influence of these technological factors on the phenolic and volatile compounds obtained in the olive oils.

Table 1. Experimental design and responses for Picual virgin olive oil

	Actual factors (coded factors)			Responses***			
Design points*	Diameter** (mm)	Temperature (ºC)	Time (min)	Total HPLC phenols (mg/kg tyrosol)	Total Folin phenols (mg/kg caffeic acid)	DPPH (µmol/kg)	Total LOX volatiles (mg/kg)
1	5.5 (0)	30 (0)	60 (0)	411	415	1109	12.2
2	5.5 (0)	20 (-1)	90 (+1)	357	344	844	12.4
3	4.5 (-1)	30 (0)	90 (+1)	508	467	1306	12.4
4	6.5 (+1)	30 (0)	90 (+1)	474	462	1393	11.7
5	6.5 (+1)	40 (+1)	60 (0)	747	761	2298	7.21
6	5.5 (0)	20 (-1)	30 (-1)	351	404	979	11.2
7	6.5 (+1)	20 (-1)	60 (0)	356	374	910	13.4
8	5.5 (0)	40 (+1)	90 (+1)	851	866	2668	7.03
9	4.5 (-1)	40 (+1)	60 (0)	758	762	2581	7.45
10	5.5 (0)	40 (+1)	30 (-1)	760	862	2662	7.40
11	6.5 (+1)	30 (0)	30 (-1)	380	422	1229	10.1
12	4.5 (-1)	30 (0)	30 (-1)	544	642	1919	9.21
13	5.5 (0)	30 (0)	60 (0)	610	724	1873	8.03
14	4.5 (-1)	20 (-1)	60 (0)	423	444	1427	10.9
15	5.5 (0)	30 (0)	60 (0)	583	639	1601	8.31
16	5.5 (0)	30 (0)	60 (0)	522	556	1636	8.60
17	5.5 (0)	30 (0)	60 (0)	415	483	1298	10.1

* Experiments were run in a random order
** Hole diameter of the hammer-crusher
*** Average of two replicates

The factors were coded according to the following transformation, Equation 2.

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (2)$$

Where x_i is the dimensionless coded value of the factor X_i, X_0 is the value of X_i at the center point or average level of the factor and ΔX the half-step change. The factors are usually coded because they provide a uniform framework to investigate the effects of factors and the coefficients of the models can be easily compared.

The experimental results were analyzed by Design-Expert v. 8.0.7.1 software (Stat-Ease, Inc., Minneapolis, MN, USA). The adequacy of the proposed model was determined by evaluating the lack of fit, coefficient of determination (R^2) and the Fisher value (F-value) obtained from the analysis of variance (ANOVA). A quadratic model for each response studied was used according to Eq. 3:

$$Y = \beta_0 + \beta_1 D + \beta_2 T + \beta_3 t + \beta_{12} D T + \beta_{13} D t + \beta_{23} T t + \beta_{11} D^2 + \beta_{22} T^2 + \beta_{33} t^2 \pm SD \quad (3)$$

Where: D is the hole diameter of the crusher (mm), T is the malaxation temperature (ºC) and t is the malaxation time (min). The predicted response (Y) was correlated with the set of coefficients (β): the intercept (β_0), linear (β_1, β_2, β_3), interaction (β_{12}, β_{13}, β_{23}) and quadratic (β_{11}, β_{22}, β_{33}). SD is the standard deviation of the model.

The statistical significance of the model and model coefficients were determined at 5% probability level (p-value = 0.05). The models for each response were expressed in terms of actual factors and without taking into account terms which were not statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of technological factors on phenolic compounds and antioxidant activity

Phenolic compounds play a very important role in VOO due to their high antioxidant activity, which contributes to the shelf life of the oils and, in addition, gives them their typical bitter taste (Zribi *et al.*, 2013).

Table 1 shows the total phenols as determined by HPLC (mg/kg tyrosol), which are the sum of the individual phenolic compounds, total phenols determined by Folin-Ciocalteau reagent (mg/kg caffeic acid) and the antioxidant potential determined by DPPH free radical scavenging activity (μmol/kg). Table 2 shows the content of individual phenolic compounds identified by HPLC (mg/kg tyrosol). Table 3 shows the models obtained for all the responses, which can be used later to predict the answers once the factors have been fixed. All the responses in Tables 1, 2 and 4 were determined in duplicate; the average values are shown in these tables as well. The software generates the regression equations after discarding the terms which are not statistically significant (p-value > 0.05); however, in order to support hierarchy, some linear terms were

TABLE 2. Responses for individual phenolic compounds by HPLC* (mg/kg tyrosol)

Design points	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
hydroxytyrosol	8.40	4.61	4.07	5.68	4.35	4.09	4.81	4.27	1.72	4.12	3.95	5.60	4.11	4.02	1.76	2.38	1.71
tyrosol	1.46	1.25	1.80	3.35	1.53	0.88	1.77	1.57	1.46	1.23	1.38	1.53	1.23	1.26	3.72	2.65	1.84
vainillin	0.89	1.73	1.46	1.29	1.86	1.23	0.63	2.91	2.38	1.71	1.54	1.01	1.45	1.01	1.55	1.89	1.97
p-coumaric acid	6.10	2.61	2.79	3.66	5.00	1.51	2.43	2.45	2.54	1.58	1.39	1.33	1.39	2.20	2.06	2.32	1.74
trans-ferulic acid	4.88	2.28	6.92	8.10	8.63	2.25	1.60	11.5	10.2	6.80	4.93	2.52	5.39	2.50	3.74	5.28	6.38
3.4-DHPEA-EDA (oleacein)	150	107	188	190	285	140	122	338	320	356	159	252	298	164	252	218	164
3.4-DHPEA-EA	64.6	51.3	78.4	83.0	192	44.1	58.0	233	179	157	52.9	87.5	111	62.4	115	104	75.9
p-HPEA-EDA (oleocanthal)	45.2	36.7	63.2	45.7	78.1	25.8	28.1	90.8	77.4	62.2	41.3	39.6	55.6	38.8	53.6	53.0	50.5
p-HPEA-EA	36.3	46.3	50.3	49.1	65.4	35.9	41.0	72.1	61.7	48.2	43.4	28.8	32.9	33.0	41.3	40.1	45.5
pinoresinol	6.23	6.07	5.47	4.86	10.9	9.35	7.92	8.45	11.4	37.0	14.6	44.1	32.5	29.2	34.0	11.8	7.48
luteolin	2.52	2.16	6.23	3.04	4.58	2.00	2.50	3.63	4.51	4.39	1.73	2.65	2.31	4.63	4.21	6.81	4.62
apigenin	3.24	2.83	4.77	3.31	3.44	2.59	3.32	4.74	5.61	8.17	1.93	3.77	3.69	2.96	2.98	2.70	2.26

3,4-DHPEA-EDA: dialdehyde form of decarboxymethyl oleuropein aglycone
p-HPEA-EDA: dialdehyde form of decarboxymethyl ligstroside aglycone
3,4-DHPEA-EA: aldehyde and hydroxylic form of oleuropein aglycone
p-HPEA-EA: aldehyde and hydroxylic form of ligstroside aglycone
* Average of two replicates

Table 3. Models (Eq. 3) in terms of actual factors and statistical parameters for the responses in Tables 2 and 4

Response	Model	*p*-value	R^2	Std. Dev.
Total HPLC phenols (mg/kg tyrosol)	1038.4 - 34.5 D - 46.0 T + 1.11 T^2	< 0.0001	0.939	47.7
Total Folin phenols (mg/kg caffeic acid)	948.1 - 37.0 D - 37.0 T + 0.97 T^2	< 0.0001	0.915	60.9
DPPH (μmol/kg)	2439.1 - 242.3 D - 42.8 T + 1.97 T^2	< 0.0001	0.939	175.2
Total LOX volatiles pathway (mg/kg)	12.49 + 0.76 D - 0.24 T	< 0.0001	0.859	0.85
***Phenol compounds* (mg/kg)**				
Hydroxytyrosol	62.66 - 13.6 D - 0.71 T - 0.43 t + 0.13 D T + 0.027 D t + 0.74 D^2 + 0.002 t^2	0.0002	0.975	0.31
Tyrosol	1.49			0.22
Vainillin	1.52			0.32
p-coumaric acid	-6.27 + 1.31 D + 0.25 T + 0.019 t - 0.045 D T	0.0008	0.913	0.19
trans-ferulic acid	-1.08 + 0.12 T - 0.067 t + 0.004 T t	< 0.0001	0.942	0.81
3,4-DHPEA-EDA	380.4 - 20.9 D - 14.7 T + 0.41 T^2	< 0.0001	0.904	28.4
3,4-DHPEA-EA	160.9 - 11.1 T + 0.29 T^2	< 0.0001	0.918	14.8
p-HPEA-EDA	18.6 - 0.55 T - 0.093 t + 0.015 T t + 0.032 T^2	< 0.0001	0.974	3.48
p-HPEA-EA	18.9 + 11.0 D - 4.62 T + 0.94 t - 0.13 D t + 0.098 T^2	< 0.0001	0.961	2.99
Pinoresinol	142.9 - 36. 6 D + 0.27 T + 0.057 t + 0.52 D T + 0.24 D t - 0.058 T t	0.0057	0.914	5.34
Luteolin	3.30			1.10
Apigenin	3.73 - 0.89 D + 0.11 T + 0.019 t	< 0.0001	0.922	0.32
***Volatile compounds* (mg/kg)**				
LOX pathway				
Hexanal	1.12 - 0.016 T + 0.001 t	< 0.0001	0.891	0.047
Hexan-1-ol	0.480 + 0.049 D - 0.006 T + 0.0005 t	< 0.0001	0.922	0.019
trans-2-hexenal	-0.124 + 1.81 D + 0.082 T - 0.049 D T	< 0.0001	0.901	0.57
trans-2-hexen-1-ol	-2.95 + 0.67 D + 0.087 T + 0.012 t - 0.018 D T - 0.002 D t	0.0002	0.926	0.045
cis-3-hexen-1-ol	3.37 - 0.82 D - 0.016 T + 0.002 t + 0.080 D^2	< 0.0001	0.915	0.048
cis-3-hexenyl acetate	0.094 + 0.45 D - 0.10 T + 0.041 t - 0.008 D t + 0.002 T^2	0.0006	0.906	0.037
1-penten-3-ol	0.376 - 0.019 D - 0.002 T	< 0.0001	0.856	0.009
1-penten-3-one	0.890 - 0.039 D - 0.001 t	< 0.0001	0.842	0.017
cis-2-penten-1-ol	0.476 - 0.008 D + 0.001 t	< 0.0001	0.906	0.007
$\frac{1}{\sqrt{trans\text{-2-Pentenal} + 0.05}}$	-13.4 + 1.04 T - 0.015 T^2	< 0.0001	0.999	0.011
Sugar fermentation				
Ethanol	-15.7 + 5.10 D + 0.58 T - 0.013 t - 0.13 D T	0.0003	0.908	0.36
Acetic acid	0.47			0.042
Other compounds				
Octane	0.239 + 0.095 D + 0.009 T + 0.010 t - 0.001 D t	0.0001	0.904	0.029
Pentan-3-one	-0.134 + 0.10 D + 0.015 T - 0.003 D T	0.0004	0.885	0.011
Octanal	4.45 - 1.48 D + 0.003 t + 0.150 D^2	< 0.0001	0.904	0.064
Nonanal	11.6 -1.82 D - 0.22 T - 0.021 t + 0.001 T t + 0.18 D^2 + 0.003 T^2	< 0.0001	0.979	0.067

D is the hole diameter of the hammer-crusher (mm), T is the malaxation temperature (ºC), t is the malaxation time (min)
R^2 is the coefficient of determination, Std. Dev. (SD) is the standard deviation

TABLE 4. Responses for individual volatile compounds by GC* (mg/kg)

Design points	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
LOX pathway																	
Hexanal	0.706	0.884	0.834	0.789	0.510	0.827	0.850	0.539	0.546	0.564	0.704	0.654	0.512	0.885	0.609	0.620	0.728
Hexan-1-ol	0.625	0.685	0.639	0.658	0.569	0.645	0.698	0.566	0.574	0.520	0.644	0.530	0.556	0.607	0.572	0.550	0.608
trans-2-hexenal	6.862	6.544	6.652	5.908	2.393	5.547	7.210	2.181	2.568	2.546	4.830	4.394	3.326	5.422	3.298	3.837	4.867
trans-2-hexen-1-ol	0.637	0.717	0.641	0.547	0.484	0.543	0.902	0.516	0.490	0.575	0.501	0.395	0.471	0.451	0.481	0.457	0.527
cis-3-hexen-1-ol	1.254	1.179	1.075	1.100	0.944	1.096	1.301	0.831	0.846	0.765	1.041	0.871	0.882	1.056	0.976	0.904	0.958
cis-3-hexenyl acetate	0.973	1.056	1.152	1.490	1.035	1.179	1.143	1.090	1.065	1.134	1.164	0.886	0.921	1.093	0.923	0.905	1.122
1-penten-3-ol	0.210	0.241	0.240	0.185	0.193	0.212	0.228	0.198	0.214	0.210	0.193	0.273	0.226	0.256	0.262	0.243	0.214
1-penten-3-one	0.534	0.590	0.649	0.566	0.602	0.650	0.632	0.610	0.646	0.634	0.614	0.716	0.667	0.669	0.664	0.601	0.603
cis-2-penten-1-ol	0.418	0.481	0.524	0.460	0.479	0.476	0.481	0.503	0.496	0.456	0.450	0.495	0.470	0.491	0.528	0.489	0.476
trans-2-pentenal	0.340	0.394	0.000	0.000	0.000	0.000	0.409	0.000	0.000	0.000	0.000	0.372	0.000	0.378	0.000	0.000	0.000
Sugar fermentation																	
Ethanol	6.833	8.330	5.265	7.700	5.370	6.922	7.387	5.585	5.739	5.953	7.826	6.568	7.306	6.095	6.013	6.504	7.621
Acetic acid	0.466	0.498	0.732	0.546	0.492	0.499	0.515	0.501	0.511	0.459	0.404	0.409	0.426	0.472	0.494	0.422	0.381
Other compounds																	
Octane	0.908	1.097	1.210	1.139	1.210	1.166	1.086	1.305	1.227	1.059	1.131	1.028	1.096	1.034	1.167	1.094	1.158
Pentan-3-one	0.296	0.349	0.341	0.310	0.297	0.330	0.384	0.000	0.305	0.277	0.301	0.298	0.303	0.316	0.351	0.330	0.320
Octanal	1.284	1.186	1.273	1.470	1.332	1.135	1.309	1.061	1.003	0.997	1.263	0.865	1.103	1.056	0.947	0.949	1.473
Nonanal	3.506	3.429	3.594	3.805	3.864	3.564	3.703	4.043	3.647	2.976	3.252	2.938	3.015	2.990	3.178	3.214	3.300

* Average of two replicates

not eliminated from the models, although they were not statistically significant. As can be seen in Tables 1 and 2, all oils meet the condition of being healthy because all of them contain more than 250 mg/kg of Hydroxytyrosol and its derivatives, according to the Commission Regulation (EU) No 432/2012.

Figure 1 shows the model surface obtained for the total HPLC phenol response; for total Folin phenols (total phenols determined with the Folin-Ciocalteau method), the surface is similar, as can be deduced from the model equation in Table 3. According to the models, the temperature during malaxation has a major influence on the presence of phenolic compounds, which increase as temperature increases. On the contrary, the phenolic compounds decrease as the diameter of the crusher hole increases, but this has less influence. Similar results were reported by other authors studying malaxation and milling separately (Vekiari and Koutsaftakis, 2002; Ranalli *et al.*, 2003). However, Ben Brahim *et al.* (2015), using Response Surface Methodology (RSM), indicated that the malaxation time does not significantly affect the phenol content.

Figure 2 shows the relationship between phenolic compound content and the antioxidant potential DPPH, both for total HPLC phenols and total Folin phenols. A very good correlation was observed in it, as is also apparent from the models in Table 3. On the other hand, from Figure 2 and Table 3 a very good correlation between both methods of quantifying total phenolic compounds in the oils is deduced, although the Folin-Ciocalteau method overestimated the content regarding HPLC.

Table 3 shows the proposed models in terms of actual factors for individual phenols; each phenol had a different model, but after examining the response surfaces, small differences were observed among them. The secoiridoid derivative class (3,4-DHPEA-EDA, 3,4-DHPEA-EA, *p*-HPEA-EDA and *p*-HPEA-EA) was clearly predominant and all of them increased with temperature. The results are similar to those obtained by other authors (Fregapane and Salvador, 2013; Gómez-Rico *et al.*, 2009; Kalua *et al.*, 2006). In agreement with other papers (Rodis *et al.*, 2002), this increase can be attributed to the increased partition coefficient between the oil and water phases, which causes an increase in the solubility of these compounds in the oil phase.

The malaxation time had a positive influence on secoiridoid derivatives from ligstroside (*p*-HPEA-EDA and *p*-HPEA-EA), but did not show a significant influence on secoiridoid derivatives from oleuropein (3,4-DHPEA-EDA and 3,4-DHPEA-EA). This is in contradiction to Ranalli *et al.* (2003), who observed a reduction in these secoiridoid derivatives with the time of malaxation, and attributed it to an increase in the oxidative reactions catalyzed by the activity of oxidoreductase enzymes present in the olive fruit such as peroxidase and polyphenoloxidase, due to the fact that olive paste was exposed to air longer when the malaxation time increased. In our case, the positive influence of malaxation time may be due to the increased activity of the β-glucosidase enzyme that hydrolyzed ligstroside and oleuropein, and the greater phenol content of the Picual variety. Figure 3 shows the response surface for the influence of the temperature and time on oleocanthal (*p*-HPEA-EDA) content, and of temperature and diameter on oleacein (3,4-DHPEA-EDA) content. For these models, factors that are not considered are not significant.

Different behaviors are also observed in other compounds; for example, pinoresinol decreased when temperature increased with a malaxation time longer than 60 minutes; and luteolin was unaltered. In addition, as seen in Table 3, some models present interaction among the factors; for example, diameter-time in alcohols (hydroxytyrosol and tyrosol) and ligstroside aglycone (*p*-HPEA-EA), or

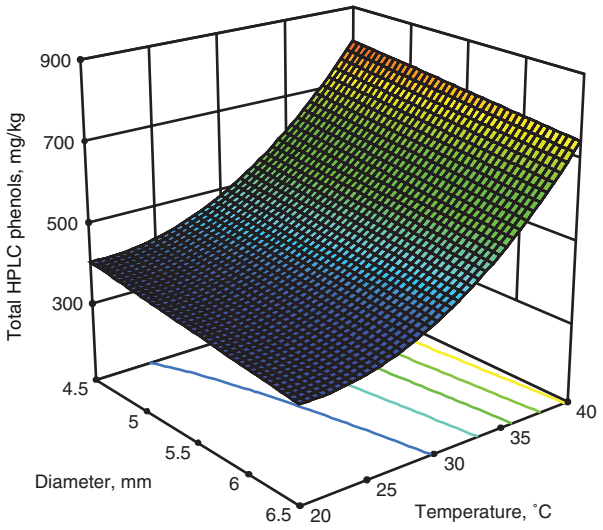

FIGURE 1. Effects of temperature and hole diameter of the hammer-crusher on total HPLC phenol content

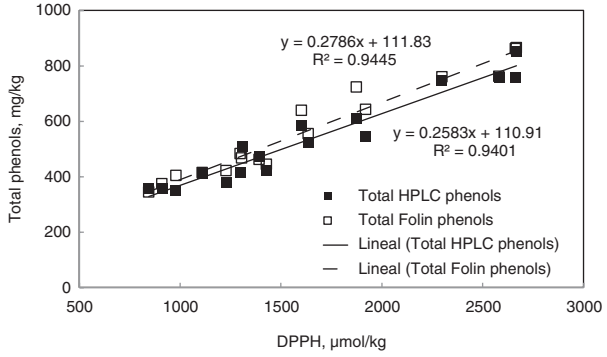

FIGURE 2. Relationship between phenolic compound content and antioxidant potential

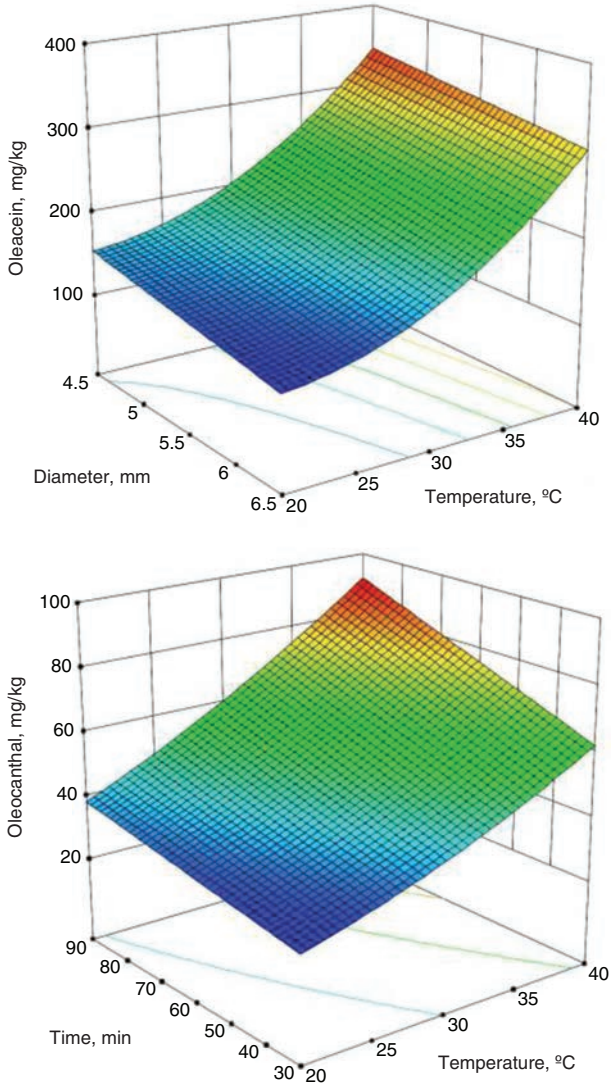

FIGURE 3. Response surfaces for oleacein and oleocanthal content

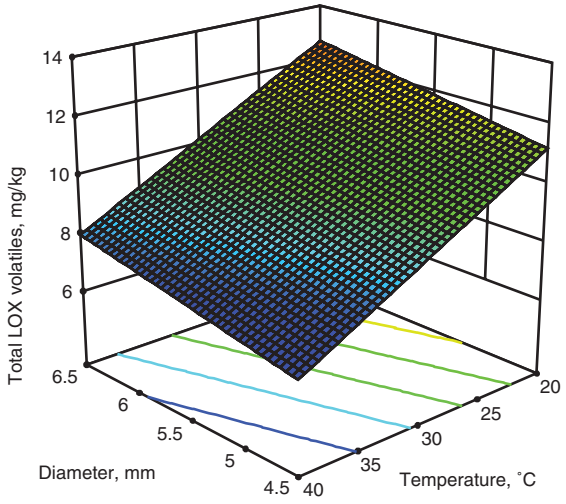

FIGURE 4. Effects of temperature and hole diameter on total LOX volatile content

diameter-time and temperature-time in pinoresinol. Finally, an interaction was observed between temperature and time in oleocanthal (*p*-HPEA-EDA), in agreement with De Torres *et al.* (2016).

3.2. Effect of technological factors on volatile compounds

Table 4 shows the volatile compound contents grouped according to the most probable precursor molecule and Table 3 shows the models. Only 16, of the 39 analytical standards used were identified in the samples because some analytical standards corresponded to compounds present in olive oils with defects, which are unwanted in extra virgin olive oils. *Trans*-2-hexenal was clearly predominant. The volatiles arising from the lipoxygenase (LOX) cascade (Angerosa *et al.*, 2004; Kalua *et al.*, 2007) decreased with temperature, but increased with the crusher hole diameter. The malaxation time was not significant. Figure 4 shows the response surface for the influence of diameter and malaxation temperature on total LOX volatile contents. The influence of temperature was observed to be much greater than that of diameter. When temperature was increased from 20 ºC to 40 ºC, the content of *trans*-2-hexenal was reduced by 62%, while the content of 1-penten-3-ol only fell by 16%. The total volatile compounds decreased by 36%, in agreement with many research papers (Fregapane and Salvador, 2013; Gómez-Rico *et al.*, 2009; Angerosa and Basti, 2001; Ranalli *et al.*, 2001), as a result of the inactivation of hydroxidelyase enzymes (Salas and Sánchez, 1999).

For most of the studied volatiles, a positive dependence was observed with malaxation time, except for 1-penten-3-one, for which one slight decrease was observed. Overall, the total volatile compounds were not significantly affected by the duration of the malaxation.

Finally, some of them increased with the diameter crusher holes: *trans*-2-hexenal, *cis*-3-hexenol, hexanol and *trans*-2-hexenol; while others decreased: 1-penten-3-ol, 1-penten-3-one and *cis*-2-pentenol. The end result would focus on an increase in total volatile compounds due to the importance of the *trans*-2-hexenal and *cis*-3-hexenol. In both cases, the maximum concentration corresponded to a diameter of 6.5 mm and a temperature of 20 ºC.

3.3. Optimal operating conditions

In order to achieve the optimal conditions of a balanced EVOO, we aimed to maximize the content in total HPLC phenols and total LOX volatiles

TABLE 5. Optimal conditions for the maximum of the main responses and prediction for some usual operating conditions

Individual response				
	Maximum value	**Diameter (mm)**	**Temperature (ºC)**	**Time (min)**
Total HPLC phenols (mg/kg tyrosol)	813.5	4.50	40.00	--
DPPH(µmol/kg)	2795	4.50	40.00	--
Total LOX volatiles (mg/kg)	12.71	6.50	20.00	--

Responses prediction				
Diameter (mm)	**Temperature (ºC)**	**Total HPLC phenols (mg/kg tyrosol)**	**Total LOX volatiles (mg/kg)**	**DPPH (µmol/kg)**
4.5	20	406	11.2	1282
4.5	25	425	10.0	1512
5.5	20	372	12.0	1040
5.5	25	391	10.8	1270
5.5	30	465	9.6	1599
6.5	20	337	12.7	798
6.5	25	356	11.5	1028
6.5	30	430	10.4	1357
6.5	40	745	8.0	2310

and, by extension, the antioxidant activity given its direct dependence with the content of total phenolic compounds (Figure 2). Table 5 shows the optimal values obtained from the derived mathematical models using the Design-Expert software. It can be observed that the optimal conditions are at the contour limits. As can be determined from the models in Table 3, and is seen in Figure 2 and Table 5, the antioxidant activity is closely linked to the total phenol content in such a way that as these were increased the antioxidant activity also increased. In contrast, the variation in total LOX volatiles was completely opposite to that of total phenols, according to the findings of Gómez-Rico *et al.* (2009) and Inarejos-García *et al.* (2011).

Therefore, obtaining balanced olive oils only depends on what is considered a balanced oil and on the operating conditions that are set to obtain the desired content in its different components. In Table 5, the total phenol content, total LOX volatiles and antioxidant activity for different operating conditions were calculated using the models in Table 3. In order to obtain an extra virgin olive oil which is balanced and of high quality from the Picual variety, it should be elaborated with a malaxation temperature between 20 and 25 ºC and preferably with a hammer-crusher hole diameter of 6.5 mm. Thus, for a hammer-crusher hole diameter of 6.5 mm 337 and 356 mg/kg total HPLC phenols were obtained for malaxation temperatures of 20 and 25 °C, respectively and, likewise, 12.7 and 11.5 mg/kg total LOX volatiles. These oils will be fragrant, healthy and, if they do not have defects, of high quality.

When the malaxation temperature is increased healthier but less fragrant oils were obtained and, on the contrary, less healthy but more fragrant oils were obtained at lower temperatures.

4. CONCLUSIONS

Temperature is the factor that has the major influence on the phenolic compounds and antioxidant activity; both of them increased as temperature increased. A good correlation between antioxidant activity and phenolic compounds was observed.

Temperature also has the major influence on the volatile compound content, thus when temperature increased from 20 to 40 ºC, total volatiles decreased by 36%. The total volatile compounds increased with the hammer-crusher hole diameter.

In order to obtain an extra virgin olive oil which is balanced and of high quality from the Picual variety, the elaboration should be carried out with malaxation temperature between 20 and 25 ºC and preferably with a hammer-crusher hole diameter of 6.5 mm. Thus, for a hole diameter of 6.5 mm 337 and 356 mg/kg total HPLC phenols were obtained for malaxation temperatures of 20 and 25 °C, respectively and, likewise, 12.7 and 11.5 mg/kg total LOX volatiles. These oils will be fragrant and nutritionally healthy.

ACKNOWLEDGMENT

This work is part of the Research Project of Excellence P11-AGR-7726. We wish to thank the Department of Economy, Innovation and Science

of the Andalusian Regional Government and the Spanish Ministry of Science and Innovation for the financial help provided.

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