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The effect of citric acid on the phenolic contents of olive oil

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ABSTRACT

Response surface methodology was used to optimise the combined effects of malaxation time (t) and aqueous citric acid solution (C_A) added at the beginning of the malaxation step on total polyphenols (*TP*) and *o*-diphenols (*OD*) content and the antiradical power (*ARP*) of the olive oil extracted from the Italian olive fruits of *Coratina* cultivar. Different tests were performed according to a 3^2 full factorial design, varying t from 30 to 90 min and the C_A from 5 to 15 ml/kg$_{paste}$. Overall optimal conditions identified by a numerical optimisation for the three responses were found to be at t = 30 min and C_A = 13.79 ml/kg$_{paste}$ under which the model predicted *TP* = 604.52 μgCAE/g$_{oil}$, *OD* = 80.44 μgCAE/g$_{oil}$ and *ARP* = 28.73 μgDPPH/μlextract. There were also linear correlations between *TP* (R^2 = 0.8176) and *OD* (R^2 = 0.8633) values of olive oil and waste water. The results of this study demonstrate that considerably short malaxation time and relatively small amounts of citric acid were required to enhance the quality of olive oil. The outcome of our study will therefore be of great value for the commercial production of olive oil with high level of polyphenols and *o*-diphenols.

1. Introduction

Several studies have shown the contribution of oxidative stress in the pathology of cancer, arteriosclerosis, malaria, rheumatoid arthritis, neurodegenerative disease and aging processes (Aruoma, 1997; Aruoma et al., 1997; Hollman, Hertog, & Katan, 1996; Meyer, Heinonen, & Frankel, 1998; Nakagami, Nanaumi-Tamura, Toyomura, Nakamura, & Shigehisa, 1995). The correlation between rancidity odours and flavours and the oxidative deterioration of fats and oils, which resulted in a decrease in the nutrition quality and safety of foods, is also fully understood (Moure et al., 2001). Compounds with antioxidant activity can preserve flavour and colour, avoid vitamin destruction in foods and more importantly protect living systems from oxidative damage (Moure et al., 2001). Several scientific studies have proved the capacity of antioxidants to protect cells from free radical damage and to preserve severe diseases (Moure et al., 2001; Saint-Cricq de Gaulejac, Provost, & Vivas, 1999; Servili et al., 2004).

Antioxidants are found in fruits such as prunes (Donovan, Meyer, & Waterhouse, 1998), berries (Abuja, Murkovic, & Pfannhauser, 1998; Heinonen, Lehtonen, & Hopia, 1998; Heinonen, Meyer, & Frankel, 1998; Prior et al., 1998) and olives (Romani, Mulinacci, Pinelli, Vincieri, & Cimato, 1999). Olive oil, one of the natural sources of antioxidants such as vitamin E, carotenoids and phenolic compounds, with a high unsaturated/saturated fatty acid ratio is claimed to have antioxidation, and bioactive compounds that have health-giving, physiological benefits and reduce the risk of chronic diseases (Aliakbarian, De Faveri, Converti, & Perego, 2008; Chiacchierini, Mele, Restuccia, & Vinci, 2007). The composition of active ingredients in olive oil can be significantly promoted by various factors including malaxation temperature, time (Servili, Selvaggini, Taticchi, Esposto, & Montedoro, 2003); and the use of microorganisms (Kachouri & Hamdi, 2004; Kachouri & Hamdi, 2006) or enzymes (Aliakbarian et al., 2008; De Faveri, Aliakbarian, Avogadro, Perego, & Converti, 2008; Vierhuis et al., 2001).

Several studies demonstrate that addition of pectolytic, hemicellulolytic, and cellulolytic enzymes during the olive oil malaxation process resulted in degrading the cell wall of the fruit and reducing the complexation of hydrophilic phenols with polysaccharides, increasing the concentration of free phenols in the olive paste and their consequent release into the oil and wastewaters during processing (Aliakbarian et al., 2008; De Faveri et al., 2008; Ranalli & De Mattia, 1997). However, limited studies have been carried out on the effect of citric acid addition on the phenolics composition of olive oils. Husken, Kooij, and Van Putte (2002) demonstrated that by mixing the olive oil with olive fruits, and an aqueous acid solution containing more that 30% (w/w) acid at 90 °C for at least 90 min, antioxidants in olive fruit were released into the oil phase. An oil with above 150 ppm polyphenol content was acquired by this method.

The aim of our study was to assess the feasibility of increasing the free phenols in the olive oil paste by simultaneous addition of citric acid during the malaxation step and control of the kneading time to hydrolyse the pectic polysaccharides, cellulosic and hemicellulosic fractions which are the major components of the cell

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wall of olive fruit (Vierhuis, Schols, Beldman, & Voragen, 2000). Free phenols in the olive paste can then be released into the oil and wastewater during the extraction process. In this study *Coratina* cultivar was used as the source of olive fruit. The acid used in this study was a food grade – water soluble acid, therefore, at the end of the process no acid residues were maintained in the oil.

Olive oil quality can be affected by malaxation time (Aliakbarian et al., 2008; Angerosa, D'Alessandro, Basti, & Vito, 1998; Angerosa, Mostallino, Basti, & Vito, 2001; Servili et al., 2003). Our previous study (Aliakbarian et al., 2008) corroborated that increasing the malaxation time from 90 min to 150 min decreased the quality of olive oil from *Coratina* cultivar in terms of *TP*, *OD* and *ARP,* which was attributed to phenolics oxidation phenomena. Aliakbarian et al. (2008) also found that at maximum concentration of enzymes (25 ml/kg_{paste}) when malaxation time was increased from 90 to 150 min *TP*, *OD* and *ARP* were decreased 20%, 40% and 17%, respectively. In this study malaxation times were selected from 30 to 90 min. Low concentrations of citric acid were also selected (between 5 and 15 ml/kg_{paste}) to create a safe and economically viable process attractive for commercial production of high quality olive oils.

Response surface methodology (RSM) is a well established statistical method that is used to evaluate the relative significance of several factors even in the presence of complex interactions (Box & Wilson, 1951; Montgomery, Runger, & Hubele, 2001). The RSM is used in food research studies to enhance or optimise phenolic compounds extraction such as evening primrose meal (Wettasinghe & Shahidi, 1999), berries (Cacace & Mazza, 2003a), olive oil (Aliakbarian et al., 2008), vitamin E from wheat germ (Ge, Ni, Yan, Chen, & Cai, 2002) and anthocyanins from blackcurrants (Cacace & Mazza, 2003b). In our study RSM method was used to assess the results of different tests according to a 3^2 full factorial design, selecting the time of the malaxation process (t) and the concentration of the citric acid (C_A) as independent variables and total polyphenols (*TP*) and *o*-diphenols (*OD*) concentrations and antiradical power (*ARP*) as response variables.

2. Materials and methods

2.1. Olive variety and extraction process

Italian olive fruits (*Coratina* cultivar) produced according to the organic agriculture rules were used as a raw material. The percentages of olive moisture (59.5 ± 1.9%), oil (19.8 ± 1.6%), and the pulp/stone ratio (2.83 ± 0.2) were determined according to the procedures described previously by Aliakbarian et al. (2008).

After manually stoning, approximately 50 g of the blended olive paste was used for the malaxation step which was carried out in a laboratory mixer at 10 rpm and at a constant temperature of 30 °C. The aqueous solution containing 30% (w/w) citric acid was added to the paste prior to the kneading step using various levels (5–15 ml/kg_{paste}) and malaxation times (30–90 min) according to the experimental design. Separations of oily must from the paste and oil from the oily must were performed by a bench-scale centrifuge using 6000 rpm for 10 min (model PK 13, ALC, Cologno Monzese-MI, Italy). A series of control tests for each malaxation time was carried out according to the above procedure, without addition of citric acid solution. The experimental results in terms of *TP*, *OD* and *ARP* in olive oil and waste water are listed in Table 1.

2.2. Oil and waste water sample analyses

Each sample of oil (5 g) was dissolved in 10 ml hexane. 1.0 ml of a 0.1 g/l syringic acid methanolic solution was added as internal standard. Samples were then extracted successively with three 12.5 ml portions of 60% aqueous methanol solution. The mixture was magnetically stirred each time for 10 min and then separated into two phases by centrifugation (3000 rpm for 10 min). The combined extracts were dried in a vacuum rotary evaporator at a temperature below 30 °C. The residue was dissolved in 1.0 ml methanol for analyses. Methanolic extracts including the internal standard were analysed for quantification of *TP*, *OD* and *ARP* concentrations. HPLC analysis was used for the olive oil polar compounds (including phenolics) lost during extraction and purification using the syringic acid methanolic solution as internal standard (Evangelisti et al., 1997). Hence, also in this case, the effects of sample preparation on phenolic compounds was assured to be the same as on syringic acid. Moreover, this compound has been often used as an internal standard when working with the olive oil (Brenes, García, Rios, García, & Garrido, 2002; Gómez-Alonso, Desamparados Salvador, & Fregapane, 2002; Gómez-Rico et al., 2007; Krichene et al., 2007). HPLC used in this study was Hewlett Packard, 1100 Series, Palo Alto, CA equipped with a C18 reverse-phase column (Model 201TP54, Vydac, Hesperia, CA) and a UV-Vis detector. The mobile phase was water/acetic acid (99:1%, v/v) (solvent A) and methanol/acetonitrile (50:50%, v/v) (solvent B), while the solvent gradient changed according to the following conditions: from 5% to 30% B in 25 min, from 30% to 40% B in 10 min, from 40% to 48% B in 5 min, from 48% to 70% B in 10 min, from 70% to 100% B in 5 min, isocratic at 100% B for 5 min, followed by returning to the initial conditions (10 min) and column equilibration (12 min). A flow rate of 1.0 ml/min was used at 30 °C. The samples (20 μl) were analysed at 280 nm.

Total polyphenol (*TP*) concentration was measured using the colourimetric Folin–Ciocalteau assay (Swain & Hillis, 1959) and a UV–Vis spectrophotometer (Model Lambda 25, Perkin Elmer, Wellesley, MA) at a wavelength of 725 nm. *TP* concentration was calibrated (R^2 = 0.999) using standard methanolic solutions of caffeic acid (10–1000 μgCAE/ml),which has been widely used as reference when working with olive oil (Aliakbarian et al., 2008; Mulinacci et al., 2001; Ranalli, Contento, Schiavone, & Simone, 2001; Ranalli, Sgaramella, & Surricchio, 1999), and expressed as microgram of equivalent caffeic acid per gram of oil (μgCAE/g_{oil}). The method response was described by the linear equation:

$$ABS_{725} = 0.002TP - 0.004 \quad (1)$$

The concentration of *o*-diphenols (*OD*) in the methanolic extract, also expressed as μgCAE/g_{oil}, was determined by the molybdate method (Gutfinger, 1981). The calibration curve (R^2 = 0.999) was made with standard methanolic solutions of caffeic acid in the range 10–250 μgCAE/ml:

$$ABS_{350} = 0.004OD + 0.001 \quad (2)$$

In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a stable free radical was used since both biological and technological roles of antioxidants depend on their interaction with oxidative free radicals (Blois, 1958). The antioxidant activity of samples was studied as their ability to reduce the DPPH radical (Brand-Williams, Cuvelier, & Berset, 1995). Moreover, such an assay has been widely used when working with olive oil (Lavelli, 2002; Rotondi et al., 2004) and, in particular, it was found out that the antioxidant activity, measured by the DPPH reduction, was related to the degradation level of olive oils, with fresh oils 3–5 times more efficient than aged oils, whereas commercial samples had intermediate efficacy (Lavelli, 2002) The DPPH concentration in the reaction medium (C_{DPPH}) was calculated from a calibration curve, whose equation, determined by linear regression using standard solutions of DPPH in the range 3–44 μgDPPH/ml was:

$$ABS_{515} = 0.023C_{DPPH} - 0.008 \quad (3)$$

with R^2 = 0.999.

Table 1
Results collected from tests planned according to the 3^2 full factorial design.

Tests	Independent Variable		Analytical parameters				
	t[a] (min)	C_A[b] (mL/kg$_{paste}$)	$TP_{waste\ water}$[c] (μgCAE/g$_{water}$)	$OD_{waste\ water}$[d] (μgCAE/g$_{water}$)	TP[e] (μgCAE/g$_{oil}$)	OD[f] (μgCAE/g$_{oil}$)	ARP[g] (μgDPPH/μlextract)
1	30	5	6183.4 (29.4)	1481.5 (–)	464.6 (4.5)	64.6 (17.0)	17.9 (10.9)
2	30	10	7785.7 (62.9)	1882.0 (25.2)	613.7 (38.0)	79.4 (43.8)	29.8 (84.8)
3	30	15	7309.8 (53.0)	2018.1 (34.2)	565.8 (27.3)	81.8 (48.2)	27.5 (70.8)
4	60	5	6550.5 (16.9)	1571.9 (11.1)	515.1 (10.9)	65.0 (13.7)	19.2 (19.6)
5	60	10	6982.2 (24.6)	1632.1 (15.3)	594.5 (28.0)	69.0 (20.6)	24.8 (54.3)
6	60	10	7002.2 (25.0)	1612.1 (13.9)	584.5 (25.8)	69.9 (22.1)	26.8 (66.7)
7	60	10	6992.2 (24.8)	1652.1 (16.7)	604.8 (30.2)	68.9 (18.6)	22.8 (41.8)
8	60	15	6673.4 (19.1)	1867.1 (31.9)	537.6 (15.7)	74.7 (30.5)	23.2 (44.2)
9	90	5	6770.7 (9.6)	1605.9 (13.8)	495.0 (1.7)	67.8 (21.8)	21.0 (28.9)
10	90	10	6463.3 (4.6)	1646.3 (16.7)	510.3 (4.9)	68.8 (23.5)	21.8 (34.0)
11	90	15	6010.3(–)	1795.6 (27.3)	465.4 (–)	170.6 (26.8)	20.0 (22.9)

The experiments were carried out in duplicate. Values between brackets refer to the percentage increase in each response with respect to its control test.
[a] t = malaxation time,
[b] C_A = citric acid concentration,
[c] TP = total polyphenols concentration in waste water,
[d] OD = o-diphenols concentration in waste water,
[e] TP = total polyphenols concentration in olive oil,
[f] OD = o-diphenols concentration in olive oil,
[g] ARP = antiradical power.

The ratio expressed in μlextract/μgDPPH was plotted versus the percentage of the DPPH concentration remained after 60 min compared to the initial one. The *ARP* value expressed as μgDPPH/μlextract is equivalent to the reciprocal of EC_{50} ($1/EC_{50}$), which corresponds to the phenolic extract concentration able to reduce 50% of the DPPH radical content.

2.3. Experimental design

The RSM was employed for modelling and analysis of the influence of malaxation time (t) and citric acid concentration (C_A), selected as independent variables, on olive oil phenolics content. In this study *TP*, *OD* and *ARP* were selected as desired responses which were assumed to be affected by the above two operative conditions.

In the RSM, the quantitative form of relationship between desired response and independent input variables can be represented by the following equation:

$$Y = F(t, C_A) \tag{4}$$

where Y is the predicted response and F is the response function (or response surface). In the procedure of analysis, the approximation of Y was proposed using the fitted second-order polynomial regression model which is called the quadratic model. The quadratic model of Y can be written as follows:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{5}$$

where Y is the desired response, β_o is the interception coefficient, βI, βii and βij are the linear quadratic and cross product terms, respectively and X_i and X_j are the coded levels of the independent variables.

The response models are built using a 3^2 full factorial design data with three levels (high, +1, low, −1 and the central point, 0) corresponding to $t = 30$, 60 and 90 min and $C_E = 5$, 10 and 15 ml/kg$_{paste}$, respectively. Each test was performed in duplicate and the central point was repeated three times (runs 5–7).

The statistical significances of the fitted quadratic models were evaluated by the F-test of ANOVA (Table 2) and the Student's t-test (Table 3) on experimental data using the "Statistica" software trial version 6.0 (StatSoft, Tulsa, OK) and the "Design Expert" software trial version 6.0.10 (Stat-Ease, Minneapolis, MN). The adequacy of the models was determined using coefficient of determination (R^2) analysis. Joglekar and May (1987) suggested that R^2 should be at least 0.80 for a good fitness of a response model. The models of each response for the full factorial design were expressed in terms of coded variables and effects being non-significant ($p > 0.05$) being stepped down from models without damaging the model hierarchy.

3. Results and discussion

3.1. Correlation between total polyphenol and o-diphenol contents of oil and wastewater

One of the major issue in large scale production of olive oil is the amount of wastewater, which is approximately 50% and 80–110% relative to the initial weight of the olives, for the traditional and continuous processes, respectively (Mulinacci et al., 2001). Wastewaters from olive oil processes also contain high value pollutant organic substances including sugars, tannins, polyphenols, polyalcohols, pectins and lipids (Lesage-Meessen et al., 2001).

The phenolic compounds from olive, once released or formed during the process, are distributed amongst the water (~53%), the oil (~1–2%) and solid phase (~45%) depending on the extraction system (Rodis, Karathanos, & Mantzavinou, 2002). While the phenolic compounds are responsible of the poor biodegradability and high phytotoxicity of the olive oil effluent (Lesage-Meessen et al., 2001), they are strong natural antioxidants with *in vitro* biological activities (Schulz & Herrman, 1980; Vinson, Su, Zubik, & Bose, 2001). It is critical to determine the polyphenol contents in each phase, and in particular in the wastewater (Table 1).

Significant linear correlations were obtained by plotting *TP* and *OD* values in the olive oil versus wastewater (Fig. 1a and b) as evidenced by the values of the coefficient of determination (R^2) found to be 0.8176 and 0.8633, respectively.

The acid treatment increased the *TP* and *OD* partition coefficients, that are expressed as the ratios of their contents in oil and wastewater ($\frac{\mu gCAE/g_{oil}}{\mu gCAE/g_{wastewater}}$). The partition coefficients were 0.08 and 0.04 for *TP* and *OD*, respectively. These values were greater than those obtained by Di Giovacchino, Costantini, Serraiocco, Surricchio, and Basti (2001), who acquired partition coefficients of 0.04 and 0.03 for *TP* using two-phase and three-phase centrifugal decanters, respectively, and 0.03 and 0.02 for *OD* using the extraction systems described above. The acid treatment, therefore resulted in increasing the concentration of phenolic compounds, particularly *TP*, into the oily phase that will be of great value for

Table 2
Results of ANOVA for the concentrations of total polyphenols (*TP*) and *o*-diphenols (*OD*) and the antioxidant power (*ARP*) by response surface quadratic model.

Source	*TP*				*OD*				*ARP*			
	SS[a]	DF[b]	*F*-Value	Prob>*F*	SS	DF	*F*-Value	Prob>*F*	SS	DF	*F*-Value	Prob>*F*
Model	25.82×10^3	4	9.23	0.0098	256.83	3	11.58	0.0042	118.61	4	8.63	0.0115
X_1	5.011×10^3	1	7.17	0.0367	58.34	1	7.89	0.0262	25.79	1	7.51	0.0338
X_2	1.479×10^3	1	2.12	0.1960	146.72	1	19.84	0.0030	26.63	1	7.75	0.0318
X_1^2	–	–	–	–	–	–	–	–	–	–	–	–
X_2^2	15.06×10^3	1	21.53	0.0035	–	–	–	–	37.94	1	11.04	0.0159
X_1X_2	4.278×10^3	1	6.12	0.0482	51.77	1	7.00	0.0331	28.25	1	8.22	0.0285
Residual	4.196×10^3	6			51.77	7			20.62	6		
Lack-of-fit	3.990×10^3	4	9.68	0.0958	49.77	5	9.95	0.0938	12.62	4	0.79	0.6255

[a] SS = sum of squares,
[b] DF = degrees of freedom.

Table 3
Results of the regression analysis for the concentrations of total polyphenols (*TP*) and *o*-diphenols (*OD*) and the antioxidant power (*ARP*)

	Source	Coefficient	Standard error	*t*-value
TP	Mean	593.79 (581.58)	77.41 (88.38)	1.32 (2.04)
	X_1	−28.90 (−28.90)	2.01 (1.02)	2.63 (1.20)
	X_2	15.70 (15.70)	12.06 (14.97)	5.73 (5.05)
	X_1^2	−30.52	0.01	−2.20
	X_2^2	−66.16 (−74.30)	0.55 (0.68)	−4.77 (−4.34)
	X_1X_2	−32.71 (−32.71)	0.07 (0.09)	−2.97 (−2.32)
OD	Mean	69.70 (70.86)	8.00 (6.33)	7.52 (8.34)
	X_1	−3.12 (−3.12)	0.21 (0.10)	−1.39 (1.39)
	X_2	4.95 (4.95)	1.25 (0.59)	2.63 (4.13)
	X_1^2	−3.18	0.001	2.22
	X_2^2	−1.07	0.06	−0.74
	X_1X_2	−3.60 (−3.60)	0.008 (0.009)	−3.16 (−2.64)
ARP	Mean	24.91 (25.19)	6.91 (5.80)	0.19 (−0.07)
	X_1	−2.07 (−2.07)	0.18 (0.07)	0.08 (1.62)
	X_2	2.11 (2.11)	0.001 (0.98)	0.57 (4.54)
	X_1^2	0.70	1.08	4.29
	X_2^2	−3.92 (−3.73)	0.05 (0.04)	−3.17 (−3.32)
	X_1X_2	−2.66 (−2.66)	0.006 (0.006)	−2.70 (−2.87)

Values given in parenthesis were calculated without taking into account the terms that the regression analysis revealed not to be significant.

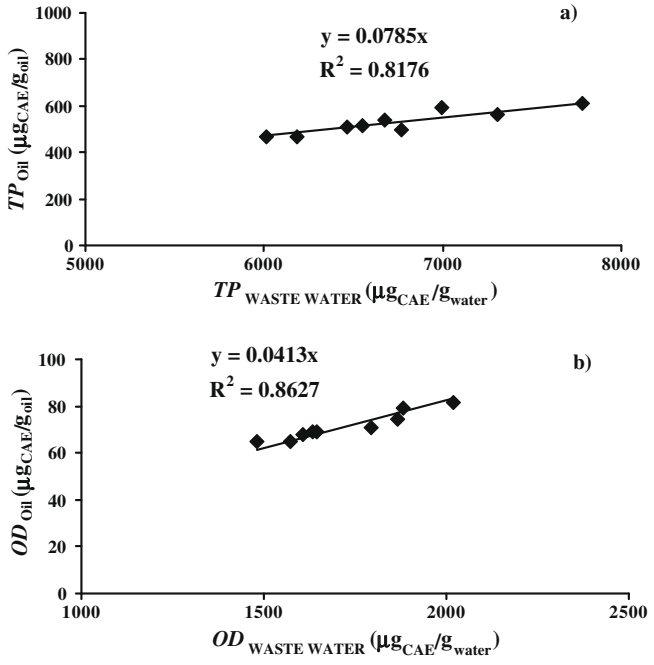

Fig. 1. Correlation between total polyphenol (a), (*TP*), and *o*-diphenol (b), (*OD*), contents of oil and waste water.

olive oil industry. The high solubility of *OD* in the aqueous phase accounted for its low partition coefficient and decreased effect of citric acid on enhancing this value (Di Giovacchino et al., 2001).

3.2. Efficiency of citric acid addition

The total amount of *TP*, *OD* and *ARP* in olive found in the literature were between 100 and 1000 μgCAE/g_{oil} (Tuck & Hayball, 2002). The percentage increase of *TP*, *OD* and *ARP* resulting from the citric acid treatment with respect to the control tests were depicted in Table 1. Within 30 min the content of polyphenolic compounds approached 445 μgCAE/g_{oil} in the control sample without using citric acid to 614 μgCAE/g_{oil} in a sample using 10 ml/kg_{paste} citric acid. The enhancement in the total amount of polyphenols was attributed to both using acid and malaxation time. For example, working with 30 min malaxation time, an increase in citric acid concentration from 5 to 15 ml/kg_{paste} led to a 23% and 31% increase in *TP* and *OD*, respectively, while raising *t* up to 90 min resulted in *TP* decrease of 3% and *OD* increases only of 5%.

3.3. Effect of malaxation time and citric acid concentration on TP, OD and ARP

The second-order models provided by RSM describing *TP*, *OD* and *ARP* concentrations as a simultaneous function of malaxation time (t) and citric acid concentration (C_A) are described by the following equations:

$$Y_1 = 581.58 - 28.90X_1 + 15.70X_2 - 74.30X_2^2 - 32.71XX_2 \quad (6)$$

$$Y_2 = 70.86 - 3.12X_1 + 4.95X_2 - 3.60X_1X_2 \quad (7)$$

$$Y_3 = 25.19 - 2.07X_1 + 2.11X_2 - 3.73X_2^2 - 2.66X_1X_2 \quad (8)$$

where Y_1, Y_2 and Y_3 are the *TP*, *OD* and *ARP* responses and X_1 and X_2 are the coded values of malaxation time (t) and citric acid concentration (C_A), respectively.

The ANOVA results calculated after withdrawal of the non-significant terms, highlighted the goodness of the fitting of the models by regression coefficients (R^2) which are 0.8602, 0.8322 and 0.8519 in the case of *TP*, *OD* and *ARP*, respectively. The significance of quadratic regression models is evident from Fisher's test value being 9.23, 11.58 and 8.63 for *TP*, *OD* and *ARP*, respectively (Table 3).

The significance of each coefficient was checked with the *p*-values (Ghodke, Ananthanarayan, & Rodrigues, 2009). The smaller the magnitude of the *p*, the more significant is the corresponding coefficient. Values of *p* less than 0.05 indicate model terms are significant. The estimated coefficient and the corresponding *p*-value suggested that, amongst the test variables used in the study, X_1 (malaxation time) and X_2 (citric acid concentration), the X_2 had a more significant effect on the *o*-diphenol ($p < 0.0030$)

contents followed by antioxidant power ($p < 0.0318$) of oil compared to *TP* ($p < 0.1960$). The interactions between two independent variables were significant in all models. The quadratic term of citric acid concentration has a larger effect on *TP* ($p < 0.0035$) followed by *ARP* ($p < 0.0159$).

The 3D response surface for total polyphenols, *o*-diphenols concentration, and the antioxidant power are illustrated in Fig. 2.

Fig. 2a shows the interaction between the malaxation time and citric acid concentration on *TP* contents of the olive oil samples. As illustrated in Fig. 2a, using a lower acid concentration (5 ml/kg_{paste}), *TP* content increased by enhancing the malaxation time. However, at a maximum level of acid (15 ml/kg_{paste}), the *TP* content reduced by increasing the malaxation time from 30 to 90 min due to the oxidation phenomena (Aliakbarian et al., 2008). The highest concentration of total polyphenols (613.74 μgCAE/g_{oil}) was obtained at 30 min malaxation time and 10 ml/kg_{paste} citric acid concentration. As shown in Fig. 2b, increasing citric acid concentration resulted in the release of *o*-diphenolic compounds in olive oil which may have been caused by degradation of the cell wall of the fruit and reduction of the complexation of hydrophilic phenols with polysaccharides. On the other hand, higher malaxation time led to a decrease in the *OD* content. For instance, using a maximum citric acid level (15 ml/kg_{paste}), the *o*-diphenolic concentration decrease from 81.4 μgCAE/g_{oil} to 70.61 μgCAE/g_{oil} by increasing the malaxation time from 30 to 90 min. The response surface used to predict the antioxidant power within the region under investigation is shown in Fig. 2c. Similar to *TP*, the quadratic C_A term is negative, making the fitted surface have a parabolic trend with maximum *ARP* values at C_A = 10 ml/kg_{paste}. Increasing malaxation time from 30 min to 90 min resulted in a decrease in *ARP* values for all three citric acid levels which confirmed the negative effect of oxidation phenomena on the olive oil phenolics. Using 10 ml/kg_{paste} of citric acid and 30 min malaxation, *ARP* approached the maximum value of 29.89 μgDPPH/μlextract.

A numerical optimisation was carried out to identify the overall optimal conditions of olive oil extraction by addition of citric acid. The three responses were then analysed by conferring the same significance or weight to them by means of the "Design expert" software. The optimum conditions predicted by model was t = 30 min and C_A = 13.79 ml/kg_{paste} under which the system achieved *TP* = 604.52 μgCAE/g_{oil}, *OD* = 80.44 μgCAE/g_{oil} and *ARP* = 28.73 μgDPPH/μlextract. The desirability of the model (0.922) indicates that only 7.80% of response was outside an acceptable region.

Finally, the linear correlation (R^2 = 0.8382) between olive oil phenolics content and their antioxidant activity in the oil confirmed the efficiency of the proposed citric acid treatment to yield a product with a potential higher resistance to oxidation with a potential longer shelf-life compared to standard oils (Ge et al., 2002; Ranalli et al., 2001; Servili & Montedoro, 2002).

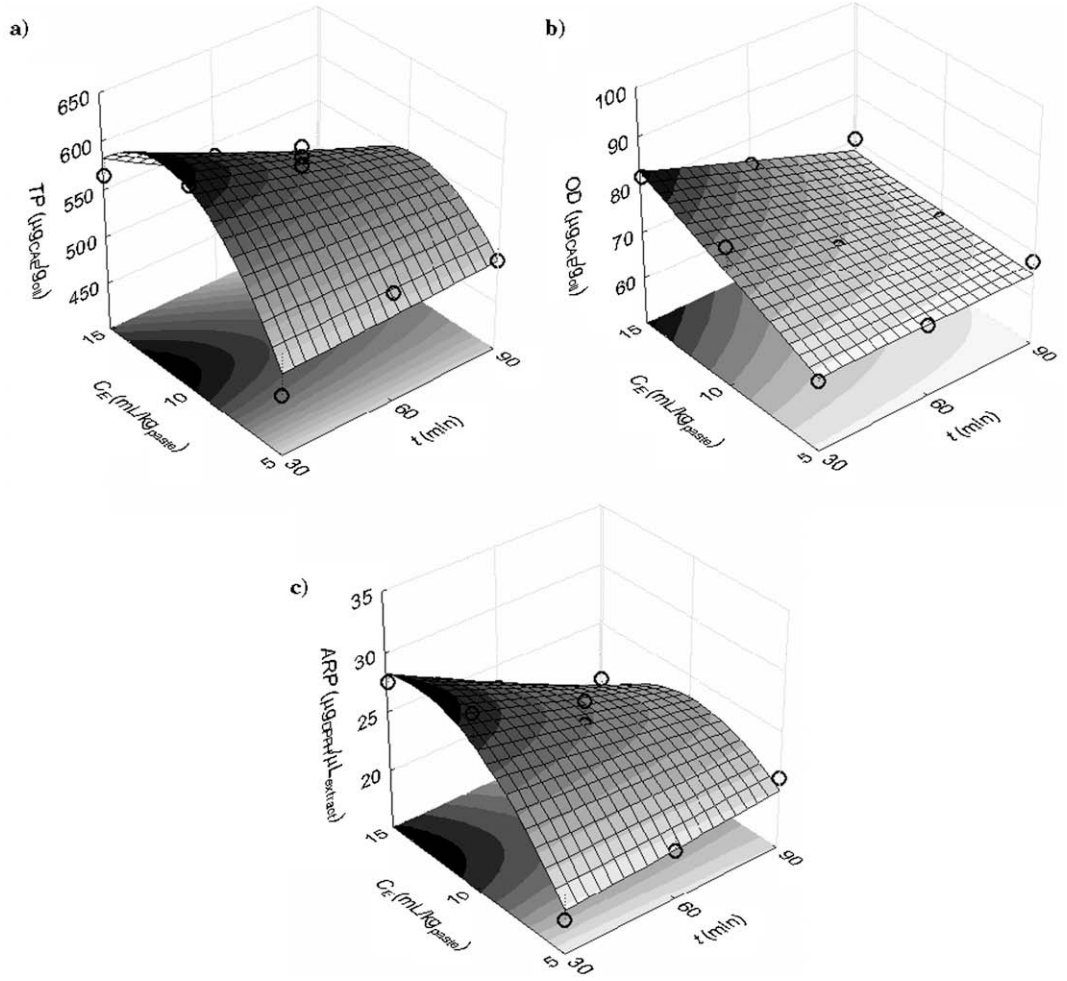

Fig. 2. Response surfaces of total polyphenols concentration (a), *TP*, *o*-diphenols concentration (b), *OD*, and antiradical power (c), *ARP*, as simultaneous functions of malaxation time (t) and citric acid concentration (C_A) according to the 3^2 full factorial design.

4. Conclusions

The model predicted that by addition of 13.79 ml citric acid per kg paste of olive the concentration of phenolic compounds will be increased 36% within 30 min at room temperature compared with the conditions that no acid was added to the malaxation process. Addition of citric acid may also decrease the malaxation time compared with conventional processes. Both these effects will be desirable from a commercial point of view as they promote the quality of olive oil in terms of total polyphenols, *o*-diphenols and their antioxidant power and enhance the extraction efficiency of olive oil from the plant.

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