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RECOVERY OF FLAVONOIDS FROM THREE-PHASE OLIVE POMACE BY AQUEOUS ETHANOL EXTRACTION

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ABSTRACT

A three-phase olive pomace (OP), the solid by-product originating from the production of olive oil, was investigated as a potential source of flavonoids. Flavonoids were extracted by an environmentally friendly procedure using aqueous ethanol as solvent. The flavonoid content of OP, expressed as quercetin equivalents (QE) per unit weight of dry material, was 25.28 ± 0.93 mg QE/g. To evaluate the effects of temperature (T), extraction time (E), liquid-to-solid ratio (R) and solvent composition (C) on the yield of flavonoid extraction (y), a Central Composite Design (CCD) coupled with Response Surface Methodology (RSM) was used. Statistical analysis of the results showed that T was the most influential factor, followed by E, R and C. A reduced polynomial model was developed by the stepwise regression method which provided an accurate description of the extraction process. Maximization of the response variable gave: y_{max} = 90.5% at T = 69.9 °C, E = 212 min, R = 36.7 mL/g and C = 43.7%. Overall, the obtained results support the use of three-phase OP as a source of flavonoids and give useful indications on the influence of process variables on their recovery.

Keywords: olive pomace, phenolic compounds, solvent extraction, waste valorization, response surface methodology (RSM).

INTRODUCTION

According to data from the International Olive Oil Council, about 3,000,000 tons of olive oil are produced annually in the world (IOOC, 2016). Over 98% of the total production is concentrated in the Mediterranean region, with Spain, Italy and Greece being the largest producers. The production of olive oil is associated with the generation of large amounts of liquid and solid wastes, namely, olive mill wastewater (OMW) and olive pomace (OP), while OMW is a dark liquid effluent containing highly polluting organic compounds such as proteins, sugars, lipids and polyphenols (Dermeche *et al.*, 2013). OP is a complex lignocellulosic material consisting mainly of olive stones, pulp residues and fruit skins (Nunes *et al.*, 2016).

Over the years, traditional olive pressing has been replaced by extraction systems based on three- or two-phase centrifugation (Roig *et al.*, 2006). In the three-phase operation, warm water is added at the centrifugation step and three outlet streams: olive oil, OMW and a relatively dry OP are produced. In contrast, in two-phase systems olive oil and a wet OP are obtained. The main drawback of the three-phase technology is the use of large quantities of warm water and hence the production of significant volumes of OMW. The two-phase system allows reduced water consumption but the wet OP produced poses difficulties for disposal, as it is very difficult to handle and dries out very slowly. Independently of the technology used, the compositional characteristics of OMW and OP, their high organic content and the fact that they are produced in large amounts during a short period of time make the environmental impact of the olive oil industry significant (Dermeche *et al*., 2013).

OP is generally used for fuel or fertilizing purposes or, to a lesser extent, as a supplement for animal feed. Recently, however, following a general trend towards the value-added exploitation of agro-industrial wastes (Mirabella *et al.*, 2014; Zuorro *et al*., 2013, 2016), attempts has been made to find alternative ways of utilization. The production of biofuels such as biochar (Hmid *et al*., 2014) and biodiesel (Che *et al*., 2012) or the use as a substrate for solid-state fermentation (Oliveira *et al.*, 2016) are just a few examples of the proposed approaches. Furthermore, the presence of high levels of phenolic compounds makes OP a potential valuable source of these substances, although only a limited number of studies have examined the feasibility of their recovery (Tercan and Seker, 2012; Zuorro, 2014; Lavecchia and Zuorro, 2015).

Phenolic compounds are an important class of secondary metabolites produced by plants to perform a variety of functions, such as protection against oxidative damage and UV radiation or defense against microbial and herbivore attacks. In the last decades, phenolic compounds have attracted increasing interest from food scientists and nutritionists due to their reported health benefits, which are attributed to their anti-oxidative, anti-inflammatory and anti-carcinogenic properties (Mushtaq and Wani, 2013). Flavonoids are a group of phenolic compounds characterized by a triple ring chemical structure and displaying high antioxidant capacity and other biologically relevant activities (Mierziak *et al*., 2014). Recently, Yahyaoui *et al*. (2014) showed that OP from a two-phase extraction system was very rich in flavonoids, with hesperidin, quercetin-3-*O*-arabinoglucoside, luteolin and quercetin being the most abundant. Flavonoid extracts from OP were also found to possess high antioxidant activity, suggesting the possibility of using them to replace synthetic antioxidants in food products, in addition to the use as functional ingredients.

The aim of this study was to investigate the recovery of flavonoids from a three-phase OP produced by an olive mill located in Central Italy. Flavonoid extraction was carried out by an environmentally friendly procedure using aqueous ethanol as solvent. To evaluate the influence of the main process variables on the flavonoid

extraction yield, a rigorous approach based on factorial design and Response Surface Methodology (RSM) was used.

EXPERIMENTAL

Chemicals and olive pomace

Ethanol, sodium hydroxide, sodium nitrite and aluminum chloride hexahydrate ($Al_2O_3 \cdot 6\ H_2O$) were purchased from Carlo Erba (Milano, Italy). Quercetin was obtained from Sigma-Aldrich (Milano, Italy). All chemicals were reagent grade and used without further purification. Aqueous solutions were prepared with deionized water.

OP was collected from a commercial three-phase oil extraction plant in Central Italy (Villa Latina, FR), placed in plastic bags and stored at –20 °C. Before performing a set of experiments, appropriate amounts of the frozen material were thawed in air at room temperature and characterized for moisture and flavonoid content.

Analytical methods

Moisture content of OP was determined by oven drying at 105 °C, while a three-stage extraction procedure with aqueous ethanol (50% v/v) as solvent (Zuorro and Lavecchia, 2013) was used to evaluate its flavonoid content. Briefly, 0.2 g of the waste and an appropriate amount of solvent (20, 10 and 5 mL in the first, second and third stage, respectively) were poured into glass flasks thermostated at 40 °C. After 60-min stirring, the flask content was filtered, centrifuged at 7,000 × g for 5 min and assayed for total flavonoids. The solid was re-extracted two additional times and the overall flavonoid content was calculated as the sum of the values obtained in each stage.

Total flavonoids were determined by the method of Zhishen *et al*. (1999) with slight modifications. Specifically, 1.5 mL of diluted sample were mixed with 0.075 mL of 5% (w/v) sodium nitrite. After 5 min, 0.15 mL of 10% (w/v) of aluminum chloride hexahydrate were added and the solution was left to react for 6 min. Then, 0.5 mL of 1 M sodium hydroxide and 0.775 mL of distilled water were added. The absorbance at 510 nm was measured using a double-beam UV-Vis spectrophotometer (UV-2700, Shimadzu, Japan) with quartz cells of 1-cm path length. A calibration curve obtained with quercetin standards was used to convert absorbance to concentration (Figure-1) and the results were expressed as quercetin equivalents (QE) per unit weight of dry OP.

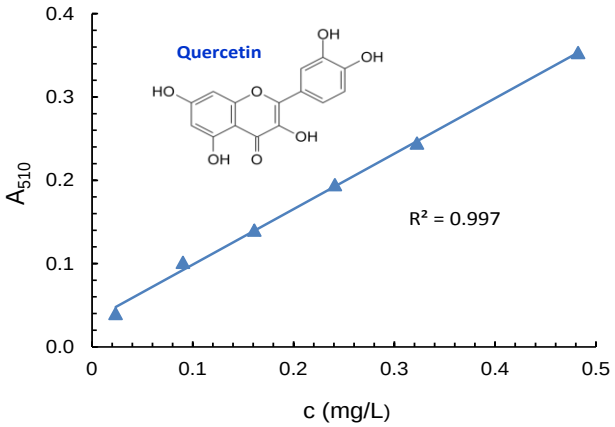

Figure-1. Calibration curve of quercetin.

Extraction procedure

Solvent extraction experiments were carried out in batch mode in magnetically stirred and thermostated (±0.1°C) screw-cap flasks, following the procedure described elsewhere (Zuorro, 2015). Briefly, 20 mL of aqueous ethanol and an appropriate amount of OP (roughly between 0.4 and 2 g) were placed into the flask. At the desired time, a sample of the liquid was taken, passed through a 45-μm nylon filter and assayed for total phenolics.

Experimental design

A Central Composite Design (CCD) was used to evaluate the effects of temperature (T), extraction time (E), liquid-to-solid ratio (R) and solvent composition (C), i.e., the volume fraction of ethanol in the ethanol–water mixture, on the recovery of flavonoids. The CCD consisted of a full 2^4 factorial design augmented by six central points and two axial points per factor at distance ±α from the central point. To ensure the rotatability of the design space, the value of α was taken as $(2^4)^{1/4} = 2$.

Factor levels were chosen based on preliminary experiments and to cover a range of values of practical interest. They are reported, in both actual (X_i) and coded (x_i) values, in Table-1. The latter were obtained by the following transformations:

$$x_i = \frac{X_i - X_{i,0}}{\Delta X_i} \quad (1)$$

where $X_{i,0}$ is the actual value of the *i*-th factor at the central point and ΔX_i is the step change value for that factor.

Table-1. Actual and coded levels of the factors used in the experimental design.

Factor	Factor level					Unit
	-2	-1	0	+1	+2	
Temperature (T)	30	40	50	60	70	°C
Extraction time (E)	60	120	180	240	300	min
Liquid-to-solid ratio (R)	10	20	30	40	50	mL/g
Solvent composition (C)	20	35	50	65	80	% v/v

The yield of flavonoid extraction (y), expressed as the percentage amount of extracted flavonoids to the total amount of flavonoids in OP, was the response variable. Overall, the CCD consisted of 30 runs, which were conducted randomly to minimize the effects of uncontrolled factors (Table-2).

The design and analysis of experiments were performed using the statistical software Design-Expert®, version 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA).

Table-2. Experimental design layout and observed responses. x_i are the coded levels of factors, y is the flavonoid extraction yield. SO and RO are the standard and the run order of experiments.

SO	RO	x_1	x_2	x_3	x_4	y (%)
1	8	–1	–1	–1	–1	29.47
2	9	+1	–1	–1	–1	58.82
3	28	–1	+1	–1	–1	34.53
4	23	+1	+1	–1	–1	61.47
5	16	–1	–1	+1	–1	31.37
6	22	+1	–1	–1	–1	65.59
7	15	–1	–1	+1	–1	41.77
8	17	+1	+1	+1	–1	81.69
9	26	–1	–1	–1	–1	36.19
10	2	+1	–1	–1	+1	39.75
11	30	–1	–1	–1	–1	16.89
12	24	+1	+1	–1	–1	64.00
13	25	–1	–1	1	+1	35.09
14	5	+1	–1	–1	+1	43.83
15	10	–1	–1	1	+1	38.69
16	6	+1	+1	1	+1	68.16
17	12	–2	0	0	0	20.33
18	13	+2	0	0	0	90.11
19	1	0	–2	0	0	35.80
20	7	0	+2	0	0	55.81
21	27	0	0	–2	0	46.40
22	20	0	0	+2	0	60.21
23	3	0	0	0	–2	43.04
24	29	0	0	0	+2	32.95
25	18	0	0	0	0	52.29
26	14	0	0	0	0	48.92
27	19	0	0	0	0	45.21
28	21	0	0	0	0	50.90
29	4	0	0	0	0	54.07
30	11	0	0	0	0	48.79
31	8	0	0	0	0	29.47
32	9	0	0	0	0	58.82

RESULTS AND DISCUSSIONS

Characterization of OP and modelling of flavonoid extraction

The moisture content of OP was about 3% (w/w) and the flavonoid content determined by the three-stage extraction procedure was 25.28 ± 0.93 mg QE/g.

To fit the CCD data listed in Table-2, different polynomial models (linear, two-factor interaction, quadratic and cubic) were used. The best result was obtained with the 2nd-order model:

$$y = a_0 + \sum_{i=1}^{4} a_i x_i + \sum_{i=1}^{4} a_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} a_{ij} x_i x_j \quad (2)$$

where y is the process response, x_i are the coded independent variables, a_0 is the intercept and a_i, a_{ii} and a_{ij} are the linear, pure quadratic and interaction regression coefficients, respectively.

The statistically significant terms in the above equation were identified by a stepwise regression procedure, where a significance level of 0.05 was considered for deleting or adding variables. The estimated model coefficients, together with the associated standard errors and 95%-confidence intervals, are reported in Table-3 and displayed in the form of Pareto chart in Figure-2.

Table-3. Regression coefficients of the reduced polynomial model (Eq. 4) with the associated standard errors (SE) and 95%-confidence intervals (95%-CI).

Coefficient	Term	Value	SE	95%-CI	
				Low	High
a_0	intercept	50.36	1.42	47.42	53.30
a_1	T	14.95	1.23	12.41	17.50
a_2	E	4.46	1.23	1.92	7.01
a_3	R	3.86	1.23	1.32	6.40
a_4	C	–3.43	1.23	–5.97	–0.88
a_{12}	T × E	4.22	1.51	1.11	7.34
a_{44}	C × C	–3.28	1.12	–5.60	–0.96

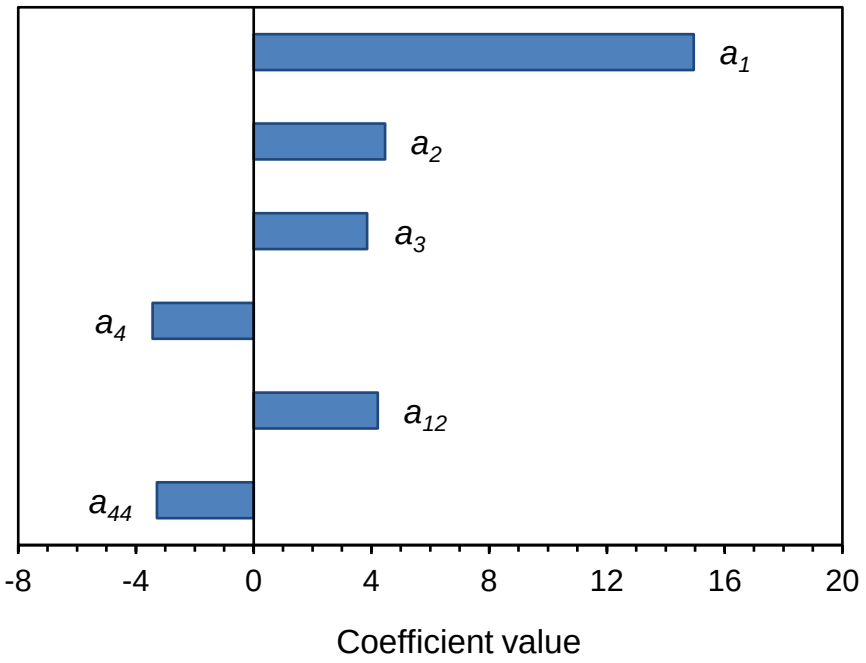

Figure-2. Pareto chart for the statistically significant model coefficients.

From the above statistical analysis, the following reduced model was derived:

$$y = 50.36 + 14.95x_1 + 4.46x_2 + 3.86x_3 - 3.43x_4 + 4.22x_1x_2 - 3.28x_4^2 \quad (3)$$

or, in terms of uncoded variables:

$$y = -11.04 + 2.28\ 10^{-1}T - 2.78\ 10^{-1}E + 3.86\ 10^{-1}R + 1.23\ C + 7.04\ 10^{-3}T\ E - 1.46\ 10^{-2}C^2 \quad (4)$$

The model provided a good fit to the data, with coefficient of determination (R^2), adjusted-R^2 and prediction-R^2 equal to 0.89, 0.87 and 0.81, respectively. A comparison between experimental and calculated extraction yields is shown in Fig. 3.

Analysis of residuals (Figure-4) indicated no apparent departures from basic ANOVA assumptions, i.e., normally distributed errors with constant variance and independent of one another. Furthermore, the lack of fit was not significant (Table-4), further supporting the adequacy of the model to describe the experimental data.

From the values of the model coefficients and from inspection of the Pareto chart, we see that:

a) the main factors temperature (T), extraction time (E), liquid-to-solid ratio (R) and solvent composition (C) were all statistically significant and their effect on flavonoid recovery increased in the order: C < R < E < T;
b) solvent composition affected the response through both a linear and a quadratic term, while a simple linear dependence was observed for the remaining factors;
c) there was a positive interaction between extraction time (T) and temperature (T), suggesting that temperature had a more pronounced effect on flavonoid recovery at higher extraction times.

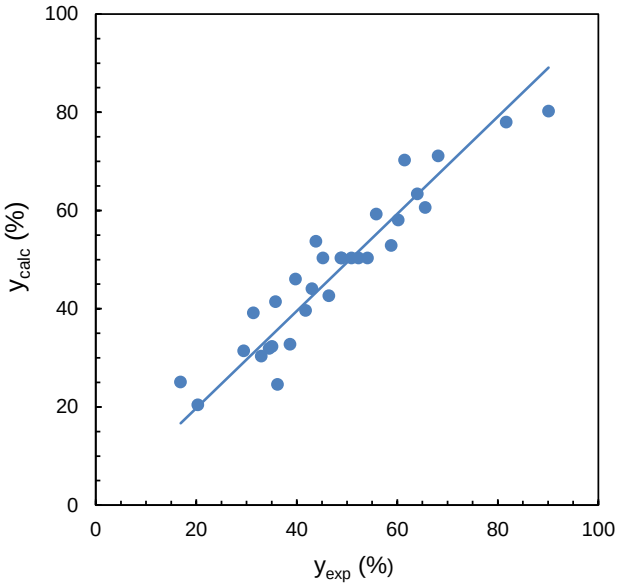

Figure-3. Comparison between experimental (y_{exp}) and calculated (y_{calc}) flavonoid extraction yields.

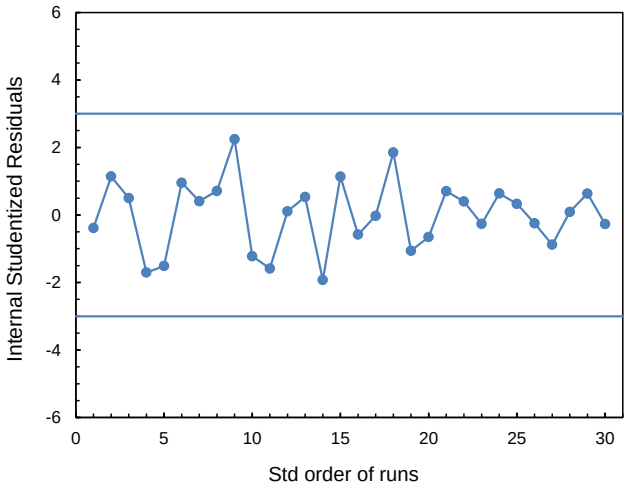

Figure-4. Internal Studentized model residuals against standard order of runs.

Table-4. Analysis of variance for the reduced polynomial model (Eq. 3). DF denotes the degrees of freedom, ΣS the sum of squares, MS the mean squares, *F* the F-value and *p* the p-value.

Source	DF	ΣS	MS	*F*	*p*
Model	6	7078.73	1179.79	32.51	<0.0001
Residual	23	834.58	36.29		
Lack-of-fit	18	786.37	43.69	4.53	0.0511
Pure error	5	48.21	9.64		
Total	29	7913.31			

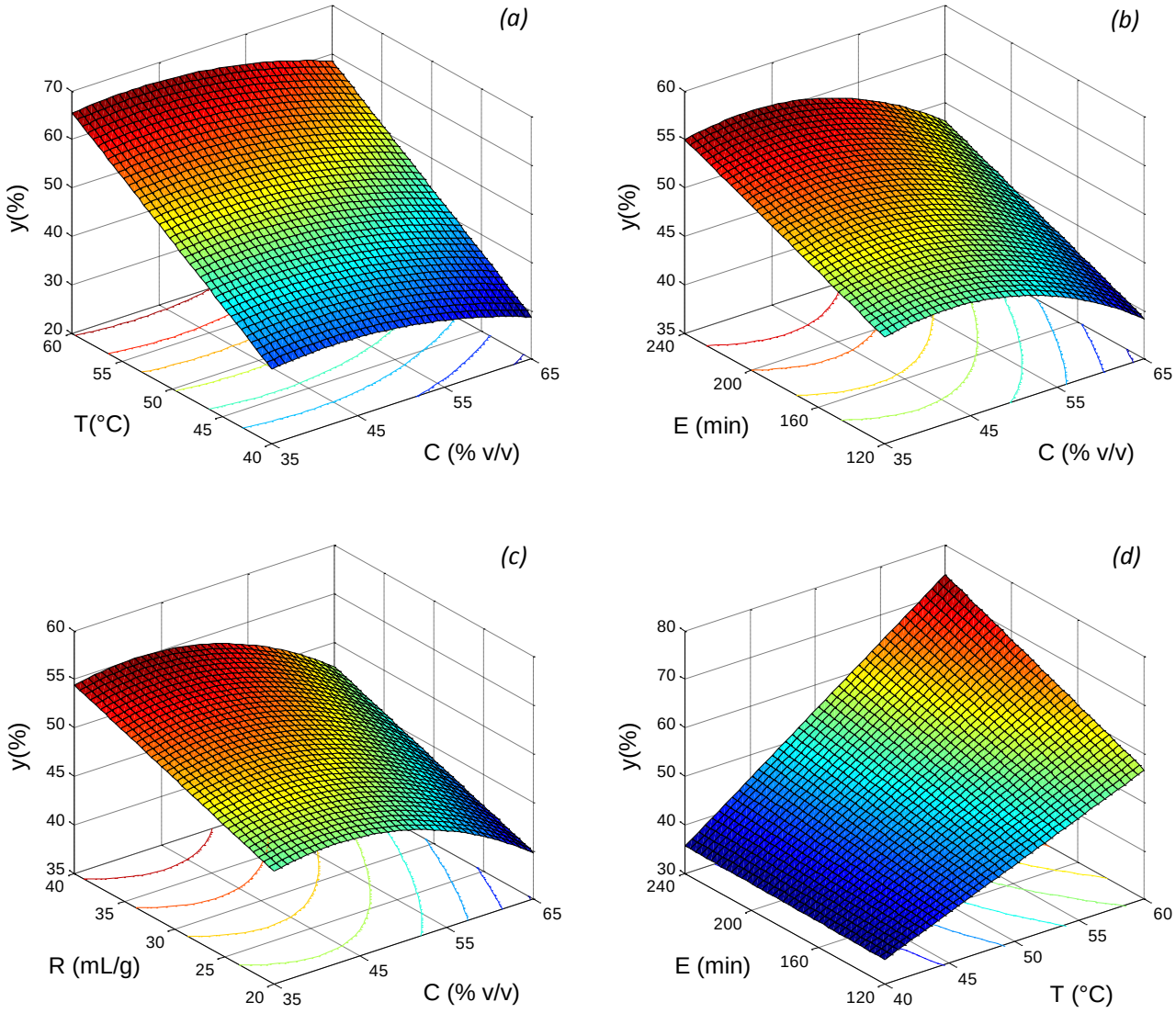

Figure-5. Response surface plots showing the effects of Temperature (T), extraction time (E), liquid-to-solid ratio (R) and temperature and solvent composition (C) on flavonoid extraction yield (*y*). For each surface plot, the levels of the other factors are held at their central values.

Analysis of response surface

Some response surface plots are shown in Figure-5. These plots were generated from Equation (4) by representing the response variable (y) as a function of two factors varying in the factorial part of the design ($-1 \leq x_i \leq +1$) while setting the others to their center-point values (T = 40 °C, E = 180 min, R = 30 mL/g, C = 50% v/v).

From Figure-5(a) and (b), the strong effect of temperature on flavonoid recovery can be easily seen. Also extraction time had a positive, though less pronounced, effect (Figure-5(b) and (d)). These effects can be explained by considering that the extraction kinetics is positively affected by temperature, mainly as a result of the thermally induced weakening of the solute-matrix interactions and/or of variations in the properties of the solvent, and that the amount of flavonoids released from OP increases with time. The positive interaction between temperature and extraction time is also evident (Figure-5(d)). In particular, in agreement with the positive value of a_{12} coefficient (a_{12} = 4.22), the influence of temperature was more significant at longer times, which can be ascribed to the fact that at these times it becomes more and more difficult to remove the residual flavonoids bound to the plant tissue.

Examination of Figure-5(a), (b) and (c) reveals the presence of an optimal solvent composition, at about 50% v/v of ethanol, at least in the factorial part of the design (C = 40–60% v/v). This result is in agreement with those of other studies on the extraction of polyphenols from plant material (Pinelo *et al.*, 2007; Panusa *et al.*, 2013; Zuorro, 2015) and can be explained by considering that the flavonoids present in OP have different affinities for ethanol and water. Accordingly, an optimal aqueous ethanol concentration can exist which maximizes the total amount of flavonoids extracted. However, it cannot be excluded that other solvent-related effects, such as a

weakening of phenolic compound-solid matrix interactions (Zuorro *et al*., 2014) or the swelling of the plant material (El Seoud, 2009), are also involved.

Finally, the positive effect of liquid-to-solid ratio (R) on flavonoid extraction (Figure-5(c)) can be attributed to the enhancement of mass transfer of released flavonoids into the solvent at higher liquid-to-solid ratios.

Optimization of flavonoid extraction

The response variable described by Eq. (4) was maximized numerically by the gradient descent method. The following result was obtained: $x_1 = 1.99$, $x_2 = 0.53$, $x_3 = 0.67$ and $x_4 = –0.42$ or, in terms of uncoded variables: T = 69.9 °C, E = 212 min, R = 36.7 mL/g and C = 43.7%. The corresponding flavonoid extraction yield was: y_{max} = 90.5%.

CONCLUSIONS

The results of this study indicate that OP from a three-phase oil production process is a rich source of flavonoids and that they can be recovered by a simple extraction procedure using aqueous ethanol as solvent. Planning the experiments according to a CCD and analysing the results by the RSM approach can help identify the effects and contribution of each process variable to the extraction efficiency. In particular, we have shown that temperature was the most influential factor, followed by extraction time, liquid-to-solid ratio and solvent composition. Optimization of the extraction process can lead to recovery efficiencies higher than 90%, further supporting the use of three-phase OP as a source of flavonoids for food, nutraceutical or cosmetic applications. Future research should be directed at analysing the recovery of flavonoids on a larger scale and at performing an accurate cost-benefit analysis. An in-depth characterization of the resulting dry extracts should also be carried out in order to identify the major flavonoid compounds and evaluate the most effective valorization strategy.

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