Optimization of Countercurrent Supercritical Fluid Extraction of Minor Components from Olive Oil

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Running Title: Optimization of CC-SFE of olive oil minor components

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ABSTRACT

A process based on the use of a semi-continuous countercurrent supercritical fluid extraction (CC-SFE) has been optimized to concentrate minor components, with functional properties, from olive oil. The optimization of the main variables involved in the supercritical extraction process (extraction pressure, temperature and sample flow rate) was performed using a surface response methodology (RSM). A central composite circumscribed design (CCCD) was employed to study the responses selected to describe the process, such as the extraction yield, the concentration of the different minor compounds (squalene, sterols and tocopherols) in the CO₂-rich upper current stream, and the recovery of these compounds from the olive oil. Parameters of the model, for each response variable, were simultaneously estimated by Partial Least Squares (PLS) regression. The statistical analysis of the results allowed obtaining mathematical models able to predict the behavior of the different responses selected as a function of the main variables involved in the process. The optimum conditions obtained, that maximized all the responses as a whole, were: extraction pressure, 234 bar; extraction temperature, 35°C and sample flow rate, 82 mL/h, working with a solvent flow rate equal to 2000 mL/h (S/F equal to 28.4 kg CO₂/kg oil). The optimum of the model was experimentally confirmed allowing around 90% recovery of squalene without significant changes on the nutritional and physicochemical properties of the oil.

Keywords: CC-SFE, nutraceutical, olive oil, optimization, RSM, squalene, sterols, tocopherols.

1. INTRODUCTION

The economic impact of the vegetable oil industry is extremely important all over the world. Spain is one of the main olive and sunflower oil producers of the world; olive oil has an extremely important socioeconomic impact being one of the most important vegetable oils consumed in the countries of the Mediterranean Basin.

In the vegetable oil industry, minor compounds (such as sterols, squalene and tocopherols) are of great interest as high added-value products because of their potential bioactivities that can help to increase the value of other food products, for example, low quality olive oil [1-3]. Squalene can be found in relatively large amounts in olive oil, representing more than 50% of its unsaponifiable fraction, and has important applications in the pharmaceutical and food industry. Its greater interest can be related to the production of functional food ingredients due to its positive impact in human health [4,5]. Tocopherols are well known as components of vitamin E, their presence has been extensively described in the olive oil [6], their interest is related to their antioxidant activity and other nutraceutical effects [7]. Also important are the effects associated to the human consumption of phytosterols, being the most important those related with cardiovascular diseases [8]. These sterols are relatively abundant in vegetable oils.

Fats and oil fractionation using supercritical carbon dioxide has been used to obtain products with improved functionality for specific applications or with better nutritional value. Among the different processes developed for vegetable oils, only deacidification has been studied in depth for olive oil [9-12]. As for minor compounds extraction from olive oil, using supercritical fluids, few studies have been conducted for example, to selectively isolate tocopherols from natural products [13-15] and squalene from deodorized distillates and olive pomace [16-18].

Previous work done in our laboratory [19-20] using a countercurrent supercritical fluid extraction column at pilot scale allowed the experimental determination of the efficiency (measured as number of transfer units (NTU) and height of a transfer unit (HTU)) and the selectivity of different random packings towards the isolation of sterols and tocopherols from olive oil. These studies were conducted to optimize, in a first step, the mass transfer of the process. Nevertheless, in order to be able to extract minor components, the concentration of these compounds in the supercritical carbon dioxide stream has to be also maximized through the study of the main variables involved in the extraction process.

Therefore, the main goal of the present work was to optimize, by means of an experimental design, the supercritical fluid extraction of minor components (squalene, sterols and tocopherols) from olive oil. The

optimization provided not only the optimum conditions for each of the responses evaluated, but also mathematical models able to properly predict the behavior of the system considering the main factors influencing the extraction process.

2. MATERIALS AND METHODS

2.1. Samples and chemicals

Spanish virgin olive oil of industrial origin and low quality (grade 2.2°) was kindly supplied by a Spanish company (Córdoba, Spain). Composition of olive oil in terms of squalene, vitamin E and sterols was: 0.458% (w/w) of squalene, 0.0205% (w/w) of vitamin E and 0.544% (w/w) of sterols. Vitamin E (97% α -tocopherol), sterols (50% β -sitosterol, 30% campesterol) and squalene (98%) were obtained from Sigma-Aldrich, St. Louis, MI, USA. Fenske rings of 3 mm of diameter (Afora, S.A., Madrid, Spain) were used as packing material for the countercurrent column. CO₂ N-38 (99.98%) was kindly supplied by AL Air Liquide España S.A. (Madrid, Spain).

2.2. Countercurrent supercritical fluid extraction (CC-SFE)

The countercurrent CC-SFE system used in this study has been described elsewhere [20]. The countercurrent SFE liquid feed pilot plant has the following features: a countercurrent extraction column (316 stainless steel, 17.6 mm. i.d. and 180 cm. length) with three levels of sample introduction (top, medium and bottom) and packed with 3 mm Fenske rings, two separator cells (270 mL capacity each), and a cryogenic trap at atmospheric pressure. During the extraction, a continuous flow of CO₂ was introduced into the column, through the bottom side. When the operating pressure and temperature were reached (approximately after 30 minutes), liquid sample was pumped at the selected flow rate during all the extraction time. The liquid sample introduction was carried out through the middle point of the packed column, located over the inlet of the CO₂, creating a countercurrent between the flow of sample (downwards) and the CO₂ flow (upwards). Based on previous work done in our laboratory [20], a solvent flow rate (CO₂) as low as possible (2000 mL/h) was selected to improve the performance of the system. The sample flow rate was optimized by including the factor in the experimental design along with the extraction conditions (extraction pressure and temperature). Cascade fractionation was achieved by setting pressures in separators 1 and 2 equal to 50% of the extraction pressure in the column and 20 bar, respectively. Temperatures in separators 1 and 2 were fixed to extraction temperature and 20 °C,

respectively. The total extraction time was 40 min for each experiment; the extractions were performed in duplicate.

2.3. Experimental Design for CC-SFE

The optimization of the CC-SFE process was carried out via the use of a Central Composite Circumscribed design (CCCD). A total of 18 experiments: 2^3 points of a full factorial design, $2 \cdot 3$ star points ($\alpha = 1.682$ distance) and 4 center points, were carried out in randomized run order. The variables selected for the optimization were: extraction pressure (P), extraction temperature (T) and sample flow rate (F). By using the above mentioned design, the variables were tested at 5 different levels; extraction pressure ranged from 65.9 (subcritical conditions) to 234.1 bar, extraction temperature from 24.8 (subcritical conditions) to 75.2 °C and olive oil flow rate from 81.8 and 418.2 ml/h. Levels of the different variables were selected based on previously published studies [9, 12]. The factor levels (coded values) and the physical values are shown in Table 1.

Table 1. Experimental and coded levels of the factors in the CCCD.

Independent Variable	Level				
	-1.682	-1	0	+1	+1.682
Pressure (P) (bar)	65.9	100	150	200	234.1
Temperature (T) (°C)	24.8	35	50	65	75.2
Sample Flow rate (F) (mL/h)	81.8	150	250	350	418.2

The responses considered to evaluate the countercurrent supercritical fluid extraction of minor components with bioactive properties from olive oil were the following: extraction yield (Y_1) (g extract / 100 g oil), amount of material extracted (as g extract / kg CO₂) (Y_2), amount of squalene extracted (as g squalene / kg CO₂) (Y_3), amount of tocopherols extracted (as mg tocopherols / kg CO₂) (Y_4), amount of sterols extracted (as g sterols / kg CO₂) (Y_5), % recovery of squalene (Y_6), % recovery of tocopherols (Y_7) and % recovery of sterols (Y_8).

Table 2 shows the experimental matrix design, with the experimental levels of the independent variables (factors), along with the results obtained for each one of the response analyzed variables.

127 Table 2. Experimental matrix design and results obtained for each one of the response variables.

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Experiment Number	Factor			Response variable							
	P	T	F	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅	Y ₆	Y ₇	Y ₈
1	100	35	150	0.492	0.317	0.015	0.134	0.003	5.086	1.516	0.784
2	200	35	150	3.936	2.537	0.14	0.694	0.021	47.523	7.871	6
3	100	65	150	0	0	0	0	0	0	0	0
4	200	65	150	2.563	1.652	0.101	0.524	0.014	34.167	5.952	4.098
5	100	35	350	0.359	0.539	0.031	0.197	0.004	4.497	0.959	0.54
6	200	35	350	1.845	2.775	0.117	0.752	0.023	16.963	3.657	2.825
7	100	65	350	0	0	0	0	0	0	0	0
8	200	65	350	1.214	1.827	0.113	0.557	0.016	16.392	2.707	1.925
9	65.9	50	250	0	0	0	0	0	0	0	0
10	234.1	50	250	2.938	3.157	0.166	0.771	0.024	33.749	5.248	4.081
11	150	24.8	250	1.595	1.714	0.082	0.56	0.016	16.706	3.817	2.69
12	150	75.2	250	0.269	0.289	0.016	0.096	0.002	3.34	0.655	0.417
13	150	50	81.8	1.971	0.694	0.056	0.273	0.006	34.886	5.671	3.369
14	150	50	418.2	0.614	1.103	0.075	0.369	0.01	9.125	1.505	0.987
15	150	50	250	0.972	1.044	0.072	0.379	0.009	14.638	2.578	1.604
16	150	50	250	0.934	1.004	0.068	0.361	0.009	13.9	2.46	1.491
17	150	50	250	1.237	1.329	0.082	0.441	0.012	16.739	3.001	2.027
18	150	50	250	0.843	0.905	0.063	0.355	0.008	12.834	2.418	1.388

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The quadratic model for each response variable (Y_i) was:

$$Y_i = \beta_0 + \beta_1 P + \beta_2 T + \beta_3 F + \beta_{11} P^2 + \beta_{22} T^2 + \beta_{33} F^2 + \beta_{12} P \cdot T + \beta_{13} P \cdot F + \beta_{23} T \cdot F + \varepsilon \quad (1)$$

where β_0 is the intercept; β_1 , β_2 and β_3 the linear coefficients; β_{11} , β_{22} and β_{33} the squared coefficients; β_{12} , β_{13} and β_{23} the interaction coefficients; and ε is the error variable, being the part of the variable that cannot be explained by the selected model. The parameters of the model, for each response variable, were estimated simultaneously by Partial Least Squares (PLS) [21] regression using the program MODDE 5.0, a Software for Design of Experiments and Optimization (Umetrics AB, Umeå, Sweden) [22]. This program allows both the creation and analysis of experimental designs. The number of significant PLS components was determined by cross-validation from the Predictive Residual Sum of Squares (PRESS) computed for each model dimension. The program automatically selects the number of PLS dimensions that gives the smallest PRESS.

2.4. LC analysis

A Varian Prostar series HPLC (USA) equipped with photodiode array detector was used for the analysis of minor components in the olive oil and in the SFE extracts. The separation column was a Kromasil C4 (Hichrom, UK) of 25 cm x 10 mm and 10 μ m particle diameter. The samples were injected with no previous treatment into the 20 μ L injection loop of the HPLC system. The mobile phase was 100% methanol at a flow rate of 4 mL/min over 30 min. Identification of compounds was achieved by comparing their retention time values and spectra with those of standards. For quantitative analysis, calibration curves were prepared by analyzing different concentration of tocopherols, sterols and squalene standards and by representing peak area (counts) vs concentration (% p/p); correlation coefficients were > 0.995 in all cases. Detection for vitamin E, sterols and squalene was performed at the maximum in the UV spectra, that is, 296 nm, 205 nm and 225 nm, respectively.

3. RESULTS AND DISCUSSION

As mentioned in the Experimental Section, several responses have been selected to describe the extraction process; among them, Y_1 and Y_2 give information about the extraction efficiency of the countercurrent system being Y_1 the extraction yield, obtained as total weight of extract per 100 g oil, and Y_2 the maximum amount of material extracted per kg of CO₂ at the extracting conditions, while the rest of the

responses provide information about the selectivity of the process towards the extraction of the minor compounds of interest found in the olive oil, such as squalene, tocopherols and sterols. The effect of the extraction conditions was evaluated by using two different responses such as the amount of each compound dissolved in CO₂ (g of minor compound per kg CO₂) and the recovery of component i, which is obtained through the equation (2):

$$\% \text{Recovery}_i = \frac{S_1 X_{1i} + S_2 X_{2i}}{F X_{Fi}} \times 100 \quad (2)$$

where S₁ and S₂ are the weight of extract obtained, respectively, in separators 1 and 2, and F is the weight of olive oil feed into the system. X_{1i}, X_{2i} and X_{Fi} correspond to the weight fraction of component i in the extracts (separators 1 and 2) and feed oil, respectively. Responses related to equation (2) provide a very complete evaluation of the process because include terms of extraction yield (S₁/F and S₂/F) and terms of enrichment of components vs feed introduced in the system (X_{1i}/X_{Fi} and X_{2i}/X_{Fi}).

As mentioned above, Table 2 shows the value responses obtained for all the experiments corresponding to the matrix design. A PLS regression was applied to estimate the parameters of the model in the equation (1) using the two significant components determined by cross-validation from the PRESS values. A summary of these results is shown in Figure 1, where the regression coefficient values for centered and scaled factors (coded factors) are shown as bar graphs for all the responses considered. In the plot, to be able to compare the coefficients, between responses, the corresponding values are normalized by dividing them by the standard deviation of their corresponding responses.

Figure 1. Plot of normalized regression coefficient values, for centered and scaled factors, obtained from PLS with two components, for the eight response variables (Y) studied.

Figure 1 shows the importance of the different terms in the model for each of the responses evaluated. As can be seen, pressure (P) has the strongest influence in the extraction process; as expected, an increase in the extraction pressure provides an important increase in the extraction yield and in the amount of extracted material (and consequently in the recovery) due to an increase in the CO_2 density. As expected, the responses from Y_2 to Y_5 are only function of pressure and temperature, being the effect to the temperature negative, that is, an increase of temperature lowers the solvating power of the CO_2 , thus lowering the density and the possibility of solubilizing certain compounds.

By comparing the diagrams of Figure 1, it can easily be seen that, when considering the responses Y_1 , Y_6 , Y_7 and Y_8 , the profile of the factors that mainly influence the responses is quite similar; this behavior seems reasonable because all of them include extraction yield, and therefore, the influence of the sample flow rate is easily justifiable. The highest yields are obtained when working at low flow rate (high solvent-to-feed ratios, S/F) because the extraction process has the limitation of the solvating power of the CO_2 at the extracting conditions; the use of high sample flow rates does not imply an improvement of extraction yield but only a saturation of the CO_2 .

For the responses dealing with extraction yield (Y_1) and % recovery of minor compounds (Y_6 to Y_8), there is also a significant interaction between pressure and sample flow rate ($P \times F$). Operating at high pressure (level +1) provides the highest yield when the sample flow rate is lower (-1).

212 The statistical significance of the estimated regression coefficients (for scaled and centered factors) along
 213 with the error bars (at 95% confidence level) for each of the response variables is shown in Figures 2 and
 214 3.

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 216 Figure 2. Estimated regression coefficients obtained from PLS, for centered and scaled factors, with error
 217 bars (at 95% confidence level) for the response variables Y₁ to Y₄.
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 220 Figure 3. Estimated regression coefficients obtained from PLS, for centered and scaled factors with error
 221 bars (at 95% confidence level) for the response variables Y₅ to Y₈.

By analyzing the Figures, the statistical importance of the factors P, T, F and P x F could be determined for most of the response variables studied; also, the no significance of other factors, such as T^2 , T x F and y P x T could be observed as well. These terms, no significantly different from zero, were excluded of the model and the PLS regression model was refitted. The results obtained using two significant components determined by cross-validation are listed in Table 3. These results include, for each response variable, the following information: the regression coefficients obtained, for unscaled factors (real values of the factors), the determination coefficient (R^2), the prediction ability of the model (Q^2), the residual standard deviation (RSD) and the P-values from the lack of fit for the model. Table 3 also includes the variable importance of the projection (VIP) values, which reflect the importance of the terms in the quadratic model analyzing all the responses as a whole. In the analysis of the results, R^2 and Q^2 values were used; these parameters provide the best summary of the fit of the model. R^2 represents the fraction of variation of the response explained by the model while Q^2 , which is calculated from PRESS value (mentioned in the Experimental Section), represents the fraction of variation of the response variable that can be predicted by the model, that is, a measure of how well the model will predict the responses for new experimental conditions within the range analyzed. Q^2 equal to 0.7 or larger indicates that the model has good predictive ability and will have small prediction errors.

Table 3. Regression coefficients, for unscaled factors, and statistics for the fit, obtained from PLS regression with 2 significant components, for each of the responses studied.

	Response variable								VIP
	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅	Y ₆	Y ₇	Y ₈	
Constant	-1.12175	-0.00043673	-0.00120655	0.0408077	0.000511428	-15.6047	-2.30096	-1.72357	
P	0.0303873	0.00339033	0.00037424	0.00121206	2.50525e-05	0.455779	0.0725197	0.0488488	2.126
T	-0.0244271	-0.0248128	-0.00125703	-0.00649829	-0.00020161	-0.273554	-0.0463902	-0.0362378	0.789
F	0.000989736	0.00410446	0.000185096	0.00103837	3.36313e-05	-0.00194093	0.000209202	0.00104318	0.721
P ²	3.02172e-05	5.09725e-05	2.43285e-06	1.31101e-05	4.16007e-07	0.000253171	4.64563e-05	4.20459e-05	0.225
F ²	1.25507e-05	-7.18388e-06	-2.17049e-07	-1.64625e-06	-6.01809e-08	0.000224325	3.45793e-05	2.13532e-05	0.246
P x F	-7.65116e-05	3.37795e-06	-4.26502e-07	-7.20773e-08	3.48105e-08	-0.00119767	-0.00018902	-0.00012463	0.477
R ²	0.964	0.978	0.949	0.966	0.970	0.953	0.967	0.964	
Q ²	0.844	0.889	0.800	0.885	0.884	0.811	0.833	0.849	
RSD	0.259	0.176	0.0139	0.0577	0.0017	3.721	0.513	0.395	
P-value (lack of test)	0.210	0.598	0.154	0.235	0.580	0.074	0.115	0.260	

R²= Determination coefficient; Q²= Predicted ability of the model; RSD=residual standard deviation; VIP= variable importance in the projection

From the results shown in Table 3, the following conclusions can be drawn: all estimated models were found to adequately describe the data (P-values of lack of fit > 0.05); from VIP values, the most important terms that influence the extraction process (and the selective extraction of minor components from olive oil using supercritical fluids) are, in decreasing order of importance P, T, F, $P \times F$, F^2 and P^2 ; the fraction of variation of the response explained by the model (R^2) was > 0.94 for all the responses; the predictive ability of the estimated model (Q^2) was > 0.80 in all cases.

Figures 4 and 5 show the contour plots for the 8 responses evaluated, as a function of pressure (P) and sample flow rate (F) and considering an extraction temperature equal to 35°C. As mentioned before, the higher values for the different responses were found working, within the experimental region studied, at maximum pressure and minimum temperatures and sample flow rates. A temperature of 35°C was selected because it was close to the minimum but slightly higher than the critical temperature for CO₂ that can favor the mass transfer properties of the extraction process. The statistical program provided the following optimum for all the responses: pressure equal to 234.1 bar, temperature equal to 35°C and sample flow rate equal to 81.8 mL/h.

Figure 4. Contour plots for the responses Y_1 to Y_4 , as a function of pressure (P) and sample flow rate (F). Extraction temperature equal to 35°C.

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265 Figure 5. Contour plots for the responses Y₅ to Y₈, as a function of pressure (P) and sample flow rate (F).

266 Extraction temperature equal to 35°C.

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268 Table 4 shows the predicted values (using the fitted models) for the eight responses studied at the

269 optimum conditions along with the lower and upper values estimated using 95% of confidence level. As

270 can be seen, extraction yields around 5% could be obtained while recoveries for the minor compounds

271 with nutraceutical properties from the olive oil were estimated as high as 73.8% for squalene, 12.2% for

272 tocopherols and 8.6% for sterols.

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Table 4. Predicted values for the eight responses studied at the optimum conditions along with the lower and upper values for 95% confidence level.

Response variable	Predicted	Lower value	Upper value
Y ₁	5.49	4.63	6.36
Y ₂	3.07	2.48	3.66
Y ₃	0.18	0.14	0.23
Y ₄	0.89	0.70	1.08
Y ₅	0.03	0.02	0.03
Y ₆	73.80	61.37	86.23
Y ₇	12.23	10.51	13.94
Y ₈	8.59	7.27	9.91

The optimum of the model was experimentally confirmed by performing 4 extractions at the selected optimum conditions. Table 5 shows the experimental values obtained along with the relative standard deviation for the 4 extractions. By comparing the results predicted by the models (Table 4) with the experimental values achieved, it can be concluded that for all the responses, except for those related to the tocopherol content, the experimental values obtained are in the range predicted by the fitted model. It is important to emphasize the high recovery achieved for squalene that reached values close to 90% while the extraction yield was also found more than 7%. As for the responses related to the tocopherol content, the low values obtained during the verification of the optimum were due to a degradation of the tocopherols in the original olive oil; this fact was associated to the storage conditions that oxidized the olive oil sample, and therefore, decreased the tocopherol content in the olive oil to be processed (from 0.0205% w/w to 0.013% w/w).

Table 5. Experimental values at the optimum conditions, obtained for the different responses studied.

Response Variable	Experimental Values	Relative Standard Deviation
Extraction yield (Y ₁)	7.26	0.45
g extract/kg CO ₂ (Y ₂)	2.56	0.16
g squalene/kg CO ₂ (Y ₃)	0.14	0.02
mg tocopherols/kg CO ₂ (Y ₄)	0.15	0.03
g sterols/kg CO ₂ (Y ₅)	0.017	0.002
% Recovery of squalene (Y ₆)	89.19	11.73
% Recovery of tocopherols (Y ₇)	3.25	0.58
% Recovery of sterols (Y ₈)	8.73	0.97

Therefore, results presented demonstrate the ability of a RSM to optimize the extraction of valuable minor compounds from low quality olive oil by using a CC-SFE process. Mathematical models provided were able to properly predict the behavior of the system considering the factors that mostly influence the countercurrent supercritical extraction process.

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FIGURE LEGENDS

Figure 1. Plot of normalized regression coefficient values, for centered and scaled factors, obtained from PLS with two components, for the eight response variables (Y) studied.

Figure 2. Estimated regression coefficients obtained from PLS, for centered and scaled factors, with error bars (at 95% confidence level) for the response variables Y1 to Y4.

Figure 3. Estimated regression coefficients obtained from PLS, for centered and scaled factors with error bars (at 95% confidence level) for the response variables Y5 to Y8.

Figure 4. Contour plots for the responses Y1 to Y4, as a function of pressure (P) and sample flow rate (F). Extraction temperature equal to 35°C.

Figure 5. Contour plots for the responses Y5 to Y8, as a function of pressure (P) and sample flow rate (F). Extraction temperature equal to 35°C.