The Supercritical carbon dioxide extraction of ω-3, ω-6 lipids and β-sitosterol from Italian walnuts: a central composite design approach

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ARTICLE INFO

Article history:
Received 30 November 2016
Received in revised form 22 February 2017
Accepted 23 February 2017
Available online 16 March 2017

Keywords:
Walnut oil
Phytosterols
PUFA
MUFA
Supercritical fluids extraction
Central composite design

ABSTRACT

A central composite design approach was used to optimize the supercritical carbon dioxide ($SCCO_2$) extraction of Italian Walnut oil, considering the effect of pressure (from 82.3 to 317.6 bar), temperature (from 309.7 to 326.5 K) and process time (from 1.5 to 6.5 h) on extraction yield, fatty acid composition and β-sitosterol content. A multiple regression analysis indicated pressure and time as major parameters influencing the extraction yield. Those parameters also induced changes in the extract compositions giving the opportunity to obtain enriched fractions of polyunsaturated fatty acid (PUFA).

Increased PUFA yields were obtained with higher pressure values but, for longer process times, extracts stated augmented amounts of saturated fatty acids.

The different solubility behavior of PUFA and other lipids in $SCCO_2$ could be exploited for the production of walnut oil fractions with high PUFA contents that can be considered as starting materials for the development of food supplements or functional foods.

1. Introduction

Walnut seeds are a good source of nutrients, particularly proteins and unsaturated fatty acids. They contain relatively high amount of oil (52–70%) with valuable nutritional properties due to the peculiar fatty acid composition characterized by the presence of monounsaturated, as oleic acid, and poly unsaturated fatty acids (PUFA) [1]. Oil composition is related to genetic, regional and seasonal variations but accepted medium values of those fatty acids are about 50% of linoleic, 20% of oleic acid and 10% of linolenic acid [2]. A further class of constituents with valuable nutritional and health promoting properties are phytosterols, mainly the β-sitosterol (representing more than the 85% of total walnut oil sterols), and campestrol (around 5%) [1,3]. Polyphenols are present in the walnut fruit as tannins, that are mainly concentrated in the epicarp and pericarp but, due to their medium polarity nature, only trace levels are detectable in oil [4]. Thus, vegetable oils with high content of unsaturated fatty acids and phytosterols may have applications in functional foods and/or in food supplements area due to their health promoting effects. Recently, there has been interest in the use of continuous, mechanical screw presses to recover oil from oilseeds, but screw pressing will not extensively replace solvent extraction in commodity oilseeds. The main reason is the lower proportion of collected oil. Benefits of screw pressing is providing a simple and reliable method of processing small batches of seed [5]. Conventional solvent extraction produces low-quality oil that requires extensive purification operations while screw pressing does not require the use of organic solvent and is able to retain bioactive compounds such as essential fatty acids, phenolics, flavonoids and tocopherols in the oils [6].

Oil extraction using supercritical fluids is an alternative method to replace conventional industrial process, such as pressing and solvent extraction, as it offers a number of advantages, including the absence of solvent residue and better retention of aromatic compounds [5,7–9]. Supercritical fluids provide high solubility and improved mass transfer rates and the extraction process can be easily manipulated by changing the main operative parameters.

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http://dx.doi.org/10.1016/j.supflu.2017.02.020

Carbon dioxide, as an environmentally friendly solvent, is the mainly used supercritical extraction agent. Extracts obtained using supercritical carbon dioxide ($SCCO_2$) are solvent-free, without any trace of toxic extraction solvents, and are thereby highly valued.

With the purpose of the optimization of the process control, the Response Surface Methodology (RSM) can evaluate the influence of multiple variables (pressure P, temperature T and time t) on output results: this approach was successfully employed to evaluate the best $SCCO_2$ extractions conditions [10–12].

RSM is a collection of mathematical and statistical approaches based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical previsions. It can be well applied when a response or a set of responses of interest are influenced by several variables. The objective is to optimize simultaneously the levels of these variables to attain the best system performance. Before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in the experimental region being studied [13–16].

In this paper, the $SCCO_2$ extraction of walnut oil was optimized by using Central Composite Design (CCD), considering both the physical parameters of extraction and the chemical composition of the obtained products.

Then chemical composition and yield of oils obtained by mechanical press were compared with the chemical profiles and yields of $SCCO_2$ extracts.

Modification in fatty acid composition and in β-sitosterol content were studied, with relationship with $SCCO_2$ extracting conditions, offering the opportunity to obtain enriched fractions with high amount in valuable compounds.

2. Material and methods

2.1. Material

Italian Walnuts were kindly supplied by SAM s.s (Sospirolo – Belluno – Italy), CO_2 (99% purity) was purchased by SIAD (Trieste – Italy) and methanol, ethanol and *n*-hexane of analytical grade were provided by Sigma Aldrich.

2.2. Method

2.2.1. Material preparation

Bulk walnuts were shelled and put in the oven at 40 °C for 15 h to gently remove the humidity, without altering the components composition. Then, the walnuts were milled by a knife mill and sieved with a 1400 μm mesh in order to homogenize the starting material and to increase its surface area.

2.2.2. Mechanical extraction

The mechanical extraction was performed by a manual hydraulic press at pressure of 3 tons for 4 h at room temperature. The oil was filtered through a gauze to avoid solid particle intrusion.

2.2.3. Supercritical extraction

The extraction process was operated using a continuous flow apparatus described previously [10]. Briefly, the extraction of the samples with $SCCO_2$ was conducted on a Separex (F) extraction equipment, connected to a high pressure pump, at predetermined pressures and temperatures. In each experiment, a sample of about 19 g was loaded into a 100 cc stainless steel extraction vessel. During the extraction process, sample was soaked in $SCCO_2$ for 30 min and extracted, for different times, at 2 L/min, measured by a flow meter at room conditions of pressure and temperature. The extract was recovered in a collecting chamber at 50 bar, to avoid the volatilization of the more volatile substances. At the end, the extract was accurately weighted after a gradual and slow depressurization.

Table 1
Coded and uncoded levels of independent variables.

Independent variables	Coded levels				
	$-\alpha$	−1	0	1	α
x_1: P (bar)	82.3	130	200	270	317.6
x_2: T (°C)	36.6	40	45	50	53.4
x_3: t (h)	1.5	2.5	4	5.5	6.5

2.2.4. FAME and β-sitosterol GC–MS analysis

GC–MS analyses were performed with a Varian 3800 gas chromatograph, equipped with auto-sampler (model 9800) and a Saturn 2100 Ion Trap Mass spectrometer. Mobile phase was Helium, and ion trap temperature was kept at 210 °C. Injector was set at 220 °C and mass spectra were acquired in the range 40–650 Da.

FAME (Fatty Acid Methyl Ester) were prepared from obtained oils with the protocol of Indarti et al. [17] with slight modifications. Briefly, triplicate oil samples of 50 mg, placed in round bottomed flasks, were accurately weighed using a balance (Mettler Toledo Classic AB204-S). Methylpendatecanoate (Sigma Aldrich) solution in hexane (10 mg/mL) was added as internal standard. Dichloromethane, methanol and sulfuric acid (10:30:0.5 v/v) were then added and the mixture was refluxed for 30 min. After, the resulting liquid was transferred to flasks kept in ice bath and containing water and diethyl ether. Flasks were vigorously shaken and the upper layer was collected, dried on anidrous sodium sulphate (Sigma Aldrich) and used for GC–MS analysis.

Samples for β-sitosterol determination were prepared by saponification in NaOH (3 M) for 30 min: liquid was extracted with diethyl ether and used for GC–MS analysis.

Agilent HP-88 (0.25 × 60 m) and HP-5 (0.25 × 30 m) columns were used for fatty acid and β-sitosterol assays respectively. For quantitative purposes, calibration curves were obtained using methylpentadecanoate and 5α-cholestane as internal standard for fatty acid and β-sitosterol analysis, respectively. Identification of fatty acid was achieved by standard references compounds and by comparison of the MS spectra obtained with NIST 2012 library of the instrument.

2.2.5. Design of experiments and statistical analysis

The Design of Experiments (DoE) purpose is the selection of the points where the response should be evaluated. In this study a five-level central composite rotatable design (CCD), with n (= 3) factors, requiring 19 experiments, was used. It was structured in 8 (2n) experimental points and 6 (2n) star points (extreme values), with an axial distance of 1.6818 ($\alpha = 2^{n/4}$) to allow the estimation of the curvature. The central point of the design was than replicated 5 times for the determination of the experimental error [14–16].

In Tables 1 and 2 extraction conditions and planned experiments are reported with coded variables. It is important to remark that the 19 experiments were conducted in a total random way: so, for example, extraction 1 was the last Run (19) done and extraction 15 was the first one.

The Response Surface Methodology, with the chosen DoE, was used to simultaneously optimize the three factors x_1 (pressure), x_2 (temperature) and x_3 (time), to attain the best system performance in terms of extract yield (Y). Then, the latter was assumed to be affected by the independent variables P, T and t.

The experimental yield, after mechanical and $SCCO_2$ extraction, was evaluated as follow:

$$Y = \frac{\text{weight}_{\text{extract}}}{\text{weight}_{\text{starting material}}} \cdot 100 \quad (1)$$

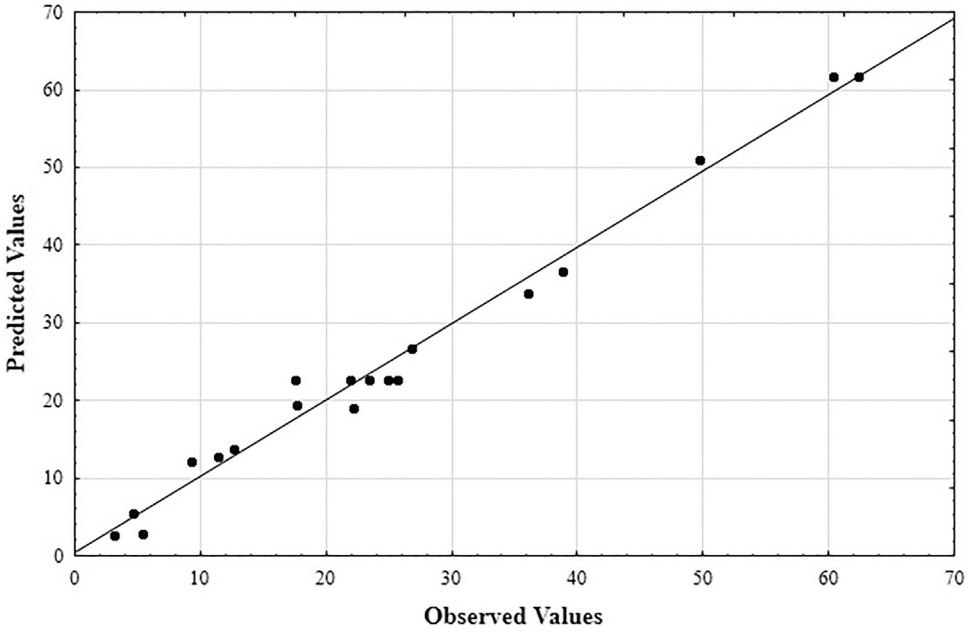

Fig. 1. Predicted vs observed values (y%).

Table 2
Central composite design experimental results.

No.	Run	x_1 (P)	x_2 (T)	x_3 (t)	P (bar)	T (°C)	t (h)	y%
1	19	−1	−1	−1	130.0	40.0	2.5	4.94
2	15	−1	−1	1	130.0	40.0	5.5	9.25
3	18	−1	1	−1	130.0	50.0	2.5	3.13
4	12	−1	1	1	130.0	50.0	5.5	4.68
5	11	1	−1	−1	270.0	40.0	2.5	26.78
6	2	1	−1	1	270.0	40.0	5.5	49.68
7	5	1	1	−1	270.0	50.0	2.5	36.09
8	4	1	1	1	270.0	50.0	5.5	62.37
9	16	−1.68	0	0	82.3	45.0	4	5.42
10	17	1.68	0	0	317.6	45.0	4	60.33
11	6	0	−1.68	0	200.0	36.6	4	22.13
12	7	0	1.68	0	200.0	53.4	4	17.65
13	13	0	0	−1.68	200.0	45.0	1.48	12.71
14	10	0	0	1.68	200.0	45.0	6.52	38.85
15	1	0	0	0	200.0	45.0	4	17.52
16	9	0	0	0	200.0	45.0	4	23.39
17	14	0	0	0	200.0	45.0	4	24.9
18	8	0	0	0	200.0	45.0	4	21.95
19	3	0	0	0	200.0	45.0	4	25.68

Then, the experimental data were fitted with a second-order polynomial equation (RSM approach):

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i x_i + \sum_{i=1}^{3} \alpha_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} \alpha_{ij} x_i x_j \qquad (2)$$

where α_0 is the constant and α_i, α_{ii}, and α_{ij} are the linear, quadratic and interactive coefficients, respectively. x_i and x_j are the factors related to P, T and t. The regression values were calculated using Matlab R2010a.

3. Results and discussions

Analyses by multiple regression were performed by using the experimental yields, reported in Table 2, to fit Eq. (2) and to identify the constant, the variable coefficients and the linear effect of variables as well as their quadratic and interaction effects by using the Student's '*T*' test and *p*-values. A small *p* value (typically ≤0.05) indicates significant model terms.

By the three independent operative conditions, only Pressure ($p = 5.77E{-}9$) and time ($p = 1.36E{-}5$) have a significant linear effect on the yield (pressure had also a quadratic effect *7.64E−3*), whereas both linear and quadratic temperature effects result to be less important with p values of 0.50 and 0.08, respectively.

By fitting equation 2, the following equation was obtained:

$$Y = 22.73 + x_1 34.99 + x_1 x_1 6.80 + x_2 0.24 - x_2 x_2 2.40 + x_3 13.56 + x_3 x_3$$
$$1.77 + x_1 x_2 8.70 + x_1 x_3 12.44 + x_2 x_3 1.76 \qquad (3)$$

In Fig. 1 the goodness of fit (GOF) is reported: the R^2 value of 0.984, indicates that over 98% of the variability in oil extract yield can be satisfied by this equation.

In Fig. 2, the response surfaces of walnut oil extract yields, generated by Eq. (3) are reported. The obtained surfaces indicatea yield extraction enhancement for pressure and time increases, while the yield variation seems to be only slightly temperature dependent.

For a single solute, higher values of pressure at isothermal conditions result to an increase of the solvent density, determining the

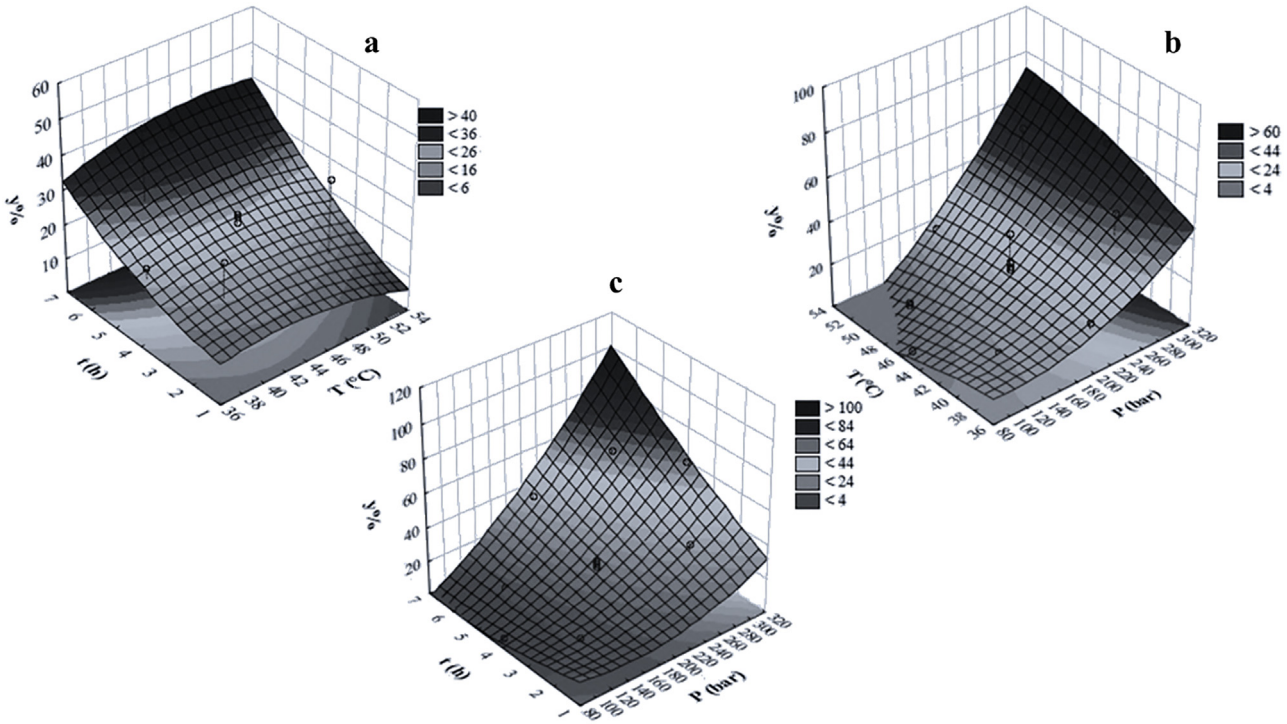

Fig. 2. Profiles of the generated surfaces: a) Temperature (T) vs Time (t), b) Pressure (P) vs Temperature (T), c) Pressure (P) vs time (t).

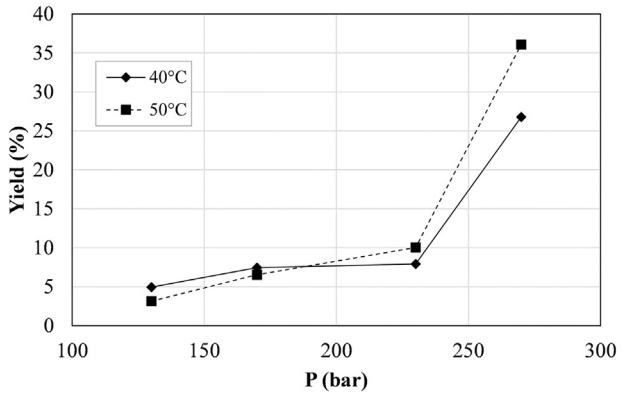

Fig. 3. Runs at 40 and 50 °C and 2.5 h of extraction time.

rise of both the solvent power and the solubility of the compound. On the other hand, at a given pressure the supercritical CO_2 density decreases with the temperature rise: usually, it implies the reduction in the CO_2 solvation power and thus the reduction in solubility, if the volatility of the solute is not very high.

However, the extraction process from a complex matrix, as is the case of natural products, is much more complicated and several factors simultaneously affect the process. Therefore, as reported in Table 2, rises of pressure and extraction time always increase the extraction yield of the walnut oils, whereas the temperature has a negative influence at lower pressures and a positive effect at higher pressures (Fig. 2b).

To verify last statement other four extractions of 2.5 h, at intermediate pressure of 170 and 230 bar and temperatures of 40 and 50 °C, were performed. The results are reported in Fig. 3 with the yields of extractions 1, 3, 5 and 7 of Table 2: in Fig. 3 it is perceptible a cross over point of the two isotherms before 200 bar.

3.1. *Quali-quantitative composition of walnut oil SCCO$_2$ extracts*

Table 3 reports the quali-quantitative compositions of the $SCCO_2$ walnut oil extracts related to the DoE central point (200.0 bar, 45 °C, 4 h), as the average data of the five extractions n. 15–19 of Table 2, and to the higher pressure DoE star point (317.6 bar, 45 °C, 4 h), extraction n. 10 of Table 2. For the DoE central point the obtained average percent yield was of 22.69 ± 3.22 SD, whereas the yield value of the star point was of 60.33%, as reported in Table 2. Both extracted oils present similar quantitative compositions, excluding the lower amounts of the Margaric Acid (1.8% at 200.0 bar and 0.2% at 317.6).

In Table 3 the fatty acid and the β-sitosterol contents, of the supercritical extracted oils, are compared with oil compositions derived by cold mechanical pressing, of Italian walnut reported in the study of Crews et al. [1] and the value of the oil analyzed in this work (MP oil). The latter presents levels of oleic (10%) and palmitic (6%) acids comparable to those of literature, whereas differences are related to the higher contents of palmitoleic acid (10.5% in MP oil and non-detectable in the Crews' analyzed samples), linolenic acid (24% compared to 12–14%) and to the lower amount of linoleic acid (48% in MP oil compared to 60–63%). The $SCCO_2$ (at 200.0 and 317.6 bar) walnut oil amounts of PUFA (Linolenic and Linoleic acids) are comparable with those reported by the MP oil, with a total concentration of about 73%. Differences are related to the higher contents of total saturated acids, with a value of 7.7%, measured for the MP oil, compared to the concentrations of 10.2% and 8.7% in $SCCO_2$ extracted oils, at 200.0 and 317.6 bar respectively.

The fatty acid (FA) contents of the $SCCO_2$ oils were analyzed to evaluate the effect of pressure, temperature and time on the extract compositions. For short extraction times of 2.5 h, not significant changes were observed in FA contents: in all the P, T conditions the amount of the total saturated (SFA) and unsaturated (UFA) acids were very close to that obtained by cold mechanical pressing. Nevertheless, differences in the relative contents of FA were stated with longer extraction times: as an example, Fig. 4 shows experimental

Table 3
Fatty acids and β-Sitosterol contents in cold mechanical pressed and supercritical (200 bar, 45 °C, 4 h) extracted oils.

	SCO_2 extracted oil		MP oil	Crews et al. [1]
	200.0 bar, 45.0 °C, 4 h	317.6 bar, 45.0 °C, 4 h		
Palmitic ac. C16:0 [% m/m]	7.3	7.4	6.3	7.3–8.1
Palmitoleic ac. C16:1 [% m/m]	6.0	6.6	10.5	n.d.–0.1
Margaric ac. C17:0 [% m/m]	1.8	0.2	0.7	n.d.–0.1
Stearic ac. C18:0 [% m/m]	1.1	1.1	0.7	2.2–2.9
Oleic ac. C18:1 [% m/m]	10.7	11.1	10.0	14.5–15.3
Linoleic ac. C18:2 [% m/m]	40.3	40.5	48.0	60.2–63.1
Linolenic ac. C18:3 [% m/m]	32.9	33.1	24.2	11.8–14.3
Sitosterol [mg/100 g]	117	118	105.3	87.7–111.0

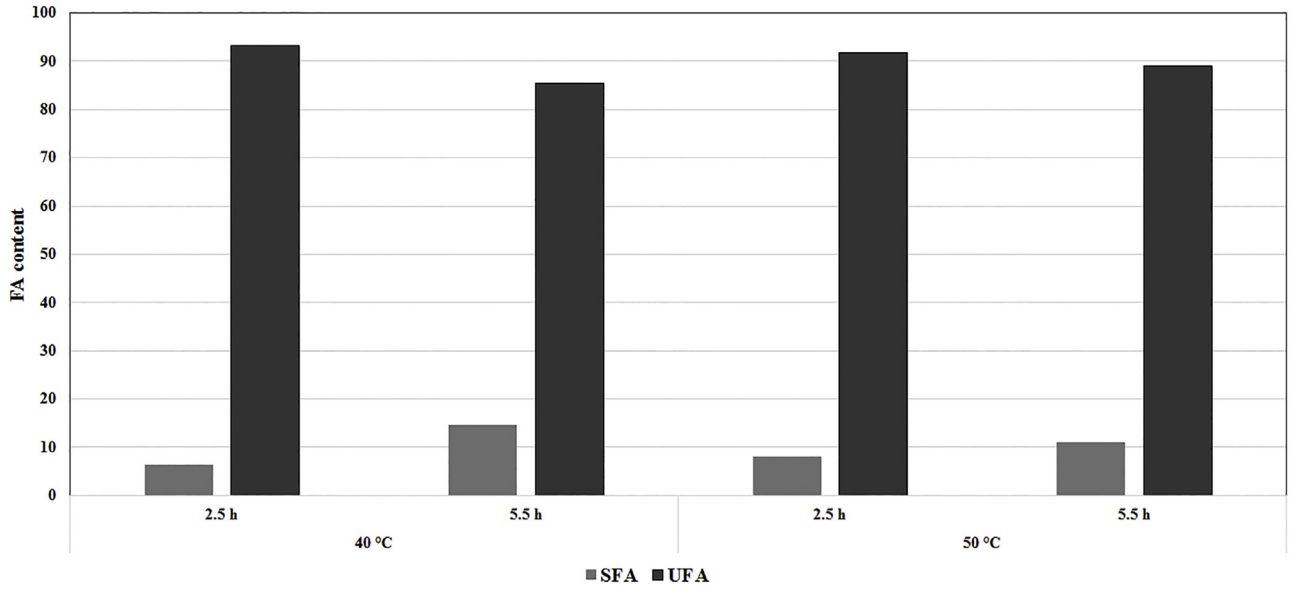

Fig. 4. FA contents of SCO_2 oils extracted at 130 bar with different times (2.5 and 5.5 h) and temperatures (40 and 50 °C).

SFA and UFA compositions, at 130 bar and 40 and 50 °C (N. 1–4 of Table 2), in which longer extraction times increase the presence of saturated acids (over 10%).

This behavior can be explained with the low solubility of saturated lipids in $SCCO_2$ due to a lower ratio of mass transfer of such constituents in the extraction fluid. Therefore, extraction at moderate pressure with short times gives a lower yield of extracted material but a higher percentage of UFA, offering the opportunity to obtain high-nutritional values fractions.

In the obtained fractions the β-sitosterol amount was also measured. MP oil contained 105 mg/100 g of β-sitosterol and this amount is comparable with literature values [1]. Due to the low content of such compound in the vegetal oils, compared to triglycerides, it was easily to appreciate possible variations in the amount related to different $SCCO_2$ conditions of extraction. Neither extraction with higher pressure, neither with longer times allowed a substantial change in the concentration of such compound respect the mild conditions extracted samples. However, as reported in Table 3, higher time of extractions of 4 h, at 200 bar and 45 °C, yield in a product with 117 mg/100 g of β-sitosterol, thus increasing of about 10% the amount of such compound compared with the MP oil. Extraction at same temperature and time but higher pressure (317.6 bar) yielded in a product presenting similar composition with the amount of β-sitosterol being 118 mg/100 g.

4. Conclusions

This paper reports about the $SCCO_2$ extraction of Italian walnut oil and the parameters optimization by central composite design. The response surface method of experimental design highlighted that rises of pressure and extraction time increase the extraction yield of the walnut oils, whereas the temperature has a negative influence at lower pressures and a positive effect at higher pressures.

$SCCO_2$ extraction of walnut oil may represent a valuable technique for obtaining and refining vegetal oils. Treatment with moderate pressure and short extraction times lead to extracted fraction enriched in unsaturated fatty acid, thus obtaining refined oils with higher nutritional and health promoting constituents amount. The different solubility, and the mass transfer of saturated and unsaturated FA derivatives can explain the observed variation of the composition due to time and pressure. Thus, the kinetic behavior of the different lipids of oil suggest a faster solubilization of unsaturated rich lipids in supercritical phase compared to saturated fractions. On the other hand, the extraction of phytosterols as β-sitosterol may need longer treatment or higher pressure values, thus offering the opportunity to obtain different oil fractions using different conditions.

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