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ORIGINAL PAPER

Effects of Supercritical Fluid Extraction Parameters on Unsaturated Fatty Acid Yields of *Pistacia terebinthus* Berries

Deniz Senyay-Oncel · Hasan Ertas · Ozlem Yesil-Celiktas

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Abstract Utilization of renewable resources and development of new processes aimed at converting these materials into value added bio-products are gaining more emphasis. The scope of this study was to optimize supercritical CO_2 extraction (SFE) parameters such as pressure, temperature and flow rate for the yields of unsaturated fatty acids from *Pistacia terebinthus* berries by a Box-Behnken statistical design. All samples were analyzed for fatty acids by GC-FID. The most effective variables were pressure ($P < 0.005$) and flow rate ($P \leq 0.005$). Maximizing the evaluative criteria for dependent variables (oleic acid, linoleic acid and linolenic acid), optimal conditions were determined to be 240 bar, 60 °C and a flow rate of 16 g/min yielding 51.2% oleic acid, 26.5% linoleic acid and 1.0% linolenic acid. The amounts of unsaturated fatty acids in SFE samples (81.3%) were higher than the hexane (74.3%) and were similar to that of cold press samples (80.1%). High concentrations of unsaturated fatty acids can indicate the utilization of the berries as a major dietary source and demonstrate challenges for industrial application of SFE as a green technology.

Keywords Box-Behnken · GC analysis · Linoleic acid · Oleic acid · Optimization · Supercritical fluid extraction

D. Senyay-Oncel · O. Yesil-Celiktas (✉)
Department of Bioengineering, Faculty of Engineering, Ege University, 35100 Bornova, Izmir, Turkey
e-mail: ozlem.yesil.celiktas@ege.edu.tr; ozlemyesil@gmail.com

H. Ertas
Department of Chemistry, Faculty of Science, Ege University, 35100 Bornova, Izmir, Turkey

H. Ertas
Center for Drug R&D and Pharmacokinetic Applications (ARGEFAR), Ege University, 35100 Bornova, Izmir, Turkey

Introduction

Availability and efficient use of existing natural resources has become imperative in modern societies subsequent to global concerns for environmental protection, public health and energy conservation, thus utilization of renewable resources is gaining more emphasis. *Pistacia* species (Anacardiaceae) is one of these resources widely distributed in the Mediterranean countries (Italy, Spain, Morocco, Greece, Tunisia, Turkey) and Asia (Jordan and Syria). Various parts of this plant have biological activities and are used in different traditional remedies such as treatment of burns, asthma and can be used as an antiseptic for bronchitis [1].

Berries of different *Pistacia* species such as *P. lentiscus* [2, 3], *P. atlantica* [4] and *P. terebinthus* [5–7] were extracted with non-polar solvents in order to obtain the oil fraction and determine the composition which is dominated by unsaturated fatty acids, particularly oleic and linoleic acids. Extracts of dried berries were reported to have hypolipidemic effect on rabbits without acute toxicity [8]. Acetone and methanol extracts of the berries of *P. terebinthus* have been studied and flavonoid contents were reported to be responsible for the antioxidant activity [9]. Furthermore, the leaves of *Pistacia* species have attracted attention as well. Antioxidant potential of the leaves besides exhibiting antimicrobial, anti-inflammatory and cytotoxic activities were reported [10, 11].

In spite of the promising composition of *Pistacia* species regarding unsaturated fatty acids, application of solvent free processes such as supercritical fluid extraction (SFE) have not been reported in the literature. SFE is a promising technique for the processing of seed oils in order to eliminate residual solvent in the final product. Furthermore, carbon dioxide shows a high selectivity to fatty acids at relatively low pressure and temperature. SFE is, therefore a challenging technology to improve the industrial processes that are based on hexane extraction.

The aim of this study was to optimize supercritical CO_2 extraction parameters such as pressure, temperature and flow rate on the yields of unsaturated fatty acids from *P. terebinthus* berries. In addition, compositions of SFE-based oils were compared with those oils obtained by Soxhlet extraction and cold press.

Materials

Plant Material

Berries were collected from *P. terebinthus* growing wild in the fields of Demirci in Manisa, Turkey and dried at room temperature for 20 days. Prior to the SFE runs, the plant material was ground using a Waring laboratory scale blender (the mean size was 500 μm) and sifted using a 10 MESH-sieve (800 μm, average). Powdered plant material was then packed in plastic bags and stored at +4 °C.

Experimental Procedure

Supercritical CO_2 Extraction

Supercritical CO_2 extraction was performed with an SFE 100 System (Thar Instruments, Inc., UK, 2006) equipped with a 100-mL extraction vessel operated in the single batch pass mode, of passing CO_2 through a fixed bed of berry particles. The extractor was filled with about 30 g of ground *P. terebinthus* berries. The independent variables were temperature (30, 55, 80 °C), pressure (100, 200, 300 bar) and flow rate (10, 15, 20 g/min). The values were settled according to the experimental design (Box-Behnken) and the extraction pressure, temperature and flow rate were automatically controlled and maintained throughout the system. When all the desired parameters were reached, the extraction was started and continued according to the total amount of consumed CO_2 during dynamic extraction under each condition (900 g). Extracts were collected from the separator outlet after releasing CO_2 from the system.

Solvent Extraction and Cold Press Oil

The oil content was determined according to the method ISO 659:1998 [12]. Ground *P. terebinthus* berries (2 g) were extracted with 300 mL of hexane (Merck) for seventeen cycles (about 6 h) using a Soxhlet (500 mL) apparatus. The extracts were evaporated to dryness at 69 °C in vacuum by a Laborato 4001, Heidolph rotary evaporator. Cold pressed oil was generously donated by the Municipality of Demirci, Manisa, Turkey.

GC-FID Analysis

Chemicals

About 2 M of potassium hydroxide (85%) used was prepared in methanol and supplied by Sigma–Aldrich (Taufkirchen Germany), whereas 2,2,4-trimethylpentane (isooctane) was supplied by Lab-Scan (Dublin-Ireland) as HPLC grade (99.5%) and methanol was from Sigma–Aldrich with high purity (99%) appropriate for gradient elution.

Preparation of Fatty Acid Methyl Esters (FAME's)

Approximately 10 mg of the sample was weighed into sample vial for analysis and methylated after addition of 2 mL of potassium hydroxide. Subsequently, the mixture was vortexed for 10 min and it was again vortexed for another 10 min after addition of 2 mL of isooctane. The vortexed sample was then centrifuged for 5 min. The supernatant sample was injected into the GC-FID system for the quantification of fatty acids.

GC-FID System

A sample solution of FAME was analyzed through a SUPELCO SP TM-2560 column, 100 m long, 0.25 mm I.D. and 0.20 μm film thickness. The column temperature was programmed initially to 140 °C and held there for 5 min, subsequently it was raised to 240 °C at a rate of 4 °C/min. It was held at 240 °C for 5 min. The injection volume for the samples was 1 μL and it was performed with a split ratio of 1:100 and constant flow operating mode at 0.7 mL/min (helium was employed as the carrier gas). The injector temperature and the heated block temperature were both set at 250 °C. The temperature for the detector base was kept constant throughout the analysis set at 260 °C. All samples were extensively analyzed by GC-FID comparing the retention times of standard reference materials (FAME-mix standards were supplied from Sigma–Aldrich Chemical Company) with those obtained for contents within the samples.

Experimental Design

Response surface methodology is a statistical method, which uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equations. In addition, this experimental methodology can generate a mathematical model [13]. The process of extraction was optimized with a Box-Behnken design for a higher yield of fatty acids from *P. terebinthus*. The factors and levels investigated in the study are shown in Table 1. The two center point runs were added to provide a measure of process stability. Experimental design, data analysis and quadratic model building were conducted using the software Design Expert (Version 7.00, Stat-Ease Inc., Minneapolis, MN, USA).

Results and Discussion

Second-order polynomial equations were used to express the extraction yields of oleic acid (mg/g dry *P. terebinthus* berries) (Y_1), linoleic acid (mg/g dry berries) (Y_2) and linolenic acid (mg/g dry berries) (Y_3) as a function of the coded independent variables (Table 1), where A, B, C represents the code of temperature, pressure and flow rate of CO_2, respectively:

$$Y_1 = 22.23745 + 0.33842A + 0.11530B + 0.82105C - 0.000228AB - 0.00476AC - 0.001882A^2 - 0.000377411B^2 - 0.057150C^2$$

$$Y_2 = 3.02400 + 0.033150A + 0.092270B + 1.56905C - 0.000011AB - 0.00172AC + 0.000375BC - 0.00020585B^2 - 0.051640C^2$$

$$Y_3 = -1.48500 - 0.038250A + 0.011570B + 0.29205C + 0.000026AB - 0.00046AC + 0.00002BC + 0.00035A^2 - 0.0000268B^2 - 0.00925C^2$$

The fitted models from the analysis of variance (ANOVA) represented the experimental data well with high correlation coefficients, R^2, ranging from 0.9791 to 0.9057. The value of model (Y_1, $P < 0.05$; Y_2, $P < 0.05$; Y_3, $P < 0.05$), was statistically significant, indicating that the model equation was adequate for mathematically predicting the yield under any combination of values of the variables.

Optimization of Oleic Acid

The effects of pressure, temperature and flow rate on the oleic acid yield of *P. terebinthus,* as well as their interactions, are shown in Fig. 1. Pressure ($P < 0.01$) was found significant, whereas temperature and flow rate ($P > 0.1$) were found not to be statistically significant. However, the interaction between the pressure and the flow rate ($P \leq 0.01$) was found significant. Thus, while the extraction yield was so susceptible even to the minor alterations of pressure, the changes in temperature and flow rate alone did not yield significant differences in oleic acid concentration. Figure 1a illustrates the effect of different combinations of temperature and pressure on the amount of oleic acid yield when the flow rate was constant at optimum value. It was apparent that maximum oleic acid was

Table 1 Experimental levels and codes of the factors used in Box-Behnken design

Exp. no	A (pressure)	B (temperature)	C (flow)	Pressure (bar)	Temperature (°C)	Flow (g/min)	Yields (%)		
							Oleic acid	Linoleic acid	Linoleic acid
1	−1	−1	0	100	30	15	43.06	23.68	1.04
2	−1	1	0	100	80	15	44.16	23.52	0.74
3	1	−1	0	300	30	15	49.46	25.55	0.98
4	1	1	0	300	80	15	48.28	25.28	0.94
5	0	−1	0	200	30	10	47.15	24.92	0.97
6	0	1	0	200	80	10	49.82	26.08	1.11
7	0	−1	1	200	30	20	48.54	24.90	0.93
8	0	1	1	200	80	20	48.83	25.20	0.84
9	−1	0	−1	100	55	10	47.34	21.10	0.00
10	1	0	−1	300	55	10	47.88	24.89	0.93
11	−1	0	1	100	55	20	39.08	21.18	0.00
12	1	0	1	300	55	20	49.65	25.72	0.97
13	0	0	0	200	55	15	51.19	26.37	0.98
14	0	0	0	200	55	15	51.19	26.78	0.97

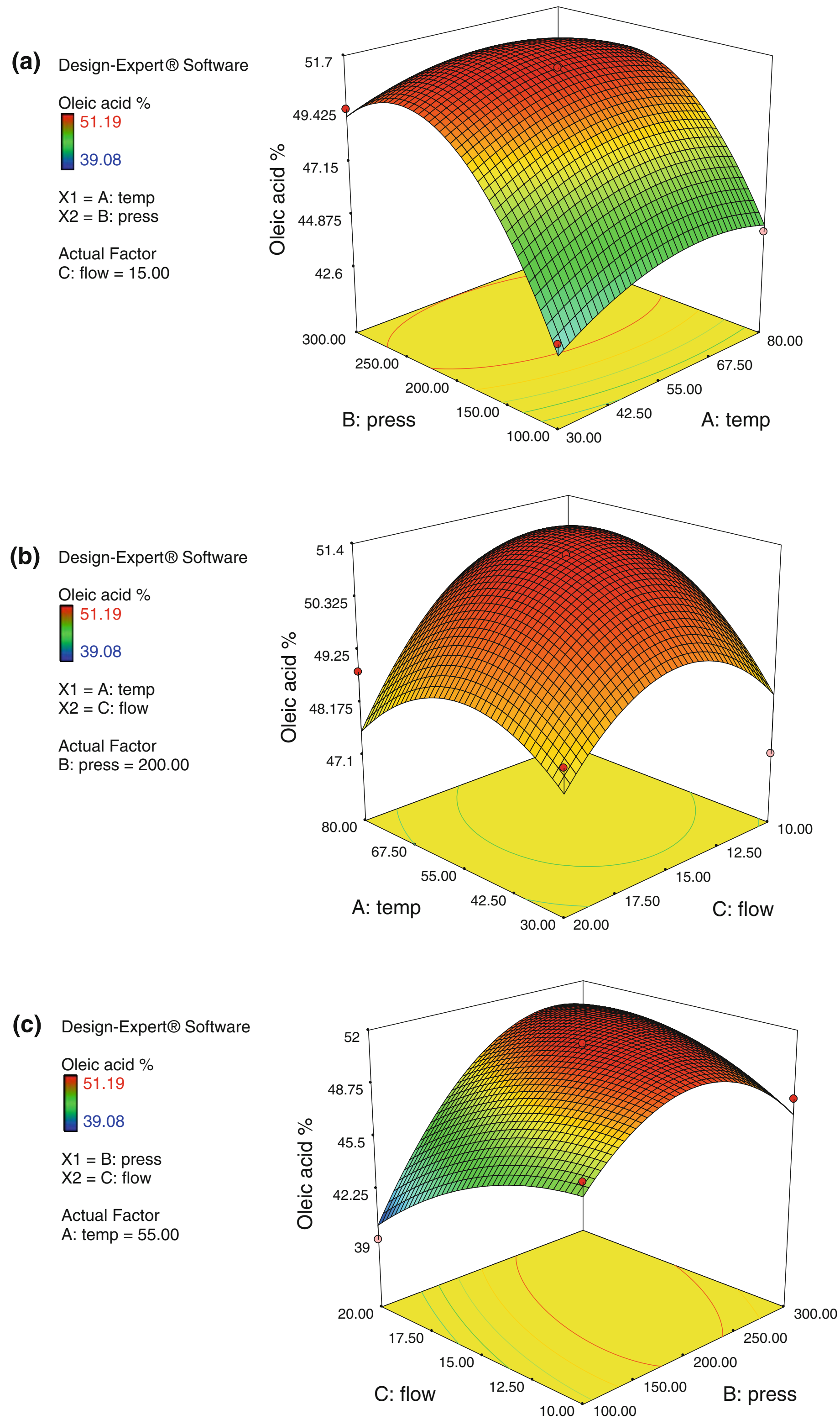

Fig. 1 3-D and contour response surface plots of oleic acid showing the effects of temperature and pressure at a constant optimum flow rate (15 g/min) (**a**), the effect of temperature and flow rate at a constant pressure (200 bar) (**b**), the effect of flow rate and pressure at constant temperature (55 °C) (**c**)

obtained by setting the temperature at 50 °C and pressure between 200 and 250 bar. When considering the relation between temperature and the flow rate, higher yields were obtained with flow rates between 12 and 20 g/min as can be seen in Fig. 1b. In regards to the interaction between the pressure and the flow rate, the maximum oleic acid yield was obtained at 250 bar pressure regardless of the flow rate at a constant temperature of 55 °C (Fig. 1c). In this study,

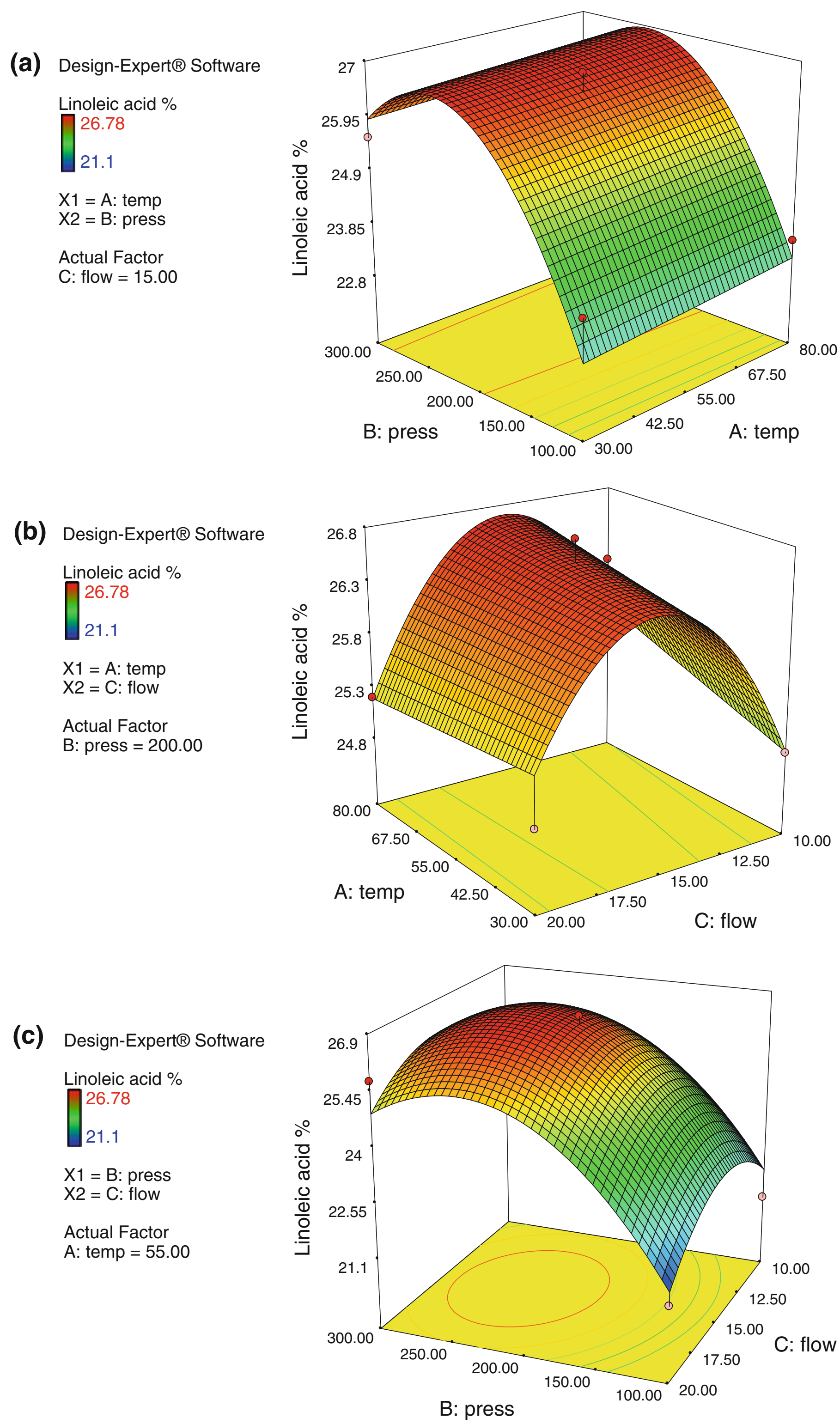

Fig. 2 3-D and contour response surface plots of linoleic acid showing the effects of temperature and pressure at a constant optimum flow rate (15 g/min) (**a**), effect of temperature and flow rate at a constant pressure (200 bar) (**b**), effect of flow rate and pressure at a constant temperature (55 °C) (**c**)

the highest amount of oleic acid was attained with a pressure of 240 bar, a temperature of 54 °C, and a flow rate of 17 g/min with a calculated value of 51.55% which is similar to the experimental value of 51.19% at operating conditions of 200 bar, 55 °C and a flow rate of 15 g/min.

Optimization of Linoleic Acid

The effects of pressure, temperature and flow rate on the linoleic acid yield of *P. terebinthus* showed a similar trend to the oleic acid as can be seen in Fig. 2. An

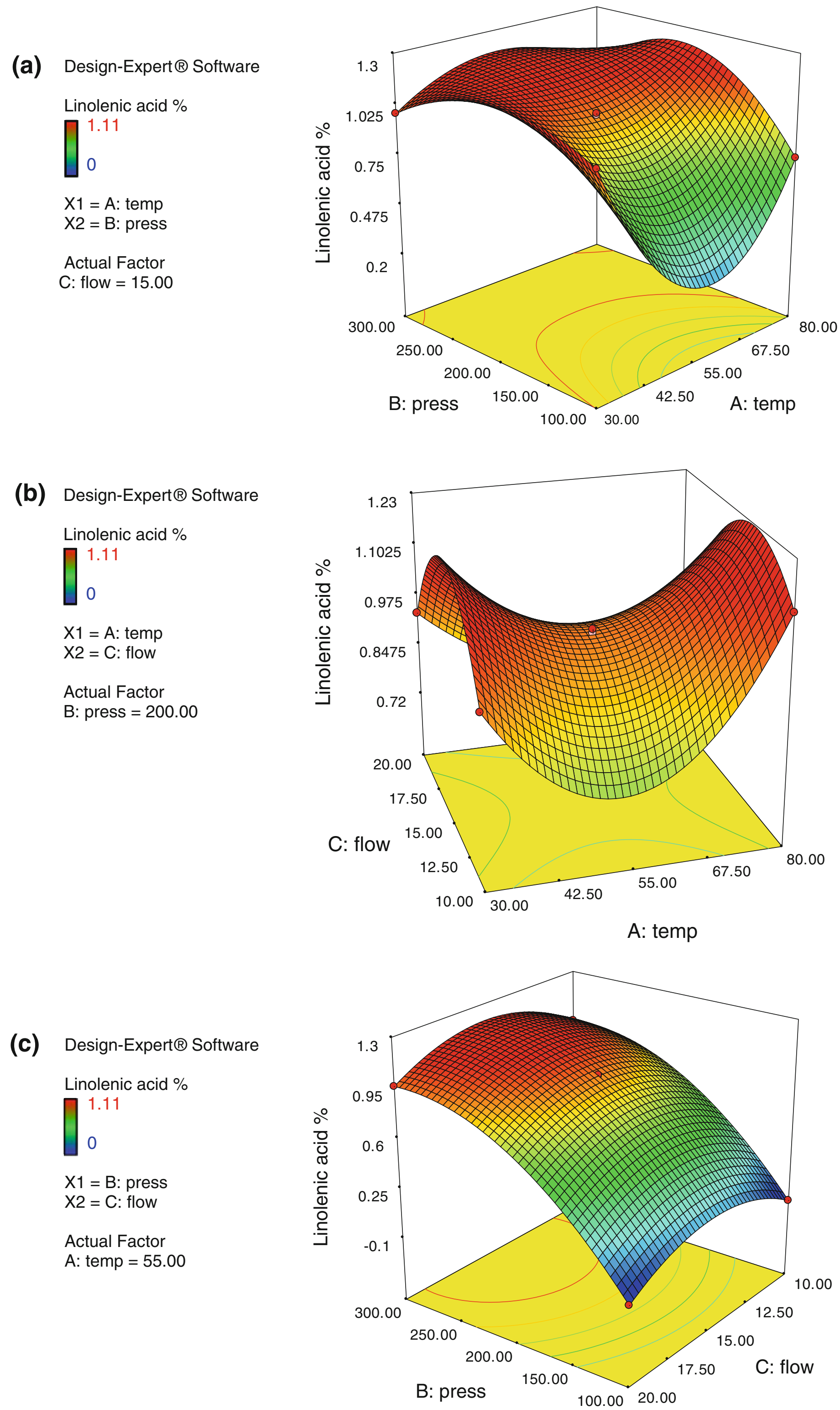

Fig. 3 3-D and contour response surface plots of linolenic acid showing the effects of temperature and pressure at a constant optimum flow rate (15 g/min) (**a**), effect of temperature and flow rate at a constant pressure (200 bar) (**b**), effect of flow rate and pressure at a constant temperature (55 °C) (**c**)

increasing amount of pressure resulted in a higher extraction yield, while the linoleic acid yield reached a maximum when the pressure was 250 bar. Temperature did not have significant effect on the linoleic acid yield ($P > 0.5$), whereas as an optimal value for flow rate was observed at 15 g/min. The highest calculated linoleic acid yield was 26.81%, obtained at 220 bar, 70 °C and 16 g/min which is similar to the experimental value under the same operating conditions which maximized oleic acid yield.

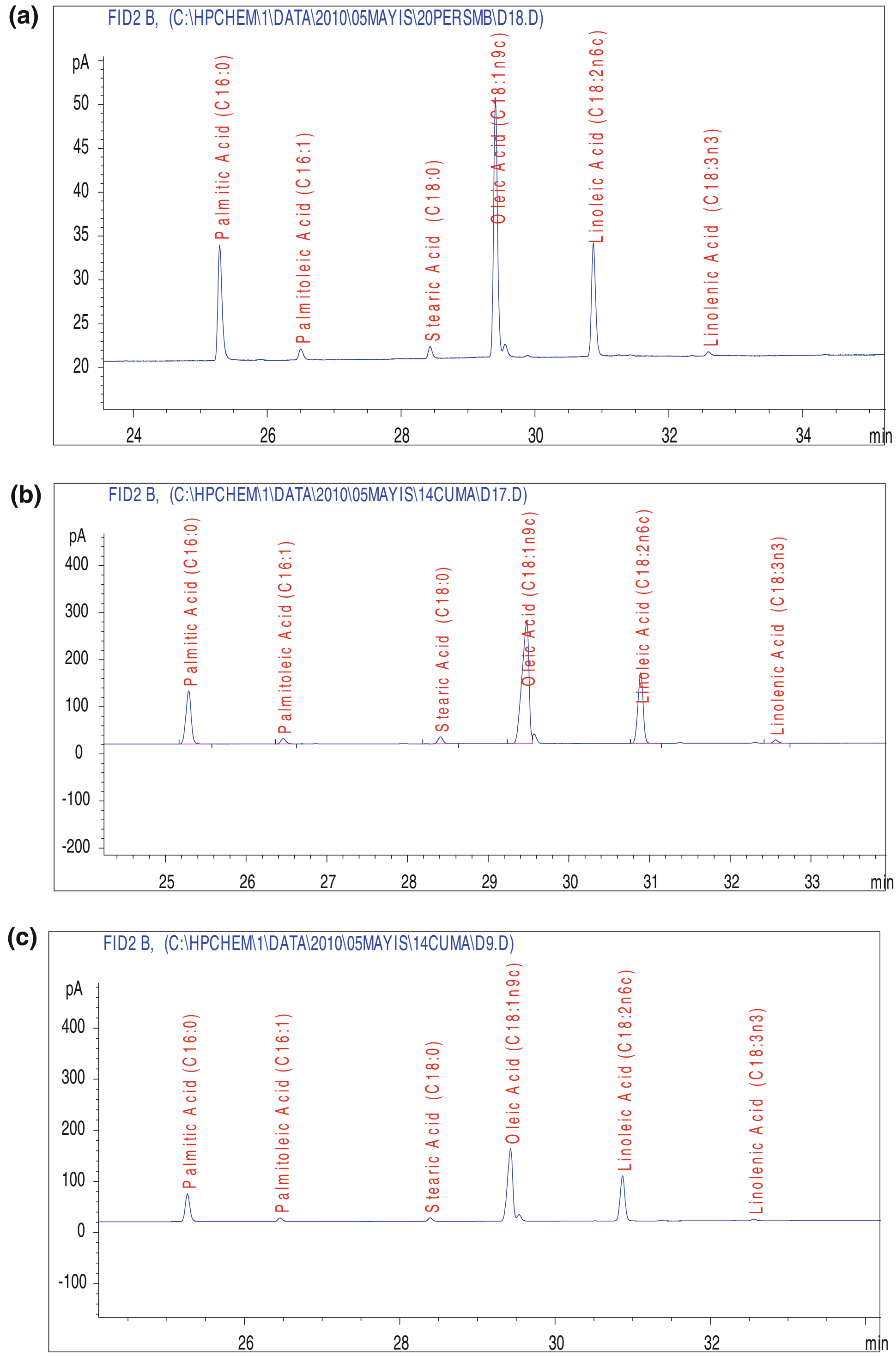

Fig. 4 GC-FID chromatograms of samples obtained with Soxhlet (**a**), cold press (**b**) and SFE (**c**) at a pressure of 200 bar, 50 °C and a flow rate of 15 g/min (c)

Optimization of Linolenic Acid

According to the model, pressure, and the flow rate have positive influences on the yields of linolenic acid, among which pressure showed the most significant effect ($P < 0.01$). Increases in the linolenic acid yield could be achieved by raising both the flow rate and pressure. As expected, the pressure dependence of the linolenic acid yield was higher than the temperature dependence and an increase in operating pressure from 200 to 250 bar and at a flow rate of about 15 g/min resulted in a steady increase in linolenic acid yield in our study. Although, the temperature did not have a significant effect ($P > 0.5$), the interactions of pressure–temperature and flow rate–temperature

Table 2 Comparison of fatty acid compositions of oils obtained by various extraction methods

Yields of fatty acids (%)	SFE	Cold Press	Soxhlet
Palmitic acid	16.2 ± 0.3	17.1 ± 0.2	22.9 ± 0.3
Heptadecanoic acid	0.0	0.1 ± 0.1	0.0
Stearic acid	1.7 ± 0.2	2.2 ± 0.1	2.4 ± 0.1
Arachidic acid	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Behenic acid	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Lignoceric acid	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Total saturated fatty acids	18.7	19.9	25.7
Palmitoleic acid	2.2 ± 0.2	1.6 ± 0.1	2.4 ± 0.1
cis-10-Heptadecanoic acid	0.0	0.1 ± 0.1	0.00
Oleic acid	51.2 ± 0.3	54.7 ± 0.4	49.4 ± 0.2
Linoleic acid	26.5 ± 0.2	22.6 ± 0.1	21.5 ± 0.2
Gondoic acid	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Linolenic acid	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
cis-11,14,17-Eicosatrienoic acid	0.1 ± 0.1	0.0	0.0
cis-13,16-Docosadienoic acid	0.1 ± 0.1	0.0	0.0
Total unsaturated fatty acids	81.3	80.1	74.3

Data represents mean values of analysis ($n = 2$) ± SEM

($P < 0.05$) were significant for the linolenic acid yield (Fig. 3).

Optimum Parameters Maximizing the Yields of Unsaturated Fatty Acids

The compositions of SFE runs under different conditions can be seen in Table 1. The highest yields of unsaturated fatty acids of *P. terebinthus* berries were 51.19% oleic, 26.78% linoleic and 0.97% linolenic acid, totaling up to 78.94% obtained at 200 bar, 55 °C and a flow rate of 15 g/min. By computation, the optimal conditions to obtain the highest unsaturated fatty acids' yield were determined as 240 bar at 60 °C and a flow rate of 16 g/min yielding 51.64% oleic acid, 26.82% linoleic acid and 1.11% linolenic acid which are also in accordance with the experimental results.

Overall, it can be concluded that pressure was the most predominant parameter followed by flow rate in order to obtain the highest yields of unsaturated fatty acids from *P. terebinthus*. The effect of pressure on supercritical CO_2 extraction of lipids from other oilseeds such as walnut [14], flaxseed [15] and daphne [16] has been reported. Our experimental results support previous findings that the yield increases with increased pressure. Thermodynamically, the increase in pressure results in an increase in CO_2 density, increasing the solvating power of the supercritical fluid, thus higher pressure is responsible for quantitative recoveries and stronger interactions between the fluid and the matrix.

Comparison of Fatty Acid Compositions of Oils Obtained by Various Extraction Methods

According to the results of GC-FID analysis, six fatty acids namely, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids comprise almost 99% of the oil samples. The chromatograms of three oils obtained from SFE, cold press and Soxhlet extraction are shown in Fig. 4. As for the contents of the samples in terms of saturated and unsaturated fatty acids, the hexane extract contained 25.7% saturated and 74.3% unsaturated fatty acids, whereas the values of cold press sample (19.9, 80.1%) were similar to that obtained by SFE (18.7, 81.3%) (Table 2) almost reaching the unsaturated fatty acid levels found in olive (83.1%) and soybean oils (83.7%) [17]. Total unsaturated fatty acids obtained under optimized conditions of SFE were higher than the values reported by Matthaus and Ozcan [7] who determined the oil content of *P. terebinthus* berries collected from 14 different locations in Turkey, likewise the values obtained with solvent extraction in various studies [5, 6].

Regarding different *Pistacia* species, Mizi and Djedaia [2] extracted the oil from *P. lentiscus* by two modes of extraction; with a mixture of CH_3OH/CH_3Cl and with a Soxhlet apparatus using ether and reported that the oil is comparable with the olive-oil in terms of unsaturated fatty acid contents. The study by Charef and coworkers [3] also validated the presence of high amounts of unsaturated fatty acids from *P. lentiscus*. Moreover, Benhassaini and coworkers [4] reported high unsaturated fatty acid contents from *P. atlantica*, besides the berries are found to be rich in protein, oil, and fiber suggesting that they may be valuable for food uses. Additionally, Fernandez and coworkers [18] developed edible films from whey protein isolate together with saturated (stearic acid) and unsaturated fatty acids (oleic and linoleic acids) and reported that film properties were improved in terms of sensitivity to moisture. The oil of *P. terebinthus* berries rich in oleic and linoleic acids can also be utilized as an edible film coating for the application of foods requiring moisture control such as fresh fruits, vegetables and bakery products in order to improve their shelf life and reduce the need for synthetic packaging.

Conclusions

In this study, supercritical CO_2 extraction of unsaturated fatty acids from *P. terebinthus* has been optimized using the Box Behnken method. The results demonstrated that the pressure and flow rate were more effective, whereas the temperature did not have a significant effect on the yield of fatty acids. The amounts of unsaturated fatty acids in SFE samples were higher than that obtained using hexane and were similar to that of cold pressed samples. Considering the synthesis of fatty acids in the body, two fatty acids are of prime importance, α-linolenic acid and linoleic acid of which humans lack the enzymes required to produce them. Consequently, these essential fatty acids must be acquired from the diet. Therefore, good sources of these fatty acids should be included in the diet. High concentrations of unsaturated fatty acids could indicate the utilization of the berries as a major dietary source.

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