scientific reports

Check for updates

OPEN

Optimizing ethanol-modified supercritical CO_2 extraction for enhanced bioactive compound recovery in hemp seed oil

Aymane Allay[1], Chaymae Benkirane[1], Abdessamad Ben Moumen[1], Marie-Laure Fauconnier[2], Hamza Bouakline[3], Jacques Nkengurutse[4]✉, Hana Serghini Caid[1], Ahmed Elamrani[1] & Farid Mansouri[1,5]

This work aimed to extract hemp seed oil using modified supercritical CO_2 with ethanol, while optimizing the overall process through response surface methodology. The effects of extraction temperature (30–60 °C), pressure (10–20 MPa), and time (120–300 min) on oil yield, total phenols (TPC), total tocopherols, oxidative stability index (OSI), total chlorophylls, total carotenoids, quality indices, and color were assessed. For a maximum yield of 28.83 g/100 g of fresh seeds, the oil was extracted at 50 °C and 20 MPa for 244 min. In addition, CO_2 modified with different proportions of ethanol (2.5–20%) under the optimized SFE conditions was also tested for enhancing phenolic compound extractability in hemp seed oil. The best proportion was 10% ethanol, which significantly increased the oil yield to 30.13%, TPC to 294.15 GAE mg/kg, total tocopherols to 484.38 mg/kg, and OSI to 28.01 h, without affecting the quality parameters and the fatty acid profile. Furthermore, the phenolic compounds in the extracted oils were analyzed via HPLC-DAD/ESI-MS^2. These findings indicated that CO_2 modified with ethanol enhanced the extraction of phenolic compounds, 26 of which were identified. Among these, the most abundant compounds were *N-trans*-caffeoyltyramine, and cannabisins A and B, with concentrations of 50.32, 13.72, and 16.11 mg/kg oil, respectively. The oil obtained by SFE with SC-CO_2 + ethanol could be valorized by evaluating its biological activities and its anti-aging, dermato-protective and antimicrobial properties for use in the cosmetics, pharmaceutical and food applications.

Keywords *Cannabis sativa* L., Phenolic compounds, Tocopherols, Oxidative stability, Supercritical fluid extraction, Co-solvent efficiency

Hemp (*Cannabis sativa* L.) seeds are often hailed as a nutritionally complete food option because of their rich nutritional profile. They generally contain 25–35% lipids, characterized by an ideal balance of fatty acids, and approximately 20–30% carbohydrates, with a significant portion of insoluble dietary fibers. In addition, hemp seeds contain 20–25% easily digestible proteins that include essential amino acids, increasing their nutritional profile. They are also rich in vitamins and minerals, making them highly nutritious[1–3].

Hemp seed oil is particularly renowned for its low levels of saturated fatty acids (SFA) and high content of unsaturated fatty acids (UFA), which reach approximately 90%. Interestingly, 70–80% of these UFA are polyunsaturated, primarily linoleic acid (C18:2n-6). This composition makes hemp seed oil a rich source of essential fatty acids, featuring a favorable n-6/n-3 ratio ranging between 3/1 and 5/1[3,4]. This ratio is beneficial for balancing dietary intake [5], making hemp seed oil an excellent option for vegetarian diets. Furthermore, the oil is known to enhance cardiovascular health, stimulate immunity and promote healthy skin, nails, and hair [1]. As a result, the demand for hemp seed oil extraction has increased.

[1]Laboratory of Agricultural Production Improvement, Biotechnology, and Environment, Faculty of Sciences, Mohammed I University, B.P. 717, 60000 Oujda, Morocco. [2]Laboratory of Chemistry of Natural Molecules, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés, 2, 5030 Gembloux, Belgium. [3]Laboratory of Applied Chemistry and Environment, Faculty of Sciences, Mohammed I University, B.P. 717, 60000 Oujda, Morocco. [4]Biology Department, Faculty of Sciences, University of Burundi, P.O. Box 2700, Bujumbura, Burundi. [5]Higher School of Education and Training, Mohammed I University, BP-410, 60000 Oujda, Morocco. ✉email: jacques.nkengurutse@ub.edu.bi

Hemp seed oil is commonly acquired through either cold-pressing or solvent-based methods. While efficient and cost-effective, solvent extraction entails prolonged extraction durations and typically employs solvents that pose hazards to both consumers and the environment[6]. Moreover, solvent-extracted oil often requires extensive refining processes, with potential solvent residues persisting in the final product. Alternatively, screw pressing offers a fairly economical option, albeit less efficient than solvent extraction does[7]. Depending on the screw press settings, Occhiuto et al.[8] noted that it is possible to extract between 60 and 80% oil from hemp seeds. The oil yield of screw press extraction could be further increased using enzymes[9]. However, a real problem with cold-processed hemp seeds is the large amount of chlorophyll extracted with the oil, which increases their sensitivity to oxidation, as it is a photosensitizer[1]. The technological maturity of this global process paves the way for the development of alternative technologies. From a sustainable development perspective, the main technological challenge facing these alternative methods is eliminating organic solvents from the extraction process.

The emergence of technologies exploiting supercritical CO_2 (SC–CO_2) as a solvent for vegetable oils dates back approximately two decades[10]. CO_2 is in a gaseous state at room temperature and atmospheric pressure, allowing for its easy removal after extraction and ensuring that extracts are free of chemical residues[11]. Moreover, this method prevents oxidation of the extracted oil since the extraction occurs in the absence of oxygen. Supercritical fluid extraction (SFE) also allows for moderate working temperatures, which is of interest for the extraction of heat-sensitive oils. While unsaturated oils such as hemp seed oil are of particular nutritional interest, they are susceptible to oxidation reactions, which accelerate the rancidity of the oil.

The oils' antioxidants, such as phenolic compounds, are naturally found in the seeds. Studying the interactions between the extraction process and the oil composition could reveal opportunities for improving oil quality by entraining antioxidant compounds from the seeds. The oil extracted by SFE (SFE-oil) contains very low levels of bioactive compounds because non-polar CO_2 has minimal solubility for polar compounds such as phenolics[6]. Adding a polar co-solvent such as ethanol could increase the selectivity and solubility of these compounds in SC-CO_2, while keeping the overall process suitable for food and pharmaceutical applications[12]. Moreover, these compounds have varying degrees of solubility in the supercritical fluids depending on pressure and temperature.

The first aim of our work was to maximize the yield and quality of hemp seed oil extracted via the SFE technique, while standardizing the optimum parameters using response surface methodology (RSM) with a Box-Behnken design (BBD). The second aim is to increase the extractability of bioactive compounds in hemp seed oil via the use a CO_2-ethanol mixture. To our knowledge, few studies have been conducted on hemp seed oil extraction using SC-CO_2[13,14]. However, no previous reports have specifically investigated the phenolic profile of the extracted SFE-oils.

This research is devoted to examining SFE parameters (namely, temperature, pressure, and extraction time) and identifying the best mixture proportion of CO_2-ethanol for extracting an oil abundant in bioactive compounds, offering high oxidative stability, without altering the yield. Therefore, we hypothesized in the present work that optimizing the extraction parameters through RSM can further increase oil recovery and that using CO_2 modified with ethanol can improve the extraction efficiency of bioactive components in hemp seed oil. For this purpose, a statistical approach using the BBD of experiment was employed, incorporating the three extraction parameters as variables to optimize the oil yield. Different extractions were subsequently carried out under optimum conditions using ethanol as a co-solvent at 4 proportions (2.5, 5, 10, and 20%). A comprehensive evaluation was carried out to examine the influence of the levels of each parameter and the four co-solvent proportions on the oil yield, total phenolic content (TPC), total tocopherol content, oxidative stability index (OSI), pigments (total carotenoids and chlorophylls), quality indices (free acidity, peroxide value (PV), and conjugated dienes (CD) and trienes (CT)), and color parameters (L*, a*, b*). In addition, a detailed HPLC-DAD/ESI-MS^2 investigation was performed to identify and quantify phenolic compounds in SFE-oil from hemp seeds with and without the use of a co-solvent.

Results and discussion

Initially, SFE was conducted utilizing SC-CO_2, excluding co-solvent, to assess the impact of extraction variables (temperature, pressure, and duration) on the yield and composition of extracted oil. The crushed seed particle size was fixed at 500 µm by sieving, and the fluid flow rate was consistently maintained at 0.25 kg/h throughout the experiment. The effects of the three variables on the SFE of hemp seed oil were assessed using a BBD in 14 experiments. The experimental results, including the oil yield, TPC, total tocopherols, OSI, total chlorophylls, total carotenoids, quality indices (free acidity, PV, and specific extinction coefficients), and color parameters, are presented in Tables 1, 3 & 4. These responses were described as a function of the studied variables using second-order polynomial equations (Table 2).

Regression coefficients and response surface analysis

The regression models for all the responses were statistically significant ($p < 0.05$), as shown by the ANOVA analysis (Table 2). The model quality was verified using the coefficients of determination (R^2) and adjusted coefficients of determination (R^2 adj). The closer these values are to 1, the stronger the correlation between the experimental and predicted data. In our results, the R^2 values for the 13 models varied from 0.94 to 0.99, whereas the R^2 adj fluctuated between 0.80 and 0.98, indicating a good fit between the predicted and experimental values. This confirms the models' suitability for predicting the 13 responses under different variable combinations. Additionally, the lack-of-fit test was non-significant, implying a strong fit between the model's predicted values and the actual observed data, except for the models related to total tocopherols, total chlorophylls, and the a* color parameter, with a significant lack of fit ($p < 0.05$). Nevertheless, these three responses still demonstrated good model performance, with R^2 and R^2 adj values of 0.99 and 0.98 for total tocopherol, 0.97 and 0.90 for total chlorophyll, and 0.95 and 0.85 for a*.

Run	Independent variables			Responses			
	Pressure (MPa)	Temperature (°C)	Time (min)	Oil yield	TPC	Total tocopherol	OSI
1	10	30	210	25.64 ± 0.45	22.77 ± 0.11	484.11 ± 7.71	10.08 ± 0.15
2	10	45	120	16.57 ± 0.36	61.18 ± 2.47	575.55 ± 9.9	13.01 ± 0.16
3	10	45	300	24.24 ± 0.18	28.23 ± 1.22	548.46 ± 2.16	9.02 ± 0.11
4	10	60	210	11.68 ± 0.23	80.87 ± 1.54	530.65 ± 16.33	11.08 ± 0.33
5	15	30	120	22.75 ± 0.17	25.79 ± 0.02	431.23 ± 4.56	9.48 ± 0.01
6	15	30	300	26.66 ± 0.31	23.52 ± 0.98	529.36 ± 5.77	9.01 ± 0.14
7	15	45	210	27.60 ± 0.20	28.12 ± 0.69	591.67 ± 2.73	10.32 ± 0.11
8	15	45	210	27.45 ± 0.38	27.68 ± 0.02	590.61 ± 13.17	10.44 ± 0.32
9	15	60	120	15.67 ± 0.21	54.37 ± 1.95	620.66 ± 5.56	12.69 ± 0.12
10	15	60	300	24.80 ± 0.14	30.55 ± 0.48	507.41 ± 4.6	8.66 ± 0.21
11	20	30	210	26.46 ± 0.40	40.27 ± 0.49	255.53 ± 9.28	7.59 ± 0.33
12	20	45	120	25.14 ± 0.64	24.92 ± 1.31	277.21 ± 3.21	8.01 ± 0.17
13	20	45	300	27.28 ± 0.51	35.70 ± 2.47	330.95 ± 3.61	9.49 ± 0.44
14	20	60	210	28.69 ± 0.22	31.58 ± 0.14	325.27 ± 13.81	9.58 ± 0.48

Table 1. Experimental results of oil yield, total phenolic content (TPC), tocopherols, and oxidation stability index (OSI) of hemp seed oils obtained under the various conditions (pressure, temperature, and time) of supercritical fluid extraction. Oil yield is expressed in g per 100 g of fresh seeds. Total phenolic content (TPC) is expressed in mg gallic acid equivalent per kg of oil (mg GAE/kg oil). Total tocopherol is expressed in mg per kg of oil (mg/kg oil). Oxidative stability index (OSI) is expressed as the induction time of lipid oxidation (hours).

Response (Y)	β_0	β_1	β_2	β_3	β_4	β_5	β_6	β_7	β_8	β_9	*P*-value		R^2	R^2 adj
											Model	lack of fit		
Oil yield	27.531	3.679	−2.584	2.855	4.049	−1.384	1.303	−1.788	−2.623	−2.430	0.0049	0.0505	0.98	0.933
TPC	27.901	−7.574	10.629	−6.033	−16.698	10.932	−5.388	9.960	6.010	−0.355	0.0006	0.0776	0.992	0.976
Total tocopherol	591.14	−118.724	35.470	1.441	5.798	20.206	−52.845	−140.68	−51.564	−17.412	0.0006	0.0255	0.993	0.977
OSI	10.38	−1.065	0.731	−0.876	0.247	1.367	−0.89	−0.437	−0.36	−0.06	0.0034	0.1466	0.983	0.944
Total chlorophyll	7.054	−2.204	2.677	1.217	−3.815	−0.543	0.119	2.443	1.413	−1.918	0.0108	0.0139	0.97	0.90
Total carotenoid	56.557	3.335	4.529	−11.816	4.097	-8.738	−11.727	−20.487	−24.395	−13.580	0.0370	0.0894	0.941	0.80
Color														
L*	44.387	1.332	−0.474	−0.409	−0.418	0.863	0.77	−0.34	0.0762	0.386	0.0394	0.0752	0.94	0.80
a*	2.812	0.376	−0.766	−2.292	1.798	−1.442	−1.72	2.659	3.394	0.0981	0.0232	0.0409	0.95	0.851
b*	18.492	0.554	1.717	0.845	1.0725	0.946	0.835	3.444	−3.911	−4.543	0.0097	0.1594	0.97	0.905
Oil quality indices														
Conjugated diene (λ 232)	1.555	−0.182	0.364	0.139	−0.314	−0.007	−0.276	0.301	0.17	−0.0532	0.0071	0.3379	0.975	0.920
Conjugated triene (λ 270)	0.429	−0.0453	0.0862	−0.0216	−0.0682	−0.0465	−0.0632	0.0312	0.03	0.0252	0.0293	0.6971	0.948	0.832
Peroxide value	3.671	−1.012	0.633	0.881	−1.295	0.573	0.231	1.195	0.324	−0.277	0.0163	0.1331	0.962	0.876
Free acidity	0.064	−0.0205	0.0417	0.003	−0.0415	0.006	−0.01	0.01	0.04	0.027	0.0211	0.9998	0.956	0.858

Table 2. Analysis of variance (ANOVA) and regression equation coefficients for each response studied of hemp seed oils extracted by supercritical fluid extraction. $\boldsymbol{Y} = \boldsymbol{\beta}_0 + \boldsymbol{\beta}_1\boldsymbol{x}_1 + \boldsymbol{\beta}_2\boldsymbol{x}_2 + \boldsymbol{\beta}_3\boldsymbol{x}_3 + \boldsymbol{\beta}_4\boldsymbol{x}_1^2 + \boldsymbol{\beta}_5\boldsymbol{x}_2^2 + \boldsymbol{\beta}_6\boldsymbol{x}_3^2 + \boldsymbol{\beta}_7\boldsymbol{x}_1\boldsymbol{x}_2 + \boldsymbol{\beta}_8\boldsymbol{x}_1\boldsymbol{x}_3 + \boldsymbol{\beta}_9\boldsymbol{x}_2\boldsymbol{x}_3$. With $\boldsymbol{x}_1$: Pressure (MPa), $\boldsymbol{x}_2$: Temperature (°C), and $\boldsymbol{x}_3$: Time (min) of extraction. β_0 is a constant; β_1, β_2, and β_3 are linear coefficients; β_4, β_5, and β_6 are interaction coefficients are β_7, β_8, and β_9 quadratic coefficients.

Effects of SFE parameters on oil yield

The experimental data revealed that the highest oil yield (28.69% of fresh seeds) was reached at the highest pressure and temperature values (20 MPa and 60 °C) during 210 min of extraction, whereas the lowest yield (11.68%) was observed at conditions of 10 MPa and 60 °C for 210 min (Table 1). The results of the ANOVA are outlined in the Pareto chart (Fig. 1a). A significant linear effect was observed for all three variables, with positive effect for pressure and time and negative effect for temperature. Additionally, a significant negative quadratic effect was observed for both temperature and time. The pressure-temperature interaction was the only significant interaction and positively impacted the oil yield. From the oil yield prediction equation, the linear effect of pressure had the highest coefficient (3.679). Therefore, pressure was the key parameter influencing oil

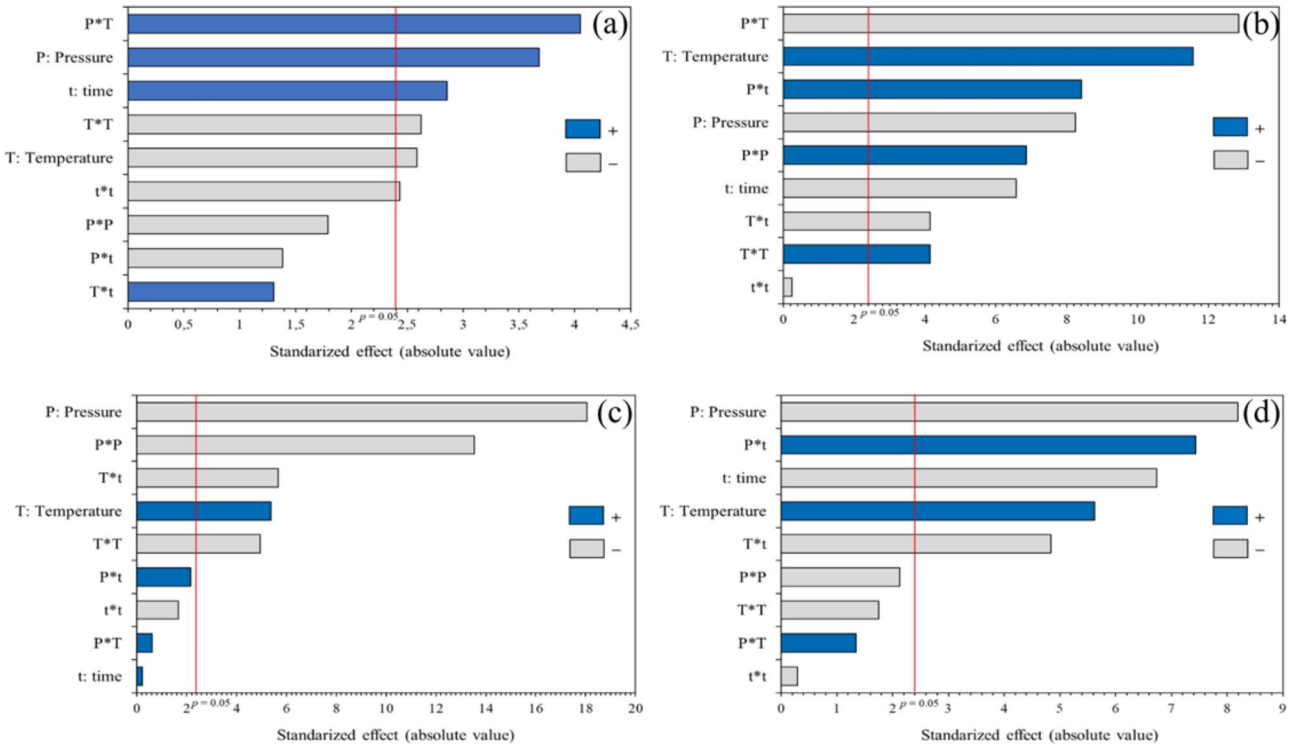

Fig. 1. Pareto chart illustrating the effects of variables and their interactions on oil yield (**a**), TPC (**b**), tocopherols (**c**), OSI (**d**) with 95% significance level.

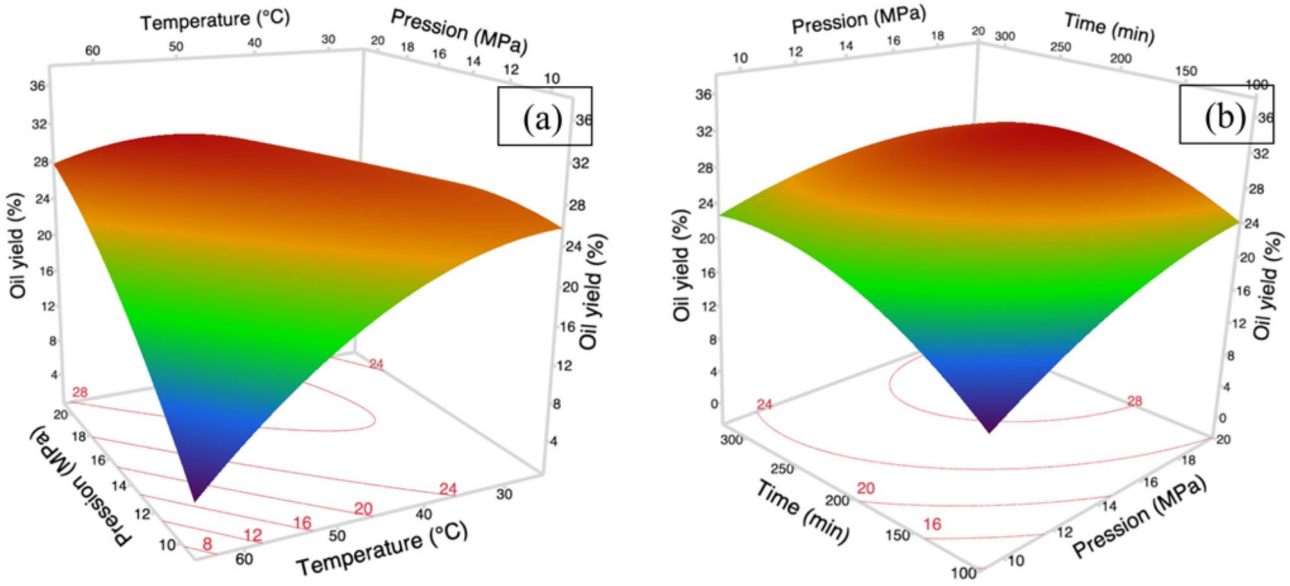

Fig. 2. Surface response diagrams and contour plots of different combined effects of the experimental design results, showing the oil yield (%) in hemp seed (*Cannabis sativa* L.) oils obtained by supercritical CO_2 extraction, as a function of the factors: (**a**). Temperature (°C) and Pressure (MPa); (**b**). Pressure (MPa) and Time (min).

extraction efficiency compared with the other factors. The three-dimensional graph presented in Fig. 2a shows the effects of pressure and temperature on the yield of SFE-oil. The surface plot demonstrated that increasing the pressure from 10 MPa to 20 MPa enhanced the oil yield. This effect could be explained by the density of SC–CO_2, which increases with pressure, increasing oil solubility in the fluid. Comparable results were noted in the studies of Devi & Khanam[13] and Da Porto et al.[15], both of which used the RSM method to optimize the oil yield of hemp

seeds. The first study, in which the pressure was varied from 20 to 35 MPa, revealed that the oil yield increased with increasing pressure to a maximum of approximately 36% at 35 MPa. The second study similarly showed that the oil yield increased as a function of pressure, reaching a peak of 20% at 30 MPa, before decreasing when the pressure further increased to 35 MPa.

Assessing the influence of temperature on SFE efficiency is more intricate than that of pressure. The pressure favored oil recovery in all the cases, whereas the temperature had a dual effect on recovery. As shown in Fig. 2a, at lower temperatures, the oil yield increases with increasing temperature, likely due to the increase in the mass transfer rate. However, the oil yield decreases at higher temperatures, presumably because of the reduction in CO_2 density[15]. However, a noticeable positive correlation was found between yield and extraction temperature when combined with pressure. At a constant extraction time of 210 min and a pressure of 10 MPa, the yield decreased as the temperature increased, dropping from 25.64 to 11.68% at 30 °C and 60 °C, respectively. Conversely, the trend reversed when extraction occurred at 20 MPa, with temperature positively impacting yield. Specifically, the yield increased from 26.46% at 30 °C to 28.69% at 60 °C. These findings are in line with those of Ferrentino et al.[16], who reported that apple seed yield decreased from 6.89 to 1.60% when the temperature was increased from 40 to 60 °C at 10 MPa. However, in the same study, the trend was reversed at a high pressure of 30 MPa, where a positive influence of temperature on yield was observed, increasing from 19.13% at 40 °C to 21.63% at 60 °C. The temperature affects oil solubility in SC-CO_2 depending on two competing phenomena: as the temperature increases, the vapor pressure of the oils increases, whereas the SC-fluid density decreases. At critical pressure, oil solubility decreases when the temperature increases, due to the predominant effect of density on solute vapor pressure. Above the critical pressure, the vapor pressure effect dominates, resulting in an increase in solute solubility. The oil solubility in SC-fluids thus reaches a minimum before increasing with temperature. This temperature-dependent change in solubility in SC-fluids is known as the crossover pressure. The compound's solubility is thus favored or disfavored by increasing the temperature, depending on whether the pressure is lower or higher than the crossover pressure.

The duration of the extraction process also plays an important role, exerting a positive linear effect on the oil yield. This effect likely arises from an increase in the SC-CO_2/seed ratio over time, which enhances the overall extraction efficiency and yield. However, for prolonged extraction times, a significant negative quadratic effect was observed. As shown in Fig. 2b, while the yield increased with time, a slight decline was noted after 240 min. Analogous results were reported in the study of Sodeifian et al.[17], which revealed a reduction in extraction yield after 130 min of extraction.

Effects of SFE parameters on TPC, total tocopherols and OSI

The extraction conditions significantly impacted the TPC of SFE-oil. The ANOVA results presented in the Pareto chart (Fig. 1b) indicate that all effects (linear, quadratic, and interaction) of the three variables were significant, except for the extraction time in the quadratic term. TPC was positively influenced by temperature but negatively affected by pressure and extraction time. The response surfaces for the TPC (Fig. 3a, b) showed that increasing the extraction temperature led to higher TPC levels. Higher temperatures can improve TPC extraction by reducing fluid viscosity, increasing diffusion coefficients, and accelerating mass transfer[18]. In addition, increasing temperature facilitates the decomposition of cell walls, thereby increasing polyphenol availability[19]. Other studies have reported similar results, with a twofold increase in the total phenol concentration as the temperature went up from 40 to 60 °C[16,19,20]. Despite the positive effect of temperature on the TPC, its combined effect with time had a negative effect on the TPC. The highest TPC concentration was obtained at high temperature (60 °C) and intermediate time (210 min). Consequently, prolonged extraction times can cause thermal degradation of bioactive compounds. Figure 3a illustrates a decrease in the TPC of the oil extracts with increasing pressure (at high temperatures). Increasing pressure generally leads to higher phenolic yields[21,22]. However, the TPC decreased due to the simultaneous increase in extraction yield. Indeed, Table 1 clearly shows that the highest TPC content (80.87 mg GAE/kg) was measured in oil extracted under conditions of 10 MPa, 60 °C, and 210 min, which resulted in the lowest extraction yield (11.68%). Pressure seems to affect the solubility of hemp seed oil more than that of phenolic compounds, as shown by the decrease in phenols in extracted oils with increasing pressure. Similarly, various studies reported that the concentration of phenols in oils from different sources decreased with increasing pressure, but the yield increased[20,23]. Nevertheless, Fig. 3b shows that at extended extraction times (> 240 min) and elevated temperatures, the TPC increased as the pressure exceeded 16 MPa. This could be attributed to the decrease in extraction yield with temperature and time, supporting the hypothesis that, although the TPC increased with all the parameters, the dilution effect of the oil in the extracts dominated the TPC of the extracts.

The total tocopherol content ranged from 255.53 to 620.66 mg/kg for oils extracted under conditions of 20 MPa at 30 °C for 210 min and 15 MPa at 60 °C for 2 h (Table 1). As for TPC, the Pareto diagram (Fig. 1c) shows that temperature and pressure had a dominant effect on the tocopherol content of the SFE-oils, whereas extraction time had no significant effect. The 3D response surface plot (Fig. 3c) of total tocopherol content as a function of extraction pressure and temperature revealed that the tocopherol concentration in hemp seed oils increased with increasing temperature, but decreased with increasing extraction pressure. Similar findings were observed by Grijó et al.[24], who reported that an increase in temperature from 40 to 60 °C, significantly increased the total tocopherol content in hemp seed SFE-oil regardless of the extraction pressure (30 or 40 MPa). However, increasing the pressure from 30 to 40 MPa significantly decreased the tocopherol concentration irrespective of temperature (40–60 °C). In contrast, Aladić et al.[25] reported a significant decrease in tocopherol content in hemp seed oil as a function of temperature and pressure. Additionally, at constant temperatures, tocopherol extraction reached its peak at 14 MPa, with a subsequent increase in pressure leading to a decrease in tocopherol concentration. Initially, the increase in total tocopherol content can be explained by CO_2 selectivity in extracting tocopherols compared with the oil parameter. This enhanced selectivity of CO_2 for tocopherols resulted in

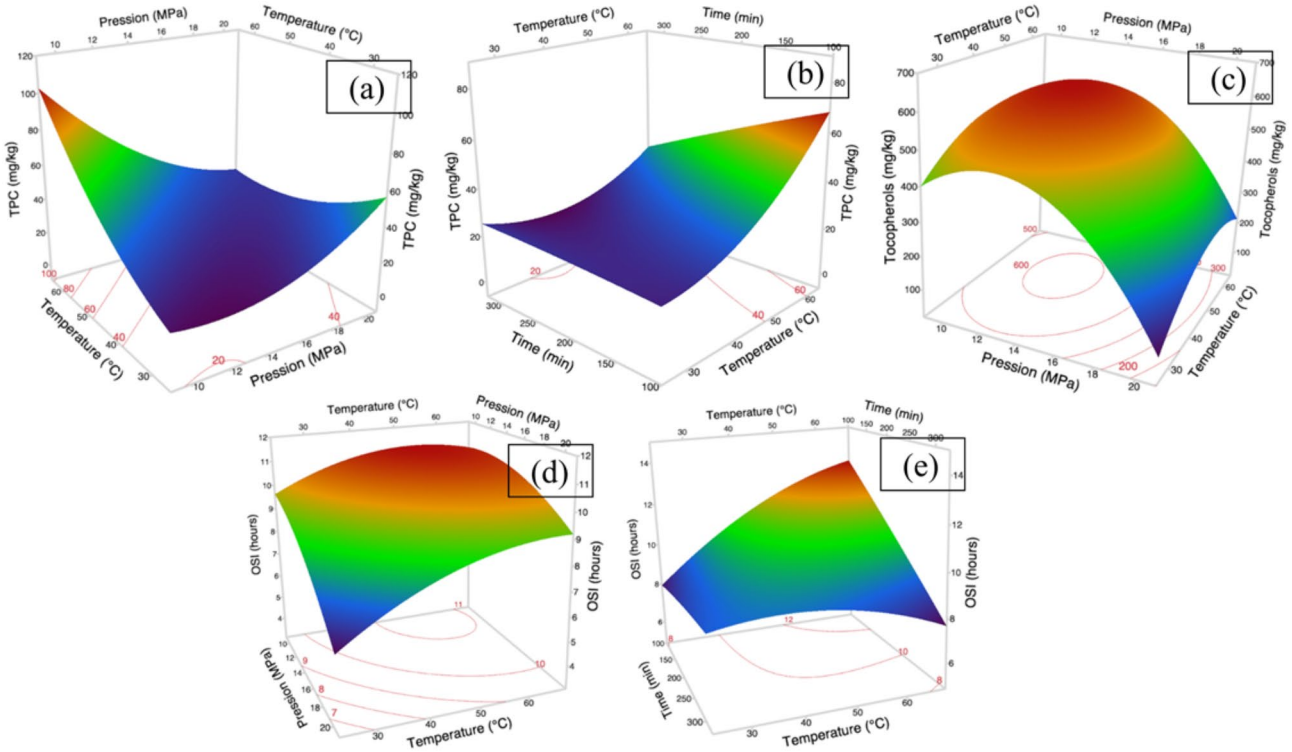

Fig. 3. Surface response plots and contour plots of different combined effects of experimental design results, showing total phenolic content (**a**,**b**), total tocopherols (**c**), and oxidative stability index (**d**,**e**) in hemp seed oils obtained by supercritical CO_2 extraction, as a function of significant factors.

higher tocopherol concentrations in the oil during the initial extraction phase, followed by a decrease in the subsequent phases[20]. Above 14 MPa, the tocopherol content decreases as the extraction yield increases. Gelmez et al.[20] observed a similar relationship between extraction yield and tocopherol content in wheat germ oils. Consequently, the findings indicate that while pressure positively impacts oil yield, it concurrently has adverse effects on the bioactive compound content of the extracted oils. The influence of temperature on tocopherol concentration can be attributed to the amphipathic properties of tocopherols, where their hydrophobic tails interact with membrane lipids while their polar head groups remain exposed on the membrane surface. This characteristic affects the kinetics of the transport and retention of tocopherols within membranes and renders their extraction more challenging. Elevated temperatures act as an external driving force facilitating the liberation of tocopherols into the extraction solvent medium, thereby leading to an augmentation of tocopherol content[26]. However, when the extraction temperature exceeds a certain threshold (55 °C), the total tocopherol content starts to decrease. Increasing the temperature may reduce the solvent density, thereby causing a lower yield of tocopherols in the oil.

The oxidative stability index (OSI), expressed as hours of induction time, was analyzed via the Rancimat method. Table 1 shows that the induction time of the SFE-oil samples under the different conditions ranged from 7.59 to 13.01 h. According to the Pareto chart (Fig. 1d), the positive linear effect of temperature and the negative linear effects of pressure and time were significant, but their interactions were significantly positive. The time-temperature interaction effect was significantly negative. The same pattern was observed for the TPC and total tocopherols. The response surface in Fig. 3d describes the influence of pressure and temperature on the oil OSI. As observed for the TPC and total tocopherols, an increase in temperature increased the induction time. The greater stability of the oil under higher pressures or temperatures could be linked to the increased dissolution of bioactive molecules in the solvent. As mentioned above, more tocopherols and phenols were released from the cell matrix with increasing temperature. These natural antioxidants scavenge free radicals and protect the oil against the formation of primary oxidation products (peroxides and hydroperoxides)[1]. Similarly, Da Porto and colleagues demonstrated that raising the extraction temperature increased the oxidative stability of hemp seed oils by increasing the yield of antioxidants, particularly tocopherols[15]. Although temperature had a positive effect, the time-temperature interaction negatively affected the oil's oxidative stability. Figure 3e shows that longer exposure at higher temperatures gave unfavorable results. This reduction in OSI could be attributed to the increase in temperature during extraction, which destroys heat-sensitive antioxidants and leads to the generation of oxidation products that accelerate oil oxidation. In addition, Fig. 3e shows that the induction time decreased with increasing extraction pressure and time, as the bioactive compound concentrations in oils extracted under these extreme conditions were low.

Run	Independent variables			Responses				
				Color				
	Pressure (MPa)	Temperature (°C)	Time (min)	L*	a*	b*	Total chlorophyll (mg/kg)	Total carotenoid (mg/kg)
1	10	30	210	42.42 ± 0.61	0.06 ± 0.23	15.79 ± 0.77	6.48 ± 0.35	15.58 ± 0.02
2	10	45	120	44.36 ± 0.28	0.40 ± 0.06	16.83 ± 0.08	7.39 ± 0.14	19.29 ± 0.08
3	10	45	300	42.43 ± 0.36	−1.61 ± 0.03	16.34 ± 0.01	11.04 ± 0.40	8.33 ± 0.07
4	10	60	210	42.58 ± 0.11	−6.74 ± 0.09	19.67 ± 0.28	20.89 ± 1.07	11.79 ± 0.13
5	15	30	120	45.41 ± 0.12	−0.33 ± 0.01	7.32 ± 0.33	3.55 ± 0.15	9.41 ± 0.16
6	15	30	300	45.52 ± 0.06	−1.17 ± 0.09	7.63 ± 0.40	5.62 ± 0.01	14.03 ± 0.14
7	15	45	210	44.44 ± 0.01	2.76 ± 0.13	18.72 ± 0.47	7.03 ± 0.25	55.67 ± 0.17
8	15	45	210	44.34 ± 0.26	2.87 ± 0.14	18.27 ± 0.16	7.07 ± 0.03	57.44 ± 0.06
9	15	60	120	45.73 ± 0.11	3.25 ± 0.22	10.78 ± 0.33	7.24 ± 0.35	46.59 ± 1.08
10	15	60	300	42.76 ± 0.11	−4.47 ± 0.04	14.43 ± 0.17	9.79 ± 0.10	4.30 ± 0.05
11	20	30	210	46.51 ± 0.54	−3.35 ± 0.12	18.53 ± 0.58	8.56 ± 0.12	3.37 ± 0.09
12	20	45	120	44.72 ± 0.23	4.61 ± 0.15	16.56 ± 0.57	5.21 ± 0.01	54.12 ± 0.17
13	20	45	300	46.24 ± 0.04	−3.18 ± 0.04	19.85 ± 0.03	6.68 ± 0.05	8.22 ± 0.05
14	20	60	210	44.99 ± 0.20	−2.95 ± 0.02	18.12 ± 0.07	7.71 ± 0.16	15.96 ± 0.06

Table 3. Experimental results of total chlorophylls, total carotenoids and color parameters (L*, a*, b*) of hemp seed oils obtained under the various conditions (pressure, temperature and time) of supercritical fluid extraction.

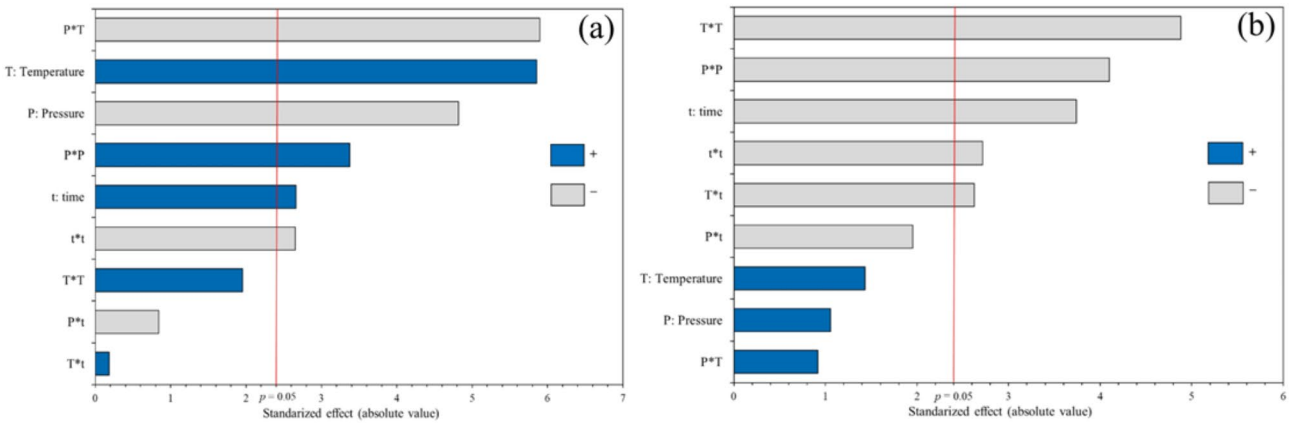

Fig. 4. Pareto chart illustrating the effects of variables and their interactions on total chlorophylls (**a**), total carotenoids (**b**), with 95% significance level.

Effects of SFE parameters on pigments and color

The pigment results presented in Table 3 show that the chlorophyll content varied between 3.55 (run 5) and 20.89 mg/kg (run 4), whereas the carotenoid content varied between 3.37 (run 11) and 57.44 mg/kg (run 8). These results clearly revealed that CO_2 was highly selective for carotenoids, with a high carotenoid/chlorophyll ratio in almost all 14 experiments. The results of the variables' effects are summarized in the Pareto diagram (Fig. 4a, b). The chlorophyll content was significantly influenced by a negative linear effect and a positive quadratic effect of pressure, alongside a positive linear effect of temperature. Only the interaction effect of temperature and pressure was significantly negative. For carotenoids, only three effects were significant: a negative linear effect of extraction time and negative quadratic effects of pressure and temperature.

The response surface depicted in Fig. 5a, b illustrates the variation in carotenoid content with respect to pressure, temperature, and extraction time. When the temperature and time were held constant (at the central point), the carotenoid content initially increased with increasing pressure, reaching a peak before decreasing. The pressure range between 12.5 and 19 MPa resulted in notably high carotenoid content. Low pressures might result in reduced carotenoid solubility in SC-CO_2, leading to decreased carotenoid content. Conversely, the enhanced carotenoid content within the 12–19 MPa pressure range could be attributed to increased CO_2 density, thereby increasing carotenoid solubility. However, the carotenoid content began to decrease at pressures exceeding 19 MPa, possibly due to a loss in CO_2 selectivity resulting from excessive pressure, leading to a dilution effect. A similar pattern was observed for temperature. A lower temperature was associated with a lower carotenoid content, whereas increasing temperature initially seemed to increase the carotenoid content before it decreased. This trend might be attributed to the dual effect of temperature on substance solubility. The

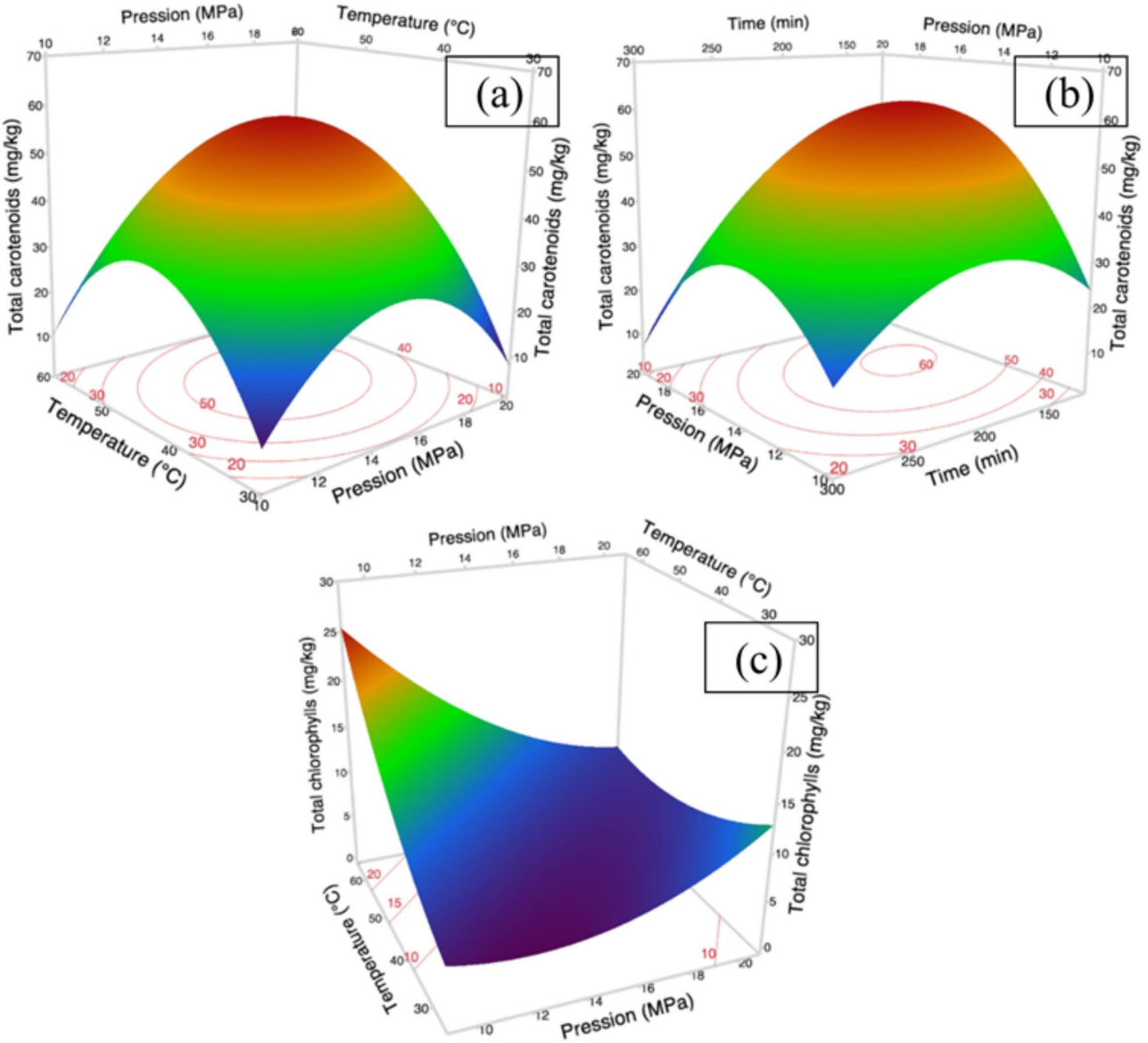

Fig. 5. Surface response plots and contour plots of different combined effects of the experimental design results, showing total carotenoids (**a**,**b**) and total chlorophylls (**c**) in hemp seed oils obtained by supercritical CO_2 extraction, as a function of significant factors.

density effect appeared to predominate between 35.50 °C and 55 °C, facilitating high-level carotenoid extraction. Beyond this range, the vapor pressure effect might impede carotenoid solubility in SC-CO_2, thus reducing the carotenoid content. Some studies in the literature, investigating various raw materials under conditions different from those proposed in our study reported similar results. They recorded that the carotenoid content in the oil was low at low pressure and temperature, increased with rising levels of these two parameters, and then sharply decreased[27,28]. Furthermore, increasing the extraction time resulted in a reduction in the carotenoid content, which was likely attributed to thermal degradation within the extraction vessel[27].

Regarding the chlorophyll content, Fig. 5c shows that SC-CO_2 extracted more chlorophylls from hemp seeds at higher temperatures and lower pressures. Similar behaviors were observed for the TPC, where the phenol content decreased with increasing pressure. This decline is attributed to the increased extraction yield, which diluted the chlorophylls in the oil. However, at higher pressures (18 MPa), the chlorophyll content in the oil began to rise, indicating that the chlorophyll content increased with pressure and temperature, but the effect of oil dilution in the extracts dominated.

Color is an important factor in attractiveness and consumer acceptability. The color parameters of the oil samples L* (lightness), a* (redness), and b* (yellowness) are presented in Table 3. The L* values in the 14 experiments ranged from 42.42 to 46.50. This parameter tends to decrease as the concentration of plant pigments in the oils increases[29]. This trend is clearly illustrated in Fig. 6a, where an increase in pressure led to higher L* values, while reducing the chlorophyll and carotenoid contents (Fig. 5a–c). The chromatic coordinates of the

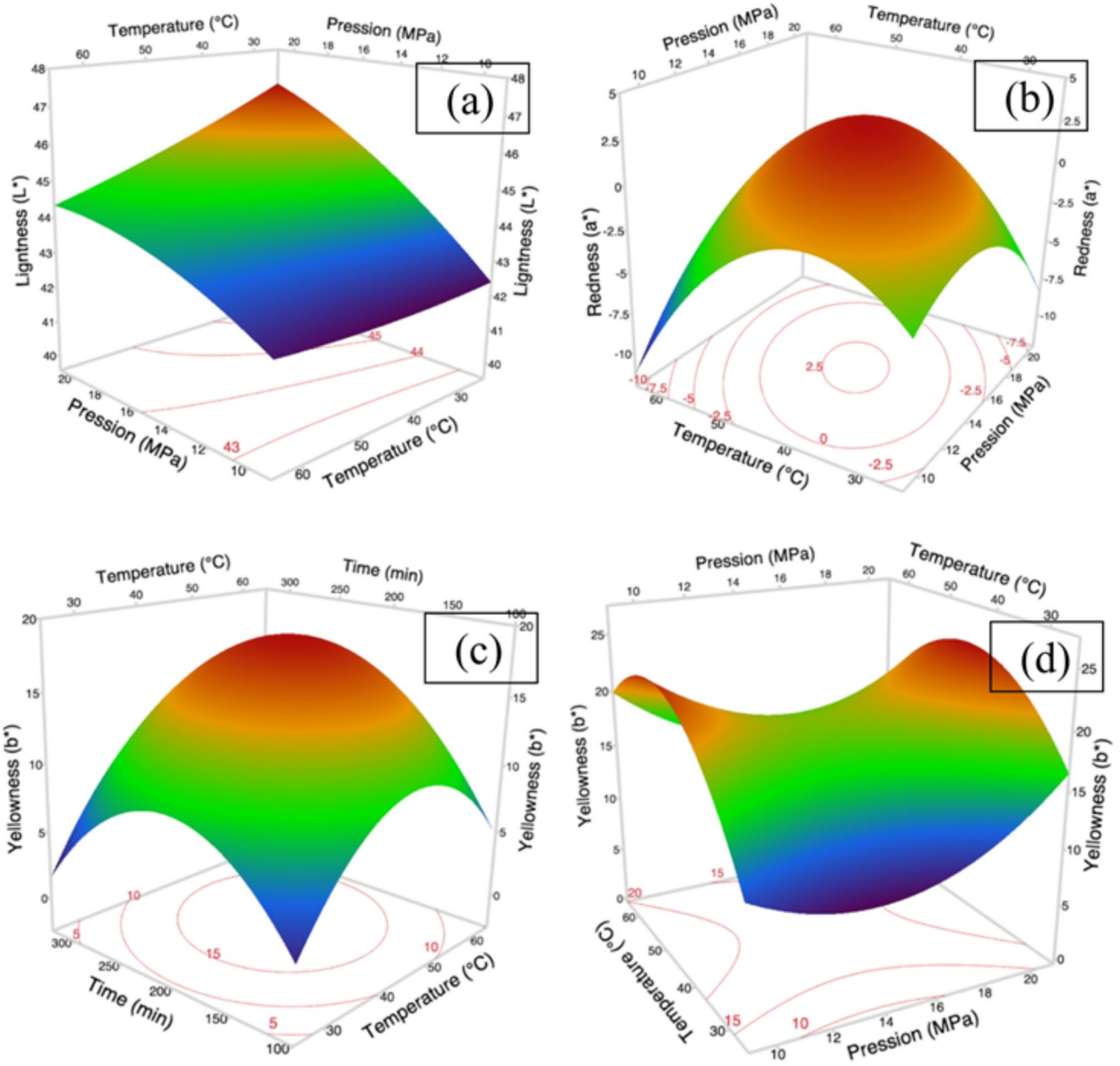

Fig. 6. Surface response diagrams and contour plots of different combined effects of experimental design results, showing the color parameters: L*(**a**), a*(**b**), and b* (**c**,**d**) in hemp seed oils obtained by supercritical CO_2 extraction, as a function of significant factors.

oil samples ranged from −6.74 to 4.61 and from 7.32 to 19.85 for a* and b*, respectively. In contrast to the L* value, the response surfaces for a* and b* (Fig. 6a, c,d) showed a similar trend to that of the pigments (mainly carotenoids). These results suggest that the increase in a* and b* values could be mainly linked to the presence of carotenoids in large quantities (in contrast to chlorophylls), which are responsible for the yellow-orange color of SFE-oils. Ramos-Escudero et al.[30] reported that higher positive a* values are associated with yellowish, orange, and reddish colors, which align with the carotenoid content. A comparative study of oils extracted from roasted and unroasted hemp seeds revealed that the decrease in chlorophyll content relative to carotenoids may account for the increase in b* value in the oil from roasted seeds[31]. Carotenoid enrichment of oils leads to improved chromatic characteristics of oils, potentially increasing product attractiveness and consumer confidence.

Effects of SFE parameters on oil quality indices

The physicochemical properties analyzed for the extracted oils, including % free acidity, PV, and CD & CT are listed in Table 4. The % free fatty acids of the extracted oils under different conditions ranged from 0.058 to 0.22%, PV varied between 1.90 and 8.36 meq O_2/kg, CD ranged from 1.44 to 2.96, and CT fluctuated between 0.36 and 0.69. Based on the Pareto chart (Fig. 7a–d), the positive linear effects of temperature and time were the most significant, whereas a notable negative linear effect of pressure was observed on the quality index

Run	Independent variables			Responses			
				specific extinction coefficients			
	Pressure (MPa)	Temperature (°C)	Time (min)	CD (λ 232)	CT (λ 270)	PV (meq O_2/Kg oil)	Free acidity (% linoleic acid)
1	10	30	210	1.50 ± 0.07	0.40 ± 0.01	3.76 ± 0.27	0.06 ± 0.01
2	10	45	120	1.75 ± 0.02	0.48 ± 0.01	5.25 ± 0.12	0.11 ± 0.01
3	10	45	300	2.18 ± 0.02	0.57 ± 0.00	6.02 ± 0.11	0.12 ± 0.00
4	10	60	210	2.96 ± 0.09	0.69 ± 0.06	8.37 ± 0.23	0.22 ± 0.02
5	15	30	120	1.01 ± 0.02	0.36 ± 0.01	2.83 ± 0.05	0.08 ± 0.00
6	15	30	300	1.706 ± 0.11	0.41 ± 0.01	3.98 ± 0.01	0.09 ± 0.00
7	15	45	210	1.51 ± 0.08	0.46 ± 0.00	3.70 ± 0.14	0.06 ± 0.01
8	15	45	210	1.60 ± 0.12	0.39 ± 0.02	3.53 ± 0.12	0.06 ± 0.01
9	15	60	120	2.19 ± 0.08	0.69 ± 0.04	2.89 ± 0.07	0.19 ± 0.02
10	15	60	300	1.78 ± 0.11	0.48 ± 0.02	4.96 ± 0.25	0.15 ± 0.00
11	20	30	210	1.72 ± 0.03	0.43 ± 0.00	4.50 ± 0.19	0.09 ± 0.01
12	20	45	120	1.44 ± 0.10	0.50 ± 0.00	1.90 ± 0.12	0.06 ± 0.03
13	20	45	300	1.84 ± 0.08	0.40 ± 0.00	4.97 ± 0.06	0.10 ± 0.02
14	20	60	210	1.92 ± 0.03	0.44 ± 0.00	3.93 ± 0.01	0.08 ± 0.00

Table 4. Experimental results of peroxide value (PV), free acidity (%), and specific extinction coefficients (conjugated dienes (CD) and trienes (CT)) of hemp seed oils extracted under the various conditions (pressure, temperature, and time) of supercritical fluid extraction.

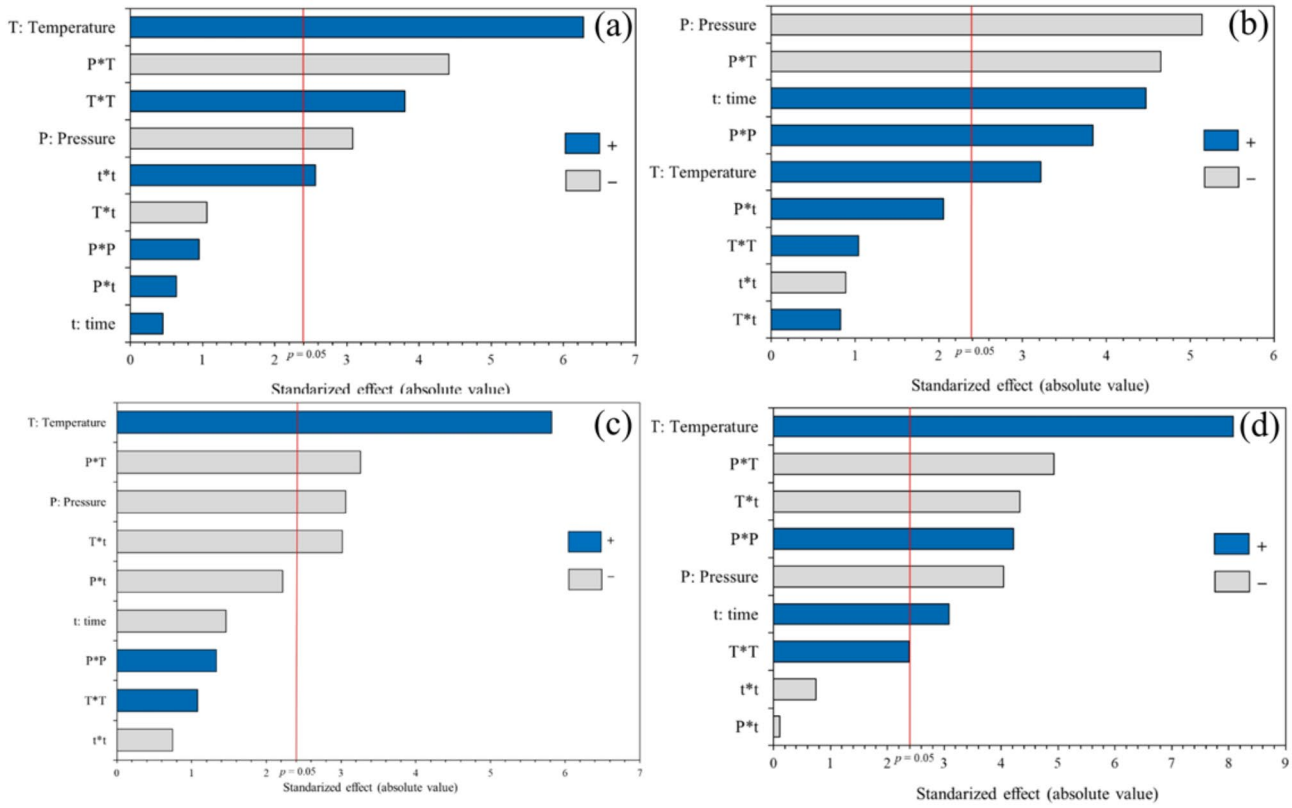

Fig. 7. Pareto chart illustrating the effects of variables and their interactions on free acidity (**a**), PV (**b**), conjugated dienes (**c**), conjugated trienes (**d**) with 95% significance level.

responses (% free fatty acids, PV, CD, and CT). Additionally, the negative interaction effect of temperature and pressure significantly impacted oil quality. The positive quadratic effect, along with the negative interaction effect of temperature and time, were significant, particularly for PV and CD. As depicted in Fig. 8a, the percentage of free fatty acids increased with rising temperature. This observation aligns with findings of Muangrat & Jirarattanarangsri[21,22] regarding SFE-oil from Assam tea seeds. The elevated percentage of free fatty acids may

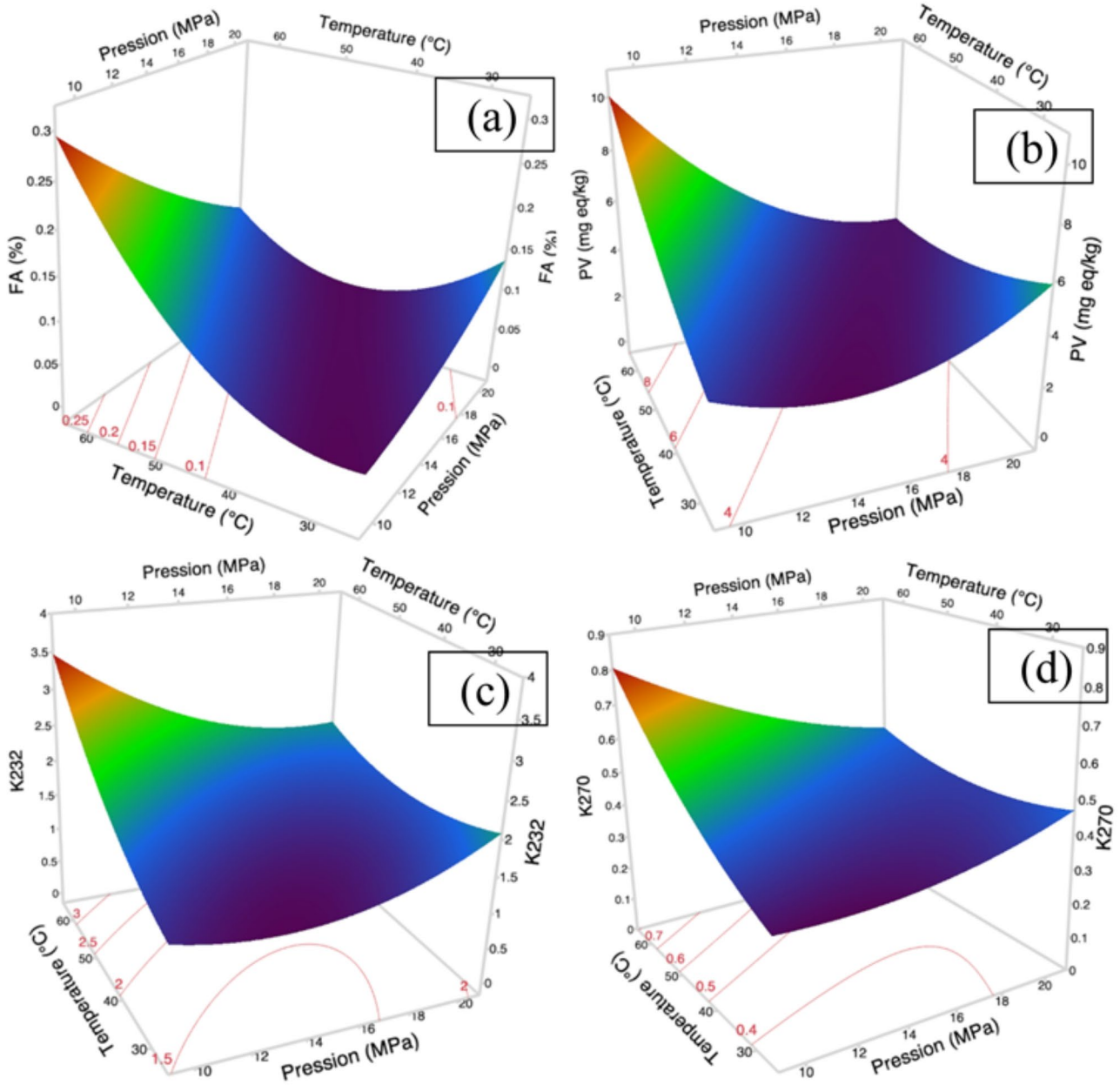

Fig. 8. Surface response diagrams and contour plots of different combined effects of experimental design results, showing free acidity (**a**), peroxide value (**b**), conjugated dienes (**c**) and trienes (**d**) in hemp seed oils obtained by supercritical CO_2 extraction, as a function of significant factors.

be related to the endogenous hemp seed enzymes, especially lipases, which release fatty acids by hydrolyzing triglyceride ester bonds. Although the optimal temperature for lipase activity is around 30 °C, it remains active even at higher temperatures such as 80 °C, indicating its heat stability[32].

Free fatty acids are detrimental to oil stability as they are rapidly oxidized and accelerate the oxidation of polyunsaturated fatty acids when exposed to high temperatures. In addition, higher pressure at low temperature increased the density of SC-CO_2, increasing its solvating power and consequently improving the solubilization of free fatty acids[33]. In this study, the primary lipid oxidation of oil samples was assessed by measuring the CD content and peroxide content. Similar to the free acidity, Fig. 8b, c show that PV and CD increased at higher temperatures and pressures and with longer extraction times. In agreement with our results, the PV of SFE-oil from Assam tea seeds increased as a function of temperature[22]. The increase in PV might be associated with the increased presence of free fatty acids, which serve as the primary substrate for oxidation reactions. Elevated extraction temperatures lead to proton loss from fatty acids, which results in the formation of free radicals. These radicals react with molecular oxygen, forming unstable peroxide radicals that further react with additional fatty acid molecules to generate hydroperoxides. Additionally, longer extraction times result in prolonged exposure of the oil to high temperatures, exacerbating oil oxidation. The secondary oxidation of the oil was assessed by

monitoring the formation of CT, which are secondary oxidation products. Hydroperoxides, as primary pre-products, undergo decomposition reactions, leading to the formation of conjugated trienoic structures (keto acids and aldehydes), which are detectable through absorbance measurements at 270 nm[34]. Similar to CD and PV, Fig. 8d shows that higher extraction temperatures and longer extraction times (>180 min) resulted in increased CT values. The oil samples extracted at relatively high temperatures and long durations presented relatively high peroxide and acid values, indicating potentially reduced stability, with relatively high CT values.

Optimization of SFE-oil from hemp seeds

The SC-CO_2 extraction conditions were optimal when the oil yield reached the maximum value. On this basis, the optimum SC-CO_2 extraction conditions predicted for hemp seed oil were: a temperature of 50.7 °C, a pressure of 20 MPa, and an extraction time of 244.2 min. The oil yield, TPC, total tocopherol, OSI, total chlorophyll, total carotenoid, quality indices (free acidity, PV, and specific extinction coefficients), and color parameters (L*, a*, b*) under these optimal conditions were 29.98%, 29.92 mg GAE/kg oil, 9.22 h, 339.16 mg/kg, 29.90 mg/kg, 7.09 mg/kg, 0.06%, 4.09 meq O_2/kg, 1.71, 0.40, and 45.20, −0.95, and 22.45, respectively. These predicted values were compared with experimental data obtained at 50 °C, 20 MPa, and 244 min. The oil yield, TPC, total tocopherol content, OSI, total chlorophyll and total carotenoid contents, free acidity, PV, CD and CT, and L*, a*, b* values were 28.83%, 30.52 mg GAE/kg, 359.26 mg/kg, 9.8 h, 31.79, 9.16 mg/kg, 0.11%, 4.65 meq O_2/kg, 1.52, 0.45, 44.55, -1.08, and 20.81, respectively (Table 5). These experimental results validated the prediction models adopted and confirmed their ability to predict responses under all conditions in the experimental field. Under these optimal conditions, the oil showed low concentrations of TPC and tocopherols, resulting in limited oxidation stability. To overcome this limitation, a second part of the study was carried out by introducing a co-solvent, in order to improve the extraction of bioactive compounds.

Effect of co-solvent addition on the yield and physicochemical parameters of SFE-oil

A second series of experiments was carried out to improve the extractability of bioactive compounds with the SFE-oil of hemp seeds. The extraction was performed under optimized conditions (50 °C, 20 MPa for 244 min) using CO_2 combined with varying ethanol proportions (2.5, 5, 10 and 20%). Ethanol is the preferred co-solvent among several options, as it is classified as a GRAS (generally recognized as safe) solvent by the US Food and Drug Administration. The results of the yield and physicochemical parameters of the oils extracted with and without co-solvent are presented in Table 5.

Oil yield and quality parameters

The oil yield increased significantly with increasing ethanol proportion, with a maximum of 30.51% at 10% ethanol, before declining at the 20% proportion level (Table 5). On the one hand, the improvement in oil may result from the increased solvent density, as well as ethanol's ability to disrupt cell walls, permitting the thorough release of cell contents[4,35]. On the other hand, the decrease in oil at 20% ethanol may be due to increased polarity, which limits lipid solubility[36]. A single study examining the effect of varying co-solvent concentrations (0, 5 and 10%) on the SFE extraction of hemp seed oil revealed that adding ethanol increased the yield, reaching a maximum of 36% using a concentration of 10%[13].

The use of ethanol did not significantly influence the free acidity and PV of the extracted oil, but significantly decreased CD and CT at 20% ethanol. The decrease in CT formation may be attributed to the strong increase in antioxidants such as phenols and tocopherols. Athanasiadis et al.[37], studied sunflower oil and reported that the addition of tocopherols resulted in a reduction in the CT value. Furthermore, the quality indices of SFE-oils with and without co-solvent were better than those reported in the literature for obtaining hemp seed oil using the cold extraction method[8,38].

Phenolic profile

The addition of ethanol, albeit at low concentrations (2.5%), increased the TPC approximately 4-fold. In addition, the TPC of the oil extracts increased from 30.52 mg/kg (without co-solvent) to 427.77 mg GAE/kg with 20% ethanol (Table 5). Similar findings reported that the use of only 5% ethanol with CO_2 in the SFE of *Clementina orogrande* peel oil increased the TPC content up to 19.51 mg/g oil, compared with a negligible polyphenol extraction using pure CO_2[39]. CO_2 behaves as a non-polar solvent at extraction pressures below 70 MPa, making it less effective in extracting highly polar compounds, such as phenolics. The maximum TPC was recorded in the oil extracted with 20% ethanol, exceeding the level reported by Aiello et al.[40] of 51.42 mg GAE/kg for hemp seed oil extracted by SC-CO_2. Moreover, this content was higher than in other studies of hemp seed oil extracted by conventional methods (solvent or mechanical techniques) from the same ecotype[3,4,31].

To further illustrate the co-solvent effect on the co-extraction of phenolic compounds with hemp seed oil, HPLC-DAD/ESI-MS^2 analysis of extracts from SFE-oils with co-solvents (2.5, 5, 10 and 20%) was carried out. The identification of compounds was conducted by comparing the precursor ion mass (MS), its fragments (MS^2) in both negative and positive modes, and its UV-Vis spectra with available literature sources. The analysis revealed the presence of 26 phenolic compounds, including 2 hydroxybenzoic acids, 7 hydroxycinnamic acids and their derivatives, and 17 lignanamides (Table 6 and Supplementary Figures S2 & S3). These compounds are commonly detected in hemp seeds and were reported for the first time in hemp seed oil extracted with a mixture of *n*-hexane, 2-propanol, and ethyl acetate[4,41–43]. These authors reported the presence of the same phenolic classes and compounds, including phenolic acids (e.g., *p*-hydroxibenzoic acid, *p*-coumaric acid), hydroxycinnamic acid amides (e.g., *N-trans*-caffeoyltyramine) and lignanamides (e.g., cannabisin A and B). Other studies have emphasized the presence of other compounds in cold-pressed hemp seed oil, mainly from the flavonoid class, such as rutin, quercetin, isorhamnetin, catechin, naringenin and apigenin (Faugno et al., 2019; Occhiuto et al.[8] Smeriglio et al., 2016).

Responses	Optimal conditions 20 MPa, 50 °C, and 244 min					
	Pure SC-CO_2		SC-CO_2 + EtOH			
	Predicted values	Observed values	SC-CO_2 + 2.5%EtOH	SC-CO_2 + 5%EtOH	SC-CO_2 + 10%EtOH	SC-CO_2 + 20%EtOH
Oil yield (%)	29.98	28.83 ± 0.17a	28.65 ± 0.14a	29.86 ± 0.12b	30.91 ± 0.16c	27.72 ± 0.13d
TPC (mg GAE/Kg oil)	29.92	30.52 ± 2.07a	121.32 ± 2.76b	203.2 ± 5.86c	294.15 ± 11.02d	427.77 ± 17.90e
Oxidative stability index (OSI) (h)	9.22	9.80 ± 0.62a	14.99 ± 0.14b	30.13 ± 0.39c	28.01 ± 0.28d	25.69 ± 0.54e
Tocophérols (mg / Kg Oil)						
γ-Tocopherol	–	292.10 ± 31.16a	402.81 ± 45.16b	406.61 ± 32.15b	412.61 ± 28.50b	576.63 ± 22.80c
α-Tocopherol	–	42.79 ± 1.64a	42.70 ± 1.39a	37.32 ± 1.06b	37.68 ± 1.36b	37.08 ± 1.88b
δ-Tocopherol	–	28.37 ± 0.12a	30.41 ± 1.10b	30.25 ± 1.67b	32.09 ± 0.4c	39.09 ± 0.40d
Total tocopherol	339.16	361.16 ± 33.88a	473.92 ± 44.34b	476.18 ± 32.55b	484.38 ± 32.52b	654.80 ± 35.01c
Total carotenoid (mg/kg oil)	29.90	31.79 ± 0.08a	18.96 ± 0.09b	15.45 ± 0.08c	14.66 ± 0.17d	11.68 ± 0.21e
Total chlorophyll (mg/kg oil)	7.09	9.16 ± 0.57a	39.03 ± 0.76b	46.00 ± 0.35c	61.03 ± 0.85d	90.72 ± 4.82e
Color						
L*	45.20	44.55 ± 0.27a	36.78 ± 0.44b	36.62 ± 0.50b	34.97 ± 0.45c	33.02 ± 0.16d
a*	−0.95	−1.08 ± 0.04a	−1.25 ± 0.03b	−3.47 ± 0.14c	−3.96 ± 0.16d	−4.75 ± 0.25e
b*	22.45	20.81 ± 0.27a	7.97 ± 0.18b	6.84 ± 0.24c	3.75 ± 0.21d	1.53 ± 0.05e
Oil quality indices						
CD (λ 232)	1.71	1.52 ± 0.03a	1.53 ± 0.12a	1.53 ± 0.07a	1.52 ± 0.09a	1.24 ± 0.04b
CT (λ 270)	0.40	0.45 ± 0.06a	0.49 ± 0.07a	0.51 ± 0.05a	0.48 ± 0.08a	0.22 ± 0.03b
PV (meq O_2/Kg oil)	4.09	4.65 ± 0.12a	4.63 ± 0.13a	4.63 ± 0.17a	4.67 ± 0.06a	4.64 ± 0.10a
Free acidity (% linoleic acid)	0.06	0.11 ± 0.03a	0.10 ± 0.02a	0.10 ± 0.03a	0.11 ± 0.02a	0.10 ± 0.01a
Fatty acids (%)						
Palmitic acid (C16:0)	–	6.09 ± 0.38a	6.61 ± 0.62a	6.71 ± 0.88a	6.39 ± 0.46a	6.38 ± 0.57a
Stearic acid (C18:0)	–	1.09 ± 0.12a	1.21 ± 0.19a	1.06 ± 0.12a	1.26 ± 0.11a	1.21 ± 0.14a
Oleic acid (C18:1)	–	21.41 ± 1.15a	20.81 ± 1.21a	20.15 ± 1.13a	19.12 ± 1.44a	19.16 ± 1.42a
Linoleic acid (C18:2 n-6)	–	49.83 ± 1.23a	49.80 ± 1.23a	50.26 ± 1.38a	50.76 ± 1.71a	50.61 ± 1.13a
γ-linolenic acid (C18:3 n-6)	–	0.89 ± 0.12a	0.94 ± 0.12a	1.10 ± 0.14a	0.95 ± 0.10a	1.01 ± 0.12a
α-linolenic acid (C18:3 n-3)	–	16.60 ± 0.95a	16.17 ± 1.17a	16.12 ± 1.13a	17.17 ± 1.14a	17.18 ± 1.36a
Arachidic acid (C20:0)	–	1.10 ± 0.11a	1.16 ± 0.13a	1.27 ± 0.11a	1.16 ± 0.19a	1.20 ± 0.12a
Eicosenoic acid (C20:1)	–	1.95 ± 0.15a	2.15 ± 0.15a	2.16 ± 0.14a	2.03 ± 0.17a	2.02 ± 0.14a
Behenic acid (C22:0)	–	0.82 ± 0.01a	0.85 ± 0.05a	0.87 ± 0.05a	0.86 ± 0.07a	0.90 ± 0.01a
Lignoceric acid (C24:0)	–	0.27 ± 0.08a	0.28 ± 0.10a	0.30 ± 0.05a	0.30 ± 0.03a	0.33 ± 0.07a
SFA	–	10.47 ± 0.80a	11.29 ± 0.91a	11.48 ± 0.85a	11.13 ± 0.69a	11.22 ± 0.52a
MUFA	–	23.37 ± 1.11a	22.97 ± 1.07a	22.31 ± 1.09a	21.15 ± 1.27a	21.17 ± 1.28a
PUFA	–	67.32 ± 1.09a	66.91 ± 1.18a	67.48 ± 1.28a	68.88 ± 1.67a	68.81 ± 1.78a
n-6/n-3 ration	–	3.05 ± 0.15a	3.14 ± 0.13a	3.25 ± 0.15a	3.01 ± 0.17a	3.01 ± 0.13a

Table 5. Effect of different ethanol proportions (2.5, 5, 10, and 20%) as a function of SC-CO_2 flow rate (0.25 kg/h), on oil yield, TPC, tocopherols, oxidative stability index (OSI), total carotenoid, total chlorophyll, oil quality indices (free acidity, peroxide value (PV), conjugated dienes (CD) and Trienes (CT)), and color (L*, a*, b*) of hemp seed oil extracted by SFE under optimal extraction conditions (20 MPa, 50 °C, and 244 min). Mean ± SD of three independent experiments ($n = 3$). abcd different superscript letters indicate significant differences ($p \leq 0.05$) within rows.

This study is the first comprehensive investigation of the phenolic composition of SFE-extracted hemp seed oil, while studying the co-solvent effect. The quantitative results depicted in Table 6 underscore the pivotal role of the proportion of ethanol to CO_2 in extracting and solubilizing phenolic compounds in the oil. The concentrations of most compounds increased with the ethanol percentage, indicating their improved solubilization due to co-solvent polarity. Notably, the highest phenol content (152.17 mg/kg) in the oil was achieved using 20% ethanol, with a spectacular richness in phenylpropanoids (phenolic group comprising hydroxycinnamic acid amides (HCAAs) and lignanamides). Specifically, *N-trans*-caffeoyltyramine emerged as the predominant compound with 50.32 mg/kg, constituting 33.06% of the total phenolic content, followed by cannabisin A and B, with 13.72 and 16.11 mg CTE/kg, respectively. Low values were recorded for phenolic acids (*p*-coumaric acid and *p*-hydroxibenzoic acid) and other lignanamides (cannabisins C, D, G, and isocannabisin N). Although differing across seed genotypes, *N-trans*-caffeoyltyramine and cannabisin A and B have been recorded as the most dominant compounds[41,42,44]. Notably, only benzoic, *p*-coumaric, and sinapic acids were quantified in the oil samples devoid of co-solvent usage. The highest sinapic acid content (3.40 mg CTE/kg) was observed in oil extracted with 2.5% co-solvent.

Phenolic compounds	Molecular formula	λmax (nm)	$[M-H]^-$ calc. (m/z)	$[M-H]^-$ found (m/z)	Optimal conditions 20 MPa, 50 °C, and 244 min				
					SC-CO_2	SC-CO_2+2.5%EtOH	SC-CO_2+5%EtOH	SC-CO_2+10%EtOH	SC-CO_2 + 20%EtOH
Unknown 1	–	264; 292	–	265.002	Nd	0.68 ± 0.05[a]	0.81 ± 0.04[b]	1.48 ± 0.27[c]	3.05 ± 0.37[d]
Unknown 2	–	292	–	325.009	Nd	0.96 ± 0.09[a]	0.56 ± 0.01[b]	1.22 ± 0.23[c]	2.51 ± 0.04[d]
p-Hydroxybenzoic acid	$C_7H_6O_3$	288; 378	137.0322	137.0317	Nd	0.63 ± 0.04[a]	0.73 ± 0.01[a]	0.93 ± 0.06[b]	1.69 ± 0.39[c]
Benzoic acid	$C_7H_6O_2$	280; 220	121.0373	121.0370	0.52 ± 0.01[a]	6.53 ± 0.05[b]	7.92 ± 0.11[c]	8.69 ± 0.12[d]	7.41 ± 0.17[e]
p-coumaric acid	$C_9H_8O_3$	230; 300; 310	163.0478	163.0480	0.77 ± 0.01[a]	1.19 ± 0.00[b]	1.37 ± 0.01[c]	2.76 ± 0.08[d]	2.17 ± 0.06[e]
N-trans-caffeoyltyramine Isomer	$C_{17}H_{17}NO_4$	200; 284; 315	298.1158	298.1160	Nd	0.41 ± 0.01[a]	0.36 ± 0.00[b]	0.99 ± 0.02[c]	1.87 ± 0.18[d]
N-trans-caffeoyltyramine	$C_{17}H_{17}NO_4$	220; 250; 294; 318	298.1158	298.1160	Nd	3.46 ± 0.07[a]	4.29 ± 0.03[b]	14.92 ± 0.86[c]	50.32 ± 2.26[d]
N-caffeoyltyramine dimer hydroxy derivative	$C_{34}H_{34}N_2O_9$	254; 340	613.2269	613.2256	Nd	0.36 ± 0.03[a]	0.40 ± 0.06[a]	0.43 ± 0.02[a]	3.94 ± 1.07[b]
Cannabisin A	$C_{34}H_{30}O_8N_2$	256	593.2002	593.2016	Nd	1.77 ± 0.04[a]	1.87 ± 0.11[a]	3.79 ± 0.15[b]	13.72 ± 1.34[c]
Cannabisin B	$C_{34}H_{32}O_8N_2$	254; 284; 314; 334	595.2159	595.2155	Nd	2.70 ± 0.03[a]	3.96 ± 0.03[b]	7.63 ± 1.01[c]	16.11 ± 1.92[d]
N-trans-coumaroyltyramine	$C_{17}H_{17}O_3N$	292; 308	282.1208	282.1215	Nd	Nd	0.32 ± 0.02[a]	0.63 ± 0.11[b]	1.67 ± 0.55[c]
Cannabisin B Isomer 1	$C_{34}H_{32}O_8N_2$	264; 284; 314	595.2159	595.2155	Nd	Nd	Nd	1.67 ± 0.2[a]	2.61 ± 0.06[b]
Cannabisin B Isomer 2	$C_{34}H_{32}O_8N_2$	268; 310	595.2159	595.2161	Nd	Nd	Nd	0.84 ± 0.18[a]	1.21 ± 0.06[b]
N-feruloyltyramine	$C_{18}H_{19}O_4N$	256; 288; 318	312.1314	312.1320	Nd	0.56 ± 0.03[a]	0.91 ± 0.01[b]	1.19 ± 0.02[c]	10.75 ± 1.00[d]
Demethylgrossamide	$C_{35}H_{34}N_2O_8$	264; 284; 314; 322	609.2315	609.2322	Nd	Nd	Tr	0.77 ± 0.09[a]	3.60 ± 0.40[b]
Cannabisin C	$C_{35}H_{34}O_8N_2$	260; 280	609.2315	609. 2317	Nd	0.42 ± 0.02[a]	0.37 ± 0.09[a]	0.44 ± 0.03[a]	1.16 ± 0.11[b]
Cannabisin C Isomer	$C_{35}H_{34}O_8N_2$	284; 322	609.2315	609.2317	Nd	0.45 ± 0.00[a]	0.63 ± 0.04[b]	0.82 ± 0.08[b]	1.96 ± 0.75[c]
Cannabisin D	$C_{36}H_{36}N_2O_8$	260; 284; 308	623.2472	623.2480	Nd	Nd	Nd	0.65 ± 0.03[a]	2.64 ± 0.18[b]
3.3'-didemethylgrossamide	$C_{34}H_{32}N_2O_8$	284; 324	595.2159	595.2160	Nd	Nd	Tr	1.63 ± 0.17[a]	5.45 ± 0.19[b]
Tri-*p*-coumaroylspermidine	$C_{34}H_{37}N_3O_6$	260	582.2688	582.2695	Nd	Nd	Nd	0.91 ± 0.12[a]	1.80 ± 0.26[b]
Cannabisin E	$C_{36}H_{38}N_2O_9$	292; 310	641.2577	641.2571	Nd	Nd	Tr	1.07 ± 0.02[a]	1.64 ± 0.04[b]
Cannabisin M	$C_{34}H_{32}N_2O_8$	288; 322	595.2159	595.2155	Nd	Nd	Tr	1.95 ± 0.31[a]	10.23 ± 0.31[b]
3.3′-demethyl-heliotropamide	$C_{34}H_{32}N_2O_8$	285; 310	596.216	595.22	Nd	Nd	Tr	3.19 ± 0.14[a]	4.31 ± 0.10[b]
Cannabisin Q	$C_{34}H_{32}N_2O_8$	284; 308	595.2159	595.2150	Nd	Nd	Tr	1.39 ± 0.08[a]	1.83 ± 0.01[b]
Cannabisin F	$C_{36}H_{36}N_2O_8$	288; 312	623.2472	623.2470	Nd	Nd	0.78 ± 0.05[a]	0.85 ± 0.04[a]	2.23 ± 0.31[b]
Isocannabisin N	$C_{35}H_{34}N_2O_8$	284; 324	609.2315	609.2306	Nd	1.28 ± 0.10[a]	1.37 ± 0.08[a]	1.88 ± 0.10[b]	1.27 ± 0.08[a]
Grossamide	$C_{36}H_{36}N_2O_8$	250; 288; 320	623.2472	623.2481	Nd	1.42 ± 0.12[a]	2.87 ± 0.14[b]	2.47 ± 0.01[c]	9.05 ± 0.53[d]
Unknown 3	–	278	–	652.354	2.27 ± 0.06[a]	1.71 ± 0.05[b]	2.41 ± 0.03[a]	2.45 ± 0.08[a]	1.54 ± 0.05[b]
Continued									
Sinapic acid	$C_{11}H_{12}O_5$	276	223.0690	223.0682	3.18 ± 0.12[a]	3.40 ± 0.26[a]	3.23 ± 0.27[a]	3.20 ± 0.31[a]	2.13 ± 0.11[b]
Total hydroxybenzoic acid					0.52 ± 0.01[a]	7.16 ± 0.15[b]	8.65 ± 0.29[c]	9.62 ± 0.35[d]	9.10 ± 0.37[cd]
Total hydroxycinnamic acid					3.95 ± 0.11[a]	4.59 ± 0.12[b]	4.60 ± 0.12[b]	5.96 ± 0.35[c]	4.30 ± 0.24[ab]

Phenolic compounds	Molecular formula	λmax (nm)	[M − H]⁻ calc. (m/z)	[M − H]⁻ found (m/z)	Optimal conditions 20 MPa, 50 °C, and 244 min				
					SC-CO_2	SC-CO_2+2.5%EtOH	SC-CO_2+5%EtOH	SC-CO_2+10%EtOH	SC-CO_2 + 20%EtOH
Total phenolic acids					4.47 ± 0.12[a]	11.75 ± 0.17[b]	13.25 ± 0.41[c]	15.58 ± 0.51[d]	13.40 ± 0.44[c]
Total hydroxycinnamic acid amides					Nd	4.43 ± 0.16[a]	5.88 ± 0.43[b]	18.64 ± 1.17[c]	66.41 ± 1.35[d]
Total lignanamides					Nd	8.40 ± 0.16[a]	12.25 ± 0.70[b]	31.47 ± 1.12[c]	81.32 ± 1.93[d]
Total phenylpropanoids					Nd	12.83 ± 0.18[a]	18.13 ± 1.78[b]	50.11 ± 2.85[c]	147.73 ± 3.37[d]
Total phenolic compounds					**6.74 ± 0.27[a]**	**27.93 ± 0.42[b]**	**35.16 ± 2.80[c]**	**70.84 ± 3.91[d]**	**168.23 ± 5.28[e]**

Table 6. Effect of different ethanol proportions (2.5, 5, 10, and 20%) as a function of SC-CO_2 flow rate (0.25 kg/h) on the phenolic composition of hemp seed oil extracted by SFE under optimum extraction conditions (20 MPa, 50 °C, and 244 min). Data, which are the mean ± SD of three independent experiments ($n = 3$), were expressed as mg/kg oil. Mean values in the same line followed by a different letter are significantly different ($p < 0.05$). *Hydroxycinnamic acid amides and lignanamides are expressed in mg *N-trans*-caffeoyltyramine equivalent per kg of oil (mg CTE/Kg oil). *Nd* Not detected, *Tr* traces, *λmax* maximum aSbsorbance peak.

Notably, the inclusion of minute amounts of ethanol (2.5%) facilitated the extraction of several phenolics, such as *N-trans*-caffeoyltyramine, cannabisin A, B, C, isocannabisin N, grossamide. The disparity between extractions with and without co-solvents might be linked to the non-polarity of CO_2, enabling the solubilization of non-polar compounds in the oil. Owing to the polarity of phenolic compounds, the use of polar co-solvents renders the SFE an efficient method for obtaining phenolic-rich oil. Conversely, several compounds were undetected at 2.5% and 5% ethanol, such as demethylgrossamide, *tri-p*-coumaroylspermidine, cannabisin E, D, M, and Q, were undetected in 2.5 and 5% ethanol but become quantifiable with a 10% ethanol proportion. Analogous results were observed in the study of Quispe-Fuentes et al.[39], where SC-CO_2 without co-solvent extracted only hesperidin and naringin from *Clementina orogrande* peel. With the use of 5% and 10% ethanol, the extraction of these two compounds was improved along with the extraction of other phenolic compounds (chlorogenic, caffeic, sinapic, *trans*-ferulic acids, and rutin), resulting in the highest phenol values with 10% ethanol. This difference can be attributed to the different polarities of each phenolic compound. Hence, every change in polarity in the oil extraction system would consequently lead to the extraction of compounds proportionally to their polarities.

Tocopherols

Compared with pure CO_2, the co-solvent significantly increased the total tocopherol content of the oil, rising from 361.16 to 654.80 mg/kg for 0% and 20% ethanol, respectively (Table 5 and Supplementary Figure S4). Tocopherols possess long unsaturated aliphatic chains that contribute to their hydrophobic nature. Additionally, the absence of highly polar groups suggests that these compounds may have relatively higher solubility in SC-CO_2. Nevertheless, irrespective of polarity, their solubility is limited due to their high molecular weight. The presence of an entrainer (ethanol) facilitates their extraction, as it can help dissolve heavier substances in CO_2[27]. Similar results were reported by Jafarian Asl et al.[45], where the use of 95% CO_2 + 5% ethanol increased the tocopherol content in rapeseed oil compared with that of pure CO_2. Specifically, the addition of the co-solvent had a significant positive effect on δ- and γ-tocopherols. The γ-isomer increased from 292.10 to 576.63 mg/kg and the δ-isomer increased from 28.37 to 39.09 mg/kg for 0 and 20% ethanol, respectively. In contrast, the α-isomer decreased significantly from 42.79 to 37.08 mg/kg when 20% ethanol was used (Table 5). This observation aligns with the structural composition of α-tocopherol, which has three methyl groups, making it more hydrophobic than γ-tocopherol, with two methyl groups, and δ-tocopherol, with only one[46]. The maximum levels of total tocopherols in hemp oil extracted by SC-CO_2 observed in the literature range from 268 to 877 mg/kg oil[24,25,40], which are similar to those reported in the present study. Moreover, the maximum total tocopherol content recorded for the oil extracted with the 80% CO_2 + 20% ethanol mixture was greater than that reported in some studies that focused on solvent or mechanically extracted hemp seed oil from the same ecotype[3,4,31].

Pigments and color parameters

The addition of ethanol to CO_2 enabled efficient recovery of total chlorophylls, which increased from 9.16 without ethanol to 90.72 mg/kg with 20% ethanol (Table 5). Conversely, it decreased the total carotenoid content, decreasing from 31.79 to 11.68 mg/kg for 0% and 20% ethanol, respectively. Chlorophylls, mainly chlorophyll b with its polar aldehyde group, are considered more polar molecules than carotenoids[47]. This polarity could explain why supercritical mixtures of CO_2 and ethanol were effective in the extraction of chlorophylls. Analogous results were reported by Georgiopoulou et al.[48], where the use of 10% ethanol increased chlorophyll extraction from green algae by SC-CO_2. CO_2 was highly selective for carotenoids, with a carotenoid/chlorophyll ratio of 3.47. In agreement with our results, Pour Hosseini et al.[49] demonstrated the selectivity of pure CO_2 for carotenoids in *Dunaliella salina* oil, with a carotenoid/chlorophyll ratio of 11.09.

The addition of ethanol also modified the color parameters of the extracted oil. It increased the value of a* (from − 1.08 to -4.75) but decreased the values of b* and L* (from 20.81 to 1.53 and from 44.55 to 30.02, respectively). This could mainly be linked to the sharp increase in chlorophyll levels, which impart a green color,

along with the decrease in carotenoid content, which responsible for the yellow color, in oils extracted with the co-solvent[29,31].

Oxidative stability index

The OSI of the oil was also significantly improved by the addition of the co-solvent, increasing from 9.80 h without the co-solvent to 30.13 h with 5% ethanol (Table 5). As a result, the oil extracted with the application of the co-solvent showed good resistance to oxidation and a more than 3-fold better shelf life than the oil extracted with pure CO_2. However, a further increase in the percentage of co-solvent (10 and 20%) led to a decrease in oxidative stability. Although co-solvents improve the solubility of bioactive compounds, they also extract some undesirable compounds from the oil, notably chlorophyll, which acts as a sensitizer. When present in large quantities, chlorophyll can cause photo-oxidation of the oil, which negatively impacts its quality and shelf life[1].

The oxidative stability of hemp seed oil depends not only on the concentration of bioactive compounds but also on their ability to donate hydrogen atoms or electrons, thereby neutralizing free radicals and interrupting oxidative chain reactions. This property is closely linked to the chemical nature and molecular structure of these compounds. To further investigate this relationship, a correlation analysis was conducted between bioactive compounds and oxidative stability to identify the compounds that most significantly contribute to oxidative stability.

The data presented in supplementary Table S1 revealed a strong correlation between oxidative stability and isocannabisin N ($r = 0.847$; $p < 0.001$) as well as γ-tocopherol ($r = 0.794$; $p < 0.001$). Moderate positive correlations were also observed with other phenolic compounds, including cannabisin F ($r = 0.698$; $p = 0.004$), benzoic acid ($r = 0.690$; $p = 0.004$), cannabisin B ($r = 0.640$; $p = 0.010$), *p*-hydroxybenzoic acid ($r = 0.626$; $p = 0.013$), grossamide ($r = 0.526$; $p = 0.044$), and *N-trans*-coumaroyltyramine ($r = 0.518$; $p = 0.048$). These results suggest that increasing the content of phenolic compounds and γ-tocopherol significantly enhances oxidative stability.

These findings align with previous reports in the literature. For instance, Porto et al.[14,25] identified γ-tocopherol as the most active tocopherol isomer in hemp seed oil due to its ability to protect polyunsaturated fatty acids from oxidation. Additionally, several studies have demonstrated the strong antioxidant activity of phenolic compounds such as benzoic acid, *p*-hydroxybenzoic acid, *N-trans*-caffeoyltyramine, *N-trans*-coumaroyltyramine, and lignanamides, including cannabisin A and cannabisin B[50–52].

Fatty acid composition

The fatty acid composition analysis (Table 5 and Supplementary Figure S5) revealed that adding ethanol to the CO_2 flow in the four tested proportions did not significantly affect the fatty acid profile of the SFE-oil samples. Moreover, the n-6/n-3 ratio was also constant. This finding aligns with the study of Cornelio-Santiago et al.[53], which reported no significant changes in the fatty acid profiles of green coffee seed oils extracted with SC-CO_2, whether with or without a co-solvent. In contrast to our results, Devi & Khanam[54], reported that adding 5–10% ethanol to the CO_2 favored the extraction of linoleic and α-linolenic acids.

SFE-oils without or with co-solvent were mainly composed of unsaturated fatty acids (UFA), with 66.91 to 68.88% being polyunsaturated (PUFA) and 21.15 to 23.37% monounsaturated (MUFA). In contrast, a small quantity (10.47–11.48%) consisted of saturated fatty acids (SFA). GC analysis identified 10 fatty acids, with linoleic acid accounting for nearly half of the total composition. (49.80–50.76%), followed by oleic (19.12–21.41%), α-linolenic (16.12–17.18%), palmitic (6.09–6.71%), eicosenoic (1.95–2.15%), arachidic (1.10–1.27%), stearic (1.09–1.26%), γ-linolenic (0.89–1.10%), behenic (0.82–0.90%), and lignoceric (0.27–0.33%) acids. The n-6/n-3 ratio ranged from 3.01 to 3.25. This fatty acid composition confirms the typical profile of hemp seed oil reported in prior research, highlighting the dominance of linoleic, oleic and α-linolenic acids (Taiffi et al., 2021; Irakli et al., 2017; Farinon et al., 2019). Compared to our results, studies on SFE-oil reported higher proportions of linoleic acid (up to 59%), α-linolenic acid (up to 18%), and γ-linolenic acid (up to 3%) and lower proportions of oleic acid (approximately 10%)[15,24,25].

Conclusion

In the present study, the effects of temperature, pressure, and time on the oil yield, TPC, total tocopherols, OSI, total chlorophylls, total carotenoids, quality indices (free acidity, PV, CD and CT), and color parameters (L*, a*, b*) of SFE-oil were examined. The BBD was used as an experimental design, and ANOVA was employed to assess the effects of the three variables. Statistical analysis revealed that the pressure variation influenced mainly the oil yield, total tocopherols, and oxidative stability, whereas temperature strongly affected the phenol, carotenoid and chlorophyll contents. The extraction time term had little influence on most of the responses studied. Process optimization indicated that 50 °C, 20 MPa and 244 min were the optimum conditions for maximum yield and good oil quality, but resulted in low levels of bioactive compounds (phenols and tocopherols). The addition of 10% ethanol increased the oil's TPC by around 8 times and total tocopherol content by around 82%, significantly enhancing the oil's oxidation stability (approximately threefold-fold) and addressing the problem of low SFE efficiency. In addition, the use of 10% ethanol also improved the oil yield by approximately 2%. Finally, HPLC-DAD-MS^2 analysis of SFE-oils showed that ethanol has a determinant role in the presence or absence of phenolic compounds, as well as in their relative quantity in the extracted oils.

Materials and methods

Reagents and chemicals

The fluid used for SFE was a food-grade carbon dioxide (CO_2) with a 99.9% purity level, ensuring minimal contamination and making the process ideal for food-related applications. Standards of tocopherols (α, β, γ and δ-tocopherols), fatty acid methyl esters (37 component mixture), and phenolic compounds, including *N*-

trans-caffeoyltyramine and various phenolic acids (gallic, benzoic, *p*-coumaric, *p*-hydroxybenzoic, and sinapic acids), were obtained from Sigma-Aldrich. LC-MS grade acetonitrile and formic acid were obtained from Sigma-Aldrich as well. Other chemicals and organic solvents were procured from Merck and were of analytical grade, meeting strict purity requirements for accurate analytical procedures in both qualitative and quantitative analyses.

Plant material

Seeds of the Moroccan *Cannabis sativa* L. ecotype were provided by the *Agence Nationale des Plantes Médicinales et Aromatiques*. The *Cannabis* plants were cultivated in the spring of 2023 in the Jebeha region, in northern Morocco. The seeds were harvested in July at maturity, when 90% had turned brown. The genetic data of the Moroccan *Cannabis* ecotype used in this study are available in our recent publication[55]. A voucher specimen (HUMPOM242) was deposited in the herbarium of Mohammed I University. After cleaning, the seeds were placed in plastic bags and stored in a refrigerator (at 2–4 °C). The water content of the seeds was 5.13 ± 0.1%, as assessed following the outlined method of Mansouri et al.[31].

Supercritical fluid extraction of hemp seed oil

A pilot-scale SC-CO_2 unit (model: HSFE-1, Shanghai Better Industry Co., Ltd, China) was employed to extract hemp seed oil. This unit comprises a 10 cm diameter extractor with a 1-liter capacity (rated for up to 50 MPa), featuring a 500 mL extraction basket sealed with porous stainless-steel discs to prevent the escape of seed powder during the process. Additionally, it included two separators (0.6 L/30 MPa), a CO_2 high-pressure pump (50 L/50 MPa), a co-solvent pump (4 L/50 MPa), and a refrigeration system, including a refrigerator, CO_2 storage tank and coils. It also features a heating water tank along with a coil heat exchanger and display instruments for CO_2 flow measurement and temperature control.

To initiate the process, 100 g of ground seed powder was sieved through a 500 µm mesh sieve and loaded into the extractor tank. As depicted in supplementary Figure S1, CO_2 from the cylinder passed through valve V2 into the purifier, cooling coil, and storage tank, where it liquefied. Following the release of air from the extractor, the liquid CO_2 was pressurized to its supercritical state moving through the heat exchanger and piston pump before being directed into the extractor at the specified pressure and temperature settings outlined in Table 1. The resulting mixture of solubilized oil in SC-CO_2 was then conveyed from the extractor to the separators via the expansion valve, which reduced the pressure. This process lowers the SC-CO_2 solvation power, allowing the separation of oil and the transition of CO_2 to its gaseous state. The gaseous CO_2 was then recycled, passed through the heat exchanger and stored in the storage vessel. During the extraction process, the pressure control valves V6, V9 and V1 regulate the pressure in the extractor and separators I & II, respectively. Measurements of temperatures and pressures in the unit were recorded, with uncertainties of ± 0.1 °C and ± 0.1 MPa, respectively. The temperature and pressure of the two separators were set at 25 °C and 5 MPa, respectively. The CO_2 flow rate was fixed at 0.25 kg/h. After the extraction process was complete, the CO_2 high-pressure pump was deactivated, and the pressure in the extractor was reversed via valve V7 to the separators and storage tank. The extracted oil was collected through the lower valve of the first separator. For CO_2 extraction with co-solvent (ethanol), adjustments were made to the two pumps to maintain a final CO_2 flow rate of 0.25 kg/h. The co-solvent was collected with the oil in glass bottles and removed under vacuum at 40 °C via a rotary evaporator. Finally, the oil was weighed and the oil yield, calculated in grams of oil per 100 g of raw matter, was determined using Eq. (1). All extraction processes were performed in triplicate, and the mean values were recorded. The collected oil was placed in dark vials, stored under nitrogen, and kept at -20 °C for 2 weeks before analysis.

$$\text{Oil yield } (\%) = \frac{\text{Weight of collected oil}}{\text{Weight of freshly ground seed}} \text{x } 100 \tag{1}$$

Experimental methodology

This research was structured in two parts. The initial part investigated the impact of pressure, temperature, extraction time, and their interactions on the yield, TPC, total tocopherols, OSI, total chlorophylls, total carotenoids, quality indices (PV and specific extinction coefficients), and color (L*, a*, b*) of SC-CO_2-extracted hemp seed oil. The subsequent part focused on the effect of co-solvent addition on the same responses and phenolic profiles of the extracted oils under optimal SFE conditions.

The first part consisted on analysis via response surface methodology (RSM) to establish the optimum conditions for extracting oil from hemp seeds by SFE. Considering data from the literature[15,13,14], the results of preliminary experiments conducted in our laboratory, as well as the specific performance of the SC-CO_2 apparatus used for hemp seed oil extraction, we selected three key variables, temperature, pressure and extraction time. These parameters were chosen because of their significant impact on oil yield, with defined ranges of 30 to 60 °C, 10 to 20 MPa, and 120 to 300 min, respectively. Experimental tests were carried out on the basis of a BBD design. A total of $N = 2k\ (k\text{-}1) + C_0$ was obtained for the 3 parameters, where k is the number of factors and C_0 is the number of center points. As shown in Table 1, the overall design comprises 14 experimental series with 2 center points and 12 edge points. A second-order polynomial model was employed to approximate the mathematical relationships among the three parameters. This model includes terms up to the second power of the independent variables, allowing it to capture potential curvature in the data and provide a more accurate fit than a simple linear model. In general, a second-order polynomial model with multiple predictors can be expressed as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j \tag{2}$$

In this model, Y represents the predicted response; X_i and X_j denote the levels of the independent variables; β_0 is a constant; and β_i, β_{ii} and β_{ij} correspond, respectively, to the linear, quadratic and interaction coefficients. By including these quadratic and interaction terms, the model accounts for nonlinear effects and interactions between the parameters, providing a more nuanced understanding of their relationships.

Once the SFE parameters had been optimized, the second part of this research was conducted to study the impact of co-solvent addition on the study responses and phenolic composition of the extracted oils. To this end, extractions were performed under optimal conditions corresponding to 20 MPa pressure, 50 °C temperature for 244 min (obtained in the first part), and ethanol was used as co-solvent at different percentages (2.5, 5, 10, and 20%). These percentages were expressed relative to a constant flow rate of CO_2 (0.25 kg/h).

Physicochemical analysis of SFE-oils

Tocopherols

Tocopherol analysis was carried out using a Shimadzu LC-6AD system (Shimadzu, Japan) equipped with a diode array detector (DAD). A solution of hexane and isopropanol (99/1; v/v) was used as the mobile phase with a flow rate of 1 mL/min to achieve separation on an Uptisphere NH_2 column (250 × 4.6 mm, particle size 5 µm), following the protocol outlined by Allay et al.[56]. The identification and quantification of tocopherols were performed at 292, 296, and 298 nm using commercial tocopherol standards (α, β, γ and δ-tocopherols). The concentration of tocopherols was determined on the basis of an external calibration curve of mixtures of α, β, γ and δ-tocopherols with concentrations of 10–360, 1–36, 50–1400, and 20–540 µg/g, respectively.

Total chlorophylls and carotenoids

The pigment content (carotenoids and chlorophylls) of the SFE-oil samples was determined according to the method reported by Aachary et al.[57]. The oil was dissolved in absolute diethyl ether (0.1 g/5mL). The solution was then thoroughly vortexed and subjected to ultrasonication for 1 min. The absorbance of the mixture was then measured at 470 nm for carotenoids and at 640 and 663 nm for chlorophylls. The carotenoid and chlorophyll contents were calculated via the Eqs. (2) and (3), respectively, and expressed in milligrams of pigment per kilogram of oil (mg/kg).

$$Chlorophylls\ a+b\ (mg/kg) = \frac{7.12\ \times\ A_{663}\ +\ 16.80\ \times\ A_{640}}{W} \tag{3}$$

$$Total\ Carotenes\ (mg/kg) = \frac{(1000\ \times\ A_{470}\ -0.52\times\ Chly\ a\ -7.25\ \times\ Chly\ b)}{226\ \times\ W} \tag{4}$$

where A refers to the absorbance at specific wavelengths (470, 640, and 663 nm) and W corresponds to the weight of the oil.

Colorimetric analysis

The color of the SFE-oil samples was objectively described based on the CIELAB parameters (L*, a*, b*). L* is a measure of the sample lightness, whereas a* and b* are the chromatic coordinates on the green-red and blue-yellow axis, respectively. After sample homogenization, measurements were made directly via a chromameter (KONIKA MINOLTA Chroma Meter CR-410 measurement area ⌀ 50 mm) with a silicon photocell detector.

Total phenolic content

SFE-oils were used to prepare phenolic extracts in 80% methanol at a 1:1 ratio (1 g oil: 1 mL 80% methanol). The extraction was performed in centrifuge tubes by shaking the mixture for 5 min. The tubes were then centrifuged at 5034 g for 10 min and the methanolic layer was recovered. To measure the TPC, 100 µL of each extract was added to 1 mL of 10% Folin-Ciocalteu reagent and 1 mL of a 10% aqueous Na_2CO_3 solution[58]. The mixture was shaken and incubated in the dark for 1 h 30 min. Absorbance was read with a UV-visible spectrophotometer at 760 nm. A calibration curve was generated with gallic acid at concentrations ranging from 10 to 120 µg/mL, and the results are presented as mg gallic acid equivalent per kg hemp seed oil (mg GAE/kg).

HPLC-DAD/ESI-MS^2 analysis of phenolic compounds

For the analysis of phenolic compounds, methanolic extracts of the oil samples were injected into an Agilent 1260 Infinity II high-performance liquid chromatography system (Agilent Technologies, USA) equipped with a diode array detector (DAD, Agilent Technologies 1200 Series). Phenolic separation occurred on a C18 column (120 × 4.6 mm, 3.5 mm particle size) and was monitored at several wavelengths (254, 280, 300, and 340 nm) following the protocol detailed in our previous publication[59]. The separated compounds were further analyzed using a Bruker Esquire HCT mass spectrometer (Germany), which features an electrospray ionization (ESI) source and functions in both negative and positive modes[59]. Esquire Control software ensured the instrument control and data acquisition, whereas ACD/labs 2021 allowed for data processing. The identification was based on comparisons of the molecular ion mass of each compound, its fragment ions, and its UV spectrum with literature data[42,44,59]. The identified compounds were then quantified on the HPLC-DAD chromatogram at 280 nm. Phenolic compound profiles were analyzed using Agilent OpenLAB CDS software. Owing to the restricted availability of standards for most phenolic compounds in hemp seeds, an external calibration curve of *N-trans*-caffeoyltyramine (2.62–84 µg/mL) was used to express all the results as mg *N-trans*-caffeoyltyramine equivalent (CTE) per kg of oil. In contrast, hydroxybenzoic acid (6.25–40 µg/mL), benzoic acid (6.25–40 µg/mL), *p*-coumaric acid (6.25–40 µg/mL), and sinapic acid (6.25–40 µg/mL) were quantified using commercial standards.

GC-MS analysis of fatty acids

Fatty acids in the SFE-oils were analyzed via gas chromatography-mass spectrometry (GC- MS). The fatty acids were first transformed to fatty acid methyl esters (FAME) using method 1 K-07 AOCS 2007 (AOCS, 2007). Specifically, SFE-oils without and with co-solvent were dissolved in n-hexane (0.1 g/mL). Then, 0.1 mL of 2 N KOH in methanol was added. After stirring and standing for 2 min, 2 mL of a saturated NaCl solution was added, and the upper phase collected. The FAME were then injected into a GC-MS 1300/TS Q8000 Evo THERMO system with an ion trap mass analyzer. Separation was performed on a TR-5 capillary column (30 m long, 0.25 mm internal diameter, and 0.25 µm film thickness) with 5% phenyl methyl polysiloxane. Injection was performed at 250 °C in fractionated mode, using an injection volume of 1 µL. The oven temperature was initially set at 120 °C for 1 min, then increased to 180 °C at 3 °C/min and held for 15 min, followed by an increase to 240 °C at 5 °C/min and held for 25 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. After separation, the FAME were introduced into the mass spectrometer, where electron impact ionization (EI) occurred at 70 eV, with an electron multiplier of 900 V and an ion source temperature of 200 °C. Mass spectra were recorded in the m/z range of 40–500, with an interface temperature of 250 °C and a scan speed of 1666 U/sec. Each sample was analyzed in triplicate, and FAME peaks were identified on the basis of mass spectra from the NIST database, literature references, and retention times of a 37-component FAME standard mixture. The results are reported as percentages of total fatty acids.

Oil quality indices

Free acidity (%), PV (meq O_2/kg), and CD and CT (λ 232 and λ 270 nm) of SFE-oils were analyzed following the official European methods of virgin olive oil CEE/2568/91[60].

Assessment of the oxidative stability index

To determine the OSI of SFE-oils, 3 ± 0.01 g of oil was oxidized in a Rancimat apparatus (Metrom Rancimat 743, Metrom Co., Basel, Switzerland). The conditions used to oxidize the oil were 20 L/h air flow and 100 °C temperature. The OSI is presented as the induction time (hour).

Statistical analysis

RSM was employed to analyze the experimental data using Statistica software version 10.0 (StatSoft Inc., USA) and JMP Pro 17 (SAS Institute Inc., USA). Regression analysis was performed to assess the linear, quadratic, and interaction effects of the independent variables, with the results visualized via a standardized Pareto chart. The regression coefficients of various models and their impacts were determined through ANOVA with a significance threshold of 5%. Furthermore, three-dimensional (3D) response surfaces were generated based on the established mathematical models to illustrate the interactive influences of significant process parameters on the responses. Moreover, to assess the effect of co-solvent percentages after SFE optimization, a single-factor ANOVA followed by Tukey's test was performed using JMP Pro 17. Finally, Pearson's correlation coefficient was used between bioactive compounds and oxidative stability of extracted oils.

Data availability

The data that support this study's findings are available from the corresponding author upon request.

Received: 1 December 2024; Accepted: 20 February 2025
Published online: 12 March 2025

References

1 Farinon, B., Molinari, R., Costantini, L. & Merendino, N. The seed of industrial hemp (*Cannabis sativa* L.): Nutritional quality and potential functionality for human health and nutrition. *Nutrients* **12** (7), 1–60 https://doi.org/10.3390/nu12071935 (2020).
2 Irakli, M., Tsaliki, E., Kalivas, A., Kleisiaris, F. & Sarrou, E. Effect of genotype and growing year on the nutritional, phytochemical, and antioxidant properties of industrial hemp (*Cannabis sativa L.*) seeds. In *Antioxid. J.* 20–25. (2019).
3 Taaifi, Y. et al. Seed composition of non-industrial hemp (*Cannabis sativa* L.) varieties from four regions in Northern Morocco. *Int. J. Food Sci. Technol.* https://doi.org/10.1111/ijfs.15136 (2021).
4 Allay, A. et al. Enhancing bioactive compound extractability and antioxidant properties in hemp seed oil using a ternary mixture approach of Polar and non-polar solvents. *Ind. Crops Prod.* **219**, 119090. https://doi.org/10.1016/J.INDCROP.2024.119090 (2024).
5 Belhaj, K. et al. Fatty acids, health lipid indices and cholesterol content of four sheep meats from moroccan pastures. *Archiv Für Tierzucht* **63**, 471–482. https://doi.org/10.5194/aab-63-471-2020 (2020).
6 Ahangari, H., King, J. W., Ehsani, A. & Yousefi, M. Supercritical fluid extraction of seed oils—A short review of current trends. *Trends Food Sci. Technol.* **111**, 249–260. https://doi.org/10.1016/j.tifs.2021.02.066 (2021).
7 Nde, D. B. & Anuanwen, C. F. Optimization methods for the extraction of vegetable oils: A review. *Processes* **8** (2), https://doi.org/10.3390/pr8020209 (2020).
8 Occhiuto, C. et al. Comparative evaluation of the nutrients, phytochemicals, and antioxidant activity of two hempseed oils and their byproducts after cold pressing. *Molecules* **27** (11). https://doi.org/10.3390/molecules27113431 (2022).
9 Latif, S. & Anwar, F. Physicochemical studies of hemp (*Cannabis sativa* L.) seed oil using enzyme-assisted cold-pressing. *Eur. J. Lipid Sci. Technol. Innovat. Resour. Lipids Oils Res.* 1042–1048. https://doi.org/10.1002/ejlt.200900008 (2009).
10 De Melo, M. M. R., Silvestre, A. J. D. & Silva, C. M. Supercritical fluid extraction of vegetable matrices: applications, trends and future perspectives of a convincing green technology. *J. Supercrit. Fluids* **92**, 115–176. https://doi.org/10.1016/J.SUPFLU.2014.04.007 (2014).
11 Parzanese, I. et al. Celiac disease: from pathophysiology to treatment. *World J. Gastrointest. Pathophysiol.* **8** (2), 27. https://doi.org/10.4291/wjgp.v8.i2.27 (2017).
12 Yerena-Prieto, B. J. et al. Evaluation of the effect of different co-solvent mixtures on the supercritical CO_2 extraction of the phenolic compounds present in *Moringa oleifera* lam. leaves. *Agronomy* **12** (6). https://doi.org/10.3390/agronomy12061450 (2022).
13 Devi, V. & Khanam, S. Comparative study of different extraction processes for hemp (*Cannabis sativa* L.) seed oil considering physical, chemical and industrial-scale economic aspects. *J. Clean. Prod.* **207**, 645–657. https://doi.org/10.1016/j.jclepro.2018.10.036 (2019a).

14 Porto, C., Da, Decorti, D. & Natolino, A. Potential oil yield, fatty acid composition, and oxidation stability of the hempseed oil from four *Cannabis sativa* L. cultivars. *J. Diet. Suppl.* **12**, 1–10. https://doi.org/10.3109/19390211.2014.887601 (2015).
15 Da Porto, C., Voinovich, D., Decorti, D. & Natolino, A. Response surface optimization of hemp seed (*Cannabis sativa* L.) oil yield and oxidation stability by supercritical carbon dioxide extraction. *J. Supercrit. Fluids* **68**, 45–51. https://doi.org/10.1016/j.supflu.2012.04.008 (2012).
16 Ferrentino, G. et al. Supercritical fluid extraction of oils from apple seeds: process optimization, chemical characterization and comparison with a conventional solvent extraction. *Innovat. Food Sci. Emerg. Technol.* 64. https://doi.org/10.1016/j.ifset.2020.102428 (2020).
17 Sodeifian, G., Ardestani, N. S., Sajadian, S. A. & Moghadamian, K. Properties of portulaca oleracea seed oil via supercritical fluid extraction: experimental and optimization. *J. Supercrit. Fluids* **135**, 34–44. https://doi.org/10.1016/J.SUPFLU.2017.12.026 (2018).
18 Santos, D. T. & Meireles, M. A. A. Optimization of bioactive compounds extraction from Jabuticaba (*Myrciaria cauliflora*) skins assisted by high pressure CO_2. *Innovat. Food Sci. Emerg. Technol.* **12** (3), 398–406. https://doi.org/10.1016/J.IFSET.2011.02.004 (2011).
19 Ahmadian-Kouchaksaraie, Z. & Niazmand, R. Supercritical carbon dioxide extraction of antioxidants from Crocus sativus petals of saffron industry residues: optimization using response surface methodology. *J. Supercrit. Fluids* **121**, 19–31. https://doi.org/10.1016/j.supflu.2016.11.008 (2017).
20 Gelmez, N., Kincal, N. S. & Yener, M. E. Optimization of supercritical carbon dioxide extraction of antioxidants from roasted wheat germ based on yield, total phenolic and tocopherol contents, and antioxidant activities of the extracts. *J. Supercrit. Fluids* **48** (3), 217–224. https://doi.org/10.1016/j.supflu.2008.11.002 (2009).
21 Buranachokpaisan, K., Muangrat, R. & Chalermchat, Y. Supercritical CO_2 extraction of residual oil from pressed Sesame seed cake: optimization and its physicochemical properties. *J. Food Process. Preserv.* **45** (9). https://doi.org/10.1111/jfpp.15722 (2021).
22 Muangrat, R. & Jirarattanarangsri, W. Physicochemical properties and antioxidant activity of oil extracted from Assam tea seeds (*Camellia sinensis* Var. assamica) by supercritical CO_2 extraction. *J. Food Process. Preserv.* **44** (3). https://doi.org/10.1111/jfpp.14364 (2020).
23 De, O., Silva, L., Ranquine, L. G., Monteiro, M. & Torres, A. G. Pomegranate (*Punica granatum* L.) seed oil enriched with conjugated linolenic acid (cLnA), phenolic compounds and tocopherols: improved extraction of a specialty oil by supercritical CO_2. *J. Supercrit. Fluids* **147**, 126–137. https://doi.org/10.1016/j.supflu.2019.02.019 (2019).
24 Grijó, D. R., Piva, G. K., Osorio, I. V. & Cardozo-Filho, L. Hemp (*Cannabis sativa* L.) seed oil extraction with pressurized n-propane and supercritical carbon dioxide. *J. Supercrit. Fluids* **143**, 268–274. https://doi.org/10.1016/j.supflu.2018.09.004 (2019).
25 Aladić, K. et al. Supercritical CO_2 extraction of hemp (*Cannabis sativa* L.) seed oil. *Ind. Crops Prod.* **76**, 472–478. https://doi.org/10.1016/J.INDCROP.2015.07.016 (2015).
26 Sun, Q., Shi, J., Scanlon, M., Xue, S. J. & Lu, J. Optimization of supercritical-CO_2 process for extraction of tocopherol-rich oil from canola seeds. *LWT- Food Sci. Technol.* **145**, 111435 (2021).
27 De Andrade Lima, M., Charalampopoulos, D. & Chatzifragkou, A. Optimisation and modelling of supercritical CO_2 extraction process of carotenoids from carrot peels. *J. Supercrit. Fluids* **133**, 94–102. https://doi.org/10.1016/j.supflu.2017.09.028 (2018).
28 Ndayishimiye, J. & Chun, B. S. Optimization of carotenoids and antioxidant activity of oils obtained from a co-extraction of citrus (Yuzu ichandrin) by-products using supercritical carbon dioxide. *Biomass Bioenerg.* **106**, 1–7. https://doi.org/10.1016/j.biombioe.2017.08.014 (2017).
29 Matthäus, B. & Brühl, L. Virgin hemp seed oil: an interesting niche product. *Eur. J. Lipid Sci. Technol.* **110** (7), 655–661. https://doi.org/10.1002/EJLT.200700311 (2008).
30 Ramos-Escudero, F. et al. Oil from three aguaje morphotypes (*Mauritia flexuosa* L.f.) extracted by supercritical fluid with CO_2: Chemical composition and chromatic properties. *Front. Sustain. Food Syst.* *6* https://doi.org/10.3389/fsufs.2022.843772 (2022).
31 Mansouri, F. et al. Laboratory-scale optimization of hemp seed roasting temperature and time for producing a high-quality pressed oil. *J. Food Process. Preserv.* **2023**, (2023).
32 Barros, M., Fleuri, L. F. & MacEdo, G. A. Seed lipases: sources, applications and properties—A review. *Braz. J. Chem. Eng.* **27** (1), 15–29. https://doi.org/10.1590/S0104-66322010000100002 (2010).
33 Gerola, A. P. et al. Properties of chlorophyll and derivatives in homogeneous and microheterogeneous systems. *J. Phys. Chem. B* **115** (22), 7364–7373. https://doi.org/10.1021/JP201278B (2011).
34 Terpinc, P., Čeh, B., Ulrih, N. P. & Abramovič, H. Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. *Ind. Crops Prod.* **39** (1), 210–217. https://doi.org/10.1016/j.indcrop.2012.02.023 (2012).
35 Bhatnagar, A. S. & Gopala Krishna, A. G. Effect of extraction solvent on oil and bioactives composition of commercial Indian niger (Guizotia abyssinica (L.f.) Cass.) seed. *JAOCS J. Am. Oil Chem. Soc.* **90** (8), 1203–1212. https://doi.org/10.1007/s11746-013-2263-7 (2013).
36 Russin, T. A., Boye, J. I., Arcand, Y. & Rajamohamed, S. H. Alternative techniques for defatting soy: A practical review. *Food Bioproc. Technol.* **4** (2), 200–223. https://doi.org/10.1007/s11947-010-0367-8 (2011).
37 Athanasiadis, V. et al. Evaluation of the efficacy and synergistic effect of α- and δ-tocopherol as natural antioxidants in the stabilization of sunflower oil and olive pomace oil during storage conditions. *Int. J. Mol. Sci.* **24** (2). https://doi.org/10.3390/ijms24021113 (2023).
38 Tura, M., Mandrioli, M., Valli, E. & Gallina Toschi, T. Quality indexes and composition of 13 commercial hemp seed oils. *J. Food Compos. Anal.* **117**, 105112. https://doi.org/10.1016/J.JFCA.2022.105112 (2023).
39 Quispe-Fuentes, I., Uribe, E., López, J., Contreras, D. & Poblete, J. A study of dried mandarin (*Clementina orogrande*) peel applying supercritical carbon dioxide using co-solvent: influence on oil extraction, phenolic compounds, and antioxidant activity. *J. Food Process. Preserv.* **46** (1), e16116. https://doi.org/10.1111/JFPP.16116 (2022).
40 Aiello, A. et al. Effects of supercritical and liquid carbon dioxide extraction on hemp (*Cannabis sativa* L.) seed oil. *Int. J. Food Sci. Technol.* **55** (6), 2472–2480. https://doi.org/10.1111/ijfs.14498 (2020).
41 Benkirane, C. et al. Phenolic profiles of non-industrial hemp (*Cannabis sativa* L.) seed varieties collected from four different moroccan regions. *Int. J. Food Sci. Technol.* **58** (3), 1367–1381. https://doi.org/10.1111/ijfs.16298 (2023).
42 Leonard, W., Zhang, P., Ying, D., Xiong, Y. & Fang, Z. Extrusion improves the phenolic profile and biological activities of hempseed (*Cannabis sativa* L.) hull. *Food Chem.* 346. https://doi.org/10.1016/j.foodchem.2020.128606 (2021).
43 Nigro, E. et al. Hempseed lignanamides rich-fraction: chemical investigation and cytotoxicity towards U-87 glioblastoma cells. *Molecules* **25** (5). https://doi.org/10.3390/molecules25051049 (2020).
44 Moccia, S. et al. Antiproliferative and antioxidant effect of Polar hemp extracts (*Cannabis sativa* L., Fedora cv.) in human colorectal cell lines. *Int. J. Food Sci. Nutr.* **71** (4), 410–423. https://doi.org/10.1080/09637486.2019.1666804 (2020).
45 Jafarian Asl, P., Niazmand, R. & Yahyavi, F. Extraction of phytosterols and tocopherols from rapeseed oil waste by supercritical CO_2 plus co-solvent: A comparison with conventional solvent extraction. *Heliyon* **6** (3). https://doi.org/10.1016/j.heliyon.2020.e03592 (2020).
46 Kurutas, E. B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutr. J.* **15** (1), https://doi.org/10.1186/s12937-016-0186-5 (2016).
47 Mandal, R. & Dutta, G. From photosynthesis to biosensing: chlorophyll proves to be a versatile molecule. *Sens. Int.* **1**, 100058. https://doi.org/10.1016/J.SINTL.2020.100058 (2020).

48 Georgiopoulou, I., Tzima, S., Louli, V. & Magoulas, K. Process optimization of microwave-assisted extraction of chlorophyll, carotenoid and phenolic compounds from Chlorella vulgaris and comparison with conventional and supercritical fluid extraction. *Appl. Sci. (Switzerland)* **13** (4). https://doi.org/10.3390/app13042740 (2023).
49 Pour Hosseini, S. R., Tavakoli, O. & Sarrafzadeh, M. H. Experimental optimization of SC-CO_2 extraction of carotenoids from Dunaliella salina. *J. Supercrit. Fluids* **121**, 89–95. https://doi.org/10.1016/J.SUPFLU.2016.11.006 (2017).
50 Chen, T. et al. The isolation and identification of two compounds with predominant radical scavenging activity in hempseed (seed of *Cannabis sativa* L). *Food Chem.* **134** (2), 1030–1037. https://doi.org/10.1016/j.foodchem.2012.03.009 (2012).
51 Yan, X. et al. Characterization of lignanamides from hemp (*Cannabis sativa* L.) seed and their antioxidant and acetylcholinesterase inhibitory activities. *J. Agric. Food Chem.* **63** (49), 10611–10619. https://doi.org/10.1021/ACS.JAFC.5B05282 (2015).
52 Zhou, Y., Wang, S., Ji, J., Lou, H. & Fan, P. Hemp (*Cannabis sativa* L.) seed phenylpropionamides composition and effects on memory dysfunction and biomarkers of neuroinflammation induced by lipopolysaccharide in mice. *ACS Omega* **3** (11), 15988–15995. https://doi.org/10.1021/acsomega.8b02250 (2018).
53 Cornelio-Santiago, H. P., Gonçalves, C. B., de Oliveira, N. A. & de Oliveira, A. L. Supercritical CO_2 extraction of oil from green coffee beans: solubility, triacylglycerol composition, thermophysical properties and thermodynamic modelling. *J. Supercrit. Fluids* **128**, 386–394. https://doi.org/10.1016/J.SUPFLU.2017.05.030 (2017).
54 Devi, V. & Khanam, S. Study of ω-6 Linoleic and ω-3 α-linolenic acids of hemp (*Cannabis sativa* L.) seed oil extracted by supercritical CO_2 extraction: CCD optimization. *J. Environ. Chem. Eng.* **7** (1), 1–10. https://doi.org/10.1016/j.jece.2018.102818 (2019b).
55 Benkirane, C. et al. Population structure and genetic diversity of Moroccan cannabis (*Cannabis sativa* L.) germplasm through simple sequence repeat (SSR) analysis. *Genet. Resour. Crop Evol.* **71**, 2037–2051. https://doi.org/10.1007/s10722-023-01754-x (2024).
56 Allay, A. et al. Combined effect of protease, hemicellulase and pectinase on the quality of hemp seed oil (*Cannabis sativa* L.) obtained by aqueous enzymatic extraction as an Eco-friendly method. *J. Oleo Sci.* **73** (7), 963–976. https://doi.org/10.5650/jos.ess24031 (2024).
57 Aachary, A. A., Liang, J., Hydamaka, A., Eskin, N. A. M. & Thiyam-Holländer, U. A new ultrasound-assisted bleaching technique for impacting chlorophyll content of cold-pressed hempseed oil. *LWT* **72**, 439–446. https://doi.org/10.1016/j.lwt.2016.05.011 (2016).
58 Mansouri, F. et al. Flavor profiles of monovarietal virgin olive oils produced in the oriental region of Morocco. *OCL Oilseeds Fats Crops Lipids* **24**, A202. https://doi.org/10.1051/ocl/2017012 (2017).
59 Benkirane, C. et al. Bioactive compounds from hemp (*Cannabis sativa* L.) seeds: optimization of phenolic antioxidant extraction using simplex lattice mixture design and HPLC-DAD/ESI-MS2 analysis. *RSC Adv.* **12** (39), 25764–25777. https://doi.org/10.1039/d2ra04081f (2022).
60 Mansouri, F. et al. H., Quality and chemical profiles of virgin olive oils of three European cultivars suitable for super-high-density planting conditions in Eastern Morocco. *Mater. Today Proc.* **13**, 998–1007. https://doi.org/10.1016/J.MATPR.2019.04.065 (2019).

Author contributions

(A) A. contributed to writing the original draft, validation, investigation, formal analysis, and data curation. C. (B) was involved in writing the original draft and conducting investigations. A. B. M. participated in validation and formal analysis. M. L. F. provided resources, secured funding, and contributed to formal analysis. H. B. was responsible for conducting the supercritical extraction. J. N. performed statistical analyses and contributed to writing, reviewing, and editing. H. S. (C) assisted with writing, reviewing, editing, and supervision. A. E. contributed to visualization, project administration, and funding acquisition. F. M. contributed to the study through writing, reviewing, visualization, supervision, methodology development, statistical analyses, and conceptualization.

Funding

This research was supported by: the Moroccan Ministry of Higher Education, Scientific Research and Innovation (VPMA2/ref.2020/1 project); the Wallonie-Brussels International (Ref. SOR/2021/493665). Also, this action benefited from the support of the Chair "Sustainable Energy" led by Mohammed VI Polytechnic University, sponsored by OCP.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-91441-x.

Correspondence and requests for materials should be addressed to J.N.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.