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Extraction technologies for hemp seed oil: a comparative study of yield, bioactive compound extractability, and oxidative stability

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An integrated comparison of four extraction methods was carried out to evaluate their performance in terms of hemp seed oil yield, physicochemical characteristics, and bioactive compound extractability. The methods included Soxhlet extraction (SOX-HPE) using a ternary solvent system (hexane/ethyl acetate/2-propanol; 4/4/2), microwave-assisted extraction (MAE) with 7.5% ethanol, supercritical fluid extraction (SFE) with 10% ethanol, and mechanical extraction (ME). Microstructural changes in the seed powders were also examined using scanning electron microscopy to better understand the mechanisms of cell wall disruption. SOX-HPE provided the highest oil yield (33.24%), followed by MAE and SFE (30.69 and 30.51%, respectively). HPLC-DAD/ESI-MS² analysis identified 29 phenolic compounds, with the highest total concentrations observed in oils from SOX-HPE and SFE (131.29 and 71.92 mg kg⁻¹ oil, respectively), including elevated levels of *N-trans*-caffeoyltyramine (39.98 and 14.92 mg kg⁻¹ oil), cannabins B (10.79 and 7.63 mg kg⁻¹ oil) and A (7.98 and 3.79 mg kg⁻¹ oil), which contributed to excellent oxidative stability (37.77 and 28.01 hours, respectively at 100 °C). SFE oil was also rich in unsaturated fatty acids (90.03%) and exhibited favorable oil quality indices. MAE produced oil enriched in tocopherols (510.66 mg kg⁻¹ oil), chlorophylls (99.68 mg kg⁻¹ oil), and carotenoids (27.31 mg kg⁻¹ oil). These findings highlight the importance of selecting appropriate extraction techniques to optimize both the nutritional quality and functional potential of hemp seed oil. In particular, SFE, especially when combined with a green polar co-solvent, emerged as an efficient and environmentally sustainable method for producing high-quality hemp seed oil. The use of less toxic polar solvents in Soxhlet extraction also demonstrated promising potential for enhancing bioactive compound recovery while reducing solvent-related hazards.

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Sustainability spotlight

This research promotes sustainable food innovation by comparing extraction techniques for producing hemp seed oil. The study evaluates the use of safer polar solvents in Soxhlet extraction and alternative sustainable approaches such as supercritical CO₂ and microwave-assisted extraction. Hemp seeds are valorized as a sustainable source of functional lipids and bioactive compounds. This work supports SDG 12, SDG 9, and SDG 3 by offering scalable extraction strategies that enhance the nutritional value of plant-based products while reducing environmental impact in post-harvest processing.

1. Introduction

The growing interest in vegetable oils as raw materials for food and industrial applications has increasingly attracted scientific attention. Consequently, research efforts have been directed

toward identifying, characterizing, developing, and utilizing these beneficial vegetable oils, alongside the exploration of cost-effective production and extraction methods. To meet the nutritional demands of the expanding global population, it is imperative to explore a broader spectrum of vegetable oils, including hemp seed oil.

Hemp seed oil is characterized by its high nutritional value and favorable fatty acid composition, comprising mainly polyunsaturated fatty acids, particularly omega-6 linoleic and omega-3 α -linolenic acids, at a healthy ratio.^{1–3} This composition confers cardiovascular and anti-inflammatory benefits. It is also rich in vitamin E, which enhances its antioxidant properties and contributes to its stability.^{2,4} Although phenolic

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compounds also possess antioxidant properties, their relatively low concentration in hemp seed oil limits their effectiveness compared to tocopherols.^{4–6} Moderate levels of chlorophylls, carotenoids, and phytosterols further increase the health-promoting attributes of the oil.⁷ Its characteristic nutty, earthy flavor makes it suitable for culinary, cosmetic, and industrial uses. However, its susceptibility to oxidation necessitates careful storage under cool, dark conditions. Sustainable cultivation practices combined with its versatile application position hemp seed oil as a valuable resource in the food, health, and industrial sectors.

Different extraction methods significantly impact the yield, quality, and chemical profile of hemp seed oil. Mechanical extraction (ME) *via* screw pressing is favored for its operational simplicity, minimal environmental impact, and ability to produce of high-quality pressed oil, although at lower yields.^{8–10} Solvent extraction frequently employs hexane as a solvent, along with polar solvents such as ethanol and ethyl acetate, which results in high oil yields with varied efficiencies in extracting bioactive compounds but raises sustainability concerns due to prolonged extraction times, high energy consumption, and the use of toxic solvents.^{11,12} Emerging extraction techniques, such as supercritical fluid extraction (SFE) using CO₂ with or without green co-solvents, and microwave-assisted extraction (MAE), combine shorter extraction durations, moderate operating conditions, high extraction efficiency, and the possibility of using greener solvents, making them particularly attractive in terms of environmental sustainability.^{13–17}

Understanding the interactions between extraction methods and oil composition is crucial for identifying opportunities to increase oil quality, particularly through the coextraction of antioxidant compounds from seeds. Few comparative studies on hemp seed oil extraction methods exist. Some have compared solvent extraction, mechanical pressing, and SFE,^{13,18,19} while others, such as Rezvankhah *et al.*,¹⁵ have examined MAE in comparison with solvent extraction. Devi & Khanam investigated several methods, such as Soxhlet extraction (SOX), SFE, percolation (PER), ultrasonication (ULT), and pyrolysis (PYR), and evaluated yield, oil composition, and economic feasibility at an industrial scale.¹⁴

In our previous studies, we investigated and optimized each of these extraction techniques separately (SOX, MAE, SFE, and ME) to identify the optimal conditions for maximizing yield and bioactive compound extraction.^{20–23} The present study builds upon this body of previous work, aiming to perform a comparative evaluation of the effectiveness of these four extraction methods in coextracting antioxidant molecules with the oil and their impact on the microstructure of the seed powder, given a comprehensive comparison in terms of yield and chemical composition, especially in terms of bioactive compound extraction.

This work aims to identify the extraction method that offers the optimal balance between oil yield, nutritional quality, bioactive compound enrichment, and oxidative stability. To this end, hemp seed oil was extracted *via* SOX, MAE, SFE and ME under the previously optimized conditions established in our published studies. The parameters assessed included phenolic

compounds, tocopherols, pigments (chlorophylls and carotenoids), color analysis (L^* , a^* , b^*), fatty acids, peroxide value, free acidity, specific extinction coefficients (conjugated dienes and trienes), and oxidative stability. Additionally, scanning electron microscopy was employed to investigate the morphological changes in the hemp seed powder following various extraction methods. This integrated approach provides novel insights that are critical for enhancing the overall quality and commercial viability of hemp seed oil.

2. Materials and methods

2.1. Chemicals and reagents

Standards, including tocopherols (α -tocopherol, β -tocopherol, γ -tocopherol and δ -tocopherol), fatty acid methyl esters (FAME), *N-trans*-caffeoyltyramine, gallic, sinapic, benzoic, hydroxybenzoic, and *p*-coumaric acids, were supplied by Sigma-Aldrich (St. Louis, MO, USA). The extraction solvents used included *n*-hexane, 2-propanol, ethyl acetate and ethanol, all of analytical grade. The fluid employed for SFE was food-grade carbon dioxide (CO₂), with a purity of 99.9%. In addition, all other chemicals and reagents used in this study were of analytical grade and were obtained from Merck Chemical Company (Darmstadt, Germany).

2.2. Plant material

In this study, experimental trials were carried out using hemp (*Cannabis sativa* L.) seeds of the 'Beldia' ecotype grown in the Jebha region of northern Morocco. Seeds were supplied by the Agence Nationale des Plantes Médicinales et Aromatiques (ANPMA). The seed water content was measured at $5.13 \pm 0.1\%$ using the standard hot-air oven method at 105 ± 1 °C until a constant weight was obtained, following the protocol described in the literature.²⁴ The fresh seeds were ground and sieved through a 500 μ m mesh sieve for use in SOX, MAE, and SFE. Seeds were stored in plastic bags at 2–4 °C in a refrigerator to preserve their quality until use.

2.3. Different hemp oil extraction methods

Four different extraction methods were used to extract hemp seed oil: SOX, MAE, SFE, and ME. The parameter levels and operating conditions for each method were previously optimized in our earlier studies.^{20–23} Each technique was individually optimized to identify the most effective parameters for maximizing oil yield, preserving its quality, and improving the recovery of bioactive compounds. The optimization focused respectively on the solvent type for SOX, the combination of pressure, temperature, time, and cosolvent (ethanol) percentage for SFE, the microwave power, extraction duration, and ethanol/hexane ratio (% EtOH) for MAE, and the pressing temperature for ME.

In the present study, these previously determined optimal conditions were applied to ensure a rigorous and fair comparison among the four methods in terms of extraction efficiency, oil composition, antioxidant co-extraction, and impact on the microstructure of the seed powder. Each extraction was



performed in triplicate, and the mean values of the obtained results were used for statistical analysis.

2.3.1. Soxhlet extraction (SOX). Approximately 30 g of crushed seeds with homogeneous granulometry ($<500\ \mu\text{m}$) were weighed, placed in a cellulose cartridge, and then inserted into the extraction chamber of a 250 ml Soxhlet apparatus equipped with a condenser and connected to a distillation flask containing 200 ml of solvent. Two experiments were carried out to extract the oil, one with *n*-hexane (SOX-H) and the other with an optimal mixture of 40% *n*-hexane, 40% 2-propanol and 20% ethyl acetate (SOX-HPE), in accordance with the conditions described by Allay *et al.*²⁰ Extractions were carried out at the boiling point of *n*-hexane and the ternary solvent mixture (HPE) over a period of 5 hours. After extraction, the solvents were removed under vacuum using a rotary evaporator maintained at 40 °C. After being weighed, the resulting oil was poured into dark vials and stored under nitrogen in a freezer at $-18\ ^\circ\text{C}$ for subsequent experiments. Both experiments were carried out in triplicate, and the mean values were recorded.

2.3.2. Supercritical fluid extraction (SFE). Extraction was performed in an SFE system described in detail elsewhere.²³ A total of 100 g of ground seeds with homogeneous granulometry ($<500\ \mu\text{m}$) were loaded into the extractor tank. CO_2 was pressurized to its supercritical state (pressure and temperature) before flowing into the extractor at a rate of $0.25\ \text{kg h}^{-1}$. Extraction was carried out under optimum conditions of 20 MPa and 50 °C for 244 min with a mixture of 90% CO_2 and 10% ethanol. The temperature and pressure of the two separators were set at 25 °C and 5 MPa, respectively. After extraction, the CO_2 was released, and the ethanol was collected with the oil in glass flasks and then evaporated in a rotary evaporator to obtain the final oil mass accurately. After being weighed, the resulting oil was poured into dark vials and stored under nitrogen in a freezer at $-18\ ^\circ\text{C}$ for subsequent experiments. The extraction was carried out in triplicate, and the mean values were recorded.

2.3.3. Microwave-assisted extraction (MAE). MAE was carried out as previously described.²¹ A total of 30 g of ground seeds with homogeneous granulometry ($<500\ \mu\text{m}$) were weighed, followed by the addition of a solvent mixture (*n*-hexane and ethanol) with a constant solvent/seed ratio of 10 : 1. The seed-solvent mixture was placed in a flask, which was then inserted into the cavity of the microwave oven and connected to a cooler to condense the solvent evaporated by the radiation. Extraction was carried out under optimum conditions with a power of 800 W, an extraction time of 13.6 min and a solvent mixture containing *n*-hexane with 7.5% ethanol. After extraction, the liquid phase was separated from the seed powder by a filter cloth, and the remaining solids were washed twice with the extraction solvent. Then, the mixture was centrifuged for 15 min at 5000g. The collected solvent mixture was evaporated in a vacuum rotary evaporator at 40 °C. After being weighed, the resulting oil was poured into dark vials and stored under nitrogen in a freezer at $-18\ ^\circ\text{C}$ for subsequent experiments. The extraction was carried out in triplicate, and the mean values were recorded.

2.3.4. Mechanical pressing extraction (ME). The extraction process was carried out *via* a single-screw extruder equipped with a motor rated at 1.7 kW, 240 V, and 4.5 A.²² One hundred grams of hemp seeds was introduced into the hopper and pressed at 100 °C. The experiment was carried out in triplicate to ensure reproducibility. After extraction by screw pressing, the crude oil was centrifuged at 5000g for 15 min to remove any remaining fine particles, resulting in a clearer oil. The clarified oil was then stored under a nitrogen atmosphere to prevent oxidation and kept in a freezer at $-18\ ^\circ\text{C}$ for later analysis. The extraction was carried out in triplicate, and the mean values were recorded.

2.4. Physicochemical characterization of the extracted oils

2.4.1. Total phenolic content (TPC). The analysis of the TPC was carried out according to the protocol described by Mansouri *et al.*²⁵ Extraction was conducted on the oil samples using a methanol/water mixture (80 : 20, v/v). The extracts were then reacted with Folin–Ciocalteu reagent, and the absorbance was measured at 760 nm. The results are expressed in mg gallic acid equivalents (GAE) per kg of oil.

2.4.2. Identification and quantification of phenolic compounds. The analysis of phenolic compounds was performed on methanolic extracts of the oils.^{20,26} The compounds were separated *via* HPLC using an Agilent 1260 Infinity II system equipped with a diode array detector (DAD) on a C18 column ($150 \times 4.6\ \text{mm}$, $3.5\ \mu\text{m}$). Elution was performed with a water/acetonitrile gradient containing 1% formic acid. UV detection was performed at 254, 280, 300, and 340 nm. Mass analyses were performed using an Esquire HCT spectrometer (Bruker Daltonics) with an electrospray ionization (ESI) source in positive and negative modes. The phenolic compounds were identified on the basis of UV-Vis, MS, and MS^2 spectra, as well as comparisons with data reported in the scientific literature on hemp seeds.^{26,27} Quantification was conducted at 280 nm using external calibration curves for *N*-trans-caffeoyltyramine, hydroxybenzoic acid, benzoic acid, *p*-coumaric acid, and sinapic acid, and the results are expressed in mg kg^{-1} of oil.

2.4.3. Tocopherol. The tocopherol content was analyzed as described by Ben Moumen *et al.*²⁸ using HPLC-DAD instrument equipped with an NH_2 Uptisphere column ($150 \times 3\ \text{mm}$, $3\ \mu\text{m}$), with detection at wavelengths of 292, 296, and 298 nm. Quantification was performed using an external calibration curve of a standard mixture of tocopherols, and the results are expressed in mg kg^{-1} of oil.

2.4.4. Pigments and color. Pigment (chlorophylls and carotenoids) analysis was carried out according to the method described by Aladić *et al.*⁸ Extraction was performed *via* ultrasonication with diethyl ether. The absorbance was measured at 663 and 640 nm for the chlorophylls and 470 nm for the carotenoids. The concentrations are expressed in mg kg^{-1} of oil.

The color of the oil samples was determined using the CIE-LAB parameters (L^* , a^* , b^*) and measured with a KONICA MINOLTA CR-410 chromameter.

2.4.5. Fatty acids. Fatty acid analysis was performed by GC-MS after transesterification of fatty acids into FAMES according



to the protocol of Allay *et al.*²³ The system used was a Thermo GC-MS 1300/TSQ 8000 Evo with a TR-5 column (30 m × 0.25 mm, 0.25 μm). The compounds were identified by comparison with the NIST database and FAME standards. The results are expressed as a percentage of total fatty acids.

2.4.6. Oil quality indices and oxidative stability. In accordance with the methods described by Mansouri *et al.*,²⁹ the free acidity (mg KOH per kg oil) was determined by titration with KOH, the peroxide value (meq O₂ per kg oil) was measured by titration with sodium thiosulfate, and specific extinction coefficients were measured at 232 nm (for conjugated dienes) and 270 nm (for conjugated trienes). The oxidative stability index (OSI), expressed in hours, was determined by the Rancimat apparatus at 100 °C under an air flow of 20 L h⁻¹.

2.5. Scanning electron microscopy (SEM)

A Quattro ESEM scanning electron microscope (FEG-Thermo Fisher Scientific) was used to examine morphological alterations in the hemp seed samples before and after oil extraction *via* different methods. The samples were coated with Au/Pd before being examined by SEM. A working distance of 10.1 mm and a spot size of 3 were used with a low-vacuum secondary electron detector. SEM images were taken at an accelerating voltage of 15 kV and a pressure of 1.05 mbar.

2.6. Statistical analysis

Statistical analysis of the differences between oils extracted *via* different methods was performed using univariate MANOVA in SPSS (v.25), with a significance threshold set at $p < 0.05$. The normality and homogeneity of the data were verified using the Shapiro–Wilk test and the Levene test, respectively ($p > 0.05$). A principal component analysis (PCA) was also performed after validating the adequacy of the data *via* the Kaiser–Meyer–Olkin (KMO) and Bartlett tests ($p < 0.05$). Finally, Pearson correlations ($p < 0.05$) between the main composition and quality variables

of hemp seed oils obtained by different extraction methods were calculated. The results are expressed as the means ± standard deviations and were visualized using GraphPad Prism 8.0 software.

3. Results and discussion

3.1. Effects of different extraction methods on oil yield

As shown in Fig. 1A, the SOX-H and SOX-HPE extractions achieved the highest yields, with values of 32.72% and 33.24%, respectively, followed by 30.69 for MAE, 30.51% (g oil per 100 g of fresh seeds) for SFE, and 21.82% for ME. Statistical analysis revealed no significant difference ($p > 0.05$) between the yields obtained by SOX-H and SOX-HPE, suggesting that the solvent mixture (40% *n*-hexane, 40% 2-propanol, and 20% ethyl acetate) is as effective as *n*-hexane alone for the extraction of hemp seed oil. This equivalence could be attributed to the ability of the ternary system to disrupt cell membranes and extract a broad spectrum of lipophilic compounds, including phospholipids.^{11,30}

Furthermore, the high efficiency of Soxhlet extraction can be explained by the thermally intensive operating conditions. Indeed, high temperature promotes the diffusion of lipid solutes and increases the solubility of lipophilic compounds in the solvent, leading to an increase in extraction yield.¹⁴ Moreover, the extended duration (5 hours) also contributed to this enhanced performance, enabling near-complete extraction of lipid constituents.¹⁵

These observations are perfectly consistent with the literature. Numerous studies have confirmed the superiority of the Soxhlet method for hemp seed oil extraction. For example, Devi & Khanam, after comparing five extraction methods (SOX, SFE, PER, ULT, PYR), reported that SOX offered the best yields.¹⁴ Similarly, Da Porto *et al.* and Aiello *et al.* reported that the oil yield extracted by SOX-H was significantly greater than that obtained *via* SFE.^{18,31} This trend was also confirmed by

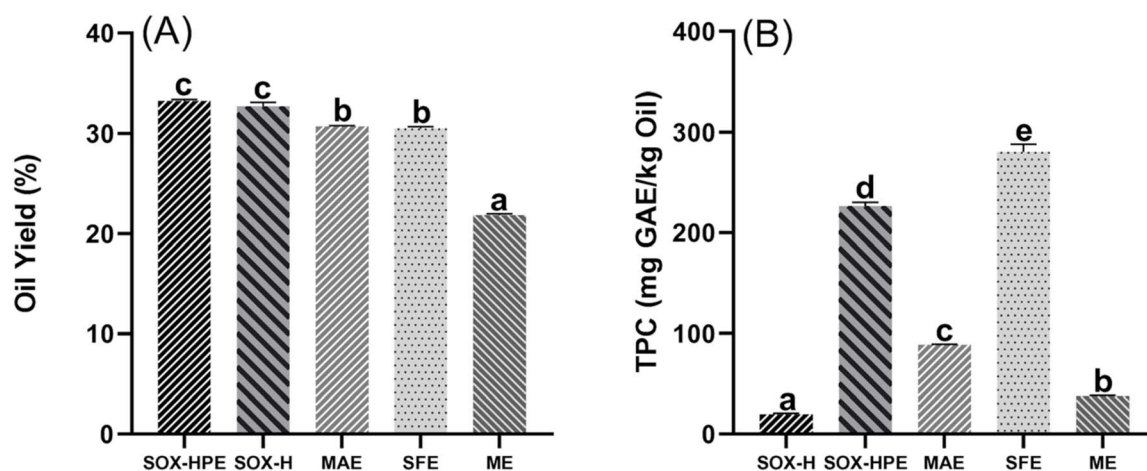


Fig. 1 Effect of different extraction methods of hemp seed oil on (A) oil yield, and (B) total phenolic content (TPC). SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C). Different letters (a, b, c, d, e) indicate significant differences ($p < 0.05$).



Rezvankhah *et al.* who highlighted a higher yield for SOX-H than for MAE.¹⁵ Finally, Crimaldi *et al.*, Aladić *et al.* and Golimowski *et al.* reported that the SOX-H method clearly outperformed cold-press extraction in terms of yield.^{8,9,32}

Although the SOX technique is the most efficient in terms of yield, it has significant drawbacks, notably, a long extraction time and elevated temperatures, resulting in high energy consumption, which limits its profitability. MAE, on the other hand, stands out for its much shorter extraction time, with a highly satisfactory yield of 30.69% achieved in just 13.60 min. This efficiency can be attributed to the disruption of cell walls by microwaves, favoring better exposure of intracellular materials to the solvent and thus improving extraction in a reduced time. Similar results were reported by Rezvankhah *et al.*, who reported that MAE offers an acceptable hemp seed oil yield of 33.91% in just 7.19 min, whereas SOX-H achieves a slightly higher yield of 37.93% in 8 h.¹⁵

Overall, compared with the SOX-H method, MAE and SFE demonstrated satisfactory extraction efficiencies, reaching 93.80% and 93.24%, respectively. These results are consistent with those reported by Aiello *et al.*,¹⁸ who reported a recovery of 93.19% for hemp seed oil compared with SOX-H, and Rezvankhah *et al.* reported a recovery of 89.40% for hemp seed oil extracted by MAE with SOX-H as a ref. 15.

In contrast, ME proved to be less efficient, with an extraction efficiency of only 66.68%. Similar results were reported by Crimaldi *et al.*,³² who observed a maximum oil recovery of 61.52% for mechanically extracted hemp seed oil. The low oil yield obtained by ME is mainly attributed to the limited mechanical disruption of the seed microstructure and the strong retention of oil within the press cake. The applied pressure primarily ruptures the outer seed coat but only partially disrupts the internal cellular compartments, particularly the oleosomes where most of the lipids are stored.^{4,33} Consequently, a significant fraction of oil remains trapped within the residual matrix, leading to incomplete recovery.³² Moreover, the absence of solvent in this process prevents efficient diffusion and solubilization of lipids through the compact and fibrous seed structure, further limiting extraction efficiency. These findings are consistent with the SEM imaging results, which show a relatively intact seed powder surface after ME, in contrast to the more pronounced cellular disintegration observed in oils extracted by SOX, MAE, and SFE.

Overall, these data confirm the advantage of the SOX method in terms of absolute yield, while MAE and SFE techniques demonstrate comparable relative efficiencies with shorter extraction times and milder operating conditions, highlighting their potential as more sustainable and economically viable alternatives.

3.2. Effect of extraction methods on the phenolic composition of hemp seed oil

Owing to their substantial variation in their polarity, phenolic compounds are influenced by the extraction method used. Fig. 1B illustrates the impact of extraction methods on the TPC content of hemp seed oils. SFE extraction resulted in

significantly ($p < 0.05$) higher TPC, reaching 294.15 mg GAE per kg oil, followed by SOX-HPE (226.43 mg GAE kg⁻¹), MAE (88.55 mg GAE kg⁻¹), ME (37.52 mg GAE kg⁻¹), and SOX-H (19.43 mg GAE kg⁻¹). This difference may be attributed to the polarity of the extraction medium. The TPC content of oil extracted by SOX-HPE with 60% polar solvents, including 40% 2-propanol and 20% ethyl acetate, and SFE with 10% ethanol as the polar solvent was almost three times greater than that obtained by MAE, where the solvent was composed of 7.5% ethanol and 92.5% *n*-hexane. Moreover, the use of pure *n*-hexane resulted in the lowest TPC content, while its combination with 2-propanol and ethyl acetate increased this content up to tenfold. In general, phenolic compounds, which are polar, are more soluble in polar solvents, which explains why increasing the polarity of the solvent facilitates the extraction of these compounds from oil.³⁴ Furthermore, Soroush *et al.* reported that increasing the percentage of isopropanol to 50% compared with that of *n*-hexane favored an increase in the phenol content in hemp seed oil extracted *via* MAE.¹⁷ On the other hand, Quispe-Fuentes *et al.* reported that increasing the percentage of ethanol in SFE extraction of *Clementina orogranda* bark oil led to an increase in the TPC compared with the use of pure CO₂.³⁵

In contrast, the oil obtained by ME exhibited a low TPC. Since ME relies solely on mechanical pressure without the use of solvents, it results in limited recovery of hydrophilic compounds such as phenolics. In comparison, polar solvents like ethanol and isopropanol enhance both lipid extraction and phenolic solubilization by disrupting cell wall integrity and modifying the polarity of the extraction medium. As demonstrated in various studies on oilseeds, these solvents break hydrogen bonds and hydrophobic interactions within the plant matrix, thereby facilitating the release of amphiphilic or partially hydrophilic molecules.^{36,37} Similar limitations in transferring phenolic compounds from seeds to oil have been reported for grape seed oil, where the extracted phenolics accounted for only 0.013–0.019% of the total seed content.³⁸

To better assess the effect of each extraction method on the transfer of phenolic compounds from the seeds to the oil, HPLC-DAD/ESI-MS² analysis was carried out on the oil extracts. Phenolic compounds were identified by comparing the precursor ion mass (MS), fragments (MS²) and UV-Vis spectra (SI Fig. S1) with data available in the literature.^{23,24,33,34} This analysis identified a total of 29 phenolic compounds: four phenolic acids (*p*-hydroxybenzoic, benzoic, *p*-coumaric, and sinapic acids), five hydroxycinnamic acid amides (HCAAs) as *N*-*trans*-caffeoyltyramine, *N*-*trans*-caffeoyltyramine isomer, *N*-*trans* coumaroyltyramine, *N*-feruloyl-tyramine, and tri-*p*-coumaroylspermidine, and twenty lignanamides as *N*-caffeoyltyramine dimer hydroxy derivative, cannabisisins (A, B, B isomer 1, B isomer 2, C, C isomer, D, E, M, Q, F, G, and O), demethyl-grossamide, 3,3-didemethylgrossamide, 3,3'-demethyl-heliotropamide, unnamed lignanamide, isocannabisin N, and grossamide (Table 1). Fig. 2 shows that, among the 29 phenolic compounds identified, the oils extracted by SOX-HPE and SFE contained all of them. On the other hand, 26 compounds were detected in the oil obtained by MAE. In contrast, oils extracted





Table 1 Phenolic composition of hemp seed oil (mg kg⁻¹ oil) obtained by different extraction methods^a

No. peak	RT (min)	Phenolic compounds	λ_{max} (nm)	[M-H] ⁻ calc. (m/z)	[M-H] ⁻ found (m/z)	SOX-H	SOX-HPE	MAE	SFE	ME
1	10.34	Unknown 1	264; 292	—	265.002	Nd	2.05 ± 0.17b	Nd	1.48 ± 0.27a	Nd
2	13.95	Unknown 2	292	—	325.009	Nd	0.47 ± 0.04a	0.56 ± 0.05a	1.22 ± 0.23b	Tr
3	14.44	<i>p</i> -Hydroxybenzoic acid	288; 378	137.0322	137.0317	Nd	0.13 ± 0.01a	2.70 ± 0.14c	0.93 ± 0.16b	Tr
4	18.99	Benzoic acid	280; 220	121.0373	121.0370	Nd	10.10 ± 0.71b	0.27 ± 0.01a	8.69 ± 0.12b	0.32 ± 0.02a
5	20.80	<i>p</i> -Coumaric acid	230; 300; 310	163.0478	163.0480	Nd	0.62 ± 0.01c	0.18 ± 0.02b	2.76 ± 0.08d	0.03 ± 0.00a
6	22.58	<i>N</i> - <i>Trans</i> -caffeoyltyramine isomer	200; 284; 315	298.1158	298.1160	Nd	3.92 ± 0.72c	0.47 ± 0.04a	0.99 ± 0.02b	Nd
7	25.42	<i>N</i> - <i>Trans</i> -caffeoyltyramine	220; 250; 294; 318	298.1158	298.1160	Nd	39.98 ± 3.47c	4.76 ± 0.16a	14.92 ± 0.86b	Nd
8	25.93	Unknown 3	250; 318; 344	—	207.000	Nd	1.52 ± 0.06a	Nd	Nd	Nd
9	26.29	<i>N</i> -Caffeoyltyramine dimer hydroxy derivative	254; 340	613.2269	613.2256	Nd	0.68 ± 0.02b	0.35 ± 0.05a	0.43 ± 0.02a	Nd
10	27.63	Cannabisin A	256	593.2002	593.2016	Nd	7.98 ± 0.79c	1.73 ± 0.11a	3.79 ± 0.15b	Nd
11	27.89	Cannabisin B	254; 284; 314; 334	595.2159	595.2155	Nd	10.79 ± 0.61c	2.43 ± 0.18a	7.63 ± 1.01b	Nd
12	28.34	<i>N</i> - <i>Trans</i> -coumaroyltyramine	292; 308	282.1208	282.1215	Nd	0.64 ± 0.02a	0.62 ± 0.01a	0.63 ± 0.11a	Nd
13	29.02	Cannabisin B isomer 1	264; 284; 314	595.2159	595.2155	Nd	5.81 ± 0.08c	0.89 ± 0.01a	1.67 ± 0.20b	Nd
14	29.48	Cannabisin B isomer 2	268; 310	595.2159	595.2161	Nd	0.74 ± 0.01a	1.34 ± 0.05b	0.84 ± 0.18a	Nd
15	29.73	<i>N</i> -Feruloyltyramine	256; 288; 318	312.1314	312.1320	Nd	10.50 ± 0.00c	0.84 ± 0.04a	1.19 ± 0.02b	Nd
16	29.98	Unknown 4	322	—	607.000	Nd	0.60 ± 0.06a	Nd	Tr	Nd
17	30.41	Demethylgrossamide	264; 284; 314; 322	609.2315	609.2322	Nd	4.06 ± 0.39c	0.25 ± 0.04a	0.77 ± 0.09b	Nd
18	31.24	Cannabisin C	260; 280	609.2315	609.2317	Nd	0.38 ± 0.01a	0.38 ± 0.02a	0.44 ± 0.03a	Nd
19	31.39	Cannabisin C isomer	284; 322	609.2315	609.2325	Nd	0.80 ± 0.07a	0.77 ± 0.02a	0.82 ± 0.08a	Nd
20	32.20	Unknown 5	284	—	609.168	Nd	0.13 ± 0.00a	0.47 ± 0.03b	Tr	Nd
21	32.34	Cannabisin D	260; 284; 308	623.2472	623.2480	Nd	0.13 ± 0.00a	0.98 ± 0.02c	0.65 ± 0.03b	Nd
22	32.82	3,3-Didemethylgrossamide	284; 324	595.2159	595.2160	Nd	0.63 ± 0.01b	0.37 ± 0.04a	1.63 ± 0.17c	Nd
23	33.12	Tri- <i>p</i> -coumaroylsperridine	260	582.2688	582.2695	Nd	0.46 ± 0.07a	0.33 ± 0.02a	0.91 ± 0.12b	Nd
24	33.42	Cannabisin E	292; 310	641.2577	641.2571	Nd	2.43 ± 0.52c	0.46 ± 0.04a	1.07 ± 0.22b	Nd
25	33.75	Unknown 6	292	—	612.262	Nd	1.04 ± 0.00a	Nd	Nd	Nd
26	35.05	Cannabisin M	288; 322	595.2159	595.2155	Nd	3.35 ± 0.11c	1.06 ± 0.04a	1.95 ± 0.31b	Nd
27	35.24	3,3'-Demethyl-heliotropamide	285; 310	595.2159	595.2160	Nd	4.01 ± 0.63b	0.17 ± 0.03a	3.19 ± 0.14b	Nd
28	35.55	Unnamed lignanamide	230–312	—	589.2661	Nd	0.51 ± 0.01b	Nd	0.25 ± 0.05a	Nd
29	35.72	Unknown 7	284	—	591.539	Nd	0.96 ± 0.02a	Nd	Tr	Nd
30	36.05	Cannabisin Q	284; 308	595.2159	595.2150	Nd	0.96 ± 0.08a	1.31 ± 0.02b	1.39 ± 0.08b	Nd
31	36.72	Cannabisin F	288; 312	623.2472	623.2470	Nd	1.03 ± 0.08c	0.20 ± 0.02a	0.85 ± 0.01b	Nd
32	37.06	Isocannabisin N	284; 324	609.2315	609.2306	Nd	0.47 ± 0.00b	0.26 ± 0.03a	1.88 ± 0.01c	Nd
33	37.65	Grossamide	250; 288; 320	623.2472	623.2481	Nd	8.74 ± 0.78c	0.22 ± 0.01a	2.47 ± 0.10b	Nd
34	37.93	Cannabisin G	290; 312	623.2477	623.2475	Nd	0.37 ± 0.33a	Nd	0.66 ± 0.02b	Nd
35	38.11	Cannabisin O	288; 312	934.3629	934.3635	Nd	2.51 ± 0.38b	Tr	0.17 ± 0.01a	Nd
36	48.31	Unknown 8	278	—	652.354	Nd	0.64 ± 0.06b	0.28 ± 0.02a	2.45 ± 0.08c	0.58 ± 0.05b
37	48.83	Sinapic acid	276	223.0690	223.0682	Nd	1.14 ± 0.16a	2.36 ± 0.12c	3.20 ± 0.31d	1.94 ± 0.23b

Table 1 (Contd.)

No. peak	RT (min)	Phenolic compounds	λ_{max} (nm)	$[\text{M-H}]^+_{\text{calc.}}$ (m/z)	$[\text{M-H}]^+_{\text{found}}$ (m/z)	SOX-H	SOX-HPE	MAE	SFE	ME
Hydroxybenzoic acid (HBA)						Nd	14.23 \pm 0.70d	2.98 \pm 0.15b	9.62 \pm 0.35c	0.32 \pm 0.02a
Hydroxycinnamic acid (HCA)						1.71 \pm 0.13a	1.76 \pm 0.17a	2.54 \pm 0.10b	5.96 \pm 0.35c	1.97 \pm 0.23a
Phenolic acids						1.71 \pm 0.13a	15.99 \pm 0.53d	5.52 \pm 0.05c	15.58 \pm 0.51d	2.29 \pm 0.24b
Hydroxycinnamic acid amide (HCAA)						Nd	28.63 \pm 0.89c	8.79 \pm 0.22a	18.64 \pm 1.17b	Nd
Lignanmides						Nd	53.55 \pm 0.95c	15.53 \pm 0.10a	32.55 \pm 1.12b	Nd
Phenylpropanoids						Nd	82.18 \pm 1.84c	24.32 \pm 0.33a	51.19 \pm 2.85b	Nd
Total phenolic compounds						2.32 \pm 0.10a	131.29 \pm 4.62e	27.01 \pm 0.59c	71.92 \pm 3.91d	2.87 \pm 0.21b

^a Data, which are the mean \pm SD of three independent experiments ($n = 3$), were expressed as mg kg^{-1} 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 $\text{mg N-trans-caffeoyltyramine}$ equivalent per kg of oil (mg CTE per kg oil). Nd: not detected; Tr: traces; RT: retention time; λ_{max} : maximum absorbance peak. SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO_2 + 10% ethanol); ME: mechanical extraction (pressing at 100 °C).

by SOX-H and ME contained only 3 to 4 phenolic compounds, highlighting the considerably lower efficiency of these methods in transferring these bioactive compounds.

The quantification results (Table 1) revealed a significant abundance of phenylpropanoids, a phenolic subgroup comprising HCAAs and lignanamides, in the extracted oils. The oils obtained by SOX-HPE had total HCAA and lignanamide contents of 28.63 and 53.55 mg kg^{-1} oil, respectively, the highest of all the methods studied, representing 62% of the phenolic compounds identified. These results are in line with previous studies on hemp seeds, which also reported a predominance of phenylpropanoids,^{26,27,39} confirming the efficient transfer of these compounds from seeds to oil.

Among the HCAAs, *N-trans-caffeoyltyramine* stood out as the most abundant compound, with concentrations of 39.98 mg kg^{-1} in oil extracted by SOX-HPE, 14.92 mg kg^{-1} by SFE and 4.76 mg kg^{-1} by MAE. In addition, *N-feruloyltyramine* and *N-trans-caffeoyltyramine* isomer were also detected in significant quantities, reaching 10.50 and 3.92 mg CTE kg^{-1} respectively, in the SOX-HPE oil. For lignanamides, cannabins A and B were the most widespread, with maximum concentrations in SOX-HPE (7.98 and 10.79 mg CTE kg^{-1}), followed by SFE (3.79 and 7.63 mg CTE kg^{-1}) and MAE (1.73 and 2.43 mg CTE kg^{-1}). These results corroborate observations in the literature, where these compounds dominate the phenolic profiles of hemp seeds.^{26,27,39} Other lignanamides, such as grossamide, demethylgrossamide, 3,3'-demethylheliotropamide, and cannabisin M, were particularly abundant in SOX-HPE oil, whereas compounds such as Tri-*p*-coumaroylspermidine and cannabins C, D, G and O were present in low quantities, or even absent, in oils extracted by SFE and MAE.

In terms of phenolic acids, the oil extracted by SOX-HPE presented the highest total content of hydroxybenzoic acids (HBAs), reaching 14.23 mg kg^{-1} . On the other hand, the oil obtained by SFE showed the highest concentration of hydroxycinnamic acids (HCAs), totaling 5.96 mg kg^{-1} . Among the phenolic acids identified, benzoic acid was the most abundant, with concentrations of 10.10 mg kg^{-1} and 8.69 mg kg^{-1} in the SOX-HPE and SFE oils, respectively. *p*-Hydroxybenzoic acid was particularly enriched in the oil extracted by MAE (2.70 mg kg^{-1}), whereas sinapic acid was detected in all oils, with the highest level observed in the SFE oil (3.20 mg kg^{-1}).

Overall, the total phenolic compounds quantified by HPLC-DAD in the oil extracted by SOX-HPE reached 131.29 mg kg^{-1} , which was significantly higher than those obtained with SFE (71.92 mg kg^{-1}), MAE (27.01 mg kg^{-1}), and the negligible amounts observed for ME and SOX-H (2.87 and 2.32 mg kg^{-1} , respectively). However, the TPC values obtained via the Folin-Ciocalteu method showed a different trend, with a higher content for SFE oil (294.15 mg GAE kg^{-1}) than for SOX-HPE (226.43 mg GAE kg^{-1}). This discrepancy can be attributed to the limited specificity of the Folin-Ciocalteu reagent, which also reacts with nonphenolic compounds such as chlorophylls.^{40,41} Consequently, the Folin-Ciocalteu results expressed in mg GAE kg^{-1} may overestimate the phenolic content.



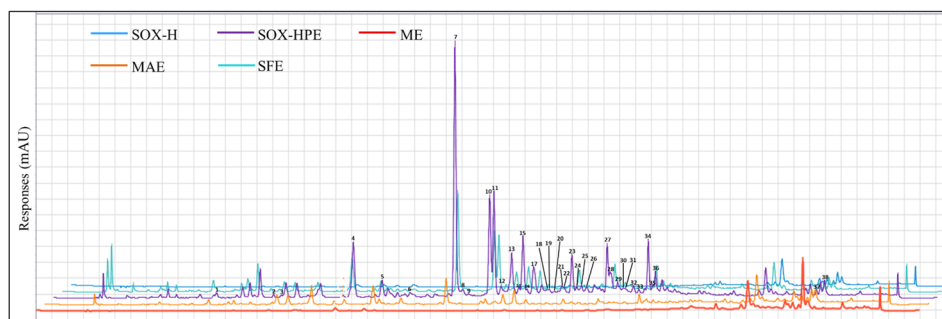


Fig. 2 HPLC-DAD chromatograms recorded at 280 nm correspond to hemp seed oil extracted using different methods, SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C). The names of the peaks correspond to the phenolic compounds in Table 1.

3.3. Effect of extraction methods on the tocopherol composition of hemp seed oil

In hemp seed oil, tocopherols are the dominant antioxidant components that can stabilize the oil against oxidation. Table 2 shows the total tocopherol content of the oils extracted *via* the different methods. The tocopherol content of the oils is clearly affected by the extraction method. The total tocopherol content ranged from 408.98 mg kg⁻¹ oil for oil extracted by SOX-H to 510.66 mg kg⁻¹ for oil extracted by MAE. γ -Tocopherol was the predominant isomer in all hemp seed oils, irrespective of the extraction method, with a maximum value of 435.13 mg kg⁻¹ observed in oil extracted *via* MAE. These results concur with those reported by Farinon *et al.*, who reported that the tocopherol content of hemp seed oil ranged from 143 to over 900 mg kg⁻¹, mainly in the form of γ -tocopherol.⁴

On the other hand, statistical analysis revealed a notable difference between methods, particularly for the δ -tocopherol isomer. The lowest content was observed in oils extracted by SOX-HPE and SOX-H (8.97 and 9.57 mg kg⁻¹, respectively), whereas the maximum value of 42.41 mg kg⁻¹ was obtained

with MAE. For the α -tocopherol isomer, the oil extracted by SFE had the highest content, reaching 39.68 mg kg⁻¹.

MAE and SFE extraction methods demonstrate superior efficiency for tocopherol recovery compared to SOX-H and ME techniques. This enhanced performance can be explained by the specific mechanisms underlying each process. MAE uses electromagnetic radiation to induce rapid disruption of cell walls, thereby increasing the exposure of intracellular compounds to the solvent. Combined with its short extraction duration (7.5 min), this approach greatly limits the thermal degradation of tocopherols. This efficiency is supported by the findings of Rezvankhah *et al.*,¹⁵ who reported a tocopherol content of 929.67 mg kg⁻¹ for MAE compared to 832.61 mg kg⁻¹ for the SOX-H method.

SFE, on the other hand, exploits the unique properties of supercritical CO₂, particularly when a co-solvent such as ethanol is added to enhance the solubilization of tocopherols. Its operation in a closed, oxygen-free system under moderate temperatures creates a protective environment against oxidative degradation, thereby preserving the integrity of thermolabile

Table 2 Effect of different extraction methods on tocopherol content, pigment content, and color parameters of hemp seed oil^a

Parameters	SOX-H	SOX-HPE	MAE	SFE	ME
Tocopherols (mg kg⁻¹oil)					
γ -Tocopherol	377.74 ± 11.03a	418.46 ± 19.41 ab	435.13 ± 12.49b	412.61 ± 28.5 ab	402.80 ± 27.60 ab
α -Tocopherol	21.66 ± 0.93a	31.18 ± 1.19b	33.12 ± 1.72b	39.68 ± 2.36c	34.28 ± 1.04bc
δ -Tocopherol	9.57 ± 0.94a	8.97 ± 0.56a	42.41 ± 1.07c	30.25 ± 1.67b	29.17 ± 0.16b
Total tocopherols	408.98 ± 11.02a	458.61 ± 20.44 ab	510.66 ± 14.97b	482.54 ± 32.52b	466.25 ± 27.69 ab
Pigments (mg kg⁻¹oil)					
Total carotenoid	1.78 ± 0.03a	19.92 ± 1.39d	27.31 ± 0.83e	14.66 ± 0.17c	12.96 ± 0.04b
Total chlorophyll	8.03 ± 0.50a	66.59 ± 4.50b	99.68 ± 0.73d	61.03 ± 0.85b	76.15 ± 0.13c
Color					
<i>L</i> *	36.21 ± 0.09b	33.33 ± 0.10a	33.54 ± 0.63a	34.97 ± 0.45 ab	35.73 ± 0.66 ab
<i>a</i> *	-1.25 ± 0.16a	-4.28 ± 0.26b	-4.01 ± 0.23b	-3.96 ± 0.16b	-6.96 ± 0.11c
<i>b</i> *	8.39 ± 0.14c	2.80 ± 0.14a	3.17 ± 0.08a	3.75 ± 0.21b	3.44 ± 0.06d

^a Mean ± SD of three independent experiments (*n* = 3). Different letters (a, b, c, d, e) indicate significant differences (*p* < 0.05) within rows. SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C).



compounds. A study by Aladić *et al.*¹³ demonstrated that SFE can yield tocopherol contents two to three times higher than those obtained by cold pressing or SOX-H extraction.

In contrast, conventional methods exhibit significant limitations. The SOX-H technique, with its prolonged extraction time (5 hours), and the ME method, involving high temperatures (100 °C), lead to greater tocopherol degradation, as reported by Rezvankhah *et al.*¹⁵ These harsh conditions accelerate the decomposition of these sensitive molecules, thus reducing their concentration in the final oil.

Moreover, the superior efficiency observed for MAE and SFE can also be attributed to the use of ethanol, which plays a crucial role in the extraction process. This polar solvent deeply disrupts the architecture of cell walls and membranes by acting on macromolecular structures and breaking the bonds that stabilize interactions between bioactive compounds and the cellular matrix.^{30,42} Similar effects have been reported in oilseeds and cereals, where ethanol and isopropanol disrupt hydrogen bonds and hydrophobic interactions within polysaccharide-protein matrices, thereby promoting the release of lipids and tocopherols.^{36,37}

3.4. Effect of extraction methods on the pigment content and color of hemp seed oil

Pigments, notably chlorophylls and carotenoids, were analyzed in hemp seed oils extracted *via* different methods using a spectrophotometer. The absorbances were measured at 640 and 663 nm to determine the total chlorophyll content and at 470 nm to determine the total carotenoid content. As shown in Table 2, the total chlorophyll and carotenoid contents of the oil extracted by SOX-H were significantly lowest at 8.03 and 1.78 mg kg⁻¹ oil, respectively, whereas the highest contents at 99.68 and 27.31 mg kg⁻¹, respectively, were observed in the oil extracted by MAE.

Methods using mixtures of polar and nonpolar solvents, such as SOX-HPE, MAE, and SFE, presented the highest pigment concentrations, suggesting that these combinations offer favorable conditions for extraction. Although pigments are predominantly apolar, several studies have confirmed the efficiency of moderately polar mixtures, such as *n*-hexane with 2-propanol or ethanol, to extract chlorophylls and carotenoids from hemp seeds or other plant matrices.^{17,43} This efficiency is often attributed to the ability of mixed solvents to penetrate plant cells, enabling optimal extraction of their contents.

The oil extracted by MAE had the highest pigment content compared with that extracted by SOX-HPE and SFE. This is could be due to the combined effect of solvent mixtures (hexane-ethanol) and the use of microwaves, which promote cell wall rupture and thereby increase pigment release. Furthermore, even without the use of solvents, the oil obtained by ME presented significant levels of chlorophylls (76.15 mg kg⁻¹), which were higher than those extracted by SOX-H, SOX-HPE, and SFE.

The high chlorophyll content observed in the oil obtained by ME can be attributed to the direct release of these lipophilic pigments, mainly localized and freely available in the seed coat.

Unlike tocopherols and phenolic compounds, which are often bound to macromolecules such as proteins or polysaccharides and require the use of solvents to break these bonds, chlorophylls are present in the chloroplasts of the green outer layers of the seed and require no solvents for extraction.⁴⁴⁻⁴⁶

Mechanical pressure, combined with temperatures of up to 100 °C, causes structural disruption of the cells and degradation of the chloroplasts, resulting in the direct transfer and accumulation of easily mobilized chlorophylls in the oil phase, a phenomenon amplified by their lipophilic nature. These observations are consistent with data in the literature: a comparative study by Aladić *et al.* showed that hemp seed oils obtained by ME had the highest chlorophyll concentrations, followed by those extracted by SFE and SOX-H.¹³

Colorimetric parameters obtained by CIELAB analyses performed on hemp seed oils extracted *via* different methods, expressed as *L** (lightness), *a** (green-red), and *b** (blue-yellow), are presented in Table 2. Overall, all samples fall within the second quadrant, characterized by negative values of *a** (indicating a green hue) and positive values of *b** (indicating a yellow hue).

Among the different methods, the oil extracted by SOX-H presented the highest values of *L** (36.21) and *b** (8.39), reflecting a lighter and slightly more yellow oil. In contrast, the oil extracted by SOX-HPE presented the lowest values for *L** (33.33) and *b** (2.80), indicating a darker, less yellow hue. For *a**, the oil extracted by SOX-H presented the least negative value (−1.25), indicating a less green hue, whereas the oil extracted by ME presented the most negative value (−6.96), corresponding to a strongly green hue.

In the absence of official colorimetric standards for unrefined oils such as hemp seed oil, the values measured using the CIELAB system were interpreted by comparison with data reported in the scientific literature. Our results fall within the typical ranges generally observed for cold-pressed and commercial hemp seed oils, the most common extraction approach for premium-quality products, with *L** values between 25 and 54, *a** between −1 and 12, and *b** between 1 and 88.^{47,48} These intervals reflect the natural variability associated with the extraction method, pigment content of the seeds, and genetic and agronomic factors.

These differences can be mainly attributed to the chlorophyll content, the primary pigment responsible for the green hue of hemp seed oil. Moyano *et al.* also reported a strong positive correlation between pigment concentration and *a** values, as well as a negative correlation with *L** and *b** in virgin olive oils.⁴⁹ Moreover, a comparative study by Mansouri *et al.*,⁵⁰ on oils extracted from roasted and unroasted hemp seeds showed that the increase in *b** value in roasted seed oils could be linked to a decrease in chlorophyll content, compensated by a higher concentration of carotenoids. These findings confirm the major influence of pigments on the colorimetric properties of vegetable oils.

Thus, color variations are not merely analytical observations but also key indicators guiding the selection of oils for specific applications. Oils exhibiting negative *a** values with low *L**, obtained through SOX-HPE, MAE, SFE, and ME, are particularly



suitable for cosmetic and nutraceutical uses due to their rich profile in bioactive compounds. In contrast, lighter-colored oils, characterized by higher L^* and lower a^* values such as those produced by the conventional SOX-H method are more appropriate for culinary purposes, offering a neutral flavor, moderate thermal stability, and a visual appearance perceived by consumers as a sign of quality and purity.

3.5. Effect of extraction methods on the fatty acid composition of hemp seed oil

The fatty acid composition of hemp seed oil is presented in Table 3. Regardless of the extraction method used, the fatty acid profile of the oils remains broadly similar, with ten fatty acids identified.

Polyunsaturated fatty acids (PUFAs) are predominant in all the oil samples, accounting for 66.86% to 68.88%, mainly in the form of linoleic (50.76–51.32%), α -linolenic (14.52–17.17%), and γ -linolenic acids (0.95–1.06%). Monounsaturated fatty acids (MUFAs) followed, with proportions ranging from 18.41% to 21.15%, and were dominated by oleic (18.23–19.12%) and eicosenoic acids (0.16–2.03%). Finally, saturated fatty acids (SFAs) accounted 9.97% to 14.48% of the total fatty acid content and were mainly composed of palmitic (6.39–10.43%), stearic (1.26–3.23%), behenic (0.28–0.86%), and lignoceric acids (0.13–0.30%). The proportions of omega-6 and omega-3 individuals vary from 51.71% to 52.34% and from 14.52% to 17.17%, respectively, with an optimal, well-balanced omega-6/omega-3 ratio of 3.01 to 3.60.

Statistical analysis revealed no significant differences ($p > 0.05$) in fatty acid composition between oils extracted by SOX-H, SOX-HPE, MAE and ME. However, the oil extracted by SFE was distinguished by significantly higher proportions of α -linolenic, γ -linolenic, eicosenoic, behenic and lignoceric acids, as well as lower proportions of palmitic and stearic acids. This composition resulted in an oil that was particularly rich in UFAs (90.03%) and low in SFAs (9.97%) compared with other oils.

These results were consistent with the data available in the literature. Devi & Khanam compared five extraction methods (SOX, SFE, PER, ULT, and PYR),¹⁴ whereas Aladić *et al.* analyzed the differences among SFE, SOX-H and cold pressing.¹³ Similarly, Rezvankhah *et al.* compared SOX-H and MAE.¹⁵ These studies show that there is no significant difference, or only minor deviations, in fatty acid composition depending on the extraction method used. On the other hand, some research has 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 (~10%). These findings suggest that the oil's fatty acid composition is primarily influenced by genotype and climatic conditions, as confirmed by several studies that have shown that the fatty acid composition of hemp oils is strongly influenced by these factors.^{1,2}

3.6. Effect of extraction methods on the quality indices and oxidative stability of hemp seed oil

The quality indices peroxide value (PV), acidity value (AV) and specific extinction coefficients (conjugated dienes (CD) and

Table 3 Fatty acid composition and quality indices of hemp seed oil obtained using different extraction methods^a

Parameters	SOX-H	SOX-HPE	MAE	SFE	ME
Fatty acids (%)					
Palmitic acid (C16:0)	10.43 ± 0.59a	10.05 ± 1.34a	10.30 ± 1.24a	6.39 ± 0.46b	10.06 ± 0.59a
Stearic acid (C18:0)	3.23 ± 0.55a	2.93 ± 0.21a	3.09 ± 0.50a	1.26 ± 0.01b	2.80 ± 0.11a
Oleic acid (C18:1)	18.50 ± 0.66a	18.69 ± 0.58a	18.23 ± 1.33a	19.12 ± 1.44a	18.76 ± 0.28a
Linoleic acid (C18:2n6)	51.32 ± 0.33a	51.13 ± 0.49a	51.04 ± 1.54a	50.76 ± 0.71a	51.27 ± 0.27a
γ -Linolenic acid (C18:3n6)	1.02 ± 0.01a	1.06 ± 0.03a	0.98 ± 0.02a	0.95 ± 0.10a	1.02 ± 0.01a
α -Linolenic acid (C18:3n3)	14.52 ± 0.54a	14.70 ± 0.44a	15.09 ± 0.62a	17.17 ± 0.34b	14.87 ± 0.20a
Arachidic acid (C20:0)	0.39 ± 0.01a	0.69 ± 0.05b	0.62 ± 0.20b	1.16 ± 0.09c	0.63 ± 0.05b
Eicosenoic acid (C20:1)	0.16 ± 0.03a	0.22 ± 0.02a	0.18 ± 0.01a	2.03 ± 0.17b	0.18 ± 0.02a
Behenic acid (C22:0)	0.28 ± 0.03a	0.39 ± 0.08a	0.34 ± 0.08a	0.86 ± 0.07b	0.27 ± 0.04a
Lignoceric acid (C24:0)	0.15 ± 0.01a	0.14 ± 0.05a	0.13 ± 0.09a	0.30 ± 0.00b	0.14 ± 0.01a
SFA	14.48 ± 0.18a	14.20 ± 0.70a	14.48 ± 0.36a	9.97 ± 0.69b	13.90 ± 0.79a
MUFA	18.66 ± 0.67a	18.91 ± 0.60a	18.41 ± 1.34a	21.15 ± 0.57b	18.94 ± 0.26b
PUFA	66.86 ± 0.87 ab	66.89 ± 0.38 ab	67.11 ± 0.50a	68.88 ± 0.67b	67.16 ± 0.48 ab
UFA	85.52 ± 0.80a	85.80 ± 0.98a	85.52 ± 0.84a	90.03 ± 0.60b	86.10 ± 0.74a
<i>n</i> -6	52.34 ± 0.32a	52.19 ± 0.46a	52.02 ± 1.52a	51.71 ± 0.81a	52.29 ± 0.28a
<i>n</i> -3	14.52 ± 0.54a	14.70 ± 0.84a	15.09 ± 1.02a	17.17 ± 0.14b	14.87 ± 0.20a
<i>n</i> -6/ <i>n</i> -3	3.60 ± 0.11a	3.55 ± 0.24a	3.45 ± 0.37a	3.01 ± 0.07b	3.52 ± 0.13a
Oil quality indices					
Conjugated dienes (K232) (CD)	1.96 ± 0.15b	2.06 ± 0.12b	2.24 ± 0.16b	1.52 ± 0.19a	1.77 ± 0.12 ab
Conjugated trienes (K270) (CT)	0.55 ± 0.02 ab	0.58 ± 0.02b	0.51 ± 0.01a	0.48 ± 0.08a	0.40 ± 0.06a
Peroxide value (meq O ₂ per kg oil)	20.80 ± 2.77d	12.56 ± 1.06c	7.68 ± 0.34b	4.67 ± 0.06a	8.30 ± 0.47b
Acid value (mg KOH per g oil)	3.91 ± 0.05c	3.09 ± 0.06b	0.26 ± 0.02a	0.22 ± 0.02a	0.18 ± 0.01a

^a Mean ± SD of three independent experiments ($n = 3$). Different letters (a, b, c, d) indicate significant differences ($p < 0.05$) within rows. SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C).



trienes (CT)) were used to compare the oils extracted *via* the different methods. The results of these indices are shown in Table 3.

The oil extracted by SFE was the best quality, with the lowest values ($p < 0.05$), a PV of $4.67 \text{ meq O}_2 \text{ kg}^{-1}$, an AV of $0.26 \text{ mg KOH g}^{-1}$, a CD of 1.52 and a CT of 0.48, followed by oils obtained by MAE and ME, which had respective values for PV (7.68 and $8.30 \text{ meq O}_2 \text{ kg}^{-1}$), AV (0.26 and $0.18 \text{ mg KOH g}^{-1}$), CD (2.24 and 1.77) and CT (0.51 and 0.40). In contrast, oils obtained *via* the Soxhlet method (SOX-H and SOX-HPE) presented the highest values ($p < 0.05$), PV (20.80 and $12.56 \text{ meq O}_2 \text{ kg}^{-1}$), AV (3.91 and $3.09 \text{ mg KOH g}^{-1}$), CD (1.96 and 2.06) and CT (0.55 and 0.58).

Despite these variations, almost all the oils extracted by the various methods comply with the Codex Alimentarius recommendations for crude vegetable oils,⁵¹ which set a maximum AV of $4.0 \text{ mg KOH g}^{-1}$ and a maximum PV of $15 \text{ meq O}_2 \text{ kg}^{-1}$. Only SOX-H-extracted oil exceeds these limits, with a PV of $20.80 \text{ meq O}_2 \text{ kg}^{-1}$.

The high values of quality indices observed in hemp seed oil obtained by SOX-H could be attributed to the high temperature and long extraction time characteristic of this method. Several studies have confirmed these observations, showing that oil extracted by SOX-H exhibited higher values of PV, AV, CD and CT than those obtained by methods such as MAE, SFE or ultrasonic-assisted extraction.^{15,52,53} In contrast, the combination of solvents such as *n*-hexane, 2-propanol and ethyl acetate in the SOX-HPE method reduced fatty acid oxidation. This can be explained by the extraction of phenolic compounds that neutralize free radicals, thus limiting the formation of peroxides and hydroperoxides.²⁰

The oil extracted by SFE was distinguished by its higher quality owing to moderate extraction temperatures (50°C) and the use of supercritical CO_2 . The latter creates an oxygen-free environment, minimizing the degradation of fatty acids into peroxides and hydroperoxides. These results were consistent with those reported by Aiello *et al.*,¹⁸ who reported that hemp seed oil extracted *via* SFE had significantly lower PV ($5.50 \text{ meq O}_2 \text{ kg}^{-1}$), CD (3.81) and CT (1.20) values than oil extracted *via* SOX-H (PV $19.50 \text{ meq O}_2 \text{ kg}^{-1}$, CD 6.32 and CT 1.25). In addition, Liu *et al.* demonstrated that pomegranate seed oil extracted *via* a subcritical extraction method with *n*-butane presented lower PV and AV values than oils obtained *via* MAE, UAE or shaking extraction with *n*-hexane and ether petroleum.⁵⁴

In addition to oil quality indices, the Rancimat accelerated oxidation test was used to evaluate and compare the oxidative stability of hemp seed oils extracted *via* different methods. This technique is based on the measurement of the induction time, which reflects the resistance of lipids to oxidation when exposed to high temperature (100°C) and constant air flow (20 L h^{-1}).

The induction time results in hours (h), presented in Fig. 3, show that the oil extracted by SOX-HPE has the best oxidative stability, with an induction time of 37.77 h, followed by oils extracted by MAE (28.60 h) and SFE (28.01 h). On the other hand, the lowest induction times were observed for oils extracted by SOX-H (13.97 h) and ME (11.70 h). Statistical analysis revealed no significant differences ($p > 0.05$) between

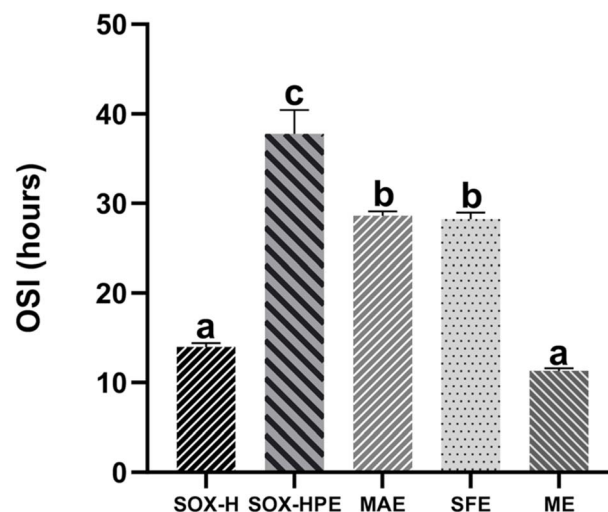


Fig. 3 Effect of different extraction methods of hemp seed oil on oxidative stability index (OSI). SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50°C , 244 min, with a mixture of 90% CO_2 + 10% ethanol); ME: mechanical extraction (pressing at 100°C). Different letters (a, b, c) indicate significant differences ($p < 0.05$).

the induction times of oils extracted by MAE and SFE or between those obtained by SOX-H and ME.

The oil extracted by ME was the most sensitive to oxidation, which could be explained by the high temperature reached during extraction by the screw press (100°C). The oil extracted by SOX-H was also highly sensitive to oxidation. This could be explained by the high SOX-H temperature and the long duration of the process.¹⁵ In contrast, the oil extracted using the SOX-HPE method exhibited oxidative stability more than three times greater than that of SOX-H oil. This shows the effect of the extraction solvent, where the mixture of *n*-hexane, 2-propanol and ethyl acetate extracted a large quantity of phenolic compounds (Table 1), which are very strong antioxidants that play a protective role against the formation of free radicals and the degradation of fatty acids.⁴ In addition, the use of a polar co-solvent such as ethanol with *n*-hexane in MAE or with supercritical CO_2 in SFE enabled the extraction of high quantities of phenolic compounds (Table 1), which slowed the oxidation rate and increased the induction time by more than 2-fold compared with oils extracted by SOX-H and ME. However, SFE resulted in higher phenolic compound contents (70.84 mg kg^{-1}) than did MAE (28.09 mg kg^{-1}) (Table 1), but the same induction time was used. This can be explained by the high unsaturated fatty acid content of the oil extracted by SFE (Table 3). The degree of fatty acid unsaturation is the main factor affecting oil stability. The richer oils are in unsaturated fatty acids, the more sensitive they are to oxidation.⁴

3.7. Principal component analysis

PCA was performed to visualize and interpret the variability of oils extracted *via* different methods in terms of yield, composition, and quality.



Before performing the PCA, pre-suitability tests were conducted to verify the statistical validity of the data. The analysis was conducted on the following variables: oil yield, total tocopherols, γ -tocopherol, total chlorophylls, total carotenoids, peroxide value, acidity value, oxidative stability index, phenolic acids, hydroxycinnamic acid amides, lignanamides, phenylpropanoids, and total phenolic compounds. The KMO index gave a score of 0.621, indicating a satisfactory partial correlation between the variables and therefore a good fit of the sample to PCA. In addition, Bartlett's sphericity test was highly significant ($p < 0.001$), confirming that the correlation matrix is not an identity matrix, which validates the existence of significant relationships between the variables.

The PCA showed that the first two principal components (PC1 and PC2) together explained 85.72% of the total variance, with 59.12% for PC1 and 26.60% for PC2, justifying their use for data interpretation. The correlation circle analysis (Fig. 4) provides a more detailed understanding of the contributions of the variables. Total tocopherols, γ -tocopherol, total chlorophylls, total carotenoids, OSI, phenolic acids, hydroxycinnamic acid amides, lignanamides, phenylpropanoids, and total phenolics are oriented in a similar direction (positive PC1 and PC2), indicating that they are strongly correlated with each other. This positive association shows that methods favoring the extraction of one group of antioxidant compounds tend to enrich the others as well. Their strong projection onto the circle attests to their major contribution to the structuring of the data. In addition, there is a strong positive and significant correlation ($p < 0.05$) between these variables (SI Table S1), indicating that methods favoring one of these groups of compounds tend to favor the others as well, probably due to similar extraction

mechanisms (interaction with plant membranes, solvent/compound affinity, *etc.*).

In contrast, the PV and AV variables are orthogonal to those of the bioactive compounds, suggesting that the richer the oil is in antioxidants, the less it is oxidized. This was confirmed by the strong significant positive correlation between these different bioactive compounds and the OSI. This relationship is consistent with the well-established protective role of polyphenols and tocopherols against oxidation.

The oil yield variable is projected intermediately. It is positively oriented on PC2 but negatively oriented on PC1. According to the correlation matrix (SI Table S1), oil yield is positively and significantly correlated ($p < 0.05$) with phenolic compounds such as HCAAs, lignanamides, and phenylpropanoids. These findings indicate that certain methods simultaneously improve the extraction of oil and these compounds, suggesting better extractability of phenolic compounds in the presence of high oil yield.

The projection of the samples on the factorial plane (Fig. 5) reveals a clear separation between the different extraction methods, illustrating the diversity of physicochemical and bioactive profiles of the oils obtained. The oils extracted by SOX-H are clearly grouped in the negative quadrants of PC1 and PC2. This position is strongly correlated with the PV and, to a lesser extent, with the oil yield and AV, reflecting quantitatively efficient extraction, but it is also associated with high levels of primary and secondary oxidation. This profile suggests that while this method maximizes lipid recovery, it compromises the oxidative stability and functional quality of the oil, as indicated by the distance of these samples from the vectors associated with bioactive compounds.

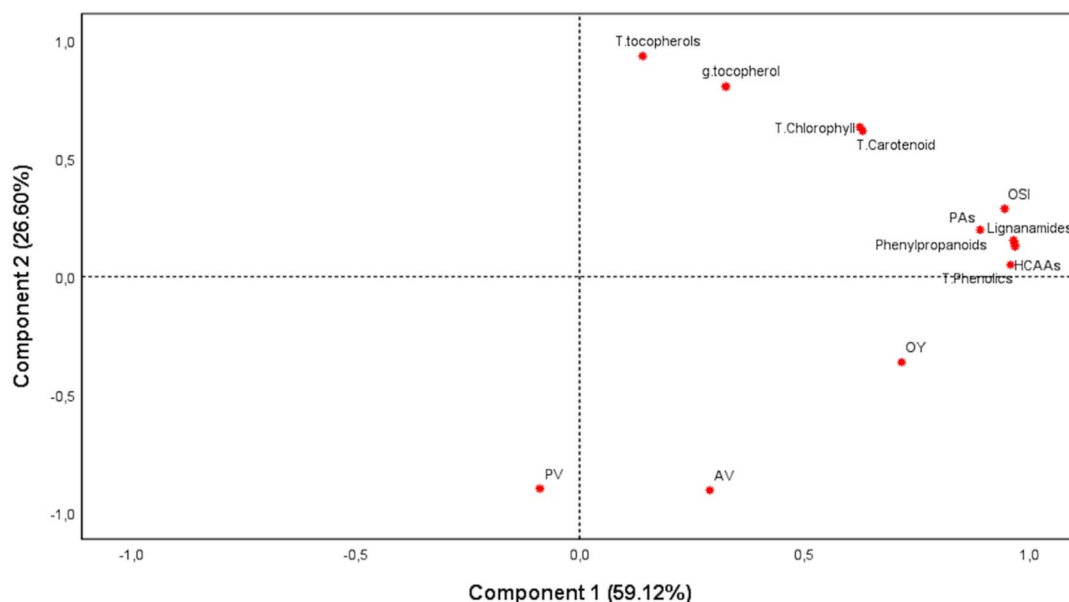


Fig. 4 Principal component analysis illustrating the distribution of key variables in hemp seed oils extracted using different methods. The variables include oil yield (OY), total tocopherols (T. tocopherol), γ -tocopherol, total chlorophylls (T. chlorophyll), total carotenoids (T. carotenoid), peroxide value (PV), acidity value (AV), oxidative stability index (OSI), phenolic acids (PAs), hydroxycinnamic acid amides (HCAAs), lignanamides, phenylpropanoids, and total phenolic compounds (T. Phenolics).



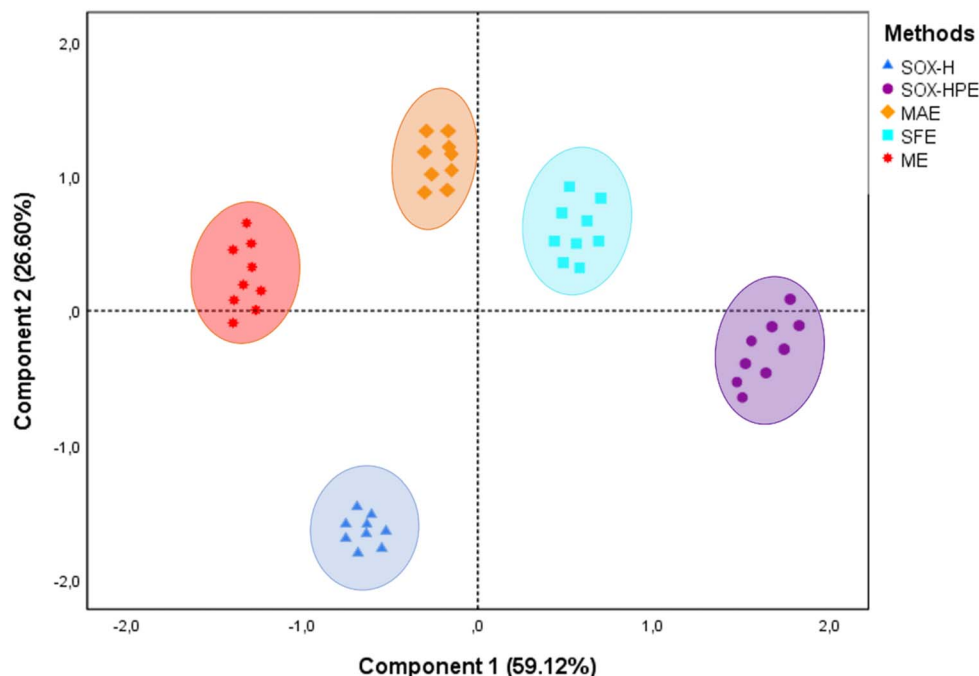


Fig. 5 Representation of the different hemp seed oil extraction methods in the factorial plane formed by the first two principal components (PC1 and PC2). SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with *n*-hexane, 2-propanol and ethyl acetate (40%, 40%, 20%); MAE: microwave assisted extraction (800 W, 13.6 min and 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C).

Conversely, oils extracted by SOX-HPE appear to be grouped in a quadrant characterized by high yields, phenolic compounds, and OSI. This grouping indicates that this method, which combines prolonged extraction and the use of polar solvents, promotes not only high oil extractability but also significant enrichment of antioxidant compounds. These compounds, particularly phenolic compounds, directly contribute to improving the resistance of the oil to oxidation, as evidenced by their correlation with the OSI. The position of these samples on the factorial plane therefore highlights a dual performance: a very high yield and a high content of functional compounds.

The oil obtained by SFE is positioned in the positive quadrant of PC1 and PC2, in association with tocopherols, pigments (chlorophylls, carotenoids), and low AV and PV. This distribution reflects a gentle extraction method, limiting oxidation phenomena while allowing good preservation of sensitive compounds. Although the yield is moderate, the nutritional quality and stability of the oil obtained by SFE make it a particularly interesting technological alternative.

The samples obtained *via* MAE occupy an intermediate position on the factorial plane, with a more marked contribution from PC2. This location reflects a high extraction efficiency of tocopherols and pigments while revealing a lower content of phenolic compounds. This positioning reflects a targeted extraction capacity for certain compounds while revealing certain limitations in terms of yield and extractability.

Finally, oils extracted by ME are at the opposite end of the spectrum of the most effective methods. Their distance from

vectors associated with bioactive compounds, yield, and oxidative stability reflect their limited overall efficiency. These samples are characterized by low concentrations of natural antioxidants, limited yield, and increased vulnerability to oxidation.

3.8. SEM analysis of the seed powder surfaces

SEM analysis revealed clear morphological differences between untreated hemp seed powder (UHS) and the residues obtained after the various extraction methods (Fig. 6). These micrographs provide valuable insights into how each extraction process affects the microstructure of the seed matrix, thereby influencing oil release and the removal of associated lipid components.

The UHS exhibited a compact and granular surface with well-defined spherical structures dispersed within the cellular matrix. These spheres most likely correspond to intact lipid droplets confined within undamaged cells, representing the natural distribution of lipids in hemp seeds prior to extraction.^{55–57} After ME, the surface appeared smoother, flattened, and densified, with the complete disappearance of the spherical lipid structures observed in the control. This transformation suggests that during pressing at 100 °C, partial fusion and coalescence of the lipid phases occurred under the combined effects of pressure and heat. The dense structure observed indicates residual oil entrapment within the compressed matrix, resulting from limited cell rupture and incomplete lipid release. Similar observations were reported by Gharsallah *et al.* who found that ground *Moringa oleifera* seeds



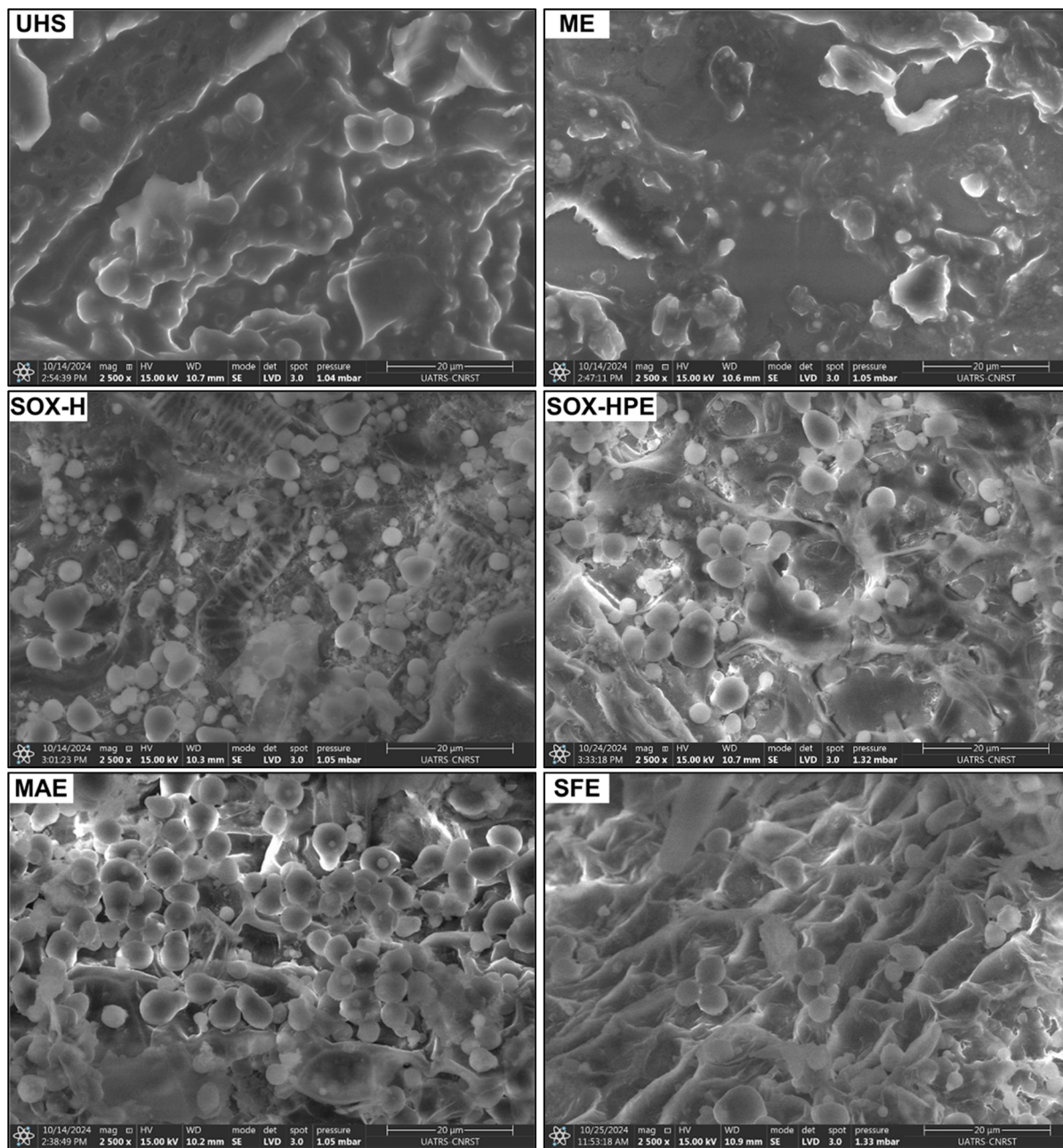


Fig. 6 Scanning electron microscopy micrographs showing the surface morphology of untreated hemp seed powder (UHS) and powders obtained after oil extraction using different techniques: SOX-H: Soxhlet extraction with *n*-hexane; SOX-HPE: Soxhlet extraction with a solvent mixture of *n*-hexane, 2-propanol, and ethyl acetate (40%, 40%, 20%); MAE: microwave-assisted extraction (800 W, 13.6 min, 7.5% EtOH); SFE: supercritical fluid extraction (20 MPa, 50 °C, 244 min, with a mixture of 90% CO₂ + 10% ethanol); ME: mechanical extraction (pressing at 100 °C).

exhibited swollen lipid cells prior to extraction, which became defatted and flattened after pressing.⁵⁶

In contrast, the SOX-H, SOX-HPE, MAE, and SFE methods induced pronounced structural damage, though with distinct morphological characteristics. The surfaces appeared fractured, porous, and exhibited significant loss of structural integrity. These findings are consistent with those of Devi & Khanam,¹⁴ who also reported the formation of deep cavities on the surfaces of hemp seeds following extraction by SOX, SFE, and ULT, resulting from complete lipid depletion.

The SOX-H and SOX-HPE powders exhibited extensive matrix fragmentation associated with high porosity and irregular cavities. In the case of SOX-H, the surface appeared highly heterogeneous, composed of lamellar fragments and dispersed cell debris, reflecting deep cell wall disorganization caused by the combined effects of heat and solvent action, which facilitated lipid migration into the solvent phase. Conversely, the SOX-HPE powder displayed more homogeneous porosity with well-defined cavities. The use of a mixture of polar and non-polar solvents appears to promote more complete



solubilization of lipido-protein components while enhancing the disintegration of the cellulosic network.⁵⁸

The powder obtained by MAE exhibited a highly altered morphology, characterized by the formation of deep cavities and marked surface agglomeration. These transformations result from the action of the high-power microwave field (800 W), which causes rapid dehydration of cellulose and internal vaporization of the cellular content. This phenomenon weakens the mechanical strength of the cell walls, leading to their rupture and thereby facilitating the release and extraction of intracellular constituents.^{59,60}

Finally, the powder obtained after SFE extraction showed a highly disrupted structure characterized by broken and porous layers, visibly deflated and oil-free surfaces, and the presence of longitudinal channels and deep fissures across the surface. These alterations reflect the mechanical stress induced by the depressurization of supercritical CO₂ on the cellular matrix, demonstrating the efficiency of this process in achieving complete oil removal.^{61,62}

These structural changes, in addition to reflecting the impact of each extraction method, also suggest the breakdown of specific chemical bonds between bioactive compounds and various intracellular constituents. In the case of the SOX-HPE, SFE, and MAE methods, the use of 2-propanol and ethanol plays an essential role in this process. These solvents have the ability to profoundly disrupt the architecture of cell walls and membranes by acting directly on macromolecular structures and causing the breakdown of bonds that stabilize interactions between bioactive compounds and the cellular matrix.^{30,42} Their action thus promotes the dissociation of protein-phenol complexes, interactions with polysaccharides, and the extraction of bound compounds.^{63,64}

Consequently, the degree of cellular disorganization induced by these solvents, combined with the effects of microwaves, pressure, or heat from the extraction processes, strongly influences not only the oil yield but also the extractability of bioactive compounds.

4. Conclusion

Various extraction methods, including Soxhlet (SOX-H and SOX-HPE), MAE, SFE and ME, were used to extract oil from hemp seeds. Among these methods, SOX-HPE presented the highest oil yield, reaching 33.24%. This efficiency was confirmed by SEM observation, which revealed a more complete extraction of lipid components.

In terms of composition, the oil obtained by MAE was the richest in tocopherols and pigments (chlorophylls and carotenoids), resulting in a characteristic green color with low lightness, low yellowness, and high greenness. On the other hand, the SOX-HPE and SFE methods stood out for their efficiency in extracting phenolic compounds. These oils were particularly rich in *N-trans*-caffeoyltyramine and Cannabins A and B, phenolic compounds specific to hemp seeds. This high antioxidant content confers increased oxidative stability to the oil, surpassing that of oils obtained by other methods. In terms of fatty acid composition, the oil extracted by SFE was

particularly rich in PUFAs, notably α -linolenic acid, and presented the lowest oil quality indices, indicating superior quality.

Author contributions

Ayman Allay: experiment, formal analysis, investigation, software, writing – original draft. Rafika El Ati: formal analysis, validation. Youssef Rbah: investigation, data curation. Marie-Laure Fauconnier: project administration, visualization. Hana Serghini Caid: validation, supervision. Ahmed Elamrani: funding acquisition, project administration, visualization. Farid Mansouri: conceptualization, methodology, writing – review & editing, supervision.

Conflicts of interest

There are no conflicts to declare.

Data availability

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

Supplementary information: MS² fragmentation spectra of the phenolic compounds identified in hemp (*Cannabis sativa* L.) seed oil, obtained by different methods as well as a Pearson correlation matrix between the main composition and quality variables of hemp seed oils obtained by different extraction methods. See DOI: <https://doi.org/10.1039/d5fb00424a>.

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