Enhancing bioactive compound extractability and antioxidant properties in hemp seed oil using a ternary mixture approach of polar and non-polar solvents

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Abstract

The present study is devoted to optimizing process parameters for solvent extraction to improve both the yield and quality of hemp seed oil. To this end, an augmented simplex lattice mixture design was carried out involving three solvents with different polarities: *n*-hexane, 2-propanol, and ethyl acetate. The findings indicated that the optimal blend for obtaining oil with a high yield and excellent quality was 40% *n*-hexane, 40% 2-propanol, and 20% ethyl acetate. This ternary mixture gave a maximum yield of 33.24% and extracted an oil very rich in bioactive compounds, enabling us to replace 60% of the hexane with two other extraction solvents, 2-propanol and ethyl acetate, which are less toxic to human health and more environmentally friendly. The ideal blend was able to extract an oil with high levels of total phenolic content (226.43 mg GAE/kg), total tocopherols (458.61 mg/kg), total phytosterols (6299.44 mg/kg), and total carotenoids and chlorophylls (19.92 and 66.59 mg/kg, respectively). Moreover, the evaluated oil exhibited significant antioxidant activity as determined by DPPH and FRAP tests (74.64 % reduction and 49.16 mg TEg/100g, respectively). This oil's richness in bioactive compounds gave it better oxidation stability. Furthermore, the phenolic profile of hemp seed oil from every solvent mixture was unveiled using HPLC-DAD/ESI-MS^2, identifying many phenolic compounds, such as *N*-caffeoyltyramine, cannabisins (A, B, C and O) isocannabisine N, demethylgrossamide, 3,3-didemethylgrossamide, 3,3-demethylheliotropamide, and grossamide. Moreover, the analyses indicated a substantial presence of hydroxycinnamic acids and lignan amides (up to 58.79 and 85.01 mg/kg, respectively) in the oil extracted with a binary mixture of 2-propanol – ethyl acetate (50-50%).

Keywords: *Cannabis sativa* L., Hempseed oil, Solvent extraction, Mixture design, Phenolic compounds, Oxidative stability.

Introduction

Cannabis sativa, a plant of profound historical significance, belongs to the Cannabaceae family and is native to central Asia (Bonini et *al.* 2018). Because of its ability to adapt to different soil and climate conditions, hemp (*Cannabis sativa* L.) has a wide geographical distribution, with use in pharmaceutical, textile, biofuel, cosmetic, and skin-care industries (Abdollahi et *al.* 2020). In Morocco, hemp has been cultivated for millennia in northern Morocco, particularly in the Rif Mountains. Its cultivation was illegal until 2020, but since the legislation has changed, it is now possible to exploit the plant and its seeds for industrial purposes.

Hemp seeds have garnered considerable scientific attention for human dietary considerations in recent years due to their interesting nutritional profile, versatile culinary, bioactive properties, and many health benefits. They are a good source of protein, fiber, and oil (Babiker et *al.* 2021; Taaifi et *al.* 2021). The oil yield varies between 28 and 35%, depending on the variety, climatic conditions, geographical region, and year of cultivation (Irakli et *al.* 2019; Taaifi et *al.* 2021). It is distinguished by a higher content (63-84%) of polyunsaturated fatty acids (PUFA), with an optimal *n*-6/*n*-3 ratio (2.5 to 5.5) (Farinon et *al.* 2020; Taaifi et *al.* 2021). Consumption of hemp seed oil has been associated with a subjective reduction in skin dryness and a reduction in the use of topical medication. It has also the potential to reduce the risks of inflammation, arthritis, hypertension, cardiovascular disease, and cancer (Babiker et *al.* 2021; Da Porto, Decorti, and Tubaro 2012). In addition, other interesting compounds, such as tocopherols, phytosterols, pigments, and polyphenols are present in hemp seed oil. These compounds contribute to enhancing nutritional value and overall sensory appeal (Mansouri et *al.* 2023; Özdemir, Bakkalbaşı, and Javidipour 2020). However, phenolic compounds are present at low concentrations, due to their low solubility in the oil. Despite the presence of

these minor compounds in the oil, hemp seed oil remains susceptible to oxidative degradation, due to the higher proportion of PUFA (Mansouri et *al.* 2023).

Different extraction methods have been employed for oil extraction from various oilseed sources (Nde & Anuanwen 2020; Okeleye & Betiku 2019), encompassing mechanical extraction, pressurized solvent extraction, ultrasonic-assisted extraction, supercritical fluid extraction, microwave-assisted extraction, Soxhlet extraction, as well as aqueous enzymatic extraction. These methods could affect not only the total yield but also the oil quality, modifying minor components possessing functional properties, which contribute to the oil's oxidation stability.

Solvent extraction is an effective, simple, and widely applicable method in large-scale industries, enabling high yields with only 0.5–0.7% oil left in the residual by-products (Alcântara et *al.* 2019). Its efficiency depends on various parameters, such as the oilseed type, extraction solvent, particle size, solid-solvent contact time, temperature during extraction, and solid/solvent proportion (Oladipo & Betiku 2019; Ramluckan, Moodley, and Bux 2014). The selection of an extraction solvent is a pivotal factor influencing the oil quality. Several desirable properties characterize an ideal solvent for seed oil extraction. Hexane stands out as the most solvent used for oilseed extraction throughout the world, due to its superior attributes compared to other nonpolar solvents, including high extraction efficiency, excellent solubilizing ability, easy recovery from the extract, and narrow range of boiling point (63–69°C) (Oladipo & Betiku 2019). Polar solvents, recognized for their ability to open cell walls and facilitate the complete extraction of cell contents, stand out as promising bio-renewable solvents for this purpose (Belyagoubi et *al.* 2022; Ramluckan et *al.* 2014). Within the polar solvent category, short-chain alcohols, specifically ethanol and 2-propanol, have gained popularity and acceptance as alternate extraction solvents thanks to their better safety profile and reduced regulatory concerns (Russin et *al.* 2011). The presence of phospholipids, with

their limited solubility in hexane, can impede the extraction process by hindering hexane's access to the oil. To overcome this challenge and facilitate the simultaneous extraction of components with varying polarities, such as phenolic compounds, a co-solvent can be introduced to elevate the polarity of the liquid phase. Numerous low-toxicity solvent mixtures, such as ethyl acetate-hexane and hexane-2-propanol, have become widely adopted in laboratory-scale oil extractions. These solvent mixtures offer the added advantage of reducing solvent recovery costs. Specifically, the hexane-2-propanol mixture has been documented to enhance extraction yield and kinetics, as reported in studies by Bhatnagar & Gopala Krishna (2013) and Okeleye & Betiku (2019).

In this context, this study attempts to identify the best solvent mixture with different polarities to extract oil from hemp seeds and evaluate how various solvent extraction mixtures impact both the yield and bioactive compound composition of extracted oil. To the best of our knowledge, this is the first study to investigate this aspect by utilizing three solvents with distinct polarities to extract hemp seed oil rich in bioactive compounds without affecting oil yield. We hypothesized in this work that *(i)* solvent mixtures with different polarities are as efficient as the conventional *n*-hexane extraction regarding oil yield and that *(ii)* hemp seed oil extracted with solvent mixture contains higher amounts of phenolic compounds with high oxidative stability index. For this purpose, a statistical approach was employed utilizing three selected solvents (*n*-hexane, ethyl acetate, and 2-propanol) to create a simplex lattice mixture design. The practicality of utilizing binary or ternary solvent mixtures was emphasized. The use of solvent mixtures provides variability in polarity, allowing compounds with varying degrees of polarity to be extracted. A comprehensive quality assessment was conducted to examine the influence of extraction solvents on the oil's chemical properties and functional groups. This assessment included the level of oil yield, tocopherols, total phytosterols, pigments (total carotenoids and chlorophylls) total phenolic content, DPPH and FRAP

antioxidant activities, and oxidation stability index. A more detailed study focused on the identification and quantification of phenolic compounds by HPLC-DAD and ESI-MS^2 techniques.

Materials and methods

1. Chemicals and reagents

The reference standards, including phenolic acids (*p*-coumaric, benzoic, *p*-hydroxybenzoic, and sinapic acids), tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol), fatty acid methyl esters (FAME), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and cholesterol, were procured from Sigma-Aldrich (St-Louis, MO., USA). Formic acid and acetonitrile were of LC-MS grade. Analytical grade *n*-hexane, 2-propanol, and ethyl acetate were used as extraction solvents, and other chemicals and reagents used in this work were also of analytical grade and from Merck Chemical Company (Darmstadt, Germany).

2. Plant material

The experimental trials conducted in this study utilized hemp (*Cannabis sativa* L.) seeds of the 'Beldia' variety grown in the Jebha region of northern Morocco in the year 2023. Seeds were supplied by the National Medicinal and Aromatic Plants Agency, Morocco. The seeds were cleaned to remove impurities and stored in plastic bags at 2 – 4°C in the refrigerator until use. Their moisture content was 5.13 ± 0.11%, determined by drying the seeds at 100 ± 3°C for 24 h.

3. Extraction of oil from hemp seeds

Three distinct solvents (*n*-hexane, ethyl acetate, and 2-propanol) were investigated for their ability to extract hemp seed oil. The characteristics of the selected extraction solvents are presented in Table 1. These solvents were deliberately selected to encompass polar solvents (ethyl acetate and 2-propanol with dielectric constants of 6.02 and 18.3, respectively) and a non-polar solvent (hexane of 1.89 dielectric constant).

First, the seeds were finely ground using a laboratory grinder and then sieved to obtain a homogenous particle size (< 500 μm). Following the defined experimental plan for different solvent mixtures (Table 2), 30 g of sieved hemp seed powder was placed in the Soxhlet apparatus with 180 mL of the extraction solvent. The experimental setup was heated using controlled heating balls operating at the respective boiling point of either a single solvent or a solvent mixture. After 5 hours of extraction, a rotary evaporator was employed to eliminate the solvent from the mixed oil-solvent based on the evaporation point of each solvent and their combinations. After weighing, the acquired oil was transferred into opaque vials and preserved under nitrogen in a freezer at -18°C for two weeks until analysis. All extractions were carried out in triplicate. The efficiency of oil extraction was calculated using the subsequent equation (1):

$$\text{Oil yield } (\%) = \frac{\text{the weight of soxhlet oil}}{\text{the weight of seed powder}} \times 100 \qquad (1)$$

4. Mixture design

To optimize the extraction process of hemp seed oil, an augmented simplex lattice design was employed, incorporating three solvents: *n*-hexane, ethyl acetate, and 2-propanol. The optimization criterion aimed to minimize prediction variance with a reduced number of experiments. Various conditions were tested, encompassing pure components representing 100% of each solvent, permutations of binary mixtures (1/2; 1/2), and ternary mixtures (2/3; 1/6; 1/6 and 1/3; 1/3; 1/3). The design, aimed at optimizing the best proportion of *n*-hexane, ethyl acetate, and 2-propanol, resulted in 11 experimental conditions (Table 2), each performed in triplicate. The chosen response parameters (dependent variables Y_i) included oil yield, total tocopherols, total phytosterols, total chlorophylls, total carotenoids, total phenolic compounds, DPPH and FRAP antioxidant activities, and oxidation stability index. All chemical analyses were carried out in triplicate for each oil sample.

5. Chemical characterization of extracted oils

5.1 Tocopherol analysis

Tocopherols were analyzed using an HPLC system equipped with a DAD detector (LC-6AD Shimadzu system). Separation of α, β, γ, and δ-tocopherols was carried out on a silica Uptisphere 120 Å NH_2 column (4.6 × 250 mm, 5 µm particle size) according to the protocol described by Ben Moumen et *al.* (2015a). Tocopherols were identified and quantified using mixed commercial tocopherol standards by comparing their retention times and peak areas at 292, 296, and 298 nm with those of the sample components. Tocopherol content was expressed as mg/kg oil. Details of the limits of detection and quantification and the linear regression for each tocopherol are included in the supplementary data (Table S1).

5.2 Total phytosterol content

The amount of total phytosterols in the oil samples was determined using the Liebermann-Burchard method (Rbah et *al.* 2024). A precisely weighed mass of 100 µg of oil was blended with 1 mL of chloroform. Subsequently, 2 mL of Liebermann-Burchard reagent was added, and the volume was adjusted to 7 mL with chloroform. The Liebermann-Burchard reagent reacted with phytosterols, resulting in a distinctive green color with measurable intensity at 640 nm. Total phytosterols content was then calculated and expressed as mg/kg oil using a cholesterol calibration curve. Details of the limits of detection and quantification and the linear regression for cholesterols are given in the supplementary data (Table S1).

5.3 Total chlorophyll and carotenoid contents

Quantification of carotenoid and chlorophyll content in the oil followed the procedure outlined by Liang et *al.* (2018). In summary, 0.1 g of the oil sample was mixed with 5 mL of diethyl ether. It was then thoroughly mixed and ultrasonically extracted for 1 minute. The absorbance was read on the spectrophotometer at the following wavelengths: 470 nm, 640 nm, and 663 nm. Contents in mg/kg oil of total chlorophylls and carotenoids were then estimated using equations (2) and (3) provided by Liang et *al.* (2018).

$$Chlorophylls\ a+b\ (mg/kg) = \frac{(7.12 \times A_{663}) + (16.80 \times A_{640})}{W} \quad (2)$$

$$Total\ Carotenes\ (mg/kg) = \frac{(1000 \times A_{470} - 0.52 \times Chly\ a - 7.25 \times Chly\ b)}{226 \times W} \quad (3)$$

where A: absorbance at wavelengths (663, 640, and 470 nm), and W: weight of hemp seed oil.

5.4 Total phenolic content

Total phenols were extracted using the method described in the literature by Ben Moumen et *al.*, (2015b) using 80% methanol. The extract was subsequently used for the Folin-Ciocalteu assay. To 0.05 mL of the concentrated extract obtained by liquid-liquid extraction, 1 mL of Folin-Ciocalteu reagent, diluted tenfold, and 1 mL of a 10% Na_2CO_3 aqueous solution were combined. After shaking, the mixture was left in darkness for 90 min before measuring absorbance at 760 nm. The results, expressed in mg of gallic acid equivalents per kg of hemp seed oil, were derived using a calibration curve as a reference. Details of detection and quantification limits and linear regression for gallic acid are given in the supplementary data (Table S1).

5.5 Identification & quantification of phenolic compounds using HPLC-DAD/ESI-MS^2

Phenolic compounds were identified and quantified by a High-Performance Liquid Chromatography system (Agilent 1260 Infinity II) equipped with a diode array detector (DAD) along with an ESI-MS^2 technique. A C18 column (150×4.6 mm) with 3.5 µm particle size was employed for the chromatographic separation. The chromatographic process involved a gradient elution using a mobile phase containing acetonitrile (A) and water (B) with formic acid (1%). The elution followed a gradient mode as per the method outlined by Benkirane et *al.* (2022). A flow rate of 600 µL min^{-1} was set, and the injection volume was fixed at 10 µL. Detection of phenolic compounds occurred at wavelengths of 254, 280, 300, and 340 nm, and UV-visible spectra for each compound were recorded between190 and 600

nm. Chromatographic data were visualized and analyzed using Agilent OpenLABCDS software. After the separation process, peaks were collected and subjected to identification using mass spectrometry (MS). The MS analysis was performed using a mass ion trap instrument (HCT Esquire, Daltonics Bruker, Deutschland) equipped with an electrospray ionization (ESI) source operating in positive and negative modes. ESI parameters included a sputtering voltage of 4500 V, a dry gas temperature of 200°C, a dry gas flow rate of 4 L/min, and a nebulizer set at 10 psi. MS^2 mass spectra were generated by isolating the precursor ion and applying a 1-10% arbitrary unit collision energy. For data treatment, the software package ACDlabs of 2021.2.1 was employed. The phenolic compounds were identified out by comparing their MS, MS^2, and UV spectra with the literature. Then, they were quantified using peak areas determined on the HPLC-DAD system measured at the 280 nm wavelength. As most of the phenolic compounds identified are less commercially available, quantification was carried out using an external calibration curve for *N-trans*-caffeoyltyramine. The results were expressed in milligrams of *N-trans*-caffeoyltyramine equivalent per kg of oil. Additionally, *p*-hydroxybenzoic, benzoic acid, *p*-coumaric, and sinapic acids were quantified using authentic standards. Details of the detection and quantification limits for each compound are shown in Supplementary data (Table S1).

5.6 DPPH scavenging activity

The 11 phenolic extracts' ability to scavenge free radicals was evaluated through the DPPH (1,1-diphenyl 2 picrylhydrazyl) test, following the methodology outlined by Benkirane et *al.* (2023). A quantity of 200 µL from each extract was blended with 2.3 mL of a DPPH solution (1.3×10^{-4} mol L^{-1} in methanol). The resultant blend was left to incubate in darkness at room temperature for 20 minutes. The reduction in DPPH absorbance at 517 nm was measured and expressed as the percentage of DPPH inhibition, utilizing the following equation (4):

$$\%\ \text{of inhibition} = \frac{DO_{DPPH} - DO_{\text{extract}}}{DO_{DPPH}} \times 100 \qquad (4)$$

5.7 Ferric reducing antioxidant power (FRAP)

The reducing capability of the analyzed samples was evaluated according to the procedure outlined by Benkirane et *al.* (2023). Specifically, 200 µL of each extract was blended with 1.25 mL of a 1g/100mL potassium ferricyanide solution and 1.25 mL of a phosphate buffer (pH 6.6) solution. After incubation (30 min, 50°C), a volume of 1.25 mL of tri-chloroacetic acid 10 % solution was added, followed by a 15-minute centrifugation at 3024 g. The resulting supernatant was mixed with distilled water (1.25 mL) and iron chloride (250 µL). Finally, the absorbance was recorded at 700 nm. To assess the reducing power of the bioactive compounds in the extracts, a calibration curve was generated using increasing Trolox concentrations. The findings are presented as milligrams of Trolox equivalent per 100 grams of oil.

5.8 GC-FID fatty acid analysis

The GC-FID analysis was employed to determine the fatty acid profile of the hemp seed oil. Before injection, the fatty acids were converted into fatty acid methyl esters (FAME) using KOH in methanol as the methylating agent, following the 1K-07 AOCS protocol 2007 (AOCS 2007). The analysis of FAME was carried out using GC-FID (GC Agilent 6890, Agilent Technologies) equipped with a BPX70 capillary column (60 m × 0.32 mm, 0.25 µm; SGE Europe). Helium served as the carrier gas at a 1 mL/min rate. The oven temperature rose from 50°C to 170°C at a rate of 30°C/min, followed by a further increase of 4°C/min to 220°C, where it remained constant for 10 minutes. Fatty acid peaks were identified by comparing their retention times with those of a Sigma-Aldrich standard. The results are expressed as a percentage of the total fatty acids.

5.9 Oxidative stability index

The oxidative stability index of hemp seed oil was assessed with the Rancimat method (Mansouri et *al.* 2019). This stability was quantified as the induction time (in hours) measured

using the Metrohm 743 Rancimat. The experimental conditions involved a sample size of 3 g, an air flow rate of 20 L/h, and a temperature of 100°C.

5.10 Peroxide value, free acidity, and ultraviolet absorbance determinations

The free acidity and UV absorbance values at 232 and 270 nm were determined according to the official methods of the European Commission for olive oil (Mansouri et *al.* 2019). The peroxide value was performed according to the Cd 8-53 method from the AOCS Official Methods of Analysis.

5.11 Statistical analysis

Regression analysis was performed on the data to evaluate the linear (eq. 1), quadratic (eq. 2), special cubic (eq. 3) and full cubic (eq. 4) models. The most appropriate model, with a confidence level of 95%, was adopted. In addition, various statistical measures, such as the coefficient of determination (R^2), adjusted coefficient of determination (R^2 adjusted), and lack of fit, were used to assess the goodness of fit of the polynomial equation derived from the analysis. The significance of the model's regression coefficients was determined by analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$. In addition, a contour plot corresponding to the established mathematical model was generated to examine the significant individual and interactive influence of the three solvents on each response. All analyses were performed using JMP Pro 15 software (SAS Institute Inc., USA) and Statistica software (version 10.0; StatSoft Inc., USA).

Linear: $$Y_i = \sum_{i=1}^{q} \beta_i x_i \quad (1)$$

Quadratic: $$Y_i = \sum_{i=1}^{q} \beta_i x_i + \sum_{i<j}^{q-1} \sum_{j}^{q} \beta_{ij} x_i x_j \quad (2)$$

Special cubic: $$Y_i = \sum_{i=1}^{q} \beta_i x_i + \sum_{i<j}^{q-1} \sum_{j}^{q} \beta_{ij} x_i x_j + \sum_{i<j}^{q-2} \sum_{j<k}^{q-1} \sum_{k}^{q} \beta_{ijk} x_i x_j x_k \quad (3)$$

290 Full cubic: $Y_i = \sum_{i=1}^{q} \beta_i x_i + \sum_{i<j}^{q-1} \sum_{j}^{q} \beta_{ij} x_i\, x_j + \sum_{i<j}^{q-1} \sum_{j}^{q} \delta_{ij} x_i\, x_j (x_i - x_j) +$

291 $$\sum_{i<j}^{q-2} \sum_{j<k}^{q-1} \sum_{k}^{q} \beta_{ijk} x_i x_j x_k \quad (4)$$

292 where Y_i is the dependent variables; x_i, x_j, and x_k are the independent variables; β_i represents
293 the linear regression coefficient, β_{ij} the binary interaction coefficient, and β_{ijk} the ternary
294 interaction coefficient.

295 **Results and discussion**

296 This study assessed the effect of three solvents (*n*-hexane, 2-propanol, and ethyl acetate) and
297 their mixtures on various response models of hemp seed oil. Hexane, ethyl acetate, and 2-
298 propanol were selected and involved in devising a simplex lattice mixture design,
299 encompassing 11 experiments. The extraction time and particle size of the ground seeds
300 remained constant across all experiments. The extraction time was set at 5 hours and the
301 particle size was determined to be less than 500 μm through sieving.
302 The outcomes of these experiments spanning oil yield, total tocopherols, total phytosterols,
303 total chlorophylls, total carotenoids, total phenolic compounds, DPPH and FRAP antioxidant
304 activities, and oxidation stability index, are outlined in Tables 2 and 4. Various regression
305 models (linear, quadratic, cubic, and full cubic) were examined to identify the best fit for the
306 experimental data. For all responses, we used the quadratic model, as the linear, cubic, and
307 full cubic models had no significant enhancement of fit against the surface. The ANOVA
308 results for the quadratic regression model predicting all responses are detailed in Table 3. All
309 quadratic models demonstrated statistical significance ($p < 0.05$) and exhibited no significant
310 ($p > 0.05$) lack of fit at a 95% confidence level, with R^2 ranging from 0.935 to 0.981. The
311 quadratic models, which express the relationship between each analytical response and the
312 three variables (solvents), were used to generate response surfaces.

313 **1. Solvent effect on oil yield**

The results presented in Table 2 reveal that the extraction solvent significantly ($p < 0.05$) influences the hemp seed oil yield. Solvent polarity is a crucial parameter determining interactions between solute and solvent (Okeleye & Betiku 2019; Oladipo & Betiku 2019). The yield of hemp seed oil using various solvent proportions ranged from 25.51 to 33.87%. Notably, the oil yield exhibited a substantial increase from ethyl acetate to 2-propanol and further to hexane. Considering the properties of each solvent (Table 1), it was observed that the oil yield increased as the polarity of the extraction solvent decreased. The elevated yield with hexane can be attributed to its lower polar index with lower boiling point compared to ethyl acetate and 2-propanol. This implies that hexane molecules penetrate the seeds more rapidly, facilitating increased oil extraction (Alcântara et *al.* 2019; Ramluckan et *al.* 2014). The dissolvability of ethyl acetate and 2-propanol, which are polar solvents, might be influenced by non-polar groups, such as fatty acid chains, leading to a reduced oil yield compared to hexane, which is a non-polar solvent. Moreover, the absence of the OH group in hexane, recognized for its interference during the oil extraction from certain oilseeds (Jayaprakasha, Singh, and Sakariah 2001), could contribute to its higher yield. Conversely, the heightened polarity and/or dipole moment associated with polar solvents may restrict lipid solubility, inducing lipid hydrolysis (Russin et *al.* 2011) and reducing oil yield in ethyl acetate and 2-propanol solvent systems. Therefore, the relatively high yield obtained with 2-propanol (31.19%), despite its high overall dielectric constant, can be elucidated by its ability to open cell walls for complete extraction of cell contents (Tir, Dutta, and Badjah-Hadj-Ahmed 2012). This polar solvent may also induce some specific interactions, potentially including the bonding of hydrogens and triglyceride ester groups (Belyagoubi et *al.* 2022).

The correlation between variations in oil yield and solvent proportions is depicted in the contour plot (Fig.1A). The contour plot representing the mixtures of the three solvents indicates that solvent combinations exhibit a superior oil extraction capacity compared to

individual solvents. The use of solvent mixtures comprising hexane, ethyl acetate, and 2-propanol (in ratios of 2/3:1/6:1/6 and 1/3:1/3:1/3; v:v:v) resulted in an increase in yield (33.87 and 33.84%, respectively) compared to hexane alone (32.72%). This phenomenon could be attributed to the solvent mixture's ability to permeate the bilayers of cell membranes and to extract a broad spectrum of constituents, in particular phospholipids and phenolic compounds (Bhatnagar & Gopala Krishna, 2013; Tir et *al.*, 2012).

2. Solvent effect on bioactive compounds

2.1 Total tocopherols

Tocopherols are characterized by their fat-soluble nature and powerful antioxidant properties. As the predominant antioxidants in hemp seed oil, tocopherols have a crucial role in protecting the oil against oxidation (Farinon et *al.* 2020). In the present study, three extraction solvents and their combinations were used to investigate how solvent polarity affects the extractability of tocopherol-rich hemp oil. The results revealed variations in the total tocopherol content of hemp seed oil extracts, ranging from 410.47 to 508.55 mg/kg (Table 2). Structurally, the existence of long unsaturated aliphatic chains gives tocopherols their hydrophobic property. In addition, the absence of strong polar moieties in their molecules would imply that the solubility of tocopherols in hexane was reasonably higher. However, irrespective of their apolar properties, their solubility in ethyl acetate and 2-propanol was significantly ($p < 0.05$) higher than in hexane. Analog results were observed in sesame seed oil and Indian Niger seed oil, showcasing a high total tocopherol content when extracted using polar solvents like isopropanol and ethanol, as opposed to apolar solvents, such as hexane, petroleum ether, and chloroform (Tir et *al.* 2012; Bhatnagar and Gopala Krishna 2013). In addition, ternary mixtures (2-propanol-hexane-ethyl acetate) and binary mixtures (ethyl acetate-hexane) gave high levels of total tocopherols. Similar results have been observed in studies on rice bran oil and sesame oil (Chen & Bergman 2005 and Tir et *al.* 2012), where

364 solvent mixtures showed a higher tocopherol extraction capacity than pure hexane. Indeed, a
365 more polar combination of extraction solvents is systematically required to improve
366 tocopherol extraction. This can be attributed to the presence of water molecules, which form a
367 barrier against the penetration of apolar solvents (Soroush et *al.* 2021). Indeed, the seeds we
368 used in our study had a moisture content of 5.13%, which could explain the obtained results.
369 The contour plot (Fig. 1B) illustrates the high levels of total tocopherols in mixtures
370 containing 2-propanol. This could be attributed to the role of 2-propanol in opening the cell
371 wall, facilitating complete extraction of plant cell contents and subsequent solubilization of
372 phospholipids, unsaponifiable and some other polar molecules, due to their inherent polar
373 nature (Bhatnagar and Gopala Krishna 2013; Tir et *al.* 2012).

374 **2.2 Total phytosterols**

375 Phytosterols are vital constituents of the unsaponifiable matter in hemp seed oil. Due to their
376 structural similarity to cholesterol, phytosterols, when introduced into the gastrointestinal
377 tract, compete with cholesterol, thus reducing its intestinal absorption (Farinon et *al.* 2020).
378 To enhance solvent penetration into lipids and augment phytosterol solubility, various solvent
379 mixtures were employed. The results revealed variations in the total phytosterols content in
380 hemp seed oil extracts, ranging from 4541.4 to 6744.7 mg/kg oil (Table 2). The predictive
381 equation (Table 3) revealed that phytosterol extraction was significantly ($p < 0.05$) more
382 effective with a pure solvent than with diverse solvent combinations. Nonetheless, polar
383 solvents, such as ethyl acetate and 2-propanol, facilitated a more substantial extraction of total
384 phytosterols compared to hexane (Fig. 1C). This outcome could be attributed to the dipole
385 moment of ethyl acetate and 2-propanol molecules. Solvation forces between these solvents
386 and the sterol core might be more favorable than with hexane. The lowest phytosterol content
387 was recorded in hexane, followed by 2-propanol, and then ethyl acetate-extracted oils
388 (4541.4, 6508.9, and 6619.5 mg/kg, respectively). The highest phytosterol levels were

obtained with the binary mixture of 2-propanol-ethyl acetate (50%-50%) and the ternary mixture (1/6; 1/6; 2/3) of hexane, ethyl acetate, and 2-propanol (6744.7 and 6631 mg/kg, respectively).

As demonstrated in a prior study, the inclusion of 2-propanol in the solvent mixture facilitates the desorption and dissolution of the lipid bilayer, leading to increased solubility of phytosterols (Tir et *al.* 2012). To date, limited research has delved into the hemp seed oil phytosterol content. Montserrat-De La Paz et *al.* (2014) and Stambouli et *al.* (2006) stated that total phytosterol contents reached 3765 and 5304.4 mg/kg in hexane-extracted oil, respectively, which are notably lower compared to the binary 2-propanol-ethyl acetate mixture with a content of 6744.7 mg/kg.

2.3 Total chlorophylls and carotenoids

The extraction of chlorophylls and carotenoids is influenced by their dissolution in a given solvent, which is associated with their respective polarities. Generally, the solubility of chlorophylls and carotenoids is primarily determined by their structural characteristics, including molecular size, presence or absence of hydroxyl groups, and hydrocarbon chain length. β-carotene and lycopene are the least polar carotenoids, but the introduction of polar functionalities (such as hydroxyl radicals) to their composition, enhances their polarity, which is the case in zeaxanthin and lutein (Aachary et *al.* 2016; Patsilinakos et *al.* 2018).

Total chlorophylls and carotenoids in hemp seed oil extracts were studied using different proportions of three solvents. As depicted in Table 2, the content of chlorophylls and carotenoids varied from 8.02 to 58.83 mg/kg and 1.78 to 34.46 mg/kg, respectively. The binary mixture (50% hexane and 50% 2-propanol) exhibited significantly ($p < 0.05$) the highest concentration of carotenoids, while a more polar mixture (1/6 hexane, 1/6 ethyl acetate, and 2/3 2-propanol) yielded the highest concentration of chlorophylls. This may be explained by the fact that carotenoids are generally non-polar, whereas chlorophylls are

414 considered to be slightly polar or amphipathic molecules (with polar and non-polar
415 characteristics) (Ashenafi et *al.* 2023; Lichtenthaler 1987). In addition, the results showed that
416 the polar solvents (2-propanol and ethyl acetate) were more effective at extracting the pigment
417 than the apolar solvent (hexane). Similar results were observed by Soroush et *al.* (2021), who
418 showed that oil extracted by microwaves using isopropanol as solvent was rich in pigments
419 (carotenoids and chlorophylls) compared with that extracted using hexane. Carotenoids and
420 chlorophylls have different polarities depending on their chemical structures and functional
421 groups. Chlorophylls a and b have a porphyrin ring structure with a magnesium ion in the
422 center and a phytol side chain. The ring confers a certain degree of polarity, while the side
423 chain is non-polar (Fiedor et *al.* 2008). Overall, chlorophylls are considered to be more polar
424 molecules, which explains their high concentration in the oil extracted with ethyl acetate and
425 2-propanol. Regarding carotenoids, β-carotene and lycopene are generally non-polar
426 molecules due to their long hydrocarbon chains composed of conjugated double bonds.
427 However, the introduction of polar functionalities, such as hydroxyl radicals, enhances their
428 polarity, as is the case for zeaxanthin and lutein (Aachary et *al.* 2016; Patsilinakos et *al.*
429 2018). For hemp seeds, Irakli et *al.* (2019) found that the seeds contained three main
430 carotenoids: lutein being the most abundant with a percentage of 72%, followed by β-carotene
431 (17%), and zeaxanthin (11%). Several studies have shown that lutein and zeaxanthin are
432 soluble in polar solvents, whereas β-carotene is soluble in non-polar solvents (Ashenafi et *al.*
433 2023; Saini and Keum 2018). This is consistent with our results where the oil extracted with
434 ethyl acetate and 2-propanol was rich in carotenoids compared to that extracted with hexane.

435 The contour plots of total chlorophylls and carotenoids (Fig. 1D-E) illustrate that the
436 combination of solvents makes a greater contribution to improving pigment extraction.
437 Furthermore, the prediction equation for carotenoids reveals that pigment extraction
438 efficiency follows the following order: 2-propanol-hexane; ethyl acetate; 2-propanol; hexane-

ethyl acetate; 2-propanol-ethyl acetate; hexane (Table 3). In addition, the efficiency of chlorophyll extraction was as follows: 2-propanol-hexane; ethyl acetate; 2-propanol-ethyl acetate; 2-propanol; hexane-ethyl acetate; hexane (Table 3). This suggests that the combination of solvents contributes more to enhancing the extraction of chlorophylls and carotenoids. This outcome is consistent with other studies reporting the efficacy of slightly polar mixtures, like 2-propanol-hexane, in extracting both chlorophylls and carotenoids from plants (Chand et *al.*, 2022; Patsilinakos et *al.*, 2018). Hence, it can be inferred that the pigments found in hemp seeds are predominantly of medium polarity.

2.4 Phenolic compounds

2.4.1 Total phenolic content

Phenolic compounds constitute a category of small molecules identified by their chemical structure presenting at least one phenol unit. These molecules exhibit diverse chemical structures, enabling their categorization into various subgroups comprising phenolic acids, tannins, flavonoids, stilbenes, coumarins, lignans, quinones, and others. Due to the substantial variation in their polarity, devising an optimal method for extracting all phenolic compounds proves challenging. Therefore, the optimization of solvent mixtures becomes imperative to improve the extraction efficiency of phenolic compounds from diverse matrices (Benkirane et *al.* 2022).

Table 4 displays the total phenolic content of the oil extracted from Moroccan hemp seeds in each run, varying from 19.43 to 376.06 mg GAE/kg across different solvent ratios. The contour plot (Fig. 2A) illustrates that solvent mixtures have significantly ($p < 0.05$) a greater capacity for phenol extraction compared to pure solvents. Table 3 outlines the regression coefficients employed for formulating the prediction equation for TPC (contour plot of total phenolic content). No antagonistic effects were observed for the diverse solvent mixtures. However, certain mixtures demonstrated more synergistic effects than others. Hexane in its

pure form had the lowest TPC, while its combination with 2-propanol and ethyl acetate resulted in 10 to 18 times higher TPC. This phenomenon could be attributed to the synergistic effects arising from the mixture of hexane, ethyl acetate, and 2-propanol (Bhatnagar and Gopala Krishna 2013; Okeleye and Betiku 2019). Plant matrices encompass various classes of bioactive substances with distinct polarities. The effectiveness of phenol extraction is notably contingent on the solubility of these compounds in a given solvent, a factor intricately tied to their polarities (Benkirane et *al.* 2022). Typically, phenolic compounds prefer polar solvents, explaining the notably low concentrations of TPC in oil extracted with hexane (solvent polarity index = 0.1). The solubility of these compounds is mainly determined by their structural characteristics, including molecular size, presence or absence of hydroxyl groups, hydrocarbon chain structure, and the methylation degree (Gheldof, Wang, and Engeseth 2002; Kuti & Konuru 2004). In addition, the specific physical properties of solvents, such as viscosity and density, have a significant impact on their efficiency in the extraction process. In general, solvents characterized by low viscosity and density exhibit strong extraction capabilities due to their enhanced diffusivity, facilitating substantial mobility of solute and solvent molecules, thereby improving extraction efficiency.

2.4.2 Identification and quantification of phenolic compounds

The identification of phenolic compounds in hemp seed oil was carried out by HPLC-DAD/ESI-MS^2. The detected compounds (Table 5 and Table S2) were characterized by matching the mass of the precursor ion (MS), their fragments (MS^2) in positive and negative modes, and their UV spectra with the existing literature (Benkirane et *al.* 2022; Leonard, Zhang, Ying, Xiong, et *al.* 2021; Moccia et *al.* 2020; Nigro et *al.* 2020). Table 5 outlined that 29 compounds were identified in hemp seed oils, comprising 2 hydroxybenzoic acids, 7 hydroxycinnamic acids and their derivatives, along with 20 lignan amides (Supplementary Fig. S1). The findings underscore the abundance of phenylpropanoids, a phenolic subgroup

encompassing hydroxycinnamic acid amides (HCAAs) and lignan amides, in hemp seed oil. These observations align with several previous research studies on hemp seeds that have noted and reported the existence of phenylpropanoids, particularly caffeoyltyramine and cannabisins (Benkirane et *al.* 2022; Leonard, Zhang, Ying, and Fang 2021; Nigro et *al.* 2020). Although literature regarding phenolic profiles of hemp seed oil is limited, a few studies have highlighted the occurrence of other phenolic compounds, including additional phenolic acids and flavonoids, such as quercetin, rutin, epicatechin, catechin, kaempferol, isorhamnetin, naringenin, and apigenin, in cold-pressed oil (Faugno et *al.* 2019; Occhiuto et *al.* 2022; Smeriglio et *al.* 2016).

The HPLC-DAD analysis was employed to quantify the phenolic compounds in hemp seed oils obtained using varying solvent proportions according to the experimental design. The quantification results revealed that the proportions of the three solvents utilized in the extraction process significantly ($p < 0.05$) influenced the presence and concentration of phenolic compounds in the resulting oil (Table 6). The variations in polarity within the oil extraction system led to distinct extraction levels for each phenolic compound, highlighting the sensitivity of the process to changes in polarity.

The highest concentrations of phenolic compounds were noticed in oils obtained through the binary mixture (2-propanol 1/2 – ethyl acetate 1/2), measuring 161.35 mg/kg of oil, and the ternary mixture (2-propanol 1/3 – hexane 1/3 – ethyl acetate 1/3), recording 155.35 mg/kg. These mixtures, prominently featuring 2-propanol, showcased the significant role of this solvent in phenolic compound extraction, which following the Folin-Ciocalteu method. Furthermore, 100% 2-propanol exhibited remarkable efficacy in extracting phenolic compounds, in stark contrast to the almost negligible amounts extracted by 100% ethyl acetate and 100% hexane. As noted in the TPC assay, the ability of 2-propanol to open cell walls facilitated the comprehensive extraction of cell contents, and its polarity facilitated the

514 solubilization of phenolic compounds. However, the total phenolic compounds of oils
515 extracted with different solvent mixtures, as determined by HPLC, were relatively low,
516 ranging from 2.32 mg/kg for oil extracted with hexane to 162 mg/kg for oil extracted with the
517 binary mixture 50% 2-propanol – 50% hexane), compared to those obtained by the Folin-
518 Ciocalteu method (ranging from 19.43 to 376.06 mg GAE/kg) for the same extraction
519 solvents. Similar results have been reported in other studies (Gheldof et *al.* 2002; Kuti &
520 Konuru 2004). The probable reason for the disparity between the two methods is that the
521 Folin-Ciocalteu method lacks specificity in the determination of phenolic compounds,
522 reacting positively with many non-phenolic compounds (Gheldof et *al.* 2002; Kuti and
523 Konuru 2004). Consequently, the results of the Folin-Ciocalteu method (expressed as mg
524 GAE/kg) may lead to an overestimation of the total phenolic content. Moreover, oils extracted
525 through the binary mixture (run 6) demonstrated a richness in lignan amides, with a content of
526 85.66 mg/kg, compared to the ternary mixture (run 9), with 73.86 mg/kg. However, the latter
527 extracted oil had a higher content of phenolic acids and HCAAs, measuring 12.42 and 61.34
528 mg/kg, compared to 9.19 and 56.95 mg/kg in the binary mixture (run 6).
529 *N-trans*-caffeoyltyramine emerged as the most prevalent compound in the extracted oils,
530 reaching concentrations of up to 42.59 mg/kg in the oil extracted with 2-propanol and ethyl
531 acetate (50-50%). This compound constituted 26.29% of the total phenolic compound content.
532 Notably, the most notable lignan amides in terms of quantity were cannabisins A and B,
533 exhibiting substantial levels of 16.78 and 16.27 mg/kg, respectively, in oils obtained through
534 the combination of 2-propanol and ethyl acetate (50-50%). Cannabisins A and B, and *N-trans*-
535 caffeoyltyramine have been identified and quantified as predominant phenolic constituents in
536 *Cannabis* seeds, as documented in earlier research (Benkirane et *al.* 2022; Leonard, Zhang,
537 Ying, Xiong, et *al.* 2021). These three compounds are acknowledged for their therapeutic
538 attributes and their anti-inflammatory and antioxidant effects (Farinon et *al.* 2020).

539 Sinapic acid was found in oils across all extraction runs, reaching its highest concentration
540 (2.1 mg/kg) in the oil obtained by combining hexane (2/3), ethyl acetate (1/6), and 2-propanol
541 (1/6). This hydroxycinnamic acid is associated with various therapeutic properties beneficial
542 for human health. However, *p*-coumaric acid, *p*-hydroxybenzoic acid, cannabisin C,
543 cannabisin D, isocannabisin N, and cannabisin G exhibited low levels or were not detected in
544 most of the extracted oils.
545 The binary mixture of polar composition (ethyl acetate 1/2 - 2-propanol 1/2), in addition to its
546 richness in phenols, gave a very strong antioxidant activity (DPPH and FRAP). The
547 antioxidant properties of phenolics stem from their chemical structure, with parameters such
548 as the extent of methylation and the number and position of hydroxyl groups playing crucial
549 roles (Vuolo, Lima, and Maróstica Junior 2019; Zeb 2021). Amongst the main identified
550 compounds in the solvent-extracted oil, *N-trans*-caffeoyltyramine exhibits a molecular
551 structure composed of two aromatic rings and three hydroxyl groups, while cannabisins A and
552 B consist of five aromatic rings with six hydroxyl groups. Hence, they offer abundant
553 hydroxyl groups for effective interaction with free radicals. *N-trans*-caffeoyltyramine along
554 with cannabisins A and B have shown robust antioxidant properties, as evidenced by their
555 ability to scavenge DPPH radicals, protect LDL against oxidation, and counteract neuro-
556 inflammatory processes *in vivo* (Farinon et *al.* 2020).

557 **3. Solvent effect on antioxidant parameters**

558 **3.1 Antioxidant activity**

559 The primary determinant of an oil's antioxidant activity is its chemical composition,
560 specifically the presence of molecules with antioxidant properties, such as phenols,
561 tocopherols, and carotenoids, as well as potential synergistic effects among these components.
562 The reactivity of antioxidants towards free radicals is intricately shaped by their chemical
563 architecture, exerting a profound influence on their antioxidant efficacy (Mansouri et *al.*

2023). As a result, reliance on a single test method to assess antioxidant activity is not recommended. On the contrary, a global approach requires the implementation of several antioxidant tests to thoroughly evaluate the intrinsic antioxidant potential of methanolic extracts of hemp seed oils extracted using different solvent mixtures. Initially, we set out to identify the most effective combination of solvents to obtain optimum antioxidant activity. To do this, we used and compared two different methods (DPPH and FRAP). The antioxidant potential of methanolic extracts, assessed using these two methods, showed a similar pattern to that of TPC, where both binary (ethyl acetate and 2-propanol) and ternary solvent mixtures showed increased efficiency in extracting antioxidant compounds compared to single-component solvent systems (Fig. 2B-C). The results revealed variations in DPPH% radical scavenging activity in the oil, ranging from 15.89 to 92.97%. FRAP test results ranged from 10.51 to 76.83 mg TE/100 g oil (Table 4). The highest TPC was observed in the methanolic extract of the oil extracted by the binary mixture (50% 2-propanol and 50% ethyl acetate) gave significantly ($p < 0.05$) the highest antioxidant activity by the FRAP test. However, this was not the case for the DPPH test, where the highest antioxidant activity was obtained experimentally with the ternary mixture (1/6 hexane, ethyl acetate, 2-propanol; 1/6, 1/6, 2/3). This may be explained by the HPLC-DAD/ESI-MS^2 analysis, showing that the total phenolics of run 6 (162.00 mg/kg) were higher than run 9 (155.35 mg/kg), but the total phenolic acids and hydroxycinnamic acid amides in run 9 (12.42 and 61.34 mg/kg, respectively) were higher than those in run 6 (9.19 and 56.95 mg/kg, respectively). Therefore, phenolic compounds, mainly benzoic acid, *p*-coumaric acid, and sinapic acid, could have a greater DPPH radical scavenging power than the other detected compounds. Using the DPPH assay, studies have shown the strong antioxidant activity of sinapic acid, *p*-coumaric acid, and benzoic acid with IC_{50} of 34, 39, and 60 µmol/mL, respectively (Szwajgier, Pielecki, and Targoński 2005).

These results suggest that the phenolic compounds present in hemp seed oil have a moderately polar nature. Previous studies have identified a notable positive correlation between total phenolic content and antioxidant potency in the seeds of seven hemp cultivars grown in Greece i.e. extracts containing higher levels of phenols indeed demonstrate the strongest antioxidant activities (Irakli et *al.* 2019).

3.2 Oxidative stability index

Fatty acid composition is the main parameter determining oxidative stability, but other minor components of the oil (pigments, tocopherols, and phenols) also contribute to oxidative stability. The effect of these components varies according to their nature, but also according to their quantity. Generally, a high linoleic acid content makes the oxidative stability of hemp seed oil unsuitable (Bhatnagar and Gopala Krishna 2013; Taaifi et *al.* 2021). In this study, we explored the oxidative stability of hemp seed oil extracts to evaluate the impact of polar solvents on the stability of the oil.

The induction time of the 11 oils extracted by different solvents showed a distinct profile, with oils extracted using polar solvents (2-propanol and ethyl acetate) showing a significantly ($p < 0.05$) higher induction time than those extracted using non-polar solvent (hexane). According to the oxidative stability index (OSI) prediction equation (Table 3), the oxidative stability of the oils extracted by different mixtures of the three solvents followed the following decreasing order: hexane-2-propanol; ethyl acetate-2-propanol; 2-propanol; ethyl acetate; hexane; hexane-ethyl acetate. The results presented in Table 4 show that the solvent characterized by the lowest oxidation stability was pure hexane (13.97 hours), while its binary combination with 2-propanol (50%-50%) exhibited the highest oxidation stability (42.18 hours), representing an approximately threefold improvement in induction time. This observation is attributed entirely to the synergic interaction of the 2-propanol-hexane

612 combination (Fig. 2D), as evidenced by the strongest regression coefficient found in all
613 antioxidant potency assays.
614 The heightened levels of oxidation stability observed for crude oils obtained using polar
615 solvents, such as 2-propanol and ethyl acetate, may be ascribed to their high content of natural
616 antioxidants, notably tocopherols, phenols, and carotenoids (Bhatnagar and Gopala Krishna
617 2013). The chemical nature of tocopherols (benzopyranols or methylated tocols), phenolic
618 compounds (aromatic hydrocarbon ring with one or more hydroxyl groups), and carotenoids
619 (hydrocarbons made up of isoprenoid units) suggests that they all have polar functional
620 groups that make them like polar lipids with an affinity for polar solvents (Bhatnagar and
621 Gopala Krishna 2013; Ramadan and Mörsel 2004).
622 **4. Optimization of solvent extraction of hemp seed oil**
623 Optimization of the responses was based on maximizing yield while ensuring good oil
624 quality. To achieve this objective, superimposed contour plots (Fig. 3) were made for the five
625 responses (oil yield, total tocopherols, total phytosterols, total carotenoids, TPC, and OSI),
626 with the proportions of the three extraction solvents (*n*-hexane, ethyl acetate, and 2-propanol).
627 The result showed that the ideal solvent combination for extracting oil with a high yield and
628 good quality was 40% hexane, 40% 2-propanol, and 20% ethyl acetate. This combination of
629 the three solvents predicted 33.36% oil yield, 231.75 mg GAE/kg TPC, 75.49% DPPH
630 reduction, 52.52 mg TE/ 100g FRAP, 39.31 h OSI, 460.01 mg/kg total tocopherols, 6390.51
631 mg/kg total phytosterols, and 20.76 mg/kg total carotenoids (Table 7). The observed values
632 for all the responses were close to the predicted values and within the confidence interval. The
633 model is therefore satisfactorily accurate and can be validated. Using the RSM method would
634 therefore allow us to define the ideal solvent mixture for extracting an optimum antioxidant-
635 rich oil from hemp seeds with a minimum number of experiments. According to the validation
636 results, the ternary mixture (40% hexane, 40% 2-propanol, and 20% ethyl acetate) gave a

maximum yield of 33.24%, which is almost similar to the yield obtained by Taaifi et *al.* (2021) with pure hexane from the same *Cannabis sativa* Beldia variety (34.44%). An optimization study of hemp seed oil extraction conducted by Stamenković et *al.* (2018) found a 30.8 % maximum yield of hexane extracted oil using a 1:10 solid-to-liquid ratio with 70°C for 15 min. In addition, the optimized ternary mixture increased the oil yield by around 1.5%, compared with hexane (32.72%), and enabled the extraction of a phenol-rich oil. The TPC was 226.43 mg GAE/kg, almost 10 times higher than that obtained with 100% hexane (19.43 mg GAE/kg). In the literature, just the TPC of cold-pressed hemp oil has been evaluated and ranged from 44 to 268 mg GAE/100g oil (Farinon et *al.* 2020).

Concerning antioxidant activities, the results of the blend were 74.64% reduction and 49.16 mg TE/kg oil for DPPH and FRAP tests, respectively (Table 7). Concerning tocopherols, the mixture (40% hexane, 40% 2-propanol, and 20% ethyl acetate) increased the tocopherol content (458.61 mg/kg) of the oil compared to hexane (410.46 mg/kg), with γ-tocopherol being the predominant isomer, accounting for 91% of the total amount of tocopherols. The other two isomers α- and δ-tocopherols were present at much lower levels (31.18 and 8.97 mg/kg, respectively). The optimal mixture yielded total tocopherol content comparable to that obtained by several authors who have worked on solvent-extracted hemp seed oil (Taaifi et al 2021; Stambouli et *al.* 2006; Farinon et *al.* 2020). Also, the mixture increased the total phytosterol concentration (6299.44 mg/kg) of the oil compared to hexane (4541.4 mg/kg). The phytosterol content was higher than that observed in the literature for oils extracted by solvent (Farinon et *al.* 2020 and Stambouli et *al.* 2006). Similarly, the carotenoid and chlorophyll contents of the oil obtained using this mixture (19.92 and 66.59 mg/kg, respectively) exceeded those obtained through hexane extraction (1.78 and 8.02 mg/kg, respectively). These values almost reached the maximum observed in run 9 of the experimental design (21.22 and 67.53 mg/kg, respectively). Pigment determination has only

been carried out in a few articles on hemp seed oil extracted by supercritical fluid or cold-pressing. According to these studies, Aladić et *al.* (2015) and Oomah et *al.* (2002) reported similar concentrations of carotenoids. In contrast, lower values were observed by Blasi et *al.* (2022).

The richness in bioactive compounds (phenolic compounds, tocopherols, phytosterols, and carotenoids) in the extracted hemp seed oil, using the optimal mixture, resulted in better oxidation stability (37.77h), almost equal to the maximum observed with the mixture of 50% hexane and 50% 2- propanol.

5. Quality and fatty acid composition of hemp seed oil extracted with the optimal mixture

The evaluation of oil quality encompasses various indices, notably free acidity, peroxide value, and specific extinction coefficients for conjugated dienes (λ232 nm) and trienes (λ270 nm), providing insights into its oxidation status. The outcomes for these parameters are outlined in Table 7. The oil extracted using the optimal mixture (hexane, 2-propanol, and ethyl acetate in a ratio of 4:4:2 v/v/v) displayed metrics, with a free acidity of 2.2 mg KOH/g, a peroxide value of 12.56 meq O_2/kg, and conjugated dienes and trienes measuring 2.06 and 0.56, respectively. These values fall below the recommended standards for crude vegetable oils (AO/WHO 2009). Additionally, the results closely align with literature reports, where a cold extraction method was typically employed for obtaining oil from hemp seeds (Occhiuto et *al.* 2022; Spano et *al.* 2020; Tura et *al.* 2023). Despite the use of a hot Soxhlet extraction method, the oil extracted with the optimal mixture demonstrated good quality. The abundance of bioactive compounds in this oil, particularly phenolic compounds, may contribute to limiting the generation of primary oxidation products.

The fatty acid composition of hemp seed oil, extracted using the optimal blend, was analyzed via GC-FID (Table 7). The resulting oil exhibited a high content of polyunsaturated fatty

acids (exceeding 65%), monounsaturated fatty acids (over 21%), and a relatively low percentage of saturated fatty acids (12.81%). Notably, linoleic acid emerged as the predominant fatty acid (49.4%), followed by oleic (19.96%), α-linolenic (15.31%), palmitic (8.24%), stearic (3.52%), and γ-linolenic (0.59%) acids. This fatty acid profile closely aligns with previous studies on hemp seed oils cultivated in Morocco (Stambouli et *al.* 2006; Taaifi et *al.* 2021) and other research conducted in various regions (Abdollahi et *al.* 2020; Farinon et *al.* 2020). Hemp seed oil emerges as a notably rich source of essential fatty acids, including linoleic and α-linolenic acid, which are vital as they cannot be synthesized by mammals and must be obtained through the diet. The ideal *n*-6/*n*-3 ratio was calculated as 3.43 (Table 7), suggesting its potential utility in reducing the *n*-6/*n*-3 ratio in dietary contexts.

Conclusion

This study utilized a simplex lattice mixture design to model and optimize the oil solvent extraction process from hemp seeds. For this purpose, the impact of three solvents (*n*-hexane, ethyl acetate, and 2-propanol) on various parameters was investigated, including oil yield, tocopherols, total phytosterols, total chlorophylls, total carotenoids, phenolic compounds, DPPH and FRAP antioxidant activities, and oxidation stability index. The quadratic model was determined to be the most appropriate for describing the extraction process, with the solvents demonstrating statistical significance. Analysis of the extracted oils revealed that the choice of solvent significantly influenced both yield and oil quality. Superimposed contour plots were employed to identify solvent proportions that maximize yield while maintaining good oil quality. The identified ideal mixture, for which all responses were satisfactory, consisted of 40% hexane, 40% 2-propanol, and 20% ethyl acetate. This ternary mixture allowed for the substitution of 60% hexane with two other extraction solvents (2-propanol and ethyl acetate) that are less toxic and environmentally friendly. This substitution did not compromise the yield and produced an oil rich in bioactive compounds, with high stability

against oxidation. The 40% remaining proportion of hexane in the optimal mixture offers an opportunity for future research to explore other solvents and their combinations that could completely replace this harmful solvent, while still extracting oil with both good yield and quality.

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Table 1. Relevant properties of the three solvents selected for the optimization of hemp seed oil extraction. (Haynes, Lide, and Bruno 2016; Snyder 1974)

Properties	**Hexane**	**Ethyl acetate**	**2-Propanol**
Type of solvent	Non-polar	Polar	Polar
Category of solvent	Hydrocarbon	Ester	Alcohol
Dielectric constant	1.89	6.02	18.3
Dipole moment	0.08	1.88	1.69
Polarity index	0.1	4.4	3.92
Boiling point (°C)	68.7	77.1	82.5
Melting Point (°C)	-96.0	-83.6	−88.5
Density (g/cm^3)	0.6606	0.902	0.785
Molar mass (g/mol)	86.18	88.11	60.095
Viscosity (cP)	0.29	0.43	2.1

Table 2. Experimental results of the design matrix for hemp seed oil extraction and corresponding responses (oil yield, total tocopherols, phytosterols, carotenoids, and chlorophylls).

Run	Independent variables			Responses				
	Hexane	Ethyl acetate	2-Propanol	Oil yield	Total tocopherols	Total phytosterols	Total carotenoids	Total chlorophylls
1	1	0	0	32.72±0.37	410.47±11.00	4541.35±13.34	1.78±0.03	8.03±0.50
2	0	1	0	25.51±0.24	508.55±18.81	6619.51±84.92	16.95±0.76	56.00±3.88
3	0	0	1	31.19±0.12	422.80±0.71	6508.87±52.39	16.86±0.35	46.86±2.73
4	1/2	1/2	0	32.49±0.17	458.21±11.34	5532.84±12.67	16.93±1.38	36.48±0.19
5	1/2	0	1/2	32.82±0.32	446.16±11.52	6484.73±18.21	21.90±0.12	56.38±0.60
6	0	1/2	1/2	30.73±0.53	478.91±8.22	6744.67±87.70	20.38±0.02	58.83±0.05
7	2/3	1/6	1/6	33.87±0.26	442.58±11.01	6072.62±12.19	16.26±0.65	50.37±0.27
8	1/6	2/3	1/6	31.37±0.23	475.82±3.50	6328.64±40.87	19.87±0.95	67.25±0.18
9	1/6	1/6	2/3	31.68±0.13	455.42±7.43	6631.04±3.97	21.22±1.51	67.54±4.63
10	1/3	1/3	1/3	33.02±0.46	475.07±0.16	6187.45±12.14	18.80±0.13	63.92±0.42
11	1/3	1/3	1/3	33.84±0.52	471.19±6.96	6231.51±17.73	18.27±0.21	64.75±0.43

Oil yield is expressed in g per 100 g of seeds (%).

Total tocopherols, phytosterols, carotenoids and chlorophylls are expressed in mg per kg of oil (mg/kg oil).

Levels vary from 0 to 1 (0–100%), where 0 refers to the absence of the solvent in the mixture, while 1 corresponds to its use as a single solvent. In each row, the sum of the levels of the three solvents is 1.

Table 3. Analysis of variance and coefficients of the quadratic model regression equation for each response studied.

Responses (Y_i)	Quadratic coefficients						*p*-values		R^2	R^2 adjusted
	β_1	β_2	β_3	β_4	β_5	β_6	Model	Lack of fit		
Oil yield	32.70*	25.65*	30.87*	15.36*	4.39	10.78*	0.00132	0.587	0.963	0.927
Total Tocopherols	410.02*	505.05*	422.85*	0.774	131.20*	59.95	0.00073	0.274	0.971	0.942
Total Phytosterols	4664.63*	6600.06*	6495.42*	284.76	3756.06*	354.04	0.00342	0.099	0.946	0.892
Total Carotenoids	2.43	17.34*	17.17*	23.81*	43.75*	6.80	0.00309	0.141	0.948	0.896
Total Chlorophylls	8.07	56.06*	46.03*	44.24	140.29*	54.20	0.00530	0.062	0.935	0.871
TPC	26.65	125.03*	303.57*	79.34	177.08	658.28*	0.00094	0.422	0.968	0.936
DPPH	16.25*	49.84*	83.23*	7.42	101.87*	125.24*	0.00068	0.146	0.972	0.944
FRAP	11.59*	36.31*	60.71*	16.83	52.41*	116.30*	0.00024	0.385	0.981	0.963
OSI	14.07*	14.48*	38.68*	2.73	65.71*	57.26*	0.00045	0.714	0.976	0.952

$Y_i = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_1 x_3 + \beta_6 x_2 x_3$, with x_1: Hexane, x_2: Ethyl acetate, and x_3: 2-Propanol

*Significant at $p < 0.05$

Table 4. Experimental results of the design matrix for oil extraction from hemp seeds and corresponding responses (TPC, DPPH, FRAP, and OSI).

Run	Independent variables			Responses			
	Hexane	Ethyl acetate	2-Propanol	TPC	DPPH	FRAP	OSI
1	1	0	0	19.43±1.13	15.89±1.50	10.51±1.84	13.97±0.62
2	0	1	0	128.77±2.27	50.55±3.18	37.36±1.23	14.51±1.04
3	0	0	1	293.88±6.29	84.58±0.36	59.70±2.30	39.96±1.08
4	1/2	1/2	0	55.66±0.89	24.34±1.02	18.92±1.18	13.08±0.97
5	1/2	0	1/2	195.61±2.44	69.01±1.46	46.37±1.83	42.18±1.13
6	0	1/2	1/2	376.06±3.40	92.71±0.56	76.83±1.21	40.40±1.03
7	2/3	1/6	1/6	150.04±1.83	53.56±1.24	36.14±1.10	28.33±0.87
8	1/6	2/3	1/6	206.47±1.65	69.11±0.53	47.45±2.28	27.45±0.71
9	1/6	1/6	2/3	357.38±3.81	92.97±0.24	71.60±1.90	41.03±1.10
10	1/3	1/3	1/3	228.98±2.44	77.67±1.18	53.12±4.30	40.34±0.53
11	1/3	1/3	1/3	203.01±0.89	75.78±1.32	49.92±0.64	36.06±0.50

Total phenolic content (TPC) is expressed in mg gallic acid equivalent per kg of oil (mg GAE/kg oil).

DPPH-radical scavenging is expressed in percentage of reduction (% reduction)

Ferric reducing antioxidant power (FRAP) is expressed in mg Trolox equivalent per 100g of oil (mg TE/100g).

Oxidative stability index (OSI) is expressed as the induction time of lipid oxidation (hours).

Levels vary from 0 to 1 (0–100%), where 0 refers to the absence of the solvent in the mixture, while 1 corresponds to its use as a single solvent. In each row, the sum of the levels of the three solvents is 1.

Table 5: Phenolic compounds identified by HPLC-DAD/ESI-MS^2 with the negative mode $[M-H]^-$ in solvent-extracted hemp seed oil

N° peak	Phenolic compounds	Molecular formula	RT (min)	λmax (nm)	$[M-H]^-$ calc. (m/z)	$[M-H]^-$ found (m/z)	error (ppm)	Mass fragments (%intensity)
1	Unknown 1	—	10.31	264; 292	—	265.002	—	265(100); 263(64); 247(13)
2	Unknown 2	—	13.94	292	—	325.009	—	256(100); 245(85); 241(35); 137(21); 138(14); 237(9)
3	*p*-Hydroxybenzoic acid	$C_7H_6O_3$	14.44	288; 378	137.0322	137.0317	-3.64	93(100); 137(89); 94(37)
4	Benzoic acid	$C_7H_6O_2$	18.97	280; 220	121.0373	121.0370	-2.47	Not fragment
5	*p*-Coumaric acid	$C_9H_8O_3$	20.79	230; 300; 310	163.0478	163.0480	1.22	119 (100); 93 (8)
6	*N-trans*-caffeoyltyramine isomer	$C_{17}H_{17}NO_4$	22.54	200; 284; 315	298.1158	298.1160	0.67	135(100);178(31); 161(20); 298(19); 284(14)
7	*N-trans*-caffeoyltyramine	$C_{17}H_{17}NO_4$	25.40	220; 250; 294; 318	298.1158	298.1160	0.67	135(100); 298(58); 161(26); 178(22); 182(17); 136(11)
8	Unknown 3	—	25.91	250; 318; 344	—	207.000	—	192(100); 207(62); 193(6); 205(6); 164(2)
9	*N*-caffeoyltyramine dimer hydroxy derivative	$C_{34}H_{34}N_2O_9$	26.24	254; 340	613.2269	613.2256	-2.11	475(100); 613(39); 595(30); 312(31); 573(17); 281(24)
10	Cannabisin A	$C_{34}H_{30}O_8N_2$	27.61	256	593.2002	593.2016	2.36	456(100); 593(42); 430(26); 291(6)
11	Cannabisin B	$C_{34}H_{32}O_8N_2$	27.87	254; 284; 314; 334	595.2159	595.2155	-0.67	485(100); 269(74); 432(68); 430(59); 322(57); 595(44); 456(11); 241(4); 202(4)
12	*N-trans*-coumaroyltyramine	$C_{17}H_{17}O_3N$	28.32	292; 308	282.1208	282.1215	2.48	145(100); 282(94); 119(73); 134(46); 162(36); 136(27); 238(8)
13	Cannabisin B Isomer 1	$C_{34}H_{32}O_8N_2$	29.00	264; 284; 314	595.2159	595.2155	-0.67	595(100); 485(41); 544(24); 432(20); 322(18); 269(14)
14	Cannabisin B Isomer 2	$C_{34}H_{32}O_8N_2$	29.47	268; 310	595.2159	595.2161	0.32	485(100); 269(64); 432(56); 456(54); 322(46); 409(34)
15	*N*-feruloyltyramine	$C_{18}H_{19}O_4N$	29.71	256; 288; 318	312.1314	312.1320	1.92	312(100); 178(96); 135(58); 297(44); 176(11); 148(7); 390(58)
16	Unknown 4	—	29.96	322	—	607.000	—	444(100); 382(98); 607(91); 470(24); 592(21); 429(19)

17	Demethylgrossamide	$C_{35}H_{34}N_2O_8$	30.39	264; 284; 314; 322	609.2315	609.2322	1.14	293(100); 283(7); 609(5); 446(4)
18	Cannabisin C	$C_{35}H_{34}O_8N_2$	31.23	260; 280	609.2315	609. 2317	0.32	499(100); 446(53); 444(51); 470(31); 609(29); 336(19); 283(16); 214(11)
19	Cannabisin C isomer	$C_{35}H_{34}O_8N_2$	31.37	284; 322	609.2315	609.2325	1.64	446(100); 609(83); 283(34); 485(16); 322(15); 571(9); 417(7)
20	Unknown 5	—	31.94	322	—	326.079	—	326(100); 325(45); 192(42); 135(19); 311(14); 147(9); 199(8)
21	Unknown 6	—	32.18	284	—	609.168	—	609(100); 560(66); 447(54); 444(42); 625(42); 211(26); 510(28)
22	Cannabisin D	$C_{36}H_{36}N_2O_8$	32.33	260; 284; 308	623.2472	623.2480	1.28	460(100); 283(34); 623(20); 268(10); 444(9); 458(8); 336(5); 350(5)
23	3.3-didemethylgrossamide	$C_{34}H_{32}N_2O_8$	32.81	284; 324	595.2159	595.2160	0.16	432(100)-269(99)-458(36)-595(22)-295(10)-338(7)-250(2)
24	Tri-*p*-coumaroylspermidine	$C_{34}H_{37}N_3O_6$	33.12	260	582.2688	582.2695	1.20	462(100); 582(87); 342(76); 316(10); 436(11); 299(4); 217(2); 533(2)
25	Cannabisin E	$C_{36}H_{38}N_2O_9$	33.41	292; 310	641.2577	641.2571	-0.93	623(100); 641(95); 489(55); 431(40); 281(21); 591(20); 312(13);604(11)
26	Unknown 7	—	33.75	292	—	612.262	—	492(100); 493(32); 476(29); 612(12); 466(6); 342(5); 283(4)
27	Cannabisin M	$C_{34}H_{32}N_2O_8$	35.05	288; 322	595.2159	595.2155	-0.67	298(100); 595(27); 431(24); 430(14); 101(11); 307(10); 467(7); 485(6); 176(1)
28	3.3′-demethyl-heliotropamide	$C_{34}H_{32}N_2O_8$	35.23	285; 310	595.2159	595.2160	0.16	107(100); 298(49); 595(23)
29	Unnamed lignan-amide	—	35.54	230-312	—	589.2661	—	426(100); 589(28); 443(7); 163(6); 261(6); 279(5); 187(3)

30	Unknown 8	—	35.71	284	—	591.539	—	230(100); 454(95); 591(53); 334(36); 455(30); 573(8)
31	Cannabisin Q	$C_{34}H_{32}N_2O_8$	36.04	284; 308	595.2159	595.2150	-1.51	298(100); 595(45); 296(4); 178(0;5)
32	Cannabisin F	$C_{36}H_{36}N_2O_8$	36.71	288; 312	623.2472	623.2470	-0.32	460(100); 623(61); 297(35); 486(29); 352(5)
33	Isocannabisin N	$C_{35}H_{34}N_2O_8$	37.05	284; 324	609.2315	609.2306	-1.47	609(100); 312(75); 296(72); 417(16); 723(17); 176(8);561(6)
34	Grossamide	$C_{36}H_{36}N_2O_8$	37.65	250; 288; 320	623.2472	623.2481	1.44	623(100); 460(77); 591(47); 297(32); 471(30); 551(23); 432(17); 486(15); 428(11); 282(11)
35	Cannabisin G	$C_{36}H_{36}N_2O_8$	37.92	290; 312	623.2477	623.2475	-0.32	589(100); 625(22); 460(14); 297(6); 486(4)
36	Cannabisin O	$C_{54}H_{53}N_3O_{12}$	38.10	288; 312	934.3629	934.3635	0.64	Not fragment
37	Unknown 9	—	48.30	278		652.354	—	293(100); 652(10); 605(7); 553(2)
38	Sinapic acid	$C_{11}H_{12}O_5$	48.81	276	223.0690	223.0682	-3.58	225(100); 223(34); 195(36); 125(35); 179(24); 221(20); 163(18); 206(16); 164(12); 155(17)

RT, retention time; λmax, maximum absorbance peak.

Table 6: Relative content of compounds identified in oils extracted by different solvents from hemp seeds.

Phenolic compounds	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run10
Hydroxybenzoic acid (HBA)										
p-Hydroxibenzoic acid	Nd	0.16±0.06	0.05±0.02	0.05±0.00	0.09±0.01	0.03±0.00	0.04±0.00	0.05±0.00	0.11±0.01	0.1±0.00
Benzoic acid	Nd	0.83±0.01	4.42±0.08	5.94±0.09	8.71±0.35	7.32±0.07	4.47±0	6.26±0.03	9.65±0.26	6.9±0.01
Total HBA	**0.00**	**0.99**	**4.47**	**5.99**	**8.80**	**7.35**	**4.51**	**6.31**	**9.76**	**7.00**
Hydroxycinnamic acid (HCA)										
p-Coumaric acid	Nd	0.54±0.00	0.14±0.00	0.41±0.01	0.51±0.02	0.28±0.00	0.31±0.00	0.25±0.00	0.66±0.01	0.52±0.00
Sinapic acid	1.71±0.13	1.33±0.05	1.65±0.80	1.48±0.61	1.65±0.78	1.56±0.66	1.07±0.00	2.10±0.06	2.00±0.05	1.49±0.76
Total HCA	**1.71**	**1.87**	**1.79**	**1.89**	**2.16**	**1.84**	**1.38**	**2.35**	**2.66**	**2.01**
Total phenolic acids	**1.71**	**2.86**	**6.26**	**7.88**	**10.96**	**9.19**	**5.89**	**8.66**	**12.42**	**9.01**
Hydroxycinnamic acid amides (HCAAs)*										
N-trans-caffeoyltyramine isomer	Nd	7.47±0.36	0.78±0.14	3.66±0.05	3.75±0.16	1.76±0.00	1.53±0.00	1.56±0.01	3.9±0.28	3.24±0.00
N-trans-caffeoyltyramine	Nd	11.27±1.24	16.99±1.15	0.52±0.01	15.9±2.58	42.59±0.14	4.60±0.02	26.75±0.59	41.15±3.79	35.04±0.85
N-trans-coumaroyltyramine	Nd	1.60±0.05	0.88±0.36	2.24±0.03	2.93±0.20	1.91±0.04	1.05±0.00	0.96±0.01	3.75±0.18	2.07±1.11
N-feruloyltyramine	Nd	4.47±0.16	4.59±0.59	1.71±0.04	5.68±0.55	9.68±0.02	2.62±0.03	6.61±0.08	10.50±0.80	8.37±0.10
Tri-*p*-coumaroylspermidine	Nd	0.74±0.21	0.66±0.06	Nd	0.61±0.15	1.01±0.02	0.67±0.03	0.40±0.02	2.04±0.20	1.47±0.02
Total HCAAs	**1.71**	**25.55**	**23.90**	**8.13**	**28.87**	**56.95**	**10.47**	**36.28**	**61.34**	**50.19**
Lignan amides *										
N-caffeoyltyramine dimer hydroxy derivative	Nd	0.61±0.03	0.31±0.02	Nd	0.60±0.19	0.57±0.01	Nd	0.49±0.00	0.56±0.01	0.41±0.03

Cannabisin A	Nd	2.06±0.31	9.70±0.69	Nd	2.31±0.42	16.78±0.14	0.67±0.01	11.31±0.50	8.68±0.95	11.05±0.17
Cannabisin B	Nd	2.51±0.26	8.49±0.58	Nd	3.12±0.48	16.27±0.07	1.03±0.01	10.45±0.41	9.96±1.02	11.21±0.17
Cannabisin B isomer 1	Nd	3.11±0.57	3.31±0.17	0.82±0.02	4.57±0.59	8.45±0.04	1.96±0.01	6.20±0.19	9.43±0.78	6.65±0.65
Cannabisin B isomer 2	Nd	Nd	0.63±0.03	Nd	0.35±0.00	1.18±0.00	0.13±0.00	0.75±0.03	0.81±0.08	0.88±0.01
Demethylgrossamide	Nd	2.33±0.03	3.18±0.28	0.72±0.01	2.40±0.26	6.21±0.01	0.89±0.01	3.91±0.11	4.93±0.40	4.00±0.08
Cannabisin C	Nd	0.42±0.05	0.52±0.05	Nd	0.49±0.01	0.70±0.03	Nd	0.52±0.02	0.55±0.04	0.57±0.01
Cannabisin C isomer	Nd	0.56±0.06	0.33±0.04	0.39±0.00	0.63±0.03	0.65±0.02	Nd	0.32±0.04	0.65±0.03	0.36±0.00
Cannabisin D	Nd	Nd	0.44±0.04	Nd	0.52±0.08	0.88±0.01	Nd	0.57±0.02	0.84±0.07	0.70±0.01
3.3-didemethylgrossamide	Nd	2.31±0.15	3.14±0.18	Nd	3.19±0.77	6.20±0.05	0.93±0	3.43±0.14	8.10±0.85	6.54±0.15
Cannabisin E	Nd	1.20±0.24	0.77±0.06	0.49±0.00	0.78±0.04	1.06±0.02	0.44±0.03	0.71±0.00	1.31±0.08	1.09±0.04
Cannabisin M	Nd	1.33±0.05	3.56±0.29	Nd	1.66±0.29	7.08±0.03	0.39±0.01	5.03±0.20	5.45±0.59	5.60±0.12
3.3′-demethyl-heliotropamide	Nd	1.57±0.23	2.67±0.12	Nd	1.29±0.19	4.96±0.01	Nd	1.38±0.00	4.85±0.51	1.96±0.04
Cannabisin Q	Nd	1.21±0.08	1.20±0.04	0.97±0.03	1.25±0.04	1.07±0.07	Nd	0.55±0.2	1.51±0.07	0.78±0.09
Cannabisin F	Nd	1.23±0.09	0.54±0.01	0.54±0.01	Nd	1.22±0.00	0.48±0.00	0.96±0.01	1.58±0.05	0.96±0.01
Isocannabisin N	Nd	Nd	0.38±0.03	Nd	0.33±0.02	0.6±0.01	Nd	0.43±0.02	0.69±0.07	0.61±0.00
grossamide	Nd	1.66±0.20	3.10±0.26	0.38±0.01	2.83±0.46	7.18±0.05	1.33±0.01	4.62±0.21	6.91±0.71	6.13±0.11
Cannabisin G	Nd	Nd	0.73±0.11	Nd	Nd	1.07±0.00	Nd	0.86±0.06	0.55±0.04	0.75±0.17
Cannabisin O	Nd	2.45±0.14	Nd	1.31±0.02	5.09±0.48	3.00±0.03	1.05±0.00	1.29±0.04	5.63±0.55	3.24±0.03
Unnamed lignan-amide	Nd	0.85±0.00	Nd	Nd	0.32±0.02	0.53±0.01	Nd	0.85±0.02	0.87±0.07	Nd
Total lignan amides	**0**	**23.10**	**43.00**	**5.62**	**31.73**	**85.66**	**9.30**	**54.63**	**73.86**	**63.49**
Total phenylpropanoids	**1.71**	**48.65**	**66.9**	**13.75**	**60.6**	**142.61**	**19.77**	**90.91**	**135.2**	**113.68**
Other compounds										

Unknown 1	Nd	1.11±0.02	0.85±0.01	0.58±0.01	1.60±0.14	1.05±0.00	0.89±0.01	1.03±0.06	1.27±0.06	1.35±0.01
Unknown 2	Nd	0.25±0.06	0.58±0.04	Nd	0.50±0.06	0.65±0.00	0.13±0.00	0.56±0.01	0.41±0.02	0.67±0.01
Unknown 3	Nd	0.64±0.05	0.73±0.24	Nd	0.58±0.17	1.87±0.03	0.55±0.01	0.90±0.01	1.02±0.07	0.93±0.01
Unknown 4	Nd	Nd	0.40±0.07	Nd	Nd	0.73±0.01	0.13±0.00	0.44±0.02	0.47±0.04	0.45±0.01
Unknown 5	Nd	Nd	Nd	Nd	Nd	0.29±0.02	Nd	0.30±0.05	Nd	Nd
Unknown 6	Nd	0.58±0.03	0.74±0.10	Nd	Nd	1.42±0.04	Nd	0.89±0.09	0.69±0.10	0.98±0.02
Unknown 7	Nd	1.06±0.19	0.58±0.14	Nd	0.84±0.07	1.01±0.00	0.87±0.01	0.74±0.02	1.28±0.10	1.09±0.01
Unknown 8	Nd	Nd	1.44±0.17	Nd	0.38±0.03	2.33±0.02	0.36±0.02	0.38±0.05	1.92±0.19	1.08±0.08
Unknown 9	0.61±0.03	1.40±0.04	0.70±0.02	0.7±0.03	0.72±0.01	0.85±0.09	0.69±0.00	0.69±0.01	0.67±0.00	0.71±0.01
Total phenolic compounds	**2.32±0.1**	**56.55±5.26**	**79.18±4.21**	**22.91±0.91**	**76.18±9.67**	**162.00±1.3**	**29.02±0.16**	**105.50±2.13**	**155.35±1.24**	**129.95±2.27**

Data, which are the mean ±SD of three independent experiments (n = 3), were expressed as mg/kg oil.

* Hydroxycinnamic acid amides and lignan amides are expressed in mg *N-trans*-caffeoyltyramine equivalent per kg of oil (mg CTE/kg oil).

Nd: Not detected

Table 7. Fatty acid composition and physico-chemical properties of the oil obtained with the optimal mixture (hexane, 2-propanol, and ethyl acetate; 40/40/20%) and data validation**.**

Responses	Observed values (mean ±SD)	Predicted values	Confidence intervals at 95%
Oil yield (%)	33.24±0.14	33.36	[32.57-34.15]
Total Phytosterols (mg Cholesterol/kg Oil)	6299.44±112.45	6390.51	[6127.03-6654]
Total Carotenoids (mg/kg Oil)	19.92±1.39	20.76	[18.55-22.97]
Total Chlorophylls (mg/kg Oil)	66.59±4.5	63.18	[55.26-83.18]
Tocophérols (mg/kg Oil)			
γ-Tocopherol	418.46±19.41	—	—
α-Tocopherol	31.18±1.19	—	—
δ-Tocopherol	8.97±0.56	—	—
Total tocopherols	458.61±20.44	460.01	[451.65-468.37]
TPC (mg GAE/kg Oil)	226.43±3.69	231.75	[195.99-267.50]
DPPH (% Reduction)	74.64±0.41	75.49	[67.79-83.18]
FRAP (mg TE/100g Oil)	49.16±1.52	52.52	[47.67-57.37]
OSI (hours)	37.77±1.66	39.31	[36.05-42.56]
Oil Quality Indices			
Peroxide value (meq O_2/kg)	12.56±1.06	—	—
Free acidity (mg KOH/g)	2.2±0,09	—	—
Conjugated dienes (λ232 nm)	2.06±0.12	—	—
Conjugated trienes (λ270 nm)	0.58±0.02	—	—
Fatty acids (%)			
Palmitic acid	8.24±1.64	—	—
Stearic acid	3.52±0.38	—	—
Oleic acid	19.96±1.21	—	—
Linoleic acid	49.4±1.52	—	—
γ-Linolenic acid	0.59±0.07	—	—
α-Linolenic acid	15.31±1.47	—	—
SFA	12.81±1.87	—	—
MUFA	21.68±1.23	—	—
PUFA	65.5±3.1	—	—
n-6	50.2±1.63	—	—
n-3	15.31±1.47	—	—
n-6/n-3	3.43±0.02	—	—

SFA: saturated fatty acid; UFA: unsaturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; n-6: PUFA n-6; n-3 : PUFA n-3.

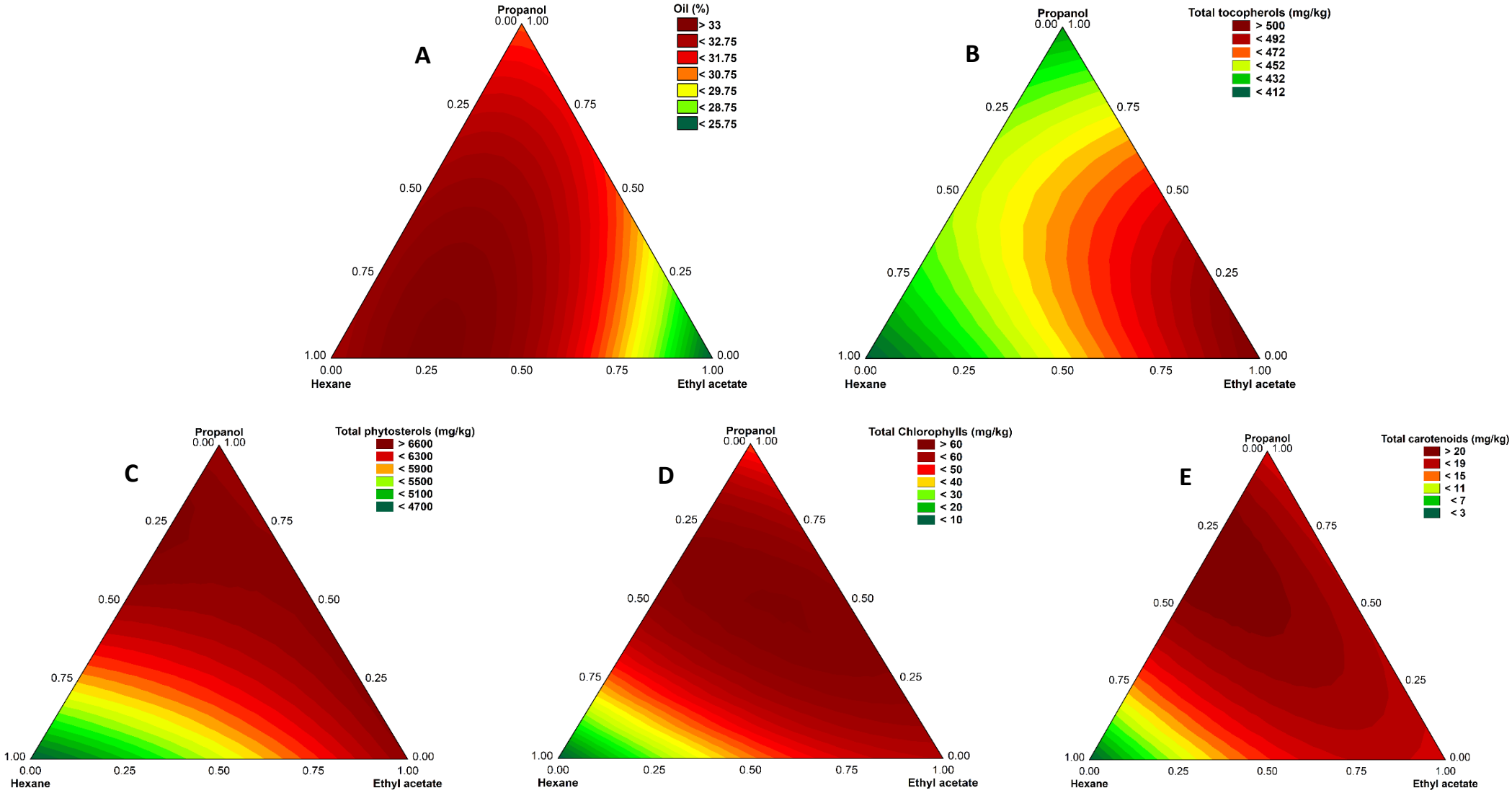

Figure 1. Contour plots of oil yield (**A**), tocopherols (**B**), phytosterols (**C**), chlorophylls (**D**) and carotenoids (**E**). The vertices correspond to pure solvents; the points on the edges correspond to binary mixtures. while the points inside the triangle correspond to ternary mixtures.

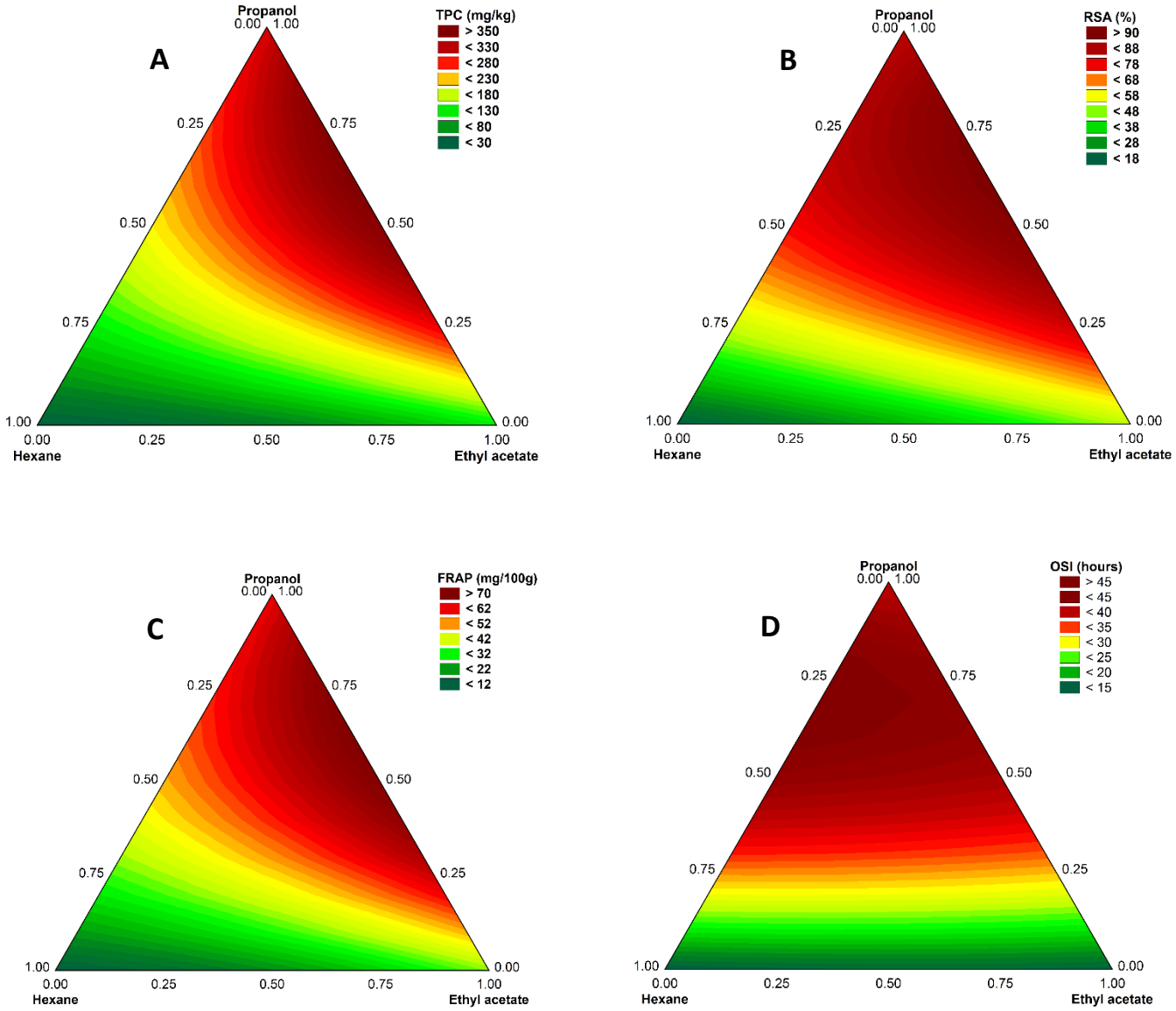

Figure 2: Contour plots of TPC (**A**), DPPH (**B**) FRAP (**C**) and OSI (**D**). The vertices correspond to pure solvents; the points on the edges correspond to binary mixtures. while the points inside the triangle correspond to ternary mixtures.

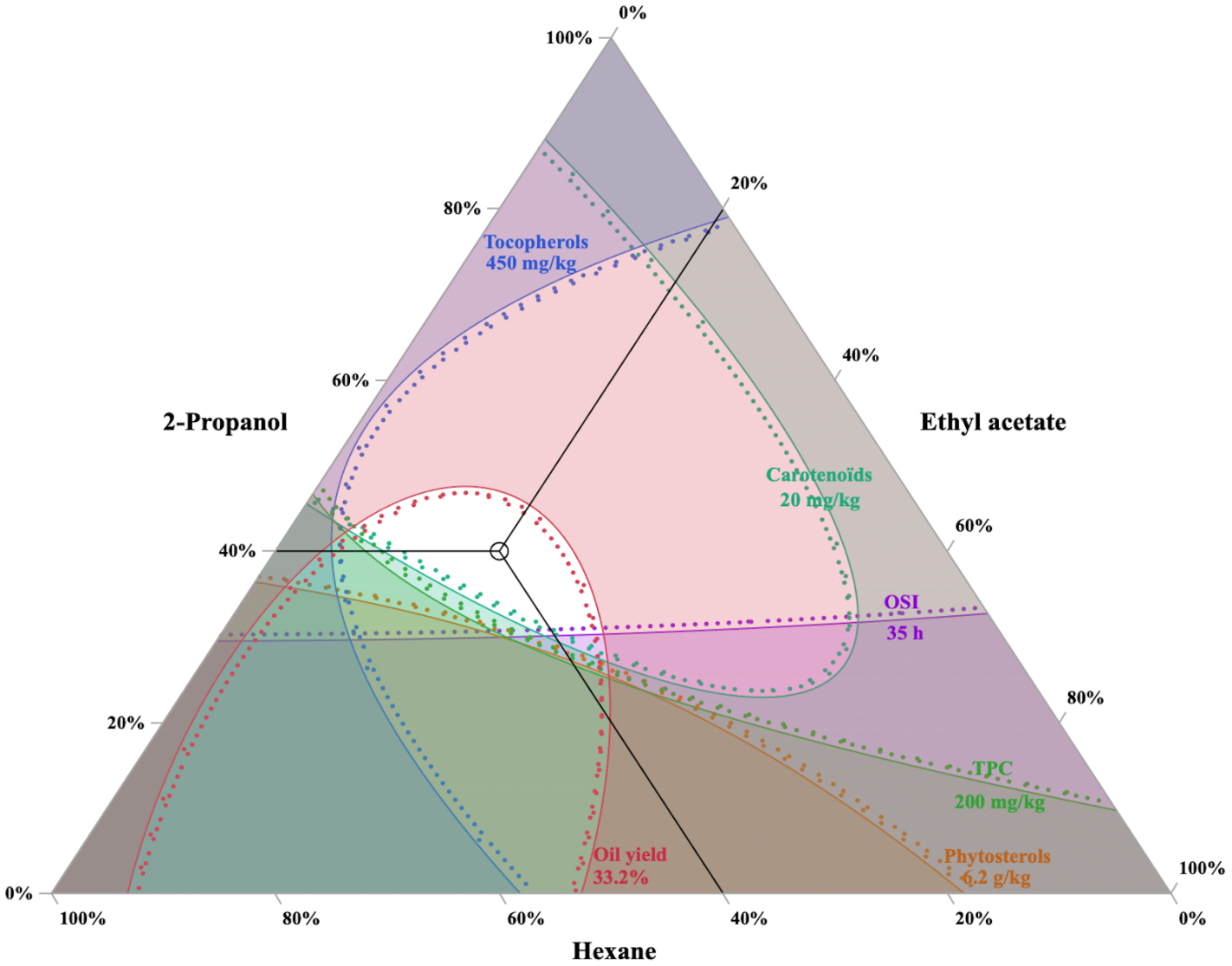

Figure 3: Superimposed contour plots of the five responses (oil yield, TPC, oxidative stability index (OSI), tocopherols, phytosterols and carotenoids). The dotted area indicates the maximum response (≥ 33.2% for oil yield, 35 h OSI, 450 mg/kg tocopherols, 6.2 g/kg phytosterols and 20 mg/kg carotenoids). The proportions of 40% hexane. 40% 2-propanol and 20% ethyl acetate were chosen to obtain the best yield with good oil quality.