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# Development of a green ultrasound-assisted method for the extraction of bioactive oils from sloe seeds: a sustainable alternative to Soxhlet extraction

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ABSTRACT

*Prunus spinosa* L. seed by-products produced during the generation of liqueur are gaining interest as an unexploited source of bioactive compounds. The objective of this study was to transform waste material into a high-value-added product by employing green procedures and solvents. Consequently, an experimental design was implemented to develop a sustainable ultrasound-assisted extraction (UAE) method, employing a mixture of ethanol and tert-butanol, for the recovery of bioactive oils from sloe seed residues. The oils were characterized in terms of fatty acid, tocopherol and polyphenol content using GC–MS, HPLC-DAD, and cLC-DAD-MS, respectively.

Furthermore, the *in vitro* antimicrobial and antioxidant capacities were evaluated using spectrophotometric methods. The optimized UAE oil extraction method was found to be sustainable, as indicated by the AGREEprep results. Furthermore, this extraction method enabled the isolation of antioxidant oils ($IC_{50}$ = 6.9 mg·$L^{-1}$) with a higher amount of bioactive compounds (oleic acid, β + γ-tocopherols, gallic acid, and 2,3-dihydroxybenzoic acid) in comparison to conventional Soxhlet extraction. Specifically, the oil from sloe seeds after maceration (NBSAM) was rich in oleic acid (73.6 %), β + γ-tocopherol (100 mg·$kg^{-1}$), gallic acid and 2,3-dihydroxybenzoic acid (271 and 25 μg·$g^{-1}$, respectively). The chemometric study, using principal component analysis (PCA), allowed the correlation between the chemical composition of the oil and its oxidative stability (lasting 21 days) and effectiveness antimicrobial (with a minimum inhibitory concentration, MIC, of 20 mg·$mL^{-1}$). In conclusion, an innovative and sustainable method for extracting seed oils from sloe residues has been developed. Moreover, NBSAM oil exhibited significant potential for feasible nutraceutical and cosmetic applications.

## 1. Introduction

*Prunus spinosa* L., commonly known as wild plum (family *Rosaceae*), is a prickly shrub that grows in uncultivated regions of West Asia, Europe and the Mediterranean [1]. The berries of this plant, which are also known as sloes, constitute an astounding little-explored source of bioactive components, including tocopherols, anthocyanins, polyphenols and fatty acids [1,2]. One contemporary form of human consumption of these fruits is the production of various beverages, including teas, jams and liqueur-based drinks, such as the traditional *Pacharán*, a homemade sloe-aromatized spirit drink originating from Navarra in Spain. However, a significant quantity of bio-residues is generated during its processing, specifically sloe seeds, which have a direct deleterious impact on the environment [3]. In this context, the valorization of this waste to obtain bioactive compounds has become a priority to minimize the environmental impact of its generation and management. It has been hypothesized that sloe seed residues have the potential to be used in the production of bioactive oils [4].

The bioactive compounds present in sloe seed oils, including fatty acids, tocopherols and polyphenols, have been demonstrated to possess significant health benefits. Concerning the fatty acid content, oleic and linoleic acids were of the greatest importance. Specifically, Atik et al. [5] and Matthäus et al. [6] reported that the content of oleic acid in sloe seed oils was found to range from 43.9 to 72.7 %, and the linoleic acid content was between 17.72 and 37.0 %. Concerning the tocopherol content, γ-tocopherol (vitamin E) was the predominant form in sloe seed oils, with concentrations ranging from 204 to 278.6 mg·$kg^{-1}$ [6,7]. Finally, phenolic compounds represent a further bioactive compound present in

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sloe seed oils, which exhibits notable bioactive potential. In particular, Atik et al. [5] found that the phenolic profile of sloe seed oils was characterized by the presence of significant quantities of vanillin (4.70 ± 0.05) mg·kg$^{-1}$, benzoic acid (4.40 ± 0.07) g·kg$^{-1}$ as well as rutin (1.10 ± 0.06) mg·kg$^{-1}$.

Despite this promising composition, the nutritional quality of sloe seed oils may be affected by the potential presence of amygdalin, a cyanogenic glucoside naturally occurring in *Prunus* seeds [8]. This compound is of particular concern, as it can degrade into cyanide during digestion, posing a risk to human health. In fact, the European Food Safety Authority (EFSA) has classified amygdalin as a toxic substance due to its metabolic conversion to hydrogen cyanide in the gastrointestinal tract [9]. Consequently, a comprehensive characterization of sloe seed oils should focus not only on their bioactive compounds and health-promoting potential, but also on evaluating the presence and concentration of amygdalin. Such assessment is essential for ensuring both the nutritional value and the safety of sloe seed oils for their broader cosmetic and nutraceutical applications.

The bioactive components present in significant quantities in oils derived from *Prunus spinosa* L. are indicative of their notable bioactive properties. Particularly, Athanasiadis et al. [4] demonstrated that sloe seed oils exhibited excellent antiradical scavenging activity (Antioxidant activity coefficient = 14.56 μmol TE·kg$^{-1}$). In addition, Sabatini et al. [1] and Veličković et al. [10] demonstrated the antimicrobial capacity of sloe seeds against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella abony* and *Escherichia coli*. These findings indicated the potential application of sloe seed oils in the nutraceutical, cosmetic and food industry.

Conventional Soxhlet extraction is frequently employed to recover sloe seed oils, using *n*-hexane as a solvent. This technique has a well-established foundation in the field of oil extraction, but it poses significant challenges due to the use of *n*-hexane, a flammable and toxic solvent. As a result, there is an increasing need to explore sustainable and environmentally friendly alternatives that align with the principles of Green Chemistry [11,12]. Among the greener and non-conventional alternatives, ultrasound-assisted extraction (UAE) has emerged as a promising green technique, offering benefits such as reduced extraction time, reduced solvent consumption, as well as simplicity and cost-effectiveness [13,14]. During the development of UAE methodologies, several factors have been identified as key determinants of efficiency, including the organic solvent, the extraction time, the temperature, the solid-to-solvent ratio, the ultrasound power and the amplitude [14]. An extensive range of organic solvents might be employed. In this regard, ethanol and tert-butanol have been identified as promising solvents due to their effectiveness in extracting bioactive oils and their status as a more sustainable alternative to *n*-hexane [15,16]. Ethanol is a highly polar primary alcohol, which enables the efficient extraction of hydrophilic compounds in oils, such as polyphenols and tocopherols [16]. In contrast, tert-butanol, a tertiary branched alcohol with lower polarity than ethanol, preferentially extracts lipophilic compounds, including vitamins, fatty acids, and triacylglycerides [15]. Consequently, a combination of these solvents represents a promising strategy for obtaining oils enriched in bioactive compounds.

From a sustainability perspective, ethanol is a safe solvent approved by the Food and Drug Administration (FDA) [16]. Although tert-butanol is flammable, it is classified as a green solvent within the food industry due to its renewable origin and favorable performance in environmental assessment frameworks such as the Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) and Multicriteria Decision Analysis (MCDA), which evaluate parameters including toxicity, biodegradability, volatility, and overall environmental impact [17,18]. Compared to other hydrophobic solvents, such as certain ionic liquids or terpenes (*e.g.*, D-limonene, α-pinene, *p*-cymene), ethanol and tert-butanol offer practical advantages, as their lower viscosity and boiling points facilitate solvent recovery and reduce energy requirements. Additionally, both solvents demonstrate superior efficiency in extracting lipophilic compounds from oils [15,17–19].

To optimize the extraction process, the application of Design of Experiments (DoE) and Response Surface Methodology (RSM) has proven to be extremely useful [20,21].

Thus, the aims of this work were: (i) to develop a sustainable ultrasound-assisted oil extraction method, using a mixture of ethanol and tert-butanol as extraction solvent, from sloe seed residues generated during *Pacharán*'s liqueur production; and (ii) to compare the developed UAE method with the conventional Soxhlet extraction using *n*-hexane. For this study, sloe seed oils were characterized using chromatographic techniques (GC–MS, HPLC-DAD and HPLC-DAD-MS) to comprehensively analyse their fatty acid, tocopherol, and polyphenol profiles. Additionally, the study encompassed the assessment of the non-nutritional amygdalin content present in oils using HPLC-MS. Subsequently, the antioxidant and antimicrobial capacities of the oils were evaluated to study the possible valorisation of the bio-residues from the liquor to obtain bioactive products. Thereafter, a Principal Component Analysis (PCA) was used to establish a correlation between bioactivities and the content of bioactive compounds. Finally, the greener assessment of both extraction methods was assessed through the novel AGREEprep software.

## 2. Materials and methods

### 2.1. Reagents, solvents, standards and bacterial strains

For all the experiments, analytical reagents and Milli-Q purified water (Millipore, Bedford, MA, USA) were required. Methanol (MeOH, ≥ 99 %), ethanol (EtOH, ≥ 99.8 %), sulfuric acid ($H_2SO_4$, 96 %), hydrochloric acid (HCl, 37 %) and *n*-hexane (96 %) of HPLC grade were acquired by Scharlab (Barcelona, Spain). In addition, for LC-MS characterization, acetonitrile (ACN) and formic acid (FA) of MS gradient quality were provided by the aforementioned trading house. 2,2-Diphenyl-1-picrylhydrazyl (DPPH, ≥ 99.9 %), dimethyl sulfoxide (DMSO, ≥99.9 %), sodium methoxide (95 %), sanitary ethanol (96 %), Tween-80, Tryptic Soy Broth (TSB) and *p*-iodonitrotetrazolium violet (INT, 98 %) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The positive control trolox (≥ 97 %) was acquired from Sigma-Aldrich (Burghasen, Germany). 2-Propanol for HPLC grade quality ($C_3H_8O$, PrOH), were purchased from Fisher Scientific (Madrid, Spain), and *tert*-butanol (*t*-BuOH) was obtained from ACS (Carlo Erba, ≥ 99 %).

α-, β-, γ- and δ-tocopherol standards were procured by Sigma-Aldrich (St. Louis, MO, USA). Tocopherol solutions were prepared to a final concentration of 1000 mg·L$^{-1}$ in methanol. The stock solutions were stable for three months at darkness conservation in the freezer at −20 °C. Working tocopherol standard solutions were prepared daily by diluting the stock solutions.

37 fatty acid methyl ester (FAMEs) mixture (SUPELCO), tridecanoic acid (C13:0, ≥ 99 %), ethyl nonanoate (> 98 %) and ethyl myristate (> 98 %) were provided by Sigma-Aldrich (Barcelona, Spain).

Caffeic acid, gallic acid monohydrate, 2, 3-dihydroxybenzoic acid, *p*-coumaric acid, *trans*-ferulic acid, kaempferol, myricetin, quercetin, rutin trihydrate, catechin, chlorogenic acid, epicatechin, naringin, and resveratrol (≥95.0 %) were obtained from Sigma-Aldrich (Madrid, Spain). Hesperidin (≥98.0 %) was provided by European Pharmacopoeia. Stock phenolic solutions (200 mg·L$^{-1}$), were mainly made in methanol but an ethanol-water mixture of 80:20 (*v/v*) was used to prepare the quercetin solution. Hesperidin was prepared in a 5 % (*v/v*) DMSO aqueous solution. The solutions were maintained in darkness at 4 °C or − 80 °C (myricetin, hesperidin, *trans*-ferulic acid, epicatechin, catechin, chlorogenic acid, and caffeic acid) for a maximum of two months. Working standard solutions were prepared daily by diluting the stock solutions as required.

Amygdalin was purchased from Sigma-Aldrich (St. Louis, USA). The stock solution (460 mg·L$^{-1}$) was prepared in MeOH, and the working standard solutions were prepared daily by appropriate dilution of the

stock solution.

*P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) bacteria strains were employed to determine the antimicrobial capacity of sloe seed oils. The positive antibiotic standard, streptomycin sulfate salt was acquired from Sigma-Aldrich (St. Louis, USA).

### 2.2. Sloe fruits raw materials

Three distinct types of sloe fruit waste (*Prunus spinosa* L.) were sourced from Hijos de Pablo Esparza Bodegas Navarra S.A. (Navarra, Spain, 2023 campaign): Spanish sloe fruits of Navarra before maceration (NSBM), Bulgarian sloe fruits before maceration (BSBM) and a mixture of sloes berries from Navarra (42 %) and Bulgaria (58 %) post-maceration (NBSAM). The latter was macerated with aniseed at a regulated temperature for a period of six months and subsequently washed with water to produce a Spanish liqueur known as *Pacharán*. Samples were stored in a freezer at −20 °C until use.

For subsequent analysis, sloe stones were meticulously separated from the mesocarp and exocarp. Then, the seeds of the stones were isolated following the methodology outlined elsewhere [22].

### 2.3. Moisture content of sloe seed samples

The moisture determination of sloe seeds was determined following the AOAC 925.10 procedure [23], and expressed as a percentage of weight (%, (*w/w*)). The samples were analyzed in triplicate.

### 2.4. Ultrasound-assisted extraction of sloe seed oils

Ultrasound-assisted extraction of sloe seed oils was performed in an ultrasonic bath with a working frequency of 50/60 Hz and a power of 150 W. NSBM seeds were used as a model to optimize the oil extraction methodology, given their control over the production process.

#### 2.4.1. Screening of UAE experimental factors

The selection of experimental factors and their corresponding levels for seed oil extraction was based on the methodology described in the literature [24–28]. In the present study, this methodology was slightly modified to suit the specific experimental conditions. As such, an experimental design was conducted using a $2^{\alpha-1}$ (where α is the number of factors) reduced factorial experimental design, incorporating four factors and two levels. The experimental factors studied in the extraction process included the ratio between t-BuOH and EtOH in the solvent mixture (0:100 and 40:60 (*v/v*)), the sample-to-solvent ratio (1/5 and 1/7.5 (*w/v*)), the duration of the extraction process (15 and 30 min), and the temperature (20 and 50 °C). In consideration of the outcomes of the preliminary tests, the upper and lower bounds of the examined parameters were determined and coded by assigning the values −1 and 1, respectively. The experimental response was the oil extraction yield, expressed as a percentage of the mass of weighed dry solid (%, (*w/w*)). Consequently, a total of eight experiments were conducted, with a sample amount fixed at 2.0000 g (Table 1).

The experimental results were subjected to a multifactorial Analysis of Variance (ANOVA). Subsequently, due to the relevant effect of the sample-to-solvent ratio on the oil recovery, the study range of this experimental factor was extended to the following values: 2/7.5, 2/15, 2/30 and 2/45 (*w/v*).

#### 2.4.2. Experimental design and optimization of the UAE process

After conducting preliminary studies, an experimental screening design was implemented, in which the experimental factors included the t-BuOH:EtOH ratio (60:40, 30:70, and 80:20 *v*/v) in the extraction solution, the extraction time (10, 15, and 20 min), and the temperature (20, 30, and 40 °C). Equal weighting was assigned to all experimental variables. The values −1, 0, and 1 were used to encode the lower, central, and upper levels of each factor. A total of 11 trials were conducted ($2^3 = 8$ experiments with three replicates at the central point), in which the response variable was oil recovery (Table 2).

**Table 1**
Experimental design and corresponding responses obtained from the reduced factorial design for ultrasound-assisted extraction of sloe seed oil.

| Experiment | Experimental factors | | | | Response |
|---|---|---|---|---|---|
| | *t*-BuOH: EtOH ratio (*v/v*); $\beta_1$ | Sample-solvent ratio (*w/v*); $\beta_2$ | Extraction time (min); $\beta_3$ | Temperature (°C); $\beta_4$ | Oil recovery (%, (*w/w*)) |
| 1 | 40:60 (−1) | 1/7.5 (−1) | 15 (−1) | 20 (−1) | 13.2 |
| 2 | 0:100 (1) | 1/7.5 (−1) | 15 (−1) | 50 (1) | 5.2 |
| 3 | 40:60 (−1) | 1/5 (1) | 15 (−1) | 50 (1) | 5.2 |
| 4 | 0:100 (1) | 1/5 (1) | 15 (−1) | 20 (−1) | 2.3 |
| 5 | 40:60 (−1) | 1/7.5 (−1) | 30 (1) | 50 (1) | 7.9 |
| 6 | 0:100 (1) | 1/7.5 (−1) | 30 (1) | 20 (−1) | 7.5 |
| 7 | 40:60 (−1) | 1/5 (1) | 30 (1) | 20 (−1) | 4.4 |
| 8 | 0:100 (1) | 1/5 (1) | 30 (1) | 50 (1) | 3.9 |

Codified values of each factor level are shown in parentheses.

The results obtained were subsequently analyzed using ANOVA and linear regression to assess the pertinence of the model and its reproducibility. Finally, extraction optimization was performed to establish the variables that maximize the experimental response. The experimental factorial design (Table 2) was intended to determine the most significant variables in the oil recovery response, as well as their interaction effects.

#### 2.4.3. Optimized UAE conditions

The optimized ultrasound-assisted extraction method comprised the weighing of 2.0000 g of sloe seed samples, followed by the addition of 45 mL of the extraction mixture *t*-BuOH:EtOH 60:40 (*v/v*). The oil was then recovered through sonication in an ultrasonic bath at 40 °C for 10 min. Subsequently, the mixture was subjected to a centrifugal process at a speed of 694.5 ×*g* for 15 min. The oil containing excess solvent was then evaporated in a concentrator at 60 °C, with a nitrogen flow of between 1.5 and 2 mL·min$^{-1}$, for a period of 1 h and 30 min. Finally, the oil recovery (%, (*w/w*)) was determined in triplicate. The resulting oils were then stored at −4 °C until use.

**Table 2**
Plan of experiments and experimental responses obtained from two-level three-variable factorial design for ultrasound-assisted oil extraction.

| Experiment | Experimental factors | | | Response |
|---|---|---|---|---|
| | *t*-BuOH:EtOH ratio (*v/v*); $\beta_1$ | Extraction time (min), $\beta_2$ | Temperature (°C), $\beta_3$ | Oil recovery (%, (*w/w*)) |
| 1 | 60:40 (−1) | 10 (−1) | 20 (−1) | 11.9 |
| 2 | 80:20 (1) | 10 (−1) | 20 (−1) | 17.3 |
| 3 | 60:40 (−1) | 20 (1) | 20 (−1) | 14.5 |
| 4 | 80:20 (1) | 20 (1) | 20 (−1) | 15.4 |
| 5 | 60:40 (−1) | 10 (−1) | 40 (1) | 35.7 |
| 6 | 80:20 (1) | 10 (−1) | 40 (1) | 30.0 |
| 7 | 60:40 (−1) | 20 (1) | 40 (1) | 30.9 |
| 8 | 80:20 (1) | 20 (1) | 40 (1) | 31.4 |
| 9 | 70:30 (0) | 15 (0) | 30 (0) | 29.5 |
| 10 | 70:30 (0) | 15 (0) | 30 (0) | 28.1 |
| 11 | 70:30 (0) | 15 (0) | 30 (0) | 28.4 |

Codified values of each factor level are shown in parentheses.

### 2.5. Conventional soxhlet extraction of sloe seed oils

Soxhlet oil extraction from NBSAM seeds was conducted succeeding the method developed by Rodríguez-Blázquez et al. [29]. The percentages in weight (%, (*w/w*)) of sloe oils were determined in triplicate.

### 2.6. Characterization of sloe seed oils

#### 2.6.1. Fatty acid analysis by GC–MS

The fatty acid composition of sloe seed oils recovered by UAE and Soxhlet extraction was determined using a gas chromatography (Agilent 6890) coupled to a mass spectrometer (Agilent 5973). This followed the internal derivatization and the GC–MS procedure developed by Rodríguez-Blázquez et al. [29] with several modifications explained bellow.

In this study, the chromatographic separation of fatty acids was conducted using a capillary chromatographic column (60 m in length, with an internal diameter of 0.25 mm and a film thickness of 0.25 μm) (DB-FastFAME Ref. G3903–63012). In addition, the column program was initiated under an isothermal condition of 100 °C for 2 min. This was followed by a heating ramp increasing at a rate of 8 °C·min$^{-1}$, starting from 100 °C and reaching a final temperature of 145 °C. Subsequently, a new isothermal condition of 145 °C was established for 20 min. This was followed by a heating ramp at a rate of 5 °C·min$^{-1}$, reaching a final temperature of 230 °C. The temperature was then held isothermally at 230 °C for 20 min. The chromatographic column was operated at a constant flow rate of 1 mL·min$^{-1}$.

The percentage of each fatty acid in sloe seed oils was determined using response factors (RF) and were analyzed in quadruplicate. This semi-quantitative analysis was performed as a method of correlating the ratio of areas between each FAME as well as the internal standard with the FAME concentration, using tridecanoic acid (C13:0) (1 mg of which was added prior to methylation). In order to ascertain the relative quantities of each FAME, a response factor of 1 was established, with the composition expressed as a percentage (%). The composition of each FAME within the samples was calculated taking into account both the individual area of each FAME and the total area of all identified analytes (with 100 % representing the culminative area of all FAMES detected).

The mean RF values, along with their standard deviations, limits of detection (LOD), and limits of quantification (LOQ), are presented in **Table S1**. Based on the obtained fatty acid profiles, heart-healthy lipid indexes—including desirable fatty acids (DFA), the hypocholesterolemic/hypercholesterolemic ratio (H/h), and the atherogenicity index (AI)—were calculated for each sample. The fatty acid profiles of sloe seed oils extracted by UAE were statistically compared using ANOVA followed by Fisher's LSD test. In contrast, the fatty acid compositions of NBSAM oils obtained by ultrasound-assisted extraction and Soxhlet extraction were compared using a *t*-Student's test.

#### 2.6.2. Determination of tocopherols content by HPLC-DAD

Tocopherol analysis in sloe seed samples was conducted by HPLC-DAD, following the methodology previously described elsewhere [29] with some modifications.

Chromatographic analysis was performed using a Jasco LC-NetII/ADC degasser, a Jasco PU-2089 Plus quaternary gradient pump, and a Jasco MD-2018 photodiode array detector. Separation of tocopherols was achieved on a Luna C18 column (5 μm, 150 × 4.6 mm, 100 Å; Phenomenex, Torrance, CA, USA) using methanol as the mobile phase at a constant flow rate of 1 mL·min$^{-1}$. UV–Vis detection was carried out at 292 nm and 305 nm. Quantitative analyses were conducted at 292 nm employing two external calibration curves: one for high concentration ranges ($n = 7$; 20.0–250 mg·L$^{-1}$) and another for low concentrations ($n = 5$; 0.5–20.0 mg·L$^{-1}$ for γ- and α-tocopherol, and 0.3–20.0 mg·L$^{-1}$ for δ-tocopherol).

LOD and LOQ were estimated by 3.3 times the background noise signal (S/N) for the former and 10 times for the latter. Precision of both retention factors (RF) and peak areas of each analyte were evaluated by the injection of standard solutions at concentrations of 5 and 50 mg·L$^{-1}$ for γ-tocopherol, 10 and 100 mg·L$^{-1}$ for α-tocopherol, and 2 and 50 mg·L$^{-1}$ for δ-tocopherol, in the same day (intra-day variation, $n = 3$) and at different days (three consecutive days, inter-day variation ($N = 9$)). Relative standard deviation (RSD, %) was used as a measure of repeatability and intermediate precision, calculated for the retention factor ($k$) and peak areas of each analyte.

Finally, samples were analyzed in quadruplicate, and results are presented as mean ± standard deviation, expressed in mg per kg of sloe seed on a dry weight basis.

#### 2.6.3. Determination of amygdalin and phenolic compounds by cLC-DAD-MS

*2.6.3.1. Extraction of phenolic compounds and amygdalin.* The phenolic compounds and the amygdalin were determined in NBSAM oils, obtained using both UAE and Soxhlet method, following the procedure explained by Bail et al. [30] with some modifications. Briefly, 600 mg of oil was mixed with 3 mL of a methanol: water solution (90:10 (*v/v*)). Then, the mixture was stirred in a vortex apparatus for 4 min at 402.48 ×*g*. The solution was centrifuged at 1528 ×*g* for 5 min (centrifuge 5804, Eppendorf). Subsequently, 2.5 mL of methanolic extract was collected for analysis. All assays were performed in triplicate for each sample.

*2.6.3.2. Chromatographic analysis.* The identification and quantification of individual polyphenols and amygdalin in sloe seed oils were carried out using cLC-DAD-MS, following the method described by Gómez-Mejía et al. [31].

The equipment employed comprised a G1376A binary capillary pump, a G1379A degasser, a G7115A diode array detector (500 nL, 10 mm pathlength) and a simple quadruple mass spectrometer (6120) equipped with an electrospray ionization source (ESI). A Synergi™ Fusion C18 capillary analytical column (150 × 0.3 mm i.d.) was also employed. The separation of the phenolic components and the amygdalin was achieved using a 4 μm Phenomenex (Torrance, CA, USA). Two distinct mobile carrier phases were utilized in this study: Solvent A, comprising 0.05 % (*v/v*) formic acid aqueous solution at pH 2.9, and Solvent B, consisting of acetonitrile. The flow rate was established at 10 μL·min$^{-1}$. Five different UV–Vis wavelengths (220, 260, 292, 310, and 365 nm) and a negative ion mode mass detection, selecting $[M\text{-}H]^-$ as the molecular ion, were employed to identify all the components. For quantitative purposes, the calibration curves were obtained by DAD or MS, in both total ions counting (TIC) or selected ion monitoring (SIM) modes, according to the superior sensitivity and/or selectivity. Furthermore, to achieve analyte focusing on the column, injection solutions were made by diluting 10–75 μL of sample aliquots, 50 μL of acetonitrile, and 15–90 μL of methanol to a final volume of 5 mL with 0.05 % (*v/v*) formic acid aqueous solution at pH 2.9. The composition of phenolic compounds in all extracts was expressed as mean ± standard deviation, in μg per gram of dry sloe seed ($n = 3$).

### 2.7. Evaluation of bioactive properties of sloe seed oils

#### 2.7.1. Antioxidant activity

The antiradical capacity of sloe seed oils was evaluated carrying out the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as described by Rodríguez-Blázquez et al. [32] with some modifications. Briefly, 30 μL of eight methanolic working solutions (0–30 μL) were prepared in DMSO with 0.5 % (v) of Tween 80 (500–531 mg·mL$^{-1}$) and subsequently combined with 270 μL of a $6{\cdot}10^{-5}$ M DPPH methanolic solution, in a 96-well microplate. The solutions were then incubated for one hour in the dark. Subsequently, the absorbances were measured at a wavelength of 515 nm. The outcomes ($n = 2$) were indicated as $IC_{50}$ values (mg·mL$^{-1}$ of oil). In addition, the oxidative stability of the oils obtained by UAE was determined using the DPPH method during a storage period of 0–21

days. This assay was conducted in duplicate for each sloe seed oil.

#### 2.7.2. Antimicrobial activity

The antimicrobial activity of sloe seed oils, obtained by ultrasound-assisted extraction, was evaluated against two Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*) and one Gram-positive strain (*Staphylococcus aureus*), following the broth micro-dilution method combined with the p-iodonitrotetrazolium chloride (INT) colorimetric assay, as described by De la Fuente et al. [33].

A 50 % (*v/v*) stock solution of each sloe seed oil extract was prepared in tryptic soy broth (TSB) containing 0.05 % (*v/v*) Tween 80. Subsequently, twofold serial dilutions (ranging from 50 % to 0.391 % (*v/v*) were prepared in a 96-well microplate. To each well, 10 μL of bacterial suspension ($1.5 \times 10^6$ CFU·mL$^{-1}$) (obtained adjusting the optical density of isolated colonies to 0.1 at 600 nm) was added. For each bacterial strain, streptomycin (0.0031–0.4 mg·L$^{-1}$) prepared in TSB with 0.5 % (v/v) Tween 80 served as the positive control. Additionally, a negative control (TSB with 0.5 % Tween 80) and a positive control (TSB inoculated with bacterial suspension) were included. The microplates were incubated at 37 °C for 24 h under constant agitation. Following incubation, bacterial growth inhibition was assessed visually by colour change after the addition of 50 μL of INT dye (0.2 mg·mL$^{-1}$) and a further 30-min incubation at 37 °C. Results were expressed as the Minimum Inhibitory Concentration (MIC, mg·mL$^{-1}$; $n = 3$).

### 2.8. Greenness assessment of seed oil extraction methods

The AGREEprep software, described by Wojnowski et al. [34], was used to evaluate the green characteristics of the UAE and Soxhlet extraction methods.

The weight of each subcriterion was adjusted according to the specific characteristics of the optimized oil extraction method. In order to optimize the UAE oil extraction method as an alternative to the Soxhlet extraction method, the following key factors were considered: the use of green solvents, the use of small amounts of sloe seed residues, and the use of more automated systems with lower energy costs during the extraction time. Consequently, given the paramount importance of the oil extraction method developed and refined in this study, criterion 3, "Sustainability, renewability, and reuse of materials", was modified to have an impact of 5, as was criterion 5, "Economy of sample size", which was assigned an impact of 4. Criteria 7 and 8, "Integration and automation" and "Energy consumption", were modified to have an impact of 3 and 5, respectively.

In alignment with the aforementioned points, **Table S2** summarises the weighting factors applied to each selected criterion within the software.

### 2.9. Statistical analysis

Data were stated as mean ± standard deviation on a dry basis and were statically analyzed by multifactorial and unifactorial ANOVA, response surface methodology, Fisher's Least Significant Difference (LSD) test, *t*-Student test and PCA by the application of Statgraphics 19 software package (Statgraphics Technologies, Inc., Rockville, MD, USA).

## 3. Results and discussion

### 3.1. Moisture content of sloe seeds

Moisture content was determined according to the procedure described in Section 2.3. A statistically significant increase in moisture content ($p$-value $< 0.05$) was observed in sloe seeds subjected to maceration (NBSAM) compared to those analyzed before maceration (NSBM and BSBM). This difference may be attributed to the maceration process, during which the fruits are stored in sealed containers and immersed in aniseed for six months to extract aromatic compounds. This prolonged exposure may promote water absorption by the seeds.

Additionally, significant differences ($p$-value $< 0.05$) were found between the moisture content of untreated seeds originating from Bulgaria (BSBM) and Navarra (NSBM). These variations could be due to a combination of climatic, edaphic, and genetic factors, as previously reported by Górnás et al. [35]. Furthermore, differences in cultivation practices may have influenced moisture retention: while the Navarra sloes were cultivated under managed conditions, the Bulgarian sloes were collected from wild-growing plants, which may have experienced greater environmental variability and lower irrigation, thereby affecting seed moisture levels.

### 3.2. Optimization of ultrasound-assisted extraction of sloe seed oils

#### 3.2.1. Preliminary assays

To optimize the UAE method for oil recovery from sloe seeds, NSBM oil was employed as a model system owing to its superior control over the production process. A reduced factorial design ($2^{4-1}$) was conducted to evaluate preliminary conditions aimed at maximizing oil recovery, as detailed in Section 2.4.1.

The experimental factors studied in the preliminary trials included the *t*-BuOH:EtOH ratio (*v/v*) in the solvent extraction, the sample-to-solvent ratio (*w/v*), the extraction time (min), and the extraction temperature (°C), selected based on their reported significant influence on ultrasound-bath assisted extraction [24–28]. The oil extraction yields (%, (*w/w*)) obtained for each of the experimental conditions are presented in Table 1.

The highest extraction efficiency was observed in experiment 4 (0:100 (*v/v*) *t*-BuOH:EtOH ratio; 1:7.5 (*w/v*) sample-to-solvent ratio; 15 min of extraction time and 20 °C of extraction temperature). However, due to the limited degrees of freedom in experimental design, multifactorial ANOVA did not reveal a statistically significant effect on the oil recovery. Nonetheless, the sample-to-solvent ratio was identified as the most influential factor, exhibiting a negative effect on the oil recovery. Conversely, the proportion of *t*-BuOH (%, *v/v*) in the extraction solvent showed a positive correlation with the oil recovery. Moreover, a significant positive interaction was found between the *t*-BuOH proportion in the extraction solvent and the extraction time, indicating that, at the initial stage, longer extraction times combined with lower *t*-BuOH proportions enhance oil yield. Furthermore, the coefficient of determination ($R^2$) for the model was 1.000, demonstrating an excellent fit to the data and a strong predictive capability for future experiments.

Subsequently, given the significant impact of the sample-to-solvent ratio on extraction yield, the range of this factor was expanded to include the following values: 2:7.5, 2:15, 2:30, and 2:45 (*w/v*). The findings demonstrated a substantial improvement in oil recovery as the ratio increased. The yields increased from (5.3 ± 0.2)%, (*w/w*) at 2:15 (*w/v*) sample-to-solvent ratio, to (31.7 ± 0.1)%, (*w/w*) at 2:45 (*w/v*) sample-to-solvent ratio. This effect can be attributed to the higher solvent-to-solid ratio, which increases the concentration gradient, thereby enhancing mass transfer and ultimately leading to greater oil extraction [14,36]. Furthermore, this observation was also confirmed by Ntalikwa et al., [36] who reported that increasing the solvent-to-solid ratio from 4:1 to 6:1 led to a higher yield of Jatropha oil.

Consequently, the optimal sample-to-solvent ratio (*w/v*) was determined to be 2.0000 g of sample to 45 mL of solvent mixture.

#### 3.2.2. Factorial experimental design

Following a thorough evaluation of extant studies in the field, a three-factor, two-level factorial screening design was implemented, incorporating three replicates at the center point (Table 2) as described in Section 2.4.2.

The resulting polynomial equation optimized and predicted by the model is shown in (Eq. (1)):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

where Y is the predicted response (oil recovery (%, *w/w*)), $\beta_0$ is the intercept, $\beta_1$, $\beta_2$, and $\beta_3$ are the linear coefficients of $x_1$ (*t*-BuOH:EtOH ratio (*v/v*)), $x_2$ (extraction temperature (°C)), and $x_3$ (extraction time (min)), and $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the interaction coefficients of the experimental factors aforementioned.

Furthermore, Table 3 shows the values of the parameters that fit the experimental data to Eq. (1) for the response evaluated.

According to the equation predicted by the model, temperature ($\beta_2 = 8.6125$) was the most influential factor, with a positive and significant effect (*p*-value $<0.05$) on the experimental response (Y, oil recovery (% (*w/w*)). This suggests that elevated extraction temperatures favor oil recovery by inducing membrane disruption and enhancing lipid diffusion. Moreover, the extraction time ($\beta_3 = -0.3375$) emerged as the only experimental factor that exhibited a negative correlation with the experimental response. Consequently, the reduction in extraction time resulted in enhanced oil recovery. Additionally, the mathematical model obtained showed a satisfactory fit, with a determination coefficient ($R^2$) of 0.8895 and a low standard error of estimation of 4.3647.

Subsequently, to optimize maximum oil extraction performance, a response surface analysis (RSA) was carried out. The resulting surface response is depicted in Fig. 1. The findings of the study indicate that the highest oil yield is achieved using a mixture of *t*-BuOH:EtOH at a volume ratio of 60:40, in conjunction with an extraction temperature of 40 °C and an extraction time of 10 min through ultrasound bath-assisted extraction. Under these conditions, the model predicted an oil yield of 35.8 % (*w/w*), which was compared with the experimental value (35.4 ± 0.5) (% (*w/w*)) using *t*-Student statistical analysis. The experimental results exhibited a high degree of concordance with the model predictions, attaining a confidence level of 95 %, thereby substantiating the model's precision.

Once the optimal extraction conditions had been determined, they were applied to the rest of sloe seeds (BSBM and NBSAM). The oil recovery for NSBM, BSBM and NBSAM seeds were (35.4 ± 0.5) (% (*w/w*)), (38.2 ± 0.5) (% (*w/w*)) and (33.0 ± 0.8) (% (*w/w*)), respectively. A statistically significant variation (*p*-value $<0.05$) was identified in the oil recovery among all sloe seeds. NBSAM seeds exhibited the lowest oil yield in comparison to NSBM and BSBM seeds. This phenomenon can be attributed to the membrane rupture that occurs during the maceration process with anise, thereby facilitating the diffusion of the lipid content and enriching the seed with hydrophilic compounds. Furthermore, the analysis revealed that Bulgarian sloe seeds exhibited the highest extraction yield, a finding that is likely attributable to the diversity of the crop and the influence of the geographical location [35].

### 3.2.3. Comparison of oil recovery using Soxhlet extraction

To compare the oil recovery (%, (*w/w*)) obtained by applying the optimized UAE method with the standard Soxhlet extraction, NBSAM seed was used, as described in Section 2.5. This sample was selected because it was the last waste generated during the production of *Pacharán* and therefore, the one with the foremost industrial interest in its respective valorization. NBSAM oil recovery obtained by Soxhlet extraction was (35.6 ± 0.2) (% (*w/w*), comparable to others reported in the literature [37,38]. Significant differences (*p*-value $<0.05$), according to *t*-Student test, were observed in the oil recovery between UAE and Soxhlet extraction methods. This outcome may be explained by the extended extraction time characteristic of Soxhlet extraction, together with the use of *n*-hexane, a non-polar solvent known to enhance the recovery of hydrophobic compounds. Notwithstanding, the oil yield using UAE was close (33.0 ± 0.8) (%, (*w/w*)) to the obtained using Soxhlet extraction. Therefore, the method optimized in this work constitutes a very attractive and efficient alternative, using sustainable and low toxic solvents, consuming less time and resources than Soxhlet extraction, offering significant economic and environmental benefits.

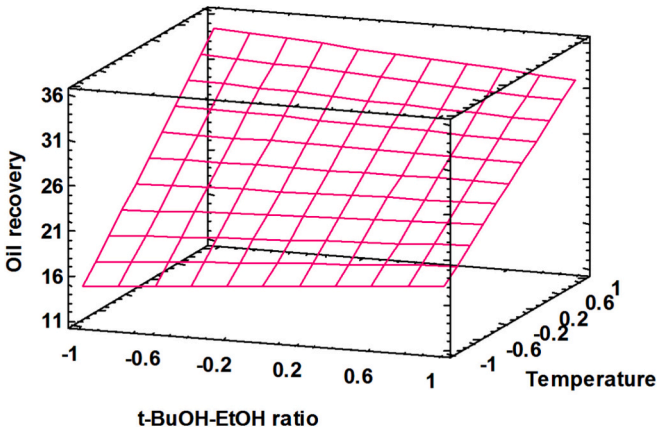



**Fig. 1.** Experimental response surface for the oil recovery response as a function of t-BuOH/EtOH ratio (*v/v*) and the temperature (°C), for an extraction time of 10 min.

## 3.3. Analysis of bioactive compound content in sloe seed oils

### 3.3.1. Fatty acid profile

Fatty acids are a class of plant-based compounds that have been demonstrated to possess a wide range of beneficial effects on the human body [37]. The composition of sloe seed oils analyzed by GC–MS are shown in Table 4. A total of 12 fatty acids were identified, and all sloe seed oils were characterized as being a rich source of unsaturated fatty acids (91.6–92.8 %), mainly oleic acid (61.0–73.6 %) and linoleic acid (18.2–29.7 %). A notable amount of palmitic acid was also detected in all of them (5.6–6.9 %). These results were consistent with the findings reported by Atik et al. [5]. For illustrative purposes, the chromatogram corresponding to the standards as well as to NSBM oil is represented, as an example, in **Fig. S1**. With a focus on assessing the nutritional quality of the oils, it is of utmost interest to determine the heart-healthy lipid indexes.

All sloe seed oils exhibited elevated concentrations of polyunsaturated fatty acids (PUFAs) (18.2–29.8 %) and monounsaturated fatty acids (MUFAs) (62.0–74.5 %), which have been demonstrated to decrease blood triglycerides, thereby reducing the risk of coronary heart disease. Moreover, sloe seed oils exhibited a high content of DFA

**Table 3**
Values of the coefficients, correlation coefficient ($R^2$), and standard error of estimation (SEE) obtained from the adjustment of the polynomial model described in Eq. (1) for the oil recovery response.

| Response | Coefficients | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\beta_0$ | $\beta_1$ | $\beta_2$ | $\beta_3$ | $\beta_{12}$ | $\beta_{13}$ | $\beta_{23}$ | $R^2$ | SEE |
| Oil recovery (%, *w/w*) | 24.827 | 0.137 | **8.612** | −0.337 | −1.437 | 0.212 | −0.512 | 0.8995 | 4.3647 |

$\beta_0$ is the intercept, $\beta_1$, $\beta_2$, and $\beta_3$ are the linear coefficients of $x_1$ (*t*-BuOH:EtOH ratio (*v/v*)), $x_2$ (extraction temperature (°C)), and $x_3$ (extraction time (min)), and $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the interaction coefficients of the experimental factors aforementioned. Coefficient factors in bold are those with a *p*-value $<0.05$, at the 95 % confidence level, and which, therefore, have a significant effect on the study response. SEE: standard errors of estimation.

**Table 4**
Fatty acid composition of sloe (*Prunus spinosa* L.) seed oils by GC–MS.

| Fatty Acids | NSBM oil obtained by UAE (%) | BSBM oil obtained by UAE (%) | NBSAM oil obtained by UAE (%) | NBSAM oil obtained by Soxhlet (%) |
|---|---|---|---|---|
| Pentadecyl acid (C15:0) | (0.017 ± 0.001) [c] | (0.013 ± 0.001) [a] | (0.015 ± 0.001) [b] | (0.020 ± 0.002) * |
| Palmitic acid (C16:0) | (6.5 ± 0.6) [b] | (6.7 ± 0.3) [b] | (5.6 ± 0.1) [a] | (6.9 ± 0.5) * |
| Palmitoleic acid (C16:1n7) | (0.9 ± 0.1) | (0.9 ± 0.1) | (0.80 ± 0.02) | (0.9 ± 0.1) |
| Margaric acid (C17:0) | (0.044 ± 0.004) [b] | (0.042 ± 0.001) [b] | (0.037 ± 0.002) [a] | (0.047 ± 0.001) * |
| Cis-10-heptadecenoic acid (C17:1n10c) | (0.121 ± 0.007) [b] | (0.111 ± 0.003) [a,b] | (0.10 ± 0.01) [a] | (0.123 ± 0.002) * |
| Stearic acid (C18:0) | (0.9 ± 0.5) | (1.29 ± 0.04) | (1.42 ± 0.09) * | (1.2 ± 0.1) |
| Oleic acid (C18:1n9c) | (64.4 ± 0.6) [b] | (61.0 ± 0.4) [a] | (73.6 ± 0.3) [c, *] | (63 ± 1) |
| Linoleic acid (C18:2n6c) | (26.9 ± 0.6) [b] | (29.7 ± 0.6) [c] | (18.2 ± 0.5) [a] | (27.2 ± 0.7) * |
| Arachidic acid (C20:0) | (0.076 ± 0.009) [a] | (0.081 ± 0.004) [a] | (0.110 ± 0.005) [b] | (0.11 ± 0.02) |
| Gondoic acid (C20:1n9c) | (0.053 ± 0.002) | (0.046 ± 0.008) | (0.05 ± 0.01) | (0.072 ± 0.009) |
| Behenic acid (C22:0) | (0.014 ± 0.003) | (0.012 ± 0.003) | (0.015 ± 0.002) | (0.028 ± 0.007) * |
| Lignoceric acid (C24:0) | (0.012 ± 0.002) [a] | (0.008 ± 0.002) [b] | (0.028 ± 0.004) [c] | (0.020 ± 0.004) |
| ∑ SFA | (7.6 ± 0.2) [a] | (8.3 ± 0.3) [b] | (7.2 ± 0.2) [a] | (8.3 ± 0.4) * |
| ∑ UFA | (92.4 ± 0.2) [b] | (91.8 ± 0.3) [b] | (92.8 ± 0.3) [b, *] | (91.6 ± 0.4) |
| ∑ MUFA | (65.5 ± 0.5) [b] | (62.0 ± 0.6) [a] | (74.5 ± 0.4) [c, *] | (64 ± 1) |
| ∑ PUFA | (26.9 ± 0.6) [b] | (29.8 ± 0.7) [c] | (18.2 ± 0.5) [a] | (27.2 ± 0.7) * |
| PUFA/SFA ratio | (3.6 ± 0.2) [b] | (3.6 ± 0.2) [b] | (2.5 ± 0.1) [a] | (3.25 ± 0.09) * |
| DFA | (93.3 ± 0.6) [a] | (93.2 ± 0.3) [a] | (94.2 ± 0.1) [b, *] | (92.8 ± 0.5) |
| AI | (0.070 ± 0.006) [b] | (0.073 ± 0.003) [b] | (0.060 ± 0.001) [a] | (0.077 ± 0.006) * |
| H/h | (14 ± 1) [a] | (13.5 ± 0.6) [a] | (16.4 ± 0.5) [b, *] | (13.2 ± 0.9) |

Results are expressed as mean values ± SD ($n = 4$). Values in the same row with different letters denote significant differences ($p$-value <0.05) among sloe oils obtained by UAE extraction method, according to one-way ANOVA and Fisher's LSD test. *Denotes significant differences ($p$-value <0.05) among NBSAM oils according to t-Student test. NSBM: Navarra sloe seed before maceration. BSBM: Bulgaria sloe seed before maceration. NBSAM: Navarra and Bulgaria sloe seeds after maceration. SFA: saturated fatty acids. UFA: unsaturated fatty acids. MUFA: monounsaturated fatty acids. PUFA: polyunsaturated fatty acids (unsaturation ≥2). PUFA/SFA: ratio between polyunsaturated and saturated fatty acids. DFA: desirable fatty acids. AI: atherogenicity index. H/h: hypocholesterolemic/hypercholesterolemic index.

(92.8–94.2 %) and low values of AI (0.060–0.077) h/H indexes (13.2–16.4), which can be attributed to their favorable fatty acid composition. This profile has been associated with a reduced risk of cardiovascular events, including atherosclerosis, myocardial infarction, and stroke, due to its positive effects on plasma lipid levels, endothelial function, and inflammation modulation [39].

In consideration of the provenance of the sloe berries, it was ascertained that NSBM oil exhibited a considerably elevated oleic acid content in comparison to BSBM oil, while the linoleic acid content was significantly lower ($p$-value <0.05). This phenomenon can be attributed to the specific form of cultivation as well as the geographical origin of the berries. In the case of palmitic acid, no statistically significant differences ($p$-value ≥0.05) were observed.

In the phase of processing sloe liqueur, minor alterations were detected in the fatty acid profile of the oils obtained by UAE. Specifically, oleic acid levels increased significantly ($p$-value <0.05) in the oil obtained from sloes from Navarra and Bulgaria after maceration with anise (NBSAM), while the palmitic and linoleic acid contents decreased significantly compared to the sloe oils before maceration (NSBM and BSBM). This fact can be attributed to the physical-chemical transformations that occur during the maceration stage. This stage may favor changes in the permeability of cell membranes and facilitate the enzymatic degradation of certain fatty acids that are more susceptible to oxidation or solvent extraction, such as palmitic and linoleic acids. The observed changes in fatty acid composition resulted in significant differences ($p$-value <0.05) in the associated cardiovascular health indexes, with NBSAM oil displaying the most favorable profile—characterized by the highest DFA value (94.2 ± 0.1) and the lowest AI and h/H ratios (0.060 ± 0.001) and (16.4 ± 0.5), respectively.

Furthermore, given the particular industrial interest in the valorization of the final seed residue produced during the *Pacharán* production process, a comparison was conducted between the fatty acid profiles of NBSAM oil obtained by the UAE method and NBSAM oil obtained by Soxhlet extraction, using n-hexane as the solvent. Statistically significant differences were identified in the major fatty acid profile of both oils ($p$-value <0.05). These findings indicate that the extraction method employed had a substantial impact on the resulting lipid content. However, minor fatty acids such as palmitoleic acid, arachidic acid, gondoic acid and lignoceric acid were the only fatty acids that did not show significant differences ($p$-value ≥0.05) in their respective content between both oils. This fact can be explained by the very low levels of these fatty acids, which makes it difficult to detect differences between extraction techniques [40]. In addition, these long-chain fatty acids are relatively stable and not significantly affected by the thermal degradation that could occur under the high temperatures used in Soxhlet extraction [40].

In addition, it was observed that NBSAM oil obtained by UAE was characterized by a higher oleic acid content and a lower linoleic acid content compared to the oil obtained by Soxhlet. It may be related to the isomerization process that C18:1n9c and C18:2n6c undergo with the temperature applied in the Soxhlet process. Furthermore, linoleic acid exhibited enhanced protection when present in complex forms (*e.g.*, phospholipids or glycerides), which Soxhlet extraction more effectively released.

Moreover, NBSAM oil obtained by Soxhlet extraction was categorized by a significant increase ($p$-value <0.05) in saturated fatty acids (SFA) content and a significant decrease in unsaturated fatty acids (UFA) content, compared to NBSAM oil obtained by ultrasound-assisted extraction. This fact may be ascribed to the greater thermal stability of SFA relative to unsaturated ones. As a result, the elevated temperatures employed during Soxhlet extraction are more likely to induce thermal degradation or oxidation of UFA, whereas SFA remain largely unaffected [29]. Furthermore, an increase in the content of SFA has been demonstrated to exert a detrimental effect on the human organism, resulting in elevated cholesterol levels [41]. This lipid profile negatively affected their respective lipid indexes which were found to be less healthy (lower DFA index and a higher both AI and H/h indexes) [42,43].

The oils obtained from the seeds through the UAE process present a remarkable fatty acid profile, particularly the NBSAM oil, which is characterized by its notably high content of UFA. It has been demonstrated that unsaturated fatty acids have a substantial impact on enhancing blood microcirculation, reducing blood viscosity and lowering cholesterol levels [44]. Therefore, NBSAM oil could feasibly be used in the nutraceutical sector for the treatment of cardiovascular and coronary disorders, either early prevention or treatment [45].

### 3.3.2. Tocopherol content

Tocopherols are a group of antioxidant compounds that have proven highly effective in interrupting oxidative chain reactions. This property

makes them particularly suitable for use as food additives [7]. Accordingly, this study is concerned with determining the tocopherol content in sloe seed oils.

The analytical characteristics of the HPLC-DAD methodology developed for the analysis of tocopherols are presented in **Table S3**. The method exhibited linear behavior ($R^2 > 0.9990$) over two concentration intervals for all analytes except β-tocopherol, whose quantification was hindered by co-elution with γ-tocopherol. With a coefficient of variation that is consistently less than 5 %, the devised approach satisfies the requirements for reproducing peak areas. Despite having a higher coefficient of variance than the preceding one, the retention factor is likewise reproducible. For an illustrative view, **Fig. S2** presents the chromatogram of the tocopherol standards solution, along with those of the NBSAM oil obtained by Soxhlet and UAE extraction.

Table 5 shows the α-, β + γ-, and δ-tocopherol content of sloe seed oils. The tocopherol content observed in this study aligns with the findings reported by Athanasiadis et al. [7]. Concerning the origin of sloe seeds, significant differences ($p$-value <0.05) were observed in the content of most tocopherols, except β + γ-tocopherol. The processing of sloe seeds during the production of *Pacharán* resulted in statistically significant differences ($p$-value <0.05) in δ-tocopherol content between sloe seed oils both before (NSBM and BSBM) ((14 ± 1) $mg{\cdot}kg^{-1}$ and (1.3 ± 0.2) $mg{\cdot}kg^{-1}$) and after maceration (NBSAM) ((20 ± 3) $mg{\cdot}kg^{-1}$).

In addition, β + γ-tocopherol was the predominant tocopherol in all sloe seed oils. Statistically significant differences ($p$-value <0.05) in its content were observed between BSBM seed oil ((87 ± 6) $mg{\cdot}kg^{-1}$) and NBSAM seed oil ((100 ± 8) $mg{\cdot}kg^{-1}$). This phenomenon could be attributed to the increased porosity of NBSAM seeds during the maceration process, which facilitated the interconversion of certain tocopherols (Morata et al., 2018). Consequently, a low β + γ/α ratio was observed in NBSAM seed oil, implying a reduced risk of obesity and higher feasible oxidative stability [29,46].

Conversely, a substantial decline ($p$-value <0.05) in tocopherol's content was evident in NBSAM oil obtained by Soxhlet extraction in comparison with NBSAM oil obtained by UAE, according to $t$-Student's test. This outcome could be attributed to the elevated temperatures and extended extraction times characteristic of Soxhlet method, which are known to facilitate the degradation of tocopherols [47,48]. These results emphasize the considerable advantages of oils obtained by UAE over those produced by Soxhlet extraction. Furthermore, the presence of bioactive compounds in the oils contributes to their high added value, given the well-established association of these compounds with potent antioxidant activity and a reduced risk of coronary heart disease [46,47].

**Table 5**
Tocopherols, amygdalin and polyphenols content in *Prunus spinosa* L seed oils.

| | NSBM oil by UAE | BSBM oil by UAE | NBSAM oil obtained by UAE | NBSAM oil obtained by Soxhlet |
|---|---|---|---|---|
| **Tocopherols content ($mg{\cdot}kg^{-1}$)** | | | | |
| α-tocopherol | (12 ± 1) $^{b}$ | (8 ± 1) $^{a}$ | (10 ± 2) $^{a, *}$ | (4.4 ± 0.1) |
| β + γ-tocopherol | (84 ± 5) $^{a}$ | (87 ± 6) $^{a}$ | (100 ± 8) $^{b, *}$ | (33 ± 1) |
| δ-tocopherol | (14 ± 1) $^{a}$ | (1.3 ± 0.2) $^{b}$ | (20 ± 3) $^{c, *}$ | (8.7 ± 0.5) |
| (β + γ)/α-tocopherol | (6.9 ± 0.3) $^{a}$ | (10.4 ± 0.9) $^{b}$ | (10 ± 2) $^{b, *}$ | (7.6 ± 0.3) |
| **Amygdalin content ($\mu g{\cdot}g^{-1}$)** | ND | ND | < LOQ | < LOQ |
| **Polyphenols content ($\mu g{\cdot}g^{-1}$)** | | | | |
| Gallic acid | ND | ND | 271 ± 17 $^{a, *}$ | 10.2 ± 0.3 $^{b}$ |
| 2,3-Dihydroxybenzoic acid | ND | ND | 25.0 ± 0.9 $^{*}$ | < LOD |
| *p*-Coumaric acid | ND | ND | < LOD | < LOQ |
| Resveratrol | ND | ND | < LOD | < LOQ |

Values are expressed as mean ± standard deviation (n = 4). Values in the same row with different letters indicate significant differences (p-value <0.05) between the oils extracted by ultrasound according to the one-way ANOVA and Fisher's LSD test. * Indicates significant differences (p-value <0.05) between NBSAM oils according to Student's $t$-test. NSBM: blackthorn seeds from Navarra before maceration. BSBM: blackthorn seeds from Bulgaria before maceration. NBSAM: blackthorn seeds from Navarra and Bulgaria after maceration. ND indicates compounds not determined. < LOD and < LOQ indicate concentrations below the detection and quantification limits, respectively.

### 3.3.3. Phenolic and amygdalin content

In recent years, phenolic compounds present in oils have attracted considerable attention due to their essential quality and nutritional properties for the human body. They are well known for their capacity to protect oils against oxidation, as well as for their function as modulators of sensory attributes such as colour, astringency, bitterness, and flavor. Moreover, existing evidence in the scientific literature indicates that these naturally occurring constituents of oils confer significant benefits in the prevention of cardiovascular diseases [49]. Notwithstanding, the quality of *Prunus* seed oils may be compromised by the presence of a non-nutritional compound known as amygdalin, which undergoes enzymatic hydrolysis in the gastrointestinal tract, resulting in the formation of glucose, benzaldehyde, and a toxic compound known as hydrogen cyanide [50]. Therefore, in the present study, the polyphenol and amygdalin content of NBSAM oils, obtained by UAE and Soxhlet extraction, was quantified using cLC-DAD-MS. NBSAM oils were selected since these seeds represent the main by-product generated during the production of *Pacharán*, and thus offer substantial potential for valorization within the framework of sustainable waste management and circular economy strategies.

The analytical parameters corresponding to the calibration curves for phenolic compounds and amygdalin are indicated in **Table S4** and **Table S5**. Particularly, due to its high sensibility, amygdalin and 2,3-dihydroxybenzoic acid were determined by Selected Ion Monitoring – Mass Spectrometry (SIM-MS). Total Ion Chromatogram – Mass Spectrometry (TIC-MS) and DAD were used to quantify gallic acid, depending on the required sensitivity of the studied oil. *p*-Coumaric acid and resveratrol were quantified by DAD. For illustrative purposes, SIM-MS chromatograms for a standard solution as well as NBSAM seed oils are represented in **Fig. S3**.

Specifically, as shown in Table 5, gallic and 2,3-dihydroxybenzoic acids were detected in NBSAM oil obtained by UAE. Conversely, the Soxhlet-extracted NBSAM oil exhibited the presence of gallic acid, *p*-coumaric acid and resveratrol. The UAE oil was found to contain the highest gallic acid content (271 ± 17) $\mu g{\cdot}g^{-1}$. A t-Student test revealed a significant difference ($p$-value <0.05) between this oil and the oil obtained by Soxhlet extraction (10.2 ± 17) $\mu g{\cdot}g^{-1}$. The presence of gallic acid in sloe oils has the potential to yield remarkable biological and pharmaceutical activities. These activities include radical scavenging, interference with cell signaling pathways, and the induction of apoptosis in cancer cells [51]. Furthermore, NBSAM oil obtained by UAE exhibited high levels of 2,3-dihydroxybenzoic acid (25.0 ± 0.9) $\mu g{\cdot}g^{-1}$, an important antioxidant which has been linked to the prevention of cardiovascular diseases [52]. Conversely, NBSAM oil obtained by Soxhlet extraction was the only sample in which *p*-coumaric acid and resveratrol were detected, albeit at concentrations below the quantification limit (2 and 33 $\mu g{\cdot}L^{-1}$).

Regarding amygdalin, this compound was detected in both oils, albeit at concentration below the limit of quantification, which was 36 $\mu g{\cdot}L^{-1}$, *i.e.*, 0.12 $\mu g{\cdot}g^{-1}$ of sloe seed. Amygdalin is harmful to the human body when its levels exceed safe thresholds in oral, intramuscular, or intravenous administration. In humans, the lethal dose of HCN is considered 50 mg, equivalent to 0.8 $mg{\cdot}kg^{-1}$ body weight [53], a value six times higher than the method's limit of quantification. The low amount of amygdalin facilitates its use in the nutraceutical and cosmetic

industries.

### 3.4. Determination of the bioactive properties of sloe seed oils

#### 3.4.1. Antioxidant capacity

The evaluation of the antioxidant potential of oils is a significant factor from both a nutritional and a quality standpoint, as it is contingent on the presence of bioactive substances such as polyphenols, tocopherols, and unsaturated fatty acids [54].

The oxidative stability curves of sloe seed oils obtained by UAE are represented in Fig. 2. NBSAM oil was the only substance to demonstrate a consistent trend, thereby indicating its notable stability against lipid oxidation. This observation is consistent with the results obtained for tocopherols, polyphenols and unsaturated fatty acids, where NBSAM oil had the highest content of antioxidant compounds, unsaturated fatty acids and tocopherols.

Moreover, the antioxidant activity of NBSAM oils obtained by UAE and Soxhlet extraction was compared. The $IC_{50}$ value of NBSAM oil obtained by UAE was found to be (6.9 ± 0.3) mg·mL$^{-1}$, which was statistically lower than the oil obtained by Soxhlet extraction ($IC_{50}$ = 24 ± 2 mg·mL$^{-1}$), indicating a significantly higher antioxidant capacity in the oil obtained by UAE. It may be attributed to the elevated levels of tocopherols and unsaturated fatty acids present in the oil obtained by UAE. In addition, the presence of 2,3-dihydroxybenzoic acid, in conjunction with elevated levels of gallic acid, contributes to the oil's capacity to counteract free radicals. It has been demonstrated that free radicals are capable of instigating lipid oxidation processes [4,51,52]. Moreover, the antioxidant capacity of NBSAM oil obtained by UAE was slightly higher than those reported by Athanasiadis et al. [4]. The findings suggest that the optimized UAE method may offer a highly effective means of recovering antioxidant oils, with potential applications as an active ingredient in cosmetic formulations of NBSAM oil.

#### 3.4.2. Antimicrobial activity

The present study examined the potential of sloe seed oils obtained by UAE. This study examined the potential of sloe seed oils obtained through ultrasound-assisted extraction in inhibiting the growth of pathogens prevalent in food and cosmetic environments [55]. These oils were selected to explore their possible application as bacterial growth inhibitors in the nutraceutical industry.

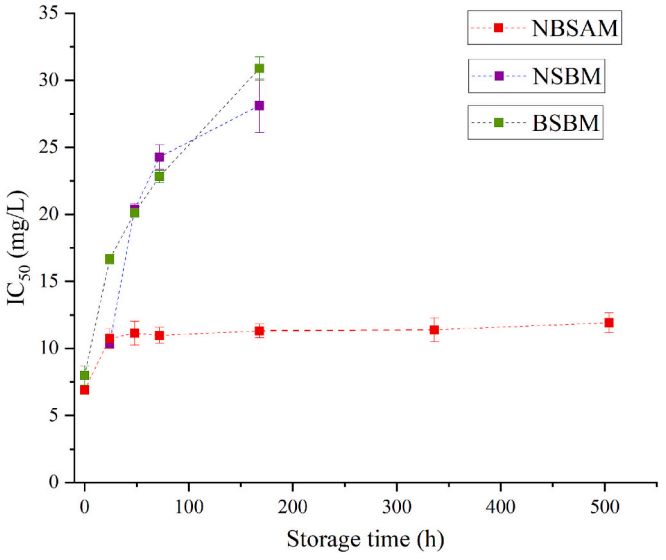



**Fig. 2.** Oxidative stability of sloe seed oils obtained by ultrasound-assisted extraction.
NSBM: Navarra sloe seed before maceration. BSBM: Bulgaria sloe seed before maceration. NBSAM: Navarra and Bulgaria sloe seeds after maceration. ND denotes non-determined compounds.

The Gram-negative bacterium *E. coli* demonstrated the lowest minimum inhibitory concentrations (MICs) in NSBM 12.5 (%, *v/v*) and BSBM (%, *v/v*). However, NBSAM oil was found to require a concentration of 25 (%, *v/v*) to inhibit bacterial growth. Regarding the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *P. aeruginosa*, it was found that only NBSAM oil demonstrated antimicrobial properties, with MIC values of 25 % and 50 % (*v/v*), respectively. In contrast, NSBM and BSBM oils required concentrations exceeding 50 % (*v/v*) to inhibit both bacteria. Consequently, the oils of NSBM and BSBM were found to be the most efficacious in the inhibition of *E. coli* bacterial growth. Whilst NBSAM oil was found to be the most efficacious in its capacity to impede the proliferation of *S. aureus* and *P. aeruginosa*. These oils have the potential to be used in the production of active packaging that can inhibit the growth of the aforementioned bacteria in food [55].

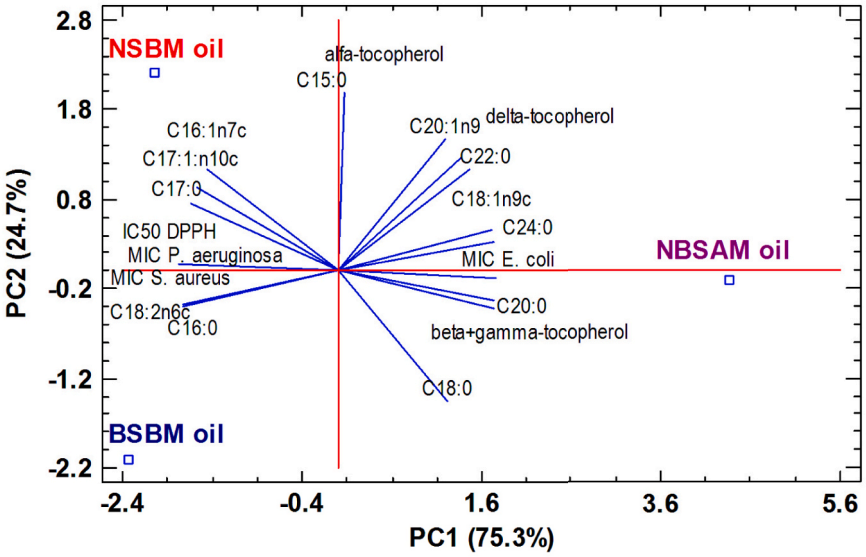
### 3.5. Multifactorial statistical analysis

With the aim of summarizing, visualizing, and correlating the composition of sloe seed oils obtained by UAE with their bioactive properties, a multivariate statistical analysis using PCA was performed. The resulting analysis enabled the reduction of the nineteen experimental variables studied to two principal components (PCs), which together accounted for 100 % of the total variability in the data (Fig. 3a).

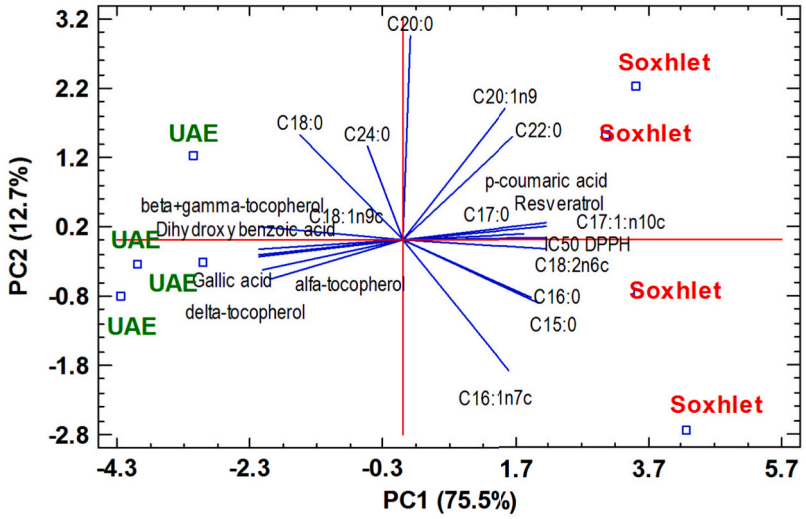
The first principal component (PC1) accounted for 75.3 % of the total variability and it was mainly related to the content of behenic acid (C22:0), β + γ-tocopherol, lignoceric acid (C24:0) and the MIC of *E.coli*. The second principal component (PC2) explained 24.7 % of the total variability and it was defined by the strong correlation with the content of α-tocopherol and pentadecyl acid (C15:0). The PCA graph indicated the presence of three homogeneous groups (Fig. 3a). The first one, formed by NSBM oil was characterized by the highest content of C16:1n7c and C17:1n10c, predominantly, and with the lowest content of β + γ- tocopherol. This specific composition rendered this oil the least effective in terms of antioxidant activity (highest $IC_{50}$ value), while exhibiting the greatest inhibitory effect against the growth of *E. coli* strain (lowest MIC value). Secondly, BSBM oil was characterized by elevated levels of C16:0 and C18:2n6c, which were positively correlated with antimicrobial activity against *E. coli*, and negatively correlated with both antioxidant activity and antimicrobial activity against *S. aureus* and *P. aeruginosa*. Thirdly, NBSAM oil was associated with the highest levels of C24:0, C18:1n9c and tocopherols, which showed a positive correlation with the antioxidant activity (lowest $IC_{50}$ value) and the most pronounced antimicrobial activity against *S. aureus* and *P. aeruginosa* (lowest MIC values).

Thus, differences were observed between the origin of the sloe berries under study and the processing to which they have been subjected. It is worth noting that all sloe seed oils obtained *via* UAE may have potential applications in the cosmetics and nutraceutical industries [56,57]. However, NBSAM oil appears to be of particular interest due to its notable antioxidant and antibacterial properties. Moreover, this oil was derived from waste generated during the production of *Pacharán*, thereby conferring it with considerable added value.

On the other hand, to facilitate a comparison of the effects of the extraction method on the composition and bioactive properties of NBSAM oils, a new PCA was performed (Fig. 3b). The resulting PCA has enabled the reduction of twenty variables to two principal components, which explained 88 % of the total data variability. The PC1 explained 75.5 % of the overall data variability and was mainly associated with content of *p*-coumaric acid, resveratrol, margaric acid (C17:0), cis-10-heptadecenoic acid (C17:1n10c), linoleic acid and DPPH antioxidant activity ($IC_{50}$). The biplot revealed the existence of two different groups with opposite characteristics (Fig. 3b). On the one hand, the NBSAM oil obtained using UAE was characterized by the highest levels of tocopherols, gallic acid, and dihydroxybenzoic acid, which provide the highest




(a)




(b)

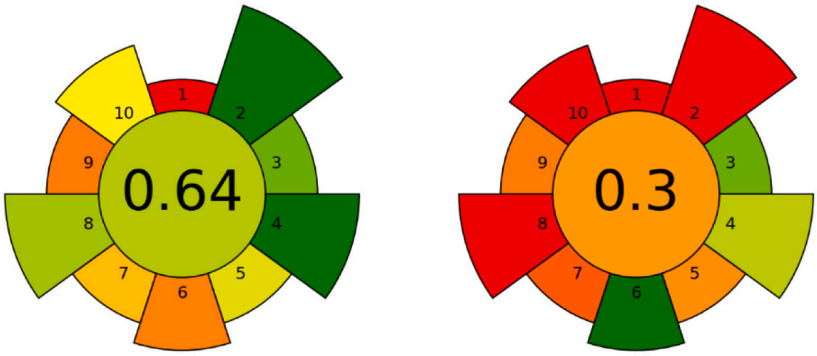
**Fig. 3.** Two-dimensional principal component analysis plot of: (**a**) sloe seed oils obtained by ultrasound-assisted extraction (scores: NSBM, BSBM and NBSAM seed oils, and loadings: fatty acid content, tocopherol composition, antioxidant activity ($IC_{50}$ value at initial time) and antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC values)); (**b**) NBSAM seed oils obtained by ultrasound-assisted extraction and by Soxhlet extraction (scores: Soxhlet and UAE, and loadings: fatty acid content, tocopherol composition, polyphenol content and antioxidant activity ($IC_{50}$ value at initial time)).

antiradical activity. Gallic acid has been reported to be a potent antioxidant compound with the capacity to induce apoptosis in cancer cells [51]. In addition, 2,3-dihydroxybenzoic acid is a well-known antioxidant compound with excellent health properties in the prevention of cardiovascular diseases.

Conversely, NBSAM oil obtained by conventional Soxhlet extraction exhibited a positive correlation with elevated concentrations of saturated fatty acids (C15:0, C16:0, and C22:0), C18:2n6c, resveratrol, and *p*-coumaric acid. A negative correlation was observed between the two variables, with the antioxidant activity demonstrating a decline in conjunction with the increase in the other variable. The negative correlation between C18:2n6c and antioxidant activity has been demonstrated Nederal et al. [58]. In addition, high amounts of SFA are attributed to a higher risk of increased blood pressure and cardiovascular disease [41]. However, the presence of *p*-coumaric acid and resveratrol in this oil, provide antioxidant properties [59,60].

### 3.6. Evaluation of the greenness of the seed oils extraction methods

The greenness of the seed oils extraction methods was performed following the procedure explained in Section 2.8. The results obtained are summarized in **Table S2** and Fig. 4, where it can be seen that the proposed UAE provides an astounding green character (overall green score = 0.64). The main impact categories of this extraction method were the use of safe extraction solvents (*t*-BuOH and EtOH), the smaller sample amount (2.0000 g per analysis), the sustainability of the process, the limit of the generation of problematic waste and operational exposure, the possible semi-automation of the process, as well as the low




(a) (b)

**Fig. 4.** Green evaluation of seed oils extraction methods: (a) optimized ultrasound-assisted extraction and (b) conventional Soxhlet extraction, using AGREEprep metric tool. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

energy demands (37.5 Wh per sample) While Soxhlet extraction presented a non-environmentally friendly approach (overall red score = 0.33). The major downsides of Soxhlet extraction process are the use of large quantities of hazardous solvents (*n*-hexane), the problematic operational exposure and the high levels of problematic residues generated. Hence, according to the AGREEprep score, the proposed sustainable method developed in this work can be considered significantly greener than the conventional Soxhlet extraction method, aligning to a greater extent with the goals of the Green Chemistry and the Circular Economy and thus demonstrating its potential application at the industrial level.

## 4. Conclusions

In this study, a straightforward, rapid, economical and eco-friendly UAE methodology has been developed as an effective alternative technique to the conventional Soxhlet method with n-hexane for the extraction of bioactive oils from *P. spinosa* L. seed liqueur by-products. The method was optimized by experimental design and response surface analysis, consisting of 2.0000 g of sloe seed sample, 45 mL of a mixture 60:40 (*v*/*v*) *t*-BuOH-EtOH as solvent extraction, 40 °C and 10 min of extraction time. The findings indicated that the extraction method employed exerts a substantial influence on the content of bioactive components present in the oil. During the study, it was demonstrated that sloe seed oil after maceration (NBSAM) obtained by UAE was of the greatest industrial interest. The composition of the sample was dominated by a high content of unsaturated fatty acids (92.8 ± 0.3)%, predominantly oleic acid (73.6 ± 0.3)%, which have been identified as contributing to healthy heart lipid indexes. Furthermore, the study identified elevated concentrations of β + γ-tocopherol (100 mg·kg-1), gallic acid, and 2,3-dihydroxybenzoic acid (271 and 25 μg·g-1, respectively). The presence of these bioactive compounds resulted in an exceptional oxidative stability of up to 20 days, and an extraordinary antimicrobial capacity against *S. aureus* and *E. coli* bacteria. In conclusion, this finding enables the extraction of bioactive oils with feasible applications in the nutraceutical and cosmetic industries.

## CRediT authorship contribution statement

**Sandra Rodríguez-Blázquez:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Lorena Fernández-Ávila:** Investigation, Formal analysis. **Esther Gómez-Mejía:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Noelia Rosales-Conrado:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization. **María Eugenia León-González:** Writing – review & editing, Visualization, Validation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.115364.

## Data availability

Data will be made available on request.

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