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ORIGINAL ARTICLE

Supercritical fluid technology for lupin hulls valorization: extraction and fractionation of lupeol

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Abstract
Extraction of lupeol from lupin hulls has been carried out using supercritical CO_2 extraction technology under different operating conditions in order to obtain value-added extracts from the raw material of industrial lupin. The operational parameters used include CO_2 pressure and flow and sequential depressurization fractionation. The highest lupeol recovery (96.8%) has been obtained using 320 bar and 50 g/min of CO_2. For sequential depressurization, the best results were obtained with a CO_2 density close to 728 kg/m^3 providing up to 92% of lupeol in the extract and an enrichment factor of 1.2. Despite this high enrichment, lupeol recovery decreases to 50% after fractionation. Better extraction recoveries would have been expected and thus further studies are necessary to improve the extraction recovery of extracts with a high lupeol composition.

Keywords By-product · Fractionation · Lupeol · Lupin hulls · Revalorization · Supercritical CO_2

1 Introduction

Food agro-industry generates around 12 to 40% of food waste at different steps in the supply chain according to the European Commission [1]. These raw materials have a strong negative impact on both environment and economy, and consequently, in the consumer population. Biomass such as husk, seeds, hulls and pulp remnants are considered by-products of food processing industry [2]. However, several studies have shown that many of these by-products have relevant contents of bioactive molecules with potential health benefits that could be extracted and used as ingredients in functional foods or nutraceutical supplements [3]. Besides, current strategies for food waste management, such as incineration, fabrication of compost or animal feeding, are not based on environmentally friendly concepts and optimum valorization. For this reason, promotion of new strategies directed to reduce the waste material and to create value-added products have become necessary for environmental sustainability and circular economy [4, 5].

Lupinus, commonly known as lupin, is a genus of plants of the legume family *Fabaceae*. Lupin seeds represent a promising nutrient source for livestock and humans [6]. The use of lupin seed flour rich in protein and fiber is spreading rapidly, and it has been used recently in bakery and meat products [7–9]. However, lupin seed hulls, which accounts for about 20% of the total weight of the seed, have been considered as by-products with increasing interest [10]. This material shows low value for animal feeding because it provides low metabolizable energy due to its high content of indigestible fiber. In our opinion, this concept of metabolizable energy should be reviewed for the optimum animal welfare.

Interestingly, some studies have shown the enormous potential of lupin hulls as a novel source of health promoting food ingredients and, in particular, as a source of dietary fiber and phytochemicals [10, 11]. The fat content of lupin hulls is very low, around 1.5 to 2.4% of the total fat content, and for this reason there is a lack of published data related to oil extraction from them. Despite this, several studies have demonstrated significant abundance of lupeol in fat isolated from lupin hulls [12, 13].

Lupeol is a triterpene alcohol associated to several bioactive properties. A large number of in vitro and in vivo studies have shown its extensive range of pharmacological effects

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and benefits for human health, such as anticancer, anti-inflammatory, antioxidant, antimicrobial, and also cardiovascular, neurodegenerative and hepatic protection effects [14, 15]. Different plants and fruits, such as olive, mango, aloe vera, oak and elm leaves, show a moderately low concentration of lupeol (3–880 µg/g) [15]. In any case, the majority of the previous studies, which have focused on the extraction of lupeol from lupin seed hulls, have shown not to be environmentally friendly due to the use of conventional organic solvents, such as the chloroform:methanol mixture or hexane [15]. In some cases, sonication and microwaves have been employed to obtain lupeol from raw materials [16].

Due to the low concentration in which bioactive compounds are found in plant tissues, an effective and selective extraction technology becomes necessary to arise an efficient isolation. In this line, special attention has been paid to the development of processes based on the application of green technologies and the use of environmentally-friendly solvents, to rule out the use of conventional and well-known toxic solvents, such as hexane [17, 18]. In recent years, supercritical fluid extraction (SFE) and, in particular, the use of carbon dioxide (CO_2) have become an interesting extraction technique, not only because CO_2 is a substance generally recognized as safe (GRAS), but also because it is used in a large number of industrial applications [19]. This technology shows interesting advantages, such as high selectivity and reproducibility, and thereby, faster mass transfer and limited extraction time, and it involves low and mild extraction temperatures [18]. In addition, supercritical CO_2 can change to the gaseous state and thus it can be eliminated from the solid extract, providing solvent-free extracts. CO_2 can still be recycled through recirculation in the supercritical system in order to avoid environmental issues as well as to decrease energy and other operational costs [20]. The selection of operational parameters, mainly temperature and pressure, has a critical impact on the final composition of the extracts, which translates into the selectivity of the extraction process [21]. The use of supercritical CO_2 is manly focused in the extraction of non-polar compounds due to its non-polar nature, and thus lupeol would be a target molecule for this type of solvent [22].

The main aim of the present work is the extraction of lupeol from lupin hulls by-products by using green technologies, in particular supercritical CO_2, to obtain value-added ingredients. In the present study, different operational parameters such as CO_2 pressure and flow have been explored. A fractionation process by sequential depressurization has been carried out for the obtention of lupeol enriched extract. The results obtained by using supercritical CO_2 extraction have been compared with those obtained by using solid–liquid conventional extraction with hexane and concepts such as composition and recovery for the different extraction processes have been discussed in depth. Supercritical CO_2 shows to be a promising and clean extraction method to obtain lupeol with high purity and good recoveries, even better than those obtained when using hexane. Moreover, this technology has the potential to be scaled-up for food industry applications.

2 Materials and methods

2.1 Materials

White lupin hulls and white lupin seeds (*Lupinus albus*) were supplied by Inveja SAS-Lup'ingredients (Haute-Goulaine, France). The material was stored under vacuum and refrigeration conditions (4 °C). For the SFE, CO_2 with a purity of 99.98% was obtained from Carburos Metálicos (Madrid, Spain). Standards of stigmasterol ≥ 95%, lupeol ≥ 98% and 1,3-diolein ≥ 99% were purchased from Merck (Darmstadt, Germany). The lupin seed oil, used as a standard for triacylglycerols, was obtained from white lupin seeds by pressing with a domestic oil expeller (Wartmann®, brand model WM-OP-1402A, Tilburg, The Netherlands). Hexane (HEX), methyl tert-butyl ether (MTBE), methanol (MeOH) and chloroform (CLF), were supplied by Macron (Avantor Performance Material, Center Valley, USA).

2.2 Methods

2.2.1 Conditioning

The grinding of the lupin hulls was carried out using an impact miller IKA-M20 (Werke Staufen, Germany). Lupine seed hulls were subjected to grinding at 20.000 rpm for 90 s, followed by 60 s at rest, and another 90 s at 20,000 rpm. The resulting flour was vacuum packaged into a bag and stored at 4 °C.

2.2.2 Granulometry

To determine the particle size distribution of the lupin hulls flour after milling, 50 g of flour were introduced into a TZA 1491 electric sieve shaker (Bunsen SA., Madrid, Spain) with four sieves with different pore sizes (> 1 mm, 1–0.5 mm, 0.5–0.25 mm, 0.25–0.125 mm) and a container, in which particles less than 0.125 mm were collected. The samples were placed for 6 min in the sieve shaker with ultrasonic vibration, with an amplitude of 1.8 mm and cycles of 9 s on and 1 s off. Finally, each fraction was collected and weighed. Granulometry is shown in Table 1.

Table 1 Granulometry (%) of lupin hulls flour

Diameter (mm)	%
> 1	8.3 ± 0.7
1–0.5	23.4 ± 0.6
0.5–0.25	39.5 ± 1.0
0.25–0.125	23.6 ± 1.1
< 0.125	5.6 ± 0.5

2.2.3 Moisture

The moisture content of lupin hulls flour was determined by gravimetry using a precision analytical balance (± 0.1 mg) after drying 10 g of samples in an oven at 105 °C until constant weight. These determinations were carried out in triplicate.

2.2.4 Determination of total fat content at analytical scale

The total fat content of lupin hulls flour was determined at analytical scale using the Folch method [23], with some modifications. In brief, 200 mL of chloroform:methanol (2:1 v/v) were added to 10 g of hulls and mixed with an Ultraturrax T18 basic IKA (Staufen, Germany) at speed between 11,800 and 16,200 rpm for 2 min, until an homogeneous mixture was obtained, that was maintained by magnetic stirring at 800 rpm during 60 min. The sample was then vacuum filtered and distilled water was added to the organic phase in a 1:5 ratio. The mixture was mixed in a vortex for 1 min and centrifuged for 5 min at 10,000 rpm. Then, the lower phase was collected and evaporated in a Büchi B-480 rotary evaporator (Uster, Switzerland) at 40 °C, under 10 mbar until constant weight. This procedure was carried out in triplicate.

2.2.5 Solid–liquid hexane extraction

Solid–liquid extraction was carried out using the method described by Piccirilli et al. [13]. Hence, 150 g of flour from lupin hulls with particle size between 250 and 500 µm were extracted 4 times consecutively with 250 mL of hexane by magnetic stirring at 900 rpm. The extraction conditions were 45 °C and 15 min each time. The solid and liquid phases were separated by vacuum filtration in a kitasato flask during 15 min and the hexane was evaporated up to an approximate a volume of 10 mL in a Büchi B-480 rotary evaporator (Uster, Switzerland) at 40 °C and 10 mbar. The volume was transferred to a 50 mL centrifuge tube, and it was left during 15 h at 25 °C for crystallization and, consequently, precipitation of lupeol. For the separation of the rest of hexane from the crystallized lupeol, a centrifuge process at 10.000 rpm during 30 min was carried out. After this, the hexane phase was recovered in a evaporate flask and the solid phase of lupeol (product 1) was washed with another 10 mL of hexane, remained at 25 °C for 15 h, and then centrifugated at 10.000 rpm during 30 min. The hexane phase of the washings (product 2) was recovered and evaporated at 40 °C, under 10 mbar until constant weight. The solid phase, rich in lupeol, was dried under nitrogen until constant weight. This procedure was carried out in triplicate.

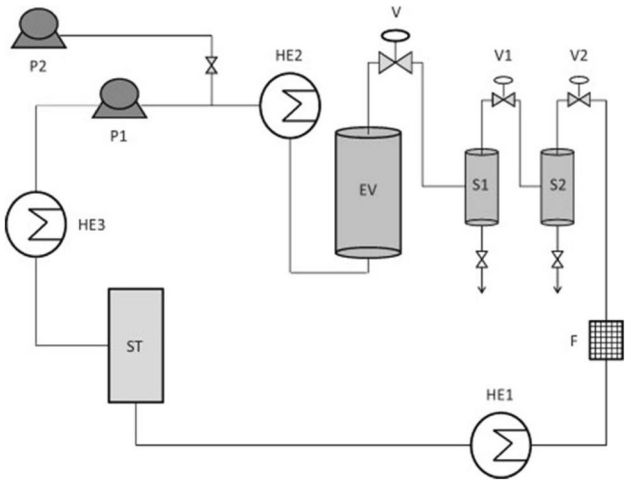

Fig. 1 Scheme of supercritical CO_2 extraction of lupin seed hulls flour. P1: CO_2 pump; P2: cosolvent pump; HE1, HE2, HE3: heat exchangers; EV: extraction vessel; S1, S2: separator cells; V, V1, V2: back pressure regulator valves; ST: CO_2 storage tank; F: filter

In the extraction processes, two main responses were evaluated:

a. Composition (wt%), defined as (weight of x-compound in a fraction/weight of entire fraction) × 100
b. Recovery (%), defined as (weight of x-compound in a fraction/weight of x-compound in Biomass) × 100

2.2.6 Supercritical CO2 extraction and fractionation though sequential depressurization

A basic extraction scheme for supercritical fluid extraction (SFE) with CO_2 is shown in Fig. 1. In brief, 150 g of flour from lupin hulls with particle size between 250 and 500 µm were used to perform a supercritical CO_2 extraction in a homemade pilot-plant device comprising a 350 cm^3 cylindrical extraction vessel (EV), and two different separators (S1 and S2), 270 cm^3 capacity each, with independent control of temperature and pressure. In general, the ratio between height and diameter of the cylindrical EV is recommended to be 5–7. The equipment design implies a batch procedure to incorporate the lupin seed hulls flour in the EV. CO_2 is first heated (HE2) up to the desired temperature and then pumped (P1) from the bottom of the EV, up to the desired extraction pressure. For adequate pumping and to prevent cavitation of

the pump, precooling of the solvent is required (HE3). In this study, the use of a co-solvent was not considered, due the relative low polarity of lupeol and the advantages associated to the use of pure CO_2.

At the exit of the extractor, the supercritical solvent with the extracted solute flows through a depressurization valve (V) to a separator 1 (S1), in which due to the lower pressure the extracts are separated from the solvent and collected. The remaining extract still soluble in CO_2 can be collected in a separator (S2), that is around 20 bar. This way permits the fractionation of the extract in two fractions (on-line) by setting suitable temperatures and pressures in the fractionation units. In this study, the SFE conditions were 180, 250, 320 bar of pressure at 40 °C during 90 min, at 50 or 100 g/min CO_2 flows. For the sequential fractionation of the extracts, the extraction was carried out at 320 bar and 50 g/min of CO_2 flow for the column, and three different CO_2 densities, 137, 642, 728, and 808 kg/m^3, were set in S1. S2 was maintained under constant conditions of 20 bar and 20 °C.

In all the CO_2 extraction and fractionation processes the composition (wt%) and recovery (%) were evaluated as explained in Sect. 2.2.5. In addition, the enrichment factor defined as the ratio between the lupeol composition in S1 and the lupeol composition in the original extract obtained at selected conditions, were calculated.

2.2.7 GC–MS method

All samples and extracts obtained were analyzed by gas chromatography (GC) coupled to mass spectrometry (MS). The GC–MS system consisted of an Agilent GC Series 6890N (Santa Clara, CA, USA) with on column injection and flame ionization detector (FID), and an Agilent single quadrupole MS detector model 5975C (Santa Clara, CA, USA), with electron energy set at 70 eV and the mass range at 50–700 *m/z*. The chromatographic separation was based on the method developed by Torres et al. [24]. A HP-5MS capillary column (7 m × 0.25 mm internal diameter × 0.25 µm) was used. The injection volume was 0.2 µL. Temperatures of injector and detector were 50 and 340 °C, respectively. The program of temperatures started at 60 °C, increasing at 42 °C min^{-1} until 250 °C. This temperature was maintained for 20 min and then increased up to 340 °C at 25 °C min^{-1}, which was maintained for 20 min. The flow rate of carrier gas (helium) was maintained at 1 mL/min. The GC–MS control and data processing was performed using Chem-Station (Agilent Technologies) software. For the compound identification, manual spectral matching was ascertained by using the mass spectral library of National Institute Standard and Technology (NIST) version 2.0.

Quantitative analysis of lipid compounds (sterols, lupeol, diacylglycerols and triacyclglycerols) using the external standard method with pure compounds. For that matter, calibration curves with each standard from 0.2 to 6 mg mL^{-1} were prepared and analyzed.

2.2.8 Statistical analysis

All experiments were carried out in triplicate and the data were expressed as mean ± standard deviation. The statistical significance of the differences between the groups was measured by one-way analysis of variance (ANOVA) and post-hoc Tukey HSD test and parametric Student´s t-test for independent samples. Statistical significance was defined at the level of $p < 0.05$. All statistical evaluations were performed using Origin (version 9.0 for Windows; OriginLab Corporation, Northampton Northampton, USA).

3 Results and discussions

3.1 Total fat content of lupin hulls

The lupin hulls had 0.76 ± 0.05% of total fat content as determined by the Folch method [23]. These values are, in general, lower than those found by Zhong et al. [7], where the total fat content ranged between 1.6 and 2.4 wt%. This difference in the total fat content can be attributed to the variety of the seeds, which in the present case were sweet lupin hulls, and to the cultivation characteristics.

3.2 Solid–liquid hexane extraction of lupeol

A solid–liquid extraction with hexane was carried out to obtain lupeol from 150 g of lupin hulls with a moisture of 6.9 ± 0.4 wt% and particle size between 250 and 500 µm, following the method of Piccirilli et al. [13]. As shown in Table 2, the products obtained were divided into product 1

Table 2 Recovery (mg/%) and composition (%) of lupeol, sterols and triacylglycerols from lupin hulls flour extracted by the solid–liquid hexane extraction method

	Biomass	Product 1 (P1)	Product 2 (P2)
Composition (wt%)			
Lupeol	72.3 ± 0.5	89.0 ± 2.1	56.8 ± 0.6
Sterols	5.0 ± 0.1	3.3 ± 0.7	9.7 ± 0.5
Triacylglycerols	22.7 ± 0.5	7.9 ± 1.6	33.5 ± 0.1
Recovery (mg* / %)			
Lupeol	819 ± 59	417 ± 35 / 51.0 ± 4.3	248 ± 40 / 30.3 ± 4.9
Sterols	56 ± 4.3	15 ± 2.4 / 27.1 ± 4.2	43 ± 9.3 / 75.7 ± 16.6
Triacylglycerols	226 ± 8.8	36 ± 5.4 / 16.1 ± 2.4	147 ± 26 / 64.9 ± 11.3

*The extracted g are from 150 g of lupin hulls flour

(P1) and product 2 (P2). The recovery of lupeol was higher in P1 (51.0%) than in P2 (30.3%). Sterols and triacylglycerols recoveries of P2 were higher than for P1. Comparing this data with those obtained through the official Folch method (considered as 100 wt%), it was observed that in terms of recovery, 81.3% of lupeol, 102.9% of sterols and 81.0% of triacylglycerols were obtained with the solid–liquid hexane extraction (P1 + P2). In terms of composition, P1 was the richest in lupeol (89.0 wt%) and P2 was richer in triacylglycerols (33.5 wt%) as compared to P1 (7.9 wt%). P2 had also higher composition in sterols (9.7 wt%) than P1 (3.3 wt%). These results are very similar to those found by Piccirilli et al. [13].

3.3 Supercritical CO_2 extraction of lupeol

A study on the combination of pressure and temperature during supercritical CO_2 extraction was conducted to obtain lupeol extracts with the highest purity and yield. Table 3 shows composition (wt%) and recovery (g/%) of the extracts obtained after supercritical CO_2 extraction from lupin hulls flour with a moisture of 6.9 ± 0.44 wt% and particle size between 250 and 500 µm, using 180, 250 and 320 bar of pressure with a combination of 50 and 100 g/min of CO_2 flow during 90 min.

Regarding composition, no significant differences ($p > 0.05$) were found in lupeol, sterols and triacylglycerol at the different conditions of CO_2 pressure and flow. The composition in lupeol was found to be between 72.3 wt% and 75 wt%, the composition in sterols was from 4.9 wt% to 5.2 wt%, and the composition in triacylglycerol was from 20.4 wt% to 22.1 wt%. Identification of lupeol in the extracts was confirmed by GC–MS analysis, as shown in Fig. 2.

As shown in Table 3, significant differences ($p < 0.05$) were observed in lupeol recovery when comparing different pressures at the CO_2 flows investigated. Thus, at 50 g/min, lupeol recovery was significantly enhanced as the pressure increased, leading values of 73.7%, 82.9% and 96.9%, at 180, 250 and 320 bar, respectively. Same pattern was observed at 100 g/min CO_2 flow between 180 and 250 bar, obtaining lupeol recoveries of 85.8% and 91.8%, respectively. However, at 100 g/min CO_2 flow no statistical differences ($p > 0.05$) were observed between 250 (91.8%) and 320 bar (87.2%).

In general, recovery of lupeol increases with increasing CO_2 flow, from 50 to 100 g/min at 180 and 250 bar, with significant increase of 17% and 10.7%, respectively. However, in the case of 320 bar no statistical differences ($p > 0.05$) were observed between both CO_2 flows. It is possible that in this particular case, CO_2 could take preferential pathways which would not happen at other pressure and flow combinations. The dead space and the axial dispersion inside the EV could create a non-ideal pattern due to the presence of preferential pathways, and this could influence negatively in the fluid phase along the extractor, resulting in a decrease in the motion forces which given the mass transfer rate causes a drop in the overall extraction efficiency [25]. The extraction efficiency is also related with the enhancement of the surface area of contact with the solvent. Although the particle size of the flour is the same for all the extraction conditions, the influence of size should be considered to avoid preferential paths [26].

Regarding the recovery of sterols, even if no significant differences ($p > 0.05$) were found for the different combinations studied, the combination 320 bar and 100 g/min represent the best for sterols recovery (94.6%). In the case of triacylglycerols, no significant differences were observed between the different treatments.

When the results obtained with supercritical CO_2 extraction are compared to those obtained with the Folch method,

Table 3 Recovery (g/%) and composition (wt%) of lupeol, sterols and triacylglycerols from lupin hulls at different conditions of pressure (180, 250 and 320 bar) and CO_2 flow (50 and 100 g/min) during the supercritical CO_2 extraction procedure. Significant differences ($p < 0.05$) in the same row are indicated with different letters

Pressure (Bar)	180		250		320		Biomass
CO_2 Flow (g/min)	50	100	50	100	50	100	
Composition (wt%)							
Lupeol	72.7 ± 1.3^a	72.3 ± 0.7^a	73.4 ± 3.2^a	73.9 ± 1.0^a	75.0 ± 1.6^a	74.9 ± 0.8^a	72.3 ± 0.5^a
Sterols	5.2 ± 0.1^a	5.1 ± 0.5^a	5.1 ± 0.5^a	5.0 ± 0.3^a	5.0 ± 0.2^a	4.9 ± 0.5^a	5.0 ± 0.1^a
Triacylglycerols	22.1 ± 1.4^a	22.6 ± 0.3^a	21.4 ± 2.8^a	21.1 ± 0.7^a	20.0 ± 1.4^a	20.4 ± 1.1^a	22.7 ± 0.5^a
Recovery (g*/ %)							
Lupeol	608 ± 18/73.7 ± 2.2^e	703 ± 21/85.8 ± 2.5^d	679 ± 42/82.9 ± 5.1^c	752 ± 4.9/91.8 ± 0.6^{a,b}	794 ± 7.4/96.9 ± 0.9^a	714 ± 46/87.2 ± 5.6^{a,b}	819 ± 59^f
Sterols	43 ± 1.2/76.2 ± 2.2^a	50 ± 7.2/88.7 ± 12.9^a	47 ± 8.3/83.7 ± 14.9^a	52 ± 3.6/90.5 ± 6.4^a	53 ± 3.9/94.9 ± 6.9^a	47 ± 4.3/84.1 ± 7.7^a	56 ± 4.3^a
Triacylglycerols	183 ± 9.9/81.1 ± 4.4^a	218 ± 14/96.5 ± 6.1^a	196 ± 40/86.7 ± 17.4^a	215 ± 7.9/95.0 ± 3.5^a	211 ± 21/93.5 ± 9.4^a	197 ± 12/87.1 ± 5.4^a	226 ± 8.8^a

*The extracted g are from 150 g of lupin hulls flour

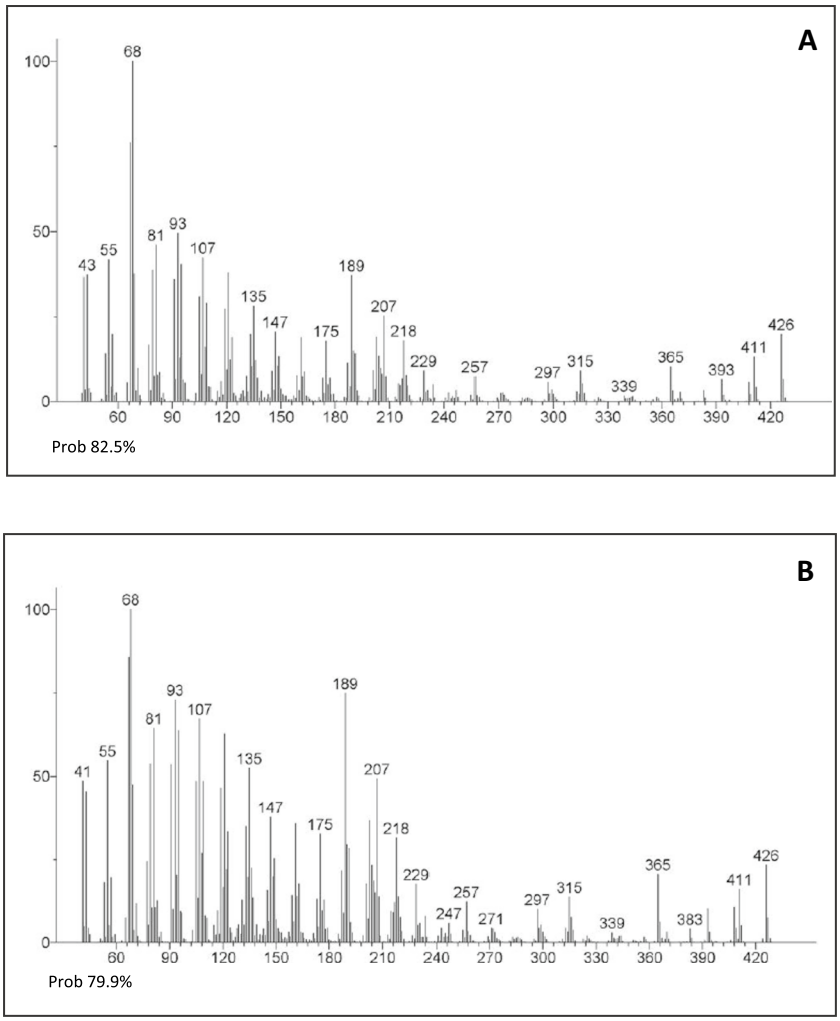

Fig. 2 Mass spectra of lupeol obtained by GC–MS. (**A**) Lupeol standard at 5 mg/mL. (**B**) Extract obtained with supercritical CO_2 at 250 bar and 100 g/min. The probability for identification according to the GC–MS library is indicated

it can be observed that the conditions 320 bar and 50 g/min of CO_2 permit to obtain the largest amount of lupeol (96.9%). Again, no significant differences were found for sterols and triacylglycerols recoveries comparing supercritical CO_2 extraction and the Folch method.

To understand the efficiency of the extraction procedure, it is important to consider the solvent-to-feed ratio, *i.e.*, the proportion of CO_2 used in relation to the amount of material being processed to obtain the target compound [20]. In the case of 50 g/min CO_2 flow, the ratio is 30:1, and in the case of 100 g/min, a ratio of 60:1 is obtained. It must be noticed that the increase in the recovery of lupeol is not substantially larger (10–11%) when the largest CO_2 flow is employed. Thus, we can conclude that the most efficient flow rate to carry out the recovery of lupeol from lupin hulls using supercritical CO_2 would be 50 g/min, since the solvent-to-feed ratio is lower, and the extraction recovery is very similar to that achieved with 100 g/min CO_2 flow.

From the comparison of the recovery and composition of the extracts obtained by supercritical CO_2 extraction and by solid–liquid extraction with hexane, it can be observed that the results are much more favorable when employing supercritical CO_2, since extracts with a similar composition are obtained in less time in comparison with the solid–liquid hexane extraction (90 min *vs.* 33 h, respectively). Besides, the recovery with the use of supercritical CO_2 extraction represents an increase of lupeol extraction yield of 19% compared to that with the hexane solid–liquid extraction.

Table 4 summarizes previous studies on supercritical CO_2 extraction of lupeol from different plant matrices with similar conditions to those used in the present study. Felföldi-Gáva et al. studied extracts from *Alnus Glutinosa* bark, where lupeol represents 14.3 g/100 g of the extract. These results were obtained at 450 bar, 60 °C, 116.7 g/min CO_2 flow and 60 min [27]. It should be noted that, even with a higher pressure, these authors attained 5.4 times lower lupeol content than that of the present study. De Souza et al. obtained 35.3 g/100 g of lupeol acetate in the extracts from *Archium Lappa* leaves through supercritical CO_2 at 200 bar, 62 °C, 50 min, 1.4 g CO_2/min and ethanol (EtOH) (2:1; EtOH w:w of solids). In this case, the use of EtOH as modifier can enhance the extraction yield, also leading to

Table 4 Literature search of supercritical CO_2 extraction to obtain lupeol from different raw materials

Biomass	Conditions	Total extract (%)	Lupeol content (g/100g of extract)	Reference
Arctium lappa leaves	200 bar, 62 °C, 50 min, 1.44 g CO_2/min, EtOH (2:1; EtOH w: w of solids)	4.8	35.3 lupeol acetate	[28]
Alnus glutinosa bark	450 bar, 60 °C, 60 min, 116.7 g CO_2/min	2.3	14.3	[27]
Grape seeds	370 bar, 65 °C, 6 h, 60 g CO_2/min	5.9–13.6	0.01–0.013	[32]
Maclura pomifera plant	350 bar, 60 °C, 6 h, 333.33 g CO_2/min	7.9	Qualitative data	[33]
Lupinus Luteus seeds	200 bar, 40 °C, 40 min, 3 g CO_2/min, EtOH 96% (1:4; EtOH:CO_2; v:v)	-	Qualitative data	[18]
Mangaba (Hancornia speciosa) pulp	300 bar, 60 °C, 210 min, 8.33 g CO_2/min, 5–10% EtOH (w:w; EtOH:CO_2)	11.1	0.13	[34]
Acacia dealbata flowers	300 bar, 45 °C, 180 min, 25 g CO_2/min, 10% EtOH (w:w; EtOH:CO_2)	1.8	18	[29]
Calendula officinalis flowers	140 bar, 40 °C, 180 min, 70 g CO_2/min	7.5	0.09	[35]
Matricaria recutita chamomile florets	250 bar, 55 °C, 2 h, 25 g CO_2/min, fractionation at 55 °C and 9 bar	18.2	23.3	[30]
Apple by-product	300 bar, 46 °C, 100 min, 10 g CO_2/min	3.3	Qualitative data of extract of triterpenes (lupeol, uvaol, erythrodiol)	[36]
V. vinifera leaves	300 bar, 80 °C, 12 g CO_2/min, 10% Ethyl acetate (w:w, Ethyl acetate:CO_2)	16.1	0.19 extract of β-amirin and lupeol	[37]

co-extraction of more polar compounds [28]. Accordingly, Casas et al. investigated lupeol extraction from *Acacia Dealbata* flowers using CO_2 at 300 bar, 45 °C, 180 min, 25 g CO_2/min and 10% EtOH (w:w; EtOH:CO_2), which provided 18 g/100 g lupeol content in the extracts [29]. In the study of Nakuerte et al., conditions of 250 bar, 55 °C, 2 h, 25 g CO_2/min were used to obtain up to 23.2 g/100 g of lupeol in supercritical extracts from *Matricaria recutita chamomile* florets [30]. In general, lupeol composition achieved of the aforementioned investigations are remarkably lower than those attained in the present work, where lupeol concentration from lupin hulls was around 75 g/100 g in the extract. This could be explained by the differences in parameters of the supercritical extraction processes (e. g. density of the CO_2) as well as by the diverse raw materials used.

One of the most relevant advantages of the present work is that the use of solvents as modifiers was not necessary. Thus, an efficient extraction of lupeol from lupin hulls flour, in terms of composition and recovery, was attained by using pure supercritical CO_2. These results would inform us about the chemical form of the lupeol to be extracted from a given matrix. Several plants produce abundant glycosylated triterpenoids (with a sugar part in their structure), which are called saponins. When this occurs, ethanol is needed as a modifier during the supercritical CO_2 extraction because it increases the molecular weight and polarity, aiding to the extraction of saponins [31]. However, as in the present study the use of modifiers was not necessary, this would indicate that lupeol from lupin hulls is present in its aglycone form.

Thus, the use of supercritical CO_2 to obtain lupeol from lupin hulls not only entails advantages in terms of composition and recovery, but also provides extracts free of solvent that have not been subjected to high temperatures. The sustainable revaluation of lupin hulls using supercritical CO_2 is evident, since not only it is possible to isolate an ingredient with high bioactive potential, but also the starting material remains intact and useful for further use for conventional purposes.

Besides, the composition of the extracts obtained by supercritical CO_2 and by the Folch method are very similar to each other. This could indicate that pure supercritical CO_2 can be as efficient as the chloroform: methanol (2:1; v:v) mixture in order to extract lupeol from this raw material, even though being solvents with different polarity. The different extraction conditions employed with supercritical CO_2 led to different amounts of total lipid content in the extracts, but without any selectivity for different lipid compounds. For a subsequent enrichment in lupeol of the extracts, strategies such as fractionation of the extract in different fractionation units in a supercritical plant could be employed.

3.4 Fractionation of lupeol extract by sequential depressurization

Once the optimal conditions for lupeol extraction from lupin hulls were achieved, the extract was fractionated in two separators S1 and S2. The aim of this fractionation was to separate lupeol and triacyclglycerols to increase the purity of the lupeol extracts. For this purpose, different supercritical CO_2 densities were used in S1 by changing the pressure and temperature. S2 was always around 20 bar to quantitatively recover the extract. At this pressure (20 bar), CO_2 has a negligible solubilization capacity and all substances dissolved in CO_2 precipitate and can be recovered in this separator.

Table 5 shows the results of the different fractionations performed. The values indicate the separator (S1 or S2) where lupeol was mostly recovered. Recovery was calculated as the amount of the identified compound out of the total of each compound extracted (S1 + S2).

Regarding the different densities used, it was observed that when the CO_2 density in S1 is close to 800 kg/m^3, the extract is soluble and almost completely recovered in S2. On the contrary, when CO_2 density is around 137 kg/m^3, the extract precipitates in S1 and only negligible amounts of extracts are recovered in S2. As can be seen, as the density of CO_2 decreases, its solubilization capacity decreases, as all the extract precipitates in S1. However, in terms of extract composition, there is no significant difference ($p > 0.05$) in lupeol composition between these densities (~ 85 wt%).

Intermediate densities (642 and 728 kg/m^3) were tested to fractionate the extracts into two different portions. Thus, at densities of about 728 kg/m^3 in S1, the total extract was fractionated into two different products. The product on S1 contained 92.5 wt% of lupeol. However, the lupeol recovery represents almost 50% of the total lupeol in the extract. This result indicates that to obtain an enrichment factor of about 1.2, 50% of total lupeol cannot be recovered in S1 and will precipitate in S2 mixed with triglycerides. Furthermore, for a density of about 642 kg/m^3 the lupeol composition was 87 wt% and 91.5 wt% of the total lupeol in the extract was recovered in this separator.

From the results shown above, a compromise must be found between purity or composition and recovery. To increase the purity of lupeol above 90 wt%, about 50% of the total lupeol remains soluble at this CO_2 density (728 kg/m^3), and precipitates in S2. It is therefore concluded that lupeol is less soluble in supercritical CO_2 than the triacylglycerols. This is explained by the fact that the purity of lupeol increases in S1. As the density is slightly reduced, the CO_2 solubilization capability decreases and vice versa.

Figure 3 shows a comparison of chromatograms of extracts obtained after solid–liquid extraction with hexane and the different supercritical fractionations mentioned above.

Fractionation of the extract represents a strategy that slightly increases the purity of lupeol, but with the drawback that a significant fraction of the total lupeol cannot be recovered in S1. It is an interesting strategy to assess the relative solubility of the two main ingredients of the extract (lupeol and triacylglycerols) and to partially eliminate triacylglycerols from the extracts, which could interfere in subsequent analyses of lupeol biological activity tests.

4 Conclusions

For the first time, lupeol has been extracted from lupin seed hulls by means of supercritical CO_2 fluid with the consequent revalorization of a waste product. These results were compared with those obtained with conventional solid–liquid extraction with hexane showing that the use of supercritical CO_2 is more advantageous. The highest lupeol recovery by supercritical CO_2 extraction

Table 5 Composition (%) and recovery (%) of lupeol and triacylglycerols by sequential depressurization as indicated. The different fractionation conditions of pressure (bar), temperature and CO_2 (°C) and density (kg/m^3) in S1 are indicated. S2 was maintained under constant conditions of 20 bar and 20 °C (density in S2: 40.8 kg/m^3). Significant differences ($p < 0.05$) in the same row are indicated with different letters

Conditions S1	Fractionation 1 S1	Fractionation 2 S1	Fractionation 3 S1	Fractionation 4 S2
Pressure S1 (bar)	53.3 ± 4.9	100.0 ± 0.0	70.0 ± 7.1	125.0 ± 35.4
Temperature (°C)	28.5 ± 4.9	38.5 ± 3.5	25.5 ± 0.7	30.7 ± 5.3
Density (kg/m^3)	137.5 ± 17.7	642.5 ± 60.1	728.5 ± 12.0	808.2 ± 17.0
Composition (wt%)*				
Lupeol	82.9 ± 2.9^a	87.0 ± 0.5^b	92.5 ± 1.6^c	84.1 ± 1.8^a
Sterols	5.0 ± 0.2^a	3.1 ± 0.7^b	1.07 ± 1.0^c	5.7 ± 0.4^d
Triacylglycerols	8.7 ± 2.7^a	6.8 ± 0.6^a	1.8 ± 1.2^b	8.3 ± 0.9^a
Recovery (%)				
Lupeol	99.4 ± 0.5^a	91.5 ± 4.1^b	47.0 ± 2.9^c	97.2 ± 0.3^d
Sterols	98.8 ± 0.1^a	90.0 ± 2.2^b	86.3 ± 1.9^c	98.9 ± 1.0^a
Triacylglycerols	96.3 ± 1.7^a	82.6 ± 20.9^a	10.8 ± 9.3^b	98.6 ± 1.3^a

*100% of composition is completed by other triterpenic alcohols

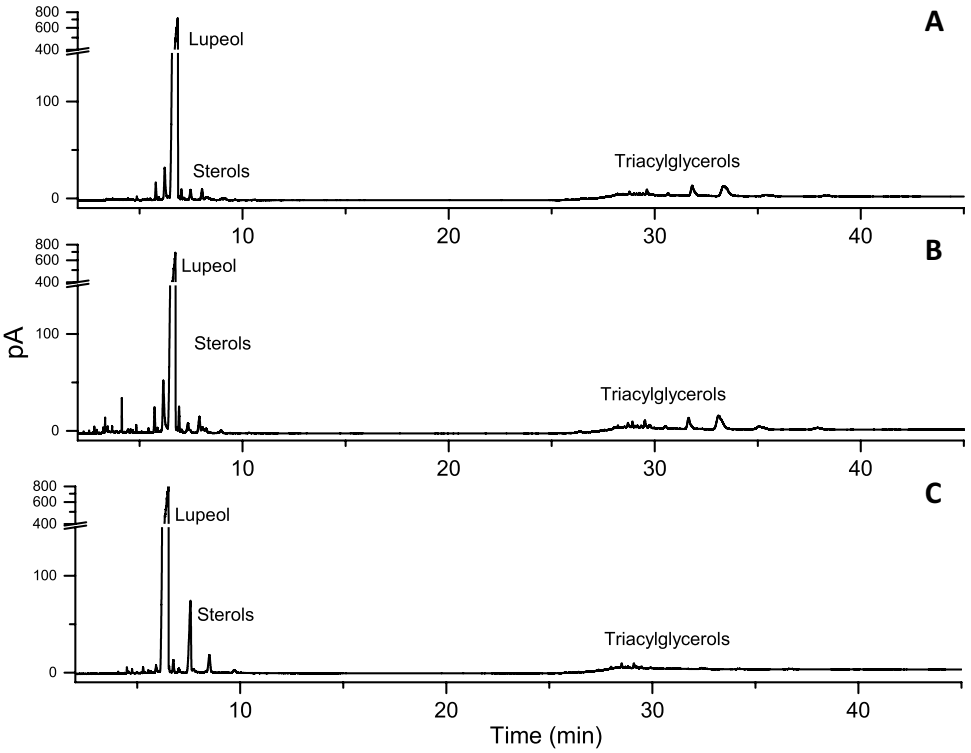

Fig. 3 Chromatograms obtained after extractions showing the identification of lupeol, sterols and triacylglycerols. **A**) Solid–liquid extraction with hexane. **B**) Fractionation 4, extract in S2. **C**) Fractionation 3, extract in S1

was 96.8%, which was achieved using 320 bar and 50 g/min of CO_2 flow rate. In terms of lupeol enrichment, sequential depressurization was also efficient in separating lupeol and triacylglycerols, thereby increasing the purity of lupeol in the extracts. A compromise between composition and recovery had to be found and it could be concluded that lupeol is less soluble in supercritical CO_2 than the triacylglycerols. Furthermore, fractionation of the extract increases lupeol composition somewhat to 92.5 wt% when the density is about 728 kg/m^3. However, a significant fraction of the total lupeol cannot be recovered. In any case, fractionation represents an interesting strategy for the evaluation of the relative solubility of the main extract ingredients (lupeol and triacylglycerols in this case) and thus partially removes triacylglycerols from the extracts. The next step would be to test the biological activity of supercritical lupeol extracts obtained from a by-product such as lupin hulls.

Author contributions LV, CB and AC: Formal analysis; Investigation; Methodology; Resources; Software; Writing-original draft; Writing—review & editing. CB: Writing-original draft. CT and GR: Funding acquisition; Project administration; Resources. CT: Investigation; Methodology; Supervision; Validation.

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Data availability Not applicable.

Declarations

Ethical approval Not applicable.

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