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Valorization of an agroindustrial soybean residue by supercritical fluid extraction of phytochemical compounds

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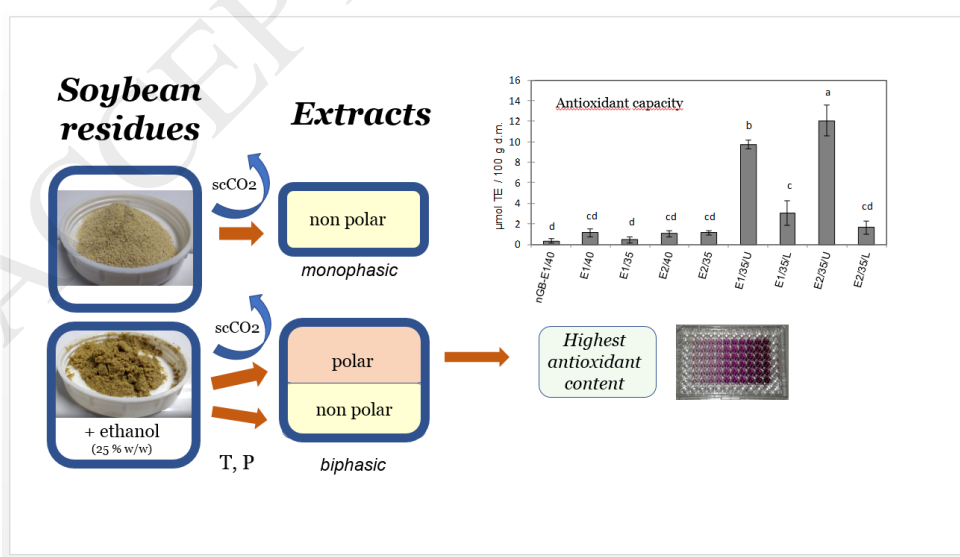
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Graphical Abstract



Highlights

- Soybean residues from pressing oil extraction are a source of phytochemical compounds.
- Valorization of these residues was achieved by using green scCO₂ extraction processes.
- Optimal extraction conditions were 40 MPa and 35°C for scCO₂ with added ethanol.
- Antioxidant extracts rich in phenols and flavonoids were obtained from two samples.

ABSTRACT

The present study proposes the valorization of an agroindustrial residue (expeller) obtained from soybean oil extraction by pressing operation. Two expeller samples, S1 and S2, were processed to obtain extracts by supercritical fluid extraction (SFE). The extractions were performed at 40 MPa and at 35 or 40°C, using CO₂ as solvent (5 kg CO₂/kg expeller). Also, the expellers were impregnated with ethanol (25% w/w expeller), and then new SFE processes at 35°C were performed for both S1 and S2. The extracts resulting from the ethanol impregnated samples showed the best antioxidant properties with values of total phenolics of 10.6 and 16.0 mg GAE/100 g d.m., flavonoids content of 65.0 and 31.3 QE/100 g d.m. and antioxidant capacity by DPPH values of 9.7 and 12.0 µmol TE/100 g d.m. for S1 and S2, respectively. Phytochemicals from soybean expellers may be recovered through green and safe technologies such as sc-CO₂ extraction.

Keywords: soybean residue, supercritical CO₂, ethanol, antioxidants, flavonoids.

1. Introduction

In recent years, due to the increase in the waste generation, people started to concern about its impact on the environment. In this context, the concept of “zero waste” has been established in society so as to encourage people to research strategies to reduce or eliminate waste production. Particularly, food wastes or agroindustrial wastes are liquid or solid residues with a high organic load, generated from different processing methods. Even though waste is removed from the production process as an undesirable material, it can be potentially reused within the food manufacturing chain. In many cases, these residues have proved to be the source of a wide variety of bioactive species, showing a great potential for promoting their treatment. Some interesting bioactive components from plant origin are phenolic compounds, carotenoids, alpha-acids, methyl-xantines [1] and vitamins [2]. Phenolics comprise flavonoids, phenolic acids and tannins, among others. These compounds have different applications in the food industry, due to their antioxidant capacity [3-5].

Nowadays, soybeans are mostly processed for oil production using two different procedures. The first one involves the use of n-hexane as solvent to extract the oil and the second one, a greener but less efficient method, uses pressure and steam to carry on the extraction. This last method is carried out by a screw press and generates a residue called soybean expeller which is usually exploited for animal feed due to its high protein content [6]. However, it is known that polyphenols and other compounds with biotechnological interest remain in a high concentration in the solid structure of the expeller after oil extraction [7]. Flavonoids, such as soybean isoflavones, are effective antioxidants because of their phenolic structure and redox potential. Main soybean isoflavones are daidzin, genistin, glycitin and their respective aglycone, acetyl and malonyl forms. It is known that these compounds are responsible for the prevention of certain types of cancers

and some chronic diseases, such as cardiovascular and Alzheimer's diseases also exerts protection against osteoporosis [7, 8]. Moreover, phenolic compounds found in soybeans could potentially be used as natural preservatives to avoid oxidative deterioration of foods or as an ingredient for functional foods and cosmetic or pharmaceutical applications. For the reasons explained above, soybean expeller becomes an interesting residue for the extraction of antioxidants and moreover, the residual protein possesses a higher nutritional value.

The extraction, fractionation and isolation of high added-value compounds from food wastes usually try to maximize the yield of the bioactive compounds, suiting the demands of industrial processing, avoiding deterioration and loss of functionality during processing, and ensuring the food grade of the final product. Among several known techniques, supercritical fluid extraction (SFE) is a modern method and environmental friendly alternative for extraction of bioactive compounds that involve the use of a gas (i.e. CO₂) above its critical temperature and pressure [9]. Particularly, supercritical carbon dioxide (scCO₂) has lower critical temperature and pressure (31 °C and 74 bar) than other supercritical solvents, such as water (374 °C and 221 bar). CO₂ is non-toxic, non-flammable, non-polluting, completely recoverable, inexpensive and inert. Moreover, its main advantages are the low solvent consumption and the absence of residues in the extract because the CO₂ is a gas at room temperature and atmospheric pressure [10]. Other advantages of the scCO₂ extractions are that extracted compounds do not suffer chemical alteration during operation [2] and that the transport properties are enhanced comparing them to the transport coefficients from other methods. Additionally, extract composition can be modified by changing process parameters (pressure, temperature and flow rate) in order to extract volatile as well as non-volatile compounds. On the other hand, a big initial investment is required due to elevated pressure [10]. Given that CO₂ molecules are non-

polar, the solubility of highly polar compounds is low. It is possible to extend and modify the selectivity and solubility of these compounds in carbon dioxide by the addition of polar species as co-solvents. Thus, the extraction yields from solid wastes may be increased due to the polar nature of most natural compounds, such polyphenols [11, 12].

Zuo, et al. (2008) studied the recovery of isoflavones from soybean meal using scCO₂ and reported an optimum extraction procedure at 40°C, 50 MPa and 80% methanol added as modifier. However, limited information is known about the use of scCO₂ extraction as an innocuous and environmentally friendly technology to obtain bioactive extracts from residues of soybean industrialization processes. The aim of this study was to evaluate the effectiveness of scCO₂ as a green solvent for the extraction of bioactive compounds with antioxidant activity from two soybean expellers. The impact of matter compaction, extraction temperature and the impregnation of expellers with ethanol on the performance of scCO₂ extraction procedures was analyzed. Moreover, the antioxidant properties of the obtained soybean expeller extracts were assessed.

2. Materials and methods

2.1. Plant material

Two soybean expeller samples from different regions of Argentina were used as agro-industrial waste. Sample 1 (S1) from Rosario (Santa Fe, Argentina) was provided by Fiddeleff SRL and not soybean specification was reported. Sample 2 (S2) was obtained from RR soybean hybrid Nidera 5008 harvested in Trenque Lauquen (Buenos Aires, Argentina). Both samples (6% w/w moisture content) were residues obtained from oil extraction processes using screw presses (mechanical method) from soybean seeds without the use of solvents.

2.2. Sample preparation

The soybean expellers were triturated in a ball mill for 6 h with cylinders of Al_2O_3 as grinding agent ($m_{\text{Al}_2\text{O}_3}/m_{\text{sample}} = 3.5$) and sifted using a sieve (45 mesh) from Zonytest (Rey & Ronzoni S.A., Argentina). Maximum particle size was 350 μm .

2.3. Supercritical fluid extraction procedures

The extraction assays were performed using a high-pressure unit (model HPU500, Eurotechnica GmbH, Germany) with an extractor volume of 500 mL and maximum pressure of 50 MPa. The experimental system consisted of a solvent reservoir, a cooling bath, several thermal resistances and valves, a pump and an extraction vessel. The schematic diagram of scCO_2 extractor unit is shown in Fig. 1. CO_2 (Gamasol S.A., 99.5 %) was employed as solvent. In each experiment, a mixture of approximately 50 g of soybean expeller and 100 g of glass beads (Marienfeld Superior GmbH, N° 4901003, $\text{Ø}=3$ mm) trapped into a filter paper was introduced into the extractor vessel. The gas was pumped into the expeller sample until pressure reached 40 MPa. The operation temperature was set for each extraction at 35°C or 40°C. Experimental conditions corresponding to each extraction assay are shown in Table 1. For extractions using a co-solvent, the expeller and the glass beads were impregnated with ethanol (Aurum, 95%) by incipient moisture method and immediately introduced into the extractor. A proportion of 16 g of ethanol and approximately 550 g of CO_2 were used. This mean that each extraction with ethanol is equivalent to use 3 % w/w of ethanol/ CO_2 . The average scCO_2 flow rate used in the extraction assays was 0.5 kg/h; it was measured with a gas meter (TG 05, Ritter GmbH, Germany). For each assay, the obtained extract mass was registered as a function of the CO_2 passage time, obtaining one extraction curve for each condition.

Extraction yield (%w/w) was calculated as the quotient between the crude extract mass (g) and the total expeller mass (g) and expressed as percentage.

2.4. Total Polyphenol Content

Total polyphenol content (TPC) of different soybean expeller extracts was measured by a colorimetric assay using the Folin-Ciocalteu reagent [13]. 1000 μL of 1:10 diluted Folin-Ciocalteu reagent (Biopack, Argentina) were mixed with 200 μL of soybean expeller extracts (diluted 5:100 with methanol). After 3 min, 800 μL of 7.5 % Na_2CO_3 were added to the mixture. After 120 min of incubation in the darkness at room temperature, the absorbance was measured at 765 nm using UV-visible spectrometer UV-1601 PC (Shimadzu Corporation, Kyoto, Japan). The blank sample was prepared using pure methanol. TPC was calculated using a standard curve of gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry matter (d.m.) Determinations were made in triplicate. All chemicals used were of analytical grade purity.

2.5. Flavonoid content

The total flavonoid contents of soybean expeller extracts were determined by a colorimetric assay following the methodology proposed by Zhishen et al. [14] with some modifications. Briefly, 200 μL of each extract (appropriately diluted in methanol) were added to 860 μL of a NaNO_2 aqueous solution (0.35% w/v). After 5 min, 60 μL of 10 % AlCl_3 were added to the reaction tube. One minute later, this mixture was combined with 880 μL of a NaOH aqueous solution (1.82% w/v). Finally, absorbance was measured at 496 nm using a UV-visible spectrometer UV-1601 PC (Shimadzu Corporation, Kyoto, Japan). The blank sample was prepared using pure methanol. Flavonoid content was

expressed as milligrams of quercetin equivalents (QE) per 100 g d.m. using a standard curve (absorbance compared with concentration). Determinations were made in triplicate.

2.6. DPPH radical inhibition

Antioxidant capacity (AC) of soy expeller extracts was determined using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical method as described by Molyneux [15]. DPPH solution was prepared by mixing 2.5 mg of DPPH radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 1.0 ± 0.02 at 517 nm. Aliquots of 60 μ L of each soybean expeller extracts (diluted 5:100 with methanol) were mixed with 840 μ L of DPPH radical adjusted solution. The blank sample was prepared using 60 μ L of pure methanol. The reaction mixtures were incubated at room temperature in the dark for 60 min. The absorbance was read at 517 nm using a UV-visible spectrometer UV-1601 PC (Shimadzu Corporation, Kyoto, Japan). Reductions in DPPH radical solution absorbance due to reaction with the expeller extracts were calculated and expressed as DPPH inhibition percentage. Moreover, a calibration curve was prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) as a standard. Finally, AC of the extracts was expressed as μ mol of Trolox equivalents (TE) per 100 g d.m. Determinations were made in triplicate.

2.7. Statistical analysis

Assays were performed in triplicate. In order to analyze results regarding TPC, flavonoid content and AC (DPPH radical inhibition) analysis of variance (ANOVA) was performed to estimate significant differences between samples and Tukey's mean test was used for comparison ($P < 0.05$) using the software InfoStat (University of Córdoba, Argentina, 2015).

3. Results

3.1. SFE procedures

Different extraction assays were performed using S1 and S2 expeller samples and varying several conditions as the use of glass beads (GB), the extraction temperature and the addition of ethanol (EtOH) as co-solvent. Extraction curves were made for each different assay showing extraction yield (% w/w) as function of specific CO₂ consumption by using supercritical CO₂. Fig. 2 shows the extraction kinetic curves of the expeller S1 with and without the use of GB and employing scCO₂ at 40 °C or 35 °C and 40 MPa. Extraction yield at these conditions was clearly improved by the addition of glass beads into powdered expeller sample. An increase of 34 % in the yield was observed in E1/40 when compared with the sample without GB (nGB-E1/40) after a scCO₂ consumption of 16 kg CO₂/kg expeller. Fig. 3 shows the extraction kinetic curves for S2 expeller samples using GB at 40 MPa, both at 40 and 35 °C. For both S1 and S2 samples, the extraction processes resulted slightly more effective when were carried out at 35 °C compared to those processes performed at 40 °C. Furthermore, significant differences were observed in the extraction yields between the assayed expeller samples. The employment of S2 allowed to obtain a yield of 3.5 % while using S1 the yield reached 2% (Figs. 2 and 3). When scCO₂ consumption was higher than 20 kg CO₂/kg expeller, the extract yield remained constant for both expeller samples. All obtained extracts consisted of a single phase when the solvent used for the extraction was pure scCO₂. In all assays, decreasing extraction rates were observed as the specific CO₂ consumption increased.

Fig. 4 shows the extraction curves from samples S1 and S2 by using scCO₂ at 40 MPa and 35 °C when EtOH was added into the expeller samples. Both ethanol impregnated samples reached a yield of approximately 10.5 % after a specific

consumption of 5 kg CO₂/kg expeller. Extracts with two liquid phases (polar upper phase (U) and non-polar lower phase (L)) were obtained by using scCO₂ plus EtOH extraction procedures. The upper phases (E1/35/U and E2/35/U) would be composed of EtOH and soluble polar compounds from soybean while the lower phases (E1/35/L and E2/35/L) would contain remaining soybean oil and other extracted non-polar substances.

3.2. Antioxidant properties of soybean expeller extracts

Antioxidant properties of expeller extracts obtained from S1 and S2 samples under different scCO₂ extraction conditions (use of GB, extraction temperature and addition of EtOH) were determined. Fig. 5 shows TPC and flavonoid content of the extracts. Biphasic extracts are represented in U (polar) and L (non-polar) phases separately (see Table 1). The highest TPC values correspond to E1/35/U and E2/35/U extracts (both polar phases) showing 10.6 ± 0.9 and 16.0 ± 1.0 mg GAE/ 100 g d.m., respectively. Significantly lower TPC values ($P < 0.05$) were shown by the rest of the extracts varying from 0.9 to 5.6 mg of GAE/ 100 g d.m. consistent with non-polar phases. Also, significant amounts of flavonoids were found only in E1/35/U and E2/35/U extracts showing values of 65.0 and 31.3 mg QE/ 100 g d.m., respectively (Fig. 5). Non-detectable levels of flavonoids were observed for the rest of the extracts.

Antioxidant capacity of the extracts determined through DPPH assay is shown in Fig. 6. In accordance with TPC results, E1/35/U and E2/35/U samples showed the highest values of antioxidant capacity. 9.7 ± 0.4 and 12.0 ± 1.0 $\mu\text{mol TE/ 100 g d.m.}$ (equal to 2.43 and 3.01 mg TE/100g d.m.) were obtained for E1/35/U and E2/35/U, respectively. These results correspond to remarkable DPPH inhibition percentages such as $39 \pm 2 \%$ and $27 \pm 3 \%$ for E1/35/U and E2/35/U extracts, respectively (data not shown). Those extracts obtained using scCO₂ as unique solvent and also those corresponding to lower

phases of extracts obtained using EtOH as co-solvent (non-polar character) had a significantly lower ($P < 0.05$) DPPH scavenging capacity compared to E1/35/U and E2/35/U extracts, with values ranging from 0.3 to 3.0 $\mu\text{mol TE}/100 \text{ g d.m.}$ A significant correlation effect was observed between TPC and antioxidant capacity of soybean expeller extracts with a Pearson coefficient of 0.97.

Regarding the effect of temperature on antioxidant properties, TPC and AC, there were no significant differences ($P > 0.05$) between samples processed at 35 and 40 °C during extraction. Also, the use of GB into expeller samples had no effect on antioxidant properties of the extracts (Figs. 5 and 6).

4. Discussion

Obtaining a high-quality extract with antioxidant power from an agroindustrial residue constitutes a technological challenge that requires a technical and economic analysis. This work demonstrated that it is possible to process soybean expeller samples at relatively low temperature and at 40 MPa of CO_2 pressure obtaining innocuous extracts with antioxidant activity.

Extraction curves obtained in this work (Figs. 2, 3 and 4) are clearly divided in three stages following extraction mechanisms described by Da Silva, et al. (2016) [16]. A first period with constant extraction rate controlled by convection mass transfer mechanism. Subsequently, a second period where the extraction rate is falling and mass transfer occurs by diffusion and convection mechanisms. Finally, the third period is mainly controlled by diffusion and extraction rate is very low.

The efficiency of the extractions with scCO_2 can be improved by the use of glass beads (GB) into expeller sample. The use of GB in the sample increases the interactions between the solvent and the powder expeller, avoiding the compaction of the powder by pressure

and producing a homogeneous flux of the solvent through the sample. GB as inert particles showed a great capacity of allowing CO₂ movement throughout the extraction vessel, without canalizations. In our study, the use of glass beads (E1/40) significantly increased extract yield compared to the assay without inert solid (nGB-E1/40). Regarding temperature effect, slightly highest extraction yields were obtained at 35°C (δ_{CO_2} = 972,26 kg/m³) compared to 40 °C (δ_{CO_2} = 956,07 kg/m³) mainly for S1 expeller. A similar effect was found by Elgndi, et al. (2017) [17] and Goyeneche, et al. (2018) [18] who carried out scCO₂ extraction processes using *Satureja montana* L., *Coriandrum sativum* L. and *Ocimum basilicum* L. leaves and radish leaves, respectively. At low pressure, lower temperatures lead to higher solubility whereas at very higher pressures an inverse behavior can be found. The solubility results from the volatility of the solute and the solvent power of the solvent. The former increases with temperature while the latter decreases due to decreasing density [19]. The effect of reduced density of the fluid may prevail over the enhanced volatility and diffusion of solute resulting in a negative effect of higher temperature on extraction yield.

In our study, it was confirmed that the use of a polar co-solvent is a key factor to increase the extraction of polyphenols by modifying the solvent power of the fluid. Among several alcohols, ethanol is the most preferable co-solvent because its low cost and generally recognized as safe (GRAS) status according to American Food and Drug Administration. It is an innocuous solvent, both at human health and environmental levels and this is a strong advantage compared to n-hexane or even methanol, particularly when SFE is applied in food, cosmetic or pharmaceutical industries. De Melo, et al. (2014) [9] stated that the addition of small amounts of EtOH may increase expressively the polarity of the scCO₂. Faster extraction curves were observed when ethanol was used as cosolvent evidenced by the high initial slope of the curves. This effect is associated to the high

solubility of ethanol in scCO₂. When the ethanol is put in contact with the matrix a process of dissolution of the polar substances in the cosolvent is started, this fact enhances the kinetic of the extraction with scCO₂. In this work, two phases were distinguished in each extract obtained using scCO₂ plus EtOH: an upper phase (lower density) containing EtOH and polar compounds such as polyphenols and a lower phase (higher density) containing oil and non-polar compounds. When the expellers were impregnated with EtOH, lower amounts of scCO₂ were necessary for the extractions (Fig. 4) compared to assays without EtOH (Figs. 2 and 3). Moreover, extraction yield became almost constant in 10.5 %, with a similar consumption of scCO₂ for both EtOH impregnated expellers (Fig. 4). In line with our results, EtOH has been employed as a co-solvent in scCO₂ extraction processes to increase the solubility of polyphenols and antioxidants from chia seed cake [20], spearmints leaves [21], grape peel [22], grape marc [23], olive leaves [24], *Eruca sativa* leaves [25], radish leaves [18].

The impact of antioxidants in foods and biological systems is well-known. In foods, fats and oils are susceptible to oxidation and leading to the development of rancid odors and flavors and formation of potentially toxic secondary compounds. Antioxidants are able to protect foods against oxidation by scavenging free radicals, chelating metals, or acting as singlet oxygen quenchers. Regarding human health, antioxidants may reduce the oxidative damage of DNA, lipids, proteins, and other molecules and prevent the development of degenerative diseases and cancer [26]. In this work, a characterization of antioxidant properties (TPC and AC) of extracts obtained by scCO₂ extraction under different conditions was performed. Folin-Ciocalteu method is commonly used to measure total phenolics (TPC) in natural products. The solubility and the response of phenolics in this assay are ruled by their chemical nature that may vary from simple to very highly polymerized substances [27]. Besides, since the basic mechanism is an

oxidation/reduction reaction, the Folin-Ciocalteu assay can be considered as an antioxidant method measuring reducing capacity of a sample [28]. Moreover, DPPH assay is a simple and highly sensitive method; it has been extensively used to study antioxidant power of food and agricultural samples [18]. DPPH radical is one of the few stable organic nitrogen radicals, which presents a deep purple color. DPPH assay is considered to be mainly based on an electron transfer reaction and measures the reducing ability of antioxidants toward DPPH radical [28]. In our study, the polar fractions of extracts obtained with scCO₂ plus EtOH (E1/35/U and E2/35/U) showed the highest TPC, flavonoids and AC values, for S1 and S2 expeller samples. Also, TPC results can be related significantly with AC obtained for the extracts. Similarly, a highly positive correlation between phenolic compound concentration determined by the Folin-Ciocalteu method and DPPH antioxidant capacity was reported by Dudonné, et al. (2009) [29] in a study with 30 plant extracts (correlation coefficient of 0.94). Phenolic substances composed of aromatic rings bearing one or more hydroxyl groups are potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals [29].

As was described in the previous section, non-polar extracts (nGB-E1/40, E1/40, E1/35, E2/40, E2/35, E1/35/L and E2/35/L) showed similar levels of AC which resulted lower than those observed in ethanolic fractions. These oil rich extracts may contain significant levels of lipophilic tocopherols associated with the antioxidant capacity of these fractions. In fact, S1 and S2 lipid composition has been previously analyzed by Gas chromatography- mass spectrometry (GC-MS). S1 expeller sample showed higher percentages of tocopherols in the analyzed profiles than S2 (data not shown). However, the different lipid compositions were not related to significant differences in the antioxidant capacities of non-polar extracts.

As far as we are concerned, the extraction of antioxidants and phenolic compounds by SFE from the residue obtained after soybean pressing operation has not been reported yet. On the other hand, soybean seed and soybean flour (previously defatted with solvents) were used as source of antioxidants in several studies with the aim to obtain bioactive extracts by conventional solvent extraction processes [7, 30, 31] or SFE [32-34]. In these previous works, antioxidant content and activity were assessed by different methods and results were expressed using different units. Hence, in many cases the comparison between studies is difficult or cannot be carried out. Unlike our study, in most of these works hexane has been first used to separate soybean oil followed by a methanolic extraction to recover phenolic compounds and flavonoids of polar nature. Thereby, Yue, et al. (2008) [7] studied the antioxidant properties of intermediate products and by-products of soybean oil refining such as crude soy oil, degummed oil, gum, and defatted soy flour extract. As a result, this last by-product showed the highest level of TPC (1.13 mg catechin equivalent/100 g) and isoflavones (55 mg/g), added to the strongest DPPH free radical scavenging capability and the highest protection of a fish oil against oxidation. In line with our results, these authors obtained a bioactive extract from a soybean solid residue with potential to be used as food antioxidant or as a nutritional supplement. However, the employment of toxic solvents to obtain this extract represents a disadvantage. Besides, Prvulovic, et al. (2017) [31] evaluated the antioxidant status of five different soybean cultivars and prepared 70% ethanolic extracts from ground seeds. The reported values for flavonoid content ranged from 23.7- 63.6 mg QE/ 100 g of seed and were similar to that observed in the present work for E1/35/U and E2/35/U extracts. This fact indicates that the used scCO₂ + EtOH procedures were effective to recover soybean flavonoids. Regarding green technologies, it should be mentioned that a novel and safe method that involves using natural deep eutectic solvents, specifically choline

chloride: citric acid (molar ratio of 1:1), was employed successfully by Bajkacza and Adamek (2017) [35] for the extraction of isoflavones from soy products prior to their determination.

Some reports described the use of scCO₂ extraction procedures to re-use agroindustrial residues and obtain extracts rich in bioactive compounds (high added value). Recently, a SFE process (20 MPa, 40 °C and EtOH at 7.5%) similar to the used in our study, was successfully employed to obtain chia seed cake extracts. It provided high yield values and free-solvent extracts, economically competitive and with a good recovery of phenolic compounds [20]. Also, Calvacanti, et al. (2016) [36] studied the valorization of defatted capuassu seeds obtained as residue of a cold-pressing process. Optimal conditions for obtaining of capuassu butter by sCO₂ extraction were 30-35 MPa and 50 °C taking into account extract composition and costs.

In general, the food groups richest in polyphenols and antioxidant content are spices and dried herbs, fruits and vegetables followed by seeds, cereals and others [37]. Thus, the soybean expeller extracts obtained in our study showed lower AC and TPC compared to those reported for extracts prepared from garlic husk by EtOH extraction (TPC of 1180 mg GAE/ 100 g d.m.) while flavonoid content (48.6 mg QE/ 100 g d.m.) was similar [38]. In addition, higher amounts of antioxidants (TPC of 1375 mg GAE/100 g d.m., flavonoid content of 1390 mg QE/100 g d.m., AC by DPPH 35.9 mg TE/100 g d.m.) were extracted from radish leaves using a similar SFE procedure (scCO₂ plus EtOH, 40 MPa, 35 °C) in a study developed by Goyeneche, et al. (2018) [18]. Also, as was described by Manna, et al. (2015) [12], seed and skin fractions of grape pomace were subjected to SFE processes (scCO₂ plus EtOH at 5%, 20 MPa, 40 °C) and significant amounts of antioxidants were extracted (82 and 299 mg GAE/ 100 g d.m.) although phenolic recovery was less efficient compared to soxhlet extractions.

Finally, the expellers under study (S1 and S2) showed significant differences in antioxidant properties, mainly between polar fractions of E1 and E2 extracts obtained by comparable SFE procedures. This fact can be attributed to differences in composition of expellers S1 and S2 obtained from two soybean cultivars grown in different geographical regions. In addition, differences in pressing operations have a significant impact on the residual oil content in the expeller and may affect the quality and quantity of phytochemicals (phenolic compounds and flavonoids). S2 allowed to obtain the extract with the highest antioxidant potential, E2/35/U.

The re-use of a soybean residue, with high content of lipids and proteins but lower content of polyphenols in the dry matter, is interesting given that it is a low-cost and easy access material. Thus, it is possible to obtain two products, a bioactive extract and a new residue (soybean solids) more concentrated in protein after scCO₂ + EtOH extraction of expellers.

5. Conclusion

The effectiveness of scCO₂ and its use along with EtOH as modifier for the extraction of bioactive compounds with antioxidant activity from two soybean expellers (residues from pressing oil extraction) was evaluated. Extractions curves for both samples at different conditions (temperature, use of glass beds, use of EtOH) were made. Yield, TPC, total flavonoids and antioxidant capacity were determined for each extract or extract fraction. According to the results, it is recommended to extract bioactive compounds with antioxidant activity from soybean residues at a temperature of 35 °C, 40 MPa and with the addition of EtOH in the sample. This procedure allowed to obtain a maximum yield, maximum bioactive compounds extraction (polyphenols and flavonoids) and maximum antioxidant capacity. Soybean expellers constitute a source of valuable components

which may be recovered through green and safe technologies and subsequently recycled inside food chain as functional additives or used for pharmaceutical applications. Therefore, future studies will be carried out to enhance the recovery of polyphenols and antioxidants from expeller samples by employing safe extraction processes and also natural deep eutectic solvents followed by the identification of these bioactive compounds.

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References

- [1] J.L. Martinez, Supercritical fluid extraction of nutraceuticals and bioactive compounds, CRC Press Taylor & Francis Group, Boca Raton, 2007.
- [2] A. Esquivel, P. Vargas-Aguilar, Uso de aceites esenciales extraídos por medio de fluidos supercríticos para la elaboración de alimentos funcionales, *Tecnología en marcha*, 20 (2007) 7.
- [3] C. Grajeda-Iglesias, E. Salas, N. Barouh, B. Baréa, A. Panya, M.C. Figueroa-Espinoza, Antioxidant activity of procatechuates evaluated by DPPH, ORAC, and CAT methods, *Food chemistry*, 194 (2016) 749-757.
- [4] S. Mazzutti, S.R. Ferreira, C.A. Riehl, A. Smania, F.A. Smania, J. Martínez, Supercritical fluid extraction of *Agaricus brasiliensis*: Antioxidant and antimicrobial activities, *The Journal of Supercritical Fluids*, 70 (2012) 48-56.
- [5] A.M. Ojeda-Sana, C.M. van Baren, M.A. Elechosa, M.A. Juárez, S. Moreno, New insights into antibacterial and antioxidant activities of rosemary essential oils and their main components, *Food Control*, 31 (2013) 189-195.
- [6] D.R. Moses, Performance evaluation of continuous screw press for extraction soybean oil, *American journal of science and technology*, 1 (2014) 238-242.
- [7] X. Yue, Z. Xu, W. Prinyawiwatkul, J. Lasso, J. King, J. Godber, Comparison of soybean oils, gum, and defatted soy flour extract in stabilizing menhaden oil during heating, *Journal of food science*, 73 (2008) C19-C23.
- [8] K.-I. Chen, M.-H. Erh, N.-W. Su, W.-H. Liu, C.-C. Chou, K.-C. Cheng, Soyfoods and soybean products: from traditional use to modern applications, *Applied microbiology and biotechnology*, 96 (2012) 9-22.

- [9] M. De Melo, A. Silvestre, C. Silva, Supercritical fluid extraction of vegetable matrices: applications, trends and future perspectives of a convincing green technology, *The Journal of Supercritical Fluids*, 92 (2014) 115-176.
- [10] L. Dominguez, M. Parzanese, *Tecnologías para la industria alimentaria, Fluidos Supercríticos*, (2012) 1-12.
- [11] P. Pereira, M.G. Bernardo-Gil, M.J. Cebola, E. Mauricio, A. Romano, Supercritical fluid extracts with antioxidant and antimicrobial activities from myrtle (*Myrtus communis* L.) leaves. Response surface optimization, *The Journal of Supercritical Fluids*, 83 (2013) 57-64.
- [12] L. Manna, C.A. Bugnone, M. Banchemo, Valorization of hazelnut, coffee and grape wastes through supercritical fluid extraction of triglycerides and polyphenols, *The Journal of Supercritical Fluids*, 104 (2015) 204-211.
- [13] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, *Methods in enzymology*, 299 (1999) 152-178.
- [14] J. Zhishen, T. Mengcheng, W. Jianming, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals, *Food Chemistry*, 64 (1999) 555-559.
- [15] P. Molyneux, The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity, *Songklanakarin J. Sci. Technol*, 26 (2004) 211-219.
- [16] R.P.F.F. da Silva, T.A.P. Rocha-Santos, A.C. Duarte, Supercritical fluid extraction of bioactive compounds, *TrAC Trends in Analytical Chemistry*, 76 (2016) 40-51.
- [17] M.A. Elgndi, S. Filip, B. Pavlić, J. Vladić, T. Stanojković, Ž. Žižak, Z. Zeković, Antioxidative and cytotoxic activity of essential oils and extracts of *Satureja montana* L.,

Coriandrum sativum L. and *Ocimum basilicum* L. obtained by supercritical fluid extraction, *The Journal of Supercritical Fluids*, 128 (2017) 128-137.

[18] R. Goyeneche, A. Fanovich, C.R. Rodrigues, M.C. Nicolao, K. Di Scala, Supercritical CO₂ extraction of bioactive compounds from radish leaves: yield, antioxidant capacity and cytotoxicity, *The Journal of Supercritical Fluids*, (2018).

[19] R. Eggers, Extraktion von Fettrohstoffen mit überkritischem CO₂, *European Journal of Lipid Science and Technology*, 96 (1994) 513-518].

[20] C. Guindani, R. Podestá, J.M. Block, M.J. Rossi, N. Mezzomo, S.R. Ferreira, Valorization of chia (*Salvia hispanica*) seed cake by means of supercritical fluid extraction, *The Journal of Supercritical Fluids*, 112 (2016) 67-75.

[21] M. Bimakr, R.A. Rahman, F.S. Taip, A. Ganjloo, L.M. Salleh, J. Selamat, A. Hamid, I. Zaidul, Comparison of different extraction methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata* L.) leaves, *Food and Bioproducts processing*, 89 (2011) 67-72.

[22] K. Ghafoor, J. Park, Y.-H. Choi, Optimization of supercritical fluid extraction of bioactive compounds from grape (*Vitis labrusca* B.) peel by using response surface methodology, *Innovative Food Science & Emerging Technologies*, 11 (2010) 485-490.

[23] C. Da Porto, A. Natolino, D. Decorti, Extraction of proanthocyanidins from grape marc by supercritical fluid extraction using CO₂ as solvent and ethanol–water mixture as co-solvent, *The Journal of Supercritical Fluids*, 87 (2014) 59-64.

[24] J. Tabera, Á. Guinda, A. Ruiz-Rodríguez, F.J. Señoráns, E. Ibáñez, T. Albi, G. Reglero, Countercurrent supercritical fluid extraction and fractionation of high-added-value compounds from a hexane extract of olive leaves, *Journal of agricultural and food chemistry*, 52 (2004) 4774-4779.

- [25] M. Solana, I. Boschiero, S. Dall'Acqua, A. Bertucco, Extraction of bioactive enriched fractions from *Eruca sativa* leaves by supercritical CO₂ technology using different co-solvents, *The Journal of Supercritical Fluids*, 94 (2014) 245-251.
- [26] B. Díaz-Reinoso, A. Moure, H. Domínguez, J.C. Parajó, Supercritical CO₂ extraction and purification of compounds with antioxidant activity, *Journal of Agricultural and Food Chemistry*, 54 (2006) 2441-2469.
- [27] T.-I. Lafka, A.E. Lazou, V.J. Sinanoglou, E.S. Lazos, Phenolic and antioxidant potential of olive oil mill wastes, *Food Chemistry*, 125 (2011) 92-98.
- [28] R.L. Prior, X. Wu, K. Schaich, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, *Journal of agricultural and food chemistry*, 53 (2005) 4290-4302.
- [29] S. Dudonné, X. Vitrac, P. Coutiere, M. Woillez, J.-M. Mérillon, Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays, *Journal of agricultural and food chemistry*, 57 (2009) 1768-1774.
- [30] D. Malenčić, J. Cvejić, J. Miladinović, Polyphenol content and antioxidant properties of colored soybean seeds from Central Europe, *Journal of medicinal food*, 15 (2012) 89-95.
- [31] D. Prvulović, Đ. Malenčić, J. Miladinović, Antioxidant Activity and Phenolic Content of Soybean Seeds Extracts, *АГРО3НАИЉЕ*, 17 (2017) 121-132.
- [32] M.c.A. Rostagno, J.M. Araújo, D. Sandi, Supercritical fluid extraction of isoflavones from soybean flour, *Food Chemistry*, 78 (2002) 111-117.
- [33] J.M. Araújo, M.V. Silva, J.B. Chaves, Supercritical fluid extraction of daidzein and genistein isoflavones from soybean hypocotyl after hydrolysis with endogenous β -glucosidases, *Food chemistry*, 105 (2007) 266-272.

- [34] Y. Zuo, A. Zeng, X. Yuan, K. Yu, Extraction of soybean isoflavones from soybean meal with aqueous methanol modified supercritical carbon dioxide, *Journal of Food Engineering*, 89 (2008) 384-389.
- [35] S. Bajkacz, J. Adamek, Evaluation of new natural deep eutectic solvents for the extraction of isoflavones from soy products, *Talanta*, 168 (2017) 329-335.
- [36] R.N. Cavalcanti, C.L. Albuquerque, M.A.A. Meireles, Supercritical CO₂ extraction of cupuassu butter from defatted seed residue: experimental data, mathematical modeling and cost of manufacturing, *Food and Bioproducts Processing*, 97 (2016) 48-62.
- [37] J. Pérez-Jiménez, V. Neveu, F. Vos, A. Scalbert, Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database, *European journal of clinical nutrition*, 64 (2010) S112.
- [38] F. Kallel, D. Driss, F. Chaari, L. Belghith, F. Bouaziz, R. Ghorbel, S.E. Chaabouni, Garlic (*Allium sativum* L.) husk waste as a potential source of phenolic compounds: Influence of extracting solvents on its antimicrobial and antioxidant properties, *Industrial Crops and Products*, 62 (2014) 34-41.

Figure Captions

Figure 1. Schematic diagram of scCO₂ extraction system.

Figure 2. Extraction yields (%) as function of specific CO₂ consumption by using supercritical CO₂ at 40 MPa from S1 expeller sample at: 40 °C without glass beads (nGB-E1/40), 40 °C with the addition of glass beads (E1/40) and at 35°C with glass beads (E1/35).

Figure 3. Extraction yields (%) as function of specific CO₂ consumption by using supercritical CO₂ at 40 MPa from S2 sample at 35°C (E2/35) and 40°C (E2/40) with glass beads.

Figure 4. Extraction yields (%) as function of specific CO₂ consumption by using supercritical CO₂ at 40 Mpa, 35°C with the addition of ethanol from expeller samples S1 (E1/35/EtOH) and S2 (E2/35/EtOH).

Figure 5. TPC and flavonoid content of soybean expeller extracts obtained by scCO₂ extraction processes from S1 and S2 expeller samples.

Primary Y axis: TPC of soybean expeller extracts (grey bars). Secondary Y axis: flavonoid content (black square symbols). U and L mean, upper and lower phase, respectively. Vertical error bars indicate standard deviation of the mean values. Means with different letters are significantly different ($p < 0.05$).

Figure 6. Antioxidant capacity of soybean expeller extracts determined by DPPH radical inhibition assay. Vertical error bars represent standard deviation of the mean values. Means with different letters are significantly different ($p < 0.05$). U and L mean, upper and lower phase, respectively.

Figure 1

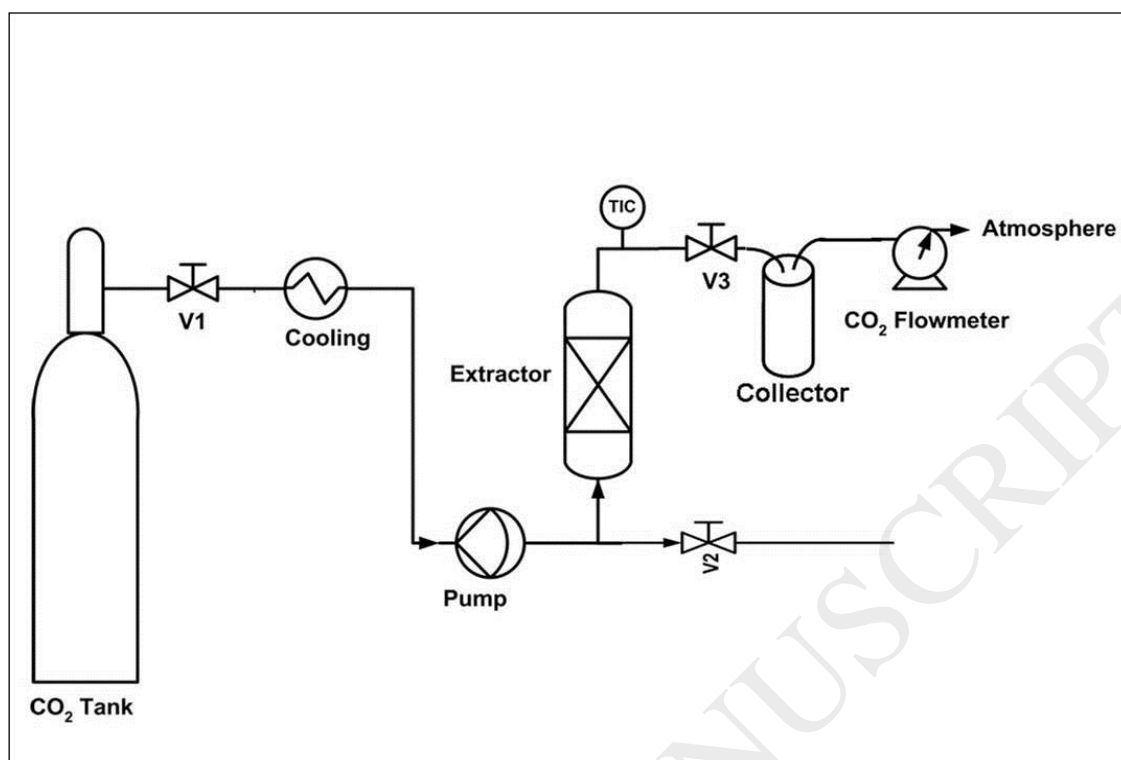


Figure 2

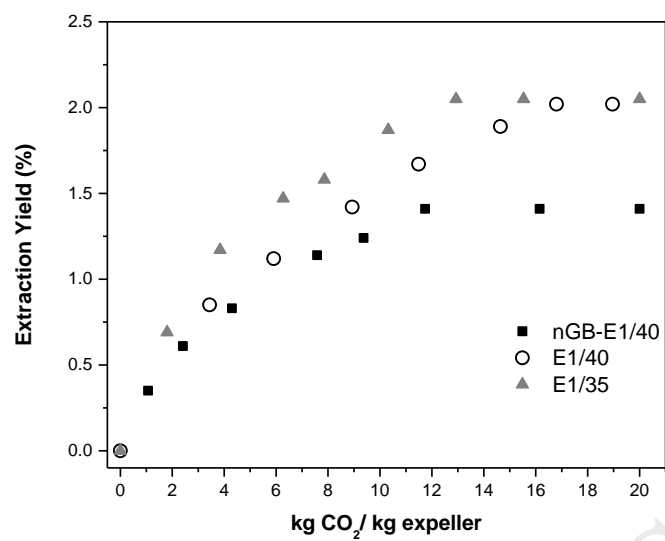


Figure 3

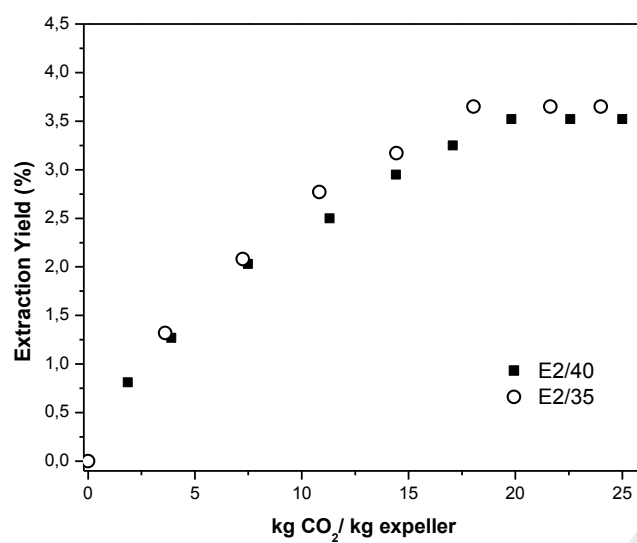


Figure 4

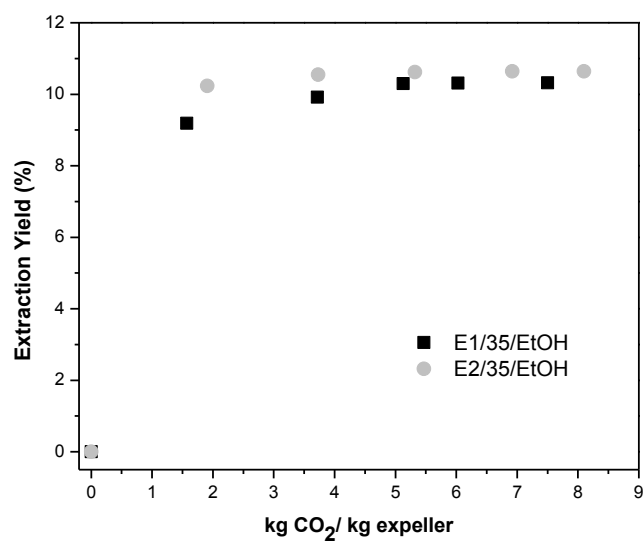


Figure 5

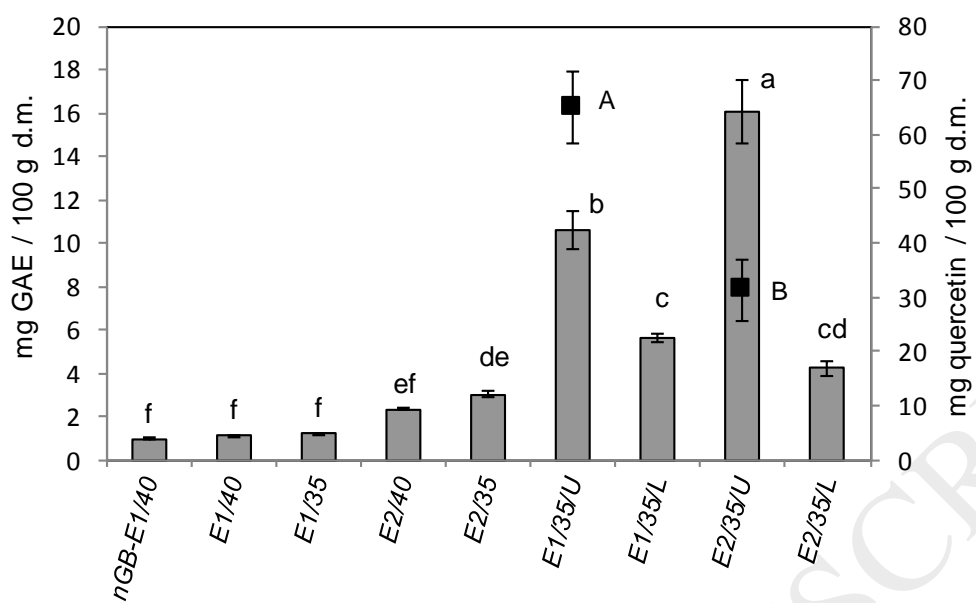
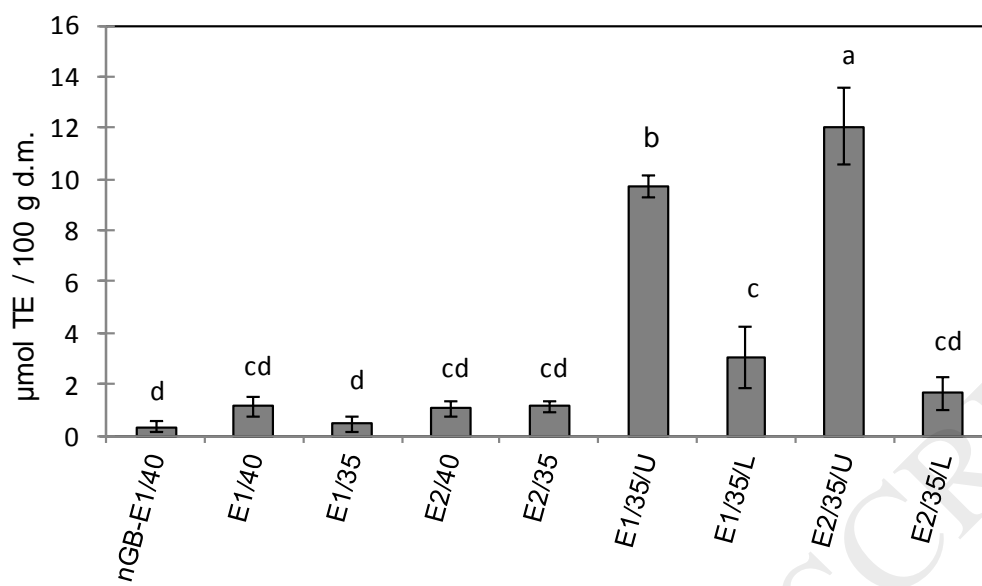


Figure 6



Tables

Table 1. Experimental conditions for the scCO₂ extraction processes.

Extract		Type	Expeller	T (°C)	Glass beads	Ethanol
nGB-E1/40		Monophasic	S1	40		
E1/40			S1	40	✓	
E1/35			S1	35	✓	
E2/40			S2	40	✓	
E2/35			S2	35	✓	
E1/35/U	E1/35/EtOH	Biphasic U: upper phase (polar)	S1	35	✓	✓
E1/35/L						
E2/35/U	E2/35/EtOH	L: lower phase (non polar)	S2	35	✓	✓
E2/35/L						