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Valorization of rice bran: modified supercritical CO_2 extraction of bioactive compounds

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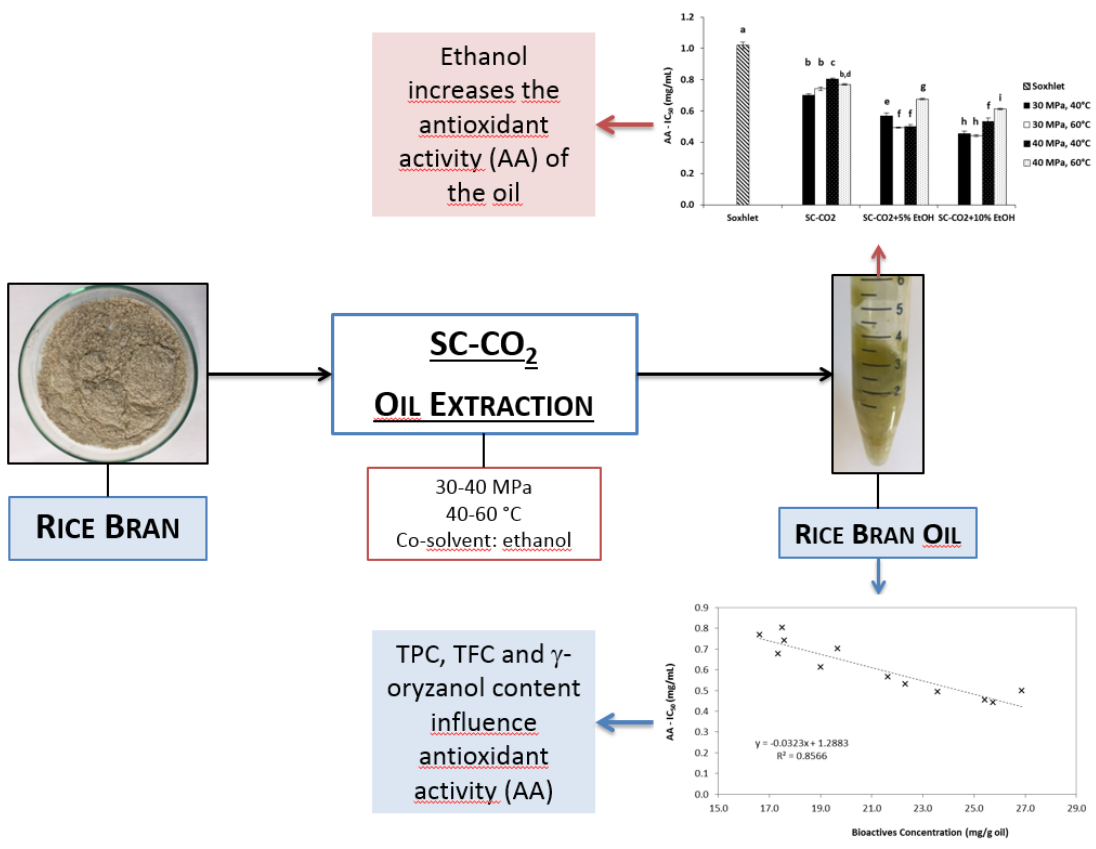
Graphical abstract



Highlights

- The valorization of rice bran was studied in this work
- Oil was extracted using SC-CO_2 and SC-CO_2 modified with ethanol
- The use of ethanol as modifier increased the antioxidant activity of the oils

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- The use of ethanol as modifier affected the fatty acid and γ-oryzanol profiles

Abstract

In this work, as a first step in a comprehensive strategy for the valorization of rice bran, the extraction of oil using supercritical CO_2 and ethanol as cosolvent has been studied. The effect of extraction temperature (40 and 60 °C), pressure (30 and 40 MPa) and amount of ethanol used (0, 5 and 10%) has been considered. The quality extracted oil has been evaluated in terms of antioxidant activity, fatty acid profile and bioactive compounds such as phenolics, flavonoids, γ-oryzanols, and tocopherols content.

Results revealed that, using neat CO_2, the best oil in terms of antioxidant activity was obtained at 40 °C and 30 MPa. However, the addition of ethanol as modifier significantly increased the amount of bioactive molecules extracted and hence the overall antioxidant activity of the oil, which was maximum at 40 MPa and 10% ethanol, regardless the temperature. The use of ethanol also affected the amount of fatty acids and γ-oryzanols extracted.

Keywords: supercritical fluid extraction; CO_2; rice bran oil; valorization; circular economy;

1. Introduction

Nowadays, Governments all around the world are boosting the transition from a linear to a circular economy. In a circular economy approach, the value of products, materials and resources is maintained in the economy

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for as long as possible, and the generation of waste minimized. Briefly described, the byproduct of a process becomes the input of a new process where it acquires new value. More specifically, the European Commission adopted a circular economy action plan *"Closing the loop - An EU action plan for the Circular Economy, COM(2015) 614"*, in the year 2015. In this plan, food waste and biomass and bio-based products (those based on biological resources) were identified as two of the main priority areas. In this sense, rice (*Oryza sativa*) bran is an excellent candidate to be valorized, for two reasons: first because it contains great amounts of bioactive molecules, such as tocopherols, oryzanols, phenolics, flavonoids (all of them with important antioxidant activity) and a complete fatty acid profile rich in unsaturated fatty acids [1]; second, because it is produced in great extent, since bulk rice has to be treated in order to remove the outer layers of the grain. In year 2016, around 740 millions of tons of rice where produced worldwide, according to FAOSTAT [2]. The 8% of this production ended up as rice bran. In the linear economy approach, 90 % of the total rice bran production is used in animal feeding [1] and only 10% for the extraction of the oil it contains (up to 22%, according to Yoon et al. [3]. This way, an important amount of bioactive molecules is underused every year.

Considering the principles of circular bioeconomy, a biorefinery approach can be a good strategy for the integral valorization of rice bran [4]. In general, a biorefinery is a facility where using diverse and complementary technologies, biomass is processed to obtain one or more bioproducts in the most sustainable way. It is possible to find in the literature examples of the conversion of rice wastes into valuable products following a biorefinery approach, such as hydroxymethylfurfural (HMF) [5,6] or

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proteins [7]. The biorefinery concept is extremely broad, and there are several classifications based on the sort of raw materials or the technologies used to process them [8]. As Nizami et al. [8] presented, two types of biorefineries are especially interesting if the recovery of bioactive molecules is intended: solvent extraction (products: primary and secondary metabolites) and supercritical conversion of biomass (products: cellulose, hemicellulose and lignin). In order to obtain the complete valorization of biomass, it is necessary to develop integrated biorefineries that use different combinations of feedstocks and technologies together at one platform to obtain multiple outcomes [9,10]. In these sense, the use of pressurized fluids (SC-CO_2 water) offers an interesting opportunity to be included in the integrated biorefinery concept. In the first step of this integrated biorefinery approach based on pressurized fluids, high added value molecules are extracted using an efficient and environmentally friendly technology such as supercritical carbon dioxide (SC-CO_2) [11]. In a second step, the defatted rice bran can be treated using pressurized hot water or supercritical water to obtain platform chemicals. Besides the recovery of high value component, Attard et al. [11] demonstrated that the biomass resulting from the SC-CO_2 extraction step, enhances the downstream process for the production of 2nd generation biofuels or platform chemicals.

SC-CO_2 technology offers a great opportunity due to its properties and ability to extract oils from natural sources, preserving the bioactive properties due to its mild operating conditions (critical temperature 31.7 °C and critical pressure around 7 MPa), non-toxicity and high selectivity to non-polar molecules. However, this non-polarity limits the extraction of polar compounds (for instance polyphenols or flavonoids),

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limitation that can be simply overcome by the addition of modifiers such as ethanol, methanol or water among others [12]. The selection of the modifier has to be done keeping in mind several aspects, as the nature of the target molecules or the final use the extract. It is well known that methanol is commonly used because it is an effective polar modifier and miscibility with CO_2 reaches 20%, but it is also known the toxicity it has, which completely advises against its use in the food industry [13]. Alternatively, ethanol is used, modifier that has lower toxicity despite presenting a lower polarity than methanol. The extraction of rice bran oil using SC-CO_2 has been previously studied [3,14–16], but to the best of our knowledge, no one has reported the use of ethanol as cosolvent in rice bran SC-CO_2 extraction processes. Therefore, it is necessary to study how the process parameters (pressure, temperature and amount of ethanol used as modifier) affect the extraction of oil from rice bran and its composition and antioxidant activity.

All in all, this work, as a first step in a comprehensive valorization of rice bran using pressurized fluids, the study of the extraction of bioactive molecules and oil from rice bran using SC-CO_2 and ethanol as cosolvent is carried out. The effect of the main process parameters temperature (40-60 °C), pressure (30-40 MPa) and percentage of ethanol as cosolvent (from 0% (neat SC-CO_2) to 10%) on the quality of the oil has been studied. The extracted oils have been completely characterized in terms of fatty acids profile and content in bioactive molecules such as polyphenols, flavonoids, oryzanols and tocopherols. The antioxidant activity provided by these bioactive molecules has been evaluated, in order to find the working conditions that maximize it.

Journal Pre-proof

2. Materials and Methods

2.1.Raw material

The raw material used in this work was rice bran kindly provided by Herba Ricemills, S.L.U. (Sevilla, Spain), which was kept at 4 °C until used in the extraction experiments.

2.2.Conventional solvent extraction

Conventional extraction, using hexane (VWR BDH Chemicals) as solvent, was carried out in a Soxhlet apparatus (Büchi B-811). In the Soxhlet extraction experiments, 5 g of rice bran (previously dried at 105 °C for 24 h) were used. 25 extraction cycles were used, followed by washing and drying stages. The extracted oil was characterized in terms of antioxidant activity, fatty acids profile and polyphenol, flavonoids, γ-oryzanols contents. This oil was used as a reference in order to evaluate the effect of the supercritical CO_2 on the properties of the extracted oil.

2.3.Supercritical fluid extraction equipment and procedure

The extraction experiments were carried out in a lab-scale plant, as shown in Figure 1. The maximum specifications of this experimental set-up are 150 °C and 50 MPa. The extractor has a length of 20 cm with ½" internal diameter. In a typical experiment, around 20 g mixed with an equal amount of Raschig rings were placed in the extractor, which was pressurized with CO_2 (Air Liquide S.A.) up to the extraction pressure.

For the neat supercritical CO_2 (SC-CO_2) extraction experiments, the experimental procedure was similar to that reported in Benito-Román et al. [17]. Two temperatures (40 and 60 °C) and two pressures (30 and

Journal Pre-proof

40 MPa) were tried, and the CO_2 mass rate was set at 0.40±0.05 kg/h. The total extraction time was 2 h, and a static extraction time of 30 min was used in each experiment. The extraction yield was expressed as the amount of oil extracted per 100 g of rice bran.

When ethanol (Merck KGcA) was used as cosolvent, a HPLC pump (Gilson 305, head SC-10, max flow rate 10 mL/min) was used. The cosolvent pump flow rate was calculated as a percentage of the CO_2 mass rate, and two levels of cosolvent were studied: low (5% of the CO_2 mass rate) and high (10%). Pressurized neat CO_2 and pressurized ethanol were put in contact in a static mixer, then heated up to the desired extraction temperature, and finally introduced in the extractor. Again, total extraction time was 2 h, and static extraction time of 30 min was used in each experiment. Due to the strong and complex interactions between the solutes of interest and the matrix, it is necessary to allow the solvent to diffuse into the matrix to alter this complex, so the solutes can be easily released [13]. The mixture ethanol-oil, collected in a vial (Figure 1, number 10), was put in contact with a nitrogen flow in order to remove the residual ethanol. Oil was then kept at -20 °C until analysis.

The oil extraction yield was calculated, and refers to the amount of oil present in the rice bran, expressed as *g of oil/100 g of rice bran*.

<Insert Fig. 1>

2.4.Analytical Methods

2.4.1. Total phenolics content (TPC)

The total phenolics content was measured using the Folin-Ciocalteu method, following the procedure reported by Ndayishimiye et al. [18],

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with some modifications. In this procedure, 0.5 mL of the oil previously dissolved in ethanol (5 mg/mL) were mixed with 0.5 mL of the Folin-Ciocalteu reagent, 5 mL of distilled water and 1 mL of sodium carbonate solution (20%). The mixture was shaken and let in darkness for 1 hour at room temperature. After that time, samples were centrifuged (13300 rpm for 10 min) and filtered (0.45 μm, pore size). The filtrate, completely clear, was measured at 750 nm using the V-750 (Jasco, Japan) spectrophotometer. A gallic acid (dissolved in ethanol) standard curve was used to calculate the total phenolic content of the oil samples, and the TPC was expressed as *mg Gallic Acid Equivalent/ g of extracted oil*.

2.4.2. Total flavonoids content (TFC)

The total flavonoids content was calculated following the procedure described in Spinelli et al. [19]. According to this procedure, 0.5 mL of the oil solution (5 mg/mL in ethanol) were mixed with 2 mL of distilled water and 0.15 mL of $NaNO_2$ solution (5%, w/v). After 6 minutes at room temperature, 0.15 mL of $AlCl_3$ (10%, w/v) were added. After 6 more minutes, 1 mL of NaOH (1M) and 1.2 mL of ethanol were added. The mixture was filtered (0.45 μm, pore size) and the absorbance measured at 415 nm (spectrophotometer V-750, Jasco, Japan). A quercetin standard curve in ethanol was used to calculate the TFC of the samples, which was expressed as *mg Quercetin Equivalent/g of extracted oil*.

2.4.3. Determination and quantification of the γ-oryzanol profile

γ-oryzanols were determined by HPLCD using a DAD detector (330 nm). Oil samples (approximately 30 mg) were dissolved in 1 mL of 2-propanol. The solutions were filtered (0.45 μm pore size) and 10 μL of this solution were

Journal Pre-proof

injected in a Zorbax XDB C18 5 μm 150 mm x 4.6 mm column. The mobile phase used consisted of acetonitrile (Merck KGaA), methanol (HiPersolv Chromanorm) and 2-propanol (Merck KGaA) in 50/40/10 proportion.

The quantification of the major γ-oryzanol components (1-Cycloartenylferulate, 2-24-Methylene-cyclo-artanylferulate, 3-Campesterylferulate, 4-β-Sitosterylferulate) is based on a study published by Mishra et al. [20]. The results are expressed in mg of each component/g of oil and the sum of each individual component was used to calculate the total mg of γ-oryzanol/g of extracted oil.

2.4.4. Determination and quantification of the tocopherol profile

Tocopherols determination was done by HPLC-DAD after isolation by solid phase extraction (SPE), following the procedure reported by Rebolleda et al. [21]. In the SPE process, silica cartridges (1000 mg/6 mL, Sep-Pak®, Waters) were used. Each cartridge was conditioned with 5 mL of n-hexane before the addition of 1 mL of oil solution (0.1 g/mL, in hexane). The elution of the analytes was done with 5 mL of n-hexane, followed by 5 mL of n-hexane:diethyleter (99:1, v/v) and 50 mL of n-hexane:diethyleter (99:2, v/v). The collected fractions were evaporated under vacuum at temperatures not higher than 40 °C. The solid residue was weighted and dissolved in 1.5 mL of n-hexane. Subsequently this solution was analysed by HPLC: tocopherols were determined following a modification of the IUPAC method [22] using a HPLC (Agilent series 1100) equipped with the software ChemStation, a degasser (G1322A), a quaternary pump (G1311A), an auto sampler (G1329A), a column oven (G1316A) and a diode array detector (G1315A). The column used was ACE 5 Silica (250 x 4.6 mm). The mobile phase was 99% hexane (A) and 1% 2-propanol (B) at

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a flow rate of 1 mL/min. An isocratic gradient was used, the total run time was 15 min and the injection volume was 50 μL. All the tocopherols were monitored at 296 nm. The column was kept at 25 °C. The individual compounds of α-, γ- and δ-tocopherols were identified and quantified using a calibration curve of the corresponding standard compounds.

2.4.5. Antioxidant activity assay

The antioxidant activity (AA) of the rice bran oil was evaluated using the $ABTS^{\cdot+}$ radical scavenging assay, in accordance with the method reported by Ndayishimiye et al. [18] with some minor modifications. In brief, the reagent $ABTS^{\cdot+}$ is obtained after the reaction between a 2.45 mM potassium persulfate ($K_2O_8S_2$, Panreac, 99% purity) with a 7 mM ABTS solution (2,2´azino-bis-(3-ethylbenzotiazolin-6-sulphonic), Sigma Aldrich) for at least 16 h at room temperature in darkness. Subsequently, the solution was diluted with ethanol in order to get an absorbance in the range from 0.7 to 0.9 at 734 nm, measured using the spectrophotometer V-750 (Jasco, Japan). Each of the extracted oils was dissolved in ethanol to get solutions with concentrations in the range 0.25 to 5 mg/mL. To 1 mL of the oil solution, 3 mL of the already prepared $ABTS^{\cdot+}$ were added. After 1 h in the dark, the absorbance was measured at 734 nm using the above mentioned Jasco spectrophotometer. The inhibition percentage was calculated for each dilution, and the inhibition percentage versus oil concentration was plotted for each oil sample. From this curve, the concentration of oil required for a 50% inhibition of the radical $ABTS^{\cdot+}$ was calculated and expressed as IC_{50} value. In this sense, the lower the concentration needed, the higher the antioxidant activity of the sample.

2.4.6. Determination and quantification of the fatty acids profile

Journal Pre-proof

The fatty acids profile was determined by the AOAC official method [23]. According to this method the fatty acid methyl esters were firstly prepared and then analyzed by gas chromatography using a Hewlett Packard (6890N Network GC System) gas chromatograph equipped with an auto-sampler (model 7683B) and a flame ionization detector (FID). Helium was used as a carrier gas at a flow rate equal to 1.8 mL/min. A fused silica capillary column (OmegawaxTM-320, 30 m x 0.32 mm) was used.

Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Aldrich). Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method [23]. Calibration curves were made for several pairs formed by internal standard+several representative chromatographic standards in order to find the corresponding response factors.

2.5.Statistical Analysis

The experimental results were analysed using Statgraphics Centurion. The LSD (Least Significant Difference) test was run to determine which means were significantly different from which others at a 95% confidence level, in order to test the significance of the experimental conditions on the studied response.

3. Experimental Results

3.1.Conventional solvent extraction

Journal Pre-proof

The oil content of the rice bran used as raw material was determined by Soxhlet extraction using hexane as solvent, and resulted to be 16.3±0.4 g/100 g of rice bran, expressed in dry basis. This oil presented total phenolics content (TPC) around 1.58±0.04 mg GAE/g of oil and total flavonoids content (TFC) around 1.01±0.09 mg QE/g of oil. The total γ-oryzanols present in the oil were 12.47±0.25 mg/g of oil, and the individual components of γ-oryzanol presented the following order of prevalence: 24-Methylene cycloartanyl ferulate (4.66±0.04 mg/g of oil) > Cycloartenyl ferulate (3.69±0.05 mg/g of oil) > Campesteryl ferulate (2.43±0.12 mg/g of oil) > β-sitosteryl ferulate (1.70±0.03 mg/g of oil). All in all, the antioxidant activity (AA) of this oil, expressed as the IC_{50} value, was 1.02±0.02 mg/mL.

Regarding the fatty acids content, the hexane extracted oil contained 784±11 mg/g of oil. The fatty acid profile was composed mainly by MUFA (41.6%) and PUFA (38.3%). More specifically, oleic (39.4%) and linoleic (37.7%) were the main fatty acids present in the oil, whereas palmitic (15.3%), was the predominant saturated fatty acid.

3.2.Supercritical CO_2 extraction

3.2.1. Determination of the extraction time

In order to determine the extraction time, the oil extraction curve was determined at 30 MPa and 40 °C. It is presented in Figure 2, where a typical SC-CO_2 extraction curve can be observed: an initial straight line (extraction is controlled by solubility) followed by a depletion zone (extraction is being controlled by the internal diffusion). Therefore the extraction rate of this second part is much lower than the initial one [17].

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From the results presented in Figure 2, the extraction time was set at 2 h, time that was considered to be enough to extract the highest amount of oil.

<Insert Fig. 2>

3.2.2. Oil extraction yield

The extraction conditions (temperature, pressure and percentage of ethanol used as cosolvent) played an important role on the extraction yield, as it is presented in Figure 3. As can be seen in this figure, the amount of oil extracted using SC-CO_2 was in all the cases lower than hexane extraction (16.3 g of oil/100 g of rice bran).

<Insert Fig. 3>

When neat CO_2 was used, the amount of oil extracted was affected by both pressure and temperature, parameters that control the density of the CO_2 and thus its solvent power. The highest yield (10.9 g of oil/100 g of rice bran) was obtained at 40 MPa and 40 °C, experimental conditions that led to the highest density of CO_2 (956.1 kg/m^3). A temperature increase up to 60 °C, involved a slight decrease in the amount of oil extracted. This result can be explained by the fact that the increase in temperature at constant pressure involves a decrease in the density of CO_2 (to 890.1 kg/m^3 at 40 MPa and 60 °C). In this case the decrease in the solvent power, due to the decrease in density, could not be compensated by the increase in the diffusion of CO_2 in the matrix and the increase in the vapour pressure of the oil. At 30 MPa, the CO_2 density varied from 909.9 to 829.7 kg/m^3, when working at 40 and 60 °C, respectively, slightly shifting the extracted oil from 9.2 to 10.2 g/100 g of rice bran. The

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obtained results are in agreement with the results presented by Tomita et al. [15] who, at 30 MPa, did not observe important differences in the amount of oil extracted, similar to the results presented in our work. These authors detected the crossover point at 30 MPa for the oil extraction from rice bran.

When ethanol was used as co-solvent, the amount of oil extracted was increased, as presented in Figure 3. The highest amounts of oil were extracted when 10% of ethanol was used. In fact, the extraction yield was 14.4 g/100 g of rice bran at 40 MPa, 60 °C and 10% ethanol. When only 5% of ethanol was used, high pressure and temperature were needed to increase the extraction yield. The addition of ethanol to the SC-CO_2 leads to a new solvent, with new properties, which interacts with the matrix and the solute in a different way. According to Lim et al. [24] the addition of ethanol as co-solvent tends to, on one hand, increase the solvent power of SC-CO_2 and, on the other, affects the matrix by swelling it. As a consequence of this swelling effect, an increase in the area in contact with the CO_2 is produced, which leads to an increase in the extraction yield. Regarding the effect of the use of cosolvent at different pressures of temperatures, Ndayishimiye et al. [18] found that the increase in the oil extracted was more stressed the higher pressure and the higher the ethanol added to the CO_2. At the lowest pressure, the addition of ethanol could not help to induce a density increase high enough to extract compounds that require higher pressures to be extracted. The increase in the oil extraction yield has been also reported by other authors using different raw materials, as can be seen in the following examples: Wejnerowska et al. [25] used SC-CO_2 and polar cosolvents (methanol, ethanol and mixtures of both) in the range from 1.3 to 20% (expressed as

Journal Pre-proof

the weight of the co-solvent with respect to weight of seeds) to increase the quinoa oil extraction rate (40-80 °C and 18-30 MPa); Fetzer et al. [26] also observed an increase of the oil extracted from baru seeds with pressure (15-25 MPa), temperature (40-80 °C) and cosolvent fraction (ethanol, added in variable ratios, from 1:1 to 1:3 (g of baru seed:g of ethanol added); Moon et al. [27] reported as well an increase of the oil extracted using ethanol as cosolvent (3%) at all the extraction conditions tried in temperatures from 45 to 55 °C and pressures in the range from 20 to 30 MPa using the plant *Asiasarum heterotropoides* as raw material; Tonato et al. [28] worked on the extraction of oil from the fungus *Nigrospora* sp. by SC-CO_2 with ethanol as cosolvent (in variable proportions in the range 30 to 70%) at 80 °C and 25 MPa, concluding that ethanol increased in all the cases the oil recovery rate and Belayneh et al. [29] pumped ethanol at concentrations of 2.5, 5 and 10% (mass rate of ethanol/mass rate of SC-CO_2) into the extraction vessel, loaded with *Camelina sativa* seeds. These authors reported a significant increase in the extraction yield at all pressure and temperature levels (35-45 MPa; 50-70 °C, respectively). This increase in oil extraction yield is, in general, attributed to the increase in the polarity of the solvent mixture as a consequence of the addition of ethanol.

3.2.3. Total phenolics content (TPC)

The total phenolics extracted under different experimental conditions are presented in Figure 4. Regarding the use of neat CO_2, no statistically significant differences were observed in the range of pressure and temperature studied: the oils extracted contained around 1 mg GAE/g of oil, which is lower than the TPC in the oil extracted using hexane. At

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40 MPa, despite the increase of the CO_2 solvent power as a consequence of the increase in density, the ability of SC-CO_2 to dissolve polar compounds is limited. These results are in agreement with those presented by other authors when using different raw materials, such as Spinelli et al. [30] from brewers' spent grain or Ndayishimiye et al. [18] from citrus peel. Phenolics extraction has been traditionally carried out using organic solvents such as methanol or ethanol among others, and more recently by pressurized hot water, which under certain conditions exhibits a polarity similar to that of the organic solvents [31]. Therefore, the addition of ethanol as cosolvent to the SC-CO_2 is expected to promote the recovery of phenolics. These compounds can be classified as free, esterified and insoluble-bound [32], so the use of ethanol as cosolvent might help to release those more strongly linked to the matrix.

The use of ethanol as cosolvent increased dramatically the extraction yield of phenolics, which proves the affinity phenolics have for polar solvents [31]. The TPC in the rice bran oil extracted in our work ranged between 1.61±0.08 and 3.42±0.12 mg GAE/g of oil, when ethanol was used as co-solvent. The statistical analysis of the results presented in Figure 4 revealed that the use of ethanol had a clear impact on the TPC of the extracted oil; however there was not statistically significant difference after increasing from 5 to 10% of ethanol. The use of 5% of ethanol as cosolvent rose the amount of phenolics extracted up to 2.80±0.09 mg GAE/g of oil at 30 MPa (40-60 °C). However, at 40 MPa the TPC decreased with temperature down to a minimum value of 1.83±0.16 mg GAE/g of oil. When 10% of ethanol was used as cosolvent, similar trends were observed. At 30 MPa, the use of 10% of ethanol led to slightly higher TPC at both temperatures (compared to the experiments in which 5% of

Journal Pre-proof

ethanol was used), whereas at 40 MPa and at both temperatures studied, a stepped decrease in the TPC was detected down to a minimum value of around 1.61±0.08 mg GAE/g of oil. This fact can be attributed to a dilution effect: it was under these experimental conditions that the highest amount of oil was recovered. The extraction conditions might have favored the extraction of other molecules resulting in a dilution of the extracted phenolics.

Belayneh et al. [29] reported trends very similar to those reported in the present work: the TPC was increased when 5% of ethanol was added to the SC-CO_2, but further increases of ethanol as cosolvent up to 10% did not increase the TPC in the *Camelina sativa* seed oil. These authors did not observe effect of pressure (35-45 MPa) on the TPC in the oil extracted, similarly to the results presented by Quitain at al. [33] using okara as raw material and to the results shown in Figure 4. Regarding the effect of temperature, Belayneh et al. [29] reported an increase in the TPC at almost all the extraction conditions. On the other hand, Fetzer et al. [26] reported a lower TPC in the SC-CO_2 plus ethanol extracted oils than the hexane extracted baru seeds oils.

From the results presented in Figure 4, it is possible to observe that the addition of ethanol as SC-CO_2 modifier increased the TPC in oils, due to the increase in the polarity of the solvent that allowed to break the strong bonds (such as dipole–dipole, dipole-induced dipole, hydrogen bonding as well as other polarity forces) between the phenolics and the matrix [13]. This fact was also reported by Benelli et al. [34] who showed that cosolvent induces a rupture in the solute/solid matrix interactions, which promotes the release of the solute. Under very specific conditions and in

Journal Pre-proof

the presence of copper, CO_2 and ethanol can react to form ethylcarbonic acids [35], that could promote the extraction of bioactive molecules. In the case that SC-CO2 plus ethanol is used as cosolvent, it seems unlikely that the formation of ethylcarbonic acids is controlling the bioactive molecules release.

<Insert Fig. 4>

3.2.4. Total flavonoid content (TFC)

Although rice bran is a source of flavonoids [1], the presence of these compounds in the oil extracted using SC-CO_2 has not been reported in the literature, due to limitations in analytical methods and the low prevalence of these compounds in rice grains [36]. Moreover, flavonoids are usually found as glycoside forms which affect their biological functions and ability to be extracted. In the present work we have been able to extract and quantify flavonoids in oils extracted from rice bran, as presented in Figure 5.

Neat SC-CO_2 provided oil with similar TFC (around 0.8 mg QE/g of oil) with a maximum value of 1.05±0.01 mg QE/g of oil obtained at 30 MPa and 60 °C, conditions that led to the lowest density of SC-CO_2 (829.7 kg/m^3). Flavonoids share similarities with phenolics, both are polar compounds, which are non-easily extracted by SC-CO_2, a non-polar molecule [37].

When ethanol was used as a modifier of the solvent power of SC-CO_2, an important increase in the TFC in oils was detected, with a maximum of 4.47±0.30 mg QE/g of oil at 40 MPa, 40 °C and 10% ethanol, as shown in Figure 5. The statistical analysis of the results indicated a limited effect of both pressure and temperature; however the extraction of flavonoids was

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favoured by the ethanol content. Unlike the results presented for the TPC, the amount of cosolvent used had a statistically significant effect on TFC in oils, being higher the higher amount of modifier used. At both co-solvent levels, the extraction of flavonoids was favoured at the highest pressure (40 MPa) and the lowest temperature (40 °C).

<Insert Fig. 5>

At 30 MPa, increases in temperature increased TFC, but at 40 MPa, the opposite trend was observed. A decrease in the phenolics content was also detected when temperature was increased at 40 MPa, conditions that led to the highest amounts of oil extracted, so the dilution effect can be behind the results observed. Spinelli et al. [30] did not find a clear effect of pressure on the extractability of TFC (15-35 MPa) in the whole range of temperatures (40-60 °C) they used to extract oil from brewers' spent grain. Under some extraction conditions they also reported a decrease in the amount of TFC extracted when using higher amounts of ethanol as modifier. No clear explanation was provided for those findings.

3.2.5. γ-oryzanol

γ-oryzanol, a complex mixture of triterpenic alcohols with ferulic acid esters of phytosterols [38], is interesting due to its antioxidant activity and bioactive properties [39]. In general, γ-oryzanol is very soluble in SC-CO_2 (solubility is around 1g/kg CO_2 at 40 MPa and 60 °C); however, in the presence of oil, the solubility in CO_2 is estimated to be around 16.9 mg/kg CO_2 under the same pressure and temperature conditions [38]. This fact points out that the solubility of γ-oryzanol falls in the presence of oil, which can be attributed to the rigid and voluminous structure of the γ-

Journal Pre-proof

oryzanol that makes it to be strongly linked with other components of the rice bran matrix [40]. All these results explain the reason why γ-oryzanol is extracted in the late stages of the SC-CO_2 extraction [3], when the oil extraction is very slowed and the extraction is diffusion controlled. It has also been reported that γ-oryzanol is soluble in different organic solvents [41]; therefore, the addition of some ethanol as co-solvent might improve the extractability of these compounds.

The experimental results obtained in this work are presented in Table 1. Compared to the Soxhlet extracted oil (12.47±0.25 mg of γ-oryzanol/g of oil), the use of SC-CO_2 (whit or without co-solvent) provided statistically significant differences in the γ-oryzanol concentrations, which were in the range from 13.19±0.07 to 20.63±0.64 mg/g of oil. The experiments done using neat SC-CO_2 revealed no statistically significant differences in the γ-oryzanol content in the oils extracted at the range of pressure and temperature studied. Regarding the γ-oryzanol profile, the following pattern was found: 24-Methylene cycloartanyl ferulate > Cycloartenyl ferulate > Campesteryl ferulate > β-sitosteryl ferulate. The extraction conditions did not alter this pattern, being increased/decreased each of the species by the same rate when the conditions changed. Other authors, such as Balachandran et al. [14] reported that 24-Methylene cycloartanyl ferulate was the main oryzanol, followed by Campesteryl ferulate and Cycloartenyl ferulate. These authors observed differences in the oryzanol profile as a function of the extraction conditions. Yoon et al. [3] reported a profile similar to ours. In other studies, it was found that the amount of γ-oryzanol extracted increases with pressure from 30 to 40 MPa (from 47 to 57 mg/g of oil) [3]. On the contrary, those authors reported a slight

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decrease in the amount of γ-oryzanol extracted when increasing the temperature. Increases in pressure involve an increase in the CO_2 density, which should favour the solvent power of the CO_2, and hence increase the extractability of γ-oryzanol [42]. Other authors [15,40,42], have also reported an increase in the γ-oryzanol extraction with pressure. Balachandran et al. [14] also reported an increase of the extraction with pressure, temperature and time, in a range of 50 to 70 °C at 35 MPa (only one experiment at 60 °C and 50 MPa, 90 min) and extraction times up to 90 min. Tomita et al. [15] studied the extraction of oil from rice bran (in a pressure range 20-40 MPa and temperature range 40-80 °C). The content in γ-oryzanol was determined, and the authors concluded that at low pressure (20 MPa) a rise in temperature produced a decrease in the amount of γ-oryzanol extracted, but at higher pressures (30-40 MPa) there was not effect of the temperature, as it was observed in our work done at 30 and 40 MPa. Similar results were obtained in our work. These authors found the highest γ-oryzanol content in the oil at 40 MPa and 60 °C (12.6 mg/g of oil). The differences among the authors can be due to operating factors, such as CO_2 flowrate, particle size or even the variety of rice used in the experimental work.

Oils extracted with SC-CO_2 and ethanol as cosolvent presented, in general, higher contents in γ-oryzanol. At 30 MPa and 40 °C, the addition of only 5% of ethanol did not increase the extractability of γ-oryzanol, compared to the neat SC-CO_2. However, at 30 MPa and 60 °C the addition of ethanol (5%) increased the amount of γ-oryzanol extracted (18.95±0.36 mg/g of oil), and further increases in the levels of cosolvent used increased even more the amount extracted (19.74±0.23 mg/g of oil).

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When the extraction was carried out at 40 MPa, in general lower amounts of γ-oryzanols were extracted, compared to the results obtained at 30 MPa. These differences were statistically significant. However, when 5% ethanol was used as cosolvent, at 40 °C the amount of γ-oryzanol extracted was the highest in all the experimental conditions tried (20.63±0.64 mg/g of oil), but at 60 °C that amount was dramatically reduced, without a clear explanation for it. According to Capellini et al. [43] the extraction of γ-oryzanol (in a conventional extraction process at atmospheric pressure) is favoured by the use of polar solvents, such as ethanol compared to the non-polar solvent like hexane. The addition of water (up to 6%) to ethanol also enhanced the extraction. These authors studied two different temperatures (60 and 80 °C), and obtained worse results in terms of γ-oryzanol recovery at the highest temperature. According to these results the polarity of the solvent plays a role in the extraction of γ-oryzanol.

In the literature, it has been possible to find only one paper that deals with the extraction of γ-oryzanol using SC-CO_2 and ethanol as co-solvent. In this paper, presented by Sookwong et al. [16], ethanol among other organic solvents, was used as co-solvent in levels up to 10 %. Under these conditions (43 °C, 37 MPa and 10% ethanol) around 11.3 mg/g oil of γ-oryzanol were extracted, with no differences between using 5 or 10% of ethanol as cosolvent. However, the extractability of γ-oryzanol was four times increased compared with the results using neat CO_2.

3.2.6. Total tocopherols

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In order to quantify the tocopherol profile of the oils extracted using SC-CO_2, it was necessary to carry out a solid phase extraction (SPE) pretreatment in order to remove compounds that interact with tocopherols and hinder their identification by HPLC. Similar problems have been reported by other authors when dealing with oils extracted from other cereals such as corn [21]. Results are presented in Table 2.

Table 2 shows that the presence of ethanol in the CO_2 enhances the extraction of tocols. Sookwong et al. [16] also reported an enhancement of the α-tocopherol recovery when using ethanol as cosolvent, being unaffected by the percentage of ethanol used as cosolvent (no difference between 5 and 10%). The increase of α-tocopherol was almost three times higher at 43 °C, 38 MPa and 60 min.

When CO_2 and 10% ethanol was used at 40 °C, the effect of pressure have been evaluated. In this case we could see how at the lower pressure, better results in terms of tocopherols extraction were obtained. Yoon et al. [3], when extracting α-tocol from rice bran did not see a significant effect of pressure (27.6-41.1 MPa) at 40 °C. Finally, Balachandran et al. [14] reported an increase of tocols concentration with temperature under isobaric conditions.

3.2.7. Antioxidant Activity (AA)

In previous sections it has been presented how the use of ethanol as cosolvent improves the overall extraction of bioactive compounds, such as phenolics or flavonoids. In this section the overall antioxidant activity of the oil extracted is analysed and results are presented in Figure 6.

<Insert Fig. 6>

Journal Pre-proof

In all cases, the SC-CO_2 extracted oil presented a higher antioxidant activity than the oil extracted using hexane (IC_{50} value was 1.02±0.02 mg/mL). More specifically, when neat CO_2 was used no statistically significant effect of pressure and temperature were observed on the AA of the extracted oils.

In general, the addition of ethanol as cosolvent increased the AA of the oils extracted, and the amount of ethanol used had a statistically significant effect. The highest AA (expressed as the IC_{50}) was detected for oils extracted at 30 MPa with 10% of ethanol (around 0.44 mg/mL). The lower values of the IC_{50} indicator mean that lower amounts of the oil are needed to reduce the activity of the radical ABTS by 50%. The antioxidant activity decreased at 40 MPa, decreased that was stepped at the highest pressure. Similarly to the trend observed for the TPC and TFC results, the lowest concentration in those bioactive molecules was observed at 40 MPa, 60 °C and 5-10% of ethanol as cosolvent, the conditions that in turn led to the highest oil recoveries. Fetzer et al. [26] reported a dramatic increase in the antioxidant activity (evaluated using the ABTS radical) of the baru seeds oils extracted with SC-CO_2 and ethanol as cosolvent compared to the hexane Soxhlet extracted oils. These authors also reported an increase of the AA with pressure (15-25 MPa), and a decrease of the AA the higher the extraction temperature (40-80 °C). They also found lower TPC contents in the SC-CO_2 extracted oils, although the overall AA was higher, which probably means that ethanol modified SC-CO_2 allowed to recover other molecules with powerful antioxidant activity, besides phenolic compounds.

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In Figure 7 (*a* and *b*), the correlation between the AA and the TPC or TFC of the extracted oils is represented. As can be seen, there is a good correlation between the phenolics content in oils and the antioxidant activity, but there is no correlation between the flavonoids content and the AA, although a certain trend can be observed: the higher the TFC, the higher the AA. Ndayishimine et al. [18] claimed than the presence of phenolics and flavonoids in extracts obtained with SC-CO_2 (an cosolvent) contribute in a great extent to the total antioxidant activity of the oils. These authors also proposed that TPC might exert more antioxidant activity than TFC, which according to Figure 7 (*a* and *b*), it is what has been observed in our work. Nevertheless, it is hard to set a complete correlation between high levels of both TPC or TFC and the antioxidant activity of a sample, since according to Huang et al. [44], the antioxidant activity is a function of the structure and interaction of phenolic compounds. Moreover, the presence of other bioactive molecules, such as γ-oryzanols will also affect the antioxidant activity of the extracted oil, and a synergistic effect of all the bioactive compounds extracted (also including tocopherols and γ-oryzanols) is expected, what will promote the total antioxidant activity of the oils [18]. The results presented in Figure 7-*c*, shows the following trend: the higher the γ-oryzanol content the higher antioxidant activity of the oil, although no clear correlation is observed.

Besides the nature of the bioactive compounds, the antioxidant activity of a sample also is affected by the method used to quantify it, because not all the bioactive molecules interact in the same way with the radical used. This fact makes difficult the comparison of antioxidant activities among authors. For instance, the ABTS assay is normally used to evaluate the antioxidant activity of hydrophilic compounds whereas DPPH is applied in

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both aqueous-organic extracts, as indicated Ndayishimiye et al. [18]. These authors tried both radicals, and in general similar trends in the antioxidant activity behaviour with the working conditions were observed.

<Insert Fig. 7>

3.2.8. Fatty acids (FA) profile

The fatty acid profile of the oil extracted from rice bran using SC-CO_2 at different pressures and temperatures was determined. Compared to the total FA extracted, the use of SC-CO_2 provided statistical significant differences compared to the oil extracted using hexane, as presented in Table 3.

When neat SC-CO_2 was used, no significant differences in the fatty acid profile were observed when changing pressure or temperature. The mayor fatty acids present in the extracted oils are oleic, linoleic and palmitic, results which were also reported by Balachandran et al. [14]. Around 18% of the fatty acids are saturated (FA), 41% are monounsaturated (MUFA) and the remaining 41% are polyunsaturated (PUFA). These results are in agreement with the results presented by Mingyai et al. [41], who found that unsaturated fatty acids were predominant in oils extracted form rice bran. The presence of unsaturated fatty acids in rice bran oil, together with other bioactive molecules, reinforces its use as health protective functional food to prevent diseases related to the high cholesterol levels. Compared to the oil extracted with the Soxhlet apparatus, the trend is to extract more unsaturated FA when using SC-CO_2. With regards to the use of SC-CO_2, when pressure is increased, the trend is to recover higher amounts of total fatty acids; however the proportion of SFA, MUFA and PUFA remains unaltered.

Journal Pre-proof

When SC-CO_2 plus cosolvent was used, some interesting results were observed. On the one hand, the use of ethanol decreased the amount of fatty acids extracted, as presented in Table 3. A decreased of 15 to 20% in the total amount of fatty acids extracted was observed when using 5% of ethanol, but when 10% of ethanol was used, no further reductions in the amount of FA extracted was observed. The statistical analysis of the results revealed that the use of co-solvent was statistically significant. However, shifting from 5 to 10% of ethanol did not show any statistically significant effect. The minimum amount of fatty acids recovered was 541±19 mg FA/g of oil, detected at 30 MPa, 60 °C and 10% ethanol, results that could be explained by a dilution effect. On the other hand, the fatty acid profile did not change significantly, being oleic and linoleic the most abundant unsaturated FA (USFA) and palmitic the most abundant saturated FA (SFA), compared to the oil extracted using neat CO_2. It is surprising that the acids most affected by the use of ethanol were oleic and linoleic acids, which were significantly reduced. In general the use of ethanol as solvent, compared to hexane, tends to increase the oils extracted from different raw materials, such as baru seeds [26] or chia seeds [45], but these authors observed slight variations in the fatty acid profile, although not clear or consistent variations (i.e. reduction of saturated fatty acids, or increase in the unsaturated fatty acids).

To the best of the authors' knowledge, there are only a few examples in the literature that present the effect of ethanol as modifier of SC-CO_2 on the composition of the fatty acid profile of the oils extracted. However, none of them refer to rice bran. The study published by Suryawanshi et al. [46], in which oil from *Argenone Maxican (L.)* seeds was extracted using SC-CO_2 and ethanol as cosolvent. These authors reported that the use of

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neat SC-CO_2 for the extraction (60 °C, 27.5 MPa) led to an oil that contained 17.7% of saturated fatty acids, whereas the use of SC-CO_2 plus cosolvent (60 °C, 35 MPa, 5% ethanol) provided an oil enriched in saturated fatty acids (24.5% of the total). Ferrentino et al. [47] also reported a fatty acid profile from brewers' spent grain extracted with and without ethanol as SC-CO_2 modifier (20-30 MPa, 40-50 °C and up to 8% ethanol). In this case the authors did no report significant changes after the use of ethanol, nevertheless only 5 fatty acids were reported and not a complete profile including SFA and USFA. Wejnerowska et al. [25] reported no significant effect of the extraction conditions regarding the fatty acid profile in quinoa oils extracted using modified SC-CO_2. Moon et al. [27] observed that in the oils extracted from *Asiasarum heterotropoides* using SC-CO_2 and ethanol as modifier, the SFA proportion in the mixture decreased, being increased the amount of MUFA and PUFA. For them, myristic acid, palmitoleic acid, and linoleic acid were the most abundant fatty acids. Finally, Tonato et al. [28] reported important variations in the fatty acids profile extracted from the fungus *Nigrospora sp.* as a consequence of the addition of ethanol as cosolvent: the addition of 50% ethanol (w/w) allowed to have 3.5 times more total fatty acids (TFA) than the addition of 30% ethanol (w/w), affecting also to the unsaturated FA/saturated FA ratio, that increased from 1.8 to 2.1. When more ethanol was used as cosolvent (70% ethanol (w/w)), the total amount of oil extracted was reduced and the fatty acid profile changed. These authors explained the results considering that the increase in the polarity prevented SC-CO_2 from having access to the non-polar solutes during the extraction step.

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4. Conclusions

In this work, it has been demonstrated that the use of SC-CO_2 and SC-CO_2 plus ethanol as cosolvent is a good alternative to extract oil from rice bran, since the obtained oil is rich in bioactive molecules with strong antioxidant activity, such as phenolics, flavonoids, tocopherols, oryzanols and unsaturated fatty acids. The supercritical extraction technology offers an effective and useful opportunity for the valorization of an extensively produced byproduct of the food industry by the recovery of oils rich in high added value bioactive molecules.

The oil extracted using neat SC-CO_2 and neat SC-CO_2 plus ethanol (5-10%) as cosolvent, in a range of pressure from 30 to 40 MPa and temperatures from 40 to 60 °C, was superior in terms of quality compared to the oil extracted with hexane in a Soxhlet apparatus. However, the oil extraction yield was lower when the supercritical technology was used. SC-CO_2 with ethanol as modifier increased the oil extracted, but still was lower than the Soxhlet hexane extraction. All in all, the working conditions that led to the best oil in terms of antioxidant activity and richness of bioactive molecules was 40 MPa, 40 °C and 5-10% of ethanol.

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Journal Pre-proof

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Figure 1. Supercritical fluid extraction plant. 1: CO_2 reservoir; 2: syringe pump; 3: cryostat; 4: bursting disk; 5: high pressure extractor; 6: oven; 7: co-solvent bottle; 8: co-solvent HPLC pump; 9: static mixer; 10: separator

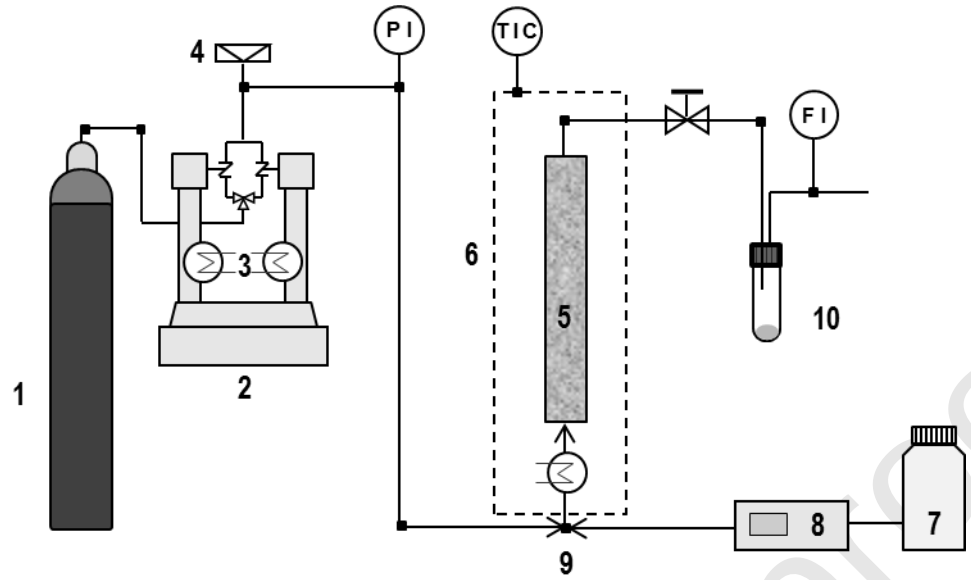


Figure 2. Extraction curve of oil from rice bran using SC-CO_2 at 30 MPa and 40 °C

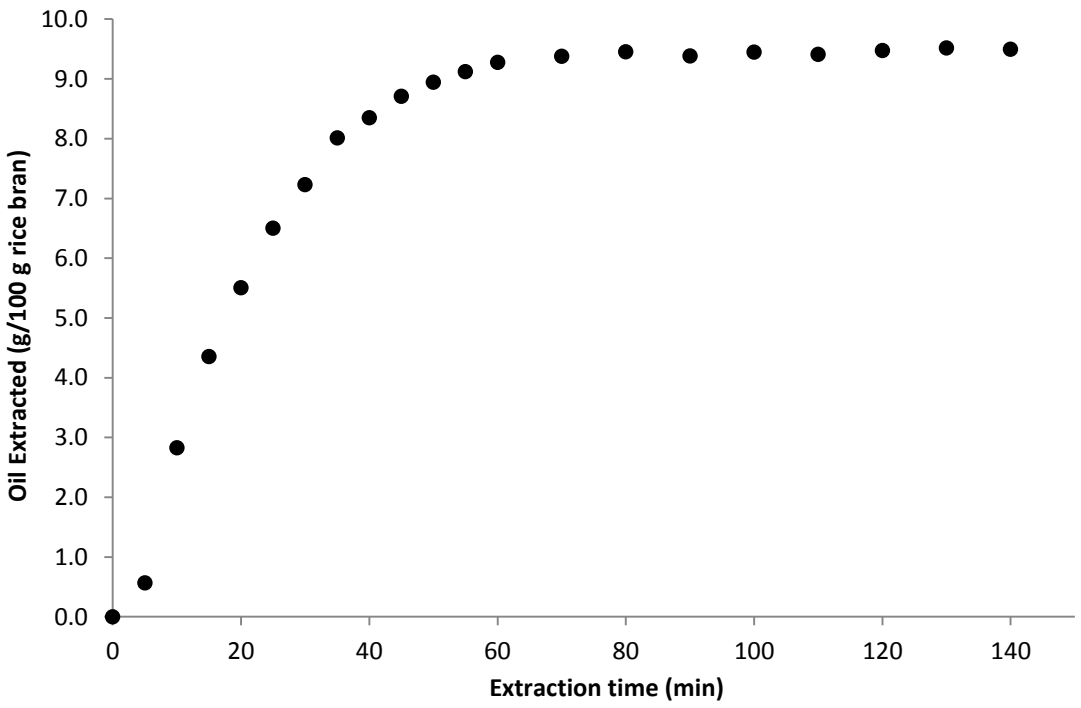


Figure 3. Amount of oil extracted from rice bran under different experimental conditions. Different letters indicate significantly different values ($p<0.05$)

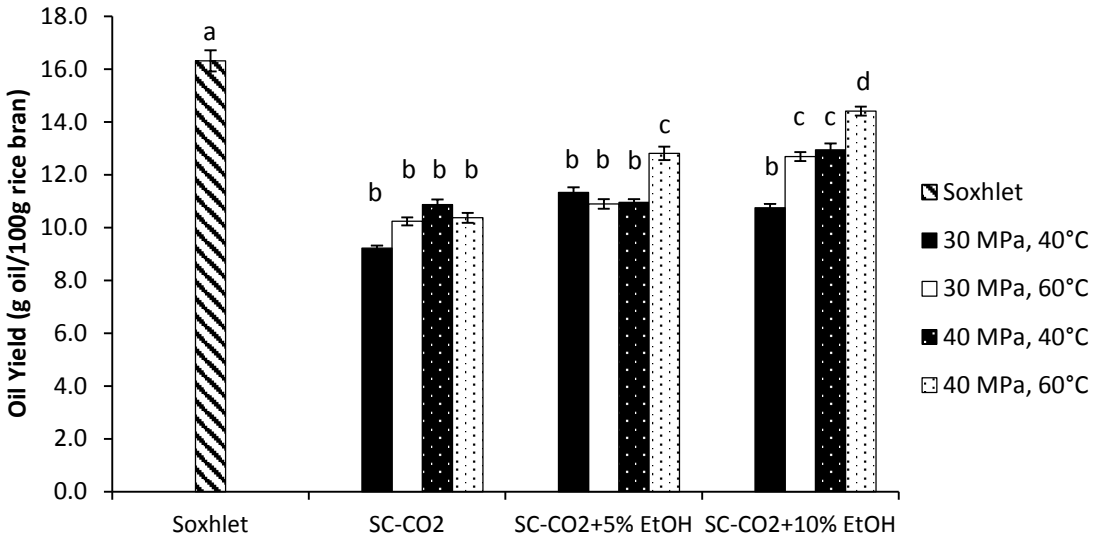


Figure 4. TPC (expressed as mg GAE/gram of oil) in oils extracted under different experimental conditions. Different letters indicate significantly different values ($p<0.05$)

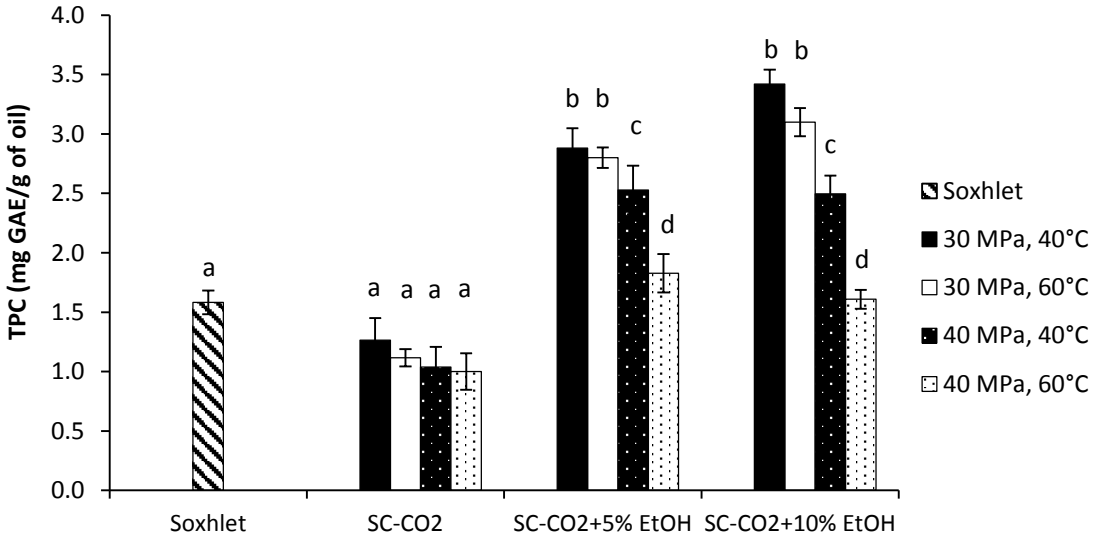


Figure 5. TFC (expressed as mg of QE/gram of oil) for oils extracted under different experimental conditions. Different letters indicate significantly different values ($p<0.05$)

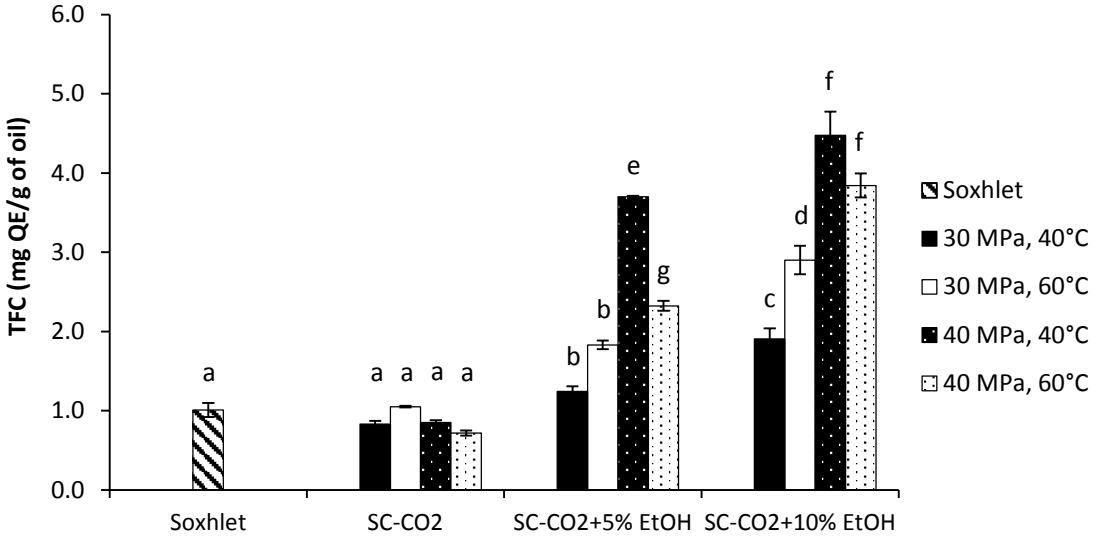


Figure 6. Antioxidant activity (expressed as the IC_{50} value) for oils extracted under different experimental conditions. Different letters indicate significantly different values ($p<0.05$)

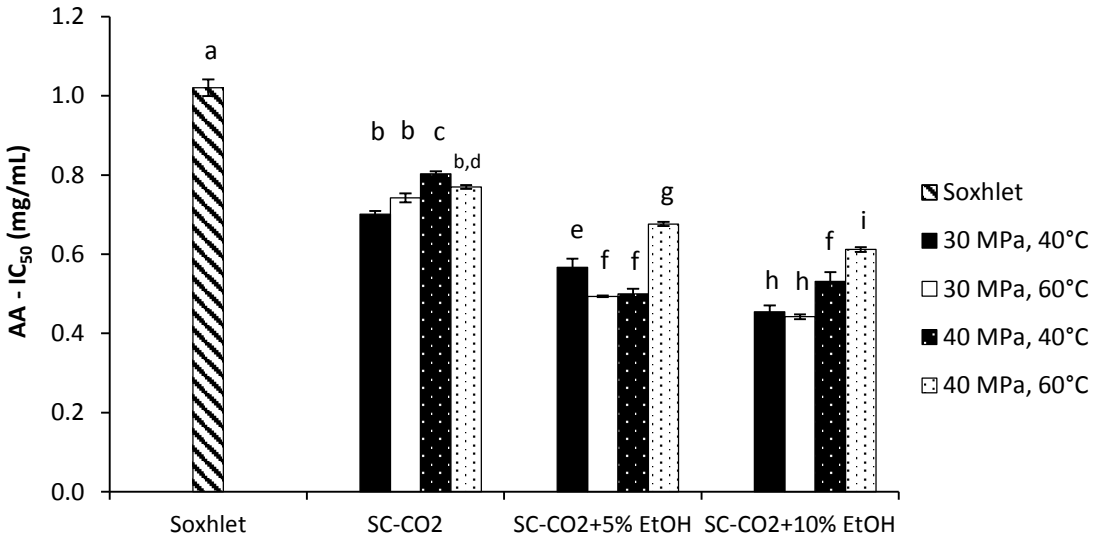
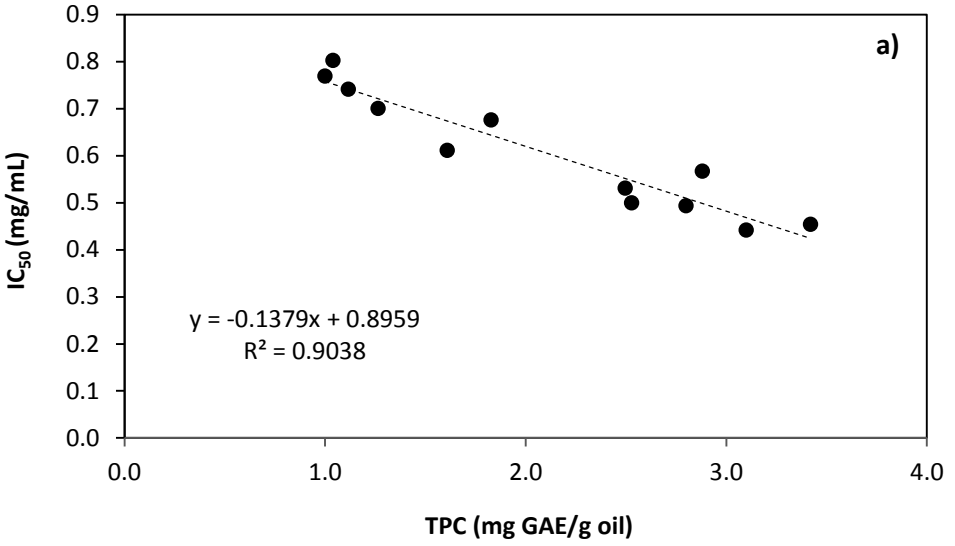
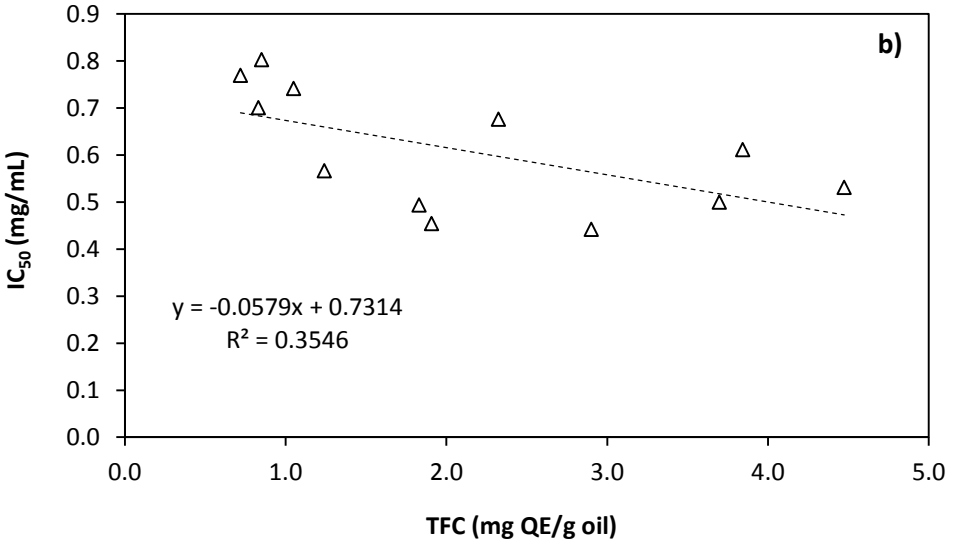


Figure 7. Correlation between the antioxidant activity and the total phenolics content (a), total flavonoids (b) and γ-oryzanol (c) content for the oils extracted under different experimental conditions


a)
y = -0.1379x + 0.8959
R² = 0.9038
IC50 (mg/mL)
TPC (mg GAE/g oil)


b)
y = -0.0579x + 0.7314
R² = 0.3546
IC50 (mg/mL)
TFC (mg QE/g oil)

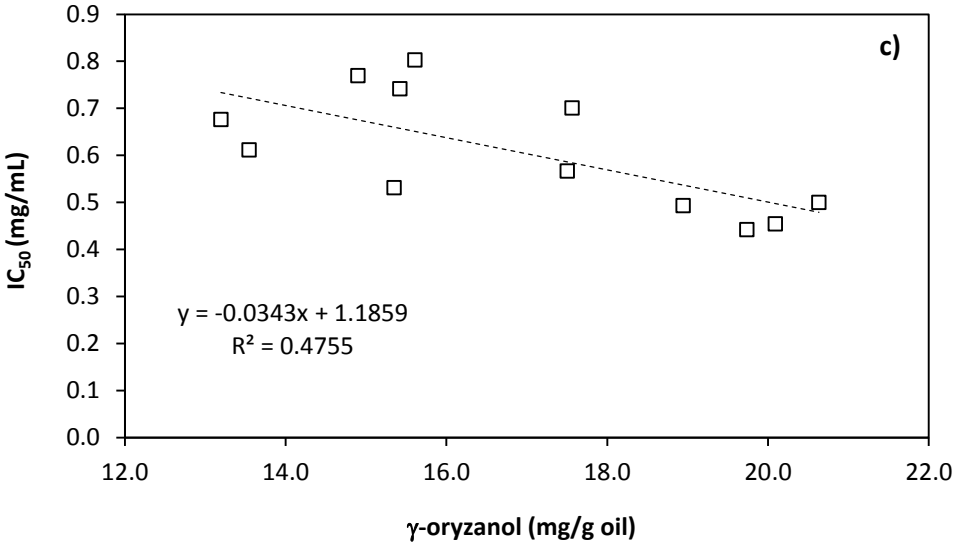

c)
y = -0.0343x + 1.1859
R² = 0.4755
IC50 (mg/mL)
γ-oryzanol (mg/g oil)

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Table 1. Concentration of γ-oryzanols in rice bran oil obtained by supercritical CO_2 extraction under different experimental conditions

			γ-oryzanol (mg/g oil)				
P (MPa)	**T (°C)**	**Solvent**	**Cycloartenyl ferulate**	**24-Methylene cycloartanyl ferulate**	**Campesteryl ferulate**	**β-sitosteryl ferulate**	**Total γ-Oryzanol**
30	**40**	**CO_2**	4.98±0.02	6.53±0.06	3.69±0.03	2.36±0.01	17.56±0.12[a]
		CO_2+5% Ethanol	4.81±0.23	6.56±0.19	3.80±0.14	2.34±0.15	17.50±0.69[a]
		CO_2+10% Ethanol	5.68±0.10	7.43±0.10	4.36±0.09	2.62±0.07	20.09±0.35[f]
	60	**CO_2**	4.25±0.23	5.90±0.24	3.29±0.17	1.99±0.13	15.42±0.78[a]
		CO_2+5% Ethanol	5.35±0.09	6.97±0.14	4.07±0.08	2.56±0.05	18.95±0.36[a,c]
		CO_2+10% Ethanol	5.15±0.05	7.68±0.01	4.67±0.02	2.23±0.17	19.74±0.23[f,a]
40	**40**	**CO_2**	4.08±0.13	5.98±0.16	3.37±0.11	2.18±0.08	15.61±0.47[b]
		CO_2+5% Ethanol	5.42±0.12	7.78±0.14	4.80±0.20	2.63±0.59	20.63±0.64[d]
		CO_2+10% Ethanol	4.09±0.07	5.74±0.09	3.48±0.12	2.04±0.21	15.35±0.23[g,b]
	60	**CO_2**	3.78±0.07	5.72±0.10	3.26±0.13	2.14±0.07	14.90±0.32[a,b]
		CO_2+5%	3.64±0.01	5.08±0.02	3.01±0.03	1.47±0.02	13.19±0.07[g]

		Ethanol					
		CO_2+10% Ethanol	3.64±0.04	5.19±0.02	3.09±0.07	1.63±0.09	13.55±0.17^{h,g}
Soxhlet			3.69±0.05	4.66±0.04	2.43±0.12	1.70±0.03	12.47±0.23^{i}

Different letters indicate significantly different values ($p<0.05$)

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Table 2. Tocopherol profile in oil extracted using SC-CO_2 under different experimental conditions

			Tocopherol (mg/g oil)				
P (MPa)	**T (°C)**	**Solvent**	α	β	γ	δ	**total**
40	40	CO_2	1.92±0.22	0.09±0.03	0.13±0.03	ND	2.14±0.28[a]
40	40	CO_2+10 % Ethanol	3.40±0.19	0.06±0.01	0.17±0.01	ND	3.63±0.21[b]
30	40	CO_2+10 % Ethanol	5.16±0.28	0.07±0.01	0.24±0.01	ND	5.47±0.30[c]

ND. Not detected. Different letters indicate significantly different values ($p<0.05$)

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Table 3. Fatty acid profile of oils extracted with SC-CO_2 under different experimental conditions

			30 MPa						40 MPa					
			40 °C			60 °C			40 °C			60 °C		
Fatty Acid (mg/g oil)		**Soxhlet**	**CO_2**	**5% EtOH**	**10% EtOH**	**CO_2**	**5% EtOH**	**10% EtOH**	**CO_2**	**5% EtOH**	**10% EtOH**	**CO_2**	**5% EtOH**	**10% EtOH**
C14:0	**Myristic**	1.7±0.0	2.2±0.0	1.7±0.1	1.6±0.0	2.1±0.1	1.7±0.1	1.6±0.1	2.3±0.3	1.8±0.1	2.0±0.1	2.4±0.2	1.8±0.1	1.9±0.1
C16:0	**Palmitic**	120±1	126±3	101±3	96±1	127±2	103±5	79±4	135±16	103±4	122±1	142±3	106±2	111±4
C18:0	**Stearic**	14±1	14±1	12±1	12±1	14±1	13±1	9±1	15±2	13±1	14±1	16±1	12±0	12±1
C18:1 n-9	**Oleic**	309±4	329±7	267±6	275±1	319±11	284±11	211±7	343±14	308±10	303±2	353±11	265±6	273±7
C18:1 n-7	**Vaccenic**	11±1	11±1	11±1	11±1	11±1	10±1	6.4±0.6	12±1	11±1	11±1	12±1	8.5±0.8	9.3±0.8
C18:2 n-6	**Linoleic cis&trans**	296±5	328±7	262±6	263±2	314±9	274±11	212±8	336±12	295±10	293±2	346±10	256±6	265±9
C18:3 n-3	**α-linoleic**	8.8±0.2	10±1	7.8±0.1	7.7±0.1	9.8±0.2	8.1±0.4	6.9±0.2	10±1	8.9±0.4	8.9±0.1	11±1	7.8±0.2	8.1±0.3
C20:0	**Arachidic**	5.5±0.1	5.4±0.2	4.6±0.1	5.0±0.0	5.6±0.3	5.0±0.2	3.8±0.2	5.8±0.7	5.2±0.1	5.4±0.4	6.0±0.2	4.7±0.1	4.7±0.3
C20:1 n-9	**Gondoic**	4.7±0.2	4.6±0.0	3.9±0.2	3.9±0.0	4.4±0.5	4.4±0.1	2.9±0.1	5.5±0.6	5.1±0.1	4.7±0.3	5.8±0.1	4.1±0.1	4.0±0.4
C22:0	**Behenic**	3.6±0.	2.0±0.	1.9±0.	2.1±0.	3.0±0.	2.9±0.	2.1±0.	2.2±0.	2.1±0.	2.4±0.	3.5±0.	2.3±0.	2.3±0.

		1	1	1	1	1	1	1	3	1	5	2	2	2
C24:0	**Lignoceric**	8.2±0.3	3.6±0.2	3.5±0.1	4.1±0.1	5.9±0.1	5.8±0.2	4.5±0.1	4.0±0.5	3.9±0.2	4.4±1.1	7.0±0.1	4.5±0.1	4.5±0.3
Saturated FA		153±2	153±4	125±3	121±2	158±2	131±6	100±4	164±19	129±4	151±2	177±4	132±2	138±6
Monounsaturated FA		326±4	347±7	282±7	290±2	335±10	299±11	212±7	362±14	325±10	320±2	373±11	279±5	287±6
Polyunsaturated FA		304±5	339±7	271±7	271±2	325±8	282±11	219±8	347±13	305±11	303±2.3	357±11	264±5	273±9
Total FA (mg/g oil)		784±11[a]	839±16[a]	678±17[c]	682±5[c]	818±14[a]	713±29[c]	541±19[d]	873±35[a]	759±25[c]	774±6[c]	907±25[b]	675±14[c]	699±22[c]

Different letters indicate significantly different values ($p<0.05$)

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