EXTRACTION OF ALKYLRESORCINOLS FROM WHEAT BRAN WITH SUPERCRITICAL CO_2

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Abstract

The supercritical fluid extraction (SFE) of wheat bran alkylresorcinols has been studied. Extractions were carried out at 40.0 MPa. The effect of particle size, static extraction pretreatment with supercritical CO_2 (SC-CO_2) and extraction temperature on the extraction kinetics was investigated. The extraction yield increased as the particle size decreased and with temperature. Extraction curves present a faster and linear initial extraction period followed by a slower extraction period. Based on these results the approximate mathematical model of Sovová was successfully applied to describe the extraction curves. The total content of alkylresorcinols was determined and compared with the alkylresorcinol content obtained by conventional organic solvent extraction. Due to the amphiphilic nature of these resorcinolic lipids, the extraction yield was

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higher for polar organic solvents than for SC-CO_2. Characterization of supercritical extracts was also performed by determining the fatty acid composition and antioxidant activity.

Keywords

Supercritical fluid extraction. Wheat Bran. Alkylresorcinols. Sovová's model

1. Introduction

Alkylresorcinols (ARs) are amphiphilic 1,3-dihydroxybenzene derivatives with a long odd-numbered alkyl chain (15-25 carbon atoms) at position 5 of the benzene ring (Ross et al., 2003). ARs are an important class of secondary metabolites that occur in bacteria, algae, mosses, fungi, animals and higher plants (Athukorala et al., 2010). ARs are mainly found in the bran fraction of cereal grains and, consequently, are largely missing in refined cereal flour and conventional cereal products. These compounds represent about 85% of total cereal grain resorcinolic lipids (Francisco et al., 2005a). Among cereal species, the bran fraction of wheat and rye presents high levels of ARs (32-143 and 36-320 mg/100 g dry matter, respectively). ARs have been reviewed as protective antioxidants in biological membranes and as having stimulant or inhibitory effects on some metabolic enzymes (Bondia-Pons et al., 2009).

Traditionally, different organic solvents have been used to extract ARs from the bran fraction of the Gramineae family (Agil et al., 2012; Mattila et al., 2005; Zarnowski and Suzuki, 2004; Zhou and Yu, 2004). However, organic solvent extraction usually requires laborious purification of the extracts.

Francisco et al. (2005a,b) reported, for the first time, the use of supercritical carbon dioxide (SC-CO_2) technology for AR extraction from cereal milling by-products, specifically from rye bran. However, in these studies none of the AR homologues were detected in the extract when pure CO_2 was used within the imposed operative conditions (35 MPa; 55 ºC-70 ºC). Therefore they proposed the use of ethanol as a polar cosolvent to improve the extractability of ARs, upon bran delipidation with pure SC-CO_2. Previous to the extraction with co-solvent a pre-extraction with pure SC-CO_2 was performed to remove a fraction that did not contain ARs. Atukorala et al. (2010) also reported ARs extraction from triticale bran by a two-step SC-CO_2 extraction since at the operating conditions (35 MPa and 70 ºC) trace amounts of ARs were detected when using pure SC-CO_2. Dey and Mikhailopulo (2009) proposed a two-step extraction process to pre-purify ARs during the extraction process from rye bran. In the first step, low concentrations of ethanol co-solvent were used and higher ethanol co-solvent concentrations were used in the second step. However, higher amounts of ARs were removed during the first extraction step. At 70 ºC and 25 MPa, by using 0.06% of ethanol as co-solvent in the first step, 1054 ng AR/g dry mater was obtained; while at lower temperatures (45ºC), by using 10% of ethanol in the second step, 381 ng AR/g dry matter was obtained. Based on these results and on previous results obtained in our laboratory, in this work the extraction capability of pure SC-CO_2 on ARs from wheat bran has been studied. Solvent power of SC-CO_2 has been improved by working at higher pressures than in previous work related with ARs extraction by using SC-CO_2.

Food industry has been always interested in the prevention of the enzymatic browning (EB) which is determined by complex oxidation reactions that are mediated by specific enzymes such as the enzyme tyrosinase (EC 1.14.18.1) that catalyzes the hydroxylation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones (Nicolas et al., 2003). Resorcinolic lipids from cereal bran have shown inhibitory activity of soybean lipoxygenases (Deszcz and Kozubek, 1997) and ARs were found to inhibit digestive enzymes and mushroom tyrosinase (Kozubek and Tyman, 1999; Ross et al., 2004).

The objective of this work is the study of the extraction capability of pure SC-CO_2 on ARs from wheat bran. Extraction curves at different operating parameters, such as particle size, static extraction pretreatment with SC-CO_2, and extraction temperature, have been obtained. The Sovova's mathematical model (Sovová, 2005) was used to describe the extraction kinetics. This way, parameters that could help to a better understanding of the extraction process have been estimated. Characterization and comparison of wheat bran extracts obtained by SC-CO_2 and conventional organic solvent extraction has been performed in terms of their AR and fatty acid profile, antioxidant activity evaluated by different methods and their inhibitory effect on tyrosinase activity.

2. Experimental section

2.1. Raw material

The wheat bran (*Triticum aestivum*, L.) was kindly supplied by HASENOSA S.A (Spain). The particle size distribution of wheat bran was determined by using a vibratory sieve shaker (Cisa model RP.09) and it is reported in Table 1. The moisture

content of the wheat bran, determined by drying in an oven at 105 ºC to constant weight, was found to be around 13 ± 1% (w/w).

2.2. Conventional solvent extraction

The conventional solvent extraction of wheat bran was carried out by using three different solvents, acetone (Merck, 99.9%), ethanol (Merck, ≥99.9%) and petroleum ether (Merck, analytical reagent). Acetone is used in most extraction procedures for ARs isolation (Zarnowski and Suzuki, 2004), ethanol is of interest since it is often used as co-solvent to modify the solvent power of supercritical CO_2 and petroleum ether was considered due to its similar polarity to CO_2.

Conventional solvent extraction experiments are summarized in Table 2. Two organic extraction methods were used: continuous shaking at room temperature and Soxhlet extraction. In the first case (R1 and R2) 4-6 grams of raw material were extracted with 50 mL of solvent (acetone or ethanol) in a glass flask during 24 h. After the extraction time, the extracts were filtered through paper filters and evaporated under vacuum using a rotary evaporator (Heibolph VV2000). In R3 and R4, Soxhlet extractions were done in a Buchi equipment (B-811 model) using 25 extraction cycles to put the sample (1 g) in contact with the solvent (acetone or petroleum ether) at its boiling temperature, followed by rinsing and drying steps. Extraction experiments were replicated twice.

2.3. Supercritical fluid extraction equipment and procedure

The extraction experiments were carried out in a laboratory SFE-plant whose P&I diagram has been previously described (Murga et al., 2003). In a SFE experiment, 6-8 grams of wheat bran were loaded in the extractor (40 mL capacity). Two syringe pumps (ISCO 260 DM), that work alternatively, provide an uninterrupted flow of CO_2

(Carburos metálicos, liquid $CO_2 \geq 99.9$ %) compressed up to the desired operating pressure, 40.0 MPa. The pressurized solvent was pre-heated up to the desired extraction temperature before entering the extractor. The extractor was held in an oven whose temperature is controlled within an accuracy of ± 0.5 ºC. The carbon dioxide flow was set to 1.5 ± 0.3 g/min. Depressurized CO_2 was quantified with a totalizer flow meter. Extraction yield was determined gravimetrically by measuring the extract weight at different time intervals.

Extraction parameters evaluated to study the extraction of wheat bran oil were: particle size, static extraction pretreatment raw material-SC-CO_2 at the extraction pressure and extraction temperature. A total of ten experiments were carried out under different extraction conditions (Table 3). Runs 5 to 8 were performed to evaluate the influence of the particle size on the extraction yield. Runs 8 to 10 were carried out to study the influence of static extraction pretreatment with SC-CO_2. Runs 11 to 14 and run 9 were carried out to determine the effect of extraction temperature. Most of the extractions were replicated twice.

2.4. Analytical methods

Determination of total AR

The total AR content in the extracted material was determined by a colorimetric method based on the use of Fast Blue RR salt (Sampietro et al., 2009). A stock solution of 0.05% Fast Blue RR reagent was used to prepare a working solution by mixing 1 part of stock reagent with 4 parts of methanol. Aliquots (20 µL) of methanol solutions of wheat bran extracts (5 mg/mL) were placed in assay tubes and made up to 200 µL with methanol. Then, 2 mL of the working solution and 10 µL of a 10% K_2SO_4 solution were

132 added to each tube. Absorbance of the reaction mixture was measured at 480 nm
133 (Hitachi U-2000 spectrophotometer) after 20 min. Olivetol (5-pentylresorcinol) was
134 used as internal standard.

135 Determination of AR profile

136 Alkylresorcinols were determined according to a modification of the method proposed
137 by Knödler et al. (2008) using an Agilent HPLC (series 1100) equipped with
138 ChemStation software, a degasser (G1322A), a quaternary pump (G1311A), an
139 autosampler (G1329A), a column oven (G1316A), a diodo array detector (G1315A) and
140 a mass spectrometry detector (G1916A) with an APcI source. The column used was
141 Kromasil C18-5 250 x 4.6 mm that operated at 25 ºC. The mobile phase was methanol
142 (A) and water (B) and the following gradient was used: 2% B to 0% B in 10 min. The
143 total run time was 100 min. The injection volume was 100 μL. All ARs were monitored
144 at 280 nm at a flow rate of 0.6 mL/min.

145 Positive-ion mass spectra of the column eluate compounds were recorded in the range
146 m/z 100‑500. Nitrogen was used both as the drying gas at a flow rate of 10 L/min and
147 as the nebulizing gas at a pressure of 380 Pa. The nebulizer temperature was set at
148 350 ºC and a potential of 4000 V was used on the capillary.

149 Individual compounds were identified by their mass spectra (Knödler et al., 2008) and
150 quantified using a calibration curve of the corresponding standard compounds (≥ 95%,
151 Sigma Aldrich): $C_{21}H_{36}O_2$ (AR-C15), $C_{23}H_{40}O_2$ (AR-C17), $C_{25}H_{44}O_2$ (AR-C19) and
152 $C_{31}H_{56}O_2$ (AR-C25). As it is shown in Figure 1, a linear relationship between the
153 number of carbons of the alkyl chain and the response factor was found and it was used
154 to calculate the response factor of the ARs that were not available.

155 Determination of fatty acids profile

156 The fatty acid profile was determined by the AOAC method (AOAC, 1995). The fatty
157 acid methyl esters were firstly prepared and then analyzed by gas chromatography (GC)
158 in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with
159 an auto-sampler and a flame ionization detector (FID). The separation was carried out
160 with helium (1.8 mL/min) as carrier gas. A fused silica capillary column
161 (OmegawaxTM-320, 30 m×0.32 mm i.d.) was used. The column temperature was
162 programmed starting at a constant temperature of 180 ºC for 20 min, heated to 200 ºC at
163 1 ºC/min, held at 200 ºC for 1 min, heated again to 220 ºC at 5 ºC/min and finally held
164 at 220 ºC for 20 min. A split injector (50:1) at 250 ºC was used. The FID was also
165 heated to 250 ºC. Fatty acid methyl esters were identified by comparison of their
166 retention times with those of chromatographic standards (Sigma Chemical Co.). Their
167 quantification was made by relating the peaks area to the area of an internal standard
168 (methyl tricosanoate) as indicated by the AOAC method (AOAC, 1995). Calibration
169 was made for several pairs formed by the internal standard + chromatographic standards
170 in order to find the corresponding response factors.

171 Determination of antioxidant capacity

172 *FRAP (Ferric Reducing Ability of Plasma) assay*

173 The FRAP assay is used to measure the reductive power of a sample (Benzie and Strain,
174 1996). It is based on increased absorbance at 593 nm due to the formation of tripyridyl-
175 S-triazine (TPTZ) complexes with ferric (II) in the presence of a reductive agent.
176 Briefly, 970 μL of FRAP reagent was mixed with 30 μL of methanol solutions of bran
177 extracts (5 mg/mL). The FRAP reagent was prepared by mixing 25 mL of 0.3 M sodium

178 acetate buffer solution at pH 3.6, 2.5 mL of TPTZ (10 mM), 2.5 mL of $FeCl_3$ (20 mM),
179 and 3 mL of water. The reaction was carried out at 37 ºC during 30 minutes and the
180 absorbance was measured at 593 nm (Hitachi U-2000 spectrophotometer).

181 Methanolic solutions of known Fe (II) obtained with different concentrations of $FeSO_4$
182 were used for calibration.

183 *DPPH assay*

184 Free radical scavenging capacity of wheat bran extracts was evaluated using 2,2-
185 diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) (Brand-Williams et al., 1995). Briefly,
186 980 µL of $DPPH^{\bullet}$ solution (50.7 µM) was mixed with 20 µL of methanol solutions of
187 bran extracts (5 mg/mL). The absorbance at 517 nm was measured (Hitachi U-2000
188 spectrophotometer) against a blank of pure methanol after the reaction was carried out
189 at ambient temperature and darkness for 60 min. Methanolic solutions of known Trolox
190 concentrations were used for calibration.

191 *Inhibition tyrosinase assay*

192 The assay was performed according to the method previously described by Chen et al.
193 (2005) with some modifications. The reaction medium (0.2 mL) contained 0.5 mM L-
194 DOPA (3,4-dihydroxy-L-phenylalanine) in 100 mM phosphate buffer (pH 7), 0.1
195 mg/mL of the enzyme tyrosinase (EC 1.14.18.1) and the wheat bran solutions in
196 dimethyl sulfoxide (DMSO). The bran extract concentrations tested were 10 and
197 20 mg/mL for extracts obtained with acetone by the Soxhlet method and 15 mg/mL for
198 extracts obtained with SC-CO_2. The absorbance at 490 nm was measured during 150 s

(Labsystems Multiskan MS microplate reader). Control assays without extract were carried out in order to determine the percentage of inhibition of the wheat bran extracts.

2.5. Statistical analyses

Statistical analysis were performed using a two-way analyses of ANOVA (Statgraphics Centurion XVI.I) and the least significant difference (LSD) test calculated to a significant level of $\alpha = 0.05$.

3. Results and discussion

3.1. Yields of conventional solvent extraction and characterization of the extracts

The results corresponding to the conventional solvent extractions are shown in Table 2. When acetone was used as solvent (R1 and R3), the shaking method resulted in higher ($p \leq 0.05$) mass of extract but in lower total AR content than the Soxhlet method. According to this result, the antioxidant activity was also higher in the extracts obtained by the Soxhlet method, in spite of the higher temperatures used in this method. This indicates that temperature could be a variable to optimize in the extraction with SC-CO_2.

There was no significant difference ($p \leq 0.05$) between acetone (R1) and ethanol (R2) extracts when comparing the total amount of extract. Zhou and Yu (2004) found that absolute ethanol at room temperature was the solvent least effective among different solvent systems (including 50% acetone solution) for extracting antioxidant agents from wheat bran fractions; while absolute ethanol in Soxhlet was a highly effective extraction method. This confirms the influence of extraction temperature on the antioxidant activity of wheat bran extracts.

When comparing acetone (R3) and petroleum ether (R4) as solvents used in the Soxhlet method, no significant difference ($p \leq 0.05$) were found between the total amount of extract obtained. However, lower levels of total AR content were obtained with petroleum ether. This suggests that non-polar solvents extract fewer ARs than more polar solvents such as acetone. Since ARs are amphiphilic compounds, their solubility in non-polar solvents is relatively low. ARs could not be quantified by HPLC for the extracts obtained by the shaking method due to experimental problems. In any case, for the rest of the extracts, it must be pointed out that the total content of AR obtained by HPLC analysis is nearly twice than by colorimetric method, concluding that the method used to determine the AR content greatly affects their quantification. Differences may be attributed to the different calibration compounds used for each method; olivetol for the colorimetric method and ARs for the HPLC method.

3.2. Influence of process parameters on the extraction yield with SC-CO_2

All the extraction experiments were performed at 40.0 MPa. First, the effect of particle size was determined (Table 3). Figure 2 shows the extraction curves obtained with bran sieved to three different sizes (R5-R7) and with whole bran (R8). The analysis of the extraction curves shows that the initial extraction rate slightly increases as particle size decreases, but after this initial period, for a fixed extraction time, extraction rates are rather similar. This can be due to the higher amount of compounds that can be extracted outside the particles due to the smaller particle size, which would decrease the importance of diffusion compared to convection (Mezzomo et al., 2009). When comparing the smallest particle size (< 500 μm, extraction time = 150 min) and the biggest particle size (> 2 mm, extraction time = 185 min) similar amount of ARs were

obtained in the extracts (Table 3). These results can be related with the work of Zarnowski and Suzuki (2004), who stated that in the extraction of ARs, grounding of grains is not necessary, because ARs are mainly located in a wax cover surrounding the grain. Landberg et al. (2007) also found no difference in AR content or homologue profile in extracts from milled and intact grains when using ethyl acetate as solvent. For the next experiments carried out in this work, wheat bran was used as received, without size screening.

The effect of the exposure time of the wheat bran to SC-CO_2 at the operating pressure without flow of the SC-CO_2 is presented in Figure 3 (R8-R10). In general, a static extraction pretreatment leads to a faster extraction of wheat bran. The extraction curves show that a static extraction pretreatment of 60 minutes improve the initial extraction rate, while longer exposure time does not. Similar results were obtained by Ivanovic et al. (2011) in the extraction of essential oil from oregano and thyme. Further extraction experiments to study the effect of extraction temperature were carried out with 60 minutes of exposure time to SC-CO_2.

The effect of extraction temperature on the extraction yield was evaluated from 40 ºC to 80 ºC at a constant pressure of 40.0 MPa (runs 11-14 and run 9). The results are shown in Figure 4 where it can be observed that the higher the temperature the higher the extraction rate. This fact indicates that, the increase of temperature increases the vapor pressure of the components to be extracted compensating the depletion in SC-CO_2 density. At a fixed extraction time, the extraction yield increases when temperature increases. Analysis of the extracts showed that the total AR content increased when the extraction temperature was increased (Table 3).

3.3. Modeling of the supercritical fluid extraction

In this work, the approximate model proposed by Sovová (2005) was used to describe the experimental extraction curves. This type of model assumes that the solute is regarded as a single pseudo compound. This simplification can lead to some errors since several components are generally involved in the extraction of complex mixtures. Additionally, according to Sovová (2012), the differences in the solubility of extract components can lead to time fractionation as it has been observed in the SFE of essential oils, that is, the more soluble components were preferentially extracted at the beginning and the least soluble components were found in higher concentrations in the extract samples from the final extraction period. In this work, the mathematical modelling of the extraction curves was done for the wheat bran extract, instead of the ARs, since these compounds represent only a low portion of the extract.

In the model of Sovová, the extraction yield is expressed as:

$$e = \frac{E}{N_m} \tag{1}$$

where E is the amount of extract (kg) and N_m the charge of insoluble solid (kg) in the extractor. The dimensionless amount of solvent consumed is obtained by:

$$q = \frac{Q\,t}{N_m} \tag{2}$$

where Q is the solvent flow rate (kg/h) and t the extraction time (h). Based on this model, the extraction curves consist of two parts. During the first one, the easily accessible solute from broken cells is transferred directly to the fluid phase, while in the second one the solute from intact cells diffuses first to broken cells and then to the fluid

phase. This leads to extraction curves with two parts each corresponding to these two mass transfer processes. When solute-matrix interactions take place, the solute never saturates the fluid phase and a smooth transition appears between the first part of the extraction curve and its end (Martín et al., 2011). Equations (3) and (4) proposed by Sovová (2005) were used to evaluate the first and second part of the extraction curve respectively:

$$e = q\frac{K\,x_u}{1+K(\gamma/r)} = qy_o \text{ , for } 0 \le q \le q_c \quad (3)$$

$$e = x_u\left[1 - C_1\exp(C_2 q)\right] \text{ , for } q > q_c \quad (4)$$

where y_o is the slope of the linear part of the curve when the experimental extraction yield, e_{exp}, is plotted vs q, which represent the initial fluid-phase concentration kg solute·kg solvent^{-1}, q_c is the relative amount of the passed solvent when all the solute in broken cells has been extracted, r is the volumetric fraction of broken cells in the particles, so called grinding efficiency, γ is the solvent-to-matrix ratio in the bed (Sovová, 2005):

$$\gamma = \frac{\rho_f \varepsilon}{\rho_s(1-\varepsilon)} \quad (5)$$

where ρ_f is the fluid density at the operating temperature and pressure, ρ_s is the bulk density and ε the bed porosity. The model has two adjustable parameters C_1 and C_2. The partition coefficient, K, and r are obtained simultaneously in the fitting procedure.

$$r = 1 - C_1\exp(-C_2 q_c) \quad (6)$$

307 The constants C_1 and C_2 of the model were obtained by minimizing the root squared
308 mean deviation between experimental and calculated yield (Langa et al., 2009):

309 $$O.F. = \frac{\sum_{i=1}^{n} \left[\left(e_{exp} - e_{calc} \right) / e_{exp} \right]_i^2}{n} \quad (7)$$

310 by using the Simplex-Nelder-Mead method. x_u is the solute concentration in the
311 untreated solid, kg solute/kg insoluble solid. In this work, x_u was evaluated numerically
312 by entering it as an adjustable parameter. As suggested by Martín et al. (2011) it would
313 not have been appropriate to obtain it from other extraction methods, in our case solvent
314 extraction methods, due to the different composition of the extracts. In any case, it must
315 be noticed that the initial value used in the fitting procedure greatly affects the value
316 obtained for x_u. In this work, the initial value considered was the value obtained by
317 extrapolation of the experimental mass of extract vs time under the most favourable
318 conditions used in this work (Martínez et al., 2003).

319 The calculated extraction curves are plotted in Figures 2 to 4. From these Figures a
320 good agreement can be observed between experimental data and model correlation. The
321 mean relative deviations (MRD) between experimental and calculated yields were
322 calculated for each extraction curve:

323 $$MRD = \frac{1}{n} \sum_{i=1}^{n} abs \left(\frac{e_{exp} - e_{calc}}{e_{exp}} \right)_i \cdot 100 \quad (8)$$

324 Fitting parameters and the values of the mean relative deviation along with some mass
325 transfer parameters are collected in Table 4. This Table also shows the $k_s a_s$ values
326 calculated according to the approximate model of Sovová (2005):

$$k_s a_s = \frac{(1-r)(1-\varepsilon)QC_2}{N_m[1-((1-r)C_2/K)]} \quad (9)$$

The value of x_u obtained in the fitting procedure remains more or less constant in all the SFE experiments and above the value reached in any of the SFE experiments. The grinding efficiency, r, increases as the particle size decreases indicating that there are probably more broken cells. The same tendency was observed by Langa et al. (2009) in the SFE of Spanish sage essential oil and by Grosso et al. (2010) in the SFE of volatile oils from different aromatic plants. In contrast, $k_s a_s$ remains more or less constant in the particle size range covered in this work. At constant pressure, $k_s a_s$ increases with temperature although some authors (Martín et al., 2011) found it to increase with CO_2 density.

3.4. Characterization of wheat bran extracts

Fatty acid profile

Extracts of wheat bran obtained with SC-CO_2 contain other compounds apart from the target ARs. The main non-polar lipids compounds in the extracts of wheat bran with SC-CO_2 were fatty acids (607 ± 32 mg/g extract). The fatty acid profile (Table 5) was mainly composed by polyunsaturated fatty acids (around 60%). The majority fatty acid is linoleic acid (C18:2 n-6) followed by oleic acid (C18:1 n-9). Within saturated fatty acids palmitic acid (C16:0) was the most common acid. Fewer amounts of α- linolenic and stearic acids were also presented. Table 5 also reports fatty acid composition of other SC-CO_2 extracts of wheat bran found in the literature (Athukorala et al., 2010; Durante et al., 2012; Kwon et al., 2010). In all cases, the majority fatty acid is linoleic acid (C18:2 n-6) followed by oleic acid (C18:1 n-9).

Comparison of extracts obtained with SC-CO_2 and organic solvents: AR content and AR profile

Extracts obtained using acetone and petroleum ether as solvents by Soxhlet method (R3 and R4) and some of the extracts obtained by SC-CO_2 extraction were analyzed by HPLC to determine the AR profile (Figure 5). No significant differences can be observed in the ARs homologue composition of the extracts obtained by conventional solvent extraction and the extracts obtained by SC-CO_2 extraction. C21:0 homologue has been found to be the most abundant, nearly 50%, followed by C19:0 (25%). Although several factors, such as genetic factors, climate, season, grain filling and soil conditions can affect the AR content (Athukorala et al., 2010), similar AR profile was also found by Landberg et al. (2007) in the extraction of wheat bran (milled) by using ethyl acetate and SC-CO_2.

Even though similar AR profile was obtained by conventional organic solvent extraction and by SC-CO_2 extraction, it must be pointed out that highest AR/extract ratio is obtained when using organic solvents, mainly polar, as acetone. From Table 3, it can be easily obtained that the AR content of the extract obtained with acetone (R3) represents as much as 22% (by HPLC). This percentage decreases down to 9% (by HPLC) when using ether petroleum (R4) as organic solvent. This fact, as it was previously explained, is due to the amphiphilic nature of ARs compounds. However the percentage of ARs obtained when using SC-CO_2 is only 6.5% at the highest temperature studied in this work. Landberg et al. (2007) reported similar values of AR content in dry extract of wheat bran (milled) with ethyl acetate (5.7 ± 0.2 %) and with SC-CO_2 (6.2 ± 0.4 %). In spite of the higher AR yield obtained by Soxhlet acetone method compared to

372 SC-CO_2, solvent extraction presents some disadvantages including long extraction
373 times, toxic waste generation and a more laborious final purification process.

374 Antioxidant activity of wheat bran extracts

375 The relationship between the antioxidant activity measured by the FRAP method and
376 the AR content of the extracts obtained under the different extraction conditions (see
377 Tables 2 and 3) suggests that the antioxidant mechanism of ARs is based on single
378 electron transfer (SET) reactions. Also, an increase of the antioxidant activity evaluated
379 by the DPPH method was observed when the AR content increased in the extracts
380 obtained with organic solvents (Table 2) what is in agreement with Korycinska et al.
381 (2009) who reported a clear relationship between antioxidant activity of breakfast cereal
382 extracts and their total amount of ARs.

383 Inhibition of tyrosinase by wheat bran extracts

384 Tyrosinase is responsible of enzymatic browning, and it may cause undesirable changes
385 in colour, flavour and nutritive value of many foods and beverages (Bajaj et al., 1997;
386 Vivar-Quintana et al., 1999). An inhibition effect of phenolic compounds from
387 *Anacardium occidentale* on the activity of this enzyme has been described (Ross et al.,
388 2004).

389 Some preliminary assays to evaluate the effect of bran extracts on tyrosinase activity
390 were carried out. Both, acetone and SC-CO_2 wheat bran extracts showed and inhibitory
391 effect on tyrosinase activity (Table 6) being slightly higher in the last one. This could be
392 due to the absence of some phenolic compounds such catechin and epicatechin whose
393 solubility in pure SC-CO_2 is reduced (Murga et al., 2000). Bajaj et al. (1997) indicated

394 the repercussion of the interactions among phenolic compounds on the EB of different
395 products. These authors showed that the presence of epicatechin inhibited or stimulated
396 the PPO actions. Epicatechin together with *p*-cumaric and ferulic acid inhibited the
397 oxidation, while combined with clorogenic acid increased the enzymatic browning.

398 **4. Conclusions**

399 SC-CO_2 extraction has been studied to obtain extracts from wheat bran fraction. The
400 influence on extraction yield and extraction quality of some SFE parameters, such as
401 particle size, static extraction pretreatment (0-135 min), and extraction temperature (40 -
402 80 ºC) at a constant extraction pressure of 40.0 MPa was studied. Temperature is one of
403 the most important parameters on the extraction yield, obtaining high amount of extract
404 as well as more AR content and antioxidant capacity when the extraction temperature
405 was 80 ºC. The extraction curves were well represented by the approximate model of
406 Sovová (2005).

407 The SC-CO_2 wheat bran extract has an important content in fatty acids, mainly
408 polyunsaturated, being linoleic acid the majority followed by oleic acid. In general, SC-
409 CO_2 extraction applied to wheat bran results in a lipophilic extract with appreciable AR
410 content and antioxidant capacity. Due to the amphiphilic nature of ARs compounds the
411 ratio AR/extract was higher when extraction was performed with polar organic solvents
412 such as acetone. It can be concluded that a valuable extract rich in ARs has been
413 obtained by SC-CO_2 extraction from a by-product such as wheat bran fraction.

414 **Nomenclature**

415 a_s = specific area between the regions of intact and broken cells (m^{-1})

416 C_1, C_2 = fitting parameters

417 e = extraction yield (kg extract·kg insoluble solid^{-1})

418 E = extract (kg)

419 k_s = solid-phase mass transfer coefficient (s^{-1})

420 K = partition coefficient

421 MRD = mean relative deviation

422 n = number of experimental data

423 N_m= charge of insoluble solid (kg)

424 O.F. = objective function

425 Q = solvent flow rate (kg·h^{-1})

426 q = relative amount of the passed solvent (kg solvent·kg insoluble solid^{-1})

427 q_c = relative amount of the passed solvent when all the solute in broken cells has been

428 extracted (kg solvent·kg insoluble solid^{-1})

429 r = grinding efficiency (fraction of broken cells)

430 t = extraction time (h)

431 x_u = concentration in the untreated solid (kg solute·kg solid insoluble^{-1})

432 y_s = solubility (kg solute·kg solvent^{-1})

433 ρ = density (kg·m^{-3})

434 ε = porosity

435 γ = solvent to matrix ratio in the bed (kg solvent·kg insoluble solid^{-1})

436 Subscripts:

437 exp = experimental

438 calc = calculated

f = fluid

s = solid

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553

554 **Table 1.** Particle size distribution of wheat bran.

Particle size, p	Mass percentage (%)
$p < 0.5$ mm	14.01
0.5 mm $< p <$ 2 mm	81.24
$p >$ 2 mm	4.75

Table 2. Experimental conditions and results obtained for conventional solvent extraction of wheat bran.

Run	Solvent-method	T (ºC)	t (h)	mg extract/ g dry bran	µg AR/g dry bran (colorimetric)	µg AR/g dry bran (HPLC)	µmol Trolox/ g dry bran	µmol Fe (II)/ g dry bran
R1	Acetone-shaking	20	24	42 ± 12^{a}	1725 ± 82^{a}	-	0.59 ± 0.03^{a}	6.4 ± 0.6^{ab}
R2	Ethanol-shaking	20	24	34 ± 1^{ab}	2236 ± 51^{b}	-	0.82 ± 0.07^{b}	5.2 ± 0.5^{a}
R3	Acetone-Soxhlet	56	≈ 3	26 ± 9^{b}	3049 ± 85^{c}	5893 ± 141^{a}	1.59 ± 0.09^{c}	10.4 ± 0.6^{b}
R4	Petroleum ether-Soxhlet	50	≈ 3	24 ± 3^{b}	1287 ± 120^{d}	2217 ± 271^{b}	0.41 ± 0.07^{d}	5.1 ± 0.5^{a}

Values represent mean (n=2) ± standard deviation (SD). Values with different letters in columns are significantly different ($p \leq 0.05$)

Table 3. Experimental conditions and results obtained for SFE of wheat bran.

Run	p (MPa)	T (ºC)	t_c (min)	Raw material	Extraction time (min)	mg extract/ g dry bran	µg AR/g dry bran (colorimetric)	µg AR/g dry bran (HPLC)	µmol Trolox/ g dry bran	µmol Fe (II)/ g dry bran
R5	40.0	40	0	p < 500 µm	150	21.3	448 ± 7	-	-	-
R6	40.0	40	0	p = 0.5-2 mm	215	19 .0	427 ± 3	-	-	-
R7	40.0	40	0	p > 2 mm	185	18.0	440 ± 10	-	-	-
R8	40.0	40	0	without sieving	110	14.3	421 ± 7	-	-	-
R9	40.0	40	60	without sieving	132	18.4	468 ± 10	840 ± 10	0.25 ± 0.01	1.42 ± 0.01
R10	40.0	40	135	without sieving	99	18.1	-	-	-	-
R11	40.0	50	60	without sieving	108	21.5	520 ± 12	912 ± 13	0.29 ± 0.09	2.66 ± 0.03
R12	40.0	60	60	without sieving	210	25.9	618 ± 21	1178 ± 13	0.29 ± 0.06	3.26 ± 0.02
R13	40.0	70	60	without sieving	162	30.6	850 ± 25	1635 ± 36	0.24 ± 0.05	4.06 ± 0.09
R14	40.0	80	60	without sieving	120	34.7	1119 ± 42	2183 ± 86	0.27 ± 0.02	4.91 ± 0.04

Table 4. Values of the parameters obtained with the approximate model of Sovová (2005) and MRD for each experiment.

Run	y_o	K	r	x_u	$k_s a_s$	C_1	C_2	q_c	MRD
R5	0.00160	0.062	0.44	0.0386	$4.5 \cdot 10^{-6}$	0.5869	0.0052	10.0	4.5
R6	0.00136	0.063	0.42	0.0344	$4.0 \cdot 10^{-6}$	0.6215	0.0072	9.6	3.6
R7	0.00111	0.048	0.34	0.0371	$6.6 \cdot 10^{-6}$	0.6993	0.0061	9.5	3.0
R8	0.00124	0.051	0.39	0.0370	$4.5 \cdot 10^{-6}$	0.6429	0.0053	10.4	6.8
R9	0.00175	0.069	0.45	0.0375	$4.8 \cdot 10^{-6}$	0.5845	0.0075	8.0	8.7
R10	0.00203	0.096	0.49	0.0375	$3.2 \cdot 10^{-6}$	0.5393	0.0070	8.4	4.6
R11	0.00186	0.072	0.48	0.0396	$1.1 \cdot 10^{-5}$	0.5754	0.0111	9.1	11.8
R12	0.00220	0.093	0.54	0.0354	$2.0 \cdot 10^{-5}$	0.6496	0.0412	8.6	4.5
R13	0.00291	0.113	0.59	0.0385	$2.9 \cdot 10^{-5}$	0.5496	0.0404	7.2	8.6
R14	0.00367	0.162	0.55	0.0395	$5.7 \cdot 10^{-5}$	0.6286	0.0626	5.4	6.4

Table 5. Fatty acid profile (g/100 g fatty acids) of wheat bran oil obtained with SC-CO_2.

Fatty acid	This work (R9-R14)	(Durante et al., 2012)	(Kwon et al., 2010)	(Athukorala et al., 2010)
Palmitic acid, C16:0	16.9 ± 0.2	19.2 ± 0.3	15.5 - 22.0	21 ± 1
Stearic acid, C18:0	1.9 ± 0.1	1.0 ± 0.1	-	5.0 ± 0.8
Oleic acid, C18:1 n-9	17.4 ± 0.4	27.8 ± 0.7	11.8 – 15.9	22 ± 2
Linoleic acid, C18:2 n-6	56 ± 1	51 ± 1	45.4 – 57.3	46 ± 6
α-linolenic acid, C18:3	5.8 ± 0.1	1.4 ± 0.1	5.7 -8.0	6.0 ± 0.6
others	2.0 ± 0.1	0.15 ± 0.01	-	-

Table 6. Tyrosinase inhibition by wheat bran extracts.

Extraction solvent	mg/mL	% inhibition
Acetone	10	11.2
	20	23.1
$SC\text{-}CO_2$	15	24.2

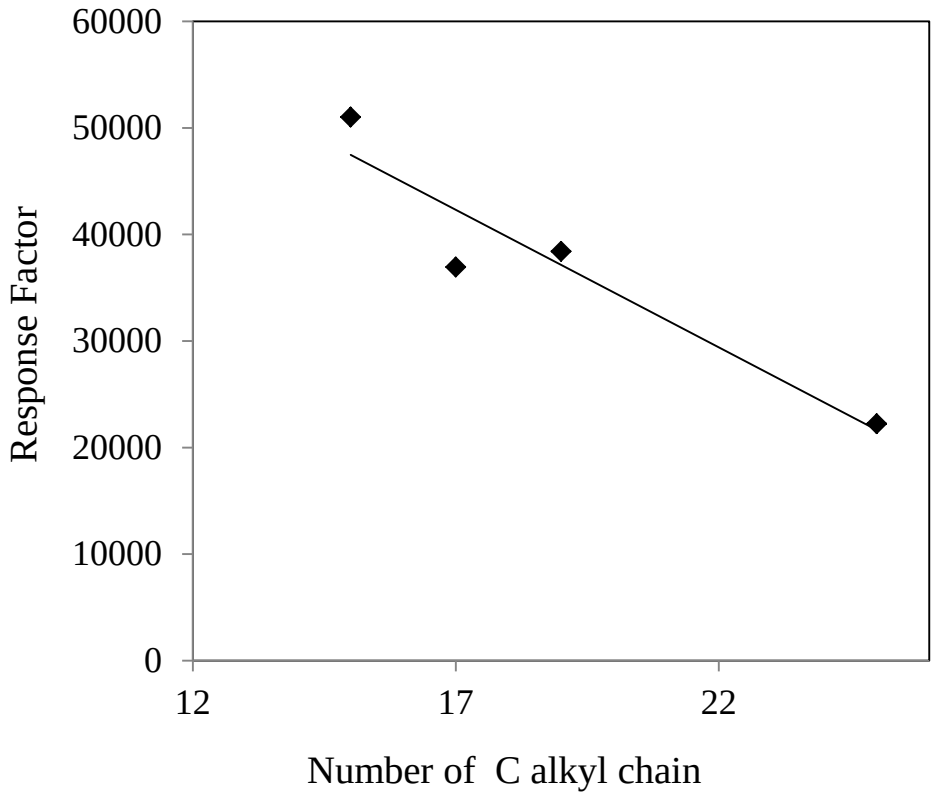

Fig. 1. Relationship between the number of carbons of the alkyl chain of AR and their response factor in HPLC.

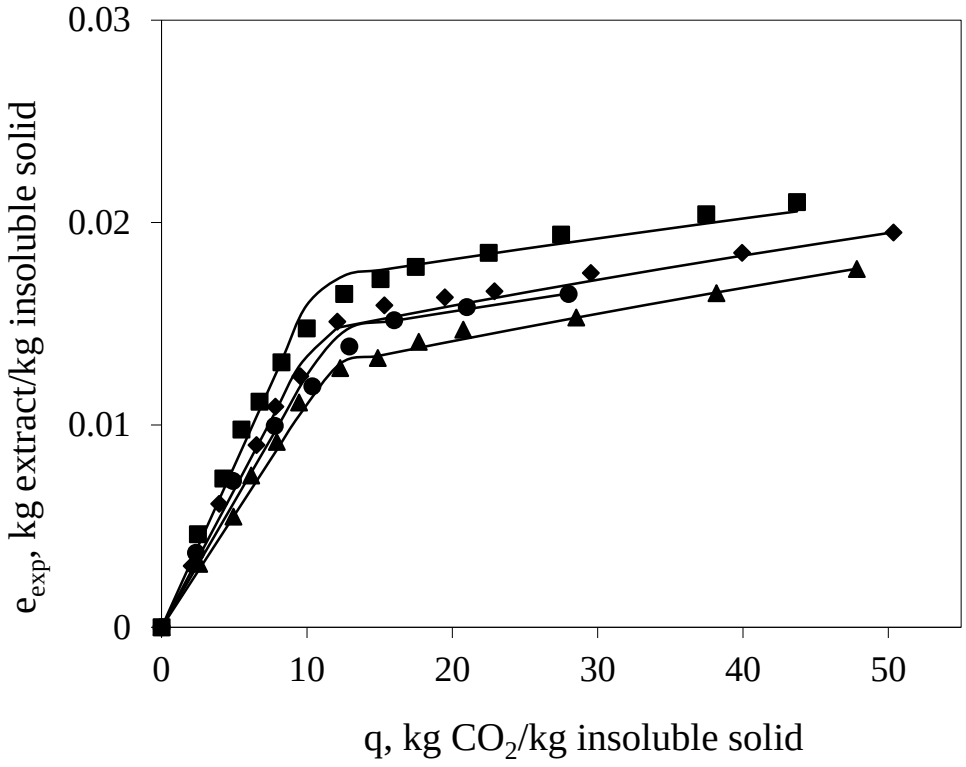

Fig. 2. Influence of particle size on wheat bran extraction yield at a constant pressure of 40 MPa and at a temperature of 40 ºC (■ < 0.5 mm; ♦ 0.5 mm- 2 mm; ▲ > 2 mm; ● without sieving). The solid lines correspond to the model of Sovová (2005).

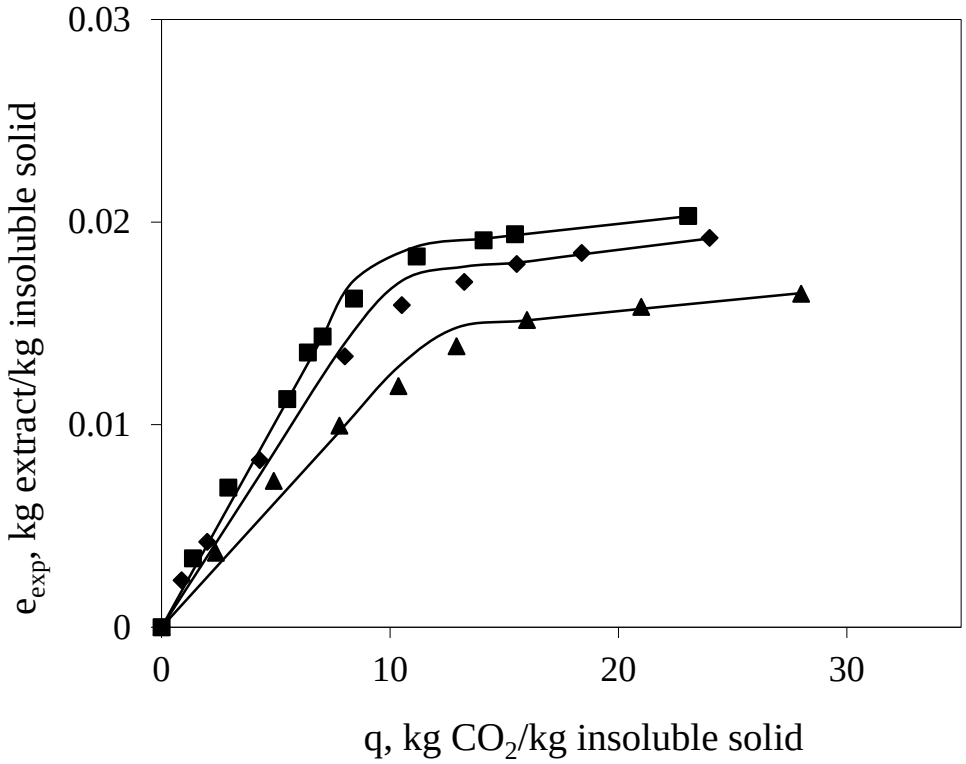

Fig. 3. Influence of static extraction pretreatment on wheat bran extraction yield at a constant pressure of 40 MPa and at a temperature of 40 ºC (■ 135 min; ♦ 60 min; ▲ 0 min). The solid lines correspond to the model of Sovová (2005).

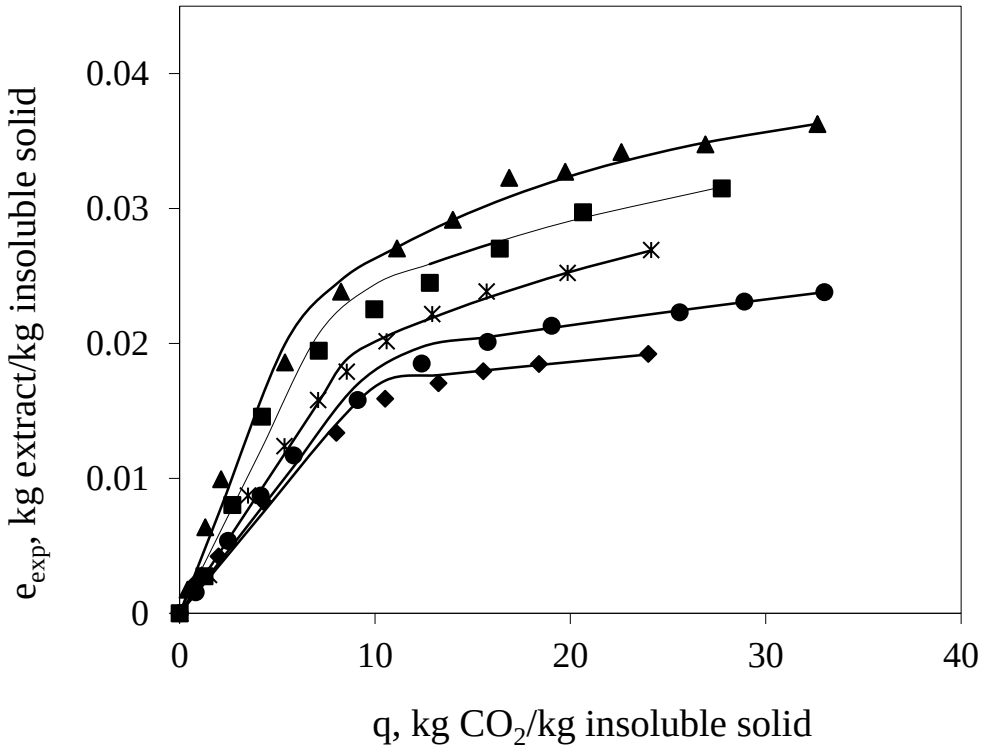

Fig. 4. Influence of extraction temperature on wheat bran extraction yield at a constant pressure of 40 MPa (▲80 ºC; ■ 70 ºC; ∗ 60 ºC; ● 50 ºC; ♦ 40 ºC). The solid lines correspond to the model of Sovová (2005).

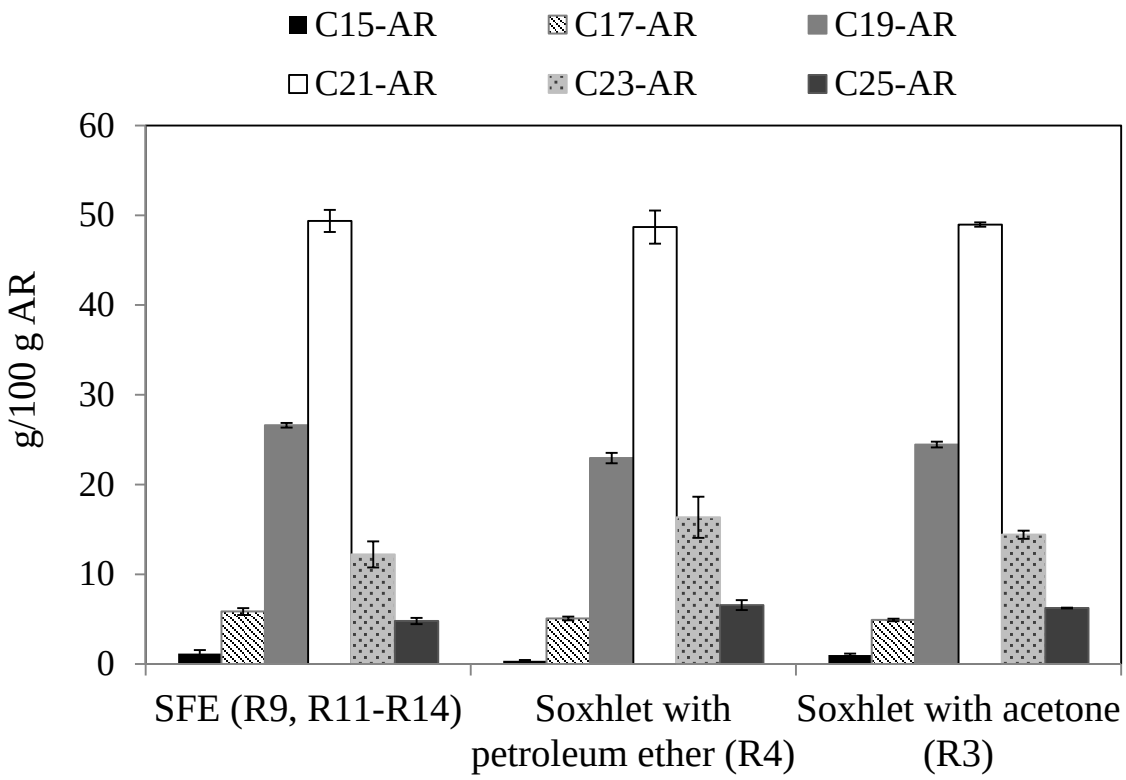

Fig. 5. AR profile obtained by HPLC for different extraction methods and solvents.