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Extraction of Rye Bran by Supercritical Carbon Dioxide: Influence of Temperature, CO_2, and Cosolvent Flow Rates

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Process parameter optimization for the supercritical CO_2 extraction of rye bran to obtain alkylresorcinols (AR) was studied by carrying out a two-level fractional design experiment. Four parameters, temperature, CO_2 flow rate, cosolvent percentage, and extraction time, presumed to influence the extraction process, were analyzed. A tentative fractionation of the crude extract was also carried out and is discussed. The best extracts were achieved when the CO_2 flow rate and extraction time or temperature and cosolvent addition were kept high. It was found that temperature increase was not statistically significant within the range of the study performed, and the extraction time was thus the most important factor. A preliminary fractionation process in two cyclone separators yielded two fractions, one rich in AR components with higher molecular weights and the other rich in AR components with low molecular weight.

KEYWORDS: Rye; extraction; alkylresorcinols; supercritical CO_2; optimization

1. INTRODUCTION

Alkylresorcinols (AR) are phenolic compounds characterized by possessing two hydroxyl groups at positions 1 and 3 of the benzene ring and a long alkyl chain (15−25 carbon atoms) attached at position 5 (*1*, *2*). AR are mostly found in numerous members of the Gramineae family (*2*, *3*). They are mainly concentrated in the bran milling fraction of cereal grains, that is, in wheat, rye, triticale, and barley (*3*−*5*), which are the main sources for human uptake.

The importance of AR resides in the wide range of their biological activities (*6*), for instance, anticancer, antimicrobial (*2*), antiparasitic, antitumor, antioxidant effects (*2*, *6*, *7*) and antileukemic properties (*8*).

Diets rich in fiber such as whole grain cereals, fruits, and vegetables are believed to be linked with a reduced risk of major chronic diseases, cardiovascular and age-related malfunctions (*9*−*13*). However, the components responsible, the metabolism, and the kinetics of absorption are not fully understood yet (*13*, *14*). In the case of AR homologues, for example, it is still difficult to tell which homologue(s) is (are) instantly metabolized and whether the quickly metabolized ones have any bioactivity.

Despite much evidence of the beneficial role of AR (*15*), more work is still required to understand conclusively their effects on health. One of the limitations to carrying out studies involving AR homologues is their unavailability in sufficient amounts due to the low yields and the time required for the isolation methods presently in use. These methods include extraction with acetone, chloroform (*2*, *16*−*18*), or ethyl acetate (*3*, *5*). Concern over solvent residues in extracted products has catalyzed a search for alternative processing methods such as the use of extraction with supercritical fluids. In comparison with the presently used methods, supercritical CO_2 (SC-CO_2) extraction has some important advantages, particularly its ability to achieve products that are completely free from processing residues (*19*).

SC-CO_2 was applied for the extraction of raw rye flakes to obtain a fraction containing AR by our research group (*20*).

SC-CO_2 is obtained by simultaneously compressing and heating CO_2 gas above its critical point (P > 7.3 MPa and T > 31 °C). In such conditions CO_2 has unique physicochemical properties, such as high density and low viscosity, that make it suitable as an extraction solvent. Supercritical conditions can in principle be obtained from any fluid, but CO_2 is the most widely used one because of its nontoxicity, low reactivity, moderate critical temperature and pressure, availability, low cost, and nonflammability. The solvent power of SC-CO_2 depends on its density, and this can be adjusted conveniently by changing the pressure and temperature or by adding cosolvents (*21*). Increasing pressure of the SC-CO_2 raises its density and allows a closer interaction of the fluid molecules with those of the compounds to be extracted. The increase of temperature raises the vapor pressure of the components to be extracted and therefore improves their extractability (*20*).

SC-CO_2 is a poor solvent for polar compounds; therefore, AR could not be extracted from raw rye flakes in the previous study, even at higher pressures and temperature, for example,

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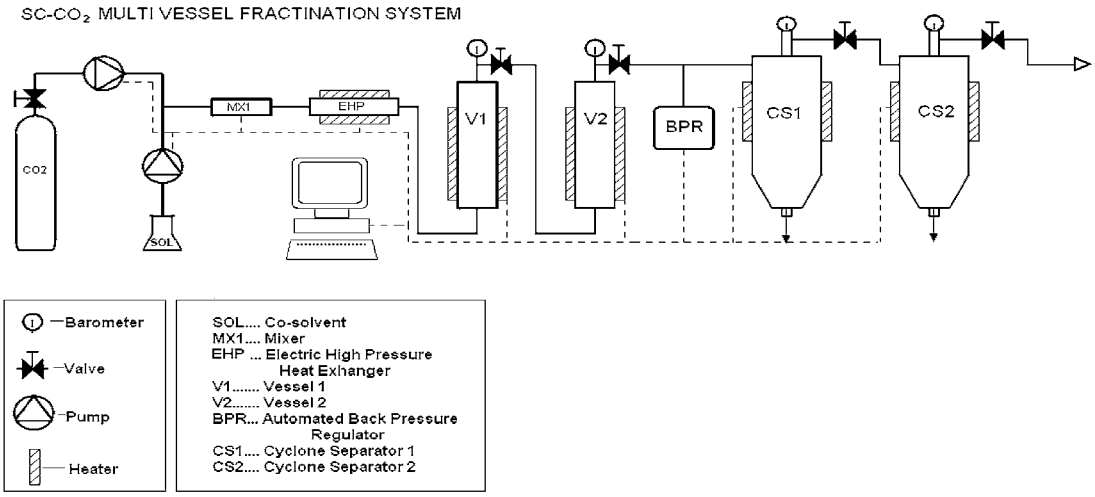

Figure 1. Schematic diagram of the SC-CO_2 system.

35 MPa and 55 °C. However, when combined with cosolvents (methanol or ethanol), SC-CO_2 proved to be more efficient than acetone, used for comparative purposes (*20*). No significant difference was achieved by using either methanol or ethanol on the extraction yield; however, ethanol is preferred in further studies due to its approval in food and pharmaceutical formulas.

A cosolvent is another solvent that is deliberately added to the supercritical fluid to enhance its solvent power, its temperature and pressure dependence, and the separation factor. Generally, a cosolvent with a critical temperature (T_c) higher than that of SC-CO_2 ($T_c = 31$ °C), such as acetone, ethanol, or methanol, ($T_c \approx 235$ °C), increases the solubility of low-volatility components (*22*).

The success of using SC-CO_2 to extract AR from raw rye flakes opens the possibilities to make these bioactive substances easily and readily available for further studies. The supercritical extraction method has been shown to be faster, more productive, and more environmentally friendly than the classical methods (*23*). The initial high investment costs involved in supercritical fluid technology can therefore be paid off in industrial process by the benefits gained by the beneficiaries of the end products.

In the previous study mentioned above, the process parameters for supercritical fluid extraction were not extensively explored. Here we present a process parameter optimization study for extraction using a two-level fractional design experiment. Four parameters that influence the extraction process, temperature, CO_2 flow rate, percent cosolvent, and extraction time, are analyzed. A tentative fractionation of the crude extract is also carried out and discussed. Because this study reports an early stage of method development, attention is concentrated on the search for optimal conditions of its applicability. Definitive quantification of individual AR homologues is not considered. This aspect remains for future studies in progress involving different fractions of rye bran and other cereal materials.

2. MATERIALS AND METHODS

2.1. Materials. Rye bran was obtained as an industrial subproduct from PPHU Vitacorn, Poznan, Poland. Acetone was purchased from Sigma. Ethanol (99.5%) and methanol (HPLC grade) were obtained from Kemetyl AB. The carbon dioxide (≥99.998%) used for extraction was from AGA Gas (Syndbyberg, Sweden). Alkylresorcinol homologues used as standards were isolated chromatographically from rye bran extracts according to the method of Kozubek and Tyman (*18*). Ultrapure water (18 Mohm) was employed in the analysis.

2.2. Methods. *2.2.1. Sample Preparation.* Batches of 30 g of rye bran were ground in a Knifetec 1095 sample mill (Foss) for 10 s under cooling and used in all extractions.

Table 1. Average of the Extracts Obtained in 8 × 4 Factor Variation during Extraction of Alkylresorcinols from Rye Bran by SC-CO_2 and Acetone

	factor analyzed[a]				recovery, mg			
trial	temp, °C	CO_2 flow, g/min	cosolvent, %	time, h	cyclone separator 1	cyclone separator 2	total	R^b
1	–	–	–	–	111.0	214.0	325.0	1.0
2	+	+	–		157.5	272.5	430.0	1.3
3	+	+	+	+	192.0	240.5	432.5	1.3
4	–	–	+	+	216.5	493.5	710.0	2.2
5	–	+	+	–	135.0	267.5	402.5	1.2
6	+	–	–	+	102.5	397.5	500.0	1.5
7	–	+	–	+	307.5	502.5	810.0	2.5
8	+	–	+	–	302.5	512.5	815.0	2.5
acetone	20			24			323.0	1

[a] High level (+): 70 °C, 10 g/min CO_2, 10% cosolvent, 4 h extracting time. Low level (–): 55 °C, 5 g/min CO_2, 5% cosolvent, 2 h extracting time. [b] R = total SC-CO_2 extract/acetone extract.

2.2.2. Extraction with Acetone. The extraction with acetone was carried out following the method described elsewhere (*17*). Twenty-five grams of ground rye bran was extracted overnight with 100 mL of acetone under continuous stirring in an Erlenmeyer flask. The slurry was then filtered through a Büchner funnel with a fiberglass filter. The sediment was additionally rinsed twice with 50 mL of fresh acetone. The filtrates were collected and centrifuged for 10 min at 3500 rpm in a Sorvall RT6000B, H 1000B (Du Pont). The supernatant solution was separated from the precipitate and the solvent evaporated in a Büchi rotavapor R-200. The extracts were weighed and kept in the refrigerator for later analysis. The extraction was performed in duplicates.

2.2.3. Extraction with SC-CO_2. The extraction with supercritical carbon dioxide was performed in an SFE-2X100F system (Thar Technology Inc., Pittsburgh, PA). The system (**Figure 1**) is equipped with two extracting vessels and two cyclone separators with nominal volumes of 100 and 200 mL. Twenty-five grams (25 g) of ground material was placed in the extraction vessel (V2) (**Figure 1**). Carbon dioxide (CO_2) was pumped in V2 up to the desired pressure. After the desired pressure was reached, the cosolvent (sol) was allowed to mix with the CO_2 stream through mix 1 as mass percentage. The mixture CO_2/cosolvent was then preheated by an electric heat exchanger (EHP) and followed to the extraction chamber V2 electrically heated. An automatic back pressure regulator (BPR) maintained the desired pressure constant in V2.

The CO_2 and cosolvent flow rates, the pressure in the pumps and V2, and all temperatures were controlled by system software from Thar Technologies, Inc. The pressures in separator cyclones CS1 and CS2 were controlled by manual back pressure regulators. The extractions were performed in duplicates following the schedule displayed in **Tables 1** and **2**. The operation pressure was maintained constant at 35 MPa as found to be optimal from the previous works (*20*). Higher pressures

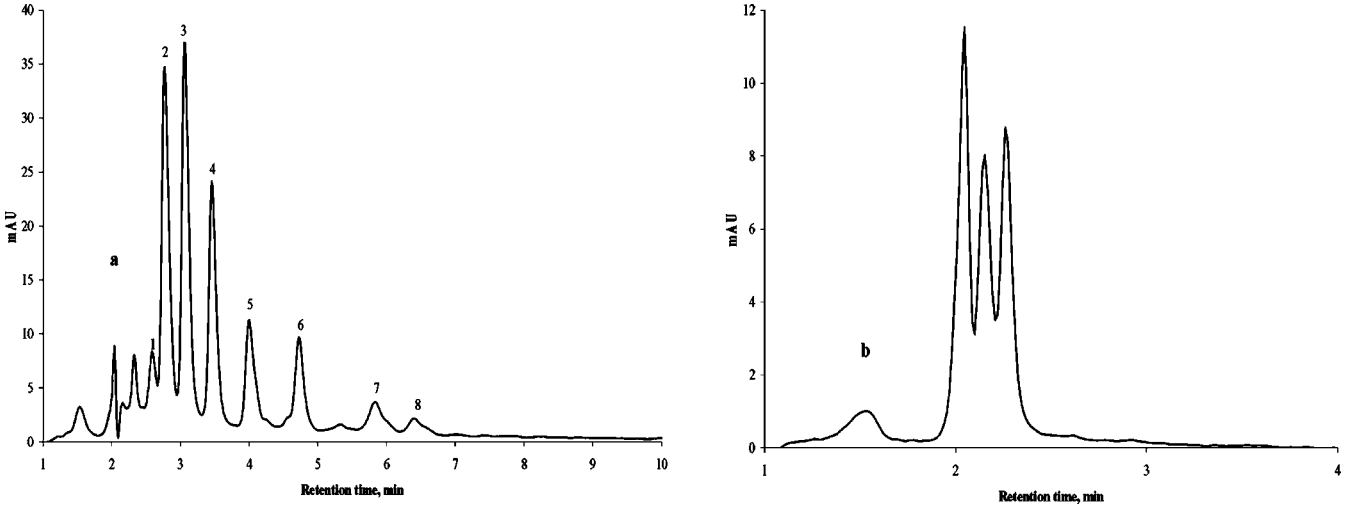

Figure 2. Chronograms of SC-CO_2 with cosolvent extract (**a**) and pure SC-CO_2 extract at 35 MPa and 55 °C (**b**). The numbers 1–8 stand for the alkylresorcinol homologues C_{15}, C_{17}, C_{19}, C_{21}, C_{23}, C_{25}, C_{27}, and C_{29} respectively.

Table 2. Chart of the Ruggedness Test

	factor analyzed[a]				total extract, mg			
trial	temp, °C	CO_2 flow, g/min	cosolvent, %	time, h	replicates		total	difference
1	–	–	–	–	320	330	650	–10
2	+	+	–	–	440	420	860	20
3	+	+	+	+	440	425	865	15
4	–	–	+	+	720	700	1420	20
5	–	+	+	–	395	410	805	–15
6	+	–	–	+	510	490	1000	20
7	–	+	–	+	790	810	1600	–20
8	+	–	+	–	795	815	1610	–20

[a] High level (+): 70 °C, 10 g/min CO_2, 10% cosolvent, 4 h extracting time. Low level (–): 55 °C, 5 g/min CO_2, 5% cosolvent, 2 h extracting time.

were not tried due to the limitation of the equipment used. The temperatures applied were above 40 °C, also on the basis of the previous findings (*20*). The cosolvent used was ethanol. The pressures in the cyclone separators 1 and 2 (**Figure 1**) were kept at 10 and 5 MPa, respectively, and the temperature at 40 °C in both. Two fractions were then collected. The CO_2 leaving the extraction process was vented via the hood to the atmosphere. The extracts obtained with cosolvent were further evaporated as described in section 2.2.2.

2.2.4. Experimental Design for SC-CO_2 Extraction. Prior to the screening of the optimal conditions for extraction, all samples were subjected to a pre-extraction with pure SC-CO_2 at 35 MPa and 70 °C for 4 h. It was observed in previous studies that pure SC-CO_2 was not able to extract AR but other lipids were obtained. When a pre-extraction with pure SC-CO_2 was performed, a fraction that did not contain AR could be removed.

After the pretreatment process, the optimization was carried out as described by Plackett and Burman (*24*). The factors were varied in eight different experiments as shown in **Table 1**. Each variable was tested at two levels: high (+) and low (−). The results obtained were then subjected to a ruggedness test according to the guidelines in ECB Analytica (*25*).

2.2.5. Checking for the Presence of AR in the Extracts. The extract fractions obtained were checked for the presence of AR with an HP 1050 HPLC (Agilent) equipped with a UV detector and an autosampler. A reversed phase column Kromasil 100-5c18 (250 × 4.6 mm) (Akzo Nobel) was used. The UV wavelength was kept at 280 nm. Twenty microliters of analyte dissolved in methanol (1 mg/mL) was injected, and the elution was conducted at ambient temperature. A linear gradient of methanol (94%) and water (6%) up to 25 min and then 100% methanol up to 50 min was used as the mobile phase at a flow rate of 1.5 mL/min. Pure alkylresorcinol homologues are not available commercially. Therefore, the observed peaks in the chromatograms of the extracted samples were identified by comparing the respective retention times with these of the pure homologues chromatographically isolated from rye bran extracts according to the method of Kozubek and Tyman (*18*).

3. RESULTS AND DISCUSSION

3.1. Yield. In classical extraction methods such as Soxhlet, several solvent systems are used in sequence to remove other classes of lipids, for example, triglycerides, phospholipids, and sterols, before ethyl acetate, ethanol, or acetone is used for the extraction of the phenolic fraction (*2*). In this way it is possible to have purer fractions for definitive quantification of the targeted AR. Because in our study the main objective is to demonstrate and find optimal conditions to fraction of extracts from the original matrixes containing AR using SC-CO_2, purification of the fraction obtained by this method was not further made; therefore, the optimization process is based on total weight of the crude extracts. Extraction of rye bran with SC-CO_2 does include substances other than the target alkylresorcinols. This explains partially the high crude extract yields obtained in the experiment. In the present study a pre-extraction of rye bran with pure SC-CO_2 was performed to remove nonpolar lipids that are extractable by the nonpolar SC-CO_2 solvent. The HPLC chromatograms obtained from the fractions obtained by SC-CO_2 with cosolvent and pure SC-CO_2 are shown in parts **a** and **b,** respectively, of **Figure 2**. **Figure 2b** is amplified to show in detail the corresponding section in **Figure 2a** matching the non-alkylresorcinolic fraction extracted with SC-CO_2 with cosolvent. It is also clear that these non-AR lipids are still coextracted when the cosolvent is added. This explains in part the great amounts of extract obtained in **Table 1**. The amounts of extracts obtained by SC-CO_2 extraction after and before the addition of cosolvent were compared gravimetrically. The fractions obtained by extraction with pure SC-CO_2 represented 2−2.5% of the combined extracts before and after the addition of cosolvent.

3.2. Identification of AR. The identification of peaks in HPLC was done by using the standard software. Internal standards of pure (>95%) C_{15}−C_{29} isolated in pure forms from rye bran fraction in the laboratory of A. Kozubek in Wroclaw, Poland, were used. Their identity was confirmed by GC-MS. Some studies concerning the isolation of AR from rye, for example, ref *5*, do not report the presence of C_{27} and C_{29} that were encountered in this study. The existence of such long-chain homologues (C_{27}−C_{29}) has, however, been previously indicated and confirmed in other papers (*18*, *26*). Such differ-

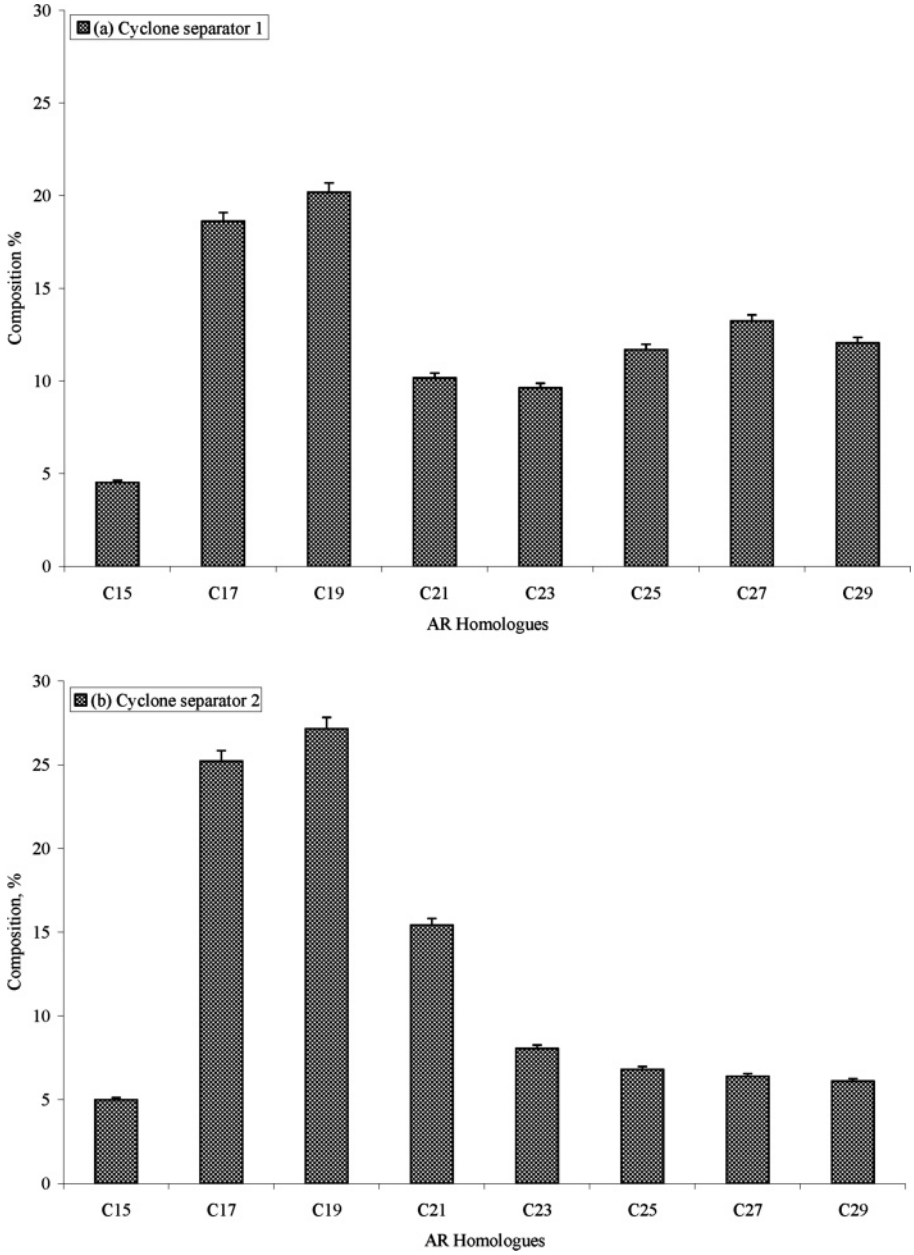

Figure 3. Composition of the fractions obtained in cyclone separators 1 (**a**) and 2 (**b**) in trial 8 expressed as relative percent of the peak area in the chromatogram.

Table 3. Results of the Ruggedness Test

	EBC method results		
factor analyzed	main effect	confidence limit	confidence interval
temp, °C	–12.5	±14.6	–27.1 to 2.1
CO_2 flow, g/min	–63.75		–78.3 to –49.2
cosolvent, %	78.75		64.2 to 93.3
time, h	125		110.4 to139.6

ences in results may be due to the difference in material source or extraction methods or protocols. The recent paper of Zarnowski and Suzuki (*27*) demonstrated, for instance, that depending on the extraction procedure and the solvent used, different amounts of AR could be obtained.

3.3. Recovery of Extracts Containing AR Using SC-CO_2. SC-CO_2 has been previously used for extracting alkylresorcinols from commercial raw rolled rye flakes (*20*). SC-CO_2 extraction was performed at different temperatures and pressures with the addition of cosolvents. At pressures >15 MPa, SC-CO_2 extraction produced greater amounts of crude extract than acetone at ambient temperature and pressure. The highest extracted amounts by SC-CO_2 were observed at 35 MPa and 55 °C with 5% w/w ethanol. At these conditions the amount of AR recovered by SC-CO_2 extraction was 80% higher than that extracted by acetone used in this study for comparison.

To establish optimal conditions for the extraction of alkylresorcinols from rye bran, a two-level factorial design experiment was established. The results obtained are summarized in **Table 1**. For comparative purposes the results on the extraction with acetone are included.

On first inspection it is observed that the highest recoveries were achieved in trials 7 and 8 ($R = 2.5$). These sets of variable combinations correspond to high levels of CO_2 flow rate and extraction time (*7*) and high levels of temperature and cosolvent percentage (*8*).

The four factors that may influence the extraction recovery when using SC-CO_2 were analyzed at two levels by a ruggedness testing method. The chart for the calculations is shown in

Table 2. **Table 3** summarizes the effects, confidence limits at 95%, and confidence intervals.

According to this test the temperature at 95% confidence is not considered to be statistically significant. An increase of the CO_2 flow rate from 5 to 10 g/min reduced the response by an average of 64, meaning that the increase of this factor does not improve the extractability.

The increase of cosolvent percentage from to 5 to 10 leads to an average increase in response of 79, and the increase of extracting time from 2 to 4 h leads to an average increase in response of 125. Cosolvent percentage and extracting time are statistically significant at the 95% confidence level. It might be noted that the magnitude of the extraction time is larger than the magnitude of the cosolvent percentage, indicating that the last factor is more important.

The results of the ruggedness test explain the best recoveries on trials 7 and 8 in **Table 2**. High CO_2 flow rates decrease the residence time of CO_2 in the extraction vessel and, therefore, the interactions between the solvent and the solute. It is logical that long extraction times were the main factor behind the high extract amounts obtained. Because the effect of temperature is not considered to be statistically significant, the only factor responsible for high extract recoveries in trial 8 (**Table 2**) is the cosolvent percentage. In the previous work (*20*) temperature had shown an ambiguous effect. Below 30 MPa, increasing the temperature from 40 to 55 °C decreased the recovery of the alkylresorcinols. At 30 and 35 MPa an increase of temperature in the same range increased the amount of the extract recovered. These results were then attributed to the higher density of the SC-CO_2 and the increase of vapor pressure of the components to be extracted, respectively. For temperatures >55 °C the same pathway as at pressures below 35 MPa is followed. Again, because the temperature increase is not significant according to the ruggedness test, the increase in the cosolvent percentage is the factor responsible for the high amounts of extracts recovered.

3.4. Preliminary Fractionation Process. On-line fractionation of the extracts obtained by SC-CO_2 was attempted in the present study. For this purpose, in all trials performed, the temperature between the cyclone separators was kept constant at 40 °C. The pressures in the first and second cyclone separators were 10 and 5 MPa, respectively. These conditions ensure different supercritical conditions in the extraction vessel and cyclone separator 1 and no supercritical state in cyclone separator 2.

The results obtained (**Table 1**) show that AR were recovered in both cyclone separators. It was also observed that the amounts of extract recovered from cyclone separator 2 (no supercritical conditions) are greater than the amounts of extract recovered from cyclone separator 1. This is understandable as at 100 MPa and 40 °C, CO_2 is in supercritical state and it still holds some solvent power. Thus, only less soluble compounds should be expected to precipitate in cyclone separator 1. In cyclone separator 2, because no supercritical state prevails, all nonvolatile extracted compounds must precipitate. HPLC analysis was performed for all of the fractions collected in cyclone separators 1 and 2. Chromatograms with eight alkylresorcinol homologues were observed (*20*). An illustrative example of the compositions in the two cyclone separators is presented in **Figure 3** based on data obtained from trial 8. From either cyclone separator it is clear that $C_{17}-C_{21}$ AR homologues are the most abundant in rye bran extract. Forty-seven percent and 27% of AR homologues with 23 carbon atoms and higher on the acyl chain have precipitated in cyclone separators 1 and 2, respectively (**Figure 3**). This should be expected because long acyl carbon chains increase the AR homologues' molecular weight and consequently decrease their solubility in the modified SC-CO_2. The presence of all AR homologues in both cyclone separators suggests that the solubility of the AR homologues in modified SC-CO_2 is not very different.

It is nevertheless possible to separate the AR homologues into two fractions, one rich in low molecular weight components and the other rich in high molecular weight ones. Either of these fractions can be used as starting material to obtain preparations of highly pure AR homologues. Depending on the final use, the fractions collected in the cyclone separators can also be considered as final products. A separation of individual AR homologues would require a series of cyclone separators that could discriminate the closely related AR homologues. Such efficient separation could also be achieved in a packed column extraction process performed with a countercurrent flow. The two-cyclone-separator fractionation process still requires further optimization. This task is in process in our group.

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