# Preparative-Scale Supercritical Fluid Extraction/Supercritical Fluid Chromatography of Corn Bran

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**ABSTRACT:** A preparative-scale supercritical fluid extraction/supercritical fluid chromatography (SFE/SFC) procedure has been developed for the removal of oil from corn bran to obtain fractions enriched with free sterols and ferulate-phytosterol esters (FPE). Operational parameters from an analytical-scale supercritical fluid fractionation technique were translated to and optimized on a home-built, preparatory-scale SFE/SFC apparatus. SFE was performed at 34.5 MPa and 40°C using supercritical carbon dioxide. These conditions did not result in exhaustive extraction of the corn bran, but yielded about 96% of the available oil. SFC was conducted in three steps, followed by reconditioning of the sorbent bed. Preparative-scale SFE/SFC of corn bran produced a fraction enriched greater than fourfold in free sterols and 10-fold in FPE, suggesting that such a scheme could be used industrially to produce a functional food ingredient.

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**KEY WORDS:** Carbon dioxide, chromatography, extraction, phytosterols, supercritical fluid.

Plant sterols (phytosterols) are complex alcohols constituted by $C_{28}$ or $C_{29}$ sterols, differing structurally from cholesterol ($C_{27}$) by the addition of an extra methyl or ethyl group on the eight-carbon side chain of cholesterol. Approximately 40–50 different known plant sterols occur naturally in several forms: in the free form, as fatty acid esters, as ferulic or *p*-coumaric esters, and as steryl glycosides, which may also be esterified with a FA. In edible oils and human diets β-sitosterol, campesterol, stigmasterol, and brassicasterol are the major plant sterols. Phytosterols usually constitute less than half of the dietary sterol intake of humans in the United States, the remainder being dietary cholesterol (1).

Phytosterols are present in low concentrations as secondary substances, but their cholesterol-lowering effects have been known since the 1950s (2). Thus, recovering phytosterols and similar high-value components is important not only from a nutritional perspective but also from a commercial point of view to add value to agricultural crops. They are also used as starting materials in the synthesis of steroids for pharmaceutical purposes, as emulsifiers in the cosmetics and food industries, and as a starting material in pesticide manufacturing; they also find individual applications in the field of liquid crystals as used in the optics industry (3).

Recently, plant sterols and plant stanols (hydrogenated forms of the respective sterols) have been incorporated into margarines and vegetable oil spreads. These food products have been shown to lower total and LDL cholesterol levels by 10 to 15% in individuals with high blood cholesterol levels (4–6). These same cholesterol-lowering compounds also have been incorporated into breakfast cereals, cereal bars, and soy beverages (7).

In the mid-1990s, Norton (8,9) reported that corn bran contained ferulate-phytosterol esters (FPE), with sitostanol-ferulate being the most abundant molecular species. Recent clinical studies have demonstrated the cholesterol-lowering properties of free and esterified sitostanol (6). Corn bran is obtained as a by-product from the dry-milling of corn and yields an oil that contains the above-mentioned cholesterol-lowering phytosterols (10). However, these FPE are present at very low levels (1.5 wt%) in the predominantly TAG-based oil. Therefore, enrichment of these moieties would be desirable because they can be used as nutraceuticals, commanding a high value in the functional foods market ($18–20/kg) (11).

Previous reports (12,13) have appeared on the methodology of supercritical fluid extraction (SFE) coupled with supercritical fluid fractionation (SFF) for the enrichment of these FPE. Carbon dioxide ($CO_2$) and ethanol (EtOH), the latter as a cosolvent, were utilized to fractionate and enrich the FPE from 1.25 to 14.5 wt% in using corn bran oil and employing a sorbent bed; however, this prior research was performed on an analytical scale.

In the present study, corn bran SFF technology has been expanded. A preparative-scale procedure has been developed for the SFE/supercritical fluid chromatography (SFE/SFC) of oil from corn bran. The oil is removed from the corn bran by utilizing supercritical carbon dioxide (SC-$CO_2$), then fractionated by on-line SFC to obtain a fraction enriched in FPE.

## MATERIALS AND METHODS

*Corn bran.* Prior to extraction, the corn bran (Illinois Cereal Mills, Inc., Paris, IL) was ground in a Model MC-170 Miracle Mill (Markson Science, Inc., Phoenix, AZ) to a powder in 20-g batches for 15 s. All of the ground corn bran was then combined and stored in a plastic container at −10°C until use. The corn bran oil used in the SFF studies was obtained using SC-$CO_2$ extraction. This procedure has been described previously (12).

*SFF.* Optimization of the SFF processing parameters (modifier percentage and extraction fluid volume) and fraction collection were performed using an Isco Model SFX 3560 (Isco, Inc., Lincoln, NE) automated extractor. The sorbent for the fractionation studies was amino-propyl bonded silica (40 μm;

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Varian Associates, Harbor City, CA), which had been determined as optimal in prior research (12). The sorbent (4.0–4.5 g) was added to a 10-mL extraction vessel, and corn bran oil (1.0–2.0 g) was applied *via* pipette to the top of the sorbent bed. The optimized fractionation procedure could be accomplished in two steps, and multiple trials were run to ascertain repeatability. The first step was accomplished with SC-$CO_2$ at 69.0 MPa and 80°C for 60 min at a flow rate of 2 mL/min (liquid $CO_2$). The extract was collected in a pressurized vial that was cooled to 10°C. The second step was conducted at 34.5 MPa and 40°C using 10 vol% EtOH/$CO_2$ for 180 min at a flow rate of 2 mL/min (liquid $CO_2$). This step required collecting the extract in two vials because of the volume of modifier. Each portion was collected for 90 min in a pressurized and cooled vial. These extracts were combined and concentrated under nitrogen at 70°C. The extracts were dissolved in hexane to a 5 mg/mL concentration and analyzed *via* HPLC.

*Preparative-scale SFE.* Six preparative-scale SFE runs were performed on 175-g batches of corn bran. SFE was conducted at 34.5 MPa and 40°C using 1200 L $CO_2$ (normal pressure and temperature) at a flow rate of 5 L/min. The corn bran extract was collected in a 250-mL round-bottomed flask and analyzed gravimetrically.

*Preparative-scale SFC.* Multiple runs were performed to optimize the SFC stage of the process. Modifier percentage (5–15 mol%) and the total volume of $CO_2$ were the parameters that were investigated to fractionate the corn bran oil and enrich the phytosterols. The chromatographic column was filled with 24 g of the amino-propyl sorbent, and corn bran oil (6.0 g) was applied to the top (inlet) of the column.

Preparative-scale SFC was accomplished in three steps and then the sorbent bed was reconditioned. The first SFC step was conducted with neat $CO_2$ at 69.0 MPa and 80°C, and a 250-mL round-bottomed flask was used for collection. The second and third steps were run at 34.5 MPa and 40°C using 10 mol and 15 mol% EtOH/$CO_2$, respectively. Fractions were collected in 500-mL round-bottomed flasks. Column reconditioning was performed with SC-$CO_2$ at 69.0 MPa and 80°C, and collection was accomplished using a 250-mL round-bottomed flask.

Ethanol collected in the fractions from the second and third SFC steps was removed under reduced pressure at 60°C. The fractions were then redissolved in hexane at a 5 mg/mL concentration, and analysis was performed *via* HPLC. The optimal modifier percentage and $CO_2$ volume were determined based on the isolation and enrichment of the FPE in the fractions.

*SFE/SFC.* The experimental apparatus employed in this study is shown in Figure 1. It is nearly identical in design and scale to a previously described unit (14) with only a few modifications. The chromatographic column hardware was identical to that described by King *et al.* (14); however, in this study, chromatography was performed with 24 g of amino-propyl bonded silica. The SFE cell (Valco Instruments Company, Houston, TX) was 5 cm i.d. × 25 cm length and had a volume of 500 mL. SFE was conducted on corn bran (175 g) with SC-$CO_2$ at 34.5 MPa and 40°C using 600 L $CO_2$ at a flow rate of 5 L/min [standard temperature and pressure (STP)]) for 120 min. The extract-laden stream was directed through the pressure-reducing regulator prior to its deposition onto the head of the chromatographic column. This allowed the pressure to be reduced to 6.2 from 34.5 MPa, allowing the extract to be concentrated at the top of the column without initially eluting any of the extract from the column. In addition, this gradual letdown of the pressure avoided freezing of the regulator from the Joule–Thompson expansion effect. After the set volume of $CO_2$ was used, the extraction was terminated and the extraction cell was bypassed. The $CO_2$ stream was then directed into the column for fractionation of the corn bran extract. SFC was performed in three steps, followed by sorbent bed reconditioning between runs. The SFC experimental parameters are listed in Table 1. Fractions were collected during each SFC step and analyzed *via* HPLC. Round-bottomed flasks were used for collection during SFC. SFC steps one and four employed 250-mL flasks, and the second and third steps used 500-mL flasks. During the SFC steps employing ethanol-modified $CO_2$, multiple collection flasks were required owing to the large volume of ethanol accumulated. Fractions were collected at equal volume intervals of $CO_2$ (500 L). The fractions collected during the second and third SFC steps were combined, respectively, and the EtOH was removed under reduced pressure at 60°C. Each fraction was then reconstituted in hexane at 5 mg/mL for HPLC analysis.

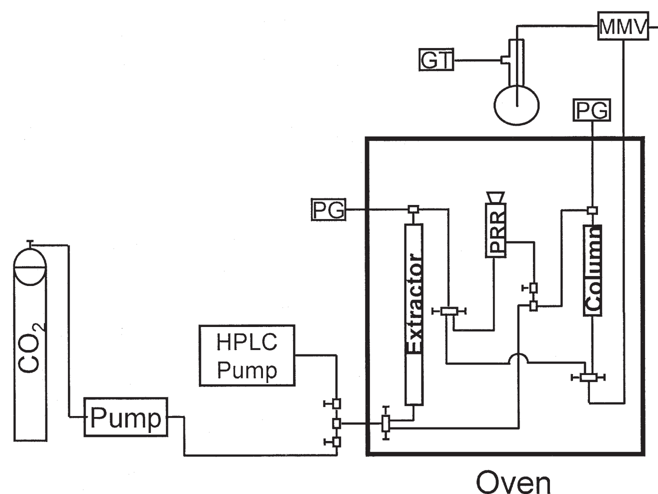


**FIG. 1.** Schematic of supercritical fluid extraction/supercritical fluid chromatography processing system. PG, pressure gauge; GT, gas totalizer; MMV, micrometering valve; PRR, pressure-reducing regulator.

**TABLE 1**
**Operational Parameters for Preparative-Scale Supercritical Fluid Chromatography of Corn Bran Oil**

| Fraction | Pressure (MPa) | Temperature (°C) | Vol. $CO_2$ (L) | Flow rate (L/min) | Ethanol (mol%) |
|---|---|---|---|---|---|
| 1 | 69.0 | 80 | 300 | 4 | $CO_2$ |
| 2 | 34.5 | 40 | 1500 | 4 | 10% EtOH/$CO_2$ |
| 3 | 34.5 | 40 | 900 | 4 | 15% EtOH/$CO_2$ |
| 4[a] | 69.0 | 80 | 400 | 4 | $CO_2$ |

[a]Sorbent reconditioning step.

*HPLC*. HPLC was used to analyze for TAG, free sterols, and FPE contained in the fractions collected during the SFF and the SFE/SFC studies. All analyses were carried out with 15-µL injections of 5 mg/mL solutions. Analyses were performed using a Spectra-Physics SP8800 pump connected to a SpectraSYSTEM AS3000 autosampler equipped with a Rheodyne 7010-151 loop injector (100 µL) (Thermo Separation Products, Inc., San Jose, CA), a Bio-Rad Model 1250424 column heater (Bio-Rad, Inc., Richmond, CA) at 30°C, an Alltech Model 500 ELSD (Alltech Associates, Inc., Deerfield, IL) at 40°C and 1.5 standard liters per minute of $N_2$, and a ChromQuest Chromatography Data System (ThermoQuest, Inc., San Jose, CA). The analytical HPLC column was a Chromsep Cartridge, Lichrosorb DIOL, 5 µm, 3 × 100 mm (Chrompack, Raritan, NJ). The mobile phase was a linear gradient of solvent A (hexane/acetic acid, 1000:1, vol/vol) and solvent B (hexane/2-propanol, 100:1, vol/vol) at a flow rate of 0.5 mL/min. The linear gradient timetable was: at 0 min, 100:0; at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0; at 50 min, 100:0 (%A/%B, respectively).

## RESULTS AND DISCUSSION

*SFF.* Previous SFF studies (12,13) using the SFE/SFC approach were performed on an analytical scale, and were designed to emulate a preparative-scale fractionation process. In this study, solute fractionation was accomplished in two steps. The first step, utilizing neat $CO_2$, removed the majority of the TG and the phytosterol fatty acyl esters. The second elution step was designed for FPE enrichment and collection and was achieved with ethanol-modified $CO_2$.

The initial fractionation experiments were performed utilizing corn bran oil and varying amounts of the amino-propyl bonded silica to check for the possibility of FPE breakthrough. These studies were necessary so that the separation column sorbent bed could be optimized for the preparative-scale SFC of the corn bran extract. It was determined that a 3:1 ratio of sorbent/oil was the minimum ratio for the FPE not to break through, avoiding collection in the first fraction. This finding was scaled up to the preparative-scale SFE/SFC system, and the sorbent/oil ratio was increased to 4:1 for this process. Thus, the FPE would be retained on the sorbent bed during the neat $CO_2$ stage, but they would elute when the ethanol modifier was introduced into the SC-$CO_2$ mobile phase.

*Preparative-scale SFE*. Before preparative-scale SFE/SFC trials were undertaken, it was necessary to conduct experiments for both the SFE and SFC stages in order to optimize the processes. The extraction cell used was chosen for the amount of corn bran that it could hold. The SFE runs yielded an average amount of extract equal to 5.85 g. This equated to an average yield of 3.49 wt% with a relative standard deviation (RSD) of 1.9%. The oil content of the corn bran was also determined in triplicate by AOCS Official Method Ac 3-44 (15), which uses petroleum ether as the extraction solvent in a Butt-type extraction apparatus. The organic solvent extraction yielded an average of 3.50 wt% with an RSD of 2.0%.

These data showed SFE yielding equivalent results to the organic solvent extraction. However, the SFE was time consuming because of the low solubility (~1 wt%) of TAG in $CO_2$ at the cited pressure and temperature. A $CO_2$ volume of 1200 L (STP) was needed for the SFE at a flow rate of 5 L/min, requiring 240 min for the extraction. To operate in an expeditious manner, it was determined to stop the SFE stage after 600 L of $CO_2$ had been used, since SFE at this point yielded ~96% of the total extract. This extraction product was then transferred to the sorbent column for the SFC stage of the SFE/SFC procedure.

*Preparative-scale SFC*. The sorbent/sorbate ratio of 4:1 was adhered to for these optimization experiments, and preparative-scale SFC was accomplished in three steps followed by a sorbent bed reconditioning. The first SFC step removed the majority of the TAG and the phytosterol fatty acyl esters. The second step was designed for maximum FPE enrichment, and the third fraction was run to elute any remaining corn bran extract from the sorbent bed, preventing extract carryover to subsequent runs. Column reconditioning purged the column of any residual ethanol and corn bran oil components and was a necessary step so that the chromatographic sorbent charge could be used multiple times for the SFC of the corn bran extracts.

The cumulative mass collected in the fractions from the SFC runs yielded an average of 4.96 g, which represented an 82.7 wt% recovery of the starting charge of corn bran oil. This is in contrast to earlier research on the analytical-scale SFF of corn bran oil, which exhibited nearly quantitative mass recovery (12). However, this result is not atypical in preparative-scale SFC as evidenced by prior investigators (14,16,17). For example, in the first two studies (14,16) involving the SFC of tocopherols, only partial recovery of the tocopherols (76 to 87%) was obtained from silica gel. Likewise, in a reported preparative-scale SFC of phytochemicals using silica as the sorbent (17), recoveries ranged from 50 to 80%, and one of the isolated compounds exhibited a 95% purity.

The mass discrepancy in the recoveries during the SFC optimization study may be partly due to limitations in the initial weighing of the corn bran oil, which was done on an analytical balance that could weigh to 0.5 g accuracy. The use of this somewhat insensitive balance was dictated by the need to weigh the mass of the chromatographic column that was utilized.

*Preparative-scale SFE/SFC*. Data from the preparative-scale SFE/SFC experiments using corn bran are shown in Table 2. The cumulative mass of the four fractions averaged 5.75 g, which is practically identical to the previously stated mass recovery of 5.85 g obtained during the preparatory-scale SFE studies. The SFE/SFC mass recovery data are more typical than the lower recovered masses noted during the preparatory-scale SFC optimization studies. This further supports the idea that error existed during the SFC optimization trials.

The first SFE/SFC fractions collected had an average mass recovery of 4.93 g, which represents 85.7% of the total extract. HPLC analyses showed that TG made up approximately 93.6% of these fractions. This finding corroborated the

**TABLE 2**
**Average Percentage Composition of Corn Bran Components After Supercritical Fluid Extraction/Supercritical Fluid Chromatography Fractionation[a]**

| Fraction | Mass[b] (g) | TAG[b] | FS[b] | FPE[b] |
|---|---|---|---|---|
| 1 | 4.93 (3.9) | 93.58 (0.6) | 0.27 (8.1) | 0 |
| 2 | 0.79 (5.0) | 6.31 (9.3) | 6.14 (8.5) | 12.88 (3.5) |
| 3 | 0.03 (10.1) | 76.21 (10.4) | 2.09 (11.9) | 2.72 (8.6) |

[a] $n = 4$.
[b] ( ) = Relative standard deviation. FS, free sterols; FPE, ferulate-phytosterol esters.

analytical-scale SFF studies using the 4:1 sorbent/sorbate ratio. In those studies, the first fractions averaged 84.7% of the total extract and TG constituted 94.3% of the fraction.

The second fraction had an average mass recovery of essentially 0.8 g, representing 13.7% of the total extract. FPE comprised almost 13% of the fraction. Thus, the FPE were enriched 10-fold from the initial corn bran oil content of 1.25%. Free sterols also showed a slight enrichment in this fraction, constituting better than 6% of the total mass. This shows a 4.5-fold enrichment of free sterols, which constituted 1.3% of the original corn bran oil.

Fraction 3 had an average mass recovery of 0.03 g, equaling 0.5% of the total extract, and consisted mainly of TG (76%). Free sterols and FPE were also present at 2.1 and 2.7%, respectively. The sorbent column reconditioning steps yielded an average mass of 0.002 g, equaling 0.03% of the total extract. As in our earlier analytical-scale corn bran SFF study (12), extract carryover from one run to the next did not seem to be problematic.

In this study, we have extended the two-step process of SFE coupled with SFC on a preparative-scale to enrich and fractionate high-value nutraceutical components. This SFE/SFC approach has been shown to be applicable for the enhanced recovery of tocopherols (14), phospholipids (18), and now FPE. By using the described process, one can extract the oil from the corn bran, fractionate the majority of the oil away from the FPE, and further enrich the FPE.

The described process uses environmentally benign carbon dioxide and a GRAS cosolvent, ethanol. It provides an alternative to conventional phytosterol extraction, which requires specialized equipment such as fractional or molecular distillation units and their attendant high energy requirements (3). Even without exhaustive extraction of the corn bran, the enrichment data are very encouraging. The reported SFE/SFC system suggests such a scheme could be used industrially to prepare a number of functional food ingredients (19).

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