

Chapter 8

Coupled Processing Options for Agricultural Materials Using Supercritical Fluid Carbon Dioxide

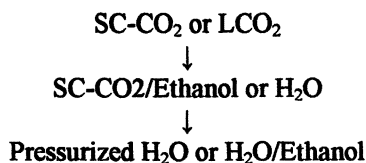
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Supercritical carbon dioxide (SC-CO₂) has been documented numerous times as a benign processing agent, particularly for the processing of agriculturally-derived substrates and foods. However SC-CO₂ alone as a medium for conducting extractions, fractionations, or reactions has certain limitations which can be overcome by coupling it with other processing options. Here combinations of multiple fluids, phases, and processing will be presented that allow a final end result to be achieved. Several examples will be presented of using different fluid compositions, including the use of cosolvents to affect the extraction or enrichment of targeted solutes from complex natural products. In addition, the coupling of supercritical fluid extraction (SFE) with supercritical fluid fractionation (SFF), and/or a reaction conducted in the presence of supercritical media (SFR) will be cited, using specific examples that produce useful industrial products.

Introduction

The application of supercritical fluids (SF) and similar media for the processing of agricultural or natural products has traditionally focused on the extraction mode utilizing carbon dioxide in its supercritical (SC-CO₂) or liquid (LCO₂) state. Beginning in the mid-1980's, options other than varying the extraction fluid's density in the SFE mode were developed, such as columnar and chromatographic techniques, which facilitated SF-derived extracts or products having more specific composition and properties. This development was followed by the advent of conducting reactions (SFR) in the presence of SFs as documented in the literature (1,2). Further examination of alternative fluids, such as subcritical water have expanded the "natural" fluid base available to the processor of agriculturally- derived products. Therefore it should be possible to process natural product matrices utilizing a series of pressurized fluids such as suggested by the sequence below:



The above sequence suggests that some degree of selective solvation should be possible, with SC-CO₂ or LCO₂ extracting non-polar solutes followed by the enhanced solubilization of more polar moieties via the addition of ethanol to the SF. Processing with pressurized water, i.e., subcritical H₂O (sub-H₂O) expands the range of extractable solutes into the polar solute range with selectivity being controlled by the temperature of extraction or addition of ethanol. Depending on the composition or morphology of the natural product being extracted, there is no reason in theory or practice that the above process could not be done in the reverse order. Therefore by combining such discrete unit processes such as SFE, fractionation (SFF), or SFR in various combinations with a matrix of extraction fluids, a number of coupled processing options can be devised yielding unique products.

A more detailed example of this coupled processing concept is cited below in Table I for the processing of citrus oils using pressurized fluids. Here six discrete unit processes are listed which include standard SFE with SC-CO₂, SFF employing stage-wise pressure reduction, SFF as practiced using column-based deterpenation (3), supercritical fluid chromatography (SFC), another variant of SFF called subcritical water deterpenation (4), and utilization of a SC-CO₂ or LCO₂ with a permselective membrane described by Towsley et al.

(5). As shown in Table I, combinations of these processes can be coupled to advantage to allow a total processing scheme to be conducted using critical fluids.

For example, a combination of processes (1) and (2) in Table I could be combined to yield a more specific composition in the final extract. Unit process 1 if conducted by sequentially increasing the extraction density when coupled with a sequence of let down pressures (unit process 2) can amplify the SFF effect. Likewise, by combining unit process 1 using SC-CO₂ followed by application of unit process 2 utilizing subcritical H₂O to deterpenate the extract from unit process 1, can yield a more specific final product from the starting citrus oil. To obtain a more enriched and/or concentrated product from the latter process, one could add on unit process 6, a supercritical fluid membrane-based separation of the aqueous extract/fractions from unit process 5 as indicated below (Table I).

Table I. Coupled Processing Options for Citrus Oils Using Pressurized Fluids

Process:	(1) SFE (SC-CO ₂)
	(2) SFF (SC-CO ₂) – Pressure Reduction
	(3) SFF (SC-CO ₂) – Column Deterpenation
	(4) SFC (SC-CO ₂ /Cosolvent)
	(5) SFF – (Subcritical H ₂ O)
	(6) SFM – (Aqueous Extract/SC-CO ₂)
Combinations:	(1) + (2)
	(1) + (2) + (3)
	(1) + (4)
	(1) + (5)
	(1) + (5) + (6)

Several other combinations of the above unit processes will be discussed shortly. The author and his colleagues have also developed several combinations of processes for the production of oleochemical industrial products (6-9). Two examples of these processes which can link discrete processing steps to advantage are discussed in the next section.

Examples of Coupled Critical Fluid-Based Processes

Recent studies in our laboratory, as well as cost considerations, suggest that there would be a great advantage to employing sub- and supercritical fluid media for multiple processing operations in a sequential fashion in a production plant. This is based on the hypothesis that the capital equipment costs are relatively high to implement a critical fluid-based process, hence multiple applications should distribute the initial costs over an entire production sequence, rather than concentrate the economics on just one unit process.

Toward this end, we have investigated tandem or coupled processes that embodied the use of pressurized fluids, namely carbon dioxide, for both extraction, fractionation and reaction. Related examples to the work described here are coupling supercritical fluid extraction (SFE) with production scale supercritical fluid chromatography (SFC) for the enrichment of high value tocopherols from natural botanical sources (10), or subcritical water hydrolysis of vegetable oils (11) followed by partition into dense carbon dioxide to produce industrially-useful mixtures of fatty acids.

Production of Monoglyceride-Enriched Mixtures

The initial example we wish to cite involves the synthesis of monoglycerides from seed oil botanical sources via glycerolysis in the presence of supercritical carbon dioxide (SC-CO₂) followed by thermal gradient fractionation using SC-CO₂ to produce highly purified monoglycerides of high economic value. Such monoglycerides command not only a premium price but are used extensively as surfactants and food additives (240 million pounds - 1984). They are traditionally made by reacting an excess of glycerol with a vegetable oil in the presence of a metal oxide catalyst at relatively high temperatures (240 -260°C) for a multi-hour period, yielding a composition that is 35-45 wt. % monoglycerides. Such a composition finds utility as a lower grade emulsifier, but in recent years has been supplanted by higher monoglyceride compositions approaching or exceeding 90 wt.%. Such enrichments are usually obtained using molecular or vacuum distillation techniques. Our method utilizes synthesis in the presence of SC-CO₂, first with CO₂ acting as the catalyst, or alternatively a lipase catalyst, to convert the raw vegetable oil to a mixture containing an intermediate level of monoglycerides, followed by further enrichment using a fractionation tower under supercritical conditions. Using this sequential manufacturing approach, we have obtained monoglyceride-containing mixtures from 35 to 95 wt.%, equivalent to those produced by vacuum distillation techniques. The described sequence of

processes makes use of only naturally-derived materials; CO₂, vegetable oils, or enzymes in producing the desired end products, and recycles not only CO₂ for further use as a reaction or fractionating medium, but intermediate glycerolysis products for further conversion to monoglycerides using the enzymatic catalyst described below.

Glycerolysis in the Presence of SC-CO₂

It is important when developing alternative processing techniques to avoid radical changes in operations, consequently we initially investigated the effect of conducting glycerolysis in the presence of SC-CO₂ (6) using a traditional batch stirred reactor approach incorporating an excess of glycerol with several different types of vegetable oils. Carbon dioxide has been shown to be an autocatalytic agent for glycerolysis conducted under ambient conditions (12), but it was not apparent that this conversion could be conducted in the presence of SC-CO₂. Consequently, a ladder of pressures (21 - 62 MPa) and temperatures (150 - 250°C) were used to optimize the glycerolysis process in the presence of SC-CO₂. Maximum production of monoglyceride was found to occur in about 3 hours at 250°C and 21 MPa; higher CO₂ pressures actually suppressed monoglyceride formation. Also, higher yields of monoglycerides were obtained at 21 MPa in the presence of SC-CO₂ than under the ambient conditions noted early. The resultant composition of the product was verified by analytical capillary SFC and found to be somewhat dependent on the starting vegetable oil. Comparison of synthesized monoglyceride mixtures in the range of 35-45 wt.% monoglyceride with an equivalent standard industrial product (Eastman Chemical Co. - Myvatex Mighty Soft Softener) indicated that the product synthesized in the presence of SC-CO₂ was much lighter than the standard industrial monoglyceride mixture, an advantage when using such emulsifiers in food compounding applications. This coupled with the zero solvent residue left in the manufactured product makes the SC-CO₂-based method particularly attractive in synthesizing glyceride compositions intended for human food use.

Perhaps of more importance, is that the SC-CO₂-based synthesis avoids the use of any inorganic catalyst that would have to be filtered out of the oil at the conclusion of a traditional glycerolysis reaction. The jettisoned CO₂ is also available for reuse, or for diversion to other manufacturing processes, such as supercritical fractionation. This makes the above reaction conditions attractive for integrating into an all supercritical fluid-based manufacturing process that is environmentally-compatible.

Enzymatic-Based Glycerolysis of Vegetable Oils in the Presence in SC-CO₂

An alternative route for conducting glycerolysis coupled with supercritical fluid fractionation is to use an enzyme-based catalyst in conjunction with SC-CO₂ to produce enriched monoglyceride mixtures. The use of enzymes for synthetic purposes in the presence of supercritical fluids has been demonstrated, often with model systems in the literature, and we have recently applied this approach for the "green" synthesis of fatty acid methyl esters (FAMES) from vegetable oils such as soybean and corn oil (13), or to synthesize margarine substitutes from the same substrates via intraesterification (14). In most of these systems, including the one to be described, the reaction is conducted by solubilizing the reactants in a flowing stream of supercritical fluid with subsequent transport over a fixed bed of enzyme catalyst. Use of the flow reactor approach provides a large degree of synthetic options to the chemist or engineer since a number of experimental variables can be easily altered and the enzyme continuously reused over multiple cycles. For this process, as well as the other examples cited above, a Novzyme 435 lipase supported on polyacrylamide resin has proven very facile for multiple types of esterifications as noted by King and Turner (15).

Initial glycerolysis experiments were run in the pressure range of 21 - 35 MPa and at temperatures between 40 - 70°C. The optimal pressure and temperature consistent with maintenance of enzymatic activity was found to be 27.6 MPa and 70°C. The vegetable oil feedstock and glycerol were each pumped into the flowing SC-CO₂ stream and the reactants mixed in a tee followed by equilibration in a mixing coil. Passage over the supported enzyme bed in the reactor produced the desired glyceride compositions, solvent-free, having improved color properties over those derived with metallic catalysts. It was found that the Novozym 435 did not lose activity over a 72 hr. period and produced glycerides having a random fatty acid distribution from a parent oil (7).

The advantage of this enzymatic/supercritical fluid mode of synthesis, besides its ecological compatibility, lies in the ability to "custom" design glyceride containing mixtures by varying the reaction conditions. First, it was observed that the resultant monoglyceride yield was dependant on the flow rate of the supercritical fluid containing the reactants throught the reaction vesssel. At low flow rates, compositions having a monoglyceride content between 80-90 wt.% could be produced, while a 20-fold increase in fluid flow rate produced glyceride mixtures that contained between 40-50 wt.% monoglyceride. Secondly, the water content of the reaction mixture was found to have an obvious effect on the monoglyceride content of the resultant glyceride mixture. Hence, if glycerol cotaining a 0.7% wt. water content was used as one of the starting reactants, then a glyceride mixture containing 84 wt.% monoglyceride

could be produced. Likewise, using glycerol with a 4.2 wt.% water content produced a 67% monoglyceride-containing mixture.

Based on reported solubilities of glycols and alcohols in SC-CO₂, it was apparent that synthesis was being conducted in a multi-phase region, where one of the components (CO₂) was in its supercritical state. Despite the lack of phase homogeneity, the glycerolysis could be successfully run in the mixed phase system. A similar observation has also been reported by Gunnlaugsdottir, et al. (16), who like ourselves, observed that a small amount of enzymatic catalyst resulted in a very high production rate of the targeted end products. Other glycols besides glycerol can also be reacted with vegetable oil substrates; e.g., 1,2-propanediol with soybean oil yields predominately monoester, which finds use in the lubricant market. This observation coupled with the above cited impact of moisture and critical fluid flow rate on the yield of monoglyceride in the product mix, offers great flexibility to the synthetic chemist or engineer for producing emulsifiers of specific composition.

Based on the above research, a patent entitled, "Monoglyceride Produced Via Enzymatic Glycerolysis of Oils in Supercritical Carbon Dioxide" was filed and granted by the U.S. patent office, as U.S. Patent 5, 747, 305 (17). This particular synthesis option is one of the two synthetic options that can be coupled with the supercritical fluid fractionation step described below.

Supercritical Fluid Fractionation of Monoglyceride-Containing Synthetic Mixtures

The use of SC-CO₂ as a reaction medium also facilitates movement and introduction of product mixtures to another stage of the production process, namely supercritical fluid fractionation of the glyceride mixtures in a high pressure column held under a thermal gradient to affect a density gradient. This method as a stand alone technique is not new, and has been used for the purification of fish oil esters (18). However coupling it with the synthetic methods just described offers an additional manufacturing dimension, including further incorporation of SC-CO₂ as a processing aid, and ultimately superior or equivalent products to those available commercially.

The technique is described in two publications (8, 18). Briefly, the CO₂ source is delivered to the column after passing through a preheater which converts it to its supercritical state, where it passes upward through a series of increasing temperature zones of 65, 75, 85 and 95°C. This decreases the density of the SC-CO₂ as it passes upwards through the column which is packed with a conventional distillation type of packing. The glyceride feedstock (from the previously described reaction sequences) to be enriched in monoglyceride is then introduced at the top of the first thermally-heated zone

so as to allow equilibration along the column. Fractionation then commences with the introduction of CO₂ flow to yield a purified monoglyceride fraction after leaving the last heated zone through an expansion valve. It should be noted that although CO₂ density and hence solvation power is lost as the fluid travels up the column into zones of increasingly higher temperature, that the vapor pressure, particularly of the more volatile components in the synthetic product reaction mixtures (i.e., the monoglycerides), facilitates their enrichment at the top of the column. This can result in glyceride mixtures that can exceed 95 wt.% in monoglyceride content.

Utilization of the fractionating tower approach when coupled with the aforementioned supercritical synthesis adds considerable additional versatility in the production of glyceride mixtures of varying composition. There is a trade off between fractionating capability and throughput, i.e., monoglyceride compositions exceeding 90 wt.% occur at lower CO₂ densities or pressures, while greater throughput is achieved at higher CO₂ densities at the expense of monoglyceride content. However, this feature does allow the production of designer emulsifiers based on monoglyceride content varying from 60 - 90 wt.% monoglyceride using SC-CO₂ densities up to 0.75 g/ml. One attractive outcome of operating the fractionating tower at peak efficiency is the ability to match or exceed the high monoglyceride content products that are achieved via the conventional vacuum distillation method. This has been verified by analytical capillary SFC and visually (their color and physical state) by inspection of the products. It is also possible by operating the fractionating column with internal reflux, to not only obtain highly purified monoglyceride fractions, but to also obtain a 90 wt.% diglyceride fraction after approximately 60% of the original charge of synthesized product has been processed on the fractionating column. However, it is most likely that the di- and triglyceride-enriched bottom fraction would be preferably transported back to the synthesis stage for further conversion to monoglyceride since it is the more economically-viable product (\$2.46/lb for the 90 wt.% monoglyceride product).

Production of Fatty Alcohol Mixtures

Fatty alcohols and their derivatives are important in many industrial processes where they are used as raw materials for surfactants and lubricants. A fatty alcohol is, in general defined as a monohydric aliphatic alcohol with six or more carbon atoms. The annual production of fatty alcohols is over 1 million metric tons. Commercially fatty alcohols are produced by one of three processes the Ziegler process, the Oxo process or by a high pressure hydrogenation of fatty acids or esters. The latter process is the only one process that uses renewable natural fats/oils whereas the two first processes utilize petrochemical

feedstocks. Depending on their application fatty alcohols are divided into subgroups. Thus fatty alcohols having eleven or more carbon atoms are usually called detergent range alcohols because they are used in the detergent industry mainly as sulfate or ethoxy sulfate derivatives. Fatty alcohols with less than eleven carbon atoms are called plasticizer range alcohols, and they are used as plasticizers and lubricants mainly in the form of ester derivatives.

Here a coupled process is described which involves the synthesis of saturated fatty alcohol mixtures from seed oil botanical sources via two discrete synthetic steps, conducted in the presence of supercritical carbon dioxide (SC-CO₂) or propane (SC-C₃H₈), followed by exhaustive hydrogenation in binary mixtures of hydrogen with the above two supercritical fluids. A number of single-step chemical reactions have been successfully conducted in supercritical fluids, such as esterifications and glycerolysis, however multiple step synthesis in critical fluids are rare. The described process is very environmentally attractive since it uses benign catalysts, recycles the gaseous reaction media and co-products, and utilizes a naturally-occurring starting substrate.

Optimization of the Transesterification Step

Successful transesterification of a naturally-derived oil such as soybean oil requires optimization of several parameters to achieve high yields of methyl esters. The use of an enzyme to catalyze the reaction required screening of several candidate lipases, among which, Novozym SP 435, a lipase derived from *Candida antarctica*, proved successful. Experiments were run to optimize the lifetime of the lipase, and a temperature of 50°C and pressure of 17 MPa proved consistent with the long term use of enzyme (please note that the enzyme could be reactivated even after utilization for over 20 consecutive runs). Addition of the methanol to the SC-CO₂ had to be adjusted to 0.8 mole fraction of fluid flow through the supported enzyme bed in order to maintain 100% enzyme activity under the above conditions. Lower flow rates of the methanol into the SC-CO₂ lowered the efficacy of the lipase, while higher flow rates of methanol also inhibited the reaction. Likewise, the volume percentage of water in the flow system must be kept below 0.05% to achieve greater than a 99% conversion to methyl esters (13) and avoid the competing hydrolysis reaction. It was found that the initial hydration level of the enzyme coupled with the water-carrying capacity of the SC-CO₂ facilitated multiple runs using the above reported lipase. The observed conversions were ascertained using capillary supercritical fluid chromatography (SFC), analysis which showed total conversion to the methyl ester moieties; and gas chromatography (GC) which

confirmed the resultant methyl ester mixture expected for soybean oil as a starting substrate, by comparison to a BF_3 /methylated soybean oil.

Optimization of the Hydrogenation Step

Full hydrogenation of methyl esters formed from vegetable oil substrates has been traditionally achieved through the use of a supported copper chromite-based catalyst. For our hydrogenations, we utilized a non-chromium hydrogenation catalyst, T-4489, from United Catalysts, Inc. The hydrogenation stage of the binary reaction sequence was initially studied separately in order to optimize the hydrogenation step. A device was constructed for generating binary fluid mixtures of hydrogen (H_2) with carbon dioxide, and later propane. This consisted of using digital flow controllers dispensing the proper amounts of each gas into a stirred autoclave, with the resultant mixture then being brought to the various desired hydrogenation pressures using a gas booster pump.

Optimization experiments were initially done using a 2^{5-1} factorial design, which included four center points, resulting in the need for 20 total experiments. The chosen experimental parameters encompassed a pressure range from 15-25 MPa, a temperature range from 210-250°C, and mole fractions of hydrogen in supercritical CO_2 and propane (C_3H_8) from 0.10-0.25. Reaction time in the supported catalyst bed and feed rates of methyl esters to the reactor were also studied in both of the above binary fluid mixtures. It was found from response surface plots, that two variables, namely the reaction temperature and mole fraction of hydrogen in the compressed fluid mixture, were critical to achieving high yields of hydrogenated product.

Using a reaction pressure of 25 MPa, the saturated alcohol mixture yield was over 97% at a 0.25 mole fraction hydrogen in SC- CO_2 (and slightly lower in SC- C_3H_8) at a temperature of 250°C. Analysis of the response surface graph for the $\text{H}_2/\text{C}_3\text{H}_8$ system indicated that a high alcohol conversion could be accomplished using lower mole fractions of hydrogen in propane than for the corresponding $\text{H}_2/\text{SC-CO}_2$ system, however the final product quality was poorer due to the appearance of more n-alkane by-product. At high mole fractions of H_2 , the $\text{H}_2/\text{SC-CO}_2$ system is superior to the $\text{H}_2/\text{SC-C}_3\text{H}_8$ system, in terms of product conversion, i.e., less alkane by-product. However, at low mole fractions of H_2 , the $\text{H}_2/\text{SC-C}_3\text{H}_8$ system gave a higher yield than the $\text{H}_2/\text{SC-CO}_2$ system. Also, the $\text{H}_2/\text{SC-C}_3\text{H}_8$ system has a five-fold greater mass throughput than the $\text{H}_2/\text{SC-CO}_2$ system. This would make the use of propane seem more attractive, however there are always more n-alkane by-products produced when conducting the hydrogenation step using propane, and its incorporation

introduces a potential flammability problem into the synthesis, despite the cited advantages of a propane-based hydrogenation system (19).

Coupling the Transesterification and Hydrogenation Stages

Utilizing the above two optimized reactions systems, with SC-CO₂ as a common solubilization and reaction agent, a coupled reaction system was constructed. The all flow reactor system required pumps for the carbon dioxide, vegetable oil, and methanol, respectively for the initial transesterification stage. Conversion to the methyl esters was achieved at a >98% level before transfer to the hydrogenation reactor.

Conversion of the methyl ester substrate dissolved in the SC-CO₂ is very rapid due to the demonstrated superior mass transfer kinetics which occur in supercritical fluid media relative to rates in condensed liquid media. Calculations of reaction times in the hydrogenation catalyst bed were between 4-9 seconds based on pulse injection experiments. Conversions for the second stage of the overall synthesis were found to average 96.5 %, yielding mixtures consisting of 90% steryl alcohol, approximately 8% palmityl alcohol, and trace levels of unreacted fatty acid methyl esters and n-alkanes. Upon depressurization of synthesized product, the methanol phase separates from the solid alcohol mixture and can be used as feed to the initial transesterification stage. Attrition and lifetime of the hydrogenation catalyst was minimized and extended by using the methyl ester substrate, which also minimizes corrosion to the entire system. Initial charges of hydrogenation catalyst could be used for over two months without requiring a change in the catalyst charge.

The above overall presented process incorporates several features of “green” processing. These are as follows:

1. The use of environmentally-compatible CO₂ as solvent and reaction medium.
2. Utilization of a natural enzyme derived from *Candida antarctica* as a lipase during the transesterification stage.
3. Use of a chromium-free catalyst during the hydrogenation sequence.
4. Incorporation of a natural, renewable resource (vegetable oil) as a starting substrate.
5. Recycling of the product methanol to feed the transesterification stage of the synthesis.

Other benefits of the described process are high yields for both stages of the synthesis using the above agents, long catalyst lifetimes under the stated

conditions, and rapid reaction conditions; particularly in the hydrogenation stage of the sequenced process. Although not demonstrated, the potential for further fractionation of the resultant saturated fatty alcohol mixture using SC-CO₂ via the use of the fractionating tower described in a previous section exists, and will be demonstrated for other lipophilic mixtures in the next section.

Development of New Critical Fluid-Based Coupled Processes

Plant sterols (phytosterols) are complex alcohols constituted by C₂₈ or C₂₉ terols, differing structurally from cholesterol (C₂₇) 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 free fatty acid (FFA). In edible oils and human diets beta-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 (20). Phytosterols are present in low concentrations as secondary substances, but their cholesterol-lowering effects have been known since the 1950s (21). 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 processing agricultural crops.

Phytosterols 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 (22). 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 (23,24). These same cholesterol-lowering compounds also have been incorporated into breakfast cereals, cereal bars, and soy beverages (25). Recent clinical studies have demonstrated the cholesterol-lowering properties of free and esterified sitostanol (24).

One approach to increase the phytosterol ester content of vegetable oils is via refining rather than isolating them from the by-products and then adding them back to the oil (26). Such a processing scheme simplifies the enrichment process and improves the economic feasibility of the production. Dunford and King (27) were able to increase the total phytosterol ester content of rice bran

oil (RBO) and corn fiber oil from <5% to over 19% utilizing the described SFF process. In general, the economic feasibility of industrial operations is higher for continuous processes when compared to batch or semicontinuous processes. Also countercurrent operations tend to be more efficient due to the larger driving force for mass transfer between solvent and solute. Thus, the objective of this new study was to examine the potential of a continuous countercurrent SC-CO₂ fractionation process for enrichment of phytosterols in vegetable oils. In the above processes, initially free fatty acids (FFA) are removed and then a phytosterol-enriched oil fraction is obtained via a second fractionation process. This particular study focused on the retention of phytosterol esters in the rice bran oil during the continuous countercurrent deacidification SFF process.

Corn bran obtained as a by-product from the dry-milling of corn and yields an oil that contains the above mentioned phytosterols (28). However, these ferulate phytosterol esters (FPE) are present at very low levels (1.5 wt%) in the predominately triacylglycerol (TAG)-based oil. Therefore, enrichment of these moieties is desired since they can be used as nutraceuticals, commanding a high value in the functional foods market (\$18–20/kg) (29).

Previous reports (30, 31) have appeared on the use of supercritical fluid extraction (SFE) coupled with supercritical fluid fractionation (SFF) for the enrichment of these FPE. Carbon dioxide (CO₂) and ethanol (EtOH), as a cosolvent, were utilized to fractionate and enrich the FPE from 1.25 to 14.5 wt% in corn bran oil employing a sorbent bed. However, this prior research was performed on an analytical scale. In the present study, SFF technology of corn bran oil has been scaled up using SFE/supercritical fluid chromatography (SFE/SFC). The oil is removed from the corn bran by utilizing supercritical carbon dioxide (SC-CO₂), and then the extract is fractionated by on-line SFC to obtain a fraction enriched in FPE.

Extraction of the berry substrates, such as elderberry or black raspberry, with sub-H₂O offers another discrete process that can be coupled with SFE using SC-CO₂ or perhaps a SFM option to enrich the aqueous extracts. Extractions of anthocyanins are frequently done with ethanol or aqueous ethanolic solvents, and must be done with care due to light-, heat-, and air-sensitivity of anthocyanins. Extraction using sub-H₂O is largely dependent on altering the extraction temperature of the fluid above its normal boiling point while under pressure, thereby changing the dielectric constant of water and hence the solvation power of the fluid [32]. For example, by adjusting temperature and pressure, the dielectric constant of the water at 20°C (~80) can be changed to a value of 48 at 100°C. This is close to the dielectric constant values for furfural (42), glycerol (47) and acetonitrile (38) at 20°C, or methanol (37.5) at 0°C. Hence, sub-H₂O offers an extraction medium that is difficult to match using GRAS (Generally-Regarded-As-Safe) organic solvents and somewhat unique in its extraction characteristics. Evidence of the use of sub-

H₂O in the literature for natural products is provided for the extraction of kava-kava (33), rosemary (34), and savory or peppermint (35).

Experimental

Three distinct processes were experimentally studied: a coupled process for deacidifying and enriching the phytosterol content of rice bran oil (RBO) by continuous countercurrent columnar fractionation, a scale up of a coupled supercritical fluid extraction (SFE)/ supercritical fluid chromatography (SFC) process for the enrichment of phytosterol in corn bran oil, and a unit process involving the subcritical water extraction of berry substrates. The experimental aspects of the first two processes are described in the literature (36, 37), and will not be repeated here. Research is currently underway to couple the described process below with other unit processes involving both subcritical water and supercritical carbon dioxide.

Subcritical Water Extraction of Anthocyanins (ANC) from Berries

The experimental apparatus used to conduct sub-H₂O extraction on berry substrates is shown in Figure 1. It consists of a modified Applied Separations Inc. (Allentown, PA) Spe-ed pumping unit feeding water from a reservoir into a extraction vessel (cell) contained in a thermo-regulated oven (Model 3710A, ATS, Inc., Butler, PA). The extraction cell was a 316 SS, 1" o.d., 9/16" i.d., approximately 55 mL in volume.

As shown in Figure 1, the water is pumped through an equilibration coil contained in the oven to bring it into its subcritical state at temperatures above its normal boiling point under pressure, and then passed through the extraction cell before exiting the oven into a cooling bath reservoir (Model 801, Polyscience, Inc., Niles, IL). Back pressure was maintained on the system with the aid of a micrometering valve which also allowed adjustment of the water flow rate. Aqueous extracts were collected after exiting the micrometering valve.

The first thermocouple in Figure 1 was connected to the temperature controller (Part No. CN4800, Omega Engineering, Stamford, CT) which regulated the oven temperature while the other thermocouples were connected to a digital meter to obtain an accurate reading of the water temperature, both before and after the extraction cell.

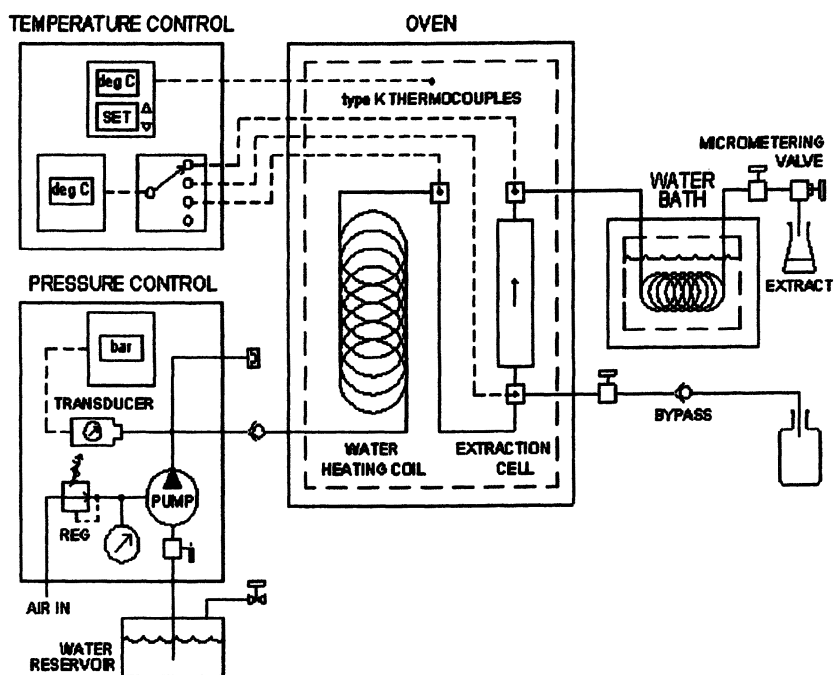


Figure 1. Subcritical water extraction system for extracting anthocyanins from berry substrates.

Extraction Procedure for Berry Substrates

Samples of various fruit berries and their by-products (pomaces) were placed in the extraction cell and the oven heated to temperatures between 110–160°C. Both deionized and Milli-Q-purified neat water as well as acidified water (0.01% HCl, pH ~ 2.3) were fed at a rate of 24 mL/min at a constant pressure of 4.0 MPa. This pressure was well in excess of that required to prevent the formation of steam within the extraction cell. Incremental samples were obtained every 60–80 g of aqueous solution expelled from the extractor over a 40 min time interval, however extracts were not taken until the cell was at the desired extraction temperature and pressure.

Color was monitored visually to an approximate equivalent of 20 ppm of cyanin-3-glucoside (a specific anthocyanin). Extract samples were analyzed by the HPLC procedure described by Skrede et al. (38). The efficiency of the sub-H₂O extraction was compared to results obtained using a 70% ethanolic extract. The control sample was extracted with 70% ethanol in water for 40 min using sonication and washed with excess ethanol to remove any remaining color from the berry substrate. Because of the extreme sensitivity of ANC's to light, heat, and oxygen; all samples were immediately prepared after extraction for injection into the HPLC as described above.

RESULTS AND DISCUSSION

Results from the columnar fractionation of rice bran oil are initially discussed in this section. For this case, one fractionation column was used to obtain the reported results, however these results may be amplified by using an even longer column with more fractionating power, or two columns operating in sequence (SFF-SFF) to accomplish both deacidification and further enrichment of the phytosterol components in rice bran oil (RBO). The reported results for the coupling of SFE-SFC have a similar purpose, namely the enrichment of phytosterol components from corn bran oil on a preparative scale. Finally, initial results on what is envisioned to be an initial stage in the multi-unit processing scheme for berry substrates, namely the subcritical water (sub-H₂O) extraction of ANC's, above the boiling point of water, are reported.

Results from the Columnar Fractionation of Phytosterols

Fractionation experiments were carried out in a continuous countercurrent mode of operation. Initially the column was filled with CO₂ and allowed to

equilibrate at the desired temperature and pressure. Then CO_2 and oil were allowed to flow and fraction collection initiated. Carbon dioxide entered the system from the bottom of the column, right above the raffinate section. In this particular study, oil was delivered into the system from the top of the column so as to allow full countercurrent contact of SC-CO_2 with the feed material. Solute-laden SC-CO_2 then proceeded upward in the column and the resultant extract was collected from the top of the column. RBO oil components, which were not solubilized significantly in the SC-CO_2 accumulating in the raffinate section of the column. The raffinate reservoir was drained in 15-min intervals to avoid overflow of the raffinate fraction into the fractionating section of the column. During a typical SFF experiment, steady state conditions were reached in the column within the first 3 hours of operation. Steady state operation of the column was ascertained by monitoring the weight and composition of the extract fraction collected in 30-minute intervals.

The fractionation experiments were carried out under isobaric and isothermal conditions over the pressure and temperature range of 138–275 bar (13.8–27.5 MPa) and 45–80°C, respectively. Carbon dioxide and oil flow rates were 2 L/min and 0.7 ml/min, respectively, as measured at ambient conditions. After the completion of the experiment the column was depressurized and residual oil drained off at the end of each run. The column was cleaned between runs at a pressure of 34.5 MPa and a temperature of 90°C by flowing CO_2 for more than 6 hours.

Crude RBO was used as starting material for the fractionation experiments. Table II shows the composition of the starting material. It should be noted that FFA composition of the crude RBO is higher (~5%, w/w) than that of the other vegetable oils such as soybean and corn oil (~1–2%, w/w) due to the presence of an active lipase in the rice plant. Hence, high phytosterol and FFA content RBO is an excellent model system applicable to this study. Table III also shows a typical raffinate composition resulting from the continuous columnar SFF process. Note that the oryzanol content of the resultant extract was increased three-fold when half of the FFA were removed from the RBO feed. The phytosterol fatty acid ester composition was also found to be higher than in the feed material, although the StE enrichment was not as significant as that found for oryzanol.

The solute loading of the SC-CO_2 increased with increasing pressure and decreasing temperature. This can be explained by the higher density of SC-CO_2 at higher pressures and lower temperatures, hence the higher solvent power of SC-CO_2 under these conditions. Therefore processing at high pressures and low temperatures requires less solvent (SC-CO_2) and reduces the processing time.

Table II. Composition of the crude rice bran oil and a typical SFF raffinate fraction obtained at 13.8 MPa and 80°C.

<i>Component</i>	<i>Crude RBO (% w/w)</i>	<i>RBO SFF Raffinate Fraction (% w/w)</i>
Free Fatty Acid (FFA)	5 +/- 0.5	2.5 +/- 0.5
Free Phytosterols (St)	0.70 +/- 0.05	0.50 +/- 0.03
Free Fatty Esters of Phytosterols (StE)	2.6 +/- 0.3	2.9 +/- 0.4
Ferulic Acid Esters of Phytosterols (FE)	1.5 +/- 0.3	4.9 +/- 0.04

However, examination of the extract compositions showed that the FFA content of the extracts was lowest at the highest pressure and lowest temperature studied as shown in Figure 2, indicating that SFF fractionation under these conditions is not suitable for efficient FFA removal from the crude oil. This is in part due to the large amount of TAG lost in the extract fraction during high pressure and low temperature processing. For example, there is a higher TAG content in the extracts at a higher pressure and lower temperature (i.e. 60% w/w TAG at 275 bar and 45°C as compared to <10% w/w TAG at 138 bar and 80°C) due to the higher SC-CO₂ density and increased volatility of TAGs. These results are similar to the data obtained from the semicontinuous process reported by Dunford & King (27). These results confirm that the deacidification process should be carried out at lower pressures and high temperatures to expedite FFA removal commensurate with lower TAG loss in the extract.

Coupled SFE/SFC Results

Previous SFF studies (30,31) 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 CO₂, removed the majority of the TAG and the phytosterol fatty acyl esters. The second elution step was designed for FPE enrichment and was achieved with ethanol-modified CO₂.

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

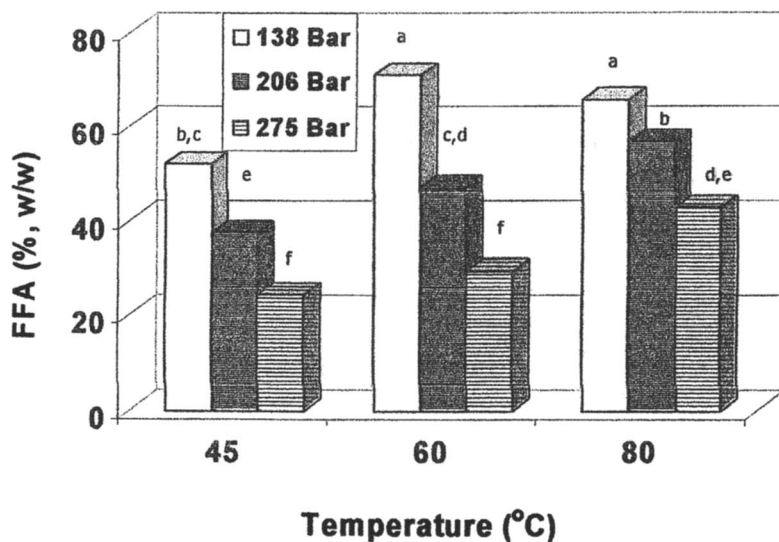


Figure 2. Effect of pressure and temperature on the FFA composition of the columnar SFF extract, i.e., bars with the same letter are not significantly different at the $P > 0.05$ level, e.g., at 138 bar, the wt. % free fatty acid (FFA) content is the same at 60 and 80°C (a), but not the same at 45°C (b,c), etc. (Reproduced from reference 36.)

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 on the sorbent column. This finding was scaled up to the preparative-scale SFE/SFC system, but the sorbent:oil ratio was increased to 4:1 for this process. Thus, FPE are retained on the sorbent bed during the neat CO₂ step, but they elute with the introduction of ethanol modifier into the SC-CO₂ 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 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 the AOCS Official Method Ac 3-44, 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%, in excellent agreement with the SFE result.

However, the SFE was time consuming because of the low solubility (~1 wt%) of TAG in SC-CO₂ at the cited pressure and temperature. A CO₂ volume of 1200 L (STP) was needed for the SFE at a flowrate of 5 L/min, requiring 240 min for the extraction. To operate in an efficient manner, it was determined to stop the SFE stage after 600 L of CO₂ had been used, since SFE at this point yielded ~96% of the available oil. It is this extraction product that 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 as described in the experimental section. 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 column could be used multiple times for the SFC step.

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 (30). However, this result is not atypical in preparative scale SFC as evidenced by prior investigators (10,39,40). For example, in the first two studies (10,39) involving the SFC of tocopherols, only partial recovery of the tocopherols (76 to 87%) was obtained from silica gel.

Coupled Preparative-Scale SFE/SFC

Data from the preparative scale SFE/SFC experiments using corn bran are shown in Table III. 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 is more typical than the lower recovered masses noted during the preparatory-scale SFC optimization studies.

Table III. % Composition of Corn Bran Components After SFE/SFC^{a,b}

<i>Fraction</i>	<i>Mass (grams)</i>	<i>TAG</i>	<i>FS</i>	<i>FPE</i>
1	4.9 (3.9)	93.6 (0.6)	0.27 (8.1)	0
2	0.79 (5.0)	6.3 (9.3)	6.1 (8.5)	12.9 (3.5)
3	0.03 (10.1)	76 (10.4)	2.1 (11.9)	2.7 (8.6)

^a n = 4

^b () = % Relative Standard Deviation

The first collected SFE/SFC fractions 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 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 (30), extract carryover from one run to the next did not seem to be problematic.

In summary, this study demonstrates a two stage coupled process of combining SFE with SFC on a preparative-scale to enrich and fractionate high-value nutraceutical components. By using the above 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. This process provides an alternative to conventional phytosterol extraction, which requires specialized equipment such as fractional or molecular distillation units and their attendant high energy requirements, and in addition, an environmentally-benign process using only CO₂ and ethanol.

Subcritical Water Extraction of Berries

Results for the sub-H₂O extraction of berries are presented below in Tables IV and V for the acidified sub-H₂O extraction of ANCs from berry pomaces, stems, and seeds at 120°C. The results in Table IV indicate that the volume of sub-H₂O required to carry out an equivalent extraction of dried elderberry seeds is much less than when using ethanol as the extraction solvent. Extraction of only 90% of the available ANCs from the same substrate (the 90%+Sub-H₂O results) yields a much more concentrated aqueous extract, but takes only 15 min versus the 40 min extraction times associated with the other results. Not only does this reduce the extraction time, but more than half the volume of the required solvent.

The above trends are also substantiated by the results shown in Table V for the extraction of black raspberry pomace by sub-H₂O. The pomace is the substance left over after the removal of the juice from black raspberries. Here, extraction with ethanol yields an approximately equivalent result to that obtained on dried elderberry seeds (Table IV). The results for extraction with sub-H₂O and 90%+ Sub-H₂O yield less total ANC than in the case of the whole dried elderberry seeds, but this is due to the reduced levels of ANCs found in all pomaces after the juice is expressed.

Table IV. Subcritical Water Extraction of Elderberry Seeds (Dry)

<i>Solvent</i>	<i>mg ANC/g-seed</i>	<i>g- ANC/g-solvent</i>	<i>Ratio</i>
Ethanol	4.76	142	33:1
Sub-H ₂ O	4.34	213	21:1
90% + Sub-H ₂ O	4.17	1853	7:1

ANC = Anthocyanin

Ratio = solvent (fluid)/substrate

Table V. Subcritical Water Extraction of Black Raspberry Pomace.

<i>Solvent</i>	<i>mg ANC/g-seed</i>	<i>g-ANC/g-solvent</i>	<i>Ratio</i>
Ethanol	4.79	141	35:1
Sub-H ₂ O	3.85	137	28:1
90% + Sub-H ₂ O	3.50	237	15:1

ANC = Anthocyanin

Ratio = solvent (fluid)/substrate

Similar encouraging results have been achieved with moist elderberry seeds, elderberry stems, and blueberry pomaces. It should be noted that the dried and moist elderberry substrates contained between 7.4 – 9.3 % moisture, while the raspberry and blueberry pomaces contained approximately 65% moisture. The above recoveries of ANCs at an extraction temperature of 120°C might seem somewhat surprising considering their inherent thermal instability, however calculations of the superficial velocity of sub-H₂O through the extraction cell are very rapid (~0.1 cm/sec), facilitating rapid mass transport of the target solutes (ANCs) from the substrate. One additional benefit of the “hot” water extraction process is the in-situ sterilization of resultant product, thereby potentially avoiding the need for thermal retorting of the final product.

Average percentages of ANCs in the final aqueous extract ranged from 8-10% for the extraction of berry seeds/stems to 2.5-4.5% from the pomaces. Although the tintorial strength of such extracts is high, it would be desirable to further concentrate these extracts for applications in the nutraceutical or functional food areas. This potentially could be accomplished by coupling a SFM process step after sub-H₂O to yield a SFE-SFM coupled process. It should be noted that the use of SFE with SC-CO₂ (neat and with cosolvents) has been reported in the literature for extracting both the oil and enriched polyphenolic

fractions from grapes (41-43). Such results suggest that by combining sequential extractions using SC-CO₂ and sub-H₂O, that an array of useful natural product extracts could be obtained, as noted by author previously (44).

Conclusions

Several total critical fluid coupled processes have been developed by combining discrete SFE, SFF, and SFR steps with SC-CO₂ and other pressurized fluids. Using this approach, a variety of useful target products can be developed that are not accessible when using one critical fluid-based unit process alone. As noted previously, another advantage of using multiple critical fluid processing steps is that it can help offset the capitalization costs that are required in constructing a high pressure processing plant, i.e., permitting more universal application of the SC-CO₂ or alternative fluid delivery and recycle system. Such combining of critical fluid-based processes offers many advantages, including the use of environmentally-compatible processing agents and extracts/products that are free of toxic solvents.

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