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Optimization of a Supercritical Fluid Extraction/Reaction Methodology for the Analysis of Castor Oil Using Experimental Design

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The aim of this work was to optimize a supercritical fluid extraction (SFE)/enzymatic reaction process for the determination of the fatty acid composition of castor seeds. A lipase from *Candida antarctica* (Novozyme 435) was used to catalyze the methanolysis reaction in supercritical carbon dioxide (SC-CO_2). A Box−Behnken statistical design was used to evaluate effects of various values of pressure (200−400 bar), temperature (40−80 °C), methanol concentration (1−5 vol %), and water concentration (0.02−0.18 vol %) on the yield of methylated castor oil. Response surfaces were plotted, and these together with results from some additional experiments produced optimal extraction/reaction conditions for SC-CO_2 at 300 bar and 80 °C, with 7 vol % methanol and 0.02 vol % water. These conditions were used for the determination of the castor oil content expressed as fatty acid methyl esters (FAMEs) in castor seeds. The results obtained were similar to those obtained using conventional methodology based on solvent extraction followed by chemical transmethylation. It was concluded that the methodology developed could be used for the determination of castor oil content as well as composition of individual FAMEs in castor seeds.

KEYWORDS: ***Candida antarctica*; castor; experimental design; hydroxy fatty acid; lipase; *Ricinus communis* L.; SFE**

INTRODUCTION

Oil from castor seeds (*Ricinus communis* L.) is highly viscous as it contains a high amount (∼90 wt %) of the hydroxy fatty acid (FA) ricinoleic acid (C18:1^{c9}-12-OH). Due to its physical properties, the oil has many industrial applications, for example, in the production of synthetic polymers, lubricants, paints, coatings, and cosmetics (*1*). The main drawback to handling castor seeds is that they are extremely toxic because of the presence of a cytotoxic lectin that inhibits protein synthesis in mammalian cells by attacking the ribosome (*2*). Castor seeds also contain a potent allergen, 2*S*-albumin, and therefore caution has to be taken when the oil is extracted from the seeds.

Conventional methodology for the analysis of oil from plant seeds usually includes solvent extraction (*3*) followed by chemical transmethylation of the triacylglycerols (*4*, *5*) or gravimetric analysis. However, in the past several years, research has been conducted on the potential replacement of organic solvent extraction with supercritical fluid extraction (SFE) using pressurized carbon dioxide as solvent (*6*−*9*). Recently, SFE was certified as an official standard method for oil extraction from soybeans as well as the seeds of cotton, canola, safflower, and sunflower (*10*, *11*).

SFE offers many advantages compared to conventional solvent extraction. These include less consumption of hazardous organic solvents, reduction in laboratory labor, higher sample throughput, gentler environment for the analytes (oxygen-free and relatively low temperature), and production of cleaner extracts. Supercritical carbon dioxide (SC-CO_2) can also be used as a medium for lipase-catalyzed reactions (*12*, *13*). The low viscosity and high diffusivity of the supercritical fluid (SF) results in faster reaction kinetics compared to organic solvents such as hexane (*14*). There are several reported applications using different lipases for transesterification (*15*), esterification (*16*), and hydrolysis (*17*) reactions in SC-CO_2. In addition, when lipase-catalyzed transmethylation is achieved simultaneously with dynamic SFE of the oil, there is a considerable advantage with reduced sample handling and shorter analysis time. This has been demonstrated in an analytical context using immobilized *Candida antarctica* lipase type B (Novozyme 435) for the determination of fat composition in meat samples (*18*), oilseeds (*19*, *20*), and vegetable oil soapstocks (*21*).

In this study, a SFE/reaction methodology was developed to convert oil to fatty acid methyl esters (FAMEs) in castor seeds for FA determination by gas chromatography. A Box−Behnken statistical experimental design was employed to evaluate the effects of temperature, pressure, methanol, and water concentra-

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tion on the yield of FAMEs from castor oil. Response surface models were also computed using multiple regression techniques to find the optimal parameters. The optimized SFE methodology was then used for the analysis of oil in castor seeds. The results obtained for the composition and total content of FAMEs from oil in the seeds were compared to those achieved using a conventional method, based on organic solvent extraction followed by acid-catalyzed transmethylation.

MATERIALS AND METHODS

Materials. Immobilized lipase, Novozyme 435 [*Candida antarctica* lipase type B, 10000 propyl laurate units (PLU)/g], was provided from Novozymes A/S (Bagsvaerd, Denmark). Castor seeds were obtained from Prof. Dick Auld, Department of Plant and Soil Science, Texas Technical University. Castor oil, ricinoleic acid methyl ester, trifluoroacetic anhydride, 3-pyridylcarbinol, 4-(dimethylamino)pyridine, and cyclohexane were obtained from Sigma-Aldrich (St. Louis, MO). Nonadecanoic acid methyl ester and GLC-68 FAME standard mixture were obtained from Nu-Chek Prep, Inc. (Elysian, MN). Heptadecanoic acid methyl ester and anhydrous acetyl chloride were purchased from Alltech (Deerfield, IL), and butylated hydroxytoluene (BHT) was obtained from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Hydromatrix was purchased from Varian Inc. (Walnut Creek, CA), and anhydrous sodium sulfate was purchased from J. T. Baker Inc. (Phillipsburg, NJ). 2-Propanol, methanol, hexane, toluene, diethyl ether, and dichloromethane were obtained from Fisher Scientific (Fair Lawn, NJ). Potassium hydroxide, sodium thiosulfate, sodium chloride, and potassium bicarbonate were obtained from Mallinckrodt Laboratory Chemicals (Philipsburg, NJ). Ethanol was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY). The water used was double distilled, and all chemicals and solvents used were of reagent grade. Carbon dioxide of 99.99% purity (Coleman grade) was obtained from Bay Airgas (Emeryville, CA).

Experimental Design and Statistical Analysis. The four experiment factors under study were methanol and water concentration, temperature, and pressure. Values for these parameters were chosen to bracket conditions known to produce FAMEs from oil via SFE (*20*). To test for curvature of the response, three levels of each experimental parameter were required: selected values were 1, 3, and 5 vol % methanol; 0.02, 0.10, and 0.18 vol % water; temperatures of 40, 60, and 80 °C; and pressures of 200, 300, and 400 bar. A Box−Behnken design was chosen to allow estimation of curvature and interaction terms to minimize the number of experiments. This design was generated from the statistical analysis software, SAS ADX (*22*), and required a total of 27 runs, including three center points.

To estimate a prediction surface, response surface regression was fitted to the data using an SAS PROC REG (*23*), with the standard model including linear and quadratic terms and interactions between pairs of factors. To obtain a clearer picture of which factors were interacting, the model was simplified by deletion of the nonsignificant terms, for example, interactive variables involving temperature. Because of the small number of runs, a significance level of $p = 0.10$ was chosen to guard against dropping terms that might be important to the model. All three techniques stepwise; forward selection and backward elimination were used to ensure which parameters were important to the model, thereby increasing confidence in the resulting model.

SFE with in Situ Methylation. *Method Development.* One gram of Novozyme 435 was mixed with 0.5 g of Hydromatrix and added to extraction cells, to form 25-mm high columns. Hydromatrix was then added on top of the enzyme preparation so that the extraction cell was almost full. Fifty milligrams of castor oil was accurately weighed directly onto the Hydromatrix. One milliliter of methanol was thereafter added on top of the oil as an entrainer to improve oil solubility in the SF. The extraction cell was then filled completely with Hydromatrix. Lipids were extracted by employing a fully automated supercritical fluid extractor, model Isco SFX 3560 (Isco Inc., Lincoln, NE). SC-CO_2 at 200−400 bar and 40−80 °C was used as extraction fluid. Methanol was dried over anhydrous sodium sulfate, and a specific amount of water was added. SC-CO_2 containing 1−9 vol % of methanol and 0.02−0.25 vol % of water was investigated in the experiment. The extraction was initiated by a 5-min static extraction followed by a 90-min dynamic extraction at a flow rate of 0.5 mL/min. Modifier was excluded for the last 5 min of the dynamic extraction. The SF flow rate was set to 1.0 mL/min during the last 30 min. FAMEs were collected in a vial with 5 mL of 2-propanol containing 0.01 wt % of BHT. The restrictor temperature was set to 80 °C and the collection temperature to 10 °C. An internal standard (nonadecanoic acid methyl ester in 2-propanol) was added to the collection vials after extraction prior to analysis. Aliquots of 50 μL of these solutions were transferred to small vials, the solvent was blown down with nitrogen, and 1 mL of cyclohexane containing 0.01 wt % of BHT was added.

Application to Castor Seeds. Novozyme 435 was prepared as described above for the method development work. Castor seeds were peeled, and the coatless seeds were thoroughly homogenized using a mortar and pestle. A seed sample of 0.10 g was accurately weighed and mixed with 1 g of Hydromatrix and 1 mL of methanol. The mixture was added to the extraction cell on top of the enzyme preparation, and the cell was filled with Hydromatrix. SC-CO_2 at 300 bar and 80 °C containing 7 vol % of methanol and 0.02 vol % of water was used as extraction fluid. All other parameters and procedures were as described above for method development.

Reference Method. Castor seeds were peeled, and the coatless seeds were thoroughly homogenized using a mortar and pestle. Seed samples of 0.01 g were accurately weighed into 10-mL glass tubes. The lipids were extracted using 2 mL of hexane/2-propanol (8:2) containing 50 μg/mL of BHT. An internal standard (nonadecanoic acid methyl ester) was added, and the extraction commenced at 55 °C for 30 min with manual shaking by hand every 10 min. The resultant extracts were filtered and dried over sodium sulfate, and the solvent was evaporated under nitrogen. The oil weight was determined gravimetrically and 0.5 mL of toluene added, and the lipids were methylated for 1 h at 80 °C using methanolic hydrogen chloride (3 wt %), as described by Christie (*5*). The resulting FAMEs were dissolved in 10 mL of cyclohexane (0.01 wt % of BHT) for GC analysis.

Analysis. Quantitative analysis was carried out by GC-FID using a Hewlett-Packard 6890 GC system with split injection connected to a 7673 automatic liquid sampler (Agilent Technologies, Palo Alto, CA). Separation was achieved on a DB-Wax column (20 m × 0.12 mm i.d., 0.18-μm film thickness) purchased from J&W Scientific, Agilent Technologies. The injector and detector temperatures were 250 and 280 °C, respectively. The column temperature program was 100 °C for 1 min, raised at 5 °C/min to 250 °C, with a final isothermal hold of 1 min. Standard solutions of a mixture of FAMEs including methyl ricinoleate at three different concentrations in the range of 40−400 μg/mL for methyl ricinoleate, and 5−150 μg/mL for the other FAMEs, were used in generating standard calibration curves. Fifty microliters of methyl heptadecanoate (1 mg/mL) was added as internal standard to each 1-mL aliquot of standard sample, and 1-μL injections were made for duplicate determinations.

Identification of peak components was achieved on a Hewlett-Packard 5890 GC system connected to a 5970A mass selective detector (Agilent Technologies). Split injection was applied, and the same type of column and temperature program as described above were used. Comparison to mass spectra of known FAMEs was used for the identification of each peak. In addition, double-bond locations for the unsaturated fatty acids were determined by interpreting spectra from picolinyl derivatives of free fatty acids (FFAs), employing the method of Christie (*5*).

RESULTS AND DISCUSSION

The number of experiments required to investigate the previously noted four parameters at three levels would be 81 (3^4). However, this was reduced to 27 using a Box−Behnken statistical experimental design. The results from this limited number of experiments provided a statistical model, which was used to identify trends for high yields from the extraction/reaction process.

Statistical Analysis. Results from the reduced regression model, using data collected from experiments using the Box−

Table 1. Parameter Estimates for Novozyme 435

variable	DF	estimate	SE	*t* value	Pr > |*t*|
intercept	1	−887.70	515.814	−1.72	0.1015
methanol	1	191.43	85.1369	2.25	0.0366
water	1	−4912.8	2128.42	−2.31	0.0324
temperature	1	2.5882	1.60894	1.61	0.1242
pressure	1	8.5742	2.82802	3.03	0.0069
pressure × pressure	1	−0.01256	0.00432	−2.91	0.0090
pressure × methanol	1	−0.52746	0.27868	−1.89	0.0737
pressure × water	1	14.491	6.96690	2.08	0.0513

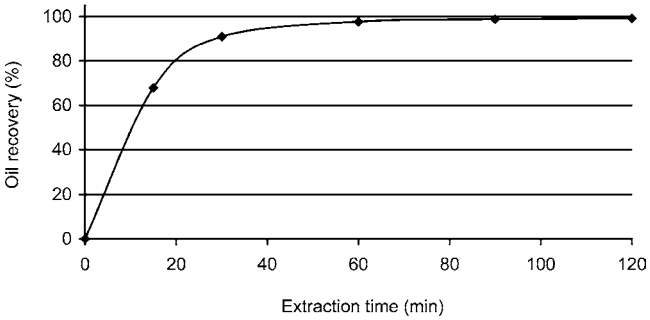

Figure 1. SFE of castor oil with collection in five separate vials after 15, 30, 60, 90, and 120 min of extraction ($n = 2$). SC-CO_2 of 80 °C and 300 bar, containing 5 vol % methanol and 0.10 vol % water, was used as SF.

Behnken design, are shown in **Table 1**. The model for Novozyme 435 has an overall significance probability of 0.0033, indicating that the model had a significant effect ($p < 0.05$) on the level of FAME yields. The R^2 for the model was 0.635, the interpretation of this number being that the model explains 63.5% of the variability among sample FAME results. Pressure appeared to have the most influence on the FAME yield as evidenced by the significant curvature and interactions with both methanol and water concentration. Temperature, with a significance probability >0.10 and which is involved in no interactions, appeared to have only little effect on the FAME yield.

Finding the Optimal Extraction/Reaction Conditions. It was hoped that the use of the Box−Behnken design with response surface regression would yield an optimum set of conditions. Initially, no maximum or minimum response could be found within the design factor ranges used. Therefore, additional experiments were performed in order to find the optimal parameter values (see Tuning of the Method).

Initially, the extraction time required for quantitative extraction of castor oil was studied by collecting extracts at different time intervals. No enzyme was used, and the oil recovery was determined gravimetrically. The results are shown in **Figure 1**. It is clear that 90 min of dynamic extraction at 0.5 mL/min is sufficient to obtain quantitative oil recovery. A flow rate of 1.0 mL/min during the last 30 min was applied in all further experiments to ensure a complete extraction, without risking loss of unreacted triacylglycerols because the concentration of analytes at the end of the extraction is relatively low.

Pressure. The density (and the solvent strength) of a SF is directly proportional to its pressure, and a higher pressure extracts fats and oils better. For the enzymatic reaction, a high solubility of the substrates is important, but at some point the enzyme activity starts to decrease with increasing pressure. This may be due to higher solubility of water in the SF, which causes drying of the enzyme, as well as lower diffusion rates of solutes at higher density (*24*).

In this study, pressures of 200, 300, and 400 bar were applied, and all of the results indicated parabolic shaped pressure curves.

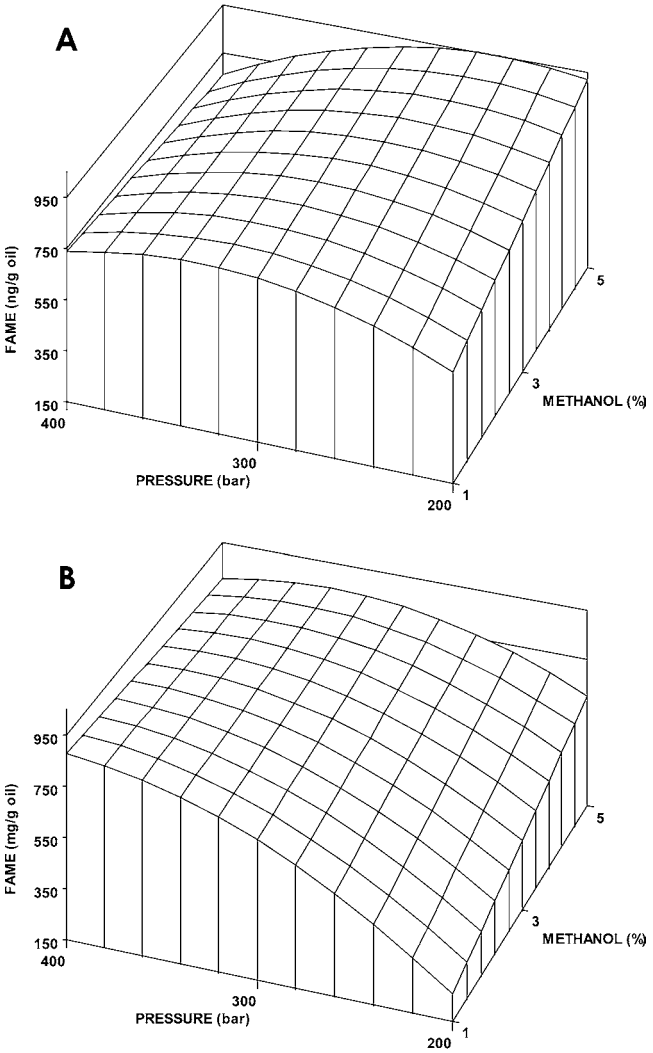

Figure 2. Response surfaces for Novozyme 435 describing the yield of total FAMEs from castor oil at constant temperature (80 °C) and water concentration: (**A**) 0.02 vol % water; **(B)** 0.18 vol % water ($n = 3$).

Some of these results are shown in **Figure 2**, which describes effects of varying pressure and methanol concentration at constant temperature (80 °C) and constant water concentration (0.02 and 0.18 vol % in parts A and B, respectively, of **Figure 2**). At the lowest water concentration studied (0.02 vol %, in **Figure 2A**), the FAME yield decreases with increasing pressure from 300 to 400 bar. This could be an effect of drying out the enzyme. At higher pressures, the solubility of water in the SF is higher, shifting the distribution of water from the enzyme preparation to the SF. Higher methanol concentration also results in increased water solubility and thereby further drying of the enzyme. With smaller amounts of water added to the SF, this drying effect is more severe (compare panels A and B of **Figure 2**).

By contrast, at the highest water concentration (0.18 vol %, in **Figure 2B**), the FAME yield generally increases with increasing pressure; the only exception is at 5 vol % of methanol, at which estimated yield decreases slightly from 300 to 400 bar. The increase in yield with increasing pressure is more pronounced at lower methanol levels. The explanation is most likely an effect of higher solvent strength of the SF. At 200 bar and 80 °C the SF density is only 0.59 g/mL, and the solubility of castor oil is limited. The solubility is decreased by higher water levels as well as by lower methanol concentration. At

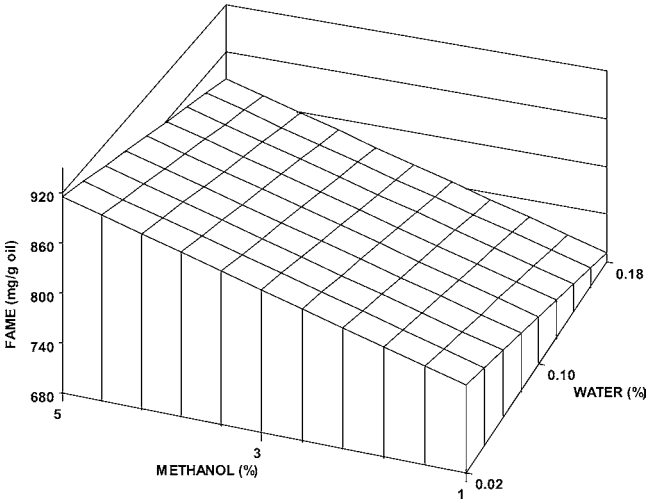

Figure 3. Response surface for Novozyme 435 describing the yield of total FAMEs from castor oil at constant temperature (80 °C) and pressure (300 bar) ($n = 3$).

the highest water level (0.18 vol %), lowest methanol concentration (1 vol %), and lowest pressure (200 bar), the FAME yield is only ~250 mg/g of oil but increases to 878 mg/g of oil whenthe pressure is increased to 400 bar.

At pressures of ~300 bar there is in most cases a maximum in FAME yield, or alternatively, the curve levels off toward higher pressures. Together with the chosen methanol level (7 vol %) and water concentration (0.02 vol %) (see below), 300 bar was selected as the optimal pressure.

Methanol Concentration. There are many aspects to consider concerning the effects of methanol concentration, with regard to both the extraction process and the enzymatic reaction. Addition of methanol to the SC-CO_2 increases its polarity. Furthermore, methanol improves the desorption of analytes from the sample matrix. In the case of castor oil, which contains triacylglycerols (TAGs) mainly composed of ricinoleic acid, an increase in the amount of methanol should improve the solubility of this relatively polar hydroxy fatty acid.

The other aspect to consider is how methanol affects the enzymatic reaction. Methanol is needed as part of the enzymatic reaction, because our goal is to perform a transmethylation reaction. However, an alcohol content that is too high may inhibit the enzyme activity (*25*) and also dry out the enzyme, because water is more soluble in a SF containing a higher amount of alcohol (*26*).

In this work, three levels of methanol concentrations were investigated; 1, 3, and 5 vol %. The response surface for FAME yield versus methanol concentration and water concentration at constant pressure (300 bar) and temperature (80 °C) is shown in **Figure 3**. The graph confirms visually that the yield is affected positively by higher methanol concentration for the selected combination of pressure and temperature, reaching a maximum at the highest methanol value (5 vol %). Moreover, because the curve does not begin to level out at the highest methanol level investigated, it appears that the span of methanol concentrations chosen for the experimental design was too narrow to attain the maximum possible extraction. Therefore, some complimentary experiments were performed afterward (see Tuning of the Method).

The relationship between FAME yield and methanol concentration is not constant across all levels of pressure studied, as shown by the interaction between methanol level and pressure in **Table 1**. This interaction can be seen in **Figure 2** and is independent of the water concentration; that is, it is the same at all three values: At the lowest pressure (200 bar) there is an increase in yield of 344 mg/g of oil from the lowest to the highest methanol concentration tested; at the intermediate pressure (300 bar), the increase in yield is only 133 mg/g of oil, and at the highest pressure tested (400 bar) a slight decrease of 78 mg/g of oil is seen. Therefore, the level of pressure to be used must be considered along with the choice of methanol concentration.

At average pressure (300 bar) the optimal methanol concentration is the highest studied (5 vol %) or even higher (see Tuning of the Method).

Water Concentration. A small amount of water is needed for the enzyme to maintain its three-dimensional structure and, thereby, also its activity. Typically, only a monolayer is needed, which is usually more than enough provided for by the manufacturer of immobilized enzymes. The enzyme preparation used in this work, Novozyme 435, contains ~1−2% (w/w) of water. However, stripping of water from the enzyme during the extraction/reaction process must be avoided. Because this can occur if dry SC-CO_2 is used (*27*), a small amount of water should be added continuously to the SF in order to maintain the enzyme activity. On the other hand, if too much water is added, side reactions may occur (such as hydrolysis) as well as denaturation of the enzyme (*28*). Water may also act as a barrier hindering the SF from reaching the active site of the enzyme (*29*). Therefore, there is an optimal water concentration for each application, which is dependent on the type of enzyme preparation, temperature, pressure, methanol concentration, sample matrix, and type of reaction studied.

In this work, water concentrations of 0.02, 0.10, and 0.18 vol % were evaluated, and some of the results are shown in **Figures 2** and **3**.

Figure 3 shows the yield at different water levels and methanol concentrations. It is clear that at 300 bar and 80 °C, the lowest water concentration gives the highest yields of FAMEs at all three methanol levels studied. As with methanol, the effect of water concentration on the FAME yield is linear, but in the case of water the relationship is negative. However, as was found with methanol, the relationship between FAME yield and water concentration is not constant across all levels of pressure studied, as shown by the interaction between water and pressure in **Table 1**. This interaction can also be seen in **Figure 2**, and is the same within the methanol concentration range studied. At the lowest pressure studied (200 bar) there is a decrease in yield of 322 mg/g of oil from the lowest to the highest water concentration tested (compare panels A and B of **Figure 2**); at the intermediate pressure (300 bar), yield also decreases, but to a lesser degree (91 mg/g of oil), and at the highest pressure tested (400 bar), an increase of 141 mg/g of oil is seen. Therefore, the level of pressure to be used must be considered along with the choice of water level. As discussed above (see Pressure), when the water concentration is increased at the lowest pressure, the solvent strength of the SF will decrease even further, resulting in lower recoveries. At the highest pressure however, where the solvent strength is high, an increase in water concentration helps by providing the enzyme with water, leading to improved FAME yields. At intermediate pressure (300 bar), the lowest water concentration (0.02 vol %) gives the highest yields.

Temperature. The temperature affects the solubility of the analytes in the SF, because it is directly correlated to the SF density as well as to the vapor pressure of the analytes. At higher pressures, the effect of increasing temperature on the extraction

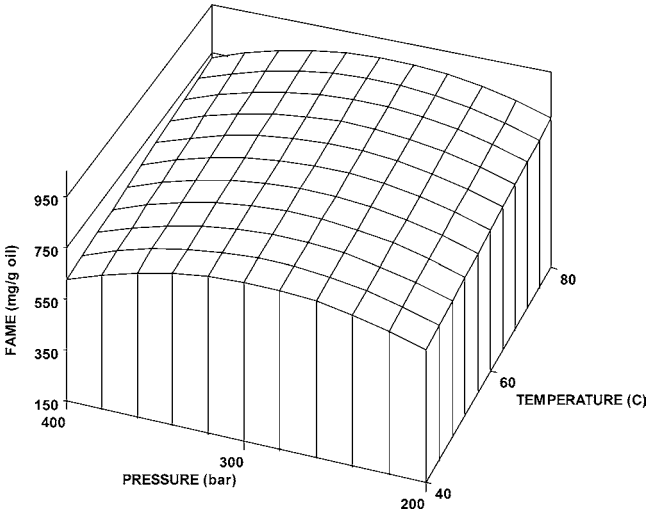

Figure 4. Response surface for Novozyme 435 describing the yield of total FAMEs from castor oil at constant methanol concentration (5 vol %) and water concentration (0.10 vol %) ($n = 3$).

yield of lipids is in general positive; the improved vapor pressure of the lipids outweighs the effect of the slight decrease in SF density. For an enzymatic reaction, a higher temperature usually leads to faster kinetics because the diffusion rates increase, but a too high temperature may result in thermal denaturation of the enzyme. Different enzymes have different optimal reaction temperatures, and these are dependent on the other reaction parameters of the system. For example, a higher temperature results in higher solubility of water in the SF and thereby lower water activity of the enzyme. The enzyme preparation used in this work, Novozyme 435, is stable in SC-CO_2 at temperatures >100 °C (*30*).

The temperature must therefore be optimized by considering both extraction efficiency and reaction performance. The result for varying pressure (200−400 bar) versus temperature (40−80 °C) at constant methanol concentration (5 vol %) and water concentration (0.10 vol %) is shown in **Figure 4**. It is obvious that the temperature is positively correlated to the FAME yield at all pressures considered, even though the effect is not big. It turned out that in all cases an increasing temperature had a slight positive effect on the FAME yield. Hence, 80 °C was chosen as optimal extraction/reaction temperature. Temperatures >80 °C were not investigated in this work because enzyme denaturation is likely to occur due to the relatively large amount of methanol and water in the system. Furthermore, at higher temperatures, a higher pressure would be necessary to maintain the same SF density, which also could cause denaturation of the enzyme.

Taking all of the results into consideration, within the ranges of parameter values studied, the highest temperature (80 °C) was considered as optimal, together with intermediate pressure (300 bar), the lowest water concentration (0.02 vol %), and the highest methanol concentration (5 vol %). This combination of parameter values gave the predicted outcome of 916 mg/g of oil according to the model in **Table 1**.

Tuning of the Method. *Methanol and Water Concentrations.* Higher concentrations of methanol were investigated, because the results obtained indicated that methanol concentrations higher than the upper level chosen in the experimental design might be beneficial for the extraction/reaction process. Therefore, methanol levels of 1, 3, 5, 7, and 9 vol % were tested, and temperature, pressure, and water concentration were held constant at 80 °C, 300 bar, and 0.10 vol %, respectively. The results are shown in **Table 2**. The data show that a methanol concentration of 7 vol % gives the highest FAME yield and was accordingly chosen as the optimal level.

Table 2. Effects of Methanol Concentration on the FAME Recovery at Constant Temperature (80 °C), Pressure (300 bar), and Water Concentration (0.10 vol %) ($n = 3$)

methanol (vol %)	FAME (mg/g of oil)	RSD (%)
1	738	5
3	804	6
5	834	4
7	841	6
9	819	5

To ensure that the lowest water concentration (0.02 vol %) was still optimal at a methanol value outside the original range of values studied, some additional experiments were performed at various water levels. The methanol concentration was kept at 7 vol %, the pressure at 300 bar, and the temperature at 80 °C. The water levels tested were 0.02, 0.10, 0.18, and 0.25 vol % of the SF. The results showed that the lowest water concentration investigated still gives the highest FAME yield. (FAME contents of 848, 841, 755, and 786 mg/g of oil were obtained for 0.02, 0.10, 0.18, and 0.25 vol % of water, respectively.) Hence, 0.02 vol % water was selected as the optimal water concentration.

Some experiments on effects of using a larger amount of enzyme (i.e., the Hydromatrix was replaced by enzyme) and a longer reaction column (i.e., the same amount of enzyme but more Hydromatrix mixed with the enzyme) were done to see if the FAME yield could be improved further. The results showed that a larger amount of enzyme made no difference and that a longer enzyme bed actually resulted in lower FAME yield (data not shown). Effects of different volumes of methanol entrainer added to the oil sample were also examined. The results demonstrated that the addition of 2 mL of methanol instead of 1 mL led to a significantly lower FAME yield (617 mg/g of oil compared to 841 mg/g of oil). A 0.5-mL addition did not give significantly higher FAME yield compared to a 1-mL addition (data not shown).

In summary, the preferred SFE/reaction methodology included the use of carbon dioxide at 300 bar and 80 °C, giving a density of 0.75 g/mL, and the addition of 7 vol % of methanol and 0.02 vol % of water. The other parameters were as described under Materials and Methods. This methodology was applied to castor seeds for the determination of composition and total content of FAMEs.

Application to Castor Seeds. Three different types of castor seeds were analyzed with regard to oil content expressed as FAMEs using the developed methodology. The results were compared to those obtained using a conventional methodology based on solvent extraction followed by acid-catalyzed transmethylation. The results are listed in **Table 3**.

The results in **Table 3** show that the SFE/enzyme methodology gives results similar to those of the conventional method. This has been demonstrated by calculating recoveries of the new method compared to the conventional one, giving values of 90−102%, with an average recovery value of 96%. The relative standard deviations (RSDs) of the two methodologies are also similar, with averages of 3.6% for the conventional method and 4.0% for the SFE/enzyme method. Hence, it can be concluded that the new methodology developed in this work is useful as an analytical protocol for the determination of FAMEs in castor seeds.

Table 3. Determination of FAMEs in Castor Seeds Using the Optimized Method (80 °C, 300 bar, 7 vol % Methanol, 0.02 vol % Water, and 90 min of Dynamic Extraction) Compared with the Conventional Methodology ($n = 3$)[a]

	conventional method		SFE/enzyme method		
castor seed no.	FAME (mg/g of seed)	RSD (%)	FAME (mg/g of seed)	RSD (%)	recovery (%)
1	545.6	6.7	556.8	4.6	102.1
2	591.1	2.8	568.6	2.5	96.2
3	542.8	1.2	486.1	4.9	89.6
av		3.6		4.0	95.9

[a] Oil contents of the seeds were approximately 56, 55, and 55% for castor seeds 1, 2, and 3, respectively.

Table 4. Composition of FAMEs in Castor Seeds Using the Optimized Method (80 °C, 300 bar, 7 vol % Methanol, 0.02 vol % Water, and 90 min of Dynamic Extraction) Compared with the Conventional Methodology ($n = 3$)

	conventional method			SFE/enzyme method		
FAME	castor seed 1	castor seed 2	castor seed 3	castor seed 1	castor seed 2	castor seed 3
C16:0	0.90	0.87	1.68	0.41	0.42	1.44
C18:0	0.83	1.00	1.53	0.78	0.90	1.35
C18:1(9)	3.10	3.55	3.68	2.60	3.19	2.99
C18:1(11)	0.21	0.36	1.09	0.35	0.34	0.51
C18:2(9,12)	4.66	3.42	4.38	4.10	4.20	3.56
C18:3(9,12,15)	0.44	0.39	0.59	0.41	0.39	0.41
C20:1(11)	0.14	0.27	0.93	0.43	0.58	0.88
C18:1(9)OH(12)	89.73	90.13	86.11	90.91	89.97	88.86

The compositions of the FAMEs derived using the two methodologies were also determined. These results are shown in **Table 4** and demonstrate that the compositions of FAMEs in the castor seeds are similar using the two methodologies. Therefore, this SFE/enzymatic methylation methodology is sufficiently accurate for use in analysis of FAME composition in castor seeds. In addition, this method may be useful for seed oils containing chemically reactive fatty acids, such as those with epoxy groups, acetylene bonds, or conjugated double bonds. These fatty acids are susceptible to reaction or oxidation under commonly employed extraction and methylation conditions. Furthermore, because the seed oil is extracted and simultaneously converted to FAMEs, this method also has potential to be used for producing methyl esters for industrial uses.

To the best of our knowledge, this is the first time a SFE/in situ methylation procedure has been used for the analysis of oil mainly composed of a hydroxy fatty acid. Moreover, this is the only analytical work using experimental design and response surfaces to evaluate effects of different extraction/reaction parameters on oil yields.

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