The Use of Immobilized *Candida antarctica* Lipase for Simultaneous Supercritical Fluid Extraction and *in-situ* Methanolysis of *cis*-Vaccenic Acid in Milkweed Seeds

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ABSTRACT: *cis*-Vaccenate was determined in milkweed (*Asclepias*) seeds employing supercritical fluid extraction (SFE) with *in-situ* enzymatic methanolysis. *Candida antarctica* lipase type B immobilized onto two different types of carrier materials—a hydrophobic polymer (Novozyme 435) and a silica-based material (NovoSample 40013)—was investigated. Several SFE parameters were investigated to find the optimal conditions, including extraction time, temperature, methanol concentration, and water level. It was shown that 90 min of dynamic extraction/reaction time was required, and that the optimal methanol and water concentrations differed for the two enzyme preparations studied. Overall, Novozyme 435 demonstrated the fastest reaction kinetics and consistently gave the highest vaccenate yields. The optimized methodology was applied on 15 species of milkweed seeds, giving an average recovery of 105 ± 7% when compared to results obtained using a conventional methodology. The average relative standard deviations were 8% for the enzyme-based method and 4% for the reference method. The proposed methodology was faster, was less laborious, and consumed less organic solvent than the reference method. Hence, SFE with *in-situ* enzymatic methanolysis is a promising methodology for the analysis of FA in oilseeds.

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KEY WORDS: *Asclepias*, fatty acids, isolation, lipase, methylation, oilseed, SFE.

Methodologies for the determination of oil expressed as FA in oilseeds usually include isolation of the lipids by solvent extraction (1–3), followed by chemical derivatization of the lipid sample to form FAME (4–7). These classical extraction and methylation methodologies are laborious and consume large volumes of hazardous organic solvent, such as petroleum ether, hexane, and chloroform. Hence, there has been extensive research focusing on the development of new, "green" extraction technologies, of which supercritical fluid extraction (SFE) probably is the most suitable alternative for oil extractions. One indication is that a method based on SFE has recently been certified as an official standard method for oil extraction from soybeans and seed from cotton, canola, safflower, and sunflower (8,9).

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SFE is an environmentally benign technique. In most cases it uses neat supercritical carbon dioxide (SC-CO_2) as the extraction fluid, and only a minimal volume of solvent for collection. Moreover, many SFE instruments are fully automatic and can handle several samples in a continuous mode, which leads to high sample throughput. In general, SFE is gentler to the analytes than many of the classical solvent-based extraction techniques, since it operates at relatively low temperatures and in oxygen-free surroundings. Another advantage is that SFE produces cleaner extracts than most of the conventional extraction techniques.

SC-CO_2 has also been shown to be a suitable medium for lipase-catalyzed reactions (10,11). Apart from the gain of avoiding organic solvents and accessing automated instruments, the low viscosity and high diffusivity of the supercritical fluid (SF) results in faster reaction kinetics compared to the same reactions in organic solvents such as hexane (12). Several types of immobilized lipases have been used in SC-CO_2 to catalyze hydrolysis (13), esterification (14), and transesterification (15) of lipids. However, in an analytical context, only immobilized *Candida antarctica* lipase type B (Novozyme 435) has been used for the production of FAME in dynamic SFE. This was done for the analysis of fat content in meat samples (16), oilseeds (17), and vegetable oil soapstocks (18). Our own experiences with lipase-catalyzed reactions in dynamic SFE include the use of several types of immobilized lipases for the hydrolysis and alcoholysis of fat and vitamin A esters for the determination of fat-soluble vitamins in foods (19,20).

This work investigated *C. antarctica* lipase type B immobilized onto two different carrier materials: a hydrophobic polymer (Novozyme 435) and a silica-based carrier (Novo Sample 40013). The first one is commercially available from Novozymes A/S and has largely been used in hydrolysis, alcoholysis, and transesterification reactions in SC-CO_2. The second one is a test preparation from Novozymes A/S and is less costly than Novozyme 435. These two enzyme preparations were tested under different supercritical conditions in order to select the most suitable enzyme preparation as well as the optimal SFE parameters for the simultaneous extraction and methanolysis of oil from milkweed (*Asclepias*) seeds. *cis*-Vaccenate was chosen as the target analyte as it has potential industrial uses, including for engineering plastics (21,22) and as an antifungal agent (23). Furthermore, vaccenic acid is converted

in mammals to CLA, which suppresses cancer and atherosclerosis, and enhances the immune system and the development of lean muscle mass (24). Several extraction/reaction parameters were investigated, such as dynamic extraction time, temperature, methanol concentration, and water concentration. The proposed methodology was used for the determination of *cis*-vaccenate in 15 different species of milkweed seeds. The obtained vaccenate levels were compared to those achieved using a conventional method, which was based on the SFE of oil from oilseeds (AOAC method 999.02) followed by acid-catalyzed transmethylation.

MATERIAL AND METHODS

Materials. Novozyme 435 (*C. antarctica* lipase type B immobilized onto a hydrophobic polymer carrier, 10,000 Propyl Laurate Units/g) and NovoSample 40013 (*C. antarctica* lipase type B immobilized onto a silica-based carrier, 20,000 Propyl Laurate Units/g) were generous gifts from Novozymes A/S (Bagsvaerd, Denmark). *Asclepias speciosa* seeds were obtained from Biogentex Laboratories Inc. (Seabrook, TX), and all other milkweed seeds used were purchased from Butterfly Encounters (Danville, CA). *cis*-Vaccenic acid methyl ester, trifluoroacetic anhydride, 3-pyridyl carbinol, 4-(dimethyl amino) pyridine and cyclohexane were obtained from Sigma-Aldrich (St. Louis, MO). Heptadecanoic acid methyl ester was purchased from Alltech (Deerfield, IL) and BHT was obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA). Hydromatrix wet support material was purchased from Varian Inc. (Walnut Creek, CA) and anhydrous sodium sulfate was purchased from J.T. Baker Inc. (Philipsburg, NJ). 2-Propanol, methanol, hexane, toluene, hydrochloric acid, diethyl ether, and dichloromethane were obtained from Fisher Scientific (Fairlawn, 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 (Colman grade) was obtained from Bay Airgas (Emeryville, CA).

Reference method. Milkweed seeds were ground to a fine powder in a coffee blender. Seed powder of 0.05 to 0.10 g was accurately weighed and mixed with 1 g of Hydromatrix wet support material. The mixture was transferred to an extraction cell, and the remaining volume was filled up with Hydromatrix. Lipids were extracted thereafter employing a fully automated supercritical fluid extractor (Isco SFX 3560; Isco Inc., Lincoln, NE). The AOAC official method for SFE of oil in oilseeds (8) was used, the only exception being that collection was achieved in 10 mL of 2-propanol (instead of on glass wool). Neat carbon dioxide was used as extraction fluid at 470 bar and 100°C (0.80 g/mL), and the extraction was performed in 5 min static mode followed by 30 min dynamic mode at a flow rate of 3.0 mL/min. The restrictor temperature was 100°C. After completion of the extraction, the collection solvent was removed by a gentle stream of nitrogen. One milliliter of toluene was added thereafter, and the lipids were methylated overnight using methanolic hydrogen chloride (5%), as described by Christie (6). The resulting FAME were dissolved in 25 mL of cyclohexane (1% BHT), and 1-mL portions were taken to vials for GC analysis.

SFE with in-situ *methanolysis.* One gram of Novozyme 435 or 1.5 g of NovoSample 40013 were mixed with 0.5 g of Hydromatrix and added to extraction cells so that they formed 25-mm-high columns. The enzyme preparations were cleaned with SC-CO_2 prior to first-time usage in order to remove components that would otherwise interfere with the chromatographic analysis (20). SC-CO_2 of 366 bar and 60°C (0.88 g/mL), containing 3 vol% of methanol, was used for 1-min static extraction and 30-min dynamic extraction at a flow rate of 2.0 mL/min. The modifier concentration was set to 0 the last 5 min of the extraction to avoid possible denaturation of the enzyme occurring owing to the rapid breakout of water molecules during depressurization (25). The cleaned enzyme preparations were equilibrated in a desiccator to a water activity of 0.43 over K_2CO_3-saturated water for at least 24 h (26).

Milkweed seeds were prepared as described above and mixed with Hydromatrix (Varian Inc.). The mixture was added to the extraction cell on top of the enzyme preparation, separated by glass wool. One mL of methanol was added as entrainer on top of the milkweed seed mixture to facilitate disruption of the matrix and desorption of the analytes (27). SC-CO_2 at 366 bar and 40, 60, and 80°C (corresponding to densities of 0.95, 0.88, and 0.80 g/mL, respectively) was used as extraction fluid. Methanol was dried with anhydrous sodium sulfate, and a specific amount of water was added. SC-CO_2 containing 1, 3 and 5% of methanol as well as 0.01, 0.03, and 0.09 vol% of water was tested in the experiments. The extraction was initiated by 5 min of static extraction followed by 90 min of dynamic extraction at a flow rate of 0.5 mL/min. Modifier was excluded the last five min of the extraction. The flow rate was set to 1.0 mL/min during the last 30 min. The collection was achieved in 10 mL of 2-propanol containing 1% of BHT. The restrictor temperature was 65°C, and the collection temperature was 5°C. The collection solvent was removed by nitrogen after the extraction, and 20 mL of cyclohexane (1% BHT) was added. Aliquots of 1 mL were transferred to small vials for analysis.

Analysis. Quantitative analysis was achieved by GC-FID using a Hewlett-Packard 6890 GC system with split/splitless 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 200 and 280°C, respectively. The column temperature program was 140°C for 1 min, 20°C/min to 170°C, held 1 min, 4°C/min to 230°C, held 1 min, 10°C/min to 260°C, and held 5 min. Standard solutions of methyl vaccenate at three different concentrations in the range of 5 to 250 μg/mL were used for generating a standard calibration curve. Fifty microliters of methyl

heptadecanoate (1 mg/mL) was added as the internal standard to each 1-mL aliquot of standard sample. One-microliter injections were used, and duplicate determinations were applied. Recovery values of the enzyme-based method were calculated by comparing the results with those obtained using the reference method.

Identification of peak components was achieved on a Hewlett-Packard 5890 GC system connected to a 5970A mass selective detector (Agilent Technologies). Split/splitless injection was applied, and the same type of column and temperature program was used as described above. Comparison to mass spectra of known FAME was used for identification of each peak. In addition, double-bond locations for the unsaturated FA were determined by interpreting spectra from picolinyl derivatives of FFA, employing a methodology described by Christie (6).

RESULTS AND DISCUSSION

General considerations. From previous experiences on working with enzymes in dynamic SFE systems, it was found that commercially available enzyme preparations contain lipid-soluble material that may interfere with the final analysis (20). Likewise, in this work it was discovered that these impurities coelute with stearate, oleate, and vaccenate, making quantification problematic. Therefore, the enzyme preparations were cleaned prior to first use.

Another experience from earlier work was that immobilized *C. antarctica* lipase can be reused at least five times without any noticeable loss in activity, with cleaning between extractions using SC-CO_2 (20). Hence, in this work enzyme preparations were used five times before discarding them.

During the entire method development, seeds from *A. speciosa* were used as a model sample. Triplicate samples were taken in all determinations.

Optimization of extraction time. To investigate how long a dynamic extraction time was needed to quantitatively extract oil from milkweed seeds, 0.15-g portions of ground *A. speciosa* seeds were prepared as described above (see the Materials and Methods section). The lowest possible flow rate was applied (0.5 mL/min), because this was known to be required to obtain quantitative yields in the enzyme-catalyzed reaction. SC-CO_2 containing 3 vol% of dry methanol was used as the extraction fluid at 366 bar. Extraction temperatures of 40 and 60°C were investigated. Several efforts were made to test 80°C as well, but the restrictor clogged for an unknown reason. Collection was achieved after 10, 30, 60, and 90 min of dynamic extraction in four separate vials containing 5 mL of 2-propanol at 5°C. After extraction, the samples were evaporated to dryness with gaseous nitrogen, and the amount of oil extracted was determined gravimetrically. The results are shown in Figure 1.

After 90 min of extraction at 0.5 mL/min, both extraction curves leveled out, indicating complete extraction. Moreover, the two curves totally overlapped, demonstrating that the oil extractability was the same for the two temperatures investigated. The most probable explanation is that the extraction kinetic is relatively unaffected by the changes in temperature, and that the lower SF solvent strength at higher temperature (lower density) is compensated for by the higher vapor pressure of the oil. Ninety minutes of dynamic extraction was applied in all further experiments, with collection in one single vial. Moreover, to minimize risks of carryover between separate extractions, a flow rate of 1.0 mL/min was applied the last 30 min of the extraction. Here, the concentration of extracted oil was relatively low, and the supercritical extraction kinetic was a more limiting factor than the kinetic of the methanolysis reaction.

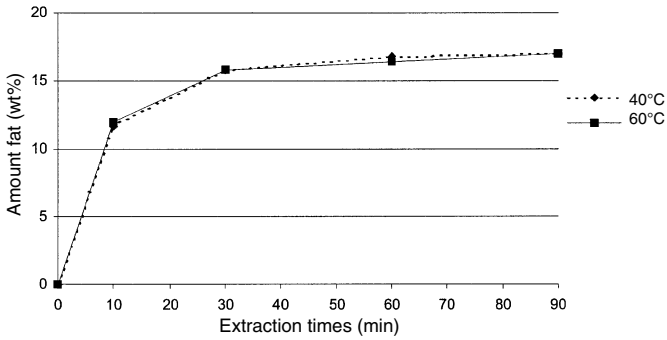

FIG. 1. Extraction profile of oil from *Asclepias speciosa* seeds at 40 and 60°C, respectively ($n = 3$). Supercritical carbon dioxide (SC-CO_2) at 366 bar containing 3 vol% of methanol was used as supercritical fluid (SF). The flow rate was 0.5 mL/min.

Optimization of temperature. It has been shown by others that temperature has a large impact on enzyme kinetics in supercritical CO_2, where higher temperatures result in faster reaction rates (28). However, too high a temperature may negatively affect the stability of the enzyme and, in the worst case, lead to thermal denaturation (29). Since fast enzyme reactions in dynamic SFE systems are essential to avoid breakthrough losses of unreacted acylglycerols, the effect of temperature on the yield of *cis*-vaccenate from *A. speciosa* seeds was investigated. Since it had already been demonstrated (see Fig.1 above) that the temperature did not affect the kinetic of the oil extraction, it was assumed that the vaccenate yields obtained in this experiment solely reflected the kinetic of the lipase-catalyzed reaction. Triplicate samples were prepared as described above (see the Material and Methods section, SFE with *in-situ* Methanolysis). SC-CO_2 containing 3 vol% of dry methanol was used as extraction fluid, at 366 bar and 40, 60, and 80°C, respectively. The results are given in Figure 2.

Figure 2 demonstrates that both types of enzyme preparations provided the highest yields at the highest temperature investigated, 80°C. The exceptionally high stability of Novozyme 435 in neat SC-CO_2 at temperatures above 100°C was demonstrated by Overmeyer *et al.* (28). However, the results in Figure 2 also show that Novozyme 435 accomplished the highest (and quantitative) vaccenate yield already at 60°C, which agrees perfectly with results obtained for alcoholysis of retinyl esters in milk powder (19). NovoSample 40013, on the other hand, required 80°C to reach its highest activity under the conditions tested. Nevertheless, the kinetics of the NovoSample 40013-catalyzed reaction were still not fast enough to accomplish quantitative

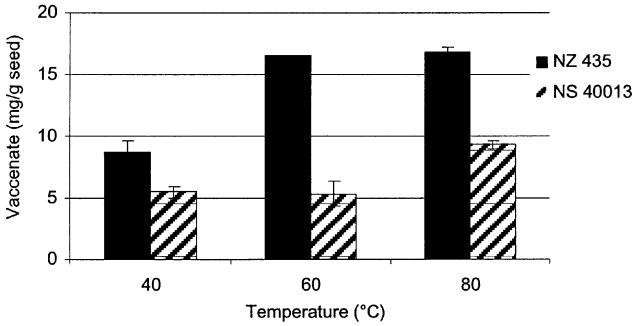

FIG. 2. Effects of different temperatures on *cis*-vaccenate yields from *A. speciosa* seeds employing Novozyme 435 and NovoSample 40013, respectively (n = 3). SC-CO_2 at 366 bar containing 3 vol% of methanol was used as SF. Reference value *cis*-vaccenate: 14.6 mg/g seed. Error bars represent relative standard deviation (RSD) values (%). For abbreviations see Figure 1.

vaccenate yields at any of the temperatures investigated. The research of Novozymes A/S also demonstrates that the initial activity of Novozyme 435 is higher than the initial activity of NovoSample 40013 (30). Consequently, Novozyme 435 remains the preferred immobilized *C. antarctica* lipase B preparation for use in dynamic SFE systems. Additionally, reaction at 60°C is preferred since a lower temperature improves the long-term stability of the enzyme. For NovoSample 40013, 80°C is the best temperature of the ones investigated.

Optimization of modifier concentration. Methanol, which naturally is a substrate in the alcoholysis reaction, was continuously added to the SF *via* a high-pressure pump. The methanol concentration should be high enough to enable quantitative methylation of the FA moieties but not too high, since that may cause inhibition of the enzyme (31). Three different methanol concentrations were investigated: 1, 3, and 5 vol%, respectively. Dry methanol was used, since it was assumed that the enzyme preparations contained sufficient water (typically a monolayer) for the first step of the reaction, which is hydrolysis of the acylglycerols. SC-CO_2 at 366 bar and 60°C was used as SF, and samples were prepared as described above. The results can be seen in Figure 3.

These results reveal that the two enzyme preparations respond differently to different methanol concentrations. It is obvious that NovoSample 40013 is more sensitive to alcohol inhibition than Novozyme 435, as a concentration of 3 vol% of methanol leads to drastically decreased recoveries of *cis*-vaccenate in the former case. Novozyme 435 on the other hand, shows a maximum at 3 vol% of methanol, as well as the lowest relative standard deviation (RSD) value.

Optimization of water level. A small amount of water is necessary to maintain the functionality of the enzyme (32). Water is initially needed in the methanolysis reaction, but the net consumption is zero (33). However, if too much water is present, the kinetically faster hydrolysis reaction will take place, in which water instead of methanol is the attacking nucleophile, giving rise to FFA instead of FAME. Hence, it is very important to optimize the water content of the extraction/reaction system carefully.

It has been demonstrated that SC-CO_2 completely free of water strips off the enzyme-bound water and thereby reversibly inactivates the enzyme (34). Therefore, the enzyme preparations were equilibrated to a water activity of 0.43 before reusing them, to ascertain whether they still possessed maximum activity after the cleaning process. A water activity of 0.43 has been shown in previous work to be appropriate for Novozyme 435, in alcoholysis and hydrolysis reactions carried out in a dynamic SFE system using SC-CO_2 containing 3 vol% of ethanol as SF (20). Triplicate samples of *A. speciosa* seeds were prepared as described above, and SC-CO_2 at 366 bar and 60°C containing 3 vol% of methanol was used as SF. Water concentrations of 0, 0.03, and 0.09 vol% were investigated for both Novozyme 435 and NovoSample 40013. The results are shown in Figure 4.

As demonstrated in Figure 4, NovoSample 40013 shows optimal activity at 0.03 vol% of water; that is, dry SC-CO_2/methanol probably renders the enzyme inactive during the course of the reaction. For Novozyme 435, on the other hand, the highest vaccenate yields were obtained using dry SC-CO_2/methanol, indicating that this enzyme preparation maintained sufficient activity throughout the extraction/reaction. However, the next time the same enzyme preparation was used

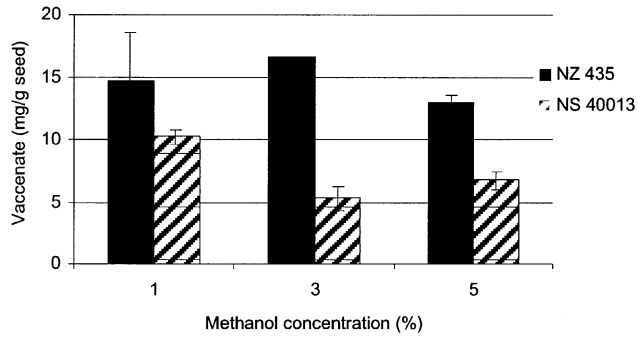

FIG. 3. Effect of methanol concentration on *cis*-vaccenate yields from *A. speciosa* seeds employing Novozyme 435 and NovoSample 40013, respectively (n = 3). SC-CO_2 at 60°C and 366 bar (density 0.88 g/mL) was used as SF. Reference value *cis*-vaccenate: 14.6 mg/g seed. Error bars represent RSD values (%). For abbreviations see Figures 1 and 2.

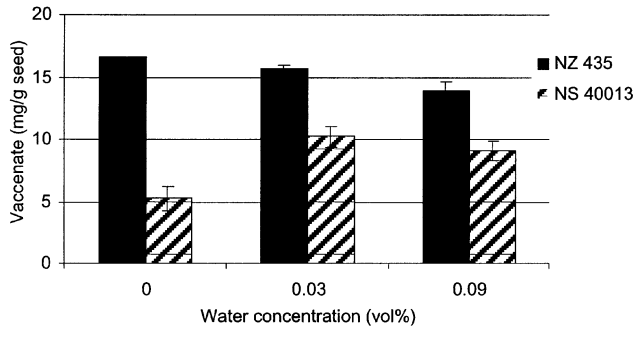

FIG. 4. Effect of water concentration on *cis*-vaccenate yields from *A. speciosa* seeds employing Novozyme 435 and NovoSample 40013, respectively (n =3). SC-CO_2 at 60°C and 366 bar (density 0.88 g/mL) containing 3 vol% of methanol was used as SF. Reference value *cis*-vaccenate: 14.6 mg/g seed. Error bars represent RSD values (%). For abbreviations see Figures 1 and 2.

(after cleaning and re-equilibration), it had lost activity, giving only about 50 to 60% vaccenate recovery. Most likely, a longer equilibration time over K_2CO_3 would be required to reactivate the enzyme. Instead, 0.03 vol% of water was used, as this amount turned out to be sufficient for maintaining the activity of Novozyme 435 for repeated usage with cleaning and 24-h re-equilibration, giving consistently high recoveries (≥100%) of *cis*-vaccenate. Considering the results given in Figures 1–4, Novozyme 435 was chosen as the best enzyme for the extraction/reaction system studied, using SC-CO_2 at 366 bar and 60°C containing 3 vol% of methanol and 0.03 vol% of water as SF.

Application to various species of milkweed seeds. The developed methodology was used for the determination of *cis*-vaccenate in 15 different species of milkweed seeds. Triplicate samples were prepared as described above, and the same samples were analyzed by the reference method. The results are listed in Table 1.

These results show that the proposed enzyme-based method gave recoveries of *cis*-vaccenate ranging from 75 to 125%, with an average recovery of 105 ± 7% ($x \pm t[s/n^{-1/2}]$). Taking into consideration that the average RSD of the new method was 8% (whereas that of the reference method was 4%), the accuracy is acceptable. The concentration of palmitate in the same samples was also determined, revealing a pattern almost identical to that found for *cis*-vaccenate, with recoveries ranging from 75 to 123% and an average recovery of 102 ± 7%. Hence, the values differing more than ±7% from the average recovery value can most likely be explained by systematic analytical error either in the reference methodology or in the new one.

To conclude, the results obtained in this work show that the optimal SFE parameters for lipase-catalyzed methanolysis largely depend on the type of enzyme carrier material. Generally, *C. antarctica* lipase immobilized to a silica-based material (NovoSample 40013) appeared to be more sensitive to methanol and water in the SF than the same enzyme immobilized to a hydrophobic polymer carrier (Novozyme 435). Moreover, NovoSample 40013 demonstrated slower reaction kinetics, with the highest obtainable vaccenate yield around 70%. However, by using Novozyme 435 and the SFE condition optimized in this work, quantitative recoveries of *cis*-vaccenate as well as palmitate were obtained from a wide selection of milkweed (*Asclepias*) seeds. The SFE methodology developed with *in-situ* methanolysis offers many advantages when compared to conventional methodologies, including less sample handling, higher sample throughput, smaller consumption of organic solvents, and faster analysis. In addition, SC-CO_2 containing methanol is gentler to the analytes than, for example, hexane, diethyl ether, and dichloromethane. Hence, the proposed methodology is a promising alternative to classical analytical methodologies and should be applicable for the determination of FA in other types of plant materials as well.

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TABLE 1
Recoveries of *cis*-Vaccenate from Various Species of *Asclepias* Seeds

	Conventional method[a]		Enzyme method[b]		
Milkweed type	Vaccenate (mg/g seed)	RSD (%)	Vaccenate (mg/g seed)	RSD (%)	Recovery (%)
A. asperula	8.7	1	9.4	8	109
A. cordifolia	17.4	8	17.2	16	99
A. curassavica	10.5	5	13.0	10	125
A. curassavica 'aurea'	9.6	3	10.9	16	114
A. erosa	13.4	1	12.3	5	92
A. fascicularis	11.8	13	12.5	16	106
A. fruticosa	10.2	4	11.3	8	111
A. incarnata spp. *pulchra*	9.1	3	8.8	3	97
A. physocarpa	11.6	5	8.7	2	75
A. speciosa 'Davis, CA'	12.6	2	12.1	4	96
A. speciosa	14.5	3	14.9	1	103
A. sullivantii	11.6	4	13.6	2	117
A. syriaca	15.9	4	16.1	1	102
A. tuberosa	10.7	3	11.6	12	108
A. viridis	7.5	7	9.1	16	120

[a]The conventional method was based on AOAC standard method 999.02, Oil in Oilseeds: Supercritical Fluid Extraction (SFE) Method (Ref. 8), followed by acid-catalyzed methylation (Ref. 6). Triplicate determinations were made. RSD, relative standard deviation.

[b]The developed enzyme-based method employed Novozyme 435 and the following SFE conditions: SC-CO_2 at 60°C and 366 bar (density 0.88 g/mL), 3 vol% of methanol, 0.03 vol% of water, 5 min static extraction followed by 90 min dynamic extraction, 0.5 mL/min the first 60 min and 1.0 mL/min the last 30 min. The collection was achieved in 10 mL of 2-propanol containing 1% of BHT. The restrictor temperature was 65°C, and the collection temperature was 5°C.

REFERENCES

1. Bligh, E.G., and W.J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*:911–917 (1959).
2. Folch, J., M. Lees, and G.H. Sloane Stanley, A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*:497–509 (1957).
3. Determination of Oil Content in Oilseeds, in *Official Methods and Recommended Practices of the AOCS*, 5th edn., edited by D. Firestone, AOCS Press, Champaign, 1998, AOCS Method Am 2-93.
4. Glass, R.L., R. Jenness, and H.A. Troolin, Simple Method for Analysis of the Fatty Acid Composition of Milk Fats, *J. Dairy Sci. 48*:1106–1109 (1965).
5. Brian, B.L., R.W. Gracy, and V.E. Scholes, Gas Chromatography of Cyclopropane Fatty Acid Methylesters Prepared with Methanolic Boron Trichloride and Boron Trifluoride, *J. Chromatogr. 66*:138–140 (1972).
6. Christie, W.W., The Preparation of Derivatives of Fatty Acids, in *Gas Chromatography and Lipids: A Practical Guide*, edited by W.W. Christie, The Oily Press, Dundee, 1989, pp. 66–84.
7. El-Hamdy, A.H., and W.W. Christie, Preparation of Methyl Esters of Fatty Acids with Trimethylsulphonium Hydroxide—An Appraisal, *J. Chromatogr. 630*:438–441 (1993).
8. Oil in Oilseeds: Supercritical Fluid Extraction (SFE) Method, in *AOAC Official Methods of Analysis*, edited by AOAC International, AOAC International, 2000, pp. 41.1.69, pp. 66–68, AOAC Method 999.02.
9. Oil in Oilseeds: Supercritical Fluid Extraction Method, in *Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th edn.*, edited by D. Firestone, AOCS Press, Champaign, 1998, AOCS Method Am 3-96.
10. Nakamura, K., Y.M. Chi, Y. Yamada, and T. Yano, Lipase Activity and Stability in Supercritical Carbon Dioxide, *Chem. Eng. Commun. 45*:207–212 (1986).
11. Chi, Y.M., K. Nakamura, and T. Yano, Enzymic Interesterification in Supercritical Carbon Dioxide, *Agric. Biol. Chem. 52*:1541–1550 (1988).
12. Kamat, S.V., E.J. Beckman, and A.J. Russell, Enzyme Activity in Supercritical Fluids, *Crit. Rev. Biotechnol. 15*:41–71 (1995).
13. Rezaei, K., and F. Temelli, On-line Extraction-Reaction of Canola Oil Using Immobilized Lipase in Supercritical CO_2, *J. Supercrit. Fluids 19*:263–274 (2001).
14. Rantakylae, M., and O. Aaltonen, Enantioselective Esterification of Ibuprofen in Supercritical Carbon Dioxide by Immobilized Lipase, *Biotechnol. Lett. 16*:825–830 (1994).
15. Liu, K.-J., H.-M. Chen, R.-C. Chang, and J.-F. Shaw, Synthesis of Cocoa Butter Equivalent by Lipase-Catalyzed Interesterification in Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 74*:1477–1482 (1997).
16. Snyder, J.M., J.W. King, and M.A. Jackson, Fat Content for Nutritional Labeling by Supercritical Fluid Extraction and an Online Lipase Catalyzed Reaction, *J. Chromatogr. A 750*:201–207 (1996).
17. Snyder, J.M., J.W. King, and M.A. Jackson, Analytical Supercritical Fluid Extraction with Lipase Catalysis: Conversion of Different Lipids to Methyl Esters and Effect of Moisture, *J. Am. Oil Chem. Soc. 74*:585–588 (1997).
18. King, J.W., S.L. Taylor, J.M. Snyder, and R.L. Holliday, Total Fatty Acid Analysis of Vegetable Oil Soap Stocks by Supercritical Fluid Extraction/Reaction, *J. Am. Oil Chem. Soc. 75*:1291–1295 (1998).
19. Turner, C., J.W. King, and L. Mathiasson, On-line SFE/Enzymatic Hydrolysis of Vitamin A Esters—A New Simplified Approach for the Determination of Vitamins A and E in Food, *J. Agric. Food Chem. 49*:553–558 (2001).
20. Turner, C., M. Persson, L. Mathiasson, P. Adlercreutz, and J.W. King, Lipase-Catalyzed Reactions in Organic and Supercritical Solvents: Application to Fat-Soluble Vitamin Determination in Milk Powder and Infant Formula, *Enzyme Microb. Technol. 29*:111–121 (2001).
21. Pryde, E.H., D.E. Anders, H.M. Teeter, and J.C. Cowan, Ozonization of Soybean Oil. The Preparation and Some Properties of Aldehyde Oils, *J. Am. Oil Chem. Soc. 38*:375–379 (1961).
22. Kleiman, R., and K.L. Payne-Wahl, Fatty Acid Composition of Seed Oils of the Meliaceae, including One Genus Rich in *cis*-Vaccenic Acid, *J. Am. Oil Chem. Soc. 61*:1836–1838 (1984).
23. Caupin, H.-J., Products from Castor Oil—Past, Present, and Future, in *Lipid Technologies and Applications*, edited by F.D. Gunstone, and F.B. Padley, Marcel Dekker, New York, 1997, pp. 787–795.
24. Yurawecz, M.P., M.M. Mossoba, J.K.G. Kramer, M.W. Pariza, and G.J. Nelson (eds.), *Advances in Conjugated Linoleic Acid Research*, AOCS Press, Champaign, 1999, Vol. 1, 480 pp.
25. Kasche, V., R. Schlothauer, and G. Brunner, Enzyme Denaturation in Supercritical Carbon Dioxide: Stabilizing Effect of Disulfide Bonds During the Depressurization Step, *Biotechnol. Lett. 10*:569–574 (1988).
26. Adlercreutz, P., Biocatalysis in Non-conventional Media, in *Applied Biocatalysis*, edited by A.J.J. Straathof and P. Adlercreutz, Harwood Academic Publishers, 2000, pp. 295–316.
27. Turner, C., and L. Mathiasson, Determination of Vitamins A and E in Milk Powder Using Supercritical Fluid Extraction for Sample Clean-up, *J. Chromatogr. A 874*:275–283 (2000).
28. Overmeyer, A., S. Schrader-Lippelt, V. Kasche, and G. Brunner, Lipase-Catalysed Kinetic Resolution of Racemates at Temperatures from 40°C to 160°C in Supercritical CO_2, *Biotechnol. Lett. 21*:65–69 (1999).
29. Chulalaksananukul, W., J.S. Condoret, and D. Combes, Geranyl Acetate Synthesis by Lipase-Catalyzed Transesterification in Supercritical Carbon Dioxide, *Enzyme Microb. Technol. 15*: 691–698 (1993).
30. Husum, T.L., T.W. Christensen, M.W. Christensen, and L.E. Andersen, Application for New Immobilized *Candida antarctica* Lipase B, Information Sheet, Oil & Fats, Novozymes A/S, Bagsvaerd, Denmark, 2001.
31. Jackson, M.A., and J.W. King, Methanolysis of Seed Oils in Flowing Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 73*:353–356 (1996).
32. Zacks, A., and A.M. Klibanov, Enzymatic Catalysis in Organic Media at 100°C, *Science 224*:1249–1251 (1984).
33. Malcata, F.X., H.R. Reyes, H.S. Garcia, C.G. Hill, Jr., and C.H. Amundson, Kinetics and Mechanisms of Reactions Catalysed by Immobilized Lipases, *Enzyme Microb. Technol. 14*:426–446 (1992).
34. Dumont, T., D. Barth, and M. Perrut, Continuous Synthesis of Ethyl Myristate by Enzymic Reaction in Supercritical Carbon Dioxide, *J. Supercrit. Fluids 6*:85–89 (1993).

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