# Enzymatic Synthesis of Oleyl Oleate in Dense Fluids

Ž. Knez[a], V. Rižner[a], M. Habulin[a] and D. Bauman[b]

[a]Faculty of Chemistry and Chemical Engineering, University of Maribor, Smetanova 17, SLO-62000 Maribor, Slovenia and [b]Pinus, Chemical Works, SLO-62327 Rače, Slovenia

**ABSTRACT:** Esterification between oleic acid and oleyl alcohol, catalyzed by the *Mucor miehei* immobilized lipase in a batch-stirred tank reactor with supercritical carbon dioxide as solvent produced higher reaction rates at supercritical conditions than in the solvent-free system. A continuous fixed-bed reactor was designed based on the results obtained from batch experiments. At 150 bar, 40°C, and with water activity 0.46% w/w, the activity of the enzyme preparation is practically unchanged when $CO_2$ was used as solvent. The addition of small amounts of water increases the conversion rate. The higher conversion also was observed at longer residence time. When *n*-butane was used as reaction medium, a decrease in conversion was observed.

*JAOCS 72*, 1345–1349 (1995).

**KEY WORDS:** Enzymes, esterification, lipase, *n*-butane, oleyl oleate, supercritical carbon dioxide.

Supercritical fluids (SCF) have been used as solvents for a wide variety of extractive applications in the last few years. Recently, supercritical solvents also have been applied as solvents in nonextractive applications, such as high-pressure micronization and chromatography, and as a chemical reaction medium as well.

Since the first reports on the use of SCF as a reaction medium (1–3), several studies on oxidation (3–6), hydrolysis (2), transesterification (1,7–14), esterification (15–30), interesterification (31–33), and enantioselective synthesis (34–36) have proven the feasibility of enzymatic reactions in SCF. The advantages of using supercritical carbon dioxide as a medium for enzymatic catalyzed reactions have been well documented (37,38). Frequently, the temperature range used for supercritical carbon dioxide in processing is compatible with the use of enzymes as catalysts. An additional benefit of using SCF along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants. However, a limitation of the process may arise from the nonpolarity of carbon dioxide, which preferentially dissolves hydrophobic compounds.

Our previous research work (39–44) on enzymatic synthesis of esters in solvent-free systems also indicates that mass transfer limitations are observed. Use of SCF decreases mass transfer limitations because of the high diffusivity of reactants in a supercritical medium, the low surface tension, and the relatively low viscosity of the mixture. The Schmidt number ($Sc = \eta/p \cdot D$, where $\eta$ is dynamic viscosity, $D$ is diffusivity, and $p$ is density) for $CO_2$ at 200 bar is 45 times lower than for water at 1 bar and 20°C. High diffusivity of SCF and low surface tension led to reduced internal mass transfer limitations for heterogeneous chemical or biochemical catalysis.

The main objectives of the present research are: (i) the comparison of an enzymatic reaction in an SCF with a solvent-free system in a batch-stirred tank reactor (BSTR); (ii) the development of an integrated production and product recovery process based on enzymatic catalysis and product fractionation with supercritical carbon dioxide; and (iii) the development of a small-scale, multipurpose enzyme reactor that incorporates a subsequent separation step for the recovery of substrate.

There are only a few reports on continuously operated systems where $CO_2$ was used as a reaction medium (15,16,22,29,31). The present article is the first report on the use of another gas, *n*-butane, for a continuously-operated enzymatic reactor. The model system used in this research was esterification of oleic acid with oleyl alcohol catalyzed by lipase from *Mucor miehei*, due to its high conversion, which was not observed for esterification with short-chain alcohols (for example, methanol and ethanol). Esterification reactions have potential uses in preparation of wax, esters, fragrances, ester oligomers, and other compounds.

## MATERIALS AND METHODS

*Enzyme preparation.* The lipase, Lipozyme™, immobilized on a macroporous anion exchange resin, was produced and kindly donated by Novo Nordisk A/S (Copenhagen, Denmark). The enzyme beads contained 10 w/w% water. Palatase 1000 L (Novo Nordisk A/S) was used in some experiments, which is a lipase of *M. miehei* in water-soluble form.

*Lipase activity.* The lipase activity was measured according to Novo Nordisk A/S (45). The activity of the Lipozyme preparation used for our synthesis was 23 BIU/g [one batch interesterification unit (BIU) corresponds to 1 micromole of palmitic acid incorporated into triolein per min at standard conditions (pH, 6.9–7.1; 40°C)]. The activity of Palatase preparation was 1000 LU/g [one lipase unit (LU) is the amount of enzyme that liberates one micromole butyric acid per min from a tributyrin substrate at standard conditions (pH, 6.9–7.1, 40°C)].

*To whom correspondence should be addressed.

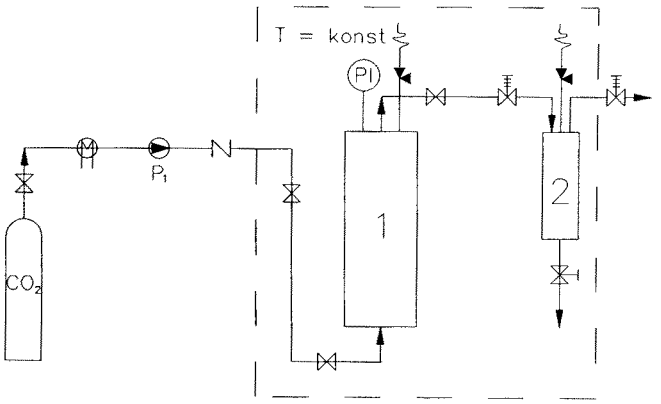


**FIG. 1.** Design of experimental apparatus for synthesis of oleyl oleate in batch-stirred tank reactor under supercritical conditions. 1, Reactor; 2, separator; $P_1$, high-pressure pump; PI, pressure indicator.

*Analytical method.* A volumetric method was used to determine the oleic acid concentration in the reaction mixture (41).

*Synthesis of oleyl oleate in a BSTR: solvent-free system.* The reaction mixture contained 47 mmol oleic acid, 47 mmol oleyl alcohol, and 1.04 g of the enzyme preparation. The mixture was stirred in a 250-mL, round-bottom flask with a magnetic stirrer and brought to the desired temperature in a water bath. Samples were taken from the reaction mixture at specific intervals, and the level of free fatty acid was determined. The concentrations of oleyl oleate were calculated from these values.

*Synthesis of oleyl oleate in a BSTR: SCF system.* The design of the experimental apparatus is shown in Figure 1. The volume of the reactor was 150 mL, designed for operation at 500 bar. The autoclave was shaken *via* an oscillating device. The whole system was placed in a constant-temperature bath.

Initially, the reaction mixture that contained 25 mmol oleic acid and 25 mmol oleyl alcohol was pumped into the reactor. Then, 0.5 g of enzyme preparation (Lipozyme$^{IM}$ or Palatase 1000 L) was added. Finally, dry $CO_2$ was pumped into the reactor up to the desired pressure. The initial concentration of reactant never exceeded its solubility limit in gas. The solubility was measured at 150 bar and 40°C. For oleic acid, it is 3.65 g/L, and for oleyl alcohol 4.2 g/L. Samples were taken out of the reactor during the reaction (same method as in Ref. 46), and the amount of free oleic acid was determined.

*Synthesis of oleyl oleate in a continuously-operated supercritical system.* The continuously-operated experimental apparatus (designed in our laboratory) is shown in Figure 2. The system consists of an air-operated, high-pressure pump (Maximator MSF 72L; Schmidt, Kranz and Co., Forge, Germany) for delivery of $CO_2$ in the system. The gaseous fluid was dried by passing it through columns packed with molecular sieves. The flow rate of $CO_2$ during these runs was varied from 0.5–1.1 L/min (measured at 20°C and 1 bar).

The substrates were pumped into the system with a high-performance liquid chromatography pump (LDC Analytical-

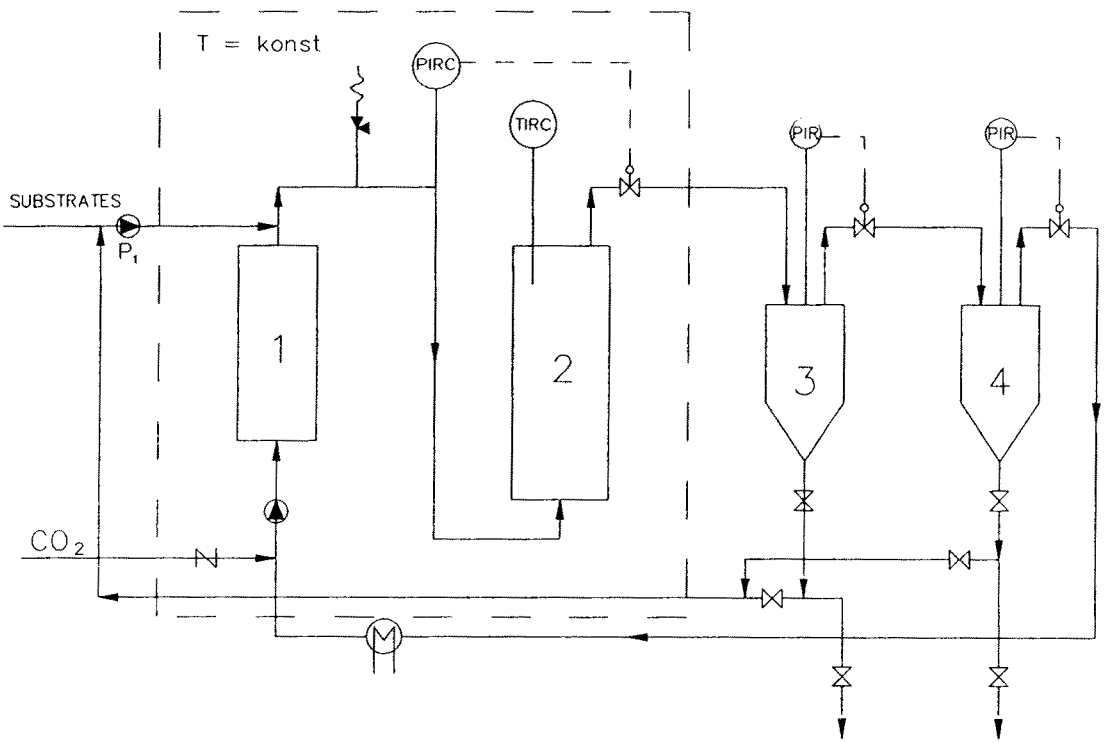


**FIG. 2.** Design of experimental apparatus for continuous synthesis of oleyl oleate under supercritical conditions. 1, Column with molecular sieves; 2, regulation enzymatic reactor; 3, separator 1; 4, separator 2; $P_1$, high-pressure pump; TIRC, temperature indication resolution control; PIRC, pressure indication regulation control.

Constametric 3000 LDC Thermo Instruments, Riviera Beach, FL) at a flow rate of 0.14–0.35 mL/min.

The $CO_2$ and substrates were equilibrated in the saturation column. The initial concentration of reactant never exceeded its solubility limit in gas. The solubility of oleic acid and oleyl alcohol in *n*-butane is much higher than in $CO_2$ (Skerget, M., and Ž. Knez, unpublished data). The reaction was performed in a fixed-bed reactor (4.5 mm i.d. × 500 mm length, 7.95 mL volume and packed with 0.5 g Lipozyme™. The substrate feed was an equimolar solution of oleic acid and oleyl alcohol. The water content of the substrates was measured by the Karl Fisher method (47).

The solute-laden supercritical carbon dioxide was depressurized through the expansion valves into separator columns S1 and S2 (volume of each 5.3 mL, length 300 mm × 16 mm i.d.), where the product and unreacted substrates were recovered. The substrates were collected in S2 and recycled (added to the feed through the pipe connecting S2 with the feed vessel). The gaseous $CO_2$ phase is finally vented to the atmosphere after flow-rate measurement through a rotameter. On pilot- or industrial-scale equipment, $CO_2$ could be condensed and recycled.

## RESULTS AND DISCUSSION

*Solvent-free system: BSTR.* Initial reaction rates for enzymatic synthesis of oleyl oleate in a solvent-free BSTR at atmospheric pressure were determined (Fig. 3). The highest value, 1.429 mmol/g/h/g of enzyme preparation, was found for 50°C. With the reduction of temperature, the initial reaction rates decreased and were 0.625 mmol/g/h/g of enzyme preparation at 40°C and 0.465 at 30°C.

*SCF system: BSTR.* Synthesis of oleyl oleate with immobilized *M. miehei* lipase (Lipozyme™) and with the water-soluble form of lipase from *M. miehei* (Palatase 1000 L) was performed at various pressures and temperatures. Some of the results are presented in Figure 4.

For enzymatic synthesis of oleyl oleate, catalyzed with Lipozyme™ in BSTR operated at supercritical conditions, the initial reaction rates were higher than those for the solvent-free system. The highest value (1.428 mmol/g/h/g of enzyme preparation) was found at 31°C at a pressure of 84.5 bar. At higher temperature and pressure (40°C, 167 bar), the initial reaction rate was lower (0.615 mmol/g/h/g of enzyme preparation). For the reaction catalyzed with Palatase 1000 L, the initial reaction rate was the lowest (0.454 mmol/g/h/g enzyme preparation), probably due to the high water activity in the system. It is evident that water activity has a strong influence on the initial reaction rates.

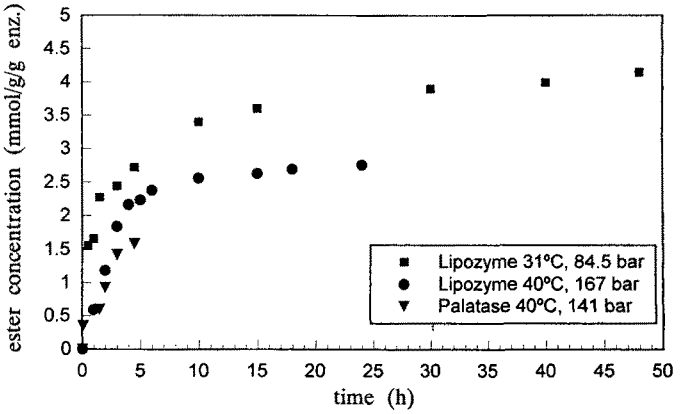


**FIG. 4.** Concentration of oleyl oleate vs. time at various process parameters for batch-stirred tank reactor operated under supercritical conditions. Abbreviations as in Figure 3. Company source for Lipozyme and Palatase listed in Figure 3.

*SCF system: continuously-operated reactor.* The dependence of the conversion to oleyl oleate vs. time is presented on Figure 5. The conversion is practically constant at reaction conditions (*P*, 150 bar; 40°C), when $CO_2$ was used as reaction medium. When *n*-butane was used (at the same conditions as mentioned previously), a decline in conversion was observed. This is probably due to deactivation of the enzyme preparation.

Water concentration in the system is one of the most important factors influencing the conversion. In our studies, the

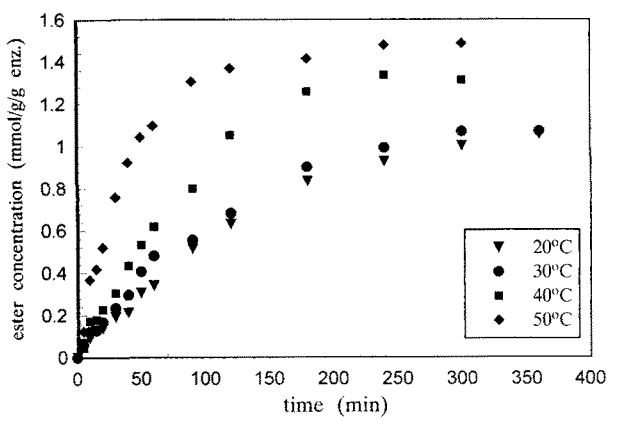


**FIG. 3.** Concentration of oleyl oleate vs. time at various temperatures in a solvent-free system (atmospheric pressure). Enz., Lipozyme (Novo Nordisk A/S, Copenhagen, Denmark).

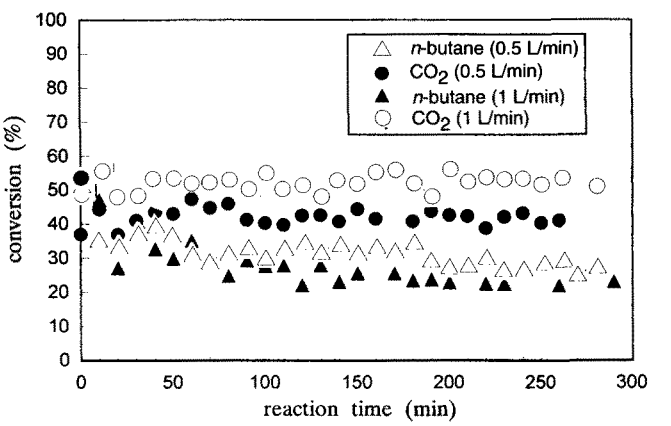


**FIG. 5.** Percent conversion vs. reaction time for continuous synthesis of oleyl oleate (*P*, 150 bar; 40°C; water activity 0.46% w/w).

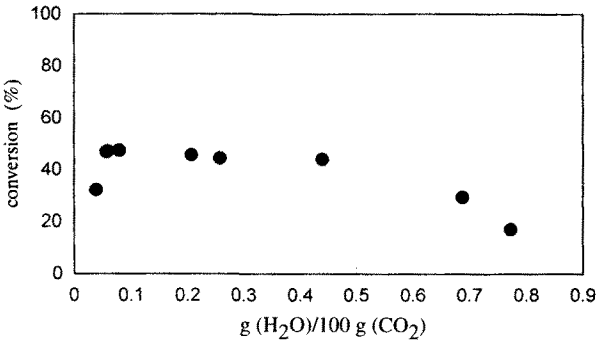


**FIG. 6.** The equilibrium conversion vs. moisture content ($CO_2$; *P*, 150 bar; 40°C).

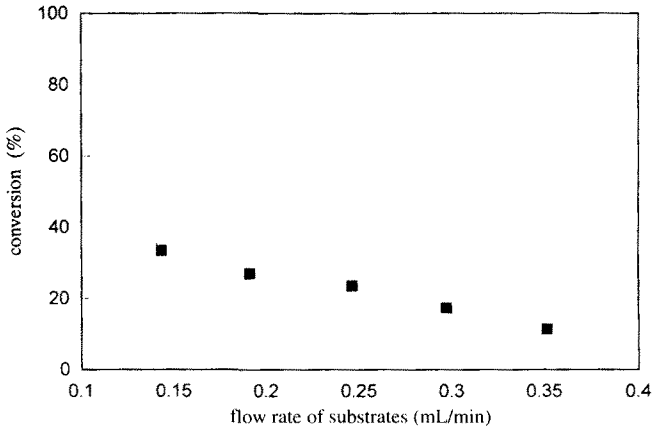


**FIG. 7.** Percent conversion vs. flow rate of substrates ($CO_2$; *P*, 150 bar; 40°C; water activity 0.46% w/w).

$CO_2$ was dried by passing it through molecular sieves, and water was added to the substrates. The high water concentrations were studied due to real industrial problems.

The equilibrium conversion vs. moisture content of the substrates (water was added to the substrates) is presented in Figure 6. The initial water concentration of the enzyme preparation was 10% by weight. Conversion is relatively high at low moisture content in the substrates but with increasing water content, conversion decreases. This phenomenon is probably due to inhibition of the enzyme preparation through the formation of carbonic acid (1,48). Another explanation for this observed phenomenon is that water is accumulated around the resin beads, and this film of water acts as a repulsive, hydrophilic barrier toward the hydrophobic fatty acid and fatty alcohol. Hydrolysis, which increases with an increase in water concentration, may be an additional reason for the decline of conversion at higher water concentrations.

The influence of the flow rates of substrates on conversion also was studied (in a relatively narrow range). Flow rate of $CO_2$ was kept constant at 0.8 L/min, and flow of the substrates was varied from 0.14–0.35 L/min. When $CO_2$ was used at 150 bar and 40°C, lower flow rates of substrates gave better yields (Fig. 7). Similar observations for other substances can be found in the literature (22,29).

On the basis of favorable results of the performed experiments, further investigations on esterification reactions, especially in nonaqueous systems, on enzymatic synthesis of chiral pure compounds will be carried out.

## ACKNOWLEDGMENT

We would like to thank Prof. Dr. Kozo Nakamura (The University of Tokyo, Tokyo, Japan) for his helpful comments on the article.

## REFERENCES

1. Nakamura, K., M.Y. Chi, Y. Yamada and T. Yano, *Chem. Eng. Commun. 45*:207 (1986).
2. Randolph, T.W., H.W. Blanch, J.M. Prausnitz and C.R. Wilke, *Biotechnol. Lett. 7*:325 (1985).
3. Hammond, D.A., M. Karel and A.M. Klibanov, *Appl. Biochem. Biotech. 11*:393 (1985).
4. Randolph, T.W., D.S. Clark, H.W. Blanch and J.M. Prausnitz, *Science 238*:387 (1988).
5. Randolph, T.W., D.S. Clark, H.W. Blanch and J.M. Prausnitz, *AIChE J. 34*:1354 (1988).
6. Randolph, T.W., D.S. Clark, H.W. Blanch and J.M. Prausnitz, *Proc. Natl. Acad. Sci. USA 85*:2979 (1988).
7. Nakamura, K., M.Y. Chi, Y. Yamada and T. Yano, *3rd World Congress on Chemical Engineering*, Tokyo, 1986, p. 945.
8. Nakamura, K., T. Hashino and T. Yano, *1st International Symposium on Supercritical Fluids*, edited by M. Perrut, Nice, I.N.P.L.A.R. Vandoeuvre, Oct. 17–19, 1988, p. 925.
9. Chi, Y.M., K. Nakamura and T. Yano, *Agric. Biol. Chem. 52*:1541 (1988).
10. Pasta, P., G. Mazzola, G. Carrea and S. Riva, *Biotechnol. Lett. 2*:643 (1989).
11. Erickson, J.C., P. Schyns and C.L. Cooney, *AIChE J. 36*:299 (1990).
12. Marty, A., W. Chulalaksananukul, J.S. Condoret and D. Combes, *High Pressure in Biotechnology*, edited by C. Balny, R. Hayashi, K. Heremans and P. Masson, John Libbey Eurotext, Montrouge, 1990, p. 461.
13. Härröd, M., M. Lilja-Hallberg and I. Elfman, *3rd International Symposium on Supercritical Fluids*, edited by G. Brunner, and M. Perrut, I.N.P.L.A.R. Nancy, Oct. 17–19, Nancy, 1994, p. 149.
14. De Carvalho, I.B., T.C. De Sampaio and S. Barreiros, *Ibid.*, p. 155.
15. Van Eijs, A.M.M., J.P.L. de Jong, H.J. Doddema and D.R. Lindeboon, *1st International Symposium on Supercritical Fluids*, edited by M. Perrut, Nice, I.N.P.L.A.R., Vandoeuvre, Oct. 17–19, 1988, p. 933.
16. Janssens, R., J.P. van der Lugt and H.M.W. Oostrom, High Pressure in Biotechnology, edited by C. Balny, R. Hayashi, K. Heremans and P. Masson, John Libbey Eurotext, Montrouge, 1988, p. 447.
17. Dumont, T., D. Barth and M. Perrut, *Abstract Handbook*, 2nd International Symposium on High Pressure Chemical Engineering, edited by G. Vetter, Erlangen, Sept. 24–26, 1990, Dechema, Frankfurt, p. 65.
18. Marty, A., W. Chulalaksananukul, J.S. Condoret, R.M. Willemont and G. Durand, *Biotechnol. Lett. 12*:11 (1990).
19. Dumont, T., D. Barth and M. Perrut, *2nd International Symposium on Supercritical Fluids*, edited by M. McHugh, Boston, May 20–22, 1991, p. 150.
20. Dumont, T., D. Barth, C. Corbier, G. Branlant and M. Perrut, *Biotechnol. Bioeng. 39*:329 (1992).
21. Marty, A., W. Chulalaksananukul, R.M. Willemont and J.S. Condoret, *Biotechnol. and Bioeng. 39*:273 (1992).

22. Marty, A., D. Combes and J.S. Condoret, *Biocatalysis in Non-Conventional Media*, edited by J. Tramper, M.H. Vermiie, H.H. Beeftink and U. von Stockav, Elsevier, Amsterdam, 1992, p. 425.
23. Bernard, P., D. Barth and M. Perrut, *High Pressure in Biotechnology*, edited by C. Balny, R. Hayashi, K. Heremans and P. Masson, John Libbey Eurotext, Montrouge, 1992, p. 451.
24. Bernard, P., D. Barth and R.M. Nicoud, *3rd International Symposium on Supercritical Fluids*, edited by G. Brunner and M. Perrut, Nancy, I.N.P.L.A.R. Nancy, Oct. 17–19, 1994, p. 137.
25. Knez, Ž., and M. Habulin, *Biocatalysis in Non-Conventional Media*, edited by J. Tramper, M.H. Vermiie, H.H. Beeftink and U. von Stockav, Elsevier, Amsterdam, 1992, p. 401.
26. Knez, Ž., and M. Habulin, *Biocatalysis 9*:115 (1994).
27. Knez, Ž., V. Rižner and M. Škerget, *3rd International Symposium on Supercritical Fluids*, edited by G. Brunner and M. Perrut, Nancy, I.N.P.L.A.R. Nancy, Oct. 17–19, 1994, p. 143.
28. Shen, X.M., T.W. de Loos and J. de Swan Arons, *Biocatalysis in Non-Conventional Media*, edited by J. Tramper, M.H. Vermiie, H.H. Beeftink and U. von Stockav, Elsevier Science Publishers, Amsterdam, 1992, p. 417.
29. Dumont, T., D. Barth and M. Perrut, *J. Supercrit. Fluids 6*:85 (1993).
30. Perrut, M., *High Pressure in Biotechnology*, edited by C. Balny, R. Hayashi, K. Heremans and P. Masson, John Libbey Eurotext, Montrouge, 1992, p. 401.
31. Douglas, A.M., W.H. Blanch and J.M. Prausnitz, *Ind. Eng. Chem. Res. 30*:939 (1991).
32. Adschiri, T., H. Akiya, L.C. Chin, K. Arai and K. Fujimoto, *J. Chem. Eng. Jpn. 25*:104 (1992).
33. Endo, Y., K. Fujimoto and K. Arai, *Developments in Food Engineering*, edited by T. Yano, R. Matsuno and K. Nakamura, Blackie Academic & Professional, London, 1994, p. 849.
34. Martins, J., T.C. Sampaio, I.B. Carvalho, M.N. da Ponte and S. Barreiros, *High Pressure in Biotechnology*, edited by C. Balny, R. Hayashi, K. Heremans and P. Masson, John Libbey Eurotext, Montrouge, 1992, p. 411.
35. Aaltonen, O., and M. Rantakyla, *2nd International Symposium on Supercritical Fluids*, edited by M. McHugh, Boston, May 20–22, 1991, p. 146.
36. Moshammer, B., B. Marr, A. Biladt and F. Froschl, *Developments in Food Engineering*, edited by T. Yano, R. Matsuno and K. Nakamura, Blackie Academic & Professional, London, 1994, p. 852.
37. Aaltonen, A., and M. Rantakyla, *Chemtech.*:240 (1991).
38. Nakamura, K., *3rd International Symposium on Supercritical Fluids*, edited by G. Brunner and M. Perrut, Nancy, I.N.P.L.A.R. Nancy, Oct. 17–19, 1994, p. 121.
39. Knez, Z., M. Leitgeb, D. Završnik and B. Lavrič, *Fat. Sci. Technol. 92*:169 (1990).
40. Knez, Ž., M. Habulin and S. Pečnik, *Proceedings of the 5th European Congress on Biotechnology,* edited by C. Christiansen, J. Munk and J. Villadsen, Munksgaard International Publishers, Copenhagen, July 8–13, 1990, p. 437.
41. Leitgeb, M., and Ž. Knez, *J. Am. Oil Chem. Soc. 67*:775 (1990).
42. Habulin, M., and Ž. Knez, *J. Memb. Sci. 61*:315 (1991).
43. Pečnik, S., and Ž. Knez, *J. Am. Oil Chem. Soc. 69*:261 (1992).
44. Krmelj, V., M. Habulin and Ž. Knez, *Fat. Sci. Technol.*, in press (1995).
45. Novo Nordisk, Analytical method AF 206/2-GB, Copenhagen.
46. Knez, Ž., and R. Steiner, *J. Supercrit. Fluids 5*:251 (1992).
47. Fisher, K., *Angew Chemie 48*:394 (1935).
48. Van Eijs, A.M.M., J.M.P. Wokke and B. Ten Brink, *Preconcentration and Drying of Food Materials,* edited by S. Bruin, Elsevier Amsterdam, 1988, p. 135.

[Received February 24, 1995; accepted June 14, 1995]