Supercritical Fluid Fractionation of Fish Oil Esters Using Incremental Pressure Programming and a Temperature Gradient

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Incremental pressure programming was demonstrated to be an effective technique for increasing the yield of 90% pure all *cis*-5,8,11,14,17-ethyl eicosapentaenoate (EPA) in the fractionation of urea-crystallized fish oil ethyl esters using supercritical fluid CO_2. The fractionations, which also produced high purity all *cis*-4,7,10,13,16,19-ethyl docosahexaenoate (DHA), were performed using a column temperature gradient. In initial experiments, the maximum temperature of the superimposed gradient was 80°C, and processing pressures ranged from 1900–2200 psi. By reduction of processing pressures, comparable yields of EPA were obtained from fractionations in which the maximum temperatures in the gradient were 70°C and 60°C.

A number of claims have appeared in both the popular and scientific literature in recent years regarding the beneficial health effects of the consumption of fish oils. The ω3 fatty acids all *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA or 20:5ω3) and all *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA or 22:6ω3) are widely thought to be the most likely of the many fatty acids present in fish oils to have beneficial physiological activity. They may also be useful in the prevention and/or treatment of a variety of illnesses—among them cardiovascular disease (1) and arthritis (2). Two recent publications provide a comprehensive overview of the clinical investigations of the therapeutic value of fish oils (3,4). While the evidence presented therein is substantial, due to the complex nature of fish oils, definitive proof of the beneficial effects of EPA and/or DHA will be difficult to establish without test materials that are highly concentrated with respect to individual ω3 fatty acids (5).

This is the fourth in a series of reports on supercritical fluid (SF) fractionation of fatty acid ethyl esters derived from fish oils (6–8). A number of conclusions were reached in the previous work.

The first of them was that carbon dioxide SF fractionation can produce fractions concentrated with respect to EPA and DHA from ethyl esters derived directly from fish oils. In agreement with several other reports (9–12), shorter-chain esters were found to be more soluble in CO_2 and were therefore collected earlier in the fractionation while longer-chain materials, accounting for the bulk of the ω3 components, are collected in later fractions. Less unsaturated components of the same carbon number as EPA (and presumably DHA) were found to have quite similar solubilities in SF CO_2; therefore, the degree to which EPA and DHA could be concentrated was limited by the presence of less unsaturated 20- and 22-carbon components. This finding is in accordance with results reported by Eisenbach (9). Recently, however, Krukonis *et al.* (13) and Krukonis (14) have suggested that by use of a SF continuous countercurrent rather than batch process, EPA could be separated from other 20-carbon esters.

The second conclusion was that application of the well-known technique of urea crystallization (15) resulted in removal of interfering saturates, monoenes, and, to a large extent, dienes and trienes. SF fractionation of the resulting mixture led to isolation of both EPA and DHA of better than 90% purity. Product yields (expressed as the wt % of the component originally present in the feedstock recovered in the 90% pure product) were approximately 70% for EPA and 80% for DHA. The fractionation was performed isobarically at 2200 psi using three heated "zones," controlled at temperatures ranging from 100°C at the top to 70°C at the lower end of the heated section of the packed column. The overall solvent-to-feed ratio (S/F), defined as the ratio of the weight of CO_2 necessary to fractionate a unit weight of esters, was found to be 450–500.

The third conclusion was that by increasing the number of heated zones from three to six, products of 90% purity can be obtained with a maximum column temperature of 80°C, although with some decrease in the yield of 90% pure products (15–20%). Operation at reduced temperatures is desirable for two reasons. First, reduction of processing temperatures lessens the possibility of decomposition of thermally sensitive polyunsaturates. More importantly, since at the processing temperatures and pressures chosen for the work discussed above, esters undergo retrograde condensation (16,17) (i.e., ester solubility in CO_2 increases with decreasing temperature), lower processing temperatures result in a lower overall S/F. In fact, it was observed that by reducing the highest column temperature from 100 to 80°C the S/F was halved, although again, there was some decrease in product yield.

All work summarized above was performed isobarically, mostly at 2200 psi. In the present work, we investigate the use of incremental pressure programming to improve product yield while attempting to minimize the S/F by operating with the highest zone temperature at 80°C or below. Incremental pressure programming involves performing the fractionation by increasing the pressure at certain predetermined points in the fractionation, in this work in increments of 50 or 100 psi. This technique is analagous to pressure (or density) gradient methods used in supercritical fluid chromatography (SFC), except that in SFC, the gradient results from a smooth, often linear, time-dependent change in pressure (18). In the present case, pressure is increased incrementally based on the changing composition of the extract during the course of a fractionation. For example, consider a mixture containing four components with significantly different solubilities in SF CO_2. The initial pressure can be chosen to be high enough to "strip" the most soluble component from the mixture but low enough to leave the bulk of the less soluble components behind. Once the most soluble component has been largely stripped from the mixture,

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the pressure can be raised to collect the second most soluble component, etc. This technique has been used for a number of applications, for example, the fractionation of complex poly(dimethyl)siloxane and perfluoroalkylpolyether mixtures using SF CO_2 and ethylene, respectively (19). Zosel (20) reported using pressure programming in combination with a "hot finger" to fractionate fish oil triglycerides and long chain α-alkenes. There are, however, no reports of the use of incremental pressure programming in tandem with a column temperature gradient.

MATERIALS

The ester feedstock was prepared by the National Marine Fisheries Service (Charleston, SC) by direct esterification of menhaden oil according to the procedure of Lehman and Gauglitz (21), followed by urea crystallization (15). Details of the process will not be discussed here but are available from Jeanne Joseph of that laboratory. The composition of the feedstock with respect to major components is given in Table 1.

EXPERIMENTAL

A full discussion of SF fractionation equipment and methodology, including details of gas chromatographic analyses of ester fractions, have been given previously (7). For easy reference, a schematic of the apparatus is shown in Figure 1. The six-foot packed column is divided

TABLE 1

Fatty Acid Profile of the Ester Feedstock and an Estimate of the Composition of the Esters With Respect to Carbon Number

Ester	GC peak area %
16:3ω4	5.2
16:4ω1	5.6
18:4ω3	7.4
20:4ω6	1.4
20:4ω3	<1.0
20:5ω3	48.9
21:5ω3	1.3
22:5ω3	1.0
22:6ω3	22.5
By carbon number[a]	
C_{16}	14.1
C_{18}	9.7
C_{20}	50.7
C_{22}	24.2

[a]Even after urea crystallization, the feedstock is still a complex mixture containing the listed as well as many minor, often unidentified components. For this reason, for example, the summed peak area percentage of 16:3ω4 and 16:4ω1 does not equal ΣC_{16} in the lower section of the table.

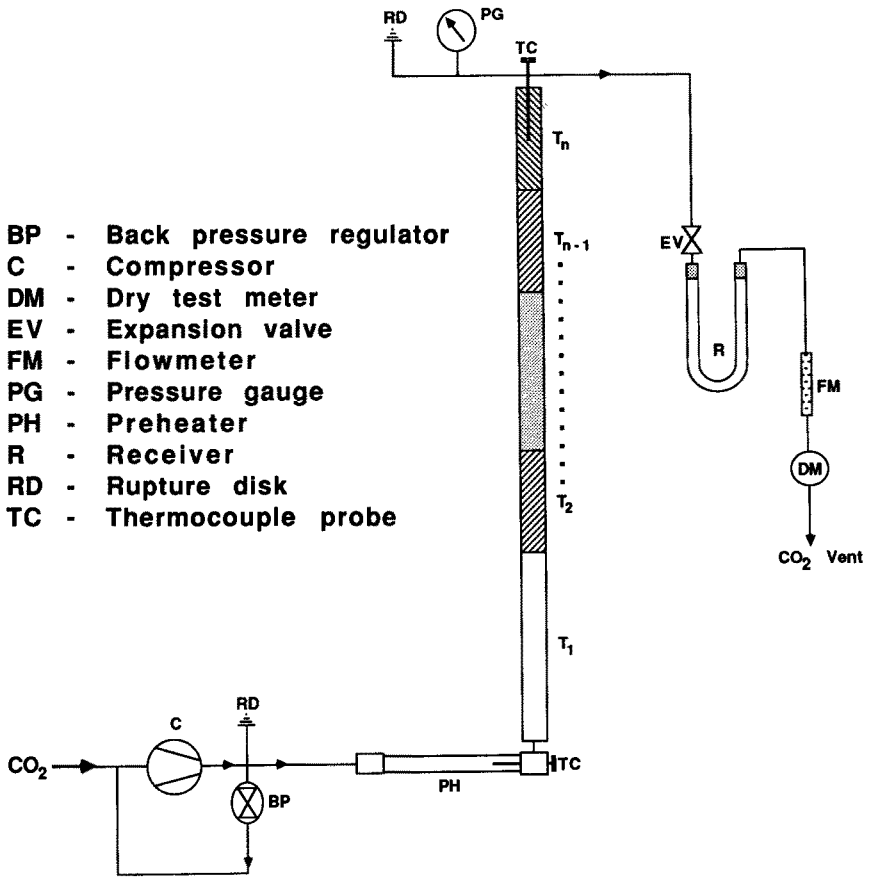

FIG. 1. Schematic diagram of the supercritical fluid fractionation apparatus. The ester charge (20–25 g) is added to the bottom of the column prior to fractionation.

into n temperatures zones established using (n-1) independently controlled heating tapes (the bottom zone is unheated). Since at the pressures and temperatures used in this work esters undergo retrograde condensation, an internal reflux is effected by the temperature gradient which increases from room temperature (T_1) at the bottom to the maximum temperature, T_n, at the top of the column.

RESULTS AND DISCUSSION

Experimental results are summarized in Table 2. Discussion of fractionations performed with $T_n = 80°C$ will be followed by a similar discussion of those fractionations performed with $T_n < 80°C$.

Fractionations in which $T_n = 80°C$. For a given column temperature gradient there are, of course, an infinite number of incremental pressure programming schemes from which to choose. Most, however, can be eliminated by balancing two practical considerations. The selectivity of the fluid, and therefore the degree of success in fractionating the mixture, is generally observed to be inversely related to pressure and thus fluid density (22). For a given set of column temperatures, the pressure should thus be minimized to effect good fractionation of the mixture. Solubility, on the other hand, is directly related to solvent density. Therefore, while a low pressure may result in good fluid selectivity, the solubility of components in the mixture may be diminishingly low, resulting in a large S/F. "Large," of course, is a relative term. The magnitude of the S/F which is considered tolerable will depend on several factors, e.g., the value of the recovered product. On the basis of previous work summarized in the introduction, a S/F of as high as 350–400 might be considered tolerable, but even then only if the yield of EPA is appreciably increased over that found previously.

In choosing the initial incremental pressure programming scheme, the urea-crystallized ester mixture (Table 1) was treated as a mixture of four components containing 16, 18, 20, and 22 carbons. Six temperature zones (not counting the bottom ambient zone) were introduced, ranging from 80°C at the top to 50°C at the bottom. It was determined experimentally that, using this temperature gradient, the 16-carbon components were recovered with a S/F of less than 400 at 1900 psi. Since esters containing 16 carbons account for about 14% of the mixture, a pressure of 1900 psi was maintained until about 14 wt % of the original charge had been collected. In fact, the pressure was maintained at 1900 psi somewhat beyond this point in order to observe the anticipated increase in the amount of solvent required to recover extract as the residual mixture became depleted with respect to 16-carbon esters and enriched in less soluble longer chain length components. The pressure was then increased to 2000 psi to "strip" the bulk of esters containing 18 carbons, i.e., the next ca. 10 wt % of the feedstock, prior to collection of the 20-carbon components at 2100 psi. Towards the end of the fractionation, when the remaining mixture consisted primarily of esters containing 22 carbons, the pressure was raised to 2500 psi for rapid recovery of these materials. The resultant fractionation curves for selected components are shown in the bottom plot of Figure 2.

The top of Figure 2 shows a plot of the pressures used in the programming scheme (right ordinate). Also shown is a plot of the "solvent-to-extract" ratio (S/E) for collection of individual fractions in the fractionation, calculated by dividing the number of grams of CO_2 necessary to collect a fraction by the fraction weight. The observed trend in the value of the S/E at various points in the fractionation is generally as anticipated. After each pressure increase, a precipitous decrease in the S/E is followed by a sometimes dramatic increase as the residual ester mixture is gradually enriched with respect to the longer-chain components. The portion of the curve generated at 2100 psi exhibits some unexpected behavior. The initial increase in the S/E is certainly expected, due to depletion of components containing 18 carbons. Furthermore, since during collection of the middle 20 wt % (i.e., from 40–60 wt %), the extract is highly concentrated (>90%) with respect to esters containing 20 carbons, one might anticipate that the S/E curve would tend to plateau, which is, indeed, observed. A hint of a second plateau is suggestive of some fractionation among 20-carbon components. However, careful examination of chromatograms for sequential fractions does not indicate significant fractionation of these components. Therefore, the observed changes of the S/E in this region are probably within experimental error.

The overall S/F for this fractionation was 340, and the yield of 90% EPA (i.e., the wt % of EPA present in the feed which was recovered in the 90% product) was 76%. Because the fractionation was terminated prematurely, DHA purity did not reach 90% and no yield figure can be given. Table 2 compares the results for this run (Exp. 3) with those of two previously reported fractionations (6,7) performed isobarically at 2200 with the top zones at 80 and 100°C (Exp. 1 and 2, respectively). The S/F for the fractionation in which incremental pressure programming was used is, as expected, higher than the analogous isobaric fractionation (Exp. 1), but about 25% lower than the isobaric fractionation with the hottest zone controlled at 100°C. The yield of 90% EPA is significantly greater than that of either isobaric fractionation.

TABLE 2

Comparison of Isobaric and Pressure-Programmed Supercritical Fractionations of Fish Oil Ethyl Esters

			Yield of 90% product				
Exp.	T_7(C)	T_2(C)	EPA	DHA	S/F	P_i(psi)[a]	P_f(psi)[a]
1	80	50	58	77	220	2200	2200
2[b]	100	70	69	81	475	2200	2200
3	80	50	76	—	340	1900	2200
4	80	40	74	87	380	1900	2200
5	80	40	85	88	340	1900	2200
6	70	40	76	74	310	1800	2100
7	70	40	80	72	310	1800	2100
8	60	40	83	67	370	1600	1900
9	60	40	87	73	330	1600	1900

[a] Initial and final pressure prior to increase of pressure to 2500 psi for rapid recovery of remaining extract. Experiments 1 and 2 taken from References 7 and 6, respectively, were performed isobarically.

[b] All fractionations except Experiment 2 were performed with six heated zones (T_1, the lowest zone, was at ambient temperature). Experiment 2 was carried out with three heated zones at 100°C, 80°C, and 70°C.

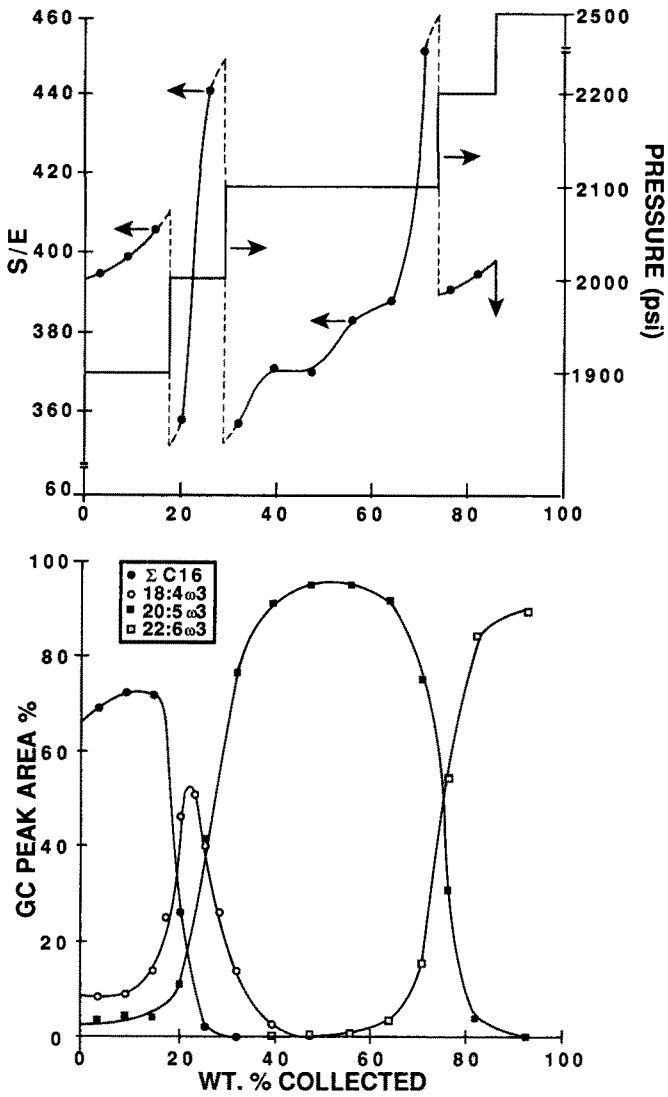

FIG. 2. Incremental pressure-programmed fractionation of fish oil fatty acid esters. Lower plot: Fractionation curves for major components in the ester feedstock (Table 1). ΣC_{16} is the sum of 16:4ω3, 16:4ω1, and a third unidentified 16-carbon ester. Upper plot: The step curve shows the pressure profile used for this fractionation (right ordinate), while the smooth curve shows the trend in the "solvent-to-extract" ratio (S/E) for individual fractions (left ordinate) defined as the weight of CO_2 necessary to collect a fraction divided by the fraction weight.

As mentioned previously, a column temperature gradient has been found to effect a superior separation, as reflected by increasing product purity and yield with an increasing number of temperature zones. Of immediate interest is the most effective gradient width, i.e., for a fixed temperature, T_n, of the top zone, what is the most effective temperature setting for the lowest heated zone (T_2 in Fig. 1)? To answer this question, some knowledge of the phase behavior of the CO_2-ethyl ester system is required. Sight glass experiments performed at 2200 psi indicated that above ca. 45°C, the CO_2-ester system consisted of a denser liquid phase primarily consisting of esters (and some CO_2) and a lighter "vapor" phase primarily consisting of CO_2, but with a quantity of dissolved esters. The ester components dissolved in this phase were found to be enriched with respect to more soluble, shorter chain components, thus indicating that above this temperature some separation of the complex mixture had been effected. Below the temperature at which the onset of this "liquid-vapor" (LV) behavior was observed, esters were found to be miscible in CO_2. Thus, for an isobaric fractionation at 2200 psi, a zone controlled at a temperature below ca. 45°C would serve no useful purpose as no enrichment would occur. Future studies will be performed to expand our knowledge of phase behavior of this system to lower and higher pressures. In the absence of such information, it seems reasonable to assume that at lower pressures the transition from one- to two-phase behavior occurs at temperatures below 45°C. Consequently, for fractionation using incremental pressure programming at pressures below 2200 psi, a gradient beginning at 40°C rather than 50°C might be more advantageous. Applying this reasoning, experiments 4 and 5 were performed with an incremental pressure programming scheme similar to that used in Experiment 3 (Fig. 1, top plot), but with a gradient in which the lowest heated zone was 40°C rather than 50°C. Although the data in Table 2 are not extensive, the wider gradient does not adversely affect product yields and, in fact, may improve them. In all subsequent experiments, the lowest heated zone was fixed at 40°C.

It might well be suggested that the isobaric fractionation in Experiment 1 is preferable to fractionations using incremental pressure programming (Exp. 3–5) by virtue of a S/F which is some 40% lower. If, however, one calculates a solvent-to-product ratio (S/P) by dividing the number of grams of CO_2 required to carry out a fractionation by the grams of product produced (the sum of EPA and DHA), the apparent advantage of the isobaric fractionation begins to vanish. For example, the S/P for Experiment 1 (490) is only 20% lower than the average of Experiments 4 and 5 (610). Additionally, given the significant effort involved in the preparation of the urea-crystallized feedstock, the superior yields (of at least EPA) from Experiment 3 further argue for the use of incremental pressure programming.

Fractionations in which T_n <80°C. By adjustment of the incremental programming scheme to somewhat lower pressures, column temperatures can be reduced further with little decrease in the yield of EPA, as shown by the results of Experiments 6 and 7 in Table 2, which are duplicate runs performed with column temperatures ranging from 40 to 70°C. The manner in which the pressure was changed in Experiment 6 is shown by the solid curve in Figure 3. The incremental programming scheme was very similar for Experiment 7. The S/F for both of these fractionations was slightly lower (310), while the yield of EPA is essentially equal to that of Experiments 4 and 5. The yield of DHA, however, is somewhat reduced. This result suggests that it may be possible to reduce column temperatures even further with no significant reduction in the yield of EPA by selecting an incremental pressure programming scheme which follows a lower pressure path.

Experiments 8 and 9 of Table 2 summarize the results of two fractionations in which the highest temperature zone was fixed at 60°C. The yield of EPA is actually slightly greater than that found in fractionations with the

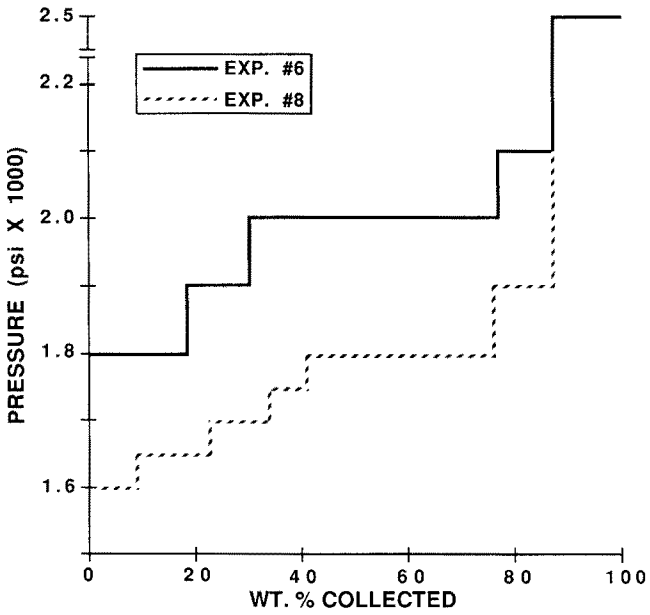

FIG. 3. Pressure programming schemes used for Experiments 6 and 8 of Table 2. Very similar schemes were used for Experiments 7 and 9.

top zones at 70 and 80°C, although the yield of DHA again appears to be reduced relative to the 80°C fractionations. To obtain this result with the top zone at 60°C, it was necessary to alter the original pressure programming scheme by increasing pressure by increments of 50, rather than 100 psi, as is shown by the dashed curve in Figure 3. The explanation for this observation lies in the fact that, as process conditions begin to approach the critical point of CO_2 (T_c = 31.1°C, P_c = 1070 psi), a smaller change in pressure is required to induce a like change in fluid density due to increasing fluid compressibility (23). A similar argument can be made for process temperatures at a given pressure. Therefore, the extent to which process pressures and temperatures can be reduced appears to be subject to practical limitations dictated by the accuracy and reliability of system components which measure and control process temperatures and pessures. Similar observations have been made for SFC near the critical point of the mobile phase (24).

The apparent decrease in the yield of DHA upon reduction of processing temperatures and pressures is not exceptionally large, and may in fact be within experimental error. With this caveat in mind, it is useful to speculate about how the yield might be improved. It is possible that, at reduced pressures, the onset of the previously discussed two-phase liquid-vapor (LV) behavior occurs below 40°C. Therefore, the yield of DHA might be increased by controlling the bottom zone at a lower temperature. Furthermore, it could well be true that the temperature at which the onset of LV behavior occurs depends on the composition of the unextracted mixture at a given point in the fractionation. This suggests that adjustment of the temperature gradient at certain stages of the fractionation might increase product yields. Confirmation of both hypotheses requires direct visual observation of phase behavior, and will be the subject of future study.

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REFERENCES

1. Leaf, A. and P.C. Weber, *N. Eng. J. Med. 318*:549 (1988).
2. Kremer, J.M., A.V. Michalek, L. Lininger, C. Huyck, J. Bigauoette, M.A. Timchalk, R.I. Rynes, J. Zieminski and L.E. Bartholomew, *Lancet 1 (8422)*:184 (1985).
3. Lands, W.E.M., *Fish and Human Health,* Academic Press, Inc., Orlando, FL, 1986.
4. Kinsella, J.E., *Seafoods and Fish Oils in Human Health and Disease,* Marcel Dekker, Inc., NY, 1987.
5. Simopoulos, A.P., *J. Nutr. 116*:2350 (1986).
6. Nilsson, W.B., V.F. Stout and J.K. Hudson, *J. Am. Oil Chem. 63*:470, Abstract 353P (1986).
7. Nilsson, W.B., E.J. Gauglitz, Jr., J.K. Hudson, V.F. Stout and J. Spinelli, *J. Am. Oil Chem. Soc. 65*:109 (1988).
8. Nilsson, W.B., E.J. Gauglitz, Jr., J.K. Hudson, and F.M. Teeny, Presented at the 194th American Chemical Society Meeting, New Orleans, LA, September 1987.
9. Eisenbach, W., *Ber. Bunsenges. Phys. Chem. 88*:882 (1984).
10. McHugh, M.A., and V.J. Krukonis, *Supercritical Fluid Extraction Principles and Practice,* Butterworths, Boston, MA, 1986, p. 191.
11. Rizvi, S.S.H., R.R. Chao and Y.J. Liaw, in *Supercritical Fluid Extraction and Chromatography,* ACS Symposium Series 366, edited by B.A. Charpentier and M.R. Sevenants, American Chemical Society, Washington, DC, 1988, p. 89.
12. Stout, V.F., and J. Spinelli, U.S. Patent 4,675,132 (1987).
13. Krukonis, V.J., C.J. Bambara, J.E. Vivian, W.B. Nilsson and R.E. Martin, Presented at the 194th American Chemical Society Meeting, New Orleans, LA, September 1987.
14. Krukonis, V.J., in *Supercritical Fluid Extraction and Chromatography,* ACS Symposium Series 366, edited by B.A. Charpentier and M.R. Sevenants, American Chemical Society, Washington, DC, 1988, p. 26.
15. Domart, C., D.T. Miyauchi and W.N. Sumerwell, *J. Am. Oil Chem. Soc. 32*:481 (1955).
16. Brule, M.R., and R.W. Corbett, *Hydrocarbon Processing 63*(6):73 (1984).
17. Debenedetti, P.G., and S.K. Kumar, *AIChE J. 34*:645 (1988).
18. Klesper, E., and F.P. Schmitz, *J. Supercrit. Fluids 1*:45 (1988).
19. McHugh, M.A., and V.J. Krukonis, *Supercritical Fluid Extraction Principles and Practice,* Butterworths, Boston, 1986, pp. 153-157.
20. Zosel, K., *Angew. Chem. Int. Ed. Engl. 17*:702 (1978).
21. Gauglitz, E.J., Jr. and L.W. Lehman, *J. Am. Oil Chem. Soc. 40*:197 (1963).
22. Prange, M.M., and W.H. Riepe, *Chem. Eng. Process. 22*:183 (1987).
23. Paulautis, M.E., V.J. Krukonis, R.T. Kurnik and R.C. Reid, *Rev. Chem. Eng. 1*:179 (1983).
24. Lee, M.L., and K.E. Markides, *Science 235*:1342 (1987).

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