# Fractionation of Menhaden Oil Ethyl Esters Using Supercritical Fluid $CO_2$

**W.B. Nilsson*, E.J. Gauglitz Jr., J.K. Hudson, V.F. Stout and J. Spinelli**
National Marine Fisheries Service, NOAA, Northwest and Alaska Fisheries Center, Utilization Research Division, 2725 Montlake Boulevard East, Seattle, WA 98112

**Supercritical fluid $CO_2$ was used to fractionate menhaden oil fatty acid ethyl esters to obtain concentrates of the esters of all *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and all *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). Separation of the ethyl esters was found to occur primarily by carbon number, thus limiting the degree to which the ethyl esters of EPA and DHA could be concentrated. Urea fractionation of whole esters in order to remove saturates, monoenes and dienes prior to fractionation with supercritical fluid $CO_2$ resulted in concentrates of EPA and DHA in purities exceeding 90%. Several criteria are given for the selection of crude oils in order to maximize both purity and yield of concentrates.**

The effect of increased dietary intake of ω3 polyunsaturated fatty acids (PUFAs) on health has received increasing attention in recent years (1). Clinical studies have noted a positive correlation between a diet high in these lipids and a decreased risk of coronary (2) and inflammatory (3) diseases. There is also evidence that ω3 fatty acids inhibit growth of certain types of tumors (4).

Fish oils are a rich source of long-chain ω3 PUFAs, the most prevalent of which are all *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA), 20:5ω3, and all *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), 22:6ω3. It has been widely assumed that EPA is the ω3 PUFA most likely to have beneficial effects on health, but there is also evidence that DHA may play a significant role (5). A major limitation in clinical studies has been the expense of individual components. Consequently, most work has been performed using fish or whole fish oils, thus making positive identification of the physiologically beneficial component(s) difficult.

There have been several attempts to isolate individual fatty acid esters by methods which include fractional vacuum distillation (6), high performance liquid chromatography (7–11) and silver resin chromatography (12). Fractional vacuum distillation requires high temperatures which can result in decomposition of PUFAs. The two chromatographic techniques involve the use of potentially toxic solvents which must be removed prior to clinical testing and, more important, they are limited with respect to possible scale-up.

An alternate method for obtaining concentrates of individual ω3 fatty acid esters is supercritical fluid extraction (13). Supercritical fluids are unique in that they can have liquid-like densities and at the same time values of transport properties (e.g., viscosity and diffusion coefficient) intermediate between those typical of liquids and gases (14). Thus they can be attractive media for extractions.

*To whom correspondence should be addressed.

The solubility of a solute in a supercritical fluid has been shown to be largely a density-driven phenomenon (15). For example, the solubility of a substance in a fluid can increase dramatically with pressure. This is especially true near the critical point where the fluid is highly compressible and a rather small increase in pressure can induce a large increase in fluid density. The dependence of solubility on temperature is somewhat more complex. At pressures near the critical point, a moderate temperature increase can cause a large decrease in fluid density resulting in a decrease in solute solubility. This is known as retrograde behavior (16). At much higher pressures, the fluid becomes less compressible and an increase in temperature induces a much less dramatic decrease in density. Thus, at higher pressures, an increase in temperature can cause an increase in solubility, i.e., "nonretrograde" behavior. It will be shown later that at the pressures investigated in this work the esters exhibit retrograde behavior.

There is growing interest in supercritical fluid extraction using carbon dioxide for applications in the food, pharmaceutical and specialty chemical industries. The critical temperature of $CO_2$ is 31.1 C. Therefore, extractions may be carried out at moderate temperatures (below 100 C) which minimize thermal degradation. In addition, no toxic solvent residue remains after extraction. A few possible applications include removal of oil from soybeans (17), polymer fractionation and monomer purification (18), fractionation of lemon oils (19), and even removal of oil from potato chips (20). Industrial decaffeination of coffee using $CO_2$ has been a reality in West Germany since 1979 (21). The reader is referred to several excellent reviews giving complete discussions of basic principles and other potential applications (22–24).

Recently, Eisenbach (25) reported a kg-scale batch $CO_2$ supercritical fluid fractionation of fatty acid ethyl esters in the retrograde region at 150 atomospheres using an internal "hot finger." His feedstock, derived from cod liver oil, reportedly contained 14.5% 20:5ω3 and 11.4% 20:1 (isomer unspecified). He found that esters were separated primarily by carbon number and reported obtaining very concentrated (90–98%) fractions of 20-carbon esters. (In this work, the stated carbon number refers to the long chain fatty acid moiety and does not include the ethyl group in the ester linkage. Compositions are reported as GC peak area %.) Since cod liver oil, however, contains a relatively large amount of 20:1, these fractions were much less concentrated with respect to EPA alone. In the case in which the composition of a fraction with respect to individual components was given, a 91.9% pure fraction of 20-carbon esters contained only 48.2% EPA. It is apparent that esters derived from an oil containing a higher percentage of 20:5ω3 relative to other 20-carbon esters would have resulted in fractions richer in EPA. With respect to this criterion, menhaden oil has a fatty

TABLE 1

**Fatty Acid Profiles of Ethyl Ester Feedstocks**[a]

| Composition | Whole esters | Composite | PUFA 1 | PUFA 2 |
|---|---|---|---|---|
| 14:0 | 7.8 | <1.0 | –[b] | –[b] |
| 16:0 | 15.6 | 2.2 | –[b] | –[b] |
| 16:1ω7 | 10.9 | 1.0 | <1.0 | <1.0 |
| 16:3ω4 | 1.1 | <1.0 | 6.3 | 5.3 |
| 16:4ω1 | 1.5 | <1.0 | 2.2 | 5.8 |
| 18:0 | 3.1 | 3.1 | –[b] | –[b] |
| 18:1ω9 | 7.6 | 5.8 | –[b] | –[b] |
| 18:1ω7 | 3.1 | 2.5 | –[b] | <1.0 |
| 18:2ω6 | 1.3 | <1.0 | <1.0 | <1.0 |
| 18:3ω3 | 1.6 | 1.2 | <1.0 | <1.0 |
| 18:4ω3 | 2.9 | 1.9 | 6.1 | 7.6 |
| 20:1ω9 | 1.2 | 2.7 | –[b] | –[b] |
| 20:4ω6 | 1.0 | 1.7 | 2.1 | 1.4 |
| 20:4ω3 | 1.5 | 3.1 | 2.6 | <1.0 |
| 20:5ω3 | 16.5 | 30.6 | 34.2 | 48.6 |
| 21:5ω3 | <1.0 | 1.6 | 2.3 | 1.3 |
| 22:5ω3 | 2.5 | 6.3 | 2.8 | <1.0 |
| 22:6ω3 | 10.9 | 26.8 | 26.9 | 22.2 |
| By carbon number[c] | | | | |
| C14 | 9.0 | <1.0 | –[b] | <1.0 |
| C16 | 32.7 | 3.8 | 14.5 | 13.1 |
| C18 | 21.3 | 16.4 | 11.7 | 9.7 |
| C20 | 21.8 | 41.8 | 39.5 | 50.5 |
| C22 | 14.5 | 36.4 | 31.8 | 25.0 |

[a]Also given is estimated composition of each material with respect to carbon number.

[b]–, Below detectable level.

[c]Most minor peaks were assumed to be due to esters with an even number of carbons and were assigned by relative retention times.

acid profile which is more advantageous for the isolation of EPA. We have therefore investigated the use of menhaden oil ethyl esters as a feedstock to obtain concentrates of the esters of both EPA and DHA using supercritical $CO_2$. Our method, though similar in principle, represents a significant modification of Eisenbach's technique.

## MATERIALS

Four separate feedstock materials were used in this work. Most work was done with a whole ester mixture selected because it was most readily available. This material was derived from a light, cold-pressed menhaden oil obtained from Haynie Products Inc., Baltimore, Maryland, in 1963. Triglycerides were converted to ethyl esters according to the procedure of Lehman and Gauglitz (26) and vacuum bleached. Molecular distillation gave a water-white material. These fatty acid esters have remained stable in cold storage (-20 C) under purified $N_2$ for more than 20 years, demonstrating that with proper treatment, polyunsaturated ester mixtures can be stored indefinitely. The composite mixture was obtained by combining a number of fractions high in EPA and DHA from several fractionations of the whole esters. PUFA 1 is a polyunsaturated fatty acid ethyl ester mixture supplied by Robert Ackman of the Technical University of Nova Scotia in Halifax. His procedure involved a urea fractionation (27) of fatty acids derived from menhaden oil, followed by esterification. Finally, PUFA 2 was obtained by urea fractionation of menhaden oil ethyl esters by the Charleston Laboratory of the National Marine Fisheries Service (Charleston, South Carolina). Details of the process are available from Jeanne Joseph of that laboratory. All ethyl ester mixtures were stored under $N_2$ at 0 C between samplings. Table 1 gives the fatty acid profiles of these four ester mixtures and an estimate of the composition of each feedstock material by carbon number. Commercial-grade carbon dioxide was obtained in 50-pound cylinders from A-L Welding, Seattle, Washington.

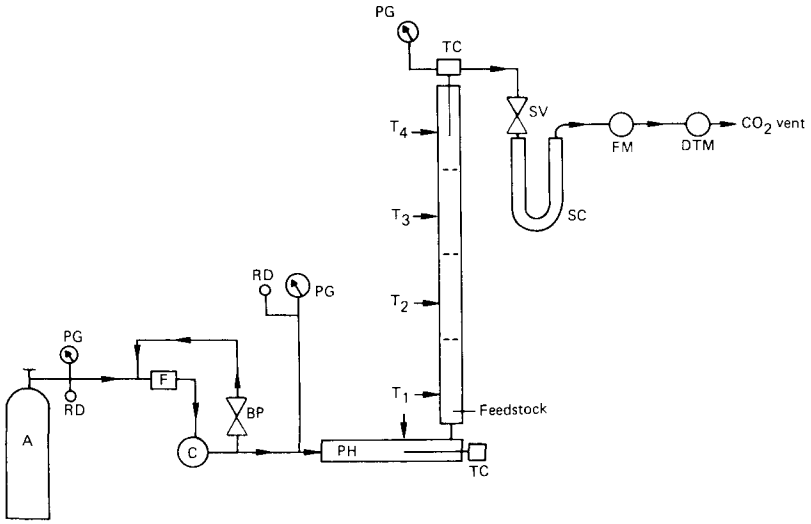


**FIG. 1. Carbon dioxide supercritical fluid extraction apparatus: (A), $CO_2$ cylinder; (PG), pressure gauges; (RD), rupture discs; (F), filter; (C), compressor; (BP), back pressure regulator; (PH), preheater; (TC), thermocouple probes (for clarity, not all probes are shown); (SV), stem valve; (SC), sample collector; (FM), flow meter; (DTM), dry test meter.**

## EXPERIMENTAL

*Extraction equipment and analyses.* A schematic diagram of the supercritical fluid extraction apparatus is shown in Figure 1. Unless otherwise noted, components were purchased from Newport Scientific, Jessup, Maryland. The heart of the system is a 10,000 psi double-ended, diaphragm-type compressor which is protected from particulate contamination by a 5-$\mu$ filter. The pressure is controlled by a back pressure regulator (Tescom, Minneapolis, Minnesota). Compressed $CO_2$ is pumped through 1/4″ O.D. high pressure 304 stainless steel (SS) tubing into a 9/16″ O.D. × 5/16″ I.D. pipe wrapped with heating tape to preheat the $CO_2$ to the desired temperature before contacting the feed material. The extraction vessel is a one-foot long 1.0″ O.D. × 11/16″ I.D. SS pipe (Autoclave Engineers, Erie, Pennsylvania). Depending on the experiment, the extractor is connected through straight couplings to up to five additional feet of pipe. The feedstock is suspended on pyrex wool at the bottom of the extractor, and the remainder of the column is filled with SS packing material. Two different packing materials were used. Initial experiments were performed with 0.375″ 304 SS ball bearings because they were immediately available. However, to lessen the possibility of channeling, later work was done using 0.16″ Propak (Scientific Development Co., State College, Pennsylvania), a 316 SS distillation packing material. By use of thermally regulated heating tapes, temperature can be varied along the length of the column. As Figure 1 shows, up to four separate column temperature "zones" ($T_1$-$T_4$), which increase from the bottom ($T_1$) to the top ($T_4$), can be introduced. Because internal thermocouples were used at the top and bottom of the column, $T_4$ and $T_1$ represent internal fluid temperatures. $T_2$ and $T_3$ are measured and controlled using thermocouple probes in contact with the outer surface of the column. Experiments in which internal and external thermocouples were placed at the same vertical location showed a difference between the column surface and internal temperatures of less than 5 C.

In a typical experiment, solute-loaded supercritical fluid $CO_2$ at $T_1$ is forced up the column through an increasing temperature profile ($T_1 \leqslant T_2 \leqslant T_3 \leqslant T_4$), causing a portion of the esters to fall out of solution. This occurs because in the pressure and temperature regime in this work, ester solubility decreases with decreasing fluid density. The introduction of a temperature profile thus results in what is analogous to rectification in a conventional fractional distillation. After leaving the column, ester-laden fluid is expanded to atmospheric pressure through a heated stem valve. The fatty acid esters are subsequently collected in a glass U-tube immersed in an ice water bath. Gaseous $CO_2$ passes sequentially through a flow meter and a dry test meter to measure flow rate and volume and is then vented.

Analyses of the fatty acid esters were performed using a Shimadzu GC9A gas chromatograph with a 30 M × 0.25 mm I.D. Durabond-225 column (J&W Scientific, Rancho Cordova, California) and flame ionization detector with helium as the carrier gas. Each run was programmed from 180 to 236 C at a rate of 2 C/min with a 9-min hold time at the final temperature.

*Method of data acquisition and presentation.* In a typical experiment, the vessel was charged with 7-20 g of ester feedstock, purged to remove air, and pressurized. Following temperature equilibration, $CO_2$ was pumped through the extractor. A large number of fractions (typically 15-35) were collected. At stages in the fractionation of special interest, the size of the fraction obtained was relatively small (0.1-0.2 g). GC analysis of each of these small fractions gives a reasonable approximation of the instantaneous composition at that

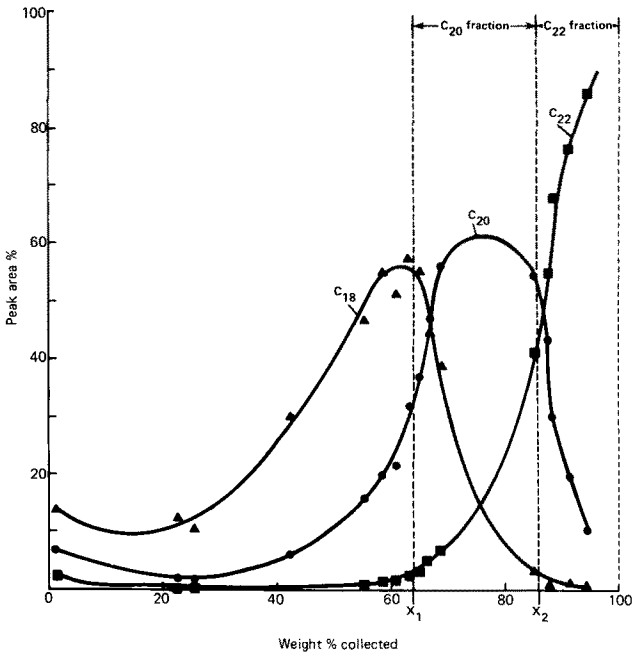


**FIG. 2. Curves for 18-carbon, 20-carbon and 22-carbon esters generated by the fractionation of whole esters using supercritical fluid $CO_2$. Conditions: 2200 psi, $T_1 = T_2 = 40$ C, $T_3 = T_4 = 100$ C, and a two-foot column. The composition of extract between $X_1$ and $X_2$ is defined as the $C_{20}$ fraction, while extract composition between $X_2$ and 100% is defined as the $C_{22}$ fraction (see text).**

point in the fractionation. The additional information provided by analyses of larger fractions allowed determination of a material balance for each major component.

Using the fatty acid profiles of small fractions, a set of fractionation curves for a given experiment can be constructed. Curves can be drawn for individual components or for fatty acids containing the same number of carbon atoms. An example of the latter representation is shown by the curves in Figure 2. These curves were generated by fractionation of the whole esters and illustrate that, to a first approximation, components are separated by carbon number. This result is in qualitative agreement with data reported by Eisenbach (25).

Fractionation curves such as those shown in Figure 2 are useful, but to facilitate rapid comparisons, the analytical data will also be used in a somewhat different manner. Consider a model ester mixture containing 60% 20-carbon esters and 40% 22-carbon esters by weight. Furthermore, assume that although esters in the model mixture may differ in degree of unsaturation, all esters of the same chain length are equally soluble. Since shorter chain length esters are more soluble, and assuming that a perfect separation is achieved, the 20-carbon esters would be recovered in 100% purity until exactly 60% of the extract has been collected. For this mixture, we define the first 60% of the extract as the "$C_{20}$ fraction." Similarly, the last 40% of the extract collected would be pure 22-carbon esters and is defined as the "$C_{22}$ fraction." In practice, of course, a perfect separation is unattainable, but determination of the EPA content of the $C_{20}$ fraction and DHA content of the $C_{22}$ fraction would provide a rigorous measure of the degree of separation under the extraction conditions chosen.

Of course, ester feedstocks are more complex than the simple model mixture discussed above, but for any real mixture the $C_{20}$ and $C_{22}$ fractions can be defined in the same manner. For example, Figure 2 shows curves obtained from a fractionation of the whole esters. For this mixture, the $C_{20}$ fraction is the extract collected between $X_1$ and $X_2$, while the $C_{22}$ fraction is that collected between $X_2$ and 100% (i.e., the remaining extract). This treatment neglects the presence of either 21-carbon esters or esters with more than 22 carbons (which is a good approximation for this feedstock material).

In a typical experiment, smaller (0.1-0.2 g) fractions were obtained near points $X_1$ and $X_2$. By extrapolation, it was then possible to determine extract composition

TABLE 2

**Dependence of the Fractionation of Whole Menhaden Oil Esters Upon Pressure and Column Temperatures[a]**

| Pressure (psi) | Experiment | Column[b] temp (C) | | $C_{20}$[c] fraction | $C_{22}$[c] fraction | | Material balance (%) | |
|---|---|---|---|---|---|---|---|---|
| | | $T_1=T_2$ | $T_3=T_4$ | % EPA | % DHA | S/F[d] | EPA | DHA |
| 2500 | 1A | 40 | 60 | 19.6 | 24.9 | 25 | 106 | 106 |
| | 2A | 40 | 80 | 31.2 | 49.0 | 110 | 97 | 103 |
| | 3A | 40 | 100 | 37.1 | 58.4 | 225 | 95 | 105 |
| | 4A | 60 | 100 | 36.8 | 50.8 | 190 | 95 | 93 |
| | 5A | 80 | 100 | 32.0 | 46.4 | 200 | 98 | 107 |
| | 6A | 100 | 100 | 28.4 | 43.4 | 225 | 101 | 103 |
| 2200 | 1B | 40 | 60 | 24.0 | 42.6 | 60 | 98 | 105 |
| | 2B | 40 | 80 | 39.9 | 53.3 | 255 | 90 | 90 |
| | 3B | 40 | 100 | 49.7 | 63.8 | 345 | 101 | 107 |

[a]Usi ng a 2-ft column, flow rate 5 standard l/min. Column packing material: 0375" stainless steel ball bearings.

[b]$T_1$-$T_4$ refer to temperatures of the four column zones defined in Figure 1.

[c]$C_{20}$ and $C_{22}$ fractions refer to extract collected between specific points in the fractionation (Fig. 2).

[d]Solvent-to-feed ratio.

of the $C_{20}$ and $C_{22}$ fractions defined above. As before, the higher the composition of the $C_{20}$ fraction with respect to EPA and the $C_{22}$ fraction with respect to DHA, the better the separation.

## RESULTS AND DISCUSSION

Data for each of the four materials in Table 1 are discussed separately.

*Whole esters.* A preliminary series of fractionation tests designed to investigate the effects of pressure and column temperatures on component separation was performed on the whole menhaden oil mixture. These tests were carried out using a 2-ft column with two temperature regions of equal length. Referring to Figure 1, this configuration corresponds to $T_1=T_2<T_3=T_4$.

The effect of temperature in the two zones is shown in Table 2 for pressures of 2200 psi and 2500 psi. At these pressures, the EPA content of the $C_{20}$ fraction and the DHA content of the $C_{22}$ fraction both increase as the upper zone temperature is increased from 60 C to 100 C while leaving the lower zone at 40 C. This result can be explained by first assuming that at both pressures extraction is occurring in the "retrograde" region. In other words, ester solubility decreases with increasing temperature. If this is true, as the temperature of the upper zone is increased, a greater portion of esters will "fall out" of the fluid phase and return to the lower zone. In essence, increasing the upper zone temperature is analogous to increasing the reflux ratio in a conventional reflux distillation (28).

The assumption that the extraction is operating in the region of retrograde solubility has not yet been proven. Data supporting this assumption are found in the values of the solvent-to-feed ratio (S/F) in Table 2. The solvent-to-feed ratio is defined as the number of grams of $CO_2$ required to carry out the fractionation divided by the number of grams of esters fractionated. Experiments 1A-3A, performed at 2500 psi, show that holding the lower zone temperature at 40 C while increasing the upper zone temperature from 60 C to 100 C increases the S/F. The increasing ratio with temperature shows that the esters are less soluble at higher temperatures. Experiments 1B-3B show that this retrograde behavior also occurs at 2200 psi.

Further inspection of Experiments 1A-3A and 1B-3B shows that for the same set of column temperatures, the fractionation at 2500 psi results in a poorer separation. The higher pressure results in an increase in solvent power, an accompanying increase in the solubility of the ester feedstock, and a decrease in selectivity.

Data discussed thus far are in qualitative agreement with work reported by Eisenbach using a packed column and internal "hot finger" (25). In that work, no data on the dependence of the separation on the temperature of the extraction vessel and column (which are analogous to the lowest temperature zone of the column for this work) were reported. In Table 2 the effect of holding the temperature of the upper half of the column constant at 100 C while increasing the temperature of the lower half of the column from 40 C to 100 C is shown for experiments at 2500 psi. A decrease in the percent of EPA in the $C_{20}$ fraction and of DHA in the $C_{22}$ fraction is observed. When both zones are at 100 C (Exp. 6A), no reflux occurs, leading to a poorer separation. As the temperature of the lower zone is decreased (Exp. 5A-3A), the separation is seen to improve, most probably due to a gradual increase in the reflux ratio as the lower zone temperature decreases.

The values of the solvent-to-feed ratio shown in Table 2 exhibit the expected trends. A decrease in pressure from 2500 psi to 2200 psi, keeping all other conditions the same, (compare 1B-3B with 1A-3A) results in a substantial increase in S/F due to decreased

TABLE 3

**Dependence of the Fractionation of Whole Menhaden Oil Ethyl Esters Upon Zone Temperature, Fluid Flow Rate[a]**

| Experiment | Column temperatures (C)[b] | | | | Flow rate (SL/min)[c] | Column length (ft) | $C_{20}$[b] fraction % EPA | $C_{22}$[b] fraction % DHA | S/F[c] |
|---|---|---|---|---|---|---|---|---|---|
| | $T_1$ | $T_2$ | $T_3$ | $T_4$ | | | | | |
| 7 | 40 | 40 | 60 | 60 | 5 | 2 | 22.6 | 33.4 | 80 |
| 8 | 40 | 40 | 80 | 80 | 5 | 2 | 39.3 | 53.7 | 170 |
| 9A | 40 | 40 | 90 | 90 | 5 | 2 | 41.3 | 57.1 | 320 |
| 9B | 40 | 40 | 90 | 90 | 5 | 2 | 38.6 | 53.5 | 300 |
| 10A | 40 | 40 | 100 | 100 | 5 | 2 | 49.4 | 57.8 | 320 |
| 10B | 40 | 40 | 100 | 100 | 5 | 2 | 47.3 | 58.7 | 370 |
| 11A | 40 | 40 | 100 | 100 | 10 | 2 | 45.4 | 59.3 | 390 |
| 11B | 40 | 40 | 100 | 100 | 10 | 2 | 42.6 | 55.9 | 400 |
| 12A | 20 | 20 | 100 | 100 | 10 | 2 | 43.0 | 55.9 | 435 |
| 12B | 20 | 20 | 100 | 100 | 10 | 2 | 44.6 | 55.7 | 380 |
| 13 | 20 | 20 | 20 | 100 | 10 | 4 | 44.6 | 57.6 | 425 |
| 14 | 20 | 20 | 20 | 100 | 10 | 6 | 46.4 | 55.1 | 360 |
| 15A | 20 | 70 | 80 | 100 | 10 | 6 | 51.0 | 58.0 | 365 |
| 15B | 20 | 70 | 80 | 100 | 10 | 6 | 51.9 | 59.5 | 380 |

[a]Column packing material: 0.16" Propak. Fractionations were done at 2200 psi.

[b]Defined in Table 2.

[c]SL, standard liters.

ester solubility at the lower pressure. In addition, there are no substantial differences in the values of S/F for experiments 3A-6A in which the upper zone temperature was held at 100 C while the lower zone temperature was gradually increased, showing that the magnitude of S/F is determined primarily by the upper zone temperature. Finally, a high solvent-to-feed ratio generally correlates with an improved separation as measured by the higher EPA and DHA content of the $C_{20}$ and $C_{22}$ fractions, respectively. The increased selectivity and better separation observed at lower pressures and higher temperatures result in a higher solvent-to-feed ratio.

Highly unsaturated esters such as EPA and DHA are readily decomposed under some conditions. Therefore, the stability of these compounds in supercritical fluid $CO_2$ under process conditions is critical to the applicability of this technique. Included in Table 2 are the material balance values for EPA and DHA. Values near 100% indicate no significant loss of these compounds due to thermal decomposition. Values of 90 to 110% were obtained for all work reported here and, for brevity, material balances are omitted in all other tabulations. (A material balance in excess of 100% is probably due to residual $CO_2$. After collection, each fraction was quickly heated to sublime most of the $CO_2$ and then weighed. To avoid autoxidation due to prolonged exposure to air, fractions were not brought to constant weight).

For the initial experiments described above, the column was packed with 0.375″ stainless steel bearings because they were immediately available. To avoid the possibility of channeling, a stainless steel distillation packing material (Propak) seemed more suitable. Results of several fractionations of the whole esters in a column packed with Propak are shown in Table 3. Data obtained using Propak confirm the trends found in fractionations using bearing packings; additional variables were examined as well. Experiments 7-10 show that the separation improves as the upper zone temperature is increased. Of particular interest are experiments 9A and 9B, for which $T_3 = T_4 = 90$ C. Eisenbach (25) reported a better separation of cod liver oil esters at 90 C than at 100 C. His results are not confirmed by data reported here, and it seems unlikely that this discrepancy is due to differences in feedstock composition. Experiments 10 and 11 show the dependence of the separation on fluid flow rate. A slight decrease in the EPA content of the $C_{20}$ fraction is observed. This presumably is due to less complete thermal equilibration at the higher flow rate. Comparison of experiments 11 and 12 reveals a rather interesting as well as convenient result. Within experimental scatter, there is no discernible difference between fractionations in which $T_1 = 40$ C and 20 C (i.e., below the critical temperature). Therefore, it is not necessary to preheat $CO_2$ prior to entry into the column. Increasing the column length from 2′ to 6′ with the top of the column at 100 C does not result in any substantial improvement, as shown by experiments 12-14. However, the use of a longer column permits the introduction of additional temperature zones such that $T_1<T_2<T_3<T_4$. In experiments 15A and 15B, column temperatures of $T_1 = 20$ C, $T_2 = 70$ C, $T_3 = 80$ C and $T_4 = 100$ C were chosen arbitrarily. It is seen that these column temperatures significantly increase the percent of EPA in the $C_{20}$ fraction.

The increase in the degree of separation upon the introduction of additional temperature zones is not without precedent. In 1949, Passino (29) described a process for purification and fractionation of vegetable and animal triglycerides. In principle, that process, known as the "Solexol" process, is quite similar to what is described in this work. The major differences are that the Solexol process was continuous and used liquid propane near the critical point. Propane in this "paracritical region," like $CO_2$, is highly compressible; an increase in temperature can result in a significant decrease in solvent density and therefore solute solubility. Passino states (29) that it is ". . . not necessary nor is it usually desirable to maintain the temperature at a constant value throughout the tower." He goes on to state rather elegantly that if there is an increasing temperature gradient, ". . . At the lowest point in the tower, an equilibrium is reached whereby certain constituents dissolve in propane, having for example, the density X. A little higher in the tower, the density of the propane is changed to some lower value, X-Y. The solubility relationships, as a result, also have changed to such an extent that an additional bottoms phase drop out; this falls downward and recontacts a higher propane density phase. It can be seen that in this way an effective internal reflux can be attained in the tower." Although, as Passino implied, a more uniform gradient probably would be more effective, the temperature zones used in this work serve the same purpose.

Inspection of Table 3 shows that S/F values under various sets of conditions are consistent with the previous discussion. The data confirm that at a given pressure S/F is determined primarily by the temperature of the upper zone and is insensitive to the lower zone temperature(s) and flow rate.

*Composite.* This material, obtained by combining latter fractions from several fractionations of the whole esters, is relatively rich in EPA (30.6%) and DHA (26.8%). Material sufficient for only two fractionations was available. Both were performed at 2500 psi using a 2′ column with temperatures of $T_1=T_2=40$ C and $T_3=T_4=100$ C. The $C_{20}$ fractions from these two experiments were 39.9% and 41.6% EPA, while the $C_{22}$ fractions contained 56.5% and 55.3% DHA, respectively. These results can be compared with those from experiment 3A (Table 2), which is a fractionation performed on the whole esters under identical conditions. Although the composite is significantly more concentrated with respect to EPA and DHA, no significant increase in the EPA content of the $C_{20}$ fraction or the DHA content of the $C_{22}$ fraction is apparent.

This result is an additional illustration that separation occurs primarily by carbon number. The whole ester mixture contains approximately 21.8% 20-carbon esters and 16.5% EPA. Therefore, about 75% of the 20-carbon esters are EPA. Similarly, although the composite is richer in EPA than the whole ester mixture, it is also more concentrated with respect to total 20-carbon esters (41.8%). Once again, only about 73% of the 20-carbon esters are EPA. Theoretically, a 100% pure 20-carbon fraction would be only about 75% EPA for either feedstock. Similar arguments can be made

with respect to DHA.

*PUFA 1.* From the data presented to this point, it is apparent that removal of the more saturated fatty acid esters from a "whole" ester mixture is necessary to obtain fractions containing EPA and DHA in higher purities. This can be accomplished by performing a urea fractionation (26) which preferentially removes saturates, monoenes, dienes and, to some extent, trienes.

The material designated PUFA 1 was obtained by urea fractionation of menhaden oil fatty acids originally containing 15.3% EPA and 9.5% DHA. After esterification the mixture contained 34.2% EPA (total 20-carbon ester content 39.5%) and 26.9% DHA (total 22-carbon content 31.8%). This feedstock (Table 1) is more promising for fractionation with supercritical fluid $CO_2$ because EPA and DHA both account for ca. 85% of the 20- and 22-carbon esters, respectively. It is, therefore, theoretically possible to obtain ca. 85% pure EPA and DHA (vs ca. 75% for the whole esters). An additional advantage is revealed by comparing the curves for PUFA 1 (Fig. 3) and the whole ester mixture (Fig. 2). The notable decrease in overlap between the 18- and 20-carbon curves is due to the somewhat fortuitous fact that, in a typical menhaden oil, the 18-carbon (as well as the 14- and 16-carbon) esters are primarily saturates and monoenes. Since these fatty acids are preferentially removed by urea fractionation, the problem of separating 18- from 20-carbon esters demonstrated in Figure 2 is significantly diminished. Consequently, the $C_{20}$ curve for PUFA 1 maximizes at about 80% vs 60% for the whole ester mixture. As Figure 3 indicates, this results in fractions containing EPA in purities approaching 70%. (The small gap indicated between the $C_{20}$ and $C_{22}$ fractions account for the 2.3% of 21:5ω3. The contribution of 21-carbon esters was neglected in Figure 2 because these are minor components in the whole ester mixture).

The reproducibility of the data is demonstrated graphically by the fractionation curves in Figure 3. Each curve is the result of three replicate fractionations. The smoothness of the curves drawn through experimental points suggests rather remarkable reproducibility. This is confirmed by the results for these experiments expressed in terms of the EPA content of the $C_{20}$ fraction and the DHA content of the $C_{22}$ fraction in Table 4, experiments 16A-16C.

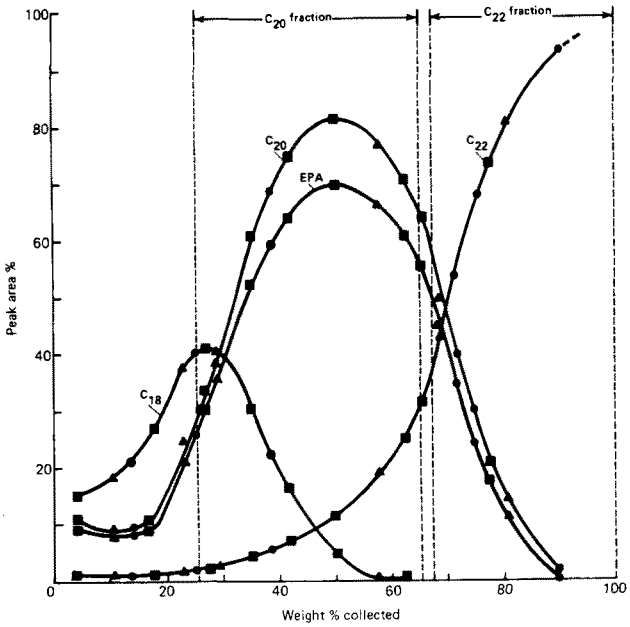


**FIG. 3. Curves for EPA and total 18-, 20- and 22-carbon esters obtained from fractionation of PUFA 1 using supercritical fluid $CO_2$. Curves are formed from three replicate runs (16A-C in Table 4) as indicated by different symbols. Conditions: 2200 psi, $T_1 = T_2 = T_3 = 22$ C, $T_4 = 100$ C, and a six-foot column.**

Also shown in Table 4 are the results of four other fractionations using a 6′ column .Introduction of additional temperature zones results in an incraese in the EPA content of the $C_{20}$ fraction from about 58% to about 64%, similar to the previous finding with the whole esters. Some of the small fractions obtained in experiments 18 and 19 contained EPA in purities exceeding 75%.

The S/F values given in Table 4 are consistently higher than those found for fractionations of the whole esters (Table 3), because of changes in the average chain length of the feedstock. As the composition of the whole ester material shown in Table 1 indicates, the shorter-chain fatty acid esters (14 to 18 carbons)

**TABLE 4**

**The Dependence of the Fractionation of PUFA 1 (see Table 1) Upon Zone Temperature and Column Length[a]**

| Experiment | Column temperatures (C)* | | | | Column length (ft) | $C_{20}$* fraction % EPA | $C_{22}$* fraction % DHA | S/F* |
|---|---|---|---|---|---|---|---|---|
| | $T_1$ | $T_2$ | $T_3$ | $T_4$ | | | | |
| 16A | 20 | 20 | 20 | 100 | 4 | 58.0 | 69.0 | 510 |
| 16B | 20 | 20 | 20 | 100 | 4 | 58.5 | 67.9 | 530 |
| 16C | 20 | 20 | 20 | 100 | 4 | 58.8 | 65.8 | 515 |
| 17 | 20 | 20 | 20 | 100 | 6 | 54.8 | 68.1 | 495 |
| 18 | 20 | 20 | 80 | 100 | 6 | 64.5 | 74.0 | 470 |
| 19 | 20 | 70 | 80 | 100 | 6 | 64.1 | 68.8 | 460 |
| 20 | 20 | 80 | 90 | 100 | 6 | 63.1 | 64.0 | 470 |

*Defined in Table 2.

[a]Column packaging material, 0.16″ Propak. All fractions 2200 psi at a 10 SL/min flow rate.

derived from menhaden oil are largely saturated and monounsaturated, while the 20and 22-carbon esters are mostly polyunsaturates. Urea fractionation removes saturates and monoenes, leaving PUFA 1 concentrated with respect to longer-chain esters. The lower solubility of these esters in $CO_2$ leads to a higher solvent-to-feed ratio.

*PUFA 2.* The importance of the fatty acid profile of the feedstock in obtaining the maximum concentration of EPA is further demonstrated by the fractionation of PUFA 2 in which EPA constitutes about 96.5% of the total 20-carbon esters (Table 1). It is thus preferable to PUFA 1 for obtaining EPA concentrates. This is confirmed by the fractionation curves for EPA shown in Figure 4. The dashed curve was obtained at 2200 psi and $T_4$ = 100 C, $T_3$ = 80 C, $T_2$ = 70 C, and $T_1$ = 22 C with approximately 7.6 g of feedstock. The maximum of the curve is about 91.5% EPA (vs a maximum of approximately 82% EPA for PUFA 1 under identical conditions). The second EPA curve shown in Figure 4 (solid curve) was generated by a fractionation under identical conditions, but with 20.0 g rather than 7-8 g of feedstock. Surprisingly, with the larger charge of esters, the maximum concentration of EPA obtained was in excess of 95%. This result has been confirmed. One possible explanation for this finding is that, for a given volume of packing material, some minimum charge is necessary to provide sufficient reflux on the column to completely "wet" the packing at all points. Complete wetting of the packing maximizes the thermal and material exchange between downflowing liquid reflux and rising ester-laden fluid, thus enhancing the fractionation (30).

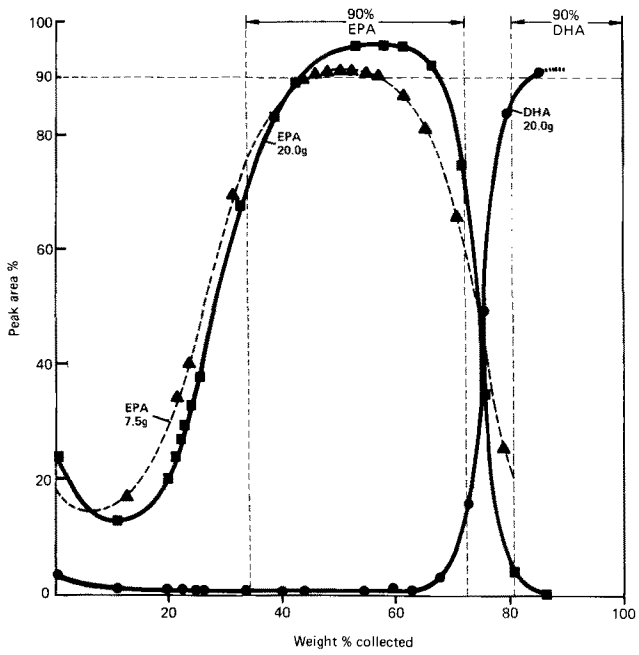


**FIG. 4. Curves generated by the fractionation of PUFA 2 using supercritical fluid $CO_2$. The dashed curve (▲) is for EPA obtained from the fractionation of 7.5 g of the feedstock. The solid curves are for EPA (■) and DHA (●) obtained from the fractionation of 20.0 g of feedstock. Conditions: 2200 psi, $T_1$ = 22 C, $T_2$ = 70 C, $T_3$ =80 C, $T_4$ = 100 C, and a six-foot column.**

For a fractionation of 20 g of PUFA 2 under these conditions, the yield of 90% pure EPA is estimated to be 37% of the total amount of extract collected. Under the same conditions, the yield of 90% pure DHA is approximately 20 wt %. Figure 4 indicates the points between which extract would be collected to obtain 90% concentration of each of the components. As found with PUFA 1, the solvent-to-feed ratios are rather high (450-500), owing to the longer average chain length of this material.

The preceding discussion indicates that the esters of EPA and DHA derived from menhaden oil can be obtained in high purity using supercritical fluid $CO_2$. Since by this technique separation of the esters occurs primarily by carbon number, it is not possible to effect a clean separation of fatty acid esters differing only in degree of unsaturation. The extent to which these components can be concentrated from whole esters is therefore limited by the presence of other 20- and 22-carbon components. Removal of saturates, monoenes and dienes by urea fractionation makes it possible to obtain EPA and DHA in purities exceeding 90%. Recent work (31), however, indicates that a continuous countercurrent rather than a batch process may be capable of separating esters of equal chain length, thus eliminating the need for urea crystallization. The yield of these highly concentrated materials is largely dependent upon the fatty acid profile of the crude oil from which the ester feedstock is obtained. Since the fatty acid content of menhaden oils can vary considerably with season, geography and fish maturity (32), selection of oil for the ester feedstock should be done with several criteria in mind. Specifically, because esters are separated primarily by carbon number and urea fractionation does not significantly remove tetra- or pentaenes, the most promising oil should contain a high ratio of EPA/20:4ω3 + ω6) and DHA/22:5ω3. In addition, because it is difficult to separate 18and 20-carbon esters cleanly, a low ratio of 18:4ω3/EPA is desirable.

## ACKNOWLEDGMENTS

This work was partially funded by a grant administered by the National Fish Meal and Oil Association. We express our appreciation to Roy Martin, scientific and technical director, National Fish Meal and Oil Association, for his confidence and support, and to Val Krukonis, Phasex Corporation, for advice and valuable discussions. We also thank Jeanne Joseph and coworkers at the Charleston Laboratory of the National Marine Fisheries Service for supplying urea fractionated menhaden oil esters.

## REFERENCES

1. Lands, W.E.M., *Fish and Human Health*, Academic Press, Inc., Orlando, FL, 1986.
2. Kromhout, J.M., E.B. Bosshieter and C. Coulander, *N. Engl. J. Med. 312*:1205 (1985).
3. 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 I* (8422):184 (1985).
4. Karmali, R., *J. Natl. Cancer Inst. 73*:457 (1984).
5. Rao, G.H.R., E. Radha and J.G. White, *Biochem. and Biophys. Res. Commun. 117*:549 (1984).

6. Ackman, R.G., P.J. Ke and P.M. Jangaard, *J. Am. Oil Chem. Soc. 50*:1 (1973).
7. Aveldano, M.I., M. Van Rollins and L.A. Horrocks, *J. Lipid Res. 24*:83 (1983).
8. Bailie, A.G., T.D. Wilson, R.K. O'Brien, J.M. Beebe, J.D. Stuart, E.J. McCosh-Lilie and D.W. Hill, *J. Chromatogr. Sci. 20*:466 (1982).
9. Halgunset, J., E.W. Lund and A. Sunde, *J. Chromatogr. 237*:496 (1982).
10. Roggero, J.P., and S.V. Coen, *J. Liq. Chromatogr. 4*:1817 (1981).
11. Pei, P. T-S., R.S. Henley and S. Ramachandran, *Lipids 10*:152 (1975).
12. Adlof, R.O., and E.A. Emken, *J. Am. Oil Chem. Soc. 62*:1592 (1985).
13. Stout, V.F., and J. Spinelli, U.S. Patent Application 879,543 (1986).
14. McHugh, M., in *Recent Developments in Separation Science*, Vol. 9, edited by N.N. Li and J.M. Calo, CRC Press, Boca Raton, FL, 1986, p. 79.
15. Chrastil, J., *J. Phys. Chem. 86*:3016 (1982).
16. Brulé, M.R., and R.W. Corbett, *Hydrocarbon Processing 63* (6):73 (1984).
17. Friedrich, J.P., and G.R. List, *J. Agric. Food Chem. 30*:192 (1982).
18. Krukonis, V., *Polymer News 11*:7 (1985).
19. Robey, R.J., and S. Sunder, paper presented at 1984 AIChE Meeting, San Francisco, CA.
20. Hannigan, K.J., *Chiltons Food Eng. July 53*:77 (1981).
21. Rizvi, S.S.H., J.A. Daniels, A.L. Benado and J.A. Zollweg, *Food Tech. 40* (7):57 (1986).
22. McHugh, M., and V. Krukonis, *Supercritical Fluid Extraction Principles and Practice*, Butterworths, Boston, MA, 1986.
23. Paulaitis, M.E., V.J. Krukonis, R.T. Kurnik and R.C. Reid, *Rev. Chem. Eng. 1*:179 (1983).
24. Williams, D.F., *Chem. Eng. Sci. 36*:1769 (1981).
25. Eisenbach, W., *Ber. Bunsenges. Phys. Chem. 88*:882 (1984).
26. Lehman, L.W., and E.J. Gauglitz Jr., *J. Am. Oil Chem. Soc. 41*:533 (1964).
27. Sumerwell, W.N., *J. Am. Chem. Soc. 79*:3411 (1957).
28. Robinson, C.S., and E.R. Gilliland, *Elements of Fractional Distillation*, McGraw-Hill, New York, 1950, p. 370.
29. Passino, H.J., *Ind. Eng. Chem. 41(2)*:280 (1949).
30. Rose, A., and E. Rose, in *Technique of Organic Chemistry-Distillation*, edited by A. Weissberger, Interscience Publishers, Inc., 1951, p. 175.
31. Krukonis, V.J., C.J. Bambara, J.E. Vivian, W.B. Nilsson and R.E. Martin, paper presented at the 194th ACS meeting, New Orleans, LA.
32. Joseph, J.D., *Mar. Fish. Rev. 47*(3):30 (1985).

[Received December 9, 1986]