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SEMI-PREPARATIVE HPLC FRACTIONATION OF CONSUMER FISH OIL (TRIACYLGLYCEROLS)

Mary Jo Wojtusik[1], Phyllis R. Brown[1]*, and Joseph G. Turcotte[2]

[1]*Department of Chemistry*
[2]*Department of Medicinal Chemistry*
University of Rhode Island
Kingston, Rhode Island 02881

ABSTRACT

A reversed-phase high performance liquid chromatographic method was developed for the semi-preparative fractionation of fish oil-derived triacylglycerols containing the esterified omega-3 fatty acids all-cis-5,8,11,14,17-eicosapentaenoic acid [EPA] and all-cis-4,7,10,13,17,20-docosahexaenoic acid [DHA]. Analytical separation conditions, such as mobile phase composition and flow rate could be directly applied to the semi-preparative mode, which was further optimized. Separation of triacylglycerol fractions was obtained in 15 minutes using flow rates of 3.0 ml/min with a mobile phase of acetone/acetonitrile (65:35, v/v). 250-mg samples of the fish oil were fractionated and multi-milligram quantities of triacylglycerols were separated, which were 65% enriched in esterified EPA and DHA; a production rate of 500 mg/hr of this enriched fraction was obtained.

INTRODUCTION

The possible health benefits of a diet supplemented with fish oils has been the focus of much recent attention. Studies in

humans and experimental animals indicate that administration of marine oils correlates with antihyperlipidemic (1-3), platelet anti-aggregatory (1,2,4-6), antihypertensive (7-9), and antiinflammatory (10-12) effects. Native fish oils, which primarily are highly complex mixtures of triacylglycerols, contain relatively large amounts of the esterified omega-3 fatty acids, 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)]. However, the potential nutritional and/or therapeutic indications of these marine triacylglycerol-derived polyunsaturated fatty acids has yet to be established unequivocally. Semi-preparative methods that result in the separation of triacylglycerol fractions that are significantly enriched in esterified EPA and DHA would provide concentrates, which then can be used for in vivo, ex vivo, and in vitro evaluation of the dietary form of derived omega-3 polyunsaturated fatty acids.

The separation of triacylglycerols of natural oils is routinely performed with nonaqueous, reversed-phase high performance liquid chromatography (RPLC) (13-21); separation is according to carbon number and degree of unsaturation (22). Nonaqueous mobile phases are used since the nonpolar triacylglycerols are not soluble in aqueous mobile phases. In the present study, an optimized analytical RPLC method was scaled-up to provide fractionation of triacylglycerols containing highly polyunsaturated molecular species. The percentages of EPA and DHA in each fraction and the production rate of triacylglycerols containing esterified EPA and DHA are reported. The separation of a

triacylglycerol fraction that is significantly enriched in the major omega-3 esterified fatty acids [EPA, DHA] is demonstrated.

MATERIALS AND METHODS

Chemicals. All solvents used in the chromatographic mobile phases and as sample diluents were HPLC grade and purchased from Fisher Scientific Company (Fairlawn, NJ). Butylated hydroxytoluene (BHT) from Aldrich Chemical Company (Milwaukee, WI) was used as an antioxidant and added to all solvents used as sample diluents.

Reference Triacylglycerols. The following simple achiral triacylglycerols were obtained from Nu-Chek Prep (Elysian, MN): tridecanoin [30:1]; trimyristin [42:0]; triolein [54:3]; trilinolein [54:6]; trilinolenin [54:9]. A reference mixture, which was used to evaluate the efficiency of the semi-preparative column, was prepared. The mixture contained triacylglycerols in the following final concentrations: tridecanoin [5 mg/mL]; trimyristin [50 mg/mL]; triolein [50 mg/mL]; trilinolein [50 mg/mL]; trilinolenin [25 mg/mL]. The individual reference triacylglycerols and the reference mixture were dissolved in dichloromethane containing BHT [100 mg/L] and stored in reaction vessels equipped with a valve system (Supelco, Bellefonte, PA); these vessels were used so that samples could be stored in a nitrogen atmosphere and at the same time permit easy withdrawal of aliquots for injection into the chromatograph. Reference standards were refrigerated (5°C) between injections.

Fish Oil Samples. The dietary fish oil supplement, Promega™ (Parke-Davis, Morris Plains, NJ) was used as the source of marine oil-derived triacylglycerols. The percentages of esterified EPA and DHA were 27.7% and 11.4%, respectively, as determined by glc analysis of methyl esters derived from transesterification of the commercial oil (from particular capsule); about 11% of minor high polyunsaturates also was present. Promega™ was dissolved in either dichloromethane or acetone that contained BHT at a final concentration of 100 mg/L. Samples were stored in valved reaction vessels, blanketed with nitrogen, and kept refrigerated between injections.

Fraction Collection. The fractions were collected from the outlet of the RI detector into glass conical test tubes. The mobile phase was removed by evaporating the solvents under a stream of nitrogen; during evaporation, the test tubes were emerged in a warm water bath.

Chromatography. The HPLC equipment used for both analytical and semi-preparative scale separations consisted of a Waters Associates (Milford, MA) M6000A pump with pulse dampeners and a M401 differential refractive index (RI) detector; a Rheodyne injector with 25-μL, 50-μL, and 2-mL sample loops distributed by the Rainin Company (Woburn, MA); and, a Recordall-5000 strip chart recorder, Fisher Scientific (Fairlawn, NJ).

The analytical reversed-phase separations were performed on a Supelcosil LC-18 column, 25 cm in length with 5-μ particles

(Supelco, Bellefonte, PA). A Whatman Magnum-9 Partisil-10 ODS-3 column, 50 cm in length by 9.4 mm i.d. was used for the semi-preparative separations. A guard column 5 cm in length packed with Supelco guard column pellicular packing material, 40-μ particle size, was used to protect both the analytical and semi-preparative columns. The Magnum-9 semi-preparative column was purged with 3 to 4 column volumes of either acetone or dichloromethane after approximately 10 injections of fish oil to prevent cumulative deposit of triacylglycerols within the column.

Mobile phases were prepared by thoroughly mixing the appropriate combinations of acetone (Ace) and acetonitrile (ACN), filtering through 0.45-μ Nylon 66 filters distributed by the Rainin Company (Woburn, MA), and degassing under a stream of helium. A mobile phase composition of 50:50 Ace/ACN (v/v) was used for both the analytical and semi-preparative fractionations of the fish oil. A 65:35 Ace/ACN (v/v) mobile phase was also employed for the semi-preparative fractionations. Flow rates of 3 to 5 mL/min were used with the Magnum-9 semi-preparative column.

Gas-liquid chromatographic analysis of Promega™ triacylglycerol esterified fatty acyl content was carried out by reacting the sample (20 mg) with trimethylanilinium hydroxide (0.2M in methanol, 0.25 mL, Regis Chemical Co., Morton Grove, IL) and diluting the solution of derived methyl esters with chloroform to a volume of 2.0 mL; injection volume was 0.5 μl of this solution. Instrument -- Perkin Elmer Model 8500 gas chromatograph; detector -- flame ionization; injector -- split capillary (split ratio =

25/1); column -- 12M x 0.22mm i.d. x 0.25 micron film thickness fused silica [(Perkin-Elmer BP-1 (100% methyl)]; carrier -- helium at 30 cm/sec; temperatures -- injector (280°C), detector (300°C), oven [100°C for 1 min, 30°/min to 190°C (hold for 1 min), 5°/min to 240°C, 30°/min to 280°C (hold for 5 min)].

RESULTS AND DISCUSSION

Analytical-Scale Separation. A mobile phase of acetone and acetonitrile (50:50, v/v) provided reasonable fractionation of marine triacylglycerol species (Figure 1). With reversed-phase columns and nonaqueous mobile phases, the elution of triacylglycerols occurs according to equivalent carbon number [ECN] (22). Triacylglycerols with the lowest ECN elute first; if two triacylglycerol molecular species have the same ECN, the species with the highest degree of unsaturation elutes first. Therefore, triacylglycerols containing the esterified omega-3 fatty acids, EPA and DHA, which are highly polyunsaturated, elute very early in the chromatogram.

A mobile phase composition of i-propanol/Ace/ACN (10:40:50, v/v) provided better resolution of peaks representing early eluting triacylglycerols when compared with a mobile phase of 50:50 Ace/ACN. However, a mobile phase containing i-propanol is not recommended for preparative-scale separations, since it is difficult to remove the alcohol from the collected fractions.

Semi-preparative Optimization. The long, narrow Magnum-9 column (50 cm x 9.4 mm i.d.) packed with analytical-size particles (10-μ)

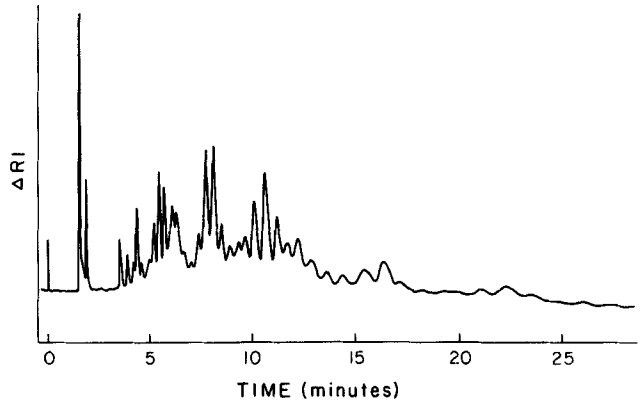

FIGURE 1. Analytical-scale RPLC fractionation of fish oil-derived triacylglycerols. Chromatographic conditions: mobile phase, 50:50 Ace/ACN (v/v); flow rate, 2.0 mL/min; column, Supelcosil LC-18, 25 cm, 5- particle size; detector, differential refractometer; attenuation, 4X.

was used since this column was very efficient. A short, thick scale-up column would not provide the versatility of the Magnum-9 because of the increased diameter; separation time is, however, sacrificed with the long, narrow column.

Unusual van Deemter curves (Figure 2) were obtained for the Magnum-9 column when evaluated with the fish-oil triacylglycerols. Flow rate was found not to affect the efficiency of the separation for later-eluting triacylglycerols (C, Figure 2). Flow rates of 3.0 and 3.5 mL/min, which provided the lowest values for the height equivalent to theoretical plate (HETP), and therefore the best separations, were used in this work.

Since adequate resolution was obtained on an analytical level with a mobile phase of 50:50 Ace/ACN, this mobile phase also was

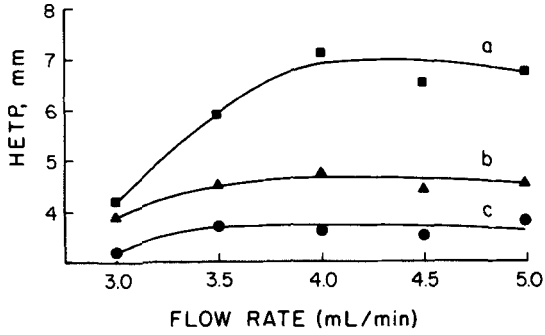

FIGURE 2. Van Deemter Curves for fish-oil triacylglycerols eluting (A) early, (B) midway, and (C) late in the chromatogram.

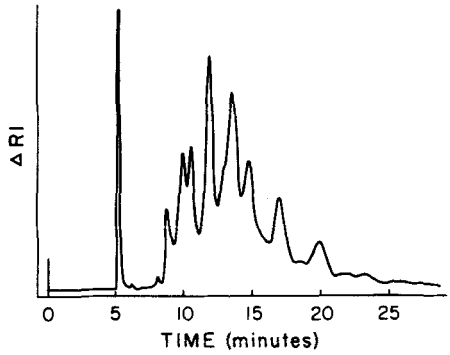

FIGURE 3. The semi-preparative RPLC fractionation of fish oil-derived triacylglycerols. Chromatographic conditions: mobile phase, 50:50 Ace/ACN (v/v); flow rate, 3.0 mL/min; column, Whatman Magnum-9 Partisil-10 ODS-3 (50 cm x 9.4 mm i.d.); detector, differential refractometer; attenuation 16X.

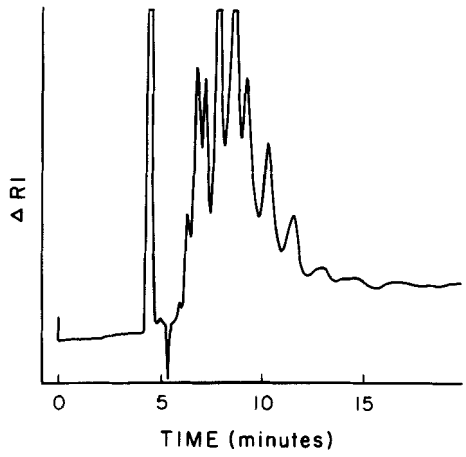

FIGURE 4. The semi-preparative RPLC fractionation of fish oil-derived triacylglycerols. Chromatographic conditions: mobile phase, 65:35 Ace/ACN (v/v); other conditions are the same as in FIGURE 3.

used with the semi-preparative column (Figure 3). Reasonable fractionation of the complex mixture of triacylglycerols was obtained in approximately 25 minutes. Since fewer peaks were seen in the semi-preparative chromatogram (Figure 3) than in the analytical chromatogram (Figure 1), and given the complexity of native fish oils, it is assumed that each peak in the semi-preparative chromatogram represents a group of chemically similar triacylglycerol molecular species.

In order to decrease the separation time, a mobile phase of 65:35 Ace/ACN (v/v) was used (Figure 4). Sufficient fractionation of the fish oil is obtained in less than 15 minutes; thus, the

production rate is increased by a factor of 2 as compared to the production rate with the 50:50 Ace/ACN mobile phase. In addition, a mobile phase with a higher percentage of acetone is removed more easily from the collected fractions.

Semi-preparative Fractionation. Three fractions were collected in the semi-preparative separation (Figure 5). Each fraction, which was collected and concentrated by removing most of the solvent, then was analyzed using the analytical RPLC column (Figure 6). In order to determine the percentages of EPA and DHA in each fraction, the three fractions were transesterified to fatty acid methyl esters. The amounts of EPA and DHA (as transesterification-derived methyl esters) in each fraction then was determined by RPLC (23). The results are shown in Table 1. Fraction A contained the triacylglycerol molecular species with the highest percentage of esterified EPA and DHA. Although Fraction B did not contain as much EPA and DHA percentage-wise as Fraction A (57% versus 73%, total), Fraction B contained the largest amount (by weight) of EPA and DHA. Fraction C contained the least amount of EPA and DHA, both by percentage and weight; i.e., 35% major esterified omega-3 fatty acids. These results indicate that a triacylglycerol fraction significantly enriched in EPA and DHA (approximately 65% after combining fractions A and B) can be obtained by collecting eluents during the first 15 to 18 minutes of the fractionation.

For scale-up, 250 mg of the fish oil supplement was injected onto the column and a 150-mg fraction of triacylglycerols that

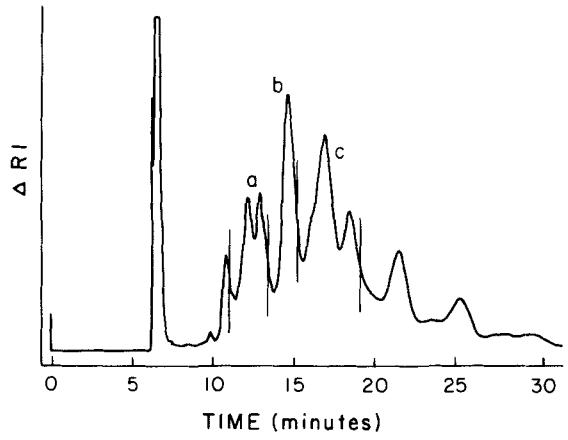

FIGURE 5. Semi-preparative fractionation of fish oil-derived triacylglycerols. Fractions A, B, and C were collected. A sample weight of 100 mg was injected onto the column. Vertical lines indicate cuts for collection of respective fractions. Chromatographic conditions are the same as in FIGURE 3.

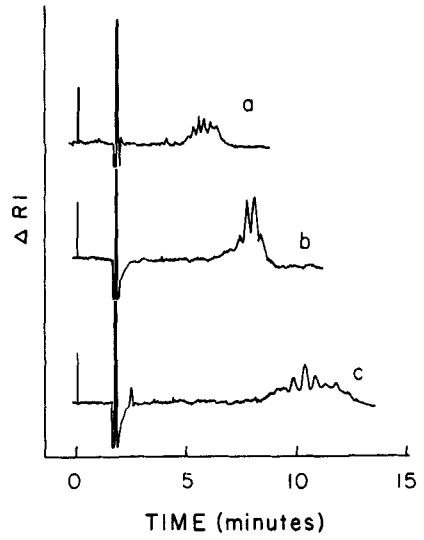

FIGURE 6. Analytical-scale fractionations of the fractions A, B, and C. Chromatographic conditions are the same as in FIGURE 1.

TABLE 1

PERCENTAGES OF EPA AND DHA IN COLLECTED FRACTIONS

Fraction	% EPA[a]	% DHA[a]
A	50	23
B[b]	39	18
C	20	15

[a]Percentages (as methyl esters) are area percents of the total area of all peaks the chromatogram.
[b]Fraction B contains five times more material by weight than fraction A.
Results are the averages of triplicate determinations.

contain high percentages of the major esterified omega-3 fatty acids was collected (Figure 7). The same mobile phase of 65:35 Ace/ACN was used and a production rate of approximately 500 mg/hour of omega-3 enriched fractions was obtained. If a second sample is injected 15 minutes after the first injection, no interference from the first sample is observed, and the production rate can be improved somewhat further.

Sample Load and Column Overload. Increasing amounts of fish oil were injected to determine the sample load that could be handled by the column. The chromatogram resulting from a 375-mg sample load is shown in Figure 8. The area of the chromatogram from 5

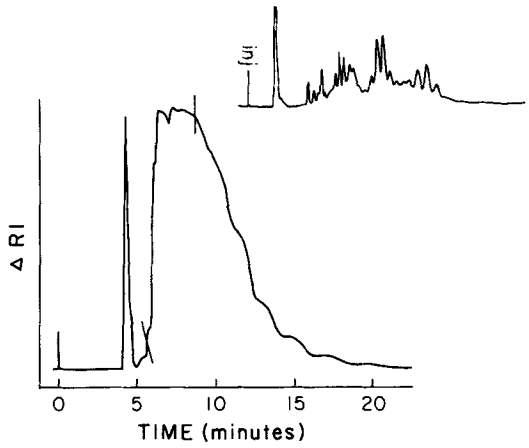

FIGURE 7. Semi-preparative fractionation of esterified EPA and DHA enriched triacylglycerol fraction. The chromatogram at the top is the analytical separation of the collected fraction. The chromatographic conditions for the analytical-scale separation are the same as in FIGURE 1. The chromatographic conditions for the semi-preparative isolation are the same as in FIGURE 4, except that the RI detector attenuation is 32X. A sample weight of 250 mg was injected.

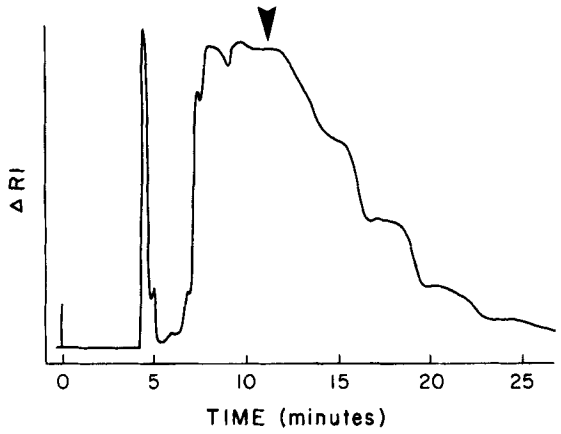

FIGURE 8. Semi-preparative fractionation of a 375 mg sample of fish oil. Chromatographic conditions are the same as in FIGURE 3, except that the RI detector response was not attenuated.

minutes to the arrow represents triacylglycerols that contain a significant percentage of esterified polyunsaturated fatty acids. Although the resolution is poor, reasonable fractionation can still be achieved to the point in the chromatogram where the arrow is drawn. Thus, the early portion of the effluent represented in the chromatogram can be collected to yield a fraction that is enriched in triacylglycerols containing esterified EPA and DHA, whereas the material eluting after this point in the chromatogram can be discarded; some material containing polyunsaturated species, however, will be lost.

The column overload condition has been suggested to occur when: the capacity factor decreases by 10%; the peak width increases; or, the number of plates decrease (24-27). A graphical method to determine the column overload has been described by Verzele and Geeraert (26) in which the column efficiency or plate number (N) versus the log of the sample weight is plotted. A graph of this type for fish oil-derived triacylglycerols is shown in Figure 9. If the graph had been extended to smaller, analytical size sample weight, constant values for plate number are observed. A relatively linear portion of the plot, but with decreasing plate numbers was obtained for logarithms of the sample weights in the range from 2 to 2.5 (Figure 9). Verzele and Geeraert (26) state that a deviation from linearity indicates column overload. Using this criterion, column overload is reached at a sample load of approximately 375 mg, which corresponds to 2.5 on the graph (Figure 9).

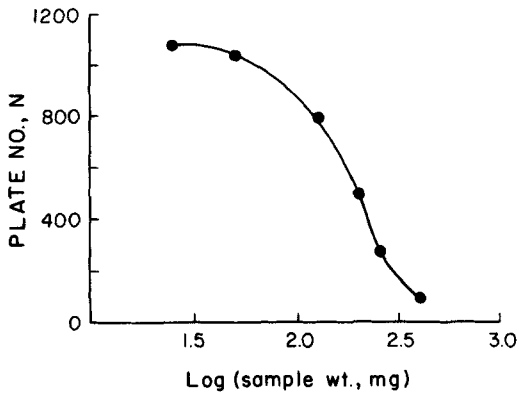

FIGURE 9. A graph of plate number (N) versus log (sample weight, mg) to describe column overload. An early-eluting component was used to calculate N. The chromatographic conditions used to generate the curve are the same as in FIGURE 3.

In conclusion, a reversed-phase semi-preparative chromatographic method was developed and used to isolate triacylglycerol fractions that were significantly enriched in esterified EPA and DHA. A production rate of 500 mg/hour can be achieved for semi-preparative separation of a triacylglycerol fraction containing approximately 65% esterified EPA and DHA, which is a higher percentage of omega-3 fatty acids than is currently available in dietary supplements. In addition, after removal of the mobile phase, a marine oil fraction is obtained that can be used directly for *in vitro*, *ex vivo*, or *in vivo* testing. The chromatographic method can be further scaled-up to produce higher production rates by using larger-scale preparative columns and packings, and higher flow rates.

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REFERENCES

1. Norum, K. R., and Drevon, C. A. Arteriosclerosis 6, 352-355 (1986).

2. Herold, P. M., and Kinsella, J. E. Am. J. Clin. Nutr. 43, 566-598 (1986).

3. Phillipson, B. E., Rothrock, D. W., Connor, W. E., Harris, W. S., and Illingworth, D. R. N. Engl. J. Med. 312, 1210-1216 (1985).

4. Dyerberg, J. in Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil, Barlow, S. M., and Stansby, M. E., eds., Academic Press, New York, 1982. pp 245-261.

5. Dyerberg, J. Nutr. Rev. 44, 125-134 (1986).

6. Bunting, S., Moncada, S., and Vane, J. R. Br. Med. Bull. 39, 271-276 (1983).

7. Lands, W. E. M. Fish and Human Health, pp. 56-62, Academic Press, New York (1986).

8. Kromhout, D., Bosschieter, E. B., and Coulander, C. de L. N. Engl. J. Med. 312, 1205-1209 (1985).

9. Lands, W. E. M. in Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil, Barlow, S. M., and Stansby, M. E., eds., Academic Press, New York, 1982, pp 267-282.

10. Lands, W. E. M. Fish and Human Health, Academic Press, New York, 1986, pp 63-82.

11. Kremer, J. M., Michalek, A. V., Lininger, L., Huyck, C., Bigauoette, J., Timchalk, M. A., Rynes, R. I., Zieminski, J., and Bartholamew, L. E. Lancet 1, 184-187 (1985).

12. Kromann, N., and Green, A. Acta Med. Scand. 208, 401-406 (1980).

13. Singleton, J. A., and Pattee, H. E. J. Am. Oil Chem. Soc. 64, 534-538 (1987).

14. Ekkehart, F., and Thiele, H. J. Am. Oil Chem. Soc. 64, 521-528 (1987).

15. Takano, S., and Kondoh, Y. J. Am. Oil Chem. Soc. 64, 380-383 (1987).

16. Sempore, G., and Bezard, J. J. Chromatogr. 366, 261-282 (1986).

17. Singleton, J. A., and Pattee, H. W. J. Am. Oil Chem. Soc. 61, 1209-1211 (1984).

18. Kimmey, R. L., and Perkins, E. G. J. Am. Oil Chem. Soc. 61, 761-766 (1984).

19. Phillips, F. C., Erdahl, W. L., Nadenicek, J. D., Nutter, L. J., Schmit, J. A., and Privett, O. S. Lipids 19, 880-887 (1984).

20. Robinson, J. L., and Macrae, R. J. Chromatogr. 303, 386-390 (1984).

21. Dong, M. W., and DiCesare, J. L. J. Am. Oil Chem. Soc. 60, 788-791 (1983).

22. The ECN is defined as the number of carbon atoms (CN) in the fatty acyl chains minus twice the number of double bonds (NDB): ECN = CN - (2 x NDB).

23. Beebe, J., Brown, P. R., and Turcotte, J. G., unpublished method.

24. Snyder, L. R., and Kirkland, J. J. Introduction to Modern Liquid Chromatography, 2nd ed., pp. 642-643, John Wiley and Sons, New York (1979).

25. Coq, G., Cretier, G., Dounet, D., and Rocca, J. Chromatographia 12, 139-147 (1979).

26. Hupe, K. P., and Lauer, H. H. J. Chromatogr. 203, 41-52 (1981).

27. Verzele, M. and Geeraert, E. J. Chromatogr. Sci. 18, 559-570 (1980).