Qualitative Analysis of Fish Oil Triglycerides with Supercritical Fluid Chromatography and Q-TOF MS

Application Note

Food Testing and Agriculture

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Abstract

A supercritical fluid chromatograph (SFC) was interfaced with a quadrupole time-of-flight (Q-TOF) mass spectrometer, with an electrospray (ESI) source in positive ion mode, to separate complex mixtures of glycerides in fish oil. Separation was achieved with several different Agilent ZORBAX StableBond C18 columns using methanol, doped with 5 mM ammonium acetate in carbon dioxide. An approximate 10:1 post-column split through a 1-m long, 50-µm stainless steel restrictor, with a make-up flow of pure methanol, was used as an interface. Other constituents of the oil included terpenoids, flavonoids, free fatty acids, phospholipids, and a number of vitamin D3 precursors.

The samples were diluted with isopropyl alcohol and injected. Plots of mass versus retention time showed almost no overlap, while resolving up to 400 components in either time or mass in as little as 7 minutes. Sensitivity was enhanced over 1,000 fold compared to UV at 230 nm. Up to half the resolved components were assigned names using several lipid databases and absolute mass. Partition number (PN) was a poor indicator of elution order.

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Introduction

Triacylglycerols (TAGs) or triglycerides are major constituents of fats and oils. They consist of a glycerol backbone esterified with three fatty acids, usually with 10 to 24 carbons each. The TAG content of most oils is complex, although fish oils are generally more complex than vegetable oils, and contain a large amount of polyunsaturated fatty acids. Detailed analysis of seed and fish oils is difficult, due to the large possible variation in the carbon number of the fatty acids at each position on the glycerol backbone, and the possible number, and location, of double bonds on each fatty acid. Many hundreds of variations are possible.

Most recent TAG analysis has been performed by either nonaqueous reversed-phase high performance liquid chromatography with a C18 stationary phase [1], or silver ion liquid chromatography [2], with complementary retention mechanisms. On C18, the partition number (PN) or elution order is said to be related to the carbon number (CN) minus two times the number of double bonds (2 × #DB) [1]. The mobile phase usually includes acetonitrile-dichloromethane mixtures, both of which are undesirable environmentally. With a silver-loaded column, the separation is based on the number of double bonds present. Both approaches have also been performed with supercritical fluid chromatography (SFC) [3-6].

Due to the complexity of such samples, two-dimensional, or comprehensive HPLC methods have been developed [7] where one dimension separates the TAGs based on the number of double bonds (silver-loaded column), while the second (nonaqueous HPLC with C18) separates based at least partly on carbon number. A variant has been demonstrated using a packed column SFC with carbon dioxide (CO_2) and a silver-loaded column as the first dimension [8], but with a nonaqueous reversed-phase HPLC second dimension.

An alternative HPLC approach [9] to achieve higher resolution involved connecting four C18, 2.7-µm porous shell particle columns in series, operated at very high inlet pressures. Run times sometimes exceeded 3 hours, but resolution was still incomplete. This development was anticipated in SFC where seven, 120-mm columns in series, with 5-µm particles, were used to chromatograph TAGs in 1996 [10], and more recently with five, 2.7-µm porous shell columns [11] with a total length of 750 mm, producing > 110,000 plates.

Purely chromatographic techniques are inadequate to fully resolve all the TAGs. An attractive alternative employs mass spectrometric detection, which can greatly improve the amount of information generated even with a relatively short column. Many different forms of SFC/MS have been used for lipid analysis [6,12-15]. However, there have been few reports on the interfacing of a quadrupole time-of-flight (Q-TOF) MS in SFC [17-19]. In the first such report [17], flow was split using 10 m of 100-µm fused silica between a T-piece mounted after the UV detector and the MS source as the splitter. More recently, di- and triglycerides in cow milk were characterized, but the interface [18] was not described.

There appear to be only a few older literature references to the use of SFC for the analysis of TAGs specifically in fish oil. Several [19-21] used capillary columns with pressure programmed CO_2 and a FID. It was thought that all TAGs with the same CN coeluted from a capillary column, regardless of the degree of unsaturation. Others used a silver-loaded packed column [5, 8].

In this work, SFC/Q-TOF MS was used for the qualitative analysis of TAGs in fish oil, with several simple interfaces to improve resolution, speed, and sensitivity.

Materials and Methods

An Agilent 1260 Infinity Analytical SFC Solution coupled to an Agilent 6540 UHD Accurate Mass Quadrupole Time-of-Flight MS with a Dual Agilent Jet Stream ESI source was used to analyze the samples. Gas temperature was 325 °C, with a drying gas flow of 10 L/min. The nebulizer pressure was 25 psi. The sheath gas temperature was 400 °C with a sheath flow of 12 L/min. Capillary voltage was 3,500 V at 5.060 µA. The fragmentor voltage was 165 V. The skimmer voltage was 45 V. The OCT1RFVpp was 750 V. The reference mass was 922.0098 (hexakis (^{1}H,^{1}H,^{3}H-tetrafluoro-pentaoxy) phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$)). The source was used in the positive ion mode. The system was controlled by Agilent MassHunter Software. Several in-house lipid databases were used to assign tentative names to many of the compounds separated.

An Agilent 1290 Infinity Pump delivered 0.5 mL/min methanol after the column, through a T-piece. Another T-piece, downstream of the make-up flow, split the flow between the backpressure regulator (BPR) and the Q-TOF MS. One meter of 50 µm stainless steel capillary mounted between the second T-piece and the needle of the ESI source was used as an approximate10:1 split. No external heater was used. In some cases, an ultra-low-volume experimental BPR was used to couple the outlet of the SFC directly to the MS source with no split.

The columns were either an Agilent ZORBAX 300SB-C18, 4.6 × 150 mm, with 3.5 µm particles (p/n 863973-902) or a ZORBAX SB-C18, 3 × 100 mm, with 1.8-µm particles (p/n 828975-302). The carbon dioxide used was bone-dry grade in a 50-pound steel cylinder, purchased from AirGas. Methanol and isopropanol were HPLC grade, purchased from VRW. Standards and ammonium acetate were > 99% pure, and were purchased from Sigma-Aldrich, Corp., St. Louis, MO.

The fish oil was purchased at a local drug store as a commercial dietary supplement in gel capsules. The oil was removed from the capsule with a syringe, then diluted either 100:1 or 1,000:1 with isopropyl alcohol, and then injected directly.

Chromatographic method

Most of the results were obtained with the ZORBAX 300SB-C18 column with 3% v/v (MeOH + NH_4COOH), held for 5.5 minutes, to 60% at 11 minutes, and then held for 1 minute. Flow rate was 3 mL/min, column temperature was 60 °C, and the outlet pressure was 140 bar. The injection volume was 2 µL. The DAD was set to 230 nm, with a 16 nm bandwidth and slit. Some results were obtained with as little as 0.5% modifier, as noted. In addition, other results were obtained using the ZORBAX SB-C18 column at 2 mL/min, 40 °C, and 140 bar outlet pressure.

Results and Discussion

A chromatogram of the fish oil obtained with the ZORBAX SB-C18 column is shown in Figure 1. This chromatogram superficially resembles chromatograms collected about 20 years earlier [21] using a capillary column, with pressure programming and FID detection. However, the older chromatogram took 2 hours to collect. Here, the separation was nearly 10 times faster. Total run time was 20 minutes, but all sample components were eluted in < 15 minutes.

Every color represented a different mass. Thus, there were many coelutions. With all filters removed, the MassHunter Software identified nearly 2,000 unique masses. Filtering out the minor components produced the chromatogram in Figure 1, which represents about 400 unique masses. The data were compared to several in-house lipid libraries. The software assigned a score, which is essentially a degree of fit. Scores greater than 90 to 95 were considered very good matches. Approximately 1/3 of the unique masses were assigned specific names based on absolute mass alone.

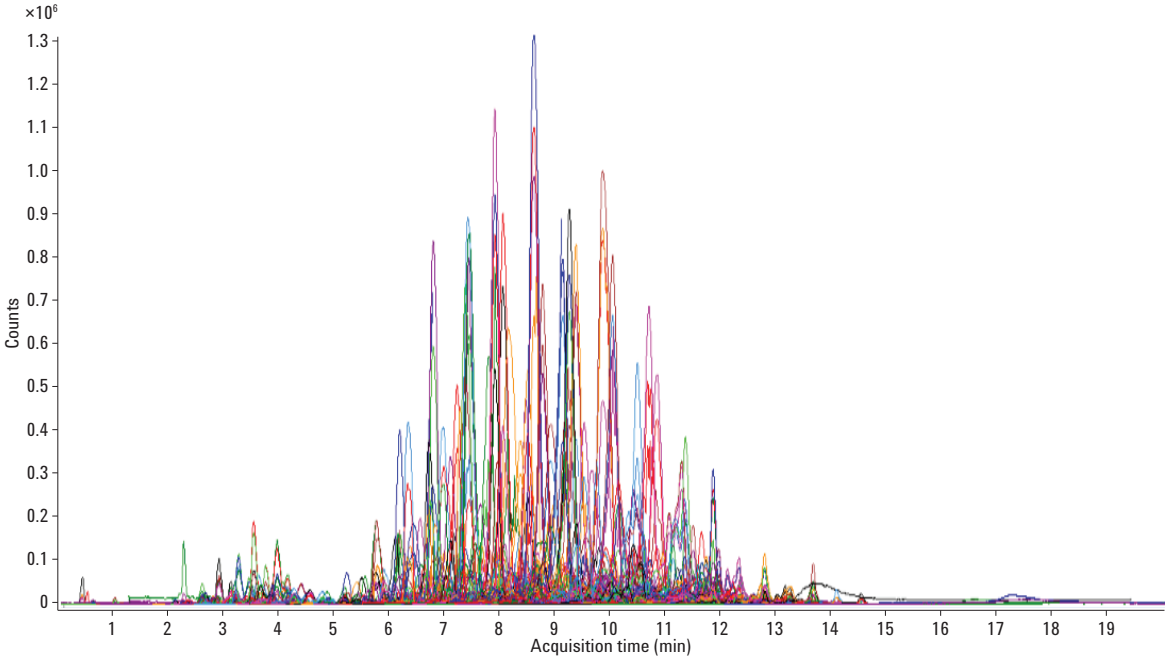

Figure 1. SFC/Q-TOF MS separation of a fish oil sample at 1 mg/mL on an Agilent ZORBAX SB-C18, 3 × 100 mm, 1.8 µm column at 2 mL/min, 40 °C, 140 bar outlet pressure. Every color represents a different mass, indicating that every apparent peak is actually the result of a large number of coelutions.

Similar data, but collected with the ZORBAX 300SB-C18 column, was better observed qualitatively by plotting mass versus retention time, as shown in Figure 2. The size of each dot represents the magnitude of the peak area. This column has lower surface area, and so retention times were shorter, and there was less chromatographic resolution compared to Figure 1. At this scale, the individual components of the oil still appeared to overlap strongly. However, expanding the scale showed remarkably few overlaps, as revealed by the insert.

The software allowed the data to be sorted according to functionality. Only triglycerides with a threshold greater than 15,000 area counts were extracted from the raw data.

Over 140 different unique masses were assigned names as triglycerides. Masses were between about 700 and 1,200. All compounds eluted between 2.5 and 7 minutes.

On C18 columns, elution order was roughly related to molecular weight. In addition to the TAGs, 42 diglycerides (DAGs) were also identified, which eluted in a fairly narrow band from 1.1 to 2.0 minutes, before the TAGs. This contrasts with another report [18] using a polar ethylpyridine column where the diglycerides eluted after the TAGs. Interestingly, a small number of vitamin D3 precursors, phospholipids, free fatty acids, flavonoids, terpenoids from C10 through C30, and even cofactor Q10, were also named.

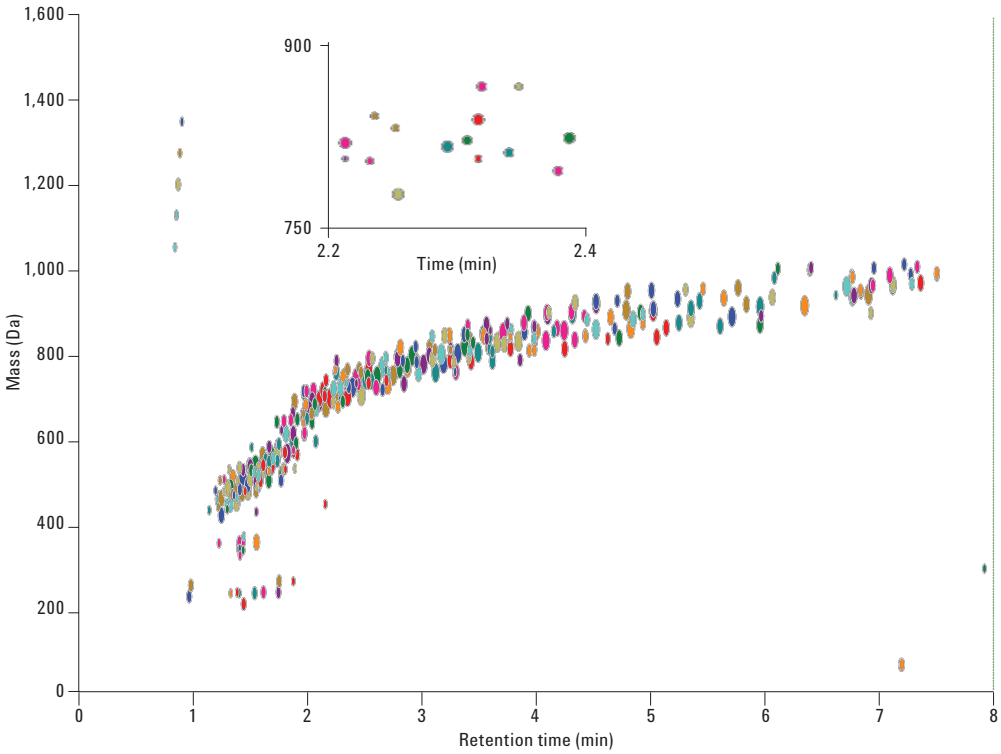

Figure 2. Plot of mass versus retention time for the same sample as in Figure 1 except using a an Agilent ZORBAX 300SB-C18, 4.6 × 150 mm column with 3.5 µm particles. Although the main plot appeared to show severe overlap, the insert revealed that the individual components were relatively well resolved.

Chromatographic resolution

The resolution between four closely related standards: linoline, linoelaidin, alpha-linolenin, and gamma-linolenin, all with 57 carbons, 6 oxygens, and either 92 or 98 hydrogens, were evaluated chromatographically using the experimental BPR with no split. The first two standards differed only in the position of one double bond on each of the three fatty acid chains (6, 9, 12 versus 9, 12, 15). The second pair differed in that in one of the pair the double bonds were all *cis*-, while on the other they are all *trans*-. They were separated with the ZORBAX SB-C18 column at 2 mL/min, 40 °C, 140 bar outlet pressure. Modifier concentration was held at 0.5% v/v for 0.5 minutes, then ramped to 5% at 10 minutes. The results shown in Figure 3 demonstrate good peak shapes and baseline resolution between both pairs. Modifier concentration was about 3% for the first pair, and nearer 4% for the second pair. These were fairly remarkable separations based on the similarity of the structures.

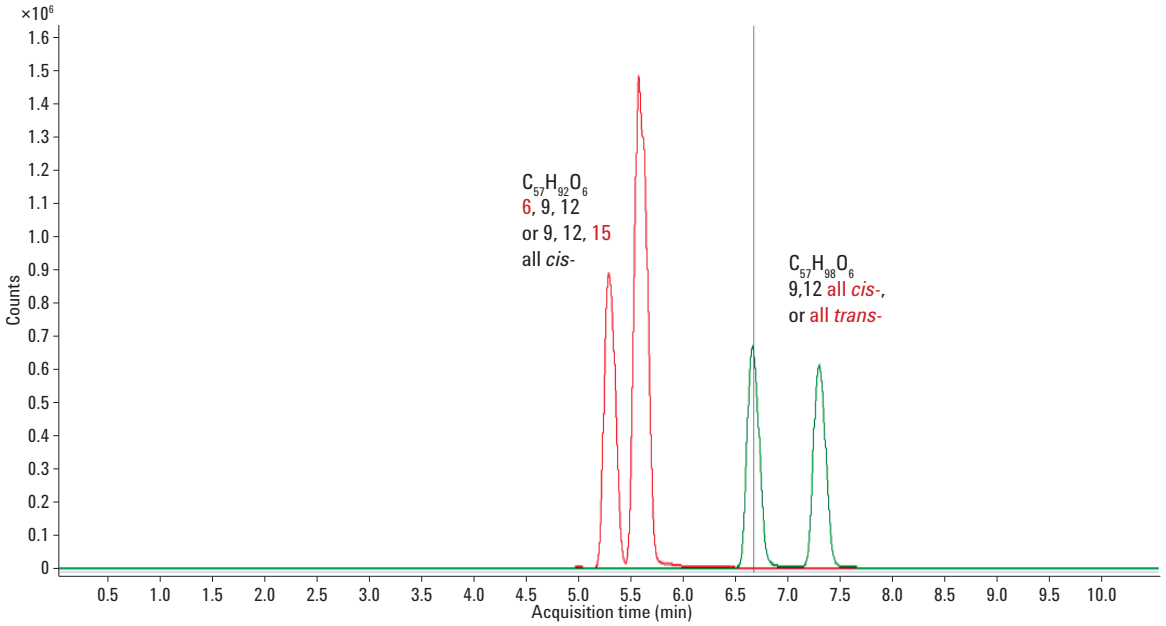

Figure 3. Four compounds, all with 57 carbons but subtle differences in double bonds, were separated chromatographically. The first two peaks differed only in that one had three double bonds on each of the three fatty acids at 6, 9, and 12 positions, while the other had double bonds at the 9, 12, and 15 positions. The second pair was identical, except that the two double bonds at 9, and 12 on each of the three fatty acids, were either all *cis*-, or all *trans*-. Agilent ZORBAX SB-C18, 3 × 100 mm, 1.8 µm column at 2 mL/min, 40 °C, 140 bar outlet pressure. Initially, isocratic flow at 0.5% v/v (MeOH + NH_4COOH) for 0.5 minutes, then 5% at 10 minutes, 20% at 20 minutes.

Elution order

The carbon numbers (CN) of many of the named peaks were plotted versus retention time, as shown in Figure 4. Clearly, retention was not proportional to CN. On C18, PN was purported to be roughly proportional to the CN – 2 × #DB [1]. A plot, shown in Figure 5, revealed virtually no correlation between retention time and PN. Dunkel *et al*. [22], proposed a slightly different equation where PN = CN – #DB, which yielded a slightly better but still poor correlation, as shown in Figure 6. At any given retention time, the PN varied by at least three units, with outliers > 5. Thus, neither version of PN is very accurate in predicting elution order, at least with fish oils.

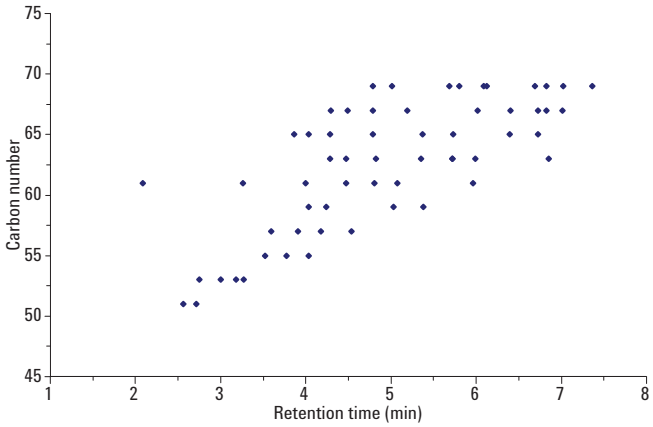

Figure 4. Plot of carbon number (CN) versus retention time. Clearly, there was very little correlation between CN (and molecular weight) and retention. Agilent ZORBAX 300SB-C18, 4.6 × 150 mm, 3.5 µm. Conditions as in Figure 2.

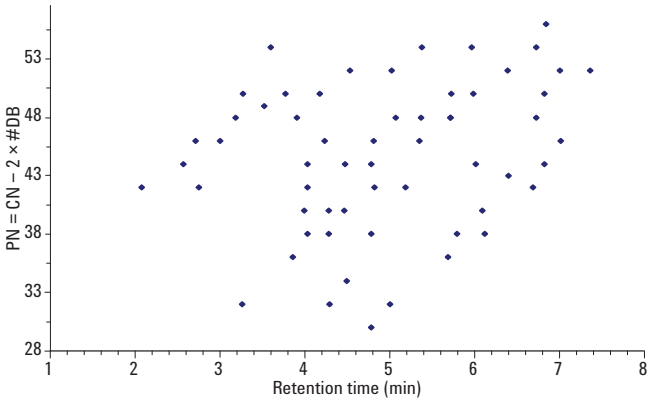

Figure 5. Plot of partition number (PN) or relative elution order. PN was even worse at predicting elution order than CN. Conditions as in Figure 2.

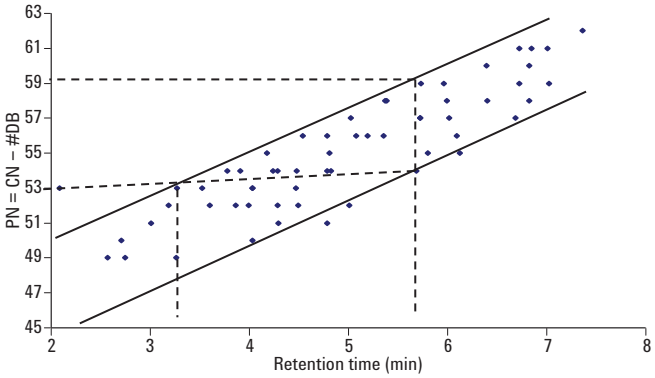

Figure 6. Plot of a variant of PN (CN – #DB) versus retention showed a better correlation than in Figures 4 or 5, but still not particularly useful. Compounds co-eluting typically can range in > 5 PN numbers. Conditions as in Figure 2.

Many compounds with the same CN but different #DB were named by the software. In the most extreme case, nine different compounds were identified with the same CN, but with 6 to 16 double bonds. A plot of retention time versus #DB for each CN is presented in Figure 7. The vertical line, marked with an A, crosses lines C57 through C67, indicating that many compounds with large differences in molecular weight, but with various degrees of unsaturation, coelute. The horizontal line marked B indicates the relative retention of all the compounds with #DB = 6, but with various CN. Only with the same #DB is retention a strong function of CN.

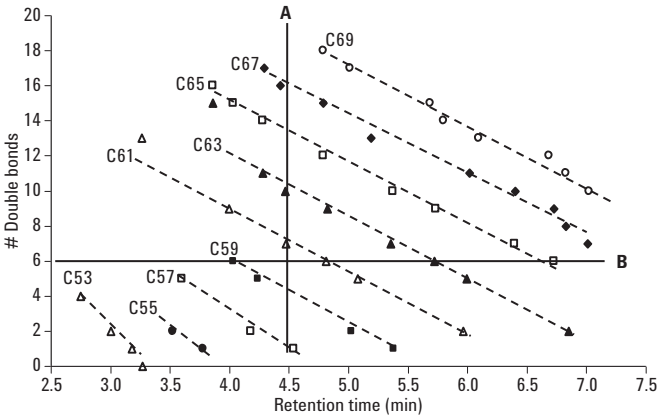

Figure 7. Plot of number of double bonds versus retention time. Each dashed line represents a single carbon number. Note the large variation in retention times due only to differences in the number of double bonds. The vertical line marked with an A shows that TAGs with C57 through C67 can co-elute, depending on the degree of unsaturation. Horizontal line B shows that retention is a strong function of CN only at the same level of unsaturation.

The software databases reported the number of double bonds on each of the three fatty acids. For example, C63 contained three different kinds of substitution. In one group, the third fatty acid (stem name or sn =3) contained a variable number of double bonds on a C22 fatty acid (C22:X, where X represents the variable number of double bonds). Nonetheless, the first fatty acid (sn = 1) was always 16:0 (16 carbons with no double bonds), and the second fatty acid (sn = 2) was always 22:0 (22 carbons with no double bonds). In another group, the third fatty acid was fixed at 22:6, the first fatty acid was fixed at 16:0, while the middle fatty acid contained a variable number of double bonds, C22:X. In the third kind of substitution, all three fatty acids were C20. A more detailed plot of this data is presented in Figure 8. Despite a minor deviation by the 20:X (sn = 1) triglycerides, the data produced a surprisingly straight line. Thus, both the chain lengths and location of double bonds varied over a fairly wide range, yet retention appeared to be almost completely due to the total number of double bonds, not their location.

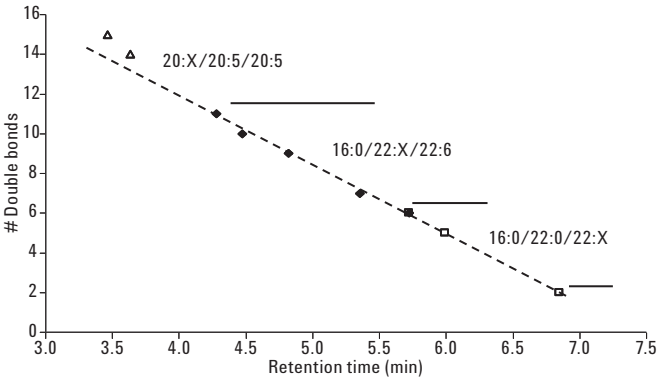

Figure 8. Plot of the number of double bonds (#DB) versus retention time for TAGs with C63, with the reported number of double bonds on the three fatty acid side chains. Only the total number of double bonds, and not the location or chain lengths of the individual fatty acids, appeared to dictate retention.

Odd CN fatty acids

Almost all the TIGs assigned a name contained fatty acids with an even number of total carbon atoms, usually between C16 and C22. Among the named peaks, with high scores, only five triglyceride families had an even carbon number, meaning at least one of the fatty acids contained an odd number of carbons (since the glyceride backbone contained three carbons). All five were between C54 and C64. In all cases, the odd fatty acid was a C17, with various numbers of double bonds. Most were located at the sn =1 position, but a few had the C17 at the sn = 2 position.

Area versus CN

The areas of all the named triglyceride peaks with the same carbon number were added, and the overall percentage at each carbon number was calculated. The results, presented in Figure 9, indicated that the most abundant carbon number was C63, with a Gaussian-like distribution at higher and lower CN, except for an outlier at C53. Such a distribution resembled the proposed distribution using capillary columns and pure CO_2. Mollerup [20, 21] found C59 or C61 to be the most abundant TAG in various marine oils from seven different species. However, Figure 9 represents actual carbon numbers versus abundance and not retention time versus abundance.

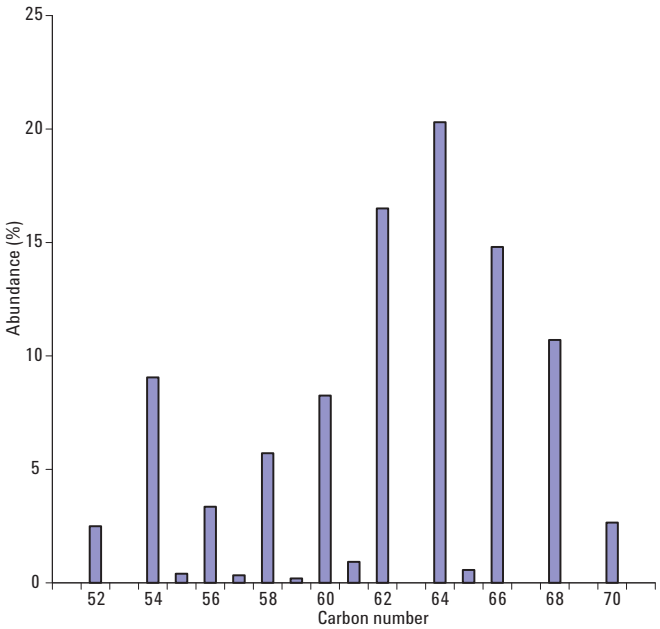

Figure 9. Plot of the sum of the area counts for all the named compounds at each CN. Notice the small amounts of TAGs with uneven carbon numbers. Conditions as in Figure 2.

Conclusions

The Agilent 6540 UHD Accurate Mass Quadrupole Time-of-Flight MS resolved at least 10 times and perhaps 100 times more components than SFC chromatography alone, with minimal overlaps and in minimal time. It appears this approach is adequate to characterize almost completely the lipid content of complex mixtures such as fish oil. The combination of SFC with a Q-TOF MS created a new level of performance in the analysis of TAGs. However, the wealth of information from a few repetitive runs can require weeks of further processing and analysis. Such data abundance requires a carefully designed analytical plan to collect only the specific information of interest.

The number of discrete compounds found was very large, yet the software was able to identify a large fraction of the components. The combined SFC/Q-TOF MS had roughly 100 times higher resolution, with >>1,000 times higher sensitivity compared to SFC/UV for these solutes. Absolute mass determinations go a long way toward definitive naming of many of the components. The ease of interfacing and the high speed of separation make SFC highly desirable for such separations. Partition number was found to be a poor indicator of elution order using two different approaches.

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