SHIMADZU
Excellence in Science

Application News

No.L492A

High Performance Liquid Chromatography

Comprehensive 2D Separation of Triglycerides in Vegetable Oil with ELSD/LCMS-IT-TOF Detection

Triglycerides, molecules consisting of a glycerol backbone to which three fatty acids are attached via ester bonds, are considered important functional components in both animal oil and vegetable oil. Triglycerides display low solubility in aqueous solvents, and their separation has typically been conducted by either silver ion-mediated normal phase analysis or reversed phase analysis using an organic solvent. However, as there are numerous molecular species consisting of combinations of fatty acids, mutual separation of the triglycerides in natural fats can be difficult using any single set of separation conditions. The Nexera-e comprehensive two-dimensional liquid chromatograph effectively achieves mutual separation of such complex components.
When conducting comprehensive two-dimensional liquid chromatography, different separation modes are generally selected for the first and second-dimension separations, and depending on the differences in separation selectivity between these dimensions, improved separation is typically seen for components that are difficult to separate in a single, one-dimensional analysis. Here, using borage oil as a sample that contains many triglycerides, micro-scale separation was conducted in the first separation using a silver column (normal phase conditions), and reversed phase separation was conducted in the second dimension by using a two-liquid gradient with non-aqueous organic solvents. Detection was conducted using a combination of an evaporative light scattering detector (ELSD) and an ion trap time-of-flight mass spectrometer (LCMS-IT-TOF). The analytical conditions are shown in Table 1.

Comprehensive Separation of Triglycerides in Borage Oil with ELSD Detection

Borage oil is a vegetable oil that is obtained from the seeds of Borago officinalis, an annual herb. Rich in triglycerides containing such fatty acid chains as linoleic acid, γ-linolenic acid, oleic acid, and palmitic acid, it offers a variety of health effects associated with these substances, such as moisturizing effect, wrinkle prevention, etc. Compared with other vegetable oils, Borage oil is rich in γ-linolenic acid, which is said to be effective in maintaining female hormonal balance. Triglycerides in natural fats and oils are generally characterized by the lengths of their alkyl chains and the number and positions of the double bonds in the alkyl chains. Triglycerides having double bonds in particular are said to possess antioxidant action, and there is considerable demand for the separation of these triglycerides depending on the presence or absence of double bonds. It is known that strong interaction is displayed by the formation of a complex comprising the double bond of an alkyl chain with a silver ion. Utilizing this property, an HPLC method in which a stationary phase impregnated with silver is relatively often used to achieve selective retention of compounds containing double bonds. Here, using the Nexera-e to achieve comprehensive separation of multiple components, a silver ion column (normal phase conditions) with strong retention for double bonds was used for the first-dimension separation, an ultra-high-speed reversed phase analytical column was used for the second-dimension separation, and an ELSD was used for detection.
The ELSD converts the target compound to fine particles by evaporating the eluent exiting the column, and by measuring the scattered light, triglycerides, which display almost no UV absorption, are effectively detected. Fig. 1 shows a comprehensive two-dimensional representation of the separation pattern (horizontal axis: separation in the first dimension with a silver ion column × vertical axis: reversed phase separation in the second dimension) generated using the specialized two-dimensional analysis software, ChromSquare. The use of comprehensive two-dimensional separation permitted difficult-to-achieve high separation using a single set of separation conditions, by which thirty-seven of the elution peaks were confirmed.

Table 1 Analytical Conditions

[Column1]	: Ag custom column (150 × 1.0 mm; 5.0 µm)
Mobile Phase	: A; 1.5 % v/v of Butyronitrile in n-Hexane B; 2.4 % v/v of Butyronitrile in n-Hexane
Time Program	: B Conc. 0 % (0 min) → 100 % (40 min) → 100 % (150 min)
Flowrate	: 0.007 mL/min (split)
Column Temp.	: 30 °C
Injection Volume	: 2 µL
Modulation Time	: 1.5 min
[Column2]	: Ascentis Express C18 column (Supelco, 50 × 4.6 mm; 2.7 µm)
Mobile Phase	: A; Acetonitrile B; Isopropanol
Time Program	: B Conc. 30 % (0 min) → 30 % (0.08 min) → 40 % (0.1 min) → 70 % (1.2 min) → 30 % (1.21 min) → 30 % (1.5 min)
Detector	: Shimadzu ELSD LT-Ⅱ
Flowrate	: 4 mL/min
Evaporative Temperature	: 58 °C
Nebulizing Gas Pressure	: 260 kPa
Detector	: LCMS-IT-TOF
Flowrate	: 2 mL/min from the 2D pump was split to 0.8 mL/min prior entering the APCI probe.
[MS Conditions]	
Ionization Mode	: APCI positive
Nebulizer Gas Flow	: 2.0 L/min
Interface Temperature	: 400 °C
Block Heater Temperature	: 230 °C
CDL Temperature	: 230 °C
Scan	: *m/z* 300-1200

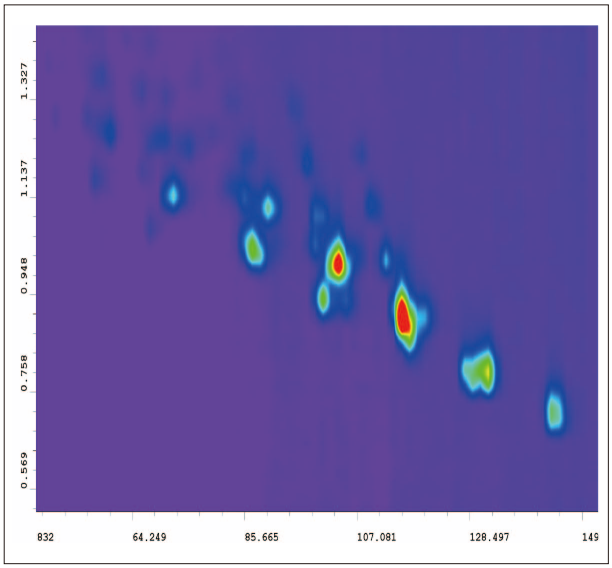

Fig. 1 Comprehensive 2D Plot of Triglycerides in Borage Oil with ELSD Detection

Comprehensive Separation of Triglycerides in Borage Oil with LCMS-IT-TOF Detection

As mentioned above, separation in the first dimension in this analysis is conducted based on the presence or absence or the difference in the number of double bonds in the fatty acid side chain. When a silver ion column is used, the greater the number of double bonds in the triglyceride, the stronger the retention will be. However, it is also possible that retention will be affected depending on the positions of the double bonds or the side chain length. In the second-dimension reversed phase separation, two-solution gradient elution is adopted in which, due to the high hydrophobicity of triglycerides, neither water nor buffer solution, etc., is used, but a non-aqueous organic solvent is used for the mobile phase. With this separation mode, elution tends to proceed in the order obtained by subtracting twice the number of double bonds from the total number of triglyceride carbon atoms, which is referred to as the partition number. The top portion of Fig. 2 shows a two-dimensional plot drawn based on the output of the LCMS-IT-TOF mass spectrometer. To facilitate identification of triglycerides using the order of elution described above, a grid is drawn superimposed on the plot. From this plot, it can be seen how separation is conducted according to the difference in the number of double bonds in the first dimension, and the difference in partition number in the second dimension.

Use of the LCMS-IT-TOF as the detector for precise mass measurement in the second dimension permits detailed qualitative analysis of the many components eluted following separation by the Nexera-e. The mass spectra corresponding to the white-circled peaks A, B and C of Fig. 2 are shown in the lower part of Fig. 2. The structural information was obtained from the peak of diglyceride with one side chain detached, and the triglyceride structure were determined as follows:

A : POP
B : OOP
C : PγLnP

Where,

P : Palmitic acid
O : Oleic acid
γLn : γ-Linolenic acid

Since these compounds each have one to three double bonds, they are eluted from the first-dimension column in this order. As lipid-related compounds often display no UV absorption, and gradient elution cannot be applied with differential refractive index detection, a combination of the Nexera-e and ELSD, or a triple quadrupole or LCMS-IT-TOF mass spectrometer can be considered essential for exhaustive analysis in this field.

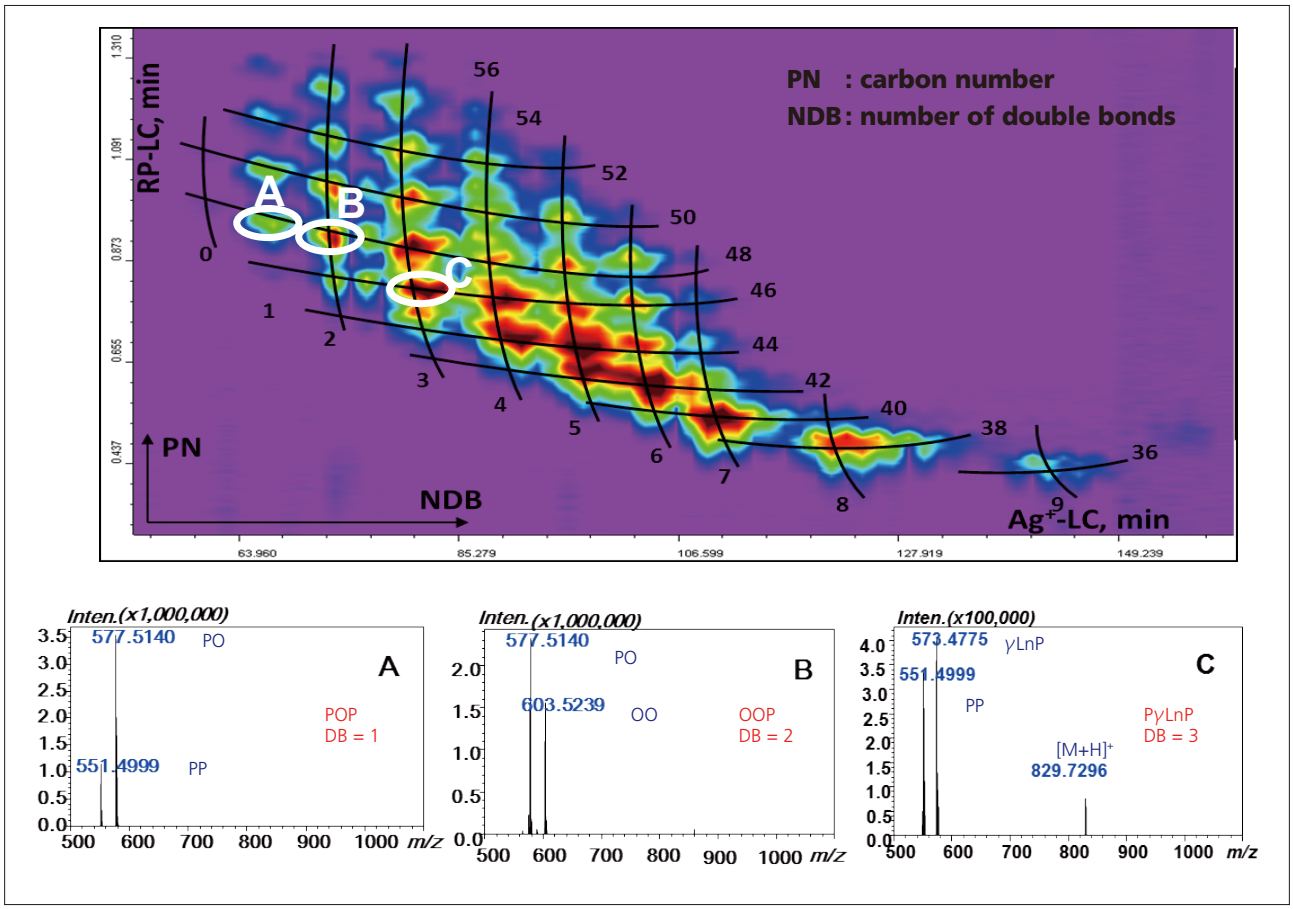

Fig. 2 Comprehensive 2D Plot of Triglycerides in Borage Oil with LCMS-IT-TOF in Addition to the Mass Spectra of Assigned Blobs

Data provided by University of Messina Prof. Luigi Mondello and Chromaleont S.r.l.

Second Edition: Feb. 2016
First Edition: May. 2015

Shimadzu Corporation
www.shimadzu.com/an/