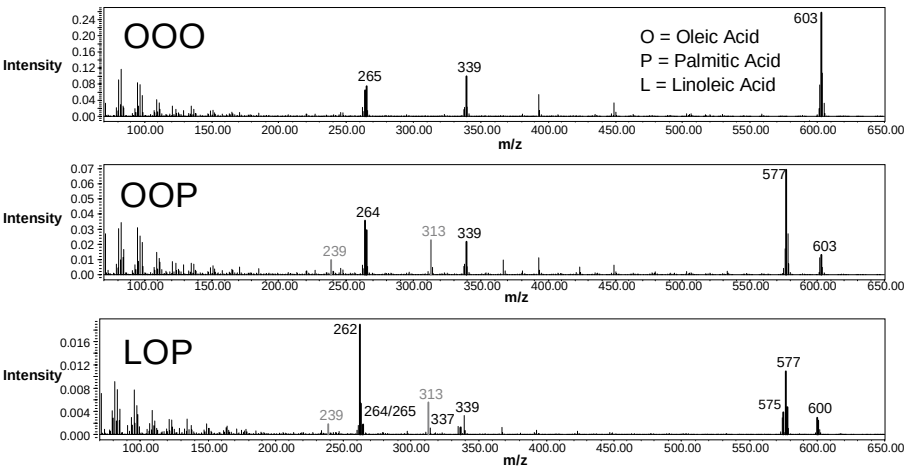
Waters Integrity System Applications

An LC/MS Method for Determining the Fatty Acid Content of Triglycerides Featuring ThermaBeam™ Electron Ionization

Highlights: The inability of typical LC detectors to positively identify compounds have led to the use of ThermaBeam LC/MS as a detection and identification alternative for triglycerides. The method featured here allows for the determination of the fatty acid content of triglycerides without time consuming fraction collection and wet chemistry.

Triglycerides are known to be difficult compounds to analyze by HPLC using traditional detectors. These materials have absorbance maxima at low wavelengths, and, coupled with the high background absorbance of commonly used solvents, the applicability of UV detectors for triglyceride analysis is limited. Since gradient elution is commonly used, RI detectors cannot be utilized. The evaporative light scattering (ELS) detector, sometimes called, confusingly, a "mass detector," is an accepted detector for the HPLC analysis of triglycerides. Good baselines and chromatographic separation can be obtained using an ELS. However, no identification of the peaks in a separation can be obtained from this type of detector. The Waters Integrity LC/MS System features a ThermaBeam interface and Electron Ionization (EI) which offers the chromatographer mass detection and positive compound identification of chromatographic peaks. The unique ThermaBeam design adds a modest amount of heat to a pneumatic nebulizer to assist the aerosol formation process (50 degrees is adequate for triglycerides). The solvent evaporates in the heated expansion region, leaving solid particles or oil droplets depending on the sample. Note that up to this point, this parallels the mechanism for ELS operation. Subsequently, though, the particles/droplets formed during ThermaBeam nebulization pass through a two-stage momentum separator, each with higher vacuum, until they enter the ion source of the mass spectrometer. Of the currently popular LC/MS interfaces, only ThermaBeam-like interfaces permit a sufficiently high vacuum in the ion source to allow electron impact (EI) ionization. EI is the accepted standard for producing mass spectra for qualitative interpretation and positive compound identification. This study describes how EI spectra can be used to determine the fatty acid composition of triglycerides.

Unsaturated Triglycerides



To determine the fatty acid content of triglycerides, EI fragmentation patterns obtained using the Waters Integrity System are studied. Unsaturated and polyunsaturated fatty acids, which are very important in triglycerides, are identified by characteristic fragmentation patterns. For example, acyl ions of unsaturates are characterized by loss of a hydrogen leading to pairs of ions at the expected mass and 1 Da less. This is seen in triolein (top spectrum on left) with the pair at m/z 264/265 Da. In OOP, the ion at m/z 264 is actually larger. This is typical for oleic acid in mixed triglycerides. Polyunsaturates are even more prone to hydrogen loss. Note that the m/z 262 for linoleic acid in LOP is much more intense than m/z 263. Examination of many triglyceride spectra allow for the development of empirical rules for expected fragmentation. These rules, which are illustrated on the reverse side of this publication, are useful together with rules for peak elution for the creation of a plan for the interpretation of triglyceride mass spectra.

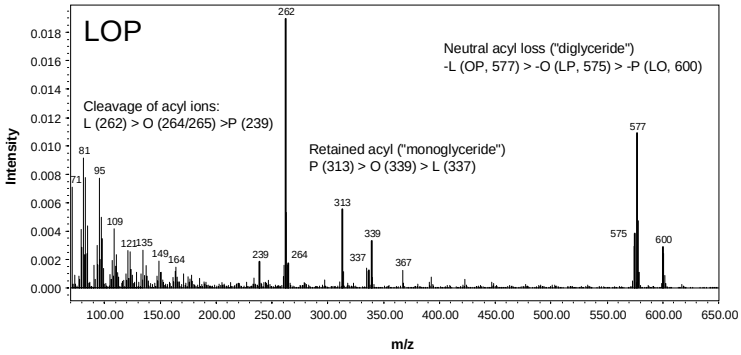
Fragmentation Rules

- Acyl losses (as ion or neutral) go as:
  - Long chain in preference to short chain.
    - E.g., stearic > myristic
  - Unsaturated in preference to saturated.
    - E.g., oleic > stearic
  - Polyunsaturated in preference to monounsaturated.
    - E.g., linolenic > linoleic > oleic

Elution Rules

- Retention time increases with increasing equivalent carbon number (ECN).
- For a given ECN, retention time increases with decreasing unsaturation.
- Elution of triglycerides in non-aqueous reversed phase chromatography is known to follow these generalizations.
- Equivalent carbon number is calculated as follows: ECN = (# of carbons in fatty acid chains) - (2 * # of double bonds in fatty acids)

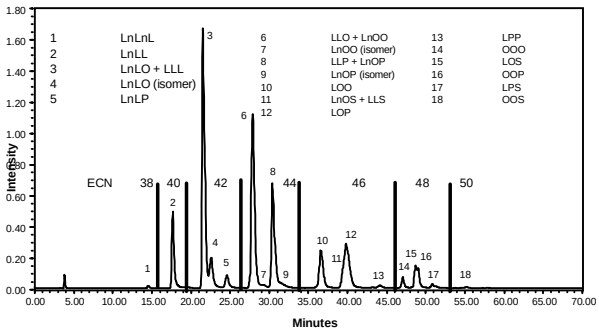
Fragmentation Illustrated



A Plan for Interpretation:

- First, obtain a good chromatogram
- Collect a mass spectrum for each peak
- Interpret unambiguous spectra first
- Catalog fatty acids by inspection of acyl and monoglyceride ions
- Use this information to assign diglyceride ions
- Deduce triglyceride composition from assigned diglycerides
- Determine ECN and unsaturation number for assigned peaks
- Return to difficult spectra (coelutions) and interpret in light of other assignments

Soy Oil - Supermarket Brand



Here is the result of applying this method to a "real" sample, soy oil purchased in a local supermarket. Note that several coelutions have been assigned. In particular, the spectrum strongly suggests the presence of two other triglycerides (peak 11, LnOS and LLS) in the upslope of peak 12 (LOP).

Combining the fragmentation and elution rules, we can outline a method for qualitatively interpreting triglyceride chromatograms. The need for good chromatography is paramount. Spectra with no ambiguities should be assigned first. The assignments must fall in place in expected elution order. Ambiguous spectra (coelutions) can be assigned based on expected ECN and nature of assigned neighboring compounds.

The Waters Integrity System featuring the ThermaBeam Mass Detector not only detects triglycerides in a straightforward manner, but allows fatty acid content to be determined using the positive compound identification capability of electron Ionization mass spectra. The mass spectra provide important structural identification evidence. Determination of fatty acid content can be obtained easily without isolation, hydrolysis, derivatization, etc.