

One-Step Triglyceride Separation by Reversed Phase HPLC Without Argentation

Triglycerides containing both saturated and unsaturated fatty acids traditionally were separated by a time consuming two-step analysis involving argentation and reversed phase chromatography. However, high efficiency reversed phase columns used with a nonaqueous mobile phase separate these triglycerides using a rapid, one-step process. The procedure separates triglycerides by both acyl chain length and degree of unsaturation. These high efficiency SUPELCOSIL reversed phase columns lend themselves to a variety of analyses.

Key Words:

- octyl
- octadecyl
- olive oil
- reversed phase HPLC
- soybean oil
- triglyceride isomers

Unsaturated Acyl Chains Complicate Triglyceride Analyses

Conventional analysis of a naturally occurring triglyceride mixture containing saturated and unsaturated fatty acids requires two steps: separation according to the total number of carbon atoms in the acyl chains (by GLC or reversed phase chromatography), and separation according to the degree of unsaturation (TLC or LC argentation chromatography). Reversed phase HPLC can readily separate saturated triglycerides according to the total number of carbon atoms in the acyl chains. Unsaturated acyl chains complicate the separation, however, because each double bond is chromatographically equivalent to shortening the chain by two carbon atoms. For example, triolein (54 acyl carbons:3 double bonds) and tripalmitin (48:0) previously have not been separable by reversed phase HPLC because both appear to have 48 acyl carbons. To separate such critical pairs, one used argentation chromatography. However, high efficiency reversed phase columns, used with nonaqueous mobile phases, permit resolution of triglycerides by acyl chain lengths and degree of unsaturation in one step, without recourse to argentation chromatography (1).

Applications of One-Step Separation for Triglyceride Analyses

Olive Oil

Olive oil contains more than 80% oleate (O)^{*} and 4-7% each of palmitate (P), stearate (S), and linoleate (L). These four fatty acids can form 20 compositionally different triglycerides. Only those ten containing one or more oleate units are expected to occur in olive oil in measurable quantity.

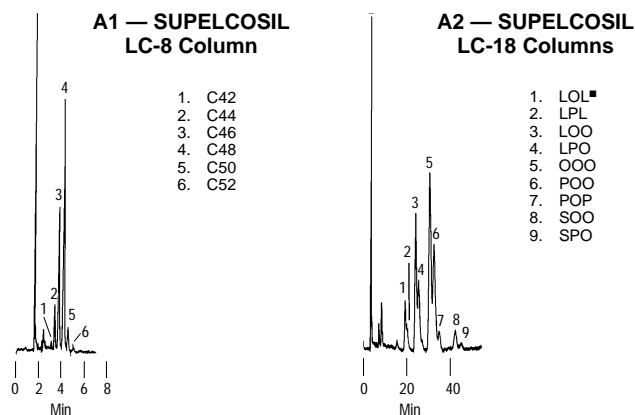
When olive oil was analyzed on a 15cm x 4.6mm ID octyl SUPELCOSIL™ LC-8 column, with approximately 10,000 theoretical plates, four main peaks were seen on the chromatogram (Figure A1). These correspond in location to saturated triglycerides with 44, 46, 48, and 50 carbon atoms, respectively. Two minor peaks correspond to saturated triglycerides with 42 and 52 carbon atoms. This is a "traditional" reversed phase separation of triglycerides.

The analysis is greatly improved by increasing the chain length of the bonded phase from octyl to octadecyl (SUPELCOSIL LC-18 column), and by either increasing the column length from 15cm to 25cm or by connecting two 15cm columns in series to obtain 17,000-20,000 theoretical plates. Under these conditions the triglycerides can be separated simultaneously by carbon number and degree of unsaturation (Figure A2), and it is possible to identify them.

Using two 15cm SUPELCOSIL LC-18 columns, eight of the ten possible oleate-containing triglycerides have been identified (Figure A2). A small amount of triglyceride containing no oleate (L₂P) also was detected. The peak corresponding to an acyl carbon number of 44 in Figure A1 has been resolved into two peaks, identified as OL₂ (54:5) and L₂P (52:4) in Figure A2. Similarly, the 46 carbon atom in Figure A1 has been resolved into four peaks for

Figure A. Comparison of Olive Oil on SUPELCOSIL LC-8 and SUPELCOSIL LC-18 Columns

Columns: A1 — SUPELCOSIL LC-8, 15cm x 4.6mm ID, 5µm particles (Cat. No. 58220-U)
 A2 — Two SUPELCOSIL LC-18, 15cm x 4.6mm ID, 5µm particles (Cat. No. 58230-U)
 Mobile Phase: acetone:acetonitrile (63.6:36.4, v/v)
 Flow Rate: 1.0mL/min
 Pump: Tracor 995
 Detector: Waters R401 refractometer
 Inj.: 10µL of 9% olive oil in mobile phase



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O₂L (54:4) and OPL (52:3) in Figure A2. Although the 48 carbon atom peak in Figure A1 probably contains four components, only three, O₃ (54:3), O₂P (52:2), and OP₂ (50:1), have been resolved in Figure A2. The triglyceride OLS (54:3) may also be present in the 48 carbon atom peak in Figure A1, but it does not appear as a separate peak in Figure A2. Because OLS (54:3) and O₃ (54:3) are identical in both total chain length and degree of unsaturation, they may not be separable using this procedure. The 50 carbon atom peak in Figure A1 consists of O₂S (54:2) and OPS (52:1), as is shown in Figure A2. The analysis, which ran for 22 minutes, was stopped before it detected the "missing" triglyceride, S₂O (54:1). It can, however, be predicted to elute after the pair O₂S (54:2) and OPS (52:1). The last small peak in Figure A1 may indicate the presence of S₂O. The order of triglyceride elution from the SUPELCOSIL LC-18 column, suggests that each double bond in an acyl chain has a somewhat greater chromatographic effect in this system than does shortening the chain by 2 carbon atoms. This effect permits the critical pairs to be separated in this system.

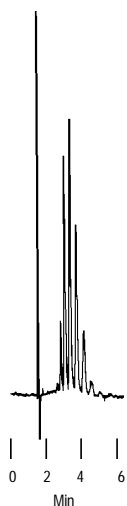
Soybean Oil

Soybean oil consists of a preponderance of triglycerides containing one or more linoleate chains. These appear as six peaks when separated on an octyl column (Figure B1) and can be resolved further on the SUPELCOSIL LC-18 column, as shown in Figure B2. Standards were not available to establish peak locations. Therefore, identification of the peaks was made on the basis of GLC of the methyl esters of isolated fractions.

Figure B. Comparison of Soybean Oil on SUPELCOSIL LC-8 and SUPELCOSIL LC-18 Columns

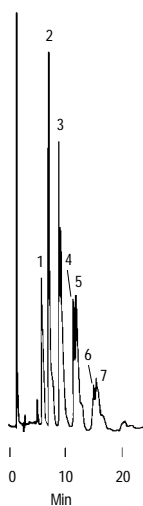
Columns: B1 — SUPELCOSIL LC-8, 15cm x 4.6mm ID, 5µm particles (Cat. No. 58220-U)
 B2 — Two SUPELCOSIL LC-18, 15cm x 4.6mm ID, 5µm particles (Cat. No. 58230-U)
 Mobile Phase: acetone:acetonitrile (63.6:36.4, v/v)
 Flow Rate: 1.0mL/min
 Pump: Tracor 995
 Detector: Waters R401 refractometer
 Inj. Vol.: 10µL of 10% soybean oil in mobile phase

Figure B1 — SUPELCOSIL LC-8 Column



1. LLL
2. LOL
3. LPL
4. LOO
5. LPO
6. LSO
7. LSP

Figure B2 — SUPELCOSIL LC-18 Columns



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Triglyceride Isomers

Single-step separation may not be possible for certain compositionally distinct triglycerides or certain positional isomers. Compositionally distinct triglycerides such as O₃ (54:3) and OLS (54:3) have the same total acyl carbon number and degree of unsaturation. Isomers such as OOP, OPO, and POO differ only in positional or chiral considerations. A means, such as that described by Brockerhoff (2), is necessary for identification of these coeluting compounds. Recent work indicates that the SUPELCOSIL LC-18 column, used with a nonaqueous mobile phase, can separate *cis-trans* isomers such as triolein and trielaidin (Figure C). The one-step separation by both carbon number and degree of unsaturation is a significant improvement in analytical capabilities and should prove to be a valuable tool for triglyceride analyses.

Figure C. *cis-trans* Isomers on a SUPELCOSIL LC-18 Column

Column: SUPELCOSIL LC-18, 25cm x 4.6mm ID, 5µm particles
 Cat. No.: 58298
 Mobile Phase: acetone:acetonitrile (63.6:36.4 v/v)
 Flow Rate: 1.0mL/min.
 Pump: Tracor 995
 Detector: Waters R401 refractometer
 Inj. Vol.: 10µL of 9% triolein and trielaidin in mobile phase



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Other Applications

The versatility of nonaqueous reversed phase HPLC may be illustrated by several examples of separations involving compounds other than triglycerides. See below.

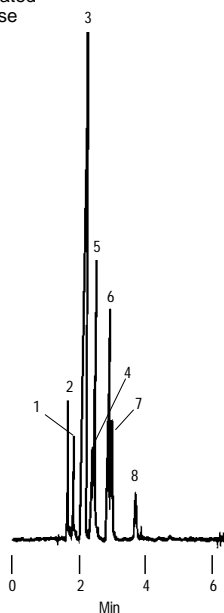
Fatty Acid Methyl Esters

A 25cm x 4.6mm ID SUPELCOSIL LC-18 column eluted with an acetone:acetonitrile (41:59, v/v) mobile phase well resolves the even numbered, saturated FAMES from C8 to C18 (Figure D). The system resolves oleate (18:1) from palmitate (16:0) and partially resolves linoleate (18:2) from myristate (14:0). The system does not separate linolenate (18:3) from laurate (12:0).

Figure D. FAMES on a SUPELCOSIL LC-18 Column

Column: SUPELCOSIL LC-18, 25cm x 4.6mm ID, 5µm particles
Cat. No.: 58298
Mobile Phase: acetonitrile:acetone (59.0:41.0, v/v)
Flow Rate: 1.0mL/min
Pump: Tracor 995
Detector: Waters R401 refractometer
Sample: 10µL of 9% C8 to C18 saturated and unsaturated FAMES in mobile phase

1. C8
2. C10
3. 12:0 + 18:3
4. 18:2
5. 14:0
6. 18:1
7. 16:0
8. 18:0



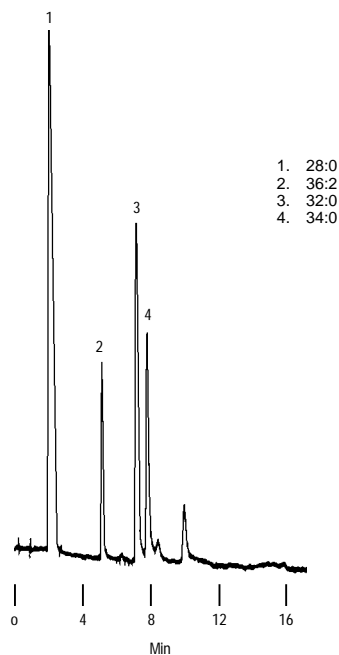
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Diglycerides

Separation of diglycerides using this technique has not been studied extensively. Nevertheless, a mixture of diglycerides containing LaP (28:0) (La=laurate), O₂(36:2), P₂ (32:0), and PS (34:0) was easily resolved using two SUPELCOSIL LC-18 columns and a mobile phase composed of acetone:acetonitrile:tetrahydrofuran (48:50:2, v/v/v) (Figure E). Baseline separation of the diolein-dipalmitin critical pair was obtained.

Figure E. Diglycerides on a SUPELCOSIL LC-18 Column

Column: SUPELCOSIL LC-18, two 15cm x 4.6mm ID, 5µm particles
Cat. No.: 58230-U
Mobile Phase: acetone:acetonitrile:tetrahydrofuran (48:50:2, v/v/v)
Flow Rate: 1.0mL/min
Pump: Tracor 995
Detector: Waters R401 refractometer
Sample: 10µL of a standard diglyceride mixture



1. 28:0
2. 36:2
3. 32:0
4. 34:0

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Mono-, Di-, and Triglycerides

Many chromatographers have been frustrated in their attempts to use reversed phase HPLC for separating mono-, di-, and triglycerides by class. Such a separation requires a refractive index detector since the mobile phase prevents detection of analytes with short wavelength UV. The use of a refractive index detector precludes the use of a gradient program. Thus, separation of these classes of compounds by reversed phase HPLC seems basically incorrect, and such separations require silica HPLC columns. Separation of mono-, di-, and triglycerides using reversed phase columns is possible, according to Dr. E. G. Perkins. The mobile phase he suggests is acetone:acetonitrile (50:50, v/v). This separation requires a flow program. He recommends a flow rate of 0.5mL/minute for 5 minutes, 1.0mL/minute for the next 10 minutes, and 4.5mL/minute thereafter. The analysis is completed in about 18 minutes. For greater reliability, however, we recommend using silica columns.

Conclusion

The ability to separate compounds on SUPELCOSIL LC-18 columns by carbon number, degree of unsaturation, and double bond configuration should find applications in other areas, such as pheromone analysis, fatty acid methyl esters, and mono- and diglycerides. Analysts who used reversed phase HPLC with non-aqueous mobile phases will find many useful applications for the technique.

Ordering Information:

SUPELCOSIL Columns

All SUPELCOSIL columns are supplied with fittings for connection to 1/16 inch OD stainless steel tubing. Five micron spherical packings are listed here. See the current Supelco catalog for more dimensions and different phases.

Description	Cat. No.
SUPELCOSIL LC-8 columns, 5µm particles	
15cm x 4.6mm ID	58220-U
25cm x 4.6mm ID	58297
SUPELCOSIL LC-18 columns, 5µm particles	
15cm x 4.6mm ID	58230-U
25cm x 4.6mm ID	58298

References

- Perkins, E.G., American Oil Chem. Soc. Meeting, San Francisco, June 1979.
- Brockerhoff, H.J., J. Lipid Res., **6**: 10 (1965).

References not available from Supelco.

Acknowledgment

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- In the shorthand representation of triglyceride structure the fatty acids are identified by letter. Their position in the molecule (1, 2, or 3) is usually indicated by the sequence of the letters.
- Shorthand notations for triglycerides in this portion of the text refer only to composition and not to fatty acid order in the molecule.

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