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W. S. Letter<sup>a</sup>

<sup>a</sup> VAREX Corporation, Burtonsville, Maryland

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# A QUALITATIVE METHOD FOR TRIGLYCERIDE ANALYSIS BY HPLC USING AN EVAPORATIVE LIGHT-SCATTERING DETECTOR

W. S. LETTER

*VAREX Corporation  
4000 Blackburn Lane  
Burtonsville, Maryland 20866*

## ABSTRACT

A rapid high-performance liquid chromatographic method for the separation and identification of several triglycerides in edible oil and fat samples using an evaporative light-scattering detector (ELSD) is described. A single 25 cm Hypersil C-18 column maintained at 35°C was used successfully to analyze and identify a variety of triglycerides from almond oil, corn oil, cottonseed oil and olive oil. Complete separation and detection of triolein, 1,2-dioleoyl-3-stearoyl-rac-glycerol, trilinolein, 1,2-dioleoyl-3-palmitoyl-rac-glycerol and 1,2-dilinoleoyl-3-oleoyl-rac-glycerol was achieved in less than 20 minutes using a binary gradient of methylene chloride and acetonitrile. No baseline drift or solubility problems were encountered using this improved method.

### INTRODUCTION

Detection of triglycerides by reversed-phase HPLC has been well documented by scientists over the past years. Methods involving the use of mostly acetonitrile, ethanol and tetrahydrofuran using UV detection of the ester bond between 200 and 230 nm seem to have been very popular in the literature (1-5). UV detection with gradient elution can provide short elution times but shifting baselines, poor sensitivity and the use of poor triglyceride solubilizing non UV absorbing solvents have made the use of them undesirable for many separations. Detection by flame ionization detectors have also been used successfully by some researchers with several good applications being demonstrated (6,7). Baseline stability and sensitivity appear to still be a problem with this type of detector. A number of separations with very long run times but impressive results were obtained through use of the R.I. detector with its non-specific response and ease of use (1,2,4,8-11). Better sample solubility through the use of UV absorbing solvents such as acetone and chloroform have contributed to its success. Unfortunately, its incompatibility with gradient analysis does not allow a user the ability to elute the triglycerides off the column in a reasonable amount of time while still maintaining adequate sensitivity. Poor baseline stability caused by temperature fluctuations is

another disadvantage associated with the R.I. detector. The evaporative light-scattering detector (ELSD) which can provide essentially flat baselines with gradient elution and can operate with many UV absorbing solvents has also been used by many researchers with success to separate a variety of triglycerides in oils and fats (1,12-17). All of these methods required long run times (ie: between 35 and 120 minutes) and did not always use solvents that could solubilize the higher molecular weight compounds.

A method which could separate all of the major triglycerides in under 20 minutes while taking advantage of better solubilizing solvents such as methylene chloride was sought and developed. This method utilizes the ELSD which allowed flat baselines to be obtained during gradient analysis and is in part based on work originally developed by Privett et al (6).

### MATERIALS & METHODS

#### Samples & Standard Triglyceride Preparation

All edible oils and fats used for sample analysis were purchased from the local grocery store and used as is without any further purification. Each sample was diluted to a concentration of 1.0 % (w/v) in equal parts of methylene chloride and acetonitrile and was stored at 4°C when not in use.

Triolein, 1,2-Dilinoleoyl-3-oleoyl-rac-glycerol, 1,2-Dioleoyl-3-palmitoyl-rac-glycerol, Trilinolein and 1,2-Dioleoyl-3-stearoyl-rac-glycerol were purchased from Sigma Chemical Company (St. Louis, MO, USA) at 99 % purity and were all used as reference standards in this work. Each of the standards was stored frozen, away from any sources of light and diluted to 0.1 % (w/v) in equal parts of methylene chloride and acetonitrile the day of use.

Both the samples and triglyceride standards were filtered through a 0.22 micron syringe filter before being placed in 2.0 mL autosampler vials for HPLC analysis.

### Solvents

All of the solvents used were purchased as B & J Brand HPLC grade from Baxter Diagnostics (Columbia, MD, USA) and were filtered through a 2 micron steel frit and thoroughly degassed with helium before use.

### Gas

Nitrogen gas for the ELSD was ultra high purity grade and purchased from Matheson Gas Products (Secaucus, NJ, USA). A regulated pressure of 80 psi was delivered to the gas inlet of the detector.

### Instrumentation

A dedicated analyzer system available from Varex Corporation (Burtonsville, MD, USA) was used for all HPLC analysis. The complete analytical system included the following instruments. A ternary low pressure mixing gradient HPLC pump, model L-6200A, from Hitachi Instruments (Danbury, CT, USA) was used to control the binary gradient and deliver solvent to the column for analysis of the standards and samples. The remainder of the automated HPLC system consisted of a Marathon autosampler with integral thermostated column compartment (Spark Holland, The Netherlands) fitted with a 20  $\mu$ L injection loop, 386/40 IBM type clone style computer with Apex 625 data acquisition, control and analysis software and Model 161-256-S CSI-2 A/D interface box all from Autochrom, Incorporated (Milford, MA, USA) and an Evaporative Light-Scattering Detector (ELSD), model ELSD IIA, from VAREX Corporation (Burtonsville, MD, USA) was used to detect the samples and standards.

The highly versatile and easy to use SlideWrite Plus graphical software from Advanced Graphics Software, Incorporated (Carlsbad, CA, USA) was used to import and reformat the raw data chromatograms for preparation of the figures used.

### Chromatographic Conditions

A Hypersil ODS 120 Å, 250 mm x 4.6 mm I.D. analytical column purchased from Alltech Associates

TABLE I

## Gradient System For Triglyceride Separation

Time (min.)	Composition of Mobile Phase	
	% A	% B
0.00	30	70
12.00	45	55
20.00	53	47
25.00	30	70

A = Methylene Chloride & B = Acetonitrile

(Deerfield, IL, USA) packed with 5 micron spherical particles was used to separate the triglycerides. No guard column was used.

The mobile phase consisted of methylene chloride and acetonitrile run as a stepped gradient described in Table I. The stronger solvent, methylene chloride, is increased first at a rate of 1.25 % per minute for 12 minutes then at 1.00 % per minute for 8 more minutes to elute all of the triglycerides from the column. The program is then quickly reversed to equilibrate the column back to the starting condition of 30 % methylene chloride and 70 % acetonitrile. The solvent flow rate was held constant at 1.00 mL / min at a controlled column temperature of 35°C throughout the separation. For best reproducibility, the injection loop (20 uL) was always overfilled exactly 3.0 times with solution for each injection.

Nebulization in the ELSD was set to 95°C drift-tube temperature (56°C Exhaust Temperature) and 50 mm flow at 26 psi of nitrogen gas backpressure flow to the nebulizer. These conditions were maintained throughout each analysis.

### RESULTS & DISCUSSION

This method detects several triglycerides in sample food oils without the need to derivatize the sample or use special mobile phase modifiers. Use of the ELSD allows the use of gradient methods as well as the use of highly volatile, UV absorbing solvents such as methylene chloride. Earlier research had shown that regardless of the detection device used the mobile phases containing acetone and acetonitrile while providing some impressive separations do have some problems fully solubilizing triglycerides with more than 46 carbon atoms in their alkyl chains (8). These triglycerides would sometimes crystalize or precipitate out inside of the tubing, column or injection valve causing either a loss of sensitivity or poor reproducibility (8). The use of a better sample solubilizing solvent system such as methylene chloride and acetonitrile allows a better range of sample solubility in the mobile phase. Detection via the ELSD allows flat baselines during the gradient method aiding in peak identification.



The ELSD, unlike some HPLC detectors does require routine maintenance in order to operate efficiently over time. Both the drift-tube and nebulizer should be cleaned every few weeks to prevent any sample from accumulating inside of the unit. If not cleaned and maintained potential baseline noise and analysis reproducibility problems can result. Routine cleaning allows the detector to operate at peak efficiency with extremely flat baselines during gradient conditions. The sometimes non-linearity of the detectors response can also be easily dealt with through the use of a third party software package capable of performing curve fits such as SlideWrite Plus. Since the response appears to be sigmoidal (18) a third order polynomial or exponential curve fit usually can be applied to the non-linear extremes of the line with good results.

Solvent backpressure to the nebulizer was measured with a simple gauge (0-30 psi range) and zero dead volume tee and provided an excellent diagnostic means of monitoring the condition of the detectors nebulizer. An increase in the backpressure of a few psi was usually indicative of contamination depositing in the nebulizer needle thus causing baseline noise and poor sensitivity to be observed. Unlike an UV or RI detector the ELSD nebulizer can occasionally become partially or completely plugged due to either particulate matter becoming lodged

in the fine needle or from highly concentrated or sticky samples building up on the inside of the nebulizer. The high pressure produced from a complete plug can overpressure either the column packing or a UV flow cell when it is desirable to operate with an UV detector in series with the ELSD. When the gauge read more than a few psi over normal operating pressure the nebulizer was promptly removed from the detector and both sonicated and backflushed with solvent. A relief valve attached to the nebulizer was preadjusted to open when the solvent backpressure on the nebulizer exceeds 25 psi and is fitted to the gauge tee to prevent any of these damaging effects from occurring. A kit containing the gauge, tee and relief valve is now available directly from the manufacturer of the ELSD and is a very useful diagnostic aid as well as safety feature not found on many other detectors. Careful attention to these maintenance details insured that the detector always operated at peak performance while these compounds were analyzed.

A reference mixture containing five triglyceride standards (0.1 % each) was injected into the column via the autosampler for comparison to the oils samples analyzed. Each triglyceride standard shown in Figure 1A was baseline resolved in under 16 minutes. A flat baseline was easily obtained throughout the total analysis time. It can also be seen that the ELSD does not

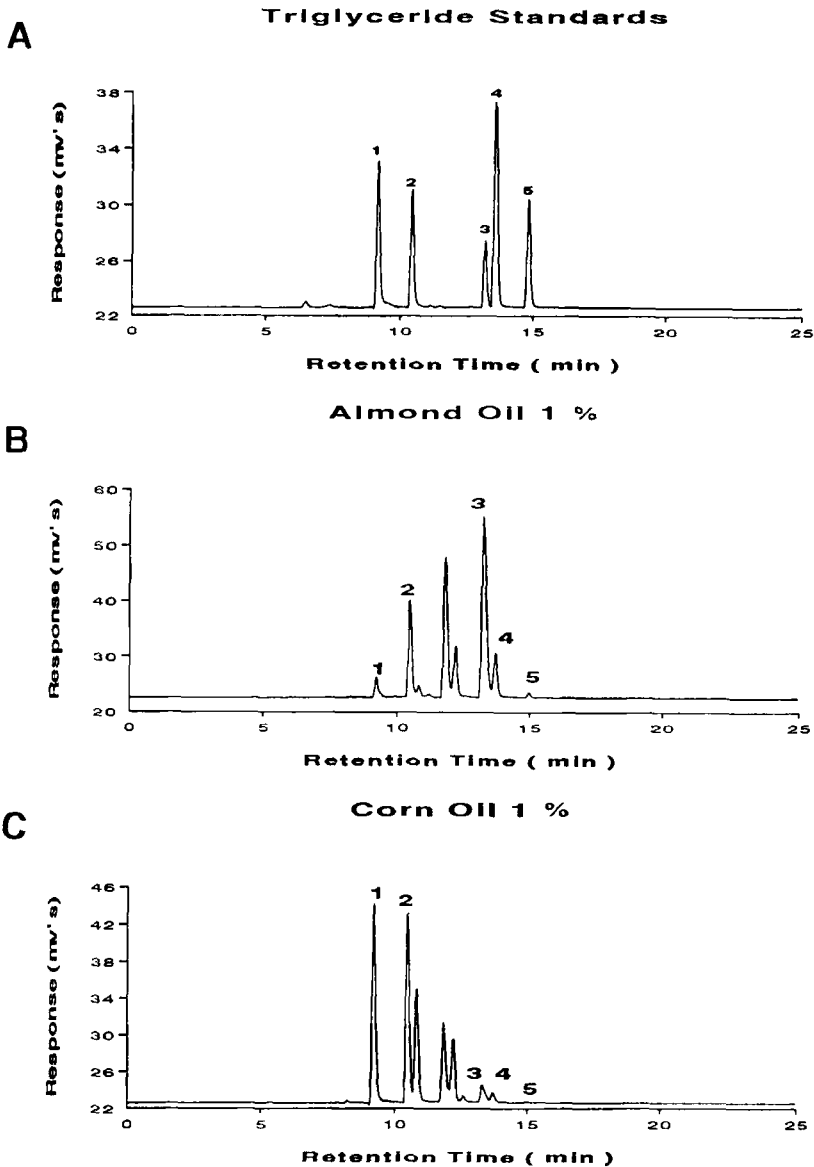
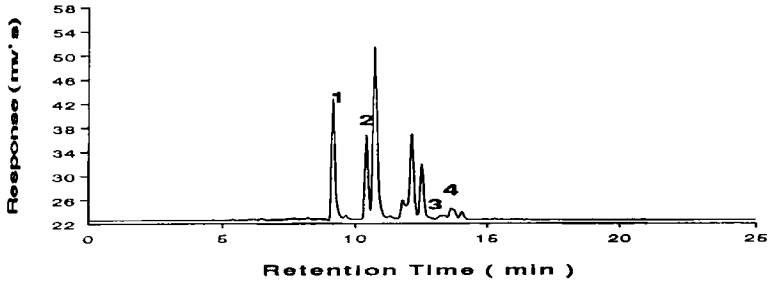


Figure 1. Triglyceride chromatograms of fats & oils using the HPLC method described in the text. Triglyceride standards (1a) are 0.1 % each. Peaks identified in the text. Triglyceride include: 1) Trilinolein, 2) 1,2-Dilinoleoyl-3-oleoyl-rac-glycerol, 3) Triolein, 4) 1,2-Dioleoyl-3-palmitoyl-rac-glycerol and 5) 1,2-Dioleoyl-3-stearoyl-rac-glycerol.

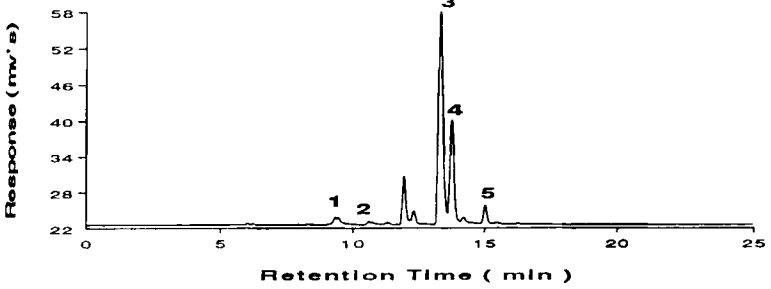
D

Cottonseed Oil 1 %



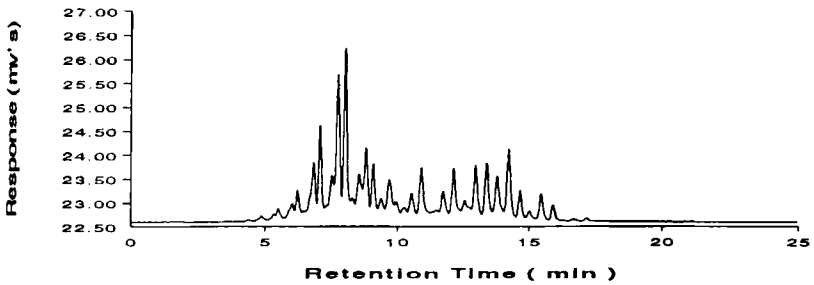
E

Olive Oil 1 %



F

Butter 1 %



respond equally to each compound at equivalent concentrations. This is mainly due to the differing physical properties of the different standards used. Differences in refractive index, viscosity, melting point and volatility can cause the detector to form different sized droplets. The resulting different sized droplets will scatter different amounts of light to the ELSD's photodetector. Most triglycerides analyzed in our lab have shown response factors of between 1 and 3 overall.

Figures 1B through 1E show clearly how the method was utilized to analyze a number of food oils for the presence of the five reference standards. All four samples were baseline resolved with excellent peak shapes. Almond oil, corn oil and olive oil were found to contain all five of the reference standards based on their retention times alone. Cottonseed oil was found to contain four of the five standards missing only 1,2-dioleoyl-3-stearoyl-rac-glycerol. Note how all of the major triglycerides were eluted in under 16 minutes for these simple oil samples. A much more complex sample of butter fat shown in Figure 1F was also analyzed using the same method already discussed. This sample was well resolved in under 20 minutes but proved to be too complex for reliable identification using this method. Perhaps a longer column or slower flow rate would yield better results for the more complex samples.

The chromatograms in the figure show how easily and rapidly one can obtain profiles of a variety of simple and even complex triglycerides in real samples. This method could prove to be very useful in obtaining qualitative comparisons of different oils to determine if they have been adulterated with other products or in evaluating rancidity in oils over time. With the appropriate calibration curves the method could be used to determine quantitatively the concentration of individual triglycerides in a variety of fat and oil samples. No attempt was made to try to quantitate any real concentrations of individual triglycerides at this time but rather to present the improved gradient method.

#### CONCLUSION

The method described utilizes a simple binary gradient with an ELSD that has been shown to separate and detect several reference triglycerides in under 20 minutes. The solvent composition containing acetonitrile and methylene chloride provided excellent solubility for the fats and oils analyzed. Use of the ELSD in the analysis of the samples resulted in the absence of temperature and baseline drift during the gradient method. The ELSD stability coupled to the use of a better solubilizing solvent were responsible for the improved results obtained in this paper. Identification of several

major triglycerides present in almond, corn, cottonseed and olive oil were made possible with this method. All peak identifications were based on retention time alone and absolute identifications of each triglyceride were not performed.

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