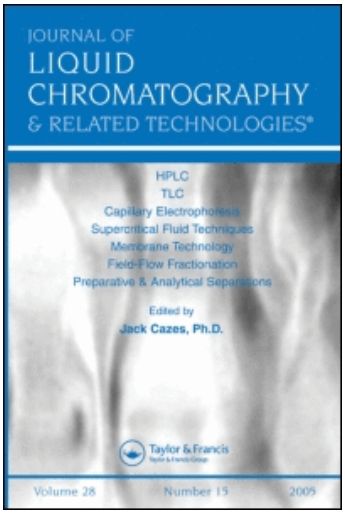
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Qualitative and Quantitative Determination of Methyl Esters, Free Fatty Acids, Mono-, Di-, and Triacylglycerols Via HPLC Coupled with a Flame Ionization Detector

W. E. Neff[a]; M. A. Jackson[a]; G. R. List[a]; J. W. King[a]
[a] National Center for Agricultural Utilization Research Food Quality and Safety Research, USDA, Agricultural Research Service, Peoria, Illinois, USA

To cite this Article Neff, W. E. , Jackson, M. A. , List, G. R. and King, J. W.(1997) 'Qualitative and Quantitative Determination of Methyl Esters, Free Fatty Acids, Mono-, Di-, and Triacylglycerols Via HPLC Coupled with a Flame Ionization Detector', Journal of Liquid Chromatography & Related Technologies, 20: 7, 1079 — 1090
To link to this Article: DOI: 10.1080/10826079708010960
URL: http://dx.doi.org/10.1080/10826079708010960

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 20(7), 1079–1090 (1997)

QUALITATIVE AND QUANTITATIVE DETERMINATION OF METHYL ESTERS, FREE FATTY ACIDS, MONO-, DI-, AND TRIACYLGLYCEROLS VIA HPLC COUPLED WITH A FLAME IONIZATION DETECTOR

W. E. Neff,* M. A. Jackson, G. R. List, J. W. King

USDA, Agricultural Research Service
National Center for Agricultural Utilization Research
Food Quality and Safety Research
1815 N. University Street
Peoria, Illinois, USA 61604

Downloaded At: 12:12 24 January 2011

ABSTRACT

High performance liquid chromatography (HPLC) with a cyanopropyl phase column coupled directly to a commercial HPLC flame ionization detector (FID) and a gradient mobile phase of tert-butyl ether and hexane proved successful to separate and quantitate mono-, di-, and triacylglycerols, free fatty acids and methyl esters. These components can occur together during glycerolysis, lipolysis, randomization, and interesterification reactions of vegetable oils like soybean. Gravimetric standards were evaluated by HPLC-FID in which each contained mono-, di, and triacylglyceols, free fatty acids and methyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids respectively. The FID response with solute weight decreased in this order: triacylglycerols, free fatty acids, diacylglycerols, methyl esters, and monoacylglycerols, respectively. The FID thus required response factors to quantitate the components of mixtures of diverse lipid species. However, for homogenous compounds the

Copyright © 1997 by Marcel Dekker, Inc.

FID response was linear and response factors were not required, for example, for a series of free fatty acids like palmitic through linolenic. The commercial HPLC-FID proved satisfactory for facile quantitation of diverse lipid species in vegetable oil reaction mixtures. Good accuracy was obtained by HPLC with FID for soybean glycerolysis products, which contained mono, di and triacylglycerols.

INTRODUCTION

Interesterification is a process used in the oils and fats industry to modify the properties of triacylglycerols (TAG). This reaction is of commercial interest for the production of margarines, shortenings, and other speciality products with desired physical properties and oxidative stability.[1-4] Depending on interesterification conditions, the side reactions of lipolysis and hydrolysis may occur.[5-7] Other reactions, such as glycerolysis to produce products like monoacylglycerols (MAG) and diacylglycerols (DAG) from vegetable oil TAG are important in the food industry. Here too, it is important to monitor the occurrence of free fatty acids (FFA), MAG, DAG, and unreacted TAG.[8] Other important food industry reactions, which require monitoring of diverse lipid species (LS) of FFA, MAG, DAG, and TAG, include vegetable oil TAG hydrolysis to produce industrially or nutritionally important FFA.[9,10]

Downloaded At: 12:12 24 January 2011

Analytical methods like thin layer (TLC)[11,12] and gas chromatography (GC)[13] and high performance liquid chromatography (HPLC)[14-21] have been used to analyze LS. TLC with FID and silica gel coated rods for LS was reviewed by Sebedio.[11] He pointed out that the GC FID response was not a linear relationship with sample amount spotted on these rods. Even for homogeneous compounds, a power function was necessary to describe FID response with respect to sample amount spotted on the TLC rod. Also, recently, Peyrou reported for TLC of oleic acid LS, the TLC-FID required power functions for calibration curves to express detector response with amount of LS spotted on the TLC rods.[12] TLC-FID response is usually linear without the need for response factors for homogeneous components of lipid mixtures.[13] However, response factors are required for GC analysis of mixtures of diverse LS. Also, to avoid thermal alteration at the elevated temperature required for GC, LS like MAG and DAG required derivatization before analysis.[13] HPLC, which does not require elevated temperature, avoids the need for derivatization and has been reported as the method of choice for LS.[13-21] Unfortunely, for HPLC of LS, detection is a problem.[14-19] Presently, the light scattering and flame ionization detectors show the most promise in LS analysis.[14-19] However,

light scattering detector response is not a linear relationship with solute amount and complex calibration curves are required for quantitation.[17,19] On-the other-hand, transport flame ionization detectors of the moving wire type[14] or rotating quartz belt[15,17] have been reported to demonstrate a linear response with solute amount without the need for response factors for homogenous LS.[14-20] We have previously described many examples of quantitative reversed phase HPLC coupled with a commercial HPLC-FID with a rotating quartz belt for TAG in vegetable oils, vegetable oil blends and products.[1-4,20] However, the rotating quartz belt FID response has not been evaluated for mixtures in which diverse LS like MAG, DAG, TAG, FFA, and methyl esters (FAME) occur together.[17]

This study describes the resolution and quantitation of identified, diverse LS with a cyanopropyl bonded phase HPLC column coupled with a commercial HPLC-FID of the rotating quartz belt design.

Downloaded At: 12:12 24 January 2011

EXPERIMENTAL[†]

Materials

Lipid standards were purchased from Nu Chek Prep, Inc.(Elysian, MN) and Sigma Chemical Co . (St. Louis, MO). All solvents were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ) and J T. Baker Inc. (Phillipsburg, NJ).

High Performance Liquid Chromatography

HPLC was performed with a Thermo Separation Products (Schaumburg, IL) (Model SP 8800) ternary solvent system with a HPLC column of bonded cyanopropyl phase, Alltech Associates (Deerfield, IL) Econosil CN, 25 cm x 4.6 mm, 5 μm. A gradient solvent program with 0.5% acetic acid (AA) in tert-butyl ether (TBE) and hexane (HEX), modified from that used by El-Hamdy and Christie[21] was used to accomplish the LS separation as follows: 2% (0.5% AA in TBE) in HEX (V:V) for 6 min., linear from 6-34 min to 100% (0.5% AA in TBE) hold 10 min then return to initial conditions 44-54 min. The flow rate was 1.0 mL/min. The 0.5% AA in TBE was required to prevent FFA being adsorbed by the HPLC column. The sample size range was 0.063 to 0.5 mg in 10 μL 0.5% AA in TBE. The HPLC detector was a commercial HPLC-FID with a moving quartz belt, Finnigan, Inc. (Austin, TX) Tremetrics Model 945. The HPLC-FID was operated with block temperature 130°C; detector gas, 140 mL/min. hydrogen; cleaning flame, 275 mL/min hydrogen;

175 mL/min oxygen; and compressed air, 0.4 cubic ft/min. The FID response was processed by a real-time computer.[22] Identification and quantitation of the HPLC-FID was based on known gravimetric mixtures of FAME; TAG; FFA, 1,2-and 1,3-DAG, and 1- and 2-MAG.

RESULTS AND DISCUSSION

A technique reported by El-Hamdy and Christie[21] for HPLC of an unidentified mixture of LS on a cyanopropyl bonded phase column gave stable retention times, resolution and symmetrical chromatogram peaks for the mixture of TAG, FAME, MAG, DAG, and FFA. These workers used a light scattering detector and did not report quantitation for the LS. HPLC detectors like those based on light scattering and ultraviolet absorption principles require complex calibration curves for quantitation.[17]

Downloaded At: 12:12 24 January 2011

We extended the cyanopropyl column resolution to more mixtures of diverse LS, which are identified. Further, we used a commercial HPLC-FID of the moving quartz belt type for quantitative analysis of LS in this study. This HPLC-FID utilized the following operational procedures: 1) the HPLC effluent is sprayed onto a rotating quartz belt, 2) the HPLC solvent is removed by high temperature and partial vacuum, 3) solutes remain on the belt, which is rotated through a FID to produce a response proportional to solute quantity, and 4) the belt is passed through a cleaning flame and returned to the starting position. There is no interface between the HPLC column exit and the detector. Previously, Christie presented a schematic of the detector.[15] The detector response is reported linear with solute concentration and does not require complex calibration curves for quantitation.[15,17]

Separation on the cyanopropyl column of LS gravimetric standards or series and reaction mixtures of FAME, TAG, FFA, DAG, and MAG is presented in Figure 1. The series for LS, which contain stearic, palmitic, oleic, linoleic, and linolenic acids exclusively, are arranged sequentially starting at the figure bottom. It is observed that the elution times for the components on the cyanopropyl column increased in this order: FAME; TAG; FFA; 1,3-DAG;1,2-DAG; 1-MAG and 2-MAG respectively. Retention time precision was an average of plus or minus 0.50 min. per LS component for triplicate analysis. Also, retention times were stable and reproducible over the two month period the cyanopropyl column was used. The LS components of the stearic acid series have the earliest retention times. The retention times for the other LS components increased in this order: palmitic, oleic, linoleic, and linolenic as the FA moiety polarity increased.

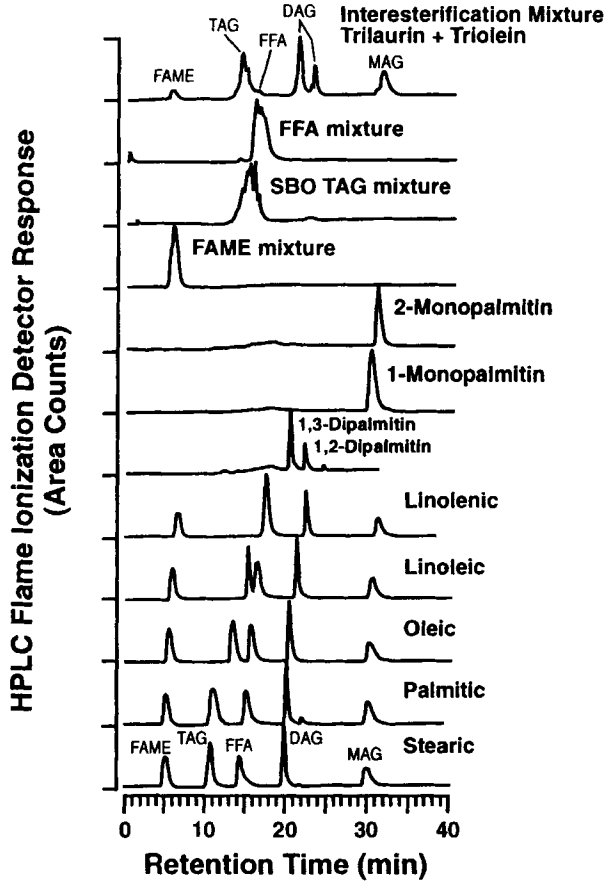


Figure 1. HPLC separation of lipid compounds with an acetic acid/methyl tert-butyl ether/hexane gradient on a cyanopropyl column coupled with an HPLC flame ionization detector.

The retention time changes are slight and in the direction expected for polar HPLC. For the LS linolenic series the TAG and FFA co-elute. Also, in Figure 1, it is observed that 1,2- and 1,3-palmitic DAG are resolved and 1 and 2 palmitic MAG are partially resolved. As, expected, the less polar of the DAG and MAG pairs eluted first. It is observed during HPLC, little DAG or MAG isomerization occurred even though the mobile phase contained acetic acid. Compared to the gravimetric standard series and model DAG and MAG, which

Downloaded At: 12:12 24 January 2011

Table 1

HPLC-FID Quantitation of Lipid Species

Gravimetric Mixture	Components	HPLC-FID Area %[a]	Weight %	Absolute Error%[b]
Stearic	FAME	18.3	20.0	1.7
	TAG	25.5	20.0	5.5
	FFA	20.8	20.0	0.8
	DAG	19.9	20.0	0.1
	MAG	15.5	20.0	4.5
			Average error	2.5
Oleic	FAME	17.9	20.0	1.8
	TAG	23.5	20.0	3.8
	FFA	21.7	20.0	0.5
	DAG	21.1	20.0	1.4
	MAG	15.8	20.0	3.9
			Average error	2.3

[a] Area % precision, standard deviation = ± 0.0 to 0.5% triplicate analysis.[23]

[b] Difference between experiment area % and weight %.

Downloaded At: 12:12 24 January 2011

gave single chromatogram peaks for each LS component, FAME (FA14:0,16:0,18:0,18:1,18:3,20:0) FFA (FA 16:0,18:0,18:1,18:2,18:3) and SBO TAG (trilinolein-mixed FA TAG-tristearin) and mixtures of DAG and MAG from an interestification reaction of trilaurin and tristearin gave somewhat broad peaks. However, these latter mixtures have LS component elution in the appropiate FAME, TAG, FFA, DAG, and MAG regions of the chromatogram to allow identification of these respected LS in samples from vegetable oil interesterification, glycerolysis, or hydrolysis reaction mixtures.

Plots of HPLC-FID response for each of the LS components in the gravimetric standards for component weight of 0.06 to 0.1 mg were linear with linear regression coefficients[23] range of 0.9916-0.9999. The FID response with component weight decreased in this order:TAG, FFA, DAG, FAME and MAG, respectively. The magnitude of the detector response per component weight or

Table 2

HPLC-FID Responce Factors[a,b] for Lipid Species

Gravimentric Mixture	Components	Response Factors
Stearic	FAME	1.09
	TAG	0.78
	FFA	0.96
	DAG	1.00
	MAG	1.29
Oleic	FAME	1.12
	TAG	0.85
	FFA	0.92
	DAG	0.95
	MAG	1.26

[a] Response factor = gravimentric component wt% ÷ HPLC=FID area %.

[b] Response factor precision ± 0.05 for triplicate analysis.

Downloaded At: 12:12 24 January 2011

response factors[24] ranged from 4.10 for TAG to 2.90 for MAG ($x10^6$ area counts per mg sample). These FID response results with respect to LS type, i.e., TAG vs. MAG, are similar to the concept of relative weight proportion of FID active carbons.[25-27] For example, MAG gave a lower detector response than TAG since MAG has fewer active carbons (methylene carbons) which are responsible for FID response.

Once linearity of detector response was established for the components of the LS gravimetric standards, the HPLC-FID quantitation was examined. Samples of palmitic, stearic, oleic, linoleic, and linolenic gravimetric series (Figure 1) with FAME, TAG, FFA, TAG, and MAG each 20% by weight were injected onto the cyanopropyl HPLC column and the FID area percent obtained by computer integration of the FID response.[22] The FID area percent and gravimetric weight percent (20 %) are compared for the LS of the stearic and linoleic gravimetric standards in Table 1. It is observed that the HPLC-FID area percent is within 1 percent of the weight percent for FFA. For FAME and DAG, the FID area percent is within 2 percent of the weight percent.

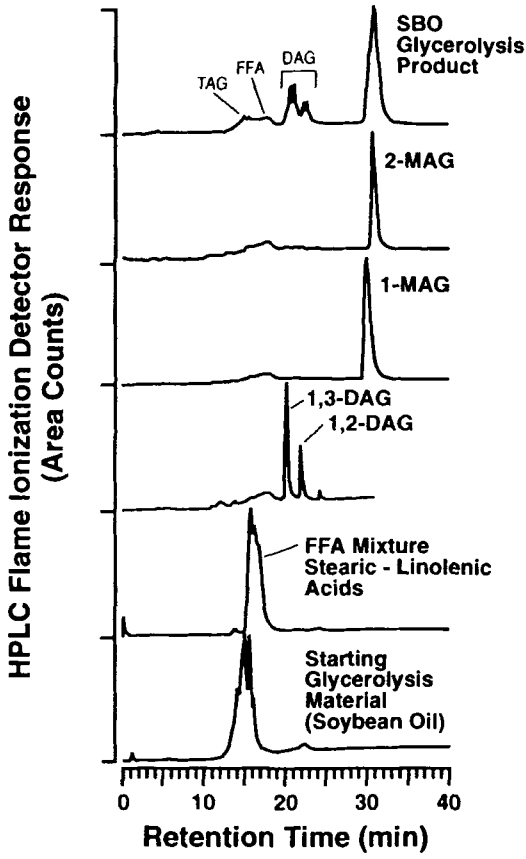


Figure 2. HPLC separation of a soybean oil glycerolysis mixture with an acetic acid/methyl tert-butyl ether/hexane gradient on a cyanopropyl column coupled with an HPLC flame ionization detector.

The HPLC-FID area is 2 percent greater than weight percent for TAG and MAG. Similar results were obtained for palmitic, linoleic and linolenic series, with average errors over all the LS components of 3 percent.

The results in Table 1 show that for good quantitation with HPLC-FID for mixtures which contain FAME, TAG, FFA, DAG, and MAG, response factors are required. Response factors calculated by dividing gravimetric weight percent by HPLC-FID area percent[24] are given for the LS of the stearic and oleic series in Table 2.

Downloaded At: 12:12 24 January 2011

Table 3

HPLC-FID Analysis of Lipid Species in a Soybean Oil Glycerolysis Mixture[a]

Analytical Method	Compounds	Uncorrected Area %	Corrected[b] Area %
HPLC-FID	TAG	1.01	< 0.1
	FFA	1.6	2.0
	DAG	26.4	21.8
	MAG	71.1	76.2
SCF[c]	TAG + FFA		1.5
	DAG		23.3
	MAG		75.3

[a] Chromatogram of glycerolysis reaction presented in Fig. 2.
[b] Uncorrected area % adjusted by average response factors given in text and the data renormalized. HPLC-FID area % precision ± 0.0-0.5%; SCF area % precision ± 1%.
[c] Supercritical fluid TAG analytical technique.[28]

Downloaded At: 12:12 24 January 2011

Similar HPLC-FID factors were obtained for the LS of the palmitic, linoleic, and linolenic gravimetric series. Since for the Ln series FFA and TAG co-elute, gravimetric standards one without FFA and one without TAG, but with the other components (25 wt % each) were analyzed to obtain response factors for TAG and FFA. Average response factors for the LS of the palmitic, stearic, oleic, linoleic, and linolenic gravimetric series were: FAME, 1.14 ± 0.6; TAG, 0.86 ± 0.12; FFA, 0.91 ± 0.09; DAG, 0.97 ± 0.05; and MAG, 1.26 ± 0.04. These response factors showed that most of the needed correction for HPLC-FID area percent was required for TAG and MAG. The other LS, FAME, FFA, and DAG had correction factors close to unity.

We utilized the above results from HPLC-FID of gravimetric standards for analysis of experimental reaction mixtures resulting from lipid transformations. In Figure 2, a reaction mixture from glycerolysis of soybean oil for preparation of MAG[28] was resolved by the cyanopropyl HPLC column coupled with FID. This chromatogram is at the top of the figure. Reference chromatograms which include the starting soybean oil and other LS standards

reversed phase solid phase extraction before HPLC analysis. We have observed that free glycerol is retained by the cyanopropyl column. Utilizing the average response factors for LS presented above, the corrected HPLC-FID area percent is given for components of the glycerolysis mixture in Table 3. It is observed, that the corrected HPLC-FID area percent are in agreement with LS composition obtained by a supercritical fluid chromatography TAG analytical method.[28] Examination of the data obtained by application of the average response factors to HPLC-FID area percent should reflect that in the glycerolysis mixture the TAG and DAG also contained mixed FA species as opposed to only single FA species in the gravimetric standards. However, good agreement was still obtained between the LS composition from HPLC-FID and the supercritical analytical methods.

REFERENCES

† Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

1. M. A. M. Zeitoun, W. E. Neff, T. L. Mounts, Rev. Franc. Corps Gras, **39**, 85-90 (1992).

2. M. A. M. Zeitoun, W. E. Neff, G. R. List, T. L. Mounts, J. Am. Oil Chem. Soc., **70**, 467-471 (1993).

3. W. E. Neff, M. A. El-Agaimy, T. L. Mounts, Ibid., **71**, 1111-1116 (1994).

4. G. R. List, T. L. Mounts, F. Orthoefer, W. E. Neff, Ibid., **72**, 379-382 (1995).

5. T. A. Foglia, K. Petruso, S. H. Feairheller, Ibid., **70**,281-285 (1993).

6. H. M. Ghazali, S. Hamidah, Y. B. Che Man, Ibid., **72**,633-471 (1995).

7. D. J. Sessa, W. E. Neff, G. R. List, M. A. M. Zeitoun, J. Food Sci. Tech, **29**, 581-585 (1996).

8. B.Yang, J. Chen, J. Am. Oil Chem. Soc., **68**, 980-982 (1991).

Downloaded At: 12:12 24 January 2011

9. Y.-S .Huang, X. Lin, P. R. Redden, D. F. Horrobin, Ibid., **72**, 625-631 (1995).

10. T. L. Bereuter, E. Lorbeer, J. Chromatog., **697**, 469-474 (1995).

11. J.-L. Sebedio, "Utilization of Thin-Layer Chromatography-Flame Ionization Detection for Lipid Analyses" in **New Trends in Lipid and Lipoprotein Analysis**, J.-L. Sebedio, E. G. Perkins, eds., American Oil Chemists' Society Press, Champaign, IL, 1995, pp. 24-37.

12. G. Peyrou, V. Rakotondrazafy, Z. Mouloungui, A. Gaset, Lipids, **31**, 27-32 (1996).

13. W. W. Christie, **Gas Chromatography and Lipids**, The Oily Press, Ayr, Scotland (1989).

14. K. Aitzetmuller, J. Chromatog., **139**, 61-68 (1977).

15. W. W. Christie, **High-Performance Liquid Chromatography and Lipids**, Pergamon Press, New York, NY (1987).

16. W. W. Christie, **Advances in Lipid Methodology-One**, The Oily Press, Ayr, Scotland (1992).

17. R. A. Moreau, "Quantitative Analysis of Lipids by HPLC with A Flame–Ionization Detector or An Evaporative Light Scattering Detector" in **Lipid Chromatographic Analysis**, T. Shibamoto, ed., Marcel Dekker, Inc., New York, NY, 1994, pp. 251-271.

18. V. K. S. Shukla, "High-Performance Liquid Chromatography: Normal Phase, Reversed Phase Detection Methodology" in **New Trends in Lipid And Lipoprotein Analysis**, J.- L. Sebedio, E. G. Perkins, eds., American Oil Chemists' Society Press, Champaign, IL, 1995, pp. 38-44.

19. W.W. Christie, "Lipid Class Separations Using High Performance Liquid Chromatography" ibid., 1995, pp. 45-48.

20. M. A. M. Zeitoun, W. E. Neff, E. Selke, T. L. Mounts, J. Liq. Chromatogr., **14**, 2685-2698 (1991).

Downloaded At: 12:12 24 January 2011

21. A. H. El-Hamdy, W. W. Christie, J. H. R. Chromatogr., **16**, 55-57 (1993).

22. R. O. Butterfield, W. K . Rohwedder, E. D. Bitner, J. O. Ernst, D. J. Wolf, H. J. Dutton, Prog. Lipid Res, **17**, 93-110 (1978).

23. R. E. Walpole, R. H. Meyers, **Probability and Statistics for Engineers and Scientists**, 3rd. edn., Macmillan Publishing Co., New York, NY, 1985.

24. M. A. Kaiser, F. J. Debbrecht, "Qualitative and Quantitative Analysis by Gas Chromatography" **in Modern Practice of Gas Chromatography**, R. L. Grob, ed., John Wiley and Sons, Inc., New York, NY, 1977, pp. 151-211.

25. R. G. Ackman, J. C. Sipos, J. Am. Oil Chem. Soc., **41**, 377-378 (1964).

26. C. D. Bannon, J. D. Craske, A. E. Hilliker, ibid., **63**, 105-110 (1986).

27. J. D. Craske, C. D. Bannon, ibid., **65**, 1190-1191 (1988).

28. M. A. Jackson, J. W. King, ibid., **73**, 353-356 (1996).

Received August 27, 1996
Accepted September 10, 1996
Manuscript 4273

Downloaded At: 12:12 24 January 2011