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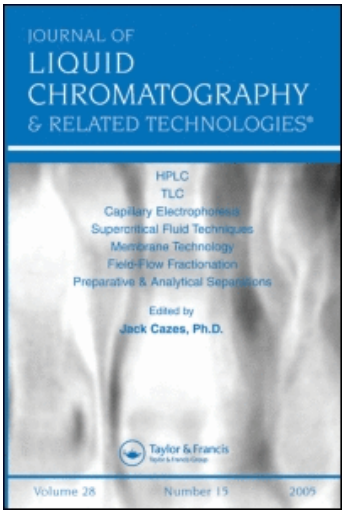

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Qualitative and Quantitative Analysis of Lipid Classes in Fish Oils by Thin-Layer Chromatography with an Iatroscan Flame Ionization Detector (TLC-FID) and Liquid Chromatography with an Evaporative Light Scattering Detector (LC-ELSD)

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Qualitative and Quantitative Analysis of Lipid Classes in Fish Oils by Thin-Layer Chromatography with an Iatroscan Flame Ionization Detector (TLC-FID) and Liquid Chromatography with an Evaporative Light Scattering Detector (LC-ELSD)

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Abstract: Combined effects of hydrogen and air flow rates on the peak response of selected neutral lipid classes (triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids, and ethyl esters) were studied to optimize and calibrate the Iatroscan Mk-6s Chromarod system for the qualitative and quantitative analysis of lipid classes by thin-layer chromatography (TLC) with flame ionization detection in fish oil during the transesterification process. Air flow rate of 2 L/min, hydrogen flow rate of 150–160 mL/min, and scan rate of 30 s/rod were found to be the optimum conditions. All samples were also analyzed by high performance liquid chromatography (HPLC) with evaporative light scattering detection. Quantitative results obtained by TLC with the flame ionization detection method were comparable to those obtained from HPLC with evaporative light scattering detection.

Keywords: Lipid classes, Fish oil, TLC-FID, Iatroscan-Mk6s, LS-ELSD

INTRODUCTION

The Iatroscan thin-layer chromatography/flame ionization detection (TLC-FID) system is an effective, simple, and rapid method for the separation and

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quantification of lipid classes in various matrices, including food and feeds, fats and oils, drugs and blood, as well as a variety of biological tissues.[1–5] This technique is a combination of the efficacy of the thin-layer chromatographic separation using a reusable silica gel-coated Chromarods instead of TLC plates, and the sensitivity of the FID. There has been a remarkable improvement in the reproducibility of this technique, which is mainly due to the positive advancements in the Chromarods, collector electrodes, spotting device, and the data acquisition and processing unit. These changes also enabled the scaling down of the sample size as compared with previous models. Although the improvement in the geometric configuration of the detector in the later version of Mark-IV and -5 models drastically improved the linearity of the FID response, multi-level calibration has still been the choice in the quantification of lipid classes.[1,6] Neutral lipids, including cholesterol, monoacylglycerol (MG), and glycolipids such as monogalactosyl diacylglycerol, digalactosyl diacylglycerol also gave curvilinear relationships with sample loads ranging from 0.5 to 10 μg, detected by Chromarods-SIII with Mark-IV.[7–10]

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The sensitivity of the FID varies, depending upon a multitude of factors including air and hydrogen flow rates, scan speed, sample load, as well as the nature of the sample.[2] The detectors of the Iatroscan versions Mark-IV and -5 are more sensitive than the earlier versions of Mark-II and -III. Although the manufacturers preset the necessary parameters, it is quite important to use optimal hydrogen and air flow rates, since they can vary depending upon the nature of the sample and the sample load. An air flow rate of 2 L/m, hydrogen pressure of 0.7 kg/cm^2 and scanning speeds of 40 s/rod have been used in the Model TH-10 analyzer to study the response and linearity of different lipid compounds.[10] However, very little information is available on the sensitivity, linearity, and reproducibility of lipid classes as determined by the Iatroscan Mark-5 system,[7] and there are no data published on the latest version, the Mk-6.

Although HPLC has been commonly used for the quantification of lipid classes, TLC and high-performance thin-layer chromatography (HPTLC) also have been used for the quantification of lipid classes.[11] Normal phase HPLC (NP-HPLC) was the most commonly used, with various detectors. In the identification and quantification of different lipid classes, including wax esters, cholesterol esters, free fatty acids, cholesterol, 1,2- and 1,3-diacylglycerols, monoacylglycerols, and triacylglycerols, NP-HPLC has been used with gradient elution with isooctane/0.1% acetic acid in tert-butylmethyl ether and an evaporative light scattering detector (ELSD).[12] This method has been calibrated with 5–150 μg/mL range of lipid standards. The variance, within-day, for the entire representative range varied from 1.9–5.1%, whereas the inter-day variances were from 7.0–26.5%. The detector response depends upon the chain length and the degree of saturation and, therefore, the quantification of an unknown compound is only semi-quantitative.[12] The intra- and inter-day precision values were reduced to 1.9–4.5 and 2.3–7.2%, respectively, by speeding up the gradient

steps and by increasing the re-equilibration time between runs.[13] HPLC, coupled to ELSD, also has been used to quantify major lipid classes in human triacylglycerol-rich lipoproteins. ELSD response was fitted to second order equations with correlation coefficients of over 0.999 for 0.25–10 μg injections.[14,15] The performance and capacity of alumina as stationary phase in an HPLC-ELSD method also has been optimized for the determination of neutral lipid classes.[16] Several other HPLC methods, combined with ELSD[17–20] and mass (light scattering) detectors,[20] UV,[19,22] and fluorescence detectors[19] have also been used for the quantitative analysis of lipid classes. Most interestingly, FID also has been used to quantify both neutral and polar lipid classes after separation by HPLC.[23,24] HPLC and TLC-FID have been used to analyze lipid classes in the production of sucrose esters; quantitative results obtained by HPLC were comparable to those obtained by TLC-FID.[25] However, there has not been a reported direct comparison of these two methods. Also, there has not been any analytical work pertaining to lipid analysis optimization on the Iatroscan model Mk-6s.

This work has been carried out, basically, to optimize hydrogen and air flow rates at a constant scanning rate for low load levels of neutral lipids for quantification through the calibration of the Chromarods system of neutral lipids in fish oil samples during transesterification, as well as to compare the quantitative TLC-FID results with LC-ELSD data.

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EXPERIMENTAL

TLC-FID

Hydrogen and Air Flow Rates

Chromarods S-III were pre-scanned twice at a hydrogen flow rate of 170 mL/min and air flow rate of 2 L/min, at a constant scan speed of 30 s/rod, using an Iatroscan Mk-6s TLC-FID system prior to the application of the samples. One microliter of the standard lipid class mixture (~2 mg/mL) of commercial standards of neutral lipids (ethyl arachidate, tripalmitin, palmitic acid, cholesterol, 1–3 dipalmitin, and monopalmitin) was applied onto Chromarods and developed in hexane/diethyl ether/formic acid (90:10:0.02) for 55 min., followed by drying at 60°C for 30 s. The rods were re-developed in hexane/diethyl ether/formic acid (65:35:0.02) for 3–5 min and dried at 60°C for 2 min. Chromarods were scanned at hydrogen flow rates of 110–170 mL/min, airflow rates of 1–3 L/min, and a scan speed of 30 s/rod.

Calibration of Chromarods

Chromarods were pre-scanned at a hydrogen flow rate of 170 mL/min and air flow rate of 2 L/min at a constant scan speed of 30 s/rod prior to the

application of the standard mixture of lipids. A mixture of commercial standards of neutral lipids, including ethyl arachidate (EE), tripalmitin (triacylglycerol or TG), palmitic acid (free fatty acids or FFA), 1,3-dipalmitin (diacylglycerol or DG), and monopalmitin (monoacylglycerol or MG) were used in different concentrations that varied from 1 to 15 mg/mL Disposable micro pipettes (1–5 μL, Drummond Microcap; Drummond, Broomall, PA.) were used for spotting samples onto rods. After focusing with acetone, rods were developed in hexane/diethyl ether/formic acid (90:10:0.02) for 55 min and dried 60°C for 30 s. Rods were then re-developed in hexane/diethyl ether/formic acid (65:35:0.02) for 3–5 min, followed by drying at 60°C for 2 min. Chromarods were scanned at a hydrogen flow rate of 160 mL/min, air flow rate of 2 L/min, and a scan speed of 30 s/rod. FID area response of each lipid standard was plotted against the sample load and the best fitted model was chosen through multi-level calibration.

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Qualitative and Quantitative Analysis of Fish Oils

Fish oil samples were taken from different steps in the process of trans-esterification of EE to TG. Oil samples were dissolved in chloroform (~5 mg/mL), 1 μL of each was spotted onto Chromarods, developed as above, and scanned under the same conditions as for the calibration standards.

LC-ELSD

Operating Conditions

Column: Waters Spherisorb S3CN 2 mm x 150 mm; Mobile phase composition: 98% Hexane/2%MTBE (v/v); Flow rate: 0.4 mL/min; Mobile phase gradient:

Time (min)	Hexane (%)	MTBE (%)
0	98	2
7	80	20
10	0	100
15	0	100
15.05	98	2
25	98	2

ELSD 2000 conditions: 30°C; 1.0 SLPM; Impactor OFF; Gain = 1.

All samples were quantified using calibration equations of appropriate standards.

RESULTS

TLC-FID

Hydrogen and Air Flow Rates

The effect of hydrogen and air flow rates on the FID response of EE, TG, FFA, DG, and MG is shown in Figs. 1–5. Each number is an average of 10 analyses (n = 10 for each data point). The FID response of all lipid classes slowly and gradually increased with increasing the air and hydrogen flow rates, while the scan rate was held constant at 30 s/rod. The FID response either leveled off or slightly increased above a hydrogen flow rate of 150 mL/min and air flow rate of 2 L/min.

Calibration of Chromarods

Calibration curves for EE, TG, FFA, DG, and MG are shown in Figs. 6A–E (each data point is an average of 10 analyses). The FID response for all compounds was linear vs. the sample load.

Qualitative and Quantitative Analysis of Fish Oils

Ten different fish oil samples, randomly taken during chemical transesterification of EE, were quantified by both TLC-FID and LC-ELSD. A typical chromatogram of TLC-FID is shown in Fig. 7; Figure 8 shows a typical chromatogram by LC-ELSD. Figures 9 and 10 show the relationship between TLC-FID data and LC-ELSD data obtained from the quantitative analysis

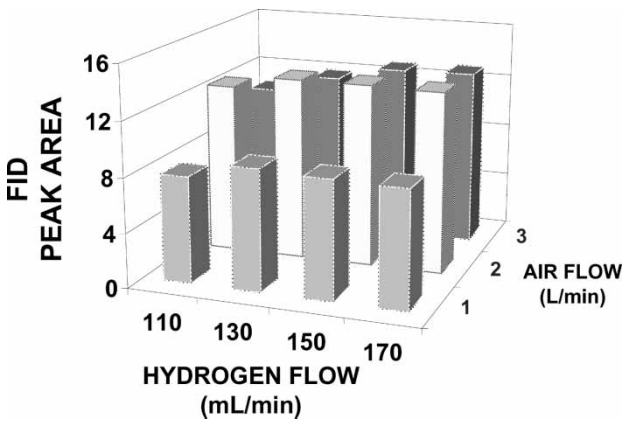

Figure 1. Effect of hydrogen and air flow rates on the FID response of EE (EE = ethyl esters).

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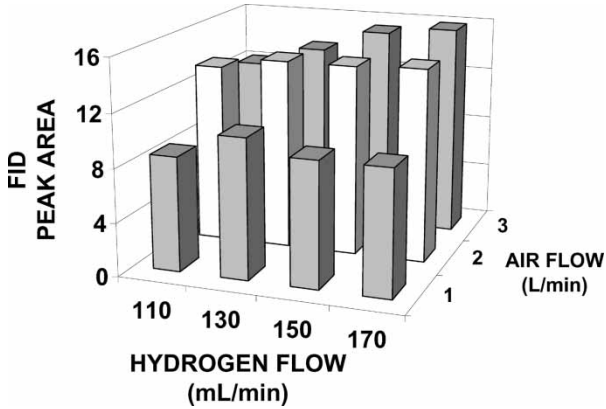

Figure 2. Effect of hydrogen and air flow rates on the FID response of TG (TG = triacylglycerol).

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of lipids in fish oil by these two methods. Variation of major lipid classes with the amount of glycerol during enzymatic tranesterification was also monitored by both methods and seemed to have a close agreement (Fig. 11).

DISCUSSION

An increase in the hydrogen and air flow rates usually leads to an increase in the detector flame temperature, with a subsequent increase in the FID response and reproducibility. The FID response of all compounds representing lipid classes consistently increased with simultaneous increase in the hydrogen

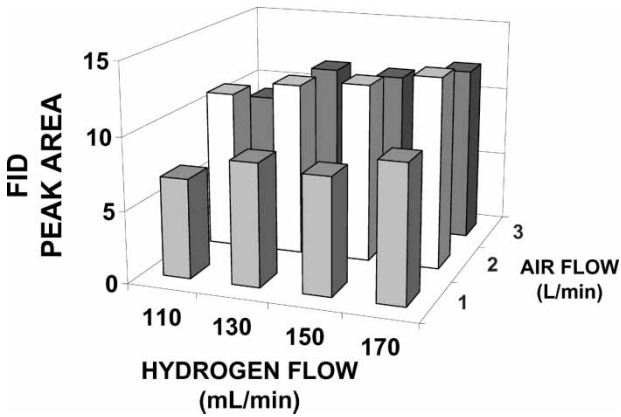

Figure 3. Effect of hydrogen and air flow rates on the FID response of FFA (FFA = free fatty acids).

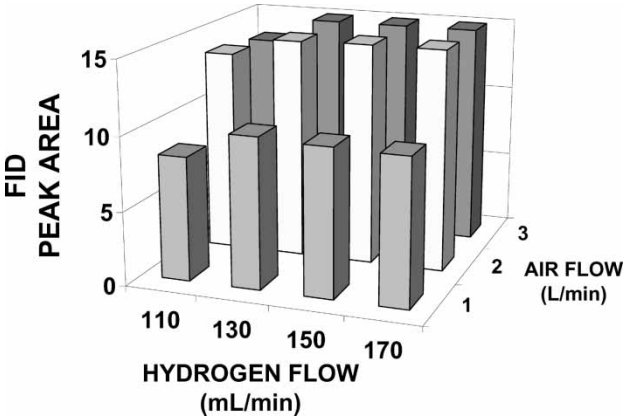

Figure 4. Effect of hydrogen and air flow rates on the FID response of DG (DG = diacylglycerol).

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flow rate at and above an air flow rate of 2 L/min. At an air flow rate of 1 L/min, the FID response was significantly lower than that at 2 and 3 L/min air flow rate and not very consistent with increasing H_2 flow. This poor response is due to a low level of ions resulting from the incomplete pyrolysis of compounds in the flame. Although these H_2/air flow rates may be sufficient to pyrolyse, significantly low sample loads ($\leq$1 mg/mL), as noticed in this study, in the routine sample screening by TLC-FID, higher flow rates may be used since higher sample loads are used. However, the upper limit of the H_2 flow rate also depends upon the scanning rate. However, high air flow rates (>2 L/min) and hydrogen flow rates (>170 mL/min) may not be advisable for routine analysis, due to the fact

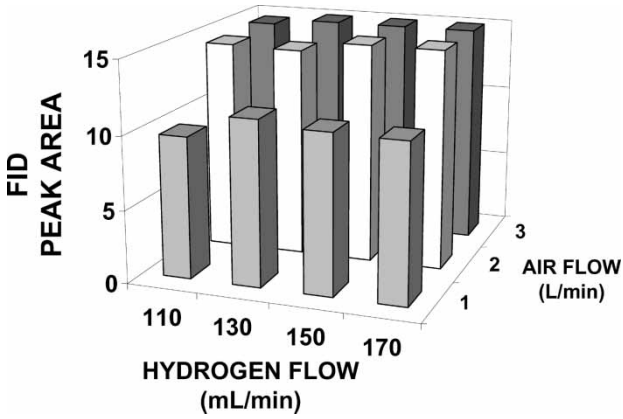

Figure 5. Effect of hydrogen and air flow rates on the FID response of MG (MG = monacylglycerol).

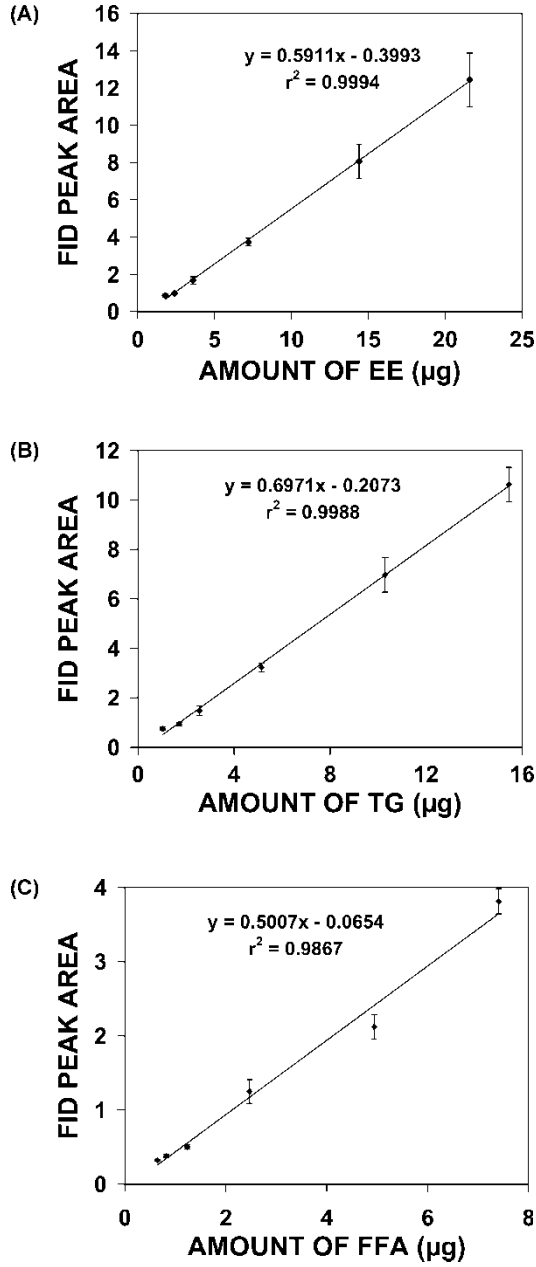

Figure 6. A) Calibration curve for EE (EE = ethyl esters); B) Calibration curve for TG (TG = triacylglycerol); C) Calibration curve for FFA (FFA = free fatty acids); D) Calibration curve for DG (DG = diacylglycerol); E) Calibration curve for MG (MG = monoacylglycerol).

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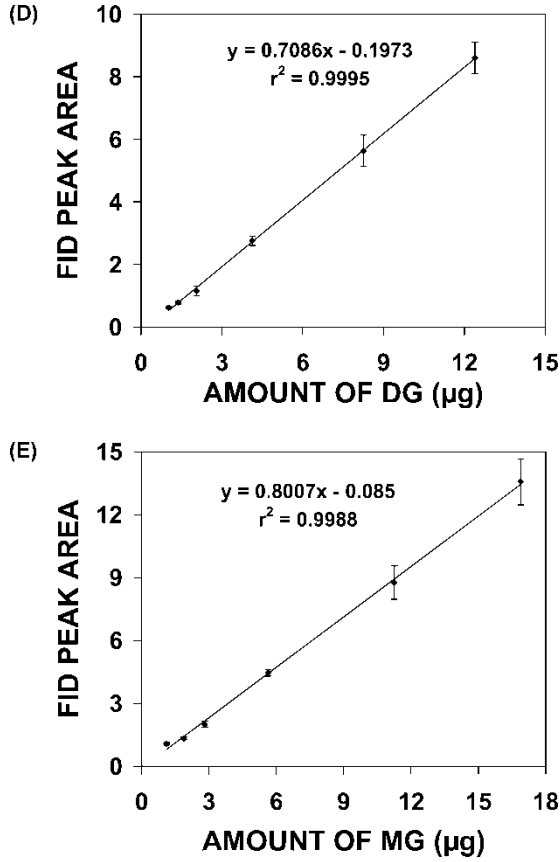

Figure 6. Continued.

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that the continuous heating of rods at high temperature may damage the thin layer, subsequently reducing the useful lifetime of the rods. Complete pyrolysis of the sample loads of 5–10 mg/mL at hydrogen flow rates of 130–150 mL/min and air flow rate of 2 L/min was noticed with no residuals found during subsequent scanning at a hydrogen flow rate of 170 mL/min and an air flow rate of 2 L/mim. These conditions may change considerably as a function of the chemical nature and chain length of the samples. The selected hydrogen and air flow rates in the present study are comparable to the conditions used in previous Iatroscan models in the quantification of lipid classes in various samples.[2]

Scanning time has a considerable effect on the FID response as well as the lifetime of the chromarods.[2] Moderate scan speed was used in the present study with other conditions optimized for hydrogen and air flow rates. Although the FID response increases with decreasing scan rate with a simultaneous increase in H_2 and air flow rates, partial sintering of glass

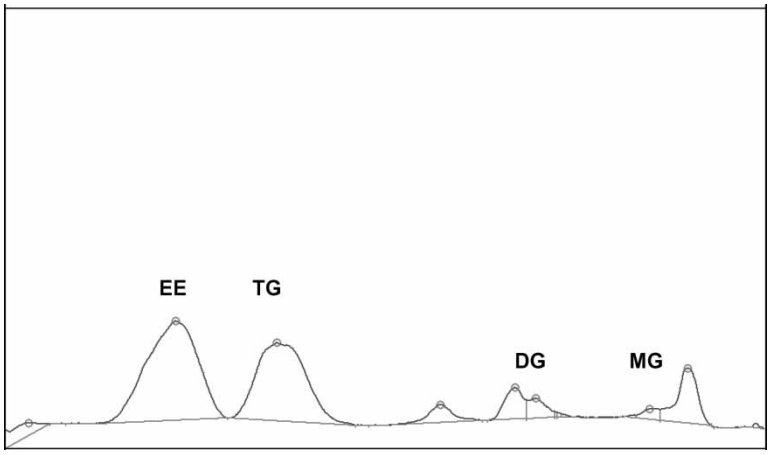

Figure 7. Typical TLC-FID chromatogram of a transesterified fish oil sample.

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particles in the frit could result in significantly lowering the useful lifetime of the rod. Therefore, optimum H_2 and air flow rates of 150–170 mL/min and 2 L/min, respectively, are recommended at a scanning rate of 30 s/rod for low sample loads. Hydrogen flow rate, even as small as 130 mL/min, could be used at an air flow rate of 2 L/min with complete combustion in the flame.

The FID response varies considerably, depending upon the type of lipid class, due to the variability in the formation of FID sensitive ions in the flame by different compounds. In quantitative analysis, this variability is minimized by using the corresponding standards that have almost the same

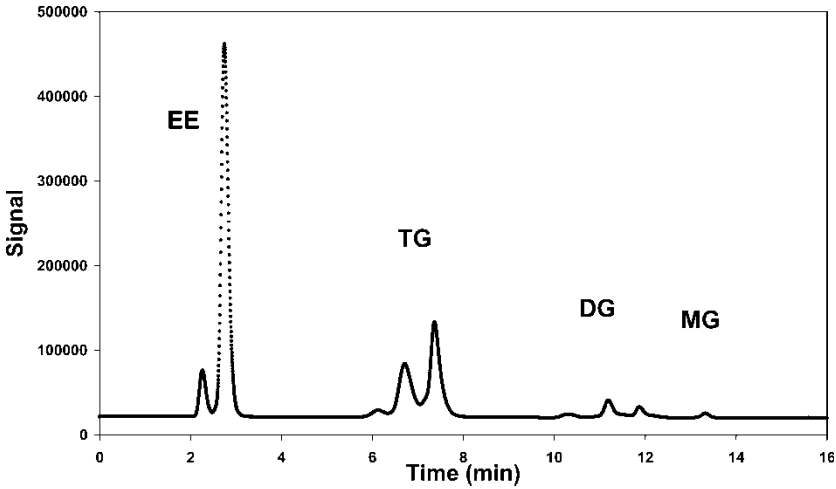

Figure 8. LC-ELSD chromatogram of transesterified fish oil.

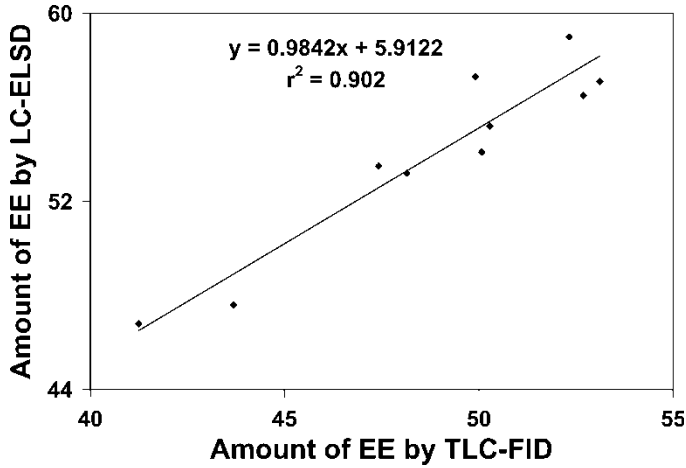

Figure 9. Relationship between TLC-FID and LC-ELSD data for EE (EE = ethyl esters).

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response. The structure-based variations could be seen even in the samples analyzed by LC-ELSD.

Although thin layers of the Chromarods are quite homogeneous, there were slight variations in the FID responses among rods for the same amount of any given lipid class (inter-rod variability). In addition, the FID response given by the same rod in repeated analyses for the same amount of any lipid class, analyzed under identical conditions, also had a

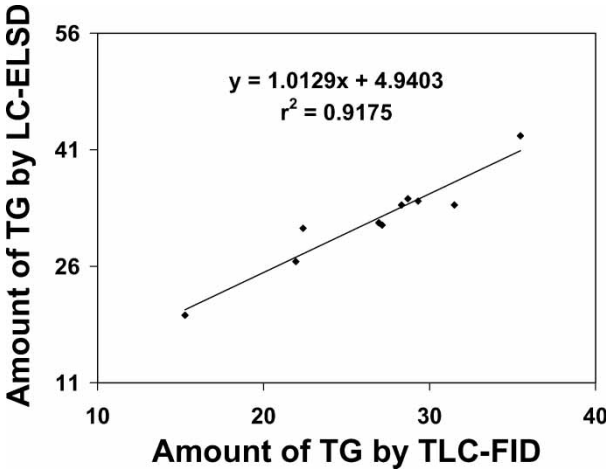

Figure 10. Relationship between TLC-FID and LC-ELSD data for TG (TG = triacylglycerol).

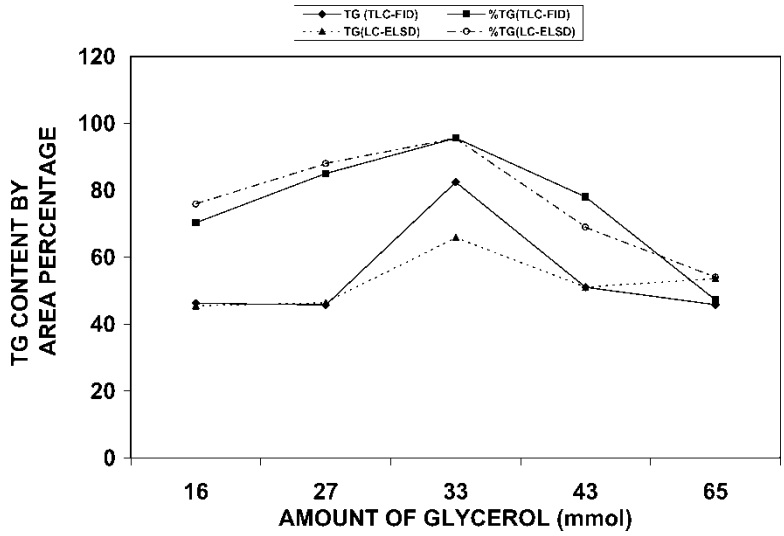

Figure 11. Change of the production of TG and percentage TG in total glycerides during the enzymatic transesterification of EE with varied amounts of glycerol (mmol glycerol/150 mmol fish oil EE) (EE = ethyl esters, TG = triacylglycerol).

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slight variation (intra-rod variability). These variabilities could be minimized by using all 10 rods as one analytical unit with repeated sample and standard analyses. However, the coefficients of variation in the lipid samples analyzed by TLC-FID were higher than those analyzed by LC-ELSD.

The chemical nature of the sample considerably affects the peak shape and area. This feature was more pronounced in the fish oil samples with highly unsaturated fatty acids analyzed by TLC-FID than by the LC-ELSD. TLC-FID gave shorter and broader peaks (Fig. 7), whereas LC-ELSD gave nice, sharp peaks with higher resolution, although all commercial standards gave similar sharp peaks for TLC-FID.

Calibration of Chromarods in the Iatroscan TLC-FID system has been a tedious process for quantification of a wide range of lipid classes with complicated derivatization of calibration equations, especially in the previous models of Iatroscan systems, due to the non-linear relationship between the FID response and the sample load.[5,26,27] Power law models ($y = ax^b$) have been the best choice and, in some cases, even squared and/or cubic terms were added to the calibration models for quantification. In previous Iatroscan models, including Mark-III and -IV, the detector response has been shown to depend on the nature of the component and to be non-linear with the sample load. Therefore, quantification of lipid mixtures using these models requires correction factors that take into account the nature of the component as well as the sample load. In the present study, all compounds

had linear FID response against the sample load and the fitted models were even slightly better than conventional Power law models. Therefore, linear calibration equations were used for the quantification of neutral lipid classes by the Iatroscan Mk-6s model. Lipid classes analyzed by Iatroscan Mark-5 also seem to have a linear response.[7]

There was close agreement between the amounts of lipid classes in randomly chosen fish oil samples during chemical transesterification determined by TLC-FID and LC-ELSD, although the coefficients of variation of TLC-FID data were higher than those of LC-ELSD. The percentage amount of ethyl esters obtained by LC-ELSD was highly significantly correlated ($p \leq 0.01$) with the amounts calculated by the TLC-FID method using linear calibration equations of appropriate external standards (Fig. 9). There was also a significant correlation ($p \leq 0.01$) between the percentage amounts of TG obtained by the LC-ELSD method and the TLC-FID method (Fig. 10). Similar significant relationships were found for DG, MG, and FFA obtained from both methods. These results clearly indicate that the TLC-FID method also could be reliably used for the quantitative analysis of lipid classes, although the calibration methods are more tedious than with the LC-ELSD method.

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The effect of the amount of glycerol on the lipid class composition during enzymatic transesterification was monitored by TLC-FID using only area percentages of the lipid classes instead of using calibration equations. The same samples were quantified by LC-ELSD. The variation in the area percentage of total TG transesterified from EE, as well as the percentage of TG in total glycerides in the reaction mixture are shown in Fig. 11. It is clear that the pattern of the distribution of these numbers is comparable to the quantitative results obtained from the LC-ELSD method, indicating that the main neutral lipid classes in some reactions can be monitored closely with the TLC-FID method using area percentages. However, discrepancies noticed at some points are mainly due the variation of response factors that were not taken into consideration by TLC-FID, since calibration equations were not used. Therefore, it is more accurate to use quantitative results derived from calibration equations of TLC-FID for a better understanding of the reaction kinetics, rather than using area percentage alone, although the data distribution patterns look similar.

CONCLUSIONS

H_2 flow rate of 150–170 mL/min and air- flow rate of 2 L/min, at a scan rate of 30 s/rod are recommended for low loads of neutral lipids. Linear calibration of TLC-FID equations can be successfully used for quantification. The coefficient of variation of the TLC-FID data are much higher than that of LC-ELSD. However, quantitative numbers obtained by both methods significantly correlated, and the relatively inexpensive TLC-FID method can be used

to monitor the variation of lipid classes during some chemical processes, especially where a large number of samples should be screened in a relatively short period of time.

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