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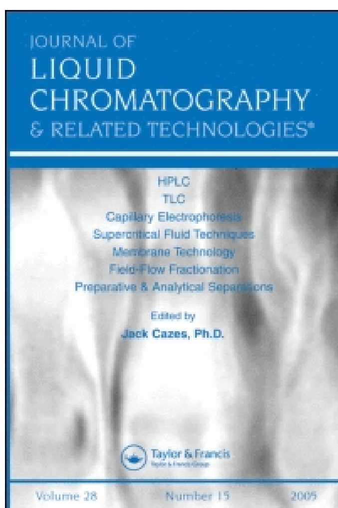

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Comparative Study of the Separation and Quantification of Lipid Classes Separated on Chromarods-A and Chromarods-SIII by Thin Layer Chromatography with Iatroscan (Mark-III) Flame Ionization Detection (TLC-FID)

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Comparative Study of the Separation and Quantification of Lipid Classes Separated on Chromarods-A and Chromarods-SIII by Thin Layer Chromatography with Iatroscan (Mark-III) Flame Ionization Detection (TLC-FID)

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Abstract: Thin layer chromatography (TLC) on Chromarods-A and −SIII were used for the separation of common lipid classes which were detected and quantified using Iatrosan Mark-III with Flame Ionization Detection (FID). Two different lots from each type of Chromarods were used and half of each lot (5 rods) was considered as 1 analytical unit. Variability of the FID response for 11 different lipid classes (neutral and polar) among rods of each lot, among different analytical units as well as the variability between 2 different lots of the same type of Chromarods were compared statistically. Variability of the response for these lipid classes by an individual rod during repeated analysis was also investigated. Calibration curves of the FID response vs. sample loads were used to develop corresponding calibration models that can be used for quantification of lipid classes separated on these Chromarods.

There was no significant difference ($p > 0.05$) in the FID response for all lipid classes separated on Chromarods-A or -SIII. However, the FID responses for all lipid classes separated on Chromarods-A were significantly different from those on Chromarods-SIII ($p < 0.01$). There was no significant difference in the FID response among analytical units of the same type of Chromarods. Curvilinear relationship of

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the FID response against the sample load was common to all lipid classes for both Chromarods-A and –SIII. Several calibration equations were derived and Power Law models ($y = ax^b$) appear to be the most convenient and appropriate for rapid quantitative analysis of lipid classes although an addition of a quadratic and/or cubic term further improved the statistical validity of the model.

Keywords: Lipid classes, Chromarods, TLC-FID, Quantification, Iatroscan-Mark-III

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INTRODUCTION

TLC-FID has been used most extensively for the analysis of simple and complex lipids of fats and oils as well as lipids in various biological systems, food products and pharmaceutical products. Numerous research studies have been carried out in these areas since the Iatroscan technology was introduced in the early 1980's. There has been a slow but steady improvement in this technology with the latest model being the Iatroscan-Mark 6. However, early models such as Mark-III and –IV are still being used successfully in many laboratories around the world. Silica-based Chromarods have been used predominantly whereas alumina-based Chromarods have been rarely used.

When comparing the TLC-FID system with other methods for the quantitative analysis of liver lipid contents in alcohol-fed rats and controls, it has been found that the TLC-FID system was a convenient method for rapid analysis of the extent of fatty liver in alcohol-fed animals.[1] Comparing the Iatroscan method for phospholipid analysis with phosphorus and gas chromatographic methods, it was found that the results of the Iatroscan method for some lipid classes were comparable to that of phosphorus or gas chromatographic techniques while for other lipid classes it gave lower values.[2]

The silica gel Chromarod Iatroscan-TH-10 TLC-FID analyzer system has been used for identifying and quantifying non-polar lipids in a variety of marine samples;[3] it was found that quantitative accuracy and reproducibility were still a major weakness in the TLC-FID system. The response of most lipids is significantly different from that which would be calculated from their ionizable carbon content, partly due to losses during volatilization before ionization and other factors.[4–6] Lipid quantification by TLC-FID can be complicated by a number of factors, including degrees of variability and variation in the amount of ionizable carbon produced during the pyrolysis of different lipid classes.[7] It has also been found that the use of an internal standard is a good method for quantification.[8] However, using a large number of samples, neutral and phospholipids of the eggs and larvae of marine fish were quantified without any internal standard and it was found suitable for small marine vertebrates and invertebrates with low to medium levels of neutral lipids consisting largely of triacylglycerols.[9] Regression lines of log rhythmic data can be used in quantifying the lipid classes in human bile.[10] In calibrating $AgNO_3$ impregnated rods for

methyl esters of fatty acid geometric isomers, and of triacylglycerols differing in unsaturation, it has been found that the detector responses of Iatroscan TLC-FID for triacylglycerols were curvilinear.[11] Two regression lines could be fitted, one for the 2–5 μg load range and another for the 5–15 μg range, and addition of quadratic and/or cubic terms improved the model. Significantly different lot-to-lot and rod-to-rod variation was recorded for most of the neutral lipid classes in the quantitative determination of neutral lipids on Chromarods-SII.[8]

Thus it appears that most of the neutral lipid classes have a curvilinear relationship between FID responses and the amount spotted over the range of 0.2 to 5.0 μg and by adding a quadratic term to the linear regression model, an improved model for quantification of lipid classes with Chromarods-III may be obtained. Non-linearity of calibration curves was quite common in earlier Iatroscan models (Mark-III, early Mark-IV) and more linearity was obtained from newer models such as Mark-5 and -6.[12–15] It is clear from the literature that quantitative analyses have been mainly performed using Silica rods (SII, SIII). However, since alumina rods also perform equally well in the separation of neutral lipids,[16,17] they may be also a choice for quantitative analysis of lipids.

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EXPERIMENTAL

Materials and Methods

Authentic standard solutions of lipid classes such as wax esters, hydrocarbons, ketone, free fatty acids, triacylglycerol, cholesterol, diacylglycerol, monoacylglycerol, phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine were used (Table 1) in this study to investigate the separation abilities of these lipids on both silica coated Chromarods-SIII and alumina coated Chromarods-A as well as for their quantification. The abbreviation of each lipid class is also given in Table 1.

All FID scans were performed on an Iatroscan Mark-III (TH 10) analyzer (Iatron laboratories, Tokyo) connected to a Spectra-Physics SP 4200 computing integrator *via* the analogue output. The Iatroscan was fitted with a push button switch to interrupt scanning anytime when required; this was especially useful during partial scanning. The Iatroscan was operated with a hydrogen flow rate of 160 mL min^{-1}, and an air flow rate of 2000 mL min^{-1}. Scan speed was set to 2 and the attenuation was 8–16.

Experimental Procedure

Lipid standards were dissolved either singly in chloroform or in some cases, especially when dissolving phospholipid standards, in a chloroform solution

Table 1. Standard lipid classes used for the separation and quantification in the Iatroscan

Lipid class	Abbreviation	Standards and suppliers
Aliphatic hydrocarbon	HC	n-nonadecane (Polyscience Laboratory)
Wax esters	WE	Palmitic acid-palmityl alcohol
Ketone	KET	2-nonadecanone (Polyscience Laboratory)
Triacylglyerol	TG	Tripalmitin (Sigma)
Free fatty acids	FFA	Palmitic acid (NU-CHEK-PREP INC.)
Free sterol	CHO	Cholesterol (NU-CHEK-PREP INC.)
Diacylglycerol	DG	1,2-dipalmitin (Serdary Research Laboratory)
Monoacylglycerol	MG	Monopalmitin (Serdary Research Laboratory)
Phosphatidylcholine	PC	L-3 phosphatidylcholine dipalmitoyl (Serdary Research Laboratory)
Phosphatidylethanolamine	PE	L-3 phosphatidylethanolamine dipalmitoyl (Serdary Research Laboratory)
Lysohosphatidylcholine	LPC	Lysophosphatidylcholine palmitoyl (Serdary Research Laboratory)

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with a minute quantity of methanol (~0.1 mL methanol/1 mL chloroform). Both Chromarods-SIII and Chromarods-A were pre-scanned twice before spotting the lipid samples, and the baseline was checked on the chromatogram to ensure the purity of the Chromarods. Standard lipid classes used for the separation and calibration are shown in Table 1. Standard solutions were spotted onto chromarods using a Hamilton syringe to cover a range from 0 to 30 μg. When free fatty acids and triacylglycerols in the mixture were not clearly separated, they were spotted separately. After the application of lipid standards, the Chromarods were focused twice to the origin in acetone and were deactivated for 10 min over a saturated solution of NaCl and then equilibrated with the vapour of the first developing solvent system for 5 min prior to development (Figures 1 and 2).

The lipid standards were separated in a stepwise sequence using the micro switch connected to the partial scanning facility of the Iatroscan.

Solvent Systems and Development

Different lipid classes were separated by developing the rods in different solvent systems. In the first development, lipid standards on both Chromarods-SIII and

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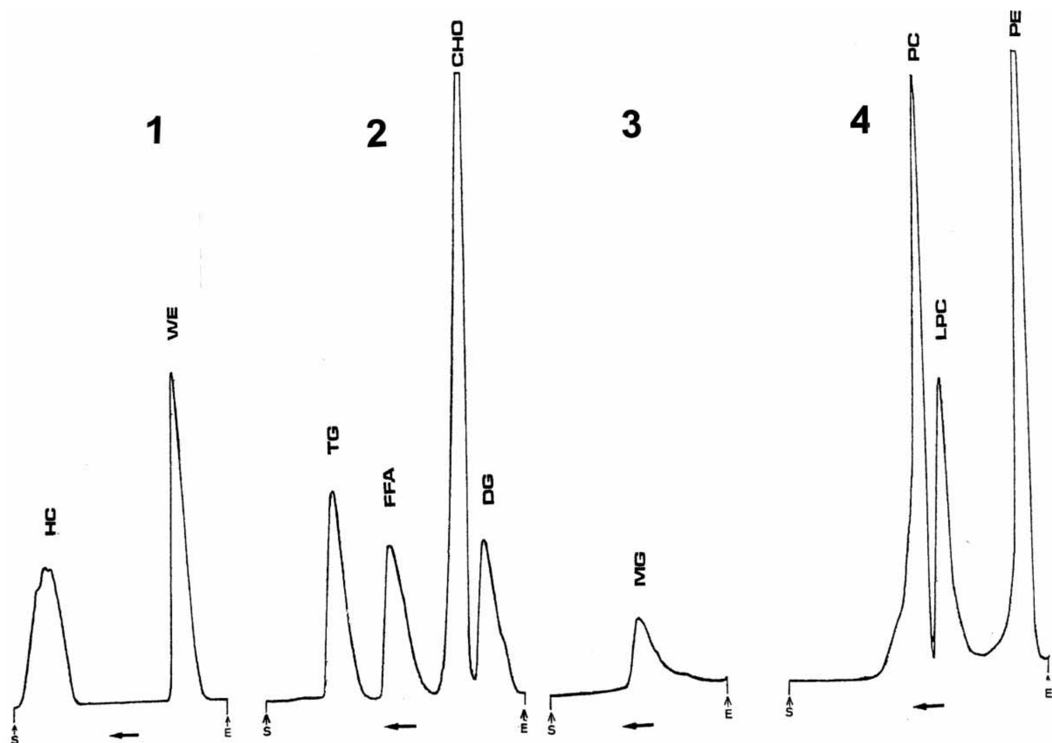

Figure 1. Lipid classes separated on Chromarods-A at different development stages (S = start, E = end, 1 = 1st development in hexane-diethyl ether-formic acid, 98:2:0.1, 2 = partial scan after 2nd development in hexane-diethyl ether-formic acid, 85:15:1, 3 = partial scan after 3rd development in 100% acetone, 4 = full scan after 4th development in chloroform-methanol-water, 60:40:10).

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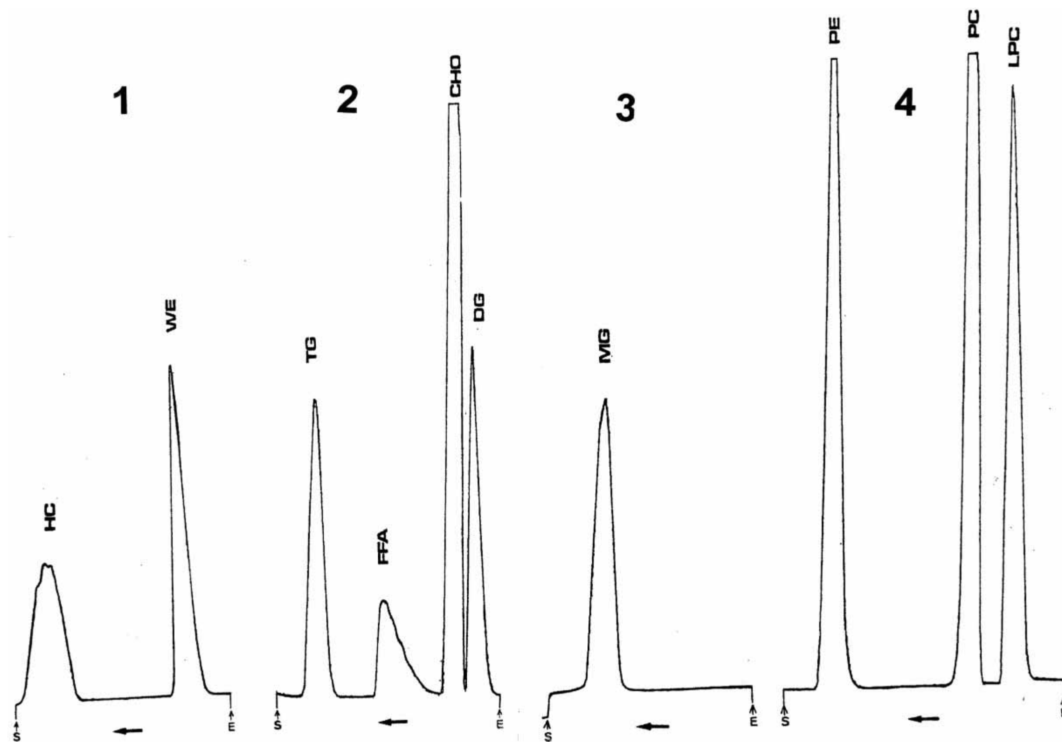

Figure 2. Lipid classes separated on Chromarods-SIII at different development stages (S = start, E = end, 1 = 1st development in hexane-diethyl ether-formic acid, 98:2:0.1, 2 = partial scan after 2nd development in hexane-chloroform-isopropanol-formic acid, 85:14.25:0.75, 3 = partial scan after 3rd development in 100% acetone, 4 = full scan after 4th development in chloroform-methanol-water, 70:30:3).

Chromarods-A were developed in a mixture of hexane-diethyl ether-formic acid (98:2:0.1) for 40 min to separate HC, WE and KET. Then the rods were air-dried for 10 min and were partially scanned to just beyond the ketone peak. Chromarods S-III were then developed in hexane-chloroform-formic acid-isopropanol (85:14.25:0.1:0.75) for 40 min whereas Chromarods-A were developed in hexane-diethyl ether-formic acid (80:20:1) for 45 min. Both types of rods were dried at room temperature for 10 min and scanned partially to the lowest point behind the DG peak. Then, Chromarods-SIII were developed in acetone (100%) for 20 min and Chromarods-A were developed twice for 30 min in acetone. After the partial scanning of both types of rods behind the MG peak, the final development was performed in chloroform-methanol-water (70:30:3) for 55 min whereas alumina rods were developed in chloroform-methanol-water (60:40:10) for 50 min.

Each development on both types of rods was repeated 3 times for 0.01, 0.1, 0.4, 1.0, and 5.0 μg concentrations of all standards in order to study the intrarod variability. Ketone was used as an internal standard. All 10 rods in one set were considered as a "lot" and 5 rods in each lot were considered to be an "analytical unit" for the calibration.

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Calibration

Area responses of FID for different concentrations of each lipid class were plotted against the concentration. The best fitted model for each plot of each analytical unit was obtained through transformations of the data, and the best fitted line was obtained by comparing the values of R^2, the F-ratio, the p-value and the characteristics of the residual plot of each data set.

Statistical Analysis

Intrarod variation was examined using the coefficient of variation for each lipid class. The interrod variability in each lot was measured by comparing the mean FID response of each rod by analysis of variance (ANOVA) *via* multiple regression using indicator variables considering repeated measurements of each rod as a predictor variable. The variation of the FID response from one lot to another was determined by ANOVA *via* multiple regression using indicator variables by comparing the mean peak areas of all 10 rods with repeated measurements. The FID response of all 10 rods in each lot was considered as a predictor variable. Unit-to-unit variation was also measured in the same manner, but repeated measurements of FID responses of 5 rods were considered as one predictor for the multiple regression. The slopes of calibration curves were compared by analysis of covariance (ANCOVAR) *via* multiple regression using indicator variables. All analyses were performed with the MiniTab 71 statistical software package on the Dalhousie University (TUNS) computer net service.

RESULTS AND DISCUSSION

Calibration

The calibration curves were prepared considering 5 rods in a lot as a "unit". The concentrations of each standard ranged from 0.01 to 10 μg except that for TG and FFA, 15, 20 and 30 μg were also used for Chromarods-SIII. Since the separation of the mixture on alumina rods was poor with increasing concentration, the maximum amount used for alumina rods was only 7 μg of each component in the mixture.

Calibration curves as second order regression lines for each analytical unit are shown in Figs. 3–10. There was a curvilinear relationship between the FID response of each lipid class and the amount of lipid spotted. Thus, all data were transformed where required to obtain the best-fitted model (some selected models are shown in Tables 2 and 3). The FID responses for many neutral lipid classes were found to have curvilinear relationships with the amount spotted and therefore, linear regression could not be used for quantifying samples with class loads below 2.5 μg since the fitted lines passed

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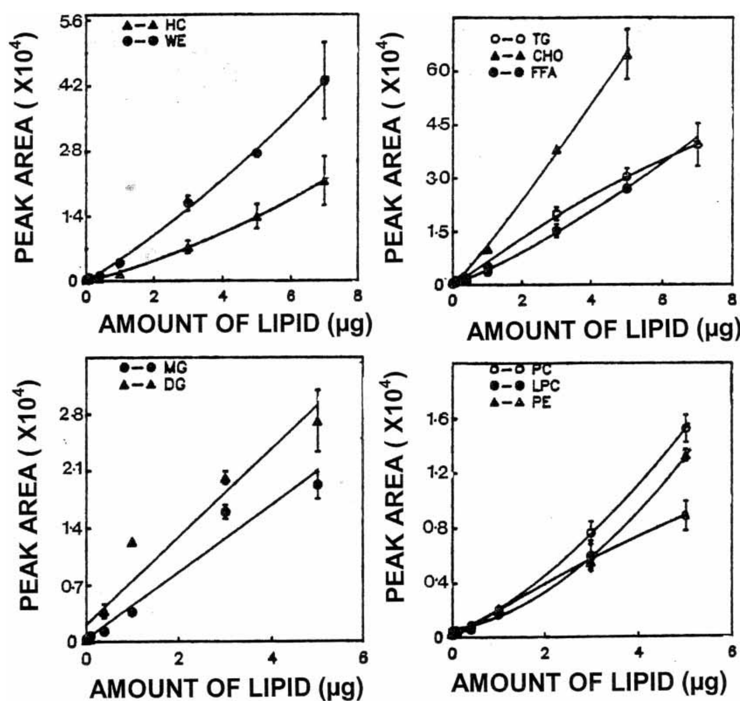

Figure 3. Calibration curves for lipid classes separated on Chromarods-A (Lot-1, Unit-1).

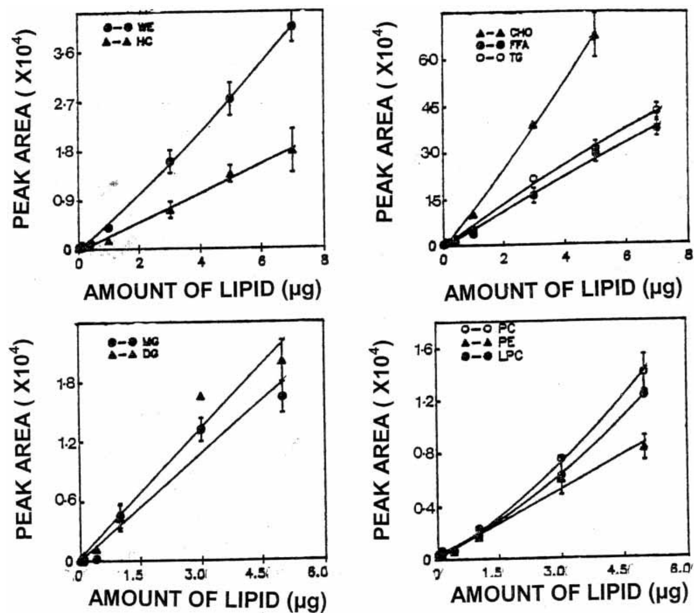

Figure 4. Calibration curves for lipid classes separated on Chromarods-A (Lot-1, Unit-2).

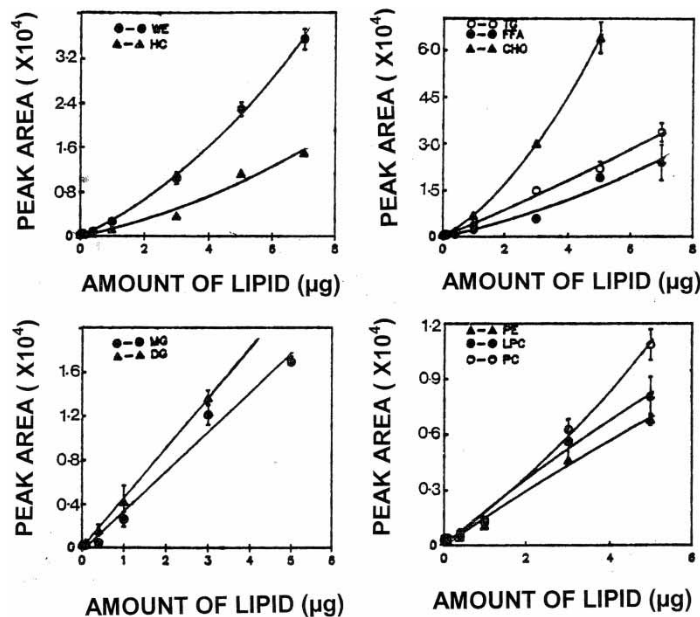

Figure 5. Calibration curves for lipid classes separated on Chromarods-A (Lot-2, Unit-1).

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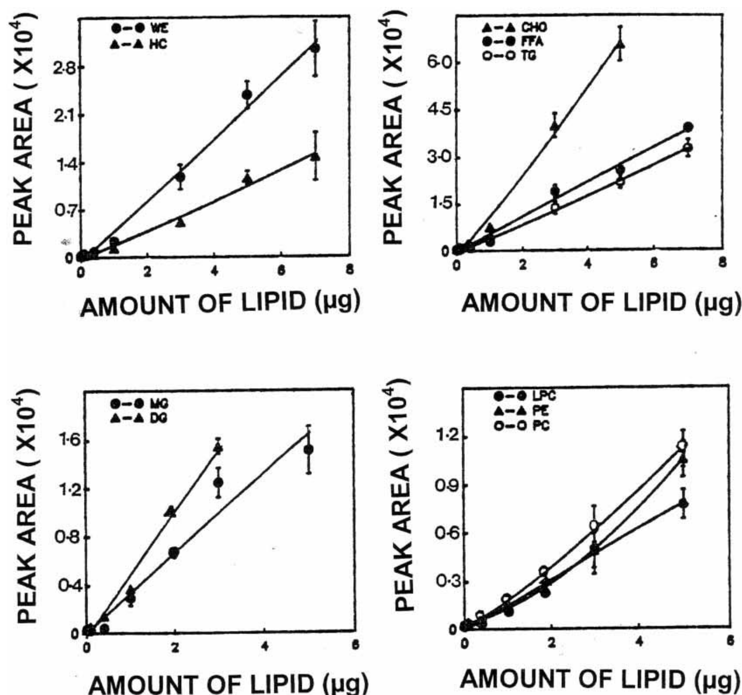

Figure 6. Calibration curves for lipid classes separated on Chromarods-A (Lot-2, Unit-2).

above the majority of the data points near the center of the range and below the majority at both ends etc.[6] Thus, quadratic regressions were found to be better for calibration.[18,19] By taking the logarithms of both sides of the power law equation ($y = ax^b$) the equation for a straight line is obtained {$\log y = \log a + b (\log x)$}. For a concentration gradient from 0.2 μg to 5.0 μg, this line could be fitted with a coefficient of determination of 97–99% for neutral lipids and an addition of a quadratic term improved the R^2 of the fitted line.[19]

In the present study, all calibration curves had curvilinear patterns and the power law model for most of the lipid classes could be fitted with a coefficient of determination of 80–90% for a concentration gradient from 0.01 to 10 μg. However, the addition of a quadratic term to the untransformed linear regression line improved the R^2 value (0.95–1.00) and F-ratio of most of the FID responses. It has also been found that the calibration curves in the range from 0.2 to 5.0 μg were distinctly curvilinear and were better described by quadratic equations when calibration methods of the Iatroscan-Chromarods system were used for marine lipid class analyses.[19] When the logarithms of dependant variables were plotted against the logarithms of

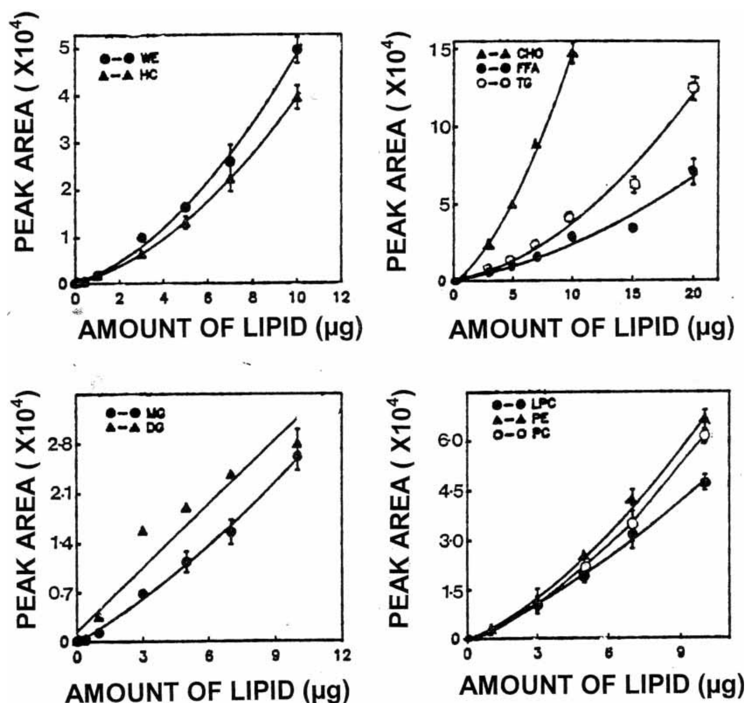

Figure 7. Calibration curves for lipid classes separated on Chromarods-SIII (Lot-3, Unit-1).

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independent variables, a combination of 2 straight lines could be clearly seen for logarithmic transformed data, one line from 0 to 1 μg with a shallow slope and the other with steeper slope passing through the 2 μg point of the independent variable.

In some cases, the addition of a cubic term further improved the distribution of residuals whereas in some situations, addition of quadratic and cubic terms to the linear regression of data with a square root transformation of the dependant variable improved not only the R^2 value (in some cases $R^2 = 1.00$), F-ratio and p-value but also the residual plot in which the residuals were randomly distributed around the zero line. However, for most applications a quadratic regression of untransformed data should be sufficient for most calibrations using Chromarods-A and -SIII.

Intra-rod Variability

Intra-rod precision of Chromarods-SIII for some neutral lipids has been studied previously[19] and it was found that the coefficient of variation

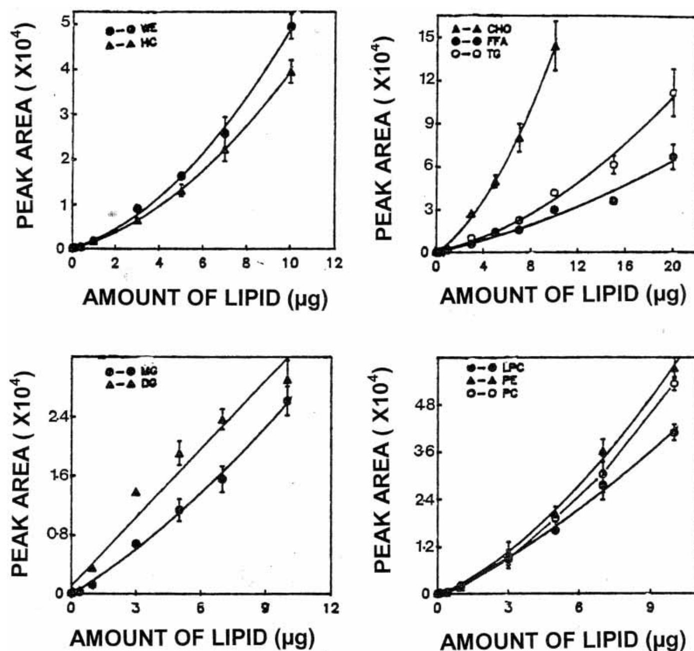

Figure 8. Calibration curves for lipid classes separated on Chromarods-SIII (Lot-3, Unit-2).

(CV) of the FID response, which is the standard deviation expressed as the percentage of the mean, was not improved by addition of an internal standard for normalization. The CV of intrarod FID responses for Chromarods-SIII varied from 9 to 19. In the present study, Chromarods of 4 different lots had different coefficients of variations and the CV varied depending upon the separation ability of the lipid classes. Intra-rod precision for all lipid classes was determined for each rod and the coefficient of variation was high for low loads but the reverse was true of high loads (Table 4). This table also clearly shows that the CV for intra-rod variability varied from one lot to the other. However, for the load level of 5 μg the CV for intra-rod variability for neutral lipids ranged from 2 to 9 for Chromarods-SIII whereas for Chromarods-A it ranged from 3 to 19. Chromarods-A usually had higher coefficients of variation than did Chromarods-SIII. Although the standard deviation of the mean FID response increased with increasing load, the CV decreased indicating higher precision at higher loads. Therefore, it is important to include FID responses of high loads in the calibration curve for the accuracy of the model. However, for Chromarods-A, CV of intra-rod precision for high load levels of phospholipids were higher than for low load levels indicating high precision for phospholipids at low loading levels.

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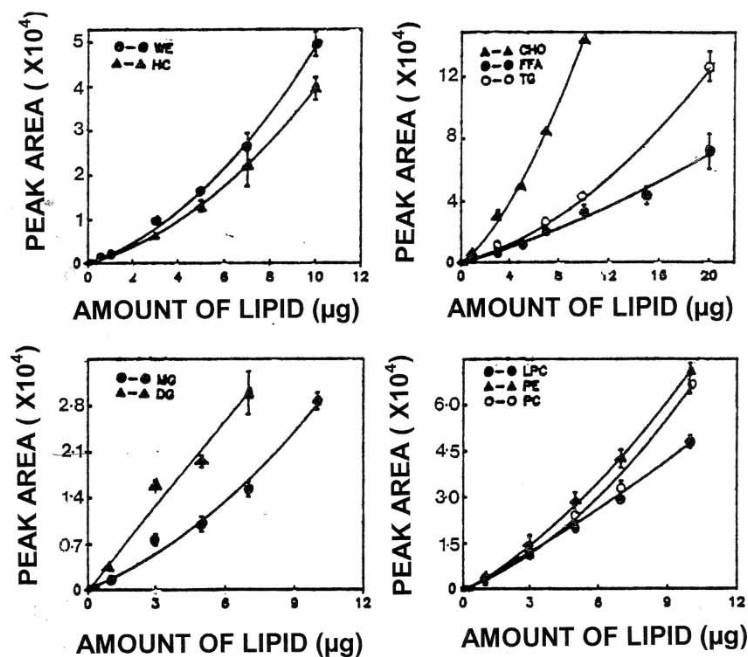

Figure 9. Calibration curves for lipid classes separated on Chromarods-SIII (Lot-4, Unit-1).

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Inter-rod Variability

In addition to the variation of FID response for the same amount of lipid class spotted on the same rod and developed in the same solvent system, the variability may differ from one rod to another. It has been reported that the use of an internal standard should reduce inter-rod variability if different rods elicit different responses as a result of the overall characteristics of individual chromarods.[19] They also indicated that the FID responses of some compounds display a deterioration in precision when their areas are normalized to that of the internal standard. The interod precision has been improved by including an internal standard for some compounds but the error was increased for cholesterol.[8]

In the present study, the CV for the inter-rod precision varied not only with the lipid class but also with the type of rod and the load levels (Table 5). It is also clear that the CV decreased with increasing load levels on both Chromarods-A and -SIII. Even when KET was used as the internal standard, the CV was not improved for either Chromarods-A or Chromarods-SIII.

Four major lipid classes (WE, TG, FFA, PC) with 1 μg loads were used to determine the inter-rod precision. Without normalizing data with an

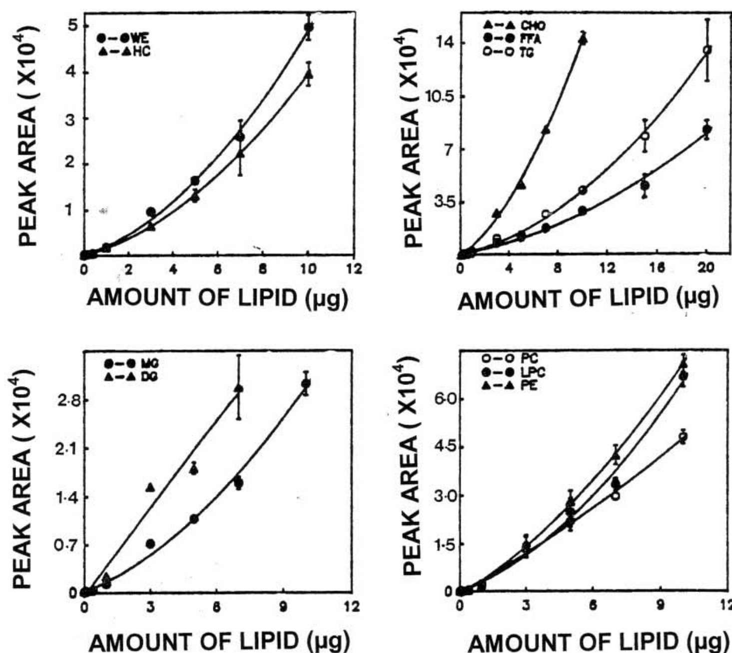

Figure 10. Calibration curves for lipid classes separated on Chromarods-SIII (Lot-4, Unit-2).

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internal standard there was no significant difference for the FID response of WE among rods in both Chromarods-A (lot 1 and 2) and both Chromarods-SIII lots (lot 3 and 4) (Tables 6, 7). However, one lot of Chromarods-SIII (lot 3) showed a significant difference among rods for TG whereas all lots of Chromarods-A and lot 4 of Chromarods-SIII did not show any significant difference for the FID response (Tables 8, 9). The FID response of FFA for different rods in the same lot also did not show any statistically significant differences. All rods of Chromarods-A lots appeared to have a similar response for FFA as indicated by a small difference in mean sum of square error (MSE) and mean sum of square regression (MSR) (Table 10). In the case of Chromarods-SIII this ratio was relatively high for both Chromarods-SIII lots (lot 3 and 4), but there was no significant difference among the FID response of individual rods in a lot (Table 11). However, this may differ or not for other lipid classes. Phosphatidylcholine was used as a representative group of polar lipids to study their differences in FID responses among rods in the same lot and the value of the F-ratio in the analysis of variance clearly showed that there was no significant difference in FID response for this phospholipid among rods in the same lot (Tables 12–13).

Table 2. Some selected quadratic and cubic calibration models for one analytical unit

Regression equation	R^2	F-ratio	p-value
HC-(Lot-1, Unit-1)			
$Y = -48 + 1938x + 164x^2$	0.99	2049.75	0.000
$Y = 116 + 1285x + 424x^2 - 24.6x^3$	1.00	2396.30	0.000
HC-(Lot-3, Unit-1)			
$Y = 121 + 1323x + 261x^2$	1.00	25464.86	0.000
$Y = 135 + 1288x + 269x^2 - 0.48x^3$	1.00	14226.06	0.000
WE-(Lot-1, Unit-1)			
$Y = -31 + 4533x + 225x^2$	0.99	993.70	0.000
$Y = -114 + 4863x + 94x^2 - 12.4x^3$	0.99	505.93	0.000
WE-(Lot-3, Unit-1)			
$Y = 193 + 1492x + 338x^2$	0.99	1232.33	0.000
$Y = -136 + 2297x + 154x^2 - 11x^3$	0.99	3022.85	0.000
TG-(Lot-1, Unit-1)			
$Y = 56 + 3561x + 227x^2$	0.99	769.11	0.000
$Y = 409 + 1705x + 1273x^2 - 139x^3$	1.00	3597.54	0.000
TG-(Lot-3, Unit-1)			
$Y = 526 + 1368x + 97.6x^2$	0.98	146.80	0.000
$Y = -900 + 3407x - 212x^2 + 10.8x^3$	0.99	132.41	0.000
FFA-(Lot-1, Unit-1)			
$Y = -31 + 4533x + 225x^2$	0.99	404.66	0.000
$Y = -114 + 4863x + 94x^2 - 12.4x^3$	0.99	204.17	0.000
FFA-(Lot-3, Unit-1)			
$Y = -5121 + 7583x + 306x^2$	0.76	10.76	0.000
$Y = 1746 - 2233x + 1185x^2 - 52.2x^3$	0.98	97.28	0.000
CHO-(Lot-1, Unit-1)			
$Y = -512 + 10042x + 267x^2$	0.99	618.80	0.000
$Y = 362 + 5449x + 2857x^2 - 344x^3$	0.99	1128.69	0.000
CHO-(Lot-3, Unit-1)			
$Y = -1333 + 6066x + 889x^2$	0.99	2290.64	0.000
$Y = -185 + 2675x + 1851x^2 - 64x^3$	1.00	3978.02	0.000

Lot-to-Lot Variability

In addition to the variability (which may not be significant) of FID responses within the rod and among rods in the same lot, there may be a considerable variation among lots. However, in the present study, 2 structurally different types of Chromarods (Chromarods-A and Chromarods-SIII) were used and lot-to-lot variability was determined by comparing the mean FID responses

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Table 3. Some selected quadratic and cubic calibration models for one analytical unit

Regression equation	R^2	F-ratio	p-value
DG-(Lot-1, Unit-1)			
$Y = -564 + 655x - 503x^2$	0.99	74.48	0.003
$Y = 499 + 971x + 264x^2 - 418x^3$	1.00	12369.30	0.000
DG-(Lot-3, Unit-1)			
$Y = -740 + 6144x - 376x^2$	0.98	125.44	0.000
$Y = -780 + 6208x + 350x^2 - 3x^3$	0.99	72.44	0.000
MG-(Lot-1, Unit-1)			
$Y = 492 + 10532x - 1069x^2$	0.99	34.64	0.008
$Y = -1206 + 19455x + 6100x^2 - 668x^3$	0.99	65.00	0.015
MG-(Lot-3, Unit-1)			
$Y = -108 + 1798x + 76.4x^2$	0.99	658.66	0.000
$Y = -340 + 2508x - 124x^2 + 13x^3$	0.99	754.22	0.000
PC-(Lot-1, Unit-1)			
$Y = 269 + 1569x + 285x^2$	1.00	4480.28	0.000
$Y = 338 + 121x + 490x^2 - 27.2x^3$	1.00	3987.41	0.000
PC-(Lot-3, Unit-1)			
$Y = 240 + 1660x + 194x^2$	0.99	540.21	0.000
$Y = 232 + 120x + 501x^2 - 30.1x^3$	0.99	644.22	0.000
PE-(Lot-1, Unit-1)			
$Y = 551 + 635x + 378x^2$	0.99	391.00	0.000
$Y = 305 + 193x - 352x^2 + 96.9x^3$	0.99	776.37	0.000
LPC-(Lot-1, Unit-1)			
$Y = -15 + 211x - 66x^2$	0.99	458.89	0.000
$Y = 168 + 115x + 477x^2 - 72.1x^3$	1.00	10086.46	0.000
LPC-(Lot-3, Unit-1)			
$Y = -628 + 289x + 297x^2$	0.99	992.60	0.000
$Y = -157 + 174x + 560x^2 - 16x^3$	1.00	4247.39	0.000

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of WE, TG, FFA and PC (1 μg level) of each lot by ANOVA *via* multiple regression. The mean FID response for WE was significantly different among 4 different lots ($p < 0.01$) (Table 14). These mean values were then compared using Bonferroni multiple comparison procedure to identify significantly different means from one another. When the mean FID responses were compared, the responses of lot 1 and 2 (Chromarods-A) were found to be different ($p < 0.01$) from the response of lots 3 and 4 (Chromarods-SIII) although there were no significant differences between lots 1 and 2 as well as between 3 and 4 ($p > 0.01$). The mean values of lots 1 and 2 were

Table 4. Mean coefficient of variation of intra-rod precision at two different loading levels

	Chromarods-A				Chromarods-SIII			
	Lot-1		Lot-2		Lot-1		Lot-2	
Lipid class	1 μg	5 μg	1 μg	5 μg	1 μg	5 μg	1 μg	5 μg
HC	17.8	3.2	13.5	10.6	18.8	3.0	6.7	5.2
WE	6.8	7.1	10.7	7.0	11.4	9.6	10.3	2.8
KET	5.4	—[a]	10.0	—[a]	11.3	—[a]	10.3	—[a]
TG	15.8	7.2	8.0	6.4	20.3	7.5	10.1	7.2
FFA	14.6	5.2	24.7	4.2	9.6	6.3	23.4	4.6
CHO	8.4	3.2	18.2	3.3	12.1	4.4	13.3	5.6
DG	23.6	5.2	27.2	4.1	10.3	4.7	21.6	3.3
MG	15.1	18.4	44.1	18.6	9.2	15.3	7.7	6.4
PC	19.9	29.0	5.8	12.9	8.9	16.9	14.8	6.7
LPC	13.7	17.6	11.6	14.5	9.1	19.3	8.9	6.8
PE	15.1	52.3	18.0	37.7	6.5	29.1	9.8	16.4

[a]Not determined.

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higher than those of 3 and 4 indicating statistically significant higher mean FID response of WE on Chromarods-A.

Table 15 shows that there was also a significant difference among the grand mean FID responses of TG on different lots ($p < 0.01$). The response

Table 5. Mean coefficient of variation of inter-rod precision at two different loading levels

	Chromarods-A				Chromarods-SIII			
	Lot-1		Lot-2		Lot-1		Lot-2	
Lipid class	1 μg	5 μg	1 μg	5 μg	1 μg	5 μg	1 μg	5 μg
HC	11.0	14.1	7.7	6.1	11.3	12.2	21.8	14.8
WE	7.5	7.1	8.1	5.5	14.7	10.6	6.9	3.3
KET	13.3	—[a]	19.6	—[a]	22.0	—[a]	28.0	—[a]
TG	7.1	7.2	8.7	7.7	10.3	7.0	12.2	5.5
FFA	10.9	7.3	12.6	8.8	13.8	8.5	22.8	15.0
CHO	9.3	9.7	10.1	9.1	19.7	6.2	19.4	5.7
DG	15.7	8.8	15.2	5.0	16.3	5.1	12.1	6.4
MG	14.9	9.3	12.2	7.4	12.6	2.2	4.2	2.7
PC	18.6	8.5	5.6	8.3	10.9	8.9	8.5	7.2
LPC	8.1	9.1	13.5	10.6	4.2	12.3	6.4	19.0
PE	15.3	4.5	13.3	17.4	2.5	23.5	5.7	48.2

[a]Not determined.

Table 6. Analysis of variance for the inter-rod variability of WE on Chromarods-A, Lot-1

Source	DF	SS	MS	F	P
Regression	9	0.16803	0.01867	1.70	0.154
Error	29	0.21908	0.01095		
Total	2	0.38711			
Source	DF	SEQ.SS			
C4	1	0.00672			
C5	1	0.00114			
C6	1	0.02976			
C7	1	0.00699			
C8	1	0.00054			
C9	1	0.00429			
C10	1	0.09497			
C11	1	0.00497			
C12	1	0.01865			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of WE on rods 1–9 respectively.

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Table 7. Analysis of variance for the inter-rod variability of WE on Chromarods-SIII, Lot-1

Source	DF	SS	MS	F	P
Regression	8	0.13076	0.01635	0.79	0.615
Error	18	0.37041	0.02058		
Total	26	0.50117			
Source	DF	SEQ.SS			
C4	1	0.01811			
C5	1	0.01140			
C6	1	0.00056			
C7	1	0.01769			
C8	1	0.04704			
C9	1	0.02427			
C10	1	0.00862			
C11	1	0.00306			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C11 = Indicator variables for the FID response of WE on rods 1–9 respectively.

Table 8. Analysis of variance for the inter-rod variability of TG on Chromarods-A, Lot-1

Source	DF	SS	MS	F	P
Regression	9	0.11679	0.01298	0.70	0.698
Error	20	0.36819	0.01841		
Total	29	0.48499			
Source	DF	SEQ.SS			
C4	1	0.03505			
C5	1	0.00001			
C6	1	0.01579			
C7	1	0.00027			
C8	1	0.01989			
C9	1	0.00125			
C10	1	0.04328			
C11	1	0.00023			
C12	1	0.00103			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of TG on rods 1–9 respectively.

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Table 9. Analysis of variance for the inter-rod variability of TG on Chromarods-SIII, Lot-1

Source	DF	SS	MS	F	P
Regression	8	0.228542	0.028568	4.11	0.006
Error	18	0.21908	0.006949		
Total	26	0.353627			
Source	DF	SEQ.SS			
C4	1	0.201333			
C5	1	0.000245			
C6	1	0.007499			
C7	1	0.000771			
C8	1	0.011542			
C9	1	0.002353			
C10	1	0.003658			
C11	1	0.001142			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C11 = Indicator variables for the FID response of TG on rods 1–8 respectively.

Table 10. Analysis of variance for the inter-rod variability of FFA on Chromarods-A, Lot-1

Source	DF	SS	MS	F	P
Regression	9	0.15422	0.01714	0.63	0.755
Error	20	0.53996	0.0027		
Total	29	0.69418			
Source	DF	SEQ.SS			
C4	1	0.0001			
C5	1	0.02757			
C6	1	0.00765			
C7	1	0.01199			
C8	1	0.01018			
C9	1	0.02221			
C10	1	0.03831			
C11	1	0.01197			
C12	1	0.02424			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C11 = Indicator variables for the FID response of FFA on rods 1–9 respectively.

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Table 11. Analysis of variance for the inter-rod variability of FFA on Chromarods-SIII, Lot-1

Source	DF	SS	MS	F	P
Regression	8	1.48515	0.18564	1.89	0.125
Error	18	1.76903	0.09828		
Total	26	3.25418			
Source	DF	SEQ.SS			
C4	1	0.36873			
C5	1	0.00739			
C6	1	0.61703			
C7	1	0.24527			
C8	1	0.08983			
C9	1	0.02981			
C10	1	0.12647			
C11	1	0.00063			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C11 = Indicator variables for the FID response of FFA on rods 1–8 respectively.

Table 12. Analysis of variance for the inter-rod variability of PC on Chromarods-A, Lot-1

Source	DF	SS	MS	F	P
Regression	9	1984771	22053	0.77	0.645
Error	20	572792	286396		
Total	29	7712691			
Source	DF	SEQ.SS			
C4	1	969242			
C5	1	38747			
C6	1	178035			
C7	1	219			
C8	1	43913			
C9	1	56304			
C10	1	605803			
C11	1	85974			
C12	1	6534			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of PC on rods 1–9 respectively.

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Table 13. Analysis of variance for the inter-rod variability of PC on Chromarods-SIII, Lot-1

Source	DF	SS	MS	F	P
Regression	8	279288	34912	0.5	0.841
Error	18	1258115	69885		
Total	26	1537414			
Source	DF	SEQ.SS			
C4	1	128725			
C5	1	1276			
C6	1	63114			
C7	1	25806			
C8	1	34993			
C9	1	295			
C10	1	4672			
C11	1	20417			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C11 = Indicator variables for the FID response of PC on rods 1–8 respectively.

Table 14. Analysis of variance for the inter-lot variability of WE

Source	DF	SS	MS	F	P
Regression	3	10.1168	3.3723	240.51	0.000
Error	110	1.5424	0.014		
Total	113	11.6591			
Source	DF	SEQ.SS			
C1	1	0.048			
C2	1	9.9369			
C3	1	0.1318			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C1-C3 = Indicator variables for the variation of FID response of WE on Chromarods-A, Lot-1; Chromarods-A, Lot-2 and Chromarods-SIII, Lot-1, respectively.

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of TG on lot 1 and 2 (Chromarods-A) was significantly higher than on lot 3 and 4 (Chromarods SIII) ($p < 0.01$). Similarly, there was also a lot-to-lot variation for FFA ($p < 0.01$) (Table 16) and the multiple comparisons showed that the mean responses of lot 1 and 2 were different from lot 3 and 4. The ANOVA table for lot-to-lot variability in FID response for polar lipids (i.e., phosphatidylcholine) shows a high F-ratio (Table 17) and the Bonferroni multiple comparison indicated that the FID response for this phospholipid on Chromarods-A was significantly lower than on the Chromarods-SIII. All neutral lipid classes had higher responses on Chromarods-A.

Table 15. Analysis of variance for the inter-lot variability of TG

Source	DF	SS	MS	F	P
Regression	3	6.188	2.0627	113.62	0.000
Error	110	1.9969	0.0182		
Total	113	8.1849			
Source	DF	SEQ.SS			
C1	1	5.0131			
C2	1	1.1521			
C3	1	0.0228			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C1-C3 = Indicator variables for the variation of FID response of TG on Chromarods-A, Lot-1; Chromarods-A, Lot-2 and Chromarods-SIII, Lot-1, respectively.

Table 16. Analysis of variance for the inter-lot variability of FFA

Source	DF	SS	MS	F	P
Regression	3	25.2756	8.5252	141.89	0.000
Error	110	6.6092	0.0601		
Total	113	32.1848			
Source	DF	SEQ.SS			
C1	1	12.2727			
C2	1	12.3388			
C3	1	0.9641			

DF = Degrees of freedom; SS = Sum of squares.
MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C1-C3 = Indicator variables for the variation of FID response of FFA on Chromarods-A, Lot-1; Chromarods-A, Lot-2 and Chromarods-SIII, Lot-1, respectively.

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Variability Among Analytical Units

It is a common practice to consider all 10 rods[6,9] or 5 similar rods[20] in a lot as one analytical unit and the mean peak area of the group of rods is taken as the FID response for any lipid class separated by TLC-FID. When a large number of samples have to be analyzed, the smaller the unit, the faster is the analysis. The intra-rod variability of such an analytical unit may be decreased by taking the average of 3 replicates on the same unit. In the present study, 5 rods of a lot were considered to be an analytical unit and the mean FID response of WE, TG, FFA and PC on each unit was

Table 17. Analysis of variance for the inter-lot variability of PC

Source	DF	SS	MS	F	P
Regression	3	5268222	1756074	14.85	0.000
Error	110	13007185	118247		
Total	113	18275408			
Source	DF	SEQ.SS			
C1	1	3284541			
C2	1	1983507			
C3	1	0174			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C1-C3 = Indicator variables for the variation of FID response of PC on Chromarods-A, Lot-1; Chromarods-A, Lot-2 and Chromarods-SIII, Lot-1, respectively.

Table 18. Analysis of variance for the inter-unit variability of WE

Source	DF	SS	MS	F	P
Regression	7	10.1848	1.4455	104.61	0.000
Error	106	1.4744	0.0139		
Total	113	11.6591			
Source	DF	SEQ.SS			
C1	1	0.0018			
C2	1	0.064			
C3	1	3.9058			
C4	1	6.0312			
C5	1	0.1538			
C6	1	0.0233			
C7	1	0.005			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of WE on analytical units 1–7, respectively.

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compared for both Chromarods-A and –SIII using analysis of variance *via* multiple regression using indicator variables. The FID response to all lipid classes significantly differed from one unit to another ($p < 0.01$) (Tables 18–21). When the Bonferroni multiple comparison test was applied, it was

Table 19. Analysis of variance for the inter-unit variability of FFA

Source	DF	SS	MS	F	P
Regression	7	25.8147	3.6878	61.37	0.000
Error	106	6.3702	0.0601		
Total	113	32.1848			
Source	DF	SEQ.SS			
C1	1	5.1487			
C2	1	7.1243			
C3	1	5.6786			
C4	1	6.7168			
C5	1	0.886			
C6	1	0.2576			
C7	1	0.0027			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of FFA on analytical units 1–7, respectively.

Table 20. Analysis of variance for the inter-unit variability of TG

Source	DF	SS	MS	F	P
Regression	7	6.30069	0.9001	50.64	0.000
Error	106	1.88418	0.01778		
Total	113	8.18487			
Source	DF	SEQ.SS			
C1	1	2.0319			
C2	1	2.98303			
C3	1	0.25765			
C4	1	0.93855			
C5	1	0.0004			
C6	1	0.0313			
C7	1	0.05786			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of TG on analytical units 1–7, respectively.

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found that the means were not different among units of the same types of rods. Thus the mean FID responses of analytical units of Chromarods-A were different from those of Chromarods-SIII, but there was no difference among units in the same lot.

Table 21. Analysis of variance for the inter-unit variability of PC

Source	DF	SS	MS	F	P
Regression	7	587375	833393	7.1	0.000
Error	106	124411658	117374		
Total	113	32	1848		
Source	DF	SEQ.SS			
C1	1	2912946			
C2	1	852675			
C3	1	862776			
C4	1	1124494			
C5	1	7			
C6	1	356			
C7	1	80496			

DF = Degrees of freedom; SS = Sum of squares; MS = Mean squares; F = F-ratio; P = Probability; SEQ SS = Sequential sum of squares; C4-C12 = Indicator variables for the FID response of PC on analytical units 1–7, respectively.

Table 22. Analysis of covariance for the comparison of FID response of WE on Chromarods-A and -SIII

Predictor	Coefficient	St. dev.	t-ratio	P
Constant	8.1457	0.2656	27.55	0.000
C2	0.2305	0.4181	0.55	0.593
C11	0.8354	0.1323	6.31	0.000
C22	−0.1082	0.1872	−0.58	0.576

C2 = Indicator variables for the FID response of WE.
C11 = Amount of WE loaded (covariate).
C22 = Interaction between C2 and C11.

Table 23. Analysis of covariance for the comparison of FID response of CHO on Chromarods-A and -SIII

Predictor	Coefficient	St. dev.	t-ratio	P
Constant	8.6851	0.3947	22.01	0.000
C2	0.4551	0.5582	0.82	0.438
C11	0.9201	0.1732	5.31	0.000
C22	−0.1417	0.245	−0.58	0.579

C2 = Indicator variables for the FID response of CHO.
C11 = Amount of CHO loaded (covariate).
C22 = Interaction between C2 and C11.

Table 24. Analysis of covariance for the comparison of FID response of PC on Chromarods-A and -SIII

Predictor	Coefficient	St. dev.	t-ratio	P
Constant	7.9176	0.3557	22.26	0.000
C2	−0.1844	0.503	−0.37	0.723
C11	0.8932	0.1561	5.72	0.000
C22	−0.3142	0.2208	−1.42	0.193

C2 = Indicator variables for the FID response of PC.
C11 = Amount of PC loaded (covariate).
C22 = Interaction between C2 and C11.

Response of Chromarods-A and Chromarods-SIII for a Range of Loading

In addition to the intra- and inter-rod variability of FID response for any lipid class at a particular loading level, the slopes of calibration curves were compared after transformation of the data to study the difference in FID

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response of Chromarods-A and –SIII for a loading-range from 0.01 to 7 μg of different lipid classes. Major lipid classes which have relatively high response were used for this comparison and the response of WE, TG, CHO, FFA and PC were compared by analysis of covariance (ANCOVA) *via* multiple regression using indicator variables. There was no significant difference between the overall responses of WE and CHO for the loading range from 0.01 to 7 μg on Chromarods-A and –SIII ($p > 0.05$) even though Chromarods-A apparently had a higher response (Table 22, 23). In the case of phospholipids, PC had a significantly different response on Chromarods-A from Chromarods-SIII ($p > 0.05$) (Table 24). It is known that different lipid classes have different responses on the same type of rod.[21,22] However, it is interesting to note that the response of TG was significantly higher than that of FFA on Chromarods-SIII whereas on Chromarods-A, FFA and TG had almost the same response.

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CONCLUSIONS

Both Chromarods-A and –SIII can be successfully used for quantitative analysis of both polar and neutral lipids. However, Chromarods-A are more recommended for the analysis of neutral lipids rather than polar lipids. The FID response demonstrated by the Iatroscan Mark-III for all compounds studied had curvilinear relationships against the sample load, especially at low loading levels. Therefore, Power Law model and quadratic equations can be used for the quantification of both neutral and polar lipids separated on Chromarods-A and –SIII and an addition of a quadratic term may be especially necessary for calibration curves with a broad range of sample loads. All 10 rods in a lot could be a better analytical unit than 5 rods or less, for quantitative analysis due to wide variability in the FID response among rods. To improve the quality and the efficacy of the analysis, all 10 new rods in a lot may be pre-screened for FID response and only those with similar FID response can be selected as an analytical unit. At least 1-2 repeated analyses may be performed to reduce the intra-rod variability further.

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