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QUANTITATIVE DETERMINATION OF NEUTRAL LIPIDS ON CHROMARODS*

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SUMMARY

Five lots of ten chromarods were spotted with 2, 4, 6, or 8 μg of cholesterol ester, cholesterol, triglyceride, methyl ester and free fatty acid and then analyzed using an Iatroscan. Rod-to-rod and lot-to-lot differences in the detector response were evident in the data. The standard deviation for the rod within lot response appeared to increase linearly as the amount of lipid applied was increased. The logarithms of the detector response data were analysed statistically to determine the relative magnitude of the rod-to-rod and lot-to-lot variances. When methyl ester was used as an internal standard or as a covariate, the variation from rod to rod and lot to lot was much smaller than in the original analysis.

INTRODUCTION

The Iatroscan has been welcomed by many workers using conventional thin-layer chromatography (TLC) as an instrument that can provide qualitative and quantitative analyses of lipids[1,2]. Careful examination of the chromatographic behaviour of lipid subclasses on the chromarods used with the Iatroscan has shown that TLC solvent systems may not be directly applicable to the chromarods. However, proper solvent selection can ensure good separation of a wide range of lipid classes[3]. This separation ability, combined with the small sample size and the speed of analysis, has led to the suggestion that the Iatroscan may be used routinely for clinical lipid analysis[4–6].

Studies of the quantitative capabilities of the Iatroscan have shown that there

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is a low precision in single estimations owing to the large coefficient of variation[6–10]. Most of this variation has been attributed to the inconsistent behaviour of individual rods[10]. Hammond[11] has recently suggested that the flame ionization detector design may be the source of quantitation problems. Initial work with the Iatroscan in this laboratory led to our questioning the uniformity of results obtained using individual rods within a single lot and also the uniformity of results obtained from different lots. This paper presents a detailed study of the quantitation of lipid subclasses using five different lots of ten chromarods. Methods of standardizing the data from the rods and lots are reported which attempt to minimize the rod-to-rod and lot-to-lot variation.

EXPERIMENTAL

The instrument and operating conditions used in this study have been reported previously[5]. Five lots of ten chromarods (type S, mean thickness of sintered coating of active absorbent, 75 μm) were received over a 7-month period. Standard solutions of 2, 4, 6, or 8 μg of cholesterol ester (CE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA) and cholesterol (C) in heptane were spotted on the rods (standards purchased from Nu Check, Elysin, MN, U.S.A.). A hexane–diethyl ether–formic acid (85:15:0.04) mixture was used as the developing solvent. The differential and integral outputs from the Iatroscan were displayed on a two-pen recorder (Fisher Recordall, Model 5000). The step height of the integration signal was taken as the detector response.

A second experiment was carried out in which 6 μg of the lipid standards (CE, ME, TG, FFA and C) were spotted on the rods from three lots, then developed and analysed. The procedure was repeated five times for each rod.

STATISTICAL ANALYSIS

Analyses of variance (ANOVA) and covariance were applied to the results from the five lots of rods in order to study the variation among lots and among rods within lots. All tests of significance were carried out at the 5% level. Because the standard errors appeared to increase approximately linearly with increasing concentration, the original data (detector responses) were transformed prior to analysis using the logarithmic transformation[12]. The transformations also facilitated comparisons of the different analyses because an ANOVA of the logarithm of a ratio is analogous to an analysis of covariance of the logs of the components with a regression coefficient of 1. The multiple regression techniques used in introducing a series of independent variables and the concepts involved in the tests for parallel lines are discussed by Snedecor and Cochran[12].

The model associated with the analyses of variance in the first experiment is:

$$Y_{ijk} = \mu + l_i + a_j + (al)_{ij} + r_{ik} + \varepsilon_{ijk}$$

where Y_{ijk} is the $ijk$the observation, μ the overall mean, l_i represents the effect of the i^{th} lot, a_j the effect of the j^{th} amount, $(al)_{ij}$ the interaction between lot i and amount j, r_{ik} the effect of the k^{th} rod in the i^{th} lot and ε_{ijk} the random error as-

sociated with the individual rods. All effects were assumed random except a_{ij}. The structure was the same in the second experiment except that replicates replaced amounts. Variance components were estimated for all random effects. It may be noted that the component for interaction in the first experiment includes two constituents, one relating to the interaction *per se* and the other to the random variation among determinations for the lot as a whole. There was no appropriate error for testing lot differences in the first experiment because one choice —the rod within lot mean square— did not include the random variation among determinations for the lots, a component in the lot mean square, while the other choice —the interaction mean square— contained the interaction component which is not included in the lot mean square. However, as the interaction mean square was the appropriate error in the second experiment, it was used in the first as well. The choice of either error term would make little difference to the interpretation of the experiment.

RESULTS AND DISCUSSION

Table I contains raw data from two lots (hereafter referred to as lots 2 and 5) of ten chromarods obtained using the Iatroscan. The data are the detector responses to the five different lipid classes (CE, ME, TG, FFA and C) when 2, 4, 6 or 8 μg of each lipid were applied to the rod. As can be seen, the average response for the ten rods from the two lots is similar in some cases (CE, 2 μg and 8 μg; ME, 4 μg and 8 μg; TG, 2 μg, 4 μg and 8 μg; FFA, 2 μg, 4 μg and 8 μg; C, 2 μg and 8 μg), but very different in others (CE, 4 μg; ME, 2 μg; FFA, 6 μg; C, 6 μg). For both lots, the standard deviation increased as the amount of lipid applied was increased. The standard deviations for lot 5 were always greater than the standard deviations for lot 2. These smaller standard deviations could reflect either greater precision in lot 2 and/or greater systematic differences among the rods of lot 5. Subsequent analyses of variances within lots showed both factors contributed to the differences in standard deviations.

The data in Table I show that there were often large differences among rods in response to a given amount of an individual lipid. This is most evident for the rods in lot 5 (CE, 2 μg, rods 5 and 8; TG, 4 μg, rods 6 and 8; FFA, 8 μg, rods 6 and 8), a result already noted in the larger standard deviations. When the responses of individual rods are examined, it is evident that in lot 5, rod 8 had a low sensitivity, whereas rod 6 had a higher sensitivity; in lot 2, rods 6 and 7 gave low responses, whereas rods 5 and 10 usually gave relatively higher responses.

In order to determine if these differences in response by the individual rods in a lot were large enough to have a considerable impact on the variation from lot to lot, data (similar to that given in Table I) were obtained for the 5 lots of 10 rods. Table II is a summary of these data. As when only lots 2 and 5 were compared, it was evident that for the five lots the mean responses to a particular amount of an individual lipid were sometimes similar and in other cases very different. In all lots, the standard deviation increased when the amount of lipid applied was increased. But lots 1 and 5 had greater standard deviations than lots 2, 3 and 4 for all lipids at the four amounts of application.

Tables III–VI show the mean square values that were obtained from the ANOVA. In addition, the estimates of the relevant variance components are also

TABLE I

DETECTOR RESPONSES TO DIFFERENT AMOUNTS OF LIPID SUBCLASSES (LOTS 2 AND 5)

Rod	*Amount 2 µg*					*Amount 4 µg*				
	CE	*ME*	*TG*	*FFA*	*C*	*CE*	*ME*	*TG*	*FFA*	*C*
Lot 2										
1	1.09	0.94	0.72	0.88	1.13	2.57	2.41	1.92	2.14	2.97
2	1.09	0.94	0.76	0.89	1.39	2.15	1.93	1.51	1.68	2.42
3	1.18	0.99	0.82	0.88	1.26	2.50	2.20	1.77	2.01	2.70
4	1.33	1.11	0.87	0.93	1.21	2.28	2.00	1.63	1.78	2.81
5	1.38	1.18	0.90	0.98	1.32	2.90	2.62	2.14	2.36	3.13
6	1.00	0.87	0.67	0.76	1.62	2.43	2.09	1.64	1.69	2.96
7	1.00	0.82	0.60	0.67	0.89	2.81	2.45	2.00	2.12	2.92
8	1.22	1.05	0.89	0.90	1.25	2.82	2.57	2.16	2.25	2.99
9	1.28	1.10	0.86	0.90	1.33	2.68	2.31	1.81	1.90	2.79
10	1.33	1.19	1.04	1.05	1.40	2.70	2.46	2.07	2.04	2.96
Mean	1.19	1.02	0.81	0.88	1.28	2.58	2.30	1.87	2.00	2.87
S.D.*	±0.14	±0.13	±0.13	±0.11	±0.19	±0.25	±0.24	±0.23	±0.23	±0.20
Lot 5										
1	0.66	0.44	0.37	0.50	0.95	1.82	1.58	1.22	1.42	2.21
2	1.03	0.50	0.50	0.70	1.03	1.43	1.10	1.00	1.05	1.88
3	1.28	0.79	0.78	0.84	1.03	2.27	1.79	1.35	1.40	2.06
4	1.60	1.13	0.98	1.04	1.19	2.84	2.58	2.22	1.93	2.38
5	2.12	1.38	1.09	1.23	1.37	2.21	2.10	1.78	1.92	2.57
6	1.07	1.16	1.37	1.50	1.70	2.50	3.04	2.99	3.37	3.43
7	1.08	0.86	0.91	0.93	1.11	2.27	2.21	2.16	2.25	2.68
8	0.65	0.42	0.40	0.43	0.84	1.58	1.27	0.90	0.90	1.85
9	1.40	1.10	0.99	1.12	1.17	3.49	3.27	2.94	3.30	3.02
10	0.99	0.90	0.89	1.06	1.12	3.02	2.62	2.44	2.63	2.63
Mean	1.19	0.87	0.83	0.94	1.15	2.34	2.16	1.90	2.02	2.47
S.D.*	±0.44	±0.33	±0.14	±0.33	±0.24	±0.65	±0.73	±0.77	±0.87	±0.50

* Standard deviation.

presented. In analysis 1, the log transformed data from all five lots of rods were analysed. In analysis 2, the detector response for each compound was divided by the response to ME before transformation (thus essentially using ME as an internal standard). In analysis 3, the transformed detector response data were analysed using the transformed ME response as a covariate. In the analysis of each lipid it was assumed that the regression relation between ME and the compound being analysed was the same for all five lots.

When the transformed data alone were analysed (analysis 1, Tables III–VI, the F ratios for the lot-to-lot differences [L/(R*L)] and the rod-to-rod differences [(R/L)/(error)] both indicated significant differences. The estimates of the variance components from the different sources tended to be similar for each lipid, although the rod component was somewhat larger for TG and FFA. The random variation inherent in the determinations (represented by the error term) were very similar for the four lipids analysed. When the data for individual lots were analysed (not shown

Amount 6 μg					*Amount 8 μg*				
CE	*ME*	*TG*	*FFA*	*C*	*CE*	*ME*	*TG*	*FFA*	*C*
4.71	4.54	3.10	3.86	4.53	6.48	6.24	4.42	4.61	6.53
4.42	3.85	3.16	3.20	4.49	6.09	5.61	4.07	4.29	5.99
4.74	4.20	3.22	3.51	4.38	6.78	6.17	3.72	5.18	6.41
4.58	4.06	2.90	3.53	4.79	6.52	5.57	4.24	4.12	6.75
5.16	4.62	3.81	3.85	5.39	7.52	6.02	4.42	5.66	6.75
4.17	3.61	2.50	2.70	3.61	6.10	5.15	3.41	3.52	4.50
4.72	4.32	3.29	3.40	4.43	7.06	5.99	4.62	4.68	6.07
4.81	4.55	3.44	3.57	4.64	6.90	6.20	4.61	5.16	6.29
4.96	4.41	3.17	3.32	4.34	6.31	5.37	3.80	4.32	6.01
5.00	4.70	3.56	3.70	4.75	6.99	6.32	4.81	4.88	6.45
4.73	4.29	3.22	3.46	4.54	6.68	5.86	4.21	4.64	6.18
±0.29	±0.36	±0.36	±0.34	±0.45	±0.46	±0.41	±0.45	±0.62	±0.65
2.56	2.37	1.78	1.92	3.10	4.48	4.14	2.58	2.92	4.38
2.13	1.95	1.56	1.75	2.99	4.00	3.40	2.34	2.42	4.81
3.44	2.82	2.06	2.09	3.00	6.38	6.03	3.04	3.80	5.28
2.63	2.46	1.57	1.72	2.63	7.49	7.38	4.10	5.25	5.82
3.42	3.37	2.81	2.98	3.95	5.98	5.83	3.60	4.09	6.74
4.10	5.32	4.13	5.18	4.11	7.48	7.12	8.81	8.16	7.97
4.27	4.18	3.29	3.46	4.08	7.53	7.15	5.61	6.03	6.66
2.58	2.27	1.58	1.62	2.60	4.58	4.09	2.05	2.26	3.56
4.14	3.87	2.86	3.10	3.29	10.00	10.47	6.17	7.72	7.24
2.59	2.00	1.50	1.45	5.96	6.88	7.27	5.42	6.20	8.42
3.19	3.06	2.31	2.53	3.57	6.37	6.20	3.88	4.52	5.88
±0.79	±1.11	±0.91	±1.17	±1.11	±1.89	±2.19	±1.54	±1.89	±1.53

here), it was apparent that lots 1 and 5 had more rod-to-rod variation than lots 2, 3 and 4.

The use of an internal standard has been proposed as one way of overcoming the systematic differences in response[7,8]. To be useful in this application, an internal standard must (i) be soluble in organic solvents, (ii) have an R_F value on chromarods that does not overlap with other compounds of interest, (iii) be non-naturally occurring and (iv) have a response similar to the compound(s) being analysed. In this study, methyl ester was chosen as an internal standard in an attempt to eliminate the lot-to-lot and rod-to-rod differences. The results are represented in analysis 2.

Comparing the estimates of the variance components from analysis 2 with those from analysis 1 for each lipid (Tables III–VI), it is apparent that most components were reduced considerably by using ME as an internal standard. In spite of the large drops in variance components, the differences among rods within lots are still significant for all compounds. Analysis 2 shows that the amount mean square remains relatively large, other than perhaps for CE, suggesting a problem in accuracy

TABLE II

MEAN* DETECTOR RESPONSE TO VARIOUS AMOUNTS OF NEUTRAL LIPIDS DEVELOPED** ON CHROMARODS FROM DIFFERENT LOTS

*Subclass****	*Amount applied (μg)*	*Lot 1*	*Lot 2*	*Lot 3*	*Lot 4*	*Lot 5*
CE	2	0.83 ± 0.17	1.19 ± 0.14	1.16 ± 0.11	1.03 ± 0.09	1.19 ± 0.44
	4	1.91 ± 0.34	2.58 ± 0.25	2.52 ± 0.13	3.14 ± 0.36	2.34 ± 0.65
	6	3.37 ± 0.71	4.73 ± 0.29	4.24 ± 0.44	4.38 ± 0.28	3.19 ± 0.79
	8	5.54 ± 0.83	6.68 ± 0.46	5.56 ± 0.71	6.08 ± 0.60	6.38 ± 1.89
ME	2	0.67 ± 0.17	1.02 ± 0.13	1.21 ± 0.09	0.93 ± 0.09	0.87 ± 0.33
	4	1.74 ± 0.40	2.30 ± 0.24	2.30 ± 0.11	2.79 ± 0.31	2.16 ± 0.73
	6	3.11 ± 0.78	4.29 ± 0.36	3.79 ± 0.41	3.76 ± 0.24	3.06 ± 1.11
	8	5.51 ± 1.13	5.86 ± 0.41	5.00 ± 0.22	5.08 ± 0.41	6.29 ± 2.19
TG	2	0.68 ± 0.20	0.81 ± 0.13	1.13 ± 0.14	0.75 ± 0.08	0.83 ± 0.14
	4	1.54 ± 0.41	1.87 ± 0.23	2.18 ± 0.18	2.29 ± 0.27	1.90 ± 0.77
	6	2.32 ± 0.71	3.22 ± 0.36	3.16 ± 0.24	2.97 ± 0.25	2.31 ± 0.91
	8	3.35 ± 0.57	4.21 ± 0.45	3.93 ± 0.18	3.89 ± 0.34	3.99 ± 1.54
FFA	2	0.71 ± 0.22	0.88 ± 0.11	1.24 ± 0.15	0.82 ± 0.07	0.94 ± 0.33
	4	1.57 ± 0.45	1.99 ± 0.23	2.54 ± 0.24	2.31 ± 0.27	2.02 ± 0.87
	6	2.42 ± 0.78	3.46 ± 0.34	3.42 ± 0.28	3.04 ± 0.21	2.53 ± 1.17
	8	3.80 ± 0.85	4.64 ± 0.62	4.21 ± 0.17	3.88 ± 0.32	4.66 ± 1.89
C	2	0.93 ± 0.15	1.28 ± 0.19	1.58 ± 0.23	1.16 ± 0.11	1.15 ± 0.24
	4	2.18 ± 0.55	2.87 ± 0.20	3.21 ± 0.51	3.46 ± 0.08	2.47 ± 0.50
	6	3.12 ± 0.54	4.54 ± 0.45	5.06 ± 1.08	4.64 ± 1.14	3.57 ± 1.11
	8	5.04 ± 0.93	6.18 ± 0.65	6.11 ± 1.11	5.28 ± 1.05	5.94 ± 1.53

* Means represent average of ten rods from a lot run simultaneously ± standard deviation.
** Developing solvent hexane–diethyl ether–formic acid 85:15:0.04.
*** CE = cholesterol ester; ME = methyl ester; TG = triglyceride; FFA = free fatty acid; C = cholesterol.

of the internal standard method at the different concentration levels, perhaps because of a non-linear relationship between the lipids and ME, a point to be discussed below. Furthermore, the significant lot* amount interactions indicate that the response patterns differ somewhat from lot to lot. The differences among lots, however, are not significant, a result which can be attributed at least in part to the relatively large amount* lot interactions (the denominator in the *F* ratio) and to the small number of degrees of freedom involved in the *F* ratio (4 and 12 in the numerator and denominator respectively).

In an attempt to determine why the use of ME as an internal standard (analysis 2) left comparatively large differences among amounts and failed to explain some of the variation among lots and among rods within lots, regression analyses (within lots) were carried out on the untransformed data from the individual lipids (C, ME, TG, FFA, CE), using the amounts applied as the independent variable. The untransformed data were used here to retain the structure depicted in Fig. 1; the fact that the coefficient of variation remained constant over the range is unlikely to have affected

TABLE III

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES ON CHOLESTEROL ESTER DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.624	0.033	0.035
Amount (A)	45	27.397	0.041	0.006
L * A	3	0.121	0.075	0.051
Rods/L	12	0.086	0.020	0.011
Error	135**	0.013	0.004	0.003
		Variance components		
Lots (L)		0.013	−0.001	0.000
L * A		0.001	0.007	0.005
Rods/L (R/L)		0.018	0.004	0.002
Error		0.013	0.004	0.003

* 1 = ANOVA of log (detector response to CE); 2 = ANOVA of log (detector response to CE/detector response to ME); 3 = ANOVA of log (detector response to CE) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.84 ± 0.05.

** 134 d.f. for analysis 3.

TABLE IV

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF CHOLESTEROL DATA

Source variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.960	0.117	0.359
Amount (A)	45	22.184	0.604	0.344
L * A	3	0.098	0.058	0.042
Rods/L (R/L)	12	0.077	0.067	0.042
Error	135**	0.018	0.027	0.018
		Variance components		
Lots (L)		0.022	0.001	0.008
L * A		0.008	0.003	0.002
Rods/L (R/L)		0.015	0.010	0.006
Error		0.018	0.027	0.018

* 1 = ANOVA of log (detector response to C); 2 = ANOVA of log (detector response to C/detector response to ME); 3 = ANOVA of log (detector response to C) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.14 ± 0.10.

** 134 d.f. for analysis 3.

TABLE V

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF TRIGLYCERIDE DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.	*Mean square*		
Lots (L)	4	0.788	0.097	0.097
Amount (A)	45	22.107	0.626	0.084
L * A	3	0.114	0.071	0.049
Rods/L (R/L)	12	0.177	0.013	0.013
Error	135**	0.016	0.005	0.004
		Variance components		
Lots (L)		0.017	0.001	0.001
L * A		0.010	0.007	0.005
Rods/L (R/L)		0.040	0.002	0.002
Error		0.016	0.005	0.004

* 1 = ANOVA of log (detector response to TG); 2 = ANOVA of log (detector response to TG/detector response to ME); 3 = ANOVA of log (detector response to TG) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.88 ± 0.006.

** 134 d.f. for analysis 3.

TABLE VI

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF FREE FATTY ACID DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.	*Mean square*		
Lots (L)	4	0.904	0.171	0.168
Amount (A)	45	21.805	0.624	0.044
L * A	3	0.143	0.054	0.043
Rods/L (R/L)	12	0.187	0.020	0.019
Error	135**	0.016	0.004	0.004
		Variance components		
Lots (L)		0.019	0.003	0.003
L * A		0.013	0.005	0.004
Rods/L (R/L)		0.043	0.004	0.004
Errors		0.016	0.004	0.004

* 1 = ANOVA of log (detector response to FFA); 2 = ANOVA of log (detector response to FFA/detector response to ME); 3 = ANOVA of log (detector response to FFA) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.94 ± 0.05.

** 134 d.f. for analysis 3.

the results to any degree. The analyses indicated that for both CE and ME there was evidence of a curvilinear relationship between amount applied and detector response (see Fig. 1). However, *ca.* 80% of the total sums of squares for both compounds could be explained by including terms for the linear and quadratic effects, as well as lot differences, in the regression equation. The regression analysis of the ME data indicated that the regression lines were not parallel from lot to lot, a result that helps to explain why the lot* amount interaction could not be completely removed using a single slope in the analyses of covariance. It was also found that the regression equations generally did not pass through zero–zero, so that some change in the relationship can be expected as the detection limits are approached.

The regression coefficients presented in Table VII reflect the patterns illustrated in Fig. 1, that is, the responses of CE, C and ME are all similar and steeper than those of FFA and TG. The difference in slopes probably reflects a difference in detector response. When using the internal standard method, it is usually assumed that the ratio of the true concentration to detector response is the same for both the standard and the unknown. Hence, if ME is to be used as a standard for FFA and TG, an adjustment will be necessary to the standard formulation.

Another problem with the internal standard method is introduced by the non-linear standard curve for ME. The ratio of the detector responses for the lipids and ME did not remain constant over the range of amounts considered. In the present context, the impact of the non-linearity could be studied by using the ME value as a covariate rather than as the denominator in the ratio. On the log scale, the closer the regression coefficient β for the covariate is to unity, the more appropriate it is to use the ratio directly. Hence the analyses of covariance were carried out on the response values of the various lipids, with the ME detector response as a covariate; the results are given as analysis 3 in Tables III–VI. Generally, the estimates of the variance components for lots and rods within lots were similar or somewhat smaller than those of analysis 2. The most noteworthy result, however, was the substantial reduction in the amount mean square for all compounds, but especially for TG and FFA.

While the β estimates for CE, TG and FFA (0.84, 0.88 and 0.94, respectively) were all near unity, the accuracy was improved by taking account of the non-linearity, probably because the slight discrepancies were exaggerated by the relatively large range of concentrations. It is interesting to note that for C, the only compound with an estimate considerably different from unity (0.14), the error was actually increased by using the internal standard method (analysis 2), *i.e.*, by assuming $\beta = 1$.

One of the difficulties associated with the analyses of Tables III–VI was that it was impossible to determine the relative contributions of interaction and random error to the lot * amount and error mean squares. In order to present the error mean square as an estimate of the random variation from determination to determination within rod, it was necessary to assume that there was no rod * amount interaction. To examine this issue more closely, a second study was carried out, in which repeated measurements were taken at the 6-μg level, thereby eliminating interaction components involving amounts. The results (not presented here) were similar to those of the first study, with the corresponding entries for analysis 3 never differing by more than a factor of 2. The estimates were especially close for TG where, for example, the rod within lot and error mean squares of the second study were 0.017 and 0.004, respectively. With the exception of the lot mean squares, the results in the second

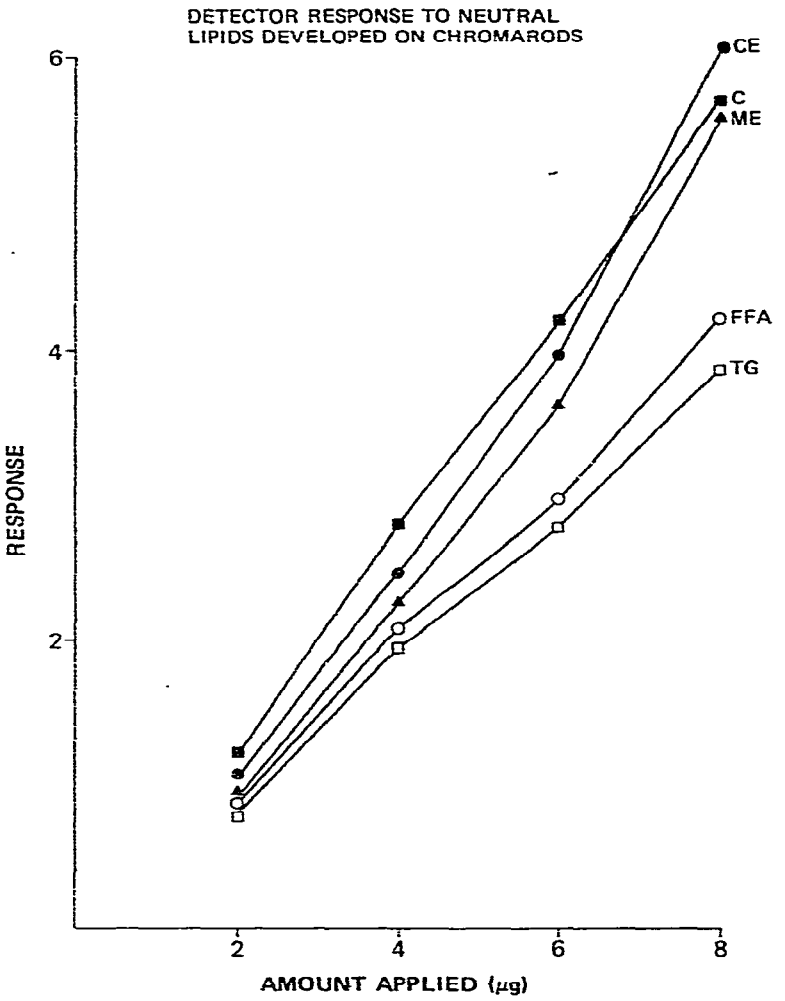

Fig. 1. Plot of detector response *vs.* the amount of lipid applied (2, 4, 6 or 8 μg) for cholesterol ester (CE), cholesterol (C), methyl ester (ME), free fatty acid (FFA) and triglyceride (TG). Each point is the mean of five lots.

study were usually somewhat smaller, suggesting that there was perhaps some interaction between amounts and lots in the original study. The fact that the replicate* lot mean squares in the second experiment were considerably larger than the corresponding error terms suggests that the random variation among replicates for a lot as a whole cannot be attributed solely to the precision of the individual rods. The estimates of β were generally somewhat smaller in this second study, the only exception being for C(0.24) but this pattern remained consistent with the estimate for C much smaller than for the other compounds.

TABLE VII

REGRESSION ANALYSIS* ON IATROSCAN LIPID DATA

Source of variation	*df.*	*CE*	*ME*	*TG*	*FFA*	*C*
			Mean squares			
Lots (L)	4	4.72	1.92	2.45	3.13	8.01
Amounts (A)	3	227.32	193.60	86.38	101.03	185.28
Linear**	1	675.63	575.03	258.26	301.63	555.22
Quadratic**	1	5.57	4.94	0.02	0.23	0.05
Cubic**	1	0.74	0.83	0.85	1.22	1.75
A * L	12	1.56	1.83	0.74	1.07	1.55
Lots * linear	4	0.97	1.85	0.66	1.04	0.99
Residual	8	1.85	1.82	0.79	1.09	1.84
Rods/L	45	0.87	1.08	0.99	1.21	1.20
Error	135	0.21	0.25	0.19	0.21	0.37
Linear*** regression coefficient		0.82 ± 0.02	0.75 ± 0.02	0.49 ± 0.02	0.54 ± 0.02	0.74 ± 0.02

* Using amount of lipid as independent variable.
** Based on orthogonal polyomials[12].
*** Based on an equation including only the linear, and not the quadratic and cubic, terms.

CONCLUSIONS

Several workers have investigated the use of an internal standard to improve on the quantitative capabilities of the Iatroscan technique[5,7,8]. However, statistical analyses to determine if the precision of the results from the rods had been increased by the use of internal standards were not included in their reports. Our results show that the variability in the measurements from the Iatroscan method can be improved by including an internal standard in the test solution. This approach does improve the precision of the method.

The use of an internal standard assumes a relationship between the concentration of the lipid and the concentration of the standard of the form:

$$\text{true concentration of lipid} = \frac{\text{detector response to lipid}}{\text{detector response to standard}} \times \text{true concentration of standard}$$

In some applications, it will be necessary to adjust this formula to allow for differences in detector response[5]. The results of the present study indicate that use of the internal standard method with Iatroscan measurements will improve precision considerably. However, it was found that the standard curves were not always linear. The effect of the slight departure from linearity was notable only in the amounts mean square, probably because of the magnitude of this term relative to the other mean squares. Hence, care should be taken to ascertain whether or not standard curves relevant in a particular application are linear. While precision is unlikely to be affected unduly by a slight lack of linearity, it may be necessary to make adjustments to

the internal standard method in order to enhance the accuracy of the method, especially if a wide range of concentrations are expected.

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REFERENCES

1 T. Okumura, T. Kadono and A. Iso'o, *J. Chromatogr.*, 108 (1975) 329–336.
2 M. Tanaka, T. Itoh and H. Kaneko, *Yukagaku (Jap. Oil Chem. Soc.)*, 25 (1976) 263–265.
3 J. K. G. Kramer, R. C. Fouchard and E. R. Farnworth, *J. Chromatogr.*, 198 (1980) 279–285.
4 D. Vandamme, G. Vankerckhoven, R. Vercaemst, F. Soetewey, V. Blaton, H. Peeters and M. Rosseneu, *Clin. Chim. Acta*, 89 (1978) 231–238.
5 D. M. Bradley, C. R. Richards and N. S. T. Thomas, *Clin. Chim. Acta*, 92 (1979) 293–302.
6 D. Vandamme, V. Blaton and H. Peeters, *J. Chromatogr.*, 145 (1978) 151–154.
7 A. Martin-Ponthieu, N. Porchet, J.-C. Fruchart, G. Sezille, P. Dewailly, X. Godaccioni and M. Delecour, *Clin. Chem.*, 25 (1979) 31–34.
8 P. Van Tornout, R. Vercaemst, H. Caster, M. J. Lievens, W. de Keersgieter, F. Soetewey and M. Rosseneu, *J. Chromatogr.*, 164 (1979) 222–227.
9 W. W. Christie and M. L. Hunter, *J. Chromatogr.*, 171 (1979) 517–518.
10 G. L. Mills, C. E. Taylaur and A. L. Miller, *Clin. Chim. Acta*, 93 (1979) 173–180.
11 E. W. Hammond, *J. Chromatogr.*, 203 (1981) 397–403.
12 G. W. Snedecor and W. G. Cochran, *Statistical Methods*, Iowa University Press, Ames, IA, 1967.