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VARIATION IN THE RELATIVE RESPONSE FACTOR FOR TRIGLYCERIDES ON IATROSCAN CHROMARODS WITH FATTY ACID COMPOSITION AND SEQUENCE OF ANALYSES*

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SUMMARY

Triglycerides, consisting of fatty acids differing in chain length and unsaturation, were partially resolved on Type S Chromarods. The relative flame ionization detection (FID) responses of equal amounts of each of the triglycerides to methyl ester internal standard were similar for single component triglycerides, and higher than for mixtures of these triglycerides. In addition, the relative FID response factor for all single and mixed component triglycerides decreased through the sequence of analyses. This would necessitate the use of different FID response factors for mixed-component triglycerides, generally found in biological samples, whereas single-component triglycerides have usually been used to obtain response factors. Increasing the scan speed and impregnating the Chromarods with copper(II) sulphate produced more consistent FID responses, reduced interrod differences, and made the FID response relate to the mass of the component rather than peak shape.

INTRODUCTION

The quantitation of lipids by use of flame ionization detection (FID) after separation by thin-layer chromatography (TLC) on Chromarods, *i.e.*, quartz rods coated with silica gel[1], has been greatly improved by the use of internal standards which reduced the rod to rod variation[2–5]. However, the relative response of the different lipid classes to an internal standard has not been found to be constant; it was shown to vary with the amount of sample applied[5,6], the ratio of sample to internal standard[7] and the scanning speed[8–10]. Mares *et al.*[11], furthermore, observed larger coefficients of variation for biological samples than for comparable amounts of model

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synthetic mixtures, a result which they attributed to the presence of unsaturated and unstable lipids in the biological samples. This was in part confirmed in a recent study by Banerjee *et al.*[12], who demonstrated that unsaturated lipids gave an enhanced FID response after exposure of the developed Chromarods to iodine vapour.

We have reported recently that molecular species within a lipid class were partially resolved on Chromarods which resulted in the spreading of the band width of the lipid class[13]. In the present communication it is shown that the FID response of triglycerides (TGs) relative to methyl ester (ME) internal standard is dependent on the fatty acid composition of the TG and on the previous usage of the rods (*i.e.*, on the sequence of analyses), offering an explanation for previous reported discrepancies in FID response. It is further shown that increasing the scan speed and impregnating the Chromarods with copper(II) sulphate, as suggested by Kaimal and Shantha[10], reduces interrod differences, makes the FID response a function of mass rather than peak shape, and makes response differences between certain TGs more consistent.

EXPERIMENTAL

Pure TGs (>99%) of 16:1 $n-7$, 22:1 $n-9$ and 22:6 $n-3$, and methyl heptadecanoate were purchased from Nu-Check-Prep (Elysian, MN, U.S.A.). Mixtures were prepared containing the ME internal standard and pairs of the TGs in the ratio of 4:0:4, 4:1:3, 4:2:2, 4:3:1 and 4:4:0 (ME:TG_1:TG_2). All lipid mixtures were dissolved in heptane to give a concentration of 8 μg/ul of total lipid.

A new set of ten Chromarods (type S) was acid washed (sulphuric acid–perchloric acid, 1:1), rinsed with distilled water and burned three times before use. One μl of the lipid mixture was spotted on the Chromarods per analysis. The five mixtures prepared from each pair of TGs were systematically analyzed. Every sample was spotted on two of the ten rods for each run, until each mixture was analyzed on every rod, a total of five runs. The spotting order is shown in Table I. This procedure was replicated twice for all three pairs of TGs, giving 30 runs in all. The rods were stored in a 55% humidity chamber (41% sulphuric acid) when not in use and acid washed once, half way through completion of the experiment.

For the second portion of this experiment, the set of Chromarods was acid washed and impregnated with copper(II) sulphate according to Kaimal and Shantha[10]. The TGs of 22:1 $n-9$, 22:6 $n-3$ and an equal mixture of both of these TGs were spotted on these rods as shown in Table I.

Details regarding the Iatroscan TH-10, Mark II, analyzer and its operating conditions were described previously[14]. The ion collector of the Iatroscan was adjusted to *ca.* 1.7 mm above the Chromarods for increased FID response[15]. Peaks were integrated using as Hewlett-Packard (Cupertino, CA, U.S.A.) 3350A Laboratory Automation System with reintegration capacity. The ratio of the integrated areas of TGs to ME represented the relative response factor.

The lipid mixtures were developed on the type S Chromarods in 1,2-dichloroethane–chloroform–formic acid (92:8:0.1) to a height of 10 cm[16]. Neither this solvent mixture nor *n*-hexane–chloroform–isopropanol–formic acid (89:11:0.55:0.055)[10] resolved ME and TGs on copper(II) sulphate-impreganted Chromarods. The developing solvent *n*-hexane–diethyl ether–formic acid (95:5:1) was used

TABLE I

SPOTTING ORDER OF METHYL ESTER (ME)–TRIGLYCERIDE (TG) MIXTURES ($ME:TG_1:TG_2$)

Three combinations of any two of the three TGs (16:1 $n-7$, 22:1 $n-9$ and 22:6 $n-3$) were mixed with the ME (methyl heptadecanoate) in the proportions indicated. In the second study only 22:6 $n-3$ and 22:1 $n-9$ TGs were used.

Analysis	*Chromarod (Type S) number*				
	1 and 6	*2 and 7*	*3 and 8*	*4 and 9*	*5 and 10*
1	4:0:4	4:1:3	4:2:2	4:3:1	4:4:0
2	4:4:0	4:0:4	4:1:3	4:2:2	4:3:1
3	4:3:1	4:4:0	4:0:4	4:1:3	4:2:2
4	4:2:2	4:3:1	4:4:0	4:0:4	4:1:3
5	4:1:3	4:2:2	4:3:1	4:4:0	4:0:4
	Chromarod ($CuSO_4$-impregnated) number				
	1, 4 and 7	*2, 5 and 8*	*3, 6 and 9*	*10*	
1	4:0:4	4:2:2	4:4:0	4:0:4	
2	4:4:0	4:0:4	4:2:2	4:2:2	
3	4:2:2	4:4:0	4:0:4	4:4:0	

which maximized the separation between ME and TGs on type S Chromarods[17,18], in order to achieve a separation on the impregnated rods.

The data were treated as a split-plot. In the analysis of variance separate estimates were obtained for error within and among TG combinations. The design included 3 × 3 Latin Squares among TG combinations as well as 5 × 5 Latin Squares within TG combinations, the former being somewhat implicated by the inclusion of an extra Chromarod (1/3 of a square).

RESULTS AND DISCUSSION

All the TGs and TG mixtures were sufficiently well resolved from the ME internal standard on the Chromarods to permit calculation of the relative peak area responses of TGs to ME. Among the TGs, the R_F values declined with a decrease in unsaturation and chain length (Fig. 1A)[13]. Sufficient data were collected from each Chromarod (four analyses per rod for each pure TG; two analyses per rod for each mixture of TGs) to determine the effect of individual rods, a factor a number of authors have concluded[5,19–21] was necessary to improve the precision.

The means of relative FID responses of total TG to ME of all 30 analyses are shown in Table II. The analysis of variance confirmed significant differences among rods as previously recognized[5,10,19–21]. The variation among rods (0.2220) was much larger than the corresponding random variation within rods (0.0167).

The relative area response among the three pairs of TGs was highly significant. However, this significance was markedly reduced by covariance analysis adjusting for time (analysis not shown), with the F ratio reduced from 61.300 to 18.173. Furthermore, when the significant replicate mean square was adjusted for the time effect,

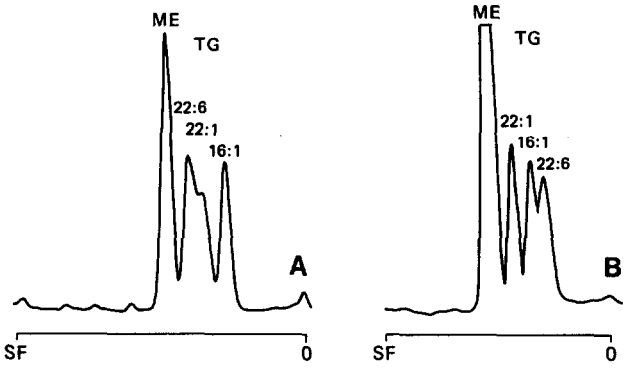

Fig. 1. Separation of a mixture of three triglycerides (TG) consisting of either 16:1 $n-7$, 22:1 $n-9$ and 22:6 $n-3$ fatty acids and a methyl ester (ME) (methyl heptadecanoate) used as internal standard. This mixture was separated on type S Chromarods using the solvent 1,2-dichloroethane–chloroform–formic acid (92:8:0.1) (A) or on copper(II) sulphate-impregnated type S Chromarods using the solvent *n*-hexane–diethyl ether–formic acid (95:5:1) (B).

TABLE II

RESPONSE FACTORS OF TRIGLYCERIDE (TG) MIXTURES TO METHYL ESTER (ME) INTERNAL STANDARD

The mixtures of each pair of TGs were spotted on the 10 Chromarods in the order shown in Table I, for a total of 5 analyses per pair of TGs. Each mixture was then analyzed in duplicate, giving a total of 30 analyses. The means are presented below and the actual data of rods 2 and 7 are plotted in Fig. 2. The relative response was calculated as the ratio of the integrated areas of total TG to ME [$(TG_1 + TG_2)$/ME]. Significance: $P > 0.05$ (NS); $P \leqslant 0.05$ (★); $P \leqslant$ (★★).

Triglyceride pair TG_1/TG_2	*Proportions of mixture* ($ME:TG_1:TG_2$)					
	4:0:4	*4:1:3*	*4:2:2*	*4:3:1*	*4:4:0*	*Mean*
	$(TG_1 + TG_2)$/ME					
22:6 $n-3$/22:1 $n-9$	1.415	1.259	1.194	1.278	1.435	1.316
22:1 $n-9$/16:1 $n-7$	1.270	1.123	1.001	1.043	1.218	1.131
22:6 $n-3$/16:1 $n-7$	1.266	1.139	1.038	1.038	1.286	1.153
Mean	1.317	1.173	1.077	1.120	1.313	

Source of variation	*Analysis of variance*			
	d.f.	*Sum of squares*	*Mean squares*	*F ratio*
Among rods (R)	9	1.9984	0.2220	13.298**
Among reps	1	0.9196	0.9196	55.074**
Among pairs of TG (P)	2	2.0472	1.0236	61.300**
R × P	18	0.4963	0.0276	1.651^{NS}
Error A	29	0.4842	0.0167	1.252^{NS}
Analyses	4	0.0312	0.0078	0.585^{NS}
Mixtures of TG (M)	4	2.8476	0.7119	53.370**
M × P	8	0.2028	0.0254	1.901^{NS}
Error B	224	2.9880	0.0133	

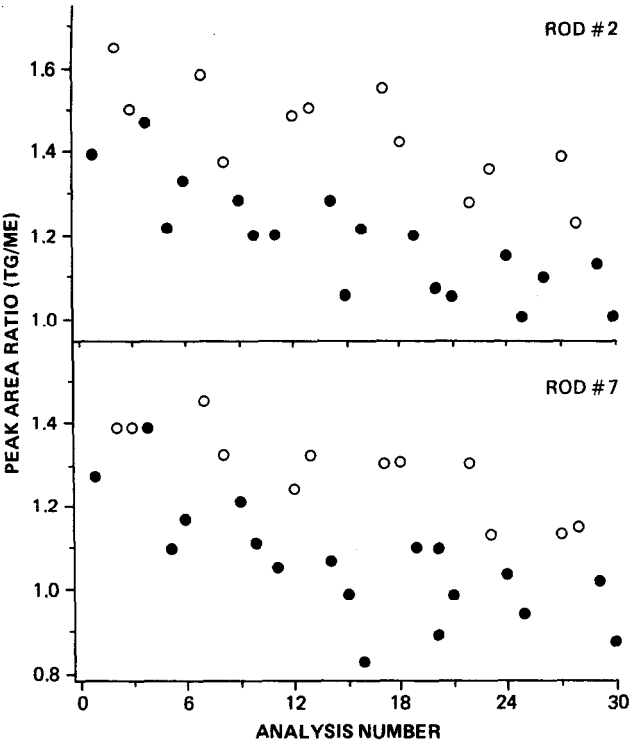

Fig. 2. The ratio of the total integrated area of triglycerides (TG) to methyl ester (ME) is plotted against consecutive numberings of analyses. Single (○) and mixed (●) component triglycerides are identified with different symbols. For identification of triglycerides see Table I.

it was also reduced, from an F ratio of 55.074 to 0.025, another indication of the large time effect. In Fig. 2 the relative FID response of TGs to ME is plotted *versus* analysis number for 2 of the 10 Chromarods. A decrease of the area response of TG to ME was clearly evident through the sequence of analyses. This is the first time where a reduction has been observed in the relative FID response of a lipid component to an internal standard with repeated use (*i.e.* sequence of analysis) of Chromarods for response factors.

The statistical analysis also showed a significant difference among the TG pairs (Table II). However, this difference was not reduced by analysis of covariance adjusting for time (F ratio of 53.370 to 47.454), indicating that this difference persisted at all times. The results of the 2 Chromarods shown in Fig. 2 clearly bear out the difference between single and multiple TG components. The TG:ME peak area ratio for a single TG component was greater than that of multiple TG components.

It is evident from these results that the FID signal in the Iatroscan system was related to the shape of the peak since the absolute amount of the TGs and ME were the same in each case. Sharp peaks, obtained for all single component TGs, gave a greater FID response than mixed TGs in which the same amount of TG was spread over a greater distance, or two separate peaks, along the Chromarods[13]. This result

indicates that different correction factors should be determined for lipid classes consisting of single and mixed molecular species when using type S Chromarods. The implications of this finding are far-reaching. Most investigators to date have determined FID correction factors for lipid classes using single molecular species and applied these same factors for the analysis of biological samples[3,6,8,11,14,17,19,22]. Based on the present findings, the FID correction factors commonly in use would appear to be too high to be applied to biological samples. The use of biologically derived lipid classes as standards for the determination of FID response factors would seem to be more reliable. Although in this study only type S Chromarods were investigated, it appears reasonable to assume that type S and S-II Chromarods would behave similarly based on an evaluation of published data.

These results suggest that not all lipid material on the Chromarod which entered the FID registered a signal. Premature loss of TGs through vaporization[12] was not considered applicable in this case since the FID response for the three single component TGs was similar. It appears that mixed TGs, in which the same amount of TGs was spread over a larger area on the Chromarod, gave a lower FID signal because relatively more TGs were totally burned without ion formation. This explanation would account for the observations that increasing the scan speed[9,10], or increasing the amount of lipid applied onto the Chromarods[5,6], increased the FID signal, since the detector effectively sensed a higher concentration of lipid material per unit of time. It would also account for the FID response observed by Parrish and Ackman[20] when they developed rods after spotting an equal amount of tripalmitin at 5 equidistant spacings along the rods at 0, 2.5, 5, 7.5 and 10 cm. Development of the rods with *n*-hexane–diethyl ether (99:1) resulted in lateral diffusion of the TG along the rod giving symmetrical peaks which were widest near the end of the rod dipped into the solvent. The broader band width TGs gave a significantly lower FID response than the sharp peaks near the top of the rods, while the undeveloped rods gave a similar FID response for all TG spots. This is to be expected, since at the same scan speed the broader peaks represented a lower relative concentration of TG per unit of time. Finally, this explanation also accounts for the lower FID response observed for biological samples as compared to single component lipids[11]. The single component lipid standards gave relatively sharper peaks on Chromarods compared to biological mixtures comprised generally of numerous molecular species which partially separated causing peak broadening[13].

Based on this hypothesis, the FID responses of single and mixed TGs should be similar if the scan speed is increased from 0.32 cm/s (gear No. 30) to 0.42 cm/s (gear No. 40). In addition, it has been reported recently that impregnating the Chromarods with copper(II) sulphate minimizes the rod to rod variation and improves the FID response of various lipid classes[10].

These two concepts were incorporated in a re-analysis of the TG mixtures with the same Chromarods following impregnation with copper(II) sulphate. Impregnating the Chromarods with copper(II) sulphate changed the chromatographic behavior of lipids, contrary to the previous report[10]. The ME and TGs could no longer be separated with the usual solvent systems of 1,2-dichloroethane–chloroform–formic acid (92:8:0.1)[16], and *n*-hexane–diethyl ether–formic acid (85:15:0.1)[18]. Even the solvent system *n*-hexane–chloroform–isopropanol–formic acid (89:11:0.55:0.055) used by Kaimal and Shantha[10] did not separate these ME and TG mixtures. A developing

TABLE III

RESPONSE FACTORS OF TRIGLYCERIDES (TGs) TO METHYL ESTER (ME) ON $CuSO_4$-IMPREGNATED CHROMARODS

The TGs of 22:-6 $n-3$ (TG_1) and 22:1 $n-9$ (TG_2), and their 1:1 mixture were analyzed on the 10 Chromarods in the order shown in Tabvle I. The FID response factor was calculated as the ratio of the integrated areas of TG to ME. Significance: $P > 0.05$ (NS); $P \leqslant 0.05$ (★); $P \leqslant 0.01$ (★★).

Chromarods	*Proportions in mixture (ME:TG_1:TG_2)*		
	4:0:4	*4:2:2*	*4:4:0*
	(TG_1 + TG_2)/ME		
1	0.72	0.47	0.37
2	0.68	0.55	0.45
3	0.64	0.50	0.46
4	0.70	0.47	0.37
5	0.69	0.45	0.43
6	0.67	0.48	0.47
7	0.60	0.42	0.39
8	0.68	0.52	0.42
9	0.64	0.47	0.44
10	0.63	0.49	0.43

Source of variation	*Analysis of variance*			
	d.f.	*Sum of squares*	*Mean squares*	*F ratio*
Among rods	9	0.0151	0.0017	1.687^{NS}
Analysis	2	0.0142	0.0071	7.119^{**}
Among mixtures of TGs	2	0.3084	0.1542	154.749^{**}
Error	16	0.0159	0.0010	

solvent was required that adequately separated ME and TG, such as *n*-hexane–diethyl ether–formic acid (95:5:1)[17,18], in order to quantitate these lipid classes (Fig. 1B). In addition, the elution order of the TGs changed from 22:6 $n-3$, 22:1 $n-9$, 16:1 $n-7$ to 22:1 $n-9$, 16:1 $n-7$, 22:6 $n-3$ (Fig. 1), clearly indicating retarded migration of polyunsaturated fatty acids, presumably by formation of metal (Cu) complexes with double bonds. There was a baseline separation of 22:1 $n-9$ TG and the other two TGs, but the TGs of 16:1 $n-7$ and 22:6 $n-3$ did not separate. For this reason, only the TGs of 22:1 $n-9$ and 22:6 $n-3$ and their 1:1 mixture were choosen to test the above hypothesis.

In addition to the qualitative changes, copper(II) sulphate impregnation also changed the FID response of the lipid classes. The FID signal of the ME increased markedly relative to the TGs (Fig. 1); in fact, it more than doubled, as seen by comparing the relative response of the same lipid mixtures in Tables II and III. In contrast, the absolute FID response of the TGs appeared to be very similar between the original and copper(II) sulphate-impregnated type S Chromarods (Fig. 1); all instrument conditions and amounts applied were kept the same. On the other hand, Kaimal and Shantha[10] reported a similar increase in the FID response of their ME (16:0) and TG mixture (14:0, 16:0, 18:0, 20:4) on type S-II Chromarods. It was also

evident from the results in Table III that the molecular species of TGs had characteristic FID response factors with improved precision (the among rods variation was not significant). The FID response of TG 22:1 $n-9$ was greater than that of TG 22:6 $n-3$. The FID response of the TG mixture was close to that predicted based on the FID responses of the individual TGs. This suggests that the FID response on copper(II) sulphate-impregnated Chromarods was related to the mass of the component, rather than to the peak shape, as was the case with unimpregnated type S Chromarods, which is in agreement with Kaimal and Shantha[10]. However, their suggestion that differences in FID response among lipid classes are now so small as to permit one to "dispense with response factors altogether under this set of conditions" may not be valid.

The results of this study have identified the fatty acid composition as one of the factors in addition to differences in concentration[5,7,19], and scan speed[10], which are causes of variation in the results of previous analyses using unimpregnated Chromarods. Increasing the scan speed and using copper(II) sulphate-impregnated Chromarods produced more consistent FID responses which were a function of the mass of the component. Response factors, however, should be determined for each lipid class, preferably with mixtures of fatty acids resembling the lipid class to be analyzed, rather than with single fatty acid components.

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