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Note

Thin-layer chromatography–flame ionization detection calibration with natural and synthetic standards

A. J. FRASER*
Natural Environment Research Council, Unit of Aquatic Biochemistry, Department of Biological Sciences, University of Stirling, Stirling FK9 4LA (U.K.)
and
C. T. TAGGART
Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1 (Canada)
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A recent paper by Kramer *et al.*[1] demonstrated that the flame ionization detector response of the Iatroscan to samples of single molecular species of triacylglycerol (TAG) was significantly higher than the detector response to equivalent weights of mixtures of two different molecular species of TAG. This observation led the authors to conclude that single-component standards should not be used for thin-layer chromatography–flame ionization detection (TLC–FID) calibration. However, it should be noted that all of the di-isomeric TAG mixtures used by Kramer *et al.*[1] contained two TAG isomers which differed markedly in terms of the chain length and/or the degree of unsaturation of their component fatty acids. The production of broad and split peaks when these samples were run on the Chromarods can therefore be explained in terms of differences in the chromatographic properties of the two isomers. Samples of natural TAG generally contain a much wider range of component isomers[2], and the chromatographic properties, and hence the shape of the peak produced on the Chromarod, of natural TAG would be expected to differ from that of, for example, a 1:1 mixture of two TAG isomers in which 22:6 ($n - 3$) and 16:1 ($n - 7$) were the sole component fatty acids[1]. The present study was therefore undertaken to assess to what extent the FID response reduction noted for di-isomeric TAG samples[1] applied to a sample of natural TAG.

EXPERIMENTAL

Preparation of standards

TAG was prepared from a TAG-rich fish oil (Marinol) containing >40% ($n - 3$) polyunsaturated fatty acids, supplied by Dr. A. Spark of the Fishing Industry Research Institute, Cape Town, South Africa. Marinol TAG was purified by TLC on a 2-mm thick TLC plate (E. Merck, Darmstadt, F.R.G.), using hexane–diethyl

* Current address: Marine Chemistry Division, Department of Fisheries and Oceans, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, Nova Scotia B2Y 4A2, Canada.

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ether–acetic acid (75:25:1, v/v/v) as the developing solvent. The TAG could be visualised without spraying and was scraped and eluted from the silica with hexane–diethyl ether (1:1, v/v). The purity of the Marinol TAG was confirmed by both conventional TLC and TLC–FID. Mono-isomeric TAG (triolein) was obtained from Sigma (Poole, U.K.) and purity was confirmed as above. Solvents were HPLC-grade (Rathburn, U.K.) and were not supplemented with antioxidant. Immediately after the purification of the Marinol TAG, standard solutions containing 2.0–12.0 mg/ml of lipid in chloroform–methanol (2:1, v/v) were prepared in triplicate. The same series of standard solutions, in triplicate, were prepared for triolein. All standard solutions were stored at −20°C under nitrogen, and were used within two weeks of preparation.

Apparatus and operating conditions

A Mk. IV Iatroscan (Iatron Laboratories, Tokyo, Japan), operated at a scan speed of 3.3 mm/s, a hydrogen flow-rate of 135 ml/min, and an air flow-rate of 1800 ml/min, was used in conjunction with a Hewlett-Packard 3390A recording integrator.

Aliquots (1 μl) of the triplicated standard solutions of each TAG were applied to five SII Chromarods using a Whatman syringe. Spotting was aided by drying under a stream of nitrogen. The standard solutions of TAG were analysed, in random order, and on the same five Chromarods, over a period of two weeks. The Chromarods were cleaned in chromic acid[3] prior to commencing the experiment, and were subsequently stored in a water-saturated atmosphere. The Chromarods were developed to the 10 cm mark on the frame in 1,2-dichloroethane–chloroform–acetic acid (92:8:0.1, v/v/v). The Chromarods were then dried at 100°C for 90 s and immediately analysed in the Iatroscan.

Statistical analysis

Analysis of variance (ANOVA) was used to identify differences among the means of the FID response to the triplicate preparations of the Marinol TAG and triolein. Differences among the means of the FID response to the range of sample loadings of Marinol TAG and triolein were also determined by ANOVA. Calibration curves were fitted to the means of each sample loading by maximising the coefficient of determination (r^2) between iterated power transforms of the mean response and sample loading. The difference between the calibration curves for Marinol TAG and triolein was assessed using an analysis of covariance.

RESULTS

Over the range of sample loadings (2.0–12.0 μg), the average FID response to triolein was 1.7-fold greater than that to Marinol TAG (Fig. 1). Analysis of variance and Duncan's multiple range test indicated that for 7 of the 10 sample loadings (5 Marinol TAG; 5 triolein), there were no significant differences among the mean FID responses to the triplicate standards. Three of the samples (Marinol TAG, 6 μg; triolein, 12 μg, 4 μg) exhibited marginal differences among the mean FID responses to the triplicate samples ($p = 0.079$, 0.020, 0.054 respectively). The mean FID responses among all sample loadings for both Marinol TAG and triolein were all significantly different ($p < 0.0001$). Analysis of covariance identified a highly sig-

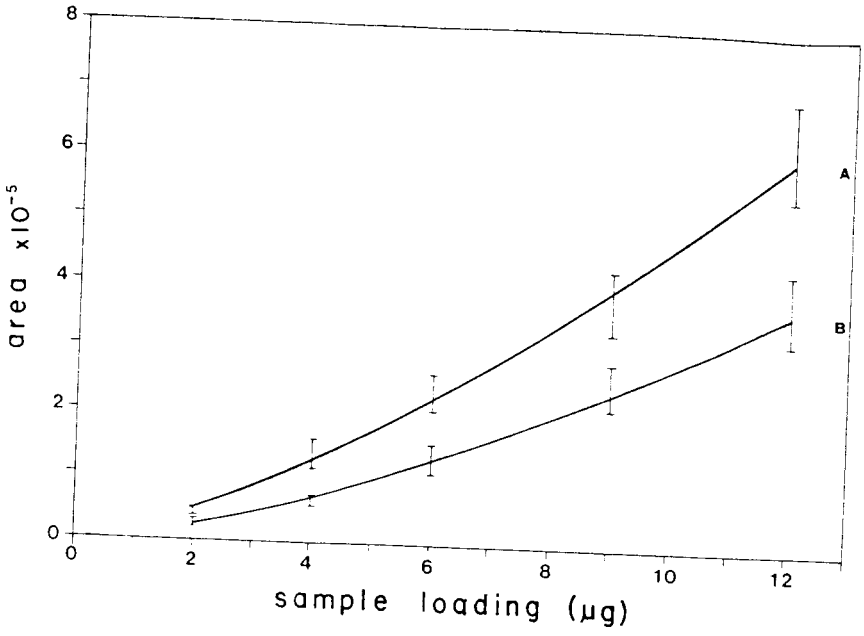

Fig. 1. Calibration curves fitted to the mean FID responses over the range of sample loadings. (A) Triolein (660.88 loading + 146.33)$^{1.48}$, r^2 = 0.999; (B) Marinol TAG (485.36 loading − 69.32)$^{1.48}$, r^2 = 0.999. Vertical bars indicate the range in FID response for the fifteen analyses of the triplicate samples.

nificant ($p < 0.0001$) difference between the Marinol TAG and triolein calibration curves (Fig. 1).

DISCUSSION

There were virtually no differences in the FID responses within each triplicate set of standards, indicating that the procedures of sample preparation and sample spotting were consistent. Thus, the different FID responses to the triolein and Marinol TAG were not a function of sample preparation or application.

A notable difference between the integrator traces produced for the two types of TAG was that the peaks produced by triolein were considerably taller and narrower than peaks produced by the same weight of Marinol. No peak splitting was observed for any samples. These differences in peak width and height can be accounted for on the basis of the variation in the molecular composition of the Marinol TAG isomers, which, as a consequence of slightly different chromatographic properties, will result in greater spreading of this TAG on the Chromarod. Ohshima *et al.*[4] demonstrated that in a polar solvent system, such as that used in the present study, separation of different molecular species of TAG is governed primarily by chain length rather than the degree of unsaturation.

In turn, Parrish and Ackman[5] have demonstrated that narrow peaks produced on Chromarods during a short development period generate a higher FID response than broad peaks produced during longer development. This phenomenon is also

discussed by Kramer *et al.*[1]. It therefore appears that, for a given weight of a particular lipid, the response of the Iatroscan FID is reduced when the lipid is spread over a greater length of the Chromarod. This phenomenon offers an explanation for the reduced FID response to Marinol TAG when compared with that to triolein (Fig. 1). However, it is important to note that the presently described difference in the FID response to Marinol TAG and triolein was considerably greater than the difference in FID response to the mono-isomeric TAG and the di-isomeric TAG mixtures used by Kramer *et al.*[1] This suggests that natural TAG may produce an even more diffuse spot on the Chromarod than di-isomeric TAG mixtures, resulting in an even greater reduction in the FID response.

The results presented above indicate that if the Iatroscan is to be used for quantitative analysis of natural lipid samples, then mono-isomeric standards should not be used. We therefore agree with Kramer *et al.*[1] in recommending that the common practice of using single component standards for TLC–FID[6–10] be replaced by the use of standards resembling the molecular species composition of the lipid classes to be analysed. Ideally, purified natural lipid should be used for calibration purposes. However, for certain lipid classes, such standards are not readily available, and the use of synthetic standards may offer the only viable alternative. On the basis of the present results it is clear that if synthetic standards are to be used, then more than two isomers of each lipid class should be present. It remains to be determined how many isomers of each lipid class need to be present before the FID response to a multi-isomeric synthetic standard is comparable to that of equivalent natural lipid.

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