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Discrimination in Recovery during Capillary GLC Analysis of Fish Oil: The Use of a Recovery Correction Factor (RCF)

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ABSTRACT

A recently proposed capillary GLC method for EPA and DHA determination in fish oils was examined. It was found that whilst the area percentage of each fatty acid showed good reproducibility when an internal standard was used, the mg/g values varied considerably. A linear relationship was observed between mg/g values of EPA and DHA and their relative recovery to C23:0, a commonly used internal standard. Following hydrogenation of the samples it became apparent that losses were likely due to the high susceptibility of polyunsaturates to oxidation. A recovery correction factor was therefore proposed to be incorporated into the calculation of analysis. This may lead to a more accurate estimation of these PUFAs.

INTRODUCTION

The desirable clinical effect of omega-3 polyunsaturated fatty acids (EPA and DHA) in marine oil is well known (Bang *et al.*, 1975; Dyerberg & Jorgensen, 1982) and a variety of EPA and DHA rich fish oil products are now widely available in the markets. The correct labelling of these products and their further clinical research require a reliable and easy-to-use assay method to determine the true levels of the EPA and DHA of the fish oils. Recently, a capillary GLC method was proposed as an international standard method for this estimation (Ackman, 1987). EPA and DHA are

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calculated either as (1) area percentage, or (2) mg/g of sample using the internal standard, C23:0. Interlaboratory collaborative studies have indicated that a relatively good repeatability (within one laboratory) can be achieved with the current method, but the reproducibility of the method between different laboratories is not satisfactory. This paper examines some of the problems associated with the method and discusses the possibility of introduction of a recovery correction factor into the method of calculation.

MATERIALS AND METHOD

Materials

Fish oil capsules were bought over the counter in the UK and all chemicals were from Sigma.

Methods

Methylation

Methylation was carried out according to the method of Joseph and Ackman (1989). Two millilitre aliquots of the internal standard (C23:0) in iso-octane at a concentration of 1 mg/ml were pipetted into a culture tube and the solvent evaporated. A sample fish oil capsule was opened and approximately 25 mg of oil content weighed out into a culture tube. To this was added 1·5 ml 0·5M NaOH. The mixture was capped tightly, mixed and heated for 7 min at 100°C. The reaction mixture was then cooled and 2 ml BF_3/MeOH was added. The mixture was blanketed with N_2, capped tightly and heated for another 5 min at 100°C. After cooling to 30–40°C, 1 ml iso-octane was added to the tube. The mixture was blanketed with N_2 and vortex-mixed for 30 s. While still tepid, 5 ml saturated NaCl solution was immediately added. After separating, the iso-octane layer was recovered. The aqueous layer was extracted once more with 1 ml iso-octane. 0·2 μl of methyl ester preparation were directly injected into GLC for analysis.

Hydrogenation

Hydrogenation was performed using a Supelco micro-hydrogenator. Three to five millilitres (30–50 mg) sample methyl esters prepared as above were evaporated with N_2 to dryness and dissolved in 5 ml methanol. This was transferred directly into the micro-hydrogenator. The hydrogenation was catalysed by 20 mg Adam's catalyst (PtO_2) for 1 h at ambient temperature. The reaction mixture was filtered through a HPLC microfilter before direct injection into the GLC.

GLC conditions

The chromatography was carried out under two conditions. Condition 1 was using a DBwax column (30 m × 0·32 mm) (Supelco) in a Perkin-Elmer Model 8420 GC. The carrier gas was helium at 20 psig. On-column injection mode was used. The oven temperature for a programmed operation was initially at 30°C, and programmed to increase to 150°C at 30°C/min, held for 25 min and increased again to 250°C at 4°C/min with a final 20 min hold. Condition 2 was using a Supelcowax 10 column (30 m × 0·25 mm) (Supelco) in a Perkin-Elmer model 8320B GC. The carrier gas was helium at 14 psig. Split injection mode (split ratio 1:50) was used. The oven temperature was initially 175°C and programmed to increase to 200°C at 1°C/min, held for 10 min, and increased further to 230°C at 2°C/min with a final 20 min hold.

RESULTS AND DISCUSSION

Discrimination between sample fatty acids and internal standard

The introduction of an internal standard into the analysis is to try to eliminate the influence of non-volatile components in oil samples and achieve a better accuracy (Ackman *et al.*, 1989). However, this may chemically exaggerate a discrimination between sample fatty acids and internal standard and statistically create more sources of error. The results in Table 1 illustrate that relative consistent results were observed under the two different chromatography conditions while the mg/g values for EPA and DHA varied considerably. The formula used to calculate the area percentage was:

$$\text{EPA}(\%) = \frac{A_E}{A_T} \times 100 \tag{1}$$

Whilst the weight estimation was

$$\text{EPA mg/g} = \frac{A_E \times \text{wt of internal standard} \times 0{\cdot}99^*}{A_{IS} \times \text{wt of sample} \times 1{\cdot}04^{**}} \tag{2}$$

where A_{IS} = area count of internal standard; A_E = area count of EPA; A_T = total area count; * = detector response factor (0·97 for DHA); and ** = correction factor for methyl ester to acid.

It can be seen that the mg/g values depend upon both A_E and A_{IS} while the % values are decided by A_E and A_T. Any possible variation in % value is due to a discrimination between EPA (or DHA) and the total fatty acids. Since EPA, DHA, and other unsaturates constitute a high proportion of fish oil, it

TABLE 1
Comparison of Analysis Results

(a) Product one

Sample	*1*	*2*	*3*	*4*	*5*	*6*
EPA mg/g	244	234	234	187	197	214
%	27·6	27·8	27·7	31·1	30·9	30·9
DHA mg/g	108	107	102	78	82	86
%	12·9	13·1	12·4	13·3	13·1	12·6
EPA %/mg/g	0·113	0·119	0·118	0·166	0·159	0·144
DHA %/mg/g	0·119	0·122	0·121	0·169	0·159	0·147

(b) Product two

Sample	*1*	*2*	*3*	*4*	*5*	*6*
EPA mg/g	129	136	132	109	109	118
%	16·1	16·0	16·2	18·3	18·7	17·9
DHA mg/g	100	106	101	78	78	84
%	12·7	12·5	12·6	13·4	13·7	13·1
EPA %/mg/g	0·125	0·118	0·123	0·168	0·172	0·153
DHA %/mg/g	0·127	0·117	0·125	0·171	0·175	0·156

N.B.: Samples 1–3 were analysed under condition 1 and 4–6 were analysed under condition 2. Each sample was prepared from single capsules from the same batch oil and results were means of triplicate GC analyses.

is expected that EPA (or DHA) would have a similar tendency to be either under- or overestimated compared with the total fatty acids. However, the internal standard, C23:0, and EPA (or DHA) are chemically very different, particularly as regards their degree of unsaturation. Thus it is unlikely that they would be underestimated or overestimated in a similar manner. Overestimating and underestimating are relative terms. More specifically, it is unlikely that 100% of each fatty acid ester can be recovered during any analysis. If one fatty acid ester has a recovery lower than average, it is underestimated, and vice versa. In view of their high susceptibility to oxidation, EPA and DHA may have a lower recovery than C23:0, particularly if care is not taken during analysis. This may be the reason why % values have a higher reproducibility than mg/g values. The use of internal standard is of benefit to the analysis, and there is no question that mg/g values reflect the fatty acid content more accurately, but this may introduce a potential discrimination in recovery, which should be carefully considered in an analytical method.

One obvious way to overcome this is to introduce a recovery factor. The only correction factor in the original formula is FID response factor, which

was proposed by Ackman and Sipos (1964) and was determined under optimised chromatography conditions, which are by no means the working condition of routine analysis. Craske and Bannon (1988) suggested an empirical correction factor to replace it. However, the empirical correction factor still only corrects differing FID response, taking no consideration of oxidation and other possible discrimination during the analysis. A more comprehensive correction factor may be needed in the calculation.

If a 100% relative recovery of EPA (or DHA) to C23:0 had been achieved, then

$$\text{EPA\% (or DHA\%)/wt EPA (or wt DHA) (mg/g)} = 0{\cdot}1 \times 100/\text{fatty acid purity in oil (\%)} \quad (3)$$

Suppose the oil contains 100% fatty acids, then EPA (or DHA)%/wt EPA (or DHA) = 0·1. Although the fatty acid content is unknown, this value should be a constant for every sample and therefore, the ratio should also be a constant wherever the sample is analysed. However, in a recent interlaboratory collaborative study, this ratio varied from 0·06 to 0·14 for the same sample (Joseph & Ackman, 1989).

If a recovery factor is introduced, then

$$\text{EPA \%/(wt EPA/RCF)} = 0{\cdot}1 \times 100/\text{fatty acid purity}$$

where RCF = recovery correction factor.

It can be more clearly seen that the variation in the ratio between different laboratories is due to each of them having a different relative recovery of EPA/DHA compared with C23:0. If the purity of total fatty acids ester is assumed to be 100% and on the basis of the range of 0·06 to 0·14 for the ratio, the recovery could be estimated to be 71% to 167%. It was found that a higher value for mg/g was significantly related to a higher relative recovery to C23:0 (Fig. 1). By plotting the relationship, the real value for EPA (or DHA) may be obtained (i.e. the value at 100% recovery).

In practice a purity of 90–95% is more realistic and if 100% recovery is achieved, then the ratio should be approximately equal to 0·11. Each laboratory should be aware of this figure. This leads to the first usage of the recovery correction factor (RCF) concept. If a laboratory has an extremely high or low value for the ratio, the analysis should be re-examined and all procedures and equipment need to be rechecked. Such attention should eliminate extreme values in analysis results.

Influence of hydrogenation on analysis

The lower recovery of sample fatty acids compared with internal standard may be produced at any stage of the analysis. The high susceptibility of EPA

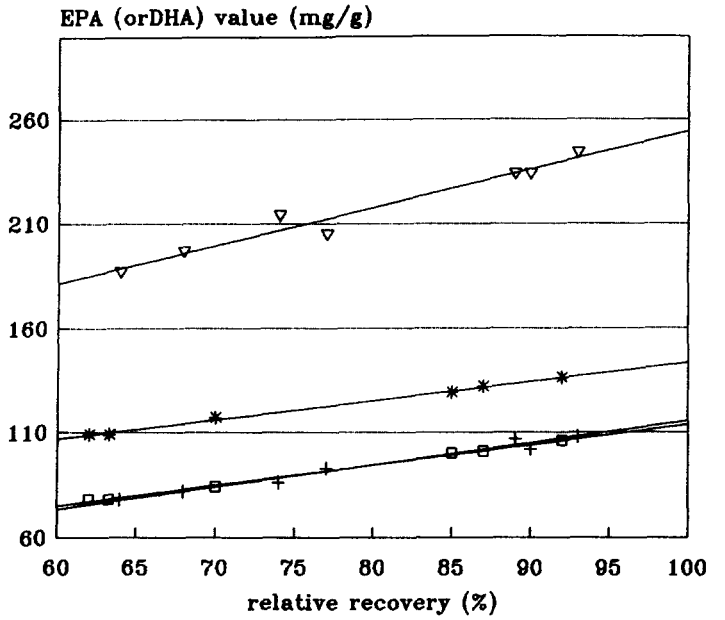

Fig. 1. Relationship between calculated EPA (or DHA) mg/g value and relative recovery to C23:0 (recovery was estimated upon fatty acid purity of 93%, which was calculated by GLC analysis after hydrogenation) ▽, EPA (product 1); +, DHA (product 1); *, EPA (product 2); □, DHA (product 2).

(or DHA) to oxidation (Cho *et al.*, 1987) seems to be a major factor. This is demonstrated by the examination of hydrogenation of different oil samples.

Table 2 shows the total fatty acid contents before and after hydrogenation of fish oil and sunflower oil, the latter representing a less unsaturated and short chain fatty acid oil. The fatty acid content of the fish oil showed an approximately 15% increase after hydrogenation while there is no

TABLE 2
Influence of Hydrogenation on Total Fatty Acid Content

	Total fatty acid content (mg/g sample) before hydrogenation	*after hydrogenation*
Fish oil	819 (38·2)	945 (17·2)
Sunflower oil	976 (36·4)	972 (50·0)

N.B.: Total fatty acid content is estimated by the following formula:

$$\text{Total fatty acid content (mg/g)} = \frac{(A_T - A_{IS}) \times \text{weight of internal standard}}{A_{IS} \times \text{weight of sample} \times 1{\cdot}04^a}$$

[a] Correction factor from methyl ester to fatty acid.

All samples were analysed under the previously described chromatography condition 1. Results were means of triplicate analysis and figures in parentheses are standard deviations of the means.

significant difference between hydrogenated and original sunflower oil. FID response factor increases as polyunsaturate fatty acid methyl esters become more saturated ones. If this is taken into consideration, the total fatty acid content increased more than 15% after hydrogenation. A logical explanation is that, after hydrogenation, there will be more chance for the sample fatty acid esters and internal standard to show similar recovery.

CONCLUSION

The major problem in capillary GLC analysis of fish oil seems to be related to the high degree of unsaturation of the oil. There is no doubt that capillary GLC has a much higher resolution than packed column GC and capillary GLC can eliminate the influence of overlapping peaks in the analysis. However, it is equally possible that the high resolution of capillary GLC means any slight change in the structure of fatty acids during analysis could lead to a large variation in results. A standard method should take this into consideration. Introduction of a recovery correction factor may be a potential solution. Not only should every effort be made in each step of analysis to prevent oxidation, but a measure of any oxidation that has occurred must be made. In the light of this, the appropriate correction factor can be determined and applied. Such a procedure will lead to a more accurate determination of the level of the PUFAs including the clinically important fatty acids EPA and DHA.

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