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# SIMULTANEOUS CAPILLARY GAS CHROMATOGRAPHIC PROFILING OF MEDIUM- AND LONG-CHAIN FATTY ACID METHYL ESTERS WITH SPLIT INJECTION

## CORRECTION FOR INJECTION-RELATED DISCRIMINATION BY THE 'BRACKETING' METHOD

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SUMMARY

Split injection-related discrimination can be a source of inaccuracy and imprecision in quantitative capillary gas chromatographic profiling methods for compounds with relatively big differences in boiling points, such as the methyl esters of medium- and long-chain fatty acids prepared from biological materials. We systematically investigated a standard containing equal masses of saturated fatty acid methyl esters, with chain lengths from $C_5$ to $C_{26}$, under different injection conditions, including injection temperature, sample volume and split ratio. Day-to-day performance was studied under one set of conditions. Normalized peak areas, reciprocal response factors, using either $C_{17}$ or $C_{23}$ as an internal standard, and 'bracketed' reciprocal response factors (peak area of each analyte divided by half the sum of the peak areas of two adjacent esters were calculated. In all experiments the bracketed reciprocal response factors were found to be closest to unity with the lowest coefficients of variation.

## INTRODUCTION

Quantitative long-chain fatty acid (LCFA) methyl ester (FAME) profiling using high-resolution capillary gas chromatography (GC) with flame ionization detection (FID) is a widely used technique in clinical chemistry [1–3], clinical biochemistry [4–7], biology [8], food chemistry [9–12] and other working areas. One of the main problems of this analysis is standardization [13], because many of the FA of biological interest, notably the polyunsaturated species, cannot be obtained in a satisfactory pure form. This inconvenience is mostly circumvented by the addition of a known amount of an odd-carbon-numbered FA, especially margarinic acid (17:0), as an 'internal calibration standard', to a standardized volume (or mass) of the biological sample before transmethylation to methyl esters. After subsequent GC analysis the analytes are quantified by relating their

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peak areas to that of the internal calibration standard, assuming that equal masses of FAME give rise to equal areas (response factors equal unity).

However, owing to the relatively big differences in boiling points of naturally occurring FA, especially when medium-chain FA (MCFA; 6:0 to 14:0) and LCFA (14:0 to 24:0) are analysed in a single GC run, one may expect boiling point-dependent discrimination when the popular split injection technique is employed [14–16]. Dependent on such parameters as injection temperature, sample volume and split ratio, the internal calibration standard technique may lead to underestimation and imprecision of those analytes with lowest and highest boiling points.

The on-column injection technique has been promoted as a method that does not suffer from such discrimination [14–16]. However, prevention of discrimination and peak distortion by this technique requires careful handling [15,16]. Other disadvantages that hamper its use in a routine setting include pollution of the analytical column with coextracted non-volatile or heat-labile components, when no precolumn is used, and the difficulty of combining it with an automated injection system. The latter problem necessitates the low-dead-volume coupling of a 0.5-mm precolumn to a 0.2-mm narrow-bore analytical column, which we found difficult to accomplish.

Recently, Van der Steege et al. [17] introduced the 'bracketing' calculation method for the quantification of MCFA in human milk, which makes use of the addition of equal masses of a series of odd-carbon-numbered MCFA and split injection. The use of half the sum of the peak areas of the two adjacent odd-carbon-numbered MCFA as an internal calibration standard area for the bracketed naturally occurring even-carbon-numbered species was found to be superior with respect to both accuracy and precision, when compared with the use of only one of each odd-carbon-numbered MCFA or 17:0. The result was that there was no need to incorporate response factors for the calculation of the final values.

This paper reports a systematic investigation of the merits of the bracketing method by analysing a standard mixture containing equal masses of 5:0–26:0 methyl esters by capillary GC with split injection. By varying the injection temperature, sample volume and split ratio, we investigated their influence on response factors and their coefficients of variation, and compared the results of the bracketing method with those obtained by using only one LCFA (either 17:0 or 23:0) as an internal calibration standard.

## EXPERIMENTAL

### *Standards and reagents*

Standard FAME (5:0–26:0) were obtained from Alltech/Applied Science (Amstelveen, The Netherlands). Reagents and other standards were obtained from Merck (Darmstadt, F.R.G.).

### *Standard solutions and priming sample*

A stock standard solution containing 500 mg each of the FAME (5:0–26:0) per litre of hexane was diluted in hexane to concentrations of 200 mg/l (standard 1), 150 mg/l (standard 2), 100 mg/l (standard 3), 66.67 mg/l (standard 4) and

50 mg/l (standard 5). For priming of the GC system we used a transmethylated human erythrocyte extract, prepared according to a previously described method [18]. Samples were stored at 4°C until analysis.

*Gas chromatography*

GC analyses were carried out on a Hewlett-Packard Model 5880 gas chromatograph (Avondale, PA, U.S.A.), equipped with a Model 7672A automatic injection system operated in the hot filled-needle mode [19], a split injector, a 50 m×0.20 mm I.D. (apolar) cross-linked methyl silicone-coated fused-silica column (film thickness 0.11 μm, Hewlett-Packard) and a flame ionization detector. The gas chromatograph was connected on-line to a Nelson Analytical Model 3000 data system (Cupertino, CA, U.S.A.). The splitter insert was filled with a glass wool beaded packing of 1 cm×4 mm, 3% OV-1 on Chromosorb W HP 80–100 mesh (Chrompack, Middelburg, The Netherlands). Split ratios ranged from 1:10 to 1:30 and injector temperatures from 220 to 280°C, dependent on the experimental setting (see below). The helium flow-rate was 0.5 ml/min and the oven temperature programme was 4 min at 80°C, 4°C/min to 280°C and 10 min at 280°C. The detector temperature was 300°C.

*Experiments*

Experiments were preceded by a two-fold injection of 2 μl of the priming sample. The data for each condition tested within one experiment were collected by serial eight-fold analysis of one of the FAME standards. Except for the study of the day-to-day performance each experiment was done within one series.

*Injection temperature dependence.* Aliquots (2 μl) of FAME standard 3 were analysed at a split ratio of 1:20, at injection temperatures of 220, 240, 260 and 280°C.

*Split ratio dependence.* Aliquots (2 μl) of indicated FAME standards were analysed at an injection temperature of 260°C, at split ratios of 1:10 (standard 5), 1:20 (standard 3) and 1:30 (standard 2).

*Injection volume dependence.* Aliquots of 1 μl (standard 1), 2 μl (standard 3) and 3 μl (standard 4) of the FAME standards were analysed at an injection temperature of 260°C and a split ratio of 1:20.

*Day-to-day performance.* Aliquots (2 μl) of FAME standard 3 were analysed at an injection temperature of 260°C and a split ratio of 1:20 on four different days. Each series was preceded by the usual priming procedure.

*Data processing*

Peak areas of the gas chromatograms, each containing 22 FAME peaks, were calculated by the Nelson data system and further processed by means of a spreadsheet program (Enable 1.0; The Software Group, New York, NY, U.S.A.) on a personal computer (Olivetti M24; Ivrea, Italy).

For each condition within one experiment we calculated the means and coefficients of variation (C.V.) of the following parameters:

(1) normalized peak areas (peak area of each analyte as a percentage of the total area of all 22 FAME);

(2) reciprocal response factors using either 17:0 or 23:0 as an internal standard (peak area of each analyte divided by that of either 17:0 or 23:0);

(3) bracketed reciprocal response factors (peak area of each analyte divided by half the sum of the peak areas of the FAME with one methylene unit less and the one with one methylene unit more; for obvious reasons not possible for 5:0 and 26:0).

For each experiment the overall means and coefficients of variation of these parameters were calculated by considering the mean (intra-parameter result) calculated for each condition within that experiment. After smoothing by a three-point moving average technique [20], the results were plotted as a function of the carbon chain-length of the FA.

## RESULTS AND DISCUSSION

### *Design of experiments and data processing for visual interpretation*

To minimize differences in the errors made during integration of FAME peak areas, the amounts of FAME that theoretically reached the column (10 ng per FAME) were held constant during testing of the various conditions within the experiments.

After repeated injections of any standard we observed a gradual, but proportional, increase of the absolute peak areas of all FAME. This phenomenon, which apparently did not influence the relative peak areas of the FAME, was explained by a priming (saturating) effect of the GC system and could be prevented by the initial injection of a transmethylated extract of biological origin.

Plotting the results of relative peak areas, reciprocal response factors and bracketed reciprocal response factors, we found consistent deviations from visual trends for some FAME (notably 12:0, 15:0, 17:0, 24:0 and 26:0; see also Fig. 1), which were explained by their lack of purity. To facilitate visual interpretation of the discrimination trend, these irrelevant deviations were filtered out by smoothing of the graphs.

### *Effects of injection parameters and day-to-day performance*

Fig. 2 shows the data of the mean normalized FAME peak areas (top) and their coefficients of variation (bottom) obtained from the experiment in which the split ratio was varied from 1:10 to 1:30. Such split ratios seem relevant to the majority of FAME analyses in clinical chemical practice. The top graph indicates poor reproducibility of normalized peak areas as a function of split ratio and considerable deviations from the theoretical value ($100/22 = 4.55\%$) especially for the lowest boiling FAME. The interpretation of the shape of the top graph in terms of processes taking place during injection has been discussed by Wang et al. [16], who studied a series of $C_{12}$–$C_{32}$ alkanes. The coefficients of variation of the relative peak areas at one split ratio (bottom) were the lowest for FAME with carbon chain-lengths of $C_{14}$–$C_{18}$ and increased with both increasing and decreasing chain-lengths. Similar trends were noted when the injection temperature and the volume were varied and when the day-to-day performance under one condition was investigated.

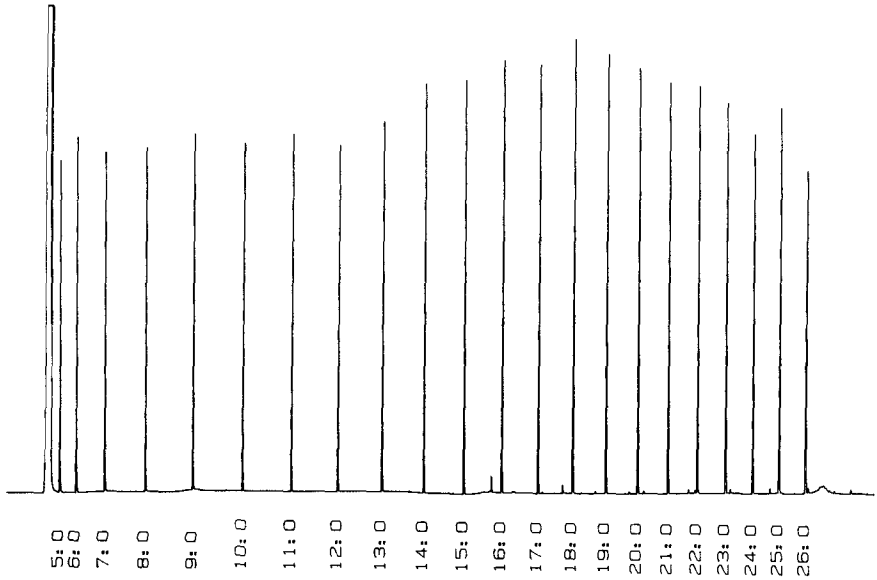


Fig. 1. Example of a chromatogram of 22 saturated FAME, prepared by capillary GC on an apolar stationary phase, using the split injection technique.

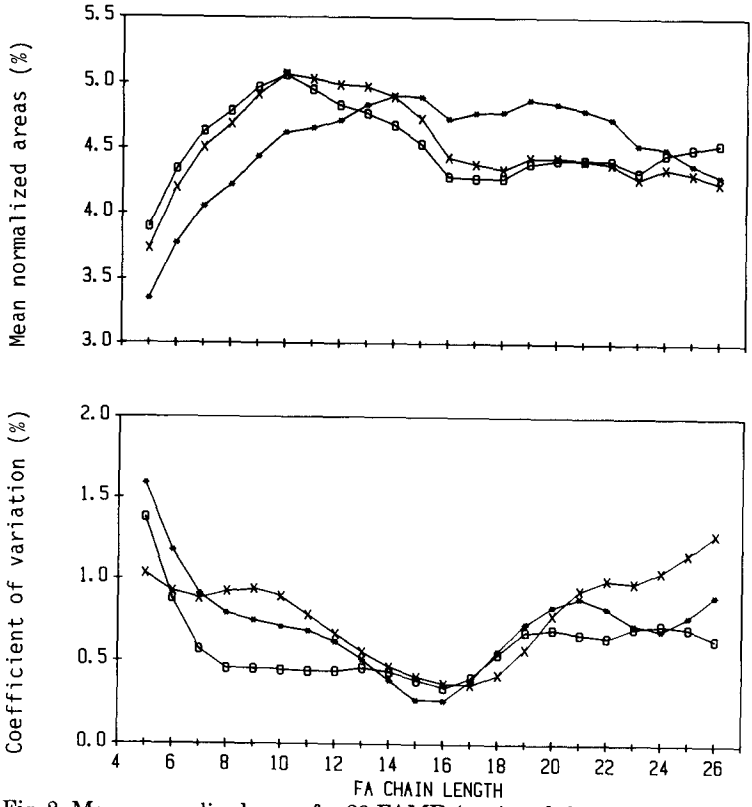


Fig. 2. Mean normalized areas for 22 FAME (top) and their coefficients of variation (bottom) determined for three different split ratios. Each point represents the mean of eight within-series determinations of a standard FAME mixture. The curves were plotted after a single three-point smoothing of the data. (*) split ratio 1:10; (×) split ratio 1:20; (○) split ratio 1:30.

TABLE I

COMPLETE SET OF MEAN RECIPROCAL RESPONSE FACTORS AND THEIR COEFFICIENTS OF VARIATION FOR 6:0, 16:0 AND 24:0, OBTAINED UNDER INDICATED CONDITIONS

Brack., bracketing method; 17:0, 17:0 as internal standard; 23:0, 23:0 as internal standard; interparameter variation indicates the mean and C.V. of the overall means of all experiments.

| Experiment | 6:0 | | | | | | 16:0 | | | | | | 24:0 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Brack. | | 17:0 | | 23:0 | | Brack. | | 17:0 | | 23:0 | | Brack. | | 17:0 | | 23:0 | |
| | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) | Mean | C.V. (%) |
| Injection temperature: | | | | | | | | | | | | | | | | | | |
| 220°C | 1.0717 | 0.12 | 0.9797 | 1.61 | 0.8956 | 2.41 | 1.0445 | 0.16 | 1.0780 | 0.32 | 0.9788 | 1.31 | 0.9185 | 0.15 | 0.9850 | 1.70 | 0.9002 | 0.59 |
| 240°C | 1.0645 | 0.27 | 0.9907 | 1.87 | 0.9040 | 1.62 | 1.0282 | 0.16 | 1.0399 | 0.44 | 0.9490 | 1.02 | 0.9044 | 0.14 | 0.9832 | 0.74 | 0.8972 | 0.22 |
| 260°C | 1.0682 | 0.05 | 1.0421 | 1.00 | 1.0011 | 1.61 | 1.0303 | 0.10 | 1.0757 | 0.15 | 1.0333 | 0.99 | 0.9109 | 0.21 | 0.9714 | 0.93 | 0.9330 | 0.26 |
| 280°C | 1.0677 | 0.13 | 0.8995 | 1.33 | 0.8444 | 1.40 | 1.0329 | 0.06 | 1.0561 | 0.21 | 0.9913 | 0.41 | 0.9015 | 0.08 | 0.9636 | 0.28 | 0.9045 | 0.14 |
| Overall | 1.0680 | 0.28 | 0.9780 | 6.03 | 0.9113 | 7.18 | 1.0340 | 0.70 | 1.0606 | 1.52 | 0.9881 | 3.54 | 0.9088 | 0.83 | 0.9758 | 1.04 | 0.9087 | 1.81 |
| Split ratio: | | | | | | | | | | | | | | | | | | |
| 1:10 | 1.0710 | 0.50 | 0.8572 | 1.42 | 0.8452 | 1.64 | 1.0342 | 0.04 | 1.0497 | 0.27 | 1.0350 | 0.72 | 0.9047 | 0.08 | 0.9138 | 0.53 | 0 9010 | 0.24 |
| 1:20 | 1.0682 | 0.05 | 1.0421 | 1.00 | 1.0011 | 1.61 | 1.0303 | 0.10 | 1.0757 | 0.15 | 1.0333 | 0.99 | 0.9109 | 0.21 | 0.9714 | 0.93 | 0.9330 | 0.26 |
| 1:30 | 1.0680 | 0.11 | 1.1053 | 1.77 | 1.0258 | 1.55 | 1.0282 | 0.07 | 1.0633 | 0.35 | 0.9868 | 0.87 | 0.9035 | 0.18 | 1.0156 | 1.13 | 0.9425 | 0.37 |
| Overall | 1.0691 | 0.16 | 1.0015 | 12.90 | 0.9574 | 10.20 | 1.0309 | 0.30 | 1.0629 | 1.23 | 1.0184 | 2.69 | 0.9064 | 0.44 | 0.9669 | 5.28 | 0.9255 | 2.35 |
| Volume: | | | | | | | | | | | | | | | | | | |
| 1 μl | 1.0651 | 0.08 | 0.9431 | 1.08 | 1.0528 | 1.28 | 1.0357 | 0.07 | 1.0673 | 0.15 | 1.1916 | 1.40 | 0.9019 | 0.09 | 0.7927 | 1.49 | 0.8848 | 0.20 |
| 2 μl | 1.0682 | 0.05 | 1.0442 | 1.00 | 1.0011 | 1.61 | 1.0303 | 0.10 | 1.0757 | 0.15 | 1.0333 | 0.99 | 0.9109 | 0.21 | 0.9714 | 0.93 | 0.9330 | 0.26 |
| 3 μl | 1.0707 | 0.07 | 1.2149 | 1.62 | 1.2914 | 1.62 | 1.0317 | 0.10 | 1.1072 | 0.31 | 1.1771 | 1.56 | 0.9054 | 0.21 | 0.9439 | 1.20 | 1.0032 | 0.24 |
| Overall | 1.0680 | 0.26 | 1.0667 | 12.90 | 1.1151 | 13.90 | 1.0326 | 0.27 | 1.0834 | 1.94 | 1.1340 | 7.72 | 0.9061 | 0.50 | 0.9027 | 10.60 | 0.9403 | 6.33 |
| Day-to-day: | | | | | | | | | | | | | | | | | | |
| Day 1 | 1.0691 | 0.17 | 1.0108 | 1.29 | 0.9405 | 1.92 | 1.0326 | 0.07 | 1.0761 | 0.21 | 1.0012 | 1.04 | 0.8973 | 0.08 | 0.9791 | 0.84 | 0.9109 | 0.40 |
| Day 2 | 1.0689 | 0.18 | 0.9529 | 2.04 | 0.8969 | 2.01 | 1.0322 | 0.07 | 1.0576 | 0.17 | 0.9955 | 0.87 | 0.8979 | 0.11 | 0.9683 | 0.98 | 0.9114 | 0.20 |
| Day 3 | 1.0689 | 0.19 | 1.0205 | 1.54 | 0.9491 | 1.43 | 1.0306 | 0.06 | 1.0611 | 0.29 | 0.9869 | 1.47 | 0.9001 | 0.09 | 0.9796 | 1.33 | 0.9109 | 0.18 |
| Day 4 | 1.0682 | 0.05 | 1.0421 | 1.00 | 1.0011 | 1.61 | 1.0303 | 0.10 | 1.0757 | 0.15 | 1.0333 | 0.99 | 0.9109 | 0.21 | 0.9714 | 0.93 | 0.9330 | 0.26 |
| Overall | 1.0688 | 0.04 | 1.0066 | 3.79 | 0.9469 | 4.52 | 1.0314 | 0.11 | 1.0676 | 0.91 | 1.0042 | 2.02 | 0.9016 | 0.70 | 0.9746 | 0.58 | 0.9166 | 1.20 |
| Interparameter variations | 1.0685 | 0.10 | 1.0132 | 4.64 | 0.9827 | 4.11 | 1.0322 | 0.24 | 1.0686 | 0.41 | 1.0362 | 2.47 | 0.9057 | 0.20 | 0.9550 | 4.88 | 0.9228 | 2.51 |

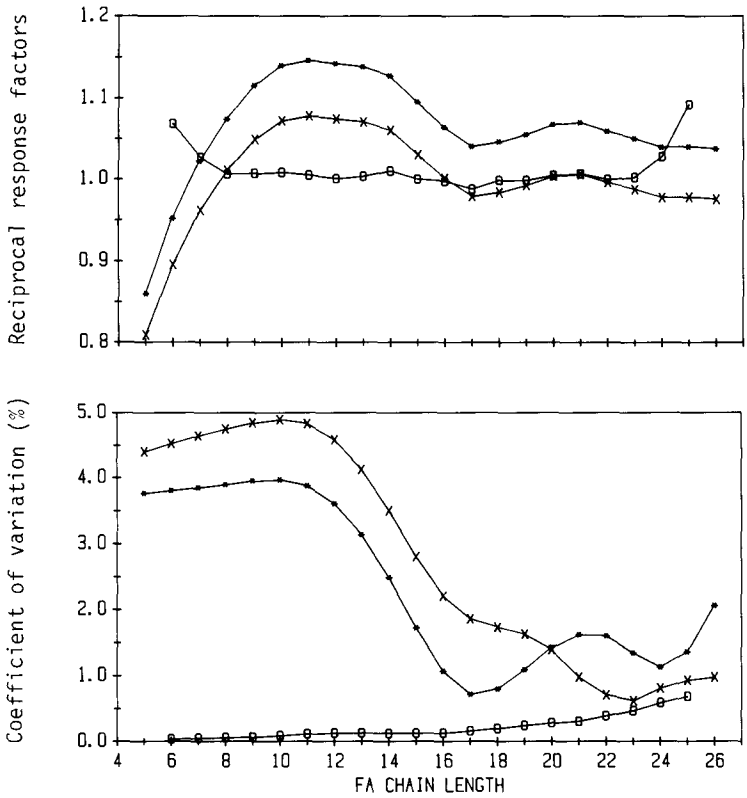


Fig. 3. Day-to-day mean reciprocal response factors (top) and their coefficients of variation (bottom) determined under one condition. Each point represents the mean of four series. Each series consisted of eight determinations of one standard FAME mixture. The curves were plotted after a double three-point smoothing of the data. (*) 17:0 as internal standard; (×) 23:0 as internal standard; (○) bracketing method.

Fig. 3 depicts the mean overall day-to-day reciprocal response factors (top) and their coefficients of variation (bottom), calculated by using the peak areas of 17:0, 23:0, or half the sum of the peak areas of the FAME with one carbon atom more and less (bracketing method) as an internal standard area. The data show considerable variance in the reciprocal response factors (top), with high coefficients of variation notably for 6:0 to 12:0 (bottom), when 17:0 and 23:0 were used as internal standards. Conversely, the bracketing method led to fairly constant reciprocal response factors, which were close to unity with the lowest coefficient of variation. The data obtained by varying the injection conditions showed a similar picture.

Table I presents the complete set of mean reciprocal response factors and their coefficients of variation for selected FAME obtained under various conditions. Statistical data on the overall means of all experiments are given as an inter-parameter variation at the bottom. The data confirm that for two FAME at the extremes of the range (6:0 and 24:0) and for one in the middle (16:0) the bracketing method is superior to the use of only one FAME as an internal standard.

*Generalization of the bracketing method for FAME analyses*

The bracketing technique is a linear interpolation method based on the assumption that, using a sufficiently small interval not wider than for instance two methylene units, an internal standard with one methylene unit less than the analyte is about as much underestimated as an internal standard with one methylene unit more is overestimated (or vice versa), owing to boiling point-dependent discrimination during injection. The method can be applied only to samples that do not contain appreciable amounts of odd-carbon-numbered FA. In a more general sense, quantification of an analyte X (see Fig. 4) by the bracketing method may be performed by dividing the area of the analyte by the weighted mean of the areas of the internal standards with one carbon atom less (Y) and more (Z), using the retention intervals (related to boiling point intervals) as weights:

$$\text{Amount X} = \frac{(a+b) \times \text{area X}}{b \times \text{area Y} + a \times \text{area Z}} \times p \text{ (kg)}$$

in which amount Y = amount Z = $p$ (kg).

Merely dealing with saturated FAME, the retention intervals $a$ and $b$ were about the same (see Fig. 1), so we refrained from incorporating the actual retention interval weight factors. However, dealing with unsaturated FAME these mass factors may be of importance for reaching the highest possible grade of accuracy and precision with this technique.

Under all circumstances it should be appreciated that the weighted bracketing method for unsaturated FAME discussed above applies only to analytes and their internal standards that elute during linear temperature programming of apolar stationary phases, which may be expected to separate compounds on the basis of their boiling points. Under isothermal conditions, the logarithm of the retention intervals (as in the establishment of Kovats' indices) may serve as a weight fac-

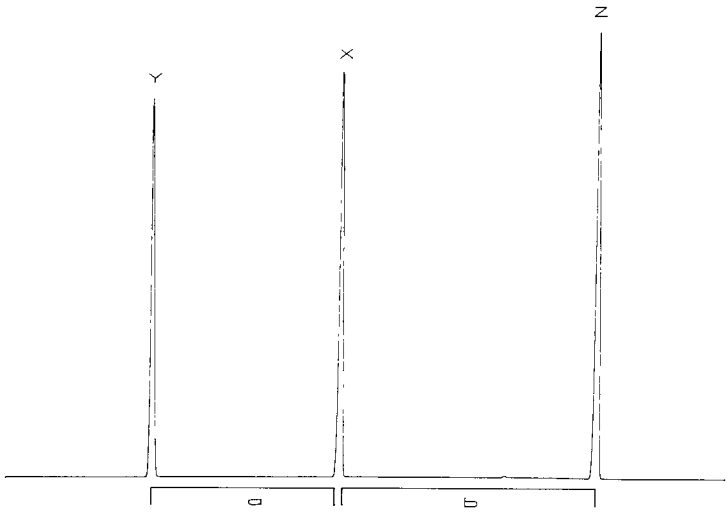


Fig. 4. Part of a gas chromatogram illustrating the general applicability of the bracketing method for analyte X and its internal standards Y and Z: $a$ and $b$ are the retention intervals between the analyte X and the internal standards Y and Z, respectively.

tor, whereas in applications that employ polar stationary phases, the use of the actual boiling point intervals may circumvent the problem of unavailability of retention intervals that merely relate to boiling points. Another, more practical, possibility is the use of retention intervals adopted from apolar stationary phases, which can be obtained from equivalent chain lengths (ECL) [21,22].

*Practical consequences for FAME analyses*

As may be determined from Fig. 3, boiling point-related discrimination of 2-$\mu$l samples injected into a 1:20 split injector at 260°C particularly affected FAME with carbon chain-lengths between $C_5$ and $C_{15}$. However, the vast majority of naturally occurring non-volatile saturated and unsaturated FA in human tissues and extracellular fluids contain chain-lengths between $C_{14}$ and $C_{24}$, which implies that their quantification by using a single odd-carbon-numbered internal standard of high purity (preferably 17:0 or 23:0 but not 15:0, see Fig. 3) may result in reasonable accuracy and precision of the assay. As estimated from our data, 10-ng amounts of $C_{14}$–$C_{24}$ FAME on-column, using 17:0 as an internal standard, may be determined with coefficients of variation between 1 and 3%.

A clear exception is human milk, which contains both MCFA and LCFA [23]. The MCFA in milk, notably 12:0 and 14:0 [12,23–27], constitute a particularly interesting fraction, as it is exclusively synthesized in the mammary gland from glucose [24–26]. This means that the MCFA/LCFA ratio in human milk grossly relates to the ratio of endogenously synthesized FA and those of extra mammary origin. Interestingly, when compared with data from 'Western' countries, we found higher relative amounts of MCFA (bracketing method applied) in milk from Curaçaon mothers [12,17]. It is unlikely that this difference points to different physiological backgrounds, as dietary habits on the island of Curaçao are essentially 'Western'. Both loss of transmethylated MCFA during sample preparation and injection-related discrimination may have resulted in an underestimation of the MCFA fraction measured by others.

We conclude that when MCFA and LCFA are simultaneously determined by capillary GC with split injection the bracketing method for MCFA leads to the highest GC accuracy and precision. The addition of odd-carbon-numbered MCFA prior to transmethylation may additionally correct for losses during transmethylation and subsequent sample processing. The bracketing method may also be applicable to other GC assays in which compounds with divergent boiling points are simultaneously determined. In situations where thermal degradation of the analytes in the column plays an important role, as is the case with high-temperature GC analyses of triglycerides [28,29], the bracketing method, using suitable synthetic triglyceride internal standards, may also be applicable for the correction for this process within well defined retention intervals, provided that the existence of a linear relationship between the degree of decomposition and the retention time can be demonstrated within that interval. Preliminary results, obtained in our laboratory using split injection on a 25 m$\times$0.25 mm I.D. WCOT Triglyceride-Analysis-Phase (0.1-$\mu$m 50% phenyl–50% methyl polysiloxane, Chrompack) and helium as a carrier gas, show that this is indeed possible for the

analysis of saturated triglycerides, but that additional correction factors are needed for those species containing polyunsaturated FA.

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