CHROM. 4113

COMPUTERIZED QUANTITATIVE ANALYSIS OF METHYL AND ETHYL ESTERS OF LONG CHAIN FATTY ACIDS BY GAS–LIQUID CHROMATOGRAPHY USING RELATIVE MOLAR RESPONSE

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(Received March 20th, 1969)

SUMMARY

1. The possibility that the relative molar response method could be applied for quantitative analyses of saturated and unsaturated fatty acid methyl esters and ethyl esters into two double bonds was verified. This method was applied to quantitative analyses of fatty acids of individual lipid classes of blood serum.

2. Computerized calculations, rather than those made manually or with desk calculating machines, of the fatty acid ester composition substantially reduced working time; while the calculation of forty equations with a desk calculating machine took six to eight hours, computerized calculations, including the printing of results, were effected in less than one minute.

3. The main advantage of this method was its ability to obtain quantitative information about the fatty acid ester spectrum which in a pure state was unobtainable and could not be analyzed either by the internal standard or by the direct calibration method.

4. The accuracy of the relative molar response method was identical with other methods when all quantitative analysis conditions were carefully controlled. For components larger than 10 % of the sample, the error was less than 2 % (relative); for components constituting 5–10 % of the sample, the error was 3–5 % (relative); and for smaller components (especially with short elution times), the error might reach 10 % (relative).

INTRODUCTION

Quantitative gas–liquid chromatographic analyses of different substances use either an internal standard or a direct calibration method[1]. The internal standard method, rather than that of direct calibration, is more frequently used in long chain fatty acid ester analyses[2–4]. Both methods require three or more standards[5] based on certain requirements[6]. The analytical conditions must be precise and be periodically calibrated in order to ensure both accuracy and reproducibility. Accurate results are influenced not only by carefully controlled column temperatures and flow rates of

the carrier gas but also by a controlled injected sample volume and flash heater temperature. However, regularity in the result is not only influenced by these parameters[7,8]; it is also influenced by the supporting material, the stationary phase, the carrier gas and the detector. Because some parameters such as carrier gas flow rate, column temperature, detector ionization voltage, etc. vary both during the analysis and daily, either a recheck or a new calibration is important. The advantage of the internal standard method is that some operations can be quantitatively checked with the internal standard during the sample preparation[2].

The standards are no longer needed when using the relative molar response method[5,9], based on the detector response size relation which is expressed by the area under the fatty acid ester peak compared with the number of carbon atoms in this acid for equimolar amounts of fatty acid esters. The relation for calculating the fatty acid methyl ester composition[5] permits a computerized evaluation of analyses. Previous computerized calculations of fatty acid ester compositions used either the current internal standard[2] or direct calibration methods[10].

INSTRUMENTS AND CHEMICALS

Instrument: Chromatograph Chrom III–IKZ (Laboratory Instruments, National Enterprise, Prague). Computer: Minsk 22. Fatty acid esters: Mixture KD (Applied Science Laboratories, Inc., Pa., U.S.A.). Mixtures of long chain fatty acid methyl esters and ethyl esters (palmitic acid, oleic acid, linoleic acid) were prepared in our laboratory from pure acids. Supporting material: Chromosorb W 80/100 mesh, acid washed (Carlo Erba, Milan, Italy). Stationary phase: DEGS (Carlo Erba, Milan, Italy). All solvents used were rectified, and their purity was checked by GLC.

ANALYTICAL CONDITIONS

Column: glass, length 2.0 m, I.D. 3 mm. Packing: 10 % DEGS on Chromosorb W 80/100 mesh, acid washed. Temperatures: oven 185°, injection port 270°. Carrier gas: nitrogen, flow rate 25 ml/min, inlet pressure 1.0 atm, atmospheric outlet pressure. Detection: flame ionization detector.

TABLE I

RESULTS OF ANALYSES OF SATURATED AND UNSATURATED FATTY ACID METHYL ESTERS IN KNOWN COMPOSITION (MIXTURE KD) MADE BY THE RELATIVE MOLAR RESPONSE METHOD

The values mentioned represent the average of five determinations. Peak areas were measured by the triangulation method. The results indicated by *A* were obtained by identical analyses, except that for the unsaturated acid esters, the *C* values for saturated acid esters with an identical number of carbon atoms were used in the calculations.

Number C: double bond number	*Actual content (Wt. %)*	*Found (Wt. %)*	*Average deviation (rel. %)*	*Found A (Wt. %)*	*Average deviation (rel. %)*
14:0	11.80	11.87	0.59	11.83	0.25
16:0	23.60	23.43	0.73	23.35	1.06
16:1	6.90	6.83	1.02	6.87	0.44
18:0	13.10	13.34	1.83	13.30	1.76
18:1	44.60	44.43	0.39	44.58	0.05

TABLE II

COMPARISON OF RESULTS OF ANALYSES FOR METHYL ESTERS AND ETHYL ESTERS OF IDENTICAL SATURATED AND UNSATURATED ACIDS

The values mentioned represent the average of five determinations. For explanation of the values indicated by *A*, see Table I.

Number C: double bond number	*Actual content (Wt. %)*	*Found (Wt. %)*	*Average deviation (rel. %)*	*Found A (Wt. %)*	*Average deviation (rel. %)*
Methyl esters					
16:0	32.00	32.65	2.0	32.40	1.3
18:1	3.00	3.08	2.7	3.08	2.7
18:2	65.00	64.26	1.1	64.47	0.8
Ethyl esters					
16:0	32.00	32.66	2.0	32.38	1.2
18:1	3.00	3.10	3.3	3.10	3.3
18:2	65.00	64.25	1.1	64.46	0.8

TABLE III

COMPARISON OF RESULTS OF IDENTICAL ANALYSES IN WHICH THE PEAK AREA OF AN INDIVIDUAL PEAK WAS INTEGRATED BOTH BY MULTIPLYING THE PEAK HEIGHT BY ITS WIDTH AT THE BASE LINE, AND BY THE TRIANGULATION METHOD

The results given in Table I (the succession of *A* values) were used for calculations in both the triangulation method and the integration method (multiplying height by width). This means that in the calculations for unsaturated components, the *C* values for saturated components with an equal number of carbon atoms were used.

Number C: double bond number	*Actual content (Wt. %)*	*Triangulation*		*Height × width*	
		Found (Wt. %)	*Average deviation (rel. %)*	*Found (Wt. %)*	*Average deviation (rel. %)*
14:0	11.80	11.83	0.25	11.73	0.59
16:0	23.60	23.45	1.06	23.53	0.29
16:1	6.90	6.87	0.44	6.76	2.07
18:0	13.10	13.30	1.76	13.00	0.76
18:1	44.60	44.58	0.05	44.92	0.71

METHOD

The relative molar response method was applied to methyl esters of long chain saturated fatty acids, and its use for a mixture of saturated and unsaturated fatty acid methyl esters (Mixture KD) was evaluated satisfactorily (Table I). Applicability of this method for ethyl esters of long chain fatty acids (ethyl palmitate, ethyl oleate, ethyl linoleate) was satisfactorily verified (Table II). The area was integrated either by triangulation or by multiplication of the peak height by the base line width. The results are compared in Table III.

To simplify data for the computer, the following original equation was used.

$$\frac{AB_1x}{10n_1} MW_1 + \frac{AB_2x}{10n_2} MW_2 + \ldots + \frac{AB_nx}{10n_n} MW_n = 100\,\% \qquad (1)$$

where

B_1–B_n are areas under peaks of individual fatty acid esters;

n_1–n_n are numbers of carbon atoms in individual fatty acid molecules;

MW_1–MW_n are molecular weights of individual fatty acid esters;

A is the theoretical area of one mole divided by the actual area of one mole of fatty acid ester;

The tenth multiple of the number of carbon atoms in a fatty acid molecule is the value of the theoretical area for one mole;

x is the number of micromoles of individual fatty acids;

n is the number of components in the mixture, of which the composition is being calculated.

The form is restated as

$$\sum_{i=1}^{n} AB_iC_ix = 100 \qquad (2)$$

where

$$C_i = MW_i/10n_i$$

The components are established by

$$D_ix = AB_iC_ix$$

and from eqn. 2 it follows that

$$x = \frac{100}{A \sum_{i=1}^{n} B_iC_i} \qquad (3)$$

After substitution into eqn. (2) we obtain

$$D_ix = AB_iC_ix = \frac{100\, B_iC_i}{\sum_{i=1}^{n} B_iC_i} \qquad (4)$$

The value of factor D_ix expressed in percentage with respect to the factor 100 is not A-dependent, therefore, A is not necessary in the calculation. Accuracy in calculating D_ix depends only on the accuracy in measuring B_i and C_i. Using a small Minsk 22 computer, the solution and printing of the results of forty equations occurred in less than one minute. A program in autocode MAT was used.

PROGRAM

```
INTEGER I:k:M
REAL A(2):B(50):C(50):D(50):X:P
FUNCTION RPRINT
REF 5
(1) P = 100
OUTDEV
LINES 15
SPACES 35
TITLE EVALUATION GLC ANALYSIS BY RELATIVE MOLAR RESPONSE METHOD +
LINES 12
TITLE 1 2 3 4 5 6 7 8 9 10 11 12 +
LINES 2
FOR I = 1:1:. 126
OUTPUT + 17
REPEAT I
LINES 5
ADR7727 = 0
(2) READ M'K
D = 0
FOR . I = 1:1:. K
READ BI'CI
D = D + DI
REPEAT I
X = P/D
FOR I = 1:1:. K
DI = DI.X
REPEAT I
WRITE M'4:0
FOR I = 1:1:. K
SPACE
WRITE DI, 2:2
REPEAT I
LINES 2
GO TO 2
(5) LINES 10
SPACES 45
TITLE CYBERNETICS DEPARTM OF COMMON MEDICINE CHUNIV +
LINES 5
STOP + 7777
START 1
```

This method was applied to the calculations of the quantitative composition of long chain fatty acid methyl esters and ethyl esters which were obtained by reesterification of individual lipid classes of blood serum, separated by both column and thin-layer chromatography.

The arrangement of input data is in praxis as follows:

$$K \quad n \quad \begin{matrix} B_1 & C_1 \\ B_2 & C_2 \\ \vdots & \vdots \\ B_n & C_n \end{matrix}$$

where

K designates pertinent chromatograms by a serial numeral;
n is the number of compounds in the analyzed spectrum;
B and C are identical to the symbols mentioned on p. 438.

EVALUATION OF RESULTS

As shown in Tables I and II, the method is applicable in quantitative analyses of methyl esters and of ethyl esters of saturated and unsaturated fatty acids; this was verified in unsaturated fatty acids having two double bonds. Calculation of unsaturated acid esters using the C_t values for saturated acids having an equal number of carbon atoms yields better results than by using C_t values obtained from the formula derived from eqn. (2) (Tables I and II).

Error in the method for components constituting more than 10% of the sample is less than 2% (relative); for components in 5–10% of the sample, error is 3–5% (relative); and for smaller components, especially fatty acid esters, with short elution times, error is up to 10% (relative). This result corroborates other observations[6]. This error may be reduced by applying a more precise method of area calculation, *e.g.* by using an electrical integrator. The use of other manual integration methods, such as cutting out and weighing individual peaks or submitting areas to perimeter proceeding, does not increase accuracy. Comparison of results obtained from identical analyses, using either triangulation or calculation of peak areas by multiplying the peak height by its base line width, demonstrates that the error is substantially the same for both methods (Table III).

TABLE IV

FATTY ACID COMPOSITION OF SOME TYPES OF LIPIDS IN BLOOD SERUM

Samples were analyzed in ethyl ester form. Acids which were found in the sample in amounts below 0.2% were not taken into consideration, since calculations showed that the resulting error was negligible.

Number C: double bond number	*Triglyceride (Wt. %)*		*1,3-Diglyceride (Wt. %)*		*1,2-Diglyceride (Wt. %)*	
12:0	0.37	0.39	—	—	—	—
14:0	3.31	3.24	1.80	1.83	2.10	2.14
15 br.	0.43	0.44	0.35	0.44	0.60	0.62
15:0	0.52	0.47	0.62	0.63	0.70	0.65
16:0	31.24	31.98	27.40	26.80	28.95	28.53
16:1	7.06	6.92	6.37	6.42	6.15	6.42
17:0	0.72	0.71	0.67	0.75	0.52	0.71
17:1	0.71	0.66	0.63	0.71	0.70	0.73
18:0	4.97	4.91	3.15	3.30	3.31	3.28
18:1	37.97	37.55	47.01	47.21	46.21	45.58
18:2	12.71	12.73	12.00	11.90	12.07	11.87

The advantage of the relative molar response method is that standards which are not easily accessible for some compounds, especially for analyses of biological materials, are not required. Furthermore, this method is rapidly calibrated and rechecked by instrumentation. On the other hand, any error in integrating the area of one peak distorts the results of all other analyzed spectrum components. It was verified, however, that if measurements of individual peak size (with accuracy of $\pm$0.2 mm), are precise, *e.g.* by slide rule, the error is not higher than that for other quantitative analyses. Requirements for measuring peak sizes and for parameter stability are identical to those in other quantitative methods[11].

A suitable example of the relative molar response method is the quantitative analysis of fatty acids contained in individual lipid classes of blood serum. Some fatty acids are found which are not readily obtained in the pure state, *e.g.* heptadecenoic acid, eicosatrienoic acid, etc. Although the level of these acids is not high, they cannot be neglected. Analytical results for some lipid classes of blood serum are summarized in Table IV. Results are given in weight percentage. Paired analytical results of the same sample demonstrate the reproducibility of this method.

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