Journal of Chromatography, 132 (1977) 543–547

CHROM. 9714

Note

Gas chromatographic analysis of lipids with the injection of aliquots of less than 0.05 μl from small samples

A. V. ZHUKOV and V. P. PCHELKIN

Lipid Biochemistry Research Unit, Institute of Plant Physiology, Academy of Sciences, Moscow (U.S.S.R.)

(First received May 24th, 1976; revised manuscript received August 31st, 1976)

The injection of aliquots on a micro-scale for column separations remains a problem in gas–liquid chromatography (GLC)[1,2], and particularly great difficulties are encountered when a sample of small volume is to be analyzed.

In our work on the extraction and fractionation of polar lipids from soybean seeds[3], it became necessary to inject micro-aliquots from small samples of fatty acid methyl esters (FAME), so that the chromatograms obtained could be used for the quantification of FAME. To this end, the response of the major peak on these chromatograms (that of methyl linoleate, H mV) in relation to the total recorder scale (10 mV) must meet the condition $5 \text{ mV} < H < 10 \text{ mV}$ (ref. 4). Methods for the injection of aliquots proposed earlier proved to be inadequate for solving this problem.

In this paper, an efficient two-step method for the injection of micro-aliquots of FAME from samples of small volume using a glass capillary is described.

EXPERIMENTAL

Reagents and materials

Reagent-grade *n*-dodecane, *n*-tetradecane and *n*-hexadecane were used without further purification. Other reagents were purified as described earlier[4].

The dimensions of the glass capillaries used for injection were length 7 mm and I.D. 0.15 mm (measured in a light microscope); if the internal diameter was less than 0.1 mm, free release of liquid from the channel was hindered.

Determination of the hydrocarbon loss in vacuo

Mixtures of 50.0 mg of *n*-alkane and 0.4 ml of benzene or the pure *n*-alkanes were kept for 30–120 min in a vacuum produced by a water-jet pump at room temperature, 35 ± 1° or 50 ± 1°. The loss of hydrocarbons was calculated as a percentage of their original weight. Completeness of the removal of benzene from its mixture with *n*-hexadecane was determined by GLC; the separation conditions were as described earlier[4], except that the column temperature was 125° and the flow-rate of the carrier gas was 2 ml/min.

Determination of FAME in small samples (<0.5 μl) and injection of aliquots into the column

To x μl of FAME* prepared from soybean seed lipids[4] and placed in a 4.0 × 0.8 cm test-tube having a silanized internal surface, v' μl of toluene were added. The capillary, with a channel volume of v'' μl, was completely filled with solution and immediately introduced into a column of a Pye argon chromatograph using a closed injection system[5]. The conditions for the FAME analysis were standard[4]; the methyl linoleate peak height (h mV) was determined visually. Toluene was evaporated *in vacuo* from the FAME solution in the test-tube at 50° and to the FAME sample v μl of *n*-hexadecane (as a benzene solution) were added; the calculation of v and possible numerical values of x, v', v'' and h are discussed below. Benzene was stripped *in vacuo* for 45 min at 35° and the capillary was filled with a solution of FAME in *n*-hexadecane; the subsequent analytical procedure was as described above. During the intervals between successive injections, the solution was kept *in vacuo* at room temperature.

RESULTS AND DISCUSSION

The high sensitivity of modern GLC detectors simplifies the task of analysis of small samples; *e.g.*, if an argon detector is used, 0.015 μl of FAME is sufficient for their composition to be determined. The most difficult step of this analysis is the removal of a suitable aliquot from such a sample[1,2]. To this end, a sample of small volume is usually dissolved in a low-boiling organic solvent[1]. However, it is impossible to maintain a constant concentration of FAME in this solution, and therefore successive aliquots would show marked differences in FAME content.

It was suggested that the concentration of the FAME solution can be maintained constant throughout the analysis if the volatile solvent is replaced by a non-volatile solvent. In preliminary experiments, it was found that *n*-hexadecane, unlike other hydrocarbons such as *n*-dodecane and *n*-tetradecane, was the most suitable as non-volatile solvent (NS) for FAME, because the loss of its weight *in vacuo* at 35° during 45 min was less than 2%. The benzene used for the preparation of a stock solution of *n*-hexadecane can be removed completely *in vacuo* with virtually no loss of NS, because the boiling points of these solvents differ markedly. Moreover, *n*-hexadecane is readily available in a pure form (Fig. 1A).

If an arbitrary amount of NS (v μl) is added to a sample to be analyzed, the concentration of the solution thus obtained, and hence the volume of the sampled aliquot will be unknown. When this volume is too small ($H<5$ mV, *cf.* Fig. 1), the resulting chromatogram of FAME will be of no use either for an exhaustive qualitative identification of the components or for an accurate determination of the quantitative composition[4]. If, on the other hand, the aliquot injected into the column contains an excess of FAME, the separation efficiency will decrease drastically, and some peaks on the differential chromatogram will extend off-scale ($H>10$ mV), thus introducing difficulties in quantification[6,7]. Thus, if v is unknown, then, in order to obtain at least one chromatogram with $5\text{ mV}<H<10\text{ mV}$ upon injection of an

* The amounts of the substances to be analyzed are expressed in volume units, because this paper is concerned with methods of sampling by glass capillaries characterized by a definite internal volume.

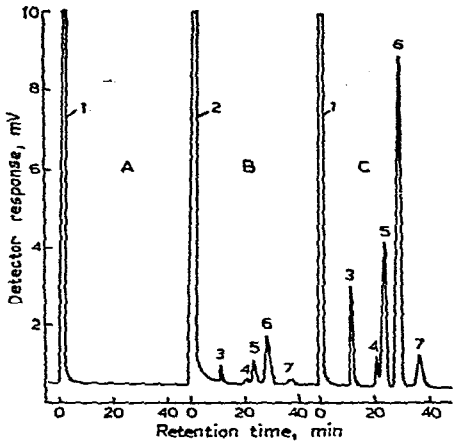

Fig. 1. Gas chromatograms of (A) *n*-hexadecane, (B) toluene solution of FAME and (C) *n*-hexadecane solution of FAME. Peaks: 1 = *n*-hexadecane; 2 = toluene; 3 = methyl palmitate; 4 = methyl stearate; 5 = methyl oleate; 6 = methyl linoleate; 7 = methyl linolenate.

aliquot of solution, it is necessary to determine by trial and error the optimal volume of an aliquot, resulting in wastage of the sample to be analyzed. It follows that the value of v should be adjusted so that the amount of FAME in v'' μl of the *n*-hexadecane solution injected into a column is such as to ensure optimal analytical results ($5\ mV < H < 10\ mV$).

To this end, it is necessary as a first step to determine the total amount of FAME in the sample, because it is usually not known beforehand. Precise weighing of 1-μl samples is fairly difficult, and therefore a special method for this determination was developed. The FAME sample was dissolved in v' μl of toluene; the use of benzene in this preliminary step was undesirable, because the concentration of this more volatile solvent in the solution would be less constant (see below). After injecting v'' μl of the toluene solution of the sample the height (h) of the chromatographic peak of the major FAME component was measured, and toluene stripped off. This h value was used as an arbitrary unit for determining the amount of FAME in the sample.

Furthermore, in order to obtain correct results it is necessary to calculate the optimal volume of NS (v value, see above). This calculation was based on the assumption that, upon repeated injections of aliquots originating from the same FAME sample but differing in the amount of FAME present, the methyl linoleate response would be inversely proportional to the volume of the respective solvent added to the sample. Some further assumptions were made: (1) the volume of the FAME sample to be analyzed is $x = 0.5$ μl; (2) preliminary sampling of small amounts of FAME as a toluene solution has no effect on the sample volume; (3) the injection of a v''-μl aliquot of a solution of FAME in *n*-hexadecane results in a methyl linoleate response of 8.5 mV (85% of total scale); (4) the width of the peak at half-height does not depend on the total height of the peak. The calculation of v follows.

It is clear that the total volume of the toluene solution of FAME is $(v' + x)$ μl (v' μl = toluene volume), the volume of FAME in 1 μl of this solution is $x/(v' + x)$ μl and the volume of FAME in the capillary filled with toluene solution is $v'' x/(v' + x)$ μl. Similarly, the volume of the aliquot of FAME in a solution of FAME in *n*-hexa-

decane is $v'' x/(v + x)$ μl. From our assumption, it follows that $h\, v'' x/(v + x) = 8.5\, v'' x/(v' + x)$ and $v = [h(x + v')/8.5] - x$.

As was mentioned above, the absolute volume of the FAME sample (x) used in the calculation is not known, because in the first step the value of x was calculated only as the arbitrary value h. However, substitution of $x = 0.3$–0.7 μl (at $v' = 20$ μl) in the equation for v has little effect on the results, and therefore the calculation was based on the assumption that $x = 0.5$ μl. Under these conditions, the value of h was usually in the range 0.5–2.0 mV. If it happened to be outside this range, this was regarded as an indication that $x < 0.3$ μl or $x > 0.7$ μl; in this instance the value of h could be restored to the range 0.5–2.0 mV by decreasing or increasing v', respectively.

Fig. 1B shows the chromatogram of FAME obtained after injection into the column of $v'' = 0.05$ μl of an approximately 3% solution in $v' = 20$ μl of toluene. It can be seen that $h = 0.9$ mV. From the above equation, $v = 1.7$ μl, and therefore v'' μl of FAME solution (*ca.* 0.015 μl of FAME) in $v = 1.7$ μl of NS were subjected to separation. The value of the methyl linoleate response in Fig. 1C ($H = 8.5$ mV) is equal to that mentioned previously. H values were used for the calculation of the accuracy and precision of the injection of aliquots of FAME. To this end, the arithmetic mean ($\bar{x}$) and relative standard deviation (S_{rel} %) were calculated from the results of ten separate experiments[8]. The data for this calculation are given in Table I.

TABLE I

CALCULATION OF $\bar{x}$ AND S

Parameter	*Expt. No.*										$\bar{x}$	S_{rel} (%)
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*		
H (mV)	8.1	8.1	7.7	7.8	7.9	8.6	8.7	9.0	7.5	8.7	8.2	± 6.05

It can be seen that $\bar{x}$ comprises 96.3% of the desired H value. Thus, the accuracy of the determination of the volume of NS necessary for the preparation of a FAME solution of optimal concentration can be considered to be satisfactory. The relative standard deviation is rather high, possibly owing to partial evaporation of toluene from the solution in the first step.

Because the volume of NS is determined by the response of the major component only, the efficiency of injection with NS does not depend on the number of components in the aliquot and their quantitative ratio. The method proposed makes possible the analysis of FAME samples of up to 0.1-μl; with a further decrease in this volume, the amount of toluene used in the first step decreased to 3 μl and thorough mixing of the sample and the solvent was no longer possible. The maximal volume of the aliquot to be analyzed is determined by the volume of the capillary used ($v'' = 0.05$ μl), and its minimal volume depends on the dilution of the sample by NS; this dilution must be made in such a way as to meet the condition $5\text{ mV} < H < 10\text{ mV}$. In this work with FAME from soybean lipids, the volume of the aliquot was as small as 0.015 μl.

Finally, another advantage of the method is that the same capillary is used for the preliminary determination of the amount of FAME present and for their final injection. As a consequence, the value of its internal volume is not present in the equa-

tion for v, and exact volumetric calibration of the capillary is unnecessary; such a calibration would be difficult, as v'' usually does not exceed 0.05 μl.

CONCLUSION

A method for removing micro-aliquots of less than 0.05 μl from small (<0.5 μl) samples of FAME and for their injection into a GLC column has been developed. The esters to be analyzed are injected into the column as a solution in a non-volatile solvent (*n*-hexadecane), using glass capillaries and a closed liquid injection system. By employing this method, it is possible to obtain many chromatograms from a small volume of sample. The arithmetic mean ($\bar{x}$) of the major peak height is equal to 96.3% of the desired value, and the relative standard deviation of separate measurements from $\bar{x}$ does not exceed $\pm$6.5%.

ACKNOWLEDGEMENT

The authors are grateful to Dr. A. G. Vereshchagin for his guidance throughout this work.

REFERENCES

1 H. P. Burchfield and E. E. Storrs, *Gas Chromatography in Biochemistry*, Mir Publishing House, Moscow, 1964, p. 69.
2 A. Oppegaard, *J. Chromatogr. Sci.*, 10 (1972) 716.
3 A. V. Zhukov and A. G. Vereshchagin, *J. Amer. Oil Chem. Soc.*, 53 (1976) 1.
4 A. V. Zhukov and A. G. Vereshchagin, *J. Chromatogr.*, 51 (1970) 155.
5 R. P. W. Scott, in *Gas Chromatography*, Foreign Literature Publishing House, Moscow, 1961, p. 178.
6 L. A. Kogan, *Quantitative Gas Chromatography*, Khimiya Publishing House, Moscow, 1975, p. 110.
7 B. A. Rudenko, A. A. Noravyan and V. E. Kucherov, *Zh. Anal. Khim.*, 23 (1968) 114.
8 A. V. Zhukov and A. G. Vereshchagin, *J. Lipid Res.*, 10 (1970) 711.