J. Lipid Research
January, 1960

# Notes on Methodology

## Gas-liquid chromatography of fatty acid methyl esters: the "carbon-number" as a parameter for comparison of columns

F. P. Woodford and C. M. van Gent
*Department of Physical Chemistry, University of Leiden, The Netherlands*

[Received for publication June 12, 1959]

► Because of the slight but perceptible volatility of stationary phases, absolute retention times of components eluted from gas-liquid chromatographic columns decrease steadily as the column is repeatedly used. This is particularly the case for the polar polymers now customarily used for the separation of unsaturated fatty acid esters in lipid analysis. Many other factors (e.g., small differences in pressure, temperature, or other operating conditions of the column) also affect the absolute retention time of a given methyl ester. For these reasons it has become the practice to quote relative retention times referred to one component of the mixture, e.g., palmitate or stearate. We have found it more convenient to express the elution sequence in different terms, obtained by the following procedure.

The retention times of the saturated straight-chain esters, at least four of which are usually present in natural mixtures, are plotted against chain-length on semilogarithmic paper and the best straight line joining them is constructed. (Identification of these peaks, usually self-evident, can be confirmed if necessary by the addition of known esters to the mixture under investigation.) A value corresponding to the retention time of any other peak can then be read off from the graph, and gives the chain length of the hypothetical saturated straight-chain ester which would be eluted at that point. The figure so obtained we designate the "carbon-number" of the ester on the stationary phase in question. Saturated esters with a straight chain have integral carbon-numbers, e.g., 15.0, 17.0 (so that in such cases the phrase has its conventional meaning), whereas esters with a branched chain and unsaturated esters have, in general, non-integral C-numbers. The carbon-number is characteristic of a particular ester on a particular stationary phase. It is, like relative retention volume, dependent on the temperature at which the column is run, but is sufficiently constant over small temperature ranges (at least 20°C) to allow comparison with other runs on the same stationary phase (including those of other authors) made in the same temperature region.

It is the practice in this laboratory to determine the fatty acid composition of fractions (phospholipids, free fatty acids, sterol esters, and glycerides) obtained by the analysis of lipids from human arteries (1) by running each sample on two columns, one containing Apiezon "L" and the other a copolymer of maleic acid, adipic acid, and ethylene glycol (MAE). The carbon-numbers of commonly occurring esters on these two stationary phases are shown in Table 1. For comparison, carbon-numbers calculated from the data of other authors are also shown. Table 1 illustrates the well-known fact that unsaturated esters are eluted before the corresponding saturated ester on Apiezon, after it on a polar stationary phase.

TABLE 1. Comparison of Carbon-Numbers (Hypothetical Chain Lengths) of Methyl Esters of Commonly Occurring Fatty Acids on Polar and Nonpolar Stationary Phases

| Methyl Ester | MAE * (185°C) | Apiezon "L" † (203°C) | Apiezon "M" § (197°C) | Apiezon "M" ‖ (197°C) | Apiezon "L" # (240°C) |
|---|---|---|---|---|---|
| Palmitoleate | 16.4 | 15.75 | 15.7 | 15.75 | 15.75 |
| Oleate | 18.3 | 17.65 | 17.6 | 17.7 | 17.7 |
| Linoleate | 18.9 | 17.5 | 17.5 | 17.4 | 17.6 |
| Linolenate | 19.65 | 17.55 | — | 17.42 | 17.6 |
| Arachidonate | 21.6 | 18.95 | — | — ‡ | 19.0 |
| Erucate | 22.2 | 21.6 | — | — | — |

* Copolymer of maleic acid, adipic acid, and ethylene glycol 1 : 3 : 4, 1 g. per 4 g. Chromosorb®.

† 1 g. per 9 g. acid- and alkali-washed Celite 545®.

‡ The $\Delta^{8,11,14,17}$-tetraenoic $C_{20}$ ester (isomer of arachidonate) investigated by these authors had a C-number of 18.85.

§ See Ref. 2. ‖ See Ref. 3. # See Ref. 4.

The behavior of saturated branched-chain esters is shown in Table 2. The figures in the last column are calculated from the relative retention times given by James and Martin (2). It can be seen that branched-chain esters are eluted before the corresponding straight-chain ester on both polar and nonpolar phases. Consideration of the carbon-numbers shows that the anteiso compounds are eluted slightly later, i.e., nearer the straight-chain ester than iso compounds.

These data enable us to assign structures to minor constituents of fatty acid mixtures with some measure of confidence: for example, the group of $C_{15}$ acids which occurs with some frequency in natural mixtures.



Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 2. CARBON-NUMBERS OF BRANCHED METHYL ESTERS ON POLAR AND NONPOLAR STATIONARY PHASES

| Methyl Ester | Carbon-Number on | | |
|---|---|---|---|
| | MAE (185°C) | Apiezon "L" (203°C) | Apiezon "M"* (197°C) |
| 4-Methylhexanoate ($C_7$ anteiso) | — | — | 6.7 |
| 6-Methylheptanoate ($C_8$ iso) | — | — | 7.45 |
| 6-Methyloctanoate ($C_9$ anteiso) | — | — | 8.65 |
| 8-Methylnonanoate ($C_{10}$ iso) | — | — | 9.55 |
| 8-Methyldecanoate ($C_{11}$ anteiso) | — | — | 10.7 |
| 10-Methylundecanoate ($C_{12}$ iso) | — | — | 11.65 |
| 10-Methyldodecanoate ($C_{13}$ anteiso) | — | — | 12.7 |
| 12-Methyltetradecanoate ($C_{15}$ anteiso) | — | — | 14.7 |
| 14-Methylpentadecanoate ($C_{16}$ iso) | 15.6 | 15.7 | 15.65 |
| 14-Methylhexadecanoate ($C_{17}$ anteiso) | 16.7 | 16.7 | 16.7 |
| 16-Methylheptadecanoate ($C_{18}$ iso) | 17.6 | 17.6 | — |
| 16-Methyloctadecanoate ($C_{19}$ anteiso) | 18.8 | 18.7 | — |
| 20-Methylheneicosanoate ($C_{22}$ iso) | 21.6 | 21.55 | — |

* See Ref. 2.

Pentadecanoate has, of course, a C-number of 15.0 on both columns; in addition, there may occur a second peak on Apiezon, C-number 14.7, whose percentage corresponds to the sum of the percentages of the two peaks on MAE with C-numbers 14.5 and 15.4. These have been therefore tentatively identified as due to $C_{15}$ branched and $C_{15}$ (mono-) unsaturated esters, respectively. Support for this identification comes from the observation that in some natural mixtures only one of the $C_{15}$ peaks occurs, having a C-number of 15.4 on MAE and 14.7 on Apiezon.

In this way several minor constituents of fatty acid mixtures have been provisionally identified on the basis of their two C-numbers alone, as shown in Table 3. The identification is based on the correlation of the results on MAE and Apiezon chromatograms of about 200 different lipid samples. The repeated appearance of peaks of the same relative magnitude on the two phases and with constant, appropriately related carbon-numbers establishes the existence and frequent occurrence of a definite unknown compound. It is then provisionally identified in accordance with the observed rules of behavior of branched and unsaturated esters; comparison with known samples where possible; and comparison with retention times reported in the literature, recalculated as carbon-numbers. Final confirmation of identity depends, of course, on further investigation of the isolated fraction or the use of hydrogenation, bromination, and ozonolysis procedures. For all the unsaturated acids shown in Table 3 the number of carbon atoms has been confirmed by fraction collection, hydrogenation, and rechromatography.

TABLE 3. PROVISIONAL IDENTIFICATION OF SOME PREVIOUSLY UNREPORTED FATTY ACIDS OCCURRING IN HUMAN ARTERY LIPIDS, BY DETERMINATION OF THE CARBON-NUMBERS OF THEIR METHYL ESTERS ON DIFFERENT STATIONARY PHASES

| Observed Carbon-Number on | | Tentative Identification |
|---|---|---|
| MAE * | Apiezon * | |
| 14.5 | 14.7 | $C_{15}$ branched |
| 15.0 | 15.0 | $C_{15}$ saturated |
| 15.4 | 14.7 | $C_{15}$ monoenoic |
| 16.6 | 16.7 | $C_{17}$ branched (? anteiso) |
| 17.0 | 17.0 | $C_{17}$ saturated |
| 17.3 | 16.7 | $C_{17}$ monoenoic |
| 20.3 | 19.6 | $C_{20}$ monoenoic |
| 20.7 | 19.4 | $C_{20}$ dienoic |
| 21.1 | 19.2 | $C_{20}$ trienoic |
| 22.0 | 22.0 | $C_{22}$ saturated |
| 22.2 | 18.8 | $C_{20}$ pentaenoic |
| 22.3 | 21.7 | $C_{22}$ monoenoic |
| 23.0 | 23.0 | $C_{23}$ saturated |
| 24.0 | 24.0 | $C_{24}$ saturated |
| 24.3 | 23.6 | $C_{24}$ monoenoic |

* Same conditions as in Table 1.

Carbon-numbers represent, of course, no difference in principle from relative retention times. We suggest, however, that they indicate more immediately and vividly the position in the elution pattern of a component under discussion (and therefore bring mistakes and anomalies more readily to light). Instead of being unattached numbers, they are closely connected with the chemical constitution of the esters; and finally, they make unnecessary the arbitrary choice of a reference compound to which retention times can be related. If retention times of fatty acid methyl esters were always expressed in this way, direct com-

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 19, 2012

parison of retention values on different phases and from different laboratories would be possible without recalculation and often from memory. Such comparisons have proved invaluable in this laboratory, and the results to which they have led will be reported subsequently.

We acknowledge with gratitude the interest and encouragement of Prof. C. J. F. Böttcher. To Drs. W. Stoffel, E. Klenk, and E. V. Truter we are indebted for the provision of certain branched and unsaturated esters.

REFERENCES

1. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-van Houte, and C. M. van Gent. *Rec. trav. chim.* **78**: 794, 1959.
2. James, A. T., and A. J. P. Martin. *Biochem. J.* **63**: 144, 1956.
3. Stoffel, W., W. Insull, Jr. and E. H. Ahrens, Jr. *Proc. Soc. Exptl. Biol. Med.* **99**: 238, 1958.
4. Lipsky, S. R., J. E. Lovelock and R. A. Landowne. *J. Am. Chem. Soc.* **81**: 1010, 1959.

Downloaded from www.jlr.org by guest, on June 19, 2012