

## THE LOSS OF METHYL ESTERS OF POLYUNSATURATED ACIDS DURING GAS CHROMATOGRAPHY

T. GERSON, F. B. SHORLAND AND J. E. A. McINTOSH

*Fats Research Division, Department of Scientific and Industrial Research, Wellington (New Zealand)*

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## INTRODUCTION

It has been found in this laboratory that the gas chromatographic analysis of mixtures containing methyl stearate, methyl oleate and methyl linoleate on Celite columns coated with polydiethyleneglycol adipate using a  $^{90}\text{Sr}$  detector gives quantitative results. However, as reported in a preliminary note by GERSON, SHORLAND AND McINTOSH<sup>1</sup>, methyl linolenate in comparison with methyl stearate showed a loss of 51%. Furthermore analysis of the methyl esters of shark liver oil before and after hydrogenation revealed a loss of 51%  $\text{C}_{20}$ -unsaturated acids (largely pentaenoic) and of 86%  $\text{C}_{22}$ -unsaturated acids (largely hexaenoic). Losses of methyl esters of polyunsaturated acids are implicit in the work of ACKMAN AND BURGHER<sup>2</sup>, who recorded the use of correction factors as did SEHER<sup>3</sup>.

The purpose of the present work was to test the effect of varying the nature of the liquid phase and of the solid support on the losses of polyunsaturated acids containing from three to six double bonds.

The solid supports tested included Celite 545, Chromosorb W, firebrick and glass microbeads. The effect of varying the polarity of Celite by precoating with dimethyldichlorosilane or with Epicote resin was also studied. The polar liquid phases examined were diethyleneglycol adipate polyester (DEGA), ethyleneglycol adipate polyester (EGA), butanediol succinate (BDS), ethyleneglycol succinate polyester (EGS), *o*-phthalic ethyleneglycol (PEG). In addition the non-polar Apiezon L (APL) was tested.

## EXPERIMENTAL

Methyl stearate (18:0) was prepared by refractionation of concentrates obtained during ester fractionation analysis and purified by crystallization from acetone. When tested by gas chromatography it was found to be free from impurities. Methyl linolenate (18:3), methyl arachidonate (20:4), methyl eicosapentaenoate (20:5) and methyl docosahexaenoate (22:6) were obtained from the Hormel Institute, Austin, Minnesota, U.S.A., and were certified to be at least 90% pure with the exception of (18:3), which was over 99% pure.

### *Test mixtures*

Test mixtures were prepared by weighing out known amounts of methyl stearate and the methyl esters of the polyunsaturated acids. The weight percentages of the polyunsaturated acids were checked by the iodine values (Wijs, 1 h). Agreement within 4% was obtained and the accepted composition of the test mixture was based on the iodine value.

### *Solid supports*

Celite 545 (30–80 mesh) and glass microbeads (approx. 80 mesh) were obtained from The British Drug Houses Ltd., Poole, England. Glass microbeads (74–53 microns) were obtained from the La Pine Scientific Company (Chicago, Ill., U.S.A.). Chromosorb W (60–80 mesh) was supplied by Johns Manville Corporation, New York, and firebrick (60–80 mesh) was obtained through the courtesy of the N.R.C. Prairie Regional Laboratory, Canada.

### *Liquid phases*

APL was obtained from Edwards High Vacuum Ltd., Crawley, Sussex, England; DEGA from Cambridge Industries Ltd., Cambridge, Mass., U.S.A.; Epicote-resin from Shell Oil Company, New Zealand, and dichlorodimethylsilane from Dow Corning Corporation, Midland, Michigan, U.S.A. EGA was prepared as described by FARQUHAR *et al.*<sup>4</sup>. BDS, EGS and PEG were obtained through the courtesy of the Prairie Regional Laboratory, N.R.C., Canada.

### *Preparation of solid supports*

Siliconized columns were prepared by treating the Celite with a 5% solution of dichlorodimethylsilane in toluene for 2–3 h. Epicote coated columns were prepared by coating an acetone solution of the resin onto Celite in a rotary evaporator. The liquid phase was subsequently applied in toluene solution, in which Epicote is only sparingly soluble.

### *Coating the solid support with liquid phase*

In general the solid supports were coated by mixing with a solution of the liquid phase and evaporating the solvent *in vacuo* on a rotary evaporator. For APL, light petroleum (b.p. 40–60°) was used. For other liquid phases, acetone was found to be more suitable. Column packings coated with EGA or with DEGA were spread in a thin layer on a tray and heated to 205° overnight before packing. One of the microbead columns was packed with solid support without the liquid phase. The liquid phase was then introduced in acetone solution and the solution allowed to drain. The solvent was afterwards removed by passing a stream of air through the column. In another experiment, a Celite column coated with 20% w/w DEGA was prepared by mixing the Celite with a solution of DEGA in acetone and allowing the solvent to evaporate over a period of a week with occasional gentle stirring.

### *Packing the column*

Small amounts of the packing, equivalent to about 6 in. in length were poured into the column. After each addition the column was rotated against a  $\frac{3}{4}$  in. hexagonal shaft driven by an electric motor to pack the particles more closely.

*Analytical methods*

The separations were carried out on columns 225 cm long and 0.6 cm internal diameter. An argon ionisation detector as described by LOVELOCK, JAMES AND PIPER<sup>5</sup> was used. Except where otherwise stated the detector voltage was 1000 V. The introduction of the sample and measurement of the peak areas were carried out as described by GERSON<sup>6</sup>. The loss of methyl esters of polyunsaturated acids was estimated by comparing their measured peak area with that calculated from the area of the methyl stearate peak which was assumed to be correct.

## RESULTS AND DISCUSSION

As already mentioned, GERSON *et al.*<sup>1</sup> using 20 % DEGA on Celite showed losses of 51 %, 51 % and 86 % for methyl linolenate, methyl C<sub>20</sub>-polyunsaturated and methyl C<sub>22</sub>-polyunsaturated esters, respectively. In the present work, a loss of 20 % only was found for methyl linolenate for this type of packing showing that the same type of packing varies in performance from column to column. It may be significant that the loss of 51 % was associated with an old column, whereas the smaller loss of 20 % was recorded with a new column. For the work now recorded the columns were freshly prepared. The results in the Tables are based on six determinations.

Tables I and II show that in the analyses of methyl linolenate and methyl arachidonate using Celite as the solid support, the losses are smaller with 20 % EGA than with 20 % DEGA, but nevertheless substantial. Although presiliconizing further reduced the loss of methyl linolenate no significant difference was noted for methyl arachidonate (see Tables I and II). The siliconized column was also tried with the methyl esters of more highly unsaturated acids but substantial losses (Table III) were incurred amounting to 42 % and 67 %, respectively, for methyl eicosapentaenoate and methyl docosahexaenoate.

On the other hand, the application of 10 % w/w Epicote to the Celite before the addition of 20 % w/w EGA minimised the losses and gave quantitative results for

TABLE I

LOSS OF METHYL LINOLENATE (18:3) ON GAS-LIQUID CHROMATOGRAPHIC COLUMNS

Mixture (wt. %)		Solid support	Liquid phase	Temp. (°C)	Mean change (%)	Standard deviation (±)	Area correction factor
18:0	18:3						
36	64	30-80 mesh Celite	20 % DEGA	205	-20	5.6	1.25
36	64	30-80 mesh Celite	20 % EGA	205	-12	5.0	1.14
38	62	30-80 mesh Celite	20 % EGA siliconized	205	-3	2.5	—
57	43	30-80 mesh Celite	20 % EGA 10 % Epicote	185	-2	5.5	—
57	43	30-80 mesh Celite	20 % EGA 10 % Epicote	205	-2	6.3	—
38	62	80 mesh glass beads	2.5 % DEGA	205	-7	2.4	1.08
38	62	80 mesh glass beads	1 % APL	205	-9	6.4	1.10

TABLE II

LOSS OF METHYL ARACHIDONATE (20:4) ON GAS-LIQUID CHROMATOGRAPHIC COLUMNS

<i>Mixture (wt. %)</i>		<i>Solid support</i>	<i>Liquid phase</i>	<i>Temp. (°C)</i>	<i>Mean change (%)</i>	<i>Standard deviation (±)</i>	<i>Area correction factor</i>
18:0	20:4						
30	70	30-80 mesh Celite	20 % DEGA	205	-40	4.3	1.67
25	75	30-80 mesh Celite	20 % EGA	205	-31	7.2	1.45
33	67	30-80 mesh Celite	20 % EGA siliconized	205	-36	3.1	1.58
35	65	30-80 mesh Celite	20 % EGA 5 % Epicote	205	-26	11.0	1.35
35	65	30-80 mesh Celite	20 % EGA 10 % Epicote	205	+ 1	8.2	—
33	67	80 mesh glass beads	0.75 % EGA	205	- 6	4.2	1.06

TABLE III

LOSS OF METHYL EICOSAPENTAENOATE (20:5) AND METHYL DOCOSAHEXAENOATE (22:6) ON GAS-LIQUID CHROMATOGRAPHIC COLUMNS

<i>Mixture (wt. %)</i>		<i>Solid support</i>	<i>Liquid phase</i>	<i>Temp. (°C)</i>	<i>Mean change (%)</i>	<i>Standard deviation (±)</i>	<i>Area correction factor</i>
18:0	20:5						
29	71	30-80 mesh Celite	20 % EGA siliconized	206	-42	5.9	1.72
29	71	30-80 mesh Celite	20 % EGA 10 % Epicote	206	-22	4.6	1.28
36	64	60-80 mesh Chromosorb W	17 % BDS	207	-45	7.1	1.82
36	64	60-80 mesh Chromosorb W 1000 V	17 % EGS	207	-46	3.6	1.85
36	64	60-80 mesh Firebrick 1000 V; 800 V	17 % PEG	207	-46 -41	5.9 5.8	1.85 1.69
29	71	30-80 mesh Celite	5 % APL	206	-50 -10	1.9 1.3	2.00 1.11
18:0	22:6						
53	47	30-80 mesh Celite	20 % EGA siliconized	205	-67	4.5	3.03
41	59	30-80 mesh Celite	20 % EGA 10 % Epicote	206	-57	6.0	2.33
41	59	30-80 mesh Celite	20 % EGA 10 % Epicote	185	-58	7.4	2.38
53	47	30-80 mesh Celite	5 % APL	205	-51	4.7	2.04

methyl linolenate and methyl arachidonate (Tables I and II). It was less effective for methyl eicosapentaenoate and for methyl docosahexaenoate (see Table III). The application of 5 % w/w Epicote to Celite was ineffective (see Table II).

The loss of methyl linolenate was considerably smaller with 1 % w/w APL on glass beads than with EGA on Celite but this trend was reversed with precoated Celite. A column using 0.75 % w/w EGA on 80 mesh glass beads gave results with methyl arachidonate (see Table II) which were almost as good as Epicote treated Celite, suggesting that this or other finely divided glass supports have potentialities in the quantitative analysis of methyl esters of polyunsaturated fatty acids.

It has been our observation that 80 mesh glass microbead columns are difficult to pack if the amount of liquid phase exceeds 2 % (w/w). Resolution on such columns was generally poor and varied with each column. No improvement was effected when using 74-53 micron glass beads.

The possibility that loss of polyunsaturated esters was due to the liquid films being destroyed during drying in the rotary evaporator was excluded by introducing the liquid phase, dissolved in acetone, into a column packed with uncoated glass beads, allowing the solution to drain off and then drying the column in a current of air. Losses on this column were similar to those on columns whose packing had been dried in the rotary evaporator. Celite columns in which the liquid phase was prepared by mixing the solid support with a solution which was allowed to evaporate slowly, with occasional gentle stirring, over a period of a week also gave similar results to those obtained by removal of the solvent on a rotary evaporator.

The effect of temperature on the losses of methyl esters of polyunsaturated acids was not extensively investigated. The losses of methyl docosahexaenoate on Celite coated with 10 % Epicote and 20 % EGA were shown to be similar at 206° and 185°.

ACKMAN AND BURGHER<sup>2</sup> from the correction factor (derived empirically by comparison of hydrogenated and unhydrogenated samples) of 1.5 for 22:6 determined on organosilicone columns of EGSS-X and EGSS-Y on siliconized Gas-chrom P (see ACKMAN AND JANGAARD<sup>7</sup>) indicated a loss of 33.3 % as compared with 57 % on Epicote-EGA columns used in the present work (see Table III).

The quantitative data presented in this paper as well as the discoloration near the inlet of the columns suggest that the losses of methyl esters of polyunsaturated acids are due to their polymerization on the column. The extent to which this occurs, however, depends on the nature of the solid support as well as of the liquid phase. The relatively good recovery of 22:6 by ACKMAN AND BURGHER<sup>2</sup> with EGSS-X and EGSS-Y suggests that these liquid phases are superior to those investigated in the present work.

Coating the Celite with Epicote reduced the loss of methyl esters of unsaturated acids without impairing resolution, while the substitution of Celite by glass beads also reduced the losses but generally decreased the resolving power. The object of producing columns of high resolving power for methyl esters of highly unsaturated acids without loss has been achieved in the present investigation for methyl linolenate, methyl arachidonate and to a less extent methyl eicosapentaenoate.

According to NOVITSKAYA<sup>8</sup> the response of the argon-ionisation detector was found to be proportional to the mass of the methyl esters of palmitic, stearic, oleic and linoleic acids while with methyl linolenate, the detector response was relatively

greater with increasing voltage within the range of 750–1500 V. In the present work the voltage generally used was 1000. Two tests were made, however, at 800 V with methyl eicosapentaenoate. The results suggest the possibility of increased response at the higher voltage but the difference was small in relation to the losses of the ester on the column.

This work indicates that each column should be tested with methyl esters of highly unsaturated acids and where necessary a correction factor applied. In addition it is considered advisable in the analysis of mixtures containing methyl esters of acids with more than three double bonds to check the fatty acid composition against that of a fully hydrogenated sample.

#### SUMMARY

Using Celite as a solid support it was found that the recovery of methyl esters of polyunsaturated acids during gas chromatographic analysis decreased with increasing unsaturation. Greatest losses were sustained with DEGA as the liquid phase. Limited tests indicated that BDS, EGS and PEG were also unsatisfactory. With linolenate, EGA on siliconized Celite proved to be satisfactory. Similarly improved results were obtained by precoating the solid support with Epicote resin, when 20% EGA as the liquid phase gave quantitative results for methyl linolenate and methyl arachidonate. The loss of methyl eicosapentaenoate was diminished from 42% to 22% on Epicote precoated columns. On the other hand the loss of methyl docosahexaenoate was decreased from 67 to 57% only. With this ester better results (51% loss) were obtained with 5% w/w APL on untreated Celite. Glass microbeads provided a more inert solid support than Celite but on account of lack of resolving power it was not examined closely.

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