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EFFECT OF TEMPERATURE ON THE SEPARATION OF THE ESTERS OF FATTY ACIDS BY OPEN TUBULAR GAS CHROMATOGRAPHY

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SUMMARY

The effect of temperature on the separation of the methyl esters of fatty acids by open tubular gas chromatography on Apiezon L, butane-1,4-diolsuccinate (BDS) and diethylene glycol succinate (DEGS) was studied. For the separation of the methyl esters of fatty acids, the determination of the effect of temperature on the variations of the equivalent carbon chain length (*ECL*) was found to be a convenient method. The values of the *ECL* were determined with a reproducibility of better than ±0.01 carbon atom. The possible errors of measurements of *ECL* values are discussed.

The *ECL* values of the methyl esters of branched fatty acids found on Apiezon L and BDS are practically independent of temperature, whereas on DEGS temperature has a definite effect.

The *ECL* values of the methyl esters of unsaturated fatty acids found on Apiezon L as well as on the polyester phases (BDS, DEGS) increase with temperature. The slope of $\delta ECL/\delta t$ increases with the increase in the number of double bonds in the molecule and with the polarity of the stationary phase. For stereoisomers, the values of $\delta ECL/\delta t$ are higher for the methyl esters of acids that have a *cis* configuration.

INTRODUCTION

The great potentiality of gas–liquid chromatography (GLC) for the separation of complex mixtures of esters of fatty acid has been well known since the classical papers[1,2] were published in this field. The use of open tubular columns makes possible the separation of extremely complex mixtures. However, identification is often difficult because of the absence of standards, which is why relations between the structure and chromatographic behaviour of separated substances are often used for their identification.

A suitable method for the identification of the separated methyl esters of branched saturated and unsaturated fatty acids has been described by LANDOWNE AND LIPSKY[3]. They identified fatty acids by using a polyester phase at two temperatures and by evaluating the temperature dependence of the separation factors.

In the case of methyl esters of branched fatty acids, it has been shown by ACKMAN[4,5] that it is advantageous to study the temperature effect by the variations of the equivalent carbon chain length.

The improved separation of the methyl esters of unsaturated fatty acids has been demonstrated for the pair of compounds eicosenoic(20:1) and linolenoic(18:3) acids by lowering the temperature[6], and for the pair of compounds eicosatrienoic(20:3) and eicosenoic(20:1) acids by increasing it[3].

The aim of this paper was to estimate the effect of temperature on the separation of the methyl esters of fatty acids at concentrations at which they occur in biological materials. Methyl esters of acids isolated from butter fat and the products of the biochemical oxidation of paraffins were used as samples. The influence of temperature on the *ECL* values of the methyl esters of branched and unsaturated acids was studied by open tubular GC on Apiezon L, butane-1,4-diol succinate and diethylene glycol succinate phases.

EXPERIMENTAL

Table I shows the instruments used and the operating conditions.

The natural acids from butter fat and the lipid fraction of the products from the biochemical oxidation of paraffins were isolated by the usual method and purified by adsorption chromatography on silica gel[7].

The standard mixtures of 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 21:0, 22:0, *cis*-18:1(9), *trans*-18:1(9), *cis,cis*-18:2(9,12) and *cis,cis,cis*-18:3(9,12,15) acids* were prepared from technical products of Lachema, Brno, Czechoslovakia and Applied Science Laboratories, State College, Pa., U.S.A.

The methyl esters were prepared by the esterification of acids with diazomethane. The hydrogenation of unsaturated acid esters was carried out with a palladium catalyst.

TABLE I

INSTRUMENTS USED AND OPERATING CONDITIONS

Conditions	*Instrument*		
	Chrom-31[a]	*Fractovap Model GI*	*Perkin-Elmer Model 900*
Column length (m)	45	45	100
Column diameter (mm)	0.25	0.25	0.25
Phase	Apiezon L	DEGS	BDS
Temperature (°C)	180–220	150–170	160–180
Pressure of nitrogen carrier gas (kp/cm²)	2.0	1.1	2.1
Efficiency (Theoretical plates)	60,000	80,000	190,000
Splitting ratio	1:100	1:200	1:250

[a] L.P., Czechoslovakia.

* In this notation, the first numeral indicates the carbon number, the second the number of double bonds, and the numerals in the brackets indicate the positions of the double bonds.

RESULTS AND DISCUSSION

The retention times of the standards and natural mixtures of the methyl esters of fatty acids were determined, under the conditions described in Table I, as the distances between leading edge of the solvent and the peak maximum of the solute. The retention data are presented as the equivalent carbon chain length[8] and were calculated by using the equation:

$$ECL_x = N + \frac{\log t'_{R,x} - \log t'_{R,N}}{\log t'_{R,N+1} - \log t'_{R,N}} \quad (1)$$

where ECL_x is the equivalent carbon chain length of the methyl ester of the fatty acid designated as x, having a retention time $t'_{R,x}$; and $t'_{R,N+1}$ and $t'_{R,N}$ are the retention times of the methyl esters of normal saturated fatty acids having carbon numbers $N + 1$ and N, respectively[9].

It can be seen that the ECL values are basically similar to the carbon numbers as calculated by WOODFORD and VAN GENT[10].

The best reproducibility of ECL values has been estimated in the literature as ± 0.02 carbon atom[11]. To reach such precision with natural mixtures, it was necessary to chromatograph the standard mixture of methyl esters of fatty acids in equal weight proportions immediately following the chromatography of the natural mixture of methyl esters of fatty acids. It can be seen from Fig. 1 that the peak width for the standard methyl esters, in equal weight proportions, increases linearly with the increase of their retention times.

On the basis of similar dependences, retention times of all overloaded methyl esters of fatty acids were recalculated.

Because the methyl esters of normal saturated fatty acids are standards for the calculation of ECL values (see eqn. 1) we have estimated their separation factors on Apiezon L, BDS and DEGS at three temperatures. The separation factors decrease with temperature and with the polarity of the stationary phase, and the results are given in Table II.

TABLE II

SEPARATION FACTORS OF FATTY ACID METHYL ESTERS ON APIEZON L AND DEGS AT DIFFERENT COLUMN TEMPERATURES

Methyl esters of acids	*Apiezon L*			*DEGS*		
	181.5°	*200.5°*	*221.0°*	*154.8°*	*165.0°*	*174.3°*
C_{13}/C_{12}	1.61	1.55	1.49	1.42	1.39	1.36
C_{14}/C_{13}	1.61	1.55	1.48	1.42	1.39	1.36
C_{15}/C_{14}	1.60	1.54	1.48	1.42	1.40	1.37
C_{16}/C_{15}	1.59	1.54	1.48	1.42	1.40	1.37
C_{17}/C_{16}	1.59	1.54	1.47	1.43	1.40	1.37
C_{18}/C_{17}	1.59	1.54	1.47	1.43	1.40	1.38
C_{19}/C_{18}	1.59	1.53	1.47	1.44	1.41	1.38
C_{20}/C_{19}	1.59	1.53	1.47	1.44	1.41	1.38
C_{21}/C_{20}	–	–	–	1.44	1.42	1.38

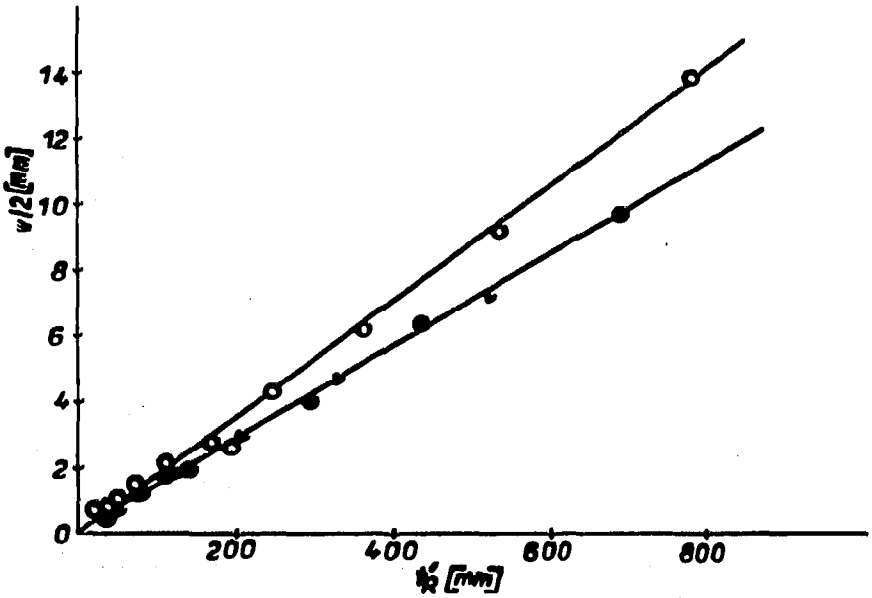

Fig. 1. Dependence of peak width at half height ($w/2$) on the retention time for the methyl esters of normal straight-chain fatty acids on an Apiezon L capillary column. ○, 221°, chart-speed of recorder = 1 cm/min; △, 181.5°, chart-speed of recorder = 16 cm/h; ●, 200.5°, chart-speed of recorder = 0.5 cm/min.

In contrast to the results of LANDOWNE AND LIPSKY[3], our separation factors for the methyl esters of normal saturated fatty acids are not constant. Therefore we have included also the methyl esters of odd-numbered fatty acids for the calculation of *ECL* values.

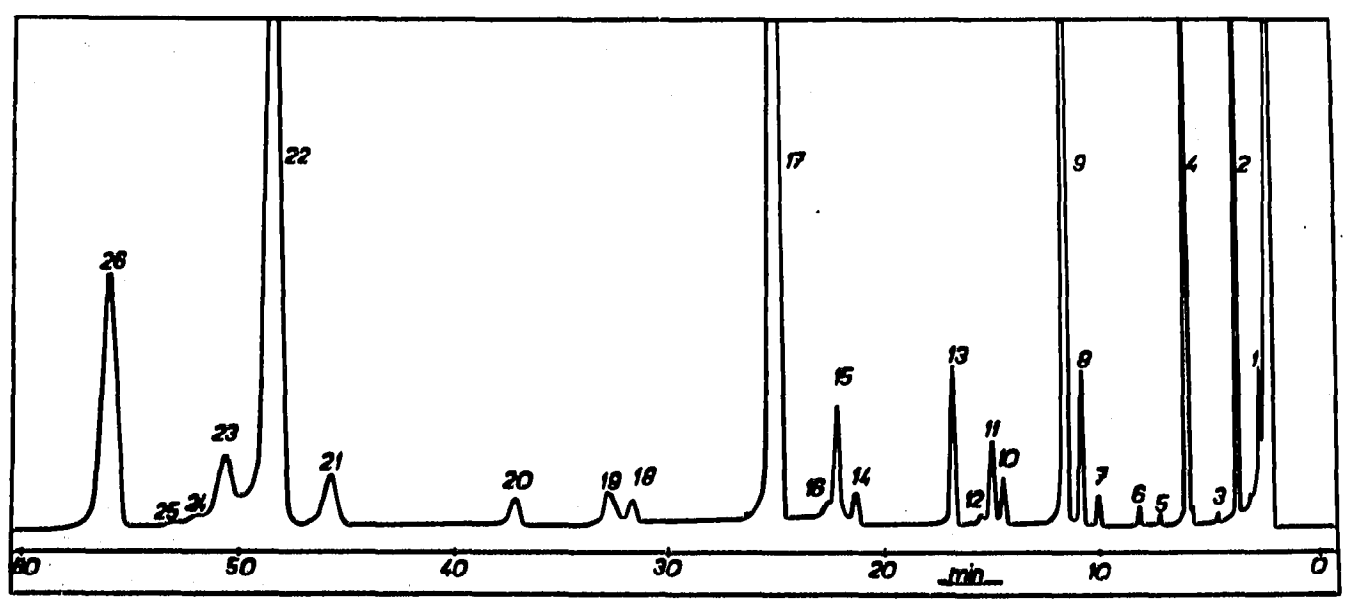

Fig. 2. Separation of the methyl esters of butter fat fatty acids on Apiezon L at 200°. 1 = 8:0; 2 = 10:0; 3 = 11:0; 4 = 12:0; 5 = *iso*-13:0; 6 = 13:0; 7 = *iso*-14:0; 8 = 14:1; 9 = 14:0; 10 = *iso*-15:0; 11 = *anteiso*-15:0 + 15:1; 13 = 15:0; 14 = *iso*-16:0; 15 = 16:1; 17 = 16:0; 18 = *iso*-17:0; 19 = *anteiso*-17:0 + 17:1; 20 = 17:0; 21 = 18:2 + 18:3; 22 = *cis*-18:1; 23 = *trans*-18:1; 26 = 18:0.

TABLE III

ECL AND $\delta ECL/\delta t$ VALUES OF THE METHYL ESTERS OF BRANCHED FATTY ACIDS ON APIEZON L, BDS AND DEGS PHASES

Methyl esters of acids	*Apiezon L*		*BDS*		*DEGS*	
	ECL, 180°	$\delta ECL/40°$	*ECL*, 160°	$\delta ECL/20°$	*ECL*, 160°	$\delta ECL/10°$
iso-13:0	12.62	<-0.01	12.55	0.00	12.53	−0.01
iso-14:0	13.62	<-0.01	13.53	0.00	13.53	−0.02
iso-15:0	14.62	<-0.01	14.53	−0.01	14.53	−0.01
anteiso-15:0	14.70	<-0.01	14.71	0.00	14.72	−0.01
iso-16:0	15.62	<-0.01	15.53	−0.01	15.53	−0.01
iso-17:0	16.61	<-0.01	16.53	−0.01	16.53	−0.02
anteiso-17:0	16.71	<-0.01	16.71	−0.01	16.72	−0.01
iso-18:0	17.61	<-0.01	17.53	−0.01	17.53	−0.01

Chromatography on Apiezon L

The great advantage of the Apiezon L phase is the good reproducibility of the *ECL* values determined in two different laboratories, which is better than 0.02 carbon atom. For example, our data for the methyl esters of oleic (17.63) and elaidic acids (17.72) estimated at 200° are in good agreement with the results of PREVOT[12] (17.62 and 17.72, respectively).

Similar results were found for the *ECL* values of the methyl esters of branched-chain acids by comparing our measurements with those of ACKMAN[4,5].

From Fig. 2, it can be seen that the methyl esters of branched and unsaturated fatty acids are eluated earlier than the corresponding straight-chain derivatives. The methyl esters of unsaturated fatty acids are eluted very close to their branched-chain analogues, which makes their identification more dificult.

The *ECL* values of the methyl esters of monomethyl branched fatty acids determined on Apiezon L are independent of temperature, *i.e.*, $\delta ECL/\delta t = 0$. The *ECL* values of the methyl esters of unsaturated fatty acids increase with increasing

TABLE IV

ECL AND $\delta ECL/\delta t$ VALUES OF THE METHYL ESTERS OF UNSATURATED ON FATTY ACIDS APIEZON L, BDS AND DEGS PHASES

Methyl esters of acids	*Apiezon L*		*BDS*		*DEGS*	
	ECL, 180°	$\delta ECL/40°$	*ECL*, 160°	$\delta ECL/20°$	*ECL*, 165.5°	$\delta ECL/10°$
10:1	9.90	0.05	10.70	0.03	—	—
12:1	11.89	0.05	12.47	0.01	12.24	0.03
12:1 (term.)	—	—	12.66	0.03	12.75	0.04
14:1 (term.)	13.80	0.05	14.47	0.04	14.75	0.04
15:1	14.72	0.04	15.25	0.05	15.45	0.03
16:1	—	—	16.30	0.01	16.63	0.04
16:1 (9)	15.71	0.04	16.38	0.03	—	—
cis-17:1 (9)	16.66	0.05	17.30	0.04	17.53	0.03
cis-18:1 (9)	17.60	0.05	18.24	0.05	18.46	0.05
cis,cis-18:2 (9, 12)	17.48	0.09	18.48	0.07	19.26	0.07
18:3	—	—	19.55	0.09	20.23	0.10

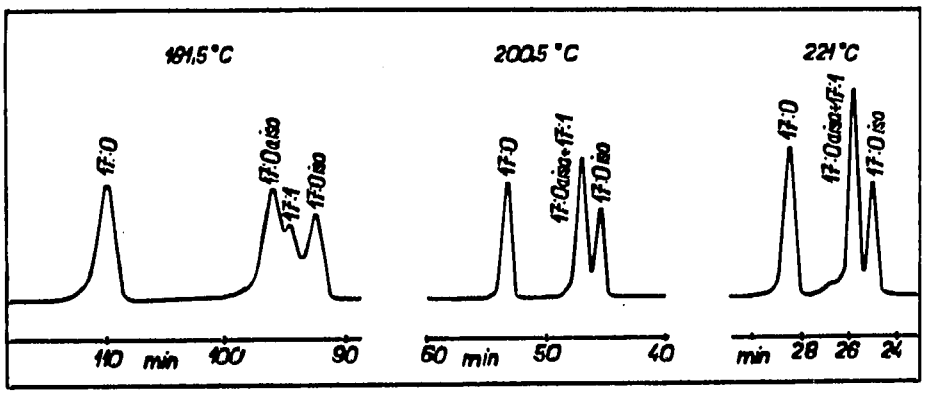

Fig. 3. Effect of temperature on the separation of the methyl esters of C_{17} acids isolated from butter fat on Apiezon L phase.

temperature ($\delta ECL/\delta t > 0$), a greater number of double bonds in the molecule and greater asymmetry of the molecule. A variation in the mobilities of the methyl esters of saturated and unsaturated fatty acids with temperature is evident from Tables III and IV and can be used for the resolution of the overlapping peaks. Fig. 3 shows the effect of temperature on the separation of 15-methylhexadecanoic-(*iso*-17:0), *cis*-hepta-9-decenoic(17:1) and 14-methylhexadecanoic(*anteiso*-17:0) acids. The resolution of these acids can be seen at 181.5°.

Chromatography on polyester phases

As a result of the polar interaction of a polyester phase with the methyl esters of unsaturated acids, these are eluted after the corresponding saturated derivatives. The retention of the methyl esters increases with the polarity of the stationary phase and with an increase in the number of double bonds in the molecule of the acid.

The great disadvantage of chromatography on polyester phases is the undefined polarity of the stationary phase, which changes with ageing of the column[12].

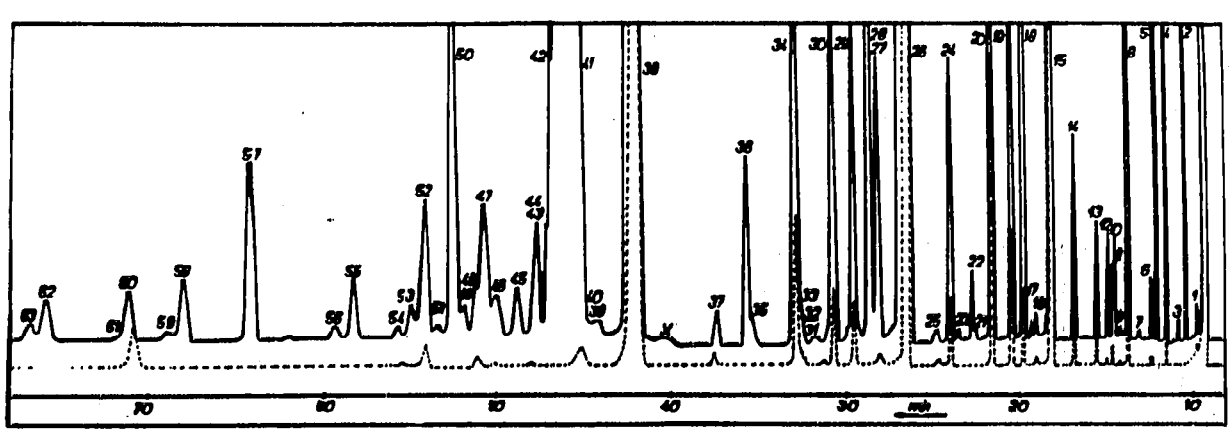

Fig. 4. Separation of the methyl esters of butter fat acids on BDS at 180°. Solid line = original sample; dotted line = sample after hydrogenation. 1 = 7:0; 2 = 8:0; 3 = 9:0; 4 = 10:0; 5 = 10:1; 6 = 11:0; 7 = *iso*-12:0; 8 = 12:0; 10 = 12:1; 11 = 12:1 + *iso*-13:0; 12 = 12:1; 13 = 13:0; 14 = *iso*-14:0; 15 = 14:0; 18 = 14:0 + *iso*-15:0; 19 = *anteiso*-15:0; 20 = 15:0; 22 = 15:1; 24 = *iso*-16:0; 26 = 16:0; 27 = *trans*-16:1; 28 = *cis*-16:1; 29 = *iso*-17:0; 31 = *anteiso*-17:0; 34 = 17:0; 36 = 17:1; 37 = *iso*-18:0; 38 = 18:0; 41 = *cis*-18:1 (9); 50 = *cis,cis*-18:2 (9,12); 52 = 19:0; 57 = *cis,cis,cis*-18:3 (9,12,15); 60 = 20:0; 62 = 20:1.

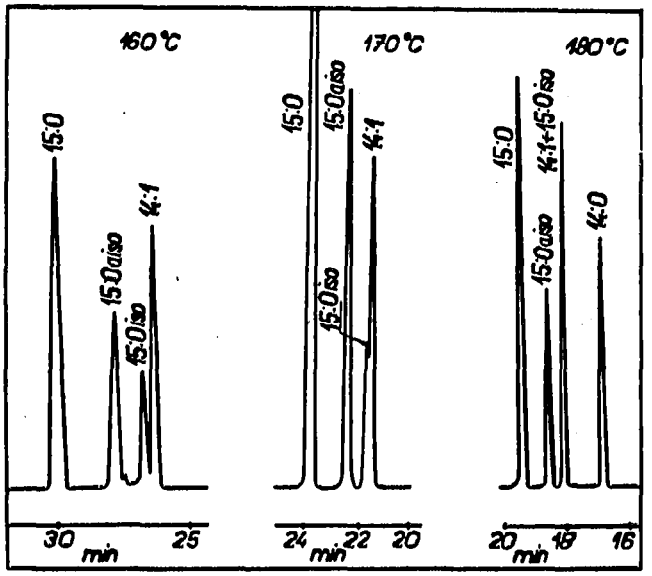

Fig. 5. Effect of temperature on the separation of the methyl esters of C_{14}–C_{18} fatty acids isolated from butter fat determined on BDS phase.

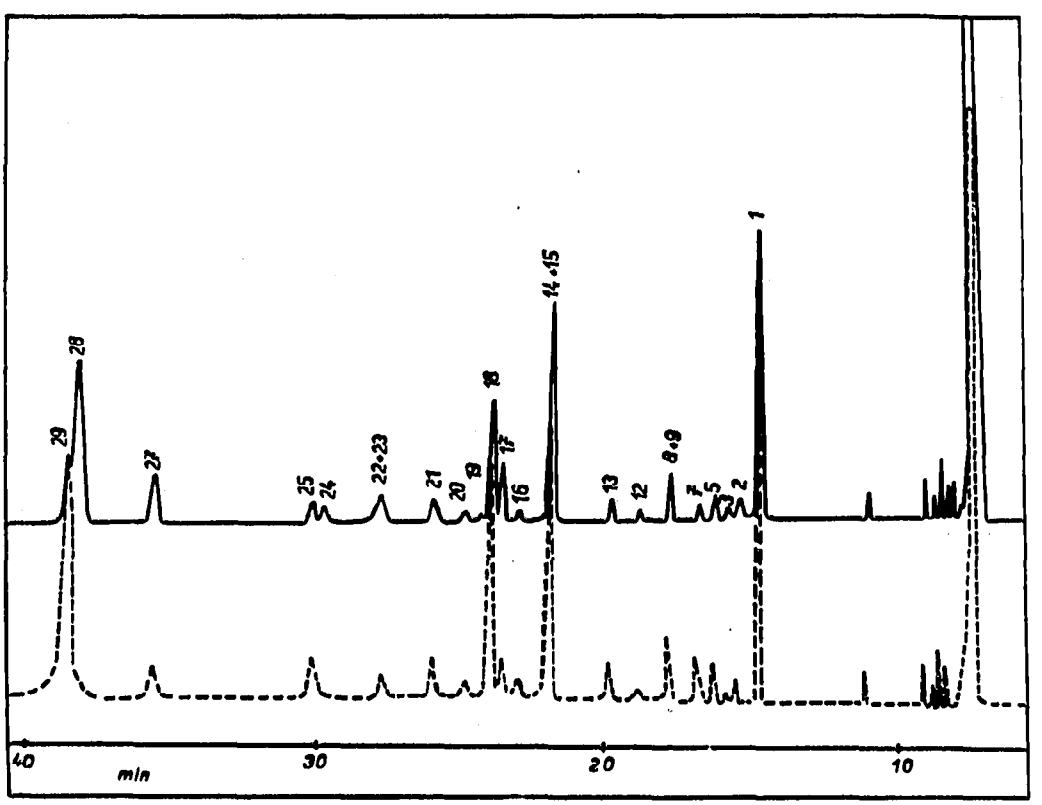

Fig. 6. Separation of methyl esters of technical oleic acid (*cis*-18:1 (9)) and elaidic acid (*trans*-18:1 (9)). The chromatogram shows a mixture of technical oleic and elaidic acids in the ratio 1:3. The dashed line corresponds to the methyl ester of technical oleic acid. The sensitivity up to peak 28 was 16 times greater than that for peaks 28 and 29. 1 = 14:0; 3 = *trans*-14:1; 4 = *cis*-14:1; 6 = *iso*-15:0; 9 = 15:0; 13 = *iso*-16:0; 17 = *trans*-16:1; 18 = *cis*-16:1; 20 = *iso*-17:0; 21 = *anteiso*-17:0; 23 = 17:0; 24 = *trans*-17:1; 25 = *cis*-17:1; 27 = 18:0; 28 = *trans*-18:1; 29 = *cis*-18:1.

The *ECL* values of the methyl esters of unsaturated acids increase with increasing temperature ($\delta ECL/\delta t > 0$) and a greater number of double bonds in the molecule. For methyl esters of fatty acids having a single double bond in the molecule, the values of $\delta ECL/\delta t$ increase with increasing distance from the centre of the molecule to the terminal CH_3 group. For a similar reason the *ECL* values of *cis* isomers are more sensitive to temperature variations than are the corresponding *trans* isomers.

Butane-1,4-diol succinate. The chromatogram of the methyl esters of fatty acids isolated from butter fat on BDS is given in Fig. 4.

The dotted line represents the mixture after hydrogenation. This procedure makes possible the identification of the saturated as well as the unsaturated acids. It can be seen that some of the methyl esters of unsaturated and branched fatty acids are not separated. These overlapping esters can be distinguished by changing the temperature. For example, Fig. 5 shows the temperature effect on the separation of tetradecenoic(14:1) and 13-methyltetradecanoic(*iso*- 15:0) acids.

In contrast to this, stereoisomers are better resolved at higher temperatures. The separation of *cis–trans* isomers on BDS at 180° is shown in Fig. 6.

The *trans* isomer (peak 28, *ECL* = 18.27) is eluated earlier than the *cis* isomer (peak 29, *ECL* = 18.32). These data do not agree with those of ACKMAN[11], who found both isomers to be unresolved at 170° (ECL = 18.20).

Diethylene glycol succinate. A chromatogram of the methyl esters of fatty acids isolated from butter fat on DEGS is given in Fig. 7. From Figs. 7 and 4, it can be shown that DEGS is more polar than BDS, which resulted in a further retardation of the elution of the methyl esters of unsaturated acids. This effect is evident from

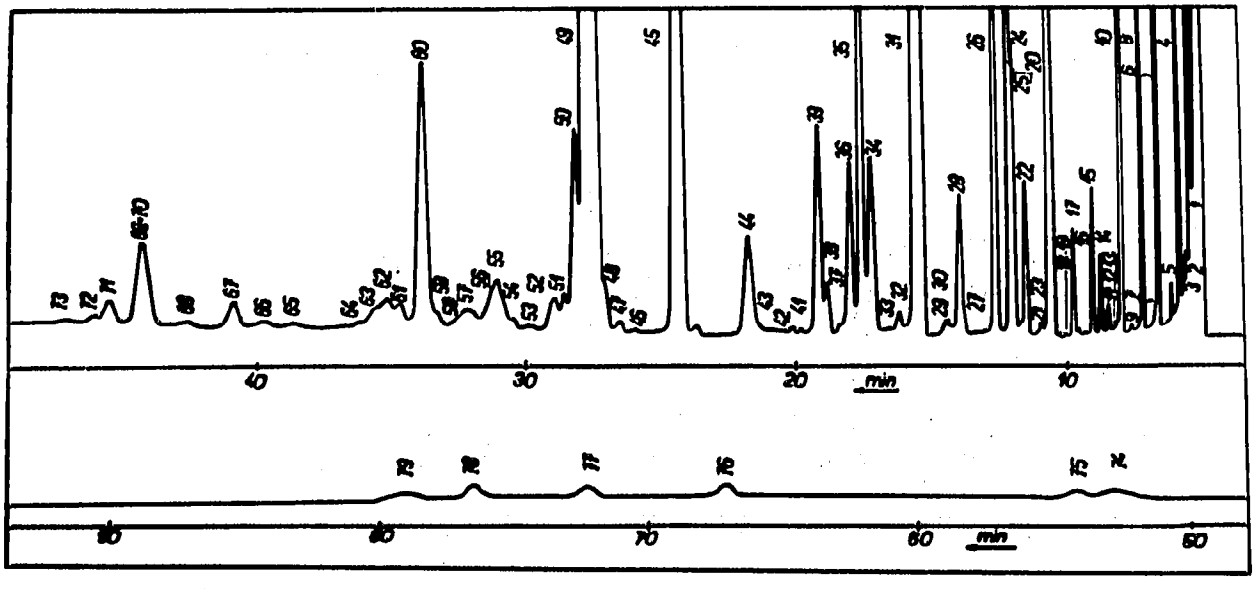

Fig. 7. Separation of the methyl esters of fatty acids isolated from butter fat on DEGS at 170°. 1 = 6:0; 2 = 7:0; 4 = 8:0; 5 = 9:0; 6 = 10:0; 8 = 10:1 + 11:0; 9 = *iso*-12:0; 10 = 12:0; 13 = *iso*-13:0; 14 = 12:1; 15 = 13:0; 16 = 13:1; 17 = *iso*-14:0; 20 = 14:0; 22 = *iso*-15:0; 24 = *anteiso*-15:0; 26 = 15:0; 28 = 15:1; 31 = 16:0; 34 = *iso*-17:0; 35 = 16:1; 36 = *anteiso*-17:0; 39 = 17:0; 44 = 17:1(9); 45 = 18:0; 49 = *cis*-18:1(9); 56 = 19:0; 60 = *cis,cis*-18:2 (9,12); 67 = 20:0; 69 = *cis,cis,cis*-18:3 (9,12,15); 70 = 20:1; 78 = 22:0.

the following examples. The methyl ester of tetradecanoic acid was eluted on BDS at 180° with the methyl ester of 13-methyltetradecanoic acid, but was eluted on DEGS at 160° unresolved from the methyl ester of 12-methyltetradecanoic acid; methyl esters of *trans*- and *cis*-hexadecanoic acid were eluted on BDS at 180° before the methyl esters of heptadecanoic acid, while on DEGS they remained unresolved and were eluted behind the methyl ester of 15-methylhexadecanoic acid; and the methyl ester of linolenic acid was eluted on BDS at 180° before the methyl ester of eicosanoic acid, but was elutated on DEGS at 175° together with the methyl ester of eicosenoic acid.

The *ECL* values of the methyl esters of monomethyl branched acids (*iso*, *anteiso*) on BDS and DEGS at 170° are in good agreement, but the temperature dependence of the *ECL* values on DEGS is nearly twice as great as that on BDS (Table III).

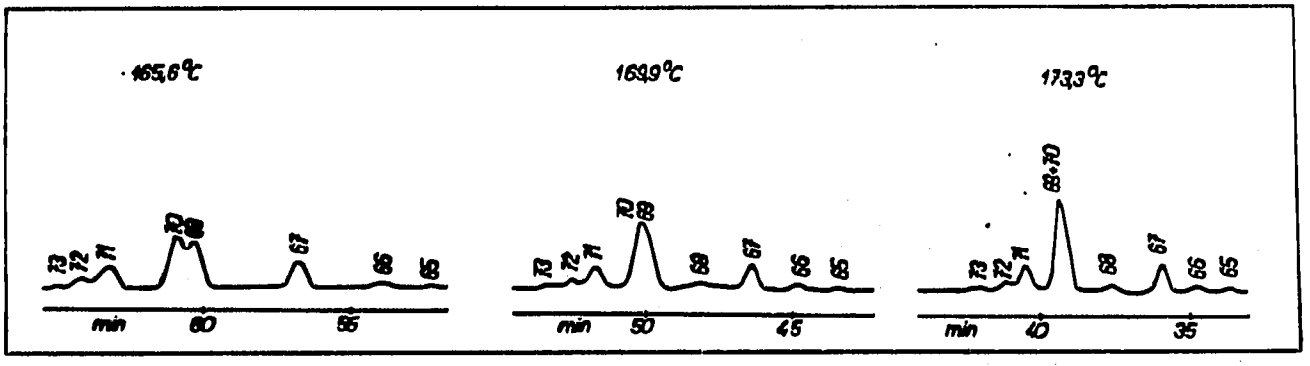

Fig. 8. Effect of temperature on the separation of the methyl esters of 18:3 and 20:1 acids isolated from butter fat on DEGS. 69 = 18:3; 70 = 20:1.

A higher temperature dependence of *ECL* values on DEGS than on BDS was found also for the methyl esters of unsaturated acids. For example, the methyl ester of oleic acid has a value of $\delta ECL/10°$ of 0.05, which is twice the value determined on BDS (Table IV).

An improvement in the separation of the methyl esters of unsaturated fatty acids by lowering the temperature is illustrated in Fig. 8.

The temperature dependence of the *ECL* values of the methyl esters of monoethylenoic isomers of fatty acids is being studied at present.

CONCLUSIONS

Separation factors for the methyl esters of normal saturated fatty acids are not constant. Therefore the methyl esters of odd-numbered fatty acids must also be included for the calculation of precise *ECL* values.

ECL values for all the methyl esters of fatty acids determined on Apiezon L and of the methyl esters of branched saturated fatty acids determined on BDS and DEGS have good reproducibility even if they are determined in several different laboratories.

The reproducibility of the *ECL* values of the methyl esters of unsaturated fatty acids determined on BDS and DEGS is inadequate as the result of the variable polarity of polyester phases as well as changes caused by ageing of the phase.

ECL values of the methyl esters of monomethyl branched fatty acids on Apie-

zon L and BDS are almost independent of temperature, but on DEGS the ECL values decrease with increasing temperature ($\delta ECL/\delta t < 0$).

ECL values of the methyl esters of unsaturated acids increase with increasing temperature ($\delta ECL/\delta t > 0$), greater asymmetry of the molecule and greater numbers of double bonds in the molecule.

A good resolution of the methyl esters of fatty acids isolated from biological materials can be achieved by using open tubular GC on the semipolar BDS phase at two different temperatures.

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