esters have progressively longer retention times when compared to 9-octadecenoic ester, and on this basis it is possible to find parallels strongly suggesting that volatility must be the determining factor. In a study of the effect of varying the acid and alcohol carbon chain lengths, while keeping the total carbon atoms constant, Lefort et al. (32) observed only slight differences in retention time on a polyester substrate where only relatively long acid and alcohol chains were involved, but an increase in retention time with a short chain in either the alcohol or the acid. It is known that the vapor pressures of esters of this type, with centrally located ester linkages, are higher than those with short chains (33); hence the shorter retention times. The opposing results of James and Scholfield for the monoethylenic octadecenoic fatty acids are thus explained.

It seems reasonable that the separation of unsaturated fatty acid esters into groups of the same structural type, with the same end carbon chain, depends on three separatory effects: a London force separation by chain length; a polar interaction with the single or methylene-interrupted double bond systems; and finally a slight modification in the volatility introduced by the length of the end carbon chain. Under these circumstances the application of the present linear plot system to any polyester seems feasible.

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An Analysis of Separation Factors Applicable in the Gas-Liquid Chromatography of Unsaturated Fatty Acid Methyl Esters on a Polyester Substrate

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Abstract

The employment of gas-liquid chromatography (GLC) separation factors between methyl esters of unsaturated fatty acids is feasible as a means of tentative identification, either between acids of one chain length and differing numbers of double bonds, or between acids of one chain length and the same number of double bonds in differing positions, provided the acid structures are appropriately grouped by end carbon chain. The modification of separation factors by temperature, chain length, number of double bonds, or position of double bonds is apparent from examination of a larger number of examples than was hitherto available. Examples of the usefulness of separation factors in identifying unknowns or predicting retention times are given.

Introduction

THE OBSERVATION has been made (1,2) that methyl esters of monoethylenic fatty acids, or of polyethylenic fatty acids with normal methylene-interrupted double bond systems, can be linearly interrelated when plotted on the basis of log retention time against number of carbon atoms in the fatty acid chain through the mutual number of double bonds and the same "end carbon chains." This correlating factor, defined as the number of carbon atoms from the center of the double bond farthest removed from the carboxyl group, to and including the terminal methyl group, is one carbon more than as originally conceived (1). This system permits the tentative identification of GLC peaks of esters of unsaturated fatty acids by extrapolation from components of a different chain length whose structure is known. The rationalization of certain literature data on this basis has permitted a listing, essentially an analysis on ethylene glycol-adipic acid polyester (EGA), of accurate retention times for methyl esters of a larger number of fatty acids with known and presumed structures than was hitherto available (2).

Systematic retention time relationships may also exist between the methyl esters of unsaturated fatty acids of one chain length and differing numbers of double bonds, or between those of the same chain length and the same number of double bonds, in different isomeric arrangements. This approach is based on the use of "separation factors" obtained by dividing the retention time or relative retention time of one fatty acid methyl ester by the lesser

retention time or relative retention time of another material of the same chain length. In this paper separation factors in general are denoted r, and retention times relative to methyl octadecanoate are denoted r_{18}. The importance attached to the end carbon chain in linearly relating acids of similar structure but different chain lengths indicated that this would be a logical basis for grouping data, and this was found satisfactory in practice. Moreover, these groupings often correspond to the structures of mixtures of acids of natural origin. Three arrangements of the end carbon chains have been studied, and the separation factors are accordingly designated types I, II, and III, as defined in detail below.

Procedure

Owing to the linear relationship of log r_{18} plots (2), certain type examples might have been sufficient to indicate the expected relationships in separation factors. However the data of Farquhar et al. (3) include a number of unknown or partly identified materials, not necessarily identifiable on the basis of the linear plot system. Their tentative identification is made possible by consideration of separation factors, and they have therefore been incorporated into those tables which are primarily intended to determine the separation factors. This in turn calls for the prediction by the linear plot system of r_{18} values for a few obscure fatty acids with structures necessary to complement the unknowns. Usually these unknowns can be correlated satisfactorily as illustrations of the value of logically determined separation factors. This is preferably accomplished through two or more different types of separation factors to reduce the possibility of erroneous identification through any error in the tabulated r_{18} values. This latter effect will produce some variation in the calculated r values even with data taken from one set of literature r_{18} values without the addition of transferred points (2).

Some of the acids not listed (2,3) have been reported in naturally occurring lipids. Thus, Klenk and co-workers report the finding of 11,14,17-eicosatrienoic and 8,11,14,17-eicosatetraenoic acids in herring oil (4); 5,8,11-eicosatrienoic and 7,10,13,16-docosatetraenoic acids in bovine adrenals (5); and 7,10,13,16-docosatetraenoic and 4,7,10,13,16-docosapentaenoic acids in brain tissue (6).

In Tables I, II, III, and IV all data taken directly from the literature are indicated in normal type, while all proposed structures and/or predicted relative retention times are underlined.

Type I Separation Factors. Table I shows in pairs those fatty acids with the same chain length and the same end carbon chain, but with different numbers of double bonds. The best correlation of separation factors is achieved when the fatty acid structures are arranged, as shown, in groups with the first double bonds in each pair falling in the same position. There is a tendency for the appropriate group separation factors to diminish as the number of double bonds increase, as the chain lengths increase, or the end carbon chains shorten. A slight loss in the separation efficiency of the polyester may cause this decrease. Thus the highest separation factor would be for 6,9-hexadecadienoate and 9-hexadecenoate, 1.17. Refer to data from (2) for 197C, end carbon chain seven. The effect of temperature is slight, although the reduction in temperature slightly increases the separation factors.

TABLE I
Separation factors of esters of fatty acids of the same chain length with varying numbers of double bonds and the same end carbon chains (type I)

	Fatty acids [a]	End carbon chain	197C		173.5C	
			r_{18}	r	r_{18}	r
22:6	4,7,10,13,16,19	3	7.75		9.55	
22:5	7,10,13,16,19	3	7.00	1.10	8.40	1.14
22:5	4,7,10,13,16	6	6.09		7.43	
22:4	7,10,13,16	6	5.50	1.11	6.40	1.16
22:5	7,10,13,16,19	3	7.00		8.40	
22:4	10,13,16,19	3	6.40	1.09	7.60	1.11
22:4	7,10,13,16	6	5.50		6.40	
22:3	10,13,16	6	5.00	1.10	5.89	1.09
22:4	10,13,16,19	3	6.40		7.60	
22:3	13,16,19	3	5.65	1.13	6.70	1.13
22:3	10,13,16	6	5.00		5.89	
22:2	13,16	6	4.38	1.13	5.15	1.15
20:5	5,8,11,14,17	3	3.85		4.33	
20:4	8,11,14,17	3	3.51	1.10	3.91	1.13
20:4	5,8,11,14	6	3.04		3.32	
20:3	8,11,14	6	2.76	1.10	3.02	1.10
20:4	8,11,14,17	3	3.51		3.91	
20:3	11,14,17	3	3.10	1.13	3.43	1.14
20:3	8,11,14	6	2.76		3.02	
20:2	11,14	6	2.45	1.13	2.66	1.13
20:2	8,11	9	2.32		2.48	
20:1	11	9	2.02	1.15	2.16	1.15
18:4	6,9,12,15	3	1.97		2.04	
18:3	9,12,15	3	1.72	1.14	1.76	1.16
18:3	6,9,12	6	1.54		1.56	
18:2	9,12	6	1.34	1.15	1.35	1.16
18:2	6,9	9	1.29		1.31	
18:1	9	9	1.12	1.15	1.12	1.17

[a] Notation after Farquhar (3).

Multiple r values covering differences due to several double bonds have not been listed. However, these are probably more accurate than those from a difference of one double bond. Thus the r values at 197C for 4,7,10,13,16,19-docosahexaenoate and 10,13,16,19-docosatetraenoate, and for 4,7,10,13,16-docosapentaenoate and 10,13,16-docosatrienoate, are both 1.21. These pairs are not related on the linear plot (2) and therefore are independently determined.

Type II Separation Factors. Table II lists in groups those fatty acids where the chain length is the same, but with differing numbers of double bonds in such positions that there are differences of three carbon atoms in the end carbon chains. At 197C there is a remarkable uniformity in the r values for the 3/6 end carbon chain pairs, and also in the 6/9 and 3/9 end carbon chain series as well, with the exception in the latter of those separation factors involving the proposed 4,7,10,13-docosatetraenoate structure. However at 173.5C the latter correlates satisfactorily with other pairs of the 3/9 and 6/9 series. Therefore, the r_{18} value at 197C may be slightly in error, but this cannot be verified by the other types of separation factors. As little as 0.10 difference would bring this ester into line. Alternatively the tentative structural identification may be wrong and the satisfactory correlation at 173.5C obtained through chance.

The separation factors for the 16-carbon acid series are not listed since the reported retention time for 6,9,12-hexadecatrienoate cannot be verified (2). However the 4/7 r of 1.22 falls between the others in the relation 3/6:4/7:6/9::1.27:1.22:1.19 at

TABLE II

Separation factors of esters of fatty acids of the same chain length, varying numbers of double bonds, and different end carbon chains (type II)

Fatty acid [a]		End carbon chain	197C r_{18}	197C r values 3/6	197C r values 6/9	197C r values 3/9	173.5C r_{18}	173.5C r values 3/6	173.5C r values 6/9	173.5C r values 3/9
22:6	4,7,10,13,16,19	3	7.75				9.55			
22:5	4,7,10,13,16	6	6.09	1.27			7.43	1.28		
22:4	4,7,10,13	9	5.30		1.15	1.46	6.17		1.21	1.55
22:5	7,10,13,16,19	3	7.00				8.40			
22:4	7,10,13,16	6	5.50	1.27			6.40	1.31		
22:4	10,13,16,19	3	6.40				7.60			
22:3	10,13,16	6	5.00	1.28			5.89	1.29		
22:2	10,13	9	4.20		1.19	1.52	4.83		1.22	1.57
20:5	5,8,11,14,17	3	3.85				4.33			
20:4	5,8,11,14	6	3.04	1.27			3.32	1.30		
20:4	8,11,14,17	3	3.51				3.91			
20:3	8,11,14	6	2.76	1.27			3.02	1.30		
20:2	8,11	9	2.32		1.19	1.51	2.48		1.22	1.58
18:4	6,9,12,15	3	1.97				2.04			
18:3	6,9,12	6	1.54	1.28			1.56	1.31		
18:2	6,9	9	1.29		1.19	1.53	1.31		1.19	1.56
18:3	9,12,15	3	1.72				1.76			
18:2	9,12	6	1.34	1.28			1.35	1.30		
18:1	9	9	1.12		1.19	1.54	1.12		1.21	1.57
16:3	6,9,12	4	0.904				0.860			
16:2	6,9	7	0.640				0.685			

[a] Notation after Farquhar (3).

197C and correspondingly at 173.5C.

An independent determination of these values for this series is possible from the data of Stoffel and Ahrens (7), obtained on EGA at 197C. It is assumed that their data for the hexadecatetraenoate and hexadecatrienoate represent the predominant isomers, the 6,9,12,15 and 6,9,12 forms respectively. The r values from these data for end carbon chain ratios of 1/4, 4/7 and 1/7 are then respectively 1.22, 1.23 and 1.50. Applying these r values to the probable r_{18} for 6,9-hexadecadrienoate, 0.740 (2), then the r_{18} for 6,9,12,15-hexadecatetraenoate at 197C is 1.11, in near agreement with the listed 1.06 and the reported

TABLE III

Separation factors of esters of fatty acids of the same chain length, the same number of double bonds, and different end carbon chains (type III)

Fatty acid [a]		End carbon chain	197C r_{18}	197C r	173.5C r_{18}	173.5C r
22:5	7,10,13,16,19	3	7.00		8.40	
22:5	4,7,10,13,16	6	6.09	1.15	7.43	1.13
22:4	10,13,16,19	3	6.40		7.60	
22:4	7,10,13,16	6	5.50	1.16	6.40	1.13
20:4	8,11,14,17	3	3.51		3.91	
20:4	5,8,11,14	6	3.04	1.15	3.32	1.18
20:3	11,14,17	3	3.10		3.43	
20:3	8,11,14	6	2.76	1.12	3.02	1.14
18:3	9,12,15	3	1.72		1.76	
18:3	6,9,12	6	1.54	1.12	1.56	1.13
22:4	7,10,13,16	6	5.50		6.40	
22:4	4,7,10,13	9	5.30	1.04	6.17	1.04
20:2	11,14	6	2.45		2.66	
20:2	8,11	9	2.32	1.05	2.48	1.07
18:2	9,12	6	1.34		1.35	
18:2	6,9	9	1.29	1.04	1.31	1.03
18:2	11,14	4	1.45		1.48	
18:2	8,11	7	1.33	1.09	1.34	1.11
16:2	9,12	4	0.810		.760	
16:2	6,9	7	0.740	1.09	.685	1.11
16:1	9	7	0.634		.....	.....
16:1	7	9	0.625	1.01	.....	.....

[a] Notation after Farquhar (3).

nonseparation from the octadecanoate peak (3). The same arguments confirm (as 0.910) the r_{18} for 6,9,12-hexadecatrienoate of 0.904 as listed by Farquhar et al. (3) and impossible to correlate by linear plot (2).

Type III Separation Factors. The r values listed in Table III are based on the same chain length and the same number of double bonds and fall into clearly defined groups depending on a 3/6, 6/9, or 4/7 end carbon chain ratio. The individual groups cover a fairly wide range of values, but in both the 3/6 series and 6/9 series there is an indication that the maximum r value is obtained where the acid structure with the most highly centralized arrangement of double bonds has the shorter r_{18} value. There are, however, not enough data to generalize.

Discussion

On the basis of this interrelation of esters of highly unsaturated fatty acids through separation factors, the probable structures for a number of the more highly unsaturated unknowns listed by Farquhar et al. (3) at 197C can be predicted. Thus the 22:5 ester with r_{18} of 6.09 should be 4,7,10,13,16-docosapentaenoate; the unknown with interpolated r_{18} of 5.50 will probably be 7,10,13,16-docosatetraenoate; and the 22:4 material with r_{18} of 5:30 could be 4,7,10,13-docosatetraenoate.

Recent evidence (15) that marine oils contain hitherto unreported amounts of polyunsaturated C_{21} acids suggests that the latter unknown could be a heneicosapentaenoate. See linear log plot (2). Thus the discrepancies in the type II separation factors for this material (Table II) are explained, and 4,7,10,13-docosatetraenoate can be predicted to have an r_{18} of 5.10 at 197C.

Of the remaining unidentified materials, that with r_{18} of 4.11, stated to be a 20-carbon acid, must be abnormal in structure. Ruling out an eicosahexanoate structure as unlikely, it must be provisionally considered to be an eicosapentaenoate ester with an end carbon chain of 1 or 2; see rules (2). The material with r_{18} of 6.60 is also unidentifiable, although stated

to have a docosapentaenoate structure, and must also be abnormal with an end carbon chain of 4 or 5. Comparison of the type III separation factors with those isomers with the next longer normal end carbon chains (Table IV) indicates a possible similarity between the structures of the unknowns. The separation factors listed for the 16-carbon acids are the only comparable ones available, and are dubious since the r_{18} values are not necessarily accurate, as well as being based on an extremely different chain length. While not excluding the shorter end carbon chain possibilities, i.e., 1 and 4 respectively, the comparison seems to eliminate the alternative possibilities with respective end carbon chains of 2 and 5. The solution of the structure of these two unknowns by physical and chemical means would be desirable in the study of separation factors, as well as an answer to the obvious question of whether they might be artifacts originating during GLC or preliminary handling.

Examination of these materials on a linear log plot (2) suggests the simple possibilities that the material with r_{18} of 4.11 may be a heneicosatetraenoate related to 5,8,11,14-eicosatetraenoate and that with r_{18} of 6.60 a tetracosenoate.

The unknown with 20 carbon atoms (3), r_{18} 2.34 at 173.5C and 2.18 (by interpolation) at 197C may be a non methylene-interrupted diene (2) or an isomer of 11-eicosenoate. Unfortunately, the type III separation factors for monounsaturated acids are not available; but on a 6/9 ratio basis (assuming 14-eicosenoate), r values would approximate respectively 1.08 and 1.08 from 11-eicosenoate. These are moderately close to the values for the 8,11 and 11,14 dienes (Table III).

The type I separation factors (14-eicosenoate from 11,14-eicosenoate) are, respectively, 1.14 and 1.13, in agreement with the general values (Table I). Considering the general absence of exotic dienes from marine oils (7), an isomer of 11-eicosenoate is an attractive possibility for this unknown, although 13-eicosenoate appears more likely for biological reasons that 14-eicosenoate. Dogfish liver oil is reported to contain 9-eicosenoic acid (8), but the type III separation factor for either the 9 or 13 isomers from the 11-isomer might be expected to be lower than that observed. Cf. Table III; see also discussion (2).

As a further example of the application of separation factors, assuming an average type II 3/9 separation factor of 1.52, the 5,8,11-eicosatrienoic acid reported by Stoffel and Ahrens (9) as a constituent of menhaden oil, and by Klenk and Eberhagen (5) as a constituent of bovine adrenals, would have r_{18} of $3.85 \div 1.52 = 2.53$. Nothing is reported in this region by Farquhar et al. (3), but this very minor component might have fallen under the 11,14-eicosadienoic peak, r_{18} of 2.45.

Separation factors should be relatively independent of concentration of substrate, although if this concentration is greatly reduced there is a possibility of modification of the factors through interaction with the support (16). The type II r values from the data of Daniels and Richmond (10) for 9-octadecenoate, 9,12-octadecadienoate and 9,12,15-octadecatrienoate, determined on 2.3% EGA at 177C, are for 3/6, 6/9 and 3/9 ratios respectively 1.27, 1.20 and 1.52. These are lower than the values given in Table II for EGA at a concentration of 20–25% (173.5C) although very close to the 197C data and to values obtained with another EGA type polyester, Reoplex 400, at 197C, respectively 1.26, 1.19 and 1.50 (3). Despite the general scattering of literature values, these values for low substrate concentration are in fair agreement with other data. Cf. (11).

TABLE IV

Comparison of the type III separation factors between unknowns of presumed abnormal structure and possible positional isomers

Fatty acid [a]		End carbon chain	197C r_{18}	197C r	173.5C r_{18}	173.5C r
20:?	?	1-2	4.11		4.75	
20:5	5,8,11,14,17	3	3.85	1.07	4.33	1.09
22:5	?	4-5	6.60		8.15	
22:5	4,7,10,13,16	6	6.09	1.08	7.43	1.09
16:4	6,9,12,15	1	1.11		1.10	
16:4	4,7,10,13	3	1.08	1.03	1.07	1.03
16:3	6,9,12	4	0.904		0.860	
16:3	4,7,10	6	0.860	1.05	0.805	1.07

[a] Notation after Farquhar (3).

The type II separation factor differences, such as those obtained at 197C for 9-octadecenoate and 9,12-octadecadienoate, r 1.19, and for 9,12-octadecadienoate and 9,12,15-octadecatrienoate, r 1.28 (Table II), have been ascribed to the influence of vapor pressure (2, 12). In general lower temperatures evidently improve all separations slightly.

The type II separation factor for *cis,cis*-9,12-octadecadienyl acetate and *cis*-9-octadecenyl acetate, separated at 184.8C on an EGA polyester (13), is 1.21, the same as the value for the methyl esters of the corresponding acids on EGA at 184.5C (3). The assessment of the effect of interchanging the acid and alcohol moieties of saturated esters (14) has been discussed elsewhere (2). This suggests a wide potential application of suitable correlated separation factors. However the present study is intended to investigate the correlation of structure and separation factors under particular conditions and the comparative use of separation factors by different workers would require careful consideration of many factors.

The use of separation factors complements the linear log plot relationship since one employs fatty acids of the same chain length and the other fatty acids of differing chain lengths. The application of these procedures is intended only as a guide to the further identification of GLC peaks through isolation, degradation, etc. However, with known or standardized mixtures of fatty acids it provides a ready means for predicting peak overlap, and identifying differences caused by column age or changes in operating conditions.

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