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SOME NEW OBSERVATIONS ON THE EQUIVALENT CARBON NUMBERS OF TRIGLYCERIDES AND RELATIONSHIP BETWEEN CHANGES IN EQUIVALENT CARBON NUMBER AND MOLECULAR STRUCTURE

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SUMMARY

A further development, giving more reproducible results, of the procedure for calculating equivalent carbon numbers (ECNs) is presented. The reproducibility is listed. ECNs define the order of elution of triglycerides and give information as to where the peak of a defined triglyceride will appear in the chromatogram. The ECN values of triglycerides are, to a reasonable degree, equal to the sum of their partial ECN values (fatty acid ECN values). They thus reflect the contribution of each fatty acid to the chromatographic properties of the total triglyceride molecule. A list of basic data is presented and the changes in ECN values are described, together with the corresponding changes in the triglyceride molecule.

INTRODUCTION

As early as 1950 Martin[1] reported the relationship between the free energy of the separation process in chromatography and the structure of the chromatographed molecule. The relationship is frequently discussed in later papers[2–5].

In 1954 Ray[6] described the linear relationship between the logarithms of retention times (t_R) and carbon numbers (CN) of the members of a homologous series. This relationship can be used to identify the components/peaks in a chromatogram; however, it is necessary to compensate for the day-to-day variations in the chromatographic process and to achieve this normally two methods are used.

According to the first method, the retention times are expressed as relative retention times (RRT), with reference to one of the peaks in the chromatogram (in lipid analysis usually the peak of triolein). All the peaks are thus characterized in relation to one defined point of the chromatogram.

Goiffon *et al.*[7,8] presented a system based on RRTs, used in high-performance liquid chromatography (HPLC). The system shows close correlation between the molecular structures of defined triglycerides and their retention times.

Perrin and Naudet[2] presented a table of RRTs for a series of triglycerides, and Stolyhwo *et al.*[9] discussed the features of the system. Sempore and Bezard[3] found the parallelism between of RRTs and ECNs, and presented a linear regression plot of RRTs *versus* ECNs.

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In the second method two standards (in lipid analysis saturated triglycerides are preferred) are added to the sample. The peaks in the chromatogram are then related to the line, represented by the two points. The system of Kovats indexes, developed for the gas chromatography (GC) of structurally similar substances such as pharmaceutical products, uses the two-point principle[4,10].

In the area of lipid analysis the same principle has been used for identification of fatty acids in GC. In this way it is possible to characterize all the peaks in the chromatogram at once, using only two standard substances. As standard substances saturated fatty acids were used, and the values obtained were called "equivalent chain lengths" (ECL)[11,12].

It was shown that the same principal relationships were valid even in reversed-phase (RP) HPLC. However, only a few papers dealing with retention indices in RP-HPLC have been published[5,13–17].

In this laboratory, we have developed a system similar to ECLs for the identification of triglyceride components/peaks in RP-HPLC of glyceride oils and fats[18–21]. In order to emphasize the similarity the units were called "equivalent carbon numbers" (ECNs), and the base in this case was the homologous series of saturated triglycerides.

This paper develops further the ECN system, based on three standard points, to obtain a more exact position of the reference line. This is a continuation of earlier work, in which mainly graphical results were presented[19]. The aim is to present a list of ECN values of some triglyceride model substances, and show some of the relationships between the variations in the ECN values and the structural changes in triglyceride molecules by the use of figures and tables.

The term equivalent carbon number (ECN) in this and in all previous publications from this laboratory is definitely not identical with the term "partition number" (PN), though it is used in that way by many workers. This is evident because the ECN values are not integers, contrary to PNs, just as ECNs are relative (equivalent) numbers[22].

The notion of PNs, sometimes also called ECNs, was useful in the early days of the technique, when resolving power was relatively limited[3,22–24].

EXPERIMENTAL

Apparatus

An Optilab 593I liquid chromatograph, Tecator, a refractive index detector equipped with a 10-mm measuring cell and a Rheodyne injection block with a 10-μl loop were used. Two 25 cm Hibar RP-18 columns (Merck, ser.nos. 410 728 and 204 551, 5 μm particle diameter, 4 mm I.D.) were coupled in series and thermostated to 30°C. An HP reporting integrator 3390A was also used.

Materials

Model triglycerides, purity 99%, were purchased from Larodan (Malmö, Sweden).

Propionitrile for synthesis (Merck) was distilled before use and before each reuse from Siccapent (Merck). Toluene p.a. (Merck) was used without further purification).

Fatty acid symbols

La = Lauric acid (C12:0); M = myristic acid (C14:0); P = palmitic acid (C16:0); Po = palmitoleic acid (C16:1,9c); Pe = petroselinic acid (C16:1,6c); St = stearic acid (C18:0); O = oleic acid (C18:1,9c); El = elaidic acid (C18:1,9t); L = linoleic acid (C18:2,9c,12c); Ln = linolenic acid (C18:3,9c,12c,15c); Ad = arachidic acid (C20:0); Ao = *cis*-11-eicosenoic acid (C20:1,11c); Be = behenic acid (C22:0); E = erucic acid (C22:1,13c).

Procedure

Volumes of 10 μl of working solution (*ca.* 0.6% in propionitrile–toluene, 3:2, v/v) were injected into the column. The mobile phase was propionitrile at a flow-rate of *ca.* 0.5 ml/min.

Calculation of ECN values

An AB800 microcomputer (Luxor, Sweden) was used, with our own BASIC program, which can be obtained on request.

RESULTS AND DISCUSSION

Definitions and calculations of ECNs

According to the principal definition of ECNs[19], the retention times of the triglycerides studied are related to the retention times of saturated triglycerides. This gives a basis for the uniform consideration of the data.

The basic relation between the logarithms of the corrected retention times and the ECNs of the saturated triglycerides (by definition equal to the carbon number) is linear, as shown in Fig. 1. Theoretically, for construction of the line only two points

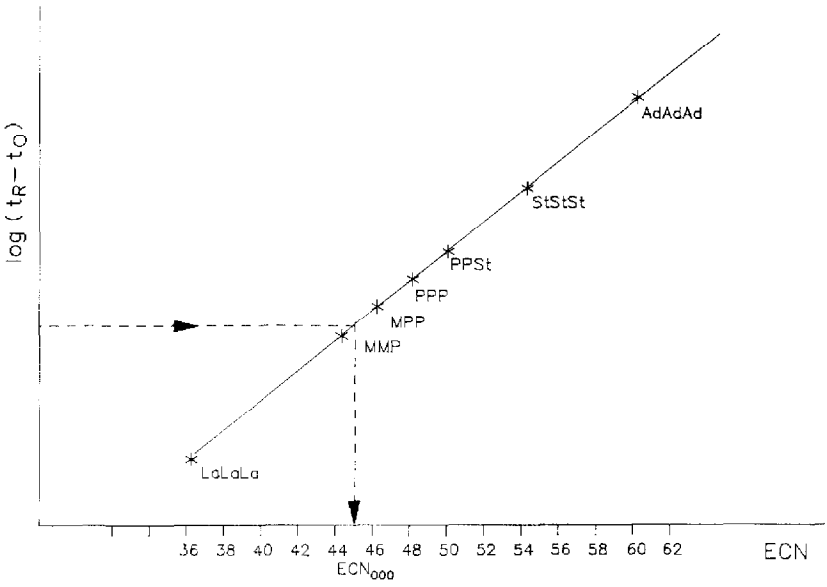

Fig. 1. Plot of log $(t_R - t_0)$ *versus* the carbon number of saturated triglycerides and the interpretation of ECN_{000}.

are necessary, but it is not possible to determine exactly the value of t_0 from the chromatogram and therefore a third point is needed before the exact position of the line can be determined. The procedure using three standards gives very reproducible ECN values and can be used for the determination of the void volume of the column system, t_0.

In this laboratory a spiking solution is used, containing trilaurin and tristearin as outer points for the definition of the standard line and trimyristin as the third point defining t_0 and thus the position of the line.

In principle, the above-mentioned considerations can be expressed by three equations:

$$\log(t_{R,LaLaLa} - t_0) = k \cdot CN + b$$

$$\log(t_{R,MMM} - t_0) = k \cdot CN + b$$

$$\log(t_{R,StStSt} - t_0) = k \cdot CN + b$$

where t_0 (dead time), k and b are the three unknowns. The computer program calculates t_0 and ECN values.

A similar standard solution containing a further component, triolein (OOO), is used to test colums and to characterize their polarity, which is expressed as ECN of triolein. Only pairs of column giving in combination $ECN_{OOO} = 45.16 \pm 0.02$ are used in this work. Table I lists polarity values for eighteen tested Merck Hibar RP-18 columns.

Relation of ECN values to structural changes in the triglyceride molecule

ECN values of some triglycerides, their reproducibilities and other molecular characteristics are presented in Table II. The values were determined experimentally by spiking the standard or sample solutions with LaLaLa, MMM and StStSt.

The ECN values of triglycerides can be considered as sums of "partial" fatty acid ECN values and can thus be calculated by addition with reasonable accuracy[19]. The partial ECN values for fatty acids are presented in Table III. These can also be used as preliminary information concerning unknown peaks.

TABLE I

POLARITIES OF SOME HIBAR RP-18 COLUMNS EXPRESSED AS ECN_{OOO}

Serial No.	*ECN_{OOO}*	*Serial No.*	*ECN_{OOO}*
110 445	45.15	410 728	45.13
204 551	45.16	410 800	45.10
208 749	45.05	415 207	44.82
303 452	45.11	415 208	45.32
308 514	45.12	415 212	45.32
308 532	45.09	415 242	45.21
308 572	45.12	415 277	45.21
314 884	45.19	420 509	45.24
314 894	45.11	423 251	45.26

TABLE II

ECN VALUES OF DIFFERENT TRIGLYCERIDES AND THEIR REPRODUCIBILITY

Triglyceride type	*ECN*			*CN*	*Double bonds*	
	Value	*S.D.*	*Number of determinations*		*Number*	*Composed of*
LaLaLa[a]	36.00			36	0	
MMP[a]	44.00			44	0	
MPP[a]	46.00			46	0	
PPP[a]	48.00			48	0	
PPSt[a]	50.00			50	0	
PStSt[a]	52.00			52	0	
StStSt[a]	54.00			54	0	
AdAdAd[a]	60.00			60	0	
MPO[b]	45.17	0.015	10	48	1	0 + 0 + 1c
PPO[a]	46.97	0.044	10	50	1	0 + 0 + 1c
PPEl[a]	47.42	0.003	9	50	1	0 + 0 + 1t
PStO[a]	48.99	0.008	7	52	1	0 + 0 + 1c
StStO[a]	50.97	0.006	6	54	1	0 + 0 + 1c
StStEl[a]	51.36	0.003	9	54	1	0 + 0 + 1t
StOAd[a]	52.80	0.009	11	56	1	0 + 0 + 1c
StOBe[a]	54.72	0.006	9	58	1	0 + 0 + 1c
OAdAd[b]	54.68		1	58	1	0 + 0 + 1c
MPL[b]	42.79	0.017	7	48	2	0 + 0 + 2c
MOO[a]	44.10	0.005	9	50	2	0 + 1c + 1c
PPL[b]	44.54	0.054	10	50	2	0 + 0 + 2c
POO[a]	46.05	0.008	7	52	2	0 + 1c + 1c
PStL[b]	46.46	0.039	6	52	2	0 + 0 + 2c
StOO[a]	47.99	0.005	6	54	2	0 + 1c + 1c
StOEl[a]	48.37	0.005	9	54	2	0 + 1c + 1t
StStL[a]	48.45	0.013	7	54	2	0 + 0 + 2c
StElEl[a]	48.78	0.004	9	54	2	0 + 1t + 1t
OOAd[a]	49.92	0.007	16	56	2	0 + 1c + 1c
OOBe[a]	51.79	0.018	11	58	2	0 + 1c + 1c
MMLn[a]	38.56	0.003	9	46	3	0 + 0 + 3c
PoPoPo[a]	39.50	0.006	9	48	3	1c + 1c + 1c
POL[b]	43.65	0.021	8	52	3	0 + 1c + 2c
OOO[a]	45.17	0.032	7	54	3	1c + 1c + 1c
StOL[b]	45.57	0.063	8	54	3	0 + 1c + 2c
PePePe[a]	46.33	0.004	9	54	3	1c + 1c + 1c
ElElEl[a]	46.27	0.007	11	54	3	1t + 1t + 1t
StStLn[a]	46.42	0.000	9	54	3	0 + 0 + 3c
OLAd[a]	47.49	0.008	11	56	3	0 + 1c + 2c
AoAoAo[a]	50.51	0.010	9	60	3	1c + 1c + 1c
EEE[a]	55.93	0.013	7	66	3	1c + 1c + 1c
PLL[b]	41.09	0.022	7	52	4	0 + 2c + 2c
OOL[b]	42.79	0.017	7	54	4	1c + 1c + 2c
LLL[a]	38.18	0.005	7	54	6	2c + 2c + 2c
LnLnLn[a]	32.34	0.044	23	54	9	3c + 3c + 3c

[a] ECN values using synthetic model triglycerides.
[b] ECN values by analysis of fractions.

TABLE III

PARTIAL ECN VALUES OF FATTY ACIDS

Partial ECN values of saturated fatty acids are equal their carbon numbers.

Fatty acid	*Partial ECN value*
Po	13.12
O	15.05
El	15.43
L	12.73
Ln	10.81
Ao	16.83
E	18.64

ECNs and CNs of the triglycerides, when plotted, give the diagram presented in Fig. 2. The family of almost parallel lines indicates that each change in the triglyceride molecule causes a particular change in ECN, as it will be shown in the following text. The diagram can thus give the first indication about the possible composition of an unknown fraction/peak, displaying the tentative carbon numbers and numbers of double bonds (NDB) and their composition.

The lines on this diagram can also be expressed as regression lines. The calculated regression parameters are listed in Table IV. The lines in the table are specified by the degree of unsaturation and the combination of double bonds in the molecule. The

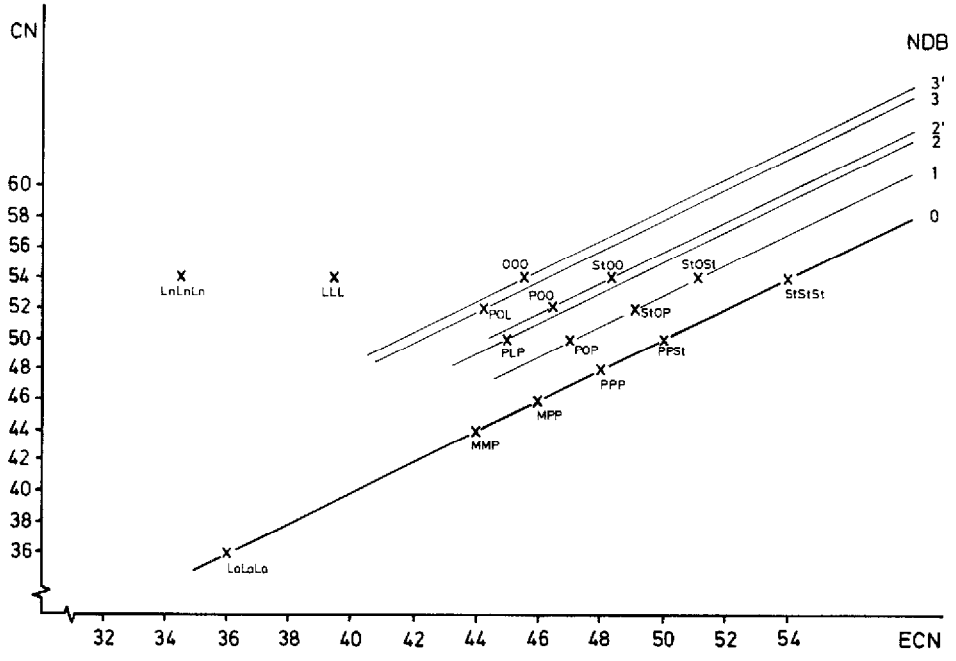

Fig. 2. Graphical representation of the relationship of ECN values to carbon numbers (CN). Curve: 0 = saturated triglycerides; 1 = one monounsaturated fatty acid; 2 = two unsaturated fatty acids; 2′ = one diunsaturated fatty acid; 3 = three monounsaturated fatty acids; 3′ = one monounsaturated and one diunsaturated fatty acid. Two 25 cm Hibar RP-18 columns, 4 mm I.D.; mobile phase: acetone–acetonitrile (64:36, v/v).

TABLE IV

REGRESSION PARAMETERS FOR THE LINES IN FIG. 2

Line combination of double bonds	*Slope*	*Intercept*	*n*	*Correlation*
0 + 0 + 0	1.00000	0.00000	7	1.00000
0 + 0 + 1	1.03539	1.30878	7	0.99984
0 + 1 + 1	1.03890	4.16420	5	0.99997
0 + 0 + 2	1.02822	4.21171	3	0.99997
1 + 1 + 1	1.03093	7.43300	2	–
0 + 1 + 2	1.04167	6.53125	3	1.00000
0 + 0 + 3	1.01911	6.70318	2	–

parameters are calculated using only triglycerides containing saturated and unsaturated fatty acids with methylene-interrupted double bonds in the *cis*-configuration starting from the 9-position and upwards, *i.e.* the natural vegetable oil fatty acids. It can be seen that the slope parameters of the lines are virtually equal and the lines are thus parallel, with the slight exception of the line for saturated triglycerides. The other parameter, the intercept, increases as expected with the number of double bonds. In addition, significant differences in this parameter are observed even for triglycerides with different double-bond combinations in the molecule.

According to the definition, the ECN values of saturated triglycerides are equal to their carbon numbers. Their ECN values thus increase by two units with a change of two in carbon number. The differences in ECNs with a change of two carbon atoms in unsaturated triglycerides are somewhat lower than two, and statistically significant. From Table V it can be seen that the mean difference is -1.94 and that the mean value has a total S.D. of 0.04. In this table the differences are presented in groups according to the degree and combination of unsaturation, and show no deviation between the groups.

These results confirm the observation, based on the regression line analysis, that the lines for the unsaturated triglycerides are reasonable parallel to each other, but not to that for the saturated triglycerides, which has a slightly lower slope parameter than the unsaturated ones.

The changes in ECN through the incorporation of one or more double bonds in a saturated triglyceride molecule are presented in Table VI. When the first double bond is introduced into the saturated triglycerides the differences between the ECN values rise with increasing carbon number. This is explained by the lower slope of the saturated triglyceride line in comparison with the line for unsaturated triglycerides (Table VI, column 2). Those figures are therefore not used in any calculation.

The real lowering of ECN by introduction of one double bond can be estimated from the introduction of a second and further double bonds in an already unsaturated triglyceride, and this value is *ca.* -2.89 ECN, with an S.D. of 0.08 ECN (Table VI).

The examples in the Table VII, however, show that the increment of one double bond, through an exchange of one monounsaturated fatty acid for a diunsaturated one, produces an ECN change that is lower than that in the case discussed above, *i.e.* -2.47 ECN, with an S.D. of 0.07 ECN. Only one measurement can be presented for

TABLE V

DIFFERENCE IN ECN AFTER CN CHANGE OF 2

Triglycerides	*Change in ECN*	*Triglycerides*	*Change in ECN*		*Triglycerides*	*Change in ECN*		
			0+1+1	*0+0+2*		*1+1+1*	*0+1+2*	*0+0+3*
MPO to PPO	−1.96	MOO to POO	−2.00		(PoPoPo to OOO)/3	−1.94		
PPO to PStO	−1.97	POO to StOO	−1.94		POL to StOL		−1.92	
PStO to StStO	−1.94	StOO to OOAd	−1.93		StOL to OLAd		−1.97	
StStO to StOAd	−1.94	MPL to PPL		−1.95	(MMLn to StStLn)/4			−1.96
StOAd to StOBe	−1.86	PPL to PStL		−1.92				
StOAd to StOBe	−1.86	PStL to StStL		−1.97				
Mean (total)	−1.935							
S.D. (total)	0.037							

TABLE VI

DIFFERENCE IN ECN WITH INCREMENT OF ONE DOUBLE BOND BY ONE MONOUNSATURATED FATTY ACID (CN CONSTANT)

Triglycerides	*Change in ECN*	*Triglycerides*	*Change in ECN*	*Triglycerides*	*Change in ECN*	*Triglycerides*	*Change in ECN*
MPO to MPSt	−2.99	POO to PStO	−2.94	OOO to StOO	−2.82	POL to PStL	−2.81
PPO to PPSt	−3.03	StOO to StStO	−2.98			StOL to StStL	−2.88
PStO to PStSt	−3.01	OOAd to StOAd	−2.88			OOL to StOL	−2.78
StStO to StStSt	−3.03	StOEl to StStEl	−2.99				
StOAd to StStAd	−3.20						
StOB to StStBe	−3.28						
OAdAd to StStBe	−3.32						
Mean total ($n=8$)		2.89					
S.D.		0.079					

TABLE VII

DIFFERENCES IN ECN WITH CHANGES BY ONE DOUBLE BOND THROUGH THE CHANGE FROM LOWER TO HIGHER UNSATURATED FATTY ACID (CN CONSTANT)

Triglycerides	*Change in ECN*	*Triglycerides*	*Change in ECN*
MPL to MPO	−2.42	StStLn to StStL	−2.02
PPL to PPO	−2.43		
PStL to PStO	−2.53		
StStL to StStO	−2.52		
PLL to POL	−2.56		
OOL to OOO	−2.38		
OLAd to OOAd	−2.43		
Mean	−2.47		−2.02
S.D.	0.068		

the exchange of one diunsaturated fatty acid for a triunsaturated one, which gave a still lower difference, −2.02 ECN. These observations can be related to the effect of the positioning of the double bond in the fatty acid chain, shown in the Table VIII.

When the double bond moves along the fatty acid chain in a triglyceride, the ECN value changes. This is shown in Table VIII by three examples, which explain why tripetroselinin (3 × C18:1,6c), triundecanoin (3 × C20:1,11c) and trierucin (3 × C22:1,13c) do not fit the line for triunsaturated triglycerides containing three isolated double bonds (1 + 1 + 1), as defined by tripalmitolein (3 × C16:1,9c) and triolein (3 × C18:1,9c). PoPoPo and OOO, like most naturally occurring fatty acids, have their first double bond just in the 9 position.

It was not possible to find fatty acids C20:1,9c and C22:1,9c, or triglycerides based on them, to measure the ECN values experimentally. Instead the values were calculated from the regression line. The values are therefore, to some degree, uncertain. Tripetroselinin was measured experimentally as standard. Nevertheless, the results show that the displacement of one double bond in the vicinity of C-9 of a fatty acid chain in a triglyceride to a vicinal carbon atom produces a change in ECN of between 0.07 and 0.13 units.

No attempt was made to explain this effect, because of the lack of basic experimental data. However, the values presented can partially explain the observed low

TABLE VIII

DIFFERENCES IN ECN WITH CHANGES IN THE POSITION OF THE DOUBLE BOND ALONG THE FATTY ACID (FA) CHAIN

Triglycerides	*Difference*	*Difference per FA*	*Difference per FA and per carbon atom*
(PePePe to OOO)/3	+1.16	+0.39	+0.13
(AoAoAo to 3 × C20:1,Δ9)/3	−0.47	−0.16	−0.08
(EEE to 3 × C22:1,Δ9)/3	−0.88	−0.29	−0.07

TABLE IX

DIFFERENCES IN ECN AFTER ADDITION OF BROMINE TO THE OLEIC ACID DOUBLE BOND IN MONOUNSATURATED TRIGLYCERIDES

Triglyceride	*ECN*		
	Not treated (both isomers)	*Bromine added*	
		Symmetrical	*Unsymmetrical*
PPO	46.97	44.70	44.91
PStO	48.99	46.71	46.93
StStO	50.97	48.65	48.88
StOO	47.99	44.02	44.10

differences in ECN when a monounsaturated fatty acid is exchanged for a diunsaturated, or a diunsaturated for a triunsaturated one.

Each new double bond in C_{18} fatty acids, for example, is created in a position three carbon atoms further away from the carboxyl group, which represents an ECN difference of 0.21–0.33 units. This does not explain the whole difference of 0.42 (2.89 minus 2.47), but possibly the greater part of it.

It was shown[21] with four isomeric pairs (rac-PPO and rac-POP, rac-PStO and rac-POSt, rac-StStO and rac-StOSt, and rac-StOO and rac-OStO) that the position of the unsaturated fatty acid in the triglyceride has no measurable influence on the ECN value.

A special case, however, is the change in ECN with addition of bromine to the oleic acid double bond in monounsaturated triglycerides. Bromine addition was performed directly in pentane solution[21,25]. Table IX shows the results obtained with four isomer pairs. The addition of bromine enhances the polarity of the triglyceride, shortens the retention time and reduces the ECN value. The two possible positions where the unsaturated fatty acid can be placed are thus not equivalent. The difference of more than 0.20 between the ECN of the two isomers is sufficient to allow the peaks of the isomer pairs to be half separated at *ca.* 40 000 and nearly completely separated at 80 000 theoretical plates.

The transition from a *cis* to a *trans* double bond (Table X) enhances the retention time and thus the ECN value by 0.40 units per double bond. Trielaidin thus has a lower polarity than triolein.

TABLE X

DIFFERENCE IN ECN WITH CHANGE FROM A *cis* TO A *trans* DOUBLE BOND

Triglycerides	*Change in ECN*	*Triglycerides*	*Change in ECN*	*Triglycerides*	*Change in ECN*
PPO to PPEl	−0.45	StOO to StOEl	−0.39	(OOO to ElElEl)/3	−0.37
StStO to StStEl	−0.39	(StOO to StElEl)/2	−0.40		
Mean total ($n = 5$)			−0.40		
S.D.			0.03		

TABLE XI

VARIATION IN ECN VALUE AS MOLECULAR STRUCTURE CHANGES

Change in molecular structure	*Difference in ECN*	*S.D.*
Increase of carbon number by 2 in sat. triglycerides (defin.)	2.00	–
Increase of carbon number by 2 in unsat. triglycerides	1.94	0.04
Increment of one double bond from sat. to monounsat. fatty acid	2.89	0.08
Increment of one double bond from monounsat. to diunsat. fatty acid	2.47	0.07
Increment of one double bond from diunsat. to triunsat. fatty acid	2.02	–
Addition of bromine to the double bond in monounsat. triglyceride	2.29 (sym.) 2.07 (unsym.)	– –
Moving of one double bond from carbon atom in the vicinity of Δ^9	0.07–0.13	–
Change of one *cis*-double bond to one *trans*-double bond	0.40	0.03

CONCLUSIONS

Equivalent carbon numbers are based on the same log t_R *versus* carbon number relation as ECL values and Kovats indices. In our laboratory ECN values are used in the HPLC of lipids, but they can be used with other homologous series of organic compounds. The calculation, using only three standard points, gives the ECN values for all peaks in a chromatogram.

ECN values define the order of elution of triglycerides from a modern RP-HPLC column. The ECNs denote the hypothetical carbon numbers of hypothetical saturated triglycerides and thus give a direct indication of the position of a peak in the chromatogram. For example, the peak of triolein, OOO (ECN = 45.17) will appear between the peaks of trimyristin, MMM (ECN = 42.00) and tripalmitin, PPP (ECN = 48.00); or, more exactly, between the peaks of MMP (ECN = 44.00) and MPP (ECN − 46.00).

It was demonstrated that ECN values are very stable and can be used directly as characterizing parameters for the components in a given HPLC triglyceride peak. Arranged in a diagram as in Fig. 2, the ECN values can give the first tentative information about an unknown peak (carbon number, and the number and positions of double bonds).

The ECN value of a triglyceride is the sum of the partial fatty acid ECN values. These can then give further initial information about the qualitative content of a peak.

The measured changes in ECN values in relation to the changes in the molecule under the reported experimental conditions are as listed in Table XI.

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