

Paper I

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Identification of fatty acids in gas chromatography by application of different temperature and pressure programs on a single capillary column

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Abstract

A method for the identification of fatty acid methyl esters (FAME) based on the analysis of shifts in equivalent chain lengths (ECL) is described. The method is based on two-dimensional retention data achieved on one capillary column. Various temperature and pressure programs are applied on the same cyanopropyl column and the shifts in the ECL values are analysed by multivariate methods. The chain length, number of double bonds, and the double bond positions can be determined with high accuracy. The same procedure is suitable for determination of the number of *trans* and *cis* double bonds in *trans* fatty acids, and for detection of artefacts in fatty acid methyl ester chromatograms.

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1. Introduction

Equivalent chain lengths (ECL) [1,2] is the most established method of reporting relative retention times of fatty acid methyl esters (FAME). The calculation of ECL values is analogous to the calculation of Kovats' indices (KI) [3], which is widely applied for reporting relative retention times of other organic compounds. Both KI and ECL were originally developed for isothermal gas chromatography and are based on the linear relationship between the logarithm of the adjusted retention times (t'_R) and the number of

carbons in homologous series [4]. The ECL concept uses the saturated straight chain FAME as reference compounds. By definition 18:0 has an ECL value of 18, 20:0 has an ECL value of 20, etc. Only the carbons in the fatty acid chain of the FAME molecule are counted. The relationship between $\log(t'_R)$ and ECL for the saturated FAME is found by linear regression or by the formula:

$$\text{ECL}_{(x)} = z + \frac{\log t'_{R(x)} - \log t'_{R(z)}}{\log t'_{R(z+1)} - \log t'_{R(z)}} \quad (1)$$

where z is the carbon number in the saturated FAME eluting immediately before the analyte of interest, x , and $z + 1$ is the number of carbons in the saturated FAME eluting immediately after analyte x .

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Today FAME are usually analysed using temperature programming where the linear relationship between $\log(t'_R)$ and ECL is no longer valid. Consequently, ECL values cannot be calculated from the formula described above, but direct relationships between the retention time and ECL can be established using non-linear regression [5,6].

The fractional chain length is defined as the difference between the ECL value of the actual FAME molecule and the ECL value of the unbranched saturated molecule with the same number of carbons. Thus, FCL is calculated by the following formula:

$$\text{FCL}_{(x)} = \text{ECL}_{(x)} - \text{ECL}_{(z)} \quad (2)$$

where x is the compound of interest, and z is the saturated fatty acid with the same number of carbons.

Although some variation occur as a function of column temperature [7–11], ECL and FCL values are recognised as being characteristic for a certain FAME molecule when analysed on a specific stationary phase. ECL and FCL values are therefore used for identification of unknown fatty acids. Since the introduction of the ECL and FCL concepts, numerous lists of these values for the most common fatty acids have been published for a large variety of stationary phases.

It has been shown that fatty acids that are structurally similar with respect to the number and position of double bonds have similar differences in $\log(t'_R)$ relative to their saturated analogues [12]. From Eqs. (1) and (2) it can be seen that these fatty acids will also have similar FCL values. The influence of the double bonds is additive, with small deviations for methylene-interrupted double bonds [13–15]. Estimates of ECL and FCL values can therefore be calculated for a certain FAME molecule if the values have not been previously reported. A drawback of using FCL values, or similar identification methods, is that the number of carbons in the molecule is often unknown because of severe overlap between chromatographic regions with different chain lengths. This is particularly a problem with fish oils, which contain a large variety of highly unsaturated long chain fatty acids. On the most polar cyanopropyl columns the highly unsaturated 22:6 n -3 may elute after 26:0 [7]. Unknown compounds in this region of the chromatogram may therefore have chain lengths from C22 to C26.

Even though the ECL value may be characteristic for a fatty acid molecule, it is not unique; the degree of overlap in ECL values between different compounds is extensive in FAME analyses. The general advice is therefore to achieve two-dimensional data by analysing unknown samples on two or more stationary phases with different polarity, ideally with co-injections of reference compounds. James [12] showed that scatter plots of $\log(t'_R)$ values obtained on two columns with different polarity may be of high diagnostic value, both for the identification of unknown structures from retention times, and for prediction of retention times of fatty acids with known structure. An identification procedure based on the differences in KI on four different stationary phases has also been developed [16]. Although these methods may provide precise and accurate information about the fatty acid structure, practical implications of applying more than one column limit their use.

It has been shown that the polarity of cyanopropyl phases is highly temperature dependent [17]. Significant increase in ECL values of unsaturated fatty acids with increasing column temperature has been observed [7–10]. In this work the potential for fatty acid identification based on shifts in ECL values, achieved under different temperature and pressure programs on the same cyanopropyl column, has been investigated.

2. Methods

2.1. Instrument parameters

All analyses were performed on a HP-5890 GC equipped with split/splitless injector, electronic pressure control (EPC) [18], HP-7673A automated liquid sampler, and HP-5972 MS detector (Agilent Technologies, Palo-Alto, CA). The system was equipped with G1034C MS Chemstation software. BPX-70, $L = 70$ m, i.d. = 0.25 mm, $d_f = 0.25$ μ m (SGE, Ringwood, Victoria, Australia) was chosen as analytical column. Helium, 99.996% was used as carrier gas.

2.2. GC-programs

For the analysis of mixtures of fatty acids with chain length up to C24, temperature programmed gas chromatography is required to achieve good resolution

Table 1
Levels of start temperature (*A*), temperature gradient (*B*), and column flow (*C*) used in GC programs

Level	<i>A</i> (start temperature) (°C)	<i>B</i> (temperature gradient) (°C/min)	<i>C</i> (flow ^a (pressure ^b)) (cm/s (kPa))
Low	160	2	18.0 (55.1)
Medium	175	3	22.0 (89.6)
High	190	4	26.0 (124.8)

^a Estimated by Chemstation software.

^b Pressure at 60 °C, increased with temperature to keep constant carrier gas velocity.

within acceptable run times. With EPC, programming of the column flow is also an option to achieve this goal.

Temperature programs with linear gradients were used. To induce changes in ECL values, three different initial temperatures, temperature gradients, and column flows were applied. The applied temperatures and flows are given in Table 1. The samples were injected at an oven temperature of 60 °C that was kept constant for 4 min. The temperature was increased by 30 °C/min to start temperature *A*, followed by a gradient of *B* (°C/min) until the last compound had eluted. The injector pressure was increased with oven temperature to give a constant velocity of *C* (cm/s). The levels of the parameters *A*, *B* and *C* are given in Table 1. The different programs will be referred to in the form *A–B–C*. The samples (0.5–1 µl) were injected in splitless mode. The split valve was opened after 4 min. Injector temperature was 250 °C and MS transfer line temperature 270 °C.

2.3. Samples

GLC-461 (Nu-Chek Prep, Elysian, MN) was used as FAME reference mixture. This mixture contains FAME with 8–24 carbons and 0–6 double bonds. Approximately 0.1 µg FAME in hexane was injected (3 ng of each compound).

The fish oil sample was of unknown origin. Three drops of oil were converted to FAME according to AOCS method Ce 1b-89 but with the following modifications: reaction times of 10 min were applied both for alkali (NaOH) and acid (BF₃) catalysed esterification, and 2 ml of methanolic NaOH (0.5 N) and BF₃ (12%) were applied.

cis/trans reference compounds were prepared by isomerisation with paratoluenesulphonic acid, and fractionated on HPLC as described elsewhere [6].

Squalane (99%) and squalene (97%) were purchased from Aldrich Chemie (Steinheim, Germany). Fatty acid ethyl esters (>99%) were purchased from Nu-Chek Prep (Elysian, MN).

2.4. Calculations

The peak apex was used to determine retention times. ECL values were calculated from the retention times of the saturated fatty acids (C14–C24), using polynomial regressions of different orders in Microsoft Excel. Principal component analysis (PCA), partial least squares (PLS) regressions and multiple linear regression (MLR) were performed in Unscrambler 7.0 (CAMO, Oslo, Norway). PCA and PLS were performed on unweighted and mean centred variables.

3. Results and discussion

3.1. Selection of GC-programs

In the initial study the reference mixture GLC-461 was analysed by combinations of all parameters given in Table 1. The full 3³ design required 27 different GC programs. Both third or higher order polynomial equations, and geometric equations ($y = ax^b$) gave acceptable *R*² values for the regressions between ECL and *t*_R. Third order polynomials were applied. (*A note of caution:* Microsoft Excel does not show enough decimals when the regression formula is displayed. Dividing all values with 100 before regression and correcting the formula afterwards will solve this problem.)

An identification procedure based on the retention times in 27 chromatograms is of no practical value because of the large amount of work and time necessary to acquire the data. It was therefore necessary

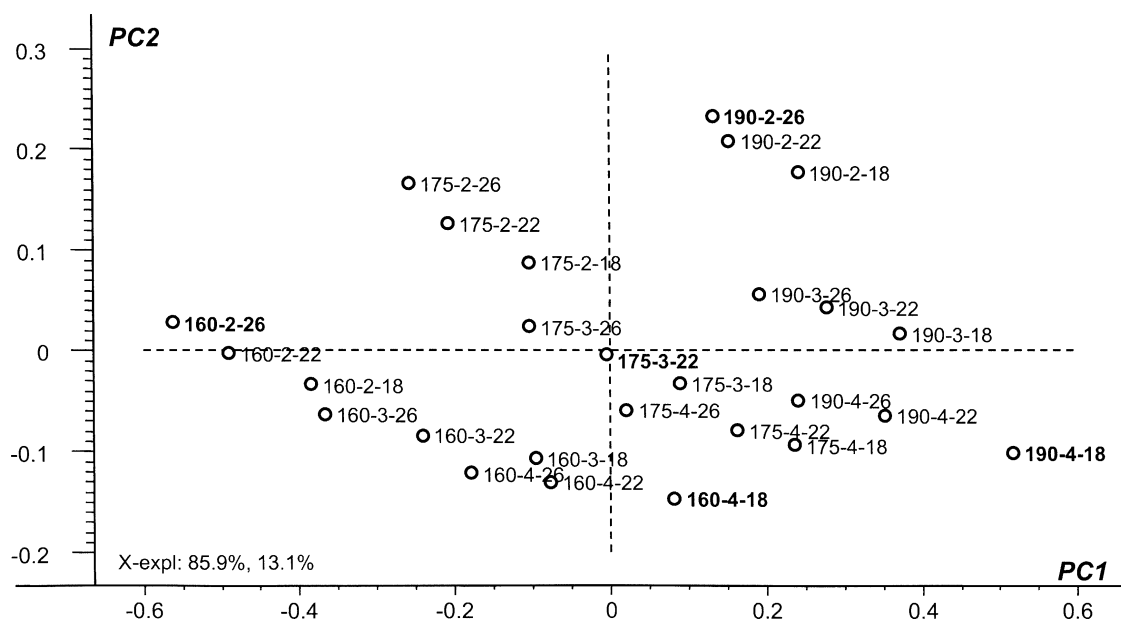


Fig. 1. PCA score plot illustrating the similarities in ECL patterns between the 27 GC programs from the 3^3 design described in Table 1. The 27 C14–C24 fatty acids in reference mixture GLC-461 were used as variables. Programs in boldface were selected for identification of fatty acid structure.

to select a few of the GC programs that had the most unique ECL patterns. This was done by PCA on the data matrix with the 27 programs as objects and the fatty acids as variables. The scores of the two first principal components (PC1 and PC2) are plotted in Fig. 1. Together the two components explain 99% of the variation in the original data matrix. GC programs that are close in the score plot have similar ECL patterns. The different GC programs are grouped in a quadrangular shape, where the objects with the extreme values along PC1 and PC2 define the corners. These programs, 160-2-26, 190-4-18, 160-4-18, and 190-2-26, have the most unique ECL patterns and were therefore applied in subsequent analyses. The program in the centre of the plot and 3^3 design, 175-3-22, was also included to increase stability of the solutions. Examples of fatty acid identification by application of these programs are given below.

3.2. Identification of long chain PUFA

The ECL values from the reference mixture and unknown compounds in analysed fish oil are given in Table 2. Only the region from C18 to C24 is consid-

ered. The retention times for all five programs were converted to ECL values by second order regressions applied on 18:0, 20:0, 22:0, and 24:0. The most unsaturated C22 fatty acids elute after 24:0, and their ECL values are calculated by extrapolation of the functions. If 25:0 or 26:0 are not present in the reference mixture, polynomials of higher order than two must be applied with care.

It can be seen from Table 2 that there is a connection between the fatty acid structure and the variations in ECL: highly unsaturated compounds show larger variation than the less unsaturated compounds. PCA was used to analyse the data in Table 2, using the five programs as variables and the fatty acids as objects. The score plot is shown in Fig. 2 and reveals two clear trends related to the structure of the fatty acids: the molecules are positioned according to their chain length and degree of unsaturation. There is also a small systematic difference between the $n-3$, $n-6$ and $n-9$ series. Fatty acids with similar values along PC1 will have similar ECL values in most cases, and may be confused when the identifications are based on retention characteristics. Most of these critical pairs, e.g. 22:1 $n-9$ and 20:5 $n-3$, are well separated along PC2.

Table 2
Calculated ECL values for long chain polyunsaturated fatty acids analysed with five temperature/pressure programs

	160-2-26	160-4-18	175-3-22	190-2-26	190-4-18	Average	Max–min
18:0	18.006	18.000	18.009	18.027	18.009	18.010	0.027
18:1	18.324	18.374	18.375	18.388	18.422	18.377	0.097
18:2	18.918	19.019	19.007	19.011	19.094	19.010	0.176
U1 (18:2)	19.125	19.237	19.221	19.219	19.307	19.222	0.181
18:3 <i>n</i> -6	19.314	19.451	19.433	19.440	19.554	19.438	0.240
U2 (18:3)	19.551	19.701	19.683	19.689	19.810	19.687	0.259
18:3 <i>n</i> -3	19.675	19.816	19.795	19.799	19.918	19.801	0.243
20:0	19.970	19.985	19.972	19.943	19.973	19.968	0.043
U3 (18:4)	20.064	20.249	20.229	20.245	20.389	20.235	0.325
20:1	20.316	20.383	20.371	20.361	20.425	20.371	0.109
U4 (20:2)	20.752	20.866	20.853	20.859	20.946	20.855	0.194
20:2	20.960	21.063	21.052	21.062	21.144	21.056	0.183
20:3 <i>n</i> -6	21.363	21.508	21.494	21.522	21.624	21.502	0.261
20:4 <i>n</i> -6	21.630	21.813	21.792	21.830	21.958	21.804	0.328
20:3 <i>n</i> -3	21.743	21.880	21.865	21.898	21.987	21.875	0.245
22:0	22.022	22.008	22.024	22.049	22.022	22.025	0.041
U5 (20:4 <i>n</i> -3)	22.130	22.322	22.302	22.348	22.467	22.314	0.337
22:1	22.372	22.420	22.424	22.465	22.479	22.432	0.107
20:5 <i>n</i> -3	22.424	22.651	22.617	22.665	22.809	22.633	0.385
22:2	23.008	23.108	23.096	23.135	23.183	23.106	0.175
U6 (21:5 <i>n</i> -3)	23.537	23.791	23.726	23.735	23.908	23.739	0.371
22:4 <i>n</i> -6	23.735	23.949	23.888	23.883	24.037	23.898	0.302
24:0	23.989	23.993	23.991	23.990	23.992	23.991	0.004
U7 (22:5 <i>n</i> -6)	23.935	24.190	24.109	24.080	24.277	24.118	0.342
U8 (22:4 <i>n</i> -3)	24.130	24.364	24.281	24.228	24.424	24.285	0.293
24:1	24.320	24.410	24.369	24.320	24.414	24.367	0.094
22:5 <i>n</i> -3	24.479	24.773	24.652	24.538	24.819	24.652	0.339
22:6 <i>n</i> -3	24.694	25.029	24.877	24.711	25.055	24.873	0.361

The fatty acids are sorted according to the average ECL values. Unknown samples from the fish oil are denoted U, tentative identifications from Fig. 2 are given in parentheses.

The trends seen in Fig. 2 are similar to the trends seen when retention data from two columns with different polarity are compared [12].

Unsaturated fatty acids can be identified by their positions in Fig. 2 relative to the fatty acids in the reference mixture. The identifications have been controlled by mass spectrometry [19,20]. Two of the unknowns, U1 and U4, were identified as dienes, one 18:2 and one 20:2 isomer. Their identities as methylene-interrupted dienes were confirmed by mass spectrometry. U2 was identified as an 18:3 fatty acid. The mass spectrum indicated that this was the *n*-4 isomer and confirmed the chain length and the number of double bonds.

Three compounds were identified as tetraenes. U5 and U8 were identified as 20:4 *n*-3 and 22:4 *n*-3 by their positions in the plot. These identifications were

also confirmed by their mass spectra. From Fig. 2 U3 is identified as an 18:4 isomer. The mass spectrum confirmed the chain length and the number of double bonds, and indicated that this is the *n*-4 isomer. Two pentaenes were found. U6 was positioned in the middle between 20:5 *n*-3 and 22:5 *n*-3 and was identified as 21:5 *n*-3, which is frequently reported in marine lipids [21,22]. The *n*-3 structure of these isomers was confirmed by the mass spectra. U7 lays on the C20 *n*-6 line, but deviates slightly from the pentaene line. The deviation from the pentaene line can be caused by a small difference between *n*-6 and *n*-3 isomers; the line is drawn on the basis on *n*-3 isomers only. A similar difference can also be seen for the tetraenes. The identity as 22:5 *n*-6 was confirmed by the mass spectrum.

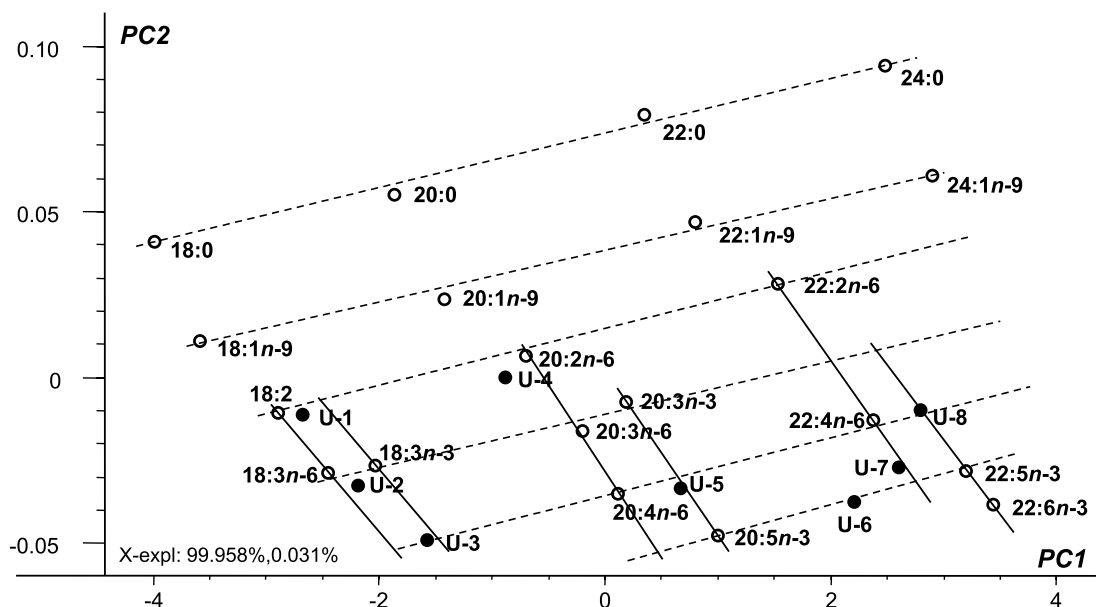


Fig. 2. Score plot from PCA with the C18–C24 fatty acids as objects. Variables were the five programs selected from Fig. 1. Open circles are fatty acids in the reference mixture GLC-461. Closed circles are fish oil fatty acids identified as described in the text. Broken lines indicate the number of double bonds; whole lines indicate the *n*-3 and *n*-6 series.

3.3. *Trans* fatty acids

Geometrical isomers of the common 18:1–18:3 fatty acids were fractionated by silver ion HPLC [6], and each fraction was analysed by the five GC programs. ECL values determined by third order polynomial regressions are listed in Table 3. The reference mixture was analysed twice to get a rough picture of the reproducibility. Between the two analyses of the reference mixture there were several days and the column had also been taken out and re-installed in the oven. The difference between the two injections of the all-*cis* isomers indicated that drift in ECL values can be ignored during the period of analysis.

The PCA score plot is shown in Fig. 3. In this plot all fatty acids have the same chain length. The fatty acids are positioned according to the number of *trans* double bonds and total number of double bonds. There is also a clear difference between 18:3 *n*-6 and 18:3 *n*-3 isomers. All groups with the same number of *cis* and *trans* double bonds are resolved. For the trienes, there are overlaps in retention times between the isomers with 1, 2, or 3 *trans* double bonds, but the groups are separated by PC2.

On cyanopropyl columns a *trans* double bond has an effect on the retention of approximately a half *cis* double bond: *trans* 18:1 *n*-9 is positioned in the middle between 18:0 and *cis* 18:1 *n*-9; *cis*–*trans* 18:2 *n*-6 is positioned between all-*cis* 18:2 *n*-6 and all-*trans* 18:2 *n*-6. From this rule one could expect the *cis*–*trans* 18:2 *n*-6 isomers to show similar behaviour to all-*trans* 18:3 *n*-6. These two fatty acids have similar retention times and are separated by only 0.01–0.06 ECL units, but are well separated by PC2 in Fig. 3.

3.4. Artefacts

Compounds that are not FAME frequently occur in FAME chromatograms. The method was therefore tested for its ability to discriminate between FAME and other molecules. For obvious reasons, it is not possible to test all potential artefacts that may occur in a FAME chromatogram. Three types of compounds were analysed: saturated hydrocarbons, highly unsaturated hydrocarbons, and fatty acid ethyl esters (FAEE). Retention times were converted to ECL values by fourth order polynomial regression. The PCA score plot is displayed in Fig. 4. The distance between

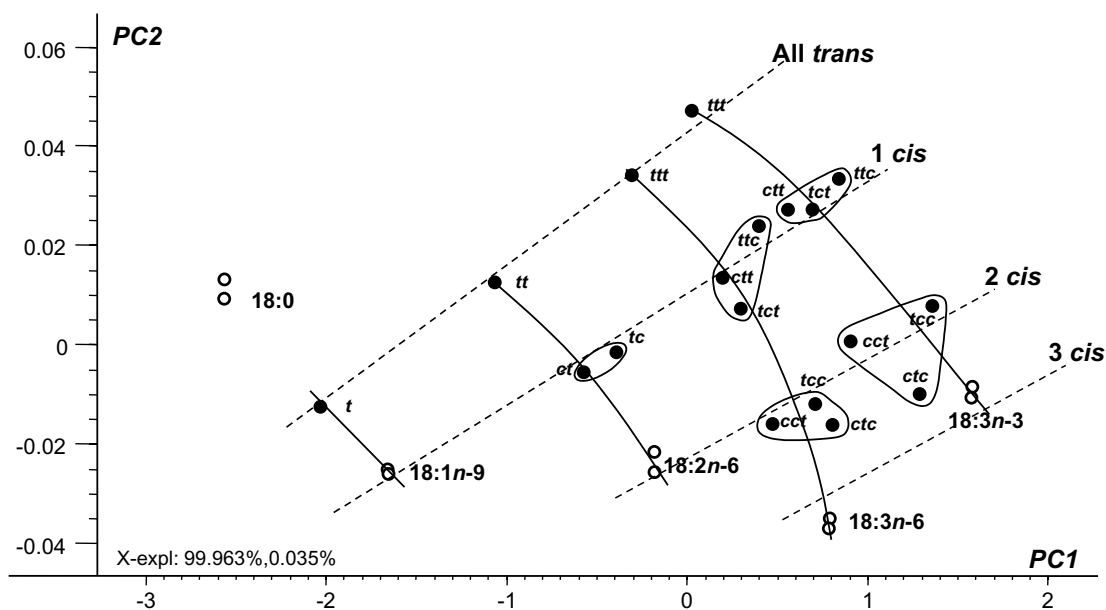


Fig. 3. Score plot from PCA with C18 fatty acids as objects. Variables were the five programs selected from Fig. 1. Open circles are fatty acids in the reference mixture GLC-461. Closed circles are *trans* isomers produced as explained in Section 2. Broken lines indicate the number of *cis* double bonds; whole lines mark series of positional isomers.

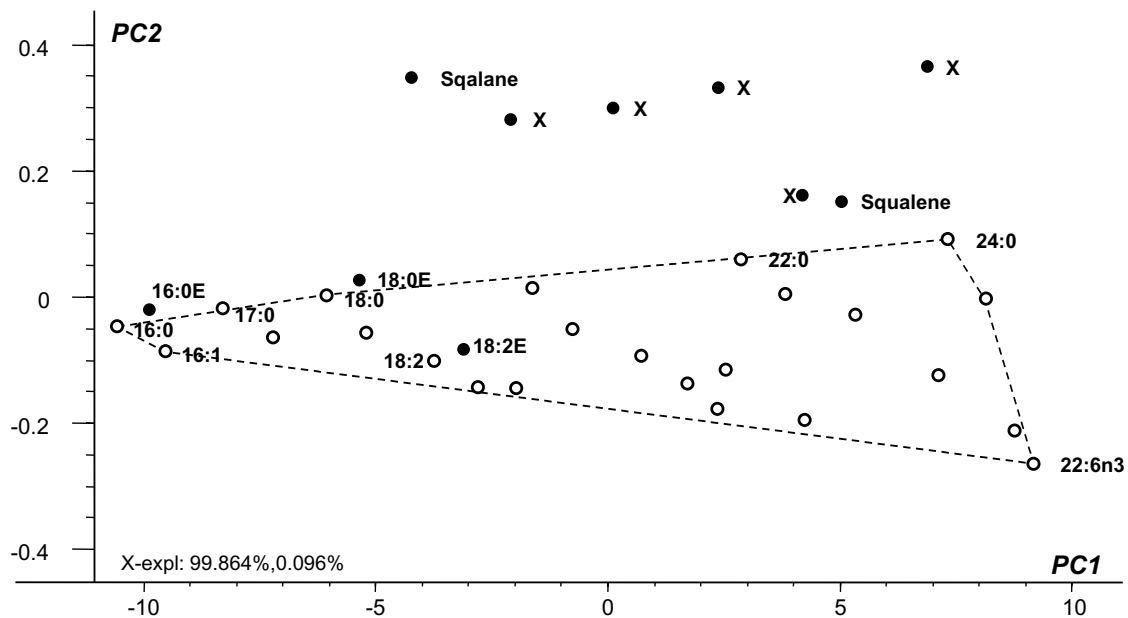


Fig. 4. Score plot of PCA with FAME in the reference mixture GLC-461 (open circles) and compounds that are not FAME (closed circles) as objects. Variables were the five programs selected from Fig. 1. The broken line marks area covered by FAME. E denotes ethyl ester, X denotes unknown (see text).

Table 3

Calculated ECL values for *trans* fatty acids analysed with varying temperature/pressure programs

	160-2-26	160-4-18	175-3-22	190-2-26	190-4-18	Average	Max–min
18:0	18.032	18.014	18.027	18.009	18.012	18.019	0.023
18:0	18.036	18.016	18.026	18.015	18.010	18.021	0.026
18:1 <i>n</i> -9 <i>t</i>	18.238	18.241	18.260	18.254	18.274	18.253	0.036
18:1 <i>n</i> -9 <i>c</i>	18.387	18.406	18.428	18.437	18.458	18.423	0.071
18:1 <i>n</i> -9 <i>c</i>	18.385	18.405	18.431	18.440	18.456	18.423	0.070
18:2 <i>n</i> -6 <i>tt</i>	18.664	18.667	18.694	18.707	18.712	18.689	0.047
18:2 <i>n</i> -6 <i>ct</i>	18.860	18.884	18.914	18.940	18.957	18.911	0.097
18:2 <i>n</i> -6 <i>tc</i>	18.937	18.961	18.994	19.020	19.038	18.990	0.100
18:3 <i>n</i> -6 <i>ttt</i>	18.997	19.002	19.029	19.054	19.052	19.027	0.057
18:2 <i>n</i> -6 <i>cc</i>	19.010	19.059	19.085	19.122	19.149	19.085	0.139
18:2 <i>n</i> -6 <i>cc</i>	19.010	19.055	19.086	19.124	19.154	19.086	0.144
18:3 <i>n</i> -3 <i>ttt</i>	19.152	19.151	19.182	19.208	19.206	19.180	0.056
18:3 <i>n</i> -6 <i>ctt</i>	19.199	19.227	19.259	19.295	19.308	19.258	0.109
18:3 <i>n</i> -6 <i>tct</i>	19.237	19.274	19.301	19.338	19.360	19.302	0.123
18:3 <i>n</i> -6 <i>ttc</i>	19.286	19.316	19.344	19.380	19.389	19.343	0.104
18:3 <i>n</i> -6 <i>cct</i>	19.290	19.346	19.372	19.422	19.453	19.376	0.163
18:3 <i>n</i> -3 <i>ctt</i>	19.362	19.392	19.420	19.460	19.469	19.421	0.108
18:3 <i>n</i> -3 <i>tct</i>	19.412	19.451	19.474	19.517	19.526	19.476	0.114
18:3 <i>n</i> -6 <i>tcc</i>	19.391	19.452	19.481	19.527	19.561	19.482	0.170
18:3 <i>n</i> -6 <i>ccc</i>	19.404	19.482	19.509	19.563	19.613	19.514	0.209
18:3 <i>n</i> -6 <i>ccc</i>	19.406	19.487	19.516	19.567	19.612	19.518	0.206
18:3 <i>n</i> -6 <i>ctc</i>	19.426	19.490	19.518	19.573	19.605	19.522	0.179
18:3 <i>n</i> -3 <i>ttc</i>	19.477	19.510	19.537	19.578	19.590	19.538	0.112
18:3 <i>n</i> -3 <i>cct</i>	19.481	19.535	19.563	19.615	19.642	19.567	0.161
18:3 <i>n</i> -3 <i>ctc</i>	19.644	19.707	19.736	19.789	19.841	19.744	0.197
18:3 <i>n</i> -3 <i>tcc</i>	19.679	19.745	19.764	19.819	19.853	19.772	0.175
18:3 <i>n</i> -3 <i>ccc</i>	19.753	19.842	19.859	19.917	19.966	19.867	0.213
18:3 <i>n</i> -3 <i>ccc</i>	19.757	19.840	19.867	19.918	19.966	19.870	0.209

The fatty acids are sorted according to the average ECL values. The geometry of the double bonds are given from the carbonyl end to the methyl end, e.g. 18:2 *n*-6 *ct* is *cis*-9, *trans*-12 octadecadienoic acid.

squalane and the FAME group is large. Squalene has more similar behaviour to FAME than squalane, but is still separated from the FAME group. Some smaller peaks, marked with “x” in Fig. 4, were seen in the chromatograms of squalane and squalene. These are probably compounds of similar structure and are also well separated from the FAME group. Squalane, squalene and the five compounds with similar values on PC2 could also be detected as outliers by having large residual variance. The high residual variance means that their retention behaviour does not fit well to the principal component model. It should be noted that both squalane and squalene are highly branched hydrocarbons. Since *n*-alkanes or *n*-alkenes were not tested, it is not possible to conclude if the deviations relative to FAME are caused by the branches in the carbon chain, or by absence of the ester group.

The FAEE are more similar to FAME than the alkanes, deviating only by one carbon in the ester alcohol. The two saturated FAEE, 16:0 and 18:0, are isomers of 17:0 and 19:0 FAME. Their positions in the plots indicate lower polarity of FAEE than of the corresponding FAME isomers. The saturated FAEE are positioned very close to the FAME area, the 18:2 FAEE are positioned well inside the FAME area, close to the 18:2 FAME.

3.5. Multivariate regression

The evaluation of the retention data described above is based on graphical interpretation of two-dimensional plots. Graphical methods are prone to bias caused by the viewer’s subjective interpretation, and there is no objective estimate of the accuracy

of the method. In addition, information may be lost when the five original variables are explained by only two principal components. An alternative strategy is to predict the chain length and number of double bonds from multivariate regression models based on the ECL data.

PLS regressions with full cross validation [23] were performed with chain length and number of double bonds as dependent variables, and the ECL data as independent variables. The dataset applied for Fig. 2 was used, and the purpose was to achieve models that gave correct predictions for the C18–C22 PUFA in the reference mixture and fish oil sample. The regressions were based on the fatty acids in the calibration sample only.

The cross validation results for the PLS calibration of chain length indicated that a model based on two PLS components would give the most accurate predictions. The calibration was based on all fatty acids in the reference mixture and the standard error of prediction (SEP) was 0.23. Bias was negligible and the residuals had normal distribution. Since the number of carbons can only have integer values, the predictions are rounded to the nearest integer. The prediction will therefore fail if the absolute error of prediction is above 0.5. The risk of failure can be estimated from the SEP of the cross validation results. SEP is the standard deviation of the residuals. With normally distributed residuals and SEP of 0.23, approximately 3% of the predictions have absolute errors above 0.5.

Application of the calibration model for prediction of the long chain PUFA gave the results listed in Table 4. All chain lengths in the reference mixture was correctly predicted; the chain lengths of the analytes in the fish oil sample agreed with the interpretation of Fig. 2 and information from the mass spectra.

A similar model for the prediction of number of double bonds gave SEP of 0.27 for a three component model, which was the optimum found by cross validation. The SEP was reduced to 0.21 when monoenes and saturated fatty acids were excluded from the calibration set. This increased accuracy may be explained by the different behaviour of methylene-interrupted polyenes compared to monoenes. It has been shown that methylene-interrupted double bonds have stronger interactions with the stationary phase than the isolated double bonds in monoenes [13–15]. The predictions of the number of double bonds also agreed with previous

Table 4
PLS predictions of chain length and number of double bonds based on the ECL values in Table 2

Sample	Chain length		Double bonds	
	Predicted	Rounded	Predicted	Rounded
18:2 <i>n</i> -6	17.93	18	2.01	2
18:3 <i>n</i> -6	17.92	18	2.97	3
18:3 <i>n</i> -3	18.26	18	3.02	3
20:2 <i>n</i> -6	19.95	20	2.02	2
20:3 <i>n</i> -6	19.89	20	3.12	3
20:4 <i>n</i> -6	19.75	20	4.09	4
20:3 <i>n</i> -3	20.38	20	2.84	3
20:5 <i>n</i> -3	20.18	20	4.93	5
22:2 <i>n</i> -6	22.09	22	1.74	2
22:4 <i>n</i> -6	21.87	22	3.99	4
22:5 <i>n</i> -3	22.09	22	5.20	5
22:6 <i>n</i> -3	22.02	22	5.85	6
U-1	18.09	18	2.13	2
U-2	18.04	18	3.24	3
U-3	18.16	18	4.17	4
U-4	19.66	20	2.22	2
U-5	20.20	20	4.18	4
U-6	21.26	21	4.98	5
U-7	21.74	22	4.76	5
U-8	22.20	22	4.12	4

Predictions are rounded to nearest integer. Unknown compounds from the fish oil are denoted U.

findings (Table 4). With an SEP of 0.21 the risk of prediction failure is approximately 2%. The model based on only PUFA predicted correct values also when applied on monoenes, but negative values (≤ 0.5) when applied on the saturated fatty acids.

3.6. Application of two programs

The information achieved from only two GC programs was also examined. The two programs that had the largest differences in ECL values were 160-2-26 and 190-4-18 (Fig. 1). Plots of ECL values of one of the programs against the difference in ECL between the two programs (Δ ECL) resemble the score plots in Figs. 2–4. The plot for the fish oil sample is given in Fig. 5, and should be compared to the score plot in Fig. 2. The two plots have the same main trends, but the lines explaining the chain length and the number of double bonds are less parallel and extrapolated with lower accuracy in Fig. 5 than in Fig. 2. An example can be seen for U3, which has a large distance

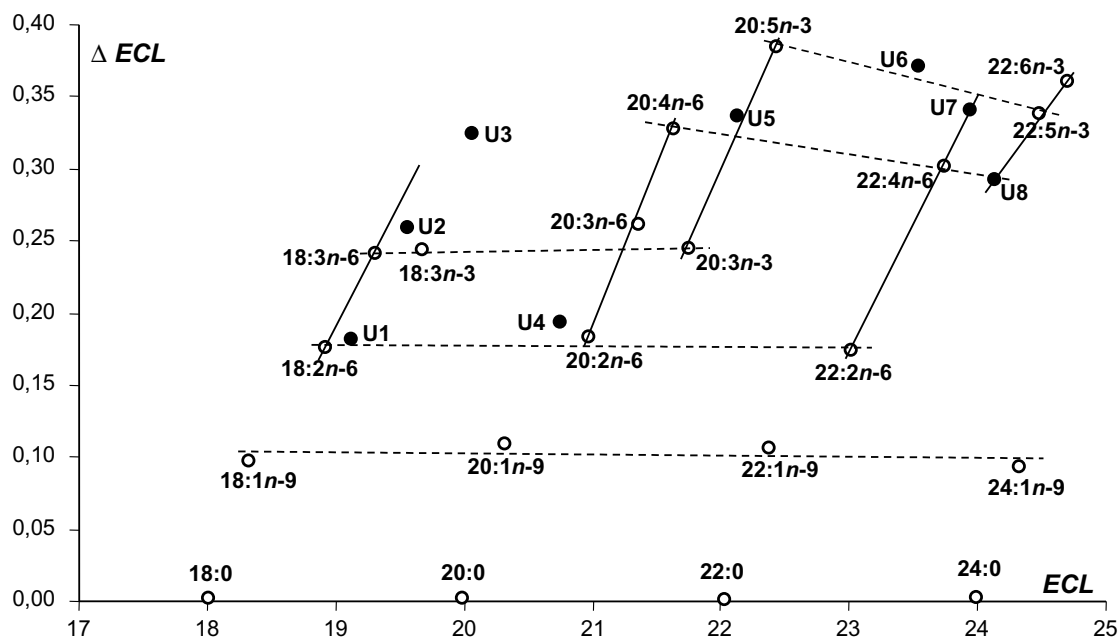


Fig. 5. Differences between ECL values at 160-2-26 and 190-4-18 (Δ ECL) plotted against ECL values at 160-2-26. Open circles are fatty acids in the reference mixture GLC-461. Closed circles are fish oil fatty acids identified as described in the text. Broken lines indicate the number of double bonds; whole lines indicate the n -3 and n -6 series.

to the tetraene line in Fig. 5. It can also be seen that the lines of tetraenes and pentaenes are far from parallel to the lines of monoenes to trienes. 22:6 n -3 is also very close to the pentaene line.

The two variables plotted in Fig. 5 were used in a multiple linear regression for the prediction of chain length and number of double bonds in a similar way as PLS was used for the predictions based on five GC programs. Cross validation results for the prediction of chain length gave SEP of 0.25, which mean that the prediction will fail in approximately 5% of the cases. The chain lengths for all compounds in the reference mixture and in the fish oil sample were correctly determined. SEP for the MLR prediction of the number of double bonds was 0.42. The number of double bonds was incorrectly predicted for 22:5, 22:6, U4 and U7. In addition, negative values were reported for two of the saturated fatty acids. Limitation of the samples to only PUFA did not increase accuracy, SEP was 0.47 and the model failed to predict the number of double bonds in 22:5, 22:6, U2, U3, and U7. Because the residuals were not normally distributed the risk of prediction failure could not be estimated from SEP, but

the large number of fatty acids that was incorrectly predicted suggests that these models have too low accuracy to be of any practical value.

In addition to the accuracy, robustness of the method is another reason to use more than two different GC programs. In some chromatograms, the retention time of an unknown component may not be accurately determined because of co-elution with other peaks. In these cases the results from that program can be left out, and PCA or PLS regressions can be based on the remaining four programs without loss of accuracy. Another problem that may occur is that two peaks may be confused. The elution order of the peaks changes, and with several peaks of equal size and similar retention times, errors can occur. In such cases the unknown object will usually be poorly fitted to the PCA or PLS model, and a warning will be given in form of unusually high residual variance.

At our laboratory this method has been applied with a variety of samples. The method is in principle independent of the detector used, but has proven to be a valuable supplement to mass spectrometric detection because the two techniques to some extent give

complementary information. Peaks that are too small to produce mass spectra of the required quality are identified from the ECL values. Larger peaks are identified from mass spectra and their ECL values may be included as known compounds in the PCA or PLS models, and thereby increase the accuracy of the identification of the small peaks. Another advantage is that mass spectra without interference can usually be achieved even in very complex samples, because peaks that co-elute in one of the five programs will be resolved in other programs.

As long as the gas chromatograph is equipped with autosampler, and controlled by external computer software capable of using several programs in the same sequence, there is not much work involved in running the analyses. When the number of unknowns in a sample is low, the analysis of the retention data can be performed within an hour. Drift in ECL values with column ageing, which is frequently observed for polar stationary phases, is no problem for the method as long as the analyses of the unknown samples and the reference mixture are not too distant in time. If no overlap with the unknown compounds occur, spiking of the saturated reference compounds into the unknown sample is a good alternative.

Other polar cyanopropyl phases may be used since they show similar increase in ECL values with increasing temperature as BPX-70 [7–10]. In our laboratory we have achieved comparable results with a 100m SP-2560 column (Supelco, Bellefonte, PA). PEG (polyethylene glycol) is another stationary phase that has found widespread use in FAME analysis. The polarity of these columns is practically unaltered by temperature [17,24] and the changes in ECL values are small [8,11], it is therefore unlikely that PEG columns can be applied in a similar manner.

4. Conclusions

The fatty acid chain length and number of double bonds can be predicted with high accuracy from ECL values obtained with various temperature and pressure programs on the same capillary column. Graphical interpretation of PCA score plots will also indicate double bond positions. The same method is suitable for

determination of the number of *trans* and *cis* double bonds in *trans* fatty acids and for detection of artefacts in FAME chromatograms.

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