# Paper III

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# Properties of *trans* isomers of eicosapentaenoic acid and docosahexaenoic acid methyl esters on cyanopropyl stationary phases

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**Abstract**

The *trans* isomers of 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) methyl esters were prepared by isomerisation with paratoluenesulfinic acid (PTSA) in dioxane. The isomers were fractionated by silver ion liquid chromatography with baseline resolution between the isomers with different number of *trans* double bonds. The fractions were analysed by GC–MS and the gas chromatographic properties of the EPA and DHA isomers with one and two *trans* double bonds were investigated on BPX-70 and SP-2560 cyanopropyl stationary phases. Different temperature and pressure programs were applied to introduce variations in retention indices of the isomers. The retention indices of all the *trans* isomers showed a strong linear correlation to the retention indices of the equivalent all-*cis* isomer, but the slopes for corresponding linear regression lines varied with the number of *trans* double bonds in the molecule. The regression lines were used to predict optimal conditions for the separation of *trans* isomers from the corresponding all-*cis* isomers. For DHA on BPX-70, and for EPA on both columns, it was possible to find windows where isomers with one *trans* double bond can be resolved from the corresponding all-*cis* isomers with $R_s > 1.0$. In general, BPX-70 seems to have a more suitable selectivity for the analysis of these isomers than SP-2560. Two-dimensional fatty acid retention indices (2D-FARI) were found to be suitable for identification of *trans* geometry in polyunsaturated fatty acids (PUFA). Although there were substantial overlaps in the range of retention times between the all-*cis* isomers and isomers with one and two *trans* double bonds, 2D-FARI separated the isomers into distinct groups according to the number of *trans* double bonds.


*Keywords:* Gas chromatography; *Trans* fatty acids; Cyanopropyl phases; Silver ion liquid chromatography; Marine fatty acids; Two-dimensional fatty acid retention indices; Equivalent chain lengths

## 1. Introduction

The presence of *trans* fatty acids (TFA) in food is believed to have negative health effects [1], and it is therefore of interest to be able to determine their levels both in food and biological tissues. The term '*trans* fatty acids' covers a wide range of fatty acids with large variations in structure and properties. TFA in food has three major sources, partial hydrogenation of fats, high-temperature processing of edible oils, and the natural occurrence of TFA in ruminant meat and diary products. TFA in ruminant and hydrogenated fats are mainly monoenes and dienes [2,3].

*Trans* geometry can also be introduced in polyunsaturated fatty acids (PUFA) by thermal isomerisation occurring in high-temperature processes, such as deodorization [4]. Thermal isomerisation is almost exclusively geometrical isomerisation, leading to isomers with the double bonds in the same position as in the original fatty acid [4]. Research in this field has mainly focused on isomerisation of alpha linolenic acid (ALA) 18:3 n-3, which has been extensively studied together with the spectroscopic and chromatographic properties of the isomers formed [4–10]. Although isomerisation has been discovered in heated fish oil esters [11], less work have been performed on the highly unsaturated fatty acids present in fish oil and other marine lipids, where the two most important PUFA are eicosapentaenoic acid (EPA), 20:5 n-3, and docosahexaenoic acid (DHA), 22:6 n-3. EPA and DHA are more unsaturated than ALA, and may therefore be more vulnerable to thermal isomerisation.

Highly polar cyanopropyl phases are today dominating in the GC analysis of *trans* fatty acids, basically because the *cis–trans* selectivity has been reported to be good [12–14]. For monoenes and dienes, *trans* isomers elute well ahead of the corresponding

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*cis* isomers [14], but the resolution pattern may be more complicated with isomers of PUFA, especially where the *trans* bonds are in positions far from the methyl end of the carbon chain [8,10,15].

Another feature with cyanopropyl columns is that the polarity of the column shows a stronger dependence on temperature than observed for other common stationary phases [13,16]. By combining information from several temperature programs, this shift in polarity can be utilised for identification of fatty acid structure, including the double bond geometry [17]. The polarity shifts also give large flexibility when optimal elution patterns are sought, a feature that is useful for solving difficult resolution problems [5].

The present work deals with the chromatographic properties of *trans* isomers of EPA and DHA analysed on cyanopropyl columns. The focus is mainly on the isomers with one *trans* double bond, which are the isomers most likely to be formed by thermal processing, but data for isomers with two *trans* double bonds are also given. EPA and DHA were isomerised and fractionated by silver ion LC (Ag-LC). The fractions were analysed by GC under varying chromatographic conditions and the retention data was applied to predict optimal resolution windows for the analysis of EPA and DHA *trans* isomers.

## 2. Methods

### 2.1. Materials

Fatty acid methyl esters (FAME) of EPA and DHA (>99% pure) were purchased from Nu-Chek Prep (Elysian, MN, USA). The fatty acid methyl esters were isomerised by heating 5 mg of the all-*cis* isomer with 5 mg paratoluenesulfinic acid (PTSA) in 1 mL dioxane for 1 h at 60 °C (10 mg PTSA in 1 mL dioxane was applied to produce the all-*trans* isomers). The isomerisation was terminated by the addition of 1 mL 1 M NaOH, and the isomers were extracted by 1 mL isooctane. Further details about the isomerisation procedure are given elsewhere [10,18].

### 2.2. Fractionation

Half the extract (500 μL) was injected onto an LC system equipped with a 4.6 mm × 250 mm silver ion column (ChromSpher 5 Lipids, Varian, Middelburg, The Netherlands), a fraction collector and a light scattering detector. The solvent flow was 1.5 mL/min and the following gradient program was applied: solvent A: hexane; solvent B: acetone; solvent C: 10% acetonitrile 90% acetone. 0–5 min: 100% solvent A, 5–9 min: gradient 100% A to 80% A/20% B, 9–10 min gradient 80% A/20% B to 100% B, 10–30 min: gradient 100% B to 100% C, 30–46 min: 100% C. Additional details about the chromatographic system can be found elsewhere [19]. The LC peaks with isomers containing 0–2 *trans* double bonds were divided into fractions with 0.5 min intervals. Fractions containing all isomers with the same number of double bonds were also collected manually (Fig. 1).

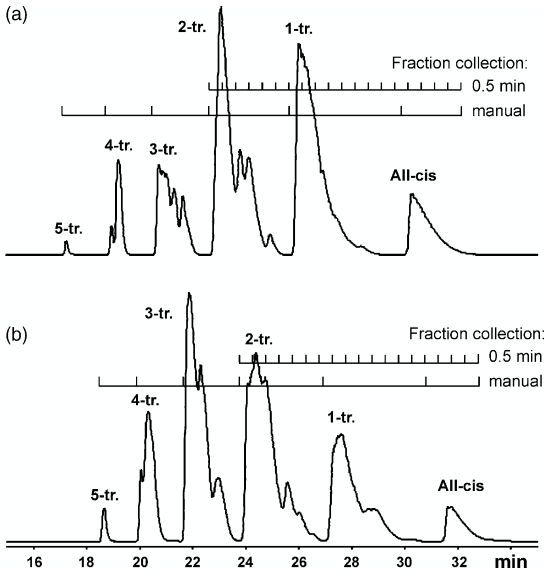


Fig. 1. Silver ion LC chromatogram and fractionation pattern of isomerised EPA (a) and DHA (b) on a ChromSpher 5 Lipids column. Linear gradients of acetone and acetonitrile in hexane were applied as mobile phase (1.5 mL/min). The fractions were collected manually or every 0.5 min. Further details are given in Section 2.2.

### 2.3. Gas chromatography

The LC fractions were analysed on an HP-5890 GC equipped with split/splitless injector, electronic pressure control, HP-7673A autosampler, HP-5972 MS detector, and G1034C MS Chemstation software. BPX-70, $L = 70$ m, I.D. = 0.25 mm, $d_f = 0.25$ μm (SGE, Ringwood Australia), and SP-2560, $L = 100$ m, I.D. = 0.25 mm, $d_f = 0.20$ μm (Supelco, Bellefonte, PA, USA) were used as analytical columns. Helium, 99.996% was used as carrier gas.

Different temperature and pressure programs were applied, five for BPX-70 and three for SP-2560. The samples (0.5 μL) were injected at an oven temperature of 60 °C that was held 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 was eluted. The injector pressure was increased with the 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 numbering in Table 1 is equal to the numbering used in Ref. [20], where further details can be found (Programs 6 and 7 were not used in this study). Injections were performed in splitless mode. The split valve was opened after 4 min. Injector temperature was 250 °C and MS transfer line temperature was 270 °C. The mass spectrometer was used in SIM mode where ions of 55, 74, 79, 80, 91 and 93 amu were recorded; additional details are given elsewhere [19,20].

### 2.4. Retention indices

For the analysis of FAME, equivalent chain length (ECL) is usually the preferred retention index system. The saturated

Table 1
Chromatographic conditions for the applied GC-programs together with ECL values and peak widths at half height for all-*cis* EPA and DHA

| Program | Column | *A*, start temperature (°C) | *B*, temperature gradient (°C/min) | *C*, flow[a] (cm/s) | EPA | | DHA | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ECL | Whh[b] | ECL | Whh[b] |
| 1 | BPX-70 | 160 | 2.0 | 26 | 22.624 | 0.026 | 25.034 | 0.026 |
| 2 | BPX-70 | 160 | 4.0 | 18 | 22.898 | 0.038 | 25.386 | 0.039 |
| 3 | BPX-70 | 175 | 3.0 | 22 | 22.818 | 0.031 | 25.260 | 0.033 |
| 4 | BPX-70 | 190 | 2.0 | 26 | 22.804 | 0.029 | 25.193 | 0.030 |
| 5 | BPX-70 | 190 | 4.0 | 18 | 23.018 | 0.040 | 25.483 | 0.040 |
| 8 | SP-2560 | 145 | 2.5[c] | 18 | 24.115 | 0.028 | 26.826 | 0.029 |
| 9 | SP-2560 | 145 | 1.5 | 24 | 23.804 | 0.023 | 26.438 | 0.024 |
| 10 | SP-2560 | 145 | 1.0 | 24 | 23.667 | 0.020 | 26.274 | 0.021 |

[a] Estimated flow. Pressure conditions are given in [20].
[b] Peak with at half height given in ECL units.
[c] 5 min isothermal after 255 °C.

unbranched FAMEs are used as calibration series and the ECL value is by definition set equal to the number of carbons in the fatty acid carbon chain [21]. The analysed fractions were spiked with small amounts of all the unbranched saturated methyl esters with chain length from C12 to C28 (not including 23:0). The relationship between ECL and retention time (at the peak apex) was calculated by stepwise second order regressions according to Ref. [20]. The applied programs have a linear relationship between ECL and retention time from C22 to C28 [20], the given ECL values are therefore equivalent to values calculated by van den Dool and Kratz formula [22] or by linear regression.

From the relationship between ECL values and retention times, the ECL value for every mass spectral scan was calculated from the corresponding time. By replacing the time-scale of the chromatograms by the ECL scale, peak retention and peak widths can be expressed in ECL units instead of minutes.

Because the ECL values on cyanopropyl phases varies considerably with chromatographic conditions and the state of the column [5,13,17,20], unknown compounds cannot be identified from ECL values alone. More accurate information about the fatty acid structure can be gained from two-dimensional fatty acid retention indices (2D-FARI) and retention index maps [20]. The 2D-FARI values are calculated by combining retention indices from different temperature and pressure programs. Regression is used to align the ECL values of the compounds in a reference mixture to a set of two-dimensional reference values. The regression model is then applied to project the ECL data for other compounds to the same 'map'. The reference mixture CLC-461 (Nu-Chek Prep) spiked with 22:3 n-3 and additional saturated fatty acids were used as reference. Further details about the 2D-FARI procedure can be found elsewhere [20].

### 2.5. Multivariate curve resolution

In cases where there are significant differences between the spectra of co-eluting compounds, multivariate curve resolution techniques can be applied to resolve the overlapping peaks [23,24], even small differences in mass spectra between geometric isomers of PUFA can in certain cases be resolved [25]. Curve resolution was applied to one set of co-eluting *trans* isomers (see Table 2), and to cases where the *trans* isomers co-eluted with saturated references. Initial estimates were calculated by the modified Borgen method [26] and refined by alternating regression [23].

### 2.6. Software

Multivariate curve resolution, integration and calculation of peak widths, linear regressions and calculation of ECL and 2D-FARI values were performed in an in-house written program, 'Q (2-05)', programmed in Matlab 6.5 (Mathworks, Natick, MA, USA).

### 2.7. Definitions and nomenclature

Geometric isomers of EPA and DHA are denoted by E and D, respectively, followed by the number of *trans* isomers, and an additional letter to distinguish between isomers with the same number of *trans* double bonds. Thus, E0 is the all-*cis* EPA isomer, E1a and E1b are EPA with one *trans* double bond, and E2 denotes EPA with two *trans* double bonds. Isomers with one or two *trans* double bonds may be referred to as '1-*trans*' and '2-*trans*' isomers. Other fatty acids than EPA and DHA are referred to by common fatty acid notation.

## 3. Results and discussion

### 3.1. Elution patterns

Silver ion chromatograms of isomerised EPA and DHA are shown in Fig. 1a and b. Silver ion chromatography separates FAME according to the number of double bonds, and has high *cis–trans* selectivity. One *cis* double bond gives roughly the same retention as two *trans* double bonds [27]. In this case, the injected compounds differ only in the geometry of the double bonds and baseline resolution was achieved between fractions containing isomers with different number of *trans* double bonds.

GC–MS chromatograms (Program 10) of the LC fractions containing one and two *trans* double bonds are shown in Fig. 2a and b. While most *trans* isomers of monoenes, dienes and n-3 trienes elute well ahead of the corresponding all-*cis* isomer

Table 2
Regression data, correlation coefficients, and 2D-FARI values for *trans* isomers (and interferents) of EPA and DHA analysed on SP-2560 and BPX-70 columns

| Code | FA | SP-2560 Regr.[a] ($y = ax + b$) | | | BPX-70 Regr.[a] ($y = ax + b$) | | | 2D-FARI | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | a | b | $R^2$ | a | b | $R^2$ | FARI-A | FARI-B | *n* |
| E0 | All-*cis* EPA | 1.000 | 0.000 | 1.0000 | 1.000 | 0.000 | 1.0000 | 20.093 | 4.836 | 3 |
| E1a | 1-*trans* EPA | 0.842 | 3.415 | 0.9999 | 0.862 | 2.807 | 0.9988 | 20.146 | 4.133 | 3 |
| E1b | 1-*trans* EPA | 0.873 | 2.960 | 0.9999 | 0.887 | 2.490 | 0.9996 | 20.320 | 4.253 | 3 |
| E1c | 1-*trans* EPA | 0.887 | 2.796 | 0.9998 | 0.900 | 2.386 | 0.9996 | 20.482 | 4.320 | 1 |
| E1d | 1-*trans* EPA | 0.895 | 2.655 | 0.9999 | 0.901 | 2.368 | 0.9995 | 20.484 | 4.325 | 2 |
| E2a | 2-*trans* EPA | 0.723 | 6.171 | 0.9997 | 0.707 | 6.190 | 0.9981 | 20.421 | 3.380 | 3 |
| E2b | 2-*trans* EPA | 0.751 | 5.657 | 0.9997 | 0.748 | 5.494 | 0.9992 | 20.538 | 3.578 | 2 |
| E2c[d] | 2-*trans* EPA | 0.759 | 5.523 | 0.9997 | 0.752 | 5.415 | 0.9991 | 20.543 | 3.599 | 2 |
| E2d | 2-*trans* EPA | 0.761 | 5.630 | 0.9994 | 0.734 | 5.906 | 0.9994 | 20.677 | 3.515 | 4 |
| E2e | 2-*trans* EPA | 0.783 | 5.156 | 0.9998 | 0.758 | 5.499 | 0.9999 | 20.746 | 3.630 | 2 |
| E2f | 2-*trans* EPA | 0.800 | 4.780 | 0.9996 | 0.803 | 4.464 | 0.9993 | 20.619 | 3.850 | 1 |
| E2g | 2-*trans* EPA | 0.769 | 5.521 | 0.9997 | 0.754 | 5.582 | 0.9990 | 20.748 | 3.607 | 1 |
| E2h | 2-*trans* EPA | 0.775 | 5.444 | 0.9997 | 0.750 | 5.694 | 0.9992 | 20.776 | 3.592 | 1 |
| E2i | 2-*trans* EPA | 0.810 | 4.642 | 0.9997 | 0.800 | 4.648 | 0.9999 | 20.743 | 3.833 | 3 |
| E5 | all-*trans* EPA | 0.394 | 13.758 | 0.9992 | 0.283 | 15.490 | 0.9891 | 21.210 | 1.329 | 1 |
| 22:0[b] | 22:0 | – | – | – | 0.000 | 22.000 | – | 22.031 | −0.048 | 1 |
| 22:1[b] | 22:1 n-9 | – | – | – | 0.263 | 16.444 | 0.9977 | 21.759 | 1.219 | 2 |
| 22:2[b] | 22:2 n-6 | 0.454 | 12.663 | 0.9999 | 0.458 | 12.721 | 0.9993 | 21.956 | 2.150 | 2 |
| 24:0[b] | 24:0 | 0.000 | 24.000 | – | – | – | – | – | – | 2 |
| Code | FA | SP-2560 Regr.[a] ($y = ax + b$) | | | BPX-70 Regr.[a] ($y = ax + b$) | | | 2D-FARI | | |
| | | a | b | $R^2$ | a | b | $R^2$ | FARI-A | FARI-B | *n* |
| D0 | All-*cis* DHA | 1.000 | 0.000 | 1.0000 | 1.000 | 0.000 | 1.0000 | 22.012 | 5.752 | 3 |
| D1a | 1-*trans* DHA | 0.879 | 2.797 | 0.9995 | 0.852 | 3.348 | 0.9995 | 22.110 | 4.899 | 3 |
| D1b | 1-*trans* DHA | 0.908 | 2.324 | 0.9993 | 0.884 | 2.816 | 1.0000 | 22.273 | 5.062 | 3 |
| D1c | 1-*trans* DHA | 0.952 | 1.285 | 0.9994 | 0.935 | 1.700 | 0.9998 | 22.283 | 5.355 | 2 |
| D1d | 1-*trans* DHA | 0.922 | 2.171 | 0.9995 | 0.896 | 2.717 | 0.9994 | 22.462 | 5.090 | 3 |
| D1e | 1-*trans* DHA | 0.931 | 2.044 | 0.9994 | 0.906 | 2.573 | 0.9992 | 22.530 | 5.173 | 3 |
| D2a | 2-*trans* DHA | 0.777 | 5.375 | 0.9994 | 0.719 | 6.545 | 0.9990 | 22.382 | 4.130 | 2 |
| D2b | 2-*trans* DHA | 0.829 | 4.141 | 0.9995 | 0.773 | 5.401 | 0.9994 | 22.421 | 4.429 | 2 |
| D2c | 2-*trans* DHA | 0.813 | 4.681 | 0.9990 | 0.750 | 6.022 | 0.9999 | 22.542 | 4.293 | 3 |
| D2d | 2-*trans* DHA | 0.814 | 4.738 | 0.9992 | 0.752 | 6.073 | 1.0000 | 22.647 | 4.288 | 4 |
| D2e | 2-*trans* DHA | 0.811 | 4.863 | 0.9988 | 0.814 | 4.599 | 1.0000 | 22.532 | 4.663 | 3 |
| D2f | 2-*trans* DHA | 0.843 | 4.040 | 0.9993 | 0.814 | 4.599 | 1.0000 | 22.532 | 4.663 | 3 |
| D2g | 2-*trans* DHA | 0.848 | 4.032 | 0.9993 | 0.809 | 4.815 | 0.9998 | 22.634 | 4.628 | 1 |
| D2h[c] | 2-*trans* DHA | 0.828 | 4.640 | 0.9995 | 0.749 | 6.365 | 0.9994 | 22.881 | 4.270 | 3 |
| D2i | 2-*trans* DHA | 0.878 | 3.333 | 0.9993 | 0.828 | 4.448 | 0.9998 | 22.701 | 4.734 | 2 |
| D2j[c] | 2-*trans* DHA | 0.831 | 4.606 | 0.9995 | 0.769 | 5.887 | 0.9989 | 22.842 | 4.386 | 3 |
| D2k | 2-*trans* DHA | 0.836 | 4.533 | 0.9991 | 0.769 | 5.951 | 0.9994 | 22.889 | 4.382 | 2 |
| D2l | 2-*trans* DHA | 0.871 | 3.664 | 0.9991 | 0.813 | 4.915 | 0.9993 | 22.834 | 4.637 | 3 |
| D6 | all-*trans* DHA | 0.418 | 14.607 | 0.9971 | 0.285 | 17.033 | 0.9981 | 23.339 | 1.604 | 1 |
| 22:5[b] | 22:5 n-3 | 0.889 | 2.435 | 1.0000 | 0.898 | 2.284 | 0.9998 | 22.110 | 4.899 | 3 |

[a] Correspondence between ECL value of current isomer (*y*) and the ECL-value of the all-*cis* isomer (*x*).
[b] Other fatty acid interfering with *trans* isomers of EPA/DHA.
[c] Compounds D2h and D2j were resolved by multivariate curve resolution (see methods section); compound D2i appeared in different LC fractions.
[d] The peak contains several isomers that could not be separated on any of the conditions applied.

[8,13,14], this is not the case for *trans* isomers of EPA and DHA, where *trans* isomers are observed on both sides of the corresponding all-*cis* isomer. In both cases, only two of the 1-*trans* isomers elute before the corresponding all-*cis* isomer.

Isomerisation with PTSA gives approximately equal amounts of isomers with the same number of *trans* double bonds [28]. From the number and size of the GC peaks, it is obvious that some peaks contain more than one isomer. Five EPA isomers with one *trans* double bond should be expected, four peaks are seen in the chromatograms in Fig. 2a. From the amounts it can be deduced that the peaks marked as E1a, E1b and E1c are pure, while E1d contain two isomers. Likewise, six isomers of DHA with one *trans* bond should be expected (Fig. 2b). The peaks marked as D1a, D1b, D1c and D1d can be expected to be pure, while D1e contains two isomers. D1c is lower than the other compounds because the isomer elute at the end of the LC fraction, which was cut too early for complete recovery of this isomer.

The chromatograms of the isomers with two *trans* double bonds are more complex. Ten EPA isomers with two *trans* double bonds are possible. Eight peaks are seen in the EPA chromatogram (Fig. 2a, bottom); the chromatographic areas indicate

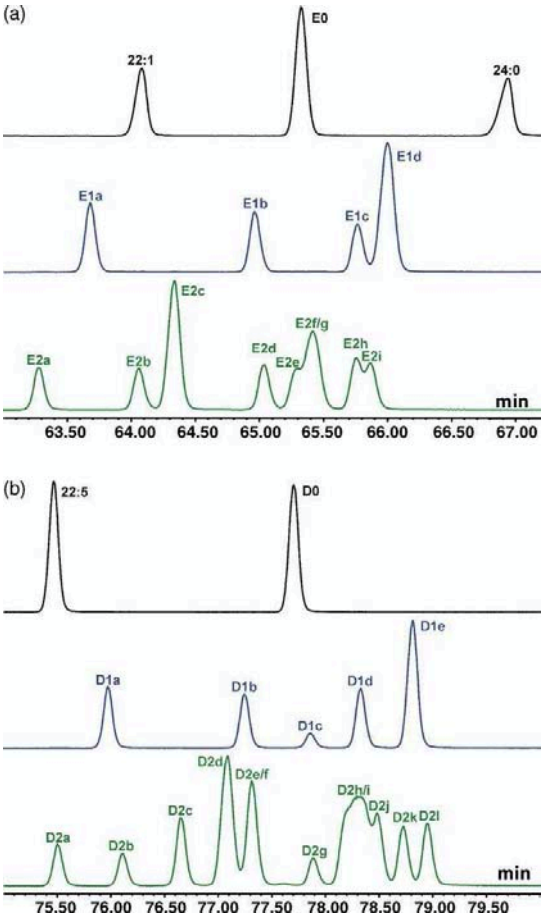


Fig. 2. GC separation of EPA (a) and DHA (b) isomers on a SP-2560 column. The all-*cis* isomers and possible interferents are shown in the upper chromatograms. The 1-*trans* and 2-*trans* isomers are shown in the middle and lower chromatograms, respectively. Chromatographic conditions are as described for Program 10 (Table 1) and in Section 2.

that E2c and E2f/g contain two isomers each. Fifteen 2-*trans* isomers of DHA are possible; ten peaks are seen in the chromatograms. The peak size indicates that D2d, D2e/f and D2h/i contain more than one isomer each.

### 3.2. Retention characteristics

Because some of the isomers that overlap in GC have slightly different elution times in Ag-LC, analysis of the fractions collected in 0.5 min intervals allowed a more detailed picture of the retention characteristics. An initial screening of all 0.5 min fractions was applied to select the fractions of interest. Empty fractions and fractions that appeared to be identical to other fractions were excluded. In general, every fraction of the 2-*trans* LC peaks and every second fraction of the 1-*trans* LC peaks were selected.

The selected fractions, plus the all-*trans* isomers, and the reference mixture containing the all-*cis* isomers were analysed by the programs given in Table 1. For all isomers, the different chromatographic conditions gave large differences in ECL values. On both columns, highly linear correlations were found between the ECL values of the various *trans* isomers and the ECL value of the corresponding all-*cis* isomer. Correlation coefficients and regression lines are given in Table 2.

### 3.3. Two-dimensional fatty acid retention indices (2D-FARI)

The retention data acquired on the BPX-70 column can be used to calculate 2D-FARI values as described in Section 2 and in Ref. [20]. The 2D-FARI plot is shown in Fig. 3, and the values are also listed in Table 2. The 2D-FARI values are suitable for identification purposes. The calculation method is robust towards changes in the column characteristics, the two dimensions give less overlap than one-dimensional retention data, and the number of carbons and double bonds in *cis* isomers can be read directly from the two indices. For accurate estimation of ECL values, the separation must be large enough to allow accurate estimation of the peak maximum. Because of peak overlap on all programs on the BPX-70 column, a single pair of 2D-FARI values is given for each of the following overlapping peaks: E2b/c, E2e/g/h, D2e/f, and D2h/j.

The 2D-FARI plot shows that the *trans* isomers behave different from ordinary *cis* fatty acids. The all-*trans* isomers are positioned far from the all-*cis* isomers, and the isomers with one and two *trans* double bonds appear in separate groups along the gradients from all-*cis* to all-*trans*. While the *cis* isomers are positioned inside the vertical columns corresponding to the number of carbons in the molecule, the *trans* isomers are displaced in the horizontal direction; all-*trans* EPA has a $FARI_A$ value above 21 and all-*trans* DHA has a $FARI_A$ value above 23. Both isomers have $FARI_B$ values similar to *cis* monoenes.

The explanation for this displacement is that *trans* unsaturated FAME have a lower response to increased column temperature than a *cis* unsaturated FAME with similar polarity. The 2D-FARI calculation method 'rotates' the values to fit the *cis* isomers. The difference in response between *cis* and *trans* double bonds can be illustrated by comparing the ECL values for the all-*cis* and all-*trans* isomers with *cis* 20:1 and 22:1. The fractional chain length (FCL) is a rough measurement for polarity of a FAME and is the fatty acid chain length subtracted from the ECL value, thus the FCLs of saturated FAMEs are zero. The FCLs of both monoenes are 0.39 on Program 1 and 0.50 on Program 5. The corresponding values are1.90 and 2.01 for all-*trans* EPA, and 2.62 and 3.02 for all-*cis* EPA. From the FCL values, it can be seen that all-*trans* EPA is far more polar than 20:1. However, if the differences in FCL between the two programs are considered, ΔFCL for all-*trans* EPA is 0.11, which is identical to ΔFCL for the monoenes and far less than ΔFCL for the all-*cis* isomer (0.39). Similar differences are observed for the corresponding DHA isomers. The FCL values are 2.18 and 2.31 for the all-*trans* isomer and 3.03 and 3.48 for the all-*cis* isomer, giving ΔFCL of 0.13 for the all-*trans* and 0.45 for the all-*cis* isomer. From these results, it seems that the effect of increased column temperature on BPX-70 is more complicated

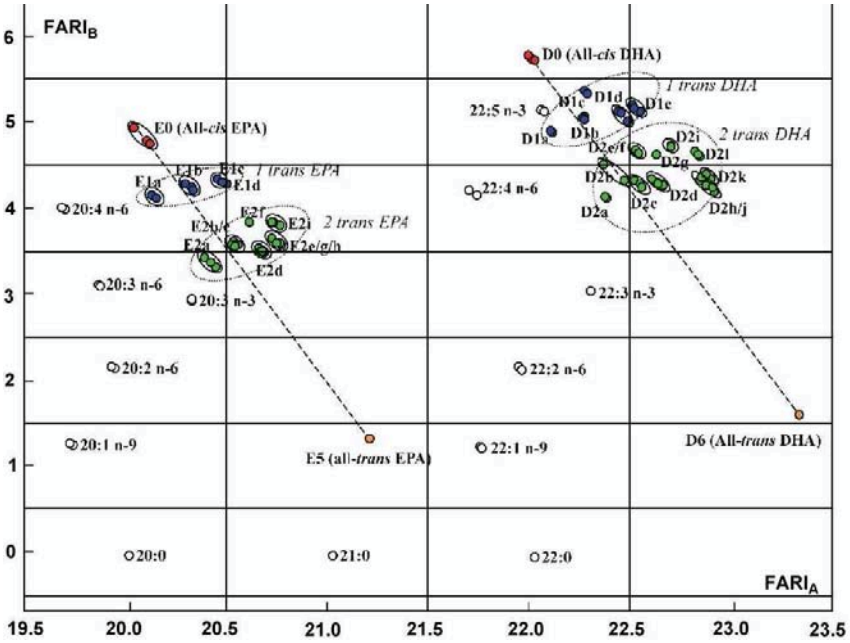


Fig. 3. 2D-FARI map of the analysed EPA and DHA isomers together with saturated FAME and common *cis* isomers (open circles). The 2D-FARI values are calculated as described in Ref. [20]. Dashed lines mark the gradients from the all-*cis* to the all-*trans* isomers.

than a general increase in column polarity, because it has a very different effect on *cis* and *trans* double bonds.

### 3.4. Resolution windows

Based on the regression data given in Table 2, the elution patterns can be visualized by line-plots as shown in Fig. 4a–d where the ECLs for each isomer are plotted against the ECL for the corresponding all-*cis* isomer. The regression lines for isomers with the same number of *cis* and *trans* double bonds are nearly parallel, and that the slopes of the gradients decrease with increasing number of *trans* double bonds. A practical consequence of these patterns is that overlaps between isomers with the same number of *trans* double bonds cannot be resolved by manipulation of the temperature and gas flow on the same column. However, peak overlaps between isomers with different number of *trans* double bonds may be avoided if chromatographic parameters are optimized. Although the ECL values and resolution patterns are quite different between the two columns (Fig. 4), the range of the slopes is nearly the same (Table 2), which indicates that similar values may also be achieved on other highly polar cyanopropyl phases.

To be able to quantify the amount of *trans* EPA and DHA, resolution of the isomers from the corresponding all-*cis* isomer must be achieved. It is also advantageous to avoid overlap with other interferents, but with mass spectrometric detection, the difference between the spectra will often be large enough to resolve such overlaps by multivariate curve resolution methods [25,29]. The plots show that it will be extremely difficult to separate all 1 and 2-*trans* isomers from the all-*cis* peaks. Since, only 1-*trans* isomers can be expected to be formed in significant amounts in samples subjected to thermal isomerisation, the discussion of optimal resolution windows focuses on resolution between the all-*cis* isomer and the 1-*trans* isomers.

For EPA isomers on SP-2560 (Fig. 4a), E0 elutes between E1b and E1c. The largest distance between these isomers is found in the window between Programs 8 and 9. From the regression lines in Table 2, a theoretical optimum is found when $ECL_{E0}$ is 23.904, where the distance between E0 and the nearest E1 peak is 0.085 units. The peak resolution, $R_s$, is estimated from the peak width of the all-*cis* isomer (Table 1); $w_h$ of 0.025 (average of Programs 8 and 9) gives $R_s$ of 2.0.

For the DHA isomers, the critical resolution is between D0 and D1c. Fig. 2b shows that the best separation is found at low polarities. At the conditions used in Program 10, which has the lowest $ECL_{D0}$, the separation between these two isomers is only 0.024 ECL units. $w_h$ of 0.021 at these conditions (Table 1) gives $R_s$ of only 0.7. Accurate quantification of a small peak eluting on the edge of a large peak requires $R_s$ above 1.0 [30]. A slight improvement in the resolution can be expected with temperature gradients below 1.0 °C, giving lower $ECL_{D0}$. However, it is unlikely that sufficient resolution of these two isomers can be achieved on this column.

Similar to the SP-2560 column, E0 on BPX-70 elutes between E1b and E1c. According to the regression lines in Table 2, the optimal resolution for the EPA isomers is found for $ECL_{E0}$ of 22.827, which is close to the ECL achieved with Program 3. The distance between E0 and the nearest E1 isomer is 0.100 ECL units. With $w_h$ estimate of 0.031 (Program 3), $R_s$ is calculated to be 1.9. Overlap between E1a and isomers of 22:1, which are

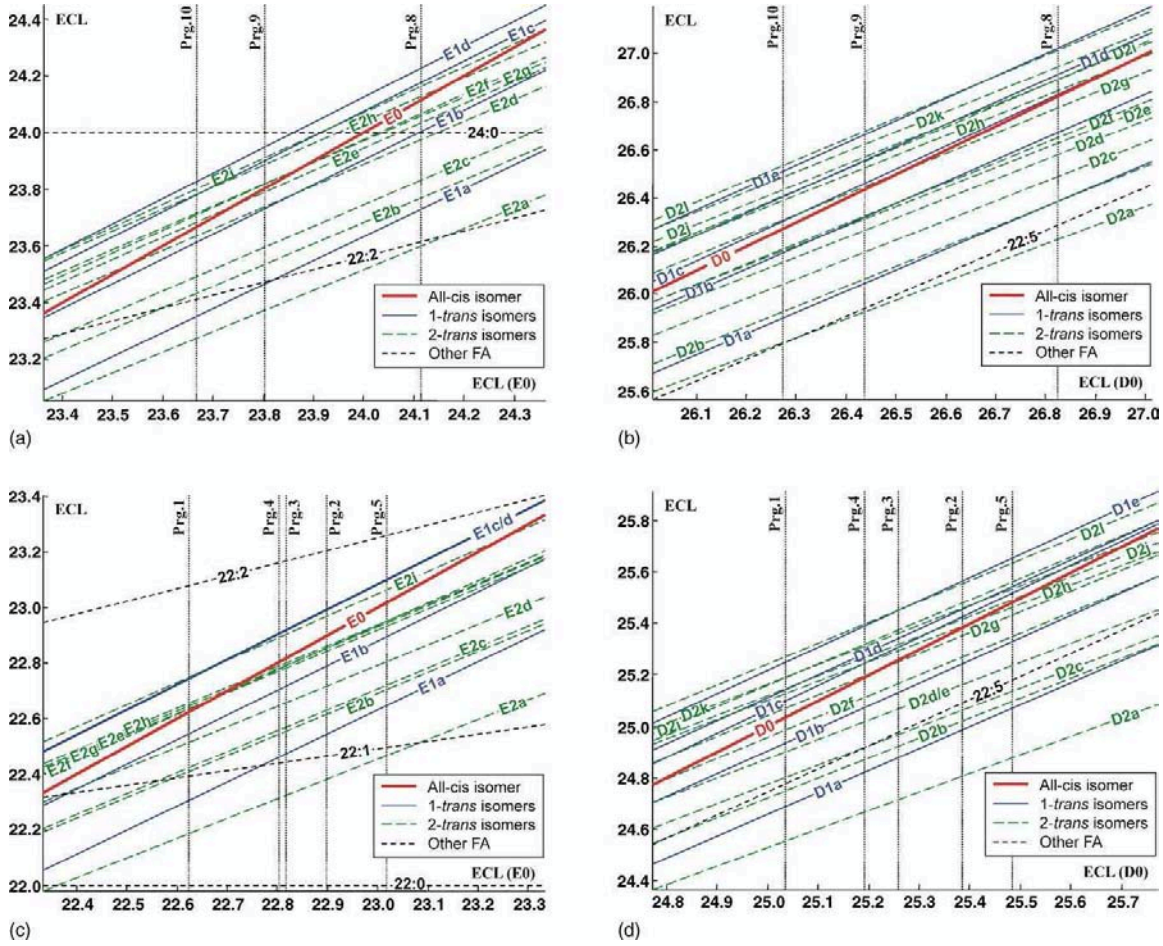


Fig. 4. ECL of *trans* isomers and possible interferents (vertical axis) plotted against ECL of the corresponding all-*cis* isomer (horizontal axis). Vertical dotted lines mark the ECL values of the all-*cis* isomers achieved with the applied programs (Table 1): (a) EPA isomers on SP-2560; (b) DHA isomers on SP-2560; (c) EPA isomers in BPX-70; (d) DHA isomers on BPX-70. The lines are based on the regression formulas given in Table 2.

abundant in some marine lipids [31], may be a problem (Fig. 4c). However, the resolution between E0 and E1b/c is large enough to allow minor adjustments to resolve overlaps between the E1a and 22:1 isomers.

Also on BPX-70 is the most critical overlap for resolution of the DHA isomers between D1c and D0. However, because of the lower polarity of the column, the distance between the two isomers is larger than achieved with SP-2560. The distance between the two isomers increases with decreasing $ECL_{D0}$. At Program 1, which has the lowest $ECL_{D0}$ of the five programs, the distance is 0.073 ECL units; $w_h$ of 0.026 gives $R_s$ of 1.6. $ECL_{D0}$ of 24.94 has been achieved with a temperature gradient of 1.0 °C and column flow of 26 cm/s [20]. Assuming similar $w_h$, $R_s$ increases to 1.8.

The main intention of this work is to indicate where good resolution windows can be found and where they are not found. Since, the properties may vary significantly from column to column and change with time [20], optimization of the chromatographic conditions may be necessary in each specific case, also because the interferents may vary. Because large variations in levels between the all-*cis* isomers and the *trans* isomers can be expected, it may be necessary to inject excessive amounts that will lead to broadening of the all-*cis* peaks. This will give poorer resolution between the all-*cis* isomers and the isomers eluting after all-*cis*. It may therefore be advantageous to select conditions that give slightly lower ECL of the all-*cis* isomers than the predicted theoretical optima.

The regressions in Table 2 have been applied to find suitable programs for the studies of thermal isomerisation of EPA and DHA on BPX-70. Separate programs were applied for EPA and DHA isomers. An ECL value of E0 of 22.63, near the predicted optimum, gave resolution to the nearest E1 peak of 0.09 ECL units, which is sufficient for baseline resolution (see discussion above). The largest deviation between actual and predicted $ECL_{E1}$ values was 0.02 units. Baseline resolution between D0 and D1 isomers could be achieved by a program with ECL of the all-*cis* isomer of 24.85, which gave a distance to the nearest D1 isomer of 0.08 units. Even though this program had

$ECL_{D0}$ values outside the range of the $ECL_{D0}$ values for the programs applied for the calculation of the regression lines (all $ECL_{D0}$ above 25), the largest deviation between predicted and real $ECL_{D1}$ was only 0.01 units. This shows that accurate predictions are achieved by extrapolation of the regression lines to regions near the investigated range. Additional details will be published elsewhere.

## 4. Conclusions

Shifts in the apparent polarity, which is a temperature effect observed on cyanopropyl columns, effects *cis* double bonds more than *trans* double bonds. It is therefore possible to move isomers with different number of *trans* double bonds relative to each other by manipulation of the temperature and flow conditions. Feasible resolution windows were predicted from the correlation between the retention indices of 1-*trans* isomers and the corresponding all-*cis* isomers. $R_s$ above 1.0 can be achieved for EPA on SP-2560, and for both EPA and DHA on BPX-70. In general, BPX-70 seems to have a more suitable selectivity for the analysis of EPA and DHA *trans* isomers than SP-2560.

Two-dimensional fatty acid retention indices (2D-FARI) are suitable for identification of *trans* geometry in polyunsaturated fatty acids. Although there is substantial overlap in range of retention times between the all-*cis* isomers and isomers with one and two *trans* double bonds, they are separated by 2D-FARI into distinct groups according to the number of *trans* double bonds.

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