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ARTICLES

Mathematical Method for the Prediction of Retention Times of Fatty Acid Methyl Esters in Temperature-Programmed Capillary Gas Chromatography

ALEXANDRE G. TORRES, NÁDIA M. F. TRUGO, AND LUIZ C. TRUGO*

Laboratório de Bioquímica Nutricional e de Alimentos, Dep. Bioquímica, Inst. Química, Universidade Federal do Rio de Janeiro, C.T. Bloco A, Lab. 528, Ilha do Fundão, CEP 21949-900, Rio de Janeiro, R.J., Brazil

An accurate method for identification of fatty acids in complex mixtures analyzed by temperature-programmed capillary gas chromatography is described. The method is based on a mathematical approach using regression curves obtained by plotting the relative retention times of fatty acid methyl esters (FAMEs) analyzed in isothermal and gradient temperature conditions. The method was applied to a complex biological sample (human milk), and it was possible to identify 64 fatty acids, including branched-chain and other fatty acids for which reference standards were not readily available. The identities of the majority of the peaks were confirmed by mass spectrometry. The relative residuals and the relative differences between estimated and measured relative retention times of individual FAMEs varied from 0.03 to 3.15% and from 0.0 to 2.9%, respectively. The method is useful for identification of fatty acids in routine analysis.

KEYWORDS: Fatty acid methyl esters; temperature-programmed gas chromatography; relative retention times; qualitative analysis

INTRODUCTION

Peaks obtained in gas chromatography can be identified based on graphical or mathematical relationships that exist between the retention times, or indices, of compounds from homologous series and some characteristics of their molecular structure (*1*−*3*). Hydrocarbons are probably the substances with the most predictable and precise chromatographic behavior, a characteristic that enables the use of the Kováts index for the identification of unknown substances in gas chromatography and the tabulation of retention data (*2*, *4*). Similarly to the hydrocarbons, the fatty acid methyl esters (FAMEs) show a highly predictable behavior in gas chromatography, as reviewed by Ackman (*1*). The relative retention times (r) of saturated FAMEs are exponentially related to the number of carbon atoms in their parent fatty acids. The linear relationship between the log r and the number of carbon atoms in the saturated fatty acid chain of FAMEs remains the basis of the majority of the graphical procedures for FAME peak identification (*1*). The presence of substituents such as a methyl side chain, or a single double bond, or systems of several methylene-interrupted double bonds does not hinder such relationships (*1*). When substituents are present, each series (with fixed number and position of a substituent) of parent fatty acids will provide a different equation for such a relationship. These linear relationships may be graphically analyzed by plotting the number of carbon atoms in the parent fatty acid of the methyl ester against log-transformed retention data, generating curves for each analysis condition for each series of fatty acids in the main classes of saturated, branched chain, monounsaturated, and polyunsaturated (*1*). Through regression analyses the best curve that fits to the data points and the equation for each function are easily calculated, making peak identification more rapid and accurate.

Although these procedures for the identification of FAMEs are useful and of practical interest they show an important limitation. The linear relationships between the number of carbon atoms and the log r are true only in the case of isothermal analyses, therefore limiting their use for complex samples or for samples with fatty acids with a wide range of chain lengths and boiling points. For these samples, analysis by temperature-programmed gas chromatography (TPGC) results in better resolution of peaks and allows the analysis of virtually all fatty acids in a single chromatographic run, with the main exception of fatty acids with trans double bonds, which requires the use of previous separation methods (*5*). However, in TPGC there is no apparent relationship between the number of carbon atoms and the log r. Therefore, it seems appropriate to search for

* To whom correspondence should be addressed. Telephone: +55 21 2562-7351. Fax: +55 21 2295-6876. E-mail: lctrugo@iq.ufrj.br.

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mathematical procedures for the identification of peaks from compounds of homologous series in TPGC analysis.

The need for expanding mathematical methods for the identification of peaks on TPGC has already been identified and studied for monoterpenes, halogenated pesticides, and *n*-alkanes, among others (*6*−*8*), but due to the requirement of relatively sophisticated and time-consuming mathematical analyses, these procedures are still of limited practical use, especially for routine analyses. Additionally, to our knowledge there are no mathematical or graphical methods for the identification of FAME in TPGC described in the literature.

We propose a convenient mathematical method developed for the identification of FAME in TPGC. Such a method would be useful for the practical analysis of FAME in complex samples, for identification of fatty acids for which standards are of limited commercial availability, and as a tool for confirmation of peak identification. The analysis of a complex and highly variable biological sample, such as human milk, in which fatty acids have a wide range of chain lengths, and numbers and position of substituents (*9*), would benefit from such a method.

The aims of the present work were to develop a method for the calculation of retention times of FAMEs in temperature-programmed capillary gas chromatography, and to demonstrate its application for the analysis of a complex biological sample such as human milk.

MATERIAL AND METHODS

Gas Chromatography. A Shimadzu GC14-B gas chromatograph equipped with a flame ionization detector (FID), a split/splitless injector, and a poly(ethylene glycol) capillary column (30-m length and 0.32 mm i.d.; Omegawax-320, Supelco Co., Bellefonte, PA) were used for all analyses. Helium was used as the carrier gas, and column pressure was set to attain a carrier gas speed of 25.0 cm/s. The injector was operated at 280 °C in the split mode at a 1:40 split ratio, and with a septum purge at 4.0 mL/minute. The column oven was operated at three different temperature conditions, one isothermal and two temperature-programmed gradients: isothermal (isot), 210 °C; gradient 1 (grad1), 200 °C (held 10 min) + 1.5 °C/min to 210 °C (held 20 min); gradient 2 (grad2), 120 °C (held 3 min) + 5 °C/min to 180 °C (held 2 min) + 2 °C/min to 230 °C (held 10 min). A standard mixture (37-component FAME mix, 47885-U, Supelco) was analyzed in the three different temperature conditions. It contained the methyl esters of the following fatty acids: 4:0; 6:0; 8:0; 10:0; 11:0; 12:0; 13:0; 14:0; 15:0; 16:0; 17:0; 18:0; 20:0; 21:0; 22:0; 23:0; 24:0; 14:1*n*-5; 15:1*n*-5; 16:1*n*-7; 17:1*n*-7; 18:1*n*-9t; 18:1*n*-9c; 20:1*n*-9; 22:1*n*-9; 24:1*n*-9; 18:2*n*-6c; 18:2*n*-6t; 20:2*n*-6; 22:2*n*-6; 18:3*n*-6; 18:3*n*-3; 20:3*n*-6; 20:3*n*-3; 20:4*n*-6; 20:5*n*-3; and 22:6*n*-3.

The retention time of each FAME, relative to that of the stearic acid methyl ester, was calculated as described by Ackman (*1*), according to the following equation:

$$r_{18:0} = (\mathrm{tr}_{(x)} - t_0)/(\mathrm{tr}_{(18:0)} - t_0) \quad (1)$$

where $r_{18:0}$ is the relative retention time of the FAME, $\mathrm{tr}_{(x)}$ is the retention time of the FAME, t_0 is the solvent front time (or dead volume), and $\mathrm{tr}_{(18:0)}$ is the retention time of methyl stearate.

At least five replicates of retention data of FAMEs from the standard mixture were used for the calculation of the coefficients of variation. The average $r_{18:0}$ for the replicate analyses were calculated for each FAME, and were used in the mathematical procedures.

Milk Samples. Ten breast milk samples were obtained after informed consent from full lactating mothers in a public day-care clinic in Rio de Janeiro (Brazil). Total fat content was estimated in all samples by the crematocrit method (*10*) immediately after collection. Aliquots from 5 of the 10 samples were separated for fatty acid analysis and to test the application of the mathematical procedure described in this paper. All of the 10 samples were then pooled and divided in aliquots for the calculation of the inter- and intra-assay variability (6 replicates; weighted averages of coefficients of variation 1.9 and 1.4%, respectively) of the whole analytical procedure for fatty acid determination. The sample pool was also used as a reference sample for branched-chain fatty acids (*a*14:0; *i*15:0; *a*15:0; *a*16:0; *i*17:0; *a*17:0; and *i*18:0), 12:1*n*-7; 18:1*n*-7, and 22:5*n*-3 (methyl esters which were employed in the development of the mathematical procedure), in addition to the FAMEs present in the standard mixture. For this purpose the pooled sample was analyzed as described in Gas Chromatography/Mass Spectrometry.

All aliquots were stored frozen (−20 °C) in glass tubes until analysis. All samples and fatty acid methyl esters standards were handled in glassware rinsed in dilute alcoholic potassium hydroxide, deionized water, 13% HNO_3, and deionized water, in sequence.

Prior to analysis, milk samples were defrosted in a water bath (37−38 °C), carefully homogenized to avoid fat losses, and immediately divided into 1.0-mL aliquots in glass tubes with Teflon-lined screw caps. Lipid extraction was carried out by the method of Bligh and Dyer (*11*), with methanol/chloroform (2:1, v/v) containing 0.1% (v/v) BHT, essentially as described in Jensen et al. (*12*). Lipid residues were stored in chloroform/methanol (10:1, v/v) under N_2 at −20 °C until transesterification and analysis. Transesterification was carried out according to the method of Kramer et al. (*13*). The resulting fatty acid methyl esters were extracted with hexane and injected into the gas chromatograph. Milk samples were analyzed in the grad2 column-temperature condition, and the pooled sample was also analyzed in the isothermal condition, as described for the standard mixture. All solvents used were of chromatographic grade (Merck; EM Science).

Gas Chromatography/Mass Spectrometry. The identification of the methyl esters of the fatty acids not present in the standard mixture, and used for the development of the mathematical procedure described herein (branched-chain fatty acids, and 12:1*n*-7, 18:1*n*-7, and 22:5*n*-3), was achieved through GC/MS analysis of the pooled milk sample. For this purpose, the pooled milk sample was analyzed at two column-temperature conditions: isot and grad2. This analysis was carried out in a Shimadzu GC17-A gas chromatograph interfaced to a QP5050A MS system. The gas chromatograph was operated at the same conditions described above for GC analysis. The mass spectrometer was equipped with an electron-impact ion source operated at 240 °C and 70 *e*V. The mass spectral data were collected from 40 to 390 amu at 1 scan/sec. The interpretation of mass spectra comprised the analysis of relevant specific fragments, such as the molecular ion, the ion resulting from the McLafferty rearrangement (*m*/*z* 74), and others of diagnostic relevance for each FAME series (*14*), and the comparison with the NIST database (similarity indexes ≥80% were considered). For the identification of the branched-chain FAMEs the fragments of diagnostic importance were those characteristic of methyl esters of most *iso* or *anteiso* branched-chain fatty acids. The mass spectra of these FAMEs presented the fragments at *m*/*z* 55, *m*/*z* 74, *m*/*z* 87, *m*/*z* 97, *m*/*z* 143, *m*/*z* 157, and *m*/*z* 199. Furthermore, the branched-chain FAMEs presented the following fragments: *a*14:0 (M^+ *m*/*z* 242, $[M - 61]^+$ *m*/*z* 181); *i*15:0 (M^+ *m*/*z* 256); *a*15:0 (M^+ *m*/*z* 256, $[M - 61]^+$ *m*/*z* 195); *a*16:0 (M^+ *m*/*z* 270, $[M - 61]^+$ *m*/*z* 209); *i*17:0 (M^+ *m*/*z* 284); *a*17:0 (M^+ *m*/*z* 284, $[M - 61]^+$ *m*/*z* 223); *i*18:0 (M^+ *m*/*z* 298). For the other three FAMEs (12:1*n*-7, 18:1*n*-7, and 22:5*n*-3) the fragments of diagnostic importance were the following: 12:1*n*-7 (*m*/*z* 55, *m*/*z* 74, *m*/*z* 83, *m*/*z* 97, M^+ *m*/*z* 212, $[M - 32]^+$ *m*/*z* 180); 18:1*n*-7 (*m*/*z* 55, *m*/*z* 69, *m*/*z* 74, *m*/*z* 83, *m*/*z* 97, M^+ *m*/*z* 296, $[M - 32]^+$ *m*/*z* 264); 22:5*n*-3 (*m*/*z* 79, *m*/*z* 91, *m*/*z* 108, *m*/*z* 175, *m*/*z* 275, *m*/*z* 315). To discriminate between isomers of monounsaturated FAMEs with double bonds at different positions (1*n*-9, 1*n*-7, and 1*n*-5) it was necessary to evaluate the elution order together with the MS data.

GC/MS was also used for confirmation of the identities of the majority of the FAME peaks (10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 18:0, 19:0; *a*14:0, *i*15:0, *a*15:0, *i*16:0, *a*16:0, *i*17:0, *a*17:0, *i*18:0; 12:1*n*-7, 14:1*n*-5, 16:1*n*-9, 16:1*n*-7, 16:1*n*-5, 17:1*n*-9, 18:1*n*-9, 18:1*n*-7, 18:1*n*-5, 19:1*n*-9, 20:1*n*-9; 18:2*n*-6, 18:3*n*-3, 18:4*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3) present in the five individual milk samples. Their identities were first determined through the use of the mathematical method and then confirmed by GC/MS with the same approach described in the above paragraph.

Mathematical Regressions. Relative retention times ($r_{18:0}$; eq 1) of FAMEs obtained in GC analyses of the standard mixture and of the pooled milk sample were used for the regression analyses. The $r_{18:0}$ obtained at isot were plotted against the $r_{18:0}$ obtained using grad1 and grad2, fitting nonlinear regressions. The nonlinear regressions were calculated separately for each class of fatty acids. The following classes were used in the regression analyses with grad1: saturated 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, 24:0; and unsaturated 16:1*n*-7, 18:1*n*-9, 18:1*n*-7, 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:1*n*-9, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3. For the nonlinear regressions with grad2, the methyl esters of the following fatty acids were used in addition to those used in grad1: saturated 10:0, 11:0, 12:0, 13:0; unsaturated 12:1*n*-7, 14:1*n*-5, 20:2*n*-6, 22:1*n*-9; and branched-chain *a*14:0, *i*15:0, *a*15:0, *a*16:0, *i*17:0, *a*17:0, and *i*18:0.

For the FAME classes analyzed at grad1 (saturated and unsaturated), relative retention times from isot plotted against those from grad1 fitted a hyperbolic function. For the branched-chain FAMEs analyzed at grad2 the plot of isot data against those of grad2 also generated a hyperbolic function, according to the following general equation:

$$r_{18:0gradA} = \alpha \times r_{18:0isot}/(\beta + r_{18:0isot}) \quad (2)$$

Polynomial quotient functions were obtained for both the saturated and unsaturated FAMEs when data from isot were plotted against those from grad2, according to the general equation

$$r_{18:0gradB} = [\alpha_1 \times r_{18:0isot}/(\beta_1 + r_{18:0isot})] + [\alpha_2 \times r_{18:0isot}/(\beta_2 + r_{18:0isot})] \quad (3)$$

where $r_{18:0gradA}$ in eq 2 is the relative retention time of the saturated or unsaturated FAME at grad1, or of the branched-chain FAME at grad2; $r_{18:0gradB}$ in eq 3 is the relative retention time of the saturated or unsaturated FAME at grad2; and $r_{18:0isot}$ is the relative retention time of the FAME at isot.

To assess the relative differences between the data points and regression curves (relative residuals), the measured relative retention times ($r_{18:0}$) of FAME at grad1 and grad2 were compared with the estimated $r_{18:0}$ at each gradient, obtained by using the measured $r_{18:0isot}$ in eq 2 or 3. The FAMEs used for these calculations were those used in the generation of the nonlinear regression curves.

To ultimately associate relative retention times of the FAMEs at gradient conditions with structural characteristics of their parent fatty acids, allowing the identification of unknown peaks, the number of carbon atoms (Cn) of the fatty acids and the log $r_{18:0}$ of their corresponding FAMEs in the isothermal analyses were associated by the least-squares method. Linear regressions were obtained according to the following general formula:

$$\log r_{18:0isot} = \alpha \times Cn + \beta \quad (4)$$

For each of four classes of fatty acids (saturated, 1*n*-9 monounsaturated, *iso*, and *anteiso* acids) the following equations were calculated:

$$\text{saturated FAME: } \log r_{18:0isot} = 0.1309 \times Cn - 2.357 \quad (5)$$

$$\text{monounsaturated 1}n\text{-9 FAME: } \log r_{18:0isot} = 0.1282 \times Cn - 2.271 \quad (6)$$

$$\textit{iso}\text{ FAME: } \log r_{18:0isot} = 0.1308 \times Cn - 2.422 \quad (7)$$

$$\textit{anteiso}\text{ FAME: } \log r_{18:0isot} = 0.1280 \times Cn - 2.357 \quad (8)$$

Coefficients of determination (r^2) of the linear regressions for the saturated, monounsaturated (1*n*-9), *iso*, and *anteiso* FAMEs were respectively, 0.9999, 1.000, 1.000, and 1.000. The standard errors for the α and β terms for the above equations were, respectively: saturated, 0.0004 and 0.0062; monounsaturated, 0.0006 and 0.0129; *iso*-, 0.0003 and 0.0043; *anteiso*-, 0.0008 and 0.0123.

For calculation of the equations for all the other series of unsaturated fatty acids, the slope (α coefficient) of the monounsaturated 1*n*-9 (eq 6) was used, because unsaturated fatty acids generate nearly parallel curves (*1*). For the calculation of the β coefficients in the equations, the $r_{18:0}$ at isot of the shortest unsaturated FAME of the corresponding series present in the standard mixture was used as a starting point. For example, for the calculation of the β-coefficients in the equations for the monounsaturated 1*n*-7 and the polyunsaturated 2*n*-6 FAME the $r_{18:0isot}$ of 16:1*n*-7 and 18:2*n*-6, respectively, were used as starting points.

Table 1. Coefficients of Variation (CV%) of Relative Retention Times ($r_{18:0}$) of FAMEs at Different Column Temperature Conditions

	min–max (CV%)[a]	mean CV%[b]
	isothermal	
saturated	0.00–2.17	1.16
monounsaturated (1*n*-9)	0.47–1.34	1.07
branched-chain	0.28–2.88	0.92
	gradient 1	
saturated	0.00–0.40	0.18
unsaturated	0.21–0.47	0.34
	gradient 2	
saturated	0.00–0.34	0.25
unsaturated	0.05–0.83	0.33
branched-chain	0.12–0.36	0.28

[a] Values represent means of $r_{18:0}$ of five replicates for each FAME. [b] Values represent means of $r_{18:0}$ of all FAMEs in each class.

Application of the Mathematical Procedure. Identification of FAME peaks present in the chromatograms of breast milk samples which were not present in the standard mixture was carried out using the regressions described in the previous section. The GC analyses of the five individual milk samples in grad2 were used in this procedure. The first step was to search for fatty acids previously reported to be present in breast milk (*9*, *15*, *16*) within the different classes. Their predicted relative retention times at isothermal condition ($r_{18:0isot}$) were estimated using the linear eqs 5−8 or the equations derived for the unsaturated FAME using the α coefficient of eq 6, according to each of the FAME classes and series. These predicted values were used in the nonlinear eqs 2 or 3 to estimate their $r_{18:0gradA}$ or $r_{18:0gradB}$, respectively, which were then matched to the measured relative retention times of peaks in the chromatograms, allowing the identification of the majority of the sample peaks that were not present in the standard mixture. For the tentative identification of unknown peaks (i.e., peaks present in the chromatograms of the samples but not present in the standard mixture and not yet reported in the literature for human milk) the same approach was used. In this case, the possible fatty acid alternatives were deduced from the presence of known peaks eluting close by and tested in the corresponding equations as described above. When these tests resulted in more than one alternative, the criteria to decide which FAME matched the chromatographic peak were the closeness between the predicted and measured $r_{18:0}$ values and the elution order.

Reliability of the mathematical procedure for predicting the identity of the FAME was tested by calculating the relative differences between their estimated and their measured $r_{18:0}$ values at grad2. The FAMEs used for these calculations were those present in the milk samples. Their estimated $r_{18:0gradA}$ or $r_{18:0gradB}$ values were determined with eqs 2 and 3, respectively, using the estimated $r_{18:0isot}$ previously predicted by use of the linear equations as described above in this section for the identification of FAME peaks present in the chromatograms of the milk samples and not present in the standard mixture.

RESULTS

The chromatographic analysis from which the proposed mathematical method was based was reliable and highly reproducible in both isothermal and temperature-programmed GC. The variability (CV%) of $r_{18:0}$ of FAMEs of the different classes (saturated, unsaturated, and branched-chain), analyzed in different column temperature conditions (isot, grad1, and grad2) are presented in **Table 1**. The CV% reported in the table refer to the FAMEs used to derive the linear and nonlinear

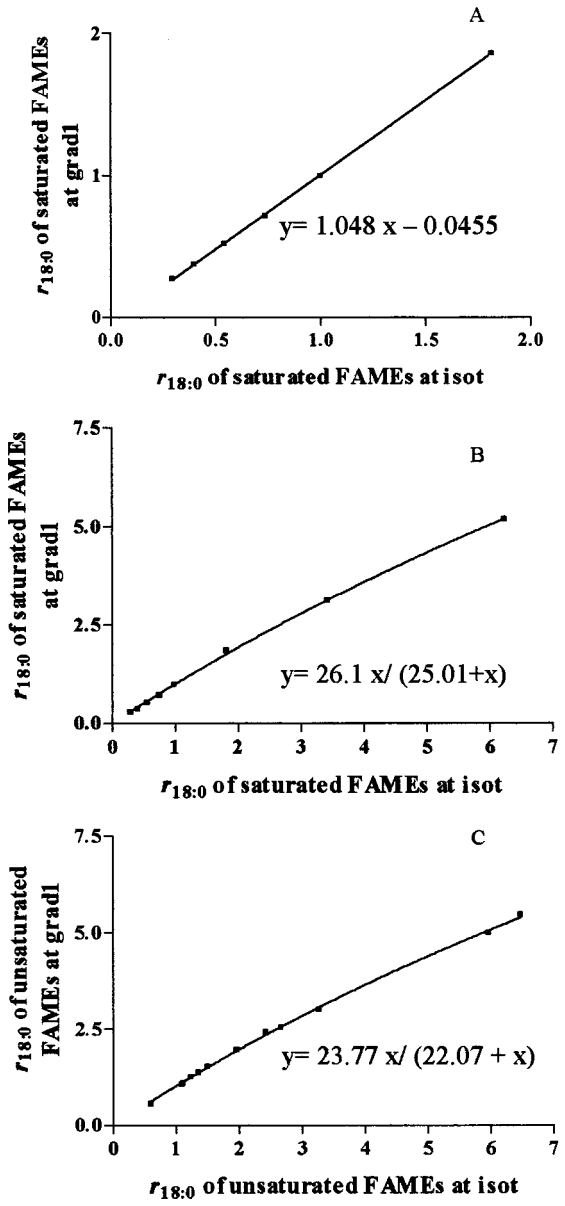

Figure 1. Plots for the regression analyses of $r_{18:0}$ of FAMEs at isot against respective $r_{18:0}$ at grad1, for saturated (A) and (B), and unsaturated (C) FAMEs. (A) Saturated fatty acids in the regression, 14:0/15:0/16:0/17:0/18:0/20:0; $r^2 = 0.9999$; standard deviation of residuals (Sy.x) = 0.0051; standard error of coefficients α (SE_α) and β (SE_β) = 0.0041 and 0.0039, respectively. (B) Saturated fatty acids in the regression, 14:0/15:0/16:0/17:0/18:0/20:0/22:0/24:0; $r^2 = 0.9998$; absolute sum of squares of residuals (ASSR) = 0.0033; Sy.x = 0.0236; $SE_\alpha = 1.989$; $SE_\beta = 2.304$. (C) $r^2 = 0.9999$; ASSR = 0.0036; Sy.x = 0.0190; $SE_\alpha = 1.384$; $SE_\beta = 1.530$.

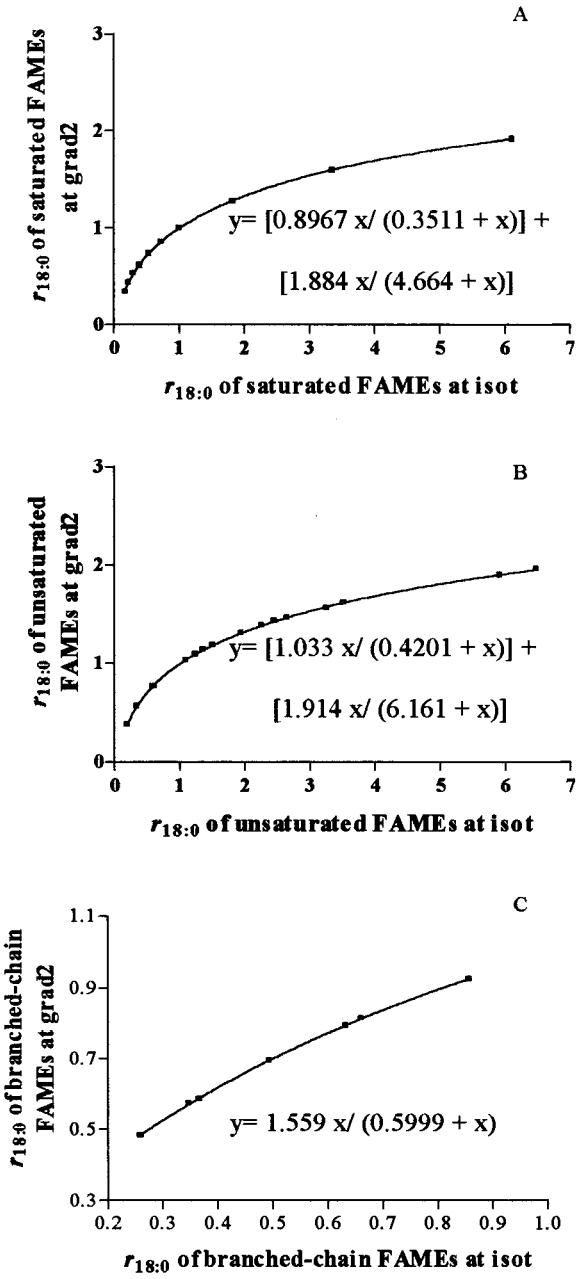

Figure 2. Plots for the regression analyses of $r_{18:0}$ of FAMEs at isot against respective $r_{18:0}$ at grad2, for saturated (A), unsaturated (B), and branched-chain FAMEs (C). (A) $r^2 = 1.000$; absolute sum of squares of residuals (ASSR) = 0.0000; standard deviation of residuals (Sy.x) = 0.0025; standard error of coefficients α ($SE_{\alpha1}$ and $SE_{\alpha2}$) and β ($SE_{\beta1}$ and $SE_{\beta2}$) = 0.0831, 0.0432, 0.0358, and 0.7402, respectively. (B) $r^2 = 1.000$; ASSR = 0.0000; Sy.x = 0.0025; $SE_{\alpha1} = 0.0703$; $SE_{\alpha2} = 0.0432$; $SE_{\beta1} = 0.0336$; $SE_{\beta2} = 0.9059$. (C) $r^2 = 0.9996$; ASSR = 0.0001; Sy.x = 0.0036; $SE_\alpha = 0.0441$; $SE_\beta = 0.0324$.

equations (see Mathematical Regressions in Material and Methods). The average values for CV% of $r_{18:0}$ were mostly smaller than 1.0.

The plots, equations, and regression parameters for the data of $r_{18:0}$ of FAMEs at isot plotted against their respective $r_{18:0}$ values at grad1 and grad2 are shown in **Figures 1** and **2**. In grad1 (**Figure 1**), the saturated FAMEs generated two different curves, one defined by a linear (**Figure 1A**) and the other by a nonlinear (hyperbolic; **Figure 1B**) function, depending on the FAME used for calculations. The linear regression was obtained when the methyl esters of fatty acids lower than C22 were used for calculations. This regression resulted in better parameters of fitting and in lower differences between estimated and measured retention data (0.10 to 1.99%) than did the nonlinear regression (0.35 to 13.11%). However, if fatty acids with chain lengths higher than C20 are to be analyzed, the nonlinear regression, which generated relative differences between predicted and measured values of 1.35% for 22:0 and 24:0, should be used.

The series of unsaturated FAMEs were analyzed together for the generation of the curves (grad1 and grad2; see Mathematical Regressions) and for the application of the derived equations

Table 2. Relative Residuals: Distances (%) between the Regression Curves[a] and the Data Points (measured $r_{18:0}$) for the FAMEs Used to Generate the Regression Curves at Temperature-Programmed Column Conditions

	saturated FAME	unsaturated FAME	branched-chain FAME
	gradient 1		
negative			
lowest difference	–0.13	–1.01	–
highest difference	–1.99	–2.01	–
positive			
lowest difference	+0.10	+0.11	–
highest difference	+1.10	+3.15	–
mean of absolute values of differences	0.66	2.03	–
	gradient 2		
negative			
lowest difference	–0.11	–0.04	–0.30
highest difference	–1.23	–2.07	–2.67
positive			
lowest difference	+0.32	+0.03	+0.08
highest difference	+1.32	+0.99	+1.19
mean of absolute values of differences	0.66	0.46	0.89

[a] Refer to **Figures 1** and **2** for regression equations.

(grad2; see Application of the Mathematical Procedure). The unsaturated FAMEs in grad1 (**Figure 1C**) generated a hyperbolic equation by nonlinear regression of data points. In grad2 (**Figure 2**), the saturated and unsaturated FAMEs (**Figure 2A** and **2B**, respectively) generated equations of polynomial quotients by nonlinear regressions of the data points. For the branched-chain FAMEs, a hyperbolic curve fitted to the data points (**Figure 2C**). Alternatively, the nonlinear regressions for gradients 1 and 2 could be calculated with all the FAME classes (saturated, unsaturated, and branched-chain) together. The curves and equations thus obtained (one for each gradient; data not shown) are similar to those obtained when data of the FAME classes were plotted separately (**Figures 1** and **2**). However, the latter procedure resulted in better regression parameters for the quality of fitting and, therefore, was used for calculation of the relative residuals and for the prediction of identity.

For determination of the relative residuals (see Mathematical Regressions), the distances between the data points and the regression curves were calculated. The lowest, highest, and mean values of relative residuals (%) for fatty acids with chain lengths >C12 are shown in **Table 2**. For fatty acids with chain lengths lower than 12 carbon atoms the relative residuals were of greater magnitude (14.5 to 20.9%).

A representative chromatogram obtained after analysis at grad2 of a breast milk sample is shown in **Figure 3**. The mathematical method used allowed the confirmation of identification of 41 FAMEs in the breast milk samples. These FAMEs were either present in the standard mixture or in the reference pooled milk sample (*a*14:0, *i*15:0, *a*15:0, *a*16:0, *i*17:0, *a*17:0, *i*18:0, 12:1*n*-7, 18:1*n*-7, and 22:5*n*-3, identified by mass spectrometry and used to generate the nonlinear equations). Furthermore, the method allowed the identification of 23 FAMEs, of which 13 were reported to be present in breast milk (*9*, *15*, *16*) and 10 were not yet reported, in addition to *a*14:0, *a*16:0, and 12:1*n*-7 identified in the pooled milk sample as described above. **Table 3** shows the measured $r_{18:0}$ values of the FAMEs present in the milk samples and their $r_{18:0grad\mathrm{A}}$ or $r_{18:0grad\mathrm{B}}$ values estimated using the mathematical

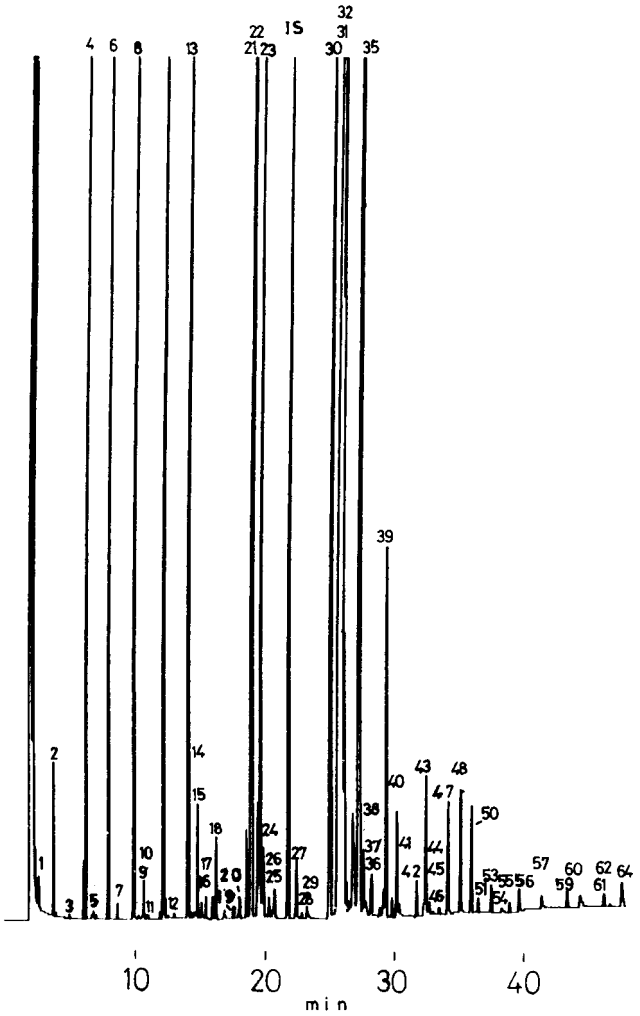

Figure 3. Representative chromatogram of a breast milk sample after analysis at grad2 [120 °C (held 3 min) + 5 °C/min to 180 °C (held 2 min) + 2 °C/min to 230 °C (held 10 min)] column temperature condition. Retention data of the FAMEs and the fatty acid corresponding to the FAME peak numbers in the chromatogram are presented in **Table 3**. The retention time of BHT is 12.4 min. Methyl esters of 11:0 and 17:0 were used as internal standards for quantitative analyses (data not shown).

procedure proposed in the present paper (see Application of the Mathematical Procedure in Material and Methods). This procedure was shown to be accurate for the prediction of identities of fatty acids in human milk. The relative differences between measured and estimated $r_{18:0}$ of these FAMEs varied from 0.0 to 2.9% (absolute value) for the fatty acids with more than 12 carbon atoms, except for *a*20:0 (5.9%). The average of the relative differences was 1.0% (absolute value) for the fatty acids with more than 10 carbon atoms evaluated (**Table 3**).

GC/MS confirmed the identities of the majority of the FAMEs reported in **Table 3**: 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 18:0, 19:0; *a*14:0, *i*15:0, *a*15:0, *i*16:0, *a*16:0, *i*17:0, *a*17:0, *i*18:0; 12:1*n*-7, 14:1*n*-5, 16:1*n*-9, 16:1*n*-7, 16:1*n*-5, 17:1*n*-9, 18:1*n*-9, 18:1*n*-7, 18:1*n*-5, 19:1*n*-9, 20:1*n*-9; 18:2*n*-6, 18:3*n*-3, 18:4*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3. For the thirteen fatty acids not yet reported in the literature to be present in breast milk, the mass spectra were analyzed with special reference to the fragments with higher diagnostic importance (*14*). The identities of six (*a*14:0, *a*16:0, 12:1*n*-7, 16:1*n*-9, 16:1*n*-5, and 18:1*n*-5) out of

Table 3. Retention Data of FAMEs from the Breast Milk Samples and Prediction of Identity: Differences (%) between Estimated ($r_{18:0gradA}$ or $r_{18:0gradB}$) and Measured ($r_{18:0}$) Relative Retention Times of FAME

fatty acid	peak[a]	t_r (min)[b]	$r_{18:0}$[b]	$r_{18:0gradA\ or}\ r_{18:0gradB}$	difference (%)
		saturated, straight chain			
6:0[c,d]	1	2.730	0.032	0.074	+131
8:0[c,d]	2	3.850	0.080	0.129	+61.1
9:0[d]	3	4.900	0.125	0.168	+34.4
10:0[c,d]	4	6.324	0.186	0.218	+17.2
11:0[c,d]	6	8.121	0.263	0.278	+4.70
12:0[c,d]	8	10.081	0.347	0.349	+0.6
13:0[c,d]	11	12.252	0.440	0.432	–1.8
14:0[c,d]	13	14.305	0.528	0.526	–0.4
15:0[c,d]	18	16.453	0.620	0.630	+1.6
16:0[c,d]	21	19.253	0.740	0.743	+0.4
18:0[c,d]	30	25.338	1.000	0.995	–0.5
19:0 [d]	37	28.705	1.145	1.134	–1.1
20:0[c,d]	42	32.042	1.288	1.282	–0.5
21:0[c,d]	49	35.636	1.442	1.436	–0.4
22:0[c,d]	55	39.184	1.594	1.596	+0.1
23:0[c,d]	58	41.914	1.711	1.758	+2.7
24:0[c,d]	62	46.885	1.924	1.915	–0.5
		saturated, branched chain			
*i*12:0	7	8.704	0.299	0.295	–1.3
*a*13:0[d]	10	11.178	0.394	0.394	+0.0
*a*14:0[c]	12	13.255	0.483	0.486	+0.7
*i*15:0[c,d]	16	15.356	0.573	0.571	–0.3
*a*15:0[c,d]	17	15.706	0.588	0.591	+0.5
*i*16:0[d]	19	17.806	0.678	0.684	+1.2
*a*16:0[c]	20	18.180	0.694	0.702	+1.0
*i*17:0 [c,d]	25	20.560	0.796	0.800	+ 0.6
*a*17:0[c,d]	26	21.027	0.816	0.816	+0.0
*i*18:0[c,d]	28	23.594	0.926	0.917	–1.0
*a*18:0	29	23.734	0.932	0.929	–0.3
*a*19:0[d]	34	26.698	1.059	1.036	–2.2
*a*20:0	40	30.082	1.204	1.133	–5.9
		monounsaturated			
10:1*n*-7[d]	5	7.211	0.224	0.237	+5.8
12:1*n*-7[c]	9	10.945	0.384	0.375	–2.3
14:1*n*-7	14	14.772	0.548	0.560	+2.2
14:1*n*-5 [c,d]	15	15.052	0.560	0.562	+0.4
16:1*n*-9	22	19.650	0.757	0.779	+2.9
16:1*n*-7 [c,d]	23	19.860	0.766	0.784	+2.3
16:1*n*-5	24	20.233	0.782	0.787	+0.6
17:1*n*-9 [d]	27	22.660	0.886	0.903	+1.9
18:1*n*-9 [c,d]	31	26.044	1.031	1.032	+0.1
18:1*n*-7 [c,d]	32	26.255	1.040	1.038	–0.2
18:1*n*-5	33	26.371	1.045	1.041	–0.4
19:1*n*-9 [d]	38	29.032	1.159	1.170	+0.9
20:1*n*-9 [c,d]	43	32.742	1.318	1.313	–0.4
20:1*n*-7	44	32.976	1.328	1.319	–0.7
20:1*n*-5	45	33.256	1.340	1.323	–1.3
22:1*n*-9 [c,d]	56	39.907	1.625	1.622	–0.2
24:1*n*-9 [c,d]	63	47.562	1.953	1.944	–0.5
		polyunsaturated			
18:2*n*-6 [c,d]	35	27.491	1.093	1.094	+0.1
18:3*n*-6[c,d]	36	28.448	1.134	1.134	0.0
18:3*n*-3[c,d]	39	29.615	1.184	1.184	0.0
18:4*n*-3 [d]	41	30.805	1.235	1.238	+0.2
20:3*n*-9[d]	46	33.746	1.361	1.359	–0.2
20:2*n*-6[c,d]	47	34.446	1.391	1.382	–0.6
20:3*n*-6[c,d]	48	35.403	1.432	1.425	–0.5
20:4*n*-6[c,d]	50	36.220	1.467	1.467	0.0
20:3*n*-3[c,d]	51	36.780	1.491	1.481	–0.7
20:5*n*-6	52	37.317	1.514	1.512	–0.1
20:4*n*-3[d]	53	37.713	1.531	1.540	+0.6
20:5*n*-3[c,d]	54	38.600	1.569	1.581	+0.7
22:2*n*-6[c,d]	57	41.681	1.701	1.696	–0.3
22:4*n*-6[d]	59	43.455	1.777	1.786	+0.5
22:5*n*-6[d]	60	44.435	1.819	1.832	+0.7
22:5*n*-3[c,d]	61	46.279	1.898	1.903	+0.3
22:6*n*-3[c,d]	64	47.772	1.962	1.948	–0.7

[a] Numerical order of peak elution (refer to **Figure 3**). [b] Values represent averages of at least two replicates of analysis (at grad2) for each of the five individual breast milk samples (average t_0= 1.983 min). [c] Fatty acids of FAMEs present in the standard mixture or identified by mass spectrometry and used in the regression analyses. [d] Fatty acids already reported to be present in breast milk samples (*9*, *15*, *16*).

these thirteen FAMEs were confirmed as follows: 16:1*n*-9, M^+ *m*/*z* 268, $[M - 32]^+$ *m*/*z* 236, $[M - 74]^+$ *m*/*z* 194; 16:1*n*-5, M^+ *m*/*z* 268, $[M - 32]^+$ *m*/*z* 236, $[M - 74]^+$ *m*/*z* 194; 18:1*n*-5, M^+ *m*/*z* 296, $[M - 32]^+$ *m*/*z* 264. For the other three FAMEs, the identification by MS was described in the Gas Chromatography/Mass Spectrometry section (in Material and Methods). The knowledge of the elution order, as well as the mathematical method described herein, helped to confirm these identifications.

DISCUSSION

TPGC analysis of fatty acids results in better resolution of chromatographic peaks and generally in shorter analysis times, but the use of mathematical procedures for qualitative analysis in TPGC is still a matter of controversy (*7*, *17*), in contrast to isothermal analysis, in which retention indices, such as the Kováts index and the equivalent chain lengths (ECL) are widely applied (*2*, *18*). The main disadvantages of the Kováts index and the ECL are that they are applicable only to isothermal analysis, and the calculations require the injection of hydrocarbons, or straight, odd and even chain saturated FAMEs, for the ECL, together with the sample (*2*), rendering these methods more time-consuming than that of the relative retention times, thus limiting their use for routine analyses.

Methods for the calculation of retention indices in TPGC have been proposed, and they have been tested in different laboratories (*7*, *8*, *17*). Although they seem to work well, they still have the disadvantage of the need for mixtures of reference compounds (hydrocarbons), and the mathematical calculations are more complex than those involved in isothermal analysis. Therefore, we propose a less complex and less time-consuming mathematical method for the identification of FAMEs on TPGC.

The relationships between $r_{18:0}$ at isothermal and temperature gradient analyses were represented by conic functions. Several factors may have contributed to the number of α and β coefficients in the polynomial equations, which defines subtle differences in the shape of the curves. The number of temperature rates of the gradients in each run can be related to the number of coefficients: two coefficients (α and β; hyperbolic function) for one rate, and four coefficients (α_1, β_1, α_2, and β_2; polynomial quotient function) for two different rates in the gradients. Other factors such as the initial and final temperatures, among others, could also have influenced the number of coefficients.

Differences in the structures of the FAMEs did not influence their behavior in the nonlinear mathematical regressions, as all classes of FAMEs analyzed generated one single curve for the same analytical conditions (data not shown). However, the curves fitted better to the data points when the main classes of FAMEs analyzed (saturated, unsaturated, and branched-chain) were plotted separately. The parameters for the evaluation of the goodness of fitting (r^2, ASSR, and Sy.x; **Figures 1** and **2**) and the values of the relative residuals (**Table 2**) show that the curves determined in regression analyses fitted very well to the data points, with relative residuals close to or below 2.0%.

Similarly to the linear relationships between the number of carbon atoms and log *r* of homologous series (*1*, *2*), the FAMEs used as the first data points in each regression were distant to the regression curves (**Table 2**), especially the saturated FAMEs, which resulted in loss of accuracy for fatty acids with chain lengths lower than C12. Therefore, equations should not be used for the prediction of $r_{18:0}$ of FAMEs eluting close to the first FAME used in each regression. The discrepancies between the curves and the measured results for the first data points in the

curves are common to other mathematical methods (*1*), but their reasons have not been specifically addressed. In the case of 6:0; 8:0, and 9:0 these discrepancies could also be related to the fact that they were not bracketed, as the first saturated FAME used for the calculation of the nonlinear regressions was 10:0.

According to the coefficients of variation of the relative retention times of FAMEs analyzed at isothermal and gradient 2 conditions shown in **Table 1** (minimum 0 and maximum 2.9%), the differences between estimated $r_{18:0gradA}$ or $r_{18:0gradB}$ and measured $r_{18:0}$ (prediction of identity of the FAME from breast milk samples; **Table 3**) that were below 3% can be considered as being within the error expected for the analysis. Considering these differences, the method was accurate for identification of 57 FAMEs (differences $< 3\%$) in the breast milk samples. Although differences between estimated and measured $r_{18:0}$ of $\geq 3\%$ made peak assignments of 7 FAMEs less obvious, they did not limit the application of the method since the knowledge of the elution order of FAMEs in the column chosen (*1*) and the knowledge of the sample fatty acid composition improve the correctness of FAME identification. Among the FAMEs presenting differences of $\geq 3\%$ those showing the highest differences were the saturated with short chain lengths, as commented above. However, they elute in a region of the chromatogram that is of simple analysis where they are practically the only peaks present. In the absence of standards for confirmation, the identification of FAMEs with differences between measured and estimated $r_{18:0}$ of $\geq 3\%$ were considered tentative.

A versatile, faster, and less expensive alternative would be to plot retention data of all the FAME classes available in just one graph to perform nonlinear regression analysis. This method could be applicable when a standard mixture containing fewer FAME standards is available, or in the case of less complex samples. However, more criteria and sample knowledge would be necessary in this case to avoid misidentifications, because predictions could be less accurate.

The general procedure to apply the mathematical method described herein for the identification of fatty acid methyl esters in complex samples should be as follows below.

Standards. (1) Analyze a standard mixture using one isothermal column temperature and one temperature gradient, as appropriate for the sample fatty acid composition. This standard mixture should contain the main fatty acids (as methyl esters) representative of the different classes and series which are possibly present in the samples. (2) Calculate $r_{18:0}$ (eq 1) for each FAME present in the standard mixture at both isothermal and gradient conditions. (3) Calculate the nonlinear regressions (eqs 2 and/or 3) for the FAMEs grouped in classes (saturated, unsaturated, and branched-chain) to obtain the relationships (α and β coefficients) between their $r_{18:0isot}$ and $r_{18:0grad}$ for each class as described in Mathematical Regressions. For these regressions use $r_{18:0}$ data of at least five FAMEs in each class to obtain accurate results. (4) Calculate the linear regressions between Cn and $r_{18:0isot}$ (eq 4) to obtain the appropriate regressions for each class of fatty acids (saturated, monounsaturated 1*n*-9, branched-chain) as described in Mathematical Regressions, as was the case of eqs 5−8. Calculate β coefficients for the equations for other classes and series of unsaturated FAMEs.

Samples. (1) Analyze a sample in the optimized temperature gradient and calculate the $r_{18:0}$ (eq 1) of the peaks not present in the standard mixture. (2) Peak assignment in the sample chromatogram can be carried out through the use of the equations defined with the standards, in a manner similar to that described for peak identification in the breast milk samples (see Application of the Mathematical Procedure in Material and Methods).

CONCLUSIONS

The method described in this paper is a valuable mathematical tool specifically designed for the identification of fatty acid methyl esters analyzed in temperature-programmed gas chromatography. It is the first report to demonstrate the mathematical prediction of FAME relative retention times in TPGC. In addition, it uses less complex calculations than those reported in the literature for TPGC analysis of other compounds. The application of the method was exemplified by the analysis of breast milk. The method described allowed the identification and/or confirmation of more than sixty fatty acids in human milk samples. The determination of the composition of human milk fatty acids is of concern for the mother's and infant's health, and comprehensive databases from different populations worldwide are recognizably required (*9*).

The proposed method can be suitable to the analysis of other complex biological samples such as marine fish oils, mammal fats, algae, and bacteria. Moreover, it would be of interest for the practical analyst for routine analyses of processed foods, infant formulas, and ruminant fats, among others.

ABBREVIATIONS AND NOMENCLATURE

Anteiso, methyl branching at the third carbon atom from the methyl end of the fatty acid; ASSR, absolute sum of squares of residuals; ECL, equivalent chain length of a fatty acid; FAME, fatty acid methyl ester; iso, methyl branching at the second carbon atom from the methyl end of the fatty acid; isot, isothermal analyses; Cn, number of carbon atoms in the parent fatty acid of the FAME; grad, temperature-programmed analyses for gradients 1 (grad1) and 2 (grad2); r^2, coefficient of determination; $r_{18:0}$, retention time of a fatty acid methyl ester, relative to that of methyl stearate; $r_{18:0grad}$, measured relative retention time of the FAME obtained through gradient analysis in either gradient 1 or gradient 2; $r_{18:0gradA}$, relative retention time of the FAME at gradients 1 or 2, used in the hyperbolic function; $r_{18:0gradB}$, relative retention time of the FAME at gradient 2, used in the polynomial quotient function; $r_{18:0isot}$, relative retention time of the FAME at isothermal analysis; Sy.x, standard deviation of residuals; TPGC, temperature-programmed gas chromatography; $tr_{(x)}$, retention time (min) of substance *x*; t_0, dead volume, or the solvent front time (min); $tr_{(18:0)}$, retention time (min) of methyl stearate.

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