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Predicting conjugated linoleic acid (CLA) composition in Brazilian dairy products by multiple regression analysis based models

Juliana C. Nunes · Alexandre G. Torres

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Abstract The analysis of conjugated linoleic acids (CLA) in dairy products is of concern for human health, but requires a combination of different analytical methods that are laborious and time consuming. The aims of the present study were to develop mathematical models based on data of the CLA and fatty acid composition of Brazilian dairy products and to apply the developed models for the calculation of CLA based on a basic analysis of fatty acid composition. The fatty acid composition of five types of dairy products was determined by gas chromatography (GC); analysis of CLA was achieved by silver-ion high-performance liquid chromatography and GC-mass spectrometry. Step-wise multiple regression analysis was performed to model the composition of CLA. All the six significant models of CLA composition (total CLA; total *cis*, *trans*, *trans,cis* CLA; *cis*-9,*trans*-11 CLA; *trans*-7,*cis*-9 CLA; *trans*-11,*cis*-13 CLA; and total *trans,trans* CLA) were physiologically consistent, had appropriate goodness of fit, and were fairly robust. According to the results of Pearson correlation analysis between estimates and experimental data ($r=0.78\text{--}0.91$ and $0.0000 < P < 0.0028$), the models could be used to rank dairy products according to the estimates of total CLA, *trans*-7,*cis*-9 CLA; *trans*-11,*cis*-13 CLA; total *cis,trans*, *trans,cis* CLA; and total *trans,trans* CLA, but not for *cis*-9,*trans*-11. The adjusted regression models allowed the estimation of CLA composition in a variety of Brazilian dairy products.

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基于多元回归分析预测巴西乳制品中的共轭亚油酸 (CLA)组成

摘要： 乳制品中共轭亚油酸(CLA)有利于人类健康,但是乳中CLA的分析方法非常复杂。本研究基于巴西乳制品中CLA和脂肪酸组成数据建立数学模型,以此用于预测乳制品中CLA和脂肪酸组成。采用气相色谱法测定了乳制品中五种类型脂肪酸组成, Ag^+ -HPLC和 GC-MS法测定了CLA组成。所建立的6个CLA组成模型(总CLA、总cis,trans和trans,cis CLA、cis-9, trans-11 CLA、trans-7, cis-9 CLA、trans-11, cis-13 CLA和总trans,trans CLA)具有非常好的相关性,较好的拟合度和准确度。根据预测值和实际值($r=0.78-0.91, 0.0000 < P < 0.0028$)的Pearson校正分析,除了cis-9,trans-11CLA外,此模型可以预测总CLA、trans-7,cis-9 CLA、trans-11,cis-13 CLA、总cis,trans, trans,cis CLA和总trans,trans CLA的组成。研究结果表明,校正的回归模型可以用于不同种类巴西乳制品中CLA的预测。

Keywords CLA composition · Whole UHT milk · Butter · Cheese · Mathematical modeling

关键词 CLA共轭亚油酸组成 · 全脂UHT超高温灭菌奶 · 黄油 · 干酪 · 数学模型

Abbreviations

<i>a</i>	<i>anteiso</i> branch
Ag^+ -HPLC	silver-ion high-performance liquid chromatography
CLA	conjugated linoleic acid
FAME	fatty acid methyl ester
GC	gas chromatography
GC-FID	gas chromatography with flame ionization detector
GC-MS	gas chromatography-mass spectrometry
<i>i</i>	<i>iso</i> branch

1 Introduction

Conjugated linoleic acid (CLA) occurs naturally in a variety of foods as positional and geometric isomers of linoleic acid (C18:2 *cis*-9, *cis*-12) with conjugated double bonds (Ha et al. 1987). Individual CLA isomers present several biological activities in animal models including anticarcinogenic properties, modulation of immune system, alteration of plasma lipid levels and changes in body composition (Whigham et al. 2000; Pariza et al. 2001), and these biological effects of CLA are isomer specific (Noone et al. 2002). The determination of the contents of total and individual CLA isomers in dairy products, which are the major natural food sources of CLA (Ritzenthaler et al. 2001), is a relevant issue as these compounds may have nutraceutical effects on the health of consumers of dairy products. However, a comprehensive analysis of CLA in dairy products requires a combination of different analytical methods to ensure accurate separation and quantification of the isomers. The complementary use of silver-ion high-performance liquid chromatography (Ag^+ -HPLC), gas chromatography with highly polar capillary columns coupled to a flame ionization detector (GC-FID) or a mass spectrometer (GC-MS) is necessary to allow detailed characterization of positional and geometrical CLA isomers (Roach et al. 2002;

Christie et al. 2007). Therefore, these analyses are laborious, generally time consuming, and require at least two analytical instruments.

CLA isomers are synthesized through two major pathways in ruminant animals, one that occurs in the rumen and the other that occurs in endogenous tissues, such as the mammary glands. CLA are by-products of the first steps of rumen biohydrogenation that can be directly absorbed by the cows. In contrast, *trans*-octadecenoates, especially *trans*-11-18:1 and *trans*-7-18:1, are more advanced intermediates in the rumen biohydrogenation, that are absorbed by the animal and might be converted to CLA in selected tissues through enzymatic Δ^9 -desaturation (Collomb et al. 2006). Endogenous synthesis by Δ^9 -desaturation is recognized as the major source of CLA in the ruminant organism as a whole, and is therefore responsible for the high levels of *cis*-9,*trans*-11 and *trans*-7,*cis*-9 isomers found in dairy products (Corl et al. 2001, 2002). These two major pathways of CLA synthesis are metabolically related and depend on environmental and individual factors, such as the composition of the cow's diet and genetic polymorphisms of Δ^9 -desaturase (Collomb et al. 2006).

CLA present in dairy products are metabolically dependent on three major factors: (1) the amounts of dietary unsaturated fatty acids available in the rumen, especially 18:2*n*-6 and 18:3*n*-3; (2) the activity of rumen bacteria; and (3) the endogenous activity of enzymatic Δ^9 -desaturation. Therefore, the contents of CLA isomers in dairy products are associated with markers of selected dietary unsaturated fatty acids (Collomb et al. 2004), of rumen bacteria (Sieber et al. 2004) and of Δ^9 -desaturation in dairy products (Lock and Garnworthy 2003). Metabolically consistent bivariate correlations of fatty acids in dairy products occur because of the strong metabolic associations of CLA with other fatty acids related to the rumen metabolism. However to our knowledge, up to date, multiple regression analysis has not been applied for the investigation of metabolically sound associations of CLA with other fatty acids of industrialized dairy products. We hypothesized that it will be possible to model the contents of CLA isomers in dairy products, and that the contents of metabolically relevant fatty acids in the samples will be significant predictors. Once developed, these multiple regression models could be applied to estimate contents of total CLA and specific CLA isomers, based on data of the basic fatty acid composition, reducing the time, complexity, and instrumentation required for the determination of detailed CLA composition in dairy products.

Considering the potential importance of finding a reliable method to estimate CLA contents in dairy products, the aims of the present study were to develop multiple regression models of the contents of total and individual CLA isomers in dairy fat, to apply the adjusted models for the calculation of total CLA and the major CLA isomers in varied dairy products, and to suggest a step-by-step protocol for the application of the proposed approach.

2 Materials and methods

2.1 Reagents

All organic solvents used were of chromatographic grade (Tedia, Brazil). A commercial mixture of fatty acid methyl esters (FAME; 37-component FAME mix; Supelco,

Co, ref 4885-U) was used for identification of FAME peaks. Standards of CLA isomers *cis*-9,*trans*-11; *trans*-10,*cis*-12; *cis*-10,*cis*-12; and *trans*-10,*trans*-12 were kind gifts from Dr. Robert Adlof (Formerly of the Agricultural Research Service, Food Quality & Safety Research Unit, US Department of Agriculture, Peoria, IL, USA). CLA standards were used as representative of the classes of CLA isomers *cis*, *trans*; *trans,cis*, *cis,cis*; and *trans,trans* and for determination of total CLA contents (milligrams per gram fat) in dairy products through external calibration in Ag⁺-HPLC analyses.

2.2 Samples

At least two of the most consumed Brazilian commercial brands for the following dairy products were obtained in local supermarkets, during winter: whole ultra-high temperature processing (UHT) milk, *Prato* cheese ripened for 4 weeks and butter from unfermented cream. A total of four, three, and two samples of butter, whole UHT milk, and *Prato* cheese, respectively, were analyzed. For each brand, two production lots were purchased and considered as independent samples. To allow for a thorough homogenization of samples in order to obtain representative aliquots (AOAC 1984a, b, c) of the dairy products analyzed, multiple packages from the same production lot for each of the dairy products had their contents combined, as follows: for milk, two packages of 1.0 L were combined and heated to 37 °C; for butter, three packages of 200 g were combined and fused at 37 °C; and for *Prato* cheese, three packages of 300 g were combined and blended in a food processor. The samples were frozen at -20 °C under N₂ until analysis.

The data used for the multiple regression analysis, consisting of fatty acid and CLA composition of dairy products, were from samples of whole UHT milk, *Prato* cheese and butter. Multiple regression models were developed for the contents of total and individual CLA isomers in dairy fat, as detailed below, in Section 2.4.

In addition to the samples included in the regression matrix, one sample of each of the following dairy products was also analyzed in order to test the application of the multiple regression models: *Mozzarella* cheese ripened for 2 weeks, nonripened *Minas Frescal* cheese, and whole UHT milk. These samples were homogenized and stored as previously described, and were used as examples of the application of the developed models. The dairy products used to test the application of the multiple regression models were from various seasons, as specified below, in order to test the applicability of the models. *Mozzarella* cheese was produced during summer, *Minas Frescal* cheese during autumn and whole UHT milk during winter. In the major areas producing milk in Brazil, the average rainfall is higher during spring and summer, compared to the dryer seasons of autumn and winter. Consequently, the pasture availability is altered during the year accordingly, and increased pasture availability is observed in the rainy months (Carvalho et al. 2003); therefore, in this period, cows are fed pasture. In contrast, during autumn and winter, cows are fed forage, which presents a different fatty acid composition when compared to pasture (Carvalho et al. 2003). In the present work, the diet of the lactating cows was not controlled, and our interest was to investigate commercial samples of dairy products, produced over varied seasons. The effects of seasonality on CLA composition in dairy products are generally indirect and are attributable to the cows' diets.

2.3 Analysis of fatty acids

Lipid extraction and derivatization of fatty acids Total lipids were extracted in triplicate by the method of Hara and Radin (1978) with appropriate adaptations for each food matrix, as described in Nunes and Torres (2010). FAME was prepared by base-catalyzed transesterification as described by Kramer et al. (1997).

Analysis of FAME by gas chromatography For determination of basic fatty acid composition, FAME were analyzed in a GC14-B gas chromatograph (Shimadzu, Japan) equipped with a split/splitless injector operated with a split ratio of 1:30, a polyethylene glycol capillary column (30 m length and 0.32 mm i.d.; Omegawax-320, Supelco Co., EUA), and a FID. Helium was used as carrier gas, at 25 cm/s, set at the beginning of the run. Temperatures of injector and detector were 260 and 280 °C, respectively. The oven temperature was held at 40 °C for 3 min, and then programmed at 2.5 °C/min to 180 °C, followed by a second gradient at 2.0 °C/min up to 210 °C, and then held for 25 min. Gas chromatographic peaks of FAME of the samples were identified by comparison with the retention time data of standards. The mathematical method described by Torres et al. (2002) was used as a complementary tool for the identification of fatty acids in samples. The areas of the peaks were corrected by the theoretical response factors of Ackman and Sipos for the FAME responses of the FID as described by Wolff et al. (1995), and area percent of FAME was converted to weight percent of fatty acids. The contents of individual fatty acids were expressed as g.100 g⁻¹ total fatty acids.

Separation of CLA methyl esters by Ag⁺-HPLC For analysis of CLA composition of the dairy products (Kramer et al. 1999; Sehat et al. 1999), Ag⁺-HPLC separation of CLA methyl esters was performed as detailed by Nunes and Torres (2010). We used a KNAUER HPLC chromatograph (KNAUER, Germany) equipped with an UV detector (KNAUER, Germany) operated at 233 nm and a ChromSpher 5 Lipids analytical silver-impregnated column (250×4.6 mm id; Varian, Inc.), and its corresponding guard column (Varian, Inc.). Total CLA contents (milligrams per gram fat) were determined by external standardization and CLA isomers contents of the two main groups (*cis,trans* and *trans,cis* CLA, and *trans,trans* CLA) were calculated separately by internal normalization and expressed as percent of total CLA. Since the *cis,trans/trans,cis* isomers are the major CLA present in dairy products and the ones with known bioactivity in humans, the proportions of *cis-9,trans-11*; *trans-7,cis-9*; and *trans-11,cis-13* isomers were also determined from the Ag⁺-HPLC analysis. The identification of the CLA isomers was confirmed by GC-MS of dimethylloxazoline derivatives (Christie et al. 2007; Sehat et al. 1998) after separation in a cyanopropylmethylarylpolydimethylsiloxane capillary column (60 m length and 0.25 mm i.d.; HP-88, Agilent J&W, EUA), as previously described (Nunes and Torres 2010).

2.4 Statistical analysis

Descriptive analysis was used to calculate averages, standard deviations, and standardized coefficients of kurtosis and skewness. All variables used in the regression

analysis matrices were normally distributed. Fatty acid variables were included, according to biological relevance, in the multiple regression matrices as determinants of the contents of CLA isomers in dairy products.

Concerning biological relevance, the following criteria were adopted for inclusion of independent variables in the model matrices:

- 1- The $C_{14:1}:C_{14:0}$ ratio was used as a proxy of Δ^9 -desaturation, as this product/substrate ratio is a good indicator of Δ^9 -desaturation when assessed through the fatty acid composition of milk fat (Lock and Garnworthy 2003).
- 2- The unsaturated fatty acids 18:1, 18:2*n*-6, and 18:3*n*-3 were included in the multiple regression matrices since these fatty acids are substrates for the synthesis of CLA (Collomb et al. 2006), and cows fed diets rich in unsaturated fatty acids tend to secrete milk simultaneously high in CLA and unsaturated fatty acids.
- 3- The branched chain fatty acids *anteiso*-C15:0 and *iso*-C17:0 were included in the multiple regression matrices, given that they are produced by rumen bacteria, especially by *Butyrivibrio fibrisolvens*, which also produces CLA (Vlaeminck et al. 2006). Thus, the sum of contents of these two branched chain fatty acids was included as a determinant of the contents of CLA in dairy products.

In addition to the biological criteria adopted for selection of independent variables, Pearson correlation analysis was used to confirm association between the independent variables and the CLA of each model. Stepwise multiple regression analysis was performed to model the contents of the following CLA-related variables in dairy products, as dependent variables: total CLA; total *cis,trans*; *trans,cis* CLA; total *trans,trans* CLA; *cis*-9,*trans*-11 CLA; *trans*-7,*cis*-9 CLA; and *trans*-11,*cis*-13 CLA. The goodness of fit of the models was evaluated through *P* values and standard errors of the coefficients, and the *P* value, adjusted R^2 and mean relative error for each model. For all the regression analyses, the number of observations was always from 7- to 14-fold higher than the number of variables initially included in the models' matrices (Kachigan 1991).

The adjusted models were applied to estimate the values of each CLA-related variable of the samples included in the regression matrices, in order to assess the relative residuals and the accuracy of the estimates. In order to validate the adjusted models, they were applied to a set of samples different from the ones used to adjust the models, and the estimates were compared to their corresponding experimental results. Additionally, Pearson and Spearman's (ranks) correlation analyses were performed between estimated and experimental values of all data to investigate the consistency of the results. Data were analyzed with Statgraphics v.7.0 (Statistical Graphics Corporation/Plus Ware, USA) and GraphPad Prism v 4.0 (GraphPad). Two-sided *P* values < 0.05 were considered as statistically significant.

3 Results and discussion

A total of 35 fatty acids were identified in the dairy products analyzed by gas chromatography in a 30 m polyethylene glycol capillary column (Table 1). Average contents of fatty acids were within the ranges reported internationally for dairy fat

Table 1 General fatty acid composition (g.100 g⁻¹ total fatty acids) of Brazilian dairy products: whole UHT milk, butter, Prato cheese, Mozzarella cheese, and Minas Frescal cheese (n=21)

Fatty Acids	Mean	SD	Min	Max
Saturated				
4:0	1.26	0.86	0.03	2.24
6:0	1.70	0.40	0.48	2.21
8:0	1.20	0.15	0.88	1.52
10:0	3.00	0.77	1.96	4.75
11:0	0.06	0.02	0.04	0.10
12:0	3.52	0.83	2.58	539
14:0	11.83	1.43	10.30	15.00
15:0	1.22	0.11	1.09	1.48
16:0	30.26	1.97	26.45	34.13
18:0	11.30	0.99	9.63	13.34
20:0	0.22	0.07	0.12	0.38
22:0	0.07	0.02	0.03	0.11
23:0	0.05	0.02	0.03	0.09
Branched chain				
<i>i</i> 11:0	0.35	0.07	0.27	0.51
<i>i</i> 13:0	0.15	0.02	0.13	0.19
<i>i</i> 14:0	0.17	0.04	0.13	0.25
<i>i</i> 15:0	0.42	0.06	0.34	0.52
<i>a</i> 15:0	0.66	0.08	0.54	0.78
<i>i</i> 17:0	0.58	0.06	0.45	0.71
<i>a</i> 17:0	0.56	0.02	0.51	0.61
<i>i</i> 18:0	0.09	0.02	0.05	0.15
Monounsaturated				
12:1	0.09	0.01	0.07	0.12
14:1	1.12	0.09	0.94	1.26
15:1 <i>n</i> -6	0.30	0.03	0.25	0.36
16:1	1.94	0.19	1.61	2.35
17:1	0.40	0.08	0.31	0.72
18:1	23.89	1.86	20.05	27.00
20:1 <i>n</i> -9+CLA <i>t,t</i>	0.27	0.09	0.19	0.52
Polyunsaturated				
18:2 <i>n</i> -6	1.93	0.36	1.42	2.75
18:3 <i>n</i> -6	0.16	0.04	0.10	0.25
18:3 <i>n</i> -3	0.45	0.14	0.28	0.80
20:3 <i>n</i> -6+21:0	0.10	0.07	0.03	0.29
20:3 <i>n</i> -3	0.14	0.06	0.07	0.31
20:5 <i>n</i> -3	0.06	0.04	0.03	0.19
CLA				
CLA <i>c,t</i>	0.79	0.20	0.54	1.40

SD standard deviation; *Min* minimum value; *Max* maximum value; *i* iso branch; *a* anteiso branch; *t,t* *trans*, *trans* CLA isomer group; *18:1* consists of all isomers of 18:1, with *cis* or *trans* double bonds; *c,t* *cis,trans* CLA isomer group

(Lawless et al. 1999; Eiffert et al. 2006; Seçkin et al. 2005; Prandini et al. 2007), except for the levels of butyric acid in some samples, which were relatively low compared to previous reports. These differences were possibly promoted by sample concentration with N_2 after methylation, that may have caused selective evaporation of butyric acid methyl ester, which is highly volatile. However, selective evaporation of butyric acid during sample preparation did not affect the mathematical models proposed herein, since this fatty acid was not included in any of the models as a significant predictor, and there is currently no strong reason to believe that butyric acid and CLA would be physiologically associated in the cow.

Six different models of CLA composition in dairy fat were developed through multiple regression analysis (Table 2). All models were physiologically sound since there are known metabolic associations between all the pairs of dependent and independent variables retained in the adjusted models (Table 2). In addition, models were consistent with the results of Pearson correlation analysis, since the coefficients in the models had the same signal observed for the Pearson correlation coefficients. All multiple regression models developed were significant (ANOVA P for total model=0.0000; Table 2) and had mean relative errors between 0.5 and 21 % (Table 2), indicating satisfactory fitting to the data. The values of adjusted R^2 of all models indicated that the selected independent variables could explain more than 85 % of the variation in the contents of each dependent variable. Additionally, all variables retained in the developed models had statistically significant coefficients ($P < 0.05$; Table 2), and in some cases P value was 0.0000, indicating highly significant independent variables.

In order to further analyze the goodness of fit of the proposed regression models, the relative residuals were calculated for all data points within the six multiple regression models (Table 3). Average values of relative residuals were lower than 11 % for all CLA-related variables investigated (Table 3). The lower average residuals observed for the major CLA components (total *cis,trans*, *trans,cis* CLA) and the higher average residuals observed for the minor CLA components (total *trans,trans* CLA) are statistically consistent. Considering the data presented in Tables 2 and 3, as a whole, it can be concluded that the application of the models proposed herein would produce satisfactory estimates and might serve to rank dairy products according to the model-based estimates of CLA composition and levels.

The next step was the test of the models' applicability, through the estimation of CLA contents in three different dairy products. The samples investigated were dairy products consumed in Brazil, of which two types were different from those included in the regression matrices (Mozzarella and Minas Frescal cheeses). Relative differences between experimental and estimated values of CLA isomers and groups of isomers for these three dairy products were on average lower than 10 % (Table 4). These results indicated that the developed models could produce satisfactory estimates for total *cis,trans*; *trans,cis* CLA; *cis-9,trans-11* CLA; and total *trans,trans* CLA, and moderately satisfactory estimates for total CLA and *trans-7,cis-9* CLA. Estimates for these values had relative differences to experimental results that were lower than 20 % for all individual variables in each sample. In contrast, the regression model was not sufficiently robust to estimate the contents of the minor CLA isomer *trans-11,cis-13*, that had relative differences as high as 28 % in Minas Frescal cheese (Table 4). These differences are possibly attributable to the effect of noise in the

Table 2 Multiple regression models of the contents of total CLA, CLA isomers, and groups of isomers in UHT whole milk, Prato cheese, and butter

Dependent variable	Independent variables ^a	P value ^b	Coefficient	Standard error of the coefficient	Weight in the model ^c	Adjusted R ² (%)	ANOVA P for the model ^d	Mean relative error (%) ^e
Total CLA (mg.g ⁻¹ fat)	<i>a</i> 15:0+17:0	0.0000	11.37	1.138	14.09	97.54	0.0000	13.43
	C _{14:1} /C _{14:0}	0.0000	-89.06	15.09	-8.015			
Σ <i>cis,trans,trans,cis</i> CLA (g.100 g ⁻¹ total CLA)	Constant	0.0000	77.01	1.641	-	85.87	0.0000	0.507
	C _{14:1} /C _{14:0}	0.0000	126.3	16.94	11.37			
	18:1	0.0072	0.246	0.082	5.889			
<i>cis</i> -9, <i>trans</i> -11 CLA (g.100 g ⁻¹ of total CLA)	18:1	0.0000	3.616	0.057	86.57	99.46	0.0000	6.679
<i>trans</i> -7, <i>cis</i> -9 CLA (g.100 g ⁻¹ total CLA)	C _{14:1} /C _{14:0}	0.0000	126.6	17.93	11.39	98.62	0.0000	9.259
	18:1	0.0013	-0.260	0.070	-6.224			
<i>trans</i> -11, <i>cis</i> -13 CLA (g.100 g ⁻¹ total CLA)	18:3 <i>n</i> -3	0.0000	4.224	0.698	1.985	97.89	0.0000	12.50
	<i>a</i> 15:0+17:0	0.0030	0.978	0.290	1.213			
Σ <i>trans,trans</i> CLA (g.100 g ⁻¹ total CLA)	<i>a</i> 15:0+17:0	0.0014	2.974	0.799	2.946	94.73	0.0000	21.06
	18:3 <i>n</i> -3	0.0445	4.119	1.921	1.936			

Equation of the multiple regression models: dependent variable=constant+independent variable₁ × coefficient₁+independent variable_n × coefficient_n

^a Independent variables: contents of fatty acids as g.100 g⁻¹ total fatty acids

^b P value for each coefficient, expressing the significance of each coefficient in respective model

^c Weight=coefficient of the independent variable × average content in the samples of the independent variable; ^d P value for the model, expressing the significance of the whole model

^e Mean relative error=(estimate of mean absolute error × 100 %)/average value of dependent variable

Table 3 Assessment of the relative residuals of the multiple regression models developed for CLA in whole UHT milk, Prato cheese, and butter

Dairy product	Relative residuals (%) ^a of data points of CLA in the multiple regression models					
	Total CLA	Σ <i>cis,trans</i> ; <i>trans,cis</i> CLA	<i>cis-9,trans-11</i> CLA	<i>trans-7,cis-9</i> CLA	<i>trans-11, cis-13</i> CLA	Σ <i>trans,trans</i> CLA
Prato cheese A	+9.25	-0.47	+6.40	-12.7	+9.03	+21.6
Prato cheese B	-1.81	-0.45	+7.77	-10.8	-12.7	+6.10
Butter A	+4.31	-0.22	+5.57	+5.50	+5.34	+4.11
Butter B	+16.9	+0.31	+5.85	+4.80	+5.82	+10.9
Butter C	-5.05	+0.91	+2.57	-3.07	+4.15	-2.74
Butter D	-18.7	-0.74	-3.52	+2.34	+7.22	+17.8
Whole Milk A	-7.47	+0.34	-7.39	+15.1	-7.28	-15.4
Whole milk B	-8.36	-0.51	-6.12	+6.37	+3.41	-6.41
Whole milk C	-2.75	-0.90	-9.71	+12.1	+9.82	-7.50
Average \pm SD ^b	8.29 \pm 5.61	0.54 \pm 0.25	6.10 \pm 2.03	8.09 \pm 4.38	7.20 \pm 2.78	10.3 \pm 6.21

^a Relative residual=(estimate-experimental value) \times 100 %/experimental value

^b Average value based on absolute values of relative residuals

model, since *trans-11,cis-13* is the minor CLA isomer analyzed, and therefore the estimate error have a relatively high weight on the estimate. Possibly, differences between estimated and experimental values observed for CLA in Minas Frescal cheese and Prato cheese were not related to manufacturing or ripening of dairy products, as recently reviewed (Bisig et al. 2007). In addition, Minas Frescal cheese and Prato cheese were produced during the dry seasons and Mozzarella cheese was

Table 4 Results of the application of the developed multiple regression models to estimate total CLA and major CLA isomers of Brazilian dairy products, and assessment of the validity of the models, through relative differences between estimates and experimental values

CLA-related variables (units)	Whole UHT milk		Mozzarella cheese		Minas Frescal cheese	
	Estimate	R.D.	Estimate	R.D.	Estimate	R.D.
Total CLA (mg.g ⁻¹ fat)	6.74	-11.8	5.78	-13.9	4.68	-18.2
Σ <i>cis,trans</i> ; <i>trans,cis</i> CLA (% ^a)	97.2	+2.63	94.7	+0.53	96.7	+1.84
<i>cis-9,trans-11</i> CLA (% ^a)	89.4	+3.53	83.1	-4.19	88.1	+5.64
<i>trans-7,cis-9</i> CLA (% ^a)	7.37	+8.30	5.96	+14.3	6.93	+0.51
<i>trans-11,cis-13</i> CLA (% ^a)	2.73	+6.80	2.77	-7.88	2.85	-27.5
Σ <i>trans,trans</i> CLA (% ^a)	5.58	+5.40	5.21	+5.07	5.28	+5.86
Average RD ^b	-	6.41	-	7.65	-	9.93

RD relative difference=(estimate-experimental value) \times 100 %/experimental value

^a Percent of total CLA

^c Average of absolute values of relative differences

produced during a rainy season; therefore, it is unlikely that the seasonality might have influenced the accuracy of the estimated values.

In addition to the relative differences between estimates and experimental values, both Pearson and Spearman's ranks correlation analyses were used to investigate if the application of the models would serve to rank the samples according to CLA levels. For these analyses, average experimental values and corresponding estimates from all the dairy products analyzed were included in the correlation matrix, generating a total of 12 data points (Fig. 1). We observed significant P and r values higher than 0.77 for five of the six isomers and groups of isomers (Fig. 1), indicating the consistency of the majority of the models to estimate CLA levels in dairy products. The exception was *cis-9,trans-11* CLA, that did not present significant correlation between estimates and experimental values, for all samples taken together (Fig. 1). This was possibly due to the low ranges of variation of contents of *cis-9,trans-11* CLA in the samples. Due to that, the samples analyzed were not easily discriminated based on their contents of *cis-9,trans-11* CLA, which were very similar. Furthermore, results of correlation analyses indicate that the models could be applied to rank the dairy products according to their levels of total CLA and specific isomers and groups of isomers, with the exception of the important *cis-9,trans-11* CLA (Fig. 1).

In order to estimate the contents of CLA in dairy products using the rationale developed in the present study, we suggest two approaches. The first is to directly apply the models developed in this study (Table 2) to the basic fatty acid composition data of dairy products, in order to estimate contents of the CLA isomers and groups of isomers in Table 2, and to rank dairy products according to contents of total CLA; *trans-7,cis-9* CLA; *trans-11,cis-13* CLA; and total *trans,trans* CLA. A major limitation of this approach is that it would only result in reasonably satisfactory estimates of CLA isomers and groups of isomers in dairy products from cows with backgrounds similar to the ones used in the present work, concerning factors related to geography, herds' genetics, and feeding practices. When these factors are considered, the direct application of the models in Table 2 could be used in cases when it is not possible due to practical reasons to develop new models.

The second is to develop multiple regression models, based on the experimental data of CLA and basic fatty acid composition from an appropriate subset of samples. In this case, in order to generate adequately robust models that would produce satisfactory estimates of CLA isomers and groups of isomers, the subset of samples should be representative of the dairy products to be analyzed, and should take into account the major factors affecting CLA composition, such as sample origin, herds' genetics, and animal feeding practices. This approach is detailed below.

(1) At least two brands of each type of dairy products of interest should be analyzed by GC-FID in order to obtain the basic fatty acid composition. The total number of observations should be at least fivefold higher than the number of independent variables included in the regression matrices (Kachigan 1991). For this analysis, a polar capillary column should be used, preferably with a polyethylene glycol phase. A standard mixture of fatty acid methyl esters should be analyzed for identification of FAME peaks. We recommend using the mathematical method described by Torres et al. (2002) and/or the equivalent chain lengths (Mjøs and Grahl-Nielsen 2006), as complementary means for the identification of branched-

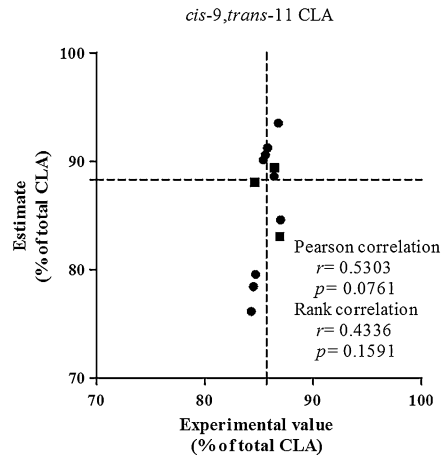
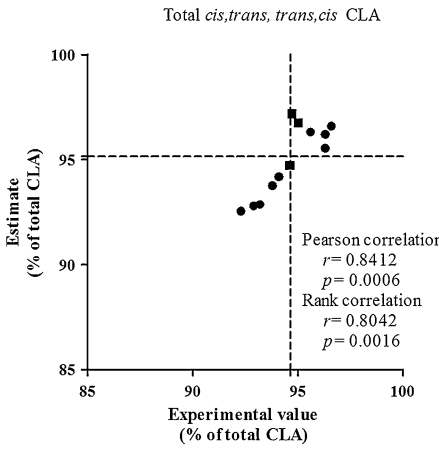
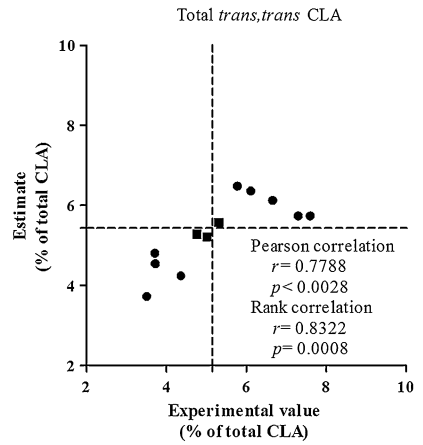
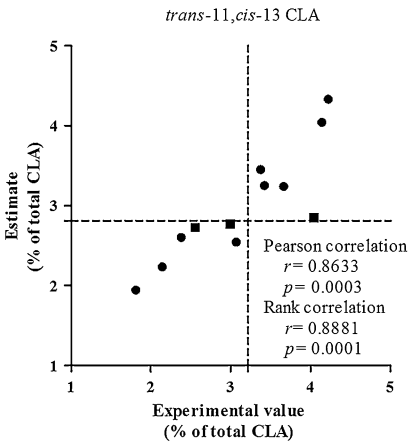
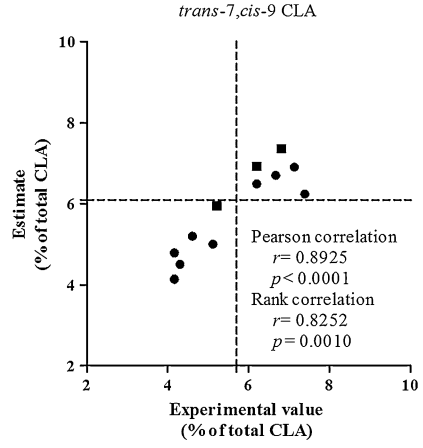
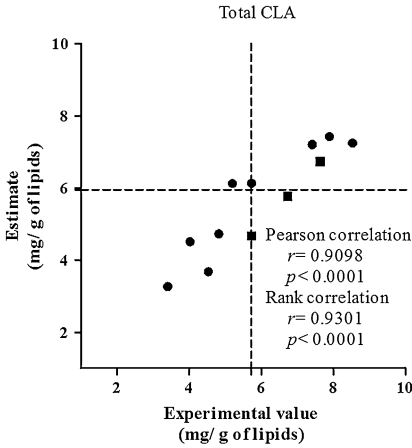


Fig. 1 Correlations between estimates and experimental values of total CLA, CLA isomers and groups of isomers in Brazilian dairy products. Estimates were determined by application of multiple regression models of Table 2. *Filled circle* data from dairy products included in the multiple regression model matrices ($n=9$); *filled square* data from dairy products different from the ones used to develop the regression models ($n=3$). Correlation coefficients (r) and P values of each of Pearson and rank correlation analyses are presented in the graphs

chain fatty acids in samples. (2) Determine the CLA composition by mass spectrometry coupled to gas chromatography with a long and highly polar capillary column, and determine the contents of CLA isomers by Ag^+ -HPLC. For a detailed description of methods of CLA analysis, the following references could be consulted (Christie et al. 2007; Nunes and Torres 2010). (3) Select independent variables physiologically associated to CLA composition in milk fat. As a starting point, use the independent variables of Table 2. Results of Pearson correlations between fatty acids and CLA help to confirm that the chosen variables are consistent. (4) With an appropriate statistical software, run the stepwise multiple regression analysis of the variables selected. (5) Evaluate the quality of fit through the interpretation of results, as presented in Table 2 and through the evaluation of the relative residuals (Table 3). (6) To estimate the CLA-related variables, the developed models should be applied as exemplified below.

Example taken from model in Table 2: estimate of total CLA in a sample of dairy product,

$$\text{Total CLA} = [11.37 \times (a_{15:0} + i_{17:0})] + (-89.06 \times C_{14:1}/C_{14:0}) \quad (1)$$

Where total CLA is the estimate of total contents of CLA (milligrams per gram fat) in the dairy product, $(a_{15:0} + i_{17:0})$ is the sum of contents ($\text{g } 100 \text{ g}^{-1}$ total fatty acids) of *anteiso*-C15:0 and *iso*-C17:0, and $C_{14:1}/C_{14:0}$ is the ratio of myristoleic-to-myristic acid.

We did not test the rationale proposed in this study to data of dairy products from other regions around the world with more varied climates and dietary regimens of the cows, and also with cows varying in genetic backgrounds. However, since the mathematical models developed were all biochemically sound and statistically consistent, application of the rationale proposed to dairy products from other countries supposedly would work satisfactorily, when appropriately tested. The direct analysis by GC-FID, GC-MS, and Ag^+ -HPLC should be the most appropriate approach to assess CLA contents of dairy products and should be performed whenever possible. We believe that the proposed mathematical approach might prove a useful complementary tool to assess CLA contents of dairy fat, which could be used when there are limitations for the direct analysis through chromatographic techniques.

4 Conclusions

We presented a rationale to develop biochemically sound multiple regression models applicable for the estimation of the CLA composition of dairy products. The developed regression models were satisfactorily robust, allowing the estimation of CLA contents of varied dairy products. The rationale of model fitting could be applied to a

subset of samples and consequently would allow the estimation of CLA contents of a larger set of samples, if sample origin, animals' genetics, and feeding practices are taken into account in sample selection.

Alternatively, the models presented in this study (Table 2) could be directly applied to the data of basic fatty acid composition in Brazilian dairy products, and allow for direct estimates of CLA composition. Therefore, the developed regression models might be a valuable simplified approach applicable for assessing CLA composition in a variety of dairy foods, based on basic data of fatty acid composition, thus reducing the time and labor required for CLA analysis, or used as a complementary tool to aid characterisation of CLA isomers and groups of isomers of dairy fat.

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