

Measurement of Conjugated Linoleic Acid (CLA) in CLA-Rich Soy Oil by Attenuated Total Reflectance–Fourier Transform Infrared Spectroscopy (ATR–FTIR)

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Conjugated linoleic acid (CLA) isomers in oils are currently measured as fatty acid methyl esters by a gas chromatography–flame ionization detector (GC–FID) technique, which requires approximately 2 h to complete the analysis. Hence, we aim to develop a method to rapidly determine CLA isomers in CLA-rich soy oil. Soy oil with 0.38–25.11% total CLA was obtained by photo-isomerization of 96 soy oil samples for 24 h. A sample was withdrawn at 30 min intervals with repeated processing using a second batch of oil. Six replicates of GC–FID fatty acid analysis were conducted for each oil sample. The oil samples were scanned using attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR), and the spectrum was collected. Calibration models were developed using partial least-squares (PLS-1) regression using Unscrambler software. Models were validated using a full cross-validation technique and tested using samples that were not included in the calibration sample set. Measured and predicted total CLA, *trans,trans* CLA isomers, total mono *trans* CLA isomers, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA had cross-validated coefficients of determinations (R^2_v) of 0.97, 0.98, 0.97, 0.98, 0.97, and 0.99 and corresponding root-mean-square error of validation (RMSEV) of 1.14, 0.69, 0.27, 0.07, 0.14, and 0.07% CLA, respectively. The ATR–FTIR technique is a rapid and less expensive method for determining CLA isomers in linoleic acid photo-isomerized soy oil than GC–FID.

KEYWORDS: Conjugated linoleic acid (CLA); GC–FID; ATR–FTIR; partial least-squares regression analysis; photo-isomerization; soy oil

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term used for the geometric and positional isomers of linoleic acid found primarily in dairy and meat products of ruminants (1). CLA isomers are found to be anticarcinogenic (1, 2) and anti-atherosclerotic (3) and helpful in normalizing the impaired glucose tolerance (4) and reducing body weight (5). A new technique involving photo-isomerization of linoleic acid to CLA has been developed, which produces CLA-rich soybean oil containing up to 23% total CLA (6). Approximately, 75% of the total CLA in the photo-isomerized oil consisted of the most stable *trans,trans* CLA isomers, while the remaining 25% consisted of the *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA isomers, *trans*-9,*cis*-12-CLA, and *cis*-10,*trans*-12 (7).

The CLA content of CLA-rich oil is currently being measured as fatty acid methyl esters (FAMES) using a gas chromatography–flame ionization detector (GC–FID) technique (8). However, this method of analysis is time-consuming and, hence, is unsuitable for rapid or real-time detection. Hence, the development of a method to rapidly identify CLA isomers is of great

interest. Attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR) has been used previously for the quantitative determination of fatty acids in food systems (9). CLA isomers have typical absorption patterns in the mid region of the infrared spectrum. The *trans,trans* CLA isomers produce a peak at 988 cm^{-1} , and the *cis,trans* CLA isomers produce two peaks at 981 and 947 cm^{-1} , respectively (10).

Previously, Xiuzhu et al. (11) developed a method to measure the free fatty acid content and peroxide value (PV) of edible oils by FTIR using spectral reconstitution. De Greyt et al. (12) developed a FTIR method for measuring low *trans* levels present in the physically refined oils using an absorbance peak height at 967 cm^{-1} . Li et al. (13) described a method based on partial least-squares (PLS) regression to determine the *trans* fat in edible oils and fats using the FTIR spectra of oil samples. A similar quantitative technique involving PLS to rapidly determine the iodine value of the palm oil samples using the FTIR spectra has been described by Che Man et al. (14). The FTIR technique has also been used for measuring the *cis,trans* content in the oils (15).

Lavillonniere et al. (16) determined the CLA composition of 12 different cheese samples using a combination of different analytical techniques involving reversed-phase high-performance liquid chromatography (RP-HPLC), gas chromatography–mass

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spectrometry (GC–MS), gas chromatography–Fourier transform infrared spectroscopy (GC–FTIR), and silver nitrate thin-layer chromatography (AgNO₃–TLC). Christy et al. (10) developed a FTIR method based on the PLS regression analysis to predict the levels of total CLA and the total *trans* content of olive oil spiked with CLA isomers and beef fat.

Currently, the GC–FID technique (8) is the only known method for the measurement of total mono *trans* isomers of CLA, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA isomers. Because this process requires a minimum of 20 min for the sample preparation and 2 h for analysis, the method becomes time-consuming and, hence, is unsuitable for real-time analysis for a large number of samples. Hence, a rapid method for the CLA analysis in the CLA-rich soy oil system should be developed. The objective of this research is to evaluate the potential of ATR–FTIR and develop a direct, rapid, online, inexpensive, and nondestructive method to measure individual CLA isomers and total CLA in CLA-rich soy oil produced by photo-isomerization.

MATERIALS AND METHODS

Materials. Refined, bleached, and deodorized (RBD) soy oil (Wesson, ConAgra, Irvine, CA) containing 52% linoleic acid and 6% linolenic acid, as measured, was obtained from a local grocery store (Fayetteville, AR) and was stored at 4 °C. Re-sublimed iodine crystals were used as a catalyst (EM Science, Cherry Hill, NJ). Sodium methoxide and anhydrous sodium sulfate (EMD Chemicals, Darmstadt, Germany) were used for methyl ester preparation. Heptadecanoic acid methyl ester (17:0; Sigma-Aldrich) was used as a standard for GC.

Photo-irradiation of Soy Oil Samples. Soy oil was photo-irradiated using the photo-irradiation unit described by Jain and Proctor (6) and method adapted from Lall et al. (17). A total of 48 borosilicate vials (10 mL) filled with 7 mL of soy oil and 0.35% iodine catalyst were attached to an illuminated glass frame of a photo-irradiation unit using transparent tapes with three UV/vis lamps of 450 W each (Ace Glass, Inc., Vineland, NJ) placed on the water-jacketed side of the laminar flow unit. The area of the glass plates allowed space for 50 vials to be isomerized simultaneously. A sample was withdrawn every 30 min for 12 h with repeated processing using a different batch of oil. A total of 96 samples were collected and used in this study.

Preparation of Fatty Acid Methyl Esters. The method of Christie et al. (18) was used to prepare CLA isomer concentrations.

Analysis of CLA Methyl Esters by GC–FID. For each sample, methyl esters were analyzed by GC using the method of Ma et al. (19).

Collection of ATR–FTIR Spectra. An ATR–FTIR spectrum of each oil sample was collected using the OMNIC software on an Impact 410 instrument (Nicolet, Madison, WI) in the absorption mode. The instrument was equipped with an interferometer and has a deuterated triglycine sulfate (DTGS)–KBr detector. The mirror has a velocity of 0.6329 cm/s and a resolution of 4 cm^{−1}. Each sample was subjected to 128 scans, with a data spacing of 1.928 cm^{−1}. The background sample was collected using the same instrumental and environmental conditions and was collected every 120 min. The sample holder was cleaned after collection of each sample by methanol and water. Samples were scanned in a wavenumber range of 4000–650 cm^{−1}.

Statistical Analysis. The ATR–FTIR chemometric models were developed for quantifying the total CLA, total mono *trans* isomers of CLA, *trans,trans* CLA isomers, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA isomers in CLA-rich soybean oil.

Calibration Model Development and Data Analysis. Statistical analysis of the spectral data involves multivariate regression analysis because of the large number of statistical variables. Multivariate analysis was performed using Unscrambler 9.6 software (CAMO ASA, Oslo, Norway) (20). The calibration set consisted of 73 randomly selected samples with CLA levels ranging from 0.38 to 25.11%. The FTIR spectra (dependent variables) of oil samples were imported into the Unscrambler as numerical data. Total CLA, total mono *trans* CLA isomers, *trans,trans*

CLA isomers, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA levels were used as independent variables. The spectral data were pretreated for outlier detection, mean centering and weighing by their standard deviations. The entire spectral region was used to develop PLS calibration models. PLS models were also developed using first and second derivatives (nine-point Savitzky–Golay derivatives) to determine the best fit of the same data.

The calibration model was subjected to full cross-validation to evaluate the calibrated models. In the full cross-validation, one sample was left out of the calibration model and isomers were predicted by the model based on the remaining sample set. The prediction residual for each prediction was then calculated. This step was repeated so that each calibration value was excluded once to test if its removal had seriously affected the model. A root-mean-square error (RMSE) was then calculated. Martens' uncertainty test was also performed by the Unscrambler to assess the stability of the regression results and to identify the perturbing samples and significant *x* variables. This test was performed along with the cross-validation technique and is based on the jack-knifing principle.

Validation of the Calibration Models. A sample set of the remaining 23 photo-irradiated soy oil samples that was not included in the calibration set was used to validate the calibration models. ATR–FTIR spectra of the validation oil samples were obtained as described earlier. CLA percentages were predicted using the prediction method provided by the Unscrambler software. Results were evaluated on the basis of root-mean-square error of calibration (RMSEC), root-mean-square error of validation (RMSEV), coefficient of determination of calibration (*R*_c²) and validation (*R*_v²), and the relative prediction discrimination index value (RPD, where RPD = SD_c/SEC) (SD_c is the standard deviation of each isomer of CLA in the calibration set, and SEC is the standard error of calibration). RPD was used as an indicator of the predictive ability of the model. Greater RPD values indicate that the range of variation in the values of CLA isomers is significantly greater than the prediction error. RPD values > 2.0 are usually acceptable (21). The weighted regression coefficient profile of the calibration model shows the regions of the oil spectrum that correlated with the CLA isomers.

RESULTS AND DISCUSSION

ATR–FTIR Spectra. Figure 1A shows a typical ATR–FTIR spectrum of control soy oil, and Figure 1B shows the spectrum of CLA-rich soy oil. The peak at 3010 cm^{−1} represents the –C–H stretch α to the double bond of an unsaturated fatty acid, while the peaks at 2926 and 2853 cm^{−1} are characteristic of the asymmetric and symmetric –CH₂ stretches of the fatty acid hydrocarbon chains (22). The ester carbonyl produces characteristic carbonyl stretching peaks between 1750 and 1735 cm^{−1} and also between 1300 and 1100 cm^{−1} from coupling of C=O and C=C stretches (22). Asymmetric bending vibrations of –C–H are observed at 1450 cm^{−1}, which is near the scissoring band in the spectra of hydrocarbons occurring at 1465 cm^{−1} (22). The broad peak between 1420 and 1300 cm^{−1} is produced by carboxylate ions (22). The peak at 720 cm^{−1} is produced by the methylene rocking vibrations, where all of the methylene groups rock in phase, which is a characteristic of the long chain fatty acids (22). In Figure 1B, the peak at 988 cm^{−1} is characteristic of *trans,trans* CLA and the peaks at 981 and 947 cm^{−1} represent the *cis,trans* CLA isomers (10) produced because of the formation of CLA, which are not evident in the spectrum of the control soy oil.

Figure 2 is an enlarged view of the fingerprint region of conjugated dienes in control and photo-isomerized soy oils containing 10% total CLA. The *trans,trans* CLA isomers produce a peak at 988 cm^{−1}, while the peaks at 981 and 947 cm^{−1} are produced by the *cis,trans* isomers (10). Because the *trans,trans* isomers of CLA are the most stable thermodynamic form, they are the highest concentration of isomers produced during photo-isomerization and, hence, give the largest peak.

Calibration Models. Model statistics of the measured and predicted values of the validation results of total CLA, total mono *trans* CLA isomers, *trans,trans* CLA isomers, *trans*-10,*cis*-12

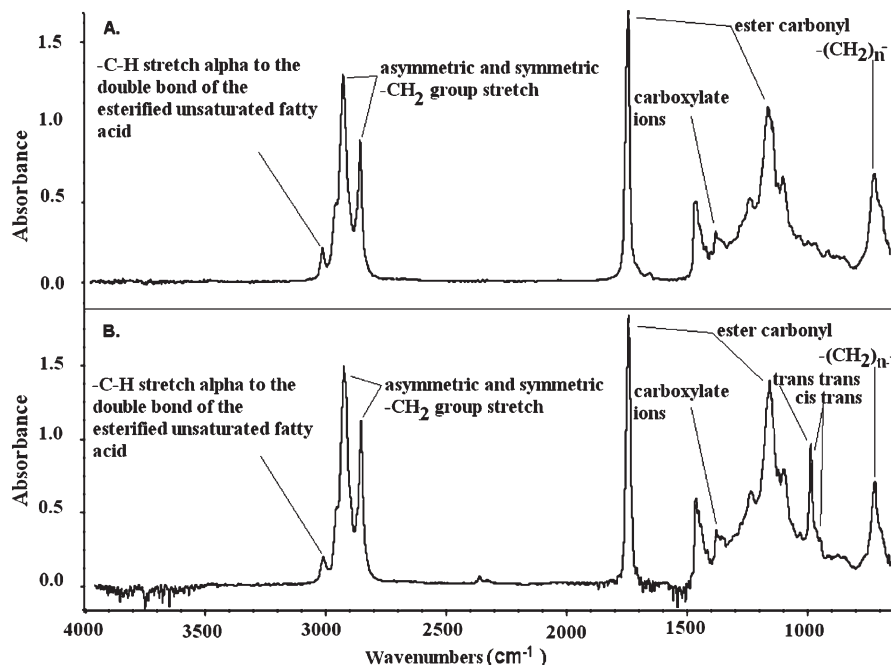


Figure 1. ATR-FTIR spectra of the 4000–650 cm^{-1} region of (A) control soybean oil and (B) photo-isomerized soybean oil containing 10% total CLA.

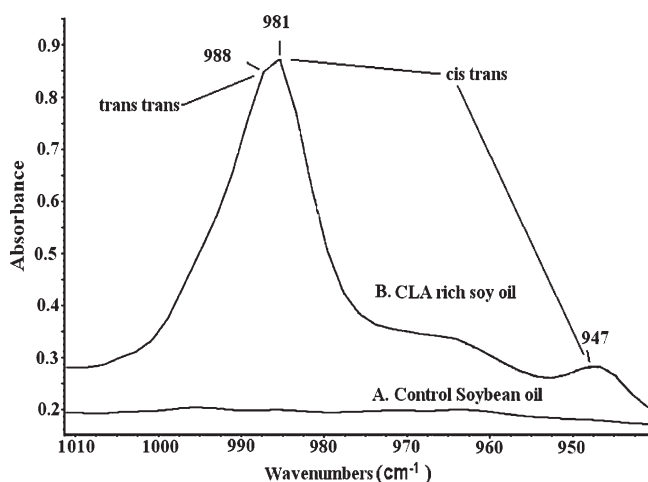


Figure 2. ATR-FTIR spectra of the 1010–900 cm^{-1} region of (A) control soybean oil and (B) photo-isomerized soybean oil containing 10% total CLA.

CLA, *trans*-9,*cis*-11 and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA are presented in **Table 1**. The level of total CLA in the oil samples ranged from 0.38 to 25.11% of the composition of the oil. *trans,trans* CLA isomers, total mono *trans* CLA isomers, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA were 0.22–18.23, 0.14–7.8, 0.057–1.76, 0–3.30, and 0.044–3.30%, respectively. The coefficient of determination (R^2) of the PLS regression models were used to signify the fit of the data. Results show that models provided accurate predictions, having R^2 values representing a good fit of the data. The R^2 value for all of the calibration models (R^2_c) was 0.99, and the R^2 value for the validation models (R^2_v) ranged from 0.97 to 0.99. RMSE is the measure of the accuracy of the model and is based on the size of the residuals in the prediction. The RMSE values for both the calibration and validation models are presented in **Table 1**. The models developed for all of the CLA isomers had RMSEC and RMSEV values ranging from 0.03 to 0.51 and from 0.07 to 1.14% CLA, respectively. All of the models

had high RPD values tabulated in **Table 1**, which are indicative of a good model.

Figures 3 and 4 show the weighted regression coefficients of the absorbance intensities at wave numbers in the mid infrared region from 4000 to 650 cm^{-1} of the PLS regression analysis of the various CLA isomers. The significant regression coefficients contributing to the models were identified by the Unscrambler 9.6 software. In the ranges of 3018–3010 and 988–941 cm^{-1} , the $-\text{C}-\text{H}$ stretch α to the double bond of the esterified unsaturated fatty acid and the CLA isomers showed positive regression coefficients, respectively. The long chain of the fatty acids showed a negative correlation in the range of 723–698 cm^{-1} .

Validation of the Calibration Set. A validation test set of 23 photo-isomerized oil samples was used to validate the previously developed models. The level of CLA in the test set of samples was previously determined using GC-FID analysis. The measured CLA levels (by GC-FID) were plotted against predicted CLA levels (using the developed models) by JMP 7.0.2 software to determine the linear correlation of the data based on the equation of the line, coefficient of determination (R^2_p), and the root-mean-square error of prediction (RMSEP). The statistics of these linear correlations are tabulated in **Table 2**. The predicted and measured CLA levels were highly correlated, with correlations of determination (R^2_p) ranging from 0.94 to 0.96 for total CLA, *trans,trans* CLA isomers, total mono *trans* isomers, *trans*-10,*cis*-12 CLA, *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and *cis*-9,*trans*-11 CLA, respectively. These results indicate the suitability of the models for prediction of CLA levels. The entire process of prediction including the ATR-FTIR scan of the oil sample takes about 10 min, which is rapid when compared to GC-FID.

Predictive models were also developed using the first and second derivative of the spectra of the oil samples. These models had an inferior predictive ability than those presented. Also, the weighted regression did not correlate with the CLA isomers specifically.

Bernuy et al. (23) recently developed chemometric models using correlations based on the FT-Raman spectrum of the oil sample and the Ag^+ -HPLC with UV detection at 233 nm.

Table 1. Model Statistics for the Prediction of CLA Isomers in Photo-isomerized CLA-Rich Soy Oil

parameters ^b	models ^a					
	total CLA	trans,trans CLA	total mono trans CLA	trans- 10,cis-12 CLA	trans-9,cis-11 CLA and cis-10,trans-12 CLA	cis-9,trans-11 CLA
Calculated						
min value	0.38	0.22	0.14	0.057	0	0.044
max value	25.11	18.23	7.86	1.76	3.30	3.30
R^2_c	0.99	0.99	0.99	0.99	0.99	0.99
RMSEC	0.51	0.32	0.15	0.03	0.08	0.03
SEC	0.51	0.32	0.15	0.03	0.08	0.03
bias	2.13×10^{-7}	1.43×10^{-7}	1.52×10^{-7}	2.55×10^{-7}	-2.64×10^{-8}	-1.98×10^{-8}
SD _c	6.58	4.73	1.88	0.46	0.88	0.56
Validated						
R^2_v	0.97	0.98	0.97	0.98	0.97	0.99
RMSEV	1.14	0.69	0.27	0.07	0.14	0.07
SEV	1.14	0.69	0.28	0.07	0.14	0.07
bias	0.09	0.05	0.03	0.006	0.02	0.07
RPD	12.9	14.7	12.5	15.3	11	18.7

^a Models comprising 73 samples. ^b R^2_c , coefficient of determination of calibration; RMSEC, root-mean-square error of calibration (% CLA); SEC, standard error of calibration; SD_c, standard deviation in the values of the calibration set data; R^2_v , coefficient of determination of validation; RMSEV, root-mean-square error of validation (% CLA); SEV, standard error of validation; RPD, relative ability of prediction determinant (=SD_c/SEC).

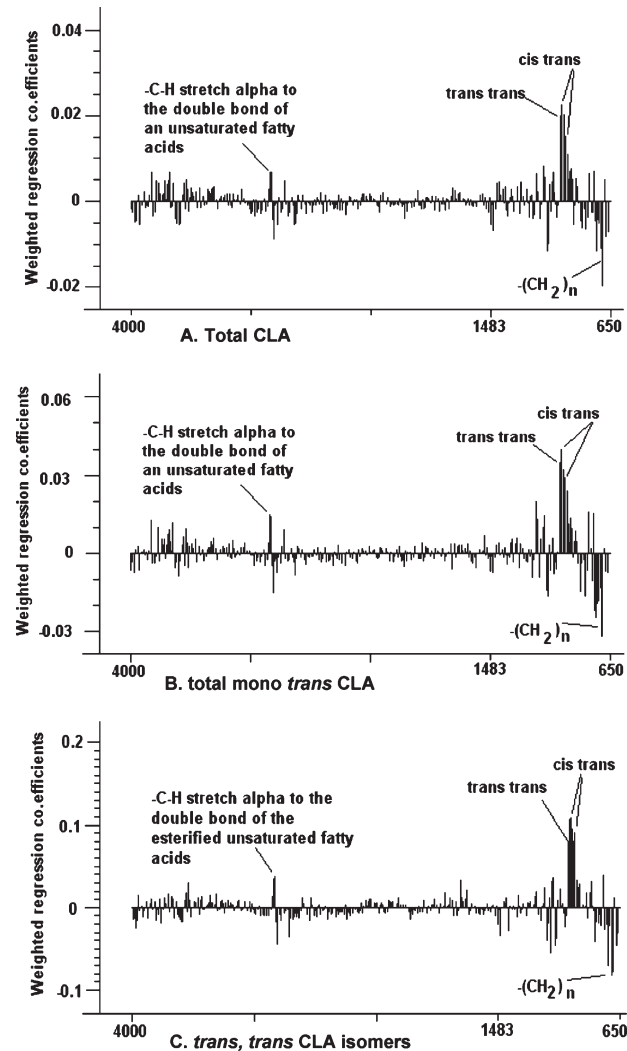


Figure 3. Weighted regression coefficients for the full cross-validated model for (A) total CLA, (B) total mono trans CLA, and (C) trans,trans CLA isomers in CLA-rich soy oil.

The model had a high degree of determination of 0.97 and a lower standard error of prediction of 0.19. The relative ability of

prediction (RPD) value for the model was not specified. The developed models were based on a limited number of samples ($n = 22$) and used a narrower range of CLA ranging from 0.05 to 3.25%, whereas the models developed in this paper used 73 samples with CLA levels ranging from 0.39 to 25.11%. Also, the developed models were limited to the measurement of total CLA levels present in the soy oil system and not for the prediction of the levels of individual CLA isomers, which are developed in the oil after the photo-isomerization process.

Christy et al. (10) developed predictive models using a correlation based on the double derivatization of FTIR spectra of the oil and the percentage of trans fat and CLA present in the oil based on the level of artificial fatty acids added to the oil system. Although these models predicted the levels of total trans and the total CLA present in the oil system with good precision, the breakdown of the trans and CLA content into their individual components was not possible using this method.

This study shows that ATR-FTIR spectra of oil identified the change in the level of conjugation in the oil after photo-isomerization of the oil. The chemometric models developed to measure CLA using FTIR also distinguished between the geometrical isomers of CLA. Using this technique, a rapid (10 min) method was developed suitable for evaluation of large oil samples with total CLA content ranging from 0.38 to 25.11% without any sample preparation. The FTIR-PLS method has potential as a rapid and reliable online technique to predict the level of total CLA and its individual isomers in the photo-isomerized soy oil.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; ATR-FTIR, attenuated total reflectance-Fourier transform infrared spectroscopy; GC, gas chromatography; FID, flame ionization detector; PLS, partial least-squares; RMSEC, root-mean-square error of calibration; RMSEV, root-mean-square error of validation; RMSEP, root-mean-square error of prediction; SEC, standard error of calibration; SEV, standard error of validation; SD_c, standard deviation in the values of the calibration set data; RP-HPLC, reversed-phase high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; AgNO₃-TLC, silver nitrate thin-layer chromatography.

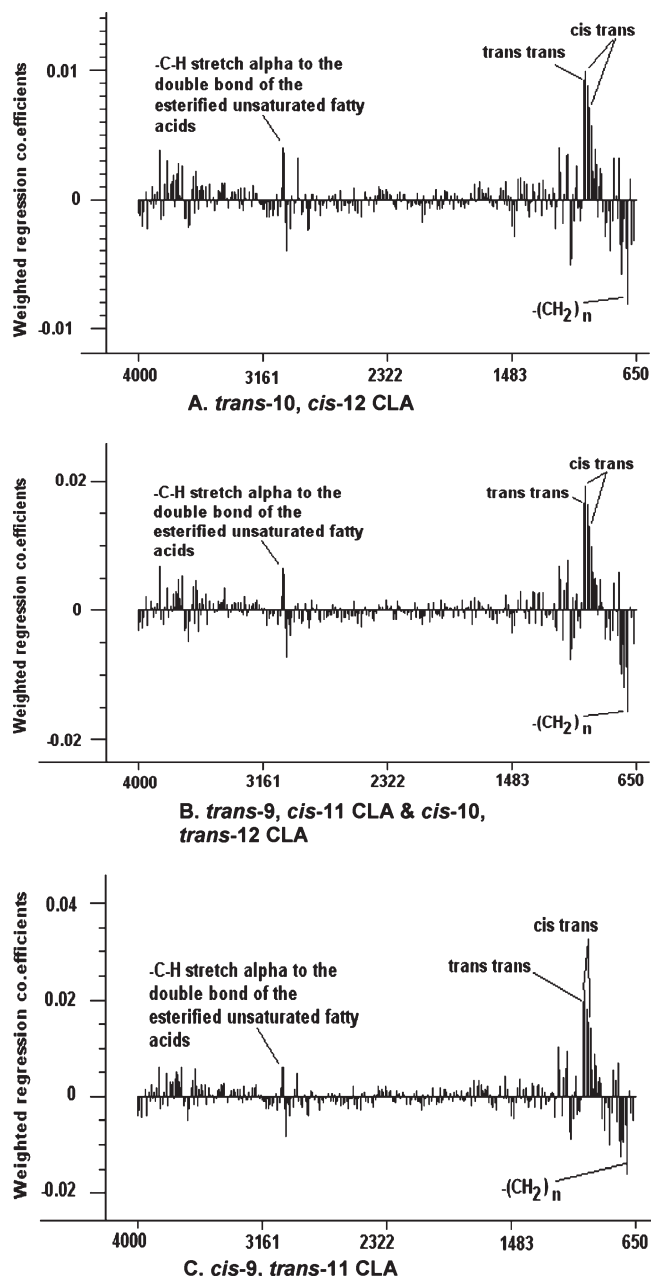


Figure 4. Weighted regression coefficients for the full cross-validated model for (A) *trans*-10,*cis*-12 CLA, (B) *trans*-9,*cis*-11 CLA and *cis*-10,*trans*-12 CLA, and (C) *cis*-9,*trans*-11 CLA in CLA-rich soy oil.

Table 2. Statistics of the Linear Plot of the Measured versus Predicted Values of the CLA Isomers Based on the Developed Models

models ^b	parameters ^a			
	<i>n</i>	<i>R</i> ² _p	line equation	RMSEP
total CLA	23	0.98	$y = 0.99x + 0.002$	0.76
<i>trans,trans</i> CLA	23	0.97	$y = 0.99x + 0.038$	0.61
total mono <i>trans</i> CLA	23	0.98	$y = 1.02x - 0.042$	0.21
<i>trans</i> -10, <i>cis</i> -12 CLA	23	0.98	$y = 1.01x - 0.008$	0.048
<i>trans</i> -9, <i>cis</i> -11 CLA and <i>cis</i> -10, <i>trans</i> -12 CLA	23	0.97	$y = 1.03x - 0.020$	0.121
<i>cis</i> -9, <i>trans</i> -11 CLA	23	0.98	$y = 1.01x - 0.004$	0.056

^a *n*, number of unknown samples not included in the calibration set; *R*²_p, coefficient of determination of prediction; line equation, equation for the linear fit of the data; RMSEP, root-mean-square error of prediction (% CLA). ^b Models presented in Table 1.

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