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ARTICLE

Determination by Fourier Transform Raman Spectroscopy of Conjugated Linoleic Acid in I_2-Photoisomerized Soybean Oil

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The potential of Fourier transform (FT)-Raman spectroscopy to quantify the total conjugated linoleic acid (CLA) content was evaluated to find a technique for the routine control of CLA synthesis by chemical procedures. The calibration and validation samples were obtained by photoisomerization of linoleic acid contained in soybean oil. The catalyst was iodine (I_2), and the light source was the green line (514.5 nm) of an argon ion laser. The criteria to select the best partial least-squares (PLS) calibration model were a low standard error of prediction (SEP), a high correlation coefficient (*R*), and the selection of relevant variables of the Raman spectrum to reduce spectral interferences. The total CLA content of the 22 samples ranged from 0.05 to 3.28% of total lipids. The best PLS calibration model was obtained with three optimal factors, a SEP of 0.22, and a *R* of 0.97. This calibration model was obtained after baseline correction of the CC stretching region (1642−1680 cm^{-1}), which contained sufficient spectral information for reliable CLA quantification.

KEYWORDS: FT-Raman spectroscopy; photoisomerized soybean oil; conjugated linoleic acid; multivariate calibration

INTRODUCTION

"Conjugated linoleic acids" (CLA) is a generic term for a group of conjugated isomers of linoleic acid (*cis*-9,*cis*-12−18:2). They are composed of positional (from positions 6,8−18:2 to 12,14−18:2) and geometric (trans−trans, cis−trans, trans−cis, and cis−cis) isomers (*1*). These fatty acids are naturally found in ruminant animals and in food products derived from them such as dairy products and meat. In cow's milk fat rumenic acid (*cis*-9,*trans*-11−18:2) is the main CLA isomer, and it accounts for nearly 75−90% of total CLA. There are also more than 20 minor CLA isomers, which are at least 1 order of magnitude smaller in quantity (*2*).

Some CLA isomers have elicited considerable interest because of their range of positive health effects such as antiobesity, antiatherogenic, anticarcinogenic, antidiabetic, and immune system enhancement (*3*). As a result, there is increasing interest in producing CLA as a food ingredient and health supplement because of the aforementioned benefits associated with the consumption of some CLA isomers. Whereas the CLA isomers of ruminant origin are enzymatically produced, resulting in the formation of a relatively specific CLA isomeric profile, commercial production is carried out by chemical synthesis, which produces different mixtures of isomers. The alkali isomerization of linoleic acid produces blends that are composed of two major CLA isomers and some minor amounts of cis−cis and trans−trans isomers (*4*). The CLA blend prepared by dehydration of ricinoleic acid contains numerous additional CLA isomers (*5*), and the CLA synthesized by I_2 photoisomerization of linoleic acid produces an even more complex CLA profile, including large amounts of trans−trans isomers (*6*).

An important parameter in the monitoring of the industrial CLA production is the total content of CLA. UV spectroscopy is a technique that could be used to control this parameter online. If there is no interfering absorbance at 233 nm, UV spectroscopy gives an excellent indication of the total conjugated double bonds (*7*). The limitation of the aforementioned technique is its scarce molecular information, which limits its application in the field of edible oil analysis. Raman spectroscopy is a very effective technique for the study of molecular vibrations, thus providing a high content of molecular information. Associated with multivariate analysis, Raman spectrometry is a powerful tool capable of extracting quantitative chemical information. In the field of edible oil analysis, Raman spectroscopy has been used in the structural analysis of edible oils (*8*), the classification of oil and fats (*9*), the determination of the cis/trans isomer composition (*10*), the determination of free fatty acid content (*11*), the determination of the oxidative stability (*12*), and the determination of the total unsaturation in oils and margarines (*13*). All of these parameters could be simultaneously determined in a few seconds. In addition, dispersive Raman spectrometers using a NIR excitation laser are readily adaptable to the control of industrial processes, because they are ideal for optical fiber transmission. Thus, the instrument may be readily and remotely coupled to a personal computer, allowing real-time reaction monitoring.

In a previous work (*14*), we reported for the first time the quantification of the *cis*-9,*trans*-11−18:2 isomer by FT-Raman

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spectroscopy in cow's milk fat. The development of a straightforward procedure was possible by the high concentration of this CLA isomer, which has an enzymatic origin. The presence of other isomers in this dairy blend did not strongly affect the analysis, because they are at least 1 order of magnitude smaller in quantity as compared to rumenic acid. The chemical synthesis of CLA, which produces complex blends of positional and geometrical isomers without a dominant isomer, requires new tools for the routine analysis of commercially produced CLA. The aim of the present work is to evaluate the potential of FT-Raman spectroscopy to quantify the total CLA synthesized by I_2 photoisomerization of linoleic acid in soybean oil.

MATERIALS AND METHODS

Samples. A total of 22 samples were synthesized by photoisomerization of linoleic acid contained in soybean oil. Resublimed iodine crystals (Merck, Whitehouse Station, NJ) were used as catalyst, and the light source was the green line of an argon ion laser Innova 99 (Coherent, Santa Clara, CA). The soybean oil (Sigma-Aldrich, St. Louis, MO) contained 54.37% of linoleic acid and 5.59% of linolenic acid, as measured by GC. A total of 200 mL of soybean oil was placed in a 500 mL beaker wrapped with aluminum foil and then heated at 60 °C to facilitate iodine dissolution. The catalyst was added at levels of 0.25 and 0.5% (w/w) with nitrogen flushing to avoid oxidation. Both solutions and a sample without iodine were placed in 220 mL capped tubes, purged with nitrogen, and then exposed to the green line (514.5 nm) of the argon laser. This line was selected because of the iodine maximum absorption at 520 nm (*15*). The monochromatic beam of 0.5 W was spread with a 25 mm plano-concave lens (Edmunds Optics, Barrington, NJ) and then collected and focused with a 50 mm plano-convex lens (Edmunds Optics). The 1 cm diameter beam crossed the tube at dark to avoid further reaction by visible light. Periodic samples of 3 mL were collected every 7 h for 70 h and then poured into plastic vials wrapped with aluminum foil. Before a sample was taken, the reaction mixture was carefully shaken to ensure homogeneity. The sample vials were purged with nitrogen and stored at −18 °C. The commercial standards of CLA isomers (*cis*-9,*trans*-11−18:2, *trans*-10,*cis*-12−18:2, *cis*-9,*cis*-11−18:2, and *trans*-9,*trans*-11−18:2) were acquired from Matreya (Pleasant Gap, PA). They were the only CLA isomers commercially available at the beginning of this study.

Raman Spectroscopy. The FT-Raman spectra were recorded by using a System 2000R spectrometer (Perkin-Elmer, Boston, MA). The samples were exposed to the 1064 nm (9394 cm^{-1}) line from an Nd:YAG laser source with a nominal power of 750 mW. An indium−gallium−arsenide (InGaAs) detector and 180°-backscattering geometry were used. All of the spectra were recorded with a resolution of 4 cm^{-1} and averaged over 100 scans to get a good signal-to-noise ratio. Data collection and data transfer were managed through Perkin-Elmer Spectrum software.

High-Performance Liquid Chromatography (HPLC). The quantification of CLA methyl esters in the samples was carried out using an Ag^+-HPLC (Gilson, Villiers le Bel, France) with UV detection at 233 nm. Three ChromSpher Lipids columns (250 mm × 4.6 mm i.d., stainless steel; 5 μm particle size; silver ion impregnated) were connected in series. They were purchased from Chrompack International (Middelburg, The Netherlands) and used as received. The carrier liquid was acetonitrile and hexane (0.1:99.9, v/v), the flow was 1.06 cm^3/min, and the oven temperature was 25.1 °C. Identification of the CLA peaks in the Ag^+-HPLC chromatogram was based on comparison with the peaks of the available CLA standards and the relative order of elution given by Sehat et al. (*16*). Under the aforementioned conditions, the relative order of elution of CLA isomers is maintained constant, and the geometric and positional CLA isomers from 8,10−18:2 to 11,13−18:2 are resolved. The methyl esterification of CLA was performed by treatment of a 500 mg sample with 10 mL of KOH (0.1 M) in methanol during 1 h at 70 °C. The addition of 4 mL of HCl (1.2 M) in methanol and further incubation during 15 min at the same temperature completed the methyl esterification. The extraction of FAME was done after the addition of 20 mL of hexane and 10 mL of demineralized water. A standard curve was plotted with the aim of calculating the concentration of the individual CLA isomers in the reaction mixture. The curve was constructed by serial dilution of the *cis*-9,*trans*-11−18:2 CLA methyl ester in hexane and was linear over the range studied (0.025−1%). The total CLA content resulted from the addition of all the individual CLA isomers that showed concentrations lower than 1%.

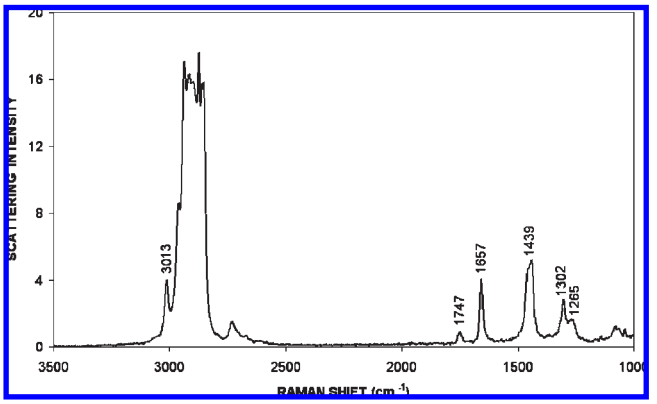

Figure 1. Raman spectrum of soybean oil.

Data Treatment. The software Unscrambler 9.6 (Camo, Oslo, Norway) was used for multivariate data analysis. Predictive equations were developed using partial least-squares (PLS) regression. All calculations were performed on the calibration set and then applied to the validation set, representing, respectively, two-thirds and one-third of the total samples. The total CLA content of the calibration set ranged from 0.05 to 3.28% with a mean value of 1.69% and a standard deviation of 1.23%. The validation set had a mean value of 2.02% and a standard deviation of 0.91%, and its total CLA content ranged from 0.63 to 2.91%. A sample could not appear in both sets, meaning that the sets were independent. Spectral preprocessing options were investigated in conjunction with the modeling to minimize the standard error of prediction (SEP). These options included first derivative, multiplicative scatter correction (MSC), and baseline correction. The criteria to select the best PLS calibration model were a low SEP, a high correlation coefficient (R), and the use of relevant variables of the FT-Raman spectrum to reduce spectral interferences.

RESULTS AND DISCUSSION

Chromatographic Analysis. The separation and quantification of the geometric and positional CLA isomers from the I_2-photoisomerized soybean oil were possible with the use of Ag^+-HPLC. The total CLA content of the 22 samples ranged from 0.05 to 3.28% with a mean value of 1.74% and a standard deviation of 1.15%. There was no production of CLA isomers in samples with 0% iodine, even when exposed to the laser for 70 h. The total CLA increased linearly with time in the 0.25 and 0.5% iodine samples during the irradiation.

Analysis of Raman Spectra. The standards of CLA isomers (*cis*-9,*trans*-11−18:2, *trans*-10,*cis*-12−18:2, *cis*-9,*cis*-11−18:2, and *trans*-9,*trans*-11−18:2) and the sample of soybean oil were analyzed by FT-Raman spectroscopy. **Figure 1** shows the spectrum of pure soybean oil. Around 3013 cm^{-1}, the scattering band of the C−H stretching vibration of ethylenic groups can be seen. The ester CO stretching vibration can be observed at 1747 cm^{-1}. The band at 1657 cm^{-1} can be assigned to the CC stretching vibration, wheras the C−H deformation vibration of the CH_2 groups can be seen at 1439 cm^{-1}. The band of the in-phase CH_2 twisting motion can be observed at 1302 cm^{-1} and the band of the in-plane C−H deformation from CC groups at 1265 cm^{-1} (*9*, *17*). The spectra of the four pure CLA isomers were analyzed to select relevant variables, thus reducing spectral interferences. The range between 1630 and 1680 cm^{-1} was selected due to its distinct and well-resolved bands. This region is dominated by the vibration (ν) mode of the CC stretching, which gives rise to sharp and narrow Raman bands. **Figure 2** displays the ν(C═C) bands arising from the four standards of CLA isomers and the pure soybean oil. The

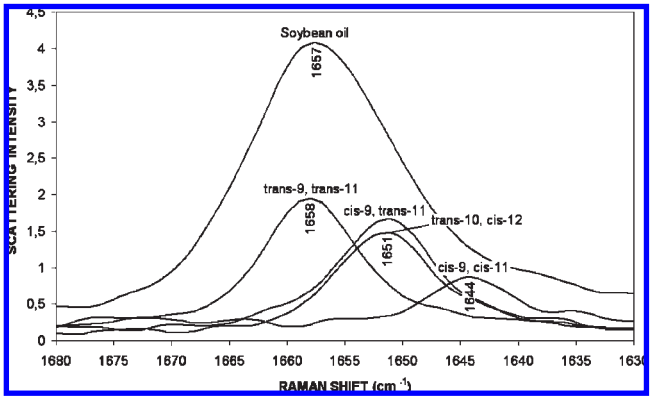

Figure 2. ν(C═C) bands of soybean oil and the four standards of CLA isomers.

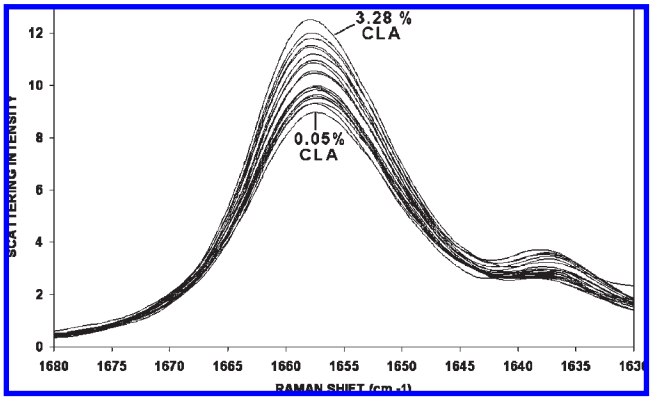

Figure 3. Evolution of the ν(C═C) band during the I_2 photoisomerization of soybean oil.

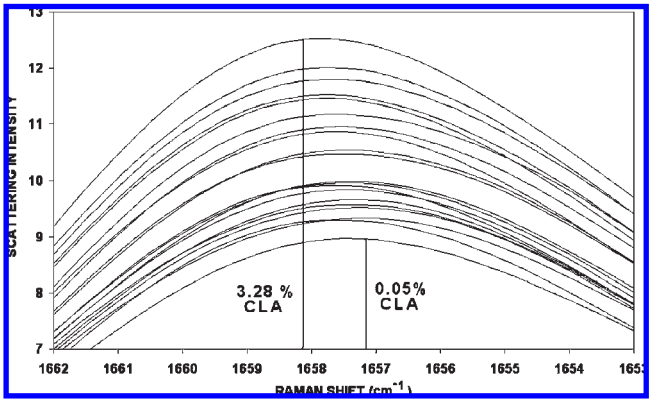

Figure 4. Shift of the ν(C═C) band during the I_2 photoisomerization of soybean oil.

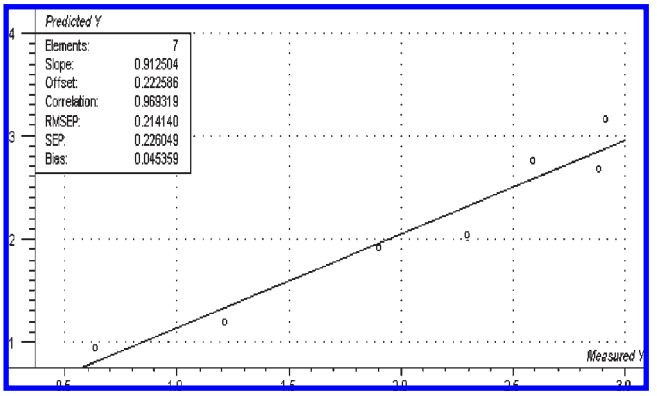

Figure 5. Validation PLS scatter plot of the calibration model obtained with the ν(C═C) band.

strongest ν(C═C) band at 1657 cm^{-1} arises for a sample of non-photoisomerized soybean oil, and it is associated with the stretching vibration of the cis CC groups. The conjugated double bonds of the geometric CLA isomers show distinctive ν(C═C) bands, except for the *cis*-9,*trans*-11−18:2 and *trans*-10, *cis*-12−18:2 isomers with a maximum at 1651 cm^{-1}. The *trans*-9, *trans*-11−18:2 isomer presents a single ν(C═C) band at 1658 cm^{-1}, and the *cis*-9,*cis*-11−18:2 isomer presents the corresponding band at 1644 cm^{-1}. To our knowledge, this is the first report of the ν(C═C) bands arising from cis−cis and trans−trans geometric isomers of CLA by Raman spectroscopy.

Figure 3 displays the evolution of the ν(C═C) band during the I_2 photoisomerization of soybean oil. We can observe that the strong ν(C═C) band of the unconjugated cis isomers from linoleic acid completely overlaps the ν(C═C) bands of the conjugated isomers. The first and second derivatives were calculated to better resolve this band, but no shoulders were revealed. However, the significant increase of the intensity in the ν(C═C) band during the I_2 photoisomerization can be correlated to the simultaneous formation of CLA isomers. The formation of some conjugated oxidation byproduct can be inferred from the scattering band around 1638 cm^{-1}. Indeed, Higuchi et al. (*18*) assigned the band around 1638−1640 cm^{-1} to the ν(C═C) vibration of the CC−CO groups. This band does not interfere with the ν(C═C) band of the I_2-photoisomerized soybean oil, and its intensity depends of the irradiation time. Under much more drastic conditions of oxidation (*19*, *20*), this band has been reported to be notoriously bigger. **Figure 4** shows a small shift to higher values for the ν(C═C) band during the photoisomerization. This could be explained by the fact that the trans−trans isomers, the most abundant in the mixture, have their maximum intensity at higher values than the ν(C═C) band of unconjugated cis isomers.

Multivariate Analysis. A total of 22 samples were used for the multivariate analysis, 11 after treatment with 0.25% iodine and 11 after treatment with 0.5% iodine. PLS models were constructed by using two regions of the FT-Raman spectrum. The partial Raman spectrum with 2401 (3500−1100 cm^{-1}) *X* variables and the ν(C═C) band with 39 (1642−1680 cm^{-1}) *X* variables were correlated with the total CLA concentration, which was used as *Y* variable. One of the 22 samples was removed after being detected as an extreme outlier in the calibration set during the development of PLS models. The best PLS prediction result of the partial spectrum was obtained for the data without pretreatment. The pretreatments did not help to reduce the model errors. This model was constructed with five factors and produced a SEP of 0.19 and an *R* of 0.97. The rise of the mean value of the reference samples explains the increase of the SEP with respect to a previous publication from our group (*14*). The most precise PLS model constructed with the ν(C═C) band gave validation statistics comparable with the best PLS model of the partial spectrum. This model was obtained after baseline correction with a SEP of 0.22 and a *R* of 0.97. It is the simplest model because the number of optimal factors is 3. These three factors capture 98.88% of the cumulative variance for the *X* block (spectral variability) of the model. The first factor alone is responsible for 90.07% of the spectral variability, indicating that it is the most important factor in the model. The other factors are less relevant, but they are needed to improve the results. The scatter plot of the validation set in **Figure 5** shows a close correlation between the Ag^+-HPLC values and the values predicted by this model. Despite the fact

that the precision of the best calibration model with the partial spectrum is somewhat better than that obtained with the ν(C═C) band, we selected the last one as the most appropriate for the total CLA determination because of the advantages of the use of a reduced region.

Conclusion. The overall results demonstrate that the information contained in the Raman spectrum of I_2-photoisomerized soybean oil can be used for the determination of total CLA. The selected PLS calibration model was developed using the ν(C═C) region (1642−1680 cm^{-1}), which contains sufficient spectral information for reliable measurement of total CLA without the interference of other compounds. The conjunction of this result and the aforementioned capacity of carrying out fast and simultaneously the determination of several other parameters, underscore the strength of Raman spectroscopy in the analysis of edible oils. Further studies should be directed to compare the sensitivity and reproducibility of dispersive Raman and FT-Raman spectrometers. If the performances of the two types of spectrometers are equivalent in this determination, the dispersive instruments should be advantageous for the online process control of CLA manufacture because they are cheaper, faster, and more robust than the FT instruments.

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