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Determination of the Conjugated Linoleic Acids in Cow's Milk Fat by Fourier Transform Raman Spectroscopy

MARC MEURENS,*,† VINCENT BAETEN,‡ SHOU HE YAN,† ERIC MIGNOLET,† AND YVAN LARONDELLE†

Unité de Biochimie de la Nutrition, Faculté d'Ingénierie biologique, agronomique et environnementale, Université catholique de Louvain (UCL), Croix du Sud 2 (bte 8), 1348 Louvain-la-Neuve, Belgium, and Département Qualité des Productions agricoles, Centre wallon de Recherches agronomiques (CRA-W), Chaussée de Namur 24, 5030 Gembloux, Belgium

The collective term "conjugated linoleic acid" or "CLA" generally refers to a mixture of conjugated positional and geometric isomers of linoleic (*cis*-9,*cis*-12-octodecadienoic) acid. In nature, these isomers are mainly formed in the rumen by biohydrogenation of polyunsaturated fatty acids. This study concerns a first trial of CLA determination in cow's milk fat by Raman spectroscopy. The spectra of pure *cis*-9-oleic, *cis*-9,*cis*-12-linoleic, *cis*-9,*trans*-11-linoleic, and *trans*-10,*cis*-12-linoleic acids have been examined in comparison with the spectra of selected milk-fat samples containing between 0 and 3% of CLA. The trial of CLA determination by Raman spectroscopy on cow milk fat has reached its objective with the two following results. First, the examination of the Raman spectra allows to identify three specific Raman signals of the chemical bonds associated to the cis,trans conjugated C=C in the rumenic and *trans*-10,*cis*-12-octodecadienoic acids at 1652, 1438, and 3006 cm^{-1}. Second, the calibration of Raman spectrometer for the CLA determination has indicated that these three specific signals suit very well for the accurate and reliable measurement of CLA concentration in milk fat. To our knowledge, the present study is the first successful attempt to determine the CLA content of milk fat by a spectrophotometric method.

KEYWORDS: Raman spectroscopy; cow's milk fat; *cis,trans*-unsaturated fatty acids; conjugated linoleic acid; rumenic acid

INTRODUCTION

Recently, the interest in the analysis of conjugated linoleic acids (CLA) has considerably increased because of the numerous potentially positive effects of different CLA isomers on human health. The collective term CLA generally refers to a mixture of conjugated positional and geometric isomers of linoleic (*cis*-9,*cis*-12-octadecadienoic) acid formed in the rumen by biohydrogenation of polyunsaturated fatty acids (*1*).

The *cis*-9,*trans*-11-linoleic acid isomer referred to as rumenic acid is generally considered as the major biologically active form among the CLA isomers found in milk fat. Moreover, milk fat from ruminant animals represents the major dietary source of CLA (*2*) In this way, the increase of CLA concentration in dairy products has become an important objective in animal nutrition research. In several countries, farmers and dairy companies are already in the process of commercializing dairy products that are naturally enriched in CLA through the use of specific rations to feed the lactating animals. The development of such activities calls for appropriate analytical methods. The determination of milk fatty acid profile, and in turn of its CLA content, is traditionally made by fat extraction followed by gas chromatography (GC) analysis. This analytical technique gives good results but allows the analysis of only 10−15 samples per day. To find a technique that is less tedious and time consuming, it has been decided to test the potential of fast spectrophotometric techniques. The present paper concerns a first trial of CLA determination in milk fat by Raman spectroscopy.

In the literature, several papers have discussed the potential of spectrophotometric techniques to study polyunsaturated fatty acids. Already in 1939, Moore observed the ultraviolet absorption at 230 nm of the conjugated double bonds from the polyunsaturated fatty acids. Later, in 1952, Jackson et al. found that the trans,trans conjugated isomers of linoleic acid are characterized by a strong infrared absorption band at 988 cm^{-1}, whereas the cis,trans and trans,cis conjugated isomers can be distinguished using the absorbances at 948 and 982 cm^{-1}. No characteristic absorption band was found for the cis,cis conjugated isomers. On the basis of that information, Smith et al. (*3*) found that the C18−C20 polyunsaturated fatty acid fraction of milk fat has strong infrared absorption bands at 948 and 982 cm^{-1}. Conjugated trans,trans dienes were later detected in a

* To whom correspondence may be addressed. E-mail: meurens@bnut.ucl.ac.be.
† Université Catholique de Louvain.
‡ Centre Wallon de Recherches Agronomiques.

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concentrated unsaturated ester fraction of milk fat with the use of ultraviolet and infrared spectroscopy (*4*) By use of differential infrared spectroscopy, Bartlet and Chapman (*5*) found that conjugated and isolated trans unsaturations were present in a constant ratio in milk fat. They used this characteristic as a basis for determining adulteration in milk fat. Currently, standardized spectroscopic methods for determining the trans isomers content of fats are routinely used (*6*, *7*). The standardized determination of trans unsaturated fatty acids by infrared spectroscopy is based on the fact that the isolated trans isomers give rise to a weak absorption around 969 cm^{-1} due to the CH out-of-plane deformation band. Isolated trans fatty acids and conjugated linoleic acids in edible oils and fats have been for the first time simultaneously determined by Christy et al. (*8*) using infrared spectroscopy and chemometrics.

Raman spectroscopy has been also investigated in the analysis of the polyunsaturated fatty acids. In contrast to the ultraviolet and infrared spectroscopy based on light absorption, the Raman spectroscopy is a measurement of a light-scattering phenomenon. When a laser illuminates matter, elastic collisions between photons and molecules of sample result in Rayleigh radiations scattered at the incident frequency, whereas concurrent inelastic collisions, resulting from vibrational transitions of chemical bonds in compounds, produce a small fraction of Stokes and anti-Stokes radiations at different frequencies. The Stokes radiations occur at lower frequencies than the Rayleigh radiations, whereas the anti-Stokes occur at higher frequencies. The abscissa of the Raman spectrum is scaled in wavenumber shift between the Stokes radiations, which are here named Raman scattering bands, and the Rayleigh radiations considered as the reference radiations at 0 cm^{-1}. There is a direct proportionality between the intensity of the Raman scattering bands and the concentration of the chemical bonds whose the vibrations are concerned.

By use of Raman spectroscopy already thirty years ago, Bailey and Horvat[9] determined the cis,trans isomer content of edible vegetable oils (0−23%) by measuring the intensities of C═C stretching fundamentals near 1656 and 1670 cm^{-1} that are associated, respectively, with cis and trans configurations of unsaturated fatty acids. The use of Raman spectroscopy has also been proved to be successful in the determination of total unsaturation of fatty acids in oils and margarines (*10*, *11*). Ozaki et al. (*12*) have used the Raman bands near 1656 and 1670 cm^{-1} to estimate the level of cis and trans unsaturation in a wide range of fat-containing foodstuffs. However, to our knowledge, no publication exists about the analysis of conjugated fatty acids by Raman spectroscopy.

MATERIAL AND METHODS

Samples. Different fatty acid standards have been obtained from different companies and stored at −18 °C before analysis: the *cis*-9-oleic acid, the *cis*-9,*cis*-12-linoleic acid, and the mixture (named CLA standard) of rumenic (*cis*-9,*trans*-11-linoleic) and *trans*-10,*cis*-12-linoleic acids (ref. O5507) were supplied by Sigma (St Louis, MO); the rumenic acid (ref 1245) and the *cis*-10,*trans*-12-linoleic acid (ref 1249) were supplied by Matreya (Pleasant Gap, PA). The undecanoic acid methyl-ester (ref 623110) used as internal standard in GC was supplied by Alltech (Deerfield, IL).

Fifty anhydrous milk-fat samples were selected from a sample bank including more than 300 samples. They were chosen to cover a concentration range between 0.02 and 2.68% CLA with a mean value of 1.11% and a standard deviation of 0.59%. The material included in the sample bank was produced in the frame of different experiments of cows' feeding aiming at changing the fatty acid profile of dairy products (*13*). The milk-fat samples were stored in optimum conditions

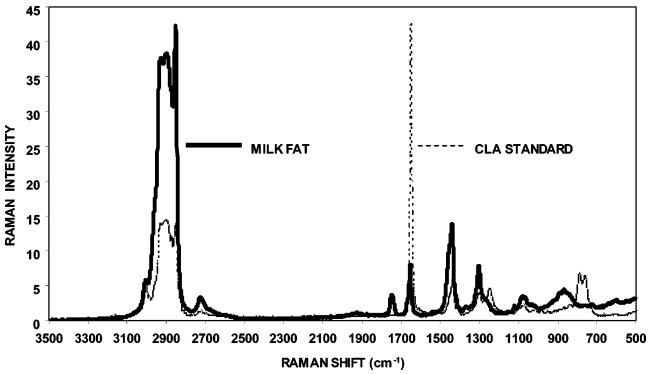

Figure 1. Raman spectra of milk fat and CLA standard.

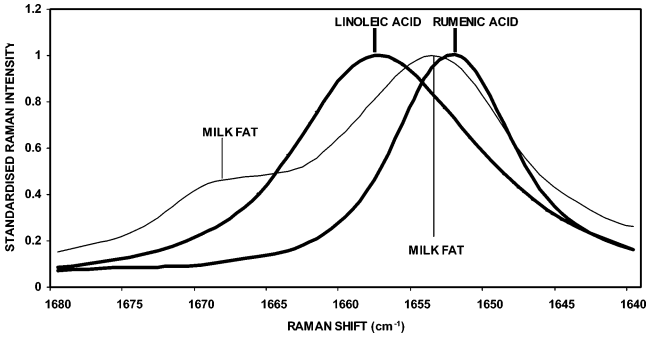

Figure 2. Raman spectra of milk fat (mean spectrum) and linoleic and rumenic acids.

for a long-term preservation. Indeed, they were put in tainted glass vials, which were then filled with nitrogen and stored at −18 °C. All the milk-fat samples were obtained from the skimming of milk using a cream separator Elecrem (Vanves, France) type 125. The separated cream was churned with a household appliance Kenwood (model Chef KM300, Tokyo, Japan). The butter obtained was then melted at the temperature of 45 °C in a water bath before centrifugation at 350 g for 10 min (Gerber Instruments, Drachten, The Netherlands). The remaining traces of water present in the oily phase were removed by treatment with natrium sulfate.

GC. The fatty acids profile of the milk-fat samples was determined by gas chromatography according to an adaptation of the European Commission guideline (*14*) for the analysis of dairy fat. In the adapted protocol, the methyl-esterification of the fatty acids, either in the free form or included in triglycerides, has been performed by treatment of 500 mg of each sample with 10 mL of KOH (0.1 M) in methanol during 1 h at 70 °C, followed by addition of 4 mL of HCl (1.2 M) in methanol and further incubation during 15 min at the same temperature. The extraction of the fatty acid methylesters (FAME) was done after addition of 20 mL of hexane and 10 mL of demineralized water and addition of undecanoic acid methyl-ester as internal standard. The chromatograph used was a Carlo Erba (Milano, Italy) GC 6000 Vega Series 2 with a SGE (Austin, TX) BPX70 column. The content of each fatty acid is expressed as percentage of the total fatty acids detected.

Raman Spectroscopy. Fourier-transform Raman (FT-Raman) spectra were acquired on a Perkin-Elmer (Boston, MA) NIR-FT-Raman spectrophotometer 2000R equipped with a Nd:YAG laser source emitting at 1064 nm (9394 cm^{-1}). The 180° backscattering refractive geometry and an InGaAs detector have been used. The spectrometer was managed through the Spectrum for Windows software of Perkin-Elmer. The spectral data were obtained with a resolution of 4 cm^{-1} and a nominal laser power of 750 mW. For each spectrum, 50 scans were co-added and averaged in order to get a good signal-to-noise ratio. The different fatty acid standards were diluted to 10% (weight/weight) with carbon tetrachloride (in order to have sufficient volumes of expensive products) and were introduced in NMR tube Series 500 from Sigma-Aldrich (Bornem, Belgium) before Raman analysis. Their spectra are presented in **Figures 1 and 2** after subtraction of the solvent spectrum. The anhydrous milk-fat samples were melted in a water bath

at the temperature of 45 °C and introduced using Pasteur pipets in classical test tubes having an internal diameter of 10 mm. All the Raman analyses were performed using a thermostabilized sample holder designed to maintain the sample at a constant temperature of 45 °C.

Data Treatment. Single linear regression was carried by Excel 7.0 (Microsoft Corporation, Richmond, VA) between the CLA concentrations and the spectral data of the 50 milk-fat samples Stepwise multiple linear regression (MLR) and cross-validated partial least-squares (PLS) calibrations were carried by NSAS 3.30 (NIR Systems, Silver Spring, MD) and Unscrambler 7.6 (Camo, Oslo, Norway). The MLR and PLS models were established on a calibration set of 36 samples randomly chosen among the 50 milk-fat samples. Afterwrds, the two calibration models were tested in prediction on a validation set constituted by the 14 remaining milk-fat samples.

RESULTS AND DISCUSSION

Raman Spectroscopy. The Raman spectrum of a milk-fat sample containing 2.68% of CLA is displayed in Figure 1. The spectrum presents well-resolved bands with various scattering intensities. Part of the scattering bands are assigned to the stretching vibration of the C−H bonds of the ethylenic groups near 3005 cm^{-1}, of the methylenic groups near 2925 and 2855 cm^{-1}, and of the methylic groups near 2960 and 2895 cm^{-1}. Scattering bands are also assigned to the stretching vibration of the C═O bonds near 1750 cm^{-1}, to the stretching vibration of the C═C bonds near 1650 cm^{-1}, to the bending vibration of the C−H bonds of methylenic groups near 1440 and 1305 cm^{-1}, and to the bending vibration of the C−H bonds of ethylenic groups near 1270 cm^{-1}. The last part of the spectrum includes scattering bands that are usually attributed to the vibrations of the C−C and the C−O bonds. Figure 1 also includes the spectrum of a CLA standard (mixture of rumenic acid and *trans*-10,*cis*-12-CLA) in order to compare its specific spectral features with the milk-fat spectrum. An intense scattering band, which seems to be specific for the conjugated linoleic acids, is observed around 1650 cm^{-1}. According to Bailey and Horvat (*13*), in this part of the spectra, the C═C stretching bands are normally associated near 1670 cm^{-1} with the trans isomers and near 1656 cm^{-1} with the cis isomers. The spectra of milk fat, rumenic acid, and linoleic acid, normalized to have a maximum scattering intensity in the 1640−1680 cm^{-1} range equal to 1, are displayed in **Figure 2**. The milk-fat spectrum presents two partly overlapping scattering bands, whereas the linoleic and rumenic acids present only one scattering band in this region. The two scattering bands of the milk-fat spectrum correspond to the trans isomers for the scattering band located near 1670 cm^{-1} and to the cis isomers for the scattering band located near 1654 cm^{-1}. At 1658 cm^{-1}, the unique scattering band of the linoleic acid spectrum corresponds to the cis isomers of the two unsaturated bonds (C_9═C_{10} and C_{12}═C_{13}) of this fatty acid. Near 1652 cm^{-1}, the unique scattering band of rumenic acid does not correspond to the scattering bands observed either for cis isomers or for trans isomers of unsaturated fatty acids. Such a unique scattering band at 1652 cm^{-1} has also been observed on the spectrum of the *trans*-10,*cis*-12-linoleic acid. The fact that the two bands at 1658 and 1670 cm^{-1} corresponding, respectively, to the cis and trans configurations of isolated double bonds are replaced by a unique band when the double bonds are conjugated was already reported by Schrader (*15*), but the wavenumber observed for the conjugated double bonds was 1640 instead of 1652 cm^{-1}. The reason for this difference of wavenumber may be due to the fact that the conjugated diene studied by Schrader is isoprene (2-methyl-butadiene) with a trans,trans configuration. **Figure 3** presents the 1660−1648 cm^{-1} spectra of all the milk-fat samples analyzed. The maximum intensity of these spectra is

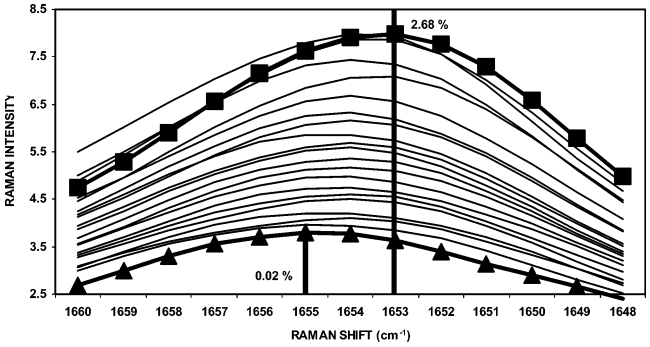

Figure 3. Raman scattering bands of the milk-fat samples observed near 1654 cm^{-1}.

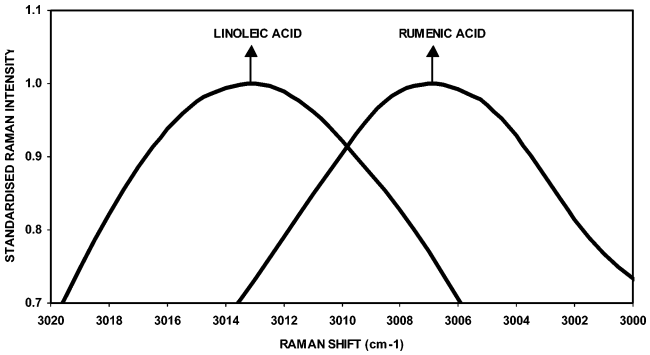

Figure 4. Spectral profiles of rumenic and linoleic acids near 3000 cm^{-1}.

shifted from 1655 to 1653 cm^{-1} when the CLA content increases from 0.02 to 2.68%. Such a shift corresponds to the fact that the cis,trans conjugated band covers more and more the right part of the isolated cis band. The discovery of the strong cis,-trans CLA band at 1652 cm^{-1} and the observation of the band shift from 1655 to 1653 cm^{-1} in function of the CLA concentration of milk fat have suggested to study the correlation between the scattering intensities in this region of the Raman spectrum and the CLA concentration. The linear regression between the percentage of CLA determined by GC and the Raman scattering intensities gave high correlation coefficients ($R > 0.7$) in the zone 1670−1640 cm^{-1}, with the highest value ($R = 0.906$) exactly at 1652 cm^{-1}. With the exception of the *trans*-11-octodecenoic acid ($R \approx 0.7$), the other unsaturated fatty acids of the analyzed milk-fat samples did not present such high correlation coefficients in this wavenumber zone. Two other interesting observations were done in comparison at other wavenumbers the spectral profile of rumenic acid with the spectral profiles of linoleic and oleic acids, the main cis-unsaturated fatty acids of milk fat. In Figure 3, the compared profiles around 1440 cm^{-1} present a peak of rumenic acid (1438 cm^{-1}) well separated from a peak of oleic acid (1441 cm^{-1}). In **Figure 4**, the compared profiles around 3010 cm^{-1} present a peak of rumenic acid (3006 cm^{-1}) well separated from a peak of linoleic acid (3014 cm^{-1}). So, the comparaison of the spectral profiles of rumenic acid with oleic and linoleic acid and and milk fat reveals specific Raman signals of the cis,trans CLA at 3 different wavenumbers (1652, 3006, and 1438 cm^{-1}).

Spectrometer Calibration. The calibration of the FT-Raman spectrometer was tested with two different chemometric methods: the MLR and the PLS regression. The used MLR algorithm (NSAS 3.30) operated a stepwise selection of the Raman data that gives the best multiple correlation with the CLA concentration of 34 calibration samples randomly chosen among the 50 milk-fat samples. As we can foresee it from the comparison of spectral profiles in **Figures 1−3**, 1652 cm^{-1} was selected as

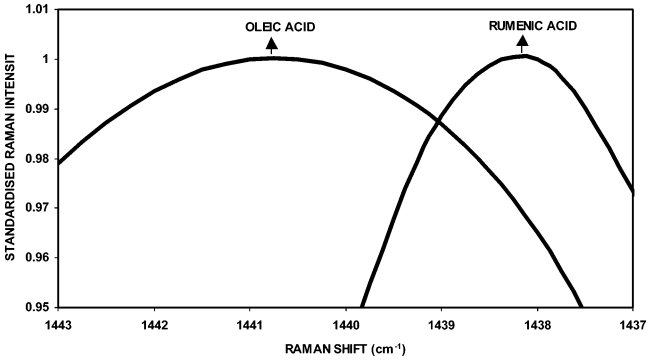

Figure 5. Spectral profiles of rumenic and oleic acids near 1440 cm^{-1}.

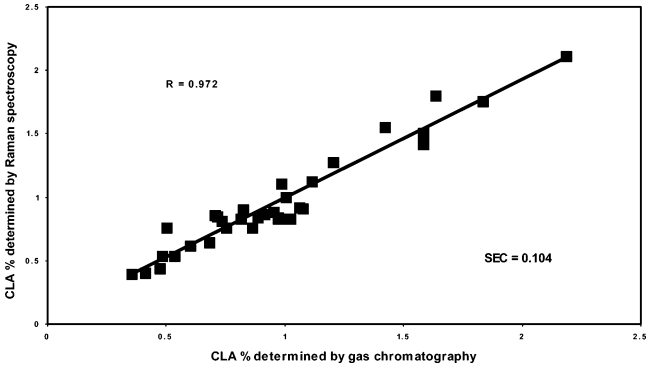

Figure 6. MLR calibration graph.

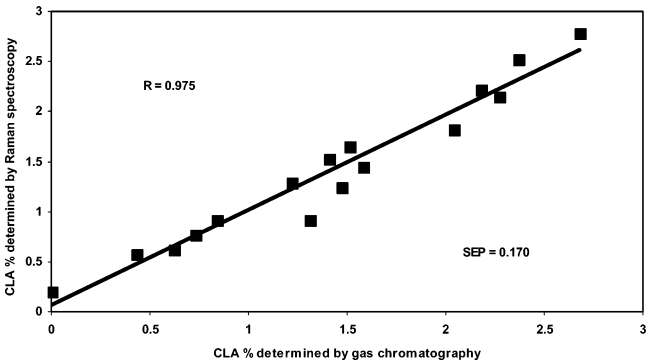

Figure 7. MLR validation graph.

first Raman data because it is the wavenumber where the correlation is the highest with the coefficient $R = 0.906$. The second selected wavenumber was 3004 cm^{-1} not very far from where a C−H band of rumenic acid peaks distinctly from the homologous band (3014 cm^{-1}) of linoleic acid, as we can see it in **Figure 4**. The third wavenumber was selected at 1438 cm^{-1}, exactly where another vibration C−H band of rumenic acid peaks distinctly from the homologous band (1441 cm^{-1}) of linoleic acid, as we can see it in **Figure 5**. So the three spectral terms of the MLR equation were selected at wavenumbers where there are specific signals of cis,trans conjugated dienes. We did not add more than three terms in the regression equation in order to avoid the overfitting problem when the number (*34*) of calibration samples is so limited and when we cannot apply the cross-validation control. The MLR calibration graph is displayed in **Figure 6** with a correlation coefficient $R = 0.972$ and a standard error of calibration (SEC) = 0.104 while the MLR validation graph is displayed in **Figure 7** with a correlation coefficient $R = 0.975$ and a standard error of prediction (SEP) = 0.170. The used PLS algorithm (Unscrambler 7.6) operated a four-segment cross validation to select four factors for a model presenting a correlation coefficient $R = 0.954$ and SEC = 0.126 in calibration and $R = 0.951$ and SEP = 0.170 in validation. The comparison of prediction performances of the two models indicate a significant advantage of accuracy for the MLR model using only three spectral data, whereas the PLS model uses four factors condensing all the spectral data and is funded on a cross-validation control.

Conclusion. The presented trial of CLA determination by Raman spectroscopy on milk fat has reached its objective with the two following results. First, the examination of the Raman spectra allows to identify three specific Raman signals of the chemical bonds associated to the cis,trans conjugated C═C in the rumenic and *trans*-10,*cis*-12-octadecadienoic acids at 1652, 1438, and 3006 cm^{-1}. Second, the calibration of Raman spectrometer for the CLA determination has indicated that these three specific signals suit very well for the accurate and reliable measurement of CLA concentration in milk fat. It is now interesting to compare the performances of Raman spectroscopy with those of ultraviolet and infrared spectroscopy in the analysis of cow milk CLA.

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