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Performance Comparison of UV and FT-Raman Spectroscopy in the Determination of Conjugated Linoleic Acids in Cow Milk Fat

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The determination of conjugated linoleic acids (CLA) in cow milk fat was studied by using UV (210–250 nm) and Fourier transform (FT)-Raman (900−3400 cm^{-1}) spectroscopy in order to determine the best spectrophotometric technique for routine analysis of milk fat. A collection of 57 milk fat samples was randomly divided into two sets, a calibration set and a validation set, representing two-thirds and one-third of the samples, respectively. All calculations were performed on the calibration set and then applied to the validation set. The CLA content ranged from 0.56 to 4.70%. A comparison of various spectral pretreatments and different multivariate calibration techniques, such as partial least-squares (PLS) and multiple linear regression (MLR), was done. This paper shows that UV spectroscopy is as reliable as FT-Raman spectroscopy to monitor CLA in cow milk fat. The best calibration for FT-Raman was given by a PLS model of seven factors with a standard error of prediction (SEP) of 0.246. For UV spectroscopy, PLS models were also better than MLR models. The most robust PLS model was constructed with only one factor and with SEP = 0.288.

KEYWORDS: Raman spectroscopy; cow milk fat; UV spectroscopy; conjugated linoleic acid; multivariate calibration

INTRODUCTION

In recent years, conjugated linoleic acids (CLA) have attracted a strong interest in food science and medicine because some of the isomers are believed to have important physiological functionality as demonstrated in various experimental designs with mice, rats, or pigs. CLA refers generically to a class of geometric and positional conjugated dienoic isomers of linoleic acid (*1*). Isomers of CLA have been reported to have a wide range of beneficial effects, such as antiobesity, antiatherogenic, antidiabetic, and anticarcinogenic properties (*2*). The rumenic acid (*cis*-9,*trans*-11-octadecenoic acid), which is quantitatively the most important CLA isomer in milk fat, is known to have a powerful biological activity (*3*). Milk, cheese, and meat from ruminants contain more CLA than foods of nonruminant origin (*4, 5*). Feed management in ruminants has been shown to have a major impact on the CLA amount as well as on the profile of CLA isomers found in dairy products and other animal fats (*6, 7*). Feeding strategies can thus be used to improve the fatty acid profile in milk in order to better suit human dietary concerns. The development of such activities calls for appropriate analytical methods. The most widely used method to quantify conjugated linoleic acids in milk fat is the measurement by gas chromatography (GC) of fatty acid methyl esters (FAME) (*8*). This approach is expensive and time-consuming. In the case that numerous analyses have to be performed on a routine basis, cheaper and fast techniques are clearly needed. It is thus of utmost importance to test the capabilities of fast techniques, like spectroscopy, which are less tedious and time-consuming.

UV spectroscopy is reported as the first spectrophotometric method used to analyze conjugated linoleic acids. Development in electronics and optics over recent years has enabled the rapid spread of compact, relatively low-cost, and powerful UV spectroscopic analysers. Khanal and Dhiman (*9*) have given a detailed account of the history of conjugated double-bonds analysis by UV spectroscopy. They refer to Booth who, in 1935, noted that the fatty acids of milk fat showed a greatly increased absorption in the UV region at 230 nm when cows were turned out to pasture after winter, establishing for the first time the presence of conjugated fatty acids in milk. They also allude to some developments that have occurred in the following decade, especially by Moore in 1939, who concluded that the absorption at 230 nm is the result of two conjugated double bonds, and by Hilditch in 1941 and Jasperson in 1945, who suggested that conjugated unsaturation occurs with polyunsaturated fatty acids with 18 carbon chains.

In 1970, conjugated dienes formed by oxidation in liposomes were evaluated by measurement of the increase in absorbance in the UV range (*10*). In 1986, Lezerovich (*11*) showed that the differential of the first-derivative spectrum, a second derivative, gives a more sensitive and accurate estimate of the conjugated diene content of fatty acids. The improved resolution

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showed narrower bands with minima rather than maxima values. Czauderna and Kowalczyk (*12*) found that there are subtle differences in the absorption maxima for the geometric isomers. They reported a maximum of 231.9 nm for the trans−trans isomer, 234.3 nm for the cis−trans (or trans−cis) isomer, and 235.4 nm for the cis−cis isomer.

Vibrational spectroscopy comprises two complementary spectroscopic methods, namely IR and Raman spectroscopy. IR spectroscopy has already been used to determine CLA concentration. In 1952, Jackson (*13*) found that trans-trans conjugated isomers of linoleic acid are characterized by a strong IR absorption band at 988 cm^{-1}, whereas the cis-trans and trans-cis conjugated isomers can be distinguished by using the absorbance at 948 and 982 cm^{-1}. No characteristic absorption band was found for the cis-cis conjugated isomers. Isolated trans fatty acids and CLA in edible oils and fats were simultaneously determined by Christy et al. (*14*) by using Fourier transform (FT)-IR spectroscopy. Our group recently reported the development of a FT-Raman method as a tool for quantification of conjugated linoleic acids in cow milk fat (*15*). To our knowledge, no other attempt has been made to use the capabilities of this technique for CLA quantification purposes.

The aim of the present study is to compare the performance of FT-Raman spectroscopy with that of UV spectroscopy in the analysis of cow milk CLA in order to determine the best spectrophotometric technique for the routine analysis of milk fat.

MATERIAL AND METHODS

Samples. A total of 57 anhydrous milk fat samples were selected from a sample bank including more than 300 samples. They covered a concentration range between 0.56 and 4.70% of CLA with a mean value of 1.63% and a standard deviation of 0.91%. The material included in the sample bank was produced in the frame of different cow feeding experiments aiming at changing the fatty acid profile of dairy products (*16, 17*). The milk fat samples were stored in optimum conditions for a long-term preservation. Indeed, they were put in tinted glass vials, which were then filled with nitrogen and stored at −18 °C. Milk fat samples were obtained, as described by Meurens et al. (*15*), from skimming of milk by using a Elecrem cream separator (Vanves, France) type 125. The separated cream was churned with a Kenwood household appliance (model Chef KM300, Tokyo, Japan). The butter obtained was then melted at a temperature of 45 °C in a water bath before being centrifuged at 350*g* for 10 min (Gerber Instruments, Drachten, The Netherlands). Remaining traces of water present in the oily phase were removed by treatment with natrium sulfate.

UV Spectroscopy. The samples were diluted with a ratio of 1:10000 in HPLC grade hexane (Sigma-Aldrich, St. Louis, MO) and placed into 1.00 cm quartz cells. Absorbance of these solutions was measured between 210 and 250 nm against pure hexane as blank. A D-2000 deuterium lamp (Ocean Optics, Dunedin, FL), which produces a stable output from 190 to 400 nm, was used as light source. The transmitted light was transported by a 400 μm diameter quartz fiber (Avantes, Eerbeck, The Netherlands) and collected by a PC2000 plug-in spectrometer (Ocean Optics) that contained a L2 lens, a 600-lines grating, and a high sensitivity 2048-element linear charge-coupled-device-array detector. Operation of the spectrometer and collection of spectra were performed by using the OOIBase32 spectrometer operating software (Ocean Optics).

Raman Spectroscopy. Spectra were registered by using the Perkin-Elmer System 2000R spectrometer (Boston, MA) equipped with an Nd:YAG laser source emitting at 1064 nm (9394 cm^{-1}). An indium−gallium−arsenide (InGaAs) detector and 180°-backscattering geometry were used. The spectrometer was managed through the Spectrum 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 coadded and averaged in order to get a good signal-to-noise ratio. The anhydrous milk fat samples were melted in a water bath at a temperature of 45 °C and placed into nuclear magnetic resonance tubes having an internal diameter of 10 mm. All Raman analyses were performed by using a thermostabilized sample holder designed to maintain the sample at a constant temperature of 45 °C.

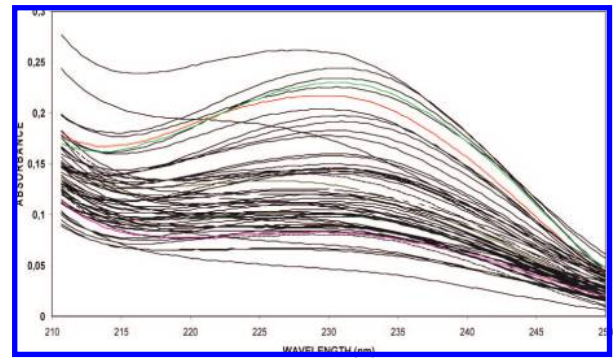

Figure 1. UV spectra of cow milk fat samples between wavelengths 210 and 250 nm.

Gas Chromatography. The fatty acids profile of milk fat samples was determined by GC according to an adaptation of the European Commission guidelines (*18*) for the analysis of dairy fat. In the adapted protocol, the methyl esterification of fatty acids, either in the free form or included in triglycerides, is performed by treatment of a 500 mg 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 FAME is done after addition of 20 mL of hexane, 10 mL of demineralized water, and undecanoic acid methyl−ester as internal standard (ref 623110), which was supplied by Alltech (Deerfield, IL). The chromatograph used was a Carlo Erba (Milano, Italy) GC 6000 Vega Series 2 instrument with a SGE (Austin, TX) BPX70 column. The content of each fatty acid is expressed as a percentage of the total fatty acids identified.

Data Treatment. Multivariate analysis was used for quantitative analysis. Stepwise multiple linear regression (MLR) and partial least-squares (PLS) calibrations were carried out with the softwares NSAS 3.30 (NIR Systems, Silver Spring, MD) and Unscrambler 9.6 (Camo, Oslo, Norway). The samples were randomly divided into two sets. All calculations were performed on the calibration set and then applied to the validation set, representing two-thirds and one-third, respectively, of the total samples. A sample could not appear in both sets, meaning that spectral selection was designed blockwise. Spectral preprocessing options were investigated in conjunction with the modeling to minimize the standard error of prediction. Options included multiplicative scatter correction (MSC), which improves the linearity of the relation between constituents and spectral values, and first derivative, which enhances spectral information and reduces baseline drift. Goodness of fit of the prediction models was evaluated according to the following criteria: robust models with low standard error of prediction (SEP) and high correlation coefficient (*R*).

RESULTS AND DISCUSSION

Analysis of Spectra. Figure 1 displays UV spectra of anhydrous milk fat samples. Conjugated dienes exhibit a distinct UV maximal absorbance in the region of 230–235 nm, whereas isolated double bonds continue absorbing until 215 nm. As it can be seen, the absorption bands of both compounds overlap, hindering the resolution of the mixture by conventional spectrophotometry. The usual method for resolving mixtures, which has been applied to the present case, is the multivariate regression.

Figure 2 shows a typical FT-Raman spectrum of anhydrous milk fat sample. This spectrum clearly reveals some characteristic Raman scattering bands such as the C−H stretching vibration of the bonds of ethylenic groups at 3005 cm^{-1}, the

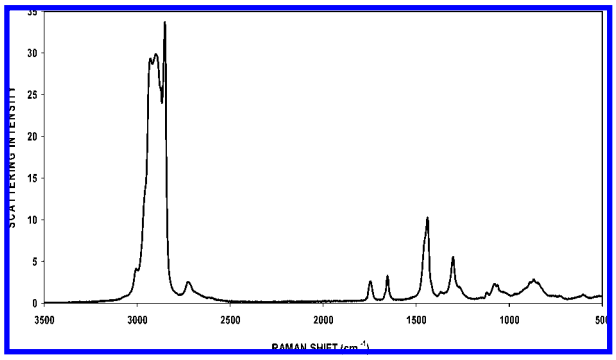

Figure 2. Raman spectrum of cow milk fat.

Table 1. Validation Statistics of PLS Calibration Models for UV Spectroscopy

calibration model	SEP[a]	R^b
without pretreatment 7 PC[c]	0.214	0.933
without pretreatment 1 PC[c]	0.288	0.881
model using first derivative	0.308	0.890
model using second derivative	0.372	0.787

[a] SEP: standard error of prediction. [b] *R*: correlation coefficient. [c] PC: partial components.

C−H asymmetric stretching at 2925 cm^{-1}, the C−H symmetric stretching at 2855 cm^{-1}, the C═O stretching in an ester at 1743 cm^{-1}, the C═C stretching around 1660 cm^{-1}, the CH_2 scissoring deformation at 1439 cm^{-1}, the in-phase methylene twisting motion at 1304 cm^{-1}, and the in-plane C−H deformation in an unconjugated cis-double bond at 1270 cm^{-1}.

UV Analysis. Calibration models, constructed with PLS regression, consisted of 40 (210–250 nm) *X* variables, and the CLA concentration was used as the *Y* variable. The undesirable systematic variations in the data, such as baseline drift and random noise, were considered. To reduce this kind of problems, we tried several mathematical tools such as MSC, first-order derivative, and second-order derivative. Statistical indicators of the calibration performances are shown in **Table 1**. A seven-factor model was selected by the software Unscrambler as the most precise. It was obtained by analyzing UV spectra without pretreatment with SEP = 0.214 and R = 0.933. The seven factors accounted for 100% of the spectral variability. Each factor explains a certain portion of the overall spectral information in the model, and this information is given as a percentage of the explained variance for the spectral part of the model. *X*-loadings of the first and second factors are displayed in **Figure 3**. The first factor alone is responsible for 96% of the spectral variability, indicating that it is the most important factor in the model. The other factors are less relevant to improve the model. The *X*-loading of the first factor shows a peak with maximal values between 231 and 234 nm, which is exactly where conjugated dienes exhibit a characteristic absorbance band as described in **Figure 1**. This is in accordance with the specific signal of conjugated diene isomers in this region. By taking into account the importance of the first factor, a one-factor model was constructed to obtain a more robust model with SEP = 0.288 and R = 0.881. **Figure 4** shows a close correlation between the values predicted by the model and the GC values in the validation set. PLS calibration modeling for UV spectroscopy is an appropriate method for the CLA determination in cow milk fat.

A MLR approach was also tested by combining the three wavelengths (231.9, 234.3, and 235.4 nm) reported by Czauderna and Kowalczyk (*12*) and 5 wavelengths (217.9, 234.9,

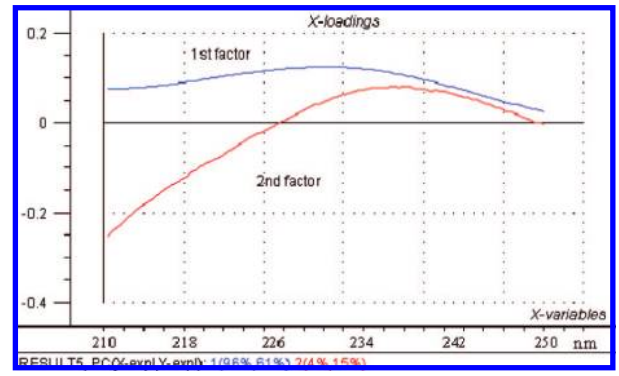

Figure 3. PLS loading vectors of the best CLA calibration model for UV spectroscopy.

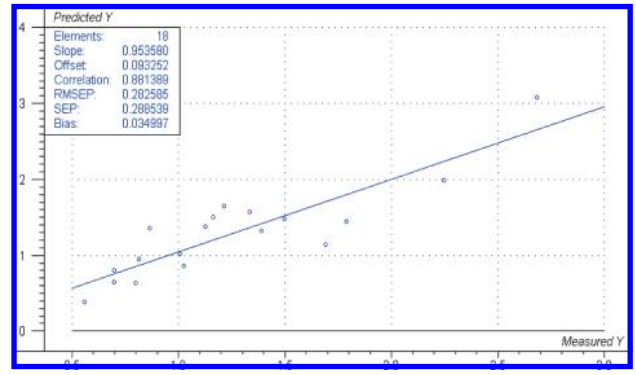

Figure 4. Validation PLS scatter plot of the most robust CLA calibration model for UV spectroscopy.

Table 2. Validation Statistics of MLR Calibration Models for UV Spectroscopy

calibration model	SEP[a]	R^b
without pretreatment	0.232	0.912
model using first derivative	0.304	0.872
model using second derivative	0.628	0.562

[a] SEP: standard error of prediction. [b] *R*: correlation coefficient.

237.8, 239.9, and 240.32 nm) automatically selected by stepwise MLR with the NSAS software. The models were tested with the same spectral pretreatment options as the corresponding PLS models. The results revealed that the most precise MLR model, a three-wavelength one, gave validation statistics comparable to those obtained with the most precise PLS model. The first and the second wavelengths (234.3 and 235.4 nm) were those proposed by Czauderna and Kowalczyk, and the third wavelength (239.96 nm) was the one of the stepwise selection. The selected wavelengths are higher than 234 nm, whereas the first PLS factor, in the PLS analysis, locates information between 231 and 234 nm (**Figure 3**). We should consider other PLS factors, such as the second one which locates most information above 234 nm, to understand the modeling. **Table 2** shows the best MLR validation statistics. They were chosen by considering the least-squares criterion and the highest multiple correlation. The SEP is 0.232 and R is 0.912 for the spectra without any pretreatment. The different pretreatments did not help to reduce the model errors; in fact, with MSC, they became worse. The prediction performance suggests that the best MLR calibration model developed with three wavelengths is appropriate, but its SEP of 0.232 is not better than the SEP of 0.214 of the seven-factor PLS calibration model. Nevertheless, the difference of

Table 3. Validation Statistics of PLS Calibration Models for Raman Spectroscopy

calibration model	SEP[a]	R^b
without pretreatment 6 PC[c]	0.246	0.936
model using first derivative	0.252	0.941
model using second derivative	0.267	0.913

[a] SEP: standard error of prediction. [b] *R*: correlation coefficient. [c] PC: partial components.

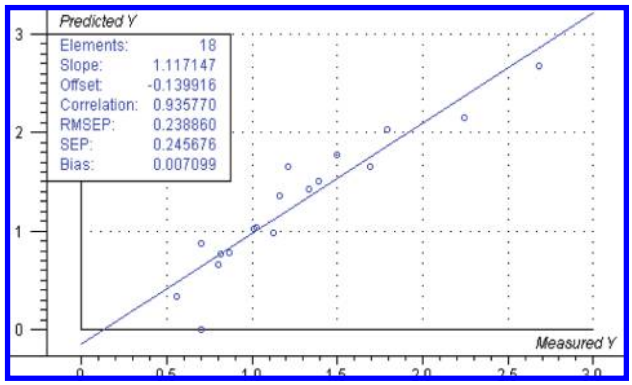

Figure 5. Validation PLS scatter plot of the best CLA calibration model for Raman spectroscopy.

Table 4. Validation Statistics of MLR Calibration Models for Raman Spectroscopy

calibration model	SEP[a]	R^b
model without pretreatment	0.381	0.865
model using MSC	0.377	0.855
model using second derivative	0.392	0.870

[a] SEP: standard error of prediction. [b] *R*: correlation coefficient.

precision is not significant, even with the one-factor PLS model, so this last one is maybe preferable for UV spectroscopy because of its robustness.

Raman Analysis. The PLS models were constructed with 2400 (3500 − 1100 cm^{-1}) *X* variables. When comparing the different spectral pretreatments, it can be observed that they do not significantly improve the PLS calibration models. The PLS validation statistics with the original, first-derivative, and second-derivative spectra as input are summarized in **Table 3**. The best prediction results are obtained for the data without pretreatment. The SEP value is 0.246, *R* is 0.936, and the number of optimal factors is six. These six factors describe 99% of the spectral variability of the model. The first factor alone describes 73% of the spectral variability, indicating that it is the most relevant; the remaining factors are needed to improve the results, meaning that there are interactions and other interfering phenomena, which are modeled by these additional factors. **Figure 5** shows the plot of the predicted data versus the GC data for the 18 milk fat samples of the validation set. The best SEP for Raman spectroscopy increased from 0.170, in our previous publication (*15*), to 0.246, in this work. This can be explained, in part, by the widening of the calibration set range.

To construct MLR models from Raman data, we applied a stepwise selection approach to determine the wave numbers that give the best multiple correlation with CLA concentration. As explained in our previous publication (*15*), the first wave number selection is 1652 cm^{-1} because it is the wave number where the correlation is the highest; the second wave number is 3004 cm^{-1}, and the third wave number is 1438 cm^{-1}. **Table 4** summarizes the overall prediction results of the models. As we can see, normalized spectra slightly improved the results. This model produced a SEP of 0.377 and an *R* of 0.855, suggesting that the calibration developed is appropriate but not better than PLS models for Raman data.

The precision of the best FT-Raman calibration model (SEP = 0.214) is somewhat better than the precision of the most robust PLS calibration model (SEP = 0.288) for UV spectroscopy. Working with specific geometric conjugated isomers could improve the Raman calibration by selecting relevant variables of the Raman spectrum and reducing spectral interferences.

CONCLUSION

This study confirms the high potential of UV and FT-Raman spectroscopy combined with multivariate calibration in the quantitative analysis of CLA in cow milk fat. By analyzing statistics of PLS and MLR correlation models, it is established that UV spectroscopy coupled with PLS regression analysis is as reliable as FT-Raman spectroscopy to monitor CLA in cow milk fat. UV spectroscopy has some advantages over FT-Raman spectroscopy because, in addition to the low cost of the supporting equipment, the specificity and sensitivity of the absorption band around 234 nm make it possible to work with very simple and robust PLS and MLR models.

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