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Evaluation of FT-NIR and ATR-FTIR Spectroscopy Techniques for Determination of Minor Odd- and Branched-Chain Saturated and *trans* Unsaturated Milk Fatty Acids

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Supporting Information

ABSTRACT: Determination of nutritionally important *trans* MUFA, CLA, and OBCFA milk fatty acids (often present in amounts lower than 1.0 g/100 g of total fat) using fast and nondestructive analytical methods would enhance their use as diagnostic tools in dairy herd and human health management. Here, PLS regression using ATR/FTIR spectra indicated potential for determination of *trans*-11 C18:1 and *trans*-12 C18:1 ($R_{cv}^2 \geq 0.80$), and *trans*-9 C18:1 in very minor concentration ($R_{cv}^2 > 0.82$), as well as *anteiso* C15:0 ($R_{cv}^2 = 0.57$) and *iso* C17:0 ($R_{cv}^2 = 0.61$). Furthermore, the main *cis*-9,*trans*-11 CLA isomer was predicted well despite the high *trans* MUFA concentration. Differentiation between the CLA and the *trans* MUFA signals was evident (based on specific *cis*/*trans* bands), and branched-chain saturated fatty acid methyl esters revealed specific *iso* and *anteiso* ATR/FTIR absorbance bands. None of the minor FA PLS results with FT-NIR showed interesting potential, except satisfactory predictions for *trans*-9 C18:1 and *cis*-9,*trans*-11 CLA. Overall, ATR/FTIR resulted in better calibrations and provided more specific information for determination of minor milk fatty acids.

KEYWORDS: *ATR, FTIR, FT-NIR, milk fat, trans fat, CLA, OBCFA, spectroscopy*

INTRODUCTION

In addition to the already established nutritional concerns for *trans* monounsaturated fatty acids (*trans* MUFA) and the beneficial biological effects of conjugated linoleic acids (CLA) for human health,[1] these two groups of milk fatty acids together with another group of specific odd- and branched-chain saturated fatty acids (OBCFA) have emerged as important nutritional biomarkers for dairy herds.[2,3] The microbial origin of OBCFA and their direct relationship with the duodenal flow of microbial biomass, as well as the potential of specific OBCFA isomers to monitor the nutrients produced during digestive processes, would allow their utilization as excellent diagnostic tools of rumen function.[4−6] Furthermore, the monitoring potential of *trans* MUFA, CLA, and OBCFA for early diagnosis of digestive disorders in cows, such as diagnosis of subacute ruminal acidosis (SARA), has recently been identified as isomer specific.[3] For example, a sudden increase in *trans*-10 C18:1, which is part of the *trans*-10,*cis*-12 C18:2 biohydrogenation pathway,[7] has been associated with low ruminal pH; thus, both fatty acids might be indicative of SARA. In addition, the *cis*-9,*trans*-11 C18:2 isomer was recently discovered to be an effective SARA predictor.[3] Moreover, drops in the *iso* C13:0 to *iso* C16:0 concentrations in milk fat were also indicative of SARA induction. The latter is thought to be caused by an OBCFA productivity shrinkage (production of total OBCFA) from ruminal cellulolytic microbiota and/or lower competitiveness of the cellulolytic bacteria relative to the rest of the microbial community (decrease in the cellulolytic/total bacteria ratio).

Thus, the ability to follow the variations of these interesting isomers in milk fat would be a valuable noninvasive strategy for detection of altered ruminal environment. Still, implementation of the latter requires reliable and effective analytical techniques, which can simultaneously quantify and differentiate minor milk fatty acids.

It would not be trivial to mention that gas chromatography (GC) and high-performance liquid chromatography are the reference methods, as they are still the gold standard and the most widely used analytical techniques for determination of almost any lipid species. However, the effectiveness for disease prevention using this strategy based on quantification of minor fatty acids would be drastically enhanced by the availability of fast, cheap, and nondestructive analytical methods, such as spectrophotometry techniques. Developing spectroscopy-based methods for the simultaneous determination of several different

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types of minor fatty acids with different chemical structures would be of major importance for animal as well as human nutrition.

Fourier transform near-infrared (FT-NIR) and attenuated total reflectance infrared (ATR/FTIR) are well-established spectroscopy techniques, both of which are widely used in the food industry.[8] ATR/FTIR is famous as the only spectroscopy analytical technique to be approved as an official AOCS (Cd 14d-99) method for quantifying total *trans* fats,[9,10] due to the unique property to detect the absorption of the C—H deformation vibration from a *trans* C═C bond in the vicinity of 966 cm^{-1}.[11] The sensitivity of the ATR/FTIR technique allows accurate determination of the total monounsaturated *trans* content in various naturally occurring nonruminant fats, vegetable and hydrogenated oils, and fats (e.g., canola, margarine and lard), but it has not been extensively tested with more complex fat matrices, such as dairy fat.

FT-NIR is the more widely used technique in various niches of the food industry, due to its property of being a strong absorption signal from many types of molecules and the ability for transmission of the NIR radiation by optical fibers over long distances. However, the former feature has also brought FT-NIR the legacy of low specificity and inability to differentiate between individual components in various homogeneous matrices, due to combinations and overlaps of broad bands from fundamental and overtone chemical vibrations (generally overlapping vibrational bands that are nonspecific and non-resolved).[12,13] Nevertheless, FT-NIR was previously reported as a viable technique for determination of the complete fatty acid profile in soybean, olive, and flaxseed oils, as well as in different mixtures of shortening and lard.[14] The latter has permitted FT-NIR to be used for rapid screening/monitoring of fat products and might allow *trans* FA determinations for regulatory labeling purposes.[14] The use of FT-NIR in bovine milk fatty acid analyses has not been investigated.

Milk fat is a complex matrix and, in addition to the many different types of fatty acids, correlations between biohydrogenation intermediates are inevitable. Thus, having a technique with differentiation ability is of great importance. Here we evaluated both FT-NIR and ATR/FTIR spectroscopy techniques for the quantification of individual and grouped *trans* MUFA, CLA, and OBCFA in a data set of 75 milk samples subselected from a collection of more than 1000 milk samples to cover the full biological concentration span of these milk fatty acids.

MATERIALS AND METHODS

Sample Selection. The sample storage and sample selection methodology were previously described.[15] Briefly, a total of 100 milk samples were selected from a sample bank (n = 1033) of six different cow feeding experiments aiming at changing the fatty acid (FA) profile of dairy products. The sample subset was selected using a genetic algorithm applied to cover the naturally occurring range of several milk fatty acids of interest (different odd- and branched-chain saturated fatty acids and different *trans* C18:1 and *cis*/*trans* C18:2 unsaturated isomers) in high, mid, and low concentration ranges.[16] The milk fat was extracted using a previously described methodology involving dichloromethane/ethanol.[17]

Fatty Acid Standards. Two *trans* monounsaturated *trans*-9 and *trans*-10 C18:1 fatty acid methyl ester (ME) standards, three branched-chain saturated *iso* C14:0, *iso* C15:0, and *anteiso* C15:0 ME, one odd-chain saturated C15:0 ME, and two triacylglyceride (TAG) standards (trielaidin and tristearin) were purchased from Larodan (Larodan Fine Chemicals, Malmo, Sweden) and Nu-Check (Nu-Check Prep, Inc., Elysian, MN, USA).

GC Reference Data. Quantification of *trans* fatty acids (TFA) and fatty acid groups using spectral data requires precise gas—liquid chromatography (GC) reference data for the construction of mathematical models. Identification and quantification of TFA through GC have been greatly improved with new highly polar, long capillary columns, but direct GC without prior fractionation could show overlapping between different *trans-n* and *cis-n* C18:1 positional isomers and *trans-n* C16:1 coelution with specific branched chain saturated, and *cis-n* C16:1 monounsaturated FAs, which might result in an underestimation of the total TFA content.[15,18] Here, we used the temperature dependency of the polarity of cyanopropyl phases[18] to mathematically deduce concentrations of overlapping fatty acids using two different temperature programs without prior fractionation. A similar approach was described before.[15,19−21] After extraction, all samples (sufficient quantity for 92 of the 100 selected milk fat samples) were methylated,[17] and the fatty acid methyl esters (FAME) were analyzed by GC according to the method of Vlaeminck et al.[2] (first temperature program) and by an isothermal (T = 180 °C) (second) temperature program. A different separation with the second temperature program allowed the quantification of most individual FAs[15,20] (Table 1). Due to an influence from the cows' diet, the total *trans* monounsaturated fatty acids (*trans* MUFA) were present in very high concentrations and ranged from 1.5 to 21 g/100 g in the milk samples.[23] Total conjugated linoleic fatty acids (CLA) maximally represented 2.0 g/100 g of milk fat (Table 1). Furthermore, insufficient GC resolution prevented reliable separation of individual *trans*-6 C18:1, *trans*-7 C18:1, and *trans*-8 C18:1 FA isomers, but allowed accurate determination of the *trans*-6 + 7 + 8 C18:1 sum. The cross-correlation results between the individual fatty acids and the major fatty acid groups indicated a high correlation (R^2 > 0.95) between *trans*-10 and *trans*-11 C18:1 and *trans* MUFA and between *cis*-9, *trans*-11 C18:2 and CLA as well as between C15:0 and OBCFA and C15:0 and ODD.

Vibrational Spectroscopy Analysis. *ATR/FTIR Spectroscopy.* All attenuated total reflectance Fourier transform mid-infrared (ATR/FTIR) spectra were acquired on a Vertex 70 − RAM II Bruker spectrometer (Bruker Analytical, Madison, WI, USA) operating with a Golden Gate diamond ATR accessory (Specac Ltd., Slough, UK). The internal reflection element was a small, non-temperature-controlled Type IIa diamond prism allowing a sampled diameter of approximately 2.0 mm. The optically dense medium was in contact with two ZnSe focusing lenses, one used to focus the incident infrared radiation and the second one to collect the reflected infrared radiation. The optical bench included an interferometer with a RockSolid configuration, KBr substrate beam splitter, and a deuterated triglycerin sulfate (RT-DLaTGS) detector. The OPUS 6.5 software for Windows of Bruker Instruments was used for instrument management, spectra acquisition, and OPUS JCAMP to JCAMP-DX file transformation. All milk fat samples were stored in vials selected by CRA-W in a previous Fourier transform Raman analysis.[22] Prior to ATR/FTIR analysis, the milk fat vials were taken out of storage, and the milk fat was kept in a melted state at a minimum of 30 min prior to spectra acquisition in a 38 ± 1 °C water bath, accompanied by gentle shaking by hand to ensure homogenization. One drop of each milk fat sample in liquid form was placed on top of the optical medium, and spectra were immediately acquired [room temperature (~24 ± 2 °C)], as well as selected FA standards (~0.1 g/sample) were analyzed after freezing at −80 °C. The spectra were collected against air as a background over the wavenumber range of 4498−500 cm^{-1} with a resolution of 4 cm^{-1}. For each spectrum, 64 scans were co-added and averaged to obtain a good signal-to-noise ratio. A total of 2074 data points were recorded from 500 to 4498 cm^{-1}. Because of very low milk fat quantity in 14 and extraction solvent contamination in 3 of all 92 selected samples,[15] ATR/FTIR spectra were available for 75 milk fat samples. For each milk fat sample, ATR/FTIR spectra were acquired in duplicate by taking a new milk fat drop, and the average of the two spectra was used for chemometrical analysis.

Table 1. Concentration Statistics of Individual and Grouped *trans* Monounsaturated (*trans* MUFA), Conjugated Linoleic (CLA) and Odd- and Branched-Chain Saturated (OBCFA) Fatty Acids in Milk Fat (g/100 g of FAME, n = 75)

fatty acid	min	max	av	SD
trans-4 C18:1	0.006	0.074	0.020	0.010
trans-5 C18:1	0.006	0.108	0.018	0.015
trans-6 + 7 + 8 C18:1	0.105	1.079	0.254	0.157
trans-9 C18:1	0.113	1.155	0.232	0.181
trans-10 C18:1	0.113	11.74	0.945	2.320
trans-11 C18:1	0.170	4.925	0.95	0.908
trans-12 C18:1	0.125	1.424	0.316	0.220
trans-15 C18:1	0.023	0.515	0.152	0.103
trans MUFA[a]	1.475	21.01	4.538	4.168
cis-9,*trans*-11 C18:2	0.005	1.958	0.437	0.405
trans-10,*cis*-12 C18:2	<0.001	0.038	0.013	0.008
CLA[b]	0.056	2.041	0.472	0.446
C15:0	0.462	2.590	1.440	0.345
C17:0	0.301	0.777	0.434	0.101
odd[c]	1.110	4.430	1.840	0.548
iso C13:0	0.012	0.048	0.029	0.008
iso C14:0	0.033	0.115	0.068	0.019
iso C15:0	0.116	0.283	0.187	0.027
iso C17:0	0.155	0.467	0.265	0.054
iso[d]	0.572	1.100	0.766	0.100
anteiso C13:0	0.005	0.025	0.012	0.004
anteiso C15:0	0.167	0.533	0.406	0.070
anteiso C17:0	0.228	0.520	0.357	0.063
ante[e]	0.533	1.050	0.775	0.115
branched[d,e]	1.230	2.030	1.540	0.181
OBCFA[c,d,e]	2.341	5.940	3.380	0.597

[a]Individual *trans* FA reported as well as all *trans* C14:1 and all *trans* C16:1 are included in the sum of trans monounsaturated fatty acids (*trans* MUFA). [b]Individual FA reported as well as *trans*-9,*cis*-11 C18:2 and *trans*-11,*cis*-13 + *cis*-9,*cis*-11 C18:2 are included in the sum of conjugated linoleic acids (CLA). [c]Individual odd FA reported as well as C5:0, C7:0, C9:0, C11:0, C13:0, C19:0, C21:0, and C23:0 are included in the sum of odd-chain fatty acids (ODD). [d]Individual *iso* FA reported as well as *iso* C16:0 and *iso* C18:0 are included in the sum of iso branched chain fatty acids (ISO). [e]Individual *anteiso* FA reported are included in the sum of ante branched-chain fatty acids (ANTE).

FT-NIR Spectroscopy. The Fourier transform near-infrared (NIR) spectra of the 75 milk fat samples were acquired using a Bruker Optics Matrix-F instrument equipped with a thermoelectrically cooled InGaAs diode detector and a Bruker Optics N261 diffuse reflection fiber optic probe (Bruker Analytical). The microprocessor-controlled optical bench included an interferometer with a RockSolid configuration, quartz substrate beam splitter with proprietary coating, and an air-cooled tungsten NIR excitation source (12 V, 20 W) with a HeNe laser at a frequency of 633 nm. Milk fat samples and pure FA standards (~0.1−0.5 g/sample) were taken out of storage, left to rest until reaching room temperature (~21 ± 2 °C), and analyzed in vials selected by CRA-W in previous Fourier transform Raman spectroscopy analysis[22] with PE-caps (Klaus Ziemer GmbH, Mannheim, Germany). The spectra of all milk fat samples were collected against air as a background with a resolution of 8 cm^{-1}, and a total of 780 data points were recorded over the wavenumber range of 4000−10000 cm^{-1} (2500−1000 nm). For each milk fat sample, FT-NIR spectra were acquired in duplicate, and the average of the two spectra was used for chemometrical analysis.

Data Treatment. Data incorporation, treatment, and partial least-squares (PLS) regression were performed similarly to previous methodology.[15] The OPUS spectra files were converted to the JCAMP-DX file system, which was imported into The Unscrambler v. 9.1 software (CAMO, Trondheim, Norway). The ATR/FTIR spectra were reduced from 2074 to 1241 variables by removing selected regions, for example, the noisy region between 500 and 560 cm^{-1} and regions identified as carriers of −OH chemical information (1020−1070 and 3050−4000 cm^{-1}), as well as minor absorbance from the background (2000−2450 cm^{-1}). The observed −OH absorption signal did not originate from the milk fat samples, but was attributed to moisture/trace of ethanol solvent used to clean the surface of the ATR crystal between analyses. Although a drying fan was used, it appeared that some ethanol moisture/vapors could be trapped between the milk fat and the crystal surface, which was apparent in the ATR/FTIR spectra of six samples. As a precaution, these regions not related to FA information were removed, and all mathematical treatments or investigations were performed with the remaining 1241 variables. The preprocessing methods of choice were a combination of linear baseline correction and multiplicative scatter correction (MSC) or a linear baseline correction and standard normal variate (SNV). Both MSC and SNV compensate for undesired variation in the scattering intensity caused by various multiplicative effects, thus the choice of using either pretreatment would be subjective.[24] Standard PLS regression was carried out in combination with the uncertainty testing "jackknife" procedure from The Unscrambler program, which is used to select significant variables that correlated with the measured parameter.[24−26] Furthermore, PLS regression was performed after ATR/FTIR or FT-NIR spectra transformation according to three different strategies: after MSC or SNV preprocessing only, MSC/SNV followed by first Savitzky−Golay derivative, or MSC/SNV followed by second Savitzky−Golay derivative (using a 1,1,2 derivative treatment, which indicates a smoothing factor with the number of data points in a running average on the left, the secondary smoothing on the right, and the polynomial order of the derivative, respectively). A total of six prediction models for each of the individual *trans* isolated and conjugated fatty acids (*trans*-4 C18:1, *trans*-5 C18:1, *trans*-6 + 7 + 8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1, *trans*-11 C18:1, *trans*-12 C18:1, *trans*-15 C18:1, *cis*-9,*trans*-11 C18:2 and *trans*-10,*cis*-12 C18:2), and both *trans* MUFA and CLA FA groups, as well as each of the individual OBCFA (*iso* C13:0, *anteiso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0 and C17:0) and ODD (sum of C5:0, C7:0, C9:0, C11:0, C13:0, C15:0, C17:0, C19:0, C21:0, C23:0), ISO (sum of *iso* C13:0, *iso* C14:0, *iso* C15:0, *iso* C16:0, *iso* C17:0, and *iso* C18:0), ANTE (sum of *anteiso* C13:0, *anteiso* C15:0, and *anteiso* C17:0), BRANCHED (sum of ISO and ANTE), and OBCFA (sum of odd and branched) FA groups were built when using either ATR/FTIR or FT-NIR spectra of all 75 milk samples. In addition, to better evaluate the prediction performance for minor fatty acids in their most common concentration range (below 1.0 g/100 g total fat) and to avoid overfitting based on discontinuous data set distribution, milk fat samples with high amounts of specific individual fatty acids (such as *trans*-10 C18:1, *trans*-11 C18:1, and *trans*-12 C18:1) were removed and new PLS models were constructed using only the remainder. For all PLS models, validation was carried out using systematic cross-validation with 3 folds and 25 units per segment. The optimal number of PLS factors used for the regression was determined from the minimum residual validation variance. The cross-validation coefficient of determination (R_{cv}^2), the root-mean-square error of cross-validation (RMSECV, g/100 g FAME), the number of PLS factors, intercept, slope, and bias parameters of the best prediction models for individual and grouped FA are presented in Tables 2 and 3 for ATR/FTIR and FT-NIR, respectively. All other results are available as Supporting Information.

RESULTS AND DISCUSSION

Although manufacturers are implementing different dietary strategies for the increase in the amounts of beneficial fats,[27] following the health status of animals is always of importance for quick prevention of productivity and quality drops. The latter requires fast and cost-effective analytical methods, which could determine fatty acids of interest ex situ or desirably in situ. For this purpose, ATR/FTIR and FT-NIR spectroscopy

Table 2. Partial Least-Squares (PLS) Cross-Validation Coefficient of Determination (R_{cv}^2), RMSECV, Intercept, Slope, and Bias Regression Parameters of the Best ATR/FTIR Models for Prediction of Individual and Grouped *trans* Monounsaturated (*trans* MUFA), Conjugated Linoleic (CLA), Total *anteiso* Saturated (ante), and Odd- and Branched-Chain Saturated (OBCFA) Fatty Acids[a]

fatty acid	*n*	g/100 g	R_{cv}^2	RMSECV[b]	#PLS	intercept	slope	bias	pretreatment[c]
trans-5 C18:1	75	max[d]	0.513	0.011	3	0.009	0.509	<0.001	SNV
trans-6 + 7 + 8 C18:1	73	<0.50	0.653	0.048	7	0.052	0.785	0.002	MSC + 2nd
trans-9 C18:1	70	<0.42	0.837	0.026	3	0.030	0.842	<0.001	MSC + 1st
trans-9 C18:1	75	max	0.848	0.070	2	0.038	0.834	0.001	SNV + 1st
trans-10 C18:1	75	max	0.896	0.743	2	0.092	0.910	0.007	MSC + 1st
trans-11 C18:1	66	<1.20	0.795	0.097	5	0.135	0.801	0.002	MSC
trans-11 C18:1	75	max	0.933	0.233	5	0.073	0.905	0.019	MSC/SNV
trans-12 C18:1	73	<0.91	0.814	0.066	1	0.058	0.802	<0.001	MSC/SNV + 2nd
trans-12 C18:1	75	max	0.753	0.109	2	0.079	0.739	0.003	MSC/SNV + 1st
trans MUFA low	63	<4.60	0.880	0.215	4	0.372	0.886	0.001	SNV + 2nd
trans MUFA mid	70	<8.10	0.945	0.255	6	0.200	0.939	0.015	MSC/SNV + 2nd
trans MUFA high	75	max	0.985	0.509	3	0.085	0.978	0.016	MSC/SNV + 1st
cis-9,*trans*-11 C18:2	75	max	0.894	0.124	7	0.098	0.821	0.003	SNV + 1st
CLA	75	max	0.942	0.095	5	0.042	0.922	0.001	SNV + 1st
anteiso C15:0	75	max	0.571	0.046	4	0.167	0.589	<0.001	SNV + 2nd
ANTE	75	max	0.588	0.077	7	0.199	0.742	0.001	MSC + 2nd
iso C17:0	75	max	0.604	0.034	5	0.085	0.678	0.001	MSC
OBCFA	75	max	0.514	0.423	6	1.301	0.621	0.021	SNV + 2nd

[a]All other results are reported in the Supporting Information. [b]Root mean square error of cross-validation (RMSECV) in g/100 g fatty acid methyl esters (FAME). [c]Indicates ATR/FTIR spectra pretreatment with multiplicative scatter correction (MSC) or standard normal variate (SNV) followed by first Savitzky−Golay (1st) or second Savitzky−Golay (2nd) derivative transformation. [d]Indicates the maximum concentration in g/100 g FAME of the corresponding fatty acid (FA) or FA group as reported in Table 1.

Table 3. Partial Least-Squares (PLS) Cross-Validation Coefficient of Determination (R_{cv}^2), RMSECV, Intercept, Slope, and Bias Regression Parameters of the Best FT-NIR Models for Prediction of Individual and Grouped *trans* Monounsaturated (*trans* MUFA) and Conjugated Linoleic (CLA) Fatty Acids[a]

fatty acid	g/100 g	R_{cv}^2	RMSECV[b]	#PLS	intercept	slope	bias	pretreatment[c]
trans-9 C18:1	max[d]	0.680	0.102	3	0.073	0.670	0.003	SNV + 2nd
trans-10 C18:1	max	0.845	0.907	6	0.175	0.851	0.034	SNV + 1st
trans-11 C18:1	max	0.766	0.438	6	0.135	0.813	0.045	SNV + 2nd
trans MUFA	max	0.901	1.311	7	0.153	0.931	0.136	SNV + 2nd
cis-9,*trans*-11 C18:2	max	0.709	0.206	7	0.128	0.750	0.005	MSC/SNV + 1st
CLA	max	0.720	0.208	7	0.129	0.750	0.009	MSC + 1st

[a]PLS results using all pretreatment combinations are reported in the Supporting Information. [b]Root mean square error of cross-validation (RMSECV) in g/100 g fatty acid methyl esters (FAME). [c]Indicates FT-NIR spectra pretreatment with multiplicative scatter correction (MSC) or standard normal variate (SNV) followed by first Savitzky−Golay (1st) or second Savitzky−Golay (2nd) derivative transformation. [d]Indicates the maximum concentration in g/100 g FAME of the corresponding fatty acid (FA) or FA group as reported in Table 1.

techniques were considered. Looking at the number of scientific publications, it becomes evident that FT-NIR has a predominant impact in the food industry. However, ATR/FTIR would seem a more logical choice, because it possesses enhanced spectral absorbance features, such as from specific unsaturated C=C bonds, and has already been established as an official method for the quantification of total *trans* fat content in foods and dietary supplements.[11] Here, the interest is in the simultaneous determination of several fatty acids with similar structures present in the complex milk fat matrix. Thus, the evaluation of both most widely used spectrophotometrical methods for their ability to produce fatty acid specific signals and in turn to determine various fatty acids present in different high, mid, and low concentration combinations is necessary.

Fatty Acid Standards. *trans MUFA Standards.* The general differences between the FT-NIR spectra of pure triolein and trielaidin standards in the vicinities of 4500−4700 cm^{-1} (2200−2130 nm) and 5600−5900 cm^{-1} (1785−1695 nm) (attributed to the differences in signals from the *cis* and *trans* C=C bonds) were previously reported.[14] Here two *trans* monounsaturated fatty acid methyl ester standards, as well as trielaidin and tristearin pure standards, were analyzed using FT-NIR and ATR/FTIR spectroscopy techniques. The second Savitzky−Golay derivative FT-NIR spectra of pure tristearin and trielaidin standards were compared to establish specific contributions from the isolated *trans* C=C bond (Figure 1). A striking difference between the spectra of both TAG was in the vicinity of 4705 cm^{-1}, where trielaidin and not tristearin showed increased absorption. The latter is a combination region, which might be specific to absorbance from C—H vibrations in isolated *trans* C=C bonds.

Furthermore, analyses of the pure standards with ATR/FTIR spectra confirmed an enhanced absorbance feature from nonconjugated *trans* C=C bonds in the vicinity of 966 cm^{-1} (from the C—H out of a plane deformation band), but no differences in the peak position or absorbance intensity were evident between the two *trans*-9 and *trans*-11 methyl ester positional isomers (Figure 2). Other bands around 1245 and

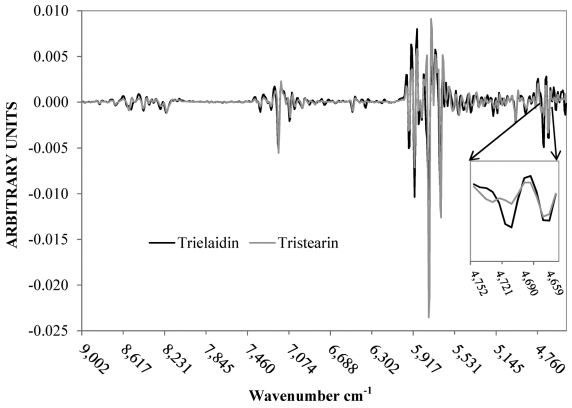

Figure 1. Second Savitzky−Golay derivative FT-NIR spectra of trielaidin (black line) and tristearin (gray line) in the 4500−9000 cm^{-1} region (*x*-axis:, wavenumbers (cm^{-1}); *y*-axis, arbitrary units).

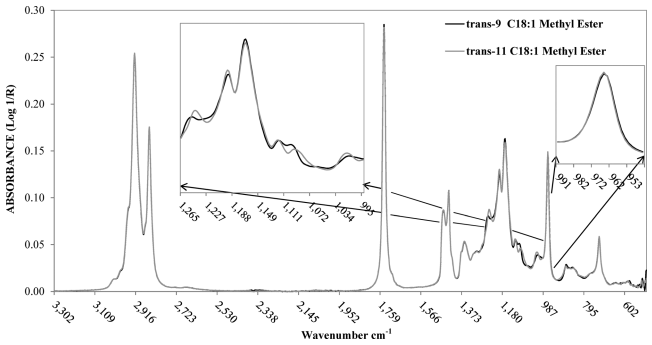

Figure 2. Multiplicative scatter corrected (MSC) ATR/FTIR spectra of *trans*-9 C18:1 (black line) and *trans*-11 C18:1 (gray line) ME in the 500−3300 cm^{-1} region (*x*-axis, wavenumbers (cm^{-1}); *y*-axis, absorbance units).

1000−1130 cm^{-1} showed clear absorbance intensity and shift perturbations, which might be isomer specific. In addition, the shape of the 966 cm^{-1} band was very broad when liquid spectra of the standards was considered; thus, partial overlap with previously reported absorption bands from conjugated *cis* and *trans* C═C bonds in *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA is suspected.[28] These features might complicate determinations of CLA isomers in low quantities, especially in the presence of high *trans* MUFA content (corresponding to an increase in band broadness and intensity).[29]

Moreover, all standards showed drastic differences between the ATR/FTIR absorption spectra obtained at liquid or solid physical phases. The crystalline spectra of the trielaidin indicated the appearance of new peaks, peak shifts, and absorption increase in specific ATR/FTIR wavebands, which are believed to contain extensive structural and vibrational information (Supporting Information, Figure SM1). The most striking feature (believed to be of most importance) was the concurrent absorbance increase and peak shift of the isolated *trans* C═C waveband in the vicinity of 966 cm^{-1} (shifted to 960 cm^{-1}). Moreover, a closer examination of the latter revealed narrower band shape compared to the liquid phase band. Detailed investigation is required for origin identification of all newly reported vibrational features (Supporting Information, Table SM1).

OBCFA Standards. Limited information is available for specific FT-NIR and ATR/FTIR absorbance features related to branched (ISO and ANTE) and odd-numbered (ODD) saturated fatty acids. Here pure *iso* C14:0, *iso* C15:0, *anteiso* C15:0, and C15:0 fatty acid methyl ester standards were analyzed at room temperature. Overall, the ATR/FTIR spectra of the *iso* C15:0, *anteiso* C15:0, and C15:0 standards were similar, except the appearance of two new peaks in the 700−920 cm^{-1} region and shifting of bands with absorbance intensity increases in the 1330−1400 and 1475−1435 cm^{-1} regions (Figure 3). Unique signals related to *iso* and *anteiso*

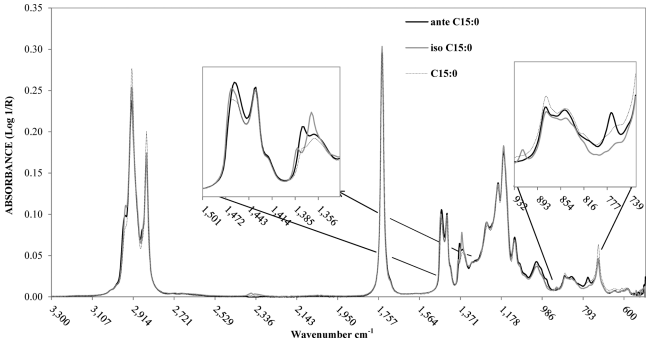

Figure 3. Multiplicative scatter corrected (MSC) ATR/FTIR spectra of *anteiso* C15:0 (black line), *iso* C15:0 (gray line), and C15:0 (dashed line) ME in the 500−3300 cm^{-1} region (*x*-axis, wavenumbers (cm^{-1}); *y*-axis, absorbance units).

branching were observed in the vicinities of 920, 1366, and 1466 cm^{-1} and 770, 1375, and 1462 cm^{-1}, respectively (Figure 3). Literature in regard to the origin of the vibrational modes in the lower wavenumber region (below 920 cm^{-1}) for branched alkanes is scarce; however, they are believed to be caused by the FA chain C−C skeletal rocking vibrations [the band position difference between *anteiso* C15:0 and *iso* C15:0 (770 and 920 cm^{-1}, respectively) might be caused by the difference in the position of the extra CH_3 methyl group at the end of the FA chain, which in turn influences the mobility of the FA chain].[30,31] Absorbance in the vicinity of 1350−1395 cm^{-1} (often in the vicinity of 1365 cm^{-1}) is known to be associated with the C−H symmetric bending vibrations in $-CH_3$.[30,31] The appearance of this more intense absorbance band in the ATR/FTIR spectra of branched-chain, but not in straight-chain, standards is due to the presence of an extra methyl group at the end of the fatty acid chain; within branched methyl ester standards, the *iso* and *anteiso* configuration position of this extra methyl group causes differences in absorbance intensity and band position at 1366 and 1375 cm^{-1} for *iso* C15:0 and *anteiso* C15:0, respectively (Figure 3). The 1475−1435 cm^{-1} absorption region is related to the asymmetrical deformation vibration of $-CH_3$ in branched alkanes.[30,31] Furthermore, no shifts in these *iso* bands between the spectra of *iso* C14:0 and *iso* C15:0 were evident, and only minor differences between the spectra of both fatty acids in C−H (higher in *iso* C15:0) and C═O (higher in *iso* C14:0) vibrations due to differences in the fatty acid chain length were present (Supporting Information, Figure SM2). These results might help in differentiation between *anteiso*, *iso*, and straight-chain fatty acids, but specificity for saturated fatty acids of similar nature within the ISO, ANTE, or ODD groups apparently seems low.

Fatty Acid Determinations. Most *trans* isolated (*trans* MUFA) and conjugated (CLA) unsaturated as well as odd- and branched-chain saturated (OBCFA) fatty acids (FA) of interest are present in minor concentrations (lower than 1.0 g/100 g of total fat) in bovine milk fat. Hence, their quantification using a

spectroscopy technique is a challenge. This is mainly due to the property of spectrophotometrical methods producing spectral information based strictly on the physical/molecular characteristics of all FA present in the sample. Thus, FA with similar chemical structure characteristics, such as the *trans* isolated and conjugated FA of interest, could have a significant overlap of spectral bands issued from the wide range of vibrations types from the different fatty acid isomers. These specific absorbances are dependent on the types and concentrations in which the corresponding fatty acids are present. For instance, previous research indicated that both *trans*-10 C18:1 and *cis*-9,*trans*-11 C18:2 CLA are important biomarkers, which might reflect the viability status of the ruminal microbial population. The latter is influenced by a drop in ruminal pH values, which in turn could drive the animal into acidotic status.[3] For this, it would be important to possess a spectroscopy methodology, which is able to differentiate between both types of fatty acid groups (*trans* MUFA versus CLA) when they are present in low, as well as in higher, than "normal" quantities. Typical average concentrations of CLA in milk fat are 0.3−0.6 g/100 g with cows fed a total mixed diet, but the levels of CLA in milk can vary widely among herds[32,33] and could reach an average of 1.1 g/100 g of CLA in milk fat with cows consuming pasture only.[34] Also, it has been shown that the concentrations of CLA in milk are affected by different dietary fatty acid sources and could drastically vary between individual animals, reaching a maximum of 5.17 g/100 g in milk fat with cows consuming a sunflower oil based diet.[35] The current data set contained both milk fat samples with very high *trans* MUFA (∼21 g/100 g) and high CLA contents (∼ 2.5 g/100 g); thus, we attempted to evaluate the prediction performance within those naturally occurring fatty acid concentration ranges. In such cases, it has already been established that in the presence of at least 1.0 g/100 g conjugated unsaturation in total fat, the official *trans* FA determination method is not applicable anymore.[9] However, that does not necessarily mean that quantification is impossible, but rather not comparable to officially applied methods. Thus, determinations with lower performance could still have implications for disease screening or monitoring.

PLS Accuracy. In general, FT-NIR spectroscopy with PLS regression resulted in complex prediction models (high number of PLS factors) and performed poorly in the determination of individual fatty acids, and the ATR/FTIR technique was superior (higher cross-validation coefficient of determination, R_{cv}^2, and lower root-mean-square error of cross-validation, RMSECV, g/100 g FAME).

trans MUFA Determinations. The ATR/FTIR spectroscopy method predicted well ($R_{cv}^2 \geq 0.90$) all major individual *trans* monounsaturated fatty acids (*trans* MUFA) as well as the *trans* MUFA group in high, mid, and low concentrations (Table 2). The regression coefficient plots for all models were similar and indicated variables in the 960−970 cm^{-1} band as main predictors. Whereas derivative transformation did not improve the high concentration range models, the best low *trans* MUFA model (less than 4.6 g/100 g in total fat) was with second Savitzky−Golay derivative spectra transformation. No variables in the 930−950 and 980−990 cm^{-1} wavenumber ranges (associated with absorbance from conjugated C=C) were picked as contributors (Figure 4). It seems that the milk fat matrix does not cause major difficulties for prediction of *trans* MUFA fatty acids in this concentration range, even in the presence of CLA.

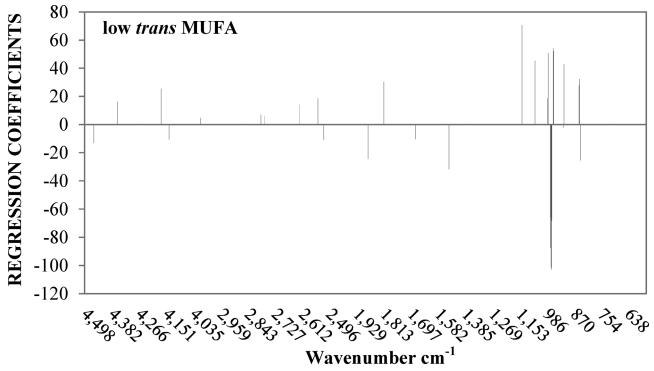

Figure 4. PLS regression coefficients of the best model (as indicated in Table 2) for prediction of the *trans* monounsaturated (*trans* MUFA) group in low concentration (<4.6 g/100 g in total fat) using ATR/FTIR spectra in the 500−4500 cm^{-1} absorbance region (*x*-axis, wavenumbers (cm^{-1}); *y*-axis, regression coefficient value).

Furthermore, satisfactory performance was evident for some of the individual fatty acids in minor concentrations (lower than 1.0 g/100 g in total fat), such as *trans*-11 C18:1 (R_{cv}^2 = 0.795, RMSECV = 0.097 with five PLS factors) and *trans*-12 C18:1 (R_{cv}^2 = 0.814, RMSECV = 0.066 with one PLS factor). Whereas *trans*-11 C18:1 was mainly determined from a specific signal in the vicinity of 960−970 cm^{-1} (Figure 5) without the

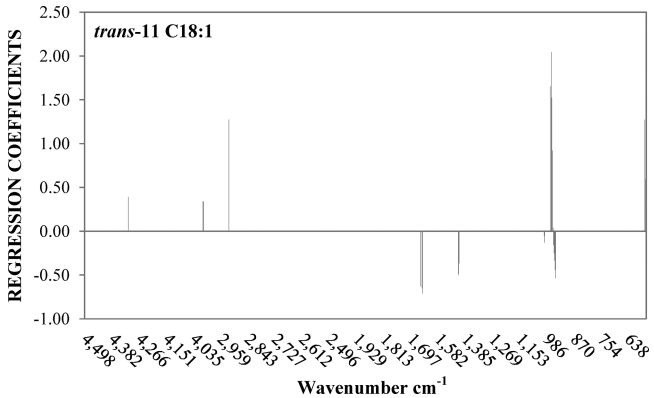

Figure 5. PLS regression coefficients of the best model (as indicated in Table 2) for prediction of the *trans*-11 C18:1 fatty acid in low concentration (<1.2 g/100 g in total fat) using ATR/FTIR spectra in the 500−4500 cm^{-1} absorbance region (*x*-axis, wavenumbers (cm^{-1}); *y*-axis, regression coefficient value).

need for derivative transformation, the *trans*-12 C18:1 prediction was due to a high reference data cross-correlation with the total *trans* MUFA group. Except for *trans*-9 C18:1 and the *trans*-6 + 7+ 8 C18:1 sum (Table 2), all other ATR/FTIR models for fatty acids in very minor concentrations (lower than 0.50 g/100g in total fat) resulted in R_{cv}^2 < 0.50 (Supporting Information, Table SM2). The ATR/FTIR calibrations for the *trans*-6 + 7 + 8 C18:1 sum and the *trans*-9 C18:1 FA were also largely influenced by the cross-correlation with the total *trans* MUFA group. Moreover, distinction between the *trans*-10 and *trans*-11 MUFA isomers is important from both animal and human health perspectives. However, the current results showed that milk fat samples rich in *cis*-9,*trans*-11 CLA could also be rich in *trans*-11 C18:1 (which is part of the CLA biohydrogenation pathway, a well-established correlation), and the high abundance of both fatty acids in the current milk fat

data set hindered ATR/FTIR determination of *trans*-10 C18:1 in low concentration (Supporting Information, Table SM2).

The FT-NIR validation models for the *trans* MUFA group with an extended concentration range (21.0 g/100 g FAME) were successful ($R_{cv}^2 \geq 0.88$, Table 3) using both first and second Savitzky−Golay derivative transformations, but were much more complex (more PLS components) and resulted in drastically higher RMSECV compared to the ATR/FTIR method. The most important regression coefficients for the best *trans* MUFA model were in the vicinities of 4713 (2122 nm), 4568 (2189 nm), 5550−5590 (1802−1789 nm), 5650−5700 (1770−1754 nm), and 5932−5970 cm^{-1} (1686−1675 nm), as well as to some extent the 7070−7090 cm^{-1} (1414−1410 nm) region from the methylene C−H stretching and bending combination and the 8450−8500 cm^{-1} (1183−1177 nm) region from the second-overtone methylene C−H bond stretching vibration[36] (Figure 6). The latter might be specific

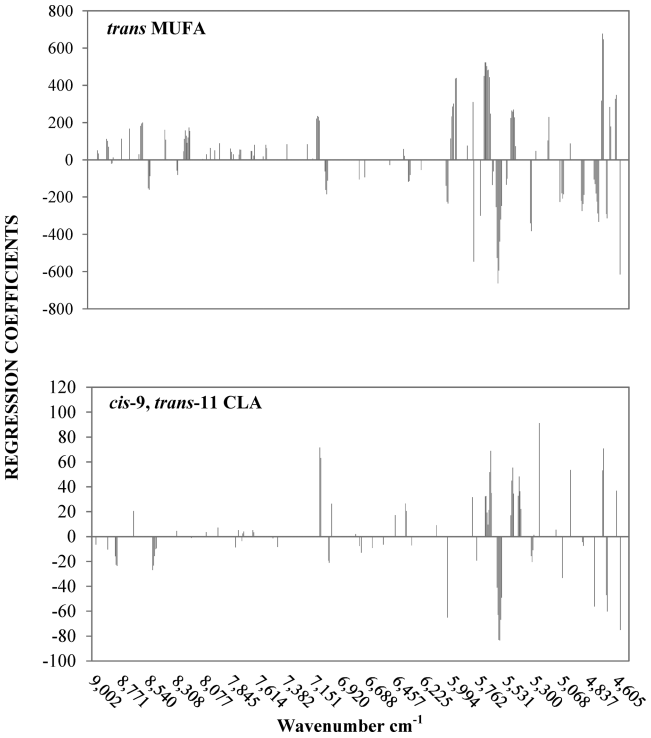

Figure 6. PLS regression coefficients for prediction of the *cis*-9,*trans*-11 C18:2 isomer and *trans* monounsaturated (trans MUFA) group using FT-NIR spectra of all milk fat samples (n = 75) in the 4500−9000 cm^{-1} (2220−1110 nm) absorbance region (x-axis, wavenumbers (cm^{-1}); y-axis, regression coefficients value).

absorption bands from the isolated *trans* C=C bonds (Figure 6), as based on the second Savitzky−Golay derivative FT-NIR spectra of the pure *trans* and saturated FA standards, which showed marked differences in the 4650−4750 cm^{-1} (2150−2105 nm) (combination region specific for isolated *trans* C=C) and 5600−5900 cm^{-1} (1785−1695 nm) (first overtone of methyl and methylene C−H symmetric and asymmetric stretching vibrations) regions, as well as some minor differences in the 8478 and 8620 cm^{-1} (1180−1160 nm) bands (possibly due to the second-overtone stretching vibration from methylene C—H in the isolated *trans* C=C bond).[37] Nevertheless, this good performance might also be a consequence of the saturated fatty acids (SFA) signal overlap in the 5520−5900 cm^{-1} (1812−1695 nm) region, which as shown with the pure TAG FT-NIR spectra is dominated by the absorption of tristearin (Figure 1). This is further supported by the observation that the regression coefficients of these 5520−5900 cm^{-1} (1812−1695 nm) bands were negatively correlated to the *trans* MUFA group concentration. The same spectral region had opposite regression coefficients in the prediction models for SFA (data not shown), which indicates the negative reference data cross-correlation between both fatty acid groups. In addition, after samples with high *trans* MUFA content were removed, the PLS models' performance significantly deteriorated in both mid (<8.1 g/100 g FAME) and low (<4.2 g/100 g FAME) concentrations (Table 3), and no *trans* MUFA specific variations in any NIR regions were detectable. Poor performance was also evident for all individual *trans* MUFA in minor concentrations (<1.0 g/100 g FAME). These very poor FT-NIR predictions for the minor individual *trans* fatty acids and for the mid and low *trans* MUFA group were indicative of the method's low sensitivity for determination of isolated *trans* C=C bonds in the milk fat matrix.

CLA Determinations. In general, the ATR/FTIR method allowed for successful prediction of the total CLA group and the *cis*-9,*trans*-11 C18:2 isomer, resulting in R_{cv}^2 of 0.94 and 0.89 and RMSECV equal to 0.095 and 0.124, respectively. It was suspected that CLA prediction performance was influenced by the high reference data cross-correlation (R^2 = 0.86) with the total *trans* MUFA group, but the better PLS model performance could not be attributed only to the latter. For example, the regression coefficients for the best model of the CLA group were different from the *trans* MUFA group (but similar to the regression coefficients for the best *cis*-9,*trans*-11 C18:2 model, 941−945, 933, 987−989, 868, 887, and 951 cm^{-1}) and appeared in the vicinities of 939−945, 978, 945, 986−991, 1134, 1161, and 962 cm^{-1} (Figure 7). In addition, the CLA group and the *cis*-9,*trans*-11 C18:2 fatty acid had insignificant (R^2 < 0.50) cross-correlation with the gas chromatography reference data of the saturated, *cis* monounsaturated, and OBCFA fatty acid groups. Thus, although part of the *cis*/*trans* absorption was buried in the high, sloping part of the spectrum[18] and despite the high *trans* MUFA abundance, successful determination of this important isomer based on a specific signal was possible (Figure 7). The best predictions were using first Savitzky−Golay derivative transformation and were based on a specific ATR/FTIR absorbance mainly in the vicinity of 939−945 cm^{-1}, as well as based on significant variables with large regression coefficients in the vicinity of 987−991 cm^{-1} (Figure 7). The emerging of the latter as important wavenumbers is not a surprise, as the signal from pure *cis*/*trans* CLA absorbance in the vicinity of 945 and 985 cm^{-1} was already reported.[11] Furthermore, to evaluate the prediction performance in low concentration, samples with high content of rumenic acid were removed (only samples with a content lower than 0.96 g/100 g in total milk fat were kept, n = 68) and PLS regression was performed using the best original ATR/FTIR *cis*-9,*trans*-11 C18:2 model combination with SNV pretreatment and first Savitzky−Golay derivative transformation. Although the narrow concentration range results deteriorated (R_{cv}^2 = 0.73, RMSECV = 0.085 with seven PLS components) compared to the best full data set calibration and no contributing variables with significant regression coefficients in the 985−990 cm^{-1} region were selected, some specificity from the 943 cm^{-1} band was still evident. Indeed, the two main determining wavebands were around 943 and 1456 cm^{-1}, and no variables in the vicinity of 985−990 cm^{-1} were considered

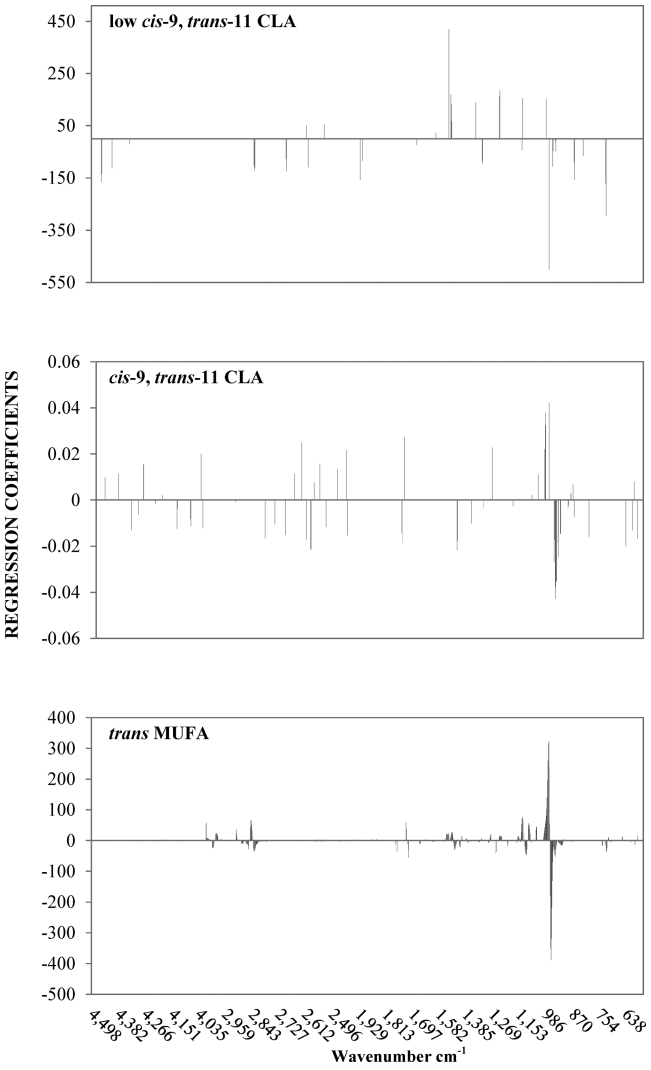

Figure 7. PLS regression coefficients of the best models (as indicated in Table 2) for prediction of the *cis*-9,*trans*-11 C18:2 isomer and *trans* monounsaturated (*trans* MUFA) group using ATR/FTIR spectra in the 500−4500 cm^{-1} absorbance region (*x*-axis, wavenumbers (cm^{-1}); *y*-axis, regression coefficients value).

(Figure 7). Because the latter absorbance band is buried in the low sloping part of the shoulder between 970 and 1100 cm^{-1}, it seems that detection of *cis*-9,*trans*-11 C18:2 signal variations in such low fatty acid concentration from this region is complicated. Nevertheless, on the basis of the pure *trans* MUFA standards, improving these CLA quantifications might be possible by narrowing the bandwidth of the *trans* MUFA band, when milk fat samples are analyzed at freezing conditions (Supporting Information, Figure SM1). Hence, the detection limit should be further investigated after optimization of the analytical procedure.

The *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA isomers have been previously reported to exhibit *trans*/*cis* and *cis*/*trans* isomer specific FTIR absorption peaks in the vicinity of 1420−1470 and 720−750 cm^{-1} from the CH_2 rocking and the CH_2 scissoring modes, respectively.[28] Thus, differentiation between both fatty acids would seem feasible. However, quantification of *trans*-10,*cis*-12 C18:2 with the current data set using ATR/FTIR spectra was not successful, possibly due to a combination of two factors: (1) the very low concentrations of this fatty acid and (2) the numerous other saturated and unsaturated fatty acids from the complex milk fat matrix also contributing to the absorption signal in these two regions. The current data set is believed to cover the full naturally occurring concentration ranges of the fatty acids of interest. Determination of *trans*-10,*cis*-12 C18:2 was also not possible using the FT-NIR method. The latter might be achieved by using a different kind of spectroscopy technique, which has already shown promise in the determination of total CLA.[23,38,39]

Contrary to the ATR/FTIR results, the FT-NIR spectra showed no specific *cis*/*trans* conjugated C=C signal, and the somewhat satisfactory total CLA (R_{cv}^2 = 0.72 and RMSECV = 0.208) and *cis*-9,*trans*-11 C18:2 (R_{cv}^2 = 0.71 and RMSECV = 0.206) performance was probably due to high cross-correlation with the reference data of the *trans* MUFA group. The latter is confirmed by the almost complete regression coefficient overlap from the best FT-NIR spectra models for CLA and *trans* MUFA (Figure 6).

OBCFA Determinations. Another important group of milk fatty acids, the OBCFA, have also emerged as viable nutritional biomarkers. With the currently established parameters for SARA detection, they could contribute to the discrimination of high-risk animals (*iso* C13:0, *iso* C16:0), as well as indicate drops in ruminal pH (*iso* C14:0, *iso* C15:0).[3] Currently, PLS regression in combination with FT-NIR or ATR/FTIR spectra was not able to detect sufficient variations in vibrational information specific to OBCFA. ATR/FTIR spectroscopy showed some potential only for the *anteiso* C15:0 and *iso* C17:0 FA, but to a limited extent (Table 2 and Figure 8). All *anteiso* C15:0 PLS models resulted in $R_{cv}^2 > 0.50$ and RMSECV $\leq$ 0.050 (Supporting Information, Table SM3) using second Savitzky−Golay derivative transformation as the best pretreatment (only four PLS components, Table 2). The reference data for *anteiso* C15:0 had very low cross-correlation (R^2 < 0.19)

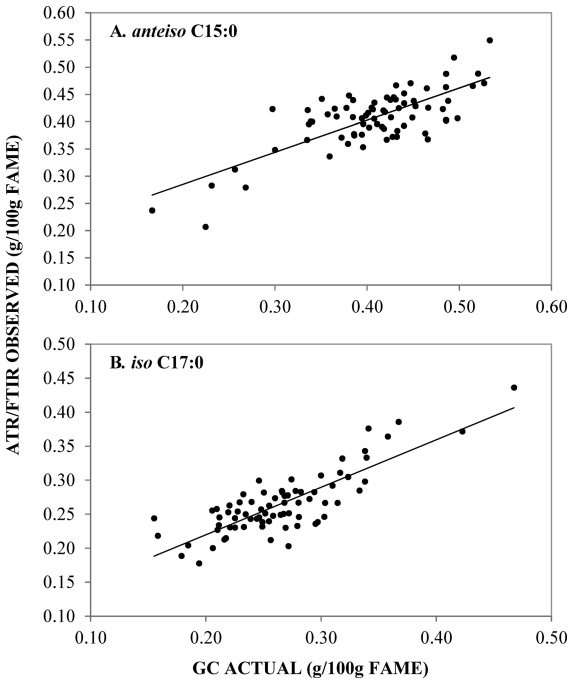

Figure 8. Validation PLS plots of the most robust prediction models (as indicated in Table 2) using ATR/FT-MIR spectroscopy for (A) *anteiso* C15:0 and (B) *iso* C17:0 fatty acids in g/100 g fatty acid methyl esters (FAME).

with any of the main FA groups (SFA, *cis* MUFA, *trans* MUFA, PUFA, or CLA). All PLS prediction parameters for *iso* C17:0 were similar (Supporting Information, Table SM3), and the best modeling performance was with MSC pretreatment and without derivative transformation (R_{cv}^2 = 0.604 and RMSECV = 0.034, Table 2 and Figure 8). The better prediction for *iso* C17:0 compared to any other *iso* branched-chain saturated FA in all PLS models might indicate detection of information related to this fatty acid. The latter was confirmed by ruling out gas chromatography data cross-correlation with the main fatty acid groups (SFA, *trans* MUFA, *cis* MUFA, PUFA, or CLA), and the cause of the better *iso* C17:0 determinations was further evaluated by removing all absorption bands from the mid-infrared spectra, which might be directly related to unsaturation (removed all variables within the 3000−3050 and 900−1000 cm^{-1} regions). A new PLS model using the remaining regions (560−899, 1001−1019, 1071−1999, 2451−2999, and 4000−4500 cm^{-1}) and the best original pretreatment combination for this fatty acid (MSC only) was constructed. This new model did not decrease in performance (R_{cv}^2 = 0.67, RMSECV = 0.031, and six PLS components). In the latter, the most contributing wavebands (with significant regression coefficients for the reference data of *iso* C17:0) were in the vicinity of 685−725, 1705, 1464, 845−849, 1160, 1088, 1350, 2972, and 2890 cm^{-1}. The 700−800, 1330−1400, and 1475−1435 cm^{-1} regions also showed differences in absorbance between the ATR/FTIR spectra of the pure *anteiso*, *iso*, and straight saturated methyl ester standards (Figure 3). Absorbance bands in the lower wavenumber region (below 920 cm^{-1}) are known to be associated with the FA chain C−C skeletal rocking vibrations, and absorbance in the vicinity of 1350−1395 cm^{-1} is associated with the C−H symmetric bending vibrations in $-CH_3$.[30,31] In addition, absorption bands of medium intensity in the vicinity of 1175−1120 and 1100−1040 cm^{-1} are known to be associated with C−C−C stretching vibrations, and the 1475−1435 cm^{-1} absorption region is related to the asymmetrical deformation vibration of $-CH_3$ in branched alkanes.[30,31] The similarities of these wavebands with the regression coefficients from the PLS models confirm the presence of information specific to branched fatty acids in the ATR/FTIR spectra of milk fat samples. However, both *iso* C17:0 and *anteiso* C15:0 FA had the highest concentration ranges and total amounts within the ISO and ANTE groups (≥0.50 g/100 g in total fat), which explains the better performance compared to any other individual branched-chain saturated FA. Thus, ATR/FTIR absorbance related to branching in saturated fatty acids might only be detectable at levels >0.50 g/100 g in total milk fat in similar or wider concentration ranges (Figure 8).

Despite the possibility for determination of *anteiso* C15:0 and *iso* C17:0, these two fatty acids have not emerged as useful biomarkers, and other *iso* branched-chain fatty acids, such as *iso* C13:0 to *iso* C16:0, have been suggested as nutritionally important. The latter were indicative of low ruminal pH and SARA risk,[3] but are present in much lower amounts in milk fat. The current milk fat data set is believed to contain the full naturally occurring concentration span of all OBCFA; thus, in tasks requiring these fatty acids as nutritional markers, more accurate analytical methods should be employed as diagnostic tools.[15] In addition, further optimization of the current method by testing different analytical conditions (such as various recording temperatures) might improve specificity of the mid-infrared spectra for certain fatty acid vibrational modes (Supporting Information, Figure SM1). The latter was already demonstrated using a different kind of vibrational spectroscopy technique, such as Fourier transform Raman spectroscopy.[23]

The FT-NIR spectra in combination with PLS regression were not able to produce any sufficient models for the prediction of individual or grouped OBCFA of interest (R_{cv}^2 < 0.50). In general, although better than FT-NIR, the ATR/FTIR method showed limited capability for minor fatty acids when milk fat is analyzed at liquid phase.

MSC versus SNV Pretreatments. The current evaluation of SNV or MSC pretreatments was based on the various literature recommendations [the use of either SNV or MSC for spectra pretreatment is indicated as "a matter of taste" (The Unsrcambler 9.2, 2004)[24] and other manuals (WinISI III, FOSS, 2005) recommend MSC only or no scatter correction],[40] as well as on the assumption that the correction ability of the different pretreatments might vary depending on the spectroscopy type and the sample presentation technique. For example, FT-NIR is a secondary vibrational spectroscopy technique, whereas FTIR exhibits mostly fundamental signals;[40] thus, they might require different scatter correction methods. The results suggest that PLS models constructed using FT-NIR and ATR/FTIR spectra with either SNV or MSC (Tables 3) did not differ; thus, both pretreatment algorithms could be used for construction of calibration models. The only drawback of MSC is the need for saving the pretreatment model, which contains the reference spectra (the mean spectra of the corresponding calibration data set used for correction).

Future Perspectives. Overall, diffuse reflectance FT-NIR (in the 4500−9000 cm^{-1} range) in combination with PLS regression was not able to reveal sufficient vibrational information related to any of the minor milk fatty acids of interest. This is in contrast to previous reports with common oils and mixes of shortening and lard, but the latter is probably due to the complex milk fat matrix and low specificity of the FT-NIR technique when many fatty acids with similar molecular vibrations are present. The ATR/FTIR is the more preferable method for determination of minor odd and branched saturated and *trans* isolated and conjugated fatty acids in bovine milk fat. In addition, ATR/FTIR spectra of samples analyzed at crystalline conditions might reveal additional fatty acid specific information and allow improvements in detection and differentiation. The drawback of the current research is that we were not able to control temperature precisely, which renders any spectra obtained for the current samples of lesser value for future industrial calibrations. Thus, improvement in methodology without complication of the analytical procedure is needed. It is not surprising that GC is still the current method of choice for quantification of milk fatty acids, despite its rather long and complicated procedure. Thus, the search for a method that combines the speed of a spectroscopy technique with the amount of information available from gas chromatography for the determination of minor OBCFA, *trans* MUFA, and CLA remains.

■ ASSOCIATED CONTENT

Supporting Information

Additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

ATR/FTIR, attenuated total reflectance Fourier transform infrared; FT-NIR, Fourier transform near-infrared; FA, fatty acid; MUFA, monounsaturated fatty acids; MSC, multiplicative scatter correction; PLS, partial least-squares; SNV, standard normal variate; CLA, conjugated linoleic acids; SFA, saturated fatty acids; ODD, odd-numbered saturated fatty acids; OBCFA, odd- and branched-chain fatty acids

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