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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · OCTOBER 2013

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Rapid Determination of Total Conjugated Linoleic Acid Content in Select Canadian Cheeses by ^{1}H NMR Spectroscopy

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Supporting Information

ABSTRACT: The application of ^{1}H nuclear magnetic resonance (NMR) spectroscopy to the measurement of conjugated linoleic acid (CLA) content in the lipid fraction of dairy products is both a novel and inviting alternative to traditional methods such as gas chromatography (GC), which can require time-consuming sample derivatization. In this work, a newly developed, rapid, and reliable lipid extraction protocol was combined with simple, nondestructive ^{1}H NMR spectroscopic analysis to measure the total CLA content in CLA standards and in various Canadian cheeses from conventional, organic, and grass-fed dairy sources. The total CLA concentrations (mg/g cheese) obtained using these new extraction and analysis methods were consistent with amounts found using the modified Folch extraction and GC analysis (correlation coefficient of 0.948). Results showed that cheeses from exclusively grass-fed dairy cows were significantly higher in total CLA content than either conventional or organic cheese.

KEYWORDS: *conjugated linoleic acids, lipid extraction, nuclear magnetic resonance spectroscopy, quantitative analysis, gas chromatography, dairy, cheese*

INTRODUCTION

Interest in conjugated linoleic acids (CLAs) has increased considerably over the past two decades due to their potential health benefits with respect to cancer,[1−5] obesity,[6] atherosclerosis,[7,8] strengthening of the immune system,[9] and calcium/bone metabolism.[10,11] CLAs are defined as a group of positional and geometric isomers of linoleic acid[12−15] and are naturally found in meat and dairy products that originate from ruminants.[13,14,16] Milk, cheese, and other dairy products are the major sources of CLAs in the human diet.[17−19] The *cis*-9,*trans*-11 isomer (*c*-9,*t*-11 CLA; also known as rumenic acid) is the predominant isomer, accounting for up to 90% of the total CLAs found in dairy products, whereas the *trans*-10,*cis*-12 isomer (*t*-10,*c*-12 CLA) can account for approximately 5% of total CLAs in dairy (see Figure 1).[20−22] Both of these isomers are available commercially and were used as standards to develop the new ^{1}H nuclear magnetic resonance (NMR) analysis method reported in this work. Dairy products also contain the *trans*-7,*cis*-9 isomer (*t*-7,*c*-9 CLA), which can account for up to 12% of total CLAs in milk-based products;[23−25] however, the *trans*-7,*cis*-9 CLA isomer is not available commercially. Unlike *trans*-fatty acids, *trans*-CLAs appear to have great potential benefits on human health.[1−11] Therefore, the ability to quickly and accurately quantify CLA content in dairy products such as cheese would be extremely valuable to the industry and could be used in a potential marketing strategy to health-conscious consumers.

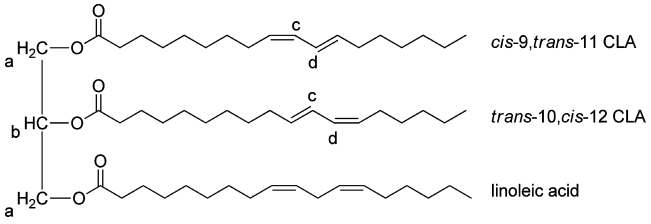

Figure 1. *cis*-9,*trans*-11, *trans*-10,*cis*-12, and linoleic acid isomers of conjugated linoleic acid in a triglyceride molecule.

Gas chromatography (GC) methods currently dominate the literature for the determination of CLA content in dairy.[26−32] However, these methods can be laborious due to required sample derivatization steps, which involve significant amounts of reagents and solvents. Therefore, development of a rapid method that provides the necessary analytical information with minimal sample preparation would be advantageous. NMR spectroscopy is one such analytical tool that avoids sample derivatization and offers the benefit of short data acquisition times. Maria et al. have previously shown ^{1}H NMR spectroscopy to be a useful technique for measuring the amount of CLAs in beef,[33] and Schripsema utilized ^{1}H NMR in the identification of the CLA content in butter and margarine products.[34] NMR spectroscopy has also been combined with chemometric analysis by Schievano et al. to distinguish between Asiago cheeses from different production chains.[35] However, there are no papers in the literature describing the use of ^{1}H NMR for the quantification of total CLA content in assorted cheeses (Cheddar, mozzarella, feta, Gouda) from various source classifications (conventional, organic, and exclusively grass-fed

Received: June 15, 2013
Revised: September 18, 2013
Accepted: September 20, 2013
Published: September 20, 2013

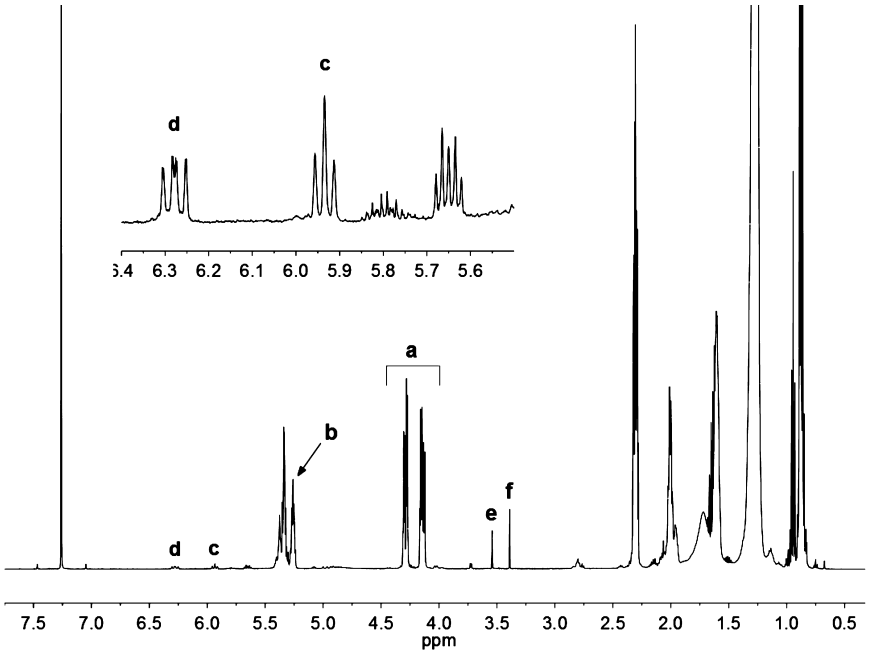

Figure 2. ^{1}H NMR spectrum of lipids extracted from cheese sample Gouda5 (500 MHz, 128 scans, $CDCl_3$).

dairy). Similarly, there are no published studies that have reported the development of ^{1}H NMR spectroscopic analysis for quantifying total CLA content using commercially available CLA standards.

Dairy makes up nearly 11% of all organic food sales in Canada, for a value of approximately $290 million.[36] Consumers are willing to pay substantially more for organic dairy products on the basis of the belief that organic items are superior to conventional products.[36] However, there are very few scientific studies that illustrate any differences in nutritional quality between organic and conventional cheese, especially in Canada.[37] According to Canadian Organic Dairy Standards, requirements for organic dairy certification include production without the use of pesticides, synthetic fertilizers, or antibiotics, as well as providing access to certified organic pasture for grazing throughout the growing season, weather permitting.[38] Increased access to pasture should alter the CLA content present in organic dairy cows and the amounts found in any dairy products derived from their milk.[39,40] We speculate that due to nutritional differences in cow feed, organic dairy products may have intermediate amounts of CLAs in comparison to exclusively grass-fed and conventional dairy products.

Herein, we describe a new rapid method to extract lipids and measure CLA content in select Canadian cheeses by ^{1}H NMR spectroscopy, showing it to be both reliable and consistent with the traditional lipid extraction and GC methods. Additionally, we determine the absolute CLA content in several conventional and organic cheeses using these new methods, and then compare the amounts to those found in cheeses from exclusively grass-fed sources.

MATERIALS AND METHODS

Sampling. Twenty-eight cheeses of four varieties (eight Cheddar, eight mozzarella, five feta, and seven Gouda) from across Canada were purchased at various supermarkets and specialty stores in southern British Columbia and stored at 4 °C until analysis (see Supporting Information Table S1 for brands and production locations). CLA standards (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Solvents, reagents, and dimethoxyethane (DME) standard were purchased from Sigma-Aldrich (Oakville, Canada), Fisher Scientific (Ottawa, Canada), or VWR (Mississauga, Canada).

Lipid Extractions. Lipid extractions for ^{1}H NMR analysis were carried out as follows: 150 mg of cheese was finely chopped into ∼2 mm^3 pieces and placed into a 20 mL vial, to which 1.5 mL of $CDCl_3$ was added. The resulting suspension was homogenized for 30 s (VWR homogenizer VDI25, speed setting 3) and then filtered through Kimwipe (Kimberly-Clark, Mississauga, Canada) directly into an NMR tube. Each block of cheese was analyzed in replicates of five. Modified Folch extractions for GC analysis were carried out following general literature procedures.[41] Approximately 1 g of cheese was finely chopped into ∼2 mm^3 pieces and homogenized in 5 mL of methanol for 15 s before the addition of 5 mL of chloroform and homogenization for an additional 15 s. The homogenizer was rinsed with 10 mL of a 1:1 chloroform/methanol mixture before the final volume was adjusted to 20 mL with the same 1:1 solution. Potassium chloride solution (0.88%, 9 mL) and one drop of 6 M hydrochloric acid were then added, and the mixture was shaken vigorously and centrifuged (Eppendorf, 5 min, 734*g*). The bottom organic layer was separated, and the aqueous layer was washed with 10 mL of chloroform and separated as above. The chloroform fractions were combined, dried over anhydrous sodium sulfate, filtered through cotton, and evaporated to dryness. These lipid-containing extracts were then redissolved in 15 mL of chloroform and stored at −20 °C until GC analysis. Each block of cheese was analyzed in replicates of five.

^{1}H NMR Measurements. All ^{1}H NMR spectra were acquired with a Bruker Avance III Ultrashield 500 MHz NMR spectrometer using $CDCl_3$ as solvent and residual protons as internal reference (7.26 ppm). Spectra were recorded at room temperature with 65536 data points, 128 scans at a spectral width of 5000 Hz, relaxation delay of 2.5 s, and acquisition time of 6.55 s. An exponential line broadening (0.30 Hz), automatic phase correction, and baseline correction (degree of polynomial equal to 5) were applied to each spectrum.

Quantitative Analysis. A known quantity (0.0155 mg) of DME was added as internal standard in 0.25 mL of $CDCl_3$ to each NMR tube. CLA concentrations were obtained by averaging the peak areas of the ^{1}H NMR signals at 5.93 and 6.28 ppm (peaks c and d in Figure 2) as normalized to the methylene signal of the internal standard

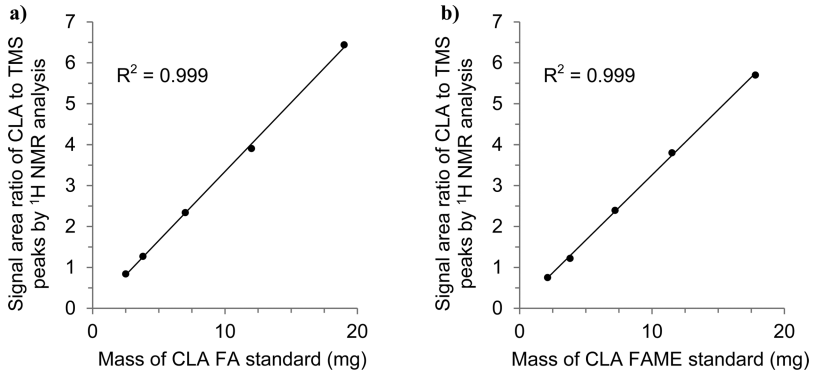

Figure 3. Estimated linear regressions of signal area ratio of CLA to TMS peaks by ^{1}H NMR analysis versus (a) mass of CLA FA standards and (b) mass of CLA FAME standards.

(DME) at 3.54 ppm (peak e; area = 4).[33,34] Schripsema used a third CLA signal (5.65 ppm) for quantification, along with the aforementioned peaks at 5.93 and 6.28 ppm,[34] whereas other authors used only two peaks as we have described.[33] We found considerable variability in the CLA content measured when the area of the peak at 5.65 ppm was also used in the analysis. To confirm the linearity of the standard curve, a series of samples with identical CLA concentrations and various DME concentrations were prepared and analyzed by ^{1}H NMR. A correlation coefficient of 0.997 indicated an excellent linear relationship between the concentration of internal standard and the integrated area of peak e in the ^{1}H NMR spectra (Supporting Information Figure S1). No significant variation in CLA amounts was observed by ^{1}H NMR analysis on different days or with different operators.

The CLA content (mg/g cheese) in each sample was calculated from eq 1:[42,43]

$$\text{total CLAs (mg/g cheese)} = \left(\frac{I_{CLA}}{I_{DME}}\right)\left(\frac{H_{DME}}{H_{CLA}}\right)\left(\frac{MW_{CLA}}{MW_{DME}}\right)\left(\frac{M_{IS}}{M_{Ch}}\right) \quad (1)$$

I_{CLA} = average signal area of CLA peaks c and d, I_{DME} = signal area of DME methylene peak e, H_{DME} = number of protons of DME methylene (4H), H_{CLA} = number of protons of CLA signal (1H), MW_{CLA} = molecular weight of CLA triglyceride (879.4 g/mol), MW_{DME} = molecular weight of internal standard DME (90.12 g/mol), M_{IS} = mass internal standard (0.0155 mg), and M_{Ch} = mass cheese (g). It is important to consider the possibility of up to three CLA groups esterified to the same glycerol moiety; therefore, the molecular weight of CLA triglyceride reflects the maximum possible number of CLAs linked to glycerol.[34,44]

Gas Chromatography Analysis. The lipids obtained by Folch extraction were derivatized into fatty acid methyl esters (FAMEs) before being analyzed by GC using a Varian 3800 gas chromatograph coupled with a flame ionization detector (FID) and CTC Analytics Combi-Pal autosampler. An SP-2650 fused silica column (100 m × 0.25 mm i.d. × 0.2 μm, Supelco Inc.) was used for chromatographic separation. Base-catalyzed methylation by sodium methoxide was employed to avoid isomerization of CLAs.[45] The FAMEs were injected at a concentration of 1−2 mg/mL and at a split flow of 1:75. Helium was used as a carrier gas at a constant 1 mL/min flow rate under the following temperature program: held at 45 °C for 4 min, increased to 175 °C at 13 °C/min, held for 41 min, increased to 215 °C at 4 °C/min, and maintained for 35 min (total analysis time = 86 min). The injector and detector temperatures were held at 250 °C.

Statistical Analysis. Linear regressions and correlation analyses were performed on the extraction methods, as well as NMR and GC results. In addition, an analysis of variance comparing the total CLA content among the varieties and classifications of cheese was performed, and a post hoc Tukey's test was used for pairwise comparisons (Minitab Inc., 2013). Significance was defined by a P value <0.001.

RESULTS AND DISCUSSION

Figure 2 shows a typical ^{1}H NMR spectrum of lipids extracted from cheese (including the DME internal standard). Signals attributed to the protons of the conjugated double bonds of CLAs typically appear between 5.0 and 6.5 ppm (signals c and d; used for measuring CLA content). These signals do not overlap with any other resonances representative of fatty acids and because the integral of a given peak in a ^{1}H NMR spectrum is directly proportional to a corresponding number of resonant nuclei, peak areas can be used for the determination of total CLA content. Signals between 0.9 and 3.0 ppm are characteristic aliphatic hydrogen resonances for the CLAs and other fatty acids, whereas the triglyceride signals are found as a pair of doublets at approximately 4.2 ppm (a) and a multiplet at 5.3 ppm (b). Peaks at 3.54 ppm (signal e) and 3.39 ppm (signal f) represent the methylene and methyl signals of the internal standard, dimethoxyethane, respectively. Both the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers have the same chemical shifts in the 5.0−6.5 ppm region, as confirmed by our NMR analysis of commercial CLA standards, and we believe the commercially unavailable *trans*-7,*cis*-9 CLA isomer would also exhibit the same chemical shifts in this region, as confirmed by NMR prediction software (CambridgeSoft, 2010). Because the majority of total CLAs in dairy products are one of these three isomers,[20−25,46] determination of CLA content by analysis of this ^{1}H NMR region can be considered representative of the total CLA content in a particular dairy product.

To establish the feasibility of using ^{1}H NMR to measure total CLA content, a quantitative analysis of CLA fatty acid (FA) and CLA FAME standards (90% purity, equal mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers) was performed. Standard curves were constructed along a linear range (from ∼2 to ∼20 mg of CLA standards; see Figure 3), and the signal area ratio of CLA to tetramethylsilane (TMS) was determined by ^{1}H NMR. The ratio of the peak areas between CLA and TMS was determined by normalizing the average peak areas of signals 5.93 and 6.28 ppm to the external reference (TMS; area = 1). Regression analyses on the data from both the CLA FA and CLA FAME standards show R^2 values of 0.999 (see Figure 3), indicating a near-perfect linear relationship between the mass of CLAs present and the average integral of peaks c and d in the ^{1}H NMR spectra.

A second analysis was carried out with the CLA FAME standard to correlate the signal area ratio of CLA to TMS obtained by ^{1}H NMR with the CLA content determined by GC. The same standard samples used to determine CLA

content by ^{1}H NMR (normalized to TMS) were then subjected to GC analysis. The results from the FAME standard analysis demonstrate a correlation coefficient of 0.999 (see Figure 4),

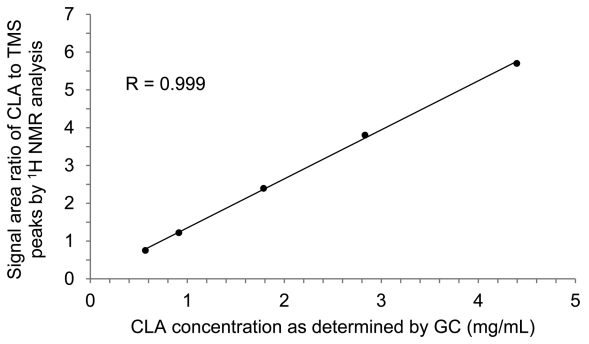

Figure 4. Correlation between signal area ratio of CLA to TMS peaks by ^{1}H NMR analysis and CLA concentration by GC analysis of CLA FAME standards. Trendline was added using Excel 2010.

indicating a near-perfect linear relationship between the signal area ratio of CLA to TMS determined by ^{1}H NMR and the amounts of CLAs observed using GC analysis. Base-catalyzed methylations are not suitable for esterifying free fatty acids,[47] and although this derivatization step was attempted on the free CLA FA standards, no signals indicative of CLA FAMEs were observed by GC.

Before applying the new lipid extraction protocol developed in this work to our cheese samples, we performed trials to verify that the extraction efficiency of CLAs by the new rapid method was consistent with the commonly used modified Folch method. Lipids were extracted in triplicate using our new extraction method, as well as the modified Folch method, from five different Gouda cheeses with various amounts of CLAs. The lipid fractions obtained from these two methods were then analyzed by ^{1}H NMR, and the corresponding signal area ratios of CLA to triglyceride peaks (normalized to 100) were determined. Whereas earlier analysis of CLA FA and FAME standards required the use of TMS as an external reference, the CLA signals of these lipid fractions were normalized to the triglyceride peaks a in their respective ^{1}H NMR spectra.[33,34] Figure 5 shows a correlation coefficient of 0.992 for these data, indicating a very strong linear correlation relationship exists

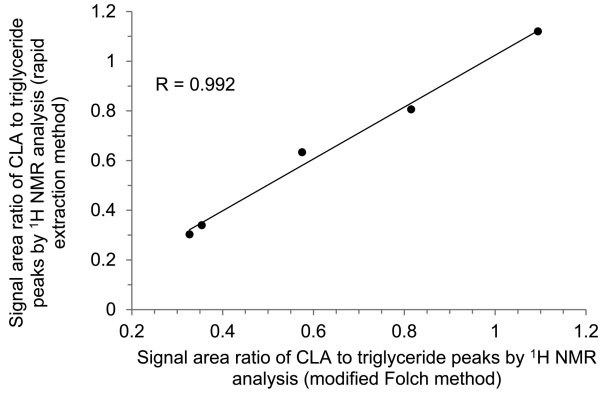

Figure 5. Correlation between signal area ratio of CLA to triglyceride peaks by ^{1}H NMR analysis using the rapid extraction method and the modified Folch method. Trendline was added using Excel 2010.

between the CLA ^{1}H NMR peak integration ratios obtained via the two extraction methods.

Having developed rapid lipid extraction and analysis protocols for the determination of total CLA content in cheese, it was also necessary to demonstrate that data acquired by these methods correlate well to data acquired by traditional methods, namely the modified Folch extraction and GC analysis protocols. Of the 28 different cheeses from four varieties (Cheddar, mozzarella, feta, and Gouda) collected for this study, 25 were analyzed by both the novel methods reported here and the Folch/GC analysis methods to examine this correlation. A quantitative analysis was performed to measure the total CLA concentration in milligrams per gram of cheese. DME was chosen as the internal standard, as it does not have the volatility issues often encountered with TMS and has previously been used as an internal standard in quantification of fatty acids by NMR.[42,43] Figure 6 shows the plot of total CLA

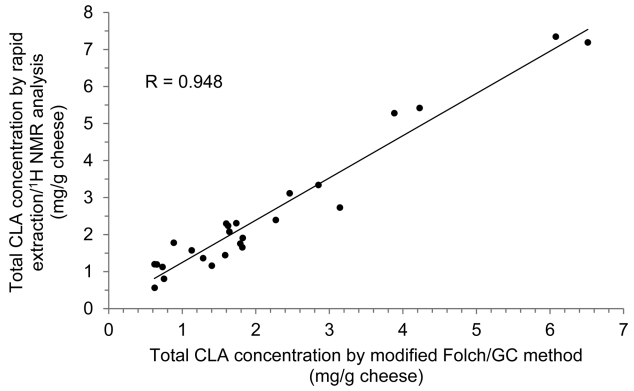

Figure 6. Correlation between total CLA concentrations determined by rapid extraction/^{1}H NMR and modified Folch/GC. Trendline was added using Excel 2010.

content determined by our ^{1}H NMR method versus the amount of CLAs measured by GC. A correlation coefficient of 0.948 suggests good agreement between data obtained by this rapid extraction/NMR analysis method and the modified Folch extraction/GC analysis method. Using the 500 MHz spectrometer and experimental conditions detailed earlier, the quantification limit was 0.003 mg of total CLAs. The advantage of using these new methods is the reduced amount of reagents needed and the shorter analysis times required to determine the total concentrations of CLAs in various cheeses. This avoids time-consuming Folch-style extractions, sample manipulations, and derivatizations (methylations), which may explain differences in values between the two methods. Furthermore, the acquisition of all ^{1}H NMR spectra was performed at room temperature, eliminating the need for elevated temperatures that may lead to double-bond isomerizations and sample degradation.

Our new extraction and ^{1}H NMR methods were used to determine the total concentration of CLAs in an assortment of cheeses from across Canada (Figure 7). Four commonly available cheese varieties were investigated: Cheddar, mozzarella, feta, and Gouda. In addition, feta and Gouda cheeses from exclusively grass-fed sources were also analyzed, but Cheddar and mozzarella varieties were unfortunately unavailable in our region. The data representing the three classifications of cheese met the requirements for tests of normality and equality of variance. Figure 7 clearly shows that cheeses from grass-fed

dx.doi.org/10.1021/jf402627q | J. Agric. Food Chem. 2013, 61, 9915−9921

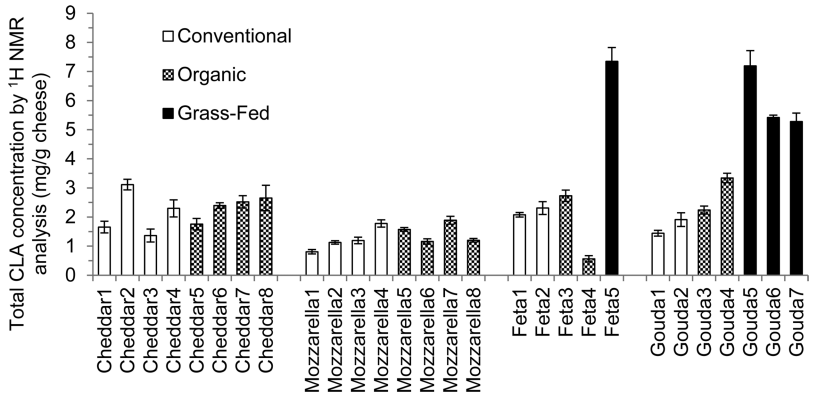

Figure 7. Total CLA concentrations by rapid extraction and ^{1}H NMR analysis methods in a variety of Canadian cheeses from conventional, organic, and grass-fed dairy sources.

sources have significantly higher average total CLA concentrations than cheeses from either conventional or organic sources (F = 56.14, P < 0.001, $n_{\text{grass-fed}}$ = 4, n_{organic} = 12, $n_{\text{conventional}}$ = 12). Pairwise comparisons showed that cheeses from exclusively grass-fed sources were significantly different from cheeses from both conventional (Tukey's test, T = 10.159, P < 0.001) and organic (Tukey's test, T = −9.611, P < 0.001) sources. However, the amounts of CLAs in cheeses from conventional and organic sources were not found to be statistically different from one another (Tukey's test, T = 0.775, P = 0.722). Interestingly, Figure 7 does appear to show a small difference in the average total amount of CLAs in cheeses from organic and conventional sources, with organic having somewhat intermediate concentrations between grass-fed and conventional sources. Increasing sample size within the organic and conventional classifications might result in a statistically significant difference in average total CLA content, which a rigorous and conservative statistical analysis of post hoc testing using Tukey's test does not currently show. Cheese from organic sources might contain higher average total amounts of CLAs compared to amounts in cheese from conventional sources due to the amount of forage consumed by the source dairy cows, any seasonal variations in diet, or differences in cheese processing and production.

In conclusion, we have developed a new rapid lipid extraction protocol and ^{1}H NMR analysis technique that can be used to determine total CLA content in cheese. We have shown the reliability of this approach and its correlation to data acquired by the commonly used modified Folch extraction method and GC analysis. The new methods provide absolute values of total CLAs like the modified Folch method with GC analysis; however, our combined extraction and NMR methods are quicker and more efficient. A complete sample analysis can be performed in <25 min and requires little sample preparation, reagent, and solvent. This new rapid extraction and ^{1}H NMR analysis approach has potential application in the dairy industry as a screening technique for total CLA concentrations in large numbers of cheese samples and in the screening of CLA content in other dairy products.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information about the brands, fat percentage, production location, and lot numbers of the various cheeses analyzed; standard curve of the internal standard (DME). This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

Funding for this project was provided by the Canada-BC Ranching Task Force Initiative.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the Thompson Rivers University Faculty of Science for use of their facilities and instrumentation and Nancy Flood for her assistance with the statistical analysis.

■ ABBREVIATIONS USED

CLAs, conjugated linoleic acids; NMR, nuclear magnetic resonance; GC, gas chromatography; FA, fatty acid; FAME, fatty acid methyl ester; TMS, tetramethylsilane; DME, dimethoxyethane

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