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ARTICLE

Fast and Simple Nuclear Magnetic Resonance Method To Measure Conjugated Linoleic Acid in Beef

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Conjugated linoleic acids (CLAs) are a group of linoleic acid isomers that are naturally found in food products originating from ruminants (meat and dairy). These acids have received special attention in recent years due to their potential human health benefits. Research efforts have been proposed to increase the CLA content in beef to improve public health. However, because there are more than 30 million beef cattle used each year by the American food industry, it will be necessary to ensure their content in a large number of samples. Therefore, it is important to have an inexpensive and rapid analytical method to measure CLA content in food products. Because gas chromatography (GC), a current popular method for measuring CLAs, is slow, this paper describes a nuclear magnetic resonance spectroscopy (^{1}H NMR) method that is potentially >10 times faster than the GC method. Analyses show a correlation coefficient of 0.97, indicating the capacity of NMR to quantify the CLA content in beef samples. Furthermore, the method proposed herein is simple and does not require sophisticated sample preparation.

KEYWORDS: Nuclear magnetic resonance spectroscopy; conjugated linoleic acid; beef

INTRODUCTION

Conjugated linoleic acids (CLAs) have received special attention in recent years because of their potential human health benefits, which include anticarcinogenic, antidiabetic, antiadipogenic, and antiatherogenic properties and effects on bone mineralization, immune system, and body composition (*1*−*9*). Moreover, it has been recognized that CLAs inhibit chemically induced carcinogenesis in many tissues, including mammary, gland skin, forestomach, and intestine, and it has been shown that CLAs inhibit the proliferation of hepatoma, prostate, colon(*4*), and breast cancer cells in vitro (*5*).

CLAs are a group of linoleic acid isomers naturally found in meat and dairy products that originate from ruminants (*2*−*6*). These fatty acids are synthesized by ruminal bacteria or by the desaturation of *trans*-vaccenic acid in animal tissue via steroyl-CoA-desaturase (*7*). *cis*-9,*trans*-11-CLA is the predominant isomer, representing 75−90% of the total CLA in ruminant fat (*8*). The CLA content is related to various inherent properties of the animals, including breed, age, sex, and type of muscle and also to feeding conditions, including basal diet and lipid supplements (*9*). CLA content can vary by > 10-fold, from approximately 0.12 to 1.25% (g/100 g of fatty acids) (*10*). Research efforts to increase the CLA content in food have been proposed to improve public health (*10*), but it will be necessary to ensure their content in a large number of samples. Each year > 30 million beef cattle are processed by the American food industry and > 250 million are consumed worldwide; therefore, it is important to develop a low-cost and rapid analytical method to measure CLA content in food products. The standard method of measuring CLA content in food is the analysis of fatty acid methyl esters using gas chromatography (GC). This method is quite slow, involves several steps, and takes 1 h to complete. Therefore, to analyze one sample of each animal slaughtered in the United States per year would require > 3000 years when using a single instrument. Aside from the time-consuming and labor-intensive nature of the GC analysis itself, an additional drawback is the need for substantial volumes of organic solvents and corrosive reagents that are difficult to dispose of and hazardous.

In this paper, we describe a method to measure CLA content using proton nuclear magnetic resonance spectroscopy (^{1}H NMR), which can be substantially faster than GC methods. This method can be a quantitative spectroscopic tool because the intensity of a resonance line in the ^{1}H NMR spectrum is directly proportional to the number of resonant nuclei measured (*11*). Furthermore, this method is much simpler and does not require sophisticated sample preparation.

MATERIALS AND METHODS

The *cis*-9,*trans*-11 CLA standard was acquired from Sigma-Aldrich (St. Louis, MO). Beef and subcutaneous samples were acquired in local markets in São Carlos, SP, Brazil. Three samples from sirloin steak (loin), rump cover (loin), silverside (round), and chest lead (chuck) were

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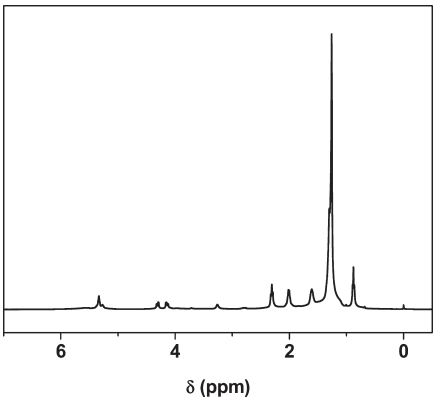

Figure 1. ^{1}H NMR spectrum of sirloin steak fat in $CDCl_3$ at 400 MHz.

analyzed, in addition to 14 samples of beef and subcutaneous fat (SCF) from sirloin steak (loin). The beef and SCF samples were ground and freeze-dried. The fats for GC analyses were extracted with chloroform from 0.3 g of freeze-dried beef (intramuscular fat, IMF) or 0.1 g of SCF. The same amounts of freeze-dried beef and SCF were extracted with 0.8 mL of deuterated chloroform for NMR analysis.

^{1}H NMR Spectra. The NMR spectra of fats were acquired using two NMR instruments: a Varian model INOVA 400 spectrometer with a magnet of 9.4 T (Palo Alto, CA) and an Anasazi EFT90 with a permanent magnet of 2.1 T (Indianapolis, IN). The ^{1}H spectra were performed at 400 MHz in $CDCl_3$ with a 10.5 μs 90° pulse width, a 2 s acquisition time, a 3 s recycle delay, and a 6.4 kHz spectral width. These spectra were acquired using either 4 or 128 scans. The ^{1}H spectra at 90 MHz were performed in $CDCl_3$ with a 23.8 μs 90° pulse width, a 1.7 s acquisition time, and 0.1 s recycle delay using 256 scans.

The CLA was quantified by integrating the peaks areas at 6.30 and 5.90 ppm, using the glycerol peaks at 4.2 ppm as an internal standard with a relative area equal to 100. The quantification limit in NMR is dependent on signal-to-noise ratio that is dependent on the number of scans, flip angle, line broadening, sample concentration, and magnetic field strength (*11*). Using the 400 MHz spectrometer and experimental conditions above, the quantification limit was 0.2 g/100 g of fatty acids.

Chromatography Analysis. Fatty acid content was determined by GC using fatty acid methyl esters that were prepared by a transesterification reaction (*12*). The analyses were performed in a Shimadzu CG-14B model machine equipped with a split (1/100) injector and a flame ionization detector. The capillary column used was a melted silica OMEGAWAX250 (30 m × 0.25 mm × 0.25 μm). The programmed temperature of the column was 50 °C for 2 min followed by a heat increase of 4 °C/min until the temperature reached 220 °C. This temperature was then maintained for an additional 25 min. The injector temperature was 250 °C, and the temperature of the detector was 280 °C. The speed of the gas drag (H_2) was 1 mL/min, and the volume of the injection was 1 μL. The correlation coefficients between NMR and GC and between IMF and SCF CLAs content were calculated on Origin 8.0 software.

RESULTS AND DISCUSSION

Figure 1 shows a typical ^{1}H NMR spectrum of beef fat with aliphatic hydrogen signals from 0.50 to 3.00 ppm and glycerol signals from 4.00 to 4.50 and 5.30 ppm. The unsaturated signal is at 5.34 ppm. The weak signals of hydrogen in the conjugated double bonds of CLA are observed in expansions of the region between 5.00 and 6.50 ppm (**Figure 2**). This figure shows the four NMR signals from unsaturated hydrogen in a sample of pure *cis*-9,*trans*-11-CLA (**a**) and the CLA signals in a sample of beef fat with >1% CLA (**b**) and in a sample of beef fat with <0.5% of CLA (**c**).

Figure 3 correlates the average area of the CLA signals at 6.30 and 5.90 ppm, as normalized by the glycerol signals at 4.20 ppm (area = 100), and the amount of CLA measured by GC. This figure shows a correlation coefficient of 0.97 for 12 samples, indicating the capacity of NMR to quantify the CLA content in the beef samples.

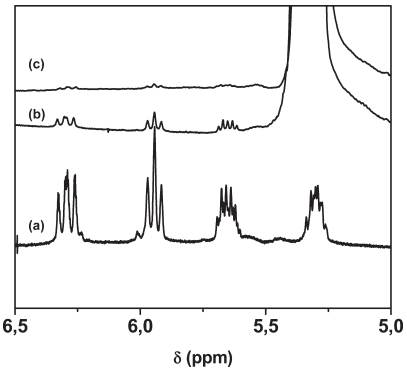

Figure 2. ^{1}H NMR spectra of pure *cis*-9,*trans*-11-CLA (**a**) and of beef fat samples with high (**b**) and low CLA contents (**c**) at 400 MHz.

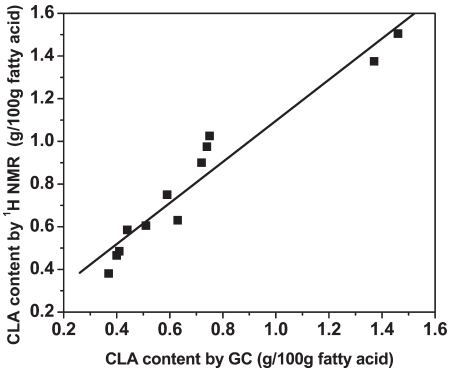

Figure 3. Correlation between CLA content in sirloin steak cuts measured by the GC and ^{1}H NMR methods.

The ^{1}H NMR analyses were performed with 128 scans each, which is approximately 10 min per analysis. In addition, we have also tested this method using only four scans per analysis, which takes approximately 20 s. We verified that it is possible to obtain usable NMR spectra without shimming and locking the magnetic field for every sample. We shimmed the magnet for one sample and then used the same setting for several additional analyses. This is possible because the CLA signals used in our analyses were very well-defined and did not overlap with other signals, even in poorly resolved spectra. In the ^{1}H NMR analysis method, the time-consuming process is the insertion and ejection of the sample, which can be automated (*13*−*15*). With this procedure, the analysis can take only a few minutes for each sample, making it >10 times faster than GC analysis.

We also analyzed the correlation between the CLA content in IMF and SCF obtained from 14 samples of sirloin steak (**Figure 4**). The results show that the average CLA content is about 25% higher in SCF than in IMF. In addition, there was a good correlation (r = 0.85) between the CLA content in SCF and IMF samples, indicating that it is possible to measure the CLA content in SCF and then use these data to estimate the IMF content. This correlation allows for the possibility of analyzing the CLA content in a beef sample by extracting the fat from a small piece (0.1 g) of raw SCF, obtained without drying, using 0.8 mL of deuterated chloroform.

The CLA content in different beef cuts was analyzed by ^{1}H NMR (g/100 g of fatty acids). In the samples analyzed, the highest amount of CLA was observed in sirloin steak (loin) at 1.3 ± 0.5%. We observed intermediate CLA content in rump cover (loin), 0.8 ± 0.3%, and silverside (round), 0.8 ± 0.3%. The lowest content was in chest lead (chuck) at 0.5 ± 0.2%. These differences in CLA content were also reported by Turk and Smith in a recent study about beef produced in the United States (*16*); however, our

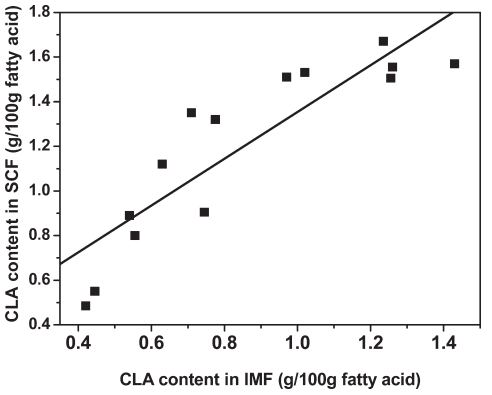

Figure 4. CLA content in subcutaneous (SCF) and intramuscular fat (IMF) in sirloin steak as determined by ^{1}H NMR.

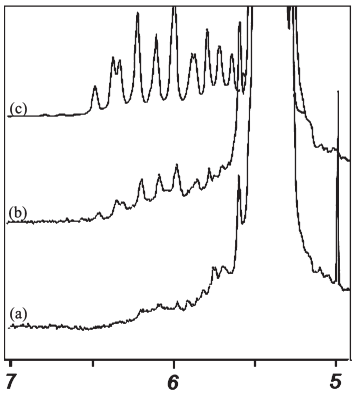

Figure 5. ^{1}H NMR spectra of pure *cis*-9,*trans*-11-CLA (**c**) and of beef fat samples with high (**b**) and low (**a**) CLA contents at 90 MHz.

results show significantly higher CLA content than those observed by Turk and Smith. This difference may be indicative of the diet effect, as most beef cattle in Brazil are pasture-fed and U.S. cattle are fed with silage. Alternatively, this may indicate the need to measure the CLA content in each cut instead of using a single measurement for each animal, which would increase the number of analyses to be performed.

To further lower the cost of the analysis, we investigated the use of a 90 MHz spectrometer based on a permanent magnet. This equipment is 2−3 times cheaper than the 400 MHz instrument and does not have the ongoing maintenance requirements of liquid nitrogen and helium. Because of these considerations the 90 MHz spectrometer is easier to use in an industrial environment. In addition, working at 90 MHz versus 400 MHz allows for a higher throughput of samples.

Figure 5 shows the 90 MHz spectrum of pure *cis*-9,*trans*-11-CLA (**c**). In this spectrum, one can see the second-order coupling of the hydrogen in unsaturated carbon and beef fat samples with high (**b**) and low CLA contents (**a**). Although it is not possible to integrate the CLA signals without the interference of other fat peaks on this instrument, the 90 MHz spectrometer can still be used to grade the CLA content in beef fat according to three general levels, low, medium, and high content, which may be useful for the food industry.

The ^{1}H NMR spectra at 400 and 90 MHz were not able to discriminate the other CLA isomers present in beef fat. However, the CLA isomers can be discriminated by ^{13}C NMR (*17*), which has higher spectral resolution than ^{1}H NMR.

We conclude that ^{1}H NMR can be used as a simple and fast method for measuring CLA content in beef. This method has the potential to be at least 10 times faster than the standard GC method, and it can be used to control and ensure the CLA content not only in beef but also in other food products, including dairy and meat from other ruminants.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; GC, gas chromatography; NMR, nuclear magnetic resonance; SCF, subcutaneous fat; IMF, intramuscular fat.

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