Conjugated α -Linolenic Acid Isomers in Bovine Milk and Muscle

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ABSTRACT

Conjugated linolenic acids (CLnA) are octadecatrienoic fatty acid isomers with at least 2 conjugated double bonds. Various CLnA isomers occur naturally, and some isomers could be formed by ruminants from dietary α -linolenic acid. Ruminant biohydrogenation of polyunsaturated fatty acids gives rise to the formation of numerous metabolites having conjugated or nonconjugated structures. The objectives of this study were to identify and characterize CLnA isomers in milk fat and muscle lipid extracts from cattle fed a high-forage diet. The analysis of total fatty acid methyl esters revealed levels of total CLnA of 0.39% in a single milk lipid extract and 0.34% in a single muscle lipid extract. Fatty acid methyl esters were fractionated by argentation thin-layer chromatography. A fraction containing dienolic fatty acids as well as CLnA isomers was isolated and analyzed. The double bond positions of CLnA isomers (*cis*-9, *trans*-11, *cis*-15 and *cis*-9, *trans*-13, *cis*-15 18:3) were confirmed by mass spectrometry of their 4,4-dimethyloxazoline derivatives. Mass spectra of the *cis*-9, *trans*-13, *cis*-15 18:3 isomer was characterized by an intense ion at *m/z* 236 attributable to the formation of 2 stabilized allylic radical fragments, whereas this intense ion corresponding to the stabilized radical fragments was located at *m/z* 262 for the *cis*-9, *trans*-11, *cis*-15 18:3 isomer. The gap of 12 amu between *m/z* 250 and 262 confirmed the occurrence of a double bond in position Δ^{13} . Configuration of the double bonds of standards having similar mass spectra and gas-liquid chromatographic retention times was confirmed by ¹H nuclear magnetic resonance. We also showed that both CLnA isomers were found in the muscle lipid extract, whereas only the *cis*-9, *trans*-11, *cis*-15 18:3 isomer was

identified in the milk lipid extract. This study appears to be the first to identify 2 CLnA isomers in bovine muscle lipid extract.

Key words: conjugated α -linolenic acid, mass spectrometry, bovine milk, bovine muscle

INTRODUCTION

Conjugated linoleic acids (CLA) appear to have a wide range of health benefits for various metabolic disorders in animal models (Belury, 2002; Nagao and Yanagita, 2005; Bauman et al., 2006; Navarro et al., 2006), whereas the effects in humans are still debated (Wahle et al., 2004; Tricon and Yaqoob, 2006). Among the CLA, 2 major isomers, *cis*-9, *trans*-11 18:2 (rumenic acid), and *trans*-10, *cis*-12 18:2, have been studied extensively. Research reports indicate that the *cis*-9, *trans*-11 18:2 isomer is found in milk and beef to a greater extent than is the *trans*-10, *cis*-12 18:2 isomer (Fritsche et al., 1999). Conjugated linoleic acid in cattle is strongly related to the fatty acid profile of the feed lipids (Chouinard et al., 2001; Noci et al., 2007). Formation of the *cis*-9, *trans*-11 18:2 isomer follows 2 routes. The first is ruminal, in which linoleic acid is converted into rumenic acid by Δ^{12} -*cis*, Δ^{11} -*trans* isomerase (Kepler and Tove, 1967). The second involves the oxidation of vaccenic acid (*trans*-11 18:1) to rumenic acid by Δ^9 -desaturation catalyzed by the stearyl-coenzyme A desaturase present in the mammary gland and in animal tissues (Griinari and Bauman, 1999).

Kepler et al. (1966) demonstrated that α -linolenic acid could be converted into the partially conjugated fatty acid, which was tentatively assigned the structure of *cis*-9, *trans*-11, *cis*-15 18:3. Confirmation of this structure (Destailats et al., 2005b), and the identification of several other minor compounds formed during the biohydrogenation process of α -linolenic acid (Lor et al., 2005a,b) has led to a proposed biohydrogenation pathway (Destailats et al., 2005b). However, some metabolites of this series of reactions remain to be identified, such as the *cis*-9, *trans*-13, *cis*-15 18:3 isomer.

The main objective of this study was to analyze milk and muscle lipid extracts from cattle fed a high-forage

Received March 1, 2007.

Accepted May 24, 2007.

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diet to identify the occurrence of the *cis*-9, *trans*-13, *cis*-15 18:3 isomer and to confirm the proposed biohydrogenation pathway. Pasture grass and grass silage were chosen for their high α -linolenic acid content (Boufaïed et al., 2003). The second objective was to evaluate the concentrations of the *cis*-9, *trans*-11, *cis*-15 and the *cis*-9, *trans*-13, *cis*-15 18:3 isomers in milk and muscle lipid extracts.

MATERIALS AND METHODS

Samples and Reagents

The samples used in this study were obtained from bovines fed a high-forage diet (silage or pasture) containing α -linolenic acid. Bovine muscle lipids were obtained from an Angus steer fed grass silage supplemented with a mineral mix. Silage was made from a grass sward containing orchardgrass, quackgrass, and weeds (70:20:10), and was treated at harvesting with formic acid (2.5 kg/1,000 kg of fresh matter). A section of the right longissimus muscle (from the 10th to 14th ribs) was taken 6 d after slaughter (a time corresponding to the normal aging period before grading of carcasses in the beef industry; Berthiaume et al., 2006), stripped of its fat and superficial muscle tissue, and then ground, vacuum-packed, and frozen at -20°C . Bovine milk fat was obtained from a nursing Simmental $\times$ Hereford crossbred cow (124 DIM) grazing pasture composed of timothy grass, tall fescue, white clover, Kentucky bluegrass, quackgrass, and weeds, supplemented with ground full-fat raw soybeans at the rate of 2 kg/d. A milk sample was obtained by hand milking 16 h after the calf had been separated from its dam; the sample was then frozen at -20°C until further analysis.

A standard mixture of equimolar levels [as determined by both GLC and silver-ion HPLC (**Ag-HPLC**)] of conjugated α -linolenic acids (**CLnA**, *cis*-9, *trans*-11, *cis*-15 and *cis*-9, *trans*-13, *cis*-15 18:3; 75 wt %) was kindly provided by Naturia Inc. (Sherbrooke, Quebec, Canada). 2-Amino-2-methyl-1-propanol was purchased from Aldrich Chemicals (Milwaukee, WI).

Lipid Extraction

Extraction of milk lipids was performed with hexane:isopropanol (3:2, vol/vol) according to a literature procedure (Wolff, 1995) with slight modifications. Briefly, a representative sample (~ 3 g) was dispersed in isopropanol (15 mL) and, following addition of hexane (30 mL), a second dispersion was carried out. The suspension was then filtered, transferred into a separatory funnel, and washed with a saturated aqueous solution of sodium chloride. The organic phase was dried over anhydrous sodium sulfate and filtered, and the solvents

were removed in a rotary evaporator at 45°C under vacuum. The lipid extract was stored at -18°C until used. Extraction of bovine muscle lipids was carried out according to the method of Folch et al. (1957).

Preparation of Fatty Acid Methyl Esters

Bovine milk and muscle lipids were quantitatively extracted and fatty acid methyl esters (**FAME**) were prepared by base-catalyzed methanolysis to prevent degradation of the conjugated fatty acids (Nuernberg et al., 2007). Methylation of fatty acids in the milk and muscle lipid extracts (~ 20 mg in 2 mL of hexane) was carried out in sealed tubes with 0.4 *N* sodium methoxide in methanol (0.5 mL). After homogenization, the mixture was held at 40°C for 15 min, cooled to room temperature, and washed with water, and FAME were extracted with hexane (2×5 mL). The organic extracts were combined, dried over anhydrous sodium sulfate, filtered, and kept under N_2 in closed vials at -18°C until analysis or separation by silver-ion thin-layer chromatography (**Ag-TLC**).

Fractionation of FAME by Ag-HPLC

The CLnA FAME standard mixture was separated by Ag-HPLC to provide pure analytical standards for structural determination. Separation involved the use of a ChromSpher Lipids column impregnated with silver nitrate (Varian, Middleburg, the Netherlands, 5 mm, 250×4.6 mm i.d.). The isocratic mobile phase was composed of 87% hexane and 13% of a mixture of hexane and acetonitrile (100:2, vol/vol), where the solvent containing the acetonitrile was under constant agitation. Elution (1 mL/min) was performed at 30°C with UV detection at 230 nm (Series 1050, Hewlett-Packard, Palo Alto, CA). Fractions of individual CLnA isomers were collected manually.

Fractionation of FAME by Ag-TLC

Fatty acid methyl esters (20 mg) prepared from milk and muscle lipids were fractionated by TLC on silica gel plates impregnated with silver nitrate (Wolff, 1995). Fractionation was performed according to the number and configuration of double bonds by using a mixture of hexane and diethyl ether (80:20, vol/vol) as the mobile phase. At the end of the chromatographic runs, the plates were sprayed with a solution of 2',7'-dichlorofluorescein and viewed under UV light. The mixture of CLnA methyl esters was used as a standard. A band corresponding to diunsaturated fatty acids ($R_f = 0.52$) was scraped off and transferred into a test tube, and methanol (1.5 mL), hexane (2 mL), and an aqueous

solution of sodium chloride (5%, wt/vol; 1.5 mL) were successively added, with thorough mixing after each addition. After being allowed to stand for ~1 min, the hexane phase was withdrawn and the sample was concentrated prior to GLC analysis.

GLC Analysis of FAME

Analysis of total FAME and Ag-TLC fractions was performed on a gas chromatograph (5890 Series II, Hewlett-Packard), equipped with a 60-m fused-silica BPX-70 capillary column (SGE, Melbourne, Australia, equivalent to 70% cyanopropyl; 60 m to 0.25 mm i.d., 0.25 μ m film thickness), and connected to a ChemStation (Hewlett-Packard). Injection (splitless mode) and detection (flame-ionization) were performed at 250°C. Two oven temperature programs were used in this study. The first program, which was used to screen the fatty acid profiles in milk and meat fats and in the Ag-TLC fractions, was as follows: 60°C isothermal for 1 min, increased to 170°C at 20°C/min, and held at this temperature for 45 min. The inlet pressure of the carrier gas (H_2) was 300 kPa at 170°C. A second temperature program was used to separate the 2 CLnA isomers: 120°C isothermal for 180 min, increased to 220°C at 10°C/min, and isothermal for 20 min at this temperature. The inlet pressure of the carrier gas (H_2) was 220 kPa at 220°C.

Preparation of 4,4-Dimethyloxazoline Derivatives

4,4-Dimethyloxazoline (DMOX) derivatives of fatty acids were prepared by using a modified literature procedure (Garrido and Medina, 1994). Fatty acid methyl esters (10 mg) prepared from both milk and muscle lipid extracts were converted into DMOX derivatives by using 2-amino-2-methyl-1-propanol (500 μ L) as reagent under a nitrogen atmosphere at 150°C overnight. The temperature was accurately controlled to prevent sigmatropic rearrangements of conjugated fatty acids, which readily occur at higher temperatures (i.e., $\geq 170^\circ\text{C}$; Destailats and Angers, 2002). After the reaction, the tubes were cooled to room temperature and the DMOX derivatives were extracted by using a mixture of diethyl ether:hexane (1:1, vol/vol; 5 mL) followed by 5 mL of water saturated with sodium chloride. The organic phase was withdrawn and dried over anhydrous sodium sulfate. Samples were adjusted to the appropriate concentration for analysis.

Analysis of DMOX Derivatives by GC-MS

4,4-Dimethyloxazoline derivatives were analyzed by GC-MS [Hewlett-Packard model 6890 Series II gas

chromatograph attached to an Agilent (Palo Alto, CA) model 5973N selective quadrupole mass detector] under an ionization voltage of 70 eV at 230°C, and connected to a computer with a Hewlett-Packard ChemStation. The injector (splitless mode) and the interface temperatures were maintained at 250°C, whereas H_2 was used as the carrier gas under constant flow (1 mL/min). Gas-liquid chromatographic separation was performed on a BPX-70 capillary column (SGE; 60 m to 0.25 mm i.d., 0.25 μ m film thickness). The temperature programming mode was similar to the one used for GLC separation of the 2 CLnA isomers.

Nuclear Magnetic Resonance Analysis

The single standard isomers obtained after successive Ag-HPLC separations (~1 mg) were dried under a stream of nitrogen. Deuterated chloroform ($CDCl_3$, solvent reference $\delta^1H = 7.27$ ppm and $^{13}C = 77.0$ ppm) was used to dissolve the standards before nuclear magnetic resonance (NMR) analysis. Nuclear magnetic resonance 1H and ^{13}C spectra were performed as well as 2-dimensional gradient-selected correlation spectroscopy and gradient-selected heteronuclear single quantum correlation to confirm double bond configurations and the position of individual CLnA standard isomers isolated by HPLC. Nuclear magnetic resonance spectra were obtained on a Varian Inova spectrometer (Varian Inc., Palo Alto, CA), running at 400 MHz for proton analysis and 100 MHz for carbon analysis, and composed of an automated triple broadband lead line with a Z-gradient. The spectra (1H and ^{13}C) of *cis*-9, *trans*-11, *cis*-15 and *cis*-9, *trans*-13, *cis*-15 18:3 fatty acids were similar to those already reported in the literature (Matikainen et al., 2003).

RESULTS AND DISCUSSION

The total CLnA contents were 0.39 and 0.34 g/100 g of total fatty acids in milk and bovine muscle lipid extracts, respectively. In a previous study, the resolution of the 2 CLnA isomers had not been determined (Destailats et al., 2005b) and the single peak containing both CLnA isomers was located after CLA and linoleic acid elution and before 20:1. However, in this study, we developed GLC conditions suitable for the separation of the 2 CLnA isomers (Figure 1). The difficulty of separation might be due to the close similarities of their structure. Nevertheless, the *cis*-9, *trans*-11, *cis*-15 18:3 isomer was predominant in both muscle and milk lipid extracts, whereas the *cis*-9, *trans*-13, *cis*-15 18:3 isomer was identified only in muscle lipid extract. In muscle lipid extract, *cis*-9, *trans*-11, *cis*-15 18:3 represented 68.8% of the total identified CLnA, whereas *cis*-9, *trans*-13, *cis*-15 18:3 accounted for only 31.2%.

Figure 1. Gas-liquid chromatograms obtained by using a 60-m BPX-70 capillary column of (A) standards of *cis*-9, *trans*-11, *cis*-15 18:3 and *cis*-9, *trans*-13, *cis*-15 18:3 fatty acid methyl esters (FAME); (B) silver-ion thin-layer chromatography (Ag-TLC) fraction of FAME prepared from milk lipids and (C) Ag-TLC fraction of FAME prepared from bovine muscle lipids (see the material and methods section for experimental conditions).

We confirmed the structure of both isomers by GLC-MS and compared their mass spectra with those of single CLnA standards. The formal structural confirmation was achieved in part by using GC-MS analysis of the DMOX derivatives (Figure 2) prepared from the milk and muscle lipid extracts and from pure CLnA standards. The use of azo-derivatives is a necessary

step before structural determination of lipid fatty acids by GC-MS, because mass spectra of FAME, the usual derivatives used in GLC fatty acid analysis, lack sufficient information to identify structural isomers. This is mainly due to the carboxyl group being highly sensitive to fragmentation and double bond migration (Christie, 1998). However, stabilization of the carboxyl group by the formation of a derivative containing a nitrogen atom results in mass spectra that allow structural determination for most fatty acids. The mass spectra of DMOX derivatives of trienoic fatty acids (Figure 2) indicated a molecular ion at m/z 331, confirming both the carbon chain length and the number of double bonds. In mass spectrum A the *cis*-9, *trans*-11, *cis*-15 18:3 acid isomer was identified and found to be identical to published data (Winkler and Steinhart, 2001; Destailats et al., 2005b). In mass spectrum B, the intense ion at m/z 236 confirmed the formation of 2 stabilized allylic radical fragments attributable to the bis-methylene interrupted structure (Christie, 1998; Wolff and Christie, 2002). The gap of 12 amu between m/z 250 and 262 showed the occurrence of a double bond in position Δ^{13} , whereas this gap was between m/z 236 and 248 for the other isomer. Moreover, gaps of 12 amu between m/z 196 and 208 and between 276 and 288 confirmed the location of the double bonds in positions Δ^9 and Δ^{15} , respectively (Christie, 1998). A sequential gap of 14 amu from 196 to 126 indicated the presence of methylene groups from C_8 to C_3 . Moreover, the ion intensities between m/z 236 and 262 were very different in both mass spectra, with a ratio of 1:4 for the CLnA standard mixture.

Partial structural determination was reported in the literature for both CLnA isomers, as their 4-methyl-1,2,4-triazoline-3,5-dione adducts, which suggested either a *cis-trans* or a *trans-trans* conjugated system (Destailats et al., 2005a). To confirm the configuration of the conjugated double bond system, we investigated isomer configurations by proton NMR with pure single CLnA isomers isolated by Ag-HPLC. The NMR spectrum of the 9, 11, 15 isomer showed that there were 4 vinylic proton types in the molecule. The first, at $\delta = 5.42$ ppm, is for hydrogen on carbons 9, 15, and 16 of the molecule. Four different hydrogens were observed with a signal appropriate for a more deshielded vinylic proton, which represented a nonsymmetric *cis-trans* system. Vinylic coupling between H-11 and H-12, $J = 15.23$ Hz, indicated a *trans* configuration, whereas coupling between H-9 and H-10, $J = 11.13$ Hz, showed a *cis* configuration. This identification is in agreement with previous reports for *cis*-9, *trans*-13, *cis*-15 18:3 (Matikainen et al., 2003). Moreover, the results of 2-dimensional NMR gradient-selected correlation spectroscopy and gradient-selected heteronuclear single

Figure 2. Mass spectra of the 4,4-dimethyloxazoline derivative of (A) *cis*-9, *trans*-11, *cis*-15 18:3 and (B) *cis*-9, *trans*-13, *cis*-15 18:3 isomers prepared from a bovine muscle lipid extract (see the material and methods section for experimental conditions).

Table 1. Identified monounsaturated, conjugated, and nonconjugated metabolites coming from the biohydrogenation or degradation or both of fatty acids in bovine milk and muscle¹

Fatty acid	Bovine milk	Bovine muscle
Monounsaturated		
18:1 <i>cis</i> -9	27.28	26.90
18:1 <i>trans</i> -11	3.24	1.17
18:1 Δ^{13}	X ²	X
18:1 <i>cis</i> -15	0.16	0.08
Nonconjugated polyunsaturated		
18:2 <i>cis</i> -9, <i>cis</i> -12	2.08	3.07
18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.68	2.02
18:2 <i>trans</i> -11, <i>cis</i> -15	0.25	0.25
18:2 <i>cis</i> -9, <i>trans</i> -13	ND ³	X
Conjugated polyunsaturated		
18:2 <i>cis</i> -9, <i>trans</i> -11	2.53	0.33
18:2 <i>trans</i> -13, <i>cis</i> -15	ND	ND
18:3 <i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15	0.39	0.23
18:3 <i>cis</i> -9, <i>trans</i> -13, <i>cis</i> -15	ND	0.11

¹Data are qualitative and are expressed in grams per 100 g.

²X = peak identified by GC-MS by using the long temperature program, but the exact concentration is not known because of fractionation by thin-layer chromatography.

³ND = not detected.

quantum correlation experiments confirmed both position and configuration of the *cis*-9, *trans*-11, *cis*-15 and the *cis*-9, *trans*-13, *cis*-15 18:3 double bond systems in CLnA.

Modification of the fatty acid composition of ruminant milk and meat fats according to seasonal and geographical parameters has been well documented (Wolff, 1995). Looor et al. (2005a) previously demonstrated an increased level of biosynthesis of the intermediate *cis*-9, *trans*-13 18:2 in lactating cows fed linseed oil containing more than 50% of α -linolenic acid. In the present study, we identified intermediates originating from ruminal biohydrogenation of α -linolenic acid, completing the biohydrogenation pathway (Table 1) proposed by Destailats and coworkers (2005b). We confirmed by GC-MS the occurrence of *cis*-9, *trans*-11 18:2 and *trans*-11, *cis*-15 18:2 isomers in bovine muscle and milk, whereas the *cis*-9, *trans*-13 18:2 isomer was exclusively identified in the bovine muscle lipid extract. We were unable to detect the isomer *trans*-13, *cis*-15 18:2 CLA in milk and in meat. This could be explained by the low production of this isomer from α -linolenic acid in the rumen or the high reduction rate to octadecenoate or to stearate in the rumen, or both. We also confirmed the occurrence of 18:1 Δ^{13} and *cis*-9, *trans*-13 18:2 in the samples by using TLC and GC-MS. However, we did not succeed in determining their concentrations, mainly due to sample manipulation related to their separation by TLC. The *trans*-13, *cis*-15 18:2 isomer has previously been synthesized and analyzed by Ag-HPLC and GLC, as reported by many authors (Belury, 2002; Delmonte et al., 2005;

Bauman et al., 2006). However, Lock and Bauman (2004) reported that this fatty acid does not occur in milk lipids, which reinforces the argument that CLA intermediates are not accumulated and are readily reduced by rumen bacteria. Moreover, the *cis*-9, *trans*-13, *cis*-15 18:3 isomer produced in the rumen and detected in the muscle did not appear to be present in detectable amounts in milk lipids under the present analytical conditions.

In conclusion, we showed the occurrence of the 2 CLnA isomers in a muscle lipid extract, whereas the *cis*-9, *trans*-13, *cis*-15 18:3 isomer was detected only in bovine muscle lipids. We characterized the double bond positions by GC-MS and confirmed the configuration of the conjugated diene system by NMR. Further investigations are required to confirm the absence of the *cis*-9, *trans*-13, *cis*-15 18:3 isomer in a milk lipid extract from cows fed a high-forage diet or α -linolenic acid.

ACKNOWLEDGMENTS

The authors are grateful to Fonds Québécois de Recherche sur la Nature et les Technologies (FQRNT) and Naturia Inc. (Sherbrooke, Canada) for a PhD scholarship to M. Plourde. They also wish to thank Rachel Gervais for her assistance regarding the bovine milk and muscle samples.

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