Gas Chromatographic Method for Analysis of Conjugated Linoleic Acids Isomers (c9t11, t10c12, and t9t11) in Broth Media as Application in Probiotic Studies

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Abstract

A gas chromatography (GC) procedure is assayed for analysis of conjugated linoleic acid (CLA) isomers *cis*-9, *trans*-11-octadecadienoic (c9t11); *trans*-10, 12 *cis*-octadecadienoic (t10c12); and *trans*-9, *trans*-11-octadecadienoic (t9t11) in culture broth by GC using NaOH–BF3 in methanol for methylating and a long capillary (100 m) high-polarity column. Repeatability of the method is assessed; the coefficient of variation for CLA isomers ranges from 4.62 for c9t11 to 8.19 for t9t11. Recovery ranges between 88.01 and 89.76, with a mean value of 89.06 for all CLA isomers studied. This method may be considered advantageous for analysis of CLA isomers in probiotics cultures samples.

Introduction

Conjugated linoleic acid (CLA) is a generic name for a mixture of isomers of linoleic acid with conjugated double bonds. The configuration of the double bonds may exhibit several possible positions and different geometric isomers (*cis* or *trans*). Double bounds of CLA are mainly found at positions 9 and 11 or 10 and 12 (1), whereas isomers having double bonds at other positions have also been reported (2). CLA has gained considerable attention in recent years because of its many beneficial effects, including anticarcenogenic activity (3,4), antiatherogenic activity (5,6), the ability to reduce the catabolic effects of immune stimulation (7,8), and the ability to reduce body fat (6). Of the individual isomers of CLA, c9t11-octadecadienoic acid has been implicated as the most biologically active because it is the predominant isomer incorporated into the phospholipids of cell membranes (9). The c9t11-CLA isomer is also the predominant isomer found in the diet.

Food products from ruminants, particularly dairy products, are the major dietary source of CLA for humans. CLAs are intermediates in the biohydrogenation of linoleic acid, and it is generally accepted that CLAs in ruminants originate from the incomplete biohydrogenation of unsaturated fatty acid by rumen bacteria (10). However, it has been demonstrated that cows can also synthesize CLA from *trans*-11-octadeceno acid, another intermediate in the rumen biohydrogenation process (11). Dietary sources of CLA include milk fat, natural and processed cheeses, meat products, and plant oil (12,13). Animal sources are richer in CLA than plant sources, and, in general, foods from ruminants contain more CLA than foods from nonruminants, and considerable research has been conducted on the CLA content and isomer distribution in cow's milk. A number of factors have been shown to influence CLA concentration in bovine milk fat, including lactation number (14), dietary restriction (15), and feed allowance (15,16). Dairy products are one of the major dietary sources of CLA. Among the different isomers of CLA, c9t11-octadecadienoic represents more than 90% of the total CLA in milk fat. In general, the analysis for CLA isomers requires their conversion to derivates that can be separated from the other fatty acids using gas chromatography (GC) (17,18), silver-ion high-performance liquid chromatography (HPLC) (19,20), or Fourier transform IR spectroscopy for measuring total CLA isomers (21,22). Thus, it is important to have standardized methodologies for separating and quantitating individual CLA isomers in complex mixtures such as foods, biological materials, and manufactures products. The aim of this work describes a GC method for the determination of CLA isomers (c9t11, t10c12, and t9t11) in MRS broth with the purpose of studying the capacity of different probiotics lactic acid bacterias to produce CLA. The repeatability and recovery of the analytical procedure were also assessed.

Experimental

Standards and reagents

Heptadecadienoic acid, Tween 80, and 14% boron trifluoride in

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methanol were supplied by Sigma Chemical Co. (St. Louis, MO). Conjugated high purity (> 98%) linoleic acid standards (c9t11, t10c12, and t9t11) and linoleic acid were purchased from Matreya (State College, PA). All reagents and organics solvents used were analytical grade.

MRS broth

MRS broth (Difco Laboratories, Detroit, MI) supplemented with different concentrations of CLA isomers (10, 25, 100, and 250 µg/mL) and linoleic acid (0.2%) was added as aqueous solution in 1% (v/v) Tween 80 and was incubated at 37ºC for 24 h.

Lipid extraction

Following incubation, the samples were centrifuged at 23,500 × *g* for 10 min at 5ºC, after centrifugation the pellet was discarded, and 6 mL of supernatant was taken for lipid extraction. The culture medium was mixed with 60 µL heptadecanoic (64.4 mg heptadecadienoic–10 mL hexane) and 12 mL of isopropanol by vigorously shaking; 9 mL of hexane was then added, and the mixture was shaken for 3 min before being centrifuged at 4000 rpm for 5 min at 5ºC. The upper layer was then collected and filtered through sodium sulphate in a filter paper. After filtration, the filter was also washed with 7 mL of hexane. The lipid fraction of the sample was collected in a 25-mL pear-shaped flask and dried under nitrogen gas at 70ºC in a Zymartk Turbo Vap LV evaporator (Hopkinton, MA).

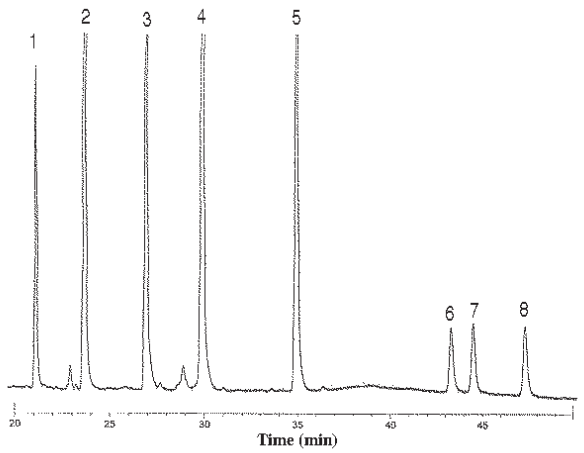

Figure 1. Representative GC chromatogram of CLA isomer (c9t11, t10c12, and t9t11) in MRS broth. Peak identifications: (1) C16:0, (2) C17:0, (3) C18:0, (4) C18:1, (5) C18:2, (6) c9t11, (7) t10c12, and (8) t9t11.

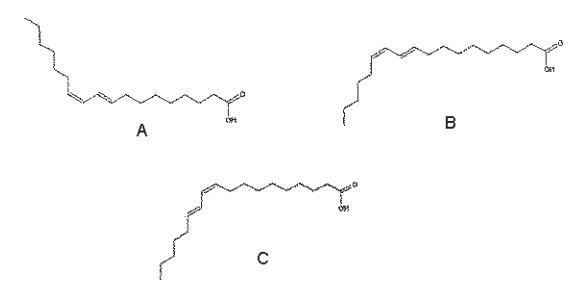

Figure 2. Geometrical and positional CLA isomers: (A) c9t11, (B) t10c12, and (C) t9t11.

Preparation of fatty acid methyl ester

Methanolic sodium hydroxide (1N) (100 µL) was added to the lipid fraction, and the mixture was stirred for 1 min and left at 70ºC in a water bath for 15 min. The free fatty acids were next methylated with 200 µL of 14% boron trifluoride in methanol at room temperature for 30 min. Then, 0.2 mL hexane and 0.1 mL water were added, samples were vortexed thoroughly, and centrifuged at 1000 rpm for 5 min. The organic layer was stored in a vial at –20ºC for further quantitation by gas–liquid chromatography (GLC).

GC analysis

CLA methyl esters were analyzed on a Hewlett-Packard 5890 (Palo Alto, CA) equipped with a flame ionization detector. Analyses were performed with a CP Sil 88 column (100-m × 0.25-mm i.d.) containing 100% cyanopropyl siloxane stationary phase (0.20-µm film thickness) (Chrompack, Varian, Walnut Creek, CA) for the separation of the CLA isomers. The conditions used were 190ºC isothermal temperature, 250ºC injector temperature, and 250ºC detector temperature. The split ratio was 1:50, and the carrier gas was helium with a head pressure of 28 psi. The injection volume was 1 µL, and the CLA peaks were identified by comparison with the retention times of the reference standards. The areas of CLA peaks were calculated as µg CLA/mL culture using heptadecanoic acid as the internal standard for determining the content of the CLA isomers.

Results and Discussion

The GLC technique using long capillary columns has been successful in quantitating total CLAs as fatty acid methyl esters (FAME). This method separated, with a good resolution, CLA isomers, *cis/trans*, and *trans/trans* isomers based on the chain length and number of double bonds. The advantages of methylating CLA are that the FAME derivates allow for GLC identification and quantitation of individual CLA isomers. The FAME including the CLA can be quantitated by GLC by adding an internal standard such as heptadecanoic acid (C17:0) into the lipid mixture before methylation (23). The methylation procedure chosen will depend on the type and chemical composition of material to be methylated. In our study, we used two stages for methylation, first an alkali-catalyzed hydrolysis using NaOH–methanol for esterified CLA isomers, followed by BF3–methanol-catalyzed methylation for free CLA isomers, which appears to have the advantage for methylating CLA isomers. It did not isomerize conjugated double bonds (24,25). Figure 1 shows a chromatogram of CLA FAME of a mixture of CLA standards in broth media, and Figure 2 shows the geometrical and positional conjugated linoleic acid isomers c9t11, t10c12, and t9t11.

Heptadecanoic acid, which is not present in the sample, is well defined as the internal standard and does not interfere with a correct quantitation of the different CLA isomers. The response factors were calculated in a mixture of c9t11, t10c12, and t9t11 with a ratio of 1:1 (w/w) with respect to the internal standard heptadecanoic acid. The response factors were 0.96, 0.94, and 0.91, respectively.

The repeatability of the analytical method for CLA isomers (c9t11, t10c12, and t9t11) was tested in a media broth using heptadecanoic acid as the internal standard. Table I shows the individual mean values and standard deviations of five replicate analyses of media broth for the determination of CLA. Under our conditions, the method offered a good repeatability with coefficient of variation (CV) ranges from 4.62 for c9t11 to 8.19 for t9t11. For recovery analysis, known amounts of the individual CLA isomers were added to a culture broth sample in which individual CLA had been determined previously using heptadecanoic acid as the internal standard, substracting the initial amounts of each individual CLA isomers (c9t1, t10c12, and t9t11) present in the sample (0.8, 0.5, and 0.4 µg of CLA/mL of MRS, respectively). Experiments were conducted in triplicate. Table II shows the initial CLA as determined by the proposed method and the percentage recovery values. The overall mean recovery calculated was in the range 88.01–89.76%; the recoveries were good being higher than 89%. The values found in our study were slightly higher than those reported by Kin et al. (11) using KOH–ethanol and HCl as the methylation reagent. They found 83% recovery of CLA and had a CV 6.6 for repeatability using a capillary GLC analysis. However, similar results were reported by Jiang et al. (26). The recovery of the CLA for the analytical procedure was 89.4%, and the coefficient of variation for the overall reproducibility of the CLA quatitation method was 3.6% using KOH–ethanol and HCl as the reagent for methylation with HPLC–GLC analysis. It was, therefore, that under the given conditions the GLC method assayed in our study does not appreciably understimate for individual CLA-octadecanoic isomers. In conclusion, the GLC method assayed in this work using NaOH–BF3 in methanol as methylation reagents with a long capillary column offer a good repeatability and recovery for determining individual CLA-ctadecadienoic isomers (c9t11, t10c12, and t10t12) in culture media and may be considered advantageous for analysis of CLA isomers in probiotics cultures samples.

Table I. Repeatability of CLA Isomers (c9t11, t10c12, and t9t11) Spiked in Broth Media

CLA isomers	Concentration (µg/mL)*						
	1	2	3	4	5	X†	RSD (%)‡
c9t11	9.71	9.34	10.29	9.90	10.51	9.95	4.62
t10c12	6.32	5.85	6.80	5.91	6.28	6.23	6.10
t9t11	4.21	3.83	3.98	4.50	3.64	4.03	8.19

* Mean value of three replicates.
† Mean values.
‡ Real standard deviation.

Table II. Recovery of CLA Isomers (c9t11, t10c12, and t9t11) in Broth Media*

CLA isomers	Recovery (ug/mL)†					
	10‡	25‡	100‡	250‡	X (%)§	RSD (%)**
c9t11	8.90	22.04	89.72	230.35	89.76	1.91
t10c12	8.11	22.18	89.53	231.98	88.01	5.67
t9t11	8.53	22.50	91.39	228.97	89.50	3.44

* Mean value of three replicates.
† Values adjusted for background amounts in broth.
‡ Amounts added to broth.
§ Mean values.
** Real standard deviation.

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References

1. Y.L. Ha, N.K. Grimm, and M.W. Pariza. Anticarcinogenics from fried ground beef: heat-altered derivatives of linoleic acid. *Carcinogenesis* **8:** 1881–87 (1987).
2. W.W. Christie, G. Dobson, and F.D. Gunstone. Isomers in commercial samples of conjugated linoleic acid. *Lipids* **32:** 1231 (1997).
3. P.C. Bayoval, C. Corre, C. Dupuis, and E. Roussel. Effects of free fatty acids on propionic acid bacteria. *Lait* **75:** 17–29 (1995).
4. Y.C. Ha, J.M. Storkson, and M.W. Pariza. Inhibition of benzo (a) pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res*. **50:** 1097–1101 (1990).
5. K.N. Lee, D. Kritchevsky, and M.W. Pariza. Conjugated linoleic acid and atherosclerosis in rabbit. *Atherosclerosis* **108:** 19–25 (1994).
6. M.W. Pariza, Y. Park, M. Cook, K. Albrigh, and W. Liu. Conjugated linoleic acid (CLA) reduces body fat. *FASEB J.* **10:** A560 (1996).
7. M.E. Cook, C.C. Miller, Y. Park, and M.W. Pariza. Inmune modulation by altered nutrient metabolism: nutritional control of inmune-induced growth depression. *Poult. Sci.* **72:** 1301–1305 (1993).
8. T.Y. Lin, C.W. Lin, and C.H. Lee. Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic. *Food Chem*. **67:** 1–5 (1999).
9. C.M. Ip, M. Singh. H.J. Thompson, and J.A. Scimeca. Conjugated linoleic acid suppreses mammary carcinogenesis and proliferative activity of the mammary gland in rat. *Cancer Res*. **54:** 1212–15 (1994).
10. M.L. Kelly, J.R. Berry, D.A. Dwyer, J.M. Griinari, P.Y. Chouinard, M.E. Van Amburgh, and D.E. Bauman. Dietary fatty acid sources affect conjugated linoleic acid concentrations in milk from lactating dairy cows. *J. Nurt.* **128:** 881–85 (1998).
11. Y.J. Kim and R.H. Liu. Selective increase in conjugated linoleic acid in milk fat by crystallization. *J. Food Sci*. **5:** 792–95 (2000).
12. Y.L. Ha, N.K. Grimm, and M.W. Pariza. Newly recognized anticarcinogenic fatty acids: identification and quantification in natural and processed cheeses. *J. Agric. Food Chem.* **37:** 75–81 (1989).
13. N.C. Shanta, E.A. Decker, and Z. Ustunol. Effect of processing conditions and food additives on the formation of conjugated linoleic acid in processed cheese. *Inst. Food Technol.* **A57:** 139–41 (1991).
14. C.F. Stanton, F. Lawless, G. Kjellmer, D. Harrington, R. Devery, J.C. Connoly, and J.J. Murphy. Dietary influences on bovine milk *cis*-9, *trans*-11-conjugated linoleic acid content. *J. Food Sci.* **62:** 1083–86 (1997).
15. C. Ip, S.F. Chin, J.A. Scimeca, and M.W. Pariza. Mammary cancer prevention by conjugated dienoic derivatives of linoleic acid. *Cancer Res.* **51:** 6118–24 (1991).
16. F. Lawless, J.J. Murphy, D. Harrington, R. Devery, and C. Stanton.

Elevation of conjugated *cis*-9, *trans*-11-octadecadienoic acid in bovine milk because of dietary sulementation. *J. Dairy Sci.* **81:** 3259–67 (1998).
17. S.A. Werner, L.O. Luedecke, and T.D. Shultz. Determination of conjugated linoleic acid content and isomers distribution in three cheddar type cheeses: effects of cheeses cultures, processing, and aging. *J. Agric. Food Chem.* **40:** 1817–21 (1992).
18. F. Lavillonniere, J.C. Martin, P. Bougnoux, and J.L. Sebedio. Analysis of conjugated linoleic acid isomers and content in French cheeses. *J. Am. Oil Chem. Soc.* **75:** 343–52 (1998).
19. N. Sehat, M.P. Yurawecz, J.A.G. Roach, M.M. Mosoba, J.K.G. Kramer, and Y. Ku. Silver ion high performance liquid chromatographic separation and identification of conjugated linoleic isomers. *Lipids* **33:** 217–21 (1998).
20. P. Juaneda, J.L. Sebedio, and W.W. Christie. Complete separation of geometrical isomers of linoleic acid by high performance liquid chromatography with silver ion column. *J. High Res. Chromatogr.* **17:** 321–24 (1994).
21. M.M. Mossoba and R.E. McDonald. Applications of capillary GC-FTIR spectroscopy to fatty acid analysis. *Inform* **4:** 854–59 (1993).
22. L. Alonso, M.J. Fraga, M. Juárez, and P. Carmona. Fatty acid composition of Spanish shortenings with special emphasis on trans unsaturation content as determined by fourier transform infrared spectroscopy and gas chromatography. *J. Am. Oil Chem. Soc.* **79:** 1–6 (2002).
23. M.P. Yurawecz, K.G. John, and Y. Ku. *Advances in Conjugated Linoleic acid Research*, Vol. 1. AOCS Press, Champaign, IL, 1996, pp. 64–82.
24. S. Koritala and W.K. Rohwedder. Formation of an artifact during methylation of conjugated fatty acids. *Lipids* **7:** 274–78 (1972).
25. P. Yurawecz. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total. *Lipids* **32:** 1219–28 (1997).
26. J. Jiang, L. Bjoerck, R. Fonden, and M. Emanuelson. Occurence of conjugated cis-9, trans-11- octadecanoic acid in bovine milk: effects of feed and dietary regimen. *J. Appl. Microbiol.* 8**5:** 95–105 (1996).

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