Determination of Conjugated Linoleic Acid (CLA) Concentrations in Milk Chocolate

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The fatty acids from a series of milk-chocolate-based confectionery samples were analyzed as methyl esters by GC to determine the presence and amount of conjugated linoleic acid (CLA). A single peak corresponding to the 9-*cis*,11-*trans* isomer and ranging from less than 0.1% to nearly 0.2% of the total fatty acids, corresponding to up to 0.3 mg per g of chocolate, was observed. One of the chocolate extracts and a milk extract were subjected to silver ion HPLC and GC-MS in order to confirm the identity of the major isomer and tentatively identity minor isomers.

Keywords: *Chocolate; confectionery; CLA; conjugated linoleic acid; lipid analysis; GC*

INTRODUCTION

Since the discovery that CLA has potential health benefits, notably anticarcinogenic activity, there has been great deal of interest in the quantitation of conjugated linoleic acid in various foodstuffs (*1*). CLA is not a single compound but the collective name for several geometrical and positional isomers of an 18-carbon conjugated dienoic acid. Both the c*is*-9,*trans*-11 and *trans*-10,*cis*-12 isomers are biologically active, but they induce different effects (*2*). The same isomers are the major products formed from alkaline isomerization of high linoleate vegetable oils. *cis*-9,*trans*-11-Octadecadienoic acid (18:2) is the major natural isomer, and there have been numerous reports of its presence in various foods including meat, cheese, milk, and others, but nothing in milk chocolate products (*3*−*6*). Cocoa and its resulting products provide a complex matrix for analysis, with an extensive chemical composition making for a potentially challenging analytical protocol.

This note describes the determination of CLA in selected confectionery products.

MATERIALS AND METHODS

Most standards and reagents were purchased from Sigma-Aldrich Company Ltd. (Poole, U.K.). Solvents were HPLC or Distol grades and were supplied by Fisher Scientific U.K. (Loughborough, U.K.). 2-Amino-2-methyl-1-propanol was from Janssen Chimica (Hyde, U.K.), and c*is*-9,*trans*-11 18:2 methyl ester was from Matreya Inc. (Pleasant Gap, PA). Samples used in this study were obtained from Hershey Foods Corporation and are commercially available milk chocolates.

Extraction of Fat. Approximately 10 g of chocolate was ground and Soxhlet extracted with isohexane (2 × 250 mL) for 2 × 1.5 h. The bulk of the solvent was removed by rotary evaporation, and then the sample was dried under nitrogen to constant weight.

Derivatization Procedures. The fatty acids were released from the extracted fat samples as methyl esters by sodium methoxide-catalyzed transesterification. The methyl esters were converted to dimethlyoxazoline (DMOX) according to the method of Fay and Richli (*7*).

Silver Ion High-Performance Liquid Chromatography. A Spectra-Physics model 8700 solvent delivery system was used (Spectra-Physics Ltd, St. Albans, U.K..), together with a Cunow model DDL 21 detector (Severn Analytical Ltd, Shefford, U.K.). A stream-splitter (approximately 10:1) was inserted between the column and the detector to enable collection of fractions. A column (4.6 × 250 mm) of Nucleosil 5SA (HPLC Technology Ltd, Macclesfield, U.K.) was converted to the silver ion form as described by Christie (*8*). For micropreparative purposes, 0.5 mg of methyl esters was applied to the column in 5 μL of 1,2-dichloroethane. The column was eluted with a gradient of dichloromethane−1,2-dichloroethane−acetonitrile (50:50:0.001, by volume) (Solvent A) and dichloromethane−1,2-dichloroethane−acetonitrile−methanol (45:45:5:5, by volume) (Solvent B). After 20 min of 100% A, there was a linear gradient to 50% A−50% B over 5 min, and 50% A−50% B was maintained for 15 min. The flow rate was 1 mL min^{-1}. A mixture containing methyl stearate, methyl oleate, and 9*c*,11*t*-octadecadienoic acid methyl ester was run as a standard.

Analytical Gas Chromatography. Total fatty acid methyl esters of the extracted fat were analyzed on a Hewlett-Packard model 5890 Series II capillary gas chromatograph linked to an HP 3365 Chemstation (Hewlett-Packard Ltd., Stockport, U.K.). The instrument was fitted with a split/splitless injector (a split ratio of 50:1 was used) and a capillary column of fused silica coated with CP-Sil 88 (0.25 mm i.d. × 100 m in length, 0.2 μm thickness; Chrompack U.K. Ltd, London). The temperature was held at 160 °C for 3 min, temperature-programmed at a rate of 2 °C min^{-1} to 220 °C, and then held at this temperature for an additional 20 min.

Hydrogen was the carrier gas at a flow rate of 1 mL min^{-1}, and pressure programming was used in constant-flow mode.

Gas Chromatography-Mass Spectrometry. Fatty acid DMOX derivatives were subjected to GC-MS on a Hewlett-Packard 5890 Series II Plus gas chromatograph, fitted with an on-column injector and a BPX70 (0.32 mm × 50 m, 0.25 μm) capillary column, connected to a Hewlett-Packard 5989B MS Engine quadrupole mass spectrometer. The column temperature was held at 80 °C for 3 min, temperature-programmed to 160 °C at a rate of 20 °C min^{-1} and then to 260 °C at a rate of 2 °C min^{-1}. Helium was the carrier gas at a

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Table 1. Fatty Acid Composition of Milk Chocolate and Milk Fat Samples

	milk chocolate sample						milk
sample no.	1	2	3	4	5	6	fat
extracted fat (%)	19.3	19.6	17.3	24.6	21.5	14.3	100.0
6:0−12:0	1.6	0.8	1.0	1.5	1.4	1.2	7.9
14:0	2.1	0.9	1.3	1.8	1.7	1.5	9.9
16:0	25.6	25.2	25.4	25.4	25.3	25.4	28.8
16:1	0.5	0.3	0.4	0.5	0.4	0.4	1.7
18:0	31.4	33.8	32.9	31.4	31.6	32.5	13.0
18:1	31.0	32.2	31.9	31.3	31.3	31.6	21.4
18:2	2.9	3.0	3.0	3.0	2.9	3.1	2.9
18:3α	0.3	0.2	0.3	0.3	0.3	0.3	0.6
20:0	1.0	1.1	1.1	1.0	1.0	1.0	0.2
other	3.4	2.4	2.6	3.6	4.0	2.9	13.1
CLA	0.18	0.09	0.07	0.15	0.13	0.12	0.54

flow rate of 2 mL min^{-1}, and pressure programming was used in constant-flow mode. The mass spectrometer was operated in electron impact mode at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

A single CLA peak with a retention time similar to that of a 9-*cis*,11-trans 18:2 methyl ester was detected by GC analysis of the total fatty acid methyl esters derived from the extracted fat of the chocolate samples. The amount varied from 0.07 to 0.18% of the total fatty acid methyl esters (Table 1). The dairy fat sample contained 0.54% of this component.

One of the chocolate samples (sample 1) and the dairy fat were subjected to further study. By GC analysis in the presence of an internal standard (methyl tricosanoate added to the fat prior to transesterification), 1.4 and 4.1 mg of CLA per g of fat, respectively, were measured. Therefore, the chocolate contained 0.3 mg of CLA per g of chocolate.

The position of the double bonds in CLA can be determined from the mass spectra of the DMOX derivatives (*9*). The presence of 9,11−18:2 was verified in both samples by GC-MS of the DMOX derivatives of the total fatty acids. The diagnostic features of the mass spectrum were a molecular ion at *m*/z 333, gaps of 12 amu between *m*/*z* 196 and 208 and between *m*/*z* 222 and 234 to locate the double bond positions, and intense allylic cleavage ions at *m*/*z* 182 and 262 (and an intense ion at *m*/z 276). The enhancement of the ions at *m*/*z* 234 (*10*) and *m*/*z* 248 (*11*) at the front and tail ends, respectively, of the 9,11−18:2 peak indicated the possible presence of 7,9- and 8,10- isomers in at least the dairy fat. Cis/trans CLA fractions were concentrated from both samples by silver ion HPLC and were analyzed by GC-MS as the DMOX derivatives; the chocolate sample was also shown possibly to contain 7,9- and 8,10−18:2 isomers. *trans*-7,*cis*-9 18:2 has been detected in cow milk as well as cheese, beef, human milk, and adipose tissue (*10*), and *trans*-8,*cis*-10 18:2 has been found in cheese (*11*). The presence of other minor CLA isomers cannot be ruled out, but the amounts in the total fatty acid samples were too small to be detected.

These results demonstrate the existence of CLA in samples of milk-based chocolate products using a variety of analytical techniques. A sample of dairy fat was also analyzed, with the concentration reported within the range reported by other investigators (*12*). These initial data should be useful in clarifying the position of milk chocolate's contribution of CLA in the overall diet.

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