The objective of this work was to obtain concentrated natural conjugated linoleic acid (CLA) from milk fat by urea crystallization. Milk fat was hydrolyzed to provide free fatty acids, followed by crystallization with different ratios of urea. The profiles of fatty acids achieved by urea crystallization showed different fatty acid compositions. Long-chain unsaturated fatty acids, including CLA, were concentrated after crystallization. The highest amount of CLA was achieved by the first crystallization with a 2:1 urea/fatty acid ratio. CLA was elevated 2.5-fold. The C18:1/C18:0 fatty acid ratio was increased from 2 to 51, and stearic acid (C18:0) was decreased 17-fold.

Key Words: conjugated linoleic acid, crystallization, milk fat, dairy, functional food

INTRODUCTION

FOOD IS NO LONGER PERCEIVED ONLY AS a collection of nutrients, but also as having effects on human health and disease prevention (Stanton et al., 1997). Almost 35% of all cancer mortality in the United States has been associated with diet (Doll and Peto, 1981). Thus, dietary modification is a practical strategy for the prevention of cancer. Dietary recommendations include reduced animal fat consumption, which has declined during the past 20 years. There has been, however, an increase in consumption of fats from plant sources. Some animal-derived fatty acids, including conjugated linoleic acid (CLA), and the n-3 polyunsaturated fatty acids (PUFAs), such as α-linolenic (18:3, n-3), eicosapentaenoic (EPA; 20:5, n-3), and docosahexaenoic (DHA; 22:6, n-3), appear to be associated with the inhibition of carcinogenesis (Ha et al., 1987; Ip et al., 1991; Layton et al., 1995). Among them, CLA has been suggested to be more effective than the n-3 fatty acids and to be one of the most effective known dietary anticarcinogenic compounds from animal sources (Ip et al., 1994). CLA has been shown to inhibit cancers in several different animal tissues including mammary (Ip et al., 1991, 1996), colon (Liew et al., 1995), forestomach (Ha et al., 1987), and skin (Belury et al., 1996). Epidemiological evidence suggests that increased milk consumption has been linked to a reduced breast cancer risk (Knekt et al., 1996). Moreover, CLA can lead to modified plasma lipids and lipoprotein cholesterol levels. A CLA-enhanced diet reduced low-density lipoprotein (LDL) in rabbit plasma (Lee et al., 1994) and fatty streak formation in hamsters (Nicolosi et al., 1997). This suggested that CLA may have an effect on the prevention of human atherosclerosis. In addition, pigs fed CLA deposited less subcutaneous fat and gained more lean tissue than those fed sunflower oil, suggesting that CLA may also function as a fat-and-lean repartitioning agent (Dougan et al., 1997).

CLA refers to a group of naturally occurring isomers of linoleic acid containing a conjugated double bond system between carbon numbers 9 and 12. CLA occurs predominantly in animal fats of milk and meat from ruminants. Only cis-9, trans-11 octadecadienoic acid has been shown to incorporate into the forestomach phospholipids (Ha et al., 1990) and rat mammary tumor (Ip et al., 1991), suggesting that the cis-9, trans-11 isomer may be the only active isomer in animal fat. The cis-9, trans-11 isomer is hypothesized to be generated by the intestinal flora of ruminants (Kepler et al., 1966). The trans-10, cis-12 isomer has also been shown to incorporate into the hepatic tissue when mice were fed a mixture of CLA isomers (Belury and Kempa, 1997). In humans, the main dietary sources of CLA are dairy products, which contain about 3 to 8 mg of CLA/g of fat (Lin et al., 1995). Ip et al. (1994) showed that 0.1% CLA in a diet was sufficient to result in a 50% reduction in mammary tumor yield in rats given a low dose of a carcinogen. A 350-g rat fed a diet with 0.1% CLA would consume about 0.015 g of CLA/day. This would be equivalent to a daily CLA intake of 3 g for a 70-kg human, about three times higher than the estimated normal consumption of 1 g of CLA/person/day (Ha et al., 1989). Therefore, an increase of CLA concentration in dairy products may help consumers in the prevention of cancer.

Milk fat is a mixture of triglycerides containing different fatty acids, characterized by the carbon chain length and number of double bonds. About 60% to 65% (by weight) of the fatty acids in milk fat are saturated (Haraldsson, 1984). Accordingly, the CLA level was hypothesized to be increased via selective elimination of the saturated fatty acids in the milk fat by crystallization.

Many studies with synthetic CLA have shown anticancer activity in animals. Such CLA contained 40% of active isomer (cis-9, trans-11). The composition of natural CLA is different from that of synthetic CLA. Natural CLA in the milk fat contains about 5 mg of CLA/g fat, with 90% from cis-9, trans-11 (Ip et al., 1991). Little research has been published to determine the effects of natural CLA due to its low concentration in common foods. In addition, dairy foods traditionally have been regarded mainly as important sources of nutrients in the western diet to provide protein, energy, calcium, and vitamins. However, there has been a considerable decrease in overall consumption of dairy foods during the past 20 years (Kinsman et al., 1986). Consumption of whole milk (3.5% fat) and butter has declined while consumption of low-fat products has increased (McBean et al., 1986). Since a high intake of saturated fat has been correlated with adverse health effects, such as coronary heart disease, there have been continuing efforts to reduce fat content in many foods (USDA, 1985).

The objective of this work was to determine whether the concentration of natural CLA in milk fat could be effectively increased by urea crystallization. This would notably increase the nutritional value of milk and other dairy products. A higher concentration of natural CLA would also make it possible to study any protective effects of natural CLA in the prevention of cancer.

MATERIALS & METHODS

Reagents and materials

All chemicals and solvents were analytical grade. Hexane, ether and methanol were from Fisher Scientific Co. (Fair
Crystallization

Saponification of butterfat

A 5-g butterfat sample, including 5 mg of heptadecanoic acid (C17:0) as an internal standard, was dissolved in 50 mL of 1 M potassium hydroxide in 99% ethanol and hydrolyzed at 80 °C for 2 h. After the solution was cooled, 50-mL water was added. The mixture was extracted twice with 20 mL hexane. The hexane extracts were combined and washed twice with 5 mL water. The aqueous solution was acidified with 35 mL of 2 N HCl and extracted with hexane three times. The hexane layer was washed with 10 mL water and dried over Na2SO4. Fatty acids were recovered with a rotary evaporator.

Crystallization

The dried fatty acid sample (4 g) was dissolved in a mixture of 50 mL ethanol and 10 mL methanol. Then 4 g or 8 g of urea [urea/fatty acid ratio 1:1 (UFR1) or 2:1 (UFR2), respectively] was added into the mixed alcohol solution and dissolved at 70 °C. The solution was allowed to crystallize at 4 °C for 8 h. Crystals were removed by gravity filtration on a Buchner funnel. To recover the CLA-containing fraction, the aqueous solution was acidified with 2 N HCl to adjust pH to 4 to 5 and extracted three times with 100 mL hexane. The hexane extract was dried over Na2SO4 and evaporated to dryness in a rotary evaporator. The dried fatty acids were weighed to determine crystallization yield. For the second stage extraction, the liquid phase was recrystallized with a concentration of urea used at the first stage.

GC analysis

Derivatization of isolated fatty acids was performed as previously described by Christie (1989), except 1% sulfuric acid was used as the catalyst instead of 1 M HCl. Briefly, samples (15 to 30 mg), including 1 mg of internal standard (heptadecanoic acid), were derivatized with 2 mL of 1% H2SO4 in methanol. The mixture was heated for 1 h in a 20-mL glass tube at 70 °C and then 5 mL of 5% NaCl was added. The esters were extracted twice with 2 mL hexane. The hexane layer was washed with 4 mL of 4% potassium bicarbonate solution and dried over Na2SO4. The solution was filtered and the solvent removed under reduced pressure in a rotary evaporator.

The samples were analyzed for CLA isomers and total fatty acid profile in a HP5890 gas chromatograph (GC) with a flame ionization detector (FID) and HP3932 integrator. Fatty acid methyl esters were separated by a Supelcowax-10 fused silica capillary column (60 m × 0.53 mm i.d., 0.5 µm film thickness; Supelco Inc., Bellefonte, Penn., U.S.A.) with 2.4 mL/min helium flow. GC conditions were as follows: injector 200 °C; oven programmed, held at 40 °C for 5 min, then increased to 220 °C at 20 °C/min, held for 40 min; detector 250 °C. One µL of sample containing 0.5 to 5 µg of CLA/µL was injected into the column in the splitless mode.

Quantification of CLA

The CLA peak was identified by comparison with the retention time of cis-9, trans-11 octadecadienoic acid standard. The CLA content was expressed as mg/g fat. An internal standard (heptadecanoic acid) was used to determine the recovery of fatty acids in the samples. The recovery of CLA was 83% (n = 6) and C17:0 (n = 10) was 80%. Repeatability for CLA was evaluated as the coefficient of variation (C.V. = 6.6%) for the 5 samples.

RESULTS & DISCUSSION

Enrichment in CLA by crystallization

The crystallization of milk fat was performed with urea/fatty acid ratios of 1:1 (UFR1) and 2:1 (UFR2) to provide the highest yield of CLA possible. At each concentration, crystallization was performed 2 times. The yield of total fatty acid in urea/fatty acid ratio 1:1 (UFR1) was 54.4% and 18.5% in the first and second crystallization, respectively (Table 1). UFR1 showed a decreased amount of CLA at the first crystallization (4.1 mg/g fat), but CLA concentration was increased up to 12.5 mg/g fat at the second crystallization, which was 2.3 times higher than control milk fat (4.9 mg/g fat; Table 1).

The yield of total fatty acids in the urea/fatty acid ratio 2:1 (UFR2) was 26.6% and 16.3%, in the first and second crystallization, respectively. The amount of CLA was increased from 5.0 mg/g fat to 11.5 and 12.7 mg in the first and second crystallization, respectively, in UFR2 (Table 1). There was little difference in CLA concentration between the first and second crystallization of UFR2 (Table 1). Considering the difference in yields between the first and second crystallization, the second was considered unnecessary in UFR2. When the urea/fatty acid ratio was higher than 3:1, the CLA level was decreased (data not shown). Different ratios of urea/solvent caused little difference in the yield of total fat and CLA.

Enrichment of CLA in milk fat depends on the urea/fatty acid ratio. Concentration of CLA and yield were inversely correlated with urea/fatty acid ratio. Comparing with the second crystallization of UFR1, CLA concentration was increased as much as UFR2 showed at the first crystallization, but yield of CLA was higher in the UFR2. Therefore, the first crystallization of UFR2 was an optimal condition for selective increase in CLA enhancement in milk fat (Table 1).

Fatty acid profile

The total fatty acid profile was determined from the integration of the peak area of the GC profile for each fatty acid, and the composition of the fatty acids after crystallization was determined (Table 1; Fig. 2). Note that the concentration of the long-chain saturated fatty acids (carbon number higher than 14) was selectively reduced by urea crystallization. Saturated fatty acid C18:0 was decreased by the crystallization. C18:0 was decreased 2.1-fold in the first crystallization and 118-fold in the second crystallization in UFR1, whereas the amount of C18:0 in UFR2 was decreased 17-fold in the first crystallization and 118-fold in the second crystallization. The ratio C18:1/C18:0, an indicator of the elimination of saturated fatty acids, was 2.0 in the control and increased to 5.7 in the UFR1 and 51.3 in the UFR2 after the first crystallization (Fig. 1). At the second crystallization, C18:1/C18:0 ratio was increased to 138 in UFR1 and 360 at UFR2 (Fig. 1). However, the other long-chain saturated fatty acids were decreased more effi-
cienly at UFR1 than UFR2 in the second crystallization (Table 2). Palmitic acid (C16:0), the most abundant saturated fatty acid in milk fat, did not change appreciably at the first crystallization, but decreased up to 35 times at the second crystallization in UFR1 (Table 2). Palmitic acid (C16:0) was decreased by 3.5 times only at the second crystallization of UFR1 and did not change in UFR2 at either crystallization (Table 2).

Long-chain saturated fatty acids were efficiently eliminated from the milk fat after crystallization, because urea forms a complex with high-carbon-number alkanes, it does not form a complex with unsaturated fatty acids.

Conversely, unsaturated fatty acid concentration was increased. Palmitoleic acid (C16:1) was increased 2 times and oleic acid (C18:0) was increased 1.5 times at the first crystallization of UFR2 (Table 2). Linoleic acid and linolenic acid were also increased 2-fold (Table 2). The second crystallization of UFR2 did not increase the concentration of unsaturated fatty acids, whereas concentration of the unsaturated fatty acids was increased at the second crystallization of UFR1 (Table 2). Oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) were not included in the urea complex and separated completely in the first crystallization of UFR2. The second crystallization did not increase the concentration of long-chain unsaturated fatty acids. However, unsaturated fatty acids were also decreased depending on the concentration of urea used (Table 2). Linoleic acid and higher unsaturated fatty acids were decreased in the urea/fatty acid ratio >3:1 (data not reported).

The most abundant unsaturated fatty acid in milk fat is oleic acid, and it was enhanced with the polyunsaturated fatty acids. Sola et al. (1997) showed that an oleic acid-enriched diet lowered lipid peroxidation, and HDL rich in oleic acid was less easily oxidized than nonenriched HDL. Linoleic acid and linolenic acid also increased after crystallization. Enhancement of unsaturated fatty acids in milk fat would probably lead to increased development of functional foods.

Moreover, short- and medium-chain fatty acids, with carbon numbers <14 were concentrated 2 to 3 times (Table 2). Butyric acid (C4:0) was not detectable after crystallization due to losses during evaporation. Short- and medium-chain fatty acids do not contribute to energy deposition in adipose tissue. They are rapidly hydrolyzed by lipase from triacylglycerols, pass mucosa cells without intracellular esterification, and are directly transported to the liver (Schweitzer and Schmidt-Wilcke, 1993).

**Advantages of urea crystallization**

Fatty acid crystallization by urea has been applied to marine oils to extract saturated fatty acids from polyunsaturated fatty acids enabling the enrichment of n-3 fatty acids such as EPA and DHA (Medina et al., 1995, Bordier et al., 1996). The appropriate urea/fatty acids ratio was 3:1 for the n-3 fatty acids (Medina et al., 1995). Oleic acid concentration decreased to a large extent and linoleic acid remained unchanged at a 2:1 ratio. For milk fat, however, oleic acid was not largely eliminated. To provide higher CLA concentration, other purification methods should be applied together. CLA was concentrated in the noncom-

**Table 2—Fatty acid profile (% of total fatty acids)**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control milk fat</th>
<th>UFR1 1st</th>
<th>UFR1 2nd</th>
<th>UFR2 1st</th>
<th>UFR2 2nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:0</td>
<td>3.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>6:0</td>
<td>1.2</td>
<td>2.2</td>
<td>2.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>8:0</td>
<td>1.2</td>
<td>1.4</td>
<td>7.7</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>10:0</td>
<td>2.8</td>
<td>3.2</td>
<td>7.9</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>12:0</td>
<td>3.2</td>
<td>3.6</td>
<td>7.9</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>14:0</td>
<td>10.2</td>
<td>11.7</td>
<td>2.8</td>
<td>12.9</td>
<td>12.6</td>
</tr>
<tr>
<td>14:1</td>
<td>0.9</td>
<td>1.0</td>
<td>5.6</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>28.0</td>
<td>25.5</td>
<td>0.8</td>
<td>8.1</td>
<td>8.1</td>
</tr>
<tr>
<td>16:1</td>
<td>1.5</td>
<td>2.0</td>
<td>4.2</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>18:0</td>
<td>11.8</td>
<td>5.5</td>
<td>0.1</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>24.1</td>
<td>31.2</td>
<td>13.8</td>
<td>35.9</td>
<td>36.0</td>
</tr>
<tr>
<td>18:2</td>
<td>2.8</td>
<td>3.8</td>
<td>14.8</td>
<td>6.0</td>
<td>6.4</td>
</tr>
<tr>
<td>18:3</td>
<td>0.4</td>
<td>0.5</td>
<td>2.3</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>CLA</td>
<td>0.5</td>
<td>0.4</td>
<td>1.3</td>
<td>1.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

n.d. = not detected

**Fig. 1**—Changes of C18:1/C18:0 ratio in milk fat by urea crystallization.

**Fig. 2**—Fatty acid composition of control and crystallized milk fat at urea/fatty acid ratio 2:1.
plexed fraction with urea, and the fraction was easily separated by filtration. Considering that milk fat contains much more cis-9, trans-11 CLA isomer than synthetic CLA, concentrated natural CLA should be expected to show more efficient anticancer effects than synthetic CLA. It may also have a synergistic effect in anti-carcinogenesis by the increase in other unsaturated fatty acids. The known active cis-9, trans-11 CLA is the major isomer in the butter fat, but the amount of CLA in dairy products is generally lower than the minimum level to show beneficial anticancer effects. Therefore, it would be necessary to concentrate CLA in the milk fat. We used this technique to concentrate CLA from high CLA milkfat (28.4 mg/g fat) from a feeding study (Kelly et al., 1998). CLA in the high CLA butter was increased from 28.4 to 70.6 mg/g fat, which was 29 times higher than the CLA in normal milkfat.

Urea complexation is a promising method for fatty acid separation as it enables handling of large quantities of material with simple equipment, and requires low cost organic solvents. Fatty acids were fractionated in the urea crystallization mainly by the degree of unsaturation, but fractionation was also influenced by chain length. The saturated and monoolefinic fatty acids could much more easily be removed as urea inclusion compounds than the polyunsaturated fatty acids. The mild conditions would not be expected to affect molecular structures of the highly unsaturated fatty acids (Grompone, 1992).

**CONCLUSION**

**RESULTS**

Showed the potential to concentrate natural CLA to a biologically effective level. Future research should consider attaining maximal yield of CLA in the milk fat combining with other purification methods. Crystallization could be used to produce large amounts of relatively low cost CLA for trials of clinical effectiveness.

**REFERENCES**


Grompone, M.A. 1992. Enrichment of omega-3 PUFAs by urea complexation is a promising method for fatty acid separation as it enables handling of large quantities of material with simple equipment, and requires low cost organic solvents. Fatty acids were fractionated in the urea crystallization mainly by the degree of unsaturation, but fractionation was also influenced by chain length. The saturated and monoolefinic fatty acids could much more easily be removed as urea inclusion compounds than the polyunsaturated fatty acids. The mild conditions would not be expected to affect molecular structures of the highly unsaturated fatty acids (Grompone, 1992).