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Conjugated linoleic acid concentrations in processed cheese containing hydrogen donors, iron and dairy-based additives

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Hydrogen donors (butylated hydroxytoluene, propyl gallate, cysteine, ascorbic acid) and dairy additives (sodium caseinate, sweet whey powder and nonfat dry milk) are capable of increasing the conjugated linoleic acid content in processed cheese. Propyl gallate was the most effective hydrogen donor tested, increasing the total and 9 *cis*, 11 *trans* (9*c*, 11*t*) CLA contents 1·59- and 1·42-fold, respectively. Among the dairy additives sodium caseinate at a concentration of 6% was the most effective, increasing the total CLA concentration 1·65-fold compared to processed cheese with no additive. Nonfat dry milk and whey powder produced 1·57- and 1·56-fold increases in CLA concentration, respectively. Lipid oxidation systems such as Fe^{2+} and Fe^{3+}/ascorbate were also effective at increasing the CLA content in processed cheese. At 500 ppm Fe, the increase in total CLA was 1·56-fold for Fe^{2+} and 1·44-fold for Fe^{3+}/ascorbate.

INTRODUCTION

Conjugated linolenic acid (CLA), an isomer of linoleic acid, is reported to have anticarcinogenic properties. CLA has been shown to inhibit mouse skin carcinogenesis induced by 7,12-dimethylbenz(*a*)anthracene, when applied on the skin surface (Ha *et al.*, 1987). Mammary tumours in rats induced by 7,12 dimethylbenz(*a*)-anthracene and mouse forestomach neoplasia induced by benzo(*a*)pyrene are suppressed by dietary CLA (Ha *et al.*, 1990; Ip *et al.*, 1991). The 9 *cis*, 11 *trans* (9*c*, 11*t*) octadecadienoic acid isomer is incorporated into the phospholipids of mice, thereby suggesting that it alone could be the active CLA isomer (Ha *et al.*, 1990). CLA is a minor component of a number of foodstuffs, including dairy products, meats and vegetable oils (Riel, 1963; Parodi, 1977; Ackman *et al.*, 1981; Brown & Snyder, 1982; Ha *et al.*, 1987, 1989; Fogerty *et al.*, 1988; Aneja & Murthi, 1990, 1991; Shantha *et al.*, 1992). If CLA is an effective anticarcinogen in humans, then processing conditions could be used to design foods to contain high concentrations of this potential micronutrient.

Aneja and Murthi (1990) have shown that the CLA content in clarified butter (ghee) varies depending on the method of its preparation. An increase in the processing temperature from 110°C to 120°C is shown to produce more than a two-fold increase in CLA concentrations in ghee. Higher concentrations of CLA have also been reported in processed cheese, such as 'cheese whiz', compared to natural cheeses (Ha *et al.*, 1989). In an earlier publication (Shantha *et al.*, 1992) we have shown that an increase in processing temperature and addition of whey protein concentrate produced a significant increase in CLA concentration in processed cheese. The microbial fermentation of milkfat during yoghurt formation has also been shown to increase CLA concentration (Aneja & Murthi, 1990, 1991). The above observations suggest that a manipulation of processing conditions and the use of different additives could increase the CLA concentration of processed dairy foods.

In the present study we report the effect of various hydrogen donors such as butylated hydroxytoluene (BHT), propyl gallate (PG), cysteine and ascorbic acid to increase CLA formation in processed cheese. Dairy additives such as sodium caseinate (Na Cas), nonfat dry milk (NFDM) and sweet whey powder have also been assessed for their ability to increase CLA concentration in processed cheese, as have lipid oxidation systems including Fe^{2+} and Fe^{3+}/ascorbate.

MATERIALS AND METHODS

Cheddar cheese (total and 9*c*, 11*t* CLA concentrations 3·56 ± 0·23 and 2·86 ± 0·22, respectively) and nonfat dry milk were purchased from a local grocery. Sodium caseinate was donated by Erie Foods International, Inc.,

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Erie, IL. Sweet dairy whey powder was donated by Land O' Lakes, Inc., Minneapolis, MN. All chemicals and solvents were reagent grade. Pure 9*t*, 11*t* octadecadienoic acid which was used as a reference was purchased from Matreya, Inc., Pleasant Gap, PA.

Preparation of processed cheese

Processed cheese was prepared as mentioned in an earlier publication (Shantha *et al.*, 1992). The formulation included 58% cheese (34% fat), 0·625% sodium tripolyphosphate, 0·625% sodium citrate, 0·6% sodium chloride and 40·15% water, resulting in a final fat concentration of 20%. The various additives—butylated hydroxytoluene (BHT, 0·02% of fat content), ascorbic acid (AH_2 0·5%), propyl gallate (PG, 0·02% of fat content), cysteine (0·5%), sodium caseinate, nonfat dry milk, sweet whey powder (0–6%), ferrous or ferric chloride/ascorbate (50–500 ppm Fe, 0·5% AH_2)—replaced equivalent amounts of water. The various additives were mixed with the cheese, salt, emulsifiers and water and stored at 80°C for 10 min under atmospheric conditions. All samples were prepared from the same lot of cheddar cheese.

Lipid analysis

Fat contents of the cheeses were determined by the Babcock method (AOAC, 1990). Fat was extracted from cheese, processed cheese and the various dairy additives using sulfuric acid (5 N) digestion followed by extraction with ether/heptane (1 : 1, v/v) as described by de Jong and Badings (1990). Peroxide values of extracted fat were determined by thiosulfate titration (AOAC, 1990). Conjugated linoleic acid for use as a reference standard was prepared by alkaline isomerization of pure linoleic acid (AOAC, 1990). Fat was transesterified to fatty acid methyl esters (FAME) using boron trifluoride in methanol (Ackman *et al.*, 1989).

Gas chromatographic analyses of fatty acid methyl esters were performed in a Perkin Elmer Auto GC using a SUPELCOWAX-10 fused silica capillary column (60 m × 0·75 mm i.d., phase thickness 0·25 μm; Supelco, Inc., Bellefonte, PA). The GC analysis was temperature-programmed from 75°C to 220°C at 20°C/min and held at 220°C for 45 min. Other parameters were split injection, helium (carrier gas) 11 psi; injection port temperature 250°C; and detector temperature 250°C. TURBOCHROM software (PE-NELSON, Cupertino, CA) was used for data analysis. The CLA peaks were identified by comparison with the retention time of the reference standard as previously described (Shantha *et al.*, 1992). Typical chromatographs for the CLA isomer standards and for processed cheese are shown in Fig. 1. CLA concentrations were calculated as mg/g of fat using the formula

$$\text{CLA (mg/g)} = \frac{(A_x)(W_{I.S.})(CF_x)}{(A_{I.S.})(W_S)(1{\cdot}04)} \times 1000$$

where A_x = area of CLA isomer, $A_{I.S.}$ = area of internal standard, CF_x = theoretical calculated correction factor for CLA based on I.S., $W_{I.S.}$ = weight of internal standard added to the sample (in mg), W_S = sample weight (in mg), and 1·04 = conversion factor necessary to express results as mg fatty acid/g fat, rather than as methyl esters (Shantha & Ackman, 1990).

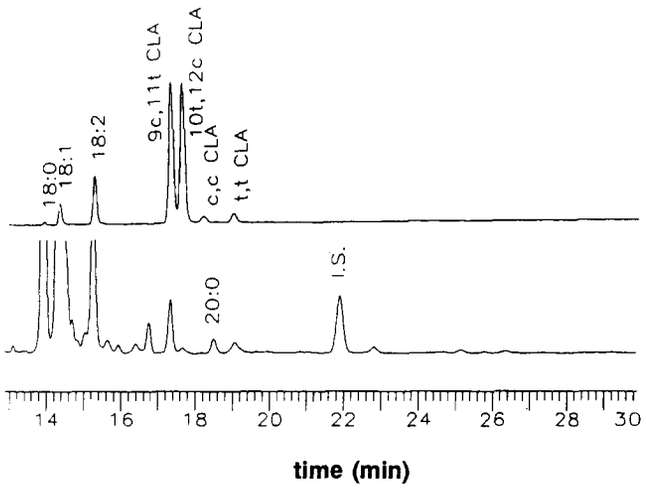

Fig. 1. Typical gas chromatogram for conjugated linoleic acid isomer standards (upper) and processed cheese (lower). Experimental conditions are outlined in the Materials and Methods section.

All processed cheese samples were prepared three times and the fatty acid methyl esters of each were injected twice into GC using an autosampler. Experiments were conducted as completely randomized factorial designs which included treatment, replicate (treatment), concentration, and treatment × concentration interaction. Replicate (treatment) was used as the error term for testing differences among treatments. If the *F* test was significant, the least significant procedure was used to determine the difference between means at the 5% level of significance (Snedecor & Cochran, 1989). Statistical calculations were conducted using GLM SAS (1985).

RESULTS

Table 1 shows the effect of various hydrogen donors on the concentrations of CLA in processed cheese. BHT, AH_2, cysteine and PG produced a significant increase in the concentration of CLA as compared to processed

Table 1. Effect of antioxidants and reducing agents on CLA concentrations in processed cheese

Treatment	CLA concentration (mg/g fat)[e]	
	9 *cis*, 11 *trans*	Total CLA
Control	3·30[a] (0·19)	4·14[a] (0·24)
BHT	3·77[b] (0·24)	4·80[b] (0·28)
Ascorbate	4·28[c,d] (0·09)	5·88[c] (0·04)
Cysteine	4·09[b,d] (0·12)	5·71[c] (0·18)
Propyl gallate	4·69[c] (0·18)	6·60[d] (0·32)

[abcd] Means with different superscripts in the same column differ significantly ($p < 0{\cdot}05$).
[e] Values represent means with S.E.M. in parentheses ($n = 6$).

Table 2. Effect of various dairy additives on CLA concentrations in processed cheese. Total and 9 *cis*, 11 *trans* (9*c*, 11*t*) CLA concentrations in processed cheese with no additives were 3·3 (0·19)^{a,A} and 4·14 (0·24)^{x,W} mg/g fat respectively

Concentration (%)	CLA concentration (mg/g fat)[e]					
	Additive					
	Sodium caseinate		Nonfat dried milk		Whey powder	
	9c, 11*t*	Total	9*c*, 11*t*	Total	9*c*, 11*t*	Total
1·5	3·86 (0·15)^{b,B,c}	5·60 (0·19)^{y,X}	4·22 (0·09)^{c,C}	6·04 (0·21)^{y,z,X}	3·80 (0·06)^{b,B}	5·93 (0·04)^{y,X}
3·0	4·33 (0·14)^{d,C}	6·43 (0·14)^{z,Y}	3·99 (0·24)^{b,B}	5·98 (0·40)^{y,X}	3·70 (0·22)^{b,B}	5·34 (0·24)^{x,X}
4·5	4·25 (0·17)^{c,d,C}	6·65 (0·19)^{z,Z}	3·38 (0·20)^{b,B}	4·68 (0·27)^{x,X}	4·35 (0·05)^{c,C}	5·93 (0·39)^{x,y,Y}
6·0	4·40 (0·11)^{d,B}	6·85 (0·25)^{z,X}	4·53 (0·12)^{c,b,B}	6·51 (0·18)^{z}	4·27 (0·06)^{c,B}	6·44 (0·16)^{y,z,X}

^{a,b,c,d} Means for 9*c*, 11*t* CLA concentrations within a column with different superscripts are significantly different ($p < 0·05$).
^{A,B,C} Means for 9*c*, 11*t* CLA concentrations within a row with different superscripts are significantly different ($p < 0·05$).
^{x,y,z} Means for total CLA concentrations within a column with different superscripts are significantly different ($p < 0·05$).
^{W,X,Y,Z} Means for total CLA concentrations within a row with different superscripts are significantly different ($p < 0·05$).
[e] Values represent means with S.E.M. in parentheses ($n = 6$).

cheese without hydrogen donors (4·14 ± 0·24 mg/g fat total CLA and 3·30 ± 0·19 mg/g fat of 9*c*, 11*t* CLA). Propyl gallate was the most effective, increasing the total and 9*c*, 11*t* CLA contents 1·59- and 1·42-fold, respectively, compared to processed cheese with no hydrogen donors. Ascorbic acid and cysteine increased the total CLA by 1·42- and 1·38-fold and the 9*c*, 11*t* CLA by 1·30- and 1·24-fold, respectively; while BHT showed the lowest activity, producing 1·14- and 1·16-fold increases in 9*c*, 11*t* and total CLA, respectively, as compared to processed cheese with no hydrogen donors.

Among the dairy additives, sodium caseinate was more effective than whey powder (WP) or nonfat dry milk (NFDM) in increasing the CLA concentration of processed cheese (Table 2). At a concentration of 3%, NFDM and WP exhibited similar activity, producing 1·4- and 1·3-fold increases in total CLA concentration, respectively. Sodium caseinate (3%) was more effective ($p < 0·05$) than NFDM and WP, with total CLA increasing 1·55-fold compared to processed cheese without dairy additive. Increasing the concentration of dairy additives from 1·5% to 6% seemed to result in increased formation of CLA, although differences in total CLA concentrations in the presence of 1·5% vs 6·0% additive were not always statistically different.

Table 3 compares the effect of ferrous (Fe^{2+}) and ferric (Fe^{3+})/AH_2 lipid oxidation systems to increase CLA formation in processed cheese. Addition of 50 ppm Fe^{2+} or 50 ppm Fe^{3+} plus AH_2 (0·5%) produced a significant increase in CLA concentration as compared to processed cheese with no added iron. There were no significant differences in CLA concentration between the various concentrations (50–500 ppm) of Fe^{2+} or Fe^{3+}. The increase due to added Fe^{2+} ranged from 0·97 to 1·33 mg/g fat for 9*c*, 11*t* CLA and from 1·63 to 2·32 mg/g fat for total CLA as compared to processed cheese with no added iron. For Fe^{3+}/AH_2 the increases in CLA content ranged from 0·88 to 1·22 mg/g fat of 9*c*, 11*t* CLA isomer and from 1·67 to 2·12 mg/g fat of total CLA.

DISCUSSION

CLA formation in dairy foods, such as ghee, yoghurt and processed cheese, seems to be influenced by processing. The mechanism proposed for the formation

Table 3. Effect of ferrous ions (Fe^{2+}) or ferric ions (Fe^{3+}) and ascorbate (AH_2, 0·5%) on CLA concentrations in processed cheese. Total and 9 *cis*, 11 *trans* (9*c*, 11*t*) CLA concentrations in processed cheese with no additives were 3·30 (0·19)^{a} and 4·14 (0·24)^{x} mg/g fat, respectively

Concentration (ppm)	CLA concentrations (mg/g fat)[c]			
	Treatment			
	Fe^{2+}		Fe^{3+}/AH_2	
	9*c*, 11*t*	Total	9*c*, 11*t*	Total
50	4·34 (0·15)^{b}	6·11 (0·18)^{y}	4·18 (0·06)^{b}	5·81 (0·09)^{y}
200	4·27 (0·02)^{b}	5·77 (0·01)^{y}	4·52 (0·08)^{b}	6·14 (0·24)^{y}
350	4·63 (0·22)^{b}	6·24 (0·05)^{y}	4·44 (0·14)^{b}	6·26 (0·19)^{y}
500	4·55 (0·19)^{b}	6·46 (0·27)^{y}	4·21 (0·06)^{b}	5·98 (0·11)^{y}

^{a,b} Means for 9*c*, 11*t* CLA concentrations within a column or row with different superscripts are significantly different ($p < 0·05$).
[c] Values represent means with S.E.M. in parentheses (n = 6).
^{x,y} Means for total CLA concentrations within a column or row with different superscripts are significantly diffferent ($p < 0·05$).

of CLA includes initial formation of linoleic acid radical, which rearranges to form conjugated dienyl radical. The dienyl radical can then abstract a proton from a hydrogen donor to give rise to conjugated linoleic acid (Ha *et al.*, 1989). If the proposed mechanism is correct, then any additive or conditions which could facilitate the initial formation of linoleic free radical or act as a hydrogen donor would produce an increase in CLA concentration. Hydrogen donors (PG, BHT, AH_2, cysteine and dairy additives) and a free radical initiating system (Fe^{2+} or Fe^{3+}/AH_2) were evaluated for their ability to increase CLA formation in processed cheese.

PG and BHT, which were added to legally allowable limits (0·02% of fat content), showed different CLA forming activity, PG being more effective than BHT. The observed differences in the CLA forming ability of these antioxidants could in part be related to their differing solubility characteristics. Cysteine and ascorbic acid, well-documented proton donors, gave similar increases in CLA concentration. The ability of ascorbic acid and cysteine to increase CLA formation can be envisioned in two ways. Ascorbate and cysteine could act as hydrogen donors; alternately they could react with the iron originally present in Cheddar cheese (0·68 mg/100 g cheese; USDA, 1976) serving as a free radical initiating system (Kanner *et al.*, 1977) to promote the formation of linoleic acid radicals.

Dairy based additives were found to increase the concentration of CLA in processed cheese. The CLA contents of NFDM (<1% fat), whey powder (1·2% fat) and sodium caseinate (2% fat) were 0·6%, 0·7% and 0·7% of the total fatty acid methyl esters, respectively (data not shown). Therefore, the maximum amounts of CLA contributed to the processed cheese by NFDM, whey powder and sodium caseinate at 6% concentration would be 0·018, 0·026 and 0·042 mg/g fat, respectively, whereas the increases in total CLA observed in processed cheese containing these additives were about 2·37, 2·3 and 2·71 mg/g fat respectively. This indicates that the observed increase in CLA was not solely due to the fat contributed by NFDM, whey powder or sodium caseinate.

Na Caseinate produced the highest concentration of total CLA in processed cheese, although there was no significant difference between the CLA concentrations in processed cheese containing 6% Na Caseinate, NFDM or whey powder. Factors which could contribute to differences between the dairy based hydrogen donors to increase the CLA concentration could be their differing composition. These additives differ in their protein content, with Na Caseinate containing 93% protein, followed by NFDM containing 36% protein and whey powder with 12·5% protein content. The ability of sodium caseinate to promote CLA formation suggests that casein is capable of donating hydrogen to linoleic acid radical, resulting in the formation of CLA. While the NFDM would also contain casein, the concentration is much lower. Previous work with whey protein concentrate (WPC) indicated that whey proteins were not very effective at promoting CLA formation. A low molecular weight fraction (MW < 5000) of whey protein concentrate was more effective than whey proteins in increasing the CLA concentration when added at levels >4·5% (Shantha *et al.*, 1992). Colbert and Decker (1991) have reported that a low molecular weight fraction (MW < 5000) of acid whey is an effective antioxidant. Therefore the ability of NFDM to increase CLA content could be due to both casein and the unidentified low molecular weight components, while the activity of powered whey would be primarily due to the low molecular weight components.

Iron was added to the processed cheese to promote free radical formation either as Fe^{2+} or as Fe^{3+}/ascorbate, since the reduced state is the active prooxidant. Iron was chosen because of its good prooxidant activity and potential nutritional benefits. Lipid peroxides were not detected in any of the iron treated sample, indicating that the iron was not promoting the formation of oxidative products associated with rancidity. Several antioxidants in milk could be responsible for controlling the formation of lipid peroxides, including casein, superoxide dismutase, peptides, amino acids, tocopherol and carotenoids (Allen & Wrieden, 1981; Richardson & Koryzka-Dahl, 1983; Colbert & Decker, 1991). The conjugated linoleic acid which has been reported to have antioxidant properties (Ha *et al.*, 1990) could also help to prevent the formation of lipid peroxides. There was no significant difference in CLA value between the Fe^{3+}/ascorbate treatment and ascorbic acid alone (Table 1). Thus it is not clear whether the increase in CLA concentration in the presence of Fe^{3+}/AH_2 system was due to the added ascorbic acid or the ferric ions. However, addition of ferrous ions did increase CLA concentrations, suggesting that iron-catalyzed free radical initiation does increase CLA formation.

The ability of all the additives tested to increase the concentration of the biologically active 9*c*, 11*t* CLA isomer (Ha *et al.*, 1990) was similar to the trends observed for increases in total CLA content, indicating that the additives were equally increasing the formation of all CLA isomers. High levels of 9*c*, 11*t* isomer in the base cheese itself (*c.* 80% of total CLA) can be explained by the presence of a greater amount of 9*c*, 11*t* CLA isomer in milk from which the cheese was prepared. The high levels of 9*c*, 11*t* isomer in milkfat have been suggested to occur through enzymatic isomerization of linoleic acid by the rumen microbes (Smith *et al.*, 1991). However, the various processing methods, being chemical in nature, produce an increase in all the isomers of CLA and not necessarily the 9*c*, 11*t* CLA isomer.

Hydrogen donors, dairy additives and prooxidants such as iron are capable of producing a significant increase in CLA levels in processed cheeses. The maximal increase in CLA seen for any of the additives tested was 1·65-fold for processed cheese containing 6% sodium caseinate. However, a considerable amount of the original linoleic acid (14–18 mg/g fat) is not

converted to CLA during processing. This suggests that other limiting factors are involved in the formation of CLA. One possible factor is the surface area of the fat in the cheese. Since the formation of CLA would require the interaction between the fat and additives, increasing the surface area could result in an increase in CLA formation. Further investigation is needed to better understand physical factors which influence the ability of milk fat to interact with additives capable of increasing the formation of conjugated linoleic acid.

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