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Effect of β-Cyclodextrin on *trans* Fats, CLA, PUFA and Phospholipids of Milk Fat

L. Alonso · P. Cuesta · S. E. Gilliland

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Abstract The aim of this study was to evaluate the effect of β-cyclodextrin (β-CD) on *trans* C18:1 fatty acid isomers, conjugated linoleic acid (CLA), polyunsaturated fatty acids (PUFA) and phospholipids in pasteurized milk. The individual *trans* C18:1 isomers were not significantly affected by the β-CD. *trans*-11 C18:1 (vaccenic acid) was found to be the major isomer (1.31 ± 0.12%) followed by *trans*-15 C18:1 (0.35 ± 0.06%). Individual *trans* linoleic acids did not show differences from the effect of β-CD, representing the high amount of the isomer *trans*-11 *cis*-15 C18:2 (0.433 ± 0.087%). The main CLA isomer *cis*-9 *trans*-11 C18:2 (rumenic acid) did not show differences between the control milk (0.672 ± 0.080%) and β-CD milk (0.663 ± 0.074%). PUFA and omega-3 and -6 fatty acids were not also significant by the effect of β-CD. Total phospholipids were not significantly affected by effect of the β-CD (0.023 ± 0.001% vs. 0.022 ± 0.001%). β-CD is a effective oligosaccharide for cholesterol removal from pasteurized milk and does not significantly affect the lipid components of the milk fat.

Keywords β-Cyclodextrin · Milk fat · *trans* C18:1 isomers · CLA · PUFA · Phospholipids

L. Alonso · P. Cuesta · S. E. Gilliland
Food Agricultural Products Center and Department of Animal Science, Oklahoma State University (OSU), Stillwater, OK 74075, USA

L. Alonso (✉)
Instituto de Productos Lacteos de Asturias, Consejo Superior de Investigaciones Científicas (CSIC), Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain
e-mail: lalonso@ipla.csic.es

Introduction

Milk contains approximately 3.4% total fat. Milk has the most complex fatty acid composition of edible fats. Over 400 individual fatty acids have been identified in milk fat. However, approximately 15–20 fatty acids make up 90% of the milk fat. The major fatty acids in milk fat are straight chain fatty acids that are saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids [1]. Positive human health effects of CLA, *trans*-11 C18:1 (vaccenic acid), PUFA and omega-3 and -6 fatty acids have been recognized [2]. They inhibit cancer and the development of atherosclerosis in animals [3] and omega-3 fatty acids also have a role in preventing certain cardiovascular diseases [4]. Recent studies have given considerable evidence that phospholipids can have a positive nutritional effect on human health, such as reduction in the risk of cardiovascular diseases [5].

In the food industry, phospholipids are used as emulsifiers or emulsion stabilizers when they are complexed with protein [6]. Five major classes of phospholipids are found in milk fat, and their approximate percentages are: phosphatidylcholine (35%), phosphatidylethanolamine (30%), sphingomyelin (25%), phosphatidylinositol (5%) and phosphatidylserine (3%) [7, 8].

Improving the nutritional value and manufacturing properties of milk fat may be achieved by altering the diet of the cow. The relationship of the fatty acid composition of milk fat and human health, for example coronary heart disease, has received considerable attention [9]. Feeding lipid protected from ruminal hydrogenation increased the unsaturated fatty acid composition of the milk lipid, and consumption of the polyunsaturated dairy products by young adults lowered plasma cholesterol in the high subgroup consisting of individuals with the highest initial

plasma cholesterol levels. Consumption of dairy products higher in unsaturated fatty acids by humans lowered total and low density lipoprotein cholesterol [10]. Methods for reducing cholesterol in fats have been developed, including blending in vegetable oils [11, 12], extraction by distillation and crystallization [13], adsorption with saponin and digitonin [14], degradation of cholesterol by enzymes from microorganisms [15, 16] and removal by supercritical carbon dioxide [17, 18]. Currently, the most effective method for reducing cholesterol content in dairy product is by using β-CD powder [19–22]. β-CD is a cyclic oligosaccharide consisting of seven glucose units and are produce from starch using an enzyme, i.e., cyclodextrin glycotransferase, to break the polysaccharide chain and form cyclic polysaccharide products and is used to remove cholesterol. The molecule is doughnut shaped and its central portion is a circular hydrophobic space similar in diameter to a cholesterol molecule, giving the molecule its affinity for non-polar molecules such as cholesterol. The radius of the cavity is such as to accommodate a cholesterol molecule almost exactly, conferring the high specific nature of β-CD ability to form an inclusion insoluble complex with cholesterol which can be removed by centrifugation. Particularly, β-CD easily absorbs cholesterol at a temperature as low as or lower than 4 °C, so that it is effective in maintaining the quality of milk. The main objective of this study was to investigate the effect of the β-CD on the milk fat composition (*trans* C18:1 fatty acids, CLA, PUFA and phospholipids) when pasteurized milk is treated with β-CD.

Materials and Methods

Materials

Pasteurized cow's milk was purchased from a local grocery store. Milli-Q water was obtained with a Milli-Q Plus ultra pure water system from Millipore (Milford, MA, USA). β-CD and all solvents and reagents were of analytical grade from the Sigma Chemical Co., (St. Louis, MO).

Standards

An anhydrous milk fat with a certified fatty acid composition (reference material BCR-164, obtained from the Commission of the European Communities, Brussels, Belgium) was used to determine the fatty acid methyl esters' (FAME) response factors. Tentative identification of *trans* C18:2 and *trans* C18:3 isomers was done by comparing the equivalent chain length values of FAME obtained with those of reference oils: partially isomerized linseed oil FAME, refined rapeseed oil (BCR 686), partially hydrogenated sunflower seed oil (BCR-688) and a blend of palm oil and partially hydrogenated sunflower seed oil (BCR-687), which had served as test material in the research project SMT4-CT97-2144 of the European Union. Besides this test material, FAME pure isomers (*cis*-9, *cis*-13, *trans*-9, *trans*-11, *trans*-13 C18:1) and PUFA mixtures (C18:2 mixture: *trans*-9 *trans*-12 + *cis*-9 *trans*-12 + *trans*-9 *cis*-12 + *cis*-9 *cis*-12; C18:3 mixture: *trans*-9 *trans*-12 *trans*-15 + *trans*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *trans*15 + *cis*-9 *trans*-12 *trans*-15 + *cis*-9 *cis*-12 *trans*-15 + *cis*-9 *trans*-12 *cis*-15 + *trans*-9 *cis*-12 *cis*-15 + *cis*-9 *cis*-12 *cis*-15) supplied by Supelco (Bellenfonte, USA) were also used as standards. PUFA were also identified by comparison with known standards: menhaden reference oil, and Supelco 37 component FAME mix (Supelco, Bellenfonte, USA). Phospholipids standards, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine were purchased from the Sigma Chemical Co., (St. Louis, MO).

Lipid Extraction

For analysis of FAME and *trans* C18:1 fatty acids, lipids were extracted following a procedure described by the International Standard Method for milk [23] using *n*-pentane and diethyl ether (1:1, v/v) after first adding an ammonium hydroxide aqueous solution (25%) to the milk. The lipid extract obtained was stored in amber vials, exposed to a stream of N_2 and frozen at −20 °C until analysis. FAME were prepared by base-catalyzed methanolysis of the extracted lipids using 2 N KOH in methanol as described by International Standard Method for milk [24].

Silver Argentation Thin Layer Chromatography (Ag-TLC)

FAME was fractionated according to the number and geometry of double bonds by TLC following the Precht and Molketin procedure slightly modified by Alonso et al. [25]. Briefly the TLC glass plates (20 × 20 cm) with silica gel (Merck, Darmstadt, Germany) were incubated with a 20% aqueous solution of silver nitrate (Merck, Darmstadt, Germany) for 16 h, partially air-dry, and activated at 120 °C for 30 min. A 45-µl amount of the solution of FAME (100 mg/ml) was applied to the activated TLC glass plates in a narrow band. The plates were developed twice in a saturated chamber in hexane and diethyl ether (9:1, v/v) with 15 cm of migration. At the end of the chromatographic runs, the plates were air dried and sprayed with a 0.20% ethanol solution of 2′,7′dichlorofluorescein (Merck, Darmstadt, Germany), and the bands were visualized under UV light. The band corresponding to the *trans* monoenic

fatty acid methyl esters, that was previously identified by elaidic acid methyl ester (*trans*-9 C18:1) running in Ag-TLC, was scraped into a flask. Stearic acid methyl ester (20 µl of a solution 6 mg/10 ml of hexane) was added as the internal standard to calculate the individual content of *trans*-18:1 isomers. The FAME were extracted with 80 ml diethyl ether in four extractions, and the solvent was evaporated under vacuum and stream of nitrogen. The residue was dissolved in 300 µl of hexane and 0.2 µl of FAME solution was used for GC analysis.

GC-FID Analyses

FAME were analyzed on an Agilent Technology 6890 chromatograph (Palo Alto, CA) with an FID detector. Fatty acids were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm id × 0.2 µm film thickness, Chrompack) using the method described by Alonso et al. [25]. The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C/min to 170 °C, held there for 55 min, and then temperature-programmed at 10 °C/min to 230 °C and held there for 33 min. Helium was the carrier gas with a column inlet pressure set at 30 KPa and at a split ratio of 1:20. The injector temperature was set at 250 °C and the detector at 270 °C. The injection volume was 0.5 µl.

Individual *trans* C18:1 FAME were analyzed using the same system and column but with the following chromatographic conditions: the initial temperature of 100 °C was maintained for 3 min, then raised to 160 °C at a rate of 7 °C min and held for 62 min, then raised to 220 °C at a rate of 2 °C and held for 20 min until the end of the analysis. The split ratio was 1:50 and hydrogen was the carrier gas with a head pressure of 15 psi. The injector and detector temperatures were 250 °C.

GC-Mass Spectrometry

Analysis was performed on an Agilent Technology 6890 N gas chromatograph coupled with a 5975 B mass spectrometer detector (MSD) (Agilent, Palo Alto, CA). Manual tuning of the MSD with perfluorotributylamine was used to adjust relative abundance for *m*/*z* 69, *m*/*z* 219, and *m*/*z* 502. The MSD was run in the scan mode (*m*/*z* range 33–250 with a threshold of 100 and sampling of 3 scans/s). Ultrapure helium was passed through moisture and oxygen traps and was used as the carrier gas. Column and GC operating conditions were the same as the FAME, the interface line at 280 °C. The electron energy and multiplier voltage of the quadrapole were 70 eV and 1,670 V. The fatty acids were identified by comparing their mass-spectral data to the mass-spectral database in the library Wiley 138, incorporated into the MS Chemstation software.

Solid Phase Extraction (SPE)

A lipid sample (500 mg) was dissolved in 1 ml of chloroform-methanol (2:1, v/v). 1 ml of the fat solution was applied to different SPE cartridges. A silica gel bonded column (Supelclean LC-SI, 6 ml volume, 1 g sorbents, Supelco Bellenfonte, USA) was used. After conditioning with hexane, the non-polar lipids were eluted with 5 ml of hexane-diethyl ether (8:2, v/v) and 5 ml hexane-diethyl ether (1:1, v/v). The recovery of phospholipids was performed by using two different conditions: the first with 4 ml of methanol and the second with 2 ml of methanol plus 2 ml of chloroform–methanol–water (3:5:2, v/v/v). The recovered fraction was dried under a gentle stream of nitrogen and it was redissolved in 0.3 ml of chloroform–methanol (2:1, v/v) before injecting into the HPLC system.

HPLC Analysis

The HPLC analysis of phospholipids was performed using a Waters System (Alliance HPLC System 2695 separation module) coupled to a 2424 evaporative light scattering detector, data acquisitions and analysis were performed with a computer using the software version Empower 2 (Waters, Milford, MA). Separation was carried out on an Extrasil silica (150 × 4.0 mm i.d., 3 µm particle size) with a precolumn (2 × 4.0 mm) from Tracer Analitica (Teknokroma, USA). Phospholipids were separated by chromatography with isocratic elution with isopropanol-hexane-water (55:37:8, v/v/v). The flow rate of the eluent was 1 ml/min and the column temperature was 35 °C. The volume of sample injected was 20 µl. The temperature of the detector was 80 °C and the gas flow was 10 ml/min. Compounds were identified by comparing the retention times of the sample peaks with those of the phospholipid standards.

Gross Composition of the Milk

Total fat, total protein, lactose and total solids of the milk were determined using a milk tester food processing equipment (Foss electric milko-scan 104) previously calibrated with a reference sample.

Statistical Analysis

All experiment were carry out three times and each experiments was analyzed two times. Experimental data were treated by analysis of variance (ANOVA) using the statistical software SAS (version 8.02, SAS Institute Inc, Cary, NC, USA). Differences among treatments were determined by statistical analysis using a Student *t* test where ($P < 0.05$) was considered statistically significant.

Table 1 Total fat (%), total protein (%), lactose (%) and total solids (%) for control pasteurized milk and pasteurized milk with 0.6% β-cyclodextrin

Sample	Fat	Protein	Lactose	Total solids
Control milk	3.73 ± 0.25	3.23 ± 0.18	4.65 ± 0.32	12.79 ± 0.36
0.6% β-CD milk	3.85 ± 0.22	3.20 ± 0.26	4.74 ± 0.38	12.62 ± 0.31

Values are means ± SEM of three replicates analyzed individually in duplicate

Results and Discussion

A number of studies have indicated the importance of cholesterol reduction in dairy products [26, 27]. Cholesterol can be removed from milk and dairy products by β-CD, a cyclic oligosaccharide consisting of seven glucose unit. At the present, there are no studies of the effect of β-CD on the lipid fraction of the milk fat components report in the literature; however did reduce free cholesterol in pasteurized milk fat by >95% with respect to the control milk [19]. Table 1 shows the composition of the control milk and β-CD treated milk. Total fat, total protein, lactose and total solids did not differ significantly ($P > 0.05$) between the control and β-CD milk.

A capillary GC chromatogram of the individual *trans* C18:1 isomers of treated and untreated pasteurized milk with β-CD is shown in Fig. 1. The equivalent chain length values were determined for each *trans* C18:1 isomers by using individual standards. Almost all *trans* C18:1 isomers were sufficiently separated to allow for an individual quantitation except for the *trans*-6-8 C18:1 and *trans*-13-14 C18:1 isomers, which were respectively quantified as a group. The average content of total *trans* C18:1 isomers in milk fat before and after treatment with 0.6% β-CD was 3.12 ± 0.19% and 2.97 ± 0.23%, respectively. Individual *trans* C18:1 proportion in control milk and 0.6% β-CD treated milk for these isomers are presented in Table 2.

Table 2 *trans* Fatty C18:1 isomers composition of the total C18:1 *trans* of the control pasteurized milk and pasteurized milk with 0.6% β-cyclodextrin

Fatty acid	Control milk	0.6% β-CD milk
trans C18:1		
trans-4–5	0.02 ± 0.01	0.02 ± 0.01
trans-6–8	0.02 ± 0.01	0.02 ± 0.01
trans-9	0.23 ± 0.04	0.19 ± 0.03
trans-10	0.35 ± 0.05	0.39 ± 0.07
trans-11	1.31 ± 0.12	1.26 ± 0.14
trans-12	0.26 ± 0.03	0.22 ± 0.05
trans-13–14	0.29 ± 0.04	0.31 ± 0.06
trans-15	0.41 ± 0.06	0.36 ± 0.07
trans-16	0.23 ± 0.04	0.20 ± 0.05
Total *trans* C18:1	3.12 ± 0.19	2.97 ± 0.23

Values are means ± SEM of three replicates analyzed individually in duplicate

Vaccenic acid was found to be the major *trans* C18:1 isomer and it comprised 1.31 ± 0.12% followed by the *trans*-15 C18:1 (0.46 ± 0.06%). No differences ($P > 0.05$) were found for each *trans* C18:1 isomers when milk was treated with 0.6% β-CD.

Table 3, shows mean values of the polyunsaturated fatty acids (PUFA) of control pasteurized milk and milk treated with 0.6% β-CD. Individual *trans* linoleic acid between control and treated milk were not statistically different ($P > 0.05$). No difference ($P > 0.05$) in the linoleic acid C18:2(n-6) content for control milk (1.836 ± 0.068%) and treated β-CD milk (1.819 ± 0.058%) was observed. In this study, the three conjugated linoleic acid isomers analyzed were *cis*-9 *trans*-11 C18:2, *cis*-11 *trans*-13 C18:2 and *trans*-10 *cis*-12 C18:2. The main biologically active CLA isomer, i.e. *cis*-9 *trans*-11 C18:2 (rumenic acid), was not significantly different ($P > 0.05$) between the control milk (0.672 ± 0.080%) and β-CD milk (0.663 ± 0.074%). The

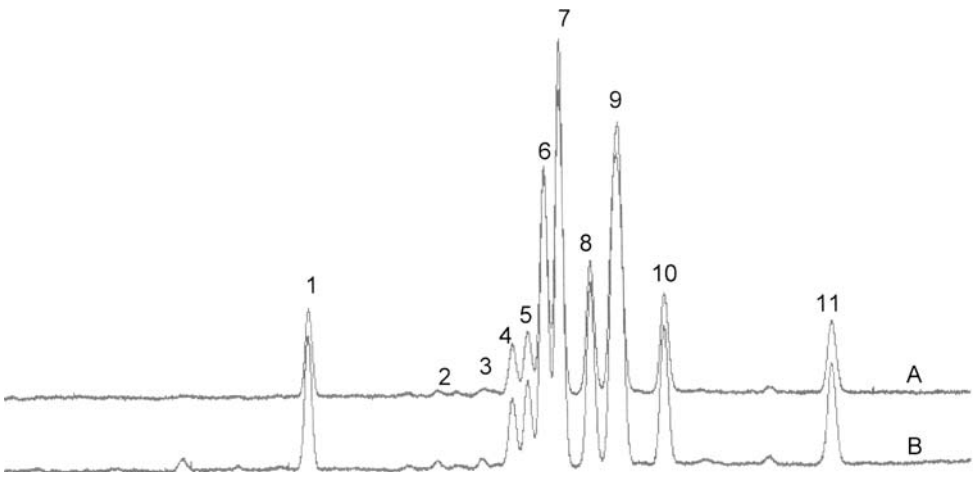

Fig. 1 Chromatogram of *trans* C18:1 fatty methyl ester isolated by Ag-TLC. *A* control milk; *B* milk treated with 0.6% β-CD. Peak identification (*1*) stearic acid, (*2*) *trans*-4 C18:1, (*3*) *trans*-5 C18:1, (*4*) *trans*-6-8 C18:1, (*5*) *trans*-9 C18:1, (*6*) *trans*-10 C18:1, (*7*) *trans*-11 C18:1, (*8*) *trans*-12 C18:1, (*9*) *trans*-13 C18:1, (*10*) *trans*-14-15 C18:1, (*11*) *trans*-16 C18:1

Table 3 Polyunsaturated fatty acids composition (g/100 g fat) of control pasteurized milk and pasteurized milk with 0.6% β-cyclodextrin

Fatty acid	Control milk	0.6% β-CD milk
Linoleic		
C18:2-*cis*-9, *trans*-13	0.182 ± 0.051	0.174 ± 0.047
C18:2-*trans*-8, *cis*-13	0.053 ± 0.011	0.050 ± 0.009
C18:2-*cis*-9, *trans*-12	0.081 ± 0.015	0.083 ± 0.013
C18:2-*trans*-11, *cis*-15	0.433 ± 0.087	0.412 ± 0.069
C18:2-*cis*-9, *cis*-15	0.024 ± 0.011	0.021 ± 0.009
C18:2(n-6)	1.836 ± 0.068	1.819 ± 0.058
Linoleic conjugated		
C18:2-*cis*-9, *trans*-11	0.672 ± 0.080	0.663 ± 0.074
C18:2-*cis*-11, *trans*-13	0.009 ± 0.002	0.007 ± 0.001
C18:2-*trans*-10, *cis*-12	0.021 ± 0.001	0.019 ± 0.001
Linolenic		
C18:3(n-6)	0.152 ± 0.006	0.167 ± 0.008
C18:3(n-3)	0.315 ± 0.016	0.332 ± 0.192
Arachidonic		
C20:2(n-6)	0.031 ± 0.004	0.027 ± 0.006
C20:3(n-9)	0.018 ± 0.004	0.020 ± 0.003
C20:3(n-6)	0.062 ± 0.007	0.055 ± 0.005
C20:4(n-6)	0.064 ± 0.008	0.068 ± 0.007
C20:5(n-3)	0.036 ± 0.005	0.033 ± 0.004
Docosahexanoic		
C22:2(n-6)	0.002 ± 0.001	0.002 ± 0.001
C22:4(n-6)	0.002 ± 0.001	0.002 ± 0.001
C22:5(n-6)	0.003 ± 0.001	0.004 ± 0.001
C22:5(n-3)	0.065 ± 0.013	0.056 ± 0.015
C22:6(n-3)	0.032 ± 0.007	0.038 ± 0.005
Total		
Conjugated linoleic Total n-3	0.702 ± 0.076	0.689 ± 0.071
Polyunsaturated Total n-6	0.441 ± 0.012	0.403 ± 0.016
Polyunsaturated	2.156 ± 0.0978	2.142 ± 0.085

Values are means ± SEM of three replicates analyzed individually in duplicate

same patterns were observed for the total CLA in control milk (0.702 ± 0.076%) and treated milk (0.689 ± 0.071%).

The concentrations for omega 3 and 6 PUFA also were not significant ($P > 0.05$) for the treatment with β-CD. The concentration of each individual arachidonic and docosahexanoic acid was very low in milk fat (Table 3). The high concentrations were for the linolenic acids C18:3(n-3) (0.315 ± 0.016%) and C18:3(n-6) (0.152 ± 0.006%). No differences ($P > 0.05$) were found in the C18:3 (n-3) and C18:3 (n-6) contents of β-CD treated milk (Table 3).

Table 4 Short (SCFA, C4-C10), medium (MCFA, 12-C14) and long chain (LCFA, C18-C22) fatty acid composition (g/100 g fat) of control pasteurized milk and pasteurized milk with 0.6% β-cyclodextrin

Fatty acids			
Sample	SCFA	MCFA	LCFA
Control milk	13.19 ± 0.55	43.41 ± 0.59	43.42 ± 0.41
0.6% β-CD milk	12.81 ± 0.61	44.10 ± 0.69	43.59 ± 0.59

Values are means ± SEM of three replicates analyzed individually in duplicate

In relation to the chain length, short (C4–C10), medium (C12–C14) and long (C16–C22) fatty acids were not significantly ($P > 0.05$) different between groups (Table 4), when the milk was treated with β-CD. There are no reports in the literature showing the effect of treating milk with β-CD on milk fatty acid composition. Chen et al. [28], using supercritical fluid extraction with carbon dioxide for fractionating milk fat to remove cholesterol, observed that the fractionated milk fat had considerable differences in fatty acid composition compared to the control milk fat. The amounts for short and medium chain fatty acids reported by these authors were 40 and 10% lower, respectively, in extracted milk compared to the control milk fat. Similar results were found by Gonzalez et al. [17] in a study on solubility of fatty acids in ewe's milk cream using supercritical fluid carbon dioxide.

There are no data in the literature concerning phospholipid composition in milk treated with β-CD. Table 5 shows the phospholipid composition of control raw milk and raw milk treated with β-CD. Analysis of variance did not reveal any significant difference ($P > 0.05$) in relative composition of the different phospholipid classes among the control milk and β-CD treated milk. Phosphatidylethanolamine was the most predominant phospholipid followed by phosphatidylcholine and sphingomyelin.

Table 5 Phospholipid (PL) composition, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM) of control pasteurized milk and pasteurized milk with 0.6% β-cyclodextrin

Sample	Phospholipids					
	PL (% of sample)	PE (% of PL)	PI (% of PL)	PS (% of PL)	PC (% of PL)	SM (% of PL)
Control Milk	0.023 ± 0.001	35.46 ± 0.28	3.89 ± 0.19	5.38 ± 0.48	16.95 ± 0.67	12.61 ± 0.42
0.6% β-CD Milk	0.022 ± 0.001	34.12 ± 0.33	3.96 ± 0.25	5.59 ± 0.56	15.62 ± 0.59	13.74 ± 0.61

Values are means ± SEM of three replicates analyzed individually in duplicate

These three species of phospholipids represent more than 80% of the total phospholipids in the dairy samples [29].

One of the reasons why the β-CD did not affect these components of the milk fat could be based on the fact that β-CD specifically forms an inclusion complex with cholesterol. The central cavity of β-CD is hydrophobic, giving the molecule its affinity for non-polar molecules such as cholesterol. The radius of the cavity is such as to accommodate a cholesterol molecule almost exactly, conferring the highly specific nature of the β-CD ability to form an inclusion complex with cholesterol. They are therefore accessible to β-CD in the aqueous phase forming the insoluble inclusion complex which can be remove by centrifugation [30, 31]. Results from the present study suggest that the treatment of pasteurized milk with β-CD did not affect the *trans* C18:1 fatty acid isomers, CLA, PUFA and phospholipids compositions in the milk fat. Therefore, this process can be applied to milk for making low cholesterol dairy products without altering any nutritional properties of the milk fat.

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