Milk Cholesterol Reduction at Pilot Station Level Using Beta-Cyclodextrin

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Abstract
The objective of this study was to investigate the possibility of cholesterol elimination from milk using beta-cyclodextrin at pilot station scale, because there is very little information considering this level of milk processing. β-cyclodextrin is a nontoxic, edible and chemically stable compound with affinity for nonpolar molecules like cholesterol, giving the opportunity for obtaining food products that can prevent the appearance of cardiovascular diseases. By applying of this procedure, the cholesterol reduction percentage was calculated as approximately 84 %, similar to the values reported by the specialty literature. Except fat and freezing point values, the other parameters values increased with an approximate percentage of 8, indicating a concentration phenomenon due to milk pasteurization. Fat concentration value decreased with 5.9 %, due to the cholesterol inclusion in the beta-cyclodextrin molecule and its elimination at the bottom of the centrifugal separator. However the variation of the parameters is insignificant, so this process could be considered efficient to be applied at a pilot station level.

Keywords: beta-cyclodextrin, cholesterol, milk.

Introduction
According to World Health Organization (2019), cardiovascular diseases are ranked as the number 1 cause of death globally, being predominant in low- and middle-income countries. Prevention can be accomplished by addressing to the behavioural risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol. Also, consumers are more aware of the nutrition and quality of the consumed food, increasing the demand for "healthy" foods (Balthazar et al., 2017). So, a diet with a high content of fat, salt, sugar and a low percentage of complex carbohydrates, fruits and vegetables increases the risk of cardiovascular diseases (https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds), http://www.heartcharter.org/about-charter/why.aspx).

There are many studies that present experiments on humans and animals which conclude that a high level of plasma cholesterol can be related to an increased intake of cholesterol and saturated fat (Lee et al., 1999; Oh et al., 2001). The main source of cholesterol in food is the raw material from animal origin (meat, milk and eggs) (Maskooki et al., 2013). Because, animal origin food is a very important source of nutrients and also, consumers became more concerned about their eating habits, many studies were developed in order to find adequate ways to reduce cholesterol level. Maskooki et al. (2013) have described a number
of ways for eliminating cholesterol, like the use of: supercritical CO$_2$, organic solvents, saponin, etc., but they all imply a series of disadvantages (remnants of the organic solvents, residues, etc.). Further researches had opened the way to the use of β-cyclodextrin (β-CD), a nontoxic, edible and chemically stable compound which demonstrated its efficiency in cholesterol elimination (Munro et al., 2004; Hwang et al., 2005; Jung et al., 2005; Kwak et al., 2005; Kim et al., 2006; Kim et al., 2007; Astray et al., 2009; Dias et al., 2010; Alonso et al., 2015; Bhatia et al., 2019).

Cyclodextrins are inexpensive starch derivatives obtained by enzymatic ways (Dias et al., 2010). β-cyclodextrin is a cyclic oligosaccharide with 7 glucose units, with doughnut shape and a circular hydrophobic space in its center which generates its affinity for nonpolar molecules like cholesterol (Alonso et al., 2009; Sakr Sally, 2017). Analyzing the crystal structure of the cholesterol in the β-cyclodextrin, Christoforides et al., (2018), showed the formation of a 2:1 host: guest inclusion complex, the cholesterol being axially encapsulated in a head-to-head β-cyclodextrin dimer and tightly bound by numerous van der Waals and C-H···O interactions to the inner dimeric host cavity.

Thus, Lee and his coworkers (1999) conducted a study to determine the optimum conditions of the cholesterol reduction process from homogenized milk with 3.6 % fat, by applying β-cyclodextrin. Beta-cyclodextrin at concentrations of 0.5% to 1.5% ensured cholesterol removal in percentages of 92.2 to 95.3%, when mixed at 10°C for 10 minutes. Mixing time (5 to 20 minutes) and the variation of the centrifugation time (5 to 20 minutes) did not significantly affect the elimination of cholesterol. However, cholesterol reduction was improved by increasing centrifugal forces.

In the study conducted by Maskooki et al. (2013), different concentrations (0, 0.5, 1 and 1.5%) of β-cyclodextrin were mixed with raw and also homogenized milk with different fat contents (1, 2.5 and 3%), at two different mixing temperatures: 8°C and 20°C, working in laboratory conditions. At each treatment, cholesterol residues, fats, proteins, lactose, non-fat solids, density and ash content of milk were measured. Significantly reduced cholesterol content was observed in both raw milk and especially in the homogenized milk. The maximum cholesterol reduction was reached at the level of 1% β-cyclodextrin.

Most of the researches studied the cholesterol elimination process in laboratory conditions and very few are found in the literature that refer to this process applied on commercial scale. So, Alonso et al. (2009) observed that the added β-cyclodextrin (0.4, 0.6, 0.8 and 1.0 %) eliminated from 65.42% to 95.31% of cholesterol at 4°C in 20 minutes. Treatment of milk with 0.8% and 1.0% β-cyclodextrin was not superior compared to the percentage of 0.6. The best rate of cholesterol elimination was observed at 6 hours of treatment. The process was applied on commercial scale, considering 3 production capacities: 100 L, 15 000 L and 50 000 L of milk. This study did not evaluated the way in which the main milk characteristics are affected by the β-cyclodextrin addition. Also, Alonso et al. (2018) managed a cholesterol reduction at commercial scale of 97% for the β-cyclodextrin treated ewe’s milk and studied the characteristics of the Manchego cheese obtained of it, showing that there is no significant difference between this cheese and a control one.

Considering the current state of knowledge regarding the cholesterol reduction of milk by using β-cyclodextrin, this research objective is to evaluate how the milk characteristics can be influenced by using this cholesterol reduction procedure at a pilot station level, working at a capacity of 30 L and applying the pasteurization process.

**Materials and methods**

Raw milk was purchased from the local market and high purity beta-cyclodextrin from Chengdu Healthlife Biotechnology Co., China.

**Cholesterol removal**

By applying the slightly modified technique of Alonso et al. (2009), 30 L of raw milk was treated with 0.6% beta-cyclodextrin, stirred for 25 minutes at 4°C and left static at the same temperature for 8 hours. Then it was passed through the BORALSAN cream centrifugal separator (100-18 model, with a capacity of 100 L/h), where the beta-cyclodextrin-cholesterol residue was eliminated, pasteurized at 67°C for 30 minutes in a pasteurization and coagulation vat, cooled down and packed.

**Cholesterol extraction and determination**

This phase was made according to the slightly modified method applied by Maskooki et al. (2013), as follows: 10 ml of raw or treated milk and 50 ml 2M KOH alcoholic solution were saponified.
at 60°C for 30 minutes. After cooling to room temperature, cholesterol was extracted with 50 ml of hexane, when the lower layer was removed and the upper one containing the extracted cholesterol was retained. Then, this layer was evaporated until dryness and the extract was redisolved with 10 ml of 2 propanol, passed through the 0,2 µm Chromafill syringe filters and injected into the chromatograph.

Cholesterol content was determined using YL9100 HPLC with UV-VIS detector at 212 nm. It consisted of the following components: vacuum degassing system of the solvent, quaternary pump for 4 solvents, Eclipse XDB-C18 column filled with silica gel with chemically grafted octadecyl groups, of the dimensions 250 x4.6 mm (length x diameter), with pores of 5 microns.

The operating conditions were: isocratic elution using a mobile composition phase of 75% methanol and 25% isopropanol HPLC purity, a flow rate of 1 mL / minute, temperature in the thermostated column compartment of 30°C and the manual injection using a Hamilton syringe.

**Physical-chemical analysis**

Physical-chemical analysis were performed using the LactoStar FTIR Milk Analyzer (Funke Gerber). This is a measuring device based on physical properties, which adopts a combined thermo-optical procedure.

**Statistical analysis**

All experiments were replicated 3 times. The experimental data were analysed for variation by using Statgraphic programm to compare the average values of the physical-chemical characteristics of the raw milk and the milk treated with beta-cyclodextrin and subjected to pasteurization. Box and wiskers plots were used to display the shape of the distribution for the individual values of the analysed parameters.
Results and discussions

Before the samples analyze, a standard scale of known cholesterol concentrations was accomplished, considering: 10, 20, 50 and 100 mg cholesterol/L.

Following the injection of the cholesterol standards, several corresponding areas were obtained, based on which a calibration line was drawn and by means of the regression analysis using the Excel program, the equation of the line was generated, as shown in the figure 1.

When injecting the milk samples, the cholesterol peak occurrence time had very close values, such as 7.56 minutes for the raw milk and 7.55 minutes for the beta-cyclodextrin treated milk, as one can see in the figure 2 and 3.

The areas generated by the chromatograph and the cholesterol concentration values determined based on the standard scale equation are presented in the table 1.

Based on the values obtained for the cholesterol concentration of the raw milk and the beta-cyclodextrin treated milk, the cholesterol reduction percentage due to its inclusion in the beta-cyclodextrin molecule was calculated as about 84 %, similar to the values reported by the specialty literature (Lee et al., 1999; Alonso et al., 2009; Maskooki et al., 2013; Alonso et al., 2018).

The two types of milk were also physically-chemically analyzed, using the LactoStar device. The obtained values are presented in the table 2.

![Figure 3. Cholesterol peak occurrence for the treated milk](image-url)

Table 1. Average area values and the corresponding cholesterol concentrations

<table>
<thead>
<tr>
<th>Milk type</th>
<th>Average area values, mVxs</th>
<th>Medium cholesterol concentration, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>455.94</td>
<td>100.19</td>
</tr>
<tr>
<td>Milk treated with 0.6 % beta-cyclodextrin</td>
<td>83.47</td>
<td>16.85</td>
</tr>
</tbody>
</table>

Table 2. Physical-chemicaly characteristics of raw and pasteurized β-cyclodextrin treated milk

<table>
<thead>
<tr>
<th>Type of milk/milk component</th>
<th>Fat, %</th>
<th>Protein, %</th>
<th>Lactose, %</th>
<th>Non fat dry substance, %</th>
<th>Freezing point, °C</th>
<th>Mineral salts, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>4.07±0.02&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>3.39±0.03&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>4.49±0.04&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>12.62±0.09&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>-0.564±0.003&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>0.687±0.03&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
</tr>
<tr>
<td>Pasteurized β-cyclodextrin treated milk</td>
<td>3.83±0.04&lt;sup&gt;&lt;b&gt;a&lt;/sup&gt;&lt;/b&gt;</td>
<td>3.66±0.02&lt;sup&gt;&lt;b&gt;b&lt;/sup&gt;&lt;/b&gt;</td>
<td>4.86±0.02&lt;sup&gt;&lt;b&gt;b&lt;/sup&gt;&lt;/b&gt;</td>
<td>13.66±0.06&lt;sup&gt;&lt;b&gt;b&lt;/sup&gt;&lt;/b&gt;</td>
<td>-0.607±0.002&lt;sup&gt;&lt;b&gt;b&lt;/sup&gt;&lt;/b&gt;</td>
<td>0.747±0.01&lt;sup&gt;&lt;b&gt;b&lt;/sup&gt;&lt;/b&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>Average ±SD (standard deviation)

<sup><b>a,b</b></sup>Different indices in the same column denotes a statistically significant difference (95 % confidence level); the method used to discriminate among the means was Fisher's least significant difference (LSD) procedure.
Table 2 presents the composition of raw milk and the changes that occur during pasteurization and treatment with β-cyclodextrin. The main components protein, lactose, non-fat dry substances and mineral salts of the raw milk were lower compared to the pasteurized β-cyclodextrin treated milk, with about 8% (7.96% for protein, 8.24% for lactose and non-fat dry substance and 7.8% for mineral substances). The increase of these components can be explained by the water evaporation and the concentration that occurs by thermal treatment during the pasteurization process in the pasteurization vat. Only the fat content of the β-cyclodextrin treated milk decreased by 5.9% due to cholesterol removing during β-cyclodextrin treatment and also the freezing point value, strengthening the observation that the milk suffered a concentration phenomenon during pasteurization.

The average values of milk main components for three independent samples of raw milk and pasteurized β-cyclodextrin treated milk were statistically compared by Box and Whisker Plot using Statgraph program showing the same variation trends (figure 4).

**Conclusion**

The originality of the study consists in the application of the cholesterol reduction procedure at a pilot station level, which also involves a pasteurization process, as well as in the analysis of the characteristics of the obtained milk.

Following the cholesterol reduction procedure by including it in the beta-cyclodextrin compound, a decrease in milk cholesterol content was obtained by approximately 84%.

Except fat and freezing point values, the other parameter values increased with an approximate percentage of 8, indicating a concentration phenomenon due to milk pasteurization. Fat concentration value decreased with 5.9%, due to the cholesterol inclusion in the beta-cyclodextrin molecule. This complex is eliminated at the bottom of the centrifugal separator during the separation procedure. The freezing point value decreased indicating once more that the concentration took place during pasteurization.

Anyway, the percentage in which the characteristics of the milk treated with beta-cyclodextrin were affected is insignificant compared to the percentage of cholesterol reduction in milk, thus this cholesterol eliminating procedure can be justified for the industrial application.
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References