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Decrease of cholesterol content in milk by sorption onto β-cyclodextrin crosslinked with tartaric acid; considerations and implications

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Abstract: Elevated cholesterol intake can induce the development of cardiovascular diseases in man, especially with long term animal origin foods consumption. Therefore, this work deals with the possibility of cholesterol content decrease in milk applying β-cyclodextrin crosslinked with tartaric acid (βCDcTA) as a removal agent. Evaluation of statistic data on food consumption in the Slovak Republic in 2018 aimed at total cholesterol daily intake and effects of "milky" cholesterol content decrease on total cholesterol balance. During the experiments, various amounts of βCDcTA addition to milk were studied resulting in optimal 5 % addition resulting in the cholesterol content decrease by 85.4 % in comparison to original cholesterol content. For monitoring purposes, an HPLC method analysing cholesterol content in saponified milk was employed. The food consumption data analysis showed that total per capita daily cholesterol intake was 369.8 mg, from which 86 mg was assigned to the cholesterol contained in milk and dairy products while the application of cholesterol removal procedure could decrease the total per capita daily cholesterol intake to 296.3 mg ("milky" cholesterol amount equal to 12.6 mg), which in below the recommended value of 300 mg daily intake still valid in the Slovak Republic. This approach might prove as a meaningful step to weaken health problems associated with high long term intake of cholesterol contained in foods of animal origin.

Keywords: cholesterol intake, milk, HPLC, health, crosslinked β-cyclodextrin, cholesterol-free foods, annual consumption

Introduction

Cholesterol is a soft, waxy compound found among the lipids or fats in the bloodstream and in all cell membranes. It is a non-saponifiable lipid, essential in maintaining vital functions of organisms, as it participates in the formation of cell membranes, several hormones, vitamin D, and bile acids needed for food fat digestion (Parish et al., 2002). On the other hand, elevated intake of cholesterol contained in foods of animal origin could result in serious cardiovascular diseases (CVD) such as arteriosclerosis and stroke. Larsson et al. (2012) analysed data such as education, weight, height, cigarette smoking, physical activity, family history of myocardial infarction, aspirin use, alcohol consumption, and diet of 34,670 women and stated that dietary cholesterol can be positively associated with the risk of total stroke and cerebral infarction. On the base of such information and real CVD situation in Slovak population, cholesterol daily intake recommended value has been set to 300 mg/adult person in the Slovak Republic by health authorities (Official Report of the Ministry of Health of the Slovak Republic, 2015). To the contrary, contemporary guidelines for CVD risk reduction from the American Heart Association and American College of Cardiology as well as "2015–2020 Dietary Guidelines for Americans" have already not issued explicit guidance for dietary cholesterol intake due to inconsistencies in the evidence base and the inherent difficulty in conducting and interpreting studies to isolate the independent effect of dietary cholesterol on CVD (Carson et al., 2020). Similarly, according to the recent European Society of Cardiology and European Atherosclerosis Society guidelines for the management of dyslipidaemias, the key initiating event in the atherogenesis is the retention of low-density lipoprotein cholesterol (LDL-C) and other cholesterol-rich apolipoprotein containing lipoproteins within the arterial wall (Mach et al., 2020). Indeed, evidence has confirmed that retention of LDL-C and other cholesterol-rich apolipoprotein containing lipoproteins within the arterial wall is the key initiating event in atherogenesis (Ference e al., 2017). One way or another, limitation of cholesterol content in foods could be one of the key issues targeting health problems associated with elevated blood cholesterol content. One of effective procedures limiting the blood cholesterol content is focusing on health dietary patterns such as the Mediterranean-style diet (Fung et al., 2005), or Dietary Approaches to Stop Hypertension (DASH) (Appel et al., 1997) which are inherently associated

with lowered blood cholesterol content due to daily intake of <300 mg of cholesterol (Carson et al., 2020). Another approach to lowering cholesterol content in diet can be based on technological processes during raw materials` treatments resulting in minimised cholesterol content in comparison with the original matrix. Alonso et al. (2009) demonstrated that beta cyclodextrin (β-CD) addition of 0.4 %, 0.6 %, 0.8 % or 1.0 % removes cholesterol from milk in the range between 65.4 % and 95.3 %, when maximum cholesterol removal was seen within 6 h of treatment with β-CD-cholesterol complex removal by centrifugation. Han et al. (2005) removed cholesterol in homogenised milk with β-cyclodextrin crosslinked with adipic acid and in this case, cholesterol removal of above 90 % was observed. Also, Lee et al. (2012) studied the use of crosslinking β-CD for cholesterol removal in milk and cream and concluded that cross-linked β-CD with 15 % adipic acid showed better β-CD recovery and reuse, and higher cholesterol removal rate at repeated recycle than cross-linked β-CD with 2 % adipic acid. Methods for cholesterol content determination in food can be divided into three major categories: classical chemical methods based on the Abell-Kendall protocol, fluorometric and colorimetric enzymatic assays, and analytical instrumental approaches such as gas and liquid chromatography (Li et al., 2019). The most appropriate and frequently applied sample treatment before HPLC include direct saponification followed by the extraction of unsaponifiable residue into a non-polar solvent. Direct saponification is preferred due to simple conversion of non-polar fatty acid esters to polar products with the following effective removal by multiple extraction with n-hexane (Bauer et al., 2014; Albuquerque et al., 2016; Kolarič and Šimko, 2020a). Alternatively, a mixture of polar and non-polar solvents has been proposed to provide more efficient cholesterol extraction from various food matrix where cholesterol is usually bound by many other biological compounds such as lipoproteins, proteins, and phospholipids (Dinh et al., 2011). The first aim of this work was thus to study conditions of cholesterol removal from milk by application of β-CD crosslinked with tartaric acid (βCDcTA) while the second aim was to consider total cholesterol daily intake per capita, its removal from milk and dairy products and effect of the removal on total cholesterol balance in Slovak population.

Materials and methods

Sample

Cow milk (3.5 % fat, Rajo a.s., Bratislava, Slovakia) was bought in a local market.

Chemicals

All solvents and chemicals were of analytical grade. Cholesterol standard was from Sigma-Aldrich with the purity of ≥99 %. Chloroform, n-hexane, ethanol, and sodium sulphate anhydrous were purchased from Centralchem s.r.o. (Bratislava, Slovakia). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemical (Loughborough, UK). Tartaric acid was provided by Lachema Brno (Czech Republic).

Sample preparation

The amount of 0.5 g of milk was refluxed with 15 mL of 1 mol/L methanolic solution of KOH for 15 min. Then, cooled matter was extracted twice with a mixture of n-hexane and chloroform (1:1, v/v) to obtain 15 mL of total extract. To increase the polarity of saponifiable residue, 10 mL of deionised water was added. To avoid the formation of emulsion during the extraction, 1 mL of ethanol (96 %) was added to the saponified matter. Then, the extract was filtrated through anhydrous Na_2SO_4, and evaporated using a rotary vacuum evaporator (Heidolph, Germany) until dryness; the residue was dissolved in 5 mL of methanol, filtered using a syringe PTFE filter with a 0.2 µm membrane (Agilent Technologies, Santa Clara, CA, USA), and analysed by HPLC.

HPLC conditions

HPLC was performed using an Agilent Technologies 1260 infinity system (Agilent, Santa Clara, CA, USA) equipped with a vacuum degasser, quarterly pump, autosampler, and a UV-DAD detector operating at 205 nm. Isocratic elution was performed at the flow rate of 0.5 mL/min mobile phase composed of acetonitrile/methanol 60:40 (v/v). The injection volume was 10 µL and the temperature was set at 30 °C. Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 µm particle size, Agilent, Santa Clara, CA, USA) was used as stationary phase with the guard column Zorbax SB-C18 (4.6 × 12.5 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA). At these conditions, cholesterol was eluted in 2.2 min. Data were recorded and treated using the OpenLab CDS software, ChemStation Edition for LC, and LC/MS systems (product version A.01.08.108).

Preparation of crosslinked βCDcTA

βCDcTA was prepared according to Han et al. (2005) as follows: 10 g of β-CD were mixed with 8 mL of distilled water and the suspension was magnetically stirred at room temperature for 2 hrs. Then, 0.2 g of tartaric acid was added, pH was adjusted to pH = 10 with 1 mol/L NaOH and the

reaction mixture was stirred for 1.5 hrs; the product was then readjusted to pH = 5 with acetic acid. Finally, βCDcTA was recovered by filtering through Whatman paper No. 2, washed three times with 15 mL of distilled water, dried at 60 °C for 20 hrs and passed through a 100 mesh sieve.

Cholesterol removal

Each sample of 10 g of milk was placed in a beaker and different amounts of βCDcTA (1.0, 2.0 or 5.0 %) were added. The mixtures were stirred at 150 rpm using a magnetic blender (MM1, Laboratorní přístroje Praha, Czech Republic) for 15 min at 23 °C and centrifuged at 166 g (5430 R Eppendorf, Hamburg, Germany) for 10 min. After centrifugation, the milk supernatant was analysed for cholesterol content. All procedures were duplicated.

Acquisition of statistical data

Statistical data on annual food consumption in the Slovak Republic were obtained from the latest annual report — Food Consumption in the Slovak Republic in 2018 (Sitárová, 2019).

Results and Discussion

Determination of cholesterol content

For the determination of cholesterol content in milk, an in-house validated HPLC method was applied (Kolarič and Šimko, 2020b). At the given conditions, chromatographic separation of cholesterol was sufficient which was confirmed by UV spectra of the cholesterol standard and cholesterol in milk. As follows from Figure 1, the spectra are practically identical with the peak ratio parameter approaching 1.

For illustration, chromatographic record of cholesterol standard analysis and milk analysis are shown in Figures 2 and 3.

Cholesterol removal degree

Efficiency of cholesterol removal in dependence on βCDcTA addition is shown in Table 1.

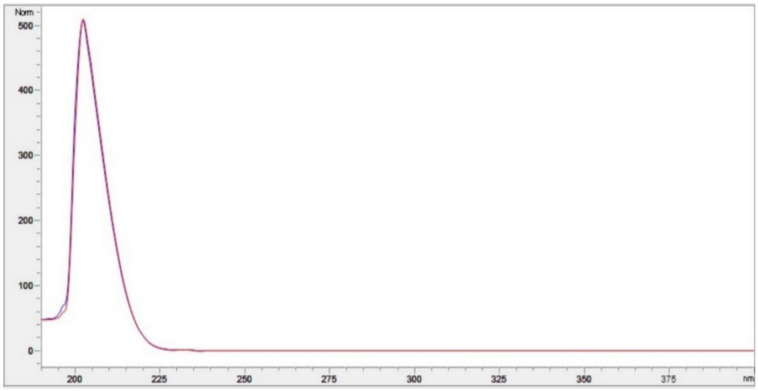

Fig. 1. Comparison of UV spectra of cholesterol standard (red line) and cholesterol in milk (blue line).

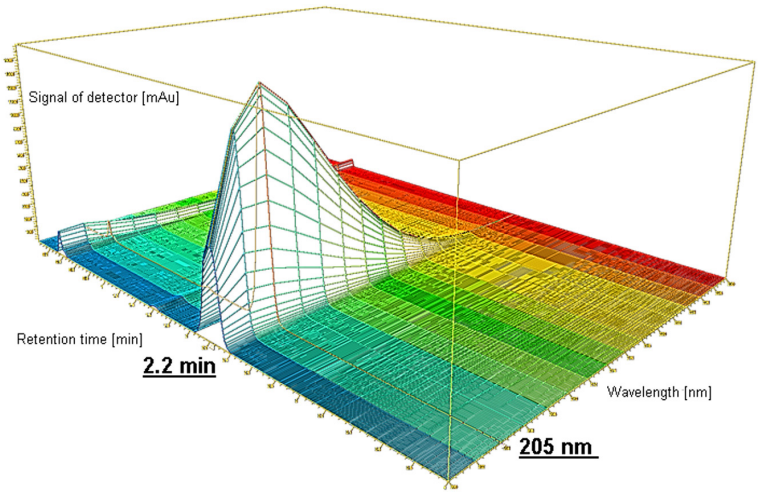

Fig. 2. 3D HPLC record of cholesterol standard analysis.

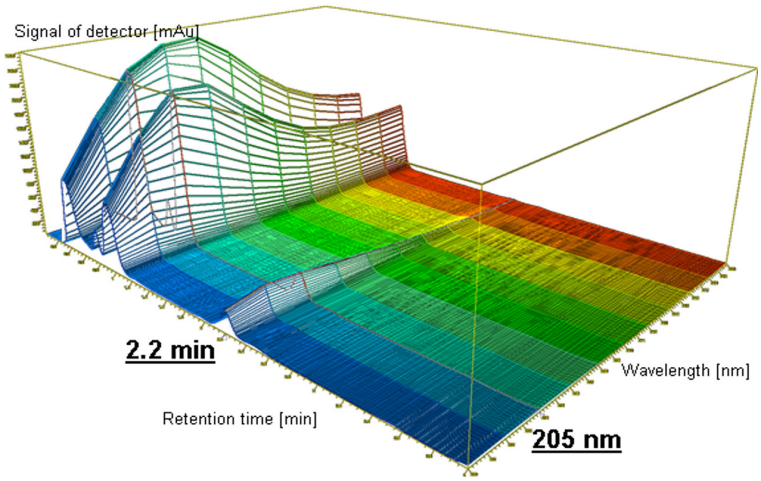

Fig. 3. 3D HPLC record of cholesterol in milk analysis.

Tab. 1. Removal of cholesterol from milk by βCDcTA addition.

βCDcTA addition [%]	Signal [mAU.s]	Cholesterol content [mg/kg]	RSD [%]	Cholesterol removal degree [%]
0	660.02 ±59.60	92.78 ±9.57	10.3	–
1	405.53 ±64.86	31.23 ±4.92	15.8	66.3
2	209.63 ±18.89	15.58 ±1.39	8.9	83.2
5	175.90 ±20.50	13.57 ±1.57	11.6	85.4

Tab. 2. Per capita food consumption in the Slovak Republic in 2018, consumption by commodity and calculated original and reduced cholesterol content.

A	B	C	D	E	F	G
Commodity	Consumption per capita* [kg]	Cholesterol content** [mg/kg]	Cholesterol daily intake by commodity [mg]	Cholesterol daily intake by commodity [%]	Reduced cholesterol daily intake [mg]	Reduced cholesterol daily intake by commodity [%]
Milk (including dairy products without butter)	171.1	130.0	60.9	16.5	8.9	3
Butter	3.4	2 690.0	25.1	6.8	3.7	1.2
Lard	3.3	950.0	8.6	2.3	8.6	2.9
Pork meat	35.4	690.0	66.9	18.1	66.9	22.6
Beef meat	5.2	720.0	10.3	2.8	10.3	3.5
Poultry meat	22.2	730.0	44.4	12.0	44.4	15.0
Fish	5.5	630.0	9.5	2.6	9.5	3.2
Eggs	13.7	3 840.0	144.1	39.0	144.1	48.6
Sum			369.8	100.0	296.3	100.0

*data taken from Sitárová, 2019.
**data taken from Frida, 2020.

As it follows from the measured data, intensity of cholesterol removal depends on the amount of βCDcTA added to treated milk. Thus, while at the 1 % addition of βCDcTA, the cholesterol content decreased by 66.3 %, at the 5 % addition, it decreased by 85.4 %. These results are a little worse in comparison with data published by Han et al. (2005) who were able to decrease cholesterol con-

tent in the range of 92.1 to 93.1 %. On the other hand, cholesterol decrease achieved by Kim et al. (2004) was in the range of 79.4 to 83.3 % when applying β-CD crosslinked with epichlorohydrin.

Evaluation of statistical data

Primary data on to average food consumption in 2018 were obtained from Sitárová (2019). As follows from Table 2 column A, these statistical data show per capita consumption of milk (as milk itself and dairy products) and butter, what is critical for the evaluation of "milky" cholesterol data. However, also other food commodities participate on total cholesterol intake. To estimate the total per capita daily cholesterol intake in Slovak population, data on cholesterol content in individual food commodities were taken from the Danish food database Frida (2020). Cholesterol content in individual commodities is shown in column C while daily per capita cholesterol intake in mg and % are displayed in columns D and E. As it can be seen from these data, per capita daily cholesterol intake in 2018 reached the value of 369.8 mg, which is really high as this includes total Slovak population including suckling infants, vegetarians or even vegans. Therefore, it is reasonable to assume that daily cholesterol intake in some social groups exceeds the recommended value of 300 mg by twice, which is seriously threating health, or even life considering long duration of this situation. Hence, decreasing cholesterol content in milk and its products by 85.4 %, enables decreasing the per capita daily cholesterol intake from 369.8 mg to 296.3 mg (column F), which is below the recommended value and the portion of cholesterol intake from milk and dairy products would decrease from 23.3 % to 4.2 % (column G) in total cholesterol balance. For comparison, mean cholesterol intake of the overall US population had been relatively constant at ~290 mg/day in 2001–2014 (Xu et al. 2018). So, by removal of cholesterol from milk and dairy products, health effects of dairy products can be considerably strengthened while the consumption of full-fat dairy products contributes to higher intakes of significant nutrients such as calcium, vitamins D and K and other bioactive compounds (Loran et al., 2018).

Conclusion

Summarising the results, information, data, and findings obtained in this study, the following conclusions can be postulated:

a) Cholesterol content in milk (and dairy products produced from milk) can be effectively decreased by βCDcTA addition
b) The addition of 5 % of βCDcTA results in cholesterol content decrease by 85.4 % in treated milk
c) Application of such treated milk in food production can decrease the total cholesterol average daily intake per capita in Slovak population from the current value of 369.8 mg to 296.3 mg, weakening health problems associated with long duration of high intake of cholesterol contained in foods of animal origin.

Conflict of interest

Authors declare no conflict of interest. The funders had no role in the design of the study, collection and interpretation of data, writing of the manuscript, or in the decision to publish the results.

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