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Evaluation of sterol composition in different formulations of cocoa milk as milk fat purity indicator

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Abstract:
Based on available national standards, the level of phytosterols in cocoa milk should not exceed 3% of sterol compounds. The aim of this study is to control the quality of the product and evaluate the sterol composition application as an indicator of cocoa milk fat authentic. Therefore, the first part of the present study aimed to examine the sterol composition of ingredients, including milk, cocoa powders. For this purpose, the level of phytosterols in products determined. After the preparation of samples, their sterol composition examined using gas chromatography. Results showed that the level of cholesterol and phytosterols in milk was 99.24 and 0.76, respectively. The examination of the sterol profile of cocoa powder revealed that the largest portion of sterol compounds belongs to beta-sitosterol, stigmasterol and campesterol have the highest level among sterol compounds, respectively. In the second part of this study, the effect of adding various amounts (2, 5, and 8%) cocoa powders was examined on the level of phytosterols in milk due to determine the optimal amount of these compounds to observe standard ranges. As expected, by increasing the percentage of cocoa powders in the formulation of cocoa milk, the level of phytosterols increased.

Keywords: authenticity; cholesterol; phytosterols; cocoa powder; flavored milk; cocoa milk.

Practical Application: Determination of cocoa milk authenticity by increasing the percentage of cocoa powders in the formulation of cocoa milk.

1 Introduction

Authentication is defined as the determination of nature and purity of foods based on their ingredients or genome that is an important matter in the food industry based on legal requirements, economic reasons, quality assurance, usage of safe products, and religious reasons (Kamm et al., 2002). Today, due to the use of alternative fats in various products, including dairy products, fat authentication is essential in these products. Milk fat has a significant role in economic, nutritional, physical, and chemical properties in dairy products (Contarini et al., 2002).

About 3.9% of the milk belongs to fat (Ahmadpour et al., 2014). It consists of 98% triacylglycerol and 2% other fats, including acylglycerols such as diacylglycerol, monoacylglycerol, free fatty acids, phospholipid, and sterols, and is rich in fat-soluble vitamins and essential fatty acids (Ntakatsane et al., 2013). Over years, the authentication of milk fat was performed by analyzing fatty acid profiles. However, because of the similarity between the profiles of fatty acids in some vegetable oils and milk fatty acid profile, its sole use is not a good criterion for evaluating the purity of milk (Derewiaka et al., 2011). In foods, phytosterols can exist in different forms and generally, an alkaline hydrolysis step is still used to separate the phytosterols from triacylglycerols, the main components of vegetable oils (Cossignani et al., 2018).

Because of the noted issues, researchers of the food industry now consider the analysis of trace amounts of fat compounds such as sterols as the basis for milk fact authentication. Animal fats, including milk, mostly contain cholesterol, while phytosterols either do not exist in animal-based products or have trace amounts. Among different types of sterols in phytosterols, beta-sitosterol is the main sterol compounds and a very good criterion for detecting the use of vegetable fats (Kamm et al., 2002).

Based on the issues discussed above, the use of vegetable fats in some dairy products is allowed up to certain limits. One of these dairy products is flavored milk, including cocoa milk. Cocoa beans are a good source of phytosterols (200 to 300 mg per 100 g of fat) which can be found in free or esterified forms. The most prevalent phytosterols in cocoa beans are beta-sitosterol and stigmasterol, comprising 59 and 22% of the total sterol of cocoa beans. The levels of campesterol, cycloartenol, 24-methylene cycloartenol, delta-5 avenasterol, and sitostanol are much lower (Oracz et al., 2014).

Various studies have authenticated the fats in different products. Raftani Amiri & Salmani (2017) examined the industrial dairy products in Kermanshah, Iran, using liquid and gas chromatography and reported that phytosterols (including beta-sitosterol and stigmasterol) are found in 33% of yogurts, 26% of butter, 25% of animal fats, and 13% of milk samples. Similarly, Sadeghi et al. (2018) reported that total amount of phytosterols (mostly beta-sitosterol, stigmasterol and campesterol) in some of the dairy products sold in Iraninan market exceeded the national standard limit of 5%, implying possible substitution of milk fat

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with vegetable oils. Moreover, Kamm et al. (2002) detected the palm oil in milk fat at the 5% and 10% levels using sterol profiles using gas chromatography. The review of the literature indicates that no study has yet been conducted on the authentication of fat in flavored milk (cocoa- and coconut-flavored milk). Therefore, the present study aimed to discover the effect of using different levels of cocoa powders on the sterol composition in milk and for this purpose determines the optimal amount.

2 Materials and methods

2.1 Materials

Cocoa powder (Delphi and Bensef brands), and sugar purchased from local stores. Pasteurized and homogenized milk purchased from Damdaran Company, Iran. Standards for cholesterol, brassicasterol, campesterol, stigmasterol, beta-sitosterol, and reagent for silylation purchased from Sigma-Aldrich-Germany, while sodium azide and chemical solvents ordered from Merck-Aldrich-Germany.

2.2. Methods

Preparation of various treatments of cocoa- and coconut-flavored milk

To prepare different samples of cocoa milk, 15% of the weight of the milk with 2.5% fat first heated in a heated water bath up to 20 °C. Then, cocoa powder (at 2, 5, and 8 wt %) and sugar (7%) separately added to the milk and homogenized using a magnetic stirrer. The resulting mixture warmed up to 50 °C for 20 min, added to the total volume of milk, and stirred using the magnetic stirrer for 20 min. Sodium azide (0.04%) added to all samples in order to prevent microbial growth (Ostadzadeh et al., 2012).

Determination of the sterol profile

15 gram of the sample weighed. Soaping performed according to standard 12228-1 using alcoholic potassium hydroxide (International Organization for Standardization, 2014) For this purpose, the non-saponifiable component extracted using diethyl ether. Afterward, the sterol composition was separated from nonsaponifable matter by thin-layer chromatography (TLC). Sterols then turned into silyl derivatives. Gas-liquid chromatography performed using the capillary column and all sterols were identified by comparing their retention time with that of the standard reference sample. As a purification method, the TLC ensures the removal of possible other compounds and impurities in the non-saponifiable matter (Institute of Standards & Industrial Research of Iran, 2008).

Thin-layer chromatography

After the extraction of non-saponifiable materials, these components (e.g. alcohols, sterols, tocopherol, and hydrocarbons) Ghachourlo et al. (2006) identified and separated using TLC from Merck-Aldrich-Germany. The plate used for TLC was made of silica gel with the dimensions of 20 cm x 20 cm and a thickness of 0.25 (Institute of Standards & Industrial Research of Iran, 2008). The chromatography solution (400 µL) containing non-saponifiable materials stained on silica plates activated by sodium hydroxide on a continuous line using a microsyringe. As the next step, the plate placed inside the developing tank containing diethyl ether and hexane (35:65) so that the solvent would reach the 10 mm of the upper limit of the plate. Then, the plate removed from the tank and the solvent was allowed to evaporate at room temperature. Next, the 2′,7′-dichlorofluorescein solution sprayed on the plate. After the evaporation of solvent and examination of the plate with an ultraviolet lamp, the sterol band scratched with a scalpel, the powder transferred to a 5mm tube, and 2mm of chloroform added to it. Then, sterols turned into silyl derivatives. The reagent for silylation, consisting of pyridine, hexamethyldisilazane, trimethylchlorosilane, added to the test-tube containing the sterol fraction, avoiding all absorption of moisture (Institute of Standards & Industrial Research of Iran, 2008).

Gas chromatography

Sterol compounds identified using Younglin gas chromatography device (model 6100, South Korea) equipped with FID detector and TRB-5 capillary column. The length and internal diameter of the column were 60 m and 0.25 mm, respectively.

The GC condition for identification sterols according to ISO 12228-1 (International Organization for Standardization, 2014) was performed as fallow:

Hydrogen was used the carrier gas and its flow rate was set at 36 cm/s. The temperature of detector/injector had been adjusted at 320° C and the programming temperature for column was done from 245 °C to 265 °C at 5 °C/min then 40 min isothermal at 265 °C. The injection volume was1 µL. The run time for sterol determination lasted 50 min.

Statistical analysis

To examine the effects of cocoa powders (each at 2, 5, and 8% levels) on the level of phytosterols in milk, completely randomized design incorporated. Results analyzed in SAS 9.3. Means compared using Duncan's multiple range test at the significance level of 95%. All samples tested in triplicate and all graphs are drawn in Microsoft Excel.

3 Results and discussion

3.1 Sterol compounds in milk

Results of the analysis of sterol compounds in milk are presented in Table 1. According to this table, the sterol composition of milk composed of cholesterol (99.24%) and phytosterols (0.76%). Similar to these results, all other studies introduce cholesterol as the highest sterol in milk, along with a trace amount of other sterols, including phytosterols (Park et al., 2007). In various studies, variable levels of milk cholesterol are reported. This difference can be attributed to the type of milk used, level of milk fat (Jensen et al., 1991), cholesterol analysis method, and processes applied to milk (Park, 2000). Contarini et al. reported that sterols in milk are cholesterol (262 mg per 100 g of fat) and beta-sitosterol (2.3 mg per 100 g of fat) (Contarini et al., 2002).

Park et al (Park et al., 2007) analyzed the sterol composition in goat and sheep milk and concluded that sterol comprises a small part of the total fat in milk, and the level of cholesterol, lathosterol, desmosterol, dihydrolanosterol, and lanosterol were 341.8, 1.47, 1.39, 2.25, and 9.75 mg per 100 g of fat in goat milk, and 288.4, 1.81, 0.41, 4.15, and 6.86 mg per 100 g of fat in sheep milk, respectively. They stated that the levels of cholesterol reported for goat and sheep milk varies because of different breeds and different analysis techniques. Moreover, they noted small peaks in the chromatogram resulting from gas chromatography, with a retention time equal to that of beta-sitosterol and campesterol, indicating the presence of phytosterols, although these compounds had not been detected using mass spectroscopy. Jensen et al. (1991), stated that the highest amount of sterol in cow milk belongs to cholesterol, ranging from 10 to 20 mg/dL, depending on the fat content of milk.

3.2 Sterol compounds in cocoa powders

The analysis of sterol profiles in cocoa powders (Table 1) revealed that beta-sitosterol, stigmasterol, and campesterol comprise the largest portion of the identified sterol compound in cocoa powders samples, respectively. The sterol composition of two samples of cocoa powder was largely similar (Table 1), showing no significant difference. Similarly, results of all reports indicate that beta-sitosterol is the highest sterol compound in the profile of vegetable fats sterols (Verleyen et al., 2002; Menéndez-Carreño et al., 2008). Verleyen et al (2002) showed that the sterol composition in coconut comprises campesterol, stigmasterol, delta-5 avenasterol, and sitosterol (7.8, 12.5, 0, and 48.6 mg per 100 g, respectively). Moreover, Kochhar (1983) determined the sterol composition in cocoa butter and concluded that the level of cholesterol, brassicasterol, campesterol, stigmasterol, beta-sitosterol, delta-5 stigmasterol, delta-7 stigmasterol, and delta-7 avenasterol were 1-2, trace, 8-11, 24-31, 59-63, 3, 1, and trace (%). The differences in the levels of sterols in cocoa powders between the present study and other sources may be attributed to a difference in the genus of plants used for making powder or processing and storage conditions. However, there are similarities between oils from different plant sources in terms of sterol type. For instance, in an attempt to identify phytochemical compositions of two edible plant oils, Yoshime et al. (2018) observed that beta-sitosterol was the most abundant sterol of both pomegranate seed oil (PSO) and bitter gourd seed oil (BSO) followed by campesterol and stigmasterol.

3.3 Sterol compounds in cocoa milk

Results of sterol composition in cocoa-flavored milk containing various amounts of cocoa milk (Table¬ 2) revealed the cholesterol and beta-sitosterol respectively comprise the highest percentage of sterol composition in different samples of cocoa-flavored milk. By increasing the percentage of cocoa powder in cocoa-flavored milk, the level of cholesterol decreased, while the level of campesterol, stigmasterol, and beta-sitosterol increased by wt% (Table 2). In other words, the results of the present study indicated that increasing the percentage of cocoa powder in cocoa-flavored milk formulation increases the percentage of phytosterols (Figure 1). The percentage of phytosterols in milk containing 2, 5, and 8% of cocoa powder was 8.31, 16.83, and 24.59%, respectively (Table 2). Similar to the results of the present study, Soha et al. (2015) investigated the level of sterols in butter samples and reported that the level of cholesterol in pure butter is 99.71% which was reduced by 96.61, 98.48, and 97.98% by an increase in the amount of palm olein, palm seed oil, and coconut to butter. However, the level of phytosterols increased from 0% in pure butter up to 1.81, 1.67, and 2.16%, respectively, in samples containing vegetable oils. The gradient of

Table 1. Sterol composition of milk, cacao and coconut powders.

Sterol	Milk	Cocoa powder 1 (Delphi)	Cocoa powder 2 (Bensef)
Cholesterol (%)	99/24 ± 0/38	2/22 ± 0/94^{a}	2/82 ± 1/30^{a}
Brassicasterol (%)	-	0/60 ± 0/12^{a}	0/52 ± 0/03^{a}
Campesterol (%)	0/11 ± 0/01	10/46 ± 0/25^{a}	10/36 ± 0/21^{a}
Stigmasterol (%)	0/65 ± 0/39	27/18 ± 0/70^{a}	27/10 ± 1/01^{a}
Clersterol (%)	-	0/08 ± 0/02^{a}	0/3 ± 0/17^{a}
Beta-Sitosterol (%)	-	57/12 ± 0/85^{a}	56/73 ± 0/85^{a}
Delta 5avenasterol (%)	-	1/73 ± 0/13^{a}	1/72 ± 0/4^{a}
Other sterols (%)	-	0/61 ± 0/53^{a}	0/46 ± 0/4^{a}

Note: Values with different superscripts in the same row differ (P < 0.05). (An average of 3 replicates ± SD).

Table 2. Sterol composition of cocoa milk samples.

	Cocoa Powder 1 (Delphi)			Cocoa Powder 2 (Bensef)		
	2%	5%	8%	2%	5%	8%
Cholesterol (%)	91/69 ± 0/15^{a}	83/17 ± 0/19^{b}	75/41 ± 0/29^{c}	92/38 ± 0/13^{a}	81/82 ± 0/12^{b}	80/02 ± 0/13^{c}
Campesterol (%)	0/82 ± 0/11^{b}	1/76 ± 0/12^{a}	1/56 ± 0/22^{a}	1/04 ± 0/08^{b}	0/94 ± 0/08^{b}	1/64 ± 0/13^{a}
Stigmasterol (%)	0/98 ± 0/08^{c}	3/69 ± 0/15^{b}	6/06 ± 0/13^{a}	1/99 ± 0/11^{c}	6/97 ± 0/08^{a}	5/03 ± 0/10^{b}
Beta-Sitosterol (%)	6/18 ± 0/15^{c}	10/72 ± 0/12^{b}	16/97 ± 0/12^{a}	4/56 ± 0/06^{c}	9/32 ± 0/09^{b}	12/94 ± 0/14^{a}
Other sterols (%)	0/33 ± 0/40^{a}	0/65 ± 0/19^{a}	00 ± 00^{b}	0/04 ± 0/06^{b}	0/94 ± 0/22^{a}	0/37 ± 0/33^{a}

Note: Values with different superscripts in the same row differ (P < 0.05). (An average of 3 replicates ± SD).

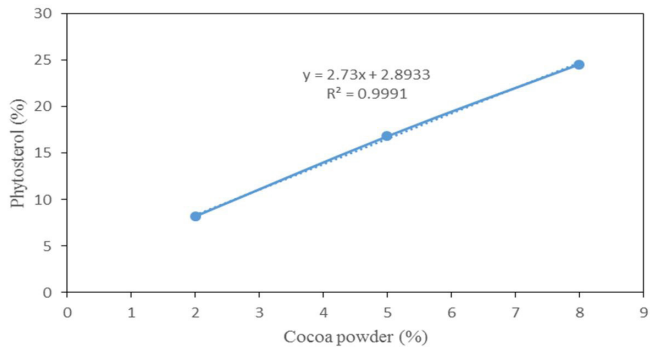

Figure 1. Comparison Phytosterols in cocoa milk samples.

increase for phytosterol in cocoa- and coconut-milk was higher compared to butter samples containing vegetable oils, which can be attributed to the matrix of initial food and the type of vegetable oil added. In addition, Contarini et al (2002) stated that the level of cholesterol and delta-5 cholesterol decreased by adding the level of margarine to milk (2-20%), while the level of brassicasterol, campesterol, stigmasterol, beta-sitosterol, and delta-5 avenasterol increased. In a different study, Keklik et al. (2018) reported that frying different meat products with olive oil as a plant-derived fat source did not significantly impact cholesterol content of the final products.

Other studies effectively used chromatography to determine the nature of fat in dairy products.

All the reviewed studies show that gas chromatography can be used to determine the nature of fat in dairy products, thereby determine the optimal amount of cocoa powder in the formulation of flavored milk. In order to determine the maximum limit of cocoa powder to achieve 3% phytosterol in cocoa-flavored milk (Institute of Standards & Industrial Research of Iran, 2014), a linear equation fitted to the data resulting from the measurement of phytosterol level (Figure 1) in samples containing different levels of cocoa powder (Equation 1).

$$y = 2/73x + 2/8933 \quad (1)$$

In this equation, y is the level of phytosterol (%) and x is the level of cocoa powder (%). Based on this equation, 0.04% cocoa powder should be used to achieve 3% of phytosterol in the formulation of cocoa-flavored milk. In this regard, Rambo et al. (2020) managed to accurately predict the chemical composition of coconut and coffee samples through regression models built using near infrared (NIR) spectroscopy data. The effectiveness of NIR spectroscopy for authentication and characterization of fats in cow and buffalo ghee (anhydrous milk fat) has been successfully proved as well (Antony et al., 2018). It is worthy to mention that there are also other promising analytical methods such as polymerase chain reaction (PCR) assay whose accuracy and reliability as authentication tools have been reported for meat product (Wang et al., 2019).

4 Conclusion

Results of the present study showed that the level of cholesterol and phytosterols in milk were 99.24 and 0.76 to prepare flavored samples. The determination of sterol profiles showed that the highest sterol compound in cocoa powder was beta-sitosterol. By adding these powders to the formulation of flavored milk, the level of phytosterols increased as expected. In general, results of this study can have application in the optimization of cocoa milk formulation in order to observe the standard for the maximum allowable level of sterol in these products.

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