Original Research Article

Nutritional evaluation of cheeses: cholesterol and its oxide 7- ketocholesterol

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ABSTRACT

The present study sought to identify and quantify the contents of cholesterol and 7-ketocholesterol in cheese samples (Mozzarella, Prato and Minas Frescal). Cholesterol and 7-ketocholesterol were identified by comparing the retention time of the sample peaks with the retention time of the standard peaks, and the characteristic wavelength was determined for each substance by using reversed phase high-performance liquid chromatography. The Prato cheese showed a higher cholesterol content, followed by the Mozzarella and Minas Frescal cheeses. Presence of the oxide 7-ketocholesterol was not found, indicating that processing did not result in cholesterol oxidation.

Keywords: Chromatography, Cholesterol oxidation, Dairy product, Oxides, Processing, HPLC.

1. INTRODUCTION

In the current stage of economic development, the food industry has been noted for its dynamics. However, certain ambiguities emerge as a result. On the one hand, it preserves foods; on the other hand, it adds substances not naturally present in foods, which cause problems to human health.

Transformations of natural foods, such as those of animal origin, have been studied in this industry to evaluate effects and possible consequences on consumption [1, 2]. In this sense, the dairy industry is particularly relevant because it turns milk into derivatives that require operations for production and marketing. Operations such as heat (above 120 ºC), shelf life and light can cause changes to the physical and chemical structure of milk, especially fat [3, 4, 5], non-polar globules comprised of triacylglycerides, cholesteryl esters, retinol esters, phospholipids, proteins, cholesterol and others [6, 7, 8].

The cholesterol content of milk ranges between 12-14 mg 100 g [9, 7]; when milk is processed, it may present alterations, e.g., being degraded into cholesterol oxides, as an auto-oxidation response [10, 1, 2, 5]. Cholesterol in dairy products can undergo oxidations, generating cholesterol oxides.

Many are the products that result from cholesterol oxidation, such as 7-ketocholesterol, 20-hydroxycholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6-cholesterol-epoxides (α and β) and cholestanotriol among others. However, the product most commonly associated with oxidation is 7-ketocholesterol, formed during heating of the

α and β isomers of 7-hydroperoxy-cholesterol, which is quite commonly found in foods and is therefore used as a possible indicator of cholesterol oxidation [11, 12, 13, 14].

Fresh milk and fresh dairy products have a low probability of cholesterol oxide formation, as the medium is liquid and the oxygen content is low [13]. Dairy products may have increased resistance to auto-oxidation of cholesterol, even after prolonged periods of adverse conditions. This may be possibly due to the low transitional characteristics of metal content, low cholesterol and saturated fat levels [15, 11].

Studies have indicated the formation, although small, of cholesterol oxides in milk heated for different times and to different temperatures, as well as cheeses and butters subjected to high temperatures during processing [16, 17, 18, 5].

In the dairy industry, heating is widely used, and it is important for verifying the presence of cholesterol oxides. Studies have shown that these compounds are more hazardous to arterial cells than cholesterol, and they are directly related to atherosclerosis, coronary diseases and mutagenic activity [19, 20, 14, 21].

Humans can absorb cholesterol oxides after their consumption in foods. They are found mainly in products such as eggs, meat, dairy and seafood. However, it was observed that fresh foods presented only traces of these compounds or did not exhibit them [11, 19].

There have been several studies on cholesterol determination in foods. Colorimetry, gas chromatography (GC) with or without a combination with mass spectrometry and high performance liquid chromatography (HPLC) are some of the most used methods [22, 8].

The analysis by gas chromatography has been used for a long time as the best method for determination of cholesterol in foods. This analysis requires the extraction of lipids, saponification and derivatization. However, the steps of extraction and saponification are complex and derivatization reagents are unstable and increase the time of analysis. Additionally, cholesterol may be destroyed thermally as a result of the high temperatures required during the separation of GC, and undesirable compounds may also be formed [23, 24, 25]. Thus, cholesterol determination by GC was gradually replaced by HPLC. Although HPLC has the disadvantages of poor absorption of cholesterol in low wavelengths, decreasing detection and increasing difficulty in quantification, as well as the need for large volumes of solvents for analysis, sample preparation for use in HPLC is simpler and can basically use saponification and choice of solvent extraction [23, 25]. Moreover, HPLC uses lower temperatures and shorter times of analysis [24].

Determining cholesterol and its oxides simultaneously is a complex task, mainly because cholesterol is unstable and the structure of its oxides is very similar to that of cholesterol itself. Thus, methods must be developed carefully for greater detection of compounds and reduced appearance of undesirable compounds. Some studies can be found in the literature for the simultaneous determination of cholesterol and its oxides by HPLC with different extraction alternatives ([24, 26, 25].

Thus, the conduction of this study sought to assess cholesterol, as well as the cholesterol oxide 7-ketocholesterol in cheese samples subjected to different processing temperatures.

2. MATERIAL AND METHODS

2.1 Acquisition of the samples

Samples of Prato, Mozzarella and Minas Frescal cheeses were purchased from two dairy farms; they have different production processes (Table 1). Samples of Minas Frescal cheese were purchased in the municipality of Itapetinga-BA, Brazil in the months of September and October 2013, and samples of Mozzarella and Prato cheese were purchased from a dairy farm in Nova Venecia-ES, Brazil in October. The cheese samples were collected already packaged, and then frozen until analysis. The experiments were conducted at the State University of Southwest Bahia, Juvino Oliveira Campus, in Itapetinga-BA, Brazil, in the LAPRON (Natural Products Research Laboratory), CEACROM (Center of Chromatographic Studies and Analysis) and Laboratory of Milk and Dairy Products.

Table 1. Processing conditions of the Prato, Mozzarella and Minas Frescal cheeses

Variables	Cheeses		
	Prato	**Mozzarella**	**Minas Frescal**
Milk temperature	32 ºC	33 ºC	33 ºC
Heating temperature	41 ºC	41ºC	36 ºC
Coagulation time	40 min.	30 min.	30 min.
Mixing time	20 min.	15 min.	15 min.
Cooking time	60 min.	45 min	45 min.
Number of grains	03	02	02
Pressing time	17 min.	15 min.	-
Curd stretching temperature	-	87 ºC	-
Curd stretching time	-	3 min.	-
Total time	90 min.	75 min.	60 min.

2.2 Physico-chemical determinations of the cheeses

Physico-chemical analyses were performed of moisture, ash, fat, total nitrogen, fat in the dry extract (FDE) and total dry extract (TDE). All analyses followed the methods described by [27].

2.3 Extraction of cholesterol and 7-ketocholesterol from the cheeses

Acquisition of the unsaponifiable matter was carried out by saponification of cheese fat and subsequent extraction with hexane [28], with modifications [29]. All reagents used in these steps were analytical grade ones.

For this purpose, approximately 0.5 g of the cheese samples was weighed and supplemented with 4 mL of a 50% KOH aqueous solution and 6 mL of ethyl alcohol. The samples were vortexed for 1 minute and left at rest for 22 hours in the dark at room temperature. After this time, 10 mL of distilled water and 10 mL of hexane were added, followed by vortexing for 5 minutes. There occurred phase separation and the hexane phase was collected, which was dried in a rotary evaporator at room temperature. When only the extract remained, it was dissolved in 2 mL of the mobile phase (HPLC purity grade) and filtered through a syringe filter (0.45 µm) and stored in identified eppendorfs until the time of analysis.

2.4 Chromatographic analysis

The experiment used a Shimadzu, LC-20AD Prominence liquid chromatograph (Japan), with a quaternary solvent system, injection valve with 20µL sample loop, column oven and a diode array detector using a C_{18} analytical column (Restek C 18, 15 cm × 6 mm id × 5µm).

An isocratic binary solvent consisting of acetonitrile and isopropanol (85:15 v/v) was used as mobile phase. Solvent flow-rate was 2 $mL.min^{-1}$, where the oven temperature was adjusted to 40 ºC aiming to get complete resolution of chromatographic peaks with a total run of 12 min. The solvents in use were chromatographic grade, filtered and degased before use.

Injections were performed in triplicate and chromatographic data of the cholesterol and 7-ketocholesterol were processed with the LCSolution® software (Shimadzu).

2.5 Identification of the analytes

The identification of cholesterol and 7-ketocholesterol was tentatively performed by comparing the retention time of commercial standards of cholesterol (Cholesterol, cod. C8667, Sigma-Aldrich®, Saint Louis, Missouri, USA) and 7-ketocholesterol (5-Cholesten-3β-ol-7-one, cod.C2394, Sigma-Aldrich®, Saint Louis, Missouri, USA) and also by the characteristic wavelength of each substance. The chromatograms were processed at 202 nm for cholesterol and at 227 nm for 7-ketocholesterol.

2.6 Validation of the analytical method

Validation of the proposed method was demonstrated by means of laboratory assays with standards, performed as routine at the Center of Chromatographic Studies and Analysis [30, 25]. The analytical method was validated for selectivity, linearity and range of application, repeatability, intermediary precision, recovery, limits of detection and quantification, which were carried out according to the [31], which is based on the international norms of IUPAC (International Union of Pure and Applied Chemistry).

Recoveries were evaluated in triplicate by adding to cheese samples aliquot standard solutions of the analytes for reaching the following concentrations: 100, 200 and 300 µg mL^{-1} for 7-ketocholesterol. The injection of control samples was performed without strengthening the standard.

2.7 Quantification of cholesterol

The quantification of the cholesterol was performed by external standardization and conducted by integrating the areas of the peaks obtained by injection of standard solutions and calculating the concentrations using the straight-line equation obtained from the standard curve of the cholesterol standard, constructed between 2 and 2000 mg L^{-1}.

2.8 Experimental design and statistical analysis

The study used a completely randomized design with three repetitions in triplicate. ANOVA was used for the HPLC results comparing the means by Tukey's test at 5% probability.

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3. RESULTS AND DISCUSSION

3.1 Physico-chemical analyses

The treatments exerted effects on the study variables (*P=.05*), except for FDE and protein content. This result is probably due to differences in cheese production techniques (Table 2).

Table 2. Average concentrations of the physic-chemical variables of Mozzarella, Prato and Minas Frescal cheeses

Analysis	Cheese		
	Mozzarella (n=9)	**Prato (n=9)**	**Minas Frescal (n=9)**
Protein (%)	$24.43^{a} \pm 1.59$	$22.67^{a} \pm 0.76$	$24.18^{a} \pm 0.78$
FDE (%)	$46.95^{a} \pm 1.58$	$48.44^{a} \pm 1.20$	$48.67^{a} \pm 0.92$
Fat (%)	$27.08^{b} \pm 0.53$	$28.58^{a} \pm 0.34$	$22.17^{c} \pm 0.47$
Water content (%)	$42.29^{a} \pm 0.97$	$40.97^{a} \pm 0.84$	$54.45^{b} \pm 0.95$
Ash (%)	$4.06^{a} \pm 0.34$	$3.36^{b} \pm 0.33$	$3.07^{b} \pm 0.38$
TDE (%)	$57.71^{a} \pm 0.97$	$59.03^{a} \pm 0.84$	$45.56^{b} \pm 0.95$

**FDE: Fat in the Dry Extract; TDE: Total Dry Extract. Means followed by different letters in the same line differ at 5% probability by the Tukey Test.*

The cheeses presented FDE percentages ranging from 46.95% to 48.67%. According to legislation, Mozzarella cheese is classified in relation to fat content as extrafatty, fatty to semifatty cheese, with fat percentages ranging from 25 to more than 60% fat; Prato cheese is classified as fatty cheese with fat percentages ranging from 45% to 59.9%. In turn, Minas Frescal is a semifatty cheese (FDE 25%-44.9%). Among the cheeses being studied, Minas Frescal was the only one outside the standards established for fat content [32, 33, 34].

The cheeses presented different classifications in relation to water content, according to legislation. The Minas Frescal cheese is very rich in water and should have a water content higher than 55%. Therefore, this was the only one that did not fit into the established conditions. The Mozzarella and Prato cheeses presented water contents of 42.29% and 40.97%, respectively, classified as cheeses with average water content [35], thus meeting the requirements of legislation.

TDE was determined by subtracting the water content found in the cheeses from the full portion (one hundred percent). The Minas Frescal cheese presented the lowest TDE, differing from the others (*P=.05*). This result was expected, since there was also a significant difference (*P=.05*) for the water content parameter between cheeses; in addition, this cheese has very high water content.

3.2 Cholesterol

The Prato cheese presented the highest cholesterol content compared to the other evaluated cheeses, even when showing similar FDE content, followed by Mozzarella, which differed from Minas Frescal. However, the cholesterol/fat ratio showed no difference among the cheeses (*P=.05*) (Table 3).

Table 3. Cholesterol content (mg 100 g^{-1}) and cholesterol/fat ratio (mg g^{-1}) for the Mozzarella, Prato and Minas Frescal cheeses

Variables	Cheese		
	Mozzarella	**Prato**	**Minas Frescal**
Cholesterol content (mg 100 g^{-1})	$98.52^{b} \pm 6.46$	$111.50^{a} \pm 7.04$	$78.75^{c} \pm 5.39$
Cholesterol/Fat ratio (mg g^{-1})	$3.15^{a} \pm 0.25$	$3.38^{a} \pm 0.26$	$3.07^{a} \pm 0.21$

**Means followed by different letters on the line differed at 5% probability by the Tukey test.*

According to Brazilian institutions, the cholesterol content of Minas Frescal cheese varies from 41-62 mg 100 g^{-1} [36, 37], and these values are lower than those observed in this study. This is a fresh cheese, typically Brazilian, whose production is not standardized; therefore, it is manufactured in different ways among dairy facilities, sometimes with whole milk and sometimes with semi-skimmed milk, which can change the final cheese water content. Thus, the lack of standardization can modify the fat content of the cheese, which in turn may influence the cholesterol content since it is attached to the membrane of fat globules [38.

Reports indicate cholesterol contents between 80-99 mg 100 g^{-1} for Mozzarella cheese in Brazil ([39, 36, 37], similarly to the findings in this study. In turn, international reports indicate variations between 46.25 and 71.4 mg 100 g^{-1} [9, 40], which are lower values than those found in the present study. The difference between the cholesterol levels in Mozzarella cheese reported in Brazilian and in international studies is probably due to differences in manufacturing techniques, as well as higher fat content of milk in Brazil.

Among the cheeses being studied, Prato cheese showed the highest cholesterol content ($p<0.05$), 111.50 mg 100 g^{-1} (Table 3), although it showed similar FDE content and fat content of the raw material (although significantly greater), slightly greater than those of Mozzarella cheese (Table 2). Reports from international literature on cholesterol in Prato cheese are unavailable, probably because it is a typical Brazilian cheese whose production technique was adapted from cheeses like Danbo (Danish) and Gouda (Dutch).

Studies indicate that cholesterol positively correlates with fat content in cheeses [41, 38, 40]; thus, the higher the fat content the higher the cholesterol content. In this study, Prato cheese showed the highest cholesterol content, although there was no difference ($P=.05$) in FDE content.

According to the [42], there is no daily quantity established for cholesterol consumption; however, intake should be minimized. In turn, the [43], by means of the CFR (Code of Federal Regulations Title 21), proposed that the daily value for cholesterol intake in a 2,000 calorie diet should be up to 300 mg.

The Prato cheese showed more than one third of the recommended amount of cholesterol intake in only 100 grams of cheese. Mozzarella and Minas Frescal cheese showed 98.52 and 78.75 mg of cholesterol per 100 g of cheese, respectively. This indicates that intake of the three cheeses must be accompanied by a balanced diet, so as not to exceed the daily recommendation.

3.3 Cholesterol oxides

Cholesterol oxides have many effects on human health [44, 45, 46, 47], which makes the results of this study positive, since the cheeses being studied are not sources of cholesterol oxides.

The recovery (sample together with the standard) indicates the effectiveness of the results observed in chromatography. It was found that the recovery curve (Figures 1, 2 and 3) overlaps the standard curve, confirming that the sample cheese did not interfere in the retention time of the 7-ketocholesterol peak in all cheeses, while each sample presented peaks at distinct moments for the standard.

Presence of the oxide 7-ketocholesterol was not detected in Mozzarella cheese (Figure 1). Among the evaluated cheeses, this cheese was expected to present oxides in function of curd stretching of the mass required for acquiring ideal consistency. This curd stretching occurs in water at 87 ºC for approximately 3 minutes.

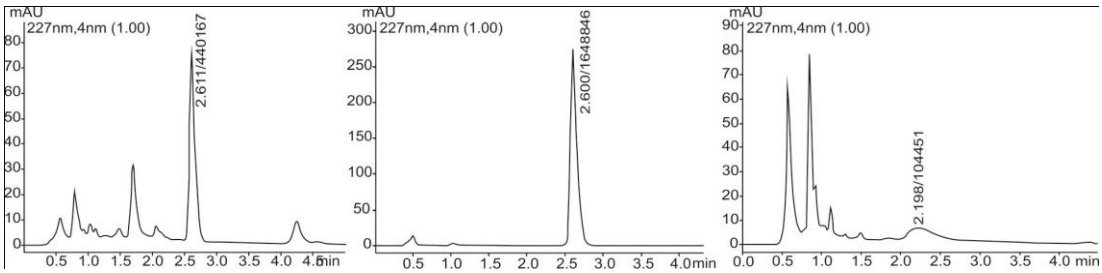

Fig. 1. From left to right: chromatograms of the Mozzarella cheese samples with the standard 7-ketocholesterol (concentration 100 µg mL^{-1}), the standard 7-ketocholesterol and pure Mozzarella cheese.

Behavior of the Prato cheese was the same as the one observed in Mozzarella cheese, i.e., presence of the oxide 7-ketocholesterol was not detected in the samples, because peaks in the chromatograms of the pure standard differed from the peak observed in the Prato cheese samples (Figure 2).

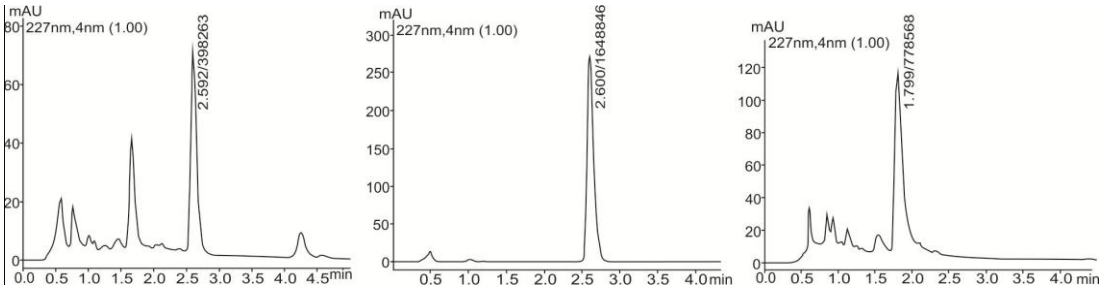

Fig. 2. From left to right: chromatograms of the Prato cheese samples with the standard 7-ketocholesterol (concentration 100 µg mL^{-1}), the standard 7-ketocholesterol and pure Prato cheese.

Among the study cheeses, Minas Frescal is the only fresh cheese, and it was not expected to present cholesterol oxides (Figure 3), since fresh dairy products have a low probability of cholesterol oxide formation ([17, 13]. Thus, the same behavior of the other evaluated cheeses was observed in this cheese, i.e., no indications were detected of cholesterol oxides.

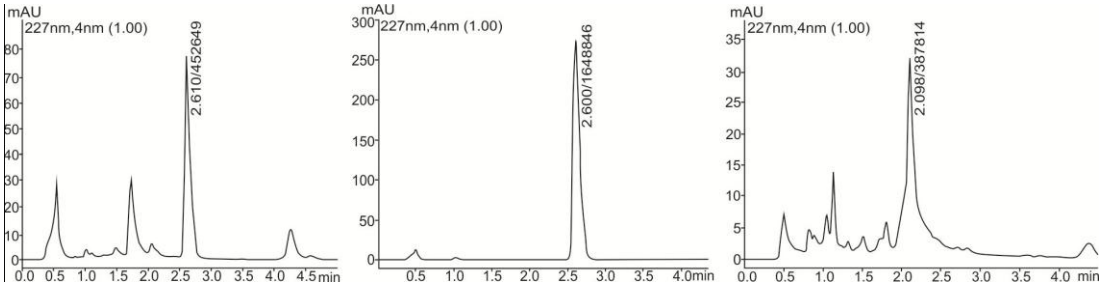

Fig. 3. From left to right: chromatograms of the Minas Frescal cheese samples with the standard 7-ketocholesterol (concentration 100 µg mL^{-1}), the standard 7-ketocholesterol and pure Minas Frescal cheese.

When studying the effect of processing on cheeses under similar conditions to those of this study, [1] observed cholesterol oxides in them.

The effects on cholesterol oxidation to form cholesterol oxides in dairy products is indicated at temperatures exceeding 120 ºC [3, 18]; however, the exposure time to temperatures may elevate cholesterol oxide levels as well [3, 2]. Of the cheeses evaluated, only the Mozzarella mass is exposed to temperatures higher than that of pasteurization (87 ºC), which could exert some effects on cholesterol content. However, this was not demonstrated under the conditions of the present study. On the other hand, it was suspected that the cholesterol observed in the milk used for producing the Prato cheese would also suffer some effect because of the exposure time of 60 minutes to the cooking temperature of 41 ºC. In light of the data, this hypothesis was not confirmed. In turn, the temperature of milk processing to produce Minas Frescal cheese was 36 ºC, a low temperature compared to the temperature at which an effect on cholesterol is observed, above 120 ºC [3], therefore, cholesterol oxidation was not expected as a function of temperature.

Generally, cholesterol-containing foods, when exposed to heat, air or light during processing and storage, may present cholesterol oxides as a response to auto-oxidation [10, 1, 2, 5]; however, in dairy products, auto-oxidation of cholesterol occurs moderately even in adverse conditions. Factors such as the modest cholesterol level and high level of saturated fat can explain this behavior [15, 17].

It is important to highlight that cholesterol cannot be the primary cause of atherosclerosis because it has low reactivity [21]. Lesions to blood vessel walls can be caused by oxidation products formed during processing, preparation and storage of food products. When these lesions are already formed, cholesterol is deposited on the walls of blood vessels, causing atherosclerosis [47].

The absence of 7-ketocholesterol can therefore be considered as a positive result. Although it is considered the least toxic cholesterol oxide, its presence in food is a reference for evaluation of cholesterol oxidation [11, 48] and these oxides cause undesirable reactions in humans, including cytotoxicity, apoptosis and proinflammatory effects, and they are also associated with chronic diseases, such as atherosclerosis and neurodegenerative processes [47].

The cheeses used in this work were submitted to processing temperatures below 80 ºC and the presence of 7-ketocholesterol was not observed, corroborating the reports of [49].

Compared to non-oxidized cholesterol, cholesterol oxides have greater pathogenic and toxic effects as a result of their ability to overcome the lipophilic membrane [44, 45, 46], and they can cause imbalance in the ratio of oxidation and redox biochemical reactions, working at all levels of the body, from signaling of diseases to regulation of inflammation, apoptosis and fibrosis [47].

Consumer products that might contain cholesterol oxides in their composition should be observed more carefully. As mentioned, the cheeses have different methods of processing and different manufacturing temperatures, which can promote the formation of these

compounds. Cholesterol oxides can be absorbed by the human diet, and they may also be formed endogenously in the body [17, 18] and perform different functions, including obligatory intermediates in of bile acid synthesis, and they can be considered a transport means for cholesterol. However, the binding of cholesterol oxides in atherosclerosis is still uncertain, hence the level of cholesterol oxides in food products should be kept to a minimum [18].

The results of this study indicated that consumption of the Mozzarella, Minas Frescal and Prato cheeses did not result in intake of cholesterol oxides, helping to clarify that dairy products should not be treated as enemies of health, but due to their complexity should be viewed holistically and not in a segmented manner, as they have been.

4. CONCLUSION

Pasteurization of milk increased cholesterol content; however, further studies are needed to clarify this effect.

Presence of the oxide 7-ketocholesterol was not detected in the analyzed cheese samples, indicating that processing did not result in cholesterol oxidation.

Consumption of Minas Frescal, Prato and Mozzarella cheeses did not lead to intake of cholesterol oxides.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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