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Influence of Microwaving and Conventional Heating of Milk on Cholesterol Contents and Cholesterol Oxides Formation

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Abstract: The effect of Microwaving and heating treatments (pasteurization and boiling) of milk on cholesterol content and cholesterol oxides formation were studied and evaluated. The 7-ketocholesterol were not detected (ND) in all raw milk samples. On the contrary, heating milk led to formation of cholesterol oxidation products (COPs), mostly, 7- ketocholesterol in different quantities. UHT milk prepared from milk powder heated at $140 \pm 1.0^\circ\text{C}$ for 4 sec was found to have the highest value of 7-ketocholesterol ($80.97 \mu\text{gg}^{-1}$), followed by microwave heated milk for 5 min ($50.29 \mu\text{gg}^{-1}$), whereas the lowest value was in milk pasteurized at $85 \pm 1.0^\circ\text{C}$ for 16 sec ($2.163 \mu\text{gg}^{-1}$).

Key words: 7-ketocholesterol, microwave, gas chromatography, cholesterol oxidation products

Introduction

Milk lipids may undergo chemical and physical changes during processing and storage such as autoxidation and formation of trans fatty acids (Semma, 2002).

Cholesterol oxidation products (COPs) have been found in many foods due to the autoxidation of cholesterol in presence of light, heat and pro-oxidants (Kumar and Singhal, 1991). Many of them showed some biological activities such as enzymatic inhibition of cholesterol biosynthesis, mutagenicity and atherosclerosis (Kumar and Singhal, 1991; Tavani *et al.*, 1997).

Milk and milk products usually undergo different changes during their preparation (boiling and Micro waving) or processing, which may include moderate or severe heat treatments that can lead to undesirable changes in lipids or proteins. Cooking and reheating of foods by microwave ovens are widely used in food preparation in millions of kitchens throughout the world. Food heating by microwave results from the conversion of microwave energy into heat by friction of water molecules vibration due to rapid fluctuation in the electromagnetic field (Potter and Hotchkiss, 1996; Decareau, 1992).

The trend of using the microwave oven in food processing could be attributed to the speed of heating and energy saving. Although microwave oven is widely used as a means of food preparation, insufficient information is available on the consequences of microwave heating on the composition and nutritional quality of the food. Some studies revealed that microwave heating affect fat oxidation and fatty acid isomer formations (Albi *et al.*, 1997a,b).

Therefore, the objective of this study was to evaluate the effect of Micro waving and conventional heating (boiling and pasteurization) of milk on cholesterol contents and cholesterol oxides formation.

Materials and Methods

Raw Cow's milk used in the study was obtained from the bulk tank of two dairy plants: Danish Jordan Dairy Company (DJD) and Al-Sanabel Dairy Co. (SDC). Whereas, UHT milk samples produced from powder milk (reconstituted) was purchased from the local market of KDD (Kuwaiti Danish Dairy Co.) brand name.

Heat Treatments of Milk and Milk Products: The raw cow's milk obtained from the two selected sources were subjected to different heat treatments as shown in Table 1.

Microwave heat treatments: Two milk samples of approximately 1L each were heated in a microwave oven (Galanz, 800 Watts, WD800B, Korea) at 80% power in Pyrex saucepan at $96.3 \pm 1.0^\circ\text{C}$ for 5 min.

Conventional heat treatments (gas cooker): Two milk samples (ca. 1L) was placed in Pyrex saucepan, and boiled on a gas cooker for 5 min at $95.5 \pm 1.0^\circ\text{C}$.

UHT reconstituted milk: UHT milk sample produced from powder milk (reconstituted) produced by Kuwaiti Danish Dairy Co., Kuwait (KDD) were purchased from the local market for comparison (production date 05/01/03 and expired on 05/07/03).

Milk fat extraction and analysis: Lipids were extracted from the milk and milk products samples with chloroform and methanol as described by Bligh and Dyer (Bligh and Dyer, 1959) with some modifications regarding sample weight, solvent volume and centrifugation speed and time. Approximately 70 g of cheese, yogurt or labaneh and 100 ml of fluid milk products were homogenized with 100ml methanol and

Table 1: Cow's milk produced by different heat treatments

Source	Type	Temperature (°C)	Time	Product
DJD	Tube pasteurization	85±1.0	16sec	Pasteurized milk
		140±1.0	4 sec	UHT
	Lab scale boiling	97.5±1.0	5 min	Boiled milk
	Microwave boiling	96.8±1.0	5 min	Microwave boiled milk
SDC	Lab scale boiling	97.5±1.0	5 min	Boiled milk
	Microwave boiling	96.8±1.0	5 min	Microwave boiled milk

100 ml chloroform using a Hamilton Beach Scovel homogenizer (NSF, USA) for 2 min at a medium speed. Then to the mixture, 100 ml of chloroform were added and the mixture was rehomogenized for an additional 2 min. The homogenate was centrifuged at 4000 rpm for 20 min using Haeraeus centrifuge (Haeraeus Christ, GmbH, Osterode/Harz, OJ3, Germany). The upper layer (methanol and water layer) was removed through aspiration. The middle and the lower layer (chloroform layer and precipitated protein layer) were filtered through a filter paper to separate precipitate particles. The chloroform-lipid extracts were again filtered through anhydrous sodium sulfate (Na_2SO_4) and the Na_2SO_4 was rinsed 3 times with 30 ml chloroform 10 ml each. The lipid extracts were dried under nitrogen using rotoevaporator (LABOROTA, 4001 WB, Heidolph, Germany) with 150 rpm at 50°C and stored for analysis in 5 ml vials (brown glass) under nitrogen at -18°C. The lipid samples were then used for the analysis of cholesterol content and cholesterol oxidation products (COPs) mainly 7-ketocholesterol.

Cholesterol and cholesterol oxides were determined for the extracted lipids by GC: Cholesterol, 7-ketocholesterol and 5 α -cholestane standards were from SIGMA, Inc., Ethyl acetate was HPLC grade (J.T. Baker Chemical Co. Phillipsburg, N.J.), potassium hydroxide was from GCC Laboratory Reagent (85%), anhydrous sodium sulfate from SDS (fine chemical limited, Boisar), and methanol HPLC grade from Lab Scan, UK. Chloroform was from GCC (Gainland chemical Co., UK), pyridine (analytical reagent grade) from CBH, Chlorotrimethyl silane (CH_3)₃SiCl was obtained from Fluka (Switzerland) and Hexamethyl disilazane $\text{C}_6\text{H}_{19}\text{NSi}_2$ was from Janssen, US. Accurately 60 to 200 mg of the lipid extract was weighed into a 25 ml screw capped test tube; 10 ml of 1M KOH in methanol and 20 ul of 5 α -cholestane solution (4ug/ul) as internal standard (IS) were added to the sample. The mixture was shaken until its free of dispersed fat particles and placed in a shaking water bath (Memmert, Germany) set at 27°C for 18 to 24 hr. Ten milliliters of distilled water were added to the saponified mixture, which was transferred to 50 ml separatory funnel fitted with a Teflon cap. Unsaponifiables were extracted three times with 10, 5, 5 ml of diethyl ether (98%, Laboratory grade, GCC, England), and the pooled diethyl ether extracts were washed once with 5ml of 0.5M KOH and 5 times with 5 ml distilled water. The ether extract was dried and

filtered using Whatman No.1 filter paper and dried over anhydrous sodium sulfate (Na_2SO_4). The Na_2SO_4 and the filter paper were washed twice with 5 ml diethyl ether to minimize the losses due to the transfer steps. The combined filtrates were concentrated under nitrogen in dark to about 1 ml and dried under nitrogen (ultra pure) after being transferred into a 5-ml vial and stored at -18°C.

Trimethylsilylation (TMS) of cholesterol and cholesterol oxides: The trimethylsilyl derivatives (TMS) of cholesterol and cholesterol oxides was carried out according to the method used by Pie *et al.* (1990) with some modification regarding the derivatization condition (time and temperature). The dried nonsaponifiables extracts were dissolved in 100ul pyridine (CBH, Nottingham, UK) and mixed for 30 sec by vortex mixer. A 100ul of each hexamethyldisilazane (Janssen, Belgium) and trimethylsilylchloride (Fluka, Switzerland) was added and mixed for another 20 sec. The vial was placed in a water bath for 40 min, and then cooled to room temperature. The mixture dissolved in 2ml distilled water and extracted 4 times with 1ml hexane (GC grade, Lab Scan, Dublin). The hexane layer was evaporated under extra pure nitrogen gas. The derivatized (TMS) cholesterol and cholesterol oxides were redissolved in 100 ul of hexane (GC grade).

Recovery determination: Quantitative recovery of cholesterol, 5 α cholestane and 7- ketocholesterol was performed using 10g starch sample previously washed with chloroform and precisely spiked with 100 μ l of 5 α -cholestane (4ug/ul), 7- Ketocholesterol and cholesterol (2ug/ul) using 100ul Hamilton syringe (Hamilton, USA). The nonsaponifiables in the spiked and nonspiked starch samples were extracted and analyzed by the same procedure followed in sample analysis.

The detection limits and calibration curves of cholesterol, 7-ketocholesterol and 5 α -cholestane were found to be about 1 ug g⁻¹ for each cholesterol, 7-ketocholesterol and 5 α -cholestane. This concentration agreed with the results obtained by Regueirio and Maraschellio (Regueiro and Marachiello, 1997) who found values ranging between 0.1 to 1 ug g⁻¹ for cholesterol oxides. The recoveries percentage of cholesterol and 7-ketocholesterol as shown in Table 2 were 98.1 and 95.5 for cholesterol 7-ketocholesterol, respectively.

Table 2: Recoveries of cholesterol and 7-ketocholesterol (addition of 200 ug)

Determination	Cholesterol Recovery (%)	7-ketocholesterol Recovery (%)
1	98.1	97.5
2	96.3	98.0
3	100.9	96.3
4	97.0	93.0
Mean Recovery	98.1	95.5
SD	2.7	2.3
CV	2.1	3.8

SD = Standard deviation, CV = Coefficient of Variability

Gas chromatographic analysis: The derivatized sterols (trimethylsilylated cholesterol and cholesterol oxides) were analyzed on gas chromatograph supplied with an split-splitless injector port and flame ionization detector. A TRB-5(95% dimethyl-5% diphenyl polysiloxan) capillary column (25m x 0.25mm i.d.; phase thickness, 0.25µm; Teknokroma, Barcelona, Spain). The GC conditions used were; 280°C oven temperature, 300°C injector port temperature, and 310°C detector temperature. One microliter of the derivatized sample was injected at a split ratio of 50:50 into the capillary column. Flow rate was set at 1.4 ml/min of N₂ carrier gas. The COPs peaks were-identified compared with the retention time of the reference standard. The COPs content of milk and milk products samples was determined using the internal standard techniques (IS) of 5α-cholestane and the units of measurement are expressed as ug/g for the COPs and as percent (%) for the cholesterol (Lin *et al.*, 1995; Sander *et al.*, 1988).

Statistical analysis of experimental data: Experiments were conducted using completely random design to find the effect of different treatments. Data were analyzed using the analysis of variance (ANOVA) procedure of SAS institute Inc., Cary, NC, USA 1998 version seven software. Duncan's multiple range tests were applied to determine significance between different treatments.

Results and Discussion

The effects of different heating methods of milk on cholesterol and 7-ketocholesterol levels are shown in Table 3. In general, no significant effect of heating of milk on cholesterol level was observed with the exception of the UHT milk prepared from reconstituted powdered milk before and after storage. Along with that, commercial storage of milk had also no significant effect ($p > 0.05$) with the exception of UHT reconstituted milk where storage significantly ($p < 0.05$) lowered the cholesterol concentration. For example, the cholesterol percentages of raw, milk pasteurized (95±1.0°C for 15 min), microwave heated and UHT milk were 0.293, 0.283, 0.275, and 0.285 %, respectively. The insignificant decrease in cholesterol content of the heat treated milk samples compared to that of raw milk are probably due to oxidation of cholesterol and formation of cholesterol oxides (Rodriguez-Estrada *et al.*, 1997).

The decrease in the cholesterol content of the reconstituted UHT compared to that of the fresh raw milk may be due to the fact that this product contains stabilizers and emulsifiers, as indicated on the label of the package (carageenan, guar gum, vegetable mono and diglycerides).

These compounds are probably able to bind (complex) some lipid components such as cholesterol and cholesterol oxides thus lowering their availability for solvent (chloroform) that is used to extract the milk fat.

Furthermore, the significant ($p < 0.05$) decrease in cholesterol contents of UHT milk prepared from reconstituted milk powder compared to that of UHT prepared from fresh cows milk could be partially explained by the increase in the level of 7-ketocholesterol. The values of 7-ketocholesterol for UHT reconstituted milk was 80ugg⁻¹ compared to 8.708 ugg⁻¹ found in UHT from fresh cow milk. The relatively high level of cholesterol oxides is probably due to the drying process including exposure to heat and oxygen.

The concentrations of 7-ketocholesterol of milk presented in Table 3 showed that 7-ketocholesterol is not detected in raw milk and is formed upon all types of heat treatments and during storage. The concentration of cholesterol oxidation ranged between 3.125 ugg⁻¹ in milk pasteurized at 85 ± 1.0°C for 16 sec and 80.97 ugg⁻¹ in UHT prepared from reconstituted milk powder at 140± 1.0°C. The results indicate that 7-ketocholesterol was significantly affected ($p < 0.05$) by heat treatment. The 7-ketocholesterol content in raw and heated milk samples ranged from < 1.0 (ND) to 15.363 ugg⁻¹.

Microwave heating of raw cows milk samples show a significant ($p < 0.05$) increase in 7-ketocholesterol compared to those heated by conventional methods (boiling and/or tube, plate or batch pasteurization). For example, 7-ketocholesterol values for pasteurized (85 ± 1.0°C for 16 sec), boiled (96.3±1.0°C for 5min), UHT and microwave heated milk sample were 3.125, 15.363, 8.708 and 50.029 ugg⁻¹, respectively. Microwave heating seems to be highly detrimental to quality compared to the other heating method due to its unique heating mechanism. These results were in agreement with those obtained by (Albi *et al.*, 1997a,b); Yoshida and Kajimoto (1994) and Yoshida *et al.* (1991) that heating of oil samples in microwave oven enhances lipid oxidation.

The formation of COPs as a result of heating is expected because heating positively increases lipid oxidation in the presence of air, prooxidant and radicals that enhance the formation of cholesterol oxides (Kumar and Singhal, 1991; Morgan and Armstrong, 1992).

Conclusions: Cholesterol oxides in particular 7-ketocholesterol, which are considered carcinogenic, were not detected in fresh milk, while all of the applied heating treatments led to the formation of cholesterol oxides at different levels. Conventional heating of milk (pasteurization and boiling) caused formation of these oxides with significant differences. Flash pasteurization

Table 3: Effect of heat treatment of and refrigerated storage of milk on formation of 7-ketocholesterol¹

Treatment	Cholesterol (%)	7-Ketocholesterol (ugg ⁻¹ fat)
Raw cow's milk	0.295 ^a ±0.017	ND
Milk Pasteurized at 85±1.0°C for 16 sec.	0.292 ^a ±0.01	2.613 ^a ±0.806
Milk pasteurized at 85±1.0°C for 16 sec and stored for 3 days ² .	0.291 ^a ±0.058	5.520 ^a ±0.186
Milk pasteurized at 95°C for 5 min.	0.290 ^a ±0.023	11.733 ^d ±8.119
Milk pasteurized at 95±1.0°C for 15 min.	0.292 ^a ±0.043	16.328 ^e ±1.717
Milk pasteurized at 85-90°C for 2 min.	0.293 ^a ±0.023	3.142 ^a ±0.694
Milk boiled at 96.3±1.0°C for 5min.	0.278 ^a ±0.017	15.363 ^d ±1.922
Milk boiled in microwave oven at (80% power) 95.8 ± 1.0°C for 5min.	0.282 ^a ±0.036	50.029 ^b ±1.089
Milk heated at 140±1.0°C for 4 sec (UHT) ³ .	0.285 ^a ±0.006	8.708 ^{de} ±1.399
Reconstituted milk powder (UHT) ⁴ .	0.260 ^b ±0.006	80.97 ^a ±1.232

¹Values represent means ± SD (n = 4). Means values in the same column with different superscript letters are significantly different (p ≤ 0.05) according to (ANOVA) Duncan's Multiple range test. ²Commercial refrigeration at 5.0 ± 1.0°C. ³Ultra high pasteurization temperature of fresh cow's milk provided by Danish Jordan Dairy Company (DJD). ⁴Milk prepared from cow's milk powder after reconstitution KDD brand name (Kuwaiti Danish Dairy Company), purchased from local market.

gave the lowest level followed by low temperature long time pasteurization (63±1.0°C for 30 min), pasteurization for 5 min at 95±1.0°C, and boiling for 5 min without significant differences between them. On the contrary, microwave heating milk caused a significant increase in the level of the COPs, which puts a big question mark on the use of microwave oven in food processing and preparation. The oxidation level in UHT milk produced from milk powder (reconstituted milk) was significantly higher than those of UHT produced from fresh milk. This draws our attention towards unsuitability of the use of milk powder in the production of UHT milk (multi-heating effect).

The ability of cholesterol extraction from milk mixed with carageenan and guar gum, was reduced. This is an indication of the formation of hydrophilic interaction(complexes), which were not extractable with organic solvent (chloroform).

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