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Free Thiol Content Analysis in Heated Milk

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ABSTRACT

Chemical changes, denaturation of whey protein, lysine content decrease and hydroxymethylfurfural (HMF) formation, occur during pasteurization and ultra high temperature (UHT) processes. These changes act as heat indices of milk and milk products. In the detection of an adulteration of reconstituted milk in milk samples, hydroxymethylfurfural is the index used routinely as a reference. In this study, free thiol contents of raw milk, reconstituted milk and mixed milk samples after pasteurization and UHT processes, were determined. The results revealed that free thiol content can differentiate between raw milk and reconstituted milk. The significant correlation between the percentage of reconstituted milk in mixed samples and free thiol is $r^2 = 0.98$. Thus free thiol content could be a new indicator for detection of reconstituted milk in raw milk.

Key words: hydroxymethylfurfural, HMF, thiol content, reconstituted milk

INTRODUCTION

After heat treatment of milk, many chemical changes significantly affect the nutritional and sensorial qualities of milk and milk products (Curda *et al.*, 1997). Heat indices are parameters that change upon heat treatment of milk samples. These include undenatured whey protein (Villamiel *et al*., 1999), β - lactoglobulin(Arteaga *et al*., 1994; Morales *et al*., 1995), lactulose(Morales *et al*., 1995) (Indyx *et al*., 1996), hydroxymethylfurfural (HMF) (Keeny and Basette,1959; Morales and Perez,1998) and furosine (van Boekel,1998). These indices can be used to evaluate the adulteration of raw milk samples by milk powder or reconstituted milk (van Boekel,1998). The change in HMF concentration is the indicator recently used for this purpose (Rehman *et al.,* 2000). In recent years, the adulteration of drinking milk with imported milk powder or reconstituted milk has become a significant problem in Thailand. This study was undertaken to try to find a new quicker, cheaper and sensitive method to detect the adulteration of fresh milk.

In the present work, the free thiol content of whey protein isolated from heat treated milk is determined. β - lactoglobulin, one of the major proteins in whey, is composed of 162 amino acid residues and has two disulfide bonds (Cys 66 – Cys 160 and Cys 106 – Cys 119) and a free thiol group at cys 121. In the native protein, this free thiol is completely buried under the C- terminal of a segment of α - helix (Kazuko *et al*., 2000). After heat treatment on milk, free thiol changes due to interactions between reactive free thiol groups that lead to formation of polymer or new substances (Arno *et al*., 2000; Creamer *et al*., 1998). Hence free thiol content is a potential indicator of heat treatment of milk. Free thiol groups react rapidly with 5, 5’-dithiobis (2-nitrobenzoic acid, DTNB)

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under alkaline condition to form a stable yellow products(Stapelfeldt *et al*., 1997) enabling rapid quantitative assay using spectrometric techniques. In this study, free content of undenatured whey protein isolated from pasteurized low temperature long time (LTLT), high temperature short time (HTST) and ultra high temperature(UHT) raw milk and reconstituted milk samples was determined. The content of free thiol in mixed milk model systems (raw milk containing 5 –90 % reconstituted milk) was also determined and the relationship between free thiol content and percentage of reconstituted milk was examined. These results were compared with those obtained using the standard indicator, free HMF content.

MATERIALS AND METHODS

Preparation of milk samples

Whole raw milk samples were sampling from four dairy centers, Kasetsart University Dairy Center, Nong Pho Dairy Center, Muark Laek Dairy Center and Ban Bunk Dairy Center. Whole milk powder samples were purchased from general supermarket in Bangkok. Milk samples 1000 ml of whole raw milk, reconstituted milk and mixed milk model systems (wholemilk : reconstituted milk) were subjected to different methods of heat treatment, pasteurized LTLT, pasteurized HTST and UHT. Reconstituted milk was prepared by dissolving whole milk powder in distilled water, 12.5 % w / v concentration. Mixed milk model systems were prepared by combining unheated whole raw milk to create samples covering the range 5 – 90% reconstituted milk.

Pasteurized LTLT and HTST treatments

Twenty milliliter aliquots were placed in stoppered Pyrex test tube (100 × 15 mm), heated in water bath (UIC Mammert) at 60 – 65°C for 30 min. (LTLT), or 82 – 85°C for 2 – 5 seconds (HTST) then immediately cooled in an ice bath to reduce temperature to 10°C (modified from Morales and Perez,1998).

UHT treatment

Twenty milliliter aliquots were heated in tightly stoppered stainless steel test tubes (200 ×10 mm) in a silicone oil bath (BUCHI oilbath B 490) and the temperature held at 150°C for 2 seconds and then cooled immediately as described above (modified from method of Morales and Perez, 1998). All sample sets were stored at 4°C before analysis.

Determination of free thiol content in raw milk, reconstituted milk and mixed milk model systems.

Milk samples from above were incubated at 25°C. The casein protein was precipitated as described in UDY (1956) as follows:0.77 ml acetic acid (33.3 %v/v) was added to 25 ml samples, incubated at 45°C for 15 m, then mixed with 0.77 ml of sodium acetate solution (27.35 %w/v). After thorough mixing, casein and some denatured whey protein were removed by filtration through Whatman filter paper No.40. The filtrate (undenatured whey protein fraction) was collected and analysed for free thiol content using modified method described by Ewart (1988). One hundred microliters of undenatured whey protein was mixed with 100 μl of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in ethanol, 200 μl of phosphate buffer pH 8.0 and 100 μl of 1 g/L sodium dodecyl sulphate (SDS). This solution was diluted to 1 ml with deionised water, the absorbance measured against a blank at 412 nm using Ultraviolet Visible Spectrophotometry (Shimadzu UV 2401PC). Free thiol content was calculated from molar extinction coefficient 13,600 M^{-1} cm^{-1}. (Stapelfeldt *et al*., 1997)

Determination of free hydroxymethylfurfural (HMF) in mixed milk model systems

Ten ml aliquots were deproteinized with 5 ml of 40% trichloroacetic acid (TCA) and 5 ml of

0.3 N oxalic acid solution as described by Morales and Perez (1998) followed by filtration through Whatman filter paper No. 42. Sample filtrates were again filtered through millipore cellulose membrane filters with pore size 0.45 mm and made up to 25 ml volume with 4 % trichloro acetic acid solution. These sample filtrates were analysed by HPLC (Hewlett Packard series 1100), equipped with a reverse phase C_{18} column (Spherisorb ODS – 2 250 × 10 mm, 5 μm particle size) using an isocratic solvent system of water:acetonitrile (95 : 5) with flow rate 0.55 ml / min. HMF was detected with a Diode Array Detector (DAD G1315 A) at 280 nm. HMF peak sample areas were determined by comparison to HMF peak of an external standard HMF solution.

Statistical analysis

Data obtained from the chemical analysis was evaluated statistically by descriptive parameters, one way ANOVA. Differences in means between model systems were analysed by Least Significant Difference (LSD) test to provide the confidence intervals and significant factors were identified by F-test (Snedecor and Cochran, 1980). All of the statistical procedures were performed at a significant level of 95 % ($p < 0.05$).

RESULTS AND DISCUSSIONS

Free thiol contents in raw milk and reconstituted milk

After casein protein in pasteurized LTLT, HTST and UHT in raw milk and reconstituted milk samples were separated and undenatured whey protein isolated filtrates were analyzed for free thiol content. The result showed that, the mean values of free thiol content in heated raw milk samples were higher than in heated reconstituted milk samples in all heat treatment processes (Table 1). Free thiol content was the highest in pasteurized LTLT, and lowest in UHT milk samples in both sample systems. This result corresponded with the observation of Curda *et al*. (1997). We concluded that free thiol content depends on temperature and time in processing. Free thiol content of reconstituted milk was quite low due to heat treatment in the production of the milk powder. After milk has passed through high heat temperature, reactive thiol reacted with each other or other substance in milk as described by Arno *et al*. (2000). New product is formed similar as in Creamer *et al*. (1998). From this above reason, we detected a low free thiol content in high heated milk product as in UHT milk. Therefore, free thiol content can be used as an index to differentiate, identify the type and determine milk sample quality.

Comparison of free thiol and free hydroxymethylfurfural contents in mixed milk model systems.

Free thiol content decreased with increasing reconstituted milk content in all mixed milk systems (Table 2). Statistical analysis showed a strong negative correlation between free thiol content and reconstituted milk content (R^2 in the range 0.97 – 0.98) (Figure 1). A linear relationship between free thiol content and reconstituted milk content presented in each heat treatment. A strong positive correlation between free HMF and reconstituted milk content occurs in each heated milk system (Table 2). R^2 values range from 0.95 - 0.98 (Figure 2). Thus, a reference index using HMF showed a linear relationship with reconstituted milk content agreeing with Rehman *et al*. (2000). Since HMF product occurred from the reaction between ε -NH_2 of lysine and lactose in milk, Maillard reaction (van Boekel,1998) depend on heating temperature too as referred by Morales and Perez (1998). Reconstituted milk showed a higher content of HMF than in raw milk (van Boekel, 1998). High content of reconstituted milk in sample must be detected free HMF in high content. There is a strong negative correlation between free thiol and free HMF, with a correlation coefficient of - 0.98 (Figure 3). Regression analysis demonstrated a linear relationship between these two parameters.

Free HMF content (μM) is 39.44 - 2.04 × free thiol content (μM). This indicated that free thiol content is an equally effective indicator of the adulteration of milk samples with reconstituted milk as the routinely used indicator, free HMF content. Based on this study, the result showed that free thiol content is a superior indicator when the portion of reconstituted milk is low. Repeat analyses were performed on a mixed milk sample comprising 5 % reconstituted milk (Table 3). The error of detection of the adulteration of reconstituted milk were 0.5 –2 % and 0.09 – 2 % by measuring free thiol content in system of each processing and measuring free HMF, respectively. The detection of adulteration by free thiol method in pasterized LTLT milk had higher accuracy than detection with free HMF method. But free thiol method showed a lower accuracy in detection an adulteration in UHT milk than analysis with free HMF method. Since HMF formed in higher content at high temperature which is easy to detect, but at

Table 1 Mean values of free thiol content in heated raw milk and reconstituted milk samples.

Heating process	Number of samples	Free thiol content (μM)	
		Raw milk*	Reconstituted milk*
Pasteurization LTLT	40	13.69 ± 0.92	4.85 ± 0.45
Pasteurization HTST	40	11.50 ± 0.22	2.26 ± 0.32
UHT	40	10.05 ± 0.05	1.02 ± 0.07

* = mean ± standard deviation
LTLT = Low Temperature Long Time
HTST = High Temperature Short Time
UHT = Ultra High Temperature

Table 2 Mean values of free thiol content, free hydroxymethylfurfural content in heated mixed milk model systems.

Reconstituted milk content in sample (%)	Heating Process					
	Pasteurization LTLT		Pasteurization HTST		UHT	
	Free thiol (μM)	Free HMF (μM)	Free thiol (μM)	Free HMF (μM)	Free thiol (μM)	Free HMF (μM)
5	11.86 ± 1.60	13.89 ± 0.31	11.02 ± 1.25	14.56 ± 0.53	10.95 ± 0.79	15.81 ± 0.23
10	10.90 ± 1.06	16.76 ± 0.34	9.95 ± 0.96	20.02 ± 0.09	9.56 ± 1.21	20.83 ± 0.10
30	9.90 ± 1.57	20.04 ± 0.98	8.41 ± 1.22	24.02 ± 0.86	7.86 ± 0.42	26.21 ± 0.44
50	8.75 ± 0.90	27.67 ± 0.64	4.25 ± 0.65	27.66 ± 1.71	4.68 ± 1.00	28.82 ± 0.16
70	6.85 ± 0.40	30.77 ± 0.96	3.03 ± 0.66	34.49 ± 0.24	2.32 ± 1.13	31.71 ± 0.09
90	4.95 ± 1.32	34.77 ± 0.96	1.42 ± 0.62	36.55 ± 1.30	1.05 ± 0.05	37.16 ± 0.96

Value in table = mean ± standard deviation, number of samples = 40, HMF = hydroxymethyl furfural
LTLT = Low Temperature Long Time
HTST = High Temperature Short Time
UHT = Ultra High Temperature

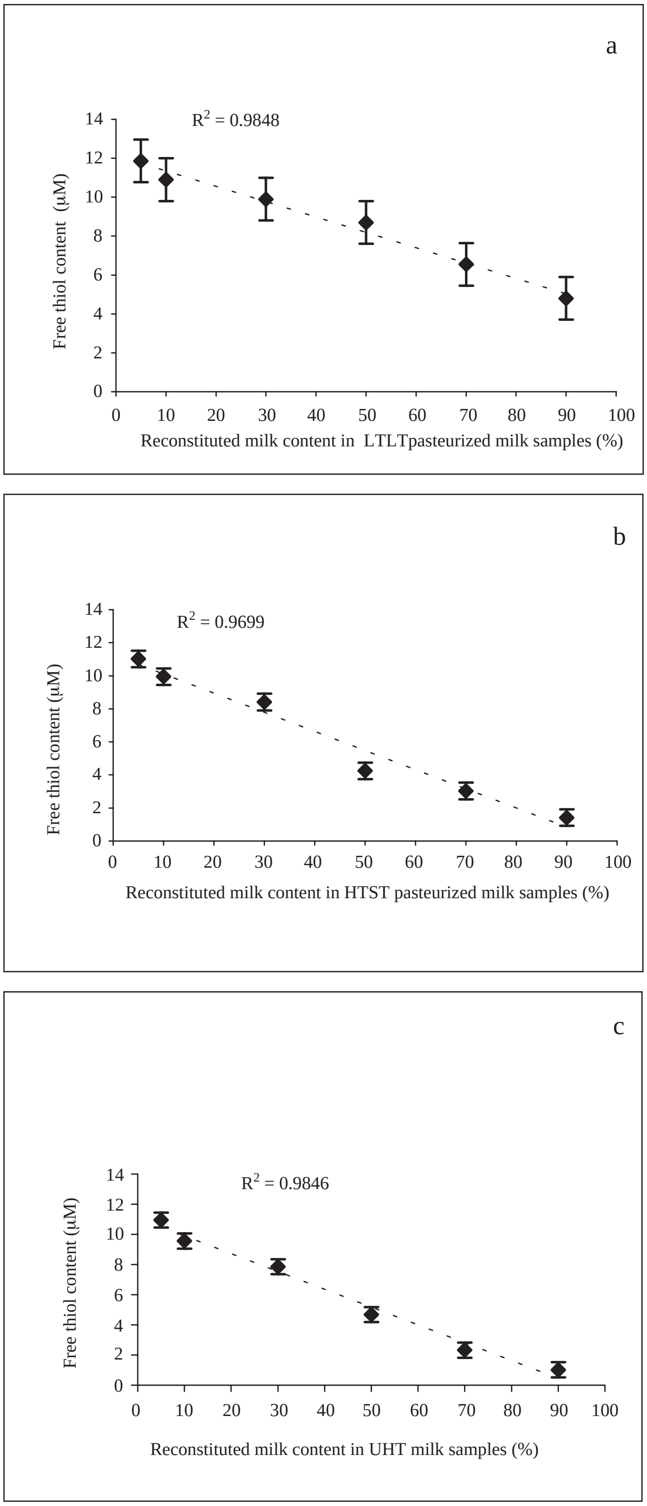

Figure 1 Relation between reconstituted milk content and free thiol content in heated mixed milk model systems, a) LTLT pasteurization, b) HTST pasteurization and c) UHT.

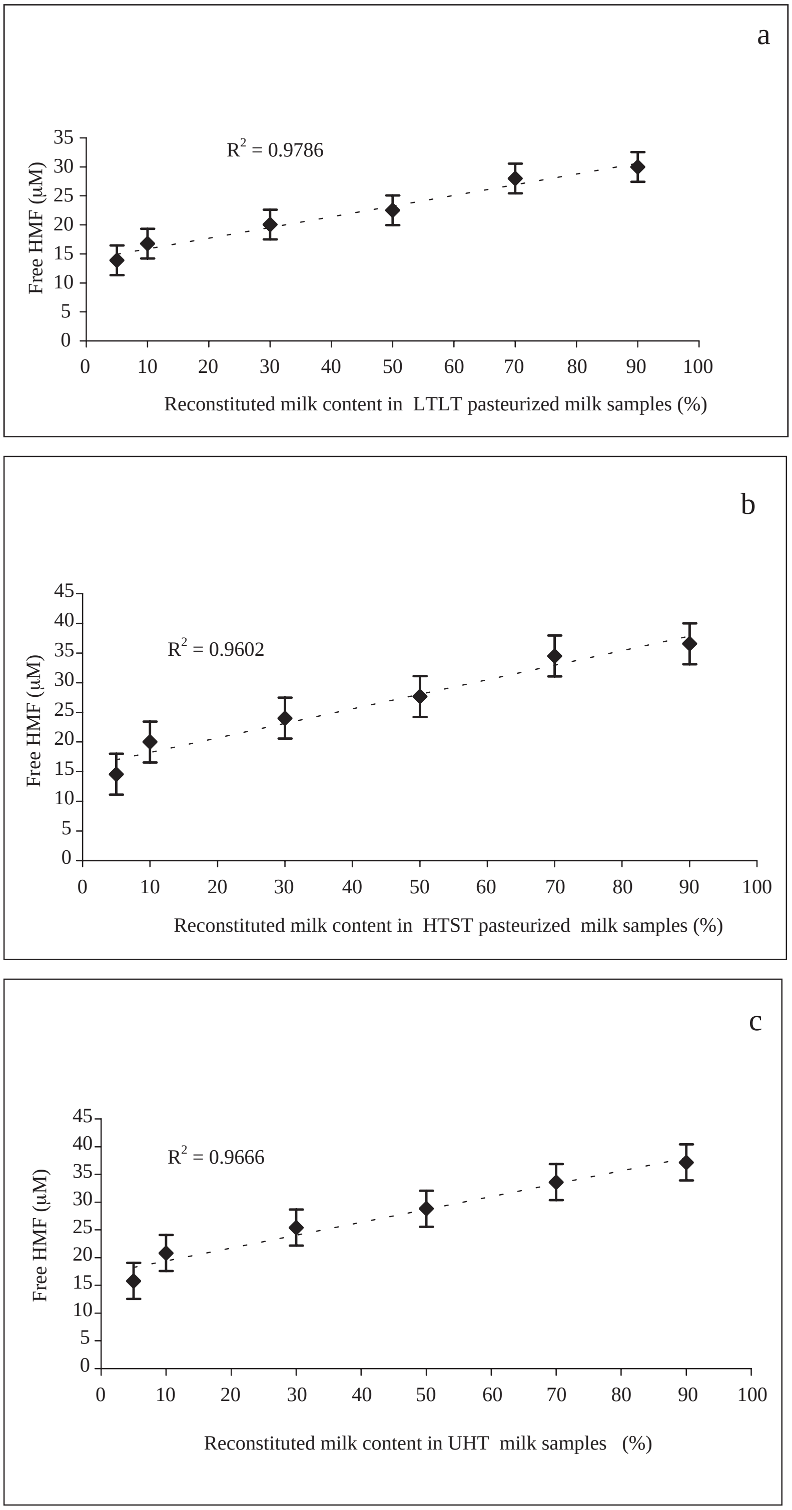

Figure 2 Relation between free hydroxymethylfurfural (HMF) and reconstituted milk content in mixed milk model systems, a) LTLT pasteurization, b) HTST pasteurization and c) UHT.

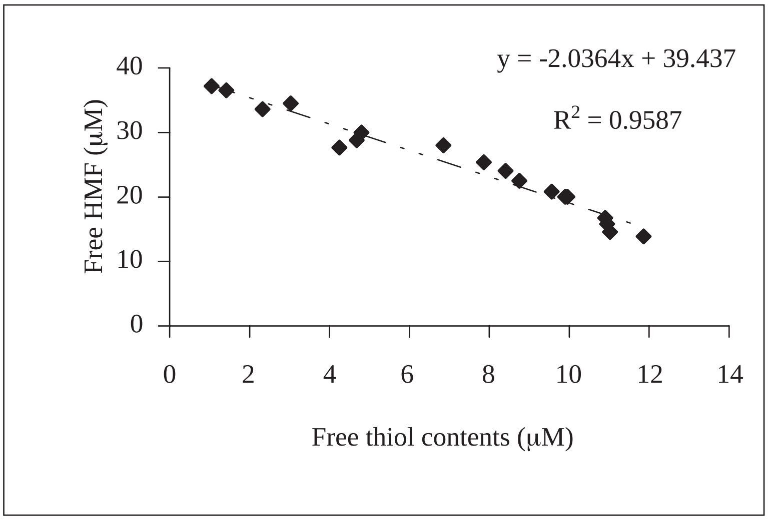

Figure 3 Correlation between free thiol contents and free hydroxymethylfurfural contents in heated mixed milk model systems.

Table 3 The value of reconstituted milk content measured by free thiol and hydroxymethylfurfural in milk samples.

Heating process	True reconstituted milk content (%)	Reconstituted milk content (%) Thiol method	Reconstituted milk content (%) HMF method
Pasteurization LTLT	5	4.67	2.20
Pasteurization HTST	5	5.50	5.09
UHT	5	3.59	5.10

Number of samples = 20
LTLT = Low Temperature Long Time, HTST= High Temperature Short Time, UHT = Ultra High Temperature
HMF = Hydroxymethylfurfural

low temperature the amount of free thiol was higher than at high heat process and gave a high accuracy to detect at pasteurized LTLT, this agrees with Moller *et al*. (1998) and Owusu (1998).

CONCLUSION

Free thiol content in heated whole raw milk samples was significantly higher than in reconstituted samples. There was a strong negative correlation between free thiol content and reconstituted milk content with R^2 0.97 – 0.98. Free HMF content and reconstituted milk content in sample also showed a high positive correlation with R^2 0.95 – 0.98. After statistical treatment of data, free thiol content highly correlated with free HMF content with correlation coefficient - 0.98 at 95 % confidence level. Thus free thiol is a valid parameter for detecting the adulteration of whole milk by reconstituted milk. This method is more sensitive than detection using free HMF content in low heat treated as in pasteurized LTLT milk samples and also easier sample preparation, quicker and less expensive method.

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