

*Full Length Research Paper*

# Discovery of proteolysis in high temperature treated milk by Reverse phase high performance liquid chromatography (RP-HPLC)

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**Proteolysis of UHT milk during storage is one of the problems affecting the dairy industry worldwide. Native enzymes have been implicated as being the main cause of spoilage of such milk. In the current study, reverse phase high pressure chromatography (RP-HPLC) method was used to detect proteolysis by native enzymes in high temperature heated milk. The aim of this research was to assess susceptibility of milk to proteolysis by native enzymes, after being subjected to various temperature-time processing conditions. Samples of raw and heated milk were clarified prior to analysis. Clarification was carried out to obtain pH 4.6 and 6% TCA soluble extracts, which were injected into RP-HPLC after filtration. This method confirmed that raw milk and milk processed at 85°C /15 s were the most proteolysed, indicating that the high temperatures employed during this study (110, 120, 130 and 142°C for 2s) inactivated the native enzymes. The RP-HPLC method is a useful method for the detection of proteolysis in milk.**

**Key words:** Reverse phase-high performance chromatography (RP-HPLC), proteolysis, milk, plasmin, heat-treatments.

## INTRODUCTION

Heat treatment of milk is used for pasteurisation/sterilization and to promote desirable physical characteristics in the protein or food system (Raikos, 2010). During heat treatment, chemical, physical and biochemical reactions take place. These changes are significant because they influence nutritional, sensorial and microbiological aspects of milk. The reactions include Maillard reactions, denaturation and aggregation of whey protein, and formation of complexes between whey proteins, caseins and fat globules (Corredig and Dalgleish, 1999).

Milk contains various proteolytic enzymes which degrade milk proteins. These proteases are either secreted into the

the milk during milk synthesis or originate from bacteria (Fox and McSweeney, 2003). Normally, psychrotrophic bacteria would not be a serious problem in heat treated milk, but their proteases survive UHT treatment and are readily able to degrade  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$ -caseins, causing proteolysis (Sorhaug and Stepaniak, 1997), and furthermore these heat-stable proteases can affect the PL system. Bovine milk contains several proteases including plasmin, plasminogen, plasminogen activators, thrombin, cathepsin D, acid milk proteases and aminopeptidase (Fox and Kelly, 2006). Plasmin (EC 3.4.21.7), occurs in the highest amount and is one of the most heat resistant

enzymes in milk (Datta and Deeth, 2003). It is part of a complex system consisting of plasminogen (PG), plasminogen activators (PA), plasminogen activator inhibitors (PAI), plasmin (PL) and plasmin inhibitors (PI) (Cruden and Kelly, 2003). It is a heat resistant enzyme with optimum activity at 37°C and pH 7.4 and hydrolyses mainly  $\beta$ - and  $\alpha_{s2}$ -caseins, and more slowly  $\alpha_{s1}$ -casein (Bastian and Brown, 1996). The rate and extent of proteolysis is determined by the types and activities of the proteolytic enzymes present (Pereda et al., 2008). These proteases have been studied from the perspective of their physiological significance in milk, their effect on the processing of dairy products, and their nutritional and antimicrobial roles (Aslam and Hurley, 1998). Proteolytic enzymes are of great importance to the dairy industry because they are responsible for imparting desirable or undesirable properties to dairy products through changes in flavour and texture (Nilsen, 2002).

The aim of the current study was to investigate susceptibility of milk processed at various temperature-time profiles to proteolysis by native enzymes during storage, with raw milk as a control. The study was also aimed at observing changes (if any) in protein breakdown products by reverse phase high pressure liquid performance (RP-HPLC).

## MATERIALS AND METHODS

Unless otherwise stated, all materials were from Fisher (Fisher Scientific UK Ltd, Leicestershire, UK). Raw milk was obtained from the Centre of Dairy Research (CEDAR), University of Reading, UK. It was processed on an APV junior UHT plate heat exchanger (APV, Crawley, UK), with two stages of heating involving hot water (80°C) and steam (112-142°C) as described (Browning et al., 2001). A constant flow rate was used, giving a residence time of 2 s in the holding section at 110, 120, 130 and 142°C but 15 s at 85°C. Homogenisation took place between the heating stages at about 170 bars. These temperature-time combinations were selected based on studies of plasmin inactivation. The lowest (85°C/ 15 s) was chosen so as to mimic pasteurisation, whereas others were in a range where inactivation of plasmin could occur and therefore it would be interesting to monitor changes in proteolysis with time.

After cooling, the samples were stored at 2°C for 2 days. The six batches of milk samples were treated with sodium azide (0.05%) to prevent bacterial contamination. They were then dispensed in sterile bottles in a laminar flow hood cabinet followed by incubation at 37°C for 28 days. Sampling for analysis was done on days 0, 3, 7, 14, and 28. Clarification to obtain pH 4.6 and 6% TCA soluble extracts was carried out as detailed below.

Clarification procedures were carried out after incubation for all milk samples studied. Prior to clarification, all milk samples were heated and held at 100°C for 10 min to denature the whey proteins. For isoelectric precipitation at pH 4.6, 50 mL warm water (40°C) was added to 5 mL of milk followed by 0.5 mL 10% (w/v) acetic acid. After standing for 10 min, 0.5 mL 1 M sodium acetate was added and placed in cold water for 10 min before filtration through Whatman no. 41 filter paper and washing and making up to 100 mL. The clear extracts obtained were further filtered by 0.2  $\mu$ m milli-pore filter before being subjected to the RP-HPLC methods.

To obtain 6% TCA soluble extracts, 5 mL of 12% (w/v) trichloro-

acetic acid (TCA) was added to an equal volume of milk (raw, pasteurised/ UHT). The test tubes were vortexed for 2-3 min and left at room temperature for 1 h. The solutions were vortexed again for 2-3 min followed by filtration through Whatman no 41. The filter paper was washed with water and the volume of the supernatant made up to 10 mL with distilled water. Filtration was further carried out by 0.20  $\mu$ m Millipore filter.

Analysis by RP-HPLC was performed on Dionex chromeleon equipment consisting of a P580 pump (Dionex Corporation, Munich, Germany) with a photodiode array detector (Dionex PDA-100), an automated sample injector and a C18, 5  $\mu$ m, 80Å, 150 x 4 mm STH 585 version 2.5 HPLC column compartment with reverse phase column (SGE 150 GL4-C-P-8/5, Melbourne, Australia) at 40°C. Data analysis was computed by the Chromeleon Datasystem soft-ware v. 6.50 SP4 Build 1000. The flow rate was 0.75 mL/min and detection was by a UV/Vis detector at 210 nm. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC grade water whereas Solvent B was 0.1% (v/v) TFA in HPLC-grade acetonitrile. The volume injected was 50  $\mu$ L. During the first 25 min, solvent B was increased from 15 to 35%. After 5 min, it was increased to 100% solvent B and held for 10 min. The column was then equilibrated at 15% solvent B for 10 min in readiness for the next sample injection. The peptides were quantified by integration of peak areas and expressed as the sum of peak areas.

Statistical analysis was carried out by using Statistical Package for Social Sciences (SPSS version 16). General Linear Model of analysis of variance (ANOVA) was used to determine statistical differences between means. Least square differences LSD (LSD) and Duncan's multiple range tests were used to determine values that were statistically different ( $P < 0.05$ ).

All analyses were carried out in triplicate, and results are expressed as mean  $\pm$  standard deviation (SD).

## RESULTS

### pH 4.6 soluble extracts

Proteolysis increased with storage time and differed among samples (Table 1). From day 0 to 28 of incubation, pH 4.6 soluble extracts of milk heated at 85°C had the highest peak area which was statistically different ( $p < 0.05$ ) from all other samples. The second highest peak was from raw milk, followed by milk heated at 110°C/ 2s and finally the last three milk samples had more or less similar peak areas over the storage period (Table 1).

Significant statistical differences in proteolysis ( $p < 0.05$ ) were observed for raw milk, samples heated at 85 and at 110°C on each day of analysis. Figures 1 and 2 show chromatograms of pH 4.6 soluble extracts of raw milk and milk processed at high temperatures which had been incubated at 37°C for 7, 14 and 28 days. From each of these chromatograms, a high peak was observed at around 34 min for all samples, which had previously been linked to activity by plasmin.

From days 7 to 14, pH 4.6 soluble extracts of raw milk samples and of samples heated at 85, 110 and 120°C had higher peak areas which were statistically different ( $p < 0.05$ ) from the rest of the samples. On day 28 however, only raw milk, samples heated at 85 and 110°C were significantly different in proteolysis from each other and

**Table 1.** Peak areas of pH 4.6 soluble extracts of raw milk and milk processed under various temperature – time conditions and incubated at 37°C for 28 days to examine the effect of proteolysis on storage time by the RP-HPLC.

Incubation time (days)	Treatments	Total peak areas of pH 4.6 soluble extracts of milk samples (m AU*min)	
day 0	Raw milk	21.2 ±1.69	a A
	Heated at 85°C	23.7±2.20	b F
	Heated at 110°C	19.6±2.77	a K
	Heated at 120°C	20.9±1.75	a P
	Heated at 130°C	19.7±1.04	a T
	Heated at 142°C	20.8±1.68	a X
day 3	Raw milk	48.4±4.77	c B
	Heated at 85°C	50.5±8.18	c G
	Heated at 110°C	36.2±2.30	d L
	Heated at 120°C	23.6±0.92	e Q
	Heated at 130°C	23.2±2.36	e U
	Heated at 142°C	21.0±0.51	e X
day 7	Raw milk	57.6±2.77	f C
	Heated at 85°C	68.6±3.70	g H
	Heated at 110°C	40.8±1.94	h M
	Heated at 120°C	24.0±1.49	i G
	Heated at 130°C	20.8±2.03	j UV
	Heated at 142°C	21.6±0.52	ij X
day 14	Raw milk	70.3±7.21	k D
	Heated at 85°C	252.9±12.12	l I
	Heated at 110°C	44.3±4.07	m N
	Heated at 120°C	26.5±2.48	n R
	Heated at 130°C	23.2±1.13	n V
	Heated at 142°C	21.3±1.07	n X
day 28	Raw milk	212±8.15	o E
	Heated at 85°C	3159.43	p J
	Heated at 110°C	60.4±1.89	oQ
	Heated at 120°C	29.7±2.07	r S
	Heated at 130°C	25.8±2.78	rs W
	Heated at 142°C	22.7±2.57	s X

Different lower case letters on the same column show significant differences ( $p < 0.05$ ) per day whereas different uppercase letters on the same column show significant differences ( $p < 0.05$ ) per sample; The experiment was replicated 3 times ( $N=9$ ); Error bars indicate standard deviations; The pH 4.6 soluble extracts were diluted ( $\times 20$ )

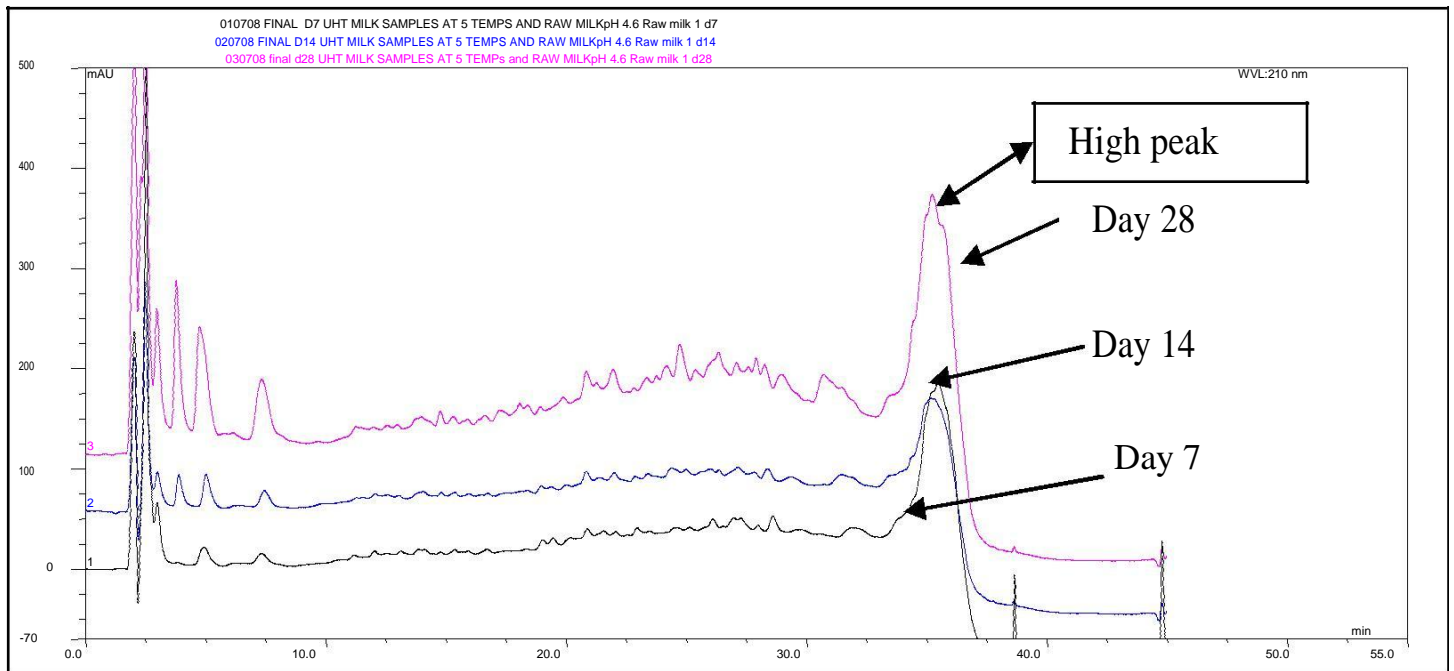
from the remaining samples (milk heated at 120, 130 and 142°C) which were not significantly different ( $p > 0.05$ ).

### 6% TCA soluble extracts

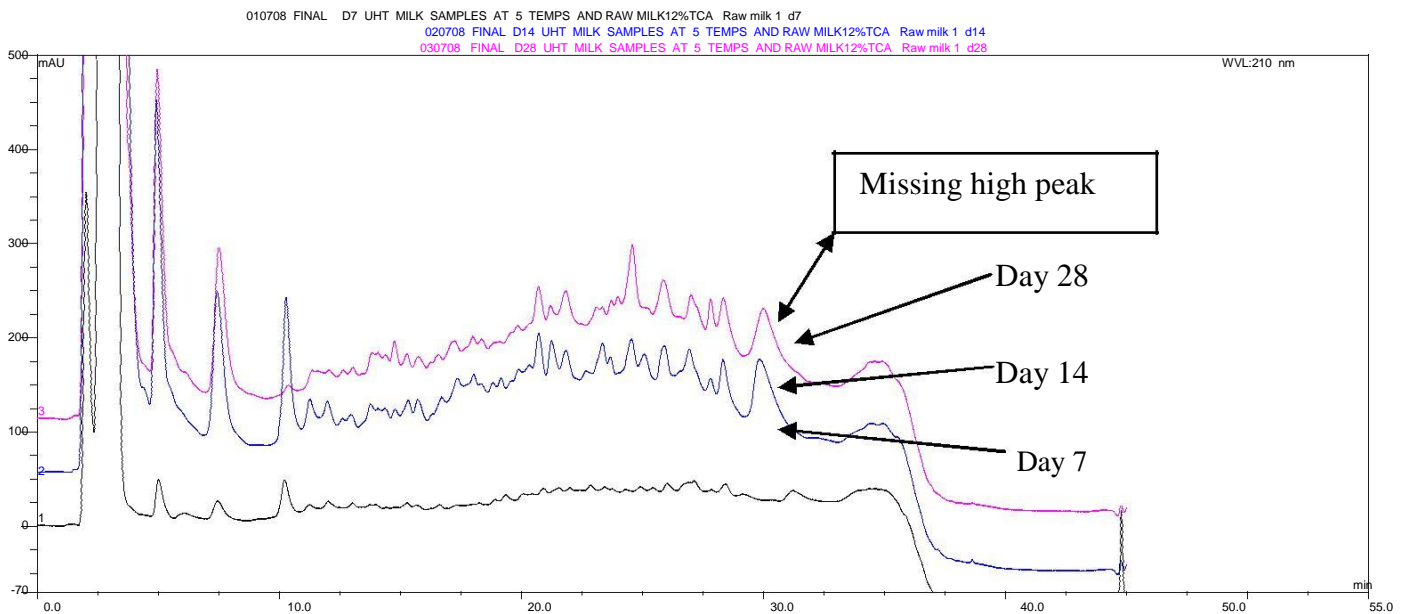
The 6% TCA soluble extracts indicated that although there were significant differences in proteolysis on day 0, the peak areas were generally low as indicated in Table 2. On day 3, statistical differences in proteolysis ( $p < 0.05$ ) were

observed between the raw milk samples heated at 85 and at 110°C which had higher peak areas than samples processed at higher temperatures (120, 130 and 142°C).

From days 7 to 14, 6% TCA soluble extracts of raw milk samples and of samples heated at 85, 110 and 120°C had higher peak areas which were statistically different from the rest of the samples. On day 28 however, raw milk, samples heated at 85 and at 110°C were significantly different in proteolysis from each other and from the remaining samples



**Figure 1.** Sample chromatogram of pH 4.6 soluble extracts of raw milk incubated at 37°C for 7, 14 and 28 days.



**Figure 2.** Sample chromatogram of 6% TCA soluble extracts of raw milk incubated at 37°C for 7, 14 and 28 days.

( $p < 0.05$ ).

Based on the day of incubation, it was evident that raw milk, milk heated at 85 and at 110°C shows significant differences ( $p < 0.05$ ) in proteolysis (Table 2). Proteolysis in the samples of milk heated at 130 and 142°C was low

and varied from day to day. The 6% TCA soluble extracts of milk heated at 85°C had the highest activity than any other sample for all incubation days followed by 6% TCA soluble extracts of raw milk. The same trend was observed for pH 4.6 soluble extracts.

**Table 2.** Peak areas of 6% TCA soluble extracts of raw milk and milk processed under various temperature – time conditions and incubated at 37°C for 28 days to examine the effect of proteolysis on storage time by the RP-HPLC.

Incubation time (days)	Treatment	Total peak areas of 6% TCA soluble extracts of milk samples (m AU*min)	
day 0	Raw milk	32.1±2.00	a A
	Heated at 85°C	33.9±1.25	b F
	Heated at 110°C	29.4±1.02	c K
	Heated at 120°C	29.2±1.25	c P
	Heated at 130°C	27.9±1.75	c U
	Heated at 142°C	25.9±1.57	d Y
day 3	Raw milk	125 ±5.01	e B
	Heated at 85°C	137±3.37	f G
	Heated at 110°C	51.9±3.50	g I
	Heated at 120°C	36.2±3.26	h Q
	Heated at 130°C	33.6±2.40	hi Y
	Heated at 142°C	29.8±1.72	i Y
day 7	Raw milk	134±3.89	j C
	Heated at 85°C	161±6.83	k H
	Heated at 110°C	60.7±3.43	l M
	Heated at 120°C	47.2±2.97	m R
	Heated at 130°C	39.9±3.93	n W
	Heated at 142°C	40.8±7.77	n Z
day 14	Raw milk	237±7.10	o D
	Heated at 85°C	442±6.04	p I
	Heated at 110°C	79.0±2.63	o N
	Heated at 120°C	60.0±7.29	r S
	Heated at 130°C	50.6±3.04	s X
	Heated at 142°C	49.0±2.85	s Z
day 28	Raw milk	330±9.61	t E
	Heated at 85°C	791 ±11.60	u J
	Heated at 110°C	90.9±7.93	v O
	Heated at 120°C	53.3±3.92	w T
	Heated at 130°C	50.2±3.72	w Z
	Heated at 142°C	49.8±2.85	w Z

Different lower case letters on the same column show significant differences ( $p < 0.05$ ) per day whereas different uppercase letters on the same column show significant differences ( $p < 0.05$ ) per sample; the experiment was replicated 3 times ( $N=9$ ); Error bars indicate standard deviations; the 6% TCA soluble extracts were diluted ( $\times 2$ ).

## DISCUSSION

### Effect of storage time on pH 4.6 soluble extracts

Based on the results shown in Table 1, it is apparent that significant levels of breakdown products were observed on each sampling day. Higher proteolytic products concentration observed in raw milk samples were probably due to the presence of the native enzymes, which resulted in proteolysis of the samples. However, the decrease in peak areas as temperatures increased especially at temperature

higher than 110°C, is probably due to destruction of plasmin and plasminogen activator. This in turn, might have decreased plasmin activity on the caseins and hence decreased proteolysis. During severe heat treatments such as UHT processing, decreased plasmin activity is due to thiol-disulphide interactions between disulphide groups in plasmin and reactive SH groups of  $\beta$ -lactoglobulin during the unfolding and denaturation that occurs at high temperatures (Grufferty and Fox, 1986; Kelly and Foley, 1997). Other changes that were described in association with severe heat treatments include denaturation of whey

proteins especially  $\beta$ -lactoglobulin, leading to the formation of  $\beta$ -lactoglobulin- $\kappa$ -casein complex example through interaction with  $\kappa$ -casein (Datta and Deeth, 2003; McMahon, 1996).

As presented in chromatograms (Figure 1), a high peak was observed at around 34 min for all samples. The peak was highest in pH 4.6 soluble extracts from samples heated at 85°C followed by those from 110°C. It is documented that during pasteurisation, plasminogen activator inhibitors, being heat labile, are destroyed causing more plasminogen to be converted to plasmin resulting in increased proteolysis (Grufferty and Fox, 1988; Richardson, 1983). Several studies regarding the inactivation of plasmin have been documented. A study was reported where pasteurisation at 72°C for 15 s and 63°C for 30 min increased proteolytic activity by 30-40 and 8-24%, respectively, after incubating at 37°C for 3-6 days (Noomen, 1975). Increased proteolysis after pasteurisation was also reported (Andrews, 1983; Andrews and Alichanidis, 1983). Several authors have documented that pasteurisation destroys the plasminogen activator inhibitors and thereby more plasminogen is converted to plasmin by the plasminogen activators (Grufferty and Fox, 1988; Nielsen, 2000; Datta and Deeth, 2001; Richardson, 1983).

At higher temperatures however (>110°C), less activity was observed probably due to destruction of plasmin itself.

The peaks also revealed that the highest rates of proteolysis occurred in the pH 4.6 soluble extracts of milk heated at 85°C and of raw milk than any other sample.

### Effect of storage time on 6% TCA soluble extracts

The first four milk samples in Table 1 shows significant differences ( $p < 0.05$ ) in proteolysis at each day of incubation. Proteolysis in the last two samples was low and varied from day to day. The 6% TCA soluble extracts of milk heated at 85°C had the highest activity than any other sample for all incubation days followed by 6% TCA soluble extracts of raw milk. This was also observed for pH 4.6 soluble extracts (Table 1).

The peak at 34 min which was apparent in all pH 4.6 soluble extracts was not so evident in 6% TCA soluble extracts samples (Figure 1). The current results are in agreement with the previous studies (Lopez-Fandino et al., 1993; Datta and Deeth, 2003). The authors explained that unlike bacterial proteases which break down proteins into smaller peptides, plasmin forms large peptides which are precipitated by TCA and hence would not appear in 6% TCA soluble extracts, but in pH 4.6 soluble extracts. Higher peak areas were observed for 6% TCA soluble extracts of samples heated at 85°C followed by raw milk sample. All the other samples had quite low peak areas which progressively decreased with increasing heating temperature (Figure 1). Another study revealed that it was impossible to determine precipitation threshold in relation to peptide size as pepti-

des containing 7-30 residues may be soluble, insoluble or partially soluble at the various TCA concentrations (Yvon et al., 1989). These authors suggested that interactions between TCA and the pep-tides induce an increase of the hydrophobicity of the pep-tides which leads to aggregation through hydrophobic interactions. It is likely that the larger peptides had been broken down during storage and leading to the formation of smaller peptides and amino acids which were soluble in TCA in addition to other peptides broken down by TCA itself.

The pH 4.6 and 6% TCA soluble extracts were correlated to assess their association or relationship. The two extracts were strongly correlated ( $R^2 = 0.93$ ) indicating that the RP-HPLC method is useful for the detection of proteolysis in milk in both extracts.

Although results from this study confirmed that milk heated at 85°C for 15 s indicated higher proteolysis than raw milk, the TNBS method indicated higher proteolysis in raw milk than milk heated at 85°C (Chove et al., 2013). This could be explained as being due to the difference in the measurement principles of the methods. Le et al. (2006) explained that the HPLC method measures the absorbance at a given wavelength of separated peptides and amino acids, the response of which is based on mass and therefore the sensitivity for detection of larger peptides is greater than that for small peptides. The TNBS method measures the free amino groups, thus the higher the amino groups the greater the absorbance, irrespective of the amino acid composition (Beeby, 1980). The latter method is therefore more sensitive to small peptides than large peptides and proteins (Le et al., 2006).

### Conclusion

The current study described the effect of high temperature processing of milk samples and its susceptibility to proteolysis during storage. Milk heated at higher temperatures (>85°C) had lower proteolysis than raw milk and samples heated at 85°C possibly due to destruction of the plasmin enzyme, which is a heat resistant enzyme. It was also shown that higher proteolysis in milk heated at 85°C for 15 s than raw milk was probably due to inactivation of plasmin and plasminogen inhibitors. Thus, it may be concluded that the higher the temperature employed for heating the milk, the less susceptible to proteolysis are the samples. This means that UHT milk processed using the temperature-time combinations employed in this study, is less susceptible to proteolysis than raw and milk heated at pasteurisation temperatures such as 85/15s. RP-HPLC method is a useful technique to detect proteolysis in milk heated at high temperatures.

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