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Research

DETECTING A RAPID METHOD FOR MEASURING THE PROTEOLYTIC ACTIVITY OF RAW AND UHT MILK

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CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT:
The relation between age gelation and proteolytic activity was investigated in this study, as proteolysis is considered to be one of the principal factors that cause gelation. Two different methods for measuring the proteolytic activity of milk samples were applied; the measurement of absorbance at 280 nm and the trinitrobenzene sulfonic acid (TNBS) method. The milk samples used were raw and UHT cow's and goat's milk. Proteolysis was also induced in UHT milk by inoculating cells of four different strains of *Pseudomonas fluorescens* into the milk and the changes taking place were observed. It has been found that the proteolytic activity of raw milk was not affected by a refrigerated storage for 10 days and only after this period it was gradually increased. The higher proteolytic activity of goat's milk in comparison with cow's milk during storage and its increased susceptibility to gelation were also established. Furthermore, the importance of storage temperature and the different effect of the four strains of *P. fluorescens* on proteolytic activity and pH of the UHT milk were shown. Finally, it has been demonstrated that there is a certain level of proteolytic activity in milk that leads to gelation when it is exceeded.

KEY WORDS: UHT milk, proteolysis, age gelation, plasmin, bacterial proteinase

INTRODUCTION

One major concern about UHT milk is age gelation. After weeks to months storage, there is a sudden sharp increase in viscosity accompanied by visible gelation and irreversible aggregation of the micelles into long chains forming a three-dimensional network. The actual cause and mechanism is not yet clear, however, some theories exist: i) Proteolytic breakdown of the casein: bacterial or native plasmin enzymes that are resistant to heat treatment may lead to the formation of a gel. ii) Formation of kappa-casein-ß-lactoglobulin complexes. iii) Chemical reactions: polymerization of casein and whey proteins due to Maillard type or other chemical reactions (Datta and Deeth, 2001).

As mentioned by Datta and Deeth (2001), the gel which forms in UHT milk is a three-dimensional protein matrix formed by the whey proteins, especially β-lactoglobulin, interacting with casein, mainly κ-casein, of the casein micelle. These interactions, which are favoured by the high temperature during the UHT process, result in the formation of β-lactoglobulin-κ-casein complexes (βκ-complexes).

The formation of βκ-complexes alters the conformation of the κ-casein in the casein micelle and the association between κ-casein and other caseins (e.g. α_{s1}-casein) gets weaker. The disruption of these interactions, with enzymatic and non-enzymatic mechanisms, results to the release of κ-casein along with the attached β-lactoglobulin. These released βk-complexes aggregate into a three- dimensional network of cross-linked proteins and when a critical concentration of them is

reached a gel of custard-like consistency is formed (Datta and Deeth, 2001).

According to Datta and Deeth (2001) the development of age gelation in different milk samples occurs after different periods of storage. This indicates that there are many factors which can affect the phenomenon of gelation. These include: i) Mode and severity of heat treatment; ii) Proteolysis (enzymatic mechanism); iii) Non-enzymatic reactions; iv) Milk production factors; v) Microbiological quality of raw milk; vi) Storage temperature; vii) Additives; viii) Fat content.

Proteolysis measurement during storage is an important tool for determining quality and potential shelf life of UHT treated milk (McKellar, 1981). Most of the methods used for measuring proteolysis in UHT milk during storage belong to two groups. As stated by AlKanhal (2000), the first group includes methods that measure aromatic amino acids in trichloroacetic acid (TCA)-soluble peptides and the second group of proteolysis detection methods measure free amino groups (FAG) in TCA-soluble peptides. The methods used during this study were from the first group the measurement of absorbance at 280 nm and from the second group the TNBS method.

The aim of this study is to investigate methods which might be useful for rapid detection of raw milk with a high proteolytic activity in order to prevent it from being used for UHT treatment and to study the phenomenon of age gelation during storage of UHT milk. To achieve that, it will mainly focus on the proteolytic activity of milk and the following objectives will be pursued: i) To use an experimental procedure to detect the presence of proteolytic enzymes in raw milk. ii) To determine types of changes that occur when proteolysis takes place. iii) To look at milk's susceptibility to proteolysis during storage. iv) To observe changes taking place when proteolysis is induced in UHT milk. v) To do measurements in milk samples subjected to age gelation.

MATERIALS AND METHODS

Measurement of absorbance at 280 nm

The following procedure is a variation of the method which was developed after preliminary work in this project.

Bulk raw cow's milk stored refrigerated (4˚C) was used on a daily basis until it became sour. One portion of milk was used straight after removal from the refrigerator while the second portion of the milk was subjected to an incubation step in order to enhance its proteolytic activity. The pH of the milk was measured before and after the incubation step. This incubation step involved two hours in a water bath of 40˚C. The same procedure was then followed for all milk samples (with or without an incubation step):

The initial step of the procedure is to obtain a clear solution by precipitating the micelles and denatured whey proteins of the milk and removing them by filtration. Precipitation of micelles and denatured whey proteins was evaluated by three different methods: i) by mixing a portion of milk with a double portion of TCA (7.5%) solution, ii) by adding 4 g of ammonium sulphate for 20 ml of milk and iii) by adjusting the pH of milk to 4.6 (isoelectric point) using 5M HCl solution.

The solutions were then filtered through Whatman Number 1 filter paper, and clear solutions were obtained in all cases. The absorbance of these solutions at 280 nm was then measured with a Pye Unicam PU 8600 UV/VIS spectrophotometer using acryl-cuvettes (No./REF 67.740) (distilled water was used as a standard). The rest of the filtered solutions (after the removal of 1 ml) were then put in test tubes and inserted in boiling water for 10 minutes. These went turbid, the amount of turbidity being proportional to the amount of undenatured whey protein. They were then filtered with the same filter paper to remove the undenatured whey protein. The remaining solutions were also diluted, using 1 ml of the solutions and 9 ml of distilled water and finally, the absorbance of these solutions at 280 nm was measured with the same spectrophotometer and cuvettes (distilled water was used as a standard). The differences in absorbances of the filtered solutions before and after incubation were considered as indicators of the proteolytic activity of the milk.

Trinitrobenzene Sulfonic Acid Method (TNBS)

The first step of this method is the incubation step, where milk is placed in a water bath of 40˚C for two hours, as in the previous method. The pH of the milk was measured before and after the incubation step. Then, 4 ml of 7.5% TCA solution was mixed with 2 ml of milk. The mixture was shaken well and filtered through Whatman Number 1 filter paper. 0.2 ml of the filtered liquid was taken and mixed with 2 ml of 1M potassium borate buffer (pH 9.2) and 0.8 ml of 5 mM TNBS. The mixture was incubated at 25˚C for 30 minutes in the dark. After incubation, 0.8 ml of 2M monobasic sodium phosphate (containing 18 mM of sodium sulphite) and 5 ml of distilled water were added. The absorbance of this mixture at 420 nm was measured using the same cuvettes and spectrophotometer as in the previous method (distilled water and the reagents were used as a standard).

Three different kinds of experiments took place using this method, which were measuring the

proteolytic activity of different milk samples. The milk samples were:

i) Raw cow's milk and raw/UHT goat's milk

ii) UHT and pasteurised cow's milk with *Pseudomonas fluorescens*. A culture of *P. fluorescens* NCDO 1524 was diluted to a broth and the solution had a concentration of 10^7-10^8 bacteria/ml. The initial purpose was to inoculate a certain number of *P. fluorescens* bacteria to the UHT and pasteurised milk, in order to give a concentration of 10^4-10^5 bacteria/ml in each container

iii) UHT cow's milk with 4 different strains of *P. fluorescens*. Cultures from 4 different strains of *P. fluorescens* were diluted to 8 broths (2 broths for each strain) and the broths had a concentration of 10^7-10^8 bacteria/ml. The 4 different strains of *P.fluorescens* were: *P. fluorescens* NCIMB 701 274 (414), *P. fluorescens* NCIMB 701 527 (415), *P. fluorescens* NCIMB 702 085 (416), *P. fluorescens* NCIMB 702 675 (417). The purpose was also to inoculate *P. fluorescens* bacteria to the UHT samples, so that all samples have a concentration of 10^4-10^5 bacteria/ml.

For all the above operations all reasonable precautions were taken in order to eliminate post processing contamination and contamination by other bacteria. All experiments were carried out in triplicates and the mean values of the measurements were taking into account.

RESULTS AND DISCUSSION

Measurement of absorbance at 280 nm

Raw cow's milk (Sample 1), was stored at 4˚C and analysed regularly. The results obtained before and after incubation are illustrated in tables 1 and 2 respectively:

Table 1: pH and absorbance of raw cow's milk (S1) before incubation during storage

pH and Absorbance before incubation during storage							
Raw milk age	Days	1st	3rd	7th	9th	10th	13th
	pH	6.75	6.71	6.62	6.66	6.7	6.55
Before Boiling	TCA	0.269	0.327	0.282	0.289	0.275	0.282
	Am. Sul.	0.94	0.661	0.804	0.82	0.889	0.815
	pH 4.6	1.562	1.483	1.364	2.315	1.546	1.514
After Boiling	TCA	0.246	0.282	0.256	0.321	0.225	0.226
	Am. Sul.	0.569	0.719	0.52	0.475		
	pH 4.6	0.824	0.851	1.297	0.889	0.95	0.819

As far as the pH of the milk during refrigerated storage is concerned, it can be concluded that it is almost constant for a period of time after which it starts to decrease rapidly. The pH of the milk even when it was 9 days old had remained within the normal limits (pH: 6.66) which suggests that the milk was of good microbiological quality and that there was a limited growth of psychrotrophic bacteria. However, after 10 days of storage the pH started to decrease significantly, which suggests that after this period of time the population of psychrotrophic bacteria started to increase.

Moreover, the incubation step, which involved two hours in a water bath of 40˚C, resulted in a considerable decrease of the pH. At this temperature the growth of microorganisms present in raw milk is at its highest due to being the optimum temperature for them and this causes the fall of the pH.

As far as the absorbance of the milk sample at 280 nm is concerned, it is obvious that the three different methods of clarifying the sample have different effects on the measurements. The samples clarified with TCA had the lowest values, whereas the ones clarified with ammonium sulphate had higher values and these adjusted at pH 4.6 had the highest values. This can be explained by the fact that these values are proportional to the amount of

proteins and peptides present in the three different filtrates.

Table 2: pH and absorbance of raw cow's milk (S1) after incubation during storage

pH and Absorbance after incubation during storage							
Raw milk age	Days	1st	3rd	7th	9th	10th	13th
	pH	6.55	6.41	6.43	6.58	6.5	5.84
Before Boiling	TCA	0.254	0.303	0.397	0.322	0.26	0.304
	Am. Sul.	1.016	0.966	0.768	2.152	0.676	0.958
	pH 4.6	1.69	1.48	1.972	1.93	1.877	1.855
After Boiling	TCA	0.247	0.275	0.249	0.234	0.232	0.217
	Am. Sul.	0.534	0.581	0.517	1.17	0.444	0.551
	pH 4.6	0.883	1.016	0.785	0.918	0.844	0.781

The peptides from bacterial proteolysis are relatively small and soluble in TCA and at pH 4.6, whereas peptides from plasmin hydrolysis are larger molecules, which are soluble at pH 4.6 but are precipitated by TCA (Andrews, 1978a and b). Therefore, changes in peptides in TCA filtrates are indicators of bacterial proteinases while changes in peptides in the pH 4.6 filtrates may arise from plasmin and/or bacterial action (Datta et al., 1999). The fact that the absorbances in pH 4.6 filtrates are high and in TCA filtrates are low suggests that the proteolytic activity in raw milk is mainly caused by the action of plasmin. Additionally, for ammonium sulphate it can be concluded that some of the peptides that are precipitated by TCA remain soluble in ammonium sulphate and therefore there is a greater fraction of soluble peptides in ammonium sulphate than in TCA. This explains why its measurements are between the ones of TCA and pH 4.6.

In addition, there is an almost constant difference for all the filtrates between the absorbances before and after boiling. This difference is caused by the undenatured whey proteins which are precipitated after the boiling. For TCA filtrates this difference is very small which proves that only a small fraction of undenatured whey proteins is soluble in TCA. For ammonium sulphate filtrates it is greater than in TCA and so we can consider that a big percentage of them is soluble in ammonium sulphate, whereas for pH 4.6 filtrates the difference is even greater, which means that the vast majority of or all of them are soluble in pH 4.6.

However, the most interesting observation that can be made from the previous results is that using absorbance at 280 nm for all three methods, the proteolytic activity of the milk seems to remain constant during refrigerated storage. We would expect that after 10 days of storage and after the pH started to fall, the bacterial proteolysis would be increased due to the growth of microorganisms. On the contrary, the proteolytic activity in the TCA filtrates before and after boiling remains constant and so the bacterial proteolysis is stable throughout storage. The same conclusions can also be made by the ammonium sulphate filtrates. Only the pH 4.6 filtrates have a slight increase in the proteolytic activity, which is more obvious after the incubation. This can be explained by the gradual conversion of plasminogen to plasmin during storage, which is

additionally favoured by the conditions of incubation.

Finally, it should be mentioned that although the incubation step affected significantly the pH values, this was not the case for the proteolytic activity. It was expected that the incubation would increase the proteolytic activity, as the enzymatic reaction rate is highest at 40˚C. However, the absorbances obtained for both samples were only slightly higher after incubation than before incubation and this was mainly for the pH 4.6 filtrates. The TCA and ammonium sulphate filtrates had about the same absorbances before and after incubation. This proves that the incubation step favours the activity of plasmin and the conversion of plasminogen to plasmin and does not affect the bacterial proteinases. The small increase is explained by the limited time (2 hours) of the incubation which restricts the rise of the activity of plasmin.

Trinitrobenzene Sulfonic Acid Method (TNBS)

1.Raw cow's milk and raw/UHT goat's milk

Raw cow's and goat's milk, UHT goat's milk prepared in the department, gelled commercial UHT milk and normal commercial UHT milk were obtained for analysis and their absorbances after the incubation step are illustrated in table 3.

Table 3: Absorbance of milk samples during storage (standard: reagents).

Absorbance during storage (standard: reagents)					
Days of experiment	1st	2nd	3rd	4th	5th
Raw cow's milk S1	0.256	0.401*			
Raw cow's milk S2	0.198	0.265	0.222	0.234	0.245
Raw cow's milk S3	0.225	0.268	0.226	0.267	0.227
Raw goat's milk	0.296	0.388	0.382	0.399	0.396
UHT goat's milk	0.285	0.372	0.349	0.352	0.342
Expired commercial UHT goat's milk	0.404*	0.49*	0.442*	0.417*	0.43*
Commercial UHT goat's milk	0.308	0.304	0.307	0.310	0.321

(*) = gelled

For raw cow's milk (Sample 1 or S1), it can be concluded that although on the 1st day of experiment it had a slight higher absorbance and therefore proteolytic activity than the other raw cow's milk samples, on the 2nd day the difference was greater and had as a result the gelation of the milk after the 2 hours incubation. This means that its proteolytic activity after the incubation step had reached a level which causes gelation. However, after the 2nd day this milk sample was not examined anymore because it went sour.

For the raw cow's milk (Sample 2 or S2) we can consider that its proteolytic activity remained almost constant throughout the experiment and presented only a small increase. We cannot be sure about the cause of this increase. Taking into account the results from the previous experiment we can estimate that it is probably caused by conversion of plasminogen to plasmin during storage.

For the raw cow's milk (Sample 3 or S3) it can be seen from table 3 that its behaviour is almost identical with the one of the previous milk. This means that they have similar levels of proteolytic activity, although it is 6 days fresher than the previous one. It is not possible to make any estimation about the proteolytic activity of these two samples after a theoretical UHT treatment and the period of time after which they are going to gel, but it is obvious that as raw milk samples, they present the same levels of proteolysis. This is controversial with the findings of Datta and Deeth (2001), which support that the storage of raw milk, before the UHT treatment, at low temperature (<4˚C) should last for less than 48 hours in order to minimize the growth of psychrotrophic bacteria and consequently, the amount of bacterial proteinases produced in the milk. Further research should be done to reach reliable conclusions.

For the raw goat's milk it can be concluded that its proteolytic activity was higher than the raw cow's milk and presented a small but constant increase during the experiment. As already mentioned in the previous experiment, goat's milk tends to have higher bacterial and somatic cell counts than cow's milk and therefore the bacterial proteinases and plasmin have a higher concentration, which makes it more susceptible to gelation. The increase of proteolysis could be due to an increased activity of either bacterial or indigenous (plasmin) proteinases.

This raw goat's milk was UHT treated and its proteolytic activity was examined for the same period of time. It can be concluded from table 3 that there is a difference between the proteolytic activity of the raw and the UHT treated milk, which means that the UHT treatment led to a decrease of proteolysis. Manji and Kakuda (1988) reported that no residual plasmin activity and 19% original plasminogen remained in indirectly (plate) processed milk (145˚C for 3 seconds). On the contrary, Mitchell and Ewings (1985) found that bacterial proteinases and especially the ones from *Pseudomonas fluorescens* are extremely heat stable with $D_{140^{\circ}C}$ ranging from 2 to 300 seconds. Considering the above we can conclude that the decrease of proteolysis was probably due to the reduction of plasmin activity after the UHT treatment.

The commercial UHT goat's milk examined showed lower levels of proteolytic activity than the previous sample. A reason for that may be the more severe temperature and time conditions that are used in the commercial UHT plants, which results in a greater reduction of proteolysis by the UHT treatment. Another reason may be the better microbiological quality of the commercial milk before the UHT process because the UHT goat's milk was stored in a refrigerator for 11 days before its UHT treatment.

Finally, the expired commercial UHT goat's milk which had already gelled showed the higher level of proteolysis in comparison with all the other milk samples. This was expected as it was the only milk sample that presented gelation even before the incubation step. Its absorbance was about 0.950 or above (data not shown), using distilled water as a standard, and 0.400 or above, using just the reagents as a standard. The only milk sample that reached or exceeded these values was the raw cow's milk (S1) that was gelled after the incubation step. This proves that a milk sample that shows the above values after the incubation step is at the edge of developing gelation.

2.UHT and pasteurised cow's milk with *Pseudomonas fluorescens*

UHT and pasteurised cow's milk inoculated with *P. fluorescens* were used for this experiment and their pH and proteolytic activity were measured during storage (table 4).

Table 4: Absorbance of milk samples during storage (standard: reagents).

Absorbance during storage (standard: reagents)										
Days of experiment	1st	2nd	5th	6th	7th	20th	21st	22nd	23rd	33rd
Pasteur. at room	0.338	1.067*	1.948*							
Pasteur. at 8˚C	0.14	0.137	0.288	0.288	0.338	1.104*		1.14*	1.091*	1.244*
Pasteur. at 2˚C						0.691*	0.805*	0.655*	0.931*	
UHT at room	0.489	0.671	1.384	1.367	1.541	1.869	1.94	1.902	1.871	1.853
UHT at 8˚C	0.148	0.144	0.392	0.452	0.446	1.309	1.405	1.408	1.461	1.493
UHT at 2˚C						1.12	1.079	1.112	1.138	
Past. at 8˚C (blank)	0.170									1.524*
UHT at room (blank)	0.208									0.096
UHT at 8˚C (blank)										0.151

(*) = gelled

As far as the pH of the milk samples before and after incubation is concerned, it was observed that the incubation step had only a very small effect on the pH of the samples and most of the times it resulted to a minor decrease (data not shown).

The pH of the UHT milk samples seemed to be more stable during storage than the pasteurised milk and presented only a slight decrease over a period of 33 days. This decrease was also probably due to the addition of the cells of *P. fluorescens*, as the UHT milk samples that were not inoculated with the bacteria had on the 33rd day almost the same pH as they had on the 1st day (data not shown).

Moreover, it should be mentioned that the pH of the UHT milk samples was not affected by the temperature at which the samples were stored. The pH values of the samples at 8˚C and at 2˚C were practically equal while the pH values of the samples at room temperature were from a little lower to even higher than the previous, depending on the day of the experiment (data not shown).

As far as the absorbance of the milk samples is concerned, it can be seen from table 4 that all milk samples presented an important increase of their proteolytic activity. The first milk sample that showed clearly this increase was the pasteurised milk that was kept at room temperature. From the 2nd day of the experiment its absorbance was really high and it was the only sample that was gelled even before the incubation step. This is explained by the fact that room temperature favours the growth of microorganisms and consequently of *P. fluorescens* which produce the proteinases and increase the proteolysis in milk.

The UHT milk that was kept at room temperature showed almost the same behaviour in terms of its proteolytic activity. This sample had the higher absorbance on the 1st day in comparison with the other samples and after 4 days of storage its absorbance was extremely high. The increase in proteolytic activity continued until the 20th day of storage and then remained stable until the end of the experiment. This increase comes in accordance with the findings of Datta and Deeth (2001) who observed a dramatic increase in proteolytic activity with increasing storage temperature of UHT milk. However, although its proteolytic activity was really high from the 5th day, it had not gelled before or after the incubation step. It had some few gelled pieces of milk, the number of which was gradually increased during storage, but it did not gelled during the whole experiment.

The proteolytic activity of the pasteurised and the UHT milk that were kept at 8˚C was, as expected, lower than the previous two samples. Although their first two measurements were about the same, after the 5th day the proteolysis level in UHT milk was slightly higher. This difference was found to be a little bigger on the 20th day and then remained stable until the end. In contrast to their proteolytic activity, the pasteurised milk was gelled on the 20th day even before the incubation step, while the UHT milk did not show gelation.

The fact that the UHT milk samples at room temperature and at 8˚C did not gel although their proteolytic activity was high enough, can be explained by the mode and severity of the heat treatment used in commercial UHT plants. Datta and Deeth (2001) suggested that in commercial processing of UHT milk, raw milk is commonly preheated, which causes denaturation of β-lactoglobulin, increase of its stability and eventually delays gelation during storage. Moreover, as McMahon (1996a and b) concluded, the more severe heat treatment applied in commercial UHT plants delays gelation due to enhancement of the extent of chemical cross-linking within micelles, which makes the UHT milk less susceptible to gelation.

3.UHT cow's milk with 4 different strains of *P. fluorescens*

UHT cow's milk inoculated with 4 different strains of *P. fluorescens* was used for this experiment and its pH and proteolytic activity was measured during storage. The pH of the blank samples and the samples with the 4 different strains of *P. fluorescens* after incubation is shown in table 5.

The absorbances of the milk samples obtained with the TNBS method after the incubation step are illustrated in table 6.

There were 5 different kinds of milk samples (1 blank and 4 inoculated with different strains of *P. fluorescens*) that were kept at room temperature and at 8˚C, and the pH of all these samples was measured before and after incubation for 8 days. Therefore, the pH of all these samples cannot be examined separately and only the most important observations will be discussed.

The pH values of all samples during storage are similar for the same sample under the same conditions before and after the incubation step. A very small decrease was observed in most cases, which was practically insignificant.

As far as the effect of the addition of *P. fluorescens* on the pH of the samples, we can see that the pH was affected by these bacteria and each strain affected with a different way the pH of the samples during storage. The former can be proved by the fact that the pH of the milk samples that were not inoculated with cells of *P. fluorescens* was almost constant during the whole experiment.

Table 5: pH of milk samples after incubation during storage

pH after incubation during storage								
Days of experiment	1st	2nd	3rd	6th	7th	8th	9th	10th
pH of blank at room	6.62	6.63	6.62	6.66	6.68	6.63	6.67	6.66
pH of 414 at room	6.52*	6.42*	6.44*	6.43*	6.44*	6.13*	6.49*	6.47*
pH of 415 at room	6.59	6.52*	6.48*	6.47*	6.53*	6.54*	6.54*	6.53*
pH of 416 at room	6.54*	6.41*	6.44*	6.23*	6.46*	6.39*	6.49*	6.41*
pH of 417 at room	6.61	6.56	6.59	6.60*	6.63	6.62*	6.69*	6.62*
pH of blank at 8°C	6.62	6.62	6.6	6.68	6.67	6.61	6.68	6.65
pH of 414 at 8°C	6.62	6.58	6.48	6.41*	6.51*	6.40*	6.48*	6.48*
pH of 415 at 8°C	6.62	6.59	6.58	6.48*	6.54	6.49	6.56*	6.55
pH of 416 at 8°C	6.64	6.58	6.50*	6.40*	6.50*	6.48*	6.50*	6.47*
pH of 417 at 8°C	6.62	6.58	6.57	6.50*	6.56	6.56	6.57*	6.54

(*) = gelled

Table 6. Absorbance of milk samples during storage (standard: reagents)

Absorbance during storage (standard: reagents)								
Days	1st	2nd	3rd	6th	7th	8th	9th	10th
blank at room	0.162	0.179	0.177	0.187	0.155	0.207	0.17	0.136
Milk 414 at room	0.537*	1.638*	1.451*	2.003*	2.028*	2.008*	1.9*	1.996*
Milk 415 at room	0.138	0.503*	0.465*	1.221*	0.998*	1.219*	1.46*	1.408*
Milk 416 at room	0.468*	1.764*	1.31*	1.994*	2.023*	2.003*	1.944*	2.01*
Milk 417 at room	0.136	0.255	0.233	0.579*	0.347	0.44*	0.463*	0.742*
blank at 8°C	0.138	0.158	0.129	0.159	0.188	0.146	0.168	0.153
Milk 414 at 8°C	0.116	0.136	0.371	1.609*	0.938*	1.118*	1.789*	0.894*
Milk 415 at 8°C	0.133	0.137	0.139	0.481*	0.321	0.352	0.334	0.353
Milk 416 at 8°C	0.128	0.14	0.559*	1.854*	0.699*	0.76*	1.824*	1.129*
Milk 417 at 8°C	0.123	0.143	0.205	0.654*	0.236	0.241	0.334	0.362

(*) = gelled

In general, strains 414 and 416 of *P. fluorescens* resulted in a gradual decrease of the pH of the milk samples at room temperature and at 8°C. Their behaviours were in most cases completely identical and consequently we can consider that both of them have the same effect on the milk samples, as far as their pH is concerned.

Strains 415 and 417 had different effect on the pH of the milk samples accordingly with the temperature that the samples were kept. At room temperature the samples inoculated with the strain 415 presented an almost constant pH throughout the experiment, whereas at 8°C the pH was decreased

after the first few measurements, then it was increased and finally it reached its initial values. On the contrary, at room temperature the samples with the strain 417 showed a gradual increase of the pH, while at 8˚C they showed the same behaviour as the previous samples, by having a decrease on the first days and an increase at the end.

Finally, it should be mentioned that there seems to be a correlation between the samples that gelled and their pH. In most cases, when the pH of the samples was falling below 6.5 these samples showed gelation after the incubation step. This can be explained by the fact that this fall of the pH was due to the growth of *P. fluorescens*, which resulted to an increase of the concentration of the bacterial proteinases that cause gelation. However, there were also some exceptions to this, mainly for the samples inoculated with the strain 417, which although it gelled its pH was gradually increasing. Probably the growth of this strain of *P. fluorescens* is accompanied by an increase in pH and that is why its pH was increasing while it was gelled.

As far as the proteolytic activity of the milk samples is concerned, it is obvious from the above results that the samples with the higher levels of proteolytic activity were the ones that presented the lower pH values. This means that these samples had the more intense growth of *P. fluorescens*.

The proteolytic activity of the milk samples (blank) that were not inoculated with cells of *P. fluorescens* remained constant both at room temperature and at 8˚C. All the other milk samples, in which *P. fluorescens* was added, showed an increase of their proteolytic activity.

It should be mentioned that there was a significant difference in the absorbances and therefore in the proteolysis levels among the same samples, depending on the temperature of storage. The samples stored at room temperature presented considerably higher levels of proteolysis in comparison with the samples stored at 8˚C. Room temperature favours the growth of *P. fluorescens* while 8˚C seems to delay its growth. In addition, there should be some other factors that also favour the proteolytic activity in higher temperatures because as already mentioned (Datta and Deeth, 2001), there is a dramatic increase in proteolytic activity with increasing storage temperature of (non-inoculated with *P. fluorescens*) UHT milk.

The different strains of *P. fluorescens* had a different effect on the proteolysis levels of the milk samples. The effect of the strains 414 and 416 was about the same while the strains 415 and 417 showed completely different behaviour. It is previously mentioned that the strains 414 and 416 had the same effect on the pH of the milk samples. Besides that, the proteolytic activity of the milk can be considered that was affected with a similar way by these two different strains.

Even from the 1st day of the experiment the milk samples with the strains 414 and 416 that were kept at room temperature showed a significant increase of their proteolytic activity and both of them gelled after the two hours incubation. This increase continued until the 6th day and after that the proteolytic activity remained stable. On the contrary, the other samples did not show any increase on the 1st day.

On the 2nd day the sample with the strain 415 at room temperature showed an increase and gelled after the incubation step. The sample with the strain 417 showed increased level of proteolysis after the 6th day, where was also gelled.

For the samples kept at 8˚C the first sample that gelled was the one with the strain 416 on the 3rd day. On the 6th day both samples with the strains 414 and 416 gelled and their proteolytic activity was really high. All the other samples at 8˚C had not gelled until the end of the experiment. Their proteolytic activity showed a gradual increase but it did not reach the necessary levels to cause gelation.

Taking into account all the absorbances in comparison with the samples that gelled, we can estimate that the mid-point of gelation for a UHT milk sample is when its absorbance is reaching the value of 0.860 (standard: distilled water) (data not shown) or 0.440 (standard: reagents). These values are close to the values found in the previous experiments.

Comparison and evaluation of methods

The two methods used were the modified method of measuring the absorbance at 280 nm and the trinitrobenzene sulfonic acid method (TNBS). It has been already mentioned that the first method measures aromatic amino acids in trichloroacetic acid (TCA)-soluble peptides while the second method measures free amino groups (FAG) in TCA-soluble peptides.

The measurement of absorbance at 280 nm is a simple to perform and inexpensive method of measuring the proteolytic activity in milk. However, sometimes it produced values that were out of range compared with the previous and the following results. This was probably due to variations during the application of the method or other factors that influenced the results. Its major drawback is so the susceptibility of the method to interference.

The TNBS method is also relatively simple and inexpensive method to perform for measuring the proteolytic activity in milk. The results obtained with this method presented a greater consistency in

comparison with the previous method, which proves that it is less susceptible to possible variations and is more sensitive and accurate. However, as mentioned by Fields (1971), it suffers from high background absorbance of the reagents. Considering the results obtained with this method, using distilled water as a standard, or just the reagents as a standard, it is recommended that by using the reagents as a standard, the influence of the background absorbance will be reduced and the accuracy of the method will be improved. In a recent study done by Chove et al. (2011), four methods were selected to assess their suitability for the detection of proteolysis in milk by plasmin and the TNBS method was recommended for use in routine laboratory analysis on the basis of its accuracy, reliability and simplicity.

To conclude, the TNBS method is suggested as a more effective method of measuring the proteolytic activity of milk than the measurement of absorbance at 280 nm, as the latter is a "crude" method, less suitable for this kind of experiments.

CONCLUSION

Both methods used, the measurement of absorbance at 280 nm and the trinitrobenzene sulfonic acid (TNBS) method were simple and inexpensive to perform and their daily application is considered possible in a typical dairy lab. However, the repeatability of results with the TNBS method was found to be better than the measurements of absorbance at 280 nm. That is why the TNBS method is suggested as a more sensitive and accurate method to measure the proteolysis level in milk.

As far as the proteolytic activity of raw cow's milk is concerned, it was observed that during refrigerated storage the level of proteolysis remained almost constant and only a slight increase took place after its 10th day of storage, probably caused by conversion of plasminogen to plasmin and not by further production of bacterial proteinases.

Raw goat's milk was found to have higher levels of proteolytic activity than cow's milk, which also showed a small but gradual increase during the experiment. This was attributed to the higher bacterial and somatic cell counts of goat's milk which favour proteolytic activity in milk.

Furthermore, the addition of cells of *P. fluorescens* in UHT milk has been found to affect significantly its proteolytic activity and pH. The different strains of *P. fluorescens* had a different effect on the proteolysis levels in milk, but all of them resulted in its increase.

In addition, proteolytic activity of milk was considerably affected by the temperature of storage. All samples stored at room temperature presented significantly higher levels of proteolysis in comparison with the samples stored at 8˚C.

Finally, it is suggested that there seems to be a correlation among gelation, pH and proteolytic activity. In most cases, when the pH of the samples was below 6.5 these samples showed gelation after the incubation step. It should also be mentioned that using the TNBS method and when proteolysis in milk was exceeding a certain level, gelation occurred. Therefore, a UHT milk sample with primary an absorbance, using the TNBS method, above 0.860 (standard: distilled water) or 0.440 (standard: reagents) and secondary a pH value below 6.5 should be considered as a milk that reached the limit of its shelf life and cannot be used anymore for human consumption.

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