# Enzymatic and/or chemical dosages coupled with the clarification of the sample applied to the screening evaluation of heat load in commercial milks

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Heat treatment is essential for hygienic safety and for extending shelf-life of milk. Heating of milk affects principally its physicochemical, nutritional and organoleptic properties. The most important changes are the decrease in whey protein solubility and the decrease in stability of casein micelles. Maillard reactions are also important and undesirable consequences.

## Heat treatments

Therefore heat treatment of milk should meet the minimally required time/temperature combinations in order to obtain a product without important heat damage.

Diverse heat treatment are used in dairy industry. Thermisation, pasteurisation and direct UHT-treatment are mild treatments and produce only a denaturation of whey proteins or enzymes. The indirect-UHT and principally in-bottle sterilisation may also cause changes in casein micelle structure and improve also the formation of "new" substances.

## Assessment of heat treatment

The principal methods proposed for evaluation of heat damages in commercial milks can be classified in two types:

1. The first one quantifies directly or indirectly the whey protein denaturation and the inactivation of enzymes resulting of mild heat treatment: (Indices or indicators type 1)

Due to their varying thermal resistance, some indigenous milk enzymes can be used as indicators of severity or effectiveness of heat treatment of milk and milk products. Enzyme

assays, though limited to heat treatment up to the mildest of ultra high temperature (UHT) treatment, have the advantage to be rapid and simple. Measurement of lactoperoxidase, catalase and alkaline phosphatase activities have been widely used to evaluate the degree and efficiency of mild heat treatments. They provide information about the thermal history of the product and are used in pasteurisation control.

The impact of the mild heat treatment can be also estimated by the quantitation of residual native β-lactoglobulin, or of residual free SH groups and disulphide bonds.

2. The second one measures the consequences of the Maillard and associated reactions resulting of drastic heat treatments (Indices or indicators type 2)

The compounds formed in the Maillard and associated reactions often used as indicators of heat damage are hydroxymethylfurfural (HMF), furosine, lactulosyllysine, carboxy-methyllysine and free fluorescent intermediary compounds. Good correlations exist between HMF, furosine and lactulose contents. They are good indicators of heat damage in drastic UHT and in-bottle sterilised milk.

Some of the coloured compounds resulting from non-enzymatic browning, such as melanoidins and humic acids, have maximal U.V. absorption between 265 and 300 nm but still absorb at 340 nm. This last wavelength was chosen in our laboratory because there is no significant interference by milk components and the Clarifying Reagent (see below). $A_{340}$ is well correlated with HMF.

However many of these usual analyses applied to milk and dairy products are time-consuming or require sophisticated equipment.

## The Clarification of milk and its application in routine analysis of milk and dairy products

Milk is a complex biological liquid with heterogeneous composition. It is at once a solution of whey proteins, a colloidal suspension of micellar casein and an emulsion of fat in this aqueous mixture. Consequently, milk is opaque to the light. Therefore, in analytical dairy laboratory, milk turbidity due to micelles of phosphocaseinate and fat globules calls for sample pre-treatment by precipitation, centrifugation and filtration before colorimetric or spectrophotometric measurement. These preliminary steps are time-consuming, tedious and often costly, they become useless if the sample is rendered transparent. That allows direct spectrophotometric measurements on the samples or on the reaction mixtures.

Clarifying Reagent (Linden, et al, 1987) is a patented mixture of organic solvents and

detergents with an apparent pH >13 and a very low absorbance between 340 and 800 nm. The solvent of the proteins disperse the micelles of casein and the detergents decrease the superficial tensions. The alkaline pH is responsible of the breakdown of intra and inter molecular bonds, the shifting of calcium out of the micelles and the increase of the hydratation of the polypeptide chains. Thus the Clarifying Reagent solubilizes casein micelles and fat globules and allows direct spectrophotometric measurement to be made without the preliminary precipitation and filtration steps required in many protocols. The patent is now closed and a review is submitted.

Many applications of milk clarification, such as the chemical measurement ($NH_2$- and SH-groups, ammonia, $A_{340}$) or enzymatic measurements (alkaline phosphatase, proteases, plasmin, plasminogen-activators, lipase, N-acetyl-ß-D-glucosaminidase, lactoperoxidase, and $\gamma$-glutamyl transpeptidase activities) can be used in routine analysis. We have recently developed the ammonia measurement in milk by means of glutamate deshydrogenase with NADH as coenzyme (manuscript in preparation).

## Question : to have a better assessment of heat treatment

At the present time there is no single method that allows the complete characterisation of all types of heat-treated milk. Measurement of whey protein denaturation and enzymes inactivation makes possible to differentiate between raw, pasteurised and UHT milks. Products of heat damage such as lactulose, furosine and HMF seem suitable for characterising and for estimating the severity of UHT and in-bottle sterilisations.

A series of investigations were performed on all types of milk. Raw milk samples were from individual fresh raw milks collected on a farm. Commercial pasteurised-, UHT-, and in-bottle sterilised milk samples were purchased at the local market and also collected from european countries.

"Camembert" and "Emmental" cheese samples were from local markets.

Pasteurisation treatment is generally carried out at 72~75°C for 20~50 s on plate heat exchangers. UHT treatment after a heat pre-treatment is performed by direct, or by indirect heating on plate or tubular heat exchangers at 140~145°C for 2~6 s. In-bottle sterilisation is carried out at 120°C for 15~20 min after pasteurisation and UHT treatments.

The exact heat load was never known for any of these heat treatments.

Successive studies have concerned many indicators of type 1 (alkaline phosphatase, $\gamma$-glutamyl transpeptidase, lactoperoxidase, -SH) and many indicators of type 2 (ammonia, $A_{340}$, CE-lactulose measured by capillary electrophoresis, HPLC-lactulose, $A_{340}$). During the optimising of these protocols we have shown that each well correlates with the corresponding reference method (r=0.98 or 0.99).

# The one-dimension evaluation of the heat load of raw milk and commercial milk

All these used indicators are not discriminant for all classes of samples of raw, pasteurised, direct-UHT, indirect-UHT, unlabelled-UHT and in-bottle sterilised milk.

A precedent study on all the types of heated milk have shown that SH amounts was unsatisfying to differentiate raw from pasteurised milk, and drastic UHT from in-bottle sterilised milks. Moreover, SH groups amount in milk presents individual and seasonal variabilities.

The studied enzymes as alkaline phosphatase, $\gamma$-glutamyl transpeptidase, lactoperoxidase differentiate effectively raw milk and pasteurised milk samples. Nevertheless, enzymes present some inconveniences:

- the reactivation of alkaline phosphatase during the storage of the heated milk
- the individual and seasonal variations characterising alkaline phosphatase and lactoperoxidase.

The $\gamma$-glutamyl transpeptidase does not present these last inconveniences. This enzyme is very interesting because its sensibility to the temperature is ranged between those of the alkaline phosphatase (the most thermolabile) and the lactoperoxidase (the most thermoresistant). Residual activities show that alkaline phosphatase is useful between 60 and 69°C, that $\gamma$-glutamyl transpeptidase between 68 and 69°C and that lactoperoxidase between 73 and 80°C during 1 minute heating.

In an other study, the $\gamma$-glutamyl transpeptidase allows to differentiate Camembert labelled "made with raw milk" from an other one made "with pasteurised milk". Its significantly discriminating power is 2 times that of alkaline phosphatase. The results are not significantly different when experiments with these 2 enzymes are carried out on Emmental. This last cheese has a large size and the cooling of the paste is quicker on the extern part compared to the middle; this leads to a variability of the samples for the same cheese, the comparison between the 2 type of cheese-making becomes not easy for Emmental.

During the following studies using ammonia, lactulose and $A_{340}$ that all the classes of milk are significantly differentiated ($P<0.001$). Only a few experiments show significant limits at $P<0.01$ and $P<0.05$. Non significant differentiation at this last limit are obtained between indirect-UHT and unlabelled UHT (with ammonia, CE-lactulose and $A_{340}$), between pasteurised and direct-UHT (with A340).

## Research of a better assessment: the 2-dimensions evaluation of the heat load of raw milk and commercial milk

The use of both indices may be useful for a better characterisation of milk samples, and a more precise evaluation of heat severity. The theoretical principle is to confront a first indicator type 1 essential for low heat treatments, and a second indicator type 2 characterising the more drastic heat treatments.

The first confrontation $A_{340}$ (type 2, Maillard or associated reactions) versus SH content (type 1, soluble protein denaturation) has been carried out on 130 milk samples. All the heat treated milks are differentiated, excepted raw and pasteurised samples which have a partial common area. This can be partly explained because raw milk samples are individual milk samples collected overall a year. SH content of raw milk depends on the season, these variations in SH content are minimised in commercial milk samples because they are treated in bulk. Pasteurised and UHT milk samples also have a small common area. It can be due to a too severe pasteurisation treatment which may produce nearly same heat damage than those observed in mild UHT treatment. These two measurements permit a characterisation of heat load of unknown samples when compared to mean values obtained for each class of milk samples.

The following experiments has been realised on classes of milk containing from 5 to 12 samples. We consider that ammonia is an indicator type 1 or an intermediary between type 1 and type 2.

The confrontation Ammonia (type 1) versus $A_{340}$ (type 2) gives a good correlation between the two parameters (0.97) but does not allow to separate pasteurised and UHT milk samples.

The confrontation Ammonia (type 1) versus CE-lactulose (type 2) gives a good correlation between the two parameters (0.95) and allows to better separate pasteurised and UHT milk samples.

The last studied confrontation Ammonia (type 1) versus HPLC-lactulose (type 2) is only carried out on UHT milk samples. The correlation between the two parameters is good (0.95). This well separates direct-UHT from indirect-UHT milk samples.

## Conclusion

The clarification of milk is simple, not time-consuming and requires not sophistically apparatus. All the chemical and enzymic protocols well correlate with the corresponding reference methods.

The study on the evaluating the heat load of commercial milk samples shows that it is

today possible to discriminate raw and pasteurised milks only by enzymic dosages. On the other hand the $\gamma$-glutamyl transpeptidase seems to be very useful for milk and cheese. Ammonia measurement presents some interest to be applied on heated milk samples.

It will be very interesting to discriminate raw, thermised and pasteurised milks. It is perhaps possible by using the confrontation of ammonia and a thermolabile enzyme activity or by using the ratio of residual activities of thermolabile enzyme activity / thermoresistant enzyme activity (i.e. $\alpha$-fucosidase / alkaline phosphatase or better $\alpha$-fucosidase / $\gamma$-glutamyl transpeptidase).

## Principal References On The Clarifying Reagent And Its Application


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