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Chemical markers for monitoring heat damage of processed milk: Review

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Abstract
The heat load indicators or chemical markers have an important role to play for controlling the nutritional and organoleptic quality of liquid milk in the future. Since dairy companies are obviously not aware of the negative effects caused by overheating of liquid milk, there is an urgent need for establishing obligatory threshold levels (limits) for market milks regarding TTIs (e.g. acid-soluble β-Lg, furosine, and lactulose). However, amongst the known heat load indicators furosine and lactulose are good thermal indicators of heat damage since high correlations were observed in their levels in most studies.

Keywords: Milk, Heat indicators, Heat treatment, Quantitative determination

1. Introduction
Nowadays, milk is nearly always thermally processed before consumption. The main purpose of heating is to make milk safe for human consumption (by killing pathogenic bacteria) and to extend its shelf life. Heating causes a significant loss of organoleptic and nutritional quality (e.g. vitamin destruction, precipitation of calcium phosphate, denaturation of whey proteins, and Malliard reaction). Furthermore, an undesirable precipitation of denatured proteins and minerals can be formed on the walls of heat exchangers. The reason is heating of milk that causes different modifications in the physico-chemical state of its components, leading primarily to the denaturation of certain protein fractions (enzymes, whey proteins and the formation of Malliard reaction products). Several heat-induced changes related to these modifications have been developed in recent years to determine the quality of milk. These so called heat-damage markers indicators can be used to control and check the heat treatments given to milk. However, only some compounds are suitable as chemical markers of the heat treatment intensity and they are called heat load indicators or time temperature integrators (TTIs) that are used to quantify the impact of thermal processes on milk (e.g. the enzymes alkaline phosphatase and lactoperoxidase; the whey protein β-lactoglobulin β-Lg; Hydroxy methyl furfural, HMF; lactulose; and furosine) (Mayer *et al.*, 2010) [36].

2. General principle
Chemical indicators are either physical or chemical devices used to monitor one or more of the process parameters of the sterilization cycle. Evaluation of heat treatment is possible if irreversible changes are induced in the product. The most interesting are (bio)-chemical reactions. Two types of chemical reactions may be used to assess heat treatments:

- The degradation, denaturation and inactivation of heat-labile components, e.g. whey proteins or enzymes ("Type 1"-indicators);
- The formation of "new" substances, e.g. lactulose or products of the Malliard reaction ("Type 2"-indicators).

However, these indicators have also many limitations. Upon heating of a dairy product heat damage depends on both duration as well as intensity (temperature) of heating. The assessment of only one indicator does not allow distinguishing between a prolonged heat treatment at relatively low temperature and a short heat treatment at a higher temperature. In most cases more than one heat indicator will be used. Also pH as well as concentration of the constituents of the products will influence the chemical and bio chemical heating reactions. Moreover, the range of possible heat treatments, from pasteurisation to in bottle sterilisation is far too broad for the use of just one intrinsic indicator. Finally, a good intrinsic indicator requires a relative simple analytical method (Jarita *et al*., 2015) [28].

3. Chemical Markers or Heat Load Indicators

Type 1-indicators

These are components that can be denatured or inactivated by heating. Two important categories are enzymes and whey proteins.

A. Enzymes

Milk contains enzymes that have the ability to catalyse specific chemical reactions. Enzyme activities can be considerably affected by thermal processing as indicated in Table 1. Enzymes are heat labile and loss of activity is in most cases easily to detect by a simple colour reaction. Alkaline phosphatase and lactoperoxidase are two important intrinsic indicators for monitoring heat damage of pasteurized milk. Since alkaline phosphatase is stable to temperatures slightly higher than those required to destroy milk pathogens, the control of the activity of this enzyme is the most important indicator for evaluating the hygienic safety of pasteurized milk. This means that pasteurized milk must be negative for the phosphatase test. Determination of the activity of lacto peroxidase, which is a rather stable endogenous enzyme, can be used as a simple test for the determination of the upper limit of pasteurization. Pasteurized milk must show a positive lacto peroxidase reaction and must be labelled as "highly pasteurized" when negative result is obtained. Therefore, the use of lactoperoxidase test distinguishes between the two treatments viz. pasteurization and high temperature pasteurization. Standard methods for alkaline phosphatase and lactoperoxidase tests include those based on spectrophotometry and fluorimetry. Other native enzymes can also be used to evaluate heat load in milk e.g. α-fucosidase, phosphodiestrase, α-mannosidase, but their practical use is much less extended.

a. Alkaline phosphatase:

Alkaline phosphatase (ALP,) is a phosphor monoesterage that catalyses the hydrolysis of phosphoric monoesters. The enzyme is widely used in stabilising adequate pasteurization of milk; inactivation of ALP takes place at temperatures slightly higher than necessary to kill M. tuberculosis, S. senftenberg or L. monocytogenes (Girotti *et al*, 1994) [22]. Remaining ALP activity after pasteurization points to improperly operating pasteurization units or possible contamination by raw milk (Murthy *et al*., 1990) [43].

Mostly, the ALP activity is quantified spectrophotometric ally with phenyl phosphate or p-nitrophenyl phosphate as substrate. The disadvantage of these methods is their relative complexity and long incubation time. Dependent on the source, their ALP detection limit is equivalent to about 0.1–0.2 to 0.5% of the raw milk level, which seems to be adequate in practice (Schlimme *et al*., 1994) [55].

Nevertheless, a rapid, more sensitive procedure has been developed, based on the fluorometric measurement of the commercial substrate Fluorophos. This technique allows estimation of ALP at very low levels in the equivalent of 0.006–0.01 % raw milk (Schlimme *et al*., 1996) [56].

Two major isoenzymes of ALP have been identified, a- and b-phosphatase, which are present mainly in the milk plasma and in the membranes of fat globules respectively (Girotti *et al*., 1994; Walstra and Jeness, 1984) [22, 59]. The difference in ALP allocation is reflected by the ALP activity. About 30–40 % of ALP activity is situated in the fat globule membrane. ALP activity is consequently lowered in raw skimmed than in raw whole milk (Claeys *et al*., 2002a; Schlimme and Thiemann, 1992) [9, 54].

Next to milk fat content, the ALP activity in raw milk is also reported to depend on the breed of cow, the stage of lactation (increasing at the end of lactation), the volume of milk produced and the age of cow (decreasing with age) (Girotti *et al*., 1994; Schlimme and Thiemann, 1992) [22, 54]. Moreover, seasonal variation in ALP activity has been observed; activity increased during summer to remain high in autumn, and to decrease again in the middle of winter (Schlimme and Thiemann, 1992) [54].

Although ALP is widely applied as an indicator of efficient pasteurization, only few detailed quantitative kinetic studies on thermal inactivation of ALP have been published. Thermal inactivation of ALP follows first order kinetics. Independent of the ALP assay applied, reported z-values range between 5 and 6.7 °C (Claeys *et al*, 2001; Murthy *et al*., 1990) [8, 43] and between 8.5and 9.5 °C (Schlimme *et al*., 1996) [56].

Some authors suggest the ALP test to be less sensitive when applied to skim milk since because of its lower fat content skimmed milk tends to have less residual ALP activity (Walstra and Jeness, 1984) [59]. However, it observed comparable inactivation kinetics in milk with different fat content and although initial ALP activity was lower in skimmed than in whole milk, fat content seemed not to affect the ALP test result for pasteurized milk substantially (Claeys *et al*., 2002a) [9].

Next to fat, also sugar has been reported to alter heat inactivation characteristics of ALP by increasing the heat stability (Sanders & Sager, 1948). More specific, removal of lactose during ultrafiltration increased ALP inactivation. Thus, use of ALP to measure the efficiency of pasteurization of dairy products with modified composition has to be undertaken with some caution.

b. g-Glutamyltransferase

g-Glutamyltransferase (GGT) catalyses the transfer of g-glutamyl residues from glutathione and other g-glutamyl containing peptides to amino acids or peptides (Baumrucker, 1979) [5]. GGT is more abundant in milk than ALP rendering an enzymic assay based on residual GGT activity more sensitive. GGT is also more heat resistant than ALP, but less than lactoperoxidase (Andrews *et al*., 1987; Martin *et al*., 1989) [2, 35]. This fact and the good correlation that exists between reduction in GGT activity and destruction of streptococci (Patel and Wilbey, 1994) [48], demonstrates the potential of GGT to describe the temperature region between that covered by ALP and lactoperoxidase as well as to define the limit between pasteurized and high temperature pasteurized milk (Herrmann et.al, 1996; Zehetner et.al., 1995) [24, 61].

For the quantitative determination of GGT a commercial test kit is available based on the procedure of Baumrucker (1979) [5]. In the assay the enzyme acts on the specific substrate, l-g-glutamyl-r-nitroanilide, transferring the glutamyl group to the receptor, glycylglycine. The r-nitroaniline thus released is quantified spectrophotometrically (McKellar *et al*., 1991) [37]. About one quarter of the GGT activity is located within the cream and three quarters within the milk serum (Baumrucker, 1979) [5]. Like ALP, GGT inactivation kinetics can be described by a first order reaction, and are characterized by an Ea-value of 457–473 kJ/mol (Andrews *et al*., 1987; Zehetner *et al*., 1995) [2, 61].

c. Lactoperoxidase

Lactoperoxidase (Lpo) is present in the milk serum at a concentration of about 0.03 g/l, although variations have been

reported. The enzyme catalyzes the oxidation of indigenous thiocyanate by hydrogen peroxide to yield highly reactive, short-lived antimicrobiological substances, mainly hypothiocyanate. Lpo has been suggested as a possible enzyme to monitor thermal processes higher than 72 °C and is used to make a distinction between pasteurized and high temperature pasteurized milk (Griffiths, 1986) [23] Though, there are some limitations on the suitability of Lpo as a TTI for liquid milk. Firstly, a cyclic variation of Lpo activity is observed with a maximum in summer (August) and minimum in winter (February) probably explicable by change in feed of cows during the year (Olszewski and Reuter, 1992) [47]. Secondly, its thermo stability is strongly dependent on the nature of the medium (buffer <milk or whey) and influenced by pH, casein, b-lg and ionic strength (Herna´ndez *et al*., 1990) [25]. Thirdly, Lpo is sensitive for irreversible photochemical inactivation and may regenerate during storage (Herna´ndez *et al*., 1990; Olszewski and Reuter, 1992) [25, 47]. Finally, a validated analytical procedure to quantify Lpo activity is not available yet. Existing methods for estimating peroxidase activity are spectrophotometrically assays based on different donors such as p-phenylenediamine (Aurand *et al.,* 1956) [4] and ABTS (2, 20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (Herna´ndez *et al*., 1990) [25], of which the latter is most commonly used. Thermal inactivation of Lpo follows first order kinetics with a z-value situated between 3.7 and 4.3 °C (Olszewski and Reuter, 1992) [47].

d. Whey Proteins

Several physical and chemical changes occur during thermal processing of milk, and these changes (denaturation) affect the functional and sensory properties of milk. During the heating process, whey proteins containing sulfhydryl residues undergo various changes resulting in the formation of -

- A protein complex between beta-lactoglobulin and kappa-casein, with consequent modification of rennet coagulation behaviour and heat stability,
- Typical off flavours and
- Unusual amino acids (lysinoalanine).

Whey proteins show different thermal stabilities:alpha-lactalbumin> beta-lactoglobulin> bovine serum albumin >immunoglobulins. The whole whey protein fraction, as well as its individual components, may be used as indicators of thermal treatment. While denaturation of alpha lactalbumin can be a good parameter to describe high-temperature treatments, such as sterilization, denaturation of beta-lactoglobulin is useful to describe thermal treatments from pasteurization to UHT processing. Most important indicators are the whey protein nitrogen index (WPNI) and the heat number.

The WPNI is the amount of undenaturated whey protein N (soluble in saturated NaCl) expressed as milligrams per gram of milk powder or liquid milk and can be determined by a turbid metric detection. The heat number is expressed as the percentage of nitrogen insoluble at pH 4.8. Monitoring consumption milk in order to make the distinction between pasteurized milk and UHT milk is often carried out by determination of acid soluble β-lactoglobulin. Chromatographic techniques allow these determinations with high precision and accuracy but variations in the absolute and relative concentrations of β-lactoglobulinin the milk may be a drawback (Mortier *et al*., 2000) [41]. The quantitative determination of acid-soluble β-Lg has been proposed to distinguish between different categories of heat-treated milk. A minimum content of 2600 mg per L for pasteurized milk, of 2000 mg per lit for high-pasteurized milk, and of 50 mg per L for UHT milk is within the limits proposed by the International Dairy Federation (Mayer et. al., 2010, Tamime, 2009) [36, 57]. The proposed data are indicated in Table 1 for various heat treated samples.

e. β-Lactoglobulin

The whole whey protein fraction (e.g. whey protein nitrogen index) as well as its individual components can be used for the classification of heat treatments. β-lactoglobulin (**β** -lg) is the most abundant whey protein; on average, milk contains about 3.3-3.5 g β-lg per litre (Schlimme *et al*., 1996) [56]. β-Lg is a globular protein containing two intra molecular disulphide bonds and one thiol group. At room temperature and milk pH the protein is predominantly present as a dimer. β-Lg is important as it can have a marked influence on the functional properties of milk products. Moreover, β -lg plays an important role in fouling of heat exchangers (Wilbey, 1996) [60]. The quantitative determination of undenaturedβ-lg has been proposed for distinguishing between different categories of heat-treated milk. A minimum content of 2600 mg/l β -lg for pasteurized milk and of 2000 mg/l β-lg for high-pasteurized milk is within the limits proposed by the International Dairy Federation. As a clear-cut distinction of UHT milk from in-bottle sterilized milk, a minimum content of 50 mg/l for the latter has been proposed (Schlimme *et al*, 1994; Wilbey, 1996) [55, 60].

In literature, conflicting results exist regarding the kinetics of β-lg denaturation, possibly reflecting the complexity of the process. Several researchers have reported first order kinetics (Claeys *et al*., 2001; Harwalkar, 1986) [8, 26], while others reported second order kinetics (Harwalkar, 1986) [26], 1.5 order kinetics (Dannenberg and Kessler, 1988; Schlimme *et al*., 1996) [13, 56] or consecutive first order reaction kinetics (Harwalkar, 1986) [26].

As to the effect of milk fat content on β-lg denaturation, views diverge. Some observed a protective effect (Pellegrino, 1994) [49], whereas others observed no effect of milk fat on β-lg denaturation (Lo´pez-Fandin *et al*, 1993) [34]. Still others observed a synergistic effect of fat on β-lg denaturation although the concentration of β-lg in crude whole and skimmed milk was comparable (De Koning *et al*., 1990) [15]. This positive correlation between milk fat content and β-lg denaturation was suggested to be related to the binding of β-lg on the fat globule surface (Claeys *et al*., 2002a) [9]. When a **β** -lg molecule denatures, it can follow three possible pathways: (i) it can bind to another denatured serum protein molecule, (ii) it can react with micellar k-casein, or (iii) it may bind to a protein on, or adsorb to the surface of a fat globule. From the rate of the reaction with fat globules, the latter pathway appears to be favoured (Corredig and Dalgleish, 1996) [11].

Similar to ALP inactivation, lactose content has been reported to have a protective effect on β-lg denaturation (Pearce, 1989) [51]. Two mechanisms are held responsible for this stabilization. Firstly, sugars and similar substances tend to maintain or increase the hydration of protein molecules, enhancing the water structure in the immediate surroundings of the protein molecules and contributing to their stability. Secondly, irreversible aggregation of the whey protein with casein is strictly hindered by means of protein-lactose complexes formed during heating.

B. Type 2-indicators

Type 2 indicators are based on the formation of 'new' substances. During heat treatment of milk, lactose is involved in the Malliard reaction and isomerisation and subsequent degradation reactions. The most studied chemical reaction in heat-treated milk is, undoubtedly, the Malliard reaction, in which amino groups (mainly casein bound lysine residues) and reducing sugars (mainly lactose) are the main reactants. The Malliard reaction consists of several steps, strictly dependent on temperature, pH, water activity, and type of sugar and amino group involved. Some of the end products considered as important Heat.

Load indicators are described below and their reported values are shown in Table 1.

a. Lactulose

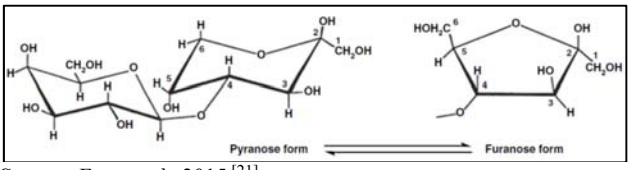
Lactose can isomerize to lactulose in heat treated milk. Lactulose isomerization is catalysed by free amino groups of casein and is strictly dependent on time of heating, heating temperature, and ph. Normally, lactulose does not occur in fresh milk and highly pasteurized milk, but only in UHT and sterilized milk. Lactulose content is therefore considered a suitable indicator of heat treatment (De Block *et al*., 1996) [14]. Lactose-derived compounds can be used to evaluate more intensive heat treatments i.e. direct and indirect UHT treatments and sterilization. Among the sugars derived from lactose, lactulose undoubtedly represents the most widely studied index for differentiating heated milks and for evaluating the heat load to which milk was subjected. Lactulose is a very interesting indicator for the study of heating of milk and milk products and the determination methods are accurate and precise (De Block *et al*., 1996) [14]. Lactulose is formed by isomerization of lactose during heating of milk and has been proposed as an analytical index to distinguish UHT from sterilized milk. UHT milk should have a lactose upper limit of 600 mg/L. Lactulose is not found in pasteurized milks, although values up to 82 mg/L have been reported in commercial samples (Tamime, 2009) [57]. A variety of methods have been reported to be used for lactulose determination e.g. GC, HPLC, CE, enzyme methods, colorimetry and continuous flow amperometry. Determination of lactulose allows distinction between pasteurized milk, UHT milk and sterilized milk.

Published data describe lactose isomerization into lactulose as an irreversible zero order (Claeys *et al*., 2001) [8] or first order reaction (Schlimme *et al*., 1996) [56] with Ea-values between 105.6 and 118 kJ/mol and between 118.3 and 125 kJ/mol respectively. In fact, the reaction is reversible, as expected for an isomerization reaction and lactulose degrades further to galactose and other constituents. Furthermore, lactose also isomerizes into very small amounts of epilactose and both lactose and lactulose are involved in the Malliard reaction (Berg and van Boekel, 1994; O'Brien, 1997) [6, 45].

Milk pH has been reported to affect lactose isomerization. In the pH range between 6.7 and 6.8 the variation of lactulose content is minimal, while below pH 6.7 the lactulose content decreases and above pH 8 the lactulose content increases substantially (Nangpal and Reuter, 1990) [44]. Soluble citrate and phosphate were found to catalyse the formation of lactulose, presumably by acting as bases (Andrews and Prasad, 1987) [3]. Probably, variation in the phosphate content of milk (86–103 mg/100 ml) is too small to affect the rate of lactulose formation in commercially processed milks sufficiently as a result of which the applicability of lactulose as a TTI could be invalidated (Florence *et al*, 1985) [20]. There are no published data on the variability in the citrate content of milk. When the calcium content of milk is increased by 50 ppm, formation of lactulose is depressed, probably due to the increased complex formation of citrate and phosphate with calcium, thus reducing the levels of phosphate and citrate available to catalyse lactulose formation (Olano *et al*., 1987) [46].

Also on the effect of milk storage on lactulose formation, contradictory data have been reported. Nangpal and Reuter (1990) [44] observed even at 20 °C a systematically increase of lactulose concentration, which was independently of the initial concentration. Andrews and Prasad (1987) [3] on the other hand, observed no effect of milk storage at room temperature on the lactulose concentration. There is probably a balance between formation and degradation of lactulose. This balance depends on external and compositional factors, which can explain the different observations during storage.

Determination of the lactulose concentration allows the differentiation of UHT from in-bottle sterilized milk, and of directly from indirectly processed UHT milk (Schlimme *et al*., 1996) [56]. Tentative proposals being considered include lactulose levels of > 100 mg/l and > 600 mg/l for UHT and sterilized milk respectively (Schlimme *et al*., 1994) [55]. However, an overlap between lactulose levels in UHT and sterilized milk has been reported rendering measurement of lactulose alone insufficient as an index of heat treatment (Wilbey, 1996) [60].



Source: Fox, *et al*., 2015 [21]

Fig 1: Chemical structure of lactulose

Significance of Lactulose in Health

Lactulose is sweeter than lactose and about 60 % as sweet as sucrose. It is not metabolized by oral bacteria and hence is not cariogenic. It is not hydrolysed by intestinal β-galactosidase and hence reaches the large intestine where it can be metabolised by lactic acid bacteria, including Bifidobacterium spp. and serves as a bifidus factor. For this reason, lactulose has attracted considerable attention as a means of modifying the intestinal microflora, reducing intestinal pH and preventing the growth of undesirable putrefactive bacteria. It is now commonly added to infant formulae to simulate the bifidogenic properties of human mil. Lactulose is also reported to suppress the growth of certain tumor cells. (Fox *et al*, 2015) [21].

Significance of measurement of lactulose

Determination of lactulose allows distinction between pasteurized milk, UHT milk and sterilized milk, improper addition of reconstituted milk powder Methods used for lactulose determination:

1. Gas Liquid chromatography(GC)
2. Thin-layer chromatography (TLC)
3. High-performance liquid chromatography (HPLC)
4. Enzyme methods,
5. Colorimetry and
6. Continuous flow amperometry.
7. Rapid determination by micro dialysis and biosensors.

Khan *et al*., (2006) [31] developed a method based on hydrolysis of lactulose under acidic conditions followed by reaction of the hydrolysed product with resorcinol, giving absorption peaks at 398 and 480 nm. A simple and rapid flow system was developed for the determination of lactulose in milk samples, which is based on the hydrolysis of lactulose to galactose and fructose by the enzyme β-galactosidase immobilized in a reactor (Moscone *et al*., 1999) [42].

Amine *et al*. (2000) [1] developed an enzymatic spectrophotometric assay for the determination of lactulose in milk samples by the hydrolysis of lactulose to fructose and galactose, and fructose dehydrogenase reacts with fructose in presence of a tetra zolium salt giving a coloured compound which can be detected spectrophotometrically at 570 nm.

b. Hydroxy Methyl Furfural (HMF)

Components formed by the Malliard reaction can also be used as intrinsic indicators for monitoring heat damage. One of those products is Hydroxy methyl furfural (HMF) which is suited as an indicator for severe heat treatments (UHT and sterilization). HMF is an intermediate compound formed in Malliard reaction in heated milks. HMF can be formed by whatever reducing sugar and can also be used as heating indicator for sugar containing lactose free dairy products. Using HMF as a marker, Ferrer *et al*. (2000) [18] observed differences in various UHT milk samples. Free HMF was found to be present only in some stored UHT milk samples, levels ranging from 8.24 to 50.9 µg/100 ml of milk, probably due to lower milk quality. Several factors possibly affecting the use of HMF as a marker have been investigated. In particular, the total HMF level in milk appears to be affected by milk fat concentration. The HMF level in commercial UHT milk (stored below 50 °C) is also related to temperature and time of storage, and increases with higher temperature (Keeney and Bassette, 1959) [30]. As for kinetics of formation of HMF, most studies in model systems and milk reported zero-order kinetics with an Ea-value between 104 and 135.1 kJ/mol (Peri *et al*, 1988) [52].

According to Berg and van Boekel (1994) [6] milk fat content has no effect on total HMF formation in UHT-milk. Jime´nez-Pe´rez *et al*. (1992) on the other hand, observed a protective effect of milk fat on total HMF formation, after pasteurization as well as after direct UHT and sterilization. Varying fat contents did not influence associated values. Next to fat content, it has been reported that HMF values increase with increasing dry matter concentration (Fink and Kessler, 1986) [19] and initial lactose concentration in the product (Kind and Reuter, 1990) [32].

Although there is formation of HMF from its precursors during storage of milk, HMF content remains constant between refrigeration and room temperature (Fink and Kessler, 1986; Jime´nez-Pe´ rez *et al*., 1992) [19, 29]. This has been attributed to the fact that part of the HMF generated during storage is lost through oxidations or other transformations. At temperatures 530 °C the equilibrium is moved towards HMF formation (Jime´nez-Pe´rez *et al*., 1992; Morales *et al*., 1996) [19, 39].

HMF is a recognised indicator for distinguishing in bottle sterilized milk (HMF values of 30–140 mmol/l) from UHT-milk (1–10 mmol/l) (Fink & Kessler, 1986; Morales *et al*., 1996) [19, 39]. Directly heated UHT milk has lower HMF values than indirectly heated milk, probably because the warming up and cooling down periods in direct UHT are virtually instantaneous so that the amount of heat this milk receives is usually less than the amount of heat received by indirectly heated UHT milk (Fink and Kessler, 1986) [19]. Another explanation is that during direct UHT saturated steam is injected into the preheated milk, diluting the milk and thus reducing the concentrations of reactants susceptible to heat damage (Kind and Reuter, 1990; Nangpal and Reuter, 1990) [32, 44]. The suitability of HMF as TTI for heat treated milk is however questioned because of the variable amounts of HMF measured in raw milk (3.6–7.3 mmol/l) (Burton, 1984) [7].

These variable HMF amounts detected in raw milk are fact generated by the acidic digestion preceding the spectrophotometric or chromatographic analysis, since HMF should not be present in unheated milk (Morales *et al*., 1996) [40]. Sterilization processes are the origin of HMF in dairy products and may be connected with their colour change (browning). Burton (1984) [7] studied changes of HMF concentration during storage of infant milk. The authors stored samples for up to nine months at different temperatures (20, 30, 37 °C). Free and total HMF (free HMF compounds plus the potential HMF compounds derived from other browning intermediates by heating sample with oxalic acid at 100 °C for 25 min) were analysed. Powdered infant milk had more HMF present than corresponding liquid milks (34.7 and 12.2 µg/kg (w/v), respectively, after 9 month, 37 °C). In this case, zero-order kinetics of HMF formation was established regardless of milk type and storage temperature (Burton, 1984) [7].

In traditional Indian dairy products (*Dudhchurpi*), HMF levels are highly correlated with the sensory attributes of the product. A strong positive correlation was found between HMF content and colour, texture, flavour as well as overall appearance (Jarita *et al*., 2015) [28]. HMF concentration was also measured in several infant milk-based formulas (Morales *et al*., 2000) [40]. In most cases, the mean concentration of HMF was 29.5 µg/kg (w/v). In two samples it was found to be 296.6 and 247.2 µg/kg (w/v) (Morales *et al*., 2000) [40].

The influence of different temperatures on HMF formation during the storage of UHT milk was studied by Mayer *et al*. (2010) [36]. There were no significant differences in HMF concentration in milk stored at 4 and 8 °C, but storage at room temperature caused a two fold increase in its amount when compared with freshly sterilized product. HMF concentration was strongly correlated with milk colour changes (Mayer *et al*., 2010) [36]. Traditionally, HMF is determined by a colorimetric method, but this method has low specificity. At present, capillary electrophoresis and RPHPLC appear to be the most powerful techniques for HMF determination.

c. Furosine

The early stage of the Malliard reaction can be monitored through the amount of furosine in milk. Malliard reaction products, such as lactulose lysine, results from heating and it are transformed into furosine by acid hydrolysis. Furosine is a very interesting Malliard product and can be obtained by the acid hydrolysis of heated milk or milk Furosine can also be used for monitoring the heat treatment of liquid milk. Furosine is present in concentrations of 3-5 mg/100g protein in raw milk and 5.2-7.5 mg/100g protein in pasteurized milk. In sterile milk samples furosine level varies within a wide range, but it is usually higher in milk processed by indirect than by direct UHT treatment. A furosine content of 8 mg per100 g protein has been suggested as upper limit for pasteurization, of 20 mg per 100 g protein for high pasteurization, and of 250 mg per 100 g protein for UHT processing (Mayer *et al*., 2010) [36].

However, overlapping of values in milk submitted to different heat treatments is the main difficulty encountered when using furosine as chemical indicator to distinguish among commercial sterilized milks. Moreover, the furosine level may increase during storage of UHT milk. High concentrations of furosine are also formed during the production of milk powder due to favourable reaction condition during this process. Therefore there is a considerable higher ratio of furosine to lactulose for milk powder than for market milk. Determination of this ratio allows demonstrating improper additions of reconstituted milk powder in milk (pasteurized milk and UHT milk). Addition of reconstituted milk powder during the production of market milks will lead to abnormally high furosine values for pasteurized milk and to an abnormally high ratio of furosine to lactulose for UHT milk. Finally, furosine determination can also be used for the detection of milk powder addition during cheese production. Cheeses normally produced from raw or pasteurized milk would have elevated furosine contents if milk powders were used during production (Mortier *et al*., 2000) [41].

Studies dealing with furosine formation kinetics, reported a zero order reaction with activation energies between 81.6 kJ/mol and 104.1 kJ/mol (De Rafael *et al*., 1997) [16]. The formation of furosine is highly dependent on protein concentration (positively correlated), and is therefore expressed as mg/100 g protein (Montilla and Olano, 1997; Rattray *et al*., 1997) [38, 53].

Similar for whey protein denaturation and lactulose formation, Pellegrino (1994) [49] observed a higher furosine formation in skimmed than in whole UHT milk, which was explained by a difference in heat load between whole and skimmed milk. Fat content would affect viscosity of milk and thus hinder the heat transfer of the process. However, excluding heat transfer phenomena by working with a small sample size, we observed a positive correlation between fat content and furosine concentration (Claeys *et al*., 2002b) [10].

During heating, denatured serum proteins initiate binding to micellar k-casein via disulphide bonds. Native fat globule proteins contain cysteine and can therefore also interact with denatured serum protein when milk is heated. This kind of interaction could explain the protective effect of milk fat on lysine blocking and on furosine formation when whole milk is heated (Morales *et al*., 1996) [39]. It has to be remarked however, that according to our results the effect of milk fat on furosine formation appeared to be insignificant in the context of process impact quantification (Claeys *et al*., 2002b) [10].

Formation of furosine has been observed during milk storage at room temperature. This can lead to misinterpretation of the actual heat load when applying furosine content as an index of heating. The amount of furosine newly formed was however independent of the furosine content immediately after heating and negligible at 4 °C (Rattray *et al*., 1997) [53].

Furosine is a useful indicator of lysine damage in milk products as well as a quality parameter to identify the presence of reconstituted milk powder in raw or pasteurized milk (Corzo *et al*., 1994) [12]. Furosine has also been proposed as a useful index for heat-induced changes in milk products; a furosine content of 8 mg/100 g protein has been suggested as upper heating limit for pasteurization, of 20 mg/100 g protein for high-pasteurization and of 250 mg/100 g protein for UHT (Schlimme *et al*., 1996) [56].

Furosine can be determined by a number of RP-HPLC methods using different columns, anion-exchange chromatography with pulsed amperometric detection, CE and GC. Since furosine is partially degraded during gas chromatography analysis, this method cannot be recommended for routine analytical application. The international standard is based on an ion-pair RP-HPLC system.

Significance of Furosine

In the case of dairy products, as a result of Malliard reaction, mainly amino groups of lysine (which is a component of proteins in milk) are reduced. High furosine content is a symptom of advanced degradation of proteins, and therefore a considerable decrement of lysine and lactose in the products. This decrease of nutritive values is especially unfavourable in the case of Infant formula, as they are the only source of proteins and carbohydrates and carbohydrates during the early infant stage of children nourished by bottle (Dorota Martysiak *et al*., 2007) [17].

Significance of Furosine determination

1. Furosine determination used for the detection improper addition of reconstituted milk powder in milk(Pasteurized milk and UHT milk), as Addition of reconstituted milk powder during the production of market milks will lead to abnormally high furosine values for pasteurized milk and to an abnormally high ratio of furosine to lactulose for UHT milk.
2. Furosine determination can also be used for the detection of milk powder addition during cheese production. (Jarita *et al*., 2015) [28].

Methods used for Furosine determination

1. RP-HPLC methods using different columns,
2. anion-exchange chromatography with pulsed amperometric detection,
3. Capillary electrophoresis (CE) and
4. Gas chromatography(GC)

d. Lysinoalanine (LAL)

Lysinoalanine (LAL) is an isodipeptide formed during protein cross linking through the reaction of dehydroalanine with lysine residues. (Hilton and Lewis, 2017) It is caused by intense heating, especially alkaline pH, and during storage of long life product such as UHT milk (Hilton and Lewis, 2017).

Veronika Faist *et al*., (2000) [58] used a modified RP-HPLC method with dansyl chloride derivatisation without solid-phase extraction for the determination of lysinoalanine (LAL) from market samples of liquid milk and cheese as well as to human milk from healthy volunteers. In raw milk, pasteurised and FSH high-pasteurised milk products LAL was quantified for the first time. The LAL contents analysed in raw and pasteurised milk ranged from 4 to 24 and 17 to 69 mg kg−1 crude protein, respectively. Compared to that, UHT-treated milk and sterilised milk showed higher LAL levels up to 186 and 653 mg kg−1 crude protein, respectively. Among all of the dairy products analyzed, highest amounts of LAL in the corresponding groups were found in sterilized milk, calcium-caseinate, processed cheese and imitation mozzarella cheese. (Veronika Faist *et al*., 2000) [58].

Significance of Lysinoalanine (LAL) in health

The formation protein – protein cross link a alkali treated proteins decrease their digestibility and biological value. Both Digestibility and (and PER) and net protein utilisation (NPU) decrease with increase in Lysinoalanine content. The decrease in digestibility is related to the liability of trypsin to cleave the peptide bond in the lysinoalanine cross link. Moreover the

steric constraints imposed by the cross link also prevents the hydrolysis of other peptide bonds in the alanine neighbourhood of the lysinoalanine and similar cross link. Evidence suggests that free Lysinoalanine is absorbed in the intestine, but it is not utilized by the body and most of it is extracted in the urine. Some lysinoalanine is metabolized in the kidney. Some studies found that 100 ppm pure or 3000 ppm protein bound lysinoalanine exhibits nephrocytomegaly (i.e. Kidney disorder). However such nephrotoxic effects not found in other animal species. At the level encountered in food, protein bound lysionalanine apparently does not cause nephrotoxicity in humans. Nevertheless, minimisation of lysinoalanine during alkali processing of protein is a desirable goal.

Significance of Lysinoalanine (LAL) determination

Lysinoalanine (LAL) was proven to be a suitable and sensitive indicator to assess the heat treatment intensity given to milk which was heated without and with the addition of dairy-based substitutes, like caseinates. (Veronika Faist *et al*., 2000) [58].

Methods used for Lysinoalanine (LAL) determination

1. Ion-exchange chromatography
2. Gas chromatography(GC)
3. Reverse Phase- High-performance liquid chromatography (RP-HPLC)

Lysinoalanine (LAL) can be analyzed by Ion-exchange chromatograph (Hilton and Lewis, 2017) and GC/MS after derivetization of both amino and carboxy group (Hilton and Lewis, 2017). However amino acid analysis and Reverse Phase- High-performance liquid chromatography have become the method of choice. (Hilton and Lewis, 2017)

The initial steps in all method are acid hydrolysis (6 M HCL at 100 °C for 23 h) of the protein to release amino acid and isodipetides including LAL, lanthionine, and histidinoalanine. After neutralization of hydrolysate (Hilton and Lewis, 2017) determined LAL on an amino acid analyzer with fluorometric detection.

e. Multi-component TTI-approach

In most cases distinction of different heat-classes of liquid milk cannot be achieved by one TTI alone due to overlap of values and to the wide range of heat load applied on liquid milk within the same heat-class. This wide range can partly be explained by variability in design of industrial plants, in working and heating conditions, in milk recycling and in other factors. So, for the differentiation of different classes of sterilized milk, it was suggested to couple relatively heat-labile components (protein, enzyme) with heat-stable components (chemical compound formed), e.g. a-lactalbumin or β-lg with lactulose. Due to slight variations caused by severe heating, the residual low concentration of the whey proteins cannot be used as a precise indicator. For milk thus labelled as 'UHT' it was proposed that two criteria, a β-lg content higher than 50 mg/l and a lactulose content equal or lower than 600 mg/l, must be fulfilled. Otherwise, milk has to be designed as 'sterilized'. Another example is coupling of the chemical compound furosine with the heat-labile proteins b-lg and/or Lpo with the objective of identifying small amounts of reconstituted milk powder in pasteurized milk (Pellegrino *et al*, 1995) [50].

Coupling of two chemical compounds, namely furosine and lactulose, can also offer some potential since the early Malliard reaction and lactose isomerization have different kinetics and are affected differently by milk composition and heating conditions. Because the formation of furosine is faster than that of lactulose, an abnormal shift from the established correlation line between lactulose and furosine indicates incorrect thermal processing or poor storage conditions. Since milk powder contains a considerable amount of furosine compared to lactulose, coupling of both TTIs allows a better distinction between genuine and adulterated milk as well. The detection of added milk powder based only on furosine determination has an important limitation seeing that there is a great range in the amount of furosine formed with the varying time and temperature limits in e.g. the UHT process (Corzo *et al*., 1994; Montilla *et al*., 1997) [12, 38].

4. Conclusion

Milk heat treatments can produce very complex effects among milk constituents. Simultaneous study of several heat-induced parameters improves the classification of industrial processed milks and provides deeper knowledge of what actually happened in heat-treated milk. As a result, the quantitative determination of heat load indicators has been proposed to distinguish between different categories of heat-treated milk. Generally, the development of Chemical markers or TTIs answers to the need of industry towards optimisation and a higher economic efficiency, the consumers demand for microbiological safe and qualitative products, and the requirements of legal authorities (criteria for authenticity, HACCP, etc.). Finally, it has to be remarked that when reflecting the notion of TTIs to a novel technology such as high pressure processing, the complexity of the problem increases from a two-dimensional (time and temperature) to a three-dimensional domain (time, temperature and pressure).

Table 1: Assessment of heat load indicators

	Raw	Therm.*	Past.*	High Past.*	UHT Direct	UHT Indirect	Steri.* milks	References
Temperature (°C)	< 40 <	65 for few mins	72 for 15 s	127 for 3-4 s	150 for 3-4 s	138 for 3-4 s	121 for 15-20 mins	
Phosphatase	+	+	-	-	-	-	-	Zehetner et. al. (1996) [62]
Peroxidase	+	+	+	-	-	-	-	
Native β-Lactoglobulin (mg/l)	3600	3400	3000	1800	800	200	Nil	Pellegrino *et. al.* (1995) [50]; Kondal-Reddy *et. al.* (1999) [33]
Lactulose (mg/l)	< 10	< 10	< 10	< 100	< 200	200 to 600	> 600	De Block *et. al.* (1996) [14]
Furosine (mg/100g protein)	< 10	< 10	< 20	< 100	< 200	100 to 250	200 to 500	Ferrer et. al. (2000) [18]

* Past. = Pasteurized, Therm. = Thermized, Steri. = Sterilized

4. References

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