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Kinetics of hydroxymethylfurfural, lactulose and furosine formation in milk with different fat content

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In the context of the general applicability of hydroxymethylfurfural (HMF), lactulose and furosine as time-temperature integrators (TTIs) for thermal processing of milk, the influence of milk fat content was studied. Formation kinetics were analysed for milk with fat content of 4·0± <0·1%. In previous experiments, it was observed that, under isothermal and non-isothermal heating conditions, formation of the three chemical compounds could be described by pseudo-zero order kinetics. Since the kinetic model was known, the experimental design could be simplified. Data were analysed by a non-linear regression procedure and results were evaluated by construction of joint confidence regions and temperature time tolerance (TTT-) diagrams. Formation kinetics of HMF and lactulose was not affected by milk fat content. Regarding furosine, significant differences were observed between kinetic parameters in whole, semi-skimmed and skimmed milk. The observed differences however were negligible in the context of process impact evaluation.

Keywords: Milk fat, hydroxymethylfurfural, lactulose, furosine, kinetics.

Thermal processing of milk not only guarantees a microbiologically safe and shelf-stable product, but can also cause substantial changes in the nutritional, organoleptic and/or technological properties, of which, many have been extensively reviewed in literature (Burton, 1988; Schaafsma, 1989). To evaluate the adequacy of the wide range of heat processes used in the dairy industry, knowledge of the process impact (in terms of food safety and quality) on milk is required. Moreover, knowledge of the impact could also provide a reliable basis for any legislative proposal on milk quality and for controlling milk authenticity in terms of processing.

Due to limitations of *in situ* methods, it is current practice to quantify the effect of a heat treatment on food products by use of physical mathematical methods or by use of time-temperature integrators (TTIs) (Van Loey et al. 1996). The latter are heat sensitive components extrinsic or intrinsic to the food product, which show a time- and temperature-dependent, easily measurable and irreversible change that can be correlated to the changes of a target attribute of a food (a safety or quality parameter) undergoing the same temperature exposure. One of the major advantages of TTIs is that they allow calculation of the process lethality or the reduction of a product quality parameter without any knowledge of the actual time-temperature profile of the heating process. As the definition implies, identification of TTIs requires an extensive study of their kinetics (Taoukis & Labuza, 1989; Hendrickx et al. 1994; Van Loey, 1996).

In this context we analysed formation kinetics of hydroxymethylfurfural (HMF), lactulose and furosine in detail (Claeys et al. 2001). Kinetics could be described by a pseudo-zero order model, *i.e.* concentration increased linearly as a function of time (up until a certain time). A first assessment of kinetic parameters was made by isothermal experiments. Next, estimated parameters were evaluated under dynamic temperature conditions since industrial thermal processes are generally characterized by a non-isothermal heating up and cooling down phase. However, if TTIs are to be used as *general* markers for heat processed milk, kinetics need to be evaluated for variability associated with the raw material as well.

Among factors that influence thermally induced chemical changes in milk are: (i) protein and sugar concentration, *e.g.* formation of furosine is, in contrast to lactulose, highly dependent on protein concentration, and is therefore expressed as mg/100 g protein (Montilla & Olano, 1997; Rattray et al. 1997); (ii) salt concentration and pH. Citrate and phosphate were found to catalyse the formation of

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lactulose, presumably by acting as bases (Andrews & Prasad, 1987); (iii) process conditions or the heat load applied. It has been demonstrated that direct ultra-high-temperature (UHT) heating by steam injection generally causes a smaller degree of lysine damage compared with an indirect UHT heating system, probably due to dilution of the milk during direct UHT with consequently a reduced concentration of reactants and additionally different heat transfer conditions (Nangpal et al. 1990; Dhen-Müller et al. 1991).

Concerning the effect of milk fat on formation of HMF, lactulose and furosine, reported results are scarce and contradictory (de Koning et al. 1990; Berg, 1993; Pellegrino, 1994; Morales & Jiménez-Pérez, 1999). Therefore, the objective of this work was to determine to what extent milk fat content influences their formation kinetics. If so, milk fat content should be taken into account when HMF, lactulose and furosine are used as intrinsic TTIs for evaluating thermal processed milk.

Materials and Methods

Milk

Pooled whole and skimmed milk were purchased at the Society for Milk Quality (Lier, Belgium). Semi-skimmed milk was obtained by mixing equal volumes of whole and skimmed milk. The milk was divided into small portions (50 ml) and stored frozen at −40 °C prior to thermal treatment.

Thermal treatment

Samples of milk in closed test tubes (pyrex, 16 × 160 mm) were immersed in a thermostated oil bath at a constant temperature. At different preset times, samples were taken from the oil bath and immediately cooled in ice water to stop further formation of the chemical compound studied.

Analytical Procedures

Total HMF content was quantified spectrophotometrically at 443 nm (Pharmacia LBK-Biochrom) using thiobarbituric acid (TBA) as a substrate following the method described by Keeney & Bassette (1959). Concentration of lactulose was determined using a D-glucose/D-fructose test combination of Boehringer Mannheim (1995). Formation of furosine was measured by RP-HPLC (Amersham Pharmacia Biotech; reversed-phase furosine dedicated column, 250 × 4·6 mm, Metal-Free, Alltech) essentially according to the method of Resmini et al. (1990), with the exception that 100 µl of hydrolysate was injected instead of 10 µl in order to increase reproducibility and accuracy of measurements.

Milk fat and protein content of whole and skimmed milk were analysed by the Society for Milk Quality (Lier, Belgium) based on the Röse–Gottlieb gravimetric principle (Belgian Norm, 1988) and the Kjeldahl method (International Dairy Federation, 1993) respectively.

Experimental Approach

Generally, when determining kinetics under isothermal conditions, at each temperature the experimental domain is divided in different preset time intervals. However, when the kinetic model is known, most reliable parameters (smallest confidence domains) are obtained when the number of samples (n) is equal to the number of parameters (p). For n larger than p, the optimum is reached by repeating the data points of the standard design ($n=p$). Slope and intercept of a curve for example, will be more accurate when replicates are made at the two end points of the observation domain. The number of replicates per measurement should be at least 5 to make a reasonable estimation of the variance possible (Fedorov, 1972; Duggleby, 1981; Endrenyi, 1981; Atkinson & Bogacka, 1997).

Data-analysis: Kinetic Parameter Estimation

In previously performed experiments, it was observed that HMF, lactulose and furosine formation reached a plateau upon prolonged heating, and could as such be described by a fractional conversion model (Claeys et al. 2001). Taking only the first phase of the model into consideration, a linear relation between concentration and treatment time was observed, and kinetics could be simplified to a pseudo-zero order model:

$$C = C_0 + k_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] t \qquad (1)$$

with C_0 the initial concentration, C the concentration of the chemical compound formed at treatment time t, E_a (J/mol) the activation energy quantifying the effect of temperature on the reaction rate, R the universal gas constant (8·314 J/mol,K) and k_{ref} the reaction rate constant at reference temperature T_{ref}. Based on eqn (1), data were analysed by means of a global non-linear regression procedure on response values. Accuracy and precision that could be associated with the estimated parameters were evaluated by constructing 90% joint confidence regions (Draper & Smith, 1981; Johnson, 1992).

All statistical procedures were carried out using the SAS software package 6.12 (SAS Institute, Cary, NC 27513, USA).

Results and Discussion

Since the kinetic model of HMF, lactulose and furosine formation was known, sampling could be simplified as stated in the *Experimental Approach* section. Doing so, reliability and accuracy of results could be increased and amount of experimental work reduced (Fedorov, 1972; Duggleby, 1981; Endrenyi, 1981). To allow for the calculation of E_a, two temperatures were chosen, corresponding to the two extremes of the temperature domain analysed during former experiments (Claeys et al. 2001). Formation

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Table 1. Kinetic parameters k_{ref} (reaction rate constant at reference temperature, T_{ref}) and E_a (activation energy) for the formation of hydroxymethylfurfural, lactulose and furosine in heated whole, semi-skimmed and skimmed milk. (See Data Analysis section for details)

	Values for k_{ref} and E_a ± standard error for $n=28$		
	Whole milk	Semi-skimmed milk	Skimmed milk
Milk composition			
Fat content, g/l	40·2	50/50 mix	0·5
Protein content, g/l	33·6		35·7
Hydroxymethylfurfural; T-domain: 90–120 °C; T_{ref}: 105 °C			
k_{ref}, µmol/l per min	0·771 ± 0·021	0·794 ± 0·01	0·815 ± 0·022
E_a, kJ/mol	116·4 ± 2·2	113·4 ± 1·1	110·5 ± 2·2
Lactulose; T-domain: 90–120 °C; T_{ref}: 105 °C			
k_{ref}, mg/l per min	31·7 ± 1·2	31·2 ± 1·0	28·2 ± 0·8
E_a, kJ/mol	109·2 ± 1·6	110·8 ± 1·5	113·6 ± 1·5
Furosine; T-domain: 90–130 °C; T_{ref}: 110 °C			
k_{ref}, mg/100 g protein per min	9·64 ± 0·14	8·95 ± 0·11	8·61 ± 0·07
E_a, kJ/mol	88·4 ± 0·9	92·8 ± 0·8	91·3 ± 0·5

of HMF and lactulose was studied at 90 °C and 120 °C, formation of furosine at 90 °C and 130 °C. At each temperature, sampling proceeded at two end points of the observation domain and was repeated seven times. To exclude an initial phase of non-isothermal heating as a consequence of warming up of the milk in the vials, the first sampling point was taken at 3 min when temperature in the vials had reached a constant value (as verified experimentally). The mean of the concentrations measured in the corresponding milk samples was used as reference (C_0 in eqn (1)).

Kinetic parameters k_{ref} and E_a were computed by one step non-linear regression and are summarized in Table 1. Differences in kinetics between whole, semi-skimmed and skimmed milk were evaluated by means of 90% joint confidence regions (Fig. 1). These are a more accurate representation of the statistical confidence associated with the model parameters than the commonly applied individual confidence intervals, because they take the possible correlation between the simultaneously estimated parameters into account. When correlation between parameters is high, a parameter pair may be well within the rectangular domain defined by the separate 95% confidence intervals, but may be very unlikely to occur since it is very far outside of the 90% joint confidence ellipse. Individual confidence intervals are suitable only for describing the limits of a single parameter without regard of the other parameter (Haralampu et al. 1985; Van Loey, 1996). Next, temperature time tolerance (TTT-) diagrams were constructed, in which curves connect those time temperature conditions leading to the same level of HMF, lactulose or furosine formation (Fig. 2).

Based on a 95% individual confidence level, no significant differences were observed for HMF formation kinetics in whole, semi-skimmed and skimmed milk (Table 1). However, 90% joint confidence regions are better tools of comparison since kinetic parameter values were highly correlated. In Fig. 1a, 90% joint confidence regions clearly overlap indicating similar formation kinetics for HMF in three types of milk. Seemingly, milk fat content has no influence on HMF formation.

This can also be illustrated by a practical-oriented example. Morales et al. (2000) measuring average HMF amounts in on-line heat-treated milk samples, observed a mean HMF value of 2·5 µmol/l in pasteurized milk, 5·6 and 8·7 µmol/l in directly and indirectly UHT-heated milk respectively, and 22 µmol/l in sterilized milk. Considering the latter amount, corresponding time temperature combinations are presented by the TTT-diagram in Fig. 2a. From this diagram it is clear that when the same processing temperatures are applied to whole, semi-skimmed and skimmed milk, similar processing times are needed to attain the same level of HMF formation.

Similar conclusions were drawn by Berg (1993), who examined HMF formation in UHT-milk with 0·1, 1·5, 3·0, 4·2 and 4·6% fat. In contrast however, are the observations of Morales & Jiménez-Pérez (1999). Studying HMF formation in lactose/caseinate model systems and in milk with different fat contents, they observed a negative effect of milk fat content on total HMF formation in both systems, after pasteurization as well as after direct UHT and sterilization. A different behaviour was found for formation of free HMF, which was enhanced at higher fat concentrations. Total and free HMF are produced through different pathways. While free HMF is formed only through decomposition of lactulosyllysine in the Maillard reaction, total HMF can also be formed by degradation of lactose. Formation of free HMF is small compared to formation of total HMF, and probably not a reliable measure for the occurrence of the Maillard reaction. In spite of the protective effect of fat on total HMF formation observed by Morales & Jiménez-Pérez (1999), E_a values of total HMF formation did not differ for varying fat contents, which is in line with our observations.

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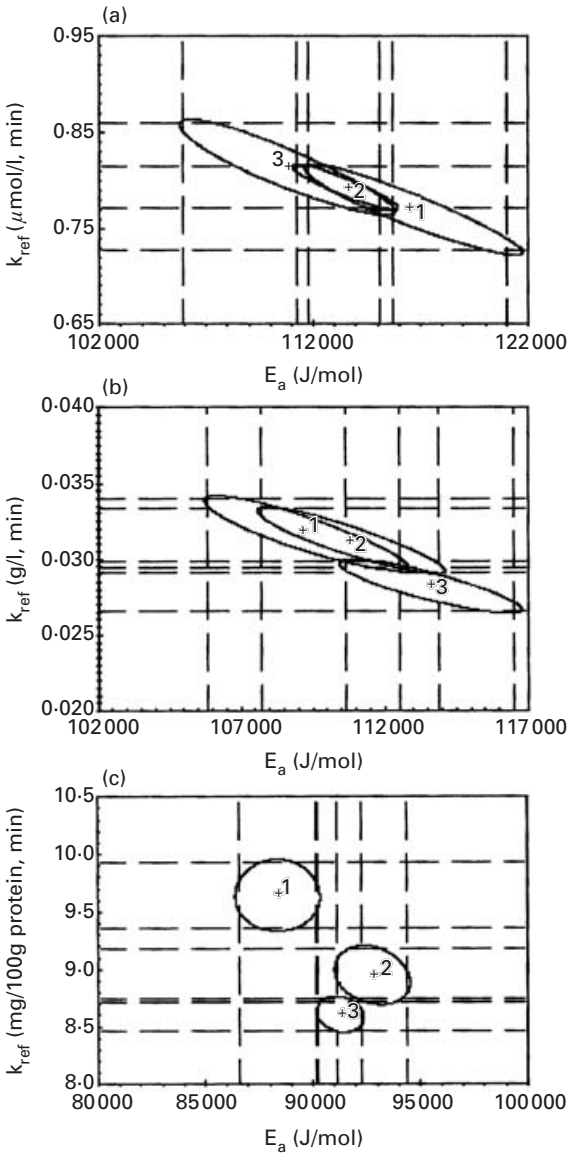

Fig. 1. Individual 95% confidence intervals (- -) and 90% joint confidence regions (indicated by ellipses) for the simultaneously estimated kinetic parameters k_{ref} (reaction rate constant at reference temperature T_{ref}) and E_a (activation energy) analysed in heat-treated whole (1) semi-skimmed (2) and skimmed (3) milk for (a) hydroxymethylfurfural (T_{ref} = 105 °C; correlation between k_{ref} and E_a: (1) 0·929, (2) 0·918, (3) 0·906), (b) lactulose (T_{ref} = 105 °C; correlation between k_{ref} and E_a: (1) 0·892, (2) 0·906, (3) 0·858), and (c) furosine (T_{ref} = 110 °C; correlation between k_{ref} and E_a: (1) 0·005, (2) 0·222, (3) 0·175). For details, see Data Analysis section.

Like HMF, formation kinetics of lactulose were comparable in whole, semi-skimmed and skimmed milk (Table 1) as indicated by the 90% joint confidence regions in Fig. 1b. Consequently, it seems that milk fat content does not have to be taken into account when using lactulose as a heat marker.

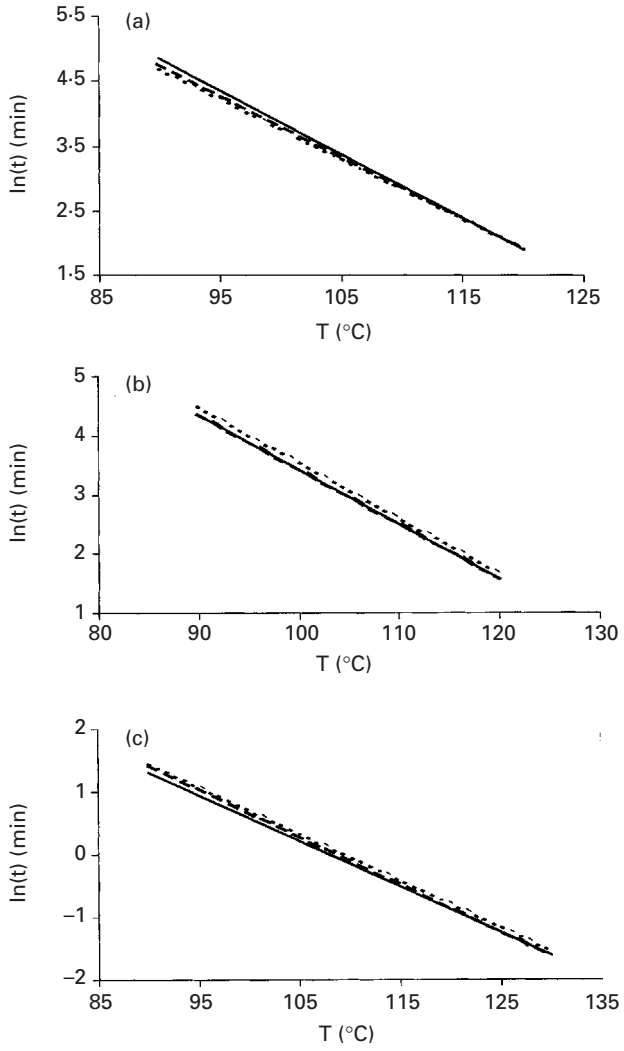

Fig. 2. Time temperature tolerance diagram for formation of (a) 22 μmol/l hydroxymethylfurfural as lower heating limit for sterilization, (b) 600 mg/l lactulose as upper heating limit for UHT, and (c) 8 mg/100g protein furosine as upper heating limit for high-pasteurization of whole (—) semi-skimmed (- - -) and skimmed (…) milk.

This can be verified by the TTT-diagram in Fig. 2b. In the application of lactulose as a heat marker, it is suggested that lactulose concentration should be below detection limit in pasteurized milk and may not exceed 50 mg/l in highly pasteurized milk. However, these criteria are under discussion due to limitations in sensitivity of the detection methods. Under UHT conditions an upper limit of 600 mg lactulose/l milk has been proposed (Schlimme et al. 1994; Wilbey, 1996). Time temperature combinations leading to such lactulose concentration in whole, semi-skimmed and skimmed milk are presented by the curves in the TTT-diagram. In order to achieve the same lactulose level in three types of milk, associated processing temperatures differ less than 1 deg C when the same processing time is considered.

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Results reported in literature on the effect of milk fat content on lactulose formation are rather contradictory and contain no explicit kinetic data. In agreement with our results are those of Berg (1993), Andrews (1984) and Geier & Klostermeyer (1983), who observed no effect of milk fat content on the formation of lactulose during heating. Contrary are the observations made by de Koning et al. (1990), who reported substantially higher lactulose formation (40–50%) in milk with 3% fat compared to milk with 1·5% fat subjected to the same UHT treatment. Pellegrino (1994) on the other hand, observed a protective effect of fat against heat-induced formation of lactulose. The difference between lactulose formed in whole and in skimmed milk was more apparent after a direct UHT process (19–31%) than after an indirect UHT process (3–5%), making the author suggest that – next to the major role of the type of process – the effect of fat will be more effective against low heat damage.

With regard to furosine formation, based on a 95% individual confidence kinetic parameter values differed significantly in whole, semi-skimmed and skimmed milk (Table 1). As depicted in Fig. 1c, 90% joint confidence regions of simultaneously estimated parameters k_{ref} and E_a are separated. Since correlation between k_{ref} and E_a was low, 90% joint confidence ellipses coincide approximately with the rectangular domain formed by the individual 95% confidence intervals associated with each parameter. Furosine formation seemed to proceed faster (higher $k_{110\,^{\circ}C}$ value) and to be less temperature sensitive (lower E_a) in milk with a higher fat content. Since E_a values differ, Arrhenius curves will intersect at a point where the effect of milk fat on furosine formation is inverted. In the temperature region studied, there appears to be a positive correlation between fat content and furosine formation, while above that region (>130 °C) an opposite trend is expected (assuming extrapolation is justified).

A possible explanation for the fact that an effect of fat was observed on furosine and not on HMF or lactulose formation kinetics is the difference in the pathway involved. In heated milk lactulose is mainly formed by lactose isomerization (Lobry de Bruyn – Alberda van Ekenstein or LA transformation), while furosine is formed during acid hydrolysis of lactulosyllysine, an Amadori product of the Maillard reaction. HMF is a result of both the Maillard reaction and the isomerization reaction. Although, in milk the route of HMF formation is mostly the LA transformation (Berg, 1993; Morales et al. 1997). The early Maillard reaction and lactose isomerization are affected differently by milk composition and heating conditions (Berg, 1993; van Boekel, 1998). During heating, denatured serum proteins initiate binding to micellar κ-casein via disulphide bonds. Native fat-globule proteins contain cysteine and can therefore interact with denatured serum protein. When milk is heated, the amount of proteins associated with the fat globules increases (Morales et al. 1995). This kind of interaction could explain the somewhat lower temperature sensitivity of furosine formation in whole compared with skimmed milk. In case of lactulose formation, it is mainly the salt system that is responsible for most of the lactulose formed during thermal processing of milk and not so much the protein fraction (Montilla & Olano, 1997).

Although significant differences were observed between kinetic parameter values for furosine formation in whole, semi-skimmed and skimmed milk, in practice they seem to be negligibly small as can be illustrated by the TTT-diagram in Fig. 2c. Furosine concentrations found in samples of Belgian consumption milk ranged from 4 to 7 mg/100 g protein for pasteurized milk, from 35 to 109 mg/100 g protein for direct UHT-heated milk, from 168 to 180 mg/100 g protein for indirect UHT-heated milk, and from 220 to 372 mg/100 g protein for sterilized milk (Van Renterghem & De Block, 1996). Although differences in processing conditions are clearly reflected by the furosine content, an overlap in furosine content is observed between the different heat classes, except for pasteurized and UHT-heated milk. The TTT-diagram in Fig. 2c represents those temperature time combinations leading to a furosine content of 8 mg/100 g protein, a level that has been discussed by the European Union as a limit for peroxidase-positively pasteurized milk (Clawin-Rädecker & Schlimme, 1995). From this diagram it is clear that the influence of fat on furosine formation is fairly insignificant; at an equal processing time processing temperatures differ less than 1·5–2 deg C for formation of the same amount furosine in whole, semi-skimmed and skimmed milk, which is an acceptable deviation in the context of process impact quantification.

Pellegrino (1994) however, observed a similar protective effect of milk fat on furosine formation as she did for lactulose formation. The protective effect was explained by a difference in heat load between whole and skimmed milk. Fat content would affect viscosity of milk and so hinder the transfer of heat. This explanation does not agree with our observations, since fat content did not have any affect whatever on HMF or lactulose formation kinetics, which have a higher temperature sensitivity than furosine.

The main objective of this study was to evaluate to what extent milk fat content jeopardizes the applicability of HMF, lactulose and furosine as intrinsic TTIs. To diminish as much as possible other factors next to fat content, a single batch of milk was used. Based on data from literature no unambiguous conclusion could be drawn; milk fat content has been reported to have a synergetic as well as antagonistic effect, or no effect at all on the formation of the chemical compounds studied in this paper. From our results milk fat content appeared to have no effect on HMF or lactulose formation. Although a significant difference was observed for furosine formation kinetics in whole, semi-skimmed and skimmed milk, the difference was too small to be relevant. HMF, lactulose and furosine thus seem generally applicable as intrinsic TTIs for the evaluation of consumption milk.

However, given that milk composition is subjected to seasonal variation and that fat content is not the only variable, variations observed in this study need to be evaluated

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in a broader framework. Further experimental work on this topic will be undertaken.

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Erratum: In Claeys W, Ludikhuyze L & Hendrickx M 2001 Formation kinetics of hydroxymethylfurfural, lactulose and furosine under isothermal and non-isothermal conditions. *Journal of Dairy Research* **68** 287–301 the units of lactulose in Figs 2 & 3 should read g/l rather than mg/l.

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