*Food Chemistry* **43** (1992) 351–358

# Determination of free carbohydrates and Amadori compounds formed at the early stages of non-enzymic browning

**A. Olano, G. Santa-Maria, N. Corzo & M. M. Calvo**

*Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain*

**I. Martinez-Castro**

*Instituto de Química Orgánica (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain*

**&**

**M. L. Jimeno**

*Centro Nacional de Química Orgánica (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain*

α-N-acetyl, ε-N-(1-deoxy-D-lactulos-1-yl)-L-lysine (ALL) was prepared as a model of protein-bonded lactose, and characterized by NMR. An HPLC method was designed to determine it. Milk model systems containing lactose, α-N-acetyl-L-lysine and salts were heated at 120°C (in the liquid state) or freeze-dried and stored at 45°C and 0·44 $a_w$. Free carbohydrates formed were determined by GC, and ALL by HPLC. Lactulose was formed at higher levels than ALL when heating, whereas the opposite was true during the storage of lyophilizates. It was shown that enolization is the main pathway of lactose degradation at high temperature, and that addition to amino groups predominates at low temperatures and $a_w$.

## INTRODUCTION

One of the causes of deterioration of milk is the interaction of lactose with other components. Various pathways then ultimately lead to the development of brown discoloration and loss of nutritive value (Burton, 1984). Maillard reaction between the ε-amino group of lysine and lactose leads to the formation of lactuloselysine (ε-N-(1-deoxy-D-lactulos-1-yl)-L-lysine), an important precursor to the formation of coloured products. Besides, enolization of lactose catalyzed by the milk salt system (Martinez-Castro *et al.*, 1986; Andrews & Prasad, 1987) which proceeds by the Lobry De Bruyn–Alberda Van Ekenstein (L–A) transformation initiates its degradation which contributes considerably to browning of milk.

Simultaneous determination of the main components formed from lactose via these two types of reaction would be useful in the study of the transformations of lactose during the processing of dairy products.

Although the chemical changes undergone by lactose during heat treatment or storage of milk have been studied for many years, most of the papers deal with either the Maillard reactions (Turner *et al.*, 1978; Hurrell *et al.*, 1983; Erbesdobler & Zucker, 1966) or the L–A transformation to give lactulose, epilactose and galactose (Martinez-Castro & Olano, 1980; Andrews, 1985; Olano *et al.*, 1989). Richards (1963) studied the changes in dried skim milk during storage taking into account both the base-catalysed degradation of lactose and the breakdown of the Amadori rearranged complex; he estimated 1-amino-1-deoxy ketoses as 5-hydroxymethyl furfural.

Direct determination of Amadori compounds in milk is at present very difficult because they are linked to proteins. Acid hydrolysis gives secondary products such as furosine and pyridosine (Finot *et al.*, 1968; Finot & Mauron, 1969); enzymatic hydrolysis gives

*Food Chemistry* 0308-8146/92/$05.00 

lactulose lysine (Möller *et al.*, 1977) but the procedure is too tedious to be used as a control method.

Model systems are of great value when the complexity of the food makes the study of reaction products difficult. In the present work we have chosen N-α-acetyl-L-lysine (acetyl lysine) as a model for casein-bonded lysine, in which only the ε-amino group is free. When this amino acid reacts with lactose, α-N-acetyl-ε-N(1-deoxy-D-lactulos-1-yl)-L-lysine (acetyl lactulose-lysine, ALL) is formed. We have studied the formation of free carbohydrates (lactulose, epilactose and galactose) by GC and that of ALL by HPLC, in model systems containing lactose, N-α-acetyl lysine and salts, in order to estimate the relative extent of Maillard and L–A reactions during heating and storage.

## EXPERIMENTAL

ALL was prepared according to Dworschak *et al.*, (1983) by heating N-α-acetyl-L-lysine (10%) and D-lactose (38%) at 100°C for 5 h in phosphate buffer, 0·1 M. Clean-up on ion-exchange columns and TLC were carried out according to Finot & Mauron (1969), using bidimensional plates of cellulose. The first eluent was methyl ethyl ketone + acetic acid + water (50 + 30 + 20) and the second was pyridine + acetic acid + water (90 + 10 + 20). The isolated product was examined by elemental analysis and NMR.

### NMR

$^1$H NMR spectra were recorded on a Bruker AM-200 spectrometer operating at 200 MHz, using deuterium oxide as solvent. Acquisition parameters were: sweep width 2·5 kHz; flip angle 30°; acquisition time 3·3 s and data size 32 K. In order to assign and determine chemical shifts, 2D scalar shift correlated $^1$H NMR experiments were performed. 2D experiments were carried out with the same spectrometer using the COSY-90 pulse sequence.

Decoupled and DEPT $^{13}$C NMR spectra were obtained on a Varian XL-300 instrument, operating at 75 MHz, using deuterium oxide solvent and dioxane as internal standard. Acquisition parameters for the decoupled $^{13}$C spectra were: sweep width 16 kHz, flip angle 52°, acquisition time 1 s and data size 32 K. The DEPT experiment was performed with $^1J$(C, H) = 145 Hz and 2 s of relaxation delay.

### Preparation of model systems

Model systems consisted of a 0·1 M phosphate buffer solution containing acetyl lysine (0·5%) and lactose (5%). After dissolving the reagents in the buffer, the solution was adjusted to the desired pH with 1N HCl. Another model system (SMUF) was prepared as before but replacing the phosphate buffer by a solution which simulates the saline system of milk (Jenness & Koops, 1962). Aliquots (10 ml) of both solutions at different pH were heated in a silicone oil bath at 120°C for a stated period in closed vessels; other portions (1 ml) were freeze-dried and placed in desiccators over a saturated $K_2CO_3$ solution (0·44 $a_w$) and stored at 45°C for periodical analysis.

### Colour

Absorbance of solutions was measured at 440 and 260 nm with a DV-70 spectrophotometer from Beckman.

### HPLC

The chromatographic system consisted of a Waters 510 pump, a Waters U6K injector, a Waters 410 differential refractometric detector and a Perkin-Elmer 024 recorder. A Waters Carbohydrate column 10 μm, 3·9 × 300 mm was used. The mobile phase was methanol + water (80 + 20 v/v), flow rate 0·7 ml/min at 1000 psi. Samples were filtered through a 0·2 μm Millipore filter prior to injection and degassed in an ultrasonic bath.

Linearity of the system was tested by constructing calibration curves over the range 1–20 μg of ALL.

### GC

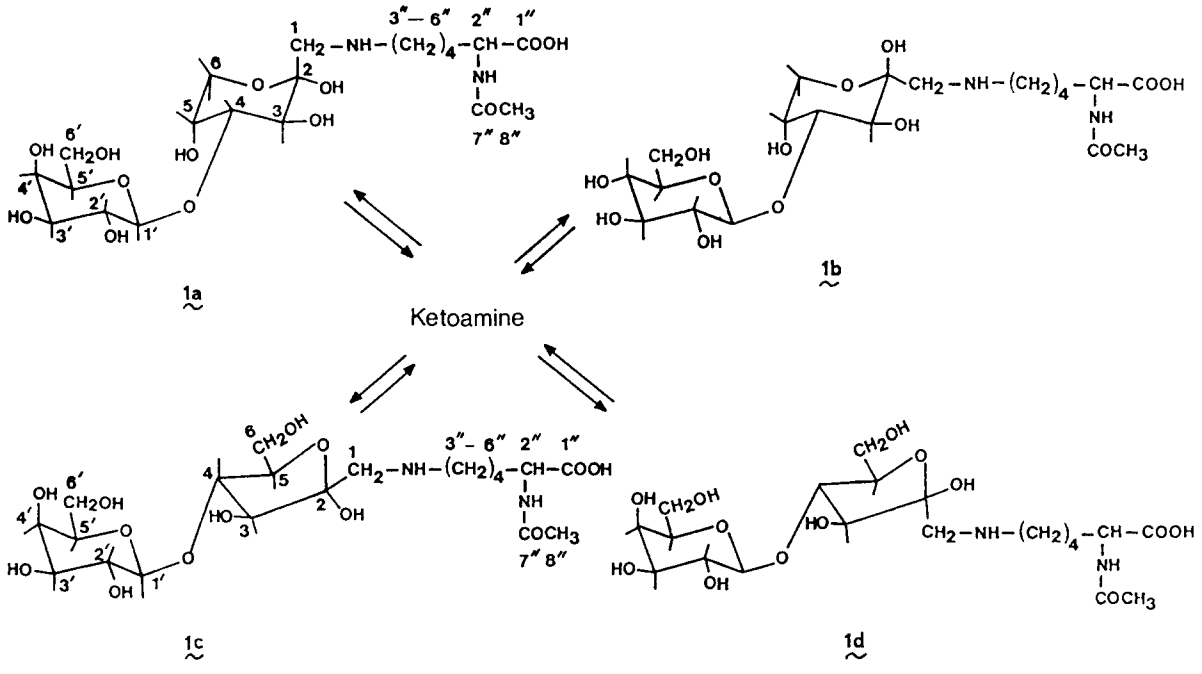
The sample (0·5 ml) was mixed with 0·5 ml (0·5 mg) of phenyl-β-glucoside (internal standard), diluted to 5 ml with methanol, kept for 1 h at room temperature and filtered. One ml of the filtrate was evaporated under vacuum at room temperature and persilylated with TMSI. GC analyses were performed on a Sigma 3B apparatus (Perkin-Elmer) equipped with a 3 m × 1 mm s/s column packed with 2% OV-17 on leached Volaspher A-2 120/140 mesh (Merck). Temperatures of injector and detector were 300°C; oven was held at 200°C for 2 min and then programmed at 15°C/min until 270°C. Carrier gas was nitrogen at 2 ml/min.

## RESULTS

The isolated ALL showed a single spot in TLC and a single peak in HPLC. Elemental composition was: C : 43·1%; H : 7·0%; N : 5·0% (Calculated C : 45·3%; H : 7·2%; N:5·3%).

### NMR

ALL, as other Amadori compounds (Röper *et al.*, 1983) mutarotates in solution to give an open and four cyclic tautomeric forms:




The $^1$H NMR spectrum is complex due to overlapping of the bands corresponding to the different tautomeric forms.

2D-homonuclear COSY experiments afforded the assignation of protons from the aminoacid chain and the protons from the main tautomeric form of the sugar moiety (Table 1).

The anomeric protons region showed an intense doublet at 4·50 ppm, J(H1'H2') = 8 Hz and other less intense at 4·40 ppm J(H1'H2') = 7·5 Hz. The COSY experiment showed that the first doublet corresponded to two signals, one of H1' from the main tautomer, related to a signal of H2' at 3·59 ppm, and the other coming from a minor tautomer, related to a signal of H2' at 3·51 ppm; the doublet at 4·40 ppm was correlated with the proton H2' at 3·54 ppm from another minor tautomeric form, whose intensity was similar to the above mentioned.

The study of the bidimensional correlation diagram allowed the assignation of the other signals from the main tautomeric form (see Table 1). By comparison of measured δ and J values with those of lactulose (Bruyn et al., 1975) it can be deduced that the main form of ALL in deuterium oxide is the β-pyranose tautomer (about 65%). Other tautomers can be roughly estimated as 18% each.

The $^{13}$C NMR spectroscopy allowed determination of the population of all tautomers. The $^{13}$C spectrum was also complex because of the overlapping of three tautomeric forms (1b, 1c, 1d). Assignments were performed by means of DEPT experiments of 90° and 135°, and by comparison with literature data of similar compounds (Röper *et al.*, 1983; Bock *et al.*, 1984).

The six signals appearing at the anomeric carbons region were assigned to the three mentioned tautomeric forms; C1' (101·85 ppm) and C2 (96·18 ppm) corresponded to 1b; C1' (103·79 ppm) and C2 (100·51 ppm) to 1d; C1' (104·19 ppm) and C2 (103·08 ppm) to 1c.

**Table 1. $^1$H NMR chemical shifts, ppm, and coupling constants, $H_2$, for the main tautomer of ALL (1b) in $D_2O$**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1b | H1' | H2' | H3' | H4' | H5' | $H6'_A$ | $H6'_B$ |
| | 4·50 | 3·59 | 3·69 | 3·88 | 3·66 | 3·7 | 3·8 |
| | J(H1', H2') | J(H2', H3') | J(H3', H4') | J(H4', H5') | J(H5', $H6'_A$) | J(H5', $H6'_B$) | J($H6'_A$, $H6_B$) |
| | 7·8 | 10·5 | 3·1 | 1·0 | 3·8 | 7·5 | N/O |
| | $H1_A$ | $H1_B$ | H3 | H4 | H5 | $H6_A$ | $H6_B$ |
| | 3·27 | 3·27 | 3·82 | 4·07 | 4·17 | 3·96 | 3·75 |
| | J($H1_A$, $H1_B$) | J(H3, H4) | J(H4, H5) | J(H5, $H6_A$) | J(H5, $H6_B$) | J($H6_A$, $H6_B$) | |
| | — | 9·7 | 3·2 | 1·0 | 1·5 | −12·2 | |
| | CH 2" | $CH_2$ 3" | $CH_2$ 4" | $CH_2$ 5" | $CH_2$ 6" | $COCH_3$ | |
| | 4·08 | 1·6[a] | 1·38 | 1·7[a] | 3·07 | 1·98 | |

[a] Assignment may have to be reversed.
N/O: not observable.

**Table 2. $^{13}C$ NMR Chemical shifts, ppm, for the 1b, 1c and 1d tautomers of ALL**

| | 1b | 1c | 1d |
|---|---|---|---|
| C1 | 53·68 | 52·80 | 51·42 |
| C2 | 96·18 | 103·08 | 100·51 |
| C3 | 69·12 | 82·48 | 77·85 |
| C4 | 78·01 | 86·08 | 84·62 |
| C5 | 67·40 | 82·28 | 81·20 |
| C6 | 64·36 | 62·85 | 61·62 |
| C1' | 101·81 | 104·19 | 103·79 |
| C2' | 71·59 | 71·54 | 71·48 |
| C3' | 73·43 | 73·57 | 73·37 |
| C4' | 69·50 | 69·33 | 69·27 |
| C5' | 76·26 | 76·15 | 76·15 |
| C6' | 62·01 | 61·90 | 61·90 |
| C1" | 176·64[a] | 176·64[a] | 176·64[a] |
| C2" | 55·41 | 55·41 | 55·41 |
| C3" | 31·73 | 31·73 | 31·73 |
| C4" | 23·14 | 23·14 | 23·14 |
| C5" | 25·55 | 25·55 | 25·35 |
| C6" | 49·06 | 48·95 | 48·87 |
| C7" | 174·48[a] | 174·48[a] | 174·48[a] |
| C8" | 22·69 | 22·69 | 22·69 |

Using $D_2O$ as the solvent and dioxane as internal reference (diox = 67·4 ppm relative to TMS).
[a] Assignment may have to be reversed.

Two other weak signals at 103·0 and 97 ppm could be assigned as C1' and C2 respectively from 1a. Data are shown in Table 2.

As the $^{13}C$ chemical shifts from fructofuranose rings appear at lower field than those of fructopyranose rings, we can assign the 1b form as α-N-acetyl-ε-N(1-deoxy-β-D-lactulopyranos-1-yl)-L-lysine, the 1d form as α-N-acetyl-ε-N(1-deoxy-β-D-lactulofuranos-1-yl)-L-lysine, the 1c form as α-N-acetyl-ε-N(1-deoxy-α-D-lactulofuranos-1-yl)-L-lysine, and the 1a form as α-N-acetyl-ε-N(1-deoxy-α-D-lactulopyranos-1-yl)-L-lysine. The equilibrium composition (calculated from the intensity of C1' signals) was: β-pyranose (1b), 65%; α-furanose (1c), 18%; β-furanose (1d), 15% and α-pyranose, (1a) 2%. This composition agrees with literature data for similar compounds (Neglia *et al.*, 1983; Röper *et al.*, 1983).

## HPLC

An HPLC chromatogram of a standard mixture is shown in Fig. 1. The ALL peak (including all tautomers, as is usual in this type of separation) was separated from those of sugars and acetyl lysine. Addition of phosphate to the mobile phase as recommended by Takeoka et al. (1979) was not necessary, because the α-carboxyl group of ALL is acetylated. Least square analysis of the calibration curve resulted in the following regression equation:

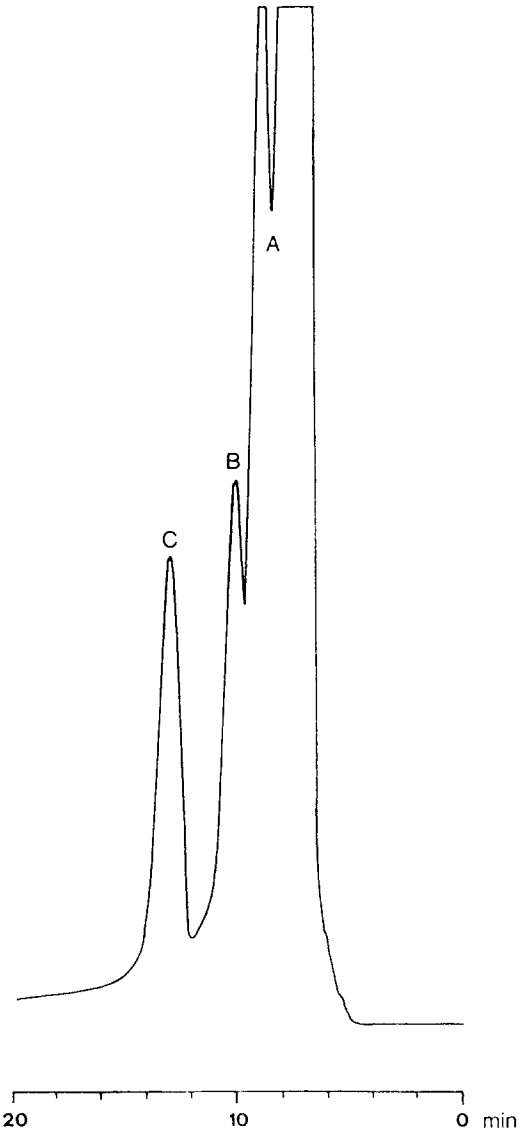



**Fig. 1.** HPLC of a standard mixture showing the separation of ALL from sugars and acetyl lysine. Peak A includes buffer and lactose; B, α-acetyl lysine; C, ALL.

$$\mu g\ ALL = 0{\cdot}946 + 1{\cdot}510 \times \text{area of ALL } (\pm 1{\cdot}080)(r = 0{\cdot}97)$$

The precision, calculated from five repeated analyses of a sample, gave a coefficient of variation for ALL of 5·71%.

## Heating

Colour increased with pH (Fig. 2) becoming dark brown in the samples heated at pH 6·5 and 7.

Figure 3 shows the formation of ALL during heat treatment (120°C) of model systems at different pH values. ALL level reached a maximum and then decreased. The maximum value found was 87 mg/100 ml after 50 min heating at pH 6·0. The shape of the curves seems to indicate that formation of ALL increase with pH, but the product formed is degraded at rates which also increase with pH and time.

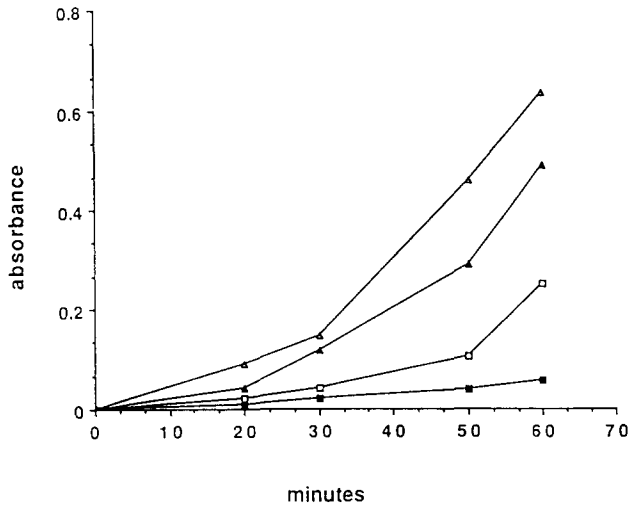



**Fig. 2.** Colour developed during heating (120°C) of model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0) measured as the absorbance at 440 nm.

The amount of lactulose, galactose and epilactose formed increased with pH and time of heating, attaining values up to 1773, 469 and 153 mg/100 ml respectively (Fig. 4). The ratio lactulose/galactose decreased with the time within the range 3·5–10·4. Heating of ALL in buffers without lactose gave only very small quantities of galactose (1–2 mg/100 ml).

When lactose was heated with acetyl lysine in a saline system similar to that of milk (SMUF) the isomerization was considerably lower than in phosphate buffers and the ALL formed was too small to be measured.

The amount of isomeric disaccharides formed was in all cases considerably higher than that of ALL; thus, it can be supposed that L–A reaction is predominant in conditions similar to sterilization of milk.

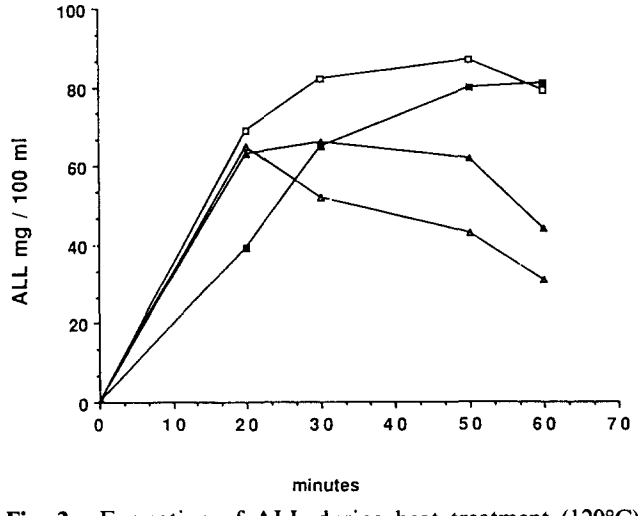
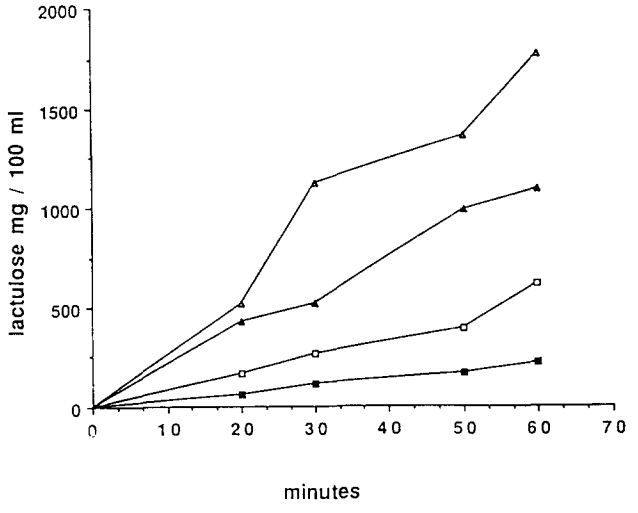
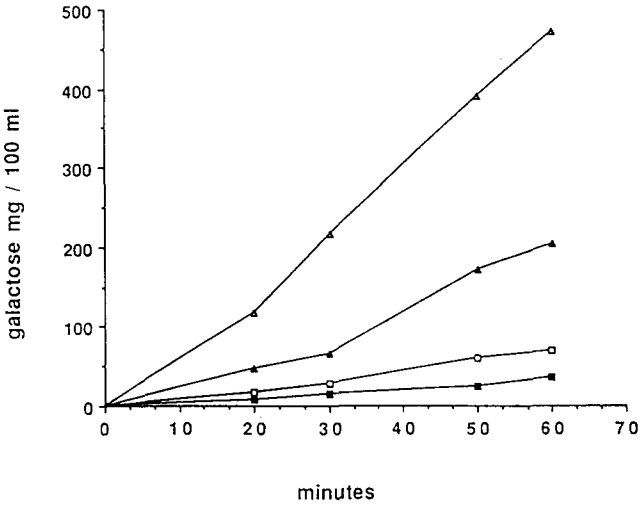


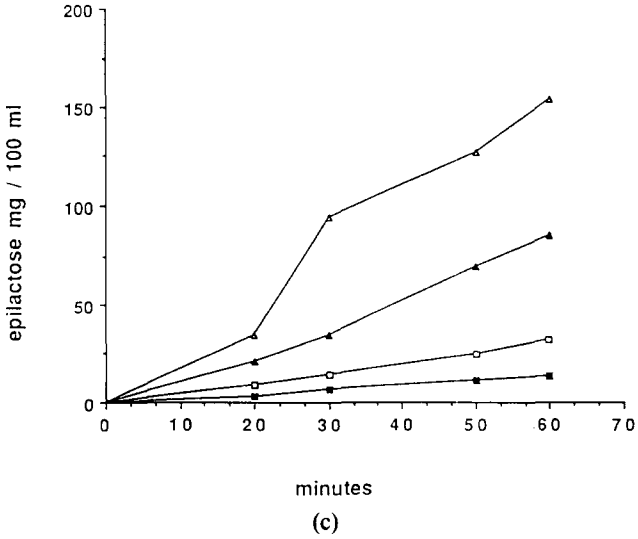

**Fig. 3.** Formation of ALL during heat treatment (120°C) of model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0).




(a)




(b)




(c)

**Fig. 4.** Carbohydrates formed during heating (120°C) of model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0).

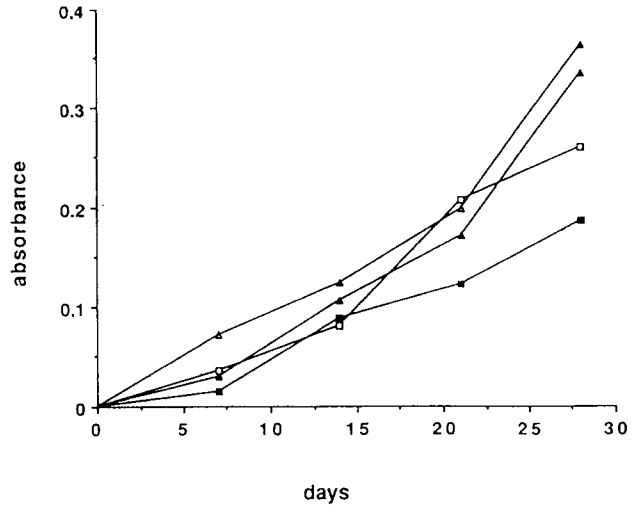



Fig. 5. Colour developed during storage of freeze-dried model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0) measured as the absorbance at 440 nm.

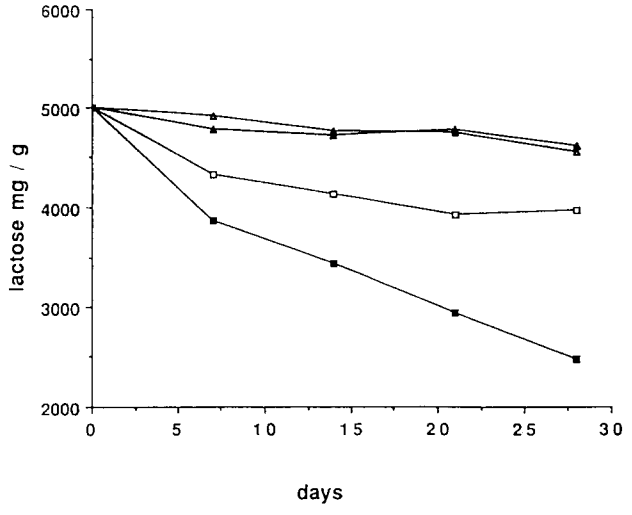



Fig. 7. Degradation of lactose during storage of freeze-dried model systems. Open symbols: pH 5·5: filled symbols, pH 7·0. Triangles: without acetyl lysine; squares: in the presence of acetyl lysine.

## Storage

As storage affects mainly powdered dairy products, the assays of storage were conducted on freeze-dried systems, in order to obtain some data about transformations of lactose at low $a_w$ levels.

The lyophilizates stored at 45°C and 0·44 $a_w$ developed a more intense colour in the presence of aminoacid (Fig. 5).

High levels of ALL were formed in freeze-dried phosphate buffer systems during the first week of storage (Fig. 6). Small differences due to pH were found except for pH 5·5 which showed a considerably lower ALL content. During the second week, a marked decrease was observed and the amounts of ALL present after a month were within the range 17–25 mg/g regardless of the pH. Richards (1963) found that 1-amino-1-deoxy-2-ketoses in stored dried milk (estimated as 5-hydroxymethyl furfural) increased rapidly to reach a maximum value and then declined.

Degradation of lactose in the absence of acetyl lysine was too small to find differences with pH (Fig. 7). The presence of the amino acid caused a considerable lactose degradation, which increased with pH and storage time. At pH 5·5 20% of lactose was degraded after a month of storage, whereas only 50% of lactose remained unaltered at pH 7 in the same period of time.

The amounts of free sugars formed during storage of samples were lower than during heat treatment (Fig. 8). In the absence of the aminoacid, lactulose decreased slightly (about 30%) whereas epilactose and galactose levels were very low. These results agree with those of Richards (1963) who found a rapid increase in the galactose content until a maximum was reached, and then a decline; lactulose level was much lower than that of galactose.

When SMUFs were stored at pH 7, galactose was the only sugar formed, and was present in very small amounts. Storage of freeze-dried SMUF solutions of ALL in the absence of lactose produced also small quantities of galactose. When lactose in SMUFs was replaced by galactose, 50% of the carbohydrate was lost in the absence of the aminoacid, and it was completely consumed in the presence of acetyl lysine (Fig. 9). This is due to the fact that monosaccharides are more reactive than disaccharides towards amines (Hashiba, 1982).

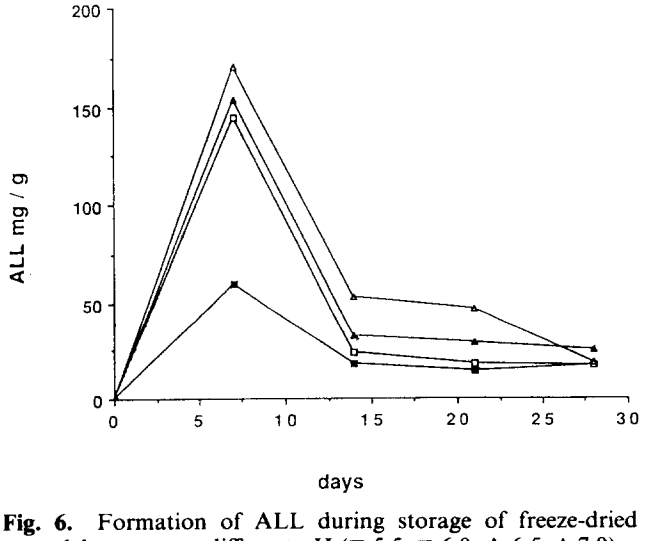
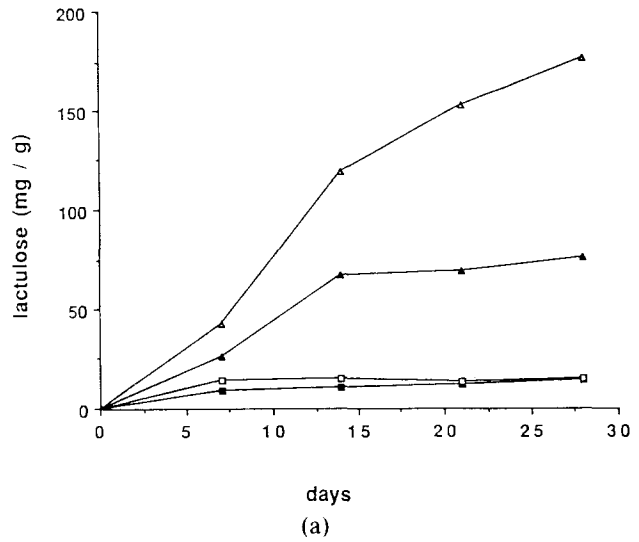
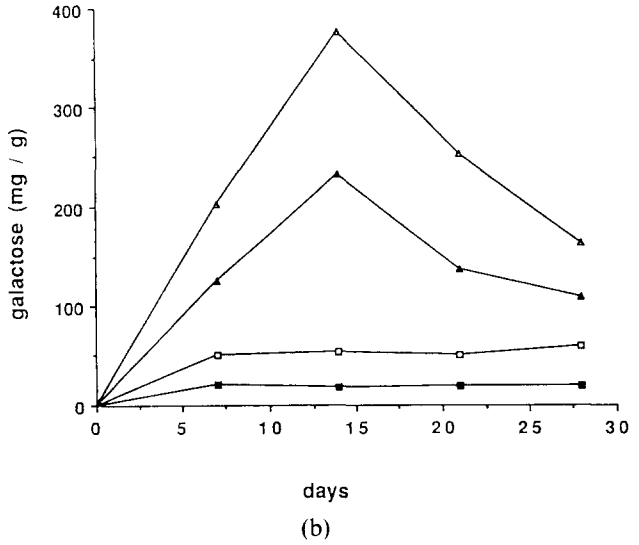


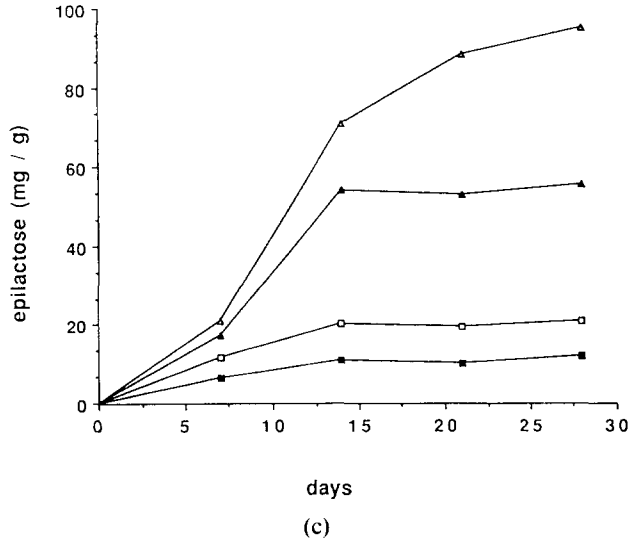

Fig. 6. Formation of ALL during storage of freeze-dried model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0).




(a)




(b)




(c)

**Fig. 8.** Free carbohydrates formed during storage of freeze-dried model systems at different pH (■ 5·5; □ 6·0; ▲ 6·5; Δ 7·0).

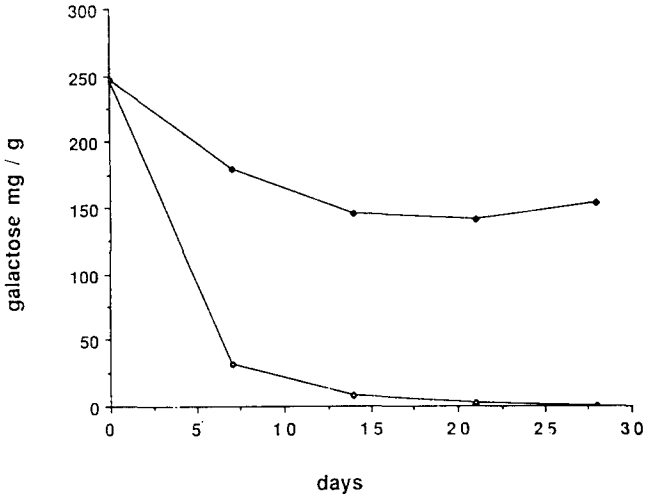



**Fig. 9.** Degradation of galactose during storage of freeze-dried model systems (SMUF). ♦ without acetyl lysine; ◊ in the presence of acetyl lysine.

## DISCUSSION

From the above results the relative importance of both Maillard and L–A reactions in different treatments is clearly shown.

The predominance of L–A transformation during heating at 120°C is clear since the main component formed was lactulose which is derived from this reaction. This conclusion agrees with existing data about furosine and lactulose reported by different authors. Erbesdobler & Dehn-Müller (1989) indicate that in commercial UHT processing (135–150°C for 4–16 s) furosine values between 0·5 and 1·5 mg/100 ml milk (i.e. about 1·5–4·5 mg/100 ml lactulosyl lysine, using the calculation proposed by Hurrell et al., 1983) can be expected, whereas Andrews (1986) found that the lactulose range in UHT milks is 5–75 mg/100 ml.

On the other hand, the Maillard reaction contributes to a greater extent under storage condition of dried products, ALL being formed in higher quantity than free sugars.

The relative proportions of sugars found during storage of model systems were very different from those formed during heating. The accumulation of galactose was noteworthy, which can be explained in part by degradation of ALL in a further stage of browning. Another part can derive from degradation of free disaccharides present, most probably lactulose, which is enolized faster than lactose (Isbell *et al.*, 1969).

## ACKNOWLEDGEMENTS

This work has been supported by Comision Interministerial de Ciencia y Tecnología (Project ALI-0158/88). The authors thank M. J. Rodriguez for technical assistance.

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