Furosine is a useful indicator in pre-baked breads

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Abstract: There is increasing demand for pre-baked bread. Bakers can meet consumer requirements for breads of specific flavour and colour by pre-baking an amount of bread in the morning and completing the baking process in the afternoon. This type of product is of special interest to sandwich bars, restaurants and large communities. In order to obtain an indicator of utility to monitor the processing of pre-baked bread, the browning indicators furosine, hydroxymethylfurfural (HMF) and glucosylisomaltol were analysed in two independent assays of bread pre-baked at 175 °C for between 5 and 30 min. No furosine was detected in raw or fermented dough. In the pre-baking stage, furosine increased from 1.5- to 5-fold between 5 and 30 min. The furosine values obtained in the two independent experiments were similar. HMF and glucosylisomaltol were only detected after 15 min, which is the time period commonly used for processing by the industry. Thus, among these indicators, furosine is the only one with utility for monitoring the pre-baking process. On the other hand, a study of the baking of pre-baked bread at 220 °C showed that HMF and glucosylisomaltol can be used to monitor the latter process.

INTRODUCTION

Bread is the most important food in the Spanish household and represents most of the production of commercial bakeries. Bread making involves three steps: dough mixing (flour, water, yeast and salt), dough fermentation and baking. The method to produce pre-baked bread has been known for some time, but the professional baker has frequently run into serious problems when using this system. There is currently an increased demand for this type of bread, although the market remains small and largely comprised of baking terminals, sandwich bars, restaurants and large communities. A baker can pre-bake an amount of bread in the morning and, without freezing it, complete the baking process in the afternoon. In addition, there is rising consumer demand for commercial pre-baked breads for easy preservation and home baking.

The method for pre-baking bread is divided into two steps. The dough is prepared by the traditional method, with some modifications. When the structure of the bread has developed after the first baking, it is taken out of the oven, cooled and later frozen or placed in a container under vacuum conditions. After the first step the bread presents a white colour and its humidity content and density are higher. After baking for 10 or 15 min in the second step, its appearance is identical to that of traditional bread.

The chemical reactions involved in bread making are essentially the Maillard reaction and caramelisation. The Maillard reaction is favoured in foods with high protein and carbohydrate content and intermediate moisture content at temperatures above 50 °C and at a pH of 4–7.1 This reaction produces changes in colour (melanoidins), flavour (aldehydes and ketones), functional properties and nutritional value (blocking or destruction of lysine).2,3

The early stages of the Maillard reaction can be evaluated by determining the furosine (ε-N-(furayl)methyl)-L-lysine) amino acid formed during acid hydrolysis of the Amadori compounds fructosyl-lysine, lactulosyl-lysine and maltulosyl-lysine, which are produced by the reaction of ε-amino groups of lysine with glucose, lactose and maltose.4 Furosine determination has been used in cereals to control the processing of pasta,5 bakery products,6 baby cereals7,8 and bread.9

The intermediate stage of the Maillard reaction can be followed by hydroxymethylfurfural (HMF) determination.10 HMF is also formed by the degradation of sugars at high temperatures1 and has been used to evaluate the heat treatment of cereal products.9,11–13

Glucosylisomaltol, an intermediate indicator of the Maillard reaction, was recently used to control the storage of baby cereals.14

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The objectives of the present study were to determine browning intensity by measuring different indicators and to assess which of them were useful to monitor pre-baked bread processing, ensuring a standard product.

**MATERIALS AND METHODS**

**Samples**
A variety of raw doughs, fermented doughs and pre-baked and baked breads were obtained from a bread-making company. The bread formula used by the company comprised wheat flour (80 kg), water (50 l), baker’s yeast (1.5 kg) and NaCl (1.2 kg). The breads weighed from 90 to 110 g. Fermented dough was baked at 175 °C to produce pre-baked bread. The pre-baked breads were baked at 220 °C.

The samples analysed were: raw dough mix; dough fermented at 22 °C (for 30, 60, 90, 120 or 150 min); and pre-baked breads from two independent experiments using different pre-baking times (experiment I: 5, 10, 15, 25 or 30 min; experiment II: 5, 8, 11, 14, 17, 20 or 25 min).

The fermented dough used for the pre-baked bread was fermented for 120 min. The breads that were pre-baked for 15 min in experiment I were baked at 220 °C for 14, 25 and 30 min. Each treatment was carried out in duplicate and there were duplicate analyses of each sample.

All the breads were rapidly cooled to −8 °C. Before the analysis the products were thawed at room temperature for 30–60 min. The samples were then divided into very small portions, left overnight at room temperature to reduce the water content, and ground into small-size particles (<500 µm).

**Furosine determination**

**Reagents**
A standard stock solution containing 1.2 mg ml⁻¹ furosine (Neosystem Laboratoire, Strasbourg, France) was used to prepare the working standard solution.

**Apparatus**
The liquid chromatographic system used in this study consisted of a Perkin Elmer 250 chromatograph (Norwalk, CT, USA) with a Waters 717 Plus autosampler (Milford, MA, USA) and a Perkin Elmer 235 diode array detector. Data were collected using the Perkin Elmer 1020 software data system.

**Procedure**
Furosine determination was performed following the method described by Guerra-Hernández and Corzo. A 500 mg sample, weighed with analytical accuracy, was hydrolysed with 4.5 ml of 7.95 M HCl at 110 °C for 24 h in a Pyrex screw-cap vial with a PTFE-faced septum. High-purity N₂ gas was bubbled through the solution for 2 min. The hydrolysate was filtered through a medium-grade paper filter. A 0.5 ml portion of the filtrate was applied to a Sep-Pak C₁₈ cartridge (Waters, Milford, MA) pre-wetted with 5 ml of methanol and 10 ml of water, eluted with 3 ml of 3 M HCl and evaporated under vacuum. The dried sample was dissolved in 1 ml of a mixture of water, acetonitrile and formic acid (95:5:0.2).

**Chromatographic conditions**
A 50 µl aliquot of the resulting solution was separated in a reverse phase C₁₈ column (Spherisorb ODS2, 5 µm, 250 mm × 4.6 mm id; Phenomenex, Torrance, CA, USA). Duplicate samples were analysed. The mobile phase consisted of a solution of 5 mM sodium heptane sulphonate (Sigma, St Louis, MO, USA) with 20% acetonitrile (Panreac, Barcelona, Spain) and 0.2% formic acid (Panreac). The elution was isocratic and the flow rate was 1.2 ml min⁻¹. The UV detector was set at 280 nm.

Calibration of the chromatographic system for furosine determination was by the external standard method. The calibration was performed by adding increasing quantities of furosine standard, within the expected concentration range, to a previously hydrolysed wheat flour sample. A calibration curve was constructed by plotting the measured absorbance in units of area versus µg of added furosine. The equation for the curve was \( Y = 9756878.61X - 36197.42 \) (range 0.025–0.25 µg); \( r² = 0.9999 \).

**HMF and glucosylisomaltol determination**

**Reagents**
Analytical reagent-grade chemicals were used. The clarified solution was composed of 15% (w/v) potassium ferrocyanide (Merck, Darmstadt, Germany) (Carrez I) and 30% (w/v) zinc acetate (Merck) (Carrez II). A standard stock solution containing 200 mg l⁻¹ 5-(hydroxymethyl)furfural (Merck) was used to prepare the working standard solutions (0.02–0.5 mg l⁻¹).

**Apparatus**
The liquid chromatographic system consisted of a Waters 600 chromatograph with a Waters 717 Plus autosampler and a Konic 200 UVIS detector (Reno, NE, USA) set at 284 nm. Data were collected using the Waters Millennium software data system.

**Procedure**
The HMF and glucosylisomaltol determinations were performed following the method described by Garcia-Villanova et al.. The ground sample (0.4 g) was weighed into a 10 ml centrifuge tube to which 7 ml of deionised water was then added. The tube was shaken vigorously for 1 min and the sample was then centrifuged for 10 min at 5000 rpm. The same procedure was followed once more. The supernatants were clarified with 0.5 ml each of Carrez I and II solutions. The resulting mixture was centrifuged for 10 min at 5000 rpm and the supernatant solutions were separated. A 2 ml aliquot of this solution was filtered through a 0.2 µm disc filter before injection.
Chromatographic conditions
A 20 µl aliquot of the filtered solution was separated by reverse phase liquid chromatography in a C18 column (Spherisorb S5 ODS2, 250 mm × 40 mm id; Sugelabor, Madrid, Spain). Duplicate samples were analysed. The mobile phase was water/acetonitrile (95:5) (Panreac). The elution was isocratic and the flow rate was 1 ml min⁻¹. The glucosylisomaltol and HMF were completely separated out in 8 and 10 min respectively and the run time was 15 min.

The external standard method was used for calibration. The HMF solution concentration and the height of the peak obtained were considered as the variables to obtain the linear regression equation. The concentration range was 0.16–3.97 µmol l⁻¹. The linear regression equation used was (n = 7)

\[ Y = 292.17X - 0.27 \]

where \( Y \) is the peak height and \( X \) is the HMF concentration. The correlation coefficient was \( r^2 = 0.9999 \).

The molar absorptivity coefficient of HMF was considered to be similar to that of glucosylisomaltol.

Moisture, protein and pH determinations
Moisture determination was carried out by the gravimetric method (AOAC 925.10), protein determination by the Kjeldahl method (AOAC 920.87) and pH determination by the potentiometric method (AOAC 943.02).¹⁷

Statistical analysis
The Sigma package (Horus Hardware SA, Madrid, Spain) was applied to study differences between treatments and parameter correlations.

RESULTS AND DISCUSSION
Pre-baking
Experiment I
The method developed by Guerra-Hernández and Corzo⁷ was used to determine levels of furosine in the breads. No furosine was detected in raw dough or after 150 min of fermentation at 22 °C. Fig 1 depicts the HPLC chromatograms of furosine (a) in fermented dough and (b) in fermented dough at different pre-baking times. The furosine contents are shown in Table 1. The pre-baking experiment was performed twice at 175 °C in an industrial rotary steam oven. Each sample was analysed in duplicate.

Furosine values ranged from approximately 5 mg per 100 g protein at 5 min to 370 mg per 100 g protein at 30 min. The increases ranged from 1.5- to 5-fold and were statistically significant (P < 0.01).

Table 1. Furosine and HMF contents obtained during pre-baking at 175 °C for 5–30 min (experiment I)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Furosine µg g⁻¹ dry matter</th>
<th>HMF mg per 100 g protein</th>
<th>HMF (mg kg⁻¹ dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.6 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>3.8 ± 0.1</td>
<td>4.6 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>75.8 ± 2.8</td>
<td>92.3 ± 3.4</td>
<td>0.08 ± 0.001</td>
</tr>
<tr>
<td>25</td>
<td>218 ± 8.1</td>
<td>266 ± 9.9</td>
<td>0.24 ± 0.004</td>
</tr>
<tr>
<td>30</td>
<td>304 ± 11</td>
<td>370 ± 14</td>
<td>0.42 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>302 ± 11</td>
<td>368 ± 14</td>
<td>0.34 ± 0.006</td>
</tr>
</tbody>
</table>

ND, not detected.

Figure 1. Chromatograms of furosine (a) in fermented dough and (b) in fermented dough pre-baked for 10 and 15 min.
The method developed by García-Villanova et al.\textsuperscript{12} was used to determine levels of HMF in the breads. The HMF contents obtained are shown in Table 1. No HMF was detected until after 15 min of pre-baking. At 15 min the HMF content was very low, approximately 0.07 mg kg\textsuperscript{-1}. At 30 min the HMF content rose to 0.40 mg kg\textsuperscript{-1}.

The moisture content in this process ranged from 41.8% at baseline (0 min) to 31.2% at 30 min and the pH decreased from 6.4 to 5.7.

Industrial pre-baking conditions for commercial bread are 14–15 min at 175°C. After this time the bread has developed structure, while the flavour and colour are similar to those of the original dough. During this time period the water content decreases by 7% and the pH shows no significant modification. Under these conditions the first stages of the Maillard reaction are favoured, so that furosine acts as a sensitive indicator for short pre-baking periods. The furosine levels increased throughout the experiment. When water losses were lower (2.3%), the HMF content was greater.

**Experiment II**

In this experiment the pre-baking was done using shorter time intervals. The furosine and HMF contents are shown in Table 2. The furosine content ranged from 5 mg per 100 g protein (mean of two breads) at 5 min to 150 mg per 100 g protein after 25 min. The loss of water content was 16%.

As in experiment I, no HMF was detected until after 14 min, which is the pre-baking time used by the bread-making company.

According to the results of experiments I and II, furosine is a more useful indicator than HMF to monitor the pre-baking of breads.

### Table 2. Furosine and HMF contents obtained during pre-baking at 175°C for 5–25 min (experiment II)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>µg g\textsuperscript{-1} dry matter</th>
<th>mg per 100 g protein</th>
<th>HMF (mg kg\textsuperscript{-1} dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.1 ± 0.1</td>
<td>5.0 ± 0.12</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>3.9 ± 0.1</td>
<td>4.8 ± 0.12</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>8.2 ± 0.2</td>
<td>10.0 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>7.8 ± 0.2</td>
<td>9.5 ± 0.24</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>19.0 ± 0.7</td>
<td>23.1 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>19.0 ± 0.8</td>
<td>15.8 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>42.0 ± 1.6</td>
<td>51.2 ± 1.9</td>
<td>0.08 ± 0.0011</td>
</tr>
<tr>
<td>18</td>
<td>34.0 ± 1.3</td>
<td>41.4 ± 1.6</td>
<td>0.08 ± 0.0011</td>
</tr>
<tr>
<td>19</td>
<td>57.2 ± 0.2</td>
<td>69.7 ± 0.2</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>56.0 ± 0.2</td>
<td>69.2 ± 0.2</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>21</td>
<td>93.0 ± 3.5</td>
<td>113 ± 4.3</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>22</td>
<td>81.0 ± 3.0</td>
<td>98.7 ± 3.6</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>23</td>
<td>140 ± 5.2</td>
<td>170 ± 6.3</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>24</td>
<td>102 ± 3.8</td>
<td>124 ± 4.6</td>
<td>0.28 ± 0.01</td>
</tr>
</tbody>
</table>

**Baking**

Furosine has proven to be a useful indicator during pre-baking treatment, when early browning stages are produced. The furosine content decreases during the advanced Maillard reaction.\textsuperscript{18–20} In order to determine if this fall in content occurs near the longest pre-baking time, a further experiment was conducted using a higher temperature and longer times. The breads that were pre-baked for 15 min in experiment I were baked at 220°C for 14, 25 and 30 min. The time established by the industry for commercial bread is 14 min, although the bread presented good organoleptic characteristics (flavour and colour) after 25 and 30 min.

Table 3 shows the results obtained after 14 min of baking at 220°C: the furosine content increased from approximately 90 mg per 100 g protein (value obtained after 15 min of pre-baking at 175°C) to 257 mg per 100 g protein. Between 15 and 30 min there was a further increase. Other authors\textsuperscript{14} who baked pre-baked breads (190°C) for 30 min showed an increase in furosine content for the first 20 min followed by a slight decrease at 30 min. Although their results are not identical to ours, they showed that stronger treatments than those used in pre-baking are needed before the furosine content falls, confirming the value of furosine in the pre-baking process.

The HMF content (Table 3) was increased at all time periods studied, from 4.44 to 7.1 mg kg\textsuperscript{-1} dry matter. These results are consistent with findings by Ramirez-Jimenez et al.\textsuperscript{9} HMF may be a useful indicator to evaluate browning intensity induced by baking.

Glucosylisomaltol is a novel indicator of non-enzymatic browning in baby cereals.\textsuperscript{21} This compound was not detected in commercial pre-baked breads, and longer baking times or higher temperatures are probably required for its generation. The results obtained during baking ranged from 6.12 to 10 mg kg\textsuperscript{-1} dry matter (Table 3). These findings are similar to those obtained by Guerra-Hernández et al.\textsuperscript{21} in a previous baking experiment. Like HMF, glucosylisomaltol may be a useful indicator to evaluate browning during the baking process.

**CONCLUSION**

Furosine, an indicator of early stages of the Maillard reaction, is not detected in bread fermentation but

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>µg g\textsuperscript{-1} dry matter</th>
<th>mg per 100 g protein</th>
<th>HMF (mg kg\textsuperscript{-1} dry matter)</th>
<th>Glucosylisomaltol (mg kg\textsuperscript{-1} dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>211 ± 1.1</td>
<td>257 ± 13.4</td>
<td>4.44 ± 0.06</td>
<td>6.12 ± 0.21</td>
</tr>
<tr>
<td>25</td>
<td>440 ± 1.7</td>
<td>536 ± 20.7</td>
<td>5.11 ± 0.11</td>
<td>7.37 ± 0.21</td>
</tr>
<tr>
<td>30</td>
<td>523 ± 0.8</td>
<td>637 ± 9.7</td>
<td>7.10 ± 0.17</td>
<td>10.0 ± 0.1</td>
</tr>
</tbody>
</table>
increases during pre-baking. Furosine is therefore an adequate indicator to monitor the manufacture of pre-baked bread. HMF and glucosylisomaltol, intermediate indicators of the Maillard reaction, are useful to monitor bread baking but not the manufacture of pre-baked bread.

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REFERENCES