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Glucosylisomaltol, a New Indicator of Browning Reaction in Baby Cereals and Bread

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Glucosylisomaltol is proposed as a new indicator of the browning reaction in baby cereals and bread. The glucosylisomaltol was synthesized from maltose and proline, purified by semipreparative HPLC, and characterized by NMR, high-resolution mass spectrometry, and GC-MS analysis. Analysis of glucosylisomaltol, previously separated from cereals by centrifugation, was carried out by reversed-phase HPLC with UV detection in isocratic elution with water/acetonitrile (95:5). Mean recovery of glucosylisomaltol by the standard addition method was 96.9%. The relative standard deviation and detection limit were 1.56% and 0.14 mg/kg, respectively. This compound was identified in samples by the similarity of the t_R and UV spectra to those of synthesized glucosylisomaltol. Moreover, the glucosylisomaltol from samples, previously separated by semipreparative HPLC, was acetylated and then separated and confirmed by GC-MS. Glucosylisomaltol was determined in baby cereals stored at 32 and 55 °C for 1 year and at 25 and 55 °C for 1 month at a water activity of 0.65. The amount of this indicator increased during storage from 0.48 to 7.7 mg/kg. The glucosylisomaltol was also determined in prebaked bread by heating at 190 °C for 30 min. The amount of this compound increased from nondetectable to 20.9 mg/kg after 30 min of baking. Glucosylisomaltol is a useful indicator to control the browning reaction during baby cereal storage and the baking of bread.

KEYWORDS: Glucosylisomaltol; nonenzymatic browning; baby cereals; bread

INTRODUCTION

Baby cereals are an important energy source for the nutrition of infants in Mediterranean countries and form the basis of their weaning−feeding from the age of 3−4 months. The manufacture of baby cereals can involve toasting, germination, boiling, hydrolysis, and drying stages (*1*, *2*). These processes improve the dispersibility in liquids and the starch digestibility, which are desirable because of the limited ability of the pancreas of a 3−4-month-old baby to digest starch (*3*). The commercial product has greater sweetness, less syneresis, and adequate organoleptic (color, flavor) and hygienic conditions, but the nutritional value is reduced. A high proportion of reducing sugars, principally glucose, maltose, and maltodextrins, are produced (*4*) during baby cereal manufacture, which react with amino groups of proteins in the Maillard reaction. The baby cereals have a long shelf life and can usually be consumed up to 2 years after their manufacture. Storage duration and conditions, as well as the particular composition of these cereals, affect the progress of the Maillard reaction that is initiated during their processing (*5*, *6*).

Dehydrated fruits and caramel are commonly included in baby cereals. The dehydration process that these ingredients undergo in their processing produces nonenzymatic browning, mainly due to carbohydrate degradation, which produces hydroxymethylfurfural (*6*, *7*). Thus, although hydroxymethylfurfural is probably the best known indicator of the extent of the browning reaction, it cannot be used to monitor modifications in color, flavor, or nutritional value during the manufacture and storage of baby cereals. Thus, another indicator of the intermediate stages of the browning reaction must be found to enable control of baby cereal processing. Moreover, furosine, an indicator of the first steps of the Maillard reaction, is not useful to follow up these products during extended storage (*8*).

The heating of lactose or maltose, carbohydrates with 1,4-glycosidic bonds, with primary or secondary amines in buffered aqueous solution for a prolonged time produces isomaltol derivatives (*9*). When model system solutions (pH 6.5) of sugars (fructose, glucose, maltose, or maltotriose) in the presence or absence of one amino acid (glutamine, asparagine, or arginine) are heated at 80 °C for 12 h, only maltose solutions containing amino acids, especially glutamine, produce glucosylisomaltol (*10*). Baby cereals contain a high proportion of maltose and glutamine (*4*), so glucosylisomaltol may be present. Glucosylisomaltol could, therefore, be useful to control the manufacture (toasting and drying) and storage conditions of baby cereals, which contain a high proportion of maltose, generated during hydrolysis (*4*), and a high proportion of the amino acid

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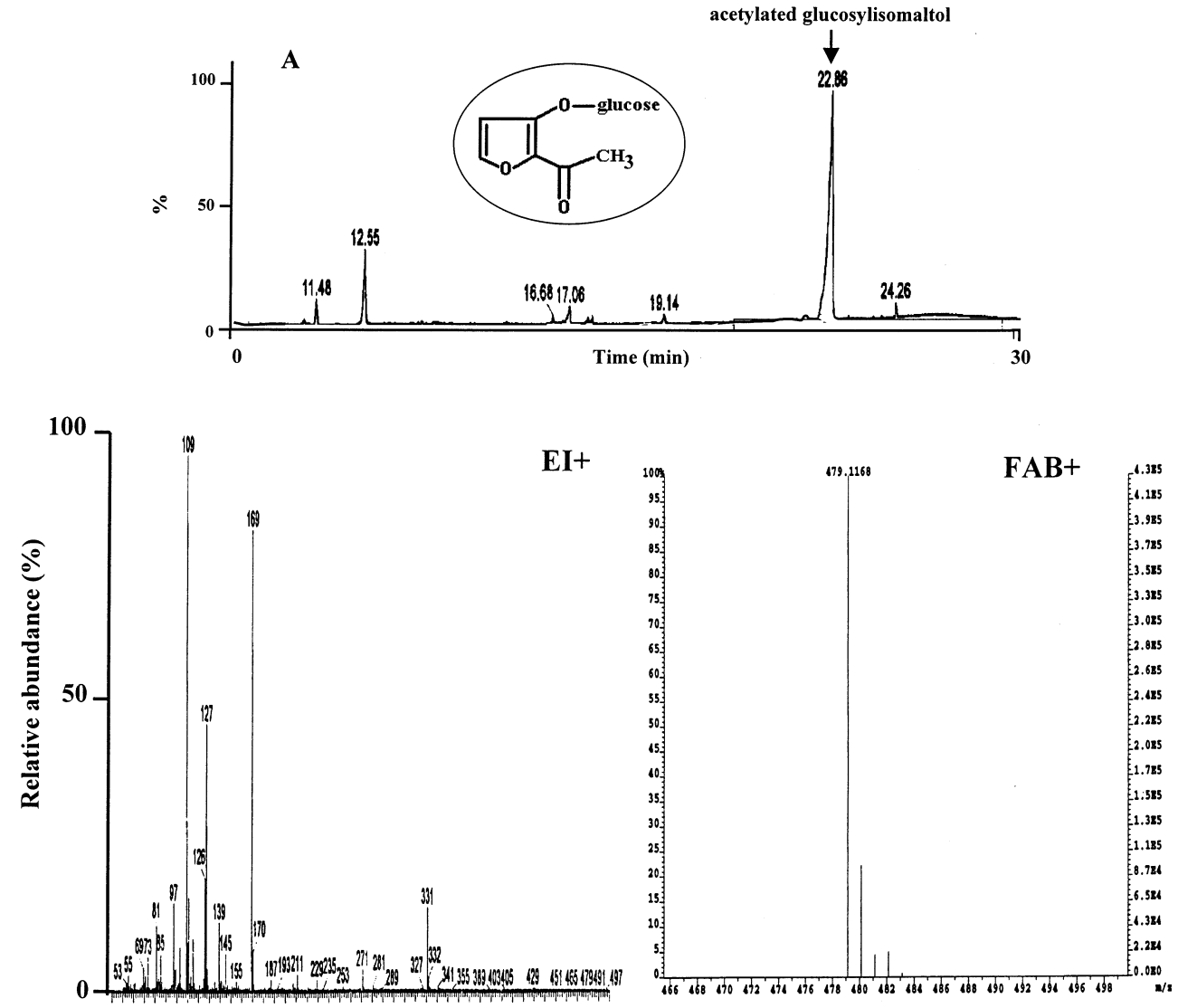

Figure 1. Chromatogram, glucosylisomaltol structure (A), and mass spectra (EI+ and FAB+) of acetylated synthesized glucosylisomaltol.

glutamine. For the same reason, it could be useful to control the baking process during bread manufacturing. The present study aimed to investigate the presence of glucosylisomaltol produced by the advanced Maillard reaction and to determine its utility in the control of baby cereal processing and storage and the baking of bread.

MATERIALS AND METHODS

Synthesis and Purification of Glucosylisomaltol. 3-*O*-α-D-Glucopyranosyloxy-2-furylmethyl ketone (glucosylisomaltol) was prepared according to the method reported by Goodwin (*11*); 17.9 mmol (6.45 g) of maltose hydrate (Sigma, St. Louis, MO), 35.7 mmol (4.11 g) of L-proline (Merck, Darmstadt, Germany), 8.8 mL of triethylamine (Panreac, Barcelona, Sapin), and 100 mL of absolute ethanol (Panreac) were stirred under reflux for 24 h at 70−80 °C. The solvents were evaporated under reduced pressure, and the residue was dissolved in 5 mL of water. The aqueous solution of the residue was continuously extracted with ethyl acetate for 36 h. The extract was evaporated under reduced pressure, and the syrup obtained was dissolved in water, passed through a 73 × 2.5 cm column of Dowex H^+ (50Wx1; 100 mesh) (Sigma), and eluted with water at 0.7 mL/min. The first 100 mL was discarded, and 240 mL was recovered in 24 tubes. The contents of 24 tubes were monitored by thin-layer chromatography (TLC) on silica gel plates with 0.25 mm thickness (Merck). The TLC was performed with 80% methanol/ethyl acetate (v/v). The spots were detected by spraying with iodine solution and 5% sulfuric acid in ethanol. A compound with an R_f value of 0.7 was obtained from the 3rd to 21st tubes. The contents of these tubes were combined and then lyophilized.

Subsequently, a small amount of the lyophilized residue was dissolved in water, and 10 μL was injected in an analytical chromatograph (Dionex AX-300) equipped with a variable-wavelength UV detector (LDC Analytical SM400). A furosine-dedicated 250 mm × 4.6 mm i.d. C_8 column (Alltech Associates Europe) was used at a flow rate of 1.2 mL/min in isocratic conditions. The mobile phase was 0.27% KCl/0.01% acetic acid with detection at 280 nm. The high-performance liquid chromatogram (HPLC) revealed a single peak (80%) at 3.9 min.

After confirmation of the presence of a compound at high concentration, probably glucosylisomaltol, the lyophilized residue was dissolved in water and purified with solid phase extraction (SPE). One milliliter was passed through a Sep-Pak C_{18} cartridge, sorbent/mass ratio 360 mg/50 mg (Millipore), prewetted with 5 mL of methanol and 10 mL of water; it was washed with 1 mL of methanol/water (10:90) and eluted with 1 mL of methanol/water (30:70). The compound was isolated by preparative chromatography (Waters 2690 with a Waters 996 photodiode array detector). The column used was a 350 × 7.8 mm i.d. Pre Nova-Pak HR C_{18} (Waters), and the flow rate was 2 mL/min with detection at 280 nm. Under these experimental conditions, the compound eluted at 9.4 min. Fractions of ∼1.5 mL were manually collected for each injection between minutes 9 and 10.

The identity of the isolated and lyophilized compound was confirmed by high-resolution mass spectrometry (MS), gas chromatography−mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR).

Characterization of Glucosylisomaltol. *NMR Analysis.* ^{1}H NMR was determined in D_2O on a Bruker AM-300 (300.13 MHz). The chemical shifts for glucosylisomaltol were δ 2.48 (s, 3H, Ac), 3.45−3.90 (m, 5H), 4.38 (t, 1H), 5.62 (d, 1H, $J = 3.5$ Hz, H═), 6.72 (d, 1H, CH-4), and 7.67 (d, 1H, CH-5). The data obtained were consistent with a previous report (*11*).

High-Resolution Mass Spectrometry. Accurate mass determination was carried out using an AutoSpec-Q mass spectrometer arranged in an EBE geometry (Micromass Instruments, Manchester, U.K.) and equipped with an fast atom bombardment (FAB) (LSIMS) source. The instrument was operated at 8 kV accelerating voltage, and Cs^+ was used as primary ion. Mass spectrum (LSIMS): *m*/*z* 311.0743 (M^+ + Na), calculated for $C_{12}H_{16}O_8Na$, 311.0743 (deviation +0.1 ppm). Mass spectrum for acetylated glucosylisomaltol (LSIMS): *m*/*z* 479.1168 (M^+ + Na), calculated for $C_{20}H_{24}O_{12}Na$, 479.1165 (deviation −0.4 ppm).

Gas Chromatography−Mass Spectrometry. GC-MS was performed on a Platform II mass spectrometer (Micromass Instruments) coupled with a Carlo Erba gas chromatograph (TermoInstruments) and equipped with an EI source at 70 eV. The analysis was carried out using a 30 m × 0.25 mm i.d., 0.25 μm, HP-5MS capillary column (Hewlett-Packard, Palo Alto, CA) inserted directly into the ion source. The temperature program was as follows: initial temperature, 60 °C; raised at 10 °C/min to 300 °C; then isothermal for 15 min. The carrier gas was helium at a flow rate of 1 mL/min.

The compound was acetylated with 100 μL of acetic anhydride, 50 μL of *N*-methylimidazole, and a catalytic amount of 4-*N*,*N*-(dimethylamino)pyridine in a sonic bath at 55−60 °C for 45 min. Two milliliters each of water and dichloromethane were then added. The organic layer was filtered through anhydrous sodium sulfate, and 2 μL was injected.

Determination of glucosylisomaltol. *Baby Cereal Samples.* Samples of baby cereals denominated "seven cereals" were obtained from a dietetic products company. According to the label information the samples contained 80% of a mixture of cereal flours (wheat, rice, barley, rye, oat, corn, and millet), as well as soy flour, sucrose, caramel, vitamins, minerals, and flavors. The commercial samples (packed under nitrogen atmosphere) were stored by the company at 32 or 55 °C for 1, 3, 6, or 12 months. These temperatures are used by the industry to control normal and accelerated storage conditions. Identical samples were kept in our laboratory at 25 or 55 °C for 1, 2, 3, or 4 weeks in an air atmosphere with controlled water activity ($A_w = 0.65$). To maintain this water activity, the moisture of cereals was controlled according to the procedure described by Salmarch and Labuza (*12*), placing the samples in a Petri plate on the upper shelf of a desiccator containing saturated sodium nitrite solution. Additional samples were also stored in our laboratory at 28 °C for 1 or 4 months. The samples were analyzed before their storage and again after storage conditions. Solid samples were stored at −50 °C until their analysis. All samples analyzed corresponded to the same batch.

Bread Samples. Six slightly baked sticks of dough (prebaked bread) of the same brand and batch were locally purchased already packed into plastic bags under vacuum conditions and stored at room temperature. The label information stipulated household baking conditions of 220 °C for 12−15 min. The ingredients listed on the label were wheat flour, water, baker's yeast, salt, enzymes, emulsifiers, and dough-conditioning agent. The weight ranged between 112 and 128 g, and the moisture was 32%. In a laboratory experiment, the different prebaked breads were baked at 190 °C for 10, 15, 20, 25, and 30 min. The samples were analyzed before baking and again after baking conditions. Solid samples were stored at −50 °C until their analysis.

Sample Extraction. The ground sample (0.6 g) was weighed into a 10-mL centrifuge tube to which 7 mL of deionized water was then added. The centrifuge tube was shaken vigorously for 1 min, and the sample was then centrifuged for 10 min at 5000 rpm. The same procedure was followed twice more. The supernatants were clarified with 0.5 mL each of Carrez I (potassium ferrocyanide, 15% w/v) (Merck) and Carrez II (zinc acetate, 30% w/v) (Merck) solutions. The resulting mixture was centrifuged for 10 min at 5000 rpm. The solution was diluted to a total volume of 25 mL with deionized water. A 1 mL aliquot of this solution was applied to a Sep-Pak C_{18} cartridge, sorbent/mass ratio 360 mg/50 mg (Millipore), prewetted with methanol (5 mL) and water (10 mL), cleaned with 1 mL of methanol/water (10:90), and then eluted with 1 mL of methanol/water (30:70). The purified liquid was filtered through a 0.20 μm disk filter before HPLC injection. Samples were analyzed in duplicate.

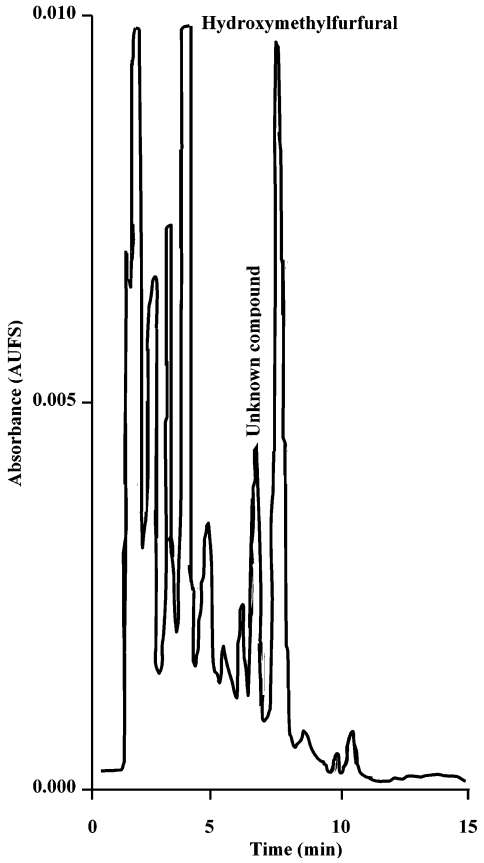

Figure 2. Chromatogram of hydroxymethylfurfural determination in baby cereal samples.

Chromatographic Conditions. The liquid chromatographic systems used in this study consisted of a Konic model 500A (Barcelona, Spain) with a 20 μL injection loop chromatograph, a UV Konic detector model 200 UVIS (Reno, NV) at 280 nm, and a Hewlett-Packard integrator model 3394A (Avondale, PA).

Twenty microliters of purified solutions was separated on a 250 mm × 4 mm i.d. Spherisorb S5 ODS2 reverse-phase C_{18} (Sugelabor, Madrid, Spain) column. The mobile phase was acetonitrile/water (5:95) (Panreac), and the flow rate was 1 mL/min. Calibration of chromatography systems for glucosylisomaltol determination was made by the external standard method. Hydroxymethylfurfural standard stock solution containing 1.59 mmol/L of 5-(hydroxymethyl)furfural (Merck) was used to prepare the working standard solutions of 0.16−3.96 μmol/L. The hydroxymethylfurfural concentration and height of the peak obtained were considered as the variables to obtain the linear regression equations ($Y = 292.17X - 0.27$; $n = 6$) ($r^2 = 0.9995$).

RESULTS AND DISCUSSION

Synthesis of Glucosylisomaltol, Identification, and Characterization. Glucosylisomaltol synthesis produced a yellow-brown residue. Partial purification was achieved by a clarification step with SPE. Complete purification of glucosylisomaltol was obtained with a semipreparative column under isocratic conditions. The isolated compound was analyzed by MS with EI+ and FAB+, giving molecular ions at *m*/*z* 288 and 311, respectively. The mass spectrum (LSIMS) was consistent with the molecular formula $C_{12}H_{16}O_8Na$. The GC-MS analysis of the acetylated compound showed a retention time of 22.8 min. The molecular ions obtained with EI+ and FAB+ were *m*/*z* 456 and 479, respectively (**Figure 1**). The mass spectrum

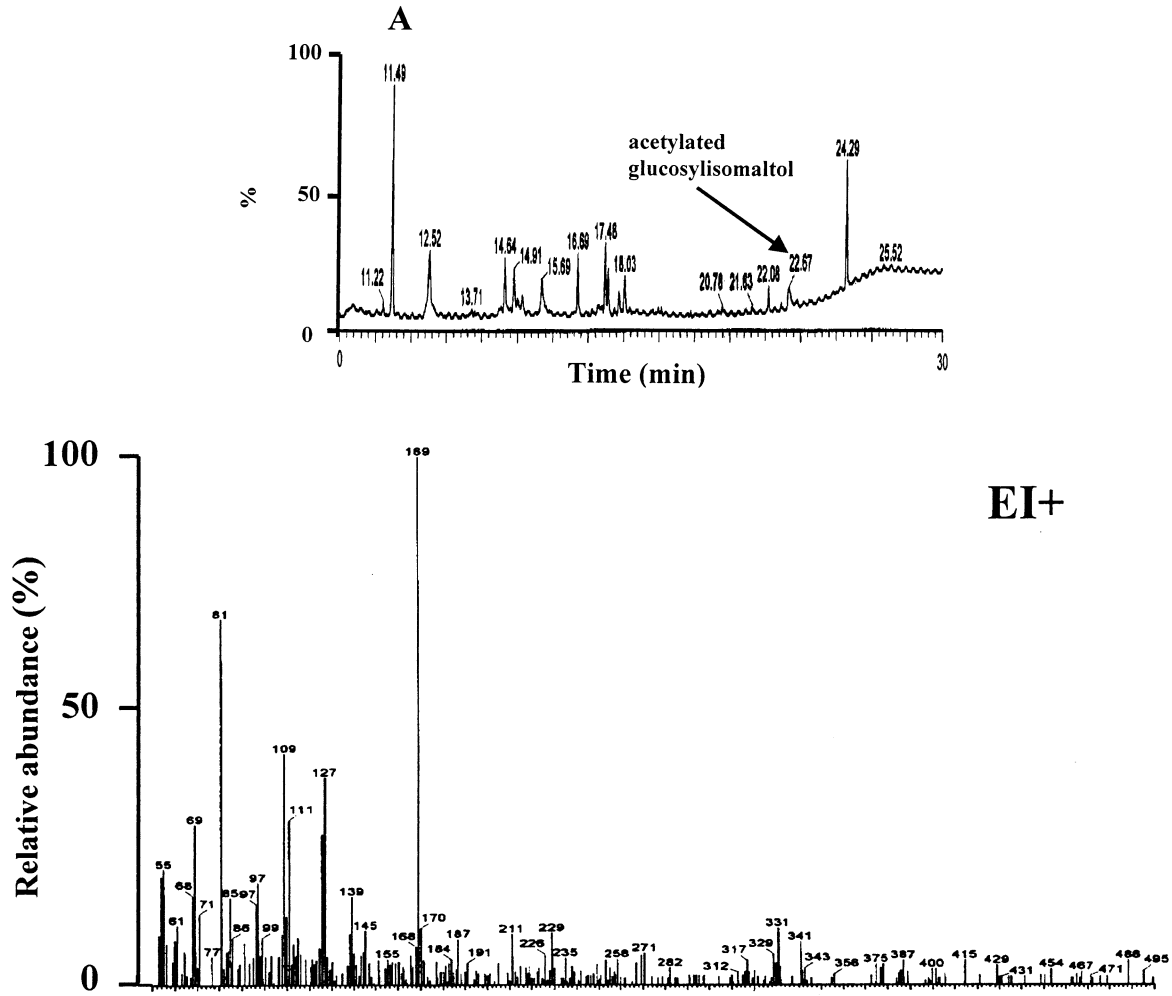

Figure 3. Chromatogram (A) and mass spectrum (EI+) of acetylated glucosylisomaltol obtained in baby cereal samples.

(LSIMS) corresponds to $C_{20}H_{24}O_{12}Na$. These molecular weights correspond to glucosylisomaltol.

^1H NMR analysis of the purified sample confirmed the structure.

Glucosylisomaltol Identification in Samples. Previous studies of browning reaction in baby cereals (*8*) showed, during chromatographic separation of free hydroxymethylfurfural, an unknown peak at ~7 min. The formation of unknown compound increased linearly with storage time of the cereal and also increased proportionally with greater temperature (**Figure 2**). The synthesized glucosylisomaltol had the same t_R as this unidentified compound.

Glucosylisomaltol was identified in samples by the similarity of its t_R and UV spectra to those of synthesized glucosylisomaltol. In addition, the glucosylisomaltol of the samples, previously separated by semipreparative HPLC, was acetylated and then separated and confirmed by GC-MS. The t_R and mass spectra of the synthesized and sample-derived glucosylisomaltol were the same (**Figure 3**).

HPLC Method Optimization. We used the same HPLC method as previously used to evaluate hydroxymethylfurfural in baby cereals (*13*), with slight modifications. A mixture of acetonitrile/water (5:95) was used as mobile phase. Other proportions had been assayed (2:98, 6:94, 7:93, 10:90, and 20:80) in preliminary studies, and better resolution was obtained between glucosylisomaltol and hydroxymethylfurfural, the principal interference compound in cereal samples, when a higher proportion of acetonitrile was used. However, when >10% of acetonitrile was used, other compounds interfered with glucosylisomaltol. When the proportion of acetonitrile was ≤2%, the t_R of glucosylisomaltol was greater than that of hydroxymethylfurfural, the converse of the usual situation. This fact could be used to determine the possible presence of glucosylisomaltol in samples.

To improve the resolution, the samples were purified with SPE after the separation of soluble compounds by centrifugation. A methanol/water concentration with >20% (v/v) methanol is necessary to elute the glucosylisomaltol in a Sep-Pak C_{18} cartridge. The typical chromatogram of a sample (bread) displayed in **Figure 4** shows the glucosylisomaltol to be completely separated from hydroxymethylfurfural. In the baby cereals and bread studied, no interfering peaks were present at the retention time of glucosylisomaltol.

Glucosylisomaltol Quantification in Samples. The compound separated by reverse-phase C_{18} HPLC was determined by its UV absorption at 280 nm. The external standard method was used for the glucosylisomaltol determination. The spectrophotometric response factor of hydroxymethylfurfural was considered to be similar to that of glucosylisomaltol.

The precision of the entire assay procedure was evaluated for "seven cereal" samples ($n = 7$). The relative standard deviation (RSD) was 1.56% for a mean value of glucosylisomaltol of 7.68 mg/kg. The accuracy was tested by the addition of glucosylisomaltol to the "seven cereal" sample with the lowest glucosylisomaltol level (0.48 mg/kg), followed by recovery assays on this sample. The amount of glucosylisomaltol added

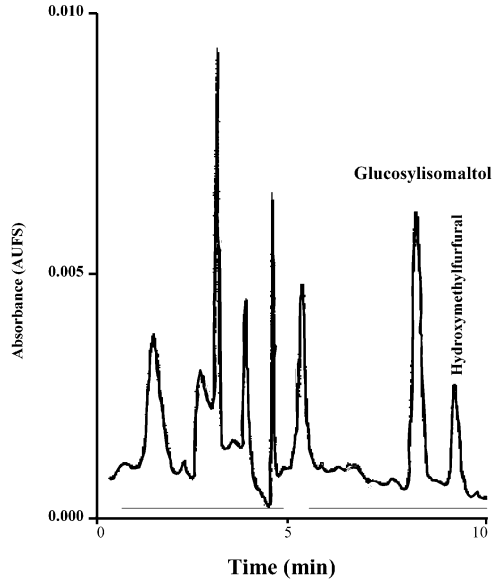

Figure 4. Chromatogram of glucosylisomaltol in prebaked bread baked for 30 min.

Table 1. Changes in Glucosylisomaltol Contents (Milligrams per Kilogram) during Storage of Baby Cereals (Seven Cereals)[a]

laboratory conditions				industrial conditions		
time (weeks)	25 °C/A_w = 0.65	55 °C/A_w = 0.65	28 °C	time (weeks)	32 °C	55 °C
0	0.48 ± 0.00	0.48 ± 0.00	0.48 ± 0.00	0	0.48 ± 0.00	0.48 ± 0.00
1	0.68 ± 0.00	2.30 ± 0.07		4	0.57 ± 0.00	0.87 ± 0.04
2	0.89 ± 0.03	3.33 ± 0.03		13	0.84 ± 0.00	1.71 ± 0.00
3	1.12 ± 0.00	5.68 ± 0.02		26	1.16 ± 0.03	2.30 ± 0.08
4	1.23 ± 0.00	7.69 ± 0.08	0.52 ± 0.03	52	1.60 ± 0.03	4.54 ± 0.29
16			0.80 ± 0.03			

[a] $n = 2$.

ranged from 1.35 to 5.15 mg/kg. The recovery range was 95.4−99.5%, and the mean value was 96.9%. The detection limit was 0.14 mg/kg (calculated as signal-to-noise ratio of two).

Table 1 displays the content of glucosylisomaltol in baby cereals ("seven cereals") stored in our laboratory and by the manufacturer. The initial content of glucosylisomaltol (0.48 mg/kg), generated during the manufacture of the baby cereals, increased during storage. At 55 °C/A_w = 0.65, the increase was 16-fold the initial content.

The results obtained in "seven cereals" samples stored under industrial conditions (with nitrogen) were similar. After storage for 1 year at 32 °C (room temperature during the summer in Mediterranean countries), the content of glucosylisomaltol increased ~3-fold.

Previous studies on the processing of baby cereals showed a slight formation of hydroxymethylfurfural after toasting, with an increase proportional to the heat treatment (*6*). In identical samples, the furosine content was very low (*5*), because the low sugar reducing content was not favorable for the Maillard reaction. When baby cereals are hydrolyzed, a high proportion of reducing sugar is formed (*4*) and the drying process, the last stage of baby cereal manufacturing, promotes the Maillard reaction and a high proportion of furosine is formed (*5*). In this drying stage, there is no relationship between the hydroxymethylfurfural and the browning process because of the presence of caramel. The glucosylisomaltol content is very low (0.48 mg/kg), because this compound arises from the advanced Maillard reaction. Furosine is therefore a more sensitive indicator to control the drying process, the last stage of baby cereal manufacturing. The shelf life of baby cereals is very long, and the intrinsic and extrinsic conditions are not favorable to furosine formation (*8*), whereas advanced products of the Maillard reaction, such as glucosylisomaltol, are formed.

Table 2. Evolution of Glucosylisomaltol (Milligrams per Kilogram of Dry Matter) during Baking of Slightly Baked Bread Sticks[a]

0 min	10 min	15 min	20 min	25 min	30 min
nd[b]	0.82 ± 0.02	1.78 ± 0.07	3.60 ± 0.00	8.64 ± 0.00	20.9 ± 0.45

[a] $n = 2$. [b] nd, not detected.

We also explored the behavior of glucosylisomaltol in bread. **Table 2** shows the results obtained when slightly heated (prebaked) commercial bread was baked at 190 °C for 30 min. Before the baking, no detectable amounts of glucosylisomaltol were found in these breads. After 10 min of baking, 0.82 mg/kg of this compound was detected, rising to 20.9 mg/kg after 30 min. There was a linear correlation between the presence of glucosylisomaltol and baking time ($r^2 = 0.6821$). The hydroxymethylfurfural content of these samples ranged between 0.06 and 19.6 mg/kg (*14*). The correlation between glucosylisomaltol and hydroxymethylfurfural contents was $r^2 = 0.9986$. Thus, similar to hydroxymethylfurfural, glucosylisomaltol could be a useful indicator to evaluate the intensity of browning in baking processes.

Glucosylisomaltol, a soluble compound arising from the advanced Maillard reaction, was detected and quantified in baby cereals and bread. The relationship found between the amount of glucosylisomaltol and the storage conditions of the baby cereals and the baking of bread demonstrates its utility to control the manufacture and storage of these products.

ABBREVIATIONS USED

A_w, water activity; SPE, solid phase extraction; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; MS, high-resolution mass spectrometry; GC-MS, gas chromatography−mass spectrometry; NMR, nuclear magnetic resonance; FAB, fast atom bombardment; UV, ultraviolet; RSD, relative standard deviation.

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