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Generation of Furosine and Color in Infant/Enteral Formula-Resembling Systems

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The extent of the Maillard reaction was studied by measuring furosine and color formation in infant and enteral formula-resembling model systems prepared by mixing calcium caseinate, laboratory-obtained or commercial whey protein with lactose or dextrinomaltose (ingredients similar to those used in infant and enteral formula manufacture) and heating the mixture at 100, 120, or 140 °C for 0−30 min. The furosine determination was performed by HPLC and the color determination by measuring colorimetric parameters *L**, *a**, and *b** in a reflection photometer. The first steps of the Maillard reaction could be followed by furosine determination when initial ingredients had low thermal damage. Hence, furosine may be an indicator of low thermal damage in ingredients with <100 mg/100 g of protein. At the concentrations used in these model systems, similar to those in infant and enteral formulas, furosine values (indirect measure of lysine losses) were higher in lactose than in dextrinomaltose systems, in which only glucose, maltose, maltotriose, and maltotetraose among all of the sugars present showed reactivity with casein. Finally, the advanced steps could be followed by color determination when the initial ingredients had high thermal damage or the model systems were heated at high temperature or for a long time. Among the parameters assayed, *b** was the most sensitive.

KEYWORDS: Milk proteins; lactose; dextrinomaltose; furosine; color

INTRODUCTION

Enteral and infant formulas are nutrient solutions administered into the gastrointestinal tract orally (infant and some enteral formulas) or through a tube (*1*). They are usually formulated using a specific combination of proteins (caseins, whey, and soy proteins), carbohydrates (mostly dextrinomaltose in enteral or lactose in infant formulas), fats, vitamins, and minerals. The wide range of commercial infant and enteral formulas with varied nutrient components provides all of the nutrients needed by infants and patients with different diseases.

Liquid formula manufacture involves the blending, pasteurization, homogenization, and sterilization of the materials. Heat treatment similar to that used in milk sterilization facilitates the manufacture of the products, guarantees their safety, and prolongs their storage life.

One of the most important modifications induced by heating and long storage conditions is the Maillard reaction, which involves amino acids and reducing carbohydrates and can produce a loss in nutritive value (*2*, *3*).

The early stages of the Maillard reaction can be evaluated by determination of furosine [ϵ-*N*-(furoylmethyl)-L-lysine], an amino acid formed during acid hydrolysis of Amadori compounds (fructosyl-lysine, lactulosyl-lysine, and maltulosyl-lysine) produced by reaction of ϵ-amino groups of lysine with glucose, lactose, and maltose (*4*).

Furosine is a useful indicator of heat damage in milk. It can be used as a criterion to distinguish between ultrahigh-temperature (UHT) milk, pasteurized milk, and in-container-sterilized milk (*5*). Prolonged heating or inadequate storage increases the furosine level in milk (*6*−*8*), infant formulas (*9*−*13*), and enteral formulas (*14*, *15*).

The development of color is an extremely important and obvious sign of the extent of the advanced Maillard reaction (*16*). The colors produced range from pale yellow to very dark brown, depending on the type of food and the extent of the reaction (*17*). Browning is desirable in some types of food, such as bakery products, cocoa, and coffee, whereas it is undesirable in sterilized milk products and dried milk products (powder milk, powder whey). Milk and milk-based systems darken with heating intensity (temperature and holding time) and storage.

The quantitative measurement of the browning rate is used as an indicator of heat treatment severity (*18*, *19*) and to evaluate the efficiency of industrial milk processing (*20*). The browning rate can be determined by various methods, including optical measurements (*17*) or analytical techniques such as thin-layer chromatography (*21*), RP-HPLC (*22*), and IE-HPLC (*23*). The measurement of absorbance at 420 nm has been used for optical

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analysis in sugar−amino acid model systems (*24*, *25*). Spectrophotometric tristimulus color measurements were taken to assess color changes in model solutions (*17*, *26*) and milk (*27*, *28*).

Because of the complexity of the Maillard reaction, most studies in the literature have been confined to model systems. Many of these models cannot be directly applied to systems resembling liquid milk, such as enteral and infant formulas, because of the different reaction conditions (a_w, moisture, pH, etc.). The present experiments were designed to use furosine and color indicators to study the early and advanced stages of the Maillard reaction in heated sugar−milk protein solutions under conditions (concentrations and heat treatments) similar to those applied in the manufacture of enteral and infant formulas.

MATERIALS AND METHODS

Samples. The calcium caseinate, commercial whey proteins isolated (WPI), dextrinomaltose, and lactose were provided by a Spanish dietetic product company. Whey proteins with minimal heat damage (laboratory whey proteins) were obtained in our laboratory from raw milk. Briefly, 5 L of raw milk was skimmed by centrifugation at 4000 rpm (14000*g*) for 10 min at 4 °C. Casein was then precipitated by acidification to pH 4.6 with 6 N HCl and centrifugation. The obtained whey was frozen at −80 °C and lyophilized. To remove the lactose, the lyophilized whey was dissolved in 500 mL of deionized water with 2% thymol (Sigma-Aldrich, Madrid, Spain) as preservative and dialyzed at 4 °C for 10 days using a size exclusion membrane of 8000 Da (Sigma-Aldrich). The water was changed on alternate days. Finally, the delactosed whey was again lyophilized.

Model systems were performed by dissolving commercial calcium caseinate, commercial whey proteins, or laboratory whey proteins (4% w/v) with lactose (7% w/v) or dextrinomaltose (12% w/v) in 100 mL of 0.1 M phosphate buffer, pH 6.5. Two aliquots of 10 mL were then placed in Pyrex screw-cap vials, which were immersed in a glycerol bath kept at 100, 120, or 140 °C from 0 to 30 min. The samples were then cooled in an ice bath and stored at −50 °C until analysis. The heating times reported exclude the heating-up period, which was estimated at 2 min. Other model systems were performed by dissolving calcium caseinate (4% w/v) and the sugars included in commercial dextrinomaltose, that is, glucose, maltose, maltotriose, maltotetraose, and maltopentaose (0.6% w/v), in order to study their formation of furosine.

Chemicals. Methanol, acetonitrile, and formic acid (HPLC grade) were obtained from Panreac (Barcelona, Spain). HCl, sodium heptanesulfonate, thymol (analytical grade), and glycerol (heavy grade) were also obtained from Panreac. Furosine was from Neosystem Laboratories (Strasbourg, France).

Equipment/Materials. Model systems were heated in an Omron E5 J glycerol bath (Barcelona, Spain). A Sorvall RC-5B (DuPont Instruments, Paris, France) was used for centrifugation and a Flexi-dry μP (FTS Systems, Manchester, U.K.) for lyophilizing. The high-pressure liquid chromatograph consisted of a Perkin-Elmer model 250 pump (Norwalk, CT) with a Waters 717 autosampler (Milford, MA) and a Perkin-Elmer model 235 diode array detector. Data were collected with a 1020 software data system (Perkin-Elmer). Furosine was separated using a Spherisorb ODS2 5 μm (250 mm × 4.6 mm i.d. Phenomenex, Torrance, CA) column. Color determination was performed by using a reflection photometer CM-2002 (Minolta Canada Inc., Mississauga, ON) with an illumination area of ∅ 11 mm and d/8 geometry in standard ISO 7724/1.

Liquid Chromatograph Operating Conditions. The mobile phase consisted of a solution of 5 mM sodium heptanesulfonate with 20% acetonitrile and 0.2% formic acid. The elution was isocratic, and the flow rate was 1.2 mL/min. The UV−vis detector was set at 280 nm. Calibration of the chromatographic system for furosine determination was by the external standard method. A standard stock solution containing 1.2 mg/mL of furosine was used to prepare the working standard solution. The calibration was performed by adding increasing quantities of furosine standard, within the expected concentration range, to a previously hydrolyzed raw milk sample. The curve was constructed in units of area against micrograms of added furosine. The equation for the curve was $Y = 1.111 \times 10^7 X - 148949.06$ (range, 0.0096−1.1500 μg), $r^2 = 0.9999$.

Furosine was determined following the methods described by Delgado et al. (*29*). One milliliter of the sample was hydrolyzed with 7 mL of 9.08 M HCl at 120 °C for 23 h in a Pyrex screw-cap vial with a PTFE-faced septum. High-purity N_2 gas was bubbled through the solution for 2 min. The hydrolysate was filtered with a medium-grade paper filter. A 0.5 mL portion of the filtrate was applied to a Sep-Pak C_{18} cartridge (Millipore, Bedford, MA) prewetted with 5 mL of methanol and 10 mL of deionized water and was then eluted with 3 mL of 3 M HCl and evaporated under vacuum (*5*). The dried sample was dissolved in 3 mL of a mixture of water, acetonitrile, and formic acid (95:5:0.2), and 50 μL of the resulting solution was analyzed by HPLC. Duplicate analyses of duplicate samples were carried out ($n = 4$).

Color determination was performed by measuring the colorimetric parameters L^*, a^*, and b^* using the CIE "C" illuminant, which represents average daylight minus the ultraviolet component. Before the analysis, the reflection photometer was always calibrated with a blank. After calibration, 3 mL of sample was added to a 1 cm glass vial fitted to the photometer output for the assay. The CM-2002 averaged the color of three different readings. Three replicate analyses were carried out. The yellowing index (YI) was calculated with the following formula (*27*):

$$\mathrm{YI} = 142.86 b^*/L^*$$

Statistical analysis of data was performed by analysis of variance (Microcal Origin 5.0, Microcal Software, Northampton, MA). Student's *t* test was used to compare means, and the level of significance was set at 99.9%.

RESULTS AND DISCUSSION

Furosine Study. Furosine content was assayed in model systems with different protein sources: calcium caseinate, commercial whey proteins donated by a local company, or whey proteins obtained in our laboratory. The concentration selected (4% w/v) corresponds to the usual proportion of protein in enteral formulas. Commercial dextrinomaltose (12% w/v) or lactose (7%) was used as carbohydrate source, at concentrations similar to those used in enteral and liquid infant formulas. The protein and carbohydrate contents of these ingredients were previously assayed (*30*). The calcium caseinate was 100% pure protein, whereas the commercial and laboratory whey proteins had protein contents of 87.8 and 96.1 g/100 g of protein, respectively. In addition, dextrinomaltose contains 0.5% protein. Regarding the carbohydrate content, the whey proteins obtained at our laboratory had only 0.25% glucose, whereas the commercial whey proteins contained 1.5% glucose, 0.25% galactose, and 3.5% lactose. Finally, dextrinomaltose contained 4.25% glucose, 4.30% maltose, and 4.60% maltotriose, and lactose had 0.63% glucose and 0.14% galactose.

Table 1 shows the results obtained for the model systems with calcium caseinate and dextrinomaltose or lactose. Heating at 100 °C produced a statistically significant increase ($P < 0.001$) in model systems with lactose at all of the times assayed except in the model system with dextrinomaltose, in which the increase was not significant between 5 and 10 min, between 15 and 20 min, and between 25 and 30 min. At 120 °C, there was a greater formation of furosine, which stabilized after 25 min, probably because the rate constant of Amadori compounds formed after the initial stage of the Maillard reaction is similar to the degradation rate of advanced Maillard products. At this temperature, the changes between different times were signifi-

Table 1. Furosine Content (Milligrams per 100 g of Protein) in Casein Model Systems

	lactose			dextrinomaltose		
time (min)	100 °C	120 °C	140 °C	100 °C	120 °C	140 °C
0	60.3	60.3	60.3	60.9	60.9	60.9
5	92.5	199	275	92.7	147	215
10	114	254	358	105	192	257
15	150	289	369	130	259	251
20	174	341	363	141	308	235
25	188	371	340	172	324	201
30	198	364	316	175	297	194

Table 2. Furosine Content (Milligrams per 100 g of Protein) in Laboratory Whey Protein Model Systems

	lactose			dextrinomaltose		
time (min)	100 °C	120 °C	140 °C	100 °C	120 °C	140 °C
0	49.9	49.9	49.9	49.6	49.6	49.6
5	58.6	274	547	59.6	145	256
10	83.3	410	590	76.0	214	269
15	98.2	474	537	88.8	281	237
20	128	550	515	91.3	304	212
25	141	604	454	99.7	296	200
30	164	579	435	113	273	182

Table 3. Furosine Content (Milligrams per 100 g of Protein) in Commercial Whey Protein Model Systems

	lactose			dextrinomaltose		
time (min)	100 °C	120 °C	140 °C	100 °C	120 °C	140 °C
0	1254	1254	1254	1277	1277	1277
5	1661	918	748	1688	947	659
10	1520	755	621	1554	683	462
15	1318	646	558	1418	508	346
20	1151	621	506	1359	458	305
25	1026	574	471	1209	429	282
30	900	554	451	1034	370	251

cant ($P < 0.001$) in both model systems. At 140 °C, this indicator was useful up to only 10 min of heat treatment because of the stabilization of furosine levels at longer heating times. The furosine content of the model systems with lactose was higher than that in model systems with dextrinomaltose.

Table 2 shows the furosine content of model systems with whey proteins obtained in our laboratory and dextrinomaltose or lactose. The behavior of these model systems was similar to that observed in the model systems with caseinate.

Commercial whey protein model systems produced furosine, but the amounts of detected furosine increased only up to 5 min of heating at 100 °C (**Table 3**). The subsequent decrease was greater when the time and temperature of heating increased. At 120 and 140 °C, the Amadori compounds were degraded or participated in the advanced stages of the Maillard reaction, so that the furosine levels decreased. This decrease was lower in model systems with dextrinomaltose than in those with lactose due to the lower degradation rate of Amadori compounds in the dextrinomaltose system that has lower reducing power.

Commercial dextrinomaltose contains amounts of mono-, di-, and oligosaccharides that contribute to the reactivity of these ingredients. To determine the reactivity of these carbohydrates, model systems prepared with calcium caseinate (4% w/v) and glucose, maltose, maltotriose, maltotetraose, or maltopentaose (0.6% w/v) were heated at 120 °C for 30 min. This temperature was selected as the usual sterilization temperature of enteral formulas. The results show a statistically significant increase

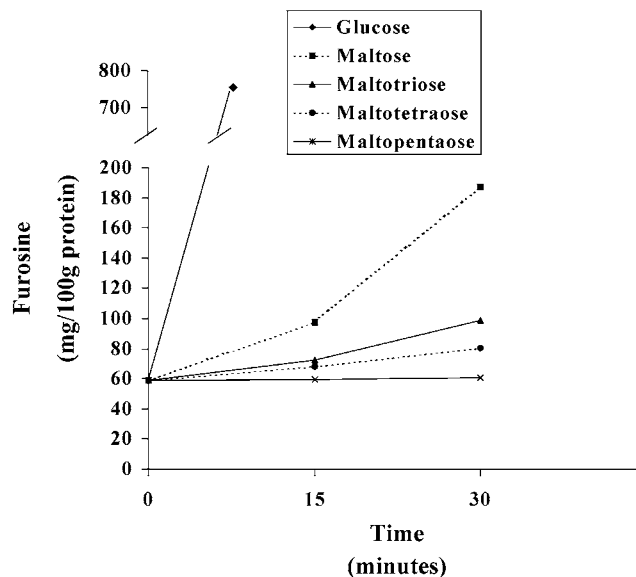

Figure 1. Furosine content (mg/100 g of protein) in casein model systems with different dextrinomaltose components heated at 120 °C.

($P < 0.001$) at 15 and 30 min of heating in all of the systems assayed except for the system with maltopentaose (**Figure 1**). As expected, the system with the lowest molecular weight of carbohydrate had the highest furosine content. The slope values ranged between 0.73 and 23.14 for maltotetraose and glucose, respectively. According to the label information, commercial dextrinomaltose has ~20% (w/w) of reactive carbohydrates (glucose, maltose, maltotriose, and maltotetraose), and commercial enteral formulas have a carbohydrate content of >10% (w/v); this means that enteral formulas have at least 2% (w/v) reactive carbohydrates.

Liquid infant and enteral formulas are prepared with casein, whey proteins, and lactose and/or dextrinomaltose. Both foods include a sterilization step during their manufacture, usually at 120 °C for not longer than 10 min. In similar treatment conditions (120 °C/10 min), more furosine was generated than destroyed only in the model systems with a low initial furosine content, whether prepared with lactose or dextrinomaltose. Therefore, furosine is useful as a heat damage indicator only in formulas with casein and/or whey proteins with low heat damage.

Enteral formulas that contained ingredients with high furosine content showed a decrease in furosine content during manufacture (*15*). However, furosine was generated during storage (*14*).

Color Study. Color was measured by reflectance, and its precision was established in model systems with dextrinomaltose. The standard deviations for L^*, a^*, and b^* parameters are shown in **Table 4**. The low deviation found indicated that these parameters could be used to study color changes during the heat treatment of model systems.

Color determination was carried out in the same model systems that were assayed for furosine content (**Tables 5–7**). In general, L^* changes showed no correlation with the visually observed browning. Even at 140 °C, the L^* value indicated a white color, whereas browning of the model system could be observed. Parameters a^*, b^*, and YI changed according to the visually observed color with longer time and higher temperature of heating. Parameters b^* and YI were the most sensitive and showed statistically significant changes ($P < 0.001$) in the model systems at temperatures of ≥120 °C for all of the times assayed. The YI parameter even revealed color changes in model systems

Table 4. Color Parameter Repeatability[a]

model system	parameter	mean	SD	CV (%)
casein–dextrinomaltose (120 °C/30 min)	L^*	23.11	0.180	0.78
	a^*	−0.35	0.028	4.20
	b^*	2.71	0.069	2.55
laboratory whey proteins–dextrinomaltose (140 °C/30 min)	L^*	44.53	0.119	0.27
	a^*	5.47	0.169	3.08
	b^*	20.75	0.166	0.80
commercial whey proteins–dextrinomaltose (100 °C/5 min)	L^*	41.45	0.185	0.44
	a^*	2.54	0.056	2.22
	b^*	0.55	0.091	1.63

[a] $n = 8$.

Table 5. Color Parameters in Casein Model Systems

temp (°C)	min	lactose L^*	lactose a^*	lactose b^*	lactose YI	dextrinomaltose L^*	dextrinomaltose a^*	dextrinomaltose b^*	dextrinomaltose YI
room		19.17	−0.49	−5.04	−37.56	21.12	−0.38	−4.87	−32.94
100	5	20.33	−0.73	−5.09	−35.77	23.52	−0.46	−5.31	−32.25
	10	20.93	−0.78	−5.08	−34.67	22.80	−0.51	−4.60	−28.82
	15	20.83	−0.80	−5.07	−34.77	22.68	−0.49	−4.41	−27.78
	20	23.90	−1.08	−5.18	−30.96	22.01	−0.50	−4.23	−27.46
	25	22.20	−0.95	−5.05	−32.50	20.91	−0.48	−4.15	−28.35
	30	23.85	−0.97	−4.30	−25.76	21.00	−0.52	−4.00	−27.21
120	5	24.67	−1.09	−3.08	−17.84	22.58	−0.48	−4.05	−25.62
	10	24.40	−1.34	−2.18	−12.76	22.07	−0.61	−3.14	−20.33
	15	24.49	−1.30	−1.03	−6.01	21.37	−0.70	−1.85	−12.37
	20	24.59	−1.23	0.52	3.02	25.99	−0.80	−0.10	−0.55
	25	23.74	−1.03	1.75	10.53	25.01	−0.59	1.83	10.45
	30	22.95	−0.75	2.88	17.93	23.66	−0.31	2.65	16.00
140	5	18.72	−0.82	2.05	15.64	20.04	−0.62	−3.08	−21.96
	10	17.23	−0.65	2.65	21.97	18.10	−0.64	0.01	0.08
	15	14.42	1.34	6.16	61.03	16.00	1.12	6.26	55.89
	20	14.90	1.50	6.03	57.82	17.60	1.49	6.45	52.35
	25	26.73	1.39	8.35	44.63	28.29	1.67	9.67	48.83
	30	31.45	3.33	13.12	59.60	32.06	3.57	14.22	63.36

Table 6. Color Parameters in Laboratory Whey Protein Model Systems

temp (°C)	min	lactose L^*	lactose a^*	lactose b^*	lactose YI	dextrinomaltose L^*	dextrinomaltose a^*	dextrinomaltose b^*	dextrinomaltose YI
room		24.37	−1.39	−4.55	−26.67	24.31	−1.04	−6.46	−37.96
100	5	56.70	−2.43	−2.87	−7.23	60.17	−2.01	−2.70	−6.41
	10	58.37	−2.44	−2.61	−6.39	60.73	−2.01	−2.85	−6.70
	15	58.86	−2.53	−2.58	−6.26	61.12	−2.00	−2.63	−6.15
	20	58.84	−2.45	−2.46	−5.97	61.33	−2.03	−2.58	−6.01
	25	59.27	−2.57	−2.42	−5.83	61.06	−2.03	−2.75	−6.43
	30	59.34	−2.57	−2.32	−5.59	61.16	−2.05	−2.61	−6.10
120	5	56.73	−2.27	−1.05	−2.64	62.68	−1.72	−1.42	−3.24
	10	60.06	−2.40	−0.35	−0.83	61.95	−1.87	−0.82	−1.89
	15	59.45	−2.46	0.71	1.71	62.02	−1.85	0.55	1.27
	20	57.94	−2.33	2.37	5.84	60.69	−1.75	2.40	5.65
	25	56.36	−1.96	4.76	12.07	59.57	−1.38	4.66	11.18
	30	55.36	−1.52	5.76	14.86	57.87	−0.89	6.31	15.58
140	5	55.96	−2.35	1.79	4.57	58.11	−1.76	1.69	4.15
	10	47.92	−0.50	10.07	30.02	52.33	−0.43	8.22	22.44
	15	44.30	2.85	15.88	51.21	49.68	1.82	14.24	40.95
	20	42.62	4.38	18.19	60.97	45.94	3.02	15.77	49.04
	25	42.59	5.56	20.09	67.39	45.25	4.38	18.89	59.64
	30	42.62	5.83	20.60	69.08	44.35	5.60	20.92	67.39

with lactose at 100 °C. The a^* parameter showed statistically significant changes ($P < 0.001$) only at 140 °C.

Model systems with caseinate and our laboratory whey proteins (with low thermal damage) showed similar b^* increments at sterilization temperatures (120 °C), whereas commercial whey proteins (damaged proteins) showed higher increases at 120 °C only in the first 15 min. At 140 °C, the b^* increase for commercial whey proteins was lower than that obtained in caseinate and laboratory whey proteins, although the final values were very similar. The systems with commercial whey proteins also showed b^* changes at 100 °C. In these types of model systems with thermally damaged ingredients, the Maillard reaction is advanced prior to the ingredient mixing step.

Table 7. Color Parameters in Commercial Whey Protein Model Systems

temp (°C)	min	lactose L^*	lactose a^*	lactose b^*	lactose YI	dextrinomaltose L^*	dextrinomaltose a^*	dextrinomaltose b^*	dextrinomaltose YI
room		24.34	−1.39	−1.44	−8.45	23.31	−1.15	−0.89	−5.45
100	5	39.70	−2.42	−1.92	−6.91	43.48	−2.21	−0.97	−3.19
	10	41.70	−2.53	−0.54	−1.86	44.11	−2.16	0.60	1.94
	15	41.54	−2.30	1.25	4.30	44.16	−1.86	2.44	7.89
	20	41.16	−1.98	3.25	11.28	43.62	−1.48	4.22	13.82
	25	40.55	−1.53	4.90	17.26	43.26	−1.01	6.15	20.31
	30	39.99	−1.15	6.48	23.15	42.79	−0.63	7.67	25.61
120	5	39.43	−1.82	3.21	11.63	44.49	−1.38	4.97	15.96
	10	37.53	−0.52	8.94	34.03	42.57	0.07	10.34	34.70
	15	36.19	0.24	11.49	45.36	41.55	0.94	13.13	45.14
	20	35.61	0.77	12.97	52.03	40.74	1.45	14.54	50.99
	25	35.39	1.09	14.14	57.08	40.43	1.87	16.00	56.54
	30	35.43	1.29	14.61	58.91	40.18	2.21	16.96	60.30
140	5	36.68	−0.68	7.19	28.00	44.40	−0.34	9.10	29.28
	10	35.10	0.67	12.45	50.67	42.36	1.32	14.40	48.56
	15	35.60	2.53	16.73	67.14	40.00	3.44	18.37	65.61
	20	37.68	3.55	18.50	70.14	39.63	4.19	20.30	73.18
	25	39.11	4.54	20.24	73.93	39.23	4.83	21.20	77.20
	30	38.09	5.38	21.66	81.24	38.10	5.26	21.43	80.35

The b^* and YI parameters may be useful to monitor the heat treatment of formulas prepared with damaged ingredients and dextrinomaltose, even at low temperatures (100 °C). Sterilization temperatures and times (120 °C for 10 min) are required before significant changes are observed in either parameter for ingredients with low thermal damage.

The reflectance of model systems with caseinate and lactose, at lower concentrations than used in the present experiments but with a similar heat treatment (*17*), showed a different behavior for E [$E^* = (L^{*2} + a^{*2} + b^{*2})^{1/2}$] and chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$]. This could be due to the different concentrations used and the different origin of the ingredients.

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