RESEARCH ARTICLE

Effect of L-asparaginase on acrylamide mitigation in a fried-dough pastry model

Kristína Kukurová[1], *Francisco J. Morales*[2], *Alena Bednáriková*[1] *and Zuzana Ciesarová*[1]

[1] VÚP Food Research Institute, Bratislava, Slovak Republic
[2] Instituto del Frío, Consejo Superior de Investigaciones Científicas, Madrid, Spain

A dough resembling traditional Spanish rosquillas was used as a model to represent classical fried-dough pastry to investigate the effects of asparaginase and heat treatment on amino acid levels and acrylamide mitigation. Wheat-based dough was deep fried at 180 and 200°C for 4, 6, and 8 min. Two recipes were formulated by addition of different asparaginase levels (100 and 500 U/kg flour) to the dough. The temperature/time profile of the frying process, moisture, sugars, amino acids, acrylamide, and some indicators of the Maillard reaction (hydroxymethylfurfural, color, free fluorescence compounds, and browning) were determined to investigate the extent of the reaction and the effect on reactants. At the both levels of asparaginase used, 96–97% of the asparagine present was converted to aspartic acid, and consequently the acrylamide level was very efficiently reduced (up to 90%). The asparaginase also affected the content of glutamine and glutamic acid in dough, resulting in a 37% increase in glutamic acid compared with the untreated sample. Concerning color, browning and Maillard reaction parameters, no significant changes between untreated and enzymatically treated samples were observed, pointing out the potential industrial and domestic enzyme application.

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1 Introduction

Acrylamide has been shown to be a carcinogen in experimental animal trials and may have a carcinogenic effect in humans [1]. Recent results published in 2008 from long-term studies on acrylamide exposure of humans have shown that consumption of high acrylamide food is linked with a slightly higher occurrence of human ovarian, endometrial, breast, and kidney cancer [2–4]. Several researchers have established that the main pathway of acrylamide formation in foods is linked to the Maillard reaction, and, in particular, the amino acid asparagine [5–8].

Correspondence: Dr. Zuzana Ciesarová, VÚP Food Research Institute, Priemyselná 4, P. O. Box 25, 824 75 Bratislava 26, Slovak Republic
E-mail: ciesarova@vup.sk
Fax: +421-2-5557-1417

Abbreviations: FIC-F, free-fluorescence intermediate compounds; **HMF,** hydroxymethylfurfural

Many strategies have been adopted to minimize acrylamide formation in different food commodities, as compiled in the CIAA-Acrylamide Toolbox [9], and recently reviewed by Friedman and Levin [10]. The formation of acrylamide during baking and frying of cereal products is closely related to both moisture content and baking temperature/time (thermal input). If products are prepared to the same final moisture but with less color development, less acrylamide is formed although longer baking times are required [11, 12]. Alternative baking technologies such as air impingement and infrared radiation heating or steam baking seemed to be promising tools for acrylamide mitigation [13]. Among others, replacing ammonium bicarbonate with alternative raising agents and inverted sugars, spraying with glycine, or addition of inorganic salts (Na^+, Ca^{2+}, chloride, carbonate, and propionate) and organic acids (citric and tartaric) in the recipe decreased acrylamide content [10–16]. However, the potentially negative impact on the sensorial properties of the product such as flavor, texture, less browning [17], and on the formation of other undesirable compounds [18] may lead to less consumer acceptance. The changes may even

affect the technology of the process, for instance, by reducing yeast fermentation properties in bread [9].

Nevertheless, the research confirms that asparagine rather than sugars is the key determinant of acrylamide formation in cereal products. Higher level of asparagine in different cereal types can cause the higher level of acrylamide in products. It is still unclear whether changes in agricultural practice could consistently reduce the content of asparagine in different varieties of grain. Selection of crop varieties with lower asparagine would theoretically lead to the reduced acrylamide formation, but this has not proved to be a practical tool for commercial production. However, the extent of milling of grain can also affect the asparagine. Current experience suggests that it is not yet possible to specify low-asparagine wholegrain. Using less whole meal and more endosperm is effective for a low-asparagine diet; on the other hand, sensorial and nutritional properties of products are altered [9].

A very promising way of eliminating acrylamide in unfermented dough is a reduction of free asparagine by asparaginase, which hydrolyses asparagine to aspartic acid and ammonia. Patents related to the use of asparaginase have been filed by several companies (*e.g.* Frito Lay, Procter & Gamble, DSM, Novozymes); nevertheless, experiments conducted so far have been restricted to laboratory and bench-scale trials. Incubation of mashed potato, potato flakes, rye flour, and wheat flour with asparaginase prior to heating decreased the acrylamide content in model systems by over 90% [8, 15, 19]. According to the review by Gordon [12], asparaginase is a promising method of acrylamide reduction especially in biscuits and crisp bread. Much effort has been put into lowering of acrylamide levels by the enzyme's producers, although a legal mandate for use in food is still needed because the enzyme is not yet licensed for food use in all countries of EU. Dossiers are being gathered for application in France and Denmark. In most other European countries, the enzyme can be applied as a processing aid without any additional safety assessments. For the US market, the enzyme producers have applied and received generally recognized as safe status from the FDA. However, the applicability of the asparaginase approach to fried-dough pastries has not been investigated yet.

Based on our previous experience in the application of asparaginase as an effective tool for mitigation of acrylamide formation in potato products [20], and investigation of heat-induced contaminants in traditional Spanish fried products [21], this investigation aimed at evaluating the usefulness of the asparaginase strategy for deep-fried pastries, and at describing other Maillard reaction products and effects that accompany thermal treatment of foods (hydroxymethylfurfural (HMF), color, free fluorescence compounds, and browning). Experiments (preparation and frying conditions) were designed to resemble the preparation of a traditional Spanish deep-fried pastry, *rosquillas*.

2 Material and methods

2.1 Chemicals and standards

The following materials were used: L-asparaginase (Novozymes, Denmark); L-asparagine (Asn); *S*-(+)-aspartic acid (Asp); *S*-(+)-glutamine (Gln); *S*-(+)-glutamic acid (Glu); D-glucose (Glc); D-fructose (Fru); sucrose (Suc); sorbitol (Sigma, St. Louis, USA or Merck, Schuchardt, Germany); acrylamide, purity 99% (Sigma-Aldrich, Steinheim, Germany); *d3*-acrylamide (2,3,3-*d3*-2-propenamide) and *d3*-glutamic acid (Isotope Laboratories, Cambridge, USA); ACN; methanol (HPLC-grade, Scharlau, Barcelona, Spain or Sigma-Aldrich); perfluorooctanoic acid (Sigma-Aldrich); acetic acid p.a. (Lachema, Brno, Czech Republic); deionized water; wheat starch (Merck); flour (Valpan artesanos; Santa Rita Harinas a.s., Spain); sunflower oil and sugar (supplied by a food company in Spain).

2.2 Simulated cereal matrix

One gram of a cereal matrix consisted of 0.0076 g asparagine, 0.1256 g glucose, 0.1140 g fructose, and 0.7600 g wheat starch. This dry matrix was mixed in 0.76 mL water. L-Asparaginase was added in to the matrix at concentrations of 100, 500, and 1000 U/kg dry weight.

2.3 Dough preparation and frying conditions

Dough was prepared according to a basic recipe containing 323 g wheat flour, 46 g glucose, 46 g fructose, and 185 mL water. Two levels of L-asparaginase in the dough, 100 U/kg flour (E100) and 500 U/kg flour (E500), were tested. All ingredients were thoroughly mixed using a lab-scale dough-blender (Mod. HR1570, Philips, The Netherlands), kept for 15 min at $37 \pm 2^{\circ}C$ in an oven (Memmert, Germany) for enzyme incubation and cooled for 5 min at $0^{\circ}C$, and then left to reach ambient temperature. In the next step, the dough was formed to rolls (10.0 ± 0.5 g and 10 ± 0.2 cm), and subsequently fried at 180 or $200^{\circ}C$ for 4, 6, or 8 min in high-oleic sunflower oil as commonly used by the industrial scale. Four units (containing 10 g dough *per* unit) were placed in the frying basket (domestic fryer, Taurus, Spain; capacity: 3 L; 1200 W) to allow free movement during frying. Two independent experiments were performed for each formulation and temperature/time combination. The sample:oil ratio was kept constant within a range of 0.013 g/g. The temperature of the fryers had been calibrated previously with external thermocouples (type K, 0.1 mm) and the temperature profile was continuously recorded by a data logger and evaluated for precision. After frying, samples were dried on paper, cooled to ambient temperature, ground, put into containers, and stored at $-20^{\circ}C$ until analysis.

2.4 Moisture

The moisture content was determined by a gravimetric method as described in AOAC-925.10 [22]. Samples were homogenized in a household cutter (Moulinette, Moulinex, Paris, France) and 2-g samples were weighed into Chopin dishes. They were dried in a convection oven (Digiheat, JP-Selecta, Barcelona, Spain) until they reached a constant weight after three consecutive readings at $105 \pm 1^{\circ}C$. Analyses were carried out in duplicate.

2.5 Protein determination

Samples (0.800–1.000 g) were analyzed by the AOAC 992.15 [23] procedure for a total protein content by heating to $105 \pm 1^{\circ}C$ in a LECO model FP-2000 protein/nitrogen analyzer (Leco Instruments, Madrid, Spain) calibrated with EDTA (Dumas method). The nitrogen-to-protein conversion factors used were 5.70 for wheat. Results were expressed as g protein/100 g product.

2.6 Color measurement

The color of ground samples was evaluated as reflectance using a spectrophotometer CM-3500d (Konica Minolta, Japan) according to the CIELab. The system gives the values of three-color components: the luminosity L^* (–black to +white component) and the chromaticity coordinates, a^* (+red to –green component), and b^* (+yellow to –blue component). A powdered sample (10 g) was added into a glass petri dish (diameter 5 cm) according to Morales *et al.* [24]. The sample was illuminated with D65 artificial daylight (10° standard angle) under conditions suggested by the manufacturer. Each color value reported was the average of three determinations. The color difference between processed and unprocessed sample (ΔE index) was calculated from the equation: $\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$, ΔL^* being the brightness difference, Δa^* the redness difference, and Δb^* the yellowness difference.

2.7 Browning measurement

Browning indices of the samples were determined after an appropriate dilution based on their absorbance at 360 and 420 nm measured in a spectrophotometer (Shimadzu UV-1603, Duisburg, Germany) using a 1-cm path length cell. Absorbances were corrected for turbidity by subtracting the absorbance at 550 nm. The browning index was defined as the OD difference between 420 and 550 nm, and between 360 and 550 nm, respectively. The results were expressed as OD/g samples after the correction for dilution.

2.8 Fluorescence measurement

Free-fluorescence intermediate compounds (FIC-F) of aqueous sample extracts were measured at an excitation wavelength of 347 nm and an emission wavelength of 415 nm as described by Delgado-Andrade *et al.* [25] with some minor modifications. The results were expressed as fluorescence intensity units *per* gram of sample after the correction for dilution. A fluorescence detector $RF^{-1}0AXL$ (Shimadzu, Japan) was used.

2.9 Acrylamide determination by LC/ESI-MS-MS

Extraction to 0.2 mM acetic acid and further pre-extraction to ethyl acetate to avoid quenching the chromatography signal by the presence of salts in the system were performed according to Gökmen and Şenyuva [26] with some modifications. The detailed procedure is described by Ciesarová *et al.* [27]. LC/ESI-MS-MS analysis was performed with a HPLC system 1200 series (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6410 Triple Quad detector equipped with ESI interface.

2.10 Amino acid determination by LC/ESI-MS-MS

To determine the amino acids, the method without derivatization according to Özcan and Şenyuva [28] with some modifications was used. The procedure is described in detail by Ciesarová *et al.* [27]. The LC/ESI-MS-MS analyses for quantification of four free amino acids (Asn, Asp, Gln, and Glu) were performed using an HPLC system 1200 series (Agilent Technologies) HPLC system 1200 series (Agilent Technologies) coupled with an Agilent 6410 Triple Quad detector equipped with ESI interface. The analytical separation was performed on a Purospher® STAR RP-8ec (150 mm × 4.6 mm, 3-μm particle size) column using an isocratic mixture of 100 mL ACN and 900 mL aqueous 0.05 mM perfluorooctanoic acid at a flow rate 0.5 mL/min at ambient temperature. All parameters of the ESI-MS-MS system were based on in-source generation of the protonated molecular ions of the four amino acids measured and the internal standard (*d3*-Glu), as well as collision-induced production of amino acid-specific fragment ions for multiple reaction-monitoring experiments.

2.11 Sugars determination by HPLC/refractive index

Contents of glucose, fructose, and sucrose were determined using HPLC with a refractive index detector PU 4003 (Pye Unicam, Cambridge, UK) on a Polymer IEX Ca^{2+} form column (250 mm × 8 mm, 8-μm particle size, Watrex, Germany) at 90°C. Deionized water was used as a mobile phase with a flow rate of 0.5 mL/min. Sample volumes of

20 μL were injected. Each sample of 1.000 g was twice extracted with 10 mL deionized water. Sorbitol was used as an internal standard. The mixture was mixed by a vortex mixer for 2 min and centrifuged at 1910 × *g* for 10 min. Final extract was filtered through a 0.45-μm pore size nylon syringe filter before injection.

2.12 HMF measurement

HMF content was measured chromatographically as described by Rufián-Henares *et al.* [29] after three consecutive sample extractions in 0.1% formic acid.

2.13 Statistic

For the statistical purposes, analysis of variance (ANOVA, one-factor analyses) was used at a significance level 0.05. For all determinations, at least three replicates of the sample were analyzed.

3 Results and discussion

Simple model systems containing sugars and asparagine or more complex systems resembling a specific food formulation are often applied for the investigation of acrylamide formation in heated food products [19]. These models offer the advantages of reproducibility, simplicity, and feasibility to identify the key factors for acrylamide formation as well as to find ways for its elimination. The appropriate doses and conditions of enzyme application were initially determined in a simulated cereal matrix before their application in fried-dough pastry. This model comprised reducing sugars, L-asparagine, wheat starch, and water in a ratio simulating that of fried-dough pastry formulas. The content of free asparagine in flour depends on the variety of cereals and the extraction during milling. According to the literature, asparagine is the limiting factor in the formulation for acrylamide formation in cereal products [9]. In addition, differences between cultivars as well as agricultural aspects (fertilization and farming system) may play a role in this context. The concentration of free asparagine in wheat flour varies widely, *e.g.* 0.18–0.19 [14], 0.14–0.17 [30], and 0.15–0.4 g/kg [31]. In the simulated model matrix, the concentration of asparagine was set at 0.1 g/kg based on the average asparagine value calculated on the analysis of six wheat flours marketed in the Slovak Republic [32].

During heating of the cereal matrix on a thermoblock at 190°C, a maximum concentration of acrylamide was achieved in 15 min as shown in Fig. 1. These conditions were set for further experiments with the asparaginase application. Although the rate of acrylamide degradation after 15 min of heating was rapid for this type of food product, prolonged heating is unrealistic in classical fried-dough pastries due to overheating and subsequently consumer unacceptability of the product. In the model matrix, three different concentrations of asparaginase (100, 500, and 1000 U/kg dry matter) were tested, and resulted in 25, 90, and 96% decrease of acrylamide content, respectively (Fig. 2).

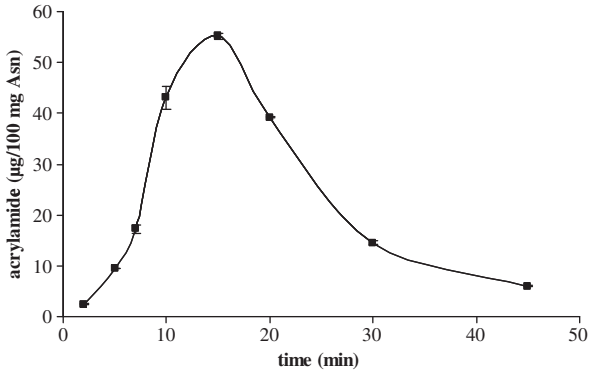

Figure 1. Formation of acrylamide during heating of a simulated cereal matrix at 190°C on a thermoblock.

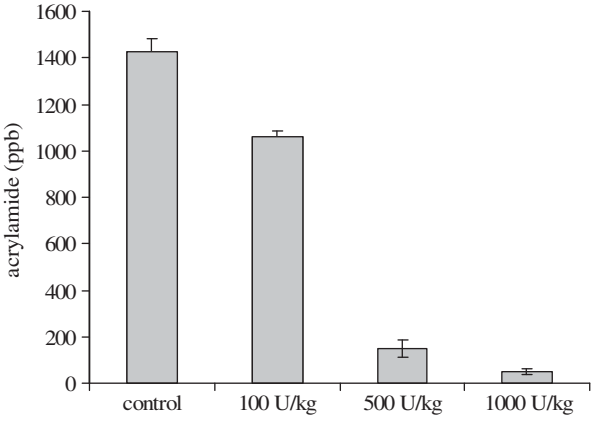

Figure 2. Acrylamide content (μg/kg) of a simulated cereal matrix after heating at 190°C for 15 min on thermoblock using three concentrations of L-asparaginase (100, 500, and 1000 U/kg dry matter; control sample: no enzyme addition).

Nevertheless, the situation in genuine food products is more complicated since the presence of factors such as pH, water activity values, and other components that affects the behavior of enzyme and the formation of acrylamide should be taken into consideration. These factors can stimulate or suppress the Maillard reaction process and consequently the final acrylamide content; therefore, the differences between the simulated cereal matrix and real samples can be expected.

For that reason, samples were prepared according to a simplified recipe of fried-dough pastry, based on traditional Spanish *rosquillas*, with and without addition of asparaginase. Samples were deep fried at 180 and 200°C for 4–8 min. These frying temperatures and frying times are usually applied to obtain a crispy product that is still moist inside without an excessive oil intake. Generally, color and crust formation are

Table 1. Acrylamide content in *rosquillas* samples after L-asparaginase treatment of the dough

Temperature of frying (°C)	Time of frying (min)	Control (μg/kg)	E100 (μg/kg)	E500 (μg/kg)
180	4	49 ± 8	<LOQ	<LOQ
	6	83 ± 7	<LOQ	<LOQ
	8	129 ± 1	<LOQ	<LOQ
200	4	74 ± 13	<LOQ	<LOQ
	6	108 ± 14	<LOQ	<LOQ
	8	215 ± 13	<LOQ	<LOQ

Control, no enzyme; E100, 100 U enzyme/kg dry matter; E500, 500 U enzyme/kg dry matter; and LOQ (20 μg/kg).

used as end point markers of the process but there is no specific information in the literature that discusses this fact. As expected, acrylamide was formed during frying of the dough used as control (range 49 ± 8–215 ± 13 μg/kg; Table 1). Acrylamide content is strongly related to thermal input, and its final concentration is a result of concurrent formation and elimination (observed at temperature higher than 170°C). In this study, increasing of frying temperature by 20°C resulted in a 30–60% increase of acrylamide, and doubling the frying time led to a nearly three times higher acrylamide content. However, a dramatic impact on acrylamide was recorded when using enzyme (both E100 and E500) in the dough preparation. Acrylamide content in the final product was lower than the LOQ (20 μg/kg) for all temperature/time combinations applied. The excellent results of the application of enzyme (originated from *Aspergillus oryzae*) in concentration as low as 100 U/kg, resulting in >90% acrylamide decrease in real samples, indicate the enormous potential for economically improving the manufacturing of cereal products. In previous studies on the application of asparaginase in cereal products, a lower effect of asparaginase was achieved. Vass *et al.* [33] obtained a 70% acrylamide reduction in crackers, and Amrein *et al.* [15] reported only a 55% reduction in gingerbread using asparaginase. These findings were in part caused by an insufficient enzyme concentration (4 U/kg of dough in Amrein's study). The method of enzyme application is also crucial. The addition of enzyme in the aqueous solution used for dough preparation is more effective than mixing with dough as reported by Amrein [15] due to better homogeneity in the system.

The content of amino acids (asparagine, aspartic acid, glutamine, and glutamic acid) and sugars (glucose, fructose, and sucrose) was determined in the raw material (flour), dough (with and without enzyme addition after incubation), and final samples. The predominant amino acids in wheat grain and flour are glutamic acid (Glu), proline, glycine, leucine, phenylalanine, valine, arginine, serine, and also aspartic acid (Asp) [34]. Asparagine (Asn) is not only the major substrate for acrylamide, but also a trace contribution of glutamine (Gln) and Asp has been described by Sanders *et al.* [35]. Wheat flour used in the doughs contained 117.07 ± 2.62 mg/kg Asn, 152.55 ± 5.61 mg/kg Asp, 71.66 ± 0.46 mg/kg Gln, and 135.42 ± 2.74 mg/kg Glu. Table 2 summarizes the impact of the enzyme during maturation of dough on the profile of the determined amino acids, showing the drastic decrease of Asn. Asparaginase is able to convert up to 96% of Asn to Asp when used at a concentration of 100 U/kg flour and 97% with 500 U/kg flour, leading to final acrylamide elimination in both tested concentrations. In parallel, a 37% increment in Glu content in the dough was observed when the higher concentration of enzyme was used. Although there is no direct explanation for this effect, it may be that the substrate specificity of the L-asparaginase was more diversified and it is reasonable to expect the presence of glutaminase activity in the asparaginase or activity to other substrates [36–38]. Final content of amino acids in samples was lowered during deep frying at 180 and 200°C as shown in Fig. 3.

As expected, the sugar content decreased during deep frying, and was slightly lower in enzymatically treated samples during the first stages of frying (data not shown). However, the addition of asparaginase did not affect the initial levels of sugars in the dough (Table 2) and consequently did not affect their reactivity during the frying process.

Besides the evaluation of the temperature of frying and the impact of asparaginase addition on final content of acrylamide, the moisture values and further markers of browning and color developing by determination of total FIC-F, ΔE value, A_{420}, and A_{360} were investigated.

The losses of moisture during frying at both temperatures (180 and 200°C) were similar for all systems; the moisture values decreased from 11.3% after 4 min to 3.2% after 8 min frying at 180°C, and from 10.5% after 4 min to 2.2% after 8 min frying at 200°C. Simultaneously, more significant differences were observed in color changes at different temperatures. ΔE value changed in control sample from 62.2 to 59.8 at 180°C and from 58.8 to 49.5 at 200°C, respectively. Consequently, enzyme had no obvious influence on total color change during frying according to CIELab expressed as ΔE value. Average ΔE values, calculated in enzymatic and non-enzymatic samples, declined from 64.1 to 59.7 using a temperature of 180°C and from 57.5 to 50.0 at 200°C.

HMF is a classical chemical marker of the extent of the Maillard reaction in thermally processed foods and particularly in cereal products [39]. The HMF contents after frying at 180°C for 8 min were 58.4 ± 0.6 mg/kg, 43.6 ± 2.0 mg/kg, and 68.4 ± 11.7 mg/kg for control, E100 and E500 samples,

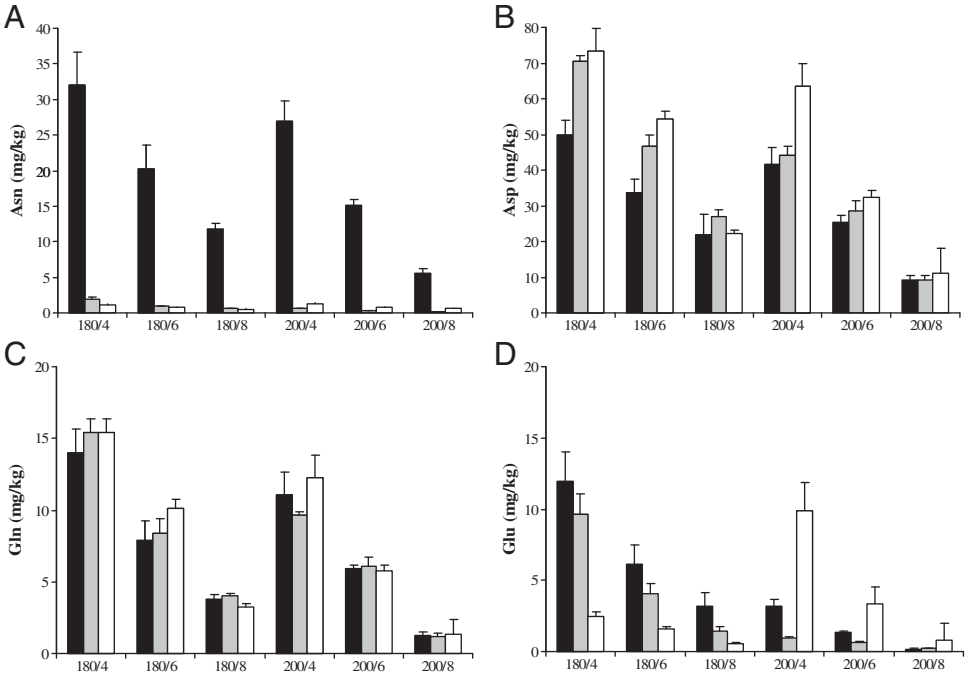

Figure 3. Amino acid contents (mg/kg) in fried-dough samples after heating under different conditions. (A) asparagine (Asn), (B) aspartic acid (Asp), (C) glutamine (Gln), and (D) glutamic acid (Glu). ■ Control sample (no enzyme addition); ■ E100 sample (asparaginase addition 100 U/kg dry matter); □ E500 sample (asparaginase addition 500 U/kg dry matter).

Table 2. Sugars and amino acids contents in flour and maturated dough.

	Sugars (g/kg) RSD (1.5–5.8%)			Amino acids (mg/kg) RSD (0.5–4.7%)			
	Suc	Glc	Fru	Asn	Asp	Gln	Glu
Wheat flour	18.1	1.6	0.5	117.07	152.55	71.66	135.42
Control Dough	14.9	83.7	88.1	15.85	127.78	156.50	23.08
E100	12.6	77.8	82.2	0.64	142.00	169.24	24.01
E500	12.1	81.9	87.4	0.41	167.33	158.22	31.62

Control, no enzyme; E100, 100 U enzyme/kg dry matter; E500, 500 U enzyme/kg dry matter. Minimum and maximum RSDs are given in parentheses.

respectively. Increasing of frying temperature by 20°C led to rapid HMF formation to 328.7 ± 1.3 mg/kg after 8 min of frying in the control samples and similar increases in enzymatically treated samples (Fig. 4).

FIC-F are also considered to be markers of the extent of the Maillard reaction as precursors of formation of end products. FIC-F increased with frying time and temperature, but were not significantly affected by enzyme addition (Fig. 5) except for the sample treated with E500 at 200°C for 4 min as compared with the control sample. Browning was also determined at 420 and 360 nm (Fig. 6), since this parameter is associated with specific Maillard reaction products in cereal foodstuffs [25]. In general, there were no significant differences between treated and untreated samples except for the sample treated with E500 at 200°C for 4 min and at 180°C for 8 min as compared with the control sample.

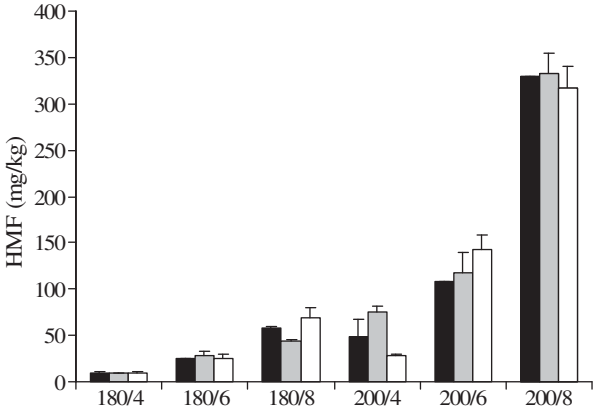

Figure 4. HMF (mg/kg) content in fried-dough samples after heating under different conditions. Labels are same as in Fig. 3.

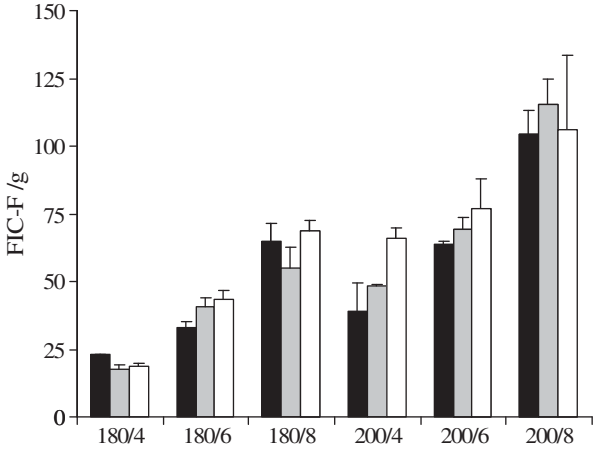

Figure 5. FIC-F (fluorescence units *per* gram of sample) in fried-dough samples after heating under different conditions. Labels are same as in Fig. 3.

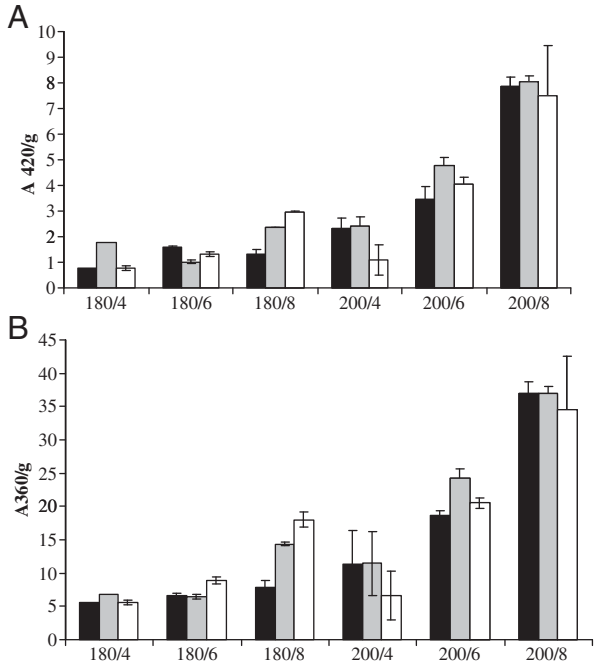

Figure 6. Browning measured as absorbance at 420 nm (A, absorbance units *per* gram of sample) and at 360 nm (B, absorbance units *per* gram of sample) in fried-dough samples after heating under different conditions. Labels are same as in Fig. 3.

4 Concluding remarks

The application of asparaginase during dough preparation for fried dough pastries reveals an effective strategy for acrylamide reduction. Mitigation is effective even at low enzyme concentration giving a cost-effective added value for its industrial application. Fried-dough pastries were prepared under realistic temperature/time conditions that are used to give products that find consumer acceptance. Maillard reaction is responsible for most of the organoleptic and textural properties in the fried-dough pastry appreciated by consumers. The investigation of several markers of the extent of Maillard reaction shows that this remains invariable, suggesting that significant modifications in the quality of the final product are not to be expected. Thus, using asparaginase, in *rosquillas*, as a representative of deep-fried dough products is a very effective method for acrylamide mitigation without any apparent negative impacts on final product quality.

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