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Modelling the effect of asparaginase in reducing acrylamide formation in biscuits

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Abstract

The influence of asparaginase on acrylamide formation as well as colour development in short dough biscuits was studied. In particular, asparaginase concentration, incubation time and temperature were changed according to an experimental design. As acrylamide formation resulted to vary significantly between biscuits obtained by using the same ingredients and process, a mixed effect model was used to model variation of acrylamide concentration. By contrast a fixed effect model was used for colour polynomial analysis. Within the range of study, the overall results allowed to find the best conditions to minimise acrylamide formation. It can be suggested that acrylamide development is minimum at intermediate asparaginase concentrations and lowest time and temperature of incubation. Asparaginase addition did not affect significantly the colour of the final product, although the quadratic term of the incubation temperature slightly did.

Keywords: Acrylamide, Asparaginase, Biscuits, Colour, Modelling.

1. Introduction

Acrylamide is a toxic and probably human carcinogen molecule (IARC, 1994) that can form in heated foods as a consequence of the reaction between asparagine and a carbonyl source via Maillard-type reactions (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Zyzak et al., 2003; Becalski, Lau, Lewis, & Seaman, 2003; Yaylayan & Stadler, 2005). Important acrylamide dietary sources are staple foods such as potato derivatives, cereal products and coffee (IRMM, 2005; FDA, 2006). Many potential routes have been identified to reduce acrylamide levels in foods. These are relevant to agronomical and technological strategies (CIAA, 2009). Among the latter are interventions based on precursor consumption, i.e. fermentation and asparaginase pre-treatments.

Asparaginase pre-treatment of raw potatoes and doughs has been claimed to reduce effectively acrylamide levels without altering the appearance and taste of the final product (Zyzak et al., 2003; Ciesarová, Kiss, & Boegl, 2006; Hendriksen, Stringer, Ernst, Held-Hansen, Schafermayer, & Corrigan, 2006; Ciesarová, Kukurová, Bednáriková, Marková, & Baxa, 2009; Kukurová, Morales, Bednáriková, & Ciesarová, 2009; Capuano et al, 2009; Ciesarová, Kukurová, & Benešová 2010). Also, the effectiveness of asparaginase in reducing acrylamide is testified by a number of patent applications concerning different processed foods, such as snack foods, chips, dough foods, etc. (Elder, Fulcher, & Leung, 2006; Elder, Fulcher, Kin-Hang Leung, & Topor, 2007; Corrigan, 2008; de Boer, 2008). As it is well known asparaginase catalyzes the hydrolysis of asparagine into aspartic acid and ammonia, thereby specifically removing a key precursor for acrylamide formation. The commercial enzyme based on cloning of *Aspergillus oryzae* has received the generally recognized as safe status from the US and has been given a favourable evaluation by the Joint FAO/WHO Expert Committee on

Food Additives (JECFA, 2007). It is now permitted for use in the United States, Australia, New Zealand, and Denmark. In Canada, where enzymes used in food applications may be considered food additives, amendments to the Regulations that will allow the use of asparaginase as a food additive has been proposed (Health Canada, 2009). In 2008, the Standing Committee on the Food Chain and Animal Health authorised the use of asparaginase for bakery product manufacturing in two EU Member States, as a processing aid, since the manufactures have stated that the enzyme is inactivated during heat processing such as baking (SANCO, 2008).

Studies have shown that asparaginase activity is affected by enzyme dose, reaction time, temperature and pH at which the reaction occurs (Hendriksen, Kornbrust, Østergaard, & Stringer, 2009). In particular, the *A. oryzae* asparaginase was shown to be most active in the neutral pH range and at temperatures up to 60 °C. Furthermore, the enzyme activity is influenced by the contact with the substrate. In fact, a limited mobility of substrate and enzyme would be responsible for incomplete hydrolysis of asparagine and only partial reduction in acrylamide formation. In this regard the food decompartimentalisation as well as the water content of the reaction environment could greatly affect asparaginase efficacy in reducing acrylamide formation. In fact, great reductions of acrylamide content could be achieved by using relatively low asparaginase concentrations in formulated foods (up to 1000 U/kg), such as bakery products and potato-based snacks, as a good enzyme distribution in the system can be reached. On the contrary, very high concentrations of asparaginase (>10000 U/L of pre-frying dipping solution) are necessary to obtain a significant reduction of acrylamide in fried potatoes (Pedreschi, Kaack, & Granby, 2008). However, also in this case, any technological operation which favours the substrate diffusion and its contact with the enzyme can lead to a greater reduction of acrylamide levels. This is the case of potato pieces treated with

asparaginase after blanching (Hendriksen et al., 2009). In fact, blanching reduces the integrity of the potato, weakening the cell wall and membrane thereby improving the substrate-enzyme contact. Besides, especially when asparaginase is added to food formulations, the water content should be sufficiently high for mobility of reactants and facilitate the contact between enzyme and substrate (Amrein, Schoenbaechler, Escher, & Amadò, 2004; Hendriksen et al., 2009). For this reason the enzyme resulted more effective when added in the aqueous phase of the dough preparation instead of in the mixture.

No fundamental modelling of the effect of asparaginase on this process is available. Due to the complexity of the relationship between the environmental variables involved in the enzymatic process and acrylamide formation, ii) the effective enzymatic activity and iii) the final acrylamide concentration achieved, a study of these relationship is needed to find the best conditions to minimise acrylamide formation.

Natural variability in baking processes is an influencing factor and acrylamide formation has been seen to vary significantly between items with similar ingredients and cooking procedures (Levine & Smith, 2005). Evenmore, Bråthen and Knutsen (2005) showed how variability played an important role in starch systems affecting the final value of acrylamide after a baking process. If this process is to be scaled-up this variability needs to be assessed so that tolerances may be established within the normal ranges of variability of an industrial process (Aguirre, Frias, Barry-Ryan, & Grogan, 2008).

The aim of this study was to investigate the influence of asparaginase in reducing acrylamide formation in short dough biscuits. The effect of asparaginase addition on browning development was also investigated. In particular, asparaginase concentration,

incubation time and temperature were modulated according to a three variable, three level central composite design.

2. Materials and methods

2.1. Sample preparation

Short dough biscuits were prepared according to the slightly modified formulation by Gallagher, Kenny and Arendt (2005). The formulation consisted of flour, margarine (Unigrà, Italy), sucrose (Carlo Erba, Milano Italy), water, glucose (Carlo Erba, Milano Italy), salt (Carlo Erba, Milano Italy), asparagine (Sigma-Aldrich, Italy) and baking powder (sodium hydrogen carbonate, disodium diphosphate, dried starch) (Cameo, Italy). The non-flour ingredients were added to the recipe at 40, 35, 20, 5, 0.7, 0.1 and 0.5% flour weight, respectively. Different levels of asparaginase (Novozymes A/S, Denmark, 3500 U/g) in the range from 100 U/kg of flour to 900 U/kg of flour were added to this recipe according to a three-factor, three-level face centered cube central composite design (CCD) (Table 1). To assure a homogeneous distribution in the dough, asparaginase was dispersed in the aqueous phase before to be added to the dry ingredients. After mixing and a 30 min resting time at 4 °C, the dough was sheeted to 0.3 cm thickness, cut to a diameter of 7 cm and left in a thermostatic cell at different incubation temperatures and times according to the CCD (Table 1). In particular, the incubation temperature ranged from 20 °C to 54 °C and the incubation time from 10 min to 30 min. According to literature data, the enzyme is active within these variable ranges (Hendriksen et al., 2009). In addition, the chosen temperature and reaction time may roughly correspond to the resting temperatures and time generally applied for the dough at industrial level. The samples were baked in an air-circulating oven (Salvis Thermocenter, Oakton, Vernon Hills, IL, USA) at 200 °C up to a final moisture of 2%.

Biscuits prepared without asparaginase addition were taken as a control. In order to assess the variability of this process, simulating an industrial situation, this process was repeated with 6 different batches of dough biscuits.

2.2. Analysis of acrylamide

Acrylamide determination was carried out according to the method of Anese, Bortolomeazzi, Manzocco, Manzano, Giusto and Nicoli (2009). Briefly, 1000 µL of an aqueous solution of 2,3,3[$^2H_3$] acrylamide (d_3-acrylamide) (0.20 µg/mL) (Isotec, Sigma-Aldrich, Italy) as internal standard and 15 mL of water Milli Q (Millipore, Italy) were added to 1 g of finely ground biscuit weighed into a 100 mL centrifuge tube. After extraction at 60 °C for 30 min under magnetic stirring, the mixture was centrifuged at 12000 x g for 15 min at 4 °C (Beckman, Avanti Centrifyge J-25, Palo Alto, CA, USA). Aliquots of 10 mL of the clarified aqueous extract were cleaned-up by solid phase extraction (SPE) on an Isolute Env+, 1 g (Biotage, Sweden). The volume of the eluted fraction was reduced under vacuum, to about 1.5 – 2 mL by using a rotary evaporator at a temperature of 80 °C and filtered through a 0.45 µm membrane filter before the HPLC-MS analysis. LC-ESI-MS-MS in positive ion mode analyses were performed by a Finnigan LXQ linear trap mass spectrometer (Thermo Electron Corporation, San Josè, CA, USA) coupled to a Finnigan Surveyor LC Pump Plus equipped with a thermostated autosampler and a thermostated column oven. The analytical column was a Waters Spherisorb ODS2 (250 x 2.0 mm, 5 µm). Elution was carried out at a flow-rate of 0.1 mL/min, in isocratic conditions, at 30 °C using as mobile phase a mixture of 98.9% water, 1% methanol and 0.1% formic acid (v/v/v). Full scan MS/MS was carried out by selecting the ions at *m/z* 72 and *m/z* 75 as precursor ions for acrylamide and d_3-acrylamide respectively. The area of the chromatographic peaks of the extracted ion at

175 *m/z* 55, due to the transition 72 > 55, and at *m/z* 58, due to the transition 75 > 58 were
176 used for the quantitative analysis. The quantitative analysis was carried out with the
177 method of the internal standard. The relative response factor of acrylamide with respect
178 to d_3-acrylamide was calculated daily by analyzing a standard solution. For each run,
179 analyses were made in duplicate on six replicated experiments. Acrylamide
180 concentration was expressed as ng/g of dry matter.
181
182 *2.3. Analysis of colour*
183 Colour analysis was carried out on sample surface using a tristimulus colorimeter
184 (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300
185 measuring head. The instrument was standardized against a white tile before
186 measurements. Colour was expressed in L* (lightness/darkness), a* (redness/greenness)
187 and b* (yellowness/blueness) scale parameters. The total colour change in the L*, a*, b*
188 colorimetric space, ΔE^*, was then calculated from Eq. (1) (Clydesdale, 1978):
189
190 $$\Delta E^* = (((L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* . b_0^*)^2)^{1/2}) \qquad \text{Eq. (1)}$$
191
192 where L^*, a^*, b^* are the actual colour values and L_0^*, a_0^* and b_0^* are the colour values
193 for a control sample, i.e. obtained without asparaginase addition.
194 For each run, analyses were made at least in triplicate on three replicated experiments.
195
196 *2.4. Determination of total solid content*
197 Total solid content was determined by gravimetric method by drying the samples in a
198 vacuum oven (1.32 kPa) at 75 °C until a constant weight.
199

2.5. Polynomial equations and statistical analysis

Modelling was aimed at describing the variation of acrylamide concentration and colour data as a function of the variables of the central composite design.

In the case of acrylamide analysis, coded variables were used to model this variation:

$$x = \frac{x - \bar{x}}{\Delta x / 2} \qquad \text{Eq. (2)}$$

where x is the explanatory variable normalised, $\bar{x}$ is the average of the variable and Δx is the range between the máximum and mínimum value of x.

The lme and lme4 libraries of the R software package (R Development Core Team, 2009) were used to fit a mixed effects model with the following components:

1. A fixed effect component containing a second order polynomial model to the dependant variables.
2. A random effect component that contains the effect of variability on the acrylamide formation.

This resulted in the following model:

$$y = B_0 + \sum B_i x_i + \sum B_{ii} x_i^2 + \sum B_{ij} x_i x_j + Zb + \varepsilon, \quad \varepsilon \sim \mathrm{N}(0, \sigma^2),\ b \sim \mathrm{N}(0, \sigma^2 \mathrm{E}) \qquad \text{Eq. (3)}$$

where B_0 is a constant and B_i, B_{ii}, B_{ij} are regression coefficients of the model and x_i and x_j are independent variables in coded values. The vector b represents the random effects, i.e. variations due to random nature associated to a Z model matrix with a relative variance-covariance matrix E which contains possible predictors influencing the

variability of the acrylamide concentration. N denotes the multivariate normal distribution (Pinheiro & Bates, 2000).

In the case of colour analysis, Statistica for Windows (Statsoft Inc., 1993) was used to fit the second order models to the dependant variables using the following equation:

$$y = B_0 + \sum B_i x_i + \sum B_{ij} x_i x_j + \sum B_{ii} x_i^2 \qquad \text{Eq. (4)}$$

The criteria for eliminating a variable from the full regression equation was based on R^2, standard error to estimate (SE) and significance F-test (and the derived p values).

3. Results and discussion

Table 2 shows acrylamide mean values and the corresponding standard deviations of short dough biscuits added with asparaginase according to the CCD. For each run of the CCD, the results of acrylamide concentration were based on two replicated analyses on six different batches of short dough biscuits, i.e. made using the same recipe and process. Although repetitions within each batch were good with standard deviations ranging from 0.1 to 10, results among batches of each run varied greatly. For instance, the acrylamide levels in asparaginase-treated made with 500 U/kg of flour and a 20 min incubation time at 20 °C (run 12) ranged from 49 to 120 ng/g_{dm}, with an average of 90 ng/g_{dm} and a coefficient of variation of 25%. Similar experimental variations were found by Hendriksen et al. (2009) in semisweet biscuits. The variation components arising from the analysis of acrylamide or from replicates of the same experimental conditions with the same batch of biscuit were not big enough to contribute to this 25% of variation and therefore it was concluded that the origin of this arised from the

247 preparation and processing of different batches of biscuit. In the light of a possible
248 variation between batches of biscuits been prepared, data were analysed by using a
249 mixed effect model (Pinheiro & Bates, 2000). The two components of the model were
250 a) fixed effects, that included all the effects of the variation of the dependent variables
251 (asparaginase dose, incubation temperature and time) which affect the acrylamide
252 concentration in a statistically significant manner; b) random effects, which include the
253 variation in final acrylamide concentrations that are due to changes among batches of
254 the biscuit dough.

255 Table 3 shows the result of the polynomial mixed effect regression. All non-significant
256 fixed terms were deleted form the model following an iterative process until the model
257 was satisfactory. As it can be seen in the table there are two quadratic effects, pointing
258 to two variables with possible minima-maxima. No significant correlation between
259 estimates was found, indicating that these parameter estimates and the standard errors
260 were estimated accurately. Fig. 1 diagnostic plots indicate that the residuals variance
261 was constant and that it followed approximately a normal distribution.

262 Fig. 2 shows the importance of the different regression variables in the acrylamide
263 formation. As it can be seen, within the margins of the present study, the variable that
264 has a bigger effect is the concentration of asparaginase introduced, followed by the
265 temperature effect. The incubation time of the asparaginase infused dough seems to be
266 the least important variable, being found mainly at the bottom of the Pareto chart. This
267 points to the result that within the present conditions, variations in temperature and/or
268 asparaginase will affect a bigger change in the final acrylamide concentration of the
269 biscuits. These results are in agreement with those reported by Hendriksen et al. (2009)
270 for semisweet biscuits incorporated with asparaginase. Models including random effects

depending on the temperature, asparaginase inoculation and incubation time were built, however none of them contributed to increase the quality of the fit, as the log-likelihood ratio test did not proof significant for any of the models ($p<0.05$). As a result, the batch-to-batch variation was modelled as a random intercept process. The importance of this random process can be seen in the magnitude of the standard deviation of it compared to any of the normalised coefficients in the model.

The batch-to-batch variability can be attributed to a non homogeneous distribution of the enzyme in the dough. This in turn can be ascribable either to the fact that very small quantities of the enzyme are incorporated to the other ingredients of the dough so that it cannot be uniformly distributed, or to a matrix effect. In the latter case, it can be suggested that the presence of fat contributes to create hydrophobic zones where the activity of the aqueous enzymatic suspension is limited.

The matrix effect could also explain the lower average percentage reductions of acrylamide levels due to asparaginase activity as compared to most of the data reported in the literature. In fact, we found that asparaginase contributed to reduce acrylamide concentration by 27 to 70% (Table 4), while the reductions reported in the literature are on average higher than 85-90% (Zyzak et al., 2003; Ciesarová et al., 2006; Kukurová et al., 2009; Capuano et al., 2009). These differences in asparaginase efficiency can be attributable to the different complexity from the compositional standpoint of the systems considered. In fact, the above mentioned papers deal with food model systems, i.e. made with starch, sugar and water, whereas, in the present study, biscuits obtained by using additional ingredients, such as fat and salt, were considered. It must be pointed

294 out that our results are in agreement with those of Hendriksen et al. (2009), who also
295 considered real food systems.

296 From Table 3 are two possible minima of acrylamide, in the asparaginase concentration
297 and with the incubation time. In Fig. 3 and 4 contour plots of the acrylamide in respect
298 of the experiment variables are presented. It can be seen that there is a minimum of
299 acrylamide formed at intermediate asparaginase concentrations (500 U/kg of flour) the
300 lowest incubation time and temperature.

301 It is interesting to note how high values of asparaginase seem to increase greatly the
302 final amount of acrylamide found and that excessive asparaginase inoculation followed
303 by high incubation times may actually increase the average acrylamide in the biscuits,
304 in comparison with low additions of asparaginase. However, within the range of the
305 study, if the final inoculums of asparaginase is well controlled and the incubation
306 temperature is kept to a minimum, it is reasonable to admit that there will be a decrease
307 of acrylamide concentration in the biscuit no matter what the incubation time will be.

308 Fig. 5 shows a stochastic simulation of the final levels of acrylamide under 500 U/kg of
309 asparaginase with 10 min of incubation time at 20 °C, which are the conditions
310 providing a minimum acrylamide concentration in the region of study. It can be seen
311 that even with the variation between batches of biscuits, the optimal conditions can
312 ensure that concentrations of acrylamide will not go over 138 ng/g_{dm} with a coverage of
313 95% of all batches. This is in contrast with the worse conditions observed (maximum
314 asparaginase added, maximum temperature of incubation at the shortest incubation
315 time) which produce an acrylamide 282 ng/g_{dm} with coverage of 95% of all the batches
316 of bread and samples.

Besides its feasibility and compatibility with the existing industrial process, acrylamide mitigation in biscuits by means of asparaginase addition has to take into account its effects on the sensory properties of foods. To this regard, the effect of asparaginase addition on the colour of the biscuits was studied. The results of the polynomial regression of total colour change data are shown in Table 5. In this case the mixed effect modelling was not included in the design because the colour data variability could be assigned to a single experimental error term. A random distribution of residuals was found. As it can be seen, the total colour change was significantly affected only by the quadratic term of the incubation temperature, while neither the asparaginase concentration nor the time influenced this parameter. This result is in agreement with observations made by other Authors who did not found any significant change in colour between asparaginase untreated and treated food systems (Capuano et al., 2009; Kukurová et al., 2009). It is likely that the temperature adopted during dough incubation may influence the development of non-enzymatic browning reactions: the higher the incubation temperature the higher the rate of the reaction that leads to the formation of brown molecules. It is worth to noting that, due to the low temperature estimate value, in the range of incubation temperatures compatible with the enzyme activity (20 to 60 °C), the total colour change can vary in a narrow range of values, i.e. between 2 and 8.

4 Conclusions

This study showed a strategy to assess the benefit of asparaginase in reducing the acrylamide formation in biscuits for managing the industrial process. The model used in this study allowed for the assessment of the effect of the natural variability associated to the baking process in final acrylamide concentration, besides the variation of the selected dependent variables. It can be concluded that, within the range of study, an

intermediate asparaginase concentration of 500 U/kg combined with the lowest time and temperature of incubation effectively reduced acrylamide formation in short dough biscuits without affecting the colour of the final product.

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Caption for figures

Fig. 1. Residual against fitted values (left) and residual quantiles against standard normal quantiles (right) plots of the polynomial mixed effects model.

Fig. 2. Effects plot for the fixed part of the polynomial model.

Fig. 3. Dependence of acrylamide concentration with asparaginase concentration and incubation time at the lowest incubation temperature.

Fig. 4. Contour of the dependence of acrylamide concentration with the normalised asparaginase concentration and incubation temperature at the lowest incubation time.

Fig. 5. Uncertainty assessment of the variability of acrylamide for the optimal conditions of intermediate asparaginase content, minimum incubation temperature and minimum incubation time.

Table 2

Acrylamide mean values and corresponding standard deviations of short dough biscuits added w
asparaginase according to the CCD

Run	Acrylamide concentration (ng/g_{dm})	Standard deviation
1	142.1	59.8
2	111.2	39.2
3	200.4	40.3
4	201.8	31.0
5	137.0	43.4
6	203.3	8.2
7	229.1	25.3
8	140.0	31.8
9	143.1	32.6
10	161.5	61.1
11	223.9	4.6
12	90.0	21.4
13	135.9	3.9
14	110.0	46.2
15	101.1	12.3

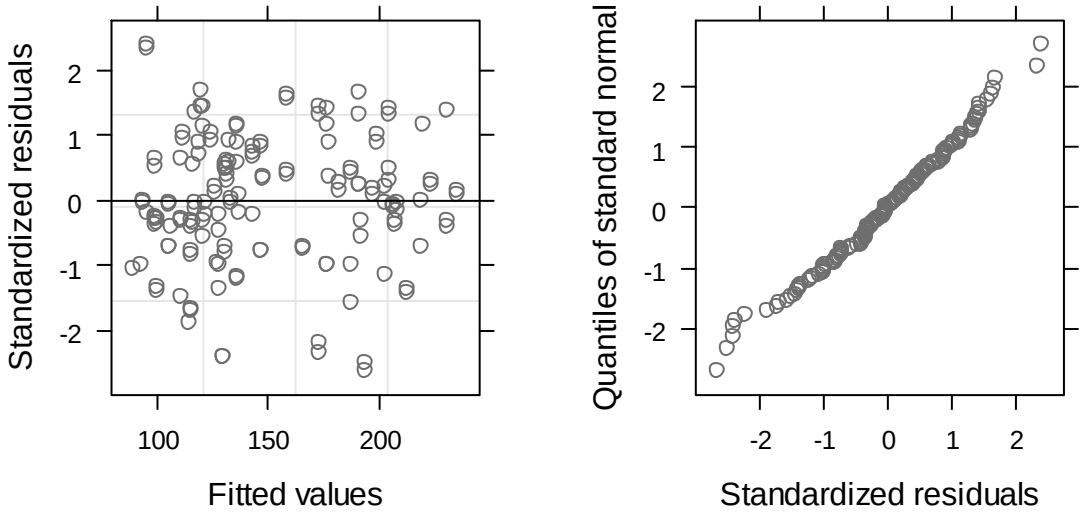

Fig. 6. Residual against fitted values (left) and residual quantiles against standard normal quantiles (right mixed effects model.

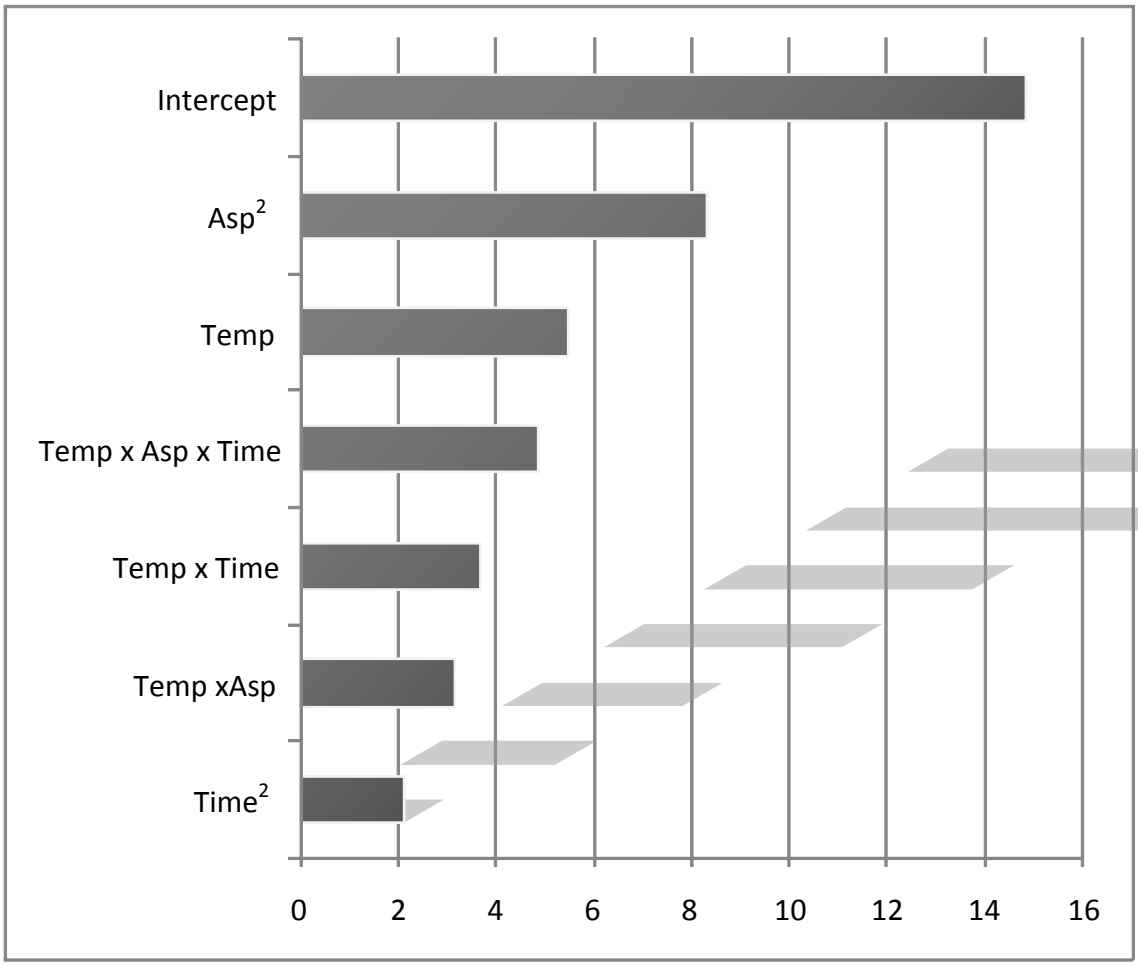

Fig. 7. Effects plot for the fixed part of the polynomial model.

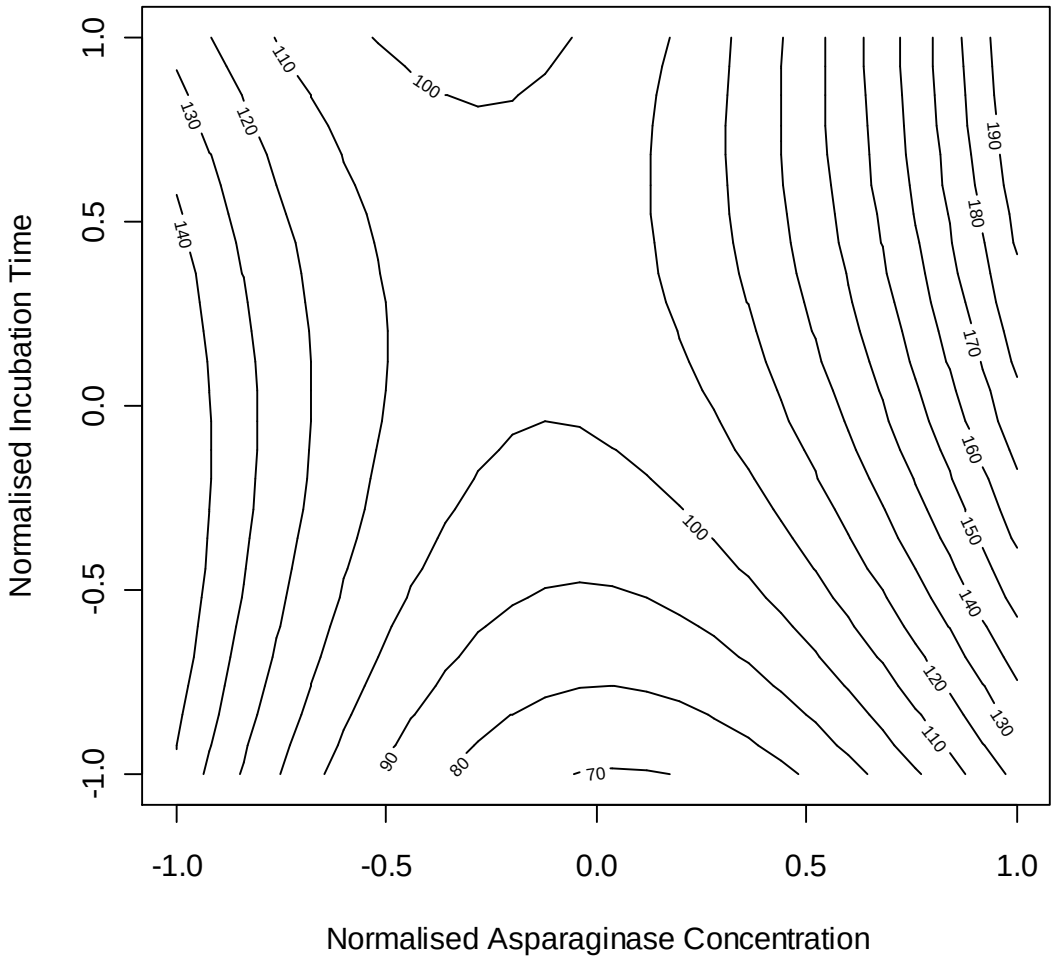

Fig. 8. Dependence of acrylamide concentration with asparaginase concentration and incubation time temperature.

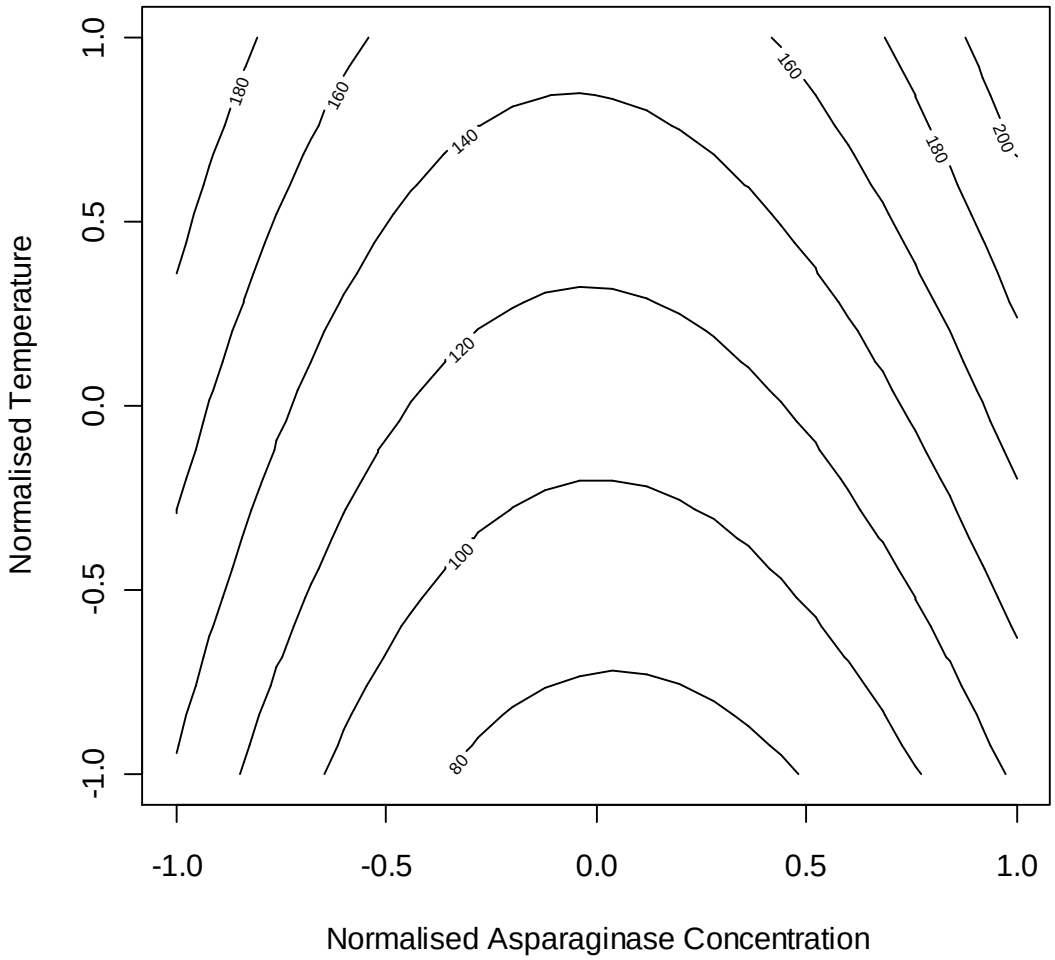

Fig. 9. Contour of the dependence of acrylamide concentration (ng/g_{dm}) with the normalised asparag
incubation temperature at the lowest incubation time.

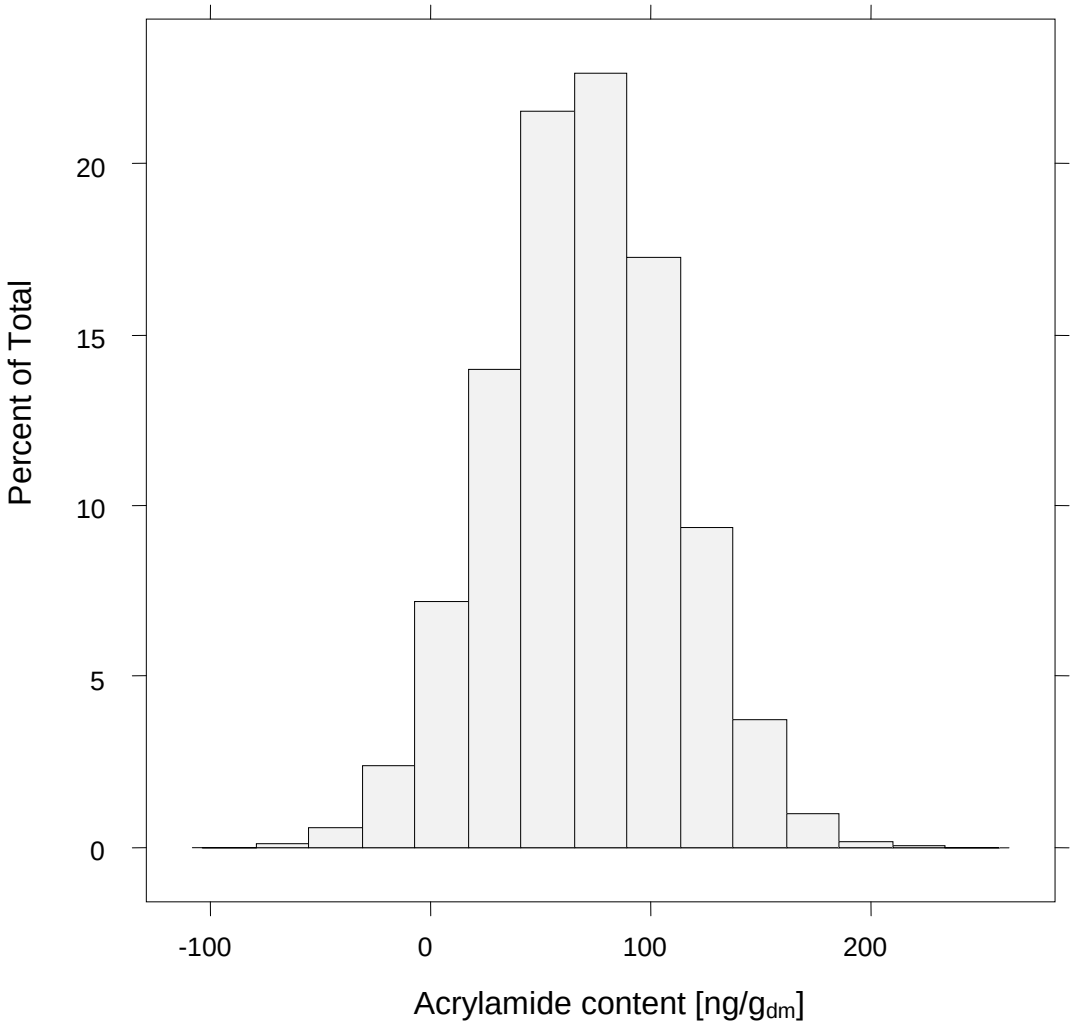

Fig. 10. Uncertainty assessment of the variability of acrylamide for the optimal conditions of intermedia minimum incubation temperature and minimum incubation time.

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