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RESEARCH ARTICLE

Effect of low-temperature long-time pre-treatment of wheat on acrylamide concentration in short dough biscuits

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In April 2002, unexpected high levels of the neurotoxic and suspected carcinogen acrylamide (AA) were found in many heated foods, mainly represented by cereal and potato derivatives. Since then, due to the great consumption of dietary sources of AA among people of different ages and in different countries, worldwide efforts have been carried out to reduce the formation of the toxic molecule in foods. In this paper, the effect of a low-temperature long-time pre-treatment of wheat grains on AA formation in biscuits was investigated. Wheat grains were subjected to heating at 100°C for 8 h and subsequently milled. The obtained flour was used to prepare biscuits that were compared for AA content, texture and color with control samples obtained by using flour from unheated wheat. The low-temperature long-time pre-treatment was responsible for a great decrease (up to 42%) in AA levels in the biscuits, without causing significant changes in the color and texture parameters. As the pre-treatment did not cause any change in sugar and asparagine concentrations, such a reduction in AA concentration can be attributed to a difference in the thermal effect generated in the biscuits obtained by using the unheated and pre-heated flours. In fact, as the heating pre-treatment caused a 2% moisture decrease in the flour, less time at the same temperature was required to obtain biscuits with comparable moisture contents.

Keywords:
Acrylamide mitigation / Biscuits / Heat pre-treatment / Thermal effect / Wheat

1 Introduction

As known, a wide range of cooked foods contains the suspected carcinogen acrylamide (AA) at levels between a few parts *per* billion (ppb) and in excess of 1000 ppb [1, 2]. This includes potato derivatives, roasted coffee and bakery products such as bread, crisp bread, biscuits, crackers and breakfast cereals. As the toxicological data suggest that AA might be carcinogenic for humans, efforts have been carried out to identify possible routes to reduce AA levels in foods and thus consumer exposure. With reference to the cereal products, the suggested technological interventions for AA mitigation are relevant to pre-treatments as well as formulation and/or process changes (http://www.ciaa.be) [3]. As asparagine rather than sugars is the key factor of AA formation in cereal products [4, 5], pre-treatments are aimed to reduce such a precursor. Among these is the use of lower gassing yeast or asparaginase [3]. The addition of lower gassing yeast results in a faster decomposition of asparagine and in a negligible increase in overall gas [6]. Asparaginase, whose production has been recently developed based on cloning of *Aspergillus oryzae*, is claimed to significantly reduce AA levels by converting asparagine into aspartic acid without altering the appearance or taste of the final product [7]. Replacing ammonium bicarbonate with alternative raising agents and/or avoiding the use of fructose represent successful formulation interventions to mitigate AA [8, 9]. With reference to

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Abbreviations: AA, acrylamide; **LTLT,** low-temperature long-time

the former, combinations of ammonium bicarbonate, sodium bicarbonate and acidulant are often required to obtain products with acceptable rheological and sensory properties [3]. Heating temperature and time, relative humidity during baking and modality of heat transfer are regarded to be important process factors influencing AA formation. It has been demonstrated that the adoption of proper combinations of time and temperature as well as the application of higher relative humidity during baking resulted to be effective strategies to keep low the AA levels in foods [10–14]. The use of deck ovens, in which heat is transferred by conduction and radiation mechanisms, instead of convection ovens, based on forced air circulation, resulted in being advantageous to reduce AA content in bakery products [13]. Also the combination of dielectric heating with conventional heating in the last stages of the baking process is responsible for a great inhibition of AA in cereal derivatives [15].

It is worth to note that each strategy may present limiting factors for their applicability depending on the type of product and industrial setting. Therefore, the most suitable intervention should be chosen taking into account the compatibility with the existing industrial process and formulation, the impact on food sensory and nutritional properties, the regulatory compliance and costs.

In this paper we studied the influence of a low-temperature long-time (LTLT) pre-treatment as a strategy to reduce AA concentration in short dough biscuits. In particular, whole-wheat grains were subjected to heating at 100°C for 8 h and then milled. The obtained flour was then used to prepare biscuits that were compared for AA content, texture and color with control samples obtained by using flour from unheated wheat.

2 Materials and methods

2.1 LTLT pre-treatment

Wheat (*Triticum aestivum* L.) grains with initial moisture of $9.9 \pm 0.1\%$ were purchased from a local mill and heated at 100°C for up to 8 h by using an air-circulating oven (Salvis Thermocenter, Oakton, Vernon Hills, IL, USA). The treated wheat was threshed by using a threshing machine (Vignoli Oleomeccanica, Trident, Italy) and subsequently milled (Tecator mod. Cyclotec 1093, Sweden) in order to obtain flour (hereafter indicated as heated flour) with particle size of about 500 μm. Analogously, unheated flour was obtained from the same wheat grains that were not subjected to the LTLT pre-treatment.

2.2 Sample preparation

Short dough biscuits were prepared according to the slightly modified formulation by Gallagher *et al.* [16], by using either the pre-heated or unheated flours. Besides flour, the formulation consisted of shortening (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, 0.05 and 0.5% flour weight, respectively. After mixing and a 30-min resting time at 4°C, the dough was sheeted to 0.3 cm thickness and cut to a diameter of 7 cm. Additional dough was prepared by using pre-heated flour having the same water content of the dough from unheated flour (control sample). The samples were baked in an air-circulating oven (Salvis Thermocenter) at 170°C up to a final moisture of 2%.

2.3 Determination of temperature and thermal effect

Temperature changes during the LTLT pre-treatment and baking were measured by a copper-constantan thermocouple probe (Ellab, Denmark). In the latter case, time-temperature changes were detected at the coldest sample point, which coincided with the geometrical centre of the sample.

The thermal effect F (min) was computed using the following equation [17]:

$$F = \int_0^t 10^{(T-T_r)/z} \cdot \mathrm{d}t$$

where T_r is the reference temperature, which was chosen equal to 100°C, T is the actual temperature of the treatment (°C), t is the time of the treatment and z represents the increase in temperature that causes a tenfold increase in the reaction rate, which was chosen equal to 32°C, which is the value usually adopted for chemical reactions.

2.4 Analysis of AA

AA determination was carried out following the method of Anese *et al.* [18]. Briefly, 1000 μL of an aqueous solution of 2,3,3[$^2H_3$] AA (d_3-AA) (0.20 μg/mL) (Isotec, Sigma-Aldrich) as internal standard and 15 mL of water Milli Q (Millipore, Italy) were added to 1 g of finely ground sample. The extraction was carried out at 60°C, for 30 min, under magnetic stirring. The mixture was then centrifuged at $12\,000 \times g$ for 15 min at 4°C (Beckman, Avanti Centrifuge J-25, Palo Alto, CA, USA). The clarified aqueous extract was cleaned up by SPE on an Isolute Env+, 1 g (Biotage, Sweden). The volume of the purified sample was reduced, under vacuum, to about 1.5–2 mL by using a rotary evaporator at a temperature of 70°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, 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 × 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). In these conditions the retention time of AA and d_3-AA was about 10 min. A time-programmed valve was used to discard the eluate from the column for the first 7.5 min in order to eliminate the compounds with retention times shorter than AA. At 12.5 min the column flow was again diverted and the mobile phase changed to 100% methanol in order to clean the column from eventually strong retained compounds. Full scan MS/MS was carried out by selecting the ions at *m/z* 72 and 75 as precursor ions for AA and d_3-AA, respectively. The area of the chromatographic peaks of the extracted ion at *m/z* 55, due to the transition 72 > 55, and at *m/z* 58, due to the transition 75 > 58, were used for the quantitative analysis. The quantitative analysis was carried out with the method of the internal standard. The relative response factor of AA with respect to d_3-AA was calculated daily by analyzing a standard solution.

2.5 Analysis of sugars

Sugars in flours were determined by HPLC according to the method of Langemeier and Rogers [19] with minor modifications. Aliquots of 10 g of flour were weighed in a 100 mL Erlenmeyer flask together with 40 mL of 60% ethanol and heated at 85°C for 20 min. After cooling, the mixture was filtered through a paper filter, the flask and the filter were rinsed with small portions of water and the filtrate was brought to a volume of 50 mL with distilled water in a volumetric flask. An aliquot of the extract was filtered through a 0.22-μm Millipore filters prior HPLC analysis. HPLC analysis was performed by using a Jasco 880-PUi liquid chromatograph (Jasco, Japan) equipped with a manual 20 μL loop injector and a Shimadzu RID-10A refractive index detector (Shimadzu USA Manufacturing, USA). Chromatographic separation was performed by using a 250 × 4.6 mm, 5 μm Alltima Amino column (Grace Division Discovery Sciences, Lokeren, Belgium) in isocratic condition. The mobile phase consisted of 70% acetonitrile and 30% water, with a flow of 1.2 mL/min. Standard solutions of glucose, fructose, sucrose and maltose were used for quantification. Sugar concentrations were expressed as milligrams of sugar *per* gram of dry matter.

2.6 Analysis of free asparagine

Asparagine was determined by an external laboratory (Neotron Spa, Modena, Italy) by using an in-house optimized HPLC method. In brief, aliquots of 1 g of sample were extracted with 30 mL of a mixture obtained by mixing 0.7 g of citric acid, 700 mL of ethanol, 10 mL of mercaptoethanol and water to a final volume of 1000 mL. The extraction was carried out by sonication for 5 min followed by stirring for 15 min at room temperature. The extracts were then cleaned up on a cation-exchange resin AG 50WX8 (BIO-RAD) column. The purified extracts were finally diluted with borate buffer (pH 10) and subjected to precolumn fluorescence derivatization using a 0.1% methanolic solution of ortophtalaldehyde at ambient temperature [20]. The quantitative analysis was performed by HPLC by using a Perkin Elmer series 200 LC pump equipped with a series 200 autosampler and a Perkin Elmer series 200 fluorescence detector. The excitation and emission wavelengths were 330 and 400 nm, respectively. The chromatographic separation was performed on a 5-μm Gemini C18 analytical column (250 mm × 3 mm id, Phenomenex). The eluent system used was a mixture of 0.1 M $NaHCO_3$, pH 7.5/acetonitrile (95:5) (solvent A), and acetonitrile (solvent B) with the following gradient program: 5 min isocratic, 5% B; 35 min, 20% B; 45 min, 30% B; 57 min, 48% B; 58 min, 60% B; 59 min, 80% B. The mobile phase flow rate was 0.65 mL/min. The (LOQ) was 0.005 g/100 g.

2.7 Color analysis

Color analysis was carried out on sample surface using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before measurements. Color was expressed in L^*, a^* and b^* scale parameters and a^* and b^* were used to compute the hue angle ($\tan^{-1} b^*/a^*$) [21].

2.8 Firmness measurement

An Instrom (Instron Series 4200, Instron, UK) equipped with a Kramer-shear cell was used to measure resistance to compression of at least seven biscuits, with 2% moisture content, sampled for each batch. Samples were placed parallel between two stainless plates and compressed at 100 mm/min. The cutting implement was allowed to travel the height of the biscuit, cutting through the sample, stopping 5 mm away from the plate.

2.9 Determination of water activity

Water activity was determined by means of a dew-point measuring instrument (AQUA LAB, Decagon, Pullman, WA, USA) at 25°C. Measurements were carried out on aliquots of the entire product previously ground.

2.10 Determination of total solid content

Total solid content was determined by gravimetric method by drying the samples under vacuum (1.32 kPa) to constant weight, according to Association of Official Agricultural Chemists [22]. With respect to the official method, drying was carried out at 75°C instead of 100°C to avoid losses due to non-enzymatic browning and pyrolysis reactions.

2.11 Statistical analysis

Analyses were carried out at least twice on two replicated experiments; therefore, each value is the average of at least four analyses. Coefficients of variation, expressed as the percentage ratio between the standard deviations and the mean values, were lower than 15 for AA, 5 for color values, 10 for water activity and firmness, and 6 for total solid content.

One-way analysis of variance was carried out and differences among means were assessed by using the Tukey test (STATISTICA for Windows, 5.1, Statsoft, Cary, NC, USA). Means were considered significantly different at $p<0.05$.

3 Results and discussion

The influence of the LTLT pre-treatment on water content and activity as well as on color of flour is presented in Table 1. Data are compared with those of the unheated flour. As expected, the LTLT pre-treatment caused a significant decrease of moisture and water activity. On the contrary, color parameters of the heated flour were not significantly different from those of the unheated one. Besides, the unheated and heated flours contained approximately the same amount of sucrose, *i.e.* $5.3\pm0.5\,mg/g_{dm}$ flour and $5.8\pm0.5\,mg/g_{dm}$ flour, respectively. Glucose, fructose, maltose and asparagine were not detectable in both unheated and heated flours, in our conditions of analysis.

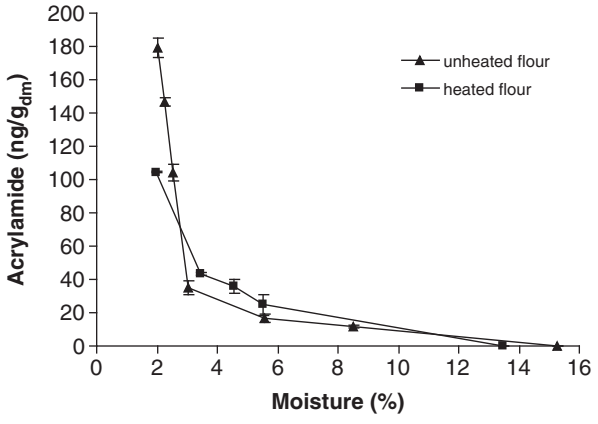

Figure 1. Changes in AA concentration of short dough biscuits prepared by using the heated and unheated flours as a function of moisture.

Figure 1 shows the changes in AA concentration of the short dough biscuits prepared by using the heated and unheated flours as a function of moisture. In both cases, AA concentration was fairly low (less than $50\,ng/g_{dm}$) until the moisture content of the biscuits fell below 3%, in agreement with literature reports [12, 23]. Below this value, changes in AA concentration differed according to the type of flour used. In fact, AA greatly increased in the biscuits obtained by using the unheated flour up to $180\,ng/g_{dm}$ at a moisture content of about 2%. By contrast, at the same moisture, AA formation in the biscuits prepared by using the heated flour was 42% less. As the LTLT pre-treatment did not cause appreciable changes in sugar and asparagine concentrations, together with the fact that sucrose, glucose and asparagine were added to both formulations in the same amount, these differences in AA formation can be traced back to the difference (about 2%) in initial moisture between the unheated and heated flours (Table 1). As the same amount of water was added to the formulations (20 g/100 g flour), also the moisture contents of the dough prepared from the heated and unheated flours differed approximately 2% (Table 2). As a consequence, the baking time needed to obtain biscuits with the same final moisture was different (Table 2). In particular, 7 and 9 min of baking were required to obtain 2% moisture biscuits from the heated and unheated flours, respectively. Such a difference in baking times corresponded to a difference in the thermal effect F (Table 2). As shown, at each moisture, the F values of the biscuits obtained from the heated flour were approximately 40–50% less than those of the biscuits prepared from the unheated flour. Therefore, it can be suggested that the less-intense baking process was responsible for a lower generation of AA. These results are confirmed by data on AA concentration of a control sample prepared by using heated flour added with the same amount

Table 1. Moisture, water activity and color of heated and unheated flours

	Moisture (%)	Water activity	Color L*	a*	b*
Unheated flour	$9.3\pm0.1^{a)}$	$0.32\pm0.02^{a)}$	$85.6\pm0.4^{a)}$	$1.9\pm0.13^{a)}$	$11.1\pm0.69^{a)}$
Heated flour	$7.6\pm0.1^{b)}$	$0.11\pm0.01^{b)}$	$84.2\pm2.1^{a)}$	$1.8\pm0.09^{a)}$	$10.5\pm0.30^{a)}$

(a, b) Different letters in the same column indicate significant difference ($p<0.05$) by Tukey test.

Table 2. Baking times at 170°C, moisture, and thermal effects of short dough biscuits prepared by using unheated and heated flours

	Baking time (min)	Moisture (%)	Thermal effect (F, min)
Unheated flour	0	15.3	0
	5	8.5	7.1
	6	5.6	11.9
	7	3.0	16.4
	7.5	2.5	19.3
	8	2.3	22.0
	9	2.0	26.8
Heated flour	0	13.5	0
	4	5.5	5.1
	5	4.5	7.0
	6	3.4	10.0
	7	2.0	13.7

of water as that of the biscuits obtained from the unheated flour. At a moisture level of 2% after baking, such a control sample had an AA level comparable (*i.e.* 175 ng/g_{dm}) with that of the biscuits from the unheated flour. As *per* our knowledge, no data are present in the literature on the influence of the thermal effect on AA formation. Nevertheless, several studies dealt with the impact of temperature-time regimes on AA formation [5, 10, 11, 13, 14]. In particular, these investigations outlined a significant interaction between baking time and temperature and showed that a proper choice of these process parameters (*i.e.* generating a moderate thermal input) could be an effective way for AA minimization during baking.

Figure 2 shows the changes in lightness and hue angle of the short dough biscuits obtained from the heated and unheated flours as a function of moisture. No significant differences ($p > 0.05$) in L^* and hue angle were found at each moisture between the biscuits obtained by using the heated and unheated flours. Also, no significant differences ($p > 0.05$) in firmness were found between the two types biscuits with a 2% moisture content, the forces recorded to fracture the biscuits obtained from the unheated and heated flours being 1893 ± 185 N and 1619 ± 105 N, respectively. This means that the LTLT pre-treatment allows to keep low AA levels in the biscuits without causing color and texture changes with respect to the sample prepared from the unheated flour.

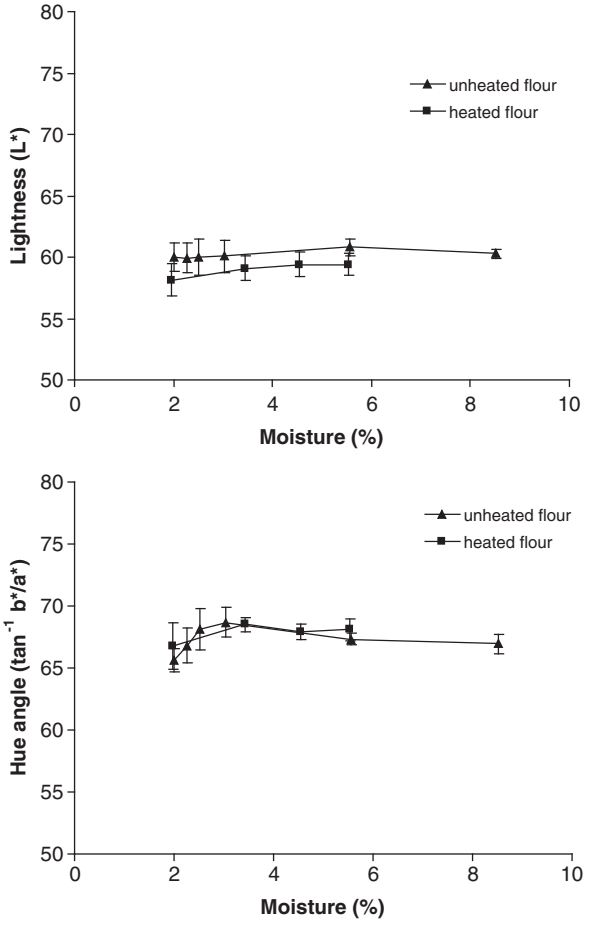

Figure 2. Lightness and hue angle values of short dough biscuits prepared by using the heated and unheated flours as a function of moisture.

4 Concluding remarks

From the toxicological point of view, bakery products together with potato derivatives are the most important sources of AA. These findings are particularly relevant for young people because the ratio between daily intake and body weight is less favorable for these consumer categories [24]. For example, crackers, biscuits and cakes account for about 19% of total AA in the diet of children and adolescents in The Netherlands, and for 17 and 36% in that of adolescents in Belgium and Germany, respectively [25].

The results reported here clearly show that the LTLT pre-treatment of wheat could be regarded as a reliable strategy to reduce AA formation in biscuits and likely in other cereal products. In fact, such a pre-treatment could be easily exploited at the industrial level. The pre-heated flour could represent a semi-manufactured product whose use does not require any change in formulation and process except a profitable reduction of baking times.

The authors have declared no conflict of interest.

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