Reactivity of acrylamide with coffee melanoidins in model systems

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Abstract

Coffee and its substitutes have been described as complex matrices for acrylamide (ACR) analysis due to both analytical interferences and ACR instability in the matrix. Melanoidins are multifunctional and biochemically active polymers which are formed in large extent during coffee roasting. Model systems composed of ACR (elimination studies) or glucose-asparagine (ACR formation/elimination studies) with/without melanoidins was heated at 180 _C. Washed sea sand and cellulose microcrystalline were used as matrix. Coffee melanoidins had a direct influence on the fate of ACR under heating, while the effect was not observed at room temperature. In addition, ACR decrease was also related to the reaction time and the initial amount of melanoidins in the media, where clearly a dose-response was observed. In contrast, pH (from 3.5 to 7.0) had no significant effect on ACR reactivity towards melanoidins. It is hypothesized that nucleophilic amino groups of amino acids from the proteinaceous backbone of melanoidins react via the Michael addition reaction with ACR, although the exact mechanism is unknown. Then, melanoidins could modulate the reaction pathways of ACR formation and elimination during coffee roasting and serve as acrylamide-mitigation substance.

1. Introduction

Melanoidins are brown anionic nitrogenous polymers formed during the final stage of the Maillard reaction (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). Melanoidins formation is a direct consequence of the thermal process applied to food, such as roasting, baking or toasting. Although their chemical structure remains largely unknown, there is increasing evidence that arabinogalactan-like carbohydrates, proteins, phenols and Maillard reaction compounds are constituents of coffee melanoidins (Bekedam, Schols, van Boekel, & Smit, 2007). There is a growing interest in coffee melanoidins, since they are not biologically inert food constituent and may exert various technological, nutritional, biological and health effects (Rufián-Henares & Morales, 2007; Somoza, 2005). In addition to their contribution to colour, texture (foam stability) and antioxidant capacity (Delgado-Andrade & Morales, 2005), melanoidins play an important role in the binding of nutritionally important metals (Morales, Fernandez-Fraguas, & Jiménez-Pérez, 2005), undesirable dietary compounds (Solyakov, Skog, & Jägerstad, 2002) and odourants (Hofmann & Schieberle, 2002). Moreover, their chelating properties towards metal ions further contribute to the antioxidant and antimicrobial properties of melanoidins in food (Rufián-Henares & De la Cueva, 2009). In summary, melanoidins reactivity is a relevant issue in food science from a technological (Petracco et al., 1999), safety (Jägerstad, Skog, & Solyakov, 2002), nutritional (O'Brien & Morrissey, 1997) and physiological (Somoza, 2005) points of view.

Acrylamide (ACR) is a processing contaminant with potential harmful consequences to humans (i.e. EFSA, 2010). ACR is naturally formed during roasting of green coffee beans, and it is found at levels of 200 mg/kg (maximum 958 mg/kg) and 188 mg/kg (maximum 1047 mg/kg) in roasted and instant coffee, respectively (EFSA, 2010). The contribution of coffee to the dietary daily intake of ACR is significant in countries with high coffee consumption, thus, level of 30% is reached in Scandinavian countries (Dybing et al., 2005). Since the presence of naturally formed ACR in foods was detected, different mitigation strategies have been

attempted (CIAA, 2009). Nowadays, there is not an effective strategy of ACR mitigation or alternative process applicable for coffee and its derivatives (EFSA, 2010). In addition, there is some controversy regarding the measurement of ACR in coffee since values decrease with storage time and temperature (Delatour, Périsset, Goldmann, Riediker, & Stadler, 2004; Hoenicke & Gatermann, 2005; Wenzl, Klaffke, Mothar, Palavinkas, & Anklam, 2005). In this context, Baum et al. (2008) confirmed that close to 90% of ACR remained firmly bound to the coffee matrix and it was hypothesized that ACR might be integrated with material eluting into the brew together with colouring material. In view of their multifunctional properties, anionic behavior and high abundance in coffee, melanoidins could have a direct implication in the ACR formation and elimination during coffee roasting. This investigation examines the role of coffee melanoidins in the formation and elimination of ACR.

2. Materials and methods

2.1. Chemicals

D-glucose, L-asparagine, acrylamide, and cellulose microcrystalline were purchased from SigmaeAldrich (Madrid, Spain). All other chemicals were purchased from Aldrich (Milwaukee, WI), or Merck (Darmstadt, Germany) and were of analytical grade. The water used was double-distilled (18.2 meqX/cm) using a Milli-Q System (Millipore Ibérica, Madrid, Spain).

2.2. Isolation of coffee melanoidins

Coffee melanoidins were prepared following the recommendation of the COST-919 group for coffee melanoidin analysis (COST-919, 1998) and as described elsewhere (Delgado-Andrade & Morales, 2005). Roasted coffee beans (Coffea arabica) were provided by a local factory; a moderate degree of roasting was applied, producing a weight loss of 16.2% (w/w) dry matter, in relation to green coffee weight. Ground coffee (100 g) was stirred in 300 mL of distilled water at 75 °C for 5 min. The solution was filtered and an aliquot of filtrate was de-fatted with dichloromethane (2 x 200 mL). The coffee brew was then subjected to ultrafiltration (Amicon ultrafiltration cell model 8400, Amicon, Beverly, MA, USA) with a 10 kDa nominal molecular mass cut-off membrane. The retentate corresponding to melanoidins was completed to 200 mL with water and washed again at least three times, after which the high molecular weight fraction was freeze dried and stored.

2.3. Preparation of model systems

2.3.1. Study of acrylamide elimination (static system)

Double-washed sea sand (30 mg _ 0.1 mg) was carefully placed at the bottom of a Pyrex test tube (100 x 15 mm). Then, 50 mL of melanoidin solution (20 mg/mL) and 50 mL of ACR solution (corresponding to 10000, 5000, 2000, 500 and 100 mg/L) were added. The melanoidin solution was replaced by buffer (0.2 M in phosphate buffer 0.1 M, pH 6.8) for control samples. In parallel, a model system with cellulose microcrystalline was designed as a polymer control instead of melanoidins.

2.3.2. Study of formation/elimination of acrylamide (dynamic system)

Double-washed sea sand (30 mg, _0.1 mg) was carefully placed at the bottom of a Pyrex test tube (100 x 15 mm). Then, 20 mL of asparagine solution (0.2Min phosphate buffer 0.1 M, pH 6.8), 20 mL of glucose solution (0.2 M in phosphate buffer 0.1 M, pH 6.8) and 50 mL of coffee melanoidin solution (20 mg/mL) were added. The melanoidin solution was replaced by buffer for control samples.

2.3.3. Effect of pH on acrylamide formation

Double-washed sea sand (30 mg, 0.1 mg) was carefully placed at the bottom of a Pyrex test tube (100 x 15 mm). Then, 20 mL of asparagine solution (0.2 M), 20 mL of glucose solution (0.2 M) and 50 mL of coffee melanoidin solution (20 mg/mL) were added. Both asparagine and glucose were dissolved in phosphate buffer 0.1 M pH 7.0, distilledwater, sodium citrate 0.1MpH 5.5 or sodium citrate 0.1 M pH 3.5 as necessary. Each melanoidin solution was replaced by the corresponding buffer for control samples.

2.3.4. Effect of staling on acrylamide elimination

Double-washed sea sand (30 mg, _ 0.1 mg) was carefully placed at the bottom of a Pyrex test tube (100 x 15 mm). Then, 50 mL of melanoidin solution (20 mg/mL) and 50 mL of ACR solution (500 mg/L) were added. The melanoidin solution was replaced by buffer for controls. After thermal treatment, the samples were rapidly reconstituted with 2 mL water, mixed and kept at room temperature. An aliquot (50 mL) was taken every 5 min for 60 min.

2.4. Heat treatment

The samples were heated in Pyrex test tubes (100 x 15 mm). Heat treatments were performed in a thermostated polyethylene glycol bath (GFL 1086, Großburgwede, Germany) equipped with an Omron E5J temperature controller (Omron Electronics, CA, USA) at 180 °C for 2, 4, 6 and 12 min. After thermal treatment, the samples were immediately cooled in ice water. Thermal treatments were carried out in parallel in open and hermetically closed screwcapped tubes to minimize the effect of evaporation.

2.5. Analysis of acrylamide

The samples were reconstituted with 2 mL water and 200 mL were then ultrafiltrated using Vivaspin 500 disposable units (Sartorius Stedim Biotech, Göttingen, Germany) in order to remove the melanoidins. Finally, 50 mL of the ultrafiltrated ACR solution was injected into an Accela 600 HPLC system (Thermo-Fisher Scientific, Palo Alto, USA) and analyzed in accordance with Knol et al. (2005) and Barber, Hunt, LoPachin, and Ehrich (2001) with minor modifications. Such method consists in the use of a reversed-phase column specially designed for separation of small molecules in highly aqueous solutions and a mobile phase composed of water:methanol (99:1) with a counter ion (heptane sulphonic acid) in order to elute compounds like acrylamide or its metabolite glycidamide. In our study, the reversed-phase columnwas replaced by a gel-permeation column (Discovery Bio GPC 150, 30 cm x 4.6 mm, 150Å, Supelco, Madrid, Spain) thermostated at 25 °C to avoid interference with the retained melanoidins. In addition, the mobile phase was water 100% delivered at 1 mL/min. ACRwas detected at 210 nm in a PDA detector equipped with a 5 cm flowcell, which also improve the detection limit compared with the former method. The method was linear between 50 and 5000 mg/L, presenting 3.1% precision and a detection limit of 20 mg/L.

2.6. Statistical analysis

Results are expressed as mean values _ standard deviation. All experiments were carried out in triplicate. Means were compared by one way analysis of variance (ANOVA) and Student's t-test at a significance level of P-values < 0.05. All analyses were carried out with Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA).

3. Results and discussion

ACR content in roasted coffee decreases during storage following a temperature-dependent model (Hoenicke & Gatermann, 2005; Lantz et al., 2006), and its variability during storage is probably due to ACR instability in the matrix (Wenzl et al., 2005). Recently, Baum et al. (2008) stated that ACR may Therefore, melanoidins are proposed in the present investigation as a realistic candidate to modulate ACR levels in

coffee. In a preliminary step, different proportions of ACR/melanoidins (from 0.008 to 0.403 mass ratio) were incubated at room temperature and 30 °C for up to 18 h in a semi-dry reaction media.

ACR content decreased slightly in the presence of the highest concentration of melanoidins, but differences were not statistically significant in any case (data not shown). Accordingly, it was expected that the reactivity of ACR towards melanoidins could be mediated by thermal treatment. A static system to evaluate the decrease was designed with standard solutions of ACR and heated at 180 °C in the presence or absence of water soluble coffee melanoidins. As expected, the ACR content in the controls (without melanoidins) progressively decreased with heating time. After 6 min of heating, initial levels of ACR decreased by 18.5% in control samples. Evaporation and/or polymerization are assumed to be the main causes of ACR decrease in the control as reported by others (i.e. Adams, Hamdani, Lancker, Méjri, & Kimpe, 2010; Claeys, de Vleeschouwer, & Hendrickx, 2005).

In addition, presence of degradation products was not observed in the chromatograms in any experiment. Therefore, the effect of melanoidins on ACR content was double checked for evaporation by comparing the respective controls without melanoidins in both open and closed systems. ACR content decrease was related to the reaction time and the initial amount of ACR in the reaction media. Fig. 1 shows the percentage of ACR decrease in the presence of coffee melanoidins in open vials; this value increased linearly up to 6 min of heating, after which therewas no further increase, probably due to excessive loss due to evaporation/polymerization. Decrease of ACR was more effective at the lower mass ratio ACR:melanoidin although similar trend was observed in all the experiments. Temperature in the vial was monitored during the heating every 30 s and the lag time was nearly 2 min (Fig. 1). Cellulose microcrystalline was used as an additional polymer control. Table 1 summarised the effect of presence or absence of cellulose in the reaction media on the ACR decrease. The amount of ACR remained without significant changes at the different levels of ACR tested.

The melanoidin:ACR ratio used in the model systems is in the range expected in roasted coffee. Literature describes that 100 g of classical roasted coffee contains about 7.2 g of melanoidins, being close to 1.8 mg/mL in filtered coffee (Fogliano & Morales, 2011). According to EFSA (2010), average content of acrylamide in roasted coffee is 200 mg/kg Alves, Soares, Casal, Fernandes, and Oliveira (2010) estimated that acrylamide content in serving coffee is 42.3 mg/L. Average melanoidin:ACR mass ratio in brewed coffee is expected to be between 9000:1 to 40000:1. For instance at the intermediate level of 2000 mg/L of ACR, the final melanoidin:ACR ratiowas 10000:1 (or 0.1 mg/g ACR:melanoidin), being in the range expected in brewed coffee.

In the following experiments, reference time of heating was set at 6 min. It was observed that the initial concentration of ACR influenced the rate of disappearance, being more efficient at the lower ACR/melanoidin mass ratio (Fig. 2). ACR decrease of 49% was recorded at the 0.005 ACR/melanoidin mass ratio (mg/g). This ratio was determined by weight rather than on a molar basis because the molecular weight of melanoidin is still unknown. These experiments confirmed that coffee melanoidins mediated the ACR decrease in model systems heated at 180 °C in a dose-response trend.

ACR and melanoidins are not naturally present in green coffee beans. Both are concomitant consequences of roasting but formed at different stages of the process. Hence their concentration might vary during roasting. ACR is rapidly formed from its precursors but its content is a balance between formation and evaporation or degradation reactions. ACR losses are enhanced at more severe heating conditions (Lantz et

al., 2006), but melanoidins are end products of the Maillard reaction. Then, it is expected that the ability of melanoidins to react with ACR will also change during thermal treatment.

Traditionally, researchers have used glucose-asparagine model systems to evaluate the kinetics of ACR formation/elimination under different conditions (Claeys et al., 2005; Knol et al., 2005). A dynamic system with ACR precursors (glucose þ asparagine) was designed, and formation and elimination of ACR took place simultaneously. In controls (without melanoidins), ACR was rapidly formed, reaching values of 339 +- 31, 5342 +- 48, 8264 +- 124, and 7426 +- 111 mg/L for open tubes and 546 +- 65, 8326 +- 121, 12364 +- 132, and 10985 +- 109 mg/L for closed tubes at 2, 4, 6, and 12 min, respectively. A lag-phase was always observed, during which ACR was not formed since reaction intermediates were generated first. Subsequently, ACR content increased exponentially with time to a maximum concentration, after which ACR again decreased. In the presence of melanoidins (1 mg), the formation of ACR decreased to 28% in closed systems and to 41% in open ones, after 6 min of heating. ACR formation did not only depend on its reactivity towards melanoidins but also on the effect of melanoidins on the chemical pathway leading ACR. Intermediate precursors of the reaction might also interact with the multifunctional residues of melanoidins, then lowering the reaction rate. In addition, glucose could be consumed by reacting with amino residues in the proteinaceous complex of the melanoidins, through the formation of Maillard reaction products. On the other hand, asparagine could react with arabinosyl residues of the arabinogalactaneprotein complex (Bekedam, Schols, van Boekel, & Smit, 2006). The network of the potential reactions involved is rather complex. Nevertheless, the contribution of these two events cannot be evaluated at this point.

Fig. 3 depicts the ACR decrease in the glucose/asparagine model system according to melanoidin concentration in the reaction media. A dose-response effect was again observed in the decrease of ACR, in line with the former results with a standard solution of ACR. The highest level of ACR decrease (55.6%) was in open systems at the highest concentration of melanoidins. Again, the reduction in ACR content mediated by melanoidins cannot be attributed solely to an addition effect; it would be caused by the participation of melanoidins in the chemical pathway of ACR formation. However, these mechanistic aspects are beyond the scope of the present study.

The precision of the method was investigated in the asparagineglucose model system. Four samples containing 20 mg/mL of melanoidins were heated to 180 °C for 6 min, extracted and analyzed. ACR content ranged from 4798 to 4932 mg/L. The RSD ranged from 2.0 to 3.8%, reflecting the good precision of the method.

Table 2 summarized the effect of pH on the decrease of ACR mediated by melanoidins. As expected, ACR formation is reduced at low pH and enhanced at higher pH levels (De Vleeschouwer, van der Plancken, van Loey, & Hendrickx, 2006). Increased proton concentration at lower pH will results in a higher amount of protonated amino groups of asparagine. Because the nucleophilic unprotonated amino group of asparagine is required in the first step of ACR formation through the Maillard reaction, the formation reaction will be blocked. Final pH levels did not change significantly after 6 min of heating. This finding is in line with those of previous studies (Knol et al., 2005). ACR decrease was relevant in the presence of melanoidins, compared with controls, at all the pH values assayed. ACR decrease in the presence of melanoidins was regardless to the pH levels.

To gain more insight into the stability of ACR in the presence of coffee melanoidins, an experiment was designed to resemble the staling of the coffee brew. After heating, water is rapidly added, kept open and

cooled down at ambient temperature for 1 h Fig. 4 depicted the ACR profile during the staling of the solution and the temperature decay. In closed vials, differences between initial and final ACR content were not statistically significant ($P < 0.05$). However, in samples heated in the open system, ACR decreased slightly during the first 20 min and no further differences were observed at more prolonged times. Likely in the closed system, the interaction between ACR and active residues of melanoidins reached a steady state more rapidly than with open vials. In the open system, ACR concentration also varied during staling likely due to evaporation.

4. Conclusions

ACR reacts with coffee melanoidins during heating at 180 °C of a low-moisture model system, and consequently a net decrease in ACR content is clearly observed. Then, melanoidins contribute to the instability of ACR in the matrix ACR is an electrophilic a,b unsaturated carbonyl and can react with nucleophilic groups such as amino-, hydroxyl- and sulfhydryl- groups via Michael addition reactions (Friedman, 2003). Coffee melanoidin is a negatively charged polymer constituted by carbohydrates, protein and polyphenols (Bekedam et al., 2007) and its polarity and number of reactive residues decrease with increasing thermal treatment (Morales, 2002). Then, nucleophilic amino groups arising from the proteinaceous residue of the melanoidin skeleton are probably involved in the elimination of ACR during coffee roasting. This hypothesis is supported by earlier studies, in which amino acids with nucleophilic side chains considerably decrease levels of the free ACR, due to Michael-type addition reactions producing the corresponding 3-(alkylamino)-propionamide (Adams et al., 2010; Koutsidis et al., 2009; Zamora, Delgado, & Hidalgo, 2010). In addition, it is known that the addition of free amino acids other than the ACR precursor asparagine, such as glycine, lysine and cysteine, can reduce ACR concentrations in thermally treated foods (Brathen, Kita, Knutsen, & Wicklund, 2005; Rydberg et al., 2003). But the exact mechanisms of ACR degradation and adduct-formation in food products remain unknown (Adams et al., 2010).

Our results are also in accordance with those observed for the interaction of coffee melanoidins with odour active compounds, such as thiols (Hofmann & Schieberle, 2002), harmful heterocyclic amines (Solyakov et al., 2002) and other low molecular weight compounds of technological interest such as saccharine (Chockchaisawasdee & Ames, 2001). In consequence, it is plausible to conclude the existing chemical interaction between coffee melanoidins and ACR but the binding site is only active under heating, and thus additional energy is needed to complete the reaction. Although it is known that asparagine is the limiting factor for ACR formation during coffee roasting (Bagdonaite, Derler, & Murkovic, 2008), our results show that the effect of soluble coffee melanoidins in modulating ACR content in the coffee brew should be considered. Further research is underway to identify the specific contribution and mechanisms of low and high molecular weight populations of melanoidins in reactivity towards ACR.

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FIGURES AN TABLES

Table 1
Percentage of acrylamide decrease in the closed reaction media (180 _C) heated for 2, 4, 6, and 12 min with or without presence of cellulose microcrystalline at different levels of acrylamide (10000, 5000, 2000, 500, 100).

Heating time	10000		5000		2000		500		100	
	Without	With	Without	With	Without	With	Without	With	Without	With
0	100.3	100.3	100.9	100.6	100.2	100.4	100.6	100.6	101.1	103.1
2	100.3	100.1	101.5	100.2	98.9	100.8	99.0	101.8	97.0	96.0
4	100.2	100.3	100.1	100.3	99.9	99.9	100.2	99.6	103.0	98.8
6	99.1	99.2	99.3	99.3	98.7	98.9	97.8	97.0	96.2	95.1
12	98.1	98.5	98.1	98.0	98.1	98.2	95.6	946	94.0	93.0

Table 2

Acrylamide formation in asparagine-glucose model (dynamic system) with or without the presence of melanoidins (20 mg/mL) and heated to 180 °C for 6 min at different pH levels.

pH	Without melanoidins		Melanoidins (20 mg/mL)	
	Closed system	Open system	Closed system	Open system
	Acrylamide μg/L	Acrylamide μg/L	Acrylamide μg/L	Acrylamide μg/L
3.5	9261 ± 108	6124 ± 78	7008 ± 103	3598 ± 89
5.5	10948 ± 106	7427 ± 92	8163 ± 109	4352 ± 103
6.8	12364 ± 132	8264 ± 124	8954 ± 110	4854 ± 94
7.0	12478 ± 98	8427 ± 106	9432 ± 101	5027 ± 111

FIGURE 1. Time course of acrylamide decrease during heating of an acrylamide/melanoidin model system at 180 °C. Acrylamide:melanoidin ratio (mg/g) of 0.5 (B), 0.25 (C), 0.1 (,), 0.025 (-), and 0.005 (A). Dashed line denoted temperature profile in vials.

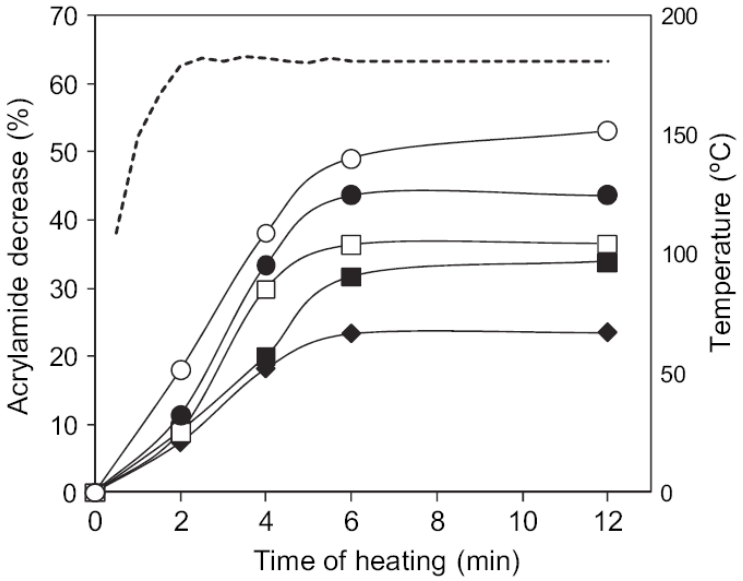

FIGURE. 2. Dose-response decrease of acrylamide in presence of melanoidins in a static system (acrylamide standard solution) heated to 180 °C for 6 min.

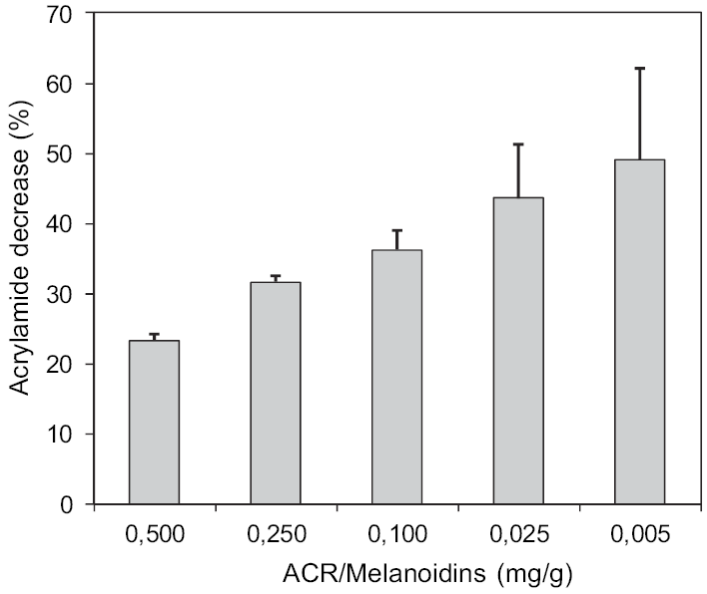

FIGURE 3. Decrease of acrylamide in presence of melanoidins in a dynamic system (glucose-asparagine model) heated to 180 °C for 6 min. Solid bar (closed vials), open bar (open vials).

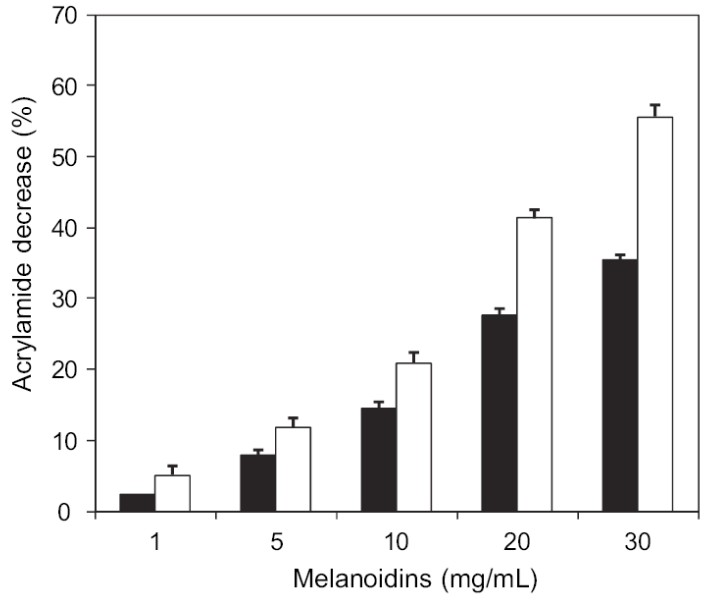

FIGURE. 4. Evolution of acrylamide content during staling at room temperature. Samples containing 500 mg/L and 10 mg melanoidins were previously heated in open (,) and closed (-) vials at 180 °C for 6 min. Bars indicate error in two independent experiments. Dotted line denotes temperature decay.