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A study on the formation and stability of ascorbigen in an aqueous system

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Ascorbigen, one of the most important indole derivatives of *Brassica* vegetables, is formed by an enzymatic degradation of indole glucosinolate glucobrassicin followed by a subsequent spontaneous reaction between indole-3-carbinol and L-ascorbic acid. Experiments, carried out in model solutions simulating *in vitro* conditions during vegetable processing, indicated the increasing rate of ascorbigen formation with decreasing pH of solution. Ascorbigen was shown to be very labile at higher temperatures and its enhanced stability in acidic medium was evident. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Epidemiological studies have clearly shown a negative association between the intake of *Brassica* vegetables and tumour incidence (Benito *et al.*, 1990; Chyou *et al.*, 1990). The anticarcinogenic effect of these vegetables may, to a large extent, be explained by the presence of naturally occurring indoles, as demonstrated by a number of studies with experimental animals (Bradlow *et al.*, 1991; Grubbs *et al.*, 1995).

Special attention is focused on indole-3-carbinol and ascorbigen, which are considered to be the most potent anticarcinogens. Precursors of these compounds are indole glucosinolates (glucobrassicins) found in over the family *Brassicaceae* (McDanell *et al.*, 1988). Major sources of these beneficial substances in human diet include cabbage, Brussels sprouts, cauliflower and broccoli, containing 100–1500 mg of glucobrassicins per kg of fresh weight (Fenwick *et al.*, 1989).

The formation of ascorbigen, an indole-containing derivative of L-ascorbic acid, proceeds in two consecutive steps. The first step involves enzymatic hydrolysis of glucobrassicin which is followed by a spontaneous reaction of the arisen intermediate (indole-3-carbinol) with L-ascorbic acid.

The breakdown of glucobrassicin (**1**, GB) (Scheme 1), induced by plant tissue disruption (i.e. by culinary procedures), is catalysed by the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). The enzymatic degradation of glucobrassicin is a rather fast process (McDanell *et al.*, 1987) and its rate depends only on the activity of myrosinase. Depending on the hydrolysis conditions, especially pH value (Virtanen, 1965) and presence of metal ions (Searle *et al.*, 1984), two indole products are formed. The major product in neutral medium is indole-3-carbinol (**3**, I3C) (Bradfield and Bjeldanes, 1987) generated via the unstable skatylisothiocyanate (**2**), whilst in acidic solutions 3-indolylacetonitrile (**4**, IAN) predominates (Latxague *et al.*, 1991). The former compound then reacts with L-ascorbic acid, if present, producing ascorbigen (**5**, ABG) (Gmelin and Virtanen, 1961; Kutáček *et al.*, 1969).

Indole-3-carbinol (**3**, I3C) is unstable in aqueous solutions (Leete, 1959) due to its tendency to form methylideneindolenine (3-methylidene-3*H*-indole cation, **6**) (Scheme 2). The reaction, generating this highly stabilised carbonium ion, is initiated by protonation of I3C which is followed by elimination of water (Sunberg, 1970).

At physiological pH, L-ascorbic acid (**7**) occurs largely in the form of C-3 anion (**8**) (Edgar, 1974). Positive conjugation effect is induced by the polarity of the C-3–O-3 bond and π-system C-2, C-3 and O-3 is reorganised causing the conversion of enolform to oxoform. Alkylation of L-ascorbate (**8**) by (**6**) proceeds at C-2 to produce a new carbon–carbon bond, unlike the C–O bond normally produced by the reaction of alkylation agents with other types of acid groups (e.g. carboxylic acids). Finally, the ultimate ring closure involving C-3 carbonyl group and C-6 hydroxyl group leads to the formation of two thermodynamically favoured 5-membered rings in E (envelope) conformation (Fodor *et al.*, 1983).

Both I3C and ABG are unstable compounds and can be transformed into a series of different products. A great number of experiments were recently conducted to

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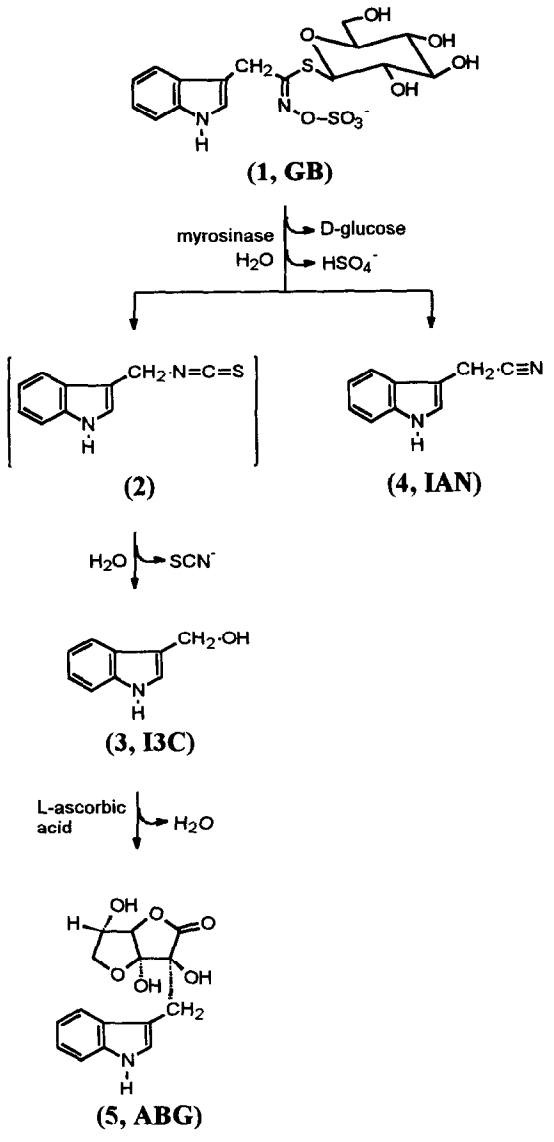


Scheme 1. Pathways of enzymatic breakdown of glucobrassicin. (**1**, GB) glucobrassicin; (**2**) skatylisothiocyanate; (**3**, I3C) indole-3-carbinol; (**4**, IAN) 3-indolylacetonitrile; (**5**, ABG) ascorbigen.

evaluate the transformation of I3C in an acidic environment and complex mixtures accounting up to 24 compounds were identified (Stresser *et al.*, 1994). The major compounds were 3,3′-diindolylmethane, 2-(3-indolylmethyl)-3,3′-diindolylmethane, 5,11-dihydroindolo [3,2-*b*]arbazole, to name a few (Bjeldanes *et al.*, 1991; de Kruif *et al.*, 1991; Grose and Bjeldanes, 1992). However, the conversion of I3C strongly depends on pH value (Amat-Guerri *et al.*, 1984).

The mechanism of ABG (**5**) degradation (Scheme 3) in acidic medium (and also in the stomach) involves the release of L-ascorbic acid and formation of methylideneindolenine (**6**). The 3-indolylmethyl moiety binds to another molecule of ABG (the addition of **6** on any other nucleophilic compound is possible as well) to form ascorbigen-dimer (**9**), ascorbigen-trimer (**10**) (Aleksandrova *et al.*, 1992) and 5,11-dihydroindolo[3,2-*b*]carbazole (**11**) (Preobrazhenskaya *et al.*, 1993). Recent investigation of ABG transformation in mild alkaline media (under physiological conditions) showed production of indole-derived carbohydrates 1-deoxy-1-(3-indolyl)-α-L-sorbopyranose (**12**) and 1-deoxy-1-(3-indolyl)-α-L-tagatopyranose (**13**) via opening of lactone ring and decarboxylation (Preobrazhenskaya *et al.*, 1996).

The qualitative and quantitative composition of products arising from GB is primarily influenced by reaction conditions during processing of *Brassica* vegetables. Detailed knowledge of factors affecting these processes is necessary since the formed products posses distinct physiological properties. The aim of this study was to investigate the kinetics of ABG formation and stability in media of different pH and under different temperatures.

EXPERIMENTAL

Chemicals

Indole-3-carbinol and L-ascorbic acid were purchased from Aldrich (Steinheim, Germany) and Farmakon (Olomouc, Czech Rep.), respectively. HPLC-grade acetonitrile and ammonium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany).

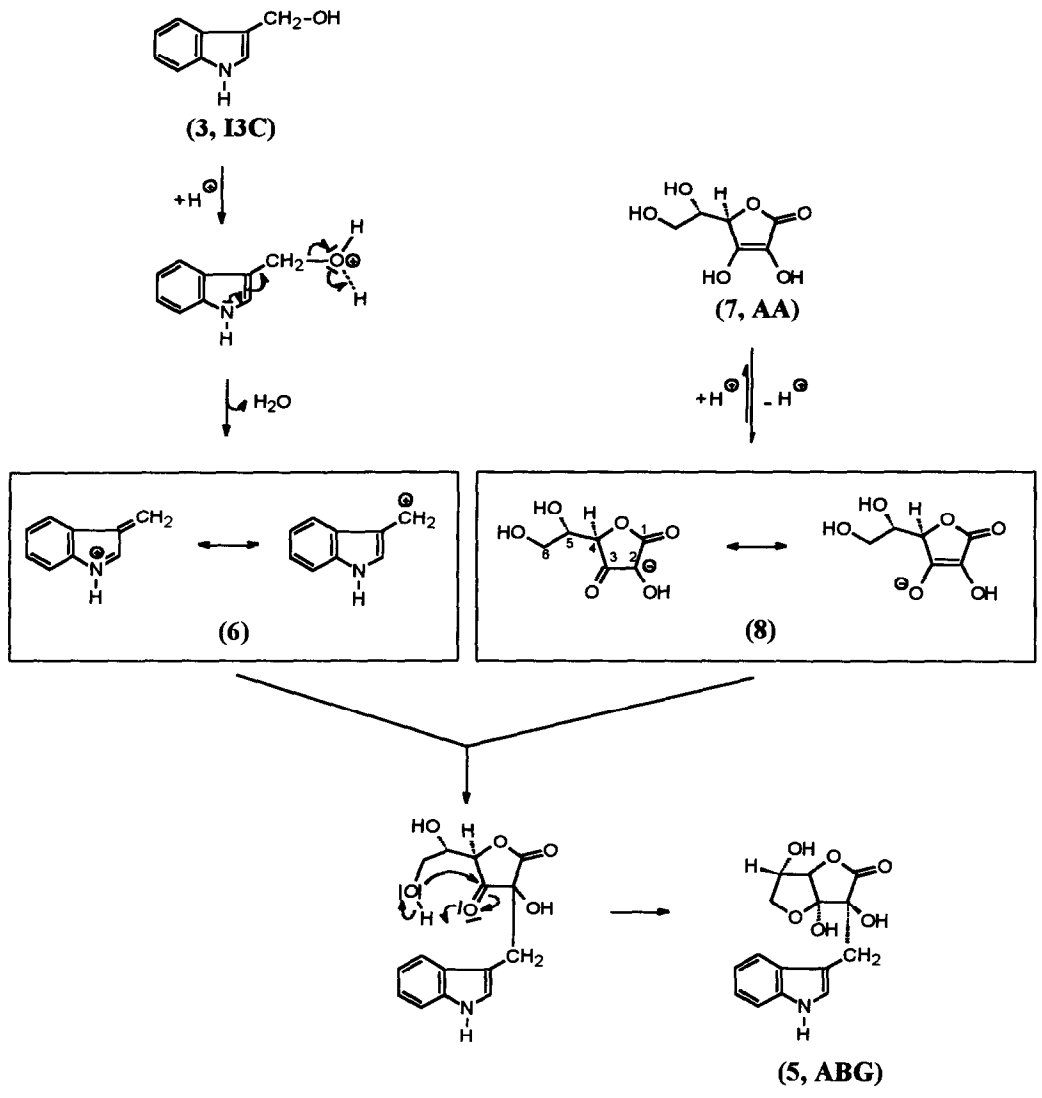
Methods and instruments

Method A

Analysis of indole compounds was carried out on a Constametric® 3200 (Thermo Separation Products, Riviera Beach, USA) high-performance liquid chromatograph. An aliquot amounting 20 μl was injected via an autosampler on Nova Pak® C18 reverse-phase column (250×4·6 mm I.D., particle size 4 μm, Waters, Milford, USA). An isocratic system consisting of 40% acetonitrile in water was used under a constant flow rate of 1 ml min^{-1}. Eluted compounds were detected at 280 nm by a variable-wavelength UV detector.

Method B

The instrument used for the determination of L-ascorbic acid was a SP8810 (Spectra-Physics, San Jose, USA) high-performance liquid chromatograph equipped with an UV detector (Hewlett–Packard, Palo Alto, USA) set at 254 nm and a Rheodyne 7125 injector (Rheodyne, Cotati, USA) with 10 μl injection loop. The separation was carried out on Nova Pak® C18 reverse-phase column (250×4·6 mm I.D., particle size 4 μm) connected with a pre-column Guard-Pak® Nova Pak® C18 (Waters, Milford, USA). The eluting solvent was aqueous 2% $NH_4H_2PO_4$ of pH 2.5 (adjusted by 4M H_3PO_4) at the constant flow rate of 0.5 ml min^{-1}.



Scheme 2. Supposed reaction mechanism of ascorbigen formation. (**3**, I3C) indole-3-carbinol; (**5**, ABG) ascorbigen; (**6**) methylideneindolenine (two extreme transition states); (**7**, AA) L-ascorbic acid; (**8**) L-ascorbate.

Software

For the calculation of rate constants Enzfitter V. 1.0 (R. J. Leatherbarrow, Cambridge, UK) was used.

Synthesis of ascorbigen

Ascorbigen was synthesised by the method of Kiss and Neukom (1966). Its identity and purity (>99.5%) was confirmed by means of NMR and HPLC, respectively.

Formation of ascorbigen

L-ascorbic acid (440 mg, 25 mmol) was dissolved in 1000 ml of a phosphate–citrate buffer (buffers of pH value ranging from 3 to 7 were used) and 100 ml of this stock solution was transferred into a 250 ml Erlenmeyer flask containing 36.8 mg (2.5 mmol) of indole-3-carbinol. The mixture was stored at 25°C under stirring. An aliquot of 0.6 ml was taken from the mixture, diluted with 0.4 ml of acetonitrile and used for HPLC determination of ascorbigen, indole-3-carbinol (method A) and L-ascorbic acid (method B).

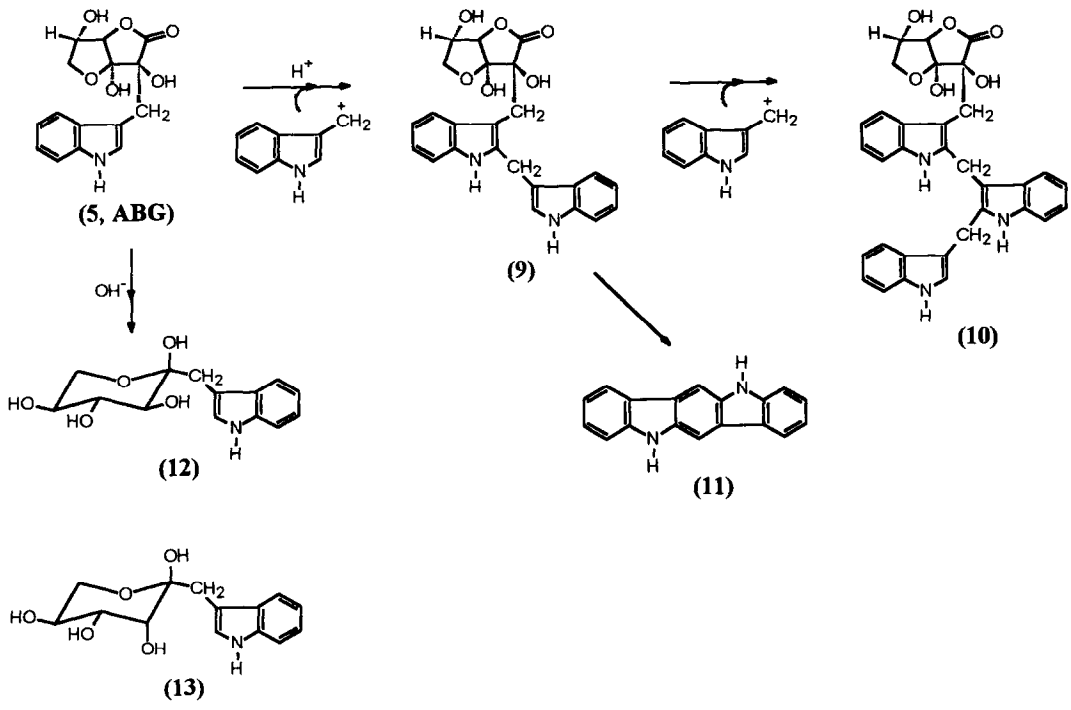
Degradation of ascorbigen

The synthesised ascorbigen (76.4 mg, 2.5 mmol) was dissolved in 100 ml of phosphate–citrate buffer (pH 3–7), heated at different temperatures (25–80°C) and analyzed as described above.

RESULTS AND DISCUSSION

Formation of ABG

Formation of ABG at 25°C was followed in aqueous buffers of different pH values consisting of I3C and AA in a molar ratio of 1:1. Natural pH of *Brassica* vegetables ranges from about 5.0 to 6.5. More acidic buffers



Scheme 3. Different mechanisms of ascorbigen degradation. (**5**, ABG) ascorbigen; (**9**) ascorbigen-dimer; (**10**) ascorbigen-trimer; (**11**) 5,11-dihydroindolo[3,2-*b*]carbazole; (**12**) 1-deoxy-1-(3-indolyl)-α-L-sorbopyranose; (**13**) 1-deoxy-1-(3-indolyl)-α-L-tagatopyranose.

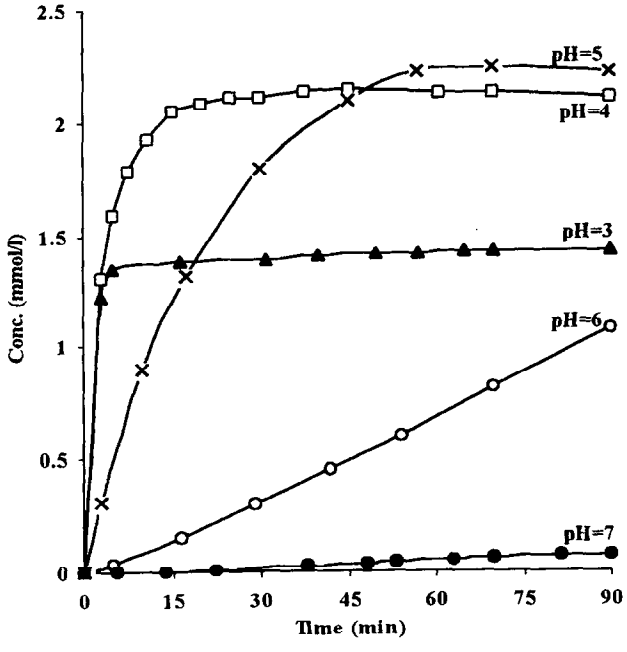
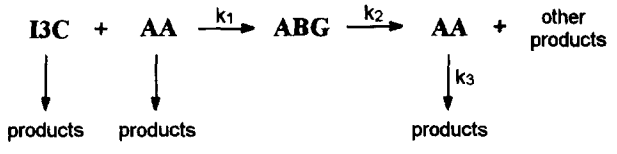


Fig. 1. Rate of ABG formation in solutions of different pH values.



Scheme 4. Investigated reaction scheme. (I3C) indole-3-carbinol; (AA) L-ascorbic acid; (ABG) ascorbigen.

were chosen in order to be able to observe ABG formation under conditions often encountered during culinary and industrial processing of vegetables.

The reaction rate of ABG formation was strongly dependent on the pH of the medium (Fig. 1). Maximum yields of ABG (54% of the theoretical amount) in solutions of pH 3 were achieved after 5 min, in pH 4 (82%) after 15 min and in pH 5 (89%) after 57 min. In solutions of higher pH value (pH 6 and 7) the reaction proceeded slowly and the maximum yield of the reaction product was not achieved even after 90 min.

It should be mentioned that both I3C and AA as well as ABG may also undergo the degradation *per se* (Scheme 4).

I3C is a labile compound and in addition to its reaction with AA, leading to the formation of ABG, it enters side reactions such as the formation of polyindolylmethanes and ABG polymerization. The latter reaction was observed especially in acid solutions of pH 3 and 4. No side reactions of AA proceeded in acid solutions so that its concentration remained practically constant after reaching the maximum concentration of ABG. However, side reactions (e.g. autoxidation) of AA were significant in slightly acidic and neutral solutions. The investigation of these side reactions was not the point of this study. Figure 2 shows the typical chromatographic trace involving all the observed indole compounds. The major product in the medium of pH 3 was ABG, followed by ABG-dimer (13% of ABG amount) and ABG-trimer (11%), similar chromatograms were

obtained in solutions of pH 4 (7 and 2%, respectively) and in solutions of pH 5 only ABG-dimer (3%) was detected. No ABG-polymers were detected in solutions of pH 6 and 7, respectively.

It can be concluded that maximum yield of ABG after 60 min of reaction is obtained in the pH interval 4.5–5 rather then at pH 4 as observed by Kiss and Neukom (1966). The yield of ABG is lower in more acidic media (< 4) due to the increased formation of ABG-polymers and the rate of ABG synthesis is slower in more alkaline media (> 5) (Fig. 1).

The rate constants of the individual reactions given in Scheme 4 obeyed first order reaction kinetics. The calculated rate constants k_1 indicated that the reaction rate of ABG formation considerably increased with decreasing pH (Table 1).

Decomposition of ABG

Stability of the synthesized ABG was investigated in solutions of different pH values (3–7) and at temperatures ranging from 25 to 80°C. Both the pH value and temperature significantly influenced the stability of ABG, but the effect of temperature was considerably higher.

Thermal treatment, often used for vegetables processing, greatly accelerates the degradation of ABG (Fig. 3). It is very probable that cooking of vegetables at 100°C results in very fast decomposition of this compound.

ABG was relatively stable during the first 2 h in solutions of pH 3 to 6 stored at 25°C and only 3–5% of this compound were lost. ABG degradation was much faster in solutions of pH 7 where 25% of the original amount of ABG decomposed. The amount of ABG decomposed in solution of pH 3–6 after 10 h at 25°C was 12–20% (and 75% in solutions of pH 7). The highest stability of ABG was observed in solutions of pH 4, whilst in more alkaline media its stability dramatically decreased. This trend was evident at higher temperatures as well. For instance, after 20 min of heating at 80°C the following decrease in concentration of ABG was observed: 54, 39, 56, 95 and 100% in solutions of pH 3, 4, 5, 6 and 7, respectively. This conclusion seems to be in accordance with the findings of Šanda *et al.* (1962).

The degradation of ABG in acidic solutions (pH 3 and 4) was accompanied by simultaneous formation of ABG-dimer and ABG-trimer. Degradation of ABG in

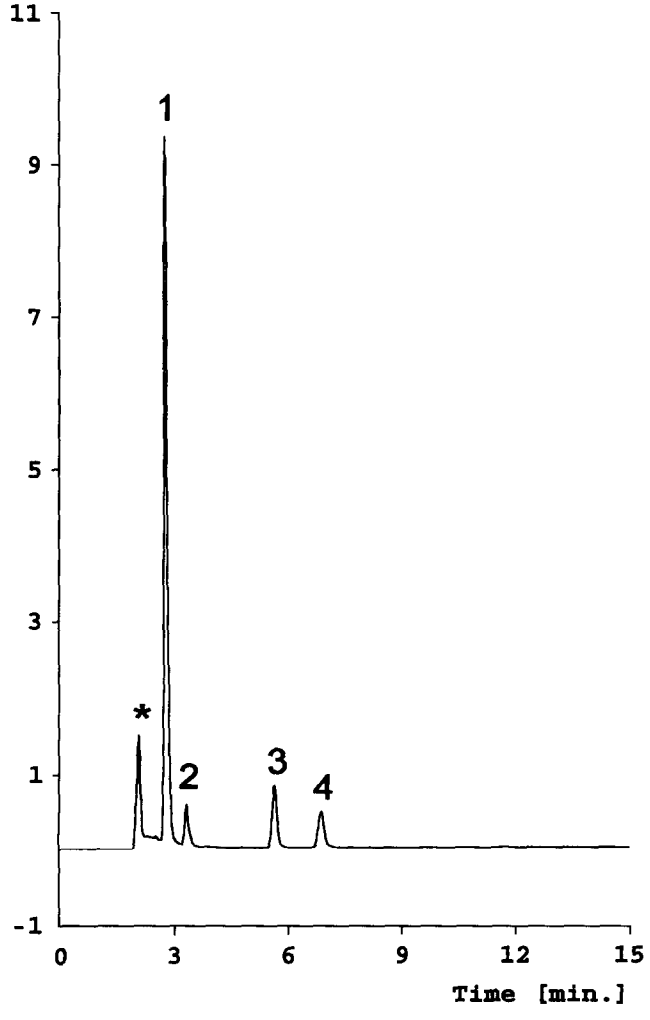


Fig. 2. HPLC chromatogram of AA and I3C reaction mixture of pH 3 after 30 min. For analysis conditions, see Experimental. Peaks: 1 = ABG, 2 = I3C, 3 = ABG-dimer, 4 = ABG-trimer; * = AA, buffer components.

Table 1. Effect of pH value on the rate constant k_1 of ABG formation

Temperature (°C)	Rate constant (k 10^6) in s^{-1}				
	pH 3	pH 4	pH 5	pH 6	pH 7
25	6740	2690	651	86.2	5.68

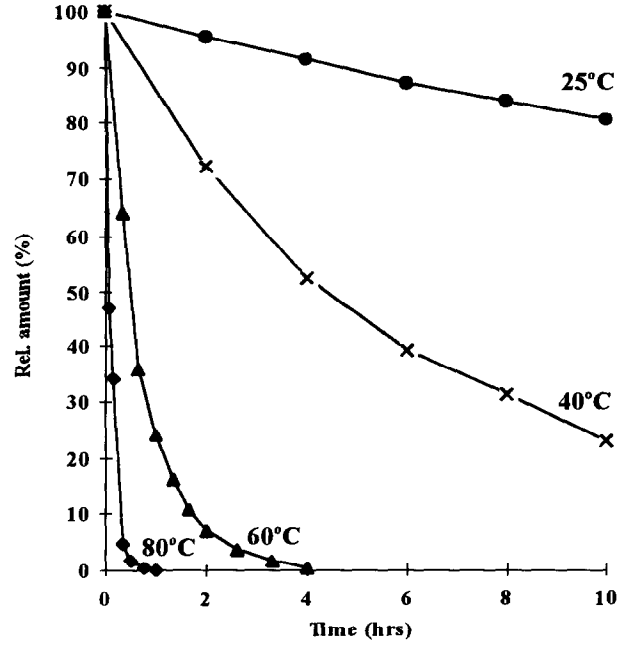


Fig. 3. Comparison of ABG degradation at different temperatures (in solutions of pH 6).

Table 2. Effect of pH value on the rate constant k_2 of ABG degradation

Temperature (°C)	Rate constant (k 10^6) in s^{-1}				
	pH 3	pH 4	pH 5	pH 6	pH 7
25	3.36	2.26	2.33	5.73	37.3
40	13.2	8.90	13.2	43.1	265
60	90.5	55.5	115	394	1950
80	482	285	624	2160	3450

Table 3. Effect of pH value on the rate constant k_3 of AA degradation

Temperature (°C)	Rate constant (k 10^6) in s^{-1}				
	pH 3	pH 4	pH 5	pH 6	pH 7
25	5.78	16.2	27.3	42.2	1630
40	12.4	28.5	78.3	153	8500
60	49.1	73.3	316	779	22000
80	140	244	875	3040	24000

solutions of higher pH value led to a series of different products, probably derived from ABG, AA and I3C. These products were not identified in this study. Some of them may show various biological activities, as they arise under physiological conditions.

The calculated rate constants of ABG degradation are given in Table 2. These data demonstrate the above presented results, i.e. faster degradation of ABG at higher temperatures and much smaller effect of pH on ABG degradation (Table 2). The amount of AA released from ABG in solutions of different pH correlated with the decrease of ABG concentration. The AA amount released in acidic solutions (at pH 3 and 4) remained practically constant, while the concentration of this compound decreased in less acidic solutions due to autoxidation. The rate constants of AA formation are presented in Table 3.

Based on the above presented results it seems that the rate of ABG formation in *Brassica* vegetables will differ according to the pH value of the plant tissue. ABG arises faster in vegetables having lower pH value and the already formed ABG remains relatively stable. On the other hand, higher quantities of IAN arise at the expense of I3C from GB with decreasing pH, even at pH 5 (Bradfield and Bjeldanes, 1987; Hrnčiřík and Velíšek, 1997). It may results in lower yields of ABG.

Higher amounts of ABG in *Brassica* vegetables based culinary products may be achieved by minimising their heat treatment (lower temperature and shorter time of the treatment).

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