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Thermally Induced Degradation of Aliphatic Glucosinolates: Identification of Intermediary Breakdown Products and Proposed Degradation Pathways

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ABSTRACT: In *Brassica* vegetables, heating processes lead to thermally induced degradation of glucosinolates (GSLs), resulting in the formation of nitriles and isothiocyanates (ITCs). To date, the mechanism is not yet satisfyingly elucidated. Thermally induced degradation of the model GSL sinigrin was studied in dry as well as aqueous medium at different pH values and temperatures. The influence of the presence of iron ions and plant matrix (broccoli sprouts powder) on the degradation was studied as well. Next to the degradation of the GSL, the formation of nitrile and ITC and the release of sugar derivatives were investigated. Because D-glucose and ITC are main thermal breakdown products under aqueous conditions, hydrolysis seems to be the initial step in the degradation pathway during cooking. In contrast, under dry conditions, the desulfo-sinigrin was identified as a main intermediary thermal breakdown product for the first time. Further, degradation of the desulfo-GSL results in the release of D-thioglucose and the corresponding nitrile. Iron(II) ions and plant matrix influence the thermal stability of the GSL and favor the formation of nitriles.

KEYWORDS: *Sinigrin, broccoli, processing, D-thioglucose, isothiocyanate, nitrile*

INTRODUCTION

Glucosinolates (GSLs) are secondary plant metabolites that occur in members of the order Brassicales including *Brassica* crops, such as cabbage, rape, or broccoli. The consumption of *Brassica* vegetables is thought to be beneficial for human health because of breakdown products of the GSLs. The latter being released enzymatically from GSLs by endogenous myrosinase after tissue disruption.[1,2] Dependent upon the structure of the GSLs and the reaction conditions [e.g., pH value, presence of iron(II), and specific proteins], nitriles, isothiocyanates (ITCs), thiocyanates, oxazolidine-2-thiones, and epithionitriles are formed.[3,4] Several beneficial health effects, such as antimicrobial, anti-inflammatory, or anticarcinogenic properties, are attributed to ITCs.[2,5−7] Especially, sulforaphane (4-methylsulfinylbutyl-ITC), deriving from the GSL glucoraphanin and present in high concentrations in broccoli, is thought to be a strong anticancer agent by inhibiting phase-I enzymes and inducing phase-II enzymes as well as apoptosis.[8−11]

In general, food processing severely influences the GSL profiles in vegetables and, therefore, the level of their corresponding health-promoting breakdown products. Freezing, chopping, and cooking can influence the GSL and breakdown product contents as a result of not only enzymatic breakdown, inactivation of enzymes, and leaching (into the cooking water) but also thermally induced degradation.[12−14] Thermal treatment of *Brassica* vegetables mainly affects indole GSLs, but aliphatic GSLs are degraded as well.[15−17] In previous studies, it was shown that nitriles were the main thermally induced degradation products of GSLs in *Brassica* vegetables and model systems.[18−21] At high temperatures, also the formation of ITCs has been observed when heating GSLs on a gas chromatography (GC) column.[22] Factors such as the pH value, presence of iron(II), and plant matrix (kind of vegetable and water content) affect the thermally induced degradation.[17−19,23−25] Iron(II) was shown to degrade aliphatic GSLs, such as sinigrin (Figure 1, structure **1**), even at room temperature, forming the corresponding 3-butenylnitrile (**2**) but not the allyl-ITC (**3**). Further, next to traces of D-glucose (**4**), the release of 1-β-D-thioglucose (**5**) was detected.[21,26,27] At 100 °C and 0.1 equiv of iron(II), the degradation was completed after 90 min.[28] To date, there is no information available about the reaction pathway(s) that the GSLs undergo when being degraded thermally and which sugar (derivatives) will be released. Such data might be of interest either to help preserve contents of (intact) GSLs when processing *Brassica* vegetables or to force a controlled formation of specific breakdown product patterns. Therefore, the aim of the present study was to identify factors influencing the thermally induced degradation of aliphatic GSLs (e.g., pH value, iron ions, and plant matrix) and to make a proposal for the mechanism(s) involved. Special regard was given to intermediates and sugars

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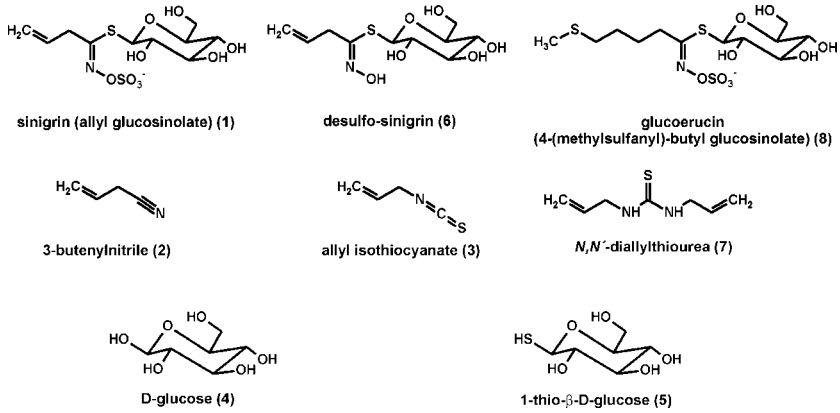

Figure 1. Chemical structures of the GSLs and their breakdown products as applied in this study.

formed. The main hypothesis was that the desulfo-GSL is a major intermediate in the thermally induced degradation of GSLs.

MATERIALS AND METHODS

Chemicals. Allyl-ITC (**3**) (≥99%), benzonitrile (99%), 3-butenyl nitrile (**2**) (98%), 1,3-diallyl-2-thiourea [*N*,*N*′-diallylthiourea (**7**)], and iron(III) chloride hexahydrate (≥99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Vitamin C (L-ascorbic acid) (≥99.5%) was purchased from Fluka (Sigma-Aldrich). D-(+)-Glucose (**4**) (≥99%) and iron(II) sulfate heptahydrate (≥99%) were purchased from Merck (Darmstadt, Germany). Arylsulfatase, isolated from *Helix pomatia*, was purchased from Roche-Diagnostics GmbH (Mannheim, Germany). 4-(Methylsulfanyl)butyl-GSL potassium salt [glucoerucin (**8**)] was purchased from PhytoLab GmbH and Co. KG (Vestenbergsgreuth, Germany). Sinigrin monohydrate (**1**) (ROTICHROM CHR) was purchased from Roth (Karlsruhe, Germany). Trifluoroacetic anhydride (≥99%) and 1-thio-β-D-glucose sodium salt hydrate (**5**) (≥99%) were purchased form Acros Organics (Thermo Fisher Scientific, Geel, Belgium). 4-Hydroxybenzyl GSL (sinalbin) was provided by the Leibniz-Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V. (Grossbeeren, Germany). 4-Hydroxybenzyl-GSL was extracted and purified from white mustard seeds (*Sinapis alba*) according to a modified method by Thies.[29] The purity was ≥99%, according to high-performance liquid chromatography with diode array detection (HPLC−DAD). All solvents were of HPLC grade. Water was of Milli-Q quality.

Material. Broccoli (*Brassica oleracea* var. *italica*) sprouts cv. Calabrese were provided by the Leibniz-Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V. Seeds were sown on water-soaked fleece in plastic trays filled with perlite. They were atomized daily until germination using a water sprayer. Water was given as needed for optimal sprout growth. Trays were kept in a greenhouse at about 24 °C during the day and 20 °C during the night, with a relative humidity of about 75%. Supplemental irradiation of 150 μmol m^{-2} s^{-1} was provided to give a 16 h photoperiod. They were harvested being 12 days old, lyophilized, and ground to a fine powder.

Thermal Treatment of Sinigrin. *Cooking of Sinigrin.* Sinigrin (**1**) dissolved in an aqueous buffer solution (4.8 μmol mL^{-1}), containing 50% water and 50% of the buffer solutions (pH 5.3, 8.878 g of KH_2PO_4 L^{-1} of water and 0.255 g of $Na_2HPO_4{\cdot}2H_2O$ L^{-1} of water; pH 8, 0.336 g of KH_2PO_4 L^{-1} of water and 11.431 g of $Na_2HPO_4{\cdot}2H_2O$ L^{-1} of water), was sealed in 10 mL glass ampules. Sealed samples were treated in a behrotest ET 2 thermoblock (Behr Labortechnik GmbH, Düsseldorf, Germany) at 100 and 130 °C (pH 5.3 and 8) for 0, 30, and 60 min. The influence of iron on GSL degradation was tested by adding 0, 0.004, or 0.04 μmol/mL iron(II) sulfate heptahydrate (simulating the soluble and total iron concentration when cooking 160 mg of broccoli sprouts in 4 mL of water, as reported previously[18]) or 0 and 0.04 μmol/mL iron(III) chloride hexahydrate to compound **1**. As the buffer solution, sodium acetate buffer was used (pH 5.3, 6.3 g of $C_2H_3NaO_2$ L^{-1} of water and 0.04 mol L^{-1} acetic acid). Additionally, the influence of vitamin C in combination with iron(II) was tested by adding 0.3 mg mL^{-1} vitamin C to 0, 0.004, or 0.04 μmol mL^{-1} iron(II) sulfate (the vitamin C concentration of the broccoli sprouts was simulated[30]). Sealed samples were treated at 100 °C and pH 5.3 for 0 and 30 min. To test the influence of the matrix on GSL degradation, 20 mg of freeze-dried broccoli sprouts powder was weighed in a crimp vial and preheated for 1 min to 100 °C. A total of 4.8 μmol of compound **1** and 1 mL of preheated (approximately 100 °C) water were added prior to sealing the vial. Because of the broccoli sprouts powder, the pH shifted to 5.3 and remained constant during the treatment period. Sealed broccoli sprouts powder−sinigrin (**1**) samples were treated at 100 °C for 10 and 45 min. Control samples were analyzed as well. All treatments were carried out in triplicate.

Dry Heat Treatment of Sinigrin. A total of 4.8 μmol of compound **1** was sealed in 10 mL glass ampules and treated at 100 and 130 °C for 10 and 45 min in the heating element. Unheated compound **1** served as the control. Prior to further analysis, 1 mL of water was added to the samples. To test for the influence of the matrix, 20 mg of freeze-dried broccoli sprouts powder was weighed in a glass ampule. A total of 4.8 μmol of compound **1** was added and mixed with the broccoli sprouts powder, and the vial was sealed. Broccoli sprouts powder−sinigrin samples were treated at 130 °C for 0, 10, and 45 min. All treatments were carried out in triplicate.

Heat Treatment of Glucoerucin. Glucoerucin (**8**) (1.6 μmol mL^{-1}) was cooked at 130 °C at a pH value of 5.3 for 60 min, analogous to compound **1**. Additionally, it was treated under dry conditions (0.25 μmol) at 130 °C for 45 min, as described above for compound **1**.

Preparation and Thermal Treatment of Desulfo-GSLs. To test whether desulfo-GSLs are intermediates during the thermal degradation of GSLs, a pure desulfo-GSL sample was prepared. HPLC samples from a previous study (stored at −30 °C), containing desulfated GSLs from broccoli sprouts (extracted and desulfated, as reported previously[18]), were homogenized. To remove the arylsulfatase, to exclude an influence of this protein, samples were filtered through centricon 10 microconcentrators [10 000 molecular weight cut-off (MWCO); Amicon GmbH, Witten, Germany]. The activity of the enzyme was tested to evaluate the successful removal of the enzyme. Therefore, 70 μL of compound **1** (3.2 mM) was added to 700 μL of unfiltered or filtered sample, and the formation of desulfo-sinigrin (**6**) was followed over a period of 7 h with HPLC−DAD, described in the Quantitation of Intact GSLs as Desulfo-GSLs by HPLC−DAD section. Additionally, the Bradford protein assay was performed to prove the absence of proteins in the desulfo-GSL preparation.[31] The pH of the desulfo-GSL solution was adjusted to pH 5.3 and 7.0 with a solution containing 11.87 g of $Na_2HPO_4{\cdot}2H_2O$ L^{-1} of water. A total of 1 mL of the solutions was treated in a sealed GC vial at 130 °C for 0 and 30 min in an oven (Typ T6, Heraeus Instruments, Hanau, Germany). All treatments were carried out in triplicate.

Analysis of GSLs and Breakdown Products. *Quantitation of Intact GSLs as Desulfo-GSLs by HPLC−DAD.* A total of 100 μL of treated compound **1** (200 μL for compound **8**) solution, 100 μL of a 1 mM stock solution of the internal standard 4-hydroxybenzyl GSL (sinalbin), and 2 mL of water were mixed and directly applied on a 150 μL DEAE−Sephadex A-25 ion-exchanger column, prepared, and washed according to the method by Mewis et al.[32] Next, 100 μL of a purified arylsulfatase solution were applied to the column and left for 12 h before the desulfo-GSLs were eluted with 1.5 mL of water. Samples containing broccoli sprouts powder were first extracted for 10 min in a methanol/water mixture (7:3, v/v; T = 75 °C). After centrifugation at 18620*g* for 5 min, the supernatant was restocked with water to 2 mL. A total of 200 μL of this extract was then applied on the Sephadex A-25 ion-exchanger column, and a total of 100 μL of 4-hydroxybenzyl-GSLs (1 mM) was added. The samples were desulfated, as described above in this section. Desulfated extracts were separated and analyzed by HPLC−DAD [HPLC pump LC-9A, degasser DGU-4A, gradient mixer FCV-10 AL, photodiode array ultraviolet−visible (UV−vis) detector SPD-M6A (all Shimadzu, Duisburg, Germany), and JASCO automatic sampler AS-950 (Gross-Umstadt, Germany)], as described previously.[16] Separation was carried out on a ProntoSIL Spheribond ODS2 column (3 μm, 125 × 4 mm) (BISCHOFF Chromatography, Leonberg, Germany) and a gradient of 0−20% acetonitrile in water from 2 to 34 min, followed by 20% acetonitrile in water until 40 min, and then 70% acetonitrile for 10 min until 50 min. The flow rate was 0.7 mL min^{-1}, and GSLs were detected at a wavelength of 229 nm. The GSL content was calculated using 4-hydroxybenzyl-GSL as the internal standard and the response factor (RF) of compound **6** in relation to desulfo-4-hydroxybenzyl-GSL. The RF was calculated using a response curve (linear regression, R^2 = 0.9997) (0.002−0.5 μmol of compound **1**/0.1 μmol of 4-hydroxybenzyl-GSL by mixing the standards, desulfating, and analyzing them, as described above). The RF of compound **1** was calculated to be 1.43, and the limit of detection (LOD) of the HPLC system was reached by injecting a solution of 0.43 nM desulfated compound **1**.

Analysis of Thermally Formed Desulfo-GSLs Using HPLC−DAD. A total of 900 μL of treated compound **1** (800 μL for compound **8**) samples or the treated desulfo-GSL samples were filled in a vial and directly (without desulfation) subjected to HPLC−DAD analysis, as described above, to test for the thermally induced formation of the desulfo-GSLs. Desulfo-sinigrin (**6**) and desulfo-glucoerucin were quantitated by external calibration. A total of 0.002−0.5 μmol of compound **1** (0.002−0.05 μmol of compound **8**) was desulfated and analyzed, as described in the Quantitation of Intact GSLs as Desulfo-GSLs by HPLC−DAD section ($R^2_{\text{ds-sinigrin}}$ = 0.9997, and $R^2_{\text{ds-glucoerucin}}$ = 0.998). Thermally induced degradation of the thermally treated desulfo-GSL samples was quantitated by comparing their peak areas prior to and after the thermal treatment.

Analysis of Volatile Breakdown Products Using Gas Chromatography with Flame Ionization Detection (GC−FID). For the determination of the thermally induced breakdown products of sinigrin (**1**) and the desulfo-GSLs, a modified GC−FID method reported previously was used.[18] To 1 mL of the treated compound **1**, compound **8**, or broccoli sprouts powder−sinigrin mixture or to 2 mL of the treated desulfo-GSLs, 2.5 mL of methylene chloride was added and transferred to a 10 mL glass tube and 100 μL of the internal standard benzonitrile (2 mM) was added. The tubes were sealed, and breakdown products were extracted and analyzed by GC−FID, as reported by Hanschen et al.[18] Briefly, a Hewlett-Packard 5890 A series II plus gas chromatograph (Böblingen, Germany) equipped with a BP-5 column (30 m × 0.25 mm × 0.25 μm film; SGE GmbH, Griesheim, Germany) and helium as a carrier gas (1.8 mL min^{-1}) were used for the determination of the volatile breakdown products. Splitless injection of 1 μL of the sample at 190 °C and a temperature gradient starting at 35 °C for 3 min and rising up to 230 °C at 9 °C min^{-1} were used for the separation of the compounds. The analyte content was calculated using benzonitrile as the internal standard and the RF of each compound relative to benzonitrile. The RFs were determined for 3-butenylnitrile (**2**) (RF = 1.88) and allyl-ITC (**3**) (RF = 2.38) using a dose−response curve prepared in methylene chloride (1−2 mM ITC or nitrile/2 mM benzonitrile; linear regression, R^2 = 0.999 for the nitrile and R^2 = 0.991 for the ITC; linear range, 0.1−3 mM for compounds **2** and **3**). The LOD of the GC−FID system was reached by injecting a benzonitrile solution of 3 μM.

Derivatization and Analysis of Sugar Derivatives Using GC−FID. A GC−FID approach was developed for the parallel determination of D-glucose (**4**) and D-thioglucose (**5**) released from compound **1**, resulting from the thermally induced degradation. A modified method by König et al. was used for the derivatization and separation of the sugars.[33] A total of 100 μL of treated compound **1** samples [pH 5.3, 130 °C; pH 8, 100 °C; and 0−0.04 μmol mL^{-1} iron(II), pH 5.3, 100 °C] were dried under a continuous nitrogen flow. A total of 325 μL of methylene chloride, 150 μL of trifluoroacetic anhydride, and 25 μL of pyridine were added, and the vial was sealed and vortexed for 30 s. After 3 h of derivatization at room temperature, the samples were diluted with 500 μL of methylene chloride and subjected to GC−FID analysis. The GC−FID system described in the Analysis of Volatile Breakdown Products Using Gas Chromatography with Flame Ionization Detection (GC−FID) section was used with modified GC conditions: the temperature program started at 110 °C for 6 min, heating to 160 °C with 3 °C min^{-1}, followed by a 2 °C min^{-1} increase until 180 °C, which was then held for 1 min. The injector was set to 230 °C. Sugar derivatives were identified by comparing their retention times and quantitated by external calibration with compound **4** (0.05−2 mM; linear regression, R^2 = 0.992) and compound **5** (0.02−1.4 mM; linear regression, R^2 = 0.998). The LOD of the method was reached by injecting a solution of 0.02 mM derivatized compound **5** (0.03 mM compound **4**); linear range was 0.02−5 mM for compounds **4** and **5**. Because compound **4** gives two peaks, the individual peak areas were summed, because the height of these peaks is dependent upon the time that they had to reach the equilibrium of the mutarotation. Compound **5** also mutarotates under neutral conditions[34] but gives only one peak.

Analysis of *N,N′*-Diallylthiourea by HPLC−DAD. Sinigrin samples already used for the determination of thermally formed desulfo-GSLs were also analyzed for *N,N′*-diallylthiourea (**7**), resulting from allyl-ITC (**3**) breakdown. The HPLC−DAD system described in the Quantitation of Intact GSLs as Desulfo-GSLs by HPLC−DAD section was used with a slightly modified solvent gradient, consisting of 1−35% acetonitrile in 0.05% orthophosphoric acid from 2 to 10 min, followed by 70% acetonitrile in 0.05% orthophosphoric acid until 34 min, and holding at this percentage for 6 min. A total of 20 μL of sample was injected into the HPLC. UV detection was conducted at a flow rate of 0.5 mL min^{-1}, a column temperature of 30 °C, and a wavelength of 244 nm. Compound **7** was quantitated using an external calibration curve (0.001−0.2 mM; linear regression, R^2 = 1.000). The LOD of the HPLC system was 1 ng, and the limit of quantitation was reached when injecting 3.1 ng of this substance.

Statistical Analysis. All data were statistically analyzed by one-way analysis of variation (ANOVA) with a significance level of $p \leq 0.05$ using Origin Pro 8.0 (OriginLab Corporation, Northampton, MA). Differences between the treatments were evaluated with Tukey's honest significant difference (HSD) test. Two-way ANOVA was performed to test for interactions between pH values and temperatures on the thermal degradation of sinigrin (**1**) and the formation of breakdown products as well as to reveal interactions between vitamin C and iron(II) (Origin Pro 8.0). Data in figures were presented as the mean ± standard deviation of a 3-fold analysis.

RESULTS AND DISCUSSION

Thermal Degradation of Sinigrin and Formation of Degradation Products. Thermal degradation of the aliphatic GSL sinigrin (**1**) was monitored, and the influence of temperature, medium [dry/aqueous (pH)], iron(II) or iron(III) ions, as well as matrix on the formation of the breakdown products was studied. Next to the temperature of 100 °C, the temperature of 130 °C was selected for some models to be able to study the breakdown of compound **1** under conditions

where it is stable at the lower temperature. Besides compound **1**, 3-butenylnitrile (**2**), allyl isothiocyanate (**3**), formation of desulfo-sinigrin (**6**), and *N*,*N*′-diallylthiourea (**7**), with the latter being a thermally induced breakdown product of the ITC,[35] have been analyzed. The structures and compound numbers of the studied substances are displayed in Figure 1, and the chromatographic separation is presented in panels A–C of Figure 2. Additionally, the release of sugars [D-glucose (**4**)/D-thioglucose (**5**)] was quantitated. Therefore, the sugars have been derivatized to their corresponding trifluoracetyl derivatives. Pure compound **5** was thermally not stable when treated separately at pH 5.3 and 100 °C (1 mM solution), and the formation of compound **4** was detected (data not shown). However, in the GSL samples, compound **5** seemed to be thermally more stable, probably because of the lower concentrations being present or the other reaction conditions being applied. Because of the thermal instability of this sugar, the determination of the sugars was preferably performed at 100 °C (pH 8.0, treatments with iron). However, if there was only slight degradation of compound **1** at this temperature (Table 1), the determination of the sugars was carried out at 130 °C (pH 5.3, dry heat). Because compound **1** partly degraded during the drying step before the sugar derivatization, probably because of the buffer solutions (no degradation occurred when it was suspended in water), untreated samples were always analyzed as controls.

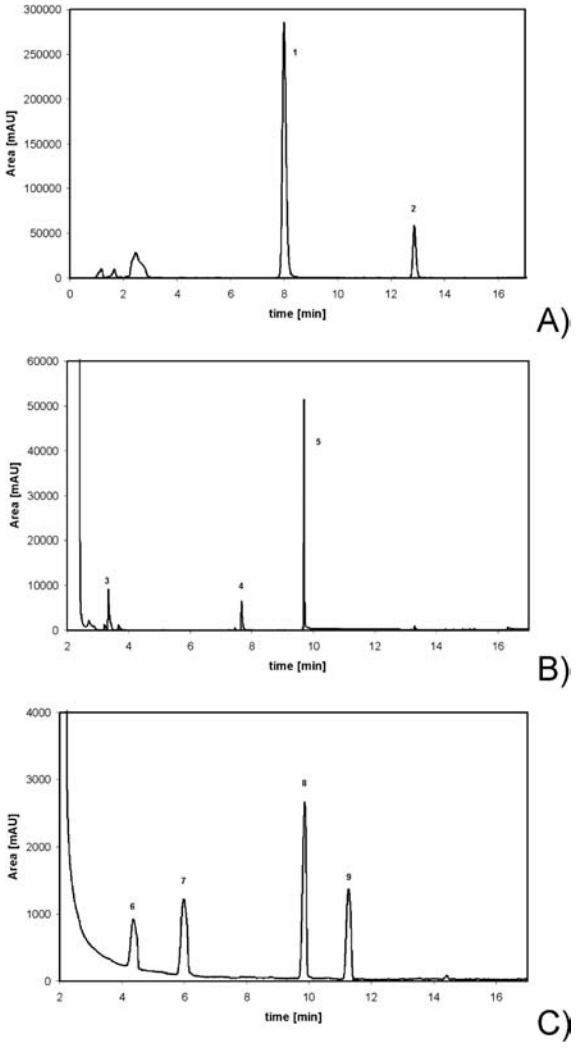

Figure 2. (A) HPLC chromatogram of desulfo-GSLs (229 nm) of a cooked sinigrin sample (100 °C, 60 min, and pH 8.0): (1) allyl-GSL (sinigrin) and (2) 4-hydroxybenzyl-GSL [sinalbin, internal standard (IST)]. (B) GC–FID chromatogram of breakdown products of a cooked sinigrin sample (100 °C, 60 min, and pH 5.3): (3) 3-butenylnitril, (4) allyl-ITC, and (5) benzonitrile (IST). (C) GC–FID chromatogram of trifluoroacetyl derivatives of D-glucose and D-thioglucose of standard mix (1 mg mL^{-1}): (6) D-glucose peak 1, (7) D-glucose peak 2, (8) D-thioglucose, and (9) peak of remaining derivatization reagent.

Thermal Degradation of Sinigrin: Influence of Aqueous Medium. Table 1 summarizes the main results of the thermal treatments of compound **1**. The nitrile/ITC ratios are given to demonstrate the distribution of the breakdown products. If the nitrile/ITC ratio is >1, higher concentrations of compound **2** than compound **3** are formed. Additionally, the overall recovery of the breakdown products was displayed to demonstrate their stability [sum of the recovery rates of the ITC (**3**) and the nitrile (**2**), with the recovery rate being the percentage of the detected breakdown product in relation to the degraded amount of compound **1**]. The D-thioglucose/D-glucose ratio was listed to illustrate the distribution of the formed sugars to gain evidence for the reaction pathways involved. If the D-thioglucose/D-glucose ratio is >1, the dominating sugar is compound **5**.

Influence of the Temperature and pH Conditions. Compound **1** was cooked at pH 5.3, simulating the native pH conditions of broccoli sprouts. At 100 °C, compound **1** was stable, although some compound **3** (0.009 μmol/mg compound **1**) and traces of compound **2** were already detectable and increased significantly with the treatment time (Table 1).

To enhance the thermal degradation and identify the sugar derivatives formed by thermal degradation under acidic conditions, compound **1** was additionally treated at 130 °C. Results are presented in Figure 3A. At 130 °C and after 1 h, 15% of compound **1** was degraded. Compounds **3** and **4** were the main breakdown products after cooking at pH 5.3, with their concentrations increasing significantly with the treatment time. The concentrations of compound **2** increased significantly as well (Figure 3A). At 130 °C, both volatile breakdown products (compounds **2** and **3**) were significantly detected in higher amounts compared to the treatment at 100 °C. Compound **5** was also detected, with its concentration already decreasing after 30 min, because of thermal instability. Additionally, compound **7**, being a thermal degradation product of compound **3**,[36] was detected in small amounts after the treatment at 130 °C. The chromatogram and the corresponding DAD spectrum are displayed in Figure 4A.

4-(Methylsulfanyl)butyl-GSL [glucoerucin (**8**)] was similarly degraded after 1 h compared to compound **1** (78% left over). Here, the nitrile was the main thermal breakdown product (nitrile/ITC ratio = 3.9). Probably, the ITC of this GSL is more labile compared to compound **3**, and therefore, the nitrile is found in higher amounts.

ITCs as thermally induced breakdown products have been reported before, but nitriles were the major breakdown products.[18,20−22] The ITC seems to derive from the GSL by a pathway similar to the enzymatically catalyzed breakdown. Hydrolysis of the GSL with release of compound **4**, resulting in the thiohydroximate-*O*-sulfonate aglucon (or a compound with a similar structure), is a precondition for the formation of the

Table 1. Influence of the Thermal Treatment on the Thermal Stability of Sinigrin and the Formation of Breakdown Products

thermal treatment of sinigrin									
	medium								
temperature (°C)	pH value	buffer	dry/wet	additional	treatment time (min)	sinigrin breakdown[a] (% of initial content)	nitrile/ITC[b] ratio	overall recovery[c] of nitrile/ITC (%)	thioglucose/glucose ratio
100	5.3	phosphate	wet		30	nd	0.3	>100	
					60	nd	0.3	>100	
100	5.3	phosphate	wet	broccoli sprouts powder	10	24.7 ± 10.6	16.1	8	
					45	14.3 ± 11.3	8.4	35	
130	5.3	phosphate	wet		30	3.6 ± 3.2	0.3	>100	0.5
					60	14.9 ± 6.1	0.3	81	0.1
100	5.3	acetate	wet	no Fe^{II}/mL	30	1.5 ± 3.0	2.2	>100	2.1
100	5.3	acetate	wet	0.004 μmol of Fe^{II}/mL	30	4.8 ± 5.9	2.4	65	2.6
100	5.3	acetate	wet	0.04 μmol of Fe^{II}/mL	30	5.5 ± 2.7	3.1	58	5.0
100	5.3	acetate	wet	0.004 μmol of Fe^{II}/mL + vitamin C	30	4.6 ± 2.2	38.3	39	
100	5.3	acetate	wet	0.04 μmol of Fe^{II}/mL + vitamin C	30	15.2 ± 2.5	32.9	17	
100	8.0	phosphate	wet		30	10.2 ± 8.8	6.2	35	1.2
					60	24.9 ± 10.2	5.4	20	0.6
130	8.0	phosphate	wet		30	92.9 ± 5.0	7.2	5	
					60	99.2 ± 0.7	6.3	5	
130			dry		10	11.7 ± 7.0	>100	20	24.5
					45	60.4 ± 3.6	16.1	15	8.4
130			dry	broccoli sprouts powder	10	33.1 ± 8.8	0.8	8	
					45	78.8 ± 8.4	8.4	15	

[a]Sinigrin breakdown: difference between the concentrations of the untreated and treated samples. The errors of both data were summed to give the error of the sinigrin breakdown value. [b]ITC = isothiocyanate. [c]The overall recovery is the percentage of the overall detected breakdown products (ITC + nitrile) in relation to the amount of degraded sinigrin.

ITC, because it is formed by a Lossen-type rearrangement.[37] Because of compound **5** also being a non-negligible thermally induced breakdown product of compound **1** under acidic conditions, other pathways are also involved mandatorily.

Under basic conditions (pH 8.0), compound **1** was significantly much more labile compared to the acidic conditions, with 25% of the initial concentration decreasing after 1 h at 100 °C (Figure 3B) and 93% being degraded after 30 min at 130 °C. Treatment at the higher temperature significantly reduced the thermal stability of compound **1** but did not affect the detected concentrations of the volatile breakdown products. Two-way ANOVA revealed that there is an interaction of the pH value and temperature during the thermally induced degradation of compound **1** as well as during the corresponding formation of the volatile breakdown products (compounds **2** and **3**) (see Table 2). It seems that the hydroxyl ions are able to catalyze the hydrolysis/breakdown reaction better than protons. At pH 8.0, the main released sugar was compound **4**, comparable to the acidic medium. However, under the basic conditions, significantly more compound **2** was formed. At 100 °C, a higher pH value resulted in significantly higher concentrations of compound **3** after 30 min of treatment but did not affect its amount after 60 min. On the contrary, at 130 °C, compound **3** was formed in significantly smaller amounts in basic medium. Because the recovery of detected breakdown products was much lower under basic conditions compared to the acidic medium and decreased with a higher temperature (Table 1), it is supposed that the ITC was more labile in the basic medium, as reported previously.[38] Thermal breakdown of compound **3** results in the formation of compound **7** and several volatile breakdown products.[35,38] Strengthening this hypothesis, at 130 °C, compound **7** was found in concentrations being more than 3 times higher than those of compound **3** (0.055 μmol of compound **7**/mg of compound **1** and 0.016 μmol of compound **3**/mg of compound **1** after 60 min at pH 8.0 and 130 °C). Because two molecules of compound **3** are necessary for the formation of one molecule of compound **7**,[38] it can be proposed that the ITC is the main thermal breakdown product formed under basic conditions. This hypothesis is also supported by the high amounts of compound **4** (compared to compound **5**) being produced after 60 min. However, the corresponding nitrile is found in higher amounts because of the thermal instability of the ITC. This is consistent with the hypothesis that basic conditions will favor the ITC formation, because the Lossen-type rearrangement of the aglucon is inhibited by protons.[39] All in all, the thermally induced degradation of compound **1** will mainly result in the formation of compounds **3** and **4** under aqueous conditions.

Thermal Degradation of Sinigrin: Influence of Dry Medium. When compound **1** was treated under dry conditions at 130 °C, it was significantly more labile compared to aqueous conditions at pH 5.3 but more stable compared to pH 8.0 (Figure 3C). Compound **2** was the main thermally induced breakdown product of compound **1**, and compound **5** was released in high amounts, with both compounds **2** and **5** increasing significantly with the treatment time. The formation of compound **6** was observed and represented 20% of the degraded compound **1** after 10 min of roasting (7% after 45 min) (Figure 3C). Figure 4B shows the chromatogram and DAD spectrum of the non-desulfated sample, containing the thermally induced compound **6**.

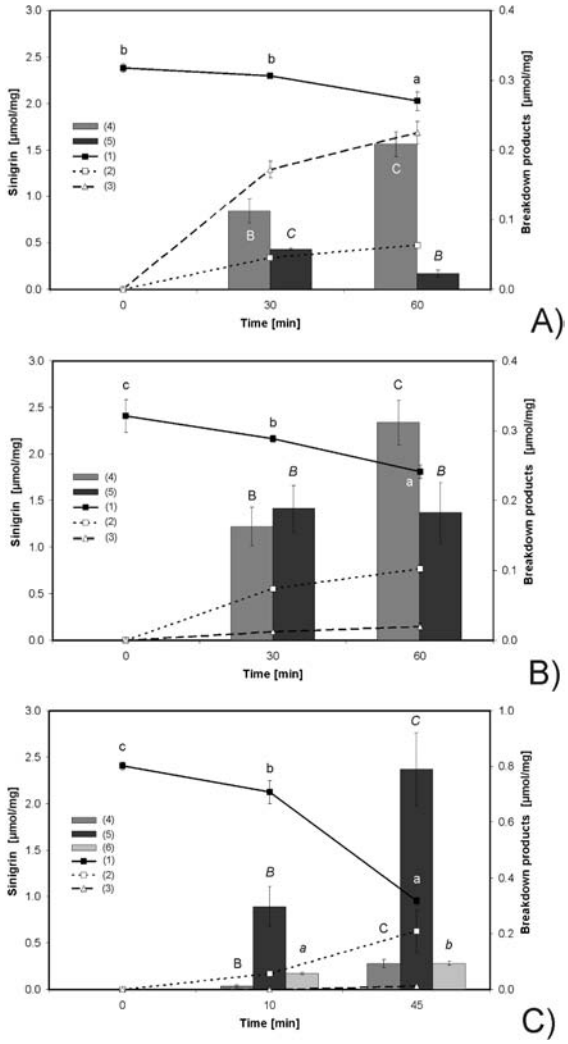

Figure 3. Thermally induced degradation of sinigrin (allyl-GSL) and formation of breakdown products. (A) Aqueous degradation at a pH value of 5.3 and 130 °C. (B) Aqueous degradation at a pH value of 8.0 and 100 °C. (C) Degradation under dry conditions at 130 °C. GSL breakdown and desulfo-GSL formation were analyzed by HPLC–DAD, and formation of breakdown products was analyzed by GC–FID. Results (breakdown products and sinigrin) were expressed in μmol mg^{-1} sinigrin. Different letters in each bar indicate significant differences between the concentrations of the nonvolatile compounds (sinigrin, small letters; desulfo-sinigrin, small italic letters; D-glucose, capital letters; D-thioglucose, italic capital letters; $p \leq 0.05$ by Tukey's HSD test). For compound numbers, refer to Figure 1.

After dry heat treatment of compound **8**, the formation of the corresponding desulfo-GSL was observed as well. Therefore, under dry conditions, aliphatic GSL, in general, will form the desulfo-GSL as a thermal breakdown product under dry conditions.

A degradation pathway resulting in the formation of the ITC is dependent upon the hydrolysis reaction of the GSL with the release of compound **4** and the formation of an instable aglucon. The presence of water is mandatory for this mechanism. Consistently, under dry conditions, only very low concentrations of ITC resulted from the thermal treatment. The influence of the water content on the thermally induced degradation of GSL in broccoli was already discussed by Oliviero et al.[24] In that study, GSLs in plant samples with low water content (13%) were more stable at temperatures up to

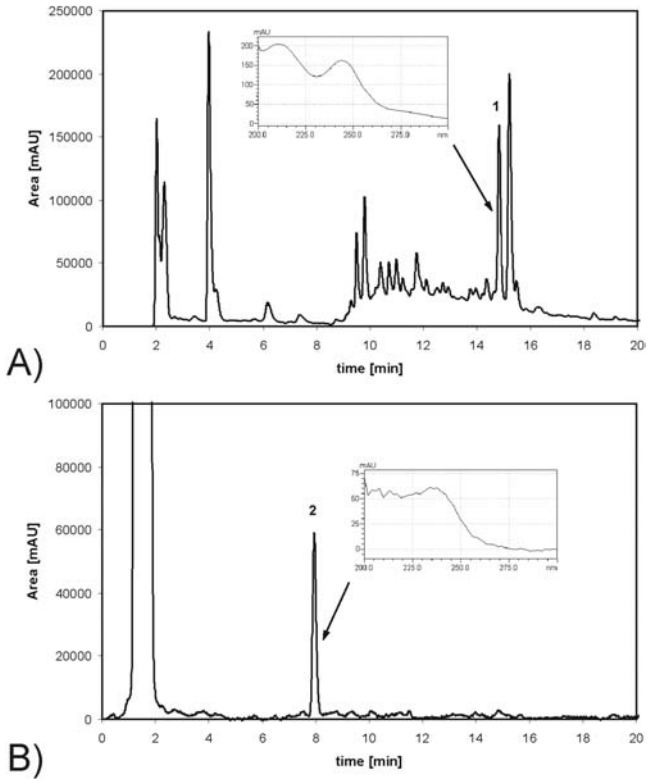

Figure 4. HPLC chromatogram and DAD spectra of the nonvolatile breakdown products of sinigrin. (A) *N*,*N*′-Diallylthiourea (1) (at 244 nm) in a cooked sinigrin sample (130 °C, 30 min, and pH 8.0). (B) Desulfo-sinigrin (2) (at 229 nm) in a roasted sinigrin sample (130 °C and 10 min).

100 °C compared to those with higher water content. The reaction seems to be diffusion-limited. However, at 120 °C, the degradation rate of the GSL in the low-water samples was higher than in samples with higher water content, because of the activation energy of the dry sample. The reaction rate would be less diffusion-limited, and the thermodynamic activity of water is increased.[24] For the formation of desulfo-GSL, water is needed as well. It might be the case that desulfo-GSL is formed out of a further intermediate or the humidity of the air could already be sufficient for the reaction.

Desulfo-GSLs as Intermediates of the Thermally Induced Degradation of GSLs. The fact that compound **3** formation is only marginal and compounds **2**, **5**, and **6** were predominantly formed leads to the hypothesis that desulfo-GSLs are important intermediates during the thermally induced degradation of GSLs and are further degraded to form nitriles and compound **5**. Such a pathway could also explain the presence of compound **5** in the cooked samples. Therefore, this hypothesis was re-evaluated by heating a protein-free mixture of desulfo-GSL to 130 °C at pH 5.3 or 7.0 [containing compound **6**, desulfo-3-(methylsulfanyl)propyl-GSL (desulfo-glucoiberverin), desulfo-3-(methylsulfinyl)propyl-GSL (desulfo-glucoiberin), desulfo-4-(methylsulfanyl)butyl-GSL (desulfo-glucoerucin), desulfo-4-(methylsulfinyl)butyl-GSL (desulfo-glucoraphanin), desulfo-3-indolylmethyl-GSL (desulfo-glucobrassicin), and desulfo-4-methoxy-3-indolylmethyl-GSL (desulfo-4-methoxyglucobrassicin)]. The thermally induced breakdown products were tentatively identified by GC–FID. Desulfo-GSLs were not stable, and after 30 min at pH 5.3, 50% of the total concentration was degraded. Indole desulfo-GSL and

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Table 2. Two-Way ANOVA for Sinigrin Treatments and the Formation of Breakdown Products[a]

treatment	treatment time (min)	*F* value	*p* value
Influence of pH and *T*			
compound **1**			
pH (5.0/8.0)	30	401.6	<0.0001
T (100 °C/130 °C)		307.2	<0.0001
pH × *T*		193.8	<0.0001
pH (5.0/8.0)	60	165.0	<0.0001
T (100 °C/130 °C)		110.7	<0.0001
pH × *T*		45.5	0.0001
compound **2**			
pH (5.0/8.0)	30	258.6	<0.0001
T (100 °C/130 °C)		69.3	<0.0001
pH × *T*		11.5	0.009
pH (5.0/8.0)	60	760.8	<0.0001
T (100 °C/130 °C)		148.0	<0.0001
pH × *T*		134.0	<0.0001
compound **3**			
pH (5.0/8.0)	30	1696.8	<0.0001
T (100 °C/130 °C)		1895.9	<0.0001
pH × *T*		1885.5	<0.0001
pH (5.0/8.0)	60	211.8	<0.0001
T (100 °C/130 °C)		214.2	<0.0001
pH × *T*		232.5	<0.0001
Influence of Vitamin C and Fe^{II}			
compound **1**			
vitamin C (0 and 0.3 mg mL^{-1})	30	31.6	0.0001
Fe^{II} (0, 0.004, and 0.04 μmol mL^{-1})		30.9	<0.0001
vitamin C × Fe^{II}		13.6	<0.0001
compound **2**			
vitamin C (0 and 0.3 mg mL^{-1})	30	48.0	<0.0001
Fe^{II} (0, 0.004, and 0.04 μmol mL^{-1})		42.4	<0.0001
vitamin C × Fe^{II}		12.3	0.0012
compound **3**			
vitamin C (0 and 0.3 mg mL^{-1})	30	484.9	<0.0001
Fe^{II} (0, 0.004, and 0.04 μmol mL^{-1})		1.8	0.2120
vitamin C × Fe^{II}		2.1	0.1693

[a]For compound numbers, refer to Figure 1.

methylsulfanyl desulfo-GSL were least stable, and 74% of desulfo-3-(methylsulfanyl)propyl-GSLs was degraded after 30 min at pH 5.3 (130 °C). At a pH value of pH 7.0, the aliphatic desulfo-GSLs were more labile and, altogether, 80% of desulfo-GSLs degraded within 30 min. All four nitriles of the sulfur-containing aliphatic desulfo-GSLs as well as 3-butenylnitrile were detected. ITC formation was not observable. The formation of desulfo-GSLs as intermediates was already described by Shahidi and Gabon when treating mustard seeds rich in compound **1** with a solvent mixture of methanol/ammonia/water−hexane.[40] They detected compound **5**, and its dimer formed in higher amounts than compound **4**. Whereas compound **2** was the main breakdown product, compound **3** was formed only in minor amounts,[40] comparable to the findings of the present study under dry conditions.

Influence of the Matrix and Iron on the Thermal Stability of GSLs. *Influence of the Matrix.* In a previous study, differences in the thermal stability of isolated GSLs and GSLs in "a broccoli sprouts plant matrix" were revealed.[18] Therefore, the thermal degradation of compound **1** was studied in the presence of small amounts of broccoli sprouts powder [containing 0.88 μmol of compound **1** g^{-1} of dry weight (DW), with an increment of initial compound **1** content of 0.7%]. The broccoli sprouts powder matrix reduced the thermal stability of compound **1**. After 10 min of cooking with the sprouts, there was significantly more degradation than after 30 min without the sprouts. However, after 45 min, this effect was not significant compared to pure compound **1**. The formation of compound **2** was significantly increased by cooking compound **1** with the broccoli sprouts powder, and higher amounts of compound **2** were found after 10 min compared to 30 min without broccoli sprouts powder. The nitrile was the dominating thermal breakdown product, as reported for sulfur-containing aliphatic GSLs in broccoli sprouts (Table 1).[18] Under dry conditions, the presence of broccoli sprouts powder significantly reduced the thermal stability of compound **1** as well (Table 1).

Influence of Iron. As reported previously, the broccoli sprouts powder contains small amounts of iron [0.92 μmol of total iron g^{-1} of DW and 0.09 μmol of soluble iron g^{-1} of DW (in water, pH 2.7)].[18] Because iron(II) is able to degrade GSL in a non-enzymatic mechanism, it was hypothesized that compound **1** is destabilized because of iron(II) present in the broccoli sprouts powder and degraded partly in an iron(II)-catalyzed degradation mechanism, forming mainly compounds **2** and **5**.[26,27] Therefore, the influence of iron(II) and iron(III) on the thermally induced degradation of compound **1** was studied. The addition of 0.04 μmol of iron(II) mL^{-1} led to a higher susceptibility toward thermal treatment and significantly enhanced the formation of compound **5**, whereas there was no significant effect for compound **2** or **3** (Figure 5 and Table 1).

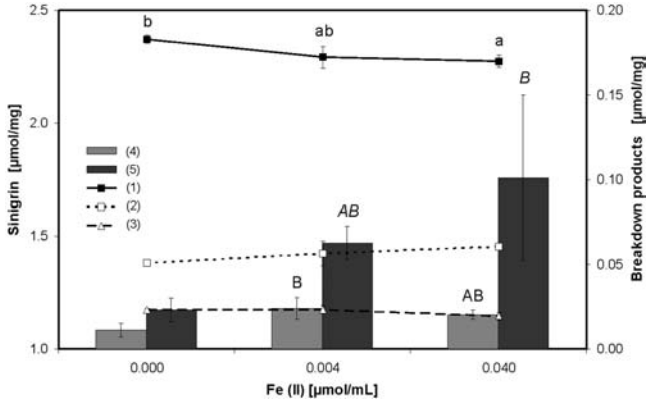

Figure 5. Influence of iron(II) on the thermally induced degradation of sinigrin. Results (breakdown products and sinigrin) were expressed in μmol mg^{-1} sinigrin. Different letters in each bar indicate significant differences between the concentrations of the nonvolatile compounds (sinigrin, small letters; D-glucose, capital letters; D-thioglucose, italic capital letters; $p \leq 0.05$ by Tukey's HSD test). For compound numbers, refer to Figure 1.

Supplementation with 0.04 μmol of iron(III) mL^{-1} did not affect the thermally induced degradation of compound **1** any way, which is in agreement with previous reports.[27] The effect of the "broccoli sprouts powder matrix" on the thermal stability of compound **1** was significantly more pronounced than the addition of 0.04 μmol of iron(II) mL^{-1}, although the overall iron of the 20 mg of broccoli sprouts powder contributed only to a overall iron concentration of 0.02 μmol of iron mL^{-1}. Therefore, other constituents of the plant matrix seem to influence the thermal stability significantly. Hence, also the

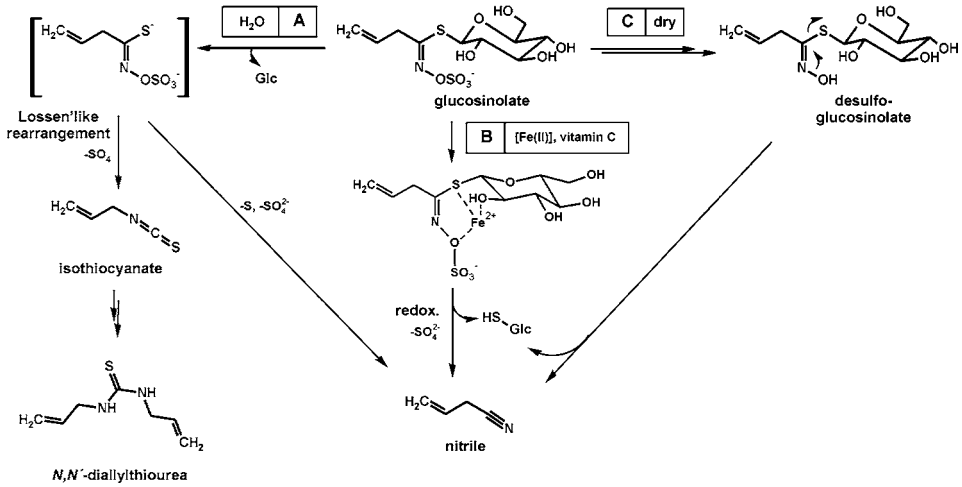

Figure 6. Suggested pathways of the thermally induced degradation of sinigrin. The iron(II)-catalyzed degradation mechanism of sinigrin is based on the mechanism proposed by Bellostas et al.[27]

influence of vitamin C on the iron(II)-catalyzed degradation was tested by adding 0.3 mg mL^{-1} vitamin C to the 0.004 and 0.04 μmol of iron(II) mL^{-1}, simulating the vitamin C concentration caused by an addition of 40 mg of broccoli sprouts powder mL^{-1}. Vitamin C alone did not affect the thermally induced degradation of compound **1**. In combination with iron(II), the concentrations of compound **2** increased with each iron(II) level significantly. At the level of 0.04 μmol of iron(II) mL^{-1}, compound **1** was significantly more labile with vitamin C than without [85% was leftover compared to 95% at the 0.04 μmol of iron(II) level without vitamin C]. To our knowledge, this is the first report of vitamin C influencing the iron(II)-catalyzed degradation of GSLs. Supporting this observation, two-way ANOVA revealed that there is a significant interaction of the presence of vitamin C on the iron(II)-catalyzed thermally induced degradation of compound **1** and the formation of compound **2** (Table 2). Therefore, vitamin C is able to have a synergistic effect on the iron(II)-catalyzed breakdown of GSLs, probably because of redox cycling of iron(III).[41]

In Figure 6, possible pathways for the thermally induced GSL degradation are proposed. In aqueous medium, hydrolysis should be the most important thermal degradation mechanism for aliphatic GSLs (pathway A). This pathway is, likewise to the enzymatically catalyzed breakdown, characterized by a release of compound **4** and leads to the corresponding ITC and nitrile, with their amounts being dependent upon the pH value. Because the ITC is not stable, it will be further thermally degraded to form products such as *N*,*N*′-di(alkyl)thiourea and volatile substances such as di(alkyl)di- and di(alkyl)-trisulfides.[35,42] Because iron(II) influenced the thermally induced degradation, GSL might follow the second pathway, including the iron(II)-catalyzed degradation (pathway B in Figure 6). A mechanism was supposed by Bellostas et al.,[27] involving the formation of a complex and the oxidation of iron(II) to iron(III), resulting finally in the release of compound **5** and the corresponding nitrile (Figure 6). GSLs with a hydroxyl group at the C-2 of the side chain additionally form the thionamide.[27] Indeed, in the present study, the addition of iron(II) slightly reduced the thermal stability of compound **1** and significantly enhanced the formation of compound **5**. Additionally, it was observed that the "broccoli sprouts powder matrix" affected the thermal stability of compound **1** even more, probably because of redox cycling of iron(III) to iron(II) by other constituents of the plant matrix, such as vitamin C or even polyphenols.[41] The present study revealed that vitamin C is able to have a synergistic effect on the iron(II)-catalyzed breakdown of GSLs and enhances the degradation. Therefore, the iron(II) content of *Brassica* vegetables seems to influence the thermal stability of GSLs and shifts the thermally induced degradation toward a nitrile formation according to pathway B (Figure 6). However, a third mechanism seems to exist, in which the GSLs degrade to the desulfo-GSLs under dry conditions. Therefore, pathway C is postulated (Figure 6) with the desulfo-GSLs as intermediates, because of the fact that hydrolysis is negligible under dry conditions. Comparatively, this pathway results also in the formation of nitriles, because nitriles were shown to be the thermal breakdown products of desulfo-GSLs. All in all, the reaction conditions, such as pH, temperature, matrix, presence of iron(II), vitamin C, and water content, have a distinct impact on the thermally induced degradation of the GSL and the breakdown product(s) formed. Further studies are needed to evaluate the impact of these factors on the thermally induced breakdown of GSLs in different *Brassica* vegetables and different GSLs to preserve plant constituents along the food-processing chain and their health-promoting effects.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

desulfo-GSL, desulfo-glucosinolate; DW, dry weight; GSL, glucosinolate; ITC, isothiocyanate; LOD, limit of detection

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