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ARTICLE

Detection and Structural Characterization of Thermally Generated Isoflavone Malonylglucoside Derivatives

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Malonylglucoside isomers were identified by high-performance liquid chromatography with ultraviolet/mass spectrometric detection and nuclear magnetic resonance (NMR). The formation and interconversions of the isomers were monitored in heated buffer and soymilk systems. Two positional or steroisomers of malonylgenistin and malonydaidzin, showing similar UV spectra and molecular weights yet different fragmentation patterns, were detected. NMR characterization of the malonylgenistin isomer revealed its structure to be 4″-*O*-malonylgenistin, suggesting a malonyl migration from the glucose-6 position to the glucose-4 position. Interconversions of malonylgenistin and its isomer were observed in both buffered and soymilk systems. While malonylgenistin partially converted to the isomer upon thermal treatment, conversion of the isomer to malonylgenistin was more spontaneous. The malonylgenistin isomer represented 6−9% of the total calculated genistein content in soymilk heated at 100 °C for various periods of time. Consequently, disregarding the content of malonylglucoside isomers in processed soy matrices can lead to isoflavone underestimation and misinterpretation of the biological contributions.

KEYWORDS: Isoflavones; malonylglucosides; malonyl isomers; isoflavone interconversions; processing conditions

INTRODUCTION

Each of the genistein, daidzein, and glycitein isoflavones exist in up to four different forms, namely, aglycone, non-conjugated glucoside, acetylglucoside, and malonylglucoside (**Figure 1A**). The effect of their chemical structures on bioavailability and physiological contributions is not yet fully understood. However, it is well-agreed that the total amount of the biologically relevant isoflavones is one of the main determinants of the nutritional value of soy products. Therefore, identifying the fate of isoflavones upon processing is essential for the accurate determination of their total content in the final product.

Processing conditions, namely, pH, temperature, and time, have a substantial influence on the profile of isoflavones (*1*), mainly converting the malonylglucosides to their more heat-stable non-conjugated β-glucosides (*2*−*4*). Malonylglucosides are the most abundant and the most thermally labile isoflavone forms (*2*, *5*). In addition to conversions between isoflavone forms, processing can also result in a "loss", a reduced total amount of isoflavones. Measured losses of isoflavones were usually assumed to be a result of leaching into waste stream (*6*−*8*), dissolving in aqueous alcohol solutions used in the production of certain soy products, such as soy protein concentrates and isolates (*9*), or binding to the protein matrix (*3*, *5*). Jackson et al. (*8*) observed a 65% loss in the total amount of isoflavones after processing raw soybeans. Although mass balance studies explained some of the loss, about 20% loss remained unexplained (*8*). Further studies showed that isoflavone losses upon processing are potentially attributed to complete degradation and/or derivatization of isoflavones in a way that cannot be detected following standard analytical approaches (*10*−*13*). Thermal processing at elevated pH caused up to 30 and 15% loss in the form of undetectable degradation products in closed buffered (*14*) and soy systems (*15*), respectively.

While it is still debatable whether or not conversions between known isoflavone forms affect bioavailability, undetected derivatives are labeled as "loss" and, thus, are assumed to have no biological relevance. However, without knowing the structures of these undetected derivatives, statements about their biological relevance are not possible. Therefore, it is crucial to differentiate between complete degradation and the formation of potentially bioavailable derivatives.

Recent studies reported the presence of malonylglucoside isomers in soybean, soy molasses, and various soy products (*16*, *17*), but complete structural information for the isomers was not given. Griffith and Collison (*16*) also observed the formation of the isomers in standard solutions of malonylglucosides left at room temperature for several hours. Careful examination of the chromatograms presented by various researchers investigating the isoflavone content in soybeans and various soy products revealed the presence of these malonylglucoside isomers; however, they were not discussed (*3*, *18*, *19*). Although these isomers can significantly contribute to the total isoflavone content, information about their structures is not available. Also, isomer formation and interconversions between these isomers and the parental malonylglucosides under various processing conditions have not yet been

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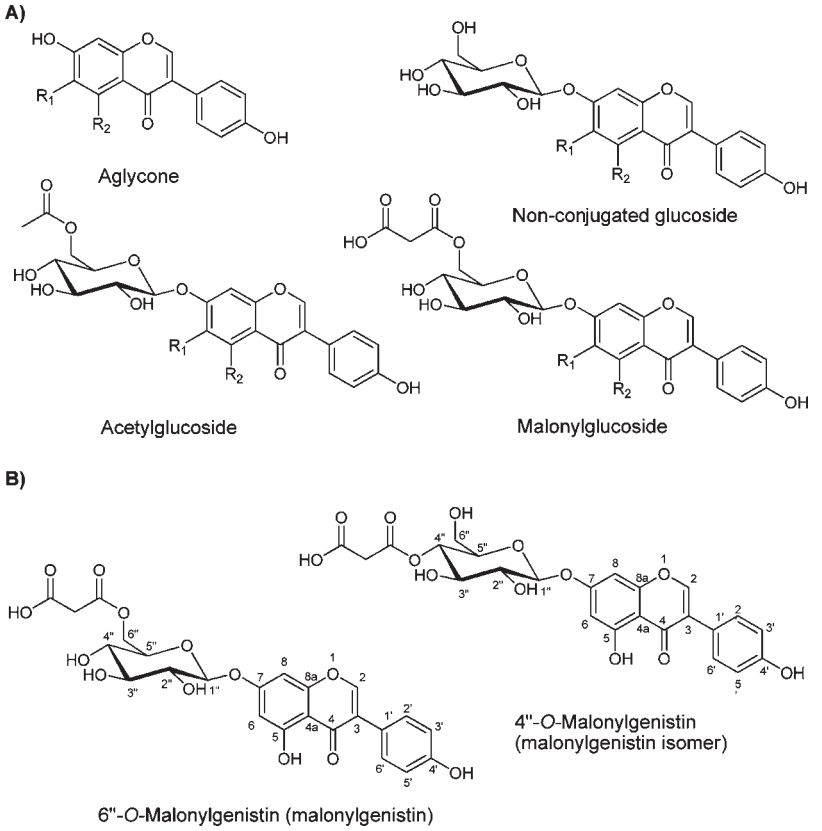

Figure 1. (A) Structures of the 12 known isoflavones categorized as aglycone, glucoside, acetylglucoside, and malonylglucoside. R_1 can be −H in the case of daidzein and genistein or $-OCH_3$ in the case of glycitein, while R_2 can be −H in the case of daidzein and glycitein or −OH in the case of genistein. (B) Structures and numbering system of 6′′-*O*-malonyl-genistin (malonylgenistin, MGin) and 4′′-*O*-malonyl-gensitin (malonylgenistin isomer, MGin isomer).

studied. Disregarding the content of the isomers can lead to erroneous results during the calculation of the loss in isoflavones. Consequently, studies that reported total isoflavone contents of soy foods might have underestimated isoflavone content up to 10−15%.

Therefore, the objective of this work was to detect, identify, and monitor the formation and conversion of isoflavone malonylglucoside isomers during thermal processing of malonylgenistin and malonyldaidzin in buffered and soymilk systems.

MATERIALS AND METHODS

Materials. High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol (MeOH) were purchased from Fisher Scientific (Hanover Park, IL). Isoflavone standards malonyldaidzin, acetyldaidzin, actetylgenistin, malonylglycitin, and acetylglycitin were purchased from Wako Chemicals (Richmond, VA). Genistein, genistin, malonylgenistin, daidzein, and daidzin were purchased from LC Laboratories (Woburn, MA). Glycitin and glycitein were purchased from Indofine Chemical Company (Hillsborough, NJ). Standard solutions of 500 mg/L were prepared using 80% (v/v) aqueous MeOH. Soy grits were kindly provided by Soylink (Product 27707-006, Oskaloosa, IA).

Thermal Treatment of Malonylglucosides in Buffered Systems. The thermal treatment of malonylglucosides was carried out in triplicate following a two-factor design completely crossed, with pH (two levels) and time (seven levels) as the independent factors, while the temperature was held constant at 100 °C. Malonylglucoside (malonylgenistin and malonyldaidzin) solutions were prepared by adding 40 μL of the respective malonylglucoside standard solution (500 mg/L) to 960 μL of phosphate buffer (0.001 M) at either pH 7 or 8, in a 1.5 mL screw cap Eppendorf tube. After vortexing, the Eppendorf tubes were placed in a water bath maintained at 100 °C (±1 °C) for 1, 5, 10, 15, 30, 45, or 60 min. After the thermal treatment, the content of each tube was transferred quantitatively to a 10 mL Erlenmeyer flask and diluted with 4 mL MeOH to make up a 4 mg/L malonylglucoside solution. Appropriate controls were prepared for each treatment. Each of the control and thermally treated malonylglucoside solutions were membrane-filtered (0.45 μm) and immediately subjected to HPLC analysis. Because isoflavones vary in molecular weights, concentrations are reported in mol/mL (nmol/mL) for accurate calculations of interconversions and loss. Percent interconversions between detected known isoflavone derivatives and percent loss in known isoflavone derivatives were calculated using the equations by Mathias et al. (*14*).

HPLC/Ultraviolet (UV) Analysis. A Shimadzu HPLC system, equipped with a SIL-10AF auto-injector, two LC-20AT high-pressure pumps, a SPD-M20A photodiode array (PDA) detector, and a CTO-20A column oven, was used. The column used was a 250 × 4.6 mm inner diameter, 5 μm, YMC pack ODS AM-303 RP-18 column, with a 20 × 4 mm guard column of the same material (YMC pack ODS AM). Isoflavone analysis was achieved as outlined by Ismail and Hayes (*20*), with minor modifications (column temperature maintained at 35 °C, absorbance monitored over a wavelength range of 190−370 nm, and integration for quantitation purposes performed at 256 nm). A seven-point external calibration with standard solutions (0.1, 0.5, 1.0, 2.0, 4.0, 8.0, and 10.0 mg/L) containing all 12 forms of isoflavones genistein, daidzein, and glycitein was performed.

HPLC/Mass Spectrometry (MS) Analysis. Solutions (20 mg/L) of thermally treated isoflavone malonylglucosides were analyzed by HPLC/MS. A Spectrasystem P4000 HPLC system consisting of a SN 4000 model quaternary pump and a UV 600LP-type PDA was used to analyze the isoflavones based on the method outlined above. The eluate from the HPLC column was split, and 10% of the flow was passed into an electrospray ionization (ESI) interface of a LCQ classic mass spectrometer (ion-trap analyzer, ThermoElectron, San Jose, CA). The ionization conditions were as follows: heated capillary temperature, 225 °C; sheath gas (N_2, 99.99%, flow rate, 7.25 L/h); nebulizing pressure, 73.5 psi; spray voltage, 4 kV; capillary voltage, 16.7 V; and positive-ion spectra were recorded over a m/z range of 150−1000. Tandem mass spectrometry (MS/MS) was employed to study the fragmentation pathway of the new derivatives as well as the known isoflavone forms. The precursor ions ($[M + H]^+$) were isolated and analyzed by collision-induced dissociation with 100% helium as the collision gas, and the daughter ion spectra were

recorded. The relative collision energies were set to a value at which ions of interest were produced in measurable abundance (varying from 9 to 31% in increments of 2).

Extraction of the Malonylgenistin Isomer from Soy Grits. Ground soy grits containing the isomer of interest (*17*) were used to extract the malonylgenistin isomer with 53% (v/v) aqueous acetonitrile solution, as outlined by Murphy et al. (*5*), without acidification. Extracts were centrifuged at 13750*g* for 10 min at 15 °C, and the supernatant was filtered through Whatman No. 42 filter paper. Acetonitrile from the filtrates was evaporated using a rotary evaporator at 37 °C for 15 min. The concentrated extracts were stored at −20 °C in amber glass bottles until further analysis.

Semi-preparative Isolation of the Malonylgenistin Isomer. To isolate the isomer of interest on a semi-preparative scale, the Shimadzu HPLC system described above was used, however, equipped with a semi-preparative 250 × 10 mm inner diameter, 5 μm, YMC pack ODS AM-12S RP-18 column, with a 10 × 10 mm guard column of the same material (YMC pack ODS AM). The separation method outlined by Ismail and Hayes (*20*) was modified by calculating a scale-up factor to ensure the same linear velocity of the mobile phases as used in the analytical run. The calculated flow rate after scale-up was 3.5 mL/min. An aliquot (300 μL) of the isoflavone extract was filtered through a 0.45 μm syringe filter and injected onto the column. A linear HPLC gradient was used. Solvent A was HPLC-grade water, and solvent B was acetonitrile, both containing 0.1% (v/v) glacial acetic acid. The initial gradient concentration was 15% solvent B, which was linearly increased to 18% in 25 min, kept constant for 5 min, linearly increased to 30% in 10 min, kept constant for 3 min, linearly increased to 90% in 2 min, and kept constant for 8 min, followed by column equilibration steps. The temperature was maintained at 45 °C. Absorbance spectra were monitored over a UV wavelength range of 190−370 nm. The fraction containing the malonylgenistin isomer was collected and lyophilized (Virtis Freezemobile 12EL). Several runs were performed, and the obtained amounts of the malonylgenistin isomer were pooled. A portion of the freeze-dried fraction was diluted with 80% (v/v) aqueous MeOH to prepare a standard solution of the isomer (∼120 mg/L), and its identity was confirmed. The remaining isomer fraction was stored at −80 °C for further analysis using nuclear magnetic resonance (NMR) and for thermal treatments. The same separation procedure was employed to isolate malonylgenistin for NMR analysis.

NMR Analysis of the Malonylgenistin Isomer. NMR experiments were carried out on a Bruker 700 MHz Avance spectrometer (Rheinstetten, Germany) equipped with a 5 mm TXI proton-enhanced cryoprobe. Structure identification was performed using the usual array of one- and two-dimensional NMR experiments [^{1}H, H,H correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)]. Carbon data were taken from the less time-consuming 2D experiments HSQC and HMBC instead of performing 1D ^{13}C experiments. Malonylgenistin (6″-*O*-malonylgenistin; **Figure 1B**) was measured in MeOH-d_4 and dimethylsulfoxide (DMSO)-d_6, and the malonylgenistin isomer (4″-*O*-malonylgenistin; **Figure 1B**) was measured in MeOH-d_4 only. Chemical shifts (δ) were referenced to the central solvent signals [MeOH-d_4: δ_H 3.31 ppm and δ_C 49.0 ppm; DMSO-d_6: δ_H 2.50 ppm and δ_C 39.5 ppm (*21*)]. *J* values are given in hertz. NMR assignments follow the numbering shown in **Figure 1B**.

6″-*O*-Malonylgenistin (700 MHz, DMSO-d_6): malonylated β-D-glucose: H1″, 5.12 (d, J = 7.7 Hz); H2″, 3.28; H3″, 3.33; H4″, 3.19; H5″, 3.75; H6″, 4.35 and 4.12; malonyl-CH_2, 3.37; aglycone: H2, 8.40 (s); H6, 6.46 (s); H8, 6.71 (s); H2′/H6′, 7.39 (d, J = 8.1 Hz); H3′/H5′, 6.83 (d, J = 8.1 Hz). 6″-*O*-Malonylgenistin (176 MHz, DMSO-d_6): malonylated β-D-glucose: C1″, 99.3; C2″, 72.7; C3″, 76.1; C4″, 69.4; C5″, 73.6; C6″, 63.9; malonyl-COOR, 167.0; malonyl-CH_2, 41.6; malonyl-COOH, 167.5; aglycone: C2, 154.6; C3, 122.5; C4, 180.5; C4a, 106.0; C5, 162.2; C6, 99.4; C7, 162.6; C8, 94.3; C8a, 157.1; C1′, 120.9; C2′/C6′, 130.2; C3′/C5′, 115.0; C4′, 157.4.

6″-*O*-Malonylgenistin (700 MHz, MeOH-d_4) (nd = not determined): malonylated β-D-glucose: H1″, 5.03 (d, J = 7.4 Hz); H2″, 3.50; H3″, 3.50; H4″, 3.3; H5″, 3.77; H6″, 4.55 and 4.27; malonyl-CH_2, nd; aglycone: H2, 8.15 (s); H6, 6.51 (s); H8, 6.71 (s); H2′/H6′, 7.40 (d, J = 8.2 Hz); H3′/H5′, 6.85 (d, J = 8.2 Hz). 6″-*O*-Malonylgenistin (176 MHz, MeOH-d_4): malonylated β-D-glucose: C1″, 101.0; C2″, 74.2; C3″, 77.1; C4″, 70.9; C5″, 75.1; C6″, 64.8; malonyl-COOR, 168.7; malonyl-CH_2, nd; malonyl-COOH, nd; aglycone: C2, 155.0; C3, 124.1; C4, 182.1; C4a, 107.9; C5, 163.6; C6, 100.7; C7, 164.1; C8, 95.5; C8a, 159.0; C1′, 122.4; C2′/C6′, 130.8; C3′/C5′, 115.7; C4′, 158.5.

4″-*O*-Malonylgenistin (700 MHz, MeOH-d_4): malonylated β-D-glucose: H1″, 5.13 (d, J = 7.7 Hz); H2″, 3.58; H3″, 3.75; H4″, 4.90; H5″, 3.75; H6″, 3.78 and 3.62; malonyl-CH_2, nd; aglycone: H2, 8.16 (s); H6, 6.54 (s); H8, 6.73 (s); H2′/H6′, 7.40 (d, J = 8.2 Hz); H3′/H5′, 6.85 (d, J = 8.2 Hz). 4″-*O*-Malonylgenistin (176 MHz, MeOH-d_4): malonylated β-D-glucose: C1″, 100.9; C2″, 74.2; C3″, 75.5; C4″, 72.3; C5″, 75.5; C6″, 61.5; malonyl-COOR, 169.0; malonyl-CH_2, nd; malonyl-COOH, nd; aglycone: C2, nd; C3, 125.0; C4, nd; C4a, 108.0; C5, 164.0; C6, 100.7; C7, 164.6; C8, 95.4; C8a, 159.1; C1′, 122.9; C2′/C6′, 131.0; C3′/C5′, 115.9; C4′, 158.8.

Thermal Treatment of the Malonylgenistin Isomer. Thermal treatment of the malonylgenistin isomer was carried out in triplicate, following a single-factor experimental design with time (four levels) as the independent factor, while the pH and temperature were held constant (pH 8 and 100 °C). Because of faster conversion rates of the isomer, the time intervals chosen were between 1 and 7 min. Isomer solutions were prepared, in triplicate, by adding 15 μL of the isomer standard solution (∼120 mg/L) to 235 μL of phosphate buffer (0.001 M) at pH 8, in a 1.5 mL screw-cap Eppendorf tube, and placed in a water bath maintained at 100 °C (±1 °C) for 1, 3, 5, or 7 min. After the thermal treatment, the Eppendorf tubes were kept in an ice bath for 15 min to bring the solutions back to room temperature (22 °C). The content of each tube was transferred quantitatively into a test tube and diluted with 500 μL of MeOH to a final concentration of ∼2.4 mg/L. A non-heated control solution was prepared accordingly. The control and thermally treated isomer solutions were membrane-filtered (0.45 μm) and immediately subjected to HPLC analysis.

Preparation of Soymilk. Soymilk was prepared from soy grits. Soy grits (5.9 kg) were ground in a grinder (MZM/VK7, Fryma, Switzerland) after the addition of seven parts of water (60 °C). The insoluble portion (okara) was then removed using a desludger unit (9749, Westfalia Clarifier, Centrico, Inc., Northvale, NJ). The total solids content was adjusted to 7% by the addition of water. The pH of the soymilk was close to neutral.

Thermal Treatment of Soymilk. The heat treatment of soymilk was carried out in triplicate with time (five levels) as the independent factor, while the temperature was held constant at 100 °C. Aliquots (12 mL) of soymilk were dispensed into 2 mL glass ampules that were sealed and placed in a water bath at 100 °C (±1 °C) for 2, 5, 10, 30, or 60 min. The contents of the ampules of each treatment were pooled, frozen at −20 °C, and lyophilized (Virtis Freezemobile 12EL). A non-heated control sample was treated accordingly. The freeze-dried samples and control were stored at −80 °C until further analysis.

Extraction of Isoflavones from Soymilk. Isoflavones were extracted from freeze-dried samples following the method outlined by Murphy et al. (*5*), however, using 0.05 g of sample instead of 2 g (*22*) and without the addition of acid to the extraction solvent. Extracts were centrifuged at 13750*g* for 10 min at 15 °C, and the supernatant was filtered through Whatman No. 42 filter paper. Acetonitrile from the filtrates was evaporated using a rotary evaporator at 37 °C for 15 min. The concentrated extracts were redissolved in 80% (v/v) MeOH and stored at −20 °C in amber glass bottles until analyzed by HPLC/PDA and HPLC/MS, following the methods outlined above.

Statistical Analysis. Analysis of variance (ANOVA) was carried out using SPSS 15 for Windows (*23*). When a factor effect or an interaction was found significant, indicated by a significant F test ($p \leq 0.05$), differences between the respective means (if more than two means) were determined using Tukey−Kramer multiple means comparison test.

RESULTS AND DISCUSSION

Conversion and Loss of Malonylglucosides as a Function of pH, Temperature, and Time. Upon thermal treatment, de-esterification of malonyldaidzin (MDin) and malonylgenistin (MGin) into their respective glucosides (**Figure 1A**), daidzin and genistin, respectively, was observed. In comparison to the control, the concentrations of both MDin and MGin significantly decreased as the thermal treatment prolonged. The decrease of MDin and MGin was accompanied by an increase in the formation of daidzin and genistin, respectively (**Tables 1** and **2**). The rate of conversion was pH-dependent, with a more pronounced conversion at pH 8

Table 1. Mean Concentrations (nmol/mL) of Din, MDin, ADin, Total Known Isoflavone Derivatives, and Total Loss in Known Din Derivatives in Samples of Malonyldaidzin Subjected to pH 7 and 8 and at 100 °C for Treatment Times Ranging from 0 to 60 min

pH	time (min)	Din[a]	Mdin[a]	Adin[a]	total known isoflavone derivatives[b]	total loss in known isoflavone derivatives[c]
7	0 (control)	0.21 g A	8.56 a A	0.08 ef A	8.85 a A	
	1	0.23 g A	8.43 a A	0.07 f A	8.74 a A	0.11 d B
	5	0.65 f B	7.51 b A	0.09 e A	8.25 bc A	0.59 c B
	10	1.28 e B	6.92 c A	0.11 d A	8.32 b A	0.52 c B
	15	1.65 d B	5.67 d A	0.12 d A	7.44 d B	1.41 a A
	30	3.00 c B	4.15 e A	0.15 c A	7.30 d B	1.54 a A
	45	4.36 b B	3.40 f A	0.16 b A	7.93 c A	0.92 b B
	60	5.69 a B	2.83 g A	0.18 a B	8.70 a A	0.15 d B
8	0 (control)	0.25 g A	8.76 a A	0.08 abc A	9.09 a A	
	1	0.25 g A	8.05 b B	0.073 cb A	8.38 bc B	0.71 cd A
	5	1.17 f A	6.92 c B	0.085 abc A	8.17 cd A	0.92 bc A
	10	2.23 e A	5.66 d B	0.09 ab B	7.99 de B	1.11 ab A
	15	3.34 d A	4.49 e B	0.093 a B	7.93 de A	1.16 ab A
	30	5.12 c A	2.56 f B	0.09 ab A	7.78 e A	1.31 a B
	45	6.13 b A	1.67 g B	0.09 ab B	7.90 de A	1.19 ab A
	60	7.55 a A	0.98 h B	0.076 bc A	8.61 b A	0.48 d A

[a] Din, daidzin; Mdin, malonyldaidzin; Adin, acetyldaidzin. [b] Total detected known isoflavones (Din + Mdin + Adin). Aglycone daidzein was not detected. [c] Loss in total known isoflavones. Means in each column with different lowercase letters are significantly different within each pH across the treatment times, and means in each column with different capital letters are significantly different within the same treatment times across pH 7 and 8, according to Tukey–Kramer multiple means comparison test ($p \leq 0.005$; $n = 3$).

Table 2. Mean Amounts (nmol/mL) of Gin, MGin, AGin, Total Known Isoflavone Derivatives, and Total Loss in Known Gin Derivatives in Samples of Malonylgenistin Subjected to pH 7 and 8 at 100 °C for Treatment Times Ranging from 0 to 60 min

pH	time (min)	Gin[a]	Mgin[a]	Agin[a]	total known isoflavone derivatives[b]	total loss in known isoflavone derivatives[c]
7	0 (control)	0.23 g A	9.19 a A	0.12 a A	9.54 a A	
	5 min	0.66 f B	8.06 b A	0.12 a A	8.85 b A	0.69 b B
	10 min	1.18 e B	7.4 c A	0.14 a A	8.71 b A	0.83 b B
	15 min	1.7 d B	6.5 d A	0.15 a A	8.33 c A	1.2 a A
	30 min	3.05 c B	5.08 e A	0.16 a A	8.29 c A	1.25 a A
	45 min	4.13 b B	3.86 f A	0.18 a A	8.17 c A	1.37 a A
	60 min	5.11 a B	3.04 g A	0.18 a A	8.34 c A	1.2 a A
8	0 (control)	0.23 g A	9.24 a A	0.12 a A	9.58 a A	
	5 min	1.09 f A	7.35 b B	0.12 a A	8.56 b B	1.02 b A
	10 min	2.12 e A	5.93 c B	0.13 a A	8.18 c B	1.40 a A
	15 min	2.93 d A	5.18 d B	0.13 a A	8.24 bc A	1.34 ab A
	30 min	5.18 c A	2.96 e B	0.13 a A	8.26 bc A	1.32 ab A
	45 min	6.33 b A	1.95 f B	0.13 a A	8.41 bc A	1.17 ab A
	60 min	7.13 a A	1.27 g B	0.12 a A	8.53 bc A	1.05 ab A

[a] Gin, genistin; Mgin, malonylgenistin; Agin, acetylgenistin. [b] Total detected known isoflavones (Gin + Mgin + Agin). Aglycone genistein was not detected. [c] Loss in total known isoflavones. Means in each column with different lowercase letters are significantly different within each pH across the treatment times, and means in each column with different capital letters are significantly different within each treatment time across pH 7 and 8, according to Tukey–Kramer multiple means comparison test ($p \leq 0.005$; $n = 3$).

compared to pH 7. Decarboxylation of MDin and MGin into their respective acetylglucosides and conversion into the aglycones daidzein and genistein were not favored under any of the treatment conditions. The observed data are consistent with previously reported results obtained upon processing of complex soymilk systems (*5*, *15*) and model buffered systems (*14*, *24*).

Along with interconversions among the known isoflavone derivatives, a total "loss" was observed. The decrease in total known daidzin derivatives continued up to 30 min at both pH 7 and 8 (**Table 1**). Maximum loss (up to ~17% on a molar basis) was observed after 30 min of thermal treatment at pH 7. One might tend to attribute this observed loss to a complete degradation of MDin. However, after 60 min of treatment, the calculated loss was significantly lower than that calculated after 30 min of treatment. This is a clear indication that complete degradation is not the only contributor to the total measured "loss" but that formation and subsequent conversion of an intermediate isoflavone derivative have occurred.

The loss in total known genistin derivatives reached a maximum (up to ~15% on a molar basis) after 15 and 10 min of heating at pH 7 and 8, respectively (**Table 2**). Different from MDin, the loss of the known genistin derivatives reached a plateau after 10 and 15 min and did not change significantly after this. This observation does not clarify whether or not the measured loss in MGin is due to complete degradation and/or conversion to an intermediate isoflavone derivative.

Detection and Characterization of an MDin Isomer. An unidentified peak was detected in several of the MDin treatment chromatograms when compared to the isoflavone standards. The unidentified peak, labeled as $P12_{min}$ according to its elution time (**Figure 2A**), was not formed in a MeOH/buffer blank and, thus, was confirmed as an MDin derivative. The abundance of $P12_{min}$ estimated by the peak areas at 256 nm was significantly different between treatments (**Figure 3A**), indicating that MDin was converted to not only daidzin but also $P12_{min}$. In comparison to the control, $P12_{min}$ formation increased significantly after 1 min of thermal treatment time and then reached a maximum when heated for 5 min at both pH 7 and 8, after which it decreased significantly as time increased. The rate of formation and disappearance of $P12_{min}$ was higher at pH 8 than pH 7.

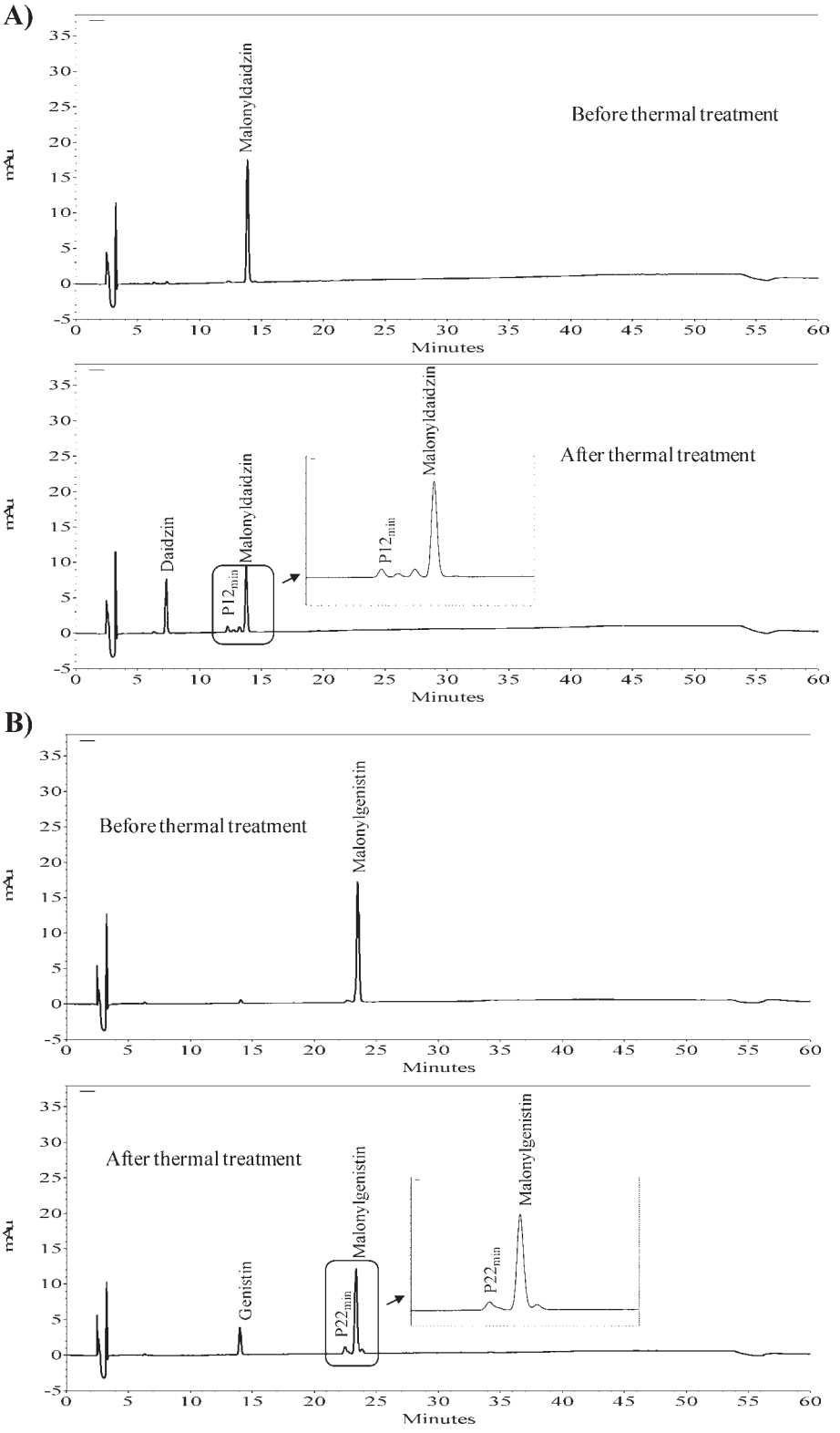

Figure 2. (A) HPLC chromatograms at 256 nm showing a malonyldaidzin isomer at a retention time of 12.03 min labeled as $P12_{min}$, which was formed upon heating a 4 mg/L malonyldaidzin solution at 100 °C for 15 min at pH 8. (B) HPLC chromatograms at 256 nm showing a malonylgenistin isomer at a retention time of 22.49 min labeled as $P22_{min}$, which was formed upon heating a 4 mg/L malonylgenistin solution at 100 °C for 15 min at pH 8.

The UV spectrum of $P12_{min}$ was similar to that of MDin, daidzin, daidzein, and acetyldaidzein with a maximum absorbance (λ_{max}) at 249 nm, indicating that $P12_{min}$ contains the aglycone daidzein in its structure. To further structurally characterize $P12_{min}$, MS was employed. HPLC/MS data revealed that $P12_{min}$ had the same quasi-molecular ion as MDin with m/z 503 $[M + H]^+$. $P12_{min}$ carried only one positive charge as indicated by resolving the isotopic envelope, determining its molecular weight to be 502. Because both $P12_{min}$ and MDin have the same molecular weight and similar UV spectra, they are potentially positional or stereochemical isomers.

To characterize the differences in the structures between the isomers, fragmentation patterns obtained by ESI−MS/MS analysis of the quasi-molecular ions of both $P12_{min}$ and MDin, at a

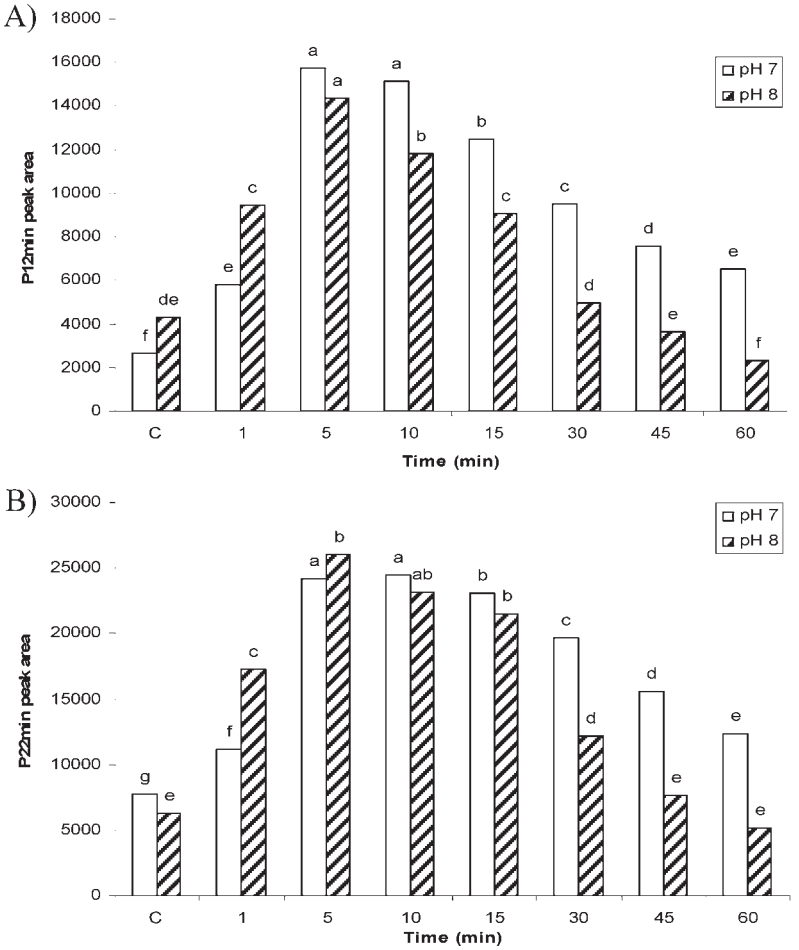

Figure 3. (A) Change in the profile of the unknown derivative $P12_{min}$ with an increasing treatment time. Data with different lowercase letters are significantly different within each pH across treatment times, according to Tukey−Kramer multiple means comparison test ($p \leq 0.005$; $n = 3$). (B) Change in the profile of the unknown derivative $P22_{min}$ with an increasing treatment time. Data with different lowercase letters are significantly different within each pH across treatment times, according to Tukey−Kramer multiple means comparison test ($p \leq 0.005$; $n = 3$).

relative collision level of 20%, were analyzed (panels **A** and **B** of **Figure 4**). The spectra for both $P12_{min}$ and MDin had m/z 255 as the base peak, representing the protonated form of the aglycone daidzein. The formation of the aglycone peak after fragmentation at an optimum collision level is a unique identifier for non-conjugated as well as conjugated isoflavones (*25*), thus confirming that both $P12_{min}$ and MDin have daidzein in their structure. Two ions with m/z 417 (protonated form of daidzin) and m/z 459 (protonated form of acetyldaidzin) were present in the fragmentation spectrum of MDin at lower relative abundance but were absent in that of $P12_{min}$. Additionally, at a collision level of 17%, it was noted that the ion with m/z 255 was formed more readily from the precursor ion of $P12_{min}$ compared to that of MDin (panels **C** and **D** of **Figure 4**). The relative abundance of m/z 503 ion was only 35 for $P12_{min}$ and close to 97 for MDin.

Detection and Characterization of an MGin Isomer. A peak that could not be assigned to any of the isoflavone standards was detected in several of the MGin treatment chromatograms and was labeled as $P22_{min}$ according to its elution time (**Figure 2B**). Running a MeOH/buffer blank confirmed that $P22_{min}$ was formed from MGin. Although the elution time of $P22_{min}$ is the same as that of the isoflavone acetylglycitin, $P22_{min}$ is not identical to acetylglycitin. The glycitin family of isoflavones does not have a hydroxyl group in the R2 position, and carries a methoxyl group at the R1 position (**Figure 1A**), which could hardly be formed under the treatment conditions employed for MGin and does not have a hydroxyl group in the R2 position. Confirmative to this assumption, the UV spectra of $P22_{min}$ and that of a glycitein isoflavone were considerably different.

The peak areas of $P22_{min}$ were significantly different between treatments (**Figure 3B**), indicating a time-dependent conversion of MGin to $P22_{min}$, with a maximum reached when heated for 5 min at both pH 7 and 8. A gradual and significant decrease in the peak areas of $P22_{min}$ was observed as the heating time increased from 15 to 60 min. Similar to what was observed for the MDin isomer $P12_{min}$, the formation and disappearance rates of $P22_{min}$ seemed higher at pH 8 than pH 7 (**Figure 3B**). This is in agreement with previous work showing that chemical conversions of isoflavones are enhanced at an elevated pH (*14*, *15*).

$P22_{min}$ and MGin had similar UV spectra with λ_{max} at 259 nm and quasi-molecular ions with m/z 519 $[M + H]^+$. Isotopic envelopes of MGin and $P22_{min}$ were similar and had similar peak spacing indicating that both compounds gained one positive charge, thus determining the molecular weight of $P22_{min}$ to be 518. ESI−MS/MS analysis revealed that the fragmentation spectra for both $P22_{min}$ and MGin had a base peak of m/z 271, corresponding to the protonated form of the aglycone genistein. However, the fragmentation spectrum of MGin had an ion with m/z 433 (protonated form of genistin), which was absent from that of $P22_{min}$ (panels **A** and **B** of **Figure 5**). Additionally, at a collision level of 17%, it was noted that the ion with m/z 271 was formed more readily from the precursor ion of $P22_{min}$ compared to that of MGin (panels **C** and **D** of **Figure 5**). The relative abundances of the ions m/z 519 and 271 were 100 and 30 and 35

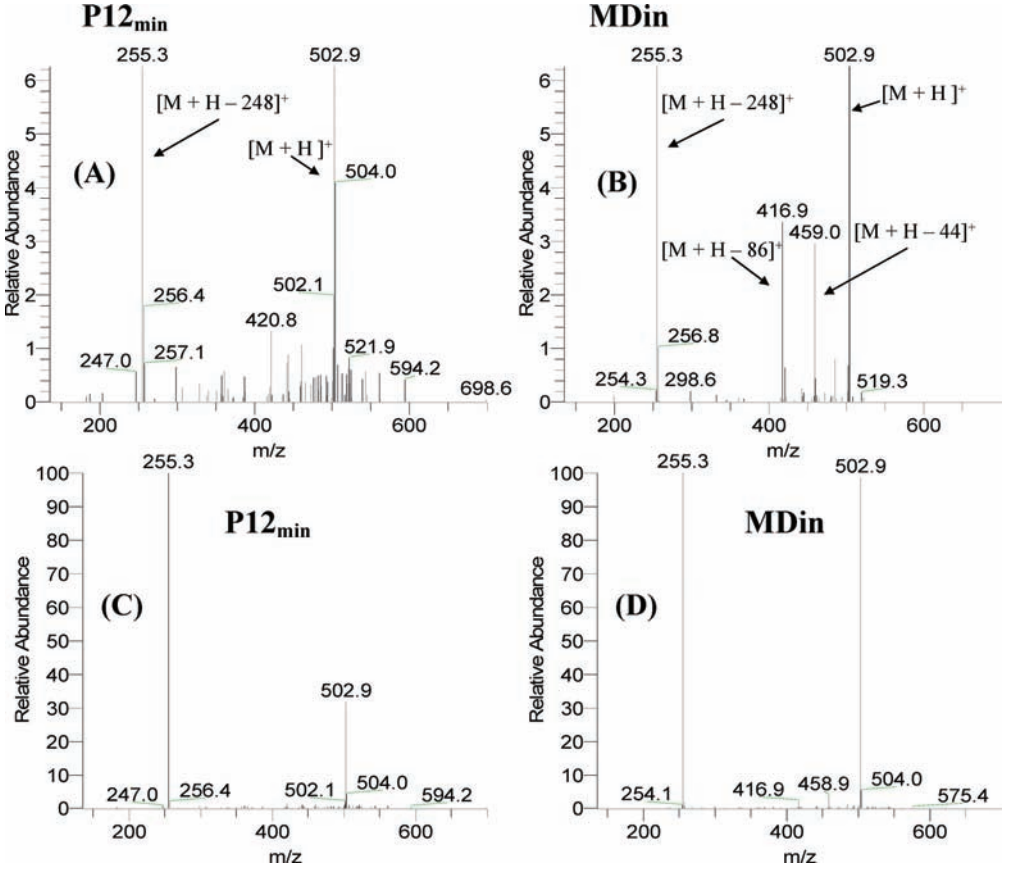

Figure 4. ESI−MS/MS analysis of the protonated forms of $P12_{min}$ and malonyldaidzin at various collision levels: (A) $P12_{min}$ at 20%, (B) malonyldaidzin at 20%, (C) $P12_{min}$ at 17%, and (D) malonyldaidzin at 17%.

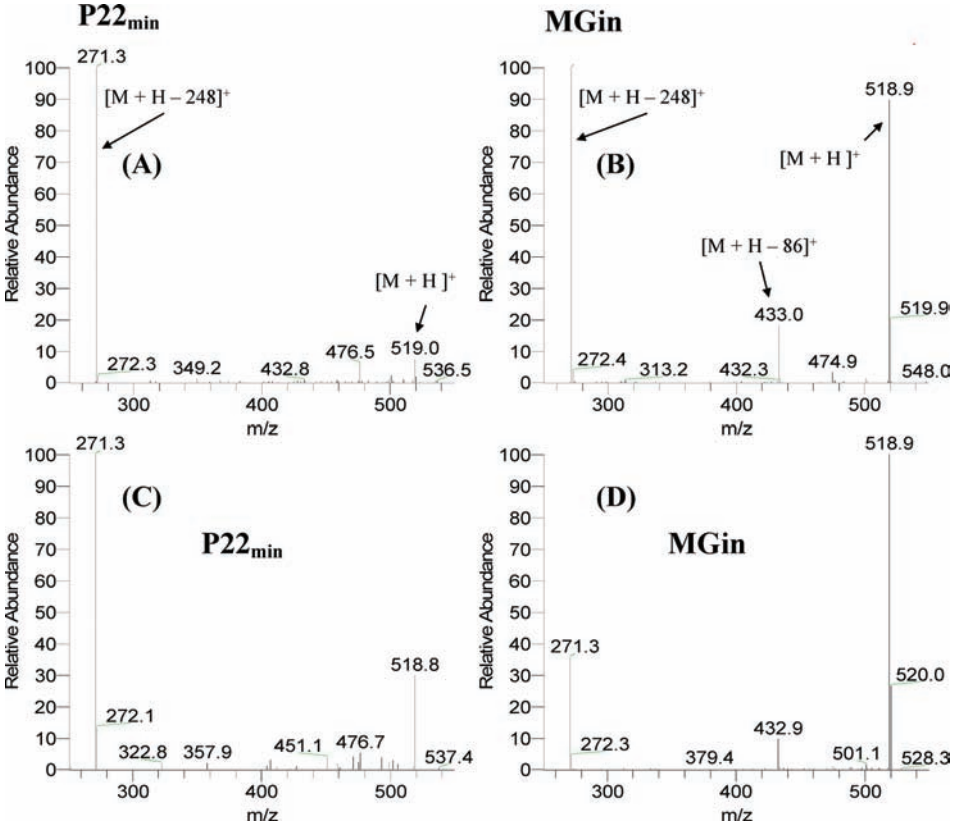

Figure 5. ESI−MS/MS analysis of the protonated forms of $P22_{min}$ and malonylgenistin at various collision levels: (A) $P22_{min}$ at 20%, (B) malonylgenistin at 20%, (C) $P22_{min}$ at 17%, and (D) malonylgenistin at 17%.

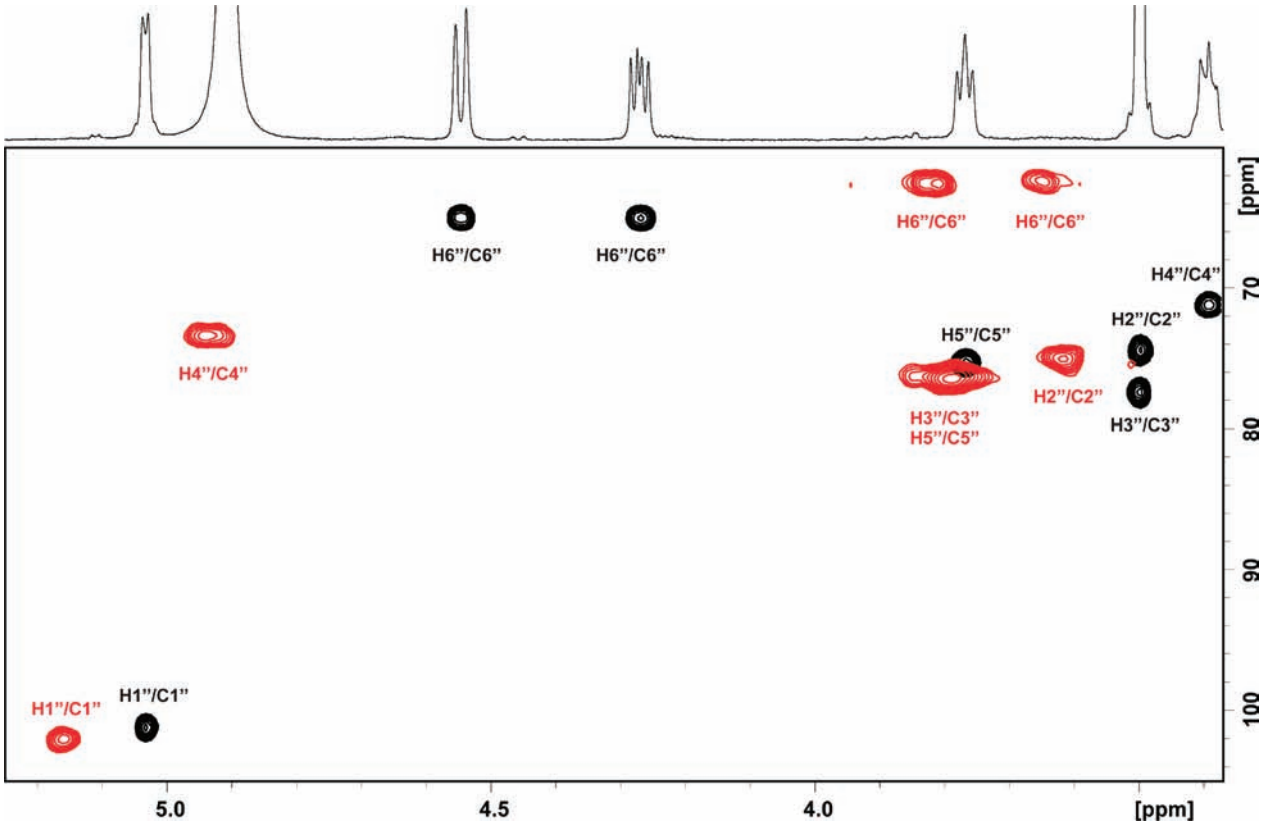

Figure 6. Overlay of the HSQC spectra (carbohydrate region) of malonyl-genistin (6″-*O*-malonyl-genistin) (black cross-peaks) and the malonyl-genistin isomer (4″-*O*-malonyl-gensitin) (red cross-peaks). The 1D proton spectrum represents 6″-*O*-malonyl-genistin.

and 100 for $P22_{min}$ and MGin, respectively. From these data, $P22_{min}$ and MGin were considered as either positional or stereoisomers. It is hypothesized that isomerization is due to migration of the malonyl group from the *O*-6-glucose position to the *O*-4-glucose position. A similar isomerization was recently demonstrated for formononetin glucoside malonate (*26*). For complete structural elucidation of the present isomer, NMR analysis was pursued. Because similar interconversions and fragmentation patterns were observed for MDin and MGin, further interconversion and structural elucidation work focused only on the MGin isomer.

Structural Elucidation of the MGin Isomer by NMR. NMR data for MGin (**Figure 1B**) were recorded in MeOH-d_4 and DMSO-d_6. The enhanced acidity of α-protons in β-dicarbonyls (keto−enol tautomerization) results in a proton−deuterium exchange in MeOH-d_4. Consequently, the malonyl-CH_2 group was not detected using MeOH-d_4 as a solvent. Using DMSO-d_6, which minimizes proton exchange, a complete NMR data set for MGin was obtained. Because of the large water signal in the carbohydrates in the diagnostic range of 3.30−3.45 ppm, the experiments for the isolated isomer were performed in MeOH-d_4 and the data were compared to that of MGin in MeOH-d_4.

Glucose proton signals were assigned using the H,H-COSY experiment. The linkage of the malonyl group to the glucose 6 position in MGin was demonstrated by cross-peaks at 4.55/168.7 and 4.27/168.7 ppm in the HMBC spectrum. The formation of an ester linkage at the glucose 6-position shifts the signals of the glucose 6-protons (4.55 and 4.27 ppm; **Figure 6**) and, although less dramatic, the 6-carbon (64.8 ppm) downfield, as also shown for 6″-*O*-acetylgenistin (*27*). The NMR spectra of the MGin isomer revealed its structure to be 4″-*O*-malonylgenistin (**Figure 1B**). The signal for the glucose 4-proton shifted downfield as did the glucose 4-carbon signal (**Figure 6**), indicating that the malonyl group is linked in this position. The HMBC spectrum shows a weak cross-peak at 4.90 and 169.0 ppm that, however, needs careful interpretation. Because the signal for the glucose 4-proton shifted extensively downfield (as also demonstrated in ref *26*), it is located underneath the water signal, making this region rather prone to fragments in the HMBC spectrum. The removal of the malonyl group from the glucose 6-position is well-demonstrated by the upfield shift of the glucose 6-proton signals (**Figure 6**). Considering all NMR experiments, the data demonstrated the malonyl group migration between positions 6 and 4 of the glucose moiety. After the first whole set of NMR experiments was performed, we recorded two more proton and HMBC spectra over the following 20 h. Especially in the proton spectra, a small but appreciable conversion from 4″-*O*-malonylgenistin (MGin isomer) back to the 6″-*O*-malonylgenistin (MGin) was noted.

Acyl migration was first noted in organic synthesis and frequently described for acetates in early and recent literature (*28*−*32*). As a general trend, migrations, which follow the direction glucose 1-position to glucose 6-position are more favored, with the 4 → 6 migration being frequently described (*31*). Intramolecular acyl migration is based on the formation of *ortho*-acid ester intermediates (*29*), which are five- or six-membered ring systems, requiring proper spatial relationships. Malonyl migration was less frequently described than acetyl migration (*26*, *33*, *34*). Wybraniec et al. (*34*) described 4 → 6 and 6 → 4 malonyl migrations on malonyl-betanins (malonyl group ester linked to a β-glucose unit) depending upon the pH conditions. In general, migrations were faster under alkaline conditions; e.g., 4 → 6 migration occurred almost instantly at pH 10.5 and 20 °C (*33*). As a result of these studies, the glucose 6-position was described as the most favored one for malonylation. Different from our experiments, the studies on 4′-*O*-malonyl-betanin and 6′-*O*-malonyl-betanin were only performed at 4 and 20 °C and not under heat. A 4 → 6 migration was also observed for the conjugated isoflavone

Table 3. Mean Amounts (nmol/mL) of MGin Isomer, MGin, Gin, Total Deteted Gin Derivatives, and Percent Loss in Detected Gin Derivatives in Samples of MGin Isomer Objected to Thermal Treatment at 100 °C and pH 8 for Treatment Times Ranging from 0 to 7 min

time (min)	isomer[a]	MGin[a]	Gin[a]	total Gin[b]	percent loss
0 (control)	4.57 a	0.20 d	0.00 e	4.77 a	0 c
1	0.50 b	3.45 b	0.26 d	4.20 c	11.8 a
3	0.29 c	3.78 a	0.50 c	4.57 ab	4.12 bc
5	0.24 d	3.15 bc	0.76 b	4.14 c	13.1 a
7	0.23 d	3.02 c	0.99 a	4.24 bc	11.2 ab

[a] Isomer, malonylgenistin isomer; Gin, genistin; MGin, malonylgenistin. [b] Total detected genistein derivatives (isomer + Gin + Mgin). Aglycone genistein was not detected. Means in each column with different lowercase letters are significantly different according to Tukey−Kramer multiple means comparison test ($p \leq 0.005$; $n = 3$).

formononetin glucoside malonate (*26*). A migration of the malonyl group to the glucose 6-position was noticed over the course of several hours in an acidic, aqueous MeOH medium while gathering NMR data for the isolated 4″-*O*-malonate isomer.

Thermal Stability and Chemical Conversions of the Isolated MGin Isomer (4″-*O*-Malonylgenistin). The MGin isomer was isolated on a semi-preparative scale, and identity and purity of the isomer fraction was determined using HPLC/PDA and HPLC/MS. The purity of the isolated MGin isomer was estimated to be ≥95%, with trace amounts of MGin contaminating the MGin isomer fraction.

Upon thermal treatment of the isomer, the formation of known genistein derivatives, namely, MGin and genistin, was observed. The formation of acetylgenistin or genistein was not observed. Considering the structural similarities between MGin and its isomer, the concentration of the isomer was determined using the calibration curve of MGin. In comparison to the control, a sharp decrease in the isomer concentration was observed after 1 min of thermal treatment (**Table 3**). The decrease in the isomer concentration was accompanied by a subsequent increase in MGin up to 3 min. As the heating time increased, a gradual decrease in MGin was observed along with a steady increase of genistin. On the basis of the observed conversion pattern of the isomer, two different scenarios are possible: (1) the isomer is converting to MGin, which undergoes de-esterification to form genistin, or (2) both the isomer and MGin are converting to genistin simultaneously. Additionally, the well-known de-esterification reaction of MGin to form genistin might also involve the formation of the isomer as an intermediate. The concentration of the isomer did not reach zero or decrease between 5 and 7 min of thermal treatment, indicating continuous interconversion between MGin and the isomer, with the forward reaction (isomer to MGin) occurring at a faster rate. To better characterize the interconversion mechanism, it is recommended to carry out thermal studies under mild heating conditions for extended periods of time.

The calculated percent loss in detected genistein derivatives reached ∼12% after 1 min of thermal treatment, dropped to ∼4% after 3 min, and then went back up to ∼11%. This trend in calculated loss indicated the formation of an intermediate derivative that escaped UV detection and with a longer heating time converted most likely to genistin. The increase in the percent loss after 5 and 7 min of thermal treatment could be attributed to complete degradation or the continuous formation and disappearance of the mentioned intermediate derivative. Further thermal studies at extended times (more than 7 min) and different temperatures are needed to characterize this phenomenon.

Interconversions between MGin and Its Isomer (4″-*O*-Malonylgenistin) in Thermally Treated Soymilk. The formation and interconversion of the MGin isomer was monitored in thermally treated soymilk. The formation of the MGin isomer and its interconversions in complex systems, such as soymilk, are potentially different from the reactions in model systems because interconversions can be influenced by various soy components, specifically soy proteins (*22*). The MGin isomer was detected in the control and all thermally treated samples. The identity of the MGin isomer as 4″-*O*-malonylgenistin in the soymilk samples was confirmed by the application of HPLC/MS and a comparison to the data of the isolated compound. As found in buffered systems, an increase in the MGin isomer was observed upon heating soymilk at 100 °C for 2 min, followed by a gradual decrease after 10 min of the thermal treatment (**Table 4**). Genistin formation started to occur after 10 min of thermal treatment and increased gradually thereafter. Insignificant changes were observed for acetylgenistin and genistein.

Table 4. Mean Amounts (nmol/g of Dry Weight) of MGin Isomer, MGin, Gin, AGin, and Total Detected Genistein Derivatives in Soymilk Samples Subjected to Thermal Treatment at 100 °C for Several Intervals of Time Ranging from 0 to 60 min

time (min)	isomer[a]	MGin[a]	Gin[a]	AGin[a]	Gein[a]	total Gin[b]
0 (control)	677.8 c	6235 a	1049 d	151.1 cd	125.2 a	8239 a
2	785.9 a	6224 a	1117 d	147.8 d	124.2 a	8399 a
5	762.5 ab	5626 b	1238 d	158.3 cd	114.4 ab	7899 ab
10	719.1 bc	5285 c	1647 c	172.1 c	107.7 b	7932 ab
30	622.5 d	4082 d	2968 b	236.6 b	119.3 b	8029 a
60	462.9 e	2657 e	3845 a	276.0 a	116.1 bc	7357 b

[a] Isomer, malonylgenisting isomer; Gin, genistin; MGin, malonylgenistin; AGin, acetylgenistin; Gein, genistein. [b] Total detected genistein derivatives (isomer + Gin + Mgin + Agin + Gein). Means in each column with different lowercase letters are significantly different across the treatment times according to Tukey−Kramer multiple means comparison test ($p \leq 0.05$; $n = 3$).

In the control sample, the MGin isomer concentration was approximately 8% of the total genistein derivatives. Under all treatment times employed, the MGin isomer represented 6−9% of the total genistein derivatives. Disregarding the MGin isomer 4″-*O*-malonylgenistin thus leads to at least a 6% (up to ∼15%, considering the MDin isomer as well) underestimation of the isoflavone concentration of a given soy food. For accurate determination of the total isoflavone content and any incurred loss in isoflavones because of processing, it is crucial to include the isomers into the analysis. Observed data indicated that the rate of interconversions between MGin and its isomer is greatly dependent upon processing temperature, time, and pH conditions. To better characterize the interconversions of the malonylglucosides and their isomers in complex systems, further studies are required covering wider ranges of temperature, pH, and time.

While the existence of isomers in soy matrices was reported earlier, the present work provided further structural characterization with full elucidation of the MGin isomer. We demonstrated for the first time that the reversible formation of the soy malonyl isomers is governed by processing parameters, namely, pH, temperature, and time. The rate of conversion of the MGin isomer to known isoflavone forms was relatively high when compared to the rate of conversion of malonylgenistin to the isomeric forms. Because the identified isomers can convert to potentially biologically relevant forms, it is crucial to include the isomers in the calculation of total isoflavone content, profile, and loss. Disregard of the isomer formation upon heating can result in overestimation of the loss in total isoflavone content and misinterpretation of the biological contributions.

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