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Stabilities of Daidzin, Glycitin, Genistin, and Generation of Derivatives during Heating†

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The soy isoflavones daidzin, glycitin, and genistin were purified from defatted soy flour using preparative-scale reverse-phase HPLC. The stabilities of the three isoflavones at different heating temperatures were investigated. Daidzin, glycitin, and genistin were lost at a rate of 26, 27, and 27% of their original concentration, respectively, after 3 min at 185 °C. At 215 °C, decreases of daidzin, glycitin, and genistin were 65, 98, and 74% after 3 min and 91, 99, and 94% after 15 min, respectively. The order of the thermal stabilities, from lowest to highest, was glycitin, genistin, and daidzin. Acetyl daidzin and acetyl genistin, daidzein, glycitein, and genistein were produced during heating at temperatures above 135 °C. The rate of binding of an acetyl group to form acetyl daidzin and acetyl genistin from daidzin and genistin was higher than the rate of loss of a glucoside group to form daidzein and genistein. However, acetyl daidzin and acetyl genistin decreased sharply at temperatures above 200 °C, while daidzein, glycitein, and genistein were relatively stable over 30 min. The stability of daidzein was higher than that of glycitein or genistein.

KEYWORDS: Isoflavones; soy; stability; heating; HPLC

INTRODUCTION

The health functions of soy isoflavones in preventing development of heart disease and cancers have been confirmed by many studies (*1*−*5*). Detailed chemical structures of soy isoflavones have been illustrated in Song et al. (*6*) and Franke et al. (*7*). Daidzein, glycitein, and genistein are the three basic chemical structures for aglycons of soy isoflavones. Also, three derivative forms from each aglycon are found in soybeans. Daidzin, glycitin, and genistin are the glucosides of daidzein, glycitein, and genistein containing a β-glucoside group. Acetyl daidzin, acetyl glycitin, acetyl genistin, malonyl daidzin, malonyl glycitin, and malonyl genistin are conjugates of the glucosides having either an acetyl or a malonyl β-glucoside.

The chemical structural differences of soy isoflavones may result in variable bioavailabilities in biological systems. Daidzein was found to have higher bioavailability than genistein in adult women (*8*). Aglycon soy isoflavones were absorbed faster and in higher amounts than their glucosides in a human study by Izumi et al. (*9*). However, the structures of soy isoflavones are not consistent during routine food processing. Factors induced in the food processing, such as enzymes in raw soy flour, heating, and additives, could affect the stabilities of soy isoflavones. Decarboxylation of malonate to acetate on the glucoside group and de-esterification of malonate or acetate to underivatized glucoside in soy isoflavones during soy food processing have been reported (*10*−*16*). Also, the glucoside group of soy isoflavone could be cleaved through the hydrolytic action of β-glucosidase or alkali to generate daidzein, glycitein, and genistein (*12*−*15*). Generally, the trend of degradation of soy isoflavone is from malonyl β-glucosides to acetyl β-glucosides to underivatized β-glucosides and finally loss of β-glucosides, yielding the basic aglycon structures of soy isoflavones.

Daidzin, glycitin, and genistin are the major form of isoflavone found in unprocessed soy flour, but they are the middle products in the degradation chain. They could be decomposed by losing the β-glucoside groups or produced by de-esterification from malonyl and acetyl glucoside soy isoflavones at the same time. Their relative thermal stabilities could not be clearly revealed in a study using soy flour or a product that potentially contains heat-induced precursors and degradation products. In this study, high-purity daidzin, glycitin, and genistin were prepared from soy flour, and their stabilities were observed during heating. This approach eliminated interferences such as precursors and enzymes, etc. The results could be useful in obtaining a more comprehensive understanding of the thermal stabilities of soy isoflavones.

MATERIALS AND METHODS

Chemicals. HPLC-grade methanol, ethanol, and hexane were purchased from Fisher Chemicals (Fair Lawn, NJ). Standards of daidzin, daidzein, glycitein, genistin, and genistein were from Sigma (St. Louis, MO).

Extraction of Soy Isoflavones. Soybeans obtained from Delta Grow Seed Co. (England, AR) were ground to flour, and the flour was defatted with hexane. A mixture of 100 mL of hexane and 50 g of the soy flour

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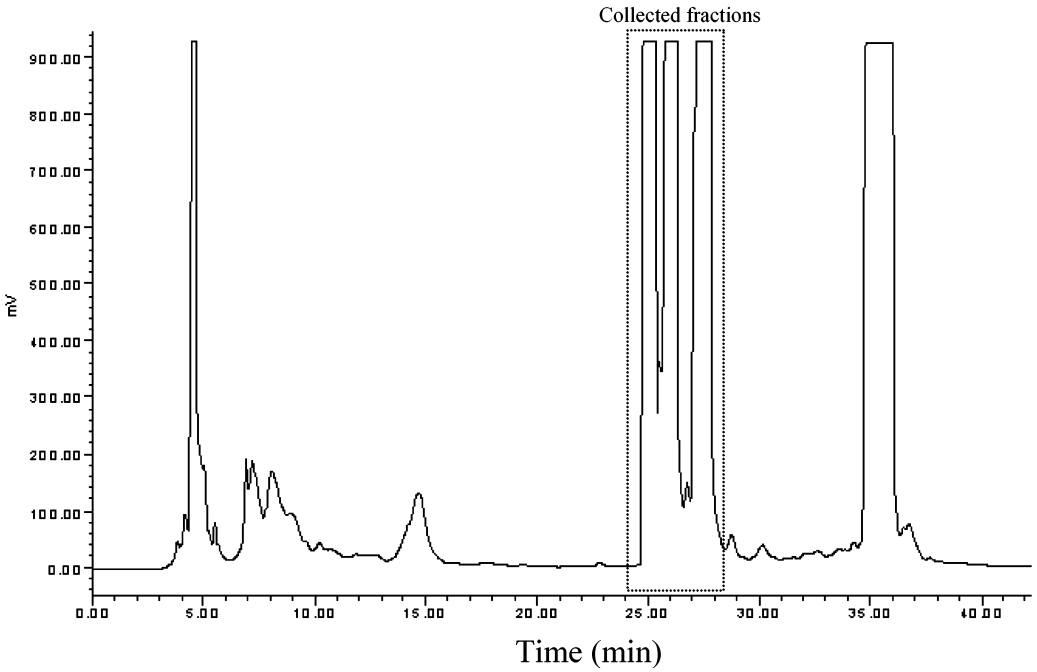

Figure 1. Chromatogram of crude soy isoflavone extract from reversed-phase preparative HPLC (column, C18; mobile phase, water/methanol 99:1 (v/v) for 20 min, 50:50 for 10 min, and 0:100 for 15 min; flow rate, 15 mL/min; UV detector, 254 nm).

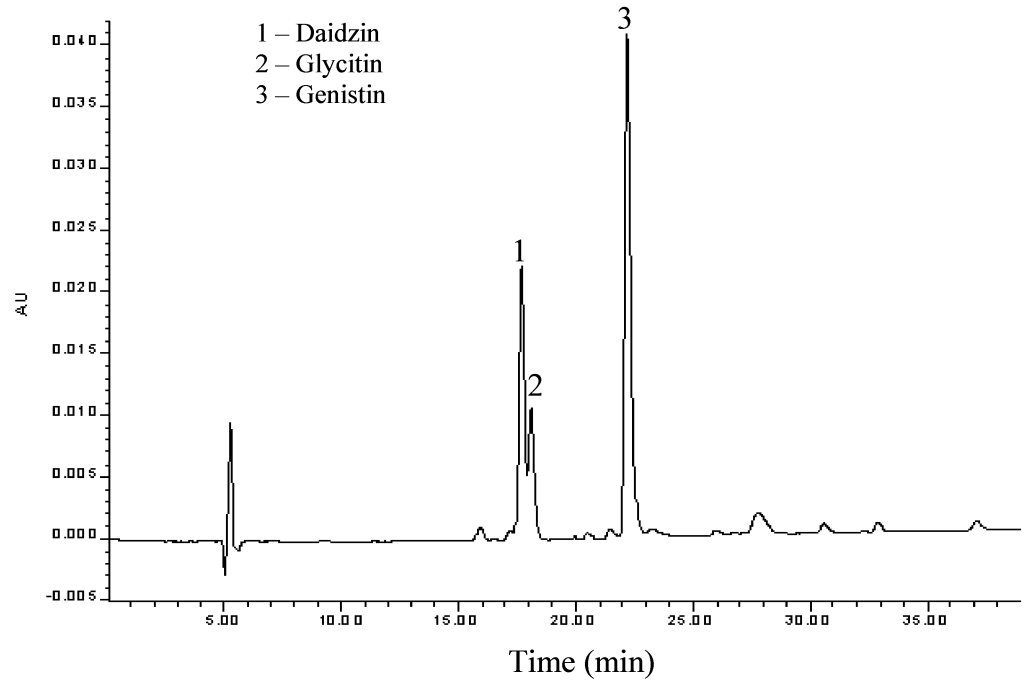

Figure 2. Chromatogram of high-purity daidzin, glycitin, and genistin from analytical reversed-phase HPLC (column, C18; mobile phase, water in methanol from 90% to 50% in 40 min; flow rate, 0.3 mL/min; UV detector, 254 nm).

was shaken at room temperature for 1 h. The supernatant was removed after the mixture was centrifuged at 1000*g* for 20 min. The defatted soy flour was dried under a hood overnight.

Methanol was used as solvent to extract isoflavones from the defatted soy flour. The defatted soy flour was mixed with 80 mL of methanol, with extraction conditions the same as those in defatting. The supernatant was placed under nitrogen flow at room temperature to evaporate the solvent until its volume was approximately 10 mL.

Purification of Daidzin, Glycitin, and Genistin Using Preparative-Scale Reverse-Phase HPLC. The preparative HPLC system consisted of a Waters (Milford, MA) PrePak RCM base packed with two 25-mm × 10-cm Prep Nova-Pak HR C18 (particle size 6 μm) cartridges and a Guard-Pak insert, a Delta Prep 4000 HPLC system, and a 481 LC spectrophotometer detector. Millennium32 Chromatography Manager (Waters) was used to record the chromatogram. The ratio of water to methanol in the mobile phase was 99:1 (v/v) for 20 min, 50:50 for 10 min, and 0:100 for 15 min. The flow rate of mobile phase was 15 mL/min, and the wavelength of the detector was set at 254 nm. One milliliter of isoflavone extract was loaded in the preparative HPLC system each time. The fractions of daidzin, glycitin, and genistin were collected and combined. Their identities were confirmed using analytical HPLC with comparison to authentic standards.

Sample Preparation and Heat Processing. The fraction obtained in the purification was divided into 1-mL aliquots and placed in test tubes. The test tubes were placed under nitrogen flow at room temperature to evaporate the mobile phase completely. This resulted in a residue of isoflavones that thinly coated the bottom of each test tube. The tubes were stored at −20 °C before use. Test tubes were

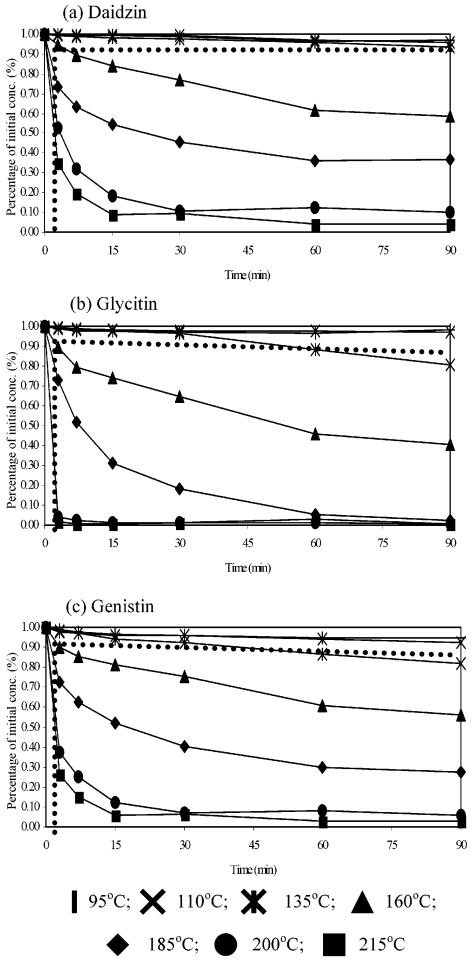

Figure 3. Percentage of the concentration changes of the three isoflavones at different heating temperatures and times. (Significant changes from initial are given to the right of and below the dotted line, $P < 0.05$.)

heated in an oil bath with a temperature controller. The temperatures were set at 95, 110, 135, 160, 185, 200, and 215 °C for each set of experiments. Two test tubes were randomly taken from the oil bath at 3, 7, 15, 30, 60, and 90 min.

Soy Isoflavones Analysis. Isoflavones in the sampled test tubes were diluted using 1 mL of methanol and quantified using an analytical HPLC system. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (3 mm i.d. × 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and Millennium32 Chromatography Manager. The mobile phase was a mixture of water and ethanol, with the percentage of water in ethanol ramped from 90% to 50% in 40 min with a constant flow rate of 0.3 mL/min. The chromatograms obtained at a wavelength of 254 nm were used to quantify the isoflavones. Glucosides and aglycons soy isoflavone peaks were verified by matching retention times with those of standards. Malonyl and acetyl conjugates were identified by comparison of UV absorption and retention time patterns with published HPLC methods (*6*, *7*, *12*, *13*).

Statistical Analysis. Each heating temperature was replicated two times. The ANOVA procedure (Excel Data Analysis, Microsoft Inc., Seattle, WA) was used to compare experimental data sampled at two different times for the same heating temperature.

RESULTS AND DISCUSSION

Purification of Daidzin, Glycitin, and Genistin Using Preparative-Scale Reversed-Phase HPLC. Although approximately 25% of soy isoflavones were lost in the hexane defatting procedure, the lipids of soy flour that could reduce the separation efficiency of preparative-scale HPLC were largely removed at the same time. To prevent any possibility of enzymatic hydrolysis that could break down the glucoside group of daidzin, glycitin, and genistin and lower their extraction yields, only methanol was used in the soy isoflavones extraction. **Figure 1** is the chromatogram of soy isoflavone extract in the preparative-scale HPLC. The three isoflavone fractions inside the dotted line frame were collected, and their chromatography in the analytical HPLC system is shown in **Figure 2**. Because most impurities were eliminated after purification in the preparative-scale HPLC, the three isoflavone peaks were the

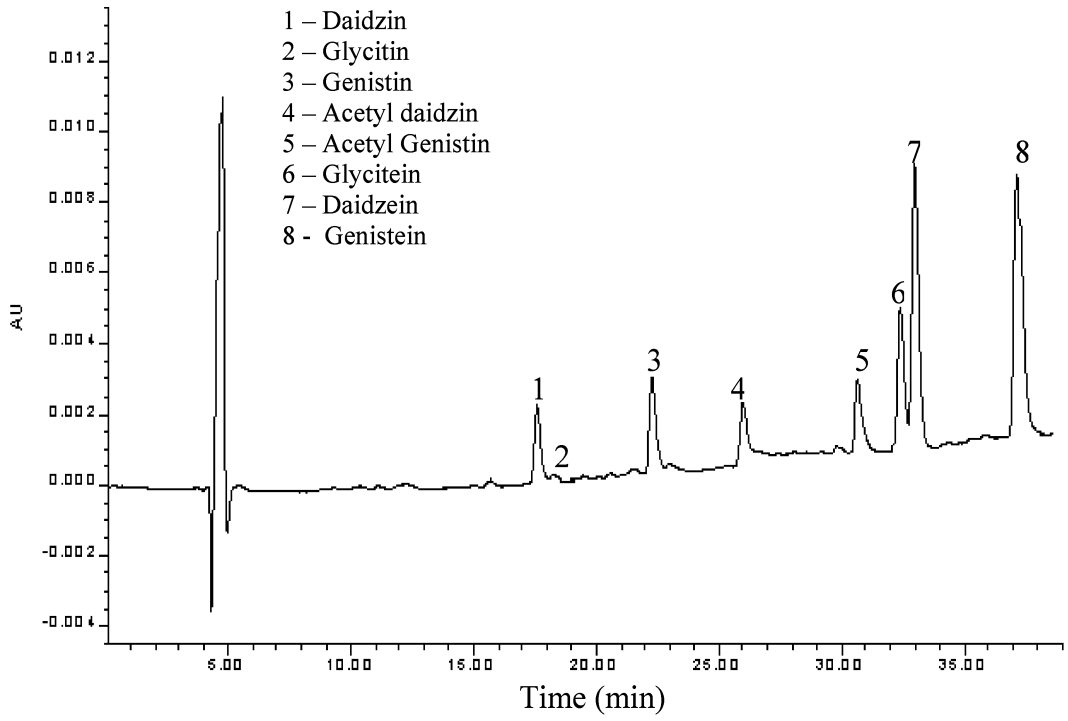

Figure 4. Chromatogram of high-purity daidzin, glycitin, and genistin heated at 200 °C for 15 min from analytical reversed-phase HPLC (column, C18; mobile phase, water in methanol from 90% to 50% in 40 min; flow rate, 0.3 mL/min; UV detector, 254 nm).

major peaks and occupied about 94% of the total peak area in the chromatogram.

Stabilities of Daidzin, Glycitin, and Genistin at Different Heating Temperatures. The concentrations of daidzin, glycitin, and genistin in each test tube were 21 ± 1, 20 ± 1, and 43 ± 2 μg/mL, respectively. The rates of degradation of the isoflavones at different heating temperatures and times are shown in **Figure 3**. At 95 and 110 °C, there was no significant change in the isoflavone concentrations within 90 min. This indicates that daidzin, glycitin, and genistin are stable at temperatures near the boiling point of water. Increasing concentrations of the three isoflavones during soy food processing were reported when soy flour was used as a sample in many studies (*10*−*14*, *16*). This would result from the de-esterification of malonyl and acetyl glucoside isoflavones to their underivatized glucosides by thermal hydrolysis. The stabilities of the three underivatized isoflavones could not be reflected directly in those studies because of the esterified glucoside isoflavones involved. Because malonyl and acetyl glucoside isoflavones were not initially present in the system used in this study, the factors influencing the concentrations of the three isoflavones are heating temperature and time only. From **Figure 3**, at 95 and 110 °C, it can be seen that the stabilities of the three isoflavones depended on their inherent heat resistance and not the dynamic balances between their degradation and production from their malonyl or acetyl forms.

At 135 °C, lower concentrations were found for glycitin and genistin after 60 min of heating and for daidzin after 90 min (**Figure 3**). The aglycon of glycitin contains −OCH_3 at the 6 position, and the aglycon of genistin contains −OH at the 5 position, each of which could contribute to molecular degradation during heating. The higher stability of daidzin may result from the lack of these groups at those positions. This suggestion could be supported by the results of Mahungu et al. (*13*), who found that the level of daidzin was stable while there was a decrease of genistin observed at similar temperatures.

When the heating temperature was increased above 160 °C, all three glucoside isoflavones decreased dramatically. The remaining concentration percentages of daidzin, glycitin, and genistin were 76.7, 64.8, and 75.6%, respectively, at 160 °C for 30 min. After 30 min at 185 °C, less than 50% of the original concentration was retained for these three isoflavones. A steady increase of these isoflavones at the expense of malonyl glucoside isoflavones was observed during both baking for 60 min and frying for 7.5 min at 190 °C (*12*). This may be because degradation of these isoflavones was less than their production from malonyl glucoside isoflavones.

The three isoflavones were dramatically decreased at heating temperatures above 200 °C. Glycitin, daidzin, genistin were reduced to 2.1, 35.1, and 26.0% within 3 min at 215 °C, respectively. Only about 10% of daidzin and genistin remained after 15 min at that temperature. The order of stabilities of the three isoflavones was glycitin, genistin, and daidzin, from lowest to highest.

Generating Products of Daidzin, Glycitin, and Genistin during Heating. Five products, daidzein, glycitein, genistein, acetyl daidzin, and acetyl genistin, were formed during heating at temperatures above 135 °C. **Figure 4** is the chromatogram of daidzin, glycitin, genistin, and their five isoflavone products after heating at 200 °C for 15 min. The molar concentration changes of the five generated products at different temperatures and times are shown in **Figure 5**. Increasing daidzein, glycitein, and genistein during high temperatures also was reported by others (*12*−*15*). It was suggested that β-glucosidase in the soy

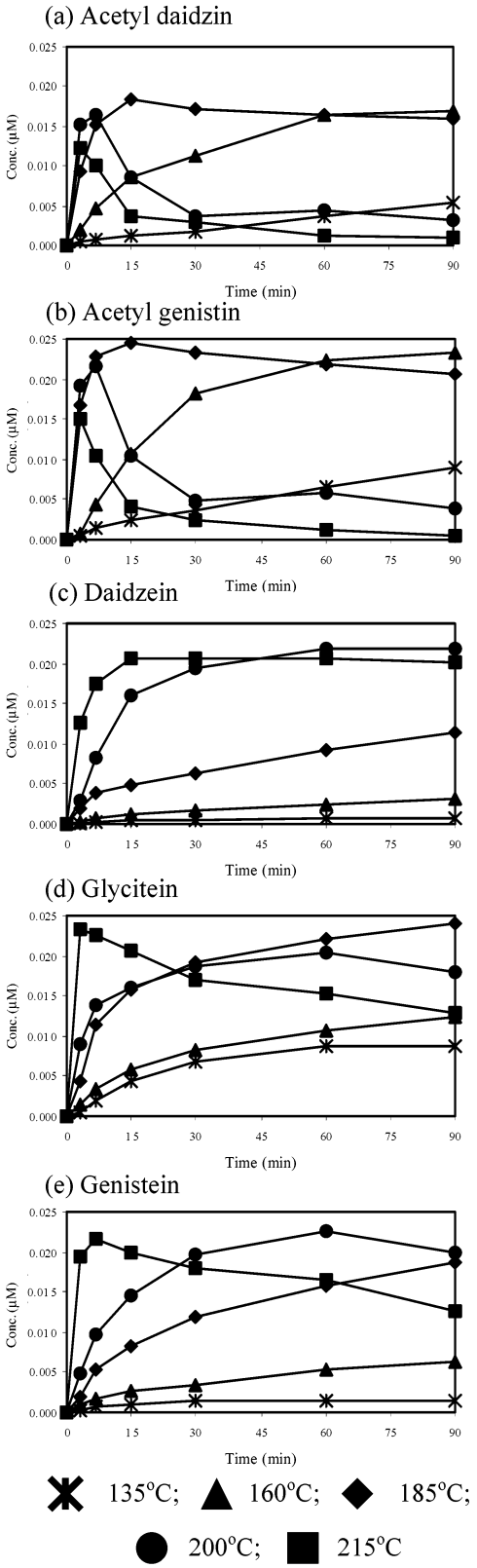

Figure 5. Concentration changes of isoflavones at different heating temperatures and times.

flour samples might be responsible for production of the aglycon isoflavones by removing glucoside groups. From **Figure 5c**−**e**, it can be seen that heating also could significantly break down glucoside groups to form these isoflavones. However, the relative production of glycitein and genistein was suppressed by their degradation after 60 min at 200 °C, while daidzein was stable until 90 min. At 215 °C, degradation started at 3, 7, and

15 min for glycitein, genistein, and daidzein, respectively. The order of their stabilities, from lowest to highest, was similar to that of glycitin, genistin, and daidzin.

An unexpected finding of this study was the generation of acetyl daidzin and acetyl genistin during heating at temperatures above 135 °C, although acetyl glycitin was not found (**Figure 5a**,**b**). Generation of acetyl daidzin and acetyl genistin during heating of soy flour is common and is believed to be due to heat-induced decarboxylation of malonyl daidzin and malonyl genistin (*12*−*15*). Because purified glucosides were used in this study, malonyl conjugates were not available for decarboxylation to acetyl conjugates. This suggests that acetyl groups, which could have been produced during thermal degradation of the original glucosides, may have acetylated daidzin and genistin during heating. Also, it was found that the generation rates of acetyl daidzin and acetyl genistin were higher than those of daidzein and genistein based on molar concentration (**Figure 5**). This indicates that the binding of the acetyl group may take place more readily than loss of the glucoside group in daidzin and genistin during heating. However, the generation of acetyl daidzin and acetyl genistin was rapidly suppressed due to degradation after 15 min at 185 °C, 7 min at 200 °C, and 3 min at 215 °C.

From this study, the thermal stabilities of daidzin, glycitin, and genistin were clearly revealed. The three isoflavones could resist heat-induced decomposition at temperatures near the boiling point of water in the absence of other factors, such as enzymes. They became unstable when heating temperatures were increased above 135 °C. They dramatically decreased at heating temperatures above 185 °C. Daidzein, glycitein, and genistein were readily produced when the glucosides daidzin, glycitin, and genistin were heated above 135 °C for 3 min. Also, it appears that acetyl daidzin and acetyl genistin may be generated from daidzin, glycitin, and genistin after their heat-induced decompositions were initiated. This may contribute to the observation in other heating studies that acetyl daidzin and acetyl genistin were highly stable and increased, while at the same time daidzin and genistin decreased during baking, frying, and high-temperature extrusion of soy flour. This information could be useful to producers of low moisture soy or soy isoflavones-supplemented food to maximize soy isoflavones during processing.

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