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ARTICLE

Changes of Isoflavone Profile in the Hypocotyls and Cotyledons of Soybeans during Dry Heating and Germination

Jian-Ping Yuan,*,†,‡ Yong-Bin Liu,‡ Juan Peng,† Jiang-Hai Wang,*,† and Xin Liu‡

†School of Marine Sciences, Sun Yat-Sen University, Guangzhou 510006, People's Republic of China, and ‡School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China

A gradient reversed-phase high-performance liquid chromatography (HPLC) method has been developed to be suitable for the separation and determination of 12 isoflavones in soybeans. Profiles of daidzein, genistein, glycitein, and their malonyl-, acetyl-, and nonconjugated β-glycosides were determined in cotyledons and hypocotyls of soybeans as affected by dry heating and germination. The results showed that the compositions and concentrations of isoflavones were remarkably different in the two parts of soybeans, and hypocotyls contained a much higher content of isoflavones than cotyledons (e.g., 7.8-fold higher). In hypocotyls, daidzein and its glycoside conjugates (59.6%) were the most abundant isoflavones, being followed by glycitein (26.6%) and genistein series (13.8%). In cotyledons, genistein and its glycoside conjugates (61.9%) were the main isoflavones, being followed by daidzein series (38.1%), and no glycitein series was found. Both hypocotyls and cotyledons contained remarkably high amounts of malonylglycosides (69.1 and 69.4%, respectively) and β-glycosides (27.1 and 25.4%), and only a very small quantity of aglycones (3.8 and 5.2%) and no acetylglycosides were detected. Acetylglycosides and β-glycosides were the thermal decarboxylation and deesterification products, respectively, of malonylglycosides, which were thermally unstable. The relative rates of decarboxylation and deesterification reactions were different in cotyledons and hypocotyls at different temperatures. During the process of germination, β-glycosides decreased, and malonylglycosides and aglycones increased, and then, malonylglycosides were the major fractions in germinating soybeans. Interestingly, the present study occasionally found a significant circadian change between malonylglycosides and aglycones with a nocturnal increase of aglycones and decrease of malonylglycosides during germination, and even aglycones became the most abundant forms at night. However, this mechanism is yet to be investigated.

KEYWORDS: Isoflavone; soybean; cotyledon; hypocotyl; dry heating; germination; HPLC

INTRODUCTION

Isoflavones, which are particularly abundant in soybeans [*Glycine max* (L.) Merrill], serve as signal molecules in the induction of microbial genes involved in soybean nodulation and also as precursors for the production of phytoalexins during plant microbe interactions (*1*, *2*). In the soybeans, free isoflavones undergo 7-*O*-glycosylation and subsequent 6″-*O*-malonylation catalyzed by glycosyltransferase and malonyltransferase, respectively, to yield malonylglycosides, which are then transported to and accumulate in vacuoles as latent forms to serve as large isoflavonoid pools (*2*−*4*). Glycosylation plays a very important role in solubilization, accumulation in vacuoles, and mobilization of isoflavonoids in legume cells (*4*), and malonylation protects glycosides from enzymatic degradation by glycosidases and helps in their intracellular transport (*2*). Therefore, malonylated products are the most abundant forms of isoflavonoids in soybeans. These isoflavone conjugates must ultimately be converted to aglycones for interactions with symbiotic or pathogenic microorganisms (*3*).

The profiles and concentrations of isoflavones in soybeans or soybean-processed foods may vary considerably depending upon variety, location, growing season, climate, cultivation practice, storage, and even processing conditions (*5*−*7*). The main isoflavones found in soybeans and soy foods exist in four chemical forms (**Figure 1**): as the aglycone forms (daidzein, genistein, and glycitein), the β-glycoside forms (daidzin, genistin, and glycitin), the malonylglycoside derivatives (6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin, and 6″-*O*-malonylglycitin), and the acetylglycoside derivatives (6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin, and 6″-*O*-acetylglycitin) (*8*−*12*). The malonylglycoside forms were present in the highest level, followed by the glycosides, the aglycones, and the acetylglycosides (*13*). Although the malonylglycosides are the predominant isoflavones in soybeans (*14*), the concentrations of the acetylglycoside, β-glycoside, and aglycone forms tend to increase during the commercial processing of

*To whom correspondence should be addressed. (J.-P.Y.) Tel: +86-20-84112299. Fax: +86-20-84112005. E-mail: yuanjp@mail.sysu.edu.cn. (J.-H.W.) Tel: +86-20-84112296. Fax: +86-20-84037249. E-mail: wangjhai@mail.sysu.edu.cn.

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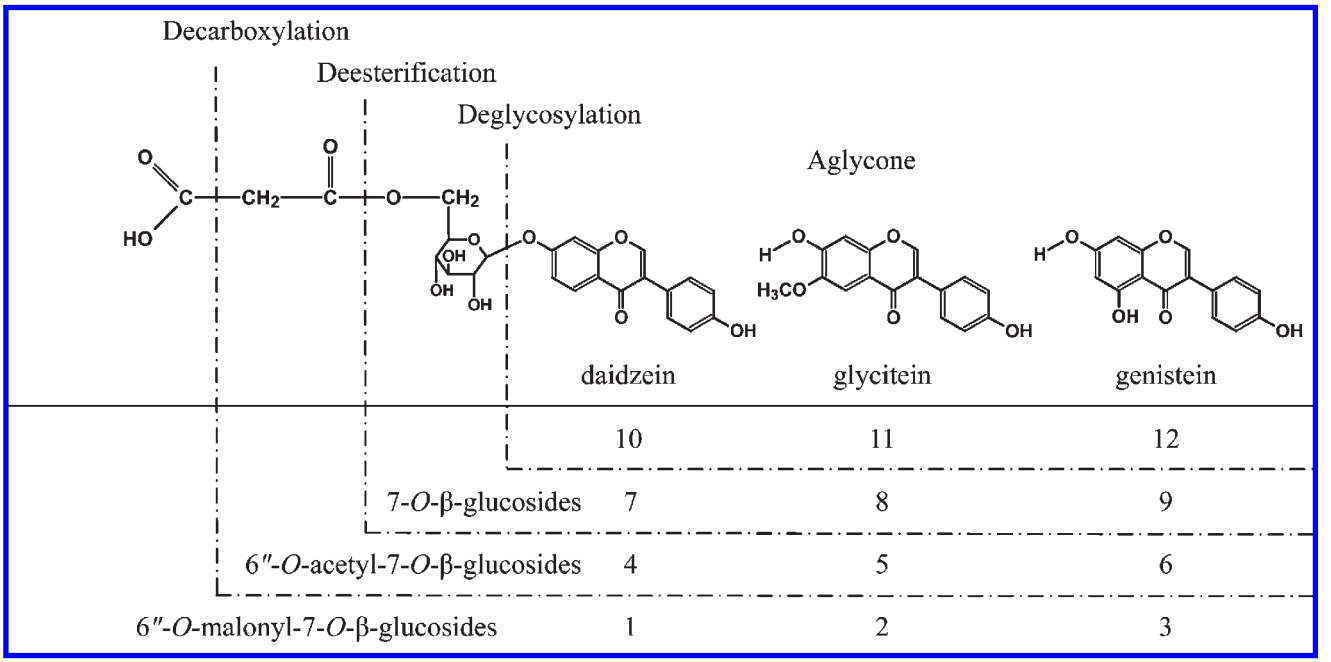

Figure 1. Structures and HPLC peak numbers of 12 isoflavones.

soybean into food products (*5*, *7*, *8*, *14*, *15*). There was a significant impact on the retention and distribution of isoflavones as a result of different processing methods. The malonylglycosides decreased dramatically with an increase in β-glycosides and aglycones after thermal processing (*16*). In addition, Phommalth et al. (*17*) found that the isoflavone content in the germinated soybeans could be increased by optimizing light treatments.

There is an increasing consumption of soybean worldwide due to its nutritional properties and the beneficial characteristics of isoflavones with estrogenlike structures and actions (*17*). Studies have shown that individuals with soybean-rich diets have significantly lower occurrences of some cancers, osteoporosis, and coronary heart disease in comparison with individuals with low soybean diets (*18*). The bioavailability of isoflavones can be affected by their chemical forms and their stabilities during processing (*19*), and deglycosylation of isoflavones may significantly affect their bioavailabilities for humans (*20*). The results have shown that the glycosides of isoflavones exhibit a greater bioavailability than their aglycones when ingested in an isolated form (*21*, *22*).

The recent study by Berger et al. (*23*) clearly showed that isoflavone synthesis and accumulation resulted from highly differentiated pathways in cotyledons and hypocotyls. To date, a very few studies have been reported on isoflavone compositions and changes in cotyledons and hypocotyls of soybeans. The major objective of the present work is to evaluate the variation of the isoflavone conjugate composition in both cotyledons and hypocotyls during dry heating and germination. In the present study, a gradient reversed-phase high-performance liquid chromatography (HPLC) method has been developed for the simultaneous separation and determination of 12 isoflavones. This HPLC method is suitable to monitor the changes of the isoflavone conjugate types in cotyledons and hypocotyls.

MATERIALS AND METHODS

Materials and Reagents. The soybeans used in this study were one yellow cultivar, purchased from a local market. Daidzein (98% purity) and genistein (98% purity) were purchased from Sigma (St. Louis, MO). Glycitein (99% purity), glycitin (99% purity), genistin (99% purity), and equol (99% purity) were purchased from LC Laboratories (Woburn, MA). Daidzin (98% purity) was purchased from ChromaDex, Inc. (Santa Ana, CA). HPLC-grade methanol was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Acetic acid used for this experimental work was of analytical grade. Water was purified using a Millipore Simplicity system (Billerica, MA).

Heat Processing of Soybeans. Soybeans were heated in a convection oven at 90, 110, 130, and 150 °C. Heated soybean samples were taken at various time intervals and analyzed for isoflavone distribution. Heat treatment experiments were repeated three times at the four temperatures. Samples were stored frozen at −20 °C until analyzed. The hypocotyls were separated from the cotyledons, and the seed coat was discarded.

Germination of Soybeans. Soybeans were soaked in distilled water for 5 h. The soaked soybeans were drained, placed on a culture dish on which moist quadruple layer gauze pads had been laid, and germinated under natural daylight and noncontrolled conditions starting at 10:00 p.m. The culture dishes were simply placed in front of a glazed window. To maintain an adequate hydration level, soybeans on gauze pads were sprinkled with distilled water 4−5 times a day. The germinating soybean samples were taken every 12 h during germination, dried in an oven at 70 °C, and stored frozen at −20 °C until analyzed. The hypocotyls were separated from the cotyledons, and the seed coat was discarded.

Extraction of Isoflavones. The raw soybean samples were carefully divided into three parts: hypocotyls, cotyledon, and seed coat, which were ground into powder, respectively, with the help of a mortar and pestle. A 0.05 g amount of the hypocotyl powder, a 0.2 g amount of the cotyledon powder, or a 0.2 g amount of the seed coat powder was accurately weighed and extracted with 10 mL of 40% aqueous methanol with the addition of a 0.5 g amount of $MgCl_2$ followed by sonication with an ultrasonic bath for 10 min and mixing up for 5 min, and then centrifugation at 10000*g* for 5 min. It has been reported that for effective extraction of isoflavones from soybeans, a certain amount of water (40−60%) in the extraction solvent was necessary to increase the propagation of ultrasonic waves, especially when the extraction was performed at lower temperatures (*24*). The extraction procedure was repeated three times, and the total extract was sampled for HPLC analysis to determine isoflavones.

HPLC. HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector from Waters Corp. (Milford, MA). Extracts were separated and analyzed by using a Waters Symmetry C18 column (4.6 mm × 250 mm, 5 μm) and a guard column (4.6 mm × 12.5 mm, 5 μm) at 30 °C. The mobile phase consisted of solvent A (0.5% of acetic acid in water) and solvent B (methanol). Used was the following linear gradient procedure: 0−7 min, 34% of B; 7−10 min, 34−38% of B; 10−15 min, 38% of B; 15−35 min,

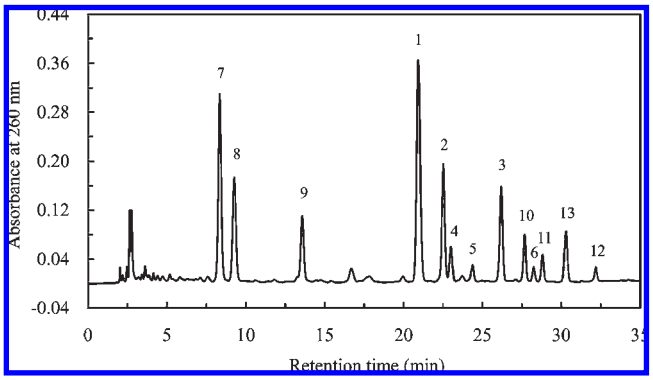

Figure 2. Typical HPLC chromatogram of 12 isoflavones (peaks 1−12; for identification of these peaks, see **Figure 1**) in the dry heating hypocotyl extract with addition of an equol standard (peak 13).

38−65% of B; and back to 34% of B within 1 min. The system was equilibrated with 34% of B for 10 min at the end of each run. The flow rate was 1.0 mL/min. The detecting wavelength was set between 210 and 400 nm, and the chromatographic peaks were measured at a wavelength of 260 nm to facilitate the detection of isoflavones. Aliquots of 20 μL were directly injected into the HPLC for the determination.

Method Validation. The stock standards of daidzein, genistein, glycitein, daidzin, genistin, and glycitin were prepared at 300 mg/L, and the additional calibration levels were prepared by a serial dilution with ethanol. The standard calibration curves were constructed using these standard solutions. The linear regression analysis was carried out by plotting the peak areas (A) against the concentrations (C) of these standard solutions in Microsoft Excel 2003. The linearity was demonstrated by a correlation coefficient (r^2) greater than 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. The isoflavone concentrations were calculated based on peak areas using calibration curves. Three replicate determinations of the dry heating hypocotyl extract with the addition of an equol standard were performed to evaluate precision, which was calculated as the relative standard deviation (RSD) for the repeated measurements. For recovery studies, known volumes of standard solutions were added to soybean powder. The spiked samples were extracted and analyzed following the described procedure. Background levels were subtracted in all recovery determinations.

RESULTS AND DISCUSSION

HPLC Analysis of Isoflavones. This study developed an HPLC method for the separation and determination of isoflavones extracted from the hypocotyls and cotyledons of soybeans. A Waters Symmetry C18 column, a mixed solvent of methanol and acetic acid aqueous solution as the mobile phase, and the gradient elution procedure were used to separate the 12 isoflavones (**Figure 1**). Although Kao et al. (*25*) suggested that the addition of a modifier such as acetic acid did not improve the separation of isoflavones, the present results showed that the addition of acetic acid (0.5%) to the mobile phase could improve the separation of isoflavones. **Figure 2** shows the typical HPLC chromatogram of the 12 isoflavones in the dry heating hypocotyl extract with adding an equol standard (peak 13), a metabolite of daidzein by human intestinal microflora (*26*), which is eluted between glycitein and genistein from the column and does not interfere with the separation of the other isoflavones.

Isoflavones all absorb UV light, and UV detection may offer sufficient selectivity and sensitivity for the determination of isoflavones. The identifications of isoflavones were achieved by comparing their retention times and spectra against the known standards. However, no malonyl and acetyl form standards were available in the present work, but the lack of these standards did not hinder the identification of these isoflavones. Using a photodiode array detector, peaks were identified by taking the spectra of each peak during elution. These isoflavones may be identified by comparing absorption spectra with those of daidzin, glycitin, and genistin because of the similarities of extinction coefficients (*25*, *27*), and many authors have also used the standard curve for glycoside isoflavones to quantify malonyl and acetyl isoflavones (*25*). Campos et al. (*27*) suggested that the malonyl derivatization of the 7-*O*-glucose did not change the absorption, but the retention time was increased. It has been established that the chromatographic behavior of different isoflavone forms on reversed-phase columns was based on their hydrophobicity (aglycone > acetylglycoside > malonylglycoside > β-glycoside) in the presence of an acid in the mobile phase to protonate the malonyl forms (*28*). The calibration curves of the peak area (A) against the concentration (C) for these isoflavone standards at 260 nm gave good linear responses over a wide range (5−300 mg/L) of concentrations (**Table 1**). The concentrations of malonylglycosides and acetylglycosides were calculated from the standard curves of their corresponding β-glycosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and their β-glycosides (*14*). The precisions were determined, and the results are shown in **Table 2**. For recovery, a known amount of isoflavones was spiked into an accurately weighed portion of the soybean powder. The recoveries are also shown in **Table 2**. The data indicated that this HPLC method was sensitive for qualitative and quantitative determination of these isoflavones.

Table 1. Linear Regression Data of Isoflavones at 260 nm

isoflavone	λ max (nm)	linear regression equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
daidzein	248.5	$y = 113646x + 70751$	0.9993	0.006	0.020
genistein	260.3	$y = 165088x + 38869$	0.9994	0.004	0.014
glycitein	257.9	$y = 104990x + 105882$	0.9997	0.007	0.022
daidzin	249.7	$y = 79241x + 188930$	0.9987	0.009	0.029
genistin	260.3	$y = 118702x - 65955$	0.9993	0.006	0.020
glycitin	257.9	$y = 75914x - 102634$	0.9995	0.009	0.031
equol	281.6	$y = 22378x - 57596$	0.9996	0.032	0.105

Table 2. Precision for the Determination of 12 Isoflavones and Equol and Recoveries for the Assay of Six Isoflavones and Equol in Extract[a]

analyte	original (mg/g)	RSD (%)	spiked (mg/g)	found (mg/g)	recovery (mg/g)	RSD (%)
malonyldaidzin	5.1175	1.5				
acetyldaidzin	2.2012	3.7				
daidzin	4.5730	1.6	3.0	7.5910	100.6	2.7
daidzein	0.4025	3.4	3.0	3.3545	98.4	2.4
malonylgenistin	1.1384	1.2				
acetylgenistin	0.4900	2.8				
genistin	0.9393	1.8	3.0	3.9123	99.1	1.8
genistein	0.0822	4.4	3.0	3.0732	99.7	2.3
malonylglycitin	1.9149	3.7				
acetylglycitin	1.0209	3.3				
glycitin	2.4726	2.4	3.0	5.5206	101.6	3.4
glycitein	0.1370	3.8	3.0	3.1010	98.8	2.8
equol	0.2514 [a]	2.1	3.0	3.2574	100.2	1.6

[a] By adding.

Distribution of Isoflavones in Soybeans. The hypocotyl, cotyledon, and seed coat samples of raw soybean, which were composed of 89.84 ± 0.06% cotyledon, 2.25 ± 0.02% hypocotyl, and 7.91 ± 0.05% seed coat, were analyzed by the developed HPLC method. The result showed that the contents of isoflavones varied in different parts of soybeans and that the distribution of isoflavones in hypocotyl, cotyledon, and seed coat had obvious differences.

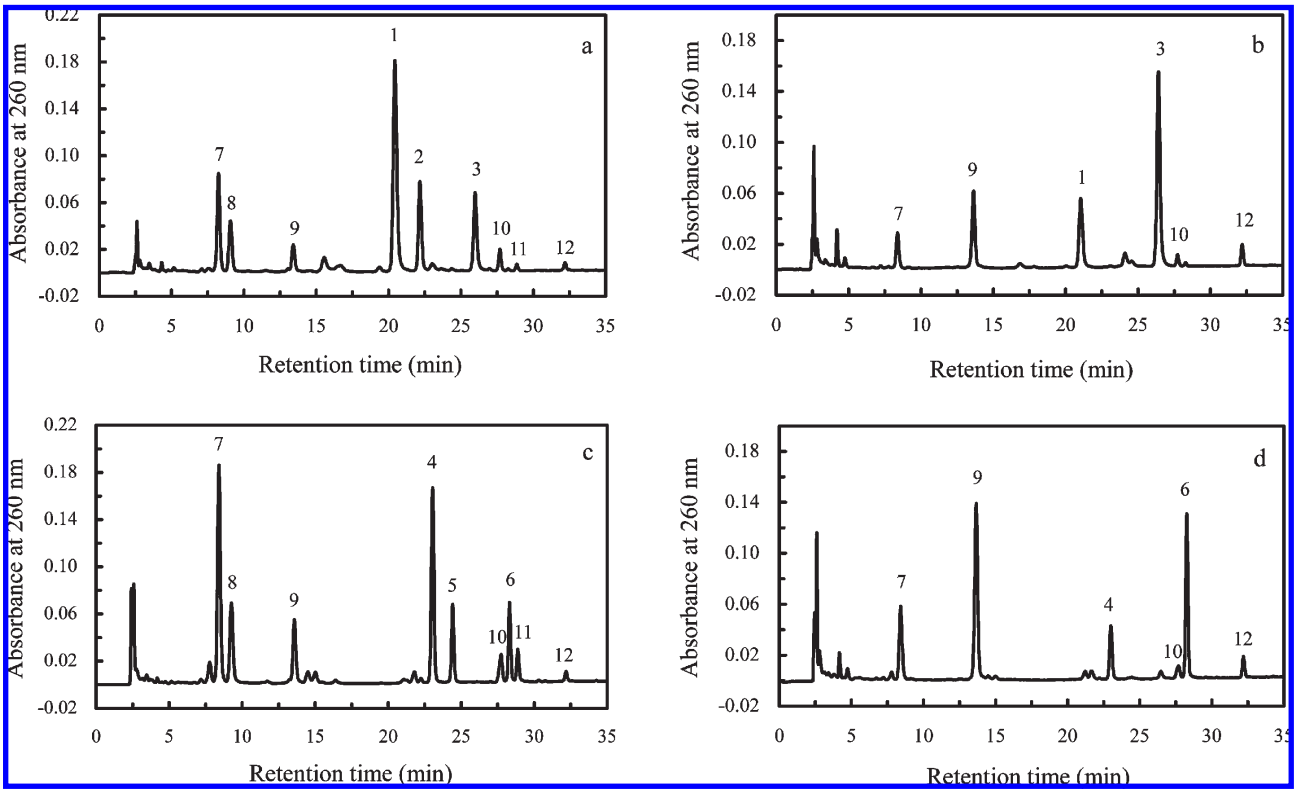

Figure 3. Typical HPLC chromatograms of the raw hypocotyl (**a**), the raw cotyledon (**b**), the fully heated hypocotyl (**c**), and the fully heated cotyledon (**d**) extracts. For identification of peaks, see **Figure 1**.

Table 3. Results of Isoflavone Analysis for the Individual Forms of Each Isoflavone and the Total Isoflavones in Hypocotyls, Cotyledons, and Seed Coat before and after Heat Processing[a]

	hypocotyls (mg/g)		cotyledons (mg/g)		seed coat (mg/g)
isoflavone	raw	heated	raw	heated	raw
malonyldaidzin[b]	8.5856	–	0.6652	–	0.0028
acetyldaidzin[b]	–	5.4663	–	0.3576	–
daidzin	2.9926	6.4743	0.2622	0.5429	0.0013
daidzein	0.3135	0.6212	0.0405	0.0751	0.0022
malonylgenistin[b]	2.0306	–	1.1199	–	0.0043
acetylgenistin[b]	–	1.3131	–	0.6617	–
genistin	0.5846	1.3701	0.3916	0.9021	0.0018
genistein	0.0755	0.1054	0.0428	0.0578	0.0024
malonylglycitin[b]	3.2166	–	–	–	–
acetylglycitin[b]	–	2.1155	–	–	–
glycitin	1.8305	2.8967	–	–	–
glycitein	0.0856	0.6563	–	–	–
total isoflavones[c]	12.3926	13.5427	1.5943	1.6601	0.0109
daidzein (%)	59.6	58.6	38.1	38.6	43.1
genistein (%)	13.8	13.1	61.9	61.4	56.9
glycitein (%)	26.6	28.3	0	0	0
malonyl (%)	69.1	0	69.4	0	40.4
acetyl (%)	0	40.6	0	38.1	0
β-glycoside (%)	27.1	49.2	25.4	53.9	17.4
aglycone (%)	3.8	10.2	5.2	8.0	42.2

[a] The mean values of three determinations are presented and the RSDs <5%. –, not detected. [b] Malonyl- and acetyl-glycosides were quantitated as glycosides. [c] Malonyl-, acetyl-, and nonconjugated β-glycosides were quantitated as aglycones, and the total isoflavones were expressed as aglycone forms (i.e., totals of genistein, daidzein, or glycitein).

Figure 3a,**b** show the HPLC chromatograms of the raw hypocotyl and cotyledon extracts, respectively. **Table 3** presents the results of isoflavone analysis for the individual forms of each isoflavone and the total isoflavones. Isoflavone contents were expressed in mg/g of sample on a dry weight basis. Malonyl- and acetyl-glycosides were quantitated and expressed as glycosides, and the total isoflavones were expressed as the free isoflavone forms (i.e., totals of genistein, daidzein, or glycitein) (*29*).

As shown in **Table 3**, total isoflavone concentrations were 12.39 mg/g in the hypocotyl, 1.59 mg/g in the cotyledon, and 0.01 mg/g in the seed coat. The results showed that soybean hypocotyl contained remarkably high amounts of total isoflavones and appeared to be the best source of isoflavones (*10*). It has been reported that hypocotyl was the part of the soybean that contained the highest concentration of isoflavones (*30*), and the concentration of isoflavones in the hypocotyl was 14.0−17.5 mg/g (*31*). In contrast, only a small quantity of total isoflavones was found in cotyledons. The present result is in agreement with the previous reports that hypocotyls contained isoflavone concentrations 6−10-fold higher than that found in cotyledons (*28*). However, Ribeiro et al. (*32*) found that the total isoflavone content in the hypocotyls (10.72 mg/g) was 25.2-fold higher than that in the cotyledons (0.43 mg/g) in BRS 213 soybean cultivar. Furthermore, the present results showed that the seed coat contained considerably lower amounts of isoflavones (0.01 mg/g) as compared to other parts of the soybean (*10*,*31*). Eldridge and Kwolek (*31*) showed that the concentration of the isoflavones was lowest in the seed coat (0.1−0.2 mg/g), whereas Ribeiro et al. (*32*) did not detect isoflavones in the seed coat.

Among the individual isoflavones, malonyldaidzin, malonylglycitin, malonylgenistin, daidzin, and glycitin were the major fractions (~93.2% of total isoflavones) in the hypocotyl. Only a small amount of genistin and daidzein and a very small amount of genistein and glycitein were detected, and no acetyldaidzin, acetylgenistin, and acetylglycitin were found in the hypocotyl (**Figure 3a**). In the cotyledon, malonylgenistin, malonyldaidzin, genistin, and daidzin were the dominant isoflavones (~94.8% of

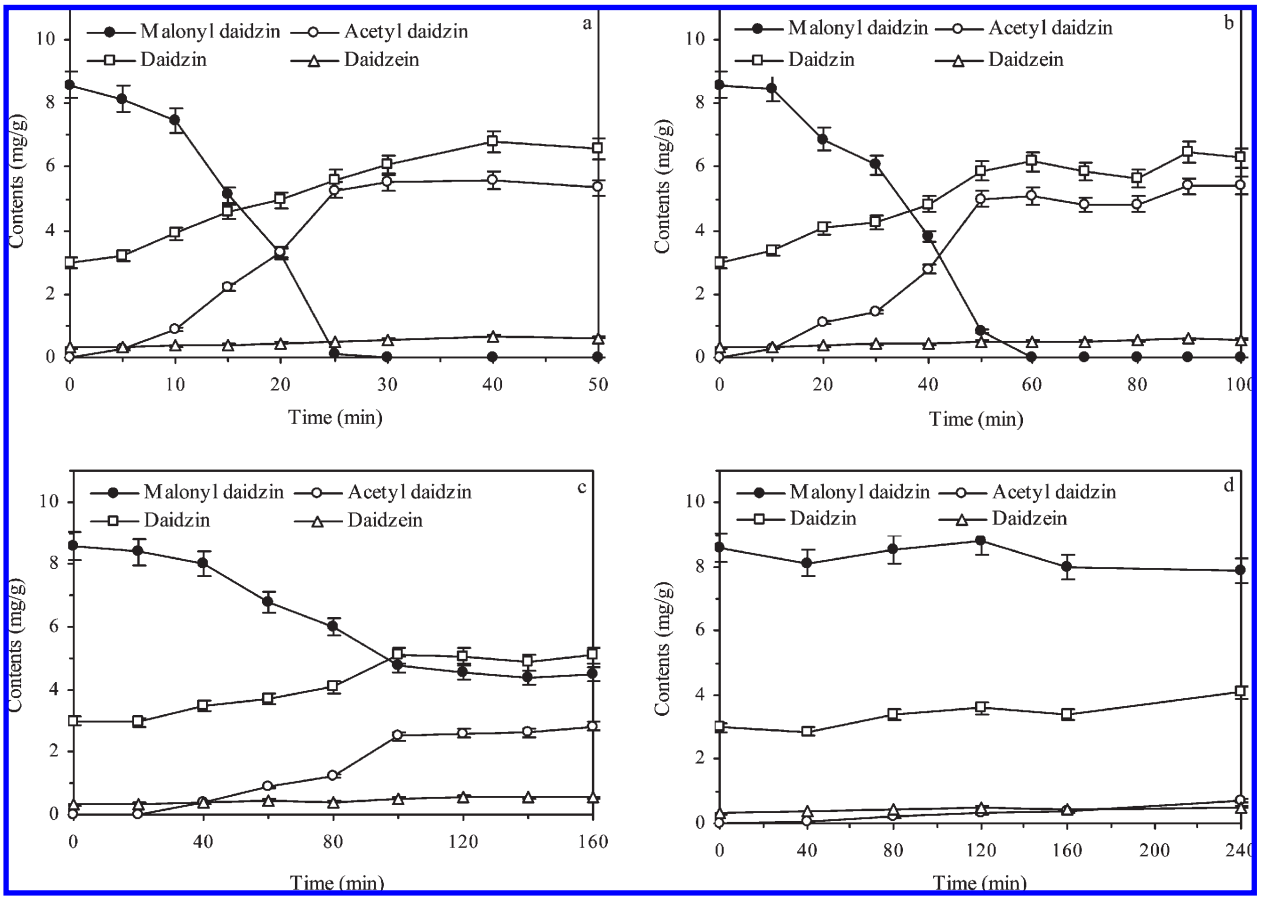

Figure 4. Changes in the contents of daidzein and its glycosides in the hypocotyls during dry heating at 150 (**a**), 130 (**b**), 110 (**c**), and 90 °C (**d**). The mean values of three experiments (RSDs < 5%) are presented.

total isoflavones). Only a small amount of genistein and daidzein was found, and no malonylglycitin, glycitin, glycitein, acetyldaidzin, acetylgenistin, and acetylglycitin were detected (**Figure 3b**).

Among four groups of isoflavones, isoflavones accumulated in the order of malonylglycoside, glycoside, and aglycone, among which malonylglycoside was the most abundant form and had the highest concentrations (8.56 and 1.11 mg/g for hypocotyl and cotyledon, respectively, quantitated as aglycones), being followed by β-glycoside (3.36 and 0.40 mg/g for hypocotyl and cotyledon, respectively, quantitated as aglycones). Aglycones were found in very small concentrations (0.47 and 0.08 mg/g for hypocotyl and cotyledon, respectively), and no acetylglycoside was detected. Malonylglycosides constituted 69.1−69.4% of the total isoflavones in both hypocotyl and cotyledon. The result is in agreement with the previous reports that malonylglycosides accounted for 57−79% of total isoflavones (*10*, *12*). β-Glycosides accounted for 27.1 and 25.4% of total isoflavones in hypocotyl and cotyledon, respectively, which are in agreement with the previous reports that glycosides accounted for 26.7−40.6% (*10*). Xu et al. (*16*) reported that the highest proportion at more than 76% of the total isoflavones was malonylglycosides, followed by β-glycosides at 19%, whereas acetylglycosides and aglycones occurred in only very small proportions.

The results showed that while the total daidzeins (7.38 mg/g), genisteins (1.71 mg/g), and glyciteins (3.30 mg/g) in hypocotyls constituted 59.6, 13.8, and 26.6% of the total isoflavone content, respectively, the total daidzeins (0.61 mg/g), genisteins (0.99 mg/g), and glyciteins (0 mg/g) in cotyledon constituted 38.1, 61.9, and 0% of the total isoflavone content, indicating that the ratio of genistein, daidzein, and glycitein forms in the hypocotyls is quite different from that found in the cotyledons (*28*). Berger et al. (*23*) investigated the differences in accumulation of total and individual isoflavones in the cotyledons and hypocotyls of soybeans and found that early isoflavone accumulation was observed in the hypocotyls, whereas the isoflavone accumulation in the cotyledons began when a plateau was reached in the hypocotyls. The isoflavone content of the cotyledons appeared to be more influenced by environment than that of the hypocotyls, which could mainly be under genotypic control (*23*). As described by Ribeiro et al. (*32*), they found that the total isoflavone content in the cotyledons was only 0.43 mg/g, while the content in the hypocotyls was 10.72 mg/g in BRS 213 soybean cultivar.

The results showed that glycitein and its conjugates (3.30 mg/g, quantitated as glycitein) were present nearly exclusively in the hypocotyls tissues at levels greater than genistein and its conjugates (1.71 mg/g, quantitated as genistein). It seems to be concentrated in the hypocotyls (*33*) and may represent an additional metabolite of particular importance to the hypocotyls (*34*). In the hypocotyls, glycitein begins its accumulation after daidzein and genistein (*23*), and daidzein appears to be a precursor of glycitein (*8*).

Change in Profiles of Isoflavones Affected by Dry Heating. It is generally accepted that isoflavones are not destroyed by heat but are rather subject to intraconversions between the different forms (*35*), and the genistein, daidzein, and glycitein forms are transformed in a similar manner (*28*). The changes of isoflavone profile in the hypocotyls and cotyledons during dry heating were investigated after whole soybeans were heated at different temperatures. The results showed that malonylglycosides in soybeans were thermally unstable and could readily be converted to their respective more heat-stable acetylglycosides and nonconjugated β-glycosides through decarboxylation and deesterification,

Table 4. Circadian Change in the Concentrations of Malonylglycosides and Aglycones in Both Hypocotyls and Cotyledons during Germination Starting at 10:00 p.m.[a]

		hypocotyls (mg/g)						cotyledons (mg/g)			
time (h)	mean length of hypocotyls (cm)	malonyl daidzin[b]	daidzein	malonyl genistin[b]	genistein	malonyl glycitin[b]	glycitein	malonyl daidzin[b]	daidzein	malonyl genistin[b]	genistein
0	0.6 ± 0.1	7.2471	0.2403	1.7156	0.0609	3.8642	0.0914	0.2486	0.0250	0.5759	0.0218
12	0.7 ± 0.2	7.8445	0.1117	2.0093	0.0195	3.7814	0.0260	0.2893	0.0132	0.6825	0.0227
24	1.4 ± 0.3	5.0357	0.9186	1.4261	0.1967	2.4667	0.3200	0.2297	0.0403	0.5468	0.0504
36	2.2 ± 0.4	6.2819	0.1712	1.5220	0.0285	2.7679	0.0477	0.2896	0.0089	0.6625	0.0100
48	2.7 ± 0.4	2.4398	1.6007	0.9522	0.4145	1.1016	0.5196	0.2374	0.0570	0.5535	0.0747
60	5.1 ± 2.0	4.2629	0.4387	1.2149	0.1122	1.2934	0.0926	0.4516	0.0046	0.8893	0.0075
72	7.4 ± 2.2	0.2986	1.4341	0.1352	0.5927	0.0955	0.4977	0.1995	0.1049	0.5597	0.1006
84	8.4 ± 2.3	3.5907	0.1820	0.7529	0.0768	0.8676	0.0577	0.4014	0.0222	0.9010	0.0178
96	9.5 ± 2.8	0.1521	1.1246	0.0672	0.4959	0.0537	0.2890	0.1453	0.1495	0.4465	0.1741
108		3.4886	0.1535	0.8767	0.0431	0.7003	0.0170	0.3466	0.0226	0.6726	0.0098
120		0.8070	1.2370	0.0673	0.4164	0.0317	0.2524	0.1158	0.1046	0.3494	0.1131

[a] The mean values of three determinations are presented and the RSDs <5%. [b] Malonylglycosides were quantitated as glycosides.

respectively. **Figure 3c**,**d** show the HPLC chromatograms of the extracts of the heated hypocotyls and cotyledons, respectively, with a complete decarboxylation and deesterification of malonylglycosides. **Figure 4** shows the changes of daidzein and its glycoside conjugates in the hypocotyls of soybeans heated at 150, 130, 110, and 90 °C (data for genistein and glycitein in hypocotyls and for genistein and daidzein in cotyledons are not shown because of their similar profiles). The results showed that the different temperatures could result in obvious differences in the degradation reaction rate of malonylglycosides. A higher temperature can make the rate of the degradation reaction markedly increase. As can be seen from **Figure 4**, when soybeans were heated at 150 and 130 °C, heating resulted in a rapid decarboxylation and deesterification of malonylglycosides with a concomitant increase in acetylglycoside and β-glycoside concentrations. No malonylglycosides were detected after heating for 25 min at 150 °C and for 60 min at 130 °C. The concentrations of acetylglycosides and β-glycosides kept increasing and then leveled off, indicating that acetylglycosides were stable to a higher heating temperature and β-glycosides were the deesterification products from malonylglycosides rather than acetylglycosides. The fact that the content of acetylglycosides was kept unchanged indicated that the degradation reaction of acetylglycoside might occur at a very low rate, especially at a lower temperature. In fact, Xu et al. (*36*) unexpectedly found that acetyl groups produced during thermal degradation of the original glycosides might have acetylated daidzin and genistin during heating and that the generation rates of acetyl daidzin and acetyl genistin were higher than those of daidzein and genistein based on molar concentration at temperatures of 135−185 °C. This indicates that the binding of the acetyl group may take place more readily than loss of the glycoside group in daidzin and genistin during heating (*36*).

When soybeans were heated at 110 °C, the degradation reaction of malonylglycosides might occur at a lower rate and malonylglycosides could not be completely decarboxylated or deesterified. Most isoflavones existed as β-glycosides and malonylglycosides, followed by acetylglycosides, whereas aglycones still occurred in only very small proportions after heating for 100 min and above. While the temperature was low, for example, at 90 °C, a smaller change in isoflavone distribution was observed, which is similar to the data published by Murphy et al. (*28*), who observed very little change in isoflavone distribution in the case of soy flour heated at 80 °C over 4 h.

In the hypocotyls, as can be seen from **Figure 4**, the decarboxylation reaction of malonylglycosides was faster than the deesterification reaction, that is, the conversion from malonylglycosides to acetylglycosides was faster than to glycosides, and the malonyl form was converted mostly to acetylglycosides, while the soy was heated at 130 and 150 °C. While heated at 90 and 110 °C, the decarboxylation and deesterification reaction rates of malonylglycosides seem to be similar. In the cotyledons (data not shown), the decarboxylation and deesterification reaction rates of malonylglycosides seem to be similar, while the soybean was heated at 90, 110, 130, and 150 °C. These data demonstrate a different mechanism for conversion of the isoflavone forms in hypocotyls as compared to cotyledons. In fact, other works have found that an interconversion of malonyl to acetyl forms, through decarboxylation, and of these to β-glycoside forms, through deesterification, can be induced by heat treatment. Grün et al. (*37*) demonstrated that when tofu in water was heated, the malonylglycosides were deesterified mostly to β-glycosides, and little decarboxylation of the malonylglycoside to the acetylglycoside was found. The effect of heat processing a liquid soy product on isoflavone distribution was evaluated in soy milk by Murphy et al. (*28*), who found that in this liquid matrix, malonylgenistin was converted almost exclusively into the β-glycoside, and little acetylgenistin was formed. The equilibrium between both reactions seems to be dependent on heat treatment conditions, such as temperatures, matrix, precursors, and enzymes, etc. (*35*, *36*).

Change in Profiles of Isoflavones Affected by Germination. It has been reported that germination changed the distribution profile of isoflavone (*35*). As shown in **Figure 3a**,**b**, in the mature beans, malonylglycosides and β-glycosides were the major fractions and only a very small amount of aglycones were detected. The present results showed that after germination, the β-glycoside contents decreased and the total aglycone content increased. Malonylglycosides were the major fractions in the germinated soybean seeds. Chiarello et al. (*35*) found that the β-glycoside contents decreased by 15 and 60% after 3 and 7 days of germination, respectively. Malonylglycosides increased by 30% after 3 days of germination but decreased again after 7 days, resulting in an accumulated loss of 30% related to nongerminated material. The total aglycone content increased by five times after 1 week of germination, whereas acetyl-conjugated forms remained at a low and constant level (*35*).

Interestingly, the present study found a significant circadian change in isoflavone distribution profile, but not always, alternating between malonylglycosides and aglycones for day and night (**Table 4**). **Figure 5** shows the HPLC chromatograms of the extracts of the hypocotyls during the germination of soybeans showing circadian rhythm. As shown in **Figure 5** and **Table 4**, in the hypocotyls sampled at 10:00 a.m. (after 12, 36, 60, 84, and 108 h of germination), the β-glycoside contents decreased and the aglycone contents increased. Malonylglycosides were the most

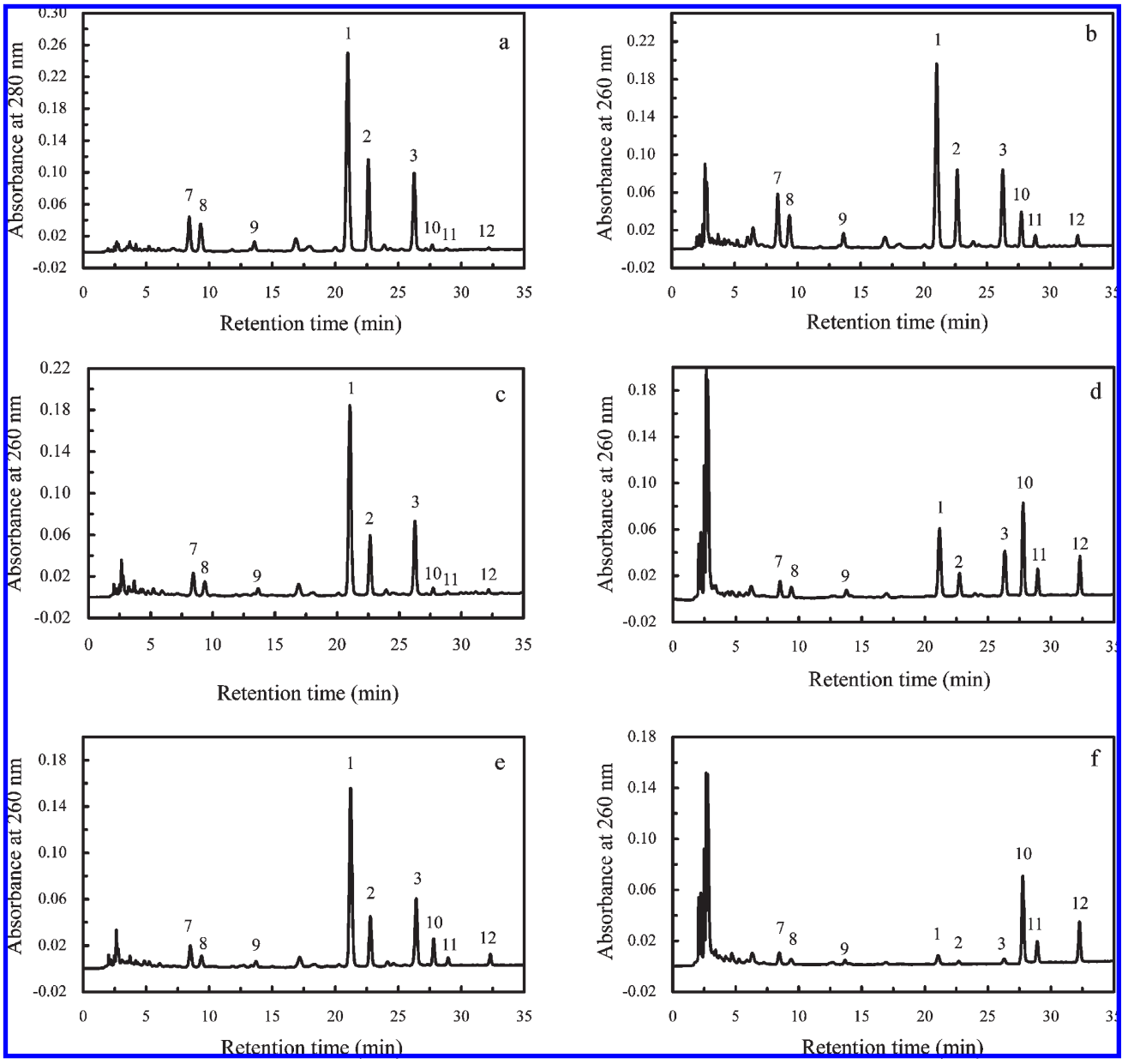

Figure 5. Typical HPLC chromatograms of the hypocotyl extracts of germinating soybeans showing the circadian rhythm after 12 (**a**), 24 (**b**), 36 (**c**), 48 (**d**), 60 (**e**), and 72 h (**f**) of germination starting at 10:00 p.m. For identification of peaks, see **Figure 1**.

abundant forms and only a small amount of β-glycosides and aglycones were detected after 60, 84, or 108 h (**Table 4**) of germination as an ordinary germination. In the hypocotyls sampled at 10:00 p.m. (after 24, 48, 72, 96, and 120 h of germination), the aglycone content markedly increased, and then, aglycones became the most abundant forms and only a very small amount of malonylglycosides and β-glycosides was detected after 72, 96, or 120 h of germination. In the cotyledons sampled at 10:00 a.m., malonylglycosides were the most abundant forms, being followed by β-glycosides, and only a very small amount of aglycones was detected after 60, 84, or 108 h of germination as an ordinary germination. In the cotyledons sampled at 10:00 p.m., although the aglycone contents also increased, malonylgenistin was the main isoflavone, being followed by genistein, β-genistin, malonyldaidzin, daidzein, and β-daidzin after 72, 96, or 120 h of germination. The circadian rhythm of isoflavone profile, with a nocturnal increase of aglycones and a decrease of malonylglycosides during germination, was found only twice in October both 2007 and 2008, and this mechanism is yet to be investigated.

Lin and Lai (*20*) reported that the ratio of aglycones to total isoflavones increased from the mature soybeans to the sprouts. The presence of aglycone isoflavones in soybeans is related to β-glucosidase activity (*32*). Ribeiro et al. (*32*) found that germination affected β-glucosidase activity, total isoflavone content, and their isomeric forms. During germination, β-glucosidase activity increased in the hypocotyls and cotyledons, while the total isoflavone content increased in the cotyledons and decreased in the hypocotyls (*32*). Soybeans have two endogenous isoforms of β-glucosidase that are able to hydrolyze β-glycoside isoflavones to their aglycone forms. These enzymes could have been activated by the soaking step prior to germination or by the germination metabolism itself (*35*).

Germination is a complex metabolic process during which the seed comes out of its latency stage and the reserved substances present in the cotyledons are broken down and used for the development and growth of the hypocotyls (*38*, *39*). Germination causes important changes in the biochemical, nutritional, and sensory characteristics and results in a substantial increase in

some biochemical constituents and biologically active components of soybeans (*40*, *41*). Therefore, the germinated soybeans are rich in some biologically active compounds, such as lecithin, phytosterols, saponins, estrogenic compounds, etc. (*40*). Those changes can vary depending on genotypes, humidity, temperature, light, the stage of germination, and seed physiological metabolism (*20*, *32*, *39*, *40*, *42*, *43*).

Like most organisms, plants have endogenous biological clocks that coordinate internal events with the external environment (*44*). Harmer et al. (*44*) found that clusters of circadian-regulated genes in pathways involved in plant responses to light and other key metabolic pathways and most of the phenylpropanoid biosynthetic pathway genes were under circadian control at the mRNA level in *Arabidopsis* (*44*, *45*). Kim et al. (*45*) found that the levels of chalcone isomerase, flavanone 3-hydroxylase, and isoflavone synthase mRNAs were under circadian control. Light supplies energy for photosynthesis as well as signals for photomorphogenesis in plants, which in turn influences allocation and use of the products of photosynthesis. Flavonoids and some phenylpropanoids have been thought to play roles in protecting against UV irradiation (*17*). Light intensities depended on the month, daytime, and weather conditions.

In conclusion, this newly developed HPLC method allows the simultaneous determination of 12 isoflavones in soybeans. From the results of distribution investigation, our findings confirmed that malonylglycoside was the most abundant form, being followed by glycoside, and aglycones were found in very small concentrations in the cotyledons and hypocotyls of dormant soybeans. The hypocotyls and cotyledons showed highly contrasted isoflavone contents and compositions, and glycitein and its derivatives were present nearly exclusively in hypocotyl tissues, indicating that isoflavone synthesis and accumulation resulted from highly differentiated pathways in the hypocotyls and cotyledons (*23*). In the germinated soybeans, malonylglycosides, which must ultimately be converted to aglycones for isoflavone-mediated symbiotic or defensive mechanisms (*3*), were the major fractions. β-Glycosides, whose contents decreased during germination, were the intermediate products of the reciprocal transformation between malonylglycosides and aglycones. The present study found a significant circadian change between malonylglycosides and aglycones with a nocturnal increase of aglycones and decrease of malonylglycosides during germination. Although this study was not able to conclusively show the specific reasons for the occasional circadian change of isoflavone profile, there was indication that the reciprocal transformation between malonylglycosides and aglycones occurred in soybeans. The hypocotyls are the part of soybean containing the highest concentration of isoflavones, especially malonyldaidzin and daidzin, which can be metabolized to the more potent estrogenic metabolite equol by the intestinal microflora (*26*). Therefore, the growing hypocotyls would be valuable source materials for various functional foods (*17*). However, a possible circadian change between malonylglycosides and aglycones should be considered because the bioavailability and clinical effectiveness are greater when ingested as glycosides rather than aglycones (*21*).

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