JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of the Application of Three Different Elicitors on Soybean Plants on the Concentrations of Several Isoflavones in Soybean Seeds

BO ZHANG,† NAVAM HETTIARACHCHY,*,‡ PENGYIN CHEN,† RONNY HORAX,‡ BRIAN CORNELIOUS,† AND DANHUA ZHU§

Department of Crop, Soil, and Environmental Sciences, 115 PTSC, University of Arkansas, Fayetteville, Arkansas 72701, Department of Food Science, University of Arkansas, 2650 North Young Avenue, Fayetteville, Arkansas 72704, and Institute of Crop Science, Zhejiang Academy of Agricultural Sciences, 198 Shiqiao Road, Hangzhou, Zhejiang 310021, China

Soybean [*Glycine max* (L.) Merr.] is a rich source of isoflavones that are often affected by biotic and abiotic factors. The objectives of this study were to evaluate the effect of various concentrations of three natural elicitors applied at different soybean growth stages on isoflavone content and to compare the efficiency of several solvent systems in isoflavone extraction and quantification. The isoflavones extracted from R96-3444 soybean using eight solvent systems were separated, identified, and quantified by a high-performance liquid chromatography (HPLC) procedure. The soybean plants were sprayed with salicylic acid, methyl salicylate, or ethyl acetate at 0, 10^{-6}, 10^{-3}, and 10^{-1} M at R1 (blooming) or R4 (full pods) growth stage. Results showed that 10^{-3} M ethyl acetate sprayed at the R1 stage significantly increased total isoflavone content and the levels of some individual isoflavones in soybean seeds. With all the elicitors that were tested, concentration was a more important factor than application time with respect to isoflavone content with lower concentrations being more effective on most isoflavones. A 53% acetonitrile solvent system was the best solvent system for extracting total isoflavone, malonyl glucosides, genistein, glycitin, genistin, acetyl-daidzin, and acetyl-genistin. The results of this study will be useful for increasing the isoflavone content in desirable soybean varieties and improving isoflavone concentration during extraction.

KEYWORDS: Isoflavones; soybean; solvent; elicitor; HPLC

INTRODUCTION

Soybean has been in Asian diets for 1000 years, and research has shown that soy intake contributes to the low incidence of breast and prostate cancer (*1*, *2*). In soybean, a number of phytochemicals are responsible for anticarcinogenic activity (*1*). Isoflavones have increasingly attracted the attention of researchers and consumers in recent years because of their association with human health (*2*, *3*). Soybean has been identified as a main source of isoflavones in plant foods for human consumption (*4*, *5*).

Twelve isoflavones, grouped into four glucoside forms (aglycones, β-glucosides, acetyl glucosides, and malonyl glucosides), have been reported to be present in soybean seeds (*3*). However, isoflavone content in soybean seeds was significantly influenced by genetic and environmental factors as well as their interactions (*6*, *7*). Japanese soybean varieties contained more malonyl-genistin than American varieties; crop year had more impact on isoflavone content than geographic locations (*6*), and high temperatures during plant growth significantly decreased isoflavone content (*8*). The stages of soybean seed maturity were shown to affect total and individual isoflavone content. The reproductive (R) period of soybean seeds has eight stages (R1−R8) beginning at the time of first bloom and ending at maturity. The total isoflavone content of NTCPR93-40 soybean at the R6 stage with green seeds reaching maximum size was significantly higher than that at the R8 stage, which is the full maturity stage with 95% of the pods reaching mature pod color, but soybean cultivar Hutcheson contained 1.26 times the total isoflavone content at the R8 stage compared to that at the R6 stage (*9*).

Biotic elicitors have been applied to soybean seeds to enhance isoflavone content (*10*). Isoflavones are involved in plant−microbe interaction because pathogens might produce elicitor molecules that increase isoflavone content in plants as a defense response (*10*). Foliar applications of biotic elicitors such as chitosan increased total isoflavone content from 16 to 96% and daidzein up to 150% (*10*). On the other hand, a series of phenolic compounds such as salicylic acid, also involved in the systemic response to pathogens, could affect metabolic processes, includ-

† Department of Crop, Soil, and Environmental Sciences, University of Arkansas.
‡ Department of Food Science, University of Arkansas.
§ Zhejiang Academy of Agricultural Sciences.

10.1021/jf0531048 CCC: $33.50
Published on Web 07/04/2006

ing the photosynthetic rate, stomatal conductance, ion uptake, and transpiration when sprayed on plants at the vegetative cotyledon stage (*11*, *12*). Roots of *Lupinus luteus* L. treated with salicylic acid exhibited increased genistein content because it improved synthesis and accumulation of secondary plant metabolites (*13*). Methyl salicylate, the methyl ester of salicylic acid, acts as a signal for plant defense response (*14*). Ethyl acetate is one of the primary metabolites produced by fungus, and it inhibited germination and growth of seeds (*15*). Methyl salicylate and ethyl acetate might be new feasible elicitors and provide greater efficiency than salicylate acid in increasing isoflavone content. However, the effects of natural elicitors applied to soybean plants at various growth stages in the field have not been evaluated.

Numerous reports indicated that the amount of isoflavone varied with the conditions during extraction. The content of aglycones increased, whereas the content of β-glucosides, acetyl glucosides, and malonyl glucosides decreased, when the soaking time and temperature of seed powder were increased during extraction (*16*, *17*). Solvent systems have been shown to have different extraction ability, and there is no uniform solvent system used in isoflavone extraction. An 80% methanol solution was used to extract isoflavones from radiation-induced soybean seeds (*18*). The acetonitrile (AcCN)/HCl system (10:2) was one of the solvent systems used for extracting isoflavones from soyfood (*4*). AcCN was shown to be a better solvent system than acetone, ethanol, and methanol for extracting isoflavones in soyfood (*19*). Moreover, the ratio of water in the acetonitrile solvent system played an important role in the amount of isoflavone extracted (*4*). Other studies showed the optimized extraction solvent for soybean seeds to be 70% ethanol or 58% AcCN (*20*, *21*). However, the more efficient solvent systems in the extraction of total and individual isoflavones need to be investigated to better differentiate among samples.

The objectives of this study were to evaluate the effects of three natural elicitors, salicylic acid, methyl salicylate, and ethyl acetate, at various concentrations applied at different growth stages on isoflavone content in soybean and to compare the efficiency of solvent systems frequently used in isoflavone extraction and quantification.

MATERIALS AND METHODS

Materials. Soybean breeding line R96-3444 from Arkansas was used in this study. R96-3444 soybean is a maturity group V line derived from the cross of PIO 9592 × KS 4895. R96-3444 soybean was selected because of its high isoflavone content (4978.7 mg/kg), which is higher than that of other breeding lines and cultivars in a preliminary screening (N. Hettiarachchy, unpublished data). R96-3444 soybean seeds were maintained in the soybean-breeding program of the University of Arkansas.

Elicitor Treatment. The field experiment was conducted at the University of Arkansas Agricultural Experiment Station (Fayetteville, AR) in 2003. The soil type was Captina silt loam with fine-silty and mesic characteristics. R96-3444 soybean was planted in a total of 108 rows (plots), 6.09 m in length and 96.5 cm between rows in a split−split plot design. Every 36 rows served as one replication with elicitors being the main plot, elicitor concentrations being the subplot, and soybean growth stage at application being the sub-subplot. The experiment unit was the 6.09 m row plot. The soybeans were planted in May as a full season crop followed by recommended management (*Arkansas Soybean Production Handbook*) throughout the growing season. Three elicitors, salicylic acid, methyl salicylate, and ethyl acetate (VWR, West Chester, PA), dissolved in distilled H_2O and diluted to four concentrations (0, 10^{-6}, 10^{-3}, and 10^{-1} M) were sprayed on R96-3444 plants at the R1 (blooming) or R4 (full pods) growth stage. When R96-3444 plants began to bloom at the end of July and reached full pods at the middle of September, 300 mL of elicitor solution was sprayed on each row, and untreated check plants were sprayed with 300 mL of distilled water using a hand sprayer. Each treatment was replicated three times with each replication in one row plot. Seeds from treated and untreated plants were harvested at stage R8 (full maturity) at mid-October and were stored at 4 °C. Seeds from untreated plants were used as a negative control for elicitor effect and as extraction materials for solvent system comparison.

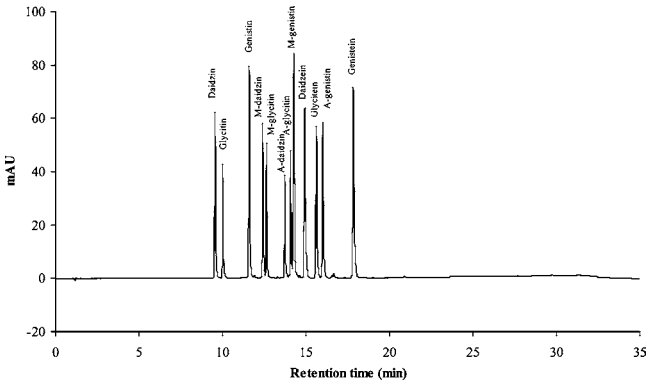


Figure 1. High-performance liquid chromatography (HPLC) profiles of 12 standard isoflavones: daidzin (9.56 min), glycitin (10.02 min), genistin (11.59 min), malonyl-daidzin (12.42 min), malonyl-glycitin (12.65 min), acetyl-daidzin (13.74 min), acetyl-glycitin (14.09 min), malonyl-genistin (14.26 min), daidzein (14.94 min), glycitein (15.62 min), acetyl-genistin (16.01 min), and genistein (17.85 min).

Isoflavone Extraction. Ten grams of seeds from each plot was ground with a Knifetec 1095 sample mill (Foss, Inc., Eden Prairie, MN) and passed through a 60 mesh sieve (W. S. Tyler Inc., Mentor, OH). Eight solvent systems were used to extract isoflavones: 53% AcCN/H_2O, AcCN/H_2O/0.1 N hydrochloride acid (HCl) (10:7:2), 53% methanol (MeOH)/H_2O, MeOH/H_2O/0.1 N HCl (10:7:2), 53% ethanol (EtOH)/H_2O, EtOH/H_2O/0.1 N HCl (10:7:2), and H_2O at 60 °C and ambient temperature. A total of 0.2 g of flour of each sample and 2 mL of solvent were mixed and shaken at 250 rpm for 2 h at ambient temperature and then centrifuged at 7000*g* for 5 min. The supernatant was used for isoflavone analysis.

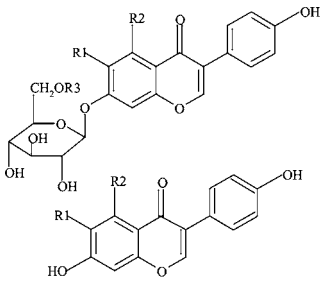
Isoflavone Analysis. After the supernatants had been filtered through a 0.2 μm PVDF Target Syringe Filter (National Scientific, Duluth, GA), 6 μL of filtrate was injected into the cartridge during HPLC for isoflavone analysis. Each sample was extracted twice separately and evaluated independently by HPLC for all 12 isoflavones (**Figure 1**). A model 1090 Hewlett-Packard (Avondale, PA) liquid chromatograph equipped with a diode array ultraviolet (UV) detector was used to analyze isoflavone content. A TSK GEL Super-ODS column (Supelco, Bellefonte, PA) was used with a flow rate of 1.0 mL/min at 37 °C. The effluent absorbance at 254 nm was used to detect the separated isoflavones. The mobile phase consisted of two solvent systems, 0.1% trifluoroacetic acid in acetonitrile (solvent system A) and 0.1% trifluoroacetic acid in Millipore water (solvent system B). Solvent system B was set up at 100% at the initial running, then changed to 50% during the first 30 min, and then returned to 100% within the last 5 min. All the standard isoflavones (genistein, daidzein, glycitein, genistin, daidzin, glycitin, acetyl-glycitin, acetyl-daidzin, acetyl-genistin, malonyl-genistin, malonyl-daidzin, and malonyl-glycitin) were purchased from LC Laboratories (Woburn, MA). Six microliters of each sample was injected for isoflavone quantification according to the isoflavone standard curve established by Xie et al. (*22*). Each sample was extracted and injected in triplicate.

Statistical Analysis. Data that were collected were expressed as the mean of three replications. Analysis of variance was performed using JMP 5.1 (SAS Institute Inc., Cary, NC). Means of isoflavone content were separated by the LSD (least significance difference) method at a $P = 0.05$ significance level. Correlations between isoflavone content and elicitor concentrations were also calculated with JMP 5.1 (*23*).

Table 1. Means of Individual and Total Isoflavone Concentrations (milligrams per kilogram) of R96-3444 Soybean Sprayed with Elicitors at Different Concentrations at Blooming (R1) or Full Pod (R4) Stage

elicitor	application time	concn (M)	isoflavone content (mg/kg)[a]																
			1	2	3	4	5	6	7	8	9	10	11	12	M-glucosides	β-glucosides	A-glucosides	aglycones	total
salicylic acid	blooming (R1)	10^{-6}	1831.3[b]	206.9	1740.5	318.1	86.5	405.4	402.4	82.7	25.7	88.7	7.7	29.8	3778.7	810.0	510.8	126.2	5225.7
		10^{-3}	1613.6	180.7	1706.4	271.9	77.6	377.2	400.8	81.4	25.3	84.3	5.1	29.8	3500.8	726.7	507.4	119.2	4854.1
		10^{-1}	1792.2[b]	204.4	1827.2	305.3	94.2	413.7	430.5	87.2	26.9	96.4	4.9	32.8	3823.8	813.2	544.5	134.1	5315.6
	full pod (R4)	10^{-6}	1719.2	206.6	1856.4	327.0	96.1	441.8	429.1	88.6	25.6	86.4	7.9	31.3	3782.3	865.0	543.3	125.6	5316.1
		10^{-3}	1522.9	211.0	1749.2	277.4	95.7	398.0	406.5	86.7	29.1	92.4	14.3[b]	32.4	3483.1	771.1	522.3	139.0	4915.6
		10^{-1}	1688.9	202.7	1779.7	285.0	89.6	398.9	410.6	83.1	27.7	91.4	9.1	28.3	3671.3	773.5	521.4	128.8	5094.9
methyl salicylate	blooming (R1)	10^{-6}	1818.3[b]	210.4	1866.0	328.5	89.8	438.9	424.9	87.9	27.9	92.2	10.2	29.7	3894.7[b]	857.2	540.7	132.0	5424.6[b]
		10^{-3}	1704.2	212.0	1756.9	316.0	90.6	413.2	411.1	88.5	27.3	96.0	10.7	30.9	3673.1	819.7	526.9	137.7	5157.4
		10^{-1}	1547.5	203.2	1708.9	291.3	91.7	397.8	396.7	85.6	26.6	91.4	10.6	30.0	3459.6	780.9	509.0	132.0	4881.4
	full pod (R4)	10^{-6}	1474.7	201.1	1719.1	286.6	91.9	411.9	402.5	86.1	27.2	90.3	10.3	31.0	3394.9	790.4	515.9	131.6	4832.8
		10^{-3}	1606.2	204.1	1805.9	327.9	93.9	435.6	415.8	88.0	27.8	93.4	11.7	32.5	3616.3	857.4	531.6	137.5	5142.7
		10^{-1}	1417.7	204.3	1640.3	273.0	93.8	382.5	380.6	83.4	28.6	88.9	14.9[b]	29.7	3262.2	749.3	492.6	133.5	4637.7
ethyl acetate	blooming (R1)	10^{-6}	1703.1	219.8	1867.1	325.6	95.2	448.4	432.2	93.1	28.7	92.1	21.9[b]	30.3	3790.0	869.2	554.0	144.2[b]	5357.4
		10^{-3}	1685.6	233.2	1884.0	317.0	104.1	432.6	435.8	90.1	27.6	88.5	22.9[b]	28.6	3802.9	853.7	553.5	139.9[b]	5350.1
		10^{-1}	1639.6	212.8	1799.9	304.7	90.6	410.0	419.1	86.2	28.9	94.7	23.8[b]	30.6	3652.4	805.3	534.2	149.0[b]	5140.9
	full pod (R4)	10^{-6}	1450.7	216.9	1703.4	301.2	100.0	407.0	396.5	86.2	31.1[b]	95.0	25.8[b]	33.4	3370.9	808.2	513.8	154.3[b]	4847.2
		10^{-3}	1598.1	233.6	1840.1	353.0[b]	111.5	473.7[b]	426.8	94.8[b]	27.9	99.2[b]	28.1[b]	34.6[b]	3671.8	938.3[b]	549.5	161.9[b]	5321.5
		10^{-1}	1588.1	219.2	1846.9	312.8	97.8	427.0	446.6[b]	90.7	29.9	98.4[b]	24.0[b]	32.8	3654.2	837.6	567.2[b]	155.3[b]	5214.3
untreated control			1551.8	189.5	1704.6	290.4	89.6	404.1	397.8	84.2	27.0	88.3	7.9	31.4	3446.0	784.0	509.0	127.6	4866.6

[a] Isoflavones were extracted with a 53% acetonitrile solvent system: (1) malonyl-daidzin, (2) malonyl-glycitin, (3) malonyl-genistin, (4) daidzin, (5) glycitin, (6) genistin, (7) acetyl-daidzin, (8) acetyl-glycitin, (9) acetyl-genistin, (10) daidzein, (11) glycitein, (12) genistein, (M-glucosides) malonyl glucosides, and (A-glucosides) acetyl glucosides. [b] The isoflavone content is significantly higher than the untreated control using the Student's *t*-test ($p < 0.05$).



No.	Isoflavone	R1	R2	R3
1	malonyl-daidzin	H	H	$COCH_2COOH$
2	malonyl-glycitin	OCH_3	H	$COCH_2COOH$
3	malonyl-genistin	H	OH	$COCH_2COOH$
4	daidzin	H	H	H
5	glycitin	OCH_3	H	H
6	genistin	H	OH	H
7	acetyl-daidzin	H	H	COH_3
8	acetyl-glycitin	OCH_3	H	COH_3
9	acetyl-genistin	H	OH	COH_3
10	daidzein	H	H	
11	glycitein	OCH_3	H	
12	genistein	H	OH	

Figure 2. Chemical structures of 12 isoflavones in soybean seed.

RESULTS AND DISCUSSION

Effects of Elicitors Applied at Different Concentrations and Times on Isoflavones in R96-3444 Soybean Seeds. Individual and total isoflavones of soybean seeds were extracted with a 53% acetonitrile solvent system from elicitor-treated and untreated plants (**Figure 2** and **Table 1**). In addition, individual isoflavones were grouped into four glucoside forms for comparison of treatment effects. Because we were interested in the effect of elicitor types, application concentration, and application time, all the means from all possible treatment combinations were compared with the mean of the untreated control. With the exception of malonyl-glycitin, malonyl-genistin, and glycitin, all other individual isoflavones, glucoside forms, and total isoflavone content were affected by combinations of elicitors × concentrations × time compared to the untreated control.

Salicylic acid significantly affected malonyl-daidzin and glycitein levels. Salicylic acid applied at 10^{-6} and 10^{-1} M at stage R1 increased the malonyl-daidzin content by 18 and 15.5%, respectively. Salicylic acid applied at 10^{-3} M at stage R4 increased the glycitein content by 81%. Malonyl-daidzin, malonyl glucosides, and total isoflavone were responsive to methyl salicylate applied at 10^{-6} M at stage R1, and glycitein was responsive to the same elicitor but at a concentration of 10^{-1} M at stage R4. Ethyl acetate had the strongest impact on isoflavone content because it enhanced most of the isoflavones, including the ones that did not exhibit a response to salicylic acid and methyl salicylate except for malonyl-glycitin, malonyl-genistin, and glycitin. Ethyl acetate at all three different concentrations applied at both R1 and R4 stages had a significantly positive impact on glycitein and aglycones. With the application of ethyl acetate, the glycitein content was increased by 2.8−3.3-fold and the aglycone content was increased by 9.6−26.9%, with 10^{-3} M at stage R4 being the most effective. When applied at 10^{-3} M at stage R4, ethyl acetate also caused a marked increase in the content of daidzin, genistin, acetyl-glycitin, daidzein, genistein, and β-glucosides. In addition, ethyl acetate resulted in a significant increase in the content of acetyl-genistin at a concentration of 10^{-6} M and in the content of acetyl-daidzin, daidzein, and acetyl glucoside at 10^{-1} M when applied at stage R4.

For subtotal isoflavone, ethyl acetate increased the content of β-glucosides, acetyl glucosides, and aglycones, and methyl salicylate increased the content of malonyl glucosides and total isoflavone (**Table 1**). Because aglycones are generated from malonyl glucosides (*24*, *25*), the level of malonyl glucosides increased in response to salicylic acid and methyl salicylate, but malonyl glucosides were possibly converted into aglycones when responding to ethyl acetate, resulting in a high aglycone content and a low malonyl glucoside content. An experiment conducted in greenhouses demonstrated that the content of daidzein was significantly increased (150%) by LCOs elicitor (*10*), and glycitein exhibited the weakest response to elicitors among aglycones (*26*). However, the results of our study indicated that glycitein was the isoflavone component most responsive to elicitors, and the glycitein content was increased by as much as 256% compared with the untreated control. The daidzein content was increased by 12.3%, which was slightly higher than the increase (10.2%) for genistein. The increase in the total isoflavone content in our study was 11.5% at best with methyl salicylate treatment at 10^{-6} M at stage R1, whereas the increase in the total isoflavone content was reported to range from 16 to 96% with elicitors [lipo-chitooligosaccharides (LCO), chitosan, actinomycetes, spores, and yeast extract] in the study by Tawaha et al. (*10*). The differences in responses of isofla-

Table 2. Summary of ANOVA for Elicitor Treatment Variables Affecting Individual and Total Isoflavone Contents (milligram per kilogram) of R96-3444 Soybean

	significance of the treatment effect[a]						
isoflavones	elicitor	time	concentration	elicitor × time	time × concentration	elicitor × concentration	elicitor × time × concentration
1	**	**	NS	NS	NS	**	NS
2	NS	NS	NS	NS	NS	NS	NS
3	NS	NS	NS	NS	NS	*	NS
4	NS	NS	NS	NS	NS	**	NS
5	NS	NS	NS	NS	NS	NS	NS
6	NS	NS	NS	NS	NS	*	NS
7	NS	NS	NS	NS	NS	*	NS
8	NS	NS	NS	NS	NS	NS	NS
9	**	NS	NS	NS	NS	NS	NS
10	NS	NS	NS	NS	NS	NS	NS
11	**	**	**	NS	NS	**	NS
12	NS	NS	NS	NS	*	NS	*
malonyl glucosides	NS	**	NS	NS	NS	**	NS
β-glucosides	NS	NS	NS	NS	NS	**	NS
acetyl glucosides	NS	NS	NS	NS	NS	NS	NS
acetyl glucones	**	NS	**	NS	NS	*	NS
total	NS	*	NS	NS	NS	**	NS

[a] One asterisk indicates a significant difference at $p \leq 0.05$. Two asterisks indicate a significant difference at $p \leq 0.01$. NS indicates no significant difference.

vones to elicitors in both studies were likely due to the different soybean genotypes that were used. R96-3444 is a high-isoflavone soybean variety that may have nearly the maximum threshold of isoflavone content for improvement. In addition, different elicitor types and environmental conditions were possible factors contributing to the discrepancies.

Effects of Elicitor Treatment Variables as well as Their Interactions on Isoflavones in R96-3444 Soybean Seeds. The ANOVA results of all the elicitor treatment combinations on individual and total isoflavone indicated that elicitor type, application concentration, spray time, and their interactions had no effect on malonyl-glycitin, glycitin, acetyl-glycitin, daidzein, and acetyl glucosides but had some effects on other isoflavone components **(Table 2)**. Elicitor type had a significant effect on malonyl-daidzin, acetyl-genistin, glycitein, and aglycones; spray time exhibited a significant effect on malonyl-daidzin, glycitein, malonyl glucoside, and total isoflavone content. However, none of the isoflavone components was affected by the interaction of elicitor type and spray time. The elicitor concentration had a significant effect on glycitein and aglycone content. These results suggested that malonyl-daidzin and glycitein levels in soybean seeds could be enhanced by manipulating both elicitor type and application time. The aglycone content could be increased by selecting the proper elicitor and optimum application concentration. In addition, the levels of acetyl-genestin and glycitein could be adjusted by choosing the right elicitor and concentration, respectively. When malonyl glucoside and total isoflavone contents are increased using elicitors, spray time is critical.

The interactions of application time and concentration and of elicitor, application time, and concentration had no significant effect on all isoflavone components except for genistein. Therefore, selection for elicitor type and concentration as well as application time can be easily done independently for improving most of the isoflavones without complication. Furthermore, the independent effect of elicitor type and concentration on several isoflavones and their interaction also played an important role in altering the concentrations of six isoflavones. It was also concluded from other studies that concentration was an important factor for isoflavone content when the elicitor was chosen (*10*, *13*). However, our study also suggested that application time is equally important for several isoflavones.

Mean Effects of Elicitor Treatment Variables on Isoflavones and Correlation among the Treatment Variables. Because the two-way interactions between the treatment variables were not significant for nearly all the isoflavone components, data across treatment were pooled to compare the mean effects of the main variables on individual and total isoflavone content (**Table 3**). No significant differences were noticed for all the isoflavone components except for glycitein and aglycones. Glycitein content was significantly affected by all three variables, elicitor, concentration, and application time, and fluctuated as much as 3-fold in concentration. In a previous study, glycitein was found to be least stable in soybean seeds induced by radiation (*18*). Presumably, glycitein might have a response to radiation treatment similar to that of elicitors. Ethyl acetate gave rise to levels of glycitein and aglycones significantly higher than those of other elicitor treatments and the untreated control. Methyl salicylic treatment also resulted in a glycitein content significantly higher than that of the untreated control. No significant difference among different application concentrations was obtained for all the individual and total isoflavone content except for glycitein and aglycones. Application of elicitors at 10^{-1}−10^{-6} M appeared to enhance the level of glycitein, and elicitors at 10^{-3} and 10^{-6} M enhanced the level of aglycones as compared to the untreated control. It has been shown that the concentration of specific elicitors has variable effects on isoflavones in different plant species. The responses of soybean and *L. luteus* were highly concentration dependent (*10*, *13*), but the concentration of elicitors had little effect on red clover (*Trifolium pratense* L.) (*27*). It has been suggested that multiple applications of elicitor may be more efficient in enhancing the plant defense response and in simulating isoflavone systhesis (*27*). Like elicitor type and concentration, application time had a significant effect on glycitein and aglycone content but not on other isoflavone components. Application of elicitors at full pod (R4) growth stage tended to have better results in enhancing

Table 3. Comparison of Mean Effects of Elicitor Treatment Variables on Isoflavone Content in R96-3444 Soybean

	isoflavone content (mg/kg)[a]																
treatment variable	1	2	3	4	5	6	7	8	9	10	11	12	M-glucosides	β-glucosides	A-glucosides	aglycones	total
salicylic acid	1694.7	202.1	1776.6	297.5	90.0	405.8	413.3	85.0	26.7	89.9	8.2b	30.7	3673.3	793.3	525.0	128.8b	5120.3
methyl salicylate	1594.8	205.9	1749.5	303.9	92.0	413.3	405.3	86.6	27.6	92.0	11.4b[b]	30.6	3550.1	809.2	519.5	134.1b	5012.8
ethyl acetate	1610.9	222.6	1823.6	319.1	99.9	433.1	426.2	90.2	29.0	94.7	24.4a[b]	31.7	3657.0	852.1	545.4	150.8a[b]	5205.2
10^{-6} M	1666.2	210.3	1792.1	314.5	93.3	425.6	414.6	87.4	27.7	90.8	14.0[b]	30.9	3668.6	833.3	529.8	135.7	5167.3
10^{-3} M	1621.8	212.4	1790.4	310.5	95.6	421.7	416.1	88.3	27.5	92.3	15.5[b]	31.5	3624.7	827.8	531.9	139.2[b]	5123.6
10^{-1} M	1612.3	207.8	1767.2	295.4	93.0	405.0	414.0	86.0	28.1	93.5	14.6[b]	30.7	3587.3	793.3	528.2	138.8[b]	5047.5
blooming	1703.9	209.3	1795.2	308.7	91.1	415.2	417.1	87.0	27.2	91.6	13.1[b]	30.3	3708.4	815.1	531.2	134.9	5189.7
full pod	1562.9	211.1	1771.2	304.9	96.7	419.6	412.8	87.5	28.3	92.8	16.2[b]	31.8	3545.2	821.2	528.6	140.8[b]	5035.9
untreated control	1551.8	189.5	1704.6	290.4	89.6	404.1	397.8	84.2	27.0	88.3	7.9	31.4	3446.0	784.0	509.0	127.6	4866.6

[a] Means followed by the same letter or no letter for elicitors, concentration, or application time are not significantly different at $p < 0.05$. Isoflavones are numbered as described in footnote a of Table 1. [b] The isoflavone content is significantly higher than the untreated control using the Student's *t*-test ($p < 0.05$).

Table 4. Individual and Total Isoflavone Contents (milligram per kilogram) in R96-3444 Soybean Seeds Extracted with Eight Solvent Systems

	isoflavone content (mg/kg)[a]							
isoflavones	AcCN/H_2O	AcCN/HCl	MeOH/H_2O	MeOH/HCl	EtOH/H_2O	EtOH/HCl	hot H_2O	cold H_2O
1	1290.3a	1289.1ab	1182.4cd	1203.8c	1252.4b	1154.9d	1010.8e	793.4f
2	141.3a	138.0ab	136.7ab	137.8ab	140.0ab	124.8b	119.4bc	97.7c
3	1524.7a	1523.6a	1359.08c	1371.2c	1466.7b	1333.3c	1158.5d	972.5e
4	273.7b	247.7c	275.4ab	252.0c	293.9ab	271.1bc	322.4a	189.1d
5	65.9a	63.4a	62.7a	62.3a	66.1a	64.1a	65.8a	47.1b
6	380.5a	368.6ab	350.8b	305.3c	374.4ab	11.6e	373.3ab	204.7d
7	364.8a	364.8a	321.2b	315.0b	328.4b	296.3b	248.4c	90.0d
8	81.4b	79.2b	71.0c	77.0b	79.3b	70.1c	104.5a	24.8d
9	31.3ab	25.7ab	19.7b	28.4ab	41.2a	24.8ab	30.4ab	23.3ab
10	91.7b	101.2b	76.4c	96.0b	80.6c	26.4d	104.8b	252.5a
11	8.8c	5.1def	5.0ef	7.7cd	7.0cde	3.5f	12.3b	20.2a
12	32.6a	38.2a	33.3b	60.7b	29.5b	29.5b	81.1c	219.0d
total	4286.8a	4244.8ab	3893.6c	3917.2c	4159.5b	3410.2e	3631.8d	2934.2f

[a] AcCN/H_2O is a 53% acetonitrile/water mixture. AcCN/HCl is an acetontrile/water/0.1 N HCl (10:7:2) mixture. MeOH/H_2O is a 53% methanol/water mixture. MeOH/HCl is a methanol/water/0.1 N hydrochloric acid (10:7:2) mixture. EtOH/H_2O is a 53% ethanol/water mixture. EtOH/HCl is an ethanol/water/0.1 N HCl (10:7:2) mixture. Hot H_2O is water at 60 °C. Cold H_2O is water at ambient temperature. Means followed by the same letter within each row are not significantly different at $p < 0.05$.

glycitein and aglycone content than at blooming (R1) growth stage and the control. The correlation coefficient between isoflavones and elicitor concentrations ranged from −0.36 to 0.29 (data not shown). Specifically, daidzein (0.29), aglycones (0.12), and acetyl-genistin (0.11) contents were positively correlated to elicitor concentration, whereas levels of other isoflavone components such as daidzin (−0.36), genistin (−0.35), and β-glucosides (−0.33) were negatively correlated with elicitor concentration. These results suggested that specific selections of elicitors and concentration are necessary for specific isoflavone component enhancement.

Extraction and Quantification of Isoflavones with Selected Solvent Systems. The eight solvent systems used in this study successfully extracted all 12 individual isoflavones from R96-3444 soybean seeds (**Table 4**). In contrast, some previous research demonstrated that acetyl-daidzin, acetyl-glycitin, and glycitein were not detected from Manokin soybean seeds when extracted with six solvents, including AcCN and MeOH (*21*). The difference in the results from the two studies may likely be due to different genotypes that were used that may have different genetic regulations on isoflavone accumulation or to environmental factors such as years and geographic locations (*6*, *7*).

Differences in the amount of glucoside forms, individual isoflavones, and total isoflavone were observed for different extraction solvent systems (**Figure 3** and **Table 4**). Four glucoside forms of isoflavones in the seeds from untreated R96-3444 soybean plants followed the same trend when extracted with all the solvent systems except for EtOH/HCl and cold H_2O.

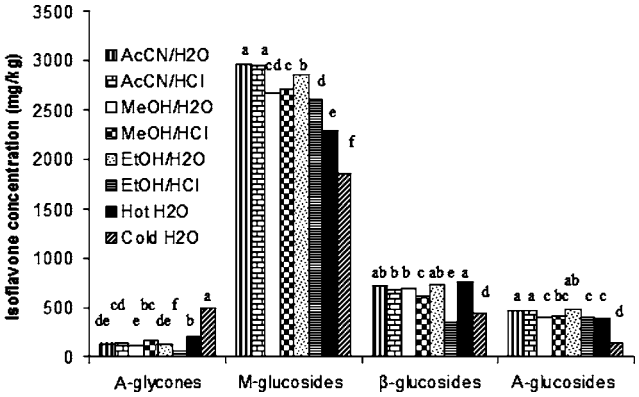


Figure 3. Total isoflavone content of the four glucoside forms extracted by eight solvent systems. Means represented by vertical bars with the same letter are not significantly different at $p < 0.05$: M-glucosides, malonyl glucosides; and A-glucosides, acetyl glucosides.

The trend for the amount of isoflavones obtained using six solvent systems (AcCN/H_2O, AcCN/HCl, MeOH/H_2O, MeOH/HCl, EtOH/H_2O, and EtOH/HCl) was as follows: malonyl glucosides > β-glucosides > acetyl glucosides > aglycones [comparable to previous reports (*28*)]. EtOH/HCl and cold H_2O extracted more acetyl glucosides and aglycones, respectively, than β-glucosides. In all the solvent systems, EtOH/HCl had the lowest extractability for β-glucosides, leading to a lower level of β-glucosides than acetyl glucosides. Cold H_2O was the most efficient in extracting aglycones, resulting in a smaller amount of β-glucosides. Previous reports have also demonstrated

the conversion among the glucoside forms (*22*). It appears that isoflavones could be converted to different forms under various extraction conditions.

The solvent systems were ranked on the basis of extraction of the total isoflavone content. AcCN/H_2O and AcCN/HCl showed superior extractability for isoflavones compared to other solvent systems. Both solvents extracted a significantly greater amount of total isoflavone, malonyl glucosides, malonyl-daidzin, malonyl-genistin, acetyl-daidzin, and genistein than other solvent systems. In addition, AcCN/H_2O and AcCN/HCl were the best in extracting acetyl glucosides, malonyl-glycitin, glycitin, genistin, and acetyl-genistin. However, AcCN/H_2O was better than AcCN/HCl because AcCN/H_2O was more efficient in extracting daidzin and glycitein than AcCN/HCl.

EtOH/H_2O was the third-ranked solvent system. The total levels of isoflavones and malonyl glucosides extracted by EtOH/H_2O were lower than those extracted by AcCN/H_2O and AcCN/HCl but higher than those extracted by other five solvent systems. EtOH/H_2O was the first in rank for extracting malonyl-glycitin, daidzin, glycitin, genistin, and acetyl-genistin. MeOH/HCl and MeOH/H_2O were the forth- and fifth-ranked solvent systems. MeOH/H_2O was less efficient for the extraction of aglycones, acetyl-glycitin, daidzein, and glycitein than MeOH/HCl but more efficient for β-glucosides, daidzin, and genistin than MeOH/HCl.

H_2O at 60 °C ranked sixth and was better than EtOH/HCl (seventh) and cold H_2O (eighth). H_2O at 60 °C had specific extractability for β-glucosides (761.5 mg/kg) and acetyl-glycitin (104.5 mg/kg), which was 5.7 and 28.4% more than those extracted by AcCN/H_2O, respectively. Cold H_2O had specific extractability for aglycones, daidzein, and glycitein, the levels of which were significantly higher than those extracted by other solvent systems. However, EtOH/HCl did not show any superior extractability for any specific isoflavone compared to other solvent systems. EtOH/HCl and H_2O appeared to be the least efficient solvent systems, but H_2O needs be considered when extracting specific isoflavones such as aglycones.

Overall, AcCN/H_2O proved to be an effective extraction solvent system for malonyl glucosides, acetyl glucosides, total isoflavone, and most individual isoflavones. AcCN/HCl had an extractability similar to that of AcCN/H_2O as a solvent system, and similar results were obtained in isoflavone extraction from soyfood (*19*). EtOH/H_2O and MeOH/H_2O were good but less efficient solvent systems than AcCN/H_2O and AcCN/HCl, which is in agreement with studies of isoflavone in soyfood (*19*). It was reported that solvent systems with pure H_2O were more efficient for isoflavone extraction than solvent systems with HCl in soyfood, including toast soy flour, tofu, tempeh, and soy germ (*16*). In our study, EtOH/H_2O was significantly better than EtOH/HCl, whereas MeOH/H_2O and MeOH/HCl had extractability similar to that for isoflavones. In addition, hot and cold H_2O may be used for specific isoflavone extraction, such as that of daidzin and daidzein.

The demand for isoflavones has increased tremendously over the past 5 years. New isoflavone products are continuously being developed and consumed (*29*). This study provided two economic and efficient ways to enhance isoflavone content for commercial use. Elicitors have the potential for increasing isoflavone content in soybean seeds when applied to plants in the field. The mechanism for isoflavone change in concentration may be related to increased rates of photosynthesis by elicitor applications (*11*). The total levels of isoflavone and specific individual isoflavones would be increased significantly with a proper combination of elicitors, concentrations, and spraying time. Solvent systems may also be manipulated to enhance the extractability for specific individual isoflavones and total isoflavone.

LITERATURE CITED

(1) Messina, M. J. Legumes and soybeans: Overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* **1999**, *70*, 439−450.

(2) Adlercreutz, H.; Mazur, W. Phyto-oestrongens and Western diseases. *Ann. Med.* **1997**, *29*, 95−120.

(3) Brouns, F. Soya isoflavones: A new and promising ingredient for the healthfoods sector. *Food Res. Int.* **2002**, *35*, 187−193.

(4) Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697−2704.

(5) Dhaubhadel, S.; McGarvey, B. D.; Williams, R.; Gijzen, M. Isoflavonoid biosyntheses and accumulation in developing soybean seeds. *Plant Mol. Biol.* **2003**, *53*, 733−743.

(6) Wang, H.; Murphy, P. A. Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994**, *42*, 674−677.

(7) Hoeck, J. A.; Fehr, W. R.; Murphy, P. A.; Welke, G. A. Influence of genotype and environment on isoflavone content of soybean. *Crop Sci.* **2000**, *40*, 48−51.

(8) Tsukamoto, C.; Shimada, S.; Igita, K.; Kudou, S.; Kokubun, M.; Okubo, K.; Kitamura, K. Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J. Agric. Food Chem.* **1995**, *43*, 184−192.

(9) Simonne, A. H.; Smith, M.; Weaver, D. B.; Vail, T.; Barnes, S.; Wei, C. I. Retention and changes of soy isoflavones and carotenoids in immature soybean seeds (edamame) during processing. *J. Agric. Food Chem.* **2000**, *48*, 6061−6069.

(10) Tawaha, A.; Seguin, P.; Smith, D. L.; Beaulieu, C. Biotic elicitors as a means of increasing isoflavone concentration of soybean seeds. *Annu. Appl. Biol.* **2005**, *146*, 303−310.

(11) Khan, W. M.; Prithiviraj, B.; Smith, D. L. Photosynthetic responses of corn and soybean to foliar application of salicylates. *J. Plant Physiol.* **2003**, *160*, 485−492.

(12) Haper, J. R.; Balke, N. E. Characterization of the inhibition of K^+ absorption in oats roots by salicylic acid. *Plant Physiol.* **1981**, *68*, 1349−1353.

(13) Kneer, R.; Poulev, A.; Olesinski, A.; Raskin, I. Characterization of the elicitor-induced biosynthesis and secretion of genistein from roots of *Lupinus luteus* L. *J. Exp. Bot.* **1999**, *50*, 1553−1559.

(14) Shulaev, V.; Silverman, P.; Raskin, I. Airbone signaling by methyl salicylate in plant pathogen resistance. *Nature* **1997**, *385*, 13−24.

(15) Hutchinson, S. A. Biological activities of volatile fungal metabolites. *Annu. Rev. Phytopathol.* **1973**, *11*, 223−246.

(16) Rickert, D. A.; Meyer, M. A.; Hu, J.; Murphy, P. A. Effect of extraction pH and temperature on isoflavone and saponin partitioning and profile during soy protein isolate production. *J. Food Sci.* **2004**, *69*, 623−631.

(17) Kao, T. H.; Lu, Y. F.; Hsieh, H. C.; Chen, B. H. Stability of isoflavone β-glucosides during processing of soymilk. *Food Res. Int.* **2004**, *37*, 891−900.

(18) Variyar, R. S.; Limaye, A.; Sharma, A. Radiation-induced enhancement of antioxidant contents of soybean (*Glycine max* Merrill). *J. Agric. Food Chem.* **2004**, *52*, 3385−3388.

(19) Murphy, P. A.; Braua, K.; Hauck, C. C. Solvent extraction selection in the determination of isoflavones in soy foods. *J. Chromatogr., B* **2002**, *777*, 129−138.

(20) Rostagno, M. A.; Palma, M.; Barroso, C. G. Pressurized liquid extraction of isoflavones from soybeans. *Anal. Chim. Acta* **2004**, *522*, 169−177.

(21) Lin, F.; Giusti, M. M. Effects of solvent polarity and acidity on the extraction effiency of isoflavones from soybeans (*Glycine max*). *J. Agric. Food Chem.* **2005**, *53*, 3795−3800.

(22) Xie, L.; Hettiarachchy, N. S.; Cai, R.; Tsuruhami, K.; Koikeda, S. Conversion of isoflavone glycosides to aglycones in SoyLife and soy meal using β-glycosidase. *J. Food Sci.* **2003**, *68*, 427−430.

(23) *JMP 5 administrator's guide to annually licensed Windows, Macintosh, and Linux Versions*; SAS Institute Inc.: Cary, NC, 2003.

(24) Rijke, D.; Zafra, A.; Ariese, F.; Brinkman, U. A.; Gooije, C. Determination of isoflavone glucoside malonates in *Trifolum pretense* L. (red clover) extracts: Quantification and stability studies. *J. Chromatogr., A* **2001**, *932*, 55−64.

(25) Kudou, S.; Fleury, Y.; Welti, D.; Magnolato, D.; Uchiida, T.; Kiamura, K.; Okubo, K. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). *J. Agric. Food Chem.* **1991**, *39*, 2227−2233.

(26) Vyn, T. J.; Yin, X.; Bruulsema, T. W.; Jackson, C. C.; Rajcan, I.; Brouder, S. M. Potassium fertilization effects on isoflavone concentration in soybean (*Glycine max* (L.) Merr.). *J. Agric. Food Chem.* **2002**, *50*, 3501−3506.

(27) Sivesind, E.; Seguin, P. Effects of foliar application of elicitors on red clover isoflavone content. *J. Agron. Crop Sci.* **2006**, *192*, 50−54.

(28) Lee, S. J.; Ahn, J. K.; Kim, S. H.; Kim, J. T.; Han, S. J.; Jung, M. Y.; Chung, L. M. Variation in isoflavone of soybean cultivars with location and storage duration. *J. Agric. Food Chem.* **2003**, *51*, 3382−3389.

(29) Uzzan, M.; Labuza, T. P. Critical issues in R&D of soy isoflavone-enriched foods and dietary supplements. *J. Food Sci.* **2004**, *69*, 77−86.

Received for review December 12, 2005. Revised manuscript received April 28, 2006. Accepted May 21, 2006.

JF0531048