FOOD COMPOSITION AND ADDITIVES

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography: Collaborative Study

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Isoflavones are biologically active compounds occurring naturally in a variety of plants, with relatively high levels found in soybeans. Twelve laboratories participated in a collaborative study to determine the aglycon isoflavone content of 8 test samples of soy and foods containing soy. The analytical method for the determination of isoflavones incorporates a mild saponification step that reduces the number of analytes measured and permits quantitation versus commercially available, stable reference standards. Test samples were extracted at 65°C with methanol–water (80 + 20), saponified with dilute sodium hydroxide solution, and analyzed by reversed-phase liquid chromatography with UV detection at 260 nm. Isoflavone results were reported as μg/aglycon/g or μg aglycon equivalents/g. The 8 test samples included 2 blind duplicates and 4 single test samples with total isoflavone concentrations ranging from approximately 50 to 3000 μg/g. Test samples of soy ingredients and products made with soy were distributed to collaborators with appropriate reference standards. Collaborators were asked to analyze test samples in duplicate on 2 separate days. The data were analyzed for individual isoflavone components, subtotals of daidzin–daidzein, glycitin–glycitein, and genistin–genistein, and total isoflavones. The relative standard deviation (RSD) for repeatability was 1.8–7.1%, and the RSD for reproducibility was 3.2–16.1% for total isoflavone values of 47–3099 μg/g.

Submitted for publication June 2001.
The recommendation was approved by the Methods Committee on Food Nutrition as First Action. *See* "Official Methods Program Actions," (2001) *Inside Laboratory Management*, November issue.

Isoflavones are a class of chemical compounds found naturally in a variety of plants such as soybeans, which contain relatively high levels. Recent studies report that isoflavones inhibit certain cancers, lessen symptoms of menopause, and contribute to improved bone density in treatments for osteoporosis (1–3).

Three parent isoflavones are found in soy: daidzein, glycitein, and genistein (Figure 1). The isoflavones also occur as the glucosides (daidzin, glycitin, and genistin; Figure 2) and the glucoside esters of the parent isoflavones. The acetyl and malonyl isoflavone glucoside esters are the most abundant forms found in soybeans, with either an acetyl or a malonyl group attached to the isoflavone glucoside at the 6″-O position, as shown in Figure 3.

Several methods reported for the determination of isoflavones use a variety of extraction procedures followed by reversed-phase liquid chromatgraphy (LC) for separation and UV or mass spectrometry for detection and quantitation. Franke et al. used acid hydrolysis, refluxing in ethanol–water (4), or enzymatic treatment of a refluxed test sample at 37°C (5) to convert all isoflavone compounds to the parent isoflavones (Figure 1). Wang and Murphy (6) extracted test samples with acetonitrile and 0.1% HCl at room temperature and analyzed the extracts by LC/UV. Barnes et al. (7) extracted test samples by using either methanol–water (80 + 20) or acetonitrile–0.1% HCl (80 + 20), both at room temperature, with analysis by LC/mass spectrometry. With the exception of methods employing enzymatic and acid hydrolysis steps, these methods of analysis determine levels of the parent isoflavones (Figure 1), the corresponding glucosides (Figure 2), and the acetyl and malonyl esters (Figure 3). Because reference standards for the glucoside esters were not commercially available until recently, estimates of their concentrations were commonly based on the assumption that the molar absorptivities of the esters were equivalent to the absorptivities of the corresponding glucosides. Reference standards for the acetyl and malonyl isoflavone esters (Figure 3) can be purchased, but they are relatively expensive and are reported to be unstable in solution (8).

The method in the collaborative study uses a mild saponification step to convert the isoflavone glucoside ester

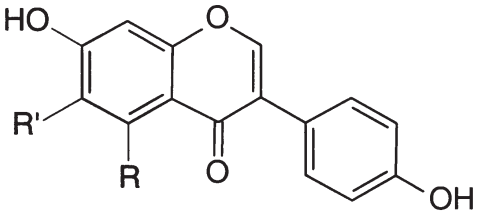

Figure 1. Structures of parent isoflavones: (a) daidzein, R = H, R′ = H; (b) glycitein, R = H, R′ = OCH_3; (c) genistein, R = OH, R′ = H.

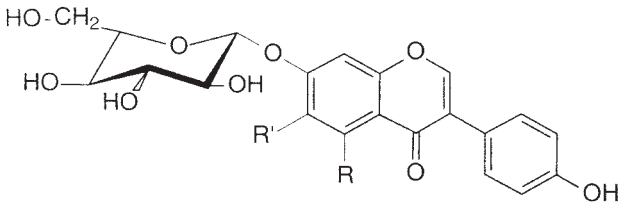

Figure 2. Structures of isoflavone glucosides: (a) daidzin, R = H, R′ = H; (b) glycitin, R = H, R′ = OCH_3; (c) genistin, R = OH, R′ = H.

forms (Figure 3) to the corresponding isoflavone glucoside forms (Figure 2), leaving the 3 parent isoflavones (Figure 1) intact. This approach allows direct comparison with stable, readily available, isoflavone parent and glucoside reference standards. Because the method leaves the isoflavone glucosides intact, it is possible to determine the relative proportions of parent isoflavone and glucoside present in test samples. Isoflavones are first extracted from the test sample with methanol–water (80 + 20) at 65°C for 2 h. Extracts are then saponified for 15 min at ambient temperature with dilute (0.13M) sodium hydroxide solution. Extracts are then filtered, centrifuged, and analyzed by using reversed-phase LC with a C_{18} column, followed by UV detection at 260 nm. Isoflavone results are reported as μg aglycon/g or μg aglycon equivalents/g.

Collaborative Study

Test samples of soy ingredients and food products containing soy were distributed to 19 academic, industrial, and commercial testing laboratories. All 19 participating laboratories were provided with 1 practice sample, 8 unidentified test samples, a vial of stock reference standard solution 5, the method, and instructions. The test samples included soy flour, soy protein isolate (blind duplicates), vegetable burger (freeze-dried), soy molasses, miso (freeze-dried), and soy beverage (blind duplicates).

Because of the cost of the isoflavone standards, a mixed solution of stock reference standards was provided to expedite completion of the study. Collaborators were required to make all necessary dilutions of the reference standards; they diluted an aliquot of the stock standard solution provided to make standard 4. Subsequent dilutions by collaborators provided all 5 working standards required in this study.

Isoflavone concentrations were provided for the practice sample and each collaborator was asked to analyze this sample to demonstrate method proficiency before proceeding with analysis of the remaining test samples. After successfully analyzing the proficiency sample, collaborators were asked to analyze each test sample in duplicate on 2 separate days. These analyses were in addition to the analyses of the blind duplicates and provided further data to estimate variability within a laboratory on a given day.

Sample Preparation and Standards

Five of the 8 test samples and the practice sample were homogenized by mixing and then placed in test sample vials for distribution to the collaborators. Soy molasses was allowed to settle for 7 days and the liquid layer was decanted to eliminate suspended material before the liquid portion was placed in test sample vials. The vegetable burger and miso were freeze-dried to enhance stability during both shipping and storage. The freeze-dried miso was mixed with dry ice before grinding. After grinding, the freeze-dried miso was allowed to equilibrate at ambient temperature for 3 days to vent CO_2 before it was placed in test sample vials. The freeze-dried vegetable burger was ground and transferred directly to test sample vials. Eight test samples, labeled A through H, were distributed to each collaborator. The test samples were assayed as received. Because the method specifies the weight of test sample to be used for extraction, based on the amount of soy protein in the test sample, collaborators were provided with recommended test sample sizes for the unidentified test samples. The recommended test sample sizes were as follows: test

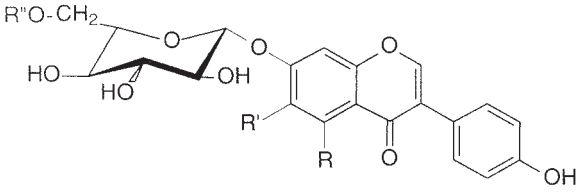

Figure 3. Structures of isoflavone glucoside esters: (a) 6″-O-[carbonyl group]-daidzin, R = H, R′ = H, R″ = carbonyl group; (b) 6″-O-[carbonyl group]-glycitin, R = H, R′ = OCH_3, R″ = carbonyl group; (c) 6″-O-[carbonyl group]-genistin, R = OH, R′ = H, R″ = carbonyl group.

Table 1. Daidzin data[a] reported as aglycon equivalents in μg/g

			Soy isolate sample		Soy beverage sample					
Lab	Day	Sample	A	E	D	H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	1301[b]	1290[b]	203	206	1029[b]	14	271	70[b]
	1	2	1248[b]	1214[b]	208	212	1039[b]	15	259	75[b]
	2	1	1270[b]	1346[b]	216	195	698[b]	17	235	79[b]
	2	2	1514[b]	1346[b]	187	202	998[b]	17	264	88[b]
2	1	1	1305	1286	225	224	1053[b]	16	266	64
	1	2	1384	1467	226	225	1196[b]	20	299	75
	2	1	1447	1443	244	249	1202[b]	19	290	70
	2	2	1445	1422	226	226	1183[b]	17	282	68
3	1	1	1287	1307	213	213	1102	17	275	68
	2	1	1363	1363	232	226	1146	18	279	67
	3	1	1322	1324	216	221	1093	16	292	69
4	1	1	1356	1350	207	209	1117	16[b]	285	129[c]
	1	2	1349	1360	213	207	1117	16[b]	286	125[c]
	2	1	1322	1358	216	214	1084	16[b]	285	132[c]
	2	2	1345	1368	212	214	1130	10[b]	281	131[c]
5	1	1	1312	1216	217[b]	191[b]	1041	17	276	111
	1	2	1281	1278	217[b]	190[b]	1047	17	276	108
	2	1	1259	1228	199[b]	150[b]	1042	18	271	100
	2	2	1266	1239	202[b]	240[b]	1036	15	269	101
6	1	1	1222[b]	1297[b]	220	246	1019	21	279	82
	1	2	1154[b]	1357[b]	216	230	1047	21	280	78
	2	1	1280[b]	1217[b]	213	191	1096	22	259	74
	2	2	1341[b]	1247[b]	212	196	1048	21	262	77
7	1	1	1295	1283	222	222	1061	18	276	73
	1	2	1286	1303	217	226	1087	18	274	71
	2	1	1296	1312	219	232	1061	19	273	74
	2	2	1302	1314	230	222	1063	18	272	76
8	1	1	1329	1271	217	217	1061	16	285	96
	1	2	1310	1303	213	223	1067	17	273	98
	2	1	1301	1258	217	222	1053	18	273	92
	2	2	1273	1294	216	221	1058	17	270	91
9	1	1	1368	1327	228	225	1070	20	290	77
	1	2	1327	1268	213	226	1088	21	292	77
	2	1	1331	1336	216	214	1098	20	283	75
	2	2	1340	1319	217	216	1108	20	290	75
10	1	1	1306	1323	206	210	1061	21	278	89
	2	1	1304	1315	209	212	1077	22	282	91
	3	1	1318	1317	205	209	1092	20	285	91
	4	1	1325	1337	209	212	1096	20	289	91
11	1	1	1210	1307	254[b]	218[b]	1123	17	273	76
	1	2	1206	1311	254[b]	222[b]	1125	17	272	76
	2	1	1320	1278	211[b]	208[b]	1060	18	290	72
	2	2	1330	1305	213[b]	210[b]	1077	18	293	72
12	1	1	1402	1418	242	224	1167	21	314	83
	1	2	1399	1404	226	232	1145	21	309	81
	2	1	1402	1376	219	231	1148	19	301	81
	2	2	1403	1381	228	221	1167	19	304	80

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

[c] Outlier by Grubbs test.

Table 2. Interlaboratory study results obtained for daidzin in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	10 (2)	1325.6	33.75	2.55	55.7	4.2	94.49	155.97	0.78
Soy beverage	10 (2)	217.9	8.51	3.90	11.45	5.25	23.82	32.06	0.74
Soy flour	10 (2)	1087.2	21.72	2.00	38.47	3.54	60.83	107.72	0.63
Vegetable burger	11 (1)	18.4	0.98	5.35	2.00	10.85	2.76	5.59	1.05
Soy molasses	12 (0)	280.1	8.92	3.18	14.12	5.04	24.97	39.53	0.74
Miso	10 (2)	80.9	3.12	3.86	12.19	15.07	8.73	34.12	1.82

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

samples A, B, E, and the practice sample, 1 g; test samples D and H, 1–2 g; test samples F and G, 2–3 g; and test sample C, 4–5 g.

A practice sample of soy flour was provided to all collaborators. The expected concentration range was provided for each isoflavone level. Laboratories were asked to proceed with the study if the results of the analysis of the practice sample were within the given range. The concentration ranges were as follows: daidzin–daidzein subtotal, 900–1100 μg/g; genistin–genistein subtotal, 1100–1300 μg/g; and glycitin–glycitein subtotal, 150–200 μg/g. All concentrations were in μg aglycon equivalents/g.

Preparation of standards involved dilution of the stock standard solution before use. Participants were instructed to transfer a 5 mL aliquot of the stock solution to a 10 mL volumetric flask, and to dilute it to volume with methanol–water (50 + 50) to make standard 4. Subsequent dilutions of standard 4 were made to prepare the remaining working standards.

Statistical Analysis

The data analysis for this summary follows the steps outlined by AOAC INTERNATIONAL (9). Each isoflavone assay was treated as an independent measurement. The method variability was determined separately for each measurement. Within-laboratory variance was estimated for each matrix and laboratory by using the restricted maximum likelihood method (REML) in SAS's PROC MIXED (10). For those cases in which there was no convergence, the estimate was derived from a method of moment estimator (MIVQUE0). The REML and MIVQUE0 methods were appropriate for estimating the variance attributed to within-day, among-days, and among-samples components with unbalanced data. Repeatability for each laboratory was determined as the sum of these components. The Cochran test was applied within each matrix to identify laboratories showing significantly greater variability than that shown by other laboratories. These laboratories were removed from further analysis. The Grubbs test for removal of laboratories with extreme averages was performed after the Cochran test. The Grubbs test for single values was followed by the Grubbs test for pairs of values (2 lowest, 2 highest, lowest and highest). No more than 2 of 12 laboratories were removed for any test sample matrix. After removal of outlying laboratories, the REML was used to estimate the among-laboratories, among-samples within-laboratory (when applicable), and among-days within-sample and within-day components of variance for each matrix. Repeatability variance was calculated as the sum of the among-samples, among-days, and within-day components. Reproducibility variance was calculated as the sum of the repeatability variance and the among-laboratories variance. The collaborative data and results of statistical analysis are shown in Tables 1–16.

Laboratories that were found to be outliers and removed from the statistical analysis are listed in Tables 1, 3, 5, 7, 9, 11, and 13. For daidzein in test sample C (vegetable burger, Table 7), the results ranged from 0 to 3 μg/g over all laboratories. To retain as much data as possible, no laboratories were removed as outliers from the statistical analysis of daidzein in this test sample, although 2 laboratories would have qualified on the basis of the statistical testing. A similar situation was found for test samples C (burger) and F (soy molasses) in the glycitein assay (Table 9). After removal of laboratory 9 from the analysis of results for test sample C (burger), the other values ranged from 0 to 3 μg/g in these 2 test samples. Statistical testing found other laboratories that qualified for removal, but none were removed. Laboratories that qualified for removal from the analysis of results for genistein in test sample C (burger, Table 11) also were retained because of the low range of values (0–3 μg/g). There were many zero values listed in the data tables, but they were mainly for glycitin, glycitein, and test sample C (burger). The glycitein family was the least abundant soy isoflavone group found, and this was reflected by the plethora of zero values. Also, test sample C (burger) had the lowest level of isoflavones by 1 order of magnitude, and therefore many zero values appeared in the data tables.

The HORRAT equation gives values that indicate whether the method is sufficiently precise for the level of analyte being measured and is based on historical collaborative study values. The HORRAT value is the ratio of the reproducibility relative standard deviation, in percent (RSD_R, %), to the pre-

Table 3. Glycitin data[a] reported as aglycon equivalents in μg/g

Lab	Day	Sample	Soy isolate sample A	Soy isolate sample E	Soy beverage sample D	Soy beverage sample H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	199	199	31	32	184[b]	0	55[b]	10[b]
	1	2	197	189	31	33	185[b]	0	56[b]	12[b]
	2	1	167	187	25	26	153[b]	0	14[b]	0[b]
	2	2	205	173	22	27	139[b]	0	7[b]	0[b]
2	1	1	206	207	30	28	208	3	51	2
	1	2	229	238	30	30	237	5	66	4
	2	1	221	221	30	30	224	3	53	3
	2	2	226	225	28	29	228	3	54	2
3	1	1	217	224	77[b]	79[b]	218	2	71	6
	2	1	227	232	88[b]	88[b]	223	2	72	6
	3	1	223	225	83[b]	85[b]	223	3	76	6
4	1	1	201	197	26	26	197	0	55	0
	1	2	201	206	28	27	199	0	55	0
	2	1	206	203	31	30	195	0	52	0
	2	2	206	206	31	32	205	0	52	0
5	1	1	231	219	33[b]	28[b]	209	3	71	12
	1	2	224	231	33[b]	27[b]	215	3	73	12
	2	1	222	215	26[b]	21[b]	207	3	62	10
	2	2	224	222	27[b]	39[b]	204	2	65	9
6	1	1	218	227	31	36	210	4[b]	66	9
	1	2	213	233	32	35	216	4[b]	67	9
	2	1	216	221	31	29	226	6[b]	58	14
	2	2	227	218	32	29	213	6[b]	64	15
7	1	1	202	199	27	27	198	2	50	2
	1	2	201	202	26	27	203	2	50	2
	2	1	204	205	26	28	199	3	50	1
	2	2	206	206	28	27	199	3	50	1
8	1	1	221	210	33	34	209	3	69	8
	1	2	214	217	32	34	210	3	66	7
	2	1	212	205	31	31	203	3	60	10
	2	2	208	210	30	31	201	3	58	10
9	1	1	217	216	32	32	209	3	57	8
	1	2	218	206	30	32	210	3	58	8
	2	1	220	219	31	31	216	4	58	4
	2	2	25	217	32	30	214	4	57	3
10	1	1	210	211	32	32	198	4	54	5
	2	1	215	217	31	31	214	4	57	5
	3	1	213	212	31	30	212	4	56	4
	4	1	218	220	30	31	220	4	66	4
11	1	1	212	235	44	37	243[b]	4	60	35[b]
	1	2	212	237	43	38	243[b]	4	59	35[b]
	2	1	213	206	33	35	205[b]	4	66	11[b]
	2	2	212	208	33	35	209[b]	3	65	10[b]
12	1	1	237	234	24	22	212	6	84	4
	1	2	236	236	20	24	207	5	82	4
	2	1	238	237	22	24	205	6	82	4
	2	2	240	236	24	23	209	6	84	4

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

[c] Outlier by Grubbs test.

Table 4. Interlaboratory study results obtained for glycitin in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	12 (0)	215.4	7.32	3.4	14.03	6.52	20.49	39.29	0.91
Soy beverage	10 (2)	30.0	2.31	7.72	4.30	14.36	6.48	12.05	1.50
Soy flour	10 (2)	210.7	6.24	2.96	9.86	4.68	17.47	27.60	0.65
Vegetable burger	11 (1)	2.8	0.44	15.75	1.71	61.17	1.23	4.78	4.46
Soy molasses	11 (1)	62.6	4.35	6.94	9.96	15.92	12.17	27.89	1.85
Miso	10 (2)	5.6	1.63	29.18	4.01	71.71	4.57	11.22	5.81

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

dicted reproducibility relative standard deviation, in percent ($PRSD_R$, %):

$$\text{HORRAT} = \frac{RSD_R, \%}{PRSD_R, \%}$$

where $PRSD_R, \% = 2C^{-0.1505}$ and C = the estimated mean concentration. HORRAT values between 0.5 and 2.0 indicate satisfactory method precision.

AOAC Official Method 2001.10 Determination of Isoflavones in Soy and Selected Foods Containing Soy

Extraction, Saponification, and Liquid Chromatography First Action 2001

(Applicable to the determination of total isoflavone content at ≥50 μg/g, individual isoflavone glucoside and aglycon content at ≥20 μg, and isoflavone family subtotals at ≥20 μg/g in soy and foods containing soy.)

See Tables **2001.10A–I** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test samples are extracted at 65°C for 2 h in methanol–water (80 + 20), and the extracts are saponified at ambient temperature with NaOH solution. The extracts are acidified, filtered, and diluted with water to methanol–water (50 + 50). The extracts are then centrifuged to clarify them and analyzed by liquid chromatography (LC). Isoflavone glucosides and aglycons are separated on a C18 reversed-phase column with a methanol–water mobile phase and determined by UV detection at 260 nm. Results are expressed in aglycon units by summing the concentrations of the aglycon isoflavones (genistein, glycitein, and daidzein) and the aglycon equivalents of the corresponding glucoside forms (genistin, glycitin, and daidzin).

B. Apparatus

(**a**) *LC system*.—With automatic sampler and 100 μL loop, binary gradient pumping system, UV detector at 260 nm, and data acquisition system.

(**b**) *Chromatography column*.—C18 reversed-phase, 200 × 2.1 mm id, or C18 reversed-phase, 200 × 4.6 mm id.

(**c**) *Balance*.—Analytical, capable of weighing to 0.00001 g.

(**d**) *Dispenser*.—Dispensing 50 ± 0.5 mL methanol–water (80 + 20).

(**e**) *Pipets*.—Dispensing 1–5 mL; with disposable tips.

(**f**) *Water bath*.—Maintaining 65°C, with shaker.

(**g**) *Orbital platform shaker*.—Holding 250 mL Erlenmeyer flasks.

(**h**) *Filter paper*.—15 cm, quantitative grade, medium porosity, fan-folded.

(**i**) *Centrifuge*.—Centrifuging 1 mL fluid at 7000 × g.

(**j**) *Microfuge tube*.—1.5 mL, disposable.

(**k**) *Vials*.—Glass, for LC autosampler, with Teflon-lined septa.

C. Reagents

(**a**) *Isoflavone standards*.—*See* Table **2001.10J**.

(**b**) *Stock standard solutions*.—Using analytical balance capable of weighing to 0.00001 g, weigh 5 mg daidzin, 5 mg genistin, 20 mg daidzein, 20 mg genistein, and 5 mg glycitein into 5 separate 50 mL low-actinic volumetric flasks. Quantitatively transfer contents of 2 mg vial of glycitin into 50 mL low-actinic volumetric flask, rinsing vial repeatedly with methanol and adding rinsings to volumetric flask. Dissolve contents of each flask in methanol and dilute to volume. Stopper each flask and mix well by repeated inversion. Store at room temperature in low-actinic glass flasks for ≤6 months.

(**c**) *Working standard solutions*.—Prepare 5 levels of working standards by diluting the volume of each stock standard shown in Table **2001.10K** in corresponding volumetric flask indicated. Add volume of water shown in Table **2001.10K**, and dilute to volume with methanol–water

Table 5. Genistin data[a] reported as aglycon equivalents in μg/g

Lab	Day	Sample	Soy isolate sample A	Soy isolate sample E	Soy beverage sample D	Soy beverage sample H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	1401	1381	395	396	1195[b]	23	93[b]	145[b]
	1	2	1341	1302	406	415	1213[b]	17	96[b]	152[b]
	2	1	1281	1417	393	380	950[b]	24	53[b]	139[b]
	2	2	1388	1328	359	391	1141[b]	22	49[b]	174[b]
2	1	1	1420	1391	423	403	1228[b]	22	83	139
	1	2	1507	1584	406	397	1432[b]	25	92	150
	2	1	1530	1533	440	434	138[b]	22	84	141
	2	2	1542	1538	405	397	1376[b]	24	84	140
3	1	1	1403	1424	402	405	1281	25	95	168
	2	1	1457	1467	433	422	1302	23	95	170
	3	1	1505	1504	413	415	1341	26	101	163
4	1	1	1447	1433	425	429	1298	22	84	154
	1	2	1435	1439	434	426	1304	21	85	151
	2	1	1475	1487	434	423	1320	21	84	154
	2	2	1484	1491	424	428	1333	18	90	153
5	1	1	1447	1341	416[b]	390[b]	1253	22	100	151
	1	2	1406	1419	419[b]	385[b]	1258	22	97	153
	2	1	1387	1369	382[b]	298[b]	1262	24	96	151
	2	2	1390	1377	388[b]	470[b]	1250	19	96	153
6	1	1	1400	1476	481	533	1325	30	103	178
	1	2	1410	1538	466	504	1329	30	103	169
	2	1	1460	1483	451	463	1356	32	102	174
	2	2	1517	1512	458	480	1296	32	101	174
7	1	1	1460	1436	446	447	1312	28	100	165
	1	2	1448	1458	457	457	1337	28	98	161
	2	1	1453	1463	435	481	1302	28	94	162
	2	2	1464	1462	453	441	1303	27	94	165
8	1	1	1458	1367	413	416	1250	20	99	161
	1	2	1415	1400	406	424	1255	22	96	162
	2	1	1407	1353	410	416	1240	23	91	156
	2	2	1375	1381	406	414	1242	23	89	156
9	1	1	1526	1528	458	461	1342	28	112	170
	1	2	1533	1455	432	462	1365	29	113	173
	2	1	1551	1529	439	435	1393	21	112	166
	2	2	1566	1513	443	429	1387	28	112	166
10	1	1	1473	1491	412	424	1323	29	95	169
	2	1	1494	1512	429	436	1350	30	94	172
	3	1	1508	1489	417	434	1354	35	96	176
	4	1	1495	1500	423	426	1346	35	96	175
11	1	1	1296	1392	490[b]	425[b]	1332	23	98	162
	1	2	1289	1394	490[b]	426[b]	1336	23	98	163
	2	1	1435	1402	393[b]	403[b]	1276	23	98	158
	2	2	1426	1413	392[b]	402[b]	1289	23	98	157
12	1	1	1495	1512	458	430	1353	27	99	169
	1	2	1491	1499	428	446	1325	26	97	166
	2	1	1502	1472	426	449	1340	25	95	167
	2	2	1502	1483	443	430	1359	25	95	163

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

Table 6. Interlaboratory study results obtained for genistin in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	12 (0)	1449.6	39.60	2.73	66.45	4.58	110.87	186.05	0.86
Soy beverage	10 (2)	430.1	14.73	3.42	28.20	6.56	41.25	78.95	1.02
Soy flour	10 (2)	1313.2	21.29	1.62	41.79	3.18	59.61	117.02	0.59
Vegetable burger	12 (0)	25.0	2.10	8.43	4.18	16.73	5.89	11.69	1.70
Soy molasses	11 (1)	96.4	2.74	2.84	7.54	7.82	7.66	21.10	0.97
Miso	11 (1)	161.6	3.23	2.00	9.98	6.17	9.05	27.94	0.83

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

(1 + 1). The approximate concentration of each isoflavone is shown in Table **2001.10L**. For standards of <99+% purity, adjust values for purity of standard accordingly. Store solutions at room temperature in low-actinic glass flasks for ≤6 months.

(**d**) *Methanol.*—LC grade.

(**e**) *Hexane.*—LC grade.

(**f**) *Acetic acid, glacial.*

(**g**) *Extraction solution.*—Methanol–water (80 + 20). Add 800 mL methanol to 1000 L volumetric flask. Add 200 mL water (do not dilute to volume), stopper, and mix well by inversion.

(**h**) *Methanol–water (50 + 50).*—Combine 250 mL methanol with 250 mL water, mix well, and filter, using vacuum, through 0.45 μm filter.

(**i**) *Mobile phase A.*—Water–methanol–acetic acid (88 + 10 + 2). Combine 3520 mL water, 400 mL methanol, and 80 mL glacial acetic acid. Mix well and filter, using vacuum, through 0.45 μm filter.

(**j**) *Mobile phase B.*—Methanol–acetic acid (98 + 2). Add 3920 mL methanol to 6 L Erlenmeyer flask. Add 80 mL glacial acetic acid, and mix well. Filter through 0.45 μm filter disk with vacuum.

(**k**) *Sodium hydroxide solution.*—2M. Weigh 80 g NaOH into 1 L volumetric flask, dissolve in water, let cool to ambient temperature, and dilute to volume with water.

D. Extraction and Saponification

Accurately weigh amount of test sample that contains ca 1 g protein, but not >5 g test sample, into 250 mL Erlenmeyer flask with ground-glass stopper. Add 40 mL extraction solution, and stopper flask. Cover stopper and neck of flask with aluminum foil, and shake flask in 65°C water bath for 2 h.

Cool to room temperature, and add 3 mL 2M NaOH. Replace aluminum foil, and shake flask at room temperature on orbital shaker for 10 min. Remove flask from shaker, and add 1 mL glacial acetic acid.

Swirl to suspend contents of flask, and pour into 50 mL graduated cylinder with ground glass stopper. Dilute to 50 mL with extraction solution and mix well.

Filter solution through quantitative-grade filter paper into 250 mL beaker. Pipet 5 mL filtrate into 10 mL graduated cylinder with ground-glass stopper. Add 4.0 mL water, and dilute to 10 mL with methanol. Stopper graduated cylinder, and invert cylinder repeatedly to mix contents.

Transfer ca 1 mL extract to 1.5 mL centrifuge tube, and centrifuge for 5 min at 7000 × *g*. Transfer clear supernatant to LC sample vial. *Note:* Do not filter supernatant through membrane filter.

E. Determination

Set LC system to flow rate of 0.4 mL/min for 2.1 mm id column and initial mobile phase composition shown in Table **2001.10M**. For 4.6 mm id column, set flow rate to 1.5 mL/min, and use same gradient. Set detector wavelength to 260 nm. Let system equilibrate by running 1 complete gradient with no injection.

Verify system performance by injecting 20 μL working standard 3, using gradient conditions in Table **2001.10M**. Verify baseline separation of daidzein and glycitein peaks.

The tailing factor for any peak should be ≤1.5. Adjust either %B or gradient times as needed to obtain required separation of all 6 components. Typical relative retention times (in min) are as follows: daidzin, 0.53; glycitin, 0.58; genistin, 0.66; daidzein, 0.89; glycitein, 0.92; and genistein, 1.00. Retention times will vary with the age and condition of the column.

Inject all working standards and each test extract. Determine area of each isoflavone peak.

F. Calculation

Determine response for each isoflavone by calculating slope (*m*) and intercept (*b*), using linear regression analysis of area counts vs response for 5 levels of each of the isoflavone standards.

Calculate concentration of each isoflavone in test sample, using following equation:

$$\text{Isoflavone, } \mu g/g = \frac{((As \times m) + b) \times 50 \times 10}{Ws \times 5}$$

Table 7. Daidzein data[a] reported as aglycon equivalents in μg/g

			Soy isolate sample		Soy beverage sample					
Lab	Day	Sample	A	E	D	H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	88	89	44[b]	53[b]	46[b]	0	18[b]	134[b]
	1	2	90	88	51[b]	49[b]	48[b]	0	20[b]	139[b]
	2	1	105	106	65[b]	52[b]	210[b]	0	1[b]	142[b]
	2	2	109	103	63[b]	61[b]	149[b]	0	1[b]	169[b]
2	1	1	52	53	20	23	6	0	2	126
	1	2	70	74	22	25	13	0	5	132
	2	1	59	56	22	23	6	0	3	142
	2	2	60	60	24	24	9	0	4	135
3	1	1	63[b]	63[b]	18	14	3	0	1	132
	2	1	64[b]	65[b]	15	14	2	0	1	131
	3	1	25[b]	43[b]	17	19	0	0	4	137
4	1	1	53	45	0[b]	0[b]	0	0	0	133
	1	2	50	48	0[b]	0[b]	0	0	0	131
	2	1	41	44	0[b]	0[b]	0	0	0	134
	2	2	44	42	0[b]	0[b]	0	0	0	134
5	1	1	67	67	24	24	12	0	11	143
	1	2	64	72	23	23	11	0	10	144
	2	1	66	79	20	17	15	0	13	140
	2	2	66	70	21	28	14	0	15	142
6	1	1	0[c]	0[c]	0[c]	0[c]	0	0	0	141
	1	2	0[c]	0[c]	0[c]	0[c]	0	0	0	132
	2	1	0[c]	0[c]	0[c]	0[c]	0	0	0	137
	2	2	0[c]	0[c]	0[c]	0[c]	0	0	0	148
7	1	1	54	52	18	19	1	0	0	156[c]
	1	2	53	54	17	17	1	0	0	154[c]
	2	1	53	54	16	20	1	0	2	156[c]
	2	2	54	55	18	20	1	0	2	159[c]
8	1	1	68	72	28	30	20	3	9	136
	1	2	73	73	27	29	21	3	9	137
	2	1	70	69	28	29	20	3	8	133
	2	2	69	70	27	27	20	3	8	135
9	1	1	52	52	18	17	1	0	2	135
	1	2	52	50	18	17	0	0	1	134
	2	1	60	60	23	22	8	1	4	137
	2	2	61	60	23	22	8	1	4	135
10	1	1	52	52	18	19	7	0	4	130
	2	1	46	58	19	20	5	0	4	131
	3	1	61	59	21	22	5	0	5	135
	4	1	58	60	18	24	5	0	3	135
11	1	1	76	93	28	29	38[b]	1	16[b]	133
	1	2	75	93	33	29	37[b]	1	15[b]	133
	2	1	87	94	27	29	27[b]	1	9[b]	132
	2	2	94	94	26	28	27[b]	1	9[b]	130
12	1	1	55	56	18	16	3	0	4	145
	1	2	55	55	15	17	3	0	4	143
	2	1	54	54	16	19	3	0	4	142
	2	2	54	54	17	18	3	0	4	139

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

[c] Outlier by Grubbs test.

Table 8. Interlaboratory study results obtained for daidzein in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	10 (2)	65.1	5.72	8.78	17.17	26.39	16.01	48.07	3.09
Soy beverage	10 (2)	19.4	1.91	9.84	8.22	42.42	5.33	23.01	4.14
Soy flour	10 (2)	5.7	2.06	36.08	6.76	118.65	5.76	18.94	9.64
Vegetable burger	12 (0)	0.4	0.21	53.78	0.90	229.91	0.59	2.51	12.47
Soy molasses	10 (2)	3.8	1.28	34.25	3.96	105.70	3.60	11.10	8.06
Miso	10 (2)	135.8	3.73	2.74	4.95	3.64	10.44	13.86	0.48

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

where *As* = peak area of isoflavone in test solution; *m* = slope from linear regression for standard response; *b* = intercept from linear regression for standard response; *Ws* = weight of test portion, g; 50 = dilution volume in **D**; 10 = second dilution volume in **D**; 5 = aliquot in **D**.

Convert concentrations of isoflavone glucosides genistin, glycitin, and daidzin to aglycon equivalents, using following equation:

$$Cae = \left(\frac{MWa}{MWg}\right) \times Cg$$

where *Cae* = isoflavone aglycon equivalents, μg/g; *MWa* = molecular weight of aglycon (Table **2001.10N**); *MWg* = molecular weight of glucoside (Table **2001.10N**); and *Cg* = concentration of genistin, glycitin, or daidzin, μg/g.

Calculate total isoflavones, μg/g aglycon equivalents/g, by summing concentrations of daidzein, glycitein, and genistein and adding this total to sum of aglycon equivalent concentrations of daidzin, glycitin, and genistin.

$$Ta = Ca(\text{daidzein}) + Ca(\text{glycitein}) + Ca(\text{genistein})$$

$$Tae = Cae(\text{daidzin}) + Cae(\text{glycitin}) + Cae(\text{genistin})$$

where *Ta* = sum of concentrations of aglycons, and *Tae* = sum of aglycon equivalent concentrations of glucosides.

$$\text{Total isoflavones, μg aglycon equivalents/g} = Ta + Tae$$

Ref. *J. AOAC Int.* **84**, 1870–1874(2001)

Results and Discussion

The data reported by collaborators are shown in Tables 1, 3, 5, 7, 9, 11, and 13, organized by isoflavone component and isoflavone total. Twelve participating laboratories analyzed 8 test samples, including 2 blind duplicates, and were asked to analyze the test samples in duplicate on 2 separate days. Duplicate runs were added to the collaborative protocol to better estimate within laboratory variability on any given day. The statistical analysis was complicated because not all of the laboratories ran duplicates each day.

The method allows for distinction between bound isoflavone forms (i.e., glucosides and glucoside esters) and unbound (aglycon) isoflavone forms. Six isoflavone components, daidzin, glycitin, genistin, daidzein, glycitein, and genistein; subtotals of daidzin–daidzein, glycitin–glycitein, and genistin–genistein; and total isoflavones were each statistically analyzed for method validity. This was undertaken because of the variety of approaches used to report isoflavone data. Typically, isoflavones are reported as total isoflavones, or subtotals of isoflavone families, or individual isoflavone components. Statistical summaries for each of the analyzed isoflavone components, subtotals, and total isoflavones are listed in Tables 2, 4, 6, 8, 10, 12, 14–16, and **2001.10I**.

The statistical summary tables list the following: mean, repeatability standard deviation (s_r), reproducibility standard deviation (s_R), repeatability relative standard deviation (RSD_r), reproducibility relative standard deviation (RSD_R), (r; $2.8 \times s_r$), reproducibility (R; $2.8 \times s_R$), and HORRAT.

The levels of isoflavone glucosides present are generally higher than those of the aglycon isoflavone forms. As a result, the isoflavone glucosides had lower RSD values (Tables 2, 4, and 6). Of the 18 different test sample sets analyzed for isoflavone glucosides, 16 had HORRAT values of <2.0. The 2 sample sets with HORRAT values of >2.0 were for glycitin (Table 4) with means of 5.6 and 2.8 μg/g. Clearly, isoflavone values at these low levels are below the limit of quantitation for individual components for this method. Mean values for glucoside isoflavones that gave HORRAT values of <2.0 were from 18 to 1449 μg/g. This result suggests that the method is capable of measuring isoflavone glucosides down to 18 μg/g.

For the aglycon isoflavone forms, 18 sample sets also were analyzed (Tables 7–12). Only 2 sets had HORRAT values of <2.0. These were the daidzein value (Table 8) and the

Table 9. Glycitein data[a] reported as aglycon equivalents in μg/g

			Soy isolate sample		Soy beverage sample					
Lab	Day	Sample	A	E	D	H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	0[b]	0[b]	0	0	0[b]	0	0	18
	1	2	0[b]	0[b]	0	0	0[b]	0	0	18
	2	1	8[b]	0[b]	0	0	60[b]	0	0	19
	2	2	35[b]	0[b]	0	0	120[b]	0	0	22
2	1	1	7	7	2	2	0	0	0	17
	1	2	8	9	3	2	0	0	0	19
	2	1	8	7	2	2	2	0	0	13
	2	2	7	8	1	3	0	0	0	13
3	1	1	6	6	1	1	1	0	0	22
	2	1	6	6	1	1	1	0	0	22
	3	1	0	3	1	1	0	0	0	27
4	1	1	0	0	0	0	0	0	0	11
	1	2	0	0	0	0	0	0	0	11
	2	1	0	0	0	0	0	0	0	10
	2	2	0	0	0	0	0	0	0	10
5	1	1	8	8	0	0	0	0	3	17
	1	2	8	9	0	0	0	0	3	17
	2	1	8	8	0	0	0	0	3	16
	2	2	8	8	0	0	0	0	3	16
6	1	1	0	0	0	0	0	0	0	21
	1	2	0	0	0	0	0	0	0	23
	2	1	0	0	0	0	0	0	0	22
	2	2	0	0	0	0	0	0	0	21
7	1	1	7	6	3[b]	7[b]	0	0	1	24
	1	2	6	2	1[b]	7[b]	0	0	1	24
	2	1	6	6	3[b]	5[b]	0	0	1	24
	2	2	6	6	4[b]	5[b]	0	0	1	25
8	1	1	10	10	5	5	4	1	2	18
	1	2	10	10	4	5	4	1	2	18
	2	1	9	10	4	4	4	1	2	18
	2	2	9	10	4	4	4	1	2	20
9	1	1	7	7	2	2	1	10[b]	1	24
	1	2	7	6	2	2	1	0[b]	1	24
	2	1	8	8	3	3	3	0[b]	2	23
	2	2	8	8	3	3	3	0[b]	2	23
	1	1	8	7	3	2	3	0	1	15
	2	1	7	7	2	3	2	0	1	17
	3	1	7	8	2	2	3	0	2	21
	4	1	7	7	2	2	2	0	2	20
11	1	1	10[b]	17[b]	0[c]	3[c]	13[b]	2	2	17
	1	2	16[b]	8[b]	4[c]	0[c]	13[b]	2	2	18
	2	1	14[b]	15[b]	7[c]	7[c]	14[b]	2	0	17
	2	2	14[b]	15[b]	7[c]	7[c]	12[b]	3	0	16
12	1	1	6	7	1	0	0	0	0	29[b]
	1	2	6	7	0	0	0	0	0	32[b]
	2	1	7	7	0	0	1	0	1	24[b]
	2	2	8	7	0	0	1	0	2	24[b]

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

[c] Outlier by Grubbs test.

Table 10. Interlaboratory study results obtained for glycitein in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	10 (2)	5.7	0.99	17.25	3.44	60.00	2.77	9.64	4.88
Soy beverage	10 (2)	1.2	0.32	25.30	1.54	122.90	0.89	4.30	7.95
Soy flour	10 (2)	1.0	0.56	56.24	1.46	147.42	1.56	4.09	9.20
Vegetable burger	11 (1)	0.3	0.09	27.95	0.71	222.73	0.25	2.00	11.73
Soy molasses	12 (0)	0.9	0.49	54.81	0.94	105.52	1.37	2.63	6.48
Miso	11 (1)	18.9	1.93	10.17	4.48	23.64	5.39	12.54	2.30

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

genistein value (Table 12) for sample G (miso). Miso is a fermented soy product in which fermentation apparently converts isoflavone glucosides and glucoside esters to the aglycon forms. Isoflavone aglycons that were present at <65.5 μg/g had HORRAT values of >2.0. With the exception of miso, the test samples contained relatively little isoflavone aglycon compared with the isoflavone glucoside. The majority of values for daidzein, glycitein, and genistein were <25 mg/g. These small peaks were apparently difficult to identify and measure consistently. The matrixes tested are natural products or processed natural products and contain materials that can produce interference peaks. These interference peaks can occlude the peak of interest and possibly be misidentified as the peak of interest. Low-level isoflavone aglycon peaks (<65 μg/g) were apparently more difficult to identify than low-level isoflavone glucoside peaks. In addition, it is apparent that some laboratories did not detect analytes present at levels of <20 μg/g.

Subtotals of isoflavone families, daidzin–daidzein, glycitin–glycitein, and genistin-genistein were also statistically analyzed. These subtotals (Tables 14–16) showed good reproducibility for high and moderate values. Subtotals with low values were not as reproducible. Test sample C (vegetable burger) for daidzin–daidzein (Table 14) had a mean of 18.8 μg/g and good reproducibility (RSD_R, 10.7%) with a HORRAT of 1.04. For test sample G (miso), glycitin–glycitein had a mean of 25.4 μg/g and an RSD of 27.4% to give a HORRAT of 2.79 (Table 15). Again for test sample C, the genistin–genistein subtotal mean was 25.3 μg/g with an RSD_R of 16.1% to give a HORRAT of 1.64 (Table 16). These results suggest that 18 μg/g is the lower limit of quantitation for the subtotal values.

The statistical results for total isoflavone (Table **2001.10I**) gave method reproducibility of 3.2–16.1%, and all HORRAT values were ≤1.80 for mean values of 47–3099 μg/g. These results demonstrate that the method is capable of measuring total isoflavones in soy and soy products down to 47 μg/g.

Collaborators' Comments

Laboratory 4.—The gradient used presented problems that were traced to mobile phase out-gassing from a mixing chamber located prepump (i.e., low-pressure side). The use of a different instrument that had 2 pumps and a post pump-mixing chamber solved the problem.

Laboratory 11.—The laboratory commented that bringing the extraction solution to volume before filtration would produce a high bias in the results. Studies undertaken by the authors' laboratory showed that a bias of approximately 2–3% existed for test samples with total isoflavone levels of 1100 μg aglycon/g. Further evaluation found that bringing the extraction solution to volume after filtration increased the complexity of the procedure and was far less repeatable within the laboratory than the method as written.

Another laboratory declined to participate in the study because of concerns that base hydrolysis would destroy the isoflavones. A study undertaken by the authors' laboratory evaluated the base hydrolysis step. Two test samples of standard solutions were taken through the method, but 1 test sample did not receive the NaOH solution for saponification. Identical recoveries were obtained for both standards, indicating that the saponification solution does not destroy the isoflavone aglycons or isoflavone aglucons. It should be noted that treatment of isoflavones with alkali at elevated temperatures does cause loss of isoflavones.

Recommendation

The results of this collaborative study show that the method for determining isoflavones by using extraction and saponification gives repeatable performance for all components with limits of detection of 20 μg/g for glucoside and subtotal components and 47 μg/g for total isoflavones. The Study Director recommends that this method be adopted First Action.

Table 11. Genistein data[a] reported as aglycon equivalents in μg/g

			Soy isolate sample		Soy beverage sample					
Lab	Day	Sample	A	E	D	H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	84	84	50[b]	58[b]	41[b]	0	15[b]	191[b]
	1	2	84	82	56[b]	55[b]	43[b]	0	17[b]	197[b]
	2	1	93	95	63[b]	47[b]	61[b]	0	63[b]	201[b]
	2	2	96	94	62[b]	55[b]	14[b]	0	49[b]	241[b]
2	1	1	44	48	28	30	4	0	3	220
	1	2	56	60	30	31	8	0	6	224
	2	1	49	54	29	29	6	0	6	234
	2	2	52	51	28	29	7	0	7	229
3	1	1	41[b]	42[b]	22	22	0	0	1	214
	2	1	43[b]	43[b]	24	24	1	0	0	214
	3	1	13[b]	29[b]	23	24	0	0	0	214
4	1	1	3	2	0	0	0	0	0	186
	1	2	4	3	0	0	0	0	0	185
	2	1	2	4	6	4	0	0	0	193
	2	2	1	2	5	5	0	0	0	194
5	1	1	50	46	30	30	7	0	2	194
	1	2	48	49	30	29	7	0	2	197
	2	1	48	48	27	22	7	0	2	193
	2	2	48	48	28	35	7	0	2	195
6	1	1	0	0	0	0	0	0	0	235
	1	2	0	0	0	0	0	0	0	221
	2	1	0	0	0	0	0	0	0	220
	2	2	0	0	0	0	0	0	0	222
7	1	1	44	43	25	25	0	0	0	202
	1	2	43	44	24	23	0	0	0	199
	2	1	44	44	24	28	0	0	0	200
	2	2	44	45	26	27	0	0	0	205
8	1	1	59	57	34	37	14	2	5	201
	1	2	57	57	33	35	15	2	5	202
	2	1	57	58	34	35	14	2	5	196
	2	2	56	56	33	34	15	2	5	198
9	1	1	46	47	27	27	1	0	0	212
	1	2	48	44	26	27	1	0	0	211
	2	1	52	53	30	30	6	0	1	212
	2	2	53	52	31	30	6	0	1	212
10	1	1	5	49	28	30	6	0	1	207
	2	1	49	49	29	31	5	0	1	209
	3	1	49	49	28	29	4	0	1	212
	4	1	47	46	26	30	2	0	0	211
11	1	1	54[b]	53[b]	44	38	52[b]	0	26[b]	212
	1	2	54[b]	59[b]	43	38	52[b]	0	26[b]	212
	2	1	100[b]	88[b]	37	40	61[b]	3	31[b]	209
	2	2	100[b]	89[b]	39	39	62[b]	3	31[b]	209
12	1	1	88	88	33	32	49	0	21	232
	1	2	87	88	31	33	47	0	20	228
	2	1	89	87	31	33	48	0	19	231
	2	2	89	87	32	32	49	0	19	224

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

Table 12. Interlaboratory study results obtained for genistein in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	10 (2)	47.8	2.87	6.00	29.45	61.60	8.03	82.47	6.89
Soy beverage	11 (1)	24.8	1.98	8.01	12.57	50.76	5.56	35.20	5.14
Soy flour	10 (2)	8.4	1.32	15.77	14.81	176.58	3.70	41.48	15.20
Vegetable burger	12 (0)	0.3	0.63	195.53	0.84	261.55	1.75	2.34	13.77
Soy molasses	10 (2)	3.4	0.62	18.35	6.21	184.84	1.73	17.39	13.87
Miso	11 (1)	210.0	3.89	1.85	13.47	6.41	10.89	37.71	0.90

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

Table 13. Total Isoflavone data[a] reported as aglycon equivalents in μg/g

			Soy isolate sample		Soy beverage sample					
Lab	Day	Sample	A	E	D	H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
1	1	1	3073	3043	723	745	2495[b]	37	452[b]	568[b]
	1	2	2960	2875	752	764	2528[b]	32	448[b]	593[b]
	2	1	2924	3151	762	700	2132[b]	41	366[b]	580[b]
	2	2	3347	3044	693	736	2561[b]	39	370[b]	694[b]
2	1	1	3034	2991	727	710	2499[b]	41	405	569
	1	2	3253	3432	716	710	2886[b]	49	468	604
	2	1	3315	3314	767	768	2821[b]	44	435	604
	2	2	3332	3304	711	707	2803[b]	44	431	587
3	1	1	3017	3065	732	734	2605	44	444	611
	2	1	3160	3175	794	774	2675	43	448	609
	3	1	3088	3128	752	764	2657	45	473	614
4	1	1	3061	3027	658	665	2612	38	423	612
	1	2	3039	3055	675	659	2620	38	426	602
	2	1	3046	3096	686	672	2599	37	422	621
	2	2	3080	3108	671	680	2668	28	423	621
5	1	1	3115	2897	720[b]	663[b]	2522	42	463	627
	1	2	3032	3057	722[b]	655[b]	2537	42	461	630
	2	1	2990	2947	654[b]	508[b]	2533	45	446	609
	2	2	3002	2964	666[b]	812[b]	2512	37	449	616
6	1	1	2841	3000	732	815	2554	55	448	666
	1	2	2777	3128	713	769	2592	55	450	632
	2	1	2956	2921	695	683	2678	60	419	641
	2	2	3085	2977	702	705	2557	59	427	657
7	1	1	3061	3019	741	746	2571	48	426	622
	1	2	3039	3063	741	758	2628	48	423	612
	2	1	3057	3085	724	795	2562	49	420	618
	2	2	3076	3088	759	742	2565	47	419	632
8	1	1	3145	2987	730	739	2558	45	469	620
	1	2	3079	3060	715	750	2572	48	451	624
	2	1	3056	2953	724	737	2534	50	439	605
	2	2	2990	3021	716	731	2540	49	432	610
9	1	1	3216	3177	765	764	2624	61	462	626
	1	2	3185	3029	721	766	2665	53	465	627
	2	1	3222	3205	742	735	2724	46	460	617
	2	2	3053	3169	749	730	2726	53	466	614

Table 13. (*continued*)

Lab	Day	Sample	Soy isolate sample A	Soy isolate sample E	Soy beverage sample D	Soy beverage sample H	Soy flour sample B	Vegetable burger sample C	Soy molasses sample F	Miso sample G
10	1	1	3054	3133	699	717	2598	54	434	614
	2	1	3115	3158	719	733	2654	56	439	625
	3	1	3156	3134	704	726	2670	59	444	638
	4	1	3150	3170	708	724	2672	59	456	635
11	1	1	2858	3097	859[b]	752[b]	2801	47	476	635[b]
	1	2	2852	3102	867[b]	752[b]	2807	47	473	636[b]
	2	1	3169	3083	709[b]	722[b]	2643	51	493	597[b]
	2	2	3176	3123	710[b]	722[b]	2675	51	495	594[b]
12	1	1	3283	3316	776	724	2784	54	522	661
	1	2	3274	3288	720	752	2727	52	513	654
	2	1	3292	3232	714	756	2745	51	503	649
	2	2	3295	3249	744	725	2787	51	508	634

[a] Samples A and E were blind duplicates, and samples D and H were blind duplicates. Samples B, C, F, and G were not run with blind duplicates.

[b] Outlier by Cochran test.

Table 14. Interlaboratory study results obtained for daidzin–daidzein subtotal in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	11(1)	375.3	40.91	2.97	65.33	4.75	114.55	182.92	0.88
Soy beverage	10 (2)	237.7	8.77	3.69	17.25	7.26	24.55	48.29	1.03
Soy flour	10 (2)	1095.3	23.37	2.13	38.52	3.52	65.43	107.86	0.63
Vegetable burger	11 (1)	18.8	0.98	5.23	2.01	10.66	2.76	5.62	1.04
Soy molasses	11 (1)	286.7	7.63	2.66	12.30	4.29	21.36	34.44	0.63
Miso	11 (1)	223.0	5.50	2.47	19.29	8.65	15.41	54.02	1.22

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

Table 15. Interlaboratory study results obtained for glycitin–glycitein subtotal in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	10 (2)	223.6	5.77	2.58	12.59	5.63	16.15	35.25	0.79
Soy beverage	11 (1)	31.7	2.77	8.74	5.42	17.11	7.76	15.19	1.80
Soy flour	10 (2)	211.6	6.29	2.97	9.93	4.69	17.62	27.80	0.66
Vegetable burger	11 (1)	3.2	0.56	17.13	2.06	63.51	1.56	5.78	4.74
Soy molasses	11 (1)	63.6	4.30	6.76	10.08	15.86	12.03	28.21	1.85
Miso	11 (1)	25.4	3.28	12.89	6.96	27.39	9.17	19.50	2.79

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

Table 16. Interlaboratory study results obtained for genistin–genistein subtotal in soy and foods containing soy by extraction, saponification, and LC

Matrix	No. of labs[a]	Mean, μg/g	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HORRAT
Soy isolate	12 (0)	1498.5	42.62	2.84	65.64	4.38	119.35	183.78	0.82
Soy beverage	10 (2)	456.1	14.99	3.29	22.46	4.92	41.98	62.88	0.77
Soy flour	10 (2)	1326.6	20.67	1.56	47.14	3.55	57.87	132.00	0.66
Vegetable burger	12 (0)	25.3	2.21	8.75	4.08	16.12	6.19	11.41	1.64
Soy molasses	11 (1)	102.0	2.95	2.89	12.10	11.87	8.25	33.89	1.49
Miso	11 (1)	371.7	5.84	1.57	18.79	5.05	16.36	52.60	0.77

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

Table 2001.10A. Interlaboratory results for daidzin in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	10(2)	1326	2.55	4.2	0.78
Soy beverage	10(2)	218	3.90	5.25	0.74
Soy flour	10(2)	1087	2.00	3.54	0.63
Vegetable burger	11(1)	18	5.35	10.8	1.05
Soy molasses	12(0)	280	3.18	5.04	0.74
Miso	10(2)	89	3.86	15.1	1.82

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10B. Interlaboratory results for glycitin in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	12(0)	215	3.4	6.52	0.91
Soy beverage	10(2)	30	7.7	14.4	1.5
Soy flour	10(2)	211	3.0	4.7	0.65
Vegetable burger	11(1)	3	15.8	61	4.5
Soy molasses	11(1)	63	6.9	15.9	1.85
Miso	10(2)	6	29.2	71.7	5.8

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10C. Interlaboratory results for genistin in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	12(0)	1450	2.73	4.58	0.86
Soy beverage	10(2)	430	3.42	6.56	1.02
Soy flour	10(2)	1313	1.62	3.18	0.59
Vegetable burger	12(0)	25	8.43	16.7	1.70
Soy molasses	11(1)	96	2.84	7.82	0.97
Miso	11(1)	162	2.00	6.17	0.83

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10D. Interlaboratory results for daidzein in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	10(2)	65.1	8.8	26.4	3.1
Soy beverage	10(2)	19.4	9.8	42.4	4.1
Soy flour	10(2)	5.7	36.1	118	9.6
Vegetable burger	12(0)	0.4	53.8	230	12.5
Soy molasses	10(2)	3.8	34.2	106	8.1
Miso	10(2)	135.8	2.7	3.6	0.48

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10E. Interlaboratory results for genistein in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	12(0)	1449.6	2.73	4.58	0.86
Soy beverage	10(2)	430.1	3.42	6.56	1.02
Soy flour	10(2)	1313.2	1.62	3.18	0.59
Vegetable burger	12(0)	25	8.43	16.73	1.7
Soy molasses	11(1)	96.4	2.84	7.82	0.97
Miso	11(1)	161.6	2	6.17	0.83

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10F. Interlaboratory results for daidzin-daidzein in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	11(1)	375	2.97	4.75	0.88
Soy beverage	10(2)	238	3.69	7.26	1.03
Soy flour	10(2)	1095	2.13	3.52	0.63
Vegetable burger	11(1)	19	5.23	10.7	1.04
Soy molasses	11(1)	287	2.66	4.29	0.63
Miso	11(1)	223	2.47	8.65	1.22

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10G. Interlaboratory results for glycitin-glycitein in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	10(2)	224	2.58	5.63	0.79
Soy beverage	11(1)	32	8.74	17.1	1.80
Soy flour	10(2)	212	2.97	4.69	0.66
Vegetable burger	11(1)	3	17.1	63.5	4.7
Soy molasses	11(1)	64	6.76	15.9	1.85
Miso	11(1)	25	12.9	27.4	2.79

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10H. Interlaboratory results for genistin-genistein subtotal in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	12(0)	1498	2.84	4.38	0.82
Soy beverage	10(2)	456	3.29	4.92	0.77
Soy flour	10(2)	1327	1.56	3.55	0.66
Vegetable burger	12(0)	25	8.75	16.12	1.64
Soy molasses	11(1)	102	2.89	11.87	1.49
Miso	11(1)	372	1.57	5.05	0.77

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10I. Interlaboratory results for total isoflavones in soy and soy-containing foods

Matrix	No. of labs[a]	Mean, μg/g	RSD_r, %	RSD_R, %	HORRAT
Soy isolate	12(0)	3099	2.92	4.11	0.86
Soy beverage	10(2)	730	3.25	4.47	0.75
Soy flour	10(2)	2635	1.77	3.18	0.65
Vegetable burger	12(0)	47	7.05	16.11	1.80
Soy molasses	11(1)	452	2.91	6.33	0.99
Miso	10(2)	622	1.78	3.18	0.52

[a] No. of laboratories retained after elimination of outliers (in parentheses).

Table 2001.10J. Isoflavone standards

Standard	Formula	CAS Registry No.	Indofine Cat. No.[a]
Daidzin	$C_{21}H_{20}O_9$	552-66-9	021096
Daidzein	$C_{15}H_{10}O_4$	486-66-8	D-O101
Genistin	$C_{21}H_{20}O_{10}$	529-59-9	021050
Genistein	$C_{15}H_{10}O_5$	446-72-0	G-103
Glycitin	$C_{22}H_{22}O_{10}$	40246-10-4	GL-002
Glycitein	$C_{16}H_{12}O_5$	40957-83-3	GL-001

[a] Cat. Nos. from Indofine Chemical Co., PO Box 473, Somerville, NJ 08876, USA; +1-908-359-6778; Fax +1-908-359-1179. Equivalent standards from other suppliers may be used.

Table 2001.10K. Preparation of working standards from dilutions of stock standard solutions

Working standard	Each stock standard, mL	Water, mL	Final volume, mL
1	1.0	6.0	200
2	1.0	6.0	100
3	2.0	12.0	100
4	4.0	24.0	100
5	4.0	24.0	50

Table 2001.10L. Approximate concentrations of individual isoflavones in working standards

Working standard	Daidzin, μg/mL	Glycitin, μg/mL	Genistin, μg/mL	Daidzein, μg/mL	Glycitein, μg/mL	Genistein, μg/mL
1	0.5	0.02	0.5	2.0	0.5	2.0
2	1.0	0.04	1.0	4.0	1.0	4.0
3	2.0	0.08	2.0	8.0	2.0	8.0
4	4.0	0.16	4.0	16.0	4.0	16.0
5	8.0	0.32	8.0	32.0	8.0	32.0

Table 2001.10M. LC pump gradient[a] for each run

			Mobile phase composition at end time	
Step	Start time, min	End time, min	%A	%B
Initial	0	0.1	90	10
2	0.1	30	40	60
3	31	31.5	0	100
4	37	37.5	90	10
5	44.5	Stop run	90	10

[a] All gradients are linear.

Acknowledgments

We are grateful to the following collaborators and their associates for their cooperation in this study:

Arti Arora, Kellogg Co., Battle Creek, MI

Sidney Cole, Sanitarium Technical Services, Cooranbong NSW, Australia

Mark Collison, ADM Research, Decatur, IL

Wayne Ellefson, Covance Laboratories, Inc., Madison, WI

Joe Gensic, Central Soya, Fort Wayne, IN

Jan Hazebroek, Pioneer Hi-Bred, Johnston, IA

Paul Johns, Abbott Laboratories, Ross Division, Columbus, OH

Stephen Klump, Ralston Analytical Laboratories, Saint Louis, MO

Stacy Lewis, Cargill Research, Wayzala, MN

Mehran Moghaddam, DuPont Genetic Technologies, Newark, DE

Patricia Murphy, Iowa State University, Ames, IA

David C. Woollard, AgriQuality NZ Ltd., Auckland, New Zealand

Table 2001.10N. Aglycon conversion factors

Isoflavone glucoside	*MWa*	*MWg*	$\frac{MWa}{MWg}$
Genistin	270	432	0.625
Glycitin	284	446	0.637
Daidzin	254	416	0.611

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