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, 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 (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 1 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.

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

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 $7000 \times g$. 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:

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

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:

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.

Table 2001.10N. Aglycon conversion factors

Isoflavone glucoside	*MWa*	*MWg*	———
Genistin	270	432	0.625
Glycitin	284	446	0.637
Daidzin	254	416	0.611

$$Cae = \frac{MWa}{MWg} \times Cg$$

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.

Total isoflavones, μg aglycon equivalents/g = *Ta* + *Tae*

Reference: *J. AOAC Int.* **84**, 1865(2001).