

# **Determination of Isoflavones in Natural Sources and Biological Fluids**

*Feifei Tian, Yongxin Zhu, Hong Long, Fuming Xie, Meloney Cregor, Chester Duda, Elsa Janle and Peter Kissinger*  
*Bioanalytical Systems, Inc., 2701 Kent Avenue, West Lafayette, IN, 47906*

## Background and Significance

Isoflavones, known as a subclass of phytoestrogen as well, are a group of plant polyphenolic compounds. They have attracted a great deal of public attention because of their potential role in the prevention and treatment of a number of chronic diseases, such as cardiovascular disease, osteoporosis, and hormone-related cancers. Human dietary exposure to isoflavones are achieved mainly by ingesting legume family (Fabaceae) plants and their products, among which soybean products are the richest source of isoflavones.

Two of the most abundant isoflavones are daidzein and genistein, which occur both in free state and as glycosides (Figure 1). They are the focus of this study, mainly because they have been proposed to be the bioactive ingredients by many studies.

## Objective

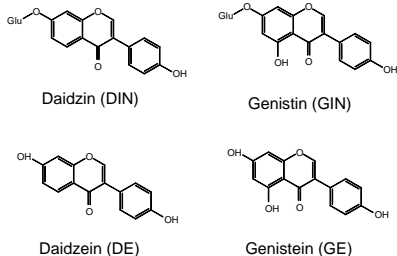


Figure 1. Structures of the isoflavones studied

Various high-performance liquid chromatography (HPLC) methods have been developed to assay the isoflavone contents in their food sources and biological fluids. The objective of this study is to demonstrate that HPLC coupled with multi-channel electrochemical (EC) detection is an alternative approach with excellent sensitivity and selectivity.

## Gradient LC Method for the Assay of Isoflavones in Natural Sources

- Column:** BAS, C8, 150x2mm, 5 $\mu$
- Flow Rate:** 0.6 mL/min
- Pump:** BAS PM-80 gradient pump
- Injection:** overflow 50  $\mu$ L loop
- Mobile Phase A:** 9.3% ACN, 5.9% MeOH, 86.7% NH<sub>4</sub>Ac buffer (25 mM, pH4.3), EDTA (0.25 mM)
- Mobile Phase B:** 19.6% ACN, 12.0% MeOH, 68.4% NH<sub>4</sub>Ac buffer (25 mM, pH4.3), EDTA (0.25 mM)

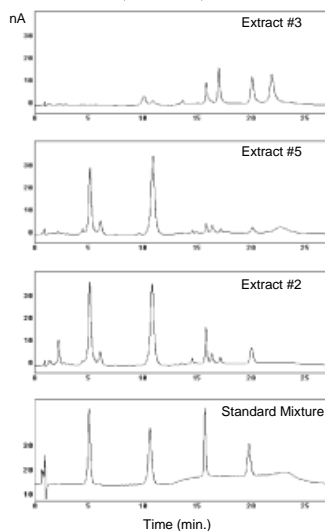


Figure 2. LC Chromatograms (950mV traces) of standard mixture and representative extracts.

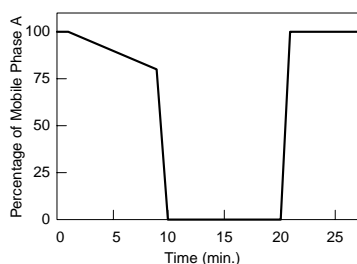


Figure 3. Gradient program.

## Stability of the Isoflavones in Mobile Phase A

Since all the standards and samples will be diluted in mobile phase A and analyzed using refrigerated autosampler, the stability of the isoflavones in mobile phase A was evaluated. A solution of four standards, 1 $\mu$ M each, was assayed at 0 min., and again at 480 min. In the meantime, it was kept in the 5  $^{\circ}$ C refrigerated autosampler. Peak heights under 950 mV trace were compared. As shown in Figure 4, none of the standards experienced significant degradation.

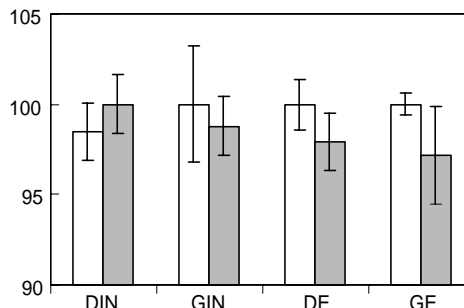


Figure 4. Stability of the isoflavones in mobile phase A, stored at 5  $^{\circ}$ C. Error bars were calculated based on three independently prepared standard solutions. Open bars: at 0 min. Filled bars: at 480 min.

## Voltammetric Characterization of Isoflavone Standards

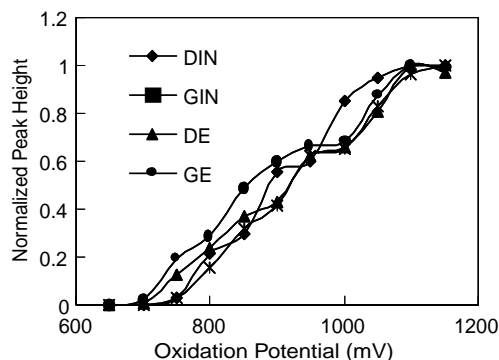


Figure 5. Hydrodynamic voltammograms (HDV) of the Isoflavone standards

A BAS Epsilon<sup>TM</sup> multi-channel EC detector was coupled with HPLC. A four channel glassy carbon working electrode was set in a radial flow configuration and referenced to a Ag/AgCl electrode. Different oxidation potentials, differing 50 mV, were simultaneously applied on the four channels. Normalized peak height was plotted against the oxidation potential (Figure 5). The multi-channel EC detection allows peak profile matching between standards and their LC retention equivalent peaks.

## Peak Profiling of Isoflavone Standards and Their Retention Equivalent Peaks (I)

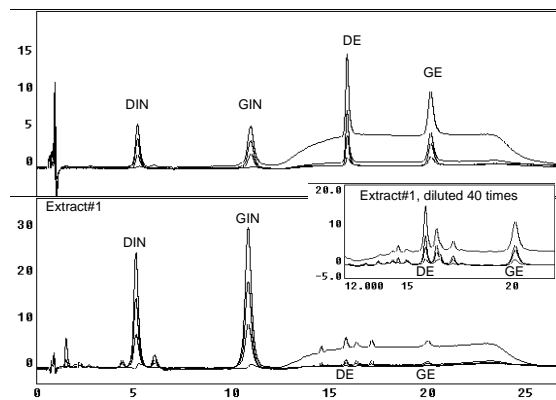


Figure 6. Chromatograms of standard mixture and representative extract. Traces with decreasing intensity are for oxidation potentials of 1100, 950, 850 and 750 mV, respectively.

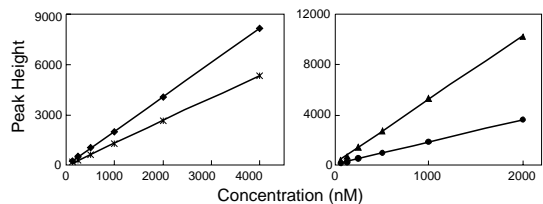
## Peak Profiling of Isoflavone Standards and Their Retention Equivalent Peaks (II)

Peak profiling at several different oxidation potentials, analogous to diode array UV profiling, is very useful for the identification of compounds of interest. The data in Table I is extracted from the chromatograms in Figure 6. It shows that peak height ratio values of the retention equivalent peaks in the extract match very well with those of the isoflavone standards.

	750mV/850mV		850mV/950mV		950mV/1100mV	
	Standards	Extract#1	Standards	Extract#1	Standards	Extract#1
DIN	.10	.11	.49	.47	.64	.63
GIN	.10	.09	.53	.52	.68	.65
DE	.32	.32	.59	.61	.62	.63
GE	.38	.38	.74	.74	.67	.67

**Table 1.** Comparison of peak height ratios for standards and retention equivalent peaks in Extract #1

## Representative Calibration Curves Used to Quantify Isoflavones in Natural Sources



Good linearity found in the range of :  
 { 125 to 4000 nM for DIN and GIN  
 { 62.5 to 2000 nM for DE and GE

Compound	Equation	R <sup>2</sup>
DIN	y=230.68x + 122.78	1
GIN	y=134.71x + 3057.2	0.9999
DE	y=51.75x + 11171	0.9996
GE	y=178.86x + 4545	0.9997

**Figure 7.** Calibration curves for isoflavones. Peak height was measured under the 950 mV trace.

## Sample Preparation for the Assay of Isoflavones from Natural Sources

Standards were dissolved in different extraction solvents and sonicated for various length of time. It was concluded that higher percentage of water and longer sonication cause degradation of DIN and GIN (data not shown). The sample preparation procedure was finalized as follows:

1. Various dry food and nutritional supplement pills, which contain isoflavones, were smashed into fine powder using mortar pestle.
2. 0.25g powder + 10 mL extraction solvent
3. Sonication for 30 min. (Sitting the vial on ice packs)
4. Transfer 200 uL suspension into a microcentrifuge filter
5. Centrifuge for 2 min.
6. Dilute the filtrate appropriately

## Isoflavone Contents in Natural Sources and Nutritional Supplements

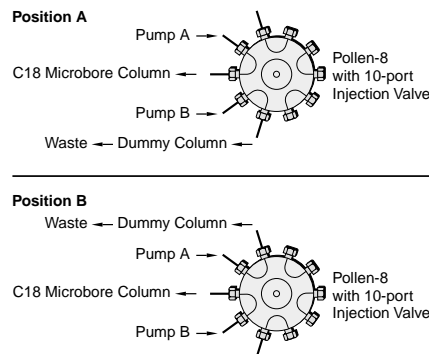
Sample	DIN	GIN	DE	GE
Puraria	211.8 (6.7)	14.0 (1.5)	182.4 (2.2)	33.2 (1.1)
Dried soybean	137.7 (13.2)	157.9 (9.7)	2.8 (0.5)	4.6 (0.3)
Soy flour	94.3 (2.5)	116.9 (13.7)	*	3.0 (0.9)
Regular rat food	34.3 (4.0)	56.2 (3.7)	*	4.9 (0.3)
NS #1	6342.6 (95.1)	10015.1 (103.6)	118.1 (3.6)	185.0 (16.8)
NS #2	9809.2 (309.8)	11532.7 (1160.7)	1209.7 (90.9)	1385.7 (77.8)
NS#3	67.1 (17.6)	198.7 (37.4)	662.5 (113.6)	1809.7 (189.2)
NS#4	990.6 (223.9)	125.0 (22.6)	453.2 (50.3)	54.0 (9.5)
NS#5	4814.5 (483.6)	7573.2 (1764.0)	286.7 (48.9)	428.7 (35.6)
NS#6	3024.0 (427.9)	5327.4 (222.6)	209.2 (28.1)	366.3 (22.6)

**Table 2.** Concentration of isoflavones ( $\mu\text{g/g}$ ) in natural sources and nutritional supplements. Standard deviations (n=3, independent sampling and sample preparation) are listed in parentheses. \*: too little for quantitation; NS: nutritional supplement.

## Why Use Microbore Column and Pump Switching for the Analysis of Isoflavones in Biological Fluids?

The biological fluids to be analyzed are rat plasma and rat brain microdialysate samples. Since they are very likely to have low concentration of target compounds and limited sample volume, using microbore column for LC becomes a sensible choice. Lower flow rate and thinner gasket, which is used in the microbore column LC/EC system also improves sensitivity by increasing oxidation efficiency. However, the four isoflavones have very different polarities, and they cannot be separated in a single run with reasonable analysis window. Therefore, a pump switching system was adopted.

## Schematic Illustration of the Pump Switching System



## LC Conditions Used for the Assay of Isoflavones in Biological Fluids

**Column:** BAS C18, 3  $\mu\text{m}$ , 100X1.0 mm

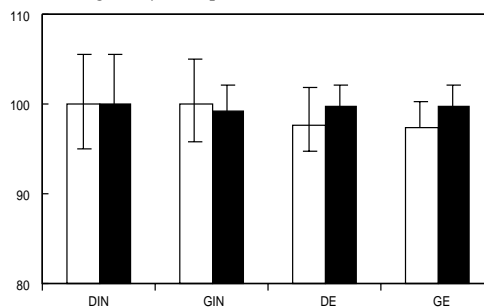
**Pump A:** 90  $\mu\text{L}/\text{min}$ ,  
4% MeOH+11.0% ACN+ 85% aqueous buffer  
(20 mM NaAc, 0.25 mM EDTA, pH 4.3)

**Pump B:** 80  $\mu\text{L}/\text{min}$ ,  
12.5% MeOH+21.0% ACN+ 66.5% aqueous buffer  
(20 mM NaAc, 0.25 mM EDTA, pH 4.3)

**Pump switching cycle:** Pump A for 17 min., then Pump B for 11 min, and then back to Pump A for 2 min.

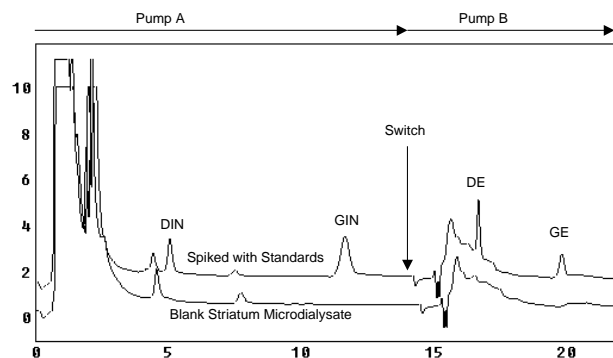
## Sample Preparation Procedures

Add 0.8 mL EtOAc into 50  $\mu\text{L}$  (spiked) plasma sample  
Vortex for 2 min. and centrifuge at 10,000 rpm for 6 min.  
Take 0.7 mL supernatant and dry under  $\text{N}_2$   
Dissolve in 20  $\mu\text{L}$  mobile phase A  
Inject by overflowing a 10  $\mu\text{L}$  loop



**Figure 8.** Stability of isoflavones extracted from rat plasma and reconstituted in mobile phase A. Open bars: at 0 min, filled bars: 480 min. Stored at 4°C.

## Can be Used to Analyze Brain Microdialysate



**Figure 9.** Representative chromatograms (850 mV traces) of blank and spiked striatum microdialysate.

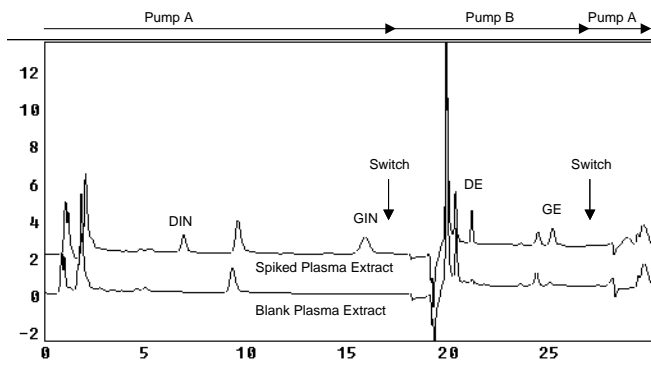
## Recovery of Isoflavone in in vitro Microdialysate

Four isoflavones were individually dissolved in MeOH at a concentration of 1 mg/mL. Then they were individually diluted in blank striatum microdialysate to yield a concentration of 1  $\mu\text{g}/\text{mL}$  and used as stock solutions. Blank Ringer's solution was perfused through 4mm brain microdialysis probe at a rate of 1  $\mu\text{L}/\text{min}$ . The perfusate was collected for 25 min per vial. Recovery was calculated by comparing the isoflavone concentrations in perfusates and those in the stock solutions.

Compound	DIN	GIN	DE	GE
Recovery	22%	30%	24%	14%

**Table 3.** Recovery of isoflavones in in vitro microdialysate.

## Can be Used to Analyze Rat Plasma



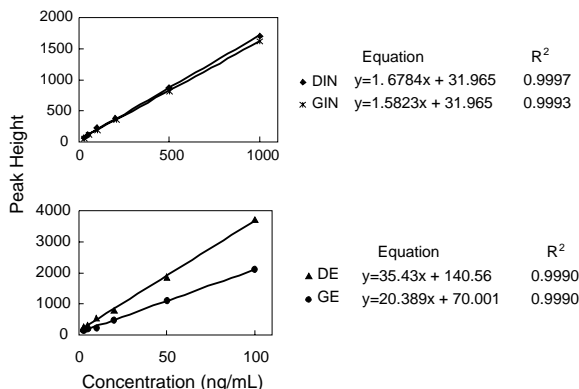
**Figure 10.** Representative chromatograms (850 mV traces) of rat plasma extracts.

### Constructing the Calibration Curves for Rat Plasma Analysis

Whole blood from several untreated rats was taken by cardiac puncture, pooled and diluted with equal volume of heparin-containing saline. It was vortexed briefly and centrifuged at 10000g for 10 min. Plasma portion was taken out and used as blank plasma.

Each isoflavone was dissolved in MeOH at a concentration of 1mg/mL, which was stored in a  $-20^{\circ}\text{C}$  freezer until being used. Standard mixture solution was made by freshly combining and diluting, with saline, the stock solution to 10  $\mu\text{g/mL}$  for DIN and GIN, 1  $\mu\text{g/mL}$  for DE and GE. The standard mixture was spiked into the blank plasma to yield a final concentration of 25, 50, 100, 200, 500 and 1000 ng/mL for DIN and GIN, and a concentration of 2.5, 5, 10, 20, 50, 100 ng/mL for DE and GE.

The spiked plasma was taken through the sample preparation procedures, and analyzed by HPLC. Typical Calibration Curves for Quantifying isoflavones in Rat Plasma



**Figure 11.** Typical calibration curves for quantifying isoflavones in rat plasma. Peak height was taken under the 850 mV trace.

### Recovery of DIN from Rat Plasma

DIN stock solution, 100  $\mu\text{M}$  in saline, was spiked into rat plasma and mobile phase A to yield two different concentrations, 5,000 and 800 nM. The spiked plasma samples were extracted according to the sample preparation procedures. The 5,000 nM mobile phase A sample and the reconstituted plasma sample was further diluted 5 times to make sure that they fell within the range of the calibration curve. The 800 nM samples were subject to HPLC analyses directly. Table 4 demonstrated that samples of the higher concentration (5,000 nM) have the same recovery rate as those of the lower concentration (800 nM).

$$\text{Recovery} = \frac{\text{Peak Height in the Reconstituted Sample}}{2.5 \times \text{Peak Height in the Corresponding Mobile Phase A sample}}$$

Concentration (nM)	800	5,000
Recovery (n=3)	74% $\pm$ 2.3%	75% $\pm$ 4.1%

**Table 4.** Recovery of DIN from rat plasma (n=3).

## Accuracy and Precision of the Method for the Assay of Isoflavones in Rat Plasma

Compound	Concentration Added (ng/mL)	Interday (n=3) Concentration Measured	Intraday (n=3) Concentration Measured
DIN	50	47.2 /3.7 /-5.6	48.5 /4.9 /-3.0
	200	210.9 /1.9 /5.4	208.8 /2.7 /4.4
	1000	980.3 /2.1 /-2.0	958.5 /6.6 /-4.2
GIN	50	46.8 /3.0 /-6.4	48.0 /4.4 /-4.0
	200	213.4 /0.5 /6.7	213.8 /2.2 /6.4
	1000	1020.6 /4.9 /2.1	999.6 /6.4 /-0.03
DE	5	5.3 /8.7 /6.0	5.3 /5.2 /6.0
	20	20.4 /6.3 /2.0	19.8 /3.9 /-1.0
	100	101.0 /1.9 /1.0	98.2 /4.3 /-1.8
GE	5	5.1 /4.2 /2.0	5.4 /10.6 /8.0
	20	20.2 /6.9 /1.0	20.6 /4.2 /3.0
	100	105.9 /6.3 /5.9	98.1 /6.1 /1.9

**Table 5.** Accuracy and precision of the method for assaying isoflavones in rat plasma. Measured concentrations are present as mean/RSD/bias in the unit of ng/mL/%/%. The quality control samples were prepared in the blank rat plasma to contain concentrations of isoflavones within the range of the calibration curves.

### Collect Rat Blood Using Culex™

Sprague-Dawley rats, weighing 280-350g, were implanted with jugular and femoral vein catheters. They are put into Ratum™ and allowed 24 hours for recovery with free access to food and water. Before dosing the drug, three baseline blood samples were collected. Drugs were then administered through femoral vein bolus injection. Blood collection start immediately by Culex, following a preset schedule. Blood sample was collected from jugular vein catheter, and each vial contained 75  $\mu\text{L}$  heparin-containing saline in addition to 75  $\mu\text{L}$  whole blood. The vials were vortexed briefly and centrifuged at 10000g for 10 min. Two 50  $\mu\text{L}$  plasma aliquots were taken, one for immediate analysis, the other for storage as a backup sample.

Culex blood collection after the drug administration was done at the following time points: 1, 6, 11, 16, 21, 26, 31, 36, 41, 46, 51, 61, and 71 min.

### Determination of DIN in Rat Plasma (I)

Two types of DIN were dosed intravenously:

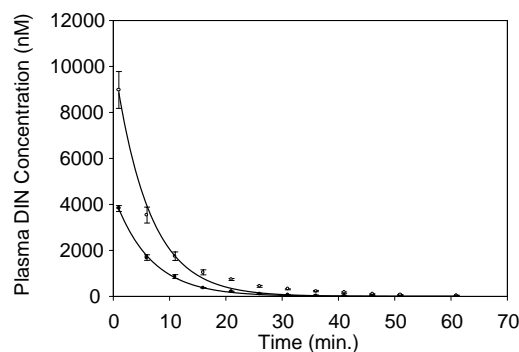
- I. Pure DIN dissolved in 20% DMSO in saline, 2mg/mL. Dosed at 5 mg/kg.
- II. 2.05 mL/kg DIN-containing mixture (in 20 % DMSO in saline). The mixture was prepared as follows:

Take 50 tablets of nutritional supplement #1, crush them into fine powder with mortar pestle, weigh 12.0 g out, soak it in 300 mL 80% MeOH/water, sonicate for 30 min. on ice pack, filter out the residual, rotorvapor dry the filtrate.

The dried mixture was quantified to contain 122mg/g DIN. Take 100 mg mixture, dissolve in DMSO and dilute with saline to yield a concentration of 20 mg/mL in 20 % DMSO/saline.

Dosing 2.05 mL/kg this mixture will deliver 5mg/kg pure DIN.

### Determination of DIN in Rat Plasma (II)



**Figure 12.** Plasma concentration of DIN vs. time. Filled circle: after i.v. administration of pure DIN 5 mg/kg. Open circle: after i.v. administration of DIN-containing mixture, at a dose which delivers 5mg/kg pure DIN. (n=4 for each administration)

## Conclusion

Dosing DIN in a form of mixture through i.v. route generates higher plasma concentration than dosing equal amount of pure DIN.