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Response to Comment on Extraction and Purification of Isoflavones from Soybeans and Characterization of Their Estrogenic Activities

We are responding to comments from Dr. Nancy Shappell of ARS-USDA on our recently published paper (*1*). Before answering Dr. Shappell's questions, we need to restate the objectives of our work. The main objective was to develop a highly efficient and low-cost method for manufacturing soy isoflavone products with high purity and efficacy. To achieve this goal, simple and low-cost strategies (i.e., antisolvent crystallization) were developed and the extraction and purification parameters of the method were optimized on the basis of the yields of isoflavones. Then HPLC analysis and MTT cell proliferation assay using MCF-7 cells were used to evaluate the purity and efficacy of the resulting isoflavone products.

Dr. Shappell raised two questions related to the method validation of HPLC analysis. The first is about the availability of the calibration curves of three isoflavone standards including genistein, daidzein, and glycitein. This information was submitted as Supporting Information with our revised manuscript on May 27, 2007, and published online on October 8, 2007. The second is about the concentrations of isoflavone standards, which had been reported in our paper (*1*) on p 6941: "Isoflavone standards were prepared in pure ethanol by serial 2-fold dilutions, and the following concentrations were obtained: from 62.5 to 500 μg/mL for genistein and daidzein and from 12.5 to 100 μg/mL for glycitein."

It was pointed out that the extraction process "before optimization" was not defined. Indeed, although we provided the general extraction conditions on p 6941, the extraction conditions before optimization used in Figure 4 of our paper (*1*) were not specified. These were as follows: soybean flour was mixed with 99.99% ethanol at the material ratio of 3:1 (mL of ethanol/g of soybean flour), and then the mixture was heated at 60 °C for 12 h.

Dr. Shappell then queried whether the evidently increased yields of isoflavones after optimization were due to hydrolyzing isoflavone glucosides into aglycones rather than improving extraction efficiency. Our experimental data, however, did not support this hypothesis. The 1-fold increase in the yields of isoflavones of the whole extraction and purification procedure after optimization [from 0.31 ± 0.03 to 0.66 ± 0.06 mg of isoflavone aglycones/g of soybean flour, shown in Figure 4 of our paper (*1*)] was mainly due to the optimization of extraction conditions, especially the increase in extraction temperature. The main difference between the extraction conditions before optimization (99.99% ethanol, 3:1, 60 °C, 12 h) and after optimization (96% ethanol, 3:1, 80 °C, 8 h) was the temperature difference. As indicated in our paper (*1*) on p 6942, the yield of total isoflavones increased significantly with increasing extraction temperature. When extraction temperature was optimized from 60 to 80 °C, the extraction efficiency was improved from 0.41 ± 0.04 to 0.87 ± 0.08 mg of total isoflavones (aglycone equivalency)/g of soybean flour (both data were from three independent experiments). About hydrolysis, the main purpose of its optimization was to reduce the amount of acid used with the precondition of almost complete hydrolysis. In the initial hydrolysis experiments (0.77 mol/L of hydrochloric acid, 60 °C, 6 h), we had already achieved almost complete conversion (88.18 ± 3.55% for genistin and 87.90 ± 4.21% for daidzin) of isoflavone glucosides into aglycones. After optimization (0.13 mol/L of hydrochloric acid, 80 °C, 6 h), we could use a much lower concentration of acid to obtain slightly better hydrolysis efficiency (90.58 ± 3.90% for genistin and 91.04 ± 3.25% for daidzin, see **Figures 1** and **2** in this response). Because there was very little change in the hydrolysis efficiency during optimization, the hydrolysis step was not the major contributor to the increased isoflavone yields by optimization. About crystallization, the ratio of antisolvent to feed mixture was 4:1 both before and after optimization and the recovery efficiency was almost unchanged (80–90% for genistein and about 70% for daidzein both before and after optimization). Therefore, the evident enhancement of isoflavone yields after optimization should be predominantly due to extraction optimization rather than hydrolysis optimization.

Another question was why "change in the product composition" induced by optimization was not cited in the Discussion. Actually, isoflavone yields and composition change induced by optimization had been discussed already in our paper (*1*) on p 6944 and two conclusions had been drawn: (1) optimization did not change the composition of our isoflavone products on the whole; (2) the main function of optimization was to improve the yield of total isoflavones.

It was suggested that we should report the effect of hydrolysis on converting soy isoflavone glucosides into aglycones so that the readers could evaluate the contribution of hydrolysis to isoflavone yields. We accept this suggestion and compare here the composition change of the extracts before and after hydrolysis by HPLC analysis. **Figure 1** of this response exhibited the chromatograms before and after hydrolysis for one representative sample: before hydrolysis, glucoside (i.e., genistin and daidzin) was the major form of isoflavones and aglycones (mainly daidzein) were only minor isoflavone components in the extract, whereas after hydrolysis, aglycones (mainly genistein and daidzein) were the predominant isoflavones. **Figure 1B** shows a large peak between the two minor peaks of daidzin and genistein after the hydrochloric acid treatment. This peak, however, was detected only when hydrolysis was conducted at a high temperature such as 80 °C, but not at lower temperatures such as 60–70 °C. This peak was not found in the final isoflavone products after crystallization [Figure 5B in our paper (*1*)]. Most likely, this peak contained some thermal degradation products from soybeans. We further calculated the hydrolysis efficiency under the optimum conditions (0.13 mol/L of hydrochloric acid, 80 °C, 6 h). The results from three indepen-

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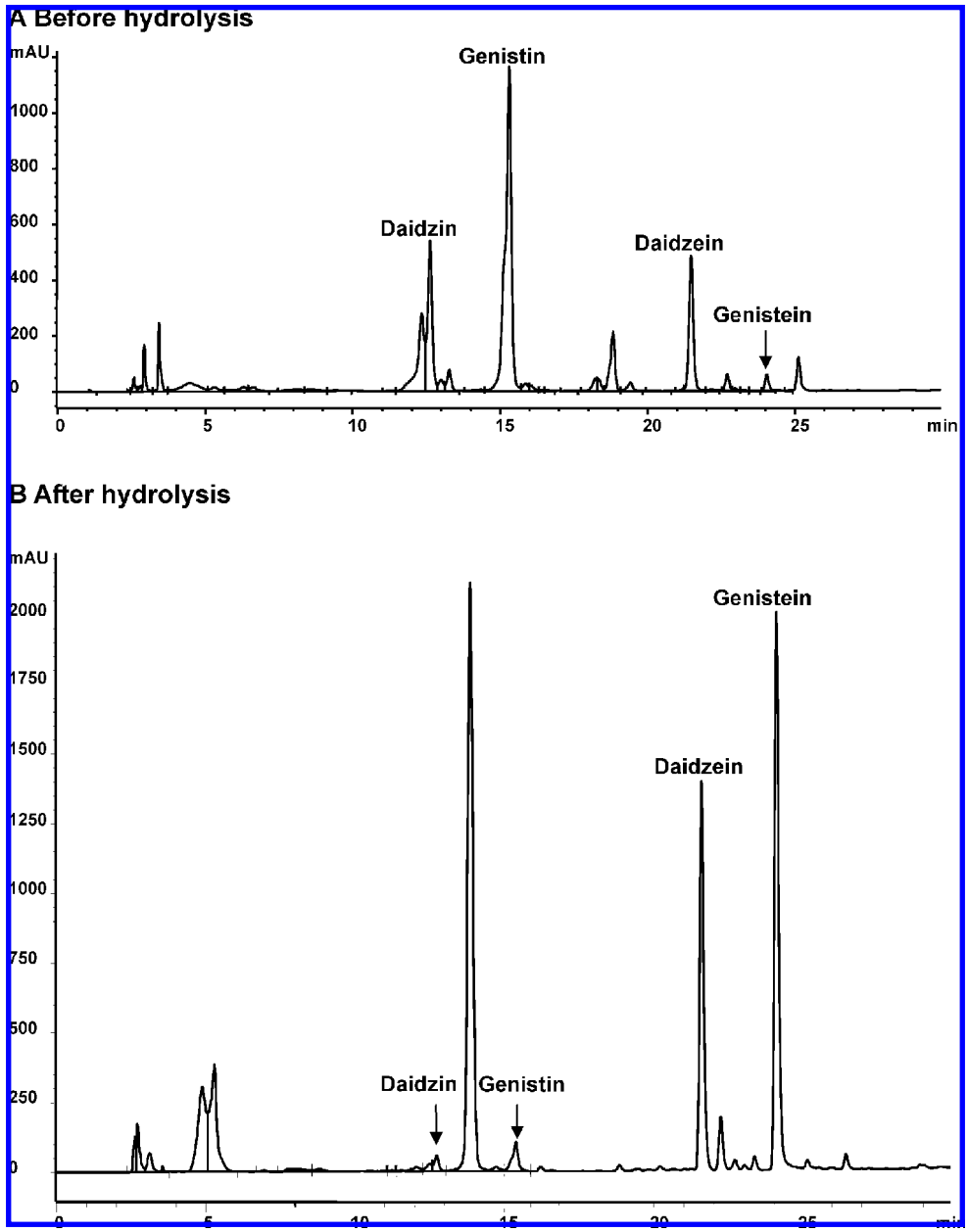

Figure 1. HPLC analysis of genistin, daidzin, genistein and daidzein in one optimized extract before hydrolysis (**A**) and after hydrolysis (**B**). The calibration curves of the four isoflavone standards by this new HPLC method are shown in the Supporting Information.

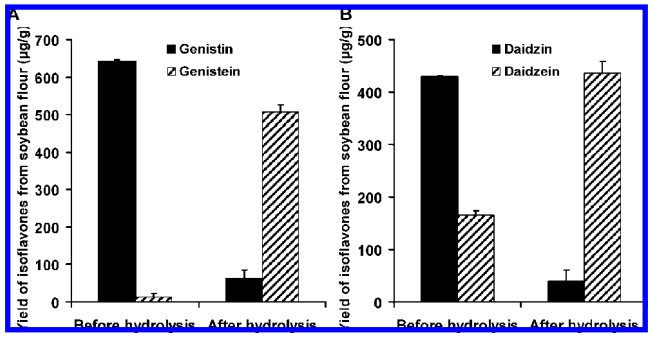

Figure 2. Yields of isoflavones from soybean flour extracts before and after hydrolysis. Data were expressed as the mean ± standard deviation of the extracts from three independent experiments.

dent experiments (**Figure 2** in this response) showed that by hydrolysis 90.58 ± 3.90% of genistin and 91.04 ± 3.25% of daidzin were degraded, and the yield was increased (36.72 ± 1.37)-fold for genistein and (1.65 ± 0.14)-fold for daidzein. The above results clearly showed that the hydrolysis process was essential for converting the glycosylated isoflavones into the corresponding aglycones. These results further support the statements and findings in our paper (*1*) on p 6946: first, the main function of hydrolysis is to convert most of the isoflavone glucosides (i.e., genistin and daidzin) into aglycones; second, in this study, >85% of genistin and daidzin was degraded by hydrolysis, whereas the contents of genistein and daidzein were increased evidently.

Dr. Shappell questioned the implications of simple composition and minor impurities of our isoflavone products based only on HPLC chromatograms. As stated in our paper (p 6944), "At 255 nm, there were only minor impurities detected in this product", we certainly agree that HPLC chromatograms at 255 nm cannot identify all of the components in our soybean isoflavone products. Regarding the safety of our products for human consumption, we had performed cell proliferation assay using human MCF-7 cells (Figure 6D on p 6946) and had not

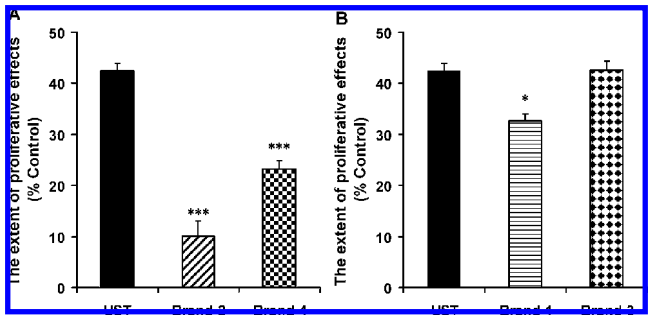

Figure 3. Cell proliferation assay: the enhanced extent of proliferative effects over control treatment in human MCF-7 cells by our isoflavone product and four commercial supplements: UST, our isoflavone product (concentration = 0.4 μg of total isoflavones/mL); brands 1−4, four commercial isoflavone supplements. All four samples were diluted to 0.4 μg of total isoflavones/mL, but brands 1 and 3 were diluted according to their actual contents of isoflavones, whereas brands 2 and 4 were diluted according to their claimed values. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$, when compared with the enhanced extent of our product using Student's two-tailed *t* test.

observed any cytotoxicity on human cells at the effective dosages used. The safety of our products should be further tested using animal models and via clinical trials.

In addition, we stress that we had performed all of the experiments at least three times. For example, five isoflavone products after optimization were used for HPLC analysis, but only the chromatograms of one sample were shown in Figure 5B on p 6945. Three optimized products were used in E-SCREEN assays, but the results of only one sample were shown in Figure 6D on p 6946. Because all of the optimized products assayed had similar composition and showed similar estrogenic activities, Figures 5B and 6D in our original paper (*1*) were both representative. To ensure the consistency of data, the data presented in Figures 5B and 6D were from "a single extraction of their product".

It was pointed out that we should provide the results of estrogenic activity comparison of our isoflavone product and four commercial supplements by E-SCREEN assay and of composition analysis of the commercial supplements. We did not include those data in the original paper because we do not think that a scientific report should include these details on commercial products. Anyway, we present the results of E-SCREEN assay in **Figure 3** of this response, which demonstrated our product had better estrogenic activity than three of the four tested commercial supplements. The composition analysis of the commercial supplements by HPLC is shown in **Table 1** of this response.

We had discussed the relative merits of our extraction and purification procedure in the Discussion of our paper (*1*) on p 6946: (1) simple and low cost; (2) high yield; (3) safe. HPLC analysis (Figure 5 on p 6945) and cell assay (Figure 6D on p 6946) revealed that the products thus obtained not only contained a high content of isoflavone aglycones but also had estrogenic activity.

Table 1. Isoflavone Contents of Four Commercial Supplements[a]

brand	genistein (mg/g)	daidzein (mg/g)	total isoflavone content (mg/g)	claimed isoflavone content (mg/g)
1	10.83	35.18	46.01	30
2	0.05	0.52	0.57	40
3	20.37	25.33	45.70	32
4	0.07	0.03	0.10	50

[a] Three to five tablets/capsules were used to prepare the sample solution for each commercial isoflavone supplement, and the average contents of isoflavones in those samples were measured in this test.

MATERIALS AND METHODS

HPLC Analysis. Isoflavone contents in all samples were analyzed using a reversed-phase C_{18} column (Phenomenex, 5 μm, 250 × 4.6 mm i.d.) on the Agilent 1100 series liquid chromatograph (including the pumping system, vacuum degasser, autosampler, and UV-DAD detector). The sample injection volume was 10 μL. The mobile phase was water with 0.1% acetic acid (A) and methanol (B). A linear gradient elution was applied from 20 to 80% B starting from 0 to 30 min, at a flow rate of 1.0 mL/min. The temperature of the column was maintained at 40 °C, and the detection wavelength was set at 255 nm, where absorbance peak areas were quantified.

NOTE ADDED AFTER ASAP PUBLICATION

Changes to the original ASAP posting of March 20, 2008, have been made in the sixth paragraph, Figure 1, and Table 1 on April 3, 2008.

LITERATURE CITED

(1) Zhang, E. J.; Ng, K. M.; Luo, K. Q. Extraction and purification of isoflavones from soybeans and characterization of their estrogenic activities. *J. Agric. Food Chem.* **2007**, *55*, 6940–6950.

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