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Evaluation of Estrogenic Activity in Diets for Experimental Animals Using in Vitro Assay

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We used a modified yeast-based human estrogen receptor α (ERα) bioassay to determine the estrogenic activity in 22 kinds of diets for experimental animals. The estrogenic activity of each diet was reevaluated by comparison with a calibration curve of 17β-estradiol. Almost all of the diets had estrogenic activity. The diets for rabbits and guinea pigs had the highest estrogenic activity compared to any other diets, including those for rats and mice. Estrogenic activity was found in dried skim milk, fishmeal, soybean meal, and alfalfa meal. In the NIH-07 diet opened for the ingredients, estrogenic activity was nearly all derived from the alfalfa meal. Multiple assays were performed to evaluate potential seasonal variations in the estrogenic potency in the raw materials of the rat and mouse diets. We found that the estrogenic activity in these raw materials changed throughout the year.

KEYWORDS: Diets; estrogenic activity; in vitro assay; genistein; daizein; coumesterol; alfalfa; soybean

INTRODUCTION

Animal diets, including those for experimental animals, are manufactured from natural raw materials, including grasses, corn, wheat, soybean, and fishes. Open formula diets include materials such as alfalfa, a typical grass, which contains the potent phytoestrogen coumestrol, known to possess estrogenic activity *in vitro* (*1*) and in vivo (*2*−*6*). Soybeans, a typical legume, contain genistein and daizein, both of which exhibit estrogenic activity in vitro (*7*) and in vivo (*8*).

Commercial animal diets wherein the formula is not disclosed are manufactured with ingredients chosen by the makers of each brand. It is difficult to calculate or infer the phytoestrogen content in such diets because the components and their proportions are unknown. In addition, the phytoestrogen content in a particular diet can vary from batch to batch, which may be caused by differences in the varieties of soybean used and their locations and conditions of cultivation (*9, 10*). It has also been reported that the phytoestrogen content in alfalfa is highest in the spring and autumn (*11*).

Boettger-Tong et al. (*12*) observed a paradoxical lack of response to exogenously administered estradiol in ovariectomized (OVX) female rats. They suspected that these results were affected by the diet, which contained high levels of genistein and daizein. In OVX rats fed with the NIH-07 (PLD) diet, which is composed of raw materials containing lower level phytoestrogens, uterine weight was smaller than that in rats fed with NIH-07 diet, suggesting that NIH-07 diet contains phytoestrogens enough to influence the uterine weight (*13*).

Although significant progress has been made on the effects of dietary phytoestrogens on experimental results, several issues remain to be clarified: (1) what kinds of diets have estrogenic activity, (2) which raw materials have estrogenic activity, (3) what is the ratio of estrogenic substances in raw materials for diets, and (4) why does the estrogenic activity in raw materials vary according to the season harvested? To address these questions, we employed yeast-based human estrogen receptor α bioassay that is widely utilized for evaluation of estrogenic activity of chemicals (*14*−*17*). This assay is useful to estimate the total estrogenic activity in materials including phytoestrogens and natural estrogens. To standardize the measurements of each diet, we calculated the estrogenic activity of the samples in comparison with a 17β-estradiol calibration curve.

MATERIALS AND METHODS

Diets and their Raw Materials. One lot each of the following diets and raw materials were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan): NIH-07 (slightly modified by Oriental Yeast Co. Ltd., solely due to material availability), NIH-07 (PLD), CRF-1, CR-LPF, MF,

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NMF, and CMF for mice and rats; RC4, LRC4, GC4, GOC4, and ORC4 for rabbits and guinea pigs; two kinds of diets for fishes (*ayu* fish and carp); DS-A for dogs; CS-A for cats; PS for monkeys; MP for pigs; ZF for herbivorous animals; PL for quails; I for insects; and XL for *Xenopus*. Most of the diets were manufactured in June, 2002, but some were manufactured between July, 2002 and September, 2002.

In addition, NIH-07 diet and NIH-07 (PLD) diet were manufactured four times a year (between October, 2001 and July, 2002), and eight kinds of their major raw materials (dried skim milk, fish meal, soybean meal, alfalfa meal, corn gluten, ground yellow shelled corn, wheat flour, and soy oil) were also obtained. These raw materials were obtained every month from February, 2002 to January, 2003.

Chemicals. 17β-Estradiol (E_2), genistein, daizein, and 2-nitrophenyl-β-D-galactosidase (ONPG) were obtained from Sigma Chemical Co. (St. Louis, MO). Coumestrol was from LKT Laboratories Inc. (St. Paul, MN). Zymolyase-20T was from Seikagaku Corporation (Tokyo, Japan). L-Histidine and L-lysine monohydrochloride were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Yeast nitrogen base without amino acids was from Becton Dickinson Microbiology Systemes (Sparks, MD). Dimethyl sulfoxide (DMSO) was from Nacalaitesque Inc (Kyoto, Japan).

Extraction of Estrogenic Substances from Diets and Raw Materials. First, 25 mL of cooled methanol and 1 M acetic acid buffer in a volumetric ratio of 9:1 were added to 0.5 g of the diet or raw materials in a 50-mL tube. They were homogenated for 2 min, sonicated for 10 min, and stirred for 30 min. After centrifugation at 4 °C for 5 min at 3000 rpm, supernatant fluids (5 mL) were transferred to a 30-mL tube and evaporated to dryness. The recovered materials in the tube were dissolved in DMSO (800 μL). Then, 12.5 μL of the prepared solution was used as a sample solution to measure estrogenic activity. E_2 dosing solutions (12.5 μL), E_2 dissolved in DMSO at 2.5×10^{-9}, 5×10^{-9}, 10^{-8}, 2×10^{-8}, and 3×10^{-8} M, were used for the calibration curve in each assay. DMSO (12.5 μL) was used for the blank. Extraction of estrogenic substance was repeated 3 times for the determination of estrogenic activity.

Reactions of Samples to Yeast. We used the yeast-based human estrogen receptor α bioassay described by Gaido et al. (1997) with minor variation. Yeast cells were grown overnight at 30 °C with vigorous orbital shaking in selective medium containing yeast nitrogen base without amino acids (6.7 g/L), plus glucose (20 g/L), lysine (36 mg/L), and histidine (24 mg/L). After overnight culture, the yeast solution (250 μL) was mixed with selective medium (1 mL) containing 5 μM $Cu(SO_4)_2$ to induce receptor production in a 15-mL tube. Then, each sample (12.5 μL) extracted from the diets or raw materials or E_2 dosing solutions (12.5 μL) for the calibration curve were added to the tubes and incubated for 4 h at 30 °C.

Measurement of β-Galactosidase. First, 525 μL of the yeast solution incubated for 4 h was transferred to a 1.4-mL tube. The tube was centrifuged at 4 °C for 5 min at 15000 rpm. The supernatant fluid was discarded. To digest yeast cells enzymatically, 800 μL of Zymolyase-20T (1 mg/mL) dissolved in Z buffer (60 μM Na_2HPO_4, 40 μM NaH_2PO_4, 10 μM KCl, and 1 μM $MgSO_4$) containing β-mercaptoethanol (270 μL/L) was added to the yeast pellet in each tube. After vigorous stirring, the tubes were incubated for 15 min at 37 °C. Then, 200 μL of ONPG (4 mg/mL) dissolved in 0.1 M phosphate buffer was added to each tube. After a 30-min incubation at 30 °C, the reaction was stopped by adding 400 μL of Na_2CO_3 (1 M). Absorbances of the solution in the tube were measured spectrophotometrically at 420 and 550 nm (DU640, Beckman Coulter Inc., Fullerton, CA). Yeast cell number was standardized by measuring light scattering at 600 nm. These procedures were performed in duplicate.

Conversion of Estrogenic Activity into E_2 Concentration and Content. Estrogenic activity was calculated as follows:

$$\text{estrogenic activity } (U) = 1000 \times (\text{absorbance } (A)_{420} - 1.75 \times A_{550})/(\text{time (min)} \times 0.05 \times A_{600})$$

where the time (min) was set at 30.

Conversion of the estrogenic activity of diets and raw materials into E_2 concentration was calculated from the calibration curve of E_2 using the software, Immuno for Windows vl.2 (Nalge Nunc International Co., Tokyo, Japan). The samples of the diets or raw materials were diluted with DMSO to be within the linear range of the assay for the estrogenic activity which ranged from 5×10^{-9} M to 3×10^{-8} M E_2. When the values of estrogenic activity of undiluted samples were lower than the lower limit (5×10^{-9} M E_2), the estrogenic activity was judged to be negative. After the conversion to E_2 concentration (μg/μL as E_2), estrogenic substance (ES) content (μg/g as E_2) in the diets or raw materials was calculated. The conversion into E_2 concentration and the calculation of ES content were based on the ratio of extraction from the diets and raw materials and on the ratio of dilution of the sample for measurement of estrogenic activity. The detection limit for ES content was 0.01 μg/g as E_2. Each value represents the mean of three extractions.

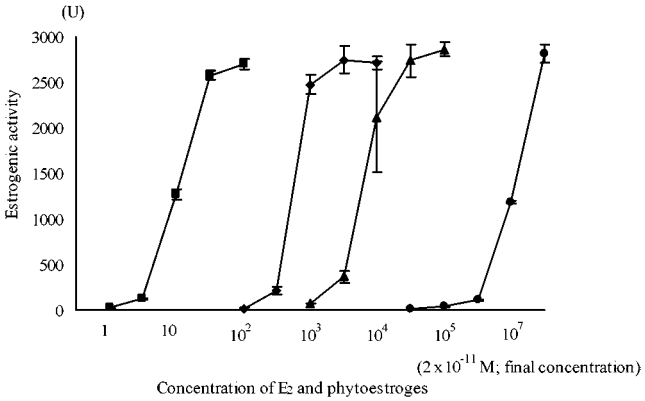

Figure 1. Estrogenic activity of coumesterol, genisutein, daizein, and E_2 each at five concentrations by the yeast assay. Estrogenic activity of coumesterol (-◆-), genistein (–▲-), and daizein (-●-) shows an E_2 (-■-)-like dose dependency. Each point for plotting represents the mean ± S. D. of three assays in triplicate.

Dose-Dependency, Recovery Efficiency, and Variation Presumption of Estrogens in Yeast Assay. To confirm dose-dependency of estrogens, we determined estrogenic activity of coumestrol, genistein, and daizein each at five concentrations, three times in triplicate by the yeast assay with E_2 as the positive control. The recovery efficiency of E_2, coumestrol, genistein, and daizein was examined as follows: These estrogens were dissolved in DMSO (25.6 nM for E_2, 1.02 μM for coumestrol, 10.2 μM for genistein, and 768 μM for daizein) then determined for E_2 activity. From the E_2 activity, ES content in 500 μL of each solution was calculated to obtain the amount of estrogens to be added. Then 500 μL of each estrogen solution was added into 24.5 mL of the extraction buffer (a mixture of methanol and 1 M acetic acid buffer in a volumetric ratio of 9:1), then mixed well. After the process extraction and measurement for E_2 activity, ES content in 25 mL of the extraction buffer containing estrogen was calculated to obtain the amount of estrogen to be recovered. The recovery rate was calculated according to the following formula: (amount of recovered estrogen/amount of added estrogen) × 100(%). The assays were repeated 3 times in duplicate. To presume variations among methods of extraction, ES were extracted from NIH-07 diet (manufactured in January 2002) as a representative material repeatedly 5 times in duplicate, and the ES was also expressed by calculating the value converted to E_2. The methods of determination and conversion to examine the recovery efficiency and variation presumption were as described above.

RESULTS AND DISCUSSION

1. Dose-dependency, Recovery Efficiency, and Variation Presumption of Estrogens in Yeast Assay. The estrogenic activity of coumestrol, genistein, and daizein showed an E_2-like dose dependency (**Figure 1**). The recovery rate of E_2, coumestrol, genistein, and daizein, was 86.8 ± 17.3, 111.0 ± 7.6, 91.9 ± 2.0, and 88.2 ± 6.8%, respectively. Extractions of estrogenic substances (ES) from NIH-07 diet and measurement of the estrogenic activity were performed 5 times, the individual values were 0.050, 0.050, 0.051, 0.052, and 0.055, with an average of

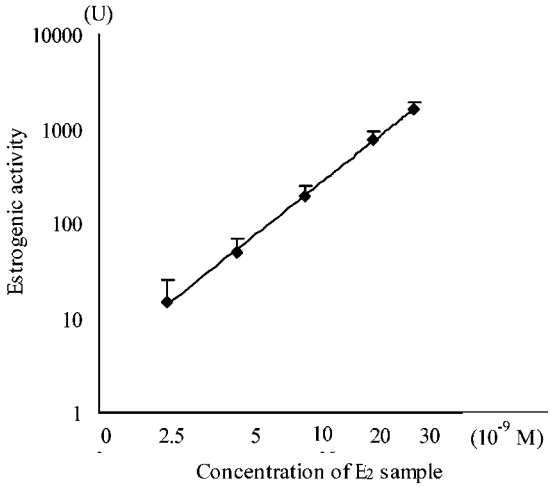

Figure 2. A calibration curve obtained from all the assays ($n = 15$) employed in the present study. Each point for plotting represents the mean ± S. D. The calibration curve has excellent linearity at an E_2 concentration range of $2.5 \times 10^{-9} - 3 \times 10^{-8}$ M when estrogenic activity and concentration of E_2 sample are shown in logarithm ($Y = 3.701 \times 10^{17} X^{1.914}$, $R = 0.9993$).

0.052 ± 0.002 μg/g, and the coefficient of variation (C. V.) was 4%.

2. ES Content in Diets for Experimental Animals. A calibration curve obtained from all the assays employed in the present study is shown in **Figure 2**. The calibration curve had excellent linearity at an E_2 concentration range of $2.5 \times 10^{-9} - 3 \times 10^{-8}$ M when estrogenic activity and concentration of E_2 sample is shown in logarithm. Except for the NIH-07 (PLD) diet, all diets contained substances having estrogenic activity (**Figure 3**). ES content was greater in rabbit and guinea pig diets as compared with any other diets. Levels of ES content in MP and ZF diets were moderate. The other diets, including the diets for rats and mice, had low levels of ES content.

3. ES Content in the Diets and Raw Materials. The overall average of ES content, expressed as E_2 content in the NIH-07 diet, was 0.07 μg/g (**Table 1**). However, estrogenic activity in NIH-07 (PLD) diet was always lower than the detection limit (0.01 μg/g as E_2). To determine which raw materials contributed to estrogenicity, each component of the NIH-07 diet was measured. The ES content was high in alfalfa (1.095−1.784 μg/g), moderate in soybean meal (0.039−0.125 μg/g), and low in dried skim milk (<0.017 μg/g) and fishmeal (<0.014 μg/g). ES content in other components was below the detection limit.

Table 1. Content of Estrogenic Substances (μg/g as E_2) in NIH-07 Diet, NIH-07 (PLD) Diet, and Their Raw Materials

diets and raw materials	Oct. 2001[a]	Jan. 2002	Apr. 2002	Jul. 2002
NIH-07 diet	0.085	0.059	0.072	0.066
NIH-07(PLD) diet	n.d.[b]	n.d.	n.d.	n.d.
dried skim milk	0.017	0.014	n.d.	0.013
fish meal (65% protein)	0.014	n.d.	n.d.	0.012
soybean meal (45% protein)	0.039	0.124	0.125	0.117
alfalfa	1.784	1.095	1.411	1.220
corn gluten	n.d.	n.d.	n.d.	n.d.
ground shelled corn	n.d.	n.d.	n.d.	n.d.
wheat (flour)	n.d.	n.d.	n.d.	n.d.
soy oil	n.d.	n.d.	n.d.	n.d.

[a] The month of manufacture. [b] n.d., less than the detection limit (0.01 μg/g as E_2).

The ratio of ES content in each ingredient to that in NIH-07 diet was calculated according to the following formula: $= (A \times B)/C$, where A represents % value of each ingredient in NIH-07 diet (See **Table 2**), B represents ES content in each raw material, and C represents ES content in the NIH-07 diet. The results showed that the ES content of the NIH-07 diet was nearly entirely derived from that in alfalfa meal (**Figure 4**). Soybean had a small contribution to the estrogenic activity of the diet. Dried skim milk and fishmeal contributed little to the estrogenic potency of the NIH-07 diet.

4. Changes in the ES Content in Raw Materials over Time. Soybean meal and alfalfa meal showed relatively high levels of estrogenic activity, whereas dried skim milk and fishmeal had lower levels throughout the year (**Table 3**). In all other raw materials, the ES content was always lower than the limit of detection. Interestingly, the ES content in alfalfa meal in January 2003 was approximately 4-fold higher than that in November 2002, while the soybean ES content in January 2003

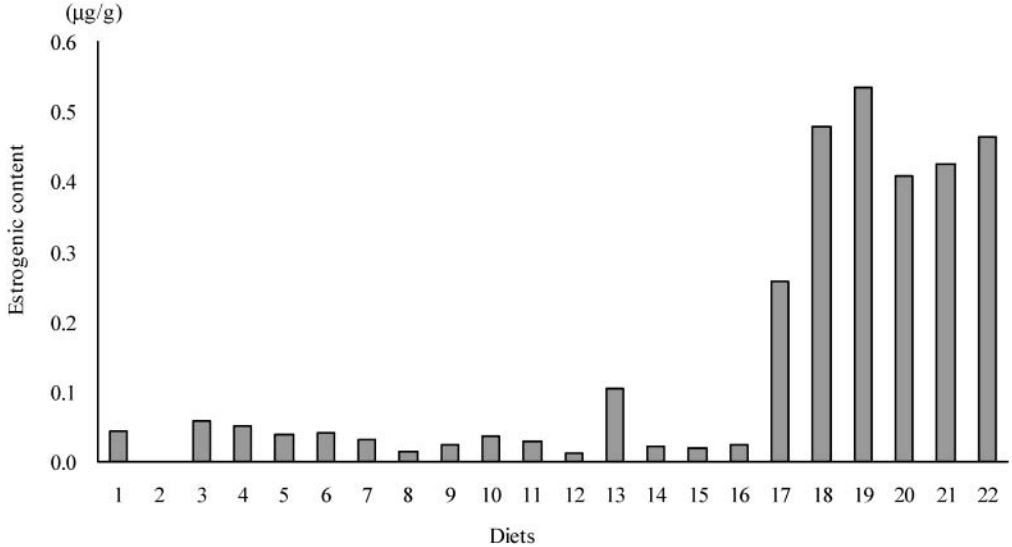

Figure 3. Estrogenic content in diets for experimental animals. Except for NIH-07 (PLD) diet, all diets contained substances having estrogenic activity (mean of 3 extractions in duplicate). 1, NIH-07; 2, NIH-07 (PLD); 3, NMF; 4, CR-LPF; 5, MF; 6, CMF; 7, CRF-1; 8, I; 9, PS; 10, DS-A; 11, CS-A; 12, XL; 13, MP; 14, PL; 15, diet for carp; 16, diet for ayu fish; 17, ZF; 18, ORC4; 19, GOC4; 20, GC4; 21, LRC4; 22, RC4.

Table 2. Ingredients of NIH-07 Diet (Slightly Modified by the Oriental Yeast Co. Ltd.)

ingredients	% by weight
dried skim milk	5.00
fish meal (65% protein)	10.00
soybean meal (45% protein)	11.75
alfalfa meal	4.00
corn gluten meal	3.00
ground yellow shelled corn	24.50
wheat flour	2.87
brewers dried yeast	2.00
dry molasses	0.75
soy oil	2.50
Salt	0.33
dicalcium phosphate	1.25
mineral mixes	1.05
vitamin mixes	1.00
total	100.00

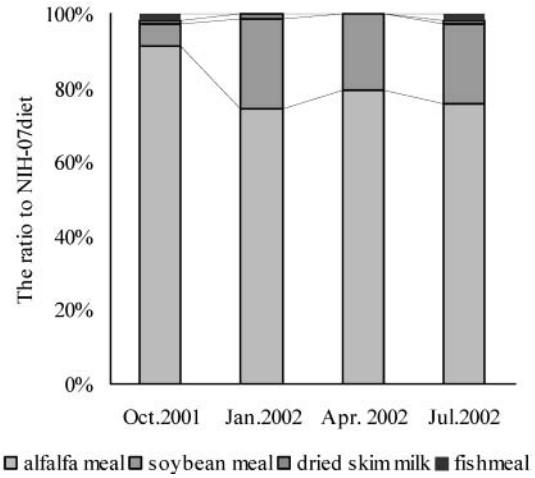

Figure 4. Changes in the ratio of estrogenic content in each ingredient by season when the diet was manufactured (mean of three extractions in duplicate). The estrogenic content of the diet is nearly entirely derived from that in alfalfa meal.

was approximately 3-fold higher than that in December 2002. These differences cannot be accounted for by the variations in extraction or measurement efficiency and must reflect authentic variations in the ES content of the materials.

We found that nearly all of the experimental animal diets contained estrogenic substances. This activity was found to reside in dried skim milk, fishmeal, soybean meal, and alfalfa meal. The majority of the estrogenic activity in the NIH-07 diet was derived from alfalfa meal, and we note that the levels of estrogenic activity in all raw materials changed throughout the year.

In dose−response analysis of several phytoestrogens, we found that the estrogenic activity of the chemicals was 1.8×10^{-2} for coumestrol, 1.8×10^{-3} for genistein, and 3.8×10^{-5} for daizein when the activity of E_2 was taken as 1.0. These values were consistent with other reports (i.e., coumestrol: 1.3×10^{-2}) (*1*). The high recovery rate (more than 80%) of E_2, coumestrol, genistein, and daizein was obtained from the recovery efficiency, and low C. V. (4%) was obtained by measurement of the NIH-07 diet performed 5 times. These data suggest that the methodology we employed for extraction and measurement is appropriate to evaluate estrogenic activity in experimental animal diets.

Phytoestrogens have been found at high levels in alfalfa meal (coumestrol, 25.3−64.8 μg/g in dry matter) (*11*) and soybean meal (genistin, 910 μg/g; genistein, 90 μg/g; daizin, 600 μg/g; daizein, 71 μg/g) (*13*). The manufacturer's information indicates that all of the diets tested (except for the NIH-07 (PLD) diet) contained alfalfa and/or soybean meal. Therefore, it follows that most of the estrogenic activity in these diets will be derived from alfalfa and/or soybean. In rat and mouse diets, (except for the NIH-07 (PLD) diet), the levels of estrogenic substances were relatively constant; the ratio of the highest (NMF diet) to the lowest (CRF-1 diet) was 1.8 times. Soy isoflavones diets have received considerably attention (18−21). It is particularly notable that alfalfa meal, which composes only 4% of all the ingredients, accounts for 80% of total estrogenicity in the NIH-07 diet. This is attributable to higher estrogenic activity in alfalfa (likely to be coumestrol) than that in soybean (maybe genistein and daizein).

The highest values for estrogenic activity in soybean meal and alfalfa meal during the year were 3 and 4 times higher than their lowest values, respectively. These results may have been derived from differences in varieties of soybean and locations of cultivation (*9*, *10*), and from differences in the coumestrol content of alfalfa during growth stages (*11*). The ES content of any diet manufactured from these materials would be expected to vary from batch to batch as the raw materials vary. Taken together, these data point to a need for studies demonstrating that the degree of estrogenic activity in the diet do not affect the results of in vivo studies. In the present study, it is unknown what kinds of substances had estrogenic activity in fishmeal and dried skim milk and why estrogenic activity of these materials was detected only in a few months in the year.

Legume plants such as alfalfa and soybean, for example, which provide animals with proteins of good quality, are used as materials to manufacture animal diet. Genistein and coumesterol are known to affect the development and physiological state of exposed animals. Such effects include increased c-fos gene in prostate (*6*), altered 5α-reductase activities in the medial basal hypothalamic preoptic area and amygadala (*22*), inhibited expression of the EGF and ErbB2/Neu receptors in rat dorsolateral prostate (*23*), inhibition of 17β-hydroxysteroid oxidoreductase Type 1 in vitro (*24*), and effects on end points (*3*, *25*−*27*). Researchers also need to be aware of the possibility that the estrogenic activity of experimental animal diets may vary from batch to batch during the year. Otherwise, these

Table 3. Changes of ES Content (μG/g as E_2) in Major Raw Materials[a]

	2002											2003
raw materials	Feb.[b]	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.
fish meal	n.d.[c]	n.d.	n.d.	n.d.	n.d.	0.012	n.d.	n.d.	n.d.	0.012	n.d.	n.d.
dried skim milk	n.d.	n.d.	n.d.	0.004	0.004	n.d.	n.d.	n.d.	n.d.	n.d.	0.013	0.009
soybean meal	0.075	0.076	0.079	0.079	0.079	0.080	0.089	0.036	0.036	0.056	0.032	0.112
alfalfa meal	1.270	1.347	1.553	1.282	1.181	1.088	1.172	0.596	0.502	0.395	1.679	1.729

[a] The estrogenic activity is always lower than the detection limit in corn gluten, yellow shelled corn, wheat flour, and soy oil for a year. [b] The month of manufacture. [c] n.d., less than the detection limit (0.01 μg/g as E_2).

estrogens might mask the estrogenic or estrogenic adverse effects of chemicals. It is particularly notable when low dose, in vivo studies are being performed. Our data may shed light on the controversy about low dose effects of estrogenic chemicals (*28*, *29*). We believe that the data presented here will be useful for researchers interested in the effects of nutrition, endocrinology, and endocrine disrupters.

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