Validation of a Novel Estrogen Receptor-Based Microtitration Plate Assay for the Determination of Phytoestrogens in Soy-Based Foods

Stephen D. Garrett,[†] Heather A. Lee,*[,†] Phöbe M. K. Friar,[‡] and Michael R. A. Morgan[†]

Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K., and Department of Food Science and Technology, University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, U.K.

A novel, nonisotopic microtitration plate assay based on the human estrogen receptor has been used to screen soy-based and soy-containing foods for their phytoestrogen content (measured as genistein equivalents). The validation of the assay for use with food extracts has been demonstrated by investigation of recoveries after acidic and enzymic hydrolysis, by investigation of matrix effects, and by comparison of results with HPLC analysis. Phytoestrogen levels in soy products analyzed ranged between 520 and 1872 μg of genistein equiv/g of soy flour, 5−282 μg/g of soy concentrates, 503−1292 μg/g of soy-protein isolates, and 108−226 μg/g of soy-based infant formulas. Samples of textured vegetable protein and bread containing soy and linseed gave values of 1114 and 68 μg/g, respectively. Comparison of results for 12 samples analyzed both by the receptor assay and by HPLC showed good correlation ($r^2 = 0.905$). The assay, which is rapid and simple to perform, is suitable for screening phytoestrogen-containing foods in order to assess human exposure to these bioactive compounds. The assay sensitivity is 3.4 μg/g, and 14 samples/plate can be analyzed in 4 h following hydrolysis.

Keywords: *Phytoestrogens; estrogenic isoflavonoids; receptor assay; screening; soy-based food*

INTRODUCTION

There is growing evidence that plant compounds with estrogenic activity (phytoestrogens) may have a role in the prevention of cancer (Messina et al., 1994), heart disease (Anthony et al., 1996), and osteoporosis (Arjmandi et al., 1996) and in the moderation of menopausal symptoms (Clarkson et al., 1998). However, phytoestrogens cause reproductive problems in a number of animal species (Setchell et al., 1987; Hughes, 1988), and concern has been expressed at exposure of young infants fed soy-based formulas containing high levels of these compounds (Setchell et al., 1997). The main groups of known phytoestrogens are isoflavones, coumestans, and lignans (Figure 1), which occur in various parts of plants. The most abundant isoflavones in foodstuffs, especially soy or soy-containing foods, are the glucosides genistin, daidzin, and glycitin (plus acetylglucosides and malonylglucosides) and their aglycones genistein, daidzein, and glycitein. The 4-methyl ether derivatives of daidzin and genistin (formononetin and biochanin A) occur in high levels in chick pea and red clover. The coumestan coumestrol is often found in sprouted legumes, whereas the lignans (secoisolariciresinol, matairesinol, and their diglucosides) are more widespread and found in the aleuronic layer of seeds (Kurzer and Xu, 1997). Following ingestion, action by bacteria in the colon may result in the generation of more estrogenically active aglucons from their glucosides and metabolites such as equol. Similarly the lignans are converted to enterodiol and enterolactone (Adlercreutz, 1995).

One important aspect of investigations into the putative beneficial or harmful effects of these compounds is the ability to measure phytoestrogens in food in order to assess dietary intakes. Perhaps surprisingly, information on dietary intakes of phytoestrogens is incomplete, particularly for Western diets. Analysis of food for phytoestrogens has been performed predominantly by HPLC (Reinli and Block, 1996), which has allowed the measurement of high levels of lignans (in linseed) and isoflavones (in soybean and soy products). More recently a GC/MS method has been developed that uses isotope dilution (Mazur et al., 1996). This method is more sensitive than HPLC (2−3 μg/100 g) and is restricted to determination of isoflavanoids in the aglucon form. It has been applied to isoflavonoids, coumestrol, and lignans in legumes (Mazur et al., 1998). Liggins et al. (1998) have also developed a GC/MS method that uses selected-ion monitoring. Rapid antibody-based methods are now used routinely in food analysis for a range of compounds from aflatoxin to peanut protein (Brett et al., 1996), enabling the rapid and cost-effective throughput of samples with good sensitivity. Although immunoassays have now been reported for daidzein (Lapcík et al., 1997; Creeke et al., 1998; Kohen et al., 1998), genistein (Lapcík et al., 1998), formononetin (Wang et al., 1994), equol (Creeke et al., 1998), and enterolactone (Adlercreutz et al., 1998), these assays have been applied to blood and urine samples rather than food. They are too specific for screening for a range of estrogenic compounds.

Using the estrogen receptor in place of an antibody allows the detection of any estrogenic substance (as defined by its ability to interact with the receptor) present in the sample in proportion to its affinity for the receptor, giving a measure of the 'total estrogenic

* Corresponding author. Tel: +44 (0)1603 255358. Fax: +44 (0)1603 507723. E-mail: heather.lee@bbsrc.ac.uk.
[†] Institute of Food Research.
[‡] University of Reading.

10.1021/jf990579o CCC: $18.00
Published on Web 09/24/1999

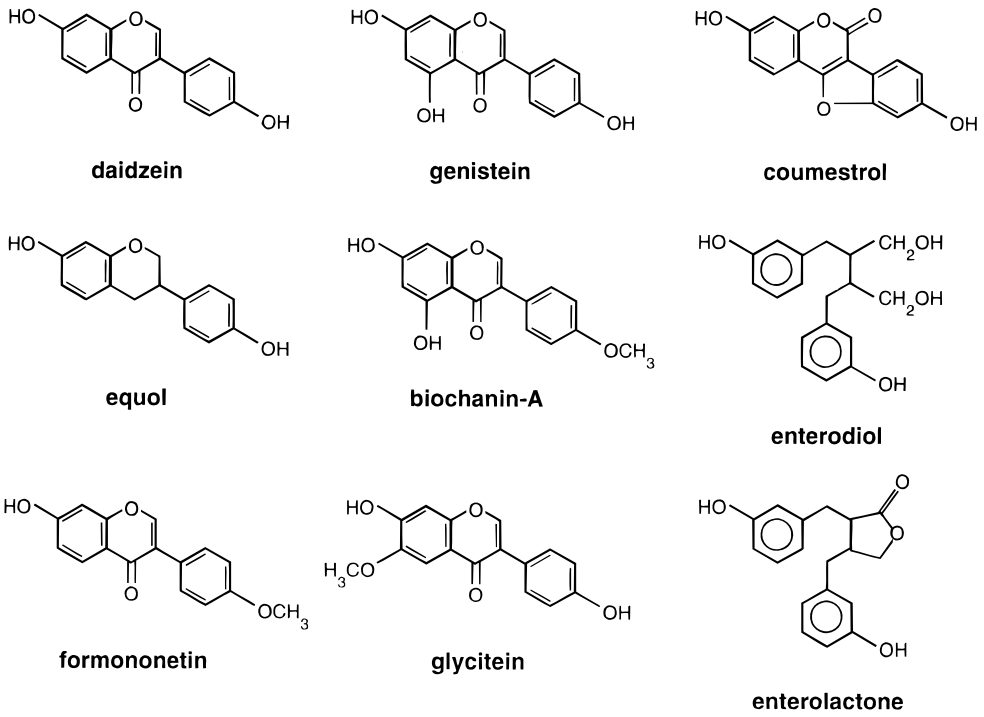

Figure 1. Chemical structures of estrogenic isoflavonoids and lignans.

load' of the food. Various receptor methods to detect estrogens have been reported (Miksicek, 1994; Häggblad et al., 1995), but these assays have used radioactive estradiol as a tracer and have not been applied to food screening. In this paper, we report the application of a novel nonisotopic receptor-based microtitration assay (Garrett et al., 1999) to the analysis of the phytoestrogen content of soy flour, concentrates and isolates and some soy-based and soy-containing foods, especially infant formulas. The assay is a combination of an ELISA for estradiol and a receptor-binding assay where the phytoestrogens compete with unbound estradiol for binding to the receptor. Phytoestrogens from a food sample are estimated by reference to a genistein standard curve, which has a working range of 20 ng/mL−2 μg/mL with within-assay and between-assay coefficients of variation of 3.4−7.0% and 5.0−19.0%, respectively. Recovery of genistein from spiked food samples, matrix effects, and validation of results from infant formulas and soy-protein isolates by HPLC are reported.

MATERIALS AND METHODS

Solvents and Standards. All solvents were HPLC grade. Isoflavonoid standards (daidzein, daidzin, genistein, genistin, glycitein, glycitin, formononetin, biochanin A, and coumestrol) were supplied by Plantech (U.K.) (Reading, U.K.) or Apin Chemicals Ltd. (Oxon, U.K.). Flavone and flavonone, used as internal standards, were purchased from Sigma Aldrich Co. Ltd. (Poole, U.K.).

Samples. The soy-based infant formulas (powders), soy flours, TVP, and bread were purchased from local retail outlets. The soy-protein isolates and soy concentrates were obtained from British Arkady (Manchester, U.K.), apart from the two isolates analyzed by HPLC, which were batches of Arcon F from Archer Daniels Midland Europort BV (Rozenburg, The Netherlands). All materials were analyzed as received.

Extraction and Hydrolysis for Analysis by Receptor Assay. Acid hydrolysis and enzymic hydrolysis were compared for efficiency and ease of use.

(*a*) *Acid Hydrolysis.* Portions of food samples (0.5 g) were extracted with hot methanol (80%; 10 mL) at 65 °C by shaking for various time intervals up to 165 min to investigate the efficiency of extraction of isoflavones (Coward et al., 1993). The samples were allowed to settle, an aliquot (5 mL) of the extract was partitioned with hexane (3 × 10 mL) to remove lipids, and the methanolic phase left to evaporate at 50 °C for 3−4 h. The dried extract was redissolved in 1 M HCl (2 mL) and heated in a boiling water bath for 2 h to allow hydrolysis to occur. Subsequently, the hydrolysate was neutralized with 6 M NaOH and diluted in phosphate-buffered saline, pH 7.4, containing 0.15% Tween 20 (PBST) and 2 ng/mL estradiol for analysis in the receptor assay.

(*b*) *Cellulase Hydrolysis.* The method was adapted from Liggins et al. (1998) to enable rapid sample throughput. Portions of food samples (2.5 g) were extracted with methanol (80%; 20 mL) by shaking at 37 °C for 2 h. After centrifuging for 20 min at 4000 g, an aliquot of the supernatant (5 mL) was removed and placed in a boiling water bath until the methanol had evaporated (approximately 5 h). Acetate buffer (5 mL, pH 5.0) was added to the remaining solution (0.5 mL) with 100 U of cellulase from *Aspergillus niger* (Sigma-Aldrich Co. Ltd., Poole, U.K.), and hydrolysis of the glucosides was achieved by overnight incubation at 37 °C. Subsequently an aliquot was taken, centrifuged to remove any solid particles, and diluted in PBST (containing 2 ng/mL estradiol) for analysis in the receptor assay.

Microtitration Plate Receptor Assay. Development of the assay has been described previously (Garrett et al., 1999). A standard solution of genistein was prepared by dissolving 1 mg in DMSO (20 μL) and making the volume up to 1 mL with methanol. Serial dilutions were then made in PBST (containing 2 ng/mL of estradiol) in the range of 2 ng−20 μg/mL. Standard or diluted sample extract (50 μL) was added to the wells of an estradiol−bovine thyroglobulin (BTG)-coated plate (Nunc Immunoplate Maxisorp; Gibco Ltd., Uxbridge, U.K.; 0.5 μg/mL) with an osmotic shock preparation of the human estrogen receptor α-ligand binding domain fused to glutathione-*S*-transferase (hERα−LBD; 50 μL) diluted in PBST containing 5% (w/v) nonfat dried milk (1:7.5). This amount of LBD is equivalent to 3.3 pmol of hER (Panvera Corp., Madison, WI). After incubation for 2 h at room temperature, anti-estradiol antibody (Serotec Ltd., Oxford, U.K.) diluted 1:5000 with PBST (50 μL) was also added to the contents of the wells,

and incubation was continued for another 2 h at room temperature. The plate was then washed five times with PBST (Denley Wellwash 5000; Denley Instruments Inc., Billingshurst, U.K.), and anti-rabbit IgG-horseradish peroxidase (HRP) labeled antibody (Sigma-Aldrich Co. Ltd., Poole, U.K.) diluted 1:1000 in PBST (150 μL) was added for 1 h at 37 °C. Finally the plate was washed again (5 times in PBST), and substrate (150 μL of 3,3′,5,5′-tetramethylbenzidine solution; Vetoquinol, Bicester, U.K.) was added. After 10 min at room temperature, the reaction was stopped by the addition of sulfuric acid (50 μL; 2 M), and the absorbances of each well were read at 450 nm (Dynatech MT5000 plate reader; Dynatech Labs. Ltd., Billingshurst, U.K.). A plot of A_{450} versus genistein concentration gave a sigmoidal curve with a working range of 20 ng−2 μg/mL (1−100 ng/well). The receptor assay has a 10-fold lower affinity for daidzein than for genistein (Garrett et al., 1999), so the daidzein content of samples is measured as one-tenth of the true daidzein value, expressed in genistein equivalents.

Extraction and HPLC Analysis. A portion of infant formula (5 g powder) was defatted at room temperature by stirring with hexane (50 mL) for 2 h. After removal of the supernatant, the process was repeated twice. Isoflavonoids were then extracted with 80% methanol (40 mL) by stirring for 2 h at room temperature, following a modified procedure based on Barnes et al. (1994). The extract was filtered, made up to 50 mL, and analyzed by reversed-phase HPLC with gradient elution using a modified procedure based on Wang and Murphy (1994). For the soy-protein isolate, the defatting step was omitted.

The column employed was a YMC-Pack ODS-AM 303 column (250 × 4.6 mm i.d., S-5 μm 120 A), protected by a Hichrom RPB guard cartridge column (10 × 0.3 mm i.d.) (all columns: Hichrom, Reading, U.K.). The injection volume of the extracts and standard solutions was 20 μL. The elution of isoflavonoids was performed at 30 °C using a mobile phase consisting of water with 0.1% glacial acetic acid (solvent A) and acetonitrile with 0.1% glacial acetic acid (solvent B) at a flow rate of 1 mL/min. Prior to running the gradient, the column was equilibrated for 10 min with solvents A and B (85/15 v/v).

The HPLC system for obtaining quantitative data was a Hewlett-Packard 1050 series module system (quaternary pump, autosampler, column heater and diode-array detector, and HP ChemStation data handling system). The analytes in the eluent were monitored simultaneously at 250 and 260 nm, and the peaks of each chromatogram were scanned between 220 and 380 nm for confirmation of identity by comparison with the spectra of isoflavonoid standards in the library. Additional confirmation of the identity of the isoflavonoids was obtained by monitoring selected ions using an LC/MS system with atmospheric pressure chemical ionization (APCI) in positive and negative mode using an HP1100 series quaternary pump (Hewlett-Packard), a Gilson 231XL autosampler (Anachem, Luton, U.K.) with a 50-μL loop, a column block heater (Jones Chromatography, Hengoed, Wales, U.K.), and a VG Platform single-linear quadrupole mass spectrophotometer (Micromass, Manchester, U.K.).

Quantification of isoflavonoids, based on peak areas at 260 nm, was achieved by using a multilevel external standards procedure and comparing—in the linear range of the calibration curve—peak areas in chromatograms of sample extracts with peak areas recorded for standards. Quantitative data for daidzin, glycitin, genistin, daidzein, glycitein, genistein, coumestrol, formononetin, and biochanin A were obtained by comparison with known standards. Concentrations of 6″-*O*-malonyl and 6″-*O*-acetyl derivatives of daidzin, glycitin, and genistin were calculated from the standard curves for the corresponding β-glucosides, as the corresponding molar absorbances remain virtually the same on acylation (Kudou et al., 1991). All data were corrected for recovery of the internal standard, flavone.

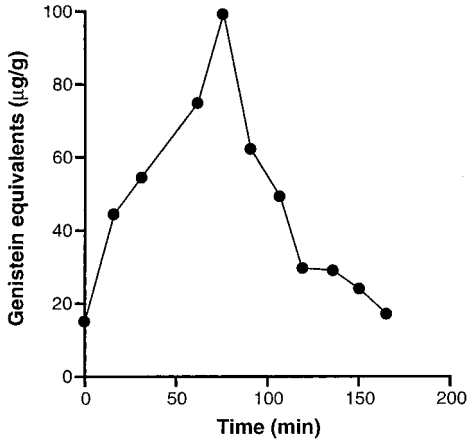

Figure 2. Efficiency of extraction for isoflavonoids as measured in the receptor assay after hydrolysis with 1 M HCl at 100 °C with increasing time.

Table 1. Recovery of Genistein following Cellulase Hydrolysis from a Soy Concentrate Sample Spiked with Genistin and Corrected for Endogenous Phytoestrogen

amount of genistin added (μg/g)	equiv amount of genistein after hydrolysis (μg/g)	genistein equiv measured (μg/g), $n = 6$	% recovery
0	0	18 ± 1	
100	62.5	87 ± 12	110.4
200	125.0	152 ± 20	107.2

RESULTS AND DISCUSSION

Plant and plant-based foods can contain isoflavones such as daidzein and genistein that are estrogenic and their glucosylated counterparts daidzin and genistin, which are much less estrogenic, if at all, but can be hydrolyzed in the gut. Further degradative metabolism can give rise to other compounds, such as equol, which is also estrogenic. In most foods (notable exceptions being fermented foods such as miso), the content of compounds that are more estrogenic is relatively low as compared to the conjugated forms. Consequently, analysis of food for estrogens is normally preceded by a hydrolysis step.

Hydrolysis for Phytoestrogen Analysis by Receptor Assay. The recovery of isoflavones from extracted portions of soy-based infant formula was measured following hydrolysis with 1 M HCl for various times from 0 to 165 min. The results after 1:100 (v:v) dilution in PBST (Figure 2) show that the optimal time for release of aglucons from their glucosides was 75 min. These findings do not agree with previously reported data (Wang et al., 1990) where extraction of genistein from soybean with 1 M HCl at 99−100 °C was optimal at 2 h, and extraction with 2 M HCl showed a decline after 70 min. This may be due to a protective effect of the soybean matrix, as Wang et al. added the acid and solvent (in this case acetonitrile) to the sample together instead of extracting first before acid hydrolysis.

An alternative method of hydrolysis using cellulase was investigated. Genistin (100 and 200 μg/g) was added to a soy concentrate sample. After hydrolysis with cellulase and analysis in the receptor assay, the results indicated recoveries approaching 100% (Table 1), which agreed with reported results of >83% (Liggins et al., 1998). This method was easier and more reproducible than acid hydrolysis and was used to analyze all of the samples.

Table 2. Results from Analysis of Three Different Types of Foods Extracted, Hydrolyzed, and Analyzed at a Range of Dilutions

infant formula powder		bread		soy flour	
dilution	genistein equiv (μg/g)	dilution	genistein equiv (μg/g)	dilution	genistein equiv (μg/g)
1:40	103	1:40	75	1:400	504
1:80	118	1:80	65	1:800	454
1:100	110	1:100	70	1:1000	599
1:200	83	1:200	58	1:2000	554
1:400	96	1:400	75	1:4000	479
1:800	118	1:800	63	1:8000	529
1:1000	129				
mean	108		68		520
SD	15		7		52
% SD	14		10		10

Table 3. Levels of Isoflavones in a Range of Soy Products Measured in the Receptor Assay

sample		μg of genistein equiv/g of matrix
soy flours	1	1258
	2	1872
	3	1558
	4	1088
	5	1360
	6	520
soy concentrates	1	282
	2	83
	3	5
	4	15
	5	14
soy-protein isolates	1	1292
	2	1140
	3	644
	4	1224
	5	503
	6	490
soy-based infant formulas	1	191
	2	177
	3	226
	4	108
textured vegetable protein		1114
bread (containing soy + linseed)		68

Matrix Effects. To assess the effect of three different food matrixes on the receptor assay, samples of infant formula, bread, and soy flour were hydrolyzed and extracted. Results for the extracts diluted from 1:20 to 1:8000 in PBST (Table 2) showed that there were no matrix effects for any of these three foods, even at the lowest dilution of 1:20, as the values obtained at all dilutions were similar. The % SD ranged from 10 to 14. This enabled the extracts to be assayed directly with no need for expensive and time-consuming cleanup procedures. Using a 1:20 dilution and a limit of detection of 20 ng/mL in the assay, this corresponds to a sensitivity of 3.4 μg/g. However, it should be possible to increase the sensitivity using lower dilutions of samples or by concentrating extracts, if required.

Analysis of Soy Products and Soy-Based Foods. The results of a range of soy flours, soy concentrates, soy-protein isolates, and other soy-based and soy-containing foods are given in Table 3. The levels of phytoestrogens measured by the receptor assay for soy flours are in the range of 520−1872 μg of genistein equiv/g (Table 3). This correlates well with values reported by other workers using HPLC: 1123 μg of genistein/g and 655 μg of daidzein/g (Franke et al., 1994), 810 μg of total genistein/g and 226 μg of total daidzein/g (Wang and Murphy, 1994), and 836−939 μg of genistein/g and 658−742 μg of daidzein/g for four varieties (Coward et al., 1993; values recalculated to represent total aglucons). The variety, cultivation practice, and storage conditions of soybeans have all been shown to have an effect on isoflavonoid levels, giving further variations in levels in soy-derived foods (Eldridge and Kwotek, 1983). Therefore, it is not surprising that a wide range of isoflavone levels have been reported.

The soy concentrates analyzed in this study were all produced by removing the soluble sugars from soy flakes or flour by extraction with hot aqueous ethanol or dilute acid, processes that also remove a high proportion of the isoflavones. The levels measured (5−282 μg of genistein equiv/g) are in agreement with other reported values for soy concentrates of 58−211 μg of genistein/g and 43−107 μg of daidzein/g (Coward et al., 1993; values recalculated to represent total aglucon levels) and 13 μg of total genistein/g (Wang and Murphy, 1994). Soy-protein isolates gave much higher levels than the concentrates but lower levels than soy flour. The isolates are prepared by extracting the protein from flakes or flour with dilute alkali followed by acid precipitation. This process appears to extract the isoflavones along with the protein to varying extents. Our results of 490−1292 μg of genistein equiv/g cover a wider range than other results in the literature, which are 273−393 μg total of genistein/g and 77−122 μg of total daidzein/g (Wang and Murphy, 1994) and 374−557 μg of genistein/g and 215−271 μg of daidzein/g (Coward et al., 1993; values recalculated to represent total aglucon levels). The value obtained in this study for textured vegetable protein (TVP) of 1114 μg of genistein equiv/g is somewhat higher than other reported values of 568-707 μg of genistein/g and 83−484 μg of daidzein/g (Reinli and Block, 1996). There are no literature reports of levels for Burgen Bread, a product introduced to the U.K. market fairly recently, but our measurement of 68 μg of genistein equiv/g would agree reasonably well with the 8% soy content of the bread.

The levels of isoflavones found in infant formulas in Table 3 (108−226 μg of genistein equiv/g dry formula powder) and in Table 4 (60−179 μg/g of genistein equiv for formula powder) agree well with the results of six American infant formula powders that contained 126−154 μg of total genistein/g and 57−78 μg of total daidzein/g (Murphy et al., 1997). Setchell et al. (1997) reported higher levels of 307−316 μg of total isoflavonoids/g comprising approximately 67% genistein and its derivatives and 29% daidzein and its derivatives, whereas Irvine et al. (1998) reported lower levels of 87 $\pm$ 6 μg of total genistein/g and 49 $\pm$ 5 μg of total daidzein/g for four soy infant formulas in New Zealand.

Correlation of Results with HPLC. Ten samples of U.K. brands of soy-based infant formulas and two batches of the soy-protein isolate Arcon F were analyzed after cellulase hydrolysis at a dilution of 1:40 and 1:10, respectively, in the receptor assay. The samples were also analyzed for total isoflavonoids, total genistein, total daidzein, and total glycitein by HPLC. The values obtained by the two methods are shown in Table 4.

The isoflavonoids in the soy infant formulas ranged from 123 to 288 μg of total isoflavonoids/g measured by HPLC and from 60 to 179 μg of genistein equiv/g measured by the receptor assay. Corresponding values for the two batches of Arcon F were 10 and 26 μg of total isoflavonoids/g for the analysis by HPLC and 6 and

Table 4. Isoflavonoid Analysis of Soy-Based Infant Formulas and Soy-Protein Isolates by Receptor Assay and by HPLC with UV Diode Array Detection

	HPLC				
sample	total isoflavonoids[a] (μg of aglucon equiv/g of powder)	total genistein[b] (μg of genistein/g of powder)	total daidzein[c] (μg of daidzein/g of powder)	total genistein + 1/10 total daidzein (μg of aglucon equiv/g of powder)	receptor assay genistein equiv (μg/g of powder)
R1	191	126	55	132	119
R2	226	132	83	140	100
R3	232	131	87	140	113
R4	226	132	83	140	103
R5	211	121	78	129	130
R6	155	94	50	99	90
R7	123	74	39	78	60
R8	288	170	102	180	179
R9	123	74	39	78	73
R10	165	101	54	106	117
RA	10	6	nd	6	6
RB	26	14	12	15	17

[a] Total isoflavonoids: sum of total genistein and related compounds + total daidzein and related compounds + total glycitein and related compounds. [b] Total genistein: genistein + genistin + 6″-*O*-acetylgenistin + 6″-*O*-malonylgenistein. [c] Total daidzein: daidzein + daidzin + 6″-*O*-acetyldaidzein + 6″-*O*-malonyldaidzein.

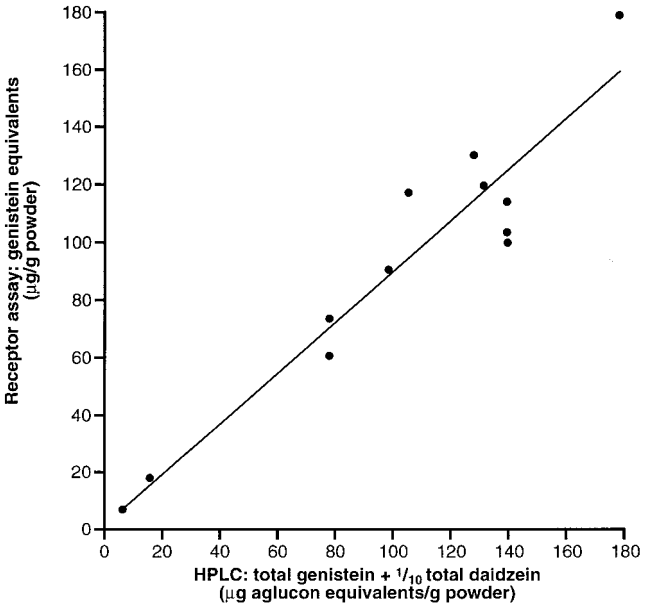

Figure 3. Correlation of results for isoflavone levels in samples of U.K. brands of soy-based infant formula and two soy-protein isolate samples obtained by the receptor assay and by HPLC. The HPLC results are expressed as the total genistein value plus one-tenth of the total daidzein value.

17 μg genistein of equiv/g for the receptor assay. If the results are compared as HPLC-determined total genistein values plus one-tenth of the HPLC-determined total daidzein values with the receptor assay values (as the receptor assay has a 10-fold lower affinity for daidzein than genistein), then the correlation is good (Figure 3; $r^2 = 0.905$, $p < 0.001$). The slope of the curve equals 0.88, and the intercept on the *Y* axis is 1.6.

We have shown that a novel, nonisotopic, microtitration plate receptor assay developed in our laboratory can be used to rapidly screen foods containing soy. Sample preparation and hydrolysis with cellulase is simple, requiring an overnight incubation but no subsequent cleanup stages. The assay takes 4 h, 14 samples can be measured (in triplicate) on each microtitration plate, and several plates can be run at the same time because of the simplicity of the procedure. The results, expressed as genistein equivalents, compare well with reported values for soy-based foods and have a very good correlation with HPLC results obtained from the same samples. To understand the effects of phytoestrogens on the endocrine system, especially in infants (Klein, 1998), we must be able to understand more about dietary exposure. This assay provides a means of screening large numbers of food samples for all estrogens, as defined by compounds that react with the estrogen receptor (either as agonists or antagonists), including compounds that may have not yet been isolated or recognized as estrogenic. Positive samples can then be subjected to more detailed analysis such as GC/MS (which will allow identification of individual aglucons) and HPLC (which will allow the quantification of the individual forms in which the estrogenic compounds occur in food) or to biological assays such as stably transfected MCF-7 cells (LeBail et al., 1998) to assess molecular activity.

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Received for review June 1, 1999. Revised manuscript received July 29, 1999. Accepted August 2, 1999. The financial support of the Ministry of Agriculture, Fisheries and Food is gratefully acknowledged, and we wish to thank Dr. Hugh Makin for helpful discussions.

JF990579O