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Sensitive high-performance liquid chromatographic method using coulometric electrode array detection for measurement of phytoestrogens in dried blood spots

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Abstract

As the epidemiological and physiological investigation of isoflavones and lignans expands, the need for sensitive methods for analyzing large numbers of samples intensifies. We have developed a method using high-performance liquid chromatography (HPLC) equipped with a coulometric electrode array detector for separation and sensitive detection of daidzein (Da), equol (Eq), genistein (Ge) and enterolactone (Enl) in dried blood spots (DBS). Detection limits ranged from 4.5 pg or 0.09 ng/mL (Eq) to 19 pg or 0.38 ng/mL (Ge) on column. Signal linearities ranged from detection limits to 200 ng/mL (Eq, Enl) and 600 ng/mL (Da, Ge) sample concentration. Correlations between DBS and serum concentrations were 0.66 (Enl), 0.88 (Eq), 0.98 (Ge) and 0.99 (Da). Intra-assay coefficients of variation (CVs) were less than 8% and inter-assay CVs ranged from 2.4 to 20.2% for Da, Eq and Ge for three levels of controls. Enl intra-assay CV was 13.6% for the low pooled control. Analytic recovery ranged from 87% (inter-assay Ge) to 98% (inter-assay Enl). DBS concentrations of Da, Ge and Eq were stable for at least 8 weeks at 4 and 25 °C, and at 37 °C for at least 5 weeks, with Enl showing greater variability at all temperatures but relative stability for 7 weeks. Measurement of samples from 135 perimenopausal Japanese women consuming habitual diets in Kyoto and Fukushima prefectures showed the former to have the expected lower concentrations of Da and Eq (416 and 87 nM) as well as Enl (49 nM) compared to the latter locale (566, 145 and 72 nM, respectively). This method could be useful in large epidemiological research or detailed physiological studies.

Keywords: Multi-channel coulometric electrode array detection; Isoflavones; Phytoestrogens; Dried blood spots

1. Introduction

Interest in functional food factors such as phytoestrogenic isoflavones and lignans continues to grow because of their many reported effects on health [1], both beneficial and adverse [2,3]. Epidemiological and clinical studies have linked the consumption of these compounds to reduced risk of reproductive cancers, menopausal symptoms, osteoporosis and heart disease [4–9]. On the other hand, potential long term effects of ingesting high concentrations of isoflavones in infant formula and the use of isoflavone supplements have been of concern [10–14]. The average intake of these compounds varies by population with Japanese consuming high levels of soy isoflavones and Finns consuming high levels of lignans, which are precursors to enterolactone [15].

However, epidemiological and physiological studies are limited by the need to perform invasive sampling procedures such as venipuncture, requiring the skills of trained medical professionals and time and travel of participants to research or clinical facilities. Additionally, blood and urine samples require rapid processing and refrigeration, increasing costs and logistical challenges. Analytical methods to measure isoflavones have been developed for foods, biological tissue, plasma, serum and urine [16], but not yet for dried blood spots (DBS). The use of DBS constitutes a relatively non-invasive blood collection method compared to intravenous serum or plasma collection and self-

Abbreviations: Da, daidzein; Ge, genistein; Eq, equol; Enl, enterolactone; Es, estriol; DBS, dried blood spot; RT, retention time; HPLC-CEAD, high-performance liquid chromatography-coulometric electrode array detection

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sampling is possible after a minimum of training. Thus, DBS can be collected in myriad environments such as participants' homes or in locales without access to immediate refrigeration. DBS have been used in newborn screening for diseases such as phenylketonuria (PKU) and congenital hypothyroidism (CH) for more than 40 years [17], and thus may be appropriate for studies of soy infant formula. DBS use in clinical and research settings has expanded to include testing for numerous diseases [17], and assays for insulin [18], estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH), sex hormone binding globulin (SHBG), estradiol (E2), testosterone (T), prolactin (PRL), androstenedione (A), dehydroepiandrosterone-sulfate (DHEA-S) and cortisol (C) for studies of the HPG and HPA axes [19] as well as C-reactive protein and Epstein Barr virus antibodies for studies of immune function [20,21] in populations throughout the world [22]. The main limitation of DBS samples is analytic, as the small blood volume necessitates sensitive assays.

Measurements of isoflavones and lignans have been conducted using gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) usually with an ultraviolet (UV) detector, and time resolved fluoroimmunoassay (TRFIA) [16]. GC–MS has excellent sensitivity, but requires extensive purification and derivatization prior to injection. Although TRFIA is a very sensitive technique [23], it is a single compound-based method useful for screening large numbers of samples and does not allow simultaneous determination of multiple analytes as do HPLC or GC–MS. HPLC-UV remains the most commonly employed method, but lacks the necessary sensitivity for measuring isoflavones in DBS. However, when HPLC is coupled with a sensitive detector such as coulometric electrode array detection (CEAD), the resultant technique is ideal for measuring multiple phytoestrogens in varied sample types [24–31].

The purpose of this study was to develop an HPLC-CEAD method for use with dried blood spots that resolves daidzein (Da), equol (Eq), genistein (Ge) and enterolactone (Enl), and can be applied in epidemiological and physiological studies of isoflavones and the metabolite equol produced by gut microflora. The main objective was to quantitatively measure isoflavones and their metabolites, but preliminary results for Enl are also included. This method is applied to samples obtained from perimenopausal Japanese women consuming their habitual diets in two different locales in Japan with distinctive dietary patterns and corresponding expected differences in their profiles of circulating isoflavones.

2. Experimental

2.1. Chemicals

Daidzein, genistein and equol were purchased from LC Laboratories (Woburn, MA). Enterolactone, estriol, estriol 3-(β-D-glucuronide) sodium salt, Trizma Base, Sodium Azide, BSA, Tween 40, and β-glucuronidase EC 3.2.1.31 type H-2 from *Helix pomatia* were purchased from Sigma Chemicals (Tokyo, Japan). Methanol, acetonitrile and distilled water (all HPLC grade), sodium chloride, sodium acetate trihydrate, acetic acid, diethyl ether and DMSO were purchased from Kanto Chemical (Tokyo, Japan). The buffer stock solutions for sample pretreatment were prepared from water purified using an Elix3, MilliQ and Gradient A10 water filtration system (Millipore Co., Bedford, USA).

2.2. Apparatus and chromatographic conditions

Analysis was performed using an eight-channel CoulArray Model 5600 HPLC detection system (ESA, Chelmsford, MA, USA) comprising two solvent pumps (Model 582), a high pressure gradient mixer, a polyetheretherketone (PEEK) pulse damper, an autosampler (Model 540), a CoulArray thermostatic chamber at 40 °C housing a MCM C18 column (No. 2363, 4.6 mm × 250 mm, 5 μM) (MC Medical, Tokyo, Japan), and two analytical cells in series, each containing four working electrodes. Multi-channel coulometric detection was performed by setting the eight electrode pairs at the following increasing voltages: 340, 450, 490, 545, 580, 620, 690 and 760 mV (versus palladium reference electrode). Final chromatographic conditions were adapted from those described by Gamache et al. [25]. Separation was performed using an isocratic elution. The mobile phase consisted of 50 mM sodium acetate buffer pH 4.8 with acetic acid, methanol and acetonitrile, 66.67:20:13.33 (v/v/v). Flow rate was 0.8 mL/min and the total run time was 60 min per sample. The injection volume was 50 μL. The system was controlled and data were acquired and processed using CoulArray software (ver. 2.0, ESA) on a Pentium-based computer. Quantitation from peak height was performed using calibration curves generated from six-point serial dilution of the standard mixture. Peaks in unknown samples were matched to those of standard compounds run in each assay on the basis of retention time (RT) (±4%) as well as response ratio between adjacent channels (±30%), and manually inspected to ensure correct assignment, particularly in cases with apparent large RT errors or poor peak ratios (usually due to incorrect peak assignment by the software in samples with very low concentrations).

2.3. Standard preparation

Stock solutions of approximately 1 mg/mL Da, Ge, Eq and Enl were prepared in MeOH and stored at −40 °C. Concentrations of the standard solutions were assessed by maximum absorbance using extinction coefficients characteristic for each compound (following Adlercreutz et al., personal communication, 2003). Concentrations were adjusted to 48 μg/mL for Da and Ge and 16 μg/mL for Eq and Enl, and then combined and serially diluted (1:1) using the HPLC mobile phase to make a set of six standards ranging from 600 to 18.75 ng/mL for Da and Ge, and 200 to 6.25 ng/mL for Eq and Enl. Aliquots of these serially diluted standards were stored at −40 °C.

2.4. Sample collection

After cleaning the finger with alcohol, samples were collected by finger-prick using a Microtainer Safety Flow Lancet (Becton Dickinson, NJ, USA) widely employed by diabetic patients to measure blood glucose. For measurement of isoflavones, at

least two drops are collected on standardized specimen collection paper (Schleicher-Schuell #903, Keene, NH, USA) used for neonatal screening and regulated by the US FDA [32], and designed to absorb blood uniformly so that concentrations are equivalent throughout the DBS. Samples are dried overnight and can remain at room temperature for at least 8 weeks for Da, Ge and Eq before refrigeration or analysis (see stability data in Section 3 below). One drop of blood is approximately 50 μL. This method uses two 9 mm diameter disks of dried blood, each equivalent to 25.11 μL of whole blood. In cases where sample collection is inadequate to obtain 9 mm diameter disks, 3.175 mm (0.125 in.) disks can be used, each equivalent to 3.125 μL of whole blood. Samples can be shipped as paper in sealed plastic bags or containers to protect against moisture, without special import permits for the USA or packaging (PHS Foreign Quarantine Regulations 42 C.F.R 71-54).

2.5. DBS pooled control preparation

Sera from individuals participating in studies of the effects of isoflavone supplements on health were combined to generate low, medium and high pools based on measured sera concentrations of Da, Eq and Ge. These were then mixed 1:1 (v/v) with washed red blood cells, prepared by washing three times with normal saline (8.6 g NaCl/L distilled water), and discarding the supernatant after centrifugation at 3000 rpm [19]. Aliquots (50 μL) were dropped by pipette onto sample collection papers, allowed to dry overnight at room temperature, and then stored frozen in an airtight container at −20 °C. This method was chosen over the alternative of spiking sera with analytes and then combining with washed red blood cells following other methods in the dried blood spot literature [19].

2.6. Sample pretreatment

Samples were punched (two 9 mm diameter disks) and eluted overnight in 0.5 mL of 0.5% BSA–Tris–buffer (pH 7.75) at 4 °C with shaking (150 rpm, 25 °C) in the first and final hours of elution. Hydrolysis is required because most isoflavones and lignans exist as glucuronide and sulphate conjugates in the blood [33]. *H. pomatia*, an edible European land snail, contains both β-glucuronidase and sulphatase enzymes and has been widely used to hydrolyze isoflavones and lignans [34,35]. The optimal amount of hydrolysis reagent added per tube, elution and incubation times, buffer pH, dilution of the sample and presence or absence of DBS throughout the sample preparation, were ascertained empirically. The sample pretreatment is detailed in Fig. 2.

2.7. DBS—serum equivalence

Matched serum and DBS samples were collected from 30 female students participating in a study of premenstrual syndrome and isoflavone supplements at Tokyo University of Agriculture. Sera were obtained by intravenous collection, and 50 μL aliquots were pipetted immediately from the tubes onto the sample collection paper. Papers were allowed to dry for 4 h and then placed in the freezer at −20 °C. Sera and DBS samples were run under the same chromatographic conditions using the same reagents and standards. Linear regression using stepwise procedures (p-value cut-offs of 0.05 for entry, 0.1 for removal) was performed to generate equations relating DBS concentrations to serum equivalent concentrations. The dependent variable was serum concentration; independent variables were DBS whole blood concentration and hematocrit (Ht).

2.8. Method validation

Limits of detection and quantification, respectively, were determined as the concentration at which the signal/noise ratio (peak height) equals 3:1 and 10:1 on the dominant channel. Response linearity for the range of interest was estimated based on calculation of the least squares regression correlation coefficient for the 7-point calibration curve for each compound. The range studied was 600–18.75 ng/mL (30–0.94 ng injected) of Da and Ge, and 200–6.25 ng/mL (10–0.31 ng injected) of Eq and Enl, with calibration curves including the origin (0,0). Chromatographic stability was evaluated by calculating the intra- and inter-assay CVs for the RTs and detector responses. Resolution (R) for the peak pairs was calculated using replicates of the standard mixture with the formula $R = (0.25)(\alpha - 1) \times \mathrm{SQRT}(N) \times k/(k + 1)$, where N is the plate number, α is the separation factor and k is the retention factor. Method precision and analytic recovery were evaluated for the four analytes (Da, Ge, Eq and Enl). Intra-assay ($N = 10$) and inter-assay precision ($N = 8$) were calculated as the percent coefficient of variation (% CV) for three pooled controls (low, medium and high concentrations). Analytic recovery was evaluated using the standard addition method at three different concentration levels and was calculated as mean recovery of the added standard compared to samples with equal volume of mobile phase (standard diluent) added as controls. Standard addition involved 50 μL addition of 600, 300 or 150 ng/mL (equivalent to 30, 15 and 7.5 ng added) of Da and Ge; 200, 150 and 75 ng/mL (equivalent to 10, 5 and 2.5 ng added) of Eq and Enl; and 2500 ng/mL (equivalent to 125 ng added) of the internal standard Es to DBS samples low in phytoestrogens after elution and prior to hydrolysis. Results were auto-corrected by the CoulArray software for recovery of the internal standard estriol at a concentration of 2500 ng/mL. Molar equivalent amounts of estriol 3-(β-D-glucuronide) sodium salt were added to each sample before hydrolysis in order to correct for loss due to hydrolysis and extraction. Following Gamache et al. [25], the glucuronide form of the internal standard estriol was used in the sample pretreatment because it is cleaved during hydrolysis, and controls for loss due to incomplete hydrolysis as well as sample loss.

2.9. Temperature stability

To assess temperature stability of the compounds in DBS, identical samples made from pooled sera and washed RBCs were submitted to three temperature conditions of varying dura-

tion: 4 °C, room temperature (approximately 25 °C), and 37 °C for 1–7 days and 1–8 weeks. Samples were considered to be stable as long as values remained within a 10% CV range of the initial values. Regression was performed to assess change in concentration for each compound over time, and *t*-tests were performed comparing the mean values for the first 7 days and the last 7 weeks.

2.10. Experimental applicability

One sample from each of 135 individuals participating in a 6-month study of menopause and regional difference in diet (Kyoto and Fukushima, regions with, respectively, 12.3% lower and 22.4% higher intake per capita of soy compared to the national average [36]) was assayed to document the range of values expected in a general population of Japanese women consuming their habitual diet. Participants collected finger-prick blood spot samples in the morning 1 day per week for 6 months. In order to maximize ability to detect equol (a metabolite of daidzein), we selected samples that followed the highest dinner intake of daidzein as estimated from dietary records for the 24-h period preceding blood collection and data on daidzein content of commonly consumed foods in Japan [37,38].

3. Results

3.1. Chromatographic parameters

The chromatographic parameters are presented in Table 1. Variation in RTs was negligible, with intra-assay CVs less than 0.11% and inter-assay CVs less than 0.61% for Da, Ge, Eq and Enl. Although all compounds of interest eluted by 39 min, the last unknown compound in DBS samples eluted at 58 min. Use of an isocratic elution permitted samples to be run one after the other with no stabilization period between samples, as is necessary for gradient elutions. An isocratic elution may also minimize changes and inconsistencies between individual sample runs. Thus, although the total run time per sample was long, run time per batch was similar to or shorter than other methods [26].

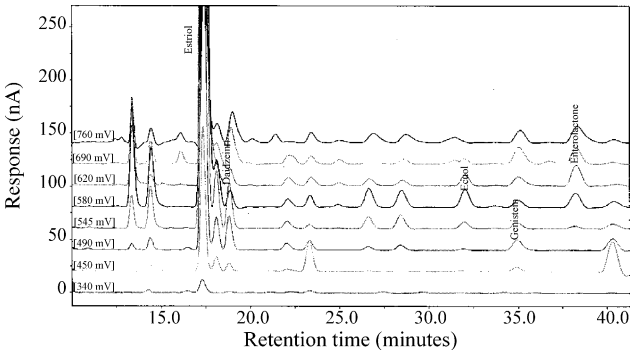

Fig. 1. Multi-channel chromatogram of the pooled sample mixture used for stability tests representing 1493, 434, 587 and 90 nM of Da, Ge, Eq and Enl, respectively. Isocratic elution was used with a MCM C18 column (4.6 mm × 250 mm, 5 μM) and a flow rate of 0.8 mL/min. Mobile phase consisted of 50 mM sodium acetate buffer pH 4.8 with acetic acid, methanol and acetonitrile, 66.67:20:13.33 (v/v/v). Detectors 1–8 are displayed at 260 nA for the region of interest (beginning with internal standard Es and ending with Enl) with corresponding potentials of 340, 450, 490, 545, 580, 620, 690 and 760 mV (vs. Pd).

Detector response intra-assay CVs were 5% or lower, but inter-assay CVs ranged from 11.6 to 14.3%, suggesting high variability between runs. Therefore, a new calibration curve was generated for each run. Peak pair resolution values ranged from 1.16 to 12.07. Critical peak pairs in this method development were enterolactone and genistein, but were adequately resolved with modifications of the mobile phase (Fig. 1 and Table 1). Critical pairs in the DBS samples were equol and a pair of unknown peaks, and daidzein and an unknown peak (Fig. 1). These were resolved by changes to the mobile phase, but caused overlap between genistein and unknown peaks, which could be resolved electrochemically because their dominant peaks were on different channels. Low peak pair resolution values between compounds of interest and unknown compounds existed in some samples, but electrochemical separation permitted accurate measurement because the quantification signals (dominant channel peak heights) were obtained from different channels. Peak heights rather than peak areas were used to reduce the influence of neighboring impurities or unknowns.

In addition to RT error, ratio accuracy was used to assess the assignment of peaks in each sample chromatogram. Ratio accu-

Table 1
Chromatographic parameters studied in low pooled control sample[a] for detection of daidzein, equol, genistein and enterolactone

Analyte	(min)	Retention time (RT)		Detection potential (mV)	Detector response CVs		R^b ($N = 10$)
		Intra-assay CV ($N = 10$)	Inter-assay CV ($N = 7$)		Intra-assay CV ($N = 10$)	Inter-assay CV ($N = 7$)	
Es (internal standard)	17.36	0.07	0.42	545	0.88	11.64	1.16
Da	18.79	0.08	0.51	545	3.53	12.89	12.07
Eq	31.96	0.10	0.60	580	2.91	13.83	2.75
Ge	34.87	0.10	0.61	490	4.50	12.65	2.51
Enl	38.23	0.11	0.57	620	5.00	14.26	

[a] Measured serum equivalent concentrations in the low pooled control were: 598 nM (Da), 69 nM (Eq), 244 nM (Ge) and 91 nM (Enl). Results for the medium and high pooled controls were similar.

[b] Resolution values were calculated from the formula: $R = (0.25)(\alpha - 1) \times \mathrm{SQRT}(N) \times k/(k + 1)$ using replicates of the standard mixture.

racy of unknown samples was calculated for each compound of interest using the following formula:

$$\frac{\text{Dominant response/leading (or following) response from the unknown}}{\text{Dominant response/leading (or following) response from the standard}}$$

If the ratios are equivalent, then the ratio accuracy value = 1. Most values were in the range of 0.8–0.95 for analytes. Accuracy ratios below this range, particularly if the RT error was great, usually indicated that the peak had been misassigned by the software (often because the sample contained very low concentrations of analyte, and a larger unknown peak was sometimes misassigned), and the analyte peaks were then manually assigned based on comparisons with standard compound RT and peak ratios.

3.2. Quantification parameters

The quantification parameters are presented in Table 2. Signal linearities as assessed by least squares regression of seven levels ranging from zero to 600 ng/mL for Da and Ge (200 ng/mL for Eq and Enl) were excellent for all of the compounds of interest with average *r* values over all runs of 0.9962–0.9995. The maximum extent of linearity was not determined because the studied range was sufficient for DBS and serum samples measured in ongoing epidemiological studies. With a 50 μL injection volume, LOD on column ranged from 4.5 pg (0.09 ng/mL, 0.37 nM) for Eq to 19 pg (0.38 ng/mL, 1.41 nM) for Ge. LOQ on column ranged from 17.5 pg (0.35 ng/mL, 1.44 nM) for Eq to 33.5 pg (0.67 ng/mL, 2.48 nM) for Ge.

3.3. Sample pretreatment

The final sample pretreatment method is presented in Fig. 2. Serum isoflavone assays typically use 200 μL to 1 mL samples [24,26], and thus we chose to use relatively large DBS samples (two 9 mm diameter punches equivalent to 50.22 μL whole blood) compared to other DBS methods.

Following previous DBS methods [19], DBS were eluted overnight (16 h) in 0.5% BSA–Tris–buffer (pH 7.75), which

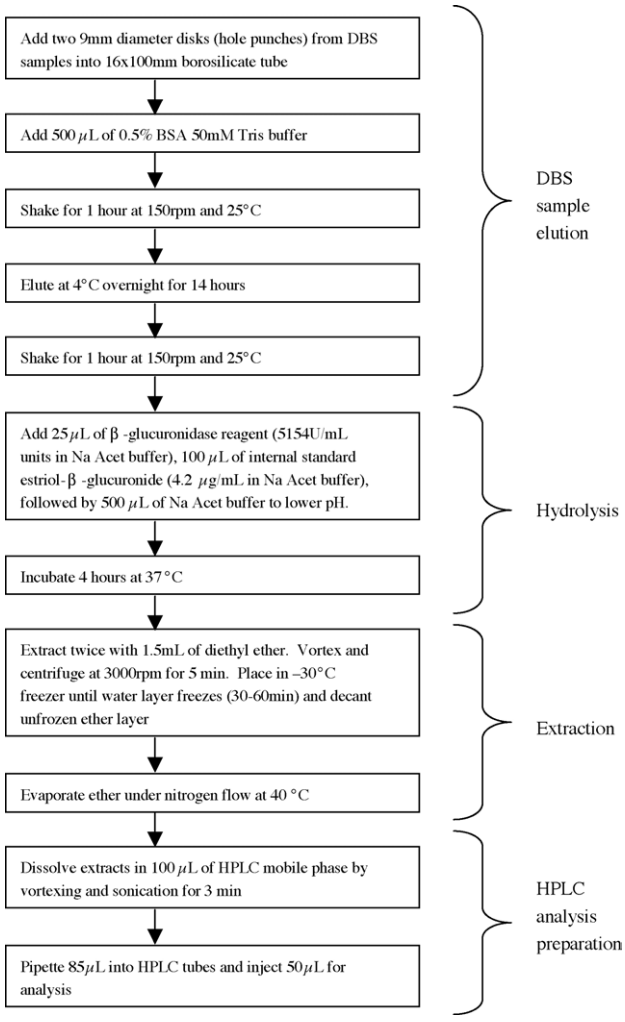

Fig. 2. Flow diagram of sample pretreatment.

Table 2
Quantification parameters for daidzein, equol, genistein and enterolactone

Analyte	Limit of detection (LOD)[a]					Limit of quantification (LOQ)[b]						Linearity (*r*)
	Measured on column				DBS serum equivalent[c] (nM)	Measured on column				DBS serum equivalent[c] (nM)	Maximum standard (μM)	
	(pg)	(fmol)	(ng/mL)	(nM)		(pg)	(fmol)	(ng/mL)	(nM)			
Da	9.0	35.4	0.18	0.71	28.4	24.0	94.4	0.48	1.89	31.2	11.51	0.9995
Eq[d]	4.5	18.6	0.09	0.37	−8.1	17.5	72.2	0.35	1.44	−6.4	2.62	0.9964
Ge	19.0	70.3	0.38	1.41	5.8	33.5	124.0	0.67	2.48	8.2	11.34	0.9962
Enl	14.0	46.9	0.28	0.94	6.2	23.5	78.8	0.47	1.58	7.4	2.52	0.9986

[a] Defined as the concentration at which the signal/noise ratio (peak height) equals 3:1 on the dominant channel.
[b] Defined as the concentration at which the signal/noise ratio (peak height) equals 10:1 on the dominant channel.
[c] DBS serum equivalent concentrations (see Table 3 for regression equations) are reported because dried blood spots are made from whole blood containing red blood cells, and thus concentrations of analytes are approximately half those in serum (which does not contain red blood cells and therefore has higher concentrations of analytes than whole blood). For ease of comparison with other studies using serum samples, concentrations from dried blood spots are usually reported as serum equivalents.
[d] DBS serum equivalent concentrations have negative values due to negative intercepts of regression equation (see Table 3).

Table 3
Serum-DBS regression equations for daidzein, equol, genistein and enterolactone

Analyte	Regression equation[a]	r^2	Concentration (nM)				N[b]
			Serum minimum	Serum maximum	DBS minimum	DBS maximum	
Da	$Y = 2.307X + 26.797$	0.99	8.510	1035.930	0.980	459.680	29
Eq	$Y = 1.581X - 8.709$	0.88	0.250	185.660	4.740	94.570	16
Ge	$Y = 2.277X + 2.559$	0.98	23.100	2166.460	15.980	994.040	30
Enl[c]	$Y = 1.825X + 4.536$	0.66	0.180	143.350	0.000	57.010	30

[a] Linear regression using stepwise (0.05 for entry, 0.1 for removal) in SPSS ver. 12. Dependent variable Y was serum concentration (nM); independent variables were X = DBS whole blood concentration (nM) and hematocrit. Equations are reported using DBS whole blood concentrations. The method was developed for use with two 9 mm diameter punches equivalent to 50.22 uL whole blood, but since samples sometimes constrain the amount used, regression equations are reported for whole blood equivalents (after adjustment for amount of sample used). Thus, if the above BS concentration (for minimum and maximum) are multiplied by 50.22/100 will get actual measured concentrations. Hematocrit did not reach significance in any models.
[b] Sample size was 30 for matched serum and blood spots. Half of samples had undetectable levels of equol (non equol-producers).
[c] Enterolactone DBS concentrations all had blank subtracted first.

has similar pH and protein content to blood. Elution of DBS and hydrolysis are the longest unattended phases of the sample pretreatment and constrain the timing of the assay. Overnight elution followed by 4-h hydrolysis yielded the highest recovery. Although most methods hydrolyze for 16 h, this is usually done for convenience as several hours are sufficient [24,29].

Extracts were redissolved by vortexing and sonication for 3 min in 0.1 mL of HPLC mobile phase [24], 85 μL was transferred to an HPLC tube, and HPLC analysis utilized an injection volume of 50 μL and a 30 μL flush volume. Because samples consist of two 9 mm punches equivalent to 50.22 μL of whole blood and extracts are redissolved in 100 μL of mobile phase for HPLC measurement, measured concentrations are 0.5022 of whole blood concentrations and thus corrections (i.e., whole blood concentration = measured concentration × 100/50.22) were performed for all samples. Concentrations of dried extracts from identical samples stored at −30 °C decreased over time (several weeks). Thus, samples were measured immediately following drying of ether extracts. During the run, samples were kept at 4 °C in the autosampler.

Serum samples were treated identically to DBS samples with the exception of the following: the treatment began with hydrolysis, 100 μL of sample were used and the additional 500 μL of sodium acetate buffer were not added.

In summary, although improvements in recovery and therefore sensitivity could possibly have been made by testing additional parameters, the goal of this method development was to obtain a procedure that would be usable in large epidemiological surveys, permitting self-sampling of blood involving minimal blood volume and single-run analysis of several phytoestrogens of interest, thus requiring a relatively uncomplicated sample pretreatment and chromatographic method.

3.4. Serum equivalence

Matched serum and DBS were obtained from 30 women and assayed under identical conditions. Correlations between blood spot and plasma values are linear and high for Da, Eq and Ge with r-squared values ranging from 0.88 for Eq to 0.99 for Da, but only 0.66 for Enl (Table 3). Serum concentrations were approximately 1.6–2.3 times greater than DBS whole blood concentrations (or, DBS are approximately 43–62% of serum concentrations) depending on compound (see Table 3). Hematocrit (Ht) did not reach significance as an independent variable in any model. Thus, results can easily be converted to serum equivalent concentrations by using the equations given in Table 3.

3.5. Method precision

Precision for the three levels of pooled control samples is presented in Table 4. Control samples were included with every batch of 36 samples. Intra-assay CVs ($N = 10$) were less than 8% for Da, Eq and Ge at all three levels of pooled controls and under 14% for Enl when measured values (not corrected for blank concentration) were used. Inter-assay CVs ($N = 8$) ranged from a low of 2.4% for Da to a high of 20.2% for Eq in the low control and 38.7% for Enl in the medium control.

Table 4
Precision of the method based on three pooled control samples

Analyte	Low control		Medium control		High control	
	Serum equivalent (nM)	Intra[a]-/inter-assay[b] (CV)	Serum equivalent (nM)	Intra[a]-/inter-assay[b] (CV)	Serum equivalent (nM)	Intra[a]-/inter-assay[b] (CV)
Da	589.2	3.3/7.7	1651.7	2.9/2.4	3543.3	5.0/9.2
Eq	69.4	3.3/20.2	562.16	3.1/13.2	1189	7.2/19.2
Ge	244.0	7.7/17.7	588.19	6.0/13.8	1163.6	5.5/14.9
Enl[c]	91.2	13.6/11.4	129.2	7.7/38.7	97.4	7.3/17.9

[a] $N = 10$.
[b] $N = 8$ for low and medium controls; $N = 9$ for high control.
[c] Using measured values, not corrected for blank ($N = 7$ for medium and high controls).

Table 5
Analytic recovery[a]

Analyte	Recovery (%)		Recovery (%)	
	Intra-assay[b] ($N = 3$)		Inter-assay[c] ($N = 3$)	
	Average	CV	Average	CV
Es	91.0	1.3	92.7	1.9
Da	91.6	2.0	95.1	6.3
Eq	94.1	17.3	96.3	14.5
Ge	91.3	7.9	87.2	7.0
Enl (corrected for blank)	93.1	18.6	97.8	4.6

[a] Method accuracy was assessed by standard addition of three levels of standard addition (7.5, 15, 30 ng Da and Ge; 2.5, 5, 10 ng Eq and Enl; 125 ng of Es for all levels per tube) added to samples (pooled controls or samples pipetted from tubes). Uncorrected for internal standard since recovery of internal standard is also reported.
[b] Intra-assay is average of triplicates at three levels.
[c] Inter-assay values are averages of recoveries at three levels for three separate runs.

3.6. Analytic recovery

The results for the analytic recovery determination are presented in Table 5. Mean intra-assay recoveries (uncorrected for internal standard recovery) ranged from 91% for Es and 91.3% for Ge to 94.1% for Eq. Mean inter-assay recoveries ranged from 87.2% for Ge to 97.8% for Enl. Intra-assay CVs were under 10% for all analytes except Eq and Enl, which were under 20%, and inter-assay CVs were under 10% for all analytes except for Eq (15%). When recoveries were corrected with the internal standard Es (which had an inter-assay percentage recovery of 93%), inter-assay recoveries for analytes ranged from 94.2% for Ge to 105.5% for Enl (data not shown).

3.7. Temperature stability

The temperature stability of analytes in DBS was determined (Table 6 and Fig. 3) by exposing an identical pooled sample to one of three temperature conditions: 4 °C, room temperature (approximately 25 °C), and 37 °C for time periods ranging from 0 to 7 days, and 2 to 8 weeks. Da, Eq and Ge remain within a 10% CV range for 8 weeks at 4 and 25 °C, and at 37 °C for 5, 8 and 5 weeks, respectively. Enl concentrations are more variable, remaining within a 20% CV range for 7 weeks at 4 and 25 °C, and within a 30% CV range at 37 °C for 7 weeks. Significant reductions (by the non-parametric Mann–Whitney test) between the mean concentrations during the first week and weeks 2–8 were found for Eq and Ge at 25 °C and for all compounds at 37 °C. Regression equations are shown in Table 6.

3.8. Measurement of samples from perimenopausal Japanese women consuming habitual diet

The DBS samples measured here were self-collected in the morning following each individual's highest dinner intake of daidzein, as estimated from dietary recalls. Although Da intake, as estimated from dietary recalls, was not significantly dif-

Table 6
Stability of analytes in DBS stored at 4, 25 and 37 °C over 8 weeks (0–7 days, 2–8 weeks)

Analyte	Average concentration of 0 day samples ($N = 4$)		Length of time within 10% CV range of average value			Significant differences between mean concentrations for 0–7 days vs. 2–8 weeks[c]			Regression equations[d]				
	(ng/mL)	(nM)	4 °C	25 °C	37 °C	4 °C	25 °C	37 °C	25 °C	R^2	37 °C		R^2
Da	379.6	1493.0	8 weeks	8 weeks	5 weeks	NS	NS	***	$Y = -1.85X + 1447.231$	0.275	$Y = -5.883X + 1473.883$		0.622
Eq	83.0	342.6	8 weeks	8 weeks	8 weeks	NS	*	**	$Y = 1.055X + 329.716$	0.522	$Y = -0.922X + 372.878$		0.291
Ge	228.4	845.4	8 weeks	8 weeks	5 weeks	NS	*	**	NS		$Y = -3.687X + 894.929$		0.568
Enl	26.7	89.5	7 weeks[a]	7 weeks[a]	7 weeks[b]	NS	NS	*	NS		NS		

[a] Within 20% CV for En.
[b] Within 30% CV for En.
[c] Significance by non-parametric Mann–Whitney test (results for ANOVA were similar); NS = non-significant.
[d] Linear regression using stepwise (0.05 for entry, 0.1 for removal) in SPSS ver.12. Y = concentration (nM); X = days at each temperature condition.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

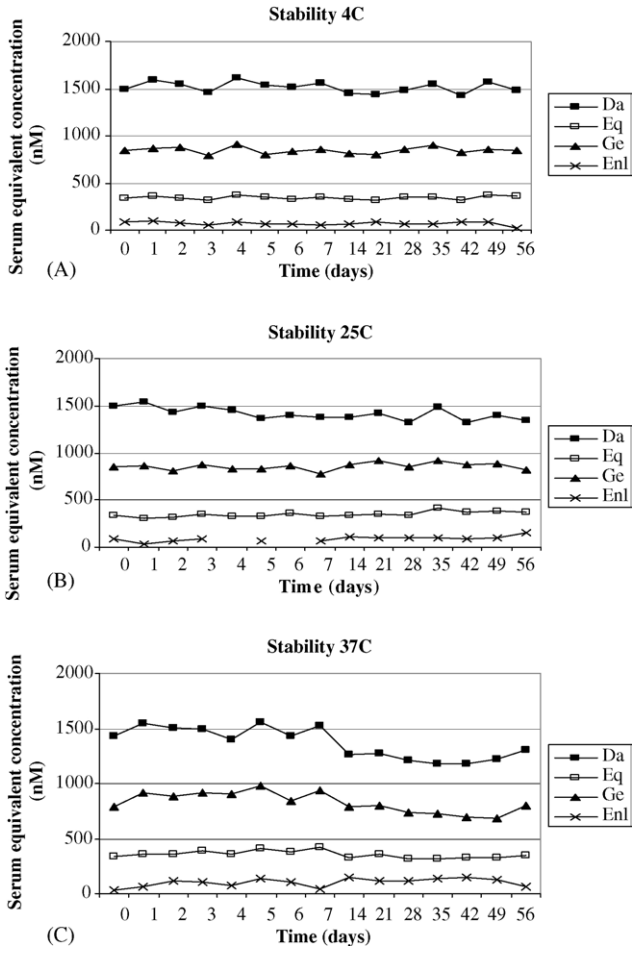

Fig. 3. Stability of Da, Eq, Ge and Enl measured concentrations (nM) in dried blood spots over 8 weeks at (A) 4 °C; (B) room temperature (approximately 25 °C) (days 4 and 6 at stability 25 °C had unusually high concentrations of Enl suggesting possible contamination or interference and are not shown); and (C) 37 °C.

ferent between Kyoto and Fukushima, Da, Eq, En and the Eq/Da ratio were significantly higher in Fukushima than Kyoto (Table 7). All values were within the analytic range of the assay.

4. Discussion

We have developed a new HPLC-CEAD method that permits quantification of the isoflavones Da and Ge, the metabolite Eq and the lignan Enl in dried blood spots. DBS sampling is minimally invasive and has many advantages over serum and urine collection for large-scale studies in naturalistic settings. HPLC, when coupled with a multielectrode coulometric array detector [24], can achieve separation and sensitive detection of phytoestrogens even in small-volume complex mixtures such as DBS without elaborate sample pretreatment.

Sample pretreatment methods were modified from several sources [19,26] and streamlined to create an efficient method for analyzing large numbers of samples. DBS samples, while exhibiting advantages over serum or urine for collection, transport and storage, require an extra sample pretreatment step involving elution into a liquid matrix in order to perform hydrolysis and extraction of compounds of interest. Advantages of DBS include: less inconvenience to study subjects, who can avoid visits to a hospital or clinic for blood collection; elimination of the expense of a technician, nurse or doctor to perform blood collection; temperature stability, which permits collection protocols under conditions when immediate refrigeration/freezing is not possible; decreased costs of sample transport and storage space; and increased safety as living organisms such as blood-borne parasites and viruses do not survive drying in the paper matrix. Dried blood spots also can be used to measure a variety of other hormones and biological parameters [18–21] and diseases of interest [17] in population studies. DBS are particularly useful for epidemiological research and detailed physiological studies such as pharmacokinetics where self-sampling at home under different experimental (e.g., dietary) conditions is desired, and as an alternative to plasma and serum samples currently used in studies of soy infant formula in infants and children [39].

The order of elution is similar to that reported in other reversed phase HPLC methods [24], except that Ge preceded Enl in this method. In many methods, separation of Ge and Enl presents a challenge. Similarly, in the development phase of this method, numerous gradients and temperature settings were tested to obtain sufficient separation of these two compounds. The final gradient chosen struck a compromise between

Table 7
Serum equivalent concentrations (nM) of phytoestrogens by region

		Da (nM)	Eq (nM)	Ge (nM)	Enl (nM)	Eq/D	Previous dinner Da intake (μmol)[b]
Kyoto ($n = 67$)	Mean	416.1	87.4	1035.1	49.25	0.52	132.6
	S.D.	516.7	163.3	1147.3	54.93	1.55	68.6
	Median	212.1	16.2	596.8	38.36	0.08	110.0
Fukushima ($n = 68$)	Mean	565.6	145.1	1058.1	71.89	0.72	127.4
	S.D.	571.4	186.2	1050.3	61.94	1.52	66.0
	Median	370.2	36.0	769.5	67.72	0.15	110.0
Significant difference by region[a]		*	**	NS	**	*	NS

[a] Significance by non-parametric Mann–Whitney test; NS = non-significant.
[b] Estimated from 24-h dietary records preceding DBS sample collection.
* $P < 0.05$.
** $P < 0.01$.

separation of analytes of interest and unknown compounds observed in DBS samples. This method gives good sensitivity and separation, with high precision and good analytic recovery over a wide range of concentrations comparable to other HPLC-CEAD methods developed for plasma and urine [26]. LOD values were similar or better for all compounds compared to most other recently published methods [24,26,30]. With HPLC-CEAD, multiple compounds can be measured in a single sample, and unknown compounds can be identified later as new standards become available, without using additional sample. For example, identification of additional metabolic pathways and intermediates such as dihydrodaidzein (an intermediate in the production of equol from daidzein), currently a topic of much interest [40], should be possible as standards become more widely available.

This study was complicated by the fact that the hydrolysis reagent β-glucuronidase, obtained from *H. pomatia*, contained measurable amounts of plant-derived compounds, particularly Enl. Hence, values in reagent blanks had to be subtracted from measured sample values. Since this method was primarily designed to measure isoflavones and the metabolite equol, the original objective was to separate Enl and Ge sufficiently to prevent Enl from the hydrolysis reagent from interfering with Ge concentration estimates. Parameters are reported here for Enl, as recent research has identified it as having potential protective health effects [41], as an important marker of intake of whole grain and fiber-containing plant foods [42], and as a major metabolite of many biologically-active phytochemicals such as sesamin, a major sesame seed lignan [43]. Analytic recovery, precision, thermostability and serum-DBS regression coefficients were worse for Enl in this study than for the isoflavones and equol, probably due to the relatively high amounts of Enl in the hydrolysis reagent compared to endogenous sample concentrations. The medium pool also appeared to have a compound that interfered with accurate assignment of Enl and led to a large inter-assay CV, although this interference was rarely observed in samples. The results of this method validation study suggest that Enl can be measured in DBS, but to improve the quantitative parameters for Enl, the hydrolysis reagent should be purified or pure enzyme (e.g., glucuronidase and sulfatase) should be used in future studies.

Pooled controls and actual samples differed in several ways because pooled controls were made so that equol would be present in detectable levels in all three controls. However, this resulted in relatively high levels of daidzein (since equol-producing subjects' sera often had high levels of daidzein). The high pool was obtained primarily from sera of women ingesting an isoflavone supplement (prepared by Fuji Oil Company Research Institute, Japan) as part of a supplement safety study, and thus levels were much higher than observed in samples from women consuming habitual diets. However, the use of these controls suggests that this method has a sensitivity range wide enough to be used with studies of supplements. Furthermore, the isoflavone supplements contained a Da:Ge ratio of approximately 3.75:1 [44] compared to typical food ratios of approximately 1:1.5 (1:2–1:2.7) [45]. Thus, Da concentrations tended to be higher than Ge in the pooled controls, in contrast to habitual dietary samples that show the reverse pattern.

Analyses of samples from women in two regions of Japan identified population differences in circulating Da, Eq and Enl that corresponded with previous survey reports of dietary intakes for those regions [36]. Although levels of these compounds were higher in Fukushima than in Kyoto, no difference was found for estimated dinner intakes of Da based on dietary records. This apparent discrepancy may be understood in terms of the pharmacokinetics of soy isoflavone metabolism [46], where breakfast intake may have contributed significantly to morning DBS sample concentrations, thus decreasing the correlation between dinner intake and DBS concentrations. Direct measures of circulating phytoestrogens may reflect population differences in actual exposure to these compounds more closely than do dietary records.

In summary, we have developed a sensitive HPLC-CEAD method for measurement of phytoestrogens in a new matrix, dried blood spots. We have also documented significant regional differences in blood concentrations of Da, Eq and Enl in DBS samples obtained from Japanese women consuming their habitual diets. Compared to plasma, serum or urine, dried blood spots have numerous advantages of easy sampling and safe and efficient storage, and when coupled with HPLC-CEAD, this method will greatly enhance the ability of researchers to conduct both large-scale epidemiological studies as well as more in-depth but less invasive physiological studies.

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