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Short communication

An investigation on the extraction and concentration of isoflavones in soy-based products ☆

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1. Introduction

Isoflavonoid genistein and daidzein have many biological effects, including estrogenic [1,2] and fungitoxic [3] activities. In vitro, they are anti-angiogenic [4] and inhibit the growth of human breast cancer [5] and prostate cancer [6] cell lines. They are less toxic to normal cells than to malignant cells and are being investigated as cancer chemopreventive agents. Genistein and daidzein are found in the subfamily papilionoideae of the Leguminosae [7]. They are relatively abundant in soy bean, primarily as their glycosides, genistin and daidzin (Fig. 1). Soy food products are popular among Asian populations and vegetarians. Urinary excretion of genistein among soy-rich Japanese diet individuals is >30 times those of traditional Western diet [8,9]. In addition, strong correlations between soy intake and reduction in breast cancer risk among Asian women and prostate cancer risk in Asian men have been documented [10,11]. The interest in the relationship between soy intake and the reduction in cancer and cardiovascular diseases [11,12] has prompted a need for the reliable quantitation of the isoflavones in soy-based food products.

Since Naim et al. [13] presented the first quantitative data on isoflavones in soy bean, several investigators [14–18] have determined the concentrations of genistein, daidzein and their glycosides in various soy products. While Eldridge [15] reported the isoflavone concentrations in various commercial soy flours, Coward et al. [17] determined their concentrations in many Asian and Western soy-based foods. However, their results were not fully validated with recovery experiments. Only Pettersson and Kiessling [14] and Coward et al. [17] reported recovery experiments. The maximum concentration of genistin in their recovery experiments was $1\ mg\ g^{-1}$ in flour and was below that found in many flour samples [15]. Therefore, it was not clear if the different daidzin concentrations in Nutrisoy flour reported by Eldridge ($610\ \mu g\ g^{-1}$) and Coward et al. ($1112\ \mu g\ g^{-1}$) were a result of extraction

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efficiency. In an attempt to determine the isoflavone concentrations in various soy products reliably, in this work existing extraction and HPLC assay methods were evaluated. From this investigation, an alternative extraction procedure and an isocratic HPLC assay were developed and validated. The method is simple and efficiently extracts isoflavones from all soy-based products. This paper reports the validated method and the concentrations of genistin, genistein, daidzin and daidzein found in many soy products.

2. Experimental

2.1. Reagents and materials

Methanol and hexane were obtained from Mallinckrodt Chemical (Paris, KY, USA), ammonium acetate from Sigma Chemical (St. Louis, MO, USA) and acetophenone from Aldrich Chemical (Milwaukee, WI, USA). The chemicals were of reagent grade and were used without further purification. Water was deionized with a Milli-Q system (Millipore, Bedford, MA, USA).

Genistin, daidzin and daidzein were purchased from Indofine Chemical (Somerville, NJ, USA). Genistein was received from the US National Cancer Institute. They were characterized by NMR and UV spectrometry and HPLC and were used as reference standards without further purification. Soy flour samples were obtained from Archer Daniels Midland (Decatur, IL, USA), Arrowhead Mill (Herford, TX, USA), Cargill Protein Products (Cedar Rapids, IO, USA), Central Soya (Fort Wayne, IN, USA) and Molly Stone Farm (Redwood City, CA, USA). Liquid soy molasses was supplied by Archer Daniels Midland. Soy milk baby formula concentrates were obtained from Mead Johnson (Evansville, IN, USA), Abbott Labs. (Columbus, OH, USA) and Nutricia (Riverside, CA, USA), soy drink and tofu from Golden Gate Tofu (San Francisco, CA, USA) and tempeh from Turtle Island Foods (Hood River, OR, USA). Several liquid soy products were lyophilized and their dry weight determined.

2.2. High-performance liquid chromatography

HPLC was performed with an IBM LC/9505 autosampler (CT, USA), an ISCO Model 2360 gradient pump, an ISCO V^4 absorbance detector (Lincoln, NE, USA) and an HP 1040 photodiode-array detection system (Hewlett-Packard, Wilmington, DE, USA). UV detection at 254 nm was applied. LC–MS was carried out with a Vestec 201XL system equipped with an LC thermospray interface (Houston, TX, USA). Data were collected and processed with a Waters Maxima 820 data station (Milford, MA, USA). Isoflavone reference standard and internal standard (acetophenone) solutions were prepared in methanol.

Separation was achieved with a Phenomenex Hypersil ODS(2), 5 μm stainless steel 316 column (250 × 4.6 mm i.d.) (Torrance, CA, USA) using an isocratic mobile phase of MeOH–buffer (0.1 M NH_4OAc, pH unadjusted), pre-mixed 35:65 (v/v), at 1.0 ml min^{-1}. Peaks were identified by peak enhancement, photodiode-array analysis and/or LC–MS analysis. Assay was accomplished with an internal standard solution (60 nl ml^{-1} acetophenone in MeOH). The buffer was prepared with deionized water. The soy extract or the reference standard solution was mixed with identical aliquots of the internal standard solution and the buffer in a 1:1:1 (v/v/v) ratio before injection.

2.3. Extraction procedures

Scheme A

For liquid soy drink and milks, 10.0 ml were mixed with 40.0 ml of MeOH in a 250 ml round-bottomed flask. For other soy products, 1.0 g was mixed with 50.0 ml of MeOH–H_2O (4:1, v/v) in individual 250 ml round-bottomed flasks. Each mixture was heated at reflux (70 °C) for 4 h. After cooling, the mixture was filtered through paper. The flask and the insoluble materials were rinsed with 40 ml of 4:1 MeOH–H_2O (4:1, v/v). The filtrate combined with the rinsings was evaporated to near dryness. The residue was taken up in 25.0 ml of water with the aid of sonication. The resulting suspension was partitioned three times with 50 ml of hexane. The remaining aqueous layer (soy extract) was analyzed for isoflavone contents by HPLC.

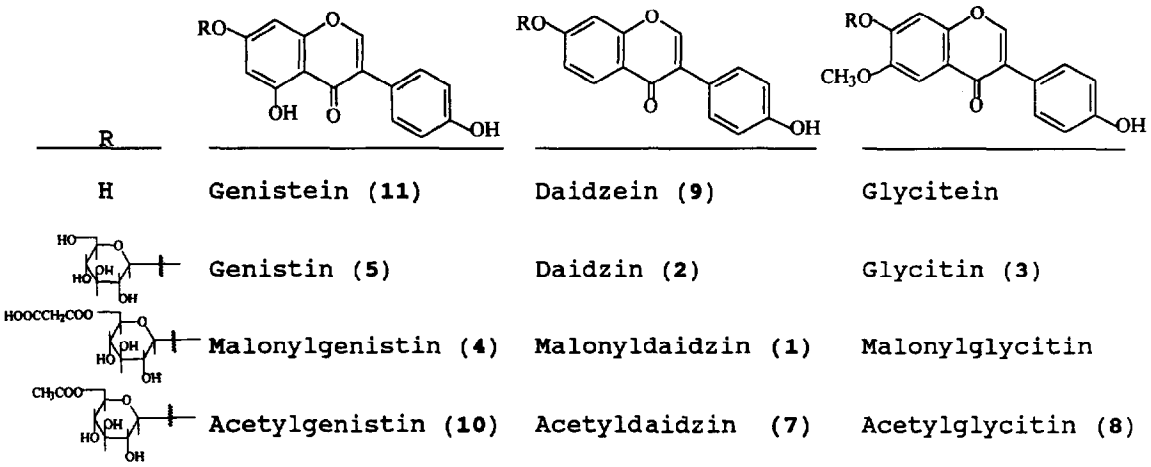


Fig. 1. Structures of major isoflavones in soy (numbers in parentheses correspond to HPLC peaks).

Scheme B

The soy samples were treated as in Scheme A up to the evaporation of the filtrate to near dryness. The residue was taken up in 25.0 ml of MeOH with the aid of sonication. The MeOH supernatent was used for HPLC assay.

2.4. Recovery experiment

Three samples of the soy product, each spiked with known quantities of reference standards, were extracted alongside three unaltered samples. The isoflavones in these six samples were determined by HPLC. The ratio of the found to the expected isoflavone contents in the spiked samples was the recovery efficiency of the extraction.

3. Results and discussion

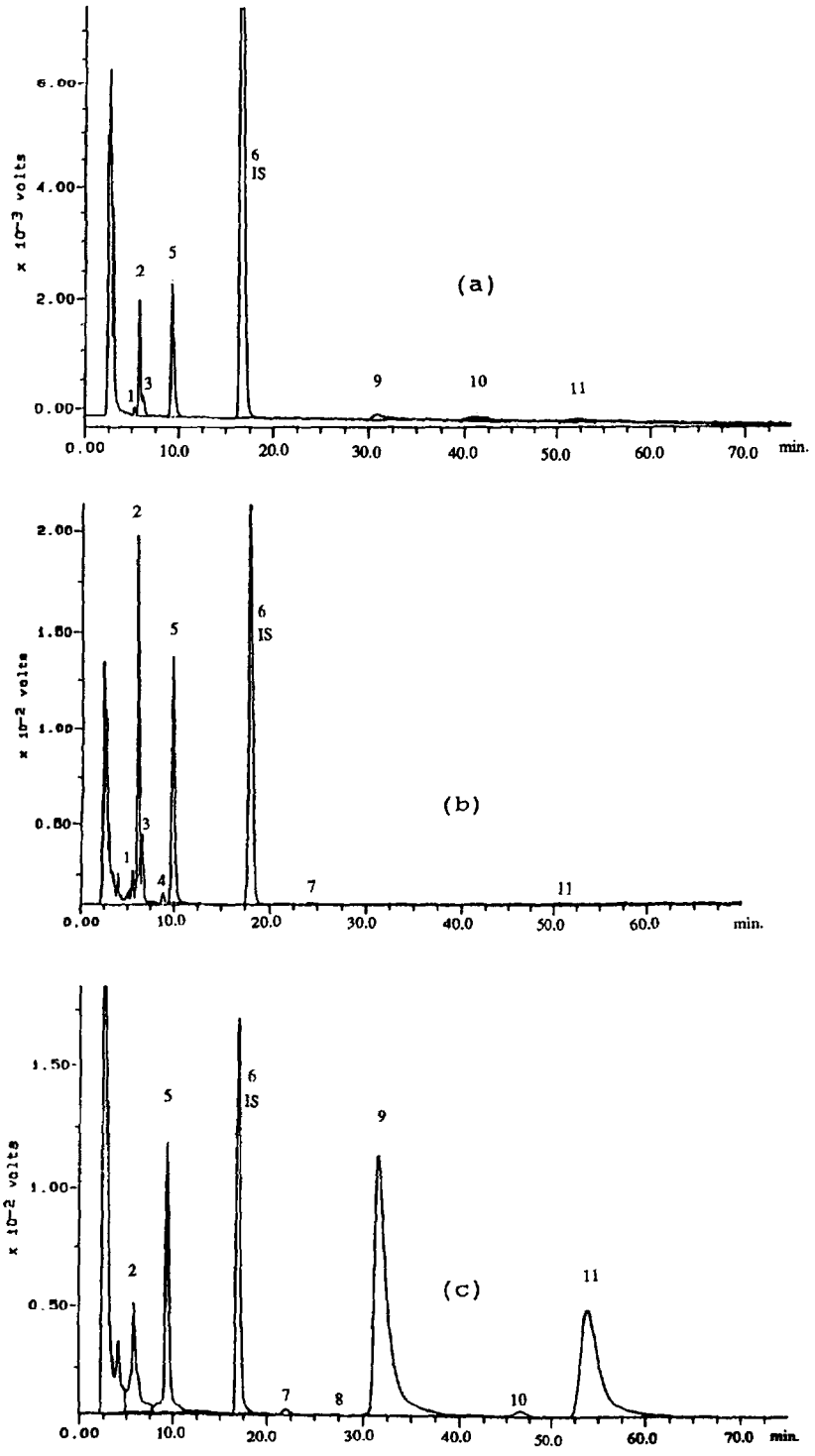
3.1. HPLC

Simultaneous separation of genistin (G), genistein (g), daidzin (D) and daidzein (d) by HPLC has been achieved with gradient elution [15,17,18], but the separation of these isoflavones by isocratic HPLC has not been reported. Aiming for simplicity and better reproducibility, an isocratic HPLC method was developed to determine G, g, D and d simultaneously. The method was modified from the isocratic separation of g and d reported by Setchell et al. [16]. In addition, an internal standard (acetophenone) was incorporated into the HPLC method for quantitation.

Fig. 2 presents the profiles of extracts of soy milk, flour and molasses obtained with the developed isocratic HPLC assay. G, g, D and d were well resolved from one another, although D was interfered with by some minor components, malonyldaidzin and malonylglycitin. The reported gradient elutions [17,18] did not fare better.

Fig. 3 compares the isocratic HPLC with the gradient HPLC of Barnes et al. [18]. In the TFA gradient (Fig. 3(b)), malonyldaidzin co-eluted with G while malonylgenistin fused with D in the NH_4OAc gradient elution (Fig. 3(c)). In addition, acetyldaidzin was too close to G for reliable peak integration. The malonylglycosides were very sensitive to the sample solution matrix during gradient elution, presumably owing to ionization of the

Fig. 2. (Overleaf.) Isocratic HPLC profiles of extracts of soy (a) milk, (b) flour and (c) molasses. Peak identification: 1, 6-*O*-malonyldaidzin; 2, daidzin; 3, glycitin; 4, 6-*O*-malonylgenistin; 5, genistin; 6, acetophenone (IS); 7, 6-*O*-acetyldaidzin; 8, 6-*O*-acetylglycitin; 9, daidzein; 10, 6-*O*-acetylgenistin; 11, genistein. Peaks 2, 5, 6, 9 and 11 were identified by spiking with reference chemicals, LC–UV and LC–MS. Other peaks were identified by LC-UV, LC–MS and relative LC mobility as compared with Ref. [18]. Extracts for milk and flour were with Scheme A and for molasses with Scheme B. See Experimental for extraction schemes and HPLC details.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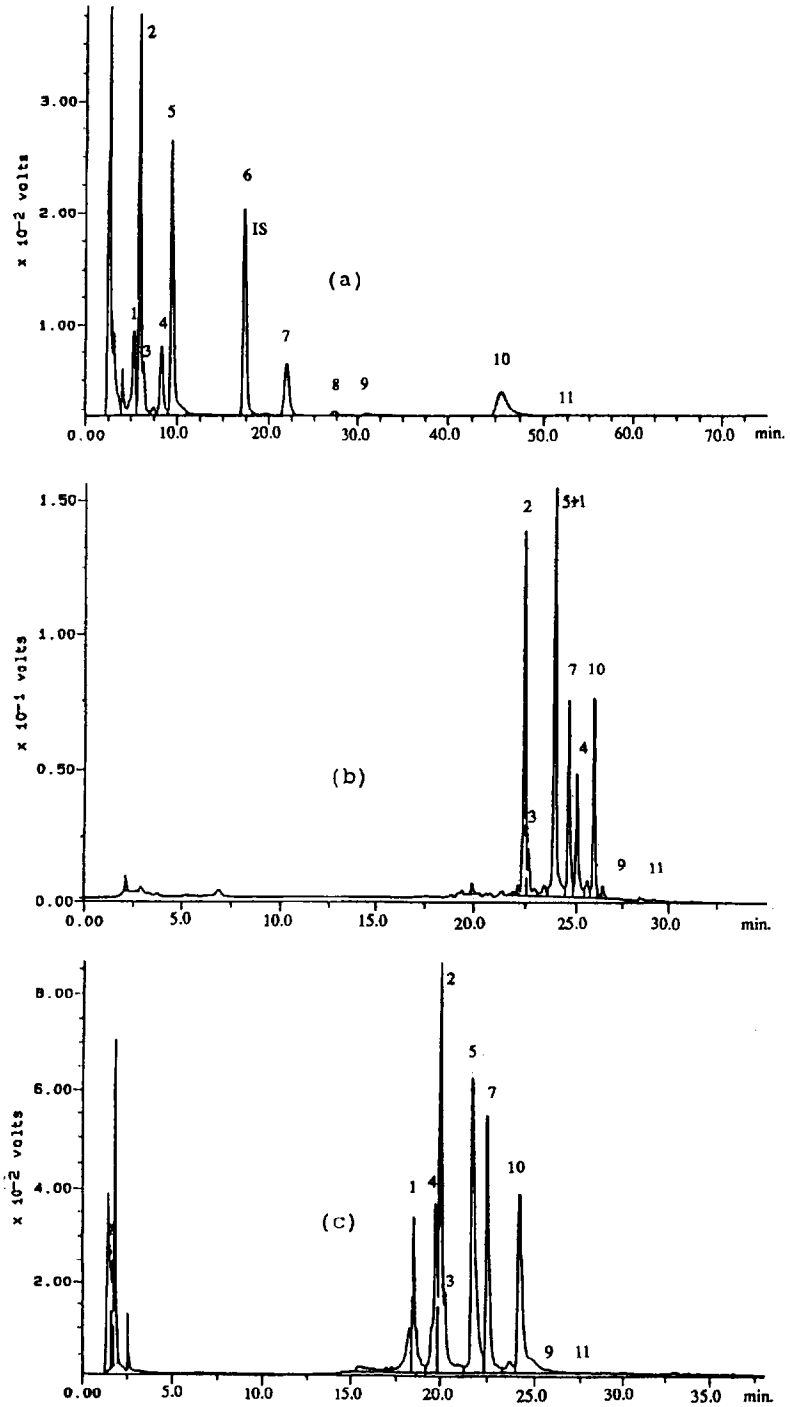

Fig. 3. HPLC profiles of a soy flour extract obtained with (a) the present isocratic elution, (b) the TFA gradient elution of Barnes et al. [18] and (c) the NH_4OAc gradient elution of Barnes et al. [18]. Extract was obtained by stirring 1 g of flour in 10 ml of MeOH–H_2O (4:1, v/v) at room temperature for 1 h. Peak identification as in Fig. 2. See Experimental and Ref. [18] for HPLC conditions.

Table 1
Validation data on the simultaneous isocratic HPLC assay of genistin, genistein, daidzin and daidzein[a]

Parameter	n[b]	Genistin	Genistein	Daidzin	Daidzein
Reproducibility (RSD) (%)	6	0.8	1.8	0.8	0.8
Linearity (r^2)	8	0.9992[c]	0.9990[d]	0.9990[e]	0.9995[f]
Accuracy (%)	8	0.8	1.8	1.9	1.9
Concentration range ($\mu g\ ml^{-1}$)[g]		4–200	0.6–240	2.5–400	1.5–263

[a] See Experimental for HPLC conditions.
[b] n is the number of data points; each data point represents single HPLC injection of individual solutions.
[c] $y = 0.007056x + 0.0087$, where x is the concentration in $\mu g\ ml^{-1}$ and y is the area ratio of analyte to internal standard peak.
[d] $y = 0.01205x - 0.0082$.
[e] $y = 0.009896x - 0.0243$.
[f] $y = 0.01744x - 0.0146$.
[g] $\mu g\ ml^{-1}$ reference standard solution.

carboxylic acid in the malonyl moiety. While the retention of other isoflavones remained the same, that of the malonylglycosides decreased when the alcoholic soy extract was diluted with the acetate buffer. The change in retention sometimes caused the malonylglycosides to co-elute with the glycosides, as in Fig. 3(b) and (c). For these reasons, it is more desirable to use the isocratic HPLC assay to determine the isoflavones in the soy extracts.

The method was validated and Table 1 summarizes the validation data. The method was reproducible (RSD < 1%), linear ($r^2 > 0.999$) and accurate (error < 2%) over a concentration range of at least from 0.5 to 200 $\mu g\ ml^{-1}$ soy extract.

3.2. Extraction

Extraction of isoflavones from soy products has commonly been achieved with hot MeOH–H_2O (4:1, v/v). Eldridge [15] reported complete extraction by stirring soy samples in aqueous MeOH for 4 h at 60 °C. After removal of the solid residue by filtration, the filtrate was directly assayed for isoflavones by HPLC without further sample treatment. To prolong the column lifetime, other investigators evaporated the filtrate to dryness, re-extracted the dry residue with H_2O [16] or H_2O–MeOH (1:1, v/v) [17], followed by hexane partitioning to defat the soy extract. Since the H_2O solubility of the aglycones is very limited, 36 $\mu g\ ml^{-1}$ for genistein, it is doubtful that the aqueous re-extraction of the dry filtrate residue would be suitable for samples with high aglycone contents.

Extraction Scheme A is essentially the procedure used by Setchell et al. [16], except 2.5 times the volume of H_2O was used in the re-extraction. Indeed, when Scheme A was applied to soy milk and flour, among others, an excellent recovery (>98%) was obtained for G. The recovery for g was good (>95%) only when the amount was less than 100 μg per sampling; beyond that level, the recovery tapered off. Table 2 summarizes the results of the recovery experiments. Scheme A, therefore, is applicable to soy samplings which contain less than 2 mg of glycosides and 100 μg of aglycones. These include soy drink, milk and most flour samples.

Table 3 presents the concentration of isoflavones in soy milk, flours and some Asian food determined using extraction Scheme A. These values are generally higher than those reported [14–18] and are probably due to differences in the extraction procedure and/or efficiencies. In the present extraction, the aqueous MeOH containing

Fig. 4. (Opposite.) Isocratic HPLC profiles of MeOH–H_2O (4:1, v/v) extracts of a soy flour obtained (a) by stirring at room temperature for 1 h, (b) by heating at 60°C for 4 h and (c) by heating at 70°C for 4 h. Peak identification as in Fig. 2. See Experimental for HPLC conditions. The difference in retention time was due to minor changes in the mobile phase composition.

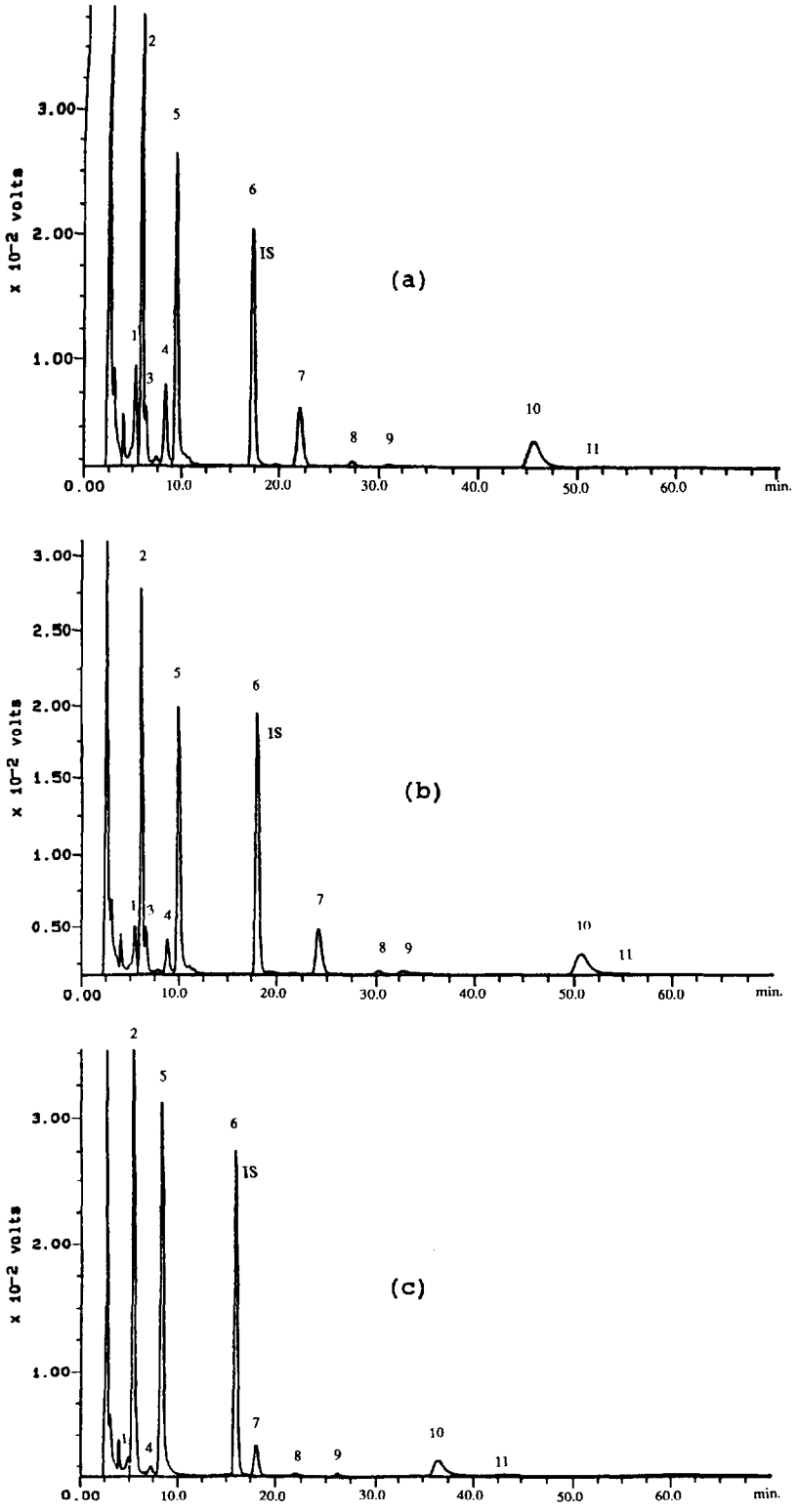
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Table 2
Extraction recoveries of Scheme A and Scheme B

Scheme	Sample	Recovery							
		Genistin		Genistein		Daidzin		Daidzein	
		%[a]	μg[b]	%[a]	μg[b]	%[a]	μg[b]	%[a]	μg[b]
A	Milk	98 ± 5	490	99 ± 13	102				
		79 ± 13	935	60 ± 7	204				
	Flour	108 ± 6	2300	87 ± 14	131	95 ± 1	2400	107 ± 8	263
B	Milk	86 ± 8	490	101 ± 14	102				
	Molasses	98 ± 9	2200	105 ± 4	3700	102 ± 2	2100	106 ± 3	3400

[a] Each recovery % is an average of single HPLC assay of extracts from three spiked samplings.
[b] Amount of isoflavone expected in each sampling of soy products after spiking.

the soy sample was heated at reflux (70 °C). It has been suggested [18–20] that malonyl and acetyl glycosides can be de-esterified to their glycosides upon heating. Indeed, as Fig. 4 shows, progressively smaller amounts of the esters are observed in the soy flour extract obtained with increasing heating during extraction. Aglycones g and d were not detected in the room temperature extracts (Fig. 4(a)) but showed up as 1–2% of its glycosides in the extracts obtained under heat (Fig. 4(b) and (c)). It is safe to assume that g and d are not present in natural soy products. The small amounts of g and d reported in Table 3 and in the literature for non-fermented soy products probably resulted from hydrolysis during extraction.

Soy molasses is a concentrate of hot aqueous EtOH extract of soy flour. When Scheme A was applied to it, the water re-extract of the evaporated filtrate was an oily viscous suspension. Hexane defatting was unable to clarify the cloudy suspension. HPLC assay of the homogenized suspension indicated that the concentrations of genistein, genistin, daidzein and daidzin were each in excess of 1 mg g^{-1} of molasses. The amount of genistein in the sample was at least ten times too much for good recovery with Scheme A. In addition, the cloudy suspension subsequently separated into a cloudy top oily suspension (2–3 ml) and a clear bottom aqueous solution (22–23 ml). The majority (>70%) of the isoflavones were trapped in the oily suspension. Thus, a different extraction scheme was needed for soy molasses or for soy products with aglycone contents higher than 100 μg per sampling.

Considering the solubility of the aglycones, H_2O was replaced with CH_3OH to re-extract the evaporated filtrate. To simplify the procedure further, the hexane partitioning step was omitted since HPLC column deterioration has not been a significant problem. Using soy flour CPP-200/20 and lyophilized molasses, which contains the highest concentration of isoflavones, the effective ratio of extraction solvent volume to sample weight was studied. The results indicated that a minimal reflux solvent (MeOH–H_2O (4:1)) volume of 20 and 40 ml g^{-1}, respectively, was needed for the flour and the molasses. The minimal volume of MeOH to re-extract the filtrate residue was 15 and 20 ml, respectively. These results let to extraction Scheme B. As Table 2 indicates, the recovery with extraction Scheme B was excellent for both the glycosides and the aglycones.

Extraction Scheme B worked well with all soy-based food products except milk. In the case of milk, lyophilized or not, the evaported filtrate was a viscous, gummy substance. This gummy substance at times hindered complete re-extraction of isoflavones by MeOH. Table 4 shows the isoflavone concentration in various soy food products determined with extraction Scheme B. The results for soy milk, drink and flour in Tables 3 and 4 are comparable, indicating that extraction efficiencies of Scheme A and B are equivalent. Except for the aglycone values in ADM Pro-fam

Table 3
Isoflavone concentrations ($\mu g\ g^{-1}$) in soy products obtained with extraction Scheme A

Soy product	Genistin	Genistein	Daidzin	Daidzein	n[j]
Milk					
Isomil[a]:					
as obtained	28 ± 2	< 1	13 ± 1	< 1	3
lyophilate	136 ± 29	< 1	73 ± 8	< 1	3
Soyalac[b], as obtained	52 ± 29	3 ± 1	18 ± 1	< 1	3
Nursoy[b], as obtained	35 ± 2	< 1	13 ± 1	< 1	3
Prosobee[c]:					
as obtained	34 ± 2	< 1	16 ± 2	< 1	3
lyophilate	129 ± 13	< 1	62 ± 7	< 1	3
Flour					
CS[d]:					
Soyafluffy	2290 ± 37	21 ± 2	1634 ± 35	12 ± 1	3
Centex	1599 ± 36	24 ± 1	977 ± 24	11 ± 1	3
Procon	19 ± 4	1 ± 1	13 ± 3	< 1	3
Promine	43 ± 24	2 ± 1	29 ± 7	< 1	3
Response	44 ± 3	< 1	27 ± 5	< 1	3
Promax	1535 ± 8	24 ± 3	704 ± 6	8 ± 1	3
Promax plus	1697 ± 59	30 ± 2	750 ± 26	31 ± 8	3
ADM[e]:					
Nutrisoy	1573 ± 41	14 ± 2	na[k]	na[k]	3
TVP	1694 ± 37	37 ± 12	1341 ± 32	20 ± 5	3
Acron-F	99 ± 2	2 ± 1	na[k]	na[k]	3
Acron-S	816 ± 86	84 ± 1	334 ± 28	27 ± 3	3
Pro-fam	504 ± 28	231 ± 5	159 ± 24	113 ± 14	3
CPP[f]:					
200/20	2112 ± 19	25 ± 2	1616 ± 12	14 ± 1	3
200/70	2034 ± 98	15 ± 1	1504 ± 101	10 ± 1	3
Arrowhead	1551 ± 3	5 ± 1	1330 ± 30	4 ± 1	3
Molly Farm	2210 ± 47	18 ± 1	1600 ± 28	8 ± 1	3
Sun Ridge Farm	1165 ± 64	3 ± 1	611 ± 38	< 1	3
Asian food					
Soy drink:					
as obtained	96 ± 6	11 ± 1	56 ± 3	7 ± 1	3
lyophilate[g]	2333 ± 92	191 ± 9	976 ± 141	134 ± 7	3
Tofu:					
hard, lyophilate[h]	1407 ± 66	178 ± 22	430 ± 26	104 ± 5	6
soft, lyophilate[i]	2043 ± 20	98 ± 23	790 ± 93	56 ± 4	3
Tempeh	239 ± 19	94 ± 8	123 ± 10	71 ± 4	3

[a] Abbot Labs., dried weight $0.27\ g\ ml^{-1}$.
[b] Nutricia.
[c] Mead Johnson, dried weight $0.28\ g\ ml^{-1}$.
[d] Central Soya.
[e] Archer Daniels Midland.
[f] Cargill Protein Products.
[g] Dried weight $0.043\ g\ ml^{-1}$.
[h] Dried weight $0.201\ g\ g^{-1}$.
[i] Dried weight $0.103\ g\ g^{-1}$.
[j] n is the number of data points; each data point represents a single HPLC assay of individual extracts.
[k] Not available.

Table 4
Isoflavone concentrations ($\mu g\ g^{-1}$) in soy products obtained with extraction Scheme B

Soy product	Genistin	Genistein	Daidzin	Daidzein	n^g
Milk					
Isomil[a]:					
as obtained	28 ± 1	<1	16 ± 1	<1	3
lyophilate	165 ± 3	<1	97 ± 7	<1	3
Prosobee[b]:					
as obtained	37 ± 2	<1	20 ± 2	<1	3
lyophilate	132 ± 10	<1	65 ± 8	<1	3
Flour					
ADM[c]:					
Nutrisoy	1354 ± 18	59 ± 7	1610 ± 36	26 ± 1	2
Arcon-S	776 ± 3	75 ± 14	583 ± 20	32 ± 6	3
CPP[d], 200/20	2144 ± 16	25 ± 2	1596 ± 9	14 ± 1	2
Arrowhead	1511 ± 107	32 ± 1	1275 ± 97	30 ± 1	3
Molasses[e]					
as obtained	1171 ± 19	1685 ± 30	558 ± 23	1809 ± 63	3
lyophilate	2051 ± 106	2953 ± 158	1036 ± 55	3194 ± 193	6
Asian food					
Tofu, hard lyophilate[f]	1548 ± 23	116 ± 14	1033 ± 20	82 ± 8	3
Tempeh	303 ± 33	79 ± 6	182 ± 16	69 ± 12	3

[a] Abbot Labs., dried weight $0.27\ g\ ml^{-1}$.
[b] Mead Johnson, dried weight $0.28\ g\ ml^{-1}$.
[c] Archer Daniels Midland.
[d] Cargill Protein Products.
[e] Archer Daniels Midland, dried weight $0.54\ g\ ml^{-1}$.
[f] Dried weight $0.201\ g\ g^{-1}$.
[g] n is the number of data points; each data point represents a single HPLC assay of individual extracts.

flour, the results for the mild, drink and flours in Table 3 are reliable, since the results in Table 4 have been fully validated with recovery experiments. The inferior results for tofu and tempeh in Table 3 suggest that Scheme B should be used for these products.

Table 5 compares the extraction efficiencies of various extraction methods. All extractions were carried out at reflux temperature (70 °C) followed by their respective procedures. Isoflavone concentrations in the final extracts were detemined with proposed isocratic HPLC assay. Since Scheme B is the Eldridge method [15] extended with a MeOH re-extraction, we compared the two only with molasses. The two methods gave similar results, as expected. For soy milk, drink and flour, Scheme B and Setchell et al.'s method [16] or Scheme A are comparable (see also Tables 3 and 4). For Asian food, Scheme B (Table 4) is more efficient than Setchell et al.'s method or Scheme A (Table 3). However, more importantly, the latter methods are not suitable for molasses, as discussed previously. The extraction method of Coward et al. [17] consistently gave lower values than those obtained with the other three methods. It is probable that the lower values reported for Nutrisoy flour by Coward et al. [17] than those reported by Eldridge [15] were the result of poor extraction efficiency.

4. Conclusion

An alternative efficient extraction scheme and an isocratic HPLC assay have been developed and validated for the determination of isoflavones in soy-based products. The method is simple and is applicable to all soy products. Using the developed method, the concentrations of genistin, genistein, daidzin and daidzein in more than 25

Table 5
Extraction efficiency comparison between Scheme B and the methods of Eldridge [15], Setchell et al. [16] and Coward et al. [17]

Soy product[a]	Isoflavone	Isoflavone per g soy product extracted (μg)			
		Scheme B	[15]	[16]	[17]
Milk, Prosobee lyophilate	Genistin	124 ± 21		128 ± 4	88 ± 6
	Genistein	< 1		< 1	< 1
	Daidzin	67 ± 12		61 ± 4	50 ± 8
	Daidzein	< 1		< 1	< 1
Flour, ADM Arcon-S	Genistin	776 ± 3			377 ± 23
	Genistein	75 ± 14			48 ± 3
	Daidzin	583 ± 20			319 ± 15
	Daidzein	43 ± 8			< 1
Flour, ADM Nutrisoy	Genistin	1354 ± 18			814 ± 55
	Genistein	59 ± 7			86 ± 13
	Daidzin	1610 ± 36			1327 ± 80
	Daidzein	< 1			< 1
Tempeh	Genistin	287 ± 37			104 ± 40
	Genistein	76 ± 6			71 ± 21
	Daidzin	182 ± 16			90 ± 30
	Daidzein	79 ± 15			54 ± 13
Molasses, ADM lyophilate	Genistin	2123	2356		1773
	Genistein	2961	2946		2252
	Daidzin	1244	1384		1069
	Daidzein	3203	3059		2594

[a] Milk, flours and tempeh were based on triplicate extractions. Molasses was based on a single extraction.

soy-based food products have been determined. The data presented have been validated with recovery experiments.

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