Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 87 (2004) 297–305
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

A simplified HPLC method for total isoflavones in soy products

José L. Peñalvo [a,*], Tarja Nurmi [a,b], Herman Adlercreutz [a]

[a] *Institute for Preventive Medicine, Nutrition, and Cancer, Folkhälsan Research Center, and Division of Clinical Chemistry, University of Helsinki, P.O. Box 63, Fin-00014 Helsinki, Finland*
[b] *Research Institute of Public Health, University of Kuopio, P.O. Box 1627, Fin-70211 Kuopio, Finland*

Received 2 July 2003; received in revised form 18 December 2003; accepted 18 December 2003

Abstract

Soybeans and soy products are the main source of isoflavones, a well-studied group of phytoestrogens with numerous biological effects. Several methods have been published to quantify soy isoflavones in food samples. We have earlier applied enzyme hydrolysis followed by acid hydrolysis to measure isoflavones as total aglycone equivalents. Alternatively, other published methods have mainly been developed to measure all 12 forms of isoflavones. In order to simplify the analyses, a new extraction method for determination of total amount of isoflavones was developed and validated. Acetyl and malonyl glucosides were hydrolysed to 7-*O*-glucosides with 80% EtOH containing 1 M HCl to remove the acetyl and malonyl groups. Aglycones and 7-*O*-glucosides were analysed with HPLC using coulometric electrode array detection.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Isoflavones; Soy; HPLC; Coulometric detection

1. Introduction

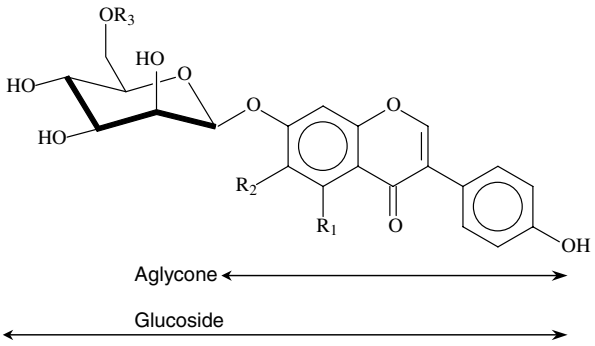
The isoflavone fraction of the soybean belongs to the family of flavonoids, a large group of plant-derived compounds often related to the induction of a physiological response in humans after consumption. More specifically, soy isoflavones are included into the so-called phytoestrogens due to their weak estrogen activity with potential protective effect against some hormone related diseases, even though a number of other biological activities and therapeutic uses have been proposed (Adlercreutz et al., 1995; Barnes, 1998; Kapiotis, Hermann, Held, Seelos, Ehringer, & Gmeiner, 1997; Kurzer, 2000).

The basic structure of isoflavone aglycone consists of a 3-phenylchroman skeleton that is hydroxylated in the 4′- and 7-positions. Depending on the substituents in the carbons 5- and 6, mainly three types of this aglycone structure, named as daidzein, genistein and glycitein, exist in soy (Fig. 1). In soybeans isoflavones occur as 7-*O*-glucosides with trivial names daidzin, genistin and glycitin respectively. In addition, all these glucosides can be esterified at the 6″-*O*-position of the glucose ring with acetyl or malonyl groups forming another six compounds commonly known as acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, and malonylglycitin.

The predominant isoflavone forms in soybeans and non-fermented soy products (soy protein, soymilk derivates, etc.) are glucosides (Naim, Gestetner, & Zilkah, 1974), and fermented soy foods (miso, tempeh) contain mainly aglycones (Coward, Barnes, Setchell, & Barnes, 1993; Wang & Murphy, 1994). After consumption, isoflavone glucosides undergo enzymatic metabolism in the small intestine to release the aglycone (Bowey, Adlercreutz, & Rowland, 2003), which is the more bioavailable isoflavone form (Izumi et al., 2000). It has also been reported that human saliva is capable of converting glucosides into aglycones (Allred, Ju, Allred, Chang, & Helferich, 2001), explaining the fact that aglycones can be absorbed to some extent in the stomach prior to the main absorption from the small intestine (Piskula, Yamakoshi, & Iwai, 1999). The hydrolysis of the glucosides in the small intestine is necessary for their absorption (Setchell

* Corresponding author. Tel.: +358-19-125-455; fax: +358-19-125-452.
E-mail address: jose.penalvo@helsinki.fi (J.L. Peñalvo).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2003.12.028



Name	Abbrev.	R1	R2	R3
Genistein	Ge	OH	H	
Glycitein	Gly	H	OCH_3	
Daidzin	Din	H	H	H
Genistin	Gin	OH	H	H
Glycitin	Glyn	H	OCH_3	H
6"-*O*-Acetyldaidzin	Adin	H	H	$COCH_3$
6"-*O*-Acetylgenistin	Agin	OH	H	$COCH_3$
6"-*O*-Acetylglycitin	Aglyn	H	OCH_3	$COCH_3$
6"-*O*-Malonyldaidzin	Mdin	H	H	$COCH_2COOH$
6"-*O*-Malonylgenistin	Mgin	OH	H	$COCH_2COOH$
6"-*O*-Malonylglycitin	Mglyn	H	OCH_3	$COCH_2COOH$

Fig. 1. Classification and structures of soy isoflavones.

et al., 2002). Only the aglycones are absorbed, and because of this the differentiation between the possible forms of isoflavones in soy products seems to be irrelevant since the physiological effects are due to the aglycones. For this reason an accurate quantification of the total isoflavone content of the soy products is important.

Several methods for soy isoflavone analyses have been published generally based on HPLC techniques with UV (Klump, McDonald, & Ballam, 2001; Wang & Murphy, 1994), DAD (Franke, Custer, Cerna, & Narala, 1995) or MS-detection (Barnes, Kirk, & Coward, 1994; Setchell et al., 2001; Wiseman, Clarke, Barnes, & Bowey, 2002). HPLC with coulometric electrode array detection (CEAD) (Nurmi, Mazur, Heinonen, Kokkonen, & Adlercreutz, 2002) and other chromatographic techniques like GC–MS have also been applied to isoflavone analyses (Liggins, Bluck, Coward, & Bingham, 1998; Mazur, Fotsis, Wähälä, Ojala, Salakka, & Adlercreutz, 1996).

The most often applied sample pre-treatment for soy isoflavones is extraction. Different solvents, various temperatures and incubation times are used. Very common extraction solvent is aqueous alcohol, ethanol or methanol (Barnes et al., 1994; Wiseman et al., 2002), and also slightly acidified acetonitrile has been used (Murphy, 1981). Few studies have compared these extraction methods, but no final conclusions have been made about which method would be superior (Barnes et al., 1994; Griffith & Collison, 2001).

These simple solvent extraction methods for isoflavones extract all different isoflavone forms as such, and detailed data about the conjugated forms and aglycones are obtained. Main disadvantage of these mentioned methods is the poor stability of the ester glucoside standards in solution and that compromises the precision of the analyses. Avoiding this fact, some methods apply enzyme hydrolysis (Liggins et al., 1998), combination of enzyme and acid hydrolysis (Mazur et al., 1996) or saponification with base (Klump et al., 2001), before the isoflavone analyses. Hydrolytic methods are directed to completely release the aglycone from the glucosides or ester glucosides, and for that several steps are required and therefore the analysis is complicated and long.

The aim of our study was to develop a simple method to analyse total isoflavones in soy products by measuring only aglycones and 7-*O*-glucosides. Our new method was planned to be a combination of simultaneous extraction and mild hydrolysis of acetyl and malonyl glucosides to avoid the measuring of all 12 forms of isoflavones or the utilization of both acid and enzymatic hydrolysis which are laborious to carry out.

2. Materials and methods

2.1. Reference standards

Because one of the objectives of this study was to analyse total isoflavones by measuring aglycones and glucosides reference standards of the acetyl and malonyl forms were needed during optimization and validation of the new method to guarantee their absence in the samples. The following 12 standards corresponding to the four different forms of the three main soy isoflavones were used. Daidzin (Din), genistin (Gin) and glycitein (Gly) were purchased from Apin Chemicals LTD (Oxon, UK). Daidzein (Da) and genistein (Ge) were obtained from Carl Roth GmbH & Co. (Karlsruhe, Germany). Acetyldaidzin (ADin), acetylgenistin (AGin), acetylglycitin (AGlyn), glycitin (Glyn), malonyldaidzin (MDin), malonylgenistin (MGin), and malonylglycitin (MGlyn) were purchased from Fujicco Co LTD. Nacalai Tesque Inc. (Kobe, Japan).

Separate standard stock solutions were made for all 12 isoflavone forms. The aglycones Da, Ge and Gly were dissolved in methanol (MeOH) and the conjugates in 80% ethanol (EtOH). Purity of the isoflavone aglycones and conjugates was checked with maximum absorption and known molar extinction coefficients as described in our previous work (Nurmi et al., 2002). Standard stock solutions were stored at 4 °C. Two different quantification standard mixtures were prepared. One containing all 12 forms of isoflavones was needed during the optimization of the sample pre-treatment conditions, and the second one containing only six standards including the aglycones and 7-*O*-glucosides was used for the final method validation.

2.2. Chromatographic conditions

Analyses were carried out by HPLC using coulometric electrode array detection (CEAD) (ESA Inc., Chelmsford, MA). HPLC consisted of two solvent pumps model 580, autosampler model 540 with cooling system and a thermal chamber for column and detector. The CEAD consisted of two detector cells each containing four measuring electrode pairs and a reference electrode made of platinum. A wide range of potentials between ±1000 mV can be applied, but for our method the eight pairs were set between 200 and 700 mV. Hydrodynamic voltamogramms were used to select the optimal oxidation potential for each isoflavone included into the assay. Individual detection potentials for aglycones and their 7-*O*-glucosides are presented in Table 1. Isoflavones were separated using an Inertsil ODS-3 column with dimensions 150 × 3 mm (GL Sciences Inc., Japan). Column was packed with end-caped particles of 3 μm. Analytical column was protected with a guard column Quick Release C_{18} (Upchurch Scientific Inc., WA) 10 × 3 mm packed with 5 μm particles. The columns were set at 37 °C in a thermal chamber together with the detector cells.

Gradient elution was needed for complete separation of the analytes. The mobile phase consisted of two eluents: (A) 50 mM sodium acetate buffer pH 5/MeOH (80:20 v/v) and (B) 50 mM sodium acetate buffer pH 5/MeOH/ACN (40:20:20 v/v/v). MeOH and acetonitrile (ACN) were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland, UK). Two different gradients (Fig. 2) were used depending on the extraction method. During the method development, the original gradient was used (gradient 1) (Nurmi et al., 2002). This was needed to quantify all 12 forms of isoflavones. Total run time was 65 min including 13 min stabilization time. Because the aim of the method development was to modify the method to quantify only six different isoflavones, the use of a shorter gradient was possible. In gradient 2 total run time was 45 min including 10 min stabilization time.

Repeatability of the gradient 2 was evaluated with intra- and interassay retention time stability. Retention times and retention time stabilities are presented in Table 1. In both gradients the flow rate was 0.3 ml/min and the injection volume 10 μl. Limits of detection (LOD) were determined from diluted standards using a signal to noise ratio (S/N) of 3:1. LOD values are described in Table 1 together with calibration ranges and correlation values for calibration linearities. Limits of quantifications (LOQ) were three times the LOD values

Table 1
Quantification parameters and gradient repeatability

Isoflavone	Detection potential (mV)	Retention time (min)	Retention time intra[a]-interassay[b](CV)	LOD[c](ng/ml)	Calibration range (ng/ml)	R^2
Daidzin	590	12.01	0.36/1.33	1.02	45.4–454.3	0.9996
Glycitin	590	13.53	1.04/1.27	0.99	42.1–421.0	0.9996
Genistin	510	19.38	0.51/1.12	0.99	43.3–433.8	0.9998
Daidzein	510	29.58	0.72/0.40	1.01	42.2–422.8	0.9982
Glycitein	590	30.52	0.99/0.37	0.40	42.1–421.0	0.9986
Genistein	510	33.25	0.87/0.26	1.00	42.6–426.2	0.9990

[a] $N = 5$.
[b] $N = 10$.
[c] LOD = Limit of detection $S/N = 3$.

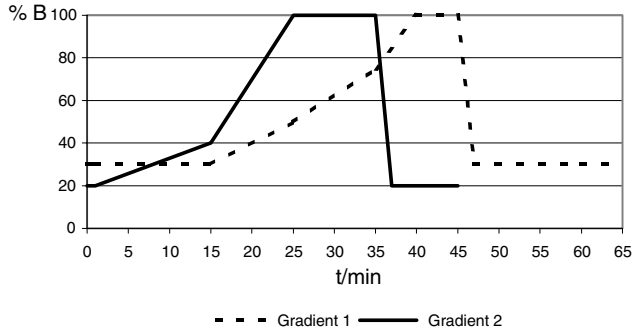


Fig. 2. Gradient profiles in the HPLC analysis of soy isoflavones.

corresponding approximately to 2–8 μg/g in sample depending on the analyte.

2.3. Sample pre-treatment

2.3.1. Samples

To include a broad range of isoflavone profiles and concentrations, three different soy isoflavone extracts named as A, B, and C were used in pre-treatment tests to evaluate the performance of the different extraction procedures in method development. After optimization, sample C was chosen as a control sample to validate the final method.

2.3.2. Comparison of different extraction procedures

Four different extraction procedures were assayed to optimize the sample pre-treatment (Table 2):

2.3.2.1. Test 1. Fifteen milligrams of the sample was weighed and 2.5 ml of 80% EtOH was added. Sample was vigorously shaken for 2 min and centrifuged 2140 × *g*. Supernatant was decanted into 10 ml volumetric flask and sample was extracted still twice applying the same procedure. Flask was filled with 80% EtOH. Before the HPLC run the sample was diluted with the mobile phase containing 20% eluent B. This extraction was applied in the beginning to quantify all 12 possible forms of isoflavones and to calculate the amount of isoflavones in aglycone equivalents. Results from this test were used for evaluation of the results in other tests.

Table 2
Extraction conditions in pre-treatment tests 1–4

Test	*T* (°C)	*t* (h)	Reagent
1	RT[a]	–	80% EtOH
2	60	2	80% EtOH
	60	4	80% EtOH
	80	0.5	80% EtOH
	80	1	80% EtOH
	80	2	80% EtOH
	100	0.5	80% EtOH
3	65	2	0.15 M[b]NaOH in 80% MeOH
4	80	0.5	0.5 M[b]HCl in 80% EtOH
		1	0.5 M[b]HCl in 80% EtOH
		0.5	1.0 M[b]HCl in 80% EtOH
		1	1.0 M[b]HCl in 80% EtOH

[a] Room temperature.
[b] Concentration in the extraction reagent.

2.3.2.2. Test 2. Fifteen milligrams of the sample was weighted and placed into a 20 ml tube with 5 ml of 80% EtOH. Mixture was incubated at different temperatures for different times: at 60 °C for 2 and 4 h, at 80 °C for 0.5, 1 and 2 h and at 100 °C for 0.5 h. Incubation conditions are presented in Table 2. After cooling, sample was shaken for 2 min and centrifuged 2 min at 2140 × *g*. Supernatant was taken into 10 ml volumetric flask and sample was re-extracted with 2.5 ml of 80% EtOH. After centrifugation supernatant were combined and the flask was filled with 80% EtOH.

2.3.2.3. Test 3. Fifteen milligrams of the sample was weighted and placed into a 20 ml tube. The sample was incubated for 2 h at 65 °C with 80% MeOH. Then the sample was saponified with sodium hydroxide, which was added to set the concentration to 0.15 M in the vial. After shaking vigorously for 10 min, 25 μl of glacial acetic acid was added to neutralize the solution. Sample was centrifuged 10 min at 2140 × *g* and supernatant was taken into the volumetric flask. Residue was re-extracted with 80% MeOH and supernatants were combined (Klump et al., 2001).

2.3.2.4. Test 4. Fifteen milligrams of the sample was weighted and placed into a 20 ml tube. Eighty percent EtOH was acidified to 0.5 or 1.0 M by adding the corresponding amount of concentrated HCl. Incubation times were 0.5 and 1 h at 80 °C with 0.5 and 1 M acid in 80% EtOH. Conditions are presented in Table 2. Five millilitres of acidified 80% EtOH was added and samples were incubated at 80 °C for 0.5 and 1 h. After incubation, samples were cooled, centrifuged and re-extracted with 2.5 ml of 80% EtOH. Supernatants were combined into 10 ml volumetric flask.

3. Results

3.1. Extraction procedures

The three samples A, B, and C were extracted with 80% EtOH (test 1) to establish the amount of different forms of isoflavones and the total amount of isoflavones. The amounts of different conjugates were also converted to corresponding isoflavone equivalents and values for total daidzein, glycitein and genistein as well as for the total amount of isoflavones in aglycones were calculated. Total amount of isoflavones in aglycone equivalents were 275, 22, and 34 mg/g in samples A, B and C, respectively, after triple extraction with 80% EtOH. Additional extractions gave no differences when

compared to the results of triple extractions. The results are presented in Table 3. Sample A contained high amount of isoflavones mainly 7-*O*-glucosides, but proportionally only some (~10%) malonyl or acetyl glucosides. Sample B was rich in acetyl glucosides (~40%), whereas sample C was rich in malonyl forms (~50%). Differences in the isoflavone profile and concentrations may require a different hydrolysis treatment.

In the pre-treatment test 2 temperatures ranged between 60 and 100 °C and incubation times from 0.5 to 4 h. Incubation at 60 °C did not show any effect on the hydrolysis of the acetyl or malonyl forms with any of the selected incubation times (2 and 4 h). Incubation at 100 °C caused losses due to an excessive boiling of the aqueous ethanol in the tubes. Incubation at 80 °C seemed to be the best option to extract isoflavones. At this temperature, 20–30% of the acetyl forms in samples A and B are hydrolysed during the first 0.5 h of the incubation. After that, no changes in the amount of acetyl forms were observed. In sample C 40% of the malonyl forms were hydrolysed, but part of it was converted to acetyl forms.

Test 3, which was the widely applied saponification (Klump et al., 2001), resulted in complete hydrolysis of acetyl forms in each sample of A, B and C. Also malonyl forms were well converted except in sample C where still 10.3% of the malonyl glucosides remained.

Test 4 was incubation with 0.5 or 1.0 M HCl in 80% EtOH at 80 °C for 0.5 or 1 h. In all conditions of test 4 the amounts of unhydrolysed acetyl and malonyl forms were very low. For the acetyl forms, complete hydrolysis to the aglycones is achieved with the acidic conditions, with no more than a 0.3% remaining in all the cases. Malonyl forms were also hydrolysed almost completely. In sample C, 5% of the malonyl glucosides remained after the hydrolysis with 0.5 M HCl in 80% EtOH. Therefore the most efficient conditions to hydrolyse acetyl and malonyl forms to aglycones and 7-*O*-glucosides were 1 M HCl in 80% EtOH with 1 h incubation at 80 °C. Under these extraction conditions, total amounts of isoflavone equivalents were 278, 28, and 32 mg/g, while the results after triple extraction with 80% EtOH were 275, 22, and 34 mg/g in samples A, B, and C respectively. The percentage amounts of remaining acetyl and malonyl forms are presented in Fig. 3. In Fig. 4, two chromatograms of the control sample C are presented. The first chromatogram was obtained after the pre-treatment with 1 M HCl for 1 h (test 4), and the second

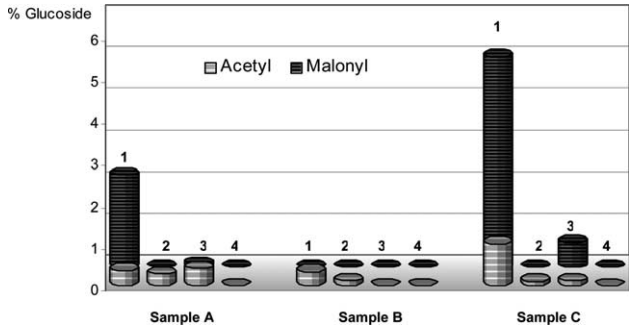


Fig. 3. The amount of malonyl and acetyl glucosides remaining after acid treatments in test 4: (1) 0.5 M/0.5 h (2) 0.5 M/1 h (3) 1.0 M/0.5 h (4) 1.0 M/1 h. Percentages were calculated from the original values presented in Table 3.

Table 3
Isoflavone profile of the control samples A, B and C

Isoflavone	Sample A (mg/g)[a]	Sample B (mg/g)[a]	Sample C (mg/g)[a]
MDin	–	–	11.2 ± 0.18
ADin	17.3 ± 0.61	10.1 ± 1.8	1.08 ± 0.02
Din	205 ± 6.8	12.8 ± 2.0	9.56 ± 0.21
Da	3.07 ± 0.13	0.32 ± 0.06	1.38 ± 0.02
MGlyn	1.21 ± 0.21	–	1.81 ± 0.05
AGlyn	0.95 ± 0.01	1.94 ± 0.36	0.06 ± 0.00
Glyn	29.7 ± 1.03	6.04 ± 1.1	1.17 ± 0.08
Gly	0.70 ± 0.01	0.36 ± 0.08	0.11 ± 0.00
MGin	–	–	14.3 ± 0.14
AGin	25.2 ± 1.1	3.50 ± 0.69	2.28 ± 0.11
Gin	160 ± 5.5	2.72 ± 0.72	12.6 ± 0.31
Ge	2.13 ± 0.12	0.10 ± 0.01	1.65 ± 0.30
Total	446 ± 15.6	37.9 ± 6.34	57.2 ± 1.20
Total Acetyl	43.4 ± 1.72	15.5 ± 2.85	3.42 ± 0.13
Total Malonyl	1.21 ± 0.21	–	27.3 ± 0.55
Total Da[b]	138	13.7	13.4
Total Gly[b]	20.7	4.21	1.85
Total Ge[b]	116	3.79	18.3
Total[b]	275	21.7	33.6

[a] Mean ± SD ($N = 3$) wet weight.
[b] Total amount of isoflavone equivalents.

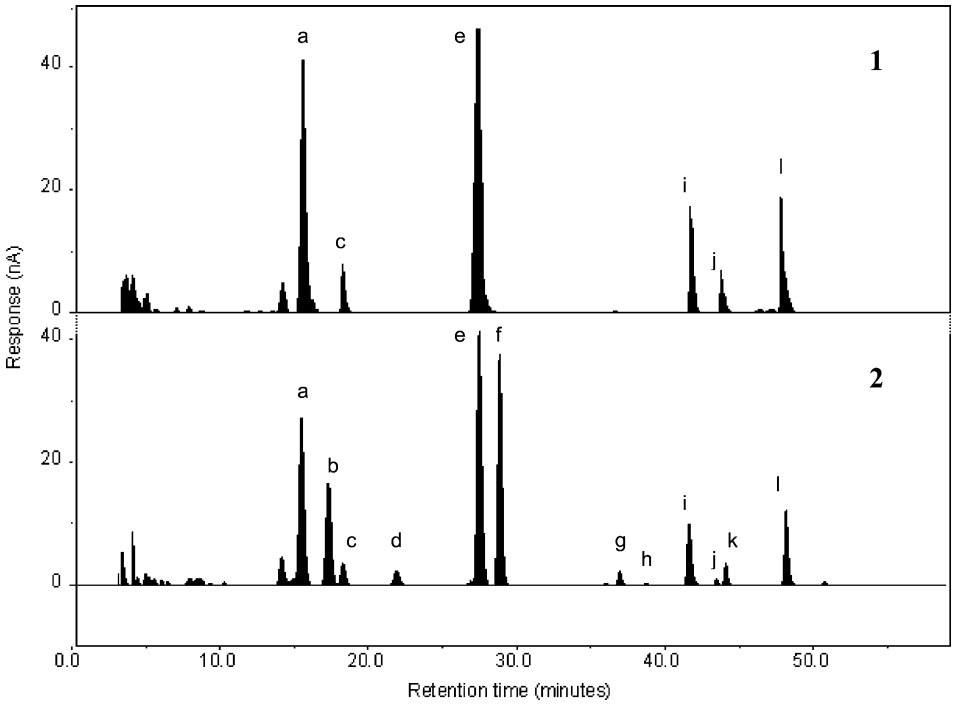


Fig. 4. Isoflavone profiles of the sample C. (1) After 1 h at 80 °C with 1 M HCl in 80% EtOH (test 4), and (2) initial situation (test 1). (a) daidzin, (b) malonyldaidzin, (c) glycitin, (d) malonylglycitin, (e) genistin, (f) malonylgenistin, (g) acetyldaidzin, (h) acetylglycitin, (i) daidzein, (j) glycitein, (k) acetylgenistin, (l) genistein.

one presents the original situation after extraction with 80% EtOH (test 1).

According to these results, 1 M HCl in 80% EtOH with 1 h incubation at 80 °C was the best choice to extract total isoflavones, and at the same time to hydrolyse the acetyl and malonyl forms. Flow diagram of the final sample pre-treatment method is presented in Fig. 5.

3.2. Validation parameters

3.2.1. Accuracy

Recovery of the isoflavones was tested by the standard addition method. The samples were spiked in triplicate with separate dilutions of each isoflavone standard corresponding approximately to 100%, 75% and 50% of the expected value in the sample C. Spiked amounts ranged from 2.4 μg/sample for Gly to 145.3 μg/sample for Din. The expected values for isoflavones and the recoveries for each level of fortification are summarized in Table 4. Recoveries ranged from 75.5% for Glyn to 119% for Da. No considerable differences have been found between recoveries at different spiked levels. Mean recovery of 7-*O*-glucosides was 87% and 114% for aglycones, and mean recovery for all the isoflavones involved in the analyses was 100%. Coefficients of variation of the recovery values ranged from 0.3% for Din to 5.4% to Gly.

The differences in recoveries found between 7-*O*-glucosides and aglycones led us to think about a possible

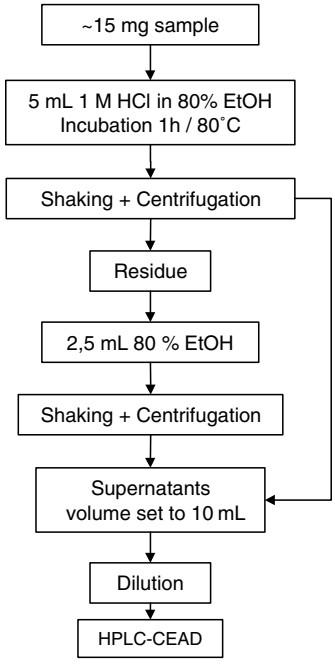


Fig. 5. Flow diagram of the final pre-treatment method for isoflavones in soy products.

hydrolysis of the 7-*O*-glucosides due to the acidified extraction media. For this reason additional tests were carried out with blank samples. Blank 1 correspond to a direct addition of the 7-*O*-glucoside standards at two different concentration levels without any matrix present

Table 4
Accuracy and precision of the method

Isoflavone	Concentration (mg/g)	Accuracy					Precision	
		Added (mg/g)	Found (mg/g)	Recovery (%)	Mean (%)	CV	Intraassay CV ($N=5$)	Interassay CV ($N=10$)
Din	19.0	20.4	36.7	93.1				
		15.7	32.5	93.4	93.1	0.30	7.4	11.5
		10.6	27.5	92.9				
Glyn	1.44	1.70	2.30	73.0				
		1.31	2.09	75.8	75.5	3.12	6.1	15.1
		0.88	1.81	77.7				
Gin	27.8	18.2	42.3	91.8				
		14.1	39.2	93.3	92.9	0.98	8.1	13.9
		9.26	34.7	93.4				
Da	2.43	2.11	5.54	122.0				
		1.55	4.80	120.5	119.7	2.30	8.7	13.8
		1.04	4.05	116.7				
Gly	0.89	0.41	1.56	120.5				
		0.31	1.40	116.0	114.8	5.46	7.2	12.3
		0.21	1.19	108.1				
Ge	3.41	2.82	6.80	109.0				
		2.22	6.08	108.0	107.9	1.20	9.7	9.5
		1.54	5.28	106.5				

Table 5
Conversion of the 7-*O*-glucosides to aglycones

Blank	Isoflavone	Total recovery (%)	CV	Conversion to aglycone (%)	CV
1[a]	Din	108	3.98	8.23	0.75
	Glyn	86.4	2.46	13.9	1.27
	Gin	99.1	5.05	9.31	0.67
2[b]	Din	102	1.22	5.56	0.25
	Glyn	89.2	3.54	11.2	1.11
	Gin	100	4.12	6.55	0.98

[a] Standards in water.
[b] Standards in wheat flour.

in the test tube, whereas blank 2 consisted in 15 mg of a isoflavone-free matrix (wheat flour) also spiked at two concentration levels. After extraction and analysis in triplicate of the blank samples, the results presented in Table 5 confirm that the 7-*O*-glucosides are partly converted to aglycones during the mild acid extraction.

3.2.2. Precision

The precision of the method was evaluated with intra- and interassay tests. Five replicates of the control sample C were analysed at the same time to assess the intraassay coefficient of variation (CV) as well as the same sample was included in 10 analytical batches to calculate the interassay CV. Both values, together with the mean isoflavone concentration for each compound after the interassay test, are presented in Table 4. Mean intra- and interassay CVs were 7.8% and 12.6% respectively with no differences in CVs between compounds at high or low concentrations.

4. Discussion

Even though many different analytical methods for soy isoflavones have been published, there was still a lack of a suitable method to measure total isoflavones with a small number of standards and with minimum steps in sample pre-treatment.

Different extraction methods for isoflavones and mild hydrolysis conditions for acetyl and malonyl glucosides were tested to develop a simple pre-treatment method to analyse total isoflavones in different soy samples. Isoflavones were simply first hydrolysed so that only aglycones and 7-*O*-glucosides were present in sample.

Triple extraction with 80% EtOH would be a suitable method if all 12 forms of isoflavones would be analysed. Main problem with this simple method is the use of labile standards for the quantification of the ester glucosides decreasing the precision of the analysis and therefore limits its application as a reliable routine method. Even though this procedure can be successfully applied for an isolated determination as it is shown in this study. Other published methods using ethanol or methanol solvents usually involves one single step extraction (Barnes et al., 1994; Coward et al., 1993; Franke, Hankin, Yu, Maskarinec, Low, & Custer, 1999), but that may cause poor recoveries if the amount of isoflavones is very high i.e. over 200 mg/g.

The saponification method (Klump et al., 2001), seemed to be very efficient, but we were not satisfied with 2 h extraction followed with saponification, neutralization and re-extraction. Also some samples seemed to be resistant to saponification. The amount of malonyls in sample C after testing was still over 10%. Isoflavone content in sample C was only moderate and therefore the amount of remaining malonyl glucosides after saponification was not expected.

Enzymatic and acid hydrolysis of the samples can solve these problems as we have proved earlier (Mazur et al., 1996; Nurmi et al., 2002), but this method involves several steps because it was developed for the measurement of a wide range of phytoestrogens in food matrices. When soy isoflavones are the phytoestrogen of interest, the method presented in this paper could be a reliable option as an accurate routine procedure due to its simpler and shorter analytical steps.

Mild acid hydrolysis and incubation completely separated the acetyl and malonyl groups of the conjugates, releasing the 7-*O*-glucosides and the free aglycones to be extracted. This pre-treatment step allows to reducing the number of ethanol extractions to one since the high temperature and acidic conditions helps to liberate the isoflavones from the food matrix. The total amount of isoflavone equivalents for samples A, B and C in Table 3 corresponded well to the values obtained after mild acid hydrolysis. All the isoflavones, which were extracted after triple treatment with 80% EtOH, were also extracted with acidified 80% EtOH and for sample B results were 40% higher than after the original extraction. The increase was due to a more efficiently extracted glycitein and daidzein. No losses of isoflavones were observed. As it has been showed in all the accuracy tests, recoveries of the isoflavones after extraction were always correct. Hydrolysis of the 7-*O*-glucosides to the correspond aglycones occurs only to some extent, and does not affect the total isoflavone content of the samples.

It has been shown that isoflavone glucosides are hydrolysed in human body before absorption (Setchell et al., 2002). Therefore, total amount of isoflavone aglycones provides sufficient information when different soy products are considered for consumption. Because any recommendations about the daily amount of isoflavones have not been given in regard of preventing diseases, the product data in soy products should be detailed and especially the amount of total isoflavone in aglycone equivalents should be given clearly.

This method development work provided finally the possibility to analyse total amount of different isoflavones by measuring only aglycones and 7-*O*-glucosides. This is an improvement in methodology for those who do not need to collect information about the conjugate pattern in soy samples.

Acknowledgements

Financial support from Phytoprevent (EU:QLK1-2000-00266) is gratefully acknowledged. This study does not necessarily reflect the views of the Commission and in no way anticipates the Commission's future policy in this area.

References

Adlercreutz, H., Goldin, B. R., Gorbach, S. L., Höckerstedt, K. A. V., Watanabe, S., Hämäläinen, E. K., Markkanen, M. H., Mäkelä, T. H., Wähälä, K. T., Hase, T. A., & Fotsis, T. (1995). Soybean phytoestrogen intake and cancer risk. *Journal of Nutrition, 125*, 757S–770S.

Allred, C. D., Ju, Y. H., Allred, K. F., Chang, J., & Helferich, W. G. (2001). Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis, 22*(10), 1667–1673.

Barnes, S. (1998). Evolution of the health benefits of soy isoflavones. *Proceedings of the Society for Experimental Biology and Medicine, 217*(3), 386–392.

Barnes, S., Kirk, M., & Coward, L. (1994). Isoflavones and their conjugates in soy foods – extraction conditions and analysis by HPLC mass spectrometry. *Journal Agricultural Food Chemistry, 42*(11), 2466–2474.

Bowey, E., Adlercreutz, H., & Rowland, I. (2003). Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. *Food and Chemical Toxicology, 41*(5), 631–636.

Coward, L., Barnes, N. C., Setchell, K. D. R., & Barnes, S. (1993). Genistein, daidzein and their b-glycoside conjugates: antitumor isoflavones in soybean foods from american and asian diets. *Journal of Agricultural and Food Chemistry, 41*(11), 1961–1967.

Franke, A. A., Custer, L. J., Cerna, C. M., & Narala, K. (1995). Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proceedings of the Society for Experimental Biology and Medicine, 208*(1), 18–26.

Franke, A., Hankin, J. H., Yu, M. C., Maskarinec, G., Low, S., & Custer, L. J. (1999). Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. *Journal of Agricultural and Food Chemistry, 47*(3), 977–986.

Griffith, A. P., & Collison, M. W. (2001). Improved methods for the extraction of isoflavones from soy-containing foods and nutritional supplements by reversed-phase liquid chromatography and liquid chromatography – mass spectrometry. *Journal of Chromatography A, 913*, 397–413.

Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y., & Kikuchi, M. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *Journal of Nutrition, 130*(7), 1695–1699.

Kapiotis, S., Hermann, M., Held, I., Seelos, C., Ehringer, H., & Gmeiner, B. M. K. (1997). Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. *Arteriosclerosis, Thrombosis and Vascular Biology, 17*(11), 2868–2874.

Klump, S. P. A., McDonald, J. L., & Ballam, J. M. (2001). Determination of isoflavones in soy selected foods containing soy by extraction, saponification, and liquid chromatography: collaborative study. *Journal of AOAC International, 84*(6), 1865–1883.

Kurzer, M. S. (2000). Hormonal effects of soy isoflavones: studies in premenopausal and postmenopausal women. *Journal of Nutrition, 130*, 660S–661S.

Liggins, J., Bluck, L. J. C., Coward, W. A., & Bingham, S. A. (1998). Extraction and quantification of daidzein and genistein in food. *Analytical Biochemistry, 264*(1), 1–7.

Mazur, W., Fotsis, T., Wähälä, K., Ojala, S., Salakka, A., & Adlercreutz, H. (1996). Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. *Analytical Biochemistry, 233*, 169–180.

Murphy, P. A. (1981). Separation of genistin, and coumestrol by gradient high-performance liquid chromatography, daizdin and their aglucones. *Journal of Chromatography A, 211*(1), 166–169.

Naim, M., Gestetner, B., & Zilkah, S. (1974). Soybean isoflavones characterization, determination, and antifungal activity. *Journal of Agricultural and Food Chemistry, 22*(5), 806–810.

Nurmi, T., Mazur, W., Heinonen, S., Kokkonen, J., & Adlercreutz, H. (2002). Isoflavone content of the soy based supplements. *Journal of Pharmaceutical and Biomedical, 28*, 1–11.

Piskula, M. K., Yamakoshi, J., & Iwai, Y. (1999). Daidzein and genistein but not their glucosides are absorbed from the rat stomach. *FEBS Letters, 447*, 287–291.

Setchell, K. D. R., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brasher, W. T., Kirschner, A. S., Cassidy, A., & Heubi, J. E. (2001). Bioavailabilty of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *Journal of Nutrition, 131*, 1362S–1375S.

Setchell, D. R. K., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S., & Heubi, J. E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *American Journal of Clinical Nutrition, 76*, 447–453.

Wang, H. J., & Murphy, P. A. (1994). Isoflavone content in commercial soybean foods. *Journal of Agricultural Food Chemistry, 42*(8), 1666–1673.

Wiseman, H. C., Clarke, D. B., Barnes, K. A., & Bowey, E. (2002). Isoflavone aglycone and glucoconjugate of high- and low-soy U.K. foods used in nutritional studies. *Journal of Agricultural Food Chemistry, 50*(6), 1404–1410.