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Isoflavone content of the soy based supplements

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Abstract

A large number of soy isoflavone products with indications of possible health effects are available on the market. Fifteen different soy based products were analyzed using high performance liquid chromatography (HPLC) with coulometric electrode array detector to determine the total amount of isoflavones in aglycones after the hydrolysis and identify the different forms of the isoflavone conjugates. The aim of the study was to evaluate how well the isoflavone content data supplied by the producers correspond to our analysis results. Only one product contained isoflavones measured in aglycones the same amount as was the value given by the producer. The total amount of the isoflavones in aglycones ranged from 0.121 to 201 mg/g. Measured amounts of isoflavones in aglycones after the hydrolysis were in general lower than the values in the product labels. Product data were often confusing and the concrete amount of isoflavones was difficult to find out.

Keywords: Isoflavones; Soy product; Supplement; Coulometric detection

1. Introduction

In the Western countries, the range of different dietary supplements available through pharmacies, health-food shops and supermarkets, has been growing many fold over the last decade. One group of these supplements is soy based health products, which contain phytoestrogens called isoflavones or/and soy protein. The basic issues in the discussion on the health effects of these supplements deal with the quantitative intake of isoflavones, the mechanisms of action and the effective concentrations in biological fluids [1–4] and in tissues [5]. It is not known, if the isoflavones or proteins or both together are responsible of the observed health effects. Results of the feeding studies are ambiguous [6]. Although some studies have assessed concentrations of phytoestrogens and associated them with certain biological effects in women, so far no definite dietary recommendations could be made [7–10]. Earlier in two papers, the analysis results of the soy based health products have been presented [11,12].

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Different analysis methods for soy isoflavones have been published [13–17]. The most popular methods apply HPLC technique [13,14] and UV-, DAD or MS-detection. Coulometric detection has been used occasionally for food isoflavone analysis [18], but the method for soy isoflavones applying HPLC with coulometric electrode array detection (CEAD) has not been published. In published HPLC methods isoflavones are usually analyzed in conjugated form and, therefore, 12 different standards are needed to determine the total amount of isoflavones. Analysis results of the isoflavones from the different laboratories vary, and any reference method for isoflavone analyses has not been selected. Recently a laboratory comparison study was organized and 24 laboratories participated. The main finding was that the reason for discrepancy in the isoflavone analysis results was differences in the standards of the conjugated isoflavones [19]. Earlier one group has corrected their results after they noticed that standards for isoflavone conjugates were impure when the original study was carried out [20].

In the present study, isoflavone content of the 15 soy based products were analyzed using an HPLC-CEAD method. Isoflavones were measured in aglycones after the hydrolysis and, therefore, only three standards were needed instead of 12. 7-*O*-glucosides were quantified when they were the only form of the conjugates present in the sample. Qualitative data about the malonyl and acetyl glucosides were collected. The aim of the study was to develop an HPLC method with CEAD to analyze soy isoflavones in aglycones after the hydrolysis and to determine whether the isoflavone content data of the supplements correspond with the quantified amounts of aglycones, which are the bioactive part of the isoflavone molecules.

2. Experimental

2.1. Standards, reference material and samples

Quantified isoflavones were genistein, daidzein, glycitein, and their 7-*O*-glucosides. Genistein was purchased from KarlsRoth GmBH (Karlsruhe, Germany). Daidzein was synthesized in the Department of Organic Chemistry, University of Helsinki, Finland. Glycitein, genistein-7-*O*-glucoside (genistin) and daidzein-7-*O*-glucoside (daidzin) were purchased from Apin Chemicals Ltd (Oxon, UK). Glycitein-7-*O*-glucoside (glycitin) was purchased from Fujicco Co Ltd, Nacalai Tesque, Inc (Kobe, Japan). The aglycones were dissolved into methanol (MeOH) and 7-*O*-glucosides into 80% ethanol (EtOH). Standard stock solutions were kept in the ultrasound bath for 10 min to confirm the complete dissolving. Quantitation standard was a mixture of the stock solutions, which was appropriately diluted with the mobile phase containing 30% eluent B before the HPLC run. All standard solutions were stored at 4 °C.

Purity of the isoflavone standards was evaluated using the maximum absorbance and the literature values for extinction coefficients at the same wavelength. Measured absorbance (A) was divided with extinction coefficient (ε) and width of the sample cell (d) using the Lambert–Beer's law of absorbance ($A = \varepsilon dc$) [21]. Calculated concentration (c) was compared with the weighed concentration and the weighed concentration was corrected when needed. Extinction coefficient for daidzein was taken from the handbook and, therefore, it can be considered as a generally accepted value [22]. The same extinction coefficient was applied for daidzin because the sugar moiety as a 7-*O*-substituent does not affect on the intensity of the absorption but may shift the absorption maximum to the different wavelength [22]. Chosen extinction coefficient for genistein was slightly higher than values generally published for genistein [23] but it was close to the extinction coefficient of genistin, which was published by Walter [24]. For glycitein two published extinction coefficients were found [25,26]. Difference for the values was 8785 U and value published by Song et al. corresponded more to the structural expectations of the value. Therefore, it was chosen and the same extinction coefficient was used for glycitin. Differences between the calculated and weighed concentrations are presented in Table 1 with the extinction coefficients and the wavelength of the maximum absorption.

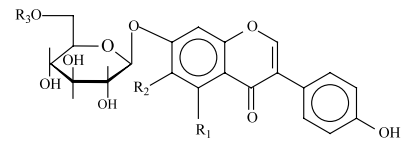
Stability of the standards was evaluated by measuring the absorbance of the stock solution always before the new quantitation standard mixture for analytical use was prepared. Any decomposing, i.e. decrease in the concentration, was not observed, but the standard stock solutions concentrated approximately 18% in a year, which was observed from the increasing concentration values. Fresh stock solutions were divided into 1 ml aliquots and stored in volumetric flasks of 1 ml. During storage volume of the aliquot was decreased and concentration was, therefore, increased. If the volumetric flask was filled into mark, the concentration was the same as was measured from the fresh standard stock solution. Pretreated samples were dissolved into the same solvent as standards and samples were analyzed immediately after the pretreatment. Therefore, any separate tests of the stability of the analytes were not carried out.

Malonyl and acetyl daidzin, genistin and glycitin, were identified using the analytes present in two soy based samples i.e. product 5 in the study and soymilk. Soymilk contained malonyl forms and product 5 contained acetyl forms. Department of Chemical Technology of Technical Research Center (VTT, Espoo, Finland) analyzed extracts of those two samples using HPLC-electrospray tandem mass spectrometry and confirmed our identification of the conjugates. The structures of the different forms of the isoflavones are presented in Fig. 1. Conjugates were extracted from the original matrices using the protocol described later in the chapter 2.4. Sample pretreatment. Fifteen soy based products were either bought from the shops or were received directly from the producers. Samples were capsules, tablets and powder.

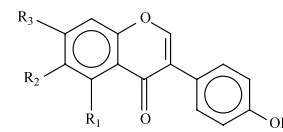
Table 1
Purity of the isoflavone standards

Compound	λ_{max} (nm)[a]	ε (1/mol cm)[b]	Difference (%)[c]
Daidzin	260	27 542[d]	−17.0
Glycitin	262	31 622[e]	−7.08
Genistin	262	39 096[f]	−17.2
Daidzein	248	27 542[g]	−15.9
Glycitein	260	31 622[h]	−39.4
Genistein	262	38 460[i]	−13.4

[a] Wavelength for the maximum absorption.
[b] Extinction coefficient.
[c] Percentage difference between the calculated concentration and the weighed value obtained with Lambert–Beer's law. See chapter 2.1 for details.
[d] Value for daidzein was used.
[e] Value for glycitein was used.
[f] Reference [24].
[g] Reference [22].
[h] Reference [25].
[i] Reference [23].



Compound	R_1	R_2	R_3	M / g/mol
Daidzin	H	H	OH	416
Malonyldaidzin	H	H	$COCH_2COOH$	502
Acetyldaidzin	H	H	$COCH_3$	458
Genistin	OH	H	OH	432
Malonylgenistin	OH	H	$COCH_2COOH$	518
Acetylgenistin	OH	H	$COCH_3$	474
Glycitin	H	OCH_3	H	446
Malonylglycitin	H	OCH_3	$COCH_2COOH$	532
Acetylglycitin	H	OCH_3	$COCH_3$	488



Compound	R_1	R_2	R_3	M / g/mol
Daidzein	H	H	OH	254
Genistein	OH	H	OH	270
Glycitein	H	OCH_3	OH	284

Fig. 1. Structures of the isoflavone aglycones and different conjugated forms of daidzein, genistein and glycitein.

2.2. Instrument

The HPLC system consisted of two pumps model 580, model 540 autosampler with refrigeration, thermal chamber for a column and a detector, CEAD, system control module and computer. Computer was Compaq Deskpro and HPLC components were all from ESA (ESA Inc Chelmsford, MA). Coulometric electrode array detector consisted of eight electrode pairs divided into two cells. Both cells are equipped with reference elec-

trode made from platinum. Potentials can be set separately on each electrode pair and the potential range is from −1000 to +1000 mV. Phenolic compounds, like isoflavones, are oxidized and the potential range for oxidation is from 0 to 1000 mV. In regular use maximum potentials are recommended to keep close to or under +700 mV to save the electrode material. Optimum oxidation potentials are determined by measuring a hydrodynamic voltamogramm of each analyte. Potential giving the maximum signal is chosen for detection and adjacent channels i.e. electrode pairs with different potentials give additional signals to confirm the identification. Signals on different channels represent an electrochemical spectrum of the analyte. In gradient separation, the coulometric electrode array detector needs certain stabilization time like column, too. In the beginning of the each run background signal is automatically set to zero with the signal of the reference electrode. Sufficient stabilization time for the detector can be observed from the stabile and non-drifting signals. Drifting and stability of the signals are also good indicators of the purity of the electrode array detector. Any contamination of the detector was not observed during the study.

2.3. Chromatographic conditions and quantitation parameters

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, (40:40:20 v/v/v). These eluents were previously used in the isoflavone analysis of the plasma samples [27]. MeOH and acetonitrile (ACN) were purchased from Rathburn Chemicals Ltd (Walkerburn Scotland, UK). Separation of the isoflavones was carried out with gradient elution using the total flow rate of 0.3 ml/min. The gradient profile is presented in Table 2. Resolution of the isoflavones was used to optimize the chromatographic separation. Resolution values were calculated using the separation factor (α), plate number i.e. column efficiency (N) and retention factor (k) by applying the formula $R = 1/4(\alpha - 1)\sqrt{N}[k/(k+1)]$ [28]. Resolutions of the peak pair daidzin–malonyldaidzin and the peak pair glycitein–acetylgenistin were lower than the recommended value, 1.5, for the baseline separation of the peaks similar in size [28]. Problems were avoided because the peak height was used for quantitation and malonyldaidzin and acetylgenistin were only identified, not quantified. Resolution values are presented in Table 3.

Table 2
Gradient profile

Time (min)	0	15	25	35	40	45	47	60
B (%)[a]	30	30	50	75	100	100	30	30

[a] Value expresses the percentages amount of the eluent B of the total flow 0.3 ml/min.

The analytical column was Inertsil ODS-3 (GL Sciences Inc, Japan). Column dimensions were 150 × 3 mm and the 3 μm particles were made from end-capped C18 material. The guard column was Quick Release C18 (Upchurch Scientific Inc WA, USA), dimensions 10 × 3 mm and packed with 5 μm particles. The column and the detector were thermostated to 37 °C and the samples were stored in an autosampler at 10 °C. Total analysis time was 60 min including the stabilizing time of 13 min. Column is completely stabilized if the R.S.D. values for the retention times are 0.5% or lower [28]. Retention time R.S.D. for inter- and intraassay runs were determined for quantified isoflavones and values are presented in Table 4. Values ranged from 0.16 to 1.1%, which were close to 0.5%.

Table 3
Resolution of the isoflavones

Peak pair	R[a]
Daidzin–malonyldaidzin	1.15
Malonyldaidzin–glycitin	1.97
Glycitin–malonylglycitin	2.48
Malonylglycitin–genistin	11.5
Genistin–malonylgenistin	1.55
Malonylgenistin–acetyldaidzin	14.7
Acetyldaidzin–acetylglycitin	4.28
Acetylglycitin–daidzein	5.97
Daidzein–glycitein	4.26
Glycitein–acetylgenistin	1.38
Acetylgenistin–genistein	10.1

[a] $R = 1/4(\alpha-1)\sqrt{N}[k/(k+1)]$, see text for details.

Table 4
Chromatographic parameters of the isoflavones

Isoflavone	Detection potential (mV)	Retention time (min)	Retention time R.S.D. inter-[a]/intraassay[b]	Detector response R.S.D. inter- [a]/intraassay[b]
Daidzin	590	10.84	1.11/0.59	8.7/3.0
Malonyl daidzin	590	11.36		
Glycitin	590	12.30	–[c]/0.31[d]	–[c]/5.2[d]
Malonyl glycitin	590	13.60		
Genistin	590	20.13	0.92/0.68	12/1.1
Malonyl genistin	590	21.37		
Acetyl daidzin	590	32.83		
Acetyl glycitin	590	34.85		
Daidzein	510	37.29	0.62/0.78	6.3/4.1
Glycitein	590	39.27	–[c]/0.16[d]	–[c]/3.8[d]
Acetyl genistin	590	40.09		
Genistein	510	44.22	0.52/0.46	12/7.6

[a] $N = 8$.
[b] $N = 23$.
[c] Not determined.
[d] $N = 5$.

Potentials on the eight electrode pairs ranged from 200 to 750 mV. Retention times and detection potentials for all 12 forms of the isoflavones are presented in Table 4 and chromatographic profile is presented in Fig. 2. Inter- and intraassay R.S.D. values of the detector response were determined for quantified analytes. Values are presented in Table 4. Limits of detection were determined with $S/N = 3$ using standards. LOD values are presented in Table 5 using the molar amount injected into column. Injection volume was 10 μl. Limits of quantitation were 3 times the LOD. Maximum ranges of the detector signal linearity were determined and included values were within the $\pm$ 5% of the calculated line. Least square estimations for the slope and the intercept are presented in Table 5 with their S.D. values. Correlations between the measured and calculated values for linearity are also presented in the same table.

2.4. Sample pretreatment

Samples were analyzed in duplicates or triplicates after the hydrolysis applying the protocol earlier published by Mazur et al. [29]. The content of 10–20 capsules was pooled in order to minimize the effect of variation between the single capsules. The capsules itself were discarded. Tablets were ground after 10–20 pieces were pooled to make a sample. Sample of 5–100 mg was swollen in 0.5 ml of water overnight at room temperature. Swollen sample was subjected to enzyme hydrolysis by adding 0.5 ml of hydrolysis reagent containing 2500 U/ml of β-glucuronidase, obtained form Helix Pomatia (Biosepra IBF/Sepracor, France), in 0.3 M sodium acetate buffer pH 4.1 at 60 °C for 2.5 h. Aglycones were extracted twice with 5 ml of diethylether shaking 2000 rpm for 2 min. Ether phase was separated after freezing the water phase (lower) in the ice bath made from EtOH and solid CO_2. Two ether phases were combined and evaporated under N_2 flow. The sample in the water phase was further hydrolyzed with 2 M hydrochloric acid 2 h at 100 °C. Aglycones were extracted twice with diethylether and the ether phases were combined and evaporated under N_2 like after the enzyme hydrolysis. Sample fractions were dissolved into MeOH, diluted with mobile phase containing 30% eluent B prior to HPLC analysis, and daidzein, genistein and glycitein were quantified. Some water phase residues of the samples with highest amounts of

isoflavones were analyzed to confirm the complete extraction after the hydrolysis steps.

For the conjugate identifications 5–50 mg sample was weighed and swollen in 0.5 ml of water overnight at room temperature. About 2 ml of EtOH was added and the sample was shaken for 2 min at 2000 rpm at room temperature. The sample was then centrifuged 10 min at 2500 rpm. Supernatant (80% EtOH) was taken into a volumetric flask and filled with 80% EtOH. Samples were diluted with the mobile phase containing 30% eluent B prior to HPLC analysis. Two reference materials (product 5 and soymilk) for the isoflavone conjugate identifications were prepared using this EtOH extraction.

Efficiency of the hydrolysis or extraction was not separately tested. Total amount of isoflavones in aglycones after the hydrolysis corresponded to the total amount of isoflavones in 7-*O*-glucosides on molar bases for the samples 1, 4, 8 and 10. This proved that hydrolysis steps were as efficient as the EtOH extraction. Similar type of solvent extractions for conjugated isoflavones have been widely applied and published [11–14,17].

3. Results

The amounts of daidzein, genistein and glycitein quantified after the hydrolysis are presented in Table 6. The total amount of isoflavones in aglycones is presented in Table 6 for each product. Values are given in mg/g to provide a uniform expression to compare the soy concentrates used to manufacture the different products. The total amount of isoflavones in aglycones per serving unit, i.e. tablet, capsule or g, is presented in Table 7 with the types of conjugation. Products 1, 4, 8 and 10 contained only 7-*O*-glucosides and it was possible to quantify the total amount of isoflavone conjugates. Results are presented in Table 7 in the same column with the type of conjugation. The amount of isoflavone conjugates in product 1 was 85% of the value given by the

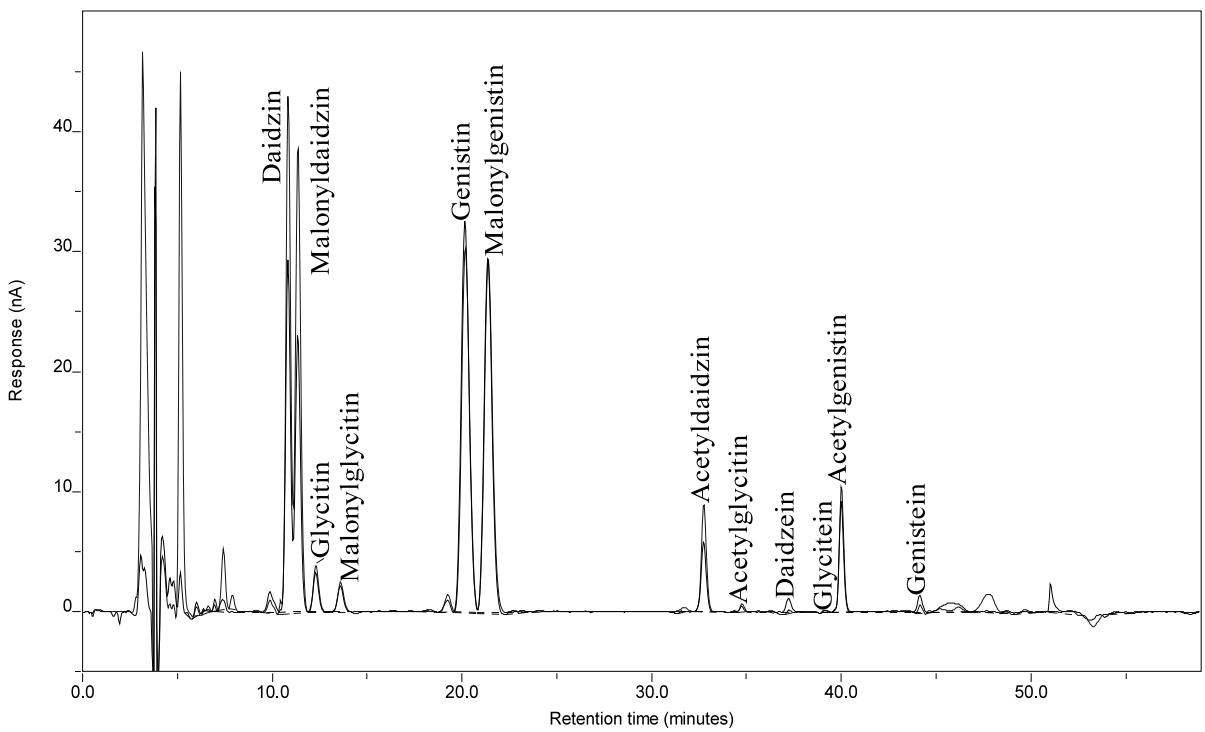


Fig. 2. Chromatographic profile of 12 isoflavone forms. Only the signals of the quantitation channels 3 and 4 were presented for clarity. Full scale on the signal axis is 50 nA.

Table 5
Quantitation parameters of the isoflavones

	LOD (fmol on column)[a]	Linearity (pmol on column)[b]	Slope ± S.D.[c]	Intercept (nA) ± S.D.[c]	r
Daidzin	73.3	147	7.515 ± 0.048	0.111 ± 1.11	1.0000
Glycitin	65.5	22.4	12.27 ± 0.457	0.456 ± 0.86	0.9995
Genistin	93.3	142	4.341 ± 0.074	0.600 ± 1.74	0.9998
Daidzein	45.7	45.6	16.39 ± 0.097	−0.483 ± 0.40	1.0000
Glycitein	38.0	76.1	10.03 ± 0.465	0.807 ± 3.14	0.9973
Genistein	93.3	46.7	7.329 ± 0.147	−0.745 ± 0.70	0.9999

[a] Determined with pure compounds, $S/N = 3$.
[b] Maximum linearity range from 0 pmol to the given value.
[c] 95% confidence interval.

producer. Corresponding values of the product 4 and 8 was 89 and 79%, respectively. The total amount of isoflavone conjugates in the product 10 was only 51% of the value given by the producer.

3.1. Evaluation of the product data

Any conjugated forms were not detected in products 2 and 9. In products 1, 4, 8 and 10 only 7-*O*-glucosides were detected and in products 13 and 15 were detected all nine forms of isoflavone conjugates. Other products contained 7-*O*-glucosides and either malonyl or acetyl forms. Different forms of conjugations were not listed in any product data. Four of the 15 soy based products were marketed without any data about the isoflavone content. In the case of two soy protein products (14 and 15) it was understandable that no isoflavone content data was presented, because the soy proteins were the actual product, not the isoflavones. Two health products (2 and 9), with no data about the isoflavone content, were marketed reasonably also without any health indications.

Products 1 and 4 were capsules containing also some other filling material than soy concentrate. In product 1 the amount of soy concentrate was 78% of the total content of the capsule. The value for the total amount of isoflavones given by the producer was 5%. Our result for isoflavones as conjugates was 4.3%, if it was taken into account that the content of the capsule was not pure soy concentrate. In product 4 the amount of soy concentrate was 16% of the total content of the capsule. On the product label a value of 40% isoflavones was given together with the word 'standardized'. In this case the data will be understood like the product would contain 40% isoflavones per capsule. After comparing our results of 7-*O*-glucosides with the values given by the producer, it was concluded that the figure of 40% refers to the isoflavone conjugate content of the soy concentrate, representing as little as 16% of the capsule content. Data of products 11–13 specified also the ratio of the individual isoflavones daidzein, genistein and glycitein. These data corresponded well to our results presented in Table 6. Data of product 10 informed that minimum amount of genistein was 70% of the total amount of isoflavones. Our result for the amount of genistein was 85% of the total amount of isoflavones even though the total amount of isoflavones even in conjugates was only 51% of the value given by the producer. Percentage amount of genistein thus corresponded to the producer data, but the total amount of isoflavone conjugates did not.

Products 3, 5–8 were marketed with exact values of the total amount of isoflavones per tablet or capsule. Products 6, 7, 10 and 11 contained isoflavones in aglycones after the hydrolysis under 50% of the value given by the producer and, therefore, even conversion into conjugates would not change the results to correspond to the values given by the producer. The total amount of isoflavones in aglycones after the hydrolysis in the product 5 was 77% of the given value. Product 5 contained a high amount of glycitein and the

product was recommended to prevent osteoporosis. It was the only product with exact and direct information about the prevention of certain disease. The measured weight of the tablet of product 5 was 50 mg lower than the tablet weight given by the producer (0.5 g). When the value is given with one decimal only, it is mathematically correct to give the value 0.5 g. However, if the measured value regularly occurs to be 0.45 g, two decimals would be more informative. Quantitative results in Table 7 were calculated using the measured weight of the tablet. Product 3 contained isoflavones in aglycones after the hydrolysis exactly the amount corresponding to the value given by the producer.

4. Discussion

In the present study HPLC method to analyze the isoflavones in aglycones after the hydrolysis was developed and 15 soy based health products were analyzed to determine whether the isoflavone content data given by the producers correspond with the measured values.

HPLC method was evaluated with different parameters. Resolution values were adequate for reliable identification and quantitation. Retention times were stable from run to run and between the assays. Detector response varied for genistein possibly due to a wider peak compared with the other isoflavones. Using the peak area gave the same result. Any specific explanation was not found. Sensitivity of the method was very high compared with the methods applying UV-detection or DAD. Linearities ranged from 350 to 2000 times the on column values of the detection limits with high correlations between the measured and calculated values. Intercepts differed from zero and S.D. values for the intercepts were high compared with the calculated intercept values. S.D. for intercepts decreased when linearity range was reduced. Presented linearity range included only the measured values differing not more than $\pm 5\%$ of the calculated line. Calibrations were made for shorter range than presented maximum linearity and for calibrations, intercepts were zero.

Purity and stability of the standards were determined using the maximum absorptions and extinction coefficients. Different values for extinction coefficients of isoflavones have been published and chosen extinction coefficients can always be criticized. The same technique had been applied also earlier [12,30]. Advantage of this

Table 6
Isoflavone concentration of the soy based health products in ground samples

Product	Product type and weight (g)	Daidzein (mg/g)[b]	Genistein (mg/g)[b]	Glycitein (mg/g)[b]	Total amount of isoflavones in aglycones (mg/g)
1	Capsule, 0.72[a]	7.07	12.5	0.948	20.5
2	Tablet, 1.63	0.045	0.076	nd	0.121
3	Capsule, 0.43[a]	15.3	28.4	2.26	46.0
4	Capsule, 0.62[a]	16.0	16.9	2.69	35.6
5	Tablet, 0.45	15.2	2.81	24.8	42.8
6	Capsule, 0.55[a]	2.69	0.614	2.81	6.11
7	Capsule, 0.63[a]	3.62	1.51	3.67	8.80
8	Tablet, 0.45	10.8	42.6	1.13	54.5
9	Capsule, 0.19[a]	0.828	0.568	0.198	1.59
10	Powder	4.28	27.0	0.334	31.6
11	Powder	13.8	23.1	3.31	40.2
12	Powder	24.2	71.5	4.71	100
13	Powder	97.3	81.5	22.0	201
14	Powder	0.196	0.569	0.048	0.813
15	Powder	0.239	0.403	0.097	0.739

[a] Weight of the capsule itself is not included.
[b] Mean value of the duplicate or triplicate.

Table 7
Analysis results for the total amount of isoflavones in aglycones, type of conjugation, and the values given by the producers

Product	Total amount of isoflavones in aglycones	Type of conjugation[a]	Values given by the producer[c]
1	14.5 (mg per capsule)	G (23.8)[b]	28.1 (mg per capsule)
2	0.142 (mg per tablet)	–	– (not given)
3	19.8 (mg per capsule)	G, M	20.0 (mg per capsule)
4	22.1 (mg per capsule)	G (35.7)[b]	40.0 (mg per capsule)
5	19.3 (mg per tablet)	G, A	25.0 (mg per tablet)
6	3.36 (mg per capsule)	G, A	9.0 (mg per capsule)
7	5.54 (mg per capsule)	G, A	12.5 (mg per capsule)
8	24.5 (mg per tablet)	G (39.4)[b]	50.0 (mg per tablet)
9	0.305 (mg per capsule)	–	– (not given)
10	31.5 (mg/g)	G (50.7)[b]	>100 (mg/g)
11	40.2 (mg/g)	G, M	100 (mg/g)
12	100 (mg/g)	G, M	200 (mg/g)
13	201 (mg/g)	G, A, M	400 (mg/g)
14	0.813 (mg/g)	G, M	– (not given)
15	0.739 (mg/g)	G, A, M	– (not given)

[a] G, 7-*O*-glucoside; M, malonyl glucoside; A, acetyl glucoside.
[b] Our HPLC–CEAD results of isoflavone conjugates in the same unit as the values given by the producer.
[c] Values based on the original information in the packages.

purity evaluation with maximum absorption and extinction coefficients was that all impurities, also those, which did not absorb on UV region, were taken into account. Impurities, which do not absorb, are ignored if only the peak area of the analyte is compared with the total peak area. The highest difference between the calculated and weighed concentration was for glycitein. Weighed glycitein was very difficult to dissolve. The calculated concentration, determined with UV-data, was the same before and after the complete dissolving. This indicated that glycitein itself was easily dissolved and the rest of the particles were impurities, which were dissolved into solvent excess. Small difference between the calculated and weighed concentrations (-7.08%) for glycitin encouraged using the higher extinction coefficient of the two published values for glycitein. Choosing the different extinction coefficients would have changed the analysis results of the isoflavones 10–20%. Then results of 7-*O*-glucosides for products 1, 4, 8 and results of aglycones for product 5 would have corresponded to the values given by the producer. On the other hand then the results of aglycones after the hydrolysis for product 3 would have exceeded the value given by the producer.

Efficiency of the extraction method for the isoflavone conjugates was not separately tested. The method of 80% MeOH extraction for the isoflavones, published by Barnes et al. [31] and later applied by Franke et al. [12], supported the idea that 80% EtOH would be suitable solvent to extract the isoflavones from the soy based matrix. The solvent solid ratio in our EtOH extraction method ranged from 500 to 50 ml/g when in the MeOH extraction method of Barnes et al. it was 10 volumes per g [13]. Earlier reported solvent solid ratio in the MeOH extraction method of Barnes et al. was 8 ml/g [31] and Franke et al. reported only the amount of sample, not the volume of the extraction solvent [12]. Wang et al. used acidified ACN and the solvent solid ratio in the method was 6 ml/g [14]. This was the method widely referred by the companies producing different soy based health products and supplements. Even though the solubility of the isoflavones would be poorer into EtOH than into MeOH or ACN, which is not likely, the solvent excess in our method would guarantee the efficient extraction. That was also confirmed when the total amount of isoflavones in aglycones after the hydrolysis was compared with the total amount of isoflavone 7-*O*-glucosides in the samples 1, 4, 8 and 10.

Analyzed molar amount of isoflavone conjugates was the same than the molar amount of isoflavones in aglycones after the hydrolysis. This comparison also confirmed the efficiency of the hydrolysis steps, which were earlier evaluated by Mazur et al. [29]. Recently it was published a new comparison of the different extraction solvents [17]. Compared extractions were 80% MeOH [31], acidified ACN [14], and 60% ACN. 80% MeOH and acidified ACN was found to be few percentages less efficient than new 60% ACN extraction. Solvent solid ratio in 60% ACN extraction was 20 ml/g and the most of the isoflavones were extracted already in few minutes.

Quantitative results of the isoflavones in aglycones measured after the hydrolysis were mainly lower than the values given by the producers. It was concluded that the values given by the producers referred to the total amount of isoflavones in conjugates. Conversion of the results to the conjugates would not change the results to correspond to the producer values. The most remarkable quantitative finding was the isoflavone content of two supplements, products 2 and 9. Those products contained isoflavones in similar amounts like two alcohol washed soy protein products, but were still marketed as an isoflavone supplements. Product 3 was manufactured to contain isoflavones in aglycones the same amount, which was the value given by the producer.

Product data available for the consumers were found from web pages of the manufacturers, from the free product leaflets and from the product packages. In general, on the web pages of the producers or marketing companies there were several links to various scientific publications and reports showing statistically significant correlation in favor of positive health effects of soy isoflavones. Product leaflets were direct prints of the web pages or vice versa. Text in the product labels itself was careful and only hints of the possible health effects were given, except in the data of product 5, which was directly recommended to prevent osteoporosis. In the studied products the amount of isoflavones was very often expressed in an ambiguous way and the units were unclear. None of the products contained the information whether the amount of the isoflavones in the product data was given in aglycones or in conjugates. According to the present results the real daily doses of isoflavones in aglycones obtained by consuming these products are only moderate or low, when compared with the amounts available in natural soy based foods [12,14,30]

5. Conclusion

The new HPLC–CEAD method is sensitive compared with the conventional HPLC–UV or DAD methods. Hydrolysis steps in the pretreatment minimize the need of different standards, which is useful for those who analyze soy samples only occasionally. On the other hand, chromatographic method was optimized to separate all 12 forms of isoflavones and method can be applied to analyze isoflavones in conjugates. Pretreatment is complicated if compared with the direct conjugate extractions with solvent. Benefit of this complicated pretreatment is that the same samples can be purified further and analyzed using GC–MS method [29], if additional data is needed.

If some health effects are expected after the soy supplementation, they are exclusively related to the active part of the isoflavone molecule, which is the aglycone without sugar moiety. The weight of the glucose unit is approximately 40% of the total glucoside weight and in the case of malonyl and acetyl forms the weight of the inactive sugar moiety increases up to 50%. Researchers, health practitioners and consumers are not always aware of these aspects. Therefore, recommendable way to express the isoflavone content of the supplements is to use the weight of the aglycones and/or the molar amounts of isoflavones. The amount of isoflavones should be connected to serving unit i.e. tablet, capsule or g of the powder. Then the amount of the supplemented isoflavones can be easily evaluated.

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