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Determination of isoflavones in nutritional supplements by HPLC with coulometric electrode array detection

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Abstract In the present study eleven soy and/or red clover based supplements were analyzed after acid hydrolysis or direct extraction with 70% aqueous ethanol for their content of total isoflavone aglucones and free aglucones plus glucosides by high performance liquid chromatography in gradient elution mode coupled with coulometric electrode array detection. The ratio of the individual isoflavones and the conjugation pattern turned out to be highly variable in the investigated products. Whereas red clover based supplements contained isoflavones (mainly formononetin and biochanin A) exclusively in free form, soy containing preparations showed variable proportions of conjugated isoflavones. The total isoflavone aglucone content in products intended for direct consumption ranged from 12.0 to 45.6 mg per capsule or tablet, whereas the content of free aglucones and glucosides was between 12.2 and 51.6 mg per capsule or tablet. The experimentally determined isoflavone aglucone content was in agreement with or higher than the manufacturer's claim in **6** of the **11** investigated products. Expression of the isoflavone content as a sum of free aglucones and glucosides raised the number of matched claims to **8**.

Keywords Phytoestrogens; Supplements; Reversed phase high performance liquid chromatography; Electrochemical detection.

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Introduction

Isoflavones, the main phytoestrogens in soy and red clover, have conquered the market in the form of nutritional supplements in the last years. Whereas classical hormone replacement therapy is associated with increased risk of mammary cancer and, contrary to earlier hopes [1], of cardiovascular disease [2], isoflavones have been promoted as multifunctional natural remedies capable of lessening climacteric troubles, of protecting against osteoporosis and arteriosclerosis, and of reducing the risk of hormone dependent cancers. Comprehensive reviews on the biological effects of isoflavones have been published [3–6].

Isoflavones occur naturally as partly malonylated or acetylated β-glucosides and, to a minor degree, as aglucones [7–12]. However, despite the impact the conjugation status may have on isoflavone bioavailability [13–15], and despite the fact that the biologically active compound is the aglucone, there is little regulation about labelling and manufacturers of nutritional supplements neglect to state whether the claimed isoflavone contents are expressed in aglucone equivalents or as a sum of free aglucones and glucosides. In addition, no information on the content of individual isoflavones is available, although it cannot be assumed that all isoflavones are comparable in their pharmacokinetics and bioavailability [15, 16].

Several analysis methods for soy and red clover isoflavones have been published. Some include cleavage of isoflavone gluco-conjugates by acid, basic,

or enzymatic hydrolysis and quantitation of the glucosides [17, 18] or of the aglucones [9, 19–25], others are based on direct extraction and analysis of the native forms by RP-HPLC [7, 10, 12, 15, 17, 26–29]. The advantage of direct extraction – performed under carefully chosen conditions to avoid the degradation of the relatively unstable malonyl- and acetylglucosides [7, 10] – is that valuable information about the native forms is obtained. However, quantitation of malonyl- and acetylglucosides is tricky because of the limited stability of the standard compounds. In addition, direct extraction of isoflavones from soy and red clover containing products gives rise to complex chromatograms, so that long LC-run times [17] and/or analysis by LC-MS are required. Therefore, the native compounds are often hydrolyzed to simpler forms. Mild basic hydrolysis can be used to remove malonyl and acetyl groups from the sugar-part of malonyl- and acetylglucosides [17]. Acid and enzymatic hydrolysis, on the other hand, are suitable for converting isoflavone conjugates directly into aglucones. As the completeness of enzymatic hydrolysis depends on whether the chosen enzyme (usually sulfatase and glucuronidase from *Helix pomatia*, cellulase, or β-glucosidases from almonds or *Escherichia coli*) is capable of cleaving conjugated glucosides (which is not necessarily the case [30]), acid hydrolysis is usually preferred.

The commonly used detection techniques for isoflavones are UV [7, 9, 10, 28, 29], UV-DAD [12, 17, 20, 26], coulometric electrode array detection (CEAD) [18, 19, 21, 23, 25] and, especially after direct extraction without acid, mass spectrometry [9, 15, 27, 29]. UV-detection is simple and reliable, but shows a lack of selectivity and sensitivity. UV-DAD is superior to UV in terms of selectivity, but still not suitable for quantitation of co-eluting compounds. A versatile alternative is CEAD which is **1**–**2** orders of magnitude more sensitive than UV-detection and selective for electrochemically active compounds. The main advantage of CEAD is that co-eluting compounds can be reliably quantitated if they are oxidized or reduced at different potentials [31]. In addition, CEAD is a useful tool for peak purity checks and furnishes information on the presence of further compounds with the same or similar electrochemically active groups. CEAD has been applied successfully to the detection of soy isoflavones [18, 19, 21, 23, 25], but not yet been used for the analysis of red clover constituents. LC-MS is the technique of choice for identification and structure elucidation purposes, but quantitation in complex mixtures may be affected by suppression or enhancement effects from matrix compounds.

The aim of the present work was to determine the isoflavone pattern in **11** different red clover and/or soy based supplements and to compare the experimentally obtained contents of both total aglucones and free aglucones plus glucosides with the manufacturer's claim. For that purpose an HPLC-method coupled with coulometric electrode array detection for the determination of the main soy isoflavones daidzein, genistein, and glycitein and for their metabolic precursors, the red clover isoflavones formononetin, and biochanin A, after acid hydrolysis or direct extraction, respectively, should be developed.

Results

Chromatographic separation and detection

Isoflavones were separated by RP-HPLC using gradient elution and detected at eight channels between +300 and +800 mV. After preliminary experiments with supplements confirming its absence, the lignan pinoresinol was chosen as internal standard because it eluted in the centre of the chromatogram and could be detected in the same channel as the soy isoflavones.

Validation of the HPLC method

Calibration functions in the investigated concentration range (20–2946 $\mu g/dm^3$) were linear with correlation coefficients $r \geq 0.9994$ for all analytes. Limits of detection ($S/N = 3$) in standard solutions ranged from 6.2 to 13.5 $\mu g/dm^3$ (62 to 135 pg on column), limits of quantitation ($S/N = 10$) from 20.7 to 45.0 $\mu g/dm^3$ (201–450 pg on column).

The repeatability of retention times (expressed as relative standard deviation, *RSD*, of 5 injections) was between 0.09 (biochanin A) and 0.40% (glycitein) intra-day and between 0.45 (biochanin A) and 1.07% (daidzein) inter-day. The intra-day repeatability of the detector response ranged from 0.81 (glycitein) to 1.84% (biochanin A) whereas the inter-day repeatability of detection was between 2.75 (daidzein) and 6.90% (glycitein). In order to compensate for the inter-day variability calibration functions were established for each analyte in the course of each analysis run.

Acid stability of isoflavone aglucones

To make sure that isoflavone aglucones are not destroyed upon hydrolysis with 1 mol/dm^3 ethanolic hydrochloric acid for 90 min an isoflavone aglucone standard mixture was investigated under the same experimental conditions. The results (recoveries: daidzein: 101.4%, glycitein: 98.7%, genistein: 96.2%, formononetin: 97.9%, and biochanin A: 100.9%) confirmed that isoflavone aglucones are stable under the chosen conditions.

Analysis of supplements

Analytes were identified by comparison of the relative retention times and the current-voltage curves (CVCs) in sample extracts with those in standard solutions. CVCs served also as purity check and for the determination of the detection potentials of the individual isoflavones. A typical coulometric electrode array chromatogram of a sample extract after acid hydrolysis is presented in Fig. 1.

To examine the recoveries in the samples isoflavone standards were added to 10 mg aliquots of sample **8** *prior to* acid hydrolysis and prior to direct extraction with 70% aqueous ethanol, respectively. The recoveries (Table 1) proved to be satisfactory for all analytes.

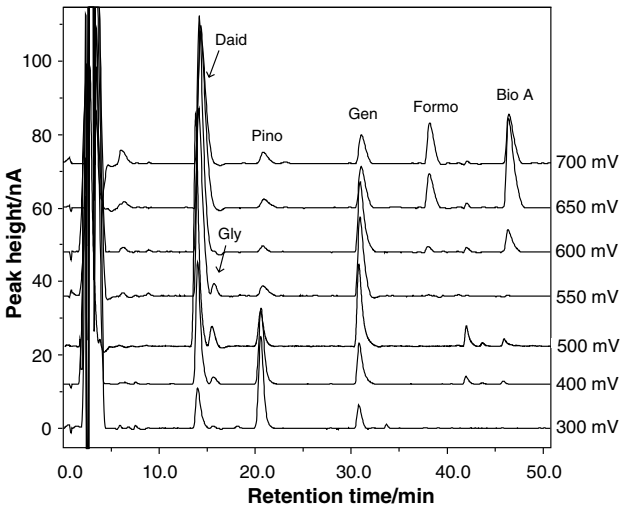

Fig. 1 Coulometric electrode array chromatogram of product **8** after acid hydrolysis. *Daid* Daidzein, *Gly* glycitein, *Pino* pinoresinol (IS), *Gen* genistein, *Formo* formononetin, *Bio A* biochanin A

Quantitative analysis

The contents of the individual isoflavone aglucones (Table 2) are expressed in mg/g tablet, capsule content, or powder in order to provide a uniform basis for comparison of the different preparations intended for consumption. The relative standard deviations of

Table 1 Recovery (%) of isoflavones upon sample preparation. Values are means of duplicate determinations for each sample preparation technique

	Daidzein	Glycitein	Genistein	Formononetin	Biochanin A
Acid hydrolysis	101.6	110.5	97.1	94.0	94.4
Direct extraction	100.9	110.7	95.1	92.1	99.2

Table 2 Content of the individual isoflavones in aglucone equivalents. Values are means of duplicate or triplicate analyses and given in mg/g capsule content. *n.d.* Not detected

Product	Daidzein	Glycitein	Genistein	Formononetin	Biochanin A	Total isoflavones
1	1.4	0.3	1.2	18.4	8.5	29.8
2	1.1	n.d.	1.8	40.6	40.5	84.0
3	0.3	0.2	0.4	9.3	6.7	16.9
4	0.9	0.5	2.2	53.5	50.0	107
5	0.9	0.5	1.1	9.8	4.0	16.3
6	20.7	2.2	12.3	20.9	21.6	77.7
7	25.2	7.3	5.9	1.9	0.5	40.9
8	20.3	3.6	7.7	10.0	9.4	51.0
9	14.8	1.4	7.0	15.4	6.6	45.2
10	262	37.5	81.7	n.d.	n.d.	381
11	9.2	3.5	10.6	n.d.	n.d.	23.3

Table 3 Content of total aglucones and free aglucones plus glucosides a) in mg per capsule or tablet and b) in % of the manufacturer's claim and actual daily intake following the manufacturer's instructions

Product	Total aglucones (mg/capsule or tablet)	Free aglucones + glucosides (mg/capsule or tablet)	Total aglucones (% of the manufacturer's claim)	Free aglucones + glucosides (% of the manufacturer's claim)	Actual daily intake (mg aglucones)
1	29.8[a]	30.3[a]	29.8	30.3	–[b]
2	40.1	40.3	100.2	100.2	40.1–80.2
3	20.5	20.9	102.7	104.1	41.1
4	45.6	46.5	114.0	114.0	45.6
5	12.0	12.2	29.6	29.9	12.0–24.0
6	18.6	20.4	92.9	97.3	37.2
7	21.3	29.7	47.4	65.7	21.3
8	21.2	24.4	84.7	97.4	42.4
9	45.4	49.3	100.9	109.5	45.4–90.8
10	381[a]	433[a]	95.3	108.2	–[b]
11	32.7	51.6	65.3	101.8	32.7

[a] mg/g; [b] Not intended for direct consumption

duplicate sample work-up and analysis ranged from 0.4 to 3.7% for the content of total isoflavones and were below 5% for individual isoflavones with contents ≥10 mg/g. Relative standard deviations ≥10% were observed only in **2** cases with contents of individual isoflavones below 1.5 mg/g.

Red clover based products (products **1**–**5**) contained mainly formononetin and biochanin A, whereas soy preparations (products **10** and **11**) were rich in daidzein, genistein, and, to a lesser degree, glycitein. Soy and red clover containing supplements (products **6**–**9**) showed variable proportions of the individual isoflavones, reflecting the ratio and the isoflavone concentrations of the original soybean and red clover extracts used to manufacture the product. The concentrations of the total isoflavone aglucones in the supplements intended for direct consumption (all except products **1** and **10**) ranged from 16.9 to 107 mg/g.

All investigated red clover products contained mainly isoflavone aglucones whereas isoflavones in soy containing preparations differed considerably in their conjugation pattern. Interestingly, the red clover isoflavones formononetin and biochanin A occurred mainly in unconjugated form in both solely red clover and soy and red clover based supplements whereas the proportions of daidzin, genistin, and glycitin, the 7-O-glucosides of the soy isoflavones, varied greatly among the different products (Fig. 2). The content of total isoflavone aglucones was significantly lower than the content of free and glucosidically bound isoflavones in all but the solely red clover based supplements (Table 3). Thus, investi-

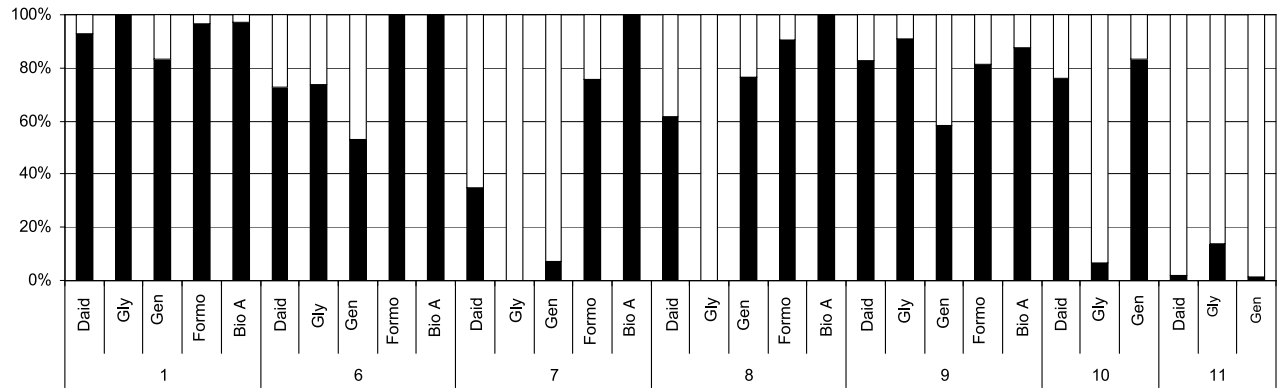

Fig. 2 Proportion of free isoflavone aglucones (■) and isoflavone glucosides (□) to the sum of free aglucones + glucosides per capsule or tablet (100%) for selected products. Product **1** is representative for the products **1**–**5**. *Daid* Daidzein, *Gly* glycitein, *Gen* genistein, *Formo* formononetin, *Bio A* biochanin A.

gated red clover and soy products could be distinguished not only by their isoflavone composition but also by their conjugation pattern.

Discussion

Sample preparation including acid hydrolysis is commonly employed in isoflavone analysis [9, 17, 19, 21, 23, 25]. Considering the conditions used in previously published methods [23, 25], we decided on refluxing in 1 mol/dm^3 ethanolic hydrochloric acid for 1.5 h. Stability tests performed with pure standard compounds under the same conditions supported previously reported data on the acid-stability of the soy isoflavones [25] and confirmed the stability of the methoxylated isoflavones formononetin and biochanin A in acidic environment. The great advantages of acid hydrolysis are that only aglucone standards are required for quantitation, that relatively clean chromatograms are obtained, and that the determined concentrations are already those of the biologically active form.

In order to investigate the conjugation pattern of isoflavones, direct extraction is required. Conditions reported in the literature differ in the type of solvent used, the sample to solvent ratio, the extraction temperature as well as in the technique, and the duration of extraction [7, 10, 12, 15, 17, 22, 26, 27]. Contrary to authors who aim at identification and quantitation of isoflavones in their native forms, our main objective in direct extraction was to determine the content of free isoflavone aglucones. Using 70% aqueous ethanol in large excess compared to the amount of sample (1 cm^3 of solvent for 4 to 6 mg of sample), aglucone recoveries close to 100% could be achieved. The supplements were well soluble in the extraction solvent, so that filtration was required only prior to HPLC analysis, thereby rendering the method fast and little error-prone due to few sample transfer steps.

As the proposed determination of the content of isoflavone glucosides does not take into account the possible presence of malonylated and acetylated isoflavone glucosides [15, 21], the actual concentrations of isoflavone conjugates might be higher in soy containing supplements where isoflavones occur both in free and conjugated form. However, malonyl- and acetylglucosides are of limited stability [10] and it is possible that they are – at least in part – transformed into the corresponding glucosides already upon extract preparation by the manufacturer. This assumption is supported by the finding that all red clover preparations contained exclusively isoflavone aglucones whereas red clover itself shows a highly complex conjugation pattern [8–11].

Experimentally determined isoflavone contents of nutritional supplements were generally lower than the manufacturer's claim in previously published papers [15, 21]. *Nurmi et al.* [21] reported that only one out of **15** analyzed products matched the claimed value if the results were presented in aglucone equivalents. They concluded that manufacturers label their products with the isoflavone content in conjugates. However, results did not correspond to the claimed values even if they were expressed in conjugates. *Setchell et al.* [15] did not correct the experimentally determined isoflavone content of soy and red clover based nutritional supplements for the aglucone equivalency and obtained a better score. **12** out of **31** investigated products were within $\pm$15% of the manufacturer's claim, **15** were below and **4** supplements contained significantly higher amounts of isoflavone conjugates than stated by the producer (experimental values up to 3.8 fold higher).

In our study, 3 of the 5 investigated red clover preparations matched the manufacturer's claim ($\pm$15%), whereas **2** products contained only $\sim$30% of the stated value. As red clover nutritional supplements contained hardly any conjugated isoflavones, expression of the isoflavone content as sum of free and glucosidically bound isoflavones did not alter the results. Two out of the **4** analyzed soy and red clover containing products deviated by less than $\pm$15% of the claimed content when experimental values were expressed in aglucone equivalents. Conversion into the conjugate content raised the score to **3** out of **4** products. However, product **9** which closely matched the manufacturer's claim on an aglucone basis came to deviate by 9.5% when glucosides were counted as conjugates. The **2** purely soy based preparations contained both isoflavone aglucones and glucosides, but in different ratios. Whereas product **10** consisted to 78% of free isoflavone aglucones, product **11** contained 94% of its isoflavones in conjugated form. Expression of the isoflavone content in conjugates yielded a slightly higher value than the manufacturer's claim for product **10**. Agreement of the experimentally determined isoflavone content of product **11** with the stated value, on the other hand, was contingent on this measure.

Comparison of experimental values with the manufacturer's claim both in aglucone equivalents and

conjugates shows that some producers label their supplements with the aglucone value whereas others state the conjugate value. Information whether the isoflavone content was given in aglucone equivalents or conjugates could not be found for any of the investigated products. In general, product information was unsatisfactory concerning the isoflavone components, the main reason why consumers buy these products. Whereas the composition of nutritional supplements was clearly stated in the ingredient list (including the percentage of red clover and/or soybean extract) neither the content of the individual isoflavones nor the ratio of aglucones to glucosides was given for any of the **9** analyzed supplements.

Both in previously published papers [15, 17, 21, 32] and in the present study the isoflavone composition of different supplements was highly variable due to the use of different plant parts with different isoflavone pattern, different extraction procedures and the variable isoflavone profile in different soybean and red clover varieties. Following the manufacturer's recommendations, between 12.0 and 90.8 mg isoflavone aglucones would be ingested per day (Table 3). Whereas 12.0 mg might not be sufficient for procuring biological effects, 90.8 mg are higher than the medium isoflavone intake in Japan and China where soy is a staple (30–40 mg) [33]. However, recently *Barnes* [33] concluded that isoflavones consumed in doses below 2 mg/kg body weight and day may be considered safe for most population groups.

To conclude, the open-minded consumer should be given the chance to compare the prices of isoflavone supplements on the basis of their content of biologically active aglucones rather than on the unknown ratio of free aglucones to isoflavone glucosides which consist to ~40% of cheap sugar. In order to enable the consumer to choose the best suited product out of the large variety available on the market, sufficient reliable and unambigous information about the isoflavone aglucone content, the conjugation pattern, and the ratio of the individual isoflavones should be provided by the manufacturer.

Experimental

Reagents and standards

Genistein (>98% purity), glycitein and biochanin A (both >97%), were purchased from Sigma (St. Louis, MO, USA), daidzein (>98% purity) and formononetin (>99%) from Indofine Chemical Company Inc. (Hillsborough, NJ, USA). Pinoresinol (96% purity) was obtained from Arbonova (Turku, Finland). Hydrochloric acid, absolute ethanol (*p.a.*), acetonitrile (Lichrosolve gradient grade), glacial acetic acid, sodium acetate trihydrate, as well as sodium hydroxide pellets were from Merck (Darmstadt, Germany). Deionized water prepared using a Barnstead EASY pure LF (Dubuque, IO, USA) was used in all experiments.

Isoflavone and pinoresinol standards (1.785 to 2.226 mg) were dissolved in 10 cm^3 absolute ethanol resulting in stock solutions containing 196.5 mg/dm^3 daidzein, 178.5 mg/dm^3 genistein, 182.2 mg/dm^3 glycitein, 178.5 mg/dm^3 formononetin, 198.3 mg/dm^3 biochanin A, and 222.6 mg/dm^3 pinoresinol. These solutions were stored in tightly sealed 10 cm^3 volumetric flasks at 4°C under exclusion of light. For the preparation of mixed standard solutions the isoflavone stock solutions were diluted 1:10 with mobile phase A and 4 cm^3 aliquots of the resulting solutions were combined in a 25 cm^3

Table 4 Product data

Product	Constituents[a]	Product type	Weight of 1 capsule or tablet (mg)[b]	Claimed isoflavone content (mg/capsule or tablet)	Recommended daily intake (No. of capsules or tablets)
1	R	powder	–	10.0%	*
2	R	capsule	477.3	40.0	1–2
3	R	tablet	1212.0	20.0	2
4	R	capsule	425.6	40.0	1
5	R	capsule	734.8	40.5	1–2
6	R (8.5%), S (8.5%)[c]	capsule	239.3	20.0	2
7	R (16.4%), S (8.2%)	capsule	521.8	45.0	1
8	R (7.4%), S (4.9%)	capsule	415.2	25.0	2
9	R (6.1%), S (24.3%)	tablet	1003.3	45.0	1 (2)
10	S	powder	–	40.0%	*
11	S	tablet	1383.0	50.0	1

[a] *R* Red clover; *S* soy; [b] Average of 5–6 capsules or tablets; [c] Percentage of soy and red clover extract on the total weight of one capsule or tablet; * Not intended for direct consumption

flask and adjusted to the mark with mobile phase A. Different volumes of the so obtained mixed stock solution were diluted with mobile phase A and 650 mm^3 of each dilution were combined with 50 mm^3 of pinoresinol (5565 $\mu g/dm^3$, internal standard), yielding **9** mixed isoflavone solutions in a concentration range from 13 to 2946 $\mu g/dm^3$.

Samples

Isoflavone supplements were purchased at local pharmacies and drugstores or provided directly from the manufacturer. A detailed description is given in Table 4. Prior to analysis the content of five or six capsules was weighed separately and pooled. Likewise, the weight of 5 or 6 individual tablets was recorded, and the tablets were finely ground by the aid of mortar and pestle and combined to one sample. The average weight of the content of one capsule or tablet was calculated. Pooled samples were stored in tightly screwed scintillation vials at 4°C.

Instrumentation

The HPLC system consisted of a Merck – Hitachi L-6200 intelligent high pressure gradient pump, a Merck – Hitachi AS-2000 autosampler equipped with a 100 mm^3 stainless steel injection loop (Rheodyne, Cotati, CA, USA), an ACE C18 column (3 μm, 150 × 3 mm I.D., Advanced Chromatography Technologies, Aberdeen, Scotland) equipped with a guard-column of the same stationary phase and an ESA Coulochem Electrode Array Detector (ESA, Chelmsford, MA, USA) controlled by a computer having installed ESA chromatographic software CoulArray® for Windows® 32 (version 1.0). The coulometric electrode array detector consisted of 2 four channel cells (each channel equipped with one porous graphite working electrode, 2 platinum counter electrodes, and 2 palladium reference electrodes).

Chromatographic separation and detection

Isoflavones were separated by RP-HPLC in gradient elution mode. Mobile phase A was prepared by mixing 230 cm^3 acetonitrile with 770 cm^3 membrane filtered (PTFE-filters 0.45 μm, Sartorius, Göttingen, Germany) 10 $mmol/dm^3$ sodium acetate buffer (adjusted to *pH* 4.8 with glacial acetic acid). Mobile phase B consisted of acetonitrile-sodium acetate buffer (*pH* 4.8; 10 $mmol/dm^3$) (50:50, *v*/*v*). Both eluents were degassed in their storage bottles by sonication for 15 min.

Standard solutions or sample extracts (10 mm^3) were injected into the chromatographic system and the compounds were separated at room temperature (24°C) at a flow rate of 0.4 cm^3/min according to the following gradient profile: 0–13 min: 0% B, 13–30 min: linear increase to 70% B, 30–40 min: linear increase to 100% B, 40–46 min: isocratic at 100% B, 46–48 min: decrease to 0% B, 48–64 min: reequilibration at 0% B. The potentials applied between working electrode and reference electrodes of the CEAD were +300, +400, +500, +550, +600, +650, +700, and +800 mV.

Calibration functions, limits of detection, intra- and inter-day repeatability in standard solutions

Calibration functions for daily quantitation ($N = 6$) were recorded in a concentration range from 20.7 to 2946 $\mu g/dm^3$ and established by linear regression of the peak height ratios of the analytes to the internal standard pinoresinol on the concentrations. Daidzein, glycitein, genistein, and pinoresinol were evaluated in channel 3 (+500 mV), biochanin A in channel 6 (+650 mV), and formononetin in channel 7 (+700 mV). The limit of detection of each analyte was calculated for $S/N = 3$ on the basis of calibration functions through the origin ($N = 9$, concentration range 13 to 2946 $\mu g/dm^3$, peak heights *vs.* concentrations). The intra-day repeatability of the chromatographic method was determined by 5-fold injection of a standard solution containing 566 $\mu g/dm^3$ daidzein, 525 $\mu g/dm^3$ glycitein, 514 $\mu g/dm^3$ genistein, 514 $\mu g/dm^3$ formononetin, 571 $\mu g/dm^3$ biochanin A, and 398 $\mu g/dm^3$ pinoresinol and calculation of the relative standard deviations of the retention times and the peak heights. The inter-day repeatability was assessed on the basis of five injections of a calibration standard containing between 796 and 884 $\mu g/dm^3$ of each analyte and 398 $\mu g/dm^3$ of pinoresinol over the period of 5 days.

Determination of the acid stability of isoflavone aglucones

A 250 mm^3 aliquot of an ethanolic solution containing 28 mg/dm^3 daidzein, 27.8 mg/dm^3 glycitein, 28.5 mg/dm^3 genistein, 28.4 mg/dm^3 formononetin, and 28.3 mg/dm^3 biochanin A was refluxed in 9 cm^3 ethanol and 1 cm^3 10 mol/dm^3 hydrochloric acid at 84°C for 90 min and diluted to 25 cm^3 with mobile phase A after *pH* adjustment to 3–4 using 3 mol/dm^3 sodium hydroxide solution. 650 mm^3 of the resulting solution were combined with 50 mm^3 of pinoresinol standard solution (5526 $\mu g/dm^3$) in an autosampler vial. The experiment was performed twice. A reference solution was prepared by pipetting a 250 mm^3 aliquot of the mixed stock solution into a 25 cm^3 volumetric flask, adding 9 cm^3 ethanol and 5 cm^3 water, and adjusting to the mark with mobile phase A. 650 mm^3 of this solution were combined with 50 mm^3 of pinoresinol standard solution (5526 $\mu g/dm^3$).

Sample preparation

For analysis by acid hydrolysis 20–30 mg of the pooled samples were weighed into a 50 cm^3 round bottomed flask, dissolved in 9 cm^3 ethanol and 1 cm^3 10 mol/dm^3 hydrochloric acid by sonication for ~30 s, and refluxed under stirring at 84°C for 90 min. After cooling to room temperature, the *pH* was adjusted to **3**–**4** with 3 mol/dm^3 sodium hydroxide solution, and the hydrolysate was diluted stepwise to 250 or 500 cm^3 (depending on the analyte concentration) with mobile phase A. Prior to HPLC analysis the solution was filtered through a 13 mm *PTFE* 0.45 μm syringe filter (Alltech, Deerfield, IL, USA) and 650 mm^3 of the filtrate were combined with 50 mm^3 of pinoresinol standard solution (5526 $\mu g/dm^3$) in an autosampler vial. Each sample was worked-up in duplicate and analyzed by HPLC once. In case the total isoflavone contents differed by more than 5%, the sample work-up and chromatographic analysis were repeated and the outlier was excluded.

For analysis by direct extraction 20–30 mg of the pooled samples were dissolved in 3.5 cm^3 ethanol and 1.5 cm^3 water in a tightly sealed 20 cm^3 glass vial equipped with a screw cap and sonicated for 90 min. Subsequent to sonication the extracts were diluted stepwise to 250 or 500 cm^3 with mobile

phase A. The further treatment and the number of samples analyzed were equal to those after acid hydrolysis.

Qualitative analysis

Isoflavones in sample extracts were identified by comparison of both relative retention times and current-voltage curves with those of reference compounds in standard solutions. Current-voltage curves were obtained by plotting the peak heights of the analytes measured in each channel against the applied potential. The principle of coulometric electrode array detection is explained in detail in Refs. [31, 34].

Quantitative analysis

The analyte concentrations in the sample solutions after acid hydrolysis or direct extraction were determined *via* external calibration functions (peak height ratios *vs.* concentrations) using pinoresinol as internal standard. Consideration of the dilution factors, the amounts of sample used for analysis, and the average content of one capsule or tablet yielded the amount of isoflavones contained in one capsule or tablet.

The content of isoflavone glucosides was calculated by subtracting the content of free isoflavones (determined by direct extraction) from the content of total isoflavone aglucones (obtained *via* acid hydrolysis) and by multiplying the difference by the ratio of the molecular weight of the glucoside to the molecular weight of the aglucone.

Determination of the recovery

The pooled sample **8** (10 mg) was spiked with the volumes of ethanolic isoflavone standard stock solutions which contained ~100% of the content of each individual isoflavone aglucone in the weighed portion as determined in previous experiments. Further sample preparation was carried out as described above. The experiments were performed in duplicate. The recovery (R) was calculated for each isoflavone individually by subtracting the amount of the respective isoflavone contained in the unspiked sample (I_{usps}) from the amount determined in the spiked sample (I_{sps}) and by dividing the difference by the amount of isoflavone added to the sample (I_{sp}) according to the following equation:

$$R = \frac{(I_{sps} - I_{usps})}{I_{sp}} \times 100$$

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