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An efficient HPLC method for the quantification of isoflavones in soy extracts and soy dietary supplements in routine quality control

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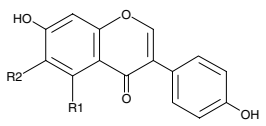
Food supplements containing soy extracts are increasingly used to treat menopausal complaints. For the quality control of such products a RP-HPLC method for the quantification of daidzein, genistein, glycitein and their respective glucosides and acetylglucosides in soy extracts and nutraceuticals was developed and validated. The extraction of the samples is very simple and avoids time-consuming handling and expensive cartridges or reagents. The compounds are quantified by internal standardization with 4′-hydroxyflavanon which is easily available at a reasonable price. On a base-deactivated C_{18} column good separation and excellent peak shape of the analytes are achieved in short time by gradient elution with water and acetonitrile. Accompanying substances do not interfere. The method was applied to the analysis of different commercial soy preparations.

1. Introduction

Numerous animal studies, epidemiologic investigations and several clinical trials suggest a high potential of dietary phyto-estrogens for the prevention of "Western diseases e.g. cardiovascular diseases, different kinds of cancer, arteriosclerosis, osteoporosis as well as for relief of postmenopausal complaints (Ososki and Kennelly 2003; Cornwell et al. 2004; Magee and Rowland 2004; Somekawa et al. 2001). Among the most important phyto-estrogens are isoflavones like daidzein, genistein and glycitein. Soy contains high amounts of glucosides, acetylglucosides and malonylglucosides of these isoflavones. The concentration of single compounds in soy products is depending on the production processes, e.g. malonylglucosides are deesterified easily (Fritsche and Steinhardt 1999).



Daidzein	R1 = H	R2 = H
Genistein	R1 = OH	R2 = H
Glycitein	R1 = OH	R2 = OCH_3

Due to the suggested effects not only the consumption of soy food but also the demand for food supplements rich in isoflavones is strongly increasing. As such products differ substantially (Chua et al. 2004), quality control of these preparations is becoming a very important task.
For the quality control of food supplements containing soy or soy extracts different analytical HPLC-methods were suggested to quantify the isoflavones. Various methods and their advantages and disadvantages were compiled by Wilkinson et al. (2002) and Wang et al. (2002).

Especially small companies producing soy food supplements cannot afford expensive instrumentation such as LC-MS (Wu et al. 2004), LC-MS-MS (Antonelli et al. 2005) or LC coupled with coulometric electrode array detection (Preinerstorfer et al. 2004) and are interested in reliable analyses at low costs.
Thus, in the presented work a respective method was developed, validated and applied to some commercial products.

2. Investigations, results and discussion

Our requirements for a method for the determination of isoflavones and their respective glycosides in soy extracts and soy nutraceuticals were simple sample preparation, short time of analysis, reasonable prices of solvents and reagents, DAD-UV detection and the quantification of by use of an internal standard.
Thus, several recently published methods were not suitable due to long time of analysis (Penalvo et al. 2004) or external standardisation (Penalvo et al. 2004; Ganzera et al. 2004; Heimler et al. 2004; Apers et al. 2004).

2.1. Sample preparation

Many different modes have been investigated for the extraction of isoflavones in soy (Wilkinson et al. 2002; Murphy et al. 2002; Xu et al. 2002; Kao and Chen 2002). To keep the extraction as simple as possible, we decided to use the method of Hsieh et al. 2004 with slight modifications, such as avoidance of defatting of soy flour (see Experimental).
The quantitative extraction of daidzein, genistein, glycitein and their 7-*O*-glucosides was proven by a recovery test for each substance. A sample of a special soy flour en-

Table 1: Recovery of added soy isoflavones

Isoflavone	Concentration calculated in %	Concentration measured in %*	Mean recovery in % (n = 2)
Daidzin	0.675	0.608	89.9
	0.750	0.677	90.4
Glycitin	0.247	0.237	95.9
	0.297	0.266	89.6
Genistin	0.133	0.124	93.2
	0.160	0.141	88.1
Daidzein	0.0438	0.0425	97.0
	0.0525	0.0505	96.1
Glycitein	0.0475	0.0430	90.5
	0.0570	0.0510	89.5
Genistein	0.0140	0.0148	105.7
	0.0166	0.0185	111.4

* mean value of twofold determination of the sample + added isoflavone amount

riched in isoflavones of known content was spiked with two different amounts of each substance. Sample preparation and HPLC were performed as described (see Experimental). The determinations were repeated twice. All mean recoveries were above 88% (see Table 1) and thus superior to those reported by Hsieh et al. (2004). Due to the minor structural differences between the isoflavonglucosides and the respective acetylglucosides a sufficient recovery of the latter compounds seems plausible from the experiments.

In repeated HPLC analyses no problems occurred due to accompanying substances.

2.2. *Chromatographic conditions*

In HPLC-analysis of isoflavones in soy usually RP C_{18} phases are used (Wilkinson et al. 2002). Due to excellent experiences with base deactivated C_{18} phases for the separation of phenolic compounds (Krenn et al. 1998, 1998a, 2002) a Hypersil BDS RP-C18 (250 × 4 mm, 5 µm) column was applied to the system. Gradient elution was performed with acetonitrile and water as mobile phase. No additional modifier was necessary (see Experimental).

Under these conditions a satisfying resolution of the analytes was achieved (see Fig. 1). Reproducibility of retention times was excellent (see Table 2). The hold-up time for calculation of the capacity factors was estimated using the clear solvent front peak.

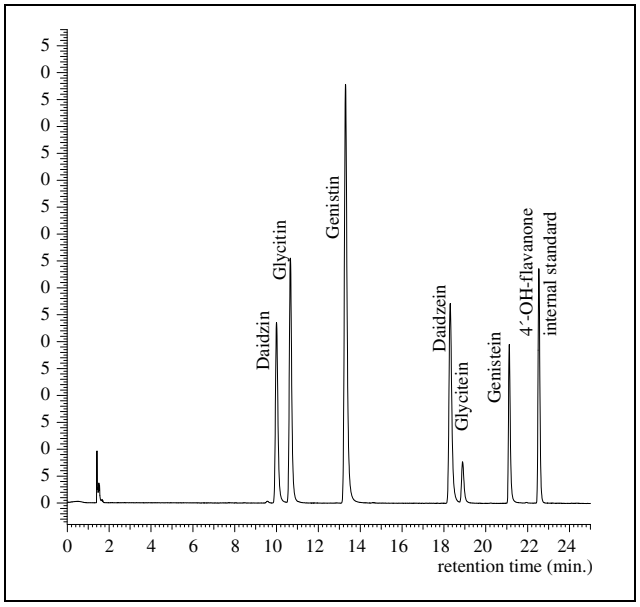


Fig. 1: HPLC of a 6 isoflavone standards and the internal standard 4′-hydroxyflavanone

Table 2: HPLC data for the isoflavones and internal standard based on interday retention times

Compound	Retention time (min)	RSD (%)	Capacity factor k′	Separation factor α
Daidzin	10.14 ± 0.13	1.28	9.23	–
Glycitin	10.77 ± 0.12	1.11	9.86	1.069
Genistin	13.40 ± 0.13	0.97	12.51	1.269
Acetyldaidzin	16.05 ± 0.10	0.62	15.18	1.214
Acetylglycitin	16.54 ± 0.09	0.64	15.68	1.033
Daidzein	18.39 ± 0,12	0.65	17.54	1.119
Glycitein	18.95 ± 0.09	0.47	18.11	1.032
Acetylgenistin	19.17 ± 0.07	0.37	18.33	1.012
Genistein	21.17 ± 0.06	0.28	20.35	1.110
6-Methoxy-flavanone (IS)	22.57 ± 0.05	0.22	21.76	1.069

Data are means from fifteen analyses

2.3. *Quantitative analysis*

Several substances e.g. formononetin (Hsieh et al. 2004), 6-hydroxyflavanone and 4′-hydroxyflavanone were tested for their applicability as internal standard and the latter proved to be the optimal one. 4′-Hydroxyflavanone elongates analysis time only marginally, is commercially available at a very reasonable price as compared to formononetin and shows a UV maximum at 252 nm, very close to the wavelength of 254 nm used for detection.

The system was calibrated for daidzein, genistein, glycitein and their 7-*O*-glucosides with a five point regression curve

Table 3: Characteristics of the analytical method derived from standard calibration set

Compound	Detection* limit (ng)	Quantification* limit (ng)	Intercept	Slope	Correlation coefficient
Daidzin	11.8	39.4	0.0000	1.2226	0.9993
Glycitin	10.7	35.8	0.0157	0.7720	0.9978
Genistin	7.9	26.0	0.0037	1.0882	0.9995
Daidzein	8.8	29.3	0.0054	0.8003	0.9998
Glycitein	9.1	30.2	−0.0024	1.1343	0.9983
Genistein	5.2	17.3	0.0023	1.4783	0.9997

* The limit of quantification was defined as amount of analyte showing a signal-to-noise-ratio of 10 to 1, the limit of detection as amount of analyte showing a signal-to-noise-ratio of 3 to 1.

Table 4: Reproducibility of the method

Compound	Inter-day (n = 15)*		Intra-day (n = 5)	
	Amount mg/g	RSD (%)	Amount mg/g	RSD (%)
Daidzin	6.05 ± 0.41	6.79	5.88 ± 0.30	5.09
Glycitin	1.99 ± 0.12	6.03	1.86 ± 0.05	2.96
Genistin	1.08 ± 0.08	7.41	0.98 ± 0.04	3.59
Acetyldaidzin	4.37 ± 0.28	6.41	4.09 ± 0.17	4.12
Acetylglycitin	1.52 ± 0.09	6.21	1.42 ± 0.05	3.69
Daidzein	0.35 ± 0.05	13.82	0.32 ± 0.02	5.75
Glycitein	0.39 ± 0.03	7.88	0.37 ± 0.01	3.05
Acetylgenistin	0.96 ± 0.07	6.89	0.88 ± 0.04	4.02
Genistein	0.11 ± 0.02	14.97	0.09 ± 0.004	4.86
total flavanone (IS)	16.82 ± 1.04	6.18	15.88 ± 0.67	4.22

* data from analyses within six weeks

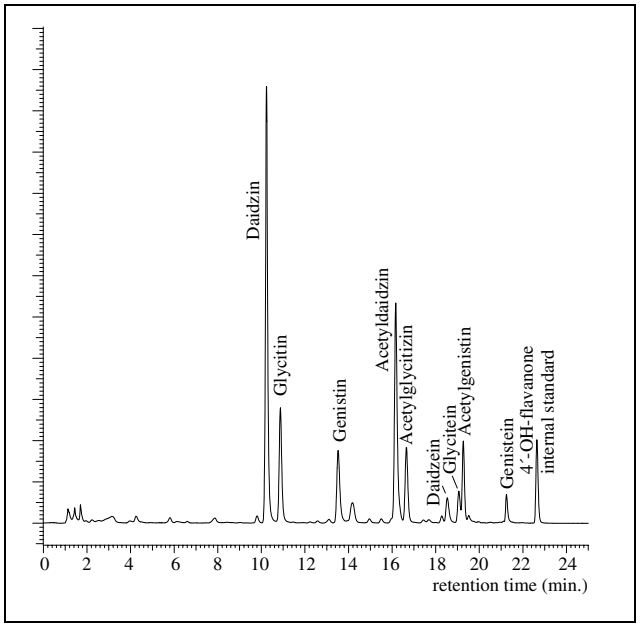


Fig. 2: HPLC of a soy sample (extraction see experimental) internal standard 4′-hydroxyflavanone

in the range of 0.05 to 0.3 mg/ml for the genins and 0.1 to 0.8 mg/ml for the glucosides. Over the selected range peak areas were linearly dependent on concentrations for all compounds with correlation coefficients of >0.9978 (see Table 3). The calibration ranges adequately covered the variations in amounts of isoflavones in the samples.

For the acetylglucosides the correction factors were calculated from those of the respective glucosides under consideration of the molecular weights.

The inter-day reproducibility of the method was determined by fifteenfold quantification of a soy flour sample over a period of six weeks. The relative standard deviations between 6.0 and 7.5% for most of the single compounds and the total isoflavone content proved satisfying accuracy and reproducibility of the method. Only for daidzein and genistein as analytes at lowest concentration in the investigated sample the deviation was higher (see Table 4).

Intra-day reproducibility after fivefold determination resulted in relative standard deviations between 3.0 and 5.7%.

For the investigated sample an analysis report (Ralston Analytical Laboratories, Saint Louis, USA) had been provided. The determined concentrations were in good agreement with this report (see Fig. 2).

The limits of detection were calculated experimentally at signal-to-noise ratios of 3 to 1 and the limits of determination of 10 to 1 (see Table 3).

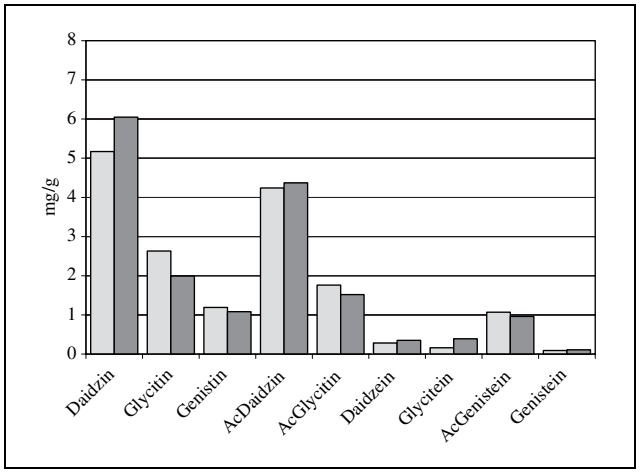


Fig. 3: Comparison of analysed isoflavone amounts in a soy flour sample with the certified contents. Ac = 6″-acetyl
Certificate; Analysed amounts

2.4. *Analysis of soy food supplements*

With the new method three food supplements were analysed. In these samples daidzin, genistin and glycitin were the main compounds. The concentrations of daidzin and genistin were comparable, the amount of glycitin was about one sixth to one fifth of the two other glucosides. Only small amounts of acetylglucosides and genins were quantified. Malonylglucosides were not detected. The determined amounts of isoflavones were between 90.7 and 108.8% of the declaration at the packages of the food supplements.

The analyses of different soy extracts resulted in varying composition of the analytes: In two samples the pattern was comparable to the one described above for the investigated nutraceuticals. In contrary, one sample contained mainly daidzein and genistein. The concentration of daidzin and genistin were approximately only two thirds of the amounts of the genines.

In the enriched soy flour which was used for the elaboration and validation of this method daidzin and acetyldaidzin were the main compounds (see Table 4).

3. Experimental

3.1. *Chemicals*

Daidzein and genistein were purchased from Sigma Chemical Co. (St. Louis, USA), glycitein from Indofine Chemical Comp. (Hillsborough, USA), daidzin, genistin and glycitin from ChromaDex (Santa Ana, USA), formononetin and 4′-hydroxyflavanone from Carl Roth AG (Karlsruhe, Germany). Acetonitrile (HPLC grade) and DMSO (analytical reagent grade) were obtained from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade.

3.2. *HPLC*

HPLC was performed on a Merck Hitachi system consisting of a LaChrom Pump L-7100, a Programmable Autosampler L-7250, an Interface D-7000 and a LaChrom Diode Array Detector L-7450 (Merck, Vienna, Austria). For the separation of the isoflavones a 250 × 4 mm I.D. Hypersil BDS-C18 column with 5 µm particles (Shandon, Runcorn, UK) was operated at room temperature. The eluent consisted of water (A) and acetonitrile (B). The gradient profile was: 0–15 min from 8 to 25% B, 15–18 min from 25 to 35% B, 18–19 min from 35 to 45% B, 19–23 min 45% B. The flow rate was 1.5 ml/min. The column was purged after every run with 100% B for 5 min followed by equilibration for 10 min with 8% B. The wavelength of detection was 254 nm.

3.3. *Standard calibration*

From stock solutions of 1 mg/ml daidzein, 1 mg/ml genistein, 1 mg/ml glycitein, 3 mg/ml daidzin, 3 mg/ml genistin and 3 mg/ml glycitin standard mixtures were prepared. For all solutions dimethylsulfoxide was used as solvent. The resulting mixtures were stored at 4 °C. Calibration graphs were obtained using five mixtures with all standards at different concentrations. 40 µl of internal standard solution (6 mg/ml 4′-hydroxyflavanone) were added automatically by the autosampling system to 500 µl of standard mixture. 5 µL volumes of these solutions were analysed in duplicate.

3.4. *Samples and sample preparation*

Different samples of soy extracts and food supplements were obtained from Melbrosin International (Vienna, Austria), a soy flour from Soya Austria (Vienna, Austria). 500 mg of soy flour were extracted with 5 ml of a mixture of acetone and 0.1 N HCl (5 + 1) under stirring for 2 h at room temperature. After centrifugation and washing of the residue the extract was evaporated under reduced pressure. The extract was dissolved in 5.00 ml methanol, to 500 µl of the solution 40 µl of standard solution

(6.0 mg 4′-hydroxyflavanone/ml) were added and 5 µl of the mixture were analysed. In analyses of extracts or food supplements with high isoflavone concentrations a solution of the extract (4 mg/ml in dimethylsuphoxide-water 3 + 1) is prepared by sonification for 15 min and centrifugation. 500 µl of this solution are mixed wiht 40 µl of standard solution as described.

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