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Fast high-performance liquid chromatography method for quality control of soy extracts

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Abstract

Soy extracts contain a mixture of isoflavones belonging to the group of phytoestrogens. In the quality control of soy the amount of isoflavones, both aglycones and glycosides, is usually determined by means of reversed-phase HPLC–UV. On conventional C_{18}-material columns, long analysis times are required in order to separate this complex mixture. In order to speed up analysis, the separation was optimized using two linked monolithic silica-based reversed-phase C_{18} columns. A spectacular decrease of the analysis time, i.e. almost three-fold, was achieved by applying a flow rate of 3–4 ml/min without loosing any separation efficiency. This analysis method for determination of isoflavones in soy extracts in less than 25 min was fully validated according to the ICH guidelines.
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Keywords: *Glycine max*; Food analysis; Isoflavones; Aglycones; Glycosides

1. Introduction

Dietary intake of soy [*Glycine max* (L.) Merr.] has wide-ranging beneficial effects on health. Owing to the growing evidence suggesting that phytoestrogens might protect against various cancers, cardiovascular diseases, osteoporosis and menopausal symptoms [1], many commercial preparations of isoflavone extracts from soy are marketed as nutritional supplements and phytotherapeutic preparations.

Isoflavones are a class of phytooestrogens found in a variety of plants including soybeans. To date, twelve isoflavones have been characterized in soybean products, i.e. the aglycones daidzein, genistein and glycitein, the β-glycosides thereof (daidzin, genistin and glycitin), and their respective acetylated glycosides, i.e. 6″-*O*-acetyl-β-glycosides and 6″-*O*-malonyl-β-glycosides. The 6″-*O*-malonyl-β-glycosides, which are the predominant natural compounds, are converted into the corresponding deacylated glycosides during processing of soybeans and soyfoods or during sample preparation and analysis [2]. Several methods for determination of the isoflavone content have been published [2–5]. After preparation of the samples, the test solutions are usually analyzed on an analytical reversed-phase (RP) C_{18} (250 mm × 4.6 mm) column by applying a mobile phase gradient starting from 15% and going in several steps to 90% acetonitrile in water (containing acid) in about 60 min. Most of the soy extracts analyzed in our laboratory contain isoflavone aglycones as well as their glycosides. Owing to the substantial difference in their concentrations, two HPLC runs are necessary to determine the total amount of isoflavones. Analyzing samples in duplicate leads to a total analysis time of about 4 h. To overcome this problem an HPLC method using monolithic silica-based reversed-phase C_{18} columns was developed and fully validated according to the ICH guidelines.

2. Experimental

2.1. Solvents, standards and standard solutions

2.1.1. Solvents

Distilled water (RiOs) prepared with a Millipore water purification system (Millipore, Bedford, MA, USA) was used. Acetonitrile (ACN, HPLC quality), methanol (MeOH,

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HPLC quality), acetic acid (analytical-reagent grade) and dimethyl sulphoxide (analytical-reagent grade) were purchased from Acros Organics (Geel, Belgium).

2.1.2. Standards

Daidzin (99.2%, HPLC purity) and genistin (99.2%, HPLC purity) were purchased from LC Labs. (Wobrun, USA), daidzein (100%, HPLC purity) and genistein (100%, HPLC purity) were obtained from Extrasynthèse (Genay, France).

Glycine max extracts were kindly donated by Biover (Brugge, Belgium).

2.2. Equipment

The analysis was performed on two linked Chromolith Performance RP-18e (100 mm × 4.6 mm) columns from Merck (Darmstadt, Germany). The apparatus used was a Beckman (System Gold 168) HPLC–diode array detection (DAD) instrument, equipped with an automatic injector (Analis, Gent, Belgium).

2.3. Methods

- Test solutions were prepared as follows

Determination of aglycones: about 200.0 mg soy extract was accurately weighed in a 50.0 ml calibration flask, and 5 ml of dimethyl sulphoxide and 20 ml of methanol were added. This solution was sonicated during 30 min. Twenty milliliters of water was added and, after cooling down to room temperature, the volume of the flask was adjusted to 50.0 ml with methanol 50% (v/v).

Determination of glycosides: 1.0 ml of the obtained test solution for the determination of the aglycones was diluted to 10.0 ml with methanol 50% (v/v).

- Reference solutions

Stock solutions of genistin or daidzin: 5.0 mg of daidzin or genistin was weighed in a calibrated flask of 10.0 ml. 7 ml of dimethyl sulphoxide was added and the resulting solution was sonicated for 10 min. After cooling down to room temperature, the volume of the flask was adjusted to 10.0 ml with dimethyl sulphoxide.

Stock solutions of genistein or daidzein: 5.0 mg of daidzein or genistein was weighed in a calibrated flask of 50.0 ml. Forty milliliters of dimethyl sulphoxide was added and the resulting solution was sonicated for 10 min. After cooling down till room temperature, the volume of the flask was adjusted to 50.0 ml with dimethyl sulphoxide.

Reference solution glycosides: 1.0 ml of daidzin stock solution and 2.0 ml genistin stock solution were pipetted in a calibration flask of 10.0 ml and filled up with methanol 50% (v/v).

Reference solution aglycones: 1.0 ml of daidzein stock solution and 1.0 ml genistein stock solution were pipetted in a calibration flask of 10.0 ml and filled up with methanol 50% (v/v).

- Chromatographic system

Mobile phase: (A) acetic acid, water (0.1:0.99); (B) acetonitrile

Time (min)	*B* (%)
0–16.5	10 → 29
16.5–18	29
18–21	29 → 90
21–23	90 → 10
23–25	10

Flow gradient

Time (min)	Flow (ml/min)
0–17	3
17–18	3 → 4
18–22.5	4
22.5–23	4 → 3
23–25	3

Ten microliters of test and reference solutions, filtered through a nylon filter (0.45 μm), were injected.

2.4. Validation

The method was validated according to the ICH guidelines on the validation of analytical methods [6,7]. All results were expressed as percentages, where n represents the number of values. For the statistical analysis Excel 2000 (Microsoft Office) was used. A 5% level of significance was selected.

Linearity: reference solutions were prepared at six concentration levels and were injected twice. The concentration levels for the isoflavone glycosides, daidzin and genistin, were within the range of 14–275 and 11–219 μg/ml, respectively, and for the isoflavone aglycones, daidzein and genistein, within the range of 0.5–26 and 0.5–25 μg/ml, respectively. For assessing the linearity the least squares line and the correlation coefficient were calculated. The calibration curves obtained were tested on the slope ($a \neq 0$) and intercept ($b = 0$) by means of Student's *t*-tests. In order to evaluate the goodness (or lack) of fit of the linear model a lack-of-fit (LOF) test [8] was performed and the residuals were graphically examined.

Precision: the repeatability and the inter-day intermediate precision were determined by analyzing six samples (100%) according to the above-described method on three different days. The standard deviation and coefficient of variation were calculated for each day. In order to evaluate whether the results obtained on the three different days were not significantly different, the results were analyzed by means

of an analysis of variance (ANOVA) single factor. Within and between days variation coefficients were calculated [9].

In order to evaluate whether the precision of the method is equal over the whole range of the method six samples weighing half of the normal mass (50%) and six samples weighing the double of it (200%) were analyzed according to the method described. The standard deviation and coefficient of variation were calculated for each level. By means of a Cochran's test the variations at these concentrations were compared with the variation at 100%. In order to evaluate whether the results obtained on the three levels were not significantly different, the results were analyzed by means of an ANOVA single factor. Within and between level variation coefficients were calculated [9].

Accuracy: a daidzin and genistin stock solution at three different concentration levels were added to the soy extract before the extraction. At each level samples were prepared in triplicate and analyzed according to the previously described method. By means of a Student's *t*-test the mean recovery percent for both isoflavones were verified to be equal to 100%.

Six samples were analyzed using both systems, i.e. on a conventional LiChrospher column and on the two coupled Chromolith Performance columns. Results were compared by means of a paired Student's *t*-test.

Specificity: peaks of daidzin, genistin, daidzein and genistein were identified based on their retention times, their UV spectra between 200 and 400 nm with respect to the reference materials, and their electrospray ionization (ESI) mass spectra. All other isoflavones were characterized on the basis of their ESI mass spectra and comparison with literature data [2–5]. LC–ESI–MS was performed in the positive ion mode on an Autospec-oa-ToF mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ionization source and coupled to a Waters 600 MS solvent delivery system (Waters, Milford, MA, USA). The LC and MS conditions optimized for the analysis of flavonoids were followed [10]. The LC column used was an Xterra RP-18 MS column (250 mm × 3 mm; 5 μm; Waters). The UV chromatogram obtained with this column were comparable to that with the LiChrospher RP-18 column. The ESI mass spectra of both aglycones and glycosides showed a protonated molecule (MH^+), which allowed the determination of the molecular mass. In addition, the ESI mass spectra of the glycosides also revealed the protonated aglycones (denoted by AH^+) due to loss of the glucose or 6″-*O*-acetylglucose residue, which allowed the characterization of the aglycone masses. The characterization of the isoflavones was performed in the same way on the Chromolith columns. LC–ESI–MS and UV spectra were recorded in the positive ion mode on an Esquire 3000 plus Ion Trap mass spectrometer (Bruker Daltronics, Bremen, Germany) coupled to an HP 1100 (DAD) liquid system (Agilent) applying a flow rate of 1 ml/min. The MS operating parameters were set using the smart parameter setting (SPS), with 380 as target mass. Mass spectra were recorded in the range *m/z* 100–500. Nitrogen was used both as nebulizing gas (207 kPa) and drying gas (300 °C; 9 l/min).

3. Results and discussion

HPLC analysis of the complex mixture of isoflavones in soy samples is generally performed on conventional particulate silica or polymer-based RP C_{18} columns [2–5]. Also in our laboratory soy extracts have routinely been analyzed on a RP C_{18} LiChrospher (250 mm × 4 mm; 5 μm; Merck) column by applying a linear gradient at a flow rate of 1 ml/min. This gradient started at 10% *B*, was increased to 29% *B* in 33 min, then to 31% *B* in 11 min, and subsequently, the column was washed with 90% *B* and equilibrated at starting conditions in about 15 min. The isoflavone glycosides, daidzin and genistin, and the isoflavone aglycones, daidzein and genistein, were used as external standards. For quantification, the contents of the 6″-*O*-acetyl-β-glycoside forms were calculated with reference to the respective glycosides, while the contents of glycitin and its 6″-*O*-acetyl-β-glycoside form and that of glycitein were calculated with reference to daidzin and daidzein, respectively. In each case, the molecular weights were taken into account for calculation of the contents.

Owing to the substantial difference in the concentration range of isoflavone aglycones and glycosides, two HPLC runs are necessary to determine the total amount of isoflavones. Analyzing samples in duplicate leads to a total run time of about 4 h.

To overcome this problem, an HPLC method applicable on basic, i.e. non-microbore equipment, using monolithic silica-based RP C_{18} columns, was developed. The existing method was transferred from the 250 mm particulate to a 10 mm monolithic column. The Chromolith Performance RP-18e column is a new type of HPLC column based on high-purity silica combined with the unique monolithic silica technology. Owing to the very high porosity of this type of column, very high flow rates can be applied at very low pressures resulting in reduction of the analysis time [11–16]. Several mobile phase gradients and flow rate gradients were tried out but the separation efficiency of the 10 mm column was not sufficient to resolve the late-eluting isoflavones, i.e. acetyldaidzin, acetylglycitin, daidzein, glycitein and acetylgenistin. In order to achieve a good separation of the late-eluting isoflavones two Chromolith Performance RP-18e (100 mm × 4.6 mm) columns needed to be linked. Running the same mobile phase gradient as applied on the particulate column at a flow rate of 1 ml/min, peaks eluted in the same order at similar retention times. The column efficiency of these linked monolithic columns (2 mm × 100 mm) was even better than that of the particulate (250 mm) column, i.e. the improvement of the resolution of the peaks of the late-eluting was remarkable. The analysis was speeded up by applying higher flow rates with parallel adaptation of the mobile phase gradient. Up till

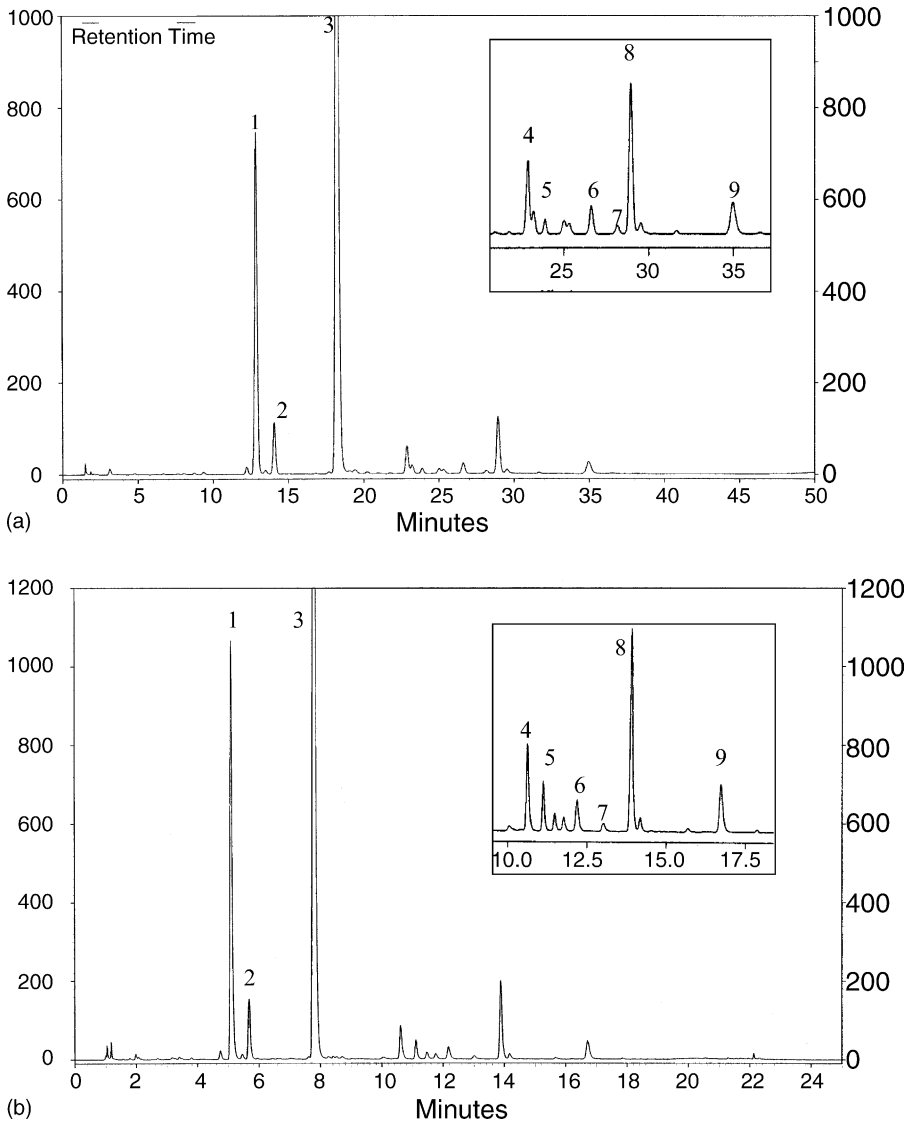

Fig. 1. Chromatograms of the separation of the isoflavones of a Soy extract on (a) a LiChrospher RP-18 (250 mm × 4 mm) column and on (b) two linked Chromolith Performance RP-18e (100 mm × 4.6 mm) columns. Identification of peaks and ESI-MS data (*m/z* values of MH^+ and AH^+): (1) daidzin (417, 255); (2) glycitin (447, 285); (3) genistin (433, 271); (4) acetyldaidzin (459, 255); (5) acetylglycitin (489, 285); (6) daidzein (255); (7) glycitein (285); (8) acetylgenistin (475, 271); (9) genistein (271).

3 ml/min the peaks were fully resolved. A flow gradient going from 3 to 4 ml/min at the end of the run for washing the column was incorporated (see Section 2), speeding up the analysis even more. A spectacular decrease of the analysis time to 25 min was achieved without loosing any separation efficiency. In Fig. 1a and b the separation of the isoflavones of the soy extract are shown on the LiChrospher and the Chromolith columns, respectively.

From environmental and economical point of view, speeding up the analysis has several concequences, both positive as negative. By applying higher flow rates, the total amount of mobile phase used is increased, i.e. about 75 ml per run on a monolithic system instead of 60 ml per run on a conventional column. On the other hand, the life time of the lamp of the UV-Vis detector is increased significantly since the time of analysis is about 3 times shorter.

This analysis method for the determination of isoflavones in soy extracts in less than 25 min was fully validated according to the ICH guidelines [6,7]. The linearity of the isoflavone glycosides, daidzin and genistin, and of the isoflavone aglycones, daidzein and genistein, was investigated. The results are shown in Table 1. Graphical examination of the residuals, the LOF test and the correlation coefficients proved the method to be linear for all of the reference compounds in the range tested. The slopes of each of the curves were significantly different from 0. The *t*-test on the intercepts revealed that point (0, 0) falls within each of the calibration curves. This implies that for routine analysis in this range of the method, the isoflavone content

Table 1
Overview of the linearity data of the isoflavone reference materials

	Daidzin	Genistin	Daidzein	Genistein
Correlation coefficient	0.9999	0.9998	0.9993	0.9997
Slope ± standard error	$(1236 \pm 6) \times 10^4$	$(1886 \pm 12) \times 100^4$	$(1926 \pm 23) \times 10^4$	$(2978 \pm 22) \times 10^4$
Intercept ± standard error	$(-4 \pm 10) \times 10^3$	$(-14 \pm 16) \times 10^3$	$(-2 \pm 4) \times 10^3$	$(-4 \pm 3) \times 10^3$
Confidence interval (95%)	$(-26$ to $19) \times 10^3$	$(-48$ to $21) \times 10^3$	$(-10$ to $6) \times 10^3$	$(-11$ to $3) \times 10^3$
F_{LOF} ($F_{crit} = 4.5$)	0.3	0.3	0.5	2.2
Concentration range (μg/ml)	14–275	11–219	0.5–26	0.5–25
Number of standards (in duplicate)	6	6	6	6

can be determined against a single standard concentration level instead of running a calibration curve each time.

In order to evaluate the precision of the method six samples were analyzed on three different days. The mean content and the between-days relative standard deviation (R.S.D.) for each of the isoflavones are summarized in Table 2. The soy extract contained a total content isoflavones of 41.1%, of which 40.4% are glycosides and 0.7% aglycones. Genistin and daidzin were the major glycosides in an amount of 25.7 and 10.0%, respectively. The precision of the method is acceptable with $R.S.D._{between\text{-}days}$ of 2.9 and 2.3% for the major compounds, daidzin and genistin, respectively, 2.6% for glycitin, 4.0, 2.9 and 4.3% for the respective aglycones, and 2.5, 2.4 and 5.0% for the respective acetyl derivatives. For both major components the ANOVA proves that, from the statistical point of view, there is no significant difference between the results obtained on three different days. Although the ANOVA is negative for most of the other isoflavones, the method can be considered precise since the $R.S.D._{between\text{-}days}$ is smaller than the R.S.D.

Table 2
Validation data of the determination of isoflavones in a soy extract

Parameter	Daidzin	Genistin	Glycitin	Daidzein	Genistein	Glycitein	Ac-daidzin	Ac-genistin	Ac-glycitin
Precision on different days									
Repeatability									
Number of replicates	6	6	6	6	6	6	6	6	6
Mean content (%)									
Day 1	9.9	25.5	1.6	0.3	0.3	0.1	1.1	1.6	0.5
Day 2	10.1	25.8	1.6	0.3	0.3	0.1	1.1	1.7	0.5
Day 3	9.8	25.7	1.6	0.3	0.3	0.1	1.1	1.6	0.5
R.S.D. (%)									
Day 1	3.3	1.8	2.3	3.0	3.3	1.0	1.7	1.2	2.5
Day 2	3.3	2.7	1.7	1.5	1.3	3.3	2.9	2.7	4.7
Day 3	2.8	2.4	0.7	2.2	2.2	4.0	2.6	0.9	2.9
Intermediate precision									
Days (n) × replicates (n)	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 x 6
$R.S.D._{between\text{-}groups}$ (%)	2.9	2.3	2.6	4.0	2.9	4.3	2.5	2.4	5.0
F_{calc} (F_{crit}= 3.68)	1.62	0.32	17.84	13.19	3.93	7.02	0.18	6.06	6.96
Precision on concentration levels									
Repeatability									
Number of replicates	6	6	6	6	6	6	6	6	6
Mean content (%)									
50	10.5	26.7	1.6	0.3	0.3	0.1	1.1	1.6	0.5
100	9.9	25.8	1.6	0.3	0.3	0.1	1.1	1.7	0.5
200	9.8	26.4	1.6	0.3	0.3	0.1	1.1	1.7	0.5
R.S.D. (%)									
50	2.0	2.4	2.6	1.2	2.2	3.1	2.5	1.2	2.8
100	3.3	2.7	1.9	3.0	3.3	4.0	2.9	2.7	4.7
200	1.6	2.9	2.2	1.7	2.8	3.7	1.6	1.8	2.7
Intermediate precision									
Levels (n) × replicates (n)	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6	3 × 6
Cochran's test ($C_{crit} = 0.707$)	0.247	0.276	0.436	0.167	0.196	0.276	0.375	0.108	0.602
$R.S.D._{between\text{-}groups}$ (%)	4.2	3.0	2.2	6.9	4.6	7.2	2.4	3.3	3.9
F_{calc} ($F_{crit} = 3.68$)	13.51	2.55	0.60	61.12	10.46	19.20	0.21	12.14	2.26

Table 3
Recovery data for daidzin and genistin

	Determined concentration (%)	Added concentration (%)	Recovery (%)
Daizin			
1.1	12.7	2.8	105
1.2	12.5	2.6	99
1.3	12.5	2.5	100
2.1	15.6	5.7	100
2.2	15.4	5.5	103
2.3	15.7	5.7	105
3.1	19.8	9.9	99
3.2	20.2	10.2	101
3.3	19.5	9.5	97
Mean (%)			101
S.D. (%)			3
R.S.D. (%)			2.6
t_{calc}			1.014
t_{table}			3.182
Genistin			
1.1	28.8	3.1	103
1.2	28.7	3.0	98
1.3	28.9	3.2	102
2.1	31.8	6.2	103
2.2	31.6	5.9	98
2.3	31.2	5.6	101
3.1	39.6	13.9	97
3.2	38.0	12.4	103
3.3	39.0	13.3	100
Mean (%)			101
S.D. (%)			2
R.S.D. (%)			2.4
t_{calc}			0.576
t_{table}			3.182

calculated by the Horwitz equation [17,18] or smaller than 5%. This R.S.D.$_{Horwitz}$ is the maximal variation allowed depending on the concentration of the compound to be determined. Experiments to investigate the precision of the whole range of the method, i.e. between 50 and 200% of the isoflavone content were performed and results are shown in Table 2. Since for each of the isoflavones C_{calc} is smaller than C_{crit}, the variation of the method can be considered equal for concentration levels within this range. Although the ANOVA between the levels is negative for most of the isoflavones, the R.S.D.$_{between\text{-}levels}$ are still acceptable and no trend, i.e. lower values at the 200% level or higher values at the 50% level, is observed. The accuracy of the method was also investigated by means of a recovery experiment, by adding reference materials to the soy extract samples at the start of the analysis (Table 3). A mean recovery ($n = 9$) of 101% (R.S.D. = 2.6%) for daidzin and of 101% (R.S.D. = 2.4%) for genistin proved that the method yields accurate results. Another way to prove the accuracy of the new analysis method employing coupled Chromolith columns was performed by injecting six samples on each of the chromatographic systems. Results obtained on both systems were proven to be equal by means of a paired Student's *t*-test (t_{crit}: 2.23, t_{calc}: 0.06).

These results show that linked monolithic silica-based RP C_{18} columns provide a good solution to overcome the long analysis times encountered in the separation of complex mixtures of natural products. The analysis was speeded up by applying a flow rate of 3–4 ml/min without loosing any separation efficiency. In contrast to the analysis on a conventional C_{18} column, peaks of late-eluting isoflavones were baseline-separated on the coupled Chromolith columns. Analyzing samples in duplicate for the determination of both isoflavone aglycones and glycosides using this system takes about 1 h 40 min instead of 4 h with conventional columns.

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