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Determination of isoflavones in soy bits by fast column high-performance liquid chromatography coupled with UV–visible diode-array detection

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Abstract

A fast determination of isoflavones (genistin, genistein, daidzein, daidzin, glycitin, glycitein, ononin, formononetin, sissotrin and biochanin A) by HPLC/UV–vis-DAD working at 254 nm is described. An Atlantis dC18 fast reversed-phase chromatographic column (20 mm × 2.1 mm, 3 μm particle size) was used at a flow rate 0.35 ml min^{-1} of a mobile phase consisted from 0.1% (v/v) acetic acid (A) at pH 3.75 and methanol. (B). A linear gradient profile was used for separation at the column temperature 36 °C. Limits of detection (LODs for 3 S/N criterion) per sample injection (5 μl) ranged from 166.2 to 17.0 fmol (9.4–1.1 ng ml^{-1}) for biochanin A and genistin, respectively. The recoveries 96–106% were obtained for the different concentrations of the isoflavones (RSDs 2–8%). The pressurized liquid extraction/HPLC/UV–vis-DAD method was used for the determination of the isoflavones in soy bits (28–962 μg g^{-1} dry weight). The proposed procedure is faster (ca. 8 min) without loosing its separation efficiency (up to 10 isoflavonoids can be determined) and sensitivity (tens to hundreds fmol).

Keywords: Isoflavones; High-performance liquid chromatography; Pressurized liquid extraction; Ultrasonic extraction; Soy bits; Foodstuffs

1. Introduction

The use of plants for large-scale production of biologically active compounds (i.e. phenylpropanoids, quinones, alkaloids), such as pharmaceuticals, foodstuffs and cosmetics is of interest from scientific and economic points of view [1]. The compounds derived from the phenylpropanoid pathways – which include flavonoids, isoflavones, lignin, coumarins and many small phenolic molecules – have a multiplicity of functions in structural support, pigmentation, defence and signalling of plants [2–4].

Isoflavones present in soybeans (i.e. genistin, genistein, daidzein and daidzin, etc.) have manifested many health benefits. Many of anticancer mechanisms (i.e. association with lower incidences of hormonally dependent cancers) were detected by in vitro experiments, including cell cultures, and in immune-suppressed mice carrying xenotransplants of human cancer cells and even phytoestrogen preventive effects to certain diseases have been discovered.

A simple shaking, Soxhlet extraction or ultrasonication using different organic solvents are prevailing techniques for the isolation of the isoflavones from soybeans and soy products. On the other hand, several new extraction procedures, such as supercritical fluid extraction, accelerated solvent extraction, etc., have been applied as alternatives of the traditional procedures in the last several years [4–18].

Analysis of isoflavones in biological samples is complicated due to low efficiency of chromatographic separation and time-consuming sample preparation. A combination of a highly effective and selective isolation/purification procedures with a highly effective and sensitive separation methods is very suitable for their quantification and identification [4–18]. A high-performance liquid chromatography (HPLC) coupled with electrochemical, UV–vis-diode-array (DAD) and/or mass spectrometric (MS) detection is commonly used

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for the determination of the isoflavones. Interest in much faster separation techniques applying fast and ultra-fast chromatographic columns has greatly increased in the last several years, especially for routine analyses, process control, etc.

The aims of this study were (i) to optimise a modified pressurized liquid (PLE) extraction procedure for the isolation of isoflavones (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin and biochanin A); (ii) to optimise and validate the HPLC/UV–vis-DAD method using an ultra fast column; (iii) to evaluate the combined procedure for determination of isoflavones in soy bits samples.

2. Experimental

2.1. Chemicals and samples

HPLC-grade acetonitrile (>99.9%; v/v) and methanol (>99.9%; v/v) were from Merck (Darmstadt, Germany). Isoflavones, flavone and all other reagents of ACS purity were purchased from Sigma–Aldrich (St. Louis, MO, USA). The stock standard solutions of isoflavones (ca. 1 mg ml^{-1}) and flavone (ca. 10 μg ml^{-1}) were prepared in an aqueous methanol (1:1, v/v) and stored in darkness at 4 °C. The working standard solutions (10–300 μg ml^{-1}) were prepared daily by dilution of the stock solutions with the aqueous methanol. All solutions were filtered through 0.45 μm PTFE membrane filters (MetaChem, Torrance, CA, USA) prior to HPLC separations. The pH value was measured using WTW InoLab Level 3 pH meter. The combined pH-electrode (SenTix H) was regularly calibrated by a set of WTW buffers (all from WTW, Weilheim, Germany).

Soy bits were purchased in the local market. The sample (2000 ± 5 mg) of soy bits was spread in a mortar and homogenised by an Ika A11 Basic grinder (IKA Werke, Staufen, Germany) to tiny particles (<0.1 mm). PLE was performed using a PSE-one extractor (Applied Separations, USA).

2.2. Chromatographic apparatus – HPLC-DAD

An HP 1100 liquid chromatographic system (Hewlett-Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an auto sampler (G1313A), a column thermostat (G1316A) and a UV–vis diode array detector (model G1315A) working at 254 nm. The isoflavones were separated on an Atlantis dC18 reversed-phase fast chromatographic column (20 mm × 2.1 mm, 3 μm particle size, Waters, Ireland). Other chromatographic conditions are given in Section 3.

2.3. Extraction procedures

2.3.1. PLE procedure

Flavone was used as a standard and was mixed with the homogenized sample (500 ± 5 mg) and left to equilibrate for 24 h. The homogenised sample was wrapped [18] into a filter paper (small envelope like format) and finally sprinkled with 3.0 g SPE-ed™ matrix, 1.5 g Florisil (15.5% MgO, 84% SiO_2, 0.5% Na_2SO_4; pH 8.5; 60–100 μm particle size) and 3.0 g Ottawa sand (Allentown, PA, USA). The mixture was placed into a 10 ml stainless steel extraction cell and extracted under controlled conditions in two steps (i) pre-heating period (5 min), the solvent hexane (elution of lipophilic compounds); temperature 145 °C, pressure 140 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen and (ii) pre-heating period (5 min), the solvent 90% aqueous methanol; temperature 145 °C, pressure 140 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen. The final extracts were collected in 60 ml glass vials with PTFE coated rubber caps and centrifuged at 4000 × *g* (Hettich, Germany). The supernatants were evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (both, IKA-Werke). The residue was reconstituted in 1 ml methanol and filtered through 0.45 μm PTFE membrane filters (MetaChem) and diluted with mobile phase prior to injection into the HPLC system to fit the concentration range of calibration curves.

2.3.2. Sonication procedure

Flavone was used as a concentration standard and was mixed with the homogenized sample (500 ± 5 mg) and left to equilibrate for 24 h. A homogenised sample was sonicated with 90% (v/v) aqueous methanol (10 ml) at the laboratory temperature for 5 min using a K5 Sonicator (UT, Czech Republic) at 38 kHz, 150 W. The obtained extract was filtered through a PTFE membrane filter (0.45 μm, 13 mm diameter, Alltech Associates, Deerfield, IL, USA) and treated as above.

2.3.3. Soxhlet extraction

Flavone was used as a standard and was mixed with the homogenized sample (500 ± 5 mg) and left to equilibrate for 24 h. A homogenised sample was used for the stirred extraction using a computer controlled commercially available fex Ika Werke 50 device (IKA-Werke) related to Soxhlet apparatus. A two steps temperature program (first step: temperature of cooling/heating block 130 °C for 30 min, cooling/heating block to 30 °C for 5 min; second step: temperature of 120 °C for 30 min, cooling to 30 °C for 5 min) was applied for isolation into a 90% (v/v) aqueous methanol (10 ml). The obtained extract was treated as above.

2.3.4. Combined sonication/pressurized liquid extraction (S/PLE)

Combined S/PLE was advantageous to use. The procedure consisted of sonication of the weighed amount of the homogenized (500 ± 5 mg) and equilibrated spiked sample wetted with 500 μl methanol for 1 min in the 2 ml Eppendorf tube. The extract was dried under nitrogen flow and the abovementioned PLE procedure was subsequently applied to increase the extraction yield.

2.4. Method validation

An accuracy, precision and recovery of the determinations of individual isoflavones (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin and biochanin A) were evaluated with soy bits samples spiked with 100 μl isoflavone standards (concentrations varying from 10 to 130 μg ml^{-1}) and 100 μl flavone (10 μg ml^{-1}) and equilibrated for 24 h. RSDs (%) of intra-day assay were performed using six homogenates. Inter-day precision was determined by analysing six homogenates over 1 week. Homogenates were assayed blindly and isoflavones concentrations were calculated from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of the individual isoflavones. Statgraphics (Statistical Graphics Corp., USA) was used for statistical analyses. Results are expressed as the means ± SD unless noted otherwise. Value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Isoflavone DAD

Recently, we have published the HPLC/UV–vis-DAD determination of isoflavones (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin and biochanin A; flavone as internal standard) in foodstuffs samples after pre-treatment by a PLE procedure [5]. The individual isoflavones were well separated on an Atlantis dC18 (150 mm × 2.1 mm, 3 μm particle size) reversed-phase chromatographic column but the overall time of analyses (approximately 40 min, Fig. 1A) was unacceptable for routine analyses. Thus, we modified the HPLC-DAD parameters to speed up the separation changing the chromatographic conditions; unfortunately without significant success.

Thus, the fast Atlantis column enabling very fast separation of the analysed isoflavone substances was tested. The recommended flow rate for the fast column (20 mm × 2.1 mm, 3 μm particle size) is 0.68 ml min^{-1} for 4 min gradient separations. Due to the problematic separation efficiencies using gradients under 6 min at the recommended flow rates, the lower flow rate was applied in development of the linear gradient profile. We applied the same mobile phase using a modified linear gradient profile from 13 up to 40% B (v/v) from start to 2 min, constant to 3.5 min, up to 50% B to 4 min and followed by negative gradient up 13% B to 6 min at a flow rate 0.45 ml min^{-1} and the temperature of the column oven set at 40 °C. The best DAD responses were again obtained at 254 nm for all the isoflavones (not shown). Ononin, daidzein and glycitein were not very well separated as could be seen from the HPLC-DAD chromatogram given in Fig. 1B. Thus, optimisation of the chromatographic parameters was necessary.

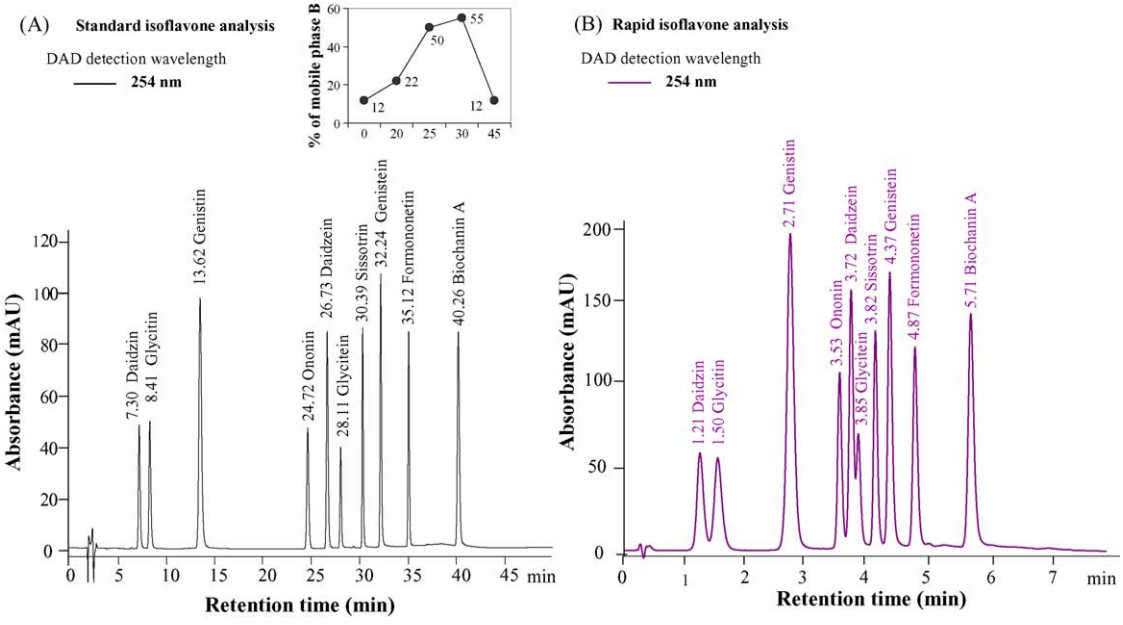

Fig. 1. HPLC-DAD chromatograms of daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin and biochanin A. HPLC parameters – mobile phase: 0.2% (v/v) formic acid (A) and acetonitrile (B); column temperature: 40 °C; detection wavelength: 254 nm; the isoflavones concentrations: 10 μg ml^{-1} (A) Atlantis dC18 reversed-phase chromatographic column (150 mm × 2.1 mm, 3.0 μm particle size, Waters, USA). A linear gradient profile from 12 up to 22% solvent A (v/v) from start to 20 min, up to 50% A to 25 min, up to 55% to 30 min and followed by negative gradient up 12% A to 45 min was used for separation. Flow rate was 0.2 ml min^{-1}. (B) Atlantis dC18 reversed-phase fast chromatographic column (20 mm × 2.1 mm, 3 μm particle size, Waters). A linear gradient profile from 13 up to 40% B (v/v) from start to 2 min, constant to 3.5 min, up to 50% B to 4 min and followed by negative gradient up 13% B to 6 min was used. Flow rate was 0.45 ml min^{-1}.

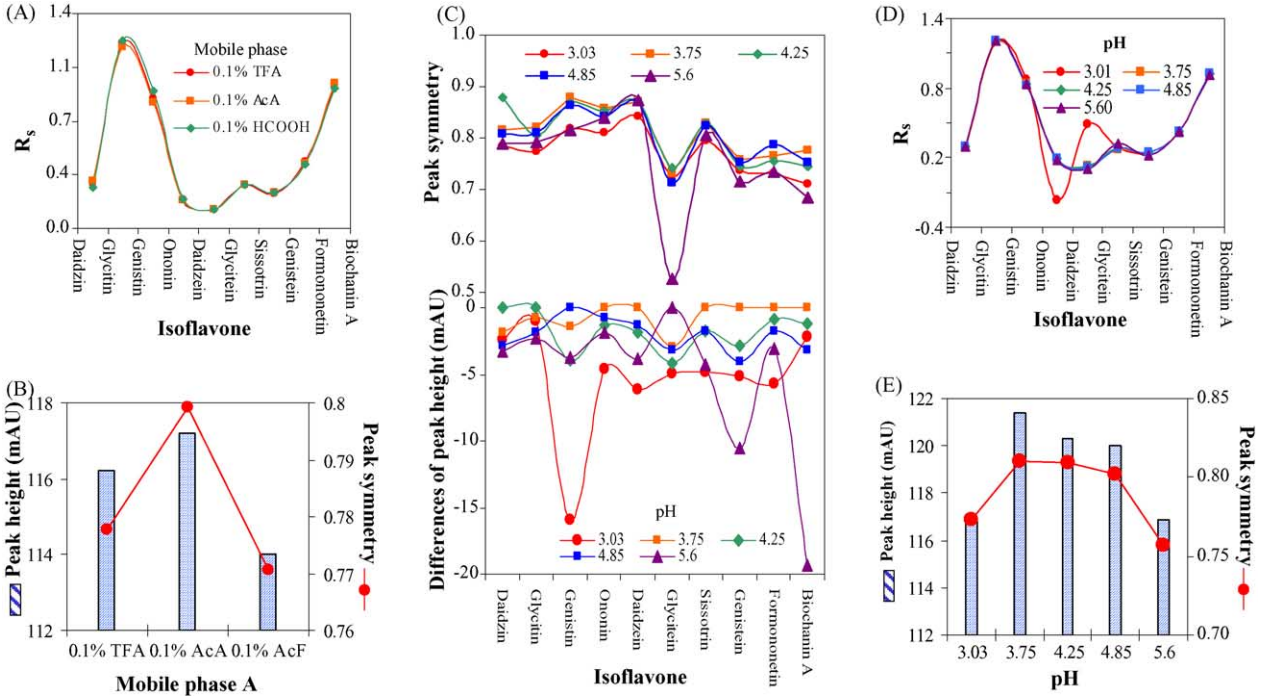

Fig. 2. Effect of different composition of solvent A in mobile phase (solvent A: trifluoroacetic acid, acetic acid or formic acid; solvent B: methanol) on (A) resolution factor, (B) average peak height and average peak symmetry. Influence of different pH values (3.03, 3.75, 4.25, 4.85 and 5.6) on (C) peak symmetry and differences of peak height, (D) resolution factor, (E) average peak height and average peak symmetry of the studied isoflavones. Averages peak height and averages peak symmetries were calculated as the sum of the peak heights and/or peak symmetries of the individual studied isoflavones divided by the number of the studied compounds. Other chromatographic conditions for Atlantis dC18 column are given in Section 3.1.

3.2. Influence of mobile phase composition

Primarily, we studied the influence of character and content of organic acids in the mobile phase (solvent A: trifluoroacetic, acetic or formic acids; solvent B: methanol) on separation and detection of the isoflavones. The basic chromatographic parameters (the resolution factor, the peak symmetry, the peak height and/or the peak area) were selected for the evaluation in our experiments. The resolution factors of the determined isoflavones are shown in Fig. 3A according to different composition of solvent A. The signals of daidzein and glycitein were still not well separated (Fig. 2A). Due to values of average peaks heights and peak symmetries, the most effective mobile phase consisted from 0.1% (v/v) acetic acid and methanol (see Fig. 2B, solvents A and B, respectively). The overall time of the isoflavones analyses was about 6 min.

3.3. Influence of pH

Values of pH markedly influence the running of chromatographic separation of many compounds thus we focused on effect of pH values of the buffer on isoflavones separation. The tested pH values of the acetate buffer were 3.03, 3.75, 4.25, 4.85 and 5.6. The differences of peak height are expressed as divergence between the highest peak height of the individual isoflavone at tested value of chromatographic parameters and detected peak height of the individual isoflavone at tested value of chromatographic parameter. The lowest tested pH value (3.03) adversely influenced the genistin signals (decrease more than 15 mA U; see Fig. 2C). This phenomenon probably related with dissociation or rather hydrolysis of genistin at the lowest tested pH value due to the well known instability of glycosides in the low pH. The higher values of pH (3.75, 4.25 and 4.85) influenced neither the quantitative running of separation nor analytical response (Fig. 2C) of all studied isoflavones. In addition, we observed the serious decrease of glycitein peak symmetry and the serious decrease of the peak heights of genistein and biochanin A at pH value 5.6 (Fig. 2C). Due to the values of the average peaks heights, the average peak symmetries and the resolution factors of the individual studied isoflavones, the most suitable pH value of the mobile phase consisted from 0.1% (v/v) acetic acid and methanol for simultaneous isoflavones determination was 3.75 (Fig. 2D and E).

3.4. Influence of gradient profile

The running of the chromatographic separation of the isoflavones at five different gradient elution profiles is shown in Figs. 3 and 4A. As can be seem from the figures, the very low changes in the gradient profile markedly influenced the separation efficiency of the studied substances. Ononin, daidzein, glycitein, daidzin and glycitin were well separated

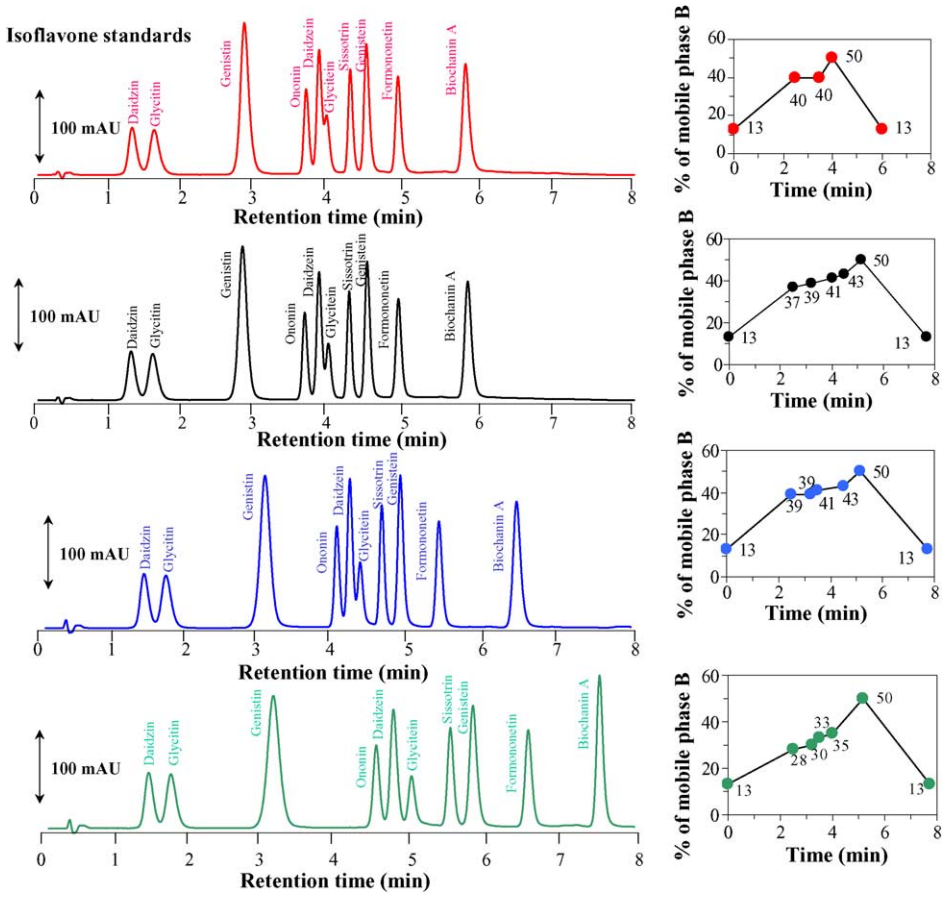

Fig. 3. Influence of different gradient profile of mobile phase on chromatographic separation of ten studied isoflavones. HPLC parameters – flow rate: 0.35 ml min^{-1}; column temperature: 36 °C. Other chromatographic conditions are given in Section 3.1.

when the amounts of methanol in the mobile phase is low at the beginning of the gradient elution (Fig. 3). That is why we selected the gradient elution that is shown in Fig. 4A. A linear gradient profile from 13 up to 22% B (v/v) from start to 2.5 min, up to 30% B to 3.21 min, up to 35% B to 4 min, up to 40% B to 4.5 min, up to 50% B to 5.14 min and followed by negative gradient up 13% B to 7.71 min was used for separation of isoflavones in the next experiments. On the other hand overall time of analyses extended about 2 min.

The shorter analyses times corresponding to the higher flow rates and consequently with the shorter re-equilibration times (ca. 1.5 min for the linear gradient profile) were the most serious advantages of the proposed fast column HPLC method. Excellent retention time reproducibility was achieved for a wide range of fast and steep gradient profiles. All of the gradients resulted in RSD values for the peak area that were well below <0.8% RSD for all isoflavones.

The studied isoflavones were well separated and easily detected (daidzin – t_R: 1.32 min, glycitin – t_R: 1.64 min, genistin – t_R: 3.14 min, ononin – t_R: 5.15 min, daidzein – t_R: 5.44 min, glycitein – t_R: 5.77 min, sissotrin – t_R: 6.32 min, genistein – t_R: 6.57 min, formononetin – t_R: 7.20 min and biochanin A – t_R: 8.06 min) at the HPLC/UV–vis-DAD conditions.

3.5. *Influence of flow rate of the mobile phase*

The flow rate of a mobile phase is a chromatographic parameter that markedly influences the running of a chromatographic separation and an analytical detection of studied compounds. We studied the influence of the different flow rates (0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 ml ml^{-1}) of the mobile phase on chromatographic separation and UV–vis-DAD detection of the isoflavones. The values of the average peaks heights, the peak symmetries and the resolution factors are shown in Fig. 5A and B. The best values of the peak height and the peak symmetry were obtained for the isoflavone separation and detection at the lowest flow rate (0.2 ml min^{-1}). On the contrary the overall time of analyses was about 20 min. To shorten the time of the analyses of the isoflavones we selected the flow rate 0.35 ml min^{-1} with overall time of analysis about 8 min. The selected flow rate was compromise between the speed of analyses and the most effective values of the peak heights and the peak symmetries. The separation efficiency was still very good and nearly baseline separation was obtained for the all isoflavones.

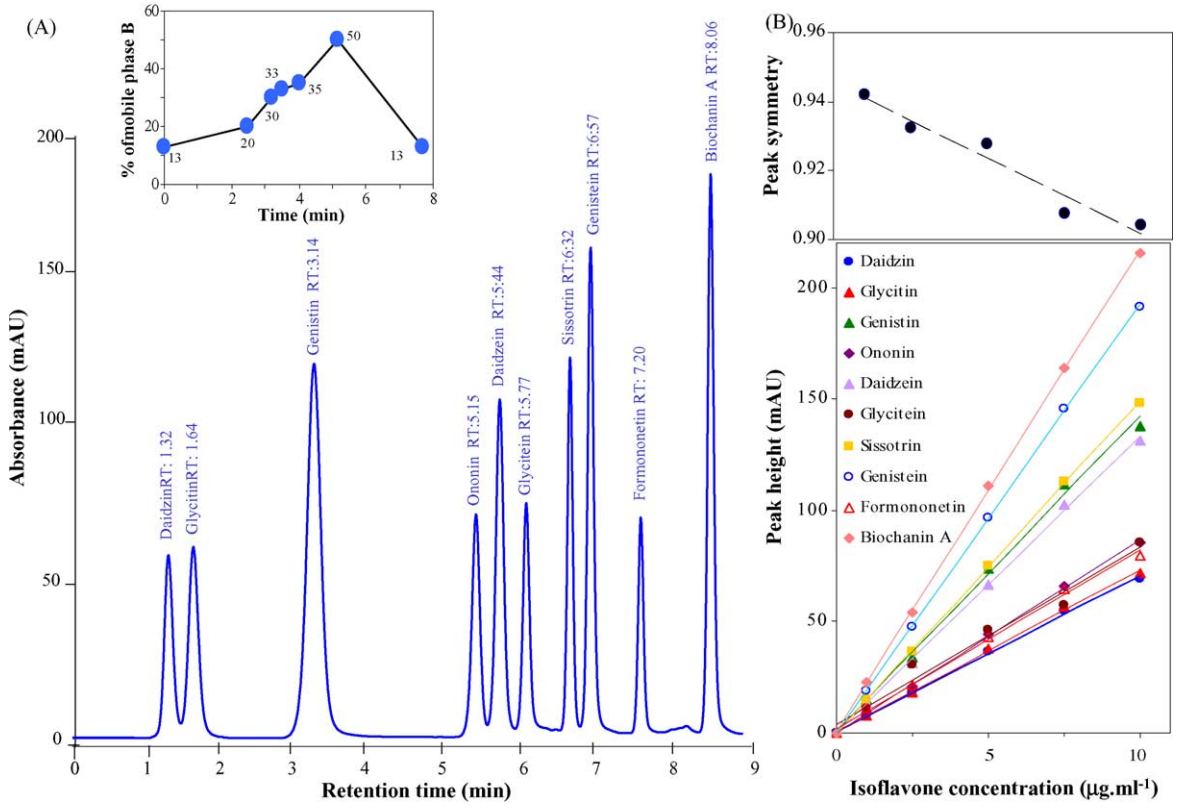

Fig. 4. (A) HPLC-DAD chromatogram of ten studied isoflavones. A linear gradient profile from 13 up to 22% B (v/v) from start to 2.5 min, up to 30% B to 3.21 min, up to 35% B to 4 min, up to 40% B to 4.5 min, up to 50% B to 5.14 min and followed by negative gradient up 13% B to 7.71 min was used. (B) Dependence of the isoflavones concentrations on peak height and/or average peak symmetry. Other chromatographic conditions are given in Section 3.1.

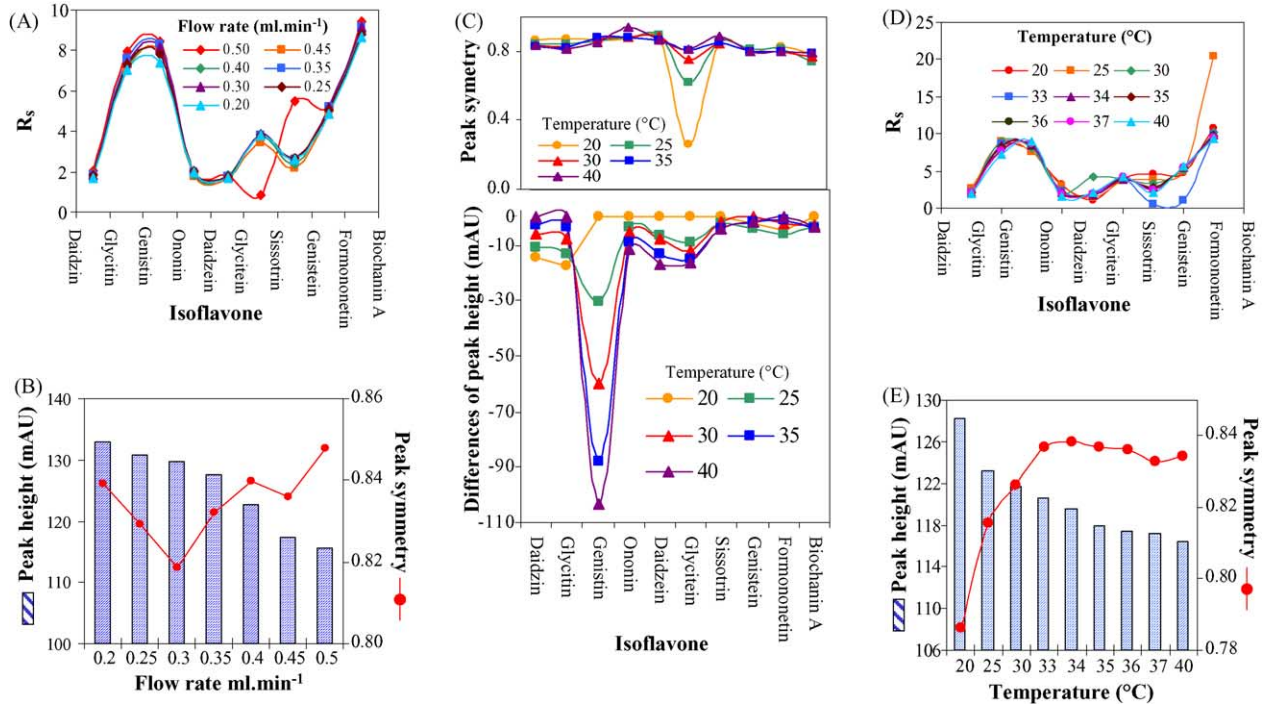

Fig. 5. Influence of different flow rates (0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 ml ml^{-1}) of the mobile phase consisted from 0.1% (v/v) acetic acid and methanol at pH 3.75 on (A) resolution factor, (B) average peak height and average peak symmetry. Effect of different temperatures (tested values was between 20 and 40 °C) on (C) peak symmetry and differences of peak height, (D) resolution factor, (E) average peak height and average peak symmetry of the studied isoflavones. Other chromatographic conditions are given in Section 3.1.

Table 1
Validation data for the determination of isoflavones ($n = 5$) at 254 nm

Isoflavones	t_R[a] (min)	Regression equation	R^2[b]	LOD[c] (ng ml^{-1})	LOQ[d] (ng ml^{-1})	RSD[e] (%)	LOD[f] (fmol)
Daidzin	1.32	$y = 7.04x + 0.40$	0.9988	3.0	10.0	2.1	36.2
Glycitin	1.64	$y = 7.27x + 0.48$	0.9980	2.9	9.6	2.3	32.4
Genistin	3.14	$y = 14.13x + 0.85$	0.9963	1.5	4.9	2.9	17.0
Ononin	5.15	$y = 8.65x + 0.14$	0.9994	2.5	8.2	1.3	46.1
Daidzein	5.44	$y = 13.31x + 0.06$	0.9990	1.6	5.2	1.5	30.7
Glycitein	5.77	$y = 7.95 + 3.91$	0.9901	2.0	6. 7	3.8	35.1
Sissotrin	6.32	$y = 14.90x + 0.02$	0.9998	1.5	4.9	1.2	25.7
Genistein	6.57	$y = 19.24x - 0.02$	0.9999	1.1	3.8	1.6	20.9
Formononetin	7.20	$y = 8.03x + 1.81$	0.9966	2.0	6.5	2.2	36.4
Biochanin A	8.06	$y = 21.61x + 0.97$	0.9997	9.4	31.5	1.9	166.2

[a] Retention times in min.
[b] Regression coefficients.
[c] Limits of detection (3 S/N).
[d] Limits of quantitation (10 S/N).
[e] Relative standard deviations ($n = 6$).
[f] Limits of detection per column injection (5 μl).

3.6. Influence of temperature

The temperature of the chromatographic column was varied (20, 25, 30, 33, 34, 35, 36, 37, and 40 °C) during initial steps of selection of optimal chromatographic conditions. The signal of genistin markedly decreased with increasing temperature (about 5 mAU per 1 °C) as can be seen from the results given in Fig. 5C probably due to genistin thermal instability. The signals of other studied compounds did not change with increasing temperature (changes about 10 mAU). On the other hand the peak symmetry of glycitein was getting worse with the decreasing temperature (Fig. 5C). The heights of the isoflavones peaks decreased with the increasing temperature but in contrary the peak symmetry and the efficiency of chromatographic separation increased (Fig. 5D and E). The signal of daidzein co-eluted with ononin/glycitein at the temperatures lower/higher then 36 °C, respectively (not shown). We preferred markedly better chromatographic separation (no co-eluting peaks) to the peak height values (sensitivity) and thus we selected the temperature 36 °C.

3.7. Calibration curves

Linear relationships between the peak heights and/or the peak areas and the isoflavones concentrations (R^2 ranged from 0.9901 for glycitein to 0.9999 for genistein) were obtained with RSDs about 1.3–3.8%. The calibration curves of the individual isoflavones are shown in Fig. 4B and their parameters are given in Table 1. The highest and the lowest limits of detections (LODs for the HPLC/UV–vis-DAD chromatographic procedure as the signal-to-noise ratio S/N = 3 criterion) were determined for biochanin A (9.4 ng ml^{-1}) and for genistein (1.1 ng ml^{-1}), respectively. After re-calculation for sample injection (5 μl volume), LODs were 166.2 and 17.0 fmol for biochanin A and genistin, respectively (see Table 1). In addition, the running of the separation of the isoflavones was negatively influenced at higher concentrations. The most effective chromatographic separation of the studied compounds was observed at lower isoflavones concentrations (under 2 μg ml^{-1}, see Fig. 4B).

3.8. Application of PLE/HPLC/DAD to the real samples

A modified PLE procedure [5,18] was used for preparation of real samples for determination of the isoflavones. The modified way of sample introduction into an extraction cell was the most important factor influencing the extraction efficiency. Unsatisfactory extraction efficiencies (40–50%) were obtained using the commonly used procedure in which the homogenized sample was mixed directly with the commercially available sorbent. The low recoveries are probably connected with the strong binding of analytes to the sorbent particles during the preparation step.

The modified procedure (wrapping the sample into a separated envelope-like space made of filtration paper, precise positioning of the envelope into an extraction cell partially filled with the sorbent and subsequent filling the cell with the

Table 2
The mean values of the amount of the extracted soy bits isoflavones (μg g^{-1}) for different extraction procedure ($n = 6$)

Isoflavones (μg g^{-1}[a])	Extraction procedure		
	Soxhlet[b,c]	Sonication[b,c]	Sonication + PLE[b,c]
Daidzin	22.7 ± 1.2	5.1 ± 0.4	37.4 ± 2.8
Glycitin	6.3 ± 0.7	2.8 ± 0.2	9.5 ± 0.5
Genistin	56.6 ± 2.5	13.3 ± 0.6	96.2 ± 4.9
Ononin	37.4 ± 2.8	19.6 ± 1.8	44.4 ± 3.2
Daidzein	2.5 ± 0.3	0.3 ± 0.1	2.8 ± 0.2
Glycitein	3.9 ± 0.3	0.8 ± 0.2	4.2 ± 0.4
Genistein	7.7 ± 0.5	1.3 ± 0.2	6.3 ± 0.7
Total	137.1	43.2	200.8

nd: not detected.
[a] Isoflavone amounts per 1 g of dry weight (10 times diluted); sissotrin, formononetin and biochanin A were not detected.
[b] Results expressed as a mean ± SD.
[c] Internal standard (flavone) was added.

Table 3
Recovery of sonication + PLE extraction procedure of isoflavones from soy bits homogenate in triplicate ($n = 6$)

Isoflavone	Homogenate ($\mu g\,g^{-1}$)[a,b,c]		Spikes ($\mu g\,g^{-1}$)[a,b,c]		Total isoflavone ($\mu g\,g^{-1}$)[a,b,c]			Recovery (%)		
Daidzin	139 ± 0.8	10.5 ± 0.8	21.3 ± 0.8	30.6 ± 1.8	147 ± 2.4	159 ± 2.6	168 ± 2.7	98	99	99
Glycitin	35 ± 0.3	10.1 ± 0.7	20.5 ± 1.3	30.8 ± 1.5	44.2 ± 1.2	55.0 ± 1.4	64.5 ± 1.8	98	99	98
Genistin	93 ± 0.6	10.5 ± 1.1	21.4 ± 1.5	30.3 ± 1.3	103 ± 1.6	111 ± 1.7	220 ± 2.0	99	97	97
Ononin	17 ± 0.1	11.5 ± 1.2	20.7 ± 1.1	31.5 ± 1.4	29.4 ± 1.1	38.5 ± 1.5	49.0 ± 1.4	103	102	101
Daidzein	25 ± 0.2	11.4 ± 0.9	19.9 ± 0.7	31.2 ± 1.6	36.8 ± 1.2	45.8 ± 1.3	58.5 ± 1.5	101	102	104
Glycitein	8 ± 0.1	10.2 ± 0.8	20.2 ± 0.9	30.5 ± 1.2	18.6 ± 1.0	29.3 ± 1.1	39.7 ± 1.3	102	104	103
Sissotrin	nd	–	–	–	–	–	–	–	–	–
Genistein	31 ± 0.5	10.3 ± 1.0	21.3 ± 1.3	29.5 ± 1.7	45.5 ± 0.9	51.8 ± 1.2	58.7 ± 1.5	98	99	97
Formononetin	nd	–	–	–	–	–	–	–	–	–
Biochanin A	nd	–	–	–	–	–	–	–	–	–

nd: not detected.
[a] Isoflavone amounts per 1 g of dry weight.
[b] Results expressed as a mean ± SD.
[c] Internal standard (flavone) was added.

sorbent seriously improved the extraction efficiencies for all tested isoflavononeses and their conjugates except of genistein. It was advantageous to use sonication (weighed amount of the homogenized sample was wetted with 500 μl methanol in the 2 ml Eppendorf tube, sonication for 1 min, and dried under nitrogen flow) prior to PLE due to increasing the isoflavones yield. The destruction of cell walls during the sonication can probably explain the observed increase in the extraction yield.

Table 4
Precision and recovery of isoflavones for soy bits homogenate analysis ($n = 6$)

Isoflavone	Isoflavone content[a,b]	Homogenate ($\mu g\,g^{-1}$)[c]	Spikes ($\mu g\,g^{-1}$)[c]	Total isoflavone ($\mu g\,g^{-1}$)[c]	Recovery (%)
Daidzin	Intra-day ($n = 6$)	37.8 ± 2.3 (6.1)	9.8 ± 0.3 (3.1)	48.9 ± 1.5 (3.1)	103
			101 ± 5 (5.0)	143 ± 6 (4.2)	103
	Inter-day ($n = 30$)	37.6 ± 4.1 (10.9)	10.1 ± 0.4 (4.0)	50.1 ± 2.9 (5.8)	103
			104 ± 9 (8.7)	149 ± 12 (8.0)	105
Glycitin	Intra-day ($n = 6$)	9.7 ± 0.3 (3.1)	10.2 ± 0.4 (3.9)	20.1 ± 1.2 (6.0)	101
			98 ± 5 (5.1)	109 ± 7 (6.4)	101
	Inter-day ($n = 30$)	9.6 ± 0.5 (5.2)	10.5 ± 0.5 (4.8)	20.7 ± 1.4 (6.8)	103
			103 ± 8 (7.8)	115 ± 9 (8.0)	105
Genistin	Intra-day ($n = 6$)	96.4 ± 3.6 (3.7)	10.4 ± 0.3 (2.9)	112.6 ± 4.7 (4.2)	105
			99 ± 6 (6.1)	207 ± 14 (6.8)	106
	Inter-day ($n = 30$)	96.6 ± 8.6 (8.9)	10.2 ± 0.6 (5.9)	111.7 ± 5.9 (5.3)	105
			104 ± 9 (8.7)	212 ± 19 (9.0)	106
Ononin	Intra-day ($n = 6$)	44.6 ± 2.8 (6.2)	9.9 ± 0.2 (2.0)	55.9 ± 2.1 (3.8)	101
			102 ± 7 (6.7)	148 ± 6 (4.1)	101
	Inter-day ($n = 30$)	44.8 ± 3.6 (8.0)	10.4 ± 0.7 (6.7)	58.2 ± 2.4 (4.1)	104
			105 ± 10 (9.5)	159 ± 8 (5.0)	106
Daidzein	Intra-day ($n = 6$)	2.8 ± 0.1 (3.6)	10.0 ± 0.4 (4.0)	13.1 ± 0.4 (3.1)	102
			103 ± 5 (4.9)	109 ± 5 (4.6)	103
	Inter-day ($n = 30$)	3.1 ± 0.3 (9.7)	10.3 ± 0.5 (4.8)	14.1 ± 0.6 (4.3)	105
			102 ± 6 (5.9)	116 ± 8 (6.9)	107
Glycitein	Intra-day ($n = 6$)	4.4 ± 0.3 (6.8)	10.5 ± 0.3 (2.9)	14.3 ± 0.5 (3.5)	96
			98 ± 7 (7.1)	100 ± 8 (8.0)	98
	Inter-day ($n = 30$)	4.6 ± 0.5 (10.9)	9.9 ± 0.5 (5.1)	14.4 ± 0.7 (5.6)	99
			104 ± 8 (7.8)	108 ± 10 (9.3)	99
Genistein	Intra-day ($n = 6$)	6.6 ± 0.4 (6.1)	10.4 ± 0.3 (2.9)	17.3 ± 0.7 (4.1)	102
			101 ± 6 (5.9)	110 ± 9 (8.2)	102
	Inter-day ($n = 30$)	6.8 ± 0.6 (8.8)	10.2 ± 0.6 (5.9)	17.7 ± 0.9 (5.1)	104
			9 ± 99 (9.1)	111 ± 10 (9.0)	105

[a] Results expressed as a mean ± SD (RSD in %).
[b] Internal standard (flavone) was added.
[c] Isoflavone amounts per 1 g of dry weight; sissotrin, formononetin and biochanin A were not detected.

The changes in the isoflavones yields according to different applied times of sonication (0–15 min) were tested before PLE procedure. In addition, we compared the isoflavones yield obtained by different extraction procedures (Soxhlet, sonication and sonication + PLE; see Table 2). The highest yields were observed at 5 min sonication (not shown) for all studied isoflavones. From the obtained results it follows that the combination of sonication and PLE was the most suitable procedure for isolation of the isoflavones from the soy bits (Table 2).

Extracts from soy bits were analysed by the combined PLE/sonication and HPLC/UV–vis-DAD chromatographic procedure. Recovery of the overall procedure was checked by the addition of the known amounts of the isoflavones to soy bits homogenates (Tables 3 and 4). The recoveries 96–106% were obtained for the different concentrations of the studied isoflavones (RSDs 2–8%, see Table 3). The repeatability of the procedure was determined using six repetitive analyses of representative samples over one week and good precision was obtained for the studied isoflavones in the samples (see Table 4).

4. Conclusion

In this work, the liquid chromatographic method for the analyses of isoflavones (daidzin, glycitin, genistin, ononin, daidzein, glycitein, sissotrin, genistein, formononetin and biochanin A) by HPLC/UV–vis-DAD was validated. The mobile phase consisting of 0.1% (v/v) acetic acid (solvent A, pH 3.75) and methanol (solvent B) at the flow rate 0.35 ml min^{-1}, column temperature 36 °C and detector wavelength 254 nm was applied. A linear gradient profile from 13 up to 22% solvent B (v/v) from start to 2.5 min, up to 30% solvent B to 3.21 min, up to 35% solvent B to 4 min, up to 40% solvent B to 4.5 min, up to 50% solvent B to 5.14 min and followed by negative gradient up 13% solvent B to 7.71 min was used for separation of the isoflavones on the Atlantis dC18 fast reversed phase chromatographic column. The combined analytical procedure was applied for the evaluation of the amounts of the isoflavones in soy bits samples. The proposed procedure is much faster (ca. 8 min) without loosing its efficiency of separation (up to 10 isoflavonoids can be determined).

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