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Technical note

Reversed-phase high-performance liquid chromatographic determination of isoflavones in plant materials after isolation by solid-phase extraction

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Abstract

A fast, sensitive (LODs ~9–17.6 n*M*) and precise (RSDs ≤8%) method combining solid-phase extraction (SPE) and RP-HPLC for isolation, purification as well as qualitative and quantitative determination of isoflavones (0.06–520 μ*M*) in plant materials is described. Plant extracts were purified and isoflavones isolated on various SPE columns. Classical sorbents were compared with new polymer sorbents as well as the wet/dry ways of extraction. Extraction efficiencies (*R*>90%) for determination of isoflavones (daidzein, genistein, formononetin and biochanin A) were evaluated and optimized on an Spe-ed ABN cartridge. Eluates were analyzed by RP-HPLC on C_{18} bonded silica using an acetonitrile–0.1% TFA gradient and UV detection at 280 nm. The method can be used for fast determination of isoflavones in plants, foods and other biological materials.

Keywords: Solid-phase extraction; Isoflavones

1. Introduction

Isoflavones include a large group of naturally occurring plant substances with estrogenic activity. They play an important role in cancer prevention, inhibit tumor initiation, oxidative damage, moderation of menopausal symptoms and other health effects. Their presence in forage legumes results in animal infertility [1].

Plant materials contain various non-polar ballast compounds (e.g., waxes, oils, sterols, chlorophyll, etc.) which can cause damage of analytical columns and interfere with the chromatographic determination. Application of preconcentration, purification and isolation steps is necessary. The most common procedures include dissolution of the sample followed by liquid–liquid extraction [1]. Solid-phase extraction (SPE) has been found as the very promising procedure [2]. The most widely used C_{18} sorbents [3–5] have several limitations [6], i.e., insufficient retention of polar compounds. High-performance liquid chromatography (HPLC) with a variety of columns and solvent systems [3–5,7] is widely used for quantitation.

In this work, different SPE methods for isolation of daidzein, genistein, formononetin and biochanin A

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from two red clover varieties were compared. Reversed-phase (RP) HPLC was applied for their quantitation after acid ethanolic extraction and SPE procedures.

2. Experimental

2.1. Apparatus and chemicals

A HP 1100 liquid chromatograph with a Zorbax SB C_{18} rapid resolution column (75×4.6 mm, particle size 3.5 μm) and a UV–Vis diode array detector (Hewlett-Packard) working at 280 nm (20 nm SBW) was used. UV spectra were recorded at the apex, both inflection points and bases of all peaks (190–400 nm, 2 nm steps) and used for identification of individual compounds.

HPLC-grade solvents (Merck), daidzein, formononetin and biochanin A (Carl Roth, Karlsruhe, Germany), genistein and trifluoroacetic acid (Sigma–Aldrich) were used. Isoflavone ethanolic stock solutions (250 μg/ml) were diluted to appropriate concentrations with mobile phase. Acetonitrile with (i) 15 m*M* phosphate buffer of pH 6.5 from 25:75 to 50:50 in 12 min, (ii) 0.5% acetic acid from 25:75 to 50:50 in 13 min and (iii) 0.1% trifluoroacetic acid (TFA) from 25:75 to 50:50 in 13 min at flow-rates of 1 ml/min and 0.7 ml/min and column temperatures 40°C and 25°C for i–ii and iii, respectively, linear gradients were compared. Each sample (10 μl) was analyzed by triplicate injections.

2.2. Plant materials

Two varieties of red clover tetraploide clover Vesna and diploide clover Start were collected in the Šlechtitelská stanice Hladké Životice (Czech Republic) in the first cutting in summer 1998. The material was dried at 105°C and immediately milled to tiny particles (<0.5 mm) just before extraction. The material (2 g) was dispersed in 10 ml of water, incubated at 37°C for 30 min and mixed with 10 ml of 3.5 *M* HCl and 80 ml of 80% ethanol. The mixture was heated to boiling point for 10 min, allowed to cool, and collected after filtration through folded rapid paper.

2.3. Solid-phase extraction

Spe-ed C_8 octyl, 200 mg, Spe-ed C_{18}/18 octadecyl, 200 mg, Spe-ed Amide 2, 200 mg, Spe-ed RP-105, 200 mg, Spe-ed ABN, 200 mg (Applied Separations, Allentown, USA) and Oasis HLB 1 ml 30 mg (Waters, Milford, MA, USA) cartridges were tested for off-line trace enrichment using the Alltech manifold system (Alltech, Deerfield, IL, USA). Ethanolic extracts (1 ml typically) diluted with 3 ml of water were used for SPE extractions.

The cartridges were conditioned with 3 ml of water (Amide 2) or 3 ml of methanol and 3 ml of water (C_{18}, C_8, RP 105, ABN and Oasis HLB). A sample was passed through the cartridge. Impurities were washed out with 3 ml of 10% (C_{18}, C_8, HLB and Amide 2) or 5% methanol (RP 105, ABN and Oasis HLB). Retained isoflavones were eluted with 3 ml of 80% methanol. The extracts were evaporated to dryness in a rotary vacuum evaporator at 39°C. The residues were dissolved in 500 μl of mobile phase and injected (10 μl) directly onto the RP-HPLC column. The results were compared with those obtained by the ''dry process'' using an unconditioned (dry) cartridge.

Recoveries were verified by the standard addition method with addition level 20 μg. Typically 1 ml of a standard solution of each isoflavone and 1 ml of a sample were mixed and diluted with water to a final volume. Isoflavone determinations were carried out for each addition level.

3. Results and discussion

The gradient profiles were optimized in order to obtain good resolution among peaks. The best selectivity, recovery, peak shape, USP plates (>11 400) and USP tailing (<1.08) from the tested mobile phases were obtained for gradients of acetonitrile and water containing 0.1% TFA. Calibration curves with extremely high linearity (r>0.9999) and low limits of detection (LODs) (9–17.6 n*M*) were obtained for all authentic standard solutions in the concentration range 0.06–520 μ*M* expected for plant extracts.

Recoveries were low for C_{18} and C_8 sorbents for both, wet and dry procedures. The extremely hydrophobic surfaces of C_8 and C_{18} make the conditioning

Table 1
Mean recoveries, R (n=3), of SPE of isoflavones of tetraploide (TP) and diploide (DP) clover for different sorbents and wet and dry procedures

	Recovery (%)											
	C_8		C_{18}		Amide 2		RP 105		ABN		HLB	
Analytes	TP[a]	DP[b]	TP[c]	DP[d]	TP[e]	DP[f]	TP[g]	DP[h]	TP[i]	DP[j]	TP[k]	DP[l]
Wet procedure												
Daidzein	87	85	86	87	92	92	97	97	98	99	94	93
Genistein	91	88	88	84	91	91	98	98	101	100	98	99
Formononetin	94	91	89	86	98	99	94	96	100	100	94	91
Biochanin A	86	87	91	88	95	93	93	93	102	102	99	98
Dry procedure												
Daidzein	41	38	34	33	90	88	85	86	90	88	90	89
Genistein	50	47	42	42	92	93	98	99	92	90	99	98
Formononetin	55	56	49	47	93	93	102	101	90	92	96	95
Biochanin A	40	40	66	64	89	89	94	95	89	88	91	90

[a] RSDs intervals for wet and dry (in parentheses) procedures [a] 3.7–5.3 (6.1–8.2)%, [b] 4.2–5.7 (6.2–8.2)%, [c] 3.3–6.7 (3.7–8.1)%, [d] 4.9–6.2 (4.2–7.8)%, [e] 2.7–5.0 (3.7–4.8)%, [f] 2.2–4.1 (2.8–4.5)%, [g] 2.9–4.3 (3.9–6.1)%, [h] 2.2–4.2 (3.2–5.8)%, [i] 2.7–4.8 (4.2–5.3)%, [j] 2.1–3.5 (4.4–5.6)%, [k] 2.5–4.0 (4.3–5.7)%, [l] 2.2–3.8 (3.9–5.5)%.

step critical to a successful extraction and must be monitored closely to prevent drying of the conditioned sorbent. New polymeric sorbents allow one to use wet and also dry procedures for the determination of isoflavones. Recoveries were greater than 90% in all cases. The best results were obtained for the wet procedure on ABN cartridge. Further advantages of the method are high reproducibility (the lowest RSDs) and low consumption of plant material (0.5–2.0 g of dry mass). The sorbents retain the polar organic compound more easily. The chemically modified polar organic groups increase the water wettability of the polymer. The sorbents do not need the conditioning step (see comparison of the results for wet and dry procedures in Table 1), thus the time of analysis is seriously shortened.

The selectivity of SPE isolation enables direct qualitative and quantitative HPLC determination of isoflavones in plant materials using Zorbax SB C_{18} rapid resolution column. The data can be useful to the breeding work of new *Trifolium* varieties with lower isoflavone concentrations, in scientific, medicinal, pharmaceutical fields and in the search for new sources of natural drugs. The method is characterized by the simplicity of the analytical procedure and relatively low cost of the reagents and equipment used.

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