Comparison of Solid-Phase Extraction Procedures for Isolation of Plant Isoflavones Prior to Liquid Chromatographic Determination

[a]B. KLEJDUS, [b]J. TŘINÁCTÝ, [a]P. HRDLIČKA, and [a]V. KUBÁŇ*

[a]*Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, CZ-613 00 Brno*
e-mail: kuban@mendelu.cz

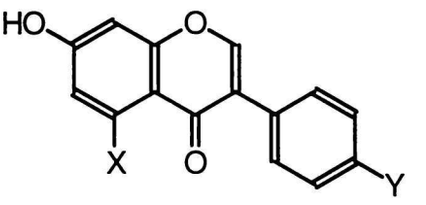
[b]*Research Institute of Animal Nutrition, Ltd., CZ-691 23 Pohořelice*

Received 26 January 2001

Solid-phase extraction (SPE) procedures for isolation of isoflavones using five different SPE cartridges and subsequent RP-HPLC separation and quantitation of daidzein, genistein, formononetin, and biochanin A were compared. Extraction efficiencies were evaluated and recoveries greater than 91—99 % were obtained using the OASIS^{TM} HLB copolymer cartridges. The isoflavones extracted with a mixture of 80 vol. % ethanol and 2 M-HCl from two varieties of red clover were quantified by diode-array RP-HPLC analysis with limits of detection LODs 10—25 nmol dm^{-3} in the concentration ranges from tenths of μmol dm^{-3} up to 0.4—0.5 mmol dm^{-3}. Higher levels (by factor 1.5—2) of all isoflavones were found in tetraploid than in diploid red clover.

Usually complicated separation and preconcentration procedures are applied for isolation of phenolic substances from plant materials prior to their chromatographic separation and subsequent identification. Efficient and fast ultrasonification, liquid/liquid extraction, and simple digestion with a solvent or a mixture of solvents can be applied [1, 2]. At present, the combination of separation and preconcentration procedures is preferred. They allow high enrichment factors and separation of interfering components (proteins, chlorophylls, waxes, oils, *etc.*) from complicated matrices of analyzed samples. Hence, solid-phase extraction (SPE) has become very popular procedure for isolation, purification, and preconcentration of organic compounds present in plant materials. In most methods, C18-bonded silica has been used as the sorbent for extraction of estrogenic isoflavones and coumestrol in animal feeds [3], for determination of estrogens in bovine blood plasma and urine [4], for determination of isoflavones from root extracts [5], and for separation and identification of phytoalexins from leaves [6]. Ethanol (60—80 vol. %) has been used as the solvent [3, 7, 8]. Addition of HCl increased the extraction efficiency of the isoflavones [2, 3, 9, 10].

In this work, different solid-phase extraction (SPE) procedures for separation of isoflavone compounds were compared using two different red clover (*Trifolium pratense*) varieties. Reversed-phase HPLC with diode-array detection was used for determination of the most common isoflavones: daidzein, genistein, formononetin, and biochanin A.



	X	Y
Biochanin A	OH	OCH_3
Genistein	OH	OH
Formononetin	H	OCH_3
Daidzein	H	OH

EXPERIMENTAL

All HPLC grade solvents – methanol, ethanol, acetonitrile, acetone, dichloromethane – were from Sigma (Steinheim, F.R.G.). Daidzein, formononetin, and biochanin A were from Carl Roth & Co. (Karlsruhe, F.R.G.). Genistein was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Isoflavones stock solutions were prepared by dissolving each substance in ethanol (250 μg cm^{-3}). Accu Bond SCX Benzenesulfonic Acid, Accu Bond C8 Octyl, and Accu Bond C18 Octadecyl (200 mg, all from J & W Scientific Fisons, Folsom, CA, U.S.A.), and OASIS^{TM} HLB 1cc 30 mg (Waters, F.R.G.) SPE cartridges were tested using an Altech manifold system (Altech, Deerfield, IL, U.S.A.).

*The author to whom the correspondence should be addressed.

Two varieties of tetraploid clover Vesna and diploid clover Start were collected in the Breeding Station Hladké Životice (Czech Republic) in the first cutting in summer 1999. The plant material was dried at 105 °C and milled (0.5 mm particles) just before extraction. The material (*ca.* 2 g) was finally dispersed in 10 cm^3 of water and incubated at 37 °C for 30 min. After incubation, 10 cm^3 of 2 M-HCl and 80 cm^3 of 80 vol. % ethanol were added. Mixture was boiled for 10 min and let to cool down, filtered through folded rapid filter paper and clean filtrate was collected. No degradation of isoflavone aglycones was observed during 30 min boiling. A significant decrease of malonate and acetate glycosides was observed, but the groups were not studied in this work.

Portions of the ethanolic extracts (1 cm^3 typically) were diluted with 3 cm^3 of water prior to the SPE extraction procedures. The procedure was repeated several times until nonsignificant increase in concentration (less than 1 rel. %) was obtained (mostly one step was sufficient for complete extraction).

SPE of Phytoestrogens from Plant Materials

Conditioning process consisted of rinsing the SCX cartridges with 3 cm^3 of dichloromethane—acetone (φ_r = 3:1). A sample was passed through the cartridge, interfering substances were washed out with 4 cm^3 of dichloromethane—acetone (φ_r = 3:1) and isoflavones were eluted with 2 cm^3 of 80 vol. % methanol. C8 cartridges were conditioned by rinsing with 3 cm^3 of methanol and 3 cm^3 of water. A sample was passed through the cartridge, the cartridge was washed with 3 cm^3 of 20 vol. % methanol and isoflavones were eluted with 3 cm^3 of methanol—acetonitrile (φ_r = 1:1). C18/1 cartridges were conditioned by rinsing with 3 cm^3 of methanol and 3 cm^3 of water. A sample was passed through the cartridge, the cartridge was washed with 3 cm^3 of 20 vol. % methanol and isoflavones were eluted with 3 cm^3 of 80 vol. % methanol. C18/2 cartridges were conditioned by rinsing with 3 cm^3 of methanol and 3 cm^3 of water (pH 2.0 with 3 M-HCl). A sample was passed through the cartridge, the cartridge was washed with 3 cm^3 of water (pH 2.0 with 3 M-HCl) and isoflavones were eluted with 3 cm^3 of methanol—acetonitrile (φ_r = 1:1). OASIS™ HLB cartridges were conditioned by rinsing with 3 cm^3 of methanol and 3 cm^3 of water (pH 2.0 with 3 M-HCl). Then a sample was passed through the cartridge, the cartridge was washed with 3 cm^3 of water and isoflavones were eluted with 3 cm^3 of methanol—acetonitrile (φ_r = 1:1).

All solid-phase extracts were evaporated to dryness in a rotary vacuum evaporator at temperature 39 ± 2 °C, the residues were dissolved in 500 mm^3 of the mobile phase and 40 mm^3 were injected directly on a HPLC column.

Apparatus and Chromatographic Conditions

The HPLC analysis was carried out on an HP 1100 chromatograph with diode-array detector (Hewlett—Packard). Compounds were separated on reversed-phase columns Hypersil ODS C18 (250 mm × 4.6 mm, 5 μm particle size, Hewlett—Packard), Supelcosil LC 18 (250 mm × 4.6 mm, 5 μm particle size, Supelco, Bellefonte, PA, U.S.A.), Nucleosil C18 (250 mm × 4.6 mm, 5 μm particle size, Merck), and Hypersil BDS C18 (250 mm × 4.6 mm, 5 μm particle size, Shandon, Cheshire, U.K.) in an isocratic mode with methanol—acetonitrile—15 mmol dm^{-3} phosphate buffer of pH 6.5 (φ_r = 42:20:38). The flow rate was 1.0 cm^3 min^{-1} and column temperature was 30 °C. The effluent was monitored with DAD at λ = 254 nm (SBW 20 nm) and λ = 360 nm (SBW 100 nm) as a reference. The acquisition of UV spectra for identification purposes was automatic at the apex, both inflection points, and the base of all peaks (λ = 190—400 nm, 2 nm steps). All analytes detected by HPLC in plant extracts were identified by comparing retention times and UV spectra with those for authentic standards analyzed in the same batch as the plant extracts and comparing UV spectra library by using match factor. The match quality is a number indicating how close sample spectrum matches the one in the spectral library. Numbers over 990 indicate good match, numbers between 950 and 990 indicate the possibility of match, and numbers below 950 indicate no match.

Quantification of Isoflavones

Calibration curves were obtained for each standard with high linearity (r > 0.9998) by plotting the peak area obtained from HPLC analysis with 40 mm^3 injections as a function of standard concentration. For this purpose the stock solutions of the standards were diluted with the mobile phase to six different concentrations. Each concentration was analyzed by triplicate injections (Table 1). Appropriate peak areas and statistical parameters of calibration curves were used for calculation of analyte concentrations in plant samples.

RESULTS AND DISCUSSION

HPLC for Analysis of Phytoestrogens

The effectivity of HPLC separation of the isoflavonoids on Hypersil ODS C18, Supelcosil LC 18, Nucleosil C18, and Hypersil BDS C18 reversed-phase columns was tested with authentic standards under isocratic conditions at 42 vol. % of methanol, 20 vol. % of acetonitrile, and 38 vol. % of 15 mmol dm^{-3} phosphate buffer with pH 6.5. Hypersil BDS C18 showed the best selectivity, recovery, and excellent peak symmetry. The USP tailing factor was less than 1.20 for all peaks and number of USP plates was greater than

Table 1. Chromatographic Performance and Calibrations Parameters on Hypersil BDS C18 Column and Library Search Result for Isoflavones

Isoflavones	RT[a] / min	k'[b]	USP tailing	USP plates	Conc. / μmol dm^{-3}	LOD[c] / nmol dm^{-3}	MF[d]
Daidzein	4.7	1.32	1.18	9223	0.2—390	17.8	995.2
Genistein	5.4	1.94	1.05	10554	0.7—435	18.7	995.3
Formononetin	9.8	4.50	1.20	10879	0.8—480	10.4	998.3
Biochanin A	15.6	8.00	1.20	11591	0.9—440	24.2	999.3

a) Retention time, *b*) capacity factor, *c*) concentration as a function of peak area units, r = 0.9998—0.9999, *d*) match factor.

Table 2. Mean Recoveries (R/%, n = 3) of SPE of Phytoestrogens of Tetraploid (TP) and Diploid (DP) Red Clover for Different Sorbents

Isoflavones	SCX[a]		C8[b]		C18/1[c]		C18/2[d]		HLB[e]	
	TP	DP	TP	DP	TP	DP	TP	DP	TP	DP
Daidzein	86	78	95	87	88	83	83	86	93	94
Genistein	83	81	95	92	91	89	93	93	96	93
Formononetin	93	85	97	96	85	86	94	93	93	92
Biochanin A	90	74	91	94	86	87	94	94	99	98

RSDs intervals for tetraploid (diploid) red clover: *a*) 3.2—9.4 (7.6—9.3), *b*) 3.1—5.2 (2.1—6.8), *c*) 4.8—9.1 (5.3—8.6), *d*) 2.1—8.3 (3.8—8.8), *e*) 2.6—6.3 (2.1—4.8).

Table 3. Content of Isoflavones (mg kg^{-1} of DM) in Red Clover Dry Matter (DM)

Isoflavones	SCX	C8	C18/1	C18/2	HLB	Average and interval (in brackets)
				Diploid		
Daidzein	130.3	159.9	132.6	175.5	199.8	159.6 (149—170)
Genistein	327.7	394.2	362.0	373.2	397.8	371.0 (360—381)
Formononetin	3568	4246	3631	4097	3631	3832.6 (3718—3947)
Biochanin A	1193	1639	1307	1574	1681	1479.7 (1404—1554)
				Tetraploid		
Daidzein	221.3	262.4	209.8	110.6	237.5	208.5 (187—229)
Genistein	641.2	704.2	648.4	682.5	697.2	647.7 (662—687)
Formononetin	6027	6289	5371	6112	5818	5923.9 (5787—6060)
Biochanin A	2689	2992	2401	2919	3155	2832.1 (2719—2945)

9200 for all mixtures (Table 1). The selectivity of this column was significantly better than for the other columns. Co-elution of biochanin A (peak No. 4) and prunetin (following peak with retention time 17.5 min) was observed when gradient elution was applied [11].

Calibration curves with high linearity (r > 0.9998) and extremely low detection limits (LODs 10—25 nmol dm^{-3} – see Table 1) were obtained for all standards in the concentration ranges from tenths of μmol dm^{-3} up to 0.4—0.5 mmol dm^{-3} typical for isoflavones in plants extracts. The typical chromatogram is given in Fig. 1*a*.

SPE Efficiencies

The extraction conditions [3] were modified in our experiments, and the mixture of 80 vol. % ethanol with 2 M-HCl was found to be the best when it was boiled with plant material for 10 min. The recoveries were greater than 91—98 % in all cases using the HLB cartridge (Table 2) while for C18, C8, and SCX sorbents the recoveries were lower (70—80 % in some cases). Lower concentrations of formononetin when using HLB can be explained by lower content of sorbent (30 mg) in the cartridge, and insufficient capacity of the cartridge for given concentrations in comparison with the C18 cartridges (200 mg).

Identification and Quantification

Analytes were identified by comparing retention times and UV spectra using match factors. The match factors were higher than 995.0 in all cases (Table 1). The measured forage levels for total amounts of daidzein, genistein, formononetin, and biochanin A in diploid and tetraploid red clover are listed in Ta-

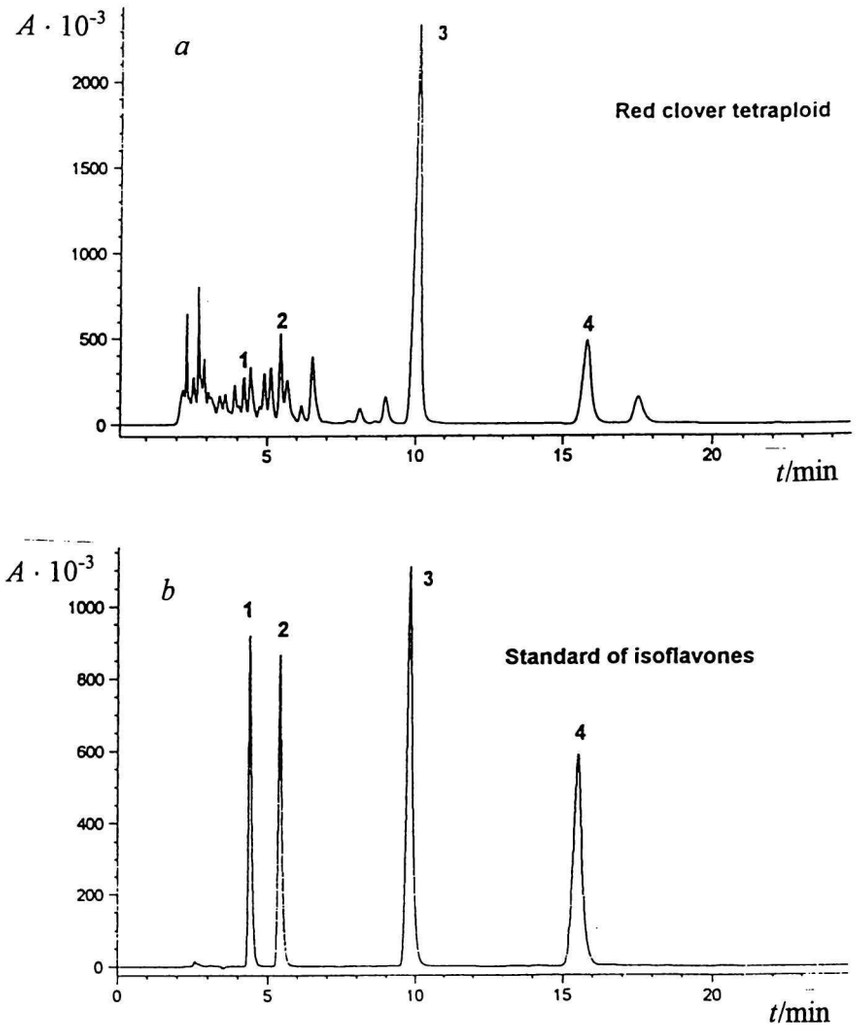


Fig. 1. Chromatograms ($A \cdot 10^{-3}$ *vs.* time in min) of isoflavones standard solutions (*a*) and SPE extracts from red clover tetraploid using OASIS^{TM} HLB cartridge (*b*) monitored at $\lambda = 254$ nm (SBW 20 nm). *1.* Daidzein; *2.* genistein; *3.* formononetin; *4.* biochanin A.

ble 3 as mean values of five separate analyses. In general, approximately 1.5—2 times higher levels of all isoflavones were found in tetraploid red clover than in diploid red clover in agreement with the literature data [10, 12].

CONCLUSION

In conclusion, OASIS^{TM} HLB copolymer was found to be the best of all tested sorbents, because of its higher recovery compared to the silica-bonded sorbents. Better chromatographic retention factors observed for the HLB sorbent correspond to the higher recoveries of polar compounds. The HLB sorbent, which is free of silanol groups, retains analytes predominantly by hydrophobic interactions. C18-bonded silica reversed-phase sorbents have several limitations. Residual surface silanol groups on C18-bonded silica pose problems for basic compounds. They are typically not well retained on traditional C18-bonded silica sorbents. In HPLC separations, the silanol groups can cause significant peak tailing of basic compounds.

Similar interactions, which cause low recoveries, occur on silica-based SPE supports. The degree of silanol interaction varies depending on the pH and ionic strength of the sample matrix. Low pH of the solutions will hydrolyze the bonded phase, while the solutions at elevated pH will dissolve the base silica. Another limitation of C18 sorbents is the fact that polar analytes may be insufficiently retained. This reduced retention can result in lower recoveries because of the analyte breakthrough during the sample-loading step. According to the increased importance of more polar compounds, C18 and C8 SPE sorbents may fail to provide satisfactory recoveries in those cases.

Proposed procedure represents an easy, fast, reproducible, reliable, and sensitive method for quantitative

determination of daidzein, genistein, formononetin, and biochanin A in forages and other plant materials and foods.

Acknowledgements. The financial support from MŠMT of the Czech Republic, Grant Reg. No. MSM 432100001, is gratefully acknowledged.

REFERENCES

1. Williams, C. A. and Harborne, J. B., *Isoflavonoids.* In *Methods in Plant Biochemistry.* (Dey, P. M. and Harborne, J. B., Editors.) Vol. *1*, *Plant Phenolics.* (Harborne, J. B., Editor.) Pp. 421—449. Academic Press, London, 1989.
2. Franke, A. A., Custer, L. J., Cerna, C. M., and Narala, K. K., *J. Agric. Food Chem. 42*, 1905 (1994).
3. Pettersson, H. and Kiessling, K. H., *J. Assoc. Off. Anal. Chem. 67*, 503 (1984).
4. Lundh, T. J. O., Pettersson, H., and Kiessling, K. H., *J. Assoc. Off. Anal. Chem. 71*, 938 (1988).
5. Pietta, P., Pierlugi, M., Manera, E., and Pierlugi, C., *J. Chromatogr. 513*, 397 (1990).
6. Edwards, C. and Strange, R. N., *J. Chromatogr. 547*, 185 (1991).
7. Murphy, P. A., *J. Chromatogr. 211*, 166 (1981).
8. Coward, L., Barnes, N. C., Setchell, K. D. R., and Barnes, S., *J. Agric. Food Chem. 41*, 1961 (1993).
9. Wang, G., Kuan, S. S., Francis, O. J., Ware, G. M., and Carman, A. S., *J. Agric. Food Chem. 38*, 185 (1990).
10. Klejdus, B., Vitamvásová, D., and Kubáň, V., *J. Chromatogr., A 839*, 261 (1999).
11. Klejdus, B., Vitamvásová, D., and Kubáň, V., *Anal. Chim. Acta, 446*, 000 (2001), in press.
12. Vetter, J., *Trifolium Species 43*, 106 (1993).