The determination of coumestrol in alfalfa (*Medicago sativa*) by capillary electrophoresis

J. Moravcová[1], T. Kleinová[1], R. Loučka[2]

[1]Institute of Chemical Technology, Prague, Czech Republic
[2]Research Institute of Animal Production, Prague, Czech Republic

ABSTRACT

High performance capillary electrophoresis (HPCE) on an uncoated fused-silica capillary column using a borate buffer at pH 9.2 as electrolyte and diode-array detection was developed for the determination of coumestrol in alfalfa. The linear detector response was established in the concentration range 0.76–140 mg.dm^{-3}, the minimum detectable limit was 0.39 mg.dm^{-3}, and migration time of coumestrol was 5 min. 3-Isobutyl-1-methylxanthin was used as an internal standard. Coumestrol was isolated by acid extraction employing a mixture hydrochloric acid-acetonitrile at 95°C for 30 min followed by solid phase extraction. Relative standard deviations of reproducibility and repeatability were 1.77% and 5.49%, respectively. Spiking recovery value of 92% was achieved. Alfalfa, variety Morava, contains 148–248 mg.kg^{-1} coumestrol in dry matter. The proposed method is useful for routine analyses.

Keywords: phytoestrogens; alfalfa; plant analysis; animal feed

Phytoestrogens are secondary plant metabolites exerting estrogenic effects on the central nervous system. They induce oestrus and stimulate growth of the genital tract of female animals. Dietary phytoestrogens are regularly consumed by farm animals and can contribute to their infertility (Farnsworth et al. 1975). Although phytoestrogens are weakly estrogenic (Welshons et al. 1990), any diet containing phytoestrogens has the potential to alter animal reproduction or hormone-dependent disease states. Moreover, phytoestrogens may also possess antiestrogenic activity and the balance between these two effects depends on the ratio of phytoestrogens to estrogens. Cattle have relatively low level of estradiol, thus the estrogenic effects could dominate. Alfalfa (*Medicago sativa*) containing coumestrol, the most potent estrogenic phytoestrogen, has been reported to cause temporary infertility in cattle (Adler 1969), cattle fed alfalfa haylage containing 37 ppm coumestrol showed clinical signs of estrogenic stimulation (Lookhart 1980). On the other hand, some beneficial effects of alfalfa diet forage have been also reported (Vanzant and Cochran 1994, Vagnoni and Broderick 1997, Broderick et al. 1999) including increased rate of growth and milk production. The content of coumestrol in alfalfa can be varied by number of factors: attack of aphids or fungal pathogens, humidity, age of the plant, temperature, and many others arising from the processing used.

Suitable analytical methods are therefore required for the detection of coumestrol in both alfalfa and animal forage containing alfalfa. Paper chromatography (PC) (Knuckles et al. 1976) or gas chromatography (GC) (Saloniemi et al. 1995) has been used exceptionally. High performance liquid chromatography (HPLC) has been frequently developed using reversed-phase C18, mostly in mixtures of methanol or acetonitrile and aqueous acids or buffers as modifiers (Lookhart 1980, Murphy 1981, Pettersson and Kiessling 1984, Setchell and Welsh 1987, Wang et al. 1990, Franke et al. 1994). Because of its speed and very high separation efficiency, high performance capillary electrophoresis (HPCE) has been introduced into determination of coumestrol (Shibabi et al 1994, Moravcová and Kleinová 2001). However, analysis of coumestrol from plant extracts is difficult, often requiring time-consuming sample handling and hydrolysis of originally presented coumestrol glucosides.

The aim of this work is the optimization of the extraction, hydrolysis, separation, and HPCE quantification of coumestrol in alfalfa.

MATERIAL AND METHODS

Sodium borate decahydrate and boric acid were purchased from Lachema (Czech Republic), and 3-isobutyl-1-methylxanthin from Sigma-Aldrich (Germany). Biochanin A, formononetin, and prunetin were a gift from Dr. Lapčík (Institute of Endocrinology, Prague, Czech Republic). 3-Methoxy-8-nitronapht-2-ol was synthesized by Dr. Šmidrkal (Institute of Chemical Technology, Prague, Czech Republic). Acetonitril and *p*-nitrophenol were obtained from Merck (Germany). Sodium hydroxide solutions (0.1 and 1.0 M) for HPCE were produced by Hewlett-Packard (U.S.A.). Solutions were concentrated under reduced pressure with a bath temperature below 40°C. Samples of alfalfa herbage were supplied by the Institute of Animal Production (Prague, Czech Republic). Alfalfa (*Medicago sativa*), variety Morava was harvested in 1998 (sample 1 and 2, second cutting) and 1999 (sample 3, first cutting, sample 4, second cutting). Coumestrol was synthesized as described previously (Moravcová and Kleinová 2001).

Sample preparation

A. Powdered alfalfa hay (0.5 g) was stirred with an extraction solution (25 mL) at 95°C under reflux. The extraction solution consisted of 66% acetonitrile or 80% ethanol (both v/v) in water. An aliquot of liquor (2 mL) was withdrawn after 30, 60, and 120 min. Solids were removed by filtration and clear filtrate was either hydrolyzed or directly analyzed by HPCE.

B. Powdered alfalfa hay (0.5 g) was soaked with water (2.5 mL) at 37°C within 30 min. The extraction solution with acetonitrile or ethanol was added (19 mL) and further procedure was identical to that described above.

C. Powdered alfalfa hay (0.5 g) was stirred with 82% acetonitrile in water (20 mL) containing hydrochloric acid (3.5 M, 5 mL) at 95°C under reflux. The mixture was sampled after 10, 20, 30, 60, and 90 min.

Acid hydrolysis

Filtrate (2 mL) obtained by procedure A or B was diluted with hydrochloric acid (3.5 M, 0.5 mL) and hydrolyzed at 95°C. The mixture was sampled after 10, 20, 30, 60, and 90 min and analyzed by HPCE.

Solid-phase extraction

The C18 cartridge (Sep-Pak® Vac 1cc, 50 mg, Waters, Ireland) was conditioned with methanol (3 mL) and water (3 mL). Hydrolyzed extracts A and B or acid extract C was passed through the cartridge. Impurities were washed with 10% methanol in water (3 mL). Retained fraction was eluted with 90% methanol in water (3 mL). Eluate was concentrated to dryness, residue was dissolved with 33% acetonitrile in water (v/v, 1 mL) and filtered through a pore-size cellulose membrane filter (0.2 μm, Sartorius AG, Germany). This clear solution was directly analyzed by HPCE.

HPCE conditions

A HP ^{3D}CE instrument equipped with diode-array detector and HP ChemStation software package were used. Prior to injection, the capillary was purged with 1 M sodium hydroxide (1 min), water (1 min), 0.1 M nitric acid (1 min), water (1 min), and filled with buffer (3 min). A replenishment of buffer was applied in all cases and the purging vacuum pressure was 2 kPa. The UV detection was performed at 220 nm with scanning between 190 and 400 nm for the identification purposes. The column temperature was kept at 40°C and the separation potential was 15 kV (positive polarity) which corresponded to the current 70 μA. Running electrolyte and standard solutions were filtered through 0.2 μm pore-size cellulose membrane filters (Sartorius AG, Germany).

Conditions A (Moravcová and Kleinová 2001): uncoated fused-silica capillary column with the extended light patch (bubble cell FD 3) (50 μm I.D. × 50 cm to the detector window, 56 cm total length), borate buffer pH 10.5 prepared from boric acid (200 mM) and sodium hydroxide (1 M).

Conditions B: uncoated fused-silica capillary column (50 μm I.D. × 50 cm to the detector window, 56 cm total length), borate buffer pH 9.2 prepared from sodium borate (50 mM) and boric acid (200 mM).

RESULTS AND DISCUSSION

During extraction, the ratio of solvent volume (milliliters) to alfalfa hay (grams) was never lower than 10 in all trials, according to the recommendation for exhaustive extraction of isoflavones (Coward et al. 1993). Aqueous ethanol and acetonitrile were chosen as extract solvents (Wang et al. 1990, Franke et al. 1994) and extraction efficiencies were optimized by varying refluxing period. Extracts of alfalfa (sample 1) were analyzed by HPCE (conditions A) allowing the separation of six isoflavones (isoformononetin, prunetin, formononetin, biochanin A, genistein, and daidzein) and coumestrol (Moravcová and Kleinová, 2001). Our results indicated that these solvent systems produced similar extraction efficiencies within the same time interval (Figure 1), the concentration of bound coumestrol reached a maximum after 30 min heating under reflux. Rehydration of dry sample prior to extraction was not helpful in shortening the extraction time in contrast to the observations described for the extraction of isoflavones (Wang et al. 1990). A 30 min extract was subsequently hydrolyzed with hydrochloric acid and the content of free coumestrol was checked by HPCE (Figure 2). Concentration of coumestrol in the extract increased with heating time, reached a maximum after 30 min, and then leveled off. The best conditions for the separate extraction and hydrolysis were estimated as follows: 80% ethanol or 66% acetonitrile, 30 min at 95°C, and a treatment with 0.7 M hydrochloric acid for 30 min at 95°C. Enzymatic hydrolysis of the extract was omitted because it was found to be slightly less effective than acid hydrolysis (Franke et al. 1994). It was found previously (Murphy 1981, Wang et al. 1990) that the addition of hydrochloric acid to methanol, acetone, acetonitrile, or ethanol can improved the extraction efficiency of isoflavones and coumestrol. We used slightly modified conditions described (Pettersson and Kiessling 1984, Klejdus et al. 1999) and we found that 30 min refluxing with 0.7 M hydrochloric acid in both 66% acetonitrile and 80% ethanol gave excellent yield for coumestrol. Thus, the acid extraction is clearly preferred over the two-step procedure due to the substantially reduced time. Further purification of extracts by solid phase extraction resulted in the remarkable reduction of analytes (Figure 3). The extraction with aqueous hydrochloric acid – acetonitrile was preferred because this system gave less impurities in the extract (Figure 3).

Interestingly, the migration time of coumestrol strongly depends on the pH of a sample solution. If the acid solution was introduced on the capillary column (Figure 2), the

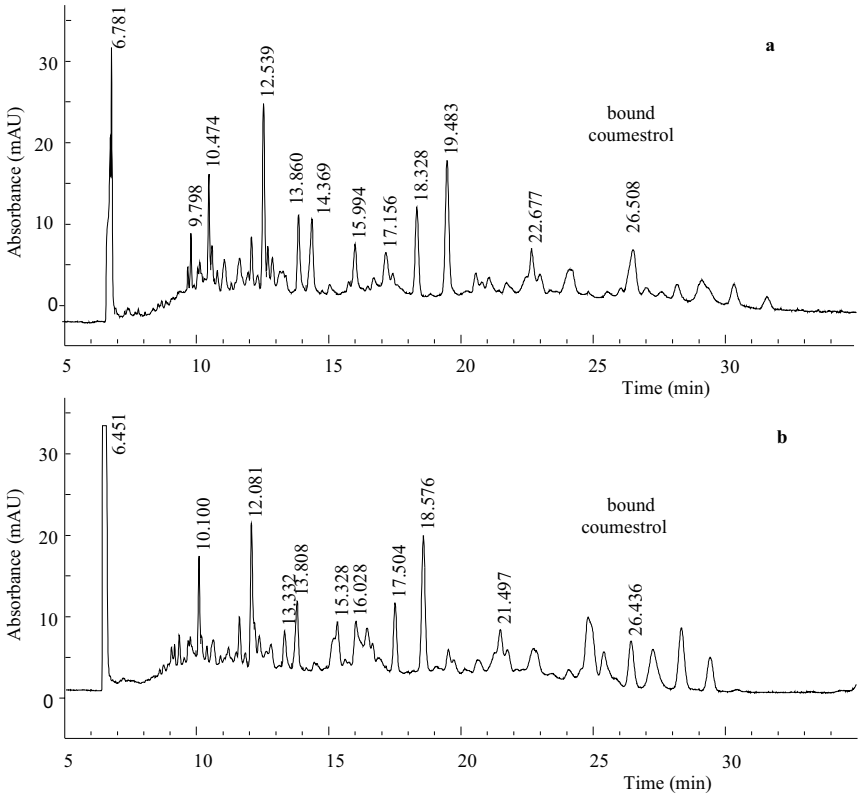

Figure 1. Electropherogram of alfalfa extracts (30 min, 95°C): a) 80% ethanol, b) 66% acetonitrile

migration time 18.5 min was obtained. Analyzing neutral filtrate after solid phase clean up, coumestrol migrates at 25 min. Moreover, the high purity of the acid extract (Figure 3b) allowed to decrease the HPCE separation time. Using a borate-working electrolyte with pH 9.2 (conditions B), excellent separation of isoflavones from coumestrol was achieved in 5 min (Figure 4). These HPCE conditions were used throughout this study. The linearity of the detector response was established at three wavelengths in the concentration range from 0.76 to 140 mg.dm^{-3} (Table 1). The minimum detectable limit calculated as a signal (S) to noise (N) ratio of 3 was 0.1 mg.dm^{-3}

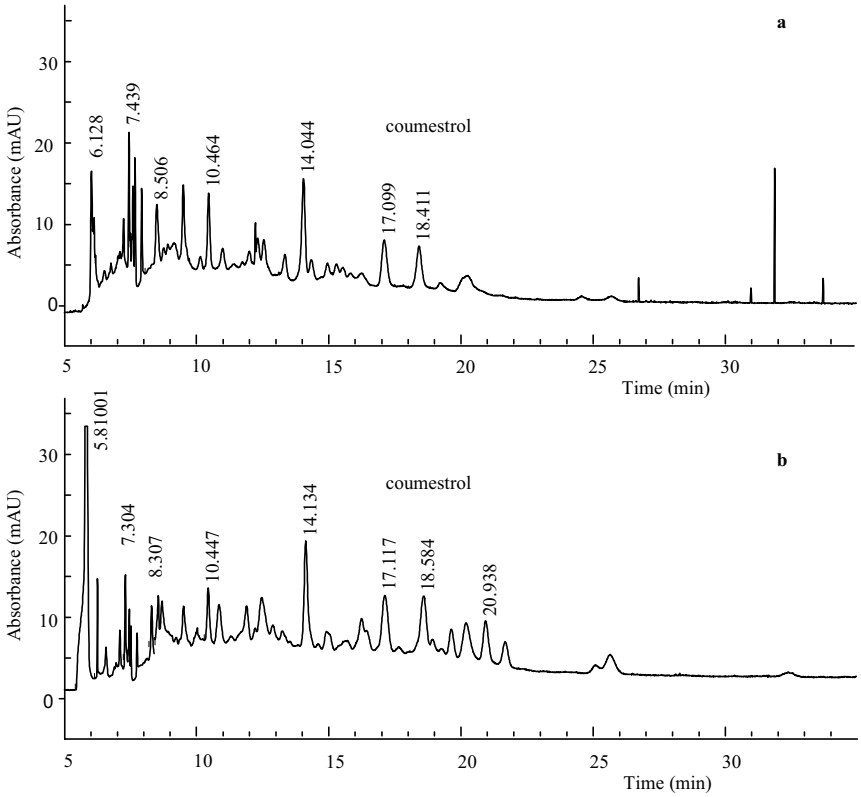

Figure 2. Electropherogram of hydrolyzed alfalfa extracts (30 min, 95°C, 0.7 M HCl): a) 80% ethanol, b) 66% acetonitrile

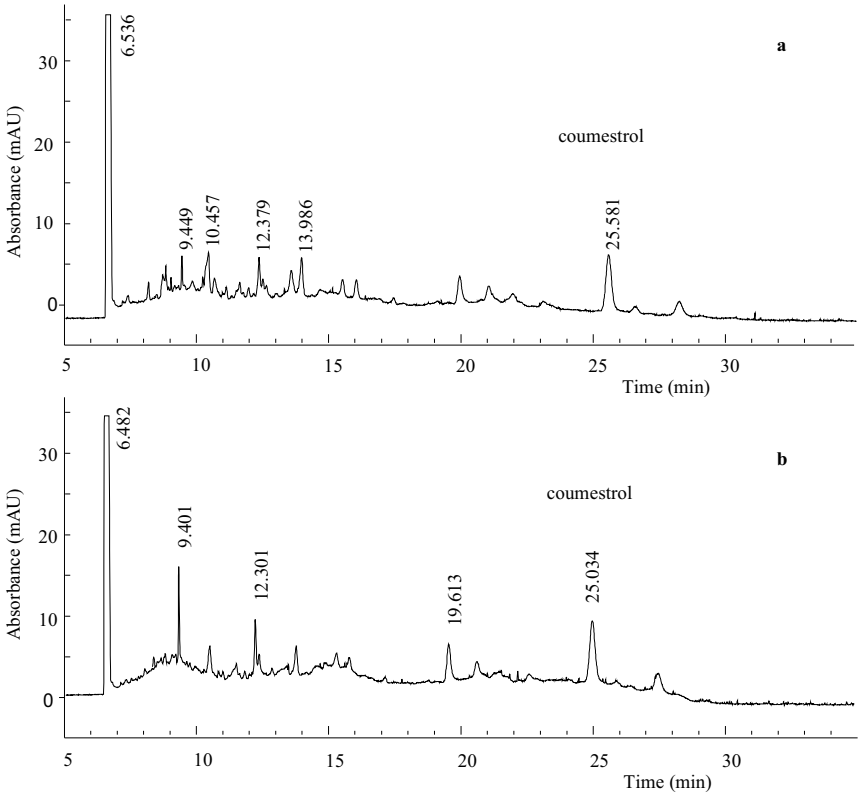

Figure 3. Electropherogram of hydrolyzed alfalfa extracts with C18 clean up: a) the purified 80% ethanol extract, b) acid extract with 0.7 M HCl in 66% acetonitrile

and the migration time of coumestrol was 5.02 ± 0.04 min. Internal standards are recommended generally for analyses therefore we searched for compounds having the potential to mimic coumestrol during sample pretreatment. Although 3-methoxy-8-nitronapht-2-ol was the best candidate, it cannot be accepted due to the commercial inaccessibility. 3-Isobutyl-1-methylxanthin (IMX) was destroyed by 70% during 30 min acid extraction therefore it had to be added into a cold extract immediately before solid phase extraction. Furthermore, impurities should be washed out with water only. *p*-Nitrophenol (PNP) (Väntinnen and Moravcová 1999) was stable under acid conditions but it was not retained enough by C18 stationary phase. We tested several other compounds (coumarin, flavone, quercetin, resorcinol) but either low stability or low affinity to C18 phase was observed. We decided to assess both reproducibility and repeatability of the HPCE method with IMX as internal standard due to its short migration time (Figure 5). The relative standard deviation (*RSD*) established for reproducibility (1.77%, Table 2) is much better than this one (8.0%) found for the HPLC determination of coumestrol in soy sprout (Wang et al. 1990), however, it contains only 2% of coumestrol presented in alfalfa. High repeatability (*RSD* = 5.49%, Table 2) confirms the validity of the proposed procedure as well. Detection limit calculated for S/N = 3 of an authentic alfalfa extract was found to be extremely low (0.39 ± 0.19 mg.dm^{-3}). This might be explaining by the low background noise observed in the proposed system when solid phase extraction was applied. Finally, the alfalfa sample (x_{av} = 237.18 mg.kg^{-1}) was spiked with coumestrol (added 70 mg.kg^{-1}), subjected to the acid extraction, and analyzed by the proposed HPCE

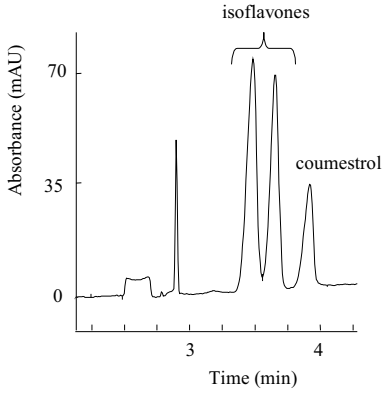

Figure 4. Electropherogram of a standard mixture of isoflavones and coumestrol (conditions B)

Table 1. The calibration results* for the HPCE analysis of a standard solution of coumestrol (conditions B)

Wavelength (nm)	$y = ax + b$		Correlation coefficient
	a	*b*	
220	5.5148	–1.5426	0.99992
280	3.1555	–1.1383	0.99987
375	5.3003	0.3233	0.99998

* calculated from two replicate analyses

Table 2. The statistic evaluation of the HPCE determination of coumestrol in alfalfa (sample 1)*

	x_{min} (mg.kg^{-1})	x_{max} (mg.kg^{-1})	x_{av} (mg.kg^{-1})	*SD* (mg.kg^{-1})	*RSD* (%)
Replicate injection of alfalfa extract (n = 9)	231.01	244.07	237.18	4.21	1.77
Replicate extraction of alfalfa (n = 9)	237.18	277.98	258.40	14.2	5.49

* cIMX = 8.5 mg.dm^{-3}

Table 3. Mean total concentration of coumestrol in alfalfa

	Coumestrol (mg.kg^{-1})	
	IMX	PNP
Sample 2	278.0	278.5
Sample 3	147.4	133.1
Sample 4	177.6	180.5

method. The recovery for coumestrol was 91.2 ± 7.8%, which corresponds to the data published previously (Pettersson and Kiessling 1984, Wang et al. 1990, Franke et al. 1994).

Employing the developed HPCE method, coumestrol was determined in alfalfa samples after 30 min acid extraction with hydrochloric acid – acetonitrile followed by a C18 clean up. Both IMX and PNP were used as internal standards, IMX was added before while PNP after solid phase extraction (Table 3). The presented coumestrol contents compare very favorably with those published formerly (147–278 mg.kg^{-1}, Lookhart 1980). Differences in coumestrol levels comparing our values with other studies (11–118 mg.kg^{-1}, Knuckles et al. 1976, 25–65 mg.kg^{-1}, Saloniemi et al. 1995) might be due to the origin of alfalfa, its genetic dispositions, or age. This fact demonstrates clearly the necessity of additional studies covering the changes in coumestrol level depending on plant species, plant part, maturation, growing conditions and processing.

In conclusion, our developed procedure represents a fast, reproducible, and sensitive method for a routine quantification of coumestrol in alfalfa. Nevertheless, the search for a more suitable internal standard will be continued.

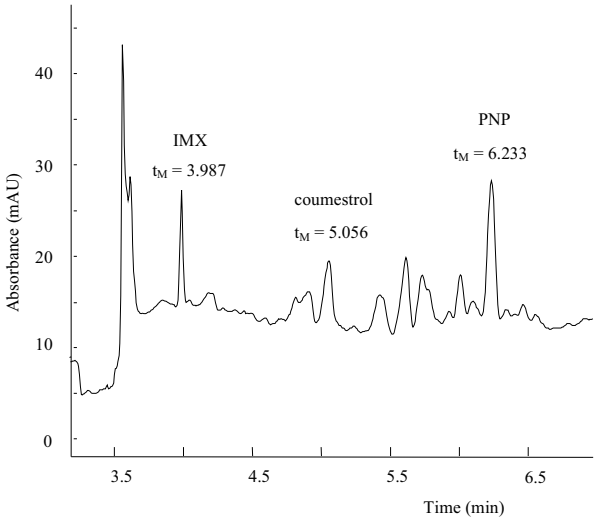

Figure 5. Electropherogram of an acid alfalfa extract with internal standards

This work was supported by the Grant Agency of the Czech Republic, project No. 523/00/0567.

REFERENCES

Adler J.H.D. (1969): Effect of estrogenic activity in forage on the reproductive processes of dairy cattle. Acta Vet. Brno, *38*: 201–213.

Broderick G.A., Koegel R.G., Mauries M.J., Schneeberger E., Kraus T.J. (1999): Effect of feeding macerated alfalfa silage on nutrient digestibility and milk yield. J. Dairy Sci., *82*: 2472–2485.

Coward L., Barnes N.C., Setchell K.D.R., Barnes S. (1993): Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. J. Agric. Food Chem., *41*: 1961–1967.

Farnsworth N.R., Bingel A.S., Cordell G.A. (1975): Potential value of plants as source of new antifertility agents II. J. Pharm. Sci., *64*: 717–754.

Franke A.A., Custer L.J., Cerna C.M., Narala K.K. (1994): Quantitation of phytoestrogens in legumes by HPLC. J. Agric. Food Chem., *42*: 1905–1913.

Lookhart G.L. (1980): Analysis of coumestrol, a plant estrogen, in animal feed by high-performance liquid chromatography. J. Agric. Food Chem., *28*: 666–667.

Knuckles B.E., de Fremery D., Kohler G.O. (1976): Coumestrol content of fractions obtained during wet processing of alfalfa. J. Agric. Food Chem., *24*: 1177–1180.

Moravcová J., Kleinová T. (2001): The determination of isoflavones and coumestrol by capillary electrophoresis. Czech J. Food Sci., *19*: 132–138.

Murphy P.A. (1981): Separation of genistein, daidzein and their aglucons, and coumestrol by gradient high-performance liquid chromatography. J. Chromatogr., *211*: 166–169.

Pettersson H., Kiessling K.H. (1984): Liquid chromatographic determination of the plant estrogens coumestrol and isoflavones in animal feed. J. Assoc. Off. Anal. Chem., *67*: 503–506.

Saloniemi H., Wähälä K., Nykanen-Kurki P., Kallela K., Saastamoinen I. (1995): Phytoestrogen content and estrogenic effect of legume fodder. Proc. Soc. Exp. Biol. Med., *208*: 13–17.

Setchell K.D.R., Wels M.B. (1987): High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical, and thermospray mass spectrometric detection. J. Chromatogr., *386*: 315–323.

Shibabi Z.K., Kute T., Garcia L.L., Hinsdale M. (1994): Analysis of isoflavones by capillary electrophoresis. J. Chromatogr., *680*: 181–185.

Vagnoni D.B., Broderick G.A. (1997): Effects of supplementation of energy or ruminally undegraded protein to lactating cows fed alfalfa hay or silage. J. Dairy Sci., *80*: 1703–1712.

Väntinnen K., Moravcová J. (1999): Phytoestrogens in soy foods: determination of daidzein and genistein by capillary electrophoresis. Czech J. Food Sci., *17*: 61–67.

Vanzant E.S., Cochran R.C. (1994): Performance and forage utilization by beef cattle receiving increasing amounts of alfalfa hay as a supplement to low-quality, tallgrass-prairie forage. J. Anim. Sci., *72*: 10598–1067.

Wang G., Kuan S.S., Francis O.J., Ware G.M., Carman A.S. (1990): A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. J. Agric. Food Chem., *38*: 185–190.

Welshons W.V., Rottinghaus G.E., Nonneman D.J., Dolan-Timpe M., Ross P.F. (1990): A sensitive bioassay for detection of dietary estrogens in animal feeds. J. Vet. Diagn. Invest., *2*: 268–273.

Received on July 16, 2001

ABSTRAKT

Stanovení kumestrolu ve vojtěšce (*Medicago sativa*) pomocí vysokoúčinné kapilární elektroforézy

Byla vypracována analytická metoda na stanovení kumestrolu ve vojtěšce využívající vysokoúčinnou kapilární elektoroforézu (HPCE) s 3-isobutyl-1-methylxanthinem jako interním standardem. Na separaci byla použita křemenná kapilára s borátovým pufrem (pH 9,2) jako pracovním elektrolytem a UV detekcí. Odezva detektoru s diodovým polem byla lineární v rozmezí koncentrací 0,76–140 mg.dm^{-3} s detekčním limitem 0,39 mg.dm^{-3} a migrační čas kumestrolu byl 5 min. Kumestrol byl extrahován za současné hydrolýzy glykosidické vazby konjugátu směsí zředěná kyselina chlorovodíková – acetonitril nebo ethylalkohol při 95°C po dobu 30 min. Surový extrakt byl dále přečištěn tuhou C18 fází. Relativní směrodatné odchylky reprodukovatelnosti a opakovatelnosti metody byly 1,77 a 5,49 %. Výtěžnost kumestrolu činila 92 % pro standardní přídavek 70 mg.kg^{-1}. Vojtěška, odrůda Morava, obsahovala 148–248 mg.kg^{-1} kumestrolu v sušině. Metoda je vhodná pro rychlé a rutinní stanovení kumestrolu v rostlinném materiálu.

Klíčová slova: fytoestrogeny; vojtěška; analýza rostlin; krmivo

Corresponding author:

Doc. Ing. Jitka Moravcová, CSc., Ústav chemie přírodních látek, Vysoká škola chemickotechnologická, Technická 5, 166 28 Praha 6-Dejvice, Česká republika, tel.: + 420 2 24 35 42 83, fax: + 420 2 24 31 10 82, e-mail: Jitka.Moravcova@vscht.cz