The Determination of Isoflavones and Coumestrol by Capillary Electrophoresis

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Abstract

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

The separation of six isoflavones (biochanin A, isoformononetin, formononetin, prunetin, daidzein and genistein) and coumestrol on an uncoated fused-silica capillary electrophoresis column was optimised using alkaline borate buffer as electrolyte and DAD detection. A baseline separation of all analytes except a pair, formononetin-biochanin A was achieved at pH 10.5 in 25 min. Detection limits were low (0.1 μg/ml) and the linearity of the detector response was established in the concentration range 0.4–60 μg/ml (180 μg/ml for coumestrol). Coumestrol was synthesized and the carbon signals in ^{13}C-NMR spectrum of both coumestrol and di-*O*-acetylcoumestrol were assigned for the first time using two-dimensional HMQC technique.

Keywords: phytoestrogens; food; feed; separation; isoflavones; coumestrol; electrophoresis

Isoflavones, coumestans and lignans comprise the three principal classes of plant phytoestrogens. These non-steroidal hormone-like compounds have recently been demonstrated to posses a broad spectrum of biological activities influencing human health (MAZUR 1998; BINGHAM *et al*. 1998; CLARKSON & ANTHONY 1998; THAM *et al*. 1997; KURZER & XU 1997). They can play a decisive role in the prevention of civilisation diseases, including cancer and atherosclerosis (MAZUR & ADLECREUTZ 1998; MAZUR *et al*. 1998). Their ability to moderate menopausal symptoms, to suppress angiogenesis or to prevent osteoporosis has been showed as well (KURZER & XU 1997). High levels of estrogenic substances in forage crops fed to cattle, sheep and hogs result in several biological effects, including increased teat length, gestation time, and uterine weight, as well as prolapsed vagina, cervix and rectum (ADAMS 1995). Beneficial effects of phytoestrogens have also been reported and include an increasing rate of growth and milk production (VAGNONI & BRODERICK 1997; BRODERICK *et al*. 1999). These and other potential effects in mammals depend on the parameters such as dose, duration of exposure, sex, stage of developments, and others. Biochanin A, isoformononetin, formononetin, prunetin, daidzein and genistein are the most important representatives of dietary isoflavones found in the *Leguminosae*. Alfalfa (*Medicago sativa*) is a rich source of coumestrol (coumestan). It is considered as suitable for human food purposes in the form of leaf protein concentrates (KNUCKLES *et al*. 1976; ELAKOVICH & HAMPTON 1984).

Available analytical techniques for phytoestrogen determination mainly include HPLC (PETERSON & KIESSLING 1984; KLEJDUS *et al*. 1999; BARNES *et al*. 1994; FRANKE & CUSTER 1994; FRANKE *et al*. 1994, 1998; GAMACHE & ACWORTH 1998). Capillary electrophoresis (HPCE) can be conveniently applied to the quantitative analysis of phytoestrogens as well. Where its use is possible, HPCE is generally faster and requires less sample handling than other techniques, the precision being potentially comparable (SHIBABI *et al*. 1994; ARAMEDIA *et al*. 1995; VÄNTINNEN & MORAVCOVÁ 1999).

A great diversity exists in both the levels and occurrence of phytoestrogens in plants and foods. Moreover, the conclusions arising from the evaluation of their bio-

Supported by the Grant Agency of the Czech Republic (project No. 523/00/0567) and the Ministry of Education, Youth and Sports of the Czech Republic (project No. 23300004).

activity have been still quite controversial, thus it is important to conduct a detailed investigation of the phytoestrogen content.

In this work we present the development of HPCE combined with diode-array detection for the separation of selected isoflavones and coumestrol in one run. This method should be used for routine analyses of the most potent phytoestrogens in food as well as plants.

MATERIAL AND METHODS

Sodium borate decahydrate, boric acid, sodium methoxide, daidzein and genistein were purchased from Sigma-Aldrich Chemie (Germany) and acetonitrile from Merck (Germany). 3-Hydroxyphenol, 2,4-dihydroxybenzaldehyde and benzyl chloride were obtained from Fluka (Germany). Biochanin A, formononetin, prunetin and isoformononetin were kindly donated by Dr. Lapčík (Institute of Endocrinology, Prague, Czech Republic). The NMR data were extracted from the spectra measured in $CDCl_3$ solution (tetramethylsilane as an internal standard) at 25°C with a Bruker AM 400 spectrometer (^{1}H, 400 MHz; ^{13}C, 100.62 MHz). Chemical shifts are given in ppm (δ-scale). Assignments of ^{13}C signals are based on APT, HETCOR and HMQC experiments. The melting points were determined with a Kofler hot block and are uncorrected. Column chromatography was performed on Silica gel 60, particle size 0.063–0.2 mm (Fluka, Germany), and TLC on silica gel according to Stahl (10–40 µm, Merck, Germany) with detection by spraying with 1% $Ce(SO_4)_2$ in 10% H_2SO_4 and subsequent mineralization or DC-Alufolien Kieselgel 60 F 254 plates (Merck, Germany) with UV detection were used. Solutions were concentrated under reduced pressure with a bath temperature below 40°C.

Synthesis and identification of coumestrol

Coumestrol (1) was synthesized from 1-(2,4-dihydroxyphenyl)-2-methoxyethan-1-on (GRAY 1998) with the overall yield 4.3 % according to the known procedure (JURD 1963, 1964). For the final purification, coumestrol was acetylated with acetanhydride in pyridine and the corresponding di-*O*-acetylcoumestrol 2 was obtained after decomposition of the reaction mixture with water followed by extraction with chloroform. The organic solvent was then evaporated and di-*O*-acetylcoumestrol was crystallized, m. p. 232°C (EMERSON & BICKOFF [1958] obtained 235–236°C). Convenient Zemplen's deacetylation of 2 with sodium methoxide in methanol gave pure coumestrol, m. p. 368°C (LASCHOBER & KAPPE [1990] found 360°C). Both compounds 1 and 2 were fully characterized by ^{1}H-NMR spectra; chemical shifts and vicinal coupling constants corresponded to the data already published for coumestrol (TAKEYA & ITOKAWA 1982; KINJO *et al*. 1987) or its diacetate 2 (LE-VAN 1984). The carbon signals in ^{13}C-NMR spectrum of both coumestrol and di-*O*-acetylcoumestrol were assigned for the first time using two-dimensional HMQC technique (Table 1).

Conditions for HPCE

During optimisation, the separation buffer consisted of sodium borate (20, 50 and 100 mM), the pH of which was adjusted to 7.6 or 8.6 by adding boric acid (200 mM). Sodium hydroxide (1 M) was used to set the pH of buffers to 9.6. More alkaline running electrolyte (pH 10.2 and 10.5) was prepared using 200 mM boric acid adjusted by sodium hydroxide (1 M). A HP ^{3D}CE instrument, an 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) and HP ChemStation software package were used. Sample injection was performed in pressure mode (150 mbar.s). Prior to the injection of an acetonitrile-water (2:1, v/v) solution of standards, 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.

Table 1. ^{13}C-NMR data for coumestrol (1) and di-*O*-acetylcoumestrol (2)

Carbon	Chemical shift (ppm)		Carbon	Chemical shift (ppm)	
	1	2[a]		1	2[a]
C-1	122.65	122.60	C-7	120.58	122.60
C-2	113.72[b]	118.87	C-8	113.98[b]	119.48
C-3	157.54	153.35	C-9	156.98[c]	149.62
C-4	103.00	111.15	C-10	98.65	106.09
C-4a	161.14	154.20	C-10a	154.61[c]	155.38
C-6	155.92	157.57	C-11a	159.44	160.28
C-6a	102.00	105.17	C-11b	104.15	110.28
C-6b	114.58	121.09	CH_3	–	21.14

[a]additional signals: 2 × CO, 168.60 and 169.30 ppm; [b,c]these signals can be interchanged

The UV detection was performed at 220 nm with scanning between 190 and 400 nm for identification purposes. The separation electric current was maintained at 20, 30, 40, 60, 70 or 80 μA and the corresponding potential varied from 15 to 25 kV (positive polarity). The column temperature was kept at 40°C. Running electrolyte and standard solutions were filtered through 0.2 μm pore-size cellulose membrane filters (Sartorius AG, Germany).

RESULTS AND DISCUSSION

Due to the low solubility of analytes, a standard mixture of six isoflavones (isoformononetin, prunetin, formononetin, biochanin A, genistein and daidzein) and coumestrol was resolved by the acetonitrile-water system. The starting separation was carried out in sodium borate buffer (200 mM, pH 8.6) according to the conditions published previously for the determination of genistein and daidzein in soy food (VÄNTINNEN & MORAVCOVÁ 1999) or of their mixture with biochanin A (SHIBABI *et al*. 1994). Although a good baseline separation of coumestrol from isoflavones was achieved, the peak of formononetin overlapped the signal of biochanin A completely while other isoflavones were only partially separated. Similarly, formononetin and biochanin A were not resolved using 25 mM ammonium acetate buffer (ARAMEDIA *et al*. 1995). Moreover, we observed the inconvenient drift of baseline even when the capillary was washed with 1.0 M solution of sodium hydroxide followed by a diluted nitric acid. In attempt to improve the resolution of the pair formononetin – biochanin A, the concentration or pH of the running buffer as well as the applied voltage were altered. The effect of sodium borate concentration on the HPCE selectivity was studied at pH 8.6 using several electrolyte systems (20–200 mM). The migration time of each analyte increased with increasing borate concentration but the separation efficiency remained rather unchanged. Simultaneously, the problems with drifting baseline were overcome by keeping the concentration of the buffer below 100 mM; the optimum concentration was established at 20 mM. More alkaline conditions (20 mM borate buffer, pH 8.6–9.7) gave longer migration times and the separation of formononetin and biochanin A was slightly more efficient. Nevertheless, the peaks of both formononetin and biochanin A were not yet resolved enough even when the voltage was changed (Fig. 1). Further increase of pH above 10 allowed achieving the best possible resolution of all phytoestrogens in 25 min as shown in Fig. 2, where

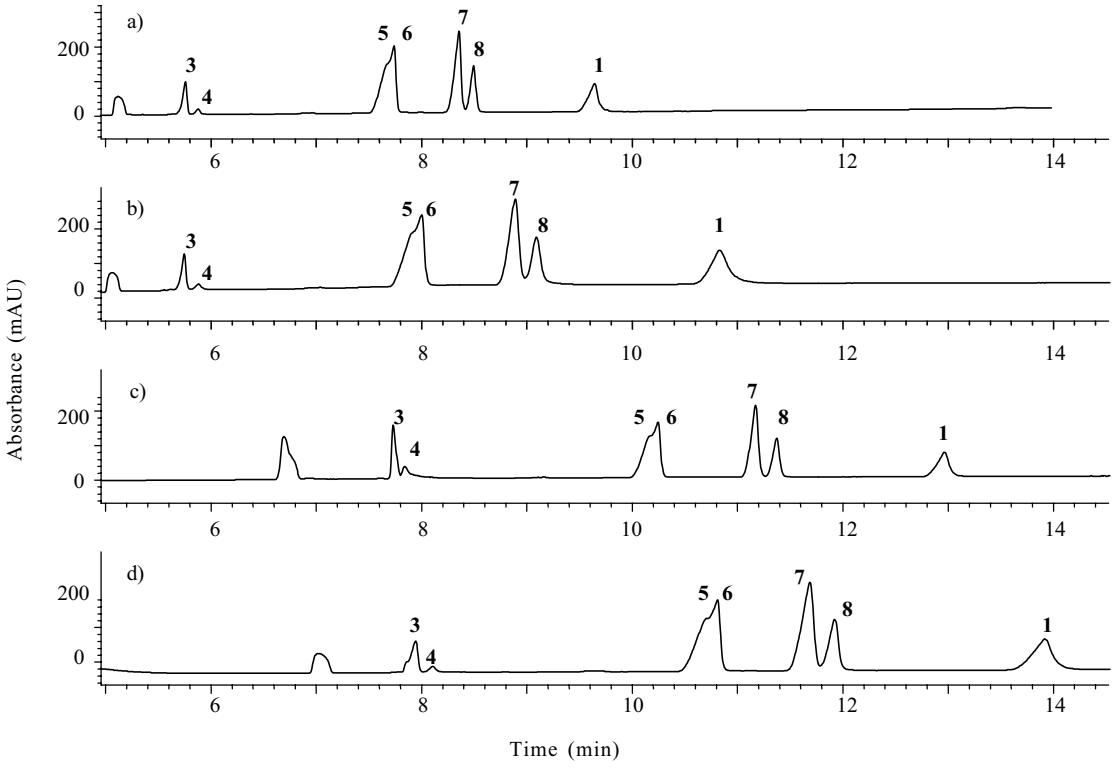


Fig. 1. Electropherogram of a standard mixture of phytoestrogens: 1 – coumestrol; 3 – isoformononetin; 4 – prunetin; 5 – formononetin; 6 – biochanin A; 7 – daidzein; 8 – genistein. Conditions: 20 mM sodium borate buffer, pH 9.6, 40°C, voltage (kV): a) 25.5, b) 23, c) 19, d) 17.5

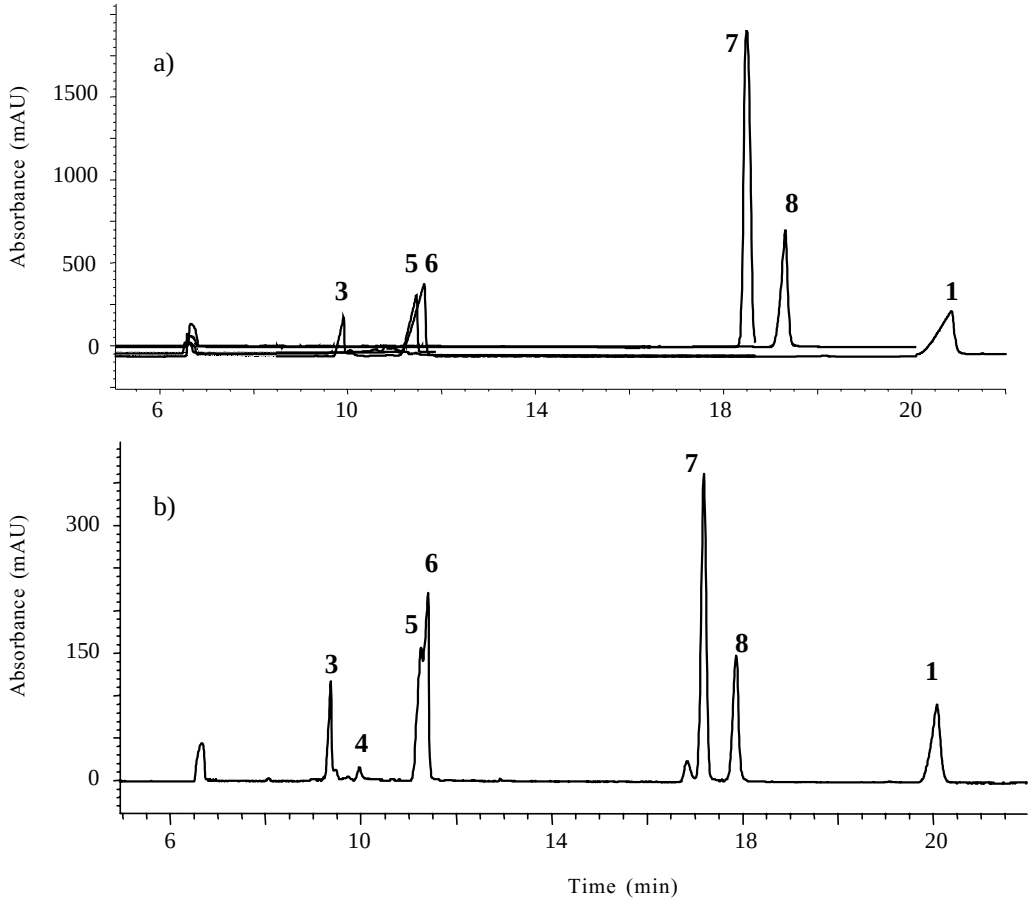


Fig. 2. Electropherogram of phytoestrogens in 200 mM boric acid adjusted at pH 10.5 by adding 1 M sodium hydroxide: 1 – coumestrol; 3 – isoformononetin; 4 – prunetin; 5 – formononetin; 6 – biochanin A; 7 – daidzein; 8 – genistein, a) injection of individual analytes, b) injection of a standard mixture

the separation of all individual analytes is compared with the electropherogram of a standard mixture.

The linearity of the detection response was established using five phytoestrogens in the concentration range expected in both food and plant extracts. The analysis was carried out twice and the means were used for calculation (Table 2). The reproducibility of migration times was calculated from ten replicate injections of a standard mixture of phytoestrogens and relative standard deviation (RSD) ranged from 1.00 to 2.03% (Table 3). The minimum detectable limit calculated as S/N = 3 was 0.1 µg/ml for all phytoestrogens except isoformononetin giving the limit of 0.28 µg/ml. Thus, the limits of detection were found to be extremely low, even three times lower than those reported in the literature (SHIBABI *et al*. 1994; VÄNTINNEN & MORAVCOVÁ 1999). This might be explained by the use of the extended light patch capillary column in this study.

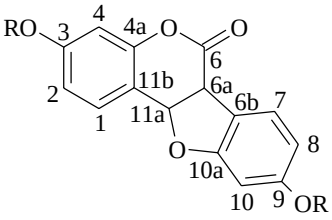
Table 2. The calibration results for the HPCE analysis of a standard mixture at pH 10.5

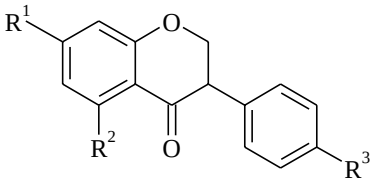
Compound	Concentration range (µg/ml)		$Y = ax + b$		Correlation coefficient
	x_{min}	x_{max}	a	b	
Formononetin	3.1	50.0	14.279	28.563	0.9979
Biochanin A	0.49	30.0	16.018	66.426	0.9950
Daidzein	0.49	60.0	10.093	6.5033	0.9986
Genistein	0.49	60.0	19.688	–3.8035	0.9993
Coumestrol	0.44	180.0	23.027	61.315	0.9998

Table 3. The migration times and noise levels for the HPCE determination of a standard mixture at pH 10.5

Compound[a]	Migration time (min)	RSD of migration time (%)	S/N[b,c]	N/B[b,d]
Formononetin	11.47	0.99	49.8	–
Biochanin A	11.64	1.02	21.8	0.0591
Daidzein	18.50	2.03	17.3	0.0357
Genistein	19.32	1.65	13.0	0.0403
Coumestrol	22.85	1.20	12.9	0.0356

[a]migration times of isoformononetin and prunetin were 9.91 ± 0.2 min and 10.65 ± 0.3 min, respectively; [b]determined for the lowest concentration in the calibration; [c]signal-to-noise ratio; [d]noise-to-baseline ratio





Compound	R	Compound	R^1	R^2	R^3
Coumestrol (1)	H	Isoformononetin (3)	OCH_3	H	OH
Di-*O*-acetylcoumestrol (2)	CH_3CO	Prunetin (4)	OCH_3	OH	OH
		Formononetin (5)	OH	H	OCH_3
		Biochanin A (6)	OH	OH	OCH_3
		Daidzein (7)	OH	H	OH
		Genistein (8)	OH	OH	OH

The worst separable pair of analytes, formononetin and biochanin A, represents a crucial problem that was not satisfactorily resolved by changing the operating parameters. It is important to note that accuracy of their determination will be influenced by a relative ratio of these analytes, as the baseline separation was not obtained. Moreover, both sample matrix and the capillary walls might suppress selectivity and thus the resolution could be further decreased. In such a case, the electrospray ionisation mass spectrometric detection with selective ion monitoring combined with HPCE will be helpful (ARAMEDIA *et al*. 1995). Another possibility is to employ a linear gradient-HPLC method investigated recently (FRANKE *et al*. 1998).

In conclusion, our developed diode-array HPCE technique represents a sensitive, easy and reproducible method covering the most common phytoestrogens. Up to six isoflavones in addition to coumestrol can be analysed in one run within 25 minutes.

Acknowledgment: The authors thank to Dr. HANA DVOŘÁKOVÁ (Central Laboratories, Institute of Chemical Technology, Prague) for NMR measurement.

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Received for publication February 21, 2001
Accepted for publication May 3, 2001

Souhrn

MORAVCOVÁ J., KLEINOVÁ T. (2001): **Stanovení isoflavonů and kumestrolu kapilární elektroforesou.** Czech J. Food Sci., **19**: 132–138.

Byla optimalizována separace šesti isoflavonů (isoformononetin, prunetin, formononetin, biochanin A, genistein, daidzein) a kumestrolu pomocí kapilární elektroforesy v borátovém pufru na nepovlečené kapiláře z taveného křemene s použitím detektoru s diodovým polem. Velice dobré rozlišení všech analytů s výjimkou obtížně separovatelné dvojice formononetin-biochanin A, která se dělila jen částečně, bylo dosaženo při pH 10,5. Analýza trvala 25 minut. Nalezené detekční limity byly nízké (0,1 µg/ml) a odezva detektoru byla lineární v širokém rozmezí koncentrací od 0,4 do 60 a v případě kumestrolu až do 180 µg/ml. Pro reprodukovatelnost migračních časů byla nalezena relativní směrodatná odchylka v rozmezí 1,0 až 2,1 %. Standard kumestrolu

byl připraven podle popsaného postupu a charakterizován byl jako di-*O*-acetylderivát. Pomocí dvoudimenzionálních NMR spekter snímaných technikou HMQC bylo možné poprvé přiřadit všechny signály uhlíkových atomů kumestrolu a jeho diacetátu.

Klíčová slova: fytoestrogeny; potraviny; krmení; separace; isoflavony; kumestrol; elektroforesa

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