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Determination of coumestrol in lucerne by ultra-high pressure liquid chromatography–mass spectrometry

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Ultra-high pressure liquid chromatography coupled with mass spectrometry (MS) was employed for the determination of coumestrol in lucerne species (*Medicago sativa* and *Medicago lupulina*) growing in Lithuania. Coumestrol was extracted with an acidified (2 mol/L HCl) methanol/water (8:2, *v/v*) solution. Optimized separations were carried out on the Acquity BEH C18 column in the gradient elution mode using the mobile phase composed of water and methanol containing 0.25% (*v/v*) acetic acid. MS detection was performed in the selected ion monitoring mode using a negative ion electrospray ionization source. The calibration curve was linear over the concentration range 0.05–5.00 mg/L. The limit of detection and the limit of quantification were 0.015 and 0.05 mg/L, respectively. The measured recoveries of coumestrol were in the range 88.5–96.1% with RSD values ranged from 4.5 to 7.2%.

Keywords: lucerne, coumestrol, liquid chromatography, mass spectrometry

INTRODUCTION

Phytoestrogens are a group of nonsteroidal polyphenolic compounds that occur naturally in a wide range of plants and induce biological responses based on their ability to bind to estrogen receptors [1]. They are generally either isoflavones (e. g. biochanin A, daidzein, genistein, formononetin) or coumestans such as coumestrol, trifoliol or repensol. Although phytoestrogens are widespread throughout the plant kingdom, they are most common in legumes such as soy, clover, lucerne and some others [2]. The common phytoestrogens found in most legume plants are isoflavones whereas in lucerne significant levels of coumestrol were found [3, 4].

Coumestrol (Fig. 1) is a coumarin-like compound with a close structural relationship to the natural estrogen estradiol. It has been reported to have up to 100-times the estrogenic activity of the isoflavones [5]. Deleterious biologic effects, among which are increased gestation time, teat length, and uterine weight, have been reported for estrogenic substances present in forage crops [6]. On the other hand, beneficial effects of such compounds have also been reported

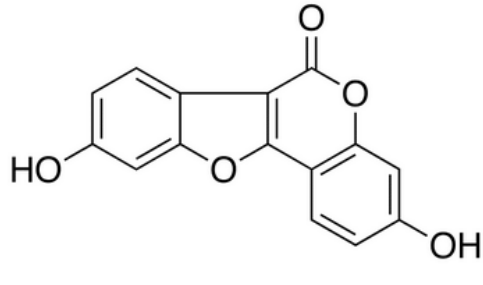

Fig. 1. Structure of coumestrol

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and include an increased rate of growth and milk production [7]. Because of the importance of phytoestrogens, the identification and quantitation of these compounds have become a hot topic in food and plant sciences [8].

Several analytical methods have been reported for the analysis of coumestrol in plant material including thin-layer chromatography [3], capillary electrophoresis [9], and high-performance liquid chromatography (HPLC) [10–12]. However, the plant matrix is rather complex and requires either a time-consuming sample clean-up procedure or an analytical technique with very high resolving power. Over the last decade, several approaches based on the use of high-temperature liquid chromatography, fused-core technology, or columns packed with sub-2 μm particles under very high pressure conditions have been developed and commercialized to improve throughput and efficiency in HPLC [13]. One of the mentioned approaches, the so-called ultra-high pressure liquid chromatography (UHPLC), is a commercially available technology that utilizes columns with sub-2 μm particles and separations at high pressures (up to 1200 bar), thereby increasing the efficiency and resolution of the separation [14]. Utilization of UHPLC coupled to mass spectrometry (MS) allows for even greater specificity and detection sensitivity.

In the present study, ultra-high pressure liquid chromatography coupled with mass spectrometry was employed for the determination of coumestrol in two lucerne species (*Medicago sativa* and *Medicago lupulina*) growing in Lithuania.

EXPERIMENTAL

All separations were carried out on a 1290 Infinity UHPLC system equipped with a diode array detector (DAD) and a 6410 triple quadrupole mass spectrometer (Agilent Technologies, USA). The Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) column (Waters, Milford USA) was employed for the separations. The mobile phase was composed of (A) water and (B) methanol/water (80:20 *v/v*) both containing 0.25% (*v/v*) acetic acid. The gradient elution program was as follows: 0–15 min, 2–100% B linear; 15–17 min, 100 2% B linear; 17–22 min, 2% B isocratic. The column temperature was maintained at 30 °C, the mobile phase flow rate was 0.25 mL/min, and the injection volume was 5 μL.

The electrospray ionization (ESI) source operated in the selected ion monitoring (SIM) mode. The nebulizer pressure, capillary voltage and drying gas flow rate were 60 psi, 4000 V and 10 L/min, respectively. The drying gas temperature was set at 280 °C. Data were acquired and processed using the MassHunter software (Agilent).

Methanol and acetic acid were of LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Coumestrol (≥95%) was also from Sigma-Aldrich. Nylon filters of 0.20 μm pore size were purchased from Carl Roth GmbH (Karlsruhe, Germany). The stock standard solution of coumestrol at 250 mg/L was prepared in aqueous methanol (1:1 *v/v*) and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solution with aqueous methanol.

The aerial parts of lucerne plants were collected in the Central Lowland of Lithuania in autumn 2014. Fresh samples were chopped, dried by lyophilization and ground in a cyclonic mill. The extraction was performed according to a slightly modified procedure described in [15]. The representative amount of sample (0.250 g) was weighed into a 20 mL glass flask with a screw cap and 10 mL of methanol/water (8:2, *v/v*) containing 2 mol/L HCl was added. The mixture was sonicated for 30 min at room temperature and then incubated in a water bath at 80–85 °C for 1.5 h with magnetic stirring. The extract was filtered through a 0.2 μm nylon syringe filter followed by analysis using a standard addition procedure.

RESULTS AND DISCUSSION

In most cases the use of liquid chromatography coupled to a specific MS detector requires effectively a very little or sometimes no chromatographic separation. Nevertheless, when dealing with complex matrices, such as plant extracts, various matrix-related ion-suppression effects can arise. In addition, lucerne extracts contain significant amounts of isoflavone formononetin which has the same molecular weight as coumestrol and may interfere with the quantification. In order to optimize separation conditions, the chromatographic profiles of lucerne extract were measured under reversed phase UHPLC conditions with different mobile phase compositions. Several binary solvent systems of acetonitrile-water and methanol-water with or without acetic acid were tested. Our preliminary studies indicated that a mobile phase comprising a mixture of methanol-water acidified with 0.25% (*v/v*) acetic acid provided good separation of coumestrol and formononetin. The acidification of the mobile phase reduced the peak tailing for coumestrol and also enhanced the ESI-MS signal response. The optimized gradient elution conditions are described in the Experimental section.

Preliminary experiments showed that the ESI-MS technique enabled the detection of coumestrol both in the positive and the negative ions mode. An intense molecular ion is registered for a protonated molecule $[M+H]^+$ (+ESI) or a deprotonated molecule $[M-H]^-$ (-ESI). Figure 2 compares the chromatograms of the coumestrol standard measured in both ESI-MS modes. As can be observed, the peak of coumestrol was approximately twice more intense using the ionization in the positive ESI mode. On the other hand, due to considerably lower background noise coumestrol showed about a 2.4-fold higher signal-to-noise ratio in the negative ESI mode. Therefore, negative ion ESI-MS detection would be preferred for the quantitative analysis of coumestrol.

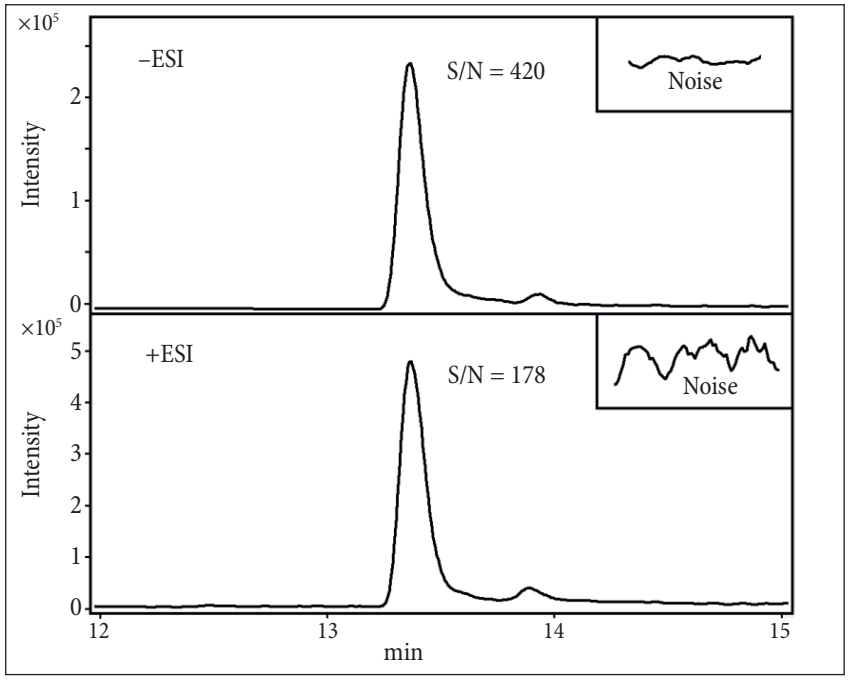

Fig. 2. UHPLC-MS chromatograms (SIM mode) of coumestrol standard (1.0 mg/L) obtained in negative ion and positive ion ESI modes. For chromatographic conditions see the Experimental section

The chromatograms of a lucerne extract obtained under optimized elution conditions by UHPLC-MS and UHPLC-UV (at 340 nm) are compared in Fig. 3. As expected, UHPLC coupled to MS offers considerably higher sensitivity providing about a 10-fold higher signal-to-noise ratio.

One significant drawback of ESI-MS is that the ionization process is greatly affected by coeluting matrix compounds [16]. The matrix effect typically results in the suppression or, less frequently, the enhancement of the analyte signal. In this work, the matrix effect was evaluated by the post-extraction spike method [17]. Lucerne extracts were spiked with coumestrol at different concentrations (0.1–1.0 mg/L) and the peak areas, after blank substraction, were compared with those obtained from pure solvent standard solutions. In both the investigated lucerne species (*Medicago sativa* and *Medicago lupulina*) the matrix suppressed the signal intensity to a great extent, ranging from 18 to 21%. Thus, external calibration by a pure solvent standard solutions cannot be used

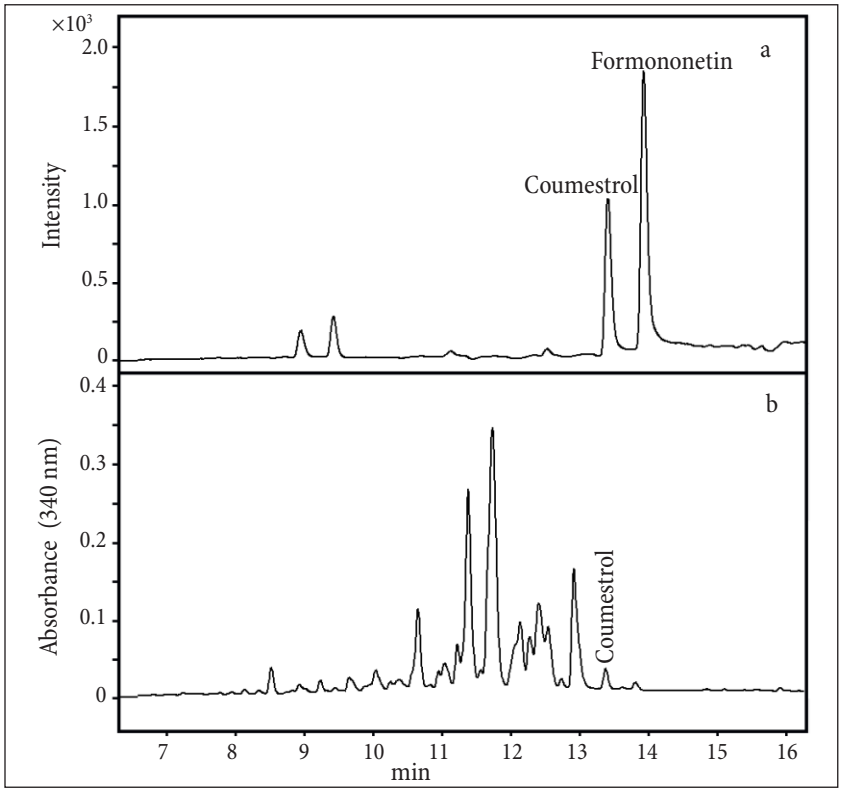

Fig. 3. UHPLC chromatograms of lucerne (*Medicago sativa*) extract obtained under optimized elution conditions using (a) MS and (b) UV (340 nm) detection modes

for quantification. Common approaches, which may be employed to reduce matrix effects, include an additional extract clean-up procedure, further dilution of the extract or a standard addition calibration procedure [18]. Clearly, an effective sample clean-up procedure is the ideal approach to remove matrix effects, but extensive sample preparation steps may be time-consuming and result in loss of the analyte. Decreasing the amount of the injected sample by extract dilution leads to reduced detectability. Thus, a standard addition calibration procedure was used in our study.

The method was evaluated for linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision. Linearity was measured with the five-point standard addition calibration curve (three replicates). LOD and LOQ were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. Because no analyte-free matrix was available, LOD and LOQ were evaluated by extrapolation of the standard addition calibration curve. The data obtained are summarized in Table 1.

Table 1. **Calibration data, LOD and LOQ for the UHPLC-MS determination of coumestrol**

Parameter	Value
Linear range, mg/L	0.05–5.00
Regression equation	$y = 74589x + 4095$
R2	0.9976
LOD, mg/L	0.015
LOQ, mg/L	0.050

Finally, three lucerne samples (two *Medicago sativa* samples collected from different locations and one *Medicago lupulina* sample) were analysed for coumestrol by the developed UHPLC-MS technique. In order to evaluate the accuracy of the method recovery tests were carried out at two concentration levels (Table 2). Figure 4 shows the overlaid SIM chromatograms of *Medicago sativa* sample extract. One chromatogram is without spike and the other two chromatograms are with 0.025 and 0.050 mg/g coumestrol spikes. The obtained recoveries were in the range 88.5–96.1% with RSD values ranged from 4.5 to 7.2%. Thus, it can be concluded that the proposed UHPLC-MS technique has shown acceptable accuracy and precision for the determination of coumestrol in lucerne plants.

Table 2. **Average values (mg/g in dry weight basis) of coumestrol determined in lucerne species (aerial parts of the plant), accuracy and precision data (n = 3)**

Lucerne species	Found, mg/g	Added, mg/g	Recovery, %
Medicago sativa 1	0.027 (5.7)a	0.025	93.4 (4.8)
		0.050	91.6 (4.5)
Medicago sativa 2	0.019 (7.2)	0.025	88.5 (5.6)
		0.050	90.9 (5.5)
Medicago lupulina	0.032 (6.4)	0.025	94.5 (5.2)
		0.050	96.1 (4.7)

[a] Values in parentheses are %RSD.

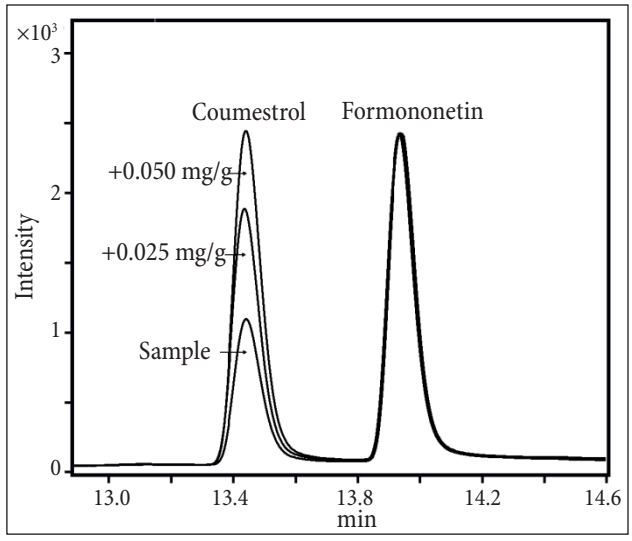

Fig. 4. Overlaid UHPLC-MS chromatograms of lucerne (*Medicago sativa*) extract without spike and with 0.025 and 0.050 mg/g coumestrol spikes

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References

1. A. Brzezinski, A. Debi, *J. Obstet. Gynecol. Reprod. Biol.*, **85**, 47 (1999).
2. T. Cornwell, W. Cohick, I. Raskin, *Phytochemistry*, **65**, 995 (2004).
3. B. E. Knuckles, D. De Fremery, G. O. Kohler, *J. Agric. Food Chem.*, **24**, 1177 (1976).
4. A. A. Franke, L. J. Custer, C. M. Cerna, K. K. Narala, *J. Agric. Food Chem.*, **42**, 1905 (1994).
5. K. Verdeal, D. S. Ryan, *J. Food Protect.*, **42**, 577 (1979).
6. A. W. H. Braden, W. H. Southcott, G. R. Moule, *Aust. J. Agric. Res.*, **15**, 142 (1964).
7. J. E. Oldfield, C. W. Fox, A. V. Bahn, *J. Animal Sci.*, **25**, 167 (1966).
8. C. C. Wang, J. K. Prasain, S. Barnes, *J. Chromatogr. B*, **777**, 3 (2002).
9. J. Moravcova, T. Kleinova, *Czech J. Food Sci.*, **19**, 132 (2001).
10. H. Peterson, K. H. Kiessling, *J. Assoc. Off. Anal. Chem.*, **67**, 503 (1984).
11. L. S. Hutabarat, H. Greenfield, M. Mulholland, *J. Chromatogr. A*, **886**, 55 (2000).
12. A. Bacaloni, C. Cavaliere, A. Faberi, P. Foglia, R. Samperi, A. Lagana, *Anal. Chim. Acta*, **531**, 229 (2005).
13. D. Guillarme, J. Ruta, S. Rudaz, J. L. Veuthey, *Anal. Bioanal. Chem.*, **397**, 1069 (2010).
14. N. Wu, A. M. Clausen, *J. Sep. Sci.*, **30**, 1167 (2007).

15. L. Taujenis, A. Padarauskas, J. Mikaliūnienė, J. Ceseviˇcienė, N. Lemežienė, B. Butkutė, *Chemija*, **26**, 107 (2015).
16. P. J. Taylor, *Clin. Biochem*., **38**, 328 (2005).
17. A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, *J. Chromatogr. B*, **877**, 2198 (2009).
18. M. Stuber, T. Reemtsma, *Anal. Bioanal. Chem*., **378**, 910 (2004).

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KUMESTROLIO NUSTATYMAS LIUCERNOJE ULTRAEFEKTYVIOSIOS SKYSČIŲ CHROMATOGRAFIJOS-MASIŲ SPEKTROMETRIJOS METODU

Ultraefektyvioji skysčių chromatografija su MS detektoriumi pritaikyta kumestroliui nustatyti Lietuvoje auginamuose liucernos augaluose (*Medicago sativa* ir *Medicago lupulina*). Kumestrolis buvo ekstrahuojamas 2 mol/L HCl parūgštintu metanolio / vandens (8:2, *v/v*) tirpalu. Chromatografinis atskyrimas atliktas Acquity BEH C18 kolonėlėje naudojant gradientinę eliuciją metanolio / vandens judria faze su 0,25 % (*v/v*) acto rūgšties priedu. MS detektuoti naudotas pasirinktų jonų monitoringo režimas ir neigiamų jonų elektropurkštuvinė jonizacija. Išmatuota kalibracinė kreivė tiesinė 0,05–5,00 mg/L kumestrolio koncentracijų intervale. Aptikimo ir nustatymo ribos atitinkamai lygios 0,015 ir 0,05 mg/L. Kumestrolio standartinių priedų išgavos iš liucernos mėginių siekia 88,5–96,1 %, o santykiniai standartiniai nuokrypiai – 4,5–7,2 %.