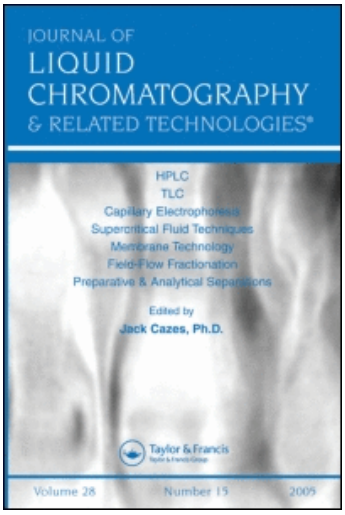

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Liquid Chromatographic Validation of a Quantitation Method for Phytoestrogens, Biochanin-A, Coumestrol, Daidzein, Formononetin, and Genistein, in Lucerne

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Liquid Chromatographic Validation of a Quantitation Method for Phytoestrogens, Biochanin-A, Coumestrol, Daidzein, Formononetin, and Genistein, in Lucerne

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Abstract: A methodology was validated to evaluate biochanin-A, coumestrol, daidzein, formononetin, and genistein in lucerne (*Medicago sativa*). Extraction done through acid hydrolysis at 80°C, followed by extraction with HLB cartridges. A C_{18} column (4.6 × 250 mm) with a 0.8 mL/min flow of methanol solution (isocratic) and detection at 260 nm was applied. Separation factors were greater than 1.1 and resolutions were superior to 2.4. Linearity was established and correlation coefficients of responses were greater than 0.995. The detection and quantification limits were between 0.12–0.17 $\mu g \cdot mL^{-1}$ and 0.37–0.53 $\mu g \cdot mL^{-1}$, respectively. The coefficients of variation were between 5 and 15%. The accurate results in lucerne were 78, 35, 20, 14, and 11 mg $(kgDM)^{-1}$ for coumestrol, formononetin, daidzein, genistein, and biochanin-A.

Keywords: Lucerne, Isoflavones, Coumestrol, LC, Validation, Quantification

INTRODUCTION

Recently, two authors made wide revisions[1,2] of different methods of phytoestrogens analysis. Even though other methods exist, liquid chromatography with UV detection is still prevailing in most laboratories.

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The phytoestrogens, biochanin-A, coumestrol, daidzein, formononetin, and genistein are secondary metabolites mainly presented as glycoside conjugates in legumes such as lucerne, clovers, or soya. Some studies[3–10] reveal the hormonal and non-hormonal effects of phytoestrogens in animal and human metabolisms.

Diets rich in phytoestrogens may affect the normal biological functions such as proliferation, differentiation, and protein synthesis in different target cells,[11] the main responsible factor being the molecular structural similarity between the endogenous estrogens and the phytoestrogens (Figure 1).

The synthesis of phytoestrogens in plants is dependent on the environment,[12,13] but the plant species variety,[14] organ, age, and nodulation[15] may influence the synthesis as well. The analysis of phytoestrogens has been mainly done and validated for soya. Other species like lucerne have very few published data and no recent validated methodologies. In Table 1, we present formononetin and coumestrol results found in the literature. The purpose of our project was to evaluate phytoestrogens in dried lucerne, and for that, we had to validate a new methodology adapted to our plant material as the published methods didn't provide adequate results in our experimental conditions.

Preliminary tests were carried out, and based on some literature, it was decided that the concentration of any of the detectable phytoestrogens in our samples would be among 10 and 90 $mg \cdot kg^{-1}$ of dry matter (DM). Some of the published results[15] were near this lower limit and others[16] gave values near the upper limit. The literature is scarce in information about the full grown lucerne phytoestrogen content. The values most commonly published are for sprouts that are known to be abundant in phytoestrogens.

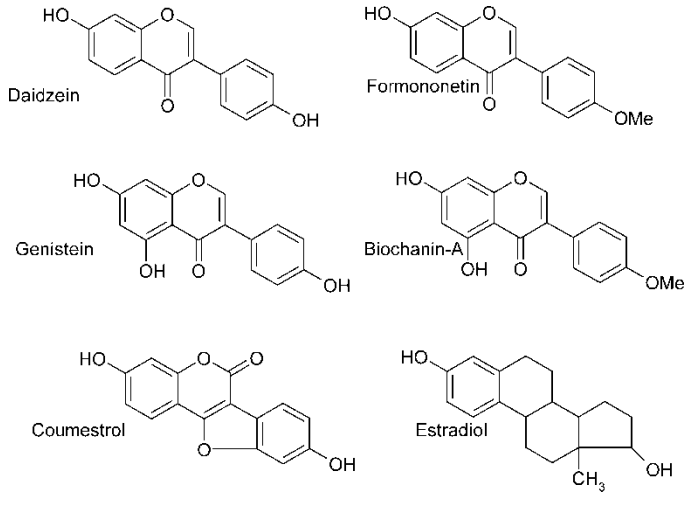

Figure 1. Phytoestrogens, daidzein, formononetin, genistein, biochanin-A, coumestrol, and estradiol with similar, molecular structure.

Table 1. Published values of phytoestrogens in lucerne (*Medicago sativa*)

Variety	Sample	Phytoestrogen	Content on the sample	Reference
Vela	Foliage	Free formononetin	Non-detectable	
		Conjugated formon	8 ± 2 nmol · g^{-1} FW[a]	
Vertus	Foliage	Free formononetin	21 ± 8 nmol · g^{-1} FW	[15]
		Conjugated formon	31 ± 3 nmol · g^{-1} FW	
Euver	Foliage	Free formononetin	Non-detectable	
		Conjugated formon	3 ± 1 nmol · g^{-1} FW	
Europe	Foliage	Free formononetin	Non-detectable	
		Conjugated formon	1 ± 1 nmol · g^{-1} FW	
Not mentioned	Sprouts	Coumestrol	46.8 mg · g^{-1} FW	[17]
		Formononetin	3.4 mg · g^{-1} FW	
Not mentioned	Fresh lucerne	Coumestrol	56 mg · kg^{-1}	[16]
	Dried lucerne	Coumestrol	84 mg · kg^{-1}	

[a]Fresh weight.

After running a few methodologies,[17–20] a new technique that gave the best results for the dried lucerne was found and validated using standard phytoestrogens. Most methods extract phytoestrogens from samples with low levels of pigments, like beans and sprouts, and when they were applied to dried lucerne, the high quantity of pigments removed, interfered with the detection. So, in this work, we present the validation of a new method to quantify five phytoestrogens (aglycones) by LC.

EXPERIMENTAL

Chemicals

Acetic acid, ammonia, chloridric acid, and ethanol were supplied by Merck, Darmstadt, Germany. Dimethyl sulfoxide (DMSO) and di-sodium hydrogen phosphate anhydrous were supplied by Fluka Steinheim, Switzerland. Methanol, (LC gradient) was supplied by SDS, Peypin, France. The phytoestrogen standards, daidzein, formononetin, and coumestrol were supplied by Fluka Steinheim, Switzerland. The genistein and biochanin-A standards were supplied by Sigma Steinheim, Germany. Purified water was obtained with a Milli-Q Plus system (Millipore, Bedford, MA, USA).

Apparatus

LC equipment consisted of a Rheodyne 7725i loop 20 μ injector, Cotati, CA, USA and prefilter; a Gilson 305 pump, dynamic mixer 811-B, and UV/Vis – 151 detector, connected to the UniPoint System version 1.71 software, Middleton, USA. A Waters Spherisorb C18 column (4.6 × 250 mm i.d. 5 μm) Milford, MA, USA, was used.

Also used were a Mettler Toledo AG 285 balance, Zurich, Switzerland, Memmert 854, oven set at 35°C Schwabach, Germany, Bandelin Sonorex RK 100 ultra-sound bath, Reagente-5 Oporto, Portugal, Selecta, Meditronic centrifuge, Barcelona, Spain, Gerhardt SV 24 water bath, Reagente-5, Oporto, Portugal, CD 7400-WPA pH meter, Cambridge, United Kingdom. The vaccum system for SPE- Dinko Mod. D95 vacuum pump, Barcelona, Spain was associated to a vacobox B-160 Buchi, Schweiz, Switzerland. Waters Oasis HLB extraction columns Vac RC 60 mg, Milford, MA, USA. were used. The nitrogen evaporation system was Reagente-5, Oporto, Portugal.

Standard Solutions

Phytoestrogen stock solutions were separately set by dissolving 5 mg standards in 50 μL of DMSO and then in 100 mL of ethanol to give solutions of 50 $\mu g \cdot mL^{-1}$.[17] Stock solutions and working standard solutions were maintained at −18°C. These were used for calibration and everyday retention time confirmation.

Calibration curves were obtained for each standard at least through 5 concentration levels. Working standards for each of them were prepared to give the following concentration: 0.5, 1, 1.5, 2, and 2.5 $\mu g \cdot mL^{-1}$ for genistein and daidzein, 0.5, 1, 2, 6, and 10 $\mu g \cdot mL^{-1}$ for biochanin-A, 0.5, 1, 2, 6, 10, 14, and 18 $mg \cdot mL^{-1}$ for formononetin and coumestrol.

Sample Preparation

Two grams of a weighed dried sample was dispersed in 10 mL of water and left over night at 35°C. Then 4 mL of 2M HCl and 32 ml of 80% ethanol were added. This mixture was heated at 80°C for 1 hour and left to cool. The ethanol lost during boiling was returned (to the initial 46 mL) and the mixture was centrifuged 10 minutes at 2000 × g. Then the 3 mL of the supernatant was diluted in 9 mL of water.

Solid-Phase Extraction (SPE)

The previous solution was submitted to an extraction procedure by SPE with HLB columns. The sorbent was conditioned with methanol (3 mL) and water

(3 mL). The solution was passed through and the impurities removed with 5% methanol in 2% acetic acid. Phytoestrogens were eluted with 3 mL of 80% methanol in a solution of 5% of ammonia hydroxide.

Liquid Chromatography

An aliquot (1 mL) of the extract was evaporated to dryness, under a nitrogen stream at 35°C and the dry residue dissolved in 1 mL of the mobile phase. After filtration, an aliquot of 20 mL was injected into the LC system and the chromatographic run was carried out at a flow rate of 0.8 mL min^{-1}, with a mobile phase of methanol and sodium phosphate at pH 8.18 (58:42, v/v). Detection was set at 260 nm. All results were the average of three replicates.

RESULTS AND DISCUSSION

Figure 2 shows a chromatogram of the phytoestrogens standards. Table 2 shows peak resolutions (R) and separation factors (α) for two different concentrations of the five standards (n = 9). The lowest R (2.38) is between formononetin and coumestrol, but a mobile phase with a pH between 8.16 and 8.18 gave acceptable peak resolutions.

The relation between the standards concentration and the detector response was linear. The line equations and the correlation coefficients (r) are presented in Table 3.

Recovery rates were evaluated on a matrix spiked with the five phytoestrogens at a concentration of 2 $\mu g \cdot mL^{-1}$. Corn flour was chosen to be the solid part of this matrix as its levels of phytoestrogens could be considered[22]

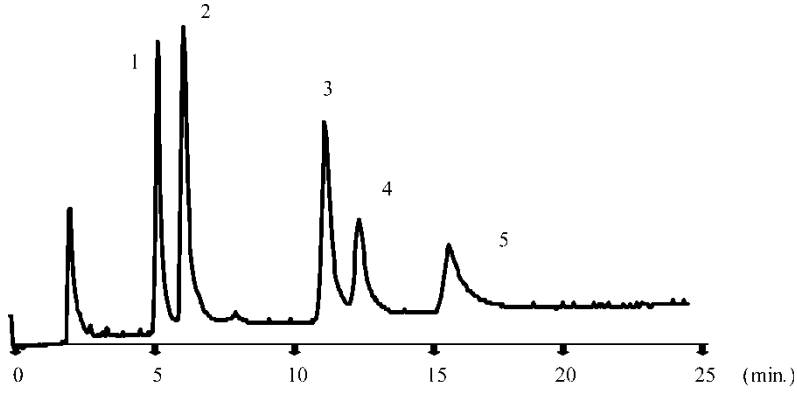

Figure 2. Chromatogram of daidzein (1), genistein (2), formononetin (3), coumestrol (4), and biochanin-A (5) at 2 $\mu g \cdot mL^{-1}$.

Table 2. Resolutions and separations factor between peaks at 1 $\mu g \cdot mL^{-1}$ and 2 $\mu g \cdot mL^{-1}$

	Separation factor[a] ($\alpha \geq 1$)				Resolution[a] ($R \geq 1.5$)			
	1 $\mu g \cdot mL^{-1}$		2 $\mu g \cdot mL^{-1}$		1 $\mu g \cdot mL^{-1}$		2 $\mu g.Ml^{-1}$	
Concentration	Mean	SD[b]	Mean	SD	Mean	SD	Mean	SD
Daidzein–genistein	1.40	0.04	1.41	0.05	6.75	0.95	6.77	1.38
Genistein–formononetin	2.50	0.12	2.48	0.24	14.15	1.44	13.55	1.51
Formononetin- coumestrol	1.11	0.02	1.12	0.03	2.38	0.27	2.46	0.27
Coumestrol–biochanin-A	1.37	0.06	1.37	0.09	6.41	1.23	6.29	1.88

[a]As calculated by [21].
[b]Standard deviation.

Table 3. Parameters of linearity and method standard deviation (SDm) and coefficient of variation (CVm)

Phytoestrogen	Equation	r^2	SDm	CVm
Daidzein	$y = 85333x + 7939.9$	0.995	0.048	3.21
Genistein	$y = 129586x + 1062.8$	0.995	0.052	3.50
Formononetin	$y = 148116x - 44098$	0.999	0.037	2.48
Coumestrol	$y = 86104x - 9569.9$	0.998	0.037	2.49
Biochanin-A	$y = 133668x - 100007$	0.998	0.045	2.25

below our detection limits. The extraction procedure, as described above, was applied to three spiked samples and a fourth was used as a blank to evaluate any residue of phytoestrogens. In the non spiked sample, the absence of phytoestrogens was confirmed. Table 4 presents mean ($n = 9$) percentages obtained for the phytoestrogens recoveries, and the accuracy achieved is considered[23] acceptable for our level of concentration (10^{-9}).

Considering that our calibration followed a linear model, the limits of detection (DL) and quantification (QL) could be calculated as $DL = 3.3 * SDm$ and $QL = 10 * SDm$[24] and their values are presented in Table 5.

The quantification limit for the biochanin-A in our method is above 0.5 $\mu g \cdot mL^{-1}$, so it is more appropriate to consider a range of work that starts above that. Therefore, we will work above 0.5 $\mu g \cdot mL^{-1}$ for daidzein, genistein, formononetin, and coumestrol, and 0.6 $\mu g \cdot mL^{-1}$ for biochanin-A.

Intermediate precision (SDi) was evaluated using 15 averages of two or more repetitions of the standard solution (2 $\mu g \cdot mL^{-1}$) obtained at 15 different days, and the result is presented in Table 6. This is a very simple evaluation of precision, just considering the variance due to a different day of work. The results achieved are suitable for food samples and analyte concentrations as low as ours (10^{-9}).

Table 4. Recovery data for each phytoestrogen at 2 $\mu g \cdot mL^{-1}$

	Recovery rate (%)	
Phytoestrogen	Mean	SD[a]
Daidzein	98.25	9.70
Genistein	83.95	7.47
Formononetin	89.12	12.30
Coumestrol	89.18	11.56
Biochanin-A	86.11	12.67

[a]Standard deviation ($n = 9$).

Table 5. Detection and quantification limits

Phytoestrogen	DL ($\mu g \cdot mL^{-1}$)	QL ($\mu g \cdot mL^{-1}$)
Daidzein	0.15	0.45
Genistein	0.12	0.37
Formononetin	0.16	0.48
Coumestrol	0.12	0.37
Biochanin-A	0.17	0.53

Table 6. Results for the evaluation of the intermediate precision

Phytoestrogen	SDi	CVi
Daidzein	10172.94	5
Genistein	12618.75	6
Formononetin	22316.73	11
Coumestrol	20317.49	15
Biochanin-A	19098.18	15

The main conclusion of this work is that it is possible to analyse the five phytoestrogens, daidzein, genistein, formononetin, coumestrol, and biochanin-A through the methodology previously described, even if some selectivity, accuracy, and precision is lost, compared to similar methods where fewer analytes are measured at the same time. Analysis time was reduced, cost of analysis cut, and correct levels of phytoestrogens in lucerne achieved.

The average phytoestrogens obtained for lucerne are presented in Figure 3 and they are the result of the analyses (27 samples) of all the plants from different origins. The highest average concentration found was for coumestrol with 99 $mg \cdot kg^{-1}$DM, followed by formononetin, daidzein, biochanin-A, and

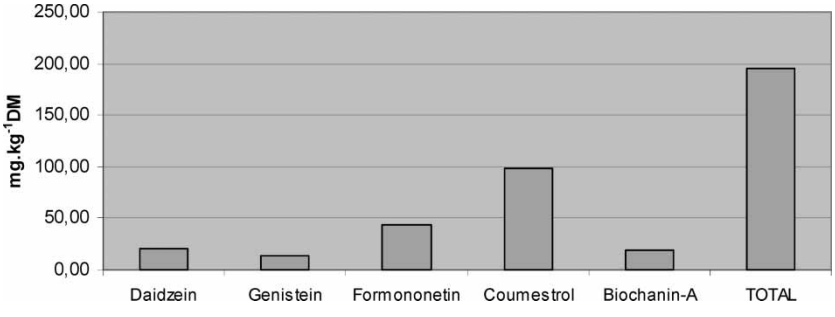

Figure 3. Average results for lucerne phytoestrogens.

genistein with 43, 21, 19, and 14 mg · kg^{-1}DM, respectively. This study is a first step of more that will be taken to evaluate environmental and genetic factors that may have an effect on the level of phytoestrogens in lucerne forrage.

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