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HPLC separation and determination of dicoumarol and other simple coumarins in sweet clover

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Abstract

Dicoumarol is a mycotoxin, that acts as a blood anticoagulant, is formed during the microbial action of molds and fungi in spoiled hay or silage containing high-coumarin plant. A HPLC-DAD method for determination of coumarins, including dicoumarol, coumarin, and 4-hydroxycoumarin was developed. Methanol and acetic acid were used as mobile phase with gradient elution. The simultaneous separation was performed using C18 type of stationary phase. The recoveries were 88.6 – 92.6 %, 91.8 – 95.0 %, and 89.7 – 94.1 % (evaluated for three concentration levels) for dicoumarol, coumarin, and 4-hydroxycoumarin respectively. The parameters of system suitability (repeatability of retention times and peak areas) were determined for evaluation of the method. The method showed a good linearity in the concentration range 0.7 – 100 $\mu g.mL^{-1}$ for dicoumarol, 0.05 – 100 $\mu g.mL^{-1}$ for coumarin and 4-hydroxycoumarin with correlation coefficients higher than 0.9885. Extracts of sweet clover herb, hay, and spoiled hay were subjected to HPLC-DAD analysis. The most abundant compound in sweet clover herb and hay extracts was coumarin. In spoiled sweet clover hay extract the 4-hydroxycoumarin was detected in addition. The formation of 4-hydroxycoumarin was also observed in the synchronous fluorescence spectra recorded at the wavelength difference of 90 nm (difference between emission and excitation wavelength).

Introduction

Sweet clover (*Melilotus albus* or *Melilotus officinalis* L.) is widespread plant in central Europe. Besides of vitamin C, allantoin, tannins, mineral salts and flavonoids it contains coumarin compounds. During the microbial action of molds and fungi (*Penicillium jenseni*, *Penicillium nigricans*) in spoiled hay or silage containing high-coumarin plant, the dicoumarol is formed (4-hydroxycoumarin is the intermediate of dicoumarol production from coumarin (Poulton *et al.* 1980). Dicoumarol, a symmetrical biscoumarin (3,3′-methylenebis(4-hydroxycoumarin; Fig. 1), is potentially toxic and as potent vitamin K antagonist and anticoagulant may cause animal death due to internal or external hemorrhaging (Stahmann *et al.* 1941; Smith *et al.* 1965; Casper *et al.* 1983). Its discovery led to the development of another modern anticoagulant drug, Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-coumarin). The European Committee prohibited the presence of dicoumarol in cosmetic products and food (Regulation (EC)

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No 1334/2008); EU Regulation (EC) No 1223/2009).

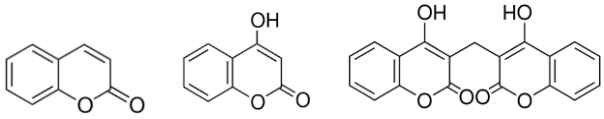


Fig. 1. Chemical structures of coumarin, 4-hydroxycoumarin, and dicoumarol (respectively).

Several analytical techniques are available for determination of dicoumarol, coumarin, and 4-hydroxycoumarin such as colorimetry (Emery *et al.* 1970), fluorescence spectrometry (Marcolan *et al.* 2011; Poláček *et al.* 2015), and liquid chromatography including thin layer chromatography (Bellis *et al.* 1967).

The literature provides several studies involving the use of HPLC with UV, MS and fluorescence detectors for determination of individual coumarin, 4-hydroxycoumarin, and dicoumarol or in group with other phenolic compounds. Only a few papers deal with simultaneous separation and determination of selected compounds in sweet clover. Table 1 summarizes some studies using HPLC for the determination of investigated coumarins in different matrices. Techniques, such as ultrasonic liquid extraction for plant and cosmetics samples (Muir *et al.* 1992; Ikeda *et al.* 2009; Xi *et al.* 2010) or accelerated solvent extraction for food sample (Vieriková *et al.* 2009) were applied for sample pretreatment.

The aim of the present work was to develop a rapid and sensitive HPLC method for simultaneous determination dicoumarol, coumarin, and 4-hydroxycoumarin in sweet clover herb and hay.

Experimental

Chemicals and sample

The standards of dicoumarol (98 %), 4-hydroxycoumarin (98 %), and coumarin (99 %), were purchased from Sigma-Aldrich (St. Louis, USA). Methanol (HPLC grade) and acetic acid (99 %, an analytical grade) were purchased from Merck (Darmstadt, Germany). The deionized water (resistivity of 18.2 MΩ/cm) was obtained from a AquaMax ultra (series 370) water purification system.

The sample of sweet clover (*Melilotus officinalis* L.; aerial parts) herb was purchased from Slovakia. The herb was dried to constant weight during 4 hour at 30 °C and powdered. The fresh herb was incubated without access to light and air at 23 °C for 14 and 21 days.

Preparation of standard solutions

The stock solutions of dicoumarol, coumarin, and 4-hydroxycoumarin were prepared by dissolving of standards in methanol (concentration of analyte 1 $mg.mL^{-1}$) and stored at 8 °C. The working standard solutions and mixed standard working solution (concentration of each analyte 100 $\mu g.mL^{-1}$) were prepared by appropriate dilution of stock solutions with methanol. The solutions were filtered through a 0.45 µm nylon membrane filter. Solutions of dicoumarol were prepared daily. Solutions of 4-hydroxycoumarin and coumarin were stable during a week.

Sample preparation

An accurately weighted portion of sample (approximately 0.1 g) was mixed with 20 mL of methanol and extracted under stirring on mechanical shaker for 60 min at 23 °C. The mixture was centrifuged for 10 min (4,000 rpm). The supernatant was used for HPLC analysis.

HPLC analysis

HPLC system (Agilent Technologies, series 1200) consisted of a binary pump, an autosampler, a column oven, a diode array detector, and a fluorescence detector. Data were acquired and controlled by the Agilent ChemStation chromatographic data system software. Separations of analytes were performed in reverse phase mode using a Symmetry C18 (150 mm x 3.9 mm I.D., 5 µm particle size; column I), Symmetry C18 (75 mm x 4.6 mm I.D., 3.5 µm particle size; column II), XBridge C18 (50 mm x 4.6 mm I.D., 3.5 µm particle size; column III) (Waters, USA) column. The isocratic and gradient elutions were performed using the mobile phases consisted of methanol with the addition 0.3 % acetic acid (A)

Table 1. HPLC methods for separation and determination of coumarin, 4-hydroxycoumarin, and dicoumarol.

Stationary phase Mobile phase	Detection	LOD	Linear range	Sample	Reference
Coumarin					
C18 acetonitrile/0.1 % formic acid gradient elution	MS	–	0.026-13.5 $\mu g.mL^{-1}$	Green tea with sweet-herbaceous odour	Yang *et al.* (2009)
C18 acetonitrile/0.1% formic acid (50/50 v/v)	DAD	0.02 $\mu g.kg^{-1}$	0.05-500 $\mu g.kg^{-1}$	Food, biscuits	Vieriková *et al.*, (2009)
4-hydroxycoumarin					
C18 methanol/phosphate buffer pH 3 (65/35 v/v)	UV (286 nm) FL (372/290 nm)	0.0121 $\mu g.mL^{-1}$ 0.0125 $\mu g.mL^{-1}$	–	Plant sample (*Morinda citrifolia*)	Ikeda *et al.* (2009)
C18 methanol/acetonitrile/ phosphate buffer pH 4 gradient elution	UV (280 nm)	0.0200 $\mu g.mL^{-1}$	0.07-4 $\mu g.mL^{-1}$	Pharmaceutical preparative	Vijayakumar *et al.* (2009)
Dicoumarol					
phenyl methanol/acetonitrile/ water gradient elution	DAD	0.2 $\mu g.mL^{-1}$	0.5-60 $\mu g.mL^{-1}$	Cosmetics	Xi *et al.* (2010)
C18 methanol/acetate buffer pH 6 (25/75 v/v)	UV (303 nm)	2 $\mu g.mL^{-1}$	–	Sweet clover hay, silage	Muir *et al.* (1992)

DAD – diode array detection; FL – fluorescence detection; LOD – limit of detection; MS – mass spectrometry; UV – ultraviolet detection.

and 0.3 % aqueous solution of acetic acid (B). The gradient profiles and flow rates used are summarised in Table 2. The injection volume was 10 µL, and column temperature was maintained at 23 °C. The diode array detector was operated in the wavelength range of 190 – 400 nm and chromatograms were obtained at 300 nm for dicoumarol and 280 nm for other compounds under study. The fluorescence detector was operated in the emission wavelength range of 270 – 500 nm and chromatograms were obtained at excitation wavelength 300 nm and emission wavelength 390 nm for detection of dicoumarol.

Table 2. The gradient profiles and flow rates for HPLC separation of target coumarins on tested columns.

Column	Flow rate [$mL.min^{-1}$]	Time [min]	A [%]	B [%]
Column I	0.8	0	65	35
		7	40	60
		10	0	100
		11	0	100
Column II	1.6	0	65	35
		2.1	40	60
		2.7	0	100
		4.0	0	100
Column III	1.6	0	65	35
		2.1	40	60
		2.7	0	100
		4.0	0	100

A – 0.3 % acetic acid; B – methanol containing 0.3 % acetic acid

Synchronous fluorescence analysis

Lumina Fluorescence Spectrometer (Thermo Scientific) equipped with a 150 Watt Ozone-free Xenon lamp was were used. Excitation and emission slits were both set at 5.0 nm. Scan speed was 200 nm/min. PMT Voltage was set at 500 V. The samples were placed in a 1 cm quartz cell.

Method validation

For the validation of the HPLC-DAD method, parameters such as linearity, limits of detection (LOD), limits of quantification (LOQ), precision, and accuracy were evaluated. The calibration curves of analytes were constructed after injection of standard solutions and obtained by plotting a graph of mean peak area versus corresponding concentration of analyte (seven concentration

levels, three replicate injections of each solution). The LODs and LOQs values were determined empirically by the injection of the series of diluted solutions of the analytes and were calculated as a signal to noise ratio of 3 and 10, respectively. The recovery tests were performed in sample of dried sweet clover spiked at three concentration levels of dicoumarol, coumarin, and 4-hydroxycoumarin. Spiked sample was held for 1 hour before extraction. Spiked and unspiked samples were treated by the same procedure. Seven independent extractions of each sample were carried out. Intra-day and inter-day precisions of the method were evaluated for seven preparations of sample within one day and three days, making triplicate injections under the working conditions and (expressed as RSD %).

Results and Discussion

HPLC separation

Reversed phase separation mode is most commonly used for HPLC separation of mixtures of coumarin derivatives including of dicoumarol as documented Table 1. The work was focused on development of HPLC method for separation and determination of three of comarins, coumarin, 4-hydroxycoumarin, and dicoumarol. Coumarin is precursor of dicoumarol and 4-hydroxycoumarin is a probable intermediate compound. (Smith *et al.* 1938) The 4-hydroxycoumarin is converted into dicoumarol in the presence of formaldehyde (Stahmann *et al.* 1941).

At the beginning of the study, a suitable analytical column, which would offer sufficient separation for quantification of analytes was estimated. Three reversed-phase C18 type analytical columns, Symmetry C18, and XBridge C18 with different particle size and column length, were tested. The mobile phases consist of methanol and acetic acid at different ratios (Fig. 2) were investigated for isocratic elution. The effectiveness of the separation was evaluated using standard solution containing coumarin, 4-hydroxycoumarin, and dicoumarol. The baseline separations of analytes were achieved, but the retention factors of dicoumarol were significantly higher in comparison to retention factors of coumarin and 4-hydroxycoumarin (higher than 7 for all tested stationary and mobile phases), which lead to longer analysis times. Gradient elution profiles shown in Table 2 were investigated, which resulted in effective separation of target compounds in analysis time lower than 15 min.

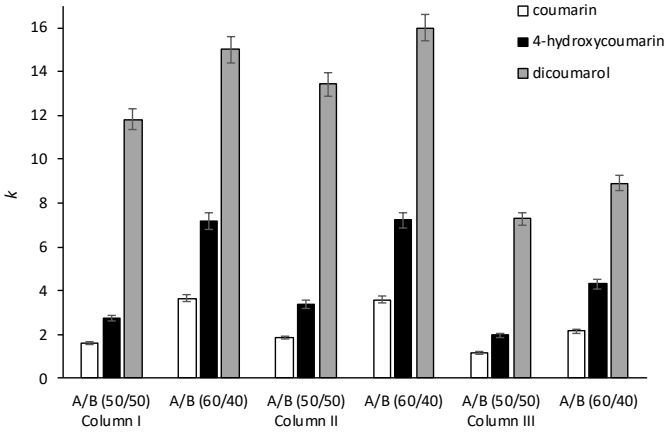


Fig. 2. Effect of mobile phase composition on retention factor of coumarins obtained for three analytical columns and isocratic elution with mobile phase A/B. A – 0.3 % acetic acid; B – methanol; flow rate 0.8 mL.min^{-1}; DAD detection.

The complete separation of analytes was achieved (resolution values higher than 2) using all tested columns as is shown in Fig. 3. The chromatographic characteristics for tested columns are summarised in Table 3.

The repeatability of the retention times and areas (determined for standard solutions at concentration level of analytes 10 µg.mL^{-1}) were lower than 1.1 % for all analytes and columns under study. All tested stationary phases were suitable for separation of coumarin, 4-hydroxycoumarin, and dicoumarol. The satisfactory resolution and symmetrical peaks of analytes were obtained. While the Symmetry C18 type of column is stable under pH range 2 – 8, the XBridge C18 stationary phase is very resistant to harsh conditions (pH 1 – 12) resulted from bridged ethylene hybrid technology. The results indicated, that fast separation using XBridge C18 was achieved, but this column was characterized by a lower column efficiency (greater HETP values) in comparison to other columns under study.

Therefore, the Symmetry C18 type column was chosen as suitable column for next analyses of real samples. The decrease of particle size and column length and use of higher flow rate (Column II) resulted in decrease of analysis time (~2.5 times in comparison with Column I)

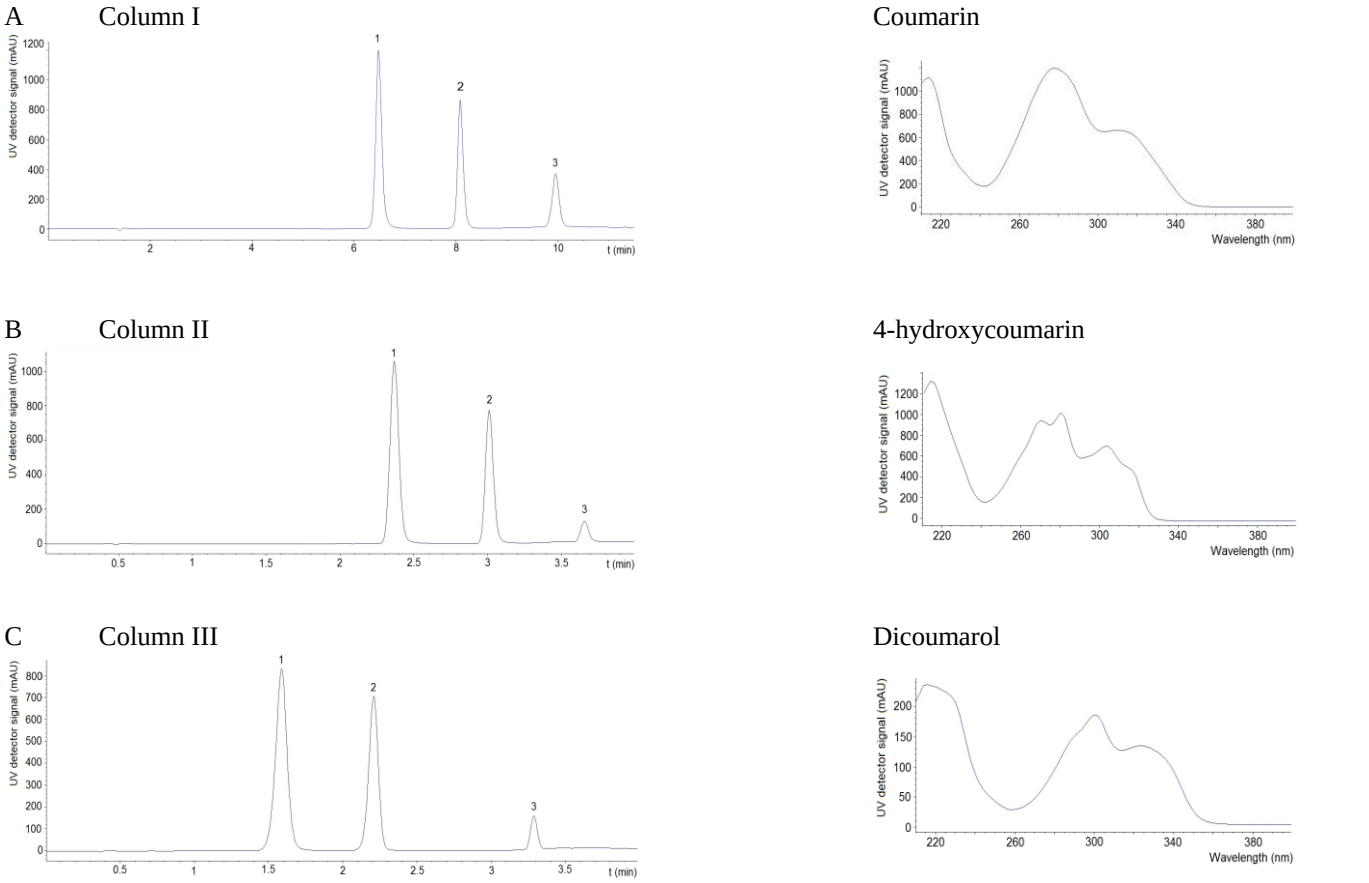


Fig. 3. Chromatographic separations of coumarin (1), 4-hydroxycoumarin (2), and dicoumarol (3) on three analytical columns and UV spectra of compounds under study. Mobile phase composition and flow rate as Table 2, DAD detection (chromatograms at 280 nm).

and finally decrease of solvent consumption.

Coumarins allow UV absorbance and some of them fluorescence too (Hroboňová *et al.* 2013). Spectrophotometric detection of coumarins was realised by DAD detector operated in the interval from 190 to 400 nm where the absorption maxima of target compounds are presented (280 nm for coumarin and 4-hydroxycoumarin, 300 nm for dicoumarol; Fig. 2). The maximum excitation and emission wavelength for fluorescence detection of dicoumarol were selected from excitation and emission spectra. As a result, the 300/390 nm (excitation/emission) wavelength pair was chosen for purpose of this study.

Validation of the method

The analytical figures of merit were determined under optimized separation and detection conditions. Parameters including LOD, LOQ, linear range, precision and accuracy were calculated to demonstrated the validation of the developed HPLC-DAD method (listed in Table 4).

The calibration curves of the three analytes were

Table 3. Retention times (tr), resolution (Rs), symmetry factor (As), and height equivalent of theoretical plate (HEPT) for HPLC separation of coumarins on tested analytical columns.

	t_r [min]	R_s	A_s	HEPT [µm]
Column I				
coumarin	6.47	7.5	0.81	11.0
4-hydroxycoumarin	8.7	10.6	0.81	6.5
dicoumarol	9.86		0.87	1.7
Column II				
coumarin	2.36	5.9	0.88	11.1
4-hydroxycoumarin	3.1	6.1	0.85	5.4
dicoumarol	3.53		0.87	2.2
Column III				
coumarin	1.59	4.7	1.7	25.0
4-hydroxycoumarin	2.22	10.3	1.8	9.3
dicoumarol	3.29		0.92	2.3

[a] mobile phase and gradient profiles as in Table 2.

Table 4. Linearity and sensitivity of the HPLC-DAD method.

Parameter	Coumarin	4-hydroxycoumarin	Dicoumarol
Linear range [μg.mL^{-1}]	0.05-100	0.05-100	0.7-100
Regression equation	y = 70165c + 3.9	y = 139610c + 11.4	y = 22050c + 38.8
R^2	0.9983	0.9896	0.9885
LOD [μg.mL^{-1}]	0.01	0.02	0.2
LOQ [μg.mL^{-1}]	0.05	0.05	0.7

created after the injection of a mixed standard solution. Results showed good linear relationship for each analyte (coefficient of determination in the range 0.9885 – 0.9983). The LODs of the method were calculated as a signal to noise ratio of 3 using the injection of series of more diluted standard solutions of analytes. The LOQs were evaluated using signal to noise ratio of 10. The LODs and LOQs of coumarin and its derivatives were in the range 0.01 – 0.2 μg.mL^{-1} and 0.05 – 0.7 μg.mL^{-1}, respectively. Since the LODs and LOQs of dicoumarol obtained for fluorescence detection were higher (2.1 and 6.5 μg.mL^{-1}, respectively) DAD detector was used in plant samples analyses.

Recovery tests of method were performed for dried sample spiked with dicoumarol levels at three concentration levels. The recovery values of dicoumarol, coumarin, and 4-hydroxycoumarin were higher than 85 %. As documented Table 5, good repeatability of the recovery was achieved (RSDs less than 4 % for all spiked concentration levels). The intra-day and inter-day precisions of developed method, determined for spiked sample were lower than 5 % and 9 %, respectively.

Analysis of real plant samples

In order to show reliability and applicability of the developed method, the extracts of sweet clover samples were analysed. Samples of herb, dried plant (aerial parts), and herb incubated for 14 and 21 days, were used in this study. Sample extracts were prepared in methanol. Comparison of retention times from HPLC and UV spectra of sample peaks and standards were used for characterization of coumarins in extracts. Analyses of dried plant and herb extracts confirmed the absence of dicoumarol and 4-hydroxycoumarin in tested samples (Fig 4A, B). The content of coumarin in dried plant sample was 3.3 – 0.1 mg.g^{-1}. The 4-hydroxycoumarin was formed in the fresh herb incubated for 14 and 21 days (Fig. 4C). The formation of 4-hydroxycoumarin was also observed in the synchronous fluorescence spectra recorded at the wavelength difference (difference between emission and excitation wavelength) of 90 nm (Fig. 5), using the recently developed method (Poláček *et al.* 2015).

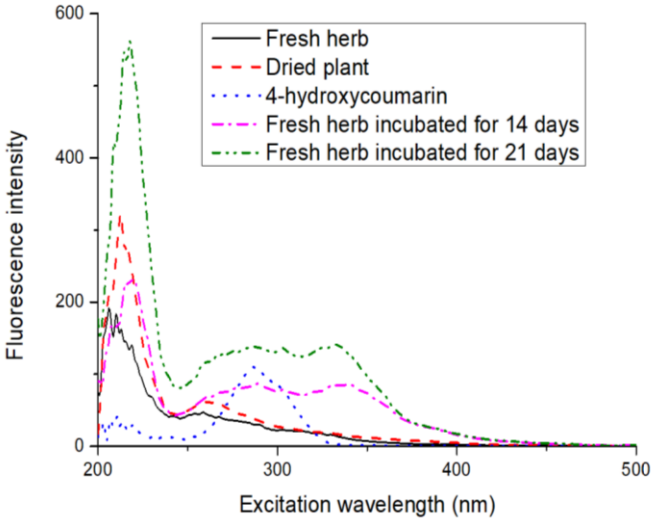


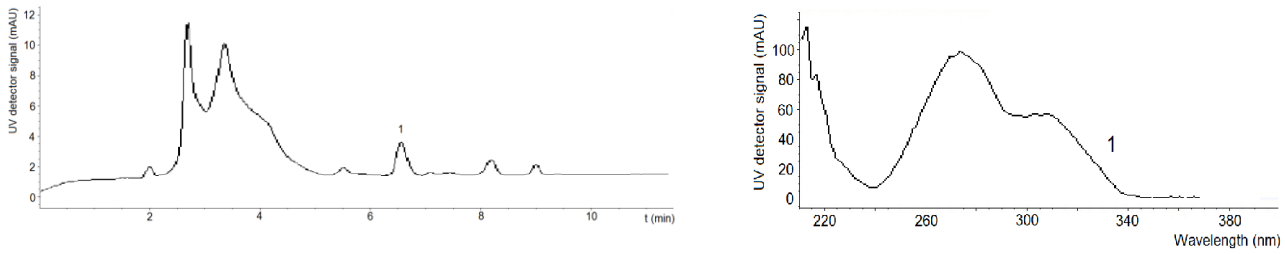
Fig. 5. Synchronous fluorescence spectra of fresh herb, dried plant, fresh herb after incubation and 4-hydroxycoumarin.

Table 5. Recovery values of extraction method for spiked dried sweet clover sample.

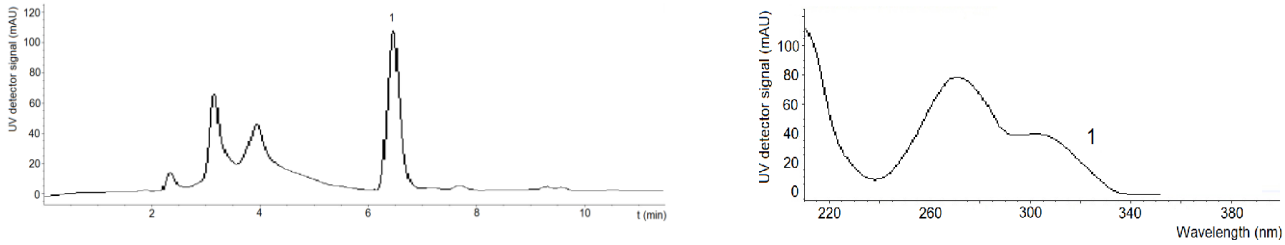
	Coumarin		4-hydroxycoumarin		Dicoumarol	
	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
C1	91.8	2.2	89.7	2.1	92.6	2.5
C2	95.0	3.0	94.1	2.6	92.5	3.1
C3	93.2	2.6	90.8	2.4	88.6	2.6

n = 7; C1 – 0.2 mg.g^{-1} of coumarin; 0.2 mg.g^{-1} of 4-hydroxycoumarin; 1.0 mg.mL^{-1} of dicoumarol; C2 – 1.0 mg.g^{-1} of coumarin; 1.0 mg.g^{-1} of 4-hydroxycoumarin; 2.0 mg.g^{-1} of dicoumarol; C3 – 2.0 mg.g^{-1} of coumarin; 2.0 mg.g^{-1} of 4-hydroxycoumarin; 10.0 mg.g^{-1} of dicoumarol.

A Meliloti herba



B Meliloti herba, hay



C Meliloti herba herb incubated for 14 (a) and 21 (b) days

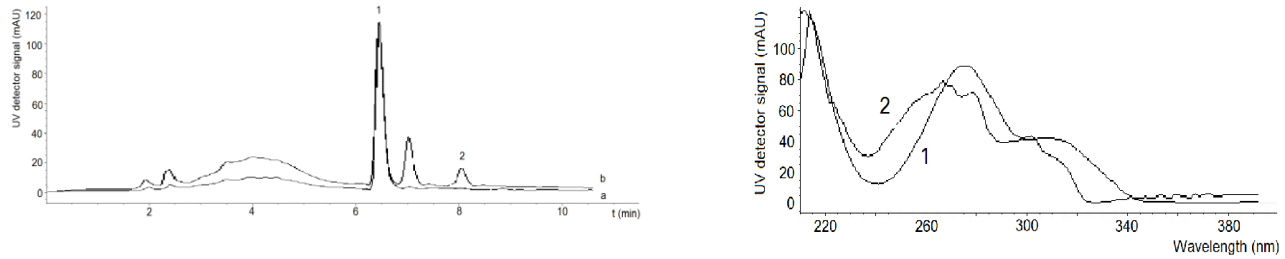


Fig. 4. Chromatograms of extracts of Meliloti herba fresh herb (A), dried plant (B), and herb after incubation (C). Stationary phase: Column I, mobile phase composition and flow rate as Table 2, DAD detection (chromatograms at 280 nm); 1- coumarin, 2- 4-hydroxycoumarin, 3- dicoumarol.

Conclusions

A simple reversed-phase HPLC-DAD method including stationary phase of C18 type and methanol-acetic acid as mobile phase with gradient elution was developed for simultaneous determination of coumarin, 4-hydroxycoumarin, and dicoumarol in extracts of sweet clover samples. The separation of coumarins by HPLC was rapid, efficient and reproducible, and the method presented acceptable results for sensitivity, linearity, precision, and accuracy. The LOD obtained by the proposed method are comparable to other methods with spectrophotometric detection. Moreover, one should note that wider linear concentration range of each compound under study was achieved. The method has potential to be used in medical plant and related products analysis, since currently there is an interest on characterization and determination of biologically active compounds in natural samples.

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