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Fast determination of colchicine by TLC-densitometry from pharmaceuticals and vegetal extracts

E. Bodoki*, R. Oprean, L. Vlase, M. Tamas, R Sandulescu

Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Emil Isac St. 13, RO-400023 Cluj-Napoca, Romania

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Abstract

Colchicine, (*S*)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo-(a(-heptalen-7-yl)-acetamide, is the main alkaloid contained in *Colchicum autumnale* (meadow saffron). There are known colorimetric, spectrophotometric, volumetric, potentiometric, voltametric, gravimetric and various chromatographic methods for quantitative determination of colchicine, each of them presenting a series of advantages and disadvantages. As an alternative, we proposed the use of a densitometric determination for colchicine allowing the determination of this alkaloid from pharmaceutical products, as well from seeds of meadow saffron. The total alkaloid extract was separated by Thin-Layer Chromatography using Silicagel $60F_{254}$ layers and a mixture of chloroform:acetone:diethylamine (5:4:1) as mobile phase. The same conditions were used for the determination from pharmaceutical products. Densitometric measurements were carried out at the absorption maximum (350 nm) of colchicine, the determinations being made by reflectance and by fluorescence. The peaks were optimized regarding to their area and shape by varying four scanning parameters (slit width and height, number of measurements and scanning speed). We established the calibration plot using pure colchicine in the range 50–600 ng mL^{-1}. The proposed method could be widely used in the pharmaceutical industry for the quick and accurate quantitative determination of colchicine because it eliminates the interferences given by other bioactive or degradation compounds. The method was characterized by validation parameters (linearity, accuracy, fidelity, sensitivity) and it was established its performances in comparison with an HPLC method and an official quantitative determination from the Romanian Pharmacopoeia X edition respectively.

Keywords: Colchicine; TLC-densitometry; Peak optimization; HPLC

1. Introduction

Colchicine is the main alkaloid contained in *Colchicum autumnale* L. (meadow saffron). The plant is considered to be very toxic to man and herbivorous animals due to the presence of alkaloids of colchicine type in all of its organs. Administered to patients with acute attacks of gout, spectacularly attenuates the pain and eliminates the inflammatory tumefaction, being a specific anti inflammatory agent used in this particular type of inflammation. The drug inhibits the migration of leucocytes to inflammatory areas, thus interrupting the inflammatory response that sustains the acute attack. The drug also presents an antimitotic action, arresting dividing cells in metaphase by preventing normal function of mitotic spindle, which helps cells divide. Nevertheless, colchicine is not a selective antimitotic agent, which is why so toxic for the organism.

There are known spectrophotometric [1], volumetric [2], potentiometric [3,4], voltametric [5,6], gravimetric [7], radio and enzyme immunoassays [8] and various chromatographic [9–11] methods for quantitative determination of colchicine, each of them presenting a series of advantages and disadvantages. Combining the chromatographic separation on a silica layer and the in situ densitometric determination of the separated compounds, results a highly efficient, quick,

* Corresponding author. Tel.: +40 264 593118; fax: +40 264 597257.
E-mail addresses: bodokie@umfcluj.ro (E. Bodoki), roprean@umfcluj.ro (R. Oprean).

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accurate and relatively inexpensive method, eliminating the possible interference given by other structurally related compounds.

2. Experimental

2.1. Reagents

Methanol, ethanol, chloroform, acetone and diethylamine were of analytical grade and were purchased from Merck, Germany, except the last one that was obtained from Fluka, Switzerland.

2.2. Samples

Colchicine was of analytical grade for biochemistry and was purchased from Merck, Germany. The stock solutions of colchicine were freshly prepared each day and were stored in the dark.

The seeds of meadow saffron were collected from the surroundings of Cluj-Napoca, Romania. The extracts were prepared using several methods, such as Soxhlet extraction, turboextraction and ultrasonication in 95% ethanol, keeping the same extraction ratio of 2.25% m/V seeds/solvant. The obtained extracts were diluted 10 times with distilled water, and the resulting solution served for injection on the HPLC system (20 μL). In TLC experiments, 5 μL of the raw ethanolic samples were applied on the plates.

The pharmaceutical samples were tablets with 1 mg declared amount of colchicine and were purchased from Fabiol SA, Bucharest, Romania. After the determination of the average weight of tablets, they were pulverized. A known amount of powder was transferred in a volumetric flask and adjusted to 10 mL with methanol. The colchicine was extracted by ultrasonication for 5 min and the suspension was than centrifuged for 3 min at 5000 rpm. About 200 μL of the supernatant were diluted to 10 mL with distilled water, which served as sample for injection on the HPLC system (20 μL). In TLC experiments, 5 μL of the supernatant served as sample.

2.3. TLC experiments

The TLC separation was performed on precoated 10 × 10 Silicagel 60 and Silicagel 60F_{254} glass plates, using a mixture of chloroform:acetone:diethylamine (5:4:1) as mobile phase, both in vertical and horizontal Desaga separation chambers, after a saturation time of 15 min. One-dimensional chromatographic separation was carried out over a path of 7.5 cm using the above-mentioned mixture, avoiding as much as possible exposure to light. All the separations were performed at ambient temperature and humidity (20–24 °C and 46–56%). For detection and quantification, scanning densitometry was performed using a Desaga CD60 densitometer, in reflectance (at 350 nm) and fluorescence modes (Hg lamp, 254 nm).

2.3.1. TLC calibration

Separate stock solutions (10 mg mL^{-1} in ethanol) were prepared. On each of the three consecutive days of validation, six calibration standards (at 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg mL^{-1}) were employed. Five sets of control samples, corresponding to 60%, 80%, 100%, 120% and 140% of the 0.3 mg mL^{-1} solution were prepared from separate weightings and stored in dark. The standard and control solutions were manually applied in 5 mm bands using calibrated Desaga capillaries (5 μL) at 1.5 cm from the bottom edge of the plate.

2.4. HPLC experiments

The high performance chromatographic separations were carried out on a HP Series 1100 HPLC system on a 3.5 μm Zorbax SB C18100 × 3 mm column. The mobile phase consisted of acetonitrile:water (75:25 v/v) and was delivered at 1 mL min^{-1}. The temperature was maintained at 42 °C during all experiments. The methanolic standard solutions of colchicine were injected automatically (10 μL) and the analytes were detected at 243 and 350 nm. The retention time for the colchicine is approximately 2.3 min.

2.4.1. HPLC calibration

Separate stock solutions (0.505, 0.628, 0.996, 1.802, 2.387 mg mL^{-1} in methanol) were prepared. From each solution, aliquots of 100 μL were transferred in 25 mL flasks and diluted with distilled water. Aliquots of 0.2 mL were diluted to 5 mL with distilled water, solutions that served for injection on the HPLC system (10 μL). Two different approaches were exploited. For the first one, five calibration standards (at 30.1, 60.2, 120.4, 200.6 and 401.3 ng mL^{-1}) were prepared. For the second approach, a larger concentration domain was investigated (30.1–24,000 ng mL^{-1}).

2.5. Spectrophotometric determination (according to X Romanian Pharmacopoeia)

The described normative allow a content of the 1 mg colchicine tablets between 90% and 110% of the declared value.

The tablets powder corresponding to about 1 mg of colchicine was shaking vigorously with 20 mL of 100% ethanol for 30 min. After centrifugation at 2000 rpm for 2 min, 10 mL of the supernatant is diluted to 50 mL with 100% ethanol. The absorbance of this solution is measured immediately at 350 nm. The absorbance for 1% solution and the light path of 1 cm is 425 at 350 nm.

3. Results and discussion

3.1. Spectral and chromatographic behavior of colchicine and its degradation products

The colchicine is strongly degraded under light exposure, the degradation products being pharmacologically inactive. The absorption spectra are completely different after 2 days of light exposure of the colchicine's solution (Fig. 1a). Because of the structural differences between the degradation products and colchicine, the chromatographic behavior is changing dramatically (Fig. 1b).

This high sensibility to light allows only one scan with the densitometer, because after each scanning the resulted peak is smaller, making it impossible to apply two-dimensional chromatography for better separation. In these chromatographic conditions the Rf of colchicine was 0.65 and for his light exposure-derivative 0.83.

Trying to increase the sensitivity and selectivity of the detection, the fluorescence spectra were measured (Fig. 2). Colchicine exhibits a natural fluorescence but the intensity of the signal is much smaller compared to the UV absorbance. In addition, for the TLC experiments the florescence mode is not suitable because the colchicine is degraded during the excitation period and the signal decrease for successive measurements.

The signal intensity is significantly improved by using fluorescence quenching on Silicagel $60F_{254}$ plates.

3.2. Peak optimization

For the UV detection, the wavelength is the main parameter that influences the peak area and height. In fluorescence quenching mode, the wavelength cannot be changed because of the florescence indicator which exhibit fluorescence at a given wavelength (254 or 365 nm). Other four operating parameters are susceptible to modify the peak shape: width (s_w) and height (s_h) of the excitation light slit, the speed of the scan expressed as the number of scans per point (n_{sc}) and distance between two successive measurements (d_m). In order to increase the peak area and height, an optimization was performed.

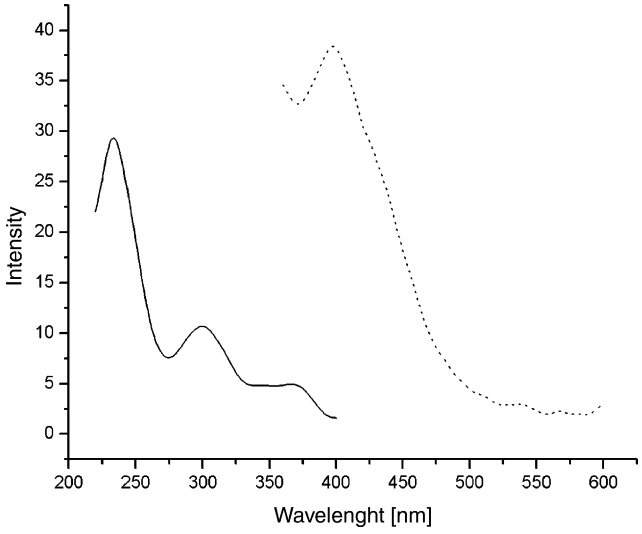

Fig. 2. Fluorescence spectra of colchicines: (—) excitation; (- - -) emission.

The influence of the four parameters over the peak area and height was investigated by using D-optimal design. The matrix contained 11 model terms (the four parameters, their interactions and a free term). Eighteen points and two central points were selected from the 288 points of a full factorial design and each measurement was performed in duplicate. The values of the four parameters were set by the densitometer software in the range indicated in Table 1.

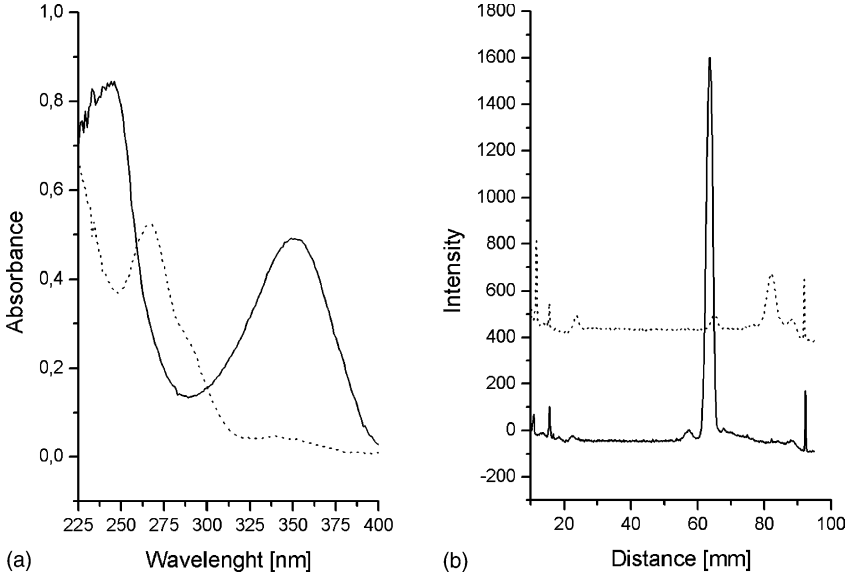

Fig. 1. Light exposure effect over the spectral (a) and chromatographic (b) behavior of colchicines: (—) colchicines; (- - -) degradation products.

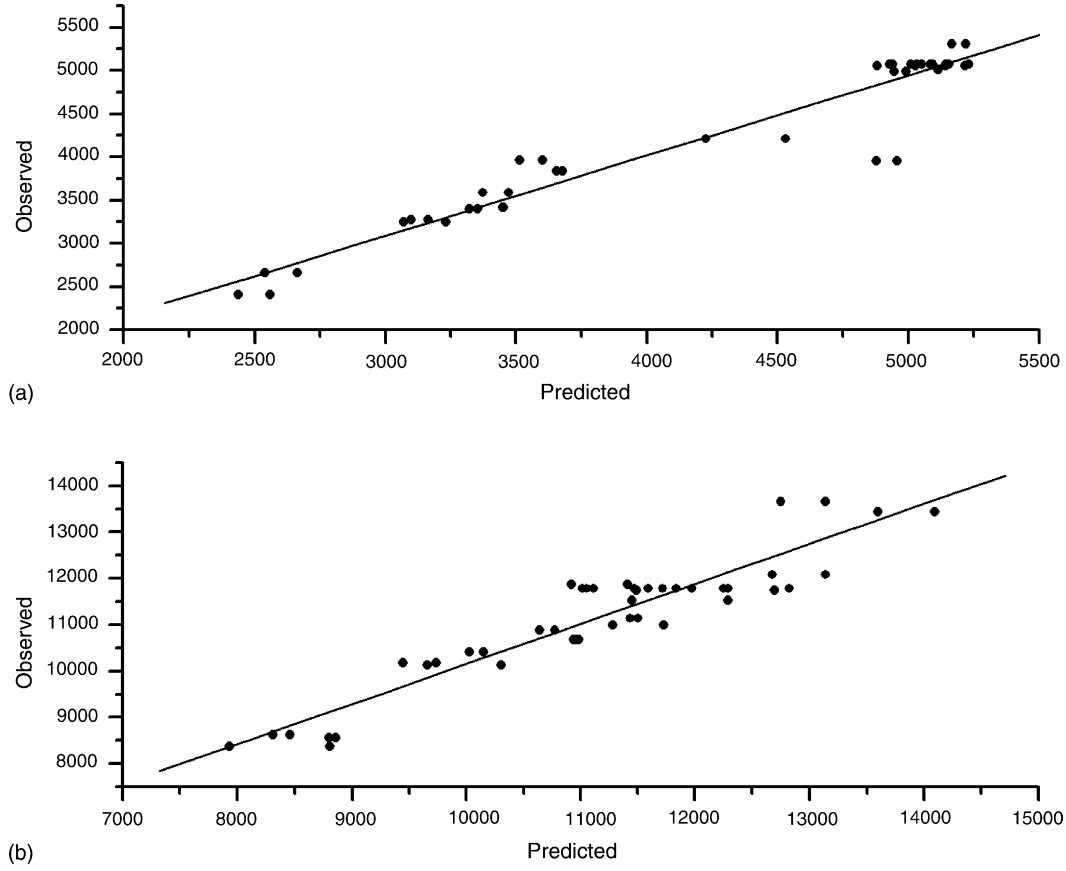

Fig. 3. Observed vs. predicted area (a) and height (b) of the peaks for MLR fitting.

The model was fitted by MLR and we found a fairy good correlation between predicted and observed values of both area (Fig. 3a, $R^2 = 0.8720$, $Q^2 = 0.7645$, reproducibility = 0.8984) and height (Fig. 3b, $R^2 = 0.9314$, $Q^2 = 0.9051$, reproducibility = 0.9913) of the peaks.

The main effect was found to be the number of scans per point for both area and height evaluations, probably because of the emission delay. Thus, maximizing n_{sc} will increase the peak height. Another important parameter was the slit height that affects the results in the same manner as n_{sc}. The other two parameter's influence is smaller than the others, even if it is statistically significant. After the optimisation, scanning parameters were chosen as presented in Table 1.

3.3. Method validation

3.3.1. TLC

Using the areas and heights of the separated peaks in the range of 0.1–0.6 mg mL^{-1}, resulted a second-degree polynomial regression, for both reflectance and fluorescence mode (Fig. 4). Unfortunately, not even for a narrow concentration domain has been achieved a linearity between the measured signal and applied quantity of colchicine.

Table 2 presents the regression curves of both reflectance and fluorescence modes for area and height, respectively.

Calibration curves were prepared in triplicate in three different days, applying 5 μL of each standard solution. Peak areas of the control and unknown samples were determined and the concentrations calculated according to peak area = $f(c)$ for both reflectance and fluorescence, because their slope is the highest over the range of the used concentrations.

The accuracy of the method was evaluated using five control samples, and the determined peak areas served for the calculation of the recovery (Table 3). Three series of control samples prepared in three different days of the validation were measured.

The intra-day repeatability and the intermediate precision on different days were calculated at five concentration levels (0.18, 0.24, 0.3, 0.36, 0.42 mg mL^{-1}) and expressed as R.S.D. (%). Three determinations ($n = 3$) were carried out for each concentration (Table 4).

Table 1
Parameter ranges for D-optimal design and their optimized values

Parameter	Range	Optimized
Slit width (s_w) (mm)	0.04–0.4	0.2
Slit height (s_h) (mm)	0.4–3	3
Number of scans per point (n_{sc}) (mm)	2–16	16
Distance between two measurements (d_m) (mm)	0.05–0.2	0.1

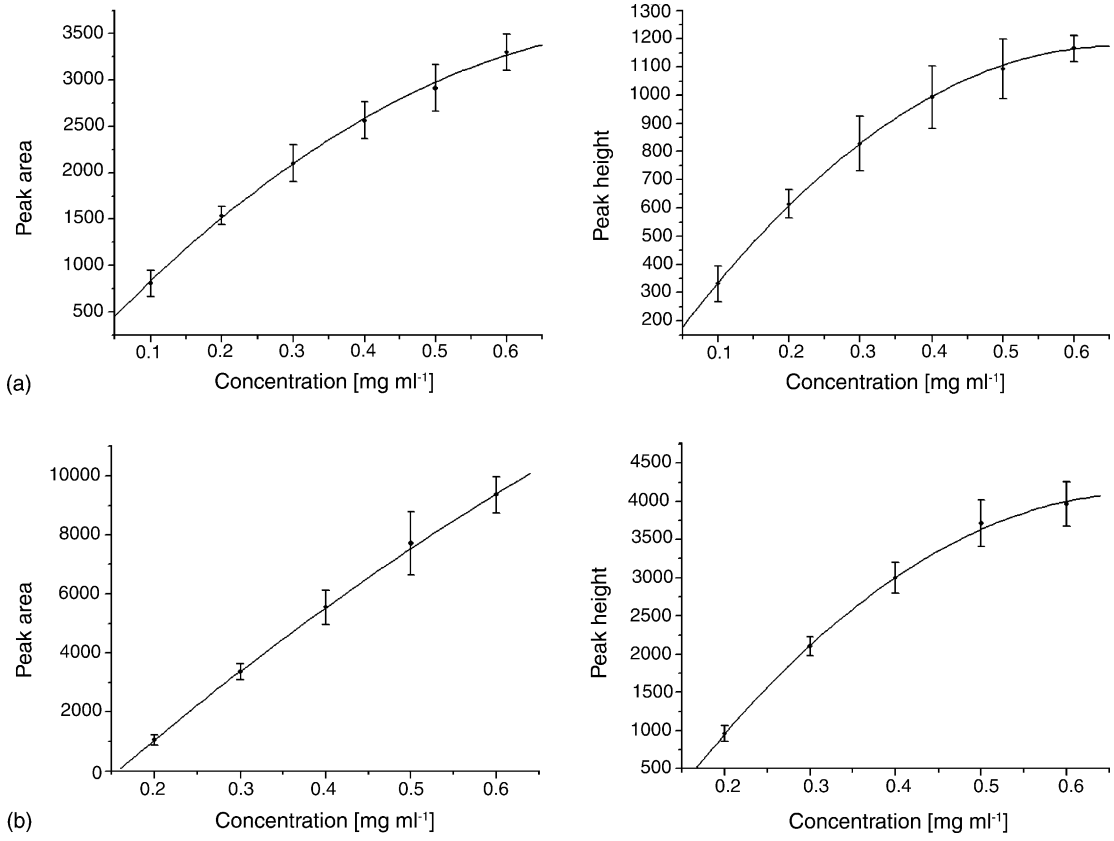

Fig. 4. Non-linear calibration of colchicines: (a) reflectance; (b) fluorescence quenching.

Table 2
Data for calibration graphs

Detection mode	Area[a]			Height[b]		
	a	*b*	*c*	*a*	*b*	*c*
Reflectance[c]	−4587.6 $R^2 = 0.9987$	8088.3	68.063	$-2688.9x^2$ $R^2 = 0.9997$	3532.2*x*	9.1633
Fluorescence[d]	$-9596x^2$ $R^2 = 0.9995$	28653*x*	−4324.1	$-13956x^2$ $R^2 = 0.9989$	18787*x*	−2254.7

[a] According to area = $a(\text{conc})^2 + b(\text{conc}) + c$.
[b] According to height = $a(\text{conc})^2 + b(\text{conc}) + c$.
[c] $n = 6$.
[d] $n = 5$.

Table 3
Accuracy testing of colchicine

Reflectance mode			Fluorescence mode		
Percentage of midrange	Nominal (mg mL^{-1})	Recovery (%)	Percentage of midrange	Nominal (mg mL^{-1})	Recovery (%)
60	0.18	98.70	60	0.18	114.44
80	0.24	104.44	80	0.24	92.08
100	0.3	102.44	100	0.3	105.66
120	0.36	97.87	120	0.36	103.98
140	0.42	99.76	140	0.42	99.12
Mean recovery (%)		100.64			103.03
RSD (%)		7.67			8.52
Cochran's test	$C_{calc} = 0.273$	$C(0.05;5;2) = 0.68$	Cochran's test	$C_{calc} = 0.664$	$C(0.05;5;2) = 0.68$
Fischer's test	$F_{calc} = 0.301$	$F(0.05;4;14) = 3.48$	Fischer's test	$F_{calc} = 0.685$	$F(0.05;4;14) = 3.48$
Confidence interval (95%)		100.64 ± 4.28	Confidence interval (95%)		104.60 ± 5.70

Table 4
Fidelity parameters

	Reflectance mode	Fluorescence mode
Repeatability (RSD)	3.62	8.26
Reproducibility (RSD)	11.29	9.99

Table 5
Colchicine determination through TLC-densitometry

Samples	TLC
Colchicine tablets, 1 mg, Fabiol SA, Bucharest (mg/tablet)	1.03 ± 0.028
Colchicine seeds—Soxhlet extraction (mg/100 g)	0.438 ± 0.022

The detection limit (LOD) and quantitation limit (LOQ) were expressed as signals, on the basis of the standard deviation (s_b) of the blank responses, as follows:

$$\mathrm{LOD} = 3s_b \qquad \mathrm{LOQ} = 10s_b$$

These signals were transformed in terms of concentration, using the calibration curve from reflectance mode, peak areas = f(c) (LOD = 0.0021 mg mL^{-1} and LOQ = 0.027 mg mL^{-1}). The standard deviation of the blank (s_b) was determined measuring six blank solutions ($n = 6$). This was only possible in reflectance mode, because in fluorescence mode there was no baseline noise recorded.

As real samples, it was determined the colchicine content of tablets and meadow saffron seeds (Table 5). The samples were treated as described earlier.

3.3.2. HPLC

Solutions of colchicines in methanol, ranging from 30.1 to 401.3 ng mL^{-1} were injected into the HPLC system in order to assess detector linearity. In the second approach a larger concentration range was investigated (30.1–24075.7 ng mL^{-1}). In both cases, peak height was plotted against the concentration of injected solutions, and the recorded response was linear ($r > 0.999$) (Table 6).

The tablets and vegetable samples were measured at 350 nm and the peak areas were used to calculate their concentration using the calibration curve from 30.1 to 401.3 ng mL^{-1} (Table 7).

Using the HPLC method, also the influence of the extraction method was investigated, comparing the most exhaustive method (Soxhlet—2.5 h) with the much faster extraction techniques (turboextraction—15 min at 6000 rpm, sonication—30 min).

Table 6
Data for calibration graphs

Range	30.1–401.3 ng mL^{-1}		30.1–24075.7 ng mL^{-1}	
Detection (nm)	243	350	243	350
Slope	0.1246	0.0650	0.1367	0.0726
Intercept	−0.2687	−0.0194	2.0702	0.4033
R^2	0.99988	0.99997	0.99999	0.99999

Table 7
Results for colchicine tablets and plant extracts

Samples	HPLC
Colchicine tablets, 1 mg, Fabiol SA, Bucharest (mg/tablet)	1.016
Colchicine seeds (mg/100 g)	
Soxhlet extraction	0.530
Turboextraction	0.479
Sonication	0.481

Table 8
Results for colchicine content of tablets by spectrophotometric determination

Samples	Colchicine tablets, 1 mg, Fabiol SA, Bucharest (mg/tablet)
Spectrophotometry	1.030

3.3.3. Spectrophotometric determination

Following the described protocol in the Romanian Pharmacopoeia X edition for the determination of colchicine from tablets, it was determined the content of the alkaloid from the single available Romanian pharmaceutical product with colchicine. The results were very similar to those obtained by the proposed TLC and HPLC method (Table 8), showing a +3% error compared to the declared value, complying with the Pharmacopoeia's normative (±10%).

4. Conclusions

The proposed TLC method can be widely used as a standard technique for rapid and accurate qualitative and quantitative determination of colchicine from different matrices, such as the analysis of plant extracts or pharmaceutical products (tablets), because the interference of so called "unknown background materials" can be more easily eliminated. Moreover, this method has several advantages over the other analytical procedures (HPLC, spectrophotometric assays), such as: simple pretreatment of the vegetal samples, low cost and high throughput of samples.

For the fluorescence mode an experimental design was performed, in order to increase the sensitivity. Four scanning parameters effects over the signal were investigated. Increasing two of them (number of scans per point (n_{sc}) and slit height (s_h)), the signal was significantly improved. The others exhibit less effect than the former two.

The TLC method's analytical performance was established and the method was validated in terms of accuracy, fidelity, detection and quantification limits. Moreover, it was compared with a spectrophotometric method, recommended by the Romanian Pharmacopoeia, and also with an HPLC method, considered to be a reference technique in drug analysis. The results obtained by the three methods show a good correlation.

Several extraction procedures (Soxhlet, turboextraction, sonication) of the meadow saffron seeds were investigated by the HPLC method. Considering the results, although the

first one takes much longer, gives the best recovery for the alkaloid, remaining the recommended procedure for quantitative determinations.

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