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Development and optimization of a stability indicating method on a monolithic reversed-phase column for Colchicum dry extract

Andreas Körner*, Silke Kohn

Solvay Pharmaceuticals GmbH, Analytical Support Department, Marketed Product Support, Hans-Böckler Allee 20, Post box 220, 30173 Hannover, Germany

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Abstract

This article presents a method developed on a monolithic reversed-phase column for separation of related alkaloids and degradation products from colchicine in dry extract preparations from Colchicum seeds. Optimization was performed using an optimization software with variation of the pH of the mobile phase and the percentage of organic modifier. This method permits for the first time a fast separation of all alkaloids including colchiceine without prior derivatization or complexation with excellent linearity, precision and low limits of quantitation which makes it a suitable method for stability testing of extracts or drug formulations containing extracts of Colchicum seeds.

Keywords: *Colchicum autumnale*; Colchicine; Colchiceine; Monolithic columns

1. Introduction

Colchicum autumnale L., commonly called Autumn Crocos or Meadow Saffron, is the best known species of Colchicum genus, belonging to the family of Colchicaceae.

Plants of this family are of pharmaceutical interest since they produce alkaloids with different therapeutic applications, mainly colchicine, 3-demethylcolchicine and colchicoside. An extract of Colchicum was first described as a treatment for gout in De Materia Medica of Padanius Dioscorides. Colchicine itself possesses anti-inflammatory properties and can efficiently exacerbate the symptoms during an attack of gout when applied in the early phase. More recently it was introduced for the treatment of the familiar Mediterranean fever [1]. Besides the alkaloid is a potent anti-mitotic agent and possesses anti-tumour activity [2].

The seeds of *C. autumnale* L., which are rich in colchicines and colchicoside are used by the pharmaceutical industry for extraction of these alkaloids since decades. Several techniques have been described for the extraction including recently published supercritical fluid extraction as a very efficient and rapid technique [3].

Colchicine is known to be sensitive to light and hydrolysis. The main degradation products described are β- and γ-lumicolchicine and colchiceine. Numerous HPLC methods can be found in literature for the control of related alkaloids in colchicine and Colchicum extracts [4–7] but especially the analysis of colchiceine turned out to be difficult [8].

Current monographs still use complexation with ferric chloride and extraction for semi-quantitative determination of this compound [9].

The work presented here deals with the characterization, quality control and stability testing of a standardized extract of Colchicum seeds and formulations thereof. The aspects for systematic development of a fast and highly selective reversed-phase HPLC method on a RP monolithic column are discussed. RP Monolithic columns which are based on highly purified silica show low dependence of plate height versus flow rate and develop a low back pressure which makes them attractive for this application due to different characteristics presented [10]. The method permits assaying of all related alkaloids and potential degradation products including colchiceine without prior derivatization. The effects of the

* Corresponding author. Fax: +49 511 857 2690.
E-mail address: andreas.koerner@solvay.com (A. Körner).

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pH value of the mobile phase and of the content of methanol and EDTA as an additive are discussed. Optimization was performed using Drylab® software.

2. Materials and methods

Initially, the method of USP 27 [11] was installed on a 250 mm × 4.0 mm LiChrospher® 100 RP 8, 5 μm column, Merck, Darmstadt, Germany. The mobile phase contained methanol (47.5%, v/v) and 0.05 M potassium dihydrogen phosphate (52.5%, v/v) adjusted to pH 5.5. The flow rate was adjusted to 1.0 ml/min and detection was performed at a wavelength of 254 nm.

The conditions for separation were afterwards adapted to a 100 mm × 4.6 mm Chromolith® RP-18e column, Merck, Darmstadt, Germany using also methanol and phosphate buffer as mobile phase. For preparation of the 0.05 M phosphate buffer 13.6092 g of potassium dihydrogen phosphate were dissolved in 2000.0 ml of water. After addition of 1.7534 g EDTA, the pH was adjusted to the pH required by addition of 1 M sodium hydroxide solution. The flow rate was adjusted to 3 ml/min and detection was performed at a wavelength of 245 nm. The sample solutions were prepared by dissolving 300.0 mg of a Colchicum dry extract preparation in 20.0 ml of methanol/water 1:1 (v/v) with addition of 2.92 g EDTA or as otherwise stated.

Drylab® software, version 2.01, Institute for Chromatography, Berlin was used for calculation of optimum separation conditions.

Standard solutions were prepared by dissolving 10.0 mg of colchicine in 20.0 ml of methanol/water 1:1 (v/v) and 1.0 mg of demecolcine, 1.0 mg of γ-lumicolchicine and 1.0 mg of colchiceine in 10.0 ml of methanol/water 1:1 (v/v). 1.0 ml of the demecolcine, lumicolchicine, and colchiceine containing solutions was further diluted with methanol/water 1:1 (v/v) to 20.0 ml.

Methanol was purchased from J.T. Baker, potassium dihydrogen phosphate from Merck, Darmstadt, Germany, sodium hydroxide was obtained from Riedel-de Haen, Seelze, Germany and EDTA from Fluka AG, Buchs, Switzerland.

Colchicine, demecolcine and γ-lumicolchicine standard were obtained from Sigma, Steinheim, Germany and further characterized as standards in house, colchiceine was synthesized from colchicine by acidic hydrolysis at Chemische Laboratorien Dr. Christoph Mark, Worms, Germany.

3. Results

Development of the method was started by adoption of the HPLC method described in the monographs of Ph.Eur. and USP 27 to a conventional C8 reversed-phase column. The injection of the dry extract and of a colchicine reference standard resulted in a chromatogram showing several peaks (Fig. 1). Three of them could be associated to colchicine, γ-lumicolchicine and demecolcine by comparison to the corresponding reference standards.

Besides the lumicolchicines (Fig. 2), which are formed by irradiation of colchicine as substance or in solution, colchiceine is regarded as a potential degradation product which might already be present in the plant and plant extracts respectively but could probably also be formed during storage by hydrolysis of colchicine (Fig. 3).

To examine, whether colchiceine could also be separated and detected by this method, acidic hydrolysis of the colchicine reference standard was performed by boiling a solution of colchicine reference standard in hydrochloric acid

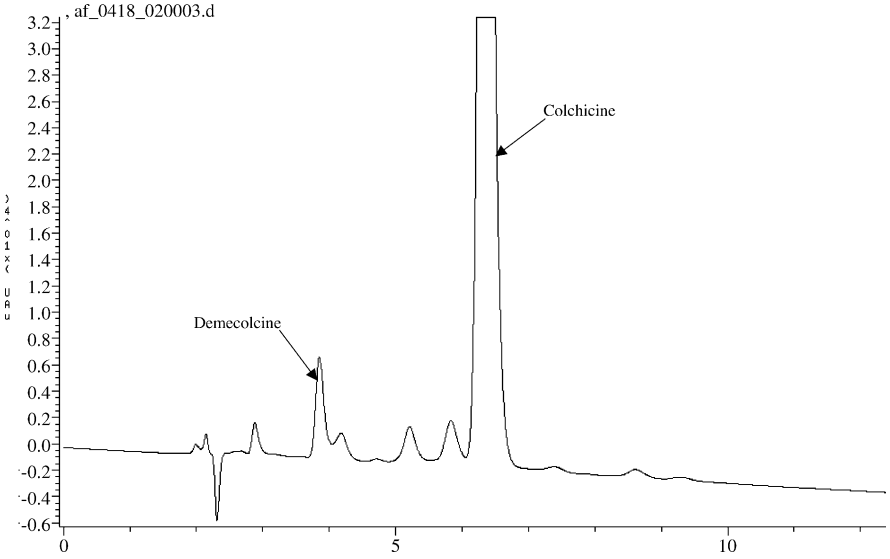


Fig. 1. Chromatogram of Colchicum dry extract on a conventional C8 reversed-phase (250 mm × 4.0 mm LiChrospher® 100 RP 8, 5 μm) column. For conditions see text.

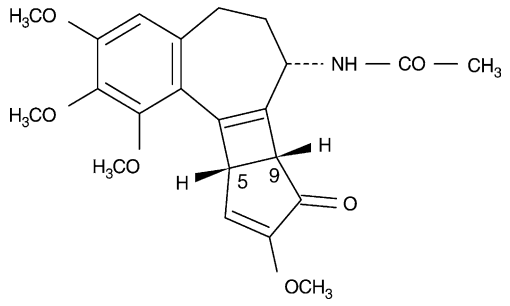
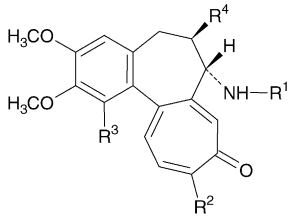


Fig. 2. Structure of γ-lumicolchicine.



R^1	R^2	R^3	R^4	
$COCH_3$	OCH_3	OCH_3	H	Colchicine
CH_3	OCH_3	OCH_3	H	Demecolcine
$COCH_3$	OH	OCH_3	H	Colchiceine

Fig. 3. Structures of colchicine, demecolcine and colchiceine.

under reflux. The resulting solutions were injected after neutralization with sodium hydroxide. Due to the structure of colchicine, which is a vinylogous ester, fast hydrolysis occurs at the methoxy-group at position 10 of the seven-membered ring. The amide function and the methoxy-groups of the aromatic ring system in contrast require harsh conditions for hydrolysis which are unlikely to occur during manufacturing of plant extracts or during storage (Fig. 3).

Table 1
Peak area of colchicine and colchiceine and related alkaloids obtained before and after hydrolysis on 250 mm × 4.0 mm LiChrospher® 100 RP 8, 5 μm column

Compound, RT (min)	Before hydrolysis		After hydrolysis	
	Peak area	Area%	Peak area	Area%
2.7	4950	0.01	3861	0.02
3.19	2495	<0.01	n.d.	n.d.
3.62	9917	0.02	634788	2.78
4.23	15487	0.03	8671	0.04
5.22	572652	1.22	657734	2.88
5.84	1182740	2.52	310539	1.36
6.33	45080293	96.08	21215637	92.83
7.78	2602	<0.01	n.d.	n.d.
8.60	41204	0.09	16761	0.07
9.3	6431	0.01	5728	0.03
Sum	46918771		22853719	

For other conditions see text.

The chromatogram after hydrolysis shows a significant loss of colchicine, but only minor products of hydrolysis and no colchiceine were found (Fig. 4). The calculation of the mass balance revealed a significant deficiency of approx. 49% (Table 1), derived from the total peak area for the hydrolyzed versus non-hydrolyzed material. Even after variation of the mobile phase composition no peak of colchiceine could be detected. The non-elution of colchiceine was consistent with

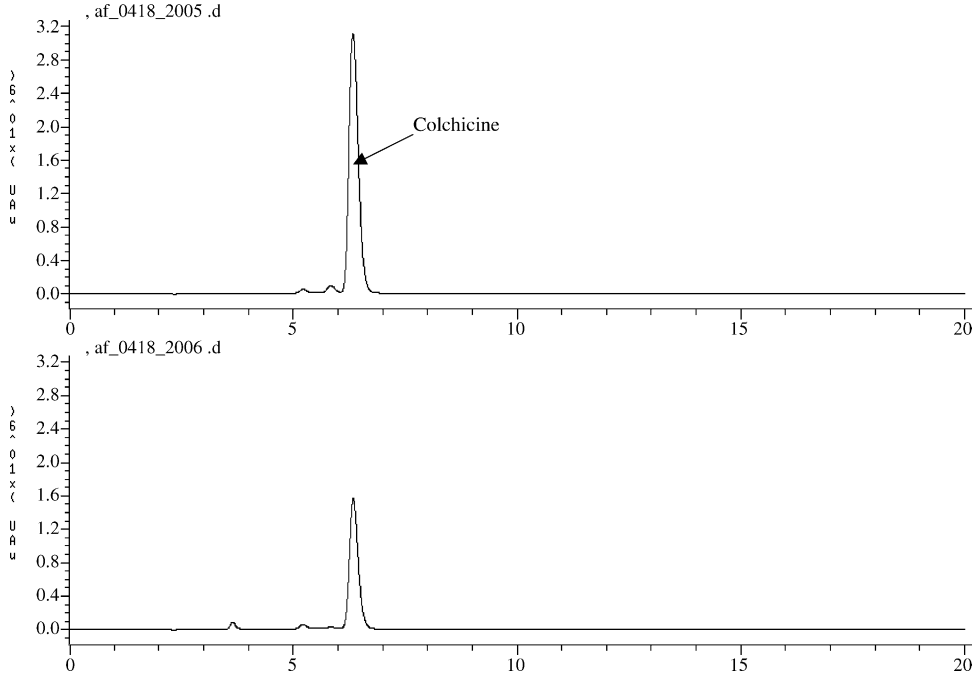


Fig. 4. Chromatogram of colchicines standard before (top) and after (bottom) acidic hydrolysis obtained on 250 mm × 4.0 mm LiChrospher® 100 RP 8, 5 μm column. For other conditions see text.

results other authors obtained before on a variety of different stationary phases. Klein and Davis [5] circumvented this problem by derivatization of colchiceine in presence of colchicines, thus obtaining the isoethyl colchicinate and ethyl colchicinate.

The behaviour of colchiceine on most silica based phases can easily be explained by its complex-forming properties due to the planar ring system and the neighborhood of the hydroxyl- and the carboxy-function at positions 10 and 9, respectively. Detection of colchiceine in most monographs is consequently based on the formation of a coloured complex of ferric-chloride in aqueous solution which can be extracted into the chloroform layer. Most older reversed-phase stationary phases contain rather high amounts of potentially complex-forming metals. Hence after addition of a complex-forming metal to the mobile phase, colchiceine could be separated on a conventional phase as the metal complex as described by Lacey and Brady using copper sulphate in the eluent to form a stable copper-colchiceine complex [8].

Since the contamination of silica-gel with metals was recognized some years ago as the main cause for tailing of basic compounds besides silanophilic interactions, synthetic silica-gel was introduced for manufacturing of efficiently end-capped reversed-phase columns.

Monolithic RP-columns were introduced in 2000. Since then, numerous separations have been published in literature. The synthesis of a monolithic column is based on synthetic silicon-dioxide. The resulting material is therefore low in metal-content and is housed in a peek-tube. Additionally, high flow rates can be applied without creating high back-pressures due to the porous structure to reduce the run-time especially with low contents of the organic modifier when higher selectivity is required. A flat dependence of the separation efficiency on the flow-rate applied is typically observed [10].

Table 2
Peak area of colchicine and colchiceine and related alkaloids obtained before and after hydrolysis on 100 mm × 4.6 mm Chromolith® RP-18e column without addition of EDTA

Compound		Peak area	
Name	RT (min)	Before hydrolysis	After hydrolysis
	0.75	7661	7689
	0.92	4380	3461
	1.92	1904	70454
	2.76	–	8914
	3.86	115609	54888
	4.28	94306	19594
Colchicine	4.68	4165813	1974882
	6.36	–	23731
Colchiceine	7.30	–	290545
Sum		4389673	2454148

4. Influence of sodium-EDTA

After adoption of the chromatographic separation to the Chromolith® RP-18e column the injection of the solution obtained after hydrolysis resulted in the chromatogram depicted in Fig. 5. The chromatogram shows the peak of colchicine and an additional peak which was identified as colchiceine. The mass-balance was calculated based on assuming identical response factors for colchicine, colchiceine and further degradation products where results are shown in Table 2. The relative response factors of colchiceine versus colchicine was verified experimentally to be approximately 1.5.

Results show, however, that the sum of peak area still decreased significantly, i.e. to approximately 56% after hydrolysis.

Besides, repetitive injections of a standard solution of colchiceine revealed a significant deterioration of the peak

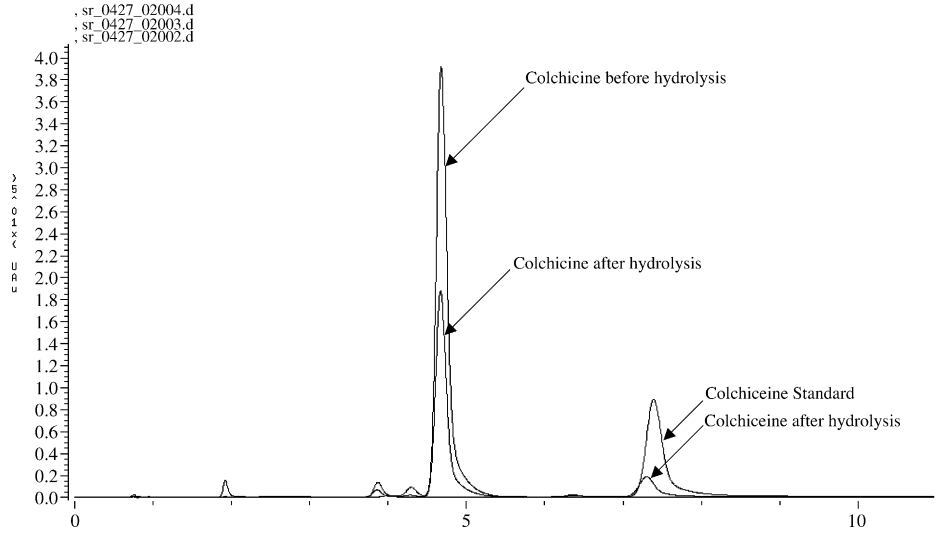


Fig. 5. Chromatogram of colchicine standard obtained on 100 mm × 4.6 mm Chromolith® RP-18e column before and after acidic hydrolysis (without addition of EDTA to mobile phase). For conditions see text.

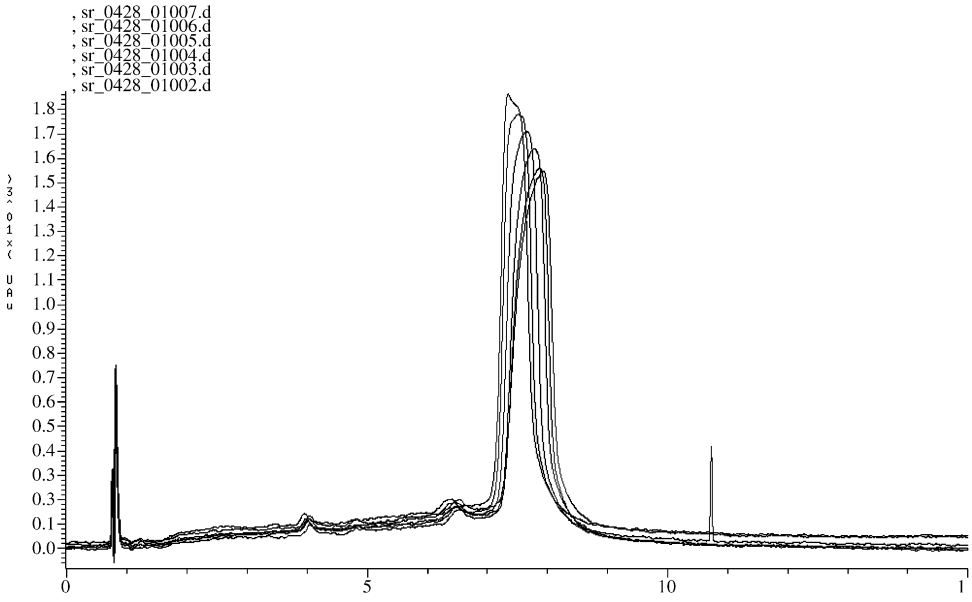


Fig. 6. Deterioration of peak-shape of colchiceine standard during repeated injections without addition of EDTA to mobile phase.

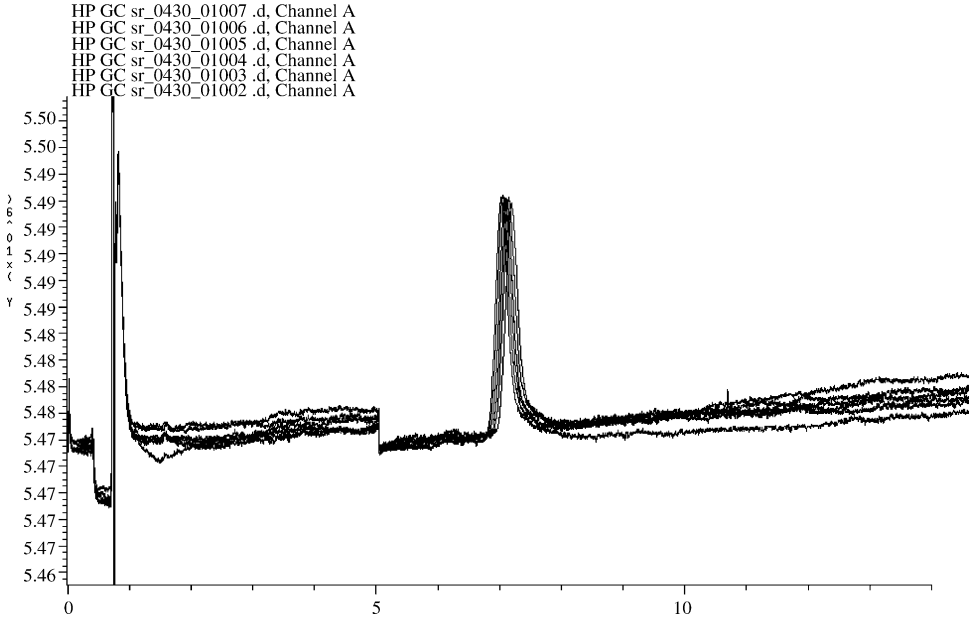


Fig. 7. Improved peak-shape of colchiceine standard during repeated injections after addition of 0.1 mM EDTA to mobile phase.

shape (Fig. 6). After addition of 0.1 mM sodium-EDTA to the solvent and the mobile phase, the peak-shape for repetitive injections could markedly be improved (Fig. 7).

5. Optimization of the mobile phase pH

For application of the HPLC method to analysis of Colchicum dry extract preparation or formulations, the chromatographic conditions had to be adapted systematically. Since dependence of retention time of colchiceine and demecolcine on the pH value of the mobile phase was expected, the pH was varied, keeping the percentage of methanol constant at 25% (v/v). Optimum conditions were calculated by the Drylab® Software. The chromatograms shown were obtained at pH values 3.0, 5.5, 6.0 and 6.8 (Figs. 8 and 9). A very complex pattern for resolution versus pH was predicted by the software (Fig. 10).

As expected from theory, the retention time of the basic compound demecolcine (approx. $pK_a = 8$) increases with increase of the pH value with deterioration of the peak shape whereas the retention time of the acidic colchiceine (approx. $pK_a = 4$) decreases. Colchicine and lumicolchicne stay almost unaffected by change of the pH value in the mobile phase.

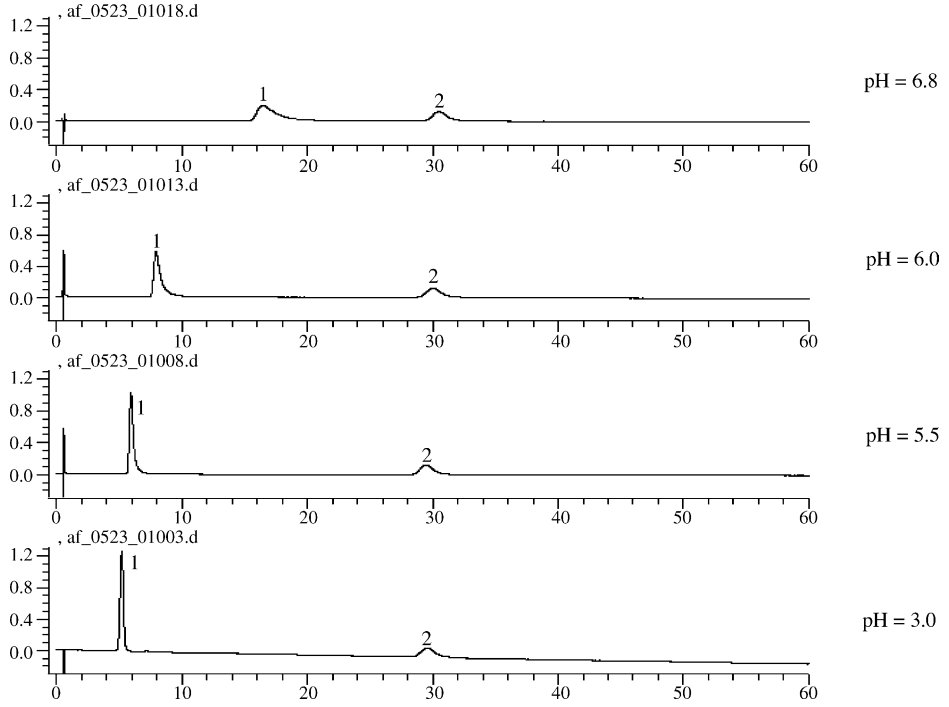


Fig. 8. Chromatogram obtained with standards of demecolcine (1) and γ-lumicolchicine at pH 3.0, 5.5, 6.0 and 6.8 with 25% (v/v) methanol in mobile phase.

Coelution of colchiceine and lumicolchicine was observed within a wide range, but maximum resolution could be verified for a pH of about 6.0 (Fig. 11).

The pH range from 3 to 5 was not attractive for separation due to worse resolution for colchicines and related alkaloids.

6. Optimization of mobile phase composition

In the next series of trials, the percentage of methanol was varied, keeping the pH constant. To calculate optimum conditions the results were entered into Drylab® software. Chromatograms are shown in Fig. 12. Maximum separa-

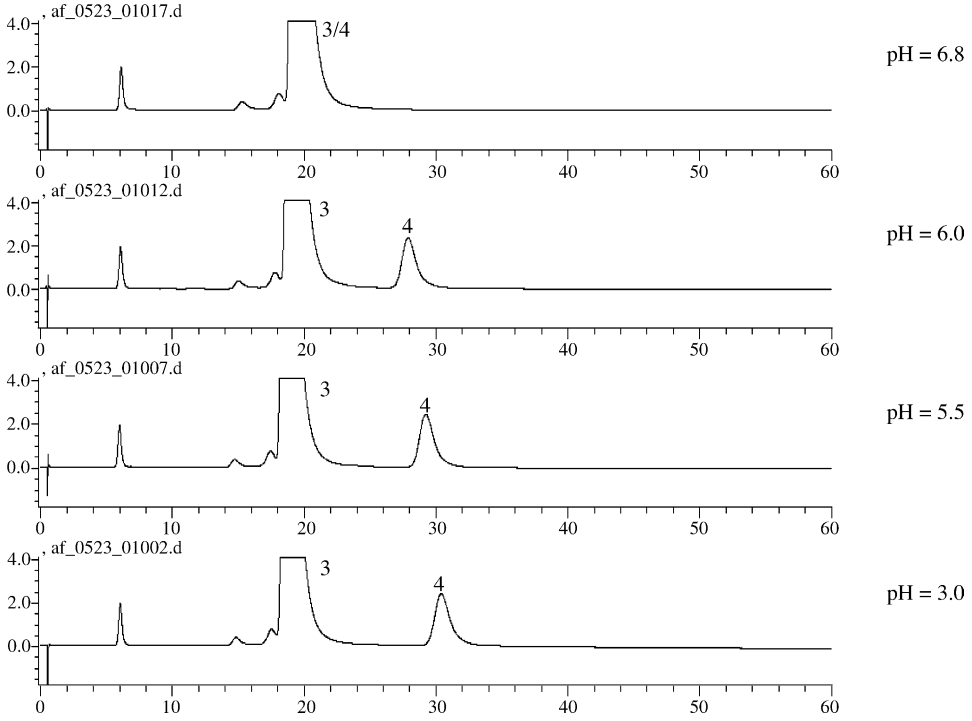


Fig. 9. Chromatogram obtained with standards of colchicine (3) and colchiceine (4) at pH 3.0, 5.5, 6.0 and 6.8 with 25% (v/v) methanol in mobile phase.

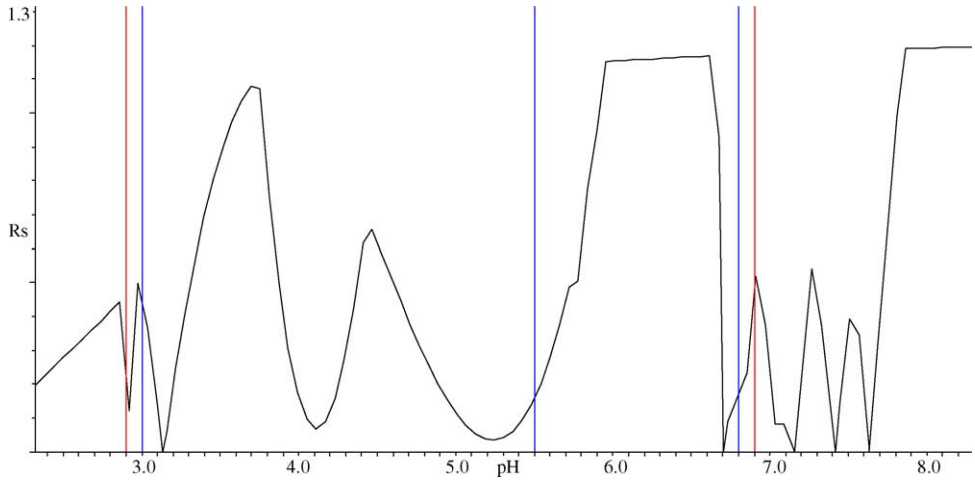


Fig. 10. Diagram obtained from Drylab® optimization showing dependence of resolution Rs on pH value of mobile phase.

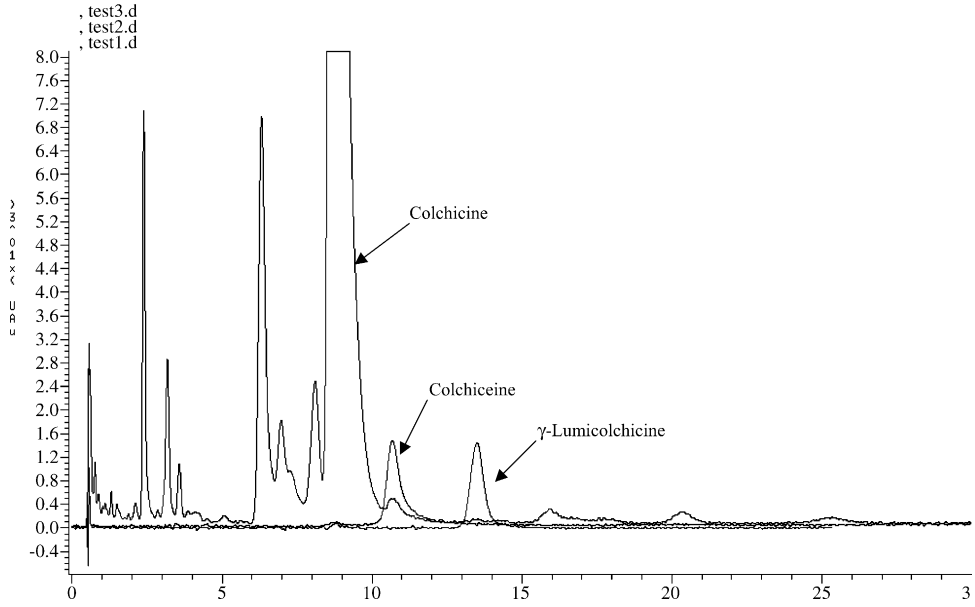


Fig. 11. Chromatogram obtained with standards and solution of Colchicum dry extract at pH 6.0, 28% (v/v) methanol.

tion was predicted within a narrow range of 25–28% (v/v) of methanol (Fig. 13).

Summarizing, the conditions for separation were adjusted to 27% methanol and 73% (v/v) of phosphate buffer, pH 6.0. The flow rate was adjusted to 3.0 ml/min, resulting in a back pressure of 14 MPa.

7. Optimization of concentration of sodium-EDTA

Taking the optimized parameters for separation, the influence of the sodium-EDTA concentration on the peak area of colchicines, colchiceine, demecolcine and γ-lumicolchicine at three different concentration levels was investigated systematically by adjusting the concentration of sodium-EDTA in the mobile phase to 0.1 (Fig. 12), 2, 3 (Fig. 14) and 4 mM. The results are shown in Table 3. Whereas a slight increase of peak area is observed for colchicine, demecolcine and γ-lumicolchicine between 2 and 3 mM, the peak area of colchiceine increases markedly and peak shape improves significantly thus lowering markedly the limit of detection.

Taking these parameters, a solution of colchicines reference standard was hydrolyzed again and peak areas were compared. The results are shown in Table 4.

This time, the sum of all peak areas was much closer before and after hydrolysis showing about 88% mass balance.

Besides colchiceine, two additional peaks were formed with a relative retention time of 0.35 and 0.44. Two minor peaks with a relative retention time of 0.8 and 0.9, respectively, degraded during hydrolysis (Fig. 15).

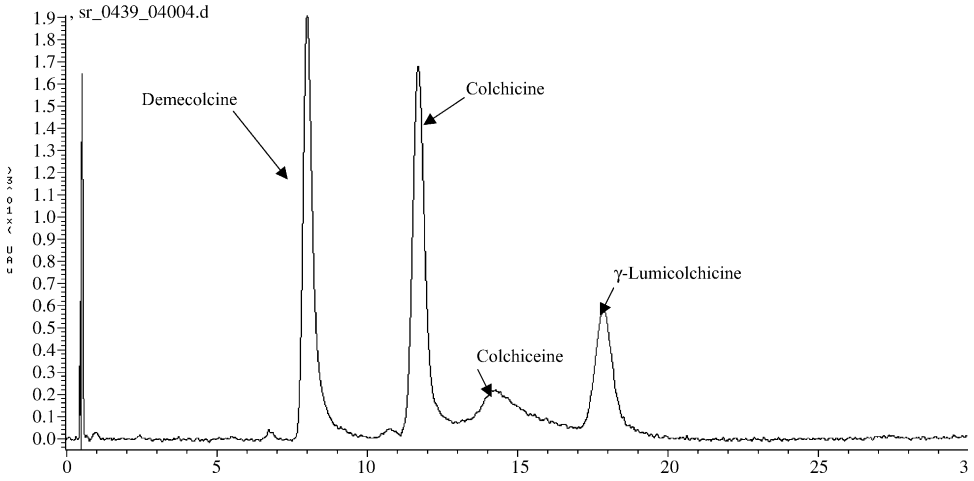


Fig. 12. Chromatogram obtained with a standard mixture of demecolcine, colchicines, colchiceine and γ-lumicolchicine with 0.1 mM EDTA in mobile phase.

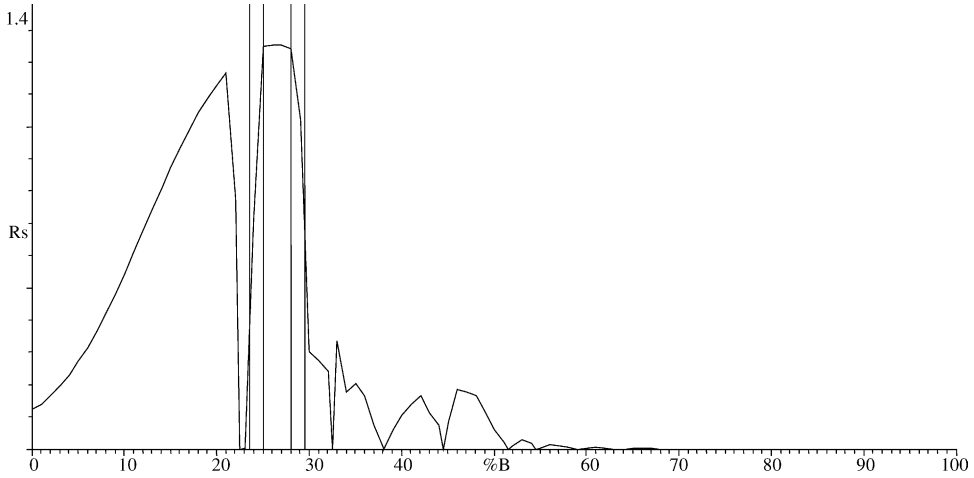


Fig. 13. Diagram obtained from Drylab® optimization showing dependence of resolution Rs on % (v/v) methanol in mobile phase.

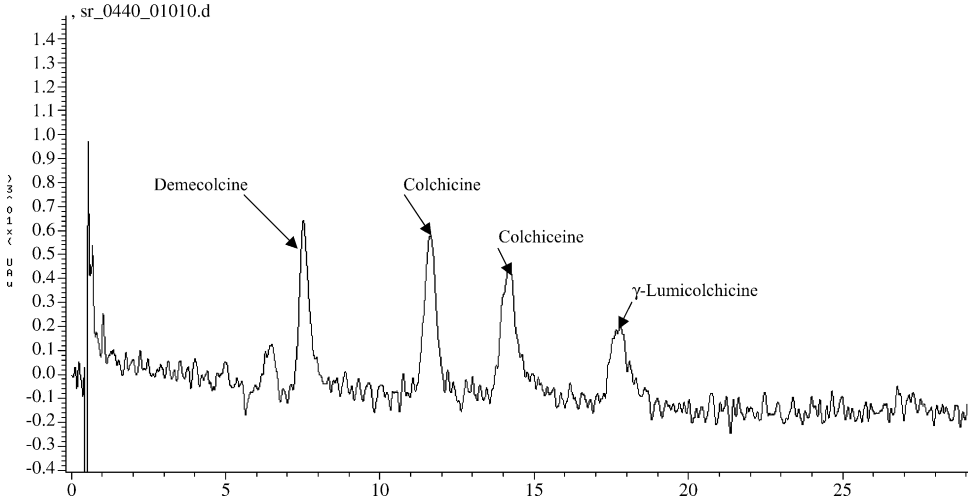


Fig. 14. Chromatogram obtained with a diluted standard mixture 0.05% (w/w) of demecolcine, colchicines, colchiceine and γ-lumicolchicine with 3 mM EDTA in mobile phase.

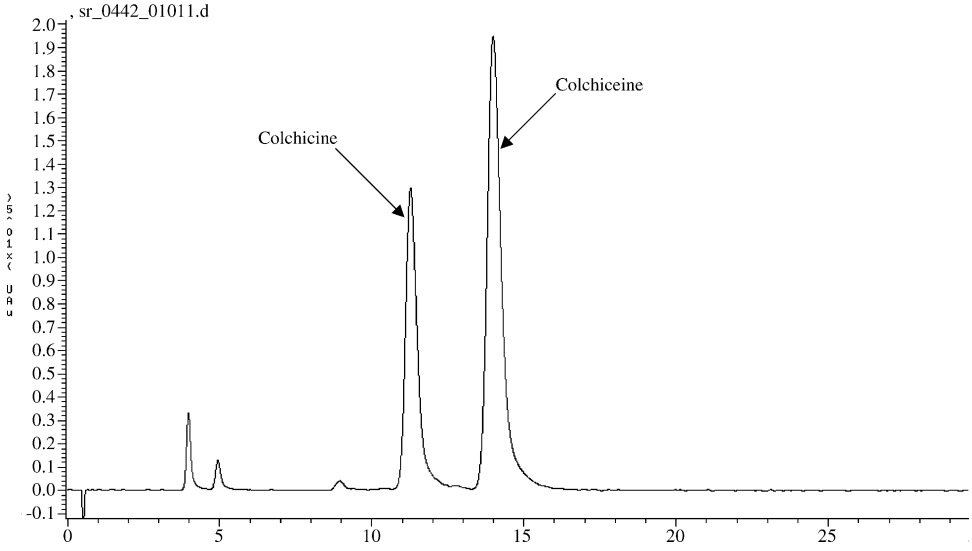


Fig. 15. Chromatogram obtained after hydrolysis of colchicine standard under optimized chromatographic conditions.

Table 3
Peak area for standards obtained vs. concentration of EDTA in mobile phase

	Concentration EDTA (mM)			
	0.1	2	3	4
0.05%				
Demecolcine	23589	15199	16501	16763
Colchicine	23468	18980	19847	21634
Colchiceine	5064	16990	20125	25907
γ-Lumicolchicine	11138	11414	13520	14633
0.10%				
Demecolcine	48302	31622	33673	35288
Colchicine	47915	38746	41413	46944
Colchiceine	11371	36213	40685	48502
γ-Lumicolchicine	23328	21307	26722	29921
0.25%				
Demecolcine	125796	81320	89586	102577
Colchicine	126198	99531	109310	121931
Colchiceine	57126	103744	114565	133535
γ-Lumicolchicine	60349	58479	65334	74906

8. Method validation

To test the suitability of the method for impurity profiling, the limit of quantitation was determined based on calculation of the signal-to-noise ratio for repetitive injections of a standard solution containing colchicine, colchiceine, demecolcine and γ-lumicolchicine at a concentration of 0.05 and 0.025% (w/w). The resulting chromatogram (0.05% (w/w)) is shown in Fig. 14. Based on the results which are tabulated in Table 5 the limit of quantitation was set at 0.025% (w/w) for demecolcine and colchicine

Table 5
Signal-to-noise ratio S/N with single peak area, standard deviation SD from six-fold injection and relative standard deviation RSD (%) for standard mixture containing 0.025 or 0.05% of each standard, respectively

	S/N	Area	SD	RSD (%)
0.05%				
Demecolcine	9.25	24351	1575	6.5
Colchicine	8.50	24740	2112	8.5
Colchiceine	6.75	19361	2229	11.5
γ-Lumicolchicine	4.25	11897	2100	17.5
0.025%				
Demecolcine	6.00	12057	886	7.3
Colchicine	5.33	13144	1067	8.1
Colchiceine	2.67	9701	1972	20.0
γ-Lumicolchicine	–	5799	2078	35.8

Table 4
Peak area and area% obtained for colchicines and colchiceine before and after hydrolysis of a colchicines standard solution on 100 mm × 4.6 mm Chromolith® RP-18e column after addition of EDTA

	RT	RRT	Before hydrolysis		After hydrolysis	
			Area	Area%	Area	Area%
	3.97	0.35	–	–	351956	3.3563
	4.93	0.44	–	–	161035	1.5354
	9.01	0.80	266548	2.224	74450	0.7102
	10.45	0.90	215296	1.7951	–	–
Colchicine	11.22	1.00	11510501	95.9826	3460398	32.9998
Colchiceine	13.95	1.24	–	–	6427198	61.2915
Sum			11992345	100.0	10475037	99.9

For conditions see text.

Table 6
Parameter estimates for regression lines calculated for colchicine and related alkaloids

Compound	γ-Lumicolchicine	Colchiceine	Demecolcine	Colchicine	
Range (%)	0.1–8.0	0.3–3.0	0.3–5.0	0.1–5.0	50–150
Range (μg/ml)	0.18–14.6	0.62–6.22	0.66–9.9	0.15–7.63	76.3–228.9
R^2	0.999	0.9994	0.9999	0.9999	0.9994
y-axis intercept	415.9	−1119.8	−1542.86	902.09	−13164
Standard error	1537.9	1296.7	886.6	663.5	73956
Slope	22968	34456	42183	55566	57059
Standard error	173.1	324.4	140.3	132.2	450.2
Root mean square error	3737.89	2346.85	1750.18	1194	87571

and approx. 0.05% (w/w) for colchiceine based on the relative standard deviation <10%, and 0.1% (w/w) for γ-lumicolchicine, based on the signal-to-noise ratio of 10:1. Results for parameter estimates of the regression-lines calculated for colchicines and the related alkaloids are tabulated in Table 6.

For repeatability, the coefficient of variation for a six-fold determination was 0.83% for assaying of colchicine content at a level corresponding to 2.25%, 3.3% for colchiceine at a level of 0.065% and 3.0% for γ-lumicolchicine at a level of 0.06%.

9. Conclusion

A highly selective HPLC method was developed for assaying of related alkaloids and potential degradation products of colchicine including colchiceine. No complexation or derivatization is required. The range validated permits quantification of colchiceine down to approx. 0.5 μg/ml, corresponding to 0.05% in the dry extract preparation. This method can be applied to dry extract preparations and tablets for release- and stability testing.

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