Capillary Electromigration Techniques for the Quantitative Analysis of Colchicine[†]

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Abstract. The separation and UV absorbance detection of colchicine by three different capillary electrophoretic methods is described. Colchicine is known as a neutral compound, being able to be determined by electrokinetic chromatography. For the first time, a non-aqueous capillary electrophoretic method is described, based on the electromigration of ionized colchicine induced by 10 mM $HClO_4$ in a mixture of methanol:acetonitrile (1:2, *v/v*) containing 60 mM ammonium formiate, opening up new perspectives in the trace analysis of the highly toxic drug from clinical and food samples using online coupling of the CE system to a mass spectrometer. For the quantitative assessment of colchicine content from meadow saffron (*Colchicum autumnale* L.) seeds and 1 mg colchicine tablets, a simple, quick and sensitive micellar electrokinetic chromatographic method was developed and fully validated according to ICH guidelines in terms of selectivity, linearity, accuracy, intermediate precision and limits of detection and quantification (95.2 ng mL^{-1}). In order to further improve the detection limits, allowing the analysis of trace levels of colchicine in biosamples and food products, an on-column preconcentration using sweeping-MEKC was investigated. Linearity of response was observed on 10–160 ng mL^{-1} colchicine, with an estimated detection limit of around 3 ng mL^{-1} colchicine. By further improving the affinity of the separation vector towards colchicine this limit could be further decreased. Preliminary application of the method for the detection of trace amounts of colchicine spiked in non-fat milk and human urine (10 ng mL^{-1}) shows encouraging results. (doi: 10.5562/cca1765)

Keywords: colchicine, capillary electrophoresis, NACE, MEKC, sweeping-MEKC

Abbreviations: COL – colchicine; IS – internal standard; CE – capillary electrophoresis; DAD – diode array detector; NACE – non-aqueous capillary electrophoresis; EOF – electroosmotic flow; SDS – sodium dodecyl sulfate; CZE – capillary zone electrophoresis; MEKC – micellar electrokinetic chromatography; MEEKC – microemulsion electrokinetic chromatography.

INTRODUCTION

Colchicine (Figure 1a), a toxic proto-alkaloid of meadow saffron (*Colchicum autumnale* L., *Colchicaceae*) and flame lily (*Gloriosa superba*, *Colchicaceae*), is one of the oldest drugs used by the human kind, mainly in the treatment of autoinflammatory diseases and gout.[1] Colchicine has anti-inflammatory, anti-mitotic, and anti-fibrotic activity.[2] It also finds applications in various other diseases like pseudogout, familial Mediterranean fever, cirrhosis of the liver and bile, and amyloidosis.[3] Its mechanism of action is due to the specific interaction with tubulin, perturbing the assembly dynamics of microtubules.[4] Therefore, besides its therapeutic value, its molecule is also an important tool in areas like plant amelioration (haploid plants), genetics and drug development.[4–6] Hence, colchicine is a lead compound for the development of other potent anti-cancer drugs.

Colchicine can also be ingested with non-therapeutic purposes with other food products, innocuously (*e.g.*, in milk, via cattle and sheep grazing on alkaloid-producing plants, or veterinary use of colchicine on cattle, accidental poisoning)[7–9] or insidiously (*e.g.*, intentional poisoning).[10,11]

Due to its genotoxicity, even at low concentrations, it could not be established for humans an acceptable daily intake (ADI) of colchicine,[12] and consequently it has been prohibited in livestock farming (Annex IV of European Council Regulation (EEC) No. 2377/90), because its residues in foodstuffs of animal origin

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constitute a hazard to consumers health, whatever the limit. Moreover, the US Centers for Disease Control and Prevention included it on the list of biotoxins of concern in case of a chemical emergency.[13]

Many studies have dealt with the development of separation techniques for the determination of colchicine, based on TLC-densitometry,[14,15] high performance liquid chromatography using UV [16–22] or MS detection.[8,23–27] Nevertheless, highly sensitive electrochemical methods of quantification are also described,[28–32] but their lack of selectivity hinders the analysis of complex matrixes (*i.e.* plant extracts, biological fluids and foodstuffs).

Comparatively, only a few papers have been dealing with the analysis of colchicine by electromigration techniques,[13,33–35] amongst which two describing the use of microchip micellar electrokinetic chromatography. One of the major drawbacks of the described electrophoretic techniques, applied to clinical drug monitoring of colchicine, toxicological screening and food analysis, is their relatively high limit of detection and quantification (the lowest theoretical LOD reported is 360 ng mL^{-1}).

As reported in literature, after a 2 mg single dose oral administration of colchicine the maximum mean plasmatic concentration (c_{max}) reached is around 0.6 ng mL^{-1}.[36] Because of an enterohepatic cycle the bile/plasma concentration ratio of colchicine can rise up to 47.[37] With therapeutic daily doses of 0.5–2.0 mg, plasma concentrations average 1 ng mL^{-1}. Anything between 5–160 ng mL^{-1} colchicine in blood, falls into the toxic or lethal levels as it has been reported on several fatal suicide cases, without being a clear correlation with the ingested drug.[38] Moreover, while colchicine plasma levels have been retrospectively determined following a lethal ingestion, no clear correlation between plasma levels and outcome has been established, although death has followed a single oral ingestion of as little as 8 mg of colchicine.[39]

Considering the above mentioned facts, the analysis of trace levels of colchicine is imposed from a great number and variety of samples encountered in the fields of pharmaceutical, clinical, forensic, food and feedstuff industry. This demand puts an extra pressure in developing cost-effective, fast, sensitive, high performance and high throughput methods of analysis of minute amounts of the proto-alkaloid.

EXPERIMENTAL

Material and Methods

Chemicals and Reagents

Analytical grade colchicine (98 %) for biochemistry, sodium hydroxide, hydrochloric acid (37 % *w/w*), acetonitrile, dichloromethane, isopropanol, acetone, petroleum ether, ethanol, methanol, SDS were all purchased from Merck, Germany. Boric acid, phosphoric acid (85 % *w/w*), ammonium formate (> 99 %), *p*-xylenolsulfonephthalein were all purchased from Sigma Aldrich. All mentioned reagents were used without any further treatment. Ultrapure water (18.2 MΩ, Barnstead EASYPure ROdi) was used for the preparation of buffers and related aqueous solutions. Aqueous buffers at various pHs were prepared by dissolving the suitable electrolyte (phosphoric acid, boric acid) in ultrapure water and adjusting with NaOH (1 M) or HCl (1 M) with the aid of a pH-meter (Consort, Belgium).

During sample preparation high purity nitrogen (5.0 purity, Linde Gaz Romania) was used for solvent evaporation.

Reference and Sample Preparation

The stock solutions of colchicine (1 mg mL^{-1}) were freshly prepared in methanol each day and were stored in the dark. Each day aliquots of the stock solution were diluted in 10 mL volumetric flasks with water or organic solvent (NACE).

Due to colchicine's neutral behavior (pK_a = 1.85 in water)[40] and high solubility in water (measured logP = 1.30 [41], estimated logP = 1.32 CSPredict software, ChemSilico LLC [42]) its sample extraction differ from those usually applied to alkaloids.[43] Moreover, the nature of the sample matrix strongly affects the extraction technique.

Vegetal samples (meadow saffron seeds) were prepared by Soxhlet extraction during 150 minutes using 95 % ethanol with an extraction ratio of 2.25 % *m/v* seeds/solvent. Before extraction, the seeds were defatted using petroleum ether, dried at room temperature and pulverized.

The pharmaceutical samples were tablets with 1mg declared amount of colchicine (Biofarm SA, Bucharest, Romania). After the determination of the average weight of tablets (0.1019 g), 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 10 min and the suspension was then centrifuged for 3 min at 5000 rpm.
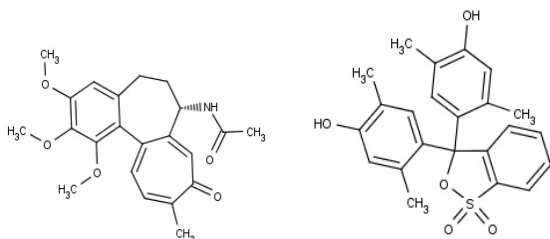
Figure 1. Structures of colchicines (a) and *p*-xylenolsulfophthaleine (b).

The obtained extract or clear supernatant was appropriately diluted with the running buffer or water and injected into the CE system for further MEKC analysis.

Spiked skim (1.5 % fat) milk samples (10 ng mL^{-1} colchicine) purchased from the local store (3 mL) were previously treated with HCl 37 % (12 µL HCl 37 % *w/w* to 1 mL milk) in order to free the bound colchicine and eliminate the most of fat and proteins.[44] After centrifugation (5 min at 5000 rpm) the supernatant was extracted by liquid-liquid extraction using dichloromethane:isopropanol (95:5, *v/v*) in a ratio of 3:10 sample/organic solvent mixture. The obtained organic phase was evaporated over dry N$_2$ and the residue was dissolved in an appropriate volume of micelle-free high conductivity buffer (100 mM phosphate buffer pH = 4.0), added to match the conductivity of the running buffer.

Spiked human urine samples (10 ng mL^{-1} colchicine) from healthy voluntaries were simply diluted with a micelle-free buffer (20 mM phosphate buffer pH = 4.0), and filtered before being injected into the CE system.

In sweeping-MEKC separations, each sample was spiked with 80 ng mL^{-1} *p*-xylenolsulfonephthalein as an internal standard.

Instrumentation and Method Description

Method development and analytical measurements were carried out on an Agilent 3D CE system (Agilent Technologies, Waldbronn, Germany). The CE system was equipped with a diode-array detector, the capillary being thermostated by air-cooling. Data acquisition was carried out by 3D-CE Chemstation Rev. A.08.03(843). Detection wavelengths in all cases were 243 and 350 nm, bandwidth of 30 nm. Both CZE and MEKC were performed using bare fused-silica capillaries (50 µm I.D., *L* = 64.5 cm,
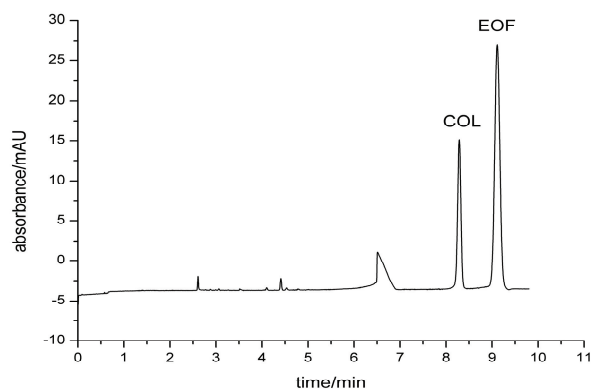
l = 56 cm, with extended light path (B.F = 3), Agilent Technologies, Germany).

The new capillary was pre-conditioned prior to use by flushing successively with 1.0 M sodium hydroxide for 20 min, 0.1 M sodium hydroxide for 20 min, de-ionized water for 20 min, and running buffer for 20 min.

At the beginning of the day the capillary was pre-conditioned with 0.1 M NaOH, water and running buffer each for 10 minutes in regular sequence. Between runs the capillary was flushed with running buffer for 3 minutes. In case of NACE, the same pre-conditioning protocol was applied, employing methanolic solutions of NaOH (1 M and 0.1 M) and pure methanol.

For aqueous CZE separations 25 mM phosphate buffer (pH = 2.50 and pH = 1.30 adjusted with HCl 1 M) and for MEKC 10 mM borate buffer (pH = 9.30) with 25 mM sodium dodecylsulphate with no organic modifier was used. Prior the analysis, all buffers and samples were passed through a 0.45 µm pore-sized syringe filter (SPARTAN 13, Whatman GmbH, Germany). Samples were loaded by hydrodynamic injection (250 mbar·s). Throughout all the analysis the capillary was thermostated at 20 °C (if not otherwise mentioned), applying a separation potential of +30 kV (465 V/cm) (if not otherwise mentioned) with a short linear ramp between 0 to 0.3 minutes.

Sweeping-MEKC was performed at 50 °C in near-zero EOF conditions created by a 5 minute flush of the capillary with HCl 0.1 M before every 8th–10th analysis. An 88.5 cm long (80 cm effective length, i.d. 50 µm, Polymicro Technologies, Germany) uncoated fused silica capillary with a BGE of phosphate buffer (20 mM, pH = 4) containing 3% (*v/v*) acetonitrile and 50 mM SDS. Samples were injected at the cathode by pressure (washing mode, 930 mbar, 36 s) and analyzed at –30 kV (reversed polarity). Throughout the separation a linear gradient (0–10 min) of 10 mbar pressure was applied from the inlet-end of the capillary.

RESULTS AND DISCUSSION

Aqueous and Non-aqueous Capillary Zone Electrophoresis

Unlike other alkaloids, colchicine behaves as a neutral compound, it is highly soluble in water, and regardless of the pH of the aqueous buffer it migrates with the electroosmotic flow. Using 25 mM phosphate buffer (pH = 2.50) a single peak at 4.47 minutes is detected corresponding to the co-eluted peaks of colchicine and solvent. Further lowering the pH of the running buffer to 1.30, the ionization of colchicine is still not observed and the co-eluted peak shows a higher migration time (12 minutes) due the further decrease of the EOF mobility.

Figure 2. Non-aqueous capillary zone electrophoresis using 10 mM HClO$_4$ with 60 mM HCOONH$_4$ in methanol : acetonitrile (1:2, *v/v*), bare fused silica capillary (*L* = 64.5 cm, i.d. 50 µm), 20 kV, 25 °C, hydrodynamic injection, detection 243 nm; Sample: colchicine and acetone (as a neutral EOF marker) in the running buffer.

Table 1. Separation parameters of colchicine by micellar electrokinetic chromatography without and with on column preconcentration

	MEKC	Sweeping-MEKC
Capillary	Bare fused silica with extended light path (B.F.= 3) $L = 64.5$ cm, $l = 56$ cm, i.d. 50 µm	Bare fused silica $L = 88.5$ cm, $l = 80$ cm, i.d. 50 µm
Preconditioning in between runs	Flushing 3 minutes with the running buffer	
Running buffer	10 mM borate buffer (pH = 9.3) with 25 mM SDS	20 mM phosphate buffer (pH = 4) with 50 mM SDS and 3 % *v/v* acetonitrile
Temperature	20 °C	50 °C
Separation potential	30 kV (465 V/cm)	−30 kV (339 V/cm)
Separation pressure	–	10 mbar (0 – 10 minute linear gradient)
Sample injection (% of the total capillary volume)	Hydrodynamic 250 mbar·s (0.54 %; ~5.92 nL)	Hydrodynamic 33480 mbar·s (60.93 %; ~1059 nL)
Detection	243/30 nm and 350/30 nm	
Linear range	200 – 2000 ng mL^{-1}	10 – 160 ng mL^{-1}
Limit of detection	31.41 ng mL^{-1}	2.84 ng mL^{-1}
Colchicine's migration time	7.64 min (RSD = 2.5 %, $n = 10$)	10.73 min (RSD = 2.10 %, $n = 10$)
Total analysis time	11 min[a]	18 min[a]

[a] capillary preconditioning comprised.

The chemical and physical properties of organic solvents are different from that of water, which could change the pK_a value of the solute and the radius of its hydration shell, thus non-aqueous capillary electrophoresis (NACE) could be used to improve separation selectivity of compounds if they are difficult to be analyzed in aqueous systems. Since, the ratio of the protonated and neutral forms of a very weak basic drug in an acidic, but totally non-aqueous medium may be smaller; the extent of electroosmotic flow is therefore expected to have a significant influence on colchicine's apparent mobility in such a medium. The nature of non-aqueous solvents proves to be critical in NACE. These solvents having a large value of dielectric constant/viscosity (ε/η) ratio could give rise to appreciable electroosmotic mobilities. In this regard, acetonitrile, with a dielectric constant of 37.5 and a viscosity of 0.34 mPa s, has perhaps the most favourable ε/η value (110 mPa^{-1} s^{-1}), even higher than that for water (78.5 mPa^{-1} s^{-1}). Other organic solvents having high ε/η values are acetone (69 mPa^{-1} s^{-1}) and methanol (59 mPa^{-1} s^{-1}).[45] Since in pure acetonitrile the solubility of electrolytes can be troublesome, as solvent for non-aqueous capillary electrophoretic separations, a mixture of methanol-acetonitrile was considered. Furthermore, as described in the literature, the fastest EOF is given by a mixture of approximatively 25–30 %, *v/v* methanol in acetonitrile, containing amonium acetate as electrolyte.[45,46] Therefore, in the following NACE assays a mixture of 1:2 *v/v*, methanol in acetonitrile was used as solvent. Ammonium formate (60 mM) was the chosen as electrolyte, whereas several organic and inorganic acids were added into the running buffer with the aim of influencing the ionization of colchicine. The molecule of colchicine could not be ionized by formic acid and trifluoroacetic acid added to the running buffer, whereas perchloric acid, at a reasonably low concentration (10 mM) allowed the determination of colchicine by NACE-CZE. The different electrophoretic behavior of colchicine in the presence of perchloric acid compared with other strong acids was attributed to an ion pairing effect, instead of a simple protonation due to the added acids. Perchloric acid acts as a strong electrolyte even in pure acetonitrile and thus can be considered fully dissociated in the used running buffer. The electroosmotic mobility ($3.3 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) was determined by injecting acetone as a neutral EOF marker, the peaks being identified based on their known UV spectra (Figure 2).

Table 2. Linear regression parameters and its statistical evaluation

Parameters	200 – 2500 ng mL^{-1}, Injection 250 mbar·s ($n = 5$, $N = 15$)	Conditions
Correlation coefficient (r)	0.9990	
Intercept	−0.0196 ± 0.0034[a]	
Slope	0.000357 ± 0.000011[a]	
Cochran's test Comparative test of the homogeneity of variances	$C_{calc.} = 0.496$ C_{theor} (0.05;5;2) = 0.683	$C_{calc.} < C_{theor.}$
Fisher's test Significant slope	$F_{calc.} = 6516.26$ F_{theor} (0.05;1;13) = 4.67	$F_{calc.} > F_{theor.}$
Fisher's test Validity of regression	$F_{calc.} = -2.71$ F_{theor} (0.05;3;10) = 3.71	$F_{calc.} < F_{theor.}$

[a] standard deviation (n=3).

Even though the described NACE method represents an absolute novelty in the CE analysis of colchicine, with the standard UV detection setup, it still does not allow the analysis of minute amounts of protoalkaloid from biological samples.

Micellar Electrokinetic Chromatography

Method Development
A 10 mM boric acid adjusted to the desired pH with 1 M NaOH was selected as running buffer. Since, in the most common working pH range (pH = 2–12), colchicine's ionization in aqueous solutions can not be significantly influenced by the pH of the buffer, its value has been set at pH = 9.30, this pH offering a conveniently high EOF (5.88 · 10^{-4} cm^2 V^{-1} s^{-1}) and consequently shorter migration time of the solute.

Based on linear solvation energy relationships, surfactant concentration could influence retention behavior of uncharged solutes by changing the phase ratio without significantly affecting selectivity.[47] The selectivity offered by sodium dodecyl sulfate (strong hydrogen-bond acid) was appropriate and by increasing its concentration only results in general increase of retention and a decrease of UV detection sensitivity, without any improvement on separation. Therefore, a concentration of 25 mM SDS was chosen as the optimized condition, since both resolution and peak shape were acceptable. The final, separation parameters are summarized in Table 1.

MEKC Method Validation
The proposed method was validated according to ICH guidelines [48] in terms of specificity, linearity, accuracy and precision. Furthermore, as an estimation of the method's analytical performance, limits of detection and quantification were also calculated.

On each of the three consecutive days of validation, five calibration standards in the range of 200–2500 ng mL^{-1} colchicine were employed. Three sets of five control samples were also prepared from individual weightings and stored in dark. The standard and control samples were injected hydrodynamically at 250 mbar·s (~ 5.64 nL). The detection and quantification was done at 243 nm. The recorded average migration time for colchicine in the optimized separation conditions was 7.64 minutes, with a relative standard deviation in the range of 0.53–2.5 %.

Specificity. The specificity of the method was assessed based on the obtained migration times and peak purity analysis offered by the DAD detector. In case of the pharmaceutical sample analysis, the assay of placebo samples assured the lack of any interference.

Linearity. Using the corrected areas (= peak area / migration time) of colchicine's peaks recorded at 243 nm a five leveled regression curve was constructed. The re-

Table 3. Statistical evaluation of method's accuracy (*n* = 5, *N* = 15)

Parameters	200–2500 ng mL^{-1} Injection 250 mbar·s	
	Nominal/ ng mL^{-1}	Recovery/ %
	200	104.95
	400	97.00
	800	100.97
	1600	99.59
	2400	99.87
Mean recovery/%		100.47
Cochran's test ($C_{calc.} < C_{theor.}$)	$C_{calc.}$ = 0.496	$C_{theor.}(0.05;5;2)$ = 0.683
Fisher's test ($F_{calc.} < F_{theor.}$)	$F_{calc.}$ = 1.06	$F_{theor.}(0.05;4;10)$ = 3.48
Confidence interval (*p* = 0.05)		100.47 ± 2.84%

gression parameters and its statistical evaluation are presented in Table 2.

Accuracy. The method's accuracy was assessed on five independent control samples, prepared in three different days, being statistically evaluated by the comparative test of homogeneity of variances (Cochran's test) and the validity of average recovery (Fisher's test) (Table 3).

Precision. Intermediate precision was established based on the obtained recoveries of six independently weighted control samples at the lower limit of linear range (200 ng mL^{-1}) on three different series prepared in three different days. The obtained data was statistically evaluated, its values being summarized in Table 4.

Limits of Detection and Quantification. The limits of detection (LOD) and quantification (LOQ) were calculated as (3.3 σ/S) and (10 σ/S), respectively, where σ is the standard deviation of the intercepts and *S* is the slope of the calibration curve. The calculated LOD and LOQ were found to be 31.41 ng mL^{-1} and 95.20 ng mL^{-1}, respectively.

The validated MEKC method was successfully applied to the quantification of colchicine from meadow saffron (*Colchicum autumnale* L.) seeds and tablets
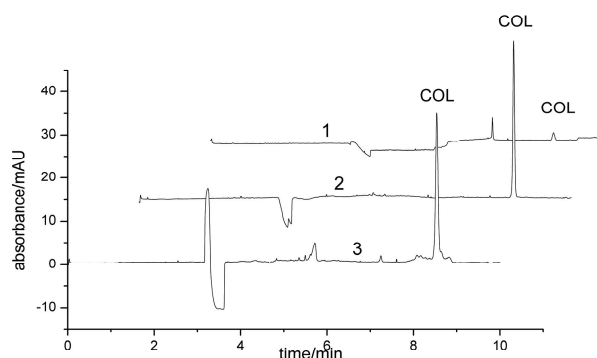
Table 4. Statistical evaluation of the intermediate precision (*k* = 3, *n* = 6, *N* = 18)

Parameters	200 ng mL^{-1}
Cochran's test Comparative test of the homogeneity of variances intra-group	$C_{calc.}$ = 0.472 $C_{theor.}(0.05;3;6)$ = 0.677 ($C_{calc.} < C_{theor.}$)
Intra-day precision (CVr)	4.84%
Inter-day precision (CVR)	6.35%

(1 mg colchicine, Biofarm S.A., Bucharest) (Figure 3). The sample extraction and pretreatment was carried out according to method described at Section 2.2. The colchicine content of Colchicum seeds and pharmaceutical sample content uniformity (per tablet) are summarized in Table 5, which are in compliance with the Romanian Pharmacopeia's normative (±10 %).[49]

Although the analytical performances of the presented MEKC method are remarkable, it is still limited to samples with a concentration of around 100 ng mL^{-1} colchicine. Therefore, pharmaceutical samples (tablets, injectable solutions) and plant extracts (aerial and underground parts) can be successfully analyzed, but is improper for the quantitative determination of trace amounts of the proto-alkaloid found in biological samples (urine, plasma) or as a residue in food and feedstuffs. Therefore, the efficiency of different on-column preconcentration techniques by sample stacking with reverse migrating vector (*i.e.* micelles)[50] in micellar electrokinetic chromatography was investigated.

In these conditions, from the cathodic end of the capillary, a large volume of micelle free sample (having a similar conductivity with the background electrolyte) is injected hydrodynamically. The capillary was previously preconditioned with the micellar running buffer, making sure that the EOF is completely suppressed. Both ends of the capillary are introduced into the micellar background solution and the separation voltage is applied with the negative polarity at the injection end. In the lack of a significant electroosmotic flow the negatively charged micelles on their way through the sample zone, towards the detection end of the capillary, sweep up the neutral analytes, stacking them in narrow bands and that are finally separated by normal MEKC. The pH of the sample and the running buffer must be kept the

Table 5. Colchicine determination through different electromigration techniques

Samples ($n = 3$)	Aqueous MEKC
Colchicine tablets, 1 mg Biofarm S.A. Bucharest (mg/tablet)	0.939 ± 0.005
Colchicum autumnale seeds, Soxhlet extraction (g/100 g seeds)	0.511 ± 0.012

lowest possible, avoiding the generation of unwanted EOF, without compromising the solubility of the surfactant. The effectiveness of the stacking is dependent on the analyte's affinity towards the micelles, the higher affinity the larger the extent of stacking.

Near-zero EOF conditions could be obtained by decreasing the zeta potential of the capillary's inner wall double-layer by an acid pretreatment. Due to the pH hysteresis effect, the re-equilibration of the surface charge on the fused silica appears to be a slow process and the silanol groups' dissociation may take several weeks at intermediate pH.[51,52] Therefore, the bare fused silica capillaries were flushed at the beginning of the day for 15 minutes by 0.1 M HCl and this acid pretreatment step has been repeated for 5 minutes at every 8th–10th analysis, assuring a near-zero EOF during all subsequent assays. The running buffer was set to pH = 4, its composition being optimized for the best signal to noise ratio and highest resolution of colchicine.

Since there is no electroosomotic flow capable of maintaining the "plug" flow in the bubble cell, using this separation technique, there is no boost of sensitivity offered by the capillaries with an extended light path. Instead of an axial compression of the separated fractions entering the detection cell, the solutes are diluted in the expanded capillary, resulting in lower signals compared with the ones obtained by the same length of a normal capillary. Therefore, for the highest sample load and separation efficiency, a long bare fused silica capillary ($L = 88.5$ cm, $l = 80$ cm, 50 μm i.d.) was used with a total inner volume of 1.737 μL.

Considering the highest gain in sensitivity, best resolution and the shortest analysis time, different compositions of the background electrolyte were tested, finally arriving to 50 mM SDS in 20 mM phosphate buffer (pH = 4) with a content of 3 % (v/v) acetonitrile. The optimum running parameters are presented in Table 1. The organic modifier was added to the running buffer for the improvement of resolution between the peaks of the added internal standard and colchicine.

Even though the injected sample volume in sweeping-MEKC is almost 180-fold higher than in simple MEKC, the gain in sensitivity is somewhat less (around 20 fold) due to the relatively high hydrophilicity of colchicine. The obtained lower limit of the linear range (10 ng mL^{-1}) is already the lowest ever reported for colchicine by an electromigration method.

Figure 3. MEKC electropherograms for the quantitative assay of colchicine from vegetal extracts and pharmaceutical samples; 10 mM borate buffer (pH = 9.30) with 25 mM SDS, bare fused silica capillary ($L = 64.5$ cm, i.d. 50 μm, B.F. = 3), 30 kV, 20 °C, hydrodynamic injection 250 mbar·s, detection 243 nm; 1 – colchicine tablets extract; 2 – standard colchicine (50 μg mL^{-1}); 3 – *Colchicum autumnale* L. seeds extract sample.

Table 6. Statistical evaluation of the two types of hydrodynamic sample injections in sweeping-MEKC

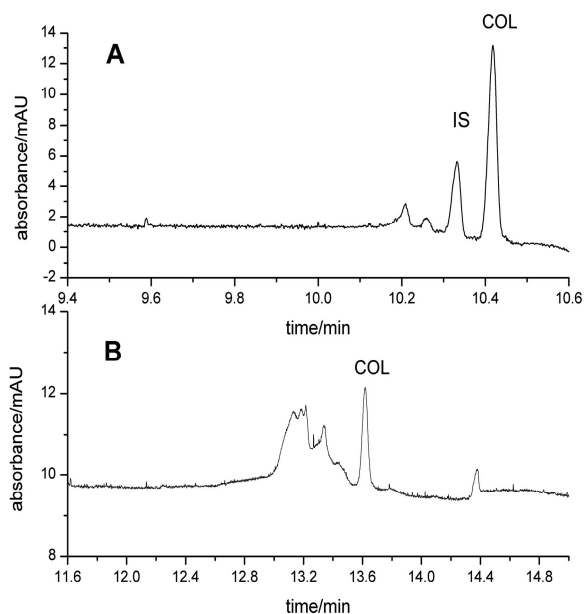| Hydrodynamic sample injection at 50 mbar for 670 s			Hydrodynamic sample injection by flushing at 930 mbar for 36 s, with the use of IS		
3 series of 5 levels of concentration (intra-day assays) 10 – 160 ng mL^{-1} colchicine					
Slope/ ng mL^{-1}	Intercept/ mAU min^{-1}	R^2	Slope/ ng mL^{-1}	Intercept/ mAU min^{-1}	R^2
0.0071454	0.048484	0.9963	0.012996	0.066698	0.9986
Average recovery/ %		96.4	Average recovery/ %		99.3
R.S.D. of recovery/ %		11.9	R.S.D. of recovery/ %		5.7
Comparative test of variances intra-group $C_{theor}(0,05;5;2) = 0,68$		$C_{calc} = 0.601$	Comparative test of variances intra-group $C_{theor}(0,05;5;2) = 0,68$		$C_{calc} = 0.629$
Confidence limits of the average recovery/ %		96.42 ± 6.04	Confidence limits of the average recovery/ %		99.35 ± 4.18

The long time taken (670 s) for the high sample volume load at the highest hydrodynamic pressure injection (50 mbar) allowed by the Agilent 3D-CE system can be overcame by flushing the capillary with the sample for 36 s with at 930 mbar. The evaluation of the obtained regression parameters by the two forms of sample injection demonstrated the lack of any statistically significant difference. However, for the assurance of the sample injection reproducibility and the method's accuracy, *p*-xylenolsulfonephthalein (*p*-xylenol blue, Figure 1b) as internal standard must be employed (Table 6). The internal standard was chosen according to its

Figure 4. Sweeping-MEKC electropherograms for the quantitative assay of colchicine from spiked skimmed milk (A) and healthy human urine (B); 20 mM phosphate buffer (pH = 4) with 50 mM SDS, bare fused silica capillary (*L* = 88.5 cm, *l* = 80 cm, i.d. 50 μm), –30 kV, 10 mbar pressure linear gradient 0 – 10 min, hydrodynamic injection 33480 mbar·s, detection 243/30 nm; IS – *p*-xylenolsulfonephthalein.

matching polarity with colchicine and its pK_a value (pK_a = 2.0), making sure that it is not a naturally occurring compound in the studied samples (vegetal extracts, urine and milk). Therefore, *p*-xylenolsulfonephthalein is in its non-dissociated form at the pH of the running buffer and is swept along by the SDS micelles similarily as colchicine.

For the further improvement of the analysis time, when most of the sample focalization has been carried out, an external pressure was applied on inlet end of the capillary (linear gradient to 10 mbar, between 0–10 minutes). Therefore, the full analysis time of colchicine using on-column sample preconcentration by Sweeping-MEKC was achieved in less than 18 minutes, with an estimated limit of detection of 2.84 ng mL^{-1}.

The developed sweeping-MEKC method was tested for the determination of colchicine from spiked urine and skimmed milk samples (10 ng mL^{-1} colchicine) (Figure 4). Using the given parameters, none of the real samples of skimmed milk (1.5 % fat) purchased from the local store contained traces of colchicine. Furthermore, urine of healthy voluntaries was spiked with standard colchicine for the assessment of recovery (96.3–101.4 %, *n* = 5). In function of the nature of the sample matrix and the used pre-treatment changes in colchicine's migration times are observed, therefore regression must be assessed by using calibration samples prepared in the analyzed matrix free of colchicine.

CONCLUSION

The performance, perspectives and limitations of three different electromigration techniques for the quantitative determination of colchicine from vegetal, pharmaceutical, food and biological fluid samples was evaluated.

For the first time, the separation of colchicine as a charged molecule was described employing non-

aqueous capillary electrophoresis. This technique opens up new perspectives in the trace analysis of the highly toxic drug from clinical and food samples; using volatile buffer additives, without the need of any pseudo-stationary phase (surfactants in MEKC) capable of inducing strong ion-suppression, therefore allowing the online coupling of the CE system to a mass spectrometer.

A validated, sensitive method by micellar electrokinetic chromatography was developed for the quantitation of colchicine from meadow saffron seeds (*Colchicum autumnale* L.) collected from the surroundings of Cluj-Napoca, Romania, the main natural source of the proto-alkaloid. Furthermore, passing a full validation, it proves to be a valuable tool for the industrial pharmacy in the quality control and content uniformity assays.

However, the main drawback of the proposed MEKC method as well as of those few capillary and microchip electrophoretic methods presented in the literature is their limited sensitivity, which hinders their use in the in clinical drug monitoring, toxicological screening and food analysis (residues). Therefore, developing a fast and highly sensitive electromigration technique with an on-column preconcentration was attempted, using sweeping micellar electrokinetic chromatography. Even though the preconcentration efficiency is not at its maximum value using SDS micelles, the preliminary results show a limit of detection of around 3 ng mL^{-1} colchicine, the lowest ever reported by capillary electrophoresis according to our knowledge. By further improving the retention factor of the separation vector (mixed-micelles or microemulsion) this limit could be further decreased, allowing the quantification of trace levels of colchicine in biological fluids or food products. Preliminary application of the method for the detection of trace amounts of colchicine spiked in skim milk and human urine (10 ng mL^{-1}) shows encouraging results.

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