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# Fingerprint analysis and synthetic adulterant search in *Hedera helix* formulations by capillary electrophoresis

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A high-performance capillary electrophoretic (CE) method has been developed for obtaining electropherograms of various extracts and the commercial formulation (fingerprints) of *Hedera helix* L used in Argentina as a cough's treatment. Also, a capillary zone electrophoresis (CZE) method was developed for the search, identification and determination of some possible adulterants. These likely adulterants are common synthetic drugs used in respiratory diseases (antitussive, decongestant and bronchodilator agents). Under optimum conditions, the analytes (ephedrine, codeine, diphenhydramine and constituents of *H. helix* formulations) were separated within less than 10 min in 20 mM sodium tetraborate buffer (pH 9.0). The present procedure was validated with respect to selectivity, linearity range, limits of detection (LOD) and quantification (LOQ), precision (repeatability and intermediate precision), solution stability and accuracy; the results obtained were satisfactory. Good linearity was obtained over two orders of magnitude and detection limits ($S/N = 3$) were better than 1.2 μg $ml^{-1}$ for all analytes. The CE methodology was successfully applied to the search and subsequent determination of ephedrine, codeine and diphenhydramine in *H. helix* extracts and its phytopharmaceutical products.

## 1. Introduction

Analyzing a herbal product for the presence of chemical markers known to be present in the preparation is a common method used in industry. Chemical fingerprinting is an additional method that can be taken into account in order to confirm or deny that a plant material is being used for the manufacture of a product (Guidelines for the Assessment of Herbal Medicines 1991; Rauchensteiner et al. 2005).

Substitution or adulteration with more toxic herbs may occur when the herb is incorrectly identified, or when a cheaper herb is supplied to replace a safer, more expensive one. Also, the addition of synthetic therapeutic substances to herbal medicines (HM) has been reported on many occasions and has been a public health concern for several years (Lau et al. 2003). Several factors may contribute to adverse effects of HM. These include consumer misuse, manufacturer abuse and contraindication, hypersensitivity and/or drug interactions. Manufacturer abuse covers the spiking of plant material and/or its derived products with synthetic substances of similar activity (Izzo et al. 2002).

*Hedera helix* L. is a climbing plant, native to Europe. The chemical constituents isolated from this plant are flavonoids, saponins, polyphenol acids, alkaloid traces, phytosterols and sesquiterpenes (Gulcin et al. 2004; Gepdiremen et al. 2005). This herb has been used because of its circulatory and expectorant properties (de Medeiros et al. 2000; Facino et al. 1995; Hofmann et al. 2003). In Argentina antitussive and expectorant syrups based on this plant are marketed. Therefore, an economical, rapid and appropriate method for the detection and assay of adulterated *Hedera helix* based formulations is needed.

The aim of this study is to develop characteristic fingerprints of *H. helix* derived products using capillary electrophoresis (CE) for recognizing and identifying marker compounds. Also, an analytical methodology for determining ephedrine, codeine and diphenhydramine in these products was developed and validated.

## 2. Investigations and results

### 2.1. *Fingerprint analysis*

The effect of buffer pH was investigated under acid and alkaline conditions. The migration behaviour of *Hedera helix* constituents and its dependence on the pH of the buffer was investigated with (a) sodium acetate buffer at pH from 4.0 to 6.0; (b) sodium tetraborate buffer at pH from 8.0 to 10.5; (c) sodium phosphate buffer at pH from 6.0 to 8.5.

The number of eluted peaks increased at lower pHs and major active components were separated more effectively with shorter times of analysis at higher pHs. As a result, two different pHs which had a clear effect on resolution

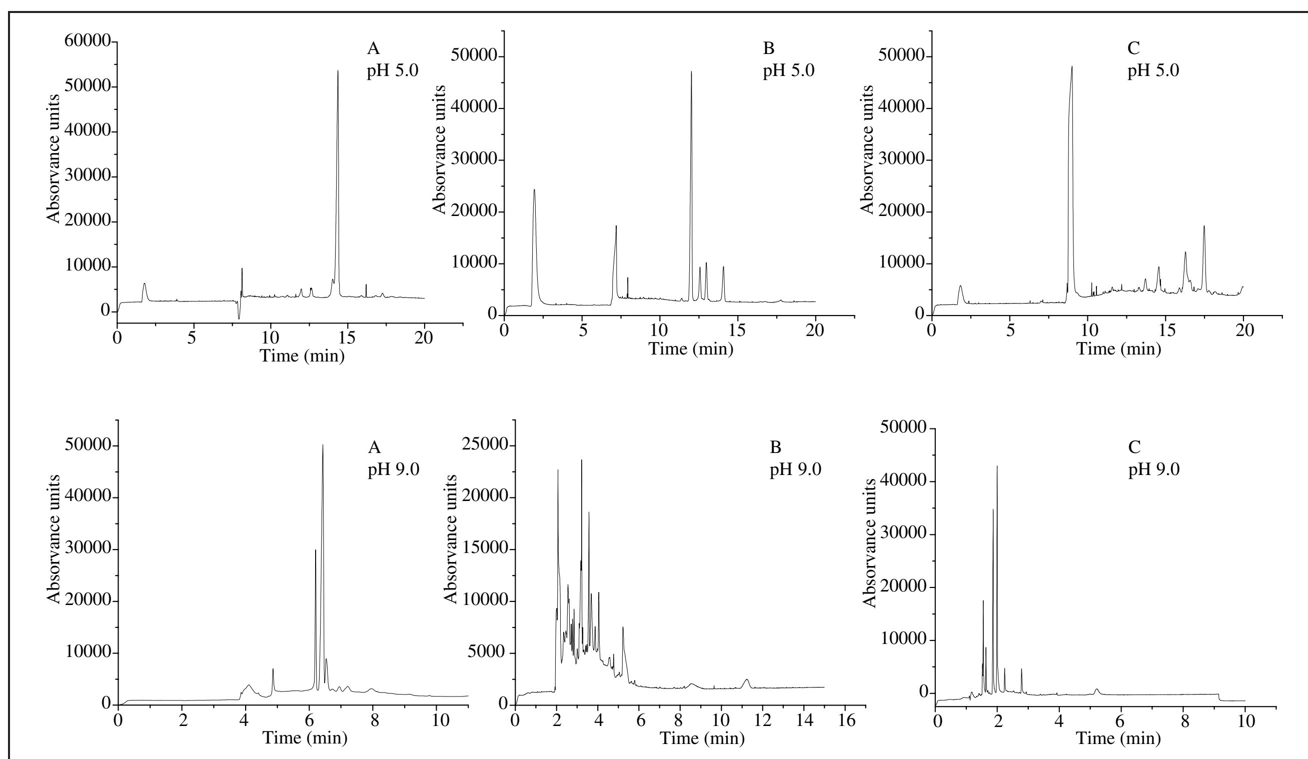


Fig. 1: Electropherograms obtained from: a) commercial syrup; b) *Hedera helix* fluid extract; c) *Hedera helix* glycolic extract. Conditions: acetate buffer 11 mM, pH 5.0 (above), tetraborate buffer 20 mM, pH 9.0 (below); capillary, 57 cm full length, 50 cm effective length, 75 μm ID, 375 μm OD; hydrodynamic injection at 0.5 Psi, 5 s; 25 kV constant voltage; 25 °C, detection by UV absorption at 212 nm

**Table 1: Quantitative parameters of the analysis of codeine, diphenhydramine and ephedrine**

| Analyte | Concentration range (μg ml$^{-1}$) | Correlation coefficient ($r^2$) | Slope ± sd[a] (95%; n = 6) | Intercept ± sd[a] (95%; n = 6) | LOQ (μg ml$^{-1}$) | LOD (μg ml$^{-1}$) |
|---|---|---|---|---|---|---|
| CDN | 4.0–100 | 0.992 | 108990 ± 6644 | 6334 ± 3721 | 3.45 ± 0.60 | 1.07 ± 0.08 |
| DP | 2.3–100 | 0.992 | 97806 ± 5860 | 4350 ± 3287 | 2.23 ± 0.70 | 0.71 ± 0.09 |
| EP | 1.5–100 | 0.994 | 54101 ± 2935 | 2819 ± 1646 | 1.40 ± 0.01 | 0.42 ± 0.05 |

sd[a] standard deviation; CDN: codeine; DP: diphenhydramine; EP: ephedrine

of peak fractions were used to separate the sample constituents. The buffer system achieved satisfactory separations at pH over 8.0 and system (b) was better than (c) under alkaline conditions, with an optimum at pH 9.0. The selected pH values for the fingerprints were buffer sodium acetate 11 mM, pH 5.0 and sodium tetraborate 20 mM, pH 9.0. The resulting electropherograms are shown in Fig. 1. The peaks of the samples were identified on the basis of their migration times and UV spectra. Peaks in the samples were monitored in the UV range 200–400 nm with a diode array detector and the peak purity was confirmed.

### 2.2. Adulterant investigation. Optimization of separation conditions

#### 2.2.1. Effect of buffer pH

Optimization of the experimental parameters was performed by investigating the influence of the buffer system, buffer concentration, applied voltage and temperature of the capillary on the separation efficiency of sample constituents.
This procedure was applied to the search for the presence of some synthetic drugs such as possible adulterants (ephedrine, codeine and diphenhydramine) and their subsequent separation and determination.

With regard to the background electrolyte (BGE) systems evaluated, it was found that the tetraborate buffer system offered higher separation efficiency than acetate. Thus, tetraborate BGE was selected for further studies.

#### 2.2.2. Effect of buffer concentration

Sodium tetraborate buffers of 10–50 mM were also tested for their use as the BGE in the separation of both sample constituents and potential adulterants. It was found that tetraborate 20 mM provided higher separation efficiency than the other concentrations. Good resolution between peaks was obtained with buffers of high concentration (over than 25 mM) but they required a long separation time.

**Table 2: Precision results**

| | Method Precision |
|---|---|
| Method Repeatability | R.S.D. (peak area): 1.73% |
| (n = 10) | R.S.D. (migration time): 0.14% |
| System Repeatability | R.S.D. (peak area): 1.58% |
| (n = 10) | R.S.D. (migration time): 0.28% |
| Intermediate Precision | R.S.D. operator (migration time): 0.93% |
| (n = 6) | R.S.D. capillary (migration time): 0.96% |
| | R.S.D. inter-day (peak area): 1.84% |
| | R.S.D. inter-day (migration time): 0.95% |

### 2.2.3. *Electrophoretic parameters*

The influence of applied voltage (10–30 kV) and the temperature of the capillary and the sample (15–25 °C) were examined. A high voltage is necessary for rapid CE analysis. It was found that when the applied voltage was higher than 25 kV, the resolution of the compounds was not improved. But when there was a lower voltage, the migration time increased. Thus, 25 kV was used as the run voltage. No significant differences were observed when the temperature was varied, and as a result, the selected conditions were: applied voltage 25 kV, capillary and sample temperature 25 °C. The best results were obtained using hydrodynamic injection with a pressure of 0.5 Psi for 5 s. The detection wavelengths chosen were 212 and 252 nm.

### 2.2.4. *Method validation*

The analytical methodology developed for the separation and simultaneous determination of ephedrine, codeine and diphenhydramine was validated for routine application in the quality control of *Hedera helix* based products.
The linear relationships between the concentrations of three compounds and the corresponding peak areas were

**Table 3: Commercial formulation[a] recovery test**

| | Base Value (mg ml$^{-1}$) | Quantity added (mg ml$^{-1}$) | Quantity found[b] (mg ml$^{-1}$) | Recovery (%)[c] |
|---|---|---|---|---|
| Aliquot I | | | | |
| CDN | – | 0.00 | 0.251 | – |
| DP | – | 0.00 | 0.252 | – |
| EP | – | 0.00 | 0.249 | – |
| Aliquot II | | | | |
| CDN | 0.251 | 0.10 | 0.354 | 100.3 |
| DP | 0.252 | 0.10 | 0.351 | 99.0 |
| EP | 0.249 | 0.00 | – | – |
| Aliquot II | | | | |
| CDN | 0.251 | 0.10 | 0.349 | 98.0 |
| DP | 0.252 | 0.00 | – | – |
| EP | 0.249 | 0.10 | 0.348 | 99.0 |
| Aliquot IV | | | | |
| CDN | 0.251 | 0.00 | – | – |
| DP | 0.252 | 0.10 | 0.350 | 98.0 |
| EP | 0.249 | 0.00 | – | – |

[a] diluted solution of the commercial formulation, Athos®
[b] mean value (n = 6)
[c] 100 × [(Found-base)/added]
CDN: codeine; DP: diphenhydramine; EP: ephedrine

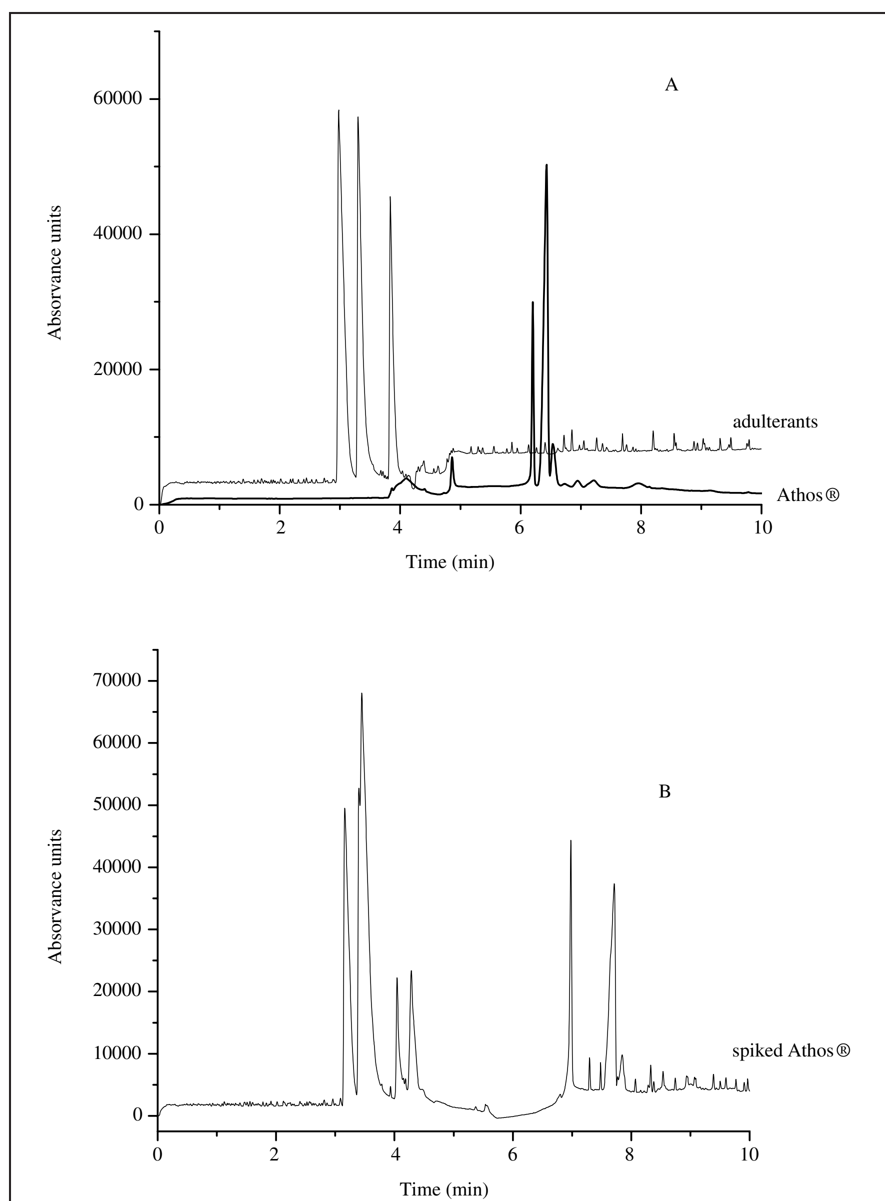


Fig. 2:
Electropherograms obtained from: A) solution mixture of adulterants and commercial syrup (Athos®); B) commercial syrup (Athos®) spiked with the solution mixture of adulterants. Conditions: tetraborate buffer 20 mM, pH 9.0; capillary, 57 cm full length, 50 cm effective length, 75 μm ID, 375 μm OD; hydrodynamic injection at 0.5 Psi, 5 s; 25 kV constant voltage; 25 °C, detection by UV absorption at 212 nm

studied. For each ingredient, a calibration graph was constructed by plotting concentration (μg $ml^{-1}$) versus corrected peak area. The slopes and intercepts of regression equations, linearity ranges, correlation coefficients and limits of detection (LOD) and quantification (LOQ) are listed in Table 1.

The method was validated for precision in relation to the migration time and the peak area of the three analytes. Precision was tested at two levels: repeatability and intermediate precision. Intermediate precision was found by changing the capillary, the operator and the day. The relative standard deviations (RSD) of the migration time and the peak area are presented in Table 2. All of these data indicated that precisions are acceptable.

Accuracy was found by performing a recovery test. Standard solutions of the analytes under study were added to different aliquots of a commercial sample of *Hedera helix* (Athos®) previously spiked with the potential adulterants. The final concentrations of ephedrine, codeine and diphenhydramine were determined applying the present methodology; the results are shown in Table 3. The values obtained indicated good accuracy for this method. The electropherograms of a real sample, a mixture of solutions of the three adulterants and a sample spiked with the drugs are presented in Fig. 2.

Selectivity was validated as recommended by the ICH Guidelines (Fabre and Altria 2001) assessing peak homogeneity or peak purity; this was achieved using a diode array detector in the UV range from 200–400 nm. It was shown that the major peaks were the common constituents of the samples without interference either from excipients in the formulation or from possible adulterants. In addition, results of recovery experiments confirmed the selectivity of the method.

As in HPLC, the stability of the solutions should be assessed at least over a period of time covering preparation and analysis. Large volumes of the mobile phase are required in HPLC, thus solutions are prepared daily; in contrast, because of the low consumption of electrolyte solution, in CE it is of major interest to test the stability of the BGE. The electrolyte used in this analysis has demonstrated good stability; it has been proved over several months.

## 3. Discussion

The fingerprints obtained showed that satisfactory separations were achieved by using sodium acetate 11 mM, pH 5.0 and sodium tetraborate 20 mM, pH 9.0 buffers. The fingerprints could help to distinguish substitutes or adulterants and further assess the differences between *H. helix* from various sources.

The potential adulterants (ephedrine, diphenhydramine and codeine) were well separated from other interference peaks commonly present in the samples and identified by spiking analyte standards. This method showed good anti-interference performance and the results indicate that the analysis of these drugs in *Hedera helix* commercial formulations can be done well by this CE method.

## 4. Experimental

The CE system employed consisted of a Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software. Detection was performed at 212 and 252 nm. The fused-silica capillaries were obtained from Micro-Solv Technology Corporation and had the following dimensions: 57 cm total length, 50 cm effective length, 75 μm ID, 375 μm OD. The temperature of the capillary and the samples was maintained at 25 °C. The pH of the electrolytes was measured by an Orion 940 pHmeter equipped with a glass-combined electrode.

Codeine hydrochloride, diphenhydramine hydrochloride and ephedrine hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO). The phytopharmaceutical samples analyzed were fluid and glycolic *H. helix* extracts supplied from a local drug store (Droguería Saporitti, Buenos Aires, Argentina) and a commercial syrup manufactured by Roemmers (Buenos Aires, Argentina). Sodium tetraborate ($Na_2B_4O_7.10\ H_2O$), sodium dihydrogen phosphate ($NaH_2PO_3$) and sodium acetate ($NaC_2H_3O_2$) were obtained from Mallinckrodt (Saint Louis, USA). The water used in all studies was ultra-high-quality water obtained from a Barnstead Easy pure RF compact ultrapure water system. All other reagents and solvents were of analytical grade quality. All solutions were degassed by ultrasonication (Testlab, Argentina). Running electrolytes and samples were filtered through 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ. USA).

The electrolyte solution (background electrolyte, BGE) was prepared daily and filtered through a 0.45 μm Titan Syringe filter (Sri Inc., Eaton Town, NJ. USA). At the beginning of the day, the capillary was conditioned with 0.1 mol. $l^{-1}$ NaOH for 5 min, followed by water for 5 min, and then with running electrolyte for 10 min before sample injection. To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with sodium hydroxide for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 4 min. Samples were pressure-injected at the anodic side at 0.5 Psi for duration of 5 s. A constant voltage (25 kV) was used for all the experiments.

Stock standard solutions containing codeine hydrochloride (CDN), diphenhydramine hydrochloride (DP) and ephedrine hydrochloride (EP) were prepared in water at concentrations of 1–1000 μg $ml^{-1}$. A combined standard solution containing codeine, diphenhydramine and ephedrine was prepared by accurately mixing the standard solutions of each drug and the resultant solution was made up to 100 ml.

Diluted solutions of the extracts and the commercial formulation were prepared as follows: 100 μl aliquots of the samples were carefully measured and diluted to 600 μl. The solutions were mixed and filtered through a 0.45 μm membrane.

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