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# CE-DAD Determination of Scutellarein and Caffeic Acid in *Abelia triflora* Crude Extract

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## Abstract

A precise, accurate, selective and sensitive capillary electrophoresis method using a diode array detector was developed for the first time for the determination of both scutellarein (SLN) and caffeic acid (CAA) in prepared *Abelia triflora* extract. Electrophoretic analysis was performed using a background electrolyte solution consisting of borax buffer (40 mM, pH 9.2) and a 200-nm detection wavelength. This method was fully validated according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. The method was linear in the concentration range 2.5–100 μg/mL and it allowed the determination of both compounds with high degree of recovery (%Er < 2%) and intra-day and inter-day precision (relative standard deviation values <2%) and method robustness was also assessed by the low values of %RSD < 2% obtained after small deliberate changes in the method parameters. The contents of SLN and CAA were calculated using both the external standard and standard addition methods. Analysis of the ethyl acetate fraction of *A. triflora* revealed that SLN and CAA were found in concentrations of 0.46 mg/g and 2.10 mg/g, respectively, in the ethyl acetate fraction and 0.29 and 1.32 mg%, respectively, in the dry plant leaves.

## Introduction

The genus *Abelia* (Caprifoliaceae) consists of about eighty species distributed throughout the Himalayas and East Asia. *Abelia triflora* R. Br. is the only species found in Pakistan. In one previous study, the methanol extract of *A. triflora* showed high toxicity in a brine shrimp lethality test (1). Previous phytochemical studies of the plant, *A. triflora* R. Br., resulted in the isolation of five compounds, two of which were isolated from the ethyl acetate fraction in good yield, namely, scutellarein (SLN) and caffeic acid (CAA) (2). CAA showed promising anticancer activity against human breast and prostate cancer cell lines (2). This is in addition to other important biological activities reported for CAA, such as antioxidant, anti-ischemia reperfusion, anti-thrombosis, anti-hypertension, anti-fibrosis, antivirus and antitumor properties (3). SLN was reported to have strong antioxidant activity as part of a study with the aim of its development as a candidate for treating ischemic cerebrovascular disease (4). As a result of these two major compounds being present in *A. triflora*, and taking in consideration their valuable biological activities, it was of great interest to us to develop a capillary electrophoresis with diode array detector (CE-DAD) method for the determination of these biomarkers in the ethyl acetate fraction of *A. triflora* leaves.

CE is a powerful tool that has been widely applied to the analysis of pharmaceuticals and flavonoids in the last 10 years. Most recent papers on the CE analysis of flavonoids are in the field of natural product research, including analysis of plants, herbs and other plant-derived products (5).

Many different high-performance liquid chromatography (HPLC) methods have been developed for determining SLN and CAA in the aerial and underground parts of medicinal plants, e.g., *Scutellaria baicalensis Georgi*. (6), *Ecliptae herba* extract (7) and *Scutellariae* (8) as well as *Radix scutellariae* (by ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS)) (9). In addition, HPLC systems equipped with different detectors has been used for the determination of CAA in Shuang–Huang–Lian formula (10), extract of burr parsley (*Caucalis platycarpos L.*) (11), crude ethanolic extracts of parsley (*Petroselini fructus*), buckthorn (*Frangulae cortex*), mint (*Mentha piperitae folium*), caraway (*Carvi fructus*) and birch (*Betulae folium*) before and after hydrolysis (12), *Thliptoceras formosanum* (13), alcoholic extract of *Securigera securidaca* flowers (14) and abnormal Savda Munziq (15). Furthermore, liquid chromatography coupled with electrospray ion-trap multistage mass spectrometry (ESI-$MS^n$) and electrospray quadrupole–time-of-flight mass spectrometry methods was established for the comprehensive analysis of the phenolic components (e.g. CAA) in the Chinese herbal prescription, Erigeron injection (16). CAA has also been identified and quantified through the analysis of *Salvia officinalis L.* leaves using thin layer chromatography and HPLC (17).

A method based on CE has been developed for the determination of CAA in the dried flower buds, leaves and stems (three medicinal parts) of *Lonicera confusa* DC. (18), in *Perilla frutescens L.* (19) and in propolis and its extract capsules (20).

In this work, we report for the first time the simultaneous analysis of the antioxidant phenolic compounds SLN and CAA from *A. triflora* crude extract using CE coupled with DAD detector. The aim of this study was to develop a simple, fast and affordable CE method for the separation of SLN and CAA. The proposed method was validated with respect to linearity, reproducibility, accuracy and recovery.

## Experimental

### Standards and reagents

Reference standards of SLN and CAA were purchased from Sigma Aldrich, St. Louis, USA (Figure 1). All reagents and chemicals used in this work were of analytical grade. Methanol and borax (disodium tetraborate decahydrate) were obtained from Winlab, UK. Sodium hydroxide was supplied by BDH Laboratory Suppliers, Poole, UK.

Deionized water was used throughout the experiments. Millipore membrane filters (0.45 µm) were purchased from Nihon Millipore (Yonnezawa, Japan).

### Plant material

The leaves of *A. triflora* were collected in 2012 from Ziarat Valley, Baluchistan province, Pakistan.

### Instrumentation and electrophoretic conditions

An Agilent capillary electrophoresis instrument (Agilent Technologies Deutschland, Waldbronn, Germany) was used in this study. The device was equipped with a DAD and Agilent ChemStation software for data handling. Electrophoretic analysis was performed using a deactivated fused silica capillary (Agilent Technologies, Santa Clara, CA) with the following dimensions: 81.5 cm total length, 70 cm effective length and 75 µm internal diameter. The temperature of the capillary and the sample was maintained at 23°C. Electrophoretic analysis was performed using a background electrolyte (BGE) solution consisting of borax buffer (40 mM, pH 9.2) and a 200-nm detection wavelength. Samples were injected into the capillary by applying pressure at the anodic side at 25 mbar for 30 s with an applied voltage of 30 kV. To ensure the reproducibility of the analysis, the capillary was washed between runs with deionized water for 2 min and then equilibrated with the running buffer for 4 min. Before sample injection, the capillary was conditioned with 0.1 M sodium hydroxide for 5 min, deionized water for 5 min and running buffer for 10 min. All solutions were filtered through 0.45 µm Millipore membrane filters (Nihon Millipore, Japan) before injection.

### Standard solutions and construction of calibration graphs

Stock solutions containing 1.0 mg/mL of SLN and CAA were prepared by dissolving accurately weighed amounts of the powders in methanol. Further dilutions were made with borax buffer solution (40 mM, pH 9.2) to obtain six working standard solutions at concentrations from 2.5 to 100 µg/mL for both compounds. Triplicate injections of each concentration were made under the specified CE conditions.

### Extraction procedure and preparation of plant extracts

Dried *A. triflora* leaves (8.0 kg) were ground up and extracted with methanol (3 × 10 L) at room temperature. The combined methanol extract was evaporated under reduced pressure to obtain a thick gummy mass (450 g). It was suspended in water and successively extracted with *n*-hexane (120 g), chloroform (90 g), ethyl acetate (50 g), *n*-butanol (80 g) and water-soluble fraction (100 g). SCN and CAA were isolated as the major compounds in the ethyl acetate fraction using several column chromatographic separations as previously described (2).

### Application of the proposed method to the analysis of plant extracts

The ethyl acetate fraction (1.1 g) of *A. triflora* leaves was weighed accurately and dissolved in 5 mL of methanol and then filtered using 0.45 µm disposable filters. The prepared methanolic extract (0.5 mL) was mixed with 0.5 of mL borax buffer (40 mM, pH 9.2). These prepared plant solutions were injected three times into the CE system under the specified conditions. In addition, a standard addition at a concentration of 25 µg/mL was performed for both SLN and CAA. The peak areas recorded for SLN and CAA in analyzed samples were used to estimate the amount of each compound found in the prepared extract (Figure 2).

## Results

### Optimization of electrophoretic conditions

A typical electropherogram of a standard mixture of STC and CAA obtained using the optimized conditions are shown in Figure 2. The peaks obtained were sharp and had complete baseline separation. In addition, they were separated within a reasonable run time (25 min): SLN was eluted at 16.5 ± 0.6 min, while CAA was eluted at 20.4 ± 0.7 min.

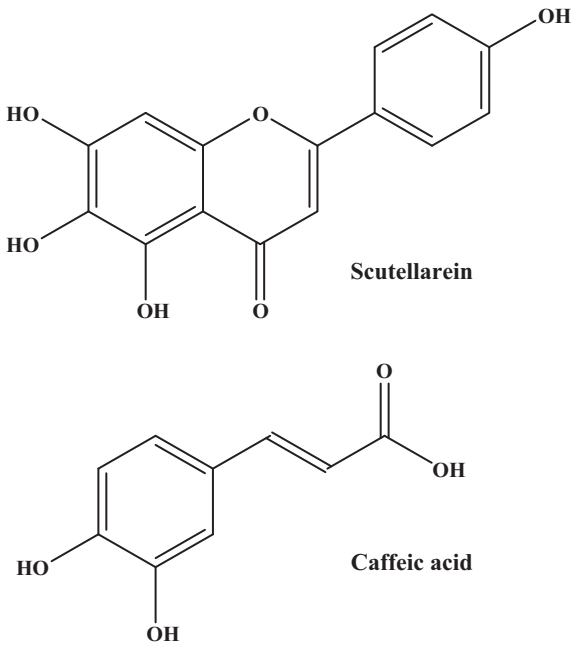


**Figure 1.** Chemical structure of the studied drugs.

## Composition of background electrolyte

The influence of the pH of the BGE buffer was investigated using 40 mM phosphate buffer in the pH range of 3.5–7 and 40 mM borate buffer in the pH range of 8.0–11. It was found that no well-shaped peaks of either SLN or CAA could be obtained until a pH of 8.0 was reached and that an increase in the buffer pH was associated with a decrease in the migration time of both analytes. Taking into consideration the separation of both analytes from interfering plant peaks with good peak shape and within a reasonable migration time, borate buffer solution (40 mM, pH 9.2) was used for further experimentation. Borate buffer solutions (pH 9.2) of different strengths (10–60 mM) were examined for their effect on separation efficiency. It was observed that 40 mM borate buffer (pH 9.2) provided the best results regarding resolution, peak shape and run time. Thus, it was applied in further experiments.

The inclusion of organic modifiers in the BGE may affect the separation by changing the partitioning equilibria of the analytes, the electro-osmotic flow and the viscosity of the BGE (21–23). It was noticed that the inclusion of methanol in the BGE (5–15%) resulted in a significant increase in the migration time of both analytes (more than 40 min) with a distortion of the peak shape. Thus, it was not included in the BGE.

## Effect of applied voltage

The effect of applied voltage on the migration of SLN and CAA was studied over a range of 15–30 kV. The highest separation efficiency with shortest analysis time was achieved with an applied voltage of 30 kV. Thus, it was applied in further experiments.

## Effect of sample injection time and applied pressure

Both sample injection time (20–40 s) and applied pressure (20–40 mbar) were evaluated for their effects on separation efficiency. It was reported that increasing the applied pressure resulted in increased separation efficiency with decreased migration time. However, an applied pressure of 30 mbar resulted in incomplete separation of both SLN and CAA. Thus, 25 mbar was used as the applied pressure. On the other hand, the sample injection time had a significant effect on the peak response and peak shape; 30 s was found to be optimum for analysis.

## Effect of capillary temperature

It was noticed that an increase in the temperature (15–25°C, stepwise 2°C) resulted in improved migration with decreased analysis time. An operational temperature of 23°C was applied since it provided a compromise between peak shapes, analysis time and most importantly resolution from the peaks of the matrix components in the plant extracts.

## Method validation

### Linearity

Under the above optimized conditions and using the method of least squares, calibration graphs were constructed by plotting the concentrations against the peak areas for both SLN and CAA. Different regression and statistical parameters were calculated. These included regression equations, intercepts, slopes, correlation coefficients and standard deviations of residuals, of intercepts, and of slopes as well as the variance ratio. The results are summarized in Table I.

### Limits of detection and quantitation

The low values (the relative standard deviation (RSD) ≤10%) of LOQ obtained for both SLN and CAA indicates the ability of the method to measure both analytes at very low concentrations and thus shows that it is suitable for analyzing their contents in plant extracts.

### Precision

Method precision was assessed at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision). Both types of precision were assessed at three different concentration levels (50, 10.0 and 5 μg/mL) of both SLN and CAA by repeating the assay three times on the same day (intra-day precision) and on three different days (inter-day precision). The RSD was <2% (Table II)

### Recovery

Recovery testing was performed in order to assess the applicability of the proposed method for the analysis of both SLN and CAA in the ethyl acetate fraction of *A. triflora* leaves. This was performed by spiking the prepared plant extract samples with known amounts of standard solutions of each of SLN and CAA at concentrations of 50, 25 and 10 μg/mL of both analytes. Spiked samples were then analyzed under the optimized conditions, and the amount recovered was calculated. The results are summarized in Table III.

### Robustness

Method robustness is defined as its ability to remain unaffected by small and deliberate changes in experimental parameters. The studied parameters included the pH of the borate buffer (9.0, 9.2 and 9.4), applied voltage (28 and 30 kV), applied pressure (23, 25 and 27 mbar), injection time (28, 30 and 32 sec) and capillary temperature (23, 25 and 27°C). Standard mixtures were analyzed under each change in the experimental conditions, and the peak area and migration time were recorded for each compound. Low values of the RSD (>2%) of migration times and peak areas indicated the robustness of the method.

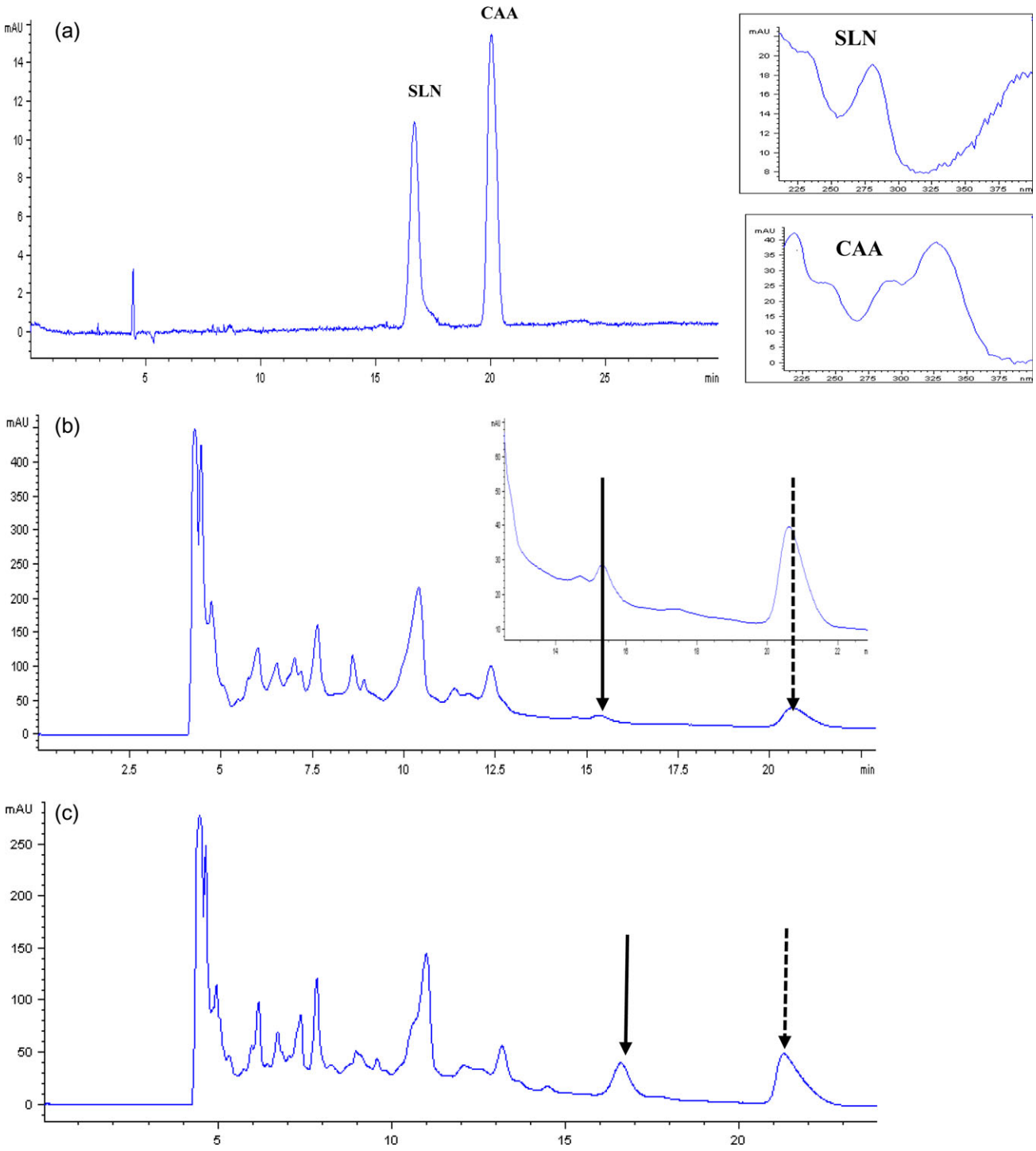


**Figure 2**. CE electropherograms of a standard mixture of SLN (25 μg/mL), CAA (25 μg/mL), (a), and their corresponding absorption spectra, (a′), (b′), prepared AbeliaTriflira extract, (b) and the corresponding spiked sample, (c). Arrows indicate migration times of the studied drugs. Spiking of plant extracts was done by a standard mixture of LUT (25 μg/mL), API (25 μg/mL).

### Stability of solutions

Solution stability was assessed using standard solutions of 25 μg/mL SLN and CAA kept at room temperature for 5 h.

### Specificity

Specificity is the ability of a method to detect the analytes of interest in the presence of all potential interfering substances that could be found when conducting actual analysis. Since *A. triflora* extract is a complex matrix with many components and their corresponding peaks, it was very important to ensure the specificity of the method for the determination of SLN and CAA in this matrix. Specificity was evaluated by spiking the analyzed plant extract samples with standard solutions of both drugs. Purity was also assessed by calculating the peak purity, a property provided by DAD.

**Table I.** Regression and Statistical Parameters for the Determination of SLN and CAA by the Proposed CE Method

| Parameter | SLN | CAA |
|---|---|---|
| Linearity range (μg/mL) | 2.5–100 | 2.5–100 |
| LOQ[a] (μg/mL) | 2.5 | 2.5 |
| LOD[b] (μg/mL) | 0.8 | 0.8 |
| Intercept | −1.80 | −0.72 |
| Slope | 7.73 | 9.027 |
| Correlation coefficient | 0.9996 | 0.9999 |
| $S_a$[c] | 5.1144 | 2.9484 |
| $S_b$[d] | 0.1088 | 0.0627 |
| $S_{y/x}$[e] | 9.1550 | 5.2781 |
| $F$[f] | 5,072 | 20,673 |
| Significance $F$ | $2.33 \times 10^{-7}$ | $1.40 \times 10^{-8}$ |

[a]LOQ: Limit of quantitation.
[b]LOD: Limit of detection.
[c]$S_a$: Standard deviation of intercept.
[d]$S_b$: Standard deviation of slope.
[e]$S_{y/x}$: Standard deviation of residuals.
[f]$F$: Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).

**Table II.** Intra-day and Inter-day Precision for the Determination of SLN and CAA by the Proposed CE Method.

| Concentration added (μg/mL) | Intra-day precision RSD[a] | Inter-day precision RSD[b] |
|---|---|---|
| *SLN* | | |
| 50 | 1.94 | 1.85 |
| 10 | 1.98 | 1.44 |
| 5 | 1.97 | 1.99 |
| *CAA* | | |
| 50 | 1.23 | 1.82 |
| 10 | 1.71 | 1.22 |
| 5 | 0.61 | 1.39 |

[a]RSD of three determinations.
[b]Data are based on assay of three replicates on three different days.

**Table III.** Recovery Results for the Determination of SLN and CAA by the Proposed CE Method.

| Concentration spiked (μg/mL) | Mean recovery (%) ± RSD[a] | $E_r$ (%)[b] |
|---|---|---|
| *SLN* | | |
| 50 | 102.17 ± 1.88 | 2.17 |
| 25 | 98.53 ± 0.95 | −1.47 |
| 10 | 98.99 ± 0.59 | −1.00 |
| *50* | | |
| 50 | 106.51 ± 1.22 | 6.51 |
| 25 | 102.15 ± 0.55 | 2.15 |
| 10 | 97.22 ± 1.58 | −2.88 |

[a]RSD of three determinations.
[b]Percentage relative error.

**Application of the CE method for the analysis of *A. triflora* extract**

The applicability of the developed CE method was extended to the quantitative analysis of SLN and CAA in the ethyl acetate fraction of *A. triflora* leaves. The electropherograms obtained by the actual analysis of prepared extracts along with spiked samples are shown in Figure 2b and c, respectively. It is obvious that several of the peaks detected are also detected in the analysis of the plant extract. However, these peaks are well separated from those of the analytes, as emphasized by the peak purity determined by DAD.

Samples of the ethyl acetate fraction of *A. triflora* leaves were prepared as described exactly in the experimental section and then analyzed in triplicate under the optimized experimental conditions. The contents of SLN and CAA were calculated using both the external standard and standard addition methods. The standard addition method was performed by spiking the prepared plant extract samples with known amounts of standard solutions of each of SLN and CAA at a concentration of 25 μg/mL of both analytes. Spiked samples were then analyzed under the optimized conditions, and the amount recovered was calculated. The same results were obtained using both the external standard and standard addition methods. Final calculations were made relative to the mass of the ethyl acetate fraction as well as to that of the dried plant leaves analyzed. Analysis of the ethyl acetate fraction of *A. triflora* revealed that SLN and CAA were found in concentrations of 0.46 mg/g and 2.10 mg/g, respectively, in the ethyl acetate fraction and 0.29 and 1.32 mg%, respectively, in the dry plant leaves.

## Discussion

### Optimization of electrophoretic conditions

Different parameters that affect the electrophoretic behavior of SLN and CAA were separately optimized in order to determine both analytes without any interference from coexisting components that could be encountered during actual analysis of plant samples. They include BGE composition, applied voltage, injection time, applied pressure and capillary temperature.

Peak purity was assessed by comparing the absorption spectra of each analyte peak with those of the corresponding standard solutions using the DAD. A detection wavelength of 200 nm was selected for the final analysis since it provided the maximum response for both compounds.

### Composition of background electrolyte

The composition of the BGE was carefully optimized in order to enable accurate determination of SLN and CAA in plant extracts without potential interference from other coexisting compounds. The BGE should also enable the elution of analytes with acceptable peak shapes and in a reasonable run time. The most important parameters involved in BGE composition include the pH, type and strength of the buffer (21–24). It is well known that the pH of BGE plays a critical role in the separation efficiency because of its effect on the magnitude of the electro-osmotic flow as well as on the ionization of analytes.

### Effect of applied voltage

Electrophoretic velocities are highly affected by the applied voltage such that increased resolution may be attained by increasing the applied voltage (21–24). Practical experimentation revealed that low voltage (<25 kV) resulted in increased migration times with excessive peak broadening.

### Effect of capillary temperature

Capillary temperature control is very important in electrophoretic separation due to the Joule heating effect, which affects the

reproducibility of the analysis. Temperature also has an effect on the viscosity of the BGE. Thus, it affects the migration of the analytes and hence the separation efficiency (21–24).

### Method validation

The method was validated according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines (25). These include linearity, limits of detection and quantitation, precision, recovery, specificity, robustness and solution stability.

### Linearity

Good linearity within the concentration ranges mentioned in the experimental section was verified by the high values of the correlation coefficients ($r$) along with small intercepts ($a$) and high variance ratios. Small values of the standard deviation of residuals ($S_{y/x}$) indicated the closeness of the points to the linear regression line (26).

### Limits of detection and quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated based on signal-to-noise ratios of 3 (LOD) or 10 (LOQ).

### Precision

The repeatability (intra-day precision) and intermediate precision (inter-day precision) were assessed. In both cases, the low values obtained for percentage relative standard deviation (RSD%) indicated a high degree of precision of the proposed method.

### Recovery

Good recovery results were obtained for both SLN and CAA in the plant extract, indicating the accuracy of the method for the analysis of both compounds in prepared plant extracts.

### Robustness

Low values of RSD (>2%) of the migration times and peak areas indicated the robustness of the method.

### Stability of solutions

The absence of any additional peaks along with unchanged peak areas in the electropherograms throughout the time of analysis indicated the stability of both compounds in their solutions.

### Specificity

The purity index calculated for both compounds was within the purity threshold, which indicated the absence of any underlying or overlapping interfering peaks. Moreover, the peaks obtained for both compounds had clear baseline separation and reasonable migration times (>25 min).

## Conclusion

This work addresses the development and optimization of a CE method with DAD for the simultaneous determination of SLN and CAA. This method was further applied to the determination of these phenolic compounds in the ethyl acetate fraction of *A. triflora* leaves. The results revealed that the proposed method is precise, accurate and sensitive with LOD of 0.8 µg/mL for both SLN and CAA. The use of DAD imparts high specificity to the method. The proposed method allowed the selective determination of both phenolic compounds in prepared *A. triflora* extract without interference from co-eluting peaks encountered in actual analysis. The results revealed that CAA is found in higher amount relative to SLN in the analyzed samples.

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