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Journal of Chromatography A, 903 (2000) 237–244

JOURNAL OF CHROMATOGRAPHY A

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Use of borate complexation for the separation of non-UV-absorbing calystegines by capillary electrophoresis

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Received 11 July 2000; received in revised form 31 August 2000; accepted 31 August 2000

Abstract

A capillary zone electrophoresis method was developed for the simultaneous analysis of seven closely related polyhydroxyalkaloids called calystegines. Successful results were obtained with a fused-silica capillary, 80 m*M* sodium tetraborate at pH 9.2 and temperature of 50°C. Detection of non-UV-absorbing calystegines was achieved through in-situ complexation with borate ions. To further improve method sensitivity, a capillary with a bubble cell was used and detection performed at low wavelength (191 nm). Effects of buffer concentration, pH and temperature on migration times and efficiency are discussed. Migration behavior of selected compounds was significantly affected by their chemical structure (i.e., number and position of hydroxy groups). Under optimized conditions, baseline separation of the selected compounds was achieved in less than 12 min. Precision was evaluated by measuring repeatability and intermediate precision of migration times and corrected peak areas. Finally, the method was applied to the qualitative analysis of calystegines in plant extracts and results were confirmed by GC–MS.

Keywords: Plant materials; Complexation; Derivatization, electrophoresis; Buffer composition; Calystegines; Polyhydroxyalkaloids; Alkaloids

1. Introduction

In the last 10 years, a large number of nitrogen-containing polyhydroxylated heterocyclic compounds have been isolated from plants. These natural products are competitive inhibitors of various glycosidases, presumably because their structure is similar to carbohydrates. The more efficient compounds are used for the treatment of various diseases including diabetes, cancer and viral infections [1]. Furthermore, these compounds exhibit additional activities, including immunomodulatory properties and inhibition of glycolipid synthesis [1]. Among these compounds, a new class of nortropane polyhydroxylated alkaloids, called calystegines (Fig. 1), has been recently isolated from different species belonging to the Solanaceae, Convolvulaceae and Moraceae [2].

Currently, calystegine determination in plant material is performed by thin-layer chromatography

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00902-X

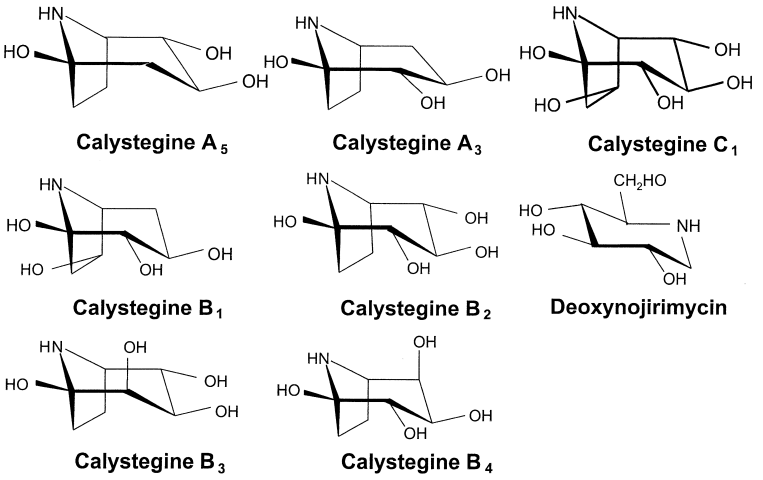

Fig. 1. Structures of investigated compounds.

(TLC) [3,4], paper electrophoresis [5] and gas chromatography coupled to mass spectrometry (GC–MS) [6]. High resolution obtained by GC, combined with structural information from MS makes GC–MS the most powerful analytical method. However, the latter requires a tedious derivatization procedure. Recently, capillary electrophoresis (CE) has evolved as an interesting alternative for the analysis of plant extracts because of its high efficiency, flexibility, accuracy and very high resolution [7–9].

Therefore, and with the expertise developed in our laboratory for tropane alkaloids analysis [10,11], the separation of calystegines was investigated by CE. However, as calystegines do not possess any chromophore or fluorophore groups, CE analysis coupled to UV detection was carried out with borate ions in the background electrolyte (BGE) solution. Complexation of borate with calystegines allows a UV detection of these compounds.

In this paper, the simultaneous analysis of calystegines with similar structures is described. Effects of borate buffer pH and concentration as well as temperature were investigated. Finally, precision of the method was determined and optimized conditions were applied to the qualitative analysis of calystegines in plant extracts. Furthermore, GC–MS experiments were carried out to confirm the presence of these compounds in the extracts.

2. Experimental

2.1. Chemicals

Standard calystegines (A3, A5, B1, B2, B3, B4 and C1) were kindly donated by Dr. N. Asano (Hokuriku University, Kanazawa, Japan). Deoxynojirimycin (internal standard) was obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and were supplied by Fluka (Buchs, Switzerland). Ultrapure water, obtained by a Milli-Q RG unit from Millipore (Bedford, MA, USA), was used for standard and sample preparation. Electrolyte solutions were filtered through a 0.45-μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Instrumentation

2.2.1. Electrophoretic procedure

CE data were generated in a HP ^{3D}Capillary Electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. A CE Chemstation (Hewlett-Packard) was used for instrument control, data acquisition and data analysis. The extended path-length capillary (Hewlett-Packard)

was 80.5 cm (length to the detector 72 cm)×50 μm I.D. (bubble factor 3). An alignment interface, containing an optical slit matched to the internal diameter, was used and the detection wavelength was set at 191 nm with a bandwidth of 2 nm.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). A constant voltage of 25 kV, with an initial ramping of 500 V s^{-1}, was applied during analysis. Temperature was maintained at 50°C unless otherwise stated. Sample injections (8 nl injection volume) were achieved using the pressure mode for 5 s at 50 mbar.

Before use, the capillary was washed with 0.1 *M* sodium hydroxide followed by water for 10 min each. To achieve high migration time reproducibility between analyses, the capillary was successively washed with 0.1 *M* sodium hydroxide for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 3 min. The carrier buffer was obtained by dissolving sodium tetraborate in water to give a concentration between 20 and 90 m*M*. For pH studies, buffer was prepared by mixing sodium tetraborate and sodium hydroxide solutions in appropriate ratio to give a pH value comprised between 9.2 and 12. All buffers were prepared using the Phoebus software 1.0 (Centre Analyse, Orleans, France).

2.2.2. GC–MS procedure

GC–MS data were obtained with a HP 5890 series II gas chromatograph and a HP 5972 mass-selective detector (Hewlett-Packard) in the electron impact ionization (EI) mode at 70 eV. Helium was used as carrier gas at a flow-rate of 1 ml min^{-1}. Injection was performed in the splitless mode at 250°C and the injected volume was 1 μl. The column was a HP5-MS fused-silica capillary (30 m×0.25 mm I.D.) coated with a phenylmethyl–silicone phase, film thickness 0.25 μm (Hewlett-Packard). The temperature program was, isothermal 100°C for 5 min, 100–270°C at 10°C min^{-1}, isothermal 270°C for 5 min. The GC–MS interface was heated to 280°C.

2.3. Standard and sample preparation

2.3.1. Standard solutions

Stock standard solutions of calystegines (4 mg ml^{-1}) were prepared in water. Working standard solutions were obtained by diluting stock standard solutions with water. Three calystegines (A5, B1 and B2) were arbitrary chosen for precision evaluation of the developed method. Deoxynojirimycin was used as internal standard at concentration of 0.3 mg ml^{-1}.

2.3.2. Sample preparation

Purified extract of transformed root culture of *Calystegia sepium* was kindly donated by Dr. A. Goldmann (Institut National de la Recherche Agronomique, Versailles, France). *Atropa belladonna* extract (aerial parts) was obtained according to the procedure described elsewhere [12]. The extract was evaporated to dryness, diluted in water and filtered through a 0.2-μm filter before injection.

For GC–MS experiments, silylation of calystegines was performed according to a procedure described elsewhere [13].

3. Results and discussion

Our first attempts to separate calystegines were carried out using high-performance liquid chromatography coupled to pulsed amperometric detection (PAD) using anion-exchange or graphitized carbon columns. Unfortunately, complete separation of investigated calystegines was not possible (results not shown). Thus, capillary zone electrophoresis (CZE) was investigated for the separation of these alkaloids.

Since calystegines possess a very low molar absorptivity, their analysis with UV detection remains a challenge. Among the different approaches developed to overcome lack of chromophore or fluorophore groups, in-situ complexation with borate ions is applied for the analysis of polyhydroxy compounds such as carbohydrates [14–16]. Indeed, complexation of borate ions with vicinal or alternate hydroxy groups under alkaline conditions gives compounds which can be detected by UV at low wavelength.

3.1. Buffer pH

Buffer pH is an important parameter to optimize in CE. Thus, pH effect was investigated between 9.2 and 12, at fixed ionic strength (50 m*M*) and temperature (40°C). It has to be noted that under alkaline

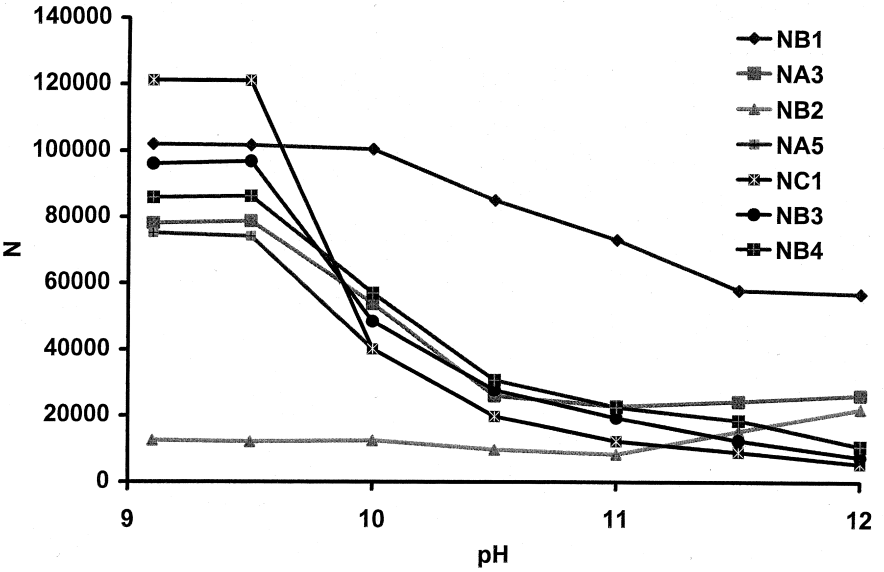

Fig. 2. pH effect on efficiency of selected compounds. Electrophoretic conditions: 50 m*M* sodium tetraborate–sodium hydroxide. Applied voltage 25 kV. Temperature 40°C. Other conditions are given in the Experimental section.

conditions, the presence of borate species allowed in-situ complexation of calystegines to form negatively charged compounds which can be separated by CE. Since experiments were carried out in cationic mode, complexed calystegines migrated after the electroosmotic flow (EOF) marker. Moreover, the presence of borate ions in the BGE resulted in UV absorbance enhancement of selected calystegines. As shown in Fig. 2, increasing pH from 9.2 to 12 resulted in a separation efficiency decrease except for

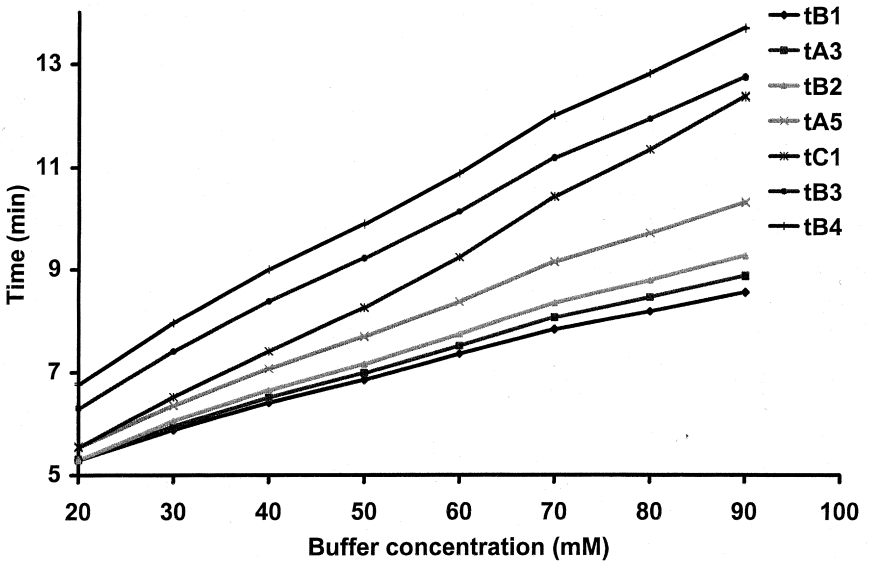

Fig. 3. Buffer concentration effect on the migration time of the selected compounds. Electrophoretic conditions: temperature 40°C, applied voltage 25 kV. Other conditions are given in the Experimental section.

B2. However, migration times were not significantly affected (data not shown). According to these results, a sodium tetraborate buffer solution set at pH 9.2, was selected for subsequent investigations.

3.2. Buffer concentration

Optimization of the method was also investigated in function of sodium tetraborate buffer concentration, from 20 to 90 m*M*. Formation of borate–polyol complexes was favored with increasing sodium tetraborate concentration, which affected calystegine electrophoretic mobilities. Consequently, migration times (Fig. 3), selectivity and efficiency increased allowing a good separation of selected compounds. The increase of migration times was probably due to a decrease of the EOF. Generally, the increase in the number of hydroxyl groups induces high probability of polyol–borate complex formation [17]. Thus, calystegines A (3 OH) migrated faster than B (4 OH) and C (5 OH). Furthermore, the migration order is greatly affected by the position of hydroxy groups within the calystegine structure. In fact, complexation with borate is favored by *cis*- rather than *trans*-oriented hydroxy groups [17]. However, at elevated buffer concentration, the generated electric current was very high, negatively affecting separation performances. As a result, 80 m*M* was selected as a good compromise for high resolution, acceptable migration times and electric current.

3.3. Separation temperature

It has been reported that temperature considerably affects resolution, efficiency and analysis time [17]. Therefore, temperature effect was investigated between 20 and 60°C in the presence of 80 m*M* tetraborate buffer (pH 9.2). As reported in Fig. 4, the migration time of all investigated calystegines decreased with increasing temperature. This behavior is mainly due to viscosity decrease at elevated temperature, resulting in higher electrophoretic and electroosmotic mobilities. Additionally, sensitivity was significantly improved at high temperature since a reduced viscosity affects the injected sample volume. Hence, temperature was fixed at 50°C for rapid separation with high efficiency. It is noteworthy that calystegine C1 exhibits a particular behavior when temperature increases (Fig. 4), which can be explained by the fact that the equilibrium constants of borate complexation depend on calystegine structure.

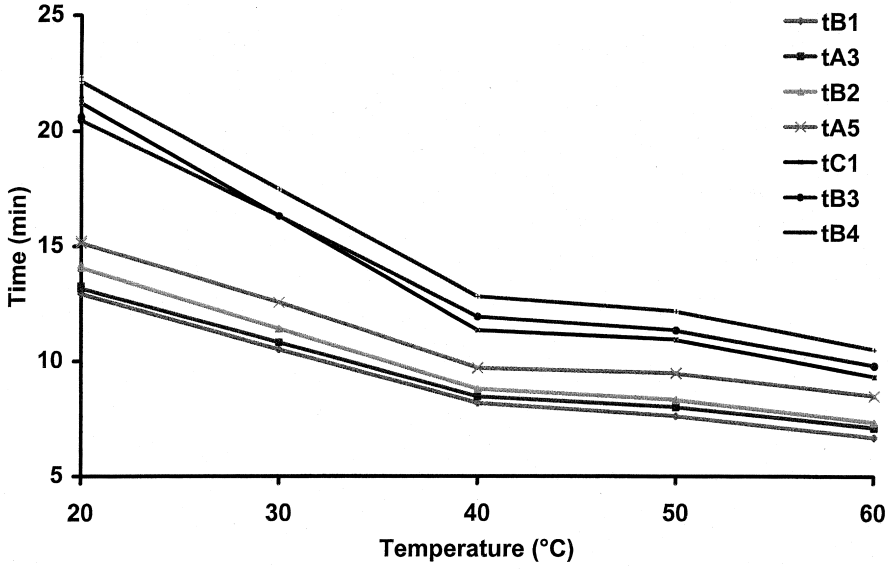

Fig. 4. Effect of temperature on selected compounds migration time. Electrophoretic conditions: 80 m*M* sodium tetraborate (pH 9.2). Applied voltage 25 kV. Other conditions are given in the Experimental section.

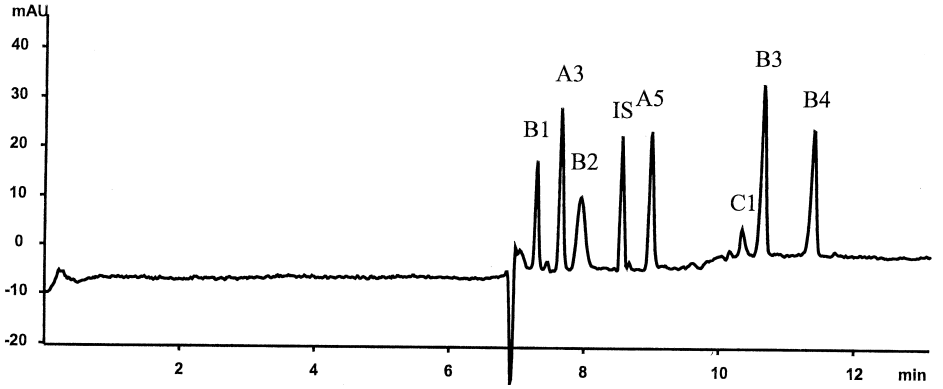

Fig. 5. Typical electropherogram of calystegines mixture (250 μg ml^{-1} each) under optimal conditions. Electrophoretic conditions: 80 m*M* sodium tetraborate (pH 9.2) at 50°C. Applied voltage 25 kV. Other conditions are given in the Experimental section.

Under optimized conditions, baseline separation of the selected calystegines was achieved in less than 12 min (Fig. 5).

3.4. Method sensitivity

The detection limits (LODs) of calystegines, defined as the lowest concentrations of analyte that can be clearly detected, were estimated as three times the signal-to-noise ratio. Estimated LOD values were 25 μg ml^{-1}, 35 μg $ml^{-1}$and 50 μg ml^{-1} for calystegines A5, B1 and B2, respectively.

3.5. Method precision

Precision was evaluated by measuring repeatability and intermediate precision of migration times and corrected peak areas. Repeatability (within-day preci-

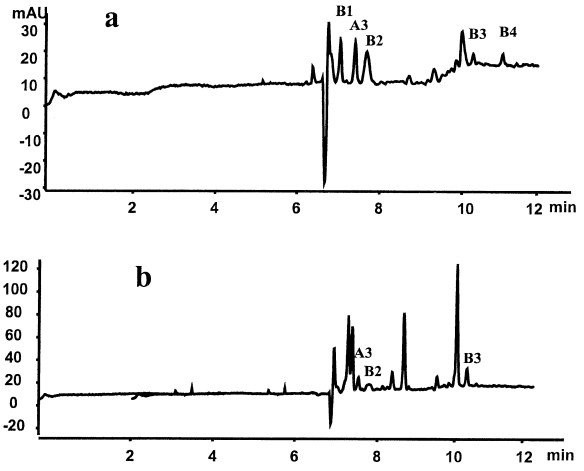

Fig. 6. Typical electropherograms obtained for: (a) *Calystegia sepium* and (b) *Atropa belladonna* extracts. Other electrophoretic conditions as in Fig. 5.

Table 1
Method precision given as RSD values

	RSD (%)		
	Calystegine A5	Calystegine B1	Calystegine B2
Repeatability			
Migration time	0.24	0.17	0.19
Relative peak area to I.S.	1.78	1.82	1.12
Intermediate precision			
Migration time	0.93	0.90	0.99
Relative peak area to I.S.	2.95	2.80	2.83

sion) of the method was determined by performing replicate injections ($n=6$) of a 400 μg ml^{-1} solution containing the three substances and the internal standard. In Table 1, relative standard deviation (RSD) values are given for migration times and peak area ratios of the selected calystegines. In all cases, RSDs were lower than 0.25% for migration time and than 1.9% for peak area ratio. Intermediate precision was also evaluated over 3 days by performing six successive injections daily. Results (Table 1) show that intermediate precision of migration time and peak area was satisfactory.

3.6. Qualitative analysis of plant extracts

Finally, the described method was applied to the qualitative analysis of purified extracts of *Calystegia sepium* and *Atropa belladonna*. As illustrated in Fig. 6a and b, calystegines can be clearly detected in such material. Identification was carried out by spiking extracts with standard calystegines.

In the *Calystegia sepium* extract, all investigated calystegines were present, except A5 and C1. The presence of A3, B1, B2 and B4 has already been reported elsewhere [5,18]. However, to the best of our knowledge, B3 is reported for the first time in this species. In the *Atropa belladonna* extract, the major compounds were A3, B2 and B3. Calystegine B1, previously reported [19], has not been identified by CE in our extract. However, it was detected in small amount by GC–MS. Nevertheless, calystegine B3, is reported in this species for the first time.

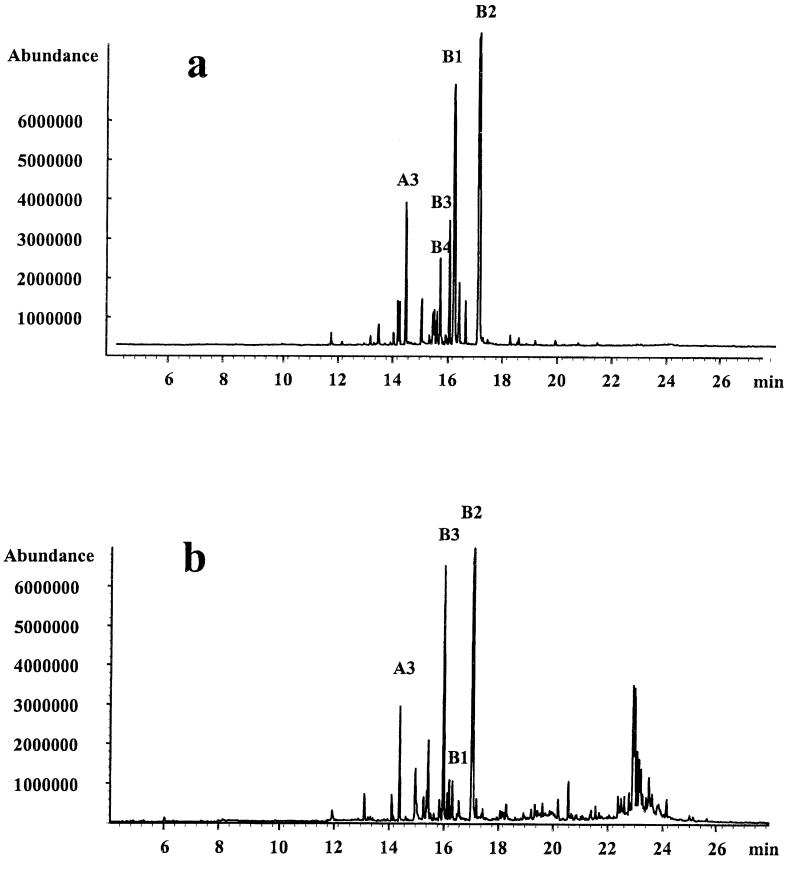

Fig. 7. Typical GC–MS chromatograms obtained for: (a) *Calystegia sepium* and (b) *Atropa belladonna* extracts. The chromatographic conditions are given in the Experimental section.

Besides the investigated calystegines, additional peaks are also present in the electropherogram, which may correspond to carbohydrates or other polyhydroxylated compounds. These results were confirmed by GC–MS experiments, as shown in Fig. 7a and b.

4. Conclusion

The potential of complexation with borate ions to simultaneously separate closely related calystegines was investigated. The separation behavior, including migration time, resolution and efficiency was significantly affected by buffer pH, buffer concentration and temperature. In particular, the number of hydroxy groups as well as their position in calystegine structure was of paramount importance. Successful results were achieved with 80 m*M* borate buffer, pH 9.2 at 50°C. Moreover, the use of extended path length capillary and detection at low wavelength allowed sensitivity improvement. Finally, optimized conditions were successfully applied to the qualitative analysis of selected calystegines in plant extracts and results were confirmed using GC–MS experiments. The described method allowed identification of calystegines not previously reported in the investigated plant material. However, despite the high resolution of the method and the possibility to use conventional UV detection, a serious sensitivity enhancement is still required. In this context, the use of other detection methods such as MS or amperometry may overcome these limitations.

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