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Simultaneous determination of scopolamine, hyoscyamine and littorine in plants and different hairy root clones of *Hyoscyamus muticus* by micellar electrokinetic chromatography

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Abstract

Hyoscyamus muticus hairy root clones were established following infection with *Agrobacterium rhizogenes* strains A4, LBA-9402 and 15834 and with *A. tumefaciens* strain C58C1pRTGus104. The accumulation of tropane alkaloids hyoscyamine, littorine and scopolamine was evaluated by micellar electrokinetic capillary electrophoresis. Littorine was reported for the first time in these clones as well as in the roots of the intact plant and confirmed by collision induced dissociation-mass spectrometry. Tropane alkaloid content in hairy roots was compared with leaves and roots of normal plants at two vegetative stages. Significant differences appeared between the alkaloid contents of the different clones. In particular, all the hairy root clones and the roots of the intact plant produced 1.5–3 and 4.5–9 times more littorine than scopolamine, respectively. The only exception was clone KB7, carrying the *h6h* gene, which overproduced scopolamine. The aerial parts of *H. muticus* plants did not contain any littorine, thus indicating different transportation or translocation mechanisms of the various tropane alkaloids.

Keywords: *Hyoscyamus muticus*; Solanaceae; Egyptian henbane; Hairy root clones; Tropane alkaloids; Scopolamine; Hyoscyamine; Littorine

1. Introduction

Tropane alkaloids, especially hyoscyamine (**3**) and scopolamine (**1**), are widely used in medicine because of their strong parasympatholytic actions. The synthetic production of these alkaloids is more expensive than their extraction from plant material and they are, therefore, currently industrially isolated from various solanaceous plants belonging to genera *Atropa*, *Duboisia*, *Datura* and *Hyoscyamus* (Bruneton, 1993). During the past years, considerable efforts have been made to develop an economically feasible in vitro production of these compounds. In this perspective, *Agrobacterium*-mediated transformed roots, so-called hairy roots, may represent a valuable alternative for the production of tropane alkaloids (Mano et al., 1989; Oksman-Caldentey and Hiltunen, 1996).

Hairy roots of *Hyoscyamus muticus* are fast growing in hormone-free medium, are genetically stable and produce high contents of hyoscyamine, the main tropane alkaloid (Sevón et al., 1998; Oksman-Caldentey et al., 1994). However, there is a large somaclonal variation between clones even of the same origin, and usually scopolamine is only found in trace amounts (Sevón et al., 1998). In addition, very little attention has been paid to other closely related tropane alkaloids due to the lack of suitable analytical methods. In particular, littorine (**4**), a positional isomer of hyoscyamine, is difficult to separate from the latter.

Recently, it has been demonstrated that the alkaloid pattern can be shifted towards scopolamine when

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introducing the *h6h* gene which is involved in the biosynthesis of scopolamine from hyoscyamine (Yun et al., 1992; Jouhikainen et al., 1999). The *Agrobacterium* strain used for transformation has also considerable effect on hyoscyamine and scopolamine production (Sevón et al., 1998; Vanhala et al., 1995). Tropane alkaloids have been determined using various chromatographic methods including TLC (De Zeeuw et al., 1994), GC (Majlat, 1982; Hartmann et al., 1986; Ballbach et al., 1977) and LC (Lund and Hansen, 1978; Gfeller et al., 1979; Fliniaux et al., 1993; Takahashi et al., 1997; Lau and Mok, 1997). However, these techniques are time consuming, may require derivatization processes or addition of ion pairing reagents. Immunological methods (Oksman-Caldentey et al., 1987a; Weiler et al., 1981) have also been used in the analysis of crude plant samples. Nevertheless, such techniques are expensive and do not allow simultaneous analysis of several related compounds. Recently, capillary electrophoresis (CE) has evolved as an interesting alternative for the analysis of secondary metabolites of plant material (Tomás-Barberán, 1995; Issaq, 1997). It offers several benefits compared to conventional analytical methods e.g. high efficiency, short analysis time, low solvent consumption and cost effectiveness. A capillary zone electrophoresis (CZE) method was developed using an uncoated fused silica capillary for the separation and the determination of various tropane alkaloids (Eeva et al., 1998). The method was applied to the dosage of hyoscyamine and scopolamine in two transgenic Egyptian henbane plants. However, under these conditions (40 mM phosphate buffer, pH 7.8), littorine was not separated from hyoscyamine leading to an overestimation of the hyoscyamine content.

The aim of the present study was to investigate the influence of various strains of *Agrobacterium* on the tropane alkaloid content of different *H. muticus* hairy root clones. Besides hyoscyamine and scopolamine, particular attention was given to whether transformed root cultures are capable of producing high contents of littorine. Furthermore, the range of alkaloids produced in hairy roots was compared to those found in the leaves and roots of the non-transformed plants at two vegetative stages.

2. Results and discussion

Due to the complexity of the plant material and the structural similarity of tropane alkaloids, a micellar electrokinetic capillary chromatography (MEKC) was selected to separate these compounds. The method was found efficient for the separation of alkaloids including scopolamine, hyoscyamine and littorine and was successfully applied to the quantitative analysis of these metabolites in various solanaceous species (Cherkaoui et al., 1997; Mateus et al., 1998, 2000). In order to investigate the effects of the different *Agrobacterium* strains on tropane alkaloid production, this electrophoretic method was applied to non-transformed root and leaf samples as well as to genetically modified root samples of *H. muticus*. The analyses were performed in the presence of homatropine (**2**) as internal standard. As illustrated in Fig. 1 and Table 1, hyoscyamine was the main alkaloid in all the transformed roots as well as in the non-transformed plants. Clones KB7, LBA-1S, A4 and C58-A were particularly rich in hyoscyamine. Furthermore, the hyoscyamine content was higher in the young leaves than in the old ones, whereas in the roots it remained very similar. Scopolamine was not detected in all the hairy root clones. Two clones (C58-A and LBA-1S) were devoid of scopolamine.

In the non-transformed plants, scopolamine content was lower in the roots and leaves of the younger plants and increased significantly when the plants matured. This is in accordance with our earlier findings (Oksman-Caldentey et al., 1987b). It is also well known that the biosynthesis of tropane alkaloids takes place in the roots (Hashimoto et al., 1991) and that they are transferred into the leaves and stored preferentially as scopolamine. Therefore, it is not surprising to find four times more scopolamine in the older leaves than in the younger ones.

In our previous paper (Mateus et al., 1999), capillary zone electrophoresis (40 mM ammonium acetate, titrated with 10% (v/v) ammonia solution at pH 8.5) was interfaced with electrospray ionization mass spectrometry (CE-ESI-MS) for the analysis of tropane alkaloids in plant extracts. Because hyoscyamine and its bioprecursor, littorine, (Robins et al., 1994), were not separated under these electrophoretic conditions, a collision induced dissociation (CID) was performed by increasing the fragmentor voltage within the MS ion focusing region. It has been shown that beside the molecular ion at m/z 290, both MS spectra showed a peak at m/z 124, which corresponds to the loss of tropic acid and phenyllactic acid for hyoscyamine and littorine, respectively. Furthermore, at a high fragmentor voltage (200 V), littorine showed an additional mass at m/z 142. Therefore, CID based on this discriminating fragment has been found a suitable tool to control the presence of littorine in plant extracts. In order to assess the presence of littorine in the studied extracts, the CE-MS method was applied and the fragmentation procedure performed by setting the skimmer voltage at 200 V. In the case of clone KB7, Fig. 2 illustrates a total ion current electropherogram under optimized CZE conditions. It has to be noted that beside hyoscyamine (**3**) and scopolamine (**1**), 6β-hydroxyhyoscyamine (**6**) and tropine (**5**), which does not possess a chromophore, are clearly detected by MS. Sub-

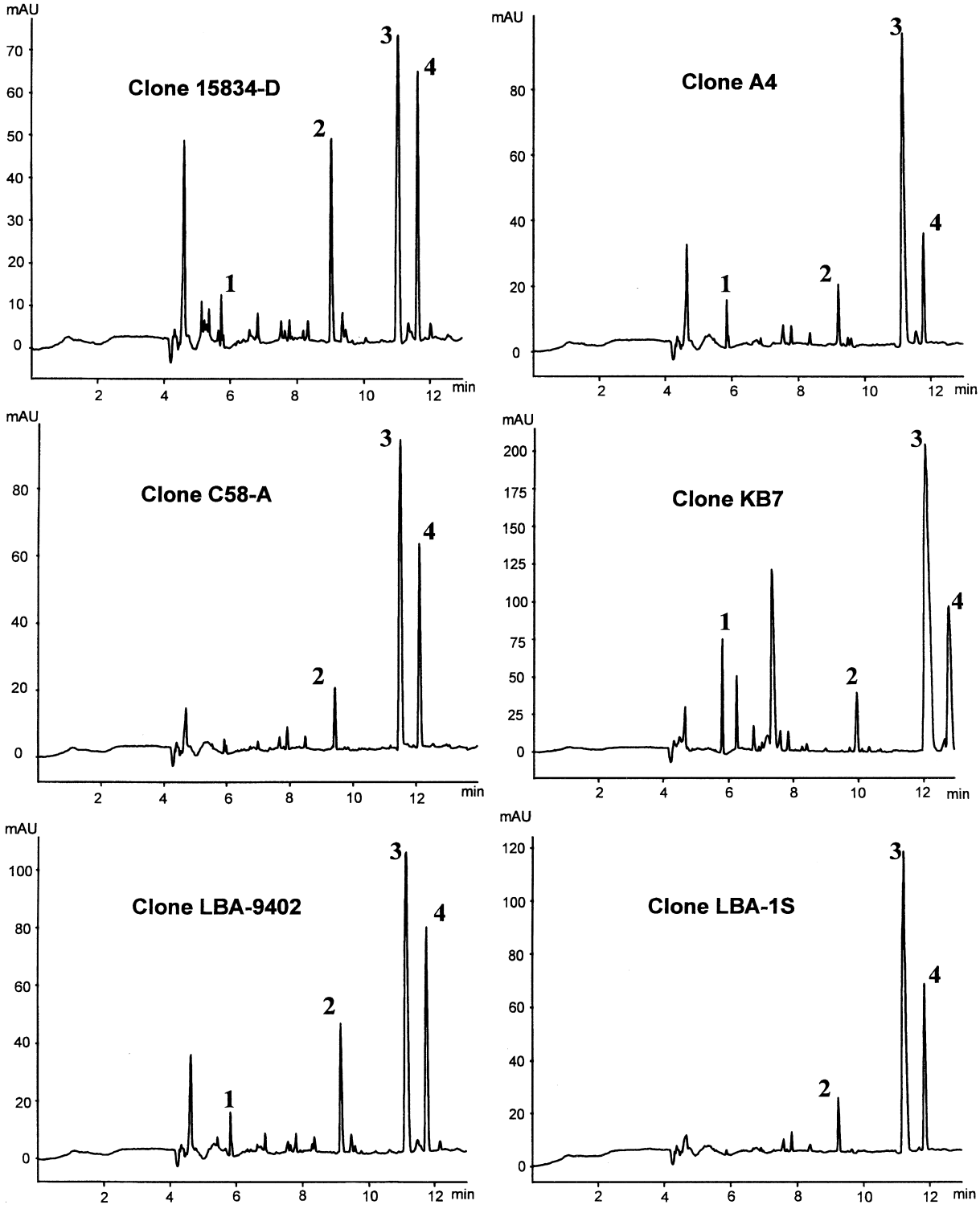


Fig. 1. Electropherograms of different clones of *H. muticus*. Detection was performed at 195 nm. Other electrophoretic conditions as described in Section 3.

Table 1
Scopolamine, hyoscyamine and littorine contents in plant leaves and roots of *H. muticus* at two vegetative stages

Sample	Scopolamine mg/kg (dry wt)	Hyoscyamine mg/kg (dry wt)	Littorine mg/kg (dry wt)
Plants before flowering			
Leaves	270	2230	n.d.[a]
Roots	130	5610	1180
Plants after seed formation			
Leaves	1000	940	n.d.[a]
Roots	260	6510	1170

[a] Not detectable (value below the quantification limit of the method).

sequently, MS spectrum of peak (**3**, **4**) exhibits a fragment ion at m/z 142, which attests the presence of littorine (Fig. 3).

Littorine was present in all the investigated clones (Fig. 1). The occurrence of this compound at low levels (approx. 100–300 mg/kg dry wt depending on the growth media) in *H. muticus* hairy roots established from *A. rhizogenes* strain 15834 only has earlier been reported (Jaziri et al., 1994). In the present study, littorine was also detected in the roots of the intact plant (Table 1). Its production was constant in the two different vegetative stages of the plant and reached about 20% of that of hyoscyamine. To the best of our knowledge, this metabolite is reported in *H. muticus* for the first time but has been found in other *Hyoscyamus* species (Hashimoto et al., 1986). We were not able to detect littorine either in the young or in the old leaves. This might indicate different transportation mechanisms to the aerial parts in various tropane alkaloids and also in different solanaceous plants or that the compound is not translocated to the leaves at all. *Agrobacterium* strains used for transformation seem to have a great importance on the tropane alkaloid contents and the alkaloid pattern. This is clearly demonstrated by the large variation of tropane alkaloid production in the different hairy root clones of *H. muticus*. All clones produced on the average 1.5–3 times more littorine than scopolamine and 2–7 times less littorine than hyoscyamine (Table 2). The only exception was clone KB7 which overproduced scopolamine because of the *h6h*-transgene (Jouhikainen et al., 1999), and gave levels exceeding those found in the intact plant (Table 1).

To conclude, micellar electrokinetic chromatography demonstrated to be suitable for the simultaneous quantitative determination of hyoscyamine, littorine and scopolamine from plant material. The presence of littorine was assessed by collision induced dissociation.

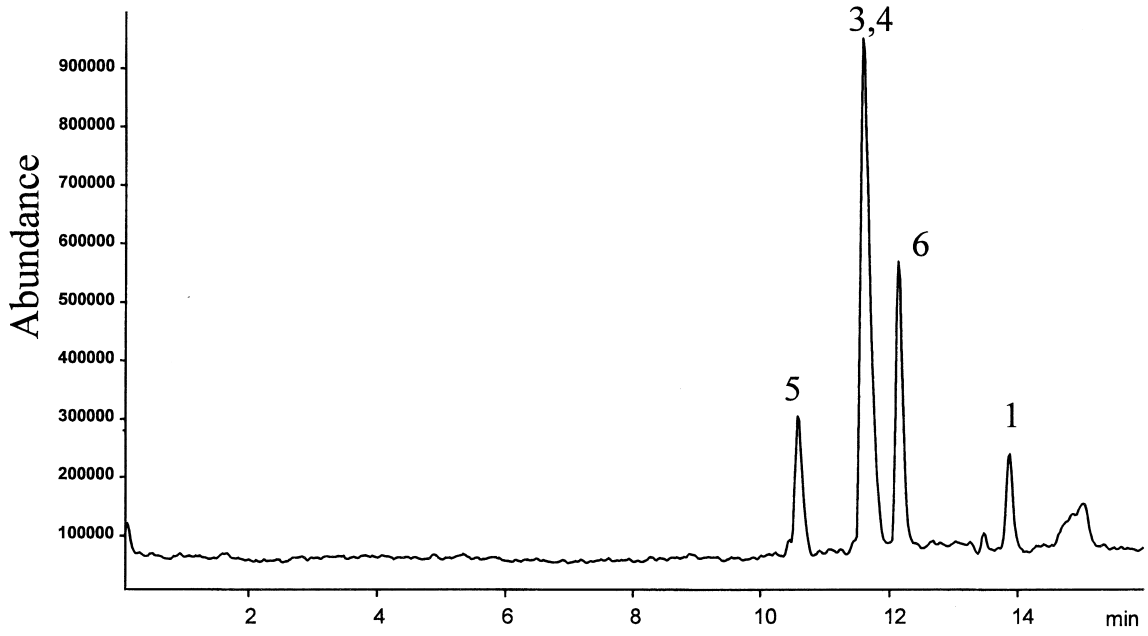


Fig. 2. CE-ESI-MS analysis of clone KB7 (total ion current electropherogram). 1 : Scopolamine, 3 : hyoscyamine, 4 : littorine, 5 : tropine, 6 : 6β-hydroxyhyoscyamine. Electrophoretic conditions were as described in Mateus et al., 1999.

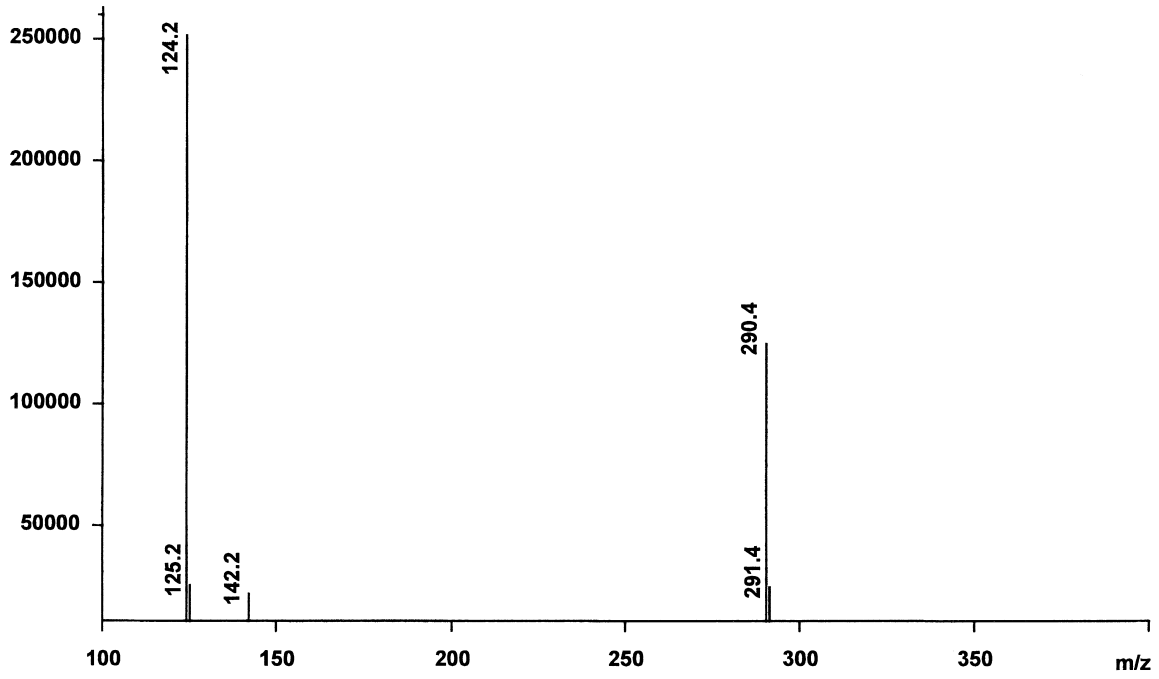
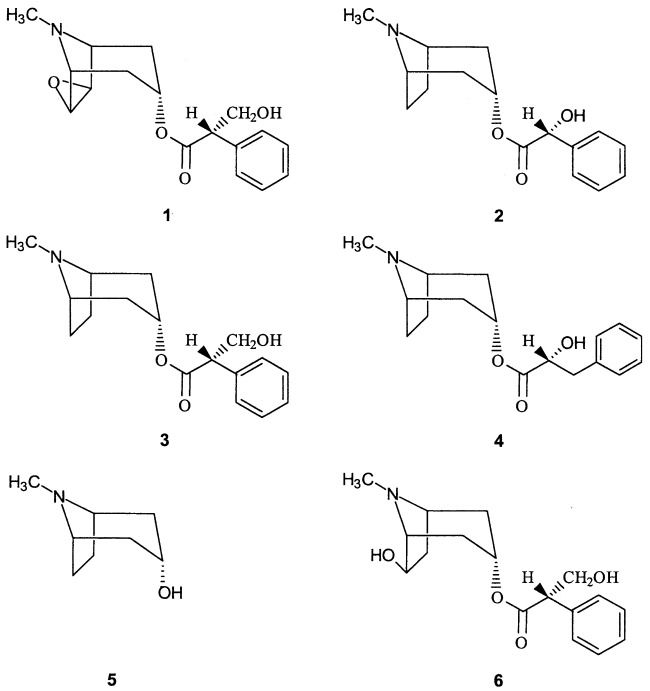


Fig. 3. CE-ESI-MS spectrum of peak (**3**, **4**) obtained at 200 V. The presence of peak at 142 indicates the occurrence of littorine in the extract.

A great variability in the alkaloid contents was observed between the different clones which is in accordance with results already published (Sevón et al., 1998; Jouhikainen et al., 1999). For the first time littorine was detected in these clones and in *H. muticus* intact roots.



3. Experimental

3.1. Plant material

H. muticus L. strain Cairo (Solanaceae) plants were grown in a greenhouse. The intact root and leaf samples were collected during the early flowering stage when alkaloid production is known to be at the maximum (Oksman-Caldentey et al., 1987b). As a comparison, leaf and root samples were also collected from the mature plant after seed formation. Lyophilized plant material was used for analysis.

The young leaves were infected with various *Agrobacterium* strains (*A. rhizogenes* A4, LBA-9402, 15834 and *A. tumefaciens* C58C1pRTGus104) to obtain the hairy root cultures. Clones A4, LBA-9402, LBA-1S, C58-A and 15834-D2 were the same as used in our previous studies, and the transformation procedure is described elsewhere (Oksman-Caldentey et al., 1994; Vanhala et al., 1995). Clone KB7 carries the 35S-*h6h* transgene that codes for the enzyme hyoscyamine-6β-hydroxylase and thus produces more scopolamine than the other clones obtained by transformation with wild type *Agrobacterium* strains (Jouhikainen et al., 1999).

The hairy root clones were grown in liquid cultures (20 ml medium in 100 ml Erlenmeyer flask). 100 ± 5 mg of root material was inoculated in the modified hormone-free B50-medium (Oksman-Caldentey et al., 1991), and the roots were cultured for 28 days in the conditions described elsewhere (Oksman-Caldentey et

Table 2
Scopolamine, hyoscyamine and littorine contents in different hairy root clones of *H. muticus*

Hairy root clones	Scopolamine mg/kg (dry wt)	Hyoscyamine mg/kg (dry wt)	Littorine mg/kg (dry wt)
KB7	1520	5860	880
15834-D2	200	1160	610
C58-A	n.d.[a]	3510	1680
A4	640	4400	920
LBA-9402	260	1810	870
LBA-1S	n.d.	5370	1930

[a] Not detectable (value below the quantification limit of the method).

al., 1991). The roots were harvested by filtering, washed twice with sterile water and lyophilized. Dry root material was used for alkaloid analysis.

3.2. Sample and extract preparation

Hyoscyamine sulfate, scopolamine hydrobromide were purchased from Sigma (St-Louis, MO, USA), homatropine and tropine from Fluka (Buchs, Switzerland). Littorine was a gift from Dr K. Shimomura (Tsukuba Medicinal Plant Research Station, Japan) and 6β-hydroxyhyoscyamine was obtained from Dr M.F. Roberts (University of London).

Stock standard solutions were prepared by dissolving each compound in methanol (1.0 mg/ml) and diluted in water to obtain standard solutions at a final concentration of 0.1 mg/ml. Water allowed sample stacking which was effective in enhancing sensitivity (increasing peak shape) by on-column preconcentration of the sample within the capillary. A peak area calibration curve for hyoscyamine, scopolamine and littorine was established over a sample concentration range of 50–125 μg/ml. Homatropine (100 μg/ml) was used as internal standard.

For alkaloid extraction, 100 mg of powdered plant or tissue culture material were extracted three times with 8 ml $CHCl_3$–MeOH-concentrated NH_4OH (15:5:1 v/v/v) with sonication for 20 min and vortex mixing at 5 min intervals to avoid aggregation of the powdered sample. After centrifugation (5000 rpm, 10 min), supernatants were collected and evaporated under reduced pressure to dryness. The residue was basified with 3 ml distilled water-concentrated NH_4OH (50:50 v/v) and applied on an Extrelut®-3 column. Alkaloids were eluted with 3×8 ml CH_2Cl_2. The solvent was evaporated to dryness and the residue diluted with water in order to obtain the considered analyte at a final concentration of about 100 μg/ml.

3.3. Analytical procedures

Electrophoresis was carried out on a HP^{3D} capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode-array detector as described elsewhere (Mateus et al., 1998). The capillary (Hewlett-Packard) was 64.5 cm long (56 cm effective length) × 75 μm ID. Detection at 56 cm from the point of sample introduction was set at 195 nm with a bandwith of 10 nm. All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 25°C and a constant voltage of 30 kV, with an initial ramping of 500 Vs^{-1}, was applied during analysis. Sample injections (ca 18 nl injection volume) were achieved using the pressure mode for 5 s at 30 mbar. Determination of hyoscyamine, scopolamine and littorine was performed using a micellar electrokinetic capillary chromatography. This method involves the use of a micellar buffer consisting of 30 mM phosphate–borate at pH 8.7, in the presence of 40 mM SDS and 16.5% acetonitrile. For each experiment, triplicate injections were carried out.

Electrospray mass spectrometry measurements were carried out in the positive ionization mode and were performed in a single quadrupole HP Series 1100 MSD. The coupling of CE instrument to the MS detector was performed according to the procedure described elsewhere (Mateus et al., 1999). In CE-MS experiments, a volatile buffer consisting of 40 mM ammonium acetate titrated with 10% ammonia solution at pH 8.5 was used.

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