Use of porous graphitic carbon column for the separation of natural isomeric tropane alkaloids by capillary LC and mass spectrometry

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Abstract

This study presents the outstanding chromatographic selectivity of a porous graphitic carbon support for the separation of four isomeric tropane alkaloids from the stem-bark of *Schizanthus grahamii* (Solanaceae). Capillary liquid chromatography coupled to mass spectrometry was studied after the appropriate selection of mobile phase composition, temperature, nature and concentration of the acidic modifier. Fragmentation behaviour by in-source collision-induced dissociation (CID) on a single quadrupole mass spectrometer, or MS^n with an ion-trap, was investigated for structural identification. The ability to differentiate the isomers by in-source CID was demonstrated and a fragmentation pathway, based on MS^n sequences together with accurate mass experiments, was proposed.

Keywords: Capillary LC–MS; Mass spectrometry; Porous graphitic carbon; Temperature selectivity; Isomeric separation; Tropane alkaloids

1. Introduction

Tropane alkaloids, with their well-known pharmalogical activities and significant therapeutic importance, are extensively used in modern medicine. This class of alkaloids is relatively widely distributed in the plant kingdom and mainly occurs in the Convolvulaceae, Erythroxylaceae, Proteaceae, Rhizophoraceae and Solanaceae families [1]. The genus *Schizanthus* [2] belongs to the latter family and presents a wide range of tropane alkaloids [3]. Indeed, the chemical evolution in *Schizanthus* runs from simple pyrrolidine derivatives to tropane ester series derived from angelic, tiglic, senecioic, itaconic or mesaconic acids leading to numerous positional and configurational isomers [4–11].

Liquid chromatography (LC) methods are widely used for the separation of tropane alkaloids [12,13] with reversed-phase (RP) supports. Nevertheless, these supports have limited selectivity towards isomeric compounds. On the other hand, normal stationary phases have a high selectivity towards compounds possessing similar chemical structures [14,15] which makes them well suited for the separation of isomeric tropane alkaloids by thin layer chromatography [16,17]. However, these normal stationary phases present many drawbacks and are rarely used in routine LC for the separation of tropane alkaloids.

Porous graphitic carbon (PGC) stationary phase [18] offers interesting alternative properties. It possesses a rigid and planar surface together with functions capable of strong charge-transfer interactions which contribute to the driving force separating closely related compounds [19,20]. In addition, its polarizable surface is virtually free of residual surface defects which makes it particularly suitable for the analysis of basic metabolites. PGC columns have already been used for the separation of closely related structures [21–23].

In this paper, a PGC packing was used for the separation of four isomeric hydroxytropane esters (Fig. 1) from *Schizanthus grahamii* Gill. by systematically evaluating different

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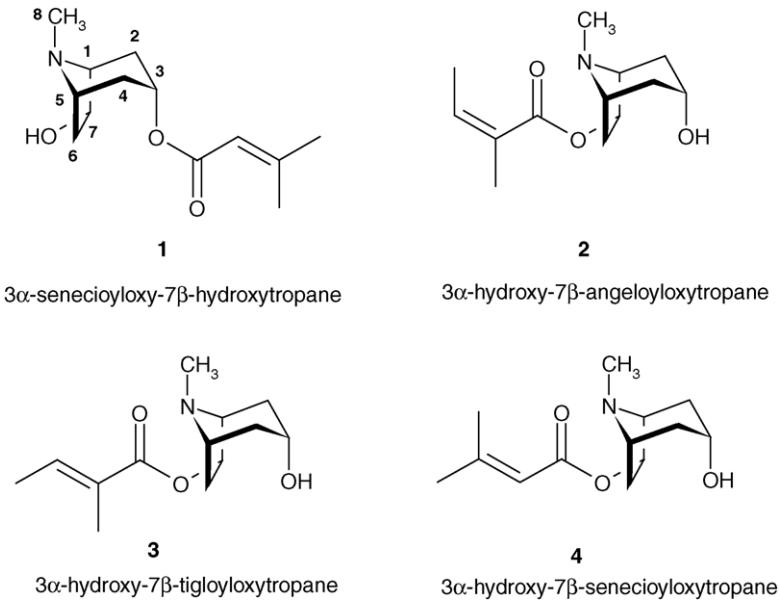

Fig. 1. Structure of the four isomeric hydroxytropane esters (MW = 239 g/mol).

chromatographic parameters such as mobile phase composition, concentration and nature of the acidic modifier, pH and temperature. Capillary LC was chosen to handle the limited sample amounts available, as well as for its flow rate compatibility with the electrospray ionisation process. In order to establish typical fragmentation pathways of the investigated tropane alkaloids, in-source collision-induced dissociation (CID) on a single quadrupole was compared to MS^n experiments performed on an ion-trap mass spectrometer. Finally, the exact mass of the precursor ion and of each fragment was determined by high resolution MS.

2. Experimental

2.1. Plant material

S. grahamii Gill. was collected in Rengo (central Chile) in January 2000. The stem-bark (2.6 kg) was extracted with ethanol at room temperature. After filtration, the alcoholic solution was evaporated to dryness. The residue was taken up in 0.1 M HCl and washed with dichloromethane. The aqueous solution was then basified to pH 12 with NH_4OH and further extracted with dichloromethane yielding to a gummy alkaline residue (6.6 g). Further purification onto an aluminium oxide column was performed according to Muñoz et al. [7] leading to the investigated purified fraction containing the four isomeric hydroxytropane esters.

2.2. Chemicals

LC grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Panreac Quimica SA (Barcelona, Spain). Formic (FA), acetic (AA) and trifluoroacetic (TFA) acids as well as ammonium formate were from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was provided by a Milli-Q Gradient A10 unit from Millipore AG (Volketswil, Switzerland).

2.3. Capillary LC-UV conditions

An UltiMate Capillary LC System was used (LC Packings—A Dionex Company, Amsterdam, The Netherlands) with a FAMOS Well Plate Micro-autosampler, a UV detector and a thermoregulated column compartment. The wavelength was set at 220 nm. Chromatographic separations were performed either on a HyperCarb capillary column (15 cm × 300 μm i.d., 7 μm/250 Å, ThermoHypersil phase packed by LC Packings) or on a PepMap C18 capillary column (15 cm × 300 μm i.d., 5 μm/80 Å, LC Packings). Acidic aqueous mobile phase (phase A) and organic mobile phase (phase B) contained from 0.05% to 0.4% (v/v) formic, acetic or trifluoroacetic acid. For basic eluents, 50 mM ammonium formate aqueous solution was adjusted to the required pH (i.e. 8.0 and 9.5) with concentrated ammonia. The organic mobile phase consisted of pure methanol. Isocratic elution was systematically carried out and conditions are specified in figure legends. Flow rate was set at 4 μl/min for both columns. Experiments were carried out at 25, 40 and 60 °C by preheating 20 cm of the LC fused-silica tubing ahead from the column inlet. Finally, 0.1 μl sample material was systematically injected onto the capillary columns.

2.4. Mass spectrometry conditions

Single quadrupole and in-source CID mass spectra were recorded on a HP series 1100 MSD (Agilent Technologies,

Waldbronn, Germany) between *m*/*z* 70 and 250 Th. Measurements were carried out in the positive ionisation mode using an orthogonal pneumatically assisted electrospray interface (ESI). The following ESI parameters were applied: electrospray voltage of 3500 V, nitrogen nebulizing gas pressure was set at 10 psi. Furthermore, the nitrogen drying gas flow rate was 5 l/min at a temperature of 250 °C.

Multiple stage MS measurements were performed with a HP 1100 Series MSD-Trap-SL (Agilent Technologies) using an electrospray interface equipped with an orthogonal microsprayer. The electrospray conditions were the same as abovementioned. A maximum trap filling time of 50 ms was chosen to limit the number of ions entering into the trap to avoid space charging effects. Ionised species were individually isolated and further fragmented. Fragmentation amplitude was set at 1.0 V to excite the precursor ion. Full scan mass spectra were taken from *m*/*z* 80 to 250 Th and specific product ions were selected to produce extracted mass chromatograms.

Accurate mass measurements were performed on a QqTOF instrument (QSTAR XL, AB/MDS Sciex, Concord, Ont., Canada) for structural characterization of MS/MS fragments. TOF-MS spectra were acquired from *m*/*z* 200 to 300 Th with an accumulation time of 1 s. Product ion spectra were recorded over the mass range of *m*/*z* 70 to 250 Th with a collision energy of 30 eV (nitrogen gas set at 5, laboratory frame). Data acquisition and QSTAR XL instrument control were performed by Analyst QS software (version 1.0 SP8).

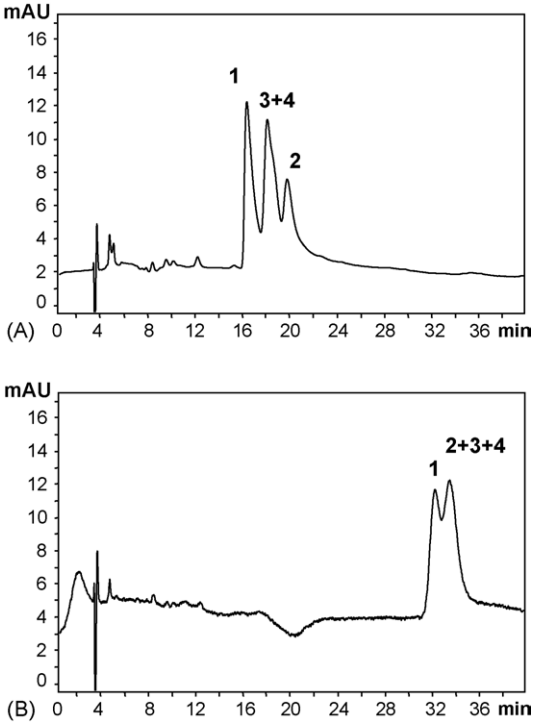

Fig. 2. Capillary LC-UV analysis of the purified plant extract on a porous graphitic column under alkaline conditions: (A) pH 8.0 at 25 °C; (B) pH 9.5 at 25 °C.

3. Results and discussion

3.1. Chromatographic separation

Separations were performed under isocratic LC conditions with MeOH as organic modifier because of its lower eluent strength compared to MeCN and because MeOH is generally more selective than MeCN. As expected, reversed phase material was unable to baseline separate the four isomeric tropane alkaloids (Fig. 1), neither in acidic nor in alkaline mobile phase (data not shown).

With the PGC column, several parameters were tested (e.g. acidic modifier, mobile phase composition, column temperature) to obtain a set of conditions which provided a baseline compound separation in the shortest period of time. This packing material can provide unusual retention mechanisms due to its rigid, planar surface with sites capable of dispersion as well as charge-transfer interactions.

Alkaline LC conditions were tested first. As shown in Fig. 2, the four isomers could neither be separated at pH 8.0 nor at pH 9.5. In the latter conditions, analysis time significantly increased since the alkaloids were mostly under their unionised form.

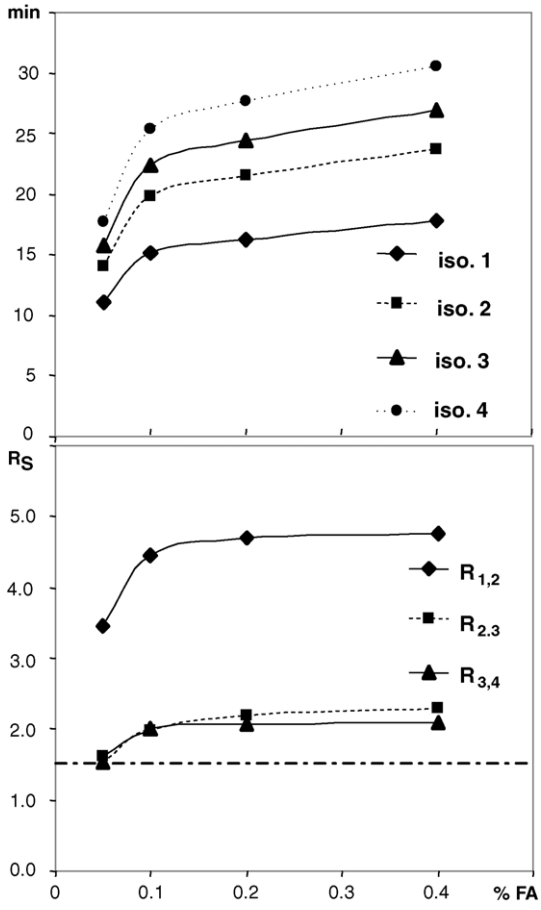

Fig. 3. Influence of formic acid (FA) concentration on separation time and peak resolution (*R*).

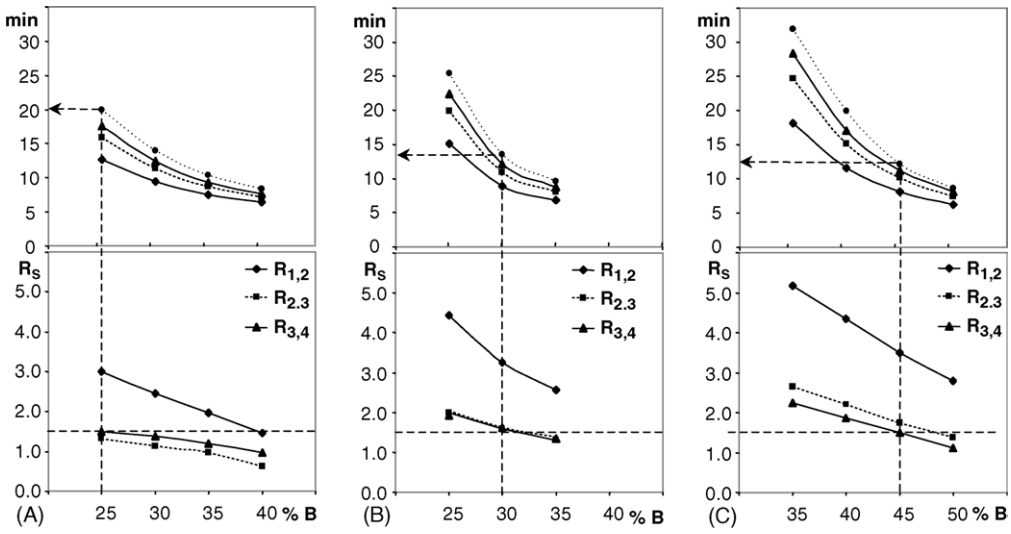

Fig. 4. Influence of the MeOH percentage on separation time and resolution (R_S) with: (A) AA; (B) FA; (C) TFA as acidic modifiers.

A complete separation of all isomers was only possible in acidic mobile phase conditions. The nature of the interactions between the graphitic surface and analytes was strongly influenced by the acidic additive which acts as an electronic modifier. Therefore, the effect of the concentration of formic, acetic and trifluroroacetic acids was evaluated. These volatile acids were preferentially chosen for further MS requirements. Fig. 3 shows that above 0.1% (v/v) formic acid, all isomers were baseline separated and higher concentration did not significantly improve resolution, while separation time increased. The same tendency was observed for acetic and trifuoroacetic acids (data not shown). Fig. 4 depicts the effect of MeOH percentage in the mobile phase on separation and puts forward the influence of the acidic modifier. Fig. 4A shows that with 0.1% acetic acid a baseline separation of the critical isomers was hardly obtained in 20 min with 25% MeOH. Formic acid (Fig. 4B) and TFA (Fig. 4C) both presented good performances, but for further ESI-MS experiment, FA was preferred to avoid eventual signal suppression effects [24]. It has to be noted that, with TFA, the critical isomeric peak pair is not the same as with AA. Moreover, TFA, known as a good ion-pairing agent, led to increased retentions and thus a higher organic solvent percentage was required for elution. Consequently, 0.1% (v/v)

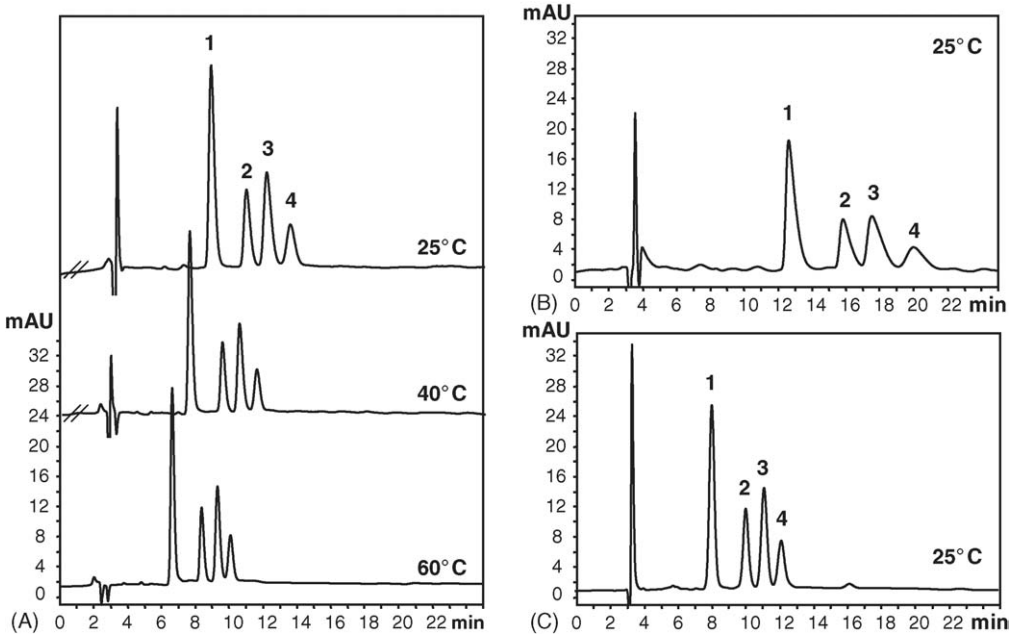

Fig. 5. Separation of the four isomers using the PGC media. (A) Conditions obtained with 0.1% (v/v) FA at 25, 40 and 60 °C; (B) 0.1% (v/v) AA at 25 °C; (C) 0.1% (v/v) TFA at 25 °C.

formic acid in 30% MeOH was selected for further experiments.

Finally, the effect of column temperature was investigated to speed up the analysis [15,25,26]. The influence on resolution was small but analysis time was significantly reduced, as reported in Fig. 5A. The efficiency, measured on the last isomer, increased from 3360 to 4880 theoretical plates when heating up from 25 to 40 °C and finally reached 6220 theoretical plates at 60 °C. Separations with AA and TFA at 25 °C are shown in Fig. 5B and C, respectively. Finally, the LC conditions obtained at 60 °C with 0.1% (v/v) FA in MeOH/water were retained to conduct MS experiments.

3.2. In-source CID experiments (single quadrupole MS)

LC–MS is a technique of choice but single quadrupole MS presents some limitations for structural identification studies. Indeed, for the tested isomeric compounds, all MS spectra were identical and showed only the protonated molecular ion at *m/z* 240 Th (Fig. 6A). Therefore, structural information

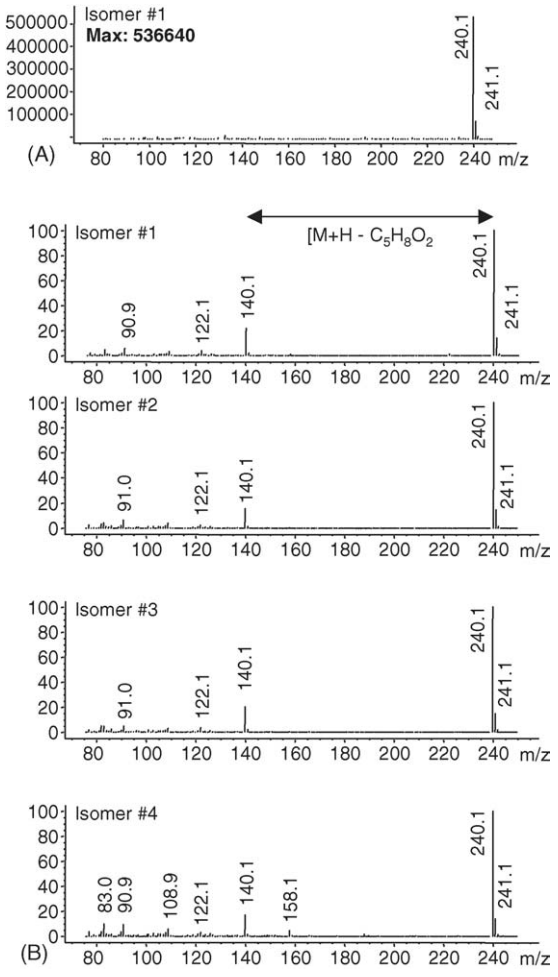

Fig. 6. (A) Single quadrupole MS spectrum of peak 1 (skimmer voltage at 80 V); (B) MS spectra of the four isomeric compounds with in-source CID (skimmer voltage at 200 V).

under these conditions was poor. In order to induce fragmentation, the skimmer voltage was increased from 80 to 200 V. The in-source collision-induced dissociation (CID) of the molecular ions lead to the formation of product ions (Fig. 6B). The latter were common to each isomer, except the fragment at *m/z* 158 Th, which was detected only in the case of the last eluting isomer. Product ion at *m/z* 140 Th was attributed to the tropane ring generated by the loss of the C5 acidic moiety (i.e. loss of 100 Da).

3.3. MS^n experiments (ion-trap MS)

MS^n experiments offer significant structural information by generating fragmentation pathways. However, no general fragmentation rules have been established when using electrospray ionisation (ESI). Nevertheless, the fragmentation pathway of the investigated alkaloids enabled to collect relevant structural information. Fig. 7A shows the extracted ion chromatogram of the pseudo-molecular ion at *m/z* 240 Th with the corresponding MS/MS spectra of the four isomers. Fig. 7B shows the extracted ion chromatogram of the fragment selected as a diagnostic ion (*m/z* 158 Th) for performing MS^3 experiments. Only isomers **2** and **4** showed a response towards this diagnostic ion which was subsequently fragmented into the tropane moiety (fragment at *m/z* 140 Th in MS^3 spectra). Further MS^4 experiments generated a fragment ion at *m/z* 122 Th by the second loss of a water molecule (data not shown). Thus, a fragmentation pathway (Fig. 8) could be proposed. Moreover, accurate mass measurements were performed with a QqTOF instrument (Fig. 8). Unfortunately, the geometrical configuration of the isomeric acid moiety could still not be determined unambiguously. So far, only peak 1 was unequivocally attributed to 3α-senecioyloxy-7β-hydroxytropane since the reference compound was at disposal. Peaks 2 and 4 did not correspond to a tigloyl derivative. This was suggested by the fragmentation pathway which showed the distinctive diagnostic ion at *m/z* 158 Th (Fig. 7). This fragment involves the neutral loss of a 82 Da moiety. Such a loss may be explained by a rearrangement that is sterically hindered for tigloyl substituted tropanes. The proposed fragmentation can be described as having two steps, including the transfer of a hydrogen atom from the olefinic methyl group to the alcoholic oxygen atom from the tropane moiety followed by the scission of the ester function (Fig. 8). The fragmentation is possible only if the methyl is orientated in a *Z* configuration. This positions an hydrogen in a six-ring arrangement (Fig. 8). In the *E* configuration of the double bond geometry (tigloyl derivatives), such a rearrangement is unlikely because hydrogen atoms are inaccessible. This proposed rearrangement lead to a stable neutral keten moiety and finally suggested that peak 3 corresponds to 3α-hydroxy-7β-tigloyloxytropane.

As reported elsewhere, the excellent separation power offered by the PGC column coupled to NMR permitted to unambiguously identify all the investigated isomers [27] and confirmed therefore the LC–MS results presented herein.

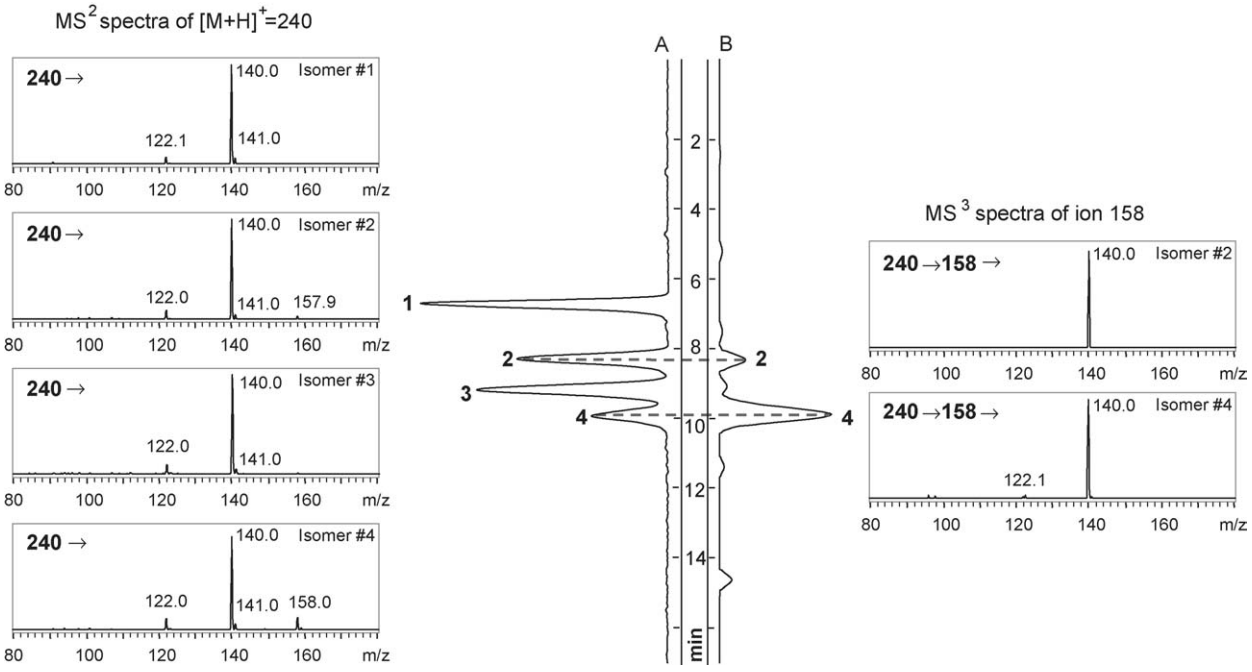

Fig. 7. Extracted ion chromatograms of (A) the isolated protonated molecular ion (*m/z* 240 Th) with MS/MS spectra of the four hydroxytropane esters; (B) the diagnostic fragment ion (*m/z* 158 Th) with corresponding MS^3 spectra.

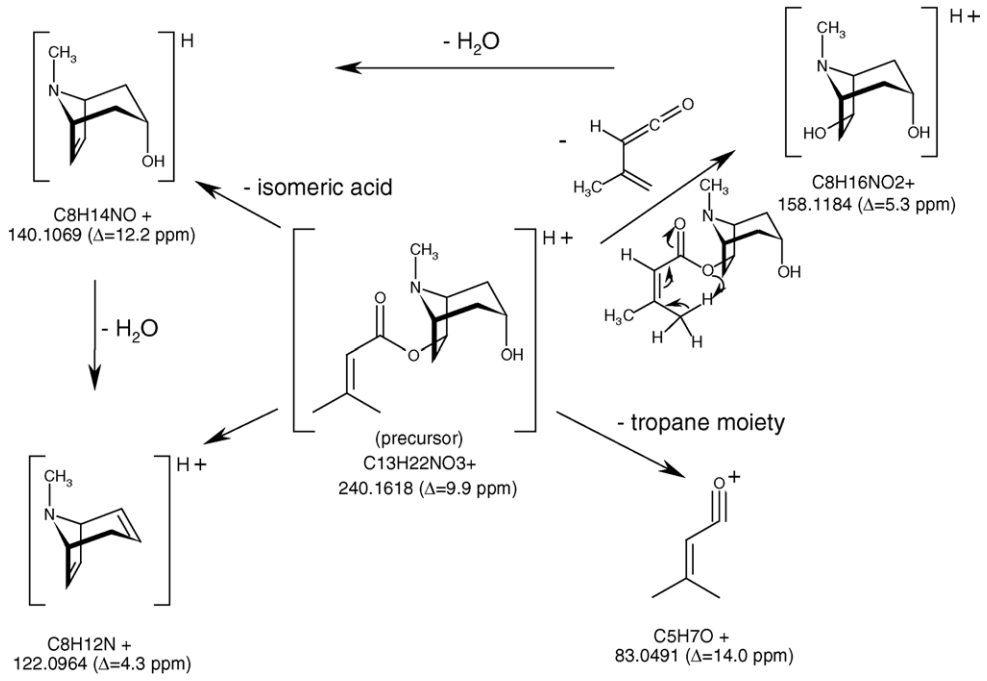

Fig. 8. Proposed fragmentation pathway of the investigated tropane alkaloids with the particular case of the senecioyl substituted isomer **4**. Accurate mass of the precursor and generated ions.

4. Conclusion

A rapid and simple LC method was developed for the separation of four tropane isomers by the systematic investigation of LC conditions. At an elevated temperature, an outstanding selectivity was obtained towards all alkaloids with a porous graphitic carbon column. The peculiar separation power in the first chromatographic dimension allowed further MS^n experiments on each individual isomer. Molecular mass was confirmed and product ions could be identified and attributed to the tropane moiety. The case study of the second and last eluting isomers pointed out that some

molecular fragments lead to distinguishable MS/MS spectra. However, even if the technique confirmed the presence of four isomeric hydroxytropane esters, it also showed some limitations as only LC-NMR could unambiguously identify all isomers. However, the inherent lack of sensitivity of NMR detection usually precludes quantitative analysis. In this respect, the developed LC–MS method offers better detection and quantitation possibilities and is sufficiently selective for the identification of these alkaloids in plant extracts.

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References

[1] P. Christen, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, vol. 22, Elsevier, Amsterdam, 2000, pp. 717–749.
[2] R.C. Pena, O. Muñoz, Biochem. Syst. Ecol. 30 (2002) 45–53.
[3] M. Lounasmaa, T. Tamminen, in: G.A. Cordell (Ed.), The Alkaloids, vol. 44, Academic Press, San Diego, 1993, pp. 1–114.
[4] O. Muñoz, Química de la flora de Chile, Universidad de Chile, Santiago, 1992.
[5] H. Ripperger, Phytochemistry 18 (1979) 171–173.
[6] G. De la Fuente, M. Reina, O. Muñoz, A. San Martin, J.P. Girault, Heterocycles 27 (1988) 1887–1897.
[7] O. Muñoz, M. Piovano, J. Garbarino, V. Hellwing, E. Breitmaier, Phytochemistry 43 (1996) 709–713.
[8] O. Muñoz, S. Cortes, Pharm. Biol. 36 (1998) 162–166.
[9] O. Muñoz, R. Hartmann, E. Breitmaier, J. Nat. Prod. 54 (1991) 1094–1096.
[10] A. San-Martin, C. Labbe, O. Muñoz, M. Castillo, M. Reina, G. De la Fuente, A. Gonzalez, Phytochemistry 26 (1987) 819–822.
[11] R. Hartmann, A. San-Martin, O. Muñoz, E. Breitmaier, Angew. Chem. 102 (1990) 441–443.
[12] B. Dräger, J. Chromatogr. A 978 (2002) 1–35.
[13] A.B. Svendsen, R. Verpoorte, Chromatography of Alkaloids, Part B: GC HPLC, Elsevier, Amsterdam, 1984.
[14] S. Hara, N. Yamauchi, C. Nakae, S. Sakai, Anal. Chem. 52 (1980) 33–38.
[15] C.F. Poole, The Essence of Chromatography, Elsevier, Amsterdam, 2003.
[16] L. Botz, L.G. Szabo, J. Planar Chromatogr. 1 (1988) 85–87.
[17] S. Nyiredy, K. Dallenbach-Toelke, O. Sticher, J. Planar Chromatogr. 1 (1988) 336–342.
[18] J.H. Knox, B. Kaur, G.R. Millward, J. Chromatogr. 352 (1986) 3–25.
[19] N. Tanaka, T. Tanigawa, K. Kimata, K. Hosoya, T. Araki, J. Chromatogr. 549 (1991) 29–41.
[20] Q.H. Wan, M.C. Davies, P.N. Shaw, D.A. Barrett, Anal. Chem. 68 (1996) 437–446.
[21] L. Gundersen, J. Chromatogr. A 914 (2001) 161–166.
[22] P.J. Houghton, T.Z. Woldemariam, Phytochem. Anal. 6 (1995) 85–88.
[23] B.J. Fish, J. Pharmaceut. Biomed. Anal. 11 (1993) 517–521.
[24] C.G. Huber, A. Premstaller, J. Chromatogr. A 849 (1999) 161–173.
[25] J.W. Dolan, J. Chromatogr. A 965 (2002) 195–205.
[26] M.C. Pietrogrande, A. Benvenuti, F. Dondi, Chromatographia 51 (2000) 193–198.
[27] S. Bieri, E. Varesio, O. Muñoz, J.-L. Veuthey, L.H. Tseng, U. Braumann, M. Spraul, P. Christen, Phytochem. Anal., in press.