Phytochemical Analysis
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# Identification of isomeric tropane alkaloids from *Schizanthus grahamii* by HPLC-NMR with loop storage and HPLC-UV-MS/SPE-NMR using a cryogenic flow probe

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Two fully automated HPLC-NMR methods are reported and compared for the structure elucidation of four isomeric tropane alkaloids from the stem-bark of an endemic Chilean plant, *Schizanthus grahamii* Gill. (Solanaceae). The first approach interfaced a conventional HPLC column to NMR by means of a loop storage unit. After elution with a mobile phase consisting of deuterated water and standard protonated organic solvents, the separated analytes were momentarily stored in a loop cassette and then transferred one-at-a-time to the NMR flow probe for measurements. The second strategy combined HPLC with parallel ion-trap MS detection and NMR spectroscopy using an integrated solid-phase extraction (SPE) unit for post-column analyte trapping. The SPE cartridges were dried under a gentle stream of nitrogen and analytes were sequentially eluted and directed to a cryogenically cooled flow-probe with an NMR-friendly solvent. The structures of the four isomeric alkaloids, 3$\alpha$-senecioyloxy-7$\beta$-hydroxytropane, 3$\alpha$-hydroxy-7$\beta$-angeloyloxytropane, 3$\alpha$-hydroxy-7$\beta$-tigloyloxytropane and 3$\alpha$-hydroxy-7$\beta$-senecioyloxytropane, were unambiguously determined by combining NMR assignments with MS data. Copyright © 2005 John Wiley & Sons, Ltd.

**Keywords:** HPLC-MS; HPLC-NMR; loop storage; cryogenic flow probe; solid-phase extraction; tropane alkaloids: isomers.

## INTRODUCTION

The genus *Schizanthus* (Solanaceae) comprises about 27 species indigenous to South America (Griffin and Lin, 2000). Among them, *Schizanthus grahamii* Gill. is endemic to Chile and grows on the southwestern slopes of the Andes. Previous phytochemical investigations showed that it accumulates several tropane derivatives, namely, tropine, 3$\alpha$-senecioyloxytropane, 3$\alpha$-hydroxy-7$\beta$-angeloyloxytropane, 3$\alpha$-senecioyloxy-7$\beta$-hydroxytropane, schizanthines C, D, E, X (San Martin *et al.*, 1987; Muñoz *et al.*, 1991) and grahamine (Hartmann *et al.*, 1990).

In recent years, the analytical approach in natural product discovery has changed and considerable efforts have been made towards a direct analysis of crude plant extracts or enriched fractions generating data with minimal sample manipulation. This approach led to the dereplication method avoiding the isolation of known or undesirable products (Wolfender *et al.*, 1998; Bobzin *et al.*, 2000). Conventional detection systems, such as UV, fluorescence and more recently evaporative light-scattering detectors, coupled with HPLC provide only limited information on the molecular structure of the separated compounds. During recent decades, the reliable coupling of HPLC and MS has resulted in a powerful analytical tool for qualitative and quantitative determination of drugs and metabolites. However, MS spectra cannot provide an unequivocal structural determination, particularly in the case of isomeric compounds or when no reference material is available. Consequently, the complementary use of HPLC-NMR coupling technologies (Albert, 1995) has become extremely attractive as it offers unparalleled structure elucidation capabilities.

Since the first paper describing HPLC-NMR coupling (Watanabe and Niki, 1978), several techniques have been developed to cope with the inherent lack of sensitivity of NMR detection. Efficient solvent suppression sequences were developed (Smallcombe *et al.*, 1995) along with reliable analyte collection techniques either in loops (Hansen *et al.*, 1999; Tseng *et al.*, 2000) or onto stationary phases (Griffiths and Horton 1998; Nyberg *et al.*, 2001), capillary HPLC-NMR with miniaturised and geometrically optimised flow probes (Lacey *et al.*, 1999; Jayawickrama and Sweedler, 2003), and cryogenically cooled probe heads (Styles *et al.*, 1984; Serber *et al.*, 2000; Spraul *et al.*, 2003). More recently, a new versatile approach including a solid-phase

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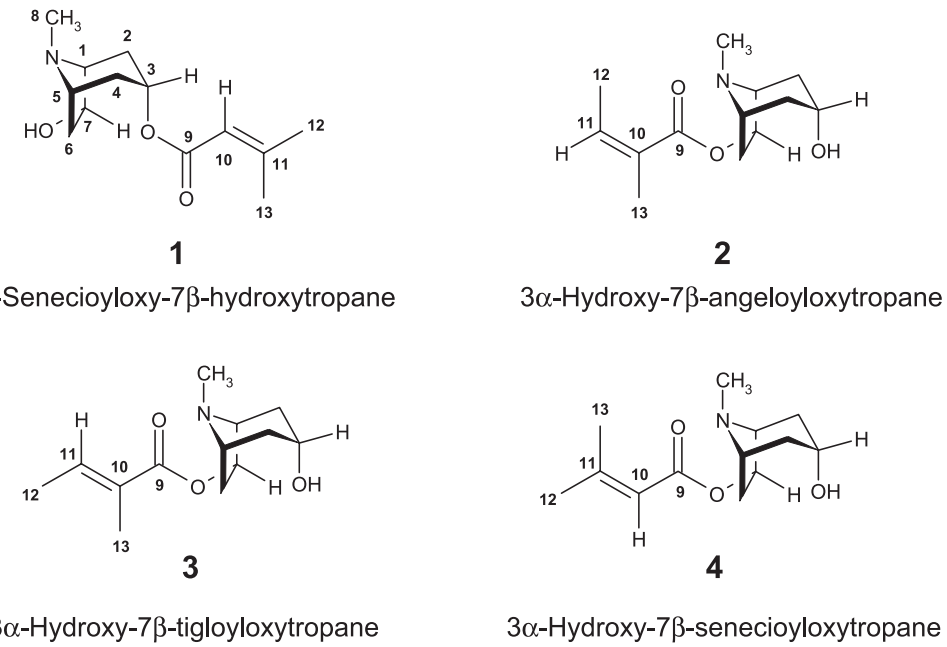


**Figure 1** Structure of the four isomeric hydroxytropane esters (molecular weight 239 g/mol).

extraction (SPE) step for post-column analyte trapping has been introduced (Exarchou *et al.*, 2003; Godejohann *et al.*, 2004; Simpson *et al.*, 2004).

In this paper, we report on two different analyte parking approaches, namely loop storage and trapping using an HPLC-UV-MS/SPE-NMR set-up to deal with the limited NMR sensitivity and overcome the short acquisition times during on-flow measurements. The latter approach uses the recently introduced cryogenic flow probe to further enhance sensitivity. Both approaches allowed the unambiguous identity of four isomeric tropane alkaloids (Fig. 1) in the stem-bark extract of *Schizanthus grahamii* Gill.

## EXPERIMENTAL

***Plant material.*** *Schizanthus grahamii* Gill. was collected in Rengo (Central Chile) in January 2000 and authenticated by Professor Fernanda Perez (Departamento de Botánica, Facultad de Ciencias, Universidad de Chile); a voucher specimen (no. 22234) has been deposited in the Faculty of Chemistry at the same University. The stem bark (2.6 kg) was extracted with ethanol at room temperature and the filtered alcoholic solution evaporated to dryness. The residue was taken up in 0.1 M hydrochloric acid and washed with dichloromethane. The aqueous solution was basified to pH 12 with ammonium hydroxide and further extracted with dichloromethane, yielding a gummy alkaline residue (6.6 g). Further purification on an aluminium oxide column was performed according to Muñoz *et al.* (1996), leading to the investigated purified fraction.

***HPLC-NMR: loop storage approach.*** HPLC and NMR were interfaced with a Bruker Biospin (Rheinstetten, Germany) peak sampling unit with 36 loops (BPSU-36), which permits loop storage measurements of up to 36 peaks within the same HPLC run [Fig. 2(A)]. The separation was monitored by UV at 220 nm and carried out on a Hypercarb® (ThermoHypersil, Bellefonte, PA, USA) porous graphitic carbon (PGC) column (125 × 4.6 mm i.d.; 5 μm particle size). Chromatography was performed on an Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series HPLC system at 25°C with deuterated water (Cambridge Isotope Laboratories, Andover, MA, USA) and acetonitrile (Riedel-de-Haen, Seelze, Germany). Both contained 0.1% formic acid. Isocratic elution with 10% acetonitrile was carried out at a flow rate of 0.9 mL/min. $^1$H-NMR spectra were recorded using a double solvent suppression pulse sequence (1D NOESY, Bodenhausen *et al.*, 1984), irradiating both residual HOD:water and acetonitrile signals. $^1$H-NMR spectra were individually acquired with a total of 256 transients on a Bruker BioSpin 600 MHz NMR magnet. The NMR flow cell had an active detection volume of 60 μL.

***HPLC-UV-MS/SPE-NMR cryogenic flow probe approach.*** The HPLC system consisted of an Agilent 1100 series quaternary pump with a UV diode array detector. The chromatographic separation was monitored by UV and MS traces and carried out on the same column as in the loop collection mode. Analysis was performed at ambient temperature using non-deuterated water and methanol (Riedel-de-Haen), both containing 0.1% formic acid (Fluka Chemie, Buchs,



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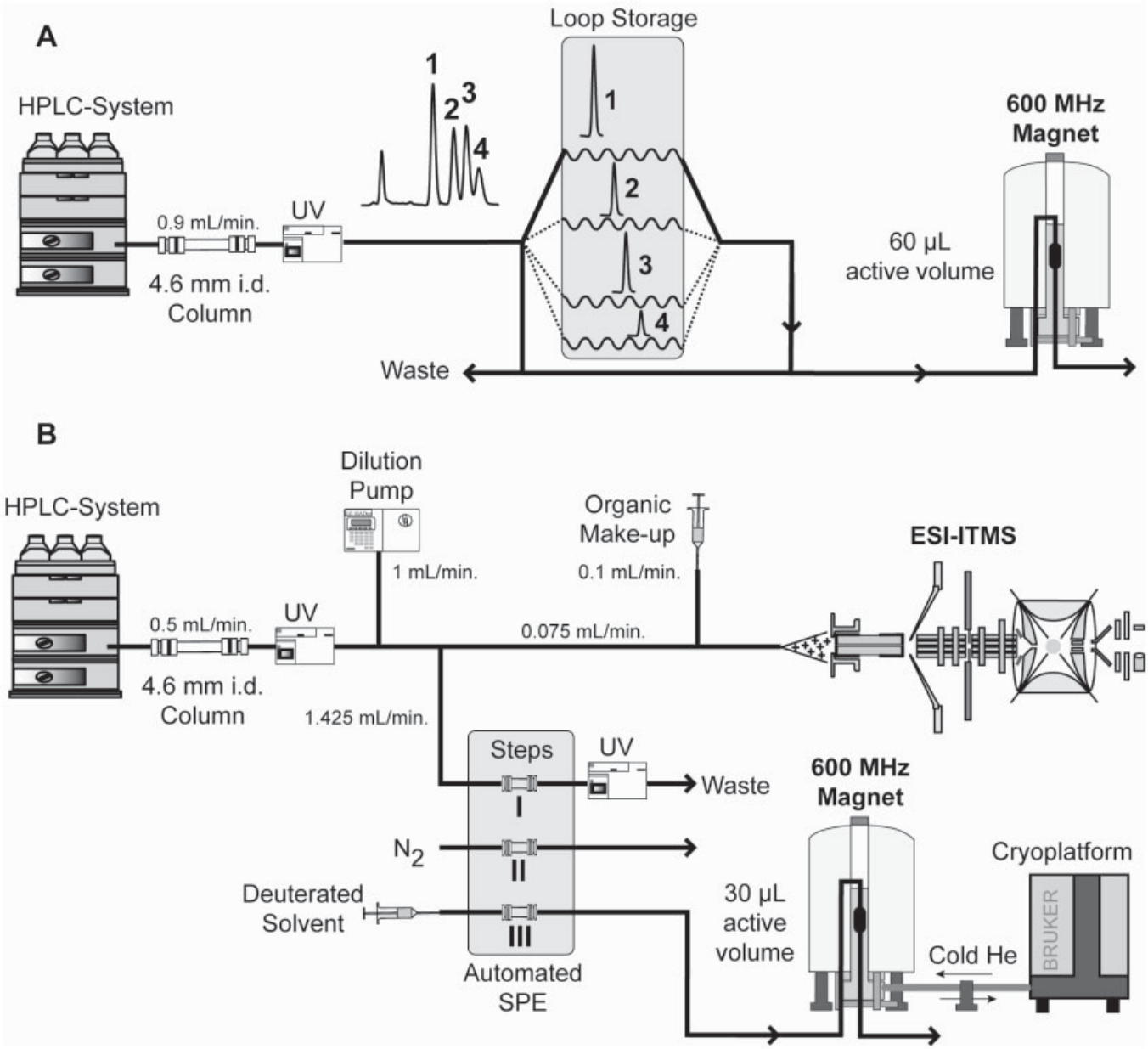


**Figure 2** Instrumental set-up for the HPLC-NMR hyphenated approaches showing: (A) peak sampling unit using storage loops; and (B) peak trapping onto SPE cartridges with parallel MS and cryogenically cooled NMR detection.

Switzerland). Isocratic elution with 20% organic solvent at a flow rate of 0.5 mL/min gave a total runtime of 30 min. A post-column make-up flow consisting of 0.1% aqueous formic acid solution was delivered at a flow rate of 1 mL/min by an additional HPLC pump (Knauer K100, Berlin, Germany). This make-up flow was used to lower the eluotropic strength of the eluent to provide suitable retention on the SPE trapping cartridges. After dilution, 5% of the total flow rate (i.e. 75 µL/min) was split and directed to the ion trap MS. However, prior to MS, a make-up flow of acetonitrile (100 µL/min) was added with a syringe pump to assist electrospray ionisation (ESI). The remaining flow rate (i.e. 1.425 mL/min) was directed to an automated Bruker BioSpin SPE unit [Fig. 2(B)].

MS experiments were performed on an Esquire 3000 plus ion trap HPLC-$MS^n$ system with an ESI interface (Bruker Daltonics, Bremen, Germany). The stainless steel capillary sprayer was set at 3.0 kV. For all experiments, the source temperature was held at 250°C, the drying gas flow rate was set at 5 L/min and the nebulising pressure at 10 psi. An MS trace was acquired in the selected ion monitoring (SIM) mode tuned on the pseudo-molecular ion $[M+H]^+$ of the isomers (*m/z* 240). Precursor ions were subjected to fragmentation, leading to the corresponding $MS^2$ fragments within a scan range from *m/z* 80 to 240. The product ion at *m/z* 158 was specifically selected to generate $MS^3$ spectra. The fragmentation amplitude and the maximum accumulation time were 0.4 V and 100 ms, respectively.



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The modified SPE, Spark Holland, Emmen, The Netherlands, unit was used automatically to collect the chromatographic peaks on HySphere GP resin cartridges (10 × 2 mm i.d.; 10–12 μm particle size) after post-column addition of 0.1% aqueous formic acid solution. An Agilent 1100 UV detector was placed after the SPE unit to measure the ability of the cartridge to retain analytes [step I in Fig. 2(B)]. After analyte loading, cartridges were dried with a gentle nitrogen gas stream for 30 min to completely remove any trace of solvent [step II in Fig. 2(B)]. Trapped compounds were then sequentially flushed with 250 μL of deuterated acetonitrile for analytes **1**, **2** and **4**, and with deuterated methanol for analyte **3**, into the NMR flow cell of the NMR magnet equipped with a 30 μL Bruker BioSpin CryoFlowProbe™ [step III in Fig. 2(B)]. This cell was positioned inside the ambient temperature bore of the probe and isolated from the closely located radio frequency coils, which were cryogenically cooled to 20 K by the cryoplatform.

$^1$H-NMR spectra at 600 MHz were acquired with 128 free induction decays (FIDs) per component. The residual non-deuterated solvent signals were suppressed by a pre-irradiation based on the one-dimensional version of a NOESY sequence. In order to process the data, a line broadening of 1 Hz was used. Two-dimensional techniques were also employed for structure confirmation of the trapped isomeric compounds. The whole system was fully automated and controlled by HyStar™ software (Bruker Daltonics) that eluted the samples in the NMR detection cell and triggered the NMR software ICONNMR™/XWINNMR™.

## RESULTS AND DISCUSSION

### HPLC-NMR: loop storage approach

During the separation of the four tropane alkaloid isomers (Fig. 1), UV detection was used to monitor the collection and storage into the loops of each individual peak by means of a switching valve. After the chromatographic separation, the contents of each loop was transferred one at a time into the NMR flow cell and subjected to a solvent suppression $^1$H-NMR experiment [Fig. 2(A)]. This approach allowed adequate NMR measurements to be performed on each separated fraction without detrimental solvent effects. Indeed, as on-flow detection suffers from lack of sensitivity, direct stop-flow measurement is often preferred in order to acquire NMR spectra. However, in the latter case, multiple stopped-flow periods are required within a chromatographic run in order to obtain an NMR spectrum of each analyte. Such a procedure may lead to undesired peak broadening owing to diffusion of retained analytes onto the column and can also cause recurring mechanical stress on the packing material. Besides, the loop storage unit is designed in such a way that the thermoregulated loop cassette can be removed from the sampling unit after the separation and may be transported for further NMR analysis.

The separation was carried out on a 4.6 mm i.d. column working with conventional organic solvents to limit costs, and with deuterated water to facilitate multiple solvent suppression and to reduce the region in the spectrum obscured by the water signal. An aliquot of 460 μg of sample material was injected onto the column. Even though methanol was the best solvent with respect to chromatographic performance [Fig. 3(A)], acetonitrile was preferred in order to avoid a strong OH signal in NMR spectra. The elution order of the four isomers was identical in both systems with some loss of resolution when acetonitrile was chosen [Fig. 3(B)].

Differentiation between the olefinic proton of each of the three isomeric moieties, namely, senecioyl (H-10), angeloyl (H-11) and tigloyl (H-11) substituents, was quite clear for the alkaloids analysed. While this proton

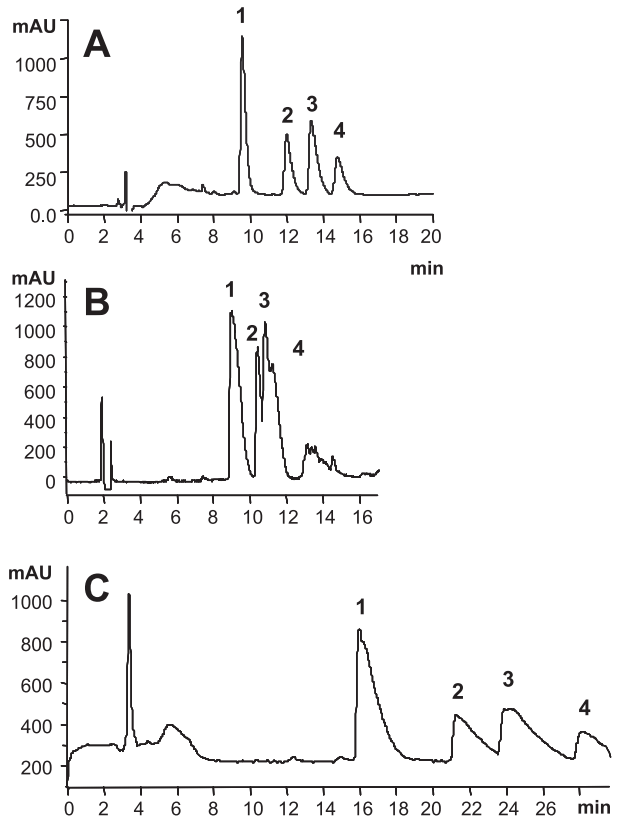


**Figure 3** Chromatographic separations of the four isomers **1–4** with injection of high sample amount, showing: (A) conventional HPLC-UV under optimal flow rate conditions (1 mL/min) and a mobile phase composed of methanol:water; (B) HPLC-UV trace (detected at 220 nm) of the separation with acetonitrile:$D_2O$ during loop sampling; and (C) HPLC-UV trace (detection at 220 nm) of the isomers in methanol:water before SPE trapping with reduced column flow rate (0.5 mL/min).




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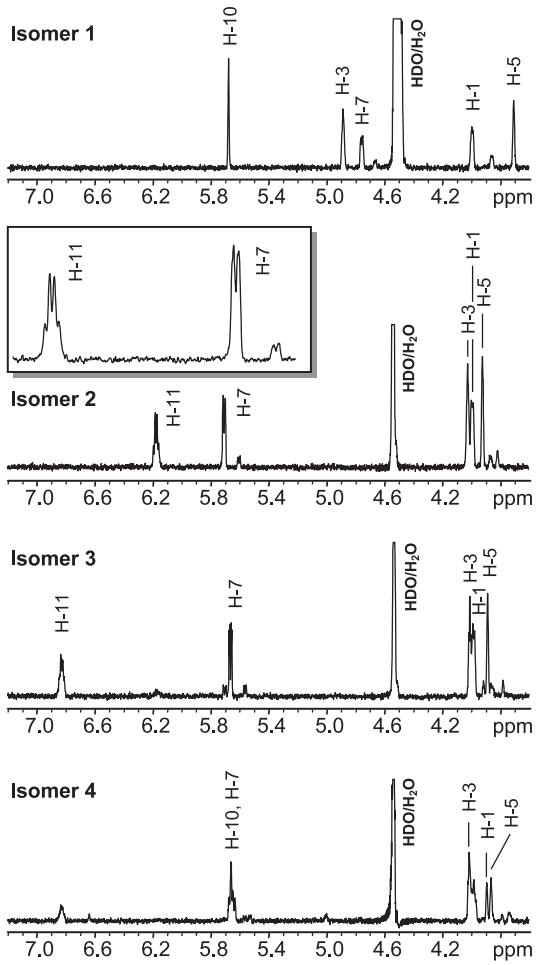


**Figure 4** $^1$H-NMR spectra at 600 MHz of the four tropane isomers **1**–**4** analysed one by one after transfer of the loop content.

showed a singlet for isomers **1** and **4** at chemical shifts of 5.68 and 5.66 ppm, respectively, isomers **2** and **3** exhibited a distinct quadruplet multiplicity at 6.17 and 6.83 ppm, respectively (Fig. 4 and Table 1). These signals not only resonated at significantly different chemical shifts, but also showed a characteristic multiplicity that permitted the unequivocal identification of the three isomeric $C_5$ acids. The olefinic hydrogen of a senecioyl moiety may theoretically give a $J^4$ long-range coupling, but in our case it resonated as a singlet for isomers **1** and **4**. It is noteworthy that, for the last isomer **4**, this specific signal overlapped with another proton (H-7). Consequently, peaks **1** and **4** could be assigned as senecioyl derivatives. The olefinic proton resonance of the *E* isomer **3** was shifted downfield relative to that of *Z* isomer **2**, mainly because of the proximity of the carbonyl group in the *E* configuration. In both cases, the multiplicity showed, as expected, a quadruplet with $J^3$ coupling constants of 7.0 and 6.3 Hz for isomers **3** and **2**, respectively. These aforementioned chemical shifts and coupling constants were in accordance with those described in the literature during the investigation of *Schizanthus pinnatus* Ruiz & Pav. and *S. litoralis* Phil. (De la Fuente *et al.*, 1988; Muñoz *et al.*, 1996) and confirmed their assignments.

The discrimination between C-3 and C-7 substitutions was established through the characteristic chemical shift of their corresponding geminal proton resonances, which clearly allowed differentiation between 3$\alpha$-senecioyl- (**1**) and 7$\beta$-senecioyl- (**4**) esterified alkaloids. Isomer **1** presented a strongly shielded H-3$\beta$ broad singlet at 4.89 ppm and a doublet ($J^3$ = 7.3 Hz) for H-7$\alpha$ resonating at 4.75 ppm, while isomer **4** showed, for these protons, a broad singlet at 4.01 ppm (H-3$\beta$) and a multiplet at 5.66 ppm (H-7$\alpha$). Similarly, the H-7$\alpha$ signal of the angeloyl derivative **2** presented a doublet ($J^3$ = 7.7 Hz) at $\delta$ 5.70 ppm, and that of the tigloyl isomer **3** exhibited a doublet of doublets (*dd*, $J^3$ = 8.5 Hz and $J^4$ = 3.0 Hz) at $\delta$ 5.66 ppm, confirming a C-7 substitution. Moreover, for the three C-7 esterified tropanes, the H-3$\beta$ signal ranged between 4.01 and 4.03 ppm and showed a broad singlet, except for **3**, which presented a triplet with a 4.0 Hz $J^3$ coupling constant.

Some slight differences could also be observed for H-1 and H-5 signals as shown in Table 1, but with a significantly different value for the C-3 esterified isomer **1**. This provided an alternative differentiation between the C-7 substituted isomers. A careful study of the four spectra indicated the presence of another discriminating chemical shift between C-3 and C-7 esterification, namely, the resonance of H-6 protons. These protons resonated at 2.76 ppm for isomer **1** and ranged between 2.94 and 2.96 ppm for the C-7 substituted esters **2**–**4** (Table 1). This is a consequence of the shielding effect of the ester proximity compared to that of the hydroxyl group. The methyl group on the amine function resonated in all cases between 2.83 and 2.89 ppm. Owing to their location under the solvent suppression peak, the methyl signals on the olefinic moiety of the alkaloids were not visible. Finally, as demonstrated, several signals were well characterised for each isomer, which not only allowed the four isomers to be distinguished but permitted their structures to be assigned unambiguously. For further comparison, we calculated a signal-to-noise ratio (S/N) of 74:1 for the H-10 singlet of isomer **1** with a 200 Hz background noise.

## HPLC-UV-MS/SPE-NMR cryogenic flow probe approach

The experimental arrangement for this set-up is outlined in Fig. 2(B). Although normally used in advance of the separation process in order to pre-concentrate or clean samples prior to their analysis, the SPE unit was here inserted after the UV detector in order to trap



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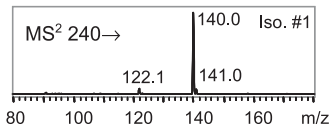
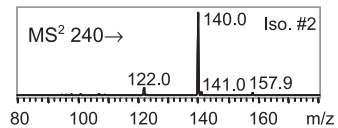
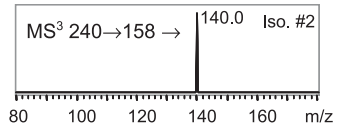
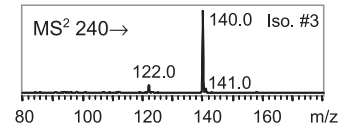
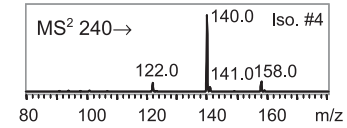
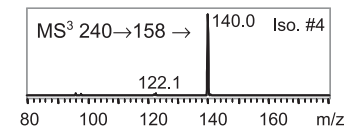
**Table 1** $^1$H-NMR (600 MHz) chemical shifts of isomers **1** to **4**: A in the loop storage configuration, B with the HPLC-UV-MS/SPE-NMR set-up, C $MS^n$ data

| Position | Isomer **1**<br>3$\alpha$-senecioyloxy-7$\beta$-hydroxytropane | Isomer **2**<br>3$\alpha$-hydroxy-7$\beta$-angeloyloxytropane | Isomer **3**<br>3$\alpha$-hydroxy-7$\beta$-tigloyloxytropane | Isomer **4**<br>3$\alpha$-hydroxy-7$\beta$-senecioyloxytropane |
|---|---|---|---|---|
| *A. Loop storage (spectra recorded in $D_2O$/acetonitrile)* | | | | |
| H-1 | 3.99 (s) | 3.99 (m) | 3.98 (m) | 3.98 (m) |
| H-2,4 | 2.1–2.3 (m) | 2.1–2.4 (m) | 2.1–2.4 (m) | 2.1–2.4 (m) |
| H-3$\beta$ | 4.89 (br. s) | 4.01 (br. s) | 4.03 (t, $J_1$ = 4.0 Hz) | 4.01 (br. s) |
| H-5 | 3.71 (s) | 3.91 (s) | 3.89 (br. s) | 3.86 (br. s) |
| H-6 | 2.76 (m) | 2.96 (dd, $J_1$ = 15.2 Hz, $J_2$ = 8.2 Hz) | 2.94 (dd, $J_1$ = 15.2 Hz, $J_2$ = 8.2 Hz) | 2.94 (m) |
| H-7$\alpha$ | 4.75 (d, $J_1$ = 7.3 Hz) | 5.70 (d, $J_1$ = 7.7 Hz) | 5.66 (dd, $J_1$ = 8.5 Hz, $J_2$ = 3.0 Hz) | 5.65 (m) |
| $NCH_3$ | 2.89 (s) | 2.85 (s) | 2.85 (s) | 2.83 (s) |
| H-10 | 5.68 (s) | | | 5.66 (m) |
| H-11 | | 6.17 (q, $J_1$ = 7.0 Hz) | 6.83 (q, $J_1$ = 6.3 Hz) | |
| *B HPLC-SPE/cryoprobe NMR (spectra recorded in $CD_3CN$ for **1**, **2**, **4** and in $CD_3OD$ for **3**)* | | | | |
| H-1 | 4.01 (m) | 4.00 (m) | 3.59 (m) | 4.00 (m) |
| H-2,4 | 2.3–2.5 (m) | 2.25–2.5 (m) | 2.0–2.3 (H-2,4) | 2.05–2.45 (m) |
| H-3$\beta$ | 5.01 (t, $J_1$ = 5.0 Hz) | 4.02 (t, $J_1$ = 4.0 Hz) | 4.00 (t, $J_1$ = 4.6 Hz) | 4.05 (m) |
| H-5 | 3.72 (s) | 3.89 (H-5, s) | 3.43 (m) | 3.85 (br. s) |
| H-6 | 2.86 (m) | 3.09 (dd, $J_1$ = 14.8 Hz, $J_2$ = 8.1 Hz) | 2.88 (dd, $J_1$ = 14.1 Hz, $J_2$ = 8.3 Hz) | 3.06 (dd, $J_1$ = 14.8 Hz, $J_2$ = 8.4 Hz) |
| H-7$\alpha$ | 4.81 (dd, $J_1$ = 8.0 Hz, $J_2$ = 2.5 Hz) | 5.86 (dd, $J_1$ = 8.1 Hz, $J_2$ = 3.3 Hz) | 5.75 (dd, $J_1$ = 7.9 Hz, $J_2$ = 2.8 Hz) | 5.80 (dd, $J_1$ = 8.4 Hz, $J_2$ = 3.3 Hz) |
| $NCH_3$ | 3.00 (d, $J_1$ = 5.3 Hz) | 2.92 (d, 5.1 Hz) | 2.67 (br. s) | 2.91 (d, $J_1$ = 5.3 Hz) |
| H-10 | 5.75 (m, $J_1$ = 1.2 Hz) | | | 5.72 (m) |
| H-11 | | 6.22 (dq, $J_1$ = 7.3 Hz, $J_2$ = 1.5 Hz) | 6.90 (qq, $J_1$ = 7.0 Hz, $J_2$ = 1.3 Hz) | |
| H-12 | | | 1.83 (d, $J_1$ = 7.2 Hz) | 1.90 (s) |
| H-13 | | | 1.86 (br. s) | 2.16 (s) |
| *C. MS data*<br>ESI-Ion trap<br>MS $[M+H]^+$<br>*m/z* 240 |  |  <br>  |  |  <br>  |

s = singlet; d = doublet; dd = doublet of doublets; t = triplet; q = quartet; m = multiplet; br. = broad.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the eluting analytes. A similar intelligence to the loop storage mode was used to select HPLC peaks for the trapping step. However, during the parking step, the entire peak associated with each analyte was collected on an SPE cartridge, whereas in HPLC-NMR only the peak maximum could be captured in a loop. Furthermore, an additional MS trace was used for peak selection. The analysis was conducted on a 4.6 mm i.d. column with non-deuterated solvents but with a reduced column flow rate (i.e. 0.5 mL/min). In order to obtain an efficient trapping, the total flow rate should not be higher than 1.5 mL/min and, since the organic percentage had to be reduced, a water make-up (1.0 mL/min) was added post-column. With non-deuterated solvents, cost was reduced and interference in MS with the alcoholic exchangeable protons was avoided. After the trapping step, the cartridges were dried with a stream of nitrogen and analytes flushed into the NMR cell with a deuterated solvent for measurements. This approach permits the use of a wider choice of solvents and buffers. Thus, HPLC-SPE-NMR can combine change of solvent prior to NMR transfer, peak concentration and multiple trapping possibilities.

Instead of increasing the signals of sample components, another way to improve sensitivity is to reduce the noise from the receiver coil and electronic amplifiers. This is the approach employed in cryogenically cooled NMR probes that operate at low coil temperature in order to decrease considerably the electronic resistance. This phenomenon is expressed by a high quality factor ($Q$), which is approximately inversely proportional to the square root of the absolute temperature. Thus, a roughly estimated S/N gain factor of 3–4 may be expected when working at 25 K (Exarchou *et al.*, 2003; Spraul *et al.*, 2003). In addition the preamplifiers are also kept at low temperature.

The $^1$H-NMR spectrum of each individual isomer is shown in Fig. 5. Slight drifts in the chemical shifts were observed due to the different NMR solvents used. Extensions of characteristic signals pointed out the resolution improvement. Indeed, in the case of 3$\alpha$-senecioyloxy-7$\beta$hydroxytropane (**1**), the above-described broad singlet of the H-3$\beta$ proton showed a distinct triplet with a coupling constant of 5.0 Hz. The appropriate S/N ratio allowed further 2D-$^1$H-NMR experiments to be recorded in reasonable acquisition times. Thus, the contour plot of COSY allowed the H-3$\beta$ signal to be correlated with two H-2,4 protons (Fig. 6). Furthermore, the H-7 signal of each isomer showed an apparent doublet of doublets instead of a doublet as observed previously with the loop storage method.

Any deuterated solvent may be employed to flush the cartridge as long as its solvent strength is adequate to elute efficiently the trapped analyte. However, for compounds present in the low μg level, the residual protons from deuterated solvents (typically 0.1%) show far higher signals than those of interest. This is briefly illustrated by the third isomer where methanol-$d_4$ was used instead of deuterated acetonitrile (Fig. 5). Indeed, in this case, the residual signal of the solvent obscured a short segment of the chemical shift range and so pointed out the importance of selecting high-purity deuterated solvents possessing few deuterated sites. In addition, particular care must be taken in the conditioning and drying process of the cartridges in order to avoid the appearance of contaminant signals.

As no commercial porous graphitic carbon cartridges were available at the time when the experiments were performed, a cartridge packed with polymerised polystyrene–divinylbenzene material (HySphere) was used in order to trap the alkaloids. A second UV detector, placed at the cartridge outlet, served to control the trapping efficiency. Unfortunately, it showed that these charged polar compounds were difficult to trap onto

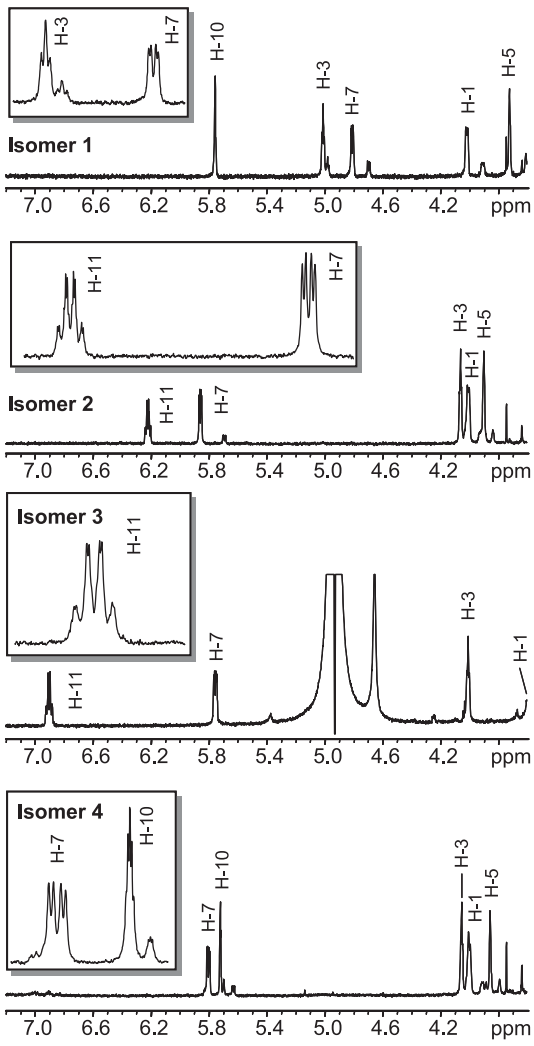


**Figure 5** $^1$H-NMR spectra at 600 MHz of the four tropane isomers **1**–**4** analysed with HPLC-UV-MS-SPE/NMR in combination with a cryoflow probe.




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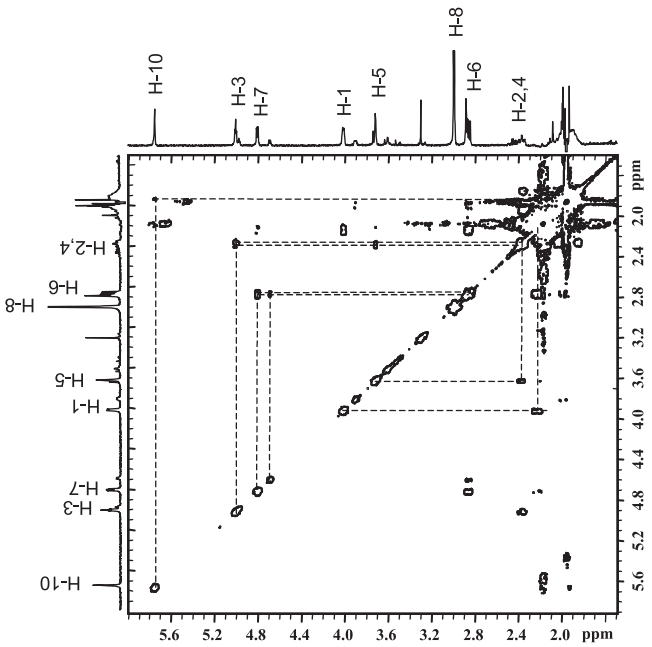


**Figure 6** COSY-$^1$H-NMR spectrum of isomer **1** using the HPLC-UV-MS-SPE/NMR set-up.

the polymeric packing material. Therefore, only a fraction of the eluting isomers could be retained on the cartridges. Although alkaloids were trapped, it was estimated that roughly 50% of the injected sample material was lost. Consequently, it was expected that four times less sample material reached the NMR probe head compared with the loop storage method. However, the S/N ratio (100:1) was 25% higher, thus demonstrating the increased sensitivity of the HPLC-SPE-NMR technique combined with a cryoprobe. Indeed, compared with the loop storage approach, roughly half the sample amount (260 μg) was injected in order to maintain good chromatographic resolution and only half the number of transients were acquired with the HPLC-SPE-NMR experiment. Finally, a roughly 4-fold sensitivity enhancement was observed. With an optimal trapping material dedicated to ionised analytes, we may even expect to double this sensitivity gain.

The parallel analysis by ion trap MS of compounds eluting with a non-deuterated mobile phase permitted an easy molecular mass determination. Further fragmentation of the pseudo-molecular ion $[M+H]^+$ at $m/z$ 240 leading to $MS^2$ spectra, provided structural information by attributing product ion at $m/z$ 140 (loss of the $C_5$ acid) to the tropane moiety (Table 1). Some differences between the isomers occurred with the appearance of an ion at $m/z$ 158, to some extent in the MS of isomer **2** but significantly in the case of isomer **4**. A subsequent fragmentation of the latter product ion led again to the tropoyl ion at $m/z$ 140 by loss of one water molecule.

HPLC-NMR, either with loop storage or with peak trapping, allowed four isomeric tropane alkaloids in a purified stem-bark extract from *Schizanthus grahamii* to be distinguished and identified. The complementary use of multiple stage MS helped in the structure elucidation process, while a cryogenic flow probe improved sensitivity. Compared with the stop flow mode, the loop storage approach avoids interrupting the chromatographic process for NMR measurements. The insertion of SPE allows the separation to be conducted with non-deuterated solvents. Through the concentration effect of the SPE, the analyte may be eluted as a sharp band into the NMR flow cell for further sensitivity enhancement.

The present study demonstrates that, if compounds cannot be efficiently trapped onto the SPE cartridges, the expected gain in sensitivity rapidly collapses and multi-trapping is ineffective. Therefore, further investigations are in progress in order to improve the trapping process of alkaloids onto suitable packing material to exploit fully the combination of the SPE set-up with cryogenically cooled probes. This will certainly permit a substantial reduction in the sensitivity gap between NMR and MS.

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