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On-line identification of tropane alkaloids from *Erythroxylum vacciniifolium* by liquid chromatography–UV detection–multiple mass spectrometry and liquid chromatography–nuclear magnetic resonance spectrometry

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Abstract

The bark of catuaba (*Erythroxylum vacciniifolium* Martius, Erythroxylaceae), a tree native to the northern part of Brazil, was investigated for its alkaloid content. With the aim of obtaining preliminary structure information on-line, the alkaloid extract was analysed by high-performance liquid chromatography coupled to diode array UV detection, to mass spectrometry and to nuclear magnetic resonance. Interpretation of on-line spectroscopic data obtained from this extract led to structural elucidation of six new alkaloids and partial identification of 18 potentially original alkaloids bearing the same tropane skeleton esterified in positions 3 and 6 by 1-methyl-1H-pyrrol-2-carboxylic acid and/or 4-hydroxy-3,5-dimethoxybenzoic acid.

Keywords: *Erythroxylum vacciniifolium*; Liquid chromatography–mass spectrometry; Nuclear magnetic resonance spectrometry; Tropane alkaloids; Alkaloids

1. Introduction

In recent times, a massive public interest in the availability of aphrodisiacs and remedies for erectile dysfunction has led to a resurgence of sales in more easily available and exotic herbal preparations [1,2]. For over a century, catuaba has been one of the most popular herbal remedies in Brazil. Catuaba has been attributed in traditional medicine with aphrodisiac and tonic properties [3,4], even though no one seems to be able to identify with certitude the plant [5]. Three species of this genus, *Erythroxylum vacciniifolium*, *E. subracemosum* and *E. catuaba*, are described in traditional medicine as catuaba which generates some confusion [5]. In the course of a study of medicinal plants from Brazil, a sample of catuaba from Paraíba, assigned the name *E. vacciniifolium*, has been investigated. This species was already studied 25 years ago, leading to the isolation and structural identifi-

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cation of three tropane alkaloids (catuabines A–C) [6,7].

In order to render the investigation of new bioactive natural products more rapid and efficient, the dereplication of crude plant extracts with LC hyphenated techniques represents a strategic element to avoid finding known constituents and to target the isolation of new bioactive products [8]. With the aim of obtaining spectroscopic information on-line, the alkaloid extract of *E. vacciniifolium* was analysed by high-performance liquid chromatography (HPLC) coupled to UV photodiode array detection (LC–DAD), to mass spectrometry (LC–MS) and to nuclear magnetic resonance spectrometry (LC–NMR). All the data obtained by these LC hyphenated techniques were separately or in partial combination processed to achieve structural identification of the compounds in the extract.

LC-ion trap (IT) multiple mass spectrometry (MS^n) in combination with high-resolution LC–time-of-flight (TOF) MS were used to ascertain molecular formulae on-line and to study fragmentation patterns [9,10]. In addition, the total number of exchangeable protons (e.g. N–H or O–H) in the detected molecules was determinated by LC–IT–MS using deuterated water (D_2O) instead of water as eluent [11,12]. On- and stop-flow LC–NMR were used in complement to LC–MS to complete on-line identification [13,14].

The detailed investigation of the main components of the alkaloid extract and the partial on-line identification of putative new tropane alkaloids from *E. vacciniifolium* by using a combination of innovative LC hyphenated techniques is presented in this paper.

2. Experimental

2.1. Plant material and extraction

The stem bark of *E. vacciniifolium* was collected in Buraquinho rain forest (João Pessoa, Paraíba, Brazil), in August 2000. A voucher specimen was deposited at the Herbarium do Laboratorio de Química de Produtos Naturais (HLPQN), Universidade Federal de Paraíba, 58059 João Pessoa, Paraíba, Brazil (JPB.-No. 152) and identified by Prof. Zoraide Maria de Medeiros Gouveia of the Department of Science of Nature, University of Paraíba, Brazil, and Dr. Douglas C. Daly, The New York Botanical Garden. Stem bark (840 g) was ground after cryocooling with liquid nitrogen, was moistened with 20 ml of concentrated NH_3 and exhaustively extracted with $CHCl_3$ (3×24 h; 3 l). After filtration of the alkaloid extracts, chloroform was removed by rotary evaporation under vacuum to give 15.7 g of $CHCl_3$ extract.

2.2. Solvents and reagents

HPLC-grade acetonitrile (MeCN) was obtained from Romil (Cambridge, UK). Deionized water was prepared using a Reinstwasser-System Clear Cartridge System (SG, Hamburg, Germany). Both the solvents were passed through Millipore filters (water: 0.45 μm HA; MeCN: 0.50 μm FH; Bedford, MA, USA). Analysis-grade triethylamine (TEA) was purchased from Fluka (Buchs, Switzerland). Deuterated water (isotopic purity: 99.8 at.% D) was obtained from Dr. Glaeser AG (Basel, Switzerland). [D_4]ammonium deuteroxide 25% (w/w) solution (isotopic purity: >99 at.% D) was purchased from Sigma–Aldrich Chemie (Steinheim, Germany).

2.3. Fractionation and isolation

The alkaloid extract (10 g) was fractionated by MPLC on a LiChroprep C_{18} (15–25 μm; Merck, Darmstadt, Germany) packed column (460 mm × 70 mm i.d.) using a MeCN (+2 mM TEA) − water (+2 mM TEA) step gradient from 5:95 to 100:0 in 3 days (flow rate: 5.0 ml/min, UV detection at 280 nm).

2.4. LC–DAD analysis

A HP-1100 HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a binary pump was used, with a photodiode array high-speed spectrophotometric detector and an autosampler, all controlled by Agilent ChemStation software. Separation was performed on a CC Nucleodur 100-5C_{18} column (125 mm × 4.6 mm i.d., 5 μm; Macherey–Nagel, Düren, Germany) equipped with a pre-column of the same material (8 mm × 4.0 mm i.d., 5 μm) using a MeCN (+2 mM TEA):water (+2 mM TEA) gradient from 5:95 to 100:0 in 34 min, including five isocratic elution steps each of 5 min at 5:95, 18:82, 23:77, 35:65 and 40:60. The resulting pH in the eluent was 11 and the total analysis time was 50 min,

including column washing and stabilization. The flow-rate was set at 1 ml/min and detection at 272 nm. UV spectra (DAD) were recorded between 200 and 500 nm.

2.5. LC–MS^n analysis

For LC–multiple stage atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS^n), the extract was analysed under the same conditions as for LC–UV. LC–APCI–MS^n was performed on a Finnigan MAT (San Jose, CA, USA) ion trap mass instrument equipped with a Finnigan APCI source. The capillary temperature was set to 150 °C, vaporizer temperature to 380 °C, corona needle current to 6.0 μA, sheath gas (nitrogen) pressure to 60 psi (1 psi = 6894.76 Pa). MS^n experiments were performed by programming dependent scan events. The first event was a full MS scan M_r (150.0–2000.0) (MS^1); during the second event the main ion recorded was isolated and selectively fragmented in the ion trap (MS^2), whilst the third event (MS^3) represented the isolation and selective fragmentation of the main ion recorded in MS^2. The collision energy was set to 35 eV.

The observation of exchangeable protons was performed using the same LC–MS^n conditions previously described except that water was substituted by deuterated water (D_2O + 2 mM TEA) as eluent.

2.6. LC–APCI–TOF-MS analysis

For the high mass accuracy measurements, the alkaloid extract was analysed under the same HPLC conditions as for the LC–MS^n. The TOF-MS experiments were conducted on a LCT spectrometer (Micromass, Manchester, UK). The APCI conditions were as follows: corona pin voltage, 5 kV; source temperature, 120 °C; vaporizer temperature, 500 °C; nebuliser gas, nitrogen; cone voltage, 30 V. MS scan time 1 s + 0.1 s interscan delay.

For accurate mass measurements, the reference compound codeine ($[M + H]^+$: 300.1594; $[M + H-18]^+$: 282.1489) (Aldrich, Buchs, Switzerland) was added post-column (0.1 mg/ml MeOH 100%). The flow rate of the lock mass was increased during the gradient elution from 0.02 to 0.1 ml/min in order to obtain a detectable signal.

2.7. LC–NMR analysis

A Varian Unity Inova 500 MHz NMR instrument equipped with a $^1H[^{13}C]$ pulse field gradient indirect detection microflow LC–NMR probe (flow cell: 60 μl; 3 mm i.d.) was used. Reversed-phase HPLC of the compounds was carried out on a Varian (Palo Alto, CA, USA) modular HPLC system, including a Varian 9012 pump, a Valco injection valve and a Varian 9050 UV detector. The separation was performed by using a CC Nucleosil 100-5C_{18} AB prepacked column (125 mm × 8.0 mm i.d., 5 μm; Macherey-Nagel) with MeCN (2 mM DH_3):D_2O (2 mM DH_3) (5:95–100:0; 80 min). The resulting pH of the eluent was 10. In order to achieve satisfactory on-flow LC–NMR detection the amount of extract injected was increased to 3.0 mg on-column. The flow rate was increased to 1.2 ml/min and the on-flow run consisted of 107 increments of 32 transients each. The total analysis time for this experiment was 80 min. During LC–NMR the UV traces were measured at 272 nm with a dual path UV detector (Varian) for monitoring the chromatographic separation. For the stop-flow experiments, the UV detector was used to trigger the stop–flow valve and trap precisely the LC-peak of interest in the LC–NMR flow cell. In the stop-flow mode, 1024 transients were accumulated for each spectrum. References of the solvent signals were set at δ 2.10 for acetonitrile. For each increment, solvent suppression was performed with the WET sequence [15]. During gradient elution the shape of the selective pulses were automatically calculated on the fly based on a scout scan recorded before each increment.

3. Results and discussion

3.1. Preliminary LC–DAD and LC–APCI–MS^n analyses

In order to obtain a preliminary idea of the compounds present in *E. vacciniifolium*, a combined LC–DAD and positive ion LC–APCI–MS analysis of the specific alkaloid extract was performed. The separation was carried out using a reversed-phase C_{18} column and a gradient of acetonitrile–water buffered with triethylamine (pH 11). The use of the alkaline buffer was required to increase the affinity of alkaloids to the reversed phase and to avoid charged molecules

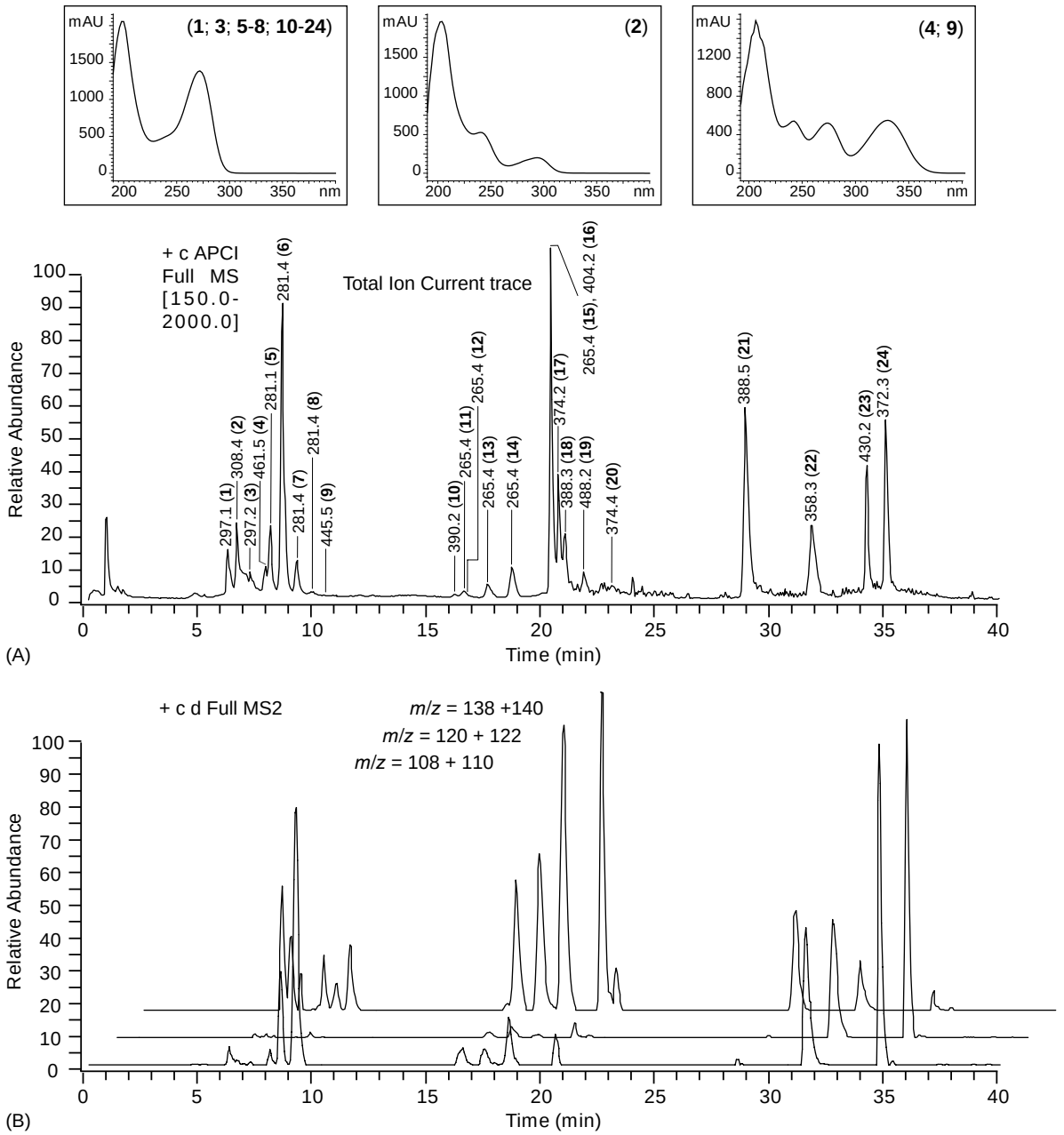


Fig. 1. (A) LC–UV–APCI–MS analysis of the crude alkaloid extract of *E. vacciniifolium*. The total ion current (TIC) trace was recorded in the positive mode between 150 and 2000 units. The UV spectra were recorded between 200 and 500 nm. (B) Selective MS–MS traces of characteristic fragment ions. (C) Complementary MS^3 traces of low-M_r fragments.

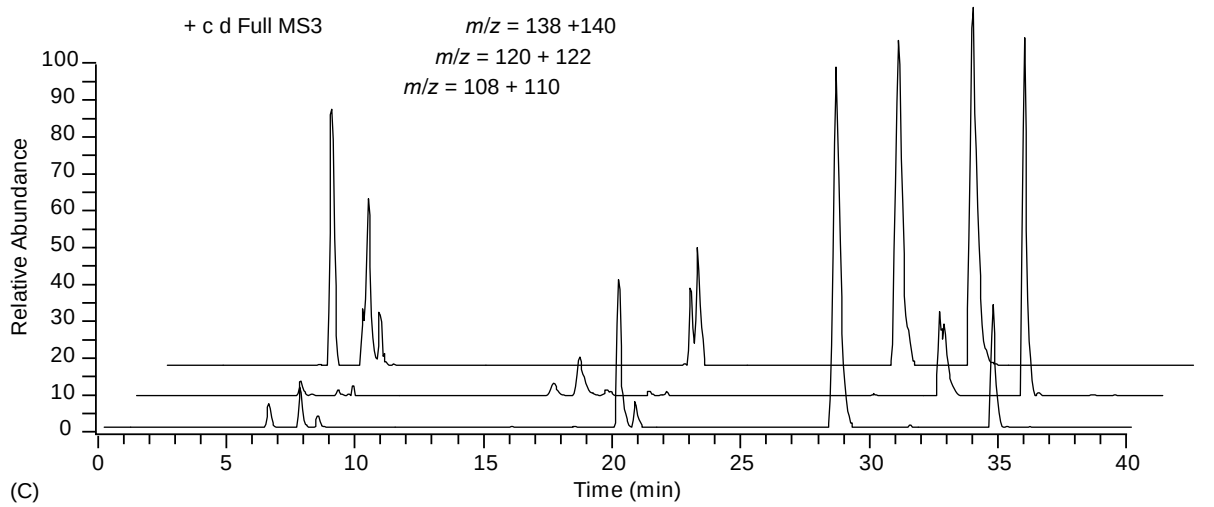


Fig. 1. (A) (*Continued*).

which could be responsible for hydrogen bonding and ionic interactions with silanol groups of the stationary phase, leading to additional retention and peak tailing.

In the LC–UV chromatogram (272 nm), a dozen main peaks and several minor peaks were recorded. Most of the constituents of the alkaloid extract had the same UV spectrum with a maximum of absorbance varying between 269 and 272 nm. Only three minor peaks presented different UV spectra: two of these peaks (**4** and **9**) had three maxima recorded at ca. 241, 271 and 329 nm; the other one (**2**) had two maxima recorded at ca. 241 and 294 nm. These observations suggested that almost all molecules shared a same chromophore and probably a similar core structure.

The LC–APCI–MS^n spectra were recorded during the same run. The total ion current (TIC) trace (Fig. 1A) was comparable to the UV trace at 272 nm. The different constituents showed protonated molecules $[M + H]^+$ in the range M_r of 265–488 including several isomers (**1** and **3**: M_r 297; **5–8**: M_r 281; **11–15**: M_r 265; **17** and **20**: M_r 374; **18** and **21**: M_r 388; Table 1). The presence of alkaloids with different numbers of nitrogen atoms was evidenced by even and odd *m*/*z* values. The MS^2 and MS^3 traces exhibited similar low-molecular mass fragments for almost all detected peaks, suggesting a common main moiety for each molecule. MS^3 was needed in some cases for recording of the characteristic low-M_r fragments not always observable in the MS–MS spectra.

In order to obtain additional structural information about the detected alkaloids, the observation of possible exchangeable protons (e.g. N–H or O–H) was undertaken by LC–APCI–MS^n using D_2O–MeCN as eluent. Under these conditions all exchangeable hydrogens were replaced by deuterium. A shift of 1 unit for all constituents was due to the presence of deuterated $[M_D + D]^+$ instead of protonated molecules $[M + H]^+$. The remaining mass difference was attributable to the number of exchangeable protons (Table 1). This analysis demonstrated that all detected compounds had at least one exchangeable proton, except **18**, **19**, **23** and **24**.

3.2. High-resolution LC–APCI–TOF-MS

For an on-line molecular formula assignment, the extract was analysed by LC–APCI–TOF-MS under identical conditions to those described for LC–MS^n. Accurate mass measurements were performed with the help of a reference compound for the lock mass. The $[M + H]^+$ of codeine at 300.1594 was used as a reference during the first 8 min of the HPLC analysis. Due to the instability of this ion with the increase of the MeCN concentration in the LC gradient, the more stable $[M+H\text{-}18]^+$ codeine fragment at 282.1489 was then employed for lock mass. Accurate masses could be measured for six constituents of the alkaloid extract (**1, 2** and **21–24**) and for their protonated molecules $[M + H]^+$, these ranged from 297.1462 to 430.2015

Table 1
LC–APCI–MS^n data of the alkaloid extract of *E. vacciniifolium*

Compound	$[M_H + H]^+$	$[M_D + D]^+$	N[a]	MS–MS	MS^3	MS^3
1	297	300	3	172 (100), 154 (25), 138 (85), 114 (10), 110 (15), 96 (5), 94 (15)	[297 → 172] 154 (85), 114 (100), 94 (30)	
2	308	312	4	156 (100), 138 (10), 110 (5), 94 (5)	[308 → 156] 138 (100), 120 (15), 110 (25), 96 (5), 94 (5)	
3	297	300	3	172 (100), 154 (10), 108 (5), 94 (10)	[297 → 172] 154 (100), 108 (5), 94 (85)	
4	461	464	3	443 (10), 263 (100), 181 (10), 138 (10)	[461 → 263] 245 (25), 182 (10), 138 (40), 108 (100), 96 (10)	
5	281	283	2	156 (100), 138 (20), 110 (5), 94 (5)	[281 → 156] 138 (95), 120 (20), 110 (20), 94 (100)	
6	281	284	3	264 (50), 156 (100), 138 (5), 112 (20), 94 (10), 82 (30)	[281 → 156] 138 (10), 112 (60), 94 (10), 82 (100)	
7	281	284	3	263 (30), 200 (30), 156 (60), 138 (50), 108 (100)	[281 → 156] 138 (100), 110 (60), 84 (50), 82 (80)	
8	281	284	3	263 (40), 156 (40), 138 (100), 108 (40), 96 (5)	[281 → 263] 245 (40), 138 (100), 108 (60), 96 (10)	
9	445	447	2	320 (25), 247 (100), 181 (85), 140 (10)	[445 → 247] 122 (100), 108 (40), 96 (20), 94 (20), 91 (20)	[445 → 320] 181 (100), 140 (15), 122 (40)
10	390	393	3	297 (30), 279 (50), 265 (85), 182 (75), 172 (85), 154 (25), 138 (100), 120 (10)	[390 → 138] 110 (100), 108 (10), 94 (15)	[390 → 172] 154 (100), 114 (60), 108 (50)
11	265	267	2	247 (5), 140 (100), 122 (10), 108 (10), 96 (5)	[265 → 140] 122 (100), 108 (20), 96 (10)	
12	265	267	2	247 (5), 140 (100), 122 (10), 108 (10), 94 (5)	[265 → 140] 122 (100), 108 (20), 94 (10)	
13	265	267	2	247 (5), 140 (100), 126 (35), 122 (15), 108 (5), 96 (10)	[265 → 140] 122 (100), 98 (30), 82 (30)	
14	265	267	2	247 (25), 203 (10), 140 (100), 122 (5), 108 (10), 96 (20)	[265 → 140] 122 (15), 110 (5), 96 (100)	
15	265	267	2	247 (5), 140 (100), 96 (20)	[265 → 140] 122 (100), 108 (5), 96 (10)	
16	404	406	2	279 (100), 182 (45), 154 (20), 138 (90), 120 (10)	[404 → 279] 243 (10), 154 (25), 108 (100), 94 (20)	
17	374	376	2	281 (100), 263 (20), 249 (10), 138 (5), 108 (5)	[374 → 281] 263 (5), 156 (100), 138 (10), 108 (5), 94 (5)	
18	388	389	1	302 (5), 263 (100), 247 (5), 202 (5), 138 (30), 122 (5)	[388 → 263] 245 (10), 203 (10), 138 (100), 108 (75), 94 (50)	
19	488	489	1	470 (20), 388 (100), 324 (85), 182 (10), 140 (25), 126 (30)	[488 → 388] 372 (100), 204 (85)	[488 → 324] 112 (35), 98 (100)
20	374	377	3	281 (15), 263 (100), 138 (20), 108 (15)	[374 → 263] 245 (15), 182 (15), 138 (30), 108 (100)	[374 → 281] 263 (40), 156 (70), 138 (100), 120 (50), 108 (65)

Table 1 (*Continued*)

Compound	$[M_H + H]^+$	$[M_D + D]^+$	N^a	MS–MS	MS^3	MS^3
21	388	390	2	263 (100), 245 (5), 138 (15)	[388 → 263] 245 (10), 182 (10), 138 (35), 108 (100), 96 (10)	
22	358	360	2	265 (50), 247 (15), 233 (100), 140 (40), 122 (20), 108 (15)	[358 → 233] 202 (20), 140 (100), 122 (85), 96 (15), 91 (20)	[358 → 265] 140 (100), 122 (10), 108 (15)
23	430	431	1	388 (15), 305 (100), 245 (10), 180 (10), 138 (5), 120 (5), 108 (5)	[430 → 305] 263 (5), 245 (25), 180 (65), 138 (5), 108 (100)	[430 → 388] 263 (100), 138 (20)
24	372	373	1	247 (100), 122 (65), 108 (25)	[372 → 247] 122 (100), 108 (45), 96 (30), 94 (20), 91 (25)	

[a] Exchangeable protons (inclusive ionizing deuteron).

(Table 2). The elemental composition of these six compounds was determined with a difference of <10 ppm between their measured and calculated masses.

Preliminary analyses showed homogeneity in the spectral data recorded for all constituents of the alkaloid extract, which suggested close structural similarities. One of the detected compounds (**22**) was particularly interesting because of its identical molecular formula and fragmentation pattern with those reported for a tropane alkaloid esterified by two pyrrolic acids. Among the alkaloids (catuabines A–C) previously reported for *E. vaccinifolium* [7], only catuabine C matched with the on-line data recorded. The putative detection of catuabine C in the extract and the common structural features shown by LC–UV and LC–MS, confirmed the possible presence of various related tropane alkaloids. This hypothesis was strengthened by chemotaxonomic data from the genus *Erythroxylum*, which is a well-known source of various tropane alkaloids. In order to confirm this deduction, the isolation of compound **22** was undertaken in view of its use as a reference molecule for further on-flow and stop-flow LC–NMR analyses.

3.3. Isolation and structure elucidation of compound 22

A single MPLC separation step performed on the alkaloid extract gave 13 fractions (A–M). Fraction L yielded pure compound **22** (813 mg). The structure elucidation of this compound was established by spectroscopic methods, including EIMS, HREI-MS, IR, ^{1}H, ^{13}C, and 2D NMR experiments. Accordingly, alkaloid **22** (catuabine D) was identified as 3α-(1-methyl-1H-pyrrol-2-yl- carbonyloxy)- 6β- (1H-pyrrol-2-yl-carbonyloxy)tropane (Fig. 2) [16]. Thus, compound **22** was a regioisomer of catuabine C (6β-(1-methyl-1H-pyrrol-2-yl-carbonyloxy)-3α-(1H-pyrrol-2-yl-carbonyloxy)tropane), differing by an inversion of the ester substituents.

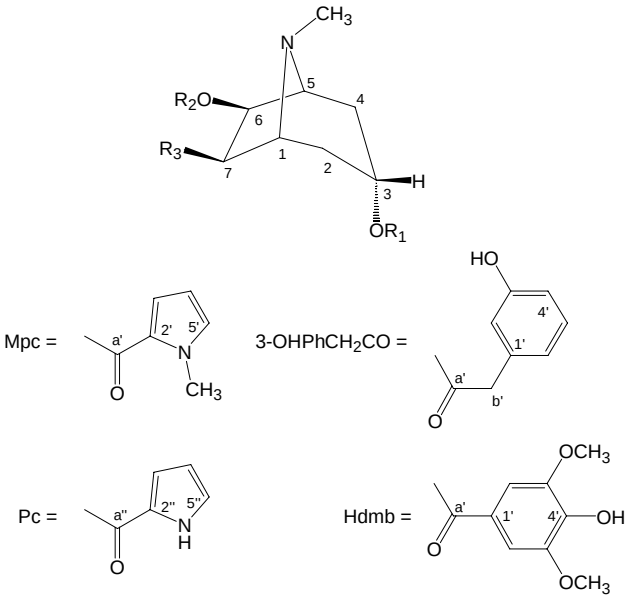
3.4. On-flow and stop-flow LC–NMR

For sensitive on-flow LC–^{1}H NMR detection, the amount of extract injected in the system was increased to 3.0 mg. The HPLC conditions were consequently optimized in order to achieve a good separation of the extract constituents. With these on-flow LC–NMR conditions, the ^{1}H NMR spectra of five main alkaloids (**14** and **21**–**24**) were successfully recorded. The proton signals of these compounds were recognizable on a contour plot of the on-flow LC–NMR experiments (Fig. 3). Two other compounds (**7** and **13**), not sufficiently concentrated to be detected on-flow, were well recorded in the stop-flow mode. The interruption of the flow during the stop-flow acquisitions did not affect the LC resolution.

3.5. Interpretation of on-line data

3.5.1. Correlation of ^{1}H NMR and LC–^{1}H NMR data of compound 22

The ^{1}H NMR resonances of **22** obtained by on-flow LC–^{1}H NMR differed by <0.2 ppm from those of the isolated compound, measured in $CDCl_3$ at 30 °C [16]. This LC–^{1}H NMR spectrum, recorded at a



Compound[a]	R_1	R_2	R_3	Other
7	H	Mpc	OH	-
13	H	Mpc	H	-
21	Mpc	Mpc	OH	-
22	Mpc	Pc	H	-
23	Mpc	Mpc	CH_3COO	-
24	Mpc	Mpc	H	-

Compound[b]	Mpc	Pc	OH	Other
1	1	-	2	N→O
2	-	-	2	3-OHPhCH$_2$CO
3	1	-	2	N→O
4	1	-	1	Hdmb
5	1	-	1	N→O
6	1	-	2	-
8	1	-	2	-
9	1	-	-	Hdmb
10	1	1	1	N→O
11	1	-	1	-
12	1	-	1	-
14	1	-	1	-
15	1	-	1	-
16	2	-	1	N→O
17	1	1	-	N→O
18	2	-	-	N→O
19	2	-	-	N→O; C_4H_9COO
20	1	1	1	-

[a] Fully identified and characterized compounds.

[b] Partially identified compounds. All compounds had a di- or trisubstituted tropane moiety mono- or diesterified by Mpc, Pc, Hdmb, 3-OHPhCH$_2$CO or C_4H_9COO. Several tropane centers had an *N*-oxide group. The structures differed in the number and position of the ester substituents or hydroxyl groups.

Fig. 2. Structures of tropane alkaloids from *E. vacciniifolium* identified on-line.

Table 2
High-resolution LC–APCI–TOF-MS data

Compound	$[M + H]^+$ measured	$[M + H]^+$ calculated	difference (ppm)	Elemental composition[a]
1	297.1462	297.1445[b]	5.6	$C_{14}H_{21}N_2O_5$
2	308.1518	308.1493[b]	8.1	$C_{16}H_{22}NO_5$
21	388.1902	388.1867[c]	8.9	$C_{20}H_{26}N_3O_5$
22	358.1760	358.1762[c]	−0.5	$C_{19}H_{24}N_3O_4$
23	430.2015	430.1973[c]	9.7	$C_{22}H_{28}N_3O_6$
24	372.1954	372.1918[c]	9.6	$C_{20}H_{26}N_3O_4$

[a] Elemental composition corresponding to the $[M + H]^+$ ions, one proton has to be deduced for calculation of the molecular formula of the different constituents.
[b] Lock mass of protonated codeine $[M + H]^+$: *m/z* 300.1594.
[c] Lock mass of the fragment of protonated codeine recalculated according to elemental composition $[M + H\text{-}18]^+$: *m/z* 282.1489.

retention time (t_R) of 42 min (Fig. 3), showed characteristic chemical shifts associated with catuabine D (**22**) (Table 3). Thus, the typical resonances for a methylpyrrole substructure were exhibited by the signals at δ_H 3.96 (N–CH_3), 6.23 (H-4′), 7.05 (H-5) and 7.21 (H-3′). A non-methylated pyrrole was associated with signals at δ_H 6.29 (H-4″), 7.12 (H-5″) and 7.09 (H-3″). The presence of the secondary nitrogen atom in this substructure was confirmed by the observation of an exchangeable proton by LC–MS^n analysis using D_2O as eluent (Table 1). The structure of the disubstituted tropane core skeleton was illustrated by three saturated methylenes at δ_H 1.79 (H_{endo}-2), 2.30 (3H, H_{exo}-2, H_{exo}-4 and H_{exo}-7) and 2.75 (H_{endo}-7). The solvent suppression around 2.10 ppm affected the signal detection of H_{endo}-4 protons. Two methine and one methyl groups linked to the nitrogen atom were also recorded at δ_H 3.38 (H-5), 3.46 (H-1) and 2.54 (N–CH_3), respectively. The last two oxygenated methine moieties showed resonances at δ_H 5.19 (H-3) and 5.75 (H-6). The multiplicity (triplet) of the H-3 signal with the coupling constant ($J = 4.4$ Hz) indicated the α-orientation (i.e. endo) of the substituent at C-3 [17–19]. The arrangement of the substituent at C-6 was established by the analysis of the coupling constants of H-6, H-7 and H-5 protons. The H-6 proton of the disubstituted tropane alkaloid showed two couplings ($J = 7.7$ and 2.8) with the two H-7 protons and it did not present any coupling with the vicinal H-5 proton. This observation implied a β-orientation (i.e. exo) of the substituent and a dihedral angle close to 90° between H-5 and H-6α [20,21].

3.5.2. Structural elucidation of compounds detected by LC–^1H NMR

The on-flow LC–NMR contour plot of the extract permitted a direct comparison of the chemical shifts of the other detected compounds with those mentioned for **22** (Fig. 3). The signals exhibited great homogeneity, corroborating the hypothesis that the major constituents of the alkaloid extract of *E. vacciniifolium* were tropanic analogues of catuabine D.

The LC–^{1}H NMR spectrum recorded for compound **24** at a retention time of 58 min exhibited similar chemical shifts to those of **22**, supporting the presence in the molecule of a tropane moiety dioxygenated at C-3 and C-6. The difference between the two compounds was in their ester substituents. In fact, two methyl groups were recorded at δ_H 3.93 (3H, s, N–CH_3) and 3.94 (3H, s, N–CH_3) in the LC–^{1}H NMR spectrum of **24**, indicating two methylpyrrole acid moieties. The presence of an additional methyl group in the structure of compound **24** was corroborated by the observation of its protonated molecule $[M + H]^+$, recorded at 372.1954 by LC–TOF-MS analysis, associated with a molecular formula of $C_{20}H_{25}N_3O_4$. Moreover, the LC–MS performed when using D_2O as the cosolvent confirmed the absence of exchangeable protons. On the basis of the above evidence, the structure of **24** was elucidated on-line as 3α,6β-di(1-methyl-1H-pyrrol-2-yl-carbonyloxy) tropane.

Spectral data of **21** (t_R = 37 min) showed that its structure was very close to that of compound **24**, with the presence of a tropanic nucleus and two methylpyrrole ester groups. However, compound **21**

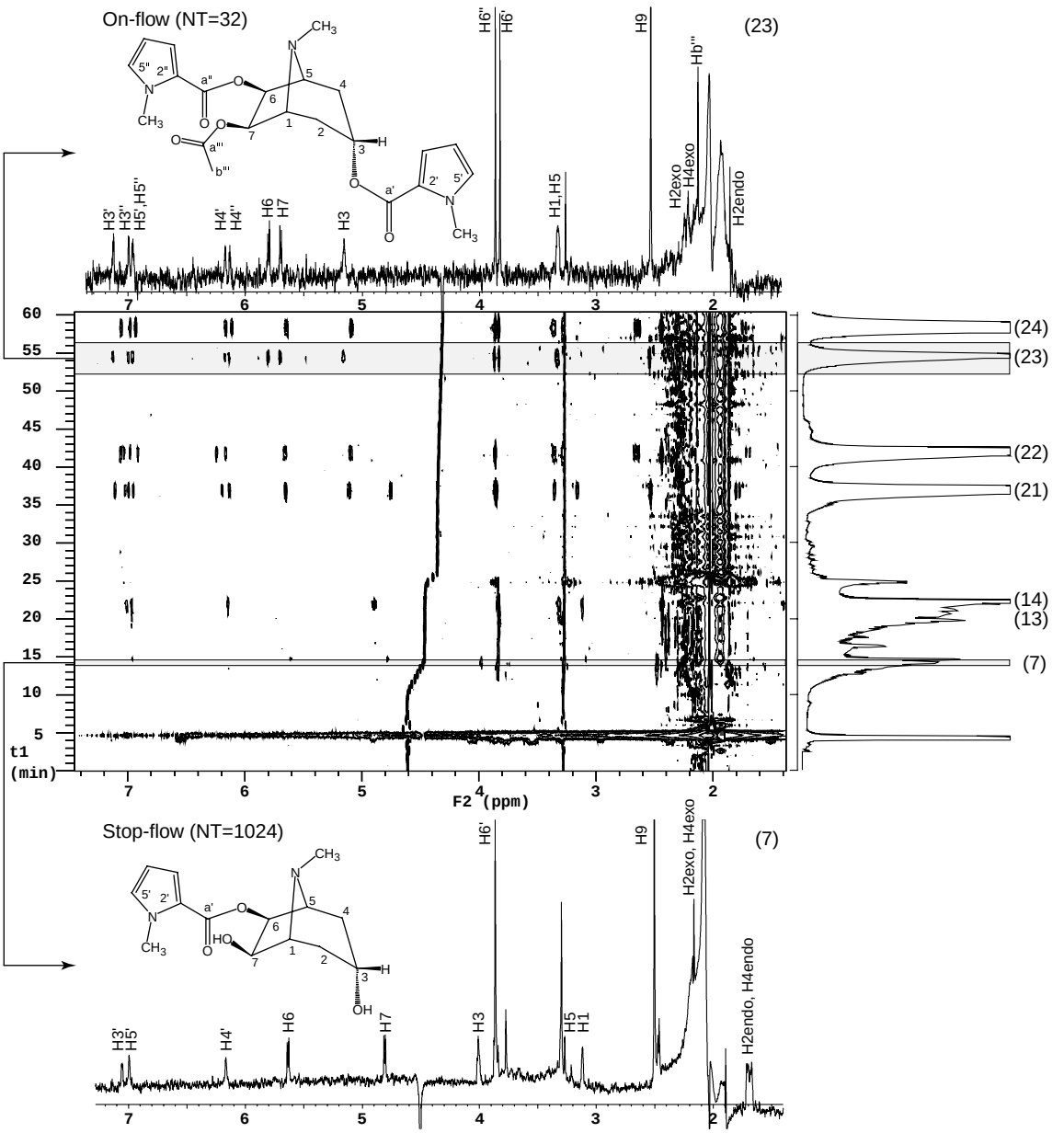


Fig. 3. On-flow LC–NMR contour plot of the alkaloid extract of *E. vacciniifolium*. On this two-dimensional plot all ^{1}H NMR resonances of the analytes appear as dots. ^{1}H NMR spectra of compounds **7** and **23** are shown as examples of resolution for on-flow and stop-flow analyses, respectively.

had an additional hydroxyl group at C-7, as indicated by the extra deshielded signal at δ_H 4.85 (H-7) and the absence of the resonance around δ_H 2.75 associated previously with the saturated methylene moiety at the 7-position for compounds **22** and **24**. The lack of any coupling constant with vicinal protons H-5 and H-1 suggested an α-orientation of protons at 6- and 7-positions, respectively. The hydroxyl group was also confirmed by the presence of one exchangeable proton in the deuterated molecule ($[M_D + D]^+$

Table 3
LC–^{1}H NMR data[a]

Proton	**7**	**13**	**14**	**21**	**22**	**23**	**24**
1	3.18 br s	3.28 br s	3.41 m	3.45 br s	3.46 br s	3.43 br s	3.46 br s
2_{exo}	2.25 m	1.98 m	1.75 m	2.32 m	2.30 m	2.35 m	2.35 m
2_{endo}	1.75 d (15.4)	1.48 d (15.4)	1.41 m	1.88 d (16.5)	1.79 d (15.4)	1.93 d (15.3)	1.82 d (15.4)
3α	–	–	1.48 m	–	–	–	–
3β	4.08 t (4.4)	4.06 t (4.4)	1.94–2.30 m	5.20 t (4.4)	5.19 t (4.4)	5.26 t (4.4)	5.19 t (4.4)
4_{exo}	2.25 m	1.98 m	4.99 m	2.32 m	2.30 m	2.32 m	2.35 m
4_{endo}	1.77 d (15.4)	1.72 d (15.4)	[b]	[b]	[b]	[b]	[b]
5	3.34 br s	3.21 br s	3.21 d (3.3)	3.25 br s	3.38 br s	3.43 br s	3.38 br s
6α	5.71 d (6.6)	5.71 dd (7.7, 2.8)	[b]	5.75 d (6.6)	5.75 dd (7.7, 2.8)	5.90 d (6.6)	5.74 dd (7.7, 2.8)
7α	4.88 d (6.6)	2.71 dd (14.8, 7.7)	1.94–2.30 m	4.85 d (6.6)	2.75 dd (14.3, 7.7)	5.80 d (6.6)	2.74 dd (14.3, 7.7)
7β	–	2.03–2.06 m	1.94–2.30 m	–	2.30 m	–	2.35 m
$N–CH_3$	2.57 s	2.53 s	2.53 s	2.63 s	2.54 s	2.64 s	2.54 s
	MPc	MPc	MPc	MPc	MPc	MPc	MPc
3′	7.13 dd (3.9, 1.1)	7.10 dd (3.9, 2.2)	7.11 dd (3.9, 2.7)	7.21 dd (3.9, 2.2)	7.21 dd (3.9, 2.2)	7.23 dd (3.9, 1.7)	7.15 dd (3.9, 1.7)
4′	6.24 dd (3.9, 1.1)	6.23 m	6.24 dd (3.9, 2.7)	6.30 dd (3.9, 2.7)	6.23 dd (3.9, 2.7)	6.27 dd (3.9, 2.7)	6.26 dd (3.9, 2.7)
5′	7.06 dd (3.9, 1.1)	7.06 m	7.06 m	7.09 m	7.05 m	7.06 m	7.03 m
$N–CH_3$	3.93 s	3.93 s	3.93 s	3.95 s	3.96 s	3.92 s	3.93 s
				MPc	Pc	MPc	MPc
3″				7.12 dd (3.9, 2.2)	7.09 dd (3.9, 2.2)	7.09 m	7.08 dd (3.9, 1.7)
4″				6.23 dd (3.9, 2.7)	6.29 dd (3.9, 2.7)	6.23 dd (3.9, 2.7)	6.21 dd (3.9, 1.7)
5″				7.05 m	7.12 dd (3.9, 1.7)	7.06 m	7.03 m
$N–CH_3$				3.96 s	–	3.96 s	3.94 s
						CH_3COO 2.24 s	

[a] The δ values given in ppm, J values in parentheses given in Hz.
[b] The solvent suppression of acetonitrile and water, respectively, affected the signal detection.

390) when compared to the corresponding protonated molecule ($[M + H]^+$ 388). The molecular formula, $C_{20}H_{25}N_3O_5$, obtained by LC–TOF-MS analysis confirmed the structure elucidation of **21** as 3α,6β-di(1-methyl-1H-pyrrol-2-yl-carbonyloxy)-7β-hydroxytropane.

LC–TOF-MS analysis of compound **23** exhibited a protonated molecule at $[M + H]^+$ 430.2015 indicating a molecular formula of $C_{22}H_{28}N_3O_6$ and implying a supplementary C_2H_2O element when compared with **21**. Furthermore, the presence of a trioxygenated tropanic skeleton and two methylpyrrole ester moieties was deduced by comparison of its LC–^{1}H NMR spectrum (t_R = 54 min) with that of compound **21**. The significant differences between the two compounds were a additional resonance for one methyl group at δ_H 2.24 and a major variation ($\Delta\delta_H + 0.95$) in the chemical shift of the signal associated with H_{endo}-7. The downfield shifted signal suggested here the occurrence of an ester substitution in the 7-position [18,20,22]. According to the molecular formula and the additional methyl group mentioned above, only an acetyl moiety could be located at this position. The presence of this acetyl group was also confirmed by the loss of 42 unit in the LC–MS spectrum. Compound **23** was thus elucidated as 7β-acetoxy-3α-6β-di(1-methyl-1H-pyrrol-2-yl-carbonyloxy)tropane.

The stop-flow LC–^{1}H NMR data (t_R = 14 min) of compound **7** showed characteristic resonances for trisubstituted tropane alkaloids, as in compound **21**, but here a single methylpyrrole ester was present. The absence of the second methylpyrrole group was confirmed by the observation of a protonated molecule at M_r 281 deduced from LC–MS^n experiments (Table 1). The similar chemical shift to other compounds associated with H_{endo}-6 at δ_H 5.71 supported the substitution of this single ester at the 6-position. The signal at δ_H 4.08, high-field shifted with respect to esterified compounds such as **21** or **23**, indicated a free alcohol at C-3 [18,20,22]. The observation of two exchangeable protons confirmed the presence of two OH groups in the alkaloid. Thus, the structure of compound **7** was elucidated as 3α,7β-dihydroxy-6β-(1-methyl-1H-pyrrol-2-yl-carbonyloxy)tropane.

The stop-flow LC–^{1}H NMR spectrum of compound **13** (t_R = 20 min) showed similar chemical shifts to **7** supporting the presence in the molecule of a single methylpyrrole ester at C-6. The LC–MS^n experiment of **13** showed a protonated molecule at M_r 265, suggesting the lack of an oxygen atom when compared to **7**. For this compound only one exchangeable proton was recorded confirming the presence of only one hydroxyl group. The NMR data of **13** exhibited a signal at δ_H 2.71, suggesting a saturated methylene moiety at the 7-position as for compounds **22** and **24**. Compound **13** was then elucidated as a 3,6-disubstituted tropane alkaloid corresponding to the structure 3α-hydroxy-6β-(1-methyl-1H-pyrrol-2-yl-carbonyloxy)tropane.

From LC–^{1}H NMR experiments, an additional compound (**14**) could be detected (t_R = 22 min). The on-flow LC–^{1}H NMR indicated that **14** possessed a single methylpyrrole ester as in **7** and **13**, and the remaining signals supported the presence of a tropanic nucleus. Its LC–MS^n analyses exhibited $[M+H]^+$ and $[M_D + D]^+$ at 265 and 267, respectively, demonstrating isomeric character with compound **13**. Despite a careful analysis of the NMR data only one deshielded tropanic proton was observed at δ_H 4.99 suggesting that the water suppression around 4.5 ppm affected the signal detection of the second oxygenated methine. The recorded values of the oxygenated methines at δ_H 4.99 and probably around 4.5 for **14** were different from those measured for the other compounds, which could implicate a variation in the oxygenated pattern of the tropanic skeleton. Accordingly, these variations impeded the complete structural elucidation of this compound.

3.5.3. *Partial identification of the minor alkaloids*

The LC–MS^n analysis of the other constituents of the alkaloid extract showed similar patterns of fragmentation for each compound, giving important information for their core structures. The selective MS^2 of each peak showed that all the different molecules shared a central structural element with fragment ions at *m/z* 140, 138, 122 or 120. Comparing this fragmentation pathway to that of the other on-line elucidated compounds and to the literature [7], these corresponded to a monohydroxylated tropane or a tropane moiety. The difference of 2 between fragments of the same type was related to the nature of their parent ions: disubstituted tropane alkaloids gave daughter ions at *m/z* 140 and 122, while trisubstituted tropane moieties gave daughter

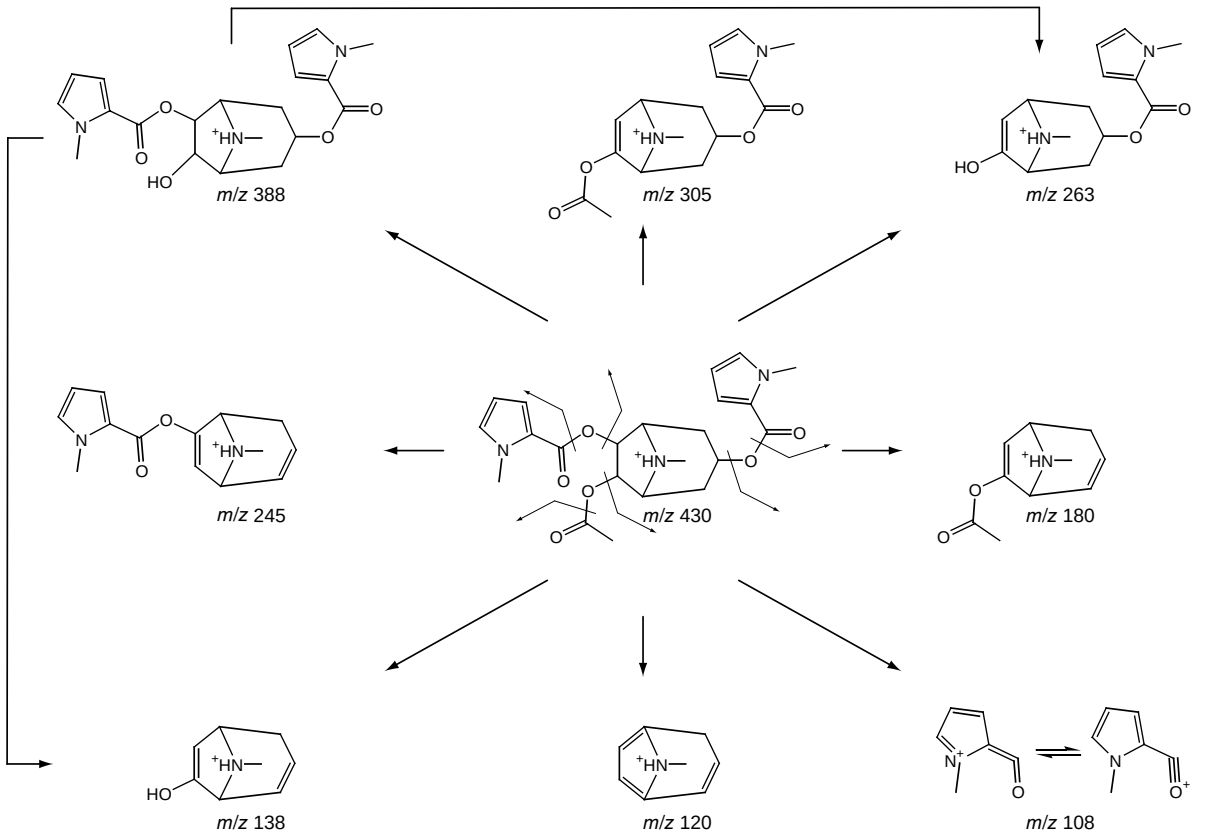


Fig. 4. Proposed fragmentation pattern for compound **23**. Due to the higher M_r, compound **23** was selected as an example. The other molecules gave similar low-M_r fragment ions.

ions at *m*/*z* 138 and 120. Two other fragment ions with low M_r (*m*/*z* 110 and 108) were also found in almost all molecules and they probably corresponded to the ester moieties. Based on the assignments made for the previously elucidated alkaloids and on literature data [7], these fragments were identified as 1-methyl-2-(oxoniomethyl)-1H-pyrrolium groups (e.g. see fragment structure of **23** in Fig. 4). As illustrated by the MS^3 experiments of ions at *m*/*z* 156 and 140, the fragments at *m*/*z* 110 and 108 could also be obtained from the fragmentation of a tri- or di-oxygenated tropane center. Due to its higher M_r, compound **23** was selected as an example to illustrate a hypothetical fragmentation pathway for the tropane alkaloids of *E. vacciniifolium* (Fig. 4). The superposition of the MS–MS and MS^3 traces at *m*/*z* 140, 138, 122, 120, 110 or 108 with the TIC trace of the extract showed that almost all detected molecules had the same daughter ions (Fig. 1A–C). This evaluation suggested that the differences among nearly all tropane molecules relied on the number and position of the ester substituents or hydroxyl groups. Blossey et al. reported the possibility of differentiating these positions in disubstituted tropane alkaloids by EI–MS, demonstrating the influence of functional groups on the fragmentation pattern of the tropane nucleus [23]. In this study, the fragments obtained by collision induced dissociation in the LC–APCI–MS^n were not sufficiently homogenous for a definitive determination of substitution positions.

The combined interpretation of LC–UV–MS^n data led to the conclusion that all compounds with an absorbance maximum around 270 nm were esterified by one or two pyrrolic chromophores, as for **7**, **13** and

21–**24** identified on-line. Comparison of these data for the minor alkaloids with those previously elucidated gave important information for their characterisation. Most of these alkaloids could be grouped in sets of isomeric structure. In many cases, molecular mass difference of 16 between these sets were recorded, indicating variation due to additional oxygen. The extra oxygen atoms could also appear in their fragment ions (e.g. *m*/*z* 263 and 247 for **21** and **24** in Table 1). This molecular ion difference occurred between compounds: **5**–**8** (each 281) and **1**, **3** (each 297); **9** (445) and **4** (461); **11**–**15** (each 265) and **5**–**8** (each 281); **17**, **20** (each 374) and **10** (390); **22** (358) and **17**, **20** (each 374); **24** (372) and **18**, **21** (each 388). In order to confirm that these oxygens corresponded to an additional hydroxyl group in the molecules, the number of exchangeable protons was calculated as mentioned before. Compounds **17** and **20** exhibited the same protonated molecule (16 more than **22**), but they had different numbers of exchangeable protons (1 for **17** and 2 for **20**). This observation suggested an additional hydroxyl group for **20** and probably the presence of an N-oxide in compound **17**. A similar deduction could also be made for compounds **18** and **21**. They had the same $[M + H]^+$ (16 more than **24**) but only **21** showed an additional exchangeable proton, suggesting the presence of an extra hydroxyl group. Therefore, compound **18** probably included a N-oxide in its structure. In the same way, five other tropane alkaloids (**1**, **3**, **5**, **10** and **16**) were deduced to contain a N-oxide in their structure in comparison with **7**, **8**, **13**, **20** and **21**, respectively. The occurrence of tropane alkaloid N-oxides in plants has previously been reported [24–27]. The ratio of N-oxides to tertiary bases in plants varies for different organs during ontogenesis. This fluctuation is an indication that N-oxides are not artifacts but that they are involved in the metabolic processes of the plant [24].

Compound **19** did not present any exchangeable protons and seemed to share a similar core structure with **18**. In fact, its most abundant fragment obtained by MS–MS (*m*/*z* 388) corresponded to the protonated molecule of **18**. After a comparison with the literature on tropane alkaloids, this fragment was assigned to the loss of an isovaleranyl moiety [28].

Compounds **9** and **4** had three absorbance maxima (241, 271 and 329 nm), indicating the presence of at least one different substituent. The MS–MS analysis of these two compounds gave typical fragment ions at *m*/*z* 247 and 263, respectively, for a tropane or a hydroxytropane esterified by a methylpyrrole, but a new daughter ion at *m*/*z* 181 appeared in the fragmentation pathway, confirming the presence of a new esterifying group. According to the fragmentation pattern of catuabine A, previously isolated from *E. vaccinifolium* [7], this fragment ion probably corresponded to a [(4-hydroxy-3,5-dimethoxyphenyl)methylidyne]oxonium. The presence of this ester was confirmed by the MS^3 experiment of **9** which gave fragment ions at *m*/*z* 181, 140 and 122 from a parent ion at *m*/*z* 320 assigned to a tropane moiety esterified by 4-hydroxy-3,5-dimethoxybenzoic acid. The trimethoxybenzoic acid ester in tropane alkaloids is relatively common and its occurrence was easily detected by MS–MS experiments giving, the typical fragment at *m*/*z* 195 for its acyl unit [17,18,21,27]. The presence of an additional hydroxyl group in **4** was also confirmed by the observation of an exchangeable proton more than in **9**.

The presence in the extract of another tropane alkaloid with an original substituent was demonstrated by the occurrence of a different chromophore for compound **2** (two maxima of absorbance at ca. 241 and 294 nm). The absence of a maximum around 270 nm suggested the lack of a pyrrolic chromophore. The MS–MS experiments of **2** gave typical fragment ions for a tropane center with a most abundant ion at *m*/*z* 156, corresponding to a dihydroxylated moiety. The loss of the substituent during the fragmentation was shown by the loss of 152 unit from the molecular ion M_r (308) of **2**. According to the literature on tropane alkaloids [27], this esterifying group could be a 3-hydroxyphenylacetic acid, but supplementary analysis is needed for unambiguous identification.

On the basis of the above interpretation of the LC–UV–MS^n spectroscopic data and by analogy with the on-line elucidated compounds, **1**–**6**, **8**–**12** and **14**–**20** were partially identified as di- or tri-substituted tropane alkaloids, mono- or di-esterified by pyrrolic, methylpyrrolic, hydroxydimethoxybenzoic, or hydroxyphenylacetic acids (Fig. 2). The proposed structures of these compounds suggested them to be new and their targeted isolation is being undertaken for complete characterisation (Zanolari et al., unpublished research) [16].

4. Conclusion

The on-line methods presented in this paper allowed complete or partial identification of 24 tropane alkaloids of *E. vacciniifolium*. For their characterisation, LC–UV and LC–MS data were acquired at the same time while the LC–NMR analysis was independently performed. A complete hyphenation of all these techniques together could be problematic because of the differences in the sensitivities of the detectors [29].

The interpretation of the data obtained by these LC hyphenated techniques permitted the structural elucidation of six new tropane alkaloids (**7**, **13** and **21**–**24**; Fig. 2). The structures of the other detected compounds were partially characterized as di- or tri-substitued tropane alkaloids, mono- or di-esterified by pyrrolic, methylpyrrolic, hydroxydimethoxybenzoic or hydroxyphenylacetic acids (Fig. 2), and they were found to be potentially new natural products. The study of this *Erythroxylum* species is relevant because of the nature of its isolated alkaloids. The pyrrole substituent is uncommon in tropane alkaloids and may play an important role in the biological activity of the compounds. In order to completely elucidate their structures and to perform a screening of their biological activities, a targeted isolation of the constituents of the alkaloid extract is being undertaken.

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