ELSEVIER

Journal of Chromatography A, 841 (1999) 165–176

JOURNAL OF CHROMATOGRAPHY A

# High-performance liquid chromatography–diode array detection–tandem mass spectrometry analyses of the alkaloid extracts of Amazon *Psychotria* species☆

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Received 6 November 1998; received in revised form 23 February 1999; accepted 26 February 1999

**Abstract**

High-performance liquid chromatography paired with UV photodiode array, electrospray MS–MS and thermospray MS detectors was investigated as a method for the analysis of alkaloids in different parts of *Psychotria* spp. collected in the Amazon. Nine products have been isolated from the vegetable material and their structures elucidated by spectroscopic means. These constituents indicate pyrrolidinoindoline and quinoline alkaloid classes. Minor components were also assigned through MS fragmentations. Place and period of collections of the plant materials are responsible for both qualitative and quantitative variations with respect to pyrrolidinoindoline and quinoline type alkaloids. 

*Keywords:* *Psychotria*; Plant materials; Alkaloids

## 1. Introduction

An ethnopharmacological survey among Amazonian ''caboclos'' identified a plant derived remedy commonly used to prepare a traditional pain killer. *Psychotria colorata* (Willd ex R & S) Muell. Arg. (Perpétua do Mato) has been found to be the main constituent of this remedy [1]. An alkaloid extract of *P. colorata* showed a marked dose dependent naloxone-reversible analgesic activity, suggesting an opioid-like pharmacological profile [2,3]. Five compounds **1, 2, 4, 12**, **15** have so far been isolated and characterized from *P. colorata* flowers (see Fig. 1 for chemical structures) [4]. Only the pyrrolidinoindoline type alkaloids **4**, **12**, **15** proved to exert analgesic effects in behavioral tests (thermal and non thermal nociceptive experiments), while the quinoline type alkaloids **1** and **2** showed no activity [5]. Bioactivity is so far dependent on structural features.

In this paper we report the characterization of the alkaloid extracts from *P. colorata* and *P. muscosa*

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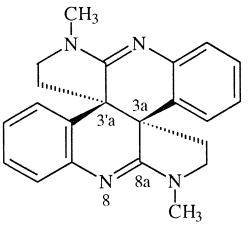
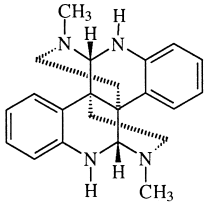
☆Part of this work was presented at the 39th Annual Meeting of the American Society of Pharmacognosy, Orlando, FL, 19–24 July 1998.

0021-9673/99/$ – see front matter 
PII: S0021-9673(99)00298-8

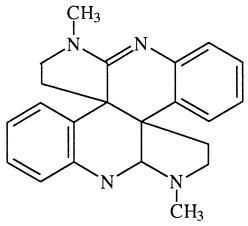


(**1**) PML 100

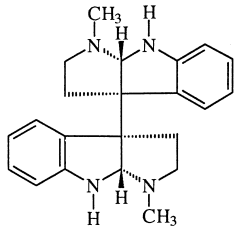
8-8a, 8'-8'a tetradehydroisocalycanthine
3a(*R*), 3'a(*R*)



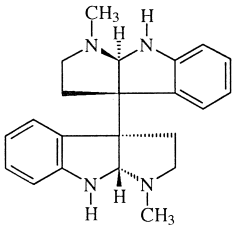
(**2**) (-)-CALYCANTHINE



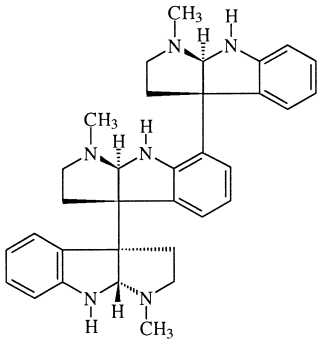
(**3**) PML 300



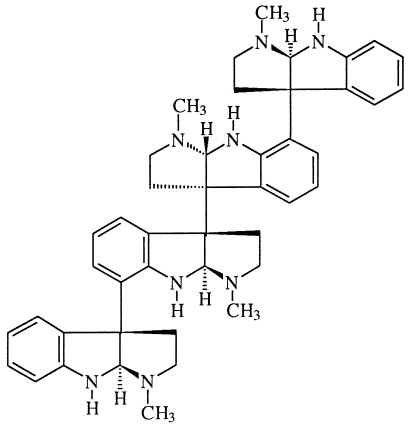
(**4**) (+)-CHIMONANTHINE



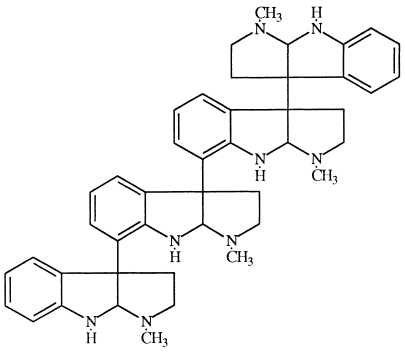
(**5**) *meso*-CHIMONANTHINE



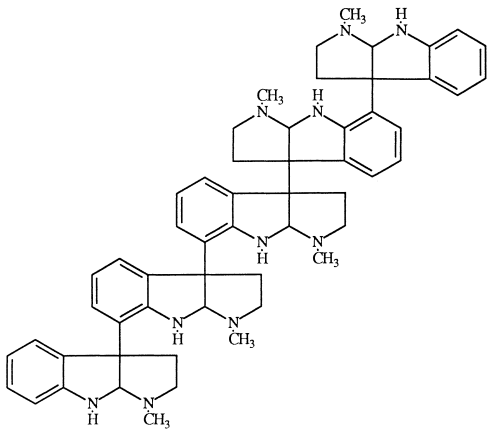
(**12**) HODGKINSINE



(**15**) QUADRIGEMINE C



(**13**) QUADRIGEMINE B



(**16**) PSYCHOTRIDINE

Fig. 1. Chemical structures of *Psychotria* alkaloids.

Table 1
Extraction and alkaloid yields of different parts of *Psychotria* spp.

| | Mass (g) | Total extract MeOH (g) (%) | Alkaloid extract (mg) (%) |
|---|---|---|---|
| *P. colorata* | | | |
| Leaves | 225 | 17.2 (7.6) | 1055 (0.6) |
| Flowers | 33 | 2.0 (6.0) | 228 (0.7) |
| Stems | 123 | 7.3 (5.8) | 213 (0.2) |
| Roots | 98 | 4.5 (4.5) | 44 (0.05) |
| *P. muscosa* | | | |
| Leaves | 30 | 2.1 (7.0) | 114 (0.4) |
| Flowers | 65 | 4.1 (6.3) | 575 (0.9) |

(Jacq.) Steyermark by high-performance liquid chromatography–diode array detection (DAD)–tandem mass spectrometry (MS–MS). The technique allows rapid identification of the structural differences among the alkaloids, allowing one to identify possible drug adulterations, and to address the isolation of active components.

## 2. Experimental

### 2.1. Plant material

*P. colorata* sp. was collected at several places around the city of Belém (Pará, Brazil) during the dry season (March to August 1993) (ZC 23, NYBG), and in February 1997 (IAN Herbarium in Belém, IAN 166.535). *P. muscosa* was collected at Benfica (near Belém) in February 1997 (IAN 166.536).

### 2.2. Standardized plant extractions

Dried material was wet with a 28% ammonium hydroxide (0.1 l/kg of dried material) and extracted six times with MeOH (10 l/kg), under $N_2$, with stirring, for 2 h. The methanol extracts were pooled, evaporated to dryness, taken up with 0.1 *M* HCl and extracted three times with diethyl ether. The aqueous phase was basified with 6 *M* aqueous NaOH to pH 10 and extracted with $CHCl_3$ (3×10 ml/g of MeOH extract). Yields are reported in Table 1.

### 2.3. Alkaloids isolation

Compounds **1**, **2**, **3**, **4**, **5**, **12** have been isolated from *P. muscosa* leaves as follows. The alkaloid extract (7.7 g) was fractionated on Sephadex LH-20 eluted with $CH_2Cl_2$. Two fractions, respectively containing low-molecular-mass compounds (fraction A, 3 g) and high-molecular-mass compounds (fraction B 3.7 g) were obtained. Fraction A was repeatedly purified on silica gel, first through a flash column chromatography (CC) (cyclohexane–EtOAc–diethylamine, 10:10:1), then through a gravity CC ($CHCl_3$–MeOH–$NH_4OH$, 9:1:0.15), obtaining **1** (345 mg), (−)-calycanthine **2** (10 mg), **3** (23 mg), hodgkinsine **12** (206 mg). Fraction B was purified by medium-pressure liquid chromatography (MPLC) on silica gel (EtOAc–diethylamine, 95:5), then by preparative reversed-phase (RP) HPLC (MeOH–water–diethylamine, 75:25:0.1), obtaining (+)-chimonanthine **4** (26 mg) and *meso*-chimonanthine **5** (29 mg).

Compounds **12**, **13**, **15** and **16** have been isolated from *P. colorata* leaves (collection of 1997) as follows. The alkaloid extract (1.3 g) was fractionated on a Sephadex LH20 eluting with MeOH. Hodgkinsine (**12**) (213 mg) was obtained, together with a crop of fractions (718 mg) which were purified by RP-MPLC (RP8, MeOH–water–diethylamine, 95:5:0.1), followed by preparative HPLC (RP18, MeOH–water–diethylamine, 83:17:0.1). Quadrigemine B **13** (10.5 mg), quadrigemine C **15** (22 mg) and psychotridine **16** (242 mg) were obtained.

The structural elucidation of compounds **1**, **2**, **4**, **12** and **15** is described in Ref. [4]. Compounds **3**, **5**, **15** and **16** were identical by $^{1}H$- and $^{13}C$-nuclear magnetic resonance (NMR), UV, MS with literature data [6–8]. Work is in progress to determine their absolute configurations.

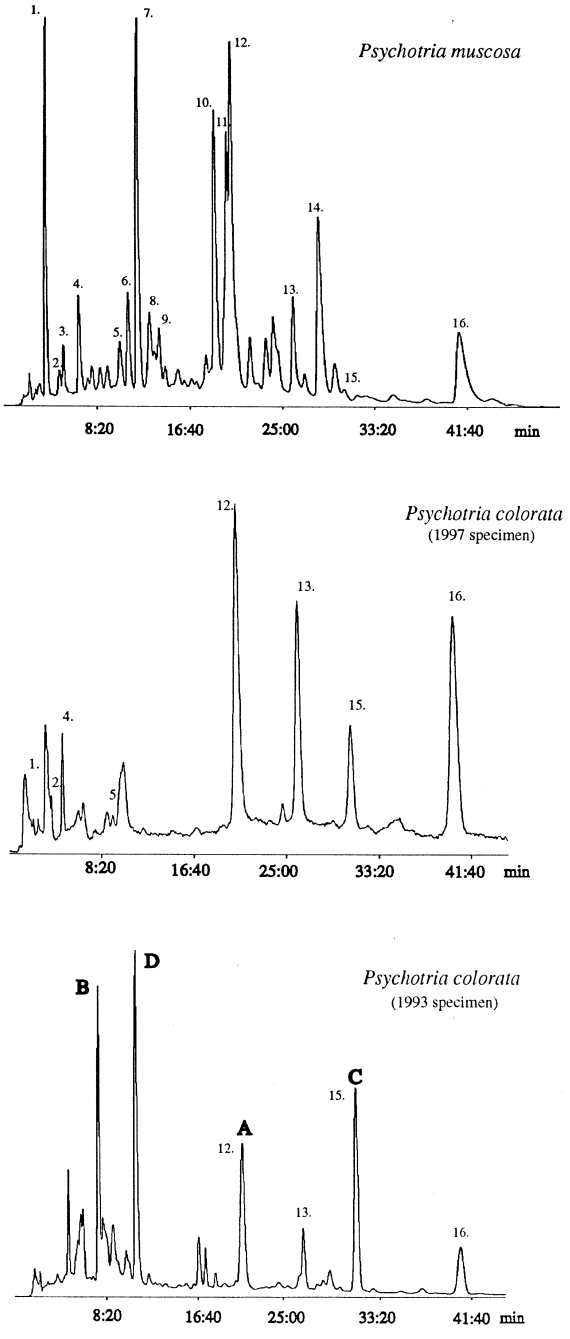



Fig. 2. HPLC–UV–Vis profiles of *Psychotria* spp. Peaks shown in Table 2.

### 2.4. Column chromatography

Column chromatography was performed with silica gel 60 (63–200 μm or 40–63 μm) or with neutral Alumina (activity I) (art 1.0177, Merck).

### 2.5. Thin-layer chromatography (TLC) analysis

TLC plates (silica gel 60 $F_{254}$) were eluted with $CHCl_3$–MeOH–$NH_4OH$ (9:1:0.15). Spots were revealed by spraying with modified Ehrlich reagent (*p*-dimethylaminocinnamaldehyde in 25% methanolic HCl) or 10% methanolic $H_2SO_4$, followed by heating, or by absorption of the UV light (254 nm).

### 2.6. HPLC–UV analysis

HPLC analyses were carried out on a Waters (Milford, MA, USA) 600-MS pump system, connected to a Waters PDA 991 detector and a NEC 386/25 personal computer. A Symmetry $C_{18}$ column (5 μm, 250×4.6 mm I.D., Waters) was used, eluted with MeOH–water–$Et_2NH$ with a linear gradient from 72:28:0.1 ($T_0$) to 85:15:0.1 ($T_{25}$) to 72:28:0.1 ($T_{40}$), at a flow-rate of 1 ml/min. Samples were filtered prior to each injection through Millex $FH_{13}$ filters (5 μm, Millipore).

### 2.7. Preparative HPLC

Preparative HPLC was carried out on a Varian 5000 pump system, connected to a L-4200 UV–Vis detector (Merck–Hitachi) and a HP 3396A integrator (Hewlett-Packard). A Symmetryprep $C_{18}$ column was used (7 μm, 150×7.8 mm I.D., Waters) eluted with the same mixture as described above, under isocratic conditions, at a flow-rate of 1.8 ml/min. Samples (400 μl, 4 mg each) were filtered before each injection.

### 2.8. HPLC–thermospray (TSP) MS analysis

The HPLC–TSP-MS system included a Waters 600-MS pump equipped with a gradient controller and a tunable UV–Vis Waters 486 detector. Injections were performed with a Waters 717 plus automatic sample injection module. The liquid chro-

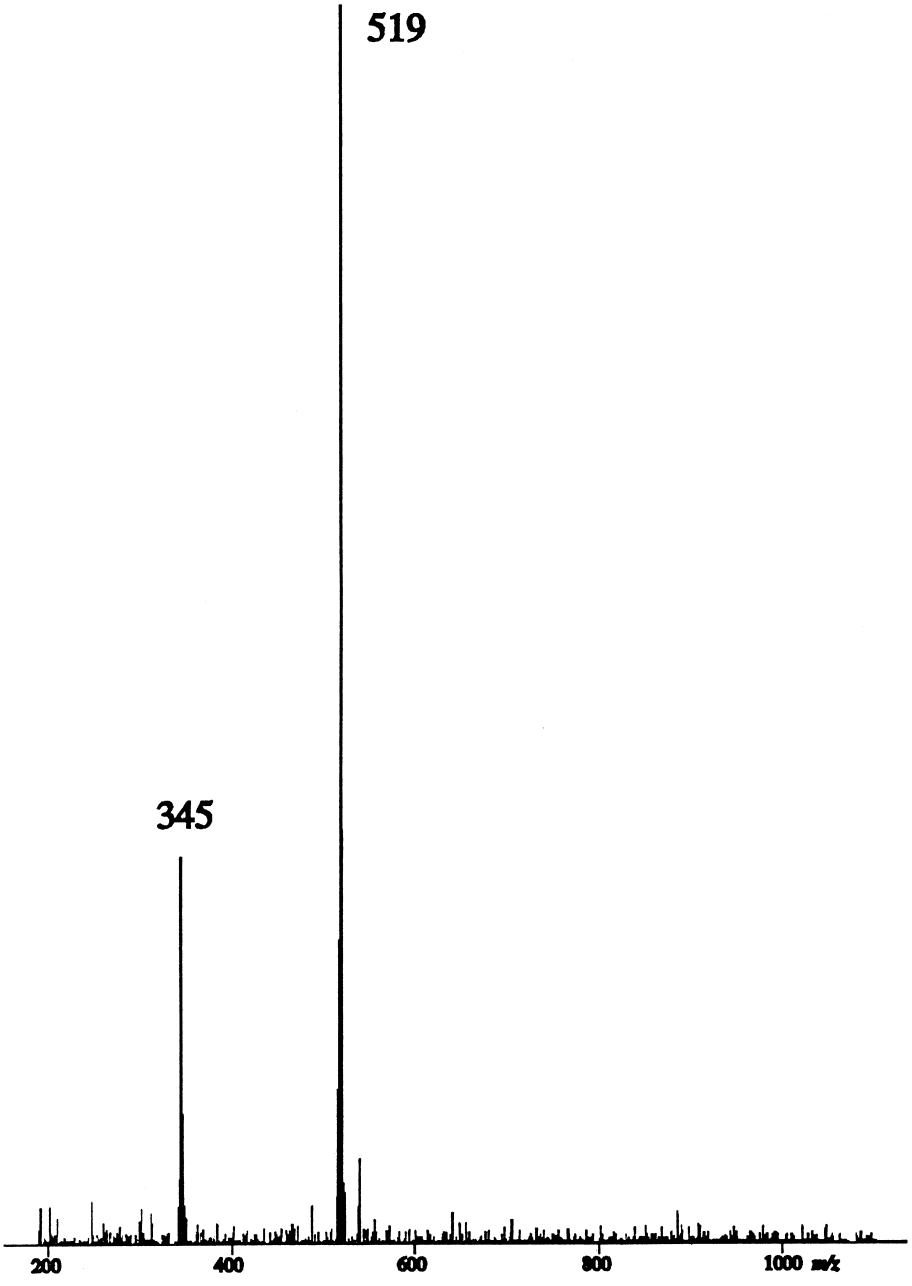



Fig. 3. Positive thermospray mass spectrum of hodgkinsine.

matography system was connected to a Finnigan MAT (San Jose, CA, USA) TSQ-700 triple quadrupole mass spectrometer equipped with a 5100 DEC station, ICIS data system and a TSP-2 TSP interface. HPLC conditions were as described in Section 2.6. MS conditions were optimized in order to achieve

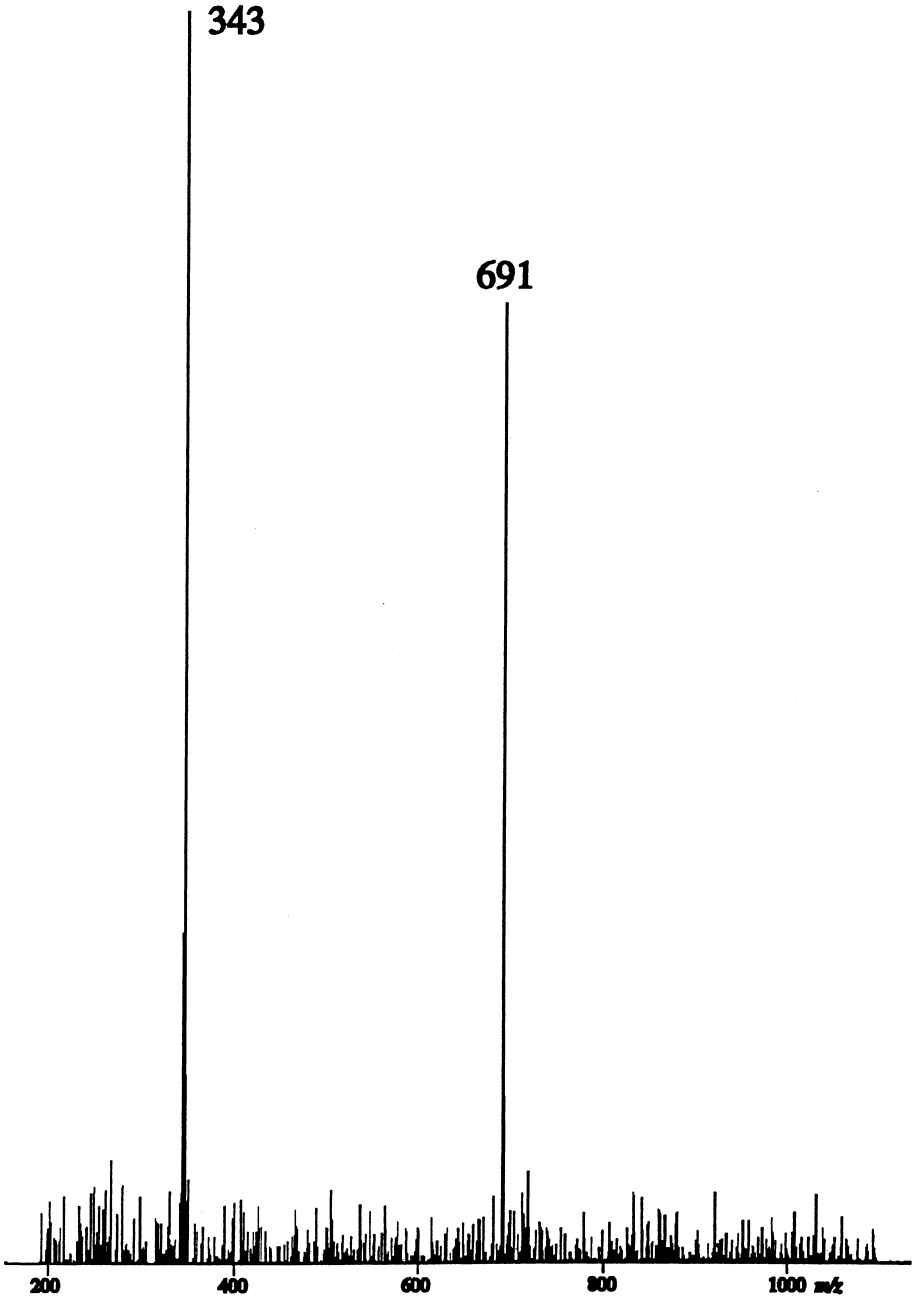



Fig. 4. Positive thermospray mass spectrum of quadrigemine C.

maximum sensitivity. Typical values were as follows: source block temperature 230°C, vaporizer temperature 75°C, repeller voltage 30 V, discharge off-mode, filament on-mode (electron energy 400 eV, emission current 350 μA). The electron multiplier and dynode voltages were set at 2000 V and 15 kV,

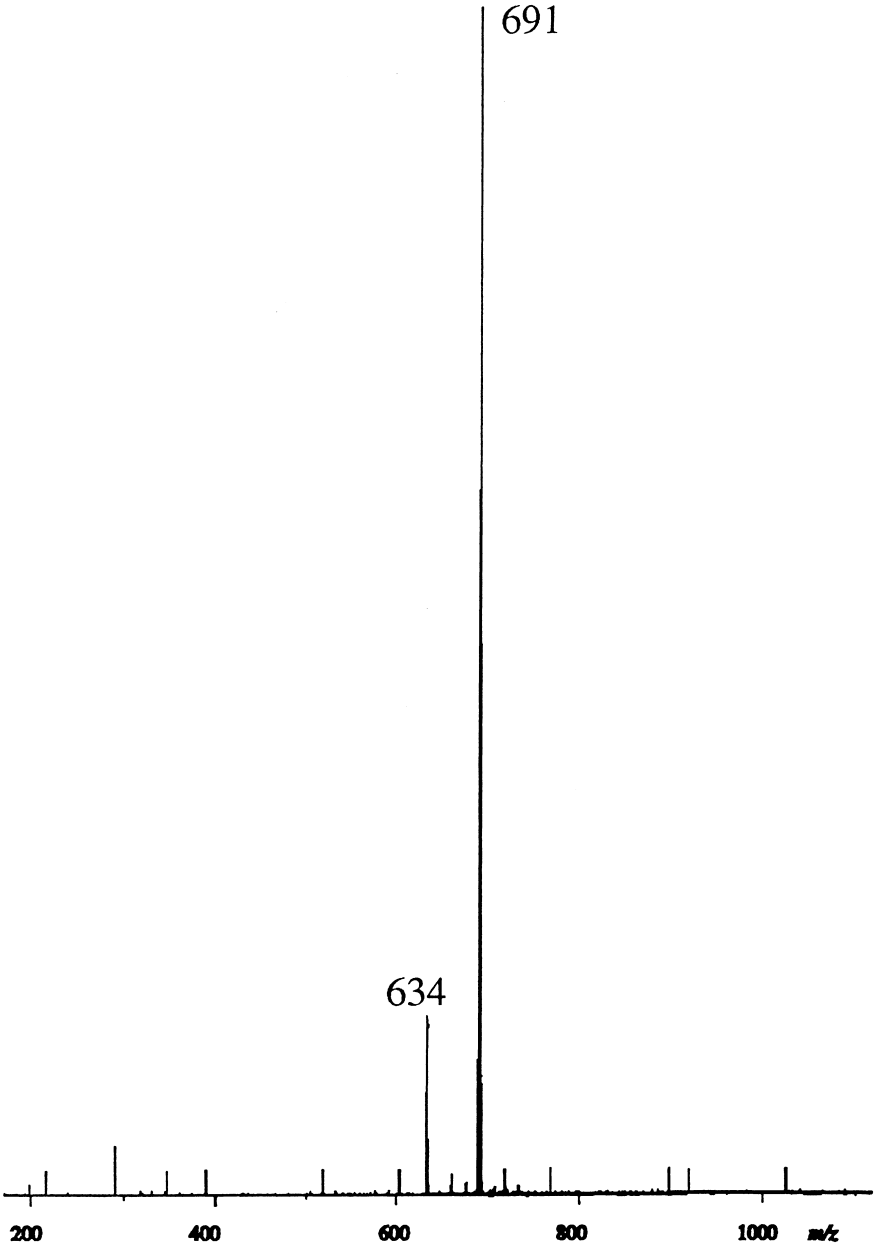



Fig. 5. Positive thermospray mass spectrum of peak D.

respectively. Preamplifier sensitivity $10^{-8}$ A/V. Positive TSP mass spectra were acquired in centroid mode scanning the Q3 analyzer from $m/z$ 200 to 900 (scan time 1 s).

### 2.9. HPLC–electrospray (ESI) MS analysis

The HPLC–ESI-MS system included a Thermo Separation Products P4000 pump (San Jose, CA,

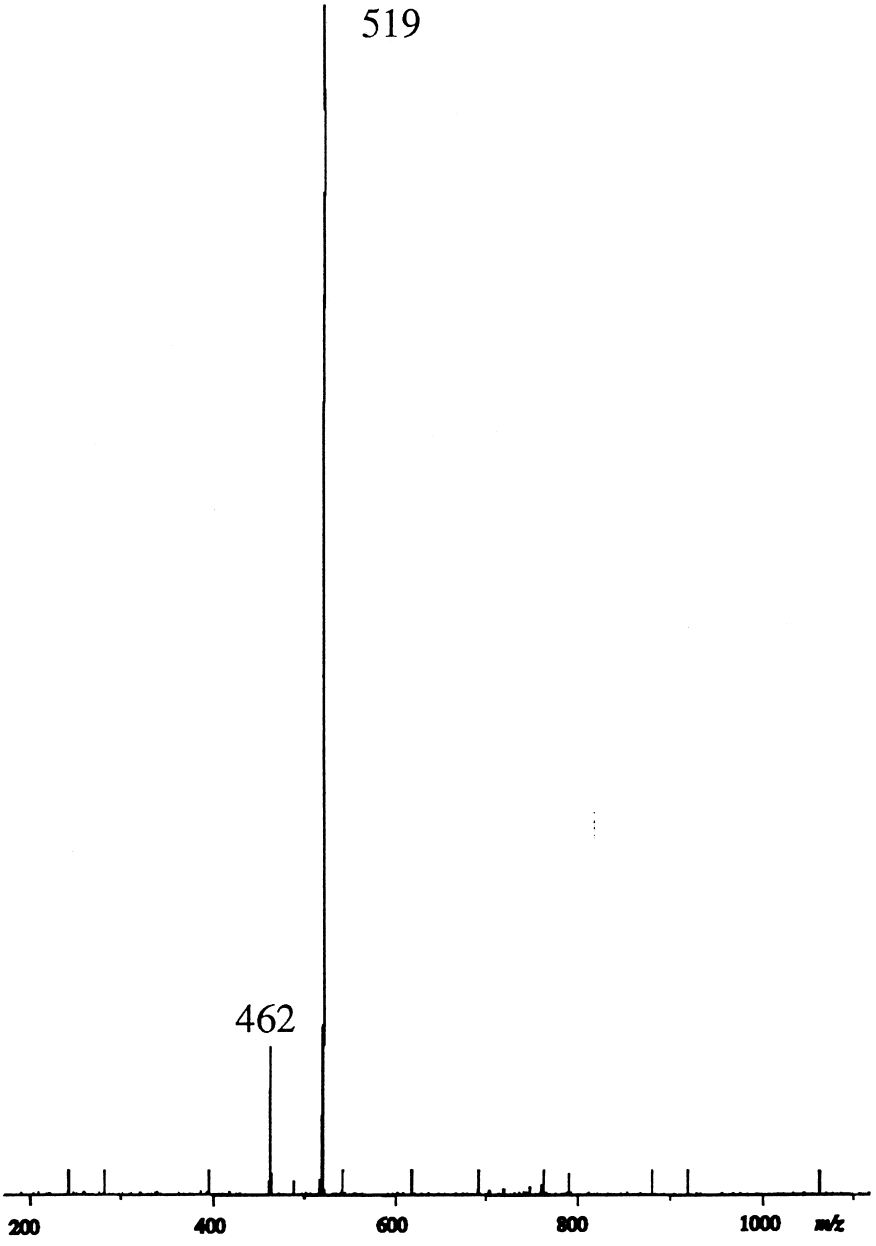



Fig. 6. Positive thermospray mass spectrum of peak B.

USA) and a tunable Thermo Separation Products UV1000 detector. The liquid chromatography system was connected to a Finnigan MAT LCQ ion trap mass spectrometer equipped with a Microsoft Windows NT data system and an ESI interface. HPLC conditions were as described in Section 2.6. ESI values were as follows: source voltage 5.0 kV, sheath gas flow-rate 60 p.s.i., auxiliary gas flow 0.2 p.s.i., capillary voltage 21 V and capillary temperature 260°C (1 p.s.i.=6894.76 Pa). Full scan mass spectra from $m/z$ 200 to 900 in the positive ion mode were obtained (scan time 1 s).

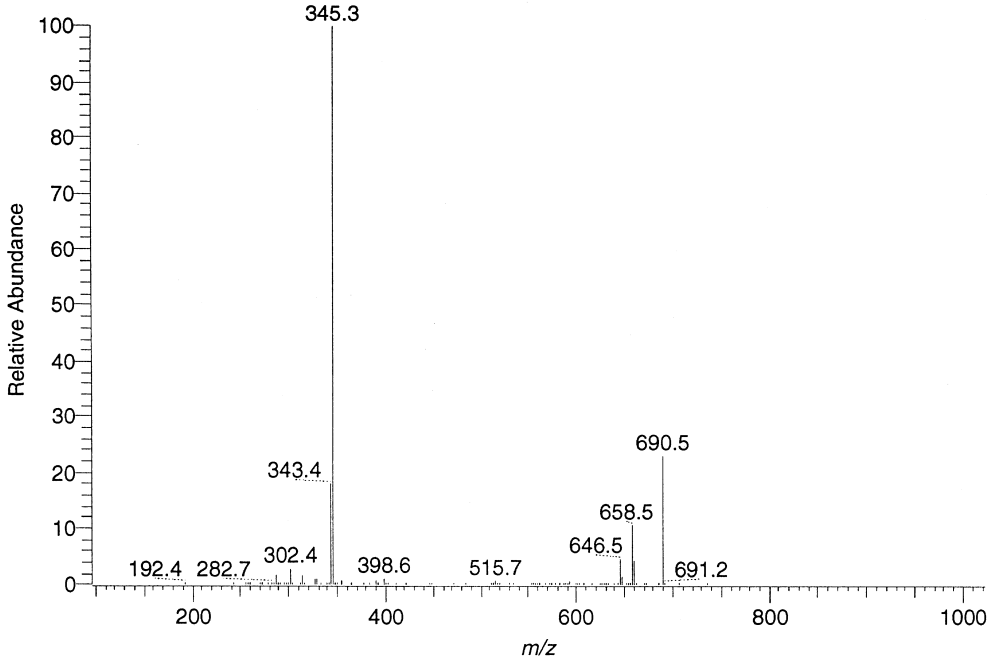



Fig. 7. Positive ESI-MS–MS spectrum of quadrigemine C (parent ion at $m/z$ 691).

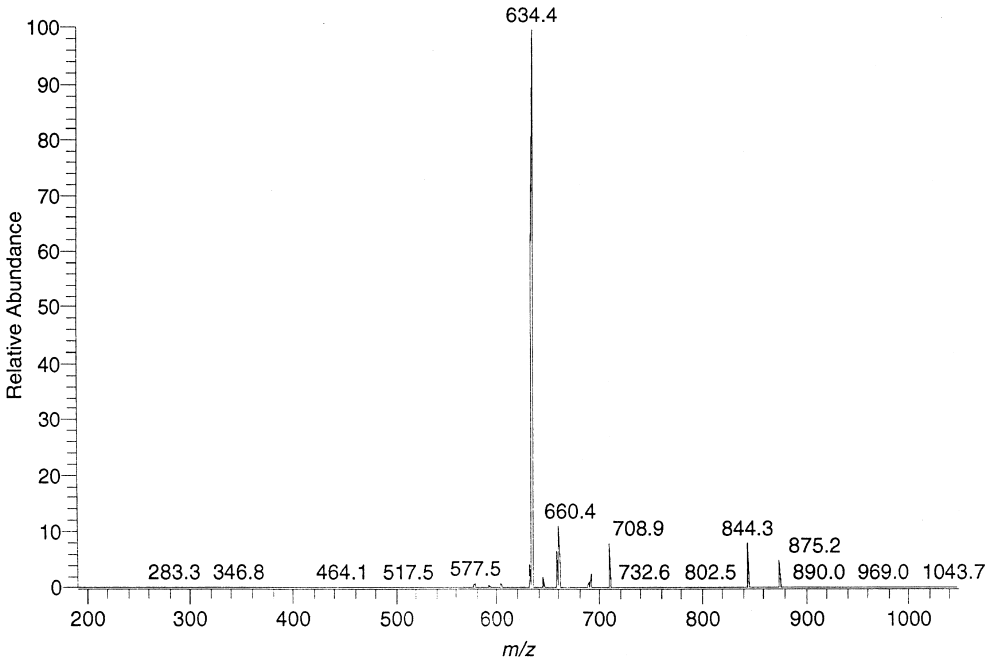
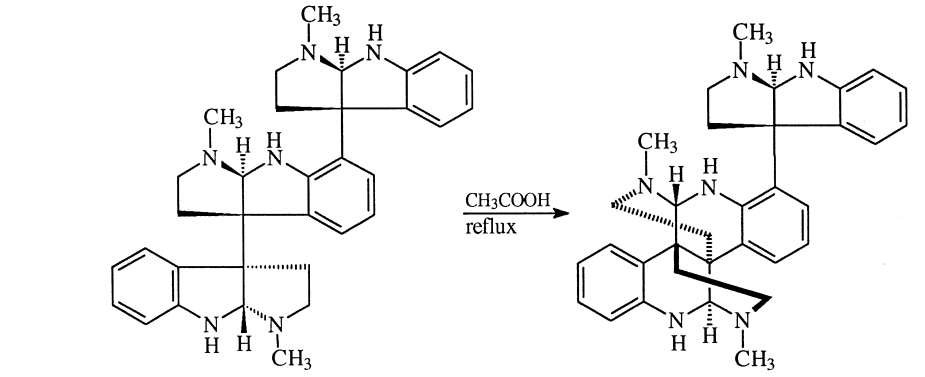
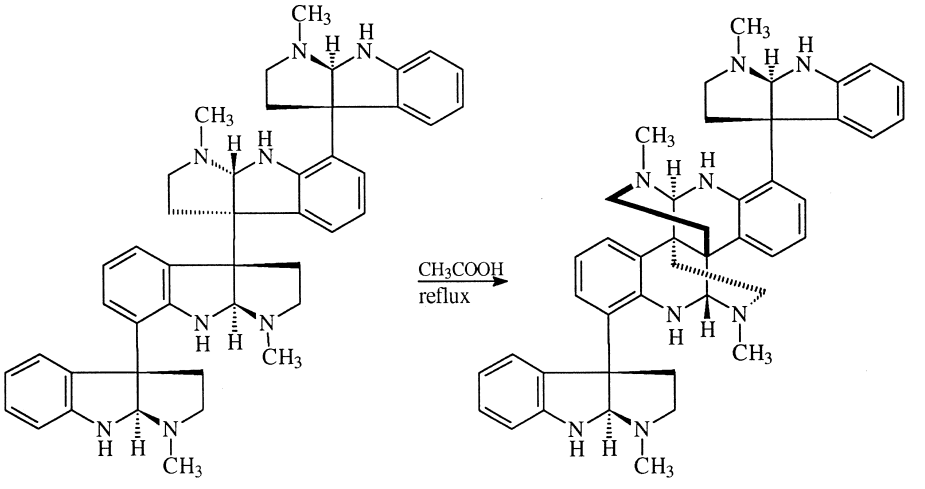



Fig. 8. Positive ESI-MS–MS spectrum of peak D (parent ion at $m/z$ 691).




(12) HODGKINSINE (17) CALYCOSIDINE




(15) QUADRIGEMINE C (18) PSYCHOLEINE

Fig. 9. Reaction scheme for the preparation of quinoline type alkaloids.

## 3. Results and discussion

The standardized extraction of different parts of *P. colorata* show identical profiles in the analysis of their alkaloid content (not shown). Table 1 reports the yields in alkaloids of the different parts: flowers give the most abundant yield.

The most abundant compounds have been isolated by means of GFC (gel filtration chromatography), RP-MPLC, CC (flash chromatography on silica gel) and final preparative RP-HPLC, and the structures were elucidated by spectroscopic means [4,6–8].

As evidenced in the chromatographic profiles (Fig. 2), *P. colorata* species, collected in different places and different seasons, show a slight difference in the less retained compounds.

Alkaloid constituents give positive thermospray mass spectra with intense protonated molecules $[M+H]^+$ and a low fragmentation. In the case of pyrrolidinoindoline alkaloids this is mainly due to

Table 2
Peak assignments for analyses of *Psychotria* spp.

| Peak No. | $t_R$ (min) | $[M+H]^+$ ($m/z$) | UV $\lambda_{max}$ (nm) | Other peaks (%) | Identification ($M_r$) |
|---|---|---|---|---|---|
| 1 | 3.49 | 343 | 276, 300 (sh) | | PML100 (342) |
| 2 | 5.00 | 347 | 248, 306 | | Calycanthine (346) |
| 3 | 5.24 | 345 | 246, 282, 304 (sh) | | PML300 (344) |
| 4 | 6.43 | 347 | 244, 302 | | (+)-Chimonanthine (346) |
| 5 | 10.21 | 347 | 244, 304 | | *meso*-Chimonanthine (346) |
| 6 | 11.05 | 519 | 248, 304 | 541, 345 (7) | Trimer (518) |
| 7 | 11.55 | 519 | 248, 304 | 541, 345 (5) | Trimer (518) |
| 8 | 13.00 | 691 (77) | 246, 306 | 519 (100) | Tetramer? (690?) |
| 9 | 13.50 | 519 | 246, 302 | 541, 344 (7) | Trimer (519) |
| 10 | 18.53 | 691 | 248, 304 | 713, 519 (20) | Tetramer (690) |
| 11 | 20.03 | 691 | 246, 306 | 713 | Tetramer (690) |
| 12 | 20.23 | 519 | 244, 304 | 541, 345 (25) | Hodgkinsine (518) |
| 13 | 25.58 | 691 | 244, 304 | 713, 517 (20) | Quadrigemine B (690) |
| 14 | 28.17 | 691 | 246, 306 | 713, 345 (10) | Tetramer (690) |
| 15 | 30.49 | 691 | 244, 304 | 345 (68) | Quadrigemine C (690) |
| 16 | 40.56 | 863 | 246, 306 | 885, 901, 518 | Psychotridine (862) |

the subsequent loss of the monomeric unit constituting the molecule. As an example, the trimer hodgkinsine **12** gives a positive spectrum (Fig. 3) with a $[M+H]^+$ ion at $m/z$ 519, and a peak at $m/z$ 345 corresponding to the neutral loss of a pyrrolidinoindoline unit (174 u). The tetramer quadrigemine C (**15**) shows a mass spectrum (Fig. 4) containing, besides the $[M+H]^+$ ion at $m/z$ 691, an ion at $m/z$ 343 due to the neutral loss of two pyrrolidinoindoline units. On the contrary, quinoline alkaloids give positive thermospray mass spectra with, beside the base peak $[M+H]^+$ ion, a fragment ion corresponding to the neutral loss of $C_3H_7N$. This is exemplified by the chromatographic peaks at retention time ($t_R$) 7.39 (**B**) and 11.00 min (**D**). Compound D (Fig. 5) shows, in its thermospray positive mass spectrum, an $[M+H]^+$ ion at $m/z$ 691, together with an ion peak and $m/z$ 634, 10% relative abundance about, corresponding to $[M-57]^+$. Compound B (Fig. 6) shows the presence of ion peaks at $m/z$ 519 $[M+H]^+$ and at $m/z$ 462 $[M-57]^+$. On the basis of these data, isomer constituents belonging to different alkaloid classes contained in *Psychotria* species may be differentiated on the basis of the fragmentation pattern of their positive thermospray mass spectra. ESI-MS–MS experiments confirmed the structural information reported above. While quadrigemine C positive ESI spectrum (Fig. 7) contains a daughter ion at $m/z$ 345 (parent ion at $m/z$ 691), corresponding to the symmetric cleavage of the molecule, compound at $t_R$ 11.00 min (**D**) gives a daughter ion (Fig. 8) at $m/z$ 634 (parent ion at $m/z$ 691), corresponding to the neutral loss of $C_3H_7N$, confirming it to be a quinoline type alkaloid (see Fig. 9 for a reaction scheme for the preparation of quinoline type alkaloids). The obvious advantage of HPLC–MS–MS is the rapid on-line differentiation between pyrrolidinoindoline type alkaloids (sequential loss of methyltryptamine units from the molecular ions) and quinoline type alkaloids, which on the contrary, show loss of $C_3H_7N$ from the molecular ion, thus avoiding time consuming isolation procedures.

**A** and **B** show the same behavior. **B** was identified as hodgkinsine **12** [4].

Because of the fact that it has been demonstrated that chimonanthine produces calycanthine by acid treatment [9], we concluded that the contemporary presence of pyrrolidinoindoline and quinoline type alkaloids was probably attributable to the different climatic conditions prior to the plant collection. A sample of hodgkinsine **12** [4] was refluxed in $CH_3COOH$ and calycosidine **17** was quantitatively recovered as reported in the literature [10]. Coinjection of B and calycosidine **17** revealed the two compounds to be different, thus hypothesizing that

compound B is not an artifact from hodgkinsine but a novel natural isomer of calycosidine whose biogenetic pathway has to be elucidated. Work is in progress to determine its absolute stereochemistry. The acid treatment of quadrigemine C gives psycholeine **18** which shows it to be different from compound **D**.

A morphologically similar species *P. muscosa* (Jacq) Steyermark was collected and the alkaloid extracts of leaves and flowers compared to those of *P. colorata*.

Considerable differences were observed among less retained compounds, which also correspond to low-molecular-mass products. The presence of a series of isomeric pyrrolidinoindoline alkaloids was deduced from the DAD–MS analysis (see Table 2). In addition, two dehydroquinoline type alkaloids **1**, **3**, biogenetically correlated to chimonanthine and calycanthine were found [9].

## 4. Conclusions

HPLC–DAD–MS–MS analysis allows one to discriminate among structurally different alkaloids present in the extracts, thus overcoming complicated isolations and structural elucidations. A preliminary analysis of *Psychotria* sp. alkaloid extracts is of benefit in addressing the isolation of the active components, and during quality control of drug preparations.

## Acknowledgements

Part of this work has been supported by MURST and CNR of Italy.

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