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The Spatial Distribution of Alkaloids in *Psychotria prunifolia* (Kunth) Steyererm and *Palicourea coriacea* (Cham.) K. Schum Leaves Analysed by Desorption Electrospray Ionisation Mass Spectrometry Imaging

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ABSTRACT:

Introduction – Species of the genera *Psychotria* and *Palicourea* are sources of indole alkaloids, however, the distribution of alkaloids within the plants is not known. Analysing the spatial distribution using desorption electrospray ionisation mass spectrometry imaging (DESI-MSI) has become attractive due to its simplicity and high selectivity compared to traditional histochemical techniques.

Objectives – To apply DESI-MSI to visualise the alkaloid distribution on the leaf surface of *Psychotria prunifolia* and *Palicourea coriacea* and to compare the distributions with HPLC–MS and histochemical analyses.

Methodology – Based upon previous structure elucidation studies, four alkaloids targeted in this study were identified using high resolution mass spectrometry by direct infusion of plant extracts, and their distributions were imaged by DESI-MSI via tissue imprints on a porous Teflon surface. Relative quantitation of the four alkaloids was obtained by HPLC–MS/MS analysis performed using multiple-reaction monitoring (MRM) mode on a triple quadrupole mass spectrometer.

Results – Alkaloids showed distinct distributions on the leaf surfaces. Prunifoleine was mainly present in the midrib, while 10-hydroxyisodepeaninol was concentrated close to the petiole; a uniform distribution of 10-hydroxyantirrhine was observed in the whole leaf of *Psychotria prunifolia*. The imprinted image from the *Palicourea coriacea* leaf also showed a homogeneous distribution of calycanthine throughout the leaf surface.

Conclusion – Different distributions were found for three alkaloids in *Psychotria prunifolia*, and the distributions found by MSI were in complete accordance with HPLC–MS analysis and histochemical results. The DESI-MSI technique was therefore demonstrated to provide reliable information about the spatial distribution of metabolites in plants. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: DESI-MSI; alkaloids; Rubiaceae; imaging

Introduction

Psychotria L. and *Palicourea* Aubl. (Rubiaceae) species are used in Brazilian folk medicine to treat several types of diseases (Delprete, 2010; Yang *et al.*, 2016). The *Psychotria* genus, which comprises approximately 1600 species, is widely acknowledged as a source of indole alkaloids as well as for its traditional indigenous use as a hallucinogenic beverage (Rivier and Lindgren, 1972). According to a recent review, 60% of the different metabolites isolated from *Psychotria* species are alkaloids, of which 87% are indole alkaloids (Klein-Junior *et al.*, 2014). In a previous study of *Psychotria prunifolia*, five indole alkaloids were isolated and among these the prunifoleine (**1**) and 14-oxoprufunifoleine (**2**) showed inhibitory activity in a time-dependent mechanism against monoamino oxidase (MAO-A), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) which are target enzymes in the treatment of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Passos *et al.*, 2013).

The genus *Palicourea* comprises approximately 600 species (Taylor, 2015). *Palicourea coriacea* (Cham.) K. Schum is known to contain the alkaloid calycanthine (**5**), glucoindole alkaloids such as 3-*epi*-strictosidinic acid and derivatives, ursolic acid and benzoyl and cinnamoyl derivatives (do Nascimento *et al.*, 2006; da Silva *et al.*, 2008).

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Phytochemical studies on *Palicourea* or *Psychotria* in recent years have further contributed to ethnobotanical, pharmacological and chemotaxonomic research (Calixto *et al.*, 2016; Yang *et al.*, 2016). However, the spatial distribution of alkaloids in the leaves or any part of the plant is not known in these genera.

In the present study, the distributions of three alkaloids in leaves of *Psychotria prunifolia* and one alkaloid in leaves of *Palicourea coriacea* were analysed by indirect desorption electrospray ionisation mass spectrometry imaging (DESI-MSI) (Takáts *et al.*, 2004). Standard histochemical staining techniques for alkaloids and HPLC–MS/MS analyses were used to validate the results obtained by DESI-MSI.

Experimental

Plant material

Forty-five fresh leaves of *Psychotria prunifolia* and *Palicourea coriacea* were collected at Bosque Saint-Hilaire in Goiás state, Brazil, in December. The leaves were placed in Falcon tubes with a piece of cotton soaked with water (three leaves per tube), and shipped to the Department of Pharmacy, University of Copenhagen where they were stored at -80°C until analysis. The voucher specimens of the *Psychotria prunifolia* and *Palicourea coriacea* were deposited at the herbarium of the Universidade Federal de Goiás (UFG) with numbers 10323 and 27153, respectively. The leaves were collected from a single adult plant of each taxon. Collection and transport were

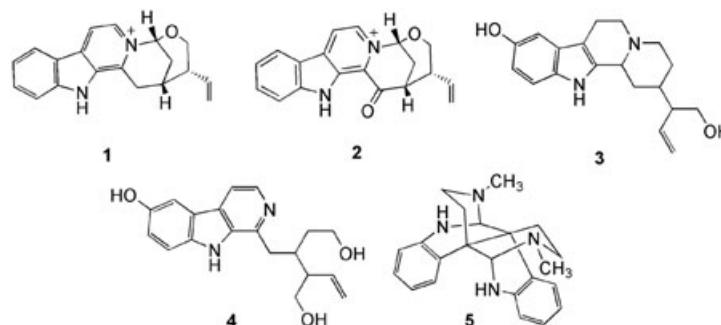


Figure 1. Structures of the alkaloids observed in this study: (1) ($M^+ = m/z$ 291) prunifoleine, (2) 14-oxoprunifoleine, (3) ($[M + H]^+ = m/z$ 313) 10-hydroxyantirrhine; (4) ($[M + H]^+ = m/z$ 327) 10-hydroxyisodeppeaninol and (5) ($[M + H]^+ = m/z$ 347) calycanthine.

Table 1. Detected and imaged alkaloids from *Psychotria prunifolia* and *Palicourea coriacea* leaves by mass spectrometry

Alkaloid	Plant	Molecular formula	Ion mode	m/z (observed)	m/z (calculated)	Error (ppm)	MS/MS (m/z)	Reference
Prunifoleine	<i>Psychotria prunifolia</i>	$C_{19}H_{19}N_2O^+$	$[M]^+$	291.1491	291.1492	-0.3	261.1387, 237.1023, 219.0918, 182.0840	(Faria <i>et al.</i> , 2010) ^a
10-Hydroxyantirrhine	<i>Psychotria prunifolia</i>	$C_{19}H_{24}N_2O_2$	$[M + H]^+$	313.1908	313.1910	-0.6	296.1644, 160.0756, 154.1226	(Kato <i>et al.</i> , 2012) ^a
10-Hydroxyisodeppeaninol	<i>Psychotria prunifolia</i>	$C_{19}H_{22}N_2O_3$	$[M + H]^+$	327.1703	327.1703	< 0.1	309, 295	(Kato <i>et al.</i> , 2012) ^a
calycanthine	<i>Palicourea coriacea</i>	$C_{22}H_{26}N_4$	$[M + H]^+$	347.2230	347.2230	< 0.1	316, 290, 285, 173	(Zhang <i>et al.</i> , 2009)

^aReference for NMR spectra data and accurate mass of these alkaloids.

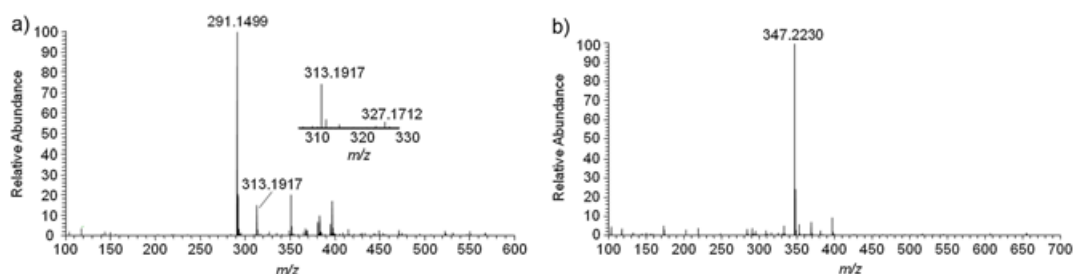


Figure 2. ESI(+)-HRMS spectra of methanolic extract of (a) *Psychotria prunifolia* and (b) *Palicourea coriacea* leaves. [Colour figure can be viewed at wileyonlinelibrary.com]

authorised by the Brazilian government through Conselho de Gestão do Patrimônio Genético (CGEN – process register number 010742/2014–0).

Chemicals

HPLC–MS grade methanol and chloroform were purchased from Th. Geyer (Roskilde, Denmark), and formic acid (ACS reagent, 99%) was purchased from Sigma-Aldrich (Copenhagen, Denmark). Porous Teflon (1.5 mm thick, medium pore size 7 mm, pore volume 36%) was purchased from Berghof (Enning, Germany).

Sample preparation for DESI-MSI experiment

Samples were prepared following the protocol of Janfelt (2015). Briefly, the porous Teflon was washed twice using a few drops of methanol and placed in a desiccator for 5 min. For imprinting, a sandwich was made with the fresh leaf on a clean piece of porous Teflon (abaxial side facing the Teflon) and covered with tissue paper (for absorption of surplus plant juice) and a thin rubber mat (20 mm × 50 mm × 1 mm) (to distribute the pressure over the entire piece of the sample material). It was mounted in a vice and pressed for 10 s. The resultant Teflon imprints were dried in a vacuum desiccator for 1 min and analysed by DESI-MSI immediately afterwards.

DESI-MS imaging analysis

DESI-MS imaging was carried out on a Thermo Scientific LTQ XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a custom-built DESI imaging ion source. The latter was based on a motorised microscope stage from Märzhäuser Wetzlar (Wetzlar, Germany) and controlled by an in-house written software as described in detail elsewhere (Thunig *et al.*, 2011). Mass spectra were collected with Xcalibur 2.0 software (Thermo Fisher Scientific) and converted to imzML files (Schramm *et al.*, 2012). Images were generated with Data Cube Explorer from AMOLF, Amsterdam (Klinkert *et al.*, 2014). The MS instrumental parameters used were as follows: 5 bar nebuliser gas (N_2) pressure, 300°C heated capillary

temperature, 5 kV spray voltage, 30 V capillary voltage, and 70 V tube lens voltage. The ion injection time was 100 ms (AGC off), and five microscans were averaged for each pixel in the image. A mixture of methanol/water (70:30, v/v) was used as the spray solvent and delivered at a flow rate of 5 μ L/min. Mass spectra were acquired in full-scan positive ion mode with a scan range m/z 250–500. The surface was scanned with a speed of 0.429 mm/s, providing a pixel size of 300 μ m. Reproducibility of the presented results was ensured by performing imaging on at least five individual leaves of each type.

Identification of metabolites by high-resolution MS by direct infusion and DESI MS/MS analysis

Half lateral of a leaf of each species was cut in small (c. 2 cm × 2 cm) pieces and extracted in methanol (c. 2 mL) for approximately 5 min at room temperature. An aliquot (100 μ L) of the supernatant was diluted in methanol (900 μ L). The sample was filtered (Millex HV, PVDF, Merck Millipore, Darmstadt, Germany) and infused into the electrospray ionisation (ESI) source of a Thermo Q-Exactive Orbitrap mass spectrometer (Thermo Scientific), using a Hamilton 500 μ L syringe at a flow rate of 0.5 μ L/min. ESI-MS analysis was performed in positive mode with capillary temperature 350°C, ionisation voltage 3.3 kV and resolution 140000. Mass to charge ratios were obtained with a mass accuracy of ≤ 3 ppm. For prunifoleine (**1**) and 10-hydroxyantirrhine (**3**) MS/MS analysis was performed on the Q-Exactive. For 10-hydroxyisodeppeaninol (**4**) the signal intensity in these experiments was insufficient for good MS/MS analysis, and therefore MS/MS for 10-hydroxyisodeppeaninol (**4**) was performed as part of the ion trap DESI-MS experiments described earlier.

Sample preparation for relative quantitation of alkaloids by HPLC-ESI(+)-MS/MS analysis

The central midrib of the leaf was dissected from leaves. The midrib and the rest of the leaves were separated, frozen in liquid nitrogen and ground in a

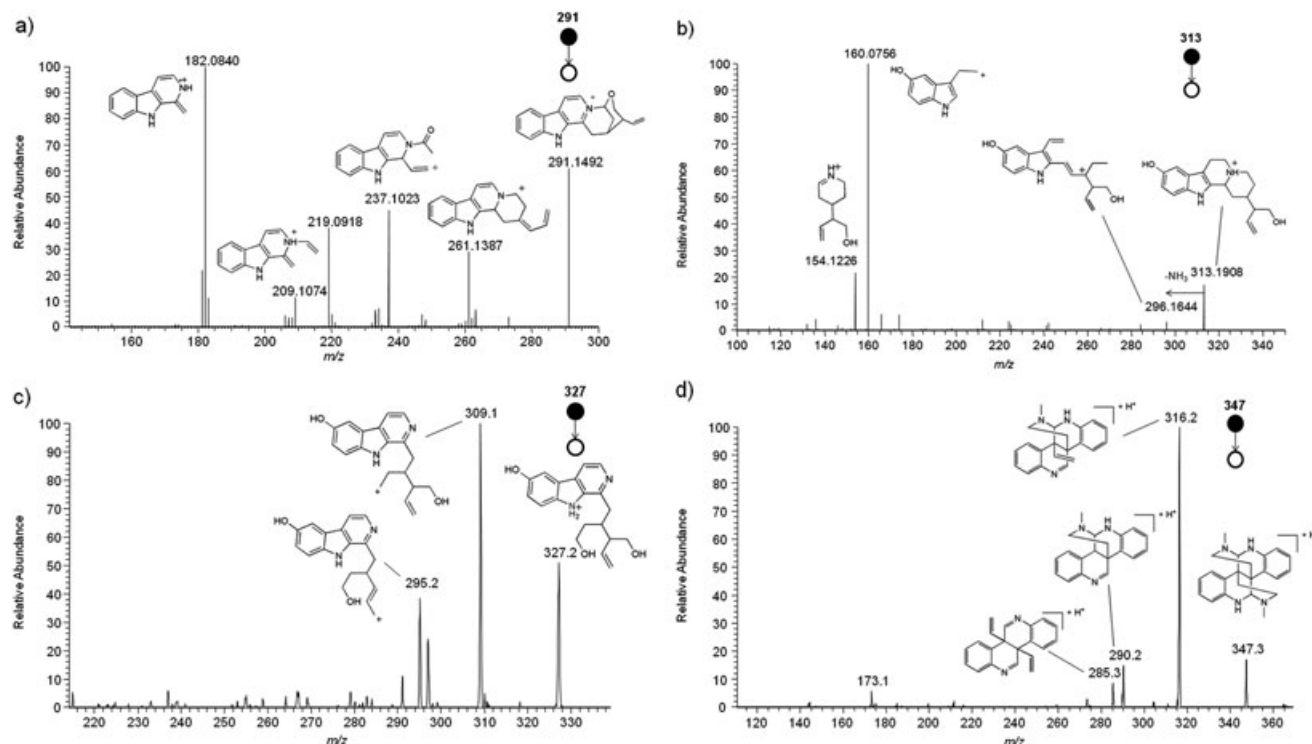


Figure 3. ESI(+)-HRMS/MS spectra of (a) prunifoleine (m/z 291.1942), (b) 10-hydroxyantirrhine (m/z 313.1917); and DESI(+)-MS/MS of (c) 10-hydroxyisodeppeaninol (m/z 327) and (d) calycanthine (m/z 347).

mortar. The resulting powder was freeze-dried (c. 3 mg of each sample) and extracted in a boiling refluxing 85% methanol in water (2.5 mL) for 3 min. Then, an aliquot (500 μ L) of each solution was centrifuged (at 12000 rpm for 5 min) and an aliquot (100 μ L) of the supernatant was diluted in methanol (900 μ L). The solution was filtered through a 0.45 μ m syringe filter (Millex HV, PVDF, Merck Millipore).

Relative quantitation of alkaloids by HPLC-ESI(+)/MS/MS analysis

HPLC-MS/MS was performed in triplicate on a Thermo-Finnigan TSQ Quantum mass spectrometer (Thermo Scientific) equipped with a Thermo Accela HPLC system. The column was a Phenomenex Kinetex 2.6u C18 50 \times 2.10 mm, and separation was performed with gradient elution using 0.1% formic acid in Milli-Q water as mobile phase A and methanol with 0.1% formic acid added as mobile phase B with a flow rate of 0.2 mL/min. The mobile phase B was raised from 30% to 70% B over 4 min, followed by re-equilibration at 30% B for 3 min. The ESI spray voltage was 4 kV in positive ion mode, sheath gas pressure 30 (arbitrary units), auxiliary gas pressure 10 (arbitrary units), ion sweep gas pressure 0, ion transfer capillary temperature 270°C and skimmer offset 0 V.

The compounds present in the plant extracts were monitored by tandem mass spectrometry in the multiple-reaction monitoring (MRM) mode using the following MS/MS transitions during the entire chromatographic run. For *Psychotria prunifolia*: prunifoleine (**1**) (m/z 291 \rightarrow 237); 10-hydroxyantirrhine (**3**) (m/z 313 \rightarrow 160) and 10-hydroxyisodeppeaninol (**4**) (m/z 327 \rightarrow 295) and for *Palicourea coriacea*: calycanthine

(**5**) (m/z 347–316). The alkaloid contents were assessed for statistically significant differences by one-tailed Student's *t* tests using GraphPad InStat 3 (GraphPad Software Inc, San Diego, CA, USA).

Histochemical analyses

The leaves for histochemical analyses were identified and collected in the wild from the same individual *Psychotria prunifolia* as used for MSI analysis. The fresh leaves were stored at +5°C for 4 h. Fully expanded leaves from the second to third node (from apex to base) were used. Leaf fragments (10 mm \times 15 mm) were collected from the apical, middle third, basal, leaf blade, and petiole areas. The samples were free-handed cut transversely using a scalpel and stained using Wagner's reagent (Furr and Mahlberg, 1981), and photomicrographs of the sections were taken using a Leica ICC50 HD microscope camera attached to a Leica DM500 optical microscope. The LAS EZ software, version 1.8.1 was used for image acquisition (Leica Microsystems GmbH, Heerbrugg, Switzerland). These assays were performed at the Biological Sciences Department at UFG (Brazil).

Results and discussion

Identification of alkaloids

To confirm the identity of alkaloids present and previously identified (Faria et al., 2010; do Nascimento et al., 2006) in *Psychotria*

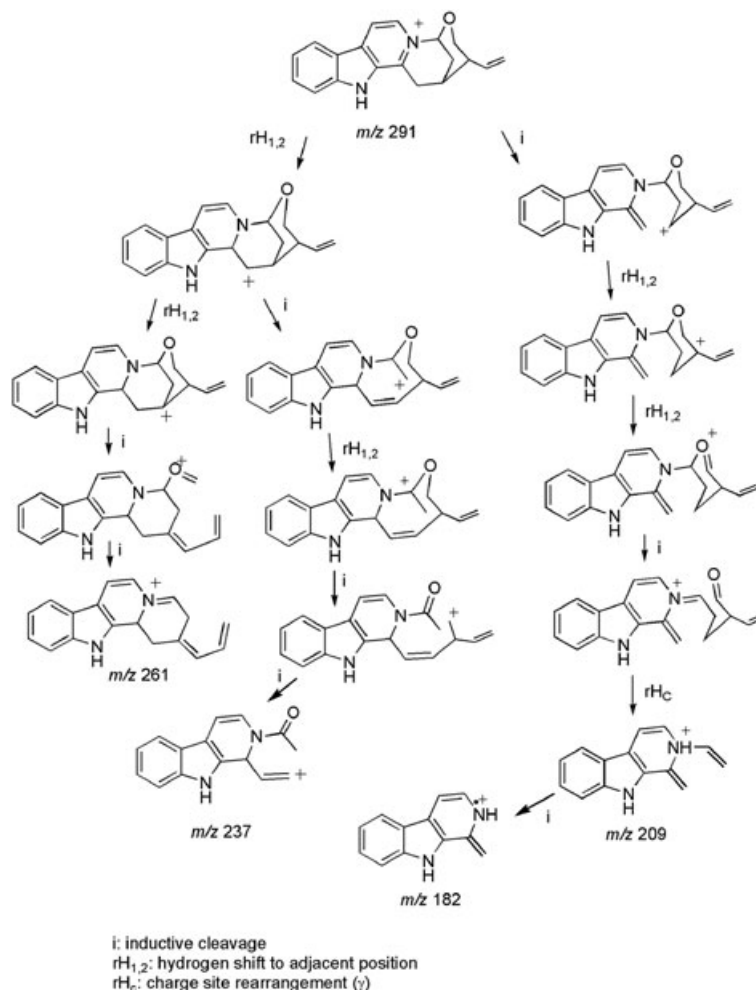


Figure 4. Molecular ion fragmentation pattern proposed for alkaloid **1** by DESI-MS/MS.

prunifolia and *Palicourea coriacea* leaves (Fig. 1; Table 1), the methanol extract from their leaves were analysed by direct infusion into an Orbitrap mass spectrometer (Fig. 2). The HRESI-(+)-MS of *Psychotria prunifolia* (Fig. 2a) is mainly characterised by the ion at m/z 291.1499, corresponding to the molecular ion of prunifoleine (**1**), whereas the HRESI-(+)-MS of *Palicourea coriacea* (Fig. 2b) presents a major peak at m/z 347.2230 $[M + H]^+$, identified as calycanthine (**5**). These compounds correspond to the most abundant alkaloids previously isolated from their respective leaves.

In addition, two less intense signals were observed in the HRESI-(+)-MS of *Psychotria prunifolia* at m/z 313.1917 and 327.1712, attributed to $[M + H]^+$ of 10-hydroxyantirrhine (**3**) and to $[M + H]^+$ of 10-hydroxyisodeppeaninol (**4**), respectively. The NMR spectra details of these alkaloids are described elsewhere (Faria *et al.*, 2010; Kato *et al.*, 2012; do Nascimento *et al.*, 2006), but MS/MS fragmentation data for these alkaloids (Figs 3–6) have not previously been presented.

The ESI-(+)-MS/MS spectrum of molecular ion **1** (M^+ m/z 291.1492, Fig. 3a) contains fragments of m/z 261.1387 of $[C_{18}H_{17}N_2]^+$ from the loss of CH_2O and m/z 237.1023 of $[C_{15}H_{13}N_2O]^+$ from the loss of a butadiene molecule ($-C_4H_6$) (Fig. 4). The ESI-(+)-MS/MS spectrum of protonated molecule **3** ($[M + H]^+$ at m/z 313.1908, Fig. 3b) contains the m/z 296.1644 fragment from the loss of NH_3 . Alternatively, the precursor ion can lose

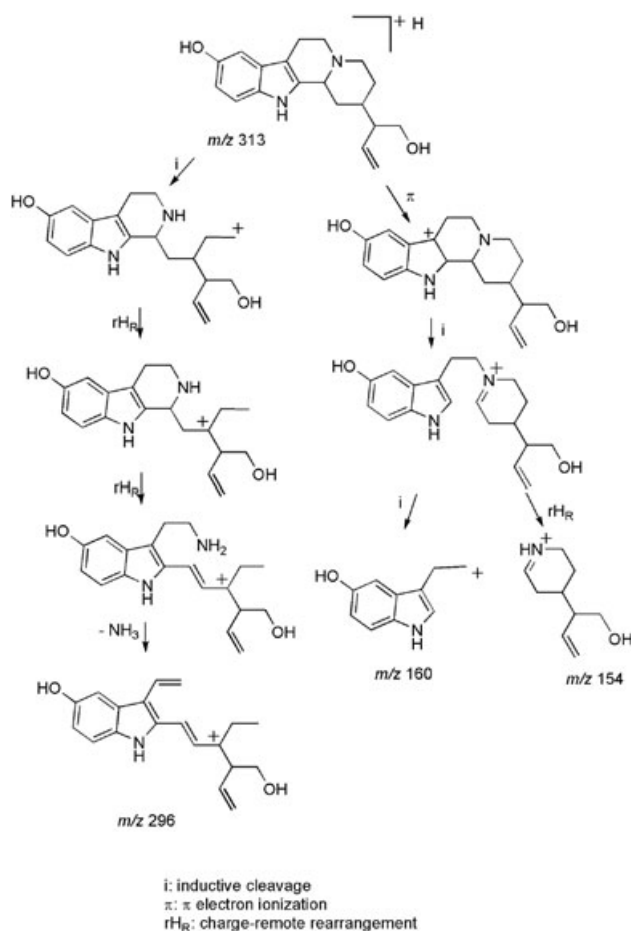


Figure 5. Protonated ion fragmentation pattern proposed for alkaloid **3** by DESI-MS/MS.

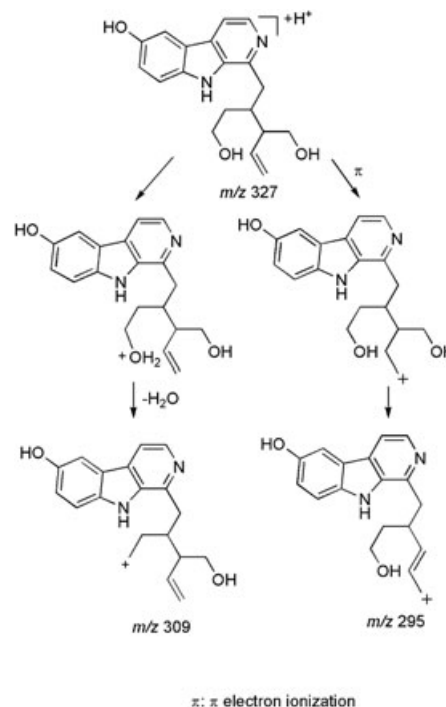


Figure 6. Protonated ion fragmentation pattern proposed for alkaloid **4** by DESI-MS/MS.

$C_9H_{15}NO$ to form the cation $[C_{10}H_{10}NO]^+$ at m/z 160.0756 or lose $C_{10}H_9NO$ to form the cation $[C_9H_{16}NO]^+$ at m/z 154.1226 (Figs 3b and 5).

Due to ion suppression, the less intense signal at m/z 327 was not stable enough for high resolution fragmentation mass spectra to be recorded. Therefore, the DESI-(+)-MS/MS spectrum of protonated molecule **4** ($[M + H]^+$ m/z 327, Fig. 3c), obtained prior to the imaging experiment, was used to identify the alkaloid 10-hydroxyisodeppeaninol, which showed fragments $[C_{19}H_{21}N_2O_2]^+$ at m/z 309 and $[C_{18}H_{19}N_2O_2]^+$ at m/z 295 from the loss of H_2O and CH_3OH , respectively (Fig. 6).

The DESI-(+)-MS/MS spectrum of protonated molecule **5** ($[M + H]^+$ m/z 347, Fig. 3d), also obtained prior to the imaging experiment, contains fragments $[C_{21}H_{22}N_3]^+$ at m/z 316 and $[C_{19}H_{20}N_3]^+$ at m/z 290 from the losses of CH_3NH_2 and $CH_3NH_2 + C_2H_2$, respectively, in accordance with data from the literature (Zhang *et al.*, 2009).

DESI-MS imaging of leaf tissue

Figure 7 shows the optical image and indirect DESI-MS images of an imprint of a *Psychotria prunifolia* leaf, revealing the distributions of the alkaloids prunifoleine (**1**, m/z 291, Fig. 7c), 10-hydroxyantirrhine (**3**, m/z 313, Fig. 7d), and 10-hydroxyisodeppeaninol (**4**, m/z 327, Fig. 7e) as well as of sucrose (m/z 381, Fig. 7f). The alkaloids prunifoleine (**1**) and 10-hydroxyisodeppeaninol (**4**) appear to be more abundant in the midrib, the latter concentrated close to the petiole, and a uniform distribution of 10-hydroxyantirrhine (**3**) can be observed in the entire leaf. The imprinted image of the *Palicourea coriacea* leaf (Fig. 8-c) also shows the homogeneous distribution of calycanthine (**5**) throughout the leaf, with less abundance in the midrib, which

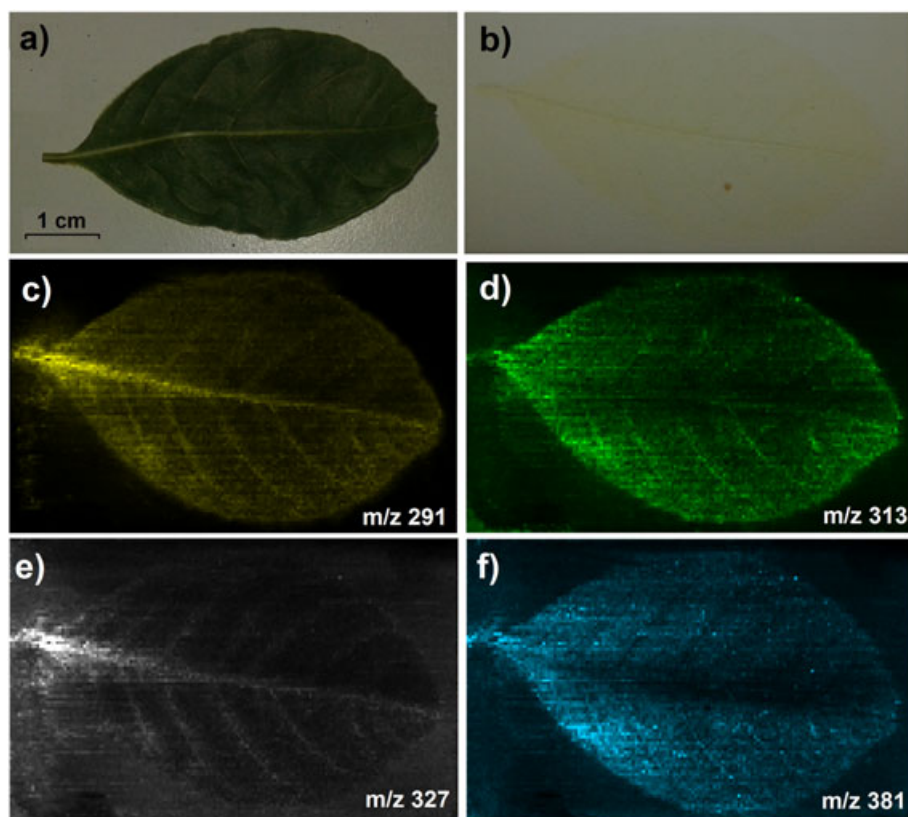


Figure 7. (a) Photograph of *Psychotria prunifolia* leaf after imprint; (b) imprint in Teflon from *Psychotria prunifolia* leaf; (c)–(f) DESI-MS images of the ions of m/z 291 = [prunifoleine] $^+$, m/z 327 = [10-hydroxyisodeppeaninol + H] $^+$, m/z 313 = [10-hydroxyantirrhine + H] $^+$ and m/z 381 = [sucrose + K] $^+$. [Colour figure can be viewed at wileyonlinelibrary.com]



Figure 8. (a) Photograph of *Palicourea coriacea* leaf after imprint; (b) imprint in Teflon from *Palicourea coriacea* leaf; (c) DESI-MS image of the ion m/z 347 = [calycanthine + H] $^+$ of *Palicourea coriacea*. [Colour figure can be viewed at wileyonlinelibrary.com]

could suggest that this alkaloid is produced in the leaf and is not transported within the plant.

Histochemical analyses

Histochemical analyses of the *Psychotria prunifolia* leaf revealed the presence of alkaloids in a brown region dyed by Wagner's reagent, mainly in the epidermis, collenchyma, main vascular bundle, and parenchyma cells from the central midrib of the leaf blade (Fig. 9A–F) and the petiole (Fig. 9G–H), confirming the localisation of alkaloids imaged by DESI-MSI. Such comparison of MSI analysis and classical histochemical staining has not previously been presented for plant tissue.

Relative quantitation of alkaloids by HPLC-ESI(+)/MS/MS analysis

DESI-MSI shows the differential localisation of alkaloids on the leaf surface, where the petiole and the midrib revealed a more abundant content of prunifoleine (**1**, m/z 291) and 10-hydroxyisodeppeaninol (**4**, m/z 327) compared to the whole leaf. Since MS imaging is normally only a semi-quantitative technique (Lee *et al.*, 2012), the alkaloid contents of *Psychotria prunifolia* were analysed by HPLC-MS for determination of relative alkaloid contents in methanol extracts from the midrib and the remaining leaf tissue.

Since no pure standards were available for absolute quantitation, the LC-MS/MS results are expressed as relative peak areas

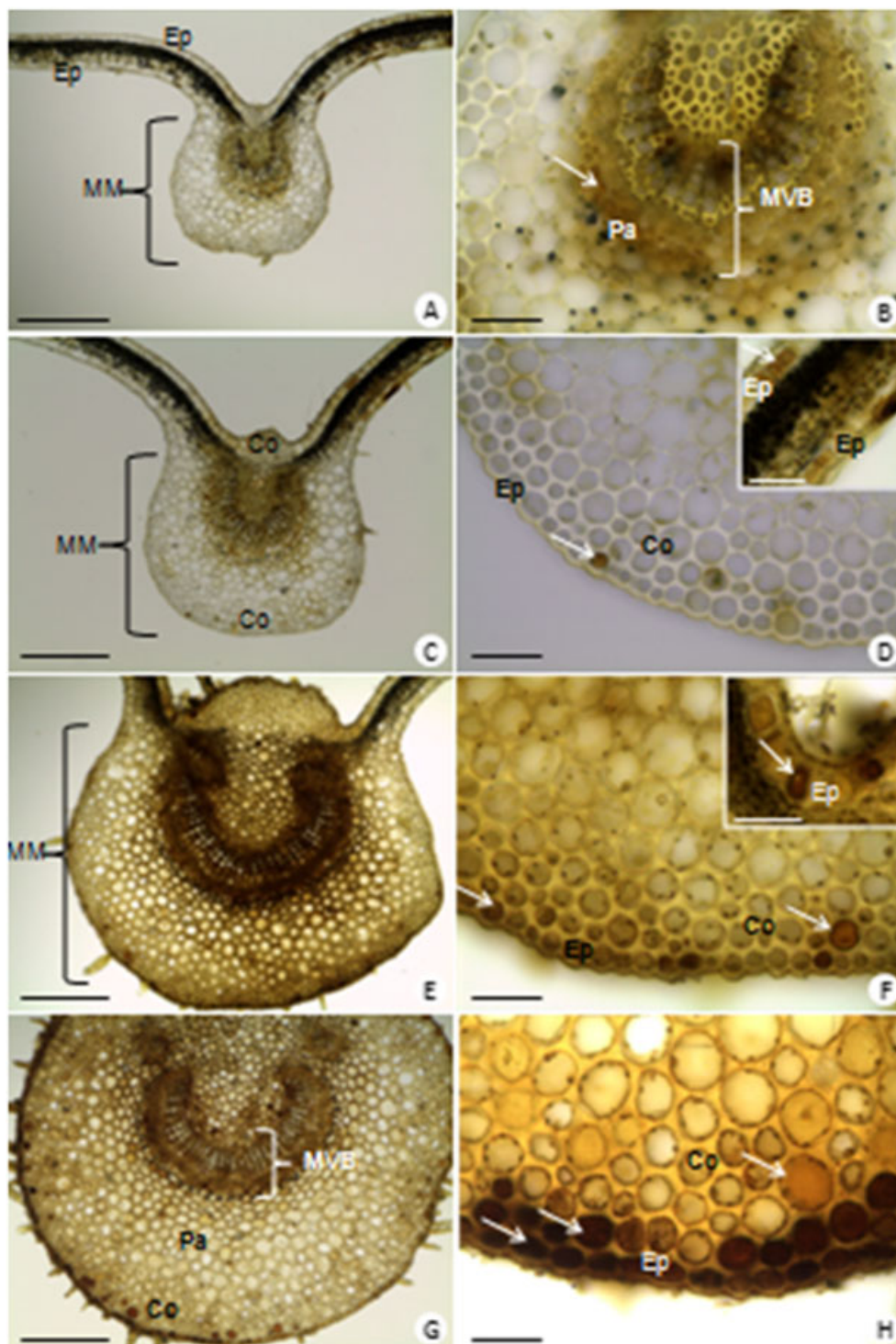


Figure 9. The transversal section of the leaf (A)–(F) and petiole (G)–(H) of *Psychotria prunifolia* under Wagner's assay. (A)–(B), (C)–(D), and (E)–(F) show apical, medial and basal regions, respectively. Inserts in (D) and (F): show details of epidermal cells (Ep). MM = main midrib; Ep = epiderm; Co = collenchyma; VT = vascular tissue. MVB = main vascular bundle. Arrow = positive result for alkaloid. Scale bar: A, C, E and G = 500 μm . B, D, F and H = 100 μm . The brown region dyed by Wagner's reagent indicates the presence of the alkaloids in these cells. [Colour figure can be viewed at wileyonlinelibrary.com]

(adjusted for the amount of leaf tissue used for extraction) of the different alkaloids found in the extracts of the two types of leaf tissue. Statistical analyses of the mean differences in alkaloid content between midrib and leaves analysed by LC-MS (Fig. 10a) showed a higher abundance of prunifoleine (**1**, m/z 291) and 10-hydroxyisodeppeaninol (**4**, m/z 327) (per gram of dried vegetal

material) in the midrib than in the remaining leaf, as well as no significant difference for 10-hydroxyantirrhine (**3**, m/z 313). For the alkaloid calycanthine from *Palicourea coriacea*, the content in the midrib was significantly lower than in the lamina leaf. The relative peak areas were converted to a percentage (Fig. 10b). These results thus confirm the findings from the DESI images.

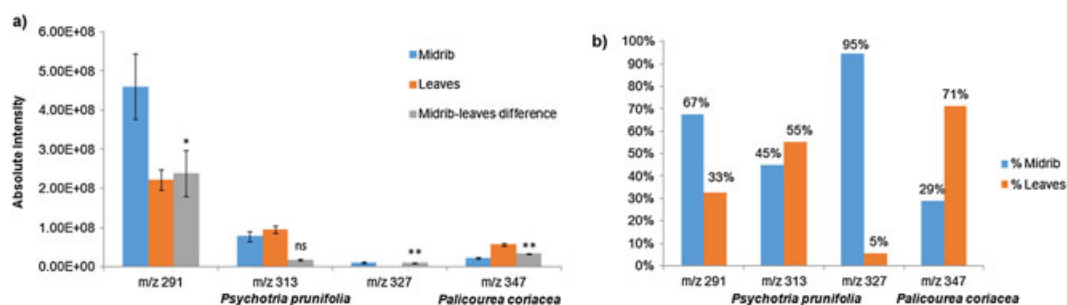


Figure 10. (a) Results of HPLC-MS analysis for the experiment separating the midrib from the leaves. Errors bars are standard deviations ($n = 3$). Asterisks indicate midrib–leaves differences that are significantly greater than zero at *95% or **99% confidence level, as calculated using a one-tailed Student's test. ns = no significant treatment effect. (b) Results of LC-MS analysis for the experiment as a percentage. [Colour figure can be viewed at wileyonlinelibrary.com]

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