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Direct analysis of *Salvia divinorum* leaves for salvinorin A by thin layer chromatography and desorption electrospray ionization multi-stage tandem mass spectrometry

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***Salvia divinorum* is widely cultivated in the US, Mexico, Central and South America and Europe and is consumed for its ability to produce hallucinogenic effects similar to those of other scheduled hallucinogenic drugs, such as LSD. Salvinorin A (SA), a kappa opiod receptor agonist and psychoactive constituent, is found primarily in the leaves and to a lesser extent in the stems of the plant. Herein, the analysis of intact *S. divinorum* leaves for SA and of acetone extracts separated using thin layer chromatography (TLC) is demonstrated using desorption electrospray ionization (DESI) mass spectrometry. The detection of SA using DESI in the positive ion mode is characterized by several ions associated with the compound – $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[2M+NH_4]^+$, and $[2M+Na]^+$. Confirmation of the identity of these ions is provided through exact mass measurements using a time-of-flight (ToF) mass spectrometer. The presence of SA in the leaves was confirmed by multi-stage tandem mass spectrometry (MS^n) of the $[M+H]^+$ ion using a linear ion trap mass spectrometer. Direct analysis of the leaves revealed several species of salvinorin in addition to SA as confirmed by MS^n, including salvinorin B, C, D/E, and divinatorin B. Further, the results from DESI imaging of a TLC separation of a commercial leaf extract and an acetone extract of *S. divinorum* leaves were in concordance with the TLC/DESI-MS results of an authentic salvinorin A standard. The present study provides an example of both the direct analysis of intact plant materials for screening illicit substances and the coupling of TLC and DESI-MS as a simple method for the examination of natural products.**
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Salvia divinorum or 'magic mint' is widely cultivated in the US, Mexico, Central and South America, and Europe and is consumed for its ability to produce hallucinogenic effects similar to those of other scheduled hallucinogenic drugs, such as LSD.[1,2] Salvinorin A (SA), a kappa opiod receptor agonist and psychoactive constituent,[3,4] is found primarily in the leaves and to a lesser extent in the stems of the plant. Chemically, it is a non-nitrogenous neoclerodane diterpenoid.[4,5] SA is one of the most potent naturally occurring psychoactive drugs known to date with an effective human dose in 200–1000 μg range when smoked.[4] *S. divinorum* is not regulated by the US Controlled Substance Act, although it is noted to be a 'drug or chemical of concern'. More recently, however, fourteen states in the US and nine other countries have enacted legislation to place *S. divinorum* under regulation.

Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/ultraviolet (LC/UV) or mass spectrometry (LC/MS) have been utilized for the identification and quantitation of SA from plant extracts and biological fluids.[6–8] The inherent limitations of these approaches are low sensitivity (LC/UV), the requirement for extensive sample preparation and relatively long analysis times. The extraction procedures commonly utilized for alkaloids involve the use of high-pH buffers which can hydrolyze ester groups common in the terpenoid-type salvinorin molecules. Solid-phase extraction has been utilized with some success; however, sample preparation times are long.[9] LC/MS techniques are generally more sensitive than LC/UV or GC/MS and good results have been obtained after the extraction of plants with organic solvents such as chloroform or acetone. These procedures are suitable for terpenoids, eliminating the possibility of sample degradation during a high-pH extraction procedure. Thin layer chromatography (TLC) is a simple and inexpensive technique which has also been used for the qualitative analysis of SA from plant extracts.[10,11] Using TLC, SA and other salvinorin analogs were extracted from plant material, separated, and visualized after reaction with vanillin – providing sensitivity down to 1 part in 50 000.[11]

Desorption electrospray ionization (DESI)-MS is an atmospheric pressure desorption/ionization (DI) mass spectrometric technique used for the analysis of samples under ambient conditions.[12] The versatility of the technique has been demonstrated in a variety of examples to date, including the analysis of intact tissues for targeted analytes such as drugs,[13] alkaloids,[14] lipids,[15–18] and glycosides.[19] The quantitative capacity of DESI when coupled to ion trap[20] and triple

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quadrupole[21] instruments has been demonstrated and lower limits of quantitation for select drug compounds in plasma were reported in the range of 10–30 ng/mL with good precision and accuracy.[21] The combination of TLC and DESI has also been demonstrated in several examples, including for the analysis of steroids,[22] dyestuffs,[23] herbal supplements,[24] and tryptic digests.[25,26] The coupling of TLC with DESI and other atmospheric DI techniques combines the simplicity and robustness of TLC with the sensitivity and specificity of mass spectrometric detection.

In this report, the feasibility of the direct examination of untreated, dry *S. divinorum* leaves for the presence of SA using DESI-MS^n is demonstrated. The coupling of TLC and DESI-MS is shown to provide additional evidence for the presence of SA in the plant leaves, enabling a simple and rapid method for screening plant material for illicit substances.

EXPERIMENTAL

Chemicals

Salvinorin A chemical standard (≥98% by HPLC) was obtained from Sigma Aldrich (St. Louis, MO, USA). Methyl *tert*-butyl ether (MTBE), hexane, acetone, acetonitrile (ACN) and HPLC grade water were obtained from Burdick & Jackson (Muskegon, MI, USA) and concentrated formic acid was obtained from J.T. Baker-Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). *Salvia divinorum* commercial leaf extracts containing 1 g each of '5x', '10x', '20x' and 14 g of dry leaves were purchased from Herbal Fire Botanicals[27] (Oskaloosa, KS, USA). Herbal Fire guarantees 12.5 mg/g SA in 5x, 25 mg/g SA in 10x, and 72 mg/g SA in 20x extracts.

Thin-layer chromatography (TLC)

Salvia divinorum samples were prepared for TLC by extracting SA from the dry leaves in acetone (1:5, w/v) for 1 min while vortex mixing, except where noted. Extraction with acetone has been reported to be more selective for SA than with other solvents such as chloroform or chloroform/methanol mixtures.[10] A volume of 5 μL of the acetone extract was pipetted onto 2.5 × 7.5 cm silica gel 60 F_{254} pre-coated TLC plates (250 μm; EMD Chemicals Inc., Gibbstown, NJ, USA) approximately 1 cm from the edge of the plate. The optimal separation of SA from endogenous plant material as determined by the highest signal-to-noise (S/N) ratio in the DESI mass spectrum was obtained with 3:1 MTBE/hexane.

DESI-MS and MS^n

All experiments were performed using an Omni Spray® DESI ion source (Prosolia, Indianapolis, IN, USA) coupled to either a Thermo Scientific LTQ™ linear ion trap mass spectrometer (San Jose, CA, USA) or a Waters LCT Premier ToF mass spectrometer (Milford, MA, USA) operated in the positive ion mode. The DESI source operating parameters were as follows: spray voltage, 3.5 kV; solvent flow rate, 5 μL/min; nebulizing gas (nitrogen, 99.9995%) pressure, 100 psi; tip-to-surface distance, 2 mm; tip-to-inlet distance, 3 mm; incident angle (relative to the surface plane), 55°. The DESI spray solvent was 80:20 ACN/water with 0.1% formic acid. Collision-induced dissociation (CID) was performed on the Thermo Scientific LTQ mass spectrometer using a normalized collision energy (CE) of 20 (arbitrary units) and an isolation width of ±1.5 Th.[28] Exact mass measurements were performed on the Waters LCT Premier in W-mode. Direct analysis of the leaf fragments was performed in the positive ion mode by pressing the dried leaves onto double-sided tape backed by a standard microscope glass slide. Due to the possibility and concern of smaller leaf fragments desorbing from the surface and entering the inlet of the mass spectrometer, only larger fragments (>10 × 10 mm) or entire leaves were analyzed.

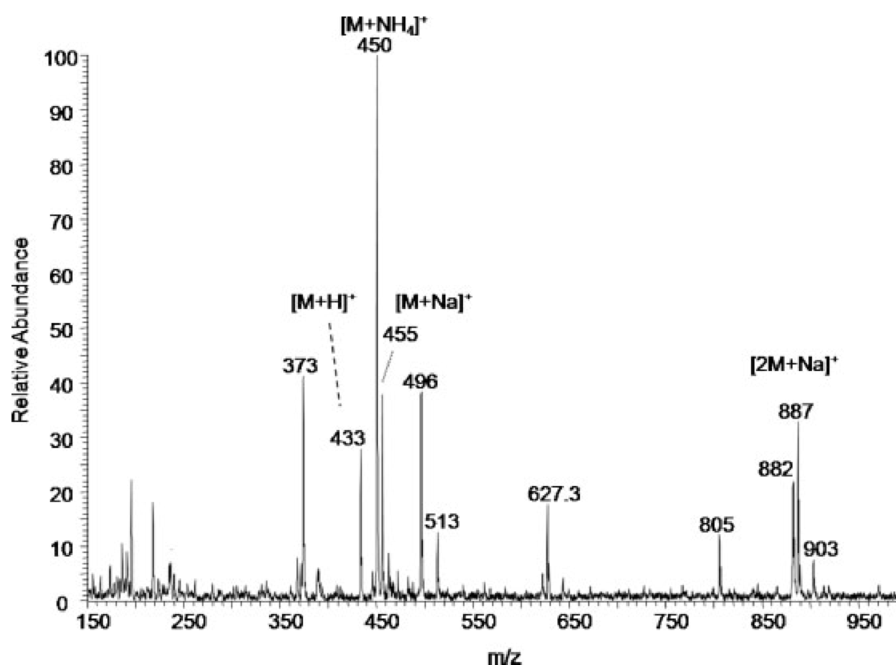

Figure 1. Positive ion DESI-MS full scan mass spectrum of salvinorin A standard prepared at 10 μg/mL (20 ng deposited onto the target) and using 80:20 ACN/water + 0.1% formic acid as the DESI spray solvent.

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Table 1. Exact mass measurements of various ions of salvinorin A

Experimental (*m/z*)	Theoretical (*m/z*)	Error (Δppm)	Ion formula
373.1660	373.1651	2.4	$C_{21}H_{25}O_6$
433.1862	433.1862	0.0	$C_{23}H_{29}O_8$
450.2138	450.2128	2.2	$C_{23}H_{32}NO_8$
882.3926	882.3912	1.6	$C_{46}H_{60}NO_{16}$
887.3440	887.3466	−2.9	$C_{46}H_{56}O_{16}Na$

DESI imaging

Imaging of a 70 mm × 23 mm area on the TLC plate was performed using a two-dimensionally automated DESI source (Omni Spray 2D, Prosolia Inc.) in the positive ion mode. The surface velocity was set to 0.5 mm/s and the y-step size to 0.5 mm, resulting in a pixel size of 0.5 mm × 0.5 mm. The Xcalibur™ 2.0.7 raw files were converted on a row-by-row basis into Analyze 7.5 format using FireFly™ data conversion software (Prosolia Inc.) for visualization in BioMAP 3.7.5 (Novartis, Basel, Switzerland).

RESULTS AND DISCUSSION

Direct analysis of *S. divinorum* leaves

The positive ion DESI mass spectrum of the authentic SA chemical standard prepared in 80:20 ACN/water + 0.1% formic acid to 10 µg/mL and deposited onto a Teflon-coated glass slide is presented in Fig. 1. The peak at *m/z* 433 is assigned to the protonated molecule of SA, $[M+H]^+$, while the peaks at *m/z* 373, 450 and 455 are assigned to the $[M+H-CH_3COOH]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions, respectively. Also present in the chemical standard are ions at *m/z* 882 and 887, which are assigned to the $[2M+NH_4]^+$ and $[2M+Na]^+$ ions, respectively. Exact mass data recorded using the Waters LCT Premier ToF mass spectrometer in W-mode provided further evidence for these assignments. All five assignments were within 3 ppm of the theoretical mass. Table 1 lists the detected ions in the SA chemical standard, along with the measurement error (Δppm). It is notable that previous examinations of terpenoid compounds by electrospray ionization (ESI) and DESI have exhibited similar prevalence for the formation of alkali (in the positive ion mode) and halide (in the negative ion mode) adducts.[19,22] The origin of the ammonium adduct is not clear but we attribute it to the presence of common ammonium-based solvents in the open air in our laboratory. Figures 2(a) and 2(b) show the MS^2 and

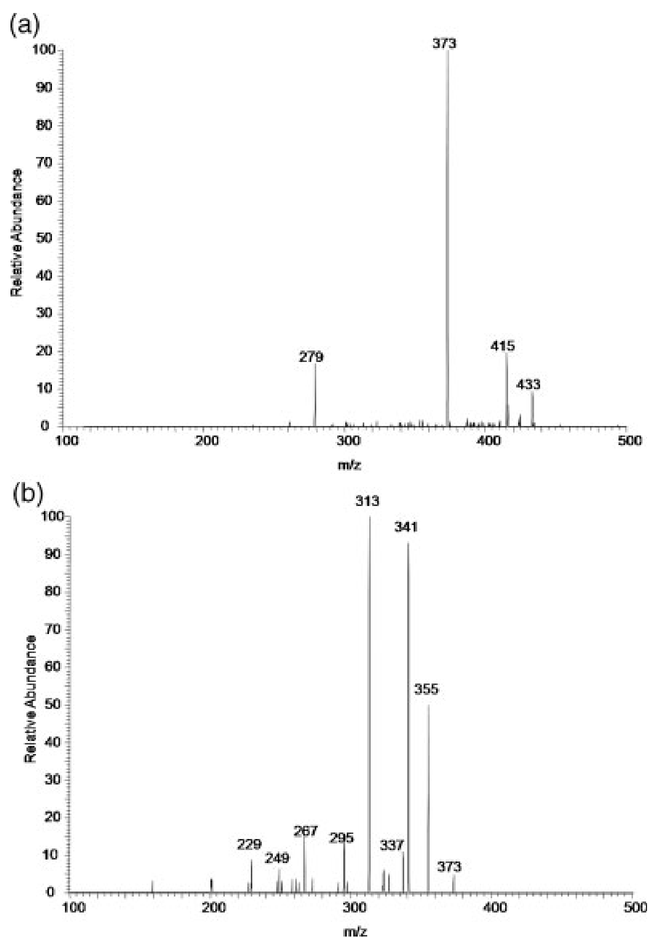

Figure 2. DESI-MS^n spectra of the protonated salvinorin A molecule at *m/z* 433 using a linear ion trap mass spectrometer: (a) MS^2 spectrum of *m/z* 433 using 20% collision energy (CE); (b) MS^3 spectrum of *m/z* 373 using 20% CE resulting from *m/z* 433 using 20% CE.

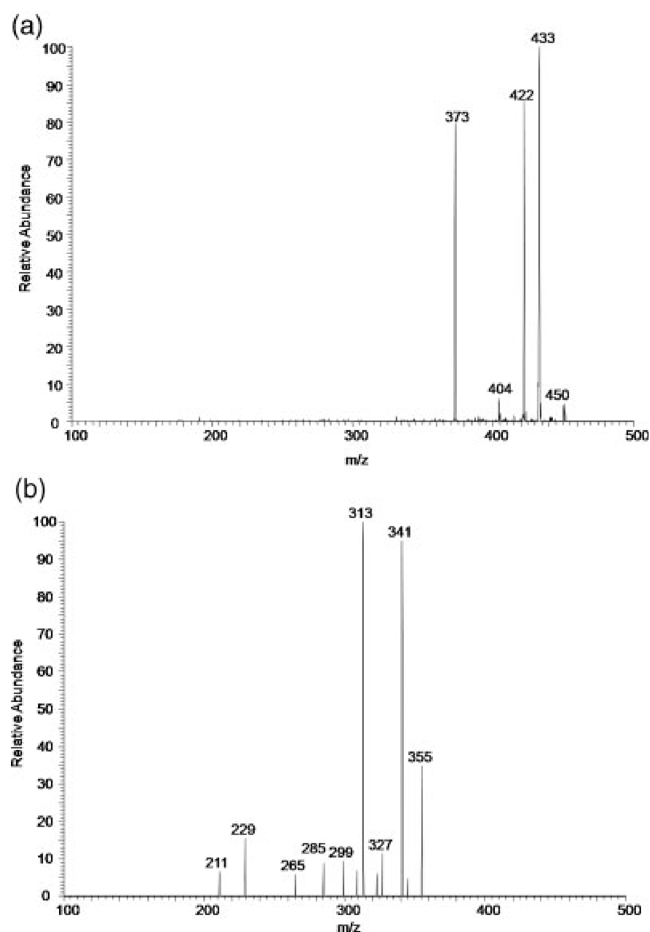

Figure 3. DESI-MS^n spectra of *m/z* 450 using a linear ion trap mass spectrometer: (a) MS^2 spectrum of *m/z* 450 using 20%CE; (b) MS^3 spectrum of *m/z* 373 using 20% CE resulting from *m/z* 450 using 20% CE.

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MS^3 spectra of the protonated SA molecule recorded using the ion trap, respectively. In concordance with previous studies by Medana *et al.*[29] using an ion trap with ESI, the MS^2 spectrum shows a loss of 60 Da (CH_3COOH) from the $[M+H]^+$ ion at *m/z* 433 to give an intense peak at *m/z* 373, in addition to a loss of 18 Da (H_2O) to give *m/z* 415. The MS^3 mass spectrum resulting from CID of *m/z* 373 shows losses of 60 Da (CH_3COOH), 32 Da (CH_3OH) and 18 Da (H_2O) resulting in the ions at *m/z* 313, 341 and 355, respectively (Fig. 2(b)). Figure 3 shows the MS^2 and MS^3 spectra for the peak at *m/z* 450, which was assigned to the $[M+NH_4]^+$ ion by exact mass measurement. MS^2 of *m/z* 450 produces product ions at *m/z* 433, 422, 404 and 373, resulting, respectively, from losses of 17 Da (NH_3), 28 Da (CO), 46 Da (HCOOH) and 77 Da, which is probably a result of consecutive losses of CH_3COOH and NH_3 (Fig. 3(a)). The complete elucidation of the fragmentation pathways of SA is not the subject of this paper; however, a more thorough examination of the fragmentation pathways of various salvinorins is provided by Medana *et al.*[29]

DESI-MS of the intact *S. divinorum* leaves was performed in the positive ion mode by pressing the dried leaves onto double-sided tape backed by a standard microscope glass slide. Analysis of the intact leaf fragments in full scan mode did not readily show ions related to SA (i.e. *m/z* 433, 450, 455, 882 and 887) or other salvinorins with sufficient signal-to-noise. Therefore, it was necessary to employ MS^n using the ion trap in order to confirm the presence of SA and other salvinorins in the leaves. The DESI-MS^2 and -MS^3 mass spectra of *m/z* 433 in the dry leaf are presented in Fig. 4. The DESI-MS^2 spectrum shows ions at *m/z* 415, 397, 373, and an intense product ion at 345 (Fig. 4(a)). Based on the results presented in Fig. 1 and those of Medana *et al.*,[29] these data suggest that other isobaric species, such as the salvinorin congeners, are present. The product ion at *m/z* 345 is not associated with the fragmentation pattern of the authentic

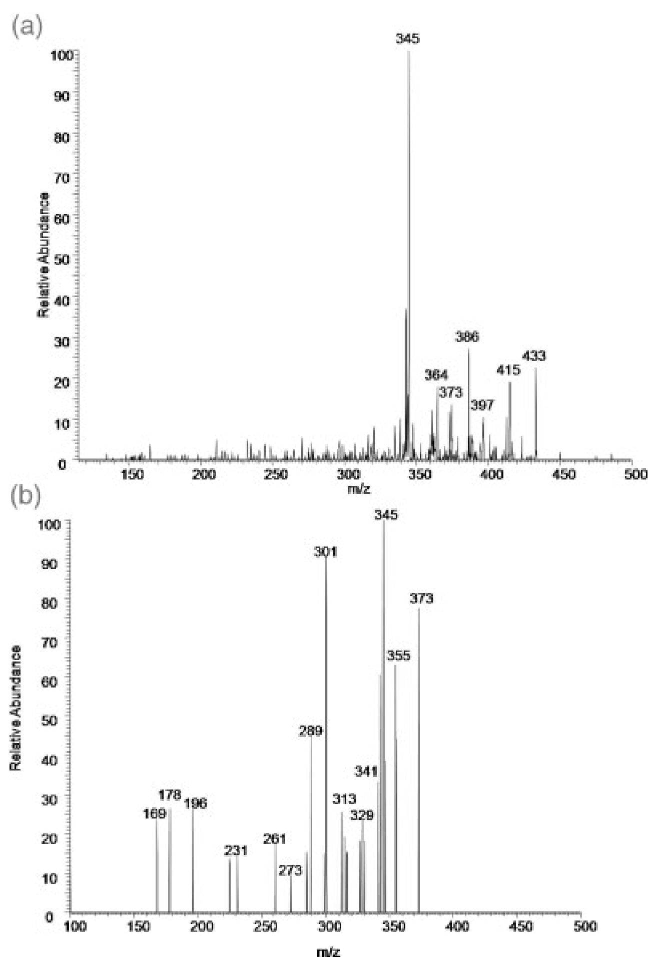

Figure 4. DESI-MS^n spectra of *m/z* 433 in intact, untreated *S. divinorum* leaf using a linear ion trap mass spectrometer: (a) MS^2 spectrum of *m/z* 433 using 20% CE; (b) MS^3 spectrum of *m/z* 373 using 20% CE resulting from *m/z* 450 using 20% CE.

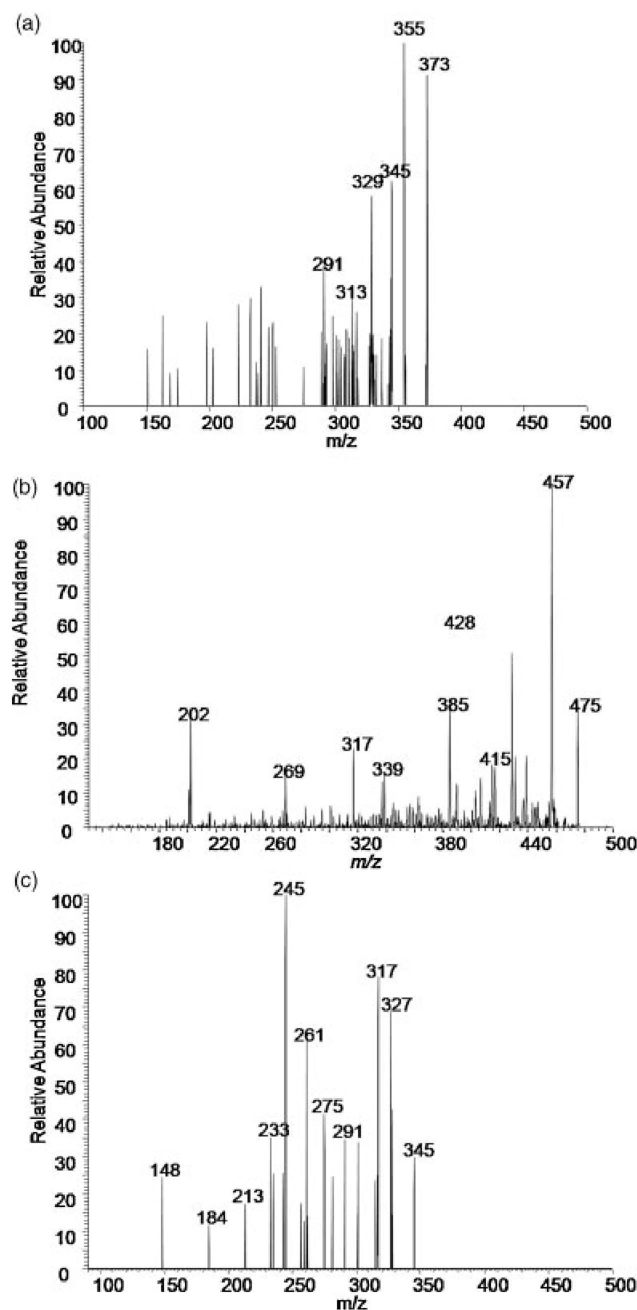

Figure 5. DESI-MS^n spectra of salvinorin and divinatorin species detected in *S. divinorum* leaves using a linear ion trap mass spectrometer: (a) MS^3 spectrum of *m/z* 373 using 20% CE resulting from CID of *m/z* 391 using 20% CE and assigned to salvinorin B; (b) MS^2 spectrum of *m/z* 475 assigned to salvinorin C; (c) MS^3 spectrum of *m/z* 345 using 20% CE resulting from CID of *m/z* 363 using 20% CE and assigned to divinatorin B.

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Table 2. Common product ions observed during DESI-MS^n of salvinorin species in *S. divinorum* leaves

Compound	Precursor ion (*m/z*) $[M+H]^+$	MS^2 (*m/z*)	MS^3 (*m/z*)	MS^4 (*m/z*)
Salvinorin A	433	373	355	337,323,309,295
Salvinorin B	391	373	355	
Salvinorin C	475	457	415	
Divinatorin B	363	345	327	

Table 3. TLC Rf values for Salvinorin analogs using 3:1 MTBE/hexane

Compound	Rf
Salvinorin A	0.49
Salvinorin C	0.64
Divinatorin B	0.85
Salvinorin B	0.95

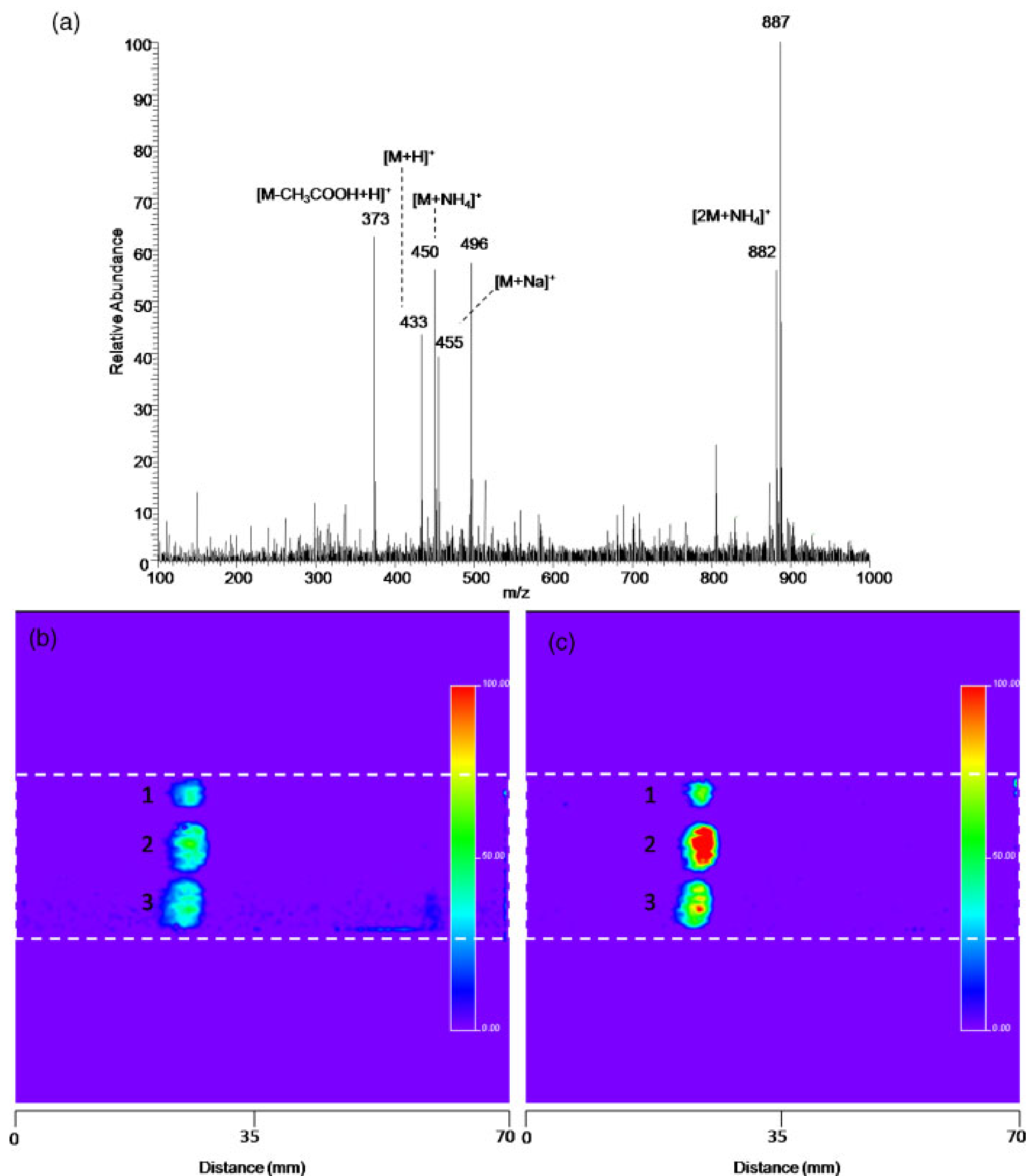

Figure 6. (a) Positive ion mass spectrum recorded during DESI imaging of the TLC plate and corresponding to the zone assigned to salvinorin A from the separation of the *S. divinorum* extract (3). (b,c) DESI-MS selected ion images (70 × 23 mm) of (b) *m/z* 455 and (c) *m/z* 887. Lane 1 corresponds to the salvinorin A chemical standard (5 mg loaded), lane 2 to the '5x' commercial leaf extract and lane 3 to the acetone extract of *S. divinorum* leaves.

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standard nor does it match previous results.[29] It is therefore attributed to endogenous molecules having ions with the same nominal mass as the precursor ion at *m/z* 433. Further, MS^3 of *m/z* 433 produced the mass spectrum in Fig. 4(b) showing product ions characteristic of the presence of SA in the leaves. Figure 5 shows positive ion MS^n spectra from precursor ions of *m/z* 391, 475 and 363 attributed to the protonated molecules of salvinorin D, salvinorin C and divinorin B, respectively, and these are consistent with previous results.[29] Table 2 shows the common precursor and product ions observed during direct examination of the leaves using DESI-MS^n. Overall, these results show through the use of tandem MS, that the detection of SA directly from raw, dry *S. divinourm* leaves is feasible. Given the biological significance of the SA molecule and the necessity for the development of rapid screening methods for its presence in the intact leaves, a complete examination of all of the salvinorin or divinatorin species was not performed and is not within the scope of the research.

TLC/DESI-MS^n

S. divinorum leaves (1 g) were ground with a mortar and pestle and extracted with 5 mL of acetone for approximately 1 min at room temperature while 0.2 g of the 5 × sage variety was extracted in 1 mL acetone over 1 min at room temperature. On the same TLC plate was deposited 5 μL of each extract and a 1 mg/mL solution of SA in acetone. The retention factors (Rfs) for the different salvinorin components separated with 3:1 MTBE/hexane on TLC are summarized in Table 3. The TLC plate was developed with 3:1 MTBE/hexane and its chemical image was recorded by rastering the surface in two dimensions with an automated DESI source in the positive ion mode coupled to the Thermo Scientific LTQ™ mass spectrometer. The selected ion images of *m/z* 455 $[M+Na]^+$ and *m/z* 877 $[2M+Na]^+$ for the chemical standard, 5x commercial leaf extract and the *S. divinorum* leaf extract are shown in Figs. 6(b) and 6(c), respectively. The positive ion DESI mass spectrum from the region on the TLC plate corresponding to SA in *S. divinorum* is presented in Fig. 6(a) and characteristically shows peaks related to SA, located at *m/z* 433, 450, 455, 882 and 887. The abundance of SA in the *S. divinorum* after extraction and separation using TLC was sufficient to permit

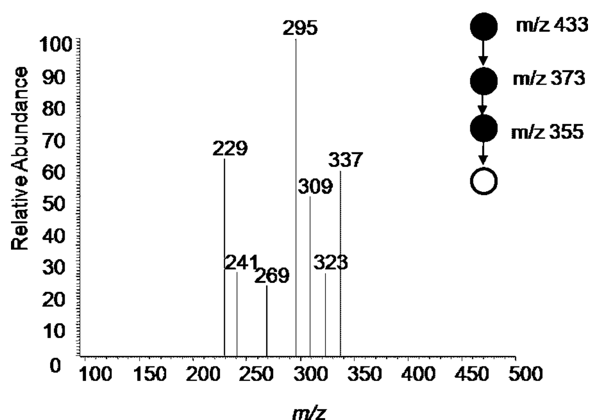

Figure 7. MS^4 spectrum using a linear ion trap mass spectrometer of *m/z* 355 using 20% CE from CID of *m/z* 373 using 20% CE resulting from CID of *m/z* 433 using 20% CE.

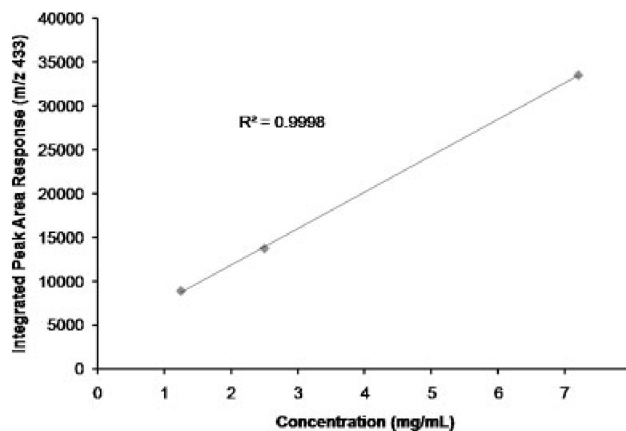

Figure 8. Calibration plot of the integrated peak area for *m/z* 433 in the commercial extracts versus concentration (grams of leaves per mL extraction solvent).

MS^4 of *m/z* 433 (Fig. 7). CID of *m/z* 355 produced ions at *m/z* 309, 337, 323 and 295, which are consistent with previously reported data.[29] It is notable that *m/z* 345 is not present in the leaf samples after TLC giving further proof that this ion is due to endogenous material in the leaf. The summation of these two approaches, direct analysis of the raw, dry *S. divinorum* leaves using DESI-MS^n and TLC/DESI-MS, provides a simple method for the qualitative analysis of suspicious plant material for the presence of SA.[29,30]

Semi-quantitative analysis of the SA in the *S. divinorum* extracts was also performed using TLC/DESI-MS The semi-quantitative analysis of *S. divinorum* leaves was performed by extracting 200 mg of each '5x', '10x', and '20x' extract standards with 1 mL of acetone, and 1 g of dry leaf with 5 mL of acetone. A volume of 5 μL of each solution was spotted onto a TLC plate and developed using 3:1 MTBE/hexane. Using the integrated peak areas associated with *m/z* 433 (Rf 0.49) recorded for each sample extract ('5x', '10x', and '20x' standards) a calibration curve was constructed. Figure 8 shows the calibration curve exhibiting an R^2 of 0.9998. Using this simple methodology and with the lack of suitable standards, the concentration of SA in the *S. divinorum* sample was estimated to be 1.6 mg/mL (0.8% w/w). This result is similar to those previously reported for the analysis of SA in *S. divinorum* samples from different geographical regions.[29]

CONCLUSIONS

The direct examination of intact, untreated leaves of the *S. divinorum* species is demonstrated in this study. The use of tandem mass spectrometry (i.e. MS^n) was essential for the detection of salvinorin A and its isomers. The use of a simple TLC protocol in combination with DESI-MS allowed for improved detection of SA in the leaf extracts. The possibility of performing semi-quantitative analysis was demonstrated using an external calibration curve and TLC/DESI-MS, although more data is needed to develop this aspect of the method further. These data illustrate the capacity of DESI-MS to provide rapid screening of plant materials for the detection of illicit substances. The method, with or without separation by TLC, should be easily extended to other plant materials or food-stuffs.

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