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Analysis of Salvinorin A in plants, water, and urine using solid-phase microextraction-comprehensive two-dimensional gas chromatography–time of flight mass spectrometry

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ABSTRACT

Salvinorin A, a psychoactive hallucinogen, and related compounds, were analyzed in plants, water, and urine using liquid–liquid extraction (LLE), solid-phase microextraction (SPME) and comprehensive two-dimensional gas chromatography–time of flight mass spectrometry (GC × GC–ToFMS). A semi-qualitative study of the extraction of Salvinorin A and analogs from *Salvia divinorum* plants by LLE showed ppb levels of Salvinorin A and several analogs in the leaves and stems of *S. divinorum* plants, much lower than expected. Quantitative analysis of Salvinorin A spiked into water and urine showed much better figures of merit for SPME than LLE, with limit of detection of about 5 ng/mL, linear range from 8 to 500 ng/mL and precision about ±10% for the SPME-based analyses using external standard quantitation. GC × GC–ToFMS was especially effective in separating the peaks of interest from matrix and chromatographic interferences.

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1. Introduction

Salvia divinorum is a member of the *lamiaceae* (mint) family of plants. It is a type of sage plant that is known to contain a psychoactive hallucinogenic drug known as Salvinorin A. The plant is used by the Mazatec in Mexico for curing and divination rituals. Recently, the plant has become popular in the United States as an alternative to marijuana and lysergic acid diethylamide (LSD). Prizinzano studied the pharmacology of Salvinorin A, a neoclerodane diterpene, and discovered three main features about Salvinorin A that distinguish it from typical hallucinogens: it is entirely natural whereas LSD is purely synthetic; it contains no nitrogen in its structure; it targets the κ opioid receptors whereas LSD targets serotonin receptors [1,2]. Fig. 1 shows the structure of Salvinorin A and its common analogs.

In the United States, Salvinorin A is not listed as a controlled substance by the Drug Enforcement Administration, nor is there any federal regulation prohibiting use of the drug [3]. Some states have classified this drug as illegal or have implemented regulations on the use of this drug. Previous work on Salvinorin A has shown that the drug can be analyzed using both LC–MS and GC–MS [2–4]. However, these studies were mainly qualitative focusing on the biochemistry of the drug with no discussion of the quantitative figures of merit. LLE with GC–MS has been employed in previous studies of Salvinorin A as the primary extraction technique for screening of biological samples and elucidation of analogs of Salvinorin A [5,6].

In this work, LLE and solid phase microextraction (SPME) was used to extract Salvinorin A from plants and spiked aqueous and human urine samples. SPME is a straightforward and sensitive method for extracting drugs of abuse from biological samples often producing higher recoveries and lower limits of detection than traditional LLE or SPE [7–15]. SPME is also capable of extracting multiple drugs of abuse and/or metabolites contained in a single matrix [14,15]. Further, SPME not only reduces sample preparation time and eliminates the need for traditional extraction solvents, it can also eliminate the need for an additional derivatization step which is often required for the analysis of drugs [16,17].

In GC × GC–ToFMS, complex mixtures are separated based on two orthogonal retention mechanisms, using two columns with different dimensions and different stationary phases [18–20]. In the most common GC × GC configuration, a non-polar first dimension column separates the mixture roughly based on vapor pressure and a polar second dimension column separates based on one or more specific interactions. Due to rapidly eluting second-dimension peaks, flame ionization or time of flight mass spectrometry are the most common detectors. Relatively few references in the literature demonstrate the use and capability of GC × GC–ToFMS for the analysis of drugs and their metabolites, but interest in this is increasing, especially for forensic and metabolomic analysis, with analytes including cocaine, amphetamines, opiates and anabolic steroids [15,21–27].

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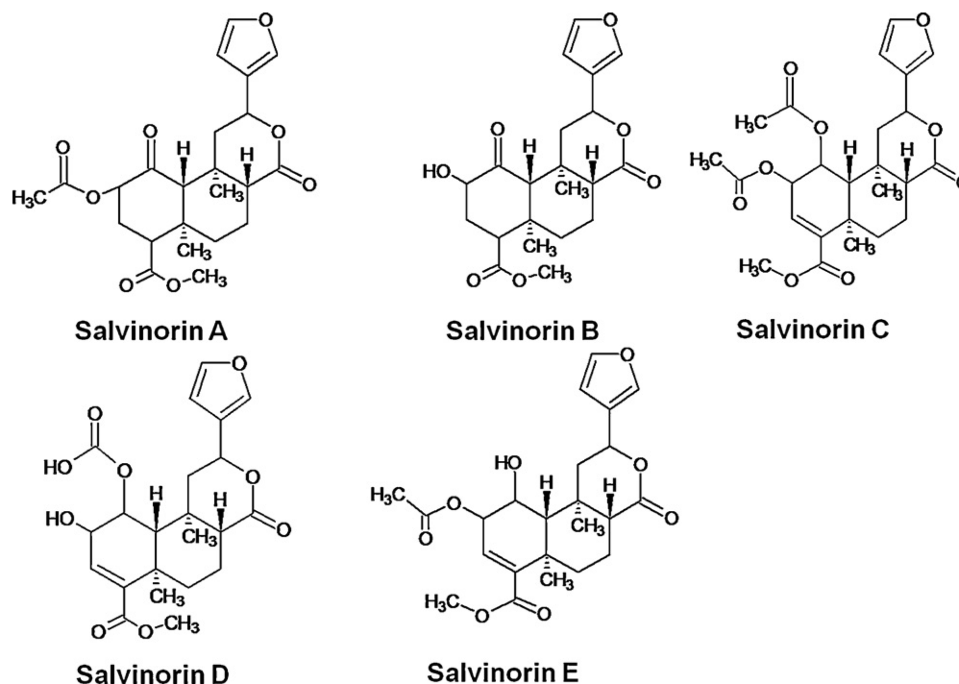

Fig. 1. Structures of Salvinorin A and analogs.

This is the first report of either SPME or GC × GC–ToFMS being used to determine Salvinorin A. *S. divinorum* leaves, stems and roots were extracted with traditional solvents, qualitatively and semi-quantitatively analyzed, while water and urine samples spiked with Salvinorin A were extracted with both liquid–liquid-extraction and automated SPME for quantitation. GC × GC–ToFMS was used for the separation and detection of Salvinorin A in all extracts.

2. Materials and methods

2.1. Materials

10 mg standard of Salvinorin A and 10 mg 'dilute N' shoot' standards were purchased from Alltech Associates, Inc. (Deerfield, IL). *S. divinorum* plants were cultivated in our laboratory. Urine was provided by volunteer humans and pooled prior to use.

2.2. Preparation of Salvinorin A stock standard

Salvinorin A (9 mg) was dissolved in 9 mL of chloroform to make a 1 mg/mL stock standard. A second 10 mg Salvinorin A standard was dissolved in 10 mL of deionized water which served as the stock solution for extraction of the drug from urine and water. The additional standards and samples described below were prepared by adding appropriate aliquots of the stock standard to water or urine to obtain the desired concentrations.

2.3. Calibration standards

For the analysis of plant material, the chloroform stock standard described above was diluted with chloroform to generate 6 non-extracted calibration standards with concentrations ranging from 120 to 8000 ng/mL. For liquid–liquid extraction, the aqueous stock standard was first diluted with deionized water to a concentration of 5000 ng/mL and this was further diluted to provide 6 calibration standards in the range of 300–5000 ng/mL. For SPME, the aqueous stock standard was diluted to 1000 ng/mL, followed by dilutions to provide 8 calibration standards with concentrations ranging from 8 to 1000 ng/mL. Duplicate standards in water and urine were prepared.

2.4. Extractions

2.4.1. Extraction of S. divinorum plants

Approximately 200 mg of leaves, stems, and roots were separated and ground using mortar and pestle. Water (15 mL) was added to both the leaf and stem samples and vortexed for 1 min immediately followed by ultrasonic extraction in an ultrasound water bath at room temperature for 30 min. Samples were vacuum filtered using a Buchner funnel and Whatman No.1 filter paper and washed with three 5 mL aliquots of water. The filtrate was collected and the plant material was discarded from each sample. Filtrates were placed into a 125 mL separatory funnel and approximately 20 mL of chloroform was added to the funnel. Samples were vigorously shaken and the funnel was vented three times. The layers were allowed to separate for approximately 15 min. The organic layer (bottom layer) was collected and evaporated to dryness under N_2 gas at 40 °C. The dried extracts were reconstituted with 0.50 mL of chloroform and then transferred to 2 mL autosampler vials for GC × GC–TOFMS analysis.

2.4.2. Liquid–liquid extraction from spiked water and urine

Aliquots of 12 and 100 μL from the aqueous stock solution were spiked into 20 mL deionized water or urine to make final spiked concentrations of 620 and 5000 ng/mL. Urine samples were vortexed for 1 min, ultrasonically mixed in an ultrasound water bath for 30 min and vacuum filtered prior to extraction. Water samples were used as mixed. The spiked water or urine samples were transferred to separate 125 mL separatory funnels. 15 mL of chloroform was added to each funnel and the funnels were shaken for 1 min and the layers were allowed to separate for 5 min. The aqueous layer was removed and discarded. A 10 mL aliquot of the organic layer was evaporated to dryness under N_2 gas at a constant temperature of 40 °C. The residue was reconstituted with 0.5 mL of chloroform and transferred to a 2 mL autosampler vial for GC × GC–ToFMS analysis.

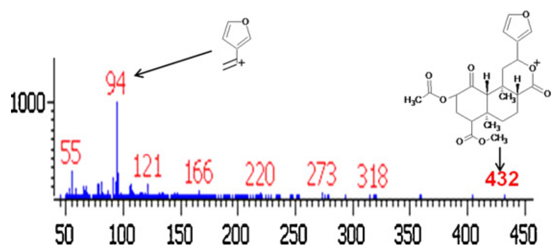

Fig. 2. Mass spectrum of Salvinorin A obtained from injected standard showing base peak (m/z 94) and molecular ion (m/z 432).

2.4.3. SPME from water and urine

An aliquot of 20 mL of the Salvinorin A aqueous stock solution was spiked into aliquots of 20 mL of water and 20 mL of urine to give 1000 ng/mL working standards, from which spiked samples at 62 and 125 ng/mL were prepared. SPME was conducted using an 85 μm polyacrylate fiber (Sigma–Aldrich, St. Louis, MO) mounted on a MPS2-Twister Model Autosampler (Gerstel, Columbia, MD). Prior to extraction, the samples were incubated at 30 °C for 10 min. The extraction time was 30 min with an agitation speed of 250 rpm at a temperature of 30 °C. Desorption from the fiber was conducted in the GC inlet, in splitless mode with a purge time of 120 s and held at a constant temperature of 280 °C. Following activation of the split purge, the fiber was further desorbed for 15 min in split mode to ensure cleanliness. Prior to each extraction, the fiber was conditioned in the heated inlet under split conditions for 10 min.

2.5. Instrumental conditions

A Pegasus 4D GC × GC–TOFMS (Leco Corporation, St. Joseph, MI) equipped with an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE), an MPS-2 Twister autosampler (Gerstel, Columbia, MD) and ChromaTOF data system (Leco, St. Joseph, MI) was used for all determinations. The column set used for the quantitative study was 15 m × 0.250 mm × 0.25 μm ZB-5 (Phenomenex, Torrance, CA) and 1.5 m × 0.250 mm × 0.25 μm DB-17 (Agilent Technologies, Wilmington, DE) as the first and second columns, respectively. For liquid injections, an injection volume of 1.0 μL was injected under splitless conditions with a 60 s purge time. The inlet temperature was held constant at 280 °C. The flow rate was held constant at 1 mL/min. The temperature ramps for the 1st and 2nd columns were 60 °C to 300 °C at 30 °C/min and 75 °C to 315 °C at 30 °C/min, respectively. The hold times at the initial and final temperatures were 2 min and 10 min, respectively. The initial temperature of the modulator was 85 °C with second dimension separation time of 8 s. The temperature of the modulator increased at a rate of 30 °C/min to a final temperature of 340 °C. The hot pulse and cold pulse times were 0.90 s and 2.10 s for each jet, respectively. The transfer line and ion source temperatures were 280 °C and 230 °C, respectively. The scan range for the mass spectra was m/z: 45 amu to m/z: 450 amu at a scan rate of 100 spectra/s. The detector voltage was set at 1450 V, 100 V higher than the optimized voltage. The total run time was 20 min.

3. Results and discussion

3.1. Initial confirmation of Salvinorin A

Prior to GC × GC–ToFMS analysis, a 1 mg/mL standard of Salvinorin A was analyzed using traditional 1-dimensional quadrupole GC–MS according to the method of Giroud [2]. The mass spectrum provided by Giroud was used as the reference spectrum. Peak for peak matching between the two spectra was performed in order to confirm the presence of Salvinorin A in the standard. Fig. 2 shows a mass spectrum for Salvinorin A from this work. The major peaks in the reference mass spectrum were at m/z: 94, m/z: 166, m/z: 273, and m/z: 432. The peak at m/z: 94 was the base peak and the peak at m/z: 432 was the peak for the molecular ion of Salvinorin A. The fragmentation pattern for each of the major peaks is discussed briefly by Giroud. The fragment for the base peak of Salvinorin A contains the THF present in the parent indicating that the positive charge on the initial radical cation, and the molecular ion would be at the oxygen in the lactone ring located adjacent to the bond connecting the THF ring to the structure. Therefore, as the molecule begins to fragment in the ion source, it must open the lactone ring via hydrogen rearrangements and or homolytic α-cleavages. This appears to result in the breaking both bonds attaching the THF ring to the rest of the molecule.

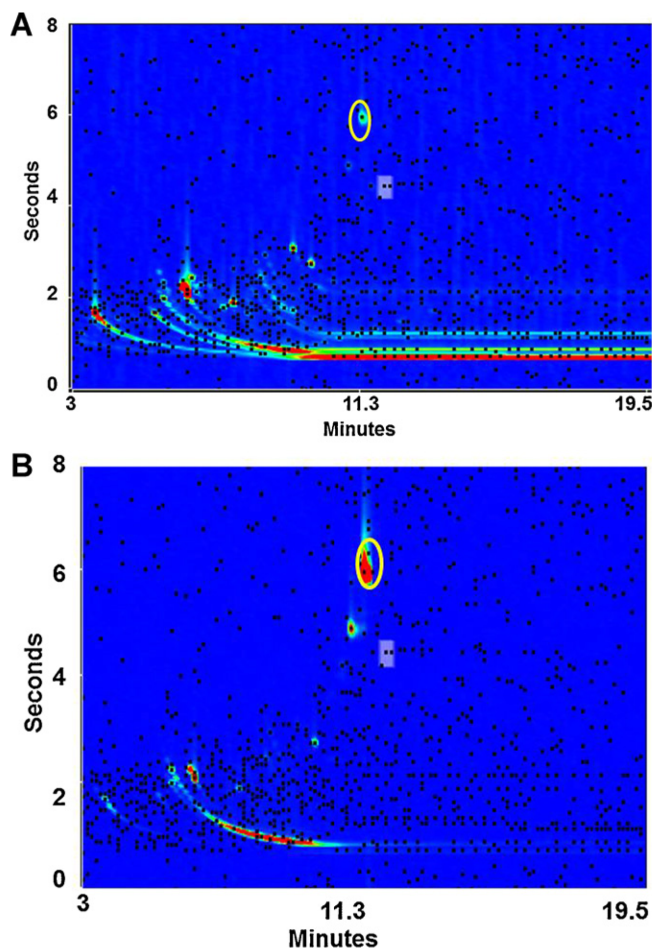

Fig. 3. GC × GC–ToFMS contour plots from SPME of 1000 ng/mL Salvinorin A in water. Plot (A) is the TIC. Plot (B) is the EIC at m/z: 94. The Salvinorin A peak is circled. The black dots represent additional peak markers as displayed by the software.

The GC × GC contour plot of a Salvinorin A standard at 1000 ng/mL in chloroform, injected by liquid injection, indicating the peak for Salvinorin A is shown in Fig. 3, demonstrating effective separation of the peak of interest from interferences such as column bleed, a strength of GC × GC. The 5% phenyl polydimethyl siloxane first dimension column is very common in drug analysis and is effective for wide ranges of analytes. RTX-200, a perfluoro polydimethyl siloxane derivative and DB-17, 50% phenyl polydimethyl siloxane, were evaluated for the second dimension, with DB-17 showing much stronger retention of the Salvinorin A, so it was chosen for further studies.

The GC × GC method was optimized by identifying the following critical parameters affecting retention and the S/N of Salvinorin

A peak: temperature ramp rate for both columns, the temperature offset between the primary and secondary ovens, the modulation period, and the MS scan rate. Each variable was changed one at a time while the other parameters were kept constant to assess the effect of each variable. With a single target analyte, a rapid, 30 °C/min, temperature program in the first dimension ensures a rapid overall analysis. The offset between the ovens was increased from 5 °C to 15 °C to decrease second dimension retention and reduce wraparound, since Salvinorin A and possible analogs are highly polar and may likely be retained on the second dimension column. The modulation time was also primarily increased to eliminate wraparound. The initial time was 5 s resulting in wraparound even with the higher oven offset, so it was increased to 8 s, yielding no wraparound and only a slight increase on the second dimension retention time. The MS scan rate was increased in order to produce smoother peaks and less noise in the mass spectra resulting in a higher S/N for Salvinorin A. The initial rate was 20 spectra/s, which yielded noisy mass spectra and a jagged appearing peak on the chromatogram for Salvinorin A. The final scan rate was 100 spectra/s. These conditions produced 1st dimension and 2nd dimension retention times for Salvinorin A of 11.5 min and 5.59 s, respectively, giving an elution temperature of 300 °C.

3.2. Extraction from S. divinorum plants

Salvinorin A was extracted from the roots, stems, and leaves of *S. divinorum* plants using LLE with chloroform. In order to estimate the amount of Salvinorin A in each part of the plant, a calibration curve for Salvinorin A was constructed using the external standard method. The curve had a linear range from 120 to 8000 ng/g with an R^2 value of 0.9998. During the method development phase, other solvents such as water, methanol, and acetonitrile were also tested, but chloroform provided the best linear range and correlation coefficient. Since chloroform is a strongly polar aprotic and hydrophobic solvent, it was able to dissolve Salvinorin A without the risk of degradation. Salvinorin A was in solution with chloroform for the duration of this research and no degradation due to chloroform was observed. The use of evaporation under a stream of nitrogen, while common and convenient, may result in some loss of analyte due to evaporation. As this is a common technique for other drugs of similar molecular size and gas chromatographic retention, this was not considered as a problem.

The highest quantity of Salvinorin A was found in the leaves of the plants, consistent with literature results, which indicate that the leaves are the primary part of the plant used for recreational use [1,2]. However, after performing an exhaustive extraction of Salvinorin A from the leaves, only approximately 60 ng/g of Salvinorin A were observed, well below the lowest point on the calibration curve. The typical dose of Salvinorin A necessary to achieve a euphoric effect is between 200 μg and 500 μg [1]. Therefore, a large quantity of our leaves would have been needed to generate the powerful euphoric effect that the drug is said to produce. It is likely that the cultivation conditions in our laboratory which were not optimized in any way, affected the plants performance. The literature also states that the leaves of the plant are often treated with additional Salvinorin A in order to make them more concentrated, thus making the euphoric effect more powerful for the user [1,2]. Wide variation in both cultivation and extraction may offer one explanation as to why the pre-concentration of Salvinorin A in the leaves is often necessary to achieve a powerful effect. The stems and roots were also extracted using the same procedure. A detectable amount of Salvinorin A was found in the stems and Salvinorin A was not detected in the roots.

Fig. 4 shows a GC × GC contour plot from one of the *S. divinorum* leaf extracts indicating the presence of Salvinorin A and analogs. The most prevalent of the analogs found were Salvinorin B and C, although pharmacology studies of the salvinorin analogs have shown that only Salvinorin A provides a hallucinogenic effect. Salvinorin A, B and C were identified by manually comparing their mass spectra to examples from Giroud [2]. Note that these are very closely related structures, as seen in Fig. 1, so their first and second dimension retention times are both quite close, but they are fully resolved. They would be much more difficult to fully resolve with traditional one-dimensional GC.

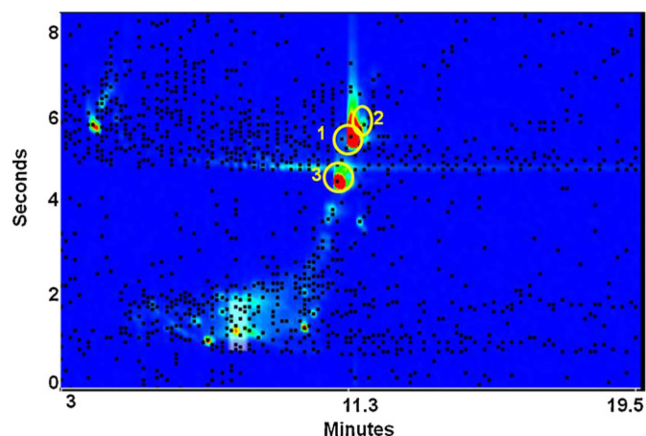

Fig. 4. GC × GC–ToFMS contour plot of *S. divinorum* leaf extract. The plot is an EIC at m/z: 94. Peak 1 is Salvinorin A at retention times of 11.5 min and 5.95 s. Peak 2 is Salvinorin C at retention times of 11.1 min and 5.50 s. Peak 3 is Salvinorin B at retention times of 11.7 min and 6.10 s. The black dots represent additional peak markers from the TIC.

Due to the unavailability of Salvinorin B or C standards, the amounts detected in the plant extracts were not quantified. However, by simple comparison of the peak areas, peak heights, and S/N ratios, it is expected that Salvinorin A is more prevalent than the other two analogs in all parts of the plant.

3.3. Extraction from spiked urine and water samples

Salvinorin A was extracted from spiked samples of urine and deionized water using both LLE and SPME. In this part of the study, the linear range, limit of detection, precision and accuracy were determined from water and urine, using external standard calibration. Analysis of spiked samples for testing the feasibility of extraction techniques for Salvinorin A is appropriate as Salvinorin A appears unmetabolized as the parent compound in human urine.

The conditions for SPME were optimized by changing the type of fiber and the extraction time, all of the other parameters were kept constant. The agitation speed was kept at 250 rpm which is the default speed for the autosampler, which agitates by moving the vial in a circular pattern around the needle. It was observed that increasing the agitation speed higher than the default setting often resulted in damage to the fiber. The incubation temperature was 30 °C; higher temperatures produced no improvement in peak area. Polydimethylsiloxane (PDMS), carboxen-divinylbenzene (CAR-DVB) and carbowax-divinylbenzene (CW-DVB) all showed little or no response for Salvinorin A, so polyacrylate (PA) was chosen as the fiber coating. In general, polyacrylate has proven effective for extracting polar analytes from polar sample matrices. The optimal extraction time was determined to be 40 min by varying the extraction time and measuring the peak area response. Longer times gave no significant improvement.

Fig. 5 shows TIC and EIC at m/z 94 GC × GC–ToFMS contour plots for the SPME extraction of Salvinorin A from spiked urine samples at the 500 ng/mL level. The top figure is a total ion chromatogram, showing full detail of the separation of both the target compound from matrix and analysis interferences. The bottom plot

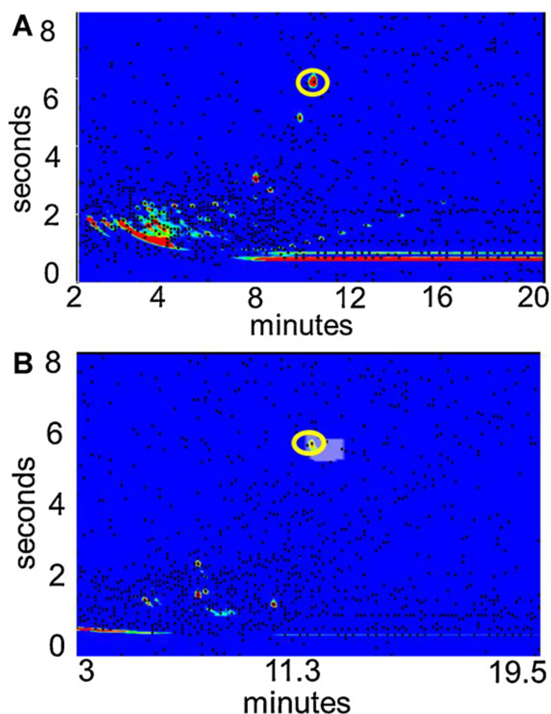

Fig. 5. GC × GC contour plots of 500 mg/mL Salvinorin A in urine by SPME. Plot (A) is the TIC. Plot (B) is the EIC at m/z: 94. The Salvinorin A peak is circled. The black dots represent additional peak markers displayed by the software.

is an extracted ion chromatogram at m/z 94, the base peak for Salvinorin A, showing a more selective viewing of Salvinorin A response. In both cases, the peak is clearly separated from interferences and readily quantifiable.

3.4. Quantitative performance

Table 1 summarizes the analytical figures of merit for the LLE and SPME extractions from both water and urine. The limit of detection (LOD) for Salvinorin A was determined using the IUPAC method with $k = 3$ [28]. SPME showed a LOD at least one order of magnitude lower than LLE. The small volume of the SPME fiber coating (approximately 1 μL) provides a potential concentration of up to 4 orders of magnitude with the 20 mL samples that were used in this work. This is significantly greater than the concentration achieved with the LLE method. Further, there is much less opportunity with the SPME technique for sample losses during sample transfer steps.

Precision was evaluated by analyzing spiked samples at the indicated concentration level (about in the middle of the expected calibration ranges) in triplicate for both LLE and SPME. For both urine and water extractions, the precision obtained by SPME was significantly better. This result was not surprising since the SPME method involves far fewer steps than the LLE method and is automated, reducing opportunities for experimental errors.

The calibration curve for the water extraction shows better linear behavior than that of the urine for concentration ranges plotted for both LLE and SPME. This is not surprising, due to matrix effects in urine. For both urine and water, SPME provided better linearity at lower concentrations, also as expected. The higher concentrations seen in the LLE curve are more typical of extractions that may be performed on plant material, while the SPME concentrations are more typical of those expected in clinical or drug testing analyses.

Extraction of concentrations higher than 1000 ng/mL using SPME was not attempted due to the risk of exceeding fiber capacity. When the calibration curves for Salvinorin A were plotted using LLE at the same low concentration range as SPME, Salvinorin A was detected, but linear behavior was not observed for the extraction from both the water and urine. Therefore, SPME is better suited for trace analysis of Salvinorin A, while LLE is better suited to the higher concentrations that may be found in extractions of plants or other Salvinorin A containing products. In the case of urine extraction, a significant decrease in the matrix effects on Salvinorin A extraction using SPME was observed, indicated by better precision from both water and urine and by the elimination of several sample handling steps in the LLE method that are directly attributable to the complexity of the matrix. These three factors helped to minimize sample loss between steps and variability between extractions.

Accuracy was evaluated by preparing five replicate spiked samples in water and urine at the indicated concentrations. As seen in Table 1, both SPME and LLE produced accurate results within the experimental error from that determination. It should be noted that in both the LLE and SPME determinations, the external standard method was used for quantitation. While the internal standard method is commonly used as a means for reporting improved precision, it was not chosen for this work. An appropriate internal standard with similar chemistry to Salvinorin A, but not present in any samples and readily available at low cost, could not easily be obtained.

Table 1
Analytical figures of merit for extraction of salvinorin a from urine and water using LLE and SPME.

	Water		Urine	
	LLE	SPME	LLE	SPME
LOD (ng/mL)	230	6	200	4
Precision (RSD)[a] (%)	16	2	24	6
Linear range				
Low (ng/mL)	300	8	300	8
High (ng/mL)	5000	500	5000	500
R^2	0.9960	0.9981	0.9587	0.9857
Accuracy (125 ng/mL)	ND	118 ± 8	ND	114 ± 12
Accuracy (620 ng/mL)	520 ± 100	ND	620 ± 150	ND

ND = not determined.
[a] SPME: 100 ng/mL; LLE 600 ng/mL.

4. Conclusions

Salvinorin A, an up and coming hallucinogen, was extracted from plants, water and urine using LLE and SPME and determined in the extracts using GC × GC–ToFMS. Semi-quantitative studies of leaves and stems of *S. divinorum* produced ppb quantities of Salvinorin A, far below expected levels due to variation in cultivation and extraction methods. Salvinorin A was not detected in the roots. Salvinorin B and C were also detected in the leaves. Spiked water and urine were determined using SPME and LLE with SPME providing superior quantitative performance. Detection limits using SPME were approximately 5 ng/mL, with a linear range from 8 to 500 ng/mL and precision of approximately ±10% using external standard quantitation. This range is appropriate for clinical or physiological samples. LLE was found more effective for higher concentrations that may be found in plant material or products containing Salvinorin A. GC × GC–ToFMS provided chromatographic separation of the closely related salvinorin analogs and separation from chromatographic and matrix interferences.

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