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RESEARCH ARTICLE

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Quantification of hordenine in a complex plant matrix by direct analysis in real time–high-resolution mass spectrometry: Application to the "plant of concern" *Sceletium tortuosum*

Meghan Grace Appley | **Megan Isabella Chambers** | **Rabi Ann Musah**

Department of Chemistry, University at Albany, State University of New York, Albany, New York, USA

Correspondence
Rabi A. Musah, Department of Chemistry, University at Albany, State University of New York, 1400 Washington Avenue, Albany, NY 12222, USA.
Email: rmusah@albany.edu

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Abstract

Recently, there has been an increase in the recreational abuse of several psychoactive plants, resulting in the United Nations Office on Drugs and Crime creating a list of "plants of concern." One such material is *Sceletium tortuosum* and products derived from it. Regulation of these materials is challenging because of their innocuous appearance, the cumbersome sample preparation steps required to render the material into a form amenable to analysis by conventional techniques, the requirement for nuanced sample analysis protocols, and lengthy analysis times. It is demonstrated here that direct analysis in real time–high-resolution mass spectrometry (DART-HRMS) can be used to not only identify *S. tortuosum* material based on the detection of characteristic biomarkers including hordenine and several mesembrine alkaloids, but also quantify the amount of hordenine present. Using hordenine-d_6 as an internal standard, a protocol, validated according to US Food and Drug Administration (FDA) Guidelines for the Development and Validation of Bioanalytical Methods, was devised for the quantification of the psychoactive component hordenine. The method was then applied to the quantification of hordenine in six commercially available products derived from the foliage and stems of *S. tortuosum*. By this method, the lower limit of quantification (LLOQ) was found to be 1 μg/ml. Observed hordenine concentrations ranged from 0.02738 to 1.071 mg of hordenine per gram of plant material. The developed technique provides an effective and quick means for the detection and quantification of hordenine in *S. tortuosum*, which can be extended to analysis of other hordenine-containing products.

KEYWORDS
DART-HRMS, hordenine, kanna, quantification, *Sceletium tortuosum*

1 | INTRODUCTION

In recent years, the recreational abuse of plant-based psychoactive substances continues to increase at an alarming rate. Whereas the marijuana variety of *Cannabis sativa* remains the most abused plant-based psychoactive substance, other psychoactive plants that fall under the category of legal highs (because their status in many countries remains unscheduled) are still very common. A subset of these have been designated as "plants of concern" by the United Nations Office on Drugs and Crime (UNODC) because of the exponential rise in their use and their potential to cause addiction.[1] When these substances are encountered in a forensic context, they can be extremely difficult to visually identify because they can appear in a broad range of both processed and unprocessed forms, and they may have the appearance of innocuous culinary products. When crime laboratories endeavor to identify them, the most

common approaches involve analysis by gas chromatography (GC)– and/or liquid chromatography (LC)–mass spectrometry (MS) after the application of significant pretreatment steps in order to render the sample into a form that is suitable for the method. However, because of the large variety of different psychoactive plants that are abused, nuanced sample processing approaches have been required, and for most psychoactive plants, there are no standard operating protocols for their analysis. The intent of most forensic GC– and LC–MS analyses is to detect one or more species-specific biomarkers (often psychoactive molecules) that can serve to reveal the identity of the plant. For example, *Salvia divinorum*-derived material is identified as such based on the detection of the psychoactive compound salvinorin A, which is a biomarker for the species. For crime laboratories, the analysis and identification of these substances is heavily time-, human-, and chemical-resource intensive, particularly when there are no immediate and independent clues as to the material's identity.

In recent years, an increasing number of crime laboratories have begun to exploit the unique capabilities of various forms of ambient ionization mass spectrometry, such as direct analysis in real time–high-resolution mass spectrometry (DART-HRMS) to triage sample unknowns and rapidly acquire information about possible sample identity. This approach is rapid because the material can often be analyzed in its native form without sample pretreatment, and the mass spectral profile obtained enables detection of a broad range of the chemicals present in their unfragmented forms, thereby providing visually apparent clues about the presence of psychoactive biomarkers of interest. Once these have been tentatively identified, the sample is then subjected to more conventional analysis by GC– or LC–MS for identity confirmation, which for complex plant matrices, remains time-consuming and expensive. What would give techniques such as DART-HRMS added value is if they could also be used to perform quantification by validated methods for the molecules of interest in the material after preliminary identification of it has been accomplished.

One of the plants of concern highlighted by the UNODC is *Sceletium tortuosum* (aka kanna). It is endemic to South Africa and has been used since prehistoric times as a mood-altering substance.[2] Other effects include decreased tension, stress, and anxiety.[3,4] At higher levels, *S. tortuosum* is used to promote euphoria and even sedation by combining it with other drugs such as phenethylamine (PEA), an approach that has been described in user forums.[5,6] Interest in the psychoactive activity of kanna[7] and kanna-derived[8] products is on the rise,[9] and its abuse in combination with Cannabis or other psychoactive agents has attracted the attention of the US Drug Enforcement Administration (DEA),[10] with a seizure of the material being reported as an intelligence alert in the DEA's Microgram Bulletin.[11]

Kanna materials are identified as such by the presence of several psychoactive molecules including hordenine and a number of genus-specific alkaloids such as mesembrine, mesembrenol, mesembrinol, and mesembrenone (Figure 1). Despite their forensic relevance, protocols for the quantification of these compounds in general, and in *Sceletium* genera in particular, are very limited. Published reports include measurement of the amounts of mesembrine alkaloids using GC–MS and reverse plasma-ultrahigh-performance liquid chromatography-photodiode array (RP-UHPLC-PDA), with lower limits of quantification of 35–68 and 2.7–3.7 μg/ml, respectively.[9,12] Each technique requires extensive sample preparation including an acid–base extraction along with lengthy sample runs not only to quantify the amount of compound present, but also to detect the compound of interest. Articles on the quantification of hordenine are even more scarce.

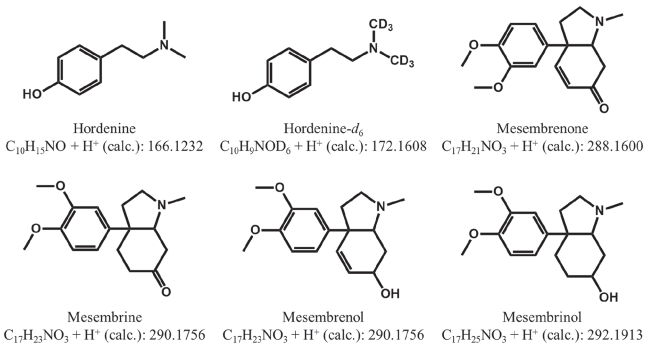

FIGURE 1 Chemical structures for relevant compounds including hordenine (analyte of interest), hordenine-d_6 (internal standard) and the mesembrine alkaloids

Hordenine is a G protein-based agonist of the D2 receptor, which has been shown to stimulate the release of dopamine, thereby resulting in feelings of euphoria.[13] Recently, this compound appeared on a list of substances banned by the US National Collegiate Athletics Association (NCAA).[14] A GC–MS procedure for the detection of hordenine in horse urine has appeared,[15] as well as another for the analysis of hordenine in beer using ultrahigh-performance LC coupled to electrospray ionization tandem mass spectrometry.[16] In this instance, the relevance of hordenine to beer is that it occurs naturally in barley (*Hordeum* spp.) malt from which beers are produced, and as such, characterization of beer has included determination of hordenine amounts.[13,17] Because of its presence in beer, hordenine detection in serum has been proposed as an approach for the forensic determination of beer consumption.[18] With the extensive sample preparation and long run times per sample associated with previously used techniques, the ability to quantify the amount of hordenine present, specifically in various complex forms of *S. tortuosum*, using a rapid technique such as DART-HRMS would be extremely beneficial. DART-HRMS has successfully been used to quantify psychoactive materials in other plants of concern including *N*,*N*-dimethyltryptamine in ayahuasca brews,[19] yangonin in kava,[20] mitragynine in kratom,[21] and mescaline in various cacti,[22] thereby adding to the growing list of validated protocols that crime labs can use to quantify psychoactive compounds by DART-HRMS. Herein, we describe the development of a validated protocol for the quantification of hordenine via DART-HRMS and demonstrate its efficient application to the determination of hordenine levels in several commercially available *S. tortuosum* products.

2 | EXPERIMENTAL METHODS

2.1 | Botanical material

Six *S. tortuosum* samples were purchased from online vendors. Fine powder and coarse powder samples were purchased from Herb Stomp (Portland, OR, USA). Foliage samples were purchased from eBay (http://ebay.com, USA). Stem samples were purchased from Schmerbals Herbals (Clemmons, NC, USA). Foliage and shredded samples were also purchased from World Seed Supply (Mastic Beach, NY, USA). Sample and vendor information is presented in Table 1.

2.2 | Chemical standards

Hordenine (analytical standard) and polyethylene glycol 600 (PEG-600) were purchased from Sigma Aldrich (St. Louis, MO, USA). Lidocaine (analytical standard) was purchased from MP Biomedicals, LLC (Solon, OH, USA). Mesembrine (analytical standard) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and hordenine-d_6 was purchased from Toronto Research Chemicals (Toronto, ON, Canada). HPLC-grade methanol was purchased from Pharmco Aaper (Brookfield, CT, USA). High-purity helium was purchased from Airgas (Albany, NY, USA).

2.3 | *S. tortuosum* extract preparation

Six extracts were made using the following *S. tortuosum* plant products: (1) Herb Stomp fine powder; (2) Herb Stomp coarse powder; (3) eBay foliage; (4) World Seed Supply foliage; (5) Schmerbals Herbals stems; and (6) World Seed Supply shredded (listed in Table 1). Plant materials that were not already fine powders (i.e., coarse powder, foliage, stem, and shredded) were pulverized using a coffee grinder (Hamilton Beach, Southern Pines, NC, USA). Roughly 200 mg of each sample was extracted four times with 5 ml of methanol, and the extracts were pooled, yielding ~20 ml of extract for each sample. Each of these samples was then filtered to remove any residual solids. When initial quantification experiments revealed that the concentration of hordenine extended beyond the upper limit on the calibration curve (e.g., for Sample 1—Table 1), a dilution step was introduced by adding methanol to yield a methanol:extract solution in a 1:1 ratio. For samples in which the amount of hordenine fell below that of the lower limit on the calibration curve (i.e., samples 3–6—Table 1), the sample was made more concentrated by evaporating a 10-ml aliquot of the extract to dryness, and the residual solid was reconstituted in 2 ml of methanol.

To confirm the efficiency of the extraction protocol and to determine the possible presence of matrix effects, three 0.200-g portions of *S. tortuosum* plant material (Sample 2) were spiked with 200 μl of a 1-mg/ml solution of hordenine-d_6. The hordenine-d_6 was then extracted using the sample protocol as outlined above, resulting in a solution with a final concentration of 10 μg/ml. A 200-μl aliquot of each of the extracts was added to 200 μl of a 10-μg/ml internal standard solution (lidocaine) for this set of experiments. The solutions were then analyzed by DART-MS and compared with a standard solution with a hordenine-d_6 concentration of 10 μg/ml (also spiked with internal standard). Comparison of the ratios of analyte to internal standard was used to assess recovery and matrix effects.

2.4 | DART-HRMS mass spectral data acquisition and data analysis parameters

A DART-SVP ion source (IonSense, Saugus, MA, USA) coupled to an AccuTOF high-resolution time-of-flight mass spectrometer (JEOL USA, Peabody, MA, USA) was used to produce DART high-resolution mass spectra. Spectra were collected using the following parameters: positive-ion mode; 250-V grid voltage; 350°C heater temperature; and 2-L/min helium flow rate. The mass spectrometer parameters were as follows: orifice 1 voltage, 20 V; orifice 2 voltage, 5 V; peak voltage, 600 V; and detector voltage, 2000 V. Spectra were collected at 1 spectrum per second over a mass range of *m/z* 60–1000. The mass spectrometer had a resolving power of 6000 FWHM and a mass accuracy of 5 millimass units (mmu). Sample introduction was performed using a 24-Pin Liquid Sampler (IonSense, Saugus, MA, USA). TssPro 3 software (Shrader Software Solutions, Grosse Point, MI, USA) was used for mass spectral data calibration, spectral averaging, background subtraction, and peak centroiding and integration. Polyethylene glycol (PEG 600) was used as a mass calibrant for all spectra. Mass spectral processing was accomplished using the Mass Mountaineer software suite (RBC Software, Portsmouth, NH, USA).

TABLE 1 Vendor information, material type, and hordenine content for the *S. tortuosum* plant materials. The results observed for the mg of hordenine per gram of dry weight material after removal of moisture are also presented

Sample	Vendor	Material type	Product label	Weighed amount (g)	Mass after removal of moisture (g)	mg/g
1	Herb Stomp	Fine powder	Kanna powder	0.2006	0.1867	1.151
2	Herb Stomp	Coarse powder	Coarse	0.2003	0.1862	0.4776
3	eBay	Foliage	Kanna	0.2007	0.1841	0.04380
4	World Seed Supply	Foliage	*Sceletium tortuosum*	0.2006	0.1823	0.05113
5	Schmerbals Herbals	Stems	Kanna	0.2003	0.1900	0.02893
6	World Seed Supply	Shredded	*Sceletium tortuosum*	0.2002	0.1961	0.08377

2.5 | Confirmation of hordenine in plant products and extracts

To confirm the presence of hordenine in the plant material and extracts, a screening was performed by immersing the closed end of a melting point capillary tube into the ground material or extract and presenting the coated surface of the tube to the DART gas stream for approximately 5 s. Three replicates of a single ground plant material or plant extract were analyzed in a single DART-HRMS run by introducing the sample three separate times into the DART gas stream. For analysis under soft ionization conditions, the samples were run using an orifice 1 voltage of 20 V. Samples were also analyzed under collision induced dissociation conditions (i.e., with an orifice 1 voltage of 60 V) to cause fragmentation that would yield a diagnostic pattern that could be used for comparison with the fragmentation patterns of authentic standards of hordenine and mesembrine acquired under identical conditions, in order to confirm compound assignments. The mass spectra were calibrated using PEG-600 and processed using the Mass Mountaineer software suite. Images of all the purchased plant materials that were analyzed are shown in Figure 2.

2.6 | *S. tortuosum* sample moisture content determination

In order to address the impact of absorbed moisture on the final hordenine concentration of the samples, the moisture content of the *S. tortuosum* samples was determined. One replicate of each plant sample (~0.200 g) was oven heated at a temperature of 100°C until constant weight (up until a total of 100 min), and the samples were then allowed to cool to room temperature (10 min) before being reweighed. Weights were measured every 20 min (six times in total). It was observed during these experiments that heating for a period of 20 min was sufficient to drive off all moisture (i.e., heating for longer than 20 min did not result in further changes to the mass of the material). The final weight of the plant material following heating was used in the determination of hordenine concentration by dry weight. The calculations of hordenine for the 20- and 100-min time intervals are shown in Table S4.

2.7 | Quantification of hordenine in *S. tortuosum* extracts

A protocol was created for the quantification of hordenine by DART-HRMS following US Food and Drug Administration (FDA) Guidelines for the Development and Validation of Bioanalytical Methods.[23] The guidelines include: (1) a selective, sensitive, and reproducible method; (2) a standard curve containing a minimum of six non-zero calibrators; (3) blank and zero calibrators that do not exceed 5% of the average response in the curve calibrators and quality control (QC) standards and do not interfere with the analyte of interest; (4) the lower limit of quantification (LLOQ) as the lowest point of the standard curve; and (5) the integration of four QC standard levels (high, medium, low, and LLOQ) into the curve. In order to validate a method, the following criteria must be met: (1) all non-zero calibrators should be within 15% of their theoretical concentrations, except for the LLOQ calibrator,

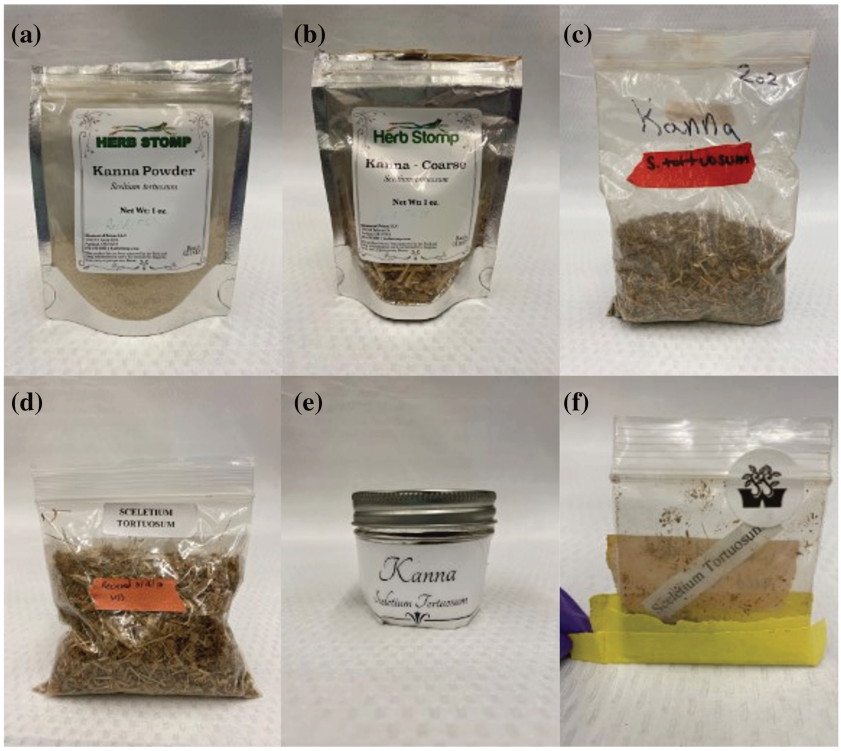

FIGURE 2 Commercially available *Sceletium tortuosum* plant materials. (a) Herb Stomp fine powder; (b) Herb Stomp coarse powder; (c) eBay foliage; (d) World Seed Supply foliage; (e) Schmerbals Herbals stems; and (f) World Seed Supply shredded plant material [Colour figure can be viewed at wileyonlinelibrary.com]

which can be within 20% of its theoretical value; (2) a minimum of six non-zero calibrators with 75% being required to meet criterion one; (3) more than 67% of the QC standards should be within 15% of their theoretical values; and (4) more than 50% of the QC standards at each level must be within 15% of their theoretical values. The R^2 values for each of the standard curves used for validation must be greater than 0.99.

To quantify the hordenine content in the various *S. tortuosum* extracts, standard curves were developed using 1 mg/ml (1000 μg/ml) stock solutions of hordenine and hordenine-d_6. Eight calibrators with the following concentrations were prepared: 20, 16, 12, 8, 6, 4, 2, and 1 μg/ml. The LLOQ, which was found to be 1 μg/ml, was then used as the lowest point on the standard curve. The internal standard of hordenine-d_6 was made by diluting the 1000-μg/ml stock solution to 20 μg/ml. Therefore, when added in a 1:1 ratio with the calibrators and extracts, its final concentration was 10 μg/ml in all standards and solutions. To satisfy the US FDA Guidelines for the Development and Validation of Bioanalytical Methods for method validation, two separately made hordenine solutions were used as the QC stock solutions. The two stock solutions were used to create two sets of QC standards (QC1 and QC2) daily, at high (18 μg/ml), medium (9 μg/ml), low (3 μg/ml), and LLOQ (1 μg/ml) concentrations on the curve. The low QC point (3 μg/ml) satisfies the requirement that it be three times higher than the LLOQ (1 μg/ml). In addition to the curve points, sample blanks (0-μg/ml hordenine and 0-μg/ml hordenine-d_6) were analyzed each day in order to confirm that *m/z* values consistent with the presence of these two compounds (i.e., within ± 0.005 of *m/z* 166.1232 and 172.1608 for hordenine and hordenine-d_6, respectively) were absent.

To allow for semi-automated analysis and quantification, a 24-Pin Liquid Sampler (IonSense, Saugus, MA, USA) was used for the creation of the standard curves, and for the quantification of hordenine in the calibrators, QC standards, and plant extracts. Aliquots (10 μl) of the calibrators, QC standards and plant extracts were dispensed into a 384-well plate. Calibration standards were analyzed in replicates of three, and QC standards were analyzed in replicates of five. The 24-Pin Sampler pins were dipped in the wells, and then, the sampler was fastened to the linear rail system. The linear rail speed was set to 0.8 mm/s. The peak area ratio (PAR) between hordenine and hordenine-d_6 protonated $[M + H]^+$ peaks at *m/z* 166.1232 and 172.1608, respectively, was plotted against the concentration of the sample to create a standard curve to confirm the concentrations of the QC standards and accurately quantify the hordenine content.

After the standard curve was validated, the hordenine content of the various plant material extracts was quantified. To remove any residual plant material, a 1-ml aliquot of each extract was centrifuged at 5000*g* for 10 min. After centrifugation, 100 μl aliquots of the extracts were spiked with 100 μl of the 20-μg/ml hordenine-d_6 internal standard solution resulting in a 1:1 dilution. As was done for the method validation experiments, the standard curve, QC standards, and plant extracts were analyzed on three different days, with the concentrations of hordenine averaged over the 3-day period.

3 | RESULTS

3.1 | Confirmation of hordenine in plant products and extracts

Before extracting hordenine from the *S. tortuosum* plant material, samples were first screened to confirm the presence of hordenine. Dried plant material was analyzed directly and without pretreatment by DART-HRMS via the capillary tube method using an orifice 1 voltage of 20 V. Representative spectra are shown in Figure 3. A peak consistent with the protonated mass (±5 mmu) of hordenine ($C_{10}H_{15}NO + H^+$) was detected at *m/z* 166.1232 in each of the *S. tortuosum* plant products. Within several of the products (foliage from eBay and World Seed Supply, and stems from Schmerbals Herbals), masses consistent with the presence of other mesembrine alkaloids, including mesembrenone (*m/z* 288.1600), mesembrine (*m/z* 290.1756), mesembrenol (*m/z* 290.1756), and mesembrinol (*m/z* 292.1910) were also detected. The structures of these compounds are shown in Figure 1.

Confirmation of the presence of hordenine and mesembrine was accomplished by determination of the presence of the diagnostic fragments associated with analysis of authentic standards by DART-HRMS under CID conditions (60 V) in plant samples analyzed under identical CID conditions. The results of these experiments are illustrated in Figure S1, which shows representative DART mass spectra acquired at 60 V for each of the plant materials analyzed (top spectra), compared with the spectra of hordenine (Figure S1a) and mesembrine (Figure S1b) (bottom spectra), rendered as head-to-tail plots. In all cases, high-resolution peaks consistent with fragmented hordenine and mesembrine were present. Due to the lack of commercially available analytical standards for the other mesembrine alkaloids (mesembrenone, mesembrenol, and mesembrinol), the presence of these structures could not be confirmed by CID. However, they were tentatively assigned based on their high-resolution masses and isotope matching.

With the observation during analysis of the bulk solid material of *m/z* values consistent with those anticipated to be present (i.e., hordenine and the mesembrine alkaloids), methanol extracts were prepared and analyzed directly by DART-HRMS. Representative spectra of the extracts of the six commercial samples (comprised of powders, foliage, stems, and shredded material) are presented in Figure 4. In all cases, similar to what was observed in the DART-HRMS analysis of the bulk solid material, protonated hordenine ($C_{10}H_{15}NO + H^+$) was again detected at *m/z* 166.1232. Furthermore, peaks consistent with the presence of mesembrenone, mesembrine, mesembrenol, and mesembrinol were also observed. For Samples 2–5, no hordenine was detected in the fourth of the four extraction rounds. For Samples 1 and 6, trace amounts were detected in the fifth extraction round (Figure S2). Quantification was performed using the pooled samples of four rounds of extraction (described below).

To determine recovery and the possibility of matrix effects, extracts of plant material spiked with hordenine-d_6 (to yield a final

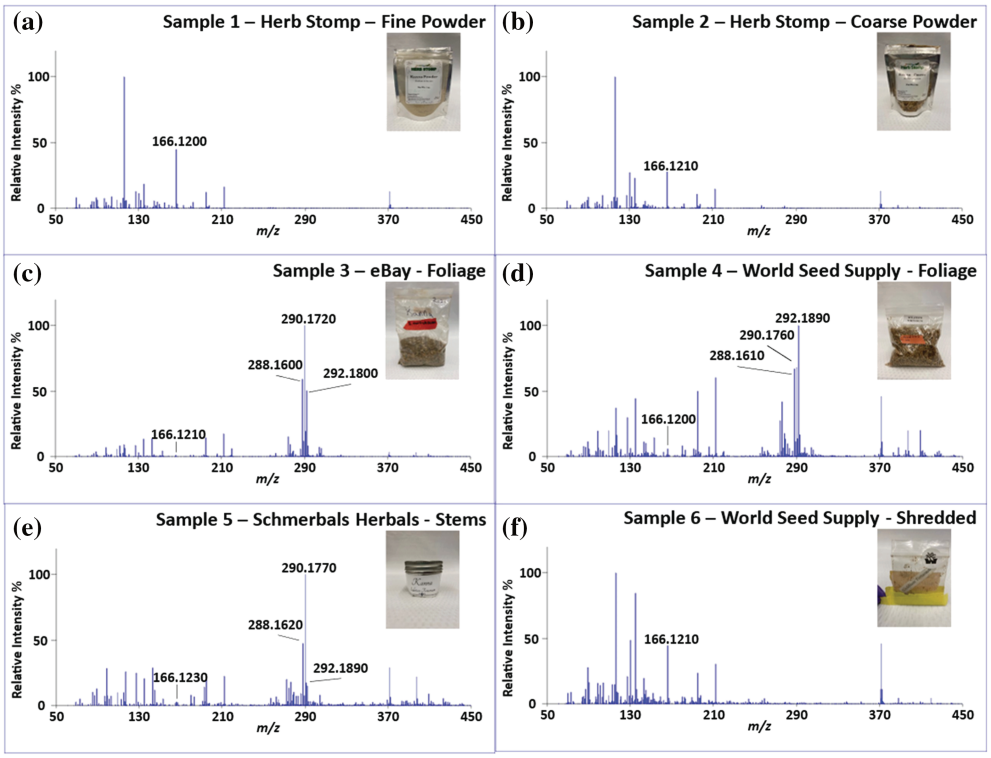

FIGURE 3 Direct analysis in real time (DART) high-resolution mass spectra obtained on direct analysis of *Sceletium tortuosum* solid material. (a) Herb Stomp fine powder; (b) Herb Stomp coarse powder; (c) eBay foliage; (d) World Seed Supply foliage; (e) Schmerbals Herbals stems; and (f) World Seed Supply shredded plant material. The peak at nominal *m/z* 166 which is representative of hordenine, is highlighted in each case. When detected, masses consistent with the presence of the protonated forms of the mesembrine alkaloids mesembrenone, mesembrine, mesembrenol and mesembrinol (at nominal *m/z* 288, 290, 290, and 292 respectively) are also highlighted [Colour figure can be viewed at wileyonlinelibrary.com]

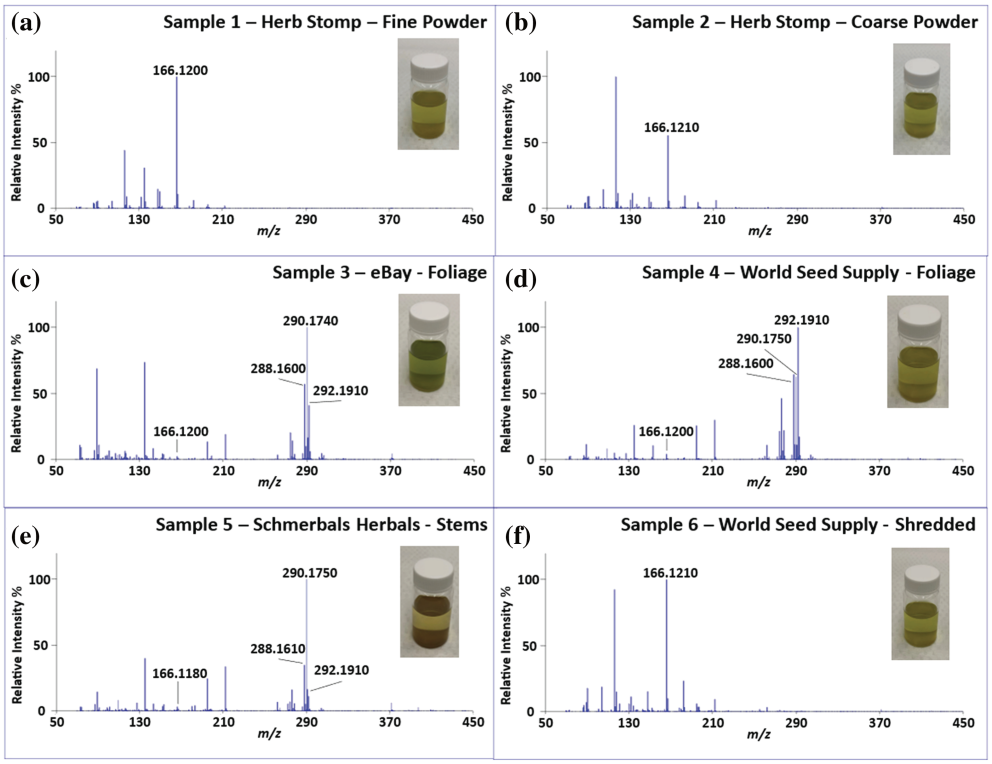

FIGURE 4 Mass spectra for the direct analysis of *Sceletium tortuosum* extracts by direct analysis in real time–high-resolution mass spectrometry (DART-HRMS). (a) Herb Stomp fine powder; (b) Herb Stomp coarse powder; (c) eBay foliage; (d) World Seed Supply foliage; (e) Schmerbals Herbals stems; and (f) World Seed Supply shredded plant material. Hordenine and the mesembrine alkaloids mesembrenone, mesembrine, mesembrenol, and mesembrinol at nominal *m/z* 166, 288, 290, 290, and 292 are highlighted. Images of the corresponding extract solutions are shown in the insets [Colour figure can be viewed at wileyonlinelibrary.com]

concentration of 10 μg/ml) were analyzed. The PARs of the analyte (hordenine-d_6 at *m/z* 172.1608) to internal standard (lidocaine at *m/z* 235.1810) for the extracts were compared with the ratio for a standard solution with a known analyte concentration of 10 μg/ml. Table S1 shows the results. The average PARs of hordenine -d_6 in the extracts were 0.443, 0.442, and 0.458, which was consistent with the PAR for the standard solution with a concentration of 10 μg/ml and a ratio of 0.449. This shows that the extraction protocol exhibited good efficiency and that there are no major matrix effects.

3.2 | Validation of a DART-HRMS protocol for quantification of hordenine

The three validated standard curves and QC standards are displayed in Figure 5. For each of the standard curves generated in this study, the concentration of each point was recalculated using the equation of the calibration curve. All of the calibrators from 1 to 20 μg/ml fell within the required limits of 15% (20% for the LLOQ) (Table S2). For the QC standards, more than 50% passed at each level, and more than 67% passed overall (Tables S3a–d). After three standard curves were completed on separate days and successfully validated according to the FDA guidelines, the method was used to determine the hordenine content in six *S. tortuosum* extracts. Sample blanks (0-μg/ml hordenine and 0-μg/ml hordenine-d_6) that were analyzed each day did not contain any peaks consistent with the presence of hordenine (*m/z* value 166.1232 ± 5 mmu) or hordenine-d_6 (*m/z* value 172.1608 ± 5 mmu). The mass spectra corresponding to the sample blanks analyzed each day are shown in Figure S3.

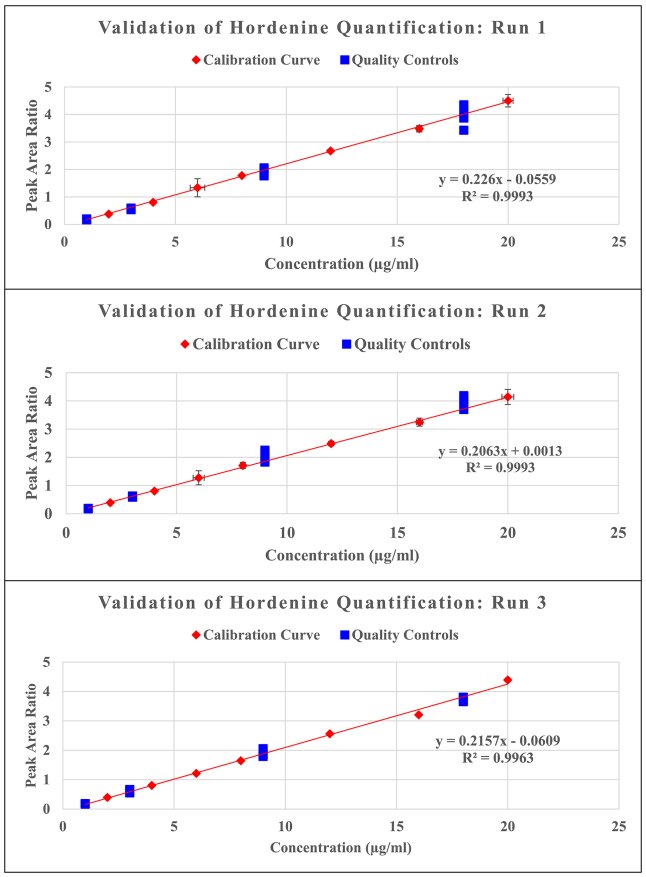

FIGURE 5 Calibration curves for the successful validation of hordenine quantification by direct analysis in real time–high-resolution mass spectrometry (DART-HRMS). The red diamonds represent calibration points, and the blue squares represent the quality control points [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | *S. tortuosum* sample moisture content determination

Table S4 shows the calculations and results for the moisture content determinations in the *S. tortuosum* samples. The mass difference between heated and unheated samples ranged from ~2% to 9% depending on the *S. tortuosum* product. The weights obtained following evaporation of the moisture were then used in the calculation of the amount of hordenine per gram of plant material. The results showed that in all cases, the loss of moisture influenced the value tabulated for the amount of hordenine present by ≤10%. This translated into underestimations of the concentrations of hordenine in the plant material of from 0.001697 to 0.07994 mg/g (if the moisture content was not taken into account). The concentrations reported in Table 1 take into consideration the moisture content of each plant material.

3.4 | Quantification of hordenine in *S. tortuosum* extracts

Aliquots of 1 ml of each of the previously mentioned extracts were centrifuged at 5000*g* for 10 min. To 100 μl of each of the supernatants, was added 100 μL of a 20 μg/mL hordenine-d_6 internal standard solution. To quantify hordenine in plant materials, the extracts were analyzed alongside a standard curve and QC standards that met the requirements outlined by the FDA guidellines. The PAR of the hordenine peak (*m/z* 166.1232) to the internal standard hordenine-d_6 peak (*m/z* 172.1608) was used to determine the concentration of the extracts using the equation from the calibration curve.

The validated standard curves used to measure the average hordenine concentrations for the six extracts are shown in Figure 6, and the concentrations, which were calculated by plugging the average PARs for each sample into the equation produced by the standard curve, are listed in Table 1. After the concentrations of hordenine in the extracts were determined, the concentrations of hordenine in the starting *S. tortuosum* plant products were calculated taking into account dilutions and reconstitutions (Table 1). The results showed that the highest concentration of hordenine detected was 1.151 mg/g in the Herb Stomp powder and the lowest (0.02893 mg/g) was in the Schmerbals Herbals stems. Because reports of the quantification of hordenine in *S. tortuosum* raw plant material are scarce, it remains unclear how typical the results observed here might be.

The results of this study can be used as a basis for determining and comparing hordenine levels not only in other *S. tortuosum* products, but also in other complex matrices that either contain hordenine naturally, or have been infused with *S. tortuosum*-derived molecules. For example, this approach could be readily applied to beer analysis to assess the presence of and quantify the amounts of hordenine which may be detected as a consequence of its presence in the barley malt from which it is made. Previous studies have shown hordenine levels in beer to range from 1.05–6.32 μg/ml,[24] which falls within the

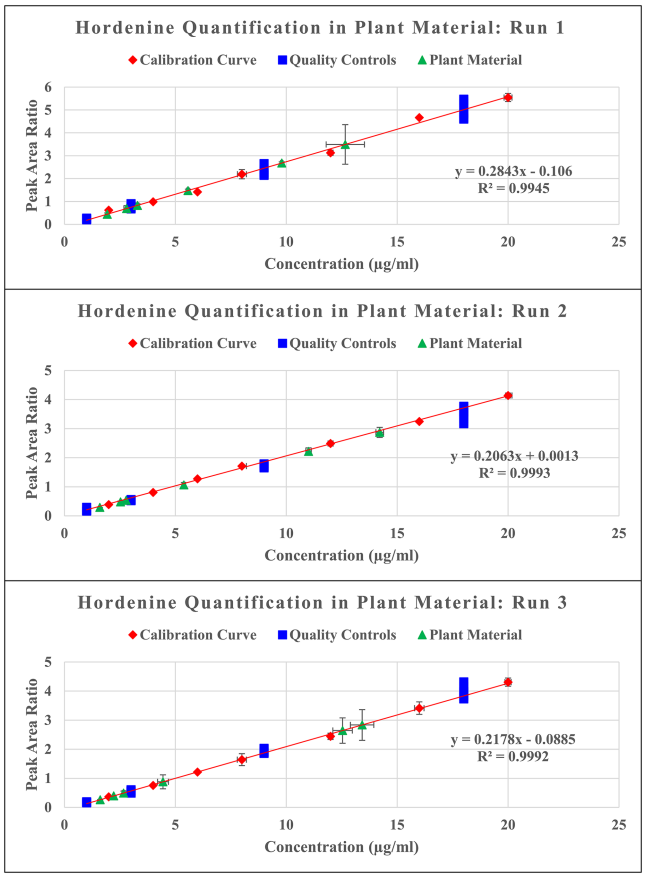

FIGURE 6 Calibration curves for the quantification of hordenine in *Sceletium tortuosum* material by direct analysis in real time–high-resolution mass spectrometry (DART-HRMS). The red diamonds represent calibration points, the blue squares represent the quality control points and the green triangles represent the average plant material points [Colour figure can be viewed at wileyonlinelibrary.com]

validated quantification range for the method developed in this study. Thus, the DART-HRMS approach, in comparison with conventional methods, provides a rapid and convenient means to not only confirm the authenticity of *S. tortuosum* (through detection of the range of *m/z* values corresponding to species-specific biomarkers such as the mesembrine alkaloids) but also for quantification of compounds such as hordenine. This method can be readily extended to other natural products of interest, particularly if deuterated internal standards are available.

4 | CONCLUSIONS

The rapid identification of *S. tortuosum* plant material and products derived from it was achieved through DART-HRMS analysis. The technique readily revealed the presence of hordenine and mesembrine alkaloid biomarkers based on the discriminating power of the high-resolution mass spectrometer used for the DART-MS analyses. This yielded molecular formula information which, along with isotope matching and the comparison of the CID spectra with those of authentic standards, enabled definitive detection and identification of hordenine. A new validated protocol that conformed to the US FDA Guidelines for Bioanalytical Method Validation was created using hordnine-d_6 as the internal standard, for the quantification of hordenine. The utility of the method was demonstrated through the quantification of hordenine in six different commercially available *S. tortuosum* products. The developed approach serves as an efficient and rapid means by which to detect and quantify hordenine and can be readily applied to the analysis of other hordenine-containing products besides *S. tortuosum* plant materials.

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ORCID

Rabi Ann Musah https://orcid.org/0000-0002-3135-4130

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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