9-1-2019

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Publication Information

Turner, Matthew W.; Rossi, Meagan; Campfield, Vannessa; French, John; Hunt, Ellie; Wade, Emily; and McDougal, Owen M. (2019). "Steroidal Alkaloid Variation in *Veratrum californicum* as Determined by Modern Methods of Analytical Analysis". *Fitoterapia, 137*, 104281-1 - 104281-7. http://dx.doi.org/10.1016/j.fitote.2019.104281

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Fitoterapia 137 (2019) 104281

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Steroidal alkaloid variation in *Veratrum californicum* as determined by modern methods of analytical analysis

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ARTICLE INFO

Keywords:
Steroidal alkaloid
Veratrum
Hedgehog pathway
Cyclopamine
Analytical methods

ABSTRACT

Veratrum californicum is a rich source of steroidal alkaloids, many of which have proven to be antagonists of the Hedgehog (Hh) signaling pathway that becomes aberrant in over twenty types of cancer. These alkaloids first became known in the 1950's due to their teratogenic properties, which resulted in newborn and fetal lambs developing cyclopia as a result of pregnant ewes consuming *Veratrum californicum.* It was discovered that the alkaloids in *V. californicum* were concentrated in the root and rhizome of the plant with much lower amounts of the most active alkaloid, cyclopamine, present in the aerial plant, especially in the late growth season. Inspired by the limitations in analytical instrumentation and methods available to researchers at the time of the original investigation, we have used state-of-the-art instrumentation and modern analytical methods to quantitate four steroidal alkaloids based on study parameters including plant part, harvest location, and growth stage. The results of the current inquiry detail differences in alkaloid composition based on the study parameters, provide a detailed assessment for alkaloids that have been characterized previously (cyclopamine, veratramine, muldamine and isorubijervine), and identify at least six alkaloids that have not been previously characterized. This study provides insight into optimal harvest time, plant growth stage, harvest location, and plant part required to isolate, yet to be characterized, alkaloids of interest for exploration as Hh pathway antagonists with desirable medicinal properties.

1. Introduction

Congenital malformations in lambs were observed in alarming frequency during the first half of the 20th century in south-western Idaho, and beginning in 1956 a detailed study of the malformed lamb problem was undertaken by researchers from the United States Department of Agriculture [1]. The affected lambs were commonly called “monkey-faced,” and were observed to have head deformities that included complete cyclopia, hydrocephalus, harelip, cleft palate, and displacement of the nose. Observations by ranchers with affected animals implicated mountain ranges with altitudes up to 10,000 ft containing alpine meadows used for grazing during the breeding season, typically in early August. Controlled breeding experiments determined the congenital anomaly was not due to genetic factors, and potential environmental elements including an excess or deficiency of nutritional factors, toxic mineral elements, or the presence of poisonous plants were assumed to underlie the observation. Field studies determined that deformities occurred in lambs from ewes that grazed during the latter part of the summer in wet seepage meadows above 6000 ft in elevation, locations where *Veratrum californicum* is commonly encountered [2]. Subsequent controlled feeding trials of fresh and dried green *V. californicum* reproduced the deformities observed in the field, and validated the teratogenic role of *V. californicum*) [3]. Following the confirmation that ingestion of *V. californicum* by pregnant ewes was responsible for the observed malformations, attention was turned toward the isolation and characterization of teratogenic compounds underlying the phenomenon of fetal malformation [4]. The teratogenic material was speculated to be steroidal alkaloids, either in the form of glycosidic derivatives or the parent alkamine. The compound responsible for the cyclopian malformations was found and given the trivial name cyclopamine [5]. Benzene extraction of *V. californicum* root and subsequent separation with paper chromatography revealed eight to ten unique alkaloids. In order to purify adequate quantities of alkaloids for additional chemical characterization, recrystallization of crude benzene extracts from acetone-water and methanol-water allowed for the enrichment of three alkaloids –veratramine, cyclopamine and an

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https://doi.org/10.1016/j.fitote.2019.104281
Received 29 June 2019; Received in revised form 26 July 2019; Accepted 27 July 2019
Available online 02 August 2019

unknown compound designated as alkaloid Q.

Feeding experiments and field observations suggested that significant variation was observed in the teratogenic effect of *V. californicum* on lambs that consumed the plant at differing growth sites and growing periods [6]. The content of cyclopamine as a function of plant part, stage of growth, and growth location was undertaken by Keeler and Binns circa 1970. In their study, benzene soluble steroidal alkaloids were isolated and total alkaloid concentrations were determined by measuring turbidity following treatment with Mayer reagent, and the concentrations of individual alkaloids were assigned based upon densitometry on photographed thin layer chromatography (TLC) plates. The TLC measurements separated three principal benzene soluble alkaloids: cyclopamine, veratramine, and alkaloid Q. The level of cyclopamine varied considerably between collection sites and stage of growth. Generally, it was determined that the concentration of cyclopamine was highest in the early growth season in the leaves, and in the late growing season in the root/rhizome. The structure of alkaloid Q was later elucidated, and was given the trivial name, muldamine [7].

Decades after the observation of the malformed lambs led to the isolation and characterization of cyclopamine, the teratogenic mechanism of cyclopamine was determined to be inhibition of the Hedgehog (Hh) signaling pathway [8]. The Hh signaling pathway is required for proper development of all animals containing bilateral symmetry and guides the formation of hands and feet, the central nervous system, and most epithelial tissues [9–12]. In mammals, the mechanism of the Hh signaling begins with the binding of one of three secreted ligands; Sonic hedgehog (Shh), Desert hedgehog (Dhh) or Indian hedgehog (Ihh) to the transmembrane transporter protein Patched (Ptch) [13]. Ptch, when not bound to one of these Hh ligands, inhibits the seven-transmembrane protein Smoothened (Smo), a G protein-coupled-like receptor. Upon binding with Hh ligands, this inhibition is inactivated, and a signaling cascade originating at Smo begins; the pathway culminates in the activation of the Gli family of zinc-finger transcription factors in vertebrates. The Gli transcription factors (particularly Gli-1 and Gli-2) up-regulate the transcription of Hh target genes, which enhance cellular proliferation and the epithelial-mesenchymal transition [14–17]. Although normal to development and several adult somatic processes, Hh signaling has been demonstrated to contribute to the pathogenesis of over twenty cancers, and is exceedingly active in basal cell carcinoma (BCC) (Chandler and McDougal, 2013) [18]. For this reason that considerable interest persists in the identification of novel Hh signal inhibitors, including those isolated from natural sources [19–22].

Few reports in the literature have used modern, highly sensitive analytical techniques to examine the full array of steroidal alkaloids in *V. californicum.* Our lab has surveyed and optimized steroidal alkaloid extraction conditions and employed state-of-the-art analytical instrumentation leading to identification of less abundant alkaloids present in *V. californicum* extracts [23]. We have also correlated extraction methods with teratogenic properties using Shh-Light II cell assays to determine how isolation of alkaloids using different chemical treatments alters the potency of cyclopamine [24]. Recently, our lab demonstrated that uncharacterized alkaloids present in *V. californicum* inhibit the Hh signaling pathway by comparing commercially available alkaloid standards to alkaloids extracted from plant specimens [25]. We first determined the concentration of four commercially available alkaloids −cyclopamine, veratramine, muldamine and isorubijervine− present in the ethanolic extract of *V. californicum*, replicated the concentration of those alkaloids observed in the crude extract in a cocktail from commercially available standards, and compared the inhibitory effect of crude extracts to the mixture of alkaloid standards. Using Shh Light II cells and a luminescence based assay, significant differences were observed for Hh pathway inhibition between the stem and root/rhizome extracts and their corresponding alkaloid standard mixtures, indicating that uncharacterized alkaloids present in plant extracts contribute to Hh signaling inhibition. In the current study, we quantify the amounts of four steroidal alkaloids −cyclopamine, veratramine, muldamine and isorubijervine− in *V. californicum* as a function of plant part, growth cycle and collection location. The molecular structure of the alkaloids quantified in this study are shown in Fig. 1. In addition to providing quantitative data for these four compounds, we provide a qualitative examination of the chemical diversity of alkaloids present in *V. californicum*. At least six alkaloids with unique molecular weight, molecular formula, and identity are reported, constituting potential drug targets for Hedgehog pathway inhibition.

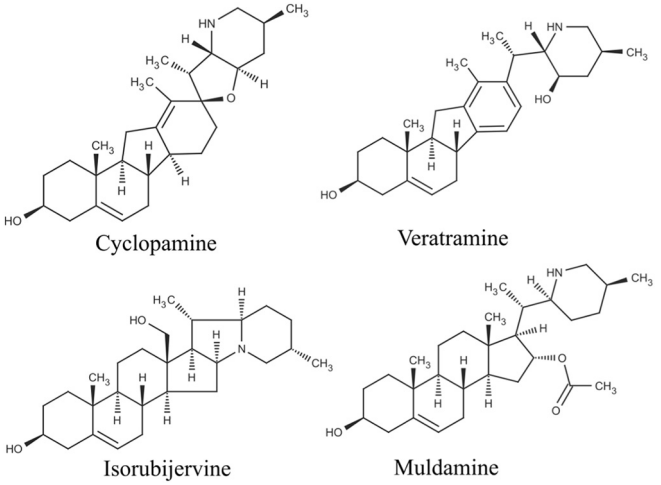

Fig. 1. Molecular structures of steroidal alkaloids cyclopamine, veratramine, isorubijervine and muldamine present in *V. californicum*.

2. Materials and methods

2.1. Materials

Plants were gathered from alpine meadows in the Boise National Forest in 2014 and stored in a freezer. All solvents were purchased from commercial sources and used without further purification. The solvents used were ethanol (95%), chloroform (HPLC grade), formic acid (MS grade), ammonium hydroxide (30% in water), and acetonitrile (HPLC grade), all obtained from Fischer Scientific (Hampton, NH, USA).

2.2. Plant collection

Plants were harvested from two stands of *V. californicum* near the Bogus Basin Mountain Resort, Boise National Forest, Idaho, USA. *V. californicum*, also known as California false hellebore, grows up to 2 m in height, has cornstalk-like stems and has large, broad elliptical leaves [26,27]. Plants were harvested at 6901 ft, elevation (N43 45.719" W 116 05.327″) beside the Shindig Trail, and at 7066 ft elevation (N43 45.858" W 116 05.090″) beside the Elk Meadows Trail. In each location, three to five full plants were harvested; the rhizome and aerial plant were separated, and transported on ice to the lab. Once in lab, plant parts were diced, followed by drying with a LabConco Freezone 4.5 freeze drying unit (Labconco Corporation, Kansas City, MO, USA), before being stored at −20 °C. Plants were harvested on May 23, 2014, July 3, 2014, and September 5, 2014 at both locations.

2.3. Biomass preparation

To prepare for extraction, the biomass was removed from the freezer, cut into even smaller pieces (1–2 cm cubes) and placed in a lyophilizer for 24–48 h to ensure dryness. Once dried the samples were submerged in liquid nitrogen, and crushed to a fine powder by mortar and pestle.

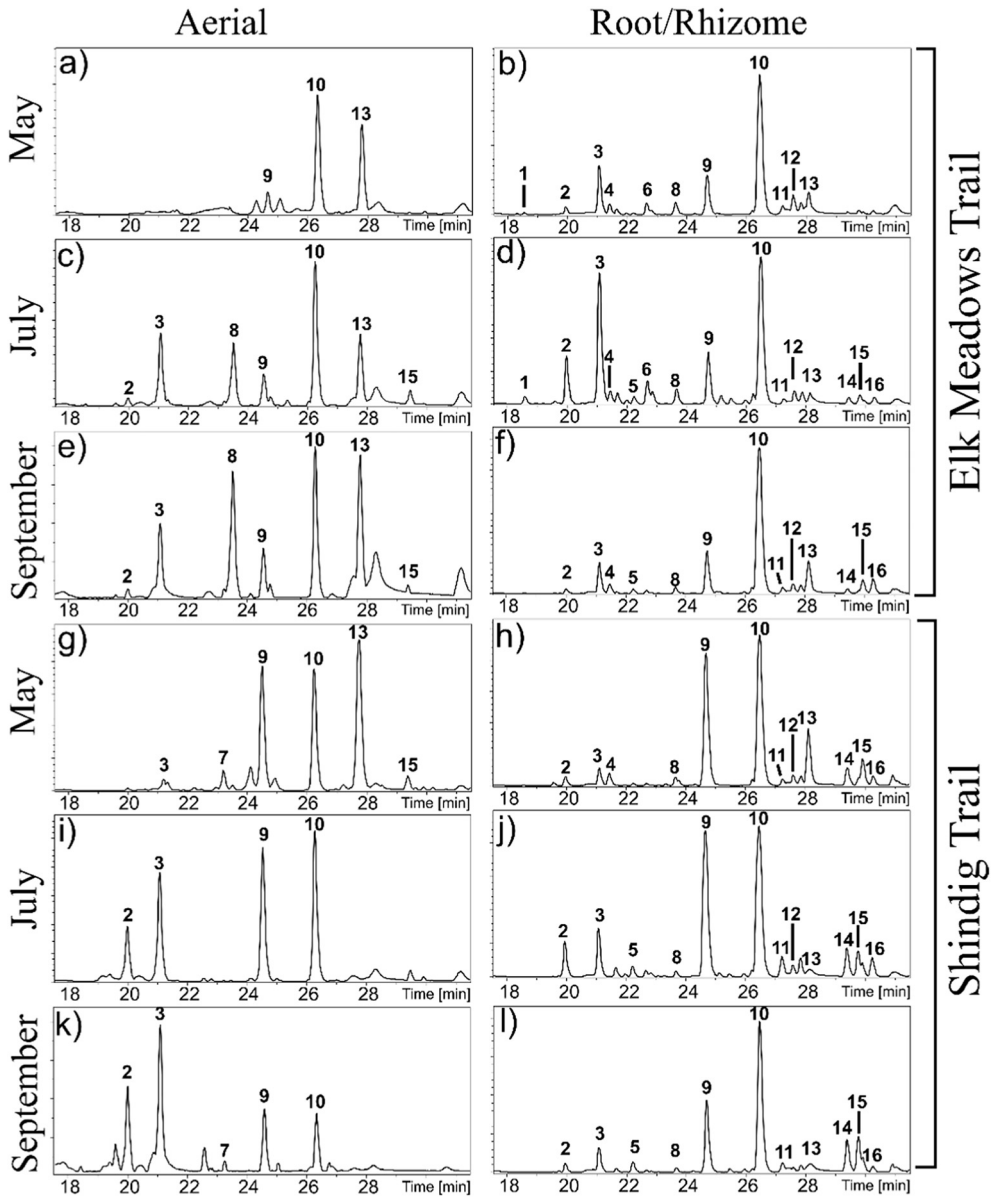

Fig. 2. Chromatograms of alkaloids extracted from the Elk Meadows (a-f) and Shindig (g-l) collection sites from aerial and roots/rhizomes of *V. californicum*. Common and unique alkaloids identified by MS are observed in each extract. Labeled peaks correspond to the data summarized in Table 1. The chromatograms displayed were generated as base peak chromatograms from data acquired on a Q-TOF mass spectrometer.

2.4. Alkaloid extraction

Approximately 2.0 g of powdered biomass was added to 100 mL of 95% ethanol and the mixture was placed in a sonicator for 30 min, followed by agitation for 24 h on a stir plate. The plant material was separated from the solvent by vacuum filtration (Whatman filter paper, 0.45 µm), and the solid material discarded. The ethanol was removed by rotary evaporation to yield the crude alkaloids. The alkaloids were dissolved in 10 mL of 95% ethanol, and the solution was warmed to 40 °C and sonicated to achieve complete dissolution. Addition of 35% aqueous ammonia achieved alkaline solvent conditions (pH ≥ 10). The alkaline solution was added directly to a supported liquid extraction (SLE) column (ChemElut, Agilent, Santa Clara, CA, USA) and allowed to adsorb for 10 min, followed by elution of alkaloids with chloroform (3 × 10 mL) using a vacuum manifold set to a pressure of 2 mbar. The chloroform fractions were combined, filtered, and evaporated to dryness. All samples were dissolved in 1 mL of 100% ethanol as a mixture of alkaloids.

2.5. Alkaloid quantification

The concentrations of cyclopamine, veratramine, isorubijervine and muldamine in alkaloid extracts were determined using an UltiMate 3000 HPLC (Thermo Scientific, Waltham, MA, USA) equipped with a Corona Veo RS charged aerosol detector (CAD) and MSQ Plus mass spectrometer (MS). HPLC separation of alkaloids was achieved using a Thermo Acclaim 120 C_{18} column (2.1 × 150 mm, 3 µm), and mobile phases consisting of 0.1% formic acid (*v*/v) in water (Buffer A) and 0.1% formic acid (v/v) in acetonitrile (Buffer B) with a flow rate of 0.3 mL/min. A linear gradient method beginning at 95% Buffer A and 5% Buffer B, up to 60% Buffer B over a 25 min run time achieved desired separation of alkaloids from the extracts. Cyclopamine (Alfa Aesar, Ward Hill, MA, USA, > 99% purity), veratramine (Abcam Biotechnology Company, Cambridge, UK, > 98% purity), isorubijervine (Logan Natural Products, Plano, TX, USA, 99% purity) and muldamine (Logan Natural Products, Plano, TX, USA, 99% purity) standards were used to create a calibration curve at concentrations of

0.1, 0.5, 1.0, 5.0 and 10.0 mM with detection recorded by CAD with the power function set to pA 1.70. Calibration curves were generated in triplicate for each alkaloid at each of the five alkaloid concentrations. The quantities of these alkaloids were determined from the alkaloid mixtures obtained from the aerial and root/rhizome extracts in triplicate. Quantification was achieved using an intra-lab validated HPLC method. Limits of detection and limits of quantification were determined from the slope and the standard deviation observed from linear calibration curves.

2.6. Alkaloid identification

To identify the steroidal alkaloids in *V. californicum* aerial and root/rhizome extracts, samples were analyzed by HPLC-MS, where the mass spectrometer was an ultra-high resolution Quadrupole Time of Flight (QTOF) instrument (Bruker maXis, Billerica, MA, USA). The electrospray ionization (ESI) source was operated under the following conditions: positive ion mode, 1.2 bar nebulizer pressure, 8 L/min flow of N_2 drying gas heated to a temperature of 200 °C, 3000 V to −500 V voltage between HV capillary and HV end-plate offset, mass range set from 80 to 800 *m/z*, and the quadrupole ion energy at 4.0 eV. Sodium formate was used to calibrate the system in this mass range. HPLC separation was achieved using a XTerra MS C_{18} column, 3.5 µm, 2.1 × 150 mm (Waters, Milford, MA, USA). The flow rate was 250 µL/min. The mobile phases were 5% acetonitrile and 0.1% formic acid in water (Buffer A) and acetonitrile and 0.1% formic acid (Buffer B). The linear gradient method was used to separate analytes starting at 5% Buffer B and increasing to 60% Buffer B over 25 min. A 1 µL sample injection volume was used. Data were analyzed with the Compass Data Analysis software package (Bruker Corporation).

3. Results

Qualitative variation was observed in the alkaloid composition of *V. californicum* by plant part, growth stage, and harvest location, as shown in the alkaloid profiles of the extracts in Fig. 2 a-l. Identification of each.

alkaloid peak was achieved by high resolution mass spectrometry and verified by elution time compared to commercially available standards. Data for the peaks labeled in Fig. 2 a–l, including retention time, *m/z*, molecular formula (MF) and alkaloid identity are summarized in Table 1. Mass spectra for peaks 1–16 are presented in Fig. 4, showing the retention time and m/z used to estimate molecular formulas provided in Table 1. Fig. 3 shows quantitative determination of the four common *Veratrum* alkaloids cyclopamine, veratramine, isorubijervine, and muldamine for which commercially available standards were available to obtain calibration curves and accurately determine alkaloid quantity. Generally, significantly higher alkaloid concentrations and alkaloid diversity are observed in the root/rhizomes in both locations as compared to the aerial plant. Alkaloid concentration is given as mg alkaloid per g of biomass used for extraction, and the standard deviation for triplicate sampling is shown. During each collection a minimum of three plant samples were collected and the standard deviation primarily reflects variation in alkaloid concentrations observed between individual plants, and to a lesser extent technical variation in extraction efficiency. The limit of quantification was determined 0.011, 0.011, 0.010 and 0.015 mg/g for cyclopamine, veratramine, muldamine and isorubijervine, respectively; and the limit of detection was determined to be 0.003, 0.003, 0.003 and 0.005 mg/g for cyclopamine, veratramine, muldamine and isorubijervine, respectively.

Table 1
Summary data of the corresponding to the peaks identified in Fig. 2.

Peak	Retention Time (min)	m/z	Estimated Molecular Formula	Alkaloid
1	18.6	576.396	$C_{33}H_{53}NO_7$	N/A*
2	20.0	572.365	$C_{33}H_{49}NO_7$	Veratrosine
3	21.1	574.381	$C_{33}H_{51}NO_7$	Cycloposine
4	21.5	414.342	$C_{27}H_{43}NO_2$	Etioline?
5	22.3	430.337	$C_{27}H_{43}NO_3$	Tetrahydrojervine?
6	22.7	574.381	$C_{33}H_{51}NO_7$	N/A
7	23.2	428.320	$C_{33}H_{53}NO_7$	Dihydrojervine?
8	23.6	576.397	$C_{33}H_{53}NO_7$	N/A
9	24.7	410.312	$C_{27}H_{39}NO_2$	Veratramine
10	26.5	412.326	$C_{27}H_{41}NO_2$	Cyclopamine
11	27.2	410.311	$C_{27}H_{39}NO_2$	N/A
12	27.6	412.326	$C_{27}H_{41}NO_2$	N/A
13	28.1	414.343	$C_{27}H_{43}NO_2$	Isorubijervine
14	29.4	416.357	$C_{27}H_{45}NO_2$	22-keto-26-aminocholesterol?
15	29.8	458.370	$C_{29}H_{47}NO_3$	Muldamine
16	30.2	398.347	$C_{27}H_{43}NO$	Verazine?

* N/A is used to designate alkaloids with identity not available.

3.1 Root/rhizome alkaloid variation. Samples collected from the Elk Meadows site showed the highest concentration of cyclopamine in the root/rhizome collected in May, with 8.69 ± 1.17 mg/g extracted; whereas the July and September collections yielded 4.14 ± 0.50 mg/g and 5.01 ± 0.50 mg/g, respectively. Interestingly, collections from the Shindig site showed the lowest rhizome concentration of cyclopamine in May at 5.20 ± 1.02 mg/g, with significantly higher concentrations observed in July and September, at 11.01 ± 1.02 mg/g and 9.39 ± 1.85 mg/g, respectively. From the Shindig location, a fluctuation in rhizome alkaloid concentration was observed for veratramine similar to that of cyclopamine, with the highest concentration observed in July at 15.35 ± 2.86 mg/g, and 6.16 ± 1.03 mg/g and 5.57 ± 2.60 mg/g for May and September, respectively. Strangely, the concentration of veratramine was much lower throughout the growth season in the samples collected from Elk Meadows, with the greatest concentration observed in July at 1.98 ± 0.20 mg/g, and 1.06 ± 0.48 mg/g 1.63 ± 0.16 mg/g observed for May and September, respectively.

From both harvest locations, muldamine and isorubijervine were less abundant in the root/rhizome than either cyclopamine or veratramine. From Elk Meadows, isorubijervine was detected above the quantification limit from each sampling period, with the greatest abundance of 1.12 ± 0.12 mg/g in September, and the lowest abundance in May at 0.44 ± 0.19 mg/g. However, from the Shindig location, only the May extract yielded a quantifiable amount of isorubijervine, measured at 0.91 ± 0.39 mg/g. However, qualitative detection of isorubijervine was observed below quantification limit from the Shindig site for both the July and September collection times. From both locations, muldamine was only present above the limit of quantification in the July and September extracts. From Elk Meadows, muldamine was not detected in the May sample, and was determined to be 0.21 ± 0.09 and 0.10 ± 0.08 mg/g for the July and September samples, respectively. The high standard deviation relative to the concentrations determined reflects that observed concentrations varied significantly between plant extracts. For the July extracts, muldamine was only detected above the limit of quantification in two of the three samples analyzed, and only one of three for the September extracts. From Shindig, muldamine was detected in all the extracts, although it was below the limit of detection for May, and July and September yielded 0.48 ± 0.20 mg/g and 0.48 ± 0.42 mg/g, respectively. Again, concentrations varied considerably between individual plants, with muldamine only detected above the limit of quantification in two of the three samples analyzed for July and one of three for September.

3.2 Aerial plant alkaloid variation. In the aerial plant at both harvest locations, the concentration of each alkaloid quantified was highest in May, and was observed to decrease progressively throughout the growth season. For the Elk Meadow location, concentrations of cyclopamine varied from 0.28 ± 0.10 mg/g in May to 0.03 ± 0.00 mg/g in September, while at the Shindig site concentrations varied from

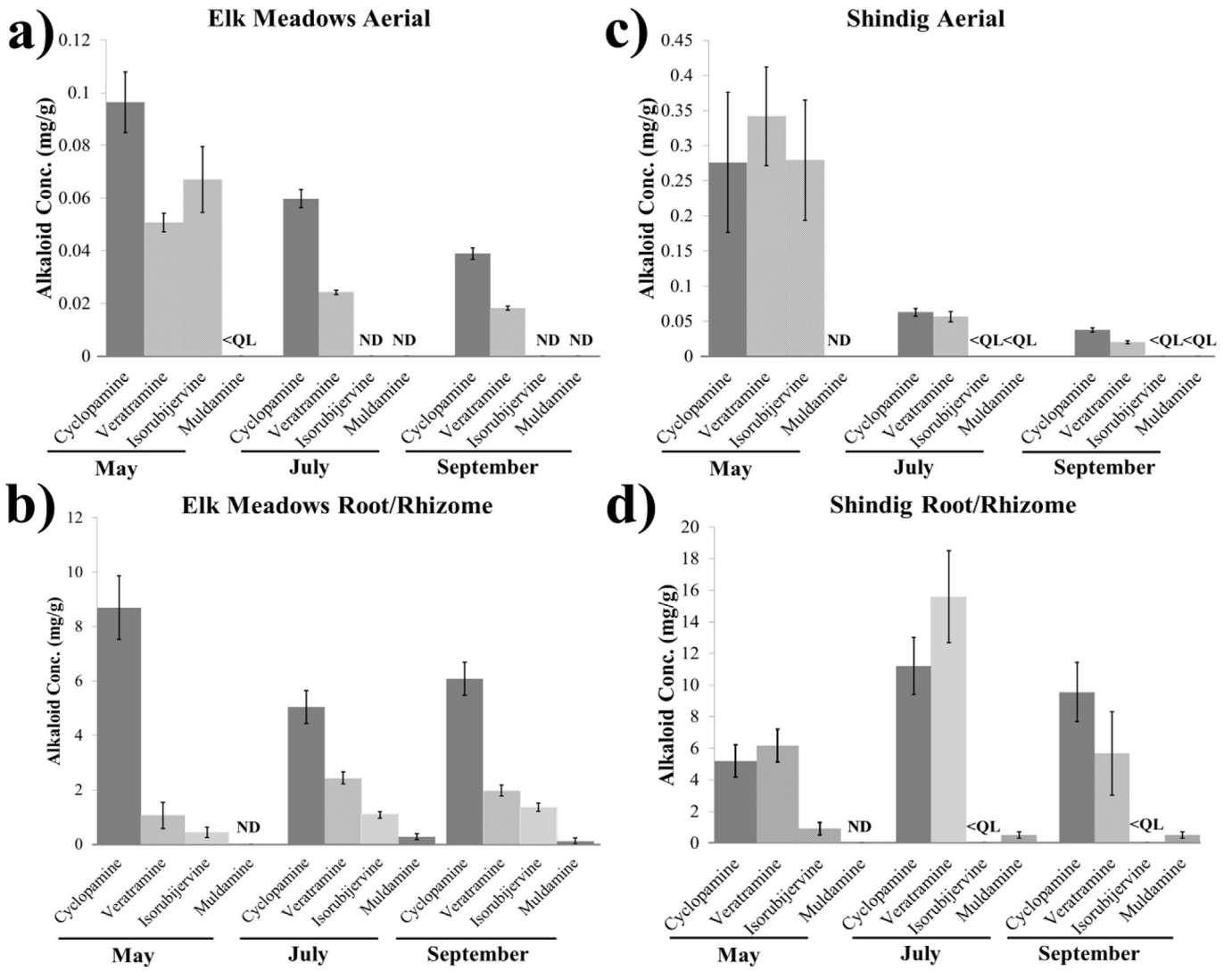

Fig. 3. Quantification of alkaloids from both harvest sites for aerial (a and c) and below ground (b and d) *V. californicum*. Alkaloid concentration is presented as mg alkaloid per g of plant biomass extracted. Significantly higher concentrations were observed in the below ground plant as compared to the aerial plant. Quantification data was generated using charged aerosol detection and calibration curves created from commercially available alkaloid standards.

0.10 ± 0.01 mg/g in May to 0.04 ± 0.00 mg/g in September. Veratramine concentrations at the Elk Meadows plot varied between 0.34 ± 0.07 mg/g in May and 0.02 ± 00 mg/g in September, and for the Shindig site, veratramine levels were determined to be 0.05 ± 0.00 in May and 0.02 ± 0.00 mg/g in September. Muldamine was either not detected or below the quantification limit in the aerial plant from both locations and during each collection period. Relatively high levels of isorubijervine were detected in the aerial plant in May at both sites, and were determined to be 0.28 ± 0.09 and 0.07 ± 0.01 mg/g for Elk Meadows and Shindig, respectively. These levels were not sustained through the growth season, as all other measurements were below the limit of quantification, or were not detected.

4. Discussion

The current investigation sought to explore the diversity of alkaloids from *V. californicum* by plant part, location, and growth stage. In order to provide a detailed analysis of the alkaloid composition of *V. californicum* based on plant part, a quantitative comparison of four alkaloids was performed for –cyclopamine, veratramine, isorubijervine and muldamine– present in the aerial portion and root/rhizome of the plant. The generation of Figs. 2 and 3, and the data presented in Table 1, consisted of analysis of 36 separate *Veratrum* samples. For each data point, three independent plant samples were harvested, solvent extracted, and analyzed by HPLC. The harvest sites were within 200 ft. elevation difference, and both locations were wet, mucky, and naturally spring fed. Elk Meadows faces west, while Shindig faces east, so sun exposure is different between the two harvest sites.

The magnitude of cyclopamine accumulated in *V. californicum* tissues reported in this study is consistent with previous recent analyses [24,28]. The observation by Augustin et al. that cyclopamine accumulates in the rhizome of the plant during the growth season is consistent with our analysis for the below ground plant at the Shindig location, in which May collections contained 5.20 ± 1.02 mg/g, and September collection yielded 9.39 ± 1.85 mg/g, an increase of ~81% over the growth season. Augustin et al. speculated that this increase, along with higher expression of biosynthetic genes in the below ground plant, indicates that cyclopamine biosynthesis occurs in the underground organs of the plant. However, from the Elk Meadows location, we observed a modest decrease in cyclopamine concentration over the growth season, with the highest value found in May of 8.69 ± 1.17 mg/g, decreasing to 5.01 ± 0.50 mg/g in September, a decrease of ~42%. The reason for the discrepancy in cyclopamine accumulation is unclear. Modest fluctuations were observed in below

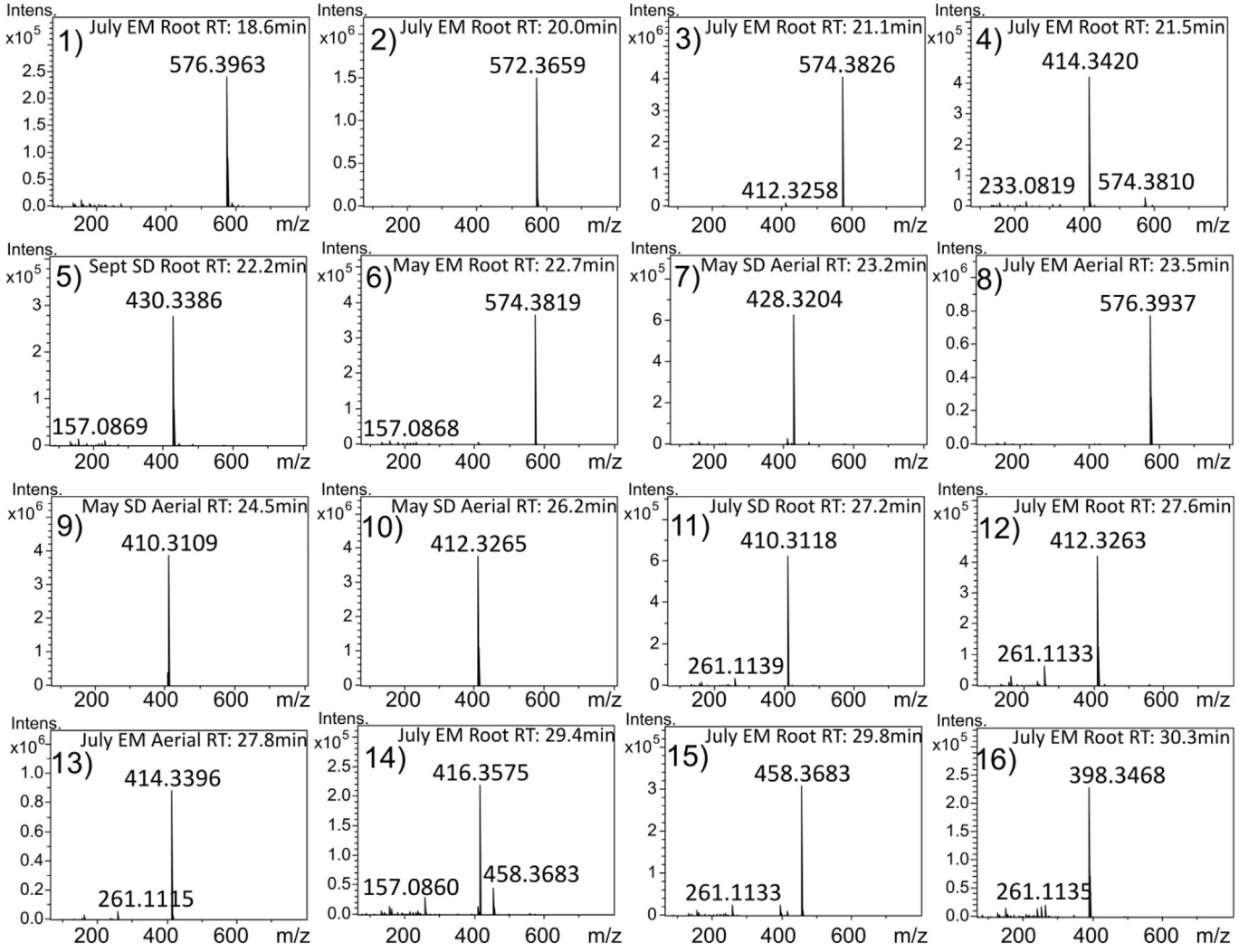

Fig. 4. Mass spectra from the peaks identified from Fig. 2 used to generate the data in Table 1. Included is the retention time (RT) and sample from which the MS data is taken. MS data was acquired using an ultra-high resolution Q-TOF mass spectrometer with mass error < 5 ppm.

ground abundance of the other alkaloids analyzed as well. From the Elk Meadows location, veratramine increased over the course of the growth season from a low in May, the highest level observed in July, and then a modest decrease in September. Similarly from the Shindig location the greatest abundance of veratramine was observed in July. However, the amount of veratramine at this time point was 7.8× greater from the Shindig location compared to the Elk Meadows location, with values of 15.35 ± 2.86 mg/g and 1.98 ± 0.20 mg/g observed from each location, respectively. From the Elk Meadows location, the content of isorubijervine increased slightly over the growth season but remained relatively low, whereas from the Shindig location isorubijervine was only quantifiable from the May collection. Muldamine remained relatively low in abundance from both locations, below or near the quantification and detection limit. In summary, alkaloid abundance and the fluctuations in alkaloid content varied considerably between collection sites. This is consistent with the findings of Keeler and Binns in their original evaluation of alkaloid content by growth stage, and is consistent with the variable teratogenic effect observed in controlled feeding trials [6].

Consistent with prior analysis of *Veratrum* alkaloid content sampled throughout the growth season, the bar graphs detailed in Fig. 3 show that aerial alkaloid quantities decrease from early season through late growth phase for the plants [6,28]. This was consistent for both locations and for all of the alkaloids quantified. Plants harvested from Elk Meadows retained ~6% of their original alkaloid content from May to September, while the Shindig aerial plants retained closer to ~27% alkaloid content. However, it is worth noting that while the quantities of cyclopamine and veratramine decreased throughout the growth season, the amount of their glycosylated derivatives –cycloposine and veratrosine– increased throughout the growth season. This suggests conversion to the glycosylated derivatives occurs during the growth season.

Qualitative differences in the alkaloid diversity are observed by plant part. The diversity of alkaloids present in the aerial plant is significantly lower than the alkaloid diversity of the root and rhizome for the same plants. As is shown in Fig. 2 and Table 1, mass spectrometry analysis identified 16 molecular ion peaks that yielded estimated molecular formulas consistent with steroidal alkaloids. Using high resolution mass spectrometry and verified by elution time compared to commercially available standards, Peaks 9, 10, 13, and 15 were definitively identified as veratramine, cyclopamine, isorubijervine, and muldamine, respectively (Fig. 2). Peaks 2 and 3 were identified as veratrosine and cycloposine based on the comparison of the accurate and exact mass for these compounds, and the high degree of mass accuracy of our Q-TOF system (mass error < 5 ppm). Peak 16, with *m/z* of 398.347 and predicted molecular formula of $C_{27}H_{43}NO$ is likely verazine, an important intermediate in the cyclopamine biosynthetic pathway [28]. Similarly, Peak 4, with m/z of 414.342 and predicted

molecular formula of $C_{27}H_{43}NO_2$ is suspected to be etioline, which is an intermediary in the biosynthetic pathway of cyclopamine, and its presence in the extract is expected [18]. Peak 15, with an *m/z* of 416.357 and an estimated molecular formula of $C_{27}H_{45}NO_2$ may be the cyclopamine biosynthetic intermediate 22-keto-26-aminocholesterol; definitive determination of this suspected assignment will require additional investigation [28]. Peaks 1 and 8 had the same *m/z* of 576.396, and estimated molecular formula, $C_{33}H_{53}NO_7$, indicating they may be isomers of one another due to the significantly difference in retention time observed by chromatographic separation. This molecular formula and variation in chromatographic retention time are consistent with glycosylated isorubijervine and etioline. Peaks 5 and 7 have m/z of 430.337 and 428.320, and estimated molecular formulas of $C_{27}H_{43}NO_3$ and $C_{27}H_{41}NO_3$. These two alkaloids are consistent with tetrahydrojervine and dihydrojervine, respectively, both of which have been reported in a prior [29]. Additionally, potential isomers of both veratramine and cyclopamine were observed as Peaks 11 and 12, respectively, with identical m/z and estimate molecular formulas, but distinct retention times for each. These compounds may be of significant biological interest, and the isolation and structural characterization of each is currently underway in our laboratory.

5. Conclusion

Veratrum californicum is a rich source of steroidal alkaloids including inhibitors of the Hedgehog signaling pathway. Although the two collection sites used in this study were relatively close together and the elevation difference between the two was not significant, quantitative variation in alkaloid amount and qualitative variation in alkaloid diversity were observed between the two sites. Additionally, considerable variation was observed between individual plants, as indicated by the relatively high standard deviation observed between triplicate biological replicates. This study provides insight into optimal harvest time, plant growth stage, harvest location, and plant part required to isolate, yet to be characterized, alkaloids of interest for exploration as Hh pathway antagonists with desirable medicinal properties.

Acknowledgments

We acknowledge financial support for Matthew Turner provided by Institutional Development Awards (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under Grants #P20GM103408 (INBRE) and P20GM109095 (COBRE in Matrix Biology). We also acknowledge support from the Biomolecular Research Center at Boise State University with funding from the National Science Foundation, Grants # 0619793 and #0923535; the MJ Murdock Charitable Trust, Idaho State Board of Education, and Research Corporation. Contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH. We wish to thank Dr. Jim Smith, curator for the Snake River Plains herbarium for his expert advice regarding *V. californicum* sampling and identification in the Boise National Forest.

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