Proﬁling of Dehydropyrrolizidine Alkaloids and their N-Oxides in Herbarium-Preserved Specimens of *Amsinckia* Species Using HPLC-esi(+)MS

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**ABSTRACT:** Species of the *Amsinckia* genus (Boraginaceae) are known to produce potentially hepato-, pneumo-, and/or genotoxic dehydropyrrolizidine alkaloids. However, the taxonomic differentiation of *Amsinckia* species can be very subtle and there seems to be marked differences in toxicity toward grazing livestock. Methanol extracts of mass-limited leaf samples from herbarium specimens (collected from 1899 to 2013) of 10 *Amsinckia* species and one variety were analyzed using HPLC-esi(+)-MS and MS/MS for the presence of potentially toxic dehydropyrrolizidine alkaloids and/or their N-oxides. Dehydropyrrolizidine alkaloids were detected in all specimens examined ranging from about 1 to 4000 μg/g of plant. Usually occurring mainly as their N-oxides, the predominant alkaloids were the epimeric lycopsamine and intermedine. Also sometimes observed in higher concentrations were the 3′- and 7-acetyl derivatives of lycopsamine/intermedine and their N-oxides. Within a designated species, an inconsistent proﬁle was often observed that may be due to natural variation, taxonomic misassignment, or nonuniform degradation due to plant collection and storage differences.

**KEYWORDS:** *Amsinckia*, herbarium samples, dehydropyrrolizidine alkaloids, HPLC-esiMS, MS/MS, lycopsamine, intermedine

**INTRODUCTION**

Plants that produce esters of dehydropyrrolizidine alkaloids and their N-oxides are well-known to have the potential to cause livestock poisonings, either via natural grazing or via livestock feed contaminated with such plants. They are also known to poison humans via contaminated diet (e.g., grains), food that naturally contains, or is naturally contaminated with, the alkaloids (e.g., some honeys), herbal medicines, or dietary supplements (e.g., some pollens). The *Amsinckia* genus (Boraginaceae), known as fiddlenoaks, comprises many species known to produce the potentially hepato-, pneumo-, and genotoxic dehydropyrrolizidine alkaloids. These include, for example, the epimeric monoesters lycopsamine, intermedine, and a methylated analogue sincamidine, and the open chain diester echiumine, which are isolated from Australian and Californian collections of *Amsinckia intermedia*. On the basis of gravimetric comparison of reduced and nonreduced plant extracts, the alkaloids were present mainly as their free bases at concentrations that ranged from 0.2 to 0.7% dry weight (dw). A GC-MS investigation of samples from 12 *Amsinckia* species collected in California showed that most were dominated by 1 or 2. While many showed a signiﬁcant presence of the C7 analogue of lycopsamine, an epimeric dehydropyrrolizidine alkaloid did not reveal any epimeric dehydropyrrolizidine alkaloid despite it being found in every sample of *A. tessellata* examined by Kelley and Seiber. This may be one instance of incorrect identification of an *Amsinckia* sp. and, along with, for example, the differentiation of *Amsinckia menziesii* and *A. intermedia*, may reﬂect an intrinsic difﬁculty in the morphologic taxonomy of the *Amsinckia* genus. Rosman differentiated *A. menziesii* from *A. intermedia*, showing that the former contained C7 and C2 mono- and diacetyl derivatives of C7 and C2. However, Kelley and Seiber, in their GC-MS investigation, equated *A. intermedia* to *A. menziesii var. intermedia*, which reﬂects the synonymous relationship recorded in CalFlora and the USDA PLANTS Database. A range of 0–0.38% of unspeciﬁed dehydropyrrolizidine alkaloids was reported for reduced extracts of *A. intermedia* collected in central Washington over a three month period, with no or only trace amounts of dehydropyrrolizidine alkaloids detected in the seeds. A recent HPLC-esi(+)MS-based investigation revealed a lycopsamine, lycopersamine-N-oxide chemotype (ca. 0.76%, dw) of *Amsinckia intermedia*, potentially associated with an intoxication of cattle in Arizona. Several other dehydropyrrolizidine alkaloids, tentatively identiﬁed on the basis of HPLC retention times and MS and MS/MS data, were present at much lower levels, i.e., acetyllycopsamine-N-oxide, echiumine-N-oxide (7NO), acetylechiumine-N-oxide, two putative dihydro analogues of lycopsamine-N-oxide, etc.
A continued concern about taxonomic differentiation based on subtle morphologic features, combined with the HPLC-esi(+)MS capacity to directly detect and quantify both the free base/N-oxide ratios, a correlation of the alkaloid profiles with the species, and a determination of the potential usefulness of herbarium-preserved specimens with respect to detection of dehydropyrrolizidine alkaloids and their N-oxides.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Methanol was reagent ACS/USP/NF grade (Pharmaco Products; Brookfield, CT). Acetonitrile was the HPLC-certified solvent (Honeywell Burdick and Jackson; Muskegon, MI), and water was Milli-Q-purified (18.2 MΩ/cm) (Millipore; USA). Formic acid, was “For Analysis” grade (>99%) (Acros Organics/Thermo Fisher Scientific; NJ). Lycopsamine, 1, and intermedine, 2, and their N-oxides (Figure 1) and lasiocarpine (all >99% pure based on HPLC-esi(+)MS and NMR analysis) were sourced from the stocks of extracted and purified pyrrolizidine alkaloids kept by the USDA/ARS Poisonous Plant Research Laboratory.

**Plant Specimens.** One or two leaves from geographically- and/or temporally differentiated specimens of annotated *Amsinckia douglasiana*, *A. tessellata*, *Amsinckia retorsa*, *A. monziesii*, *Amsinckia lycopoides*, *Amsinckia eastwoodiae*, and *A. intermedia* were harvested from the Stanley L. Welsh Herbarium, Brigham Young University. Additionally, leaf samples of similarly differentiated species of *A. eastwoodiae*, *A. douglasiana*, *A. lunaris*, and *A. vernicosa* were obtained from the Botany Herbarium and the Agronomy Herbarium of the University of California, Davis. Samples of specimens of *A. monziesii* var. *intermedia*, *A. intermedia* and *A. retorsa* were harvested from the USDA/ARS Poisonous Plant Research Laboratory Herbarium. Finally, five samples each of *A. eastwoodiae*, *Amsinckia lunaris*, and *Amsinckia vernicosa*, and four samples each of *Amsinckia grandiflora* and *A. douglasiana* were supplied by the Jepson Herbarium at the University of California, Berkeley (Table 1).

**Sample Preparation and Extraction.** The entire sample for each specimen was transferred to a weighed microcentrifuge tube (2 mL Graduated Free Standing (Fisherbrand, Pittsburgh, PA) to which was added a 4.5 mm Copperhead copper-coated steel pellet (Crosman Corporation, Bloomfield, NY). The capped tubes were then shaken using a MM301 Retsch shaker (Retsch Inc., Newtown, PA) for 5 min at 17 cps. The grinding pellet was carefully removed from the centrifuge tube that was then recapped and weighed to afford the residual plant weight (ca. 7–70 mg). Methanol (0.5 mL) was added to each tube and the powdered plant gently extracted by inversion mixing at room temperature for 16 h. After centrifugation (15000×g, 5 min), analytical samples were prepared by adding a 10 μL aliquot of the supernatant to 90 μL of a solution of 0.1% formic acid/methanol (1:1, v/v) containing lasiocarpine (ca. 10 μg/mL) as an internal standard. In some cases, a more concentrated sample was prepared by dilution of 50 μL of the supernatant with 50 μL of the formic acid/methanol solution.

**HPLC-esi(+)MS and MS/MS Analysis.** Analytical samples (5 μL) were injected using a model 1260 Infinity HPLC system (Agilent Technologies, CA) onto a 150 mm × 2 mm i.d., 4 μm, Synergi Hydro RP column (Phenomenex, Torrence, CA), fitted with a 2 mm × 4 mm i.d. AC C18 guard column (Security Guard cartridge system, Phenomenex, Torrence, CA). A gradient flow (400 μL/min) of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) was used to elute sample components from the column. Mobile phase B was held at 3% for 2 min before linearly increasing to 70% by 10 min. After holding at 70% for another 5 min, the column was re-equilibrated to 3% mobile phase B over 2 min and held for a further 7 min before the next injection. Eluate from the column was monitored using a Velos Pro LTQ mass spectrometer (Thermo Scientific, USA) in a two-scan, positive ion mode and equipped with a heated electrospray ionization (HESI)
| Table 1. Specimens of *Amsinckia* Species Harvested from Various Herbaria |
|---------------------------------------------------------------|
| **Amsinckia** | **collection** | **herbarium and voucher no.”** |
| species   | state   | county | date       |                                     |
| A. douglasiana | 1 CA    | Monterey | April 15, 1964 | UCB61045                             |
|           | 2 CA    | San Luis Obispo | March 22, 2011 | UCB8147                             |
|           | 3 CA    | Santa Clara | April 8, 1938 | UCB2007                              |
|           | 4 CA    | Monterey | April 9, 1938 | UCB2094                              |
|           | 5 CA    | San Luis Obispo | April 10, 1938 | UCB2088                              |
|           | 6 CA    | Merced | March 16, 1941 | AHUC 038259                          |
|           | 7 CA    | San Luis Obispo | March 26, 1962 | DAV 32015                            |
|           | 8 CA    | Fresno | March 9, 1952 | AHUC 038496                          |
|           | 9 CA    | Tulare | February 3, 1952 | AHUC 038426                        |
|           | 10 CA   | Monterey | May 12, 1970 | UCB7442                              |
|           | 11 CA   | Monterey | April 2, 1938 | UCB2970                              |
|           | 12 CA   | San Bernito | April 1, 1932 | UCB16,142                           |
| A. tesselata | 1 UT    | Washington | April 10, 2010 | BYU28877                            |
|           | 2 UT    | Washington | March 21, 1986 | BYU22112                             |
|           | 3 UT    | Washington | April 13, 2001 | BYU1698                              |
|           | 4 UT    | Washington | April 5, 1978 | BYU357                               |
|           | 5 UT    | Washington | April 24, 1988 | BYU23962                             |
| A. retrorsa | 1 UT    | Cache | June 27, 2005 | BYU21095                             |
|           | 2 UT    | Box Elder | June 24, 1989 | BYU6704                              |
|           | 3 UT    | Wasatch | July 19, 1983 | BYU13946                             |
|           | 4 ND    | Humboldt | June 5, 2008 | BYU15565                             |
|           | 5 WA    | Benton | April 23, 2006 | BYU49                                |
|           | 6 WA    | Adams | June 21, 2002 | PPRL2778                             |
|           | 7 leaves | WA          | June 21, 2002 | PPRL1999                             |
|           | 8 flowers | WA          | June 21, 2002 | PPRL1999                             |
| A. menziesii | 1 UT    | Washington | April 12, 1932 | BYUxxxx                              |
|           | 2 UT    | Washington | May 26, 1978 | BYU11938                             |
|           | 3 UT    | Tulelake | May 13, 1971 | BYU4097                              |
|           | 4 UT    | Rich | June 13, 1981 | BYU1272                              |
|           | 5 UT    | Rich | July 3, 1983 | BYU13877                             |
| A. lycopsoides | 1 Canada | Quebec | August 26, 1967 | BYU26446                            |
|           | 2 WA    | Benton | June 13, 1984 | BYU1086                              |
|           | 3 WA    | Klikitat | May 1, 1989 | BYU3808                              |
|           | 4 WY    | Albany | August 11, 1946 | BYU4090                            |
|           | 5 WA    | Benton | April 28, 1984 | BYU527                               |
|           | 6 WA    | Lincoln | June 11, 1958 | BYU2051                              |
| A. eastwoodiae | 1 CA    | Kern | April 5, 1953 | UCB588                               |
|           | 2 CA    | Kern | March 21, 1996 | UCB19161                             |
|           | 3 CA    | Kern | March 21, 1996 | UCB19165                             |
|           | 4 CA    | Tulare | April 14, 1938 | UCB2104                              |
|           | 5 CA    | Stanislaus | March 27, 1986 | BC31117                             |
|           | 6 CA    | Calaveras | May 9, 1967 | AHUC 33811                           |
|           | 7 CA    | Fresno | March 20, 2002 | DAV 151803                           |
|           | 8 CA    | Kern | April 21, 1965 | DAV 37826                            |
|           | 9 CA    | Kern | April 9, 1927 | UCB11616                            |
|           | 10 CA   | Fresno | March 28, 1939 | UCB15316                            |
|           | 11 CA   | Tulare | February 29, 2013 | UCB7316                        |
|           | 12 CA   | Kern | April 7 1941 | UCB286                               |
|           | 13 CA   | Contra Costa | March 27 1957 | UCB5765                             |

| Table 1. continued |
|---------------------------------------------------------------|
| **Amsinckia** | **collection** | **herbarium and voucher no.”** |
| species   | state   | county | date       |                                     |
| A. intermedia | 1 UT    | Washington | April 28, 1986 | BYU870                              |
|           | 2 UT    | Washington | May 31, 1985 | BYU15520                             |
|           | 3 UT    | Washington | March 18, 1987 | BYU17090                            |
|           | 4 UT    | Washington | April 15, 1983 | BYU21616                            |
|           | 5 UT    | Washington | June 6, 1985 | BYU1635                              |
|           | 6 WA    | Adams | May 16, 2012 | PPRL4362                             |
|           | 7 WA    | Adams | May 16, 2012 | PPRL4361                             |
|           | 8 WA    | Adams | May 15, 2012 | PPRL4363                             |
|           | 9 AZ    | Mohave | February 16, 2012 | PPRL4377                        |
|           | 10 AZ   | Mohave | February 16, 2012 | PPRL4378                        |
| A. menziesii var. intermedia | 1 AZ    | Mohave | March 13, 2012 | PPRL4364                             |
|           | 2 AZ    | Mohave | February 16, 2012 | PPRL4351                        |
| A. vernicosa | 1 CA    | San Luis Obispo | April 14, 1985 | DAV 141038                          |
|           | 2 CA    | San Joaquin | April 2, 1935 | UCB16951                             |
|           | 3 CA    | Alameda | April 21, 1935 | UCB548                              |
|           | 4 CA    | Alameda | April 3, 1937 | UCB1751                              |
|           | 5 CA    | Stoneslaus | April 13, 1940 | UCB4341                             |
|           | 6 CA    | Merced | March 20, 1938 | UCB2879                              |
| A. lunaris | 1 CA    | Contra Costa | April 14, 1986 | UCB539                               |
|           | 2 CA    | Contra Costa | April 25, 1976 | UCB507                    –2|
|           | 3 CA    | Contra Costa | April 19, 1899 | UCB21083                             |
|           | 4 CA    | Contra Costa | April 20, 1938 | UCB3178                             |
|           | 5 CA    | San Mateo | April 3, 2008 | DAV 182419                           |
| A. grandiflora | 1 CA    | San Joaquin | April 9 1938 | UCB3021                              |
|           | 2 CA    | San Joaquin | March 19 1938 | UCB2866                              |
|           | 3 CA    | San Joaquin | May 7 1938 | UCB3397                              |
|           | 4 CA    | San Joaquin | April 5 1956 | UCB6064                              |

“**DAV (Botany Herbarium) and AHUC (Agronomy Herbarium):** Campton Herbarium at the UC Davis Center for Plant Diversity. UCB: Jepson Herbarium, University of California, Berkeley. BYU: Stanley L. Welsh Herbarium, Brigham Young University.”

The full scan (m/z 200–800) was followed by a data dependent, collision-induced dissociation (CID) scan using a generic CID energy of 32%, activation Q of 0.25, and an activation time of 10.0 ms. The capillary temperature was set at 275 °C, the ionization spray voltage at 3.45 kV, the HESI source heater temperature at 305 °C, and the sheath gas flow was 40 units with an auxiliary flow of 5 units.

**Identification and Quantitation of Dehydropyrrolizidine Alkaloids.** Reconstructed ion chromatograms (RICs) displaying the mass to charge ratio (m/z) of the protonated molecule (MH+) for dehydropyrrolizidine alkaloids previously identified in *Amsinckia* spp. were used in the first instance to identify potential alkaloids (Figure 2). The retention times and MS/MS data were examined for fragment ions characteristic of dehydropyrrolizidine alkaloids or their N-oxides and, further, compared to literature reports of the suspected alkaloids.12,14

To account for intrinsic inconsistencies between analytical ion chromatograms, the peak area for a dehydropyrrolizidine alkaloid or its N-oxide was divided by the peak area of the internal standard, lasiocarpine, to afford an “adjusted area”. Every HPLC sample was analyzed twice to provide an average “adjusted area”. This process immediately highlighted any machine or user errors that could be
corrected. Relative quantitative estimates of the dehydropyrrolizidine alkaloid and N-oxide content of most samples were then based on an eight-point calibration curve (adjusted area = 1.35 \times \text{concentration in } \mu g/mL; R^2 = 0.9983) generated using seven 1:1 serial dilutions of intermedine, 2, from 14.15 to 0.12 \mu g/mL. In more dilute samples, the quantitative estimate was completed using an eight-point calibration curve (adjusted area = 0.0023 \times \text{concentration in } \mu g/mL; R^2 = 0.9993) generated using seven 1:1 serial dilutions of 2 from 2.2 to 0.016 \mu g/mL). Therefore, concentrations of alkaloids and their N-oxides are expressed as "\mu g equivalents of intermedine/g plant material".

### RESULTS AND DISCUSSION

CalFlora recognizes 11 species and five additional varieties of *Amsinckia*,\(^\text{10}\) while the USDA plants database describes 10 species with six varieties and many synonyms.\(^\text{11}\) It appears that of those, only *A. intermedia*, *A. lycopsoides*, *A. menziesii*, and *A. tessellata* are widely distributed and have been carried from their historical ranges, mainly in the states of California, Oregon, and Washington, with lesser representation east onto the Columbia Plateau and Great Basin regions. The remaining species and varieties are distributed mainly in coastal California and nearby desertic ranges, some into the Great Valley, and others along the U.S. coast northward as far as Skagway, Alaska. The weedy representatives of this genus have been spread, by whatever means (feed, autos, planes, people, etc.), widely within the U.S. and to other parts of the world including Europe and Australia.

However, the taxonomic differentiation within the genus *Amsinckia* (Boraginaceae) is somewhat suspect in many cases, due mainly to the minor morphological distinctions upon which such differentiation is based. Therefore, in an attempt to discover any useful chemotaxonomic indicators, the dehydropyrrolizidine alkaloid profiles of leaf samples collected from herbarium specimens of *Amsinckia* species, including some of doubtful assignation, were acquired from crude methanolic extracts of the samples. Because only small samples of leaves from herbarium specimens could be taken for analysis, detection of only the major dehydropyrrolizidine alkaloids present in each sample was expected. For similar reasons, replicate analyses of the same specimen were not possible and thus potential intraspecimen variation could not be accounted for or addressed.

Dehydropyrrolizidine alkaloids, usually present mainly as their N-oxides, were detected in every sample analyzed (Figure 1). The efficiencies of recovery of dehydropyrrolizidine alkaloids and their N-oxides from these small samples were not determined. However, because all samples were treated the same way, it is assumed that the relative profiles determined for each specimen will be an accurate reflection of alkaloid content. Estimated total levels of dehydropyrrolizidine alkaloids were usually quite variable within and between species and varied from about 1 to 4500 \mu g equivalents of intermedine/g plant material (Table 2). The predominant dehydropyrrolizidine alkaloids observed were the N-oxides of lycopsamine, 1, and

### Table 2. Total Dehydropyrrolizidine Alkaloid Content Summary

| Amsinckia species | no. of specimens | total dehydropyrrolizidine alkaloid content (\mu g equivalents intermedine/g dry weight plant) |
|-------------------|------------------|-----------------------------------------------------------------------------------------|
| *A. douglasiana* | 12               | 13–1412                                                                                     | 403 |
| *A. lycopsoides* | 6                | 144–1055                                                                                     | 1640 |
| *A. menziesii* var. intermedia | 2 | 2088–352                                                                                     | 2455 |
| *A. vernicosa* | 6                | 362                                                                                             | 777 |
| *A. grandiﬂora* | 4                | 69–19–23                                                                                      | 126 |

*Specimen 8 was a flower sample and is excluded from these estimates of leaf content.*
intermedine, 2, in varying relative amounts from almost exclusively 1 in specimen 2 of *A. tessellata* to almost exclusively 2 in specimen 2 of *A. lunaris* (Figure 3). In extracts that contained larger amounts of alkaloids several trace to minor levels of components were observed that revealed MS/MS profiles strongly indicative of dehydropyrrolizidine alkaloids. However, their contribution to the overall dehydropyrrolizidine alkaloid content and profile was considered negligible. Additionally, minor amounts of the 1,2-dihydro analogues of the dehydropyrrolizidine alkaloids were observed that displayed very similar MS/MS profiles, albeit with fragment ions 2 Da greater than their dehydro counterparts. The relative abundance profile of dehydropyrrolizidine alkaloids could be quite different between species and even within a designated species (Table 3). It remains to be determined whether these differences represent natural diversity or whether taxonomic misassignments have occurred. For example, specimen 1 of *A. retorsa*, collected in 2005 from Cache County, Utah (Tables 1 and 3), was an intermedine chemotype with about 95% of total dehydropyrrolizidine alkaloids identified intermedine-N-oxide, 2NO, (Figure 4A), whereas the other seven specimens of the same species collected in neighboring counties in Utah, or from sites in the states of Nevada and Washington, all showed a more even ratio of 1NO and 2NO (Figure 4B–H). Also noted are the relative differences in the N-oxides of the two 3’-monoacetylated derivatives, 5NO(1) and 5NO(2), described in detail later in the text, between the specimens of this species (Figure 4).

When comparing the specimens annotated as *A. menziesii*, *A. intermedia*, or *A. menziesii* var. *intermedia* (Table 3) it appears as though four out of five specimens of *A. menziesii* and seven out of 10 of the *A. intermedia* specimens produce both 1NO and 2NO in various relative amounts (from predominantly 1NO, through approximately equal amounts, to predominantly 2NO), whereas *A. menziesii* specimen 3 and *A. intermedia* specimens 4, 9, and 10, and both samples annotated *A. menziesii* var. *intermedia*, only produce 1NO. Therefore, it is possible that the sole production of 1 and its N-oxide can characterize *A. menziesii* var. *intermedia* and that the *A. menziesii* and *A. intermedia* specimens with the inconsistent profiles may be misassigned.

Three minor abundance ions, isobaric with lycopsamine, 1, and intermedine, 2, were deduced, on the basis of the MS/MS data, to be tessellatine, 3, and related isomers (Table 4). In particular, the base ion peak at m/z 156 in the MS/MS profile for the putative tessellatine isomers is in contrast to the base peak at m/z 138 that is observed with the C9 monoesters 1 and 2 and is consistent with a C7 monoester dehydropyrrolizidine alkaloid analyzed under these esi(+)MS conditions. A corresponding N-oxide was only observed for the putative tessellatine peak that eluted earlier than the other two isomers. The putative tessellatine (as its N-oxide) is only a major contributor in one specimen analyzed, *i.e.*, *A. eastwoodiae* specimen 1 (Tables 1 and 3). Similar to the lack of tessellatine detected in the preparative chromatography work by Cooper et al.9 no significant amount of the putative tessellatine, its isobaric isomers or any other peak that might be assigned to tessellatine on the basis of its MS/MS data were observed for any of the other samples analyzed. This included *A. grandisflora*, *A. douglasiana*, and *A. tessellata* in the section Tessellatae and which were previously reported to contain significant amounts of tessellatine, 3.

Echiumine, 7, an open chain diester dehydropyrrolizidine alkaloid more commonly observed in *Echium* species,12 has been previously reported to be restricted to the section Muricatae and has been detected in one of three specimens of *A. eastwoodiae* and four of 14 specimens of *A. menziesii* var. *intermedia* examined using GC-MS.7 In this present HPLC-esi(+)MS/MS examination, up to three closely eluting echiumine-N-oxide isomers (Figure 2) with identical MS/MS profiles were observed to occur in various relative abundances. In addition to both specimens of *A. menziesii* var. *intermedia* and one of 13 specimens of *A. eastwoodiae*, echiumine (as its N-oxide)12 has also been detected in one of eight specimens of *A. retorsa*, one of five specimens of *A. menziesii*, three of six specimens of *A. lycopsoides* and five of 10 specimens of *A. intermedia*, all in the section Muricatae, but also in three of five specimens of *A. tessellata* in the section Tessellatae.

Within some specimens examined in this study, four monoacetylated derivatives of lycopsamine-N-oxide isomers (i.e., MH+ m/z 358) were detected in various relative abundances ranging from one, two, three, or through to all four being present in a specimen. The characters of the four isomers are illustrated by comparison of the MS/MS profiles (Table 4) for m/z 358 in *A. intermedia* specimen 2 and specimen 5 (Figure 5). In *A. intermedia* specimen 2, two major monoacetylation peaks are observed at ca. 8.70 and 8.92 min. They have near identical MS/MS profiles (peaks 3 and 4, Figure 5) that include a strong loss of 60 Da (m/z 358 → m/z 298) indicative of a 3’-acetyl derivative. In *A. intermedia* specimen 5, three major monoacetylation peaks are observed at ca. 8.68, 8.73, and 8.92 min. While the third, later eluting peak is the same as described for specimen 2, the early eluting peaks (peaks 1 and 2, Figure 5) again have near identical MS/MS profiles that include a minor ion at m/z 298, a base ion peak at m/z 214, and an ion at m/z 180 indicative of a C7 acetylated derivative.14 Because of the near coelution of the peaks at ca. 8.68, 8.7, and 8.73 min, there is some mixing of the MS/MS data when all three are present, but scans at the beginning, middle, and end of the coeluting peak envelope help distinguish the three eluants. It is suggested that the four isomers represent 3’- and 7-acetyl derivatives of lycopsamine-N-oxide and intermedine-N-oxide. The largest
Table 3. Estimated Total Dehydropyrrolizidine Alkaloid Content and Relative Concentrations of Lycopsamine, 1, and Intermedine, 2, and Their N-Oxides, Along with the Levels of the N-Oxides of Other Major to Minor Dehydropyrrolizidine Alkaloids Observed in Extracts of Several Amsinckia Species

| Amsinckia species | mass extracted (mg) | dehydropyrrolizidine alkaloid (% relative abundance) |
|------------------|---------------------|-----------------------------------------------------|
|                  |                     | 1^\textsuperscript{a} | 1NO | 2 | 2NO | 3NO | 2^\textsuperscript{b} × 4NO | 5NO (1) | 5NO (2) | 2 × 6NO | 3 × 7NO | total^4 |
| A. douglasiana    |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 57.3                | 4                     |   96 | 10 |
| 2                | 49.8                | 7                     | 28  | 16 |
| 3                | 60.8                | 52                    | 33  | 15 |
| 4                | 31.8                | 48                    | 51  | 19 |
| 5                | 45.8                | 33                    | 61  |  6 |
| 6                | 15.4                | 27                    | 16  |  3 |
| 7                | 22.7                | 34                    | 63  |  3 |
| 8                | 13.7                | 10                    | 4   | 17 |
| 9                | 8.4                 | 42                    | 17  |  4 |
| 10               | 11.1                | 15                    | 81  | + |
| 11               | 21.8                | 34                    | 66  | 20 |
| 12               | 7.1                 | 67                    | 21  |  3 |
| A. tessellata    |                     | 77.2                 | 4   | 95 |
| 1                | 65                  | 12                    | 74  | + |
| 2                | 57.8                | 1                    | 69  | + |
| 3                | 59.7                | 10                   | 74  | + |
| 4                | 55.7                | 9                    | 40  |  3 |
| A. retrorsa      |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 37.7                | 95                   | 5   | 44 |
| 2                | 37.2                | 37                   | 48  |  6 |
| 3                | 30.1                | 6                    | 13  | 10 |
| 4                | 17.7                | 37                   | 27  | 32 |
| 5                | 55.7                | 2                    | 22  | 12 |
| 6                | 23.9                | 3                    | 42  |  9 |
| 7                | 11.9                | 7                    | 43  | 29 |
| 8                | 66.1                | 18                   | 31  | 28 |
| A. menziesii     |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 6.4                 | 6                    | 30  | 10 |
| 2                | 21.5                | 15                   | 14  | 24 |
| 3                | 37.1                | 11                   | 72  |  3 |
| 4                | 9.8                 | 4                    | 29  |  5 |
| 5                | 27.7                | 3                    | 26  |  6 |
| A. lycopoides    |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 49.6                | 14                   | 14  | 36 |
| 2                | 42.4                | 3                    | 19  |  8 |
| 3                | 30.3                | 3                    | 27  |  6 |
| 4                | 44                  | 9                    | 20  | 46 |
| 5                | 42.6                | 5                    | 21  | 40 |
| 6                | 41.7                | 5                    | 28  |  8 |
| A. eastwoodiae   |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 25.6                | 14                   | 13  |  5 |
| 2                | 50.2                | 2                    | 21  |  5 |
| 3                | 39.1                | 3                    | 39  |  4 |
| 4                | 58.8                | 16                   | 39  |  8 |
| 5                | 33.5                | 4                    | 34  |  6 |
| 6                | 15                  | 9                    | 27  | 17 |
| 7                | 18.5                | 3                    | 21  |  6 |
| 8                | 9.1                 | 15                   | 8   | 44 |
| 9                | 33.2                | 27                   | 20  | 24 |
| 10               | 31.5                | 29                   | 10  |  6 |
| 11               | 22                  | 2                    | 51  |  9 |
| 12               | 19                  | 3                    | 14  | 12 |
| 13               | 20.5                | 4                    | 45  |  5 |
| A. intermedia    |                     |                      |      |    |     |      |                        |         |         |        |         |        |
| 1                | 55                  | 10                   | 60  | 18 |

^1^: Dehydropyrrolizidine content.
^2^: Intermedine content.
^3^: Lycopsamine content.
^4^: Total N-oxide content.

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effect of acetylation might be expected at the epimeric C3′ position and thus have more effect on retention time of the lycopsamine- and intermedine-based compounds as observed in this HPLC system.

Even though the first five specimens annotated as *A. intermedia* were all collected in Washington County, Utah, between 1983 and 1987 (Table 1), they displayed somewhat different N-oxide profiles for lycopsamine isomers and acetyl-lycopsamine isomers (Figure 6). This may serve as a further example of the variation observed in the HPLC-esi(+)MS profiles of some of the *Amsinckia* species examined or an indication of possible misassignment on morphologic grounds. Specimens 2, 3, and 5 (Table 1) contained similar amounts and ratios of 1NO and 2NO. However, specimen 2 was found to produce both of the 3′-acetyl isomers, 5NO, whereas specimens 3 and 5 produced both of the 7-acetyl isomers, 4NO, and only the 5NO isomer 2 (Rt ca. 8.9 min). Specimen 1 produced mainly 1NO relative to 2NO, whereas specimen 4 was a lycopsamine chemotype similar to *A. intermedia* specimens 9 and 10.5

In accord with an earlier observation,9 diacetylation of 1 and/or 2 was sometimes evident as two well resolved peaks (Figure 7) with MS/MS profiles (Table 4) that included the presence of ions at m/z 180 and 214 as well as a loss of 60 Da (m/z 400 → m/z 340), consistent with acetylation at both C7 and C3′. The data did not allow specific assignment of the peaks to either diacetyllycopsamine-N-oxide or its epimer diacetylimedine-N-oxide. For example, no relative abundance correlation was observed between the similar relative abundances of 1NO and 2NO in specimen 9 of *A. douglasiana* and specimen 5 of *A. retrorsa* and their C3′ and C7 monoacltylated derivatives or the two diacetylated derivatives (Figure 7).

Four of the specimens of *A. lunaris* were collected (in 1899, 1938, 1976, and 1986) from Contra Costa County in California while the fifth was collected (in 2008) from San Mateo County in the same state. Similar to four of six specimens of *A. vernicosa*, four of the five *A. lunaris* specimens were also intermedine, 2, chemotypes with the N-oxide predominating except for the oldest specimen collected in 1899. In contrast, *A. lunaris* specimen 4, despite being collected in the same county, produced equal amounts of intermedine and lycopsamine, and their N-oxides, in addition to significant levels of the putative tesselatine-N-oxide, 3NO. Another source of contrast between the *A. lunaris* specimens was the production of the monoacltylated derivatives. The oldest two specimens (3 and 4) showed unquantified traces (confirmed by the MS/MS profiles) of the 3′-acetyllycopsamine/intermedine isomer 2,
Figure 4. Comparison of HPLC-esi(+)MS reconstructed ion chromatograms displaying (A–H) the major ions m/z 300, 316, and 358 for the specimens 1–8, respectively, of *Amsinckia retrorsa* (Table 1). Peak 1 = intermedine, 2 = lycopsamine, 1: peak 3 = intermedine-N-oxide, 2NO; peak 4 = lycopsamine-N-oxide, 1NO; peak 5 = 3′-acetyllycopsamine-N-oxide isomer 1, 5NO(1); peak 6 = 3′-acetyllycopsamine-N-oxide isomer 2, 5NO(2); peak 7 = putative tessellatine-N-oxide, 3NO.

Table 4. MS and MS/MS Data for Pyrrolizidine Alkaloids Detected in Methanol Extracts of Various *Amsinckia* Species

| pyrrolizidine alkaloid | retention time (min) | MH+(2M + H)+ m/z (% relative abundance)b | MS/MS m/z (% relative abundance) |
|------------------------|----------------------|------------------------------------------|----------------------------------|
| lycopsamine (1) and intermedine (2) | 7.0 and 6.7 | 300 | 282(0.5), 256(3), 210(2), 156(6), 138(100), 120(22), 94(48), 298(8), 272(19), 254(3), 226(28), 210(4), 172(100), 155(8), 154(7), 138(27), 137(6), 136(12), 120(2), 112(2), 108(2), 94(7) |
| lycopsamine-N-oxide and intermedine-N-oxide | 7.7 and 7.6 | 316(100)/631(17) | 298(3), 272(5), 238(3), 226(5), 192(1), 156(100), 139(1), 138(3), 120(1), 108(2) |
| putative tessellatine (3) or isomer | 6.4 | 300 | 282(1), 256(5), 238(1), 210(1), 192(2), 156(100), 139(1), 138(3), 120(1), 108(2) |
| putative tessellatine-N-oxide or isomer | 6.8 | 316(100)/631(2) | 298(3), 272(5), 238(3), 226(5), 192(1), 156(100), 139(1), 138(3), 120(1), 108(2) |
| unidentified tessellatine (3)-like isomers (no corresponding N-oxides observed) | 8.18 and 8.3 | 300 | 282(2), 256(23), 238(2), 210(56), 194(5), 184(2), 156(100), 139(16), 138(9), 122(8), 120(14), 110(3), 108(2), 94(5) |
| 3′-acetyllycopsamine and/or 3′-acetylintermedine (5) | 8.22 | 342 | 324(2), 297(20), 282(80), 187(3), 156(1), 138(100), 136(3), 120(14) |
| 3′-acetyllycopsamine and/or 3′-acetylintermedine (5) isomer | 8.5 | 342 | 324(2), 282(40), 187(3), 156(1), 138(100), 136(3), 120(14) |
| 3′-acetyllycopsamine-N-oxide and/or 3′-acetylintermedine-N-oxide | 8.7 and 8.9 | 358 (100)/715(10) | 340(10), 316(9), 298(100), 280(2), 172(15), 154(2), 138(5), 137(1), 136(3), 120(14) |
| 7′-acetyllycopsamine and/or 7′-acetylintermedine (4) | 8.6 | 342 | 324(1), 282(5), 198(3), 180(57), 162(4), 138(13), 136(1), 124(1), 120(100), 118(2) |
| 7′-acetyllycopsamine-N-oxide and/or 7′-acetylintermedine-N-oxide | 8.67 and 8.72 | 358(100)/715(5) | 340(12), 314(25), 298(6), 268(23), 252(4), 242(3), 214(100), 197(4), 180(11), 178(5), 154(13), 137(6), 136(3), 120(3) |
| 3′,7-diacyllycopsamine and/or 3′,7-diacylintermedine (6) | 9.4 and 9.65 | 384 | 366(6), 352(1), 342(2), 338(1), 324(100), 240(1), 198(2), 180(79), 162(5), 120(90), 118(2) |
| 3′,7-diacyllycopsamine-N-oxide and/or 3′,7-diacylintermedine-N-oxide | 9.63 and 9.88 | 400(100)/799(5) | 382(12), 358(11), 340(100), 322(4), 214(9), 197(2), 180(3), 137(2), 136(1), 120(1) |
| echiumine (7) | 10.4, 10.47, and 10.58 | 382 | 364(2), 338(1), 300(1), 238(2), 220(27), 138(1), 120(100), 118(2) |
| echiumine-N-oxide | 10.53, 10.58, and 10.71 | 398(100)/795(5) | 380(10), 354(22), 308(18), 298(6), 292(4), 282(2), 254(100), 238(2), 237(5), 236(1), 220(12), 218(4), 154(2), 137(4), 136(3), 120(3) |

“Where indicated by bold numbers, structures are shown in Figure 1. Depending upon the intensity of the protonated molecule (MH+), weak to moderate dimer ions (2M + H)+ were observed for N-oxides.”
Figure 5. MS/MS differentiation of four monoacetylycopsamine isomers detected at varying relative amounts in the *Amsinckia* species analyzed. Shown, for example, are the HPLC-esi(+)MS reconstructed ion chromatograms (displaying m/z 316 and 358) for *Amsinckia intermedia* specimens 5 and 2 (Table 1) and the MS/MS spectra for each pair of monoacylated N-oxide derivatives (peaks 1 and 2 = C7 acetylation (4), and peaks 3 and 4 = C3′ acetylation (5)). Also annotated are the peaks for lycopsamine-N-oxide, 1NO; intermedine-N-oxide, 2NO; and the putative tessellatine-N-oxide, 3NO.

Figure 6. HPLC-esi(+)MS comparison of N-oxide profiles of the lycopsamine isomers (m/z 316) and acetylated isomers (m/z 358) produced by: (A−E) specimens 1−5 (Table 1) identified as *Amsinckia intermedia*, all collected in Washington County, Utah, between 1983 and 1987. Peak 1 = putative tessellatine-N-oxide, 3NO; peak 2 = intermedine-N-oxide, 2NO; peak 3 = lycopsamine-N-oxide, 1NO; peak 4 = two 7-acetylycopsamine-N-oxide isomers, 4NO; peak 5 = a 3′-acetylycopsamine-N-oxide isomer, 5NO (1); and peak 6 = a 3′-acetylycopsamine-N-oxide isomer, 5NO (2).
$\textit{A. intermedia}$ var. $\textit{intermedia}$, compared to four of the five $\textit{A. menziesii}$ samples and eight of the 10 $\textit{A. intermedia}$ samples of the $N$-oxides of 1, 2, and the monoacetyl derivative(s) of 1 and/or 2. The other two only showed 1 (about 33% total dehydropyrrolizidine alkaloid content) and its $N$-oxide (ca. 61%). Of the 12 specimens annotated as $\textit{A. douglasiana}$, six were lycopsamine/lycopsamine-$N$-oxide chemotypes, whereas the other six produced both lycopsamine-$N$-oxide and its epimer, intermedine-$N$-oxide, in various relative levels.

It is clear that the application of HPLC-esi(+)MS and MS/MS to the analysis of mass-limited samples harvested from herbarium-preserved specimens is a useful approach to simultaneous profiling of the dehydropyrrolizidine alkaloids and their $N$-oxides. It is important to consider that intraspecies (as designated in this study by the herbaria) variations in profiles may reflect differences in degradation of the alkaloids and their $N$-oxides that would facilitate or support species differentiation or (2) that the observed, inconsistent profiles of dehydropyrrolizidine alkaloids within a species may reflect taxonomic misassignment. For example, it is possible that the lack of significant, or any, intermedine-$N$-oxide in samples annotated as $\textit{A. menziesii}$ var. $\textit{intermedia}$, compared to four of the five $\textit{A. menziesii}$ samples and eight of the 10 $\textit{A. intermedia}$ samples

Figure 7. HPLC-esi(+)MS reconstructed ion chromatogram comparison of lycopsamine-$N$-oxide and intermedine-$N$-oxide ($m/z$ 316) with their mono- ($m/z$ 358) and diacetylated ($m/z$ 400) derivatives detected in specimen 9 of $\textit{Amsinckia douglasiana}$ (A) and specimen 5 of $\textit{Amsinckia retrorsa}$ (B). Peak 1 = putative tessellatine-$N$-oxide, 3NO; peak 2 = intermedine-$N$-oxide, 2NO; peak 3 = lycopsamine-$N$-oxide, 1NO; peak 4 = two 7-acetyllycopsamine-$N$-oxide isomers, 4NO; peak 5 = a 3′-acetyllycopsamine-$N$-oxide isomer, 3NO (2); peak 6 = mainly 3′-acetyllycopsamine-$N$-oxide isomer, 3NO (1), with minor presence of 7-acetyllycopsamine-$N$-oxide isomers, 4NO; peaks 7 and 8 = 3′,7-diacetyl derivatives of lycopsamine and intermedine $N$-oxides, 6NO.

$\textit{A. douglasiana}$ were all quite low, ranging from 2–26 $\mu$g equivalents intermedine/g plant. In contrast to most of the other species examined the level of free base exceeded the level of corresponding $N$-oxide. Three of the $\textit{A. grandiﬂora}$ specimens were clearly lycopsamine/lycopsamine-$N$-oxide chemotypes, with the fourth presenting with a significant level of intermedine and its $N$-oxide.

The levels of dehydropyrrolizidine alkaloids observed did not uniquely reflect the date of specimen collection and herbarium-mounting. There were some inconsistencies within a species collected in the same area but decades apart, for example $\textit{A. eastwoodiae}$ specimens 4 and 11 collected in 1938 and 2013 in Tulare County, California both returned very low (ca. 1–2 $\mu$g equivalents intermedine/g plant material) total levels of dehydropyrrolizidine alkaloids. However, there were also some inconsistencies such as $\textit{A. douglasiana}$ specimens 2, 5, and 7 collected from San Louis Obispo County, California, in 2011, 1938, and 1962, respectively, that showed total alkaloid levels of about 3, 3, and 1412 $\mu$g equivalents intermedine/g plant material. Of these, only specimen 2 produced about equal levels of the $N$-oxides of 1, 2, and the monoacetyl derivative(s) of 1 and/or 2. The other two only showed 1 (about 33% total dehydropyrrolizidine alkaloid content) and its $N$-oxide (ca. 61%). Of the 12 specimens annotated as $\textit{A. douglasiana}$, six were lycopsamine/lycopsamine-$N$-oxide chemotypes, whereas the other six produced both lycopsamine-$N$-oxide and its epimer, intermedine-$N$-oxide, in various relative levels.

It is clear that the application of HPLC-esi(+)MS and MS/MS to the analysis of mass-limited samples harvested from herbarium-preserved specimens is a useful approach to simultaneous profiling of the dehydropyrrolizidine alkaloids and their $N$-oxides. It is important to consider that intraspecies (as designated in this study by the herbaria) variations in profiles may reflect differences in degradation of the alkaloids and their $N$-oxides that would facilitate or support species differentiation or (2) that the observed, inconsistent profiles of dehydropyrrolizidine alkaloids within a species may reflect taxonomic misassignment. For example, it is possible that the lack of significant, or any, intermedine-$N$-oxide in samples annotated as $\textit{A. menziesii}$ var. $\textit{intermedia}$, compared to four of the five $\textit{A. menziesii}$ samples and eight of the 10 $\textit{A. intermedia}$ samples...
may well support a differentiation between the three and indicate possible taxonomic misassignment of the samples with inconsistent profiles. Future work should also attempt to correlate the dehydropyrrolizidine alkaloid profiles of the herbarium-preserved specimens with profiles determined for collections of fresh plant from the same sites to determine any significant changes of profile with age of the preserved specimen or the potential for specimen collection and storage artifacts.

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