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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[1−3] and poisoning of humans via contaminated diet (eg., grains), food that naturally contains, or is naturally contaminated with, the alkaloids (eg., some honeys), herbal medicines, or dietary supplements (eg., some pollens).[4] The *Amsinckia* genus (Boraginaceae), known as fiddlenecks, comprises many species known to produce the potentially hepato-, pneumo-, and genotoxic dehydropyrrolizidine alkaloids.[5−9] These include, for example, the epimeric monoesters lycopsamine, **1**, intermedine, **2**, and a methylated analogue sincamidine, **8**, and the open chain diester echiumine, **7** (Figure 1) that were 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).[6] A GC-MS investigation of samples from 12 *Amsinckia* species collected mainly in California showed that most were dominated by **1** or **2**. While many showed a significant presence of the C7 analogue of **1** i.e., tessellatine, **3**, there was no evidence for the presence of sincamidine, **8**.[7] A counter-current chromatography and subsequent GC-MS investigation of *Amsinckia tessellata* collected from Tucson, Arizona, that yielded about 0.02% pyrrolizidine alkaloids[8] did not reveal any tessellatine despite it being found in every sample of *A. tessellata* examined by Kelley and Seiber.[7] 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. Roitman[9] differentiated *A. menziesii* from *A. intermedia*, showing that the former contained **1**, **2**, and the mono- and diacetyl derivatives of **1**. However, Kelley and Seiber, in their GC-MS investigation,[7] equated *A. intermedia* to *A. menziesii* var. *intermedia*, which reflects the synonymous relationship recorded in CalFlora[10] and the USDA PLANTS Database.[11] 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.[3] A recent HPLC-esi(+)MS-based investigation[5] revealed a lycopsamine, **1**/lycopsamine-*N*-oxide chemotype (ca. 0.76%, dw) of *Amsinckia intermedia*, potentially associated with an intoxication of cattle in Arizona. Several other dehydropyrrolizidine alkaloids, tentatively indentified on the basis of HPLC retention times and MS and MS/MS data,[12] 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,

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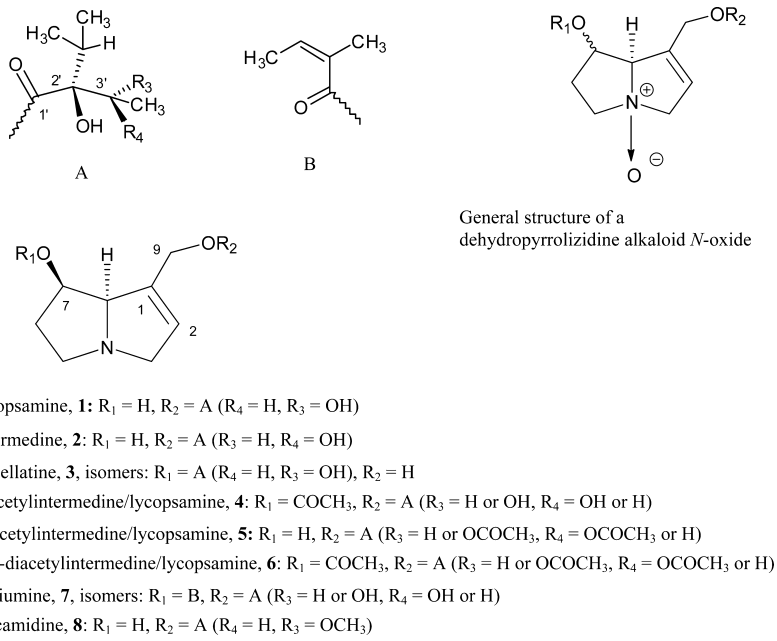

Figure 1. Structures of dehydropyrrolizidine alkaloids detected in *Amsinckia* species or discussed in the text.

and a putative deoxylycopsamine-*N*-oxide. The latter has also been observed in *Cryptantha crassipes*[13] and may correspond to the monoester dehydropyrrolizidine alkaloid amabiline previously identified in some *Amsinckia* species.[7] Also described in the same study of collections of *Amsinckia intermedia* from Arizona and Washington states were some pan-seasonal changes in dehydropyrrolizidine alkaloid levels and, in contrast to Johnson et al.,[3] the presence of **1** as its free base in the seeds of plants collected in Arizona and a significant increase in the quantity and diversity of alkaloids produced by the plants collected in Washington relative to the Arizona plants.[5] This difference once again raises the issue of potential taxonomic misassignment based upon very similar morphologic features.

A continued concern about taxonomic differentiation based on subtle morphologic features, combined with the HPLC-esi(+)MS capacity to directly detect and quantitate both the free base and *N*-oxide forms of the dehydropyrrolizidine alkaloids, prompted this current investigation of annotated herbarium specimens of *Amsinckia*. The objectives included a more accurate profiling of 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 retrorsa*, *A. menziesii*, *Amsinckia lycopsoides*, *Amskinckia eastwoodiae*, and *A. intermedia* were harvested from the Stanley L. Welsh Herbarium, Brigham Young University. Additionally, leaf samples of similarly differentiated specimens 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. menziesii* var. *intermedia*, *A. intermedia* and *A. retrorsa* 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 species	collection: state	county	date	herbarium and voucher no.[a]
A. douglasiana				
1	CA	Monterey	April 15, 1964	UCB61045
2	CA	San Louis Obispo	March 22, 2011	UCB8147
3	CA	Santa Clara	April 8, 1938	UCB2007
4	CA	Monterey	April 9, 1938	UCB2094
5	CA	San Louis 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. tessellata				
1	UT	Washington	April 10, 2010	BYU28877
2	UT	Washington	March 21, 1986	BYU2212
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	Adams	June 21, 2002	PPRL1999
8 flowers	WA	Adams	June 21, 2002	PPRL1999
A. menziesii				
1	UT	Washington	April 12, 1932	BYUxxxx
2	UT	Washington	May 26, 1978	BYU11938
3	UT	Tooele	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	Klikilat	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	BCB3117
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	UCB15136
11	CA	Tulare	February 29, 2013	UCB736
12	CA	Kern	April 7 1941	UCB286
13	CA	Contra Costa	March 27 1957	UCB5765
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	UCB664

[a]DAV (Botany Herbarium) and AHUC (Agronomy Herbarium): Crampton Herbarium at the UC Davis Center for Plant Diversity. UCB: Jepson Herbarium, University of California, Berkley. BYU: Stanley L Welsh Herbarium, Brigham Young University.

source. The first 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 of significant peaks thereby observed were compared to standards where available. Otherwise, the 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

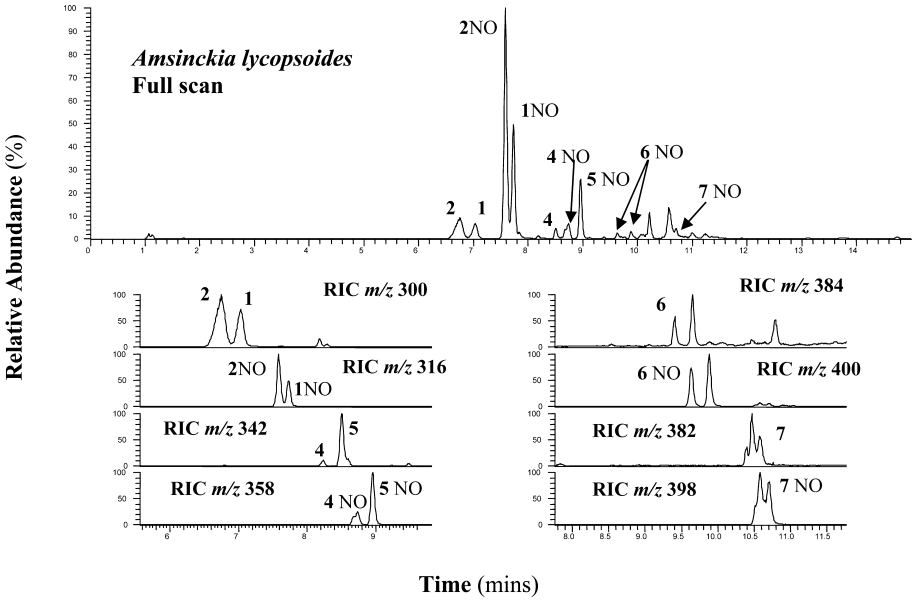

Figure 2. HPLC-esi(+)MS base ion (m/z 200−1000) and reconstructed ion chromatograms for specimen 5 of *Amsinckia lycopsoides* (Table 1). Peaks are annotated with the structure numbers (bold) (Figure 1).

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 × concentration in μg/mL: R^2 = 0.9983) generated using seven 1:1 serial dilutions of intermedine, **2**, from 14.15 to 0.12 μg/mL. In more dilute samples, the quantitative estimate was completed using an eight-point calibration curve (adjusted area = 0.0023 × concentration in μg/mL: R^2 = 0.9993) generated using seven 1:1 serial dilutions of **2** from 2.2 to 0.016 μg/mL). Therefore, concentrations of alkaloids and their *N*-oxides are expressed as "μg equivalents of intermedine/g plant material".

RESULTS AND DISCUSSION

CalFlora recognizes 11 species and five additional varieties of *Amsinckia*,[10] while the USDA plants database describes 10 species with six varieties and many synonyms.[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 μg 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

		total dehydropyrrolizidine alkaloid content (μg equivalents intermedine/g dry weight plant)			
Amsinckia species	no. of specimens	median	average	range	standard deviation
A. douglasiana	12	13	139	1−1412	403
A. tessellata	5	57	87	4−192	76
A. retrorsa	7[a]	351	640	16−2269	822
A. menziesii	5	134	405	4−1670	711
A. lycopsoides	6	144	1055	2−3991	1640
A. eastwoodiae	13	22	175	1−1216	362
A. intermedia	10	263	1061	12−4584	1557
A. menziesii var. *intermedia*	2	2088	2088	352−3824	2455
A. vernicosa	6	6	10	2−26	9
A. lunaris	5	364	607	4−1875	777
A. grandiflora	4	69	111	19−287	126

[a]Specimen 8 was a flower sample and is excluded from these estimates of leaf content.

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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

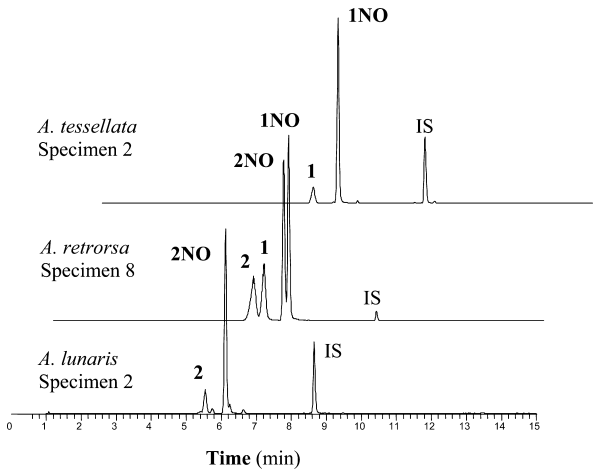

Figure 3. HPLC-esi(+)MS reconstructed base ion chromatograms showing differences in the production of lycopsamine, **1**, intermedine, **2**, and their *N*-oxides in samples of three species of *Amsinckia*. Peaks are annotated with the structure numbers (bold) (Figure 1). "IS" is the internal standard lasiocarpine.

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. retrorsa*, 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, **2**NO, (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, **5**NO(1) and **5**NO(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 **1**NO and **2**NO in various relative amounts (from predominantly **1**NO, through approximately equal amounts, to predominantly **2**NO), whereas *A. menziesii* specimen 3 and *A. intermedia* specimens 4, 9, and 10, and both samples annotated *A. menziesii* var. *intermedia*, only produce **1**NO. 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.[15] 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.,[8] 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. grandiflora*, *A. douglasiana*, and *A. tessellata* in the section Tessellatae and which were previously reported to contain significant amounts of tessellatine, **3**.[7]

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. retrorsa*, 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

		dehydropyrrolizidine alkaloid (% relative abundance)										
Amsinckia species	mass extracted (mg)	**1**[b]	**1**NO	**2**	**2**NO	**3**NO	2[c] × **4**NO	**5**NO (1)	**5**NO (2)	2 × **6**NO	3 × **7**NO	total[a]
A. douglasiana												
1	57.3	4	96									10
2	49.8	7	28		16	17	16	15				3
3	60.8	52	33		15							1
4	31.8	48	51			1						19
5	45.8	33	61			6						3
6	15.4	27	16		15							3
7	22.7	34	63									1412
8	13.7	10	40	4	17		13	1	6	3		136
9	8.4	42	17									4
10	11.1	15	81	4	+[d]	+						38
11	21.8	34	66									20
12	7.1	67	21	9	3							16
A. tessellata												
1	77.2	4	95				+		+			44
2	65	12	74								+	192
3	57.8	1	69				+		+		6	57
4	59.7	10	74						+		3	139
5	55.7	9	40									4
A. retrorsa												
1	37.7				95			5				64
2	57.2		37		48			2	14			59
3	30.1	6	13	7	17	10		34	6			16
4	17.7		37		27				32		2	2269
5	55.7	2	22	1	12			11	38	10		351
6	23.9	3	42	3	39				12			579
7	11.9	7	43	5	29				14			1145
8	66.1	18	31	18	28				4			4061
A. menziesii												
1	6.4	6	30	10	52							134
2	21.5	15	14	28	24							4
3	37.1	11	72								3	35
4	9.8	4	29	5	34		24					180
5	27.7	3	26	6	46				13			1670
A. lycopsoides												
1	49.6	14	14	36	15							2
2	42.4	3	19	10	58				1	1	1	3991
3	30.3	3	27	6	36		17		4	8		212
4	44	9	20	20	46							13
5	42.6	5	21	9	40		1		12	2	5	2035
6	41.7	5	28	8	39		2		9		10	75
A. eastwoodiae												
1	25.6	14	13		6	55						9
2	50.2	2	21	5	50	2	15				6	79
3	39.1	3	39	4	38	2	8		4			113
4	58.8	16	39	8	37							1
5	33.5	4	34	6	46		4		6			682
6	15	9	27	17	45							66
7	18.5	3	19	11	60		2		3			1216
8	9.1	15	8	44	20							22
9	33.2	27	20	24	6	19						6
10	31.5	29	10	30	6	11						14
11	22		51		19	30						2
12	19	31	14	12	3	7	17					66
13	20.5	44		56								2
A. intermedia												
1	55	10	60		10		18		+			12

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Table 3. continued

		dehydropyrrolizidine alkaloid (% relative abundance)									
Amsinckia species	mass extracted (mg)	**1**[b]	**1**NO	**2**	**2**NO	**3**NO	2[c] × **4**NO	**5**NO (1)	**5**NO (2)	2 × **6**NO	3 × **7**NO	total[a]
2	55.8	1	28	3	58			6	3			100
3	57.1	4	28	7	48		7		3	2		66
4	44.9	9	90				+		+			22
5	43.2	1	25	2	54		10		6			426
6	14.9	1	14	1	17		+	+	36	3	27	549
7	12.8	4	27	2	24		+	+	22		19	79
8	17.8		13	2			2	5	12	3	10	2094
9	26.5	4	83				+		2		1	4584
10	26.1	8	79				+		2		2	2680
A. menziesii var. *intermedia*												
1	26.6	4	93								0.3	3824
2	33.4	4	86						5		3	352
A. vernicosa												
1	57.4	6	5	47	28							5
2	16.8			99	+							16
3	25.8	15	17	34	34							6
4	57.3			95	5							26
5	43.2			83	17							3
6	36.3			99	+							2
A. lunaris												
1	33.4			5	35		54					1875
2	18.3			12	74		3		6			364
3	39.9			66	34				+			10
4	25.3	13	29	15	26	17			+			4
5	40.6			7	70			20				774
A. grandiflora												
1	12.4	33	64			3						287
2	37.7	43	54			2						19
3	26.4	51	33	9	4	4						21
4	25.5	11	82			6						117

[a]Concentration shown as μg intermedine equivalents/g plant material. [b]Structures shown in Figure 1: **1**, lycopsamine; **2**, intermedine; **3**, tessellatine; **4**, 7-acetyllycopsamine and/or 7-acetylintermedine; **5**, 3′-acetyllycopsamine and/or 3′-acetylintermedine; **6**, 3′,7-diacetyllycopsamine and/or 3′,7-diacetylintermedine; **7**, echiumine and isomers. [c]isomers with the same MS/MS profiles and eluting close together. [d]"+" indicates nonquantitated, low level detection of alkaloid.

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 acetyllycopsamine 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 **1**NO and **2**NO. However, specimen 2 was found to produce both of the 3′-acetyl isomers, **5**NO, whereas specimens 3 and 5 produced both of the 7-acetyl isomers, **4**NO, and only the **5**NO isomer 2 (Rt ca. 8.9 min). Specimen 1 produced mainly **1**NO relative to **2**NO, 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 diacetylintermedine-*N*-oxide. For example, no relative abundance correlation was observed between the similar relative abundances of **1**NO and **2**NO in specimen 9 of *A. douglasiana* and specimen 5 of *A. retrorsa* and their C3′ and C7 monoacetylated 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 tessellatine-*N*-oxide, **3**NO. Another source of contrast between the *A. lunaris* specimens was the production of the monoacetylated derivatives. The oldest two specimens (3 and 4) showed unquantified traces (confirmed by the MS/MS profiles) of the 3′-acetyllycopsamine/intermedine isomer 2,

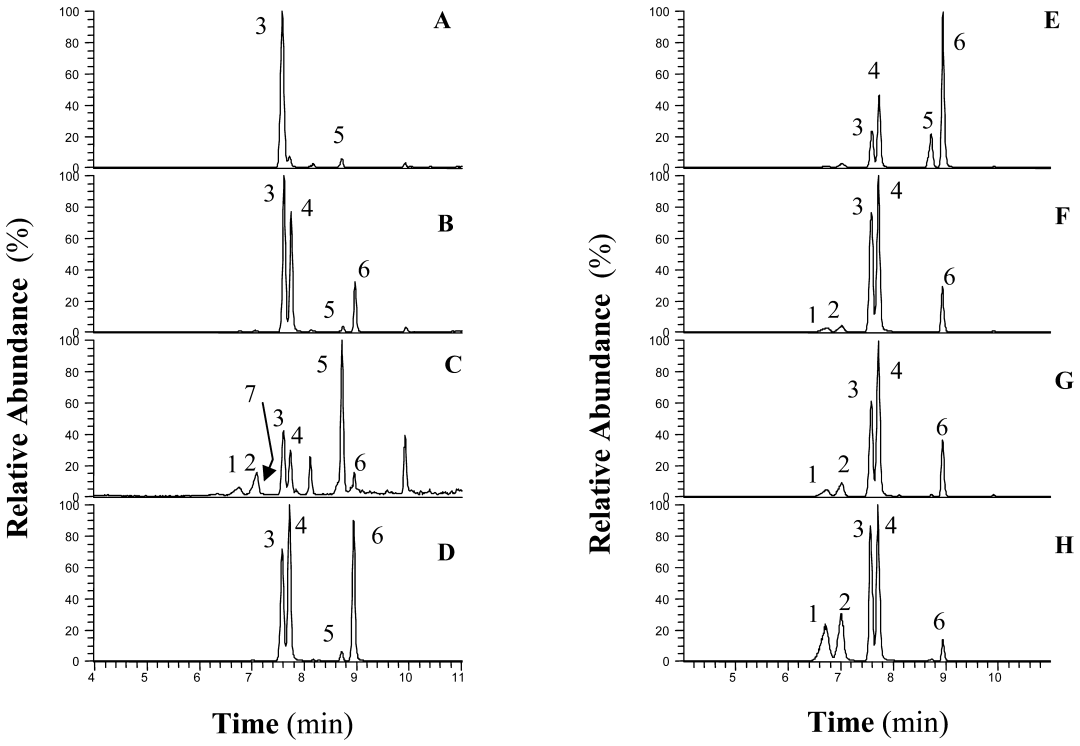

Figure 4. Comparison of HPLC-esi(+)MS reconstructed ion chromatograms displaying (A–H) the major ions *m/z* 300, 316, and 358 for the specimens 1–8, respectively, of *Amsinckia retrorsa* (Table 1). Peak 1 = intermedine, **2**; peak 2 = lycopsamine, **1**; peak 3 = intermedine-*N*-oxide, **2**NO; peak 4 = lycopsamine-*N*-oxide, **1**NO; peak 5 = 3′-acetyllycopsamine-*N*-oxide isomer 1, **5**NO(1); peak 6 = 3′-acetyllycopsamine-*N*-oxide isomer 2, **5**NO(2); peak 7 = putative tessellatine-*N*-oxide, **3**NO.

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

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),
lycopsamine-*N*-oxide and intermedine-*N*-oxide	7.7 and 7.6	316(100)/631(17)	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)
putative tessellatine (**3**) or isomer	6.4	300	282(1), 256(5), 238(1), 210(1), 192(1), 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(3), 172(100), 171(8), 170(6), 154(5), 153(2), 138(1), 137(3), 136(2), 111(1), 106(3)
unidentified tessellatine (**3**)-like isomers (no corresponding *N*-oxides observed)	8.18 and 8.3	300	300(2), 282(9), 256(23), 238(2), 210(36), 194(5), 184(2), 156(100), 139(16), 138(9), 122(8), 120(14), 110(3), 108(2), 94(3)
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)
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(3), 137(6), 136(3), 120(3)
3′,7-diacetyllycopsamine and/or 3′,7-diacetylintermedine (**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-diacetyllycopsamine-*N*-oxide and/or 3′,7-diacetylintermedine-*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(2), 120(3)

[a]Where indicated by bold numbers, structures are shown in Figure 1. [b]Depending upon the intensity of the protonated molecule (MH^+), weak to moderate dimer ions $(2M + H)^+$ were observed for *N*-oxides

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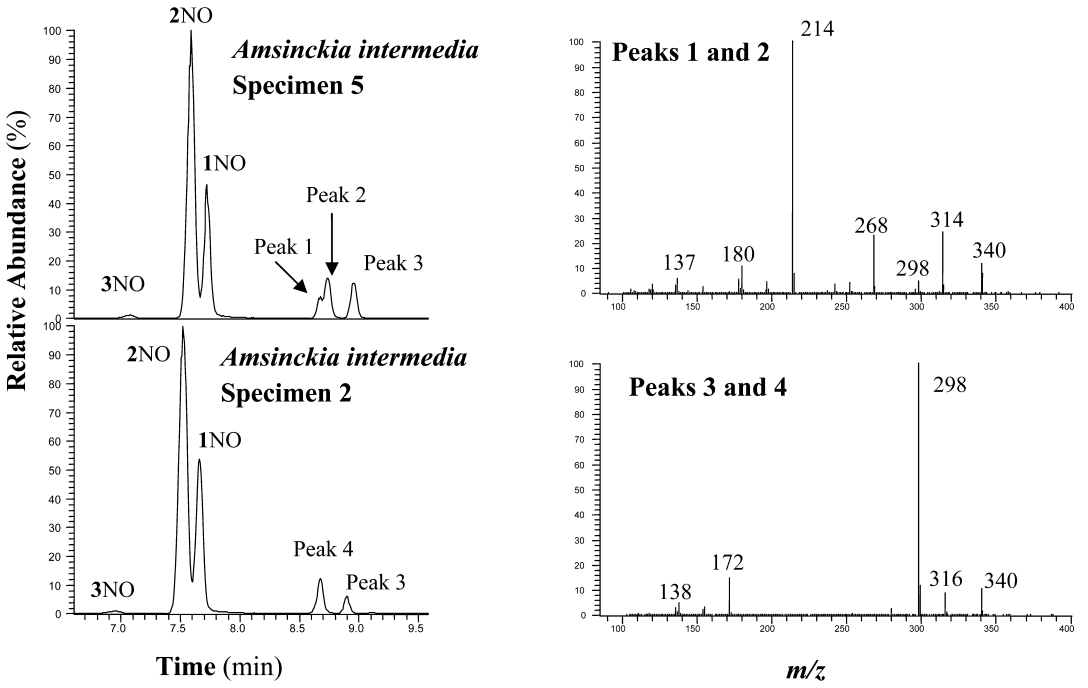

Figure 5. MS/MS differentiation of four monoacetyllycopsamine isomers detected at varying relative amounts in the *Amsinckia* species analyzed. Shown, for example, are the HPLC-esi(+)MS reconstructed ion chromatograms (displaying *m*/*z* 316 and 358) for *Amsinckia intermedia* specimens 5 and 2 (Table 1) and the MS/MS spectra for each pair of monoacetylated *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, **1**NO; intermedine-*N*-oxide, **2**NO; and the putative tessellatine-*N*-oxide, **3**NO.

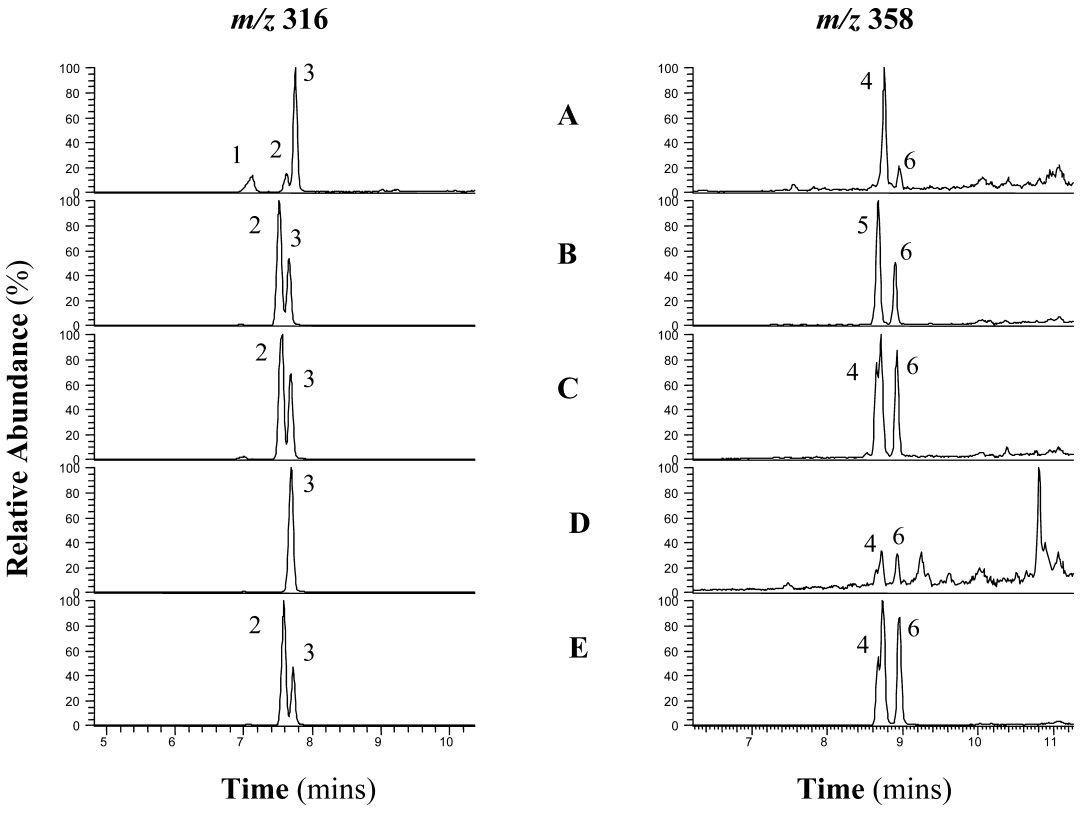

Figure 6. HPLC-esi(+)MS comparison of *N*-oxide profiles of the lycopsamine isomers (*m*/*z* 316) and acetylated isomers (*m*/*z* 358) produced by: (A–E) specimens 1–5 (Table 1) identified as *Amsinckia intermedia*, all collected in Washington County, Utah, between 1983 and 1987. Peak 1 = putative tessellatine-*N*-oxide, **3**NO; peak 2 = intermedine-*N*-oxide, **2**NO; peak 3 = lycopsamine-*N*-oxide, **1**NO; peak 4 = two 7-acetyllycopsamine-*N*-oxide isomers, **4**NO; peak 5 = a 3′-acetyllycopsamine-*N*-oxide isomer, **5**NO (1); and peak 6 = a 3′-acetyllycopsamine-*N*-oxide isomer, **5**NO (2).

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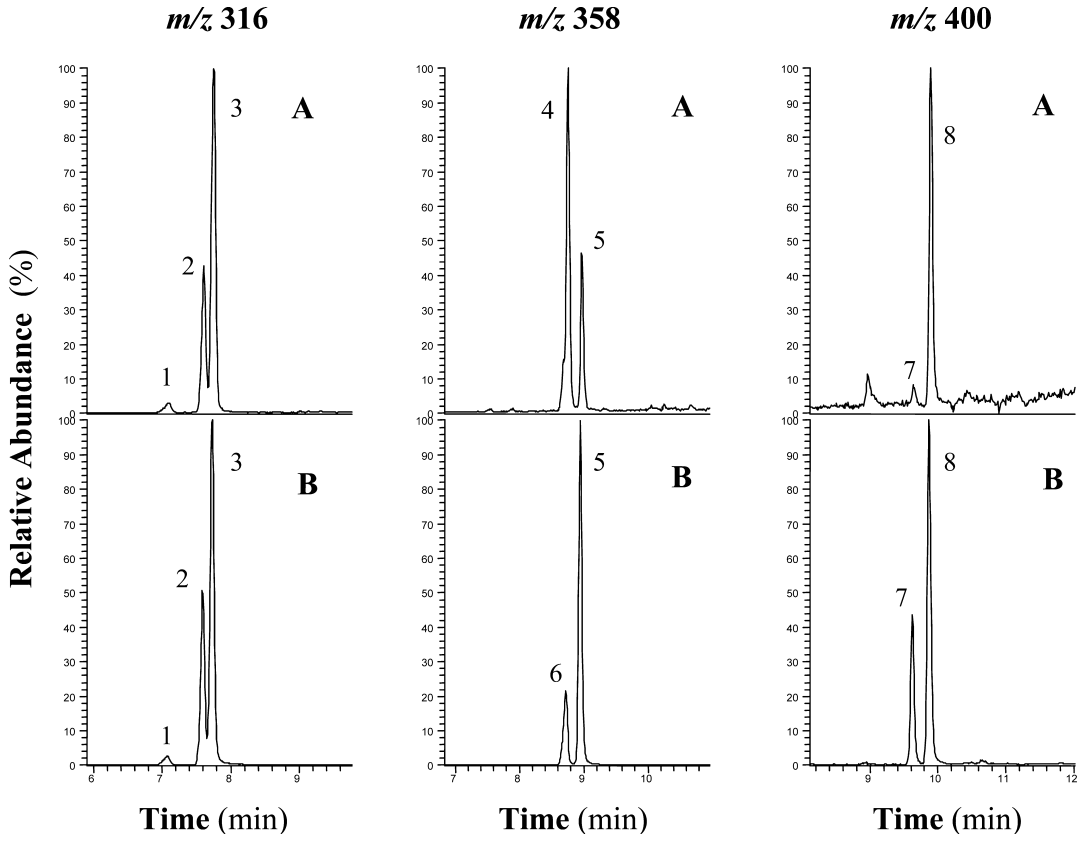

Figure 7. HPLC-esi(+)MS reconstructed ion chromatogram comparison of lycopsamine-*N*-oxide and intermedine-*N*-oxide (*m*/*z* 316) with their mono- (*m*/*z* 358) and diacetylated (*m*/*z* 400) derivatives detected in specimen 9 of *Amsinckia douglasiana* (A) and specimen 5 of *Amsinckia retrorsa* (B).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

5NO (2). Specimen 1 produced a single 7-acetyl derivative, presumably of intermedine, while specimen 2 produced both the 7-acetyl derivative, **4**NO, and the second 3′- acetyl derivative, **5**NO (2). In stark contrast, the specimen collected in San Mateo County, although still an intermedine chemotype, only produced the first 3′- acetyl derivative, **5**NO (1).

The levels of dehydropyrrolizidine alkaloids in the six specimens of *A. vernicosa* were all quite low, ranging from 2−26 μg 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 *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 consistencies within a species collected in the same area but decades apart, for example *A. eastwoodiae* specimens 4 and 11 collected in 1938 and 2013 in Tulare County, California both returned very low (ca. 1−2 μg equivalents intermedine/g plant material) total levels of dehydropyrrolizidine alkaloids. However, there were also some inconsistencies such as *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 μg 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 *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 were influenced by different collection, storage, and preservation processes. Notwithstanding this caveat, and although the dehydropyrrolizidine alkaloid data have not yet been correlated to an in-depth examination of morphology for the species examined, it seems evident that there are either: (1) few, robustly unambiguous differences in the dehydropyrrolizidine alkaloid profiles 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 *A. menziesii* var. *intermedia*, compared to four of the five *A. menziesii* samples and eight of the 10 *A. intermedia* samples

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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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