Analysis of Toxic Norditerpenoid Alkaloids in *Delphinium* Species by Electrospray, Atmospheric Pressure Chemical Ionization, and Sequential Tandem Mass Spectrometry

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A rapid electrospray mass spectrometry method was developed for screening larkspur (*Delphinium* spp.) plant material for toxic norditerpenoid alkaloids. The method was calibrated using two standard alkaloids, methyllycaconitine (**1**) and deltaline (**2**), with a recovery of 92% from spiked samples and relative standard deviations of 6.0% and 8.1% for the two alkaloids, respectively. Thirty-three samples of plains larkspur, *Delphinium geyeri*, were analyzed. Methyllycaconitine (**1**) concentration was 0.27% ± 0.08% during a 1-month period in 1997 establishing the relative risk of poisoning from the plant to be low. The method was also applied to the trace analysis (<1 ppm) of **1** in serum samples from sheep dosed different levels of the alkaloid. Electrospray ionization combined with sequential tandem mass spectrometry and HPLC coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry were used to detect and tentatively identify three new norditerpenoid alkaloids from *Delphinium nuttallianum* [bearline (**6**), 14-acetylbearline (**7**), 16-deacetylgeyerline (**8**)]. The tentative structure of the new alkaloids was predicted from the tandem mass spectra fragmentation patterns and assigning the substitution pattern for methoxy and acetyl groups at the C-14 and C-16 carbons.

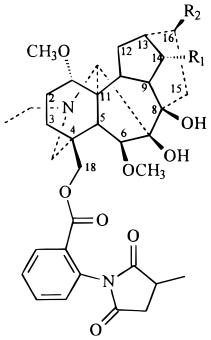
Keywords: *Delphinium; electrospray mass spectrometry; norditerpenoid alkaloids; poisonous plants*

INTRODUCTION

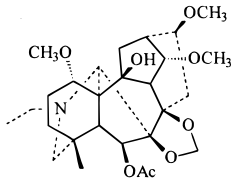
Many larkspurs (*Delphinium* spp.) found in the western U.S. are poisonous to livestock (Kingsbury, 1964; Pfister et al., 1999). For management purposes the key toxic larkspur species are categorized into three groups: (1) tall larkspurs (*Delphinium barbeyi*, *D. occidentale*, *D. glaucescens*, and *D. glaucum*) which grow in the higher mountain regions (>2000 m) among the aspen, conifer, and alpine meadow plant communities with an average mature plant height of 90−180 cm; (2) plains larkspur (*D. geyeri*) which grows primarily in the foothill and shortgrass prairie ranges with a mature plant height of 40−80 cm; and (3) low larkspurs (*D. andersonii*, *D. nuttallianum*, *D. bicolor*) which grow in the foothill and mountain ranges; typical mature plant height ranges from 10 to 60 cm.

The toxic compounds in larkspurs are C_{19} and C_{20} diterpenoid alkaloids (Jacyno, 1996 and references therein). Individual plants often contain 15 or more alkaloids, the most toxic of which contain the *N*-methylsuccinimidoanthranoyl (MSAL) functional group at C-18 (i.e. methyllycaconitine (**1**), Figure 1). These MSAL alkaloids are typically 20−100 times more toxic than lycoctonine or the methylenedioxylycoctonine (MDL) alkaloids (i.e., deltaline (**2**), Figure 1) (Manners et al., 1993, 1995). The toxic alkaloids act as potent neuromuscular blocking agents causing muscle weakness and paralysis, followed by respiratory failure and death if ingested at a lethal dose (Nation et al., 1982; Benn and Jacyno, 1983; Kukel and Jennings, 1994; Jacyno, 1996).

MSAL Alkaloids



MDL Alkaloids



Deltaline (**2**)

MSAL Alkaloids	R_1	R_2	Mol. Wt.
Methyllycaconitine (**1**)	OMe	OMe	682
14-deacetylnudicauline (**3**)	OH	OMe	668
Geyerline (**4**)	OMe	OAc	710
Nudicauline (**5**)	OAc	OMe	710
*Bearline (**6**)	OH	OAc	696
*14-acetylbearline (**7**)	OAc	OAc	738
*16-deacetylgeyerline (**8**)	OMe	OH	668
Unknown (**9**)	OAc	OH	696
Unknown	OH	OH	654

Figure 1. MSAL (*N*-methylsuccinimidoanthranoyllycoctonine) and MDL (methylenedioxylycoctonine) substituted norditerpenoid alkaloids. *Possible new compounds tentatively identified with the given common names.

The development of useful range management practices to reduce livestock losses from larkspur poisonings is dependent on understanding and assessing the po-

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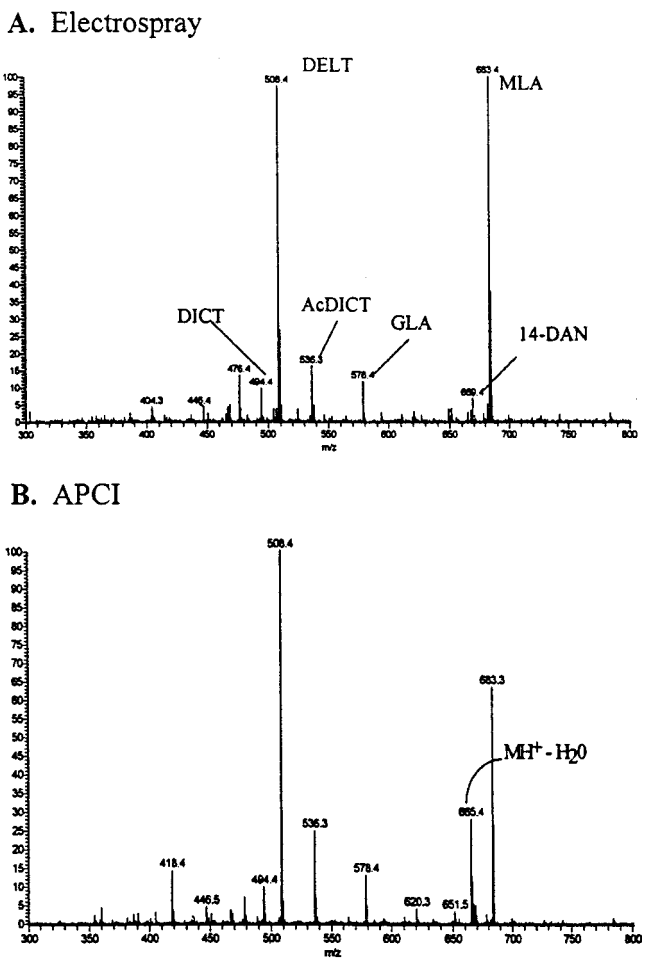

Figure 2. Comparison of (**A**) electrospray and (**B**) atmospheric pressure chemical ionization (APCI) techniques for crude alkaloid extract from a *Delphinium barbeyi* sample. DICT = dictyocarpine; DELT = deltaline (**2**); AcDICT = 14-acetyldictyocarpine; GLA = glaucenine/barbisine; 14-DAN = 14-deacetylnudicauline (**3**); and MLA = methyllycaconitine (**1**).

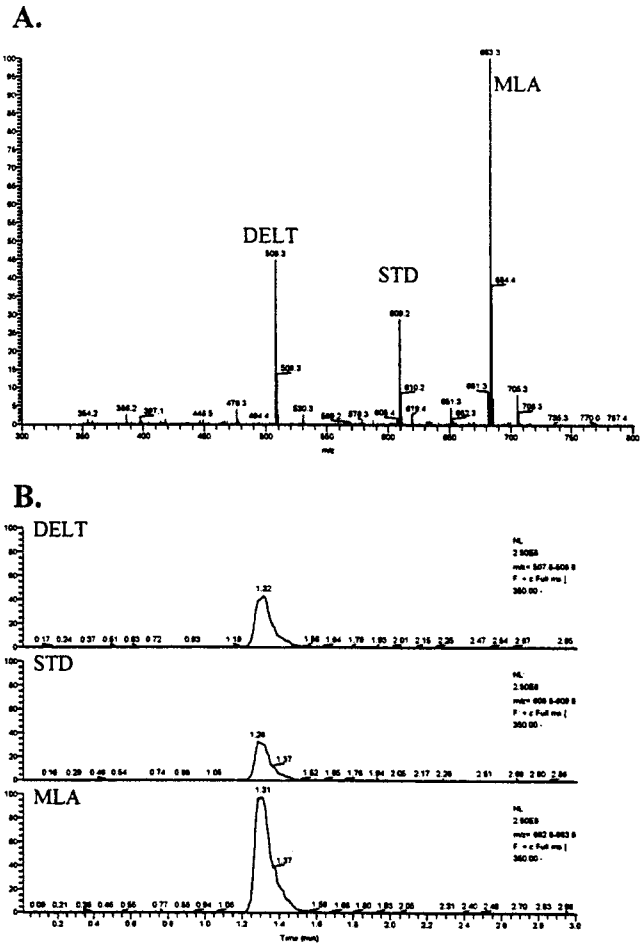

Figure 3. (**A**) Electrospray mass spectrum of a mixture of alkaloid standards deltaline (DELT) and methyllycaconitine (MLA). (**B**) Reconstructed ion chromatograms from the loop injection of the alkaloid standard mixture containing deltaline (DELT, $MH^+ = 508$), reserpine (STD, $MH^+ = 609$), and methyllycaconitine (MLA, $MH^+ = 683$).

tential toxicity of the various plants on the ranges. Ultimately this is achieved through a process of characterizing the various plant alkaloids, followed by toxicity testing, and analyses of plants sampled from the range. To help expedite this process new analytical tools are continually being sought to measure and characterize larkspur plant alkaloids.

Recently, atmospheric chemical ionization (APCI) (Wada et al., 1993; 1994) and electrospray (Marko and Stermitz, 1997; Chen et al., 1999) mass spectrometry (ESMS) have been reported as valuable analytical techniques for detecting and identifying diterpene-type alkaloids. The use of mass spectrometry for detection and characterization of toxic diterpene alkaloids provides a highly flexible analytical tool capable of providing both qualitative and quantitative information as needed. In our laboratory, some specific applications have included the use of APCI and ESMS for (1) the screening of larkspur plants for toxic alkaloids; (2) the quantitative measurement of individual alkaloids in plant samples and the serum of poisoned animals; (3) the use of sequential tandem mass spectrometry (MS^n) for characterization of alkaloid isomers and identification of new alkaloids from crude plant extracts; and (4) coupled LC/MS for detailed characterization of larkspur plant materials. It is the purpose of this paper to present examples from these four applications.

EXPERIMENTAL PROCEDURES

General Experimental Procedures. Solvents (methanol, chloroform, hexane, and 2-propanol) were HPLC grade. Acetic acid was 99.99% (Aldrich), and water was distilled and deionized. MS experiments were performed using a Finnigan LCQ (Finnigan Corp.) mass spectrometer equipped with either an electrospray (ES) or atmospheric pressure chemical ionization (APCI) source (Finnigan Corp.). ES solvent flow and HPLC flow were supplied using a HP 1100 binary pumping system (Hewlett-Packard), and sample injections were made using the HP 1100 autosampler. For electrospray, the solvent flow was 0.50 mL/min methanol/1% acetic acid (50:50) flowing into a Finnigan ES source with the following operating conditions: source voltage, 5 kV; capillary voltage, 3.7 V; capillary temperature, 250 °C; mass scan range 150−1000 amu. For LC-APCI-MS experiments, isocratic HPLC conditions were as previously described using a 250 × 4.6 mm column packed with 5 μm Spherisorb alumina (Keystone Scientific) with an isocratic flow of hexane/95% 2-propanol (85:15) at 3.0 mL/min (Manners and Pfister, 1993). The APCI source (Finnigan Corp.) was operated with source voltage 4.23 kV; vaporizer temp 450 °C; capillary voltage 23 V; capillary temperature 150 °C scanning a mass range of 350−1000 amu. For sequential tandem experiments (MS^n), collision induced dissociation (CID) spectra

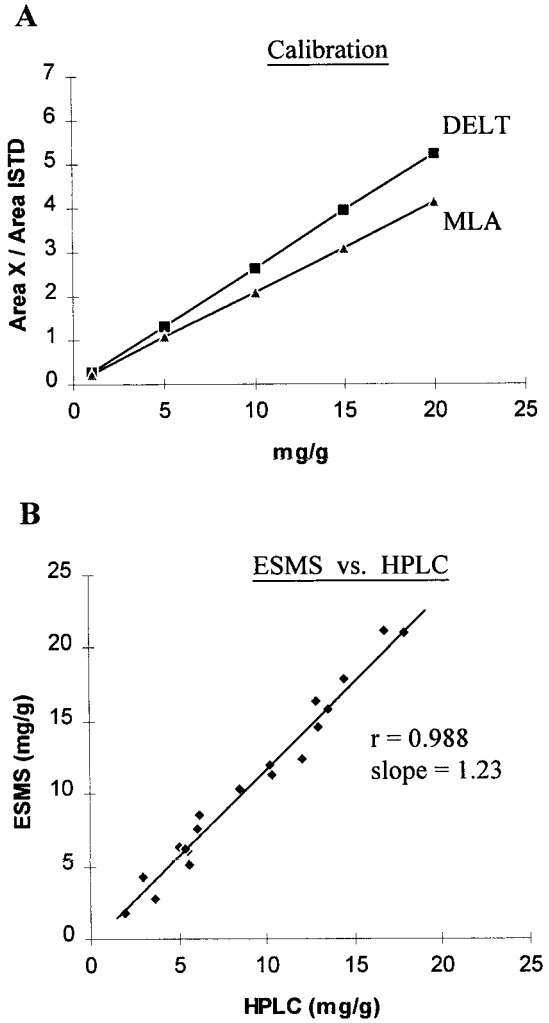

Figure 4. (**A**) Calibration of ESMS for deltaline and methyllycaconitine standards for quantitative analysis of larkspur alkaloids in plant material. (**B**) Comparison of ESMS and HPLC methods for determining total MSAL alkaloids (mg/g) in 20 tall larkspur samples (total MSAL = methyllycaconitine + 14-deacetylnudicauline + barbinine).

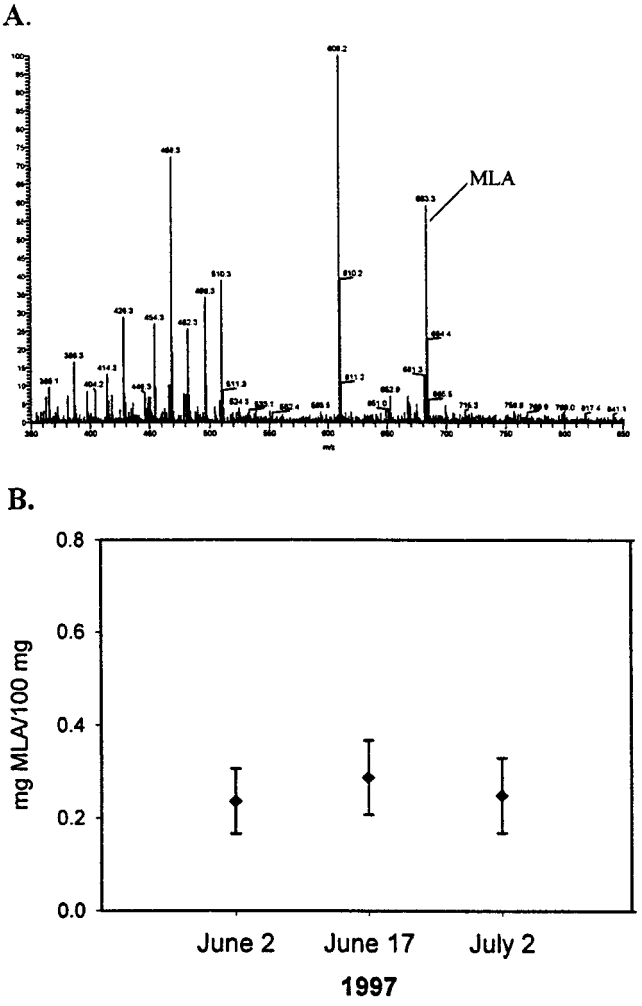

Figure 5. (**A**) Electrospray mass spectrum of alkaloids from *Delphinium geyeri* collected on the Maxwell Ranch in northern Colorado. (**B**) Results from quantitative analysis of the toxic alkaloid methyllycaconitine in *D. geyeri* plants collected on June 2 ($n = 10$), June 17 ($n = 10$), and July 2 ($n = 13$) during the summer of 1997 and measured from reconstructed ion chromatograms for methyllycaconitine using the internal standard method.

were obtained using helium as a damping gas introduced into the trap at flow rate of 1 mL/min. Collision energy was selected (using arbitrary units ranging from 10 to 30%) at a value to yield detected parent ions at 1−5% relative abundance.

Plant Collections. *Delphinium barbeyi* (PPRL collection no. 89-3) was collected July 6, 1989 near Manti, UT. *Delphinium nuttallianum* was collected east of Bear Lake, UT, May 1997 and May 1998 (Intermountain Herbarium, Utah State University, voucher no. 226055). *Delphinium geyeri* was collected north of Fort Collins, CO, on June 2, June 17, and July 2, 1997. All plants were air-dried at ambient temperatures and then ground in a Wiley Mill to pass a 1 mm screen.

Plant Extraction. For electrospray MS screening, ground plant material (100 mg) was placed in a 10 mL screw cap test tube, 5 mL of absolute methanol was added, the tube was sealed, and the sample was extracted for 1−2 h by mechanical rotation. After extraction the sample was centrifuged and a 25 μL aliquot was added to 1.0 mL of the electrospray flow solvent (50:50 mix of methanol and 1% acetic acid). For quantitative analysis the initial methanol extraction time was increased to 18 h (overnight). After the overnight extraction, reserpine (500 μg) was added as an internal standard, and the sample was mixed for 5 min and centrifuged. An aliquot (25 μL) of the extract was then added to 1.0 mL of ES flow solvent.

For liquid chromatography/mass spectrometry analysis a 100 mg sample was extracted with a mixture of 1% H_2SO_4 (4 mL) and $CHCl_3$ (4 mL) for 45 min and centrifuged, and the aqueous portion was removed. The sample was extracted a second time with 2 mL of 1% H_2SO_4 for 5 min. The two acid extracts were combined, and the pH was adjusted to 9 by the addition of concentrated NH_4OH. The sample was then extracted twice (4 mL, 2 mL) with $CHCl_3$ and filtered through anhydrous sodium sulfate, and the solvent was removed by evaporation under a stream of nitrogen on a heating block (70 °C).

Quantitative Electrospray. A set of five standards containing 0.1, 0.5, 1.0, 1.5, and 2.0 mg each of methyllycaconitine and deltaline alkaloid standards and 0.5 mg of reserpine (internal standard) was prepared in 4 mL of $CHCl_3$. An aliquot (25 μL) of each standard was added to 1 mL of ES flow solvent and a 20 μL loop injection made. The HPLC system was programmed for a 5.0 min isocratic run at 0.5 mL/min (no column inline). After injection the sample was pumped directly

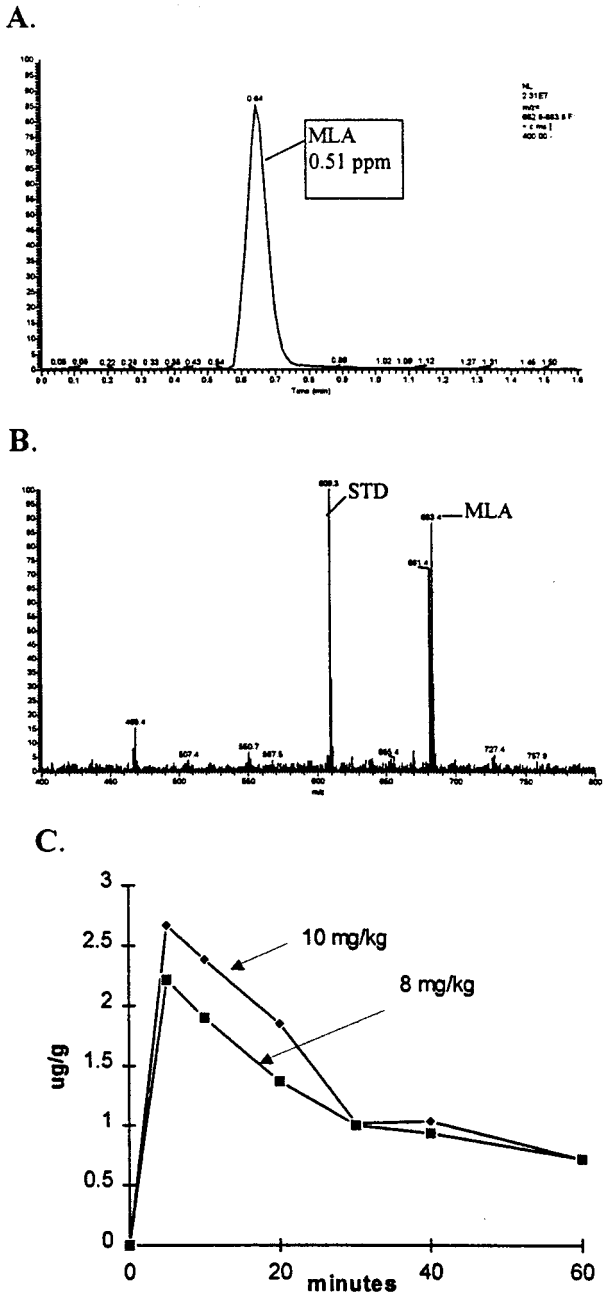

Figure 6. (**A**) Reconstructed ion chromatogram (m/z = 683) from loop injection of ovine serum sample containing 0.51 ppm methyllycaconitine (MLA). (**B**) Electrospray mass spectrum of serum extract containing MLA and possible MLA metabolite (m/z = 681). (**C**) MLA serum concentration (ppm = μg/mL) in two sheep given a single iv dose of purified MLA (10 mg/kg b.w. and 8 mg/kg b.w.) after iv injection of the purified alkaloid.

into the ES source. Analysis of individual alkaloids was completed by measuring peak area from reconstructed ion chromatograms for the protonated molecular ions of the analytes. Response (peak area analyte/peak area internal standard) versus (μg/sample amount) analyte was used to generate calibration curves for methyllycaconitine and deltaline standards. The relative standard deviation from the method was measured after replicate (n = 10) analysis of a *Delphinium barbeyi* sample (89-3) and was 8.1% for deltaline and 6.0% for methyllycaconitine. This same *D. barbeyi* plant sample (in triplicate) was spiked with the alkaloid standards, and recovery was 92%.

Sheep Serum Extraction. Two sheep were given a single dose of purified methyllycaconitine (no. 6326 = 10 mg/kg b.w., no. 6327 = 8 mg/kg b.w.). Blood samples were taken periodically for 1 h after dosage. The serum was obtained by standard procedures and retained (−20 °C) until further analyses. An aliquot (4.0 mL) of ovine serum was extracted by adding 5 drops (Pasteur pipet) of concentrated ammonium hydroxide and 4 mL of $CHCl_3$ and mixing the sample for 5 min. The samples were then centrifuged to aid layer separation, and the $CHCl_3$ portion was removed and filtered through a small plug of anhydrous Na_2SO_4 in a Pasteur pipet. The aqueous serum portion was extracted a second time with 4 mL of $CHCl_3$, the two extracts were combined, the samples were placed in a heating block (70 °C), and the solvent was removed by evaporation under nitrogen flow. Reserpine (2.0 μg) was added with 2.0 mL of the ES flow solvent and 20 μL injection made for ESMS analysis. Amount of methyllycaconitine was measured based on internal standard method and a response factor of 1.10. Serum methyllycaconitine concentrations (ppm) were then calculated as μg of methyllycaconitine/4.0 mL of sample.

Under the experimental conditions described it is important not to allow the samples to remain for long periods of time (>24 h) in the ES flow solvent. We observed transesterification of the succinimido group producing the succinimido-methyl ester (MH^+ + 32) after samples had remained in the methanol/acetic acid solvent for several days.

RESULTS AND DISCUSSION

Larkspur Plant Screening and Toxic Alkaloid Analysis. Marko and Stermitz (1997) first described the analysis of norditerpenoid alkaloids from *Castilleja* and *Delphinium* plants using electrospray mass spectrometry (ESMS). Crude alkaloids were isolated from plant material using a classical solvent extraction and acid/base partition procedures. Infusion of the isolated crude alkaloid solution into the ESMS was used to detect and characterize alkaloids from *Delphinium* plants and their transfer to *Castilleja* plants via root parasitism. Using somewhat similar methodology we developed a rapid analytical method for screening larkspur plant material in small amounts.

In brief, the dry ground plant material (≤100 mg) is extracted with methanol for several hours. An aliquot is diluted with the electrospray (ES) flow solvent and a loop injection made into the ES source and mass spectrometer. Under the ES conditions used, no fragmentation is observed, and the resulting mass spectrum is a simple array of molecular ions (MH^+) from the alkaloids. From the example in Figure 2A, the major alkaloids of *Delphinium barbeyi* were detected (methyllycaconitine (**1**), MH^+ = 683 and deltaline (**2**), MH^+ = 508) along with several of the minor alkaloids (dictyocarpine, MH^+ = 494; 14-acetyldictyocarpine, MH^+ = 536; glaucenine/barbisine, MH^+ = 578; 14-deacetylnudicauline (**3**), MH^+ = 669). The most toxic alkaloids, those possessing the *N*-methylsuccinimidoanthranoyl (MSAL) functional group at C-18 (i.e., methyllycaconitine), have higher molecular weights. Thus, a cursory scan of the mass spectrum from the region 650−750 Da can quickly identify the most toxic alkaloids in the sample. For example in Figure 2A, the most toxic alkaloids identified are **1** and **3**.

Table 1. Sequential Tandem Mass Data Collected from CID Spectra of the Protonated Molecular Ion and Then Sequentially from the Most Abundant Fragment Ion

alkaloid	(MH^+)	MS^2	MS^3	MS^4
methyllycaconitine	683	683(3), 665(13), **651**(100), 619(45), 605(15), 601(14), 587(10), 573(11), 569(4), 386(10)	650(6), 633(45), **619**(100), 605(38), 601(45), 587(25), 573(25), 569(14), 386(22), 354(11)	619(8), 601(66), **587**(100), 573(47), 569(22), 559(11), 404(10), 386(72), 354(27), 216(49)
geyerline	711	711(1), 693(4), 679(45), **651**(100), 647(6), 619(14), 601(3), 587(10), 569(2), 354(2)	651(5), 633(11), **619**(100), 601(20), 587(25), 573(12), 569(12), 541(4), 354(5)	619(1), **601**(100), 587(33), 571(35), 569(74), 541(14), 354(9), 336(11), 216(12)
bearline	697	697(1), 679(4), 665(31), **637**(100), 633(5), 619(3), 605(12), 573(9), 555(1), 340(2)	637(3), 619(11), **605**(100), 587(22), 573(28), 559(10), 555(15), 527(3), 340(5), 322(4)	**587**(100), 573(24), 559(9), 557(33), 555(66), 527(15), 340(13), 322(10), 294(6), 216(11)
14-acetylbearline	739	739(7), 721(3), 707(54), 689(2), **679**(100), 675(8), 647(12), 629(2), 615(10), 382(2)	679(26), 661(11), **647**(100), 629(15), 615(27), 601(14), 597(13), 569(4), 382(5), 216(3)	629(99), 615(34), 601(17), 599(28), **597**(100), 569(17), 382(10), 364(12), 216(11)
nudicauline[a]	711	711(8), 710(13) 693(11), **679**(100), 661(9), 647(42), 633(14), 629(10), 601(8), 414(11)	679(6), 678(15), 661(42), **647**(100), 633(55), 629(56), 615(9), 601(26), 587(10), 414(26)	647(40), 629(74), 615(45), 601(64), 587(39), 569(13), 545(16), 432(9), **414**(100), 382(11)
14-deacetylnudicauline[a]	669	668(4), 651(16), **637**(100), 619(15), 605(55), 591(17), 587(14), 573(8), 559(11), 372(16)	637(4), 619(41), **605**(100), 601(6), 591(31), 587(38), 573(21), 559(22), 555(7), 372(26)	605(30), **587**(100), 577(23), 573(70), 559(37), 555(27), 529(17), 390(16), 372(93), 340(32)
16-deacetylgeyerline	669	651(11), **637**(100), 619(21), 605(46), 591(12), 587(21), 559(9), 372(15), 354(5), 216(12)	637(9), 619(88), **605**(100), 591(37), 587(76), 569(10), 559(17), 372(31), 354(11), 216(27)	605(20), **587**(100), 577(6), 569(22), 559(23), 372(70), 354(18), 326(10), 216(42), 188(8)

[a] Mass data were not collected below 250 amu.

The identification of an individual alkaloid from the ES mass spectrum is based solely on the presence of a molecular ion and the correlation to a known molecular weight of an alkaloid previously identified as present in the toxic tall larkspur plant material being analyzed. We are confident of the assigned alkaloids in the case of tall larkspur species (*Delphinium barbeyi*, *D. occidentale*, *D. glaucescens*, and *D. glaucum*) based on prior chemical analyses of hundreds of plant samples (Pelletier et al., 1981; Joshi et al., 1988; Kulanthaivel et al., 1988; Joshi et al., 1989; Pelletier et al., 1989; Kulanthaivel et al., 1990; Manners et al., 1996, 1998). In analysis of new plant material the ES mass spectrum may provide initial evidence as to the possible alkaloid content of the plant material, but other supporting spectroscopic or chromatographic data must be obtained to verify specific alkaloid identifications. This fact is later demonstrated from the analysis of low larkspur (*Delphinium nuttallianum*) plants using tandem mass spectrometry and liquid chromatography/mass spectrometry techniques.

Analysis of larkspur plant extracts using atmospheric pressure chemical ionization (APCI) mass spectrometry yields similar results (Figure 2B). Under APCI conditions the lower molecular weight alkaloids (i.e., deltaline (**2**) and other MDL alkaloids) produce molecular ions, but the MSAL type alkaloids (i.e., methyllycaconitine) yield a significant fragment ion from loss of water [methyllycaconitine = 683 (MH^+), 665 $(MH^+ - 18)$]. Since such fragment ions could complicate the interpretation of the mass spectrum from unknown samples, ES ionization was chosen as the primary method for screening larkspur plant material and used for further development of a quantitative method for the alkaloid mixtures.

Ultimately, the toxicity of any larkspur plant material is dependent on both the type and concentration of alkaloid in the plant. We therefore made the ESMS method quantitative by extending the plant sample extraction period (overnight) and by adding an internal standard (reserpine). The method is configured to run as a 5-min analysis. One minute from the start of the run, a loop injection is made using the HPLC autosampler, and the sample is quickly introduced into the ES source producing a single peak in the total ion chromatogram. Data are collected for 2 more min to the end analysis and the next sample is processed. This method is essentially a flow injection type analysis. After the loop injection and data collection, individual ion chromatograms can be reconstructed for each alkaloid of interest and the peak areas calculated (Figure 3). In this manner compounds are separated by mass as opposed to chromatography for quantitation. A set of standards ($n = 5$) containing known amounts of two alkaloids (**1** and **2**) and the internal standard (reserpine) were prepared and analyzed using the described method. Calibration curves were plotted (Figure 4A) for the two major alkaloids and were linear ($r^2 \geq 0.990$). The precision (relative standard deviation) of the method, as measured from multiple ($n = 10$) analyses of a *Delphinium barbeyi* plant sample, was 8.1% and 6.0% for deltaline and methyllycaconitine, respectively. Average recovery ($n = 3$) from spiked plant samples was 92% for both alkaloids.

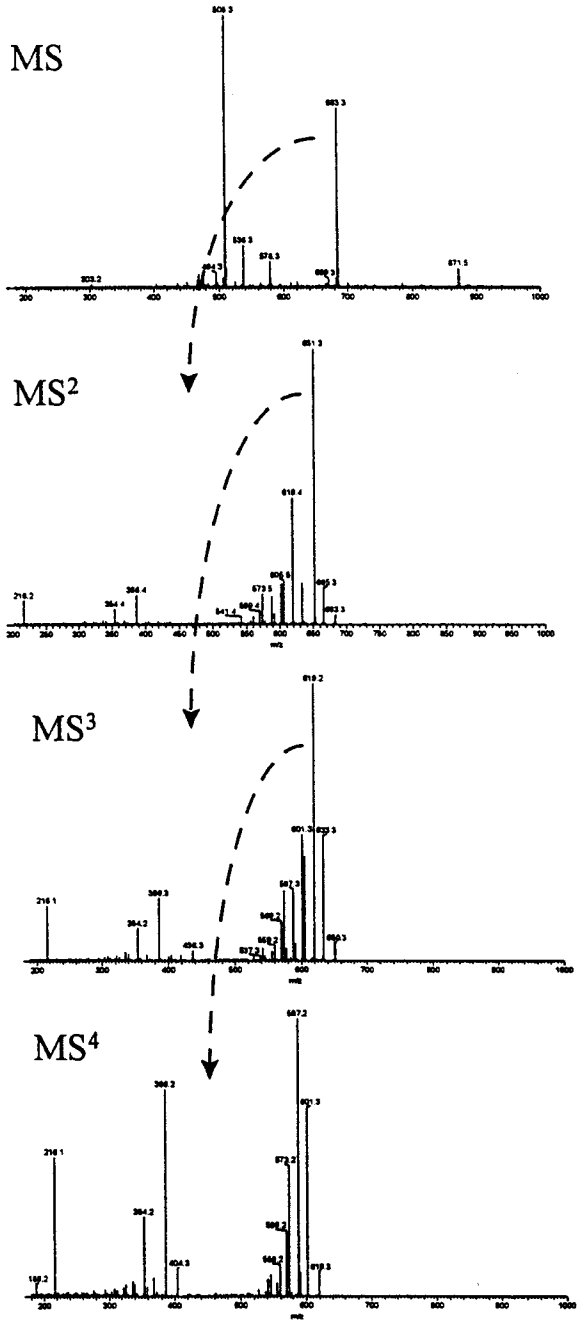

Figure 7. Tandem mass spectrometry (MS^n) analysis of the toxic norditerpenoid alkaloid methyllycaconitine ($MH^+ = 683$) showing sequential losses of methanol (− 32) at $m/z = 651$, 619, and 587.

An accurate measurement of all individual alkaloids in a plant sample becomes difficult because of the lack of appropriate standards and the required generation of calibration curves for each alkaloid. Therefore we used the following approach. Available standards of **1** and **2** were chosen as representative compounds for the two most common alkaloid types (MDL and MSAL, Figure 1) found in the poisonous larkspur plants. The measurement of each alkaloid of interest was based on these two calibration curves. For example, the amount of 14-deacetylnudicauline (**3**), a MSAL type alkaloid, would be measured from the methyllycaconitine calibration curve; and the amount of dictyocarpine, a MDL alkaloid, would be measured from the calibration curve based on the deltaline standard. Using the described method a large number of samples can be extracted overnight, aliquots and dilutions made the next day, samples loaded into the autosampler, and samples run in sequence approximately every 5 min.

Twenty samples, previously analyzed by HPLC (Manners and Pfister, 1993), were selected and analyzed using the described method. The level of methyllycaconitine, 14-deacetylnudicauline, and barbinine were measured and then summed to give total MSAL alkaloids. These values were then compared to those previously determined by HPLC (Figure 4B). As expected, the ESMS method resulted in slightly higher levels of total MSAL alkaloids as compared to the values measured by HPLC. The HPLC method requires extraction of the alkaloids using an acid/base partition procedure in which some loss of alkaloid occurs. The ESMS method uses a simple one-step methanol extraction with no chance of alkaloid loss.

In application of the method, 33 samples of *Delphinium geyeri* were analyzed to measure toxic alkaloid levels during a 1-month grazing period for an area of rangeland near Fort Collins, CO (Maxwell Ranch). A qualitative examination of the mass spectrum from individual samples (Figure 5A) identified the major toxic alkaloid as methyllycaconitine (**1**). Ion chromatograms were analyzed for all samples, and the peak areas were measured. The average toxic alkaloid concentration did not significantly change during the 1-month period (Figure 5B), and based on this analysis ($0.27\% \pm 0.08\%$, $n = 33$) the relative risk of grazing this pasture by cattle was considered to be low (Pfister et al., 1997); indeed there were no significant losses from larkspur poisoning in this area in 1997.

Serum Analysis. The analysis of toxic norditerpenoid alkaloids in serum is of importance for the investigation of toxicokinetics, detection of possible toxin metabolism, and potential diagnostic purposes. Normal-phase liquid chromatography (Manners and Pfister, 1993) and IR spectroscopy (Gardner et al., 1997) are proven methods for the analysis of plant material where typical alkaloid concentrations are > 1 mg/g. However, these methods do not have the sensitivity required for the analysis of trace levels (<1 ppm) of alkaloids in samples such as serum from animals poisoned by larkspur or laboratory animals dosed alkaloids during toxicity testing. Some success has been reported for the analysis of the alkaloid methyllycaconitine at concentrations below 1 ppm in rat plasma and brain tissue using reverse-phase chromatography with electrochemical detection (Turek et al., 1995). However, we have found ESMS to be an excellent technique for trace level analysis of toxic alkaloids in such samples as serum.

In a study to determine the minimum effective dose (ED_{50}) of methyllycaconitine in sheep, study animals were dosed the purified alkaloid by iv infusion. Blood samples were taken periodically from the animals during a 60 min time period after infusion. The serum was then isolated, extracted, and analyzed by ESMS. Reconstructed ion chromatograms for $m/z = 683$ (MH^+, methyllycaconitine) were extracted from the full scan ($m/z = 400-800$) data, and **1** could easily be detected in the sheep serum below 1 ppm (Figure 6A). The alkaloid serum levels measured for all samples taken during the first hour after dosage are plotted in Figure 6C. This preliminary data indicates a bimodal elimination of the alkaloid from the serum. In addition, a cursory examination of the full scan mass spectrum obtained from the serum samples (Figure 6B) detected

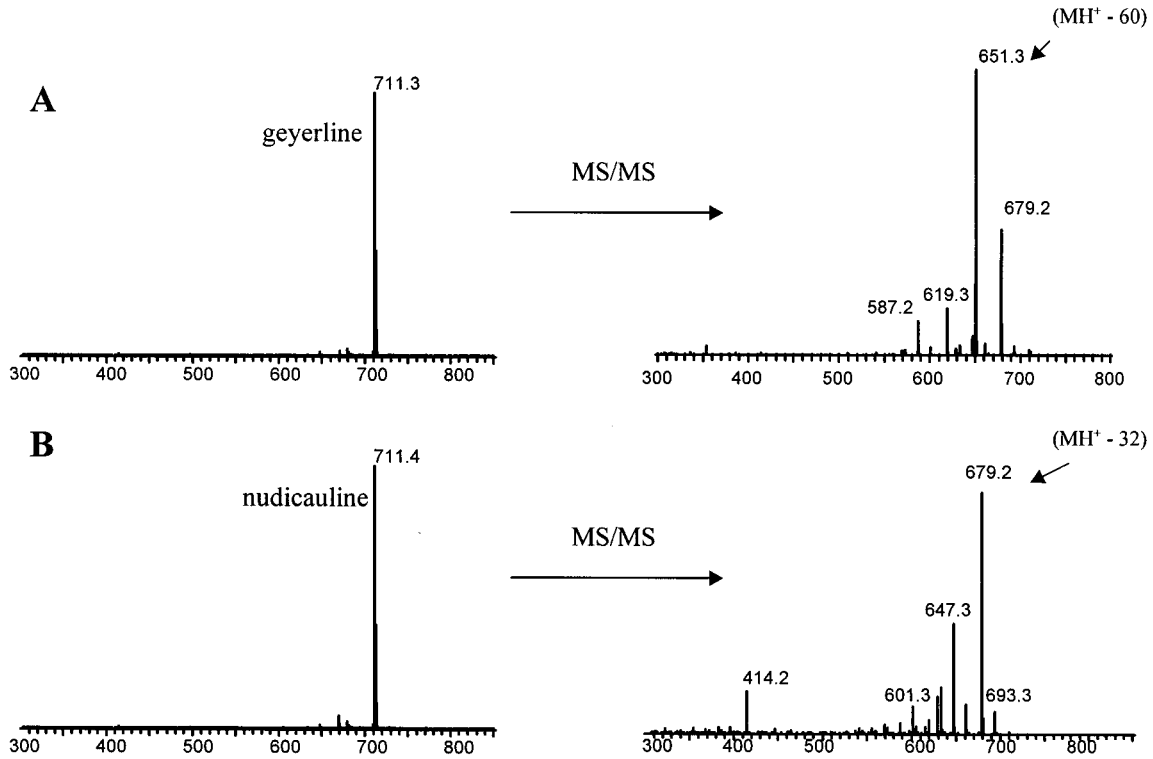

Figure 8. (**A**) Electrospray mass spectrum of the alkaloid geyerline (m/z = 711.3) and the resulting product ion spectra (MS^2) showing preferential loss of acetic acid ($MH^+ - 60$) at the C-16 position. (**B**) Electrospray mass spectrum of nudicauline (m/z = 711.4) and the resulting product ion spectra for nudicauline with the primary loss of methanol ($MH^+ - 32$).

a possible metabolite of **1** in the serum. The observed 681 ion could be the molecular ion from oxidized methyllycaconitine. A further investigation of the toxicokinetics and possible metabolism of methyllycaconitine and other toxic larkspur alkaloids is still in progress.

Sequential Tandem Mass Spectrometry. One advantage of an ion-trap mass spectrometer is its ability to perform sequential tandem mass (MS^n) experiments providing structurally informative fragment ions generated by collision induced dissociation (CID) of the molecular ion. For the toxic norditerpenoid alkaloids in larkspur the most abundant fragment ions in MS^n generated product ion scans occur from loss of functional groups (methoxy and acetate) at the oxygenated positions of the molecule (Table 1), thus the advantage of obtaining this type of structural information from ion fragmentation is apparent. Chen et al. (1999) similarly showed principal losses of H_2O ($MH^+ - 18$), methanol ($MH^+ - 32$), and acetic acid ($MH^+ - 60$) from tandem CID spectra of diterpene alkaloids from *Aconitum* species and the ability to use the muliple MS procedures for structural characterization of diterpene alkaloids in a similar manner.

The toxic larkspur alkaloid methyllycaconitine (**1**) shows sequential losses of methanol ($MH^+ - 32$, most abundant ions) for the first three tandem mass experiments (Figure 7). It is not known at which location each group is lost during the tandem experiments, but based on further examples from known compounds it is hypothesized that the functional groups at C-16 are the most labile and those at C-14 are the most stable. For example, the alkaloids nudicauline (**5**) and geyerline (**4**) cannot be distinguished based solely on their ESMS because they have the same molecular weight and therefore both produce molecular ions of 711 Da (Figure 8A,B) by ESMS. Compounds **4** and **5**, however, can be distinguished based on the MS^2 product ion spectra. Geyerline (**4**) shows preferential loss of acetic acid (m/z = 651, $MH^+ - 60$), while the major fragment loss from **5** is methanol (m/z = 679, $MH^+ - 32$). It is noted that in the MS^2 spectrum of **5** that fragmentation of the acetyl group at C-14 (m/z = 651) is not detected nor is the loss of acetic acid observed in additional sequential (up to MS^4) tandem experiments with **5** (Table 1). The data from these two alkaloids as well as others (Table 1) support the hypothesis of a preferential loss of the functional group at the C-16 position and an apparent stability of the functional group at C-14. Information gained from such MS^n experiments has allowed us to identify several possible new compounds directly from small amounts of crude methanol plant extracts.

Samples of low larkspur (*Delphinium nuttallianum*) were collected from locations in the Rocky Mountain region as part of an initial study on toxic low larkspur species in the U.S. and for comparison to work completed in Canada (Bai et al., 1994). These plants were screened using ESMS to see what toxic alkaloids might be present. Most samples were found to contain the alkaloids **1**, **3**, **4**, and **5** as the major toxic norditerpenoid alkaloids. However, the ES mass spectrum from one particular location (Bear Lake, UT) showed the presence of two new alkaloids in relatively high concentrations as evident from molecular ions observed at 697 and 739 daltons (Figure 9A). MS^n experiments were conducted on both parent ions by direct infusion of the crude methanolic extract into the ES source (Figure 9B,C).

The most abundant ion in the MS^2 product ion scan of 697 ion was m/z = 637 ($MH^+ - 60$) placing the acetate group at the C-16 position assuming our hypothesis on the fragmentation sequence is correct. The MS^3 product ion spectrum of the isolated 637 ion was similar to the MS^3 spectrum of **3** indicating two additional methoxy

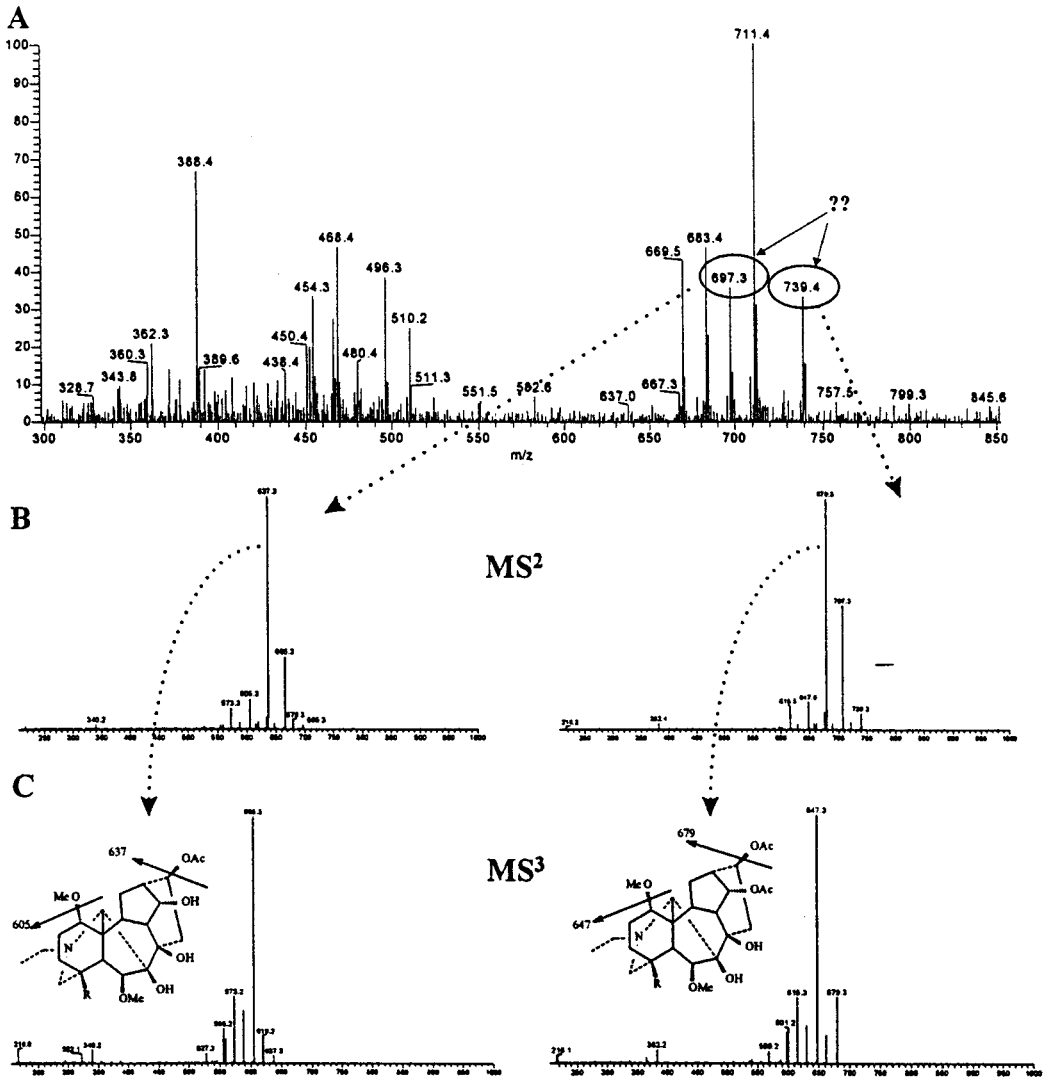

Figure 9. (**A**) ES mass spectrum of crude methanolic extract of *Delphinium nuttallianum* (Bear Lake) showing two previously unidentified alkaloids at $m/z = 697$ and 739. (**B**) MS^2 product ion spectra for $MH^+ = 697$ and 739 ions showing loss of acetic acid ($MH^+ - 60$) at $m/z = 637$ and 679. (**C**) Sequential MS^3 product ion spectra of 637 and 679 showing loss of methanol (-32) at $m/z = 605$ and 647.

groups at C-1 and C-6. As far as we know, the substitution pattern proposed for **6** has not been previously described, and the alkaloid was given the common name bearline. A similar MS^n analysis of the molecular ion 739 indicates acetate groups at C-14 and C-16 as follows. The C-16 acetate was lost in the initial MS^2 experiment. The MS^3 spectrum was then similar to that of **5** (C-1 methoxy, C-6 methoxy, and C-14 acetate) showing the sequential loss of two methoxy groups and retention of the C-14 acetate group. This compound ($m/z = 739$) was therefore identified as 14-acetylbearline (**7**). Subsequently, **6** and **7** have been isolated after a large scale extraction of plant material, and the structural identity has been confirmed by NMR spectroscopy (details will be reported in a separate publication along with experimental mouse toxicity data for the new alkaloids of *Delphinium nuttallianum*).

The combination of electrospray and sequential tandem mass spectrometry provides a relatively rapid technique for screening large numbers of small plant collections and in the case of larkspur plants can quickly identify plants possessing toxic alkaloid compounds or particular plants that might contain new alkaloids. Larger amounts of plant material can then be collected based on those containing new or interesting alkaloids and a more classical phytochemical characterization completed to identify the alkaloids and isolate and purify material for toxicity testing. Product ion spectra (CID spectra) from tandem mass spectrometry can also be used to verify the identity of specific alkaloids or the presence of isomeric compounds in larkspur plant material.

Liquid Chromatography/Mass Spectrometry (APCI). A number of HPLC methods have been used in the analysis of larkspur alkaloids (Manners and Pfister, 1993; Majak et al., 1987), and Wada et al. (1994) reported on the application of HPLC coupled to APCI mass spectrometry for the analysis of diterpene alkaloids of *Aconitum*. Since our laboratory has extensively used the normal-phase HPLC method developed by Manners and Pfister (1993), we chose those chromatographic conditions coupled to an APCI source for LC/MS type analyses.

A total ion chromatogram, taken from the LC/MS analysis of the crude alkaloid fraction isolated from low larkspur, *Delphinium nuttallianum* (Bear Lake collection), is shown in Figure 10. The previously identified toxic alkaloids from the ES screening experiment were detected from selected ion chromatograms and included **3** ($m/z = 669$), **1** ($m/z = 683$), **6** ($m/z = 697$), **5** and **4**

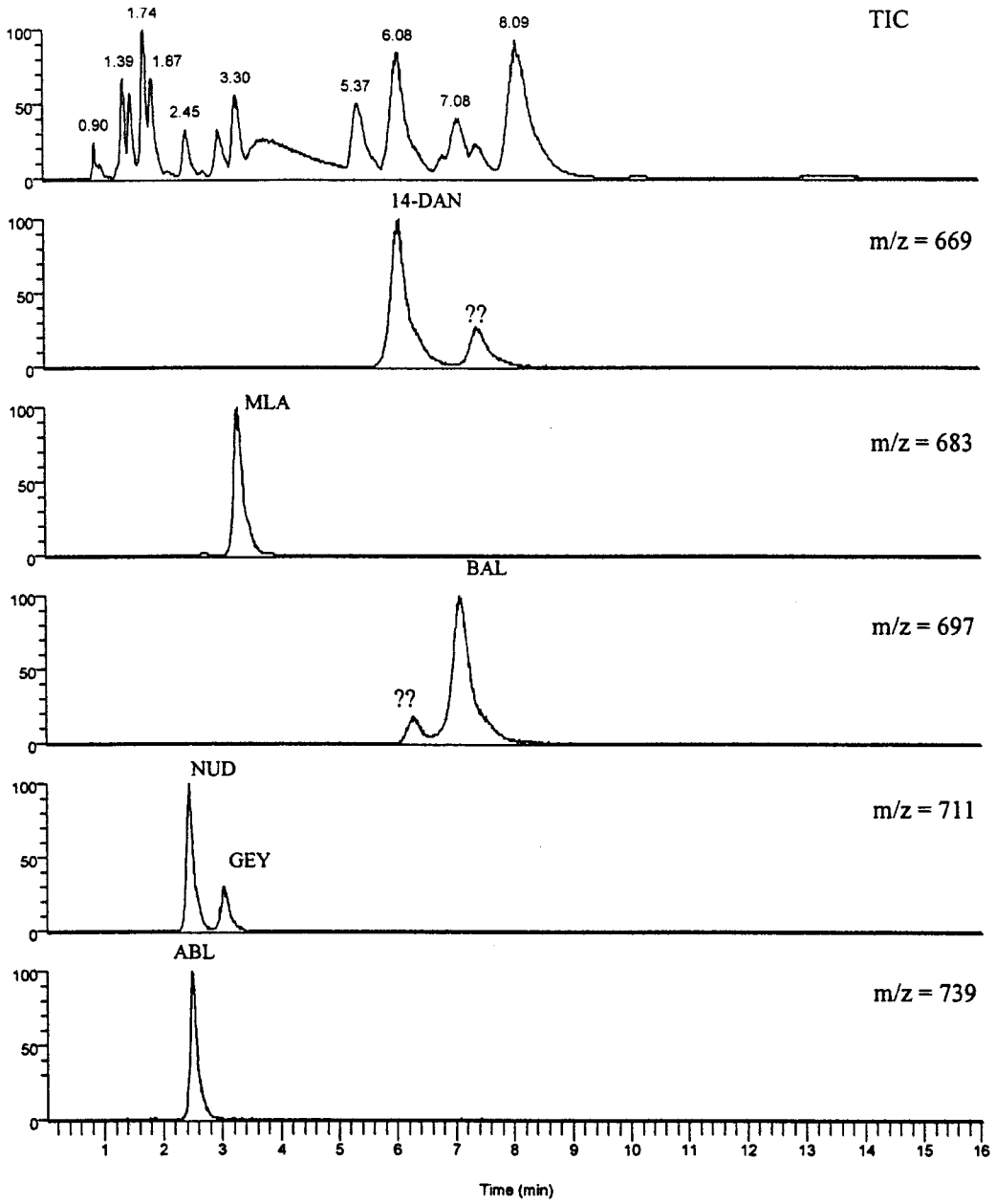

Figure 10. LC/MS analysis using APCI source and normal phase liquid chromatography and the crude alkaloid mixture isolated from *Delphinium nuttallianum* (Bear Lake). Reconstructed ion chromatograms identifying the alkaloids 14-deacetylnudicauline (DAN), methyllycaconitine (MLA), bearline (BAL), geyerline (GEY), nudicauline (NUD), 14-acetylbearline (ABL) and two unknown alkaloids (??).

(m/z = 711), and **7** (m/z = 739). In addition, two other small unknown peaks (marked as ??) were observed in the 669 and 697 ion traces. The unknown 669 compound (R_t = 7.5 min) was subsequently isolated using semi-preparative scale HPLC. The MS^n spectra of the isolated compound was very similar to that of **3** (the other peak in the 669 ion trace). Therefore a portion was acetylated and analyzed by both ES(MS^n) and LC/MS. The LC retention time and MS^n analysis of the acetylated product were identical to the known alkaloid geyerline (**4**). We have therefore tentatively identified the compound as 16-deacetylgeyerline (**8**). The other unidentified compound (MH^+ = 697) remains unknown at this time, but it is speculated that it is the C-14 acetyl, C-16 hydroxy compound (**9**).

The *Delphinium nuttallianum* plant material collected from the Bear Lake site appears to be exceptionally prolific in producing an array of toxic norditerpenoid alkaloids. Interestingly, nearly all the methoxy and acetyl substitution possibilities at the C-14 and C-16 positions were identified from this plant material (Figure 1). In a serendipitous manner this plant material provided excellent examples of several possible mass spectrometry experiments that can be conducted using ES and APCI sources, tandem MS, and HPLC procedures.

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