JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Usnic Acid in Lichen Toxic to Elk by Liquid Chromatography with Ultraviolet and Tandem Mass Spectrometry Detection

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Usnic acid is unambiguously confirmed by tandem mass spectrometry (MS/MS) in tumbleweed shield lichen, *Xanthoparmelia chlorochroa.* The lichen contains 2% usnic acid by liquid chromatography with UV quantification at 282 nm. The UV linear range for usnic acid quantification is from its 4 ng limit of detection to 2 μg injected. UV signal saturation is recognized by distortion of the usnic acid UV spectrum. Positive ion electrospray−tandem mass spectrometry offers no similar means to recognize quantification data recorded above the linear range of electrospray. Electrospray ionization capacity and matrix effects limit the reliability of tandem mass spectrometry quantification. The combination of UV quantification and MS confirmation provides a reliable analytical method for measuring usnic acid levels in plant material.

KEYWORDS: Elk deaths; liquid chromatography-tandem mass spectrometry; LC-MS/MS; tumbleweed shield lichen; *Usnea barbata*; usnic acid; *Xanthoparmelia chlorochroa*

INTRODUCTION

In February and March of 2004, the consumption of lichen browse caused the deaths of an estimated 328 elk in the vicinity of the Daley Ranch wildlife habitat area of Carbon County, Wyoming (*1*). Because of a lack of food in this area, elk were left with little else but to eat tumbleweed shield lichen, *Xanthoparmelia chlorochroa* (*2*). Autopsies found this plant in the stomachs of all of the afflicted elk, making this lichen, or one of its chemical components, a likely candidate for the observed toxicity. Symptoms of intoxication were an alert and vocal state, accompanied by the inability to rise from the ground to eat, drink, or escape predators. On the basis of the symptoms and previous livestock poisoning, the toxic agent was thought to be usnic acid. Our laboratory had experience in the analysis of botanicals for usnic acid, so we obtained suspect browse from the Wyoming State Veterinary Laboratory and confirmed that the lichen contained 2% usnic acid. Another lichen, Old man's beard, *Usnea barbata*, known to contain usnic acid, was purchased for comparative analysis with the implicated elk browse. *U. barbata* was found to contain 1.5% usnic acid.

Lichens are symbiotic organisms composed of a fungus that cultivates a photosynthesizing partner of algae or cyanobacteria. The fungus gives the lichen its shape while its partner produces food. Lichens are found worldwide and are able to survive in extreme environmental conditions. Their success is attributed to a capacity to survive in a resting state for months, a slow metabolism, and production of biologically active compounds, which offer chemical protection to the lichen (*3*).

Usnic acid, 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-(2H,9bH)dibenzo-furandione, $C_{18}H_{16}O_7$, is a yellow pigment found only in some lichens. It is a product of the secondary metabolism of the fungal partner and exists in two enantiomers that differ in orientation of the methyl group located in position 9b. Usnic acid is also called usnein or usniacin since it is not an organic carboxylic acid (*4*). Its weak acidity comes from keto−enol tautomerism (**Figure 1**). Both enantiomers possess antimicrobial and antimycotic properties. The (−)-enantiomer is a selective herbicide due to its ability to inhibit carotenoid biosynthesis (*5*). These properties give usnic acid-producing lichens an advantage over competing plant and microbial species. This protection extends to the animal kingdom because of additional properties of usnic acid. It uncouples oxidative phosphorylation in mitochondria (*6*). A study of its use to control parasites showed damage to mitochondria of treated epimastigotes (*7*). Deleterious effects of usnic acid on mammalian cells have also been reported (*8*). A 1953 report of lichen livestock poisoning in Wyoming described symptoms consistent with the afflicted elk and suggested that a toxic dose was 1% of an animal's weight for 5 days or a single dose of 3.6% of an animal's weight (*9*). It induced necrosis of cultured mouse hepatocytes through oxidative stress and disruption of the normal metabolic processes of cells (*10*). In addition to animal toxicity, usnic acid was recently associated with fulminant liver failure in humans (*11*).

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10.1021/jf052767m
Published on Web 03/09/2006

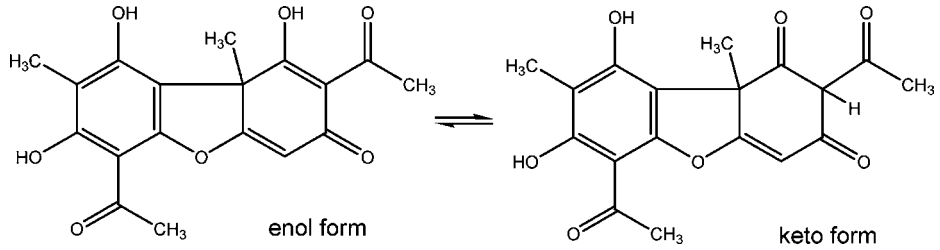

Figure 1. Enol and keto forms of usnic acid.

Lichens containing usnic acid and usnic acid itself are traditional medicine ingredients. Usnic acid is poorly soluble in water and many organic solvents (*12*). Glycols, surfactants, and complexing agents have been tried as possible nontoxic solubilization aids for usnic acid in tissue culture studies because of its poor solubility in nontoxic solvents (*13*). Therefore, traditional lichen teas, aqueous infusions, or decoctions are likely to contain minimal amounts of usnic acid. Alcohol extracts contain measurable levels of usnic acid (*14*), but direct ingestion of the solid material is a more likely route to harmful exposure. This may have allowed the tragic consequences of ingesting usnic acid to escape notice until it was popularized as a weight loss supplement (*15*).

Light, temperature, and humidity play an important role in the concentration of usnic acid in lichens. Seasonal variations in the concentration of usnic acid have been detected (*3*). If the toxic effect of usnic acid is dose-dependent, then elucidation of its role in the elk incident requires unambiguous confirmation of its presence in the suspect browse and a determination of the level of usnic acid in the browse.

The liquid chromatography−tandem mass spectrometry (LC-MS/MS) protocol applied to these analyses originally targeted toxins at parts per billion levels. Work was undertaken to determine suitable parameters for reliable quantification and confirmation of the percentage levels of usnic acid observed in the lichens. The resulting protocol for usnic acid utilizes room temperature solvent extraction of the lichen and analysis by LC with UV and MS/MS detection. Quantification is based on standard additions of usnic acid to each extract.

EXPERIMENTAL PROCEDURES

Chemicals and Consumables. Poly-DL-alanine, 98% (+)-usnic acid, and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Acetone, acetonitrile, methanol, and water were high-performance liquid chromatography (HPLC) certified grade solvents. Certified disposable pipet tips and 50 mL polypropylene conical tubes with caps were obtained from VWR Scientific Products (Buffalo Grove, IL). Maxi-Spin filter tubes, 0.45 μm PVDF, were obtained from Alltech Associates (Deerfield, IL). Amber glass autosampler vials were obtained from Agilent Technologies (Wilmington, DE). Luna 3 μm C8(2) 150 mm × 2.0 mm LC columns were obtained from Phenomenex (Torrance, CA). *U. barbata* was purchased from Stony Mountain Botanicals (Londonville, OH). Suspect lichen browse identified as *X. chlorochroa* was collected by the Veterinary Services Branch of the Wyoming Game and Fish Department from the incident area.

Caution. Usnic acid is a strong hepatotoxin associated with human liver failure. Acetic acid, acetone, acetonitrile, and methanol are hazardous. Avoid contact or inhalation exposure to these reagents.

Analytical Instruments. An Agilent 1100 HPLC equipped with a vacuum degasser, binary pump, autosampler, temperature-controlled oven, and 190−600 nm diode array detector was coupled to a Micromass Quattro triple-quadrupole mass spectrometer equipped with an electrospray source.

Preparation of Standards. A standard solution of 0.2 mg/mL usnic acid in acetonitrile was prepared using a red glass volumetric flask to protect the solution from light. Usnic acid in acetone was prepared in the same way.

Sample Preparation. Lichens were ground to pass 40 mesh screen in a model 3383-L10 Wiley mill (Thomas Scientific, Swedesboro, NJ). Portions up to 0.4 g of powdered lichen were accurately weighed into 50 mL polypropylene conical tubes to which 8 mL of acetonitrile was added. The tubes were capped and vigorously agitated for 20 min on a shaker table. Portions were centrifuged to settle solids with an Allegra 21 Beckman Coulter centrifuge using a C0650 head (Palo Alto, CA). The liquid fractions were decanted into Maxi-Spin filter tubes and spin filtered. Residual solids in the 50 mL polypropylene conical tubes were re-extracted with 8 mL of acetonitrile. The decanted liquid from the second extraction was poured into the filter tube containing the first extract. The filter tubes were then spin filtered again to combine the first and second extracts below the filter. The filtered extracts were quantitatively transferred to 100 mL volumetric flasks and diluted to volume with acetonitrile. Extracts were stored in the dark prior to transfer to amber sample vials for analysis. Aliquots of 0, 100, 200, 400, and 600 μL of usnic acid standard solution in acetonitrile plus 100 μL of acetonitrile extract and 900, 800, 700, 500, and 300 μL of acetonitrile were placed in screw-capped amber sample vials for analysis.

Analysis for Usnic Acid in Lichens by UV and Electrospray Ionization (ESI)-LC-MS/MS. The C8 column was maintained at 35 °C. Mobile phase A was 0.5% acetic acid in water. Mobile phase B was 0.5% acetic acid in acetonitrile. The gradient parameters were 40% B for 2 min, 100% B for 8−18 min, and 40% B for 24−34 min. The capillary cone voltage was 4.1 kV. The cone voltage was 20 V. The source temperature was 120 °C. The desolvation gas temperature was 250 °C. The desolvation gas flow was 700 L/h nitrogen. The cone gas flow was 165 L/h nitrogen. First and third quadrupole resolutions were 15 V. Polyalanine (0.1 mg/mL) in methanol was used to calibrate the mass scale of the instrument from 155 to 385 Da.

The argon collision gas pressure was adjusted to 3×10^{-3} mbar for MS/MS. The collision energy was varied for each monitored decomposition in multiple reaction monitoring mode (MRM). The decompositions monitored for usnic acid were 345 > 233 at 32 eV, 345 > 261 at 32 eV, 345 > 303 at 22 eV, 345 > 327 at 22 eV, and 345 > 345 at 2 eV. The ion dwell time was 0.06 s for each decomposition with 0.08 s of interchannel delay and 0.04 s of interscan delay.

Between 2 and 10 μL volumes of usnic acid solution, diluted extract, and spiked diluted extract were injected for analysis. UV and MS data were recorded for 15 min. The column was washed with 50:50 methanol/acetonitrile at the end of daily operations.

Usnic acid was confirmed in the lichen extracts by comparison of the data recorded for usnic acid standard and unspiked lichen extracts. The relative abundances of the signals recorded for the extracts at the retention time of usnic acid were within ±10% of standard usnic acid.

Quantification was by UV at 282 nm. The concentration of the usnic acid standard solution was used to determine the micrograms of usnic acid added to each portion of an extract. A plot of absorbance vs micrograms of usnic acid added to the milligrams of extract in 100 μL aliquots of extract solution provided slope and intercept values for computation of the levels of usnic acid in the lichens by the method of standard additions.

RESULTS AND DISCUSSION

Acetone (*16*), acetonitrile, alcohols, carbon dioxide (*4*), carbon bisulfide (*9*), dichloromethane/acetone (*17*), diethyl ether (*18*),

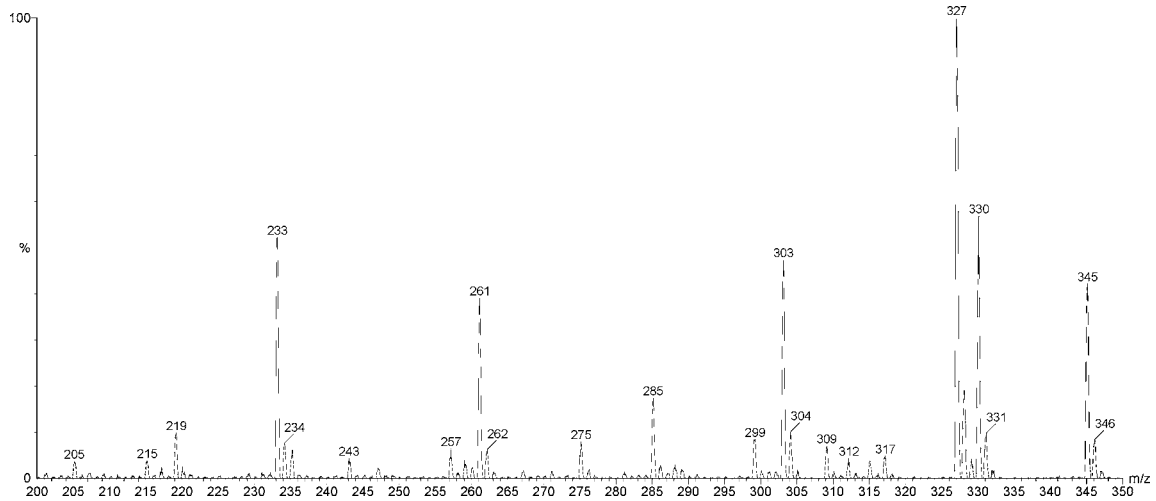

Figure 2. APCI-MS spectrum of usnic acid recorded by infusion of *U. barbata* extract into Quattro Micro LC-MS/MS. The portion was 0.3800 g of lichen extracted two times with 8 mL of 80% acetonitrile and diluted to 50 mL. Conditions: corona, 4 μA; cone, 50 V; extractor, 6 V; cone gas, 115 L/h; desolvation gas, 315 L/h at 300 °C desolvation; and 130 °C source temperatures.

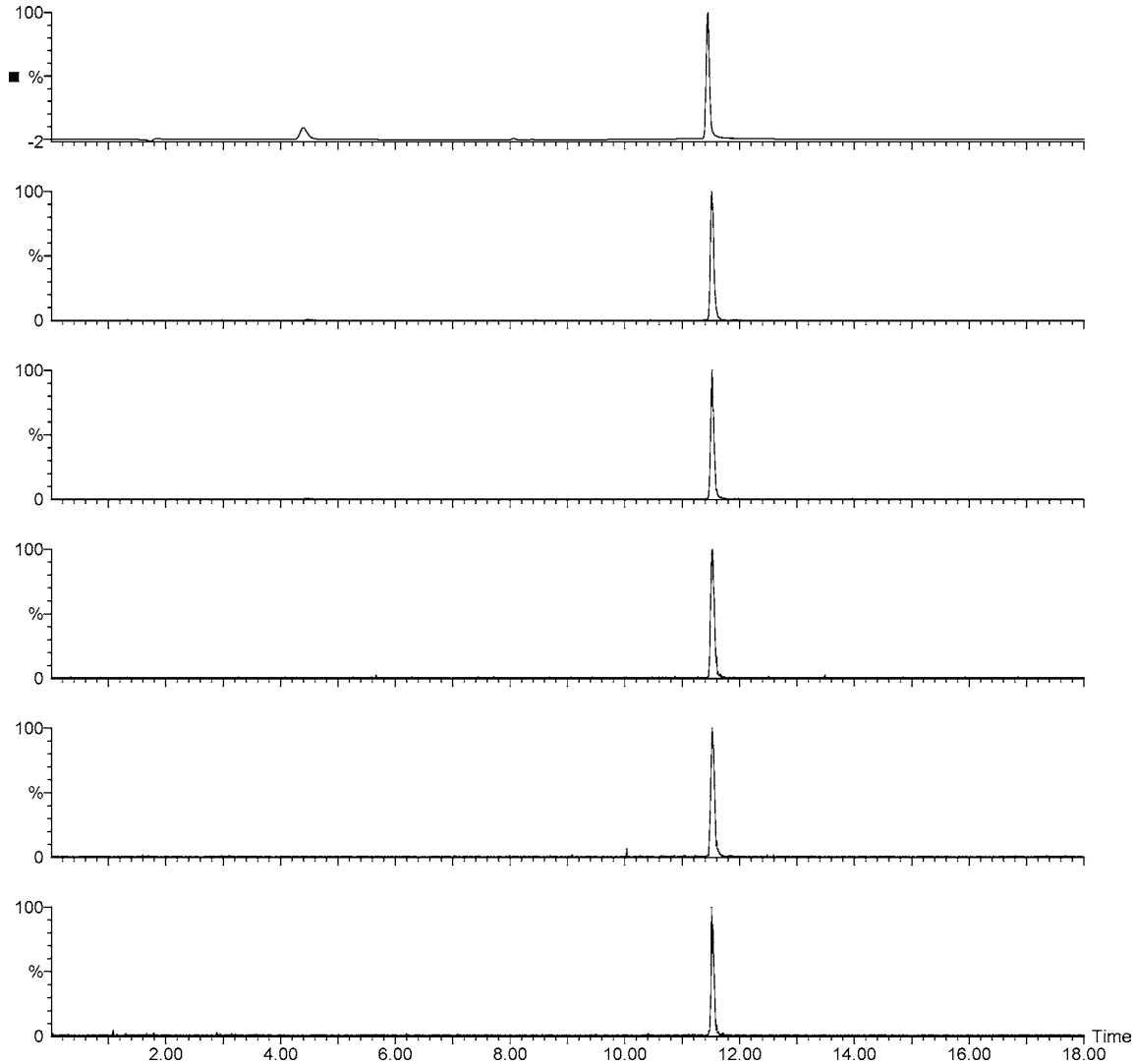

Figure 3. UV and MS/MS data for 2 μL injection (6.4 μg injected) unspiked elk browse. The top chromatogram is the UV absorbance at 282 nm. Chromatograms below the top one are MS/MS ion profiles for *m*/*z* 345, 327, 303, 261, and 233. Calculated usnic acid content, 2.16%.

and petroleum ether (*19*) have been used to extract usnic acid from lichens. It is soluble in acetone or acetonitrile at 1 mg/mL at room temperature. It is less soluble in methanol or ethanol. Repeated shaking extractions of powdered plant material with 10 mL portions of acetone or acetonitrile or column filtration with acetone or acetonitrile yielded >98% ($n = 4$) of the extracted usnic acid in the first 20 mL of solvent. Acetonitrile was chosen for further study. Solvent extraction portions were

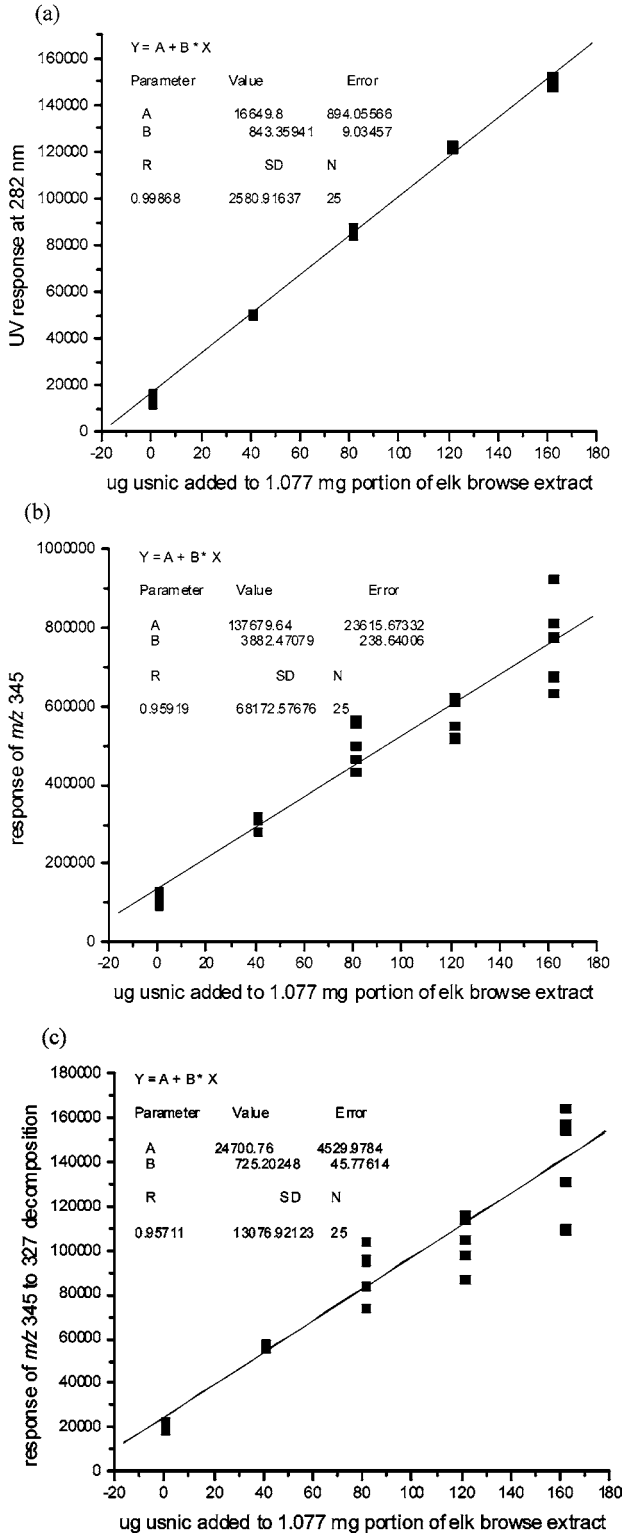

Figure 4. Standard additions data recorded for 0.2693 g of elk browse (*X. chlorochroa*) extracted 2 × 8 mL of 80% acetonitrile diluted to 50 mL. Portions (0.2 mL) equivalent to 1.077 mg were spiked with 0, 40.4, 80.8, 121.2, and 161.6 μg of usnic acid and diluted to 1.0 mL. Plots present five replicate analyses of sets (25 injections total) over 14 h. (**a**) UV response at 282 nm. (**b**) MS/MS response of *m/z* 345. (**c**) MS/MS response of *m/z* 327. Average results for the five data sets were as follows: 3.37 ± 1.49% (*m/z* 327); 3.46 ± 1.39% (*m/z* 345); and 1.83 ± 0.16% (282 nm). Data were plotted with Origin 6.0 (Microcal, Northampton, MA). Solutions change color when exposed to light for a month. Usnic acid stability in solution was not a factor in these data.

decreased to 8 mL so that two extractions would fit in one spin filter tube after 10 mL extraction portions suggested that the level of usnic acid in the lichen was 2.09 ± 0.16% ($n = 5$) in 20−500 mg lichen portions of *Xanthophylla chlorochroa* and 1.52 ± 0.18% ($n = 5$) in *U. barbata*. The maximum portion weight was decreased to 400 mg to retain the same sample: solvent ratio.

The addition of water to acetonitrile has been used to improve the recovery of pesticides from low moisture foods (*20*). The addition of 20% water in the lichen extraction step diminished the observed usnic acid content to 1.75 ± 0.07% for *X. chlorochroa* ($n = 3$) and 1.07 ± 0.07% ($n = 3$) for *U. barbata*.

Comparison of usnic acid fortified portions in which usnic acid was added either before or after extraction suggested recovery of 93% ($n = 2$) of the added usnic acid for the implicated elk browse and 94% ($n = 2$) for *U. barbata*. A soxhelt apparatus was not available for an extraction efficiency comparison, but an accelerated solvent extractor (model ASE 200, Dionex Corporation, Sunnyvale, CA) was available, so it was used to extract five portions of each lichen with acetonitrile at 100 °C and 1500 pounds per square inch. Portions of powdered lichen material ranging in weight from 0.107 to 0.466 g were mixed with Celite and placed in 10 mL pressure cells. These were preheated for 5 min, pressurized, and twice extracted with 10 mL of acetonitrile for 10 min. The system plumbing was automatically rinsed between samples. Some fine sample particulates were transported by the extraction solvent to the collection tubes. The extracts were spin filtered to remove these solids. Four of the cells leaked. Their extracts were discarded. Usnic acid levels observed in the remaining ASE test portions were 1.91 ± 0.07% ($n = 2$) for *X. chlorochroa* and 1.67 ± 0.17% ($n = 4$) for *U. barbata* and agreed with data obtained by room temperature acetonitrile extraction.

The chosen LC gradient and mobile phases eluted usnic acid in 7 min on a phenyl column, 11 min on a C8 column, and 15 min on a C18 column. The 7 min retention time was judged to be relatively close to minimally retained coextractives. The 15 min retention time was well beyond 100% B in the gradient. This led to use of the C8 column with its 11 min usnic acid retention time.

Confirmation of Usnic Acid Identity by LC-MS. The ESI mass spectrum and negative ion fast atom bombardment MS/MS fragmentation of usnic acid have been described (*21*−*23*). Usnic acid fragments in a characteristic manner during ESI-MS/MS or atmospheric pressure chemical ionization (APCI)-MS. A collision energy of 0−2 eV fails to decompose the protonated molecule. Increasing the collision energy results in fragment ions that are diagnostic for usnic acid. A collision energy of 22 eV results in losses of methyl and water to produce abundant ions at *m/z* 330 and 327. The relative abundance of *m/z* 303 is also significant at this collision energy. This fragment ion may occur through the loss of the elements of CH_2CO from the protonated molecule. A collision energy of 32 eV yields *m/z* 261 and *m/z* 233. These three collision energies are used in a MRM protocol to maximize the recorded signals of these five characteristic usnic acid ions in the positive ion ESI-MS/MS mode. These were also the principal ions observed for usnic acid in the positive ion APCI-MS mode of operation (**Figure 2**). The available instrumentation in our laboratory detected usnic acid better in the ESI mode than in the APCI mode, so ESI-MS/MS was used for these analyses.

Quantification of Usnic Acid Content by LC. The lichen samples examined contain more than 1% usnic acid by weight. Usnic acid is the principal component detected in the lichen

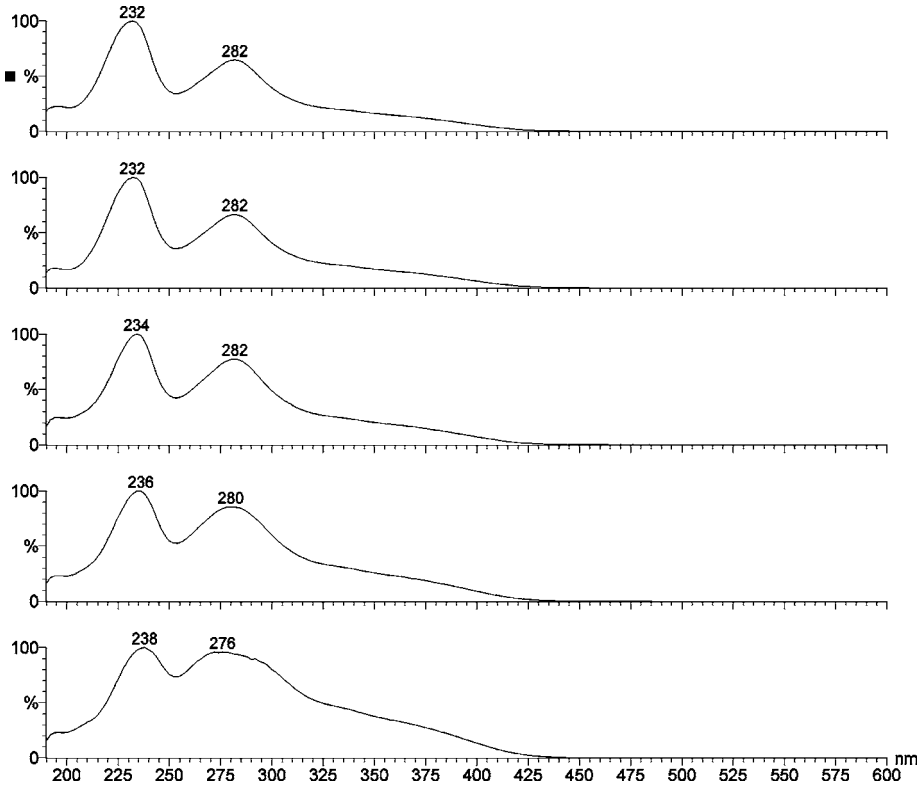

Figure 5. UV spectra recorded for standard additions quantification based on 10 μL injections of 3.04 mg/mL (30.4 μg injected) portions of elk browse extract. The top spectrum is unspiked extract. The spectra below the top were recorded for portions spiked at 41, 82, 164, and 264 μg/mL usnic acid. Spectra are distorted by signal saturation above 82 μg of usnic acid added.

extracts by both UV and MS (**Figure 3**). The ultraviolet UV spectrum of usnic acid has two maxima. The larger absorbance maximum is at 232 nm with a lesser maximum at 282 nm. The 232 nm maximum is solvent-dependent and not as unique as 282. LC with UV detection at 282 nm reliably quantifies usnic acid in the lichen extracts by standard additions of usnic acid with a limit of detection of 4 ng injected (**Figure 4a**). The UV response of usnic acid at 282 nm is linear up to 2 μg injected. The ion profiles recorded for *m*/*z* 345 and its decomposition product ion at *m*/*z* 327 do not provide reliable quantification (**Figure 4b**,**c**). ESI-MS/MS does not provide a precise or accurate measurement of usnic acid in these extracts because of ESI capacity and matrix effects.

In an electrospray source, the LC effluent passes through a capillary that is at high potential relative to a counter electrode. The electrostatic field distorts the meniscus at the tip of the capillary into a cone, which emits a stream of droplets that split into smaller droplets that yield gas phase ions. The analyte ion current plateaus with increasing concentration because an increasing fraction of the usnic acid in the droplets is not converted into gas phase ions before it is lost to the pumps. The upper limit of the linear range has been reported as 10^{-5} M for various analytes. Cole suggests that a reasonable explanation for an apparent upper limit for the linear range of 10^{-5} M is the complete filling of the droplet surface with sample. More sample in solution would not give more sample at the droplet surface, so the upper limit to sample ion abundance is reached (*24*).

Sample preparation extracts up to 0.4 g of lichen and dilutes this to 0.4 g/L for LC-MS analysis. An extract containing 2% usnic acid would yield a final solution that is 2.3×10^{-5} M in usnic acid. If 10^{-5} M is the upper limit for the linear range of the mass spectrometer, this may explain the observed differences in MS and UV quantification. To examine the possibility that extracts could be diluted into a concentration range in which MS and UV quantification provided comparable results, lichen extracts containing 3 mg/mL were prepared, spiked with usnic acid for quantification, and then diluted 5-, 10-, 100-, and 1000-fold. Ten microliter injections of the 3 mg/mL solutions (30 μg of extract injected) yielded high values for usnic acid content by MS. The average of two samples of elk browse was 6.1% based on *m*/*z* 327 and 4.5% based on *m*/*z* 345. Standard additions of UV quantification plots curved to a plateau for higher levels of added usnic acid. The 1000-fold dilution produced solutions that were too dilute for reliable UV detection and marginal MS data with MS quantifications of 5.6 and 9.5% based on *m*/*z* 327 and 2.1 and 5.8% based on *m*/*z* 345. The 100-fold dilutions resulted in values of 3.0 and 3.5% with *m*/*z* 327, 3.6 and 2.8% with *m*/*z* 345, and 2.6 and 1.4% by UV. The closest agreement for the three modes of quantification was with 10-fold dilutions. Observed values were 1.98 and 1.54% for 327, 1.84 and 1.32% for 345, and 1.87 and 2.06% for UV. The 5-fold dilutions provided quantifications that were comparable to 2 μL injections of the 3 mg/mL extracts. Average values at that concentration injected were 4.02 $\pm$ 1.12% for 327, 3.83 $\pm$ 0.99% for 345, and 2.09 $\pm$ 0.38% by UV ($n = 3$). Dilutions between 5- and 10-fold yielded the most consistent results by UV. MS data did not provide the same precision or accuracy at any dilution tested. *U. barbata* extracts behaved similarly. The influence of matrix coextractives in the absence of stable-labeled usnic acid was left as the most likely cause of scatter in the observed MS results for the diluted portions.

Usnic acid is a neutral molecule rather than a preformed ionic species. It must be protonated to form a gas phase positive ion. Electrospray is a competitive process, and the usnic acid in the extract is not alone. Matrix coextractives significantly impact the ESI process (*25*). They can enhance (*26*) or suppress (*27*) the analyte signal. Column and gradient program changes did

not improve MS quantification. Relative abundances were within ±10% of usnic acid standard in acetonitrile throughout sample sets. This suggested that coextractives influenced ionization of usnic acid in the electrospray process rather than presented isobaric interferences in the analyses.

The relative abundance information in the MS data does not provide a means to cull data points recorded outside the linear range of electrospray. The recorded UV spectra can be used to exclude saturated UV data points from the quantitifications. The UV spectrum of usnic acid distorts with signal saturation (**Figure 5**). The maxima at 232 and 282 shift toward each other. At the onset of signal saturation, the maxima are 234 and 280 and become 238 and 272 with a further increase in the usnic acid content. When the saturated standard additions data points are discarded from the 3 mg/mL data sets, UV quantifications derived from the 3 mg/mL solutions agree with those from diluted solutions ($2.1 \pm 0.02\%$, $n = 3$).

Diluting extract into the linear working range of the mass spectrometer would permit the use of stable-labeled usnic acid as an internal standard. Accurate and reliable ESI-MS/MS quantification should then be possible because labeled usnic acid has the same analytical behavior as usnic acid but can be distinguished from it by a mass spectrometer due to their difference in mass. The labeled compound coextracts with the analyte and is subject to the same ESI conditions as the analyte so it compensates for the effects of extraction and sample matrix on the analysis (*28*, *29*). Unfortunately, the synthesis of stable-labeled usnic acid is not trivial making it either unavailable or costly (*30*). Use of this approach to quantification would also encumber UV quantification of usnic acid because the labeled and native usnic acids have the same UV spectra. Discarding reliable UV quantification data in favor of a significantly more expensive assay is not cost effective.

Benefit of Confirmation of Identity by MS. Confirmation of identity by an independent analytical technique validates a tentative finding based on the chromatographic behavior of a standard and an analyte (*19*). In this case, usnic acid was tentatively identified by LC-UV in the lichens ingested by the elk. There are many compounds in the environment that absorb in the UV and may elute near the retention time of usnic acid. Each of these compounds is a potential false response in the quantitative assay of a minimally characterized environmental sample. Additional proof such as infrared spectrometry, nuclear magnetic resonance spectrometry, or MS corroborates the finding. Of these techniques, MS is readily amenable to the in-stream identification of components resolved by LC.

In conclusion, ingestion of the lichen, *X. chlorochroa*, resulted in the deaths of an estimated 328 elk in the Red Rim desert area of Wyoming. Our laboratory found that the lichens contained 2% usnic acid by weight. The pathology produced by feeding the lichens to captive elk was consistent with that observed in afflicted wild elk but differed from the effects reported for usnic acid ingestion by other species and in vitro studies (*7*, *10*, *11*). University of Wyoming researchers have since undertaken additional work to characterize the pathology of *X. chlorochroa* poisoning (*31*). The ESI-MS/MS analytical results reported here demonstrate the utility of MS in investigations of incidents of this nature.

ACKNOWLEDGMENT

We acknowledge the generous donation of lichen associated with the incident by the Wyoming Game and Fish Laboratory. It was their expertise and good field work that linked the lichen to the stricken elk.

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Received for review November 8, 2005. Revised manuscript received February 9, 2006. Accepted February 10, 2006.

JF052767M