AGRICULTURAL MATERIALS

Analysis of Salazinic, Norstictic, and Usnic Acids in *Xanthoparmelia chlorochroa* by Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry

REBECCA DAILEY, ROGER SIEMION, and MERL RAISBECK[1]
University of Wyoming, Wyoming State Veterinary Laboratory, Department of Veterinary Sciences, 1174 Snowy Range Rd, Laramie, WY 82070
CHRIS JESSE
Wyoming Department of Agriculture, Analytical Services, 1174 Snowy Range Rd, Laramie, WY 82070

The lichen species *Xanthoparmelia chlorochroa* is toxic when consumed by domestic sheep, cattle, and Rocky Mountain elk. Clinical signs exhibited by poisoned animals include red urine, ataxia, and muscular weakness that rapidly progresses to recumbency. Elk are unable to recover once becoming recumbent; however, most affected cattle can recover if offered suitable feed shortly following the onset of signs. At present, the pathogenesis and specific toxin(s) are unknown. As part of an effort to elucidate the proximate toxin, a method using ultra-performance LC coupled to MS/MS with negative-ion electrospray ionization has been developed to compare salazinic, norstictic, and usnic acid concentrations in *X. chlorochroa* collected from locales associated with lichen poisonings. Compounds were extracted from lichen samples with acetone and sonication. The stationary phase was a Waters Acquity UPLC™ BEH C_{18} (50 2.1 mm; 1.7 m particle size) column. The mobile phase consisted of an acetonitrile–water gradient. The precision of the method was confirmed by an SD below 0.4% (n = 9) for triplicate samples. LOD values were 200, 100, and 50 ng/mL for salazinic, norstictic, and usnic acids, respectively.

Xanthoparmelia chlorochroa is a vagrant, foliose lichen that inhabits high elevation plains along the Rocky Mountain range in North America (1). It has been cited as an indicator of good pronghorn antelope range (1) and an important source of forage for them during long or harsh winters (2). *X. chlorochroa*, however, has been shown to cause deleterious effects in other herbivores such as domestic sheep, cattle, and elk (*Cervus canadensis*; 3, 4). Accounts of any lichen species being toxic to mammals are extremely rare. There are a few anecdotal reports of *X. chlorochroa* poisonings during the 1930s and 1940s in Wyoming cattle and sheep (3). Two elk cases and several suspected cattle cases were submitted from throughout Wyoming to the Wyoming State Veterinary Laboratory during the past four years. The elk mortalities occurred in the Red Rim-Daley Wildlife Habitat Management Area (RRWHMA) near Rawlins, WY, in March 2004 and 2008. An estimated 400–500 elk were killed, while cases in cattle only seem to affect about 2% of the herd (4; R. Dailey, unpublished data). Clinical signs exhibited by animals poisoned by *X. chlorochroa* include red urine, ataxia, and muscular weakness that rapidly progresses to recumbency. Once recumbent, no elk and few cattle recover. No distinctive gross or histological lesions develop, and no diagnostic assay currently exists to confirm *X. chlorochroa* intoxication. At present, both the pathogenesis and toxin(s) remain unknown.

Structures for the three analytes of interest are shown in Figure 1. Usnic acid is a dibenzofurane, and salazinic acid and norstictic acid are depsidones. All three compounds have some degree of antibacterial activity (5). Usnic acid is one of the more thoroughly studied lichen compounds. It is found in several genera of lichen and exists in two enantiomeric forms (6). Several publications describe the antibacterial, antiparasitic, antiproliferative, antiviral, and hepatotoxic properties of usnic acid (6–17). More recently, the myotoxic properties of usnic acid have been described in domestic sheep, which were orally gavaged with the compound (18). High doses of (+)-usnic acid caused a severe degenerative appendicular skeletal myopathy, but it did not induce the syndrome exhibited by *X. chlorochroa*-intoxicated animals. It would seem that other compounds in the lichen, possibly in addition to (+)-usnic acid, interact to produce the specific syndrome observed in lichen-poisoned animals (18). Little is known regarding the biological activity of salazinic and norstictic acids. These compounds are responsible for the dye properties of *X. chlorochroa* and are used in chemotaxonomic identification (1). Lichen secondary metabolites are thought to act as a chemical defense for the lichen, protecting it from insects, herbivores, pathogens, and UV light (1, 19, 20).

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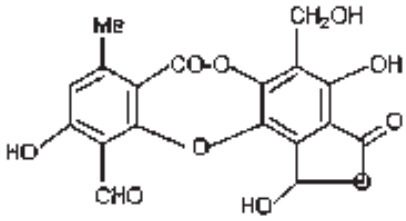
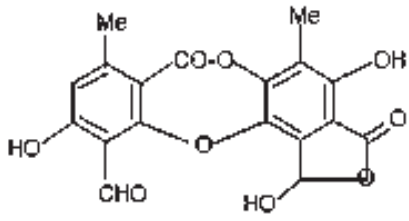
[1] Corresponding author's e-mail: raisbeck@uwyo.edu



Salazinic acid



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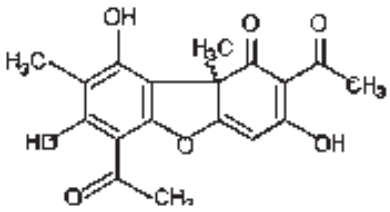

Usnic acid

Figure 1. Structures of salazinic, norstictic, and usnic acids.

HPLC has been utilized in the analysis of lichen secondary metabolites—especially oakmoss absolute, which is a lichen extract used in perfume and cosmetics (21–24). LC/MS/MS or a photodiode array detector has also been used to characterize oakmoss absolute and perfumes (25–27). A previous study using LC with UV quantification found that *X. chlorochroa* samples from the 2004 elk mortality contained 2% usnic acid by weight (28). The aim of the present investigation was to quantify several of the major secondary metabolites present in *X. chlorochroa* using RP ultra-performance LC coupled to MS/MS (UPLC/MS/MS). A review of the literature shows this to be the first reported UPLC/MS/MS method for the identification of the lichen secondary metabolites salazinic, norstictic, and usnic acids.

Experimental

Materials and Apparatus

Norstictic acid (99%) was purchased from ChromaDex Inc. (Lot No. 14529-106; Santa Ana, CA); salazinic acid (99%) was purchased from InterBioScreen Ltd (Lot No. 1N-26155, Moscow, Russia). The enantiomeric form of usnic acid found

Table 1. *Xanthoparmelia chlorochroa* collection dates and locations

Group	Collection location	Date	Latitude	Longitude
1	Red Rim-Daley WHMA[a]	03/2004	41 42.9 N	107 28.2 W
		10/2005		
		02/2007		
		02/2008		
2	McCulloch Peaks grazing allotment[b]	02/2006	44 40.6 N	108 51.5 W
3	Polecat Bench grazing allotment[b]	03/2007	44 53.2 N	108 47.3 W
4	Monolith HMA[c]	11/2005	41 13.6 N	105 38.3 W
		05/2006		
5	M. Ranch central WY[c]	05/2008	42º38.1'N	107º23.2'W

[a] Site of the 2004 and 2008 elk mortalities (ref. 4). WHMA = Wildlife Habitat Management Area.

[b] Site of a probable lichen-poisoning event involving domestic cattle (B. McKenzie, U.S. Department of the Interior, personal communication, 2006 and 2007).

[c] Site selected as earlier studies used *X. chlorochroa* from the Laramie plains (ref. 3). HMA = Hunter Management Area.

Table 2. Parent and product ion parameters

Compound	Parent ion, *m/z*	Product ions, *m/z*	Cone, V	Collision energy, V
Salazinic acid	387.06	227.3	25	20
		343.2	25	16
Norstictic acid	371.06	227.2	25	18
		327.2	25	14
Usnic acid	343.13	259.3	30	20
		328.3	30	20

in *X. chlorochroa* was determined via polarimetry in a previous study (14), and (+)-usnic acid (98%) was purchased from Sigma (Lot No. 07009LC; St. Louis, MO). All solvents were HPLC grade. Acetonitrile and water were purchased from Fisher Scientific (Springfield, NJ), and acetone was purchased from Burdick & Jackson (Morristown, NJ).

An Acquity UPLC™ system (Waters Corp., Milford, MA), consisting of an autosampler, solvent degasser, quaternary solvent delivery system, column oven, and diode array UV-Vis detection system, was coupled to a Micromass Quattro micro API triple-quadrupole mass spectrometer (Waters). The mass spectrometer was operated in negative-ion mode using electrospray ionization. Source working conditions were as follows: capillary voltage, 2.4 kV; source temperature, 120 C; desolvation temperature, 375 C; cone gas flow rate, 50 L/h; and desolvation gas flow rate, 550 L/h.

Chromatographic Conditions

Mobile phase A consisted of water and mobile phase B consisted of acetonitrile. A gradient was run from 10% B to 30% B in 6 min at a flow rate of 0.2 mL/min. The mobile phase composition then linearly increased to 95% B over 1.5 min and held for 7.5 min. Over the subsequent 5 min, the mobile phase composition was returned to 10% B. The column was equilibrated at initial mobile phase conditions for 10 min at the end of each analysis, giving a total run time of 25 min. An Acquity UPLC BEH C_{18} (1.7 m, 50 2.1 mm) column (Waters), with guard column, was operated at 50 C. The autosampler temperature was 10 C and the injection volume was 5 L.

Sample Collection and Extraction

X. chlorochroa was collected by hand from the RRWHMA in March 2004 (University of Nebraska at Omaha Lichen Herbarium No. 15631) immediately following the elk mortality and again in October 2005 (University of Nebraska at Omaha Lichen Herbarium No. 16366), February 2007 and February 2008 (University of Nebraska at Omaha Lichen Herbarium No. 16989). In addition, *X. chlorochroa* was collected from two federal grazing allotments near Cody, WY, in February 2006 and March 2007 (University of Nebraska at Omaha Lichen Herbarium No. 16368), the Monolith Hunter Management Area, south of Laramie, WY, in November 2005 and May 2006 (University of Nebraska at Omaha Lichen Herbarium No. 16367), and from a private ranch in the central portion of the state in March 2008 (Table 1). The Monolith Hunter Management Area was the only sampling location not associated with a known *X. chlorochroa* poisoning.

Lichen was allowed to dry completely and then ground using a Thomas-Wiley Mill (Model ED-5; Arthur H. Thomas

Table 3. Compound MS/MS parameters

Analyte	MW	Product ions	Declustering potential	Focusing potential	Entrance potential	CEP[a]	CE[b]	CXP[c]
Norstictic acid	372.28	217.00	–21	–350	–5	–30	–22	–46
		365				–30	–6	–58
Salazinic acid	388.28	217.6	–21	–350	–7.5	–28	–34	–38
		233.5				–28	–32	–48
Usnic acid	344.31	250.2	–21	–310	–11.5	–14	–28	–50
		305.2				–14	–38	–50

[a] CEP = Collison cell entrance potential.
[b] CE = Collison energy.
[c] CXP = Collison cell exit potential.

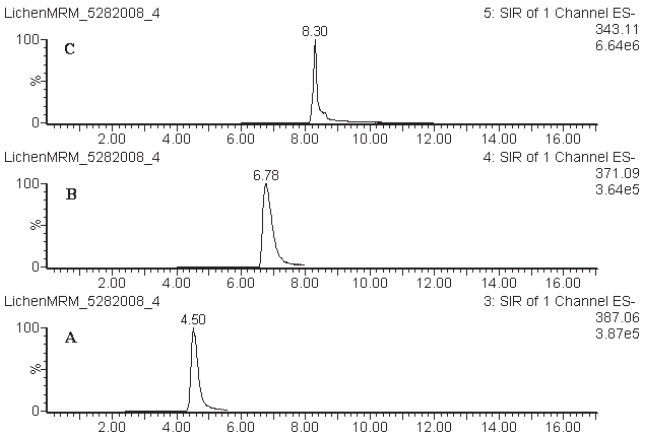

Figure 2. Chromatograms showing resolution of compounds (1 g/mL mixed standard): (A) salazinic acid, (B) norstictic acid, and (C) usnic acid.

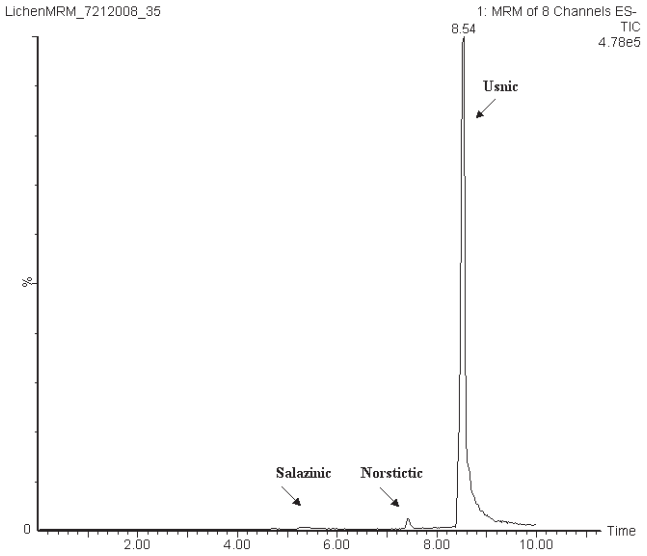

Figure 3. *Xanthoparmelia chlorochroa* extract from RRWHMA 2008.

Co., Philadelphia, PA). Once ground, lichen samples were stored in airtight containers and frozen (–70 C) until analysis. Acetone (200 mL) was added to 100 mg ground lichen, sonicated for 30 min, and allowed to sit overnight at 20 C. The following morning, the extract was filtered using a Whatman No. 4 filter (Whatman International Ltd, Maidstone, UK), 100 mL fresh acetone was added, and sonicated for an additional 30 min. The extract was filtered again, the supernatant was combined with the previous 200 mL, and then reduced to a final volume of 100 mL using a Rotavapor (R-110, Brinkmann Instruments, Westbury, NY). Lichen extracts (1 mL) were reduced to dryness and reconstituted in 8 mL water–acetonitrile mobile phase (7:3, v/v). Samples were thoroughly mixed using a vortex mixer for 1 min, then filtered using a 0.20 m PTFE filter (Advantec MFS Inc., Dublin, CA) prior to injection. The extraction process was replicated three times for each of the nine lichen samples.

MS/MS Analysis

MS/MS analysis was performed in the multiple reaction monitoring negative-ionization mode. Spectra for salazinic, norstictic, and usnic acids were obtained by infusing a mixed standard (125 ng/mL) at a flow rate of 10.0 L/min. Mobile phase composition was (50:50, v/v) (A:B) at a flow rate of 0.2 mL/min. The dwell time was 100 ms, and the interscan and interchannel delays were both set at 20 ms. Data acquisition was carried out by MassLynx software (Version 4.1; Milford, MA). The precursor and product ions for each analyte, together with the applied collision energy are reported in Table 2. Peak areas were integrated using QuanLynx software (Version 4.1; Milford, MA).

A stock solution containing 32 g/mL salazinic, norstictic, and usnic acids was prepared in acetonitrile. Standards (250, 500, and 750 ng/mL; and 1.0, 2.0, and 4 g/mL) were then created from the stock solution. The blank and standards

Table 4. Average and SD of three replicate extractions for salazinic, norstictic, and usnic acids from *X. chlorochroa*

Location	Salazinic acid, g/mL	Norstictic acid, g/mL	Usnic acid, g/mL
Red Rim-Daley WHMA 03/2004	13.41 ± 1.45	3.60 ± 0.04	8.41 ± 3.67
Red Rim-Daley WHMA 10/2005	13.98 ± 1.09	3.47 ± 0.09	14.68 ± 2.62
Red Rim-Daley WHMA 02/2007	14.46 ± 2.41	3.65 ± 0.08	15.09 ± 6.65
Red Rim-Daley WHMA 02/2008	15.07 ± 2.35	4.89 ± 0.57	18.13 ± 4.49
Monolith HMA 11/2005	17.59 ± 2.52	3.85 ± 0.05	9.74 ± 2.75
Monolith HMA 05/2006	16.51 ± 1.93	3.84 ± 0.42	14.16 ± 4.46
McCulloch Peaks grazing allotment 02/2006	14.96 ± 2.04	3.59 ± 0.12	15.77 ± 1.67
Polecat Bench grazing allotment 03/2007	12.46 ± 0.75	4.14 ± 0.03	12.17 ± 3.40
M. Ranch central WY 05/2008	11.95 ± 0.54	3.50 ± 0.02	14.46 ± 2.56

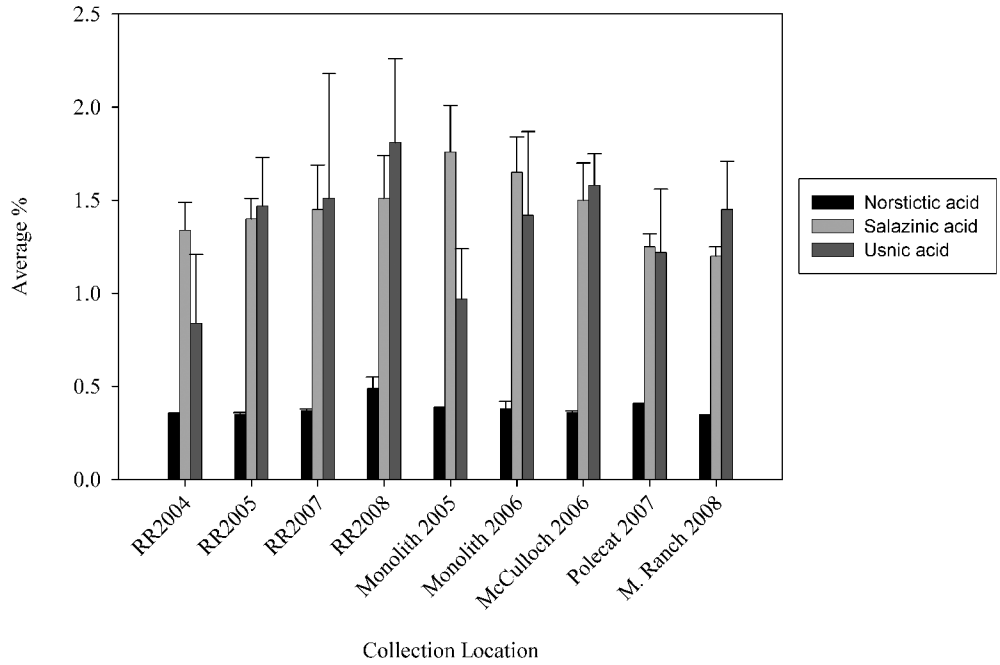

Figure 4. Average percent of three replicate extractions for salazinic, norstictic, and usnic acids extracted from *X. chlorochroa*.

were prepared in water–acetonitrile (7:3, v/v). Calibration curves were established for all three compounds using the peak areas for each analyte (Table 3). Norstictic acid was linear (r^2 = 0.9926) over the concentration range of 250 ng/mL–4 g/mL, while a quadratic equation (29) was used for salazinic (r^2 = 0.9992) and usnic acids (r^2 = 0.9994). The precision of the method was evaluated by injecting each lichen extract in triplicate.

Method Validation

A 2.5 g/mL standard, prepared as described above, was run as a sample between each set of nine lichen samples (n = 3). The average SD for each analyte was less than 0.30 g/mL. Reproducibility of the extraction method was verified by extracting all nine samples three times. The RSDs among triplicate lichen sample analyses were as follows: salazinic, 0.25%; norstictic, 0.02%; and usnic, 0.37%. Recovery experiments were also conducted by extracting spiked *X. chlorochroa* samples. The spike recovery average (n = 3) and SDs for salazinic acid were 101% ± 38.9; for norstictic, 66.1% ± 4.24; and for usnic, 135% ± 22.6. LODs determined by an S/N of 7 were 200, 100, and 50 ng/mL for salazinic, norstictic, and usnic acids, respectively.

Results and Discussion

Chromatograms of a 1 g/mL mixed standard and sample (RR2008) are shown in Figures 2 and 3. The average concentration and SD for each lichen sample are reported in Table 4 and Figure 4.

A one-way analysis of variance was used to compare each analyte by collection location using the SPSS (Version 13.0; SPSS Inc., Chicago, IL) statistical package. Norstictic acid did not pass the homogeneity of variances test (P = 0.001), so the Dunnett T3 post hoc analysis was utilized. No significant differences were observed among norstictic concentrations (P = 0.115). Both salazinic (P = 0.84) and usnic acids (P = 0.158) passed the homogeneity of variances test. No significant differences were observed between groups for usnic acid concentration (P = 0.134). Using Tukey post hoc analysis (= 0.05), both Monolith 2005 and Monolith 2006 groups contained significantly higher concentrations of salazinic acid compared to the M. Ranch 2008 and Polecat 2007 groups.

The oral effective dose (ED_{50}) for usnic acid, administered by gavage, was determined to be 485–647 mg/kg/day for seven days in domestic sheep (18). Comparing the (+)-usnic acid concentration in each of the nine *X. chlorochroa* samples to the (+)-usnic acid ED_{50}, a sheep consuming *X. chlorochroa* containing 1.81% (+)-usnic acid (the highest recorded concentration of the nine lichen samples) at 2% of its body weight/day would receive a dose of 362 mg/kg/day (+)-usnic acid. This dose of (+)-usnic acid is well below the ED_{50} of 485–647 mg/kg/day determined in sheep.

Toxicity of *X. chlorochroa* varies, as was evident in the lichen feeding study conducted with domestic sheep (30). All lichen-fed ewes displayed red urine and varying degrees of locomotor signs; however, the severity of clinical signs differed dramatically between the four lichen groups (RRWHMA 2004, RRWHMA 2005, McCulloch Peaks 2006, and Monolith Hunter Management Area 2006). Ewes fed lichen from the RRWHMA collected in 2004 were the most

severely affected group, while ewes fed lichen from the same area collected in 2005 were the least severely affected group. Both lichen groups had approximately equal salazinic and norstictic concentrations (13.9 and 13.4 in 2004; 3.5 and 3.6 g/mL in 2005). The RRWHMA 2005 group, however, contained almost twice the amount of (+)-usnic acid as the RRWHMA 2004 group (14.7 and 8.41 g/mL, respectively), despite being much less toxic to sheep. This bolsters the conclusion that other compounds in the lichen, possibly in addition to (+)-usnic acid, interact to produce the specific syndrome observed in lichen-poisoned animals.

No correlations could be made between the most toxic lichen group and concentrations of salazinic or norstictic acids. It is quite possible that these concentration variations underlie the differences observed in the toxicity of the lichen, as was observed in the *X. chlorochroa* feeding study (30).

Conclusions

The method described above is a rapid and reliable means for the analysis of salazinic, norstictic, and (+)-usnic acids in lichen. Nine *X. chlorochroa* samples collected from throughout Wyoming were analyzed. Norstictic acid concentrations were relatively similar among *X. chlorochroa* samples. Concentrations of salazinic and usnic acids, however, varied noticeably. While statistically significant salazinic acid concentration differences were observed between various sources of *X. chlorochroa*, the more relevant question would be, Are these differences biologically significant? There is very little toxicological data for salazinic and norstictic acids (i.e., no in vivo LD_{50}). Given the steep dose-response curve constructed when establishing the (+)-usnic acid ED_{50} in domestic sheep (485–647 mg/kg/day for 7 days), the differences in usnic acid concentrations between *X. chlorochroa* samples could very well be biologically significant. The differences in salazinic acid concentrations could also be biologically significant, but this is merely speculation until more toxicological data are available.

Acknowledgments

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