DOI:10.1021/jf1012456

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Tremetone and Structurally Related Compounds in White Snakeroot (*Ageratina altissima*): A Plant Associated with Trembles and Milk Sickness

Stephen T. Lee,*,† T. Zane Davis,† Dale R. Gardner,† Steven M. Colegate,† Daniel Cook,† Benedict T. Green,† Kimberly A. Meyerholtz,‡ Christina R. Wilson,‡ Bryan L. Stegelmeier,† and Tim J. Evans§

†Poisonous Plant Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1150 East 1400 North, Logan, Utah 84341, ‡Indiana Animal Disease Diagnostic Laboratory, Purdue University, 406 South University, West Lafayette, Indiana 47907, and §Veterinary Medical Diagnostic Laboratory, University of Missouri—Columbia, 1600 East Rollins Street, Columbia, Missouri 65211

Ingestion of white snakeroot (*Ageratina altissima*) can cause trembles in livestock and milk sickness in humans. The toxicity has been associated with tremetol, a relatively crude, multicomponent lipophilic extract of the plant. In this study, 11 different compounds were isolated from white snakeroot-derived lipophilic extracts from 18 collections. Six of the isolated compounds have not been previously reported to be found in white snakeroot. High-performance liquid chromatography (HPLC) analysis indicated that there are three different chemotypes of white snakeroot from the plant samples analyzed. Elucidation of these chemotypes may explain the sporadic and unpredictable toxicity of white snakeroot to livestock and humans.

KEYWORDS: White snakeroot; tremetol; *Eupatorium rugosum*; *Eupatorium urticaefolium*; *Ageratina altissima*; tremetone; dehydrotremetone

INTRODUCTION

White snakeroot (*Ageratina altissima* (L.) King & H. Rob. var. *altissima*, a member of the Asteraceae, previously named *Eupatorium rugosum* Houtt and *Eupatorium urticaefolium* Reichard), is commonly found throughout the eastern half of North America. Ingestion by livestock can sporadically and unpredictably cause a disease called trembles characterized by weight loss, listlessness, reluctance to move, muscular tremors when forced to stand and move, constipation, and apparent joint stiffness (*1*−*3*). Terminal collapse and coma can often occur in livestock several days to weeks after clinical signs appear (*1*, *2*). Consumption of milk from cows that have grazed white snakeroot can cause milk sickness, a sometimes fatal disease in humans, even if lactating cows show few signs of sickness. Historically, milk sickness was also called sick stomach or the slows because it was characterized by listlessness, leg pains and cramps, loss of appetite, vomiting, constipation, a white coating on the tongue, and an acetone odor to the breath (*3*). Milk sickness caused many deaths among Midwestern settlers during the 1800s, ultimately forcing entire settlements to be abandoned (*1*, *2*). In 1917, after nearly 100 years of investigations, white snakeroot was determined to be the cause of trembles and milk sickness (*1*).

In the late 1920s, Couch (*2*) concluded that the toxin in white snakeroot was a compound which he named tremetol. However, tremetol was later determined to be a complex mixture including at least six components referred to as terpene I, sterol I, sterol II, tremetone (**1**), hydroxytremetone (**2**), and dehydrotremetone (**3**) (**Figure 1**) (*4*). Tremetone (**1**), hydroxytremetone (**2**), and dehydrotremetone (**3**) were toxic in a goldfish bioassay and, therefore, it was concluded that these compounds were likely responsible for causing trembles and milk sickness (*4*). However, synthetic tremetone (**1**) was not toxic to white leghorn cockerels or sheep (*5*). More recently, Beier and co-workers (*6*, *7*) reported that, following microsomal activation, tremetone (**1**), but not dehydrotremetone (**2**), was toxic in vitro to murine melanoma (B16F1) cells and five other mammalian cell lines. Thus, the relative toxicity of the white snakeroot-derived benzofuran compounds and sterols to livestock and humans remains elusive.

The toxicity of white snakeroot has been difficult to study because some populations of plants appear to be nontoxic (*3*, *8*). Generalizations have been made that fresh plant from 1 to 20% of an animal's body weight must be consumed to cause death (*9*, *10*). However, this has not been correlated to physiochemical concentrations.

The objective of this study was to characterize the chemical profiles of white snakeroot from different populations. HPLC methods have allowed the isolation, identification, and quantification of 11 different compounds from plants in 18 geographically different white snakeroot populations. This information may provide insights into the sporadic and unpredictable nature of the intoxication related to white snakeroot.

MATERIALS AND METHODS

Plant Material. White snakeroot was collected from 18 different locations (**Table 1**). Composite collections of the above ground parts of at least 10 plants were collected randomly at each site except for the collection at Karst Trailhead, which was a collection of 6 plants. The plants collected

*Corresponding author [phone (435) 752-2941; fax (435) 753-5681; e-mail stephen.lee@ars.usda.gov].

 Published on Web 07/20/2010

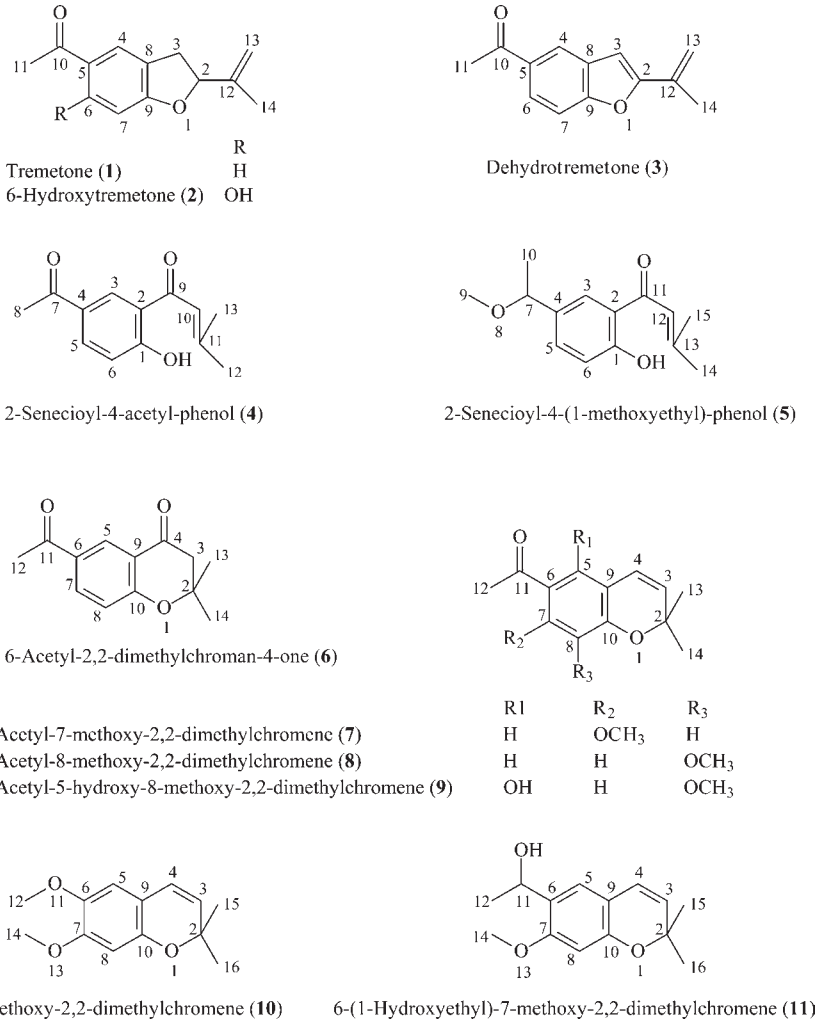

Figure 1. Chemical structures of tremetone (**1**), 6-hydroxytremetone (**2**), dehydrotremetone (**3**), dehydrotremetone (**3**), 2-senecioyl-4-acetylphenol (**4**), 2-senecioyl-4-(1-methoxyethyl)phenol (**5**), 6-acetyl-2,2-dimethylchroman-4-one (**6**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**), 6-acetyl-8-methoxy-2,2-dimethylchromene (**8**), 6-acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (**9**), 6,7-dimethoxy-2,2-dimethylchromene (**10**), and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**).

were in early to full flower. White snakeroot plants were identified by Dr. Stanley L. Welsh, curator at the Stanley L. Welsh Herbarium at Brigham Young University, Provo, UT, and Dr. David S. Seigler, Professor, Department of Plant Biology, University of Illinois, Champaign, IL. The current taxonomic classification for this plant is given as *Ageratina altissima* (L.) King & H. Rob. var. *altissima* as per the USDA, National Resources Conservation Service, Plant Database. Voucher specimens for the white snakeroot were deposited in the Poisonous Plant Research Laboratory Herbarium, Logan, UT, and accession numbers are listed in **Table 1**. The plant collections from the Vermilion Research Observatory (VRO), Trelease Woods, Brownfield Woods, Allerton Park, Interstate, and Salt Fork sites in Illinois and those from the Davies County site in Missouri were air-dried at ambient temperature. The plant collections from the Rutan Woods, Stidham Woods, Hart Woods, and Tonica sites in Illinois and those from the Veterinary Medical Diagnostic Laboratory at the University of Missouri (VMDL), Karst Trailhead, Pierpont Meadows Road, Evans Place, Shooting Star Trailhead sites in Missouri, the Wabash River in Indiana, and the Cincinnati Zoo in Ohio were freeze-dried. The dried plant material was ground using a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden) to pass through a 1 mm screen.

Extraction and Isolation of Compounds. *Method A: Extraction−Silica Gel Column Chromatography−Preparative HPLC*. Compounds **1** and **3** were previously isolated from *Isocoma pluriflora* (rayless goldenrod) (*11*). Using the same method, compounds **2** and **6** were isolated from white snakeroot collected at the Vermilion Research Observatory site and at the Trelease Woods site, respectively. For example, dry, ground, white snakeroot (1.25 kg) was extracted for 112 h by Soxhlet extraction with hexane (9 L). The extract was rotary-evaporated to a viscous dark green residue. An aliquot of the residue were redissolved in $CHCl_3$ and was adsorbed on approximately 75 g of silica (70−230 mesh, 60 Å; Sigma-Aldrich, St. Louis, MO), and the solvent was allowed to evaporate. A 30 cm × 8 cm i.d. silica (70−230 mesh, 60 Å) column was prepared by slurry packing silica (650 g) in hexane/ethyl acetate (90:10) (1.5 L). The sample, adsorbed on silica, was then added to the head of the silica column. Sand (white quartz −50 + 70 mesh; Sigma, St. Louis, MO) (2 cm) was added on top of the sample to protect the column bed. Mobile phase 1 (hexane/ethyl acetate 90:10, 1.5 L) was added to the head of the column until a first intense yellow band completely eluted. After elution of the yellow band, mobile phase 2 (hexane/ethyl acetate, 70:30, 2.5 L) was added to the column and the eluent collected. Mobile phase 2 eluent was rotary-evaporated to a dark green viscous residue, which was re-extracted with $MeOH/H_2O$ (70:30). This aqueous methanol extract was repeatedly filtered through a 30 mm nylon 0.45 μm syringe filter (National Scientific Co., Rockwood, TN) until clear and injected (2−5 mL, depending on the sample concentration) onto a Waters Prep LC2000 Preparative Chromatography System equipped with a UV−vis detector (Millipore Co., Milford, MA) monitoring λ 280 nm. The HPLC column (two 40 × 100 mm Bondapak C18 PrepPak cartridges (15−20 μm, 125 Å; Millipore Co.) connected in series) was protected with a guard column (40 × 10 mm) of the same packing material. The mobile phase was 20 mM ammonium acetate/methanol (30:70, v/v) at a flow rate of 75 mL/min. The major peaks were collected separately from multiple injections. The combined

Table 1. White Snakeroot Collection Sites, Dates, and Herbarium Accession Numbers

collection site	county	GPS coordinates	collection date (month/day/year)	accession no.
Illinois sites				
Stidham Woods	Champaign	40° 12.667′ N/88° 21.970′ W	09/01/2009	3546
Hart Woods	Champaign	40° 13.732′ N/88° 21.346′ W	09/28/2009	3519
Brownfield Woods	Champaign	40° 08.874′ N/88° 09.786′ W	09/16/2009	3558
Rutan Woods	Vermilion	40° 04.406′ N/87° 54.370′ W	09/01/2009	3545
Salt Fork	Vermilion	40° 05.536′ N/87° 49.683′ W	09/16/2009	3555
Interstate	Vermilion	40° 06.483′ N/87° 40.866′ W	09/17/2009	3562
VRO	Vermilion	40° 03.587′ N/87° 33.886′ W	09/15/2009	3550
Tonica	LaSalle	41° 12.011′ N/89° 01.236′W	08/15/2008	3614
Allerton Park	Piatt	40° 00.366′ N/88° 39.183′ W	09/16/2009	3560
Trelease Woods	Champaign	40° 08.111′ N/88° 08.390′ W	09/16/2009	3557
Missouri sites				
Davies County	Davies	39° 58.300′ N/93° 59.016′ W	09/14/2009	3553
Shooting Star Trail	Boone	38° 52.167′ N/92° 17.527′ W	09/04/2008	3571
Karst Trailhead	Boone	38° 51.984′ N/92° 18.195′ W	10/18/2007	2846
VMDL	Boone	38° 56.410′ N/92° 18.933′ W	10/01/2009	3497
Evans Place	Boone	38° 51.228′ N/92° 19.611′ W	10/01/2009	3494
Pierpont Meadows	Boone	38° 51.286′ N/92° 19.701′ W	09/04/2008	3404
Indiana site				
Wabash River	Tippecanoe	40° 25.092′ N/86° 53.959′ W	09/29/2009	3615
Ohio site				
Cincinnati Zoo	Hamilton	39° 08.683′ N/84° 30.483′ W	11/02/2009	3563

fractions, corresponding to each of the major peaks, were each evaporated to <30% the original volume and extracted three times with equal volumes of $CHCl_3$. These three $CHCl_3$ extracts were combined, dried over anhydrous Na_2SO_4, filtered, and rotary-evaporated to dryness.

Method B: Extraction−Preparative HPLC. Dry, ground plant material (40 g) was Soxhlet-extracted for 72 h with hexane (700 mL). The extract was rotary-evaporated to a viscous, dark green residue that was re-extracted with MeOH/H_2O (70:30), repeatedly filtered through a 30 mm Nylon 0.45 μm syringe filter until clear, and separated using the preparative reversed phase HPLC method described in Method A. Using this method, **7** and **11** were isolated from white snakeroot collected at the Davies County site, **8** and **9** were isolated from plant material collected at the VMDL site, **5** was isolated from plant material collected at the Evans Place site, and **10** was isolated from plant material collected at the Cincinnati Zoo.

Method C: Extraction−Preparative HPLC−Solvent Partitioning. Isolation of **4** from the Trelease Woods plant material required some modifications of method B. Immediately after the fraction containing **4** was collected using the preparative HPLC, diethyl ether was added to the fraction until the mixture separated into polar and nonpolar phases. After thorough mixing, the diethyl ether phase was collected, dried over anhydrous $MgSO_4$, and then rotary-evaporated to dryness. This procedure (HPLC fractionation and solvent partitioning) was repeated multiple times until enough **4** was collected for NMR analysis.

NMR Analysis. NMR spectra of compounds in $CDCl_3$ were recorded using a JEOL 300 NMR spectrometer (JEOL Ltd., Peabody, MA). Chemical shifts are reported in δ units (ppm) and coupling constants (J) in hertz. When necessary, assignments were confirmed using HSQC (*12*) and HMBC experiments (*13*). The NMR data for compounds **2** (*14*, *15*), **4** (*16*), **5** (*17*), **6** (*16*, *18*, *19*), **7** (*20*, *21*), **8** (*22*, *23*), **9** (*24*), **10** (*25*, *26*), and **11** (*27*, *28*) were correlated to previously reported data. ^{1}H NMR assignments were confirmed (*24*), and ^{13}C NMR assignments for compound **9** were determined using HSQC and HMBC experiments.

6-Acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (9): ^{1}H NMR (300 MHz, $CDCl_3$), δ 1.50 (6H, s, H-13, H-14), 2.53 (3H, s, H-12), 3.84 (OCH_3), 5.60 (1H, d, J = 10.13, H-3), 6.71 (1H, d, J = 10.13), H-4), 7.03 (1H, s, H-7); ^{13}C NMR (300 MHz, $CDCl_3$), δ 26.42 (C-12), 28.28 (C-13, C-14), 57.36 (OCH_3), 78.26 (C-2), 110.43 (C-9), 111.94 (C-6), 113.49 (C-7), 116.10 (C-4), 128.53 (C-3), 141.27 (C-8), 150.48 (C-10), 155.14 (C-5), 202.36 (C-11).

Analytical Scale Extraction and HPLC Analysis. Using a HPLC method developed for the quantitation of tremetone, dehydrotremetone, and 3-oxyangeloyltremetone in white snakeroot and rayless goldenrod (*11*), the concentrations of **1**−**11** were determined in the different white snakeroot collections. Dry, ground aerial plant material was weighed (100 mg) into a screw-top glass test tube (16 mL). The plant material was extracted (16 h) by mechanical rotation with hexane/ethyl acetate (70:30 v/v) (8 mL) at ambient temperature. HPLC was performed using a Shimadzu LC-20AT (Shimadzu Co., Kyoto, Japan) equipped with an autosampler and PDA detector from the same vendor and a 100 mm × 2 mm i.d., 5 μm, Betasil C_{18} column (Thermo Hypersil-Keystone, Bellefonte, PA). Samples (10 μL) in hexane/ethyl acetate (70:30 v/v) were injected onto the column and eluted with a 20 mM ammonium acetate/acetonitrile mobile phase at a flow rate of 0.4 mL/min. The mobile phase program was 20 mM ammonium acetate/acetonitrile, 65:35 v/v, for 4 min followed by a linear gradient to a composition of 65% acetonitrile at 20 min. At 21 min the composition was increased to 100% acetonitrile for 5 min. The eluant was monitored at λ 280 nm. The concentration of tremetone in plant collections was quantified against a seven-point tremetone calibration curve using previously isolated tremetone (**1**) (*11*). The standard solutions were prepared by serial dilution with hexane/ethyl acetate (70:30) over the range of 0.78−50.0 μg/mL. The calibration curve had R^2 of 0.9999. Using the isolated compounds, response factors relative to tremetone at λ 280 nm were determined to be 1.3, 0.69, 0.35, 0.87, 1.3, 1.5, 1.7, and 5.6 for 6-hydroxytremetone (**2**), dehydrotremetone (**3**), 2-senecioyl-4-acetylphenol (**4**), 2-senecioyl-4-(1-methoxyethyl)phenol (**5**), 6-acetyl-2,2-dimethylchroman-4-one (**6**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**), 6-acetyl-8-methoxy-2,2-dimethylchromene (**8**), and 6,7-dimethoxy-2,2-dimethylchromene (**10**), respectively. Response factors were not determined for 6-acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (**9**) and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**) because these compounds were not sufficiently pure due to incomplete resolution from contaminants by preparative HPLC. Chemical profiles were classified into groups by visual analysis of the HPLC chromatograms based upon the presence or absence of major peaks and their relative amounts from the HPLC analysis.

Data Analysis. MANOVA and discriminant analysis of each predetermined group as a pairwise comparison was performed using BioNumerics 4.6 (Applied Maths, Inc.) Two parameters were reported: (1) L (Wilk's lambda likelihood ratio test) is the likelihood of the obtained discrimination with the assumption that the groups are drawn from the same population. A low L value implies that the groups are likely to be drawn from different populations. (2) p is the probability that a random grouping of the groups would yield the same degree of discrimination.

RESULTS AND DISCUSSION

Isolation and Identification of Compounds. As a result of the opportunistic approach to acquisition of plant samples, some collections were freeze-dried, whereas others were air-dried by the collectors depending upon the availability of equipment. An in-depth investigation of the potential effects on phytochemical profile revealed no significant changes in qualitative or quantitative profile (unpublished data). Standard compounds were isolated on a larger

Table 2. Concentrations of Benzofurans, Phenols, and Chromenes in White Snakeroot Plant Collections

	compound concentrations[a] (μg/mg of dry weight)										
collection site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Illinois sites											
Stidham Woods	2.6	6.3	0.53								
Hart Woods	1.3	2.0	0.94								
Brownfield Woods	0.61		0.89								
Rutan Woods	0.86	5.7	0.99								
Salt Fork	4.7	2.7	0.53								
Interstate	2.0	0.079	0.43								
VRO	1.3	7.6	0.56								
Tonica	0.56	0.082	0.88								
Allerton Park	0.25	0.094	0.059				0.076				*0.027*
Trelease Woods	0.12	0.062	0.036	1.0		0.61					
Missouri sites											
Davies County	0.20		0.075				1.4	0.090			*1.1*
Shooting Star Trail	0.21	0.32	0.052	0.010	0.17	0.039	1.3	0.047			*0.69*
Karst Trailhead	0.28	0.59	0.30	0.058	0.23	0.16	2.2				*0.47*
VMDL	0.19	0.34	0.066		0.33	0.079	0.58	0.040	*0.27*		*0.55*
Evans Place	0.22	0.35	0.094	0.14	2.1	0.11	0.77				*0.87*
Pierpont Meadows	0.16	0.16	0.056	0.050	2.4	0.10	0.59				*0.44*
Indiana site											
Wabash River			0.021							9.9	
Ohio site											
Cincinnati Zoo	0.053									1.7	

[a] The concentrations in italics are expressed in tremetone equivalents.

scale from specific collections in which the compounds were shown by HPLC to be more abundant. Tremetone (**1**) and dehydrotremetone (**3**) were previously isolated from rayless goldenrod (*I. pluriflora*) (*11*). In this study, 6-hydroxytremetone (**2**), 2-senecioyl-4-acetylphenol (**4**), 2-senecioyl-4-(1-methoxyethyl)phenol (**5**), 6-acetyl-2,2-dimethylchroman-4-one (**6**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**), 6-acetyl-8-methoxy-2,2-dimethylchromene (**8**), 6-acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (**9**), 6,7-dimethoxy-2,2-dimethylchromene (**10**), and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**) were extracted from white snakeroot plant material from six different collection sites by Soxhlet extraction with hexane and isolated using reversed phase HPLC (**Figure 1**). Compounds **2**, and **4**−**11** were identified on the basis of comparison of NMR spectroscopic data with those previously reported. ^{13}C NMR assignments for 6-acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (**9**) were not found in the literature; hence, complete ^{1}H and ^{13}C NMR chemical shifts for **9** are reported herein and were assigned using HSQC and HMBC correlations.

Isolation methods without column chromatography and minimizing the use of water were used to isolate 2-senecioyl-4-acetylphenol (**4**). In the presence of silica or water **4** reacted to form 6-acetyl-2,2-dimethylchroman-4-one (**6**). This transformation of **4** to **6** under basic conditions has been previously described (*16*).

White Snakeroot Chemotypes. The concentration of tremetone (**1**) was determined from a tremetone (**1**) HPLC calibration curve. The concentrations of **2**−**8** and **10** in white snakeroot samples were determined from a tremetone (**1**) HPLC calibration curve. Adjustments to the calculated tremetone equivalents concentrations were made according to the respective UV−vis absorption response factors determined for each compound. Response factors were not determined for **9** and **11** because the isolated samples of these compounds were not pure enough, and hence their concentrations are expressed as tremetone equivalents. The concentrations (**Table 2**) of 11 compounds were determined for the 18 composite collections (**Table 1**). Three potential chemotypes were identified on the basis of the presence or absence of the major benzofuran/phenol/chromene compounds (**Table 2**). To confirm that each of these chemotypes was unique, multivariate statistical methods

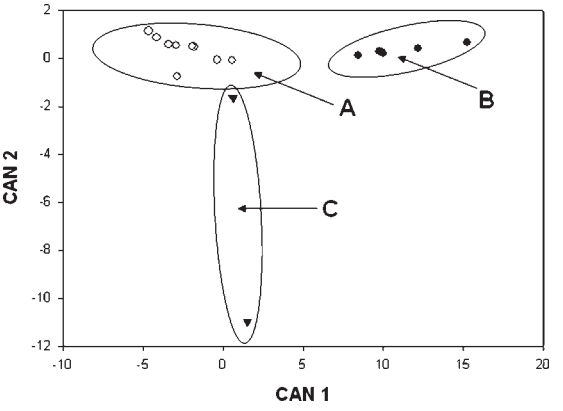

Figure 2. Plot of the first two canonical variables resulting from the discriminant analysis of the tabulated HPLC data: ○, chemotype A; ●, chemotype B; ▼, chemotype C.

(MANOVA and discriminant analysis) were used to test for grouping. An overall analysis found that there were differences between the defined groups ($p < 0.10$). A pairwise test was used to identify which groups were different. Chemotype A was different from chemotype B ($p < 0.10$, $L = 0.0897$). Chemotype C could not be statistically compared to the other two chemotypes via the MANOVA because of its small sample size ($n = 2$). Discriminant analysis was also performed as a pairwise comparison or in groups of three profiles. Discriminant analysis showed (**Figure 2**) clear separation of each group on the basis of one or more alkaloid.

Collections from Illinois representing chemotype A have similar benzofuran/phenol/chromene profiles consisting of tremetone (**1**), dehydrotremetone (**3**), and, in 90% of the collections, 6-hydroxytremetone (**2**) (**Table 2**). A representative HPLC chromatogram from plant material collected at the VRO is shown in **Figure 3A**. Concentrations of these three benzofurans were highly variable between locations. For example, the concentration of tremetone (**1**) ranges from 0.12 μg/mg in the Trelease Woods plant material to 4.7 μg/mg in the Salt Fork plant material. Two collections, Allerton

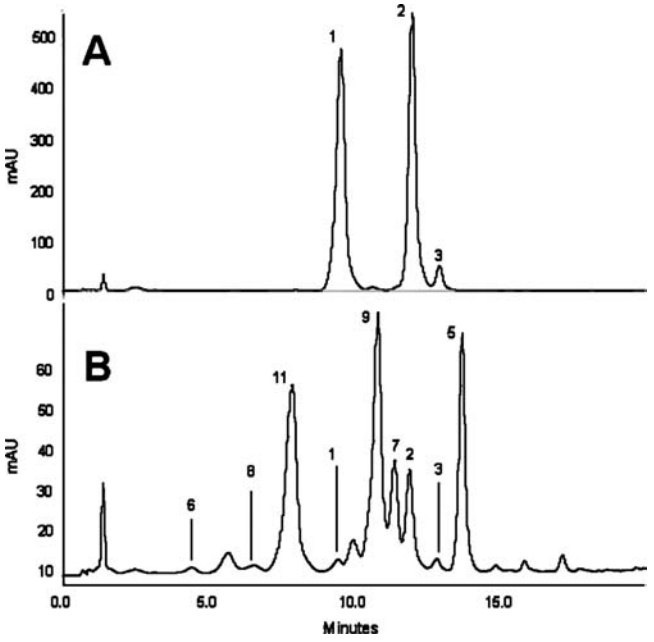

Figure 3. HPLC chromatograms of extracts from white snakeroot collected from (**A**) the Vermilion River Observatory (VRO) site and (**B**) the Veterinary Medical Diagnostic Laboratory (VMDL), University of Missouri. Peak numbers refer to compound structures in **Figure 1**.

Park and Trelease Woods, contain low concentrations of the benzofurans **1**−**3** compared to other collection sites near Champaign, IL, and also contain other phenol and chromene compounds. Allerton Park plant material contains 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**) and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**) at relatively low levels, whereas the Trelease Woods collection contains 2-senecioyl-4-acetylphenol (**4**) and 6-acetyl-2,2-dimethylchroman-4-one (**6**) at higher concentrations than the chromenes in the Allerton Park plant material. A reported incident of white snakeroot poisoning in sheep occurred near Danville, Vermilion County, IL (*7*), which is in the same county and vicinity as the Salt Fork, Interstate, and VRO collections. In this study, these collection sites had relatively high concentrations of tremetone, 6-hydroxytremetone, and dehydrotremetone. Couch reported milk sickness in Paxton, Ford County, IL (*2*). Vermilion County and Champaign County border Ford County on the east and south sides, respectively. Paxton, IL, is within 65 km of all the Vermilion and Champaign County collection sites.

Collections from near Columbia, MO, representing chemotype B have profiles that included tremetone (**1**), 6-hydroxytremetone (**2**), dehydrotremetone (**3**), 2-senecioyl-4-(1-methoxyethyl)phenol (**5**), 6-acetyl-2,2-dimethylchroman-4-one (**6**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**), and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**) (**Table 2**). A representative HPLC chromatogram from plant material collected at the Veterinary Medical Diagnostic Laboratory at the University of Missouri (VMDL) is shown in **Figure 3B**. Tremetone (**1**), dehydrotremetone (**3**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**7**), and 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**11**) were found in all collections representing this chemotype. 6-Hydroxytremetone (**2**), 2-senecioyl-4-(1-methoxyethyl)phenol (**5**), and 6-acetyl-2,2-dimethylchroman-4-one (**6**) were found in 83% of the collections representing this chemotype. Additionally, several other benzofuran/phenol/chromene compounds including 2-senecioyl-4-acetylphenol (**4**) and 6-acetyl-8-methoxy-2,2-dimethylchromene (**8**) were found in at least half of the Missouri collections, whereas 6-acetyl-5-hydroxy-8-methoxy-2,2-dimethylchromene (**9**) was only found at the VMDL site. The benzofuran/phenol/chromene profile of the white snakeroot collected at the Davies County site is the most dissimilar when compared to the other Missouri collection sites. This site is also the farthest removed in distance from the other Missouri collections.

The Wabash River and Cincinnati Zoo collections represent chemotype C. These collections both contain 6,7-dimethoxy-2,2-dimethylchromene (**10**), also known as precocene II (**10**) (*22*, *23*). Additionally, each of these collections contain only one other benzofuran ketone, one containing tremetone (**1**) and the other containing dehydrotremetone (**3**). A previous paper describes two collections of white snakeroot plant material from Cincinnati that were growing side by side. One plant contained a relatively high concentration of tremetone (type A), whereas the second plant (type B) contained less tremetone and higher amounts of the precocenes I and II (*8*). The plants collected at the Wabash River site and the Cincinnati Zoo appear to be chemically similar to that white snakeroot described as type B. More collections will need to be made to determine the chemotype of the other previously reported collection described as type A at the Cincinnati Zoo.

Whereas the present data support three distinct chemotypes for plants collected at (1) Illinois, (2) near Columbia, MO, and (3) the Wabash River and Cincinnati Zoo, a comprehensive investigation into the physiochemical profiles is ongoing and will better define any year-to-year variation within a specific population. In addition, the Allerton Park and Trelease Woods collections near Champaign, IL, and the Davies County collection 180 km northeast of Columbia, MO, may represent other chemotypes. Additional plant collections will need to be examined to determine if the plants at these sites represent different chemotypes.

Compounds **1**−**4** and **10** have been previously reported in white snakeroot (*4*, *8*, *29*−*31*). However, this is the first time that compounds **5**−**9** and **11** have been reported in white snakeroot. Compounds **7**−**9** have been previously reported in both the *Ageratina* and *Eupatorium* genera (*22*, *32*−*36*), whereas **6** has been previously reported in the *Eupatorium* genus (*29*, *31*) and **11** has been reported in the *Ageratina* genus (*27*, *34*). 2-Senecioyl-4-(1-methoxyethyl)phenol (**5**) has not previously been reported in either the *Ageratina* or *Eupatorium* genus.

Differentiating the chemotypes of white snakeroot is necessary to understand the toxicity of white snakeroot. This information will prove to be even more valuable as additional information about the relative toxicity of the benzofurans, phenols, and chromenes in animal models becomes available. For example, if tremetone is the primary toxin in white snakeroot, as has been suggested (*6*), an animal would have to eat approximately 88 times more white snakeroot from the Cincinnati Zoo than white snakeroot from the Salt Fork site to become intoxicated. White snakeroot poisoning of sheep was reported (*7*) near Danville, Vermilion County, IL, and a case of milk sickness was reported (*2*) in Paxton, Ford County, IL. Both are within 65 km of the Vermilion and Champaign County collection sites described in this study and could imply a similar chemotype containing high concentrations of tremetone (**1**), 6-hydroxytremetone (**2**), and dehydrotremetone (**3**). It will also be important to understand if the chemical differences in these chemotypes are related to genetic or environmental influences, or both. An understanding of the chemotypes of white snakeroot will allow risk assessments and management recommendations to be made to prevent livestock losses and the contamination of milk in grazing animals. In conclusion, this information provides some potential insights into the sporadic and unpredictable nature of the intoxication related to white snakeroot.

ACKNOWLEDGMENT

We thank Andrea Dolbear and Katie Lott for technical assistance as well as Steve Buck and Jenny Kroll for assistance in plant

collection. We appreciate the assistance of M. E. Barkworth, Director, and M. B. Piep, Assistant Curator, of the Intermountain Herbarium at Utah State University, Dr. Stanley Welsh of the Stanley L. Welsh Herbarium at Brigham Young University, and Dr. David S. Seigler, Professor, Department of Plant Biology, University of Illinois, Champaign, IL, for taxonomic confirmation of plant specimens.

LITERATURE CITED

(1) Moseley, E. L. Milk sickness caused by white snakeroot. *The Ohio Academy of Science*; Edwards Brothers: Ann Arbor, MI, 1917; pp171.
(2) Couch, J. F. The toxic constituent of richweed or white snakeroot (*Eupatorium urticaefolium*). *J. Agric. Res.* **1927**, *35*, 547–576.
(3) Burrows, G. E.; Tyrl, R. J. Asteraceae Dumort. In *Toxic Plants of North America*, 1st ed.; Iowa State University Press: Ames, IA, 2001; pp161.
(4) Bonner, W. A.; DeGraw, J. I., Jr. Ketones from "white snakeroot" *Eupatorium urticaefolium*. *Tetrahedron* **1962**, *18*, 1295–1309.
(5) Bowen, D. M.; DeGraw, J. I., Jr.; Shah, V. R.; Bonner, W. A. The synthesis and pharmacological action of tremetone. *J. Med. Chem.* **1963**, *6*, 315–319.
(6) Beier, R. C.; Norman, J. O.; Irvin, R. T.; Witzel, D. A. Microsomal activation of constituents of white snakeroot (*Eupatorium rugosum* Houtt) to form toxic products. *Am. J. Vet. Res.* **1987**, *48*, 583–585.
(7) Beier, R. C.; Norman, J. O.; Reagor, M. C.; Rees, M. S.; Mundy, B. P. Isolation of the major component in white snakeroot that is toxic after microsomal activation: possible explanation of sporadic toxicity of white snakeroot plants and extracts. *Nat. Toxins* **1993**, *1*, 286–293.
(8) Smith, R. A.; Lang, D. G. Are there toxic and non-toxic varieties of *Eupatorium rugosum* Houttuyn? In *Toxic Plants and Other Natural Toxicants*; Garland, T., Barr, A. C., Eds.; CAB Interntional: New York, 1998; pp 220−222.
(9) Doyle, L. P.; Walkey, F. L. White snakeroot poisoning in livestock. *Indiana Agric. Exp. Stn. Bull.* **1923**, 270.
(10) Curtis, R. S.; Wolf, F. A. *Eupatorium ageratoides*, the cause of trembles. *J. Agric. Res.* **1917**, *9*, 397.
(11) Lee, S. T.; Davis, T. Z.; Gardner, D. R.; Stegelmeier, B. L.; Evans, T. J. A quantitiative method for the measurement of three benzofuran ketones in rayless goldenrod (*Isocoma pluriflora*) and white snakeroot (*Ageratina altissima*) by HPLC. *J. Agric. Food Chem.* **2009**, *57*, 5639–5643.
(12) Bax, A.; Summers, M. F. Proton and carbon-13 assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
(13) Bodenhausen, G.; Ruben, D. J. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem. Phys. Lett.* **1980**, *69*, 185–189.
(14) Liu, Z.-L.; Tian, X. The components of *Cacalia tangutica*. *Bull. Korean Chem. Soc.* **2004**, *25*, 1078–1080.
(15) Benn, M.; Lynds, S.; Knox, E. B. A reexamination of the secondary metabolites of *Dendrosenecio kilimanjari* subsp. *cottonii*. *ARKIVOC* **2009**, 17–22.
(16) Orden, A. A.; Bisogno, F. R.; Cifuente, D. A.; Giordano, O. S.; Sanz, M. K. Asymmetric bioreduction of natural xenobiotic diketones by *Brassica napus* hairy roots. *J. Mol. Catal. B: Enzym.* **2006**, *42*, 71–77.
(17) do Nascimento, A. M.; Salvador, M. J.; Candido, R. C.; de Albuquerque, S.; de Oliveria, D. C. R. Trypanocidal and antifungal activities of *p*-hydroxyacetophenone derivatives from *Calea uniflora* (Heliantheae, Asteraceae). *J. Pharm. Pharmacol.* **2004**, *56*, 663–667.
(18) Orden, A. A.; Bisogno, F. R.; Cifuente, D. A.; Giordano, O. S.; Sanz, M. K. Comparative study in the asymmetric bioreduction of ketones by plant organs and undifferentiated cells. *J. Mol. Cat. B: Enzym.* **2008**, *51*, 49–55.
(19) Bohlman, F.; Zdero, C.; Lonitz, M. Neue guajen-derivate aus *Parthenium hysterophorus* und ein weiteres pseudoguajanolid aus *Ambrosia cumanensis*. *Phytochemistry* **1977**, *16*, 575–577.
(20) Ahluwalia, V. K.; Prakash, C.; Gupta, R. An elegant synthesis of some naturally occurring linear acetylchromenes: eupatoriochromene, methyleupatoriochromene (encecalin); evodional and methylevodiol. *Tetrahedron* **1982**, *38*, 609–611.
(21) Castaneda, P.; Gomez, L.; Mata, R.; Lotina-Hennsen, B.; Anaya, A. L.; Bye, R. Phytogrowth-inhibitory and antifungal constituents of *Helianathella quinquenervis*. *J. Nat. Prod.* **1996**, *59*, 323–326.
(22) Taylor, D. R.; Wright, J. A. Chromenes from *Eupatorium riparium*. *Phytochemistry* **1971**, *10*, 1665–1667.
(23) Pabreiter, C. M.; Willuhn, G.; Steigel, A.; Matthiesen, U. Chromenes from *Arnica sachalinesis* and *A. ampelxicaulis*. *Phytochemistry* **1992**, *31*, 1070–1072.
(24) Jain, A. C.; Khazanchi, R.; Kumar, A. Synthesis of some naturally occurring acetylchromenes. *Bull. Chem. Soc. Jpn.* **1979**, *52*, 1203–1204.
(25) Steinbeck, C.; Spitzer, V.; Starosta, M.; von Poser, G. Identification and structure of two chromenes from *Calea serrata* by semiautomatic structure elucidation. *J. Nat. Prod.* **1997**, *60*, 627–628.
(26) Adler, M. J.; Baldwin, S. W. Direct, regioselective synthesis of 2,2-dimethyl-2*H*-chromenes. Total syntheses of octandrenolone and precocenes I and II. *Tetrahedron Lett.* **2009**, *50*, 5075–5079.
(27) Fang, N.; Yu, S.; Mabry, T. Chromenes from *Ageratina arsenii* and revised structures of two epimeric chromene dimers. *Phytochemistry* **1988**, *27*, 1902–1905.
(28) Gonzalez, A. G.; Aguiar, Z. E.; Grillo, T. A.; Luis, J. G.; Rivera, A.; Calle, J. Chromenes from *Ageratum conyzoides*. *Phytochemistry* **1991**, *30*, 1137–1139.
(29) Bonner, W. A.; DeGraw, J. I., Jr.; Bowen, D. M.; Shah, V. R. Toxic constituents of white snakeroot. *Tetrahedron Lett.* **1961**, *12*, 417–420.
(30) Bohlman, F.; Zitzkowski, P.; Suwita, A.; Fiedler, L. *cis*-Kolaveninsaure und weitere inhaltsstoffe aus vertretern der tribus Eupatorieae. *Phytochemistry* **1978**, *17*, 2101–2105.
(31) Zalkow, L. H.; Ghosal, L. G.; Fleischmann, T. J. The co-occurance of desmethylencecalin and hydroxytremetone in *Eupatorium rugosum*. *Phytochemistry* **1977**, *16*, 1313.
(32) Anthonsen, T. New chromenes from *Eupatorium* species. *Acta Chem. Scand.* **1969**, *23*, 3605–3607.
(33) Gonzalez, A. G.; Barrera, J. B.; Yanes, A. C.; Diaz, J. G.; Perez, E. M. R. Chromenes and benzofurans from *Ageratina glenchonophylla*. *Phytochemistry* **1989**, *28*, 2520–2522.
(34) Banerjee, S.; Jakupovic, J.; Bohlmann, F.; King, R. M.; Robinson, H. Chromenes from *Ageratina riparia*. *Phytochemistry* **1985**, *24*, 2681–2683.
(35) Bohlmann, F.; Fielder, L. Neue nerolidol-derivate aus *Ageratina acshenbornia*. *Phytochemistry* **1977**, *17*, 566–567.
(36) Gomez, F.; Quijano, J. S.; Calderon, A.; Perales, A.; Rios, T. 2,2-Dimethylchromenes from *Eupatorium aschembornianim*. *Phytochemistry* **1982**, *21*, 2095–2097.

Received for review April 1, 2010. Revised manuscript received July 1, 2010. Accepted July 2, 2010.