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Evaluation of Drying Methods and Toxicity of Rayless Goldenrod (*Isocoma pluriflora*) and White Snakeroot (*Ageratina altissima*) in Goats

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ABSTRACT: White snakeroot and rayless goldenrod cause "trembles" and "milk sickness" in livestock and humans, respectively. The toxin in white snakeroot and rayless goldenrod was identified in 1927 and 1930, respectively, as tremetol. It was reported that the toxin in white snakeroot disappears as it is dried and that completely dried plants were incapable of producing trembles or milk sickness. Conversely, it has been reported that the rayless goldenrod toxin was not destroyed by drying and that the plant is toxic either fresh or dry. In this study the concentrations of tremetone, dehydrotremetone, and structurally similar compounds were determined in white snakeroot and rayless goldenrod before and after various drying conditions. Tremetone, dehydrotremetone, and structurally similar compounds in rayless goldenrod and white snakeroot are most stable upon freeze-drying, followed by air-drying, and least stable upon oven-drying (60 °C). Also demonstrated is that tremetone is stable and that dried white snakeroot and rayless goldenrod are capable of inducing toxicosis in livestock.

KEYWORDS: *rayless goldenrod, Isocoma pluriflora, white snakeroot, Eupatorium rugosum, Ageratina altissima, tremetone, dehydrotremetone*

INTRODUCTION

White snakeroot (*Ageratina altissima* (L.) King & H. Rob. var. *altissima*) (family, Asteraceae) and rayless goldenrod (*Isocoma pluriflora* (Torr. & A. Gray) Greene) (family, Asteraceae) cause "trembles" and "milk sickness" in livestock and humans. Milk sickness caused many deaths among Midwestern settlers during the 1800s, forcing entire settlements to be abandoned. The first documented case of milk sickness occurred in 1810.[1] However, the disease was sporadic and unpredictable, and it was not until 1917 that white snakeroot was shown to be responsible for trembles and milk sickness.[2] In the early 1900s a disease with nearly identical clinical signs broke out in the southwestern United States, and it was quickly established that the southwestern milk sickness was due to livestock ingestion of rayless goldenrod.[3]

Shortly thereafter, Couch identified the toxin in white snakeroot and rayless goldenrod as tremetol.[1,4] Later, Couch reported that tremetol rapidly disappears as white snakeroot is dried and that completely dried plants were incapable of producing trembles or milk sickness.[1,4−6] Couch suggested that toxin degradation was plant specific as he concurrently reported that the rayless goldenrod toxins were not destroyed by drying and that both fresh and dry plants are toxic.[1,4,5]

Decades later, Bonner et al.[7] and Bonner and DeGraw[8] extracted white snakeroot and separated the extract into a sterol fraction and a ketone fraction. The components of the sterol fraction were not toxic in a goldfish assay.[9] The ketone fraction was separated into three ketones, tremetone (**1**), dehydrotremetone (**2**), and hydroxytremetone, which were all toxic in the goldfish bioassay.[9] More recently, Beier and co-workers[10] isolated tremetone (**1**) from white snakeroot and reported that, upon microsomal activation, tremetone (**1**) was toxic to murine melanoma ($B16F_1$) cells and five other mammalian cell lines. They also concluded that tremetone (**1**) is most likely the toxic component in both white snakeroot and rayless goldenrod. Beier et al. also reported that tremetone (**1**) is unstable and is converted to nontoxic dehydrotremetone (**2**) in dried plant and plant extracts. The conversion of toxic tremetone (**1**) to nontoxic dehydrotremetone (**2**) was offered as an explanation for the sporadic toxicity of white snakeroot and rayless goldenrod.[10,11]

In general, subsequent review literature on white snakeroot toxicity has perpetuated these reports and their suppositions: tremetol or tremetone (**1**) are the toxic principles; white snakeroot plants lose toxicity when dried;[12−15] and tremetone (**1**) is converted to dehydrotremetone (**2**) during isolation.[13] Additionally, it is reported that rayless goldenrod contains the same or similar toxic principle as white snakeroot.[12−14]

Recently, a quantitative high-performance liquid chromatography (HPLC) method to measure the concentrations of tremetone (**1**) dehydrotremetone (**2**) and structurally similar compounds in white snakeroot and rayless goldenrod plant material was developed. Using this method it was determined that the concentrations of these compounds vary considerably between plant populations.[16,17] The concentrations of these compounds have been measured in toxicity studies in which goats were dosed with dried white snakeroot and dried rayless goldenrod.[18]

The objectives of this study were to determine the concentrations of tremetone (**1**), dehydrotremetone (**2**), and structurally similar compounds in white snakeroot and rayless

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goldenrod before and after various drying methods and to determine the best approach to dry plant material for future chemotaxonomic studies and animal dosing studies. We also evaluated the stability of tremetone (**1**) and the effect of dried white snakeroot and rayless goldenrod on goats.

EXPERIMENTAL PROCEDURES

Plant Material: Drying Studies. Rayless goldenrod was collected near Pecos in Reeves County, TX (31° 23.969′ N/103° 29.969′ W, accession no. 3476, Poisonous Plant Research Laboratory Herbarium) on June 1, 2009. White snakeroot was collected at the University of Illinois, Nettie Hart Woods Research Area, Champaign County, IL (40° 13.732′ N/88° 21.346′ W, accession no. 3515, Poisonous Plant Research Laboratory Herbarium) on June 1, 2009. The rayless goldenrod and white snakeroot collections were shipped overnight to the Poisonous Plant Research Laboratory. Plants were identified by personnel at the Poisonous Plant Research Laboratory.

Plant Material: Animal Dosing. Rayless goldenrod for animal dosing was collected on May 6, 2008, near Pecos, TX (31° 23.969′ N/ 103° 29.969′ W, accession no. 3056, Poisonous Plant Research Laboratory Herbarium), air-dried at ambient temperature, and ground August 7, 2008. Plants were identified by personnel at the Poisonous Plant Research Laboratory.

White snakeroot for animal dosing was collected on September 16–18, 2009, near Danville, IL (40° 05.563′ N/87° 49.683′ W, accession no. 3555, Poisonous Plant Research Laboratory Herbarium), dried at ambient temperature, stored, and ground February 26, 2010. Plants were identified by personnel at the Poisonous Plant Research Laboratory.

Plant Processing. The plants from both the rayless goldenrod and white snakeroot collections of June 1 were processed on June 2, 2009. Leaves were stripped off the rayless goldenrod plants and collected in a 4 L plastic utility pan. The leaves were thoroughly mixed by stirring the leaves by hand and then separated into four subsamples. Leaves from 10 white snakeroot plants were picked starting from the bottom of the stem to the top of the stem and put in subsamples 1, 2, 3, and 4 in order until all leaves were removed from the stem. Leaves from the next plant were picked from the bottom to the top of the stem and put into subsamples in the order 2, 3, 4, and 1. Leaves were picked from the bottom to the top of the third stem and put in subsamples in the order 3, 4, 1, and 2. This process was repeated until the leaves from all 10 stems were placed in the four subsamples. Subsamples 1–4 were further processed in the following manner: (1) fresh, intact leaves were ground in liquid nitrogen and approximately 300 mg was weighed into tared screw-top glass 16 mL test tubes for immediate extraction with hexane and ethyl acetate; (2) freeze-dried, leaves were put in a plastic bag, dipped in liquid nitrogen, and then freeze-dried for 48 h; (3) oven-dried, leaves were oven-dried at 60 °C for 48 h; (4) air-dried, leaves were placed in the greenhouse to dry for 7 days. Maximum daytime temperatures were 34 °C and minimum nighttime temperatures were 4.5 °C.

Fresh intact leaves were ground in liquid nitrogen using a mortar and pestle. Plant material was frozen during and after the fresh grinding process. The mortar and pestle used to grind the plant material was placed in the freezer (−80 °C) for at least an hour before being used. Leaves that were freeze-dried, oven-dried, or air-dried, under the conditions described previously, were ground to pass through a 1 mm screen using a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden).

Fresh, ground rayless goldenrod and white snakeroot leaf material was extracted and analyzed by HPLC immediately after grinding with liquid nitrogen. Immediately after injection by the HPLC system the autosample vials were recapped and stored in the refrigerator at 4 °C for 7 days. These samples (fresh-2) were analyzed again in 7 days along with the samples that were freeze-dried, oven-dried, and air-dried. After the second analysis, the contents of the autosample vials containing the extract from the fresh, ground rayless goldenrod and white snakeroot leaf material were returned to the tared 16 mL screw-top test tubes, and the hexane/ethyl acetate (70:30, v/v) was allowed to evaporate to complete dryness, after which the samples were placed in an oven (60 °C) overnight. After evaporation of the extraction solvent, the tared 16 mL screw-top test tubes were weighed, and the dry weight of the rayless goldenrod and white snakeroot leaf material was obtained.

Extraction. Fresh ground-leaf material (300 mg) previously weighed into a tared screw-top glass test tube (16 mL) and dry ground-leaf material were weighed (100 mg) into a screw -top glass test tube (16 mL). The leaf material was extracted (16 h) by mechanical rotation with hexane/ethyl acetate (70:30 v/v) (8 mL). The samples were centrifuged (5 min) and supernatants transferred (1 mL) into autosample vials for HPLC analysis. Samples from different plant-processing methods were run in quadruplicate.

HPLC. Analytical scale reversed phase HPLC was performed on a Shimadzu LC-20AT 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, USA). 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. Detection of analytes in the eluent was performed at λ 280 nm. Under these conditions tremetone (**1**), dehydrotremetone (**2**), 3-oxyangeloyl-tremetone (**3**), 6-hydroxytremetone (**4**), and 2-senecioyl-4-acetylphenol (**5**) eluted at 8.5, 12.0, 14.7, 11.0, and 9.6 min, respectively (see Figure 1 for chemical structures). The compound concentrations in leaves were quantitated against a dehydrotremetone (**2**) six-point calibration curve, prepared by serial dilution using previously isolated dehydrotremetone (**2**)[16] in hexane/ethyl acetate (70:30, v/v) over the range of 3.13–100 μg/mL.

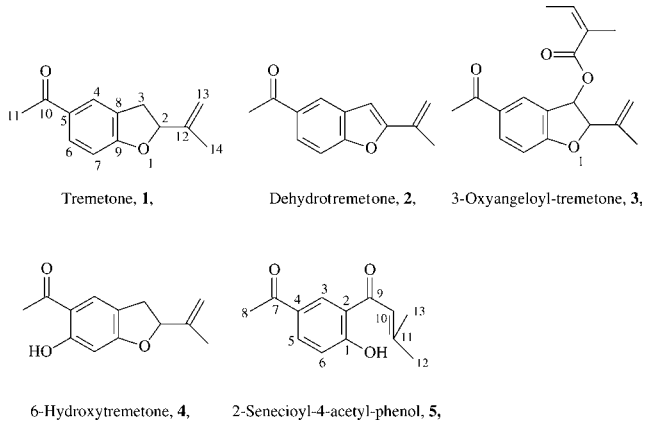

Figure 1. Chemical structures of tremetone (**1**), dehydrotremetone (**2**), 3-oxyangeloyl-tremetone (**3**), 6-hydroxytremetone (**4**), and 2-sencioyl-4-acetylphenol (**5**).

NMR. NMR spectra of tremetone in $CDCl_3$ were recorded using a JEOL 300 NMR spectrometer (JEOL Ltd., Peabody, MA, USA).

Animal Dosing. Yearling, Spanish goats in good body condition that weighed 22–34 kg were randomly divided into groups (controls or treated) for dosing. The different groups of goats were dosed intraruminally via oral gavage until they demonstrated classical clinical signs of poisoning (7–9 days). The control group was given similar amounts of ground alfalfa/grass hay via oral gavage. The dose was split and given twice per day (a.m. and p.m.). During the studies the goats had free-choice access to water and long-stem alfalfa hay ad libitum. All animal work was done under veterinary supervision with the approval and supervision of the Utah State University Institutional Animal Care and Use Committee.

Data Analysis. A two-way ANOVA was performed using Sigma Stat 3.1 whereby the extraction method and the compound were the

two factors with a post hoc test of significance using a Bonferroni correction. A p value of <0.001 was considered to be statistically significant.

RESULTS AND DISCUSSION

Rayless Goldenrod. Three benzofuran ketones, tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**), were detected in rayless goldenrod. A similar benzofuran ketone profile was observed from rayless goldenrod as reported by Lee et al.[16] There was a significant interaction ($p < 0.001$) between content of benzofuran ketones and treatment (drying method). For further comparison individual benzofuran ketone concentrations were compared between treatments and reported as the mean (± SD) in Table 1. Tremetone (**1**) and 3-oxyangeloyl-tremetone (**3**) concentrations did not vary between treatments. Dehydrotremetone (**2**) concentrations varied between treatments as decreased concentrations were observed in samples that were air- and oven-dried compared to the control sample.

Table 1. Variation in Mean Concentrations (± SD) of Benzofuran Ketones in Rayless Goldenrod with Different Drying Methods

	compound concentrations[a] (μg/mg of dry weight)		
drying method	tremetone (**1**)	dehydrotremetone (**2**)	3-oxyangeloyl-tremetone (**3**)
fresh	0.64 ± 0.05a	4.4 ± 0.2a	1.02 ± 0.04a
fresh-2	0.74 ± 0.05a	4.4 ± 0.3a	1.02 ± 0.07a
freeze-dried	0.60 ± 0.04a	4.0 ± 0.3a	1.2 ± 0.1a
oven-dried	0.54 ± 0.04a	2.9 ± 0.2c	1.05 ± 0.07a
air-dried	0.53 ± 0.03a	3.4 ± 0.2b	0.92 ± 0.08a

[a]Different letters within a column represent significance between drying methods at $p < 0.001$.

White Snakeroot. Three benzofuran ketones, tremetone (**1**), dehydrotremetone (**2**), and 6-hydroxytremetone (**4**), as well as an additional similar compound, 2-sencioyl-4-acetylphenol (**5**), were detected in the white snakeroot sample. A similar profile was observed from white snakeroot by Lee et al.[16,17] As with goldenrod there was a significant interaction ($p < 0.001$) between content of benzofuran ketones and treatment treatment (drying method). The concentrations of the individual benzofuran ketones were compared between treatments and reported as the mean (± SD) in Table 2.

Tremetone (**1**) concentrations were similar to the fresh plant material in the freeze-dried treatment but significantly less in the oven-dried treatment. Tremetone (**1**) concentrations were significantly greater in the air-dried sample compared to fresh plant material and freeze-dried sample, which is likely due to the lack of sample homogeneity. Dehydrotremetone (**2**) concentrations were statistically different in the two controls samples; however, these differences were not considered to be biologically significant and are likely due to limitations in analytical procedures. The concentrations of dehydrotemetone (**2**) in freeze-dried and air-dried treatments were statistically different from the fresh plant material but, again, were not considered to be biologically significant as a similar amount of variation is found within the fresh plant samples as is found when these two treatments and the control were compared. The oven-dried treatment was the only sample that was biologically and statistically different from the other treatments. 6-Hydroxytremetone (**4**) concentrations were not different between any of the treatments. 2-Sencioyl-4-acetylphenol (**5**) differed between treatments, but the concentration of compound present in the sample makes it difficult to determine whether the differences in treatment are due to the treatment or due to how the near-homogeneous samples were prepared from the white snakeroot leaves.

Tremetone Stability. A vial of tremetone (**1**) was isolated from rayless goldenrod and stored the majority of the time in the refrigerator at 4 °C and has been stable for 38 months over the time period from November 18, 2008, to February 9, 2012. Tremetone (**1**) from this vial has been used as an analytical standard in several studies.[16−19] A subsample of the tremetone (**1**) was dissolved in deuterated chloroform and confirmed to be tremetone (**1**) by NMR on July 12, 2011. The NMR sample was left at room temperature and was reanalyzed by NMR on July 25, October 20, and December 16, 2011. The spectra from the NMR analyses were identical.

Dried Rayless Goldenrod Dosing Experiments. Dried ground rayless goldenrod containing 0.20 μg/mg tremetone (**1**), 0.71 μg/mg dehydrotremetone (**2**), and 1.2 μg/mg 3-oxyangeloyl-tremetone (**3**) was dosed to four goats at ∼60 mg benzofuran ketones/kg body weight or at 3.0% body weight for 7 days from August 8 to 14, 2008.[18] Stiffness, reluctance to move, and trembles when forced to exercise were observed in the goats. At the time of necropsy the goats had palor and streaking in many of the large appendicular muscles as well as extensive monophasic degeneration and necrosis of skeletal muscle. The degeneration and necrosis were inconsistent as some animals and different skeletal muscle were variably affected. Microscopically, the myopathy was characterized by segmental loss of myocyte striation, hypereosinophilia, clumping and disruption of sarcoplasmic proteins, and monocytic inflammation composed mostly of debris-laden macrophages with fewer numbers of lymphocytes (Figure 2). There were early changes of myocyte regeneration seen as proliferation and rowing of myocyte nuclei.[18]

Table 2. Variation in Mean Concentrations (± SD) of Benzofuran Ketones and Phenols in White Snakeroot with Different Drying Methods

	compound concentrations[a] (μg/mg of dry weight)			
drying method	tremetone (**1**)	dehydrotremetone (**2**)	6-hydroxytremetone (**4**)	2-sencioyl-4-acetylphenol (**5**)
fresh	4.6 ± 0.1c	6.0 ± 0.1b	0.50 ± 0.01a	0.73 ± 0.04a
fresh-2	4.9 ± 0.1bc	6.4 ± 0.1a	0.78 ± 0.01a	0.78 ± 0.04a
freeze-dried	5.2 ± 0.2b	5.6 ± 0.2c	0.53 ± 0.04a	0.27 ± 0.02bc
oven-dried	3.0 ± 0.1d	3.0 ± 0.1d	0.47 ± 0.01a	0.03 ± 0.01c
air-dried	6.8 ± 0.3a	5.6 ± 0.2c	0.35 ± 0.02a	0.58 ± 0.07ab

[a]Different letters within a column represent significance between drying methods at $P < 0.001$.

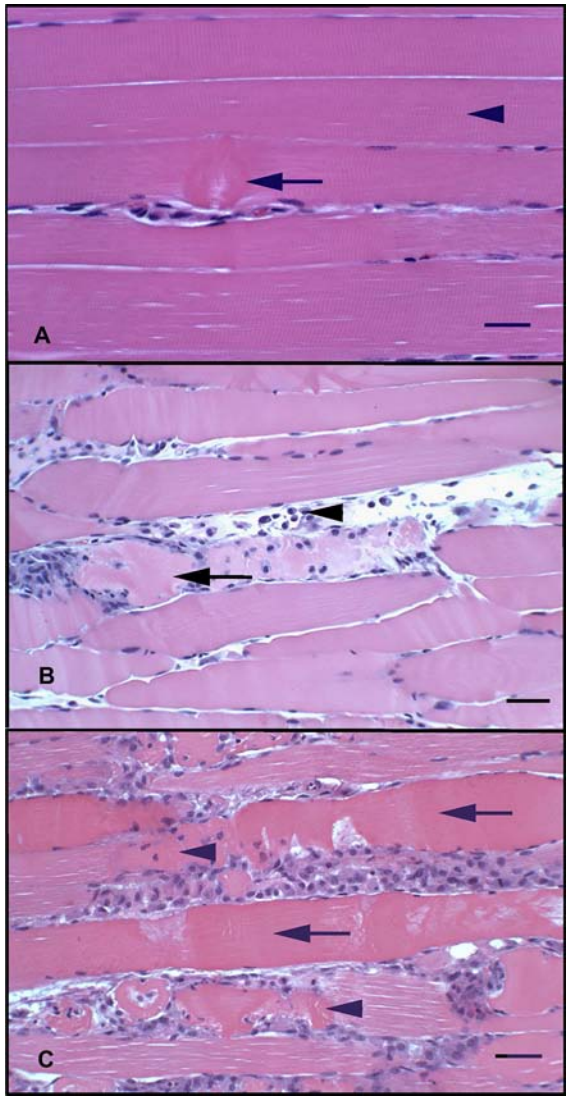

Figure 2. (A) Photomicrograph of the semitendinosus muscle of a control goat. Note the prominent striations (arrowhead) and rare contraction bands (arrow). H&E, bar = 30 um. (B) Photomicrograph of the semitendinosus muscle of a goat poisoned with rayless goldenrod (*Isocoma pluriflora*) for 7 days. Note the extensive myonecrosis with coagulation and clumping of sarcomere protein (arrow) and adjacent inflammation (arrowhead). H&E, bar = 50 um. (C) Photomicrograph of the biceps femoris muscle of a goat poisoned with white snakeroot (*Ageratina altissima*) for 9 days. This plant also induces extensive myonecrosis with coagulation and clumping of sarcomere protein (arrow) and adjacent inflammation (arrowhead). H&E, bar = 30 um.

Dried White Snakeroot Dosing Experiments. Dried ground white snakeroot containing 2.1 μg/mg tremetone (**1**), 0.53 μg/mg dehydrotremetone (**2**), 0.96 μg/mg 6-hydroxy-tremetone (**4**), and 0.72 μg/mg 6-acetyl-7-methoxy-2,2-dimethylchromene was dosed to four goats at ∼60 mg benzofuran ketones/kg body weight or at 1.7% of body weight for 9 days from April 13 to 21, 2010. Three of the four goats became sore and reluctant to move and demonstrated trembles when forced to exercise. At necropsy animals that were clinically affected had palor and streaking in many of the large appendicular muscles. Myocyte palor was confirmed histologically as extensive monophasic degeneration and necrosis of skeletal muscle. The necrosis was similar to that of rayless goldenrod-induced myonecrosis as it was also variable in location and severity. The myocyte degeneration and necrosis was also characterized by myocyte swelling, hypereosinophilia, and loss of striation. In necrotic myocytes there was coagulation and clumping of proteins with focal monocytic inflammation (Figure 2). Some lesions were more chronic, with early fibrosis and myocyte regeneration seen as proliferation and rowing of myocyte nuclei.

In summary, tremetone (**1**) and related compounds were present in all three methods of drying of rayless goldenrod and white snakeroot. The benzofuran ketones were best preserved when dried by freeze-drying, and we concluded that this is the best method for handling samples for analytical procedures. However, air-dried plant material retains the tremetone-like compounds and is more practical in cases when relatively large amounts of plant are needed for toxicology studies in animals. Furthermore, there is no evidence that tremetone (**1**) is converted to dehydrotremetone (**2**) as an isolated compound or upon plant drying. Additionally, animal dosing experiments demonstrated that air-dried rayless goldenrod and white snakeroot are toxic to goats for at least 14 and 30 weeks after collection and drying, respectively. These results demonstrate that white snakeroot and rayless goldenrod toxins are much more stable than previously reported and that air-dried plants remain toxic. Furthermore, white snakeroot and rayless goldenrod inadvertently harvested with other forages may be toxic to livestock.

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Notes

The authors declare no competing financial interest.

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