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Quantitative Method for the Measurement of Three Benzofuran Ketones in Rayless Goldenrod (*Isocoma pluriflora*) and White Snakeroot (*Ageratina altissima*) by High-Performance Liquid Chromatography (HPLC)

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White snakeroot (*Ageratina altissima*) and rayless goldenrod (*Isocoma pluriflora*) can cause "trembles" and "milk sickness" in livestock and humans, respectively. Tremetol, a complex mixture of sterols and derivatives of methyl ketone benzofuran has been extracted from white snakeroot and rayless goldenrod and is reported to be the toxic substance in plant material. In this study, the three major benzofuran ketones, tremetone, dehydrotremetone, and 3-oxyangeloyl-tremetone, were isolated from rayless goldenrod. Using these compounds as standards, a quantitative high-performance liquid chromatography (HPLC) method was developed to measure these compounds in white snakeroot and rayless goldenrod. Concentrations of tremetone, dehydrotremetone, and 3-oxyangeloyl-tremetone were found to vary considerably among the different white snakeroot and rayless goldenrod plant collections. Differences in concentrations of tremetone, dehydrotremetone, and 3-oxyangeloyl-tremetone in white snakeroot and rayless goldenrod plants may explain the historical sporadic and unpredictable toxicity of these plants to livestock and humans.

KEYWORDS: Rayless goldenrod; *Haplopappus heterophyllus*; *Isocoma wrightii*; *Isocoma pluriflora*; white snakeroot; *Eupatorium rugosum*; *Eupatorium urticaefolium*; *Ageratina altissima*; tremetone; dehydrotremetone; 3-oxyangeloyl-tremetone

INTRODUCTION

White snakeroot [*Ageratina altissima* (L.) King and H. Rob. var. *altissima* (Asteraceae), previously *Eupatorium rugosum* Houtt and *Eupatorium urticaefolium* Reichard] and rayless goldenrod [*Isocoma pluriflora* (Torr. and A. Gray) Greene (Asteraceae), previously *Isocoma wrightii* (A. Gray) Rydb and *Happlopappus heterophyllus* (A. Gray) S.F. Blake] contain a mixture of alcohols and ketones, referred to as tremetol, that cause "trembles" and "milk sickness" in livestock and humans, respectively. "Milk sickness" caused many deaths among Midwestern settlers during the 1800s, ultimately forcing entire settlements to be abandoned. Although the first documented case of milk sickness occurred in 1810 (*1*), its occurrence is sporadic and unpredictable, and it was not until 1917 that white snakeroot was unequivocally shown to be responsible for "trembles" and "milk sickness" (*2*).

In the late 1920s, after many studies, Couch concluded that the toxin in white snakeroot was tremetol (*1*). He later reported that tremetol was also present in rayless goldenrod found in the southwestern United States (*3*). However, Couch erroneously concluded that tremetol was a pure compound, when it is actually a complex mixture of sterols and derivatives of methyl ketone benzofuran that includes but is not limited to tremetone (**1**), dehydrotremetone (**2**), hydroxytremetone, dehydroxytremetone, and 3-oxyangeloyl-tremetone (**3**) (*4*, *5*). Bonner and co-workers (*4*, *6*) isolated the three ketones, tremetone (**1**), dehydrotremetone (**2**), and hydroxytremetone, from the tremetol mixture. All three compounds were toxic in a goldfish bioassay. However, questions arose when tremetone (**1**) was synthesized and did not induce toxicity in white leghorn cockerels or sheep (*7*). Beier and co-workers (*8*, *9*) reported that, upon microsomal activation, tremetone (**1**) produced a product that was toxic to murine melanoma ($B16F_1$) cells and five other mammalian cell lines. However, dehydrotremetone (**2**) was not toxic even after microsomal activation. The relative toxicity of the benzofuran ketone compounds in animal studies is unknown.

Herein, we report the development of a high-performance liquid chromatography (HPLC) method to quantify tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) in white snakeroot and rayless goldenrod (**Figure 1**). Additionally, we show that the concentrations of the benzofuran ketones (**1**−**3**) varied considerably among different plant collections of both white snakeroot and rayless goldenrod. This observation might help to explain the sporadic and unpredictable toxicity of white snakeroot and rayless goldenrod.

MATERIALS AND METHODS

Plant Material. Rayless goldenrod (*I. pluriflora*) for large-scale extraction was a collected in Palo Duro canyon, near State Highway

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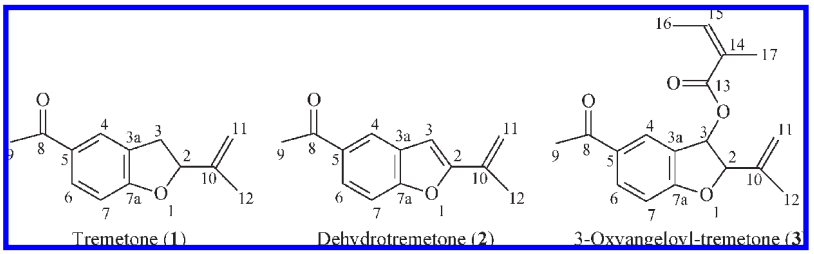

Figure 1. Chemical structures of tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**).

207, approximately 20 miles south of Claude, Armstrong County, TX, on Sept 27, 2007 at two locations at 34° 47.075′ N/101° 17.331′ W and 34° 50.090′ N/101° 25.040′ W. The plant material was identified as *I. pluriflora* by the staff at the Intermountain Herbarium at Utah State University (accession number 249123). The plant material was dried at ambient temperature for 4 months, and the leaves and stems were separated and ground to pass through a 2 mm screen.

Two small collections of rayless goldenrod were collected for analytical analysis: one in Reeves County, TX, on May 7, 2008 at 06° 42.656′ N/ 34° 74.847′ W and the second in Palo Duro canyon, near State Highway 207, approximately 20 miles south of Claude in Armstrong County, TX, on May 8, 2008 at 34° 50.090′ N/101° 25.040′ W (accession numbers 250012 and 250014, Intermountain Herbarium, Utah State University). The whole plant was kept on ice for transport and then frozen until plant parts (leaves and stems) were separated, freeze-dried, and ground with a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden), to pass through a 1 mm screen.

White snakeroot was collected from six different locations. Two collections were made in Boone County, MO, on Sept 4, 2008, east of the Veterinary Medical Diagnostic Laboratory, University of Missouri, (38° 51.228′ N/92° 19.611′ W) and then 9 miles south of Columbia, MO, on East Pierpont Meadows Road (38° 56.410′ N/92° 18.933′ W), while four collections were made in Illinois on Sept 10, 2008: The University of Illinois' Vermilion River Observatory, Vermilion County, IL (40° 03.582′ N/87° 33.847′ W), The University of Illinois' Trelease Woods research area, 2 miles northeast of Urbana, Champaign County, approximately (40° 08.111′ N/88° 08.390′ W), The University of Illinois' Brownfield Woods research area, 2 miles northeast of Urbana, Champaign County (40° 08.874′ N/88° 09.787′ W), and The University of Illinois' Nettie Hart Woods research area, 2 miles northeast of Mahomet, Champaign County (40° 13.732′ N/88° 21.346′ W). The white snakeroot collected was air-dried for 7 days and then ground, using a Cyclotec 1093 sample mill, to pass through a 1 mm screen. The plant material was identified as white snakeroot (*E. rugosum*) by Dr. Stanley L. Welsh at the Stanley L. Welsh Herbarium at Brigham Young University. Voucher specimens for the white snakeroot were deposited in the Poisonous Plant Research Laboratory Herbarium as accession numbers 3400−3405. The current taxonomic listing for this plant is given as *A. altissima* (L.) King and H. Rob. var. *altissima* as per the United States Department of Agriculture (USDA), National Resources Conservation Service, Plant Data Base.

General Experimental Procedures. Melting points were determined on a Fisher−Johns melting point apparatus (Fisher Scientific Co., Hampton, NH). Proton and Carbon 13 nuclear magnetic resonance (NMR) spectra were recorded on a JEOL 300 NMR spectrometer (JEOL Ltd., Peabody, MA) using $CDCl_3$. Chemical shifts were reported in δ units (ppm), and coupling constants (J) were reported in hertz. Optical rotations were measured on a Ruldolph Research Auto Pol IV polarimeter (Rudolph Research Analytical, Flanders, NJ). The IR spectra were recorded by applying 5 μL of 0.5 mg/mL $CHCl_3$ onto KBr powder into a micro DRIFTS cell, allowing the $CHCl_3$ to evaporate and using DRIFTS accessory (Spectra Tech, Inc., Stamford, CT) on a Nicolet Magna-IR 550 FTIR spectrometer (Nicolet Instrument Co., Madison, WI).

Extraction and Isolation of Benzofuran Ketones. Dry, ground plant material (rayless goldenrod, 1.25 kg) was extracted for 112 h by Soxhlet extraction with hexane (9 L). The extract was evaporated via rotary evaporation to a viscous dark green extract. Aliquots for silica column chromatography were prepared by dispersing the extract 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 × 8 cm inner diameter 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 (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 (90:10 hexane/ethyl acetate, 1.5 L) was added to the head of the column, and fractions (300 mL) were collected until a first intense yellow band completely eluted. After elution of the yellow band, mobile phase 2 (70:30 hexane/ethyl acetate, 2.5 L) was added to the column and collected in fractions (300 mL) until all colored bands eluted.

The fractions were analyzed by gas chromatography−mass spectrometry (GC−MS). Fractions 8−12 contained three major compounds with possible $[M]^+$ or prominent fragment ions at 202 and 200, corresponding to tremetone (**1**) and dehydrotremetone (**2**) or tremetone-like compounds. Fractions 8−12 were rotary-evaporated to dryness, reconstituted in $MeOH/H_2O$ (70:30), filtered through a 30 mm Nylon 0.45 μm syringe filter (National Scientific Co., Rockwood, TN) until clear, and injected (2−5 mL, depending upon the sample concentration) onto a Waters Prep LC2000 preparative chromatography system equipped with a UV−vis detector (Millipore Co., Millford, MA) monitoring λ at 280 nm. The HPLC column [two 40 × 100 mm Bondapak C18 PrepPak cartridges (15−20 μm, 125 Å; Millipore Co., Millford, MA)], 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. Using these conditions, the chemical constituents in the fractions were resolved into three major peaks eluting at 5.3 (tremetone), 7.6 (dehyrotremetone), and 9.8 (3-oxyangeloyl-tremetone) min. These three major peaks were collected separately from multiple injections, and the fractions corresponding to each of the three peaks were combined. The mobile phase was evaporated to < 30% of the original volume and extracted with equal volumes of chloroform (3×). The $CHCl_3$ extracts were combined, dried with anhydrous Na_2SO_4, filtered, and rotary-evaporated to dryness. Tremetone (**1**) and 3-oxyangeloyl-tremetone (**3**) were obtained as yellow oils, while dehydrotremetone (**2**) was crystallized by dissolving in MeOH, adding H_2O, and allowing the MeOH to evaporate at room temperature. The aqueous solution was filtered, and the crystals were collected. Tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) isolated were used as standards to quantify these compounds in extracts of rayless goldenrod and white snakeroot.

Tremetone (1). Yellow oil. $[\alpha]^{26.0}_D$ −52.4 (EtOH, *c* 0.44) (literature values: $[\alpha]^{24.0}_D$ −59.6 (*4*), 1962; $[\alpha]^{24.0}_D$ −59.6 (EtOH, *c* 5.5) (*7*)). UV λ_{max} (nm): 204, 227, 289. IR (KBr powder, DRIFTS) ν_{max} (cm^{-1}): 1671, 1606, 1587, 1486, 1436, 1357, 1286, 1264, 1239. ^{13}C and ^1H NMR: see **Tables 1** and **2**. EIMS (70 eV) *m*/*z* (relative intensity): 202 $[M]^+$ (57), 187 (100), 169 (10), 159 (51), 144 (35), 141 (28), 131 (34), 115 (19), 91 (14).

Dehydrotremetone (2). White crystals. mp 83−86 °C (literature values: mp 87.5−88.5 °C (*4*), mp 87−89 °C (*10*)). UV λ_{max} (nm): 254, 280 shoulder. IR (KBr powder, DRIFTS) ν_{max} (cm^{-1}): 1676, 1556, 1438, 1359, 1300, 1268, 1236, 1156. ^{13}C and ^1H NMR: see **Tables 1** and **2**. EIMS (70 eV) *m*/*z* (relative intensity): 200 $[M]^+$ (65), 185 (100), 157 (40), 128 (17).

3-Oxyangeloyl-tremetone (3). Yellow oil. $[\alpha]^{27.1}_D$ −112.7 (EtOH, *c* 0.48). UV λ_{max} (nm): 226, 273. IR (KBr powder, DRIFTS) ν_{max} (cm^{-1}): 1716, 1680, 1609, 1359, 1258, 1226, 1147. ^{13}C and ^1H NMR: see **Tables 1** and **2**. EIMS (70 eV) *m*/*z* (relative intensity): 200 (100), 185 (93), 157 (13), 128 (7), 115 (3), 55 (10). CIMS (CH_4) *m*/*z* (relative

Table 1. ^{1}H NMR[a] Data (300 MHz) for Tremetone (**1**), Dehydrotremetone (**2**), and 3-Oxyangeloyl-tremetone (**3**)

	1	2	3
H-2	5.26 dd (7.9, 9.7)		5.12 br, s
H-3	3.31 dd (7.9, 15.8)	6.66 s	6.20 s
	3.31 dd (9.7, 15.8)		
H-4	7.81 d (1.5)	8.15 s	8.05 s
H-6	7.80 dd (7.6, 1.5)	7.91 d (8.7)	7.80 d (8.6)
H-7	6.81 d (7.6)	7.45 d (8.7)	6.95 d (8.6)
H-9	2.53 s	2.63 s	2.53 s
H-11	4.93 br, s	5.21 s	4.96 s
	5.08 br, s	5.80 s	5.07 s
H-12	1.75 s	2.11 s	1.75 s
H-15			6.12 q (7.2, 7.2)
H-16			1.97 d (7.2)
H-17			1.86 s

[a] The solvent was $CDCl_3$; chemical shifts are in δ (ppm); and J values in parentheses are in hertz.

Table 2. ^{13}C NMR[a] Data (300 MHz) for Tremetone (**1**), Dehydrotremetone (**2**), and 3-Oxyangeloyl-tremetone (**3**)

	1	2	3
C-2	87.03	158.49	91.56
C-3	34.01	103.20	76.64
C-3a	129.9	129.22	125.34
C-4	125.55	122.30	128.36
C-5	130.80	132.72	131.49
C-6	130.62	125.47	132.88
C-7	108.89	111.03	110.19
C-7a	164.09	157.49	164.93
C-8	196.80	197.82	196.43
C-9	26.51	26.86	26.57
C-10	143.40	132.50	140.28
C-11	112.72	114.40	113.96
C-12	17.16	19.38	17.75
C-13			167.41
C-14			127.21
C-15			139.79
C-16			16.06
C-17			20.59

[a] The solvent was $CDCl_3$, and chemical shifts are in δ (ppm).

intensity): 329 [M + 29] (16), 315 [M + 15] (2), 301 [M + 1] (3), 233 (5), 200 (100), 185 (64), 159 (12), 131 (3).

Analytical-Scale Extraction. 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. Timed extraction experiments on rayless goldenrod and white snakeroot were carried out over a period of 24 h. The timed extraction data showed that all three benzofurans (**1**−**3**) reached maximum extraction from rayless goldenrod at 4 h. After this time, there was no increase in the concentration of benzofurans (**1**−**3**) extracted. The timed extraction data also showed that the maximum extraction for white snakeroot was accomplished in 10 h. An extraction time of 16 h (overnight) was chosen for this study. The samples were centrifuged (5 min) and transferred (1 mL) into autosampler vials for GC−MS and HPLC analysis.

GC−MS. GC−MS data were acquired using a Trace GC Ultra and Polaris Q mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a split/splitless injector and a 30 m × 0.25 mm, 0.25 μm film thickness DB-5MS (Agilent Technologies, Inc., Santa Clara, CA) column, operated in the splitless mode. The injection volume was 1.5 μL, and the injection port temperature was 250 °C. The split vent flow rate was 50 mL/min and purged after 0.80 min. The oven temperature was 80 °C for 1 min, increased 80−300 °C at 30 °C/min, and was held at 300 °C for 3.0 min. Electron impact ionization (EI) at 70 eV was used with an ion source temperature of 200 °C. Chemical ionization mass spectra were collected using methane gas (Air Products and Chemicals, Inc., Allentown, PA) to confirm molecular ions. The detector scanned the mass range from 50 to 650. Under these conditions, tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) eluted at 6.28, 6.42, and 7.62 min, respectively.

Analytical-Scale HPLC. Analytical-scale reversed-phase HPLC was performed on a Shimadzu LC-20AT (Shimadzu Co., Kyoto, Japan) equipped with an autosampler and photodiode array (PDA) detector from the same vendor and a 100 × 2 mm inner diameter, 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. Detection of analytes in the eluant was performed at λ of 280 nm. Under these conditions, tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) eluted at 9.3, 12.7, and 15.2 min, respectively. These compounds were quantified against a seven point calibration curve using the previously isolated compounds (**1**−**3**). The calibration curve was prepared in hexane/ethyl acetate (70:30) over the range of 1.56−100.0 μg/mL by serial dilution.

Spike and recovery experiments were performed by extracting and analyzing four samples of rayless goldenrod leaves from Reeves County, TX, as above. The average of these analyses were used to establish baseline concentrations of tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) in this rayless goldenrod sample. A 200 μL aliquot of a 1 mg/mL solution of **1**−**3** in methanol was transferred via a pipet into four separate screw top glass test tubes (16 mL). The methanol was evaporated from the test tube under a gentle flow of N_2 at 50 °C. After evaporation of the methanol, 100 mg of plant sample was added and the spiked sample was extracted as described with hexane/ethyl acetate (70:30, v/v) (8 mL), resulting in a spike concentration of 25 μg/mL. The same procedure was followed to prepare spiked samples, for spike and recovery experiments, using white snakeroot plant material collected at the Brownfield Woods, Champaign County, Illinois site. Limits of detection (LODs) for **1**−**3** were determined by dilutions of the lowest standard (1.56 μg/mL) that resulted in measured peak heights that were 3× the height of the baseline noise.

RESULTS AND DISCUSSION

Isolation and Identification of Benzofuran Ketones. Tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) were extracted from rayless goldenrod by Soxhlet extraction with hexane and isolated using preparative-scale normal-phase chromatography followed by preparative-scale reversed-phase HPLC. Compound **2** was crystallized from MeOH/H_2O, while compounds **1** and **3** were reduced to yellow oils. These compounds were identified on the basis of a comparison of physical and spectroscopic data with that previously reported (*4*, *5*, *7*, *9*, *10*). The isolated tremetone (**1**) and dehydrotremetone (**2**) standards were estimated to be ≥95% pure based on HPLC, GC−MS, and NMR analysis. The 3-oxyangeloyl-tremetone (**3**) standard was estimated to be ≥83% by the same methods. The major contaminant in the 3-oxyangeloyl-tremetone standard was dehydrotremetone (**2**).

Tables 1 and **2** show ^{1}H and ^{13}C NMR assignments for **1**−**3**. The ^{13}C NMR shift assignments for C-4 and C-6 were previously incomplete (*5*, *10*), and therefore, direct C−H bond correlations obtained from our heteronuclear multiple-quantum coherence (HMQC) data were used to assign and confirm all chemical-shift assignments.

Quantitative HPLC Analysis. We investigated the use of GC−flame ionization detector (FID), GC−MS, HPLC−UV, and HPLC−MS methods for development of a quantitative analytical method for tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**). Except for HPLC, we encountered limitations with all of these techniques, which prevented the development into robust analytical methods. Surprisingly, we found that GC−FID did not have detection limits low enough to

quantify our lowest two standards (3.13 and 1.56 μg/mL). HPLC−MS was evaluated using both ESI and APCI ionization sources in both positive and negative modes, and very high limits of detections were observed with all three compounds (**1**−**3**). GC−MS appeared promising in that it proved to be sensitive and selective; however, we were unable to achieve acceptable linear calibration curves. Of the methods tested, only HPLC with UV detection resulted in acceptable results.

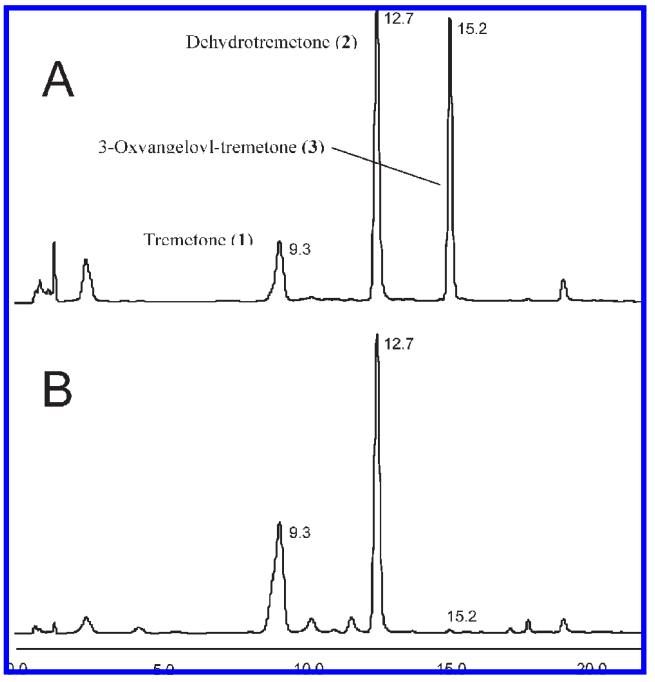

Figure 2. HPLC chromatograms of extracts from (A) rayless goldenrod and (B) white snakeroot.

Table 3. Percent Recoveries of Tremetone (**1**), Dehydrotremetone (**2**), and 3-Oxyangeloyl-tremetone (**3**) in Rayless Goldenrod and White Snakeroot Plant Material and Measured LODs for **1**−**3**

compound	rayless goldenrod% recovery	white snakeroot% recovery	LOD (ng)
tremetone	97.0 ± 2.8	92.4 ± 5.3	0.468
dehydrotremetone	104 ± 4	95.3 ± 5.0	0.156
3-oxyangeloyl-tremetone	100 ± 4	97.2 ± 1.7	0.468

Using the isolated benzofuran ketone standards (**1**−**3**), a reversed-phase HPLC method was developed for the quantitation of these compounds in both rayless goldenrod and white snakeroot plant material (**Figure 2**). Calibration curves were linear ($R^2 \geq 0.9963$) for all three compounds over the range of 1.56−100 μg/mL). Spike recoveries from rayless goldenrod ranged from 97 to 104%, while recoveries of the same compounds from white snakeroot ranged from 92 to 97% (**Table 3**). The LODs for **1** and **3** were 0.468 ng, while the LOD for **2** was 0.156 ng (**Table 3**). It was not possible to determine LOD in the presence of the plant matrix, in that rayless goldenrod and white snakeroot plant material that did not contain benzofuran ketones was not available.

Standard deviations for tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) are reported in **Table 4** for four replicates of rayless goldenrod leaves and white snakeroot. When these standard deviations are converted to percent relative standard deviations, the percent relative standard deviations for the rayless goldenrod stems are 4.9, 9.8, and 1.9% for tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**), respectively. The percent relative standard deviations for the white snakeroot plant material are 3.6, 5.1, and 20.2% for tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**), respectively. The relative standard deviation for 3-oxyangeloyl-tremetone (**3**) is low (0.0097 μg/mg); however, the measured amount of 3-oxyangeloyl-tremetone (**3**) is relatively low, which results in a higher percent relative standard deviation.

Using the HPLC method, the concentrations of compounds **1**−**3** were determined in the stems and leaves from a plant collected in Reeves County, TX, and a plant collected in Armstrong County, TX. In both plants, the leaves contained higher concentrations of the compounds of interest than the stems (**Table 4**). In addition, the plant from Reeves County had higher concentrations of all three compounds (**1**−**3**) in both the leaves and stems when compared to the same plant parts in the Armstrong County plant.

The benzofuran ketones (**1**−**3**) were also measured in white snakeroot plants collected from four locations near Champaign, IL, and two locations near Columbia, MO (**Table 4**). Unlike rayless goldenrod, where the leaves and stems are readily separable because of the stiff woody stem of the rayless goldenrod plant, the entire above-ground white snake root plant was sampled. Both compounds **1** and **2** were found in white snakeroot plants from all six locations; however, 3-oxyangeloyl-tremetone (**3**) was found in only two of the plants. The concentrations of compounds **1** and **2** in the white snakeroot plants vary from

Table 4. Measured Levels of Tremetone (**1**), Dehydrotremetone (**2**), and 3-Oxyangeloyl-tremetone (**3**) in Rayless Goldenrod and White Snakeroot Plant Samples

sample	tremetone (μg/mg)	dehydrotremetone (μg/mg)	3-oxyangeloyl-tremetone (μg/mg)
	Rayless Goldenrod		
leaves (n = 4)Reeves County, TX	0.736 ± 0.036	1.74 ± 0.17	2.71 ± 0.05
stemsReeves County, TX	0.106	0.226	0.535
leavesArmstrong County, TX	0.324	0.382	0.110
stemsArmstrong County, TX	0.0740	0.0260	0.0347
	White Snakeroot (Above-Ground Plant Parts)		
Brownfield Woods (n = 4),Champaign County, IL	2.50 ± 0.09	2.56 ± 0.13	0.0481 ± 0.0097
Hart Woods,Champaign County, IL	4.43	2.78	ND
Vermilion River Observatory,Vermillion County, IL	6.50	0.458	0.0404
Trelease Woods,Champaign, County, IL	0.641	0.344	ND
Pierpont Meadows,Boone County, MO	0.383	0.212	ND
University of Missouri Campus,Boone County, MO	0.737	0.281	ND

0.38 to 6.50 μg/mg and from 0.21 to 2.78 μg/mg, respectively. However, in general, the plant samples from the locations near Champaign, IL, had higher levels of benzofuran ketones than those near Columbia, MO. Although we were able to measure considerable variation among plants from different locations, more plants will need to be sampled during different times of the growing season before coming to any firm conclusions about the levels of these compounds from different locations. Beier et al. reported that tremetone (**1**) readily degraded to dehydrotremetone (**2**) in purified samples (*9*). However, we did not observe instability or degradation of compounds **1**, **2**, or **3** under the conditions used in this study.

As research into the cause of "trembles" in livestock continues, a robust analytical method that can be used to measure tremetone (**1**), dehydrotremetone (**2**), and 3-oxyangeloyl-tremetone (**3**) in rayless goldenrod and white snakeroot will be useful in determining the effect that these compounds have on the cause and progression of poisoning. This method will be used to identify plant material to be used in animal feeding studies, to determine the concentration of these compounds in different plant populations, and to determine accurate doses of the various compounds in feeding studies. Using this method to determine the concentrations of the benzofuran ketones and after feeding studies, we will be able to identify the toxic benzofuran ketone(s) and plant concentrations that may cause poisoning, so that management recommendations may be made to livestock owners in areas where white snakeroot and rayless goldenrod are found.

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