Evaluation of the Stability of Benzofuran Ketones in Rayless Goldenrod (*Isocoma pluriflora*) and White Snakeroot (*Ageratina altissima*) Under Different Storage Conditions

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Abstract

White snakeroot (*Ageratina altissima*) and rayless goldenrod (*Isocoma pluriflora*) 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." However, it has been suggested that the toxins in rayless goldenrod were not degraded by drying and both fresh and dry plants are toxic. Later, tremetol isolated from white snakeroot and rayless goldenrod was determined to be a complex mixture containing various benzofuran ketones including tremetone **1**, dehydrotremetone **2**, and hydroxytremetone in white snakeroot and tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** in rayless goldenrod. In this report, the stability of the benzofuran ketones in white snakeroot and rayless goldenrod was studied by measuring the concentrations of the benzofuran ketones in ground and in intact dried leaves stored at different temperatures over an approximately 6-year time period.

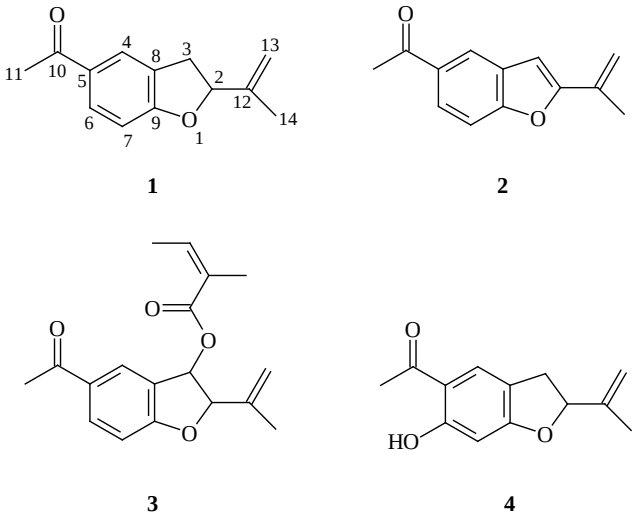
Keywords: *Ageratina altissima*, dehydrotremetone, *Eupatorium rugosum, Isocoma pluriflora*, rayless goldenrod, tremetone, white snakeroot

Introduction

White snakeroot (*Ageratina altissima* (L.) R.M. 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 (Couch 1927, Kingsbury 1964, Burrows and Tyrl 2013). In 1917, white snakeroot was shown to cause "trembles" and "milk sickness" (Moseley 1917). Poisoning in livestock is first manifest as depression, reluctance to eat, and inactivity followed by muscle tremors of the nose, flanks, and legs especially after exercise. The poisoned animal will often display rapid breathing, elevated heart rate, a stiff gait, and altered posture as affected animals are reluctant to move and stand hunched up with a flexed back. In the early 1900s, a disease with nearly identical clinical signs as trembles broke out in livestock in the southwestern United States, and it was quickly established that the southwestern "milk sickness" was due to ingestion of rayless goldenrod (Marsh 1926; Couch 1927, 1930).

Couch identified the toxins in white snakeroot and rayless goldenrod as tremetol (Couch 1927, 1930). Later, Couch reported that tremetol rapidly disappeared when white snakeroot was dried and that completely dried plants were incapable of producing "trembles" or "milk sickness" (Couch 1926,1927, 1928, 1930). Couch suggested that toxin degradation was plant specific as he concurrently

reported that the rayless goldenrod toxins were not destroyed by drying, and therefore both fresh and dry plants were toxic (Couch 1926, 1927, 1930). Later, tremetol isolated from white snakeroot and rayless goldenrod was determined to be a complex mixture containing various benzofuran ketones including tremetone **1**, dehydrotremetone **2**, and hydroxytremetone in white snakeroot (Bonner et al. 1961, Bonner and DeGraw 1962) and tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, in rayless goldenrod (figure 1) (Zalkow et al. 1962, 1979). Several studies have implicated the benzofuran ketones as the toxic principles although there is no conclusive large animal data to support this suggestion (Bonner et al. 1961; Zalkow et al. 1962, 1979; Bowen et al. 1963; Beier et al. 1987, 1993).



1 **2**

3 **4**

Figure 1. Chemical structures of tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4**.

In a recent study, the effect of different plant drying methods on the concentrations of the benzofuran ketones in white snakeroot and rayless goldenrod was evaluated (Lee et al. 2012). It was concluded that the benzofuran ketones were most stable when they were freeze dried and air dried but showed some instability when they were oven dried in a period of 7 days between collection, drying, and analysis. More recently, we surveyed several *Isocoma* spp. for their benzofuran ketone content using field and herbarium specimens (Lee et al. 2015). In general, a lower frequency of detection of several benzofuran ketones was observed in herbarium specimens compared with field collections. In particular, it was observed that dehydrotremetone **2** was present in the majority of the field collections but was conspicuously absent in many of the herbaria specimens. This observation was further investigated by sampling and analyzing a herbarium specimen that was over 5 years old and by comparing it with plant material from the same location that we had analyzed over 5 years previously. This comparison suggested that the benzofuran ketones tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** had degraded over the 5-plus years and further suggested that dehydrotremetone **2** degraded more rapidly than the other benzofuran ketones. Based on these observations, the objective of this study was to determine the stability of the benzofuran ketones in white snakeroot and rayless goldenrod in ground and in intact plant material at different temperatures over an approximately 6-year time period.

Experimental

Plant Material

Rayless goldenrod was collected May 6, 2008, near Pecos, TX, 31º23.969´ N / 103º29.969´ W, accession #3056 at the ARS Poisonous Plant Research Laboratory (PPRL) Herbarium, Logan, UT, and accession #250012 at the Utah State University Intermountain Herbarium, Logan, UT. White snakeroot was collected September 5, 2008, at Hart Woods, IL, 40º13.732’ N / 88º21.346’ W, accession #3401 at the PPRL Herbarium. Plants were identified by PPRL personnel.

Plant Processing

The plants from the rayless goldenrod collection were dried at ambient temperature, and the leaves stripped off and collected in a 4 L plastic utility pan and thoroughly mixed by stirring the leaves by hand and then separated into six subsamples: room temperature-unground, refrigerator-unground, freezer-unground, room temperature-ground, refrigerator-ground, and freezer-ground. The subsamples (approximately 2 g) were placed in re-sealable zipper plastic bags. Room temperature samples were stored in the laboratory at 21 °C, refrigerated samples were stored at 4 °C, and freezer samples were stored at –20 °C. All subsamples were stored in the dark. Plants from the white snakeroot collection were treated in the same manner as the rayless goldenrod plants and separated into the same six subsamples described above.

Rayless goldenrod and white snakeroot samples designated ground were ground to pass through a 1

mm screen using a Cyclotec 1093 sample mill (Tecator, Hoganas, Sweden) on February 17, 2009, and February 18, 2009, respectively. Rayless goldenrod and white snakeroot samples designated unground were ground the day of extraction, 1 day prior to the date of HPLC analysis.

Extraction

Dry, ground leaf material was 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 (8 mL, 70:30 v:v). The samples were centrifuged (5 min at 15,000 x *g*) and supernatant transferred (1 mL) into autosampler vials for HPLC analysis. Rayless goldenrod and white snakeroot samples from different storage conditions were run in sextuplicate (n = 6) on days 0 and 2119 and run in singlet on days 7, 21, 35, 56, 85, 140, 252, and 525.

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 x 2 mm i.d., 5 µm, Betasil C_{18} column (Thermo Hypersil-Keystone, Bellefonte, PA). Samples (10 µL) in the extraction solution were injected on to 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. The compound concentrations in leaves were quantitated against tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** standards. Purity of standards was > 95% as determined by HPLC-PDA and NMR. Six-point calibration curves of the standards were prepared by serial dilution using previously isolated **1**, **2**, **3**, and **4** (Lee et al. 2009) in hexane:ethyl acetate (70:30, v:v) over the range of 3.13 µg/mL – 100 µg/mL.

Data Analysis

A one-way ANOVA was performed using Sigma Plot 12.5 where chemical amounts in the different storage conditions were compared. A post-hoc test of significance using a Bonferroni correction was performed. A p-value of <0.05 was considered to be statistically significant.

Results and Discussion

The qualitative and quantitative benzofuran ketone profile in rayless goldenrod and white snakeroot at day 0 was similar to that previously reported by Lee et al. (2009) (figure 2). Three benzofuran ketones, tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, were detected in rayless goldenrod (table 1), while three benzofuran ketones, tremetone **1**, dehydrotremetone **2**, and 6-hydroxytremetone **4**, were detected in white snakeroot under all storage conditions and time points (table 1). Temperature, state of the plant material (ground or unground), and storage time all influenced the stability of each benzofuran ketone in rayless goldenrod and white snakeroot. Each benzofuran ketone responded to these factors independently although some general trends were observed. First, each respective compound decreased more in both species in the samples stored at 21 °C compared to the sample stored at −20 °C in ground and unground samples, while samples stored at 4 °C were generally intermediate. For example, in rayless goldenrod that was not ground, dehydrotremetone **2** amounts at day 2119 were 1.3 ±0.1, 0.49 ±0.08, and 0.10 ±0.01 µg/mg in the respective samples stored at −20, 4, and 21 °C (table 1). Second, each respective compound decreased to a greater extent in the ground sample of each species compared to the unground sample of each species stored at the same temperature. For example, in white snakeroot stored at 4 °C, tremetone **1** amounts at day 2119 were 0.71 ±0.02 µg/mg in the ground sample and 2.7 ±0.2 µg/mg in the unground sample (table 1). Third, degradation of each compound was not linear over the experiment. In general, most of the degradation of the benzofuran ketones occurred within the first 252 days (figures 3-8). For example, concentrations of dehydrotremetone **2** in rayless goldenrod were stored at 21 °C were 1.2 ± 0.1, 0.34, and 0.10 ± 0.01 µg/mg in the unground sample at day 0 (initial), day 252, and day 2119 (figure 4).

Dehydrotremetone **2** is the most rapidly degraded of the four benzofuran ketones in rayless goldenrod and white snakeroot in this study (figures 2, 4, 7). Dehydrotremetone **2** degraded in both plants under all storage conditions ($P<0.05$) with one exception, unground rayless goldenrod stored at −20 °C. For example, over 83% of the dehydrotremetone **2** in rayless goldenrod is degraded over the total study time of 2,119 days in unground plant material at 21 °C (figure 4). Of the 83% of the degraded dehydrotremetone **2**, 69% of the total loss occurs in

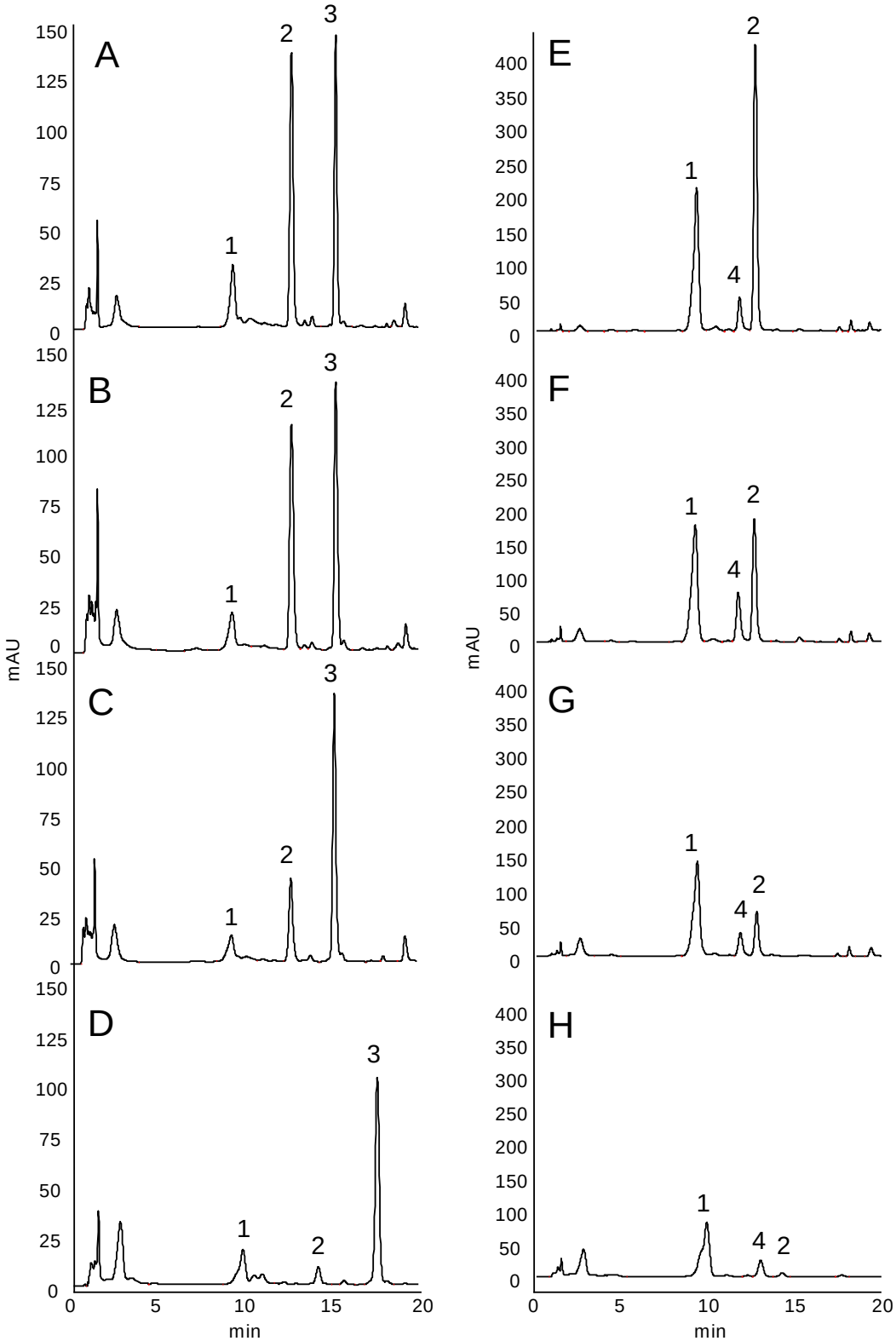


Figure 2. HPLC chromatograms of unground rayless goldenrod stored at 21 °C at (A) day 0, (B) day 85, (C) day 252, and (D) day 2119. HPLC chromatograms of unground white snakeroot stored at 21 °C at (E) day 0, (F) day 85, (G) day 252, and (H) day 2119. Annotated peaks correspond to tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4**. Peak retention times for the day 2119 chromatogram are different due to a different Betasil C_{18} column used in the analysis.

Table 1. Variation in mean concentrations (±SD) of benzofuran ketones in rayless goldenrod and white snakeroot over 2,119 days under different storage temperatures[1]

Storage Conditions	Compound Concentrations ± SD (n=6) µg/mg of dry weight		
Rayless Goldenrod	Tremetone **1**	Dehydrotremetone **2**	3-Oxyangeloyltremetone **3**
Initial	0.57 ± 0.03[b]	1.2 ± 0.1[a]	5.3 ± 0.4[b]
Unground -20 °C	0.73 ± 0.04[a]	1.3 ± 0.1[a]	6.5 ± 0.3[a]
Unground 4 °C	0.48 ± 0.08[c,d]	0.49 ± 0.08[b]	5 ± 1[b]
Unground 21 °C	0.40 ± 0.03[d,e]	0.10 ± 0.01[d]	5.3 ± 0.2[b]
Ground -20 °C	0.55 ± 0.02[b,c]	0.50 ± 0.01[b]	4.8 ± 0.1[b]
Ground 4 °C	0.41 ± 0.02[d]	0.27 ± 0.01[c]	3.66 ± 0.04[c]
Ground 21 °C	0.33 ± 0.02[e]	0.031 ± 0.002[d]	1.7 ± 0.1[d]
White Snakeroot	Tremetone **1**	Dehydrotremetone **2**	6-Hydroxytremetone **4**
Initial	5.3 ± 0.9[a]	3.5 ± 0.6[a]	1.2 ± 0.3[b]
Unground -20 °C	4.8 ± 0.2[a]	2.4 ± 0.1[b]	1.5 ± 0.1[a]
Unground 4 °C	2.7 ± 0.2[b]	0.88 ± 0.05[c]	0.68 ± 0.09[c]
Unground 21 °C	2.1 ± 0.2[b,c]	0.07 ± 0.01[e]	0.45 ± 0.07[c]
Ground -20 °C	1.75 ± 0.03[c]	0.66 ± 0.01[c,d]	0.50 ± 0.01[c]
Ground 4 °C	0.71 ± 0.02[d]	0.30 ± 0.01[d,e]	0.171 ± 0.004[d]
Ground 21 °C	0.30 ± 0.01[d]	0.0145 ± 0.0004[e]	0.040 ± 0.001[d]

[1]Different letters within a column represent significance between drying methods at $P < 0.05$.

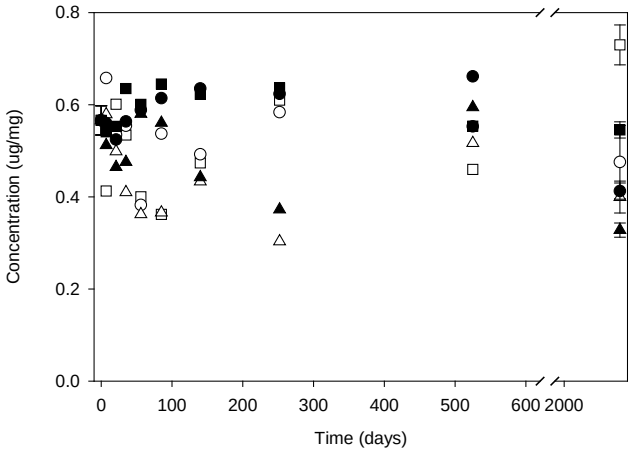


Figure 3. Variation in the concentration of tremetone **1** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

the first 252 days. Similar relative amounts of dehydrotremetone **2** degradation were observed in white snakeroot. Tremetone **1** and 6-hydroxytremetone **4** showed similar trends of degradation under similar conditions but to a lesser extent than dehydrotremetone **2**. The most stable of the benzofuran ketones was 3-oxyangeloyl-tremetone **3**. No significant degradation of 3-oxyangeloyltremetone **3** was detected in any of the samples with the exception of ground samples stored at 4 and 21 °C.

In summary, the four benzofuran ketones were most stable when stored as unground material at −20 °C while they were most unstable when stored as ground material at 21 °C regardless of the plant matrix. All benzofuran ketones showed no or minimal degradation when stored at −20 °C in both rayless goldenrod and white snakeroot with the exception of dehydrotremetone **2** in white snakeroot. These results are consistent with our observations of tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3** in herbarium specimens compared with field collections in different *Isocoma* spp. (Lee et al. 2015). The occurrence of 3-oxyangeloyltremetone **3** was the most consistently observed benzofuran ketone between herbarium specimens and recent field collections while dehydrotremetone **2** was rarely observed in the older herbarium specimens. In a previous study, minimal degradation of the benzofuran ketones under

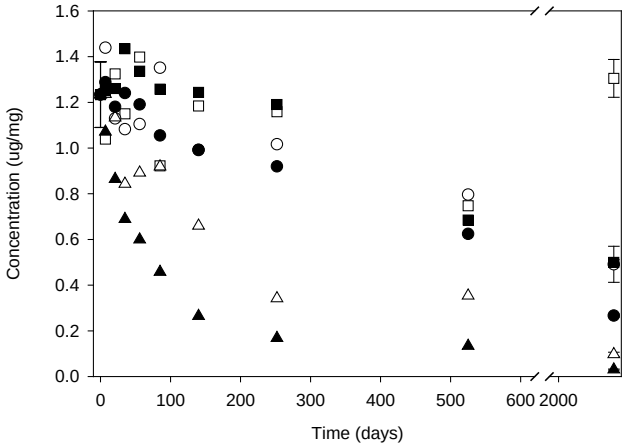


Figure 4. Variation in the concentration of dehydrotremetone **2** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

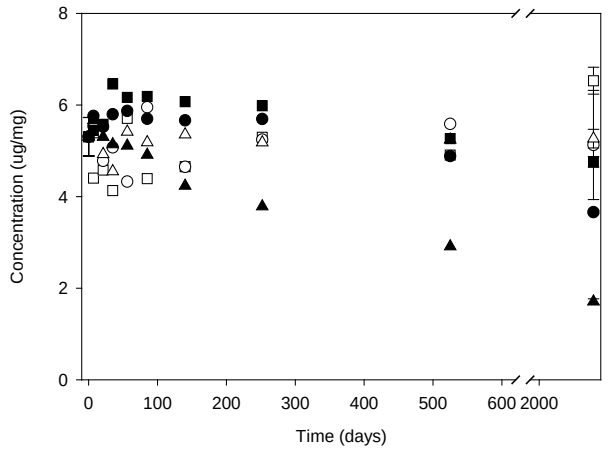


Figure 5. Variation in the concentration of 3-oxyangeloyltremetone **3** in rayless goldenrod over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

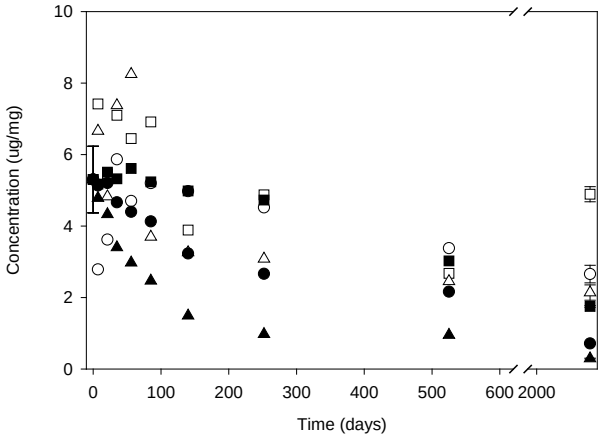


Figure 6. Variation in the concentration of tremetone **1** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

different drying conditions was observed in rayless goldenrod and white snakeroot; however, the length of time between drying, grinding, and analysis was 7 days (Lee et al. 2012). As a result, we were unable to observe the degradation of the benzofuran ketones observed here over a longer period of time.

The instability of the benzofuran ketones is due to factors within the plant as these compounds are highly stable when purified (Lee et al. 2012). Since the benzofuran ketones have been reported to contribute to the toxicity of these plants, in considering these results, plants should be stored intact before and ground just prior to animal dosing studies. Lastly, these results may contribute to the reported sporadic toxicity of these plants made by early investigators.

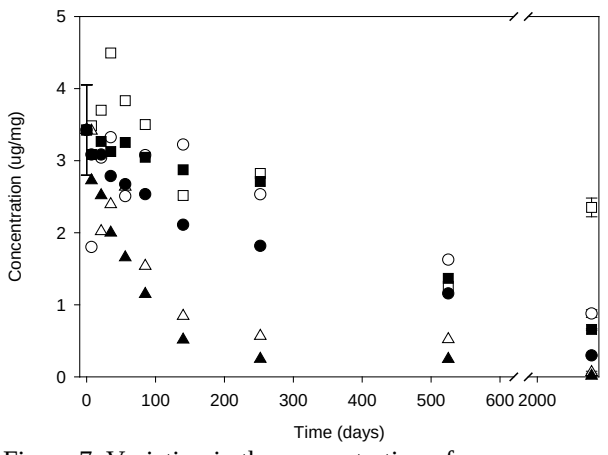


Figure 7. Variation in the concentration of dehydrotremetone **2** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

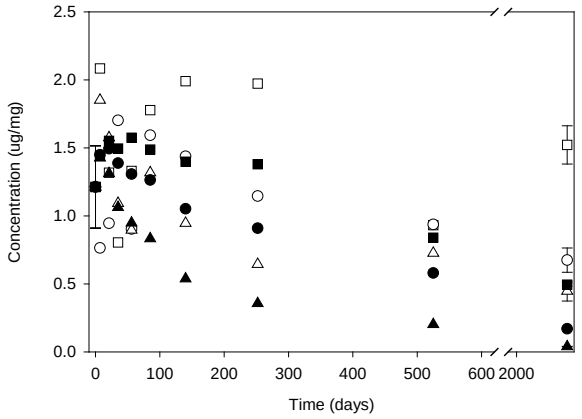


Figure 8. Variation in the concentration of 6-hydroxytremetone **4** in white snakeroot over time under different storage conditions: unground −20 °C (□); unground 4 °C (○); unground 21 °C (Δ); ground −20 °C (■); ground 4 °C (●); and ground 21 °C (▲).

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