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Gerald W. Kaufmann
Loras College

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Seasonal Variation of Tremetol Concentrations Found in White Snakeroot, *Eupatorium rugosum* Houtt (Compositae)

GERALD W. KAUFMANN

Department of Biology, Loras College, Dubuque, Iowa 52001

White snakeroot is a perennial composite whose foliage is poisonous to livestock. The poison is an unidentified substance contained in a mixture called tremetol. Most cases of poisoning occurred in late summer, and it was presumed livestock were forced to eat the plants because of a dearth of forage. Field observations indicate cattle will voluntarily eat white snakeroot and perhaps the plant varies seasonally in toxicity. Preliminary bioassy using minnows (*Notropis* spp.) exposed to extracts of plants gathered monthly in 1977 indicated the plants were most toxic in July and August. Tremetol was extracted from plants gathered monthly in 1978. The highest concentrations were from plants gathered in August and September.

INDEX DESCRIPTORS: white snakeroot, *Eupatorium rugosum*, tremetol, tremetone.

White snakeroot is an erect, branching perennial, 3-15 dm tall, of the Compositae. The distribution of the plant is coincident with the distribution of the eastern deciduous forest of North America. Plants which grow along the wooded edges or along streambanks form a taller and more dense bunch than those growing under the forest canopy. In eastern Iowa, the plants emerge in late April but do not exhibit rapid stem elongation until late June or early July. They begin to bloom in mid-July and continue until October. They are moderately frost tolerant and usually persist well into October.

White snakeroot is poisonous to livestock, causing a disease called trembles. Identification of the toxic component has not yet been completed. In 1926, James Couch isolated a viscous, straw-colored oil from white snakeroot, which produced trembles in test animals (Couch, 1927). He named this substance tremetol, and described it as a secondary aromatic alcohol with a doubly unsaturated side chain, and a molecular formula $C_{16}H_{22}O_3$. No further work was done until W. A. Bonner and J. I. DeGraw of Stanford University began to study it in 1959 (Bonner and DeGraw, 1961; Bonner et al, 1962; Bonner et al, 1964). This team discovered that tremetol was a mixture containing a sterol fraction and a ketone fraction. The ketones were the most suspect as the causes of trembles. The ketone fraction contained 48% 2-isopropenyl-5-acetyl-2, 3-dihydrobenzofuran (tremetone), 17% 2-isopropenyl-5-acetylbenzofuran (dehydrotremetone), 2.5% 2-isopropenyl-5-acetyl-6-hydroxy-2, 3-dihydrobenzofuran (hydroxytremetone), and 2% of a fraction which contained two isometric ketones ($C_{13}H_{14}O_2$). They found that tremetone, dehydrotremetone, and hydroxytremetone were toxic to goldfish. However, while crude tremetol produced trembles in chickens, tremetone was harmless, suggesting that this ketone may not be responsible for trembles.

Many white snakeroot poisoning cases occur in late summer and early fall, and are frequently associated with drought. The literature implies that livestock will not eat white snakeroot unless there is a dearth of forage (Moseley, 1906; Couch, 1927; Sniverly and Furbee, 1966). My observations indicated that livestock actually preferred white snakeroot, particularly when grazing was deferred until July. If cattle willingly eat the plant, the seasonal distribution of trembles may be associated with seasonal concentrations of the poison. The aim of this study was to ascertain whether or not white snakeroot exhibited a seasonal variation in its production of tremetol.

BIOASSAY

Fish toxicology was used as a preliminary bioassay to indicate differences in tremetol levels of white snakeroot. Plants were gathered the 10th of each month from May through September, 1977, sealed in double plastic bags and frozen. Fifty grams of leaves from each month were placed in 450 ml of absolute methanol, allowed to steep in sealed jars under refrigeration for 7 days, and the extract was then strained from the leaves through cheesecloth. Seven continuously aerated 3l aquaria were utilized at room temperature. Ten minnows (*Notropis* spp) were placed in each aquarium. Five aquaria received 21 ml of extract from one of the monthly plant collections. Twenty one ml was chosen because it produced LC50 according to methods suggested by Cairns and Dickson (1973). Two aquaria were used as controls: one received 21 ml of methanol and the other contained only water. Fish deaths were recorded daily for 96 hours. All fish in the aquaria containing the extracts from plants gathered in July and August died within 24 hours. (See Table 1).

Table 1. Total number of dead fish at the end of each 24 hour period using 21 ml of extract of white snakeroot gathered from May through September. Each acquarium contained 10 fish.

Date of plant collection	24 hr.	48 hr.	72 hr.	96 hr.
May 10	1	2	3	3
June 10	1	2	2	3
July 10	10			
August 10	10			
September 10	4	4	5	5
Methanol Control	1	2	2	2
Control	0	1	1	1

CHEMICAL ASSAY

White snakeroot plants were collected monthly from May to October, 1978, packaged, labeled, and immediately frozen. In order to prevent as much water loss as possible when removing the samples from storage, they were unpackaged in a subzero environment. Leaves and petioles were stripped from the stems and weighed. Approximately 560 to 820 grams of this material was used in each monthly extraction.

The extraction procedure used was modified from Bonner and DeGraw (1961). The quantitative nature of the study necessitated washings incorporated into all steps. Extract were performed

Table 2. Gram tremetol per gram of wet and dry weight of white snakeroot leaves gathered during 1978.

Plant Collection Date	Wet Weight of Leaves (gms)	Dry Weight of Leaves (gms)	Tremetol Isolated (gms)	Grams Tremetol per Gram Wet Weight ($x10^2$)	Gram Tremetol per Gram Dry Weight ($x10^2$)
May 20	814.02	83.81	1.30	0.16	1.60
June 17	520.73	61.53	1.07	0.17	2.05
July 15	642.71	100.34	1.49	0.15	2.32
August 12	562.15	77.28	2.17	0.28	3.82
September 9	682.92	101.52	2.22	0.22	3.25
October 7	804.24	102.76	1.90	0.18	2.36

several times to minimize losses. All steps carried out by Bonner and DeGraw *in vacuo* were done at approximately 25 mm Hg in a water aspirated rotary evaporator.

After the leaf material was weighed, it was thoroughly macerated in a Waring blender to facilitate extraction. The samples were then steeped in methanol overnight. The liquid was drained off and the leaf matter covered with methanol and refluxed for 3 hours. This process was subsequently repeated for 1.8 hours. The leaf matter was thoroughly rinsed, air dried, and weighed. The liquid portions were combined and reduced at atmospheric pressure to 1/3 volume. The resulting residue was further concentrated to 1/9 its volume.

This residue was chilled overnight to affect separation into two layers, a dark green lipid layer and a brown aqueous layer. The lipid layer (100 ml) was diluted to six times its volume, while the aqueous layer (180 ml) was diluted to twice its volume. Both were chilled for several hours. The lipid mixture separated into an aqueous layer and a lipid layer. Following extraction of these combined aqueous layers (750 ml) with two 150 ml portions of ether, the water-soluble fractions were discarded and the ether fractions distilled at reduced pressure to remove the ether. The lipid residue obtained was combined with the lipid layer.

The lipid layer (200 ml) was then extracted with 1500 ml of hot (65°C) 50% ethanol and filtered rapidly by suction. A dark tarry resin was filtered out and discarded, and the filtrate was concentrated at reduced pressure to one-eighth its volume (200 ml). The solution was next allowed to chill until separation occurred. Following separation, an aqueous layer was decanted from the lipid layer and extracted three times with 100 ml of ether. The ether was evaporated to yield a residue, which was recombined with the lipid layer. The lipid weighed from 10-20 grams.

To free the toxin from its ester combination, the lipid fraction was saponified for three hours with 4 ml of 5% methanolic KOH per gram lipid fraction. After saponification, the lipid was concentrated at reduced pressure to one-half its volume (50 ml) and diluted by two and a half times its volume. This solution was extracted with 200 ml of ether and allowed to separate overnight. The ether fraction was then collected and the aqueous fraction re-extracted with two 200 ml portions of ether. The erher extracts were combined and the ether was evaporated at reduced pressure to yield a reddish-orange residue.

Tremetol concentrations were calculated in terms of grams of tremetol per gram of wet and dry weight of plant material (See Table 2). Leaves collected in May, June, and July contained small fluctuations in tremetol concentrations. Leaves taken in August contained approximately twice the amount of tremetol compared to the earlier months, while leaves taken in September and October contained successive declines in tremetol concentrations. Dry weight concentrations are probably a more reliable index for comparison, since a recent rainfall or wilting of plants can influence wet weight.

DISCUSSION

Data from both bioassay and chemical assay indicates a rapid increase of tremetol levels in white snakeroot during the midsummer (July and August 1977) and late-summer months (August and September 1978) and subsequent cessation. The difference between 1977 and 1978 in the exact timing of peak concentrations correlates with lack of rainfall. July 1977 was hot and dry whereas July 1978 was cool and wet. Perhaps drought stress causes metabolic changes in white snakeroot, causing it to produce more tremetol. This would explain the correlation of poisoning cases with drought. Range management implications would be to allow grazing during spring and early summer and defer grazing during dry periods.

Both fish toxicology and chemical assay indicate seasonal defferences in tremetol levels. Unfortunately, tremetol levels may be independent of the level of the unknown toxin which produces trembles.

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