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Received for review September 20, 1977. Accepted March 12, 1979. This work was supported in part by Texas Agricultural Experiment Station, Project No. H-0919, Texas A&M University System.

Quantitative Analysis of Hymenoxon in Plant Tissue

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Hymenoxon was successfully extracted with ethyl acetate from ground plant material and quantitatively analyzed on a 3% OV-17 gas–liquid chromatographic column. Dimethylhymenoxon or flavone was used as the internal standard. Recovery of fortified hymenoxon from plant tissue was 100–105%. The concentration of hymenoxon was determined in *Hymenoxys odorata*, *Helenium hoopsii*, and *Baileya multiradiata*, and the hymenoxon content was related to the relative toxicity of the three plants.

Hymenoxys odorata (bitterweed) is a major poisonous range plant that is indigenous to Texas, New Mexico, Colorado, Oklahoma, Arizona, and California. Kim et al. (1975) isolated a poisonous sesquiterpene lactone, hymenoxon, from bitterweed and Ivie et al. (1975) reported the isosolation of hymenovin, an epimeric mixture of hymenoxon, from this plant. Pettersen and Kim (1976) determined the stereochemistry of hymenoxon by X-ray crystallography. Hill et al. (1977) identified hymenoxon as a constituent of two other toxic plants of compositae, *Baileya multiradiata* (baileya) and *Helenium hoopsii* (sneezeweed). In the present study, a procedure was developed for the quantitative analysis of hymenoxon in these plants.

EXPERIMENTAL SECTION

Chemicals. Hymenoxon (mp 135–136 °C) was extracted from *H. odorata* and repeatedly recrystallized by the procedure of Kim et al. (1975). Flavone was obtained from K&K Laboratories Inc., and dimethylhymenoxon was prepared according to the method by Kim et al. (1975).

Instrumentation. A Varian 2100 or a Barber Colman 5000 gas chromatograph (GC) was used to analyze extracts of plant tissues. The Varian GC was equipped with a 4.8 ft × $^1/_8$ in. glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) and helium was used as the carrier gas at a flow rate of 40 mL/min. The operating temperatures were as follows: column, 205 °C; injector, 215 °C; and flame ionization detector, 255 °C. The Barber Colman GC contained a 6 ft × $^1/_6$ in. glass column packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh). The operating temperatures were as follows: column, 210 °C; injector, 235 °C; and flame ionization detector, 250 °C. Nitrogen was the carrier gas with a flow rate of 45 mL/min.

Quantitative Analysis of Hymenoxon in Plant Tissues. Standard solutions of hymenoxon, dimethylhymenoxon, and flavone were prepared in ethyl acetate. To determine the linearity of detector response to these compounds, different concentrations of hymenoxon and dimethylhymenoxon standards were injected in triplicate on the Barber Colman GC, and different concentrations of hymenoxon and flavone standards were analyzed in triplicate on the Varian GC. The linear regression equation and the correlation coefficient of peak height vs. amount of standard applied to the column were calculated for each compound.

Standard solutions (0.161–0.241 mg/mL) of hymenoxon were made in ethyl acetate. Dimethylhymenoxon (50 μg/mL in ethyl acetate) or flavone (40 μg/mL in ethyl acetate) was added as the internal standard for the chromatographic procedure. To establish a standard curve, 1.0 mL of each hymenoxon standard was pipetted into a 15-mL centrifuge tube and mixed with 1.0 mL of the flavone internal standard or 1.0 mL of the dimethylhymenoxon internal standard. Each solution was evaporated to dryness under a stream of air in a water bath, and the residue was dissolved in 200 μL of ethyl acetate. A 2.0-μL sample of the concentrated solution was injected on the GC. The peak height of the hymenoxon peak was divided by the peak height of the internal standard peak to give the relative peak height (RPH). The linear regression equation and the correlation coefficient for the RPH vs. the milligrams of hymenoxon standard for each internal standard was calculated.

The ground, aerial portion of the plants were dried in a vacuum oven at 60 °C and stored in a desiccator. A 40–50-mg sample of the plants was weighed and transferred to a glass-stoppered test tube. Hymenoxon was extracted by mixing the plant sample with 8.0 mL of ethyl acetate for 2 min on a vortex mixer, and the particulate matter was removed from the ethyl acetate by centrifugation. A 1.0–4.0-mL aliquot of the ethyl acetate extract was transferred to a 15-mL centrifuge tube containing 1.0 mL of flavone internal standard or 1.0 mL of dimethylhymenoxon internal standard. The mixture was evaporated to dryness in a water bath under a stream of air and the residue was dissolved in 200 μL of ethyl acetate. A 2.0-μL sample of the concentrated extract was analyzed on the GC. The RPH of the unknown was determined and the amount of hymenoxon extracted was calculated from

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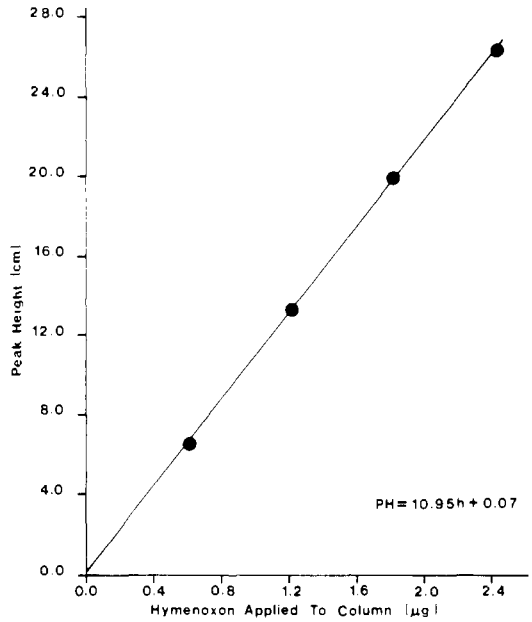

Figure 1. The relation between FID response (peak height) and the amount of hymenoxon applied to a 3% OV-17 GLC column on the Varian GC. Operating temperatures were as follows: column, 205 °C; injector, 215 °C; and detector, 255 °C (PH = peak height in centimeters, h = micrograms of hymenoxon applied to column).

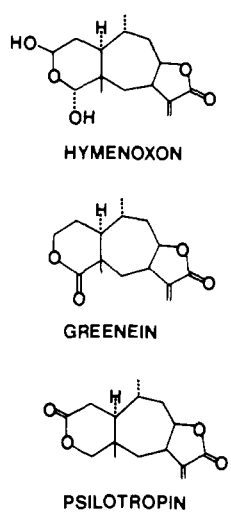

Figure 2. The stereochemical structure of three sesquiterpene lactones.

the linear regression equation of the standard curve. A standard curve was prepared for each day of analysis.

RESULTS AND DISCUSSION

A linear relationship between concentration and detector response (peak height) was obtained for hymenoxon, flavone, and dimethylhymenoxon. The correlation coefficient was greater than 0.995 for each curve. A response curve for hymenoxon on the Varian GC is presented in Figure 1.

Different operating temperatures were required to obtain the same retention time for hymenoxon on both instruments. The retention times were flavone, 5.6 min; dimethylhymenoxon, 6.9 min; and hymenoxon, 8.0 min. Moreover, two peaks in addition to hymenoxon were observed on chromatograms when the lactone was injected on the Barber Colman GC at an injection temperature of 245 °C and a column temperature of 230 °C. The retention time for the two peaks corresponded to psilotropin and

Table I. Percent Recovery of Hymenoxon from *H. odorata* Spiked with Hymenoxon

sample size, mg	hymenoxon in sample, mg	hymenoxon added to sample, mg	total hymenoxon, mg: calcd	total hymenoxon, mg: assayed	recov., %
41.22	1.04	0.483	1.52	1.52	100.0
39.99	1.00	0.483	1.48	1.49	100.7
42.18	1.06	0.483	1.54	1.61	104.5
45.18	1.13	0.724	1.85	1.88	101.6
42.64	1.07	0.724	1.79	1.79	100.0
45.78	1.15	0.724	1.87	1.89	101.1

Table II. Concentration of Hymenoxon in Three Plants of Family Compositae

plant	plant part	hymenoxon concn, %	SD[a]
B. multiradiata	flower	0.28	0.066
H. odorata[b]	flower	0.83	0.037
H. odorata[b]	aerial	1.22	0.062
H. hoopsii	aerial	0.82	0.011

[a] Mean of triplicate analysis. [b] Mature plant.

greenein. Kim et al. (1975) have reported the conversion of hymenoxon to two sesquiterpene dilactones (psilotropin and greenein) under basic conditions. Evidently, hymenoxon can be partially dehydrated to psilotropin and greenein at an injection temperature of 245 °C as the dilactones were not observed at lower temperatures (Figure 2).

The reproducibility of the extraction and analysis of five samples of immature *H. odorata* was 1.9% relative standard deviation. The recovery of hymenoxon from 40–46 mg of the plant material fortified with 0.483 mg or 0.724 mg of hymenoxon is given in Table I.

The concentration and standard deviation of triplicate analysis of the flower of mature *H. odorata* and *B. multiradiata* and the whole aerial part of *H. odorata* and *H. hoopsii* are listed in Table II. The concentration of hymenoxon was slightly higher in mature bitterweed than in mature sneezeweed. Also immature bitterweed had a higher concentration of hymenoxon than the mature plant. Of the three plants analyzed, *Baileya* contained the lowest concentration of hymenoxon.

Repeated animal feeding studies suggested that the relative toxicity of these plants was as follows: *H. odorata* (immature) > *H. odorata* (mature) > *H. hoopsii* > *B. multiradiata* (Boughton and Hardy, 1937; Lewis and Dollahite, 1960; Rowe et al., 1973; Marsh et al., 1921; Sperry et al., 1964). The current study found that the relative concentration of hymenoxon in the plants were: *H. odorata* (immature) > *H. odorata* (mature) > *H. hoopsii* > *B. multiradiata*. This observation would suggest that hymenoxon is a major toxic principle of these plants of family Compositae.

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Received for review July 18, 1977. Accepted February 26, 1979. This work was supported in part by the Texas Agricultural Experiment Station Project No. H-6255, Texas A+M University System. Technical Article no. 13538.

Degradation of Herbicide-Related Nitrosamines in Aerobic Soils

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Four ^{14}C-labeled nitrosamines were incorporated into aerobic soils, and the rate of $^{14}CO_2$ production was measured. Rates of $^{14}CO_2$ evolution were similar for *N*-nitrosodipropyl-, diethyl-, and dimethylamines. Samples of nitrosodipropylamine individually labeled with ^{14}C at carbons 1, 2, and 3 were synthesized. The rate of $^{14}CO_2$ production was independent of the position of the label. The reaction is believed to be microbiological because sterilization of the soil inhibited CO_2 production. For a few days after application, volatilization of unchanged nitrosamine from soil also occurred. It is estimated that the half-life of these nitrosamines in aerobic soils is on the order of three weeks. *N*-Nitrosopendimethalin [*N*-(1-ethylpropyl)-*N*-nitroso-3,4-dimethyl-2,6-dinitrobenzenamine] was relatively stable in soil and significant quantities could be recovered unchanged after several months.

N-Nitrosamines have been of agricultural interest for several years, in part because it has been postulated that various nitrogen-containing pesticides and fertilizers might be nitrosamine precursors (Ayanaba et al., 1973). Interest in the environmental chemistry of nitrosamines was considerably enhanced by the announcement (Fan et al., 1976; Ross et al., 1977) that four commercially acquired herbicides had contained nitrosamines, and by the subsequent investigation by the Environmental Protection Agency that revealed that several additional pesticides were also contaminated with nitrosamines (Pesticide and Toxic Chemical News, 1977). One of the obvious questions arising in assessing whether the compounds present an environmental hazard is, what are their fates in soil?

Tate and Alexander (1975, 1976) reported that *N*-nitrosodipropylamine (NDPA, **1**), *N*-nitrosodiethylamine (NDEA, **2**), and *N*-nitrosodimethylamine (NDMA, **3**) were resistant to microbiological degradation (all three of these nitrosamines have since been detected as impurities in herbicides; Pesticide and Toxic Chemical News, 1977). Kearney et al. (1977) found, in contrast, that *N*-nitrosoatrazine was rapidly degraded in Metapeake loam; only 8% of the added nitrosoatrazine could be recovered after 1 month. We recently studied the formation of *N*-nitrosobutralin in soil (Oliver and Kontson, 1978). Nitrosation of butralin was observed only when the soil was heavily amended with sodium nitrite; however, the limited amount of nitrosobutralin that did form proved to be quite persistent, and a small amount was recovered after 6 months. Fan et al. (1976) were unable to detect NDPA in soil following trifluralin application, and Elanco (Eli Lily and Co.) researchers examined soils and well water in areas with histories of high trifluralin usage, but did not detect NDPA in either medium (Amundson, 1978). Recently, Saunders et al. (1979) reported that NDPA was, in fact, degraded in both aerobic and anaerobic soils.

Because of the importance of some of the nitrosamine-containing herbicides, we have examined the degradation of four ^{14}C-labeled nitrosamines (**1–4**, Figure 1) in aerobic soils. To the extent that the experiments can be compared, our results seem to be in good agreement with those of Saunders et al. (1979).

EXPERIMENTAL SECTION

Caution. Many nitrosamines are potent carcinogens and must be handled and disposed of accordingly.

Analytical Procedures. Gas chromatography was performed on a Hewlett-Packard Model 5310 instrument equipped with a flame ionization detector. Thin-layer chromatography (TLC) was performed with 0.25-mm precoated silica gel plates, F-254, E. Merck, Darmstadt. The most common TLC solvents were CH_2Cl_2 (for **1–3**) and toluene (for **4**). No-screen medical X-ray film (NS-54 T, Kodak) was used to autoradiograph TLC plates.

Chemicals. NDMA-^{14}C (**3a**), NDEA-*1*-^{14}C (**2a**), and sodium propionate-*1*-^{14}C, -*2*-^{14}C, and -*3*-^{14}C (**5a**, **5b**, **5c**) were purchased from New England Nuclear. Pendimethalin-*1-ethyl*-^{14}C (*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine-*1-ethyl*-^{14}C) was supplied by American Cyanamid.

***N*-Nitrosodipropylamine-^{14}C (1a, 1b, 1c).** Sodium propionate-*1*-^{14}C (**5a**, 50 μCi, 2.5 mg) was suspended in dichloromethane (200 μL). The mixture was stirred and cooled, then thionyl chloride (55 μL) was added. After warming gently 0.5 h, the mixture was cooled and a solution of *n*-propylamine (150 μL) in dichloromethane (500 μL) was added dropwise. After 15 min the mixture was partitioned between dichloromethane and 2 N KOH; the organic phase was washed successively with 1 N HCl, 2% K_2CO_3, and saturated NaCl. The solution was dried ($MgSO_4$) and concentrated just to dryness. Dry tetrahydrofuran (1 mL) was added and also evaporated just to dryness, then a borane–tetrahydrofuran solution (0.5 mL, 1 M) was added to the residue and the resulting mixture was warmed under N_2 for 6 h. After cooling, concentrated HCl (5 drops) was added, then the mixture was evaporated

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