$H_5C{\equiv}CH$ (relative antifeeding percentage 64%) and 1-phenylpropyne $C_6H_5C{\equiv}CCH_3$ (96%).

The relative antifeeding activities of capillin (1; 100%) and capillarin (2; 97%) are strongly active against larva. Also, the numbers of uneaten sample disks of 1 and 2 are 10 and 8, respectively, and show the same tendency with percentage. In terms of chemical structure, 1 has a C=O carbonyl group instead of a CH_2 methylene group in a side chain of 9. 2 has a δ-lactone ring formed by cyclization of a side chain of 9 and a C≡C triple bond in a side chain. It is therefore suggested that a C=O carbonyl group and a C≡C triple bond in a side chain and a lactone ring (Rodriquez et al., 1976) are of the many factors that contribute to a compound being antifeeding active.

The relative antifeeding percentage of methyleugenol (3; 100%) is strongly active against larvae. Also, the number of the uneaten sample disk is 10 and shows the same tendency with percentage. 3 has *o*-dimethoxybenzene as a partial structure. And the contribution to antifeeding activity of this group is continuously studied in this laboratory.

ar-Curcumene (4) is weakly active (relative antifeeding percentage 85%, uneaten sample disk 5). On the other hand, the relative antifeeding percentages of γ-terpinene (5) and caryophyllene (6) are 61% and 73%, respectively. Also, the numbers of the uneaten sample disk of 5 and 6 are 2 and 3, respectively. These data show inactivity. But, bornyl acetate (7) is strongly active (relative antifeeding percentage 100%, uneaten sample disk 10).

In the present and previous studies, it was observed that 1–4 and 7–9 among whole components, which were isolated from the growing buds of *A. capillaris*, showed antifeeding activity for larvae of the cabbage butterfly. It is a very interesting point in the food chain cycle of the natural world that these components prevent insects from eating the growing buds of the plant.

Registry No. Capillin, 495-74-9; capillarin, 3570-28-3; methyleugenol, 93-15-2; *ar*-curcumene, 644-30-4; bornyl acetate, 76-49-3; caryophyllene, 87-44-5; γ-terpinene, 99-85-4.

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Quantitation of Hymenoxon and Related Sesquiterpene Lactones

Hyeong L. Kim,* Stephen H. Safe, and Millard C. Calhoun

A rapid and reproducible method for the quantitation of hymenoxon and related sesquiterpene lactones has been developed using reversed-phase HPLC. The methanol extract of a dried and ground plant sample was analyzed on an RP-8 column eluted with methanol–water (1:1, v/v) and monitored at 235 nm. The hymenoxon content of the above ground whole plant of *Hymenoxys odorata* ranged from 0.21 to 1.27% according to the site, season, and year of collection. Helenalin and mexicanin E were concentrated in the flowering heads (8.95 and 4.66%, respectively) of *Helenium microcephalum* while tenulin was concentrated in the leaves (6.14%) of *Helenium amarum*. The detection limit was 25 ng for helenalin and mexicanin E and 75 ng for hymenoxon and tenulin. The recovery rate of hymenoxon was 102.5 ± 4.5%.

Hymenoxon and related sesquiterpene lactones (Figure 1) are widely distributed among well-known livestock poisoning plants such as *Helenium*, *Hymenoxys*, and *Baileya spp.* The reported lethal dose of smallhead sneezeweed (*Helenium microcephalum*) in sheep and cattle is approximately 2.5 g/kg when the animals are force-fed the freshly ground flowering plants as a single dose whereas the LD_{50} for bitter sneezeweed (*Helenium amarum*) in sheep is 2.0 g/kg per day for 2 consecutive days (Dollahite et al., 1964, 1972). The oral LD_{50} values of dried and ground bitterweed (*Hymenoxys odorata*) in sheep range from 2.9 to 8.5 g/kg according to the site, season, and/or year of collection (Rowe et al., 1973). Sesquiterpene lactones helenalin and mexicanin E, among others, have been isolated from *H. microcephalum* (Clark, 1939; Kim, 1980); tenulin and aromaticin have been isolated from *H. amarum* (Clark, 1939; Lucas et al., 1964); hymenoxon has been isolated from *H. odorata* (Kim et al., 1975; Ivie et al., 1975a). Both hymenoxon and bitterweed (*H. odorata*) elicit common toxic effects in sheep (Terry et al., 1981); thus, the toxicity of a bitterweed sample appears to be dependent on the hymenoxon content.

When *H. amarum* is eaten by dairy cattle, tenulin is excreted in the milk, making it unpalatable due to the bitter taste (Ivie et al., 1975b). Helenalin causes electrocardiographic aberrations consistent with myocardial damage, respiratory paralysis, and progressive hypotension

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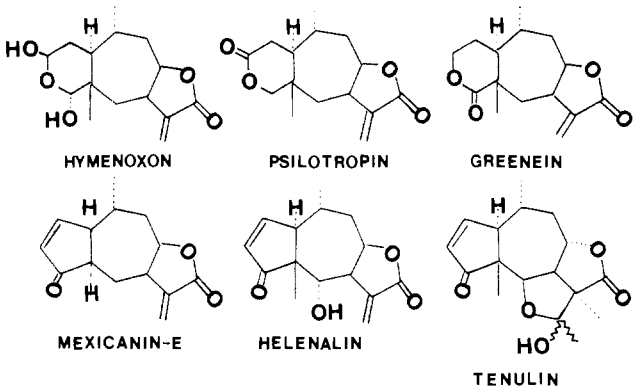

Figure 1. Structures of sesquiterpene lactones.

(Lamson, 1913; Szabuniewicz and Kim, 1972). Some of the toxic sesquiterpene lactones of plant origin including helenalin and tenulin are also cytotoxic and antileukemic (Picman, 1986). The intraperitoneal (IP) LD_{50} values of the above-mentioned sesquiterpene lactones in mice range from 3.1 mg/kg for mexicanin E to 185 mg/kg for tenulin (Kim, 1980). HPLC has been utilized for the isolation of sesquiterpene lactones (Ivie et al., 1976; Bohlmann et al., 1985), and quantitation of hymenoxon by gas chromatography (GC) (Hill et al., 1979) has also been reported. However, a discrepancy has been observed (Calhoun et al., 1985) between the hymenoxon content of bitterweed samples determined by the GC method and the toxic doses of bitterweed samples determined by feeding trials in sheep. In addition, no molecular ion peak but an $[M - 18]^+$ signal of hymenoxon has been observed by electron impact mass spectrometry (MS) or GC–MS, suggesting that hymenoxon is heat labile (Kim, unpublished observations). Reported herein is a rapid and reproducible procedure for the quantitation of helenalin, mexicanin E, tenulin, and hymenoxon in plant samples by reversed-phase HPLC.

EXPERIMENTAL SECTION

H. microcephalum was collected near Milican, TX, in June 1986; *H. amarum* was collected near College Station TX, in November 1985; *H. odorata* was collected near Sonora, TX, in various seasons from 1980 through 1986. All the samples of *H. odorata*, above-ground whole plant, were dried in a plant drier at or below 60 °C and ground with a Wiley mill using 1-mm screen. The plant parts, leaves, and flowering heads were dried at room temperature and ground with a motor and a pestle to a fine powder. The dried and ground plant samples were stored in a desiccator to reach constant weights before analysis.

Analytical standards were either isolated from appropriate plant sources or prepared by the method described (Kim, 1980), and several recrystallizations provided pure samples. Hymenoxon (mp 135–136 °C) was isolated from *H. odorata*; helenalin (mp 168–169 °C) and mexicanin E (mp 92–93 °C) were isolated from *H. microcephalum*; tenulin (mp 186–188 °C) was isolated from *H. amarum*; and psilotropin (mp 144–145 °C) and greenein (mp 176–177 °C) were prepared by heating hymenoxon in water–methanol with potassium hydroxide or calcium hydroxide. The melting point and the proton magnetic resonance (^1H NMR) spectrum of the compound isolated or prepared were identical with that reported or that on file. A collection of ^1H NMR spectra of sesquiterpene lactones has been published (Yoshioka et al., 1973). Psilotropin (or floribundin) and greenein have previously been isolated from *Hymenoxys rechardsonii* and *Hymenoxys greenei*, respectively (Herz et al., 1970). The standard solutions were prepared daily along with the plant extracts for the analyses; the concentration of each sesquiterpene lactone was determined from the standard curves prepared for each compound.

The HPLC instrument consisted of two pumps (Rainin Model HPX with 10-mL head), a dynamic mixer, an injector (Rheodyne Model 7125) with a 20-μL loop, an RP-8 (E. Merck, Darmstadt, Germany) column (5 μm, 0.4 × 25 cm) equipped with a precolumn (0.1 × 1.0 cm) packed with Perisorb RP-8 (30-40 μm, Upchurch Scientific), a variable-wavlength detector (Waters Associates Model 481), and a recorder-integrator (Hewlett-Packard Model 3390A).

Analyses of Plant Samples. A dried and finely ground sample (10–15 mg; except for bitterweed, 45–55 mg), in triplicate, was vortexed with methanol or acetonitrile (4.0 mL, HPLC-grade, Fisher Scientific) for 1–2 min, allowed to stand 5 min, decanted into a screw-capped vial, and then allowed to settle (usually for 15–20 h) until analyzed. For the comparison of the efficacy of extracting solvent, several bitterweed samples were extracted with acetonitrile (4.0 mL) or ethyl acetate (8.0 mL). Half of the ethyl acetate extract (4.0 mL) was transferred to a round-bottom flask (25 mL) and concentrated in vacuo at or below 35 °C to dryness. The residue was taken up in methanol and adjusted to a volume of 2.0 mL. The clear supernatant (1–5 μL) in methanol or acetonitrile was injected directly onto the column, and the elution was monitored at 235 nm (range = 0.2 AUFS). The mobile phase composition was 50% methanol in water (v/v), and the flow rate was 0.9 mL/min.

Recovery of Hymenoxon. Eight bitterweed samples (45–55 mg each) were taken from the same lot (collected January 1982, Sonora, TX) and a known amount (22.5 μg each) of hymenoxon in ethyl acetate (75 μg/mL) was added to five samples. These bitterweed samples were extracted with ethyl acetate and analyzed by HPLC as described above.

Isolation of Helenalin and Mexicanin E. Dried and ground flowerhead of *Helenium microcephalum* (10 g), collected in June 1986 was allowed to stand in methanol (500 mL) for 15–20 h and filtered. The plant residue was further extracted twice in the same manner. The combined methanol extract was concentrated in vacuo and partitioned with benzene and water (200 mL each), and the aqueous layer was further extracted with benzene (2 × 100 mL). The combined benzene extracts were concentrated in vacuo to about 20 mL and applied to a silica gel column (E. Merck, 70–230 mesh), 2.3 × 18 cm, packed in benzene, followed by elution with benzene and benzene–acetone. The concentrated eluates (50 mL/fraction) were analyzed by HPLC as described above, and the fractions containing mexicanin E or helenalin were combined. Yellow and green pigments (1.6 L of benzene), followed by mexicanin E (1.4 L of benzene) and a mixture of unknown compounds [0.3 L of benzene and 0.3 L of benzene–acetone (19:1)], followed by helenalin [1.45 L of benzene–acetone (19:1) and 0.45 L of benzene–acetone (9:1)], were eluted. Further elution with benzene–acetone (9:1) provided a mixture of unknown polar compounds. The yields of mexicanin E and helenalin after recrystallization from ether and benzene were 0.127 and 0.507 g, respectively.

Conversion of Hymenoxon into Psilotropin and Greenein. A bitterweed sample (50 ± 5 mg) was vortexed with EtOAc (8.0 mL) for 1–2 min and allowed to stand for 5–10 min, and the clear supernatant (4.0 mL) was concentrated in vacuo to dryness. The residue was dissolved in methanol, adjusted to 2.0-mL volume, allowed to stand overnight, and analyzed by HPLC. To the remaining clear

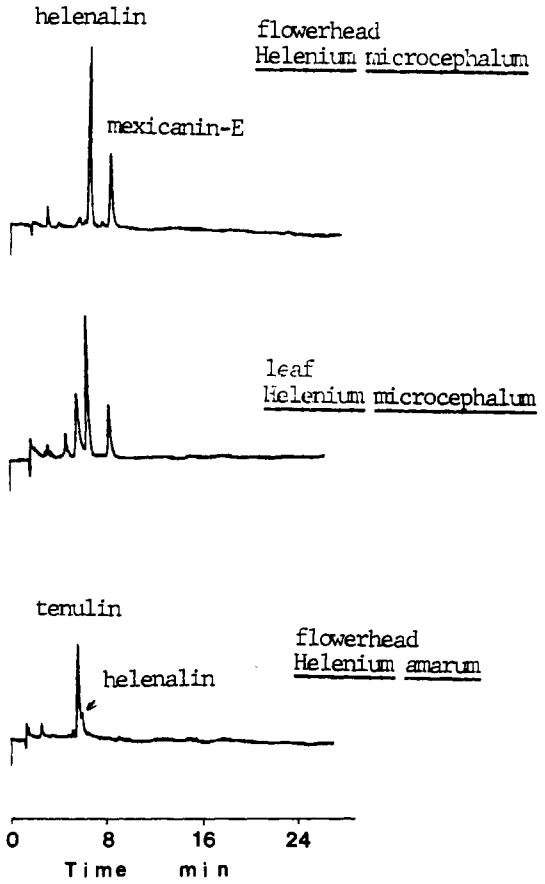

Figure 2. Chromatograms of the extract of *H. microcephalum* and *H. amarum*.

supernatant (0.5 mL) were added water (50 mL) and potassium hydroxide solution (3%, 1.5 mL), and the resultant mixture was heated at 60–65 °C, in a water bath, for 30 min. The reaction flask was cooled to room temperature, acidified with HCl (6 N), to pH 1–2, and extracted with EtOAc, 3 × 100 mL. The combined organic layers were concentrated in vacuo to dryness. The dried reaction product was dissolved in methanol, adjusted to a volume of 2.0 mL, and analyzed by HPLC. The standard solution containing 0.25–0.5 mg of hymenoxon was treated as described, and the recovery of psilotropin and greenein was examined.

RESULTS AND DISCUSSION

Hymenoxon and related sesquiterpene lactones were eluted within 10 min by reversed-phase HPLC using either an RP-8 or C18 column. The quantitation of all the above-mentioned compounds were readily accomplished on the RP-8 column eluting with 50% MeOH in water; however, the application of a C-18 column was limited to the quantitation of helenalin and mexicanin E eluting with 55–60% methanol in water. HPLC analysis of *H. microcephalum* methanol extract readily separated helenalin and mexicanin E (Figure 2; R_t 7.13 and 9.28 min, respectively); however, the separation of helenalin and tenulin was poor under the same conditions (R_t 7.13 and 6.59 min, respectively) when a methanol extract of *H. amarum* was analyzed. The detection limits for the sesquiterpene lactones varied between 25 ng (for helenalin) and 75 ng (for hymenoxon). However, the detection limit could be lowered approximately fourfold by changing the monitoring wavelength to 220 nm and adjusting the detector range to 0.1 AUFS. The mean recovery rate of hymenoxon was 102.5 ± 4.5% when a known amount (22.5 μg each) of hymenoxon was added to each bitterweed sample (five

Table I. Concentration of Hymenoxon in *H. odorata* (Whole Plant)

location	date collected	% hymenoxon (SD)[a]
1	Dec 1980	0.39 (0.02)
	Jan 1982	0.52 (0.01)
	April 1982	0.52 (0.04)
2	Dec 1980	0.43 (0.02)
	Nov 1983	1.27 (0.06)
	March 1984	0.40 (0.02)
	Nov 1984	0.53 (0.02)
	March 1985	1.02 (0.02)
	March 1986	1.01 (0.08)
3	March 1985	0.21 (0.03)

[a] Mean (SD) of triplicate analyses.

Table II. Concentration (%) of Sesquiterpene Lactones[a]

plant part	tenulin[a]	helenalin[a]	mexicanin E[b]	hymenoxon[c]
leaf	6.14 (0.11)	2.60 (0.05)	1.09 (0.04)	2.14 (0.04)
flowering head	2.75 (0.14)	8.94 (0.64)	4.66 (0.43)	2.01 (0.11)
stem	0.46 (0.02)			

[a] Mean (SD) of triplicate analyses from (a) *H. amarum* collected in November 1985, (b) *H. microcephalum* collected in June 1986, and (c) *H. odorata* collected in March 1986.

45–55-mg samples) that contained 0.53 ± 0.01% hymenoxon determined simultaneously with the "spiked" samples.

Hymenoxon appears to undergo rearrangement or conversion, yielding yet unknown products in methanol. Hymenoxon eluted as a single peak when a freshly prepared solution in either methanol or acetonitrile was injected onto an RP-8 column and eluted with methanol–water (1:1, v/v) but eluted two minor peaks within 2 h in addition to the original peak whose retention time had not changed (Figure 3). However, the size of the two minor peaks stabilized in several hours, and the integrated area ratio (approximately 1:1.2:5.9) of these three peaks remained unchanged for several days. Therefore, the quantitation of hymenoxon was routinely performed about 16–20 h after the methanol extracts of bitterweed and the standard solution of hymenoxon in methanol (25–100 μg/mL) were prepared and allowed to stand at room temperature. Solvent dependency of hymenoxon stability in solution has been observed; the proton magnetic resonance (^{1}H NMR) spectrum of hymenoxon in $(CD_3)_2CO$, $(CD_3)_2SO$, or $(CD_3)_2SO$–$CDCl_3$ (1:3, v/v) exhibits a set of well-resolved signals representing the exocyclic methylene protons, a tertiary and a secondary methyl proton, and two hydroxyl protons of hemiacetal group (Kim et al., 1976). In contrast, the ^{1}H NMR spectrum (270 MHz) of hymenoxon in $CDCl_3$ exhibits several unidentified signals including a doublet and a triplet centered at δ 6.27 and 5.67, respectively, adjacent to the two doublets of exocyclic methylene protons (Kim, unpublished observations). It is conceivable that hymenoxon is either converted to yet unknown products or formed a mixture of epimers in certain solvents such as methanol and/or chloroform-*d*, although the nature of such products or the stability of hymenoxon in various solvents has not been rigorously determined. The following reactions may demonstrate the labile nature of hemiacetal moiety of hymenoxon: (1) Hymenoxon is converted to its dimethyl ether when hymenoxon is allowed to stand in methanol with a catalytic amount of an acid. (2) Hymenoxon is converted to two dilactones, psilotropin and greenein, when hymenoxon is heated in water or methanol–water with a base (Kim et al., 1975).

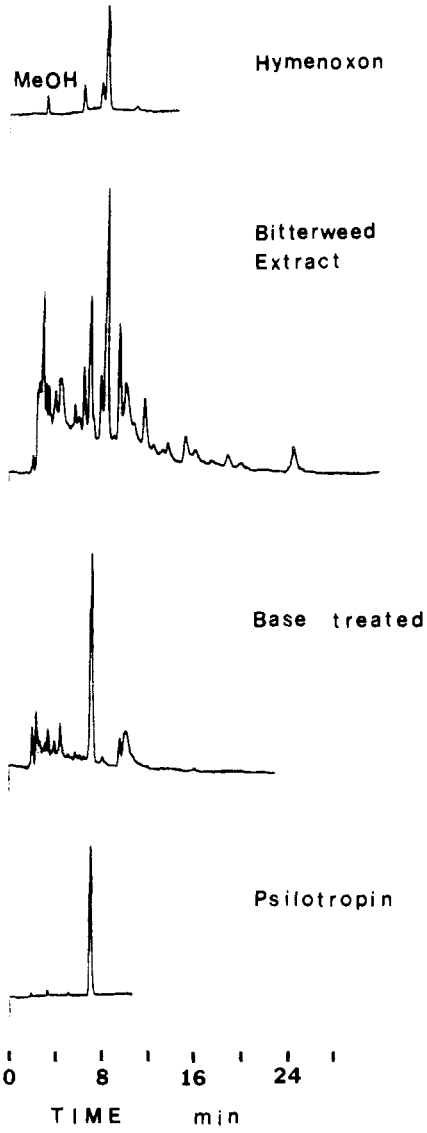

Figure 3. Chromatograms of hymenoxon bitterweed extract before and after the base treatment and psilotropin.

The concentration of hymenoxon in the above-ground whole plant varied between 0.21 and 1.27% (Table I). The concentrations of hymenoxon in the leaves and flowerheads were almost identical (Table II). The hymenoxon content appears to vary according to the site, season, and year of the plant collection; however, no definite trend is known at present regarding the relationship between the environmental factors (vide infra) or stage of growth and the toxicity of a bitterweed sample. The reported oral LD_{50} values of dried and ground above-ground portion of bitterweed range from 2.9 to 8.5 g/kg in sheep; plant samples collected during winter or early-spring months appear to be more toxic than those collected in summer or late spring (Rowe et al., 1973), and this has been in contrast to the previous observation that the toxicity of bitterweed increased slightly as the plant matured (Boughton and Hardy, 1937). It has also been observed that the toxicity of a bitterweed sample is closely related to the rainfall of a season; the plants collected during a prolonged drought are more toxic than those collected during seasons with normal rainfall (Boughton and Hardy, 1937). The minimum lethal dose (an approximate LD_{50} dose) of fresh green plant collected in a year of normal rainfall has been reported to be 13 g/kg while that of fresh green plant collected during a period of drought is 5 g/kg. (The moisture content of the plant samples collected for the feeding trials

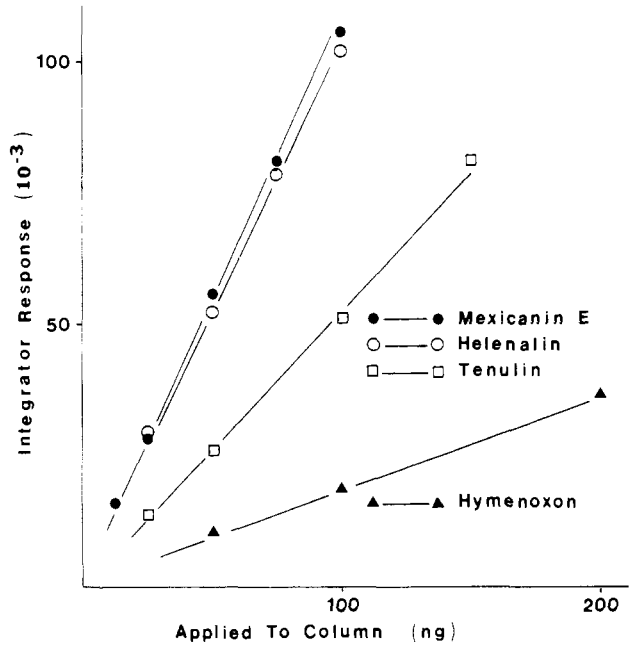

Figure 4. Relationship between the detector response (235 μm) and the amount of sesquiterpene lactones applied to the column.

is not known.) It has been speculated that the toxicity of a bitterweed sample and its hymenoxon content are dependent on the manner of collection, the drying and storage conditions, the stage of growth of the plants, the growing location, and the type of soil.

An attractive, alternate method for the quantitation of hymenoxon was attempted; hymenoxon was converted into two stable dilactones, psilotropin and greenein, by heating an aqueous solution of the sesquiterpene lactone in the presence of a base as previously reported (Kim et al., 1975; Hill and Camp, 1979). However, this method was not applicable for the quantitation of hymenoxon for the yield (377–546 μg of psilotropin/mg hymenoxon), and product ratios of the dilactones (psilotropin to greenein) were not consistent. The quantitation of individual dilactones, however, was readily achieved as the other sesquiterpene lactones; the recorder-integrator response to an increasing amount of each lactone applied to the column furnished a good correlation curve (Figure 4), the correlation coefficients being greater than 0.99.

The effect of solvents on the extraction of hymenoxon appears to be minimal; hymenoxon was readily extracted with ethyl acetate, acetonitrile, or methanol. However, acetonitrile or methanol is more convenient than ethyl acetate as solvents since the extract can be directly analyzed without further workup. Since methanol–water was the eluting solvent for HPLC analyses, methanol was also chosen for the extraction process. Methanol appeared to remove more polar substances from the plant samples tested; however, they did not interfere with the analyses.

Helenalin and mexicanin E are highly concentrated in the flowerhead of *H. microcephalum* and constituted 8.94 and 4.66%, respectively, of dried and ground flowerhead. In contrast, the concentrations of helenalin and mexicanin E in the leaf were 2.60 and 1.09%, respectively (Table II). Although it was previously concluded that the toxicity of *H. microcephalum* was primarily dependent upon the helenalin content (Witzel et al., 1976), the contribution of mexicanin E to the toxicity of this plant should be reevaluated. The reported yield of helenalin from the plant sample used for the study was 3.6%, and no other toxic fraction was found upon TLC analysis of the acetone extracts of the above-ground part of *H. microcephalum*. The

occurrence of mexicanin E in *H. microcephalum* was not known to Witzel et al. (1976) but has since been reported (Kim, 1980). Mexicanin E is about 3 times more toxic than helenalin; the intraperitoneal LD_{50} values of mexicanin E and helenalin in mice are appproximately 3.1 and 9.9 mg/kg, respectively.

The concentrations of tenulin in *H. amarum* were higher in the leaf than in the flowerhead: 6.14 and 2.75%, respectively (Table II). Helenalin was also detected in the methanol extract of *H. amarum*, but aromaticin was not detected. (Aromaticin was isolated from the plant samples collected at the same site previously by silica gel chromatography in a very low yield.) Since helenalin is more toxic than tenulin (Kim, 1980), the contribution of helenalin to the toxicity of *H. amarum* should also be considered. The intraperitoneal LD_{50} value of tenulin in mice is approximately 184.6 mg/kg. The isolation of helenalin from *H. amarum* collected in North Carolina has recently been reported (Bohlmann et al., 1985).

The HPLC method developed in this study provides a rapid and reproducible procedure for quantitating toxic sesquiterpene lactones in various plant extracts. The detection limits are 25–75 mg, and selective improvements can be achieved by changing the detection wavelength to 220 nm and by increasing the sensitivity setting of the detector. The toxicities of plant samples have previously been based on toxicant yields determined after time-consuming extraction and purification or are determined by the expensive feeding trials with grazing animals. This HPLC method will now permit relatively rapid estimation of the potential toxicity of a plant extract and can also be used in the analysis, purification, and structure determination of unidentified lactones in the complex plant extracts and partially purified fractions. This method also appears to be useful for the quantitation of heat-sensitive compounds such as hymenoxon that decompose under the conditions of GC analysis.

ACKNOWLEDGMENT

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Registry No. Hymenoxon, 57377-32-9; helenalin, 6754-13-8; mexicanin E, 5945-40-4; tenulin, 19202-92-7.

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