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Identification and Quantitation of the Alkaloids of *Lupinus latifolius*

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Lupinus latifolius is the species of lupine implicated as being responsible for a human deformity with the alkaloid anagyrine speculated to be the teratogen. Alkaloids were extracted in the present study from *L. latifolius* utilizing Soxhlet extraction. This modification proved less time consuming and significantly more efficient ($p < 0.01$) than the original method. Capillary gas chromatographic and mass spectrometric analysis of the alkaloid extract revealed six components. Anagyrine represented 86% of the total alkaloids and 1.14% of the plant dry weight. Lupanine, aphylline, and 5,6-dehydrolupanine accounted for 6.5, 3.9, and 1.9% of the total alkaloids, respectively. Aphyllidine and 4-hydroxylupanine were also identified in small quantities.

Ingestion of various species of lupine has resulted in outbreaks of acute poisoning of sheep and cattle. Couch (1926) postulated that the poisonous properties of lupines were due primarily to alkaloids. Wagnon (1960) reported that congenital crooked calf disease was suspected by California ranchers to be due to maternal ingestion of *Lupinus laxiflorus*. This congenital deformity is characterized by twisted or bowed limbs (arthrogryposis), curvature of the spine (scoliosis), twisted neck (torticollis), and/or cleft palate (Keeler et al., 1977). Shupe et al., (1967) proved that ingestion of certain species of lupines by pregnant cows resulted in crooked calf disease. Detailed studies were performed later by Keeler (1973a,b, 1976) and Keeler et al. (1976) to identify the compound responsible for the teratogenic effects of lupines. Keeler's efforts implicated the quinolizidine alkaloid anagyrine as the probable teratogen.

Kilgore et al. (1981) reported that in September 1980, a baby boy born in the mountainous back-country of northwestern California (Trinity County) was brought to the U.C. Medical Center in Sacramento with severe bilateral deformities of the distal thoracic limbs. The deformities included bilateral radial hypoplasia, curvature of the ulna, absent thumbs, and webbed fingers on one hand. On the basis of circumstantial evidence it was proposed that the child's deformities resulted from in utero exposure to anagyrine from maternal ingestion of milk from goats that had foraged on teratogenic lupines. Subsequently, Lazerson and Ortega (1984) determined that the child also suffered from red cell aplasia thought to be an anagyrine-induced stem cell defect.

The area where the goats had regularly foraged showed the presence of the perennial lupine *Lupinus latifolius*. This investigation was undertaken to identify and quantify the alkaloids present in this species of lupine.

EXPERIMENTAL SECTION

Plant Material—Collection and Preparation. Approximately 25 kg of lupine plants (*L. latifolius*) in the early preflower state was collected over an 8-h period during May 1983 in Trinity County, CA. The whole plants were spread out over a plastic tarp for 24 h prior to drying. Three portions were successively air-dried in a dehydrator at 98 °F for 24 h over a 3-day period. After air-drying, the plants were placed in large plastic bags and stored at room temperature for up to 1 week at which time they were ground with a Wiley mill containing a 2.0-mm mesh screen. The ground lupine plants were placed in plastic storage containers and stored at 6 °C.

Alkaloid Extraction. Triplicate samples of ground plant material were treated in the following manner. A 25-mL sample of 5% NH_4OH in methanol was mixed into 10 g of each sample. After air-drying, individual samples were placed in a Soxhlet extractor containing 350 mL of CH_2Cl_2. After overnight extraction the solvent containing the alkaloids was evaporated to ca. 10 mL on a rotary vacuum evaporator at 40 °C. The alkaloids were then partitioned into 150 mL of 1% H_2SO_4. The CH_2Cl_2 was discarded, and the aqueous phase was alkalinized to pH 9.5 with concentrated NH_4OH. The alkaloids were then partitioned back into 50 mL of CH_2Cl_2 followed by two additional 20-mL partitions with CH_2Cl_2. The combined CH_2Cl_2 layers were evaporated to near dryness on a rotary vacuum evaporator and transferred to test tubes for drying with a nitrogen evaporator at 40 °C. The residue was taken up in ethanol for gas chromatographic analysis.

In order to ascertain the efficiency of alkaloid extraction with the Soxhlet method, triplicate samples of ground plant material (10 g each) were also treated as described by Keeler (1973a). The only modification was that the alkaloids were repartitioned with 50 mL of $CHCl_3$ followed by two additional 20-mL partitions rather than 10 and 5 mL, respectively.

Concentration of Anagyrine Standard. Anagyrine was concentrated on a Varian Model 920 GC equipped with a 6 ft × $^1/_4$ in. (o.d.) packed stainless-steel column (10% OV-101) and a thermal conductivity detector. After injection of the plant extract, a 30-cm capillary tube, inside a Pasteur pipet, was placed in contact with the column exit port when the peak corresponding to anagyrine was recorded, for collection of the anagyrine standard. The operating conditions consisted of an injector temperature of 210 °C, column temperature of 235 °C, detector temper-

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Table I. Comparison of the Alkaloid Content of *L. latifolius* Utilizing Soxhlet Extraction vs. Keeler's Method

peak no.	alkaloid	% total alkaloid[a] (SE)		% plant dry wt[a] (SE)	
		Soxhlet	Keeler's	Soxhlet	Keeler's
I	5,6-dehydrolupanine	1.92 (0.10)	2.41 (0.06)	0.026 (0.002)	0.0298 (0.0006)
II	lupanine	6.49 (0.11)	6.25 (0.13)	0.086 (0.001)	0.076 (0.001)
III	aphylline	3.87 (0.02)	3.84 (0.09)	0.052 (0.002)	0.0047 (0.001)
IV	aphyllidine	0.78 (0.02)	0.86 (0.05)	0.0103 (0.0003)	0.0103 (0.0003)
V	4-hydroxylupanine	1.27 (0.04)	1.02 (0.09)	0.017 (0.001)	0.013 (0.001)
VI	anagyrine	85.66 (0.02)	85.63 (0.22)	1.143 (0.036)	1.004 (0.016)
	total alkaloid content			1.334[b] (0.042)	1.220 (0.008)

[a] Average and SE are based on $n = 3$. [b] Significantly greater than the total alkaloid content by Keeler's method ($p < 0.01$).

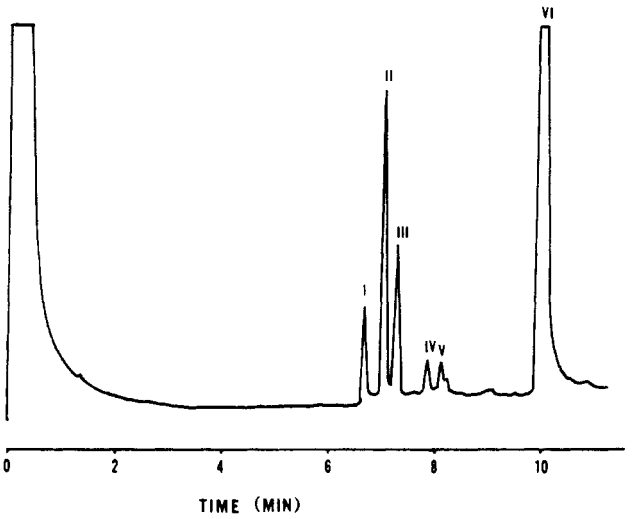

Figure 1. Capillary gas chromatogram of the alkaloids from *L. latifolius*. Peaks I–VI: see Table I for identification.

ature of 255 °C, and a helium carrier gas flow of 50 cm³/min. Injections up to 50 μL of a concentrated alkaloid solution in ethanol were possible with the thermal conductivity detector. Insertion of the capillary tube into a Pasteur pipet prevented contamination of the tube from previously condensed residue. This allowed for the collection of milligram quantities of anagyrine within several hours.

Gas Chromatography and Mass Spectrometry. The alkaloids were separated and quantified on a Tracor Model 222 GC equipped with a flame ionization detector modified with a fused silica capillary column (DB-1, 15 m × 0.25 mm (i.d.)). The operating parameters consisted of a column temperature program of 150 °C (2 min) to 220 °C at 6 °C/min, helium carrier gas flow of 0.26 cm³/min, helium makeup gas flow of 2.78 cm³/min, split ratio of ca. 1:50, and injector and detector temperatures of 300 °C. Recording was with a Hewlett-Packard Model 3390-A integrator.

Quantitation was relative to an internal standard curve of 4-aminoantipyrene (4-AA) and anagyrine. Each alkaloid extract was diluted 1:100 in ethanol, and 100 μL of each solution was pipetted into separate vials. A 100-μL portion of 4-AA at 100 ng/μL in ethanol was then added to each vial, and ca. 2 μL of each 4-AA/alkaloid solution was injected in triplicate into the GC.

Identification of the alkaloids was based on mass spectra obtained on a Finnigan 3200 (EI, 70 eV) gas chromatograph–mass spectrometer interfaced with a fused silica capillary column (DB-1, 30 m × 0.25 mm (i.d.)) programmed from 40 to 270 °C at 4 °C/min.

RESULTS

Gas Chromatography and Mass Spectrometry. The capillary GC profile of the alkaloid extract is presented in Figure 1. The six peaks, identified by mass spectrometry, correspond to (I) 5,6-dehydrolupanine, (II) lupanine, (III) aphylline, (IV) aphyllidine, (V) 4-hydroxylupanine, and (VI) anagyrine. Peak V consistently had an adjacent peak with GC, but a fragmentation pattern was not obtained with mass spectral analysis and as such remains unidentified.

The alkaloid content of *L. latifolius* as determined by a Soxhlet extraction procedure is presented in Table I. The total alkaloid content was calculated at 1.334% of the plant dry weight. Anagyrine represents the major alkaloid present, accounting for 86% of the total alkaloids and 1.14% of the plant dry weight. Lupanine, aphylline, and 5,6-dehydrolupanine represent 6.5, 3.9, and 1.9% of the total alkaloids, respectively. On a dry-weight basis there were 0.086% of lupanine, 0.052% of aphylline, and 0.026% of 5,6-dehydrolupanine. Aphyllidine and 4-hyroxylupanine were also identified in small quantities.

Table I also shows the alkaloid content of the same plant material as determined by Keeler's method of extraction. The calculated total alkaloid content is significantly less ($p < 0.01$) than that determined from the Soxhlet extraction method. The percentages of the total alkaloids were similar between the two methods, with the exception of 5,6-dehydrolupanine and 4-hydroxylupanine. It remains unclear whether these differences are a result of the differences in methods or simply experimental artifacts.

The mass spectral data are presented in Table II. The references cited are those that aided in the identification of individual alkaloids.

DISCUSSION

The extraction procedure used in this study is essentially a modification of Keeler's method (1973a). By using a Soxhlet extractor the time for alkaloid extraction was reduced by 3 days. Another advantage of the Soxhlet method is that it was a significantly more efficient ($p < 0.01$) method in analyzing the quinolizidine alkaloids of *L. latifolius*. The calculated total alkaloid contents (and standard error) were 1.334 (0.042) vs. 1.220 (0.008), using the Soxhlet vs. Keeler's method, respectively. Obviously, Keeler's method is more suitable for large-scale plant extractions, but the Soxhlet method appears more advantages when dealing with smaller quantitites of plant material.

Table II. Mass Spectral Data of the Alkaloids of *L. latifolius*

peak no.	alkaloid	M^+	characteristic ions (rel abund)	reference
I	5,6-dehydrolupaine	246	246 (100), 98 (54), 245 (44), 247 (16), 97 (15)	Cho and Martin (1971a)
II	lupanine	248	136 (100), 248 (79), 247 (72), 149 (65), 150 (55)	Cho and Martin (1971a), Schumann et al. (1968)
III	aphylline	248	136 (100), 247 (54), 220 (54), 137 (44), 138 (29)	Schumann et al. (1968)
IV	aphyllidine	246	246 (100), 136 (59), 245 (52), 134 (29), 217 (18)	Schumann et al. (1968)
V	4-hydroxylupanine	264	264 (100), 263 (52), 136 (35), 247 (28), 134 (23)	Cho and Martin (1971b); Hatzold et al. (1983)
VI	anagyrine	244	244 (100), 98 (93), 146 (46), 160 (29), 243 (26)	Keeler (1973b), Neuner-Jehle et al. (1964)

As exemplified by the GC profile (Figure 1) a DB-1 capillary column allows for an effective resolution of the quinolizidine alkaloids. Initially, a packed column (3% apiezon L) was used that allowed for the detection of four poorly resolved peaks. With use of the capillary column we were able to identify two previously undetected peaks.

Keeler (1973a) reported that lupines with an anagyrine plant concentration of over 1.44 g/kg were generally teratogenic when fed to pregnant cows. Davis and Stout (1986) reported that *L. latifolius* Agardh. contained anagyrine at 6.04 g/kg dry plant weight and 26% of the total alkaloids. The *L. latifolius* from Trinity County contained anagyrine at 11.43 g/kg dry plant weight and 86% of the total alkaloids. Both values are remarkably high relative to other reported values for lupines in general. Keeler (1973a) reported finding anagyrine levels up to 7.31 g/kg dry plant weight and 50% of the total alkaloids in *Lupinus caudatus* and 6.76 g/kg and 37% in *Lupinus sericeus*. He also reported finding anagyrine at 10.41 g/kg dry plant weight and 52% of the total alkaloids in an unidentified species from central Idaho. Davis (1982) reported finding anagyrine values ranging from 1.35 to 9.83 g/kg of dry plant weight for *L. caudatus* harvested in May 1978 while *L. sericeus* showed ranges of 3.54–5.76 g/kg for the same period. Davis and Stout (1986) also reported finding anagyrine at 10.27 g/kg dry plant weight and 53% of the total alkaloids in *Lupinus montigenus*.

Kilgore et al. (1981) point out the possibility of anagyrine producing the human deformity is circumstantial, and adequate proof may be long in coming. The presence of anagyrine at a very high percentage (86%) of the total alkaloids and plant dry weight (1.14%) supports their hypothesis. It is reasonable that a goat foraging on the plant investigated in this study could be exposed to a relatively high level of anagyrine before the acute toxic effects resulting from the total alkaloid consumption become apparent. This would allow for a higher concentration of anagyrine in goats milk relative to alkaloid profiles from lupines containing a lesser percentage of anagyrine. Currently, we are investigating the transfer of anagyrine into goats milk to obtain information on the level humans may incur.

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Identification of Several New Metabolites from Pentachloronitrobenzene by Gas Chromatography/Mass Spectrometry

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Four new metabolites derived from pentachloronitrobenzene have been detected and identified in parsnips: a tetrachlorophenyl methyl sulfoxide, two isomeric tetrachlorophenyl methyl sulfones, and a trichlorophenyl methyl sulfone. Their occurrence has been tentatively determined to have taken place in the soil over a long period after initial application with subsequent translocation to newly planted crop foliage.

The primary use of pentachloronitrobenzene (PCNB) as a soil fungicide to control *Rhizoctonia* in cotton (*Gossypium hirsutum*) and *Sclerotium* in peanut (*Arachis hypogaea*) has been widespread for some time (U.S. EPA, 1976). Definitive metabolism studies of PCNB in the peanut have been reported by Lamoureux et al. (1980, 1981) and Rusness and Lamoureux (1980). These authors concluded that three main competing reactions were responsible for the observed metabolic pathway: (1) aryl nitro reduction; (2) nucleophilic displacement of the nitro group; (3) nucleophilic displacement of a chloro group. The major product via aryl nitro reduction was also identified as pentachloroaniline (PCA), with pentachlorothioanisole (PCTA) being identified as a minor product through catabolic reaction via *S*-(pentachlorophenyl)cysteine. Translocation of PCNB and its primary

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