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Pyrrolizidine Alkaloids in Honey from *Echium plantagineum* L.

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Honey produced from stands of *Echium plantagineum* L. (Paterson's Curse or Salvation Jane) contained pyrrolizidine alkaloids to the extent of 0.27–0.95 ppm. The main alkaloid present was echimidine, with smaller amounts of 7-acetyllcopsamine, 7-acetylintermedine, echiumine, uplandicine, lycopsamine, intermedine, and a new alkaloid which is probably acetylechimidine.

Many species of the family Boraginaceae are frequented by bees as a source of nectar and pollen. The purple-flowered herb, *Echium plantagineum* L., known as Paterson's Curse in Victoria and New South Wales and as Salvation Jane in South Australia, is an annual weed forming dense stands over thousands of square miles of southeastern Australia. It is worked by apiarists for brood building in the spring and as a major source of honey (Purdie, 1968). Species of Boraginaceae are also sources of pyrrolizidine alkaloids (Bull et. al., 1968). *E. plantagineum* contains a number of alkaloids of which echiumine, echimidine (Culvenor, 1956), lycopsamine, intermedine, and 7-angelylretronecine (Frahn, 1979) have been identified. A recent, unpublished study of the alkaloids of this species by the authors has also disclosed three alkaloids recently found for the first time in *Symphytum* × *uplandicum* (Russian comfrey), 7-acetyllycopsamine, 7-acetylintermedine and uplandicine (Culvenor et al., 1980), and a new alkaloid which is probably acetylechimidine. The possibility that these alkaloids may be transmitted to honey derived from *E. plantagineum* was investigated by us in 1957 with negative results. It has now been reinvestigated, using more sensitive detection methods, following the demonstration by Deinzer et al. (1977) that pyrrolizidine alkaloids are present in honey produced from *Senecio jacobaea* L. (Ragwort) at the level of 0.3–3.9 ppm.

MATERIALS AND METHODS

Honey. Samples of ~2 kg of fresh honey produced from stands of *E. plantagineum* were obtained from four suppliers who named as the source localities Tocumwal, Young, Stockinbingal, and Southern Riverina, all in New South Wales. The samples from Young and Stockinbingal were still in the comb. A fifth sample of unknown source locality, but labeled as *Echium* honey, was purchased in an Adelaide department store.

Extraction of Alkaloids. The honey (1 kg) was diluted with water (1 L) and divided into two equal portions. One part was acidified with H_2SO_4 (23 mL of concentrated acid) and stirred with zinc dust for 4 h. Both parts were then basified with concentrated aqueous NH_3 to pH 9, saturated with NaCl, and separately extracted with chloroform. Emulsions formed and were broken by centrifuging at 2000 rpm. The extracts, after removal of solvent, were taken up in 0.5 N H_2SO_4, and the solutions washed 3 times with chloroform before basifying to pH 9 with NH_3, saturating with NaCl, and extracting with chloroform. The unreduced extract was assumed to represent the tertiary base alkaloids and the reduced extract the combined tertiary bases and *N*-oxides.

In two instances, the honey (2 kg) was diluted, adjusted to pH 9, and extracted with chloroform to give the tertiary base alkaloids. The aqueous residue was then reduced with $Zn-H_2SO_4$, filtered, adjusted to pH 9, and extracted with chloroform to give the parent alkaloids of the *N*-oxides.

Identification and Quantitation by Gas Chromatography–Mass Spectrometry (GC–MS). *GC–MS Conditions.* The instrument used was a Varian MAT-111 gas chromatograph–mass spectrometer containing a 1 m × 2 mm i.d., glass-lined stainless steel column packed with 1% SE30 (U/P) on Chromosorb W (HP), mesh size 80–100. The injector temperature was 210 °C, and the oven temperature was programmed from 150 to 220 °C at 6 °C/min. The GC–MS interface temperature was 220 °C, and the carrier gas (helium) flow rate was 15 mL/min.

Identification of Alkaloids. The alkaloid extracts were examined underivatized as well as after conversion to butylboronates and trimethylsilyl derivatives. Retention times and mass spectra were compared with those of authentic samples.

Derivatization. For butylboronation, the alkaloid extracts were taken up in pyridine containing butylboronic acid (10 μg/μL) and used immediately. For trimethylsilylation, the alkaloids were taken up in a mixture of pyridine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (1:1) and kept for 30 min–8 h at room temperature before injection.

Quantitation. The butylboronate derivatives were used for quantitation. With the exception of lycopsamine and intermedine, they give sharp, reproducible GLC peaks for the main alkaloids. The almost identical retention time (RT) of 7-acetylintermedine butylboronate (6.1 min) and uplandicine butylboronate (6.1 min) and the similar RT of 7-acetyllycopsamine butylboronate (5.9 min) (Figure 1) made it necessary for these three alkaloids to be quantitated together as group A. The GLC peaks of echiumine butylboronate (RT 8.8 min) and echimidine butylboronate (RT 8.5 min) also overlap (Figure 1), and these two alkaloids were therefore also grouped for quantitation as group B.

Quantitation was based on a comparison of peak areas with those produced by approximately equal amounts of authentic samples of either 7-acetyllycopsamine butylboronate (group A) or echimidine butylboronate (group

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Table I. Alkaloid Extraction from Honey Samples Derived from *E. plantagineum* (Paterson's Curse; Salvation Jane)

sample	source	alkaloid levels (tertiary base + *N*-oxide),[a] ppm: group A alkaloids[c]	group B alkaloids[d]	total alkaloids, ppm	proportion present as *N*-oxide, %
1	Southern Riverina, N.S.W.[e]	0.23	0.64	0.87	0
2	Stockinbingal, N.S.W.	0.01	0.45	0.46	27
3	Tocumwal, N.S.W.	0.05	0.30	0.35	50
4[b]	Young, N.S.W.	0	0.27	0.27	n.d.
5	Adelaide store	0.23	0.72	0.95	0

[a] Uncorrected for incomplete extractions of alkaloid. Figures are the means of three to five injections. Standard errors are less than 10% of the values quoted. [b] Tertiary base only. [c] Group A: 7-acetyllycopsamine; 7-acetylintermedine; uplandicine. [d] Group B: echimidine; echiumine. [e] N.S.W., New South Wales.

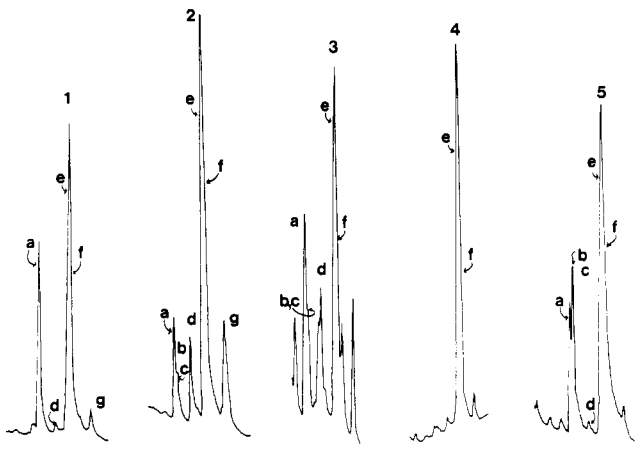

Figure 1. Typical total ion chromatograms of alkaloid extracts of the five honey samples when run as butylboronate (bb) derivatives. The components have been identified as overlapping 7-acetyllcopsamine bb (M^+, 407), (b) 7-acetylintermedine bb (M^+, 407), (c) uplandicine bb (M^+, 423), (d) an unidentified 7-angelylpyrrolizidine alkaloid, (e) echimidine bb (M^+, 463), (f) echiumine bb (M^+, 447), and (g) "acetylechimidine" bb (M^+, 505) by comparison of their mass spectra and retention times with those of authentic samples. For quantitations (by peak area), the overlapping peaks a, b, and c (group A alkaloids) were measured as one, as were peaks e and f (group B alkaloids).

B). Aliquots of the butylboronate derivatives of each extract, varying from 2 to 8% of the total (depending on the alkaloid content of the sample), were injected several times, and the means, calculated from these separate injections, were determined.

Extraction Efficiency. Honey (1 kg) produced in a district free of *E. plantagineum* was diluted and weakly acidified with H_2SO_4. An aliquot of a standard solution of echimidine (500 μg) was added to the honey solution, and the extraction and estimation were performed as above. A second portion of this honey was extracted in the same way, as a control, and found to be free of pyrrolizidine alkaloids.

RESULTS AND DISCUSSION

When gas chromatographed underivatized, the alkaloids of *E. plantagineum* (especially those which are esters of echimidinic acid) are subject to thermal decomposition which increases with use of the column. This necessitated use of two types of derivatization, trimethylsilylation, and butylboronation, as well as examination of the underivatized extracts, to identify the alkaloids present in the honey samples. Under the conditions used, lycopsamine and intermedine produced bis(trimethylsilyl) derivatives (Culvenor et al., 1980), and 7-acetyllycopsamine and 7-acetylintermedine gave mono(trimethylsilyl) derivatives (M^+, 413). Echiumine, echimidine, and uplandicine yielded mixtures of pairs of bis(trimethylsilyl) derivatives (M^+, 525, 541, and 501), presumably involving the secondary hydroxyl and one or the other of the tertiary hydroxyls in the echimidinic acid moiety. Acetylechimidine does not appear to be derivatized to any appreciable extent under these conditions. More vigorous conditions, involving MSTFA or more powerful trimethylsilylation reagents, produced the tri(trimethylsilyl) derivatives of lycopsamine (M^+, 515) and intermedine (M^+, 515) and the bis(trimethylsilyl) ethers of their 7-acetyl derivatives (M^+, 485) but resulted in degradation and multiple products from the alkaloids with echimidinic acid side chains. These latter products interfered considerably with interpretation of the data and the milder conditions were therefore preferable.

The total alkaloid content (groups A plus B) of the various honey samples was found to range between 0.27 and 0.95 ppm, with the proportion present as *N*-oxide varying from 0 to 50% (Table I). Group B alkaloids (echimidine/echiumine) were the main constituents in each sample (0.27–0.72 ppm) with group A alkaloids (7-acetyllcopsamine, 7-acetylintermedine, and uplandicine) next in prominence (0–0.23 ppm). Lycopsamine and intermedine, an alkaloid of retention time and mass spectrum appropriate for acetylechimidine, and an unidentified alkaloid were also present in most samples. The efficiency of recovery of echimidine, from a honey sample to which it had been added at the level of 0.5 ppm, was 60% from an unreduced solution and 70% from a reduced solution. Among the *Echium* alkaloids, echimidine is probably of only average difficulty in extraction from aqueous solution, so that a better estimate of the alkaloids actually present in the honey is probably obtained by increasing the levels observed by 50%. Even then, the alkaloid levels in *Echium* honey (0.54–1.9 ppm) would remain somewhat lower than those found in *Senecio jacobaea* honey in the United States (0.3–3.9 ppm) by Deinzer et al. (1977).

The significance of the alkaloid levels in *Echium* honey is not readily evaluated from the data presently available. The combined effects of the alkaloids might be expected to approximate those of echimidine present at the full group B level, since echimidine has higher acute toxicity (LD_{50} = 200 mg/kg ip in male rats; Bull et al., 1968) than lycopsamine and intermedine (>500 mg/kg) (Fowler and Schoental, 1967) and, by implication, their acetyl derivatives. Acetyl derivatives are expected to approximate the parent alkaloids in toxicity (Culvenor et al., 1976) because of ready removal of the acetyl group by liver esterases (Mattocks, 1970). The presence of part of the alkaloid as *N*-oxide has only minor influence on toxicity if the alkaloid is ingested orally, because of reduction of the *N*-oxides by the gut flora (Mattocks, 1971). Two pyrrolizidine alkaloids of higher acute toxicity than echimidine, monocrotaline and lasiocarpine, have been shown to produce moderate chronic liver damage, in pigs and rats, respectively, when administered for long periods at dose rates approximately

2–3 ppm in the diet (Hooper and Scanlan, 1977; Jago and Peterson, 1979). Much lower intake rates would result from normal human consumption of *Echium* honey such as investigated here, and there is no evidence to suggest that these lower intake rates would produce adverse effects. However, a need for further investigation of the significance of the alkaloids in *Echium* honey, and of conditions which might increase or decrease their level, is emphasized by the carcinogenicity of some pyrrolizidine alkaloids and, particularly, of the plant *Symphytum officinale* (Hirono et al., 1978) which contains alkaloids very similar to those of *E. plantagineum*.

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Soybean Peroxidases: Purification and Some Properties

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Soybean peroxidases have been purified 628-fold with a yield of 4% by combined consecutive treatments consisting of ammonium sulfate fractionation from a pH 4.5 extract of partially defatted soybeans and column chromatographies by gel filtration on Bio-Gel P-60, gradient elutions from DEAE-Sephadex A-50, affinity chromatography on Con A–Sepharose 4B, and hydrophobic chromatography on phenyl-Sepharose CL-4B. Three anionic isozymes, detected by polyacrylamide disc gel electrophoresis, differed in charge rather than in molecular size. Soy peroxidases showed optimum activity at pH 5.5 with guaiacol as the hydrogen donor, a K_m for H_2O_2 of 0.58 mM at optimum guaiacol concentration, a K_m of 5.9 mM for guaiacol at optimum H_2O_2 concentration, and competitive cyanide inhibition with respect to guaiacol with K_i = 0.15 μM. A minimum molecular weight of 37 000 was estimated for the polypeptide chain by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. At 70 °C, it took 72 min to inactivate the peroxidase isozymes 97%, and at 80 °C, these enzymes were almost completely inactivated after 15 min.

Peroxidases can contribute to deteriorative changes in flavor, texture, color, and nutrition both in raw foods such as fruits and vegetables and in processed products (Burnette, 1977; Haard, 1977). In addition to the general peroxidatic reactions, peroxidases can also catalyze oxidasic, catalatic, and hydroxylation reactions (Haard, 1977). In the oxidasic reaction (Nicholls, 1962), molecular oxygen is used as an electron acceptor rather than hydrogen peroxide. We are interested in determining the oxidasic capacity of soybean peroxidase and its possible role in generating off-flavors from endogenous lipids and phenolic constituents.

Peroxidases have been implicated in fatty acid oxidation (Stumpf and Bradbeer, 1959). Rothe (1956) proposed that an antioxidant complex aided by a peroxidase caused breakdown of lipid hydroperoxides. Täufel and Rothe (1965) established that peroxidase in oats and wheat is involved in lipid oxidation. Meanwhile, Gini and Koch (1961) observed a rapid decrease in linoleic acid hydroperoxides incubated with soy flour extracts, which they attributed to a peroxidase action. Schormüller et al. (1969) separated a guaiacol–linoleic acid hydroperoxide oxidoreductase (GLO) in soybeans from the H_2O_2-converting peroxidase by chromatography on CM-Sephadex, but they could not separate GLO from lipoxygenase. Further investigations were made by Grosch et al. (1972) on the purification of the lipoxygenase–GLO complex from soybeans and its activity on linoleic acid hydroperoxide.

Sessa (1979) reviewed the literature on generation of bitter taste in legumes from oxidation of phosphatidylcholine. Bitterness of oxidized phosphatidylcholine has been attributed in part to an unsaturated trihydroxy fatty acid moiety that was generated via a lipoxygenase–peroxidase complex isolated from soybeans (Moll et al., 1979). The oxidasic capacity of the H_2O_2-converting soy peroxidase, as distinguished from the lipoxygenase–GLO complex, with action on endogenous lipid and phenolic

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