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Solid-Phase Extraction and LC–MS Analysis of Pyrrolizidine Alkaloids in Honeys

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Strong-cation-exchange, solid-phase extraction of pyrrolizidine alkaloids and their *N*-oxides from honey samples was followed by reduction of the *N*-oxides and subsequent analysis of total pyrrolizidine alkaloids using high-performance liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. A limited survey of 63 preprocessing samples of honey, purposefully biased toward honeys attributed to floral sources known to produce pyrrolizidine alkaloids, demonstrated levels of pyrrolizidine alkaloids up to approximately 2000 parts per billion (ppb) in a sample attributed to *Echium plantagineum*. Up to 800 ppb pyrrolizidine alkaloids was detected in some honeys not attributed by the collector to any pyrrolizidine alkaloid-producing floral source. No pyrrolizidine alkaloids were detected in approximately 30% of the samples in this limited study, while some honeys showed the copresence of pyrrolizidine alkaloids from multiple floral sources such as *E. plantagineum* and *Heliotropium europaeum*. In addition, retail samples of blended honeys (with no labeling to suggest that pyrrolizidine alkaloid-producing floral sources were used in the blends) have been shown to contain up to approximately 250 ppb pyrrolizidine alkaloids.

KEYWORDS: Pyrrolizidine alkaloids; *N*-oxides; honey; LCMS

INTRODUCTION

Pyrrolizidine alkaloids (PAs) and their *N*-oxides are well established as hepatotoxins in animals and humans (*1*, *2*). In addition to hepatotoxic effects, pyrrolizidine alkaloids have been related to pneumotoxicity, genotoxicity, and carcinogenicity. However, the major effect of dietary pyrrolizidine alkaloids on humans is reported to be hepatic venoocclusive disease (*3*). Recent research (*4*) has confirmed that orally administered pyrrolizidine *N*-oxides are equally genotoxic and carcinogenic as their parent pyrrolizidine alkaloids despite the fact that they are also readily excretable (detoxifying), hepatic metabolites of the parent pyrrolizidine alkaloids.

After a comprehensive risk assessment of toxic pyrrolizidine alkaloids, the German Federal Health Bureau established regulations, on the basis of their potential to be genotoxic and carcinogenic, that restrict oral exposure to pyrrolizidine alkaloids or their *N*-oxides in herbal preparations to 0.1 μg/day with the exclusion of pregnant and lactating women for which zero exposure is recommended (*5*). In The Netherlands, there is a recommendation of 0.1 μg of pyrrolizidine alkaloids/100 g of food (*6*). On the basis of the potential to cause hepatic venoocclusive disease, the Australia New Zealand Food Authority (ANZFA), now the Food Standards Australia New Zealand (FSANZ), set a provisional exposure level of 1 (μg of pyrrolizidine alkaloids/kg of body mass)/day (*7*).

In addition to the unknown effects of long-term, low-level exposure of humans to dietary pyrrolizidine alkaloids or their *N*-oxides, the potential for synergistic or predisposing effects of other agents (e.g., copper and other hepatoxins) with pyrrolizidine alkaloids/their *N*-oxides is also a consideration and has been suggested as one possible factor in the cause of childhood cirrhosis (*8*). As such, all potential dietary sources of pyrrolizidine alkaloids/their *N*-oxides need to be investigated for the presence of the alkaloids.

Honey is just one such potential source of dietary pyrrolizidine alkaloids and their *N*-oxides. The occurrence of pyrrolizidine alkaloids in honeys, as a result of bees foraging on pyrrolizidine alkaloid-producing plants is well documented (*6*). In this study, strong-cation-exchange (SCX), solid-phase extraction (SPE) of pyrrolizidine alkaloids and their *N*-oxides from honey samples was followed by a reduction step to convert all *N*-oxides into their parent tertiary bases. The subsequent application of high-performance liquid chromatography coupled with ion trap mass spectrometry with atmospheric pressure chemical ionization (HPLC−APCI-MS) was used to analyze alkaloidal fractions.

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MATERIALS AND METHODS

Chemicals and Solvents. All chemicals and solvents used were of analytical reagent or HPLC grade purity. HPLC water was MilliQ-purified (Millipore), while sample preparation water was purified using reverse osmosis.

Honey Samples. Various honey samples, with a bias toward those with floral sources deemed to be more likely to contain pyrrolizidine alkaloids, and including two samples of a blended retail product, were supplied by Capilano's Honey (Brisbane, Australia). Each sample was numbered, and the details of each sample, including the major floral source as indicated by the apiarist, were recorded under the guidance of the Australian Bureau of Resource Sciences. In addition, each sample received a unique CSIRO Plant Toxins Research Group accession number. We purchased other retail samples from commercial outlets.

Pyrrolizidine Alkaloid Standards. Authenticated (NMR, MS) standards, isolated from plant sources, of echimidine, echiumine, lycopsamine, intermedine, heliotrine, lasiocarpine, senecionine, europine, and indicine were obtained from the collection of the CSIRO Plant Toxins Research Group.

HPLC−APCI-MS. Samples were analyzed using a SpectraPhysics autosampler and liquid chromatography system coupled to a Thermo-Finnigan LCQ ion trap mass spectrometer. Samples were injected onto a 250 × 4.2 mm i.d. Alltima C8 reversed-phase column (Alltech, Deerfield, IL) protected by a guard cartridge of equivalent adsorbent. Adsorbed compounds were eluted from the column with a gradient flow (1 mL/min) of 0.01% trifluoroacetic acid in water (mobile phase A) and 0.01% trifluoroacetic acid in acetonitrile (mobile phase B). Mobile phase A was held at 93% for 5 min before being ramped down in a linear fashion to 30% over 15 min. A reequilibration phase restored the 93% A in preparation for the next sample injection.

The mass spectrometer response was tuned to a solution of heliotrine in methanol. Mass spectrometric data were collected in the positive ion mode with a capillary temperature and voltage of 220 °C and 7 V, respectively, and an APCI vaporization temperature of 450 °C. The source voltage was 6 kV at a source current of 5 μA. The sheath and auxiliary nitrogen gas flow ratio was 76:7. Software-generated, reconstructed ion chromatograms (RICs) for those pyrrolizidine alkaloids that might be expected to be observed were integrated and subsequently quantitated against an echimidine standard calibration curve. Quantitation was calibrated against an injection standard of caffeine or 5-methoxydimethyltryptamine, stock solutions in methanol at 1 mg/mL, added to each sample.

To confirm the presence of pyrrolizidine alkaloids, MS/MS experiments were conducted using the direct infusion LC−MS mode, isolating the ion of interest (the $[M + H]^+$ adduct ion for the suspected alkaloid), and adjusting the fragmentation energy to achieve the most useful fragmentation spectrum that allowed examination for ions characteristic of pyrrolizidine alkaloids.

Sample Processing and Preparation. *SPE Extraction.* A sample of honey (25 g) was diluted with water (50 mL) and centrifuged at 25000*g* for 20 min to remove particulate matter. The supernatant was applied (approximately 1 mL/min under slight vaccum) to a preconditioned (washed with methanol and then water), 500 mg/2.8 mL Alltech "Extract-Clean" strong-cation-exchange resin column (Alltech). After loading, the column was washed with water (20 mL), and the adsorbants were then eluted with a solution of methanol in aqueous acid ($MeOH/H_2O$/concentrated HCl, 40:135:25, 20 mL) at a rate of 0.5−1 mL/min.

Reduction of Pyrrolizidine N-Oxides and Extraction of Pyrrolizidine Alkaloids. Zinc dust (300 mg) was added to the column eluate and the mixture stirred at room temperature for 3−4 h. On settling, the supernatant was decanted into a screwcap tube, made basic (pH >9.5) by adding concentrated ammonium hydroxide solution, and then extracted with chloroform (2 × 6 mL). The dried (anhydrous sodium sulfate) chloroform extract was evaporated to dryness under a flow of nitrogen at 40 °C.

Preparation of Analytical Samples. The residue from the chloroform extract was quantitatively transferred to a sample vial (2 mL) using methanol and then evaporated to dryness under a flow of nitrogen. The residue was reconstituted in an acidic methanol solution (1 mL) containing 1% acetic acid in water (500 μL), methanol (490 μL), and injection standard solution (10 μL). A small sample (10 μL) was analyzed using HPLC−APCI-MS.

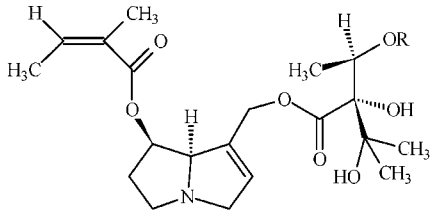
Table 1. Molecular Ions and Selected MS/MS Fragment Ions of Major Pyrrolizidine Alkaloid Markers of Various Floral Sources Determined in This Study

pyrrolizidine alkaloid	$[M + H]^+$	MS/MS fragment ions
indicine	300	256, 138, 120, 94
acetylindicine	342	324, 282, 138, 120
unidentified *H. amplexicaule* alkaloid	358	340, 138, 120
echimidine	398	380, 336, 220, 120
acetylechimidine	440	422, 380, 362, 336, 322, 220
echiumine	382	324, 238, 220, 120
uplandicine	358	340, 298, 180, 120
lycopsamine/intermedine	300	256, 156, 138, 120, 94
acetyllycopsamine/acetyl intermedine	342	324, 282, 180,120
unidentified *E. vulgare* alkaloid	480	462, 380, 322, 220, 120
heliotrine	314	256, 138, 120, 94
lasiocarpine	412	394, 336, 220, 120
europine	330	312, 272, 120
heleurine	298	280, 122
senecionine	336	308, 138, 120

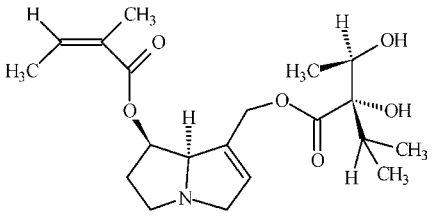
RESULTS AND DISCUSSION

Some pyrrolizidine alkaloid-producing plants to which foraging bees have access include species of the genera *Echium*, *Heliotropium*, *Senecio*, *Amsinckia*, and *Crotalaria* (*6*). The difficulties associated with analysis of low levels of pyrrolizidine alkaloids using GC−MS have been previously discussed (*9*). Thus, in this study, the SCX, solid-phase extractives of honey were reductively treated with zinc/HCl and then examined, using HPLC−APCI-MS, for the presence of pyrrolizidine alkaloids considered to be markers of specific floral sources (**Table 1**; **Figure 1**). APCI of samples, rather than electrospray ionization (ESI), was selected due to the better stability of the APCI-MS response, compared to the ESI-MS response, over the extended time period (24 h) required to complete an analytical sequence. Comparison of the retention times and mass spectrometric data ($[M + H]^+$ and MS/MS) for eluted compounds with those of authenticated pyrrolizidine alkaloids, including the observation of fragment ions characteristically associated with pyrrolizidine alkaloids (**Figure 2**), was used to identify pyrrolizidine alkaloid components of the honey extracts.

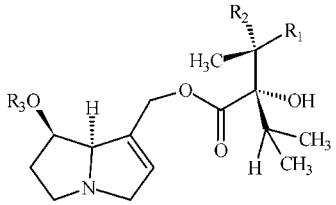
A total of 63 honey samples collected and supplied during the 1998/1999 seasons were analyzed within 4 weeks of receiving the samples. These samples were drawn from bulk honey containers (200 L) supplied by the honey collectors and prior to any processing at the packaging company. Relative to distribution in the marketplace, the sampling was purposefully biased toward "at risk" honeys, i.e., those with an attributed floral source (by the bee keeper) associated with the presence of pyrrolizidine alkaloids. Thus, 13 samples were attributed to *Echium plantagineum* (Salvation Jane or Paterson's Curse), 9 samples were listed as an *E. plantagineum* mix, 4 samples were collected in the vicinity of *Heliotropium amplexicaule* (Purple Top), 2 samples were derived mainly from *Heliotropium europaeum* (Common Heliotrope or Potato Weed), and 35 were attributed to floral sources with no known association with pyrrolizidine alkaloids. In addition, five retail samples of honey were analyzed. Three of these were samples of blended honeys, with no labeling to suggest floral sources, another was described as Leatherwood honey, and the last was described as Alpine Borage honey. The latter two are derived from bees foraging on *Eucryphia lucida* and *Echium vulgare*, respectively.



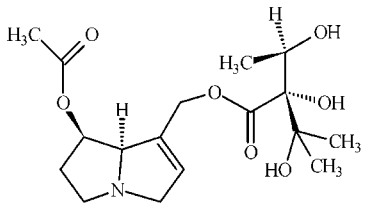
R = H : **echimidine**
R = $COCH_3$: **3'-*O*-acetylechimidine**



echiumine



R_1, R_3 = H R_2 = OH : **lycopsamine**

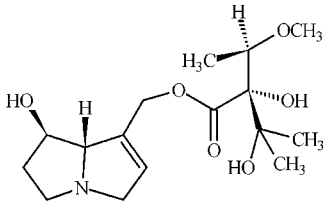


uplandicine

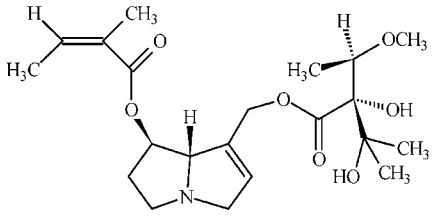
R_1 = OH R_2, R_3 = H : **intermedine** or **indicine**

R_1 = H R_2 = OH R_3 = $COCH_3$: **7-*O*-acetyllycopsamine**

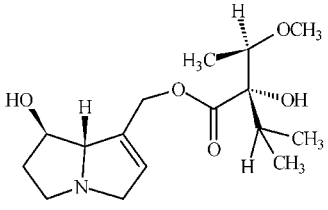
R_1 = OH R_2 = H R_3 = $COCH_3$:**7-*O*-acetylintermedine** or **7-*O*-acetylindicine**



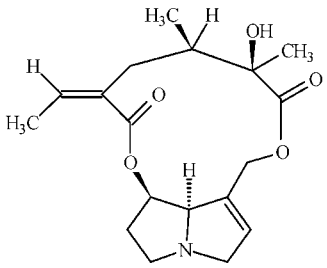
europine



lasiocarpine



heliotrine



senecionine

Figure 1. Structures of major pyrrolizidine alkaloid markers (**Table 1**) of floral sources for honeys examined in this study.

The efficiency of reduction of pyrrolizidine *N*-oxides using different reduction conditions and reagents has previously been briefly discussed (*9*). As a result, zinc/hydrochloric acid (1M) was chosen as the reduction medium for a maximum reaction time of 4 h to effect the reduction of pyrrolizidine *N*-oxides in this study. An average efficiency of reduction of the pyrrolizidine *N*-oxides, using zinc/acid, was estimated to be 80% on the basis of the reduction of the *N*-oxides of heliotrine (100 ± SD 14% recovery) and senecionine (62 ± SD 8% recovery). To estimate the recovery efficiency of pyrrolizidine alkaloids, authenticated senecionine, lasiocarpine, or echimidine was spiked into honey, in triplicate, at concentrations of approximately 0.2, 1, 2.5, 10, 25, and 50 μg/mL. Average recovery efficiencies (±SD) were 74 ± 22% for senecionine, 72 ± 27% for echimidine, and 71 ± 29% for lasiocarpine. Thus, for the analysis of samples, a conservative estimate of 70% was used for the recovery efficiency. The levels of pyrrolizidine alkaloids present in samples were quantitated against a seven-point calibration curve of authenticated (NMR, MS) echimidine over the concentration range of 0.02−25 μg/mL and are thus reported as echimidine equivalents. All calibration curves had R^2 values in excess of 0.98 and usually in excess of 0.99. Variation in detector response was accommodated by inclusion of an injection standard (5-methoxydimethyltryptamine or caffeine) with every analytical sample against which responses of all pyrrolizidine alkaloids were adjusted. In several cases, spiking of samples with authenticated standards was used to confirm identification. With near-optimal performance of all HPLC and MS parameters, peak area integration of RICs displaying specific marker ions for the expected pyrrolizidine alkaloids (**Table 1**) and quantitation against the calibration curve of authenticated standards demonstrated an apparent, overall level of detection for the method of approximately 1 μg of individual pyrrolizidine alkaloids/kg of honey (1 ppb). Taking into consideration the

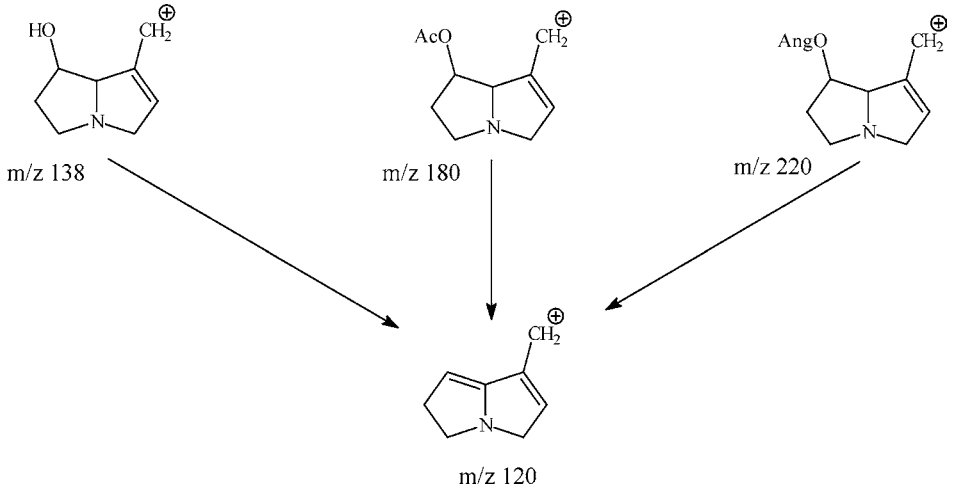


Figure 2. Some mass spectrum fragment ions characteristic of pyrrolizidine alkaloids. "Ac" represents an acetyl group, while "Ang" represents an angelyl group or one of its configurational isomers.

70% efficiency of extraction, the adjusted level of detection for this overall method was approximately 1.5 ppb.

Most samples were analyzed in duplicate, i.e., two separate aliquots (approximately 25 g each) of each honey sample. The results (**Table 2**) show that each of those 29 honeys attributed by the collector to a pyrrolizidine alkaloid-producing floral source was positive for the presence of pyrrolizidine alkaloids (ranging from approximately 33 to 2200 ppb). The identities of the pyrrolizidine alkaloids observed were consistent, on the basis of literature reports and unpublished CSIRO Plant Toxins Research Group data, with the major described floral source and sometimes indicated more than one pyrrolizidine alkaloid-producing floral source. Of the 39 honeys attributed to floral sources other than pyrrolizidine alkaloid-producing sources, or with no floral source attribution, 19 samples were positive for pyrrolizidine alkaloids, ranging from approximately 3 to 800 ppb.

The greatest quantity of pyrrolizidine alkaloids detected in the samples analyzed was approximately 2270 ppb in a sample attributed by the collector to be sourced in the vicinity of Salvation Jane (*E. plantagineum*). The pyrrolizidine alkaloids detected, including echimidine, echiumine, acetylechimidine, and the 7-*O*-acetyl derivatives of lycopsamine and/or intermedine (**Figure 3**), were clearly indicative of *E. plantagineum* being consistent with literature reports (*10*, *11*) and phytochemical analysis of locally collected *E. plantagineum* (unpublished results).

A sample that was attributed to Purple Top (a colloquial name in Australia for *H. amplexicaule*) by the collector contained approximately 1500 ppb total pyrrolizidine alkaloids. The HPLC−APCI-MS data (**Figure 4A**) showed the presence of indicine, closely related to intermedine through enantiomerization of the C9 esterifying acid, as the major pyrrolizidine alkaloid and heliotrine as a significant component. Additionally, acetylindicine was readily observed, and an unidentified pyrrolizidine alkaloid ($[M + H]^+$ 358) that coeluted with indicine was revealed via an RIC (**Figure 4B**). These observations are consistent with phytochemical examination of *H. amplexicaule* (unpublished results).

Analysis of two Potato Weed honeys indicated the presence of approximately 190 ppb total pyrrolizidine alkaloids. Potato Weed is a common name for *H. europaeum* (Common Heliotrope), and the profile of pyrrolizidine alkaloids observed (**Figure 5**) was consistent with the floral attribution of the honey. Thus, lasiocarpine, heliotrine, and europine were confirmed as the major pyrrolizidine alkaloids present via a spiking experiment in which the authenticated standards coeluted with the sample components. Also present was echimidine and a trace

Table 2. Mean Concentrations (Parts per Billion) of Pyrrolizidine Alkaloids Determined in Replicate Analyses of Duplicate Samples of Honeys Attributed to Various Floral Sources[a]

Floral Sources (PA Positive Samples/Total Samples)				
non-pyrrolizidine alkaloid-producing floral sources		retail samples		
various attributions (16/35)		unknown source (3/3)	*E. vulgare* (1/1)	*E. lucida* (0/1)
M01045I (3–3)	M01071E (25–27)	M01075A (120–126)	HON103 (1190–1263)	HON081 ND[b]
M01041M (5–7)	M01048S (53–62)	HON56B (122–135)		
M01011U (10–12)	M01076M (77–84)	HON079 (271–283)		
M01067S (10–13)	M01044W (144–167)			
M01077Y (14–15)	M01047G (177–273)			
M01014E (11–16)	M01039Y (298–314)			
M01046U (16–17)	M01040A (394–490)			
M01016C (16–20)	M01043K (711–814)			

Pyrrolizidine Alkaloid-Producing Floral Sources (PA Positive Samples/Total Samples)				
E. plantagineum (13/13)		*E. plantagineum* mix (9/9)	*H. amplexicaule* (4/4)	*H. europaeum* (2/2)
M01072Q (215–428)	M01074Q (696–757)	M01073C (25–42)	M01020O (212–415)	HON082 (121–250)
M01037A (280–401)	M01096Y (536–954)	M01004Y (42–49)	M01021A (399–449)	HON083 203
M01068E (291–435)	M01069Q (689–914)	M01038M (45–54)	M01087E (850–1143)	
M01035C (521–648)	M01008U (1026–1112)	M01095M (87–113)	M01088Q (1335–1647)	
M01009G (607–664)	M01005K (1362–1660)	M01034Q (116–151)		
M01033E (648–792)	M01006W (1533–1922)	M01094A (151–168)		
	M01007I (1914–2634)	M01003M (190–286)		
		M01036O (507–615)		
		M01023Y (705–1028)		

[a] The sample number is given with the concentration in parentheses. [b] None detected.

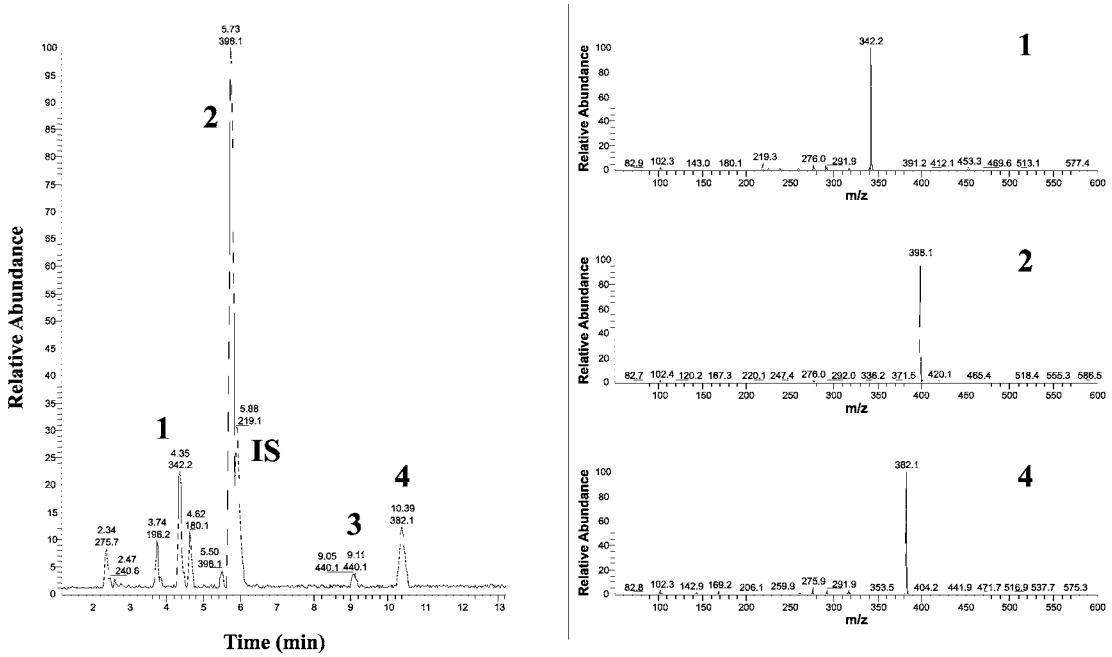


Figure 3. HPLC–APCI-MS base ion (*m*/*z* 105–500) chromatogram and some selected mass spectra for honey sample M01007I from New South Wales, Australia, and labeled with Salvation Jane (*E. plantagineum*) as the main floral source. Eluted peak 1 corresponds to acetylintermedine and/or acetyllycopsamine ([M + H]$^+$ 342), peak 2 to echimidine ([M + H]$^+$ 398), peak 3 to acetylechimidine ([M + H]$^+$ 440), and peak 4 to echiumine ([M + H]$^+$ 382). Peak IS on the shoulder of peak 2 is the injection standard ([M + H]$^+$ 219).

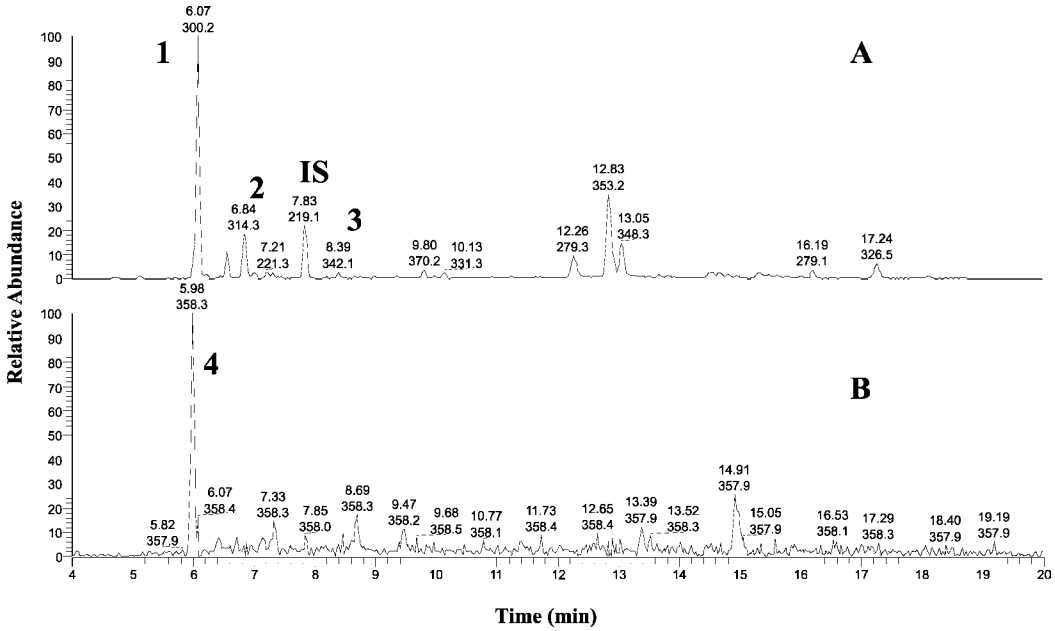


Figure 4. HPLC–APCI-MS base ion (*m*/*z* 200–500) chromatogram (**A**) and reconstructed ion chromatogram for *m*/*z* 358 (**B**) for honey sample M01088Q from Queensland, Australia, and labeled with Purple Top (*H. amplexicaule*) as the main floral source. Eluted peak 1 corresponds to indicine ([M + H]$^+$ 300), peak 2 to heliotrine ([M + H]$^+$ 314), peak 3 to acetylindicine ([M + H]$^+$ 342), and peak 4 to an unidentified pyrrolizidine alkaloid ([M + H]$^+$ 358). Peak IS is the injection standard ([M + H]$^+$ 219).

of the acetyl derivatives of intermedine and/or lycopsamine. This latter observation indicated either the contamination of the Potato Weed honey sample with another honey sample sourced from *E. plantagineum*, or that the foraging bees had simultaneous access to *H. europaeum* and smaller amounts of *E. plantagineum*.

Another indication of bees coforaging on *E. plantagineum* and *H. europaeum* was found with a honey attributed by the collector to the non-pyrrolizidine alkaloid-producing Grey Box (*Eucalyptus microcarpa*). Analysis of this honey sample yielded a total pyrrolizidine alkaloid content of approximately 760 ppb. The HPLC−APCI-MS ion profile and specific RICs (**Figure 6**) showed the presence of echimidine, echiumine, lycopsamine, and/or intermedine and their 7-*O*-acetyl derivatives indicative of *E. plantagineum* and lasiocarpine, heliotrine, and europine indicative of *H. europaeum*. This observation of pyrrolizidine alkaloids from a honey supposedly derived from a non-pyrrolizidine alkaloid-producing floral source is indicative of the potential for dispersal of pyrrolizidine alkaloids into unexpected honey supplies via natural (foraging bees) or artificial (honey-packaging companies) blending of honeys from different floral sources.

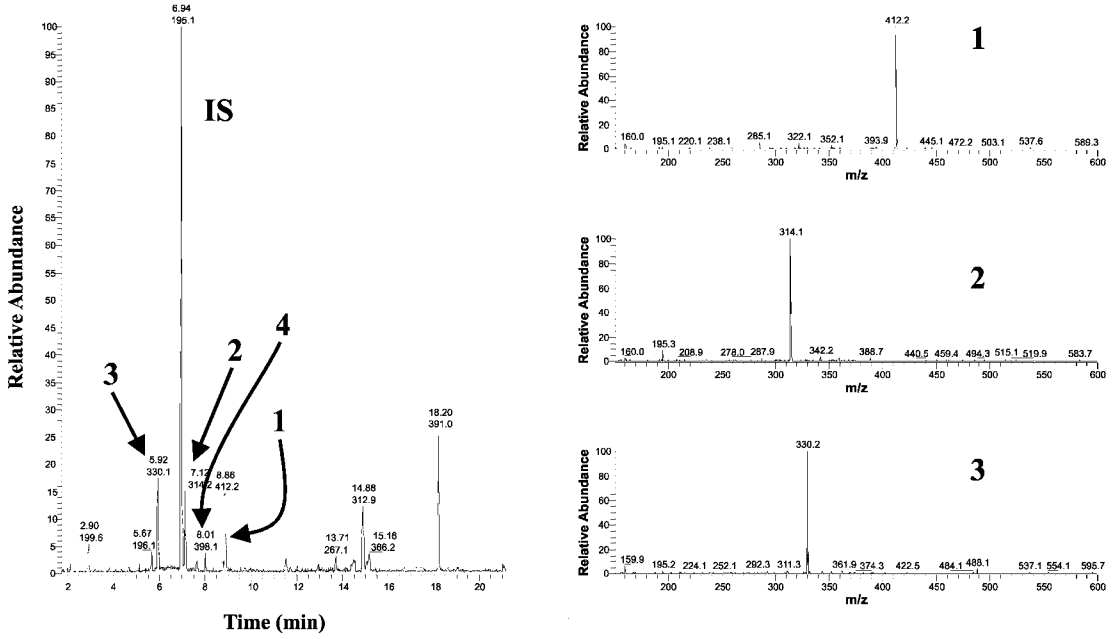


Figure 5. HPLC–APCI-MS base ion (*m*/*z* 150–500) chromatogram and some selected mass spectra for a honey sample (HON082) labeled with Potato Weed (*H. europaeum*) as the main floral source. Eluted peak 1 corresponds to lasiocarpine ([M + H]$^+$ 412), peak 2 to heliotrine ([M + H]$^+$ 314), and peak 3 to europine ([M + H]$^+$ 330). Peak 4 is echimidine ([M + H]$^+$ 398), while peak IS is the injection standard ([M + H]$^+$ 195).

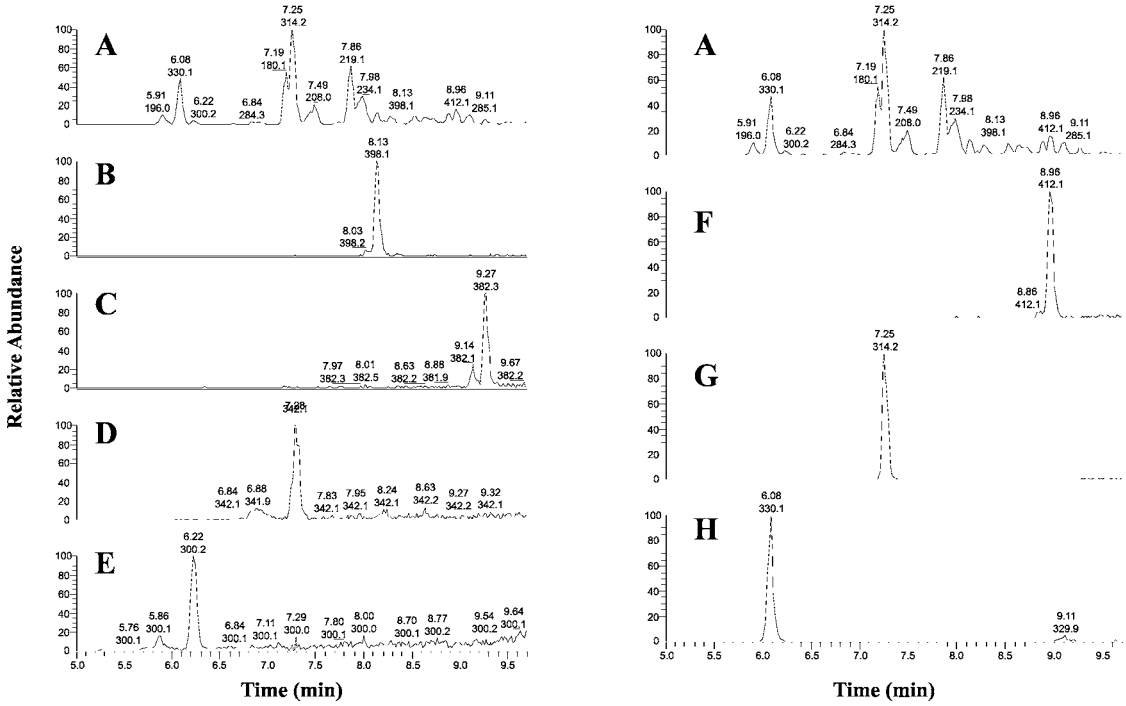


Figure 6. Presence of pyrrolizidine alkaloids from *H. europaeum* and *E. plantagineum* in honey sample M01043K collected in northern Victoria, Australia, and attributed by the collector to Grey Box (*E. microcarpa*). Base ion (*m*/*z* 150–500) chromatogram (**A**). Reconstructed ion chromatograms for the *E. plantagineum* pyrrolizidine alkaloids: (**B**) *m*/*z* 398, echimidine; (**C**) *m*/*z* 382, echiumine; (**D**) *m*/*z* 342, acetyllycopsamine and/or acetylintermedine; (**E**) *m*/*z* 300, lycopsamine and/or intermedine. Reconstructed ion chromatograms for the *H. europaeum* pyrrolizidine alkaloids: (**F**) *m*/*z* 412, lasiocarpine; (**G**) *m*/*z* 314 heliotrine; (**H**) *m*/*z* 330, europine.

Alpine Borage is a colloquial name in New Zealand for *E. vulgare*. Analysis of a retail honey sample derived from *E. vulgare* revealed (**Figure 7A**) a level of approximately 1220 ppb total pyrrolizidine alkaloids. Coelution with an authenticated standard confirmed echimidine (**Figure 7D**) as the major pyrrolizidine alkaloid present consistent with phytochemical analyses of the plant (*12*; unpublished results). Also readily observed (**Figure 7B**) was a late-eluting peak with [M + H]$^+$ 480 displaying fragmentations, including MS/MS, characteristic of pyrrolizidine alkaloids. This is an, as yet, unidentified pyrrolizidine alkaloid considered characteristic for *E. vulgare* on the basis of HPLC−ESI-MS of the whole plant extracts (unpublished results). Reconstructed ion chromatograms (**Figure 7C**,**F**) displaying ions at *m*/*z* 440 and 358 clearly showed the

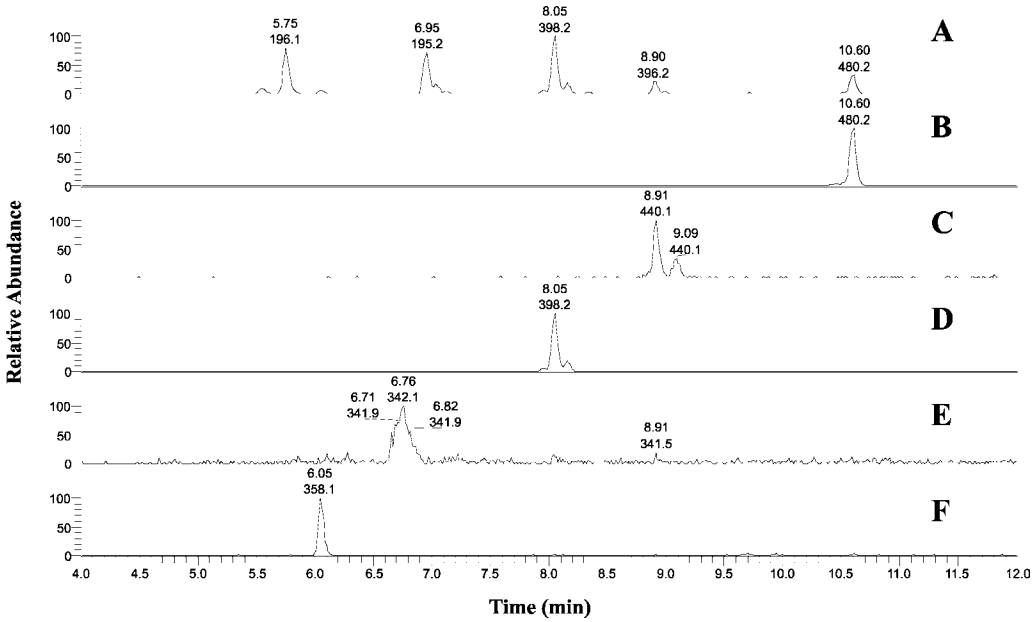


Figure 7. HPLC–APCI-MS analysis of Alpine Borage (*E. vulgare*) honey (HON103). Base ion (*m*/*z* 150–500) chromatogram (**A**). Reconstructed ion chromatograms for (**B**) *m*/*z* 480 for the unidentified pyrrolizidine alkaloid, (**C**) *m*/*z* 440 for acetylechimidine, (**D**) *m*/*z* 398 for echimidine, (**E**) *m*/*z* 342 for acetyllycopsamine and/or acetylintermedine, and (**F**) *m*/*z* 358 for uplandicine. Note that all RICs correlated with observed retention times of the standard pyrrolizidine alkaloids expected except for *m*/*z* 342 (**E**).

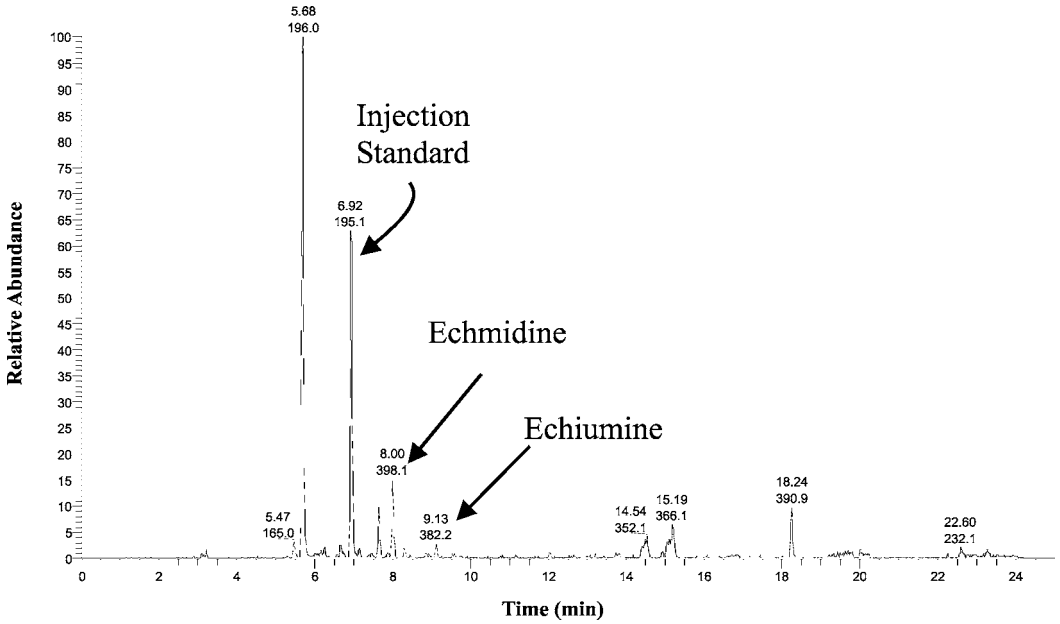


Figure 8. HPLC–APCI-MS base peak (*m*/*z* 150–500) chromatogram for a retail honey (HON079) with no labeling to indicate floral sources. The presence of echimidine and echiumine suggests *E. plantagineum* as at least one floral source.

presence of 3′-*O*-acetylechimidine and uplandicine as also observed by El-Shazly et al. (*12*). The RIC displaying *m*/*z* 342 (**Figure 7E**) did not coelute with the standard 7-*O*-acetylintermedine and/or 7-*O*-lycopsamine, and its occurrence in other honeys in isolation from known pyrrolizidine alkaloids indicated that it may be a non-pyrrolizidine alkaloid coextractive.

Three retail samples of blended honeys, with no labeling to attribute a floral source, were analyzed and shown to contain 120−250 ppb pyrrolizidine alkaloids indicative of *E. plantagineum* (**Figures 8** and **9**).

This research has demonstrated levels of pyrrolizidine alkaloids in excess of 2000 ppb in some honey samples specifically attributed to *E. plantagineum* and up to approximately 800 ppb in honeys not attributed by the collector to any pyrrolizidine alkaloid-producing floral source. No pyrrolizidine alkaloids were detected in approximately 30% of the samples in this limited survey, while some honeys showed the copresence of pyrrolizidine alkaloids from multiple floral sources such as *E. plantagineum* and *H. amplexicaule* or *H. europaeum*. Commercial, blended honeys (with no labeling to suggest that pyrrolizidine alkaloid-producing floral sources were used in the blend) have been shown to contain up to approximately 250 ppb pyrrolizidine alkaloids.

At present there are no maximum permissible concentrations (MPCs) of pyrrolizidine alkaloids in honey. However, noting that honey is only one source of dietary pyrrolizidine alkaloids to be considered, potential MPCs can be estimated by combining

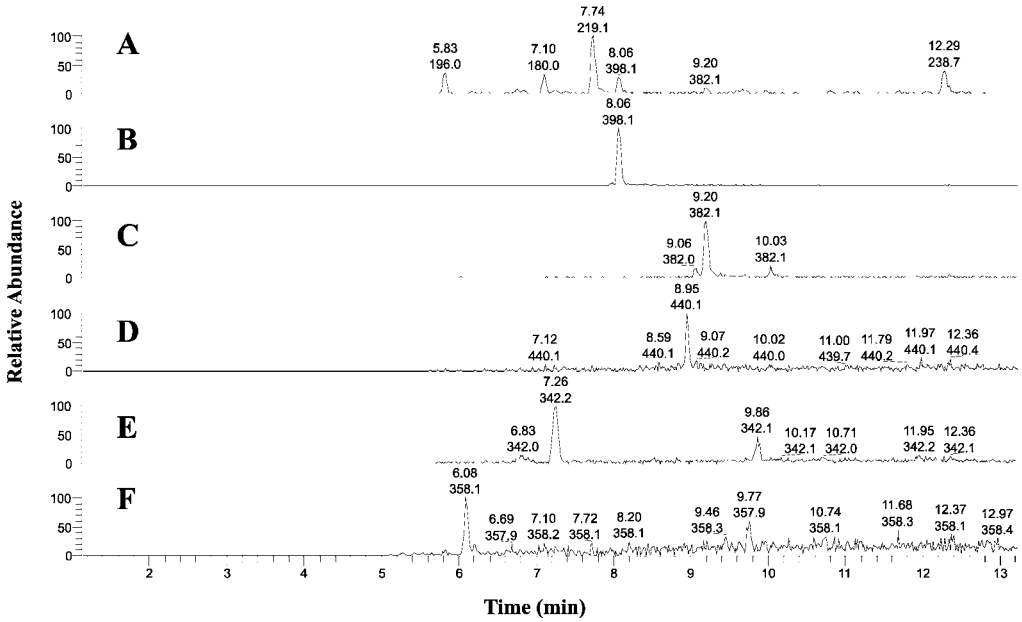


Figure 9. Final retail product of an *E. plantagineum* honey blend (M01075A) with other non-pyrrolizidine alkaloid-containing honeys: (**A**) the base peak (*m*/*z* 105–500) chromatogram, (**B**) RIC displaying *m*/*z* 398 for echimidine, (**C**) RIC displaying *m*/*z* 382 for echiumine, (**D**) RIC displaying *m*/*z* 440 for 3′-*O*-acetylechimidine, (**E**) RIC displaying *m*/*z* 342 for 7-*O*-acetyllycopsamine and/or 7-*O*-acetylintermedine, and (**F**) RIC displaying *m*/*z* 358 for uplandicine.

recommended or established provisional tolerable intakes of pyrrolizidine alkaloids with nutritional survey data that give an estimate of food consumption. For example, the 1995 Australian Nutritional Survey estimates that a 2−4 year old, 17 kg child could consume 28.6 g of honey/day (*13*), while a similar British survey estimated 30 g/day (*14*). If it is assumed that honey is the only dietary source of pyrrolizidine alkaloids, then the following MPCs can be estimated.

If the provisional tolerable daily intake (PTDI) of 1 (μg of pyrrolizidine alkaloids/kg of body mass)/day suggested by the FSANZ (formerly ANZFA) (*7*), on the basis of pyrrolizidine alkaloid-induced hepatovenoocclusive disease in humans, is accepted, then ingestion of honey containing approximately 600 ppb pyrrolizidine alkaloids will exceed the FSANZ PTDI for a 17 kg child in the 95th percentile that consumes approximately 28.6 g of honey/day. If, on the other hand, Dutch or German regulations or recommendations (0.1 μg of pyrrolizidine alkaloids/100 g of food or 0.1 μg of pyrrolizidine alkaloids/day, respectively), based on the proven genotoxic carcinogenic effects of some pyrrolizidine alkaloids in experimental animals, are extended to honey, then MPCs of approximately 3.5 and 1 ppb, respectively, might be expected. Because honey is not the only dietary source of pyrrolizidine alkaloids (*15*), these estimated MPCs will in fact be lower because the total PA intake needs to be considered.

The UK Ministry of Agriculture, Fisheries and Food (MAFF) has concluded, on the basis of the apparent lack of effect of the pyrrolizidine alkaloids in one cup of comfrey tea per day, that there is no cause for concern for an *adult* consumer of honey containing 60 ppb pyrrolizidine alkaloids (*14*). Any recommendations, however, should take note that young children are more susceptible to pyrrolizidine alkaloid intoxication (*5*, *8*, *16*) than adults, and the MPCs should be calculated to protect this more vulnerable section of the population. In addition, the recommendations need to take into account the potential for synergistic or predisposing factors, such as high levels of copper, genetic predisposition, or low levels of dietary sulfur amino acids (*17*), to exacerbate pyrrolizidine alkaloid intoxication in subsets of the population.

Of the 63 preprocessed and 5 retail honeys analyzed in this study, 13 of the preprocessed honeys and 1 retail honey would not pass the MPC calculated using the FSANZ-suggested PTI of 1 (μg of pyrrolizidine alkaloids/kg of body mass)/day. However, if the MPCs were to be based, following the logic outlined above, on the German or Dutch PTIs, all those honeys in which pyrrolizidine alkaloids were detected (44 preprocessed honeys and 4 of the retail honeys) would be excluded.

The results confirm the presence of levels of pyrrolizidine alkaloids in honeys that may be cause for concern and demonstrate the potential for widespread dispersal of pyrrolizidine alkaloids into blended honeys and honeys attributed to non-pyrrolizidine alkaloid-producing floral sources. These observations support routine analyses of honeys in the marketplace in the same manner as for chemical contaminants and residues from antibiotics, pesticides, and herbicides.

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LITERATURE CITED

(1) Stegelmeier, B. L.; Edgar, J. A.; Colegate, S. M.; Gardner, D. R.; Schoch, T. K.; Coulombe, R. A.; Molyneux, R. J. Pyrrolizidine alkaloid plants, metabolism and toxicity. *J. Nat. Toxins* **1999**, *8*, 95−116.

(2) Huxtable, R. J. Human health implications of pyrrolizidine alkaloids and herbs containing them. In *Toxicants of Plant Origin, Volume 1 Alkaloids*; Cheeke, P. R., Ed.; CRC Press: Boca Raton, FL, 1989; pp 41−86.

(3) Prakash, A. S.; Pereira, T. N.; Reilly, P. E. B.; Seawright, A. A. Pyrrolizidine alkaloids in human diet. *Mutat. Res.* **1999**, *443*, 53−67.

(4) Chou, M. W.; Wang, Y.-P.; Yan, J.; Yang, Y.-C.; Beger, R. D.; Williams, L. D.; Doerge, D. R.; Fu, P. P. Riddelliine *N*-oxide is a phytochemical and mammalian metabolite with genotoxic

activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. *Toxicol. Lett.* **2003**, *145*, 239−247.
(5) German Federal Health Bureau. Bundesanzeiger, June 17, 4805. *Dtsch. Apoth. Ztg.* **1992**, *132*, 1406−1408.
(6) Edgar, J. A.; Roeder, E.; Molyneux, R. J. Honey from plants containing pyrrolizidine alkaloids: A potential threat to health. *J. Agric. Food Chem.* **2001**, *50*, 2719−2730.
(7) Food Standards Australia New Zealand. *Pyrrolizidine alkaloids in food: A toxicological review and risk assessment*; Technical Report Series No. 2; 2001. http://www.foodstandards.gov.au/mediareleasespublications/technicalreportseries/index.cfm (accessed June 20, 2004).
(8) Tanner, M. S. Role of copper in Indian childhood cirrhosis. *Am. J. Clin. Nutr.* **1998**, *67* (Suppl.), 1074S−1081S.
(9) Beales, K. A.; Colegate, S. M.; Edgar, J. A. Experiences with the trace analysis of pyrrolizidine alkaloids using GCMS and LCMS. In *Poisonous Plants and Related Toxins*; Acamovic, T., Stewart, C. S., Pennycott, T. W., Eds.; CABI Publishing: Wallingford, U.K., 2003; pp 453−458.
(10) Culvenor, C. C. J. The alkaloids of *Echium plantagineum* L. I. Echiumine and Echimidine. *Aust. J. Chem.* **1956**, *9*, 512−520.
(11) Culvenor, C. C. J.; Edgar, J. A.; Smith, L. W. 1981. Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. *J. Agric. Food Chem.* **1981**, *29*, 958−960.
(12) El-Shazly, A.; Sarg, T.; Ateya, A.; Abdel Aziz, S.; El-Dahmy, L.; Witte, L.; Wink, M. Pyrrolizidine alkaloids from *Echium setosum* and *Echium vulgare*. *J. Nat. Prod.* **1996**, *59*, 310−313.
(13) Australian Bureau of Statistics. *1995 National Nutrition Survey*; Canberra, Australia, 1995.
(14) MAFF Food Surveillance Information Sheet 52—Surveillance for pyrrolizidine alkaloids in honey. 1996. http://archive.food.gov.uk/maff/archive/food/infsheet/1995/no52/52honey.htm (accessed June 20, 2004).
(15) Colegate, S. M.; Edgar, J. A.; Stegelmeier, B. L. Plant-associated Toxins in the Human Food Supply. In *Environmental Toxicology: Current Developments*; Rose, J., Ed.; Gordon and Breach Science Publishers: Amsterdam, 1998; pp 317−344.
(16) International Programme on Chemical Safety. *Pyrrolizidine alkaloids*; Environmental Health Criteria 80; WHO: Geneva, 1988.
(17) Cheeke, P. R. Pyrrolizidine alkaloids. *Natural Toxicants in Feeds, Forages, and Poisonous Plants*, 2nd ed.; Interstate Publishers Inc.: Danville, IL, 1998; pp 338−352.

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