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Improved Method for Extraction and LC-MS Analysis of Pyrrolizidine Alkaloids and Their *N*-Oxides in Honey: Application to *Echium vulgare* Honeys

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A method for analyzing honey samples was developed that enabled the simultaneous detection and identification of pyrrolizidine alkaloids and their *N*-oxides. Honey samples were treated with methanol or dilute sulfuric acid and then centrifuged to remove insoluble material. Subsequent strong cation exchange, solid-phase extraction of the supernatant provided a fraction that was analyzed for the presence of pyrrolizidine alkaloids and their *N*-oxides using high-pressure liquid chromatography coupled to electrospray ionization mass spectrometry. The procedure was validated using extracts of *Echium plantagineum* and authenticated standards of pyrrolizidine alkaloids and their *N*-oxides from other plant sources. Of several variations of the solid-phase extraction method assessed in this study, the best combination for generic use involved the dilution of honey with 0.05 M sulfuric acid and the subsequent application of the centrifuged solution to solid-phase extraction columns at the rate of a maximum of 10 g of honey per solid-phase extraction column. The method was applied to the analysis of nine floral honeys, five of which were attributed by the apiarist to *Echium vulgare*. Seven of the honey samples were positive for pyrrolizidine alkaloids and *N*-oxides characteristic of *E. vulgare*.

KEYWORDS: Honey; pyrrolizidine alkaloid; pyrrolizidine-*N*-oxide; *Echium vulgare*; Boraginaceae; borage; Viper's bugloss; cation exchange; solid-phase extraction; food safety

INTRODUCTION

Hepatotoxic pyrrolizidine alkaloids and their *N*-oxides are found in over 6000 plant species and can be transferred to honey produced by bees foraging on pyrrolizidine alkaloid-producing plants (*1*−*3*). The potential adverse effects of human exposure to low levels of pyrrolizidine alkaloids have resulted in several regulatory authorities imposing or suggesting maximum allowable levels of pyrrolizidine alkaloids in foods and herbal medicines (*2*). The potential for fetuses to be especially vulnerable (*4*) and for synergistic exacerbation of the effects of pyrrolizidine alkaloids (*5*) indicates that all dietary sources of pyrrolizidine alkaloids and/or *N*-oxides should be assessed for possible contribution to overall pyrrolizidine alkaloid/*N*-oxides exposure.

Recent reports on the analysis of pyrrolizidine alkaloids and their *N*-oxides in *Echium plantagineum* (Paterson's curse or salvation Jane) (*6*) and in *Symphytum* spp. (comfrey) (*7*, *8*) reinforced the very significant contribution that the *N*-oxides make to the total pyrrolizidine alkaloid content and the advantages of using liquid chromatography−mass spectrometry to simultaneously analyze for the presence of the pyrrolizidine alkaloids and their *N*-oxides. The current paper is a development on previous research in which the *N*-oxides in honeys were reduced to their parent pyrrolizidine alkaloids as part of the extraction procedure prior to quantitative analysis using high-pressure liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS) (*3*). A different selection of solid-phase extraction (SPE) media, previously applied in the analysis of *Echium plantagineum* (*6*) and pollen from *Echium vulgare* (*9*), has now allowed development of a simplified method of extraction of pyrrolizidine alkaloids and their *N*-oxides from honey samples in preparation for their simultaneous analysis using high-pressure liquid chromatography combined with electrospray ionization mass spectrometry (HPLC-ESI-MS). The method was validated against standard pyrrolizidine alkaloids and their *N*-oxides and then applied to the analysis of five honeys attributed to *E. vulgare* and four honeys with no indication that pyrrolizidine alkaloid-producing plants were utilized.

MATERIALS AND METHODS

Chemicals and Solvents. All chemicals and solvents were of analytical reagent or HPLC grade purity. HPLC water was Milli-Q-

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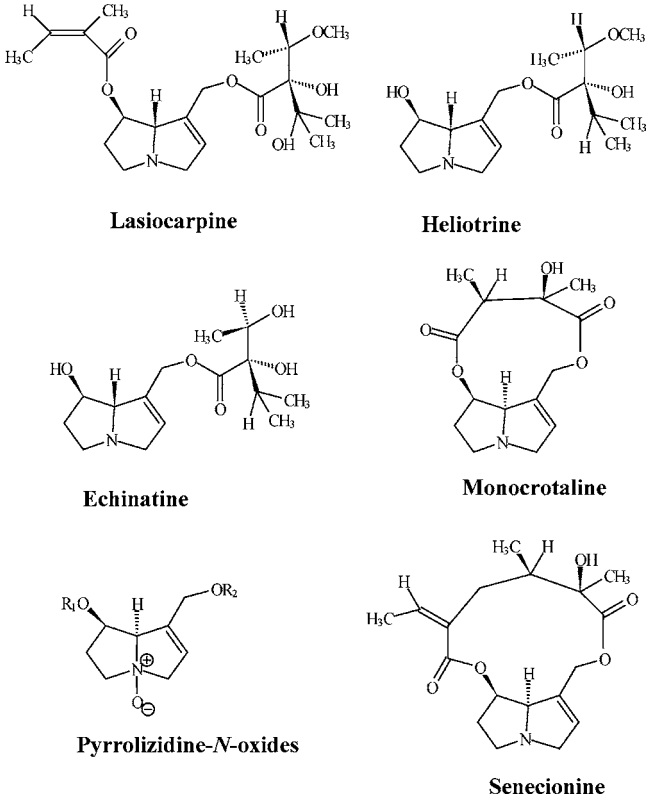

Figure 1. Structures of pyrrolizidine and pyrrolizidine-*N*-oxide alkaloid standards.

Table 1. Total Pyrrolizidine and Pyrrolizidine-*N*-oxide Alkaloid Content of Honeys

sample	honey name	wt of honey analyzed (g)	total pyrrolizidine/ pyrrolizidine-*N*-oxide alkaloid content (ppb)[a]
2002-1	borage crystalline	24.2	555 ± 13
2002-2	borage clarified	24.6	545 ± 3
2002-3	clover crystalline	24.5	none detected
2002-4	blue borage herbal	17.7	1498 ± 59
2002-5	New Zealand alpine borage	25.3	2850 ± 143
2002-6	clover blend	24.4	17 ± 1
2002-7	blue borage	26.7	697 ± 42
2002-8	rata	32.4	954 ± 23
2002-9	multifloral with manuka	27.1	none detected

[a] μg equiv of lasiocarpine/kg of honey for pyrrolizidine alkaloids plus μg equiv of lasiocarpine-*N*-oxide/kg of honey for *N*-oxides.

purified (Millipore), whereas sample preparation water was purified using reverse osmosis. Authenticated (NMR, MS) standards, isolated from plant sources, of heliotrine and its *N*-oxide, lasiocarpine and its *N*-oxide, monocrotaline, echinatine-*N*-oxide, and senecionine (**Figure 1**) were obtained from the collection of the CSIRO Plant Toxins Research Group. An extract of *E. plantagineum* was made by steeping the flowering tops, collected from a regularly infested paddock in the vicinity of Geelong, Australia, in methanol (*6*).

Extraction of Honey Samples. Various honeys (**Table 1**) were purchased from a commercial outlet in New Zealand. Duplicate samples (~20 g) of each honey were mixed with methanol or 0.05 M sulfuric acid (~30 mL or 1.5 × v/w).

Dilute Acid Extraction. The 0.05 M sulfuric acid/honey mixtures were centrifuged (>12000*g* for 15 min) to remove insoluble particulates. If the samples were applied to a single SPE column, the supernatants were kept warm (40 °C) by immersion in a water bath during application to the SPE columns. Otherwise, the supernatant, at room temperature, was divided into aliquots equivalent to <10 g of honey and each aliquot applied to a separate SPE column and recombined after processing.

Methanol Extraction. The methanol/honey mixtures were warmed at 40 °C for 10 min to achieve homogeneity. After cooling and keeping at room temperature for 2 h, the methanolic honey mixtures were centrifuged (>12000*g* for 15 min) to remove the resultant precipitate and other nonsoluble particulates. The supernatants were kept at room temperature during application to the SPE columns.

SPE of Supernatants. The supernatants from the diluted honey samples were applied to individual, Strata (500 mg/3 mL), strong cation exchange (SCX) SPE columns (Phenomenex, Torrance, CA) that had been conditioned with methanol (6 mL) followed by 0.05 M sulfuric acid (6 mL). Application flow rates were maintained up to 0.5−1 mL/min by the appropriate use of vacuum in an SPE column vacuum manifold. In the case of the methanol extraction of honey, the SCX SPE columns were prepared for sample application after the conditioning phase by a final washing with methanol (6 mL). After sample application, each loaded cartridge was washed with water (3 mL) and then methanol (3 mL) before the adsorbants were eluted with ammoniated methanol (methanol saturated at 0−4 °C with ammonia gas and subsequently stored at room temperature, 6 mL). The ammoniated methanolic eluate was immediately evaporated to dryness under a flow of nitrogen in a heating block at 30−40 °C. In the case of the 0.05 M sulfuric acid dilutions of the honey that were distributed to SPE columns at a rate of 10 g of honey per column, the ammoniated methanol eluates from each column were combined prior to evaporation. The residue from each sample was reconstituted into methanol (1 mL), containing senecionine (~5 μg) as an internal standard, to yield the analytical stock sample that was kept at −5 to −10 °C until analyzed. The samples were reconstituted in methanol, rather than a solvent more closely resembling the HPLC mobile phase, to facilitate further redox resin treatment and maintain their usefulness if other analytical techniques needed to be applied. However, tests showed no difference between analytical runs of the sample made up in aqueous acidic (0.1% formic acid for example) methanol or in methanol alone.

HPLC-ESI-MS Analysis. Samples were analyzed using a ThermoFinnigan Surveyor autosampler and liquid chromatography system coupled to a ThermoFinnigan LCQ ion trap mass spectrometer essentially as previously described (*6*, *9*). Briefly, aliquots (2 μL) of samples were injected onto a 150 × 2.1 mm i.d. Aqua C18 reverse phase column (Phenomenex) protected by a guard cartridge of equivalent adsorbent. The column was eluted (200 μL/min) with a decreasing linear gradient (93−7% mobile phase A over 15 min) of 0.1% formic acid in water (mobile phase A) into 0.1% formic acid in acetonitrile (mobile phase B). The injection of larger aliquots of the reconstituted sample, derived from smaller sample sizes of honey, is feasible but would require validation with respect to the chromatographic resolution of the analytes and the representative nature of the smaller sample from potentially heterogeneous bulk honey samples.

RESULTS AND DISCUSSION

There are several issues to consider when SCX SPE is applied to the analysis of honeys for the presence of pyrrolizidine alkaloids and *N*-oxides. To aid flow rates, the viscosity of the honey needs to be reduced with an appropriate pyrrolizidine alkaloid/*N*-oxide solvent. Blockages of the SPE columns are likely if all fine particulate matter (including pollen) and some honey components are not removed. Capture of pyrrolizidine alkaloids/*N*-oxides by the SPE column and their subsequent elution need to be efficient and reproducible.

Although most brands of SCX SPE columns evaluated were very efficient at capturing pyrrolizidine alkaloids/*N*-oxides, there were often problems in efficient release and recovery of the pyrrolizidine alkaloids/*N*-oxides. The use of the silica-based "Strata" SCX SPE columns provided efficient pyrrolizidine alkaloid/*N*-oxide capture from honey-based matrices and subsequent release into ammoniated methanol, which, being readily volatile, was easily removed under a stream of nitrogen.

Methanol, 0.05 M sulfuric acid, and mixtures thereof were all tried as diluents of the honey samples. Vigorous centrifuga-

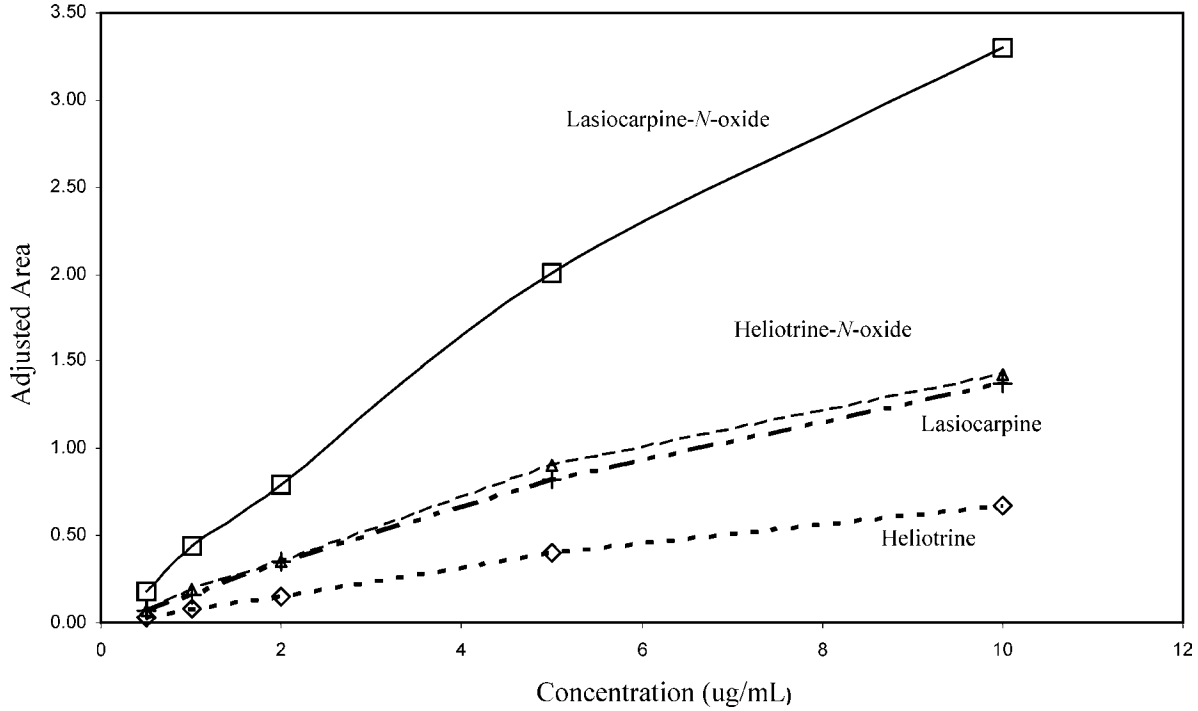

Figure 2. Typical calibration curves generated using heliotrine, heliotrine-*N*-oxide, lasiocarpine, and lasiocarpine-*N*-oxide (**Figure 1**) standards normalized against an internal standard of senecionine to give the "adjusted area". To quantitate pyrrolizidine alkaloids in samples, equations for the polynomial ($n = 2$) "best fit" curves were generated using Microsoft Excel.

tion of diluted samples was essential to help prevent subsequent SPE column blockages. Dilution of the honey with 0.05 M sulfuric acid reduced the viscosity of the sample but, even with centrifugation, was not reliable in terms of preventing blockage of columns unless the supernatant was kept at ~40 °C (immersion in a water bath) during the ensuing SPE process. However, dilution of the honey with methanol resulted in a significant precipitate (usually ~4−5% of the weight of the sample) and a clearer honey solution following centrifugation and did not lead to any column blockages with the samples tested. Dilution of honey samples with mixtures of methanol and 0.05 M sulfuric acid (100:0, 75:25, 50:50, 25:75, 0:100) resulted in a decrease in the amount of precipitate to ~2% of the weight of the honey at a methanol/sulfuric acid ratio of 50:50. At 25% methanol and less, there was no significant precipitate in the test honey.

The efficiency of the SCX SPE adsorbent to extract and retain the pyrrolizidine alkaloids/*N*-oxides from the diluted honey was assessed by applying a diluted honey sample, containing known amounts of added pyrrolizidine alkaloids and/or their *N*-oxides, to two conditioned SCX SPE columns connected in series. Once the sample had been applied, the columns were separated and each washed with water (3 mL), methanol (3 mL), and then ammoniated methanol (3 × 6 mL). The three separate washes with ammoniated methanol served to check the efficiency of elution of the pyrrolizidine alkaloids/*N*-oxides. Each resultant analytical sample was analyzed twice, using the HPLC-ESI-MS method, and the results were quantitated against standard calibration curves (**Figure 2**). In addition, every sample injection was separated by a methanol injection to check for any carry-over of analytes from one injection to the next. The inherent instability of the ESI-MS response made inclusion of an internal standard with every analytical sample essential for confident quantitation. For convenience in this study, the macrocyclic *Senecio* spp. pyrrolizidine alkaloid, senecionine (**Figure 1**), was chosen as the internal standard after initial qualitative analysis demonstrated the absence of naturally occurring senecionine. Because, following the methodology for this study, the internal standard is added immediately prior to HPLC-ESI-MS analysis rather than at the beginning of the extraction process, the choice of internal standard is restricted only by its availability and chromatographic properties. For generic use with honeys, an internal standard determined to be unlikely to be present in any honey would be preferable. To obviate the need to establish and use recovery efficiencies in calculating the final concentration of pyrrolizidine alkaloids in a sample, an internal standard with a similar extraction/recovery efficiency to the pyrrolizidine alkaloids could be added at the honey dilution stage of the analytical process.

The pyrrolizidine alkaloid class of secondary metabolites is structurally very diverse, and the ESI-MS response factors can be quite different (**Figure 2**). This observation has particular implications for quantitation when an authentic standard of the specific analyte is not available for generation of a standard calibration curve. Consequently, in this study the concentrations of isolated pyrrolizidine alkaloids are expressed in terms of equivalents of a representative pyrrolizidine alkaloid. From **Figure 2** it can be seen that the *N*-oxides are more responsive under the ESI-MS conditions employed in this study than their corresponding tertiary alkaloids. Also evident is the disparity of final concentrations that can be derived depending upon whether concentrations are expressed in terms of heliotrine equivalents or lasiocarpine-*N*-oxide equivalents, for example. The coefficients of determination (R^2) for the calibration curves shown in **Figure 2** are all >0.997.

Assessment of the recovery efficiency of the SCX SPE process involved quantitative comparison of unprocessed solutions of pyrrolizidine alkaloids or their *N*-oxides with solutions, including spiked honey supernatants, that had been subjected to the SCX SPE process both at room temperature and at 40 °C. Thus, aliquots (1 mL) of methanolic solutions (10 μg/mL) of authenticated samples of a range of pyrrolizidine alkaloids/

N-oxides from plant sources other than *Echium* spp. that is, heliotrine, heliotrine-*N*-oxide, lasiocarpine, lasiocarpine-*N*-oxide, monocrotaline, and echinatine-*N*-oxide (**Figure 1**), or a crude methanolic extract of *E. plantagineum* flowers was used to spike honey from various sources. Also spiked were volumes of methanol or 0.05 M sulfuric acid equal to the volumes used to dilute the honey samples, that is, ~30 mL. The lack of any pyrrolizidine alkaloids/*N*-oxides in the sample solvent that passed through the columns, in the water and methanol washes of the columns, or in the ammoniated methanol eluates from the second column demonstrated complete capture of the pyrrolizidine alkaloids/*N*-oxides from the aqueous acid, methanolic, and diluted honey solutions by the first column. Normalized to the internal standard response, the duplicate injections of samples for HPLC-ESI-MS analysis showed very good reproducibility between injections, with a maximum difference of ~6% observed. The blank methanol injections interspersed between the sample injections were shown to be clear of any pyrrolizidine alkaloid/*N*-oxides or indeed any carry-over, including higher concentration, non-pyrrolizidine alkaloid honey components, from the previous injection.

The results indicated similar efficiencies (85 ± SD 5% compared to the unprocessed samples) for the isolation of pyrrolizidine alkaloids/*N*-oxides from the SCX SPE-processed aqueous acid and methanol solutions. Similar recoveries from spiked honey samples diluted with 0.05 M sulfuric acid were consistently around the 70−80% level. By contrast, recoveries from spiked honey samples diluted with methanol were sample dependent, with some honey samples quite amenable to dilution with methanol and returning similar recoveries (73 ± SD 8%) to the dilute sulfuric acid or methanolic solutions of pyrrolizidine alkaloids/*N*-oxides alone. However, other honey samples when diluted with methanol returned only ~1−10% of the pyrrolizidine alkaloids/*N*-oxides added. Because the reason for this variation between the honeys is unknown, the amenability of honey samples to methanol treatment is unpredictable at this stage. This adverse effect of some honey samples on pyrrolizidine alkaloid/*N*-oxide recovery was demonstrated by adding 1, 5, 10, 15, or 20 g of an Australian-derived honey to a solution of *E. plantagineum*-derived pyrrolizidine alkaloids/*N*-oxides in methanol (30 mL). The addition of 1 or 5 g of honey had no adverse effect on recoveries, whereas addition of 10 g reduced recovery to ~50% and addition of 15 and 20 g reduced recovery to <10%.

Despite the more consistent recovery from honeys diluted with acid, the propensity for the aqueous acid-diluted honey sample to block the SPE column, although uncommon, was also unpredictable. However, two approaches were developed to manage or reduce the incidence of column blockage associated with the dilution of honey with 0.05 M sulfuric acid. First, in the event of a greatly reduced flow through the SPE column, warming of the column to ~40 °C with warm air re-established acceptable flow without any detrimental effect on pyrrolizidine alkaloid/*N*-oxide levels, as established by direct comparison of acid-diluted, spiked honey samples with dilute acid or methanolic solutions of the pyrrolizidine alkaloids/*N*-oxides, all processed at ~40 °C. Second, to reduce the incidence of column blockage, a 0.05 M sulfuric acid/honey supernatant was first divided into aliquots that represented ≤10 g of honey, that is, two aliquots for a 20 g sample of honey. These aliquots were then processed through separate SCX SPE columns, and the ammoniated methanol eluates from each were combined, evaporated to dryness, and reconstituted in methanol for analysis. Compared with the unprocessed extract of *E. plantagineum*, the two-column SCX SPE of an acid solution of *E. plantagineum* pyrrolizidine alkaloids/*N*-oxides returned a mean recovery efficiency of 95 ± SD 3.5%. Similar two-column SCX SPE of a spiked honey sample returned a mean recovery efficiency of 101 ± SD 6.2%.

To check for a compromise between ensuring a good recovery of the pyrrolizidine alkaloids/*N*-oxides (using 0.05 M sulfuric acid as the diluent) and minimizing the potential for SPE column blockage (using methanol as the diluent), an experiment was conducted in which a series of samples (20 g each) from an Australian-derived honey were spiked with *E. plantagineum* extract and diluted with methanol, 0.05 M sulfuric acid, or mixtures thereof (methanol/0.05 M sulfuric acid, 100:0, 95:5, 75:25, 50:50, 25:75, 5:95, 0:100). Diluents with 100−50% of methanol resulted in significant precipitates (4−2% of honey sample weight) and had a significant deleterious effect on the recovery of the pyrrolizidine alkaloids/*N*-oxides from the Australian honey tested, returning 10−15% recovery. At 25 and 5% of methanol in 0.05 M sulfuric acid, average recoveries of 80% were achieved but with no concomitant increase in precipitated insolubles compared to acid alone. This observation implies that reduced recovery may be linked to the production of significant precipitate. However, no pyrrolizidine alkaloids/*N*-oxides could be detected in the precipitate pellets formed during the centrifugation step and similar development of precipitates in other honeys (especially the pyrrolizidine alkaloid-positive honeys) (**Table 1**) did not significantly affect the recovery compared to recovery from spiked methanol or dilute acid solutions. The possibility still exists, however, for particular honeys to contain methanol-precipitable components capable of irreversibly interacting with the pyrrolizidine alkaloids/*N*-oxides.

In addition, the results from the recovery efficiency study showed that honey has an innate capacity to naturally reduce *N*-oxides to their parent tertiary bases. This capacity was observed when pyrrolizidine alkaloid-free honey samples were spiked with standard *N*-oxides and subsequent levels of between 5 and 14% of the respective parent pyrrolizidine alkaloids were observed. This property was also observed when pyrrolizidine alkaloid-free honey was spiked with an extract of *E. plantagineum*, which comprised in excess of 95% *N*-oxides (*6*), with a resultant increase in the respective tertiary pyrrolizidine alkaloids (**Figure 3**). Whether the reduction capacity is related to the time of exposure of the *N*-oxides to the honey or is a limited function of the volume of honey remains undefined.

The single SCX SPE column, methanol-based, and 0.05 M sulfuric acid-based extraction methodologies were applied to nine samples of honey purchased in New Zealand (**Table 1**). For this study, an estimate of 73% recovery efficiency was generically used, for both the acid- and methanol-based extractions, to adjust the levels of pyrrolizidine alkaloids/*N*-oxides isolated from the honey samples. The honeys ascribed to borage, or mixes thereof, are attributable to *E. vulgare* (Viper's bugloss) for which borage is a colloquial name in New Zealand. This is quite different from the borage, *Borago officinalis*, more commonly referred to in other parts of the world. *E. vulgare* was introduced from Europe into New Zealand and is common to abundant in drier areas of the South Island and in some drier areas of the North Island (**Figure 4**) (*10*). It provides monofloral forage opportunities for bees, as does *E. plantagineum* in Australia. Each honey sample was treated with methanol or 0.05 M sulfuric acid and centrifuged, and the supernatant was subjected to SCX SPE and subsequent HPLC-ESI-MS analysis. The analytical results showed that these New Zealand honeys,

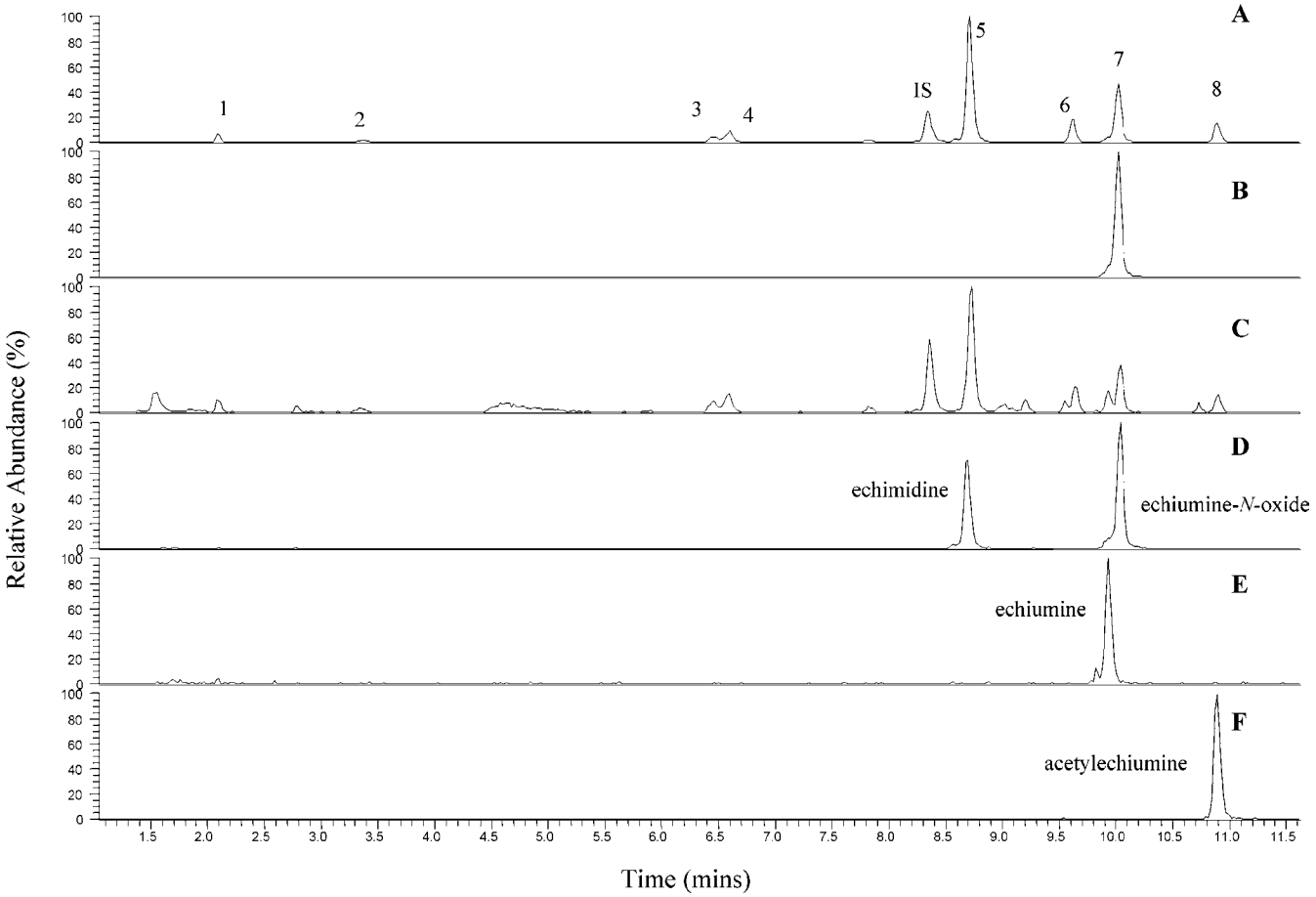

Figure 3. Natural reduction of pyrrolizidine alkaloid *N*-oxides by honey: (**A**) pyrrolizidine alkaloid profile of an extract of *E. plantagineum*, showing the presence of the *N*-oxides of leptanthine and echimiplatine (peaks 1 and 2, *m*/*z* 332), 7-*O*-acetyllycopsamine and 7-*O*-acetylintermedine (peaks 3 and 4, *m*/*z* 316), echimidine (peak 5, *m*/*z* 414), 3′-*O*-acetylechimidine (peak 6, *m*/*z* 456), echiumine (peak 7, *m*/*z* 398), and 3′-*O*-acetylechiumine (peak 8, *m*/*z* 440) (*6*) with the internal standard, senecionine (peak IS, *m*/*z* 336); (**C**) pyrrolizidine alkaloid profile of an extract of *E. plantagineum* exposed to honey, showing development of peaks attributed to the parent tertiary pyrrolizidine alkaloids eluting slightly earlier than their respective *N*-oxides; (**B**, **D**) reconstructed ion chromatograms displaying *m*/*z* 398 for the base ion chromatograms **A** and **C**, respectively, showing the formation of echimidine from its *N*-oxide; (**E**, **F**) reconstructed ion chromatograms, from chromatogram **C**, displaying *m*/*z* 382 and 424 showing the formation of echiumine and acetylechiumine from their *N*-oxides.

Figure 4. Vista of *E. vulgare* in New Zealand showing the monofloral opportunity for foraging bees. (Photo courtesy of Barrie Wills.)

in contrast to some Australian-derived honeys, were all amenable to dilution with methanol, returning slightly higher pyrrolizidine alkaloid/*N*-oxide contents than from the same samples diluted with the 0.05 M sulfuric acid.

Of the nine honey samples quantitatively analyzed in this study, only two did not have any detectable (<1 ppb) pyrrolizidine alkaloids/*N*-oxides present (**Table 1**). The pyrrolizidine alkaloids and their *N*-oxides were identified in the samples through combined assessment of their HPLC retention times, their expected molecular ion adducts ($[M + H]^+$), and their MS/MS spectra (**Table 2**), either as a direct comparison with the MS/MS spectra of standard alkaloids or by observation of fragments characteristic of pyrrolizidine alkaloid structure (*6*). The pyrrolizidine alkaloids/*N*-oxides observed were characteristic of *E. vulgare* (*3*, *9*) and included echivulgarine, vulgarine, 7-*O*-acetylvulgarine, echimidine, 3′-*O*-acetylechimidine, uplandicine, echiuplatine, echimiplatine, and leptanthine, as the tertiary pyrrolizidine alkaloid bases or as their *N*-oxides (**Figure 5**). The structures of some of these alkaloids (shown with an asterisk in **Figure 5**) remain tentative, based on mass spectrometric analysis and biogenetic considerations (*6*, *9*). The major pyrrolizidine alkaloids/*N*-oxides were easily observed in the total base ion chromatograms of some of the honey extracts (**Figure 6**), and the minor alkaloids were observed via display of their molecular ion adducts ($[M + H]^+$) in reconstructed ion chromatograms (RICs) (**Figure 6**). Although many of the pyrrolizidine alkaloids/*N*-oxides attributed to *E. vulgare* are the

Table 2. MS/MS Data for Pyrrolizidine and Pyrrolizidine-*N*-oxide Alkaloids Isolated from Honey Samples

alkaloid	molecular on adduct $[M + H]^+$	MS/MS fragments *m/z* (% abundance)
echivulgarine-*N*-oxide	496	478 (20), 396 (25), 352 (10), 338 (100), 332 (2), 254 (10), 220 (5)
echivulgarine	480	462 (43), 418 (7), 398 (14), 380 (100), 336 (28), 322 (63), 280 (3), 240 (8), 220 (77)
7-*O*-acetylvulgarine-*N*-oxide	456	438 (30), 356 (40), 338 (15), 298 (100), 214 (30), 180 (10)
7-*O*-acetylvulgarine	440	422 (34), 340 (53), 296 (22), 282 (50), 180 (100)
acetylechimidine-*N*-oxide	456	438 (18), 396 (30), 352 (12), 338 (100), 254 (8)
acetylechimidine	440	422 (240, 380 (67), 336 (22), 322 (100), 220 (53)
vulgarine-*N*-oxide	414	396 (15), 338 (2), 336 (2), 314 (35), 270 (12), 256 (100), 240 (2), 172 (18), 138 (3)
echimidine-*N*-oxide	414	396 (100), 356 (10), 352 (80), 338 (20), 254 (25), 220 (1)
vulgarine	398	380 (12), 338 (2), 336 (5), 254 (10), 240 (62), 238 (2), 138 (100), 120 (30)
echimidine	398	380 (28), 338 (16), 336 (100), 296 (10), 254 (8), 220 (48), 190 (5), 120 (84)
echiuplatine-*N*-oxide	398	380 (18), 338 (26), 336 (100), 296 (8), 254 (6), 120 (1)
echiuplatine	382	320 (12), 220 (34), 120 (100)
uplandicine-*N*-oxide	374	356 (100), 330 (1), 312 (68), 298 (12), 272 (1), 270 (3), 214 (24)
leptanthine-*N*-oxide	332	314 (100), 270 (70), 256 (28), 228 (8), 172 (35)
echimiplatine-*N*-oxide	332	314 (100), 270 (48), 256 (20), 228 (10), 172 (30)

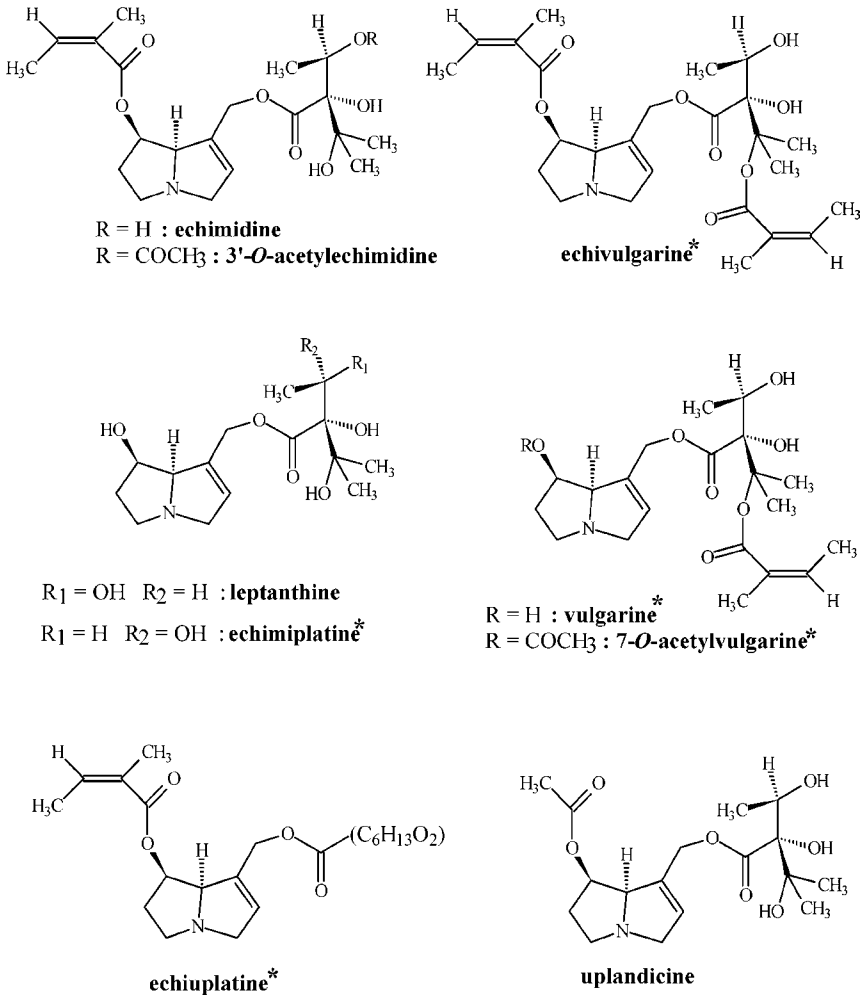

Figure 5. Structures of pyrrolizidine alkaloids (and their *N*-oxides) identified in the honey samples (see **Tables 3** and **4**). Structures labeled with an asterisk (*) are tentatively based upon mass spectrometry evidence and biogenetic considerations (*6*, *9*).

same as those found in *E. plantagineum* (*6*) (**Figure 3**), the presence of echivulgarine, vulgarine, and acetylvulgarine (and their *N*-oxides) in the *E. vulgare* profile and of echiumine (and its *N*-oxide) in the profile of *E. plantagineum* differentiates the two plant sources.

For quantitation, the RIC area response of each identified pyrrolizidine alkaloid or *N*-oxide was normalized against the area response of the internal standard, senecionine, and converted to a concentration (parts per billion) in microgram equivalents of lasiocarpine per kilogram of honey (for the pyrrolizidine alkaloids detected) plus microgram equivalents of lasiocarpine-*N*-oxide per kilogram of honey (for the *N*-oxides detected). Lasiocarpine and its *N*-oxide were chosen as the calibration standards for this study because of their similarity in structure, and presumably ESI-MS response factors, to the major pyrrolizidine alkaloids and *N*-oxides, respectively, isolated from the honeys. Thus, a more accurate estimation of concentrations in the honeys was expected. The highest level thus detected was in excess of 2500 ppb in the alpine borage sample 2002-5 for which the contributions of the individual pyrrolizidine

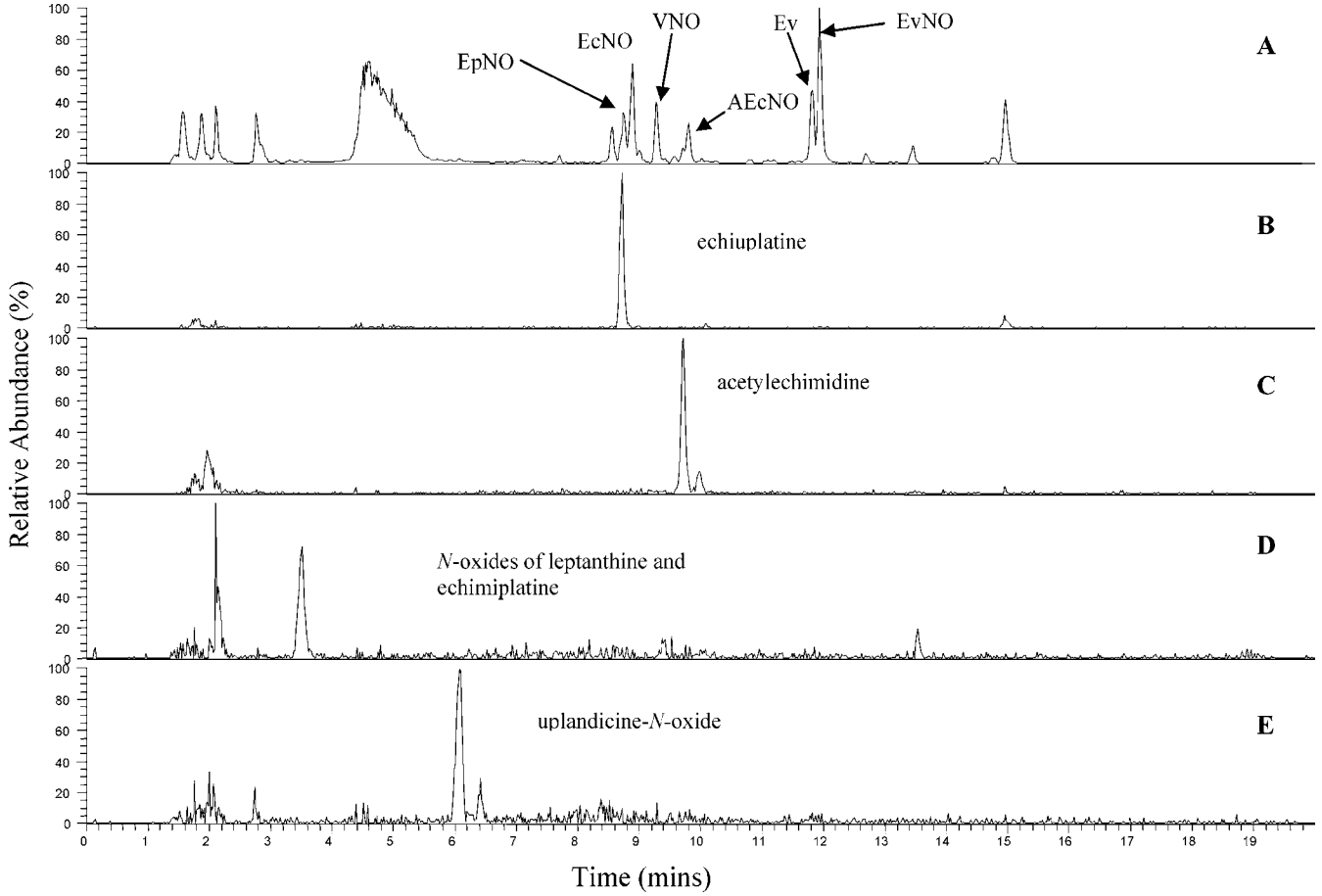

Figure 6. HPLC-ESI-MS analysis of alpine borage honey (sample 2002-5): (**A**) base ion (*m*/*z* 200–500) chromatogram showing the major pyrrolizidine alkaloids/*N*-oxides echivulgarine-*N*-oxide (EvNO, *m*/*z* 496), echivulgarine (Ev, *m*/*z* 480), acetylechimidine-*N*-oxide (AEcNO, *m*/*z* 456), vulgarine-*N*-oxide (VNO, *m*/*z* 414), echimidine-*N*-oxide (EcNO, *m*/*z*, 414), and echiuplatine-*N*-oxide (EpNO, *m*/*z* 398); (**B**, **C**, **D**, **E**) reconstructed ion chromatograms displaying *m*/*z* 382, 440, 332, and 374 for echiuplatine, acetylechimidine, leptanthine-*N*-oxide, and echimiplatine-*N*-oxide, and uplandicine-*N*-oxide, respectively.

Table 3. Typical Profile of Pyrrolizidine Alkaloids and Their *N*-Oxides Identified in a Sample of Alpine Borage Honey (Sample 2002-5)

retention time (min)	molecular ion adduct ($[M + H]^+$) (*m*/*z*)	identity	concn (ppb)[a]
11.94	496	echivulgarine-*N*-oxide	558 ± 40
11.81	480	echivulgarine	879 ± 35
10.12	456	7-*O*-acetylvulgarine-*N*-oxide	trace
9.97	440	7-*O*-acetylvulgarine	trace
9.80	456	acetylechimidine-*N*-oxide	96 ± 4
9.72	440	acetylechimidine	104 ± 7
9.27	414	vulgarine-*N*-oxide	166 ± 3
9.01	398	vulgarine	84 ± 7
8.90	414	echimidine-*N*-oxide	291 ± 9
8.84	398	echimidine	299 ± 25
8.73	398	echiuplatine-*N*-oxide	140 ± 10
8.72	382	echiuplatine	194 ± 13
6.07	374	uplandicine-*N*-oxide	15 ± 1
3.51	332	leptanthine-*N*-oxide[b]	14 ± 1
2.10	332	echimiplatine-*N*-oxide[b]	10 ± 1

[a] μg equiv of lasiocarpine/kg of honey for pyrrolizidine alkaloids or μg equiv of lasiocarpine-*N*-oxide/kg of honey for *N*-oxides. [b] Assignments might be reversed.

Table 4. Individual Pyrrolizidine Alkaloid/*N*-Oxide Concentrations in Those Honey Samples in Which Pyrrolizidine Alkaloids, or Their *N*-Oxides, Were Detected

	concn (ppb)[a] in honey sample						
alkaloid	2002-1	2002-2	2002-4	2002-5	2002-6	2002-7	2002-8
echivulgarine-*N*-oxide	nd[b]	nd	113	558	nd	nd	288
echivulgarine	293	300	584	879	8	426	172
acetylechimidine-*N*-oxide	nd	nd	20	96	nd	nd	46
acetylechimidine	nd	nd	33	104	nd	nd	24
vulgarine-*N*-oxide	nd	nd	99	166	nd	nd	69
vulgarine	34	44	59	84	nd	46	20
echimidine-*N*-oxide	nd	nd	243	291	nd	nd	154
echimidine	152	132	173	299	9	127	61
echiuplatine-*N*-oxide	nd	nd	36	140	nd	nd	66
echiuplatine	76	69	93	194	nd	98	37
uplandicine-*N*-oxide	nd	nd	18	15	nd	nd	5
leptanthine-*N*-oxide[c]	nd	nd	12	14	nd	nd	6
echimiplatine-*N*-oxide[c]	nd	nd	15	10	nd	nd	6

[a] μg equiv of lasiocarpine for pyrrolizidine alkaloids and μg equiv of lasiocarpine-*N*-oxide for *N*-oxides. [b] None detected. [c] Assignments might be reversed.

alkaloids/*N*-oxides, and the associated differences between the means and their two component analytical results, are shown in **Table 3**. The mean contributions for each of the pyrrolizidine alkaloids/*N*-oxides to the totals in all of the pyrrolizidine alkaloid-positive honeys are shown in **Table 4**. The errors in the means shown in **Table 3** for honey sample 2002-5 were typical of those observed in the other samples shown in **Table 4** (data not shown). In addition, the alpine Borage sample 2002-5 was presented as "honey in the comb". Therefore, when the product was sampled, it was inevitable that some comb wax added to the weight of the sample. Taking this into consideration would result in a higher level of pyrrolizidine alkaloids/*N*-oxides than has been reported herein.

Of the seven samples that tested positive for pyrrolizidine alkaloids or *N*-oxides, no *N*-oxides were observed in four (2002-1, 2002-2, 2002-6, and 2002-7), with echivulgarine as the major

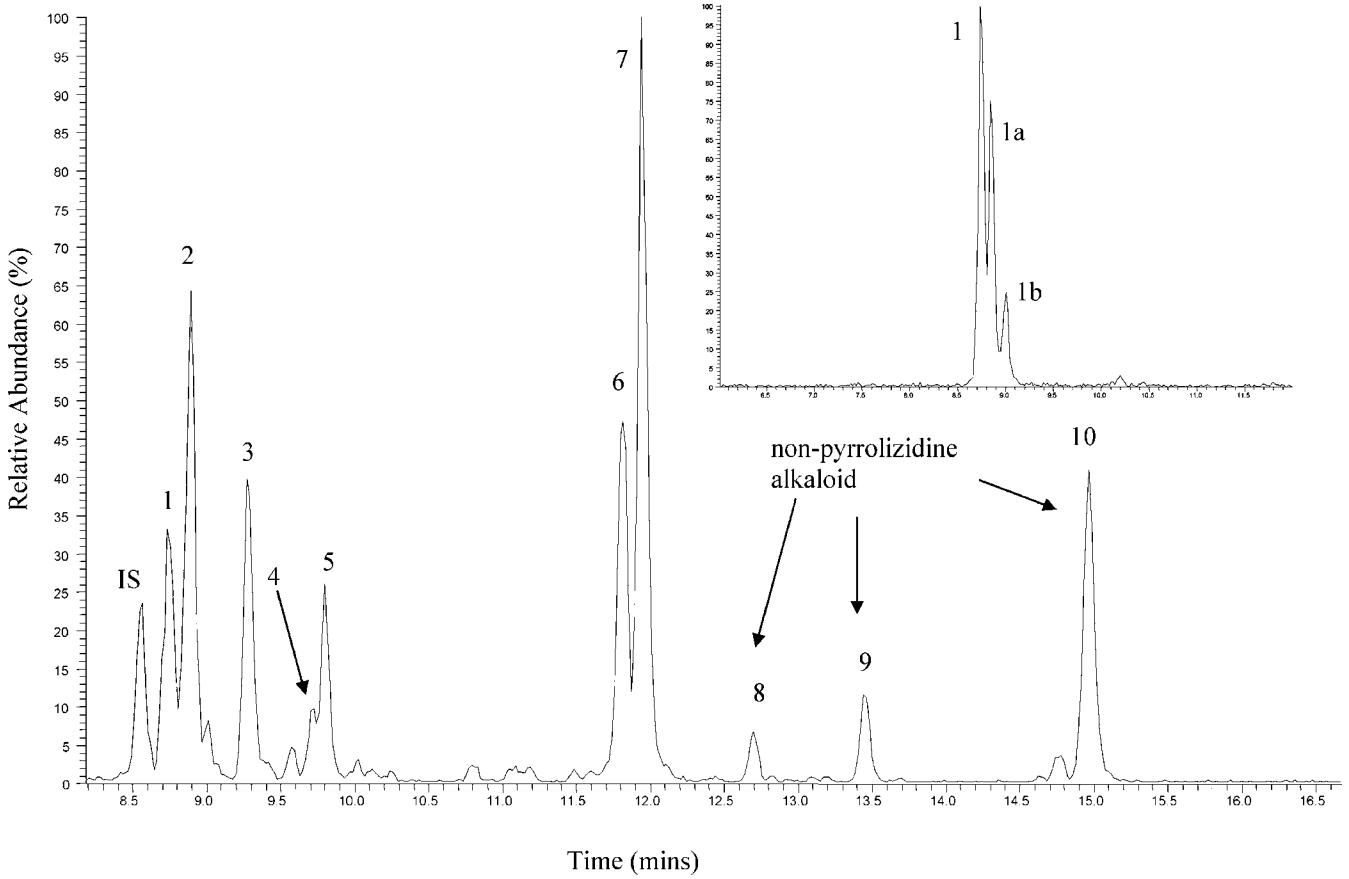

Figure 7. Expanded (t_R 8.2–15.8 min) HPLC-ESI-MS base ion (*m*/*z* 330–500) chromatogram for New Zealand alpine borage honey (sample 2002-5). Peaks: IS, internal standard, senecionine; 1, echiuplatine-*N*-oxide; 2, echimidine-*N*-oxide; 3, vulgarine-*N*-oxide; 4, acetylechimidine; 5, acetylechimidine-*N*-oxide; 6, echivulgarine; 7, echivulgarine-*N*-oxide. There is no mass spectrometric evidence for pyrrolizidine alkaloid character of peaks 8–10. The inset shows a reconstructed ion chromatogram displaying *m*/*z* 397.5–398.5 demonstrating three separate compounds with this molecular weight.

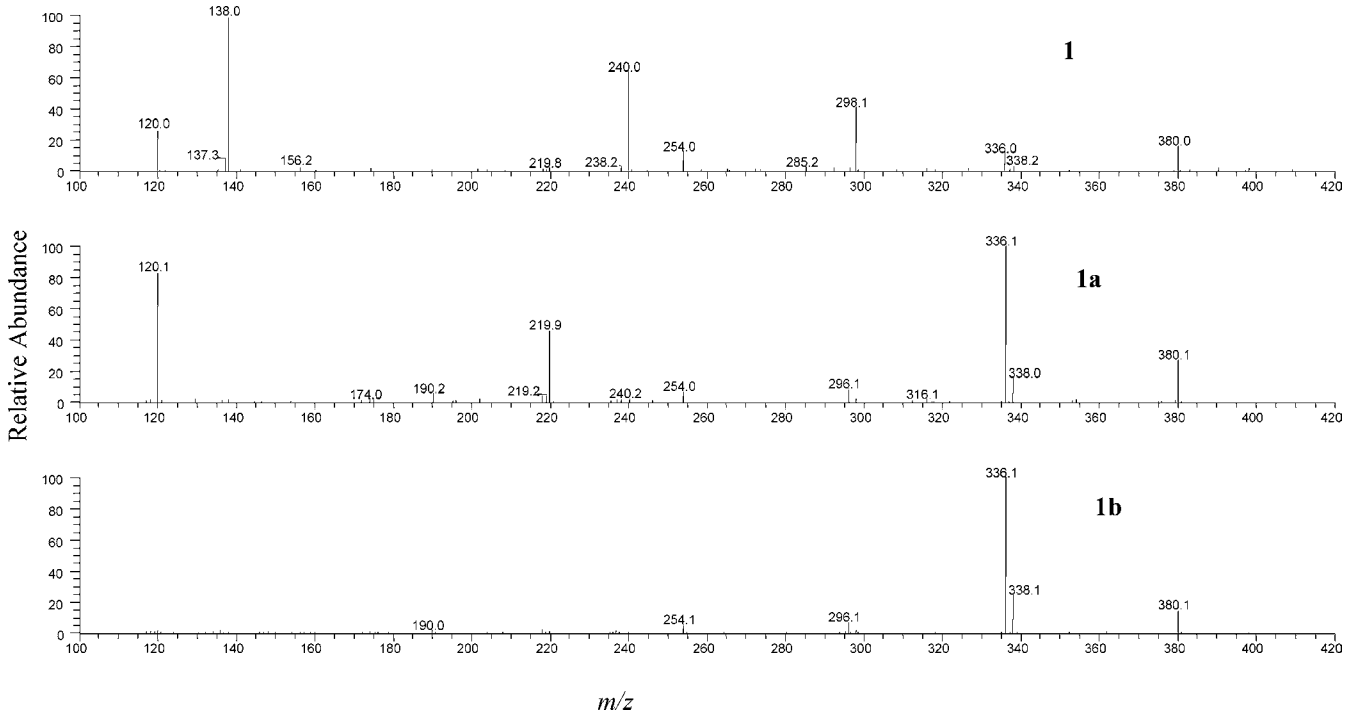

Figure 8. MS/MS spectra of *m*/*z* 398 for peaks 1 (echiuplatine-*N*-oxide), 1a (echimidine), and 1b (vulgarine) (**Figure 7**).

pyrrolizidine alkaloid. In the three other pyrrolizidine alkaloid-positive samples (2002-4, 2002-5, and 2002-8), a full suite of pyrrolizidine alkaloids/*N*-oxides was observed with echivulgarine, echimidine, and their *N*-oxides predominating. Isolation and purification of these component pyrrolizidine alkaloids/*N*-oxides at a later date will enable determination of their ESI-MS

response factors relative to lasiocarpine and lasiocarpine-*N*-oxide and appropriate adjustments to be made to the concentration estimates reported herein (**Tables 1**, **3**, and **4**).

All identified peaks were confirmed using MS/MS experiments, and the value of such data to the identification of peaks is exemplified in the case of vulgarine, echimidine, and echiuplatine-*N*-oxide, all of which have the same value for their molecular ion adduct and all elute fairly close together. An expanded view (**Figure 7**) of the base ion (*m*/*z* 330−500) chromatogram for the borage honey (2002-5), which returned the highest levels of pyrrolizidine alkaloids/*N*-oxides, shows the assignment of peaks to specific pyrrolizidine alkaloids and *N*-oxides. An RIC displaying *m*/*z* 398 showed the apparent presence of three closely eluted peaks (peaks 1, 1a, and 1b, inset of **Figure 7**). The individuality of these peaks, rather than them resulting from some undefined separation artifact, was confirmed via their MS/MS spectra (**Figure 8**; **Table 2**). The confident identification of these peaks, using MS/MS, became more important at lower levels of pyrrolizidine alkaloid content when only pyrrolizidine alkaloids and not *N*-oxides were observed. In these cases, only two peaks with molecular ion adducts at *m*/*z* 398 were observed, and the MS/MS experiments were able to clearly demonstrate their identity as echimidine and vulgarine rather than including the *N*-oxide of echiuplatine.

Of the processing methods assessed in this study, the multiple-column SCX SPE of acidified honey solutions, such that each column was used to process ≤10 g of the larger (>20 g) honey sample, is the most generically reliable. The single-column SCX SPE of methanolic solutions of 20 g samples of honey offers the advantage of flow reliability over similar processing of acid solutions of 20 g of honey samples, but the recovery efficiency can be very poor with some honeys. However, both single-column SCX SPE protocols were used successfully in this study.

Within the limitations of quantitation as lasiocarpine/lasiocarpine-*N*-oxide equivalents, it is qualitatively clear that significant amounts of pyrrolizidine alkaloids/*N*-oxides are present in some of these sample honeys, whether they are directly attributed to *E. vulgare* (borage) or not, such as the clover blend and the rata honey (attributed to a New Zealand native tree, *Metrosideros umbellata*). The potential for very high levels in honeys presented "in the comb" (e.g., sample 2002-5) (**Table 1**) would also require consideration based on these results. These observations, in conjunction with the known health concerns with pyrrolizidine alkaloids, support a need for a more widespread survey of honeys with accurate quantitation targeted at the individual pyrrolizidine alkaloids and their *N*-oxides.

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