Pyrrolizidine Alkaloids from *Echium vulgare* in Honey Originate Primarily from Floral Nectar

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ABSTRACT: Pyrrolizidine alkaloids (PAs) in honey can be a potential human health risk. So far, it has remained unclear whether PAs in honey originate from pollen or floral nectar. We obtained honey, nectar, and plant pollen from two observation sites where *Echium vulgare* L. was naturally abundant. The PA concentration of honey was determined by targeted analysis using a high pressure liquid chromatography–mass spectrometry system (HPLC-MS/MS), allowing the quantification of six different PAs and PA-N-oxides present in *E. vulgare*. *Echium*-type PAs were detected up to 0.153 μg/g in honey. Nectar and plant pollen were analyzed by nontargeted analysis using ultrahigh pressure liquid chromatography–high resolution-mass spectrometry (UHPLC-HR-MS), allowing the detection of 10 alkaloids in small size samples. *Echium*-type PAs were detected between 0.3–95.1 μg/g in nectar and 500–35000 μg/g in plant pollen. The PA composition in nectar and plant pollen was compared to the composition in honey. Echimidine (+N-oxide) was the main alkaloid detected in honey and nectar samples, while echivulgarine (+N-oxide) was the main PA found in plant pollen. These results suggest that nectar contributes more significantly to PA contamination in honey than plant pollen.

KEYWORDS: pyrrolizidine alkaloids, *Echium vulgare*, honey, nectar, plant pollen, high pressure liquid chromatography–mass spectrometry (HPLC-MS/MS), ultrahigh pressure liquid chromatography–high resolution-mass spectrometry (UHPLC-HR-MS)

1. INTRODUCTION

Pyrrolizidine alkaloids (PAs) are toxic compounds produced by plants as a chemical defense against herbivores. Many unrelated plant species produce PAs, and some of them are particularly abundant in European agro-ecosystems. These PA-containing plants mainly belong to the Asteraceae (Senecioneae and Eupatoriaceae tribes), Boraginaceae (all genera), and Fabaceae (*genus Crotalaria*) families. PAs may get into the food chain when food products are either contaminated with or derived from PA-containing plant tissues. PAs can occur as N-oxides or as free-base/tertiary forms. These two forms are both hepatotoxic and genotoxic. In plants, the N-oxides are found in higher concentrations than the corresponding free-bases (tertiary PAs). Acute poisoning or chronic exposure to PAs mostly affects liver function, since PAs are activated by nucelophilic compounds through the liver’s detoxification enzymes. The chronic intake of low PA-levels in food can lead to liver cirrhosis and cancer. Legal limits for PAs in food have not yet been established in the European Union or in Switzerland. However, the German Federal Institute for Risk Assessment (BfR) recommends an intake of not more than 0.007 μg of 1.2-unsaturated PAs per day per kg bodyweight.

Honey is one of the best studied food products with respect to PA contamination. When honeybees collect nectar and plant pollen from PA-containing plants, PAs are transferred into bee products such as honey or bee-collected pollen. PAs have been detected in honey samples from various geographical and botanical origins. PA concentrations of up to 2 orders of magnitude over the limits recommended by the BfR have been reported in monofloral honeys from *Echium vulgare* or *E. plantagineum* and from *Senecio jacobaea*. Honey is mostly composed of concentrated nectar and contains only traces of pollen. Therefore, PAs contained in nectar constitute an important potential source of PAs in honeys. However, the concentration of secondary compounds may be considerably higher in plant pollen than in nectar, and some pollen types contain particularly high amounts of PAs. Consequently, it remains unclear whether the PA content in nectar is high enough to substantially contaminate honey and, more generally, whether the PAs in honey predominantly originate from pollen or nectar.

Prior research has suggested pollen as the major source of PA contamination in honey. Contamination of honey could be caused by the liberation of PAs from pollen. Lastly, experiments with plant pollen from *Senecio vernalis* added to
PA-free honey have suggested that contamination of honey may occur through diffusion of PAs from pollen into honey.\textsuperscript{20} Unraveling the entry mechanism by which PAs contaminate honey is important for reducing risks associated with PA-containing bee products.

Two approaches can be used to examine the pathway from the different plant tissues into bee products. First, quantitative analyses of nectar, pollen, and honey may help determine which plant part is the main contributor to the total PAs found in honey. Second, differences in the PA composition (relative abundance of different PAs) found in nectar can be compared to that found in pollen. This information can be used to determine whether the PA composition in honey more closely matches that of nectar or pollen. In the present study, we performed qualitative and quantitative analysis of the different alkaloids found in \textit{Echium} nectar and pollen.

We selected \textit{Echium vulgare} as a model to study the pathway by which PAs are transferred into honey. This plant species is the only species of the genus \textit{Echium vulgare} regularly found in Switzerland. It is widely distributed in Europe and has been previously described as a major source of PA contamination of European honeys.\textsuperscript{19,21} We chose two observation sites where \textit{E. vulgare} was blooming during the bee season. These sites were located in two different climatic regions, one located to the north and the other to the south of the Alps.

### 2. MATERIALS AND METHODS

#### Chemical Reagents.

The echimidine used in this study was obtained from Phytoolab (Vestenbergsgreuth, Germany), while the heliotrine was from Latoxan (Valence, France). For plant extraction, milli-Q water was used. Formic acid and glass beads (Ø 2 mm) were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade methanol was purchased from Merck (Dietikon, Switzerland). Cyclohexane (>98% purity), sulfuric acid, and ammonia were purchased from Merck Chemicals (Darmstadt, Germany). The solvents and additives used for LC-MS were water, acetonitrile, and ULC-MS grade formic acid acquired from Biosolve (Valkenswaard, Netherlands).

#### Observation Sites.

We selected two observation sites where \textit{Echium} plants were abundant around bee colonies. The first observation site was located north of the Alps near Basel, close to the border between Switzerland and France (hereafter Basel). The other observation site was located close to Italy, along the southern flank of the Alps in the Verzasca valley (hereafter Verzasca). At the Verzasca site, two beekeepers participated in the project (hereafter Verzasca 1 and Verzasca 2). The aerial distance between these two apiaries was approximately 400 m.

**Honey and Plant Sample Collection. Honey.** In total, four samples of honey were included from Basel and six from Verzasca. In Basel, the honeys were harvested on 8 June and 27 July 2012, as well as on 29 June and 8 August 2013. In Verzasca 1, the honeys were harvested on 1 August 2012, 2 August 2013, and 1 August 2014, while in Verzasca 2 they were harvested on 30 July 2012, 2 August 2013, and 1 August 2014. Eight additional honey samples were obtained from apiaries in diverse regions of Switzerland. All of the additional honey samples were produced between 2009 and 2011.

**Plant Material.** In Basel, \textit{E. vulgare} was in blossom during June and July, while in the Verzasca valley \textit{E. vulgare} was in blossom from June until August. Samples of nectar and plant pollen from \textit{E. vulgare} were collected at the two observation sites. Samples were only collected under dry weather conditions to avoid wash-out of plant pollen from the anthers and dilution of nectar by the rain. In Basel, the samples were collected on 18 June and 4 July 2013 and on 19 June and 27 June 2014. In Verzasca, the samples were collected on 29 June and 17 July 2013 and on 6 July and 18 July 2014. In total, 20 nectar samples from Basel (\( n = 10 \) in 2013 and \( n = 10 \) in 2014), 16 nectar samples from Verzasca (\( n = 7 \) in 2013 and \( n = 9 \) in 2014), 14 plant pollen samples from Basel (\( n = 5 \) in 2013 and \( n = 9 \) in 2014), and 13 plant pollen samples from Verzasca (\( n = 4 \) in 2013 and \( n = 9 \) in 2014) were collected. On the day before any given sample collection, plants were tightly bagged with a fine mesh, and a layer of insect glue was spread around the lower part of the stem in order to prevent insect visits. Nectar was collected using a Pasteur pipet previously elongated to a capillary on a flame. The pipet was directly placed into the corolla, carefully avoiding any disruption of floral tissues. In 2013, pollen from anthers of \textit{Echium} flowers (plant pollen) was collected with metal forceps. Plant pollen was carefully removed from the surface of the anthers in order to prevent contamination of the pollen with other flower parts, especially the anthers. This procedure yielded low amounts of plant pollen. In order to facilitate collection and avoid contamination with other plant parts, plant pollen was collected in 2014 by immersing the stamens into cyclohexane.\textsuperscript{17} The cyclohexane was subsequently evaporated. For comparison, two plant pollen samples were collected from the same plant using both methods (forceps and cyclohexane). Since the two samples gave comparable results (data not shown), we concluded that the cyclohexane did not wash out PAs from the pollen, and hence that both collection methods would yield similar results. All samples were kept on dry ice during collection and subsequently stored at –80 °C until extraction.

#### Sample Preparation of Honey for Quantification of PAs with LC-MS/MS. Honey samples were prepared as described by Dübecke et al.\textsuperscript{15} Since PA-N-oxides are polar organic compounds with basic characteristics, they are soluble in polar organic solvents or in mixtures of solvents and acidified water. Ten grams of honey, together with 100 ng of heliotrine as internal standard and 30 mL of 0.05 M sulfuric acid were vigorously shaken for 20 min. Samples were then filtered overnight using a 2 mm mesh to remove particles that could block the solid-phase extraction. Clean-up was conducted using SPE SCX Cartridges (Varian) washed previously with methanol and conditioned with 9 mL of 0.05 M sulfuric acid. Samples were loaded onto the column, washed with 9 mL of deionized water, eluted into a glass vial using ammoniated methanol,\textsuperscript{18} and dried at 40 °C in an ambient air stream. Samples were then reconstituted in 1 mL of deionized water, shaken vigorously, and filtered into a 2 mL glass vial using a 0.45 μm syringe filter.

The PA concentration was determined by targeted analysis using a HPLC-MS/MS system as described in Dübecke et al.,\textsuperscript{15} allowing the detection of six different PAs or PA-N-oxides (echimidine, echimidine-N-oxide, acetylchelmidine, acetylchelmidine-N-oxide, echivulgarine, and echivulgarine-N-oxide) commonly found in \textit{E. vulgare}.\textsuperscript{17} The total PA concentration was calculated as the sum of the six different PAs. LC-MS/MS analysis was performed using an HTC PAL autosampler of CTC Analytics AG, a Shimadzu LC-system with a Thermo Hypersil Gold C18 column (50 × 2.1 mm, 1.9 μm particle size), and an Applied Biosystems API4000 QTRAP triple quadrupole mass spectrometer. Concentrations were corrected against the recovery of the internal standard. For quantification, external calibration was performed using echimidine as the standard. The limit of quantitation (LOQ) for echimidine in honey was 1 ng/g. As no further reference standards were available, echimidine-N-oxide, acetylchelmidine, acetylchelmidine-N-oxide, echivulgarine, and echivulgarine-N-oxide were indirectly quantified using the calibration of echimidine, assuming the same response factor and thus the same LOQ. A linear range was achieved from 0.5 to 100 ng/mL for the echimidine standard. Recovery of echimidine near the LOQ was 97%. Repeatability was 5.4%, as determined with six independent sample preparations measured by the same person on the same day.

#### Extraction of PAs from Nectar and Plant Pollen, and UHPLC-HRMS Analysis. Nectar. Five microfilters of nectar was directly transferred into a glass vial containing a 200 μL insert and diluted 10 times with the extraction solvent (70% methanol, 29.5% ultrapure water, and 0.5% formic acid, v/v). Plant Pollen. 1 mg of plant pollen was accurately weighed using a microbalance scale (Mettler Toledo), mixed with 100 μL of extraction solvent as described above, and transferred into a 2 mL Eppendorf tube. Five glass beads were added, and the tube was vigorously shaken at 30 Hz for 4 min to disrupt the pollen structure and to extract the
PA Concentrations in Honey. *Echium*-type PAs were found in most of the honeys collected at both observation sites. The total PA concentrations of the honey samples produced in Basel in July and August during the years 2012 and 2013 were very low, 0.003 μg/g and 0.002 μg/g, respectively (Figure 1), and near the LOQ, while no PAs were measurable in the samples harvested in June 2012 and 2013 (data not shown).

Higher concentrations of PAs were detected in the samples produced in Verzasca. Levels ranged from 0.002 μg/g to 0.153 μg/g, and varied substantially between the collection years and the two apiaries (Figure 1). With respect to the type of alkaloids, echimidine (sum of tertiary base and the corresponding N-oxide) was present in the highest concentrations, followed by echivulgarine (+N-oxide) and acetylechimidine (+N-oxide), respectively. Echimidine (+N-oxide) accounted, on average, for 72% of the total PAs found in honey. Its concentration was four to six times higher than that of echivulgarine (+N-oxide). Similar results were obtained for honey samples containing *Echium*-type PAs from various other locations within Switzerland, where echimidine (+N-oxide) was also found to be the dominant alkaloid (Table 1).

PA Concentrations in Nectar and Plant Pollen. Plant pollen contained high concentrations of PAs, while much lower concentrations of PAs were found in nectar (Figure 2). The PA concentrations of plant pollen collected in Basel ranged from 1600 to 35000 μg/g and were on average 7428 μg/g (in 2013) and 24453 μg/g (in 2014), respectively. Plant pollen from Verzasca collected in 2013 and 2014 contained PAs ranging in concentration from 500 to 12900 μg/g. Average PA concentrations of 5427 μg/g and 9661 μg/g were measured in pollen collected in 2013 and 2014. In contrast, the PA content of nectar was on average more than 500 times lower than the PA concentration in pollen. The PA concentration in nectar samples collected in Basel ranged from 4.8 to 95.1 μg/g. PA concentrations were on average 21.3 μg/g (in 2013) and 40.1 μg/g (in 2014). Nectar from Verzasca contained PAs from 0.3 to 51.5 μg/g, on average 15.7 μg/g (in 2013) and 18.0 μg/g (in 2014). The PAs in nectar and plant pollen were mainly N-oxides, and the contribution of PAs as free bases to the total amount of each type of PA was very low (data not shown).

Figure 1. Concentrations of the three main *Echium*-type PAs in honeys from the two observation sites: red, echivulgarine (+N-oxide); black, acetylechimidine (+N-oxide); blue, echimidine (+N-oxide).
in nectar and plant pollen. However, the percentage of several PA-types varied between the investigated plant matrices. In nectar, approximately half of the PA content was echimidine (+N-oxide), while the other half consisted of acetylechimidine (+N-oxide) and low amounts of vulgarine (+N-oxide), echivulgarine (+N-oxide), and acetylvulgarine (+N-oxide). In contrast, echivulgarine (+N-oxide) was the main alkaloid-type in plant pollen (63% of the total PA content). The four other alkaloid-types were present at substantially lower concentrations. Similar PA profiles were obtained with samples collected in 2013 (data not shown). In summary, the echimidine-type was the dominant alkaloid in honey as well as in nectar, while pollen contained mainly the echivulgarine-type.

4. DISCUSSION

Nectar of E. Vulgare As a Primary Source for PAs in Honey. The proportion of the different types of PAs found in honey was similar to that found in nectar, but strikingly different from that found in pollen. Echimidine (+N-oxide) was the dominant alkaloid found in honey and nectar. In contrast, plant pollen mostly contained echivulgarine (+N-oxide). This unequal quantitative distribution of alkaloids strongly suggests nectar as the primary source of PAs in honey.

Prior research has found concentrations of PAs in plant pollen of up to 14000 μg/g.17 We measured similar average PA concentrations in plant pollen from Basel in 2013 (7428 μg/g) and from Verzasca in 2013 (5427 μg/g) and in 2014 (9661 μg/g), but pollen collected in 2014 in Basel contained a higher amount of PAs (24453 μg/g). Climatic and genetic variations may affect the amount of PA produced by the plants, and thus, the PA content in pollen. Boppré et al.17 found echivulgarine-N-oxide as the major alkaloid in Echium pollen, followed by vulgarine-N-oxide, echimidine-N-oxide, and acetylechimidine-N-oxide. Similar proportions of alkaloids were found in our study, thus supporting their results. Since the total PA content of plant pollen was found in concentrations that were 1000 times higher than in honey, the authors concluded that plant pollen has the potential to be a significant source for PAs in honey.17 However, they did not investigate nectar as a potential source of alkaloids in honey, and did not report the unequal proportions of the alkaloids in honey compared to pollen. Since we integrated analyses of Echium nectar and of honeys harvested at the same locations, we obtained a more complete picture of the contamination pathway for this plant species, suggesting that floral nectar contributes more significantly to honey contamination than does pollen. Our results suggest that pollen may play a small role in the PA contamination of honey and that a small proportion of PAs may be released from pollen into honey, as previously suggested by Kempf.38,40 For example, the proportion of echivulgarine (ca. 15% of the total PAs) found in the honey from Verzasca 1 (2013; Figure 1) was higher than what would be expected from pure nectar, which
contains proportionally less echivulgarine (less than 5% of the total PAs).

**Estimation of Concentration and Dilution Factors for Nectar and Pollen during Honey Ripening.** In order to estimate an approximate factor by which the components of nectar, such as sugars and PAs, are concentrated during the process of honey ripening, we measured the water content in nectar and honey. Nectar samples contained water in the range 30% to 95%, depending on climatic conditions, with an average of 66% (standard deviation = 18.3%). honeys contained on average 17.3% (standard deviation = 1.6%) water. Therefore, PAs are concentrated during the processing of nectar to honey by a factor of approximately four (Table 2).

| Table 2. Estimation of the Contribution of PAs from Nectar and Pollen to the PA Content in Honey |
|-------------------------------------------------|---------------------------------|------------------------|
| nectar                                          | pollen                         | PA contribution to honey |
| average PA conc. (µg/g)                          | 25                             | ++++                    |
| concentration/dilution                          | 4:1                            | >1:5000                |
| PA contribution to honey                        |                                | +                      |

Honey contains traces of pollen that depend on several factors. When bees forage from flowers, they come into contact with the anthers, so that pollen may fall into nectar that is later collected. Pollen grains can also stick to the bee’s body hair. Furthermore, apicultural practices can influence the amount of pollen in honey.43 For these reasons, the pollen content of honey is variable. We therefore assessed the weight of the honey sediment, which mainly consists of pollen. Some honey also contains other components, e.g. “honeydew indicators”, such as algal cells and mold spores.43 Thus, the weight of pollen may be overestimated by this procedure. On average, our honey samples from Basel and Verzasca contained a sediment of 0.048 mg/g (standard deviation = 0.032). This value is close to the range of 0.14 to 0.2 mg/g found by Maurizio in honey from Germany and Switzerland.43 In other words, pollen in honey is usually diluted at least 5000-fold in honey (Table 2).

We measured an average total PA concentration of 25 µg/g (standard deviation = 22 µg/g) in nectar and an average total concentration of 13551 µg/g (standard deviation = 9787 µg/g) in plant pollen. Thus, the PA concentration in nectar is approximately 500 times lower than it is in plant pollen. However, during honey ripening, the concentration of nectar increases about 4-fold, while the pollen content is diluted to at least 1:5000. Therefore, despite its lower initial PA concentration, the nectar of E. vulgare plays a substantially greater role in the PA contamination of honey than does pollen (Table 2). This is in agreement with our observation of similar PA proportions of nectar and honey.

**PA Concentration in Monofloral Honeys.** Previous studies have found PA concentrations of up to 2.45 mg/g in monofloral honey of E. vulgare or plantagineum,30−32,35 and up to 3.9 µg/g in monofloral honey of S. jacobaea.33,34,39 The highest concentrations of PAs ever measured (up to 13 µg/g) were found in honey collected from hives in a field of Senecio jacobaea.31 In the present study, we measured an average alkaloid content of 25 µg/g in nectar from E. vulgare plants (Figure 2; Table 2). This value is on the same order of magnitude as the maximum PA concentrations ever measured. Therefore, the concentration of PAs in nectar is indeed high enough to explain the observed PA concentrations in honey.

The highest total PA concentration found in our honey samples was 0.153 µg/g, measured in a sample from Verzasca (Figure 1). In fact, this honey is a polyfloral honey characteristic of the area of production. In such honeys, nectar from E. vulgare can be diluted more than 150 times with nectar from other plant species, explaining the final PA observed in this sample. We analyzed the pollen grains of this honey by microscopy. The majority of the pollen (88%) originated from Castanea sativa, while only 2.8% originated from E. vulgare. Sensory and melissopalynological analyses revealed Castanea sativa, Rubus sp., Tilia sp., Rhododendron sp., and E. vulgare as other significant components of the honey. Thus, it stands to reason that a substantially higher amount of alkaloids could be expected in a monofloral honey of E. vulgare.

In conclusion, we found that most of the PAs in our honey samples were attributable to nectar, contrary to previous assumptions that proposed pollen as the primary source.17,58,40 If pollen were the main source of PAs in honey, PA contamination of honey could be reduced by passing the honey through a filtration system designed to remove pollen. However, since nectar is the main contributor of PAs, such filtration would not substantially decrease the concentration of PAs, and would thus be an ineffective technical solution to the problem. A more suitable approach to reducing PA contamination in honey would be to avoid PA producing forage plants, such as those belonging to the Boraginaceae, in large numbers near apiaries. Some PA producing plants are an important foraging source for several solitary bee species. In small numbers, these plants will usually not pose a serious contamination threat.

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**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02320.

Table S1: UHPLC-HRMS retention and mass characteristics (MS/MS ions) of Echium-type PAs. Figure S1: UHPLC-HRMS chromatograms of echimidine standard (1), PAs in pollen and nectar from Echium vulgare: echimidine-N-oxide (2), vulgarine-N-oxide (3), acetylene-chimidine-N-oxide (4), acetyl-vulgarine-N-oxide (5), and echivulgarine-N-oxide (6). Peak numbers also refer to Table S1. Figure S2: Linear range of echimidine standard in UHPLC-HRMS. Linearity was achieved from 0.005 to 4 µg/mL. (PDF)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We would like to thank the beekeepers from Basel and the Verzasca valley for providing us with bee-collected pollen and honey samples. Matteo Lucchetti is financially supported by a grant from Agroscope, Swiss Federal Research Institute for Agriculture and Food Sciences, for his doctorate studies.

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