



## Detection of pyrrolizidine alkaloids in commercial honey using liquid chromatography–ion trap mass spectrometry

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### ABSTRACT

Pyrrolizidine alkaloids (PAs) are known secondary plant metabolites which can cause hepatotoxicity in both humans and livestock. PAs can be consumed through the use of plants for food, medicinal purposes and as contaminants of agricultural crops and food. PA contaminated grain has posed the largest health risk, although any PA contamination in our food chain should be recognised as a potential health threat. For this purpose, retail honeys were tested by LC–MS/MS. The method allows for specific identification of toxic retronecine and otonecine-type PAs by comparison to reference compounds via a spectral library. In total, 50 honey samples were matched to the reference spectra within a set of tolerance parameters. Accurate data analysis and quick detection of positive samples was possible. Positive samples contained an average PA concentration of 1260  $\mu\text{g kg}^{-1}$  of honey. Good linear calibrations were obtained ( $R^2 > 0.991$ ). LOD and LOQ ranged from 0.0134 to 0.0305 and 0.0446 to 0.1018  $\mu\text{g mL}^{-1}$ , respectively.

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### 1. Introduction

Pyrrolizidine alkaloids (PAs) are naturally occurring plant toxins which have gained attention due to their hepatotoxic effects on both humans and livestock. PA-containing plants originate from the families Asteraceae, Boraginaceae and Fabaceae and are widespread in their distribution, being present in 3% of all flowering plants (Smith & Culvenor, 1981). PAs are proven hepatotoxic, carcinogenic, genotoxic, and pneumotoxic compounds (Culvenor et al., 1976; Mattocks, 1986; Schoental, 1968). Those which cause toxicity consist of a 1,2-unsaturated necine ring and a necic acid from which carboxylic esters form a macrocyclic structure (Prakash, Pereira, Reilly, & Seawright, 1999). The structure of retronecine and otonecine type PAs are given in Fig. 1.

Livestock losses due to the presence of toxic PAs have been firmly established. These losses can occur from foraging on PA-containing plants but are predominately from contaminated feed, hay or silage, as PAs in their dried state become more palatable (Candrian, Luthy, Schmid, Schlatter, & Gallasz, 1984; Stegelmeier et al., 1999; Stegelmeier, 2011). Cases of PA exposure in humans have occurred worldwide, although developing countries have

been more susceptible. The reasons behind this are twofold; firstly PA-containing plants are particularly hardy surviving long periods of drought and secondly poor crop management. Mass intoxications have arisen from the use of contaminated grain. The earliest case termed ‘bread poisoning’ dates from 1920 (Willmot & Robertson, 1920) and more recent cases have occurred in both Afghanistan and Ethiopia in 2008 (IRIN Asia, 2008a, 2008b; Molyneux, Gardner, Colegate, & Edgar, 2011). PA toxicity also arises from the direct consumption of PA-containing plants used as food, in salads, cooking spices, herbal teas and medicines. Wiedenfeld (2011) provides a comprehensive list of cases where the source of human intoxication was unequivocally identified as PAs.

Food products from animal origin such as milk, eggs and honey are prone to contain PAs. Although there are no recorded cases of human PA poisoning from these foodstuffs they should not be overlooked. Studies have shown that PAs can be carried-over into milk (Deinzer, Arbogast, & Buhler, 1982; Dickinson, Cooke, King, & Mohamed, 1976; Hoogenboom et al., 2011) from inadvertent grazing on ragwort (*Senecio jacobaea*) and into eggs from hens feeding on unfettered grain infiltrated with *Heliotropium* and *Echium* spp. seeds (Edgar & Smith, 2000).

Honey and pollen products also have been shown to contain toxic PAs. Earlier reports document the occurrence of PAs in honey from *Senecio jacobaea* (Crews, Startin, & Clarke, 1997; Deinzer, Thomson, Burgett, & Isaacson, 1977) and *Echium plantagineum* (Culvenor, Edgar, & Smith, 1981) with PA levels of up to 3900

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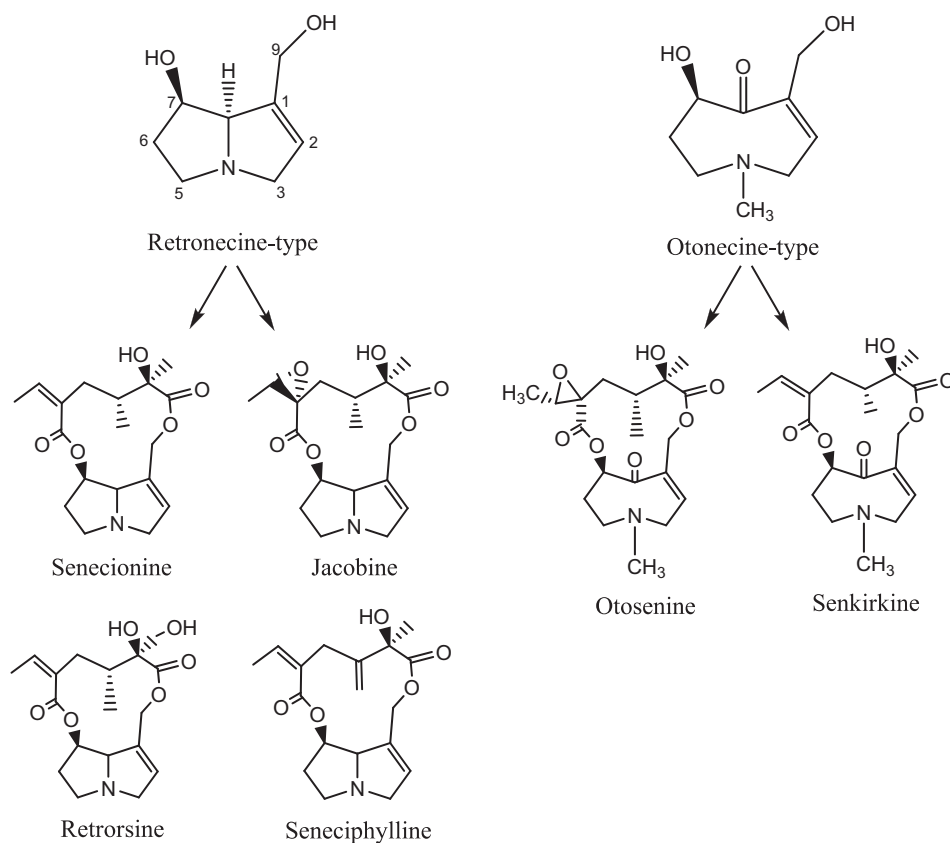


Fig. 1. Representative chemical structures for retronecine-type and otonecine-type PA toxins used within this study.

and  $950 \mu\text{g kg}^{-1}$  of honey detected, respectively. Crews et al. (1997) stated that the higher PA content honey samples were recognised by beekeepers as being unpalatable due to the presence of *Senecio jacobaea*. A study conducted (Beales, Betteridge, Colegate, & Edgar, 2004) on 63 pre-processed honey samples included a small number of retail samples ( $n = 5$ ) which were shown to contain PAs up to  $250 \mu\text{g kg}^{-1}$  of honey. A later study on 9 commercial floral honeys detected PA levels in seven samples between 170 and  $2850 \mu\text{g kg}^{-1}$  of honey (Betteridge, Cao, & Colegate, 2005). However, the most recent comprehensive studies of retail honey found upper PA concentrations of  $120 \mu\text{g kg}^{-1}$  (Kempf et al., 2008) and  $267 \mu\text{g kg}^{-1}$  in honey (Dübecke, Beckh, & Lüllmann, 2011).

There are no set maximum residue limits (MRLs) for PAs in food or feed. Guidelines or tolerable daily intakes (TDIs) have been established through several independent studies (Edgar, Colegate, Boppré, & Molyneux, 2011) but since the detection methods are not standardised the TDIs for toxic PAs range from 0.1 to  $1.0 \mu\text{g kg}^{-1}$  body weight per day or 0.1 to  $1.0 \mu\text{g/day}$  over a 6 week period. It is for this reason we undertook a concise study of retail honey ( $n = 50$ ) in order to develop a validated method for 11 PAs using LC coupled to an ion-trap mass spectrometer with automated identification based on spectral library matches. The combined use of ion-trap MS for exact fragmentation and the NIST (National Institute of Standards and Technology) algorithm based library allows for fast and robust detection of PA-positive samples.

## 2. Materials and methods

### 2.1. Reagents and chemicals

LC–MS grade water, HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Dublin, Ireland). Formic acid (99–

100% purity) was obtained from Reagecon Ltd. (Co. Clare, Ireland). Sulphuric acid (98%) and ammonium hydroxide (33%  $\text{NH}_3$  in  $\text{H}_2\text{O}$ ) were purchased from Sigma Aldrich (Wicklow, Ireland). Strong cation exchange polymeric solid phase extraction (SPE) cartridges, Strata-X-C 33  $\mu\text{m}$  (60 mg/3 mL), were acquired from Phenomenex Inc. (Cheshire, UK) and 0.45  $\mu\text{m}$  PTFE syringe filters from Lab Unlimited (Dublin, Ireland). All honey tested during this study was purchased from supermarkets within Ireland.

### 2.2. Pyrrolizidine alkaloid (PA) standards

Crotaline, retrorsine, senecionine were purchased from Sigma Aldrich (Ireland), senkirkine, seneciphylline from Carl Roth (Germany), otosenine, echimidine, lycopsamine from PhytoLab (Germany), heliotrine and trichodesmine from Latoxan (France). The PA jacobine was kindly provided by Dr. Patrick Mulder (RIKILT).

#### 2.2.1. Standard solutions

Individual primary stock solutions of PAs were prepared in methanol at a concentration of  $1 \text{ mg mL}^{-1}$ . Working standards were prepared from a concentration of  $2 \mu\text{g mL}^{-1}$  in methanol weekly. Internal standard, crotaline, was prepared from a stock solution of  $100 \mu\text{g mL}^{-1}$  in 0.05 M sulphuric acid. All primary standard solutions were stored at  $-20^\circ\text{C}$ , while weekly working standards were stored between 2 and  $4^\circ\text{C}$ .

#### 2.3. Negative control samples

Previously analysed honey which tested negative for the PAs used in this study was extracted using the same procedure described below, for use in preparing control samples and stored at

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