Food Chemistry 136 (2013) 1577–1583

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

Caroline T. Griffin [a], Martin Danaher [b], Christopher T. Elliott [c], D. Glenn Kennedy [d], Ambrose Furey [a,e,*]

[a] Team Elucidate, Department of Chemistry, Cork Institute of Technology (CIT), Bishopstown, Cork, Ireland
[b] Food Safety Department, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland
[c] Queens University Belfast (QUB), 1209 David Keir Building, Stranmills Road, Belfast BT9 5AY, United Kingdom
[d] Agri-Food and Biosciences Institute (AFBI), Stoney Road, Stormont, Belfast BT4 3SD, United Kingdom
[e] PROTEOBIO, Mass Spectrometry Centre for Proteomics and Biotoxin Research, Department of Chemistry, CIT, Bishopstown, Cork, Ireland

ARTICLE INFO

Article history:
Available online 27 February 2012

Keywords:
Pyrrolizidine alkaloids (PAs)
Food safety
Plant toxins
LC–QIT
LC–MS/MS
Spectral library
Honey
Solid-phase extraction (SPE)

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 μg 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 μg mL^{-1}, respectively.

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

* Corresponding author at: Team Elucidate, Department of Chemistry, Cork Institute of Technology (CIT), Bishopstown, Cork, Ireland. Tel.: +353 21 4335875; fax: +353 21 4345191.
E-mail address: ambrose.furey@cit.ie (A. Furey).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2012.02.112

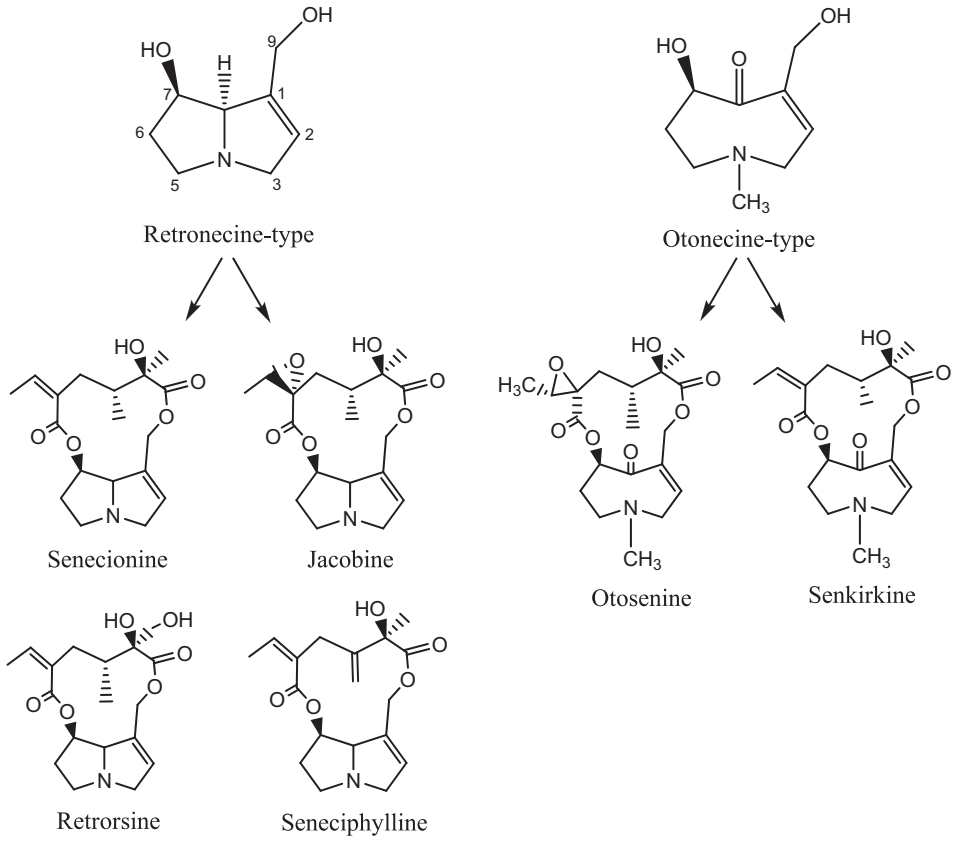

Fig. 1. Representative chemical structures for retronecine-type and otonecine-type PA toxins used within this study.

and 950 μg 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 μg kg^{-1} of honey. A later study on 9 commercial floral honeys detected PA levels in seven samples between 170 and 2850 μg kg^{-1} of honey (Betteridge, Cao, & Colegate, 2005). However, the most recent comprehensive studies of retail honey found upper PA concentrations of 120 μg kg^{-1} (Kempf et al., 2008) and 267 μg 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 μg kg^{-1} body weight per day or 0.1 to 1.0 μg/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% NH_3 in H_2O) were purchased from Sigma Aldrich (Wicklow, Ireland). Strong cation exchange polymeric solid phase extraction (SPE) cartridges, Strata-X-C 33 μm (60 mg/3 mL), were acquired from Phenomenex Inc. (Cheshire, UK) and 0.45 μm 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 mg mL^{-1}. Working standards were prepared from a concentration of 2 μg mL^{-1} in methanol weekly. Internal standard, crotaline, was prepared from a stock solution of 100 μg mL^{-1} in 0.05 M sulphuric acid. All primary standard solutions were stored at −20 °C, while weekly working standards were stored between 2 and 4 °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

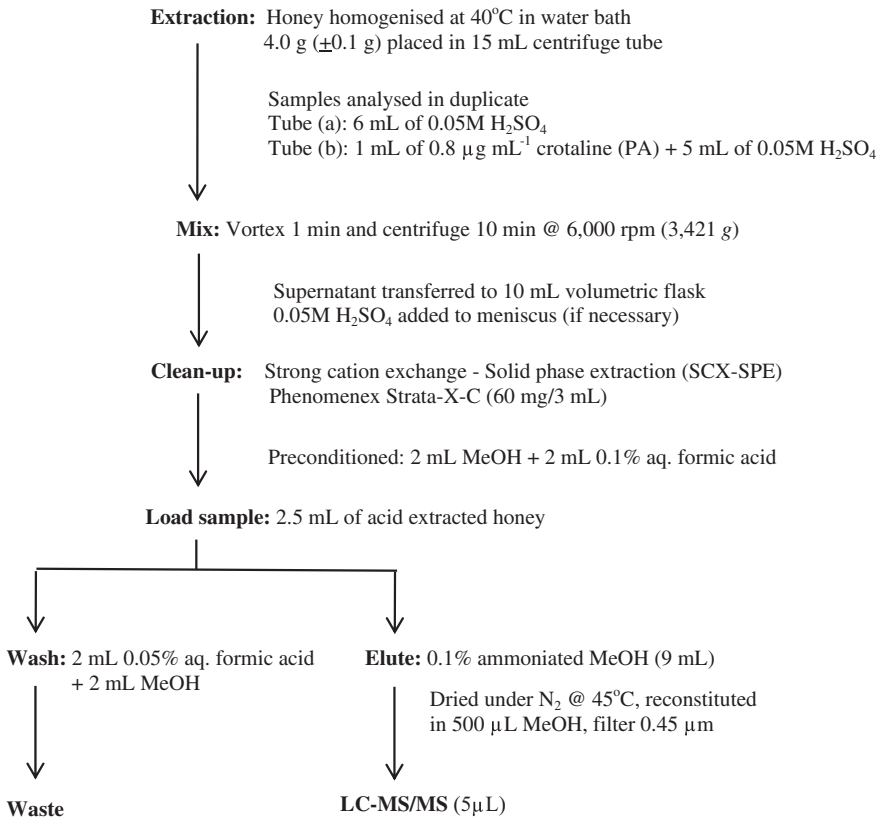

Fig. 2. A flow diagram for the overall extraction procedure.

4 °C. Prior to analysis a 0.8 μg mL^{-1} control of crotaline in blank extracted honey was prepared.

2.4. Sample preparation

Honey samples were homogenised in a water bath at 40 °C (±2 °C) prior to weighing 4 g (±0.1 g) into 15 mL centrifuge tubes in duplicate, giving '*a*' and '*b*' samples. All sample tubes '*a*' had 6 mL of 0.05 M sulphuric acid solution added. All sample tubes '*b*' had 1 mL of 0.8 μg mL^{-1} of internal standard crotaline and 5 mL of 0.05 M sulphuric acid solution added. Samples were capped and vortexed for approx. 1 min. The samples were then centrifuged at 6000 rpm (3421*g*) for 10 min and the supernatant was transferred to a 10 mL volumetric flask making up to the calibration mark with 0.05 M sulphuric acid, if necessary. Strata-X-C SPE cartridges were preconditioned with 2 mL of methanol followed by 2 mL of acidified water (0.1% formic acid). 2.5 mL of the honey solution was loaded onto the cartridge and allowed to dry for 1 min. The cartridge was washed with 2 mL of acidified water (0.05% formic acid) followed by 2 mL of methanol. The cartridge was eluted with 9 mL of 0.1% ammoniated methanol. Eluted samples were dried at 45 °C under nitrogen in a turbovap. Dried samples were reconstituted in 500 μL of HPLC grade methanol, vortexed and passed through a 0.45 μm syringe filter to prevent blockage of the analytical LC column. A flow diagram for the overall extraction procedure is shown in Fig. 2.

2.5. LC–MS/MS analysis

Separation was carried out on an Agilent 1200 LC coupled to an Agilent 6340 series ion trap mass spectrometer using a Thermo Fisher Hypersil Gold aQ C_{18} analytical column (150 × 2.1 mm, 3 μm particle size) fitted with an identical guard column (10 × 2.1 mm). The column was maintained at 30 °C and a binary gradient separation was performed using a flow rate of 0.2 mL min^{-1}. The mobile phase consisted of 0.05% formic acid in water (A) and 100% acetonitrile (B). The gradient profile was 0–2 min 20% B; 2–15 min linear increase in B from 20% to 50%; 15–17 min held at 50% B and 18–30 min post acquisition time at starting mobile phase, 20% B, to re-equilibrate the column. The autosampler needle was washed with 100% methanol between injections to eliminate carryover. All sample injections, volume 5 μL, were carried out in duplicate. The MS conditions were positive electrospray ionisation (ESI) mode with nebuliser pressure at 20 psi. Nitrogen was used as the drying gas under a flow of 10 L min^{-1} and a temperature of 350 °C. Table 1 details the retention time, MS/MS fragment ions and fragmentation amplitude obtained for each of the 11 PAs. To ensure an excess of 15 data points across a peak and reproducible integration for quantitative analysis time segments were used at 6.5 and 11.5 min (Fig. 3).

Acquisition and analysis of data was performed with Agilent ChemStation LC and 6300 Series Trap Control (version 6.2). Library building and spectral matching were conducted using DataAnalysis™ and LibraryEditor™ software (Bruker Daltonik GmbH) (see Table 2).

3. Results and discussion

3.1. Method development

The developed LC-method allows for the detection of 11 PAs within 15 min, having a period of 0–3 min initially diverted to waste to allow for elimination of matrix components (such as sugars, amino acids and minerals) which could potentially interfere with MS analysis (through source contamination or enhanced ion suppression). It was found that using 10 void volumes of the column was necessary to fully equilibrate the system before the next

Table 1
Retention times (Rt_{min}), MS/MS fragment ions and fragmentation amplitude of 11 reference PAs.

No.	PA	Rt_{min}	Precursor ion $[M+H]^+$	Product ions	Fragmentation amplitude (*V*)
1	Crotaline	4.9	326	280; 237; 138; 120	1.2
2	Lycopsamine	5.6	300	156; 138; 120; 94	1.2
3	Jacobine	7.4	352	308; 380; 155; 120	1.2
4	Retrorsine	8.0	352	324; 276; 138; 120	1.1
5	Heliotrine	9.0	314	156; 138; 120; 96	0.8
6	Trichodesmine	9.0	354	308; 222; 164; 120	1.0
7	Otosenine	9.1	382	168; 150; 122; 110	1.0
8	Seneciphylline	10.0	334	306; 280; 138; 120	1.1
9	Senecionine	12.5	336	308; 290; 138; 120	1.2
10	Echimidine	13.3	398	380; 336; 220; 120	0.5
11	Senkirkine	13.8	366	168; 150; 137; 122	1.2

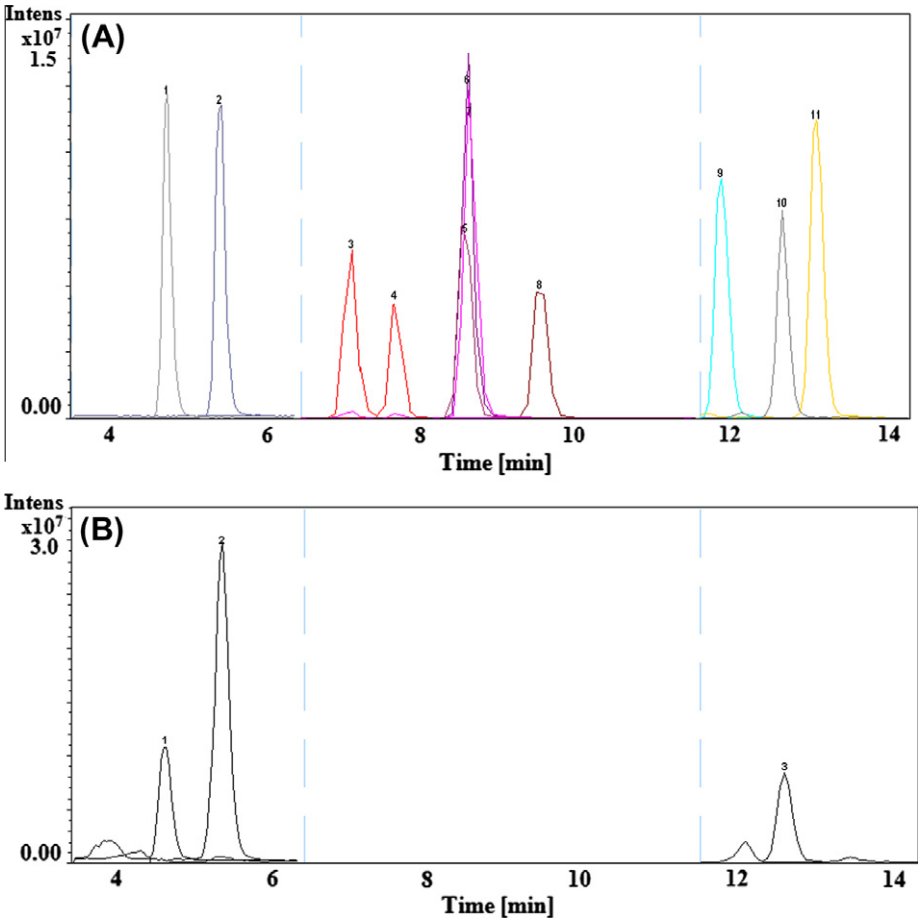

Fig. 3. (A) EIC of MRM obtained in + ESI mode for 11 reference PA compounds at a concentration of 1 μg mL^{-1}: (1) crotaline (4.9 min); (2) lycopsamine (5.6 min); (3) jacobine (7.4 min); (4) retrorsine (8.0 min); (5) heliotrine (9.0 min); (6) trichodesmine (9.0 min); (7) otosenine (9.1 min); (8) seneciphylline (10.0 min); (9) senecionine (12.5 min); (10) echimidine (13.3 min); (11) senkirkine (13.8 min). (B) EIC of MRM obtained in + ESI mode for PA-positive sample No. 24 (tube 'b') showing peaks (1) crotaline (I.S. at 4.9 min), (2) lycopsamine (5.6 min) and (3) echimidine (13.2 min). Column conditions as reported in Section 2.5.

injection giving a total run time of 30 min. Both the gradient elution and the use of a polar endcapped C_{18} reversed phase column provided good retention and separation for PAs. Solid phase extraction using strong cation exchange (SPE-SCX) had previously been shown to be effective for isolating PAs from honey (Betteridge et al., 2005). The honey matrix is a complex mixture of several groups of compounds from which PAs can be difficult to isolate. The polymeric SCX sorbent strongly retains basic compounds such as PAs, allowing for a strong organic clean-up wash without the loss of the analyte. This provides a clear advantage over C_{18} sorbents which can result in the co-elution of matrix compounds that could potentially compromise the LC–MS analysis (Mroczek, Glowniak, & Wlaszczyk, 2002).

MS conditions were initially optimised by infusing standards at a concentration of 1 μg mL^{-1} and tuning using the SmartFrag collision energy ramping tool. SmartFrag uses collision-induced dissociation (CID) voltage ramping over a range of energies, 30–200% of the amplitude, to ensure the precursor ion receives the necessary energy for maximum fragmentation with minimal ion loss from the trap. It eliminates time-consuming manual CID voltage optimisation that would normally be carried out to optimise the collision gas and energy for each individual compound since they tend to be very compound dependent (Goodley, 2000).

The MS was operated in multiple reaction monitoring (MRM) mode utilising the ion charge control (ICC) function to achieve the optimum MS/MS. This feature integrates the total ion signal

Table 2
Calibration data including regression equation, correlation coefficients, relative standard deviation, limits of detection and quantitation for the 11 reference PAs used in this study.

PA	$[M+H]^+$	Regression equation y =	R^2 =	%RSD	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Crotaline	326	$4 \times 10^7.x + 1 \times 10^6$	0.9928	6.21	0.0134	0.0446
Lycopsamine	300	$5 \times 10^7.x + 2 \times 10^6$	0.9955	7.25	0.0182	0.0608
Jacobine	352	$3 \times 10^7.x + 805892$	0.9924	7.76	0.0293	0.0976
Retrorsine	352	$2 \times 10^7.x + 383697$	0.9965	5.60	0.0250	0.0833
Heliotrine	314	$4 \times 10^7.x + 1 \times 10^6$	0.9936	7.51	0.0278	0.0928
Trichodesmine	354	$6 \times 10^7.x + 2 \times 10^6$	0.9969	7.91	0.0305	0.1018
Otosenine	382	$6 \times 10^7.x + 1 \times 10^6$	0.9973	6.99	0.0186	0.0621
Seneciphylline	334	$3 \times 10^7.x + 741832$	0.9926	6.58	0.0198	0.0660
Senecionine	336	$5 \times 10^7.x + 1 \times 10^6$	0.9930	6.46	0.0237	0.0790
Echimidine	398	$3 \times 10^7.x + 2 \times 10^6$	0.9917	8.08	0.0230	0.0765
Senkirkine	366	$6 \times 10^7.x + 2 \times 10^6$	0.9912	5.56	0.0146	0.0487

produced in each spectrum and alters the time of the next ion accumulation period to maintain the correct number of ions in the ion trap. Toxic retronecine-type PAs produce common fragments *m/z* 138 and 120 whilst otonecine-type PAs produce *m/z* 168 and 150. All PA compounds exhibit the precursor $[M+H]^+$ protonated ion.

3.2. Spectral library development

To establish a spectral PA library the 11 reference compounds were analysed through positive ESI mode. PA compounds (at a concentration of 1 μg mL^{-1}) were chromatographically analysed in methanol by (i) spiking into a blank honey sample to account for matrix effects and (ii) through direct infusion via a syringe pump. The MS acquisition settings for generating the library spectra are the same as those outlined earlier. The MS/MS and MS^3 spectra are averaged and imported to the LibraryEditor™ software. The full MS spectra are excluded as they contain too many background interferences. Each PA reference compound had one MS/MS and MS^3 spectra, CAS number, chemical formula, exact mass and structure assigned to it. Other significant parameters entered were the optimised fragmentation amplitude, product ions with abundance greater than 1%, peak width of *m/z* 0.5 and isolation width of *m/z* 1.0.

Once sample analysis has been carried out the data file will contain either MS/MS or MS^3 spectra. From the total ion chromatogram (TIC) or the extracted ion chromatogram (EIC) the user can perform an automatic integration and library search using pre-specified parameters which are saved within the data file. One such important factor used within this study was retention time, set to an acceptable deviation of ±0.2 min. If another compound with the same *m/z* precursor ion elutes outside this time window it will not be selected for spectral comparison.

The sample spectra are matched to the library spectra using a NIST algorithm which scores for *fit* (*F*), *reserve fit* (*R*), *purity* (*P*) and *match factor* (*M*). The *fit* correlates the number of spectral peaks and intensities between the sample and library entry. The *reverse fit* demonstrates the uniformity between these peaks, thus if the *reverse fit* score is low it signifies that there are too many interferences in the obtained sample spectrum. The *purity* score is representative of an amalgamation between *fit* and *reverse fit*. Incorporating the user pre-specified parameters the *match* score is returned giving an overall effective score with a maximum of 1000. Thus, a positive sample will return a *match* score ideally above 900, with 1000 indicating an exact correlation. The use of spectral libraries is excellent for fast and reliable identification of positive samples (Gómez-Romero et al., 2011) but could also provide a means to reduce variability between both intra- and inter-laboratory methods.

3.3. Calibration and linearity data

Calibration studies were conducted over a 7-point range using an external standard calibration for the 11 reference compounds by injecting in triplicate. The linearity of the method was evaluated over a range of 0.1 to 1.0 μg mL^{-1} and the measured coefficient of correlation, R^2, resulted with acceptable values all above 0.991. The limit of detection (LOD) and quantitation (LOQ) was established chromatographically from the signal-to-noise ratio (S/N) of the precursor ion using S/N = 3 and 10, respectively.

3.4. Precision, accuracy and recovery

To determine the precision and accuracy of the method, control samples ($n = 7$) were prepared in methanol at a concentration of 0.8 μg mL^{-1} and analysed in triplicate over 5 days. The measured concentrations resulted in a relative standard deviation (RSD) of 4.42%. Honey samples were analysed in duplicate with both tubes '*a*' and '*b*' having two injections each. PA concentrations in positive samples are reported as a calculated mean ($n = 4$). The percentage recovery calculated for crotaline in '*b*' samples was 87% ± 9 SD, which was considered acceptable since previous literature reported recoveries of 85% ± 5 SD (Betteridge et al., 2005).

3.5. Screening of samples for PAs

In our study, all samples were processed using the library matching criteria outlined previously in Section 3.2. Samples are taken as positive for one or more of the PAs contained within the spectral library if the match factor exceeded 900. With low LODs and high percentage recoveries (87% ± 9 SD) the method clearly identifies samples that contain the toxic PAs. Throughout the study all samples were tested with sample 'b' containing 0.8 μg mL^{-1} of crotaline, acting as an internal standard. Analysing in duplicate with sample 'a' unspiked allowed for the detection of crotaline itself, as this PA can potentially be found within honey. Crotaline, as an internal standard spiked into each sample (0.8 μg mL^{-1}), was detected in every sample '*b*' analysed and identified by the spectral library with all 50 samples returning a purity score above 950 and a perfect match score of 1000. Sample No. 24 (b) is shown in Fig. 3(b) with peaks signifying the presence of crotaline (the internal standard) and identifying two PAs; lycopsamine and echimidine.

The 50 retail samples surveyed were categorised by their origin; 9 indigenous, 8 from within the EU, 23 non-EU and 14 labelled as blends of EU and non-EU honeys. Eight samples tested positive for one or two PAs, predominantly lycopsamine and echimidine, agreeing with findings by Dübecke et al. (2011). Detected PA concentrations ranged from 182 to 4078 μg kg^{-1}, as outlined in Table 3, this concurs with previous literature reports (Culvenor et al., 1981 and Beales et al., 2004). From the 8 positive samples 6 originated from outside the EU and 2 were blends of EU and non-EU honeys. Positive samples from outside the EU had PA concentrations ranging from 190 to 4078 μg kg^{-1} whilst samples labelled as blends of both EU and non-EU honeys had a relatively lower PA concentration range of 182–634 μg kg^{-1}.

Table 3
List of positive honey samples including library search scores.

Sample No.	PA detected	Rt_{min}		Conc. μg kg^{-1} (n = 4)[b]	%RSD	Library search scores			
		Actual	Observed [a]			*Fit*	*R fit*	*Purity*	*Match*
1	Lycopsamine	5.6	5.5	331	3.55	941	949	942	1000
8	Lycopsamine	5.6	5.5	182	13.72	957	964	958	1000
11	Lycopsamine	5.6	5.6	538	8.47	997	998	997	1000
12	Lycopsamine	5.6	5.5	1700	4.81	997	997	998	1000
	Echimidine	13.3	13.2	1517	0.36	998	999	999	1000
13	Lycopsamine	5.6	5.5	1825	1.48	997	997	997	1000
	Echimidine	13.3	13.2	1746	0.11	999	999	999	1000
24	Lycopsamine	5.6	5.6	4078	0.65	995	995	996	1000
	Echimidine	13.3	13.2	1536	2.42	1000	1000	1000	1000
25	Lycopsamine	5.6	5.5	852	0.47	998	998	998	1000
	Echimidine	13.3	13.2	190	1.11	996	998	996	1000
49	Echimidine	13.3	13.2	634	12.78	997	997	998	1000

[a] Accepted only if Rt_{min} within ±0.2 min.
[b] Samples analysed in duplicate each having two injections.

While PAs are predominantly known for their hepatotoxic effects, they are also classified as genotoxic and carcinogenic. In a recent report published by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM), the panel concluded that there is a potential health risk for children who are large consumers of honey (EFSA, 2011). It is important to remember that consumption of honey is not restricted to the standalone product but may be ingested through various forms such as cereals, baked goods and food supplements, to name a few. Currently the European Union (EU) imports over half the honey it consumes on an annual basis. This is significant in light of our results in which non-EU honey had the highest level of detected PAs, 4078 μg kg^{-1}. Imposing a TDI of 1 μg kg^{-1} b.w. per day as suggested by the Food Standards Australia New Zealand (FSANZ, 2001) and assuming an average body weight of 80 kg for an adult male, ingesting 20 g of honey with a detected PA concentration of 4078 μg kg^{-1} would place said subject at the recommended TDI. EFSA has decided against the use of TDI and adopted the Margin of Exposure (MOE) approach instead. The reasoning is that PAs are deemed genotoxic and carcinogenic as well as hepatotoxic. A lower confidence limit on the benchmark dose associated with a 10% excess cancer risk ($BMDL_{10}$) was established at 70 μg kg^{-1} b.w. per day. Thus the levels found may not pose an acute toxicity risk but could contribute significantly to a chronic risk.

4. Conclusion

This study describes the application of using QIT-MS in conjunction with a spectral library to allow for fast and accurate PA detection in complex food matrices such as honey. A total of 16% of the retail honeys tested positive with half of these having a PA concentration in excess of 1000 μg kg^{-1} of honey. Using an average consumption level of 20 g of honey per serving (Kempf et al., 2008; Dübecke et al., 2011) the positive samples would exceed all suggested TDI levels, as summarised by Edgar et al., 2011. According to EFSA the possibility of acute toxicity arising from exposure to PAs in honey is low (EFSA, 2011). The PA-content in honey is lower than that contained in medicinal and herbal preparations. However, the detected levels are such that PAs in honey are a cause for concern for chronic toxicity in humans.

The method discussed here has been validated for 11 PAs and it is our intention to expand the spectral library to include a further 5 PAs and their N-oxides. As a result of the data obtained from this study a large scale retail survey (n > 300) has been initiated so that a comprehensive profile of all retail honey within Ireland can be assessed. EFSA have also called for further investigative research as their findings are based on PAs in honeys from one EU Member state only. It is becoming more evident the importance of establishing an acceptable level for toxic PAs in food and feed and to use validated analytical methods for detecting their presence. The use of spectral libraries can provide one such way of improving identification of PAs and their N-oxides in order to streamline and reduce differing results.

Acknowledgements

The authors would like to express their gratitude to Dr. Patrick Mulder (RIKILT) for supplying the jacobine reference standard. This work was funded through the Food for Health Research Initiative (FHRI) by the Irish Department of Agriculture, Fisheries and Food (DAFF) and the Health Research Board (HRB), 2008.

References

Beales, K. A., Betteridge, K., Colegate, S. M., & Edgar, J. A. (2004). Solid-phase extraction and LC–MS analysis of pyrrolizidine alkaloids in honeys. *Journal of Agriculture and Food Chemistry, 52*, 6664–6672.

Betteridge, K., Cao, Y., & Colegate, S. M. (2005). Improved method for extraction and LC–MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: Application to *Echium vulgare* honeys. *Journal of Agriculture and Food Chemistry, 53*, 1894–1902.

Candrian, U., Luthy, J., Schmid, P., Schlatter, Ch., & Gallasz, E. (1984). Stability of pyrrolizidine alkaloids in hay and silage. *Journal of Agriculture and Food Chemistry, 32*, 935–937.

Crews, C., Startin, J. R., & Clarke, P. A. (1997). Determination of pyrrolizidine alkaloids in honey from selected sites by solid phase extraction and HPLC–MS. *Food Additives and Contaminants, 14*, 419–428.

Culvenor, C. C. J., Edgar, J. A., Jago, M. V., Qutteridge, A., Peterson, J. E., & Smith, L. W. (1976). Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. *Chemico-Biological Interactions, 12*, 299–324.

Culvenor, C. C. J., Edgar, J. A., & Smith, L. W. (1981). Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. *Journal of Agriculture and Food Chemistry, 29*, 958–960.

Deinzer, M. L., Thomson, P. A., Burgett, D. M., & Isaacson, D. L. (1977). Pyrrolizidine alkaloids: Their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). *Science, 195*, 497–499.

Deinzer, M. L., Arbogast, B. L., & Buhler, D. R. (1982). Gas chromatographic determination of pyrrolizidine alkaloids in goat's milk. *Analytical Chemistry, 54*, 1811–1814.

Dickinson, J. O., Cooke, M. P., King, R. R., & Mohamed, P. A. (1976). Milk transfer of pyrrolizidine alkaloids in cattle. *Journal of American Veterinary Medical Association, 169*, 1192–1196.

Dübecke, A., Beckh, G., & Lüllmann, C. (2011). Pyrrolizidine alkaloids in honey and bee pollen. *Food Additives and Contaminants, 28*, 348–358.

Edgar, J. A., Colegate, S. M., Boppré, M., & Molyneux, R. J. (2011). Pyrrolizidine alkaloids in food: A spectrum of potential health consequences. *Food Additives and Contaminants, 28*, 308–324.

Edgar, J. A., & Smith, L. W. (2000). Transfer of pyrrolizidine alkaloids into eggs: Food safety implications. In A. T. Tu & W. Gaffield (Eds.), *Natural and selected synthetic toxins: Biological implications. ACS Symposium Series 745* (pp. 118–128). Washington, DC: American Chemical Society.

European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) (2011). Scientific Opinion on Pyrrolizidine alkaloids in food and feed.

EFSA Journal, 9(11), 2406–428. Available from: http://www.efsa.europa.eu/efsajournal.

Food Standards Australia New Zealand (FSANZ) (2001). *Pyrrolizidine alkaloids in food: A toxicological review and risk assessment.* Technical Report Series No. 2. Canberra (ACT): FSANZ. Available from http://www.foodstandards.gov.au/_srcfiles/TR2.pdf/.

Gómez-Romero, M., Zurek, G., Schneider, B., Baessmann, C., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2011). Automated identification of phenolics in plant-derived foods by using library search approach. *Food Chemistry, 124*, 379–386.

Goodley, P. Agilent Technologies (2000). *Maximizing ms/ms fragmentation* (5988-0704EN). Available from: http://www.chem.agilent.com/Library/technicaloverviews/Public/5988-0704EN.pdf.

Hoogenboom, L. A. P., Mulder, P. P. J., Zeilmaker, M. J., van der Top, H. J., Remmelink, G. J., Brandon, E. F. A., et al. (2011). Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. *Food Additives and Contaminants, 28*, 359–372.

IRIN Asia (2008a). *Afghanistan: WHO confirms 'charmak' disease in Herat Province Kabul*, 15 May 2008. Available from: http://www.irinnews.org/report.aspx?reportid=78218.

IRIN Asia (2008b). *Afghanistan: 'Charmak' disease still killing people, livestock in West Herat*, 16 December 2008. Available from: http://www.irinnews.org/report.aspx?reportid=81971.

Kempf, M., Beuerle, T., Bühringer, M., Denner, M., Trost, D., von der Ohe, K., et al. (2008). Pyrrolizidine alkaloids in honey: Risk analysis by gas chromatography–mass spectrometry. *Molecular Nutrition and Food Research, 52*, 1193–1200.

Mattocks, A. R. (1986). *Chemistry and toxicology of pyrrolizidine alkaloids*. London: Academic Press.

Molyneux, R. J., Gardner, D. L., Colegate, S. M., & Edgar, J. A. (2011). Pyrrolizidine alkaloid toxicity in livestock: A paradigm for human poisoning? *Food Additives and Contaminants, 28*, 293–307.

Mroczek, T., Glowniak, K., & Wlaszczyk, A. (2002). Simultaneous determination of N-oxides and free bases of pyrrolizidine alkaloids by cation-exchange solid-phase extraction and ion-pair high-performance liquid chromatography. *Journal of Chromatography A, 949*, 249–262.

Prakash, A. S., Pereira, T. N., Reilly, P. E. B., & Seawright, A. A. (1999). Pyrrolizidine alkaloids in human diet. *Mutation Research, 443*, 53–67.

Schoental, R. (1968). Toxicology and carcinogenic action of pyrrolizidine alkaloids. *Cancer Research, 28*, 2237–2246.

Smith, L. W., & Culvenor, C. C. J. (1981). Plant sources of hepatotoxic pyrrolizidine alkaloids. *Journal of Natural Products, 44*, 129–152.

Stegelmeier, B. L., Edgar, J. A., Colegate, S. M., Gardner, D. R., Schoch, T. K., Coulombe, R. A., et al. (1999). Pyrrolizidine alkaloid plants, metabolism and toxicity. *Journal of Natural Toxins, 8*, 95–116.

Stegelmeier, B. L. (2011). Pyrrolizidine alkaloid-containing toxic plants (*Senecio*, *Crotalaria*, *Cynoglossum*, *Amsinckia*, *Heliotropium*, and *Echium* spp.). *Veterinary Clinics of North America: Food Animal Practice, 27*, 419–428.

Wiedenfeld, H. (2011). Plants containing pyrrolizidine alkaloids: Toxicity and problems. *Food Additives and Contaminants, 28*, 282–292.

Willmot, F. C., & Robertson, G. W. (1920). Senecio disease, or cirrhosis of the liver due to Senecio poisoning. *Lancet, ii*, 848–849.