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Loss of Pyrrolizidine Alkaloids on Decomposition of Ragwort (*Senecio jacobaea*) as Measured by LC-TOF-MS

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The decomposition of toxic pyrrolizidine alkaloids in ragwort (*Senecio jacobaea*) on storage in waste bags has been evaluated by a new time-of-flight mass spectrometric detection method. The method makes progress in meeting the clear need for modern analytical methods for pyrrolizidine alkaloids and for studies into factors affecting the stability of the toxins in the uprooted plant, which might still be accessible to animals. The experiments demonstrated a rapid decomposition of the toxins in ragwort stored in bags, from 340 mg/kg to less than 40 mg/kg in four weeks and virtually complete loss after 10 weeks. The information obtained can guide effective ragwort removal procedures to safeguard grazing animals.

KEYWORDS: Pyrrolizidine alkaloids; ragwort; *Senecio jacobaea*; time-of-flight; LC-TOF-MS

INTRODUCTION

Ragwort (*Senecio jacobaea*) is a weed plant found throughout much of Europe and is native to the British Isles. It contains toxic pyrrolizidine alkaloids (PAs), which can be debilitating to grazing animals leading to fatalities through stress precipitated by liver damage. Ragwort is one of the most frequent causes of plant poisoning of livestock. It is responsible for over 90% of the complaints that the U.K. Department for Environment, Fisheries and Rural Affairs (Defra) receives about injurious weeds (*1*). Despite this the number of reported poisoning incidents in the U.K. is small. According to U.K. government figures, the number of reported incidents of ragwort poisoning in cattle in England, Wales, and Scotland were between 7 and 26 per year for the period 1985 to 1990 (*2*). It is not known what proportion of incidents go unreported.

Ragwort is a highly successful plant which can be difficult to control. It spreads through seeds and is biennial or occasionally perennial when the flowering stems are removed. Young plants appear in May or early June as low rosettes. In their second year, the rosettes mature, and from late June onward, they produce tall flowering stems, which carry dense flat topped clusters of bright yellow daisy-like flower heads. The flowering stems produce seeds in the autumn and then die.

Defra guidance (*3*) on the control of ragwort states that palatability of the weed increases when plants are conserved in hay or silage and that ragwort in silage remains highly toxic. There is cost and environmental damage caused by the disposal of ragwort by incineration, which is used to prevent ingestion of the dead plant by livestock and to prevent dispersal of seed. Advice has been issued on disposal suggesting that rotting in compost bins should continue for up to 12 months (*4*).

The compounds responsible for the toxicity of ragwort are a series of PAs. The ragwort PAs comprise an unsaturated amino alcohol system (retronecine) esterified with a series of dicarboxylic acids with branched macrocyclic chains of 5 to 10 carbon atoms, which are heavily substituted. *S. jacobaea* contains six major PAs, seneciphylline, senecionine, jacozine, jacobine, jacoline, and jaconine, the structures of which are shown in **Figure 1**. Studies of the PA content of the many *Senecio* species have shown them to exist principally as a mixture of the free bases and *N*-oxides (*5*−*8*).

Several cases have been reported of cows having been poisoned by ensiled grass that had been heavily infested with ragwort (*9*−*12*), although in these cases the silage had not been analyzed. In the few publications describing the poisoning of horses by ragwort (*13*), there have similarly been no reports of an analysis of the feed.

The European Food Safety Authority (EFSA) (*14*) has recently expressed the need for analytical methods suitable for the analyses of individual PAs as well as total PAs in plant materials. EFSA has also called for analytical surveys focused on those selected alkaloids, which have been identified as major hepatotoxins.

There have been very few published studies of the effect of either composting processes on PAs or simple alternative treatments of ragwort such as placing in black bin bags in direct sunlight in the field to give thermal or microbiological breakdown of the PAs. The characterization of composting conditions or other procedures for destruction of the toxin would allow the formulation and dissemination of advice to the public. It would reduce incineration and encourage collection and destruction of ragwort by landowners.

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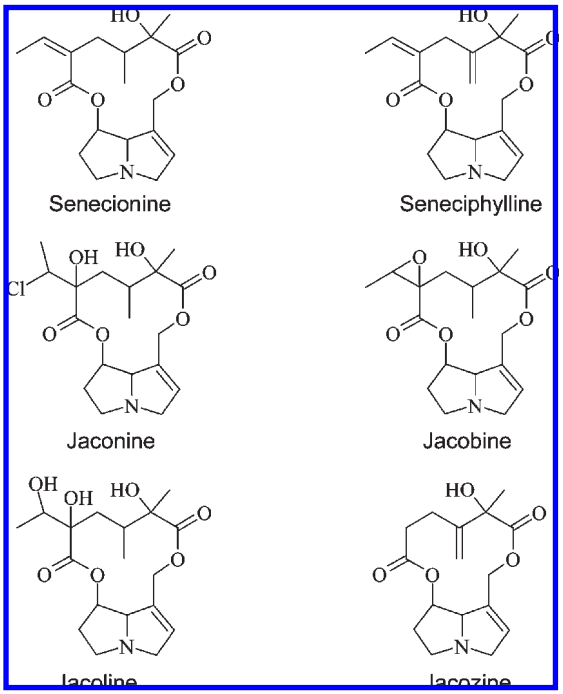

Figure 1. Major pyrrolizidine alkaloids of *Senecio jacobaea*.

Candrian et al. (*15*) measured the decomposition of PAs from *Senecio alpinus* in hay and silage, and Bradbury and Willis (*16*) the degradation of *S. jacobaea* PAs under acid conditions. Mattocks (*17*) has reported the enzymic hydrolysis of PAs to the retronecine base.

Analytical methods for PAs are based on two approaches. In the first, the *N*-oxides of the PAs are reduced with zinc dust to form the parent amines, and these are usually determined by gas chromatography. To determine the proportion of oxide and free base, the analysis has to be carried out with and without reduction of the oxides. The second approach is to use liquid chromatography with mass spectrometric detection (LC-MS). This method can detect both the free alkaloids and their *N*-oxides in the same analysis and has become the method of choice in recent years (*18*).

Mass spectrometry has been used as a detection method for PAs separated by LC, with atmospheric pressure chemical ionization (*18*−*21*) or electrospray ionization (ESI) (*22*−*26*). Time-of-flight mass spectrometry (TOF-MS) has the potential to complement these methods in providing accurate molecular mass information. The availability of accurate mass data is useful in the identification of unknown peaks that can be matched against libraries of empirical formulas. The presence of some fragment ions increases the confidence in the identification of compounds in the absence of authentic standards. Additionally, in the case of many alkaloids, including PAs, the nitrogen rule further reduces the number of possible matches to the empirical formulas.

The aims of this study were to evaluate the applicability of LC-TOF-MS instrumentation to this analysis and use it to identify the PAs and their *N*-oxides in ragwort from the U.K., and to study the effects of decomposing ragwort by bag storage on the levels of alkaloids present.

MATERIALS AND METHODS

Ragwort Samples. Ragwort plants identified as *S. jacobaea* were collected from a single colony in Yorkshire, U.K. in mid-July when flower buds were well developed. The plants were all of similar size and were mature; they had commenced flowering but had not set seed.

Ragwort plants were pulled up and kept in a loosely sealed black polythene bin liner of size 70 wide (when flat) × 90 cm tall, which was placed in the sun. The bag contained 20 plants and was loosely filled. They were sampled every two weeks for 14 weeks with two plants being removed from the bag and combined as one sample. As most of the plant material was removed during the course of the experiment, a reasonably good sample of the ragwort was obtained.

Alkaloid Standards. Senecionine was supplied by ChromaDex Inc., Irvine, USA. A stock solution of 1 mg/mL was prepared in methanol.

Liquid Chromatography. A 1200 series LC system (Agilent Technologies, Santa Clara, CA, USA) including a vacuum solvent degassing unit, a binary high pressure gradient pump, an automatic sample injector, and a column thermostat was used. LC separation was achieved on a 150 mm × 2.1 mm i.d., 3 μm, Atlantis dC18 column (Waters, Manchester, U.K.). The mobile phase was aqueous 0.1% acetic acid solution (A) and acetonitrile (B). The initial gradient condition was 80% A and 20% B changing linearly to 50% B over 20 min, held for 20 min, changed to 100% B over 15 min, and held for 10 min. After analysis, the column was equilibrated for 10 min. The flow rate was 0.3 mL/min, and the injection volume was 5 μL.

Mass Spectrometry. Mass spectrometry was performed in positive electrospray mode using a 6200 series MSD TOF instrument equipped with a dual spray electrospray source (Agilent Technologies, Santa Clara, CA, USA). The drying gas (350 °C, 9 L/min) and nebulizer gas (40 psi) were nitrogen. The capillary voltage was 4000 V, skimmer 60 V, and octopole RF voltage 250 V. Mass spectra were acquired over the range m/z 100−1100 with a spectral acquisition rate of 0.89 s per spectrum. Real time mass correction was performed using a solution including purine ($C_5H_4N_4$ at m/z 121.05087) and hexakis (1*H*,1*H*,3*H*-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$ at m/z 922.00980).

For the identification of PAs in the sample chromatograms, a database was prepared containing information on PAs known to occur in *S. jacobaea* (*8*, *27*). The database contained the empirical formulas and accurate masses for the parent PAs, their *N*-oxides, and their sodium, potassium, and ammonium adducts.

Replicated (×5) extractions and analysis of a dried ragwort sample showed that for PA levels of about 4 to about 100 mg/kg the standard deviation was less than 4 mg/kg, except for seneciphylline *N*-oxide (41 to 82 mg/kg, sd 17%). In view of the likely high variability associated with the decomposition and sampling of plants in different parts of the bag, the analytical uncertainties were considered insignificant.

Preparation of Ragwort Samples. The ragwort plants were freeze-dried and powdered. Portions (5 g) of the dried ragwort were extracted overnight with methanol in a Soxhlet extractor. The methanol was evaporated to about 80 mL and made up to exactly 100 mL with methanol. Because of the high mass accuracy of TOF-MS and the resulting ability to accurately extract the ions of interest, no cleanup was required.

RESULTS AND DISCUSSION

PAs in the Ragwort Samples. A comparison was made of the accurately determined molecular mass of the compounds detected in the ragwort samples by LC-TOF-MS with the database entries. PAs identified were jacoline, jacoline *N*-oxide, jacozine, jacobine, jaconine, jacobine *N*-oxide, seneciphylline, seneciphylline *N*-oxide, and senecionine. It was not possible to distinguish between the TOF mass spectra of PAs with the same empirical formulas, notably between jacobine and eruciflorine, between jacozine and erucifoline, or between their *N*-oxides. **Figure 2** shows the ion chromatograms for the major PAs obtained from the LC-TOF-MS of a ragwort extract with the identified PA peaks labeled. The plant extracted was from a population different from that of the decomposition samples and shows jacoline *N*-oxide, which was not seen in the test samples. The TIC traces were

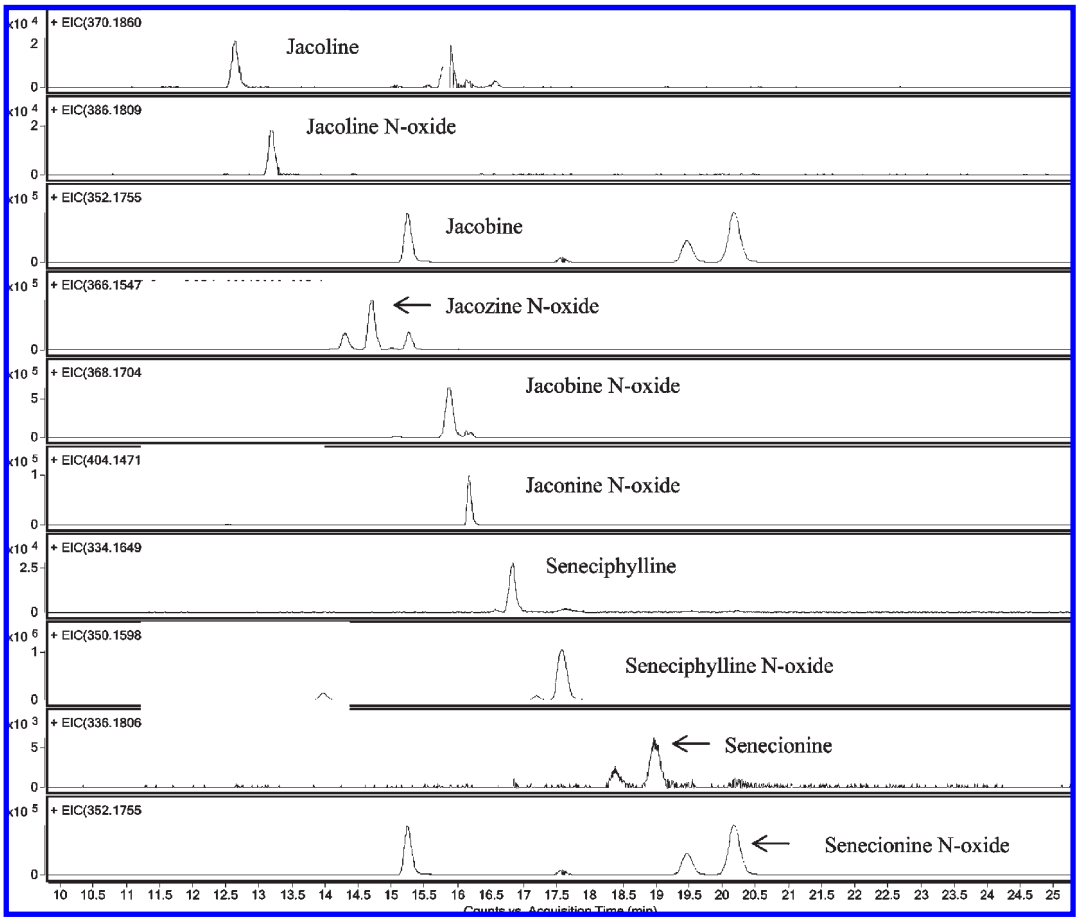

Figure 2. Chromatogram obtained from the LC-TOF-MS of an extract of fresh ragwort.

more complex with overlapping peaks. As mentioned above, no sample cleanup was applied due to the high mass accuracy of TOF-MS and the resulting ability to accurately extract the ions of interest. **Figure 3** shows confirmation of the identity of jacobine and jacobine *N*-oxide as examples. The mass spectrum of the jacobine peak shows the molecular mass of protonated jacobine (*m*/*z* 352.1747). The mass spectrum of jacobine *N*-oxide contained an ion corresponding to the molecular mass of protonated jacobine *N*-oxide (*m*/*z* 368.1698) and also a much smaller ion at *m*/*z* 735.3312 corresponding to the protonated dimer of jacobine *N*-oxide. The mass spectrum of the peak identified as senecionine *N*-oxide also contained evidence of the dimer.

The mass error associated with the measured mass spectra of all of the PAs ranged from 3 to 12 ppm. The maximum errors (11−12 ppm) were for the peaks identified as senecionine, jacozine *N*-oxide, and jaconine *N*-oxide.

The approximate concentrations of the PAs were determined by measuring the response for a portion of the zero-time sample to which a single point standard addition of senecionine had been made. The response factor for senecionine was used for the estimation of all PAs in the sample before storage in the bag. The semiquantitative results are reported in **Table 1**. Jacobine and seneciphylline were the major PAs, at levels of about 50 and 40 mg/kg dry weight, respectively, as the free base and about 130 and 70 mg/kg, respectively, as the *N*-oxides. The total PA concentration was approximately 350 mg/kg of which about 250 mg/kg was *N*-oxides.

Table 1 shows the decline in the PA concentration with storage. The decline in the level of jacobine, the major PA

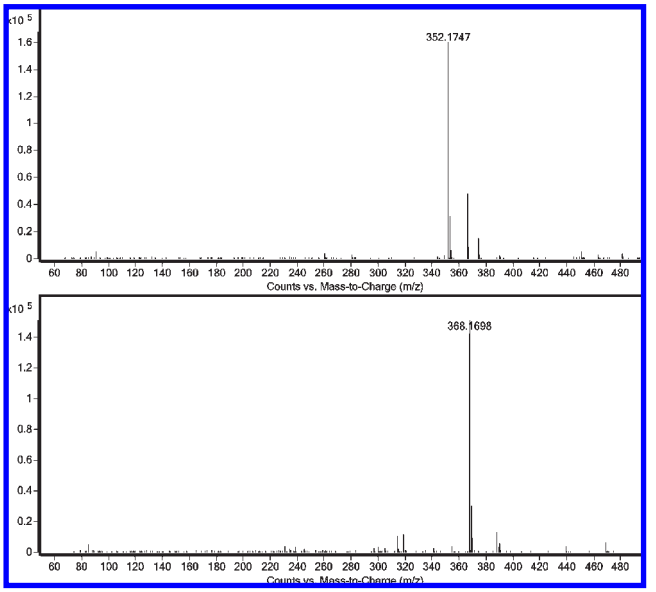

Figure 3. Mass spectrum of the peak identified as jacobine/erucifoline (**top**) or jacobine/erucifoline *N*-oxides (**bottom**).

was particularly rapid, with no jacobine and very little jacobine *N*-oxide being detected after only two weeks. Senecionine and seneciphylline were more persistent when not present as oxides. The decline in PA level is illustrated diagrammatically in **Figure 4** where the percentage of the original PA present in the fresh sample is plotted against time in the bag. The data show a reasonably close fit to an exponential line despite the high variability: each sampling

Table 1. Semiquantitative Results: Levels of PAs in the Stored Samples (mg/kg Dry Weight Basis)[a]

Week	0	2	4	6	8
jacoline	2	-	-	-	-
jacobine[b]	51	-	-	-	-
seneciphylline	37	21	19	11	4
senecionine	4	3	2	4	1
total free PAs	94	24	21	15	6
Jacoline N-oxide	-	3	-	-	-
jacozine *N*-oxide[c]	17	1	-	-	-
jacobine[b] *N*-oxide	129	6	2	1	-
jaconine *N*-oxide	3	2	-	-	-
seneciphylline *N*-oxide	68	7	9	2	-
senecionine *N*-oxide	31	1	1	-	-
total PA *N*-oxides	248	20	12	3	1
total PAs	342	44	33	19	6

[a] Note: no PAs were detected in samples from weeks 10, 12, and 14. (-) Less than 1 mg/kg. [b] Or eruciflorine. [c] Or erucifoline N-oxide.

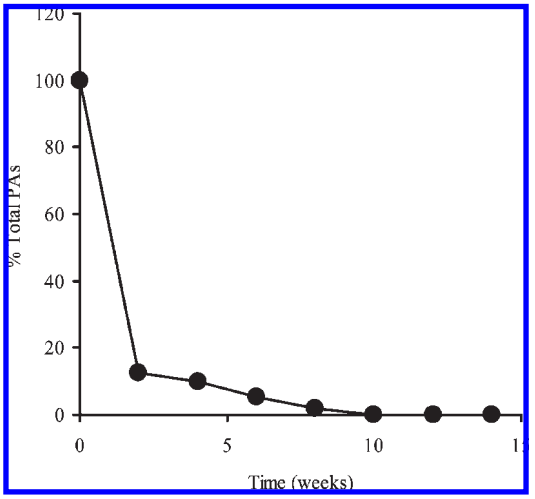

Figure 4. Decline in PA levels on bag storage.

point corresponding to a plant within a large bag. It is of interest that no increase was seen in the proportion of *N*-oxide as the reduced form of the PA decreased. This suggests either that the decomposition route is not via oxidation or that the *N*-oxides are themselves even less stable, and therefore, no accumulation is seen. The decay rates of the free bases and the *N*-oxides were similar. Bradbury and Willis (*15*) showed that in acid conditions the degradation products of *S. jacobaea* were retronecine and a dehydration product of retronecine, but these compounds were not observed in the LC-TOF-MS chromatograms.

Candrian et al. (*14*) found that PAs from *Senecio alpinus* decomposed in silage but not in hay and dried fodder. In silage, levels decreased over two months. Contrary to what was found in the current experiments, the ratio of free alkaloids to *N*-oxides was generally greater than 1. The quantitative results for the PA levels agree with the limited data presented elsewhere for *S. jacobaea*, which are restricted to a few values for flowers, where typically 400 to 1700 mg/kg were measured (*28*, *29*). PAs accumulate in seeds and flowers, and it would therefore be expected that levels in the entire plant would not reach these values. *S. jacobaea* sampled over three years from five locations on the west coast of the US contained between 0.02 and 0.91% dry weight of PAs with a mean of 0.31% of which 27% was in the form of *N*-oxides (*30*). The proportions of alkaloid present as *N*-oxide have also not been reported previously with sufficient authority to make comparison with the results presented here, which show high levels of jacobine and seneciphylline *N*-oxides. Also, it must be remembered that the levels reported here are based on an assumption of equal response factors in the LC-TOF-MS analysis and so are semiquantitative estimates.

S. jacobaea plants have been classified into three chemotypes containing PAs of the jacobine, senecionine, or erucifoline types with the divisions having a genetic basis (*31*). The plants we have analyzed contained roughly similar proportions of jacobine/eruciflorine and senecionine but cannot easily be assigned to a chemotype.

The results have demonstrated that the LC-TOF-MS instrument is useful for the determination and confirmation of PAs in weeds or other plant samples. The most valuable aspect of the LC-TOF-MS is the ability to provide accurate molecular mass information allowing the empirical formulas to be matched against a database without the need for authentic standards.

The methodology described here will, with validation, be useful toward meeting EFSA's recently expressed requirement for methods and analytical data on PAs in feed. The data obtained will add to our information on the toxins present in ragwort in the U.K. The demonstration of the loss of toxic PAs on storage of ragwort in simple bags for a brief period of summer shows this method as suitable for detoxifying the plant, demonstrating that the environmentally unfriendly practice of incineration is unnecessary. There is considerable scope for improving our understanding of the levels of PA toxins in ragwort in the U.K., their variation with geographical location, variety, season, and plant maturity, and means of removing the toxins by composting techniques similar to those described here.

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