Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows

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Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows

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Abstract

Pyrrolizidine alkaloids (PAs) are toxins present in many plants belonging to the families of *Asteraceae*, *Boraginaceae* and *Fabaceae*. Particularly notorious are PAs present in ragwort species (*Senecio*), which are held responsible for hepatic disease in horses and cows and may lead to the death of the affected animals. In addition, these compounds may be transferred to edible products of animal origin and as such be a threat for the health of consumers.

To investigate the possible transfer of PAs from contaminated feed to milk, cows were put on a ration for 3 weeks with increasing amounts (50-200 g day⁻¹) of dried ragwort. Milk was collected and sampled twice a day, faeces and urine twice a week. For milk, a dose-related appearance of PAs was found. Jacoline was the major component in milk despite being a minor component in the ragwort material. Practically no N-oxides were observed in milk, notwithstanding the fact that they constituted over 80% of the PAs in ragwort. The overall carry-over of the PAs was estimated to be only around 0.1%, but for jacoline 4%. Notwithstanding the low overall carry-over, this may be relevant for consumer health considering the genotoxic and carcinogenic properties demonstrated for some of these compounds. Analysis of the faeces and urine samples indicated that substantial metabolism of PAs is taking place. The toxicity and potential transfer of metabolites to milk is unknown and remains to be investigated.

Keywords: pyrrolizidine alkaloids, cows, milk, jacoline, ragwort, analysis, carry-over, *Senecio*

Introduction

Pyrrolizidine alkaloids (PAs) are toxic compounds present in many plants belonging to the families of *Asteraceae* (*Compositae*), *Boraginaceae* and *Fabaceae* (*Leguminosae*). Important members of the *Asteraceae* family are ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaeae*) and common groundsel (*Senecio vulgaris*). Hundreds of different PAs have been isolated and characterised from a broad range of PA-containing plants (Molyneux and James 1990; EFSA 2007; Hartmann and Witte 1995). PAs are composed of a necine base and one or two ester groups or a macrocyclic diester. Figure 1 shows some important representatives. Even within one species there may be more than 10 different PAs present (Hartmann and Witte 1995; Joosten et al. 2009, 2010). Furthermore, the composition (and concentration) may fluctuate with respect to climate and environmental conditions, the age and part of the plant, and the variety (genotype/chemotype). The PAs in ragwort and groundsel are generally of the macrocyclic diester type and in the N-oxide form, but can be easily reduced to a tertiary amine (Hartmann and Toppel 1986; Lindigkeit et al. 1997).

In a number of countries, like the Netherlands, the incidence of ragwort appears to be increasing. Due to their bitter taste, PA-containing plants are generally unpalatable and normally avoided by grazing animals in the field. However, in preserved and composed feeds, this recognition is lost and the toxic PAs may be consumed by livestock. PAs for long have been recognized as toxic for livestock (Bull et al. 1968), causing serious effects on the liver which may eventually cause the death of the animal. Nevertheless they have not been listed as undesirable substances and thus far the European Commission has not established permitted levels for PAs in animal feed stuffs.

PAs may also endanger human health either directly by consumption of PA-containing plants but also indirectly through animal derived food products. Various PAs have been shown to have genotoxic properties and to cause tumours in rodents (Fu et al. 2004; EFSA

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3 61 2007). As a result it might be assumed that there is no threshold for these effects, meaning
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5 62 that even a small dose may potentially cause tumours. EFSA did not draw this conclusion,
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8 63 based on the fact that no epidemiological studies have been performed to show that exposure
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10 64 to PAs results in increased cancer cases in humans (EFSA 2007). The National Institute for
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12 65 Public Health and the Environment (RIVM) of the Netherlands used a linear extrapolation to
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15 66 arrive at the dose corresponding to an increased risk of 1 extra cancer case per million people
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17 67 exposed, being 30 ng per person per day (virtual safe dose or VSD) (van der Zee 2005). This
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20 68 VSD is derived from a rat study with riddelliine but there are no studies to show whether a
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22 69 VSD for other PAs would be different. Regarding this very low VSD, it is essential to
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25 70 evaluate the potential risk of ragwort and related plants for the consumer.

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27 71 Various studies have been performed to study the presence of PAs in honey, showing
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29 72 significant levels in some retail honey and especially when honey is collected near fields with
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31 73 PA containing plants (Crews et al. 1997; Edgar et al. 2002; Boppré 2008). PAs may also be
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34 74 present in other food products like milk. In order to evaluate the potential transfer of PAs
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36 75 from feed stuffs to milk, Dickinson et al. (1976) dosed 4 lactating cows via rumen fistula with
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38 76 4 to 5 kg of dried ragwort (corresponding to 10 g kg⁻¹ body weight per day) for a period of 5-7
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41 77 days and then gradually decreased the dose by 50-75% after 14-26 days. The PA content of
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43 78 the dried material was 1.5 g kg⁻¹. The relatively high dosage in this experiment resulted in a
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46 79 decrease in both body weight and milk production after 5 days. Livers of the animals were
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48 80 clearly affected by the treatment. Concentrations determined in milk were in the range of
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51 81 470-840 µg l⁻¹. Due to the limited availability of standards, only a few PAs could be detected
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53 82 in the ragwort, like jacobine, seneciphylline, jacoline, jaconine and jacozine. N-oxides were
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55 83 not analysed for in this study. Jacoline was the only PA identified in the milk. Taking into
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57 84 account the amount of ragwort fed to the cows, the milk yield and the PA concentrations in
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60 85 ragwort and milk, it was calculated that about 0.1% of the PAs was transferred to the milk.

Deinzer et al. (1982) carried out a study on milk of goats dosed via a rumen cannula with 10 g dried ragwort per kg bw per day. The PA content of the ragwort was estimated at 3 g kg⁻¹ dry weight. Using a gas chromatographic method that was based on the reduction of all free bases to a single derivative of retronecine, a total PA content in the milk of 330 to 810 µg l⁻¹ was reported. The transfer to milk was estimated to be around 0.1% of the daily dose. The authors also did not consider or investigate the possible presence of N-oxides in the ragwort material, nor in the milk.

Although these studies indicate that the transfer could be relatively small, the eventual concentrations in milk may still present a considerable risk for the consumer. The VSD described above would be reached by a lifelong daily consumption of only 0.1 ml of the milk from the Dickinson and Deinzer studies. However, the animals in these studies received unrealistic high levels of ragwort. On the other hand, not all PAs were analysed, like the N-oxides which normally constitute the majority of the alkaloids present in plant material. Molyneux and James (1990) reviewed the existing data and pointed to the possibility that in addition to the free bases also the N-oxides might be transferred to milk and actually be responsible for at least a part of the toxic effects observed in animals given milk from exposed mother animals. Therefore it was decided to improve the analytical methods to determine PAs and to repeat the Dickinson study using much lower levels of ragwort, unlikely to cause adverse effects in the animals. The present paper confirms that jacoline was the major PA in ragwort transferred to the milk, although it was only a minor component in the plant material itself. At the same time the study indicates that a major part of the PAs is metabolized possibly resulting in metabolites that are still a potential health hazard to the consumer.

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Materials and Methods

Materials

Analytical samples of a number of PA tertiary amine standards (senecionine, seneciphylline, retrorsine, senkirkine, otosenine, heliotrine) were obtained from commercial sources (Phytolab, Vestenbergsgreuth, Germany; Phytoflan, Heidelberg, Germany; Latoxan, Valence, France). Jacobine, jacoline, erucifoline and florosenine were isolated from plant material by PRISNA (Leiden, The Netherlands). Integerrimine was a gift from Dr. Trigo (UNICAMP, Campinas, Brasil). Riddelliine and riddelliine-N-oxide were a gift from Dr. Chou (NCTR, Jefferson, AR, USA). Acetylseneciphylline was prepared by acetylation of seneciphylline with acetic anhydride and pyridine. N-oxides of senecionine, seneciphylline, retrorsine, integerrimine, acetylseneciphylline, jacobine and erucifoline were prepared by N-oxidation of the corresponding tertiary amines with 30% hydrogen peroxide according to Chou et al. (2003). N-oxide standards of senecionine, seneciphylline and retrorsine have recently become available from the above mentioned vendors as well. The standards were at least 90% pure according to LC-MS/MS analysis. In total a set of 20 reference standards was available for this study.

Adult plants of ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaea*) and narrow-leaved ragwort (*Senecio inaequidens*) were collected during June and July 2008 at various sites around Wageningen and Nijmegen in The Netherlands. The materials were subsequently air dried, cut into pieces, ground to 1 mm with a Peppink 200 AN grinding machine (Veerman, Olst, The Netherlands), and homogenized. In total 16.6 kg of dry material was produced, being a mixture of 84% ragwort and 16% narrow-leaved ragwort. The material was stored in the dark at room temperature.

133 *Animal treatment*

134 Three dairy cows (Holstein Friesians) were treated at the Waiboerhoeve in Lelystad (part of
135 Livestock Research, Wageningen UR). Animals were 4-5 years of age, weighed 600-700 kg
136 and were fistulated in the rumen. Cows produced around 40 litres of milk per day.
137 Throughout the experiment they received a diet of grass and corn silage (60/40 w/w) and in
138 addition soy and minerals. The treatment period consisted of five phases, each lasting one
139 week. In week 1, animals received no ragwort (adaptation/control period), in week 2 they
140 received two daily doses of 25 g, in week 3 two daily doses of 50 g, in week 4 two daily doses
141 of 100 g and in week 5 no ragwort (depletion period). The dried ragwort was introduced
142 directly into the rumen through the fistula in the morning and afternoon just after milking.
143 Milk was collected twice daily around 6.00 and 16.30. In addition, urine and faeces samples
144 were taken twice a week between 10.30-12.30, 4-6 hours after dosage of the ragwort. Milk,
145 urine and faeces samples were kept frozen at -20°C until analysis. The experimental plan was
146 evaluated by an ethical committee prior to the study. The health of the animals was closely
147 monitored by a veterinarian. One cow experienced mastitis during week 2. Another cow
148 encountered a brief period of indigestion at the end of week 4. Both cows were treated with
149 standard medication.

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151 *Chemical analysis*

152 LC-MS/MS was used for the detection and quantification of PAs in biological matrices such
153 as plant material, milk, urine and faeces. It allows the simultaneous detection of both forms of
154 PAs, the free base (tertiary amine) and the N-oxide form (Xiong et al. 2009a; Joosten et al.
155 2010; Crews et al. 2010). LC-MS/MS is a very selective and sensitive technique capable to
156 detect compounds at low concentrations. In milk, sub-ng ml^{-1} to ng ml^{-1} concentrations were
157 expected to be present, considering the relatively low dosing regime and the reported low

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3 158 transfer rate to milk. Therefore milk samples were concentrated prior to analysis. Urine and
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5 159 faeces samples were purified by an solid phase extraction (SPE) procedure to remove matrix
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8 160 interferences. PA concentrations in the plant material were relatively high. To obtain samples
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10 161 that contained PAs in concentrations matching with the linear range of the mass spectrometer,
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12 162 the plant extracts were diluted. No additional clean-up by SPE was necessary.
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17 164 *Analysis of ragwort*
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20 165 The analysis of the homogenised plant material was conducted according to the method
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22 166 described by Joosten et al. (2010). To assess the PA composition and homogeneity of the
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24 167 dried ragwort material, 14 samples (5-10 g) were randomly taken from the homogenized
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26 168 material. From each sample two subsamples of 0.5 g were taken and transferred to 50 ml test
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28 169 tubes. Heliotrine (100 µl of a 100 µg ml⁻¹ solution in methanol) was added as internal
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30 170 standard. Twenty-five ml of a 2% formic acid solution in water was added and the samples
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32 171 were extracted by rotary tumbling for 1 h. The extracts were filtered over a glass microfiber
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34 172 filter (Whatman 1820-150). A 25 µl aliquot was taken from the resulting clear extract,
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36 173 transferred to an HPLC vial and mixed with 975 µl of a 10 mM ammonia solution in water.
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38 174 The sample extracts were injected on the LC-MS/MS in a randomized order. Quantification
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40 175 was performed with internal standard correction against a 6-point calibration curve of PA
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42 176 standards (0-500 ng ml⁻¹) in a diluted extract of tansy (*Tanacetum vulgare*). The extract of
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44 177 tansy was prepared the same way as the ragwort extracts and was used to mimic a blank plant
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46 178 extract. Homogeneity was checked by means of the ANOVA method described by Fearn and
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48 179 Thompson (2001). The material was found sufficiently homogeneous. Relative standard
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50 180 deviations for the concentrations obtained were generally less than 10% for the major
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52 181 components and less than 15% for the minor components. Although the plant extracts were
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54 182 diluted 40 times to match the concentration of the major components with the linear range of
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the MS detector, many minor components could be adequately detected and quantified. The limit of quantification (LOQ) for the individual PAs and their N-oxides in dried plant material was between 0.2 and 0.5 $\mu\text{g g}^{-1}$.

Analysis of milk

The PA content in milk was assessed by means of LC-MS/MS according to an in-house validated protocol (van den Top 2007). In short, test portions of 3.0 ml of thawed milk were transferred to test tubes and stored for at least 4 hours at -20°C . After the samples were allowed to thaw at room temperature, 7.0 ml of methanol containing 0.1% formic acid was added and the samples were mixed well. The mixtures were again placed at -20°C for at least 4 hours. They were taken from the freezer and immediately centrifuged (10 min at 1950g, 4°C) to obtain clear supernatants. From the supernatants 5.0 ml was taken and evaporated to dryness under a gentle stream of nitrogen at 55°C . The dried residues were resuspended by vortex mixing in 600 μl water containing 0.1% acetic acid and subsequently centrifuged at 1950 g for 10 min. An aliquot (300 μl) was filtered over a 0.45 μm Eppendorf filter and transferred to an HPLC vial. The sample extracts were analysed using LC-MS/MS and quantified against a 5-point calibration curve of PA standards in 0.1% acetic acid in water. During the analytical sessions, duplicate determinations for some test portions were performed and to all milk test portions and heliotrine was added at 100 ng ml^{-1} as an internal standard to correct for recovery. Blanks (determination without test portion) were performed at regular intervals. Method recovery percentages per day were used to correct for recovery. The results of the duplicate determinations were within the expected variability (measurement uncertainty at validation level ranged from 44 to 67%). In the blanks no PAs were found. All samples of evening milk were analysed in single. The samples of the evening milk taken during the period of the highest dosage were analysed a second time, together with the milk

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3 208 collected in the morning. Results presented for the evening milk are the average of the
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5 209 duplicates. The LOQ in milk for the individual PAs and their N-oxides was between 0.05 and
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12 212 *Analysis of urine and faeces*
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15 213 The analysis of PAs in urine and faeces was based on an in-house validated method for the
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17 214 determination of PAs in animal forage (Oosterink and Mulder 2008). Central part of this
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19 215 method is the use of SPE for purification and concentration of the sample extracts. The SPE
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21 216 clean-up step developed for forage could be used without modifications for urine and faeces.
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23 217 Matrix matched standards together with heliotrine as an internal standard were incorporated in
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25 218 the method to correct for differences in recovery and matrix effects.
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28 219 Urine samples were thawed overnight at room temperature. Two aliquots (2 ml) of each
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30 220 sample were transferred to 10 ml test tubes and 4 ml 0.1% aqueous ammonia solution was
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32 221 added. The pH of the extract was checked with a pH-stick and adjusted to pH 10-11 if
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34 222 necessary. Following addition of heliotrine (50 μl of a 1 $\mu\text{g ml}^{-1}$ solution in methanol), the
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36 223 extracts were shaken manually and purified by solid phase extraction (SPE) over Strata-X 60
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38 224 mg, 3 cc cartridges (Phenomenex, Torrance, CA, USA). Cartridges were conditioned with 3
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40 225 ml methanol and equilibrated with 3 ml water. After application of the sample extracts, the
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42 226 cartridges were washed with 3 ml 1% aqueous formic acid solution, followed by 3 ml 1%
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44 227 aqueous ammonia solution. The cartridges were dried under reduced pressure and eluted with
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46 228 3 ml methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen at
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48 229 50°C and the dry residues were reconstituted in 1 ml water/methanol 9:1 (v/v). Subsequently,
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50 230 10 μl was injected on the LC-M/MS system. Quantification was performed with internal
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52 231 standard correction against a six-point matrix matched calibration curve of PA standards (0-
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54 232 250 ng ml^{-1}) in blank urine. For each cow individual calibration curves were constructed

using a urine sample from week 1 (pre-administration period). LOQs ranged between 0.2 and 0.5 $\mu\text{g l}^{-1}$ for most PAs. In general, the free bases were somewhat more sensitive than the N-oxides.

Deconjugation of urine samples was carried out as follows: two aliquots (2 ml) of the urine sample were transferred to 10 ml test tubes. The pH of the urine was adjusted to 4.8 with concentrated acetic acid. *Helix pomatia* extract (20 μl) (Merck, Darmstadt, Germany) was added and the sample extracts were incubated for 16 h at 37°C in a water bath. The extracts were processed as described above. For each cow an individual matrix matched calibration curve was included in the same concentration range as for the non-hydrolysed samples. The matrix matched standards were subjected to the deconjugation procedure.

Faeces samples were thawed overnight at room temperature. Samples were homogenized by hand with a spoon. Two aliquots (2 g) were transferred to 50 ml test tubes. To the test samples 40 ml 2% aqueous acetic acid was added together with heliotrine (50 μl of a 1 $\mu\text{g ml}^{-1}$ solution in methanol). The mixtures were extracted by rotary tumbling for 1 h and subsequently centrifuged at 3500 rpm for 10 min. Twenty ml of the supernatant was transferred to a 50 ml test tube and the pH was raised to 10-11 with concentrated ammonia. The extracts were purified by SPE as described above for the urine samples. The final extracts were prepared in 500 μl water/methanol 9:1 (v/v). Quantification was performed with internal standard correction against a six-point matrix matched calibration curve of PA standards (0-25 ng ml^{-1}) in blank faeces. For each cow individual calibration curves were constructed using a faeces sample from week 1 (pre-administration period). LOQs were comparable to those in urine.

Instrumentation

Two analytical methods were developed and validated independently from each other at two laboratories (RIKILT and RIVM). The two different LC-MS/MS gradients used in this study were an acidic gradient for the analysis of the milk samples and an alkaline gradient for the plant, urine and faeces samples. However, performance of both methods was comparable. The pH of the mobile phase has a pronounced effect on the elution of the PAs, most notably the N-oxides. These elute a couple of minutes before the free bases under alkaline conditions and elute just after the free bases under acidic conditions. The elution order of the specific PAs is not affected by the pH of the mobile phase.

Analyses of plant extracts, urine and faeces samples for PA content were performed on a Waters Acquity UPLC coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA), operated in positive electrospray mode. The compounds were separated on a Waters UPLC BEH C18 150 x 2.1 mm, 1.7 μ m analytical column, kept at 50°C and run at 0.4 ml min⁻¹ with an acetonitrile/water gradient containing 6.5 mM ammonia. The gradient started at 100% water and was changed to 50% acetonitrile in 12 min. Total runtime of the method was 15 min.

Analysis of milk samples was performed on a Waters Acquity UPLC coupled to a Waters Quattro Ultima Pt tandem mass spectrometer (Waters, Milford, MA, USA). Compounds were separated on a Waters UPLC BEH C18 100 x 2.1 mm 1.7 μ m analytical column, kept at 50°C and run at 0.35 ml min⁻¹ with a gradient of acetonitrile and water containing 0.1% acetic acid. The gradient started at 100% water and was changed to 15% acetonitrile in 18 min. The column was washed with 80% acetonitrile for 3 min to remove slow eluting lipids. Total run time was 25 min.

For both systems the MS/MS collision energy was optimized for each individual compound using reference standards or plant extracts when standards were not available. Two precursor product ion transitions were selected and incorporated in a multiple reaction

monitoring (MRM) method. The dwell time for each transition was set at 20 msec (Premier XE) or at 30 msec (Ultima Pt). A total of 49 transitions was monitored in a single run to include all potentially relevant PAs. In Table 1 an overview is presented of the mass spectrometric settings used for the detection of the relevant PAs. All compounds could be unequivocally characterized on the basis of retention time and fragmentation transitions. With the final method approximately 50 compounds (including 10 metabolites) were monitored. For those compounds for which no reference standard was available, a semi-quantitative (indicative) value could be obtained by comparison with a closely related analogue (often an isomer), which exhibited a similar MS/MS fragmentation spectrum. In these cases the same transitions were used. For some compounds (e.g. jaconine and its N-oxide) no closely related standard with similar MS/MS spectrum could be identified. In such cases the concentration was estimated by taking the sum of the two most intense fragments and comparing this with the sum area of the transitions selected for the most closely related standard (e.g. jacobine and its N-oxide).

Data modelling

Transfer modelling consisted of regression analysis of the PA concentration in evening milk during the exposure weeks 1, 2, 3 and 4 against the PA amount which has been administered as a single bolus dose in the rumen, i.e. the amount administered with 25, 50 or 100 g of the ragwort material. In this analysis the PA concentrations during each of the exposure weeks were averaged for each of the three individual animals (the PA concentrations were in fact considered constant during each exposure week). These averages + SD were then used as the data point in the regression analysis which resulted in a single coefficient characterising the transfer from feed to milk. Next to this coefficient the percentages of the total doses which were administered during one week transferred to milk were calculated.

The milk elimination half-life of jacoline, jacobine, jaconine, senkirine, otosenine and florosenine was determined using the evening milk concentrations on day 7 of the highest dose administered and day 1 to 7 of week 5 (no administration of ragwort material). On day 1 of week 5 also concentrations in morning milk were available and used in the calculation of the milk elimination half-life. A mono-exponential pharmacokinetic approach (decay to background concentration) was used to determine the milk elimination half-life:

$$C(t) = Bg + C_l.exp(-k_l.t)$$

C is the concentration of PAs in milk, C_l is model parameter for concentration level, k_l is a concentration decay rate and Bg is the supposed background level. The model parameters were fitted to the data with a relative error data model (an absolute error data model leading to a less optimal description of the data).

Results and Discussion

Animal status

Three cows were fed with 0, 50, 100, 200 and again 0 g of dried plant material daily, each time for a period of 7 days and divided over two daily doses. The animals were exposed to relatively low doses, not only to mimic a realistic situation, but also to prevent an intoxication of the animals. The total dose (2.5 kg) given to the animals was estimated to be less than 10% of the lethal dose (CliniTox, 2009). The health of the animals was carefully monitored during the duration of the experiment.

Milk samples taken 3 days after changing the dosage were examined for protein, fat and lactose. There were no effects of the treatment on these parameters. Protein content e.g. was 3.1 ± 0.2 % during the first week without ragwort, and 3.0 ± 0.3 , 3.3 ± 0.4 , 3.3 ± 0.3 and 3.1 ± 0.2 during the daily treatment with 50, 100, 200 and again 0 g ragwort. However, no decline in milk production or any signs of intoxication were observed. Figure 2 shows the milk production of the three cows during the entire study. One cow (#2) showed an indigestion at the end of the fourth week, being the end of the period on the highest PA-dose. This immediately affected the milk yield. Furthermore, another cow (#1) showed mastitis at the end of the second week. In both cases treatment with standard medication was successful and the animals quickly recovered. However, both events are not uncommon for dairy cattle and were very likely not related to the administration of ragwort.

The absence of an effect on the milk production clearly distinguishes our study from that of Dickinson et al. (1976) in which a rapid decline in milk production was observed, possibly affecting the excretion of PAs in the milk.

PA levels and composition in ragwort

The plant material used in this study consisted primarily (84%) of ragwort (*Senecio jacobaeae*). Analysis of individual samples of ragwort showed minor differences between the locations. The ragwort consisted primarily of jacobine, jaconine, erucifoline, senecionine and seneciphylline and their corresponding N-oxides. The remainder (16%) was dried narrow-leaved ragwort (*Senecio inaequidens*). Major alkaloids in this plant were retrorsine, senecivernine and their N-oxides. Narrow-leaved ragwort also contained small amounts of otonecine-type PAs, such as senkirkine, otosenine and florosenine.

The homogeneity of the dried and mixed plant material was assessed by ANOVA (Fearn and Thompson 2001) and was found sufficient. Table 2 shows the concentrations of the

various PAs and their N-oxides in the final material. Figure 3A shows the pattern of PAs in this material. The total concentration of PAs was 2.3 g kg^{-1} on a dry weight basis, with 82% of the PAs in the N-oxide form and 18% as free bases. Jacobine and jaconine contributed relatively strongly to the overall free base content, in accordance with Joosten et al. (2010). Jacoline and its N-oxide contributed respectively 1.0 and 0.7% to the overall PA content. Based on the overall content, during the 5 different weeks, the cows were exposed to 0, 115, 230, 460 and 0 mg PAs per day respectively, divided over two dosages per day.

PA concentrations in milk

Analysis of milk samples revealed the presence of several tertiary bases but no N-oxides. Jacoline, jacobine, jaconine, senkirkine, otosenine and florsenine were the PAs found at concentrations above the limit of quantification (LOQ). Table 2 includes the average concentrations for these PAs in the milk of the three cows during the period of the 200 g day^{-1} dose. The pattern in milk is shown in Figure 3B. When compared with the ragwort mixture (Figure 3A), a relatively high contribution of jacoline ($80.1 \pm 2.9\%$ of the total PA content; mean $\pm$ SD for the 3 cows during the highest dosage) was observed. In the ragwort mixture, the amount of jacoline free base was only 1% of the analysed PAs. Jaconine was the second most important PA in milk contributing $9.4 \pm 2.9\%$, which is more in line with the content in the plant material (5.1%). Senkirkine, otosenine and florsenine together contributed for $7.4 \pm 1.5\%$ to the PA content in milk. This is a relatively large contribution, considering the approximate 1% contribution in the plant material.

Figure 4A shows the concentration of jacoline in the milk of the three cows during the different treatment periods. Levels increased rapidly following the change to a higher dosage. There were some temporary declines in the concentrations which for cow 2 coincided with the indigestion (Figure 2). For the other two cows there was no explanation. Average total PA

levels during the 3 different dosage periods were 2.1, 5.5 and 9.7 ng ml⁻¹. When the highest total PA levels, obtained at the 200 g day⁻¹ treatment, are set at 100%, the average level in the 50 and 100 g day⁻¹ period were 26 and 55% respectively, in line with the dosage. For jacoline these figures were 22, 58 and 100%. Following termination of the treatment, total PA concentrations rapidly declined to about 30% at the end of the first and 2% at the end of the second day.

To check for a potential difference in the PA content of the morning and evening milk samples, morning milk samples of the 4th week, i.e. the period with the highest dosage, were also analysed. The average concentrations are presented in Figure 4B. The small but consistent differences found between morning (M) and evening milk (E) probably reflect the differences in time between dosage and milking (10 h for the evening milk and 14 h for the morning milk with 54% of the daily milk production in the morning milk), and suggest that the majority of transfer of the PAs to milk occurs in the first hours after dosing, after which dilution occurs with more milk being produced. Similar patterns have been found for lactose, fat and protein content of morning and evening milk (Meijer, personal comment).

Based on the milk production of about 40 litres per day and a total PA concentration of 10 µg l⁻¹ during the highest dosage level, the overall amount excreted in the milk was 400 µg PAs day⁻¹ or about 0.1% of the overall daily dose of PAs (N-oxides + free bases). This figure is similar to that calculated from the study of Dickinson et al. (1976) but that study only included the free bases. When compared to only the free-base PAs in the ragwort (18% of total), the overall transfer was 0.5%. Regarding jacoline, about 4% of this PA and its N-oxide present in the plant material ended up in the milk as the free base, or 7% when only jacoline as free base is considered. Panariti et al. (1997) dosed sheep for 5 days with about 30 mg radiolabelled seneciphylline per day and observed milk levels up to 1 mg l⁻¹. This would correspond to a transfer rate of 3%, being much higher than observed in our study for this

compound. However, the levels were based solely on the radiolabel and may as well represent metabolites including macromolecular adducts. Candrian et al. (1991) treated one lactating cow with a single dose of 547 mg radiolabelled seneciphylline and observed milk levels corresponding up to 0.1 mg l^{-1} . The overall amount of radiolabel detected in the milk was 0.16%. However, only part of this appeared to correspond to the free base and some N-oxide. Our study did show traces of hydroxylated metabolites in milk (data not shown). However, no detectable amounts of seneciphylline and its N-oxide were found in milk.

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414 *PA concentrations in urine and faeces*

Urine and faeces samples were collected on two days during each week at the end of the morning, about 4-6 hours after the dosing of the ragwort. Samples were taken directly from the cows. Table 3 shows the total PA concentrations in urine for the three cows. There was a more or less dose-related increase in the concentrations, average levels in the five different weeks being 0, 107, 201, 398 and $3 \mu\text{g l}^{-1}$. In the last week, the samples were taken 2 and 6 days after the last treatment, the samples from day 2 after treatment still showing slightly elevated levels. Most PAs present in the plant material could also be detected in the urine (Table 2). Application of a glucuronide/sulfate deconjugation step in the analysis resulted in a slightly (14%) elevated total concentration. This was primarily due to seneciphylline and erucifoline. Apparently, these PAs are excreted in urine mainly as conjugates, most likely as glucuronides. The concentrations of most other PAs were not affected by the deconjugation step, indicating that these PAs are present primarily in a non-conjugated form. This is clearly demonstrated in Figure 3C, showing the patterns of the urine samples taken during the week on the highest dosage, both before and after deconjugation. Compared to the composition of the original plant material, it is clear that the pattern in urine is rather different. Jacobine-N-oxide was by far the most important PA in urine ($38.4 \pm 4.8\%$; mean $\pm$ SD for the 3 cows)

whereas in the plant material it contributed for only 14%. On the other hand, the N-oxides of erucifoline, retrorsine and senecionine, contributing for respectively 21, 14 and 11% in the plants, made up only 3.9 ± 1.5 , 5.3 ± 2.6 and $1.2 \pm 0.1\%$ in the urine. Jacoline, a minor PA in ragwort (1%) but by far the most important PA in milk, contributed for $14.1 \pm 2.1\%$ in the urine. Also the otonecine PAs are relatively abundant in urine, contributing for 8.5% as compared to 1% in the ragwort material. The change in the pattern clearly indicates that some PAs are more efficiently metabolized than others. Alternatively, some PAs may be transformed into other ones as recently found in a study on the metabolism of adonifoline (the major PA in a related ragwort species, *Senecio scandens*) in the rat (Xiong et al. 2009b). In this study it was shown that adonifoline was metabolised to a number of hydroxylated metabolites and (dehydro)retronecine derivatives. Very recently, glucuronidation by liver microsomes was reported as a new metabolic pathway for PAs (He et al. 2010). In our chromatograms of urine samples several new peaks were present that likely represent hydroxylated metabolites of PAs (free bases and N-oxides) occurring in ragwort. Up to 10 new compounds with masses 350, 352, 366, 368 and 370 were detected, that were not present in the plant material or in the urine samples before the treatment. The most important metabolites are hydroxy-senecionine and hydroxy-integerrimine and their corresponding N-oxides. Accurate quantification was difficult, due to the absence of reference standards, but it was estimated that between 15 to 25% of the excreted PAs in urine were newly formed compounds not present in ragwort. No efforts were undertaken to detect metabolites with a dehydroretronecine moiety or related conjugates.

Since the urine was not collected quantitatively, it is difficult to determine the overall fraction of ingested PAs that was accounted for in the urine. When assuming a total urine production of 40 litres per day, roughly 17 mg of PAs would be excreted in the urine during the period on the highest dosage, as compared to an intake of about 450 mg per day. The

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3 456 identified hydroxy-metabolites would add an estimated 4 mg to this amount. The time lag
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5 457 between the dosing and the time of sampling of the urine is approximately 4-6 hours (morning
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7 458 dosing) and 18-20 h (evening dosing). It is however unlikely that the total amount excreted in
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9 459 urine will be very much higher than estimated on the basis of the 4-6 h sample. This can be
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11 460 derived from the concentrations of jacoline and the otonecine PAs senkirkine, otosenine and
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13 461 florosenine found in the collected urine. The average amount of jacoline detected in urine
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15 462 accounted for approx. 60% of the jacoline present in the ragwort. Senkirkine was present at a
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17 463 concentration which accounted for 34% of the original dose, otosenine for 37% and
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19 464 florosenine for 33%. As will be discussed below, jacoline and the otonecine PAs were also
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21 465 present in faeces in substantial amounts. Other studies have also indicated that extensive
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23 466 metabolism of PAs occurs and that only a minor part of the PAs is excreted unchanged in
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25 467 urine and faeces (Estep et al. 1990; Chu and Segall 1991)

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27 468 Total PA concentrations in faeces samples are shown in Table 3. There was more or less a
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29 469 dose-related increase in the concentrations, on average being 0, 23, 38, 65 and 4 $\mu\text{g kg}^{-1}$ in the
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31 470 5 different weeks. Table 2 shows the concentrations of the different PAs in the samples taken
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33 471 during the week on the highest dosage. The samples contained practically no N-oxides. As in
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35 472 the milk, jacoline was the most important PA, contributing for $36.1 \pm 7.3\%$ to the total PA
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37 473 content (Figure 3D). Other important PAs in faeces were jacobine ($13.2 \pm 4.6\%$), and the
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39 474 otonecine PAs otosenine ($12.4 \pm 1.1\%$), floridanine ($12.0 \pm 2.7\%$) and florosenine ($8.8 \pm$
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41 475 2.4%). Together, these components made up $82 \pm 2\%$ of the PAs in faeces, as compared to 87
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43 476 $\pm 3\%$ in the milk, $23 \pm 3\%$ in urine and only 5% in the original plant material. Based on 40 kg
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45 477 of faeces per day, the total amount excreted in faeces was estimated to be 2.6 mg,
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47 478 representing less than 1% of the original PAs during the highest dosage period. Nevertheless,
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49 479 the average amount of jacoline excreted in the faeces accounted for 19% of the amount
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51 480 present in the ragwort material, that of senkirkine for 3%, otosenine for 27%, florosenine for

14% and floridanine for 115%. Some of the metabolites found in urine were also detected in faeces, albeit at relatively low concentrations. Hydroxy-senecionine and hydroxy-integerrimine were two of the major metabolites also found in urine. The contribution of hydroxylated PA metabolites to the total excretion in faeces was estimated to be between 5 to 10%.

Although it should be clear that urine and faeces samples may not have been collected at the peak of the excretion, both the concentrations and the change in the patterns indicate intensive metabolism. The major question is whether this could result in toxic metabolites or compounds that can be transformed into toxic metabolites, knowing that the toxicity of PAs is caused by reactive metabolites. If so, the major issue would be if such metabolites could actually be present in the milk, and as such present a further risk to the consumer. The studies with radiolabelled seneciphylline indicate that metabolites may indeed be excreted into the milk (Candrian et al. 1991; Panariti et al. 1997).

Modelling of transfer to milk

As shown in Figure 4A, transfer of jacoline to milk quickly led to a rather constant, i.e. “steady state”, concentration in evening milk. In this way, for each of the four dose levels tested, an animal specific “steady state” concentration in milk was obtained. Analysing these levels with simple regression analysis led to a transfer coefficient of $3.7 \times 10^{-3} \mu\text{g l}^{-1}$ per μg of administered jacoline (see Figure 5A). After the exposure was stopped, jacoline rapidly disappeared from the milk (and thus from the animal’s body). The half-life for jacoline in milk was calculated to be 8 hours (Figure 5B).

As with jacoline the other 5 PAs detected in milk quickly reached a “steady state” (data not shown). Transfer coefficients amounted to 3×10^{-5} (jacobine), 7×10^{-5} (jaconine), 5×10^{-4} (senkirkine), and 2×10^{-4} (otosenine and florosenine) $\mu\text{g l}^{-1}$ per μg of administered compound.

506 Elimination half-lives from milk were 8 hours (jacobine), 6 hours (jaconine), 16 hours
507 (senkirkine) and 9 hours (otosenine and florosenine).

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509 *Relevance of the data for other plant species*

510 The rather selective carry-over of jacoline, jaconine and some of the otonecines suggests that
511 not all PAs and as such PA-containing plants may be of equal potential relevance for
512 consumers of milk from exposed animals. It also raises the question which properties are
513 relevant for possible transfer to milk. It seems obvious that the potential of the animal for the
514 biotransformation of individual compounds plays an essential role. The N-oxides, which were
515 prominent compounds in the ragwort material, were not detected in milk and faeces samples.
516 In urine N-oxides were present, accounting for 56% of the excreted PAs. Of the N-oxides
517 excreted jacobine-N-oxide was predominant whereas those of other PAs were almost or
518 completely absent. This indicates that either intensive metabolism or very rapid excretion via
519 urine (depletion in less than 4 h) takes place. However, preliminary studies with liver slices
520 from cows did not show significant degradation of these N-oxides. Similar was true for
521 jacoline whereas other free bases like senecionine, seneciophylline, erucifoline and jacobine
522 were substantially metabolized. In this study there were no indications for the transformation
523 of certain PAs into their N-oxides or into other PAs. Jacoline was the major or second
524 metabolite present in milk, urine and faeces. Assuming that jacoline is not formed by
525 metabolism from other PAs, around 85% of the free jacoline present in the ragwort material
526 was accounted for in milk, urine and faeces. Taking jacoline and its N-oxide together, still
527 50% could be found in these matrices. The otonecine PAs seem to be relatively stable as well.
528 Excretion percentages for these PAs vary from 35% (senkirkine) to over 110% (floridanine).

529 The potential role of the bacteria and other micro-organisms in the rumen or intestines
530 should not be ignored (Mattocks 1971; Aguiar and Wink 2005). Lanigan (1970) showed that

the N-oxide of heliotrine was effectively metabolized by ovine ruminal fluid to the free base and subsequently l-goreensine and 7-hydroxy-1-methylpyrrolizidin. The possibility that e.g. jacoline may also have been formed from other PAs is important when focussing on plants containing PAs that are transferred to milk. Furthermore it should be mentioned that the majority (82%) of the PAs in the ragwort material used was in the N-oxide form. In the studies of Dickinson et al. (1976) and Deinzer et al. (1982) the ragwort material was not analysed for N-oxide content, but it is reasonable to assume that this was not very different from our study. However, upon storage and depending on the drying conditions, the relative contribution of the free bases may become more prominent. Recent screening of animal forages in The Netherlands seem to corroborate this: often the amount of free bases was higher than that of the N-oxides (Mulder et al. 2010). Consequently, a high content of free PA bases in a specific animal feed could lead to a relatively high transfer of PAs to milk.

Conclusions

The present study investigated the potential carry-over of PAs present in ragwort and narrow-leaved ragwort to milk. The amount of ragwort given to the cows had no immediate effects on the milk production. The carry-over to milk in our study resembled that in the study by Dickinson et al. (1976), notwithstanding that in our study the ragwort dosages were 20 to 100 times lower. Similar to the study of Dickinson, the overall transfer of PAs was rather low (0.1%), but this figure may be higher for specific PAs, like jacoline (4-7% depending on whether or not the N-oxide is taken into account as a precursor) and the otonecine type PAs. Furthermore, there are strong indications for substantial metabolism of the PAs in cows, raising attention to the possible transfer of metabolites into the milk. At the highest dosage level of 200 g dried ragwort per day, the VSD in consumers would be reached at a daily intake of 2-10 ml of affected milk. This urges for more research towards the risk of specific

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PAs, like jacoline, in milk but also in other food items. Since PAs can be classified as genotoxic carcinogens and since metabolites are known to be involved in these effects, further studies are needed to investigate the potential risks of ingestion of PA-containing herbs by food-producing animals and the risk of milk consumption in specific situations.

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Legends

Figure 1. Chemical structures of pyrrolizidine alkaloids representative for ragwort species.

Figure 2. Milk production of the three cows during administration of 0 (day 1-7), 50 (day 8-14), 100 (day 15-21), 200 (day 22-28) and 0 (day 29-35) g day⁻¹ ragwort. At the end of the fourth week cow 2 showed indigestion.

Figure 3. Pattern of PAs in the ragwort used for the study (A), and milk (B), urine (C) and faeces (D) collected during the period on the highest ragwort level. In the case of urine the pattern includes both PA concentrations obtained both without (light) and with a deconjugation step (dark). Abbreviations of the different PAs are listed in Table 1.

Figure 4. Concentrations ($\mu\text{g l}^{-1}$) of jacoline in evening milk of the 3 cows dosed with 0 (day 1-7), 50 (day 8-14), 100 (day 15-21), 200 (day 22-28) or 0 (day 29-35) g day⁻¹ of ragwort (A). Solid lines represent the average steady state levels for jacoline in each period. Figure B shows concentrations of total PAs ($\mu\text{g l}^{-1}$) in morning (M) and evening (E) milk starting with the morning milk samples just before the first dose of 100 g of plant material and ending with the evening milk taken 24 hours after the last dosing. Concentrations are the average of the 3 cows $\pm$ SD.

Figure 5 Transfer model of jacoline from *Senecio* from feed to milk (A, weekly mean $\pm$ SD of the milk concentration of each individual cow: solid line ($y = 3.7 \times 10^{-3} x + 0.0721$) and the half-life in milk after exposure had stopped (B).

Table 1. MS/MS conditions used for the analysis of pyrrolizidine alkaloids. The standard that has been used for quantification of the individual compounds is indicated.

Compound	Abbreviation	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Standard used for quantification
Heliotrine (IS)	Hel	314.2	138.0	25	
Senecionine	Sn	336.2	94.0; 120.0	40; 30	Sn
Senecionine N-oxide	Sn-ox	352.2	94.0; 120.0	40; 30	Sn-ox
Integerrimine	Ir	336.2	94.0; 120.0	40; 30	Ir
Integerrimine N-oxide	Ir-ox	352.2	94.0; 120.0	40; 30	Ir-ox
Senecivernine	Sv	336.2	94.0; 120.0	40; 30	Ir
Senecivernine N-oxide	Sv-ox	352.2	94.0; 120.0	40; 30	Ir-ox
Retrorsine	Rt	352.2	94.0; 120.0	40; 30	Rt
Retrorsine N-oxide	Rt-ox	368.2	94.0; 120.0	40; 30	Rt-ox
Usaramine	Us	352.2	94.0; 120.0	40; 30	Rt
Usaramine N-oxide	Us-ox	368.2	94.0; 120.0	40; 30	Rt-ox
Riddelliine	Rd	350.2	94.0; 138.0	40; 30	Rd
Riddelliine N-oxide	Rd-ox	366.2	94.0; 118.0	40; 30	Rd-ox
Seneciphylline	Sp	334.2	94.0; 120.0	40; 30	Sp
Seneciphylline N-oxide	Sp-ox	350.2	94.0; 138.0	40; 30	Sp-ox
Spartiodine	St	334.2	120.0; 138.0	30; 30	Sp
Spartiodine N-oxide	St-ox	350.2	94.0; 138.0	40; 30	Sp-ox
Acetylseneciphylline	AcSp	376.2	120.0; 138.0	30; 30	AcSp
Acetylseneciphylline N-oxide	AcSp-ox	392.2	94.0; 118.0	40; 30	AcSp-ox
Jacobine	Jb	352.2	120.0; 155.0	30; 30	Jb
Jacobine N-oxide	Jb-ox	368.2	120.0; 296.0	30; 25	Jb-ox
Jacoline	Jl	370.2	94.0; 138.0	40; 30	Jl
Jacoline N-oxide	Jl-ox	386.2	94.0; 120.0	40; 30	Jb-ox
Jaconine	Jn	388.2	94.0; 120.0	40; 30	Jb
Jaconine N-oxide	Jn-ox	404.2	94.0; 138.0	40; 30	Jb-ox
Jacozine	Jz	350.2	94.0; 138.0	40; 30	Jb
Dehydrojaconine	DhJn	386.2	94.0; 120.0	40; 30	Jb
Erucifoline	Er	350.2	94.0; 120.0	40; 30	Er
Erucifoline N-oxide	Er-ox	366.2	94.0; 118.0	40; 30	Er-ox
Acetylerucifoline	AcEr	392.2	94.0; 118.0	40; 30	Er
Acetylerucifoline N-oxide	AcEr-ox	408.2	94.0; 120.0	40; 30	Er-ox
Senkirkine	Sk	366.2	122.0; 168.0	30; 25	Sk
Otosenine	Ot	382.2	122.0; 168.0	30; 25	Ot
Onetine	On	400.2	122.0; 168.0	30; 30	Ot
Desacetyldoronine	DesDor	418.2	122.0; 168.0	30; 30	Ot
Florosenine	Fs	424.2	122.0; 168.0	35; 30	Fs
Floridanine	Fd	442.2	122.0; 168.0	30; 30	Ot

Table 2. Concentrations of PAs in ragwort, milk, urine and faeces samples. Data on milk, urine and faeces are the averages found in the samples from the 3 cows during the period on 200 g plant material per day. Urine data are concentrations after deconjugation of the samples. SD represents the variation between the 3 cows. Empty cells indicate not detected.

Compound		ragwort (mg kg ⁻¹ dw)	milk (µg l ⁻¹)	urine (µg l ⁻¹)	faeces (µg kg ⁻¹)
Senecionine	Sn	24.8 ± 1.5		1.2 ± 0.1	0.3 ± 0.0
Senecionine N-oxide	Sn-ox	253.0 ± 20.3		5.3 ± 1.7	0.8 ± 0.4
Integerrimine	Ir	8.4 ± 0.5		0.6 ± 0.2	0.2 ± 0.0
Integerrimine N-oxide	Ir-ox	103.4 ± 6.4		6.0 ± 1.9	
Senecivernine	Sv	3.1 ± 0.3		0.1 ± 0.1	
Senecivernine N-oxide	Sv-ox	100.9 ± 11.2			
Retrorsine	Rt	9.9 ± 1.0		6.2 ± 1.9	1.4 ± 0.7
Retrorsine N-oxide	Rt-ox	336.1 ± 30.7		22.5 ± 11.3	
Usuramine	Us	1.7 ± 0.3		3.4 ± 1.6	
Usuramine N-oxide	Us-ox	44.5 ± 4.4		3.7 ± 1.8	
Riddelliine	Rd	1.7 ± 0.2		0.9 ± 0.4	
Riddelliine N-oxide	Rd-ox	22.5 ± 1.7		1.4 ± 0.3	
Seneciphylline	Sp	29.4 ± 1.8		28.2 ± 3.5	0.9 ± 0.1
Seneciphylline N-oxide	Sp-ox	189.4 ± 14.3		12.7 ± 4.6	
Spartiodine	St	1.1 ± 0.1		0.3 ± 0.0	
Spartiodine N-oxide	St-ox	5.7 ± 0.5			
Acetylsceneciphylline	AcSp	0.3 ± 0.1			
Acetylsceneciphylline N-oxide	AcSp-ox	4.8 ± 0.8			
Jacobine	Jb	84.7 ± 5.5	0.30 ± 0.11	17.2 ± 5.7	8.8 ± 4.6
Jacobine N-oxide	Jb-ox	325.4 ± 18.3		161.6 ± 33.2	0.3 ± 0.2
Jacoline	Jl	21.8 ± 1.3	7.75 ± 0.83	61.2 ± 23.3	24.2 ± 9.6
Jacoline N-oxide	Jl-ox	17.8 ± 1.4		5.9 ± 1.9	
Jaconine	Jn	119.2 ± 8.0	0.94 ± 0.39	2.3 ± 0.2	1.6 ± 0.7
Jaconine N-oxide	Jn-ox	5.8 ± 0.5			
Jacozine	Jz	2.6 ± 0.2		0.8 ± 0.4	
Dehydrojaconine	DHJn	4.9 ± 0.4			
Erucifoline	Er	77.0 ± 5.1		28.5 ± 12.0	2.9 ± 1.0
Erucifoline N-oxide	Er-ox	481.6 ± 29.9		17.5 ± 10.6	
Acetylerucifoline	AcEr	1.7 ± 0.2			
Acetylerucifoline N-oxide	AcEr-ox	29.7 ± 1.7			
Senkirkine	Sk	8.0 ± 1.6	0.40 ± 0.15	13.6 ± 1.6	1.2 ± 0.4
Otosenine	Ot	5.9 ± 0.5	0.11 ± 0.01	11.1 ± 3.4	8.0 ± 1.8
Onetine	On	1.3 ± 0.1		0.2 ± 0.1	1.3 ± 0.2
Desacetyldoronine	Desdor	1.1 ± 0.2		0.2 ± 0.0	0.3 ± 0.2
Florosenine	Fs	8.1 ± 0.8	0.21 ± 0.02	11.0 ± 3.6	5.5 ± 0.1
Floridanine	Fd	1.3 ± 0.1		0.4 ± 0.1	7.6 ± 0.6
Sum retronecine bases		392 ± 20	8.99 ± 1.31	150.8 ± 38.0	40.3 ± 14.4
Sum retronecine N-oxides		1921 ± 102		238.0 ± 54.3	1.1 ± 0.4
Sum otonecines bases		26 ± 3	0.72 ± 0.15	36.5 ± 8.4	23.9 ± 2.4
Total		2339 ± 119	9.71 ± 1.34	425.3 ± 99.5	65.2 ± 16.6

Table 3. Total PA concentrations in urine and faeces samples collected after the morning milking (potential metabolites excluded). Urine samples were treated with deconjugation enzymes.

Day	Cow 1	Cow 2	Cow 3	mean	SD
Urine ($\mu\text{g l}^{-1}$)					
2	0	0	0	0	0
5	1	0	0	0	0
9	109	80	133	107	27
12	91	66	162	106	49
16	231	111	266	203	82
19	234	137	227	200	54
22	339	219	519	359	151
26	322	526	461	436	104
30	5	10	6	7	3
33	0	0	0	0	0
Faeces ($\mu\text{g kg}^{-1}$)					
2	0	0	0	0	0
5	0	0	0	0	0
9	14	44	15	24	17
12	16	19	28	21	6
16	28	33	50	37	11
19	25	43	50	39	13
22	44	77	68	63	17
26	50	65	89	68	20
30	5	13	8	8	4
33	0	0	0	0	0

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.