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Ron Laurentius Hoogenboom, Patrick P J Mulder, Marco J. Zeilmaker, Hester J. van den Top, Gerrit J. Remmelink, Esther F.A. Brandon, Mirjam D. Klijnstra, Gerwin A.L. Meijer, Ronald Schothorst, Hans P. van Egmond

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6 2 **Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows**  
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30 12 Netherlands  
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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<sup>-1</sup>) 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*

## 36 Introduction

37 Pyrrolizidine alkaloids (PAs) are toxic compounds present in many plants belonging to the  
38 families of *Asteraceae* (*Compositae*), *Boraginaceae* and *Fabaceae* (*Leguminosae*). Important  
39 members of the *Asteraceae* family are ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaeae*)  
40 and common groundsel (*Senecio vulgaris*). Hundreds of different PAs have been isolated and  
41 characterised from a broad range of PA-containing plants (Molyneux and James 1990; EFSA  
42 2007; Hartmann and Witte 1995). PAs are composed of a necine base and one or two ester  
43 groups or a macrocyclic diester. Figure 1 shows some important representatives. Even within  
44 one species there may be more than 10 different PAs present (Hartmann and Witte 1995;  
45 Joosten et al. 2009, 2010). Furthermore, the composition (and concentration) may fluctuate  
46 with respect to climate and environmental conditions, the age and part of the plant, and the  
47 variety (genotype/chemotype). The PAs in ragwort and groundsel are generally of the  
48 macrocyclic diester type and in the N-oxide form, but can be easily reduced to a tertiary  
49 amine (Hartmann and Toppel 1986; Lindigkeit et al. 1997).

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

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

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2  
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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7 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  
16  
17 67 exposed, being 30 ng per person per day (virtual safe dose or VSD) (van der Zee 2005). This  
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19  
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<sup>-1</sup> 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<sup>-1</sup>. 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<sup>-1</sup>. 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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58 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.

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3 86 Deinzer et al. (1982) carried out a study on milk of goats dosed via a rumen cannula with 10 g  
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5 87 dried ragwort per kg bw per day. The PA content of the ragwort was estimated at 3 g kg<sup>-1</sup> dry  
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8 88 weight. Using a gas chromatographic method that was based on the reduction of all free bases  
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10 89 to a single derivative of retronecine, a total PA content in the milk of 330 to 810 µg l<sup>-1</sup> was  
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12 90 reported. The transfer to milk was estimated to be around 0.1% of the daily dose. The authors  
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15 91 also did not consider or investigate the possible presence of N-oxides in the ragwort material,  
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17 92 nor in the milk.

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20 93 Although these studies indicate that the transfer could be relatively small, the eventual  
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22 94 concentrations in milk may still present a considerable risk for the consumer. The VSD  
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24 95 described above would be reached by a lifelong daily consumption of only 0.1 ml of the milk  
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27 96 from the Dickinson and Deinzer studies. However, the animals in these studies received  
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29 97 unrealistic high levels of ragwort. On the other hand, not all PAs were analysed, like the N-  
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31 98 oxides which normally constitute the majority of the alkaloids present in plant material.  
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34 99 Molyneux and James (1990) reviewed the existing data and pointed to the possibility that in  
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36 100 addition to the free bases also the N-oxides might be transferred to milk and actually be  
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39 101 responsible for at least a part of the toxic effects observed in animals given milk from exposed  
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41 102 mother animals. Therefore it was decided to improve the analytical methods to determine PAs  
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43 103 and to repeat the Dickinson study using much lower levels of ragwort, unlikely to cause  
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46 104 adverse effects in the animals. The present paper confirms that jacoline was the major PA in  
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48 105 ragwort transferred to the milk, although it was only a minor component in the plant material  
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51 106 itself. At the same time the study indicates that a major part of the PAs is metabolized  
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53 107 possibly resulting in metabolites that are still a potential health hazard to the consumer.  
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3 108 **Materials and Methods**  
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8 110 **Materials**  
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10 111 Analytical samples of a number of PA tertiary amine standards (senecionine, seneciphylline,  
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12 112 retrorsine, senkirkine, otosenine, heliotrine) were obtained from commercial sources  
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15 113 (Phytolab, Vestenbergsgreuth, Germany; Phytoplan, Heidelberg, Germany; Latoxan, Valence,  
16  
17 114 France). Jacobine, jacoline, erucifoline and florosenine were isolated from plant material by  
18  
19 115 PRISNA (Leiden, The Netherlands). Integerrimine was a gift from Dr. Trigo (UNICAMP,  
20  
21 116 Campinas, Brasil). Riddelliine and riddelliine-N-oxide were a gift from Dr. Chou (NCTR,  
22  
23 117 Jefferson, AR, USA). Acetylseneciphylline was prepared by acetylation of seneciphylline  
24  
25 118 with acetic anhydride and pyridine. N-oxides of senecionine, seneciphylline, retrorsine,  
26  
27 119 integerrimine, acetylseneciphylline, jacobine and erucifoline were prepared by N-oxidation of  
28  
29 120 the corresponding tertiary amines with 30% hydrogen peroxide according to Chou et al.  
30  
31 121 (2003). N-oxide standards of senecionine, seneciphylline and retrorsine have recently become  
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33 122 available from the above mentioned vendors as well. The standards were at least 90% pure  
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35 123 according to LC-MS/MS analysis. In total a set of 20 reference standards was available for  
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37 124 this study.  
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43 125 Adult plants of ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaea*) and narrow-leaved  
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45 126 ragwort (*Senecio inaequidens*) were collected during June and July 2008 at various sites  
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47 127 around Wageningen and Nijmegen in The Netherlands. The materials were subsequently air  
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49 128 dried, cut into pieces, ground to 1 mm with a Peppink 200 AN grinding machine (Veerman,  
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51 129 Olst, The Netherlands), and homogenized. In total 16.6 kg of dry material was produced,  
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53 130 being a mixture of 84% ragwort and 16% narrow-leaved ragwort. The material was stored in  
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55 131 the dark at room temperature.  
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3 133 ***Animal treatment***  
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5 134 Three dairy cows (Holstein Friesians) were treated at the Waiboerhoeve in Lelystad (part of  
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7 135 Livestock Research, Wageningen UR). Animals were 4-5 years of age, weighed 600-700 kg  
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9 136 and were fistulated in the rumen. Cows produced around 40 litres of milk per day.  
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11 137 Throughout the experiment they received a diet of grass and corn silage (60/40 w/w) and in  
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13 138 addition soy and minerals. The treatment period consisted of five phases, each lasting one  
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15 139 week. In week 1, animals received no ragwort (adaptation/control period), in week 2 they  
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17 140 received two daily doses of 25 g, in week 3 two daily doses of 50 g, in week 4 two daily doses  
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19 141 of 100 g and in week 5 no ragwort (depletion period). The dried ragwort was introduced  
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21 142 directly into the rumen through the fistula in the morning and afternoon just after milking.  
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23 143 Milk was collected twice daily around 6.00 and 16.30. In addition, urine and faeces samples  
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25 144 were taken twice a week between 10.30-12.30, 4-6 hours after dosage of the ragwort. Milk,  
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27 145 urine and faeces samples were kept frozen at  $-20^{\circ}\text{C}$  until analysis. The experimental plan was  
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29 146 evaluated by an ethical committee prior to the study. The health of the animals was closely  
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31 147 monitored by a veterinarian. One cow experienced mastitis during week 2. Another cow  
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33 148 encountered a brief period of indigestion at the end of week 4. Both cows were treated with  
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35 149 standard medication.  
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45 151 ***Chemical analysis***  
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47 152 LC-MS/MS was used for the detection and quantification of PAs in biological matrices such  
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49 153 as plant material, milk, urine and faeces. It allows the simultaneous detection of both forms of  
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51 154 PAs, the free base (tertiary amine) and the N-oxide form (Xiong et al. 2009a; Joosten et al.  
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53 155 2010; Crews et al. 2010). LC-MS/MS is a very selective and sensitive technique capable to  
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55 156 detect compounds at low concentrations. In milk,  $\text{sub-ng ml}^{-1}$  to  $\text{ng ml}^{-1}$  concentrations were  
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57 157 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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19  
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  
23  
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<sup>-1</sup> 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  
31  
32 171 were extracted by rotary tumbling for 1 h. The extracts were filtered over a glass microfiber  
33  
34 172 filter (Whatman 1820-150). A 25 µl aliquot was taken from the resulting clear extract,  
35  
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<sup>-1</sup>) 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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3 183 the MS detector, many minor components could be adequately detected and quantified. The  
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5 184 limit of quantification (LOQ) for the individual PAs and their N-oxides in dried plant material  
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8 185 was between 0.2 and 0.5  $\mu\text{g g}^{-1}$ .  
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13 187 *Analysis of milk*

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15 188 The PA content in milk was assessed by means of LC-MS/MS according to an in-house  
16  
17 189 validated protocol (van den Top 2007). In short, test portions of 3.0 ml of thawed milk were  
18  
19 190 transferred to test tubes and stored for at least 4 hours at  $-20^{\circ}\text{C}$ . After the samples were  
20  
21  
22 191 allowed to thaw at room temperature, 7.0 ml of methanol containing 0.1% formic acid was  
23  
24 192 added and the samples were mixed well. The mixtures were again placed at  $-20^{\circ}\text{C}$  for at least  
25  
26 193 4 hours. They were taken from the freezer and immediately centrifuged (10 min at 1950g,  
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28 194  $4^{\circ}\text{C}$ ) to obtain clear supernatants. From the supernatants 5.0 ml was taken and evaporated to  
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31 195 dryness under a gentle stream of nitrogen at  $55^{\circ}\text{C}$ . The dried residues were resuspended by  
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33 196 vortex mixing in 600  $\mu\text{l}$  water containing 0.1% acetic acid and subsequently centrifuged at  
34  
35 197 1950 g for 10 min. An aliquot (300  $\mu\text{l}$ ) was filtered over a 0.45  $\mu\text{m}$  Eppendorf filter and  
36  
37 198 transferred to an HPLC vial. The sample extracts were analysed using LC-MS/MS and  
38  
39 199 quantified against a 5-point calibration curve of PA standards in 0.1% acetic acid in water.  
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41  
42 200 During the analytical sessions, duplicate determinations for some test portions were  
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44 201 performed and to all milk test portions and heliotrine was added at  $100\text{ ng ml}^{-1}$  as an internal  
45  
46 202 standard to correct for recovery. Blanks (determination without test portion) were performed  
47  
48 203 at regular intervals. Method recovery percentages per day were used to correct for recovery.  
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50  
51 204 The results of the duplicate determinations were within the expected variability (measurement  
52  
53 205 uncertainty at validation level ranged from 44 to 67%). In the blanks no PAs were found. All  
54  
55 206 samples of evening milk were analysed in single. The samples of the evening milk taken  
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57 207 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  
4  
5 209 duplicates. The LOQ in milk for the individual PAs and their N-oxides was between 0.05 and  
6  
7  
8 210  $0.2 \mu\text{g l}^{-1}$ .

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10 211

11  
12 212 *Analysis of urine and faeces*

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14  
15 213 The analysis of PAs in urine and faeces was based on an in-house validated method for the  
16  
17 214 determination of PAs in animal forage (Oosterink and Mulder 2008). Central part of this  
18  
19 215 method is the use of SPE for purification and concentration of the sample extracts. The SPE  
20  
21 216 clean-up step developed for forage could be used without modifications for urine and faeces.  
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23  
24 217 Matrix matched standards together with heliotrine as an internal standard were incorporated in  
25  
26 218 the method to correct for differences in recovery and matrix effects.

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28  
29 219 Urine samples were thawed overnight at room temperature. Two aliquots (2 ml) of each  
30  
31 220 sample were transferred to 10 ml test tubes and 4 ml 0.1% aqueous ammonia solution was  
32  
33 221 added. The pH of the extract was checked with a pH-stick and adjusted to pH 10-11 if  
34  
35 222 necessary. Following addition of heliotrine ( $50 \mu\text{l}$  of a  $1 \mu\text{g ml}^{-1}$  solution in methanol), the  
36  
37 223 extracts were shaken manually and purified by solid phase extraction (SPE) over Strata-X 60  
38  
39 224 mg, 3 cc cartridges (Phenomenex, Torrance, CA, USA). Cartridges were conditioned with 3  
40  
41 225 ml methanol and equilibrated with 3 ml water. After application of the sample extracts, the  
42  
43 226 cartridges were washed with 3 ml 1% aqueous formic acid solution, followed by 3 ml 1%  
44  
45 227 aqueous ammonia solution. The cartridges were dried under reduced pressure and eluted with  
46  
47 228 3 ml methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen at  
48  
49 229  $50^\circ\text{C}$  and the dry residues were reconstituted in 1 ml water/methanol 9:1 (v/v). Subsequently,  
50  
51 230  $10 \mu\text{l}$  was injected on the LC-M/MS system. Quantification was performed with internal  
52  
53 231 standard correction against a six-point matrix matched calibration curve of PA standards (0-  
54  
55 232  $250 \text{ ng ml}^{-1}$ ) in blank urine. For each cow individual calibration curves were constructed

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2  
3 233 using a urine sample from week 1 (pre-administration period). LOQs ranged between 0.2 and  
4  
5 234  $0.5 \mu\text{g l}^{-1}$  for most PAs. In general, the free bases were somewhat more sensitive than the N-  
6  
7  
8 235 oxides.

9  
10 236 Deconjugation of urine samples was carried out as follows: two aliquots (2 ml) of the  
11  
12 237 urine sample were transferred to 10 ml test tubes. The pH of the urine was adjusted to 4.8  
13  
14 238 with concentrated acetic acid. *Helix pomatia* extract (20  $\mu\text{l}$ ) (Merck, Darmstadt, Germany)  
15  
16 239 was added and the sample extracts were incubated for 16 h at  $37^\circ\text{C}$  in a water bath. The  
17  
18 240 extracts were processed as described above. For each cow an individual matrix matched  
19  
20 241 calibration curve was included in the same concentration range as for the non-hydrolysed  
21  
22 242 samples. The matrix matched standards were subjected to the deconjugation procedure.

23  
24 243 Faeces samples were thawed overnight at room temperature. Samples were homogenized  
25  
26 244 by hand with a spoon. Two aliquots (2 g) were transferred to 50 ml test tubes. To the test  
27  
28 245 samples 40 ml 2% aqueous acetic acid was added together with heliotrine (50  $\mu\text{l}$  of a  $1 \mu\text{g ml}^{-1}$   
29  
30 246  $^1$  solution in methanol). The mixtures were extracted by rotary tumbling for 1 h and  
31  
32 247 subsequently centrifuged at 3500 rpm for 10 min. Twenty ml of the supernatant was  
33  
34 248 transferred to a 50 ml test tube and the pH was raised to 10-11 with concentrated ammonia.  
35  
36 249 The extracts were purified by SPE as described above for the urine samples. The final extracts  
37  
38 250 were prepared in 500  $\mu\text{l}$  water/methanol 9:1 (v/v). Quantification was performed with internal  
39  
40 251 standard correction against a six-point matrix matched calibration curve of PA standards (0-  
41  
42 252  $25 \text{ ng ml}^{-1}$ ) in blank faeces. For each cow individual calibration curves were constructed using  
43  
44 253 a faeces sample from week 1 (pre-administration period). LOQs were comparable to those in  
45  
46 254 urine.

47 255

48 256 *Instrumentation*

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3 257 Two analytical methods were developed and validated independently from each other at two  
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5 258 laboratories (RIKILT and RIVM). The two different LC-MS/MS gradients used in this study  
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7  
8 259 were an acidic gradient for the analysis of the milk samples and an alkaline gradient for the  
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10 260 plant, urine and faeces samples. However, performance of both methods was comparable. The  
11  
12 261 pH of the mobile phase has a pronounced effect on the elution of the PAs, most notably the  
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14  
15 262 N-oxides. These elute a couple of minutes before the free bases under alkaline conditions and  
16  
17 263 elute just after the free bases under acidic conditions. The elution order of the specific PAs is  
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19  
20 264 not affected by the pH of the mobile phase.

21  
22 265 Analyses of plant extracts, urine and faeces samples for PA content were performed on a  
23  
24 266 Waters Acquity UPLC coupled to a Waters Quattro Premier XE tandem mass spectrometer  
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26 267 (Waters, Milford, MA, USA), operated in positive electrospray mode. The compounds were  
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28  
29 268 separated on a Waters UPLC BEH C18 150 x 2.1 mm, 1.7  $\mu$ m analytical column, kept at  
30  
31 269 50°C and run at 0.4 ml min<sup>-1</sup> with an acetonitrile/water gradient containing 6.5 mM  
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33  
34 270 ammonia. The gradient started at 100% water and was changed to 50% acetonitrile in 12 min.  
35  
36 271 Total runtime of the method was 15 min.

37  
38 272 Analysis of milk samples was performed on a Waters Acquity UPLC coupled to a Waters  
39  
40  
41 273 Quattro Ultima Pt tandem mass spectrometer (Waters, Milford, MA, USA). Compounds were  
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43 274 separated on a Waters UPLC BEH C18 100 x 2.1 mm 1.7  $\mu$ m analytical column, kept at 50°C  
44  
45  
46 275 and run at 0.35 ml min<sup>-1</sup> with a gradient of acetonitrile and water containing 0.1% acetic acid.  
47  
48 276 The gradient started at 100% water and was changed to 15% acetonitrile in 18 min. The  
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51 277 column was washed with 80% acetonitrile for 3 min to remove slow eluting lipids. Total run  
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53 278 time was 25 min.

54  
55 279 For both systems the MS/MS collision energy was optimized for each individual  
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57  
58 280 compound using reference standards or plant extracts when standards were not available. Two  
59  
60 281 precursor product ion transitions were selected and incorporated in a multiple reaction

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3 282 monitoring (MRM) method. The dwell time for each transition was set at 20 msec (Premier  
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5 283 XE) or at 30 msec (Ultima Pt). A total of 49 transitions was monitored in a single run to  
6  
7 284 include all potentially relevant PAs. In Table 1 an overview is presented of the mass  
8  
9  
10 285 spectrometric settings used for the detection of the relevant PAs. All compounds could be  
11  
12 286 unequivocally characterized on the basis of retention time and fragmentation transitions. With  
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14  
15 287 the final method approximately 50 compounds (including 10 metabolites) were monitored.  
16  
17 288 For those compounds for which no reference standard was available, a semi-quantitative  
18  
19 289 (indicative) value could be obtained by comparison with a closely related analogue (often an  
20  
21 290 isomer), which exhibited a similar MS/MS fragmentation spectrum. In these cases the same  
22  
23 291 transitions were used. For some compounds (e.g. jaconine and its N-oxide) no closely related  
24  
25 292 standard with similar MS/MS spectrum could be identified. In such cases the concentration  
26  
27 293 was estimated by taking the sum of the two most intense fragments and comparing this with  
28  
29 294 the sum area of the transitions selected for the most closely related standard (e.g. jacobine and  
30  
31 295 its N-oxide).

36 296

### 38 297 *Data modelling*

40  
41 298 Transfer modelling consisted of regression analysis of the PA concentration in evening milk  
42  
43 299 during the exposure weeks 1, 2, 3 and 4 against the PA amount which has been administered  
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45 300 as a single bolus dose in the rumen, i.e. the amount administered with 25, 50 or 100 g of the  
46  
47 301 ragwort material. In this analysis the PA concentrations during each of the exposure weeks  
48  
49 302 were averaged for each of the three individual animals (the PA concentrations were in fact  
50  
51 303 considered constant during each exposure week). These averages + SD were then used as the  
52  
53 304 data point in the regression analysis which resulted in a single coefficient characterising the  
54  
55 305 transfer from feed to milk. Next to this coefficient the percentages of the total doses which  
56  
57 306 were administered during one week transferred to milk were calculated.  
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59  
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2  
3 307 The milk elimination half-life of jacoline, jacobine, jaconine, senkirkinine, otosenine and  
4  
5 308 florosenine was determined using the evening milk concentrations on day 7 of the highest  
6  
7  
8 309 dose administered and day 1 to 7 of week 5 (no administration of ragwort material). On day 1  
9  
10 310 of week 5 also concentrations in morning milk were available and used in the calculation of  
11  
12 311 the milk elimination half-life. A mono-exponential pharmacokinetic approach (decay to  
13  
14  
15 312 background concentration) was used to determine the milk elimination half-life:  
16

17 313

$$20 314 C(t) = Bg + C_1 \cdot \exp(-k_1 \cdot t)$$

22 315

24 316  $C$  is the concentration of PAs in milk,  $C_1$  is model parameter for concentration level,  $k_1$  is a  
25  
26 317 concentration decay rate and  $Bg$  is the supposed background level. The model parameters  
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28  
29 318 were fitted to the data with a relative error data model (an absolute error data model leading to  
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31 319 a less optimal description of the data).  
32

34 320

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## 39 322 **Results and Discussion**

41 323

### 44 324 *Animal status*

46 325 Three cows were fed with 0, 50, 100, 200 and again 0 g of dried plant material daily, each  
47  
48 326 time for a period of 7 days and divided over two daily doses. The animals were exposed to  
49  
50 327 relatively low doses, not only to mimic a realistic situation, but also to prevent an intoxication  
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52 328 of the animals. The total dose (2.5 kg) given to the animals was estimated to be less than 10%  
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54  
55 329 of the lethal dose (CliniTox, 2009). The health of the animals was carefully monitored during  
56  
57  
58 330 the duration of the experiment.  
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3 331 Milk samples taken 3 days after changing the dosage were examined for protein, fat and  
4  
5 332 lactose. There were no effects of the treatment on these parameters. Protein content e.g. was  
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7  
8 333  $3.1 \pm 0.2$  % during the first week without ragwort, and  $3.0 \pm 0.3$ ,  $3.3 \pm 0.4$ ,  $3.3 \pm 0.3$  and  $3.1 \pm$   
9  
10 334  $0.2$  during the daily treatment with 50, 100, 200 and again 0 g ragwort. However, no decline  
11  
12 335 in milk production or any signs of intoxication were observed. Figure 2 shows the milk  
13  
14 336 production of the three cows during the entire study. One cow (#2) showed an indigestion at  
15  
16 337 the end of the fourth week, being the end of the period on the highest PA-dose. This  
17  
18 338 immediately affected the milk yield. Furthermore, another cow (#1) showed mastitis at the  
19  
20 339 end of the second week. In both cases treatment with standard medication was successful and  
21  
22 340 the animals quickly recovered. However, both events are not uncommon for dairy cattle and  
23  
24 341 were very likely not related to the administration of ragwort.  
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29 342 The absence of an effect on the milk production clearly distinguishes our study from that  
30  
31 343 of Dickinson et al. (1976) in which a rapid decline in milk production was observed, possibly  
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33 344 affecting the excretion of PAs in the milk.  
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#### 39 346 ***PA levels and composition in ragwort***

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41 347 The plant material used in this study consisted primarily (84%) of ragwort (*Senecio*  
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43 348 *jacobae*). Analysis of individual samples of ragwort showed minor differences between the  
44  
45 349 locations. The ragwort consisted primarily of jacobine, jaconine, erucifoline, senecionine and  
46  
47 350 seneciphylline and their corresponding N-oxides. The remainder (16%) was dried narrow-  
48  
49 351 leaved ragwort (*Senecio inaequidens*). Major alkaloids in this plant were retrorsine,  
50  
51 352 senecivernine and their N-oxides. Narrow-leaved ragwort also contained small amounts of  
52  
53 353 otonecine-type PAs, such as senkirkine, otosenine and florosenine.  
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57 354 The homogeneity of the dried and mixed plant material was assessed by ANOVA (Fearn  
58  
59 355 and Thompson 2001) and was found sufficient. Table 2 shows the concentrations of the  
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1  
2  
3 356 various PAs and their N-oxides in the final material. Figure 3A shows the pattern of PAs in  
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5 357 this material. The total concentration of PAs was  $2.3 \text{ g kg}^{-1}$  on a dry weight basis, with 82%  
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7  
8 358 of the PAs in the N-oxide form and 18% as free bases. Jacobine and jaconine contributed  
9  
10 359 relatively strongly to the overall free base content, in accordance with Joosten et al. (2010).  
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12 360 Jacoline and its N-oxide contributed respectively 1.0 and 0.7% to the overall PA content.  
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14  
15 361 Based on the overall content, during the 5 different weeks, the cows were exposed to 0, 115,  
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17 362 230, 460 and 0 mg PAs per day respectively, divided over two dosages per day.  
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20 363

#### 21 22 364 *PA concentrations in milk*

23  
24 365 Analysis of milk samples revealed the presence of several tertiary bases but no N-oxides.  
25  
26 366 Jacoline, jacobine, jaconine, senkirkine, otosenine and florosene were the PAs found at  
27  
28  
29 367 concentrations above the limit of quantification (LOQ). Table 2 includes the average  
30  
31 368 concentrations for these PAs in the milk of the three cows during the period of the  $200 \text{ g day}^{-1}$   
32  
33 369 dose. The pattern in milk is shown in Figure 3B. When compared with the ragwort mixture  
34  
35 370 (Figure 3A), a relatively high contribution of jacoline ( $80.1 \pm 2.9\%$  of the total PA content;  
36  
37 371 mean  $\pm$  SD for the 3 cows during the highest dosage) was observed. In the ragwort mixture,  
38  
39 372 the amount of jacoline free base was only 1% of the analysed PAs. Jaconine was the second  
40  
41 373 most important PA in milk contributing  $9.4 \pm 2.9\%$ , which is more in line with the content in  
42  
43 374 the plant material (5.1%). Senkirkine, otosenine and florosene together contributed for  $7.4 \pm$   
44  
45  
46 375 1.5% to the PA content in milk. This is a relatively large contribution, considering the  
47  
48  
49 376 approximate 1% contribution in the plant material.  
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51  
52  
53 377 Figure 4A shows the concentration of jacoline in the milk of the three cows during the  
54  
55 378 different treatment periods. Levels increased rapidly following the change to a higher dosage.  
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57 379 There were some temporary declines in the concentrations which for cow 2 coincided with the  
58  
59 380 indigestion (Figure 2). For the other two cows there was no explanation. Average total PA

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3 381 levels during the 3 different dosage periods were 2.1, 5.5 and 9.7 ng ml<sup>-1</sup>. When the highest  
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5 382 total PA levels, obtained at the 200 g day<sup>-1</sup> treatment, are set at 100%, the average level in the  
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7  
8 383 50 and 100 g day<sup>-1</sup> period were 26 and 55% respectively, in line with the dosage. For jacoline  
9  
10 384 these figures were 22, 58 and 100%. Following termination of the treatment, total PA  
11  
12 385 concentrations rapidly declined to about 30% at the end of the first and 2% at the end of the  
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14  
15 386 second day.

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17 387 To check for a potential difference in the PA content of the morning and evening milk  
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19 388 samples, morning milk samples of the 4<sup>th</sup> week, i.e. the period with the highest dosage, were  
20  
21 389 also analysed. The average concentrations are presented in Figure 4B. The small but  
22  
23 390 consistent differences found between morning (M) and evening milk (E) probably reflect the  
24  
25 391 differences in time between dosage and milking (10 h for the evening milk and 14 h for the  
26  
27 392 morning milk with 54% of the daily milk production in the morning milk), and suggest that  
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29 393 the majority of transfer of the PAs to milk occurs in the first hours after dosing, after which  
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31 394 dilution occurs with more milk being produced. Similar patterns have been found for lactose,  
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33  
34 395 fat and protein content of morning and evening milk (Meijer, personal comment).

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38 396 Based on the milk production of about 40 litres per day and a total PA concentration of 10  
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40 397 µg l<sup>-1</sup> during the highest dosage level, the overall amount excreted in the milk was 400 µg  
41  
42 398 PAs day<sup>-1</sup> or about 0.1% of the overall daily dose of PAs (N-oxides + free bases). This figure  
43  
44 399 is similar to that calculated from the study of Dickinson et al. (1976) but that study only  
45  
46 400 included the free bases. When compared to only the free-base PAs in the ragwort (18% of  
47  
48 401 total), the overall transfer was 0.5%. Regarding jacoline, about 4% of this PA and its N-oxide  
49  
50 402 present in the plant material ended up in the milk as the free base, or 7% when only jacoline  
51  
52 403 as free base is considered. Panariti et al. (1997) dosed sheep for 5 days with about 30 mg  
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54 404 radiolabelled seneciphylline per day and observed milk levels up to 1 mg l<sup>-1</sup>. This would  
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56 405 correspond to a transfer rate of 3%, being much higher than observed in our study for this  
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3 406 compound. However, the levels were based solely on the radiolabel and may as well represent  
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5 407 metabolites including macromolecular adducts. Candrian et al. (1991) treated one lactating  
6  
7 408 cow with a single dose of 547 mg radiolabelled seneciphylline and observed milk levels  
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9  
10 409 corresponding up to 0.1 mg l<sup>-1</sup>. The overall amount of radiolabel detected in the milk was  
11  
12 410 0.16%. However, only part of this appeared to correspond to the free base and some N-oxide.  
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14  
15 411 Our study did show traces of hydroxylated metabolites in milk (data not shown). However, no  
16  
17 412 detectable amounts of seneciphylline and its N-oxide were found in milk.  
18  
19  
20 413

21  
22 414 *PA concentrations in urine and faeces*

23  
24 415 Urine and faeces samples were collected on two days during each week at the end of the  
25  
26 416 morning, about 4-6 hours after the dosing of the ragwort. Samples were taken directly from  
27  
28 417 the cows. Table 3 shows the total PA concentrations in urine for the three cows. There was a  
29  
30 418 more or less dose-related increase in the concentrations, average levels in the five different  
31  
32 419 weeks being 0, 107, 201, 398 and 3 µg l<sup>-1</sup>. In the last week, the samples were taken 2 and 6  
33  
34 420 days after the last treatment, the samples from day 2 after treatment still showing slightly  
35  
36 421 elevated levels. Most PAs present in the plant material could also be detected in the urine  
37  
38 422 (Table 2). Application of a glucuronide/sulfate deconjugation step in the analysis resulted in a  
39  
40 423 slightly (14%) elevated total concentration. This was primarily due to seneciphylline and  
41  
42 424 erucifoline. Apparently, these PAs are excreted in urine mainly as conjugates, most likely as  
43  
44 425 glucuronides. The concentrations of most other PAs were not affected by the deconjugation  
45  
46 426 step, indicating that these PAs are present primarily in a non-conjugated form. This is clearly  
47  
48 427 demonstrated in Figure 3C, showing the patterns of the urine samples taken during the week  
49  
50 428 on the highest dosage, both before and after deconjugation. Compared to the composition of  
51  
52 429 the original plant material, it is clear that the pattern in urine is rather different. Jacobine-N-  
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54 430 oxide was by far the most important PA in urine (38.4 ± 4.8%; mean ± SD for the 3 cows)

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3 431 whereas in the plant material it contributed for only 14%. On the other hand, the N-oxides of  
4  
5 432 erucifoline, retrorsine and senecionine, contributing for respectively 21, 14 and 11% in the  
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7  
8 433 plants, made up only  $3.9 \pm 1.5$ ,  $5.3 \pm 2.6$  and  $1.2 \pm 0.1\%$  in the urine. Jacoline, a minor PA in  
9  
10 434 ragwort (1%) but by far the most important PA in milk, contributed for  $14.1 \pm 2.1\%$  in the  
11  
12 435 urine. Also the otonecine PAs are relatively abundant in urine, contributing for 8.5% as  
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14  
15 436 compared to 1% in the ragwort material. The change in the pattern clearly indicates that some  
16  
17 437 PAs are more efficiently metabolized than others. Alternatively, some PAs may be  
18  
19 438 transformed into other ones as recently found in a study on the metabolism of adonifoline (the  
20  
21 439 major PA in a related ragwort species, *Senecio scandens*) in the rat (Xiong et al. 2009b). In  
22  
23 440 this study it was shown that adonifoline was metabolised to a number of hydroxylated  
24  
25 441 metabolites and (dehydro)retronecine derivatives. Very recently, glucuronidation by liver  
26  
27 442 microsomes was reported as a new metabolic pathway for PAs (He et al. 2010). In our  
28  
29 443 chromatograms of urine samples several new peaks were present that likely represent  
30  
31 444 hydroxylated metabolites of PAs (free bases and N-oxides) occurring in ragwort. Up to 10  
32  
33 445 new compounds with masses 350, 352, 366, 368 and 370 were detected, that were not present  
34  
35 446 in the plant material or in the urine samples before the treatment. The most important  
36  
37 447 metabolites are hydroxy-senecionine and hydroxy-integerrimine and their corresponding N-  
38  
39 448 oxides. Accurate quantification was difficult, due to the absence of reference standards, but it  
40  
41 449 was estimated that between 15 to 25% of the excreted PAs in urine were newly formed  
42  
43 450 compounds not present in ragwort. No efforts were undertaken to detect metabolites with a  
44  
45 451 dehydroretronecine moiety or related conjugates.

52  
53 452 Since the urine was not collected quantitatively, it is difficult to determine the overall  
54  
55 453 fraction of ingested PAs that was accounted for in the urine. When assuming a total urine  
56  
57 454 production of 40 litres per day, roughly 17 mg of PAs would be excreted in the urine during  
58  
59 455 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  
6  
7 458 dosing) and 18-20 h (evening dosing). It is however unlikely that the total amount excreted in  
8  
9 459 urine will be very much higher than estimated on the basis of the 4-6 h sample. This can be  
10  
11 460 derived from the concentrations of jacoline and the otonecine PAs senkirkine, otosenine and  
12  
13 461 florosenine found in the collected urine. The average amount of jacoline detected in urine  
14  
15 462 accounted for approx. 60% of the jacoline present in the ragwort. Senkirkine was present at a  
16  
17 463 concentration which accounted for 34% of the original dose, otosenine for 37% and  
18  
19 464 florosenine for 33%. As will be discussed below, jacoline and the otonecine PAs were also  
20  
21 465 present in faeces in substantial amounts. Other studies have also indicated that extensive  
22  
23 466 metabolism of PAs occurs and that only a minor part of the PAs is excreted unchanged in  
24  
25 467 urine and faeces (Estep et al. 1990; Chu and Segall 1991)

26  
27 468 Total PA concentrations in faeces samples are shown in Table 3. There was more or less a  
28  
29 469 dose-related increase in the concentrations, on average being 0, 23, 38, 65 and 4  $\mu\text{g kg}^{-1}$  in the  
30  
31 470 5 different weeks. Table 2 shows the concentrations of the different PAs in the samples taken  
32  
33 471 during the week on the highest dosage. The samples contained practically no N-oxides. As in  
34  
35 472 the milk, jacoline was the most important PA, contributing for  $36.1 \pm 7.3\%$  to the total PA  
36  
37 473 content (Figure 3D). Other important PAs in faeces were jacobine ( $13.2 \pm 4.6\%$ ), and the  
38  
39 474 otonecine PAs otosenine ( $12.4 \pm 1.1\%$ ), floridanine ( $12.0 \pm 2.7\%$ ) and florosenine ( $8.8 \pm$   
40  
41 475  $2.4\%$ ). Together, these components made up  $82 \pm 2\%$  of the PAs in faeces, as compared to  $87$   
42  
43 476  $\pm 3\%$  in the milk,  $23 \pm 3\%$  in urine and only 5% in the original plant material. Based on 40 kg  
44  
45 477 of faeces per day, the total amount excreted in faeces was estimated to be 2.6 mg,  
46  
47 478 representing less than 1% of the original PAs during the highest dosage period. Nevertheless,  
48  
49 479 the average amount of jacoline excreted in the faeces accounted for 19% of the amount  
50  
51 480 present in the ragwort material, that of senkirkine for 3%, otosenine for 27%, florosenine for

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2  
3 481 14% and floridanine for 115%. Some of the metabolites found in urine were also detected in  
4  
5  
6 482 faeces, albeit at relatively low concentrations. Hydroxy-senecionine and hydroxy-  
7  
8 483 integerrimine were two of the major metabolites also found in urine. The contribution of  
9  
10 484 hydroxylated PA metabolites to the total excretion in faeces was estimated to be between 5 to  
11  
12  
13 485 10%.

14  
15 486 Although it should be clear that urine and faeces samples may not have been collected at  
16  
17 487 the peak of the excretion, both the concentrations and the change in the patterns indicate  
18  
19 488 intensive metabolism. The major question is whether this could result in toxic metabolites or  
20  
21 489 compounds that can be transformed into toxic metabolites, knowing that the toxicity of PAs is  
22  
23 490 caused by reactive metabolites. If so, the major issue would be if such metabolites could  
24  
25 491 actually be present in the milk, and as such present a further risk to the consumer. The studies  
26  
27 492 with radiolabelled seneciphylline indicate that metabolites may indeed be excreted into the  
28  
29 493 milk (Candrian et al. 1991; Panariti et al. 1997).

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### 35 36 495 *Modelling of transfer to milk*

37  
38 496 As shown in Figure 4A, transfer of jacoline to milk quickly led to a rather constant, i.e.  
39  
40 497 “steady state”, concentration in evening milk. In this way, for each of the four dose levels  
41  
42 498 tested, an animal specific “steady state” concentration in milk was obtained. Analysing these  
43  
44 499 levels with simple regression analysis led to a transfer coefficient of  $3.7 \times 10^{-3} \mu\text{g l}^{-1}$  per  $\mu\text{g}$  of  
45  
46 500 administered jacoline (see Figure 5A). After the exposure was stopped, jacoline rapidly  
47  
48 501 disappeared from the milk (and thus from the animal’s body). The half-life for jacoline in  
49  
50 502 milk was calculated to be 8 hours (Figure 5B).

51  
52  
53 503 As with jacoline the other 5 PAs detected in milk quickly reached a “steady state” (data  
54  
55 504 not shown). Transfer coefficients amounted to  $3 \times 10^{-5}$  (jacobine),  $7 \times 10^{-5}$  (jaconine),  $5 \times 10^{-4}$   
56  
57 505 (senkirkine), and  $2 \times 10^{-4}$  (otosenine and florosene)  $\mu\text{g l}^{-1}$  per  $\mu\text{g}$  of administered compound.



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3 506 Elimination half-lives from milk were 8 hours (jacobine), 6 hours (jaconine), 16 hours  
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5 507 (senkirkine) and 9 hours (otosenine and florosene).

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9  
10 509 *Relevance of the data for other plant species*

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12 510 The rather selective carry-over of jacobine, jaconine and some of the otonecines suggests that  
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14  
15 511 not all PAs and as such PA-containing plants may be of equal potential relevance for  
16  
17 512 consumers of milk from exposed animals. It also raises the question which properties are  
18  
19 513 relevant for possible transfer to milk. It seems obvious that the potential of the animal for the  
20  
21 514 biotransformation of individual compounds plays an essential role. The N-oxides, which were  
22  
23 515 prominent compounds in the ragwort material, were not detected in milk and faeces samples.  
24  
25 516 In urine N-oxides were present, accounting for 56% of the excreted PAs. Of the N-oxides  
26  
27 517 excreted jacobine-N-oxide was predominant whereas those of other PAs were almost or  
28  
29 518 completely absent. This indicates that either intensive metabolism or very rapid excretion via  
30  
31 519 urine (depletion in less than 4 h) takes place. However, preliminary studies with liver slices  
32  
33 520 from cows did not show significant degradation of these N-oxides. Similar was true for  
34  
35 521 jacobine whereas other free bases like senecionine, seneciphylline, erucifoline and jacobine  
36  
37 522 were substantially metabolized. In this study there were no indications for the transformation  
38  
39 523 of certain PAs into their N-oxides or into other PAs. Jacobine was the major or second  
40  
41 524 metabolite present in milk, urine and faeces. Assuming that jacobine is not formed by  
42  
43 525 metabolism from other PAs, around 85% of the free jacobine present in the ragwort material  
44  
45 526 was accounted for in milk, urine and faeces. Taking jacobine and its N-oxide together, still  
46  
47 527 50% could be found in these matrices. The otonecine PAs seem to be relatively stable as well.  
48  
49 528 Excretion percentages for these PAs vary from 35% (senkirkine) to over 110% (floridanine).

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51 529 The potential role of the bacteria and other micro-organisms in the rumen or intestines  
52  
53 530 should not be ignored (Mattocks 1971; Aguiar and Wink 2005). Lanigan (1970) showed that

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2  
3 531 the N-oxide of heliotrine was effectively metabolized by ovine ruminal fluid to the free base  
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5 532 and subsequently l-gorensine and 7-hydroxy-1-methylpyrrolizidin. The possibility that e.g.  
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8 533 jacoline may also have been formed from other PAs is important when focussing on plants  
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10 534 containing PAs that are transferred to milk. Furthermore it should be mentioned that the  
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12 535 majority (82%) of the PAs in the ragwort material used was in the N-oxide form. In the  
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14  
15 536 studies of Dickinson et al. (1976) and Deinzer et al. (1982) the ragwort material was not  
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17 537 analysed for N-oxide content, but it is reasonable to assume that this was not very different  
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19 538 from our study. However, upon storage and depending on the drying conditions, the relative  
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21 539 contribution of the free bases may become more prominent. Recent screening of animal  
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23 540 forages in The Netherlands seem to corroborate this: often the amount of free bases was  
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25 541 higher than that of the N-oxides (Mulder et al. 2010). Consequently, a high content of free PA  
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27 542 bases in a specific animal feed could lead to a relatively high transfer of PAs to milk.  
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#### 34 544 **Conclusions**

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36 545 The present study investigated the potential carry-over of PAs present in ragwort and narrow-  
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38 546 leaved ragwort to milk. The amount of ragwort given to the cows had no immediate effects on  
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40 547 the milk production. The carry-over to milk in our study resembled that in the study by  
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42 548 Dickinson et al. (1976), notwithstanding that in our study the ragwort dosages were 20 to 100  
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44 549 times lower. Similar to the study of Dickinson, the overall transfer of PAs was rather low  
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46 550 (0.1%), but this figure may be higher for specific PAs, like jacoline (4-7% depending on  
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48 551 whether or not the N-oxide is taken into account as a precursor) and the otonecine type PAs.  
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51 552 Furthermore, there are strong indications for substantial metabolism of the PAs in cows,  
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53 553 raising attention to the possible transfer of metabolites into the milk. At the highest dosage  
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55 554 level of 200 g dried ragwort per day, the VSD in consumers would be reached at a daily  
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57 555 intake of 2-10 ml of affected milk. This urges for more research towards the risk of specific  
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3 556 PAs, like jacoline, in milk but also in other food items. Since PAs can be classified as  
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6 557 genotoxic carcinogens and since metabolites are known to be involved in these effects, further  
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8 558 studies are needed to investigate the potential risks of ingestion of PA-containing herbs by  
9  
10 559 food-producing animals and the risk of milk consumption in specific situations.  
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12 560

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27 566 Food and Consumer Product Safety Authority (VWA).  
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3 649 **Legends**  
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8 651 Figure 1. Chemical structures of pyrrolizidine alkaloids representative for ragwort species.  
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12 653 Figure 2. Milk production of the three cows during administration of 0 (day 1-7), 50 (day 8-  
13 654 14), 100 (day 15-21), 200 (day 22-28) and 0 (day 29-35) g day<sup>-1</sup> ragwort. At the end of the  
14 655 fourth week cow 2 showed indigestion.  
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22 657 Figure 3. Pattern of PAs in the ragwort used for the study (A), and milk (B), urine (C) and  
23 658 faeces (D) collected during the period on the highest ragwort level. In the case of urine the  
24 659 pattern includes both PA concentrations obtained both without (light) and with a  
25 660 deconjugation step (dark). Abbreviations of the different PAs are listed in Table 1.  
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34 662 Figure 4. Concentrations ( $\mu\text{g l}^{-1}$ ) of jacoline in evening milk of the 3 cows dosed with 0 (day  
35 663 1-7), 50 (day 8-14), 100 (day 15-21), 200 (day 22-28) or 0 (day 29-35) g day<sup>-1</sup> of ragwort (A).  
36  
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38 664 Solid lines represent the average steady state levels for jacoline in each period. Figure B  
39 665 shows concentrations of total PAs ( $\mu\text{g l}^{-1}$ ) in morning (M) and evening (E) milk starting with  
40 666 the morning milk samples just before the first dose of 100 g of plant material and ending with  
41 667 the evening milk taken 24 hours after the last dosing. Concentrations are the average of the 3  
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48 668 cows  $\pm$  SD.  
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53 670 Figure 5 Transfer model of jacoline from *Senecio* from feed to milk (A, weekly mean  $\pm$  SD of  
54 671 the milk concentration of each individual cow: solid line ( $y = 3.7 \times 10^{-3} x + 0.0721$ ) and the  
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57 672 half-life in milk after exposure had stopped (B).  
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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
Spartioidine	St	334.2	120.0; 138.0	30; 30	Sp
Spartioidine 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 <sup>-1</sup> dw)	milk (µg l <sup>-1</sup> )	urine (µg l <sup>-1</sup> )	faeces (µg kg <sup>-1</sup> )
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			
Acetylseneciphylline	AcSp	0.3 ± 0.1			
Acetylseneciphylline 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

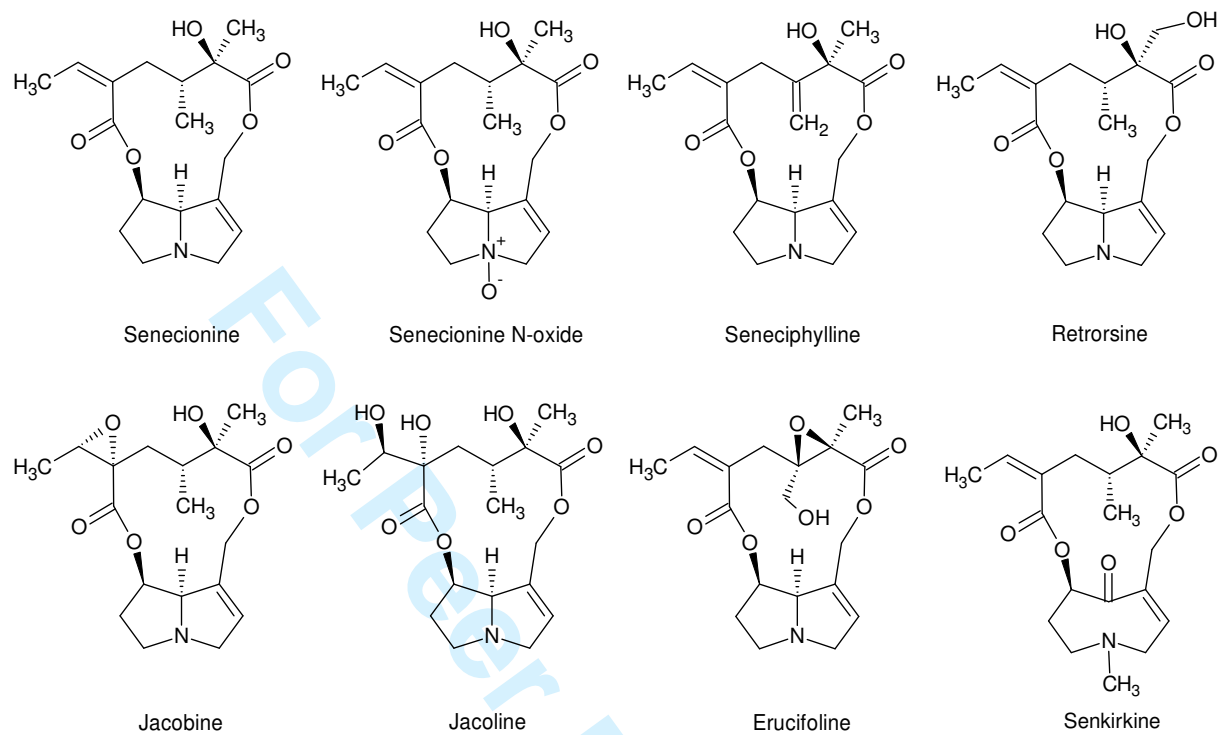


Figure 1.

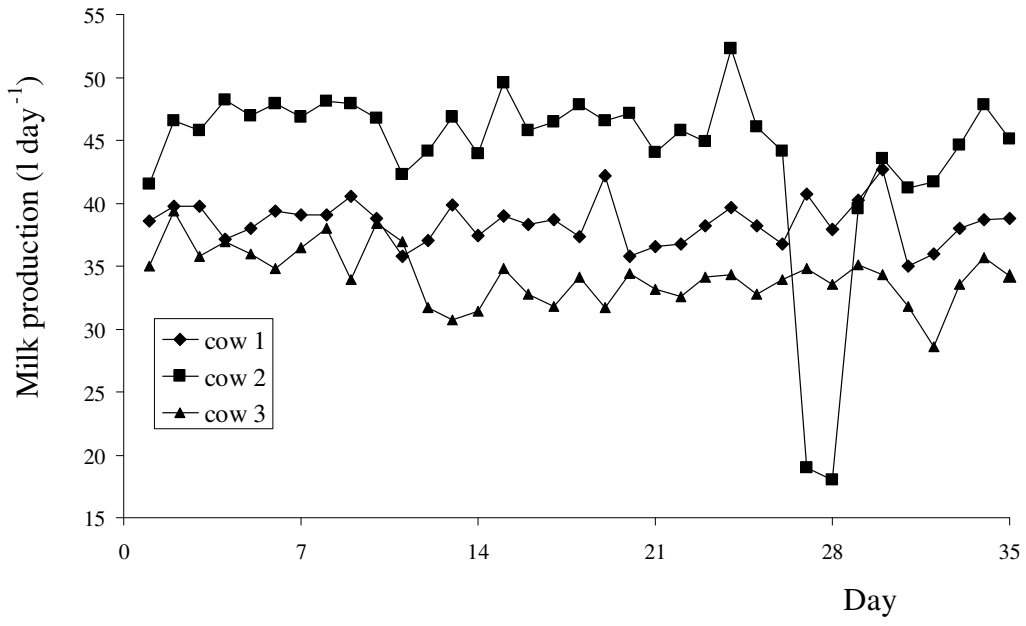
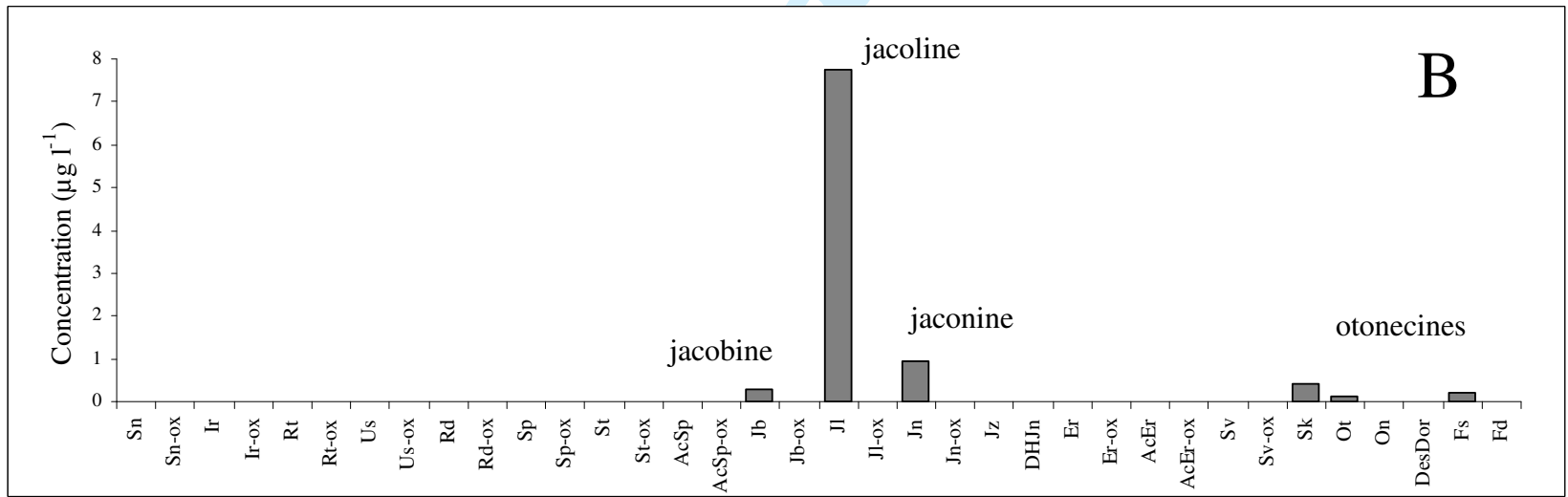
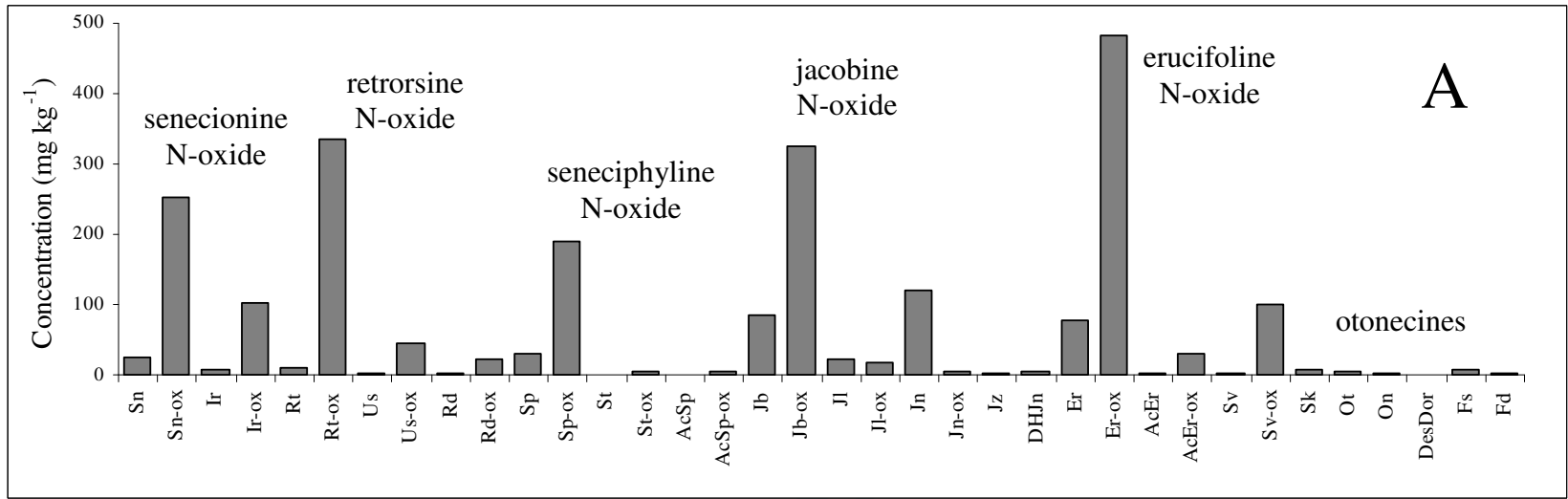


Figure 2.

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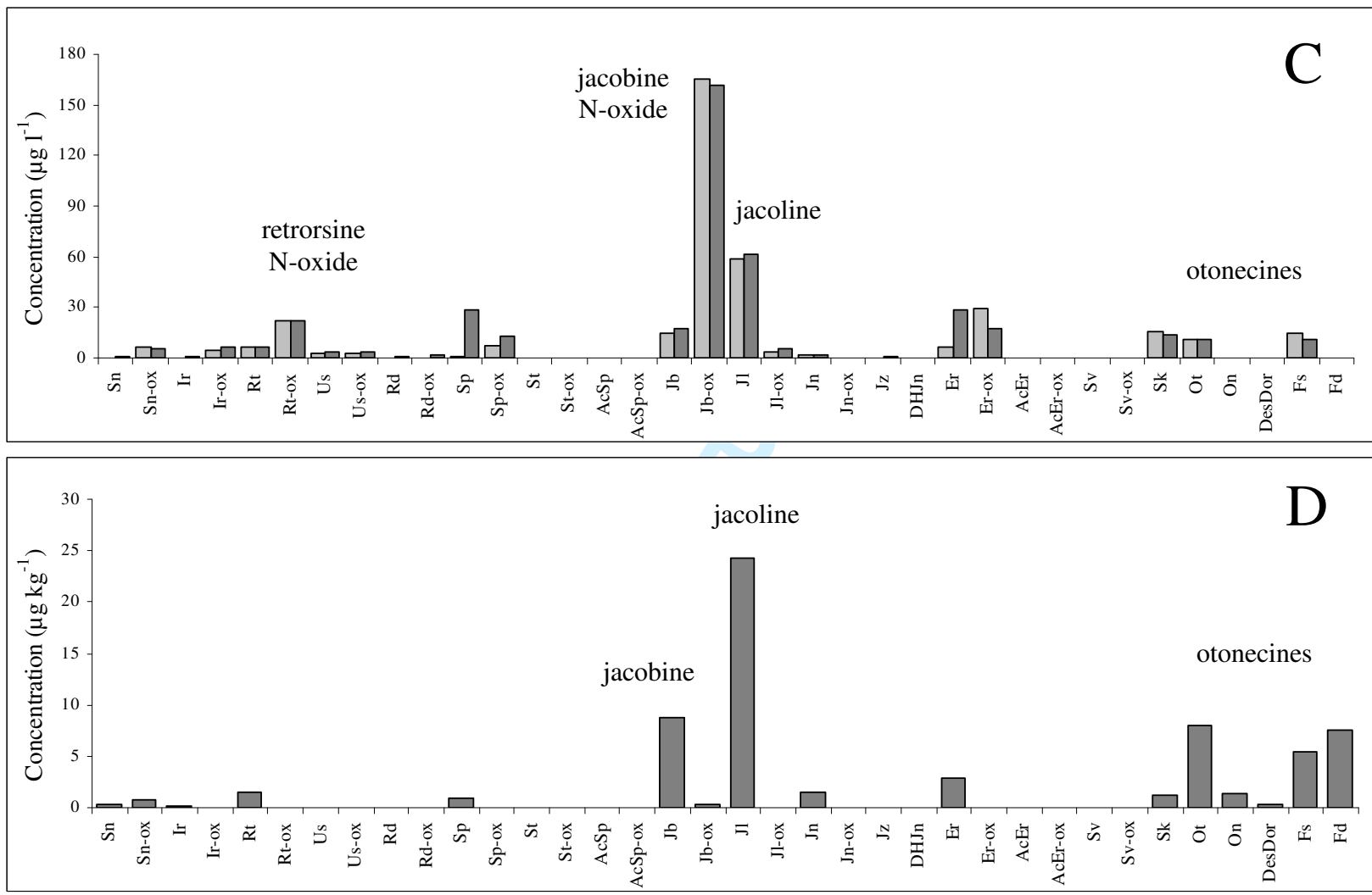


Figure 3.

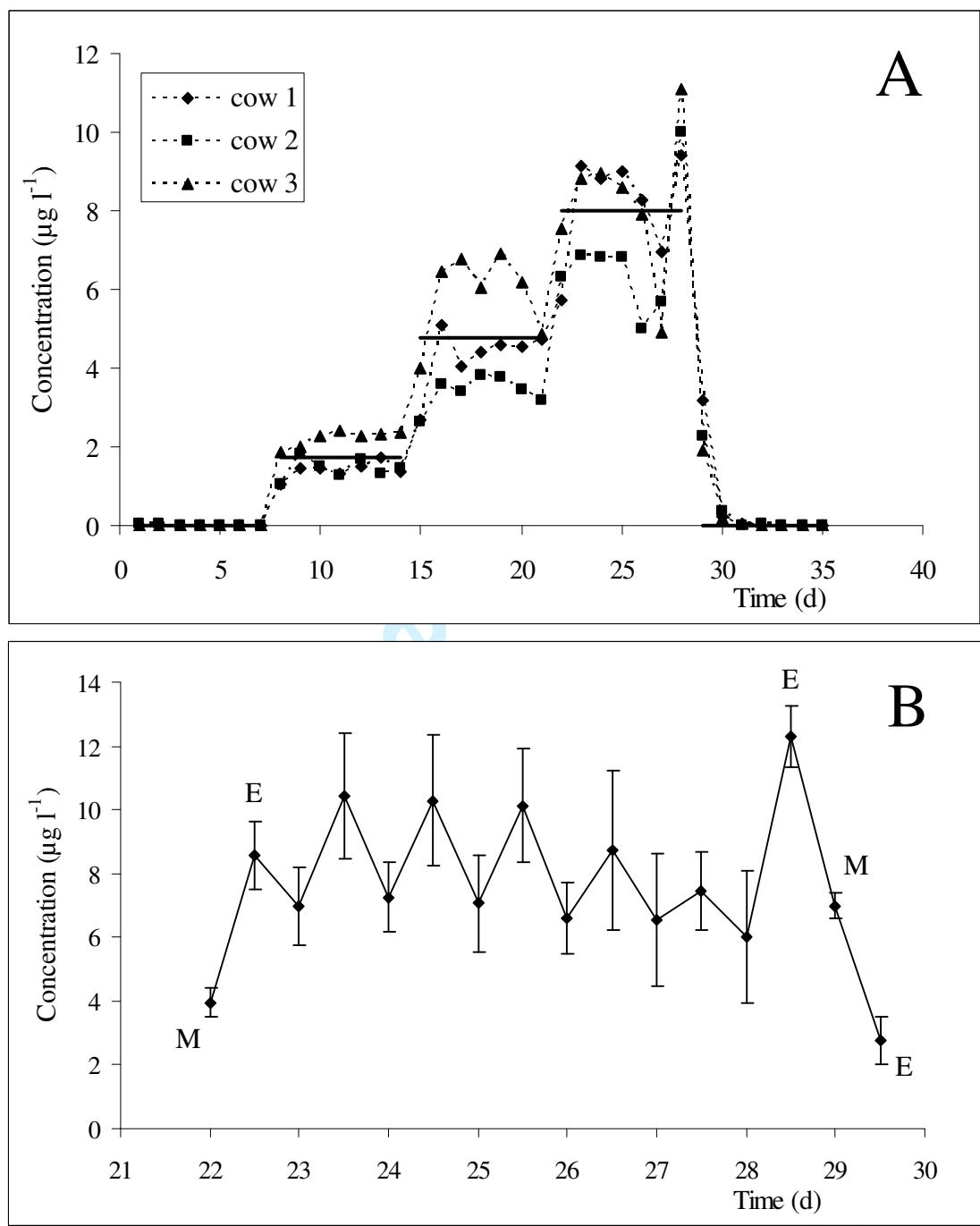


Figure 4.



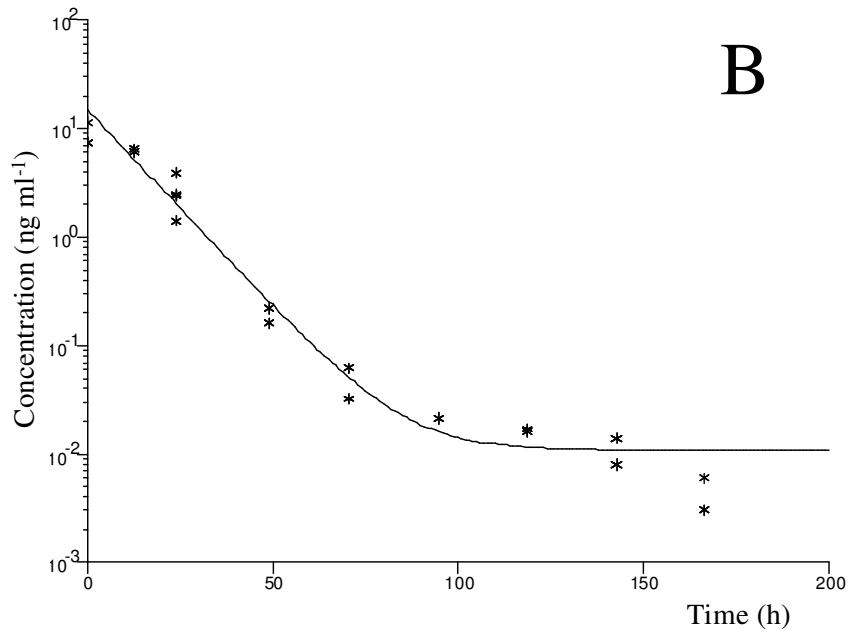
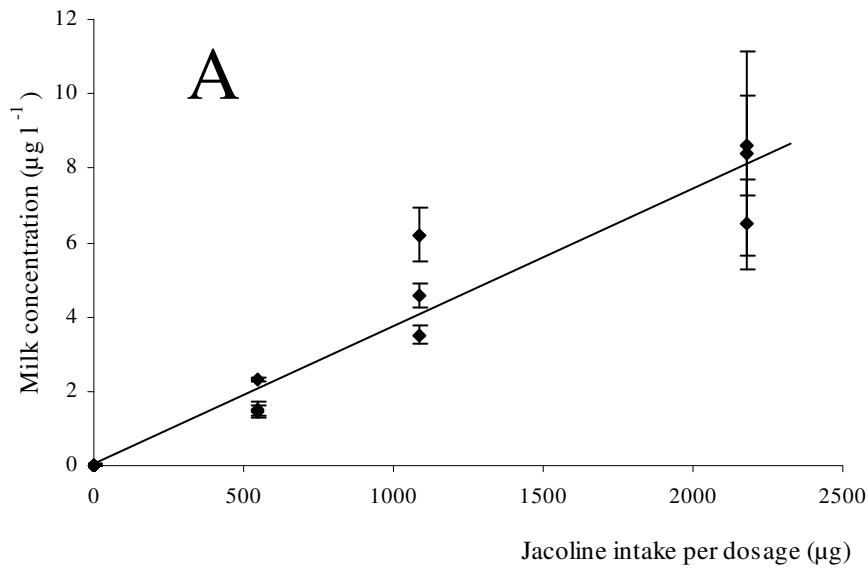


Figure 5.