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Dutch survey pyrrolizidine alkaloids in animal forage

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Summary

Pyrrolizidine alkaloids (PAs) are secondary plant metabolites produced by a number of plants from the *Asteraceae (Compositae)*, *Boriginaceae* and *Fabaceae* (*Leguminosae)* families. Many of these alkaloids have been shown to be highly toxic, causing hepatic veno-occlusive disease (VOD), liver cirrhosis and ultimately death. PAs may have also mutagenic and carcinogenic potential. Amongst livestock, cattle and horses are especially susceptible to the toxic effects of the PAs. Humans may also be at risk by the consumption of milk of livestock fed with PA-contaminated forage. Of the *Asteraceae* family, members of the Ragwort and Groundsel genus (*Senecio*) are common weeds occurring world-wide in pastures, (marginal) grasslands, arable lands and along the borders of rivers, roads and highways. *Senecio* species contain substantial concentrations of PAs. *Senecio* species have often been implicated in intoxications of livestock and sometimes humans. The European Food Safety Authority (EFSA) has recently published a scientific opinion on the presence of PAs as undesirable substance in animal feed (EFSA, 2007). Important conclusions of the EFSA report are that analytical methods for the detection and quantification of PAs in animal feed, including those from *Senecio*, are largely lacking as well as the availability of monitoring and survey data.
At RIKILT - Institute of Food Safety a (semi)quantitative method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the determination of PAs in animal feeds has been developed and validated. This method comprises 40 macrocyclic PAs (including tertiary amines and N-oxides) representative for ragwort species. The method has been used for the analysis of 147 forage samples collected in 2006-2008. Most of the samples were collected in the framework of the Dutch National Monitoring Program on animal feedingstuffs. In this study four categories of forage were considered: grass silage, hay, dried grass and alfalfa. In 31 of the 147 forage samples (traces of) PAs could be detected (LoD: 10 µg/kg). In nine samples the total PA content exceeded 100 µg/kg. In three instances the total PA content exceeded 1 mg/kg. Grass silage was practically free of PAs, in only 5% of the samples low (less than 30 µg/kg) concentrations of PAs were detected. The hay samples were free of PAs except for one sample, which contained 549 µg/kg PAs. The dried grass samples contained no or very low amounts of PAs, except for one sample that contained 288 µg/kg. In contrast, a high percentage of alfalfa samples was contaminated with PAs. Of 31 samples analysed, 23 (74%) contained at least traces of one or more PAs. In 16 samples relatively low amounts were found (between 10 and 100 µg/kg) and in four samples the PA sum was between 100 and 1000 µg/kg. Three samples (10%) contained high amounts of PAs, respectively 3.5, 3.8 and 5.4 mg/kg. The PA concentration in the alfalfa samples averaged to 455 µg/kg, which is 30 times or more the average concentration obtained for silage, dried grass and hay.
In order to link the observed PA profiles in the contaminated samples with a particular *Senecio* species, reference samples of Tansy ragwort (*Senecio jacobaea/Jacobaea vulgaris*), Common groundsel (*Senecio vulgaris*) and Narrow-leafed ragwort (*Senecio inaequidens*) were collected in the vicinity of Wageningen. Average PA profiles were constructed for these three species. Comparison with these reference profiles revealed that in most instances the forage samples were contaminated with Common groundsel. Only in two occasions there was evidence for other *Senecio* species present in the forage. The contaminated hay sample contained PAs that could not be directly linked to one of the three *Senecio* species included in this study, but a related ragwort species appears likely. The contaminated dried grass sample contained a mix of mostly Common groundsel together with a smaller amount of Tansy ragwort.

Conclusion

Common groundsel (*Senecio vulgaris*) presents the largest risk for contamination of animal forage in The Netherlands. Of the animal forage categories included in this study alfalfa production is in particular at risk. Alfalfa can be contaminated with PAs at concentrations that may potentially be toxic to livestock, especially at long-term exposure.

Recommendations

1. This study indicates that in The Netherlands Common groundsel is presenting the largest risk for contamination of animal forage. Especially alfalfa forage may contain substantial amounts of PAs. It is recommended to monitor the production of alfalfa more closely, with respect to the commercial end products as well as on site inspection of the plots where alfalfa is grown. The latter may lead to a better understanding of the specific conditions that favor contamination with Common groundsel and which measures can be taken to minimize the risk of contamination.
2. The current method covers only a small part of the PAs that could potentially be present in animal feeds. It is recommended to expand the current method to mono- and diester PAs that do occur in plants of the Boriginaceae family, like Viper's bugloss (*Echium vulgare*), Comfrey (*Symphytum spp*), Hound's tongue (*Cynoglossum officinale*) and of the *Asteraceae* family, Hemp agrimony (*Eupatorium cannabinum*). These species are (locally) quite common in The Netherlands, as well as in other parts of Europe. At the same time, the monitoring of animal forage should be extended to these PAs as well.
3. To assess the potential risk for PAs entering the food chain through transfer to milk the monitoring data should be combined with *in vitro* and *in vivo* experiments. The data currently available on milk transfer is rather limited. The transfer ratios of individual PAs (in their tertiary as well as N-oxide form) from feed to milk should be investigated, as it can be expected that differences in polarity and chemical reactivity may affect metabolism and result in different transfer ratios.

Samenvatting

Pyrrolizidine alkaloïden (PA's) zijn secundaire plant metabolieten die geproduceerd worden door een aantal planten uit de families van Composieten *(Asteraceae/Compositae)*, Ruwbladerigen *(Boriginaceae)* en Vlinderbloemigen *(Fabaceae/Leguminosae)*.Veel van deze alkaloïden zijn zeer toxisch en kunnen leiden tot hepatische veno-occlusieve ziekte (VOD), lever cirrose en uiteindelijk sterfte. Veel PA's hebben waarschijnlijk ook mutagene en carcinogene eigenschappen. Van de veestapel zijn met name koeien en paarden bijzonder gevoelig voor de toxische effecten van PA's. Mensen kunnen ook risico lopen, door de consumptie van melk afkomstig van dieren die met PA's gecontamineerd voer hebben gekregen. Leden van het kruiskruidgeslacht (*Senecio*), behorend tot de *Asteraceae* familie, zijn algemene onkruiden die wereldwijd voorkomen in weiden, (marginaal) grasland, akkerland en langs borders van wegen en rivieren. Kruiskruiden bevatten substantiële hoeveelheden PA's. Kruiskruiden zijn in het verleden vaak betrokken geweest bij vergiftigingsincidenten van veestapels en soms ook van mensen. De Europese Food Safety Authority (EFSA) heeft recentelijk een wetenschappelijke opinie gepubliceerd over de aanwezigheid van PA's als ongewenste stoffen in diervoeders (EFSA, 2007). In dit EFSA rapport wordt geconcludeerd dat analytisch chemische methoden voor de detectie en kwantificering van PA's in diervoeder, waaronder die van kruiskruiden, grotendeels ontbreken en dat er tegelijkertijd een grote behoefte is aan bruikbare monitorings data.

Bij het RIKILT - Instituut voor Voedselveiligheid is voor de bepaling van PA's in ruwvoeders een (semi)kwantitatieve methode ontwikkeld en gevalideerd die gebaseerd is op vloeistofchromatografie - tandem massaspectrometrie (LC-MS/MS). Deze methode omvat een veertigtal macrocyclische PA's die representatief zijn voor kruiskruidsoorten. De methode is gebruikt voor de analyse van 147 ruwvoeders verzameld in 2006-2008. De meeste monsters zijn genomen in het kader van het Nederlandse Nationaal Plan diervoeders. In deze studie zijn vier categoriën ruwvoeder onderscheiden: kuilgras, hooi, (kunstmatig) gedroogd gras en luzerne. In 31 van de 147 ruwvoeders werden (sporen van) PA's gedetecteerd (detectielimiet: 10 µg/kg). In negen monsters overschreef de totale PA concentratie 100 µg/kg en in drie gevallen werd 1 mg/kg overschreden. Kuilgras was praktisch vrij van PA's; slechts in 5% van de monsters werden lage concentraties (minder dan 30 µg/kg) aan PA's aangetroffen. De hooimonsters waren ook vrij van PA's, op een monster na dat 549 µg/kg PAs bevatte. De gedroogd grasmonsters bevatten geen of slechts lage hoeveelheden PA's, behalve voor een monster dat 288 µg/kg bevatte. Daarentegen bleek een hoog percentage luzernemonsters gecontamineerd met PA's. Van de 31 onderzochte monsters bevatten 23 (74%) tenminste sporen van een of meerdere PA's. In 16 monsters betrof het relatief lage concentraties (tussen 10 en 100 µg/kg) en in vier monsters lag de PA concentratie tussen 100 en 1000 µg/kg. Drie monsters (10%) bevatten hoge gehaltes PA's, respectievelijk 3.5, 3.8 en 5.4 mg/kg. De gemiddelde PA concentratie in de luzernemonsters was 455 µg/kg, wat 30 keer of meer hoger is dan de gemiddelde concentratie gevonden in kuilgras, hooi en gedroogd gras.

Om de gevonden PA profielen te kunnen relateren aan een specieke kruiskruidsoort, zijn referentiemonsters van Jacobskruiskruid (*Senecio jacobaea/Jacobaea vulgaris*), Klein (Gewoon) kruiskruid (*Senecio vulgaris*) en Bezemkruiskruid (*Senecio inaequidens*) verzameld in de omgeving van Wageningen. Gemiddelde PA profielen werden opgesteld voor deze drie soorten. Vergelijking met deze referentieprofielen toonde aan dat de ruwvoeders in de meeste gevallen verontreinigd waren met Klein kruiskruid. Slechts in twee gevallen waren er aanwijzingen voor de aanwezigheid van

andere kruiskruidsoorten. Het gecontamineerde hooimonster bevatte PA's die niet direct gerelateerd konden worden aan een van de drie onderzochte kruiskruiden. Waarschijnlijk gaat het hier wel om een verwante kruiskruidsoort. Het gecontamineerde gedroogd grasmonster bevatte een combinatie van voornamelijk Klein kruiskruid en een kleine hoeveelheid Jacobskruiskruid.

Conclusie

Klein kruiskruid (*Senecio vulgaris*) geeft het grootste risico op verontreinigde ruwvoeders in Nederland. Van de vier onderzochte ruwvoedercategoriën is het risico het grootste bij de productie van luzerne. Luzerne kan verontreinigd zijn met PA's op niveaus, die bij langdurige blootstelling mogelijk toxisch zijn voor de veestapel.

Aanbevelingen

1. Deze studie laat zien dat in Nederland Klein kruiskruid het grootste risico geeft op verontreiniging van diervoeders. Het risico is met name groot bij luzerne, waar soms aanzienlijke hoeveelheden PA's in zijn aangetroffen. Aanbevolen wordt de productie van luzerne nauwgezetter te gaan monitoren, zowel wat betreft de eindproducten als ook door het uitvoeren van veldinspecties. Dit laatste zal kunnen leiden tot een beter inzicht in de specifieke omstandigheden waaronder de verontreiniging met Klein kruiskruid optreed en welke beheersmaatregelen er getroffen kunnen worden om het risico op verontreiniging te verminderen.
2. De huidige methode omvat maar een klein deel van de PA's die potentieel aanwezig kunnen zijn in diervoeders. Het verdient aanbeveling de huidige methode uit te breiden met mono- en diester PA's die voorkomen in planten van ondermeer de *Boriginaceae* familie, zoals Slangekruid (*Echium vulgare*), Smeerwortel (*Symphytum spp*), Hondstong (*Cynoglossum officinale*) en van de *Asteraceae* familie, zoals Koninginnenkruid (*Eupatorium cannabinum*). Deze soorten zijn (plaatselijk) vrij algemeen in Nederland, als ook in andere delen van Europa. Tegelijkertijd dient de monitoring van diervoeders ook uitgebreid te worden tot deze PA's.
3. Om het risico in te kunnen schatten dat PAs in de voedselketen terechtkomen door middel van overdracht naar melk is het noodzakelijk de monitoring data te combineren met *in vitro* en *in vivo* experimenten. De gegevens die momenteel beschikbaar zijn aangaande overdracht naar melk zijn nogal beperkt. De overdrachtsverhouding van individuele PA's (in zowel hun tertiaire amine vorm als in hun N-oxide vorm) van voer naar melk zou moeten worden bepaald, omdat verwacht mag worden dat verschillen in polariteit en chemische reactiviteit van invloed zullen zijn op het metabolisme en kunnen resulteren in verschillende overdrachtsverhoudingen.

Contents

1 Introduction

Pyrrolizidine alkaloids (PAs) are the toxic principles of many plants belonging to the families of *Asteraceae/Compositae*, *Boriginaceae* and *Fabaceae*/*Leguminosae*. Hundreds of different PAs have been isolated and characterised (EFSA, 2007, Hartmann and Witte, 1995). The pyrrolizidine alkaloids are a complex group of structurally related compounds that are composed of a necine base and one or two ester groups or a macrocyclic diester. An important genus of the *Asteraceae* that contains PAs are the ragworts and groundsels (*Senecio*). The PAs in ragworts and groundsels are generally of the macrocyclic diester type. Many different PAs have been described for the *Senecio* family; even within one species there may be more than 10 different PAs present (Hartmann and Witte, 1995). 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). PAs are mainly present in the plant in a water soluble N-oxide form, but can be easily reduced to a tertiary amine form (Hartmann and Toppel, 1986). Pyrrolizidine alkaloids for long have been recognized as toxic for livestock (Bull *et al*, 1968). Nevertheless they have not been listed as undesirable substances in animal feeds, nor have they been included in the Annex of Directive 2002/32/EC. Thus far the European Union has established no Permitted Levels for any neither of the individual PAs nor as a group in animal feedingstuffs.

Due to their bitter taste PA-containing plants are generally unpalatable and as a result avoided by grazing animals in the field. In preserved and composed feeds this recognition is lost and the toxic PAs may be consumed by livestock. Consumption of substantial amounts of PAs may lead to acute intoxications, causing veno-occlusive disease (VOD) and hemorrhagic liver necrosis and death. Long-term exposure to lower concentrations may result in hepatic megalocytososis, VOD and liver cirrhosis. Besides their hepatotoxicity PAs exert genotoxic and probably carcinogenic effects (EFSA, 2007, Fu *et al*, 2004). The toxicity and carcinogenicity of individual PAs is related to some structural features: macrocyclic esters are generally more toxic than diesters and monoesters. The presence of an 1,2-unsaturated bond is essential for the occurrence of the toxic effects. The N-oxide forms requires reduction (e.g. in the intestinal tract) before bioactivation can occur. The PAs are quickly metabolised, primarily in the liver, where the most serious effects are observed. Several routes of detoxification are operating as well: oxidation of tertiary amines to the more water soluble N-oxides; enzymatic hydrolysis of the ester bonds liberating the (non-toxic) necine base and acid; and conjugation with glutathione transferase. However, oxidation of the PA to the corresponding dehydro-PA (DHP) signifies a route towards bioactivation. DHP derivatives can interact with proteins and DNA to form covalently bound adducts (Fu *et al*, 2004).

Although modern agricultural production practices have greatly reduced the risk of widespread exposure of livestock and humans to pyrrolizidine alkaloids, there is still considerable concern for incidental intoxications (EFSA, 2007). The EFSA opinion specifically states the need for suitable analytical methods and -when they become available- to use these methods to collect monitoring data for PAs (individual as well as total PA content) in feed materials. To date still very few methods are available that have been validated specifically for the analysis of PAs in feeds. Monitoring data on the occurrence of PAs in animal feeds are equally scarce. These data are highly needed to assess the potential exposure of livestock to PAs.

RIKILT has recently developed and validated an analytical method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the analysis of macrocyclic PAs in animal forages/roughages (silage, grass, hay, alfalfa), including artificially dried and processed samples. This method has been used for the analysis of 147 samples taken from the Dutch National Monitoring Plan 2006-2008, including silage, grass, hay and alfalfa samples. The results obtained in this survey are presented in this report. To identify the possible *senecio* species present in contaminated samples, averaged PA profiles were constructed for three *Senecio* species common in The Netherlands: Tansy ragwort (*Senecio jacobaea/Jacobaea vulgaris*), Common groundsel (*Senecio vulgaris*) and Narrow-leaved ragwort (*Senecio inaequidens*) (see Annex I for commonly used names of plants). The results are discussed and recommendations for further research and monitoring are proposed.

2 Material and Methods

A concise description of the used analytical methodology is presented here. The method is described in detail in RIKILT standard operation procedure RSV A1061 (in Dutch). Full details on the method development and validation will be presented in a future peer reviewed paper.

2.1 Analytical method

Analytical samples of the PA standards were obtained from various sources (commercial as well as gifts) or synthesized in-house. N-oxides were prepared by N-oxidation of the corresponding tertiary amine with 30% hydrogen peroxide according to Chou *et al.* (2003). The standards were at least 90% pure according to LC-MS/MS analysis. Some PAs were not available as standard. For these compounds a semi-quantitative (indicative) value could be obtained by comparison of the LC-MS/MS response with that of a structurally related standard. Approximately 40 compounds were included in the final method (see Annex II for chemical structures).
Analysis of the 2007 and 2008 survey samples and the analysis of plant extracts for PA profiling was performed on an Waters Acquity UPLC coupled to a Waters Quattro Premier XE tandem mass spectrometer, operated in positive electrospray mode. The compounds were separated on a Waters UPLC BEH 150 x 2.1 mm 1.7 µm analytical column run at 0.4 ml/min with an acetonitrile/water/ammonia (0.05%) gradient. Total runtime of the method was 15 min. Initial validation of the method and the analysis of the 2006 survey samples was performed on an Agilent 1100 series HPLC system coupled to a Waters Quattro Micro tandem mass spectrometer. Chromatographic separation was obtained on an Waters XBridge C18 150 x 3.0 mm 5 µm analytical column run at 0.4 ml/min with an acetonitrile/water/ammonia (0.05%) gradient. Total run time of the method was 25 min.
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 (see Annex III for instrumental details). For the Quattro Premier method the dwell time for each transition was set at 20 msec. A total of 43 transitions was monitored in a single run to include all potentially relevant PAs. All compounds could be unequivocally characterized on the basis of retention time and fragmentation transitions. For the Quattro Micro method the dwell time was set at 80 msec. Due to the lower scanning rate of the Quattro Micro compared to the Quattro Premier, fewer transitions (25) could be monitored in a single run, necessitating the use of only one parent to product ion for the minor PAs.

2.2 Sample preparation and method validation

(Prewilted) grass silage was freeze-dried upon arrival. Residual water content of the freeze-dried grass and of dry forage (hay, dried grass, processed grass and alfalfa) was determined by heating at 103°C in an oven. Dry forage and freeze-dried silage were homogenized by grinding the complete sample with a Peppink 200AN grinding machine (Veerman, Olst, The Netherlands), through an 1 mm sieve. Laboratory samples (2 g) were extracted with a 2% formic acid solution (40 ml) on a rotary tumbler

for 30 min. The samples were centrifuged for 15 min at 3500g. Of the extracts 10 ml was made alkaline (pH 10) with concentrated ammonia solution and purified and concentrated by solid phase extraction over Strata X 60 mg, 3 cc cartridges. The cartridges were preconditioned with 3 ml methanol followed by 3 ml water. After application of the extract, the cartridges were washed with 3 ml 1% formic acid solution, followed by 3 ml 1% ammonia solution. The cartridges were dried under reduced pressure and eluted with 3 ml methanol. After evaporation of the solvent the residues were reconstituted in 500 µl methanol/water (1:9). Of the sample 5 to 10 µl was injected in to the LC-MS/MS.
Samples were analysed in triplicate. Heliotrine (100 µg/kg) was spiked to the samples as an internal standard. To one of the samples no standards were spiked, one sample was spiked with a mixed solution of standards at 250 µg/kg and one sample at 1000 µg/kg. When one or more PAs were detected above the detection limit (10 µg/kg) the amount was quantified against the lower spiked sample. Samples containing PAs at an individual concentration in excess of 250 µg/kg were quantified against the higher spiked sample. In both cases internal standard correction was applied. PAs for which no reference standard was available were quantified against a structurally related standard. The reported concentrations refer to the dried samples, with correction for 12% water content.
For in-house validation of the method 30 blank samples (grass silage, hay, and alfalfa) were spiked at three levels (50, 250 and 1000 µg/kg). Recoveries obtained for grass silage (n = 22 samples) at the 250 µg/kg level averaged 98 ± 23 % for the N-oxides and 90 ± 11 % for the tertiary amines. For dry forage (hay, alfalfa) (n = 8 samples) slightly lower recoveries were obtained: 74 ± 5% for the N-oxides and 78 ± 5% for the tertiary amines.

2.3 PA composition of reference plants

Adult plants of Tansy ragwort, Common groundsel and Narrow-leaved ragwort were collected from various locations (within a 30 km radius) around Wageningen, The Netherlands. In most cases several plants were collected from each location. Plants were air-dried, grinded and homogenized. To laboratory samples (1 g) heliotrine (10 µg/g) was spiked and the samples were extracted with 50 ml 2% formic acid solution on a rotary tumbler for 30 min. Extracts were filtered over a glass microfiber filter (Whatman). An aliquot (25 µl) was diluted with water to 1 ml in an HPLC vial and 10 µl was injected in the LC-MS/MS system. Quantification was performed by comparison with a solution of standards (100 ng/ml), with internal standard correction.

3 Results

3.1 Analysis of animal forage from the Dutch National Monitoring Plan

During 2006-2008 147 samples of different categories of animal forage were analysed for their PA content. An overview of the analysed samples and (sub) categories is presented in Table 1.

Table 1 Overview of animal forages monitored during 2006-2008.

		Year			
Category	Sub category	2006	2007	2008	Total
Silage	Grass silage	15	10	14	39
	Prewilted silage	1	1	15	17
	Total silage	*16*	*11*	*29*	*56*
Hay	Hay	0	11	1	12
	Hay nature reserve	0	25	0	25
	Total hay	*0*	*36*	*1*	*37*
Grass	Dried	0	6	2	8
	Pellets, crumb	4	4	7	15
	Total grass	*4*	*10*	*9*	*23*
Alfalfa	Dried/hay	1	6	3	10
	Pellets, crumb	5	7	9	21
	Total alfalfa	*6*	*13*	*12*	*31*
Total		**26**	**70**	**51**	**147**

In Figure 1 an overview is given of the obtained results for all samples, and in Table 2 specified for each of the four main animal feed categories. In Annex IV to IX the results for the individual samples can be found. As can be seen from Figure 1, of the 147 samples analysed, 116 were free or practically free of pyrrolizidine alkaloids (containing no single PA exceeding the LoD of 10 µg/kg). Of the remaining 31 samples, 22 contained relatively low concentrations of PAs (total PA concentration between 10 and 100 µg/kg), while six samples contained substantial amounts (total PA content between 100 and 1000 µg/kg). Three samples contained high amounts of PAs (in excess of 1 mg/kg). The most heavily contaminated forage sample contained approximately 5.4 mg/kg PAs (calculated on the basis of 12% moisture).

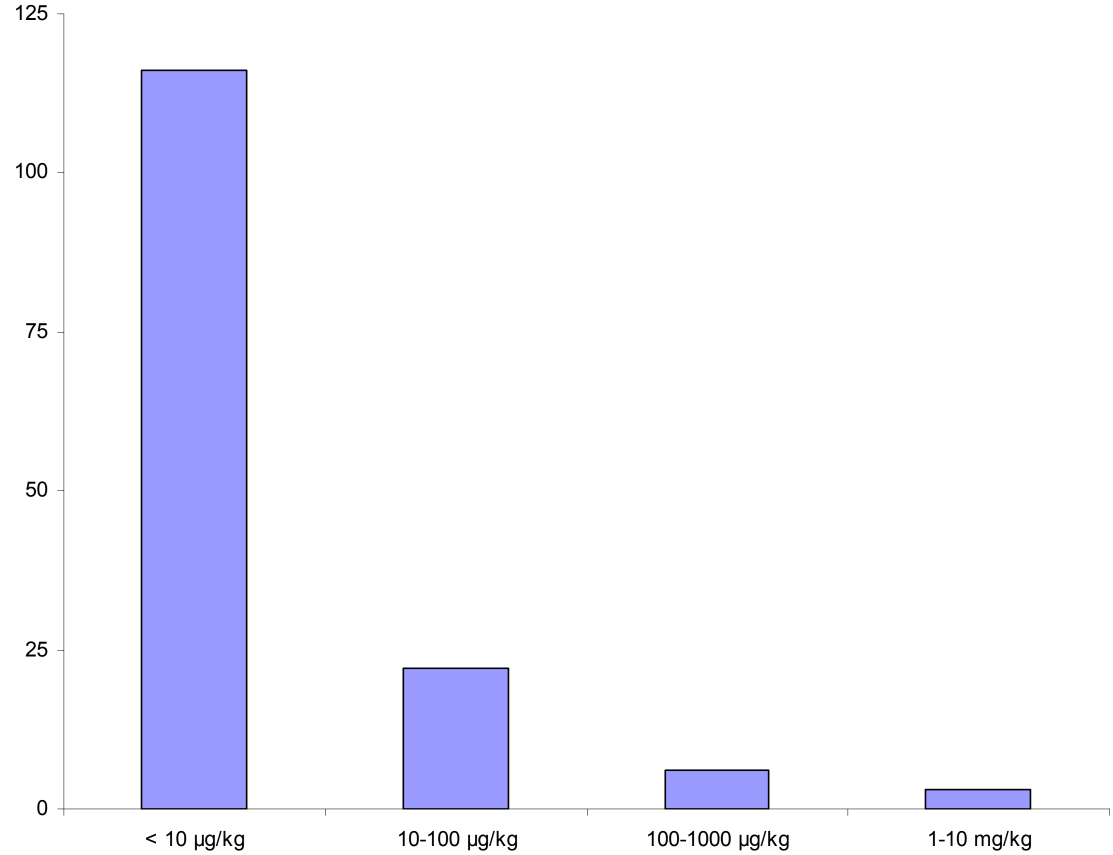

Figure 1 Bar chart showing the combined results obtained for all forage samples. The samples are classified according to the total PA concentration determined.

With respect to the different animal feed categories, large differences in PA concentrations and incidences were observed (Table 2). (Prewilted) grass silage samples constitute the largest category that was analysed. On a total of 56 samples only 3 contained measurable amounts of PAs (Table 2, Annex IV). The concentrations detected were very low, between 10 and 30 µg/kg. The average PA content for grass silage is less than 10 µg/kg.
The second category comprises 37 hay forage samples including 25 samples that were collected from nature reserves (Table 2, Annex V). These samples did not contain PAs except for one sample. In this sample (202282) significant levels of PAs were detected (see Annex VIII for the concentration of individual PAs and Annex IX for the relative profile). The total PA amount was 549 µg/kg and the PA profile consisted mostly of otosenine, florosenine and erucifoline-N-oxide. The mean PA content for hay samples is 15 µg/kg.
The third category is formed by (artificially) dried and processed (pellets, crumb) grass samples (Table 2, Annex VI). This category contained no, or very low amounts of PAs, except for one sample (217349), which contained a total of 288 µg/kg PAs. In this sample seneciphylline, senecionine, retrorsine and spartioidine were present (no N-oxides) as well as smaller amounts of jacobine, jaconine and acetylerucifoline (Annex VIII Annex IX). The average PA content for dried grass samples is 14 µg/kg.

Table 2 Overview of obtained results for the animal forages monitored during 2006-2008.

		Distribution according to PA concentration (%)				Concentration (µg/kg)	
Category	Sub category	< 10 µg/kg	10-100 µg/kg	100-1000 µg/kg	> 1000 µg/kg	Average	Maximum
Silage	Grass silage	92	8	0	0	<10	28
	Prewilted silage	100	0	0	0	<10	<10
	Total silage	*95*	*5*	*0*	*0*	*<10*	*28*
Hay	Hay	100	0	0	0	<10	<10
	Hay nature reserve	96	0	4	0	22	549
	Total hay	*97*	*0*	*3*	*0*	*15*	*549*
Grass	Dried	100	0	0	0	<10	<10
	Pellets, crumb	73	20	7	0	21	288
	Total grass	*83*	*13*	*4*	*0*	*14*	*288*
Alfalfa	Dried/hay	40	30	20	10	411	3524
	Pellets, crumb	19	62	10	10	476	5401
	Total alfalfa	*26*	*51*	*13*	*10*	*455*	*5401*
Total		**79**	**15**	**4**	**2**	**121**	**5401**

The final category comprises 31 samples of alfalfa, which is commercially available in dried or in processed (pellets, crumb) form. In contrast to the other categories analysed, most samples contained PAs at levels exceeding the detection limit (Table 2, Annex VII). Although in many cases (16 out of 31) the concentrations detected were relatively low (between 10 and 100 µg/kg), in 7 instances elevated levels were detected (Annex VIII Annex IX). In samples 174359, 175232 and 217348 high amounts were detected, respectively 3.5, 3.8 and 5.4 mg/kg. On average the alfalfa samples contained 455 µg/kg PAs.

3.2 PA content of Senecio species common in The Netherlands

In The Netherlands approximately 10 plant species can be found that produce macrocyclic PAs. Three species, Tansy ragwort (*Senecio jacobaea/Jacobaea vulgaris*), Common groundsel (*Senecio vulgaris*) and Narrow-leafed ragwort (*Senecio inaequidens*), can be considered as particularly relevant. These species are quite common in many parts of The Netherlands, can locally be present in considerable densities and are known to contain substantial amounts of PAs (Röder, 1995). To determine which *Senecio* species is present in each of the forage samples in which PAs were detected, it is important to obtain information on the PA profile of these species. In order to establish an average PA profile for these species (at least for the Central part of The Netherlands) adult plants were collected from various locations around Wageningen (in an approximately 30 km radius). (Pooled) plants extracts were subsequently analysed for their PA content. In the following sections the three plant species will be described and their PA composition presented and compared with literature data. It should be noted that in general the results reported in the literature were obtained by GC-based methods. In these methods it was required to reduce N-oxides to the tertiary amines prior to analysis. Therefore literature data available on the distribution between N-oxides and tertiary amines of individual PAs is very limited.

3.2.1 Tansy ragwort (Senecio jacobaea/Jacobaea vulgaris)

Tansy ragwort (*Senecio jacobaea/Jacobaea vulgaris*), also called Common ragwort, is a bi-annual plant (sometimes perennial) that is native to Europe but is now found in many parts of the world (often as an invasive species). The plant grows along roadsides and in pastures and can locally produce heavily invested patches, especially when the sites have been disturbed (see Annex X for photographs). The first year a rosette is formed. Typically the rosettes have deeply indented bluntly lobed leaves. In the second year during spring one or more flowering stalks (typically of 75 to 150 cm height) are produced. In Western Europe the plant flowers in June-August. The composite flower heads are arranged in flat topped clusters and have a daisy-like appearance. In the coastal areas a variant (*Senecio jacobaea dunesis*) exists in which the yellow petals are absent. The plant generally dies when the seeds are mature (September/October). When the stalk is cut before seed has been set, the plant will produce new stalks or become perennial.

The PA composition of Tansy ragwort has been the subject of a number of studies. Several chemotypes have been described that differ in PA composition (Witte et al, 1991, Hartmann and Dierich, 1998, Macel *et al*, 2004). The jacobine chemotype is common to the coastal regions of Western Europe. It typically contains significant amounts of jacobine, jaconine, jacoline and jacozine, while the erucifoline content is low or absent (see Annex II for chemical structures of the PAs). The erucifoline chemotype becomes prominent more inland. This chemotype typically contains erucifoline as major alkaloid together with acetylerucifoline, while jacobine and related PAs are only found in trace amounts. In the transition zone mixed chemotypes may be found that contain both jacobine and erucifoline in relatively large amounts.

The PA bouquet for Tansy ragwort growing in the centre of The Netherlands (the area around Wageningen) was investigated by means of LC-MS/MS analysis of aqueous extracts of adult plants. Although the composition and concentration of PAs in individual plants may be variable, by pooling a sufficient number of plants (several hundreds plants were collected from 14 different locations), an averaged composition could be established. It is evident from Figure 2 that plants growing in the centre of The Netherlands contain jacobine as well as erucifoline in significant amounts, indicating that a mixed chemotype is predominant. Besides jacobine-N-oxide and erucifoline-N-oxide, senecionine-N-oxide and seneciphylline-N-oxide are important PAs. These four PAs are on average present in excess of 10% of the total sum. Other important PAs are the corresponding reduced forms (tertiary amines) and jaconine, all constituting between 3 and 8% of the total sum. Many other PAs can be detected at lower concentrations. PAs with an otonecine backbone (e.g. senkirkine, otosenine, florosenine) are virtually absent. The average total PA content is 1.6 mg/g dry weight, which is in line with earlier reports (Röder, 1995). N-oxides constitute approximately 76% of the total PA content in air-dried plant material; tertiary amines 24%.

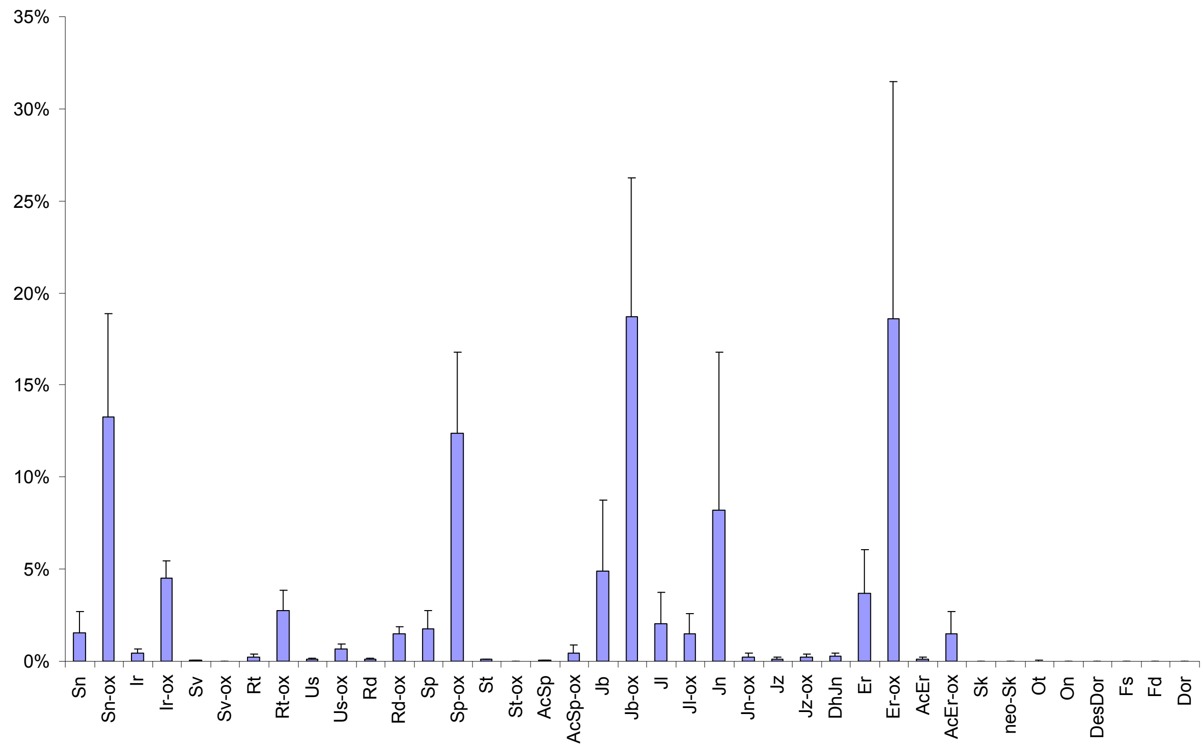

Figure 2 Relative PA composition of Tansy ragwort. Average of plants collected from 14 locations around Wageningen, the Netherlands. From each location between 10 and 100 plants were collected, air-dried and homogenized before analysis. Error bars indicate standard deviation.

3.2.2 Common groundsel (Senecio vulgaris)

Common groundsel (*Senecio vulgaris*) is a small, annual, weed that originates from Europe but now occurs world-wide in areas with a moderate climate. It grows in roadsides, (arable) fields, gardens and fallow lands. It grows between 10 and 50 cm in height and produces many disk flowers without petals (Annex X). It grows quickly after winter and flowers in April/May. Common groundsel can produce up to two or three generations yearly.

Though modest in appearance, Common groundsel produces significant amounts of PAs. According to a study of Borstel *et al* (1989), flowering heads of Common groundsel collected in Germany and Denmark contained seneciphylline and senecionine as major PAs, together making up 70-80% of the total content. Smaller amounts of integerrimine, retrorsine and senecivernine were detected as well. Hartmann and Dierich (1999) reported a very similar composition for plants grown on culture medium. They also showed that the PAs are present as N-oxides. Borstel *et al* (1989) reported a total alkaloid content of 0.6 to 1.0 mg/g on fresh weight basis. Assuming a fresh to dry weight ratio of 5 to 1, this corresponds to a total alkaloid content of 3 to 5 mg/kg, or 0.3 to 0.5%. In a study of Johnson *et al* (1985), Common groundsel, was identified as a weed that could cause problems of forage contamination in the US, particularly in dry years. An average PA content of 0.25% was calculated for plants sampled in California. Common groundsel recently was found as contaminant in mixed salad items intended for human consumption (BfR, 2007).

Based on the analysis of sample extracts of plants (all parts) collected in the area around Wageningen, an averaged composition was constructed (Figure 3). The PA-profile is dominated by seneciphylline-N-oxide (close to 40%) and senecionine-N-oxide (20%), in accordance with the reports of Borstel *et al*

(1989) and Hartmann and Dierich (1999). Other PAs, such as the tertiary amines seneciphylline, senecionine, as well as retorsine-N-oxide, integerrimine-N-oxide and spartioidine-N-oxide are present each at 5-8% of the total. The PAs typical for Tansy ragwort - jacobine and erucifoline- are not found in detectable amounts. Neither is any of the otonecine based PAs found in Common groundsel. The average total PA content is 2.7 mg/kg dry weight, or 0.27%. In air dried material on average 82% of the PAs is present as N-oxide, 18% as tertiary base.

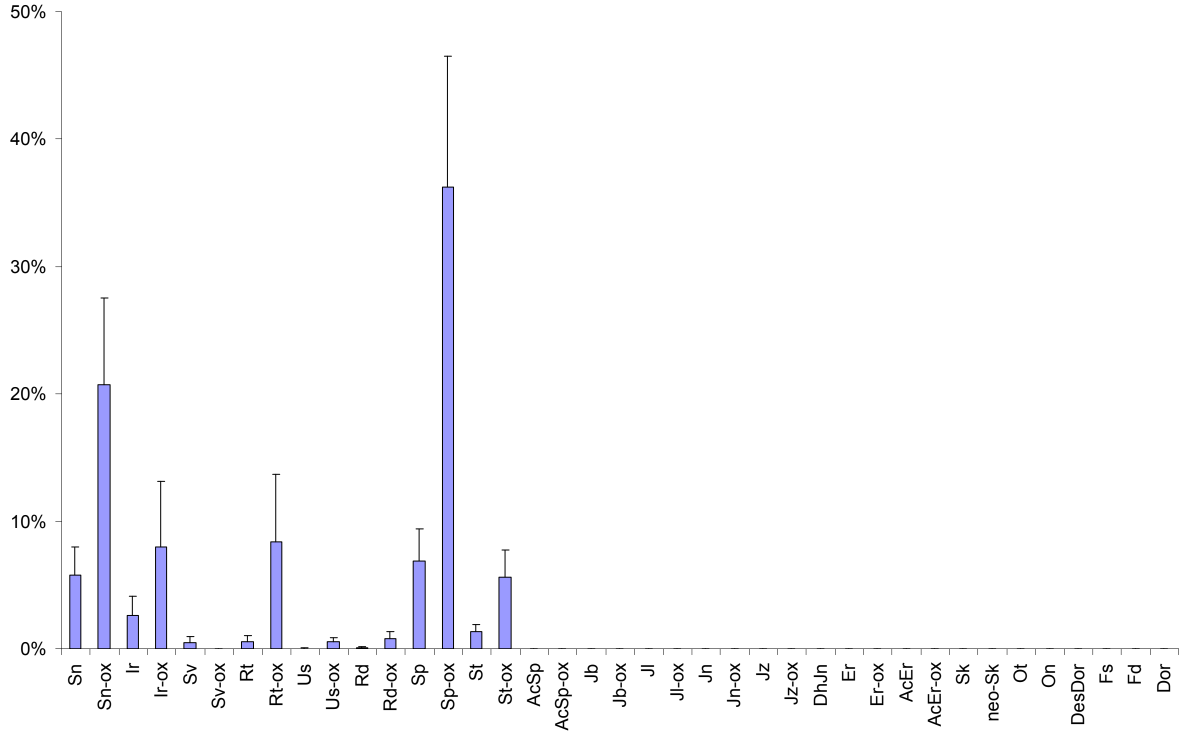

Figure 3 Relative PA composition of Common groundsel (Senecio vulgaris). Average of plants collected from 5 locations around Wageningen, the Netherlands. Error bars indicate standard deviation.

3.2.3 Narrow-leafed ragwort (Senecio inaequidens)

Narrow-leafed ragwort (*Senecio inaequidens*) is a perennial plant originating from Southern Africa, (accidentally) introduced in Western Europe by the end of the 19th century. It is currently proliferating rapidly along roadsides of highways and railroad tracks. Narrow-leafed ragwort is a shrubby, highly branched plant, growing 50-75 cm tall (Annex X). It has linear slender leaves with flower heads that appear similar to Tansy ragwort (*Senecio jacobaea*), with the flowers loosely placed at the end of the stalks. The plant flowers from June till late fall (Heger and Böhmer, 2006).
Bicchi *et al* (1991) identified senecionine, retrorsine and senecivernine as major components (all around 25%) of Narrow-leafed ragwort sampled in Italy. Seneciphylline, integerrimine, usaramine as well as the otonecine base alkaloids doronine and desacetyldoronine are important minor constituents (3 to 7% each). Hartmann and Dierich (1999) reported retrorsine and florosenine as major components (both 35%) of Narrow-leafed ragwort from Germany. Otosenine, usaramine, integerrimine and senecivernine were reported as minor compounds (5-9%). Analysis of extracts of plants collected in the area around Wageningen resulted in the following averaged composition (Figure 4). The PA profile is dominated by retrorsine-N-oxide (over 50%), and senecivernine-N-oxide (20%). Usaramine-

N-oxide, integerimine-N-oxide and senecionine-N-oxide each contribute between 5 to 10%. Small but still significant amounts of florosenine, otosenine and senkirkine are found as well. The PAs typical for Tansy ragwort - jacobine and erucifoline- are not found in detectable amounts. Similarly, seneciphylline - a major component of Common groundsel - is practically absent in Narrow-leafed ragwort. A mean concentration of 2.1 mg/kg dry weight is obtained. N-oxides constitute 96% and tertiary amines only 4% of the total content.

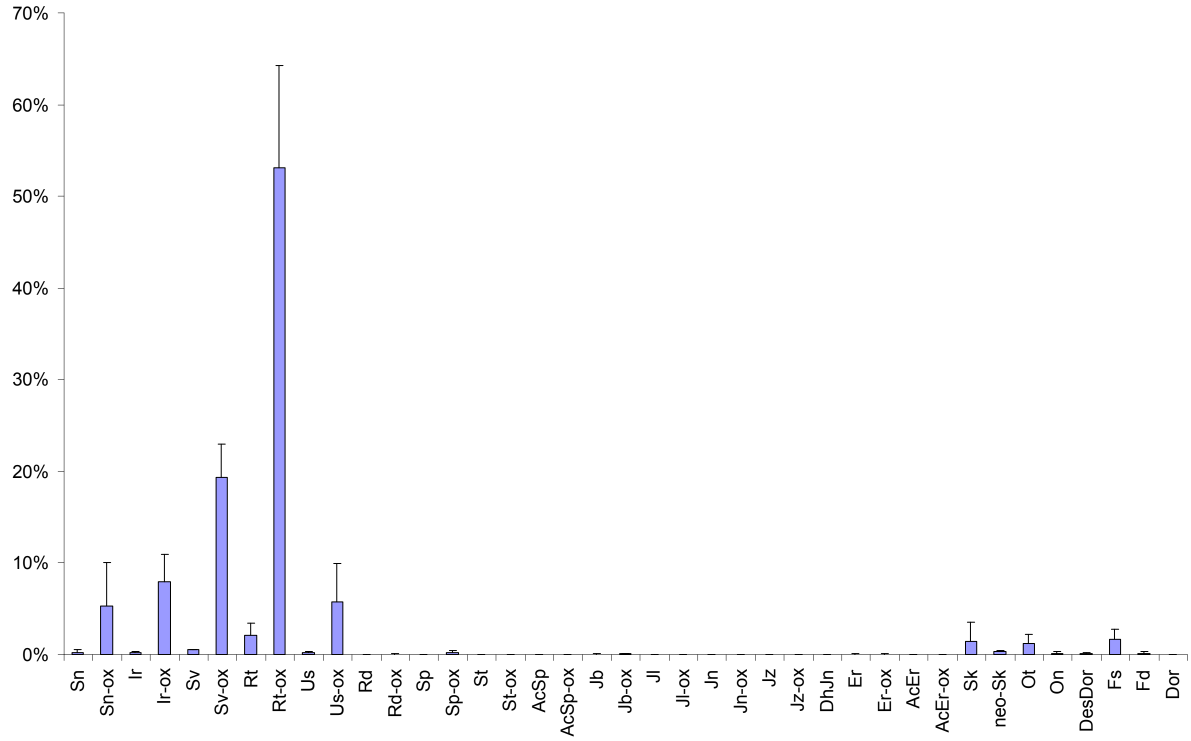

Figure 4 Relative PA composition of Narrow-leaved ragwort (Senecio inaequidens). Average of plants collected from 6 locations around Wageningen, the Netherlands. Error bars indicate standard deviation.

4 Discussion and conclusions

In the period 2006-2008 147 samples of animal forage and roughage were selected from the Dutch National Monitoring Program on animal feeds. In the same period an LC-MS/MS method was developed and validated for the analysis of PAs in animal forages. With this method the samples were screened for some 40 different PAs. Samples were classified in one of the four different main categories of animal forage that were included in this survey: silage, hay, (dried) grass and alfalfa. The results of this screening effort have been presented in Section 3. In order to link the PA profiles that were obtained for contaminated samples with particular *Senecio* species, plant samples of Tansy ragwort, Narrow-leafed ragwort and Common groundsel were collected in the vicinity of Wageningen. Analysis of these samples has resulted in averaged PA profiles for these three species.
For grass silage the results indicate that the risk of contamination with PAs is relatively small (Table 2). Only in 5% of the samples trace levels were found, which are not likely to present a risk to animals or humans (i.e. via consumption of milk). The average PA content of the samples is well below 10 µg/kg.
The hay samples were generally free of PAs as well (Table 2). Special attention was directed to the batches of hay produced from nature reserve areas. These fields may be more prone to contamination with ragwort species than plots that are in regular use for agriculture. Nevertheless, only in one sample (202282) out of 25 analysed, significant amounts of PAs (549 µg/kg) were detected (Annex IX , Annex IX). This specific sample originated from a nature reserve in the Dutch province of Zeeland. The PA profile, consisting mostly of otosenine, florosenine and erucifoline-N-oxide, does not match well with any of the profiles obtained for Tansy ragwort, Narrow-leafed ragwort or Common groundsel. Although otosenine and florosenine are components that occur in Narrow-leafed ragwort, retrorsine is notably missing in the profile of sample 202282. Moreover, erucifoline has not been reported as a component present in Narrow-leafed ragwort. Most likely the PA profile belongs to another member of the *Senecio* family. Other *Senecio* species that can be found in The Netherlands are Fen ragwort (*Senecio paludosis*), Marsh ragwort (*Senecio aquaticus*), Broad-leaved ragwort (*Senecio fluviatilis*) and Hoary ragwort (*Senecio erucifolius*). A possible candidate is Marsh ragwort which has been reported to contain erucifoline as well as otonecine PAs (Pelser *et al*, 2005). On average, the PA content of hay samples included in this survey, is quite low (15 µg/kg).
The samples belonging to the remaining categories - (artificially) dried and processed (pellets, crumb) grass and alfalfa - were taken mostly from batches produced by the drying companies located in the North, Northwest and Southwest parts of the Netherlands. Investigation of the production sites (as far as this could be retrieved from the available information) of the samples indicated that these are representative for the situation in The Netherlands. Two alfalfa samples (217347 and 223182) originated from France (Champagne region). One processed grass sample (217349) contained a significant amount of PAs (288 µg/kg, Annex VIII , Annex IX). This is the only sample of the survey that actually contained some amounts of the PAs typical for Tansy ragwort, jacobine and jaconine. However, the profile is dominated with PAs typical for Common groundsel, such as seneciphylline, senecionine, retrorsine and spartioidine. This indicates that the sample is mainly contaminated with Common groundsel and to a smaller extent with Tansy ragwort. The average PA content of the dried grass samples is only 14 µg/kg.
In contrast to the other categories, a high percentage of contaminated alfalfa samples was encountered (Table 2). In approximately 75% of the samples analysed at least traces of PAs were detected. One

third of the samples contained PAs in a concentration exceeding 50 µg/kg. Three samples (10%) contained high amounts of PAs, in the range of 3.5 to 5.4 mg/kg. The four samples (171232, 175061, 194625, 210283) that were taken from organically grown alfalfa contained no or low amounts of PAs (max 29 µg/kg). The two samples from France contained respectively 72 and 88 µg/kg. An average PA concentration of 455 µg/kg was calculated for the alfalfa samples, which is approximately 30-fold higher than the average for hay and dried grass. Interestingly, the PA-profile of all the contaminated alfalfa samples (including the French samples) is very similar (Figure 5, Annex IX). Seneciphylline, senecionine, retrorsine, spartioidine and integerrimine are the major PAs in these samples. These are PAs typical for Common groundsel (compare Figure 5 with Figure 3) and they are present in approximately the same relative ratios. The ratio between the tertiary amines and the N-oxides in the analysed alfalfa samples, however, is quite variable. In the air-dried plant material that was collected near Wageningen the N-oxides did make up over 80% of the total PA content. In some survey samples the majority of the PAs is indeed in the N-oxide form, but in some other samples the tertiary amines dominate, while in some samples both forms are present in more or less equal amounts. Possibly this is related to the drying conditions used during the manufacturing of the products. It is known that prolonged heating of plant material may result is (partial) reduction of N-oxides to the corresponding tertiary amines (Hartmann and Toppel, 1987). With the available information a distinct correlation between the tertiary base/N-oxide ratio and the way the batches were produced could not be made.

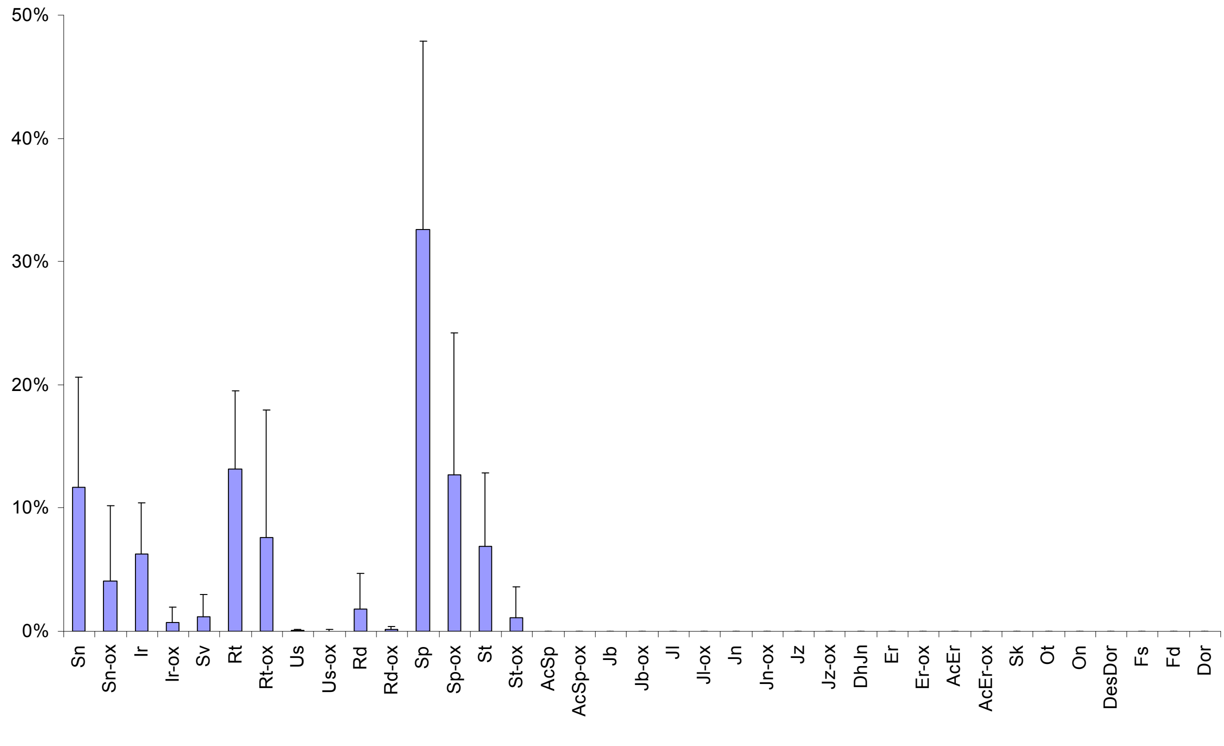

Figure 5 Relative PA composition of alfalfa samples containing more than 50 ug/kg PA (10 samples). Error bars indicate standard deviation.

Alfalfa (*Medicago sativa*) is a flowering, perennial plant belonging to the pea family (*Fabaceae*). It can grow to a height of 1 m and has a very deep root system of up to 4 m. Worldwide it is a very important crop used in agriculture, due to its high feeding value. Its ability to fix nitrogen in the soil makes it an attractive plant to improve soil quality for agricultural practice. Alfalfa is normally sown in early spring and harvested (cut) three to four times a year for the period of three years. After three years rotation with another crop is recommended, because the yields generally drop. In the Netherlands, grass and alfalfa allocated for artificially drying are typically grown within a limited radius (up to 30 km) around one of the six remaining drying companies. In the Netherlands the majority of alfalfa is grown on clay soil in the region of Groningen, Flevoland, Noord Holland and Zeeland, where the drying factories are located. Total production area is around 6,000 ha (60 km^2) divided over approximately 600 farms (Bont *et al*. 2008). Productivity per ha is approximately 14 tons on a dry weight basis, amounting to a total annual production in the Netherlands of around 85,000 tons. A comparable amount of dried grass is produced yearly in the Netherlands. Approximately 15% of alfalfa is organically grown. The dried products are sold as alfalfa hay (bales) or in the form of pellets or crumb. It may also be mixed with grass, clover and green oats. The majority of the alfalfa produced is sold on the Dutch market. It is consumed as such or as ingredient of compound feeds by livestock (especially in the dairy industry). A smaller but still significant part (15%) is purchased by owners of horses (Bont *et al*, 2008). It has been estimated that the daily portion of alfalfa in the diet of horses is in the order of 100 to 300 g, which is typically less than 5% of the total ration (Bikker, 2009). A smaller part of the horse farms and private owners may give their animals up to 2 kg daily. Production of (dried) alfalfa in the Netherlands is relatively modest compared to some other European countries, e.g. France and Spain, which produce between 1,000,000 and 2,000,000 tons annually (Bont *et al*, 2008). As a result in these countries alfalfa plays a more prominent role in the diet of livestock.

In general plants that contain pyrrolizidine alkaloids are avoided by grazing animals due to their unpalatability (bitter taste due to the presence of the alkaloids). The risk of ingestion by grazing of PA-containing plants is considered to be low under normal circumstances. However, when the plants are accidentally harvested and mixed through the forage, the animals will not recognize the plants anymore and these will likely be consumed (EFSA, 2007a). The fact that almost no traces of Tansy ragwort has been found in the investigated samples, is an indication that farmers and producers are well aware of the risk of contamination and that control in general is effective. Unfortunately, the risk of contamination with Common groundsel appears to be much less known, notwithstanding the recent interest in Common groundsel as a contaminant in salad (BfR, 2007b).
The European Union has not established legislation that specifically addresses the contamination of animal feeds with seeds and plant materials of *Senecio* species. Council Directive 2002/32/EC (2002) on undesirable substances in animal feed focuses mainly on toxic seeds of alkaloid containing plants, without specifically mentioning *Senecio* species. A maximum content of 3000 mg/kg feedingstuff (0.3%) has been established. The seeds of *Senecio* species generally constitute only a relatively minor part of the total weight of the adult plant, while all parts of the plant are toxic. By taking all parts into account and adopting an average PA content of 1-3 mg/g dry weight for the *Senecio* species discussed in this study, this would correspond to a maximum PA content of 3-9 mg/kg feedingstuff. In the current study 3 out of 147 samples did contain PAs in this concentration range. These samples were alfalfa forage, signifying the importance of further study and closer monitoring of this particular class of animal feeds.

5 Recommendations

1. This study indicates that in The Netherlands Common groundsel is presenting the largest risk for contamination of animal forage. Especially alfalfa forage may contain substantial amounts of PAs. It is recommended to monitor the production of alfalfa more closely, with respect to the commercial end products as well as on site inspection of the plots where alfalfa is grown. The latter may lead to a better understanding of the specific conditions that favor contamination with Common groundsel and which measures can be taken to minimize the risk of contamination.

2. Although in the current survey a substantial number (40) of (macrocyclic) PAs have been monitored, these PAs are linked to only a limited number of ragwort species. Many plants are known that contain non-macrocyclic PAs (mono and diester forms). These non-macrocyclic PAs may exhibit similar toxicity (Fu *et al*, 2004, EFSA, 2007), but have not been included in the current analytical method. Most notably plants of the *Boriginaceae* family, like Viper's bugloss (*Echium vulgare*), Comfrey (*Symphytum spp*) and Hound's tongue (*Cynoglossum officinale*) and Hemp agrimony (*Eupatorium cannabinum*) of the *Asteraceae* family, contain these types of PAs. These species are (locally) quite common in The Netherlands, as well as in other parts of Europe. It is recommended that suitable analytical methods are developed for the PAs present in these plants and that the monitoring of animal forage is extended to these PAs as well.

3. To assess the potential risk for PAs entering the food chain through transfer to milk the monitoring data should be combined with *in vitro* and *in vivo* experiments. The data currently available on milk transfer is rather limited and the methods that have been used in these studies are sometimes outdated (Borne, 2007). Such studies should also consider the transfer ratios of individual PAs (in their tertiary amine as well as N-oxide form) from feed to milk, as it can be expected that differences in polarity and chemical reactivity will affect metabolism and result in different transfer ratios.

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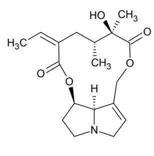
Annex I Glossary of Plant names

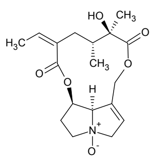
Overview of the common Scientific, English, Dutch, German and French names for the plants mentioned in this study which contain pyrrolizidine alkaloids

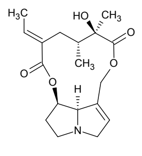
Scientific name	English name	Dutch name	German name	French name
Senecio jacobaea/ Jacobaea vulgaris	Tansy (Common) ragwort	Jakobskruiskruid	Jakobs-greiskraut (kreutzkraut)	Seneçon jacobée
Senecio vulgaris	Common groundsel	Klein (Gewoon) kruiskruid	Gemeines (Gewöhliches) Greiskraut	Seneçon commun
Senecio inaequidens	Narrow-leafed ragwort	Bezemkruiskruid	Schmalblättriges Greiskraut	Seneçon du cap
Senecio paludosis/ Jacobaea paludosa	Fen ragwort	Moeraskruiskruid	Sumpf-greiskraut	Seneçon de marais
Senecio aquaticus/ Jacobaea aquatica	Marsh ragwort	Waterkruiskruid	Wasser-Greiskraut	Seneçon aquatique
Senecio erucifolius/ Jacobaea erucifolia	Hoary ragwort	Viltig kruiskruid	Raukenblättriges Greiskraut	Seneçon à feuilles de roquette
Echium vulgare	Viper's bugloss	Slangenkruid	Natternkopf	Vipérine commune
Symphytum officinalis	Common comfrey	Gewone smeerwortel	Gemeiner (Echter) beinwell	Consoude officinale
Cynoglossum officinale	Hound's tongue	Veldhondstong	Echte hundszunge	Cynoglosse officinal
Eupatorium cannabinum	Hemp agrimony	Koninginnenkruid	Wasserdost	Eupatoire chanvrine

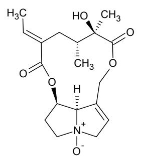
Annex II Chemical structures of pyrrolizidine alkaloids found in Senecio species

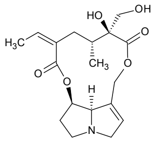
Senecionine and retorsine type

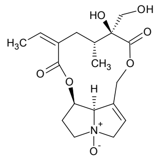

Senecionine
$C_{18}H_{25}NO_5$
335.17

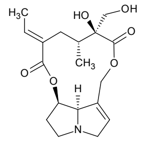

Senecionine-N-oxide
$C_{18}H_{25}NO_6$
351.17

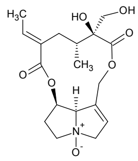

Integerrimine
$C_{18}H_{25}NO_5$
335.17


Integerrimine-N-oxide
$C_{18}H_{25}NO_6$
351.17

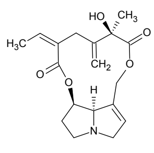

Retrorsine
$C_{18}H_{25}NO_6$
351.17

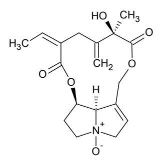

Retrorsine-N-oxide
$C_{18}H_{25}NO_7$
367.16

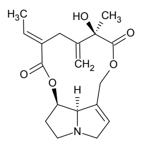

Usuramine
$C_{18}H_{25}NO_6$
351.17

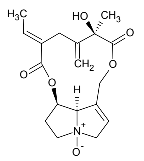

Usuramine-N-oxide
$C_{18}H_{25}NO_7$
367.16

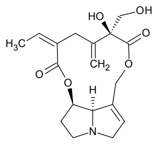
Seneciphylline and riddelliine type

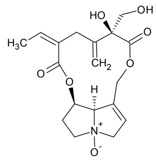

Seneciphylline
$C_{18}H_{23}NO_5$
333.16

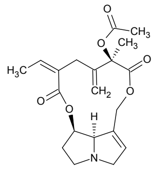

Seneciphylline-N-oxide
$C_{18}H_{23}NO_6$
349.15

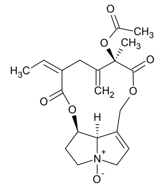

Spartioidine
$C_{18}H_{23}NO_5$
333.16


Spartioidine-N-oxide
$C_{18}H_{23}NO_6$
349.15

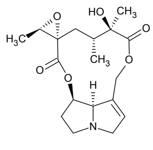

Riddelliine
$C_{18}H_{23}NO_6$
349.15

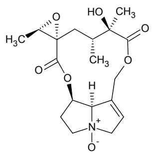

Riddelliine-N-oxide
$C_{18}H_{23}NO_7$
365.15

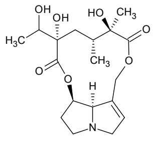

Acetylseneciphylline
$C_{20}H_{25}NO_6$
375.17

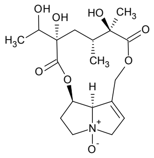

Acetylseneciphylline-N-oxide
$C_{20}H_{25}NO_7$
391.16

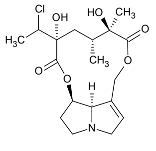
Jacobine type

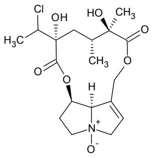

Jacobine
$C_{18}H_{25}NO_6$
351.17

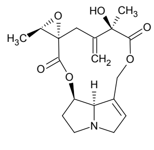

Jacobine-N-oxide
$C_{18}H_{25}NO_7$
367.16

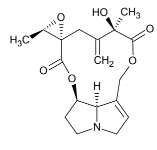

Jacoline
$C_{18}H_{27}NO_7$
369.18


Jacoline-N-oxide
$C_{18}H_{27}NO_8$
385.17

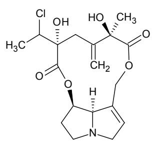

Jaconine
$C_{18}H_{26}ClNO_6$
387.14

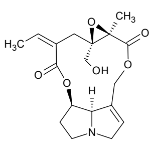

Jaconine-N-oxide
$C_{18}H_{26}ClNO_7$
403.14

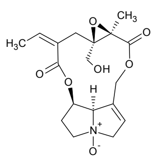

Jacozine
$C_{18}H_{23}NO_6$
349.15

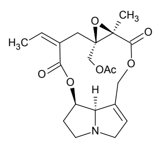

Jacozine-N-oxide
$C_{18}H_{23}NO_7$
365.15

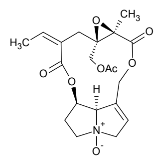
Erucifoline and senecivernine type

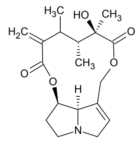

Dehydrojaconine
$C_{18}H_{24}ClNO_6$
385.13

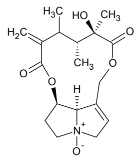

Erucifoline
$C_{18}H_{23}NO_6$
349.15


Erucifoline-N-oxide
$C_{18}H_{23}NO_7$
365.15

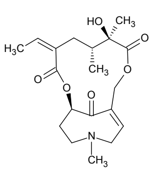

Acetylerucifoline
$C_{20}H_{25}NO_7$
391.16

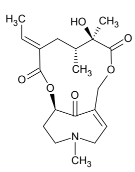

Acetylerucifoline-N-oxide
$C_{20}H_{25}NO_8$
407.16

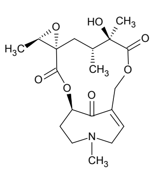

Senecivernine
$C_{18}H_{25}NO_5$
335.17

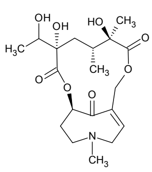

Senecivernine-N-oxide
$C_{18}H_{25}NO_6$
351.17

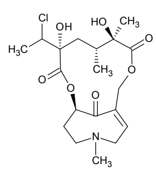
Senkirkine type

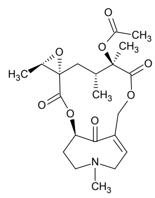

Senkirkine
$C_{19}H_{27}NO_6$
365.18

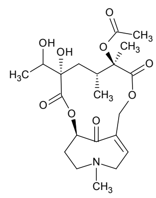

Neo-Senkirkine
$C_{19}H_{27}NO_6$
365.18

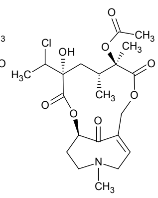

Otosenine
$C_{19}H_{27}NO_7$
38118


Onetine
$C_{19}H_{29}NO_8$
399.19

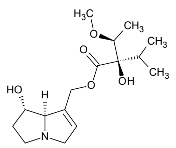

Desacetyldoronine
$C_{19}H_{28}ClNO_7$
417.16


Florosenine
$C_{21}H_{29}NO_8$
423.19


Floridanine
$C_{21}H_{31}NO_9$
441.20


Doronine
$C_{21}H_{30}ClNO_8$
459.17

Internal standard (non senecio PA)


Heliotrine (IS)
$C_{16}H_{27}NO_5$
313.19

Annex III MS/MS conditions used for the analysis of pyrrolizidine alkaloids

Alkaloid	Abbreviation	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
Heliotrine (IS)	Hel	314.2	138.0	25
Senecionine	Sn	336.2	94.0; 120.0	40; 30
Senecionine-N-oxide	Sn-ox	352.2	94.0; 120.0	40; 30
Integerrimine	Ir	336.2	94.0; 120.0	40; 30
Integerrimine-N-oxide	Ir-ox	352.2	94.0; 120.0	40; 30
Senecivernine	Sv	336.2	94.0; 120.0	40; 30
Senecivernine-N-oxide	Sv-ox	352.2	94.0; 120.0	40; 30
Retrorsine	Rt	352.2	94.0; 120.0	40; 30
Retrorsine-N-oxide	Rt-ox	368.2	94.0; 120.0	40; 30
Usaramine	Us	352.2	94.0; 120.0	40; 30
Usaramine-N-oxide	Us-ox	368.2	94.0; 120.0	40; 30
Seneciphylline	Sp	334.2	94.0; 120.0	40; 30
Seneciphylline-N-oxide	Sp-ox	350.2	94.0; 138.0	40; 30
Spartioidine	St	334.2	120.0; 138.0	30; 30
Spartioidine-N-oxide	St-ox	350.2	94.0; 138.0	40; 30
Riddelliine	Rd	350.2	94.0; 138.0	40; 30
Riddelliine-N-oxide	Rd-ox	366.2	94.0; 118.0	40; 30
Acetylseneciphylline	AcSp	376.2	120.0; 138.0	30; 30
Acetylseneciphylline-N-oxide	AcSp-ox	392.2	94.0; 118.0	40; 30
Jacobine	Jb	352.2	120.0; 155.0	30; 30
Jacobine-N-oxide	Jb-ox	368.2	120.0; 296.0	30; 25
Jacoline	Jl	370.2	94.0; 138.0	40; 30
Jacoline-N-oxide	Jl-ox	386.2	94.0; 120.0	40; 30
Jaconine	Jn	388.2	94.0; 120.0	40; 30
Jaconine-N-oxide	Jn-ox	404.2	94.0; 138.0	40; 30
Jacozine	Jz	350.2	94.0; 138.0	40; 30
Jacozine-N-oxide	Jz-ox	366.2	94.0; 118.0	40; 30
Dehydrojaconine	DhJn	386.2	94.0; 120.0	40; 30
Erucifoline	Er	350.2	94.0; 120.0	40; 30
Erucifoline-N-oxide	Er-ox	366.2	94.0; 118.0	40; 30
Acetylerucifoline	AcEr	392.2	94.0; 118.0	40; 30
Acetylerucifoline-N-oxide	AcEr-ox	408.2	94.0; 120.0	40; 30
Senkirkine	Sk	366.2	122.0; 168.0	30; 25

Annex III MS/MS conditions used for the analysis of pyrrolizidine alkaloids, continued

Alkaloid	Abbreviation	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
Neo-Senkirkine	Neo-Sk	366.2	122.0; 168.0	30; 25
Otosenine	Ot	382.2	122.0; 168.0	30; 25
Onetine	On	400.2	122.0; 168.0	30; 30
Desacetyldoronine	DesDor	418.2	122.0; 168.0	30; 30
Florosenine	Fs	424.2	122.0; 168.0	35; 30
Floridanine	Fd	442.2	122.0; 168.0	30; 30
Doronine	Dor	460.2	122.0; 168.0	35; 30

Annex IV Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in grass silage, corrected for 12% moisture

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
170081	Grass silage	<10	<10	<10	<10
173489	Grass silage	<10	<10	<10	<10
181308	Grass silage	<10	<10	<10	<10
181309	Grass silage	<10	<10	<10	<10
181716	Grass silage	<10	<10	<10	<10
181714	Grass silage	<10	<10	<10	<10
181713	Grass silage	<10	<10	<10	<10
182077	Grass silage	<10	<10	<10	<10
181712	Grass silage	<10	<10	<10	<10
181715	Grass silage	<10	<10	<10	<10
181711	Grass silage	<10	<10	<10	<10
183852	Grass silage	<10	<10	<10	<10
183853	Grass silage	<10	<10	<10	<10
184537	Grass silage	<10	<10	<10	<10
184724	Prewilted grass	<10	<10	<10	<10
184772	Grass silage	<10	<10	<10	<10
188449	Grass silage	<10	<10	<10	<10
188924	Grass silage	<10	<10	<10	<10
189077	Grass silage	<10	<10	<10	<10
190537	Grass silage	<10	<10	<10	<10
190538	Grass silage	<10	<10	<10	<10
190539	Grass silage	<10	<10	<10	<10
191494	Grass silage	<10	<10	<10	<10
202055	Prewilted grass	<10	<10	<10	<10
202275	Grass silage	<10	<10	<10	<10
202276	Grass silage	<10	<10	<10	<10
202283	Grass silage	<10	<10	<10	<10
208185	Grass silage	<10	<10	<10	<10
208186	Grass silage	<10	<10	<10	<10
209893	Grass silage	<10	<10	<10	<10

Annex IV Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in grass silage, corrected for 12% moisture, continued

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
209955	Grass silage	<10	<10	<10	<10
209956	Grass silage	<10	<10	<10	<10
209957	Grass silage	<10	<10	<10	<10
210048	Grass silage	<10	<10	<10	<10
210187	Prewilted grass	<10	<10	<10	<10
210188	Prewilted grass	<10	<10	<10	<10
211511	Prewilted grass	<10	<10	<10	<10
212139	Grass silage	<10	<10	<10	<10
212338	Prewilted grass	<10	<10	<10	<10
212339	Prewilted grass	<10	<10	<10	<10
212976	Grass silage	<10	<10	<10	<10
213606	Grass silage	<10	<10	<10	<10
215062	Prewilted grass	<10	<10	<10	<10
215308	Grass silage	<10	<10	<10	<10
217134	Prewilted grass	<10	<10	<10	<10
217135	Prewilted grass	<10	<10	<10	<10
217136	Prewilted grass	<10	<10	<10	<10
217673	Prewilted grass	<10	<10	<10	<10
217820	Prewilted grass	<10	<10	<10	<10
217821	Prewilted grass	<10	<10	<10	<10
218423	Prewilted grass	<10	<10	<10	<10
218729	Prewilted grass	<10	<10	<10	<10
221286	Grass silage	<10	28	<10	28
221287	Grass silage	<10	15	<10	15
221479	Grass silage	<10	15	<10	15
223037	Prewilted grass	<10	<10	<10	<10

Annex V Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in hay forage, corrected for 12% moisture

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
202026	Hay	<10	<10	<10	<10
202027	Hay	<10	<10	<10	<10
202030	Hay	<10	<10	<10	<10
202031	Hay	<10	<10	<10	<10
202035	Hay	<10	<10	<10	<10
202037	Hay	<10	<10	<10	<10
202038	Hay (nature reserve)	<10	<10	<10	<10
202039	Hay (nature reserve)	<10	<10	<10	<10
202040	Hay (nature reserve)	<10	<10	<10	<10
202041	Hay/Grass	<10	<10	<10	<10
202042	Hay (nature reserve)	<10	<10	<10	<10
202043	Hay (nature reserve)	<10	<10	<10	<10
202044	Hay (nature reserve)	<10	<10	<10	<10
202045	Hay (nature reserve)	<10	<10	<10	<10
202046	Hay (nature reserve)	<10	<10	<10	<10
202047	Hay (nature reserve)	<10	<10	<10	<10
202048	Hay (nature reserve)	<10	<10	<10	<10
202049	Hay (nature reserve)	<10	<10	<10	<10
202050	Hay (nature reserve)	<10	<10	<10	<10
202051	Hay (nature reserve)	<10	<10	<10	<10
202052	Hay (nature reserve)	<10	<10	<10	<10
202053	Hay (nature reserve)	<10	<10	<10	<10
202054	Hay (nature reserve)	<10	<10	<10	<10
202056	Hay (nature reserve)	<10	<10	<10	<10
202057	Hay (nature reserve)	<10	<10	<10	<10
202058	Hay (nature reserve)	<10	<10	<10	<10
202059	Hay	<10	<10	<10	<10
202273	Hay	<10	<10	<10	<10
202274	Hay	<10	<10	<10	<10
202277	Hay	<10	<10	<10	<10

Annex V Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in hay forage, corrected for 12% moisture, continued

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
202278	Hay (nature reserve)	<10	<10	<10	<10
202279	Hay (nature reserve)	<10	<10	<10	<10
202280	Hay (nature reserve)	<10	<10	<10	<10
202281	Hay (nature reserve)	<10	<10	<10	<10
202282	Hay (nature reserve)	15	236	298	549
202285	Hay (nature reserve)	<10	<10	<10	<10
223939	Hay/Grass	<10	<10	<10	<10

Annex VI Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in dried and processed grass forage, corrected for 12% moisture

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
171460	Grass pellets	<10	<10	<10	<10
178898	Grass pellets	<10	<10	<10	<10
178899	Grass pellets	<10	<10	<10	<10
178900	Grass pellets (organic)	<10	<10	<10	<10
189859	Dried grass/Hay	<10	<10	<10	<10
190160	Grass crumb	<10	<10	<10	<10
190725	Grass crumb	<10	11	<10	11
202025	Grass	<10	<10	<10	<10
202028	Dried grass	<10	<10	<10	<10
202029	Dried grass	<10	<10	<10	<10
202033	Dried grass	<10	<10	<10	<10
202034	Grass pellets	<10	<10	<10	<10
202036	Grass crumb (organic)	<10	<10	<10	<10
202284	Grass	<10	<10	<10	<10
217032	Grass pellets	<10	<10	<10	<10
217036	Dried grass	<10	<10	<10	<10
217349	Grass crumb	288	<10	<10	288
218760	Grass pellets	<10	10	<10	10
218762	Grass pellets	<10	<10	<10	<10
219596	Grass crumb	<10	<10	<10	<10
222600	Grass pellets	<10	<10	<10	<10
223042	Dried grass	<10	<10	<10	<10
223183	Grass crumb	13	<10	<10	13

Annex VII Survey results on pyrrolizidine alkaloid analysis in animal feeds (2006-2008). Indicative concentrations of PA's groups detected in alfalfa forage, corrected for 12% moisture

		Concentration (µg/kg)			
ID	Description	Retronecine Tertiary amines	Retronecine N-oxides	Otonecine amines	Total
171231	Alfalfa pellets	<10	<10	<10	<10
171232	Alfalfa pellets (organic)	19	10	<10	29
174359	Dried alfalfa	1981	1543	<10	3524
175061	Alfalfa pellets (organic)	<10	12	<10	12
175231	Alfalfa crumb	<10	14	<10	14
175232	Alfalfa crumb	3375	390	<10	3765
192044	Alfalfa	<10	<10	<10	<10
192217	Alfalfa	147	49	<10	196
192771	Alfalfa pellets	51	164	<10	215
193678	Alfalfa	16	10	<10	26
194625	Alfalfa pellets (organic)	<10	13	<10	13
194627	Alfalfa pellets	<10	19	<10	19
194677	Alfalfa	14	11	<10	25
195822	Alfalfa pellets	72	23	<10	95
197259	Alfalfa crumb	<10	<10	<10	<10
197633	Alfalfa hay	<10	17	<10	17
199057	Alfalfa crumb	<10	11	<10	11
200508	Alfalfa crumb	11	25	<10	36
202032	Alfalfa hay	260	65	<10	325
209507	Alfalfa	<10	<10	<10	<10
210283	Alfalfa (organic)	<10	<10	<10	<10
213665	Alfalfa	<10	<10	<10	<10
217033	Alfalfa pellets	29	<10	<10	29
217035	Dried alfalfa	<10	<10	<10	<10
217347	Alfalfa crumb (French)	38	34	<10	72
217348	Alfalfa crumb	4504	897	<10	5401
217436	Alfalfa crumb	<10	<10	<10	<10
218763	Alfalfa pellets	10	<10	<10	10
222599	Alfalfa pellets	139	<10	<10	139
223182	Alfalfa crumb (French)	88	<10	<10	88
224052	Alfalfa crumb	48	<10	<10	48

Annex VIII Indicative concentrations (in µg/kg) of individual PA's detected in forage samples, corrected for 12% moisture

ID	Description	Sn	Sn-ox	Ir	Ir-ox	Sv	Rt	Rt-ox	Us	Us-ox	Rd	Rd-ox
171232	Alfalfa pellets (organic)											
174359	Dried alfalfa	866	612	293	136	14	409	482	9		13	10
175061	Alfalfa pellets (organic)											
175231	Alfalfa crumb											
175232	Alfalfa crumb	203	53	233	31	176	539	55	10		231	28
190725	Grass crumb											
192217	Alfalfa	10		13			28	10			16	
192771	Alfalfa pellets	10	26	12			11	46				
193678	Alfalfa											
194625	Alfalfa pellets (organic)											
194627	Alfalfa pellets											
194677	Alfalfa	14					14					
195822	Alfalfa pellets											
197633	Alfalfa hay											
199057	Alfalfa crumb											
200508	Alfalfa crumb											
202032	Alfalfa hay	24	12	17		11	27	10			10	
202282	Hay (nature)	16						17			9	
217033	Alfalfa pellets	9					11					
217347	Alfalfa crumb (French)						16	21				
217348	Alfalfa crumb	1384	338	485	114	171	376	102		20	53	19
217349	Grass crumb	48		16		11	22				10	
218760	Grass pellets											
218763	Alfalfa pellets											
221286	Grass silage											
221287	Grass silage		15									
221479	Grass silage											
222599	Alfalfa pellets	26		10			12					
223182	Alfalfa crumb (French)	10		13			21					
223183	Grass crumb											
224052	Alfalfa crumb	10					11					

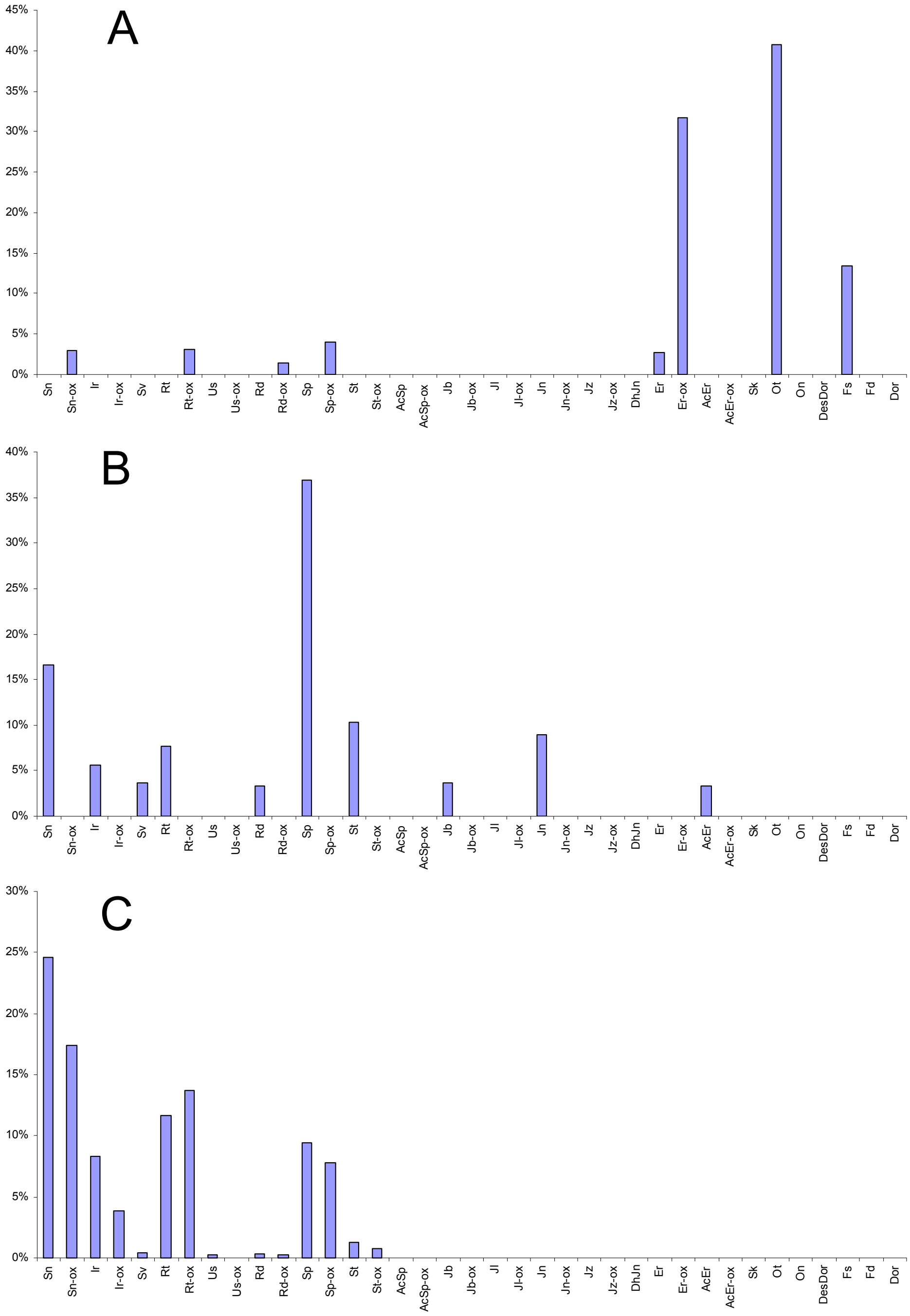
Annex VIII Indicative concentrations (in µg/kg) of individual PA's detected in forage samples, continued

ID	Description	Sp	Sp-ox	St	St-ox	Jb	Jb-ox	Jl	Jl-ox	Jn	Jn-ox	Jz
171232	Alfalfa pellets (organic)	19	9									
174359	Dried alfalfa	3531	275	45	28							
175061	Alfalfa pellets (organic)		12									
175231	Alfalfa crumb		14									
175232	Alfalfa crumb	1501	194	492	32							
190725	Grass crumb		11									
192217	Alfalfa	55	40	26								
192771	Alfalfa pellets	17	75		17							
193678	Alfalfa	16	9									
194625	Alfalfa pellets (organic)		13									
194627	Alfalfa pellets		19									
194677	Alfalfa	14	11									
195822	Alfalfa pellets	31	23	12								
197633	Alfalfa hay		17									
199057	Alfalfa crumb		11									
200508	Alfalfa crumb	11	25									
202032	Alfalfa hay	135	43	36								
202282	Hay (nature)		22									
217033	Alfalfa pellets	9										
217347	Alfalfa crumb (French)	22	13									
217348	Alfalfa crumb	1690	219	345	85							
217349	Grass crumb	106		30		11				26		
218760	Grass pellets		10									
218763	Alfalfa pellets	9										
221286	Grass silage		28									
221287	Grass silage											
221479	Grass silage		15									
222599	Alfalfa pellets	76		15								
223182	Alfalfa crumb (French)	44										
223183	Grass crumb	13										
224052	Alfalfa crumb	28										

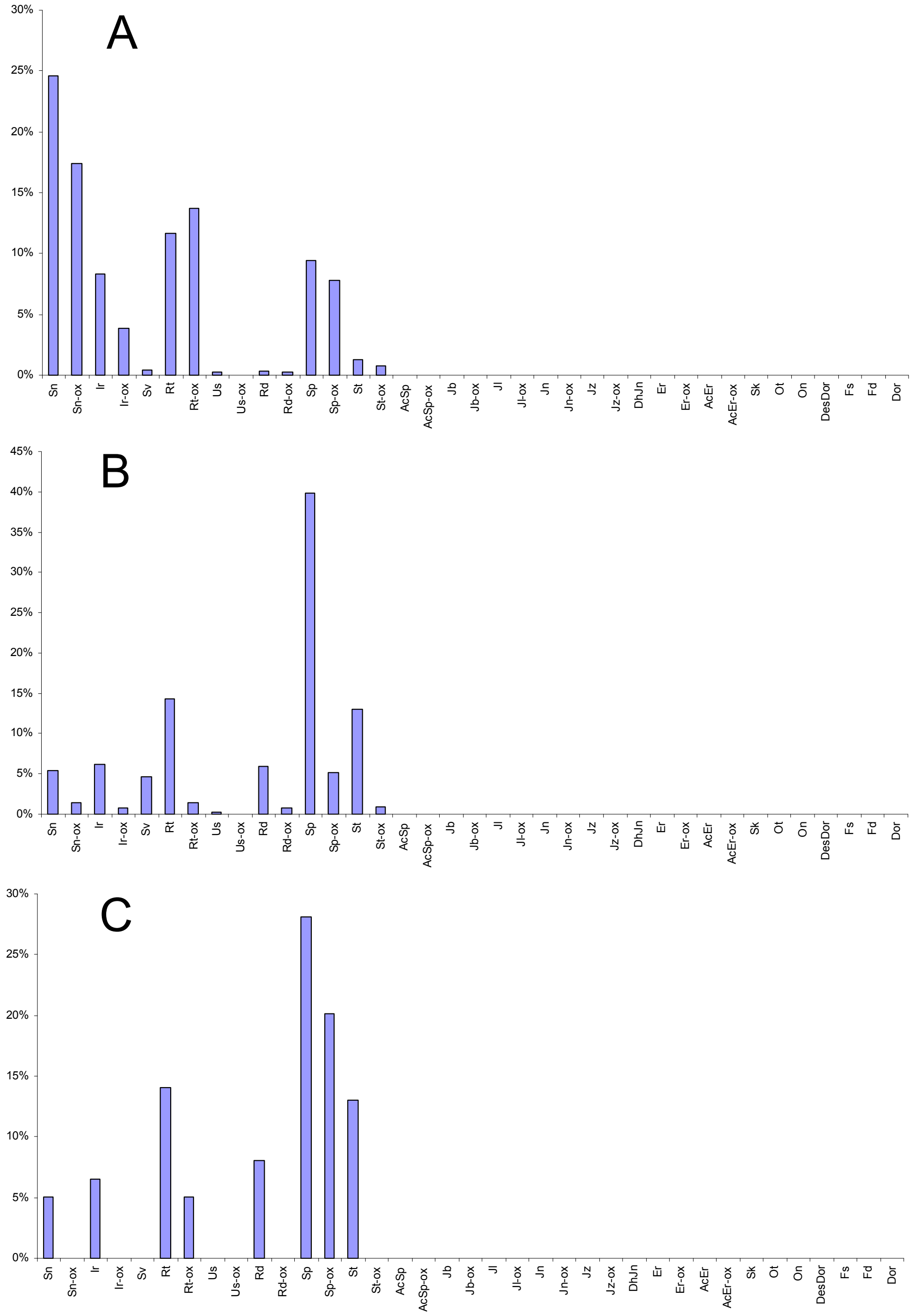
ID	Description	Er	Er-ox	AcEr	AcEr-ox	Sk	Ot	On	Des-Dor	Fs	Fd	Dor
171232	Alfalfa pellets (organic)											
174359	Dried alfalfa											
175061	Alfalfa pellets (organic)											
175231	Alfalfa crumb											
175232	Alfalfa crumb											
190725	Grass crumb											
192217	Alfalfa											
192771	Alfalfa pellets											
193678	Alfalfa											
194625	Alfalfa pellets (organic)											
194627	Alfalfa pellets											
194677	Alfalfa											
195822	Alfalfa pellets											
197633	Alfalfa hay											
199057	Alfalfa crumb											
200508	Alfalfa crumb											
202032	Alfalfa hay											
202282	Hay (nature)	15	174				224			74		
217033	Alfalfa pellets											
217347	Alfalfa crumb (French)											
217348	Alfalfa crumb											
217349	Grass crumb			10								
218760	Grass pellets											
218763	Alfalfa pellets											
221286	Grass silage											
221287	Grass silage											
221479	Grass silage											
222599	Alfalfa pellets											
223182	Alfalfa crumb (French)											
223183	Grass crumb											
224052	Alfalfa crumb											

Annex IX Relative PA profile of selected samples

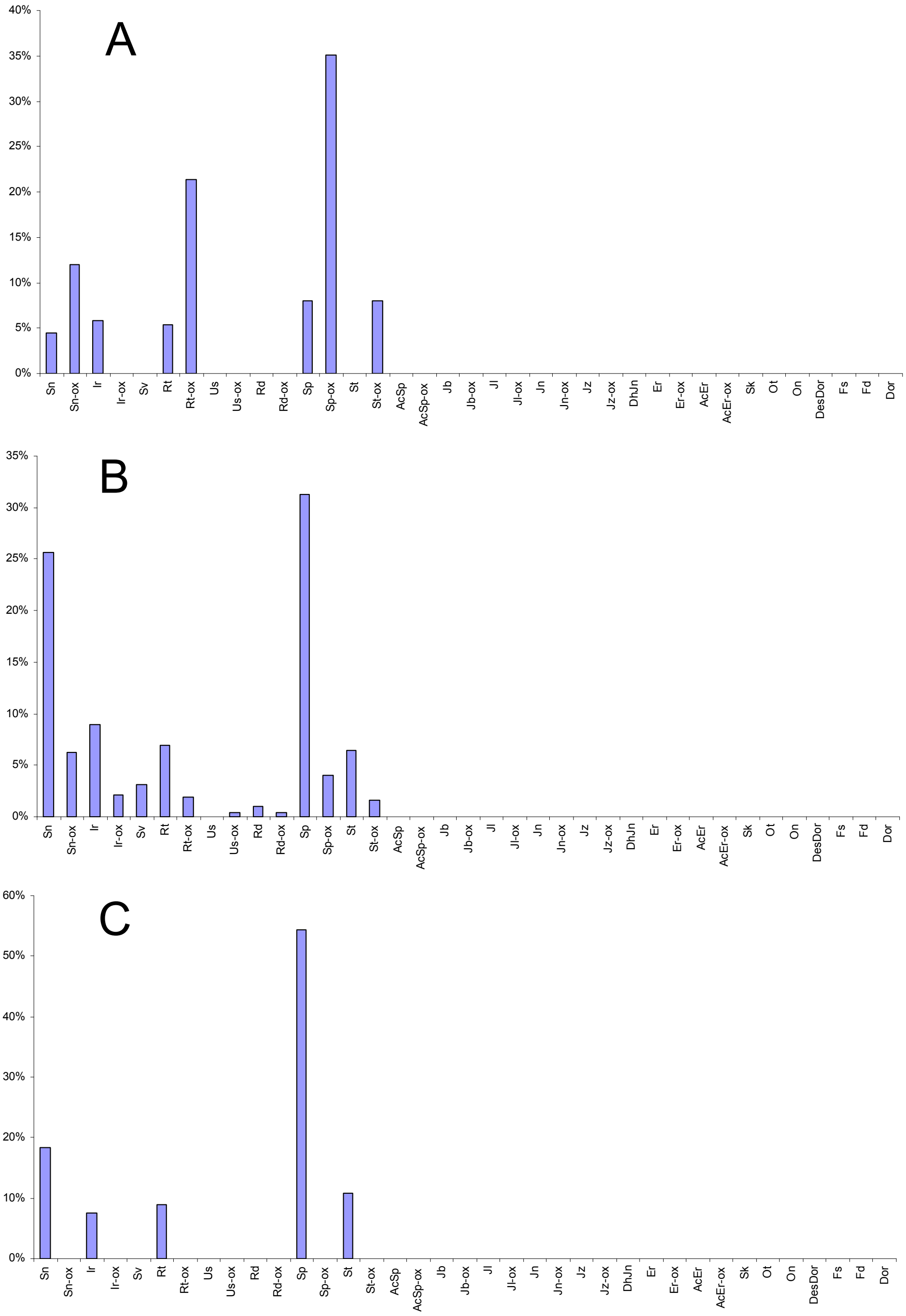
A. Sample 202282 (hay from a nature reserve); B. 217349 (dried grass) and C. 174359 (dried alfalfa)



A. sample 175232 (alfalfa crumb); B.192217 (alfalfa) and C. 192771 (alfalfa)



A. sample 202032 (alfalfa hay); B. 217348 (alfalfa crumb) and C. 222599 (alfalfa pellets)



Annex X Photographs of selected Senecio species

Patch of flowering Tansy ragwort (*Senecio jacobaea/Jacobaea vulgaris*)

First year rosette of Tansy ragwort


Flowering heads of Tansy ragwort


Common groundsel (*Senecio vulgaris*)

Narrow-leaved ragwort (*Senecio inaequidens*)