

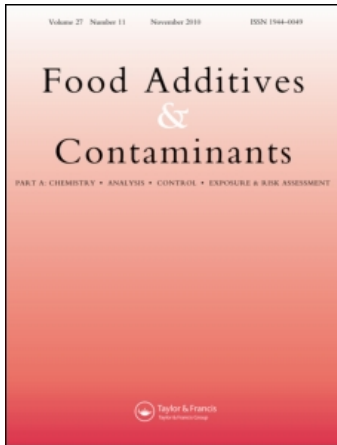
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## Plants containing pyrrolizidine alkaloids: toxicity and problems

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## Plants containing pyrrolizidine alkaloids: toxicity and problems

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Pyrrolizidine alkaloids (PA) are toxic for human and livestock. They undergo a metabolic toxication process in the liver which is the first target organ for PA poisoning. Worldwide many episodes of human PA intoxications are well reported. In many cases the reason for these intoxications has been PA contamination in food. The main tools for analysing food and fodder on PA content are based on GC and HPLC separation, followed by MS(-MS) detection. Actual incidents with toxic PA are the ‘*Jacobaea vulgaris* (syn. *Senecio jacobaea*) problem’ in Europe and the ‘*Ageratum conyzoides* problem’ in Ethiopia.

**Keywords:** gas chromatography/mass spectrometry; risk assessment; toxicology; cytotoxicity; natural toxicants; alkaloids

### Introduction

Pyrrolizidine alkaloids (PAs) possessing a 1,2-double bond in their base moiety (necine) are hepatotoxic, carcinogenic, genotoxic, teratogenic and sometimes pneumotoxic. PAs have been estimated to be present in about 3% of all flowering plants (Smith and Culvenor 1981). They mainly occur in species of the plant families Asteraceae, Fabaceae and Boraginaceae.

Several reports in the literature prove the fact that PA-containing plants are hazardous for livestock (Gilruth 1903, 1904; Bull and Dick 1959). For a long time it has also been well established that humans can be affected by toxic PA (Willmott and Robertson 1920; Steyn 1933; Bourkser 1947; Milenkov and Kizhaikin 1952; Tandon and Tandon 1975; Mohabbat et al. 1976; Mayer and Lüthy 1993; Chauvin et al. 1994). Here the main source for the intoxication was found to be PA-contaminated grain and bread.

The International Program on Chemical Safety (IPCS), a joint agency of the WHO, FAO and ILO, stated that ‘consumption of contaminated grain or the use of PA-containing plants as herbal medicine, beverages, or food by man, or grazing on contaminated pastures by animals, may cause acute or chronic disease’ (IPCS 1989).

Besides this, different medicinal plants were also found to show a hazardous potential for humans (Roeder 1995, 2000). Many investigations on herbal preparations or extracts from medicinal plants were performed and the toxic risk by the consumption of phytomedicines which contain PA plants was proved. It can be assumed that so-called ‘bush-teas’ are the

reason for several liver diseases reported in Jamaica and several developing countries from Africa and the West Indies (Bras et al. 1954, 1957, 1961). While PA poisoning is mainly a problem in developing countries because there the use of traditional medicine is common, within the last 25 years, especially in industrialised countries, the use of herbal medicine has become increasingly more common due to increased interest of people in alternative medicine which has occurred hand in hand with a greater influence of the ‘green wave’.

In addition, several food products of animal origin can also be a source for PA ingestion: milk has been shown to contain PA when milk-producing animals consume PA-containing plant material (Schoental 1959; Dickinson et al. 1976; Johnson et al. 1978; Dickinson 1980; Goeger et al. 1982; Lüthy et al. 1983; Candrian et al. 1984; Molyneux and James 1990). Human milk has also caused liver diseases in neonates and infants (Roulet et al. 1988).

Honey was shown to be another source of PA exposure; here it seems that the contamination may be due to plant pollen, which is rich in PAs, being transferred by bees into the honey (Deinzer et al. 1977; Culvenor et al. 1981; Roeder 1995, 2000; Edgar et al. 2002; Beales et al. 2004; Betteridge et al. 2005; Boppré et al. 2005). Eggs from poultry exposed to PAs in PA-contaminated grain were also shown to be a possible source of PA exposure for humans (Edgar and Smith 1999).

Recently it was shown in Germany that salads can sometimes be contaminated with PA-containing

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plants (Bundesamt für Risiokobewertung (BfR) 2007a). It was found that, especially in supermarkets, ready-packed rocket salad and salad mixtures have been contaminated by *Senecio vulgaris*, a typical weed of field crops.

While in several countries the use of PA-containing plants or preparations from them are controlled by state regulations, nothing exists in the case of regulating contaminated food and fodder. In the European Union, EFSA recommended in its opinion (EFSA 2007) that feed material which can be contaminated with PA should be monitored. More data are needed to assess human PA exposure from resulting from feed and carry-over into animal products. Meanwhile the so-called 'zero-tolerance principle' can be applied; this principle is used in cases where no safe or tolerable level can be determined based on available, valid scientific data, or if insufficient toxicological data are available. Due to their genotoxic and carcinogenic potential, this principle can be applied for PA in food and fodder (BfR 2007b).

Actually, in Middle Europe common groundsel (*Senecio vulgaris* L.) and tansy ragwort (*Jacobaea vulgaris*, syn. *Senecio jacobaea* L.) are of particular concern. Common groundsel, a common field weed, has been found as a contaminant in salads, and tansy ragwort has been discussed recently on account of its extensive expansion into pastures and meadows

which has led to a great number of intoxications in livestock (mainly horses), especially in Germany (see <http://www.jacobskreuzkraut.de>; and <http://www.izn.niedersachsen.de/servlets/download?C=39412784&L=20>). As well as the hazard for grazing animals (i.e. direct toxicity), the possibility of contaminated hay and silage and transfer of PAs into foods such as milk and milk products is under investigation and considered to become problematic.

### Toxication process of pyrrolizidine alkaloids

PA produced by *Senecio* species are ester alkaloids derived mainly from the necines retronecine and otonecine. They are carcinogenic, mutagenic, genotoxic, fetotoxic and teratogenic.

PAs themselves show a more or less low acute toxicity but *in vivo* they undergo a three-step metabolic toxication process in the liver, which is, as a result, the first target organ for the toxicity. This toxication process is well investigated (Mattocks 1968, 1972; Culvenor et al. 1969, 1971; Jago et al. 1970; Mattocks and White 1971a, 1971b; IPCS 1989).

After absorption a hydroxyl group is introduced in the necine at positions 3 or 8 by the cytochrome P-450 monooxygenase enzyme complex in the liver (Figure 1, IIa and IIb). These hydroxy-PAs (OHPAs) are unstable and undergo a rapid dehydration to the

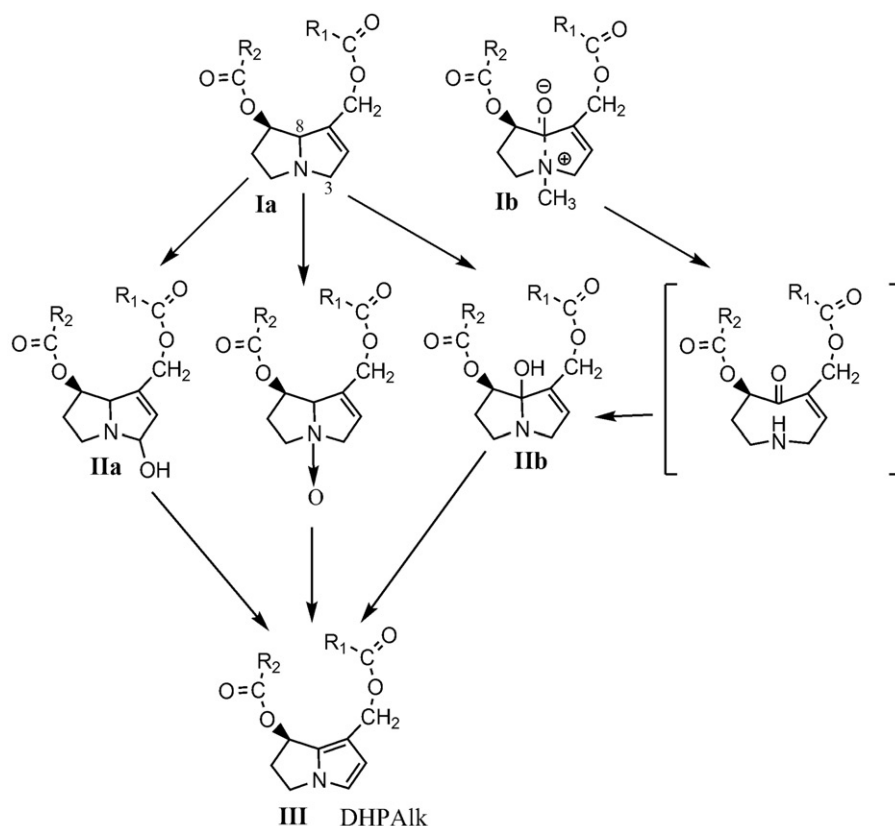


Figure 1. Enzymatic hydroxylation and didehydropyrrolizidine products.

didehydropyrrolizidine alkaloids (DHPAlk; Figure 1, III). This dehydration results in a second double-bond in the necine followed by spontaneous rearrangement to an aromatic pyrrole-system III.

PAs occur mainly as their N-oxides in the plants and these cannot be directly converted to the OHPA, but on oral ingestion they are reduced by the gut enzymes or the liver microsomes and NADH or NADPH to the free bases and therefore they show equal toxicity to that of the free bases (Mattocks and White 1971a, 1971b; Powis et al. 1979; Chou et al. 2003; Wang et al. 2005a, 2005b; Wan et al. 2005).

Otonecine-type PAs (Figure 1, Ib) are metabolised to the OHPAs (Culvenor et al. 1971; Lin et al. 1998, 2000). These otonecine PAs possess a methyl function at the nitrogen and a quasi keto function at the bridge-carbon 8. After hydroxylation of the N-methyl group it is lost as formaldehyde leaving an NH function which undergoes condensation with the C8 keto group to produce product IIb (Figure 1) which spontaneously dehydrates to the DHPAlk III.

The metabolites III are able to generate stabilised carbonium ions (Figure 2, IV and VI) by loss of hydroxy groups or ester functions as hydroxyl or acid anions. These carbonium ions can react rapidly with nucleophiles (Figure 2, VII).

*In vivo* metabolites IV and VI react rapidly with nucleophilic mercapto, hydroxyl and amino groups on proteins and the amino groups of purine and pyrimidine bases in nucleosides like DNA and RNA. These alkylated products show abnormal functions and in the case of DNA mutations are possible. As this metabolic toxification takes place in the liver it is obvious that this organ is the first target for the intoxication and results in liver damage leading to the veno-occlusive disease (VOD) in which the veins are narrowed.

#### Detoxication of PA

As well as metabolic activation, detoxification of PA also occurs *in vivo*: hydrolysis of the ester bonds in PA from type Ia or Ib by esterases leads to necic acids and to the free necines. Both are non-toxic products and – on account of their higher water solubility – can be renally excreted. The rate of hydrolysis is dependent on the level of steric hindrance of the ester linkages (see above); and it has been shown that the more highly branched the necic acids are the more resistance to hydrolysis (Culvenor et al. 1976; Mattocks 1986). This means that macrocyclic diesters (Ia and Ib) with more complex acid moieties are more hazardous on account of their lower rate of hydrolytic detoxication.

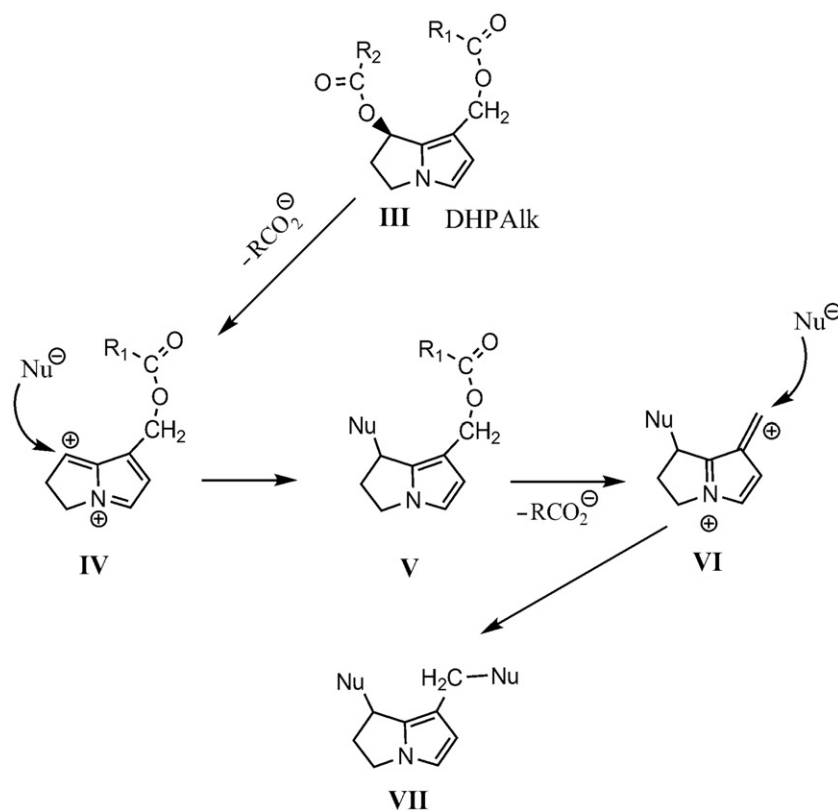


Figure 2. DHPAlk and carbonium ion building.

The N-oxides of PAs (the form occurring most commonly in plant sources) are highly water soluble and can therefore be excreted renally. Besides their natural occurrence, N-oxidation of PAs also takes place in the liver and can be seen as a detoxification process (Figure 2) (Mattocks 1968; Jago et al. 1970; Mattocks and White 1971b; Williams et al. 1989; Miranda et al. 1991). However, it has been shown that the N-oxides – besides excretion – can be converted by dehydration or acetylation followed by elimination of acetic acid to the DHPAlk (Figure 2, III) (Mattocks 1986; Culvenor et al. 1970).

### PA toxicity in humans

PA poisoning of humans can progress from an acute to a sub-acute and finally to a chronic state (McLean 1970; Peterson and Culvenor 1983; IPCS 1989; Huxtable 1989; Prakash et al. 1999; Stegelmeier et al. 1999; Fu et al. 2004). Acute poisoning is characterised by haemorrhagic necrosis, hepatomegaly and ascites; death is caused by liver failure due to necrosis and liver dysfunctions (Peterson and Culvenor 1983; IPCS 1989; Huxtable 1989; Prakash et al. 1999). Sub-acute levels are characterised by hepatomegaly and recurrent ascites; endothelial proliferation and medial hypertrophy leading to an occlusion of hepatic veins, resulting in the VOD, which can be seen as a characteristic histological sign for PA poisoning (Peterson and Culvenor 1983; IPCS 1989; Huxtable 1989; Prakash et al. 1999; Fu et al. 2004). The VOD causes centrilobular congestion, necrosis, fibrosis and liver cirrhosis, the end-stage of chronic PA intoxication.

A very important fact is that PA toxicity not only correlates with the amount and duration of uptake. It is shown that the susceptibility for PA intoxication is also dependent on gender (males seem to be more sensitive than females) and on age (children and especially neonates and foetuses show a much higher sensitivity than adults). In 2003 it was shown that the daily uptake of approximately 7 µg PA (from a herbal tea containing comfrey) during pregnancy did not show a toxic effect in the mother's liver but damaged the foetal liver in such a way that the newborn child died after 2 days (Rasenack et al. 2003).

It has also been observed that cofactors can exacerbate the PA poisoning: liver-damaging agents, bacterial or viral infections as well as medical drugs like barbiturates or metals like copper or mycotoxins like aflatoxins can increase the severity and likelihood of PA liver damage (Bull et al. 1968; Newberne and Rogers 1973; White et al. 1973; Lin et al. 1974; Tuchweber et al. 1974; Yee et al. 2000).

There is a large number of reports in the literature about different liver diseases (mainly VOD) possibly

connected with PA poisoning. But, on account of the fact that those reports mainly describe sub-chronic or chronic intoxications, in most cases the connection cannot be proven because there is often a long period between the outbreak of the liver disease and a possible ingestion of PA-containing material. Table 1 therefore lists only those cases where the source of PAs was identified and the liver disease was therefore undoubtedly caused by PA intoxication.

### PA determination

Several methods can be used for the qualitative and quantitative determination of PAs in plant material or its preparations:

- TLC: using the detection method of Dann/Mattocks; Dann 1960; Mattocks 1967), this is a quick, sure and easy method for a qualitative detection of PAs. Using TLC in a densitometric way (Bartkowski et al. 1997), it is also possible to have quantitative results; the detection limit is about 1–10 µg.
- LC-MS: different HPLC methods are described. Obviously, this is the mostly used analytical method for the determination of PAs. A great benefit is the fact that PA-N-oxides can be analysed as well as the free bases. Depending on the equipment, the detection limit is less than 0.1 µg per injection.
- GC-MS: also in the case of GC, a lot of methods are described. A GC problem is that N-oxides have to be reduced to the free bases before being analysed. This reduction procedure is described intensively and does not seem to be a limiting factor. The detection limit is similar to that in LC.

All three methods can only be used accurately in case reference material is available. The reason is that the single PAs show different detector responses in the respective analysing methods.

A further interesting – but unfortunately very specific – quantification method is the enzyme-linked immunosorbent assay (ELISA). Via a PA hemisuccinate and bovine serum albumine, enzyme-linked antigens (AG-E) can be synthesised and used for the production of antibodies (polyclonal as well as monoclonal) in rats (Röder and Pflüger 1986, 1995). This method is very sensitive and needs no complicated clean-up procedure. Unfortunately, it is sensitive only for a concrete PA structure; that means, antibodies produced by a retrorsine-AG-E can only be used for the analysis of retrorsine and senecionine, but are inactive for seneciphylline, jacobine or monocrotaline and, of course, also for all otonecine-type PAs.

Table 1. Human intoxications.

| Location and year       | Affected people                     | Observed damage                                                    | Source of PA                                                                                | References                    |
|-------------------------|-------------------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------------------|
| South Africa, 1920      | 11 adult people                     | Abdominal pain, vomiting, cirrhosis                                | <i>Senecio illicifolius</i> , <i>S. burchelli</i>                                           | Willmott and Robertson (1920) |
| Jamaica, 1954           | 23 adults                           | VOD                                                                | Bush-teas with <i>Crotalaria fulva</i>                                                      | Bras et al. (1961)            |
| South Africa, 1968      | 15 children; ten died               | VOD                                                                | Bush-teas; <i>Crotalaria</i> sp.?                                                           | Freiman et al. (1968)         |
| Venezuela, 1969         | Five-year-old girl                  | VOD                                                                | <i>Crotalaria anagyroides</i> , <i>C. pumila</i> consumed as infusion and as vegetable soup | Grases and Beker (1972)       |
| Kuwait, 1970            | Adults                              | Liver carcinoma                                                    | <i>Heliotropium ramosissimum</i> ('Ramram')?                                                | Macksad et al. (1970)         |
| Jamaica, 1970           | Six children                        | VOD                                                                | Bush-tea from <i>Crotalaria</i> and <i>Senecio</i> sp.                                      | Brooks et al. (1970)          |
| Iraq, 1970              | Nine children                       | VOD                                                                | Food contaminated by a <i>Senecio</i> sp.                                                   | Al-Hasany and Mohamed (1970)  |
| Afghanistan, 1970–1972  | 7200 people                         | VOD                                                                | Wheat contaminated with <i>Heliotropium popovii</i> , ssp. <i>gillianum</i>                 | Mohabbat et al. (1976)        |
| India, 1973             | 486 people                          | VOD                                                                | Cereals contaminated with <i>Crotalaria</i> spp.                                            | Tandon et al. (1976)          |
| Ecuador, 1973           | Woman                               | VOD                                                                | Herbal tea with <i>Crotalaria juncea</i>                                                    | Lyford et al. (1976)          |
| India, 1973, 1975       | Four males                          | Endemic ascites                                                    | Millet contaminated with <i>Crotalaria</i> spp.                                             | Krishnamachari et al. (1977)  |
| China, 1973, 1978       | Two adults                          | VOD                                                                | <i>Gynura segetum</i>                                                                       | Hou et al. (1980)             |
| India, 1974–1977        | Six people                          | VOD                                                                | <i>Heliotropium eichwaldii</i>                                                              | Datta et al. (1978)           |
| Martinique, 1975        | Two children                        | VOD                                                                | Bush-teas with <i>Crotalaria retusa</i> and/or <i>Heliotropium</i> sp.                      | Saint-Aimé et al. (1977)      |
| USA, 1976, 1977         | Four children                       | Vein congestion and necrosis of liver                              | <i>Senecio longilobus</i>                                                                   | Stillman et al. (1977)        |
| UK, 1976                | Woman                               | VOD                                                                | Maté (Paraguay tea) contaminated with PA of unknown origin                                  | McGee et al. (1976)           |
| USA, 1984               | 49-year-old woman                   | VOD                                                                | Food supplement containing <i>Symphytum</i> spp. root                                       | Ridker et al. (1985)          |
| China, 1985             | Four women                          | VOD                                                                | Herbal tea containing <i>Heliotropium lasiocarpum</i>                                       | Culvenor et al. (1986)        |
| Switzerland, 1985       | 59-year-old man and 27-year-old son | VOD                                                                | Herbal tea consisting of <i>Senecio</i> spp.                                                | Margalith et al. (1985)       |
| Switzerland, 1986       | Five-day-old baby                   | VOD                                                                | Herbal tea containing <i>Tussilago farfara</i> consumed during whole pregnancy              | Roulet et al. (1988)          |
| UK, 1986                | 13-year-old boy                     | VOD                                                                | Herbal tea containing <i>Symphytum</i> spp.                                                 | Weston et al. (1987)          |
| Tadjikistan, 1992, 1993 | 3906 people                         | Abdominal pain, hepatomegaly, ascites, alteration of consciousness | <i>Heliotropium lasiocarpum</i>                                                             | Chauvin et al. (1993)         |
| Peru, 1994              | 38-year-old woman                   | VOD                                                                | Herbal tea from <i>Senecio tephrosioides</i>                                                | Tomioka et al. (1995)         |
| Spain, 1995             | 73-year-old man                     | VOD                                                                | <i>Senecio vulgaris</i>                                                                     | Cansado et al. (1995)         |
| Austria, 1995           | 18-month-old boy                    | VOD                                                                | Herbal tea with <i>Adenostyles alliariae</i>                                                | Sperl et al. (1995)           |
| Argentina; 1999         | 23-year-old woman                   | VOD                                                                | Herbal tea containing <i>Senecio vulgaris</i>                                               | Vilar et al. (2000)           |
| Germany, 2002           | Foetus                              | VOD                                                                | <i>Symphytum</i> spp.                                                                       | Rasenack et al. (2003)        |
| Afghanistan, 2008       | 270 people (50 fatalities)          | VOD                                                                | Wheat contaminated with 'charmac' ( <i>Heliotropium popovii</i> , ssp. <i>gillianum</i> )   | Kakar et al. (2010)           |

Note: VOD, veno-occlusive disease.

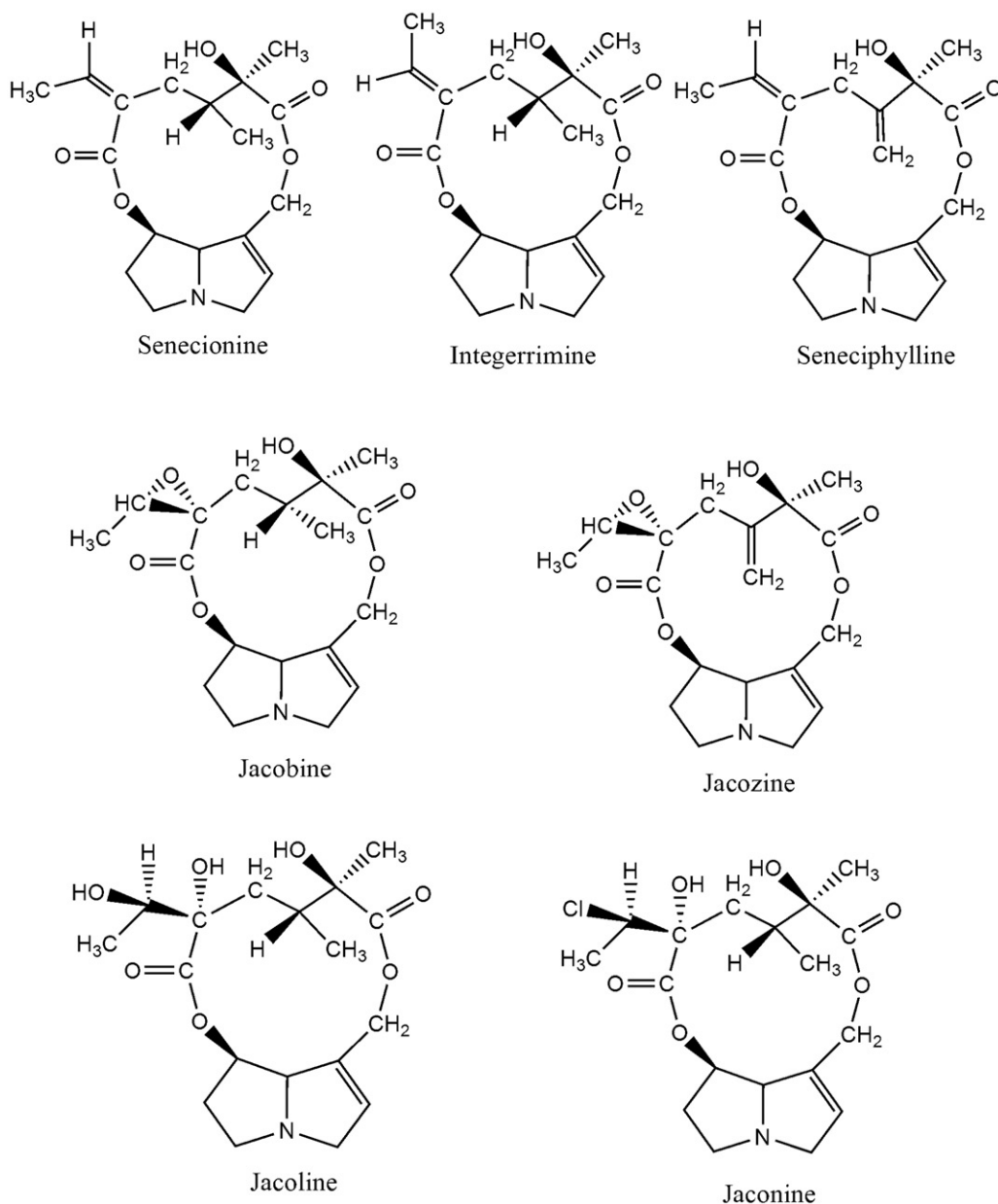


Figure 3. Structures of isolated PA from *Jacobaea vulgaris*, syn. *Senecio jacobaea*.

### Toxic PA in food and fodder

During the last three years an extensive increase of the spreading of *Senecio* species (especially *Jacobaea vulgaris*, syn. *Senecio jacobaea* L., Asteraceae) has been observed in Central Europe. This led to severe problems as this spreading includes pastures and meadows: horses, which show a high sensitivity for PA intoxication, were affected in large numbers. To date more than 100 horses have died on account of acute intoxication by *J. vulgaris* (see <http://www.jacobskreuzkraut.de/wirkung.htm>; and <http://www.jacobskreuzkraut.de>; [http://www.izn.niedersachsen.de/servlets/download? C=39412784&L=20](http://www.izn.niedersachsen.de/servlets/download?C=39412784&L=20)). As for

ruminants, intoxication in grazing animals seems to be negligible because these animals show a very low susceptibility and the amount of *Senecio* species on meadows is too low for intoxication to occur; in this case fodder such as hay or silage is responsible for the hazardous problems.

To evaluate a possible risk, plant material of *J. vulgaris* was analysed for its PA content during the vegetation period. The PAs from the plant were isolated by column chromatography and their structures elucidated by spectroscopic methods. Figure 3 shows the structures of the isolated PAs. Figure 4 shows a GC from an alcoholic plant extract.

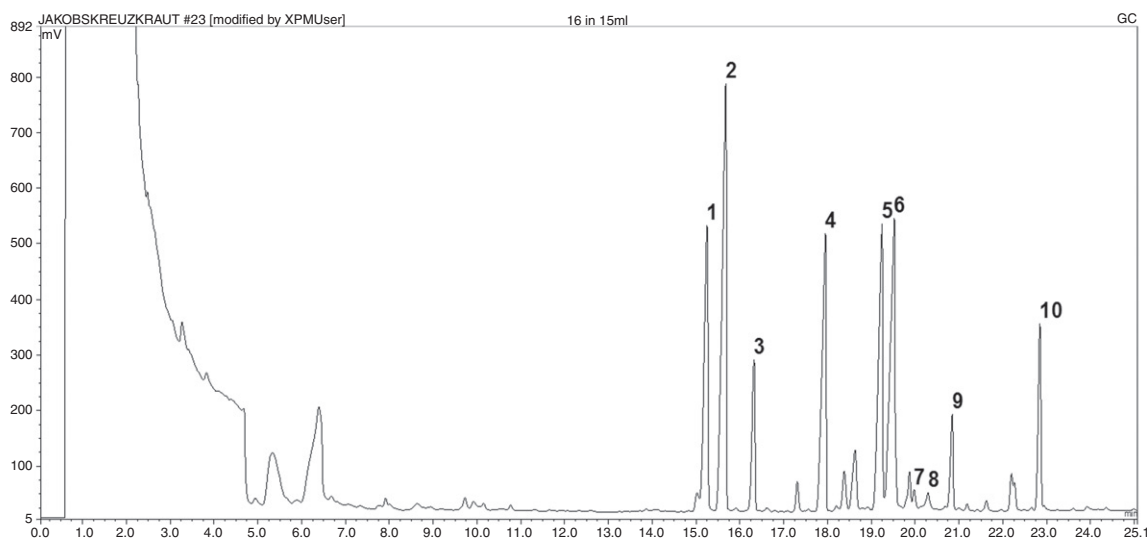


Figure 4. Gas chromatogram of the PA from *Jacobaea vulgaris*, syn. *Senecio jacobaea*: 1 = Senecionine; 2 = Seneciphylline; 3 = Integerrimine; 4 = Jacobine; 5 = Jaconine; 6 = Jaconine isomer; 7 = Jacozine; 8 = Jacoline; 9 = Erucifoline; 10 = Senkirkinine.

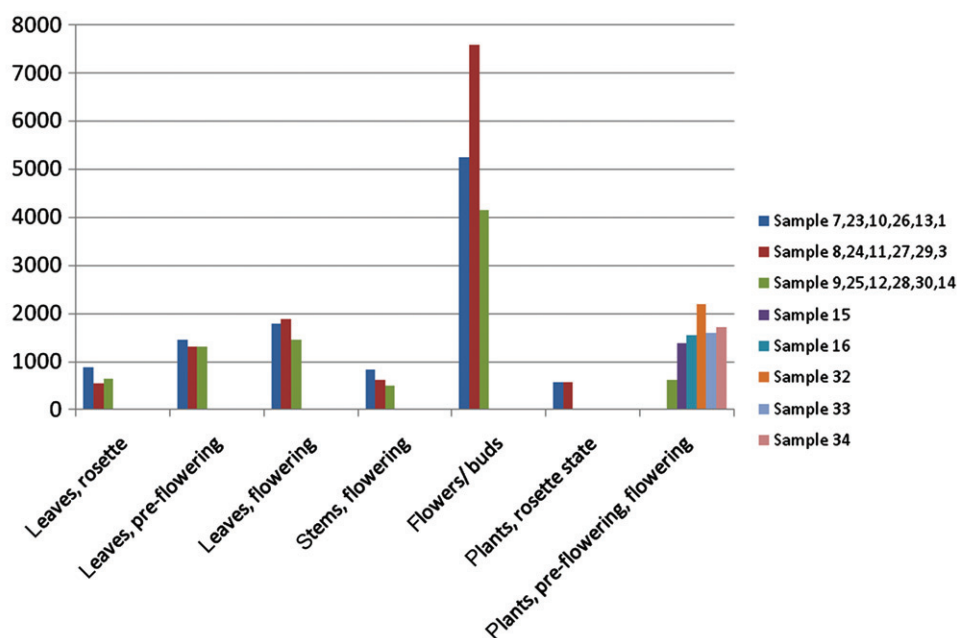


Figure 5. PAs ( $\mu\text{g g}^{-1}$  plant material) in *Jacobaea vulgaris*, syn. *Senecio jacobaea*.

The plant parts were analysed (Figure 5) and it could be shown that the PA content ranges from 0.1% to 0.2%, except in flowers, where amounts up to 0.8% were found. These data show that the application of 2–4 kg of dried plant material reach the lethal level in horses (approximately 350 kg bw).

This is of great importance in the case of hay or silage where a contamination with *J. vulgaris* of, for example, 1–5% is a possible scenario. In those cases the lethal dosage for horses will be reached in a

few days of feeding such contaminated hay (see [http://www.jacobskreuzkraut.de/toedliche\\_dosis.htm](http://www.jacobskreuzkraut.de/toedliche_dosis.htm)). In this context the question occurs whether there is a decrease during hay production and storage or during the production of silage.

Figures 6 and 7 show PA contents of hay and silage produced from *J. vulgaris*. The data show that in the case of hay no reduction of the PA level (compared with dried plants) can be observed. Contrary to this, the results for silage show a decrease in the PA level

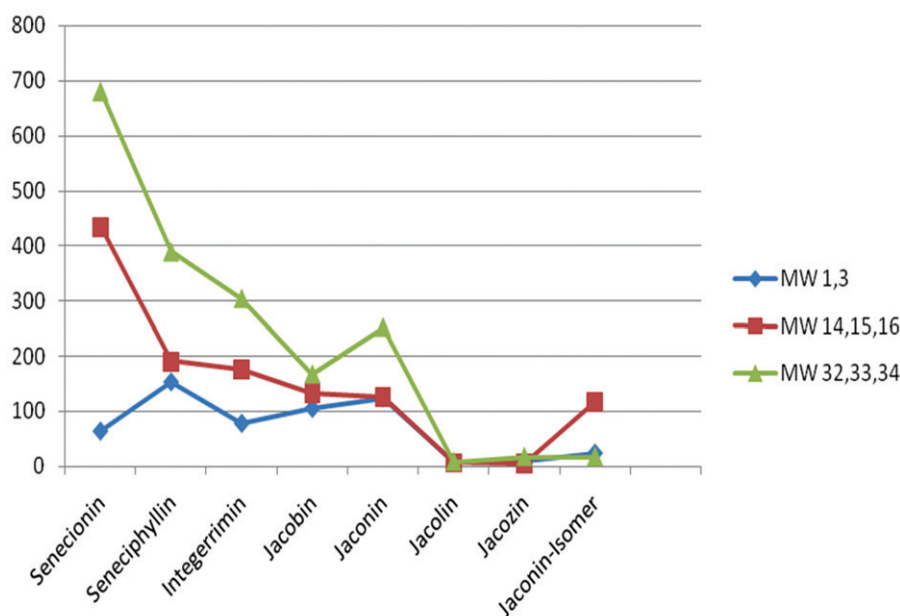


Figure 6. PAs ( $\mu\text{g g}^{-1}$ ) in hay from *Jacobaea vulgaris*, syn. *Senecio jacobaea*.

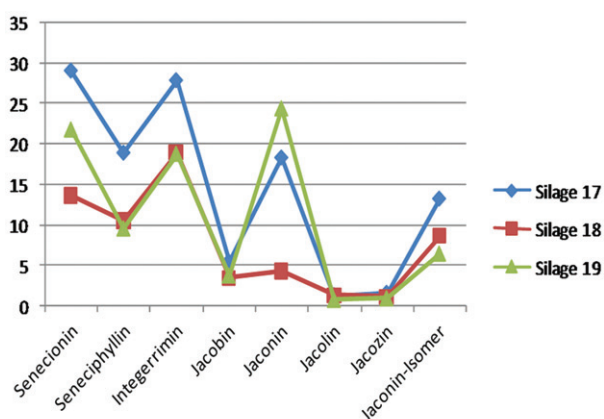


Figure 7. PAs ( $\mu\text{g g}^{-1}$ ) in silage from *Jacobaea vulgaris*, syn. *Senecio jacobaea*.



Figure 8. Salad sample (left *Senecio vulgaris*; right rucola).

down to 10%. It can be assumed that this is due to an enzymatic decomposition. Therefore, it can be concluded that in the case of hay the possible toxic risk is equal to the original plant material, whereas feeding silage to animals seems to be without any toxic risk.

Feeding contaminated material is not only hazardous for the animals themselves, but also can produce human risks, in case PAs are excreted in animal products like milk and honey.

A direct human exposure to PA was found in 2009 when salads contaminated with *Senecio* plants (here, especially *S. vulgaris*, a common weed on field crops) occurred in stores and supermarkets. Especially in the case of rucola and salad mixtures containing rucola, this problem became of high importance as it is not possible for non-experts to distinguish between salad leaves and *Senecio* leaves (Figure 8). Our own

analysis of such a salad sample (45 g) resulted in an amount of 2606  $\mu\text{g}$  PAs. Taking into consideration the fact that for herbal medicinal preparations the daily uptake limit (different in several countries) ranges from PA-free (Austria) to over  $1 \mu\text{g kg}^{-1}$  or  $1 \mu\text{g l}^{-1}$  (the Netherlands) to  $1 \mu\text{g}$  (Germany), it becomes obvious that such contaminated food shows a severe health risk.

As already mentioned, several human PA exposures have been observed due to contaminated grain and/or bread. A similar problem was found in the Tigray region of Ethiopia. In the last few years many people (especially children) have died there on account of severe liver failure. After excluding all other risk potentials (aflatoxins, etc.), a PA-containing plant was suspected as being responsible for the problems: *Ageratum conyzoides*. Several years ago this plant

was described as containing toxic PAs (Wiedenfeld and Röder 1991). The people in this region mainly use millet for the production of food, but also for beverages like beer. An inspection of the grain fields in this area resulted in the findings that indeed *A. conyzoides* is widespread and that the seeds of this weed can contaminate millet. We analysed six millet samples from different houses with respect to their PAs that originated from *A. conyzoides*. Three showed PA contamination. The sample with the highest amount came from a house where all family members had died ('Dagusha house 1'). We found 87.1 µg PAs in 182 g millet. As the normal daily consumption amounts to approximately 100 g millet/person, it becomes obvious that, consumed over a more or less longer period, such grain contamination can indeed produce the fatal problems observed in the Tigray region.

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