Phytochemical and analytical studies of feed and medicinal plants in relation to the presence of toxic pyrrolizidine alkaloids

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Jaime Becerra Jimenez

aus Mexiko

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1. Gutachter: Prof. Dr. Michael Wiese

2. Gutachter: Prof. Dr. Gerd Bendas

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Abstract

Pyrrolizidine alkaloids (PAs) are wide-spread in the plant kingdom; they are present in approximately 3 % of all flowering plants. Unsaturated PAs possess hepatotoxic, carcinogenic, genotoxic, teratogenic properties.

Senecio species are common weeds and form part of the primary vegetation in disturbed environments. The distribution and coverage of species such as *S. jacobaea* and *S. aquaticus* have remarkably increased in the last decade and these plants pose a latent risk as feed contaminants. The present work analyzed the effect on the PA-degradation of four common feed production methods.

In this work it could be established that if the starting material is contaminated with PA-containing plants, none of the herein analyzed feed production methods produces PA-free products. Hay production as well as pelleting are methods which involve only physical processes and they are the least effective methods for PA reduction. Methods involving chemical and biological processes produced a drastic reduction in the PA-content in the feed material analyzed. The reduction found in these methods might be enough to prevent acute intoxications but do not guarantee the safeness of the product for a long-term consumption.

Regarding the use of PA-containing plants as herbal remedies, the two plants here studied pose a low risk.The PAs found in both plants are pyrrolizidine alkaloids monoester, specifically lycopsamine, imtermedine and their acetylated forms. These kind of PAs are the least toxic ones, and their concentration in the plant is very low, approximately 0.001 %. In addition, the administration route is topical, which reduces the intoxication risk.

Contents

1 Introduction

The term alkaloid was first introduced by W. Meisner to describe a group of substances reacting like a base. The word alkaloid derives from the Arabic root *kali* which means soda and from the Greek word *eidos* wich means with appearance of. Within the big family of alkaloids one class consists of the pyrrolizidine alkaloids (PAs), formerly known as Senecio alkaloids. PAs are widely distributed among the plant kingdom, being present in several unrelated families such as Asteraceae, Boraginaceae, Fabaceae, Apiaceae, Convolvulaceae, Celestraceae, Proteaceae, Santalaceae, Sapotaceae, Ranunculaceae, Euphorbiaceae, Orchidaceae, Scrophulariaceae and Poaceae (Hartmann, 1999; Ober y Kaltenegger, 2009; Dreger *et al.*, 2009; Roeder, 1995).

It is assumed that PAs are present in more than 6000 plants, their occurrence has been estimated to reach 3 % of all flowering plants (Smith y Culvenor, 1981). Despite their wide distribution, their occurrence is concentrated within the families Boraginaceae in all its genera, Fabaceae (*nom. cons.* Leguminoseae) in the genus *Crotalaria* and in Astereceae (*nom. cons.* Compositae) in the tribes *Senecioneae* and *Eupatorieae* (Roeder, 1995).

Naturally occurring PAs present a great diversity, so far about 350 structures have been reported (around 700 if PA *N*-oxides are also considered) (Hartmann y Witte, 1995; Stegelmeier *et al.*, 1999). In contrast to other alkaloid groups, PAs do not show any remarkable physiological or pharmacological activity. Their importance relies on their potential (in relation to their structure) hazard to humans and animals.

1.1 Chemistry of PAs

Structurally, PAs are esters consisting of an amino alcohol known as necine and one or more acids (necic acids). The necine bases are bicyclic ring systems with a bridgehead nitrogen and a hydroxymethyl group at C-1. A second hydroxyl group commonly occurs at C-7 and in some cases additional hydroxyls have been found at C-2, C-6 and C-1 (Figure 1). Most of the naturally occurring PAs are derived from one of the four necine bases: platynecine, retronecine, heliotridine and otonecine (Mattocks, 1968; EFSA, 2007) (Figure 2). Retronecine and heliotridine are diasteroisomers at the C-7 position (Bull *et al.*, 1968; Mattocks, 1986; Rizk, 1990). Necines can be saturated or possess a double bond at the 1,2-position (Roeder, 1995; Dreger *et al.*, 2009). In this work, PAs with a saturated necine moiety are referred to as "saturated", whereas PAs from a necine with a 1,2-double bond are called "unsaturated". PAs usually occur as monoesters, open diesters or macrocyclic diesters. The esterified portions (necic acids) generally show five to ten carbon atoms and differ in the degree of chain branching, hydroxylation and unsaturation (Robins, 1989).

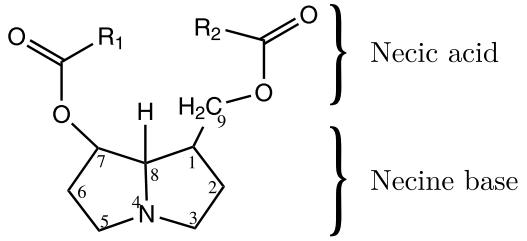

Figure 1: Structure of a pyrrolizidine alkaloid

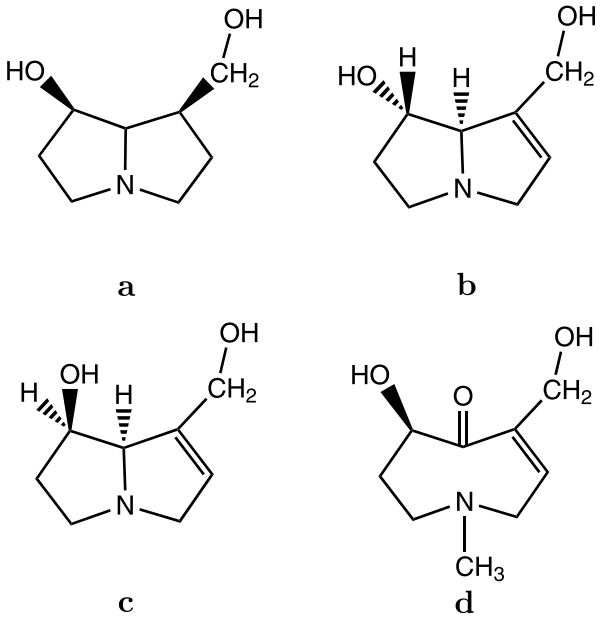

Figure 2: Principal necine bases. **a)** platynecine, **b)** heliotidine, **c)** retonecine and **d)** otenecine.

1.2 Biosynthesis

The first approaches to the biosynthesis of PAs were made through ^{14}C labeling of ornithine, acetate and propionate in the case of *Crotalaria* spp. (Nowacki y Byerrum, 1962) and ornithine for *Senecio spp.* (Hughes *et al.*, 1964; Bottomley y Gheissman, 1964; Khan y Robins, 1981). From these experiments it was established that the retronecine moiety was synthesized from ornithine and that acetate and propionate were incorporated into the necic acids. Bale y Crout (1975) showed in a double-labeling experiment using ornithine and arginine that ornithine presents a higher efficiency to produce the necine. In the same work putrescine was proposed as a putative

intermediate, this idea was later confirmed by further experiments.

Putrescine is a common precursor for all PAs, however its origin has been shown to be different and specific depending on the plant family. For *Heliotropium spp.* putrescine is always formed from arginine whereas in the genus *Crotalaria* (Birecka *et al.*, 1987) and *Senecio* it is built from ornithine (Birecka *et al.*, 1988).

In plants the polyamine putrescine is a substrate in secondary metabolism, as well as forming part of the common pool of polyamines involved in gene expression in eucaryotes and bacterias (Facchini, 2006; Jänne *et al.*, 2004). In plants and animals, polyamines take part during cell migration, proliferation and differentiation.

The biosynthesis of PAs starts from spermidine which is synthesized from putrescine and decarboxylated S-adenosylmethionine (deSAM), an aminopropyl group is transferred from deSAM to putrescine by the spermidine synthase (Graser y Hartmann, 2000). The first common pathway to PAs is the formation of homospermidine, a reaction catalyzed by the homospermidine synthase (HSS), the first specific enzyme of PA biosynthesis. HSS transfers the aminio butyl moiety of spermidine in an NAD^+ dependent reaction to the diamine putrescine which leads to the synthesis of the symmetric triamine homospermidine (Ober y Hartmann, 1999, 2000; Ober y Kaltenegger, 2009).

Further reactions, via the 1-(4-aminobutyl)-3,4-dihydro-2H-pyrrolium salt, generate the necines trachelanthamidine as well as heliotridine and retronecine (Robins, 1989; Ober y Kaltenegger, 2009; Wiedenfeld, 2013) (Figure 3).

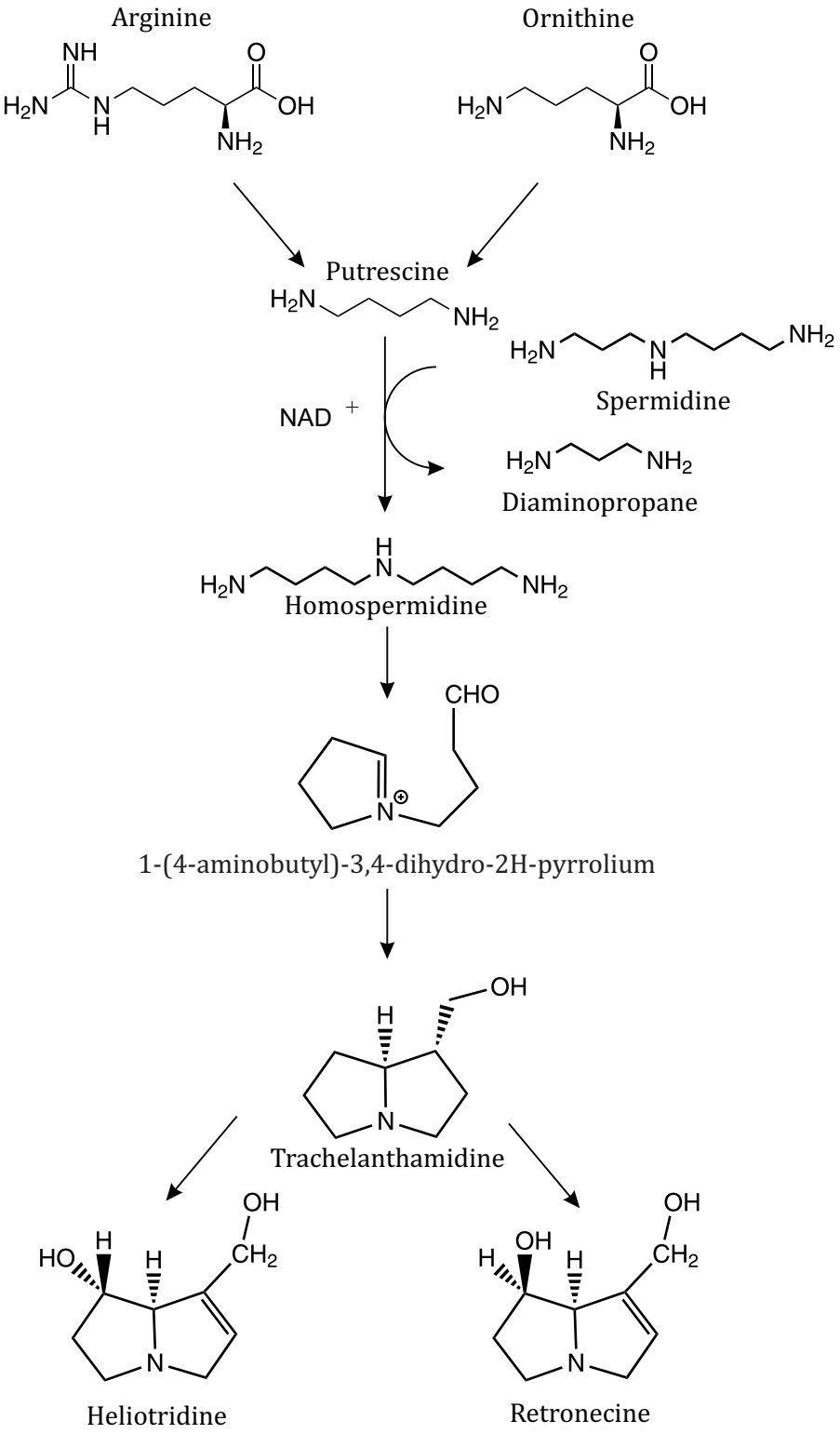

Figure 3: Biosynthesis of unsaturated necine bases heliotridine and retronecine. Modified from Wiedenfeld (2013) and Ober y Kaltenegger (2009)

Contrary to the well studied necine biosynthesis, the biosynthesis of the necic acids remains poorly studied. Crout and coll. (1966; 1966; 1967; 1970) proved that L-threonine and L-isoleucine are incorporated into the seneciphyllic acid, DL-valine is the precursor of echimidic acid and that L-isoleucine is the base of angeloylic acid. In general C^{10} acids are derived from isoleucine and variations involve L-threonin, (*2S*)-threonine, (*2S*)-valine and (*2S*)-leucine. The complete pathway of senecic acid, the acidic part in PAs rosmarinine and senecionine, is via two molecules of isoleucine (Stirling *et al.*, 1997).

The PAs synthesis site varies among species, e.g. in *Heliotropium indicum* PAs are produced in the shots, whereas in *Symphytum officinale* they are only synthesized in the roots. For the family Asteraceae basic structures are formed in the roots and further modifications take place in the leaves and inflorescences .

1.3 Toxicity and Metabolism

PA-containing plants are probably the most common poisonous plants affecting livestock, wildlife and humans. PAs are highly toxic to many animal species and have caused great livestock losses (see section 1.4.1) and human deaths (see section 1.4.2). The toxicity of PAs gained attention after their correlation with carcinogenic properties. The toxicity varies among structures and species, chronic poisoning affects mainly the liver. Other organs can be affected such as lungs and blood vessels and in some instances kidneys, pancreas, gastrointestinal tract, bone marrow and brain (Mattocks, 1986). However parent PAs, neither saturated nor unsaturated, are toxic *per se*.

Unsaturated PAs (1,2-dehydropyrrolizidine alkaloids) acquire their toxicity as a result of specific pathways in their metabolism.

1.3.1 Metabolism of pyrrolizidine alkaloids

After gastrointestinal absorption PAs are transported into the liver where, like many other xenobiotics, they are biotransformed by the incorporation of polar groups so that they can be conjugated and renally excreted (Chen *et al.*, 2010).

Saturated PAs are regarded as nontoxic, their metabolism includes *N*-oxidation reactions forming the corresponding pyrrolizidine alkaloid *N*-oxides which are water soluble and therefore prone to be excreted through urine and hydrolysis reactions producing the necine base and necine acids, also soluble and nontoxic.

Within unsaturated PAs, retronecine- and heliotridine-type pyrrolizidine alkaloids can undergo three main pathways:

1) Hydrolysis: cleavage of the ester functional groups to form the necine bases and the necine acids.

2) *N*-oxidation: formation to the corresponding pyrrolizidine alkaloid *N*-oxide

3) Oxidation: PAs oxidation takes place via two steps. a) Hydroxylation of the necine base at the C-3 or C-8 position to form the corresponding 3- or 8-hydroxynecine derivative and b) spontaneous dehydration producing a dehydropyrrolizidine derivative (Fu *et al.*, 2002a, 2004).

On the other hand, otonecine-type pyrrolizidine alkaloids present only two principal metabolic pathways since they are structurally different from retronecine- and heliotridine-type PAs in the methyl group attached to the N. These pathways can be either the hydrolysis of the ester groups, forming the corresponding necine bases and acids or the formation of didehydropyrrolizidine alkaloid (DHPA) (Lin *et al.*, 2000, 2002)(Figure 4).

Consequently, the hydrolysis is considered to be a detoxication process and the formation of the final didehydropyrrolizidine alkaloid (for retronecine- and heliotridine-type PAs via hydroxylation and for otonecine-type PAs via oxidative *N*-demethylation) is considered as a bioactivation process in the intoxication with PAs.

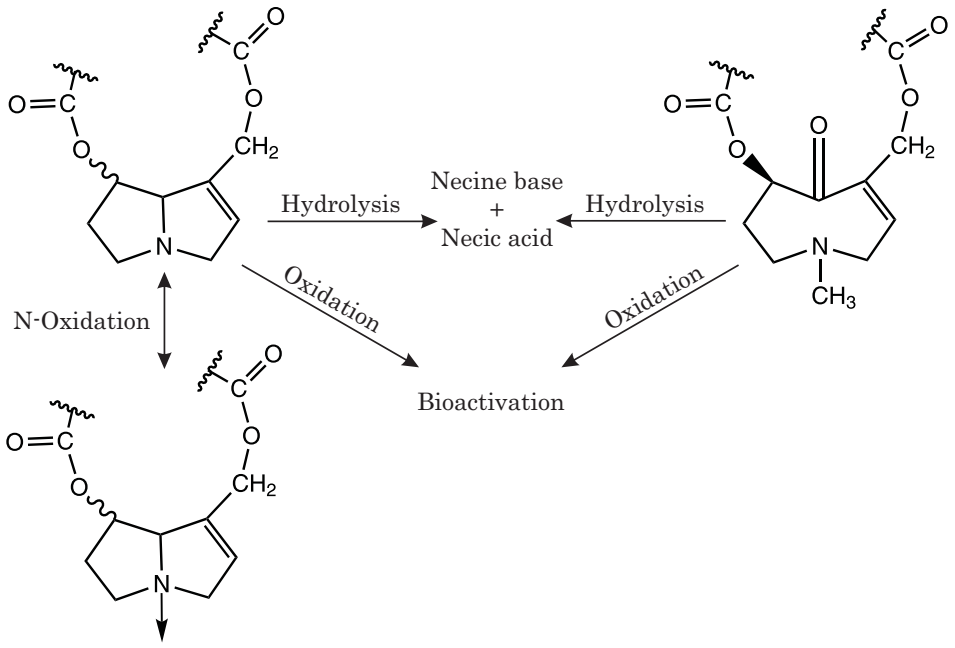

Figure 4: Metabolism of unsaturated PAs

Bioactivation

In the case of unsaturated PAs the critical step to show toxicity is the formation of the metabolite didehydropyrrolizidine alkaloid (DHPA) (EFSA, 2007; Wiedenfeld y Edgar, 2011; Wiedenfeld, 2008). The formation of the DHPA is mainly catalyzed by cytochrome P-450 monooxigenases, specifically CYP3A and CYP2B (Fu *et al.*, 2004).

For pyrrolizidine alkaloids with a retronecine- or heliotridine-type moiety, the cytochrome P-450 incorporates a hydroxyl group in the carbon adjacent (C-3 or C-8) to the nitrogen atom in the necine base to produce a hydroxy-PA. Due to the unstable nature of hydroxy-PAs, a rapid and spontaneous dehydration results in the formation of a second double-bond and the corresponding DHPA.

Bioactivation of otonecine-type PAs occurs by oxidative *N*-demethylation. The *N*-methyl group is first hydroxylated, this reaction is followed by loss of formaldehyde, leaving an NH

function which by condensation with the C-8 ketone group produces a C-8-hydroxy-PA, which, as previously described, dehydrates to a DHPA (Figure 5).

DHPAs are no longer alkaloids but high reactive aromatic systems which undergo further biotransformations. By loss of hydroxy groups or ester functions from carbons C-9 or C-7, DHPAs generate stable carbonium ions which rapidly bind to nucleophiles leading to DNA adduct formation, DNA cross linking and DNA-protein cross linking (Wiedenfeld y Edgar, 2011; Fu *et al.*, 2002a; IPCS, 1989; Mattocks, 1986). DHPAs may undergo further transformations and react with SH groups such as glutathione in a reaction catalyzed by the enzyme glutathion S-Transferase (GST) making them more water soluble and facilitating their excretion (Nigra y Huxtable, 1992; Reed *et al.*, 1992). Given the high reactivity of DHPAs, glutathion conjugation can occur non-enzymatically. GSTs isozymes often overlap in their substrate specificity (Fig.6).

The species-specific differences (as well as intraspecific and individual) in GST expression is, at least partially, responsible for the sensibility to PAs. Mice show low sensitivity and are known for the high expression of GST enzymes, indicating the importance of the competition between activating enzymes (CYP450) and detoxifying conjugation reactions (GST) (Lin *et al.*, 2002; Huan *et al.*, 1998).

As a result of extensive binding to glutathion (GSH), a relative cellular GSH deficiency accompanied with a loss in the capacity to scavenge and detoxify reactive oxygen (and nitrogen) species increases cellular oxidative stress and lipid peroxidation contributing to the vulnerability of hepatocytes (EFSA, 2007). In contrast to the 1,2-unsaturated PAs, data suggest that no metabolic activation takes place for fully saturated PAs because reactive pyrrole derivatives (DHPA) cannot be formed (Mattocks y White, 1971).

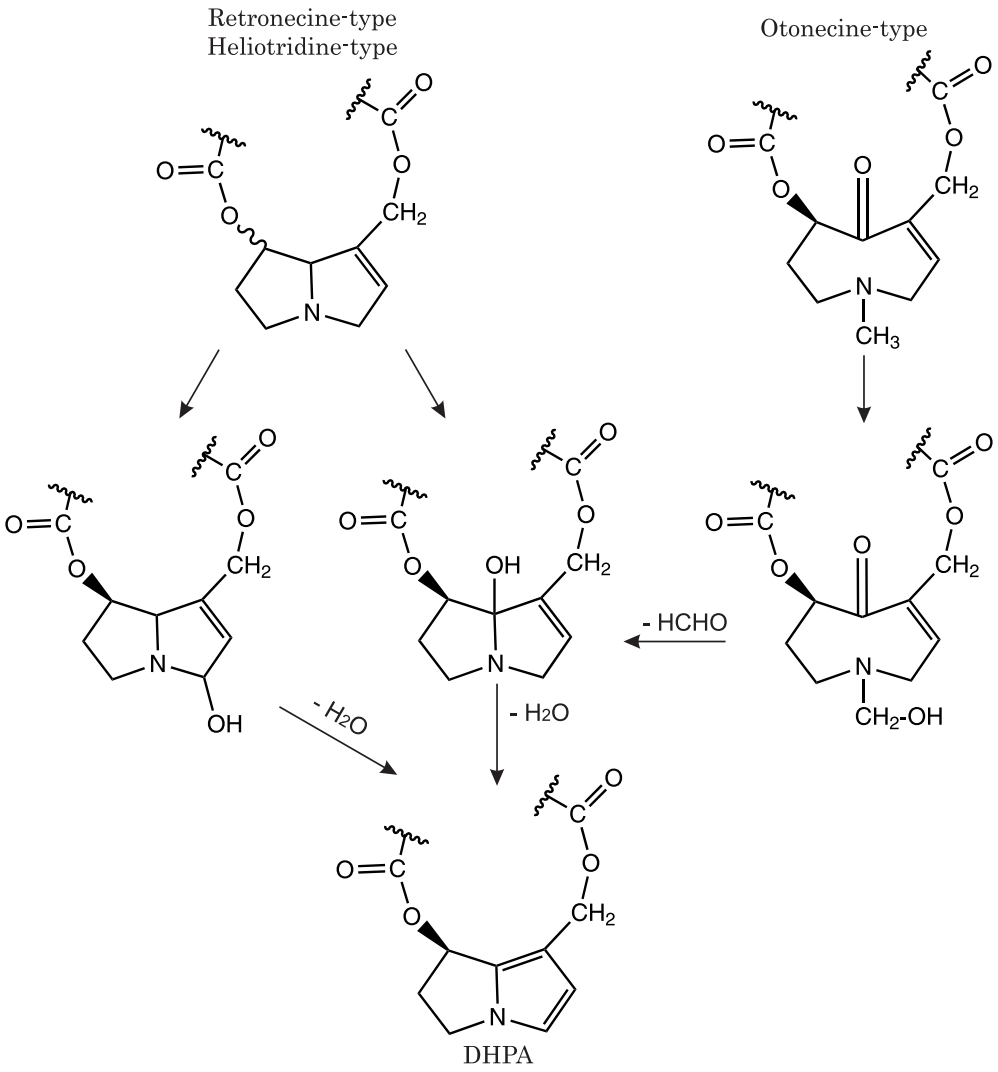

Figure 5: Formation of the DHPA

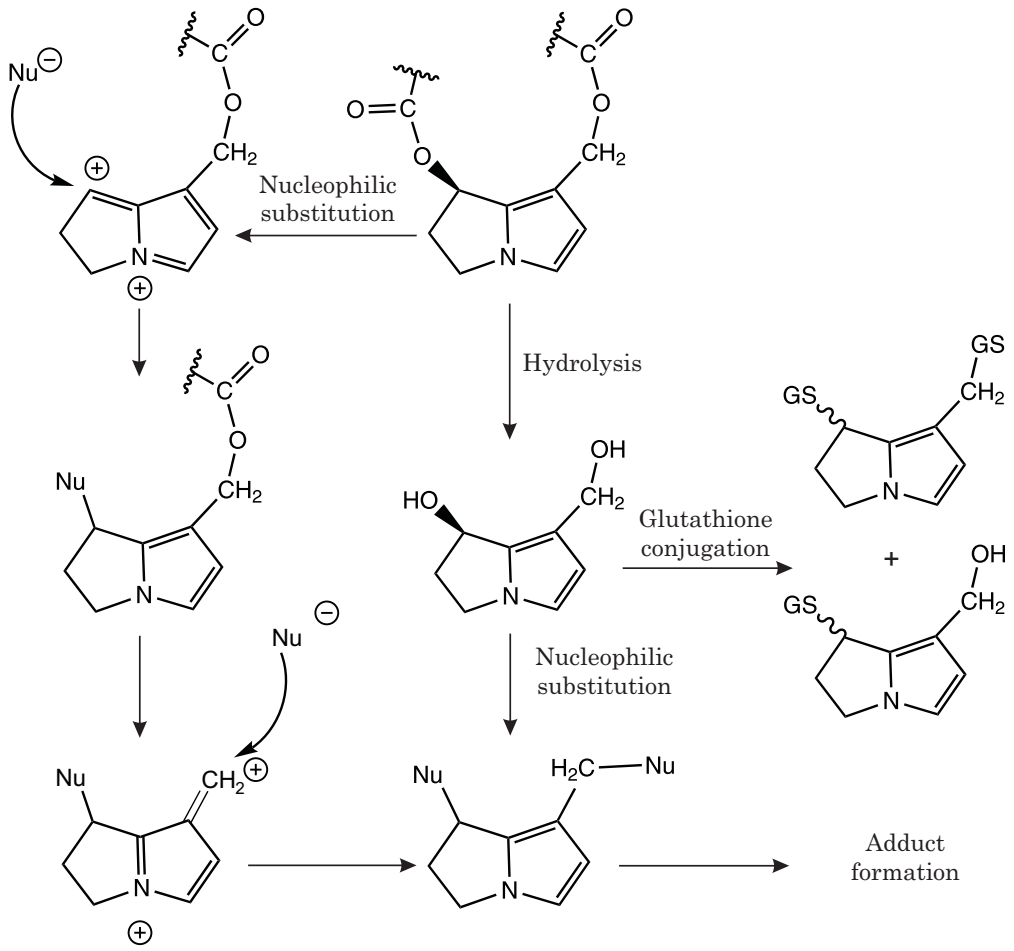

Figure 6: Reactions of the DHPA

1.3.2 Veno-Occlusive Disease

The most conspicuous manifestations of PA-poisoning are megalocytosis and veno-occlusion in liver and lungs (IPCS, 1988). Hepatic veno-occlusive disease (VOD), described first in 1920 by Willmot and Roberson, is a clinical syndrome characterized by hepatomegaly, ascites, weight gain and jaundice (Bayraktar *et al.*, 2007; Mcdonald *et al.*, 1984; Wadleigh *et al.*, 2003).

VOD is a non-thrombotic occlusion of the central veins of hepatic lobules. The first histological change in VOD is the injury of hepatic venules, which is characterized by

subendothelial oedema, red cell exudation, deposition of fibrin and factor α (von Willebrand factor -VWF-) within venular walls.

The ingestion of PAs triggers the coagulation cascade inducing a hypercoagulable state marked by the release of VWF, thrombomoduline and cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-I beta (IL-1β), endothelin-1 (ET-1), P-selectine and E-selectine. TNF-α and IL-Iβ activate the expresion of coagulation factors including tissue factor (TF) and plasminogen activation inhibitor-1 (PAI-1).

Afterwards, platelets are activated as well as the fibrosis markers such as transforming growth factor beta-I (TGF-β1) an N-terminal propeptide for type III procolagen (PIIIP). This cascade of events leads to fibrous obliteration of the affected venules (Chen *et al.*, 2010) (Figure 7).

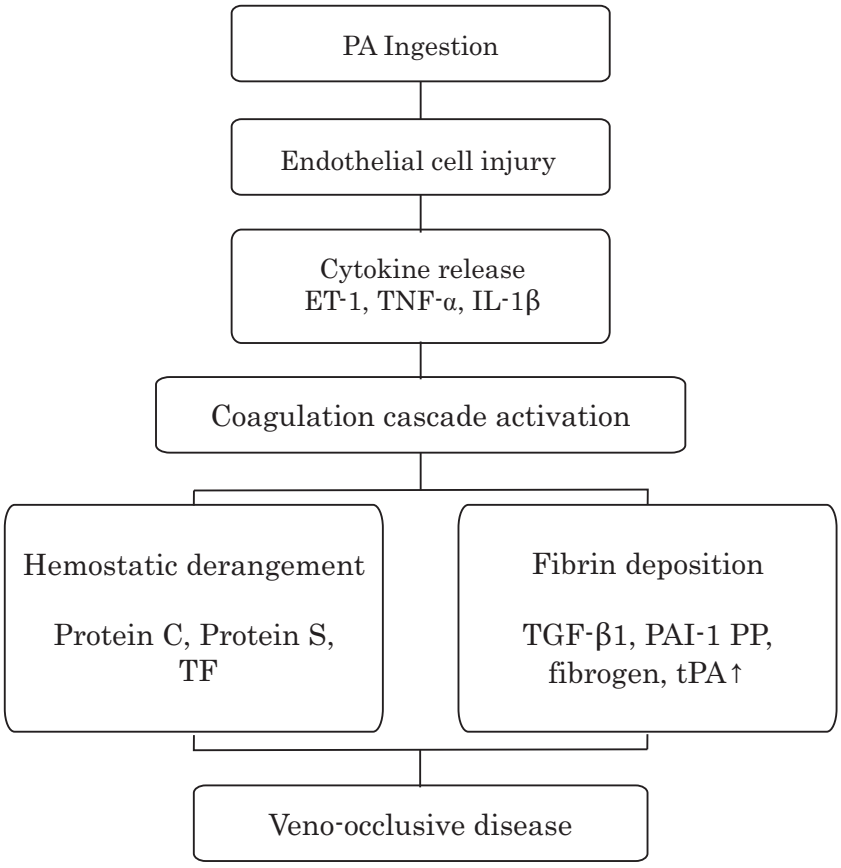

Figure 7: Coagulation pathway in pathogenesis of VOD. Adapted from Chen y Huo (2010)

1.4 Food and feed contamination with PAs

1.4.1 PA toxicity in ruminants

Intoxication with PA-containing plants has been known for a long time. *Senecio* had been suspected by farmers in Great Britain to be harmful to livestock as far back as 1787, in addition *Crotalaria sp.* was taken as the main cause of the Missouri River bottom disease in U.S.A. Similar observations were made worldwide and by the beginning of the 20^{th} century, cases of cirrhosis with a common symptomatology had been made in distant places, for example in Canada where it was known as Pictou disease or the Winton disease in New Zealand or the Schweinberger disease in the Valley of Ohm in Germany (Bull *et al.*, 1968).

The implication of plants from the genus *Senecio* as poisonous was demonstrated for the very first time by Gilruth (1903) in an experiment with *S. jacobaea* and horses. Chase (1904) described the Molteno disease in Cape Colony, South Africa, as a cirrhosis of the liver associated with the occurrence of *Senecio spp.*

Along with the cases of intoxication with *Senecio* (Asteraceae) during the first half of the 20^{th} century, plants of the genus *Cotalaria* (Fabaceae) as well as *Echium* and *Heliotropium* (both from the family Boraginaceae) were recognized as the cause of livestock poisoning (Bull *et al.*, 1956).

Although the acute intoxications were the ring alarm to call the attention on PA-poisoning, they are unlikely to occur. Animals freely pasturing tend to avoid PA-containing plants due to their bitter taste (Baker *et al.*, 1989). Consumption happens only in conditions of shortage of food (e.g. after a drought period or in high dense pasture fields) (Prakash *et al.*, 1999). More common are sub-acute and chronic intoxication, since the amount of PA

containing plants in fodder or their distribution in pasture and meadows does not reach the necessary level to trigger an acute intoxication (Molyneux *et al.*, 1988).

Substantial differences in susceptibility have been observed among species. Pigs and poultry are the most susceptible, while sheep and goats are relatively resistant to PAs; horses and cattle present an intermediate susceptibility. These differences are believed to be partially due to variations in the amount and activity of the cytochrome P450. Other factors regarding susceptibility are gender and age, young animals are more sensitive than adults and females more than males (Prakash *et al.*, 1999; Wiedenfeld, 2008; Wiedenfeld y Edgar, 2011; Fu *et al.*, 2002a, 2004).

PA poisoning is the most common plant-associated poisoning disease in livestock worldwide (Prakash *et al.*, 1999). A summary collection of intoxication cases in ruminants is presented in Table 1 to illustrate major causes and risks for animals.

Table 1: Relevant ruminant PA intoxications. Modified from Wiedenfeld y Edgar (2011)

Country	**Year**	**Animal**	**Plant**
Albania	1995	Cattle	*Senecio subalpinus*
Argentina	1994	Cows	*Senecio selloi*
Australia	1962	Sheep	*Echium plantagineum*
	1968	Sheep	*Crotalaria mucronata*
	1972	Heifers	*Heliotropium europaeum*
	1985	Calves	*Heliotropium europaeum*
	1987	Cattle	*Heliotropium amplexicaule*
	1987	Sheep	*Echium plantagineum*, *Heliotropium europaeum*
	1991	Heifers	*Senecio lautus*

Continued on next page

Table 1 – *Continued from previous page*

Country	**Year**	**Animal**	**Plant**
Bhutan	1994	Yaks	*Senecio raphanifolius,* *S. biligulatus,* *Ligularia spp.*
Brazil	1987	Cows, steers, heifers, calves	*Senecio brasiliensis,* *S. selloi,* *S. heterotrichius,* *S. crisplatinus,* *S. leptilobus*
	1987	Bovines	*Senecio brasiliensis,* *S. selloi*
	1988	Bovines	*Senecio brasiliensis,* *S. selloi*
	1993	Cows, steers, heifers	*Senecio tweediei*
	2001	Sheep	*Crotalaria retusa*
	2005	Sheep	*Senecio brasiliensis*
Canada	1969	Heifers	*Senecio jacobaea*
Mexico	1982	Sheep	*Senecio sanguisorbe*
Russia	1979	Calves	*Cynoglossum officinale*
Sudan	1981	Calves	*Crotalaria saltiana*
Switzerland	1980	Cattle	*Senecio alpinus*
The Netherlands	2002	Cattle	*Senecio jacobaea*

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Table 1 – *Continued from previous page*

Country	**Year**	**Animal**	**Plant**
UK	1917	Cattle	*Senecio jacobaea*
Uruguay	1978	Cattle	*Senecio brasiliensis*
USA	1962	Heifers	*Amsinckia intermedia,*
	1963		*Senecio vulgaris*
	1989	Calves	*Cynoglossum officinale*

1.4.2 PA toxicity in humans

The final relation of PAs as the responsible for the intoxication was established in the former USSR, where different endemic diseases caused by the consumption of PA-contaminated bread with seeds of *Heliotropium lasiocarpium* were studied (Bourkser, 1947).

Since then, although rare, several cases linking PA-containing plants to deaths have been documented. Any doubts, large scale outbreaks are the most sensational, such as the cases in central and south Asia. In Afghanistan approximately 8000 people were acutely poisoned by wheat contaminated with *Heliotropium popovii*, 3000 of them were seriously affected and several died (Mohabbat *et al.*, 1976; Tandon y Tandon, 1975; Tandon *et al.*, 1976). Another incident was reported in 1992 in Tadjikistan where four thousand people got poisoned, this time with wheat contaminated with seeds of *Heliotropium lasiocarpum* (Chauvin *et al.*, 1994).

Several more cases where PA intoxication has been confirmed involve herbal preparations. In developing countries the use of plants to treat medical problems is quite extended as part of their traditional medicine. On the other hand, in industrialized countries the so

called "green wave" has brought an increase in the use of herbal medicine in a look for alternative options. A common misconception in Western countries alludes to the idea that herbal medicines do not show any undesired side-effects. This thought has led to an increase in fatal poisoning resulting from the consumption of herbal products containing PAs such as comfrey (*Symphytum sp.*).

Acute intoxications are uncommon, nevertheless the sanitary and scientific community have called the attention to the sub-acute and chronic intoxication. The World Health Organization recognized and alerted in 1988 the health risk due to contamination of crops with plants producing PAs (IPCS, 1988) and the risk of consuming medicinal plants or herbal preparations without the proper control regulations (IPCS, 1989; ANZFA, 2001). PAs have been identified in plants used in the traditional medicine of South America (Bah *et al.*, 1994; Hirschmann *et al.*, 1987), Sri-Lanka (Arseculeratne *et al.*, 1981, 1985) and China (Zhao *et al.*, 1989; Fu *et al.*, 2002b).

Extensive reviews of PA-containing plants used for medicinal purposes have been compiled for the following regions: Europe (Roeder, 1995), China (Roeder, 2000), Mongolia, Nepal and Tibet (Roeder y Wiedenfeld, 2009), Madagascar and Mascarene Islands (Roeder y Wiedenfeld, 2011) and India (Roeder y Wiedenfeld, 2013). There is a great number of medical reports with typical liver damage caused by PAs where the the source of exposure could not be identified (Sergi *et al.*, 1999; Seibold-Weiger *et al.*, 1997; Müller-Höcker *et al.*, 1987; Price *et al.*, 1996).

Honey derived from PA producing plants has been proven to contain pyrrolizidine alkaloids, resulting from the the presence of pollen in the honey (Beales *et al.*, 2007; Betteridge *et al.*, 2005; Boppré *et al.*, 2005; Culvenor *et al.*, 1981; Dübecke *et al.*, 2011; Edgar *et al.*, 2002; Kempf *et al.*, 2008). Another source of PAs in food is milk from animals consuming PA containing plants (Deinzer *et al.*, 1982; Dickinson *et al.*, 1976; Dickinson, 1980; Goeger *et al.*, 1982b,a; Candrian *et al.*, 1991; Lüthy *et al.*, 1983), in this context, human milk

from women exposed to PAs has caused liver diseases in neonates and infants (Roulet *et al.*, 1988).

Despite the available data about the presence of PAs in eggs (Edgar y Smith, 1999) the EFSA (2011) refers that eggs and meat are unlikely to be sources for intoxications. Nevertheless, in the same work it is adverted to the reader the lack of information on this issue. Table 2 shows a sum-up of human intoxications with PAs.

Table 2: Documented Human Intoxications with PA. Modified from Wiedenfeld y Edgar (2011)

Location	**Year**	**People affected**	**Observed damage**	**PA source**	**Reference**
South Africa	1920	11 Adults	Abdominal pain, vomiting, chirrhosis	*Senecio illicifolius*, *Senecio burchelli*	Willmot y Robertson (1920)
Jamaica	1954	23 Adults	VOD	Bush-teas with *Crotalaria fulva*	Bras *et al.* (1961)
South Africa	1968	10 Children (10 died)	VOD	Bush-teas possibly *Crotalaria sp.*	Freiman *et al.* (1968)
Venezuela	1969	1 Girl (5 y.o.)	VOD	*Crotalaria anagyroides*, *C. pumila* consumed as an infusion and vegetable soup	Grases y Simon Beker (1972)
Jamaica	1970	6 Children	VOD	Bush-tea from *Crotalaria* and *Senecio*	Brooks *et al.* (1970)
Iraq	1970	9 Children	VOD	Food contaminated by *Senecio sp.*	Al-Hasany y Mohamed (1970)

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Table 2 – *Continued from previous page*

Location	**Year**	**People affected**	**Observed damage**	**PA source**	**Reference**
Afghanistan	1970-1972	7,200 People	VOD	Wheat with seeds of *Heliotropium popovii* ssp. *gillianum*	Mohabbat *et al.* (1976)
India	1973	486 People	VOD	Cereals contaminated with *Crotalaria spp.*	Tandon *et al.* (1976)
Ecuador	1973	1 Woman	VOD	Herbal tea with *Crotalaria juncea*	Lyford *et al.* (1976)
India	1973, 1975	4 Male adults	Endemic ascites	Millet contaminated with *Crotalaria spp.*	Krishnamachari *et al.* (1977)
China	1973, 1978	2 Adults	VOD	*Gynura segetum*	Hou *et al.* (1980)
India	1974-1977	6 People	VOD	*Heliotropium eichwaldii*	Datta *et al.* (1978)

Continued on next page

Table 2 – *Continued from previous page*

Location	**Year**	**People affected**	**Observed damage**	**PA source**	**Reference**
Martinique	1975	2 Children	VOD	Bush-teas with *Crotalaria retusa* and/or *Heliotropium sp.*	Saint-Aimé *et al.* (1977)
USA	1976, 1977	4 Children	Vein congestion and hepatic necrosis	*Senecio longilobus*	Stillman *et al.* (1977)
UK	1976	1 Woman	VOD	Mate (*Ilex paraguariensis*) contaminated with PA of unknown origin.	McGee *et al.* (1976)
USA	1984	1 Woman	VOD	Food supplement containing root of *Symphytum spp.*	Ridker *et al.* (1985)
China	1985	4 Women	VOD	Herbal tea with *Heliotropium lasiocarpum*	Culvenor *et al.* (1986)

Continued on next page

Table 2 – *Continued from previous page*

Location	**Year**	**People affected**	**Observed damage**	**PA source**	**Reference**
Switzerland	1985	2 Men (Father and son)	VOD	Herbal tea consisting of *Senecio spp.*	Margalith *et al.* (1985)
Switzerland	1986	5 days old baby	VOD	Mother drank during the whole pregnancy a tea containing *Tussilago farfara*	Roulet *et al.* (1988)
UK	1986	1 Boy (13 y.o.)	VOD	Herbal tea containing *Symphytum spp.*	Weston *et al.* (1987)
Tadjikistan	1992, 1993	3,906 People	Abdominal pain, hepatomegaly, ascites, alteration of consciousness	*Heliotropium lasiocarpum*	Chauvin *et al.* (1994)
Peru	1994	1 Woman	VOD	Herbal tea from *Senecio tephrosioides*	Tomioka *et al.* (1995)
Spain	1995	1 Man	VOD	*Senecio vulgaris*	Ortiz-Cansado *et al.* (1995)

Continued on next page

Table 2 – *Continued from previous page*

Location	**Year**	**People affected**	**Observed damage**	**PA source**	**Reference**
Austria	1995	1 Baby (18 months old)	VOD	Herbal tea with *Adenostyles alliariae*	Sperl *et al.* (1995)
Argentina	1999	1 Woman	VOD	Tea containing *Senecio vulgaris*	Vilar *et al.* (2000)
Germany	2002	Fetus	VOD	*Symphytum spp.*	Rasenack *et al.* (2003)

2 Aim of the work

PA-containing plants are probably the most common poisonous plants affecting livestock, wildlife and humans. PAs are highly toxic to many animal species and have caused great livestock losses.

In the last decade the native as well as introduced species of *Senecio* have increased their coverage range in Europe, specially in Germany. It has been observed that on account of this situation the contamination of food and feed occurs likewise, which increases the hazardous risk for humans.

The aim of the present work is to analyze the effect on PA degradation of four common feed production methods:

- Hay production
- Pellets production
- Ensilaging
- Composting

The direct contact with PAs by consumption of PA-containing plants has been well documented. Several plants belonging to the tribe Eupatorieae (Asteraceae) are used in different traditional medicines incluiding the folk medicine of Mexico. The taxon Eupatorieae is well known to contain pyrrolizidine alkaloids.

The present work estimates the hazardous potential of the plants *Ageratum maritimum* and *Ageratina chiapensis* in relation to their PA-content.

3 Materials and Methods

3.1 Extraction

3.1.1 Plant material

Dry material was grounded to a fine powder in a food processor (Krups). The material was extracted with methanol in a soxhlet apparatus for one week. The methanolic extract (ME) was dried under reduced pressure in a rotary evaporator (Büchi) and stored at 4°C until needed.

PAs were further extracted from the ME following the method described by Wiedenfeld (1997) with modifications. Briefly, ME was resuspended in hydrochloric acid 2.5 % v/v and first washed with dichloromethane (CH_2Cl_2) and then with diethyl ether up-to no remarkable color was observed in the organic phase. The organic phase was discarded and Zn-dust was added to the aqueous fraction and set to stir for one hour in order to reduce the *N*-oxides to free bases. After reduction the pH of the aqueous phase was adjusted to 8-9 with ammonia (25 %). Sodium chloride was added until saturation of the phase. The aqueous phase was then extracted several times with CH_2Cl_2. The organic phase was evaporated to dryness and stored at 4°C.

3.1.2 Animal material

Liver material was homogenized in methanol and then filtered with diatomaceous earth and vacuum. The methanolic extract was dried under reduced pressure and stored at 4°C.

3.2 PA isolation

3.2.1 Column chromatography

PA isolation through column chromatography was attempted in two modalities depending on the particle size of the silica gel used. In both cases silica gel was the stationary phase, in type-A columns the particle size was 0.040-0.063 mm, while in columns of the type-B the size was 0.063-0.200 mm. Mixtures of dichloromethane:methanol were use as mobile phase in a 5 %-stepwise gradient from 90:10 to 50:50.

Flow was kept at 2 ml/min and the elute was collected in 10 ml fractions and dried under reduced pressure. PA presence in the fractions was analyzed by TLC (see section 3.3.1).

3.2.2 Preparative TLC

For preparative thin layer chromatography (p-TLC) precoated glass silica gel 60 F_{254} plates (Merck, Darmstadt) were loaded with help of a Linomat IV (Camag) apparatus. A solution of dichoromethane:methanol:ammonia (85:14:1) was used as eluent.

After elution a small section of the plate was cut and developed (see section 3.3.1). Under UV_{254}-lamp along with the developed section as reference, individual PA-containing sections were scratched from the plates. Fractions were extracted several times with a solution of dichloromethane:methanol 50:50. Silica was removed by filtration and fractions were dried under reduced pressure. Samples were kept at 4°C for further analysis.

3.3 Detection

3.3.1 TLC development

TLC was used as a qualitative analysis. Presence of PAs was confirmed by developing the TLC plates according to the Dann-Mattocks method (Dann, 1960; Mattocks, 1967). Briefly, Dann-Mattocks detection method consists of 3 solutions (i.e. solution A, B and C) applied stepwise with periods of heating at 120°C for 20 minutes after each spraying.

Solution A	Hydrogenperoxide 30 %	
Solution B	Acetic anhydride	10 ml
	Petroleum ether	40 ml
	Toluene	50 ml
Solution C	p-Dimethylaminobenzaldehyde	2 g
	Hydrochloric acid 37 %	54 ml
	Ethanol	*q.s.* 100 ml

3.3.2 Sample cleaning

Before gas chromatography (GC) or GC-MS analysis, samples were cleaned up by solid phase extraction (SPE). Samples were redissolved in 1 ml CH_2Cl_2 and applied on a diol solid-phase column (Macherey-Nagel). After washing steps with CH_2Cl_2, the PAs were eluted with acetonitrile/methanol (1:1). The solvent was removed under reduced pressure and the samples were stored at 4°C until needed.

3.3.3 GC, GC-MS detection

Gas chromatography was performed on a apparatus Hewlett Packard model 5890, Series II with an NPD-Detector and an autosampler number 7673A. The data were recorded in a PC-system Chromeleon 6.40.

The GC apparatus was equipped with a Fused-Silica-Gel capillary column, Optima-5, DF-60m, 0,25 μm X ID (Macherey-Nagel). Program conditions were as follows:

Carrier gas: Helium.
Mode: splitless.
Flow: 1.5 PSI.
Injector temperature: 300°C.
Detector temperature: 300°C.
Temperature program: 180°C - 280°C; 5°C/min.; end time: 15 min.
Injection volume: 3 μl.

GC-MS analysis was done in a Hewlett Packard 5890, Series II, Quadrupol MS with an autosampler 7673 with the following conditions:

Column: CP-Sil m8, 50 m, 0,25 μm x 0,25 mm.
Temperature program: 180°C - 280°C; 5°C/min.; end time: 15 min.
MS Source temperature: 180°C.
Interface temperature: 290°C.
Injector temperature: 290°C.
Mode: Full-scan .
Injection volume: 3 μl.

3.4 Ensilage

For ensilage production *Lolium perenne* as well as *Senecio jacobaea* were collected in 2010 during their leave and flowering state respectively; both plants were pre-dried to 40 % weight of starting material.

Ensilaging was performed by C. Berendonk and K. Hünting at the Chamber of Agriculture of North Rhine-Westphalia; Kleve, Germany (Landwirtschaftskammer Nordrhein-Westfalen). The ensilaging was made according to the DLG guidelines (DLG, 2000).

For the assay, sealed 500 ml glass jars were used, eight different concentrations of *S. jacobaea* were tested (i.e. 0, 1, 5, 10, 25, 50, 75 and 100 %; n=3), the ensilaging time was 90 days.

After the ensilaging period pH was measured and 300 g samples were dried at 60°C. PAs were extracted and quantified as described in sections 3.1.1 and 3.3.3.

3.5 Pellets and Compost

The manufacturing of both pellets and compost from hay contaminated with *S. aquaticus* was part of a cooperation project with the State Office for Agriculture of Bavaria, Germany (Bayerisches Landesamt für Landwirtschaft).

The stock material, hay contaminated whith *S. aquaticus*, was produced in Öschlesee in the region of Algäu in regular hay producing fields without any particular weed control. Cut forage from 20 to 30 representative samples was homogenized and reduced to a sample of approximately 5 kg of fresh material which was later set to dry in a air-drying chamber.

The dry hay and the structural material (wood pieces) were taken for the prepatation of the compost, which was done following the guidelines of the Bundesgütegemeinschaft Kompost e.V. (Federal Compost Association) (BGK, 2006).

The structural material had a ratio of 1:3 to the hay material.

The pellets were produced using forage from the same contaminated fields. Fresh forage was loaded into a pelleting machine, which forms the pellets by drying and compressing the forage.

4 *Senecio spp.* as feed contaminants

In recent years, PA-containing plants have received special attention from food and health agencies in Europe. Their occurrence on pastures and meadows compromises both animals and humans health due to their toxic potential through PA poisoning.

Senecio species are common weeds and form part of the primary vegetation in disturbed environments. Some species such as *S. jacobaea* or *S. aquaticus* are native to Europe but some others like *S. inaequidens* were introduced to the continent; in both cases their distribution and coverage have remarkably increased in the last decade and they pose a latent risk as feed contaminants.

4.1 Botanical description

4.1.1 *Senecio jacobaea*

Biennial or perennial herb usually dying after flowering; it frequently behaves as a perennial if damaged. Single-stemmed or much branched from the base, the stems furrowed, glabrous or cottony, branching above the middle to give a flat-topped, more or less dense compound corymb. Basal leaves forming a rosette, the first formed leaves (either of seedlings or root buds) ovate, blunt, successive leaves becoming increasingly lyrate-pinnatifid with 0-6 pairs of lateral lobes. Rosette and lower stem leaves stalked, upper leaves more or less amplexicaul, pinnatifid to bi-pinnatifid with lateral and terminal lobes becoming more similar, auricles laciniate; rosette leaves are usually lost before flowering. Plants vary in the degree of crisping of the leaves and in the presence or absence of cottony hairs on the undersides. Capitula 12-25 mm diam., involucral bracts or phyllaries oblong-lanceolate (c. 13), acute and more or less glabrous with a few subulate much shorter bracts (c. 5). Ray florets 12-15, golden yellow, like the disc florets. Achenes c. 8-ribbed, 2 mm long and 0.6 mm. diam., those of the rays glabrous, those of the disc hairy. Pappus twice as long as the achenes, readily detached, especially from achenes of the ray florets.

Geographical distribution

S. jacobaea distribution is difficult to define especially at the eastern and southern limits because the plant extends its range following man's activities. The northern limits lie at 62° 30' (isolated record at Sordmoere) in Norway (absent from Iceland and Faeroes). It is present in southern Sweden and uniformly distributed in Denmark. The plant is a

continental species and extends as far east as Siberia and as far south as Asia Minor. Present in Romania, Hungary and Bulgaria and in northern Greece, and North Africa. Introduced into New Zealand, Tasmania, Australia and South Africa as well as to North and South America.

S. jacobaea presents a very plastic altitudinal distribution, it has been recorded in coastal areas as well as in altitudes as high as 1570 m in Bavaria.

In Germany *S. jacobaea* is a native plant which in the last years has increased in occurrence, in North Rhine-Westphalia presents from 3 % and up-to 10 % of the coverage (Berendonk, 2009).

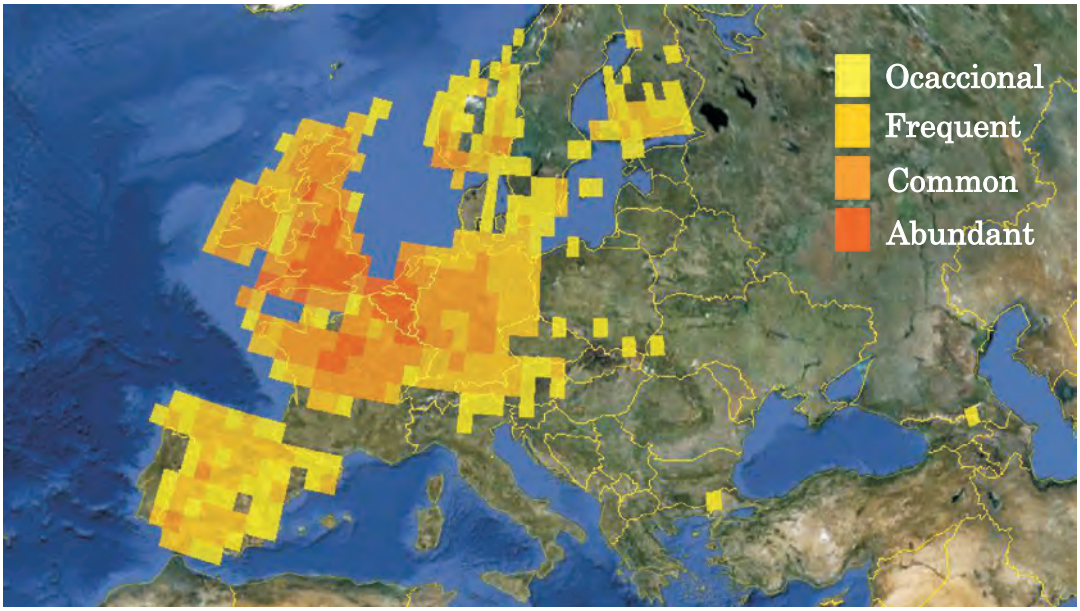

Figure 8: Distribution and abundance of *S. jacobaea* in Europe. Modified from a map generated by GBIF (2013); one degree cells plotted on Google Earth

4.1.2 *Senecio aquaticus*

A usually biennial non-stoloniferous herb with a short, more or less erect, premorse stock and erect flowering stems, 25-80 cm. Glabrous or cottony above, often reddish, with ascending branches above. Basal leaves long stalked, elliptical to ovate, undivided, or lyrate pinnatifid with a large ovate to ovate-oblong terminal lobe and 1 to several pairs of much smaller, oblong lateral lobes; lower stem leaves stalked, more or less lyrate-pinnatifid, middle and upper semi-amplexicaul, pinnatifid, with the lateral lobes directed forwards; all crenate to coarsely serrate, more or less glabrous, firm, slightly waved, often purplish below.

Heads 2.5-3 cm diameter in irregular lax corymbs. Involucral bracts narrowly acuminate, green with white margins, with a few narrower and much shorter bracts at the base. Ray-florets 12-15, golden yellow. Achenes 2.5-3 mm, all more or less glabrous; pappus about twice as long as the achene, readily falling. Very variable in shape of leaves, especially basal leaves.

Geographical distribution

Senecio aquaticus can be found in marshes, wet meadow and ditches. Common throughout the British Islands. It reaches 460 m in England and Ireland. It has been recorded in West and Central Europe from North Italy northwards to 60° 47´ N in Scandinavia and eastwards to Posen and lower Silesia (Clapham *et al.*, 1990).

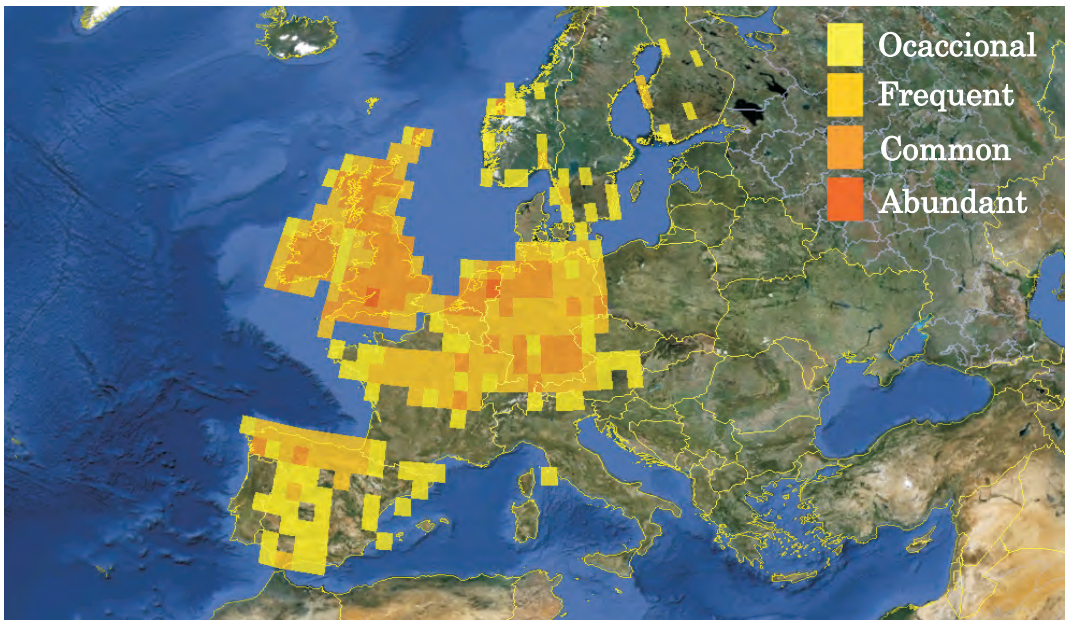

Figure 9: Distribution and abundance of *S. aquaticus* in Europe. Modified from a map generated by GBIF (2013); one degree cells plotted on Google Earth

4.1.3 *Senecio vulgaris*

An annual or overwintering herb with erect or ascending, weak, rather succulent stems 8-45 cm, glabrous or with non-glandular hairs, irregularly branched. Leaves glabrous or cottony, pinnatifid with distant, oblong, blunt, irregularly toothed lobes; lower leaves lanceolate or obovate in outline, narrowed into a short petiole, upper oblong, semi-amplexicaul, auricled. Inflorescence is a dense, terminal, corymbose cluster. Heads 8-10 x 4 mm, at first subsessile, later stalked. Involucre more or less cylindrical, inner bracts 5-8 mm usually glabrous, and often black tipped , outer bracts 8-10, 1-2 mm. Ray florets usually 10, rarely 11, yellow. Achenes 1.5-2 mm, densely hairy on the ribs; pappus white, long. Very variable in the dissection of the leaves and hairiness.

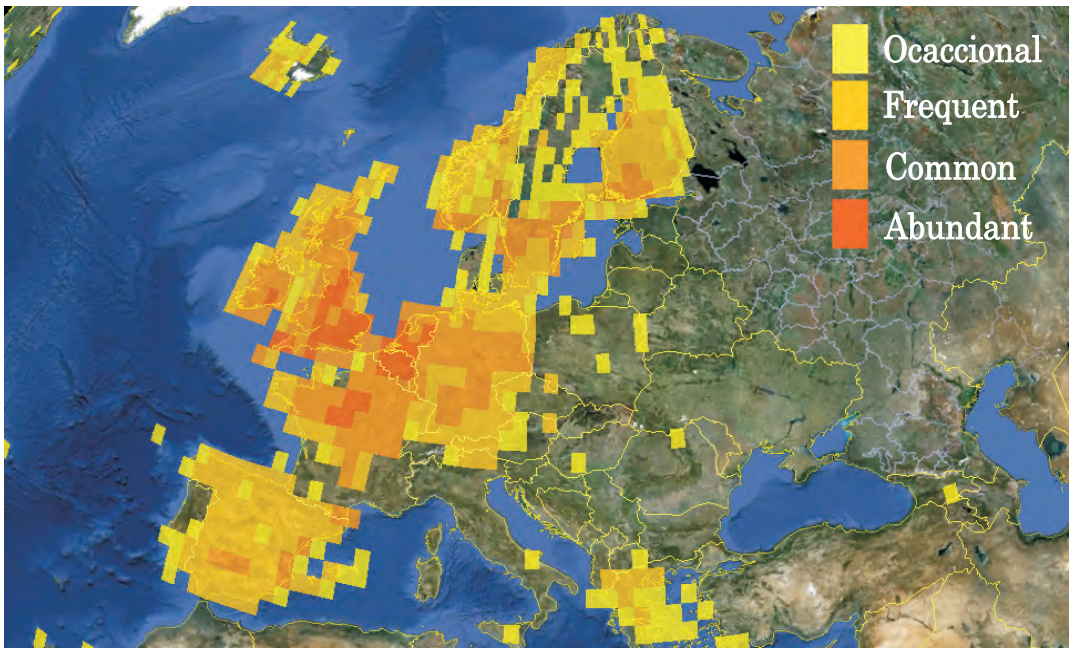

Figure 10: Distribution and abundance of *S. vulgaris* in Europe. Modified from a map generated by GBIF (2013); one degree cells plotted on Google Earth

Geographical distribution

Senecio vulgaris grows in disturbed sites, waste places, roadsides, gardens, nurseries, vineyards, landscaped areas, agricultural lands. It distributes at altitudes up to 1000 m. It is a native plant to Eurasia and introduced thoughout the United States and some regions in Mexico (FNA, 2006).

4.2 Pyrrolizidine alkaloids of *S. jacobaea* and *S. vulgaris*

In order to have a profile of the pyrrolizidine alkaloids present in both species *S. jacobaea* and *S. aquaticus*, plants were collected in the State of North Rhine-Westphalia. Plant samples were worked out as described in chapter 3: Materials and Methods. In the case of *Senecio jacobaea* 9 PAs were identified and 3 PAs were identified in *Senecio aquaticus*. The identification of the PAs was done by comparison with certified references in CG and GC-MS analysis.

The PAs senecionine, seneciphylline and integerrime were common to both species. In addition jacobine, jacozine, jacoline, jacozine isomer, jaconine and erucifoline were only found in *S. jacobaea*. The mass spectra for the 9 different PAs are given in the following Figures. The full interpretation of the cleavage pattern is given only for the pyrrolizidine alkaloids senecionine and seneciphylline. Since the fragmentation pattern of the rest of the PAs is the same when considering the necine moiety, the cleavage pattern is given up to the formation of the corresponding fragments of m/z 136 and m/z 138.

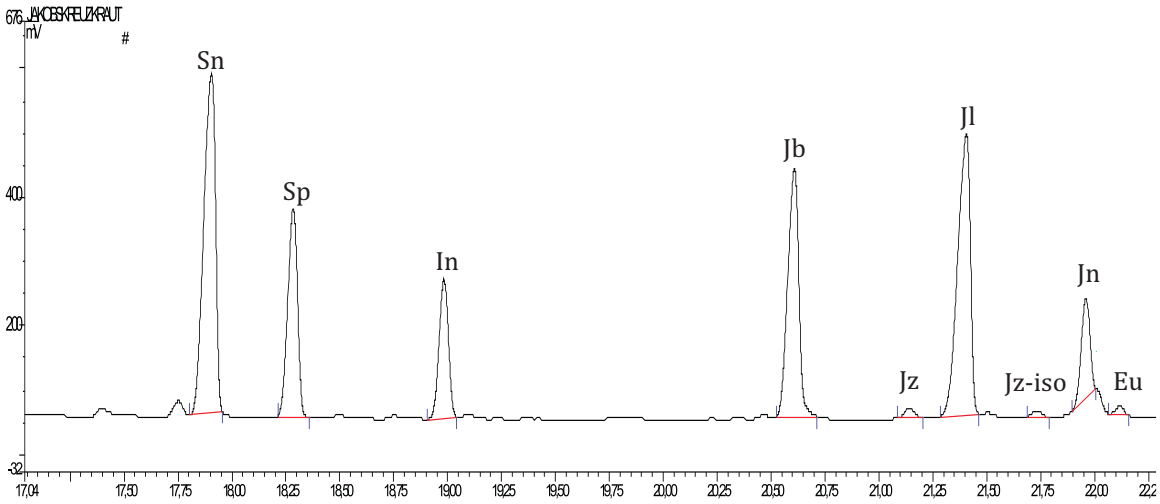

Figure 11: Gas chromatogram of the extract of *S. jacobaea*.
Sn: senecionine, **Sp**: seneciphylline, **In**: integerrimine, **Jb**: jacobine, **Jz**: jacozine, **Jl**: jacoline, **Jz-iso**: jacozine isomer, **Jn**: jaconine, **Eu**: erucifoline.

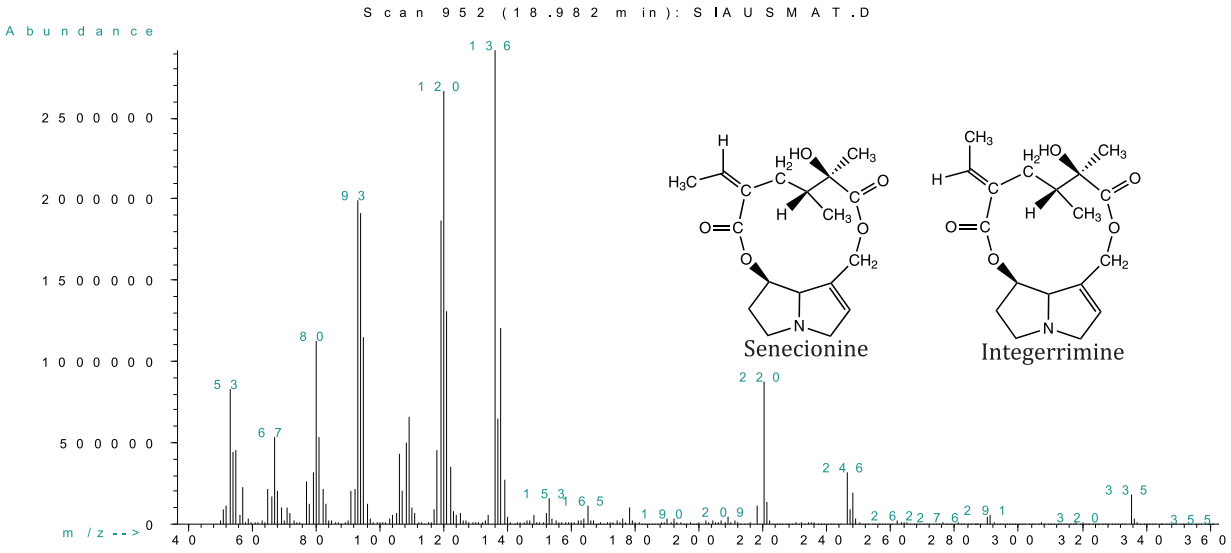

Figure 12: Mass spectrum of senecionine and integerrimine

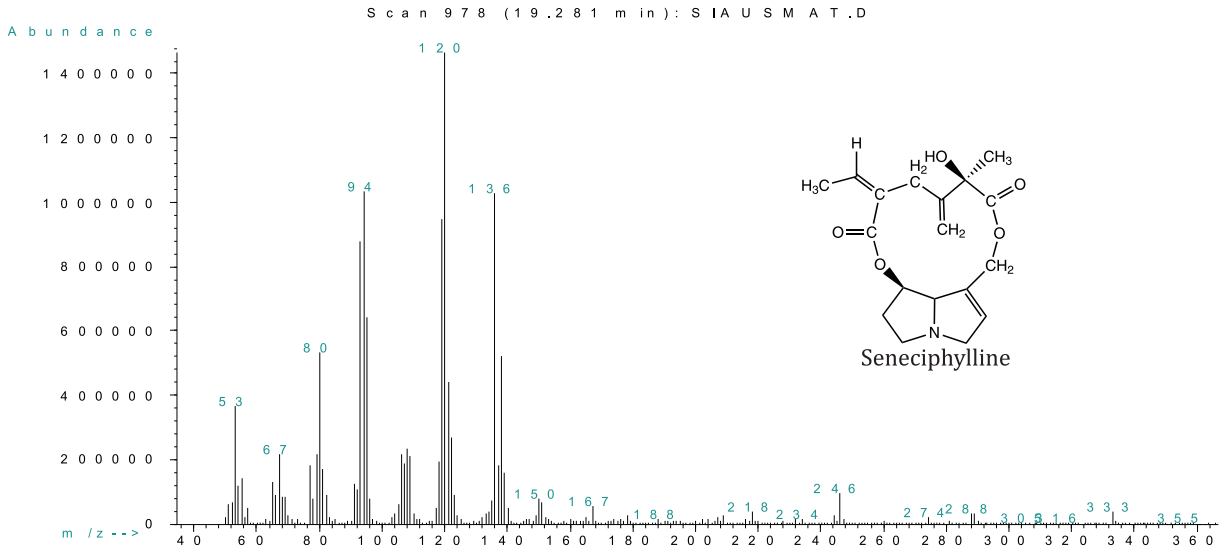

Figure 13: Mass spectrum of seneciphylline

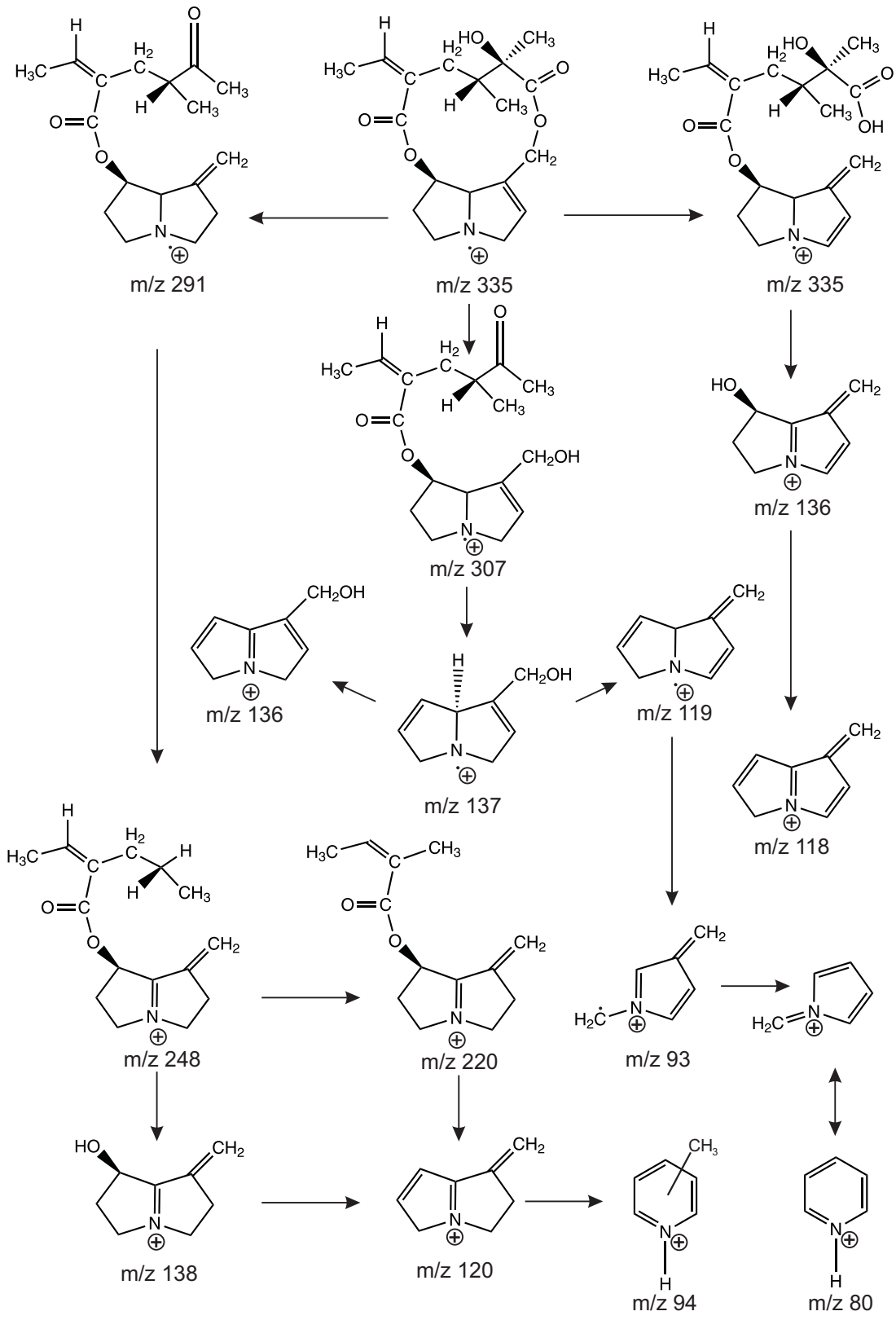

Figure 14: Interpretation of the fragmentation of senecionine

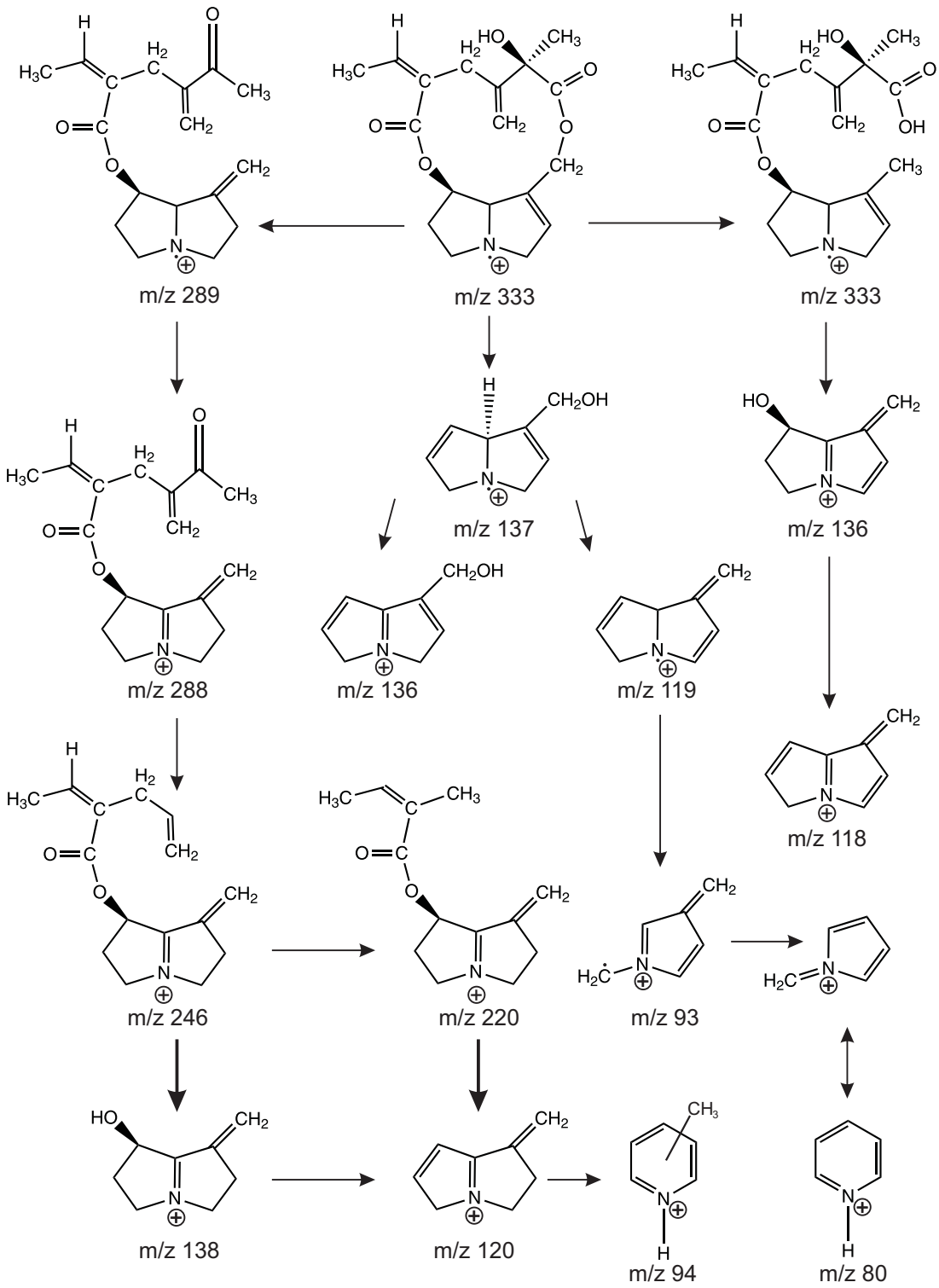

Figure 15: Interpretation of the fragmentation of seneciphylline

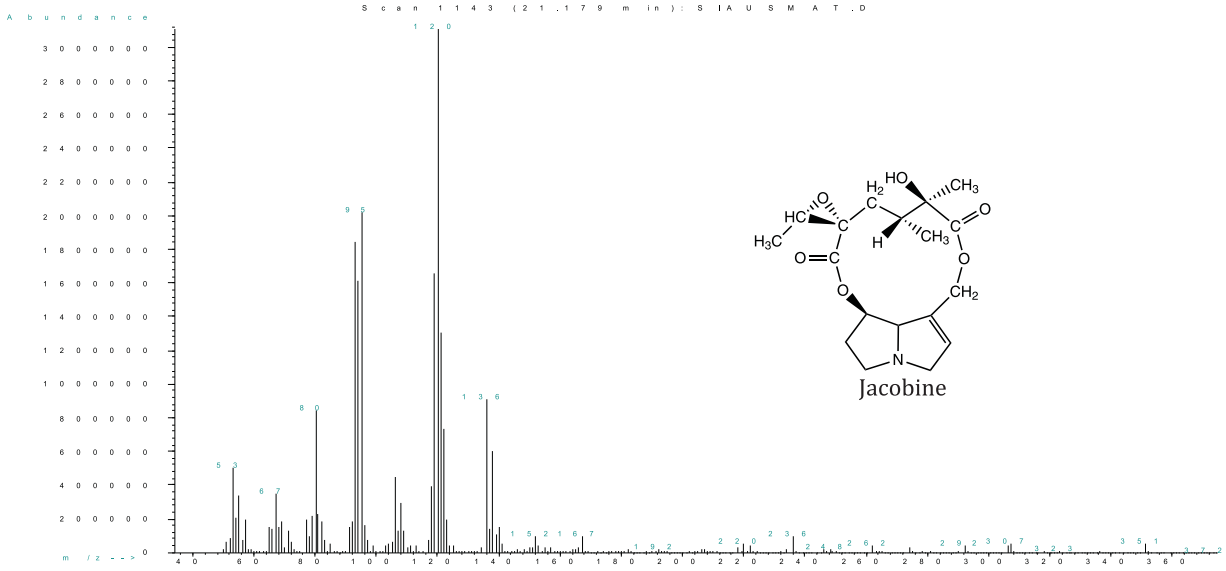

Figure 16: Mass spectrum of jacobine

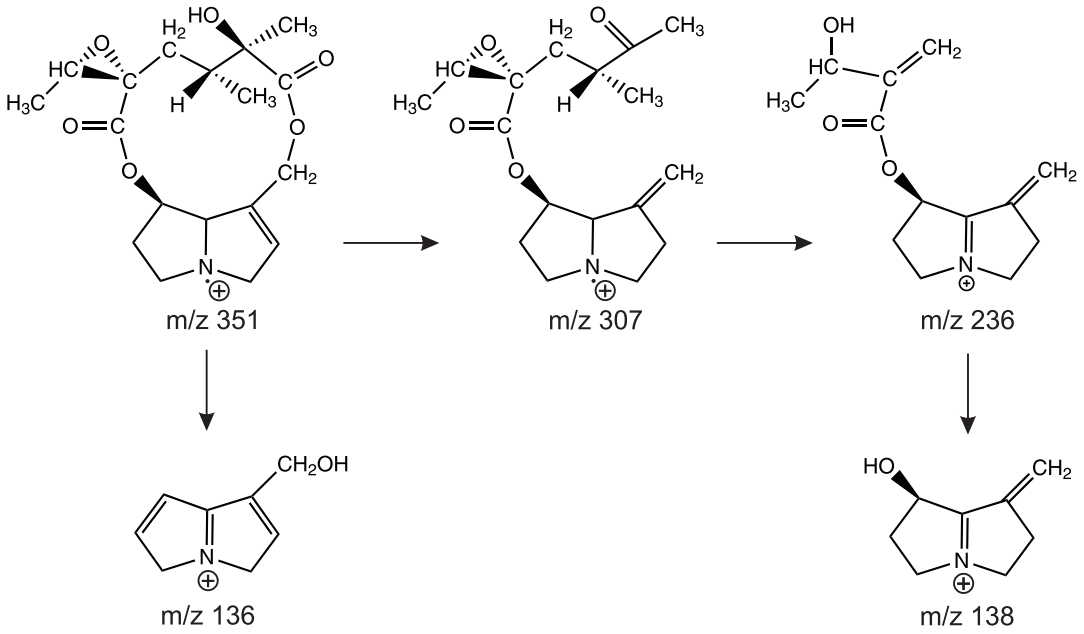

Figure 17: Interpretation of the fragmentation of jacobine

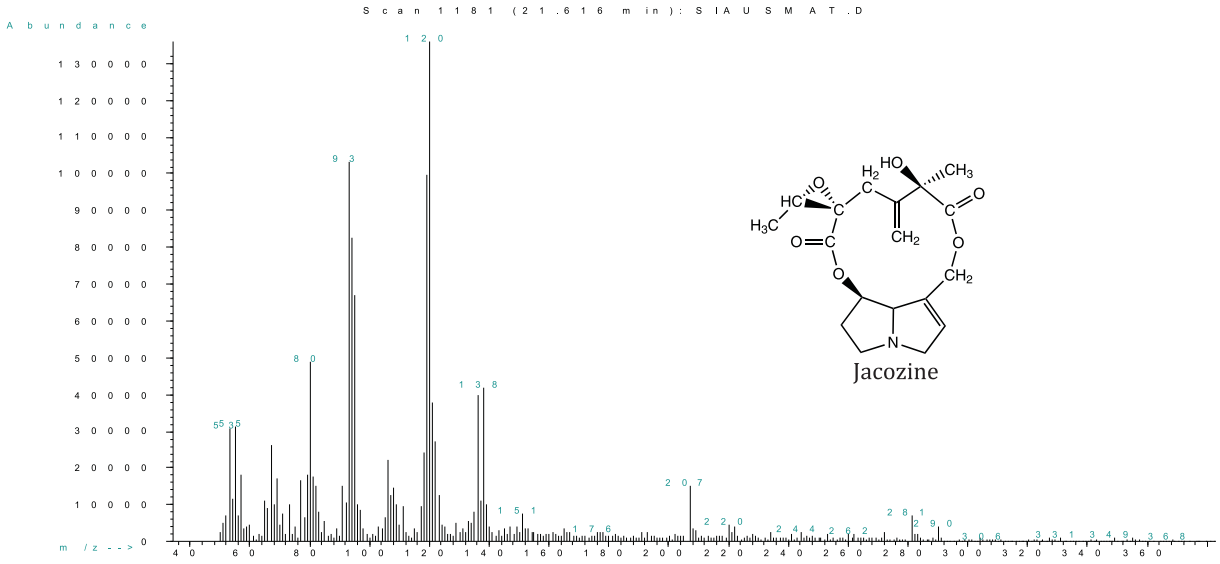

Figure 18: Mass spectrum of jacozine

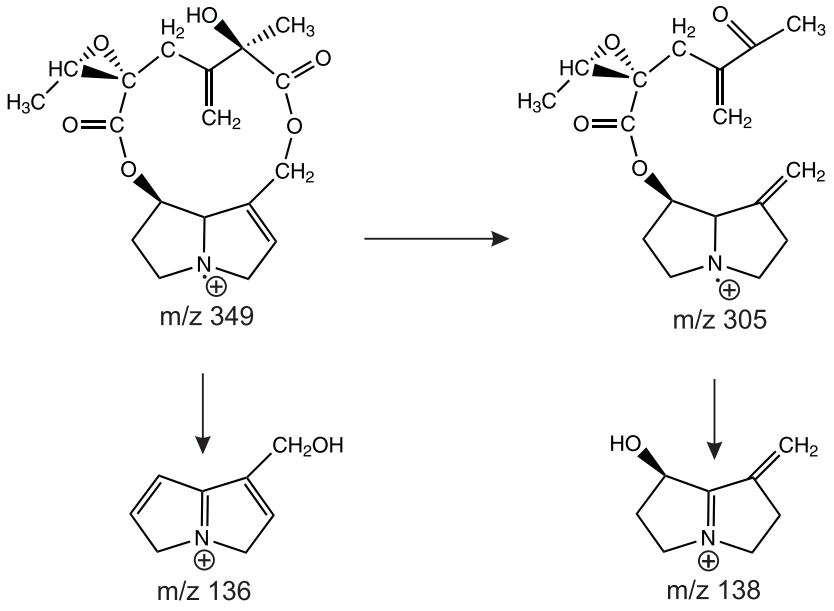

Figure 19: Interpretation of the fragmentation of jacozine

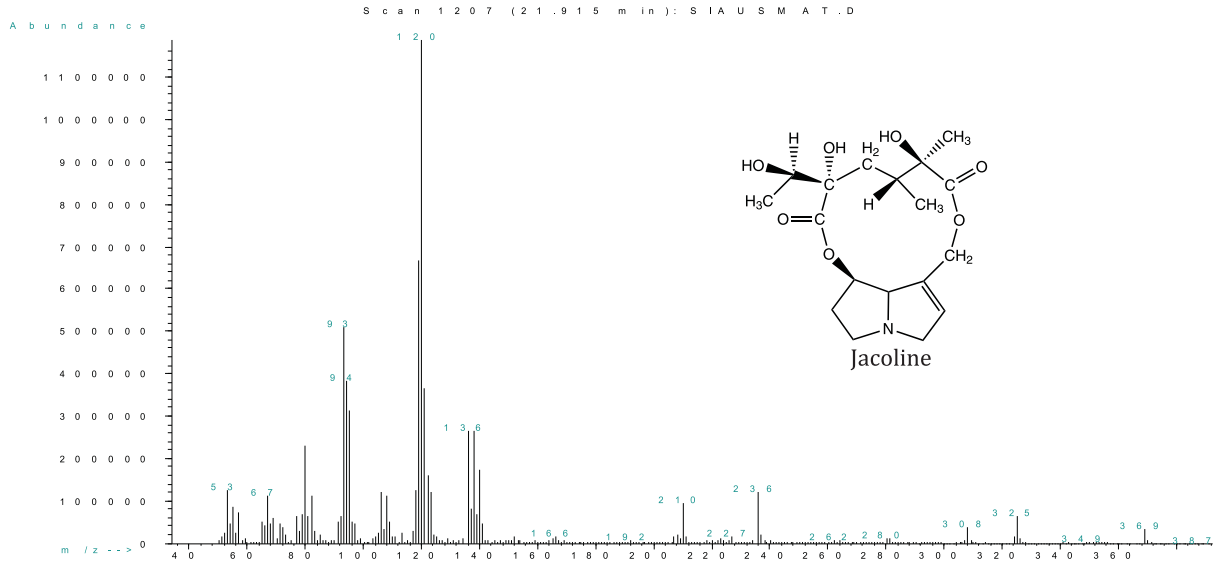

Figure 20: Mass spectrum of jacoline

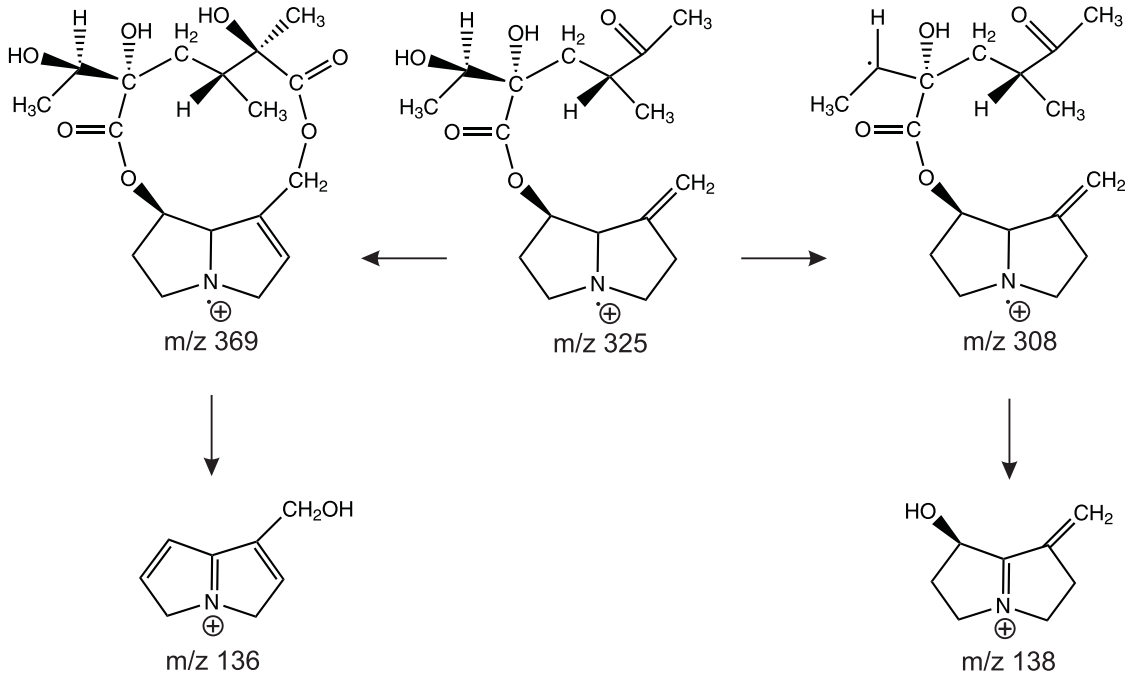

Figure 21: Interpretation of the fragmentation of jacoline

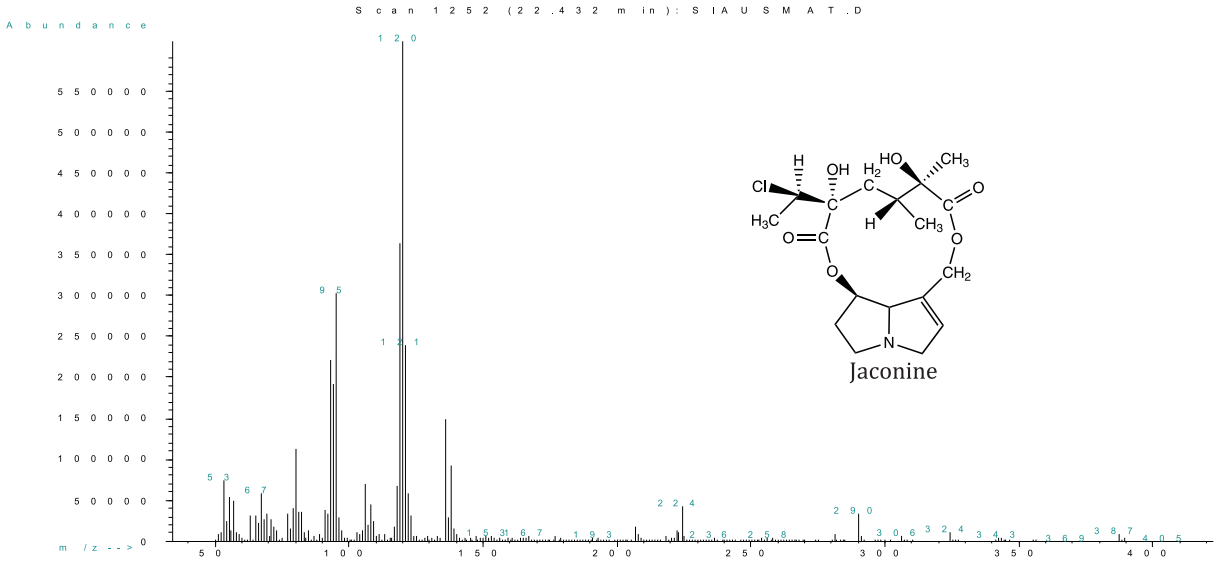

Figure 22: Mass spectrum of jaconine

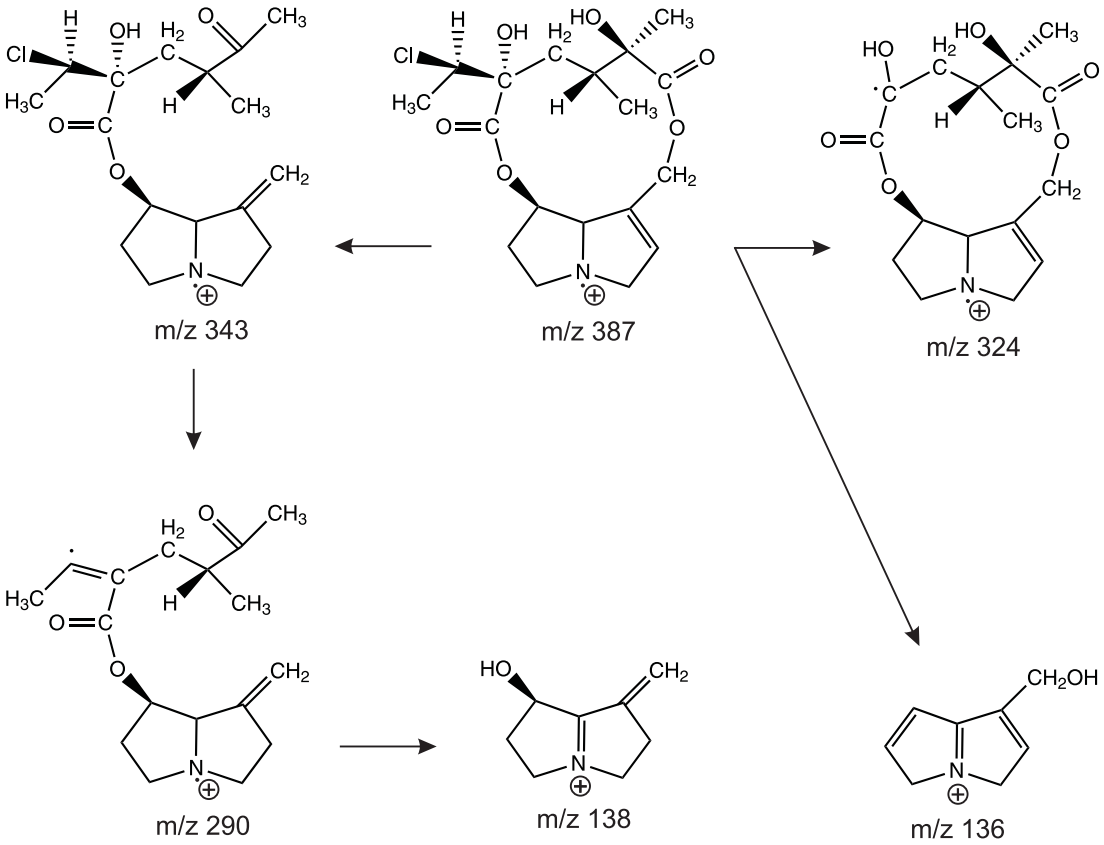

Figure 23: Interpretation of the fragmentation of jaconine

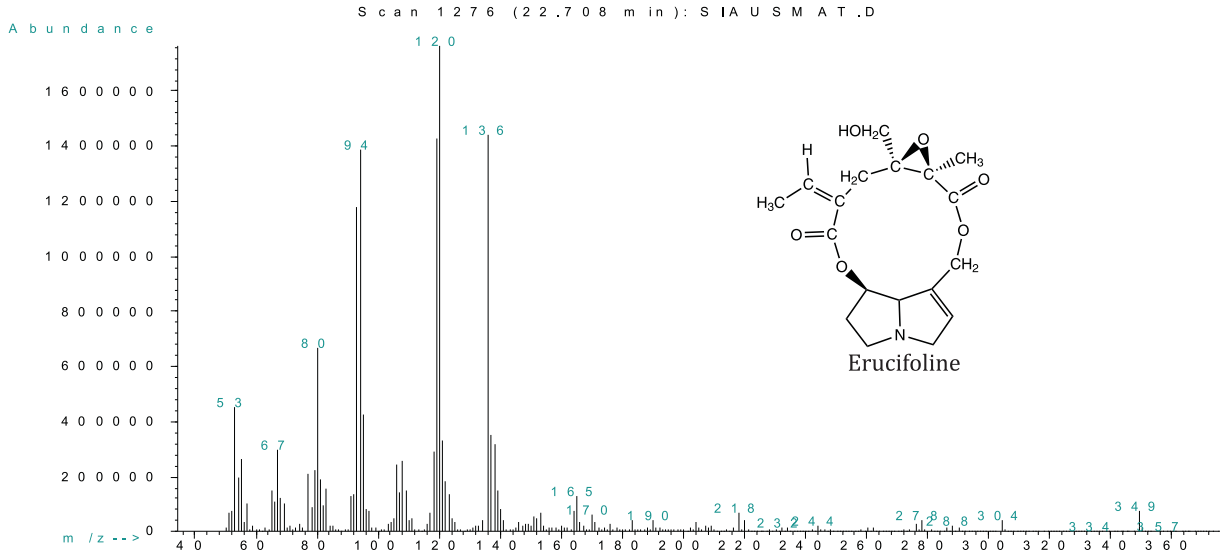

Figure 24: Mass spectrum of erucifoline

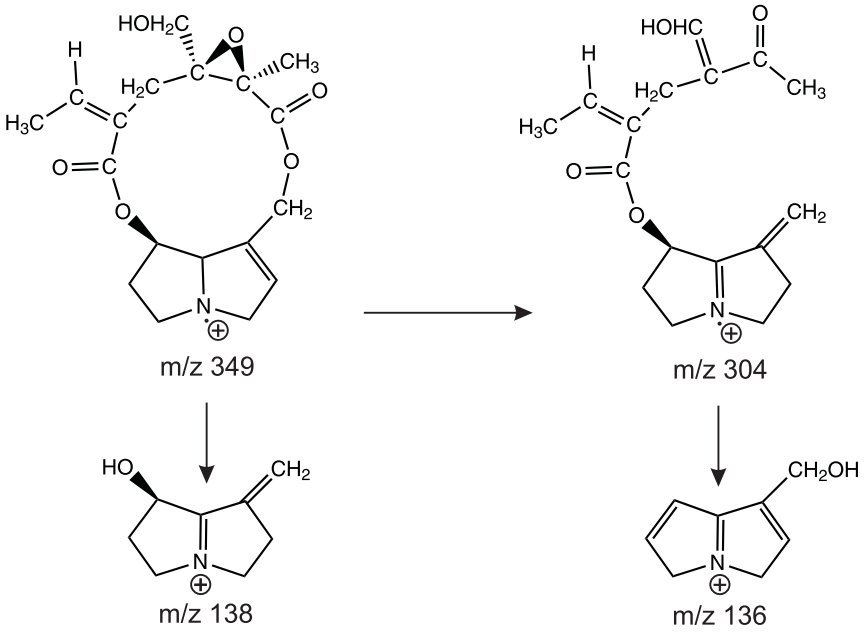

Figure 25: Interpretation of the fragmentation of erucifoline

4.3 Analysis of pyrrolizidine alkaloids in feed

4.3.1 Method

For a description of the methods used for this experiment see correspondent sections under the chapter Materials and Methods (chapter 3).

4.3.2 Results

Silage contamination with *Senecio jacobaea*

Through our cooperation with the Landwirtschaftskammer Nordrhein-Westfalen, samples of the silage with different amounts of *S. jacobaea* as well as the starting material were obtained for their analysis. Nine different PAs could be detected in all samples independently from the percentage of *S. jacobaea* in the silage. Jacobine and jaconine were the most abundant PAs (Figure 26). Mean values of the alkaloid concentration are presented in Figure 27. For comparison purposes, data were recalculated and standardized to micrograms per gram of *S. jacobaea* (Figure 28).

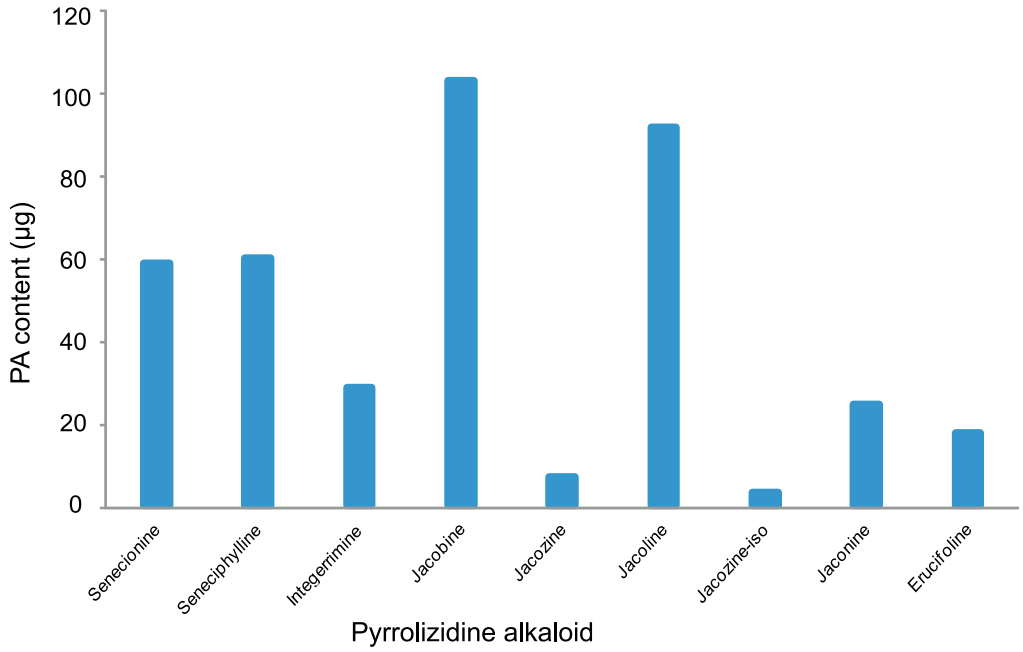

Figure 26: PA content in silage.

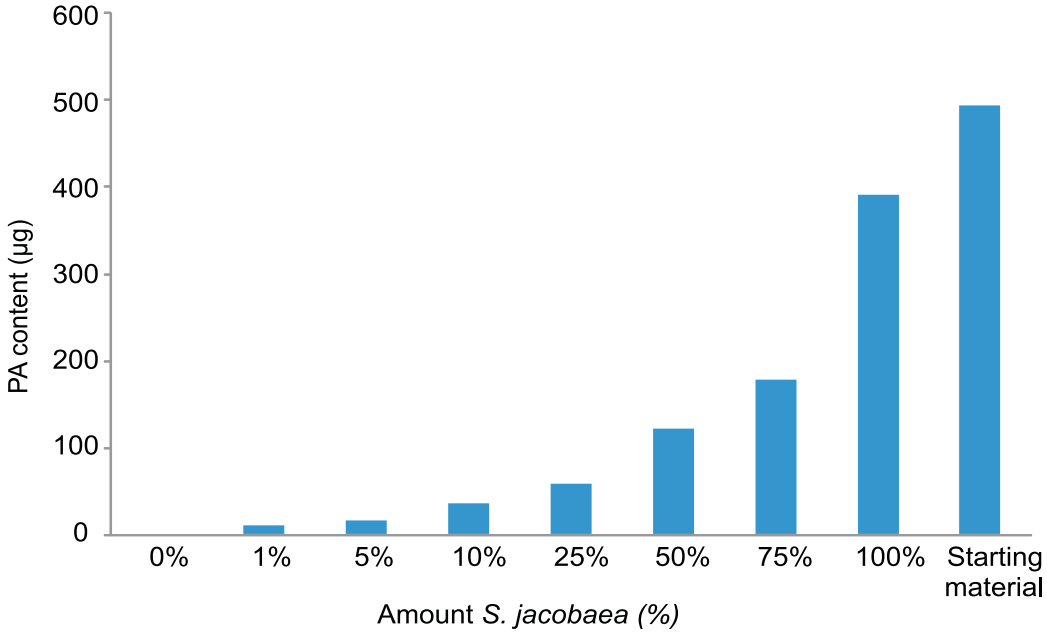

Figure 27: PA content in silage. Values presented are the arithmetic mean. n=3.

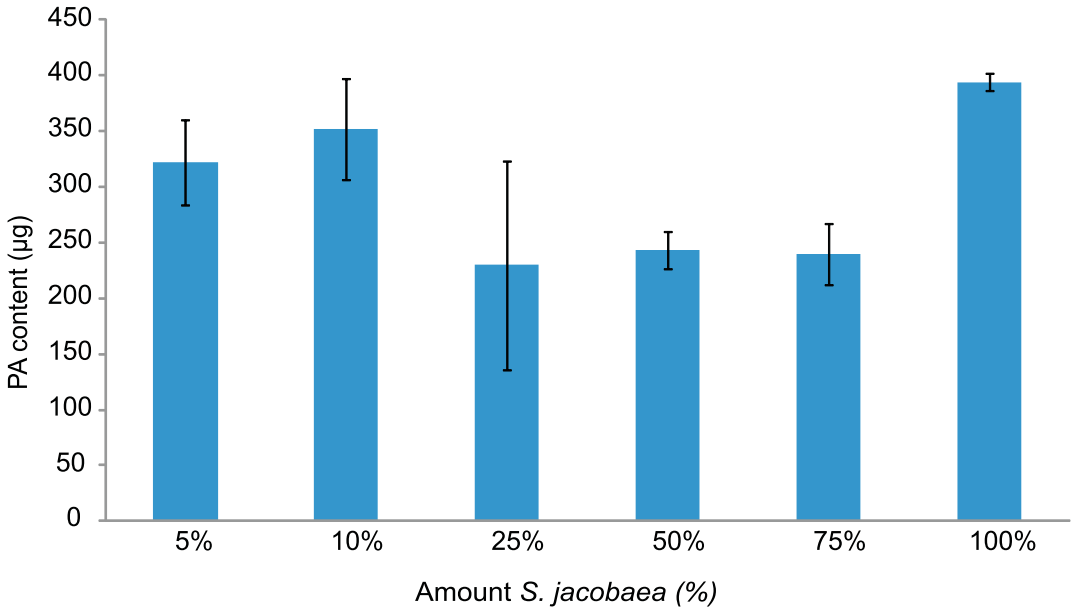

Figure 28: PA content in silage μg per gram *S. jacobaea*. Values presented are the arithmetic mean. n=3.

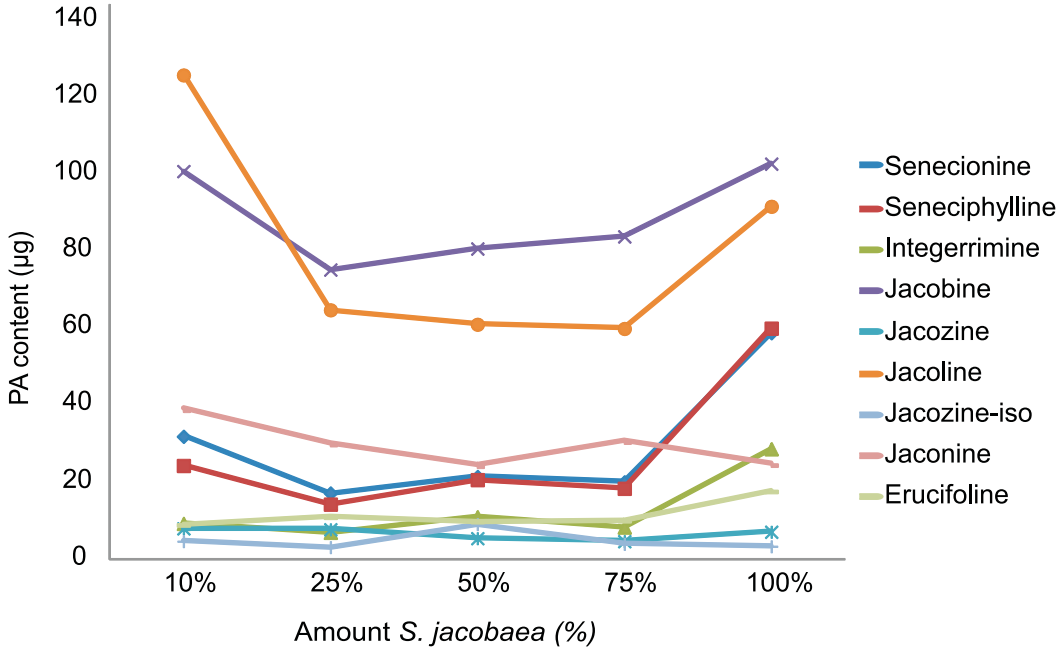

Figure 29: Single PA content in silage. Values presented are the arithmetic mean. n=3.

Individual PAs were quantified within the samples with different percentages of contamination as shown in Figure 29. This let us to analyze possible differences in degradation depending on the PA-structure.

Senecionine, seneciphylline, integerrimine as well as jacobine and jacoline showed a clear decrease while jaconine, jacozine, jacozine isomere and erucifoline remained less effected.

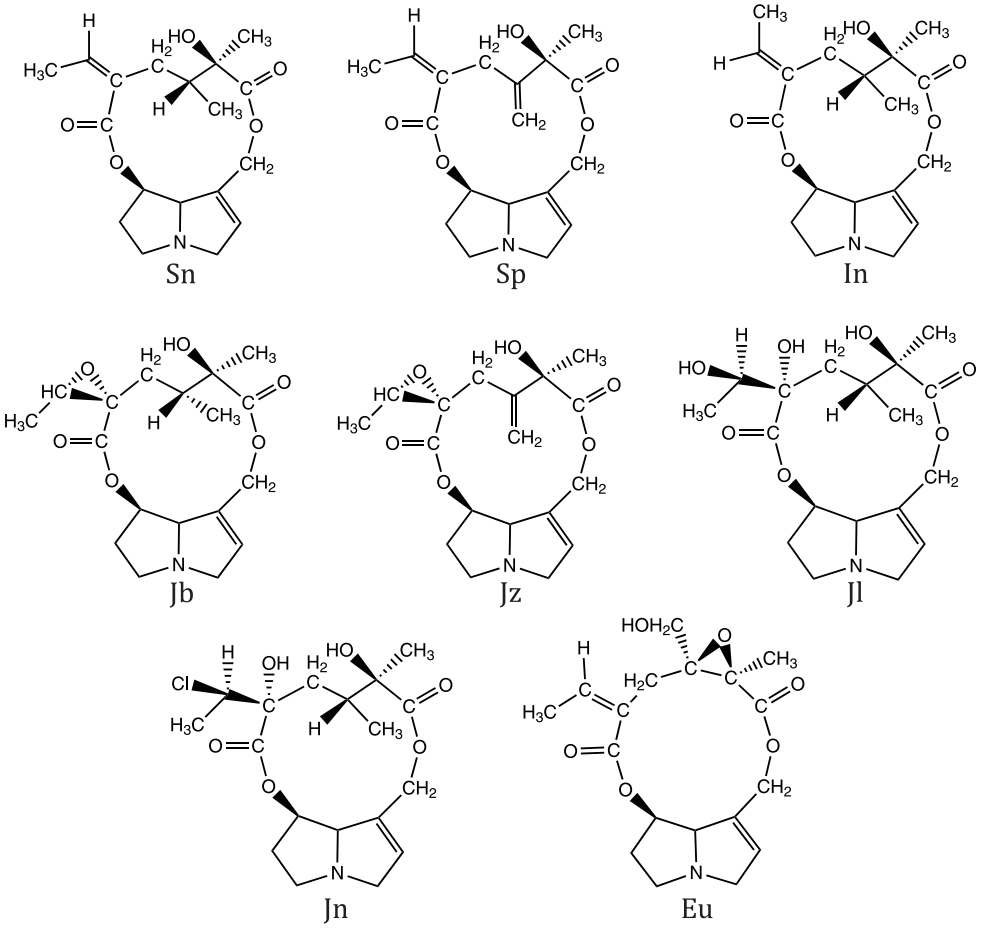

Figure 30: Structures of the pyrrolizidine alkaloids found in the silage samples.
Sn: senecionine, **Sp**: seneciphylline, **In**: integerrimine, **Jb**: jacobine, **Jz**: jacozine, **Jl**: jacoline, **Jn**: jaconine, **Eu**: erucifoline.

Hay, pellets and compost contamination with *Senecio aquaticus*

From our project partner the Bayerisches Landesamt für Landwirtschaft, nine samples were received consisting of four samples contaminated hay, three samples of pellets and two samples of compost. From the two samples of compost one included the structural material and in the other one the structural material was removed by sieving. All nine samples were independently homogenized, extracted and analyzed as described in sections 3.1.1 and 3.3.3.

All nine samples analyzed were positive for the three PAs senecionine, seneciphylline and integerrimine (Figure 31) which means that none of the three storage methods degrades the PA-content totally.

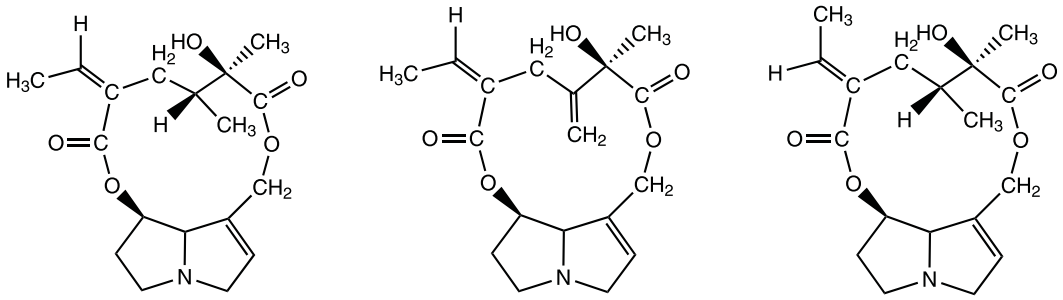

Figure 31: Structures of the PAs found in *Senecio aquaticus*

Heterogeneity of samples veils a detailed interpretation of the analyzed storage methods. Although a difference in PA-content between the hay and the compost can be seen (Table 3), a percentage estimation would result unrealistic. In general terms it can be said that pelleting produced a slight or no effect on the PA-content whereas composting exerts a more significant but not complete degradation.

Table 3: PA content in feed μg per gram of *Senecio aquaticus*

	PA μg/g			
	Senecionine	**Seneciphylline**	**Integerrimine**	**Total**
Hay 1	4,915	2,792	1,704	9,413
Hay 2	2,487	0,961	0,558	4,007
Hay 3	8,670	6,735	3,614	19,020
Hay 4	9,491	6,302	3,901	19,695
Pellets 1	0,792	0,449	0,285	1,528
Pellets 2	3,004	1,613	1,106	5,725
Pellets 3	3,555	2,011	1,485	7,051
Raw Compost	0,943	0,753	0,583	0,569
Fine Compost	0,079	0,078	0,031	0,188

4.3.3 Discussion

From the results presented in the section 4.3.2, it can be seen that after contamination of the starting material, namely forage, with PA-containing plants neither simple storage methods such as hay production or pelleting nor more complex methods involving bioactivity e.g. ensilaging or composting lead to a total degradation of the toxic PAs.

Previous works analyzing the effect of ensilaging have not reach a conclusive position. In a feeding experiment with silage made from *S. ridellii* Vardiman (1952) reported that the toxicity was greatly reduced or even lost. In a similar way Mulder *et al.* (2009) postulated the complete degradation or at least up to under measurable amounts of PAs in silage samples containing *Senecio vulgaris* or *Senecio inaequidens*.

On the other hand Donald y Shanks (1956) reported a massive outbreak of ragwort poisoning in cattle fed on silage containing *Senecio sp.* In that respect our work supports the idea that ensilaging does not eliminate the toxicity of pyrrolizidine alkaloids. Although there is a reduction in their total amount, this is not as drastic as the one reported by Candrian *et al.* (1984) who found a degradation of up to 95.5 % on silage consisting

only of *Senecio jacobaea*. In the same work, silage partially made of *S. jacobaea* had a degradation between 54.3 % and 90.9 %.

In the work here presented the PA-degradation was found to occur in silage containing 100 % and 75 % of *Senecio jacobaea*; the PA-content was reduced by 60 % when compared to the starting material. Silage samples containing 10 % to 50 % of *Senecio jacobaea* were statistically identical to those with 75 %. This means that there is a critical concentration of PAs after which no degradation process occurs.

It is important to highlight that as shown in Figure 29 different PAs are affected differently. The main degradation occurs in the alkaloids senecionine, seneciphylline, integerrimine, jacobine and jacoline, whereas in the case of jaconine, jacozine, jacozine-isomer and erucifoline the reduction can be considered zero.

During ensilaging, the pH value and the exclusion of air to generate an anaerobic environment are crucial factors for a proper enzymatic activity. A pH value of around 4 has been established to be a good marker of a stable ensilaging process (Thaysen, 2000). In the current study the quality of silage samples was measured using this marker, all the samples had a pH value of approximately 3.9, which assures that the enzymatic activity was taking part during the whole ensilaging process.

The observed degradation is the result of multifactorial conditions involving pH, temperature and enzymatic activity, all of them closely interconnected and dependent on each other. It has been proven that the degradation takes place at the ester functions, specially at position C-7. Mattocks (1986) showed that this ester function is the most reactive center in macrocyclic diester pyrrolizidine alkaloids.

It was shown by X-ray analysis (Wiedenfeld, 2008) that PAs which possess a

double-bond adjacent to the ester function at position C-7 form highly conjugated systems which facilitate the cleavage of the ester bonds. This might be the reason for the observed enzymatic decomposition of the PAs senecionine, integerrimine, seneciphylline and erucifoline, while the other PAs which do not posses such double-bond result in stronger ester bonds and in a higher stability towards decomposing attacks.

Regarding to the composting, the PA-degradation was found to be 88 %. The composting has characteristics completely different to ensilaging, starting with the oxygen conditions, which in composting takes places in an aerobic environment. Composting results in a significant loss of mass, Andersen *et al.* (2011) reports values of 55-73 % of material (including water) lost to the atmosphere. The carbon and nitrogen loss were calculated to be 63-77 % and 51-68 % respectively whereas the loss of organic matter was 66-99 %. The heavy metals as well as phosphorus and potassium were found mainly in the final compost, which means that the loss of these in the leachate is very low.

Another relevant factor in composting is the pH, which in contrast to ensilaging is more alkaline ranging from 6 to 9 under optimal conditions (Tchobanoglous *et al.*, 2003; Yamada y Kawase, 2006). In addition to this, water is constantly added during the composting process to keep a moisture content between 50 % and 70 % which have been proven to be the most suitable conditions to maintain a proper bacterial activity (Polpraset, 1996). The high content of water along with the other conditions of pH and biological activity facilitate the cleavage of the ester functions in the macrocyclic PAs, leading to their nontoxic structural components (necic acids and necine bases).

Despite the PA reduction during composting (88 %) or ensilaging (60 %), toxic pyrrolizidine alkaloids are still present in the final products, which means that once that the raw material is contaminated with any PA-containing plant, none of these methods

can be considered 100 percent safe for producing PA-free supplies for animal consumption, since chronic exposure to sub-lethal doses of PAs might lead to cause veno-occlusive disease or cancer (EFSA, 2011, 2007; Wiedenfeld y Edgar, 2011; Fu *et al.*, 2002a; IPCS, 1989; Mattocks, 1986).

Hay production and pelleting are greatly discouraged in case of suspicion of contamination with *Senecio spp.*, since these two methods, which mostly consist in drying the plant material, hardly alter the PA-content within the feed supplies.

It is important to keep in mind that, the PA-profile of a given plant varies with the place of collection and the environmental conditions. Macel *et al.* (2004) analyzed the variations in the pyrrolizidine alkaloid patterns of *S. jacobaea*. In this study different chemotypes could be identified using following 9 PAs as markers: senecivernine, senecionine, seneciphylline, integerrimine, jacobine, jacozine, jacoline, erucifoline and eruciflorine. The concentration as well as the PA-profile was shown to be related to the population of study, although hybrids were also found. Similar findings of environmental variation of the PA-profile and the effect of intra- and interspecific hybridization have been published by Joosten *et al.* (2011) and Kirk *et al.* (2010).

4.3.4 Contamination of salad with *Senecio vulgaris*

In the summer of 2009 a ready-to-eat package of rucola salad (also known as salad rocket) was found to be contaminated with another plant (Figure 32). After macroscopic and microscopic examination the alien plant was identified as *Senecio vulgaris* (Figure 33). The extraction and further analysis were conducted as described previously.

Figure 32: Contaminated pack of rucola salad

A B

Figure 33: Botanical identification of *Senecio vulgaris*. In A typical black bracts can be observed

Senecionine, seneciphylline and integerrimine , characteristic pyrrolizidine alkaloids of *Senecio vulgaris*, were found in the sample in a total amount of 2068 μg in 45 g of fresh plant material (Figure 34).

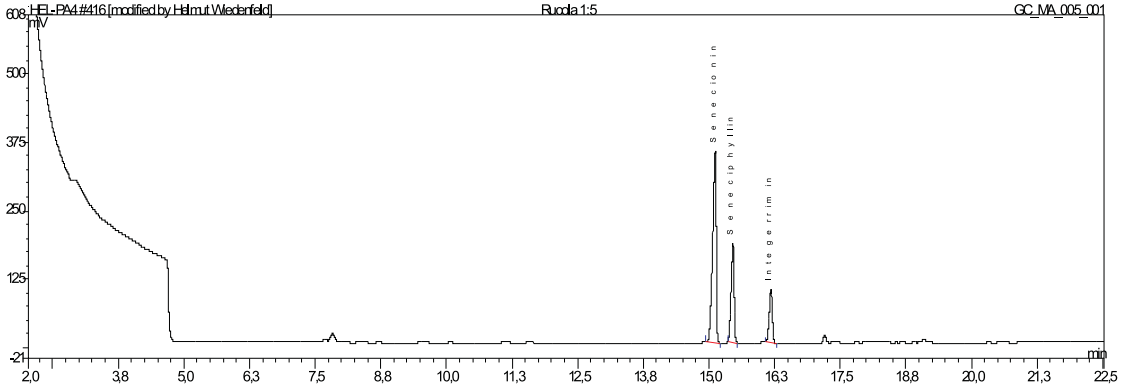

Figure 34: Gas chromatogram of sample of *S. vulgaris*

The amount of pyrrolizidine alkaloids found is not a lethal dose. There is little information about lethal doses of PAs in humans. In experiments conducted in rats it has been found that the LD_{50} of senecionine has been reported to be around 60 mg/kg b.w. Culvenor *et al.* (1969) reports 85 mg/kg b.w., Mattocks (1972) reports 50 mg/kg b.w., and more recently Wang *et al.* (2011) reports 57 mg/kg b.w.) whereas the LD_{50} of seneciphylline is 77-83 mg/kg b.w. (Bull *et al.*, 1968).

Despite the sub-lethal dose found in this incident of contamination, the case must not be overlooked, factors such as age, gender and health condition influence the final response to PAs. In this respect, children are more sensitive than adults and women more than men (Wiedenfeld, 2008). Recurrent exposure to low concentrations of pyrrolizidine alkaloids has been correlated to genotoxicity and mutagenesis (Fu *et al.*, 2002a, 2004).

This time the conditions of having the whole plant as well as the significant differences between the leaves of rucola and *S. vulgaris* (see Figure 35) allowed the easy identification of the contaminant.

A B

Figure 35: Comparison of the leaves of rucola salad and *S. vulgaris*. **A.** Rucola leaf **B.** Leaf of *S. vulgaris*

The Federal Institute of Risk Assessment of Germany (Bundesinstitut für Risikobewertung, BfR,) has made reference to cases of poisoning with *S. vulgaris* and other Senecio species in humans and animals, which resulted in hepatic injury and partly were fatal. The BfR, has advised that contamination of food with 1,2-unsaturated PAs from *S. vulgaris* should be as low as reasonable achievable (BfR, 2007; Dusemund *et al.*, 2011).

It is difficult to quantify how often and in which degree one is exposed to PAs along the food chain. The only well documented and therefore the best controlled source of exposure is honey (EFSA, 2011). However, as already discussed there are several poorly controlled sources. Even within the honey production, the enormous amount produced every year, add to the global market that involves common practices such as mixing of supplies with different origins and qualities; the control of these products is a titanic task almost impossible to be carried out.

In the best scenario the plausible risk of intoxication by pyrrolizidine alkaloids can only be prevented but not suppressed.

4.4 PA intoxications in human and animals. Case reports

4.4.1 Problem statement

Massive outbreaks of poisoning by pyrrolizidine alkaloids are uncommon, however isolated cases of acute intoxication are relatively frequent. In this work liver samples from animal or humans whose medical diagnostic (symptomatology and/or histology) led to suggest intoxication by PA-containing plants were analyzed.

4.4.2 Method

Liver samples were obtained from the physician or veterinarian and further handled as described in sections 3.1.1, 3.1.2, 3.3.1 and 3.3.3.

4.4.3 Case reports

Nine liver samples and two plant material samples were analyzed during this work. From the nine samples, four liver samples and one plant sample were positive to PA or to their metabolites. The results are presented in the following sections.

Case 1

In the autumn of 2009 in the surroundings of Hanover five newborn foals died within 3 days. A liver sample from the horse which died the last was analyzed. GC-MS analysis (Figure 36) revealed the presence of two putative PA-metabolites with retention time of 16.02. (Figure 37) and 20.36. (Figure 38). The parent PA could not be determined.

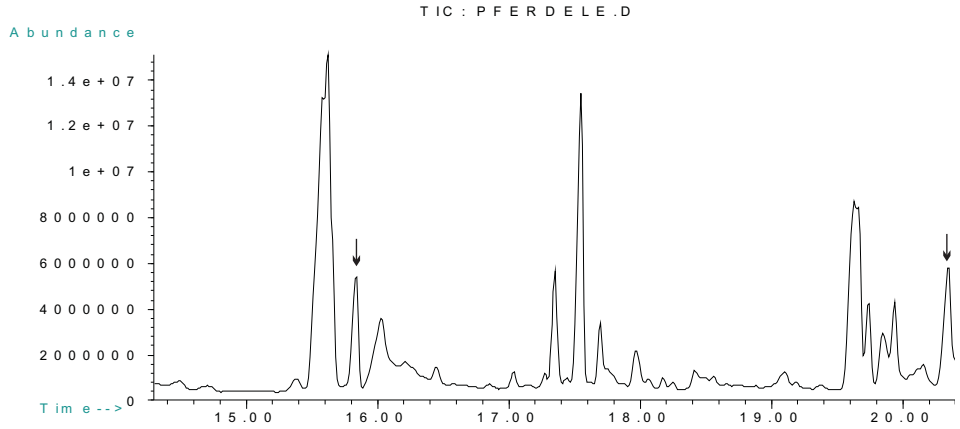

Figure 36: Gas chromatogram of the liver sample of the horse of case 1.

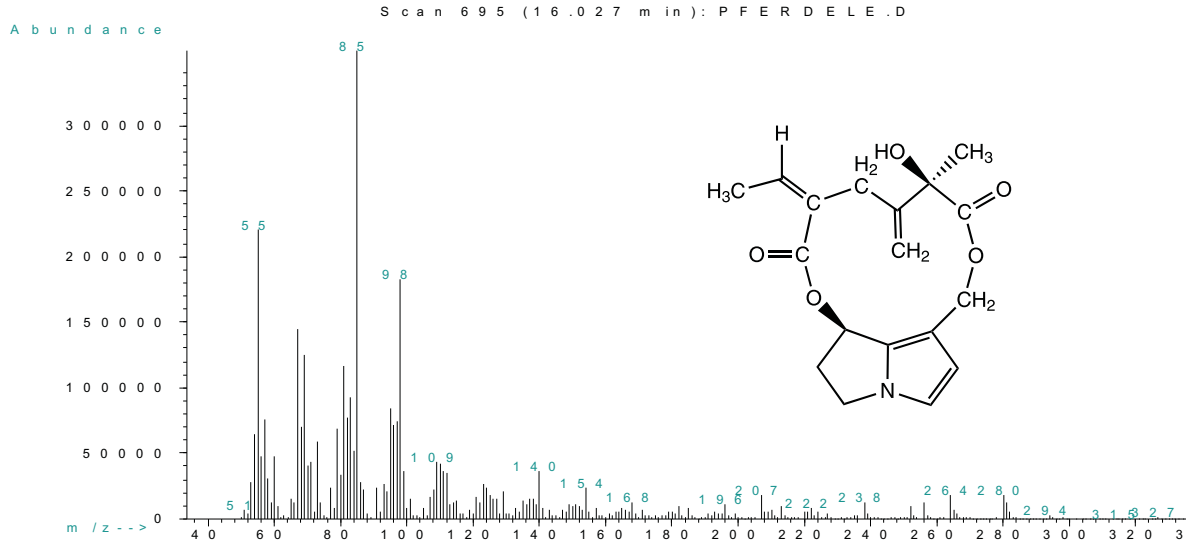

Figure 37: Mass spectrum of metabolite (dehydro-seneciphylline).

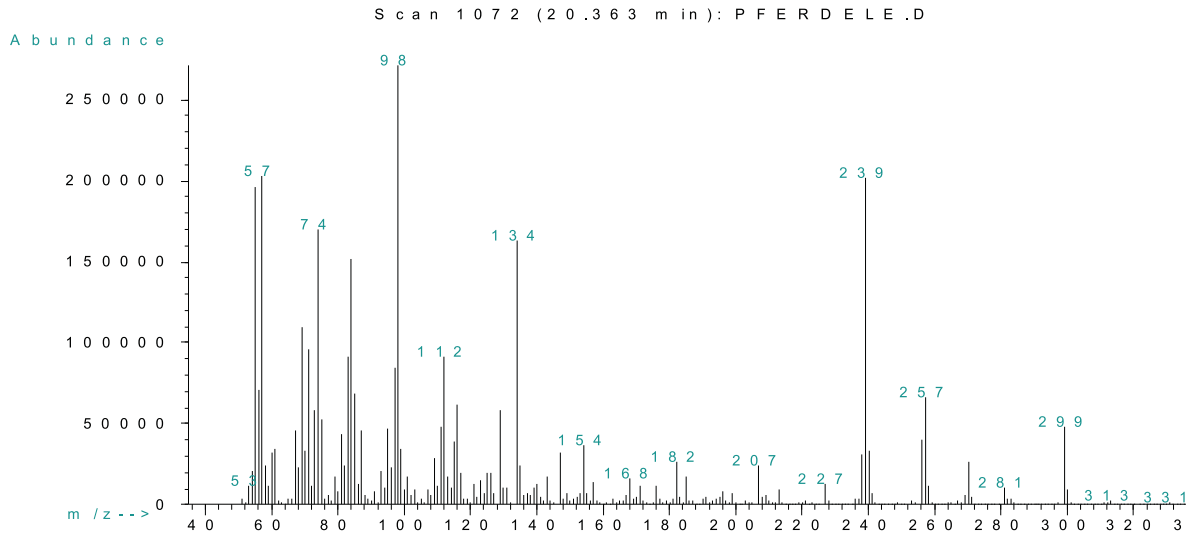

Figure 38: Mass spectrum of an unidentified PA metabolite.

Case 2

A four-weeks-old calf from Lower Saxony died after the intake of allegedly *S. jacobaea*. The mass spectrum (Figure 39) shows a peak at 15.58 with a molecular peak $[M]^+$ of m/z 341 (Figure 40), which along with the peaks at m/z 295, 281, 222 and 180 can be assigned to an acetyl-monoester PA.

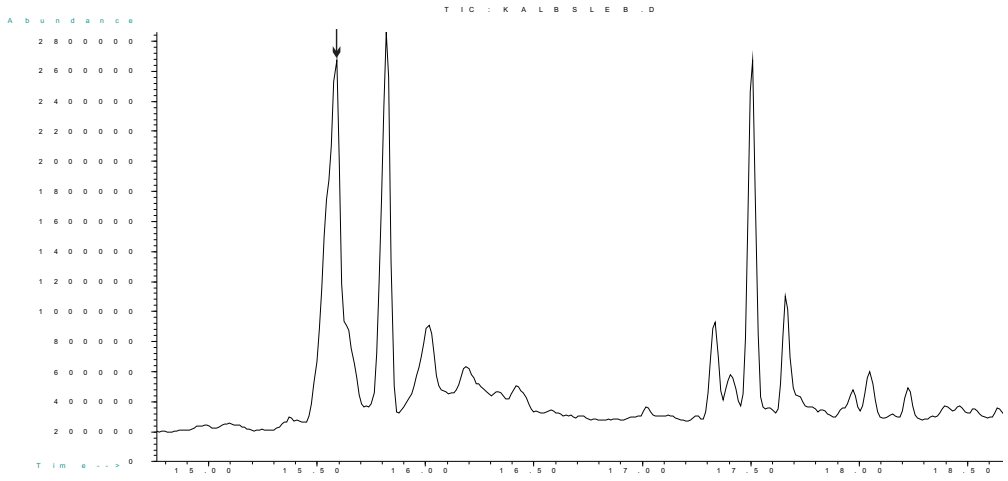

Figure 39: Gas chromatogram of the liver sample of the horse of case 2.

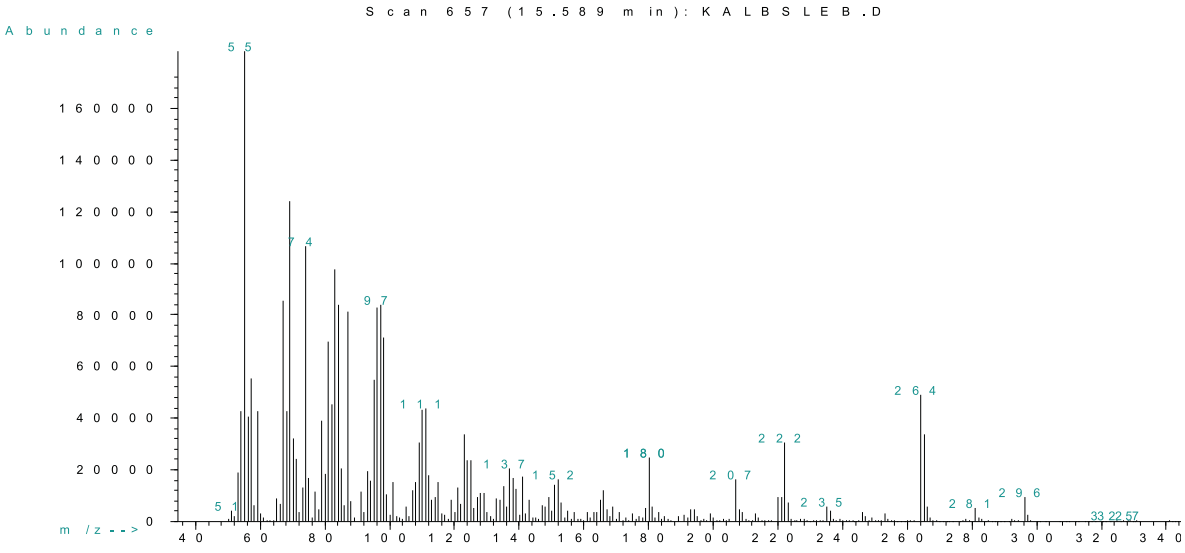

Figure 40: Mass spectrum of an acetyl-monoester PA.

Case 3

A camel from a German zoo died after showing the characteristic symptoms of PA-intoxication. One peak at 15.63 min. could be identified as acetyl-monoester PA according to the characteristic peaks of m/z 295, 281, 222 and 180 (Figures 41, 42).

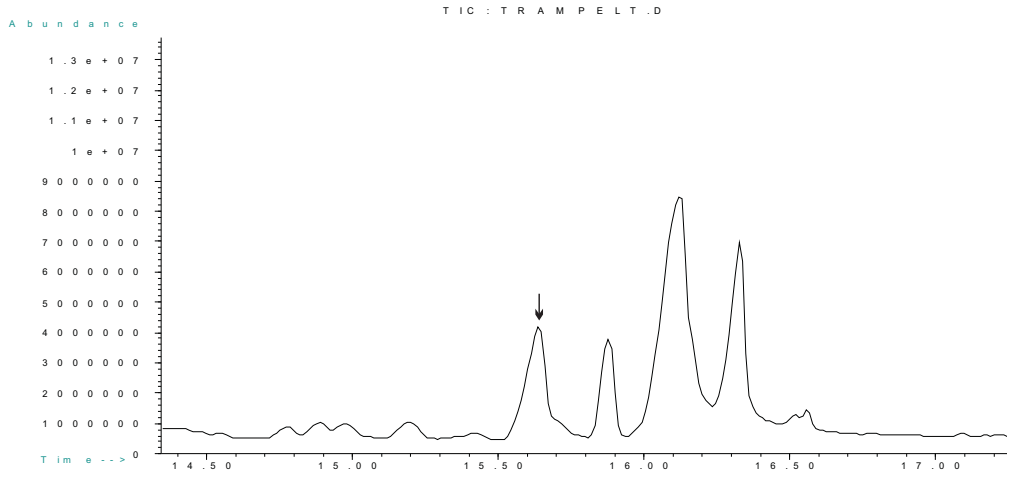

Figure 41: Gas chromatogram of the liver sample of the camel of case 3.

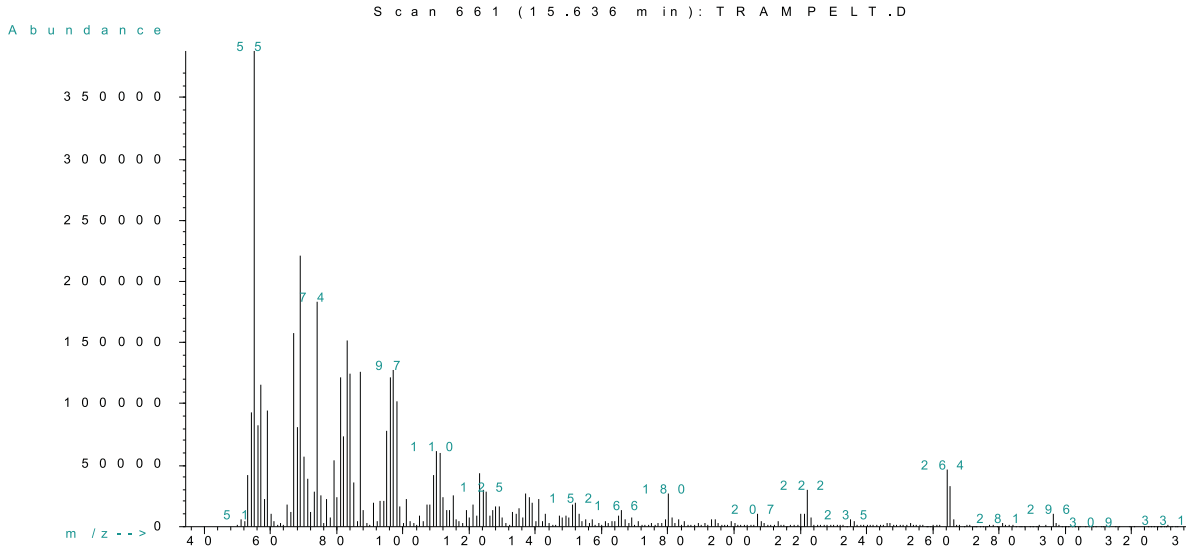

Figure 42: Mass spectrum of an acetyl-monoester PA.

Case 4

A sample of a two-months-old calf from Munich was analyzed to detect pyrrolizidine alkaloids. No free PAs could be detected although a putative PA metabolite could be identified at minute 15.60. Figures 43 and 44 show the gas chromatogram and mass spectrum of the sample respectively.

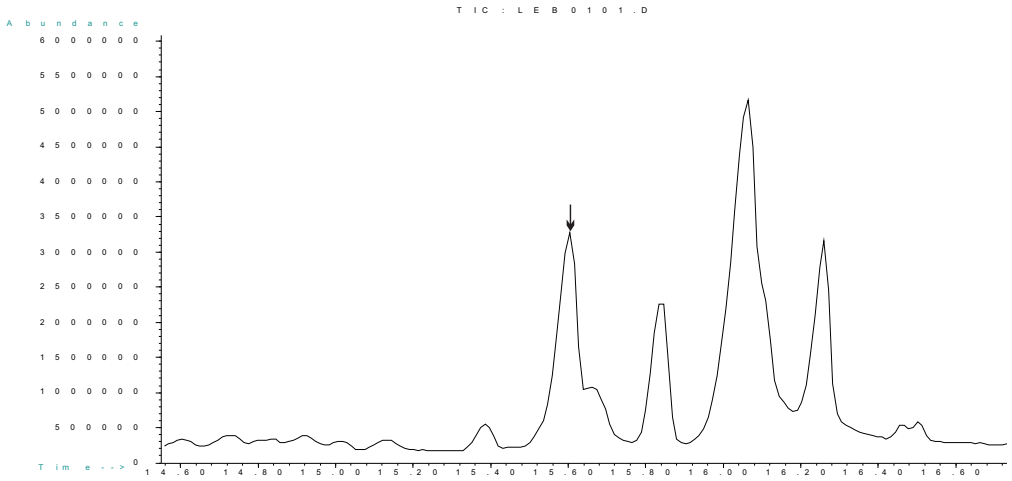

Figure 43: Gas chromatogram of the liver sample of the calf of case 4.

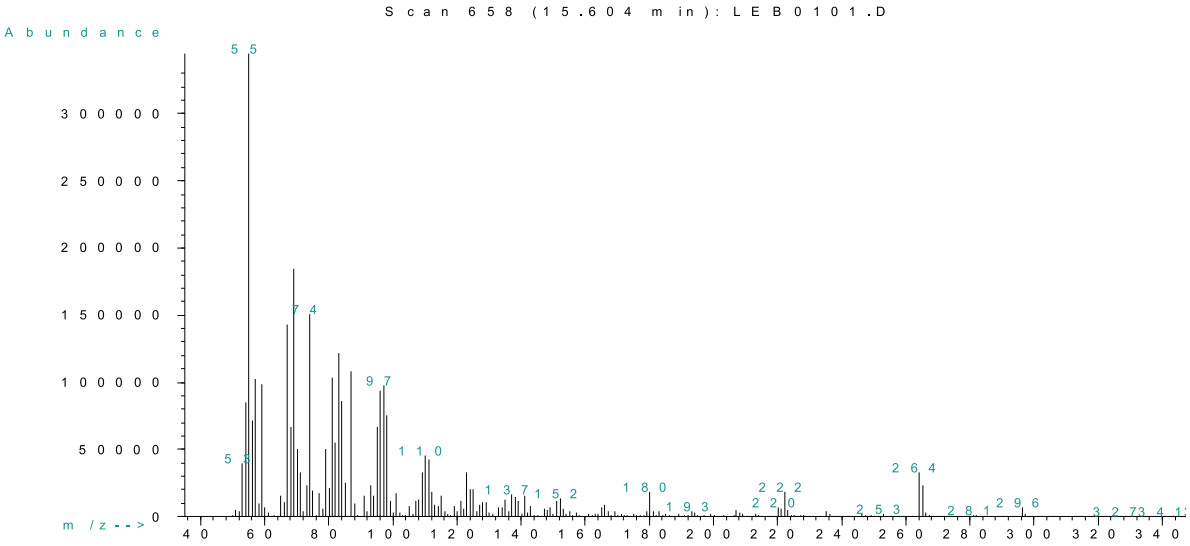

Figure 44: Mass spectrum of an acetyl-monoester PA.

The interpretation of the PAs assigned as acetyl-monoester-PAs was done based on the characteristic fragments of m/z 295, 281, 222 and 180. The Figure 45 shows a hypothetical structure for the interpretation of these mass spectra.

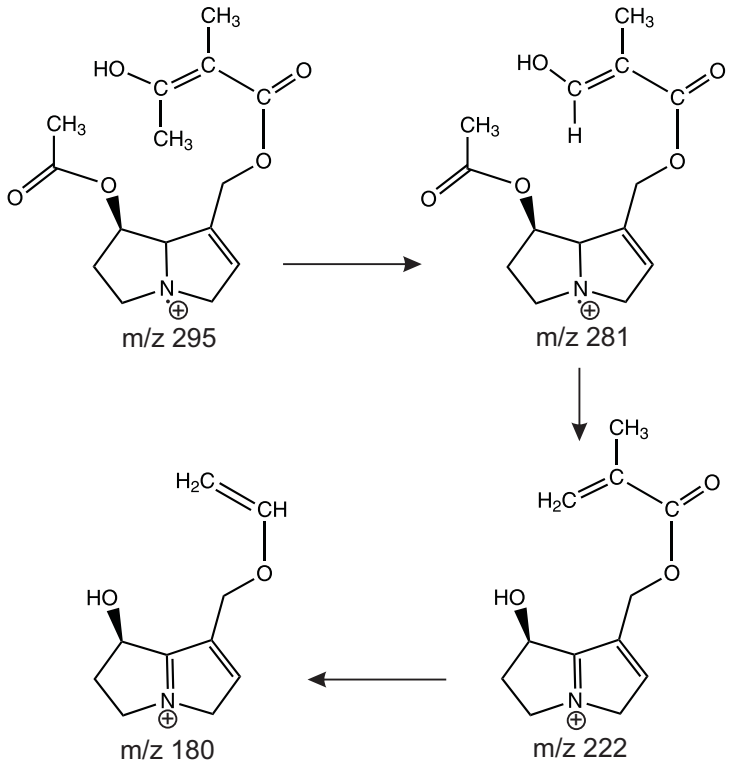

Figure 45: Interpretation of the fragmentation of a acetyl-monoester-PA.

Case 5

A female patient was admitted to the hospital "Klinikum rechts der Isar" in Munich after consumption of *Petasites albus* (Asteraceae). Clinical features of severe liver damage were observed in the patient. The plant material was analyzed in order to quantify the PA intake as well as its identity.

Two PAs could be identified at retention times 18.86 and 19.17 with molecular peaks $[M]^+$ of 335 and 333 respectively. After comparison with references the peaks identity was established as senecionine (Rt 18.86) and the main alkaloid as seneciphylline (Rt 19.17). The interpretation of the fragmentation pattern of the main peaks of both chromatograms is presented in Figure 14 (on page 41) for senecionine and in Figure 15 (on page 42) for seneciphylline.

After identification of the PAs a quantitative analysis was carried out. The intake was calculated for 10 leaves of *P. albus* with a dry weight of 26.10 g. and the PA-content was estimated as 817.50 mg (78.44 mg senecionine and 739.06 mg seneciphylline).

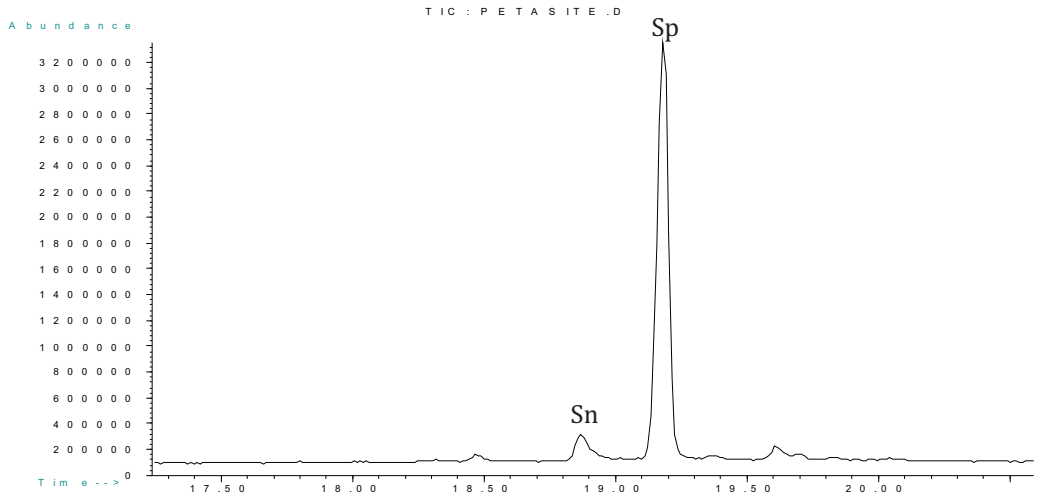

Figure 46: Gas chromatogram of the sample of *Petasites albus* of case 5.

As observed from the results presented in this section, PA-intoxication are recurrent in the veterinarian practice. Prakash *et al.* (1999) refers the PA-poisoning to be the most common plant associated poisoning disease in livestock worldwide. Despite their occurrence PA-poisoning events are often overlooked especially in cases of chronic intoxication.

As part of this research the Federal Ministry of Food, Agriculture and Consumer Protection of Germany (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV)) was contacted to require information about national statistics related with the occurrence of poisoning by pyrrolizidine alkaloids. Unfortunately there are no data available of possible or confirmed cases of PA-intoxication (also known as seneciosis) in animals.

If we consider the data given by Berendonk (2009), in North Rhein-Westphalia Senecio species could reach up to 10 % of coverage in meadows and grazing lands. The real risk of this fact can be passed over due to the fact that in summer free grazing animals avoid Senecio species because of their bitter taste, but in winter animals are fed with processed feed supplies (i.e. hay, silage, pellets) where the animals can not distinguish the taste and therefore consume the products even if they are contaminated.

The Dutch survey on pyrrolizidine alkaloids in animal forage conducted by Mulder *et al.* (2009) points out the high risk and common occurrence of PA-contamination in forage. Since the main Senecio species in Germany are the same as in The Netherlands (*S. jacobaea*, *S. vulgaris*, *S. inaequidens* and *S. aquaticus*) and their abundance is similar (see Figures 8, 9 and 10) comparable data can be expected for German animal forage.

Another fact that has to be taken into consideration in future programs is the imminent globalization of the forage market. Germany imports and exports fodders from and to all the world, that means that international legislation and standards should be set in order to guaranty the safeness of those products.

5 Pyrrolizidine alkaloids in *A. chiapensis* and *A. maritimum*

Asteraceae is the largest plant family with approximatly 24,000 species (Judd *et al.*, 1999). In this family the occurrence of PAs is well documented in species from the genus *Senecio*. In Europe several specimens of this genus were used in the past for therapeutic purposes, just to mention some; *S. jacobaea* was used until early nineteenth hundred and the use of *S. fuchsi* was discouraged as recent as 1990 (BGA, 1990). Some other related plantas such as *S. cineraria* (*Cineraria maritima*) still appear in the homeopathic pharmacopoeias (Weiss y Fintelmann, 2002).

PAs in Asteraceae are not constricted to Senecio or to its tribe Senecioneae, they are also characteristic metabolites in the tribe Eupatorieae. The medicinal use of species belonging to this tribe is widespread and their use has been recorded in Europe, Asia and America. It is impressive that *Eupatorium cannabinum*, the only specimen of this group naturally occurring in Europe, has reports of use as medicinal plant. Roeder and Wiedenfeld reported the use of at least 12 different PA-containing medicinal plants from the tribe Eupatorieae in Asia (Roeder, 2000; Roeder y Wiedenfeld, 2009, 2011, 2013).

In Mexico, like in many other developing countries, the persistent lack of proper medical services has not only permitted but promoted the survival of the traditional medicine. The history of use of medicinal plants in Mexico can be tracked as back as its first inhabitants.

The knowledge of the Mexican medicine is a enormous challenge, which was first taken up during the Colony in the 18^{th} and 19^{th}centuries. The most recent systematic review of Mexican medicinal plants reports 3103 species in 1000 genera and 183 families (Argueta Villamar *et al.*, 1994). The heterogeneity of cultures together with the rich biodiversity resulted in a vast folk medicine.

However, in this abundant selection of plants it is common to find potential hazardous plants like PA-containing plants. In the work of Argueta Villamar *et al.* (1994) appear 8 species of Eupatorieae, some of them like *Ageratum conyzoides* and *Chromolaena odorata* are well known to contain pyrrolizidine alkaloids.

Plants belonging to the tribe Eupatorieae are expected to contain PAs, nevertheless the hazardous potential can be only estimated after knowing quantitatively and qualitatively their PA content. The risk of PA-consumption is treated within this work in sections 1.4.1 and 1.4.2.

The present work estimates the hazardous potential of two Mexican medicinal plants belonging to the tribe Eupatorieae, *Ageratina chiapensis* and *Ageratum maritimum*, in relation to their PA-content.

5.1 Botanical description

5.1.1 *Ageratina chiapensis* (B. L. Rob.) King & H. Rob.

Sparingly branched or treelike shrub up to 3-4 m high, often with several stems in a clump; inflorescence with few hairs, gummy, with dark stipitate glands; foliage somewhat resinous-dotted; leaves slightly rough above, velvety to the touch beneath when young, ovate or ovate-cordate, long-petiolate, 11-18 cm long, 8-12 cm wide, acute to broadly obtuse at apex, rounded to broadly truncate-subcordate at base, pinnately veined, or the third or fourth pair of veins above the base large and strongly ascending and the blade subtriplinerved; margins prominently but rather finely serrate-dentate except at base; inflorescence a loose, rounded, or somewhat elongate panicle 15-30 cm long and wide; involucre 8-9.5 mm long, fleshy at base, campanulate, about 2-seriate, the phyllarie 15-20, nearly equal, linear-attenuate, up to 9 mm long and 1 mm wide, glandular to the tips; flowers white or nearly so, contrasting with the purplish involucre; corolla glabrous; anthers 1.5-2 mm long, obtuse at base; style-branches filiform, exserted 4-5 mm; achenes 3.5-4 mm long, slenderly columnar-clavate, substipitate, about 5-angled, strigose on the sides and angles especially above the middle; pappus white; receptacle glabrous.

Geographical distribution

In Mexico *Ageratina chiapensis* has a distribution in the states of Jalisco, Guerrero, Michoacan, Oaxaca, Chiapas and Veracruz. Outside Mexico it has only been recorded in Guatemala (Figure 47). The typical habitat of this plant is in ravines and steep wooded slopes, in pine and fir forests, 1900-2600 m on the Pacific slope.

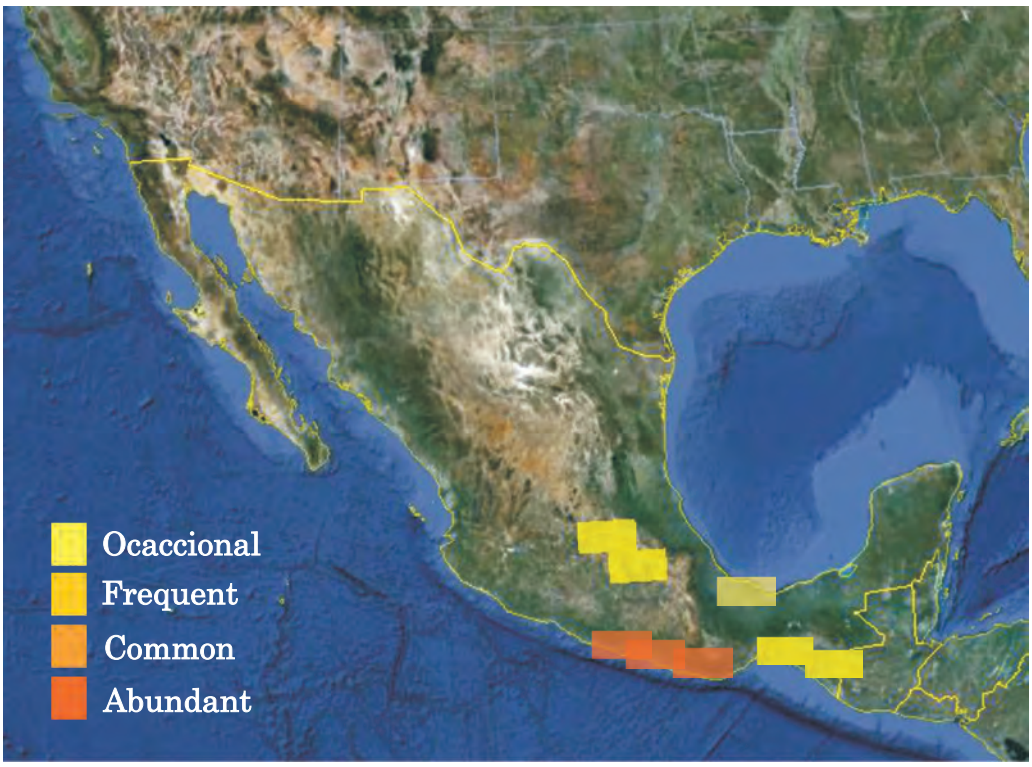

Figure 47: Distribution and abundance of *A. chiapensis* in Mexico. Modified from a map generated by GBIF (2013); one degree cells plotted on Google Earth

Ethnobotany

A. chiapensis is known in Mexico by the names of hoja de San Nicolás (St. Nicolas' leaf) and hoja santa (holy leaf). It is traditionally used in the treatment of diabetes in form of an infusion and for stomach problems such as indigestion in form of a poultice (Martínez-Alfaro *et al.*, 2001).

5.1.2 *Ageratum maritimum* Kunth

Annual or perennial shrub of 10-50 cm (semisucculent, rhizomatous, forming colonies). Stems decumbent to straggling or creeping (rooting at nodes). Leaf blades deltate-ovate to oblong, mostly 0.8-4 x 0.5-3 cm (fleshy), margins toothed, faces glabrous or glabrate. Peduncles glabrous or glabrate. Involucres approximately 3 x 3-4 mm. Phyllaries elliptic-lanceolate, glabrous or glabrate, tips abruptly tapered to nearly obtuse. Corollas lavender or blue to white. Cypselae glabrous; pappi usually blunt coronas approximately 0.1 mm.

Geographical distribution

In USA it grows in the state of Florida. In Mexico along the costal line of Quintana Roo. Other locations include Belize, Cuba and Hispaniola (Dominican Republic and Haiti).

Its classic habitat is beach sand and nearby thickets, coral soils, salt marshes, hammocks and roadsides. Altitude range 0-10 m. Phenology: all the year round.

Ethnobotany

A. maritimum is called Hawayche' in the Mayan region of Mexico. It has been reported to be used against herpes, mainly herpes labialis (Cabrera *et al.*, 1982; Martínez, 1991).

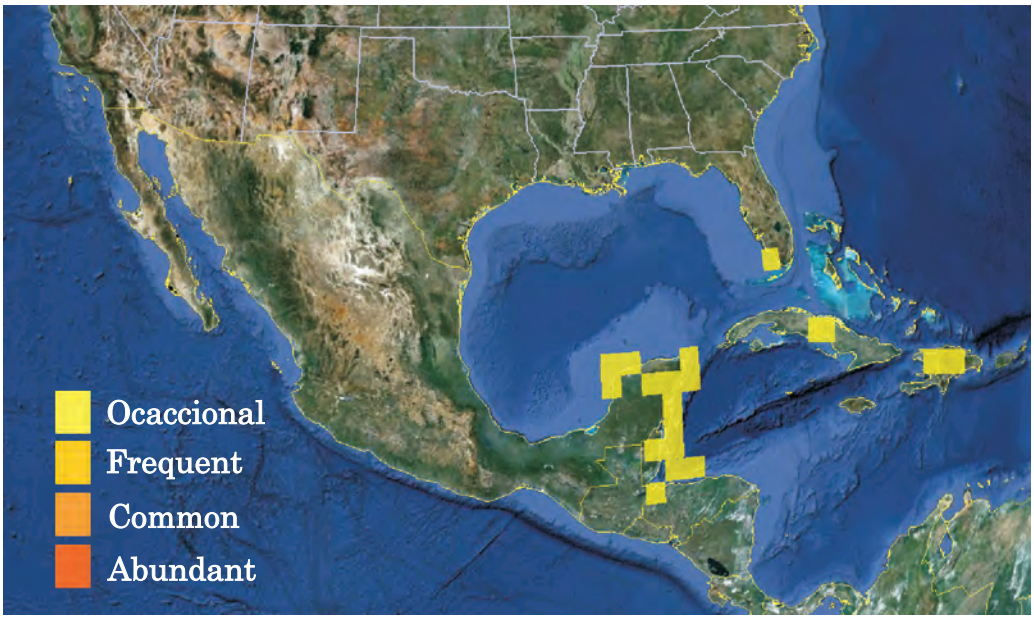

Figure 48: Distribution and abundance of *A. maritimum*. Modified from map a generated by GBIF (2013); one degree cells plotted on Google Earth

5.2 Pyrrolizidine alkaloids of *Ageratina chiapensis* and *Ageratum maritimum*

5.2.1 Method

Plant material of *A. maritimum* was collected at 19°50'15.05" N / 87°26'59.46" W, 6 km North from Punta Allen. Quintana Roo, Mexico. *Ageratina chiapensis* was collected in the state of Oaxaca, Mexico near the locality of Santos Reyes Nopala at 16° 6'37.33" N / 97° 7'0.54" W. In both cases, fresh material was set to dry in a desiccating room at 40°C and later extracted and analyzed as described in sections 3.1.1 and 3.3.3.

5.2.2 Phytochemical results and discussion

Ageratina chiapensis

The first characterization of the crude extract of *A. chiapensis* was done by TLC according to the procedure listed in section 3.3.1. Five spots corresponding to putative pyrrolizidine alkalois could be detected (Figure 49). In order to isolate the single PAs, the crude extract was applied to a column type-A and eluted as described in section 3.2.1. PAs were further isolated by preparative TLC (see section 3.2.2).

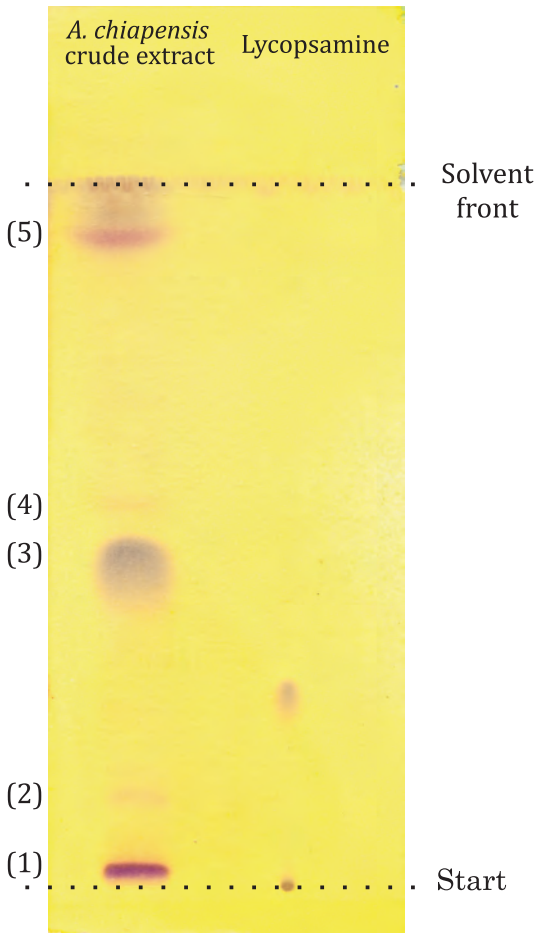

Figure 49: TLC of the crude extract of *A. chiapensis*.

After GC-MS analysis of the fractions three pyrrolizidine alkaloids could be identified by the characteristic peaks m/z 138, 120, 93, 80. These peaks correspond to the necine base of an unsaturated pyrrolizidine alkaloid (Figure 50).

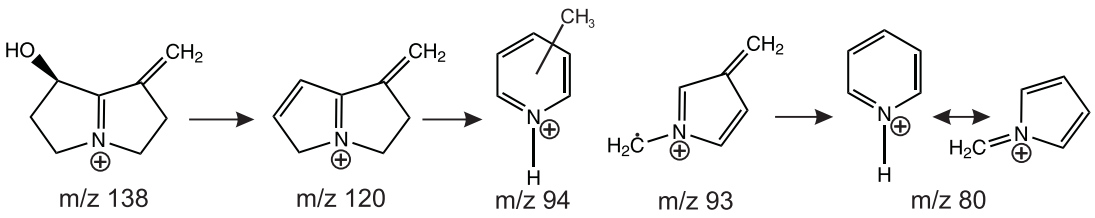

Figure 50: Characteristic fragments of the necine base of an unsaturated PA.

From the three PAs isolated (referred to in this work as PA-1, PA-2 and PA-3), PA-1 had a molecular peak $[M]^+$ of m/z 255, which can be assigned to the formula $C_{13}H_{21}NO_4$ of the 3-Hydroxy-2methyl-butyric acid-retronecinester (Figure 51). The mass spectrum and its interpretation are given in Figures 52 and 53 respectively.

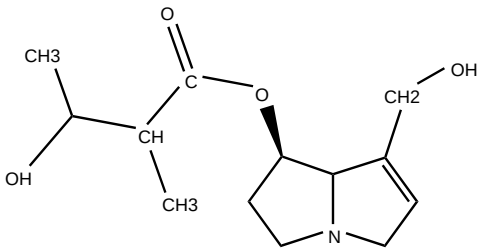

Figure 51: Structure of the isolated pyrrolizidine alkaloid PA-1

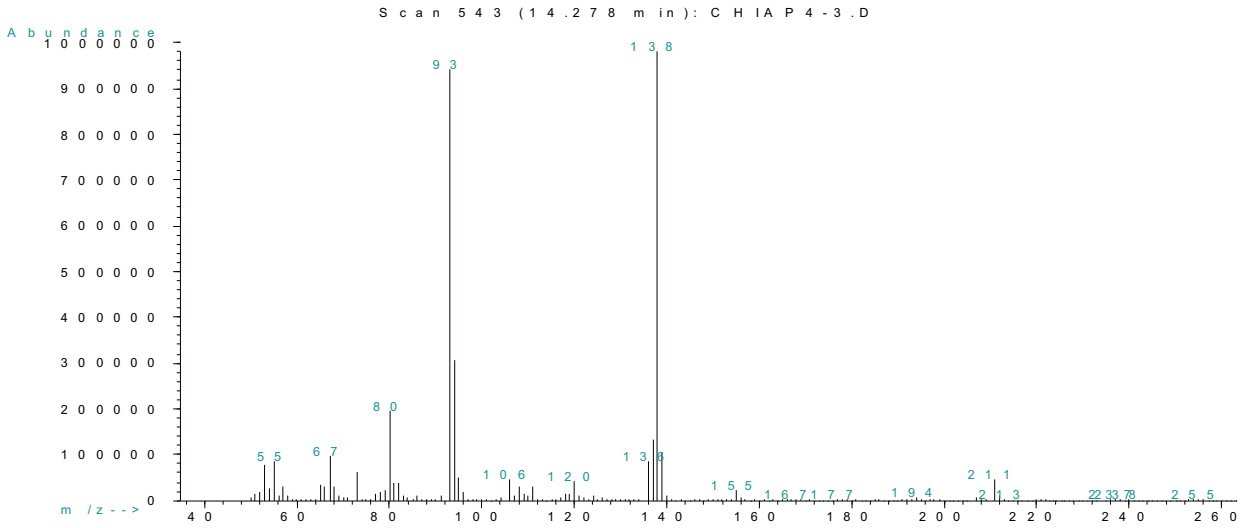

Figure 52: Mass spectrum of PA-1

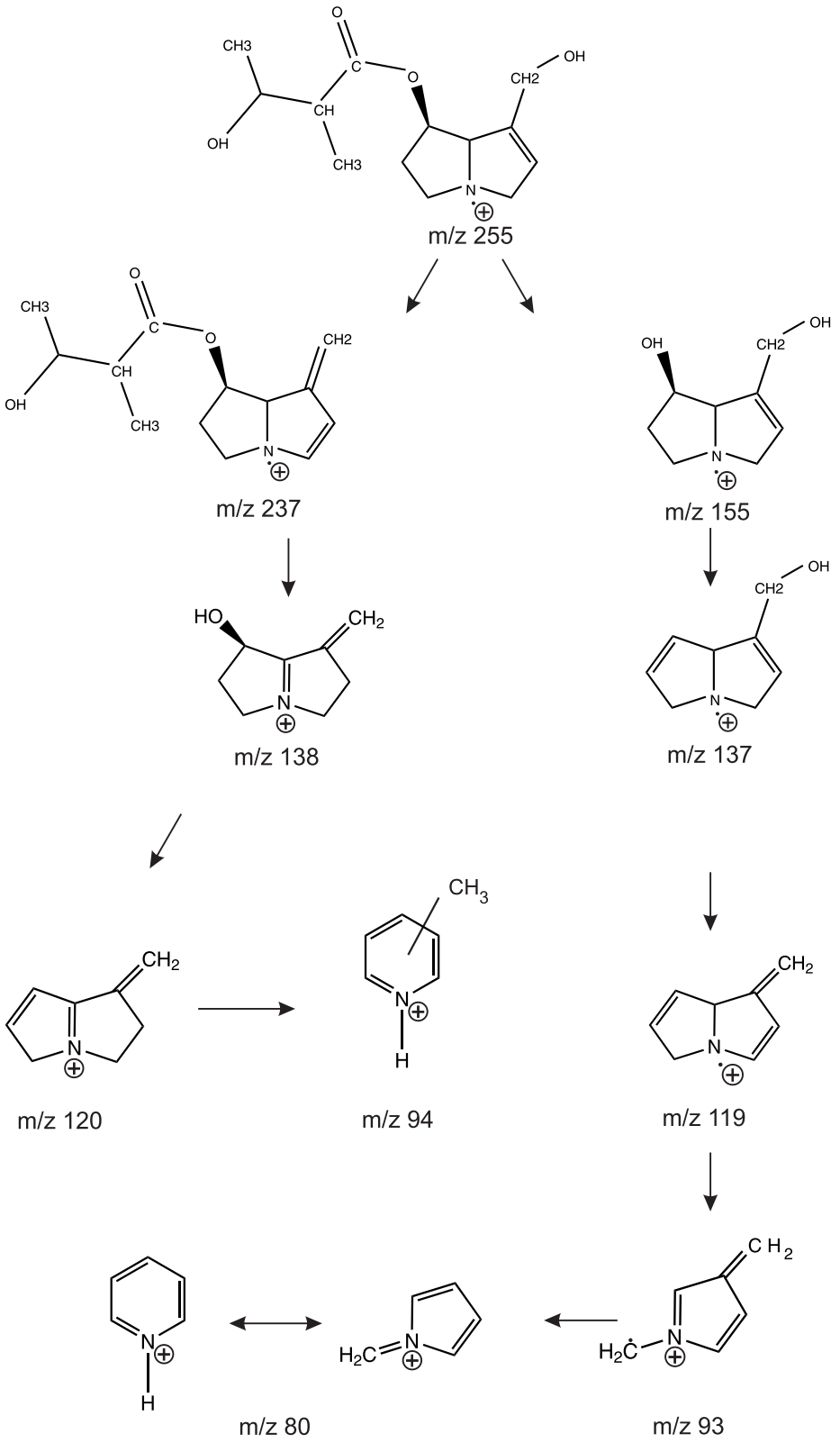

Figure 53: Interpretation of the fragmentation of PA-1

The other two PAs isolated had a molecular peak $[M]^+$ of m/z 341 and after comparison with references were identified as acetyl-lycopsamine (PA-2) and acetyl-intermedine (PA-3) (Figure 54). The mass spectrum and the interpretation of the cleavage pattern of acetyl-lycopsamine is given in Figures 55 and 56.

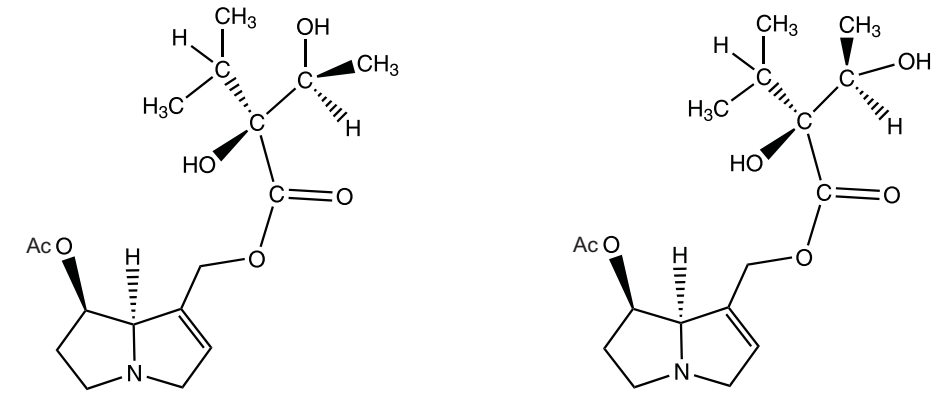

Figure 54: Structures of the isolated pyrrolizidine alkaloids PA-2 and PA-3

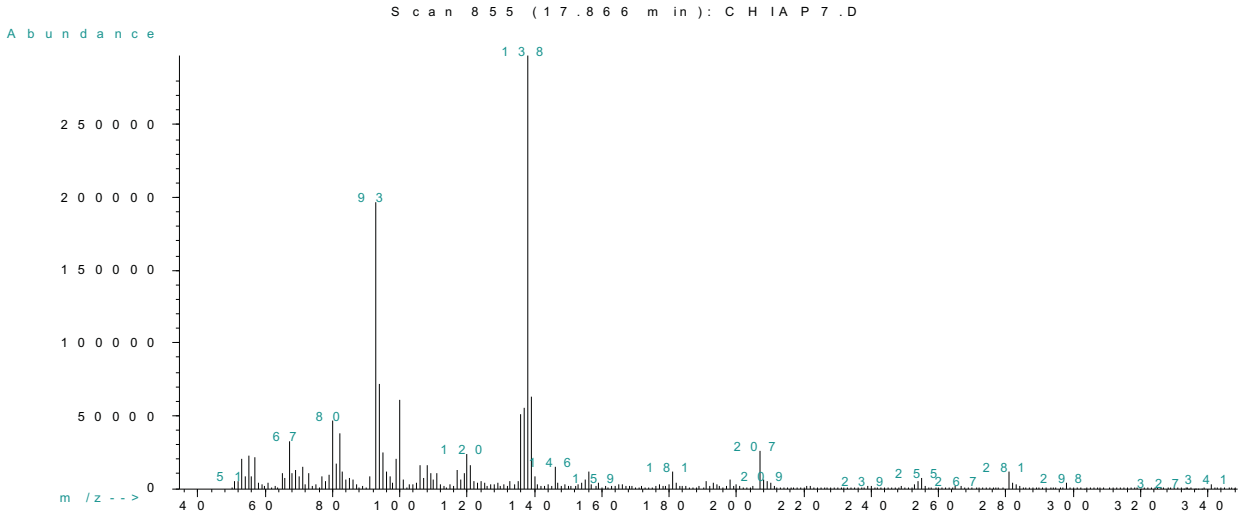

Figure 55: Mass spectrum of acetyl-lycopsamine

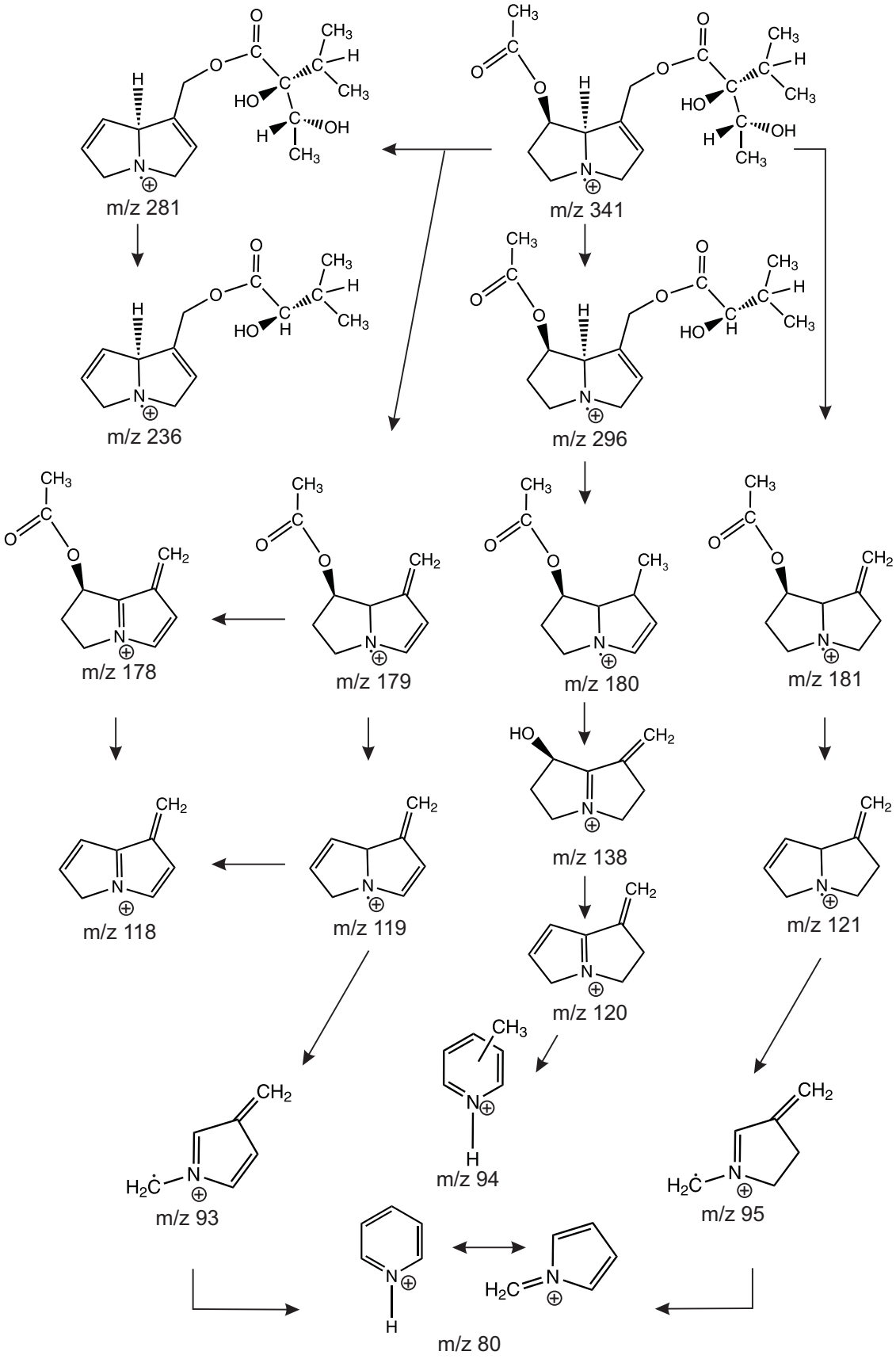

Figure 56: Interpretation of the fragmentation of acetyl-lycopsamine

Ageratum maritimum

From the crude extract of *Ageratum maritimum* the TLC analysis revealed the presence of five main pyrrolizidine alkaloids: two saturated and three unsaturated PAs (see section 1.1.

The pyrrolizidine alkaloids present in the crude extract could be identified with help of the GC and GC-MS analysis of the fractions. The saturated PAs found in *A. maritimum* had molecular peaks of $[M]^+$ m/z 237 and 281 respectively.

In a similar way, the saturated PAs had molecular peaks of $[M]^+$ m/z 299 and 241 respectively, these pyrrolizidine alkaloids where identified as lycopsamine, intermidine and the acetylated form, 7-acetyl-intermedine (Figures 57, 58, 59 and Tables 4, 5).

The alkaloids lycopsamine and intermedine differ in the configuration at C-11 and C-12. In the case of lycopsamine it shows the 11*S*, 12*S* configuration whereas intermedine shows a 11*S*, 12*R* configuration. This difference can be recognized by ^{1}H-shifts at C-12. For lycopsamine C-12: H is $\sim$3.9 ppm and for intemedine $\sim$4.1 ppm (see Table 4 and Figures 57 and 58) (Wiedenfeld y Röder, 1991). Another empirical data given by Wiedenfeld and Röder (1991) to determine the configuration of C-11 and C-12 are the values of the methyl groups C-15 and C-16. These groups show nearly identical ^{1}H-NMR data but different ^{13}C-shift values. The configuration *S,S* has a $\Delta\delta$ higher than 1.5 ppm whereas the configuration *S,R* has a $\Delta\delta$ value of approximately 0.2 ppm

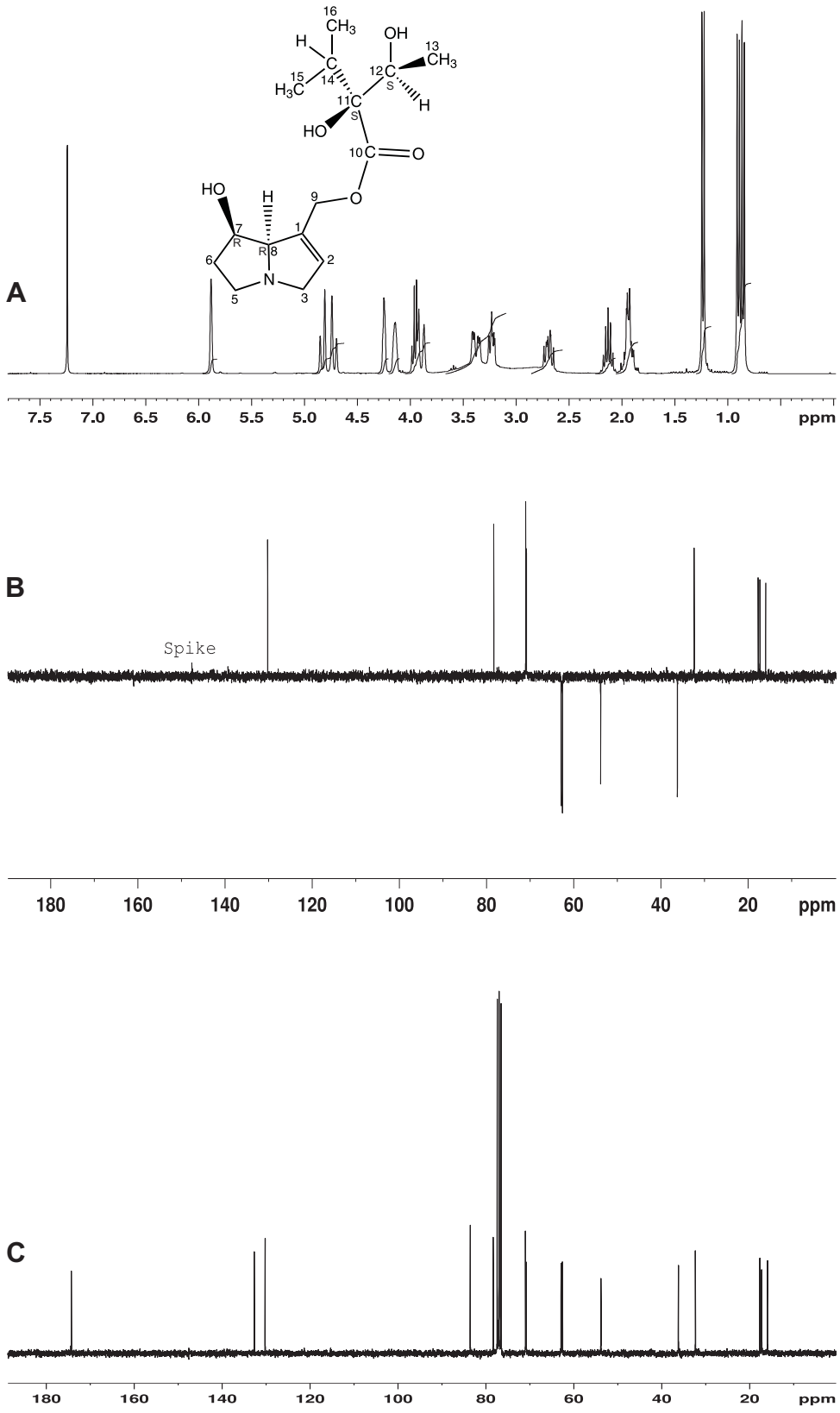

Figure 57: NMR-Spectra of lycopsamine. A) ^{1}H-NMR spectrum, B) DEPT 135 spectrum, C) ^{13}C-NMR spectrum.

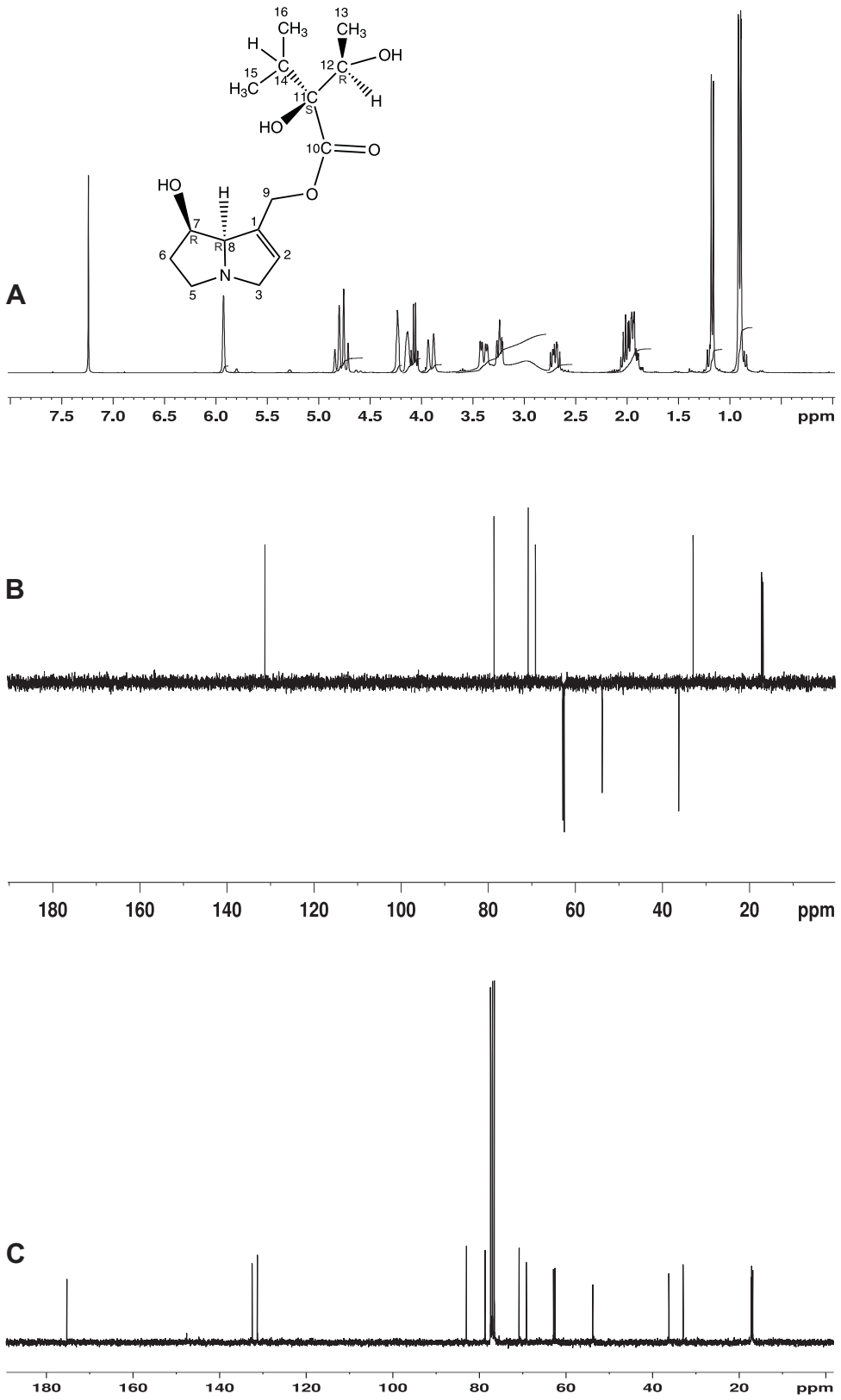

Figure 58: NMR-Spectra of intermedine. A) ^{1}H-NMR spectrum, B) DEPT 135 spectrum, C) ^{13}C-NMR spectrum.

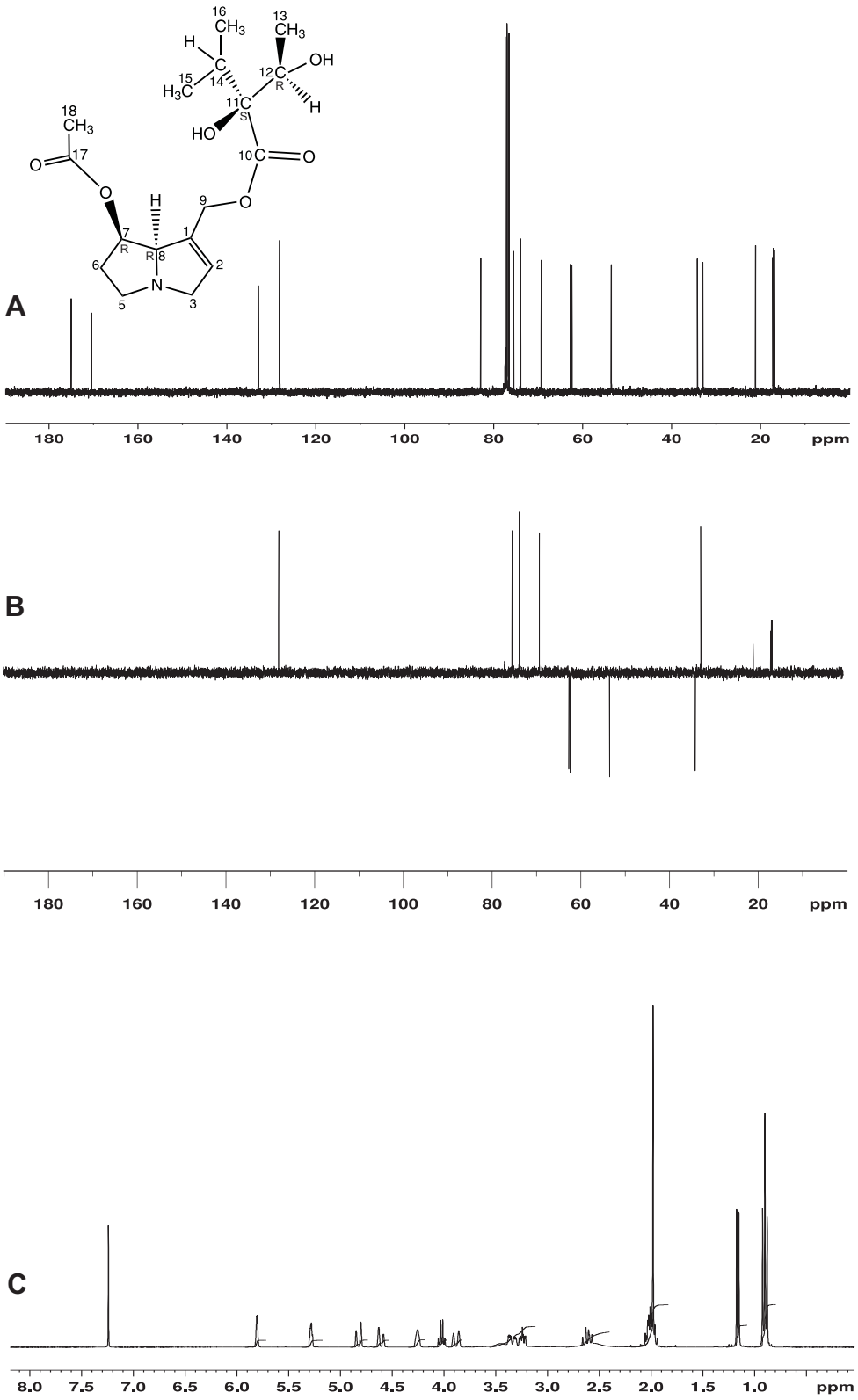

Figure 59: NMR-Spactra of Acetyl-intermedine. A) ^{1}H-NMR spectrum, B) DEPT 135 spectrum, C) ^{13}C-NMR spectrum.

Table 4: NMR data of lycopsamine and intermedine

Position	Lycopsamine		Intermedine	
	^{1}H δ(ppm)	^{13}C δ(ppm)	^{1}H δ(ppm)	^{13}C δ(ppm)
1		132.71		132.53
2	5.90	130.24	5.93	131.30
3	α:3.95 β:3.40	62.90	α:3.90 β:3.40	62.89
5	α:3.25 β:2.70	53.83	α:3.20 β:2.70	53.83
6	1.95	36.21	1.90	36.23
7	4.25	70.85	4.25	69.17
8	4.15	78.34	4.10	78.69
9	4.78	62.60	4.75	62.55
10		174.35		175.31
11		83.57		82.99
12	3.95	71.01	4.05	70.84
13	1.22	17.28	1.20	17.09
14	2.12	32.35	2.0	32.95
15	0.89	17.68	0.90	17.24
16	0.86	15.95	0.98	16.87

Table 5: NMR data of acetyl-intermedine

Possition	Acetyl-intermedine	
	^{1}H δ(ppm)	^{13}C δ(ppm)
1		132.95
2	5.81	128.14
3	α:3.90 β:3.40	62.71
5	α:3.36 β:2.60	53.54
6	1.95	34.19
7	5.30	69.32
8	4.25	75.53
9	α:4.80 β:4.63	62.41
10		175.03
11		82.94
12	4.03	73.95
13	1.17	17.09
14	2.63	32.96
15	0.90	17.22
16	0.87	16.88
17		170.43
18	2.03	21.11

The pyrrolizidine alkaloids present in the two plants studied in this work, are mainly monoesters or their acetylated forms. Within the unsaturated pyrrolizidine alkaloids the necine bases carrying a monoester substituent at position C-7 or C-9 are considered to be the least toxic. The toxicity of PAs is closely related to their structure, saturated PAs are taken as non-toxic and the toxicity in the unsaturated PAs increases from monoester $<$ diester open chain $<$ macrocyclic diester .

In this respect, it can be stated that both *A. chiapensis* and *A. maritimum* pose a low risk due to their PA-content. In addition to the structural features of the PAs of these plants, the amount of PAs contained in these plants is very low approximately 0.001 % in the dried plant material.

Another important point to consider in the calculation of the risk of using these plants is the route of administration. *A. chiapensis* as well as *A. maritimum* are mainly used in topical formulations. Martínez-Alfaro *et al.* (2001) reported the use of *A. chiapensis* for the treatment of indigestion. It is important to highlight that the administration rout is not oral but topical in form of a poultice which is applied on the abdominal area of the patient. In the case of *A. maritimum* Cabrera *et al.* (1982) and Martínez (1991) reported its topical use against herpes labialis.

The topical use of the plants together with the low PA-content and the structural features of the PAs in the plants are characteristics which led to propose that the two plants analyzed in this work pose a poor risk to the health of the user.

Traditional medicine systems are most of the time good filters for poisonous plants. The problem lies in plants such as PA-containing plants where the toxicity is not acute but chronic. For this reason the toxic potential of plants such as *A. chiapensis* and *A. maritimum* escape unadvertised and it is difficult for the healer to correlate the

administration of those plants to symptoms which may appear after several months or even years.

Despite the low toxic potential of plants belonging to the tribe Eupatorieae in the traditional medicine of Mexico, plants of this tribe are well known to be visited by honeybees. In Mexico species of Eupatorium in the Mayan region of South Mexico have been reported to be part of the honey-flora of the region. Pollen of plants such as *Eupatorium albicaule*, *Ageratina adenophora* and some other Eupatorieae species has been found in honey end-products (Alfaro *et al.*, 2010).

Pollen is the part with the highest concentration of PAs, regardless of the plant. Contamination of honey with pyrrolizidine alkaloids is a problem well known since 1977 with the first report made by Deinzer *et al.*. Subsequent works have established the substantial risk of PA-contamination of bee products (honey and pollen). The use of PA-containing plants is a common practice in the honey production by apiarists all around the world. Species from the genera *Echium, Senecio, Eupatorium, Heliotropium, Borago, Myosotis, Chromolaena, Petasites, Ageratum, Ageratina*, among others, have been detected as contaminants in honey (Edgar *et al.*, 2011, 2002; Beales *et al.*, 2007, 2004; Kempf *et al.*, 2008; Dübecke *et al.*, 2011).

The levels of PAs vary from unifloral to multifloral honeys as well as in relation to the pollen content (EFSA, 2011). In addition, the daily/yearly consumption of honey varies within population. Hence, it is difficult to make an estimation of the risk that pose the consumption of honey contaminated with PAs.

6 Summary and perspectives

The analysis of pyrrolizidine alkaloids is a hot topic in the European Union, the reports of the EFSA (2007, 2011) expose the magnitude of the problem.

The occurrence of PAs as contaminants in food and feed supplies is the the tip of the iceberg. As presented in this work, the common storing methods such as drying to produce hay, pelleting, ensilaging and composting do not eliminate the content of PAs when these are present as contaminants in the starting material. The methods involving only physical processes are the least effective in PA reduction. Ensilaging and composting are methods that include chemical and biological activities, these methods result in a drastic reduction of the PA-content as it can be seen the the results showed in chapter 4. The reduction found by these methods might be enough to prevent acute intoxications but do not guarantee the safeness of the product for a long-term consumption.

In this matter, feeding animals with contaminated supplies poses a risk that should not be taken, first because it compromises the lifes of the animals and second because it can be the starting point in the contamination of the food chain through derived animal products such as milk.

Senecio species are gaining presence all along Germany and Middle Europe, they are pioneer plants that settle easily in disturbed fields and their biological features include hybridization between species of the genus. The hybridization has been correlated to the gain of novel PAs and in some cases the increase in the amount of PAs (Kirk *et al.*, 2004, 2005, 2010).

From the results in this work, it can be established that the only way to have PA-free feed is to start from fodder free from any contamination with PA-containing plants. This is the challenge that must be undertaken in further feed-production programs. International governmental programs must be set in order to prevent or reduce fatal cases of animals which are directly translated to economic losses.

Regarding the direct consumption of PA-containing plants as herbal remedies, the two plants here studied pose a low risk. However, the population, in this case of Mexico, should be alerted and discouraged from using plants which have been proven to contain toxic compounds.

The use of herbal remedies is not restricted to developing countries, in section 4.4 in this work, one case of acute intoxication in Munich, Germany was analyzed. This kind of cases do not occur often in the medical practice. However, the so called green wave has brought a revival of the use of herbal preparations in developed countries. This more or less recent phenomena differs from the practices of traditional medicine in the lack of knowledge gained generation by generation. The WHO alerts on this matter that in developed countries the relation with the alternative practices goes from uninformed skepticism to the uncritical enthusiasm (WHO, 2002).

In a similar way, veterinarians must be able to identify the symptoms of intoxication by PAs. In order to help to understand and estimate the real impact of the contamination

by PAs in both grazing fields as well as in feed, records of fatal cases of farm animal should be kept in a national medical system. As shown in the cases presented in this work, PA-intoxication is recurrent and should not be underestimated.

In addition, due to the international trade of forage along with the spread of invasive PA-containing plants; the occurrence of fatal cases could be expected to increase unless suitable measures are undertaken against PA-contamination of food and feed products.

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- **Becerra-Jimenez, J.**, Kuschak, M., Roeder, E., Wiedenfeld, H., 2013. Toxic Pyrrolizidine alkaloids as undesired Contaminants in Food and Feed: Degradation of the PAs from *Senecio jacobaea* in silage. Die Pharmazie. 68, 1-4.

Verfassererklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst habe. Ich habe keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche gekennzeichnet.

Bonn, 08. 07. 2013 Jaime Becerra Jimenez