CHAPTER 1: LITERATURE REVIEW

1.1 Pyrrolizidine alkaloids

1.1.1 Introduction

Alkaloids are natural plant products that contain a heterocyclic nitrogen atom, are basic in character and are sometimes toxic to animals when eaten (Holstege *et al.* 1995). The biological role of alkaloids in plants is largely unknown, but is thought to have evolved as a feeding deterrent (Ober and Hartmann 1999). Direct evidence of this protective role in the plants is rare, but it is well known that many insect herbivores have developed various adaptations, even utilizing alkaloids for defense against predators (Boppre 1990; Lindigkeit *et al.* 1997).

Pyrrolizidine alkaloids are mainly present in the families Boraginaceae (many genera), Asteraceae (tribes Senecioneae and Eupatorieae), Orchidaceae (nine genera) and Fabaceae (mainly the genus *Crotalaria*) (Dharmananda 2002). More than 95% of the PA containing plants investigated thus far belonged to these four families (Ober and Hartmann 1999).

Pyrrolizidine alkaloids are a major cause of livestock losses each year and can also enter the human food chain as contaminants of grain. Contamination of grain is particularly likely to occur in parts of the world with arid climates and poor rainfall, which promote the growth of PA-containing plants, growing as weeds among cultivated crops. The first recorded instance of PA poisoning in humans was in 1920 in South Africa when many people in the Western Cape suffered from liver cirrhosis after eating bread made with wheat, probably contaminated with *Senecio burchellii* (Willmot and Robertson 1920). To date the largest reported outbreak of human intoxication by PAs was in Afghanistan in 1974 when an estimated 35 000 people were affected after grain was contaminated with *Heliotropium* plant material. Among 7 200 cases examined, 1 600 were affected and many died 3-9 months after the onset of clinical signs (Mohabbat *et al.* 1976).

Pyrrolizidine alkaloid contaminated animal products can also enter the human food chain and although these exposure incidences may not cause immediate toxic effects in humans, the effect of long-term, low-level, chronic exposure is still uncertain. The most frequently encountered source of residues is the milk of animals that have ingested PA-containing plants (Dickinson *et al.* 1976). Chickens can also transfer PAs to their eggs after eating contaminated grain (Edgar and Smith 2000) and honey has been found to contain high PA levels, up to 1 $mg.kg^{-1}$, causing a risk to those who consume large amounts of honey (Deinzer *et al.* 1977). Pyrrolizidine alkaloid residues are, however, unlikely to be present in meat from animals ingesting plants containing PAs, since the compounds are rapidly cleared from the tissues and slaughter would have to occur within a few hours after grazing on contaminated pastures (Mattocks *et al.* 1988).

1.1.2 Structures

Generally, PAs are esters of hydroxylated methyl pyrrolizidines, consisting of a necine base- and necic acid moiety. The necine base can either be 1,2-unsaturated or saturated. The unsaturated necine bases are further classified as two types; retronecine-type (or heliotridine-type, a 7(S)-isomer of 7(*R*)-retronecine) and otonecine-type alkaloids (Mattocks 1986). Pyrrolizidine alkaloid bases can also exist as *N*-oxides, which are often found together with the basic alkaloids in plants (Fig 1-1).

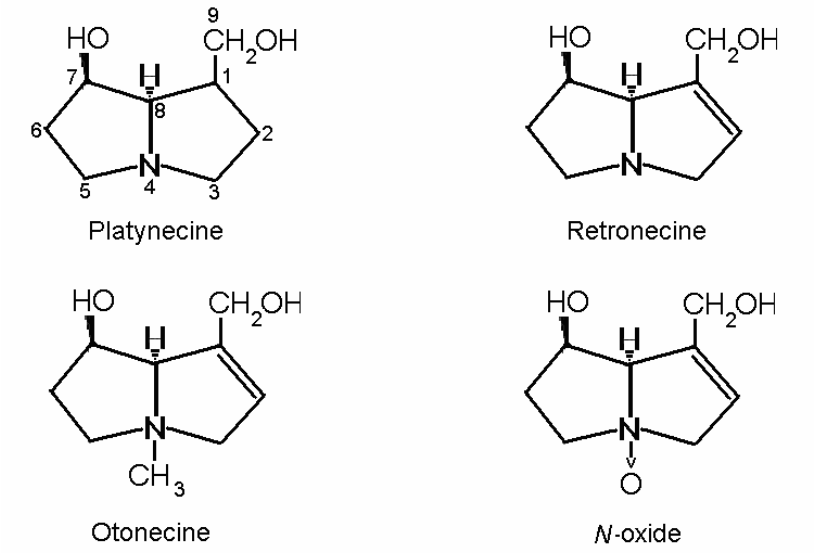

Figure 1-1: Typical structures of the different types of necine bases of PAs

Together with the *N*-oxides more than 640 pyrrolizidine alkaloid structures are possible, of which at least 350 types have already been found in nature and their structures elucidated (Röder 1995).

The acids with which the necines are esterified are called necic acids. Acid moieties often contain mono- or diester groups, with the diesters cyclic or acyclic (Fig 1-2) (Röder 1995).

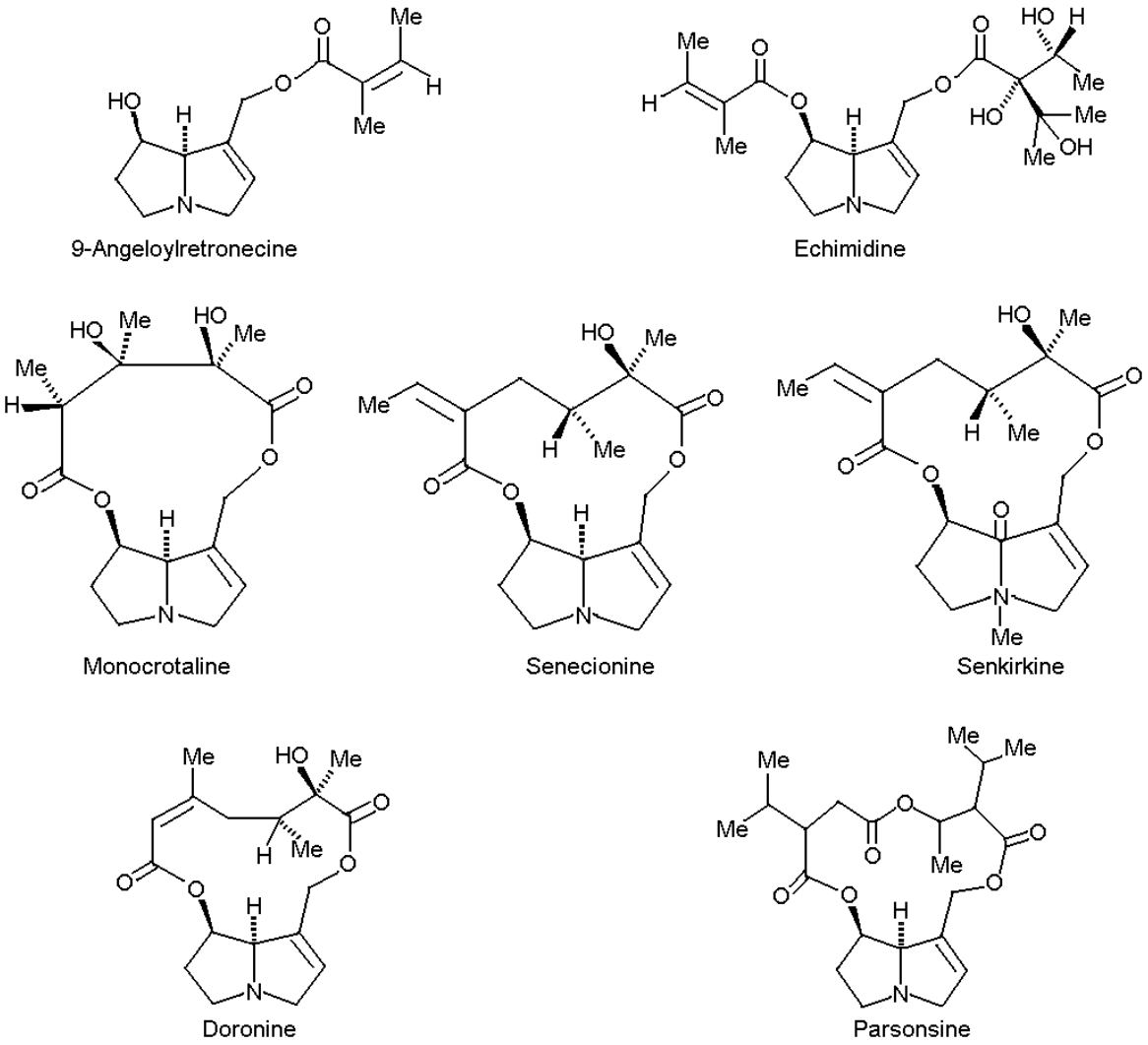

Figure 1-2: Unsaturated PAs bearing typical necic acid moieties

1.1.3 Synthesis

Pyrrolizidine alkaloids are synthesized in plants during amino acid metabolism. The necine base is derived from ornithine and arginine via putrescine and homospermidine metabolism while the acid portion is mainly derived from valine and leucine although acids from isoleucine and other compounds are known (Hartmann *et al.* 1988). Pyrrolizidine alkaloids are synthesized as *N*-oxides in the roots of most of the PA producing plants and are translocated to the aerial parts where they are converted into the species-specific alkaloids (Ober and Hartmann 1999). The conversion reactions include position-specific dehydrogenations, hydroxylations, epoxydations and *O*-acetylations. It is suggested that evolutionary variations in the activities of these enzymes, caused by mutations of the underlying genes, led to the variation in PA patterns between species (Hartmann 1997).

1.2 Absorption and biotransformation

1.2.1 Absorption

Information regarding the absorption of PAs originates from experimental work done by Swick *et al.* (1982). They measured the transfer of a mixture of PAs across isolated segments of the rabbit gastro-intestinal tract and found that PAs were transferred across ileum and jejunum, but not the stomach. Once absorbed from the small intestine PAs are transported to the liver where they are metabolized.

1.2.2 Liver activation

Saturated PAs are not converted to toxic metabolites and are quickly excreted in the urine without any adverse effects. Depending on the structure of the acid moiety, the parent alkaloids of many PAs are also chemically unreactive and much of the dose is excreted unchanged. The remainder of the PAs may become cytotoxic through its metabolism to pyrroles in the hepatic parenchymal cells (Prakash *et al.* 1999). The major metabolic routes of unsaturated PAs in the liver are (Fig 1-3): (a) hydrolysis of the ester group to the corresponding necine base, (b) oxidation of necine base to form the corresponding *N*-oxide and (c) dehydrogenation of the necine base to the corresponding didehydropyrrole. Other minor

routes are known, but these three pathways account for the major known effects of the alkaloids (Winter and Segall 1989).

1.2.2.1 Hydrolysis

Unsaturated PAs that are susceptible to hydrolysis (a in Fig 1-3) have low toxicities. These PAs are hydrolyzed by tissue esterase to highly water-soluble necine bases and acid moieties, which are readily excreted in urine within the first 24 hours.

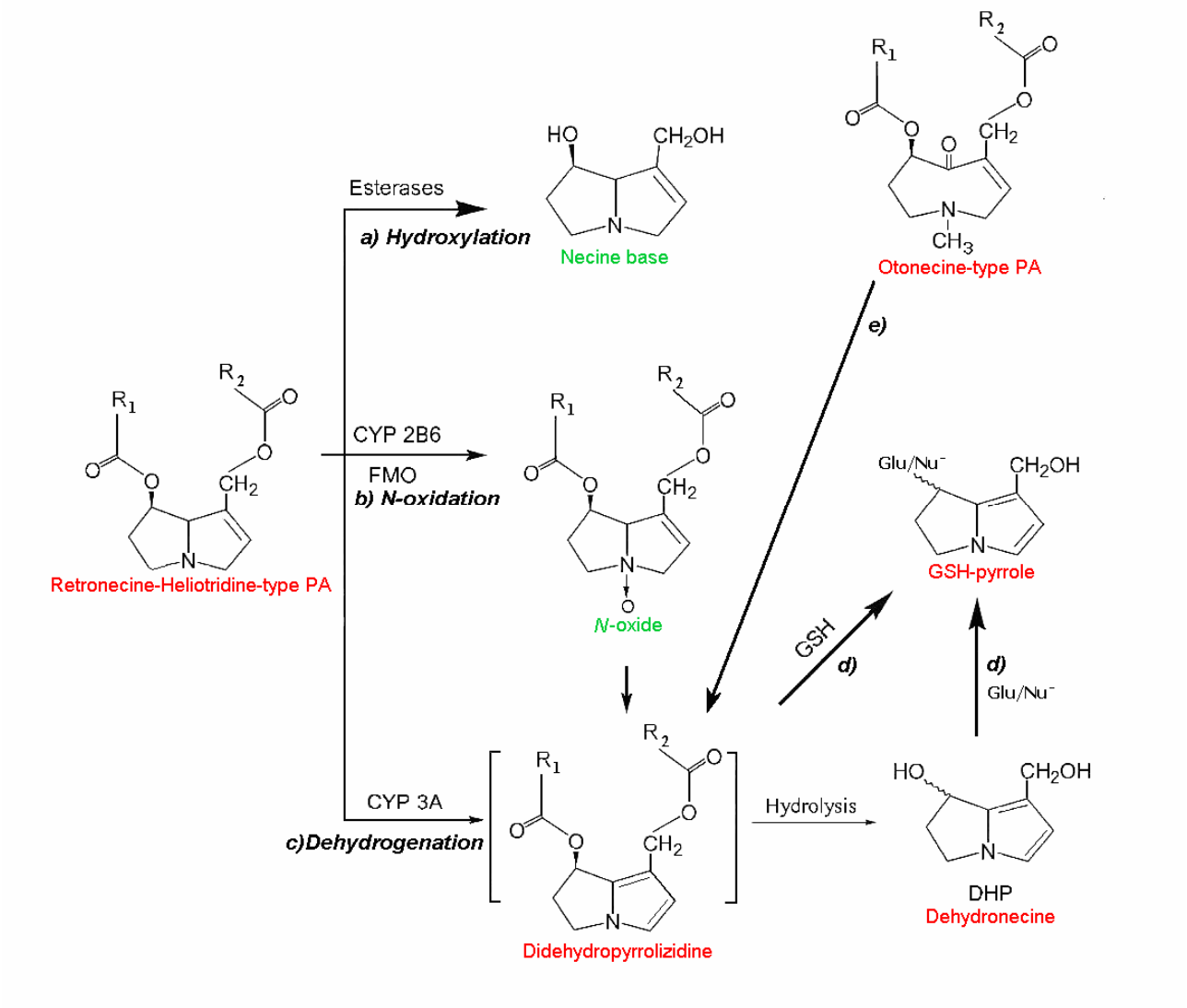

Figure 1-3: Metabolic pathway of 1,2-unsaturated PAs to toxic and non-toxic bases (adapted from Fu *et al.* 2002a)

The susceptibility of the PA molecule to hydrolysis is directly related to the structure of the acid moiety, with short and unbranched acid chain esters more easily hydrolyzed than those where

hydrolysis is sterically hindered (Mattocks 1992). Esterase activity towards monocrotaline is high in guinea pig liver and may account for guinea pig resistance towards monocrotaline poisoning (Deuker *et al.* 1992).

1.2.2.2 N-Oxidation

N-oxidation (b in Fig 1-3) of unsaturated PAs leads to the formation of non-toxic or less toxic *N*-oxides. The pathway is catalyzed by flavin-containing monooxygenase (FMO) enzymes and by cytochrome P450 FMO (Craig 2002). This pathway is also a detoxification route that produces highly water-soluble metabolites. *N*-oxides are often present in plants together with the basic alkaloids. There is no evidence that the *N*-oxides are toxic unless first converted to basic alkaloids by microbes in the gut of some animals (Mattocks 1986).

1.2.2.3 Dehydrogenation

Dehydrogenation by cytochrome P450 (c in Fig 1-3) leads to the formation of reactive dehydropyrrolizidines, which are the primary toxic metabolites responsible for acute toxicity (Castagnoli *et al.* 1997). Dehydropyrrolizidine alkaloids (DHP) may undergo further hydrolysis in the liver cells to form corresponding pyrrolic alcohols. These are also active alkylating agents, less reactive than the pyrrolic esters, but far more persistent and are referred to as the secondary toxic metabolites known to cause extensive extra-hepatic injury (Winter and Segall 1989).

1.2.2.4 Conjugation

Dehydropyrrolizidine alkaloids can in addition also react with glutathione (GSH) to form DHP-GSH conjugates (d in Fig 1-3) which are more water-soluble and subsequently excreted into the bile or sinusoidal blood and ultimately into the urine (Yan and Huxtable 1995). Alternatively the soluble GSH-pyrrole can serve as a transport vehicle to other organs such as the lungs in which toxicity can be elicited if the pyrrole is released from GSH (Cheeke 1989).

1.2.2.5 N-demethylation

Alkaloids of the otonecine-type PAs undergo *N*-demethylation with the eventual formation of a dehydropyrrolizidine alkaloid (e in Fig 1-3). Otonecine-type PAs can also be conjugated with GSH and are about 7 times less toxic than the corresponding necine, as it must first be *N*-demethylated, leading to a less effective conversion (Ge Lin 1998a).

1.2.3 Experimental evidence of liver activation

The relationship between liver activation and toxicity is well established in the literature e.g.:

- Pyrrolizidine alkaloids do not produce localized toxicity when applied to the skin or injected subcutaneously. The major site of PA metabolism is the liver and it is also the site of most damage (Schoental and Head 1955).
- Bull *et al.* (1968) injected a high dose of heliotrine into the tails of young rats. Heliotrine was present in the liver after two minutes and it was also the site with the most damage in the post-mortem specimens.
- Mattocks (1977) studied the distribution of a radioactive labeled PA analogue in rats. The highest concentration of radioactivity was seen in the liver (3.9%), spleen (0.27%) lungs (0.19%) and kidneys (0.18%). About 69% of the dose was excreted in the urine in the first day. The binding of radioactivity in the liver and lungs was more persistent than in the other organs.

Although the main site of damage is the liver, some PAs also affect other organs. There is no evidence that PAs are metabolized in tissue other than the liver, thus, damage to other organs is probably due to metabolites transported from the liver (Culvenor *et al.* 1976). To damage tissues other than the cells in which they are formed, active metabolites must cross the cell membranes and must not be metabolized while transported in the bloodstream. Estep *et al*. (1992) injected ^{14}C-labelled monocrotaline into rats and found high residual radioactivity in the red blood cells. They concluded that red blood cells may be the transport agent for hepatic-generated reactive metabolites to other organs.

1.3 Toxicity of pyrrolizidine alkaloids

1.3.1 Toxicity and structure

Primary toxic metabolites of PAs are highly electrophillic and react with nucleophillic constituents in the cell to exert their effect (Hincks *et al.* 1991). The structure of the individual unsaturated PA determines the metabolic route, which will in turn determine the toxicity of the metabolite, once formed (Fig 1-3). The rate of bio-activation also depends on substrate (PA) concentration and on the metabolizing enzyme activity of the host animal.

Among the PAs, cyclic diesters are the most toxic, with non-cyclic diesters of intermediate toxicity and the monoesters the least toxic. The amino alcohols are not toxic. The toxicity of the *N*-oxides, when first reduced to the basic alkaloid by bacteria in the gut, is of the same order as that of the basic alkaloid (Mattocks *et al.* 1988). *N*-oxides are, however, much more water-soluble and are subject to different pharmacokinetics when absorbed unchanged from the gut (see LD_{50} results listed in Table 1-1).

The steric orientation of the PA molecules and the degree of hydrophilicity appear to be the major factors governing the relative amounts metabolized through the different pathways. Factors preventing hydrolysis include a branching in proximity to the carbonyl groups and rigidity of the acid chain due to cyclic diester rings or unsaturation. Hydrophilic PAs are also more accessible to hepatic microsomal enzymes, which facilitate their conversion to pyrroles and *N*-oxides. The ratio of *N*-oxide to pyrrole varies depending on the type of ester and the enzyme activity of the animal (Winter and Segall 1989).

It is also possible that other metabolites, such as 4-hydroxy-2,3-unsaturated aldehydes may contribute to the acute hepatotoxicity of some PAs (Segall *et al*. 1985). However, this has still to be confirmed.

Table 1-1: Acute toxicity data for unsaturated pyrrolizidine alkaloids (adapted from Mattocks 1986)

Type	Alkaloid	Animal	LD_{50} mg/kg
Cyclic diesters:	Monocrotaline	Rat male	109
	Monocrotaline	Rat female	230
	Monocrotaline	Mouse female	259
	Retrorsine	Rat male	34
	Retrorsine	Rat female	153
	Retrorsine	Mouse female	69
	Retrorsine	Guinea pig	>800
	Senecionine	Rat male	50
	Senecionine	Mouse	64
	Integerrimine	Mouse	78
Non cyclic diesters:	Heliotrine	Rat female	478
	Heliotrine-*N* -oxide	Rat female	2500

1.3.2 Essential features for toxicity

Toxic PAs must firstly have a structure that can be converted to toxic metabolites, and secondly the animals` enzymes must be able to bring about the conversion. It became clear from extensive research done by Mattocks (1986) that PAs need to have certain essential features before they can be toxic. These are:

1. An unsaturated 1-2-pyrrole ring. The other ring is not essential for toxicity and can even be absent.
2. One/preferably two hydroxyl groups or substituted hydroxyl groups attached to the pyrrole ring via one carbon atom.
3. At least one of the hydroxyls must be esterified and diesters are more toxic than monoesters.
4. The acid moiety must have a branched chain.

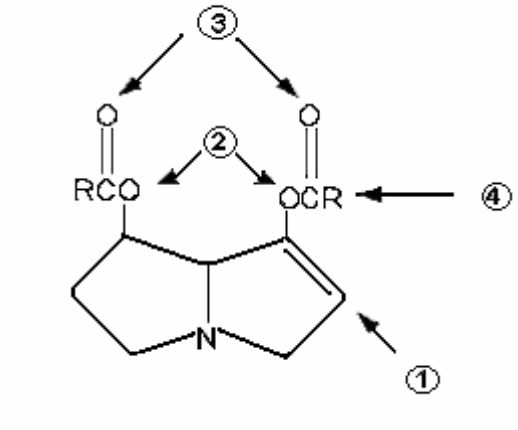

Figure 1-4: Essential structures for hepatotoxicity (Prakash *et al*. 1999)

1.3.3 Reactivity of active metabolite

The amount of didehydropyrrolizidines formed in tissues depends on the availability of substrate (PA) for activation, on the activities of cytochrome P450 enzymes (mainly members of the CYP3A and CYP2B6 subfamilies in the target site) and on the activation of detoxification pathways.

Dehydropyrrolizidine alkaloids are bi-functional intermediates with reactive electrophilic centers at C-7 and C-9 which can react with a variety of nucleophilic cellular macromolecules. Hydroxy-, mercapto- or amino groups of enzymes, globulins, haemoglobin and purine- or pyrimidine bases of DNA and RNA may function as nucleophiles (Fu *et al.* 2002a). The bi-functionality of the pyrrolic metabolites may cause cross-linkages in DNA and RNA, leading to modifications of genetic material (Thomas *et al*. 1998). These adducts may persist in tissues and generate chronic injury. The less stable metabolites are the most reactive and tend to react with nucleophiles close to the point of formation, while more stable metabolites can disperse more before exerting an effect. Thus, monocrotaline frequently damages lung tissue, whereas retrorsine, which yields more reactive pyrrolic metabolites, causes only liver damage (Mattocks 1992).

Detoxification of the pyrrolic metabolites is possible via different mechanisms (Fu *et al*. 2002a):

- Alkylation of PA metabolites with constituents such as glutathione which renders more polar products that are easily excreted in urine (d in Fig 1-3).
- Polymerization – it has been shown that metabolites that polymerase easily are also less toxic.
- Hydrolysis – pyrrolic alcohols, rather than DHP, are formed in an aqueous environment.

Monocrotaline was shown to arrest activation of cdc2 kinase in the target cells (Thomas *et al.* 1998). This inhibits the G2 phase of cell division, which is the normal checkpoint activated in the presence of DNA damage, allowing time for DNA repair. Progress to mitosis is therefore not possible, and this leads to the antimitotic affect seen in PA toxicosis.

1.3.4 Comparative responses

Animal species have different susceptibilities to the toxic effects of PAs. Horses and cattle are often poisoned after consuming moderate amounts of PA-containing plants e.g. consuming about 5% of their total body weight of *S. jacobaea* over time will cause death in cattle. Sheep and goats can consume quantities up to 300% of their body weight without showing any adverse effects (Craig 2002). These differences are partly due to variations in effectiveness of liver enzyme conversion of PAs to toxic metabolites (Prakash *et al.* 1999). The resistance of sheep and goats to PA poisoning has also been linked to detoxification by ruminal microorganisms (Craig 1995). On the other hand, differences in resistance may, however, also

be due to the balance between the formation of toxic pyrrolic metabolites and the detoxification pathway producing non-toxic *N*-oxides and DHP-GSH conjugates (Yan and Huxtable 1995).

1.4 Clinical signs and pathology of toxicosis in livestock

1.4.1 Peracute mortality

High doses of PAs can cause rapid death - within minutes of ingestion. This type of peracute toxicity is not due to cytotoxic actions, but is associated with pharmacological actions and death may be preceded by convulsions or by coma. The acute toxicity for some PAs, e.g. supine cannot be determined due to rapid mortalities (IPCS 1989).

1.4.2 Acute, subacute and chronic toxicity

The rat LD_{50} of most of the alkaloids known to be significant for human health is in the range of 34-300 $mg.kg^{-1}$ (Mattocks 1986). Factors such as sex, age and nutritional status play a role in susceptibility of the individuals.

Pyrroles act preferentially on the hepatocytes and endothelium of blood vessels during the acute phase (Prakash *et al.* 1999). In the hepatocytes the immediate action is a rapid fall in cytoplasmic protein synthesis, reaching 30% of control levels after 15 minutes (Harris *et al.* 1969). This is manifested as disaggregation of polyribosomes, and is followed by failure of pyruvate oxidation, loss of glycogen, structural damage to the mitochondria, lysosomal activity, failure of mitochondrial NAD systems and nuclear NAD synthesis, and finally necrosis (Mattocks *et al.* 1986). Some of the toxic metabolites escape and damage the endothelium of central veins, causing cell proliferation and veno-occlusive disease. More stable metabolites may once again escape via the blood stream and induce damage in other organs, especially the lungs.

Consequently, typical hepatotoxic lesions of PA toxicosis are swelling of hepatocytes, centrilobular necrosis, megalocytosis of the parenchymal cells, fibrosis, bile duct proliferation, veno-occlusion with consequently loss of liver function. These effects are mainly due to the alkylation of pyrroles with DNA, causing impaired cell division (Chojkier 2003). In longer

surviving cases the liver becomes hard, fibrotic and shrunken with subsequent signs of liver failure such as hyperbilirubinaemia, jaundice, hypoalbuminaemia, oedema and ascites.

In acute toxicity studies using laboratory animals, death commonly ensues 1- 4 days after a fatal dose. The liver is firm, congested, with deep-red, granular appearance and ascitic fluid is often present. In addition to necrosis, the sinusoids may become dilated with blood, causing compression of surrounding hepatocytes. Veins are occluded by cells of uncertain origin which may become replaced by fibrous tissue contributing to loss of function (Prakash *et al.* 1999).

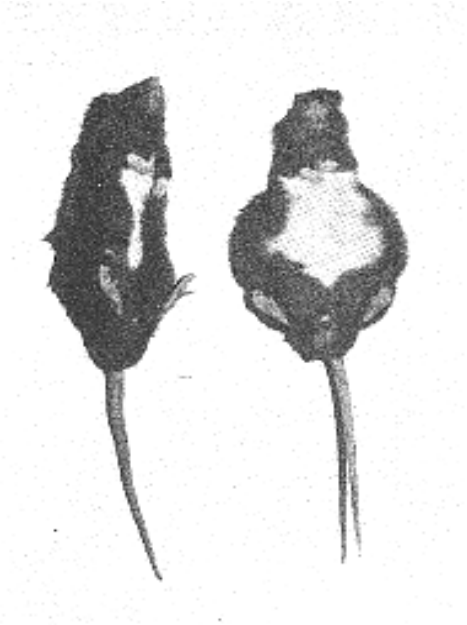

Figure 1-5: Ascites in a rat (right) due to PA intoxication, compared to a control rat (left) (Cheeke 1989)

With livestock poisonings the toxic effects of PAs are often delayed as the signs of poisoning become apparent some time after the animals have eaten PAs containing plants. In acute poisoning, death occurs within about seven days, due to severe liver damage and clinical signs include jaundice, wasting and sometimes photosensitization (Cheeke 1989).

A special feature of chronic PA-induced pathological changes is that after low level single/multiple exposure and well after the alkaloid and its soluble metabolites have been eliminated from the body, the disease is commonly progressive. Liver failure may occur suddenly, months to years after the last episode of PA exposure (Schoental and Magee 1959). It has been shown that the binding of pyrrolic metabolites to various nucleophiles is reversible,

suggesting that this constitutes a reservoir of secondary metabolites from which the active molecules could be released in a continuing or intermittent manner and maintain the progress of cell damage (ANZFA 2001).

Liver lesions associated with chronic PA toxicity in laboratory animals include the proliferation of bile ducts, various degrees of fibrosis and diffuse infiltration of the parenchyma with cells of unknown origins. Many of these changes persist after exposure to PAs is stopped and the liver never returns to normal even though the animal may appear to be in good health. The progression of chronic liver lesions is the same, whether the animal received a single sub-lethal dose, or a succession of smaller doses. Post-necrotic fibrosis is present in all the laboratory animals by day 10 with progressive enlargement of the parenchymal cells (Schoental and Magee 1959).

Ascites is often attributed to low serum albumin levels (oncotic pressure) (Cheeke 1989). Elevated serum billirubin concentration is normally only present in terminal stages and elevated activities of liver enzymes are transient i.e. increase only when actual tissue necrosis occurs. Aspartate aminotransferase can sometimes be an indicator of *Crotalaria* poisoning in livestock (Sippel 1964). Craig (2002) found that gamma glutamyltransferase increases and remains high during low level, chronic exposure. Bile ducts may account for almost half the weight of the liver in the terminal stages of chronic toxicosis. Other clinical signs include a rough hair coat, diarrhoea, prolapsed rectum, dullness, photosensitization and abnormal behavior (Cheeke 1989).

1.4.3 Other clinical effects

Certain PAs, e.g. monocrotaline, produce veno-occlusive disease of the liver as well as a sequence of changes in the lungs and heart that result in pulmonary arterial hypertension and right ventricular hypertrophy (Shubat and Huxtable 1992). Early changes in the lung include alveolar oedema and haemorrhage, causing progressive proliferation of alveolar walls and pulmonary hypertension.

Chronic lung lesions have been produced by most PAs that produce chronic liver lesions, though higher doses were required in some instances (Culvenor *et al.* 1976). Shubat and Huxtable (1992) gave various low doses of monocrotaline to rats over different time periods. They found that the threshold for monocrotaline toxicity is a function of the cumulative dose

received (>14mg.kg^{-1}) and is independent of the period over which it was administered. This study provides evidence for the cumulative effects due to chronic or intermittent consumption of PA contaminated products.

In South Africa many *Crotalaria* species cause “jaagsiekte” in equidae (Kellerman *et al.* 1988). This chronic respiratory disease in horses and mules is characterized by fever, polypnoea, dyspnoea, pulmonary emphysema, pneumonia and sometimes fibrosis or cirrhosis of the liver. Small amounts of the plant must be ingested for many weeks for “jaagsiekte” to develop. Respiratory lesions have also been reported in cattle poisoned by *C. spartioides* and sheep drenched with *C. dura*. The neurological abnormalities sometimes seen in horses (head-pressing) are attributed to the elevated blood ammonia levels, associated with hepatic encephalopathy (Cheeke 1989).

In sheep the consumption of PA-containing plants often leads to excessive liver copper concentrations, followed by a haemolytic crisis associated with chronic copper poisoning. This condition also occurs in horses, rats and rabbits. Increased copper levels are attributed to aberrations in copper absorption, copper-binding proteins or decreased excretion.

PA intoxication also affects iron metabolism and may cause decreased haematopoiesis due to an inability to incorporate iron into red blood cells. The spleen is greatly enlarged in PA poisoning, causing deposition of haemosiderin in various tissues (Anon. 1997).

PA intoxication also affects vitamin A metabolism, leading to an overall reduction of vitamin A levels. This is most likely due to suppression of synthesis by the liver of retinol binding protein (Cheeke1989).

There are also reports of kidney damage due to PA poisoning in animals, mostly attributed to monocrotaline (Hooper and Scalan 1977).

1.4.4 Clinical effects in humans

The reversibility of liver damage following PA exposure is unpredictable in man. It is reported that following an outbreak of acute PA intoxication, some fifty percent of patients will recover completely and about twenty percent will die rapidly. Some of the survivors will appear to

recover clinically, but may go on to develop cirrhosis and liver failure year's later (Stuart and Bras 1957).

Pyrrolizidine alkaloid poisonings in humans presents with nausea, acute upper-gastric pain, fever and elevated liver enzymes. This may lead to abdominal distention with prominent dilated veins on the abdomen (Fig 1-6). Mortality is usually high in the acute phase due to hepatic failure.

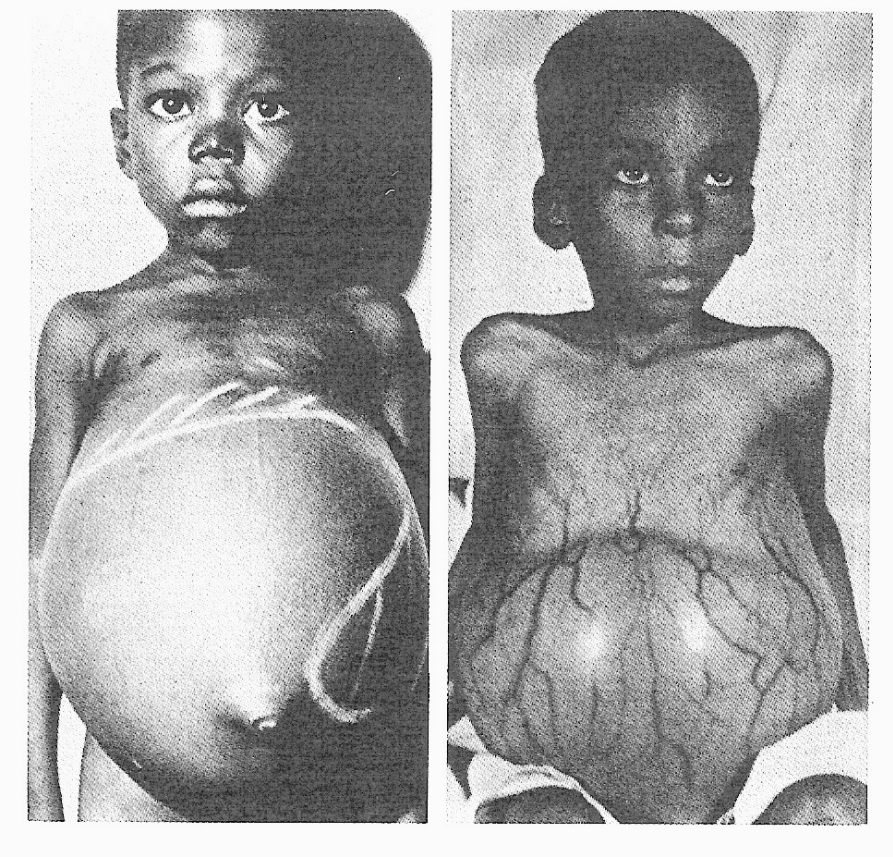

Figure 1-6: Ascites associated with veno-occlusive disease in the West Indies in infants 5 and 7 years old (Huxtable 1989)

Chronic toxic effects in humans are difficult to detect and usually require long-term epidemiological studies. There are no substantial long-term follow-up data to assess whether exposure to PAs resulted in increased incidence of chronic liver disease or cancer in populations where poisonings have occurred (IPCS 1989). With chronic ingestion of small amounts of PAs, or in survivors of acute toxicity, the disease proceeds through fibrosis to cirrhosis, that is indistinguishable from cirrhosis of other causes (Dharmananda 2002). An

unexpected finding in humans is the absence of megalocytes, which is a striking feature of chronic PA poisoning in animals. Removal of PA exposure will usually alleviate the disorder, but liver cirrhosis is not readily reversible. No incidents of primary pulmonary hypertension due to PA poisoning have yet been reported in humans (IPCS 1989).

Veno-occlusive disease is the most common cause of cirrhosis in infants in Jamaica (Bras *et al.* 1961) and the use of PA-containing plants as herbal teas is believed to be a significant aetiological factor. Chronic, low-level exposure of humans to PAs is likely to increase as alternative herbal remedies become more popular. It has been suggested that consumption of PA-containing medicinal herbs might contribute to the high incidence of chronic liver disease and primary liver cancer in Asia and Africa, especially as it may act synergistically with aflatoxins and the hepatitis B virus (Steenkamp *et al*, 2000). The risk of toxic effects due to these PAs may be particularly high in children as they are more susceptible to the effects of PAs, yet are more likely to recover than adults (Stegelmeier *et al.* 1999).

1.4.5 Carcinogenicity and teratogenicity

To date, PAs have been shown to cause cancer only in rodents and there is no conclusive evidence for malignant neoplasia in humans (IARC 1976). Epidemiological studies on survivors of large-scale human intoxications have not indicated any abnormal incidence of cancer and there are also no reports of cancer in domestic animals caused by exposure to PAs in their diet (Prakash *et al.* 1999).

Cancer due to ingestion of large quantities of PAs has, however, been reported in various laboratory experiments. Some examples are:

- Monocrotaline, lasiocarpine and heliotridine had a high mutagenic activity in the *Drosophila melanogaster* assay (Clark, 1959).
- Schoental and Bensted (1963) demonstrated that rats receiving a single large dose of PA may develop chronic liver disease and finally hepatocellular carcinoma more than 13 months after receiving the initial dose.
- More recent evidence presented by the National Toxicology Program showed that some PAs can be carcinogenic. Mice and rats were dosed with riddelline (up to 3 $mg.kg^{-1}$) by gavage for a period of up to two years. The study indicated that riddelline caused liver neoplasms and leukemia in rats and lung neoplasms in female mice (Anon 2003b).

- Fu *et al*. (2002b) lists 15 PAs known to induce tumors in experimental animals, all of which are also present in plants commonly used as Chinese herbal medicines.

In addition, teratogenic effects were produced at very high doses e.g. 50–200 mg.kg^{-1} via intraperitoneal injection of heliotrine in pregnant rats (Röder 1995).

1.5 *Crotalaria* species (rattle pods)

1.5.1 Introduction

Over 600 *Crotalaria* species are known, of which many were used as soil enrichers in the early development of farming lands all over the world (Damron and Jacob 1998). Over 300 *Crotalaria* spp. occur naturally in Africa (Arnold and De Wet 1993) and about 80 indigenous species of *Crotalaria* have been recorded in South Africa (Bromilow 1996). Mattocks (1986) list 43 South African *Crotalaria* species with unsaturated PAs including *C. juncea, C. dura, C. globifera* and *C. spartioides.*

Crotalaria spartioides (duinebos) appears to be the most hepatotoxic *Crotalaria* in SA and is regularly associated with liver failure in cattle. In 1969 a farmer used *C. spartioides* to supplement the diet of about 40 cows. Nineteen of the cows died due to fibrosis or cirrhosis of the liver (Kellerman *et al*. 1988).

Crotalaria burkeana, C. barkae, possibly *C. rhodesiae, C. steudneri* and other annual species result in transient laminitis, colloquially known as ‘stywesiekte’ in cattle and equines (Naudé *et al.* 1992). Due to the difference in clinical presentation this disease may be due to other toxins that have not yet been identified.

Feeding experiments with *C. dura* and *C. globifera* caused “jaagsiekte”, a respiratory disease, in horses at high doses (90 -180 g per day for 116 days) (Kellerman *et al*. 1988). A similar disease was induced in sheep after drenching with *C. dura* (Steyn and De Kock 1932). Marais (1944) extracted large quantities of dicrotaline from both *C. dura* (0.27%) and *C. globifera* (0.18%).

The PA content of *Crotalaria* species from other parts of the world is also known: *C. grahamiana* and *C. pallida* often cause poisoning of horses on Easter Island. The PAs found

in these plants are grahamine (2800 µg.g^{-1}), monocrotaline (100 µg.g^{-1}) and an agrahamine analogue (40 µg.g^{-1}) in *C. grahamiana.* A retrorsine analogue (13 µg.g^{-1}) and a senecionine analogue (2 µg.g^{-1}) are found in *C. pallida* (Anon. 1999).

Crotalaria spectabilis consumption by laying hens has been reported to cause a rapid decrease in egg production, with increased mortality. Feeding trials demonstrated that the adverse effect level was between 0.01 – 0.1% of the diet. Concentrations exceeding 0.3 % were fatal within 18 days (Damron 2001).

Crotalaria spectabilis and *C. retusa* contain retronecine and turneforcidine as well as monocrotaline, spectabiline and retusine (Damron and Jacob 1998). Alkaloids of trichodesmine and senecionine were identified in *C. juncea* after accidental poisoning with herbal medicine in Ecuador (IPCS 1989). Crotananine and cronaburmine were isolated from *C. nana* after grain contamination in India (Tandon *et al.* 1976). Monocrotaline and fulvine were isolated from *C. fulva* after herbal poisoning in the West Indies (Mattocks *et al.* 1988). The two main *Crotalaria* alkaloids involved in human toxicity in Australia are cronburmine and cronaburmine from *C. nana* (ANZFA 2001).

A total of 49 plant species used in Chinese herbal medicine are known to contain PAs (Fu *et al*. 2002b). It includes five *Crotalaria* spp. namely *C. albida, C. assamica, C. mucronata, C. sesseliflora* and *C. tetragona.* These species are known to contain monocrotaline and/or retrorsine.

Other *Crotalaria* species, used as medicines, have also caused toxicity in East Africa *(C. brevidens, C. incan, C. laburnifolia, C. mucronata, C. recta, C. retusa),* in Jamaica *(C. brevidens),* Asia *(C. laburnifolia, C. retusa)* and Sri Lanka *(C. verrucosa)* (Huxtable 1989).

Huxtable (1989) lists various *Crotalaria* species used as medicinal herbs in the developing world. They include *C. fulva* and *C. spectabilis* used as medicinal teas in Jamaica, *C. retusa* as herbal tea and as vegetable in Barbados, *C. laburnoides* used as herbs in Tanzania and *C. juncea* used as herbal tea in Ecuador. He suggests that PA poisoning should be considered in these countries in all human cases presenting with veno-occlusive disease.

Two epidemics of *C. nana* poisoning via contaminated grain have occurred in Mahya Pradesh in India in the 1970's, of 67 cases studied 28 died. There was, however, a poor correlation between the contamination level of the grain eaten by the affected households and the presence of veno-occlusive disease in the affected individuals (Huxtable 1989).

1.5.2 *Crotalaria sphaerocarpa*

Several of the *Crotalaria* spp. are minor weeds, but only *C. sphaerocarpa* (maize crotalaria; mielie crotalaria) poses a serious threat as it grows in close association with grain (Fig 1-7). *Crotalaria sphaerocarpa* is difficult to control as it germinates over the whole season; deep germinating plants are generally difficult to control with pre-emergence herbicides and the plant is tolerant to most post-emergence herbicides once it is past the seedling stage. The plants grow to a height of up to 1.5 m and produce large numbers of seeds (Fig 1-8).

Figure 1-7: *Crotalaria sphaerocarpa* growing between maize in the Bothaville district

Figure 1-8: Large numbers of seeds are produced by *C. sphaerocarpa*

Seed dimensions (2 x 3 mm) should allow easy removal by normal sieving, although the seedpods (3-4 x 4-6 mm) containing two seeds may not be removed that easily (Fig 1-9). Contaminated grain is downgraded due to the alleged toxicity of the seed (Eloff *et al*. 2003). *Crotalaria sphaerocarpa* is included in the poisonous plant database of the US Food and Drug Administration, Center for Food Safety & Applied Nutrition Data as a poisonous plant (Anon. 2003a). All the references used for this listing, however, originate from South Africa, the latest being Kellerman *et al.* (1988).

Figure 1-9: Seed dimensions of *C. sphaerocarpa*

Various *Crotalaria* spp. e.g. *C. spectabilis, C. retusa, C. sagittalis* and *C. mucronata* are listed by Beasley (1999) as hepatotoxic plants in the USA, but *C. sphaerocarpa* is not included in this list. Other poisonous plant databases e.g. GRIN and AGRICOLA lists *C. sphaerocarpa* as native to Africa and it is unclear whether this plant occurs in countries outside of Africa. The IPCS report (Mattocks *et al.*1988) lists plants known to contain hepatotoxic alkaloids as well as the alkaloids isolated from each source. Although the list was updated in 1988, *C. sphaerocarpa* was also not included in this list.

In the 1969 Annual Report of Onderstepoort, feeding experiments with *C. sphaerocarpa* revealed that large amounts were required to induce histological liver lesions in cattle. The investigators concluded that the risks in practice were insignificant (Anon. 1969). In an unrelated more recent study in neighbouring Mozambique, Faftine and coworkers (2000) studied forage selected by cattle on communal land over a two year period. Although *C. sphaerocarpa* was present and probably grazed by cattle, no adverse effects were reported.

Crotalaria sphaerocarpa growing in South Africa has apparently not yet been chemically analyzed and no evidence of acute or chronic toxicity has yet been found (Eloff *et al*. 2003).

1.6 Risks

1.6.1 Introduction

The risk of chemical contaminants in food should reflect the toxicity of the chemical compound as well as the potential daily intake in a normal diet. While the hazardous nature of a compound may be well known, the risk it poses to public life may be negligible if the quantity in a normal diet is below the threshold of toxicity for that substance. The exception is carcinogenic substances, which should be reduced to as low as reasonably achievable (Anon. 1995).

Determination of acute toxicity through establishing LD_{50} levels are not generally performed any more. This has to a large extent been replaced by the no-observed-adverse-effect-level (NOAEL); the highest dose administered to the animal that does not produce any adverse effects. An additional safety factor of 10 is normally added to compensate for inter-individual

variations. This is again multiplied by 10 to compensate for inter-species variations with the extrapolation to man. Once the NOAEL has been determined, an acceptable daily intake (ADI) can be calculated.

1.6.2 Grain contamination

Outbreaks of PA toxicosis typically occur when large numbers of people ingest contaminated food grains and develop veno-occlusive disease. Estimates of PA intakes during these epidemics are approximate and the amounts ingested as well as the toxicities of the different alkaloids vary considerably. Analytical methods are not standardized and it is difficult to compare results between laboratories from different parts of the world.

According to the ANZFA report (2001) levels of PAs found in various grains in Australia have ranged between <50 – 6000 $\mu g.kg^{-1}$. There has, however, never been a systematic analysis of grains entering the food supply.

Altee *et al.* (1998) reported on an episode of PA poisoning in 1994 with *Senecio* contaminated wheat in Mosul, Northern Iraq. During this outbreak 14 people were hospitalized, and two died. The other 12 patients recovered and were symptom free at a 12 month follow-up examination. Seeds were identified as *S. vulgaris.* The seeds gave a positive Ehrlich's test for PAs, but quantitative analysis was not performed.

The estimated PA intake during some *Crotalaria* outbreaks has been calculated (IPCS 1989). In an outbreak in India, millet contaminated with *C. nana* seed had an average PA content of 0.5 $g.kg^{-1}$ and the estimated daily intake by the population was 0.66 $mg.kg^{-1}$ body weight, sustained for approximately two months before disease became apparent. The PA content of wheat in an outbreak in Afghanistan, due to *Heliotropium popovii* seed contamination was 0.04 $g.kg^{-1}$. The estimated daily intake in this case was 0.03 $mg.kg^{-1}$ body weight and was sustained for approximately six months before diagnosis of veno-occlusive disease (Chauvin *et al*. 1994).

No reports could be traced on estimations of chronic exposures through grain contamination over longer periods.

1.6.3 Indirect sources

The exposure to the same toxicant in different food commodities would result in a possible market-basket effect. Related toxins or other toxins present may additively or synergistically increase toxicity. Systematic analysis of the exposure levels of PAs entering the human food chain has never been done and due to the lack of available data, it is not possible to estimate the potential dietary exposure of the general population to PAs (ANFZA 2001).

Pyrrolizidine alkaloids have been found in a variety of foods. Alkaloid levels of up to 1 $mg.kg^{-1}$ have been recorded in honey from certain hives in Australia (Culvenor *et al.* 1981). No reports of poisoning through contaminated honey could be found and it is assumed that blending and bulking reduced these PA level to non-toxic ranges.

Edgar and Smith (2000) determined PA quantities in eggs after contamination of wheat, used in chicken feed, with *H. europaeum* seeds. The PA levels in contaminated eggs ranged from 5 -168 $\mu g.kg^{-1}$ after feed grain was contaminated. The total PA concentration in the seeds was 26 $\mu g.g^{-1}$ and contained heliotrine, europine and lasiocarpine. The same PAs as well as their secondary metabolites were present in the eggs. The highest concentration of PAs in eggs was 38 µg per egg.

Pyrrolizidine alkaloids have been detected in human breast milk during PA poisoning epidemics, causing veno-occlusive disease in babies (ANFZA 2001). De Medeiros *et al.* (1999) fed *C. spectabilis* seeds to a lactating dairy goat. Milk from the goat was given to growing rats over an eight week period. The seeds had no clinical effect on the goat as goats are known to be relatively resistant to PA poisonings. The rats developed interstitial pneumonia, as well as liver and kidney damage, indicating that the monocrotaline or toxic metabolites were excreted in the milk.

In cows, experimental exposure to *S. jacobaea* led to high levels (470 – 835 $\mu g.L^{-1}$) of PAs in milk (Dickinson *et al.* 1976), but it had no adverse affects when fed to calves. It appears from this study that only the more water soluble metabolites are excreted in milk, which will directly affect the final toxicity of the milk consumed.

Although PA levels in milk, honey and eggs are often higher than the recommended safety levels, no evidence of chronic health problems in humans associated with these edible products could be found.

1.6.4 Herbal or medicinal consumption

Apart from accidental poisonings there is also increasing concern about the use of plants containing PAs in herbal medicinal preparations.

Dharmananda (2002) published an extensive review of herbal intoxications, which led to liver damage and even death in humans. The dose and duration were estimated in some of these cases e.g. for heliotrine a dose of 4-10 mg.kg^{-1} per day for 3 to 7 weeks led to veno-occlusive disease. In another case, a combination of crotanine and cronaburmine at less than 1 mg per day for several months led to the same disorder. Liver necrosis was caused by retrorsine and riddelline consumption at 0.7-1.5 mg.kg^{-1} per day for 2 weeks. From these cases the toxic dose range in humans appears to be between 0.1-10 mg PA.kg^{-1} per day.

In South Africa many *Senecio* and *Crotalaria* species are sometimes used to prepare traditional herbal medicines. Steenkamp *et al* (2000) identified 20 children at two South African hospitals suffering from veno-occlusive disease after administration of traditional remedies. In four of the cases a simple colourimetric screening test confirmed the presence of PAs in on-admission urine samples. PA poisoning by traditional medicines may be a huge problem in South Africa as these cases are often not diagnosed or reported.

1.6.5 Other factors to consider

Little research has been conducted on the effect that environmental factors have on PA concentration in plants. It is known that in the Asteraceae, biosynthesis of PAs is strictly controlled by root growth, and production ceases when root growth stops (Ober and Hartmann 1999).

Hol and co-workers (2003) investigated the effect of environment on the PA content in *S. jacobaea* plants when grown in a climate chamber. They reported that increasing nutrient levels led to a significant reduction in the PA concentration of the roots and shoots of the plant, but that the total amount of PA produced in the plant was not affected. They concluded that

change in biomass, rather than production rate, was the reason for the changes induced by nutrients.

Crotalaria juncea, known to contain toxic PAs in Equador, is regarded as a useful fodder plant in Asia and is often fed to cattle without any adverse effects (Srungbomee and Makasame, 1981). It may be possible that the PA content in a specific plant may be entirely different when growing in different areas.

Pyrrolizidine alkaloids can also be changed by heat or by enzymatic action when harvested plants are stored (Bull *et al.* 1968). Changes were also observed during the drying process in the ratios of *N*-oxide to the free base. However, the stability of the unsaturated pyrrolizidine alkaloids and *N*-oxides at high temperatures, for example, during cooking, is not known.

Other toxins may be present in plants containing PAs. In one case of *C. mucronata* poisoning of sheep in Australia, animals started dying within the first day due to acute lung damage (Laws 1968). The rapidity of onset and the atypical lung lesions found in this case suggest that toxins other than PAs were causing the mortalities (IPCS 1989).

Although evidence is still conflicting, health risks in some population groups may also be higher. Factors such as age, gender, nutritional status, as well as other synergistic effects need to be incorporated into future NOAEL.

1.7 Regulation criteria

1.7.1 Aim of regulation

Because of their known involvement in human poisoning and their potential carcinogenicity, exposure to PAs should be kept as low as practically achievable. Prevention of exposure is the only effective method of limiting toxicity and the setting of regulatory tolerance levels for certain grains may be the best method to prevent exposure.

Specific information is needed before NOAEL can be established. International standards, of allowable levels of toxins in food, are drawn up by the Codex Alimentarius Commission, under the auspices of the Food and Agriculture Organization (FAO) and the World Health

Organization (WHO). The current standards do not specify the concentrations allowed, but state that levels should not pose a threat to human health. Specific information is therefore needed in each case to set the standards. The toxicity of the substance, the mass of the seed and the concentration of the toxin must be known. The effect of environmental factors on the toxin content and the stability of the toxin during processing should also be taken into account. A safety factor should be built in to accommodate inter-individual variations and possible synergistic effects due to other compounds (Eloff *et al.* 2003).

Ideally, the levels of contaminating toxins in processed food consumed by the South African population, particularly children who are more susceptible, would be the only justifiable indicator for determining the acceptable tolerances in grains and other food commodities.

1.7.2 Regulations in other countries

The NOAEL of PAs has not yet been established in experimental animal studies. Estimates of intake causing toxic effects in humans indicate that they are more sensitive than rats and domestic animals (IPCS report 1989). The lowest known dose that caused veno-occlusive disease in a human was estimated to be 15 $\mu g.kg^{-1}$ per day, and was the result of self-medication with a comfrey (*Symphytum officinale*) preparation (Ridker *et al.* 1985).

Due to the hazards that may arise, the Federal Health Department of Germany has drastically restricted the sale of pharmaceuticals containing PAs and *N*-oxides with a 1,2-unsaturated necine skeleton. PA-containing pharmaceuticals are exempted from this restriction if a daily oral administration of 0.1 µg per day, and 10 µg per day with external application is not exceeded (Röder 1995). Similar regulations have been proposed in other countries such as Britain and Australia (Stegelmeier *et al.* 1999).

Regulations in Australia and New Zeeland are discussed in the ANZFA report (2001). The main alkaloids involved in human poisoning in Australia until 1988 were heliotrine from *Heliotropium*, echimidine from *Symphytum*, riddelline from *Senecio longilobus* and crotanine from *Crotalaria nana*. The approximate rat oral LD_{50} for these alkaloids are 300, 500, 50 and 100 $mg.kg^{-1}$, respectively. The collective data from these incidents suggest that the daily PA intake were cumulative in doses down to 33 $\mu g.kg^{-1}$ (expressed as heliotridine equivalents). From this data a tentative NOEL of 10 $\mu g.kg^{-1}$ per day is calculated. Applying a factor of 10 for

human variability sets the provisional tolerable daily intake (PTDI) for humans at 1 µg.kg^{-1} body weight per day.

Current USA guidelines restrict allowable seed levels of certain PA containing plants: *Crotalaria spectabilis, C. sagittalis* and *C. striata* require less than one whole seed per pound of product (Mattocks *et al*. 1988).

1.7.3 Regulation in South Africa

In South Africa, prior to 2002, regulation guidelines, set by the Department of Health, suggested a limit of one seed of *C. sphaerocarpa* per 10 kg of maize (Anon. 1987). The toxic seed regulation, on the other hand, allowed tolerances to be determined by the Agricultural Boards and a level of three seeds per 10 kg grain was consequently accepted. After the demise of the marketing boards in 1990, the allowable level reverted back to the regulations set by the Department of Health. This level was, however, more stringent than those of other trading partners. Due to subsequent pressure from producers, the Department of Health changed the allowable level to 10 *C. sphaerocarpa* seeds per 10 kg grain (Anon. 2002), as an interim measure, based on recommendations made by Eloff and co-workers (2003). This is the interim allowable level, provided that research confirms the validity of the assumptions made in the report.

Eloff *et al.* (2003) calculated the risk posed by grain contamination using 500 g of dry maize as the normal daily intake (two meals). They reasoned that a dose of 5 mg.kg^{-1} per day of monocrotaline (the most abundant PA in *Crotalaria* spp.) led to cancer in approximately a third of the rats in a feeding trial by Shumaker *et al*., 1976. By applying a safety factor of 10 to achieve non-toxic levels, and an additional 100 fold decrease for species differences, they estimated that 5 -10 µg.kg^{-1} per day would probably be equivalent to a NOAEL for humans. Based on findings of Marais (1944) it was calculated that PAs can contribute up to 0.05 % of the seed mass. For an average *C. sphaerocarpa* seed of 3.6 mg this calculates as 1.8 µg PA per seed. For acute toxicity a 70 kg person would have to eat 194 seeds per day (3880 seeds per 10 kg) to reach this intake. If the daily intake limit is reduced to 1 µg.kg^{-1} per day (Australian criteria for chronic exposure) the level should be set at 777 seeds per 10 kg maize. To comply with European regulation of 0.1 µg per day the level should drop to 11 seeds per 10 kg. The authors stressed that the assumptions on which this recommendation was made, still have to be tested.

1.8 Conclusion

Unsaturated PAs pose a threat to human health especially as the risk of low-level exposure has not yet been determined. Grain contamination with *C. sphaerocarpa* is a real problem in certain areas (e.g. Bothaville district) in South Africa. The toxicity of *C. sphaerocarpa* seed is, however, not known and may be very low. Previous feeding trials to prove toxicity in animals have failed and it is therefore inappropriate to investigate toxic levels via this route. The alternative is to determine the toxic PA content of *C. sphaerocarpa* seeds with an analytical method in a laboratory and to calculate the risk to humans based on daily consumption.

1.9 Aim and objectives

The aim of this study is to determine the toxicity of the PAs present in *C. sphaerocarpa* seed and to ensure that risk assessment is based on systematic, scientifically acceptable criteria. In order to achieve this, the following questions need to be answered:

- How toxic are *Crotalaria* species occurring in SA that may contaminate grain, and which PAs are present in these species?
- Is there a difference in PA levels under different environmental conditions?
- In order to minimize low-level exposure, should government only regulate levels of seed allowed in grain or rather regulate the concentration levels of toxic PAs in food products?
- Will it be possible to determine PA levels in processed grain?
- What is the threat to SA consumers of grain products imported from countries with more toxic PA contaminants?

It appears that the PA content of *C. sphaerocarpa* seeds growing in South Africa has never been chemically analyzed. Furthermore, several unpublished experiments carried out by the Veterinary Institute at Onderstepoort in 1968 could not show acute toxicity in the animals tested (Anon 1969). To repeat the toxicity studies under these circumstances was not a viable option. It is also often difficult to extrapolate toxicity results from animal studies to humans.

The main objective of this study is therefore to develop an analytical method that can distinguish between toxic and non-toxic PAs, in order to determine the toxic PA content in *Crotalaria* spp. that may contaminate grain in South Africa. The levels of toxic alkaloids will be

used as the basis for recommendations on the level of noxious seed that could be allowed in grain in future.

The objectives of the study are the following:

- Develop an HPLC-MS/MS method to distinguish between toxic and non-toxic PAs.
- Identify and quantify the toxic PAs in *C. sphaerocarpa* seed.
- Determine the concentrations of the toxic PAs in seeds from different sites.
- Determine toxic PA levels in other parts of *C. sphaerocarpa* and of different ages.
- Investigate the toxic PA content of other PA-containing plant species.
- Determine the stability of toxic PAs during the food cooking process.
- Determine the lowest level of detection (LLOD) for the determination of toxic PAs in maize meal.

The study will focus on the toxic PAs in the seeds as these are thought to be the reason for the inclusion of *C. sphaerocarpa* in the FDA Poisonous Plants Database (Anon 2003a). Compounds that meet the structural criteria for toxicity will be isolated where possible and identified in subsequent investigations. The toxicity tests of the isolated compounds in animals are beyond the scope of this study especially since many attempts to prove toxicity have already failed (Anon 1969). Other tests may be added to the study depending on the specific PAs found in the selected plants.

1.10 Envisaged results

The research may lead to a better understanding of toxic PAs that cause a variety of human, wildlife and livestock health problems in South Africa. If it could be proven that only non-toxic *Crotalaria* species occur in cultivated lands, the allowable level of seeds could drastically be increased. On the other hand, if products from other grain producing countries have a high risk of PA intoxication; it could give South African producers a competitive edge in the market.

Herbal preparations and traditional medicines containing hepatotoxic PAs may pose a real threat to humans as large quantities are normally consumed and children are often the ones who are treated in this manner. These products are also not controlled by any regulatory guidelines. The screening method for toxic PAs, developed in this study, could therefore also be used to regulate or restrict the use and sale of these products.

The study may furthermore lead to the establishment of a network of reference laboratories that could assist member states in identifying plants and their seeds suspected of containing toxic PAs. This in turn will allow regulation and restrictions on the import and sale of seeds, herbs and herbal preparations that may contain harmful pyrrolizidine alkaloids.