# PHOMOPSINS IN FOOD

## A Toxicological Review and Risk Assessment

TECHNICAL REPORT SERIES NO. 1

## AUSTRALIA NEW ZEALAND FOOD AUTHORITY
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## SUMMARY

The phomopsins are a family of mycotoxins produced by the fungus *Phomopsis leptostromiformis*. Lupins are the main host for the fungus, which is capable of infecting most parts of the plant. Infection of the vegetative parts of the plant can result in high levels of phomopsin being present on the stubbles, which is the major source of animal exposure to phomopsin. Under certain storage conditions, infected lupin seed can also exhibit significant levels of phomopsin contamination. While the majority of lupin seed is used in animal feed, lupin products are also increasingly being introduced into food for human consumption. Therefore, whole lupin seed and flour may be a source of human exposure to phomopsins, which have been shown to be stable to processing, including cooking.

**Hazard assessment**

Very limited data is available on the metabolism and kinetics of phomopsins, due in part to the lack of suitably radio–labelled phomopsins. Limited evidence from toxicity studies suggest that phomopsins may be only partially absorbed from the gastrointestinal tract, following oral exposure. Once absorbed, the phomopsins appear to be metabolised in the liver to a reactive form. Data from studies using other routes of exposure also suggests that liver metabolism is essential to the toxicity of phomopsins. There is some evidence, from animal toxicity studies, to indicate that phomopsins, or their metabolites, may be excreted via the kidneys.

The toxicity of phomopsins appears to be related largely to their ability to bind to tubulin. This results in the inhibition of important cellular functions such as spindle formation during mitosis, and the intracellular transport of lipids. Other observed effects include distortions of cell nucleus shape plus apparent disruptions to membrane systems within the cell. These toxic effects appear largely confined to the liver.

The ingestion of phomopsins has, so far, only been associated with adverse effects in animals. In particular, the ingestion of phomopsin–contaminated lupin stubble has been linked to lupinosis, a debilitating disease of sheep. Given the apparent mechanism of toxicity, however, it is reasonable to conclude that humans would also be vulnerable to the toxic effects of phomopsins.

The majority of available animal studies rely on acute or sub–chronic exposure using subcutaneous or intraperitoneal routes, or in the case of sheep, intraruminal routes. Data on oral exposure is mainly limited to reports from observations of field–affected animals, and these reports tend to be qualitative in nature.

The most common sign of toxicity seen in animals following acute and sub–chronic exposure, regardless of the exposure route, is liver toxicity. Liver failure is the most common cause of death in these animals. The most sensitive clinical indicator for this toxicity is inappetence in the affected animals. Reduced appetite has been observed in animals where gross liver damage is not yet apparent and, in sheep, has been associated with an intraruminal dose of 12.5μg/kg bw/day. This dose may approximate a LOEL for this exposure route. Animals receiving sub–lethal doses of phomopsins exhibit some capacity to recover once treatment discontinues.

The acute and sub–chronic toxicity studies have shown that a number of parameters may affect the toxicity of phomopsins. Firstly, susceptibility to the toxin appears to vary between species. For example, sheep appear to be far more susceptible than rats to the toxic effects of a given dose of phomopsins. Secondly, limited evidence, from both acute and sub–chronic toxicity studies, suggests that the toxicity of phomopsins may vary depending on the route of exposure, with a given dose of phomopsins being less toxic by the oral route. This suggests that the absorption of phomopsins by the gastrointestinal tract may be limited or that phomopsins undergo some degradation following ingestion. Thirdly, the toxicity of a given total dose of phomopsins appears to be greater if it is administered in smaller fractions over an extended period of time. This may indicate a cumulative effect.

The only data available for chronic toxicity are qualitative observations in cases of chronic lupinosis in sheep. As with acute and sub–chronic exposure, the liver appears to be the principal target organ of toxicity. The qualitative nature of the chronic studies did not enable the determination of a LOEL or NOEL for these effects.

There is very little data on which to assess the potential genotoxicity of phomopsins. Negative results have been obtained in bacterial mutagenicity assays. However, some equivocal evidence exists, from cultured mammalian cells, that phomopsins may induce chromosomal aberrations. No information was available on the *in vivo* genotoxicity of phomopsins.

Studies on the reproductive toxicity of phomopsins were not available. In a single developmental toxicity study using rats, significant embryotoxicity was observed. However, significant maternal toxicity was also observed at all dose levels tested. Therefore, it was not possible to attribute the observed embryonic deaths to the direct action of phomopsins. Additional developmental studies using more appropriate dose levels are warranted.

The carcinogenicity data reported, while not derived using an oral route of exposure, is a serious concern. Data from a sub–chronic study using the subcutaneous route indicates that there is an unequivocal association between phomopsin treatment and an increased incidence of liver cholangiocarcinomas and hepatocellular carcinomas in rats at a dose level of 30µg/kg bw/day administered for 17 weeks. The potential for carcinogenicity of phomopsins following oral exposure remains unclear and warrants further investigation.

Overall, it can be concluded that phomopsins are potent cytotoxic agents which predominantly target the liver and which are clearly liver carcinogens in the rat. Some animal species appear more vulnerable than others to the toxic effects of phomopsins. Phomopsins may also be less toxic by the oral route, although still capable of causing severe disease, e.g., lupinosis in sheep. The cytotoxic nature of phomopsins suggests that humans would also be vulnerable to its toxic effects, however, the available animal studies do not allow a determination of a safe level of dietary exposure to phomopsins.

The absence of a NOEL for phomopsins from animal studies as well as the absence of data on the potential toxicity of phomopsins in humans means that it is not possible to derive a tolerable level for human exposure.

**Dietary exposure assessment**

The survey data available for phomopsins is limited to Australian data and restricted to lupin seed only.

Surveys have found that up to 20% of harvested lupin seed can be infected by *P. leptostromiformis*. A survey of commercial lupin seed from Western Australia, Victoria and New South Wales conducted in the early 1980s found levels of phomopsins ranging from <6µg to 360µg/kg. More recently, levels as high as 4522µg/kg in seed have also been detected.

The sorting of lupin seed has been shown to be an effective means of reducing the phomopsin contamination of seed. In a survey of unsorted lupin seed from the 1991/92 harvests in Western Australia, the mean level of contamination by phomopsins was found to be 6.1µg/kg. If the seed was sorted on the basis of discolouration, the mean level of phomopsins in the clean seed was 1.3µg/kg, compared to a mean level of 355.1µg/kg in the discoloured portions.

There is no data available on the levels of phomopsins carried over to lupin flour. Therefore, it is not clear to what extent the milling process may remove phomopsin contamination. In addition, no data is available for other potential sources of exposure such as other lupin products, offal and milk. Therefore, there is insufficient survey information to enable a dietary exposure assessment to be done. However, sub–populations groups most likely to have high exposure to phomopsins would be those consuming large amounts of lupin products.

**Risk characterisation**

Phomopsins have been shown in animal studies to be potent liver toxins and carcinogens in rats. Although no direct evidence of toxicity in humans is available, their mechanism of action is such that humans are likely to be susceptible to their toxic effects. Phomopsins appear to be less toxic by the oral route than by other routes but still capable of causing severe liver disease in sheep following oral ingestion. Phomopsins also appear to be stable during cooking. The paucity of toxicity data available does not make it possible at this time to identify a NOEL in animal studies or assign a tolerable level for human exposure.

The survey data on the levels of phomopsin in food is confined to lupin seed. Phomopsin levels in food are not surveyed as part of the Australian or New Zealand Total Diet Surveys, nor are its levels routinely surveyed in other food groups such as milk, offal, meat etc. Furthermore, the extent to which lupin flour and other lupin products are included in foods is not known, therefore, a dietary exposure assessment for phomopsins is not possible.

The difficultly of establishing a tolerable level of human exposure to phomopsins, combined with a paucity of exposure data, makes it difficult to clearly characterise the

potential public health and safety risk from exposure to phomopsins in food. However, the available data suggests that phomopsins are highly toxic in all mammalian species tested and may be a health concern in humans exposed to lupins or products derived from lupins.  Given these concerns, particularly with regard to the potential carcinogenicity of phomopsins, it would be prudent to ensure that human exposure be kept as low as is reasonably achievable.

**Further work**

To further characterise the potential public health risks associated with phomopsins, further research is required on: (i) the extent of phomopsin contamination of lupin seed used for direct human consumption, flour prepared from lupin seeds, and offal from animals grazing on lupin stubble; (ii) the potential toxicity of phomopsin following long term low level exposure.

# PHOMOPSINS IN FOOD

## A Toxicological Review and Risk Assessment

## INTRODUCTION

The phomopsins are a family of tubulin-binding cyclic peptide mycotoxins produced by the fungus *Phomopsis leptostromiformis*. Lupins are the main host for the fungus and under certain storage conditions infected lupin seed can develop significant levels of phomopsin.

Certain strategies have been developed to limit contamination of lupin seed. Lupin breeding programs have been successful in producing varieties that are resistant to *P. leptostromiformis* (Cowling *et al* 1986, 1988, Allen and Cowling 1986). The resistant lines can still be colonised by the fungus but there is a significant reduction of phomopsin contamination of the seed (Than *et al* 1994). As contamination of lupin seeds by phomopsin is associated with discolouration of the seed another effective strategy has been to use commercial grading equipment to select seeds that have reduced phomopsin levels.

### Chemical and physical properties

The phomopsins are a family of macrocyclic hexapeptide mycotoxins produced by the fungus *Phomopsis leptostromiformis* (Culvenor *et al* 1977, Allen and Hancock 1989). The chemical structure of the phomopsins identified so far is shown in Figure 1. The compounds, phomopsinamine A and octahydrophomopsin A, are chemical derivatives of phomopsin A. Both have similar biochemical activity as phomopsin A. A third phomopsin, phomopsin C, has been partially identified but a full structure is not yet available (Edgar 1991).
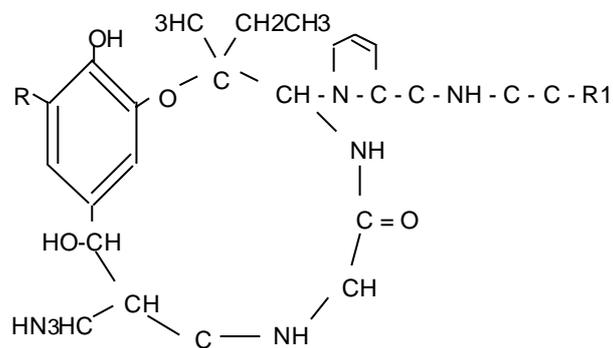
Mass spectral and X–ray crystallographic studies have shown that the phomopsins are linear peptides but with a 13–membered ring formed by an ether bridge in place of 2 hydroxyl groups (Edgar *et al* 1986, Mackay *et al* 1986). The phomopsins contain the unusual amino acid residues 3,4-didehydroproline, 2,3-didehydroisoleucine, 2,3-didehydroaspartic acid, 3,4-didehydrovaline, 3-hydroxyisoleucine and N-methyl-3-(3'-chloro-4',5'-dihydroxyphenyl) serine (Edgar *et al* 1985, Edgar *et al* 1986).

Phomopsin A is soluble in water above pH 7.5 and below pH 1.0, reasonably soluble in aqueous alcohols, but only sparingly soluble in lipid solvents. The phomopsins have been shown to be resistant to destruction by extensive processing, including cooking (Cockrum *et al* 1994)

### Sources of exposure

*P. leptostromiformis* is a saprophyte of lupins. The fungus can infect stems, leaves, pods and seeds of the plant. Infection of the vegetative parts of the plant can result in high levels of phomopsin being present on the stubbles. While this is not a concern for human exposure, it is the major source of animal exposure to phomopsin. Infected seed, under certain storage conditions, can also develop significant levels of

**Figure 1:  The chemical structure of the phomopsins**



|  | **R** | **R1** |
|---|---|---|
| **Phomopsin A** | Cl | - NHC - COOH<br>‖<br>C<br>H  COOH |
| **Phomopsin B** | H | - NHC - COOH<br>‖<br>C |
| **Phomopsinamine A** | Cl | - NH2 |
| **Octahydrophomopsin A** | Cl | - NHCH - COOH<br>│<br>CH2COOH |

phomopsin although under normal storage conditions there should be no increase in the phomopsin content because seed moisture will be below 11% and there is little likelihood of any fungal metabolism. The majority of lupin seed is used in animal feed but lupin products are being increasingly introduced into the human food supply. Therefore, whole lupin seed and flour could represent a source of human exposure to phomopsin.

Other foods that may contain phomopsin are certain horticultural products such as chestnuts and mangoes, of which *Phomopsis* spp. are significant spoilage fungi, although this spoilage may limit consumption of affected products.

Some anecdotal evidence exists that suggests that phomopsins may accumulate in the livers of animals that ingest infected plants. Survey data would be useful therefore to determine if offal may be a secondary source of phomopsin exposure to humans.

Currently, no data is available on the levels of phomopsin in lupin flour, in horticultural products or in animal offal.

## REVIEW OF TOXICOLOGY DATA

### Metabolism

No data is available from controlled experiments on the absorption, metabolism, distribution or routes of excretion of ingested phomopsin in animals. The lack of suitably radio–labelled phomopsin has contributed to this paucity of data.

*In vitro* studies using artificial rumen preparations have shown that phomopsin does not undergo any significant metabolism by rumen microorganisms (Peterson 1986).

Peterson (1986) states that at high levels of ingestion, the toxicity of phomopsin is limited by its rate of absorption (Peterson 1986), however, there is no data to substantiate this statement. Cytotoxic effects in the liver of nursling rats have been observed one hour after intraperitoneal injection of phomopsin (Peterson 1978). This suggests that once absorbed, phomopsin may undergo very rapid transport to the liver, probably through the portal vein. No data could be found on the mechanisms that might facilitate the subsequent distribution of phomopsin to other tissues. There is some evidence from kidney effects observed in toxicity studies that phomopsin, or an active metabolite of phomopsin, may be excreted via the kidneys (Peterson 1986, Peterson and Lanigan 1976, Peterson 1990).

### Postulated mechanisms of toxicity

The phomopsins are some of a number of compounds capable of binding to tubulin (Tonsing *et al* 1984) and this binding is considered to be responsible for the acute toxicity of phomopsin by preventing the polymerisation of tubulin at concentrations of less than 1 $\mu$M (Lacey *et al* 1987, Peterson 1990). This action leads to an inhibition of crucial functions such as spindle formation during mitosis and the intracellular transport of lipids.

Interference in the intracellular movement of lipids has been shown to result in the accumulation of fat in liver parenchymal cells as well as an interference with the excretion of the products of biliary metabolism. This leads to a condition known as "fatty liver". Interference with mitotic spindle formation and function results in interference to the cell cycle, principally metaphase arrest and abnormal chromatin aggregation. *In vivo* mitotic arrest in hepatocytes, induced by phomopsin, is followed, in most cases, by cell death (Peterson & Lanigan 1976). This suggests that the effects resulting from the binding of phomopsin to tubulin are not reversible. However, results from studies using cultured cells have been conflicting in this respect (Tonsing *et al* 1984, Brown & Bick 1986).

Cell membranes may also be affected by phomopsin, where changes in the activity of some membrane–associated enzymes and increased fluidity have been observed in fractions of hepatocyte plasma membrane after phomopsin treatment (Peterson 1986). A redistribution of Golgi apparatus membranes has also been observed (Tonsing *et al* 1984). It is not apparent if the direct binding of phomopsin causes such changes to membrane systems, or to what extent other intracellular membrane systems might be involved. It has been postulated that such reactions may contribute to the formation of micronuclei and the distortion of nuclear shape which are often seen following phomopsin exposure (Peterson and Lanigan 1976, Brown and Bick 1986).

**Acute toxicity**

Mice

A number of acute studies using mice exposed intraperitoneally to crude preparations of phomopsin have been done (Peterson and Lanigan 1976, Papadimitriou *et al* 1974), however, the actual phomopsin dose used in these experiments does not appear to have been quantified. Pathological examinations revealed the liver as the main target organ with major changes in the central zones of the hepatic lobes. Triglyceride accumulation began after 12–16 hours, reaching a maximum at 24 hours. A variety of observed changes in the nucleus of liver cells were directly proportional to the dose administered. Changes in the activities of various liver enzymes were also observed. Alkaline phosphatase, 5'–nucleotidase, β–glucuronidase and acid phosphatase activities were elevated, whereas, succinic dehydrogenase, glutamic oxaloacetic transaminase, lactate dehydrogenase and glucose–6–phosphatase activities were decreased. These changes persisted for several days but gradually returned to normal after 4 weeks. An increase in the number of parenchymal cells undergoing mitosis in the liver commenced after 26 hours. This reached a maximum between the second and third day and then declined. A high proportion of the cells undergoing mitosis were abnormal, and included arrested metaphases. Similar mitotic effects were also seen in the kidneys, but were largely restricted to the proximal convoluted tubules, and occurred much later than in the liver. Evidence of mitotic arrest was not observed in the duodenum, lung or spleen.

*Rats*

Lethal doses (LD$_{50}$) of phomopsin in rats, from various routes of exposure have been reported by Peterson (1986), however, the studies from which they were derived were

not cited.  The reported values are 24 – 52.5mg/kg for oral exposure, 4.4 – 8.0mg/kg for subcutaneous injection and 1.2 – 2.0mg/kg for intraperitoneal injection.

Groups of 2–week old nursling hooded male rats (5 rats/dose) were given a single intraperitoneal dose of a crude preparation of phomopsin (Peterson 1978).  This preparation was subsequently found to contain about 4% toxin on a dry weight basis, therefore, the appropriate adjustments have been made to the doses reported for the study.  Nursling rats were selected over adult rats because they were expected to exhibit a high level of mitotic activity, and hence be a better model for studying the effects of phomopsin on the cell cycle.  The doses used varied, depending on the experiment, but were within the range 0–2.64mg/kg.  Animals were killed at various intervals up to 28 days after injection.  The response in male and female rats (5 rats/sex/dose) was also compared at the dose levels of 0.026mg/kg and 0.53mg/kg at 18 hours and 7 days after injection.  No sex–specific differences were observed.  The intraperitoneal $LD_{50}$ for this study was estimated to be about 1mg/kg, causing death in 4 to 8 days.  This value is consistent with other reported $LD_{50}$ values for this exposure route (see above).  The principal effect associated with low doses of phomopsin (<0.04mg/kg) was metaphase arrest in liver parenchymal cells.  This could be seen within one hour of injection and reached its peak within 2 to 4 days after which its occurrence declined rapidly.  At higher dose (>0.17mg/kg), fatty changes and fibrosis developed in the liver and the rats became jaundiced.  The fatty changes to the liver could be observed 18 hours after injection and reached their maximum at 3 to 4 days after injection.  Fatty changes were much less evident 6 days after the injection.  Depletion of cortical cells in the thymus, depletion of haematopoietic tissue in the spleen, reduced gastric activity, and retarded growth rates were also observed.  The severity of these responses increased with increasing dosage.  Mitotic arrest was also observed in the kidney and pancreatic acinar, but only at high doses (0.7mg/kg for kidneys and 1mg/kg for the pancreas), and not in any other tissues.

*Sheep*

In sheep, the ingestion of phomopsin–contaminated lupin stubble is associated with the occurrence of a disease known as lupinosis.  The classical clinical signs of lupinosis are inappetence, loss of condition, lethargy and jaundice (Gardiner and Parr 1967, van Warmelo *et al* 1970, Gardiner 1975).  The gross pathology of lupinosis depends on whether the disease is acute, subacute or chronic.  In acute and subacute lupinosis there is generalised jaundice, and the liver is greatly swollen.  It is a bright yellow, orange or creamy colour, and the cut surface is very greasy.  The gall bladder is usually enlarged, the adrenal glands swollen and the kidneys may be dark brown in colour.  There is often ascites and oedema of connective tissues, and the caecum is frequently distended with hard, dry faecal material.  Evidence of myopathy may be seen as obvious areas of pallor or very subtle pale streaking in the skeletal and cardiac muscles.  Microscopically, phomopsin primarily affects the liver, causing mitosis–arresting and cytotoxic effects in individual cells.

Groups of desexed male sheep (1–3 sheep/dose) were given a single subcutaneous dose of phomopsin over the dose range 1.25 – 98µg/kg and observed over 28 days (Jago *et al* 1982).  All sheep given ≥ 75µg/kg died in 3–5 days.  Those given between 10 and 37.5µg/kg died in 10–26 days, except for two animals who were showing clinical signs of recovery at the end of the experiment.  All of the sheep receiving

5µg/kg or less survived the 28 days of the experiment. The first clinical sign observed in the sheep was inappetence, which rapidly progressed to anorexia in all sheep that subsequently died during the experiment. Reduction in food intake was observed within one day of administration of doses at 10µg/kg or higher, and within 3 days after 2.5µg/kg. Weight loss could not be correlated with dose. Clinical signs of toxicity were not observed in animals receiving the lowest dose of 1.25µg/kg. In sheep given lethal doses of phomopsin, both total serum protein and albumin levels fell by 10–17% within 4 days, with albumin levels continuing to fall, consistent with liver failure. Post–mortem findings were consistent with those previously described for lupinosis in sheep (see above), with the liver being the only organ with gross changes. There were too few animals to establish an $LD_{50}$ dose. Survival time was shown to have an approximate inverse relationship to dose, ranging from 3 days after 94µg/kg to 26 days after 10µg/kg.

*Conclusion*

Most of the acute studies establish a dose–response relationship for phomopsin–induced toxicity leading to death. The principal target organ for toxicity in sheep, mice and rats, following acute exposure, is the liver, although effects in other tissues and organs, most notably the kidney, have also been reported. Lesions associated with renal tissue may be indicative of urinary excretion of phomopsin or its metabolites. Liver failure is the most common cause of death. Sheep appear to be far more susceptible to phomopsin toxicity than rats on the basis of a study using subcutaneous exposure. There is some evidence from reported $LD_{50}$ values for rats that phomopsin may be less toxic if taken by the oral route. This may indicate that absorption of phomopsin from the gastrointestinal tract is limited or that phomopsin is metabolised to some extent in the gastrointestinal tract. Apart from one oral study for rats, most studies rely on subcutaneous or intraperitoneal routes of exposure, which are inappropriate for determining oral toxicity.

**Sub-chronic toxicity**

*Rats*

Groups of 10 week old male and female Long–Evans rats were administered phomopsin by subcutaneous injection at the dose level of 30µg/kg bw for 5 days per week for 2, 6 or 17 weeks (Peterson 1990). Matched groups of 4 or 5 treated and control rats were killed at scheduled intervals during the treatment period and up to 32 weeks after dosing commenced. In addition, some male and female rats were allowed to survive until about 2 years of age. Controls rats received equal volumes of physiological saline.

During the treatment period, no effect was observed on the appearance or behaviour of the rats, except for an initial temporary retardation in growth rate, which returned to normal after the fourth week of treatment. Phomopsin was shown to decrease survival times in a manner which was related to the duration of the treatment and was statistically significant for the groups treated for 6 and 17 weeks. All rats administered phomopsin for 17 weeks developed permanent, irreversible liver damage, characterised by nodular cirrhosis and extensive biliary hyperplasia, which continued to progress after the treatment ceased. In some of the rats in the 6 week

dose group, similar effects were seen, while in others, the cessation of treatment was followed by almost complete regression of the liver lesions, with only a small amount of fibrous tissue evident 2 years after the last injection. This indicates that both the biliary and parenchymal damage may become irreversible after a relatively short period of exposure to phomopsin. The livers of animals administered phomopsin for 2 weeks exhibited full recovery within a few weeks after treatment was ceased.

This study also looked at the incidence of tumours and these findings are summarised in Table 1.

**Table 1: Incidence of tumours in rats at 2 years following SC injection with phomopsin at 30µg/kg bw/day**

| Treatment Duration | No. of animals[1] | Cholangioma | Cholangiocarcinoma[#] | Hepatocellular carcinoma |
|---|---|---|---|---|
| Untreated | 59 (34M, 25F) | 0 | 0 | 0 |
| 2 weeks | 20 (20M) | 0 | 0 | 0 |
| 6 weeks | 34 (20M, 14F) | 10 | 1 (1) | 0 |
| 17 weeks | 37 (27M, 10F) | 22 | 2 (1) | 3 (2) |

[1] the number of rats older than 40 weeks at death or scheduled killing
[#] figures in parentheses are the number of tumours that had metastasised

The tumours most commonly observed in the phomopsin–treated rats were primary liver tumours. They were of two types – biliary tumours (cholangiocarcinoma and cholangioma) and liver parenchymal tumours (hepatocellular carcinoma). The appearance of these tumours was related to the treatment and their number increased with increasing duration of treatment. Tumours were first observed approximately 36 weeks after the commencement of the study. The occurrence of liver tumours following such a short exposure period suggests that some of the phomopsin–induced liver lesions, described above, may continue to progress in the absence of further exposure to the toxin. Primary liver tumours were not observed in any of the control animals. A small number of non–hepatic tumours were observed, but these did not appear to be related to the treatment.

*Sheep*

Groups of desexed male sheep (3 animals/dose) were administered single or multiple doses of phomopsin by either the subcutaneous (SC) or intraruminal (IR) routes (Peterson *et al* 1987). IR injection was used to simulate the retention of plant material in the rumen and is considered equivalent to gavage. The regimen of phomopsin doses given to the sheep, and its effects, are summarised in Table 2.

**Table 2: Schedule of administration of phomopsin and its effects on sheep**

| Route | No. of doses | Dose rate (µg/kg bw) | | No. of deaths | Liver damage | | Time (days) from first injection to: | |
|---|---|---|---|---|---|---|---|---|
| | | Per injection | Total | | No. affected | Severity | Clinical signs | Death |
| SC | 1 | 2 | 2 | 0 | 0 | –,–,– | | S,S,S |
| SC | 1 | 10 | 10 | 2 | 3 | ++,++,+++ | 3 | 4,4,S |
| IR | 1 | 125 | 125 | 0 | 0 | –,–,– | | S,S,S |
| IR | 1 | 250 | 250 | 0 | 0 | –,–,– | | S,S,S |
| IR | 5 (1 week) | 50 | 250 | 1 | 1 | +++,–,– | 7 | 9,S,S |
| IR | 1 | 500 | 500 | 0 | 3 | +++,++,++ | | S,S,S |
| IR | 1 | 1000 | 1000 | 1 | 3 | +++,+,+++ | 9 | 12,S,S |
| IR | 5 (1 week) | 200 | 1000 | 3 | 3 | +++,+++,+++ | 7 | 13,13,13 |
| IR | 20 (4 weeks) | 50 | 1000 | 3 | 3 | ++,++,++ | 22-28 | 29,31,31 |
| IR | 80 (16 weeks) | 12.5 | 1000 | 0 | 1 | –,++,– | | S,S,S |

Key: –, normal; +, mild; ++, moderate; +++, severe; S, survived to end of experiment

[#] principally jaundice and lethargy, and excluding inappetence

Mild suppression of appetite was observed in 1 out of 3 sheep administered a single SC dose of 2.0µg/kg, and was completely suppressed in all 3 sheep given a single SC dose of 10µg/kg, with two of the animals dying 4 days after exposure. The surviving sheep exhibited slow recovery of appetite. The overall clinical, biochemical and histological responses closest to the effect seen from a single SC dose of 10µg/kg resulted from a single IR dose of 1000µg/kg. The same total dose administered at daily IR rates of 50 or 200µg/kg was more toxic, resulting in the death of all the sheep. A single IR dose of 500µg/kg was associated with significant liver damage, but no deaths. Single IR doses of 125 and 250µg/kg and repeated daily IR doses of 12.5µg/kg over 16 weeks were not associated with any detectable tissue damage, but were associated with a loss of appetite. Of the four groups that received a total IR dose of 1000µg/kg (either singly or through multiple doses), treatment by single dose was less toxic than divided doses of 200 or 50µg/kg.

Apart from jaundice and small amounts of ascites in a few severely affected animals, gross pathological changes were restricted to the livers of sheep that had developed clinical disease at some stage. In sheep that died early from acute toxicity, the livers were very fatty. With lower doses of phomopsin or longer survival, liver fat was less evident, the organ was firmer and the colour changed to orange or dark ochre. A variety of lesions, based on colour and texture, were evident in the livers, extending for various depths into the organ. There was no consistency in the appearance of these lesions. Histopathological examination revealed that in sheep surviving for longer periods (> 4 days), the fatty changes in the liver were progressively replaced by fibrosis and a variable degree of proliferation of biliary tissue. Pigmented macrophages containing ceroid and haemosiderin were abundant in the fibrosed tissue. A SC dose of 10µg/kg may approximate the $LD_{50}$ for this exposure route in sheep.

*Cows*

A group of 14 Holstein–Friesian cows in mid–lactation were dosed orally with phomopsin A in water at 2.88mg/cow/day (calculated to be approximately 5.2µg/kg bw/day based on an initial average body weight of 556kg) for 8 weeks (Hough and Allen 1994). Controls were dosed orally with equal volumes of water only. Cows

were returned to pasture for 4 weeks after the exposure period before cessation of the study. Milk yield, milk composition, body weight and body condition were measured weekly for the 8 week treatment period and for 4 weeks after treatment. Liver damage was monitored by the measurement of plasma γ–glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH) activities, and plasma bilirubin concentrations were determined weekly during the 8 week treatment period. Histological examinations of the liver were not done.

Administration of 5.2μg phomopsin A/kg bw/day was not associated with any measurable effect on milk yield, fat, protein or total solids content. No significant difference was found between the treatment and control groups with respect to body weight, plasma GGT and GLDH activities or bilirubin concentrations.

*Conclusion*

The target organ of toxicity following sub–chronic exposure was shown to be the liver in both rats and sheep. Animals receiving sub–lethal doses of phomopsin exhibited some capacity to recover. For sheep, at least following acute exposure, there appears to be a difference in toxicity of the order of a 100 fold from intraruminal dosage, compared with parenteral. This suggests that gastrointestinal absorption of phomopsin may be limited. Comparable data are not available for repeated daily dosage. An intraruminal dose of 12.5μg/kg bw/day in sheep for 16 weeks, associated with loss of appetite but no observed tissue damage, appears to be close to the minimum dose producing any measurable adverse effect. An oral dose of 5.2μg/kg bw/day, administered to lactating cows for 8 weeks, was not associated with any adverse findings, however, histopathological examinations of the liver were not done in these animals. Frequent or daily doses of phomopsin appear to be tolerated less well than single doses of phomopsin. This may be indicative of a cumulative effect.

The carcinogenicity data reported while not derived using an oral route of exposure, are a serious concern. The data indicates that phomopsin is clearly associated with the occurrence of liver carcinomas in rats following subcutaneous exposure to 30μg phomopsin/kg bw/day for up to 17 weeks. The number of rats affected was shown to increase with increasing duration of treatment.

**Chronic toxicity**

There are no controlled chronic studies available for phomopsin. The only data available are qualitative observations in cases of chronic lupinosis in sheep. In these animals the liver is small, hard, coppery or tan in colour and often misshapen. The rumen contents are watery, and the abomasum and small intestine contain very little solid matter. The caecum may contain hard, dry, impacted faecal material. Ascites may be present and there may be evidence of general muscle wastage (Gardiner 1965, Gardiner 1967a).

*Conclusion*

The liver is likely to be the principal target organ of phomopsin toxicity following chronic oral exposure. There is insufficient data to enable the setting of a LOEL or NOEL.

**Genotoxicity**

Very little information could be found on the genotoxicity of phomopsin. Negative results have been obtained for phomopsin in the Ames test and in the Chinese hamster ovary chromosome aberration and HGPRT locus mutation tests (BIBRA 1986). However, Brown and Bick (1986) have shown that phomopsin can induce chromosomal aberrations consisting of chromatid and isochromatid deletions and chromatid exchanges in the Chinese hamster DON cell line.

*Conclusion*

There is some limited evidence obtained from tests with cultured mammalian cells that phomopsin may induce chromosomal aberrations.

**Reproductive toxicity**

There are no studies available on the reproductive toxicity of phomopsin.

**Developmental toxicity**

In a study by Peterson (1983), the effect of phomopsin on pregnant hooded rats and their embryos was examined. In the first experiment, 40 pregnant rats in 4 treated and 2 control groups (number per group not specified) were injected intraperitoneally (IP) with 30 or 90μg/kg bw/day on days 6–10 or 11–15 of pregnancy. In a second experiment, 25, 100, or 400μg/kg of phomopsin was administered as a single IP dose to 200 pregnant rats in 15 treated and 5 control groups (number per group not specified) on days 6, 8, 10, 12 or 14 of pregnancy. On day 20 of pregnancy the dams were killed and the foetuses examined.

A dose of 90μg/kg bw/day was associated with the death of 40% of the dams. Liver damage was also observed in all the dams that had received phomopsin treatment, regardless of the dose or duration of exposure. A single dose of 400μg/kg or a dose of 90μg/kg bw/day for 5 days was associated with high embryo lethality. Doses of 30μg/kg bw/day for 5 days were found to be associated with embryo lethality only when administered over days 6–10 of the pregnancy. Foetuses that survived the higher dose rate of 90μg/kg bw/day were severely retarded in their growth and their skeletal ossification was irregular. Notably, the livers of the foetuses were apparently unaffected, and there was an absence of metaphase arrests in any of the embryonic tissue examined. This suggests that the embryonic deaths may not be associated with direct phomopsin action on their tissues but may instead be the indirect result of maternal toxicity.

*Conclusion*

The dose levels of phomopsin used in this study were all associated with significant maternal toxicity, including death, and, therefore, were probably set too high. Because of this it is not possible to associate the observed embryonic deaths with the direct action of phomopsin. Furthermore, given that phomopsin is a proven mitotic inhibitor, the absence of metaphase arrests in the embryonic tissues suggest that

phomopsin may either have been excluded from direct contact with the embryos, or require metabolic activation which cannot be provided by an immature embryonic liver.

**Carcinogenicity**

There are no long-term studies available using the oral route of exposure. However, a sub–chronic study in rats, where a single dose level was administered to rats by subcutaneous injection for up to 17 weeks, demonstrated an unequivocal association between phomopsin treatment and the occurrence of liver tumours, which first appeared at 36 weeks.

**Human studies**

None available.

**HAZARD CHARACTERISATION**

**Establishing a NOEL in animals**

A limited number of animal studies on the toxicity of phomopsin are available. The majority of these studies rely on acute or sub–chronic exposure using subcutaneous or intraperitoneal routes, or in the case of sheep, intraruminal exposure. Intraruminal exposure involves direct injection of phomopsin into the rumen of sheep and is regarded as similar to gavage.

An intraruminal dose of 12.5μg/kg bw/day, from a sub–chronic study in sheep, was associated with reduced appetite but no observable tissue damage. This dose was the lowest tested in the study, therefore, it is not possible to determine the NOEL. This level may, however, approximate a LOEL for these effects. An oral dose of 5.2μg/kg bw/day, administered to lactating cows in a sub–chronic study, was not associated with any adverse findings. However, this finding was based solely on liver enzyme and bilirubin analyses; histopathological examinations of the liver were not done. Therefore, neither study is adequate for establishing a NOEL, although they may give some indication of where such a level may be.

**Establishing a tolerable level of exposure in humans**

Information on the effects of phomopsin in human is not available. All of the available animal studies have some limitations in relation to their usefulness in determining a safe level of dietary exposure for animals, not only because of the routes of exposure, but because they frequently only used a single dose level of phomopsin. Furthermore, no reproductive toxicity studies are available. Therefore, in the absence of human data and in the absence of a NOEL in animals, it is not possible to establish a tolerable level of exposure to phomopsin in humans.

Given the highly toxic nature of phomopsin, as seen from animal studies, it seems reasonable to conclude that the level of exposure in humans should be kept as low as is reasonably achievable.

## DIETARY EXPOSURE ASSESSMENT

### Distribution in foods

Lupin flour can be used in bread, biscuits, and pasta. Lupin hulls are also used as a fibre component in high fibre white bread and muffins. Whole seeds may also be used in a mashed form in a number of processed foods, or to produce milk and lactic beverages. There is potential, therefore, for phomopsin to be found in all these food categories.

There is no data available on the levels of phomopsins in the tissues of livestock, in milk or in other animal products.

### Levels in food

The survey data available for phomopsins is limited to Australian data and restricted to lupin seed only.

Surveys have found that up to 20% of harvested seed can be infected by *P. leptostromiformis* (Clarke and Kellock 1979, Ali *et al* 1982, Wood and Petterson 1985). A survey of commercial lupin seed from Western Australia, Victoria and New South Wales has found levels of phomopsins ranging from <6μg to 360μg/kg (Petterson *et al* 1985). Levels as high as 4522μg/kg in seed have also been detected (Than *et al* 1994).

In a survey of unsorted lupin seed from the 1991/92 harvests in Western Australia, the mean level of contamination by phomopsins was found to be 6.1μg/kg. If the seed was sorted on the basis of discolouration, the mean level of phomopsins in the clean seed was 1.3 μg/kg whereas in the discoloured portions the mean level was 355.1μg/kg. The results of this study demonstrate that seed sorting is an effective means of reducing phomopsin contamination of seed.

Survey data is only available for lupin seed. There is no data available on the levels of phomopsin carried over to lupin flour. Therefore, it is not clear to what extent the milling process may remove phomopsin contamination. As it is known that phomopsins are concentrated in the seed coat initially, special attention may need to be directed towards gathering survey data for lupin hulls, which may be included as a fibre supplement in breads.

No data is available for other potential sources of exposure such as other lupin products, offal, milk etc.

### Exposure estimates

There is insufficient survey information to enable a dietary exposure assessment to be done.

**Identification of high exposure sub–populations**

Sub–populations groups that are most likely to have high exposure to phomopsin are those who consume large amounts of lupin products.

**RISK CHARACTERISATION**

Phomopsins have been shown in animal studies to be a potent liver toxins and carcinogens in rats. Although no direct evidence of toxicity in humans is available, their mechanism of action is such that humans are likely to be susceptible to their toxic effects. Phomopsins appear to be less toxic by the oral route than by other routes but still capable of causing severe liver disease in sheep following oral ingestion. Phomopsins also appear to be stable during cooking. The paucity of toxicity data available does not make it possible at this time to identify a NOEL in animal studies or assign a tolerable level for human exposure.

The survey data on the levels of phomopsin in food is confined to lupin seed. Phomopsin levels in food are not surveyed as part of the Australian or New Zealand Total Diet Surveys, nor are its levels routinely surveyed in other food groups such as milk, offal, meat etc. Furthermore, the extent to which lupin flour and other lupin products are included in foods is not known, therefore, a dietary exposure assessment for phomopsins is not possible.

The difficultly of establishing a tolerable level of human exposure to phomopsins, combined with a paucity of exposure data, makes it difficult to clearly characterise the potential public health and safety risk from exposure to phomopsins in food. However, the available data suggests that phomopsins are highly toxic in all mammalian species tested and may be a health concern in humans exposed to lupins or products derived from lupins. Given these concerns, particularly with regard to the potential carcinogenicity of phomopsins, it would be prudent to ensure that human exposure be kept as low as is reasonably achievable.

**Further work**

In order to further characterise the potential public health risks associated with phomopsins, further research is required on: (i) the extent of phomopsin contamination of lupin seed used for direct human consumption, flour prepared from lupin seeds: and offal from animals grazing on lupin stubble: (ii) the potential toxicity of phomopsin following long term low level exposure.

## REFERENCES

Ali, S.M., Paterson, J. and Crosby, J. (1982). A standard technique for detecting seed transmitted *Phomopsis leptostromiformis* of lupins and for testing commercial seed in South Australia. *Aust. J. Exp. Agric. Anim. Husb*. **22**: 190–193.

Allen, J.G. and Cowling, W.A. (1986). Preventing lupinosis with *Phomopsis*–resistant lupins. *J. Agric. West. Aust*. **27**: 89–90.

Allen, J.G. and Hancock G.R. (1989). Evidence that phomopsins A and B are not the only toxic metabolites produced by *Phomopsis leptostromiformis*. *J Appl Toxicol*. **9:** 83–89.

BIBRA (1986). A summary of the results of mutagenicity tests with alkaloids isolated from Western Australian lupins. Report on BIBRA Project No. 30585. The British Industrial Biological Research Association, Carshalton, UK.

Brown, J. K. and Bick, Y. A. E. (1986). Comparison of cellular effects of phomopsin and colcemid in Chinese hamster cells *in vitro*. *Cytobios* **46**: 79–91.

Clarke, R.G. and Kellock, A.W. (1979). Control of seed–bourne *Phomopsis* sp. on lupins. *Aust. Plant Pathol. Newsletter* **8**: 34.

Cockrum, P.A., Petterson, D.S. and Edgar, J.A. (1994). Identification of novel phomopsins on lupin seed extracts. In: Plant–associated Toxins (Eds. S.M. Colegate and P.R. Dorling), CAB International, Wallingford, UK, pp 232–237.

Cowling, W.A., Wood, P.McR., Allen, J.G., Gladstones, J.S. and Hamblin, J. (1986). Effects of genetic resistance on *Phomopsis* incidence and potential stem and seed toxicity. In: *Proceedings of Fourth International Lupin Conference*, Western Australia Department of Agriculture, South Perth, pp 230–239.

Cowling, W.A., Allen, J.G. and Wood, P.McR. (1988). Resistance to *Phomopsis* stem blight reduces the lupinosis toxicity of narrow–leafed lupin stems. *Aust. J. Exp. Agric*. **28**: 195–202.

CSIRO (1992). CSU1–ELISA monitoring of phomopsins in lupin seed. CSIRO Animal Health.

Culvenor, C.C., Beck, A.B., Clarke, M., Cockrum, P.A., Edgar, J.A., Frahn, J.L., Jago, M.V., Lanigan, G.W., Payne, A.L., Peterson, J.E., Petterson, D.S., Smith, L.W. and White, R.R. (1977). Isolation of toxic metabolites of *Phomopsis leptostromiformis* responsible for lupinosis. *Aust J Biol Sci*. **30:** 269–277.

Edgar, J.A., Culvenor, C.J., Frahn, L., Jones, A.J., Gorst–Allman, C.P., Marasas, W.F.O., Steyn, P.S., Vleggar, R. and Wessels, P.L. (1985a). In: *Trichothecenes and Other Mycotoxins* (Ed. J. Lacey), John Wiley, New York, p 317.

Edgar, J.A., Frahn, J.L., Cockrum, P.A. and Culvenor, C.C.J. (1986). Lupinosis. The chemistry and biochemistry of the phomopsins. In: Mycotoxins and Phycotoxins (Eds. P.S. Stern and R. Vleggaar), Elsevier, Amsterdam, Holland, pp 169–184.

Edgar, J.A. (1991). Phomopsins: antimicrotubule mycotoxins. In: *Handbook of Natural Toxins*, (Eds. R.F. Keeler and A.T. Tu), Marcel Dekker Inc, pp 371–395.

Gardiner, M.R. (1965). The pathology of lupinosis of sheep, gross– and histopathology. *Pathologia Veterinaria* **2**: 417–445.

Gardiner, M.R. (1967a). Lupinosis. *Adv. Vet. Sci.* **11**: 85–138.

Gardiner, M.R. and Parr, W.H. (1967b). Pathogenesis of acute lupinosis in sheep. *J. Comp. Pathol.* **77**: 51–62.

Gardiner, M.R. (1975). Lupinosis. *J. Agric. West. Aust.* **16**: 26–30.

Hough, G.M. and Allen, J.G. (1994). Low intakes of phomopsins do not affect milk production by dairy cattle in mid–lactation. *Aust. Vet. J.* **71**: 154–156.

Jago, M.V., Peterson, J.E. and Payne, A.L. (1982). Lupinosis: Response of sheep to different doses of phomopsin. Aust. *J. Exp. Biol. Med. Sci.* **60**: 239–251.

Lacey, E., Edgar, J.A. and Culvenor, C.C. (1987). Interaction of phomopsin A and related compounds with purified sheep brain tubulin. *Biochem Pharmacol*. **36:** 2133–2138.

Mackay M. F., Donkelaar, A. and Culvenor, C.C. J. (1986). The X–ray structure of phomopsin A, a hexapeptide mycotoxin. *J. Chem. Soc. Chem. Commun*. 1219–1221.

Papadimitriou, J.M, Bradshaw, R.D., Petterson, D.S. and Gardiner, M.R. (1974). A histological histochemical and biochemical study of the effect of the toxin of lupinosis on murine liver. *J. Pathol* **112**: 43–53.

Peterson, J.E. and Lanigan, G.W. (1976). Effects of *Phomopsis rossiana* toxin on the cell cycle and on the pathogenesis of lupinosis in mice. *J. Comp. Pathol.* **86**: 293–306.

Peterson, J.E. (1978). *Phomopsis leptostromiformis* toxicity (lupinosis) in nursling rats. *J. Comp. Pathol.* **88**: 191–203.

Peterson, J.E. (1983). Embryotoxicity of phomopsin in rats. *Aust. J. Exp. Biol. Med. Sci.* **61**: 105–115.

Peterson, J.E. (1986). The toxicity of phomopsin. In: *Proceedings of the Fourth Internation Lupin Conference*. Western Australian Department of Agriculture, South Perth, pp 199–208.

Peterson, J.E., Jago, M.V., Payne, A.L. and Stewart, P.L. (1987). The toxicity of phomopsin for sheep. *Aust. Vet. J.* **64**: 293–298.

Petterson, D.S., Peterson, J.E., Smith, L.W., Wood, P.McR. and Culvenor, C.C.J. (1985). Bioassay of the contamination of lupin seed by the mycotoxin phomopsin. Aust. *J. Exp. Agric*. **25**: 434–439.

Than, K.A., Tan, R.A., Petterson, D.S. and Edgar, J.A. (1994). Phomopsin content of commercial lupin seed from Western Australia in 1991/92. In: *Plant-associated Toxins*, (Eds. S.M. Colegate and P.R. Dorling), CAB International, Wallingford, UK, pp 62–65.

Tonsing, E.M., Steyn, P.S., Osborn, M. and Weber, K. (1984). Phomopsin A, the causative agent of lupinosis, interacts with microtubules *in vivo* and *in vitro*. *Eur. J. Cell Biol*. **35**: 156–164.

van Warmelo, K.T., Marasas, W.F.O., Adelaar, T.F., Kellerman, T.S., van Rensburg, I.B.J. and Minne, J.A. (1970). Experimental evidence that lupinosis of sheep is a mycotoxicosis caused by the fungus *Phomopsis leptostromiformis* (Kuhn) Bubak. *J. South African Vet. Med. Assoc*. **41**: 235–247.

Wood, P. McR. and Petterson, D.S. (1986). *Phomopsis leptostromiformis* infection and phomopsin A content of lupin seed in Western Australia. *Aust. J. Exp. Agric*. **26**: 583–586.