TOX/2008/12

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

BRACKEN

Issue

1. Bracken is an invasive toxic fern that is common throughout the world. There are several different sub-species and varieties throughout the world. The varieties that are found in the UK are all of the *Pteridium aquilinum* subspecies *aquilinum.* The UK varieties of this subspecies are *aquilinum* (the most common), *latiusculum* and *atlanticum*. Bracken is found in all parts of the country and dense growths cover large amounts of land in Wales, Scotland and northern England.

2. Eating bracken is known to be harmful to farm animals and there is evidence that it also be harmful to humans. The varieties found in the UK are toxic and potentially fatal to animals if eaten, but there is great variability in the amount of the bracken toxin ptaquiloside, and possibly of other bracken toxins, in different varieties of bracken that are found throughout the world. There is also variation between strains within particular varieties of bracken. All parts of the bracken plant contain potentially harmful chemicals, some of which can be excreted in milk and may leave residues in meat and offal derived from animals that ate the plant. Thus there is a potential hazard to consumers.

3. At the meeting of the interdepartmental Quarterly Review of Incidents that was held on 3rd July 2007 it was agreed that the COT should be asked to advise on the consumer safety issues associated with eating foods derived from bracken-poisoned animals. It would be helpful if COT could also advise on the consumer safety of foods derived from animals that ate bracken without showing clinical signs of poisoning. The Veterinary Laboratory Agency (VLA) had identified several cases of suspected bracken poisoning in farm animals and wanted advice on what, if any, restrictions should be placed on using foods derived from the poisoned animals for human consumption. The VLA noted that there were probably many additional cases of bracken poisoning that had not been reported to them, and that there would be large numbers of animals that had eaten bracken without showing signs of poisoning. Officials from the FSA advised that the foods from bracken-exposed animals might contain residues of bracken-derived toxic substances, including genotoxic carcinogens, but there was no readily-available information on which substances presented the greatest hazard, what concentrations of them were present in foods and how rapidly the

residues were cleared from edible tissues. In the absence of such information, it was not possible to give unequivocal advice on when milk from affected animals could be safely used for human consumption or when the animals could be slaughtered.

4. The COT and its sister committees COM and COC last advised on the safety of foods derived from animals reared on bracken infested land in the Annual Reports of COM in 1993 and of COT in 1996. The Committees had considered the available information on carcinogenicity and mutagenicity of bracken, along with the results of a government-sponsored study of the transfer of bracken mutagens into milk from goats fed on bracken. They advised that the risk to consumers was very low and that further research need not be undertaken on bracken mutagens. This advice was based partly on the assumption that cattle and goats will not eat bracken if other food is available. A more detailed summary of the evaluations by COM, COC and COT is given in Section 1 of Annex A.

5. Since 1996, further information on bracken has been published. The COT is asked to review the available data on the bracken, taking particular note of the information that has become available since the last COT evaluation. The COT is requested to advise on the consumer safety of foods derived from animals that had been exposed to bracken, or to identify gaps in the data that prevent it from giving such advice.

Literature Search

6. Computer searches of the literature were performed in order to identify recent articles on the toxicology, including epidemiology, carcinogenicity and mutagenicity, of bracken and of several of the chemicals found in bracken. Articles on the amounts of these chemicals in bracken and released from bracken into aquifers or following consumption by animals, and on their pharmacokinetics were also sought. Details of these searches are given in Annex B. Further relevant articles were identified from the references cited in the articles identified by the computer search and from articles on bracken that were already on file.

Toxicology of Bracken

7. There are numerous articles available that deal with aspects of the safety of bracken and its constituent chemicals. Many of the studies were old and few of them were up to modern standards. An overview of the relevant available data is presented as Annex A. A list of reference can be found in Section 7, at the end of Annex A.

8. It has been known for over a century that bracken is toxic to farm animals. Acute poisoning by feeding on bracken can be fatal. Prolonged ingestion of sub-

lethal amounts of bracken can lead to a complex array of ailments, including hypoplasia of the bone marrow, thrombocytopaenia, leucopaenia, immunosuppression, reduced intestinal uptake of iron, thiamine (vitamin B_1) deficiency and severe internal haemorrhages. The most visible symptoms in farm animals are weakness, lack of motor coordination of back legs and neck, propensity to acquire infectious diseases, breathing difficulties and nasal, rectal and urinary bleeding. Exposure to bracken is also associated with the development of various tumours in different species. In non-ruminant species, the principal adverse effect of eating bracken is to cause a deficiency of thiamine (vitamin B_1), but this does not occur in ruminants. In cattle, exposure can cause an acute haemorrhagic syndrome and a related chronic disease called bovine enzootic haematuria (BEH) that involve changes to the blood vessels of the urinary bladder and the later development of benign and malignant bladder tumours. Syndromes similar to BEH have also been described in buffalo, sheep and deer. Bracken feeding of cattle has also been associated with a slow-developing epidermoid carcinoma of the upper digestive tract and a progressive retinal degeneration. Sheep are more prone to progressive retinal degeneration (called bright blindness or PRD in sheep) than cattle. In quail, the feeding of bracken caused reduced testis weight and reduced male fertility and feeding of a solvent extract of bracken caused adenocarcinomas of the caecum, colon and distal ileum.

9. There are several epidemiological studies of human populations in Japan, Brazil, Venezuela, Costa Rica and Wales that show an association between eating bracken and the development of cancers of the stomach and oesophagus. None of the studies is definitive. Although there is a strong association between eating bracken and cancer in cattle, the strength of association is less in human studies. The results do however consistently show an association between eating bracken and human stomach cancer. Many of the human studies were too small to show a dose-response relationship, but one Japanese study showed a higher risk of oesophageal cancer in people who ate bracken regularly than in those who ate it only rarely. Recently, a Japanese study showed an association between eating wild plants (mainly bracken) and pancreatic cancer in men.

10. Bracken caused skeletal abnormalities in the offspring of pregnant mice to whom it was fed, showing that a toxic component of bracken can cross the placental barrier.

11. There is no carcinogenicity bioassay of bracken that has been performed to modern standards, but its carcinogenicity has been investigated in numerous more limited studies in a variety of species. Feeding of bracken to various strains of mice produced a variety of neoplasms, including leukaemia, lung adenomas, intestinal tumours, bladder tumours and liver nodules. In guinea-pigs, dietary bracken caused intestinal adenomas and adenocarcinomas and transitional cell carcinomas of the bladder, and it also caused panmyelopathy of the bone marrow and haematuria. In toads, dietary bracken caused ileal adenocarcinomas

and malignant liver tumours. Most of the studies were performed in rats. Dietary bracken caused various tumours including gastrointestinal tumours, including adenocarcinomas and sarcomas, mainly in the ileum; transitional cell carcinomas of the urinary bladder; and pre-neoplastic nodules in the liver. Various experiments were performed in rats to characterise the agent causing the carcinogenicity. It was found that all parts of the bracken plant are carcinogenic, but the most potent part is the crosier (young unfolded bracken frond), which is the part of the plant eaten by humans in certain parts of the world. The traditional ways of processing bracken for human consumption (boiling in water or treatment with wood ash, salt or sodium bicarbonate) had the effect of reducing its carcinogenic potency to rats.

12. Cytogenetic analysis of peripheral blood taken from cows or people who had eaten bracken showed increased numbers of chromosomal aberrations. Mice fed Welsh bracken spores were found to have DNA-adducts in their stomach and small intestines, but not in the liver. In contrast, rats fed Brazilian bracken (a sub-species not found in the UK) did not have DNA-adducts in their stomach or ileum. Various extracts of bracken (including boiling water, acetone, methanol and ethanol extracts) were mutagenic to Ames strains of *Salmonella typhimurium*, as was milk from bracken-fed cows. It was very briefly reported that a fraction that had been isolated chromatographically from bracken was mutagenic to *Drosophila* and mice, but no details of the test were given.

Ptaquiloside

13. Two separate groups of workers in Japan and the Netherlands independently identified ptaquiloside (a norsesquiterpene glycoside of the illudane type) as the principal carcinogen in bracken. They used various solvent extraction, resin adsorption and chromatographic techniques to separate various fractions which they tested with mutagenicity or short-term carcinogenicity assays in rats to find those with the highest mutagenic/carcinogenic potency. The most purified fraction was then analysed by NMR spectroscopy to identify the chemical structure of the principal compound present. Ptaquiloside is stable when present in plant tissues, and isolated ptaquiloside readily breaks down in alkaline conditions to for its carcinogenically active form, activated ptaquiloside (APT). Both ptaquiloside and APT slowly break down in acidic conditions to form pterosin B, which is not carcinogenic. Composting bracken destroys the ptaquiloside in it.

14. Ptaquiloside might be responsible for much of the toxicity of bracken. Progressive retinal degeneration (PRD or bright blindness) has been reproduced in sheep given ptaquiloside intravenously. Haemorrhagic cystitis and haematuria have been produced in guinea-pigs (but not rats or mice) given subcutaneous ptaquiloside.

15. It has been suggested that ptaquiloside is responsible for more than half of the carcinogenic potency of bracken. There is no carcinogenicity bioassay of ptaquiloside that has been performed to modern standards, but its carcinogenicity has been investigated in more limited studies in rats. Ptaquiloside was administered as weekly oral doses of 100 to 780 mg/kg bw for 8 or 8½ weeks and then the rats received no further treatment for the rest of their lives. The highest dose caused haematuria and a loss of bodyweight and all dose levels produced tumours of the mammary gland (adenocarcinomas, papillary carcinomas and anaplastic carcinomas) and ileum (adenocarcinomas). Hyperplasia was seen in the bladders of many of the treated rats. No tumours were seen in rats that had been given weekly intravenous doses of 20.7 mg/kg bw of ptaquiloside for 10 weeks, followed by 30 weeks without further treatment, but these rats developed monocytosis and focal renal tubular necrosis.

16. Ptaquiloside was tested for mutagenicity at different pHs. It was not mutagenic in either TA98 or TA100 strain of *Salmonella typhimurium* when tested at pH 7.4 in the absence of metabolic activation, but was mutagenic in both strains if it was pre-incubated at pH 8.5. It caused chromatid exchange type aberrations in CHL-cells in the presence and absence of S9 at pHs 5.3, 7.4 and 8.3, but the genotoxic potency was greater at the higher pHs. Ptaquiloside also produced DNA-adducts *in vitro* and caused *in vitro* unscheduled DNA synthesis in a rat hepatocyte culture at pH 7.2.

17. It is thought that the reason for the higher genotoxic potency of ptaquiloside at higher pH is because under mildly alkaline conditions, ptaquiloside is converted by β-elimination into an illudane-dienone compound, which is referred to as APT (activated ptaquiloside). APT possesses a highly reactive cyclopropyl ring. It is electrophilic with a greater capacity to alkylate DNA than ptaquiloside. It is stable in mildly alkaline conditions, but under acidic conditions it is converted to a less reactive substance, pterosin B. Pterosin B is also formed from the breakdown of ptaquiloside under acidic conditions. Compounds that were chemically similar to APT but lacked an activated cyclopropane moiety were not mutagenic to *Salmonella typhimurium* TA98 or TA100 strains. Whereas no tumours were seen in rats that had been given weekly intravenous doses of 20.7 mg/kg bw of ptaquiloside for 10 weeks, followed by 30 weeks without further treatment, the same treatment with 20.7 mg/kg bw APT caused tumours of the mammary (adenocarcinomas and papillary carcinomas) and ileum (adenocarcinoma) along with elevated plasma levels of tumour necrosis factor TNFα, monocytosis, focal type II pneumonocyte necrosis and focal renal tubular necrosis. Investigation of samples of ileum from the rats from this study by ^{32}P-postlabelling showed the presence of DNA-adducts that gave a spot in thin-layer chromatography in a similar place to the adducts found when rats were treated with ptaquiloside. Other studies showed the that APT binds covalently to purine bases on DNA, opening the cyclopropyl ring of the molecule and forming adducts with the N-7 of guanine and the N-3 of adenine. Alkylation of adenine (but not that of guanine) caused cleavage of the N-

glycosidic linkage of the modified adenines to produce abasic sites on the DNA molecule. The abasic sites were unstable and breakage of the phosphodiesterpentose backbone of the DNA molecule occurred. Investigations were made of the H-*ras* and *p53* genes from the mammary glands of rats that had received weekly intravenous doses of 20.7 mg/kg bw and had been killed immediately after dosing. No mutations were found in *p53*, but there were double mutations at codons 58 and 59 of the H-*ras* gene. Mutations of the H-*ras* proto-oncogene also occurred in the ileums of calves fed bracken in their diet

18. Oral dosing with APT at 6 weekly doses of 20.7 mg/kg bw or at 3 weekly doses of 41.4 mg/kg bw did not produce any tumours when the animals were killed 30 weeks later. Possibly insufficient time had been allowed for tumours to develop. However the orally-treated rats showed necrosis of blood cell precursors in the bone marrow and had apoptotic bodies in their livers, addition to tubular necrosis of the kidneys, monocytosis and elevated plasma tumour necrosis factor TNFα.

19. It seems likely that ptaquiloside, and its more active form ATP, are responsible for much or the carcinogenicity of bracken and for at least some of its other toxic effects. Tumours are produced by a genotoxic mode of action.

Other Potentially Toxic Substances in Bracken

20. Bracken contains a large number of substances in addition to the illudanes-type sesquiterpene glycoside, ptaquiloside. The other substances detected in bracken include other illudanes and protoilludanes (such as ptaquiloside Z, isoptaquiloside, pteroside A2 and caudatoside), terpenic indanones (pterosins), thiaminases, the cyanogenic glycoside prunacin, braxin glycosides, *p*-hydroxystyrene glycosides (ptelatosides A and B), the flavinoid quercetin and its glycoside rutin, kaempferol, shikimic acid, ecdysteroids, tannins, dihydrocinnamic acids, phloretic acid, dihydroferulic acid, 2,3-butanediol, 3-methylbutan-2-ol, monomethylsuccinate, methyl-5-oxoproline, 2(3H)-dihydrofuranone and *t*-2-methylcyclohexanol. Little is known of the toxicology of many of these substances and information on the amounts in bracken are often lacking.

21. No toxicological data are available for the illudane-type substances other than ptaquiloside, but their chemical similarity to the genotoxic carcinogen ptaquiloside raises the concern that some of them might be similarly carcinogenic.

22. The indanones are found in high concentrations (approximately 24 mg/kg (w/w)) in young fronds, but they do not act as alkylating agents. Pterosin B is much less electrophilic than ptaquiloside. A range of different indanones from bracken were shown to be non-mutagenic at pH 7.4, when tested in *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of S9. They

were also non-clastogenic when tested in CHL cells in the absence of metabolic activation. Extracts containing high levels of pterosins A, B, C and others did not cause leucopaenia or thrombopaenia in calves.

23. The *p*-hydrostyrene glycoside, ptelatoside A, was tested for carcinogenicity in rats at a concentration of 1.3 mg/kg in the diet (equivalent to 0.065 mg/kg bw/day) for 109 or 125 days. At this dose, there was no evidence of any carcinogenicity. There was insufficient ptelatoside A available to test higher concentrations.

24. Quercetin was found in bracken at 570 mg/kg (dry weight), but it is also found in many other fruits and vegetables, often at higher concentrations (eg. up to 65,000 mg/kg in onions). Thus it is considered unlikely that quercetin is responsible for the toxicity of bracken. There is some evidence that quercetin might be genotoxic and it might be carcinogenic. There is also some evidence that it has anti-cancer properties. It is possible that the presence of quercetin might contribute to the overall carcinogenicity of bracken.

25. Kaempferol is chemically similar to quercetin, from which it differs by lacking one hydroxyl group. It was found at 1100 mg/kg (dry weight) in bracken. It is also found in certain other plants. Tea can contain up to 10,000 mg/kg of quercetin plus kaempferol, combined. The results of mutagenicity tests suggest that it is an *in vivo* mutagen, but a limited study in rats (400 mg/kg feed given to 6 males and 6 females for 540 days) showed no evidence to suggest that it was carcinogenic.

26. Shikimic acid was found at 1440 mg/kg (dry weight) in Welsh bracken. Shikimic acid was not genotoxic in bacterial or *in vivo* mammalian assays (mouse bone marrow assay and UDS in rat gastric mucosa). Mice given intraperitoneal doses of shikimic acid developed stomach cancer and leukaemia. The carcinogenic potency was reported to be lower if the shikimic acid were first treated with alkali. This is in contrast to bracken, which increased its carcinogenic potency when treated with alkali.

27. The tannins in bracken are mainly condensed tannins derived from procyanidin and prodelphinidin. Fronds of tropical bracken can contain up to 0.012 mg/kg of tannins. There is limited evidence to suggest that some tannins might be carcinogenic to rats, but the feeding of bracken tannins at a dietary concentration of 4000 mg/kg for up to 72 weeks did not cause any cancer in F344 or Sprague-Dawley rats.

28. Anti-thiamine enzymes, thiaminases, seem to cause most of the short- to medium-term symptoms of bracken poisoning in monogastric animals. Thiaminases types 1 and 2 have been found in bracken at activities of 3.1 and 3.5 µg thiamine destroyed/g plant material/hour, respectively. A third more heat-stable thiaminase (possibly caffeic acid, a substance that also has both pro- and

anti-cancer properties) might also play a role. Rats developed typical nervous lesions of antivitaminosis-B_1 that could be cured by thiamine (vitamin B_1). Thiaminase activity is highest in rhizomes and very young fronds. No reports of thiamine deficiency in bracken-consuming human populations have been reported. It is possible that humans are less prone to thiamine deficiency than farm animals as a result of having a more varied diet.

29. Prunacin is a cyanogenic glycoside that is found in the highest concentrations in young fronds. Not all varieties of bracken are cyanogenic as some lack either prunacin or the β-glycosidase needed to liberate hydrocyanic acid from it. Farm animals seem to avoid eating the cyanogenic varieties. Prunacin is usually present in bracken at harmless quantities, but there have been fatal cases of cyanide poisoning in animals that have been fed on young fronds of bracken.

30. Braxins A1 and A2 are β–glucopyranosides that are present in bracken rhizomes at up to 600 mg/kg. Braxins A1 and A2 were not present in bracken fronds although braxin B was detected. Ptaquiloside and braxins A1, A2 and B each caused haemorrhagic cystitis in guinea-pigs within 24 to 48 h of first exposure. Braxins A1 and A2 caused swelling of and release of histamine from mast cells *in vitro*.

31. Bracken contains several ecdysteroids which can prevent insects from moulting and developing into adults. They are not thought to be a toxic hazard to mammals.

Exposure

32. It is rare for people in the UK to eat bracken. However, in some parts of the world including Japan, Brazil, New Zealand, Canada and the USA, the young curled bracken fronds (called crosiers or fiddleheads) are eaten. Analysis of fronds and rhizomes of the bracken variety that is most common in the UK (*Pteridium aquilinum* var. *aquilinum*) found them to contain ptaquiloside concentrations of 213-2145 mg/kg and 11-902 mg/kg, respectively.

33. Inhalation exposure to spores of bracken is possible in all parts of the UK. However, the amount of bracken material inhaled is likely to be much less than the amount ingested when eaten as a food. The highest levels of spores in the air are likely to be in bracken infested areas during dry weather in July to September. Inhaled spores (about 30 µm diameter) would be expected to become trapped in the mucus of the nasopharynx and to be then swallowed.

34. There is also a potential for indirect exposure to toxins from bracken as a result of eating foods derived from animals that have eaten bracken or as a result of water-soluble substances from bracken leaching into water sources that are used for drinking water for humans and farm animals. Soils at sites where

bracken (*Pteridium aquilinum* var. *aquilinum*) was growing contained 0.22-8.49 mg/kg of the water-soluble carcinogen ptaquiloside. Laboratory experiments showed that exposure of fresh mature bracken fronds to water aerosols for 1.5 h caused 0.18-0.24% of the ptaquiloside in the fronds to dissolve into the water. The amount leaching from young fronds or decaying fronds might be greater than this, but no data are available. No information is available on the concentrations of substances derived from bracken that occur in drinking water in different parts of the country, but there is potential for contamination of private and municipal supplies as a result of run-off of water soluble substances (such as ptaquiloside) from bracken-infested land.

35. Some food producing animals eat bracken. Intensive grazing, usually by sheep or pigs, is used in the UK to clear bracken and to reduce invasion of pastures. There may also be some exposure to bracken as a result of the traditional use of bracken as bedding for animals. It is theoretically possible that animals that are exposed to bracken could have residues of harmful bracken-derived chemicals in their tissues, which could be eaten by human consumers. No information is available on the concentrations of residues of any toxic substances derived from bracken in meat and offal or their rates of depletion from edible tissues.

36. There have been 22 recent (1999-2007) documented cases of UK cattle being poisoned by bracken, although the total number of poisonings is likely to be greater than this. Toxic substances from bracken can be excreted into the milk. The milk from bracken-fed cows caused leucopaenia in calves, produced bladder cancer in mice, produced cancers of the intestines, bladder and kidneys in rats. Various solvent extracts of the milk were mutagenic to *Salmonella typhimurium* strains TA98 and TA100, and caused pulmonary adenomas in the offspring of mice that had been exposed during pregnancy. Thus it seems that toxic agents in bracken can be passed into the milk and can cross the placental barrier. There is a potential hazard from toxic components of bracken being passed into milk intended for human consumption. People who consume local unbulked milk or dairy products from bracken infested areas would be expected to be at greater risk than those drinking only bulked milk from commercial dairies.

37. Toxic components of bracken that have been detected in milk from bracken-fed cows include ptaquiloside and shikimic acid. A concentration of 0.11 mg/L of ptaquiloside was found in milk from a cow that had been fed for 7 days on 6 kg/day of fresh bracken fronds that contained 0.25 ± 0.05 mg/g of ptaquiloside. The total amount of ptaquiloside that was excreted into the milk of this cow was equal to 1.2% of the amount of ptaquiloside that it ingested. In another study two cows transferred into their milk 8.6 ± 1.2% of the ptaquiloside they ingested from 6 kg/day of fresh fronds of bracken that provided doses of 2400 to 10000 mg/cow/day of ptaquiloside. Doses of 2400, 4500, 8100 and 10000 mg/cow/day of ptaquiloside in bracken produced peak concentrations of 10, 27, 38 and 55 mg/L of ptaquiloside in the milk. After a few days the cows

refused to eat the feed containing the highest concentration of ptaquiloside. Ptaquiloside was first detected in milk at 38 h after the start of dosing and peaked at 86 h. After feeding of bracken stopped, the concentrations gradually dropped off until none was detectable at 86 h after the end of the dosing period.

38. Data from the most recent UK food surveys (1986-2002 depending on the subpopulation investigated) indicate that the sector of the UK population with the highest chronic intake of milk is infants aged 6-12 months (1992-1993 survey). It has been estimated that a UK infant having the upper 97.5th percentile chronic intake of milk (851 g/person/day for milk excluding infant formulae and breast milk) from a cow that had been fed a sub-clinical dose of 5000 mg/day of ptaquiloside in bracken could receive a dose of up to 22.8 mg/person/day of ptaquiloside (or 2.62 mg/kg bw/day for an infant weighing 8.7 kg). If intakes of breast milk and infant formulae are included in the estimate, the 97.5th percentile intake of infants is estimated to be 3.7-28.2 mg/person/day (or 0.43-3.24 mg/kg bw/day). This gives a very conservative estimate of the maximum chronic consumer intake of ptaquiloside from milk from bracken-exposed cows that show no clinical signs.

39. Since the 1992-1993 survey of intakes of foodstuffs by infants, the Government has published advice that cows' milk should not be directly fed to infants of age 1 year or less. As a consequence of this, it is conceivable that the intake of milk by infants is now less than the figures indicated by the 1992-1993 survey, and consequently the chronic intakes of ptaquiloside by UK infants that are reported in the preceding paragraph might well be overestimated. Furthermore, most consumers would consume bulked milk, where the milk from any cows receiving such high intakes would be diluted with milk from cows with low or zero intakes of ptaquiloside. It is also conceivable that pasteurisation and other processing of milk would further reduce the levels of ptaquiloside present (although there are no data to confirm this).

40. The results of UK food surveys indicate that, after infants, the subpopulation with the highest per capita chronic intake of milk is the institutional elderly and those with the highest intake in relation to bodyweight are toddlers aged 1½-4½ years. Using the same information on possible amounts of ptaquiloside in cows' milk as used to estimate the intake of infants, the 97.5th percentile intakes of ptaquiloside by the institutional elderly and toddlers have been estimated to be 2.9-22.1 mg/person/day (0.047-0.36 mg/kg bw/day) and 2.8-21.6 mg/person/day (0.19-1.49 mg/kg bw/day), respectively.

41. The above estimates of intake of ptaquiloside from milk represent the maximum intake that might be anticipated to occur as a result of drinking milk solely from cows receiving the maximum dose of ptaquiloside that will not cause clinical signs of toxicity in the animals. Milk from bracken poisoned cows might well contain higher amounts of ptaquiloside and the acute human exposure from occasionally consuming milk from poisoned cows would be expected to be

higher. Exposure to ptaquiloside and other bracken toxins might also occur from other sources, including drinking water and consumption of foods derived from bracken-exposed animals. However, no quantitative data are available on the levels of exposure from sources other than milk.

CONCLUSIONS

42. The Committee is asked to consider the following proposals for conclusions that may be drawn.

i. The eating of bracken over a long period of time can cause cancer in humans and in animals. In humans, the stomach and the oesophagus are the most common sites for such cancer to develop.

ii. The UK population does not normally eat bracken, so direct eating of bracken is unlikely to be a health hazard in the UK, unless dietary habits change.

iii. Although thiamine deficiency caused by thiaminases is the major effect of short- to medium-term exposure to bracken in monogastric animals, this effect is not known to occur in humans.

iv. Several of the chemicals in bracken have the potential to contribute to the carcinogenicity of bracken. However, the substance that appears to be responsible for most of the carcinogenicity is ptaquiloside, which needs to be converted into its active form, APT.

v. Ptaquiloside is genotoxic and carcinogenic.

vi. It is not possible to identify a dose of ptaquiloside to which consumers could be exposed without increased risk of developing cancer. This is because it is a genotoxic agent and also because the quality of the carcinogenicity studies is insufficient to allow the reliable identification of a clear NOAEL or benchmark dose level. Only high concentrations of 20.7 mg/kg bw or more were tested, so no conclusion can be made about the potency of ptaquiloside.

vii. The UK population might be exposed to harmful chemicals from bracken as a result of contamination of drinking water or by consuming residues in foodstuffs derived from animals that ate bracken.

viii. The available information is insufficient to indicate the amounts of ptaquiloside or other toxic chemicals that are present in drinking water that is collected from bracken infested areas.

ix. There is considerable uncertainty about the amount of ptaquiloside that humans might consume in milk and other foods derived from animals that ate bracken. The estimates given in paragraphs 38-40 represent the highest amounts of ptaquiloside that might be taken in by heavy consumers of milk, assuming that all of the milk came from cows with largest intake of ptaquiloside that could be eaten without them showing any clinical signs of poisoning (assuming that milk from poisoned cows would not be used for human consumption). In practice, consumer intakes are likely to be less than these estimates as a result of bulking of milk sourced from many cows and possibly also due to processing of the milk.

x. The available information is insufficient to indicate the amounts of ptaquiloside or other toxic chemicals that might occur in meat and offal derived from animals that ate bracken.

xi. No information was available on the rates of depletion of residues of bracken-derived substances in milk or edible tissues of food-producing animals. Therefore it is not possible to give clear advice on how long bracken-poisoned animals should be left before it is safe to use their milk for human consumption or to slaughter them for human consumption.

RECOMMENDATIONS

43. The Committee may wish to consider whether further research is needed to identify:

i. The amount of ptaquiloside and/or other harmful substances that can be present in drinking water that is collected from bracken-infested land.

ii. The concentrations of residues of ptaquiloside and/or other harmful substances derived from bracken that might be present in foodstuffs derived from animals that were poisoned by bracken.

iii. The rates of depletion of bracken derived residues from edible tissues and milk from animals that were poisoned by bracken.

iv. The carcinogenic potency of ptaquiloside and/or other carcinogens present in bracken.

Secretariat
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TOX/2008/12 – Annex A

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

BRACKEN: SUMMARY OF AVAILABLE DATA RELATING TO CONSUMER SAFETY OF FOODS DERIVED FROM ANIMALS EXPOSED TO BRACKEN

Contents

1. Background

In 1986, the International Agency for Research on Cancer (IARC) classified bracken in their Group 2B: possibly carcinogenic to humans. This classification was based on the conclusion that the available epidemiological evidence for carcinogenicity in humans was inadequate, but that there was sufficient evidence of carcinogenicity in animals that it should be regarded as if it were carcinogenic to humans (IARC, 1986).

In 1988, the COC was asked by The Ministry of Agriculture, Fisheries and Food (MAFF) to consider the carcinogenicity of bracken and its human health risks, following reports that bracken may constitute a human health risk. The COC was also asked to consider the possible occurrence of bracken “toxins” in the food supply. A paper (CC/88/8) reviewing the published data on the toxicology and epidemiology of bracken in relation to

carcinogenicity was prepared and was discussed at the meeting of COC that was held on 17th May 1988 (CC/MIN/88/2). Further information related to the carcinogenicity of bracken were presented to the following meeting of COC on 18 October 1988 (CC/MIN/88/3): a page of data giving the annual numbers of new cases of small bowel neoplasia reported in Scotland over the years 1971 to 1984 (CC/88/21 as amended); and a report from the Secretariat of presentations given at a meeting of the International Bracken Group on 28th September 1988 (CC/88/26). In 1990, the COC commented on an additional epidemiology study of gastric cancer in Gwynedd, Wales (Galpin, *et al*., 1990) that was presented to the Committee as CC/90/23. The study was criticised for the lack of a method for assessing exposure to bracken, the small number of subjects investigated, the use of hospital data as controls, and the lack of consideration of confounding factors, such as salt in the diet (CC/MIN/90/2).

At the time, there was little information available about the extent to which farm animals grazed on bracken and the occurrence of bracken constituents in dairy products. The available human epidemiological data were limited and the COC advised that evidence of carcinogenicity to humans was inconclusive. Carcinogenicity studies in laboratory animals in which whole bracken had been administered in the diet, in some cases accounting for up to one third of the total diet, were flawed in their experimental design, execution and interpretation. Despite these limitations, the COC concluded that these studies had demonstrated a clear trend for the increased incidence of benign and malignant tumours of the small and/or large intestine. and/or urinary bladder and that there was a need for properly conducted carcinogenicity bioassays in rats and mice. The COC also evaluated data on the active constituents of bracken and concluded that ptaquiloside, an inherent constituent of bracken, had been shown to be capable of reproducing some of the carcinogenic effects of whole bracken.

Following the conclusions of the COC, MAFF commissioned work to investigate whether mutagenic components in bracken could be passed into the milk of bracken-fed goats and thus enter the food chain. Goats had been used rather than cattle as it was expected that they would be less likely to be reluctant to graze bracken, however the results of the study showed that bracken was unappetising to goats. MAFF received the results in 1992 and asked the COM to comment on them. These data were presented, alongside the results of several recent mutagenicity studies on solvent extracts of bracken (Matoba, *et al*., 1987; Matsuoka, *et al.,* 1989; Moura, *et al*., 1988; Nagao, *et al.,* 1989; Ojika, *et al.*, 1987), in a paper (MUT/93/8) that was discussed at the meeting of COM that was held on 11th February 1993 (MUT/MIN/93/1). The Members of COM agreed the following conclusions:

(i) Solvent extracts of bracken showed mutagenic activity in bacterial assays. There was evidence that most of the mutagenic activity appeared to be due to the compound ptaquiloside, but other potentially mutagenic compounds might be present. There was evidence that mutagenic activity was expressed in mammalian cells *in vitro*. There were limited data available which suggested this mutagenic potential might be expressed *in vivo*.

(ii) Work carried out for MAFF on the possibility of bracken mutagens being transmitted to the milk of bracken-fed goats suggested that very little, if any,

mutagenic activity is present in the milk of goats exposed to UK bracken for periods of time of up to one month.

(iii) In view of the fact that cattle and goats would not eat bracken if other food is available, the Committee recommend that no further work should be carried out at present on the risk of transmission of mutagenic compounds from bracken into milk for human consumption, provided that the milk was bulked and processed centrally. (COM, 1993; COT, 1996)

Overviews of the advice of the COC and the COM were given in the Annual Reports of the COM (COM, 1993) and the COT (COT, 1996). The COT accepted the advice of the COC and COM and agreed that the risk to the human population was very low and that further research need not be undertaken on bracken mutagens (COT, 1996).

Cases of bracken poisoning tend to occur sporadically, but can involve significant losses in affected groups of cattle, usually young stock (Cranwell, 2004). There have been sporadic anecdotal reports of bracken poisoning in cattle in Great Britain that have been recorded by the Veterinary Laboratory Agency (VLA) on the Veterinary Investigation Diagnosis Analysis (VIDA) over the years 1975-2003. There were peak incidences occurring in certain years (1975, 1985, 1994, 1995), often those with long hot dry summers during which cattle may seek food that is not their normal ration (Cranwell, 2004). There were 22 cases of bracken poisoning in cattle recorded on the VIDA database between 1999 and April 2007 (Livesey, 2007). One case was a 4-year-old dairy cow, one was a 20-month-old-heifer (pregnant but not yet lactating) and the rest were beef cattle. Bracken poisoning in sheep is not recorded on VIDA as a separate diagnosis but is recorded as a general "plant poisoning" category, so information on the number of cases of bracken poisoning in sheep was not available. The cases of bracken poisoning that are recorded on VIDA are considered by VLA to be the "tip of the iceberg" of cases of clinical poisoning, as farmers and vets are not required to report cases of bracken poisoning to VLA. There are likely to be many more instances of sub-clinical exposure of food-producing animals to bracken. When VLA have investigated bracken poisoning incidents they have usually found a low incidence of clinical poisoning in the potentially exposed group of animals, but with a large proportion of animals showing severe anaemia and agranulocytosis without displaying clinical signs.

Two incidences of suspected bracken poisoning in pigs were reported in 2007 (Harwood, *et al.,* 2007). The first incident involved a group of 18 young saddleback pigs, 2 of which showed respiratory signs and were shivering. One subsequently died. The other incident involved 10 young pigs, 2 of which showed respiratory signs and 1 later died. These incidents are discussed further in Section 5.2.1.

Food-producing animals can be exposed to bracken by grazing on bracken-infested land, by intentional feeding of bracken or as a result of the unintentional presence of bracken in feed. There has been concern over the possible presence of toxic or carcinogenic bracken-derived substances occurring in foods (meat, offal, milk, etc.) that are produced by animals that have suffered bracken poisoning. At the meeting of the interdepartmental Quarterly Review of Incidents that was held on 3rd July 2007 it was agreed that the COT

should be asked to advise on the consumer safety issues associated with eating foods derived from bracken-poisoned animals.

2. Bracken varieties found in Britain

There are two main sub-species of bracken (*Pteridium aquilinum*): *aquilinum* and *caudatum* and there are several varieties within each sub-species. The sub-species *caudatum* (including varieties *caudatum, arachnoideum, esculentum* and *yarrabense*) does not grow wild in the UK, but several varieties of subspecies *aquilinum* do.

Prior to 1989, only a single formal taxon of British bracken, *Pteridium aquilinum* subspecies *aquilinum* variety *aquilinum*, was widely recognised, but more recently additional varieties (*latiusculum* and *atlanticum*) have been recognised (Wolf, *et al*., 1994). The *aquilinum* variety is the most common and is found throughout the UK. The *latiusculum* variety (common to North America and Asia) is found in native pinewood in Scotland and the variety *atlanticum* is found mostly on limestone areas of Scotland and Wales.

Other varieties of the *aquilinum* subspecies that do not grow wild in the UK include varieties *wightianum, pubescens, feei, decompositum*, *pseudocaudatum* and *africanum*.

Note that, in some documents, *Pteridium aquilinum* is referred to as *Pteris aquilina*.

3. Range of bracken

Bracken fern (*Pteridium* spp.) is a very common plant throughout the world and it is the commonest fern found in Britain. It is found on light acids soils in a variety of habitats, including woodland, heath, moorland and hill up to an altitude of about 600 m (up to 3000 m in some other countries). It was estimated that in 1993, bracken covered over 450,000 hectares (4,500 km^2) of Britain (Cooper and Johnson, 1998). Another estimate judged the area of the UK covered by bracken to be 6700 km^2, an area the size of Devon (Fullick and Fullick, 1991). The area of Scotland covered by bracken was measured by digital mapping techniques to be 63,250 hectares (632.5 km^2) (Miller, *et al*., 1989), whereas earlier estimates based on a census of Scottish farmers had given a much larger area of 180,000 hectares (1,800 km^2). In some counties of Scotland and Wales, bracken covers 6% of the land area (Taylor, 1999).

The land area covered by bracken is rising globally. Within the UK, the area covered by bracken has been reported to be increasing at an average rate of 1% per year, and is as much as 3% per year in some areas (Taylor, 1985; Miller, *et al*., 1989). However, it has also been reported that aerial photography taken in the 1970s and 1980s have shown a small decrease in bracken cover in nine out of ten English and Welsh National Parks in highland or marginal areas, with the exception being the Brecon Beacons (Pakeman, et al., 2000).

4. Human exposure to bracken

4.1. Eating of bracken

Bracken is eaten as food in Japan, Brazil, New-Zealand (Maoris) and north-eastern USA and Canada, but it is not normally eaten in the UK. It is grown commercially for food in Japan, Canada, north-eastern USA and Siberia (IARC, 1986). Japan imports over 13000 tonnes of bracken annually, in addition to the local production (Alonso-Amelot, 2002). The part of the plant commonly eaten is young curled fronds (known as crosiers, also referred to in the USA as fiddleheads).

There is also a potential for indirect exposure to toxins from bracken as a result of eating foods derived from animals that have eaten bracken or as a result of substances from bracken leaching into water sources that are used for drinking water.

4.2. Indirect exposure via milk

There have been cases of cattle being poisoned by bracken in the UK: 22 cases in cattle, including one dairy cow, were reported to the VLA between 1999 and 2007 (Livesey, 2007) although this is probably an underestimate of the number of poisonings that occurred in this period as there is no requirement for farmers to report such cases. Furthermore, it is expected that there will be many more instances of dairy cows being exposed sub-clinically to bracken. It is impractical to entirely separate farm animals from bracken, especially in upland areas where animals graze extensively. Toxic components of bracken can pass into the milk of cows that eat it (Pamukcu & Price, 1969; Evans IA, *et al.,* 1972; Pamukcu, *et al*., 1978). It is assumed that they can also pass into human breast milk. Consumers of milk or milk products might be exposed to toxic or carcinogenic components of bracken. Those who consume local unbulked milk could be exposed to higher concentrations of the harmful agents than those who drink bulked milk from commercial dairies. Evans IA (1987) suggested that consumers of acidic dairy products such as buttermilk could be at most risk because the carcinogenic agent in bracken appeared to be more stable under these conditions.

In a series of experiments by Evans, *et al.* (1972), a Friesian and an Ayrshire cow were fed twice daily supplements of up to 2 kg of pelleted dry bracken (ie. 4 kg/cow/day) for 25 days. Milk from the treated cows was found to contain a highly water-soluble substance of molecular formula $C_7H_{10}O_5$ (the same as for shikimic acid). The milk was fed to a 5-week-old male Guernsey calf (1 - 1.5 gallons per day) and blood samples were collected. There was a marked leucopaenia followed by a decreased number of platelets, that was said to be typical of the effects in calves of eating bracken. From day 34 there was blood and mucus in the faeces. After 90 days of feeding the milk from treated cows, the haematocrit value was less than 20% of the normal value, pyknotic normocytes were present and the neutrophil count had decreased to almost zero. In a supplementary experiment, extracts from the milk from bracken treated cows was also fed (at levels equivalent to the diet containing 25% bracken) to Swiss White mice throughout

pregnancy and lactation. The offspring were maintained on normal diet for 12-18 months before being killed for post-mortem. Increased incidences of pulmonary adenomas were seen in the groups of mice that were given methyl acetate extract (75% [8/12] had pulmonary adenomas), methyl acetate residue (55% [6/11]) or alkaline ether residue (20% [7/20]), but the incidence in those given alkaline ether extract (5% [1/21]) was similar to that in the control group (4% [1/27]). It was concluded that bracken contains toxic substances that can be excreted into milk and can pass the placental barrier.

Pamukcu, *et al*. (1978) fed 4 Brown Swiss cows bracken in their diet to give a dose of 1 g bracken/kg bw/day over 1.5 to 2 years. Two cows were fed normal diet as controls. All of the bracken treated cows (but none of the controls) developed papillary tumours of the urinary bladder. A series of experiments were performed in laboratory rodents that were given fresh milk in place of drinking water or powdered milk (or fractions of milk) at a concentration of 50% in their pelleted diet.

- In a carcinogenicity test of fresh and powdered milk from bracken-fed cows, groups of 10-35 Norwegian strain rats of each sex were given the following for their lifetime: Group 1 – fresh milk from bracken-fed cows; Group 2 – powdered milk from bracken-fed cows; Group 3 – fresh milk from control cows; Group 4 - powdered milk from control cows; and Group 5 – was given no milk. Tumours were produced in the intestines (adenocarcinomas, haemangiosarcomas, fibroadenomas and fibrosarcomas, mostly of the ileum), urinary bladder (papillomas and transitional cell carcinomas) and renal pelvis (transitional cell carcinomas) in the animals in Groups 1 & 2, but not in the other groups. In Group 1, the incidences were 3/34 (8.8%) intestinal tumours, 7/34 (20.6%) bladder tumours and 2/34 (5.9%) renal tumours; and in Group 2, the incidences were 6/56 (10.7%) intestinal tumours, 6/56 (10.7%) bladder tumours and 4/56 (7.1%) renal tumours. It was concluded that the carcinogenic factor in bracken is excreted into the milk and survives the processing to powdered milk.
- A carcinogenicity test of powdered milk fractions was performed in Swiss albino mice. The fractions were prepared by dissolving the powdered milk from bracken-fed and control cows in distilled water and extracting it with various solvents: pentane, methanol, benzene, diethyl ether, ethyl acetate. These extracts and the residue from the extractions were incorporated with cholesterol in a ratio of 1:3 (w/w) into pellets that were surgically implanted into the bladders of mice. Groups of 40 mice (gender not specified) were used. Mice surviving 52 weeks were killed for autopsy with the bladders being examined for tumours. Comparing the results for each fraction of milk from bracken-fed cows with the results for the same fraction of milk from control cows, the only fraction to show a significantly increased ($p<0.01$) incidence of bladder tumours was the diethyl ether extract of milk from bracken fed cows. This produced bladder tumours (mostly transitional cell carcinomas) in 41.7% (10/24) of the mice compared with 15.8% (3/19) with the diethyl ether fraction the control milk.
- Mutagenicity tests were performed on four milk fractions, extracts with pentane, chloroform and chloroform:methanol (1:1), using *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation. The chloroform-methanol extract of milk from bracken-fed cows was mutagenic to TA100, but no

mutagenicity was seen in any of the other fractions from milk from bracken-fed cows or from control cows.

The UK's Ministry of Agriculture, Fisheries and Food (MAFF) commissioned a study to investigate whether mutagenic compounds present in bracken could be passed into the milk of bracken-fed goats (Symonds, 1991; Dean, 1991; MAFF, 1996a). Three lactating goats had gastric fistulae prepared surgically to enable the administration of known quantities (15-30% by weight of the total diet, maximum dose 10 g/kg bw/day) of fresh young fronds of bracken that had been collected locally in Yorkshire. Dosing continued for 4 weeks and the goats were milked three times per week. An ethyl acetate extract of the milk from the goats was tested for mutagenicity before and during the feeding trial using *Salmonella typhimurium* strain TA100 in the absence of metabolic activation. Testing milk samples that had been spiked with a DMSO extract of bracken showed that the extract recovered about 20% of the mutagenic activity in the milk. The sensitivity of the test was reported to allow "the detection of mutagens at less than 0.5 μg/mL". Bracken samples from several different sites were tested for mutagenicity. Not all of the samples were mutagenic, so care was taken to administer only mutagenic samples of bracken to the goats. None of the samples of milk from the goats fed bracken induced mutations in TA100.

The amounts of individual chemicals from bracken that are passed into milk are covered under the headings for the individual chemicals (eg. ptaquiloside) later in this paper.

4.3. Contamination of meat and offal

Some food-producing animals eat bracken. Intensive grazing, usually by sheep and pigs, is used to control bracken and reduce invasion of pastures (Livesey, 2007). This use of animals to clear bracken and the observation that there is high incidence of sub-clinical bracken poisoning in some farm animals is evidence that bracken is palatable to some animals. The young emerging fronds in springtime are likely to be more palatable and appear to be more carcinogenic than older fronds and other parts of the plant. There may also be some exposure to bracken as a result of the traditional use of bracken as bedding for animals. It is theoretically possible that animals that are exposed to bracken could have residues of harmful bracken-derived chemicals in their tissues, which could be eaten by human consumers. No information on the amounts of residues or their rate of clearance has been found.

4.4. Contamination of drinking water

Rainwater running off from bracken infested land could contain harmful water soluble chemicals (such as ptaquiloside) from the bracken with the possible result that private or municipal sources of drinking water could become contaminated. No information is available on the amount of contamination that occurs (Evans IA, 1979; Galpin and Smith, 1986; Galpin, *et al*., 1990).

Contamination of drinking water by spores of bracken is also possible. However, in a Water Research Council survey of Scotland, very few spores were found to enter water during the sporing season in 1989 (WRc, 1989).

4.5. Inhalation of spores

Inhalation exposure to spores of bracken can occur in areas where bracken grows freely and this can also give an oral exposure as a result of swallowing spores trapped in the mucous of the respiratory tract. A single large frond of bracken can release about 1 g of spores during July to September (Evans IA, 1987; Evans and Galpin, 1990). The spores are of about 30 μm diameter and are catapulted into the air by the sporangium in hot dry weather. The spores can travel for up to hundreds of miles in the wind. Thus there is potential for low level exposure of the entire UK population, although the highest exposures will be in bracken infested areas.

Inhaled spores would be expected to become trapped in the mucus of the nasopharynx and to be then swallowed. Oral exposure to spores might also occur as a result of them contaminating drinking water. Bracken spores were carcinogenic to mice when added to their drinking water – see Section 6.2.4.1.

5. **Toxicity of bracken in farm animals**

Bracken has been shown to cause toxicity in various animal species (Alonso-Amelot and Avendaño, 2002). Its toxicity to cattle, horses and goats has been recognised for over a century. Acute bracken poisoning can cause death within a few days. Prolonged ingestion of sub-lethal amounts of bracken can lead to a complex array of ailments, including hypoplasia of the bone marrow, thrombocytopaenia, leucopaenia, immunosuppression, reduced intestinal uptake of iron, thiamine (vitamin B_1) deficiency and severe internal haemorrhages. The most visible symptoms in farm animals are weakness, lack of motor coordination of back legs and neck, propensity to acquire infectious diseases, breathing difficulties and nasal, rectal and urinary bleeding.

5.1 Ruminants:

Ruminants are unaffected by the thiaminases in bracken. Their gut microflora produces sufficient thiamine that the effect of the dietary thiaminases is insufficient to cause clinical deficiency of thiamine.

5.1.1. Cattle:

Bracken poisoning in cattle was first recognised in Britain in 1893 (Lander, 1912). Cattle will readily eat hay containing up to 30% bracken (Rosenberger, 1971). The eating of bracken, especially the young green fronds, is the cause of an acute haemorrhagic syndrome and a related chronic disease called bovine enzootic haematuria (BEH).

Acute poisoning in cattle produces severe depression of the bone marrow and leads to the "haemorrhagic syndrome" (Cranwell, 2004). There are several other conditions that can produce similar haemorrhagic conditions and these include anthrax, septocaemic pasteurellosis, pruritis pyrexia haemorrhagic syndrome (PPH), furazolidone toxicity, coumarin/warfarin poisoning, malignant catarrhal fever, type II BVDV infection, disseminated intravascular coagulation (DIC), Factor XI deficiency, and redwater fever. The most obvious signs of bracken poisoning in cattle are lethargy and pyrexia (up to 43ºC). Closer inspection of a clinical case may detect the following: dysentery or melaemia; bleeding from the nose, eyes or vagina; excessive salivation; haematuria; and petechial haemorrhages on any visible mucus membranes, skin or the anterior chamber of the eye. Animals may die from the poisoning at any time up to 6 weeks after removal from the contaminated pasture. Once the animal develops marked pyrexia, death usually follows within 1 – 3 days. Death is usually a result of terminal bacteraemia, infarctions in lung, liver, kidneys and heart, and massive haemorrhage into the gastrointestinal tract from ulcers. Post-mortem findings typically show widespread petechiae and haemorrhages throughout the carcase. Haematological investigations can be useful in confirming a clinical suspicion of bracken poisoning as an affected animal will usually have a very low platelet count ($<60 \times 10^9$/L) and a very low total leucocyte count ($<2.0 \times 10^9$/L).

Calves may show a different acute clinical syndrome to adult cattle: bradycardia and death from heart failure and a larynitic form due to laryngeal oedema (Cranwell, 2004).

When bracken was fed to heifer calves at 30% (w/w) in their diet for 24 months, there were various changes in the blood (Bhure, *et al*., 2006). In serum, total protein concentration was increased, whereas total immunoglobulin was decreased. Plasma fibrinogen and antitrypsin levels were increased. Glutathione peroxidase inactivity was decreased in polymorphonuclear cells. In erythrocytes, there was a decreased haemoglobin concentration but increased concentrations of glutathione, increased activities of catalase, glutathione-S-transferase, acetylcholine esterase, total ATPase, Na^+-K^+ATPase and Mg^{2+}ATPase, and increased. There were no significant effects on osmotic fragility, lipid peroxidation, or glutathione reductase activity in erythrocytes; on myeloperoxidase in polymorphonucleocytes; or on arginase, lactate dehydrogenase or ceruloplasmin in serum/plasma.

BEH involves changes to the urinary bladder including capillary ectasia, angiomatous cavity formation and vascularised proliferation of mesenchyme and epithelium with eventual infiltration of large parts of the bladder lumen by papillary carcinomas, transitional epithelium carcinomas, haemangiosarcomas and fibromas (Alonso-Amelot and Avendaño, 2002). Feeding of bracken to cattle caused haematuria, bladder papillomas, oesophageal and intestinal malignancies, toxicity to the liver and kidneys, and death within 6 months to 2 years from first exposure. A major acute feature of this syndrome is a panmyeloid depression of bone marrow activity that causes leucopaenia and thrombocytopaenia, resulting in widespread petechial haemorrhages (Evans, *et al*., 1954; Yamada, *et al*., 1998). These effects are often accompanied by pyrexia, damage to the gut lining and ulceration (Evans, *et al*., 1954).

Unlike acute bracken poisoning in non-ruminants, BEH is unaffected by dietary supplementation with thiamine (Fenwick, 1988).

BEH occurs in numerous areas on all continents, apart from Antarctica, and is most common on land that has not been used very long for agricultural purposes. Highly developed agricultural countries such as Denmark and The Netherlands do not have a BEH problem, but it is a serious concern in some mountainous regions of Austria, Switzerland, Germany, the Balkans and Central and South America (Rosenberger, 1971). BEH has also been reported to occur in north-western USA, central and southern Europe, Madeira, Turkey, Australia, New Zealand, China, Japan, Korea, India, Hawaii and the Azores (Alonso-Amelot, 2002; Bento, *et al*., 1999; Dawra, *et al*., 1999; Pinto, *et al.,* 1999).

A study of 123 cattle with BEH on farms in Spain followed some of the clinico-pathological changes that occurred in the disease (Perez-Alenza, *et al*., 2006). Monocytosis with otherwise normal haematological parameters represented an initial response to the consumption of bracken and was suggested as an early haematological marker of BEH. Later phases were characterised by anaemia, leucopaenia, thrombocytopaenia, monocytosis, hypergammaglobulinaemia, microhaematuria and proteinuria. In the final phase of the disease, monocyte counts returned to normal levels, but other changes persisted.

In 206 cattle kept on land in Bolivia that was infested with bracken (*Ptaquiloside aquilinum* var. *arachnoideum* and *Ptaquiloside aquilinum* var. *caudatum*), there were 22 cases of BEH (10.6%). Anaemia and leucopaenia were seen in all of the animals with BEH and raised temperatures and variable respiratory and cardiac frequencies were reported in some animals. All of the animals with BEH had bladder tumours (haemangiocarcinomas, haemangiosarcomas and fibrosarcomas) and 50% also had oesophageal squamous cell carcinomas. (Marrero, *et al*., 2001)

A study was performed in 30 cattle (26 cows; 4 bulls) that were fed a combination of fresh and dried bracken from Bolu, Turkey, (a total of up to 144 kg of fresh bracken and up to 1572 kg of dried bracken) for up to 1920 days (Pamukcu, *et al.,* 1976). A control group consisted of 14 cows. The estimated average dosages received by different groups of cows ranged from 5.6 to 12.8 g bracken/kg bw/day. Haematuria occurred from 60 days after the start of feeding bracken, and anaemia and leucopoenia occurred later. Papillomas appeared in the bladders after 1 year and carcinomas after 2.6 years. Of the 30 cattle that received bracken, 22 developed haematuria and 20 developed tumours of the urinary bladder within 5.3 years. No bladder tumours were found in any of the control cows, 8 of which died after 4 years and 6 survived 10 years.

From 652 cows that had been kept on bracken (*Pteridium aquilinum* var. *caudatum*) infested pasture in Perez Zeledon, Costa Rica, 68 (10.4 %) showed haematuria and all of the haematuric animals had bladder lesions, many of which were neoplastic (papillomas, haemangiomas and transitional cell carcinomas) (Villalobos-Salazar, *et al*., 1989).

The earliest report of the carcinogenicity of bracken was by Rosenberger and Heeschen (1960), who described neoplastic changes to the bladder mucosa of cattle (development of polyps) in an experiment in which 5 cattle were fed fresh or dried bracken for 15 months.

Cattle in upland areas of Scotland and northern England are substantially more prone to alimentary cancer than those in the immediately neighbouring lowlands (Jarrett, *et al*., 1978). The origin of the affected cattle was identified in 75% of cases and without exception they came from marginal or hill land infested with bracken. More than 70% of the UK cases came from the west coast of Scotland, particularly Argyllshire, which has 100,000 acres of bracken, 15% of the Scottish total.

Bracken feeding has also been associated with a slow-developing epidermoid carcinoma of the upper digestive tract (including tongue, mouth, oesophagus and rumen) of cattle (Fenwick, 1988). The condition has been described in Brazil, Kenya and in upland areas of northern England and Scotland (Moreira Souto, *et al*., 2006; Jarrett, *et al*., 1978; Fenwick, 1988).

Papillomaviruses have been found in the upper alimentary tract of almost all cattle affected by epidermoid carcinoma that have been examined (Fenwick, 1988). In healthy cattle, papillomas caused by papillomaviruses normally regress, but in cattle feeding bracken in upland areas of the UK there was a correlation between persistent papillomatosis and cancer (Jarrett, 1978; Jarrett, et al., 1978). The involvement of bovine papillomavirus type 4 (BPV-4) in cancers of the alimentary canal and of the urinary bladder has been demonstrated in a series of experiments in which cattle were inoculated with BPV-4 and /or fed bracken (Campo, *et al*., 1992 and 1994). Similarly in a bracken infested region of southern Italy, an examination of 1133 slaughterhouse cattle found BPV-4 in more than 60% of the histologically-confirmed oesophageal papillomas (Borzacchiello, *et al*., 2003a). BPV-4 induces benign papillomas in the upper gastrointestinal tract in cattle and these can undergo neoplastic progression to carcinoma in animals grazing on bracken (Campo, *et al*., 1999). Similarly, cattle bladder cancers are associated with the presence of another papillomavirus, BPV-2 (Campo, 1997; Borazacchiello, et al., 2003b). Expression of the genes for cyclooxygenases-1 and -2 (COX-1 and COX-2), expression of the viral oncoprotein E5 and the presence of activated H-*ras* have also been detected in bladder tumours from cattle suffering from BEH (Borzacchiello, *et al.,* 2003b and 2003c). Campo, *et al*. (1999) suggested that infection by papillomavirus and dietary exposure bracken fern or its constituent chemicals might also be involved in the carcinogenesis of upper GI tract tumours in humans.

It has been reported (Campo, 1997) that bracken-fed cattle become chronically immunosuppressed, possibly as a result of pterosins and pterosides in the plant. The effects included reductions in the number of circulating polymorphonucleocytes and lymphocytes. It was postulated that immunosuppression may be part of the aetiology of the bracken-associated cancer of the alimentary tract.

A progressive retinal degeneration that was similar to the bracken-related sheep disease "bright blindness" has been described in cattle grazing on bracken-infested land in Wales (Fenwick, 1988).

It has been suggested that immunosuppression during bracken poisoning might have caused cattle to have become susceptible to viral infection, causing malignant catarrhal fever in a group of 6 cattle that showed thromobocytopaenia and/or leucopaenia following ingestion of bracken (Twomey, *et al*., 2002).

5.1.2. Sheep and other ruminant species:

BEH also occurs in buffalo that eat bracken and a similar disease has been reported in sheep and a few isolated stags (Rosenberger, 1971).

Sheep are less prone to acute haemorrhagic effects of bracken than cattle, although deaths of sheep from this cause have been reported in Yorkshire and Australia (Fenwick, 1988). An acute outbreak of polioencephalomalacia in sheep resulting from impaired thiamine metabolism has been caused by the consumption of bracken, (*Pteridium aquilinum* var. *esculentum*) and rock fern, both of which contain thiaminase (Fenwick, 1988). Chronic exposure to bracken can also cause progressive retinal degeneration (PRD or "bright blindness") in sheep (Cooper and Johnson, 1998; Fenwick, 1988).

PRD has occurred in sheep in northern and central England and is characterised by stenosis of the blood vessels of the eye and progressive retinal atrophy. The condition has been reproduced experimentally by feeding 1 kg/sheep/day of dried bracken (50% of the total feed) to a group of 22 Dorset Horn ewes for up to 63 weeks (Watson, *et al.,* 1972): 15/22 of the ewes developed PRD, but none of the group of 22 control ewes developed the condition. The numbers of circulating platelets and leucocytes were also decreased in the bracken-treated ewes. Hirono, et al. (1993) induced PRD in lambs by administering diet containing bracken powder and also by administering ptaquiloside either by intravenous injection or by a catheter into the jejunum. The first onset of PRD was seen after 4 months of feeding bracken powder. It was concluded that PRD was caused by prolonged exposure to ptaquiloside in bracken.

In an experiment performed in north-east Yorkshire, administration of diet containing dried young bracken fronds to 8 North Country Cheviot wether lambs at a rate of 0.9 kg dried bracken/sheep/week for up to 62 months caused bladder cancer in 7 of the animals (McCrea and Head, 1981). The animal that did not develop bladder cancer died prematurely at 45 months. Two treat animals had tumours of the jaw and one had a tumour of the rumen, but these tumours had been shown in an earlier field study (McCrea and Head, 1978) to be common in this breed of sheep reared in the area of the experiment (48% incidence for jaw tumours and 20% for rumen tumours, whereas no cases of bladder tumours had been reported in the field). No tumours were found in 8 control animals when they were killed at the end of the study. Progressive retinal degeneration was found in one of the bracken-treated animals.

5.2. Non-ruminant species:

In horses, mules, pigs and other non-ruminant species, the principal effect of acute ingestion of bracken is thiamine deficiency. Thiaminase in the bracken destroys thiamine (vitamin B_1). Acute bracken poisoning in non-ruminants can be treated by removal of bracken from the diet and supplementation of the diet with thiamine and if treated sufficiently early the animals usually have a rapid and complete recovery. (Fenwick, 1988)

5.2.1. Horses:

The antithiamine action of bracken produces a condition known as "sleepy staggers" (Cranwell, 2004). Signs of bracken poisoning include anorexia, exhaustion, gait disturbance, staggering, lack of coordination, weak but rapid pulse, elevated blood pyruvate levels, cardiac irregularity and reduced blood levels of thiamine (Fenwick, 1988). Death is preceded by muscular spasms and backward inflection of the neck.

5.2.2. Pigs:

Reports of bracken poisoning in pigs are less frequent than in horses, possibly because the symptoms are less obvious in pigs (Fenwick, 1988). Prolonged exposure in pigs will produce a thiamine deficiency, which presents as sudden death with myocardial degeneration and fibrosis, pulmonary oedema, pleural effusion, pleurisy and pleural fibrosis (Cranwell, 2004).

Bracken poisoning has been produced in pigs in an experiment. Two castrated Large White pigs were fed a diet containing 25-33% dried ground rhizome of bracken. A third pig was kept as a control, being given a basal ration containing no bracken. After 20 days on the bracken diet, blood pyruvate levels began to rise in both experimental pigs. Pyruvate levels reached a peak on day 25, but the next day it dropped back to normal levels, possibly as a result of them gaining access to an external source of dietary thiamine (cattle and sheep faeces). Subsequently, from day 37, blood pyruvate levels rose once more. By day 42, both experimental pigs were "showing signs of anorexia and vomiting". One of the experimental pigs was found dead on day 55 and the other died on day 71. The control pig was killed on day 72. Post-mortem examinations were performed. The hearts of the experimental pigs appeared enlarged and mottled with discoloured patches, the lungs were oedematous and the trachea contained froth. Histopathology showed degeneration of cardiac muscle with hyperaemic areas and infiltration with eosinophils; the spleens contained high levels of iron as compared with controls; and there was polymorph infiltration of the lungs of one of the experimental pigs. The authors concluded that the feeding of bracken rhizomes to pigs caused lesions suggestive of acute heart failure and that the signs were similar to those described in thiamine (vitamin B_1) deficiency. It was suggested that the toxicity was caused by the thiaminase in the bracken (Evans WC, *et al*., 1972).

Two incidences of suspected bracken poisoning in pigs were reported in 2007 (Harwood, *et al.,* 2007). In both incidents there was circumstantial evidence of bracken consumption. The first incident involved a group of 18 young saddleback pigs, 2 of which showed respiratory signs and were shivering. One subsequently died . The other incident involved 10 young pigs, 2 of which showed respiratory signs and 1 later died. Post-mortem examinations were performed on the 2 pigs that died. Both animals had marked interlobular oedema of the lungs, particularly in the caudal lobes. There was no evidence of lung consolidation or pleurisy. The pericardial sacs contained an excessive accumulation of serous fluid. Histological examination confirmed alveolar capillary congestion and oedema with some haemorrhaging. Focal fibrinoid vasculitis was seen in the lungs of one of the two pigs that were examined. The myocardial tissue of each pig showed a moderate to severe subacute necrotising cardiomyopathy.

5.2.3. Quail:

It was briefly reported that the feeding of bracken (amount not reported) to 10 male quail caused reduced testis weight relative to bodyweight (40% less on average), reduced male fertility and a doubling of the number of non-developed eggs produced by untreated females with which the bracken-treated males were mated. (Evans IA, 1968)

6. Cancer hazard from bracken

6.1. Human epidemiology

As part of an investigation of the Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC), 46,465 men and 64,327 women aged 40-79 years were followed from 1988/1990 to 1999 (Lin, *et al*., 2006). A questionnaire was used to assess dietary intakes. During 1,042,608 person-years of follow-up there were 300 deaths from pancreatic cancer. High consumptions of pickles and of edible wild plants (mainly bracken crosiers) were associated with increased risk of pancreatic cancer. In particular high consumption of edible wild plants was significantly associated with an increased risk of pancreatic cancer in men, with a relative risk of 2.98 (95% CI = 1.46 – 6.07) in men who ate such plants almost every day. A high intake of fruit by men was associated with a low risk of pancreatic cancer (RR = 0.51). Smoking did not modify the associations with dietary habits.

Subsequent to the pioneering publication by Evans and Mason (1965) on the carcinogenic activity of bracken, there have been numerous publications demonstrating associations between exposure to bracken and cancer. The eating of bracken in Japan has been associated with high prevalences of gastric cancer (Hirono, *et al*., 1972; Haenszel, *et al*., 1976) and of oesophageal cancer (Kamon and Hirayama, 1975). Positive correlations have been identified between living in a bracken-infested area of Wales and the development of cancers, including stomach cancer (Galpin and Smith, 1986; Buckley, 1989). Similarly, in Venezuela, there was a higher prevalence of gastric cancer in people living in areas where bracken grew freely than in those living in areas where bracken did not grow (Alonso-Amelot and Avendaño, 2001).

A retrospective case control epidemiological study of 46 people with histologically-confirmed stomach or oesophageal cancer in a Brazilian community in Ouro Preto. An age- and sex-matched control group of 40 residents of Ouro Preto was used. Interviews and questionnaires revealed that 39 cases and 25 controls regularly consumed bracken crosiers of the *arachnoideum* variety (after first boiling them for about half-an-hour). The results revealed that the unadjusted risk of developing oesophageal or stomach cancer was 3.40 and 3.45 times greater (5.47 for combined risk of oesophageal or stomach cancer), respectively to a control population who did not eat bracken. The combined overall age-sex adjusted rate corrected for smoking tobacco and drinking alcohol was an increased risk developing gastric or oesophageal cancer of 3.63 (95% confidence interval = 1.24 – 10.63) for those who ate bracken. (Santos, *et al*., 1987; Marlière, *et al*, 1994 & 1999)

In bracken-infested areas of both Wales and South America, a raised prevalence of gastric cancer was identified (Galpin and Smith, 1986; Galpin, *et al.,* 1990; Alonso-Amelot, *et al.,* 1996; Alonso-Amelot, 1997; Alonso-Amelot, *et al.,* 1998; Alonso-Amelot and Avendaño, 2001). Often these areas depended on local milk production and it was suspected that people might be exposed to a carcinogen derived from bracken that was present in the milk.

In Gwynedd, North Wales, 101 histologically-confirmed gastric cancer patients were each matched by sex, age and social class to 2 hospital in-patients without cancer and 77 of the gastric cancer patients were also matched, using the same criteria, to 1 patient with a confirmed cancer of a different site, excluding oesophagus (Galpin, et al., 1990). A total of 202 non-cancer controls and 77 cancer controls were used. A questionnaire was used to determine bracken exposure and source of water in childhood. Exposure to bracken in childhood had an increased risk (RR = 2.34. $p<0.001$) compared to no exposure and length of residence in Gwynedd was also associated with increased risk (RR=2.46, $p<0.01$). Consumptions of buttermilk in childhood and as an adult were associated with increased risks (RR=1.61 and 1.86, respectively), but only the increased risk from adult exposure was significant ($p<0.05$). Evans IA (1987) suggested that consumers of acidic dairy products such as buttermilk could be at most risk because the carcinogenic agent in bracken appeared to be more stable under these conditions. The report of this study (Galpin, et al., 1990) has been seen by COC as CC/90/23 and was criticised for the lack of a method for assessing exposure to bracken, the small number of subjects investigated, the use of hospital data as controls, and the lack of consideration of confounding factors, such as salt in the diet.

In another study of people in Gwynedd (Buckley, 1989), the incidence of all incidences of stomach and oesophageal cancer (both dead and surviving patients) within the 34 districts of Gwynedd over the years 1974-1988 were compared with one-another on the basis of age of patients and region. Correlations were made of incidences of each cancer to the proportion of each region with bracken-infestation, with deep peat, with shallow peat or without a mains water supply. There were strong correlations ($p<0.001$) between stomach cancer in females and bracken infestation, oesophageal cancer in males and

bracken and oesophageal cancer in males and deep peat. There was a less strong positive correlation ($p<0.05$) between stomach cancer in males and bracken. There were negative correlations between stomach cancer in males ($p<0.05$) or females ($p<0.001$) and not being on mains water; between oesophageal cancer in females and not being on mains water ($p<0.001$); between oesophageal cancer in females and deep peat ($p<0.05$); and between stomach cancer in females and shallow peat ($p<0.05$). It was noted that although standard mortality ratios (SMR) for many of the districts of Gwynedd were greater than the national averages for Wales and England, the differences were not statistically significant. The authors concluded that the percentage of bracken cover was positively and spatially associated with incidences of cancers of the stomach and oesophagus, but that other factors might also affect incidences.

Contamination of drinking water with bracken leachates has been proposed as a potential cause of human cancer (Rasmussen, *et al.,* 2003a). However, in Wales, there was not a statistically significant correlation between the drinking of water from bracken infested watersheds and the prevalence of gastric cancer (Evans IA, 1979; Galpin and Smith, 1986; Galpin, *et al*., 1990). Galpin and Smith (1986) investigated whether there was a correlation between the drinking of water from bracken infested watersheds and the prevalence of gastric cancer in the human population in Gwynedd, Wales, over the period 1954-1977. The age-adjusted standardised mortality ratio (SMR) was used as an index of relative mortality. The SMR was calculated for each administrative district by the indirect method, for ages 35-75 years. Standard death rates were obtained from the Registrar General's Statistical Review of England and Wales. Assessment of bracken cover was made from maps of the Vegetation Survey of Wales 1961-1966. Information on water supply was provided by the Welsh Water Authority and this included details of catchments and distribution of supplies, runoff, treatment and abstraction for each source of water supply. An estimate of the potential annual average contamination (mean toxin index) was calculated for each water source by dividing the area occupied by bracken by the runoff. The results showed no statistically significant correlation between deaths from gastric cancer and the percentage of bracken in water catchments. Similarly examination of the relationship between the theoretical mean toxin index and the SMR failed to show any positive correlation.

The incidence of gastric, oesophageal and cervical cancers in humans over 1981-1986 was studied in contrasting regions of Costa Rica, a lowland bracken-free area and a highland area heavily infested with *Pteridium aquilinum* var. *caudatum*. Standardised incidence rates of cancers in the two regions were taken from the National Cancer Register. In bracken-infested areas the frequency of gastric cancer varied from high (66-108 cases age-adjusted per 100,000 inhabitants per year) to very high (<108); whereas in bracken-free areas there was a low frequency (<33). High rates of gastric cancer (2.73 times higher, with standardised incidence rates of 19.40 in lowlands and 53.02 in highlands) and oesophageal cancer (2.98 times higher, with standardised incidence rates of 1.28 in lowlands and 3.82 in highlands) were seen in the people in the highland bracken infested highland area. When the incidences for the different sexes were analysed separately, an even greater difference was seen in the incidence of oesophageal cancer in women (7.64 times greater incidence in the bracken-infested highlands than in

the lowlands). There was no significant difference between the two areas in the incidence of cervical cancer in women (1.17 times higher incidence in the highlands). The authors suggested that the high rates of gastric and oesophageal cancers were caused as a result of contamination of local milk with a substance from the bracken eaten by cows. The consumption of bracken by the local cows was confirmed by the occurrence of bovine enzootic haematuria (BEH) in the herds of cows kept on the bracken infested lands. (Vilalobos-Salazar, 1985; Villalobos-Salazar, *et al.,* 1989)

In a Venezuelan epidemiology study (Alonso-Amelot and Avendaño, 2001), a comparison was made of the numbers of certified deaths due to gastric cancer that occurred in hospitals in two areas of contrasting topography in the western region of the country: a bracken-infested (*Pteridium* spp.) mountainous area (three Andean states: Mérida, Táchira and Trujillo) and a neighbouring lowland area (Zulia state) that was essentially bracken-free. Diagnoses were obtained from death certificates of 5.5 million people who died between 1986 and 1996. Age-adjusted death rates were calculated by an indirect method using the number of deaths from all cancer types in the lowland region as the standard population. No segregation by race was included in the statistical analysis. In Venezuela as a whole and in all four states studied, the most common cause of death was heart disease and cancers were listed as the second most common. Rates of death from different cancers in the mountainous and lowlands areas over the period 1990-1996 were calculated. Table 1 summarises the average age/sex-adjusted mortality rates for different cancers in the two regions. For both the mountainous area and the lowland area, the most common site for malignancies was the uterus (cervix and endometrium). In the mountainous area the second most common site for malignancy was the stomach, but stomach cancer was much less common in the lowland area. Compared with the death rates in the lowland area, there was a marked increase in the mountainous area in the rate of deaths due to stomach cancer (3.64 times higher) and smaller increases in the rates of cancers of the prostate, lungs and mouth. The authors considered that the most likely environmental cause of the increased rate of gastric cancer deaths in the mountainous area was exposure to milk from bracken-fed cows. In the mountainous area, bracken was much more common and the prevalence of bracken-evoked bovine enzootic haematuria was high (18% of cattle in Mérida). It was postulated that ptaquiloside in the bracken that was eaten by cows was excreted in the milk, which was drunk locally and caused the gastric cancer in consumers. The possible influence of other factors, including poverty, diet, alcohol abuse and stomach infection with *Helicobacter pylori,* were considered but the authors considered the differences between the two regions with regard these factors were insufficient to be the cause of the difference in gastric cancer death rates.

Table 1: Average cancer age/sex-adjusted mortality rates per 100,000 people by affected organ in the study area (1990-1996)

Site of cancer	**Death rate (±SD)**			
	Mountainous area	**Lowland area**	**t-value**	**p-value**
Stomach	14.37 (0.67)	3.95 (0.46)	27.899	0.0000
Uterus	15.64	13.84	2.647	0.0382

	(1.36)	(0.92)		
Prostate	6.77 (1.34)	9.17 (1.38)	8.955	0.0001
Breast	4.81 (1.16)	6.67 (1.63)	3.534	0.0123
Lung	4.49 (0.83)	9.47 (0.75)	13.433	0.0000
Colon/rectum	2.58 (0.50)	3.06 (0.51)	2.676	0.0367
Mouth	1.39 (0.35)	2.64 (0.52)	3.350	0.0007
Oesophagus	1.18 (0.10)	0.99 (0.67)	0.766	0.4730

Increased prevalence of gastric cancer has also been seen in another bracken infested mountainous region of Venezuela (Sierra). The ten-year (1985-1994) average gastric cancer mortality per 100,000 people was 27.53, as compared with 11.89, in the lowland region of El Vigia which was relatively bracken-free (Alonso-Amelot and Avendaño, 2001). The prevalences of gastritis in the two areas were 1151 per 100,000 in Sierra and 1075 per 100,000 in El Vigia.

Eating of bracken was one of several dietary cancer risk factors considered in a study by Hirayama (1979). Exposures to bracken, hot tea gruel (chagayu), meat, fruit, and cigarettes were looked at in 98 patients with cancers of the oesophagus and 476 control subjects of 60 years of age or older in Nara, Wakayama and Mie prefectures of Japan. The relative risk of cancer was 2.68 (95% confidence interval = 1.38-5.21) in those who ate bracken daily and it was 1.53 (95% confidence interval = 0.90-2.62) in those who ate bracken occasionally, as compared with those who ate bracken rarely or not at all (χ^2 trend = 8.04, p=0.004). There was no significant difference between the responses of men and women. When subjects were stratified by intake of hot tea gruel, smoking and intake of meat and fruit, the increase in risk of oesophageal cancer associated with eating bracken appeared to be confined to those who took hot gruel tea and was relatively greater in those who also smoked and in those who did not eat meat and fruit each day. Information was lacking on the source of the controls used in the study and on the alcohol intakes of the subjects.

A study of 783 patients with stomach cancer and 1566 hospital controls in Hiroshima and Miyagi prefectures of Japan (Haenszel, *et al*., 1976) found a higher risk of stomach cancer in farmers than in others. The risk of stomach cancer in this study was higher than in another study (Haenszel, *et al*., 1972) that the same workers had performed in Hawaiian-Japanese people. There were associations between the eating of various foods and the risk of stomach cancer. Bracken was one of several foods that were eaten in greater amounts in the Japanese populations than in the Hawaiian group. Salted dried fish and salt-picked vegetables were other foods that were associated with increased risk of stomach cancer.

6.2. **Laboratory studies of the toxicity of bracken**

6.2.1. Repeat-dose toxicity

Rats:

Bracken fern, collected from Mukteswar Kumaon, India, was dried and incorporated into the diet of 6 albino rats (gender and strain not reported) at 25% for periods of 30, 60 or 90 days. Analyses of 2 samples of the bracken showed different amounts of ptaquiloside: 4.6 and 20.7 mg/kg. Control groups consisted of 4 rats at each time point. Bodyweight gain was decreased in all of the treated groups. At 30 days, haematology showed decreased packed cell volume and decreased total leucocyte counts, whereas at 60 and 90 days there were more extensive haematological effects including in addition increases in clotting time, erythrocyte sedimentation rates and neutrophil counts, and decreases in erythrocyte counts, haemoglobin and lymphocyte counts. Serum biochemistry showed decreased concentrations of glucose and urea, increased concentrations of creatinine, and increased activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase. Increased weights of liver (significant only at 30 days) and spleen (significant at 60 and 90 days) were seen. Histologically, the liver showed vacuolar denegerative changes in hepatocytes. The spleen showed passive congestion, hyperplasia of reticuloendothelial cells, thicken trabeculae, presence of haemosiderin-laden macrophages, free haemosiderin and the presence of megakaryocytes. Other changes reported in the bracken-fed rats included oedema in the brain, sub-epicardial haemorrhages in the heart, emphysema, hypersecretory activity in the intestines and degenerative changes in the testes. (Gounalan, *et al*., 1999a)

Guinea-pigs:

In a haematobiochemical study, a group of 6 male guinea-pigs (strain not reported) were fed for 60 days on a diet containing 30% dried bracken that had been collected in the Kumaon district of Nainital, India (Kumar, *et al.,* 2000). A control group consisted of 6 male guinea-pigs. Blood was collected on days 0, 20, 40 and 60. Feed intakes and bodyweight gains were consistently lower in the bracken-treated group than in controls. Haematological examinations on day 60 showed decreased packed cell volume, and increases in erythrocyte sedimentation rate, and counts of total leucocytes and of erythrocytes. There were decreases in serum total protein, serum albumin, blood reduced glutathione at day 60, but not at earlier timepoints. In erythrocytes there were increases in lipid peroxidation and catalase activity and a small decrease in acetylcholinesterase activity at day 60 only; and an increase in glutathione-S-transferase activity at day 40 only. Autopsies were not performed.

Rabbits:

Mixed gender groups of 6 New Zealand White rabbits were given diet containing 25% dried bracken for 30, 60 or 90 days. This study used same dried bracken that was used in the rat study described above. Control groups consisted of 4 rabbits at each time point.

There was no significant effect on bodyweight gain. Haematology at 30, 60 & 90 days showed increases in clotting time and neutrophil counts and decreases in total leucocyte counts, lymphocyte counts, erythrocyte counts, haemoglobin and packed cell volume. Serum alanine aminotransferase and alkaline phosphatise activities were raised at all time periods. In addition, serum glucose concentration was decreased and serum aspartate aminotransferase activity was increased at 60 and 90 days. Blood urea concentration was elevated at 90 days only. Liver weight was increased at all time periods and kidney weights were increased at 60 and 90 days. Histology of animals killed at all time points showed bracken-fed rabbits to have mild oedema in the brain, hyperaemia and occasionally haemorrhages in most visceral organs, degenerative changes in cardiac muscle fibres, dilated and engorged liver sinusoids, vacuolar degenerative changes in hepatocytes, haemosiderosis and depleted lymphoid follicles in the spleen, hyaline casts in renal tubules, degenerative changes in epithelial cells lining the renal tubules, and degenerative changes in the testes. In addition the mesenteric lymph nodes of rabbits killed after 60 or 90 days showed oedema and depletion of lymphoid follicles. (Gounalan, *et al*., 1999b)

Cats:

Five female cats were given 10 g of dried and ground bracken (*Pteridium aquilinum* var. *caudatum*) mixed with beef every 48 hrs, while three control cats were given beef without any bracken. Before and throughout the experiment all cats were subjected to clinical examinations, including blood biochemistry, urinalysis and haematology. Seven days after the start of administration, the bracken-treated cats showed adverse effects, including vomiting, weakness, dark coloured urine, presence of bilirubin in urine, elevated serum activities of ALT and AST and clinical signs of jaundice. The treated cats died 9-10 days after the start of treatment (2-3 days after first showing symptoms) and post-mortems showed yellowish soft liver and yellow colouration of the internal surfaces of the body cavity. Histology showed fatty degeneration of hepatocytes. (Villalobos-Salazar, *et al*., 1989)

6.2.2. Developmental toxicity:

Mice:

A group of 8 pregnant ICR-JCL mice were fed diets containing 33% dried Japanese bracken throughout gestation. Control groups of 7 pregnant mice were fed either basal diet or 33% cellulose in the diet. Maternal bodyweight gain and serum protein concentration were low in comparison with controls. Fetus weights were low and there were increased amounts of pups with extra cervical or lumbar ribs and retarded ossification or incomplete fusion of sternebrae. (Yasuda, *et al*., 1974)

6.2.3. Mutagenicity:

The results of mutagenicity studies of bracken are summarised in Table 2.

6.2.3.1. Bacterial assays;

A methanol extract of bracken was tested for mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 in the absence of an external metabolic activation system and was mutagenic to both strains (van der Hoeven, *et al*., 1983). An acetone extract of bracken was tested in *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of S9 and was mutagenic to TA98 in the presence of S9 (White, *et al*., 1983). Fukuoka, *et al*. (1978) found that boiling water and ethanol extracts of bracken (*Pteridium aquilinum* var. *latiusculum*) were mutagenic to *Salmonella typhimurium* strains TA98 and TA100 in the presence of S9 from livers of PCB-treated rats, and that ether and acetone extracts were only mutagenic if the bracken was pretreated with hesperidinase.

Ethanol and water extracts of raw and cooked curled tops and stalks of young bracken (Pteridium aquilinum) were tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535and TA1538 in the presence and absence of S9. Positive controls using sodium azide, 2-nitrofluorene, benzo(a)pyrene and 2-anthramine showed that the assay was working as expected. The ethanol-extracts from raw and cooked bracken were not mutagenic in any of the tester strains. The water extract from raw bracken was clearly mutagenic to strain TA1538 in the absence of metabolic activation but not in the presence of S9. The water extract of cooked bracken did not cause a statistically significant increase in the number of revertants of any tester strain but there was a slight non-significant increase with TA1538 in the absence of S9, which was about a tenth of the increase in the number of revertants that was seen in TA1538 exposed to water extract of raw bracken in the absence of S9. (Yoon and Lee, 1988)

Milk from cows fed bracken was mutagenic to *Salmonella typhimurium* strains TA98 and TA100 and in strain 45T of *Bacillus subtilis*, all tested in the absence of metabolic activation (Pamukcu, *et al*., 1978; İstanbulluoğlu, *et al*., 1981). However, milk from goats fed mutagenic samples of bracken was not mutagenic to *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation (Symonds, 1991; Dean, 1991; MAFF, 1996a).

6.2.3.2. Test in a virus:

An aqueous extract of bracken caused reversion to wild-type of mutant T4 rII bacteriophages (Roberts, *et al*., 1971). The bacterium *Escherichia coli* KB(λ) was used as an indicator organism. It was lysed by the revertant wild-type phage but not by the T4 rII tester phage.

6.2.3.3. Tests in insects:

A fraction of a solvent-extract of bracken that had been separated by paper chromatography and cellulose column chromatography was reported to be cause dominant lethal mutations in *Drosophila melanogaster* (Evans IA, *et al*., 1967; Roberts, *et al*., 1971). No further details were available.

6.2.3.4. *In vivo* assays:

Groups of 10 Swiss mice, including 2 control groups, were given intraperitoneal (i.p.) injections of water suspensions of various extracts of bracken that had been collected on the Brazilian savanna. The solvents used for the extracts were hexane, ethanol, hot water and cold water. 72.5 h later, the animals were given i.p. injections of colchecine. Cells were collected from the peritoneum and the bone marrow for metaphase analysis. In peritoneal cells, structural chromosomal aberrations including gaps, breaks and acentric fragments were seen with all of the extracts tested, and aneuploidy was also seen with the ethanol extract. In bone marrow cells, there were increased numbers of structural aberrations on with the hexanol and cold water extracts. Identical doses produced more aberrations in the peritoneal cells than in the bone marrow cells. (Almeida Santos, *et al*., 2006)

A fraction of a solvent-extract of bracken that had been separated by paper chromatography and cellulose column chromatography was reported to be mutagenic in an unspecified test in mice (Evans IA, *et al*., 1967). No further details were available.

DNA-adducts were detected by ^{32}P-postlabelling in the upper gastrointestinal tract (stomach plus small intestine) of six BDF_1 mice at 5 & 24 hrs after they had been given 25 mg/mouse of spores of bracken (*Pteridium aquilinum* var. *aquilinum*) by oral gavage(Povey, *et al*., 1996). The bracken spores had been collected in Anglesey, Wales. No DNA-adducts were detected in the livers of the treated mice. The same group also performed ^{32}P-postlabelling analysis of DNA adducts in tissues obtained from rats that had been given bracken (*Pteridium aquilinum* sub-species *caudatum* var. *arachnoideum*) from Ouro Preto, Brazil (Freitas, *et al*., 1999b). Groups of 4 rats of unreported gender were given 10% (w/w) of dried young fronds of bracken in their diet for up to 70 weeks, or water from cooked bracken as the only source of drinking water for up to 21 weeks. Further groups were given single oral doses of methanol extracts of fresh bracken, dried bracken or cooked bracken. Control animals were fed the diet without any additions of bracken. Stomach and ileum were taken for ^{32}P-postlabelling analysis. No DNA adducts were detected in the tissue samples from any of the groups of rats. There was a clear difference between the finding of DNA-adducts in the stomach and small intestines of mice fed Welsh bracken spores and the absence of DNA-adducts in the stomach and ileum taken from rats fed Brazilian bracken fronds. It was not clear whether this was due to a difference between Welsh and Brazilian varieties of bracken.

Peripheral blood from 13 cows (*Bos taurus* x *Bos indicus*) maintained on pasture infested with bracken in Brazil was examined cytogenetically (Moura, *et al.,* 1988). Increased frequency of chromosomal aberrations was seen in peripheral lymphocytes from these cows as compared to blood from 28 cows that had been raised on bracken-free pasture. These results were confirmed in another study (Lioi, *et al.,* 2004) in which blood from 56 cattle with BEH that had been raised on bracken-infested pastures was compared with blood from 30 control cattle that had had no access to bracken. Increased numbers of chromosomal aberrations were seen in the cattle with BEH. Urine, serum and milk from

20 of the BEH cattle and quercetin and ptaquiloside were detected in all but one of the cattle (details not reported), but these chemicals were not detected in urine, serum and milk from 5 control cows. 27 of the cattle with BEH were slaughtered and all had bladder tumours with 11 of them bovine papillomavirus type 2 (BPV-2) DNA. In a third study in cattle (Peretti, *et al*., 2006), blood from 45 cattle with BEH which had access to bracken in southern Italy was compared with blood from 15 control cattle that did not have access to bracken. In the bracken exposed BEH cattle there were increased numbers of aneuploid cells and cells with sister chromatid exchanges, chromatid breaks, chromosome breaks, fragments and gaps.

Cytogenetic analyses of peripheral blood lymphocytes taken from people who were regular consumers of bracken showed a significant increase in the number of chromosomal aberrations, including chromatid breaks and rearrangements (Santos, *et al.,* 1999; Recouso, *et al*., 2003). An exposed group of 11 men and 12 women aged 39 to 60 years from the Ouro Preto region of Minas Gerais, Brazil, had regularly consumed cooked bracken for at least 8 years. Blood samples were taken during the months of May to July, when bracken was most heavily consumed. A control group consisted of 10 subjects aged 35-63 years who did not eat bracken. There was a strong correlation between increased levels of chromosomal aberrations in lymphocytes and the consumption of bracken. There was no correlation with gender, smoking habits or alcohol consumption.

Table 2: Summary of the mutagenicity data on bracken

Test	Material tested	Result	Reference
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 (no S9 used)	Methanol extract of bracken	Positive in both strains	Van der Hoeven, *et al.,* 1983
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 with and without S9	Acetone extract of bracken	Positive in TA98 in the presence of S9	White, *et al*., 1983
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 with and without S9	Boiling water and ethanol extracts of bracken	Positive in both strains in the presence of S9	Fukuoka, *et al*., 1978
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 with and without S9	Ether and acetone extracts of bracken	Positive only after pretreatment of the bracken with hesperidinase	Fukuoka, *et al*., 1978
Reverse mutation of *Salmonella typhimurium* strains TA1535, TA1538,	Ethanol extracts from raw or cooked bracken	Negative	Yoon and Lee,1988

TA98 and TA100 with and without S9			
Reverse mutation of *Salmonella typhimurium* strains TA1535, TA1538, TA98 and TA100 with and without S9	Water extracts from cooked bracken	Negative	Yoon and Lee,1988
Reverse mutation of *Salmonella typhimurium* strains TA1535, TA1538, TA98 and TA100 with and without S9	Water extracts from raw bracken	Positive in TA1538 in the absence of S9	Yoon and Lee,1988
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 (no S9 used)	Milk from cows fed bracken	Positive in both strains	Pamukcu, *et al.*, 1978
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 and mutation of *Bacillus subtilis* strains 17A and 45T (no S9 used)	Milk from cows fed bracken	Positive in strains TA100 and 45T	İstanbulluoğlu, *et al*., 1981
Reverse mutation of *Salmonella typhimurium* strains TA98 and TA100 (no S9 used)	Milk from goats fed bracken	Negative	Dean, 1991
Reverse mutation in a bacteriophage	Aqueous extract from bracken	Positive	Roberts, *et al*., 1971
Dominant lethal mutations in *Drosophila*	Solvent extract of bracken	Positive	Evans IA, 1967 Roberts, *et al*., 1971
Metaphase analysis of peritoneal and bone marrow cells harvested from i.p.–dosed mice	Extracts of bracken using hexane, ethanol, hot water or cold water	Positive for all extracts in peritoneal cells; positive for hexane and cold water extracts in bone marrow.	Almeida Santos, *et al*., 2006
Unspecified *in vivo* test in mice	Solvent extract of bracken	Positive	Evans IA, *et al*., 1967
^{32}P-postlabelling to detect DNA adducts in liver of mice	Welsh bracken spores	Negative	Povey, *et al*.,1996
^{32}P-postlabelling to detect DNA adducts in upper GI tract of mice	Welsh bracken spores	Positive	Povey, et al.,1996

^{32}P-postlabelling to detect DNA adducts in stomach and ileum of rats	Brazilian bracken fronds	Negative	Frietas, *et al*., 1996
Cytogenetic examination for chromosomal aberrations in blood from exposed cows.	Bracken infested pasture	Positive	Moura, *et al*., 1988
Cytogenetic examination for chromosomal aberrations in blood from exposed cows.	Bracken infested pasture	Positive	Lioi, *et al.,* 2004
Cytogenetic examination for aneuploidy, chromosomal aberrations and SCE in blood from cows with BEH.	Bracken infested pasture	Positive for aneuploidy, chromosomal aberrations and SCEs.	Peretti, *et al*., 2006
Cytogenetic examination for chromosomal aberrations in blood from human consumers	Cooked young fronds of bracken	Positive	Santos, *et al*., 1999; Recouso, *et al*., 2003

Urine from rats fed bracken was not directly mutagenic to *Salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation (Hatcher, *et al.,* 1981). Solvent extracts of the urine were fractionated on a silica gel column. One of the fractions was mutagenic in TA100 but not in TA98. The authors noted that the mutagenic fraction contained quercetin amongst other substances.

6.2.4. Animal studies of the carcinogenicity of ingested bracken:

Milk from cows fed a diet containing bracken fronds was reported to be carcinogenic to mice and rats (Pamukcu & Price, 1969; Pamukcu, *et al.,* 1978).

6.2.4.1. Mice:

In a non-standard study, exposure of mice to spores from bracken produced increased incidences of gastric cancer and leukaemia (Evans IA and Galpin, 1990). Spores were suspended in water and given to 98 mice (two strains of mice, TFI and Aber, both sexes) by oral gavage. Each mouse received 200 mg of spores. The report did not report whether this was a single dose or if repeated doses were given. Water without spores was given to 49 control mice. The mice were observed for up to 2 years. Of the 98 spore-treated mice, 52 developed malignancies; 28 developed leukaemias (mainly lymphocytic) and 6 developed stomach cancers. The control animals did not develop any leukaemias or stomach cancers and only 2 of them developed malignancies (both of the mammary).

A 30% bracken diet was fed to groups of ACI mice for 260 days or to CD mice for 180 days. Hyperplastic nodules were seen in the livers of both strains of mice. (Hirono, *et al.,* 1984c)

Groups of 34 C57BL/6 mice and of 20 dd mice (groups contained unspecified numbers of males and females) were fed a diet containing 330 g/kg of dried young bracken fronds for 17 weeks. Intestinal tumours developed at the terminal jejunum in 11/34 of the C57BL/6 mice and lung adenomas developed in 7/10 of the dd mice that survived more than 37 weeks. No tumours were found in groups of control mice. (Hirono, *et al*., 1975)

A group of 40 female Swiss mice was fed a diet containing dried bracken mixed with grain in a ratio of 1:2. This diet was given every other week for 60 weeks. All of the 33 mice that survived 30 weeks or more developed lymphatic leukaemia with multiple organ involvement. In addition, 5 of these mice developed multiple epithelial tumours of the lungs. None of the mice developed intestinal or bladder tumours. No tumours were detected in any of the 38 control mice that survived 30-60 weeks. (Pamukcu, *et al*., 1972)

A group (number of animals not stated) of pregnant Swiss White mice were fed for 6 weeks, throughout gestation and lactation, on diet containing various fractions of bracken at doses that were equivalent to 25% dried bracken in the diet. The fractions used were alkaline ether extract, alkaline ether residue, methyl acetate extract and methyl acetate residue; and a control group were given just the basal diet. The offspring were kept on bracken-free diet for 12-18 months and then were killed and examined for lung tumours. There were increased numbers of pulmonary adenomas with all fractions except the alkaline ether extract (see Table 3). The authors concluded that the results showed that carcinogenic factor in bracken can pass from the exposed mother to its offspring through the placenta and/or the milk. (Evans IA, *et al.,* 1972)

Table 3: Pulmonary adenomas mice given various fractions of bracken

Fraction	No. offspring in group	No. offspring with pulmonary adenomas	% offspring with pulmonary adenoma
Control	27	1	4
Alkaline ether extract	21	1	5
Alkaline ether residue	34	7	20
Methyl acetate extract	12	8	75
Methyl acetate residue	11	6	55

6.2.4.2. Rats:

Young fronds of bracken (*Pteridium aquilinum* var. *caudatum* subs. *arachnoideum*) were collected in the Ouro Preto region of Brazil and either dried or cooked in boiling water (Freitas, *et al*., 2002). Dried bracken was fed at a level of 10% in the diet to a group of 13 female Wistar rats; water from the cooking of the bracken was fed to a group of 20 rats,

and a group of 6 rats was kept as a control group. The rats were killed 15 – 20 months later when they presented signs of poor health. At post-mortem, the animals were examined for tumours, which were then examined histologically. Of the group of 6 rats given dried bracken, 4 developed ileal tumours (adenomas and adenocarcinomas), 2 of these also developed bladder papillomas and 2 rats did not have any tumours. Of the group of 20 rats given the water from cooking bracken, 17 developed ileal tumours (adenomas and adenocarcinomas and carcinomas), 8 had bladder tumours (papillomas, adenomas and carcinomas, 8 developed tumours at other sites (cervical polyps, cervical fibromas, cystadenofibroma and uterine leiomyoma) and none were free of tumours. In the 13 controls, one rat had a cystadenofibroma.
DNA was extracted from 8 selected tumours of the bladder and intestines and from adjacent normal tissue. Exons 5-9 of the *p53* gene and exons 1 and 2 of the K-*ras* and H-*ras* genes were examined by DNA-sequencing. No mutations were found at any of these sites on the DNA from the tumours. Amplification of five microsatellite loci (*IGHE, PRLR, ADRB2, PBPC2* and *IVD*) in the malignant tumours and in surrounding normal tissue did not show any instability.

A diet containing 5% (w/w) dried young Brazilian bracken crosiers was fed to 9 male and 6 female Wistar rats for up to 70 weeks (Santos, *et al*., 1987). A control group of 6 males and 5 females were given a similar diet without the added bracken. All animals were examined for possible tumours in the gastrointestinal tract, bladder, lungs, liver and kidneys. All of the rats that were fed bracken developed tumours in the gastrointestinal tract, mainly in the ileum. Most of these tumours were malignant (adenocarcinomas and sarcomas), but some benign tumours were also found. One bracken-treated rat developed a lymphoma. No tumours were found in the control rats.
[In an earlier study by the same workers (Santos, *et al*., 1986), a higher dietary level of dried young curled fronds of bracken (33%) had been fed to 5 female and 7 male Holtzman rats for 9 weeks, but the animals had rejected the diet and only one male rat developed a tumour, an adenomatous polyp of the ileum. No tumours were found in 4 females and 7 males that were given processed (boiled and dried) bracken. The only tumour found in a control group of 6 female and 3 males was a subcutaneous adenoma in one animal (gender not reported).]

Groups of 11-25 Sprague-Dawley or ACI rats of each sex were fed for 260 days (Sprague-Dawley rats) or 180 days (ACI rats) on either control diet or a diet containing dried mature bracken fronds that had been collected in Hokkaido, Japan (Hirono, *et al*., 1984c). At the end of the study, all rats were killed for post-mortem examination and their livers were examined microscopically. Hyperplastic nodules were detected in the livers of several of the rats that had been fed bracken (1/15 in male Sprague-Dawleys, 5/14 in female Sprague-Dawleys, 4/19 in male ACI, and 0/11 in female ACI), but in none of the control rats. The nodules were considered to be pre-neoplastic lesions. In a follow-up experiment, groups of 7 female Sprague-Dawley rats were fed diets containing various fractions of a water extract of bracken that had been separated by various solvent extraction, resin adsorption and chromatographic techniques. Some of the fractions caused liver nodules and some did not and the results from this were used as part of the

process to identify the major carcinogenic components of bracken (see the section on "Characterisation of the carcinogenic agent in bracken").

A group of 15 male and 15 female Sprague-Dawley rats was fed for 260 days on a diet containing 30% bracken fronds. No tumours were found in untreated control rats, but various tumours were found in the group that was fed bracken. Mammary adenocarcinomas were seen in 8 females, mammary papillary adenocarcinomas in 13 females, ileal adenomas were seen in 14 males and 9 females, ileal adenocarcinomas were seen in 13 males and 12 females, spindle cell fibrosarcomas were seen in the ileum of 1 male and 1 female, urinary bladder carcinomas were seen in 6 males and 6 females, bladder papillomas in 3 males and 1 female and squamous cell carcinoma of the Zymbal gland was seen in 2 females. (Hirono, *et al.,* 1983)

A dietary concentration of either 30% powdered bracken for 58 weeks caused multiple ileal tumours (adenomas, fibroadenomas and adenocarcinomas) and transitional cell carcinomas of the urinary bladder in a group of 8 male and 11 female Norwegian albino rats (Pamukcu, *et al*., 1980b). A control group consisted of 9 males and 10 females. intestinal tumours (adenomas, adenocarcinomas and fibrosarcomas) were found in 7/8 males and 10/11 females; and bladder tumours (papillomas and transitional cell carcinomas) were found in 6/8 males and 8/11 females. No intestinal or bladder tumours were found in controls.

Small groups of ACI rats were given oral doses of dried bracken or aqueous extracts of dried bracken for up to 16 months. Group 1 (7 males & 7 females) was given diet containing 1 part dried bracken to 2 parts basal diet for 3 months. Group 2 (6 males & 6 females) was given a boiling water extract in drinking water (60 g extract to 1 L water) for 16 months. Group 3 was given a cold water extract in drinking water (60 g extract to 1 L water) for 16 months. Group 4 (3 males & 4 females) was given boiling water extract in drinking water (60 g extract to 1 L water) for their lifetime. Most of the rats given untreated dried bracken (Group 1) or broiling water extract (Groups 2 & 4) developed tumours in the urinary bladder (transitional cell carcinomas and squamous cell carcinomas) and ileum (adenomas, adenocarcinomas and fibrosarcomas). No tumours were found in the rats in Group 3, which was given cold water extract of bracken. (Hirono, *et al*., 1978)

An aqueous extract of bracken was tested for carcinogenicity in groups of 5 male and 5 female ACI rats. The extract was prepared by boiling 90 g of dry bracken powder three times in 1 litre of water for 3 mins and filtering off the aqueous extract. The extract was concentrated in a rotary evaporator and mixed into the diet in a proportion of 1:2 by weight. A control group of 18 rats of each sex was maintained on basal diet. The test diet was fed for 500 days. Out of the 10 test rats, 9 developed bladder tumours (transitional cell carcinomas) and 8 of these also had ileal tumours (8/10 had adenomas and 7/10 had adenocarcinomas). (Hirono, *et al*., 1978)

A series of experiments were performed on bracken that had been collected in Hokkaido, Japan (Hirono, *et al.,* 1975).

- In one experiment, groups of ACI rats of both sexes (numbers of each sex not stated) were fed for 4 months on a pelleted diet containing 33% of either unprocessed bracken (26 rats) or bracken that had been cooked in boiling water for 5-10 minutes (12 rats). After the treatment period the rats were all fed control diet and were allowed to live out their lifespan. Another group of 13 rats acted as a control. Of the 26 rats fed the unprocessed bracken, 2 died of pneumonia within 7 months and all (100%) of the 24 survivors died of multiple ileal tumours (adenomas, adenocarcinomas and sarcomas) within 14 months of the start of feeding. Six (25%) of these rats also had caecal tumours (mainly adenomas) and one had a bladder papilloma. In the group fed cooked bracken, the onset of tumours was later and the rats survived longer. In the group fed cooked bracken, 9 rats had (75%) ileal tumours (adenomas, adenocarcinomas), 1 had a rectal adenocarcinoma and 6 had bladder tumours (papillomas and carcinomas). The only tumour found in controls was a bladder papilloma in one of the rats. The authors noted that cooking reduced the carcinogenicity of bracken to the intestines, but increased the number of tumours found in the bladder, possibly as a result of longer survival.

- In another experiment, groups of 10-14 ACI rats (groups contained unspecified numbers of males and females) were fed diets containing 33% untreated bracken fern (Group A), 33% bracken immersed in water treated plus wood ash (Group B), 33% bracken immersed in water plus sodium bicarbonate (Group C), 33% bracken fern that had been pickled in salt and then immersed in water (Group D), or basal diet alone (Group E: the control group). The experimental diets were given for 17 weeks and all groups were observed for 70 weeks. The incidences of tumours in Groups A, B, C and D were 11/14, 3/12, 1/10 and 1/10, respectively. Most of the tumours were in the ileum. No tumours were found in controls (Group E)

Groups of 5-9 ACI rats of each sex were fed for 2 months on pelleted diets containing concentrations of 11, 20 or 33% of either the curled tops of young bracken fronds (crosiers) or the stems of bracken fronds with the crosiers removed. The rats were observed for up to 16 months. Tumours of the intestines and bladder were found in animals from all treated groups. At dietary concentrations of 20% or greater, there were more tumours and more variety of different types of tumour seen in the rats given bracken crosiers than in those given stems. A control group of 22 rats was not fed any bracken and did not develop any tumours. The authors concluded that the crosiers (the part of the plant sometimes eaten by humans) are more carcinogenically potent than the rest of the stems of bracken. (Hirono, *et al.,* 1973)

Groups of ACI rats were given pelleted diets containing 33% whole bracken frond (9 males and 9 females), 33% bracken rhizome (7 males and 6 females) or 33% bracken starch (8 males and 6 females) that was made from bracken rhizomes in the traditional Japanese way. There was no control group. The diets were given for 17 weeks and the animals were observed for 70 weeks. No tumours were seen in the animals given bracken

starch, but 13/18 rats given bracken fronds and all 13 of the rats given rhizomes developed intestinal tumours. In addition one animal from the frond group and one from the rhizome group developed bladder tumours. The authors concluded that the fronds and the rhizomes of bracken are carcinogenic but such carcinogenicity is not produced by feeding starch produced from bracken rhizomes. (Hirono, *et al.,* 1973)

The carcinogenicity of processed bracken, as used for human food, was investigated in groups of 5-7 ACI rats of each sex (Hirono, *et al*., 1972). Group I received pelleted diet containing 33% (by weight) unprocessed dried bracken. Group II was given 33% dried bracken that had been processed by boiling with wood ash (pH 10) for 20 hrs. Group III was given 33% dried bracken that had been processed by boiling with sodium bicarbonate (pH 9) for 20 hrs. Group IV were given 33% bracken that had been treated with a mixture of sodium chloride, burnt alum and bittern for 4 months. A control group were given basal diet with no added bracken. The diets were fed for 4 months. Most of the rats given unprocessed bracken (Group I) developed tumours of the ileum (adenomas, adenocarcinomas and sarcomas) and a few also developed bladder tumours (papilloma or carcinoma). None of the rats given processed forms of bracken (Groups II, III and IV) developed bladder tumours and the incidence of ileal tumours was much lower than in Group I: three of the Group II rats had ileal adenomas; one Group III rat had an ileal adenocarcinoma and one Group IV rat had an adenoma. None of the control rats developed tumours.

A group of 16 male and 10 female ACI rats (Group I) was fed for 4 months on a pelleted diet containing 33% dried young bracken. Another group of 6 male and 6 female ACI rats (Group II) was fed for 4 months on a pelleted diet containing 33% bracken fern that had been immersed in boiling water for 5-10 mins. A third group of 13 males and 9 females (Group III) received, for 18-27 days, the water in which the bracken had been boiled. A control group of 6 male and 7 female rats was given basal diet. The rats were observed for 70 weeks. The diets were administered for 17 weeks. In Group I, all rats died or were killed when moribund within 61 weeks. All of the 24 Group I rats that survived 30 weeks developed tumours in the ileum (16 had adenomas, 18 adenocarcinomas and 12 sarcomas) and 7 rats developed caecal tumours (6 adenomas and 1 sarcoma). In Group II, 9 rats developed intestinal tumours (7 had adenomas and 3 had adenocarcinomas), 3 rats developed carcinomas of the urinary bladder and 1 had lung adenoma. Group III had no significant difference to controls with regard to the incidence of tumours (1 breast sarcoma and 2 bladder papillomas). One bladder papilloma was seen in the control group. The authors suggested that the lower incidence of cancer in the rats given boiled bracken (75% of Group II) as compared to the unboiled bracken (92.3% of Group I) reflected a reduced carcinogenic potency that may be due to removal or destruction of some of the carcinogenic agent (Hirono*, et al.,* 1970)

A group of 17 male and 14 female rats (strain not stated) was fed 33% dried bracken in their diet. A second group of 38 males and 52 females was fed the same diet but was also given weekly subcutaneous injections of 2 mg thiamine hydrochloride (to counteract any thiaminase activity of the bracken), and a third group of 10 males and 12 females were kept as controls. The diets were given for up to 52 weeks. All of the rats that were fed

bracken (with or without thiamine injections) developed intestinal tumours. Urinary bladder carcinomas were seen in 6/17 males and 4/14 females in the group given bracken alone and in 19/36 male and 35/51 females in group given bracken plus thiamine. (Pamukcu and Price, 1969)

Rats fed diets that contained bracken developed multiple ileal adenocarcinomas (Evans and Mason, 1965). A 66/34-mixture of dried bracken fronds and Levers No. 4 rat diet was pelleted and fed *ad lib* to 20 male and 20 female rats (7-week-old, Glaxo strain, hooded Lister non-inbred). The report states that the bracken diet was fed *"for 64 days, from August 14 to November 11, 1964, inclusively and from January 8 to 18, 1965"*. These dates indicate an initial dosing period of 89 days, followed 56 days later by a second dosing period of 11 days (100 days in total), which is not consistent with the claim that the bracken was fed for 64 days. No other food was offered. No information was available on the amount of bracken eaten by the rats. A control group of 40 male and 40 female rats were given basal diet. The test and control rats were given three subcutaneous injections of thiamine (3mg in 0.2ml physiological saline) at approximately 2-week intervals to counteract any thiaminase activity of the bracken. At 346 days after the start of the experiment, all 20 males and 14 of the females that were fed bracken had died or had been euthanized as a result of their poor condition, whereas all of the control animals remained alive. Post-mortem examinations of the decedents showed multiple small tumours in the ileum of all but one of the bracken-treated rats (this female had a mammary tumour). In 10 of the animals with small ileal tumours there was also a large (2-4cm diameter) ileal tumour which in some cases protruded through the muscle wall of the intestines and adhered to surrounding tissues. Histological examination of the ileal tumours showed them to be adenocarcinomas of the mucosa. It was commented that intestinal tumours are rare in this breed of rat.

6.2.4.3. Guinea-pigs:

Bracken fern powder was fed to a group of 16 male guinea-pigs (strain not reported) at a level of dietary incorporation of 30% (w/w) for 30 months, followed by a further 24 months on normal diet that contained no bracken (Dawra, *et al*., 2002). The bracken powder was prepared from *Pteridium aquilinum* collected in a forested area of Palampur, India, and it contained 3.74 ± 0.6 mg/kg of ptaquiloside. Another group of 30 male guinea-pigs was kept as a control group. Post-mortems were performed on all decedants and on the surviving animals that were killed at weeks 52-54 of the study. Selected tissues, including bladder, kidneys, urethra, liver, heart, lungs, spleen and intestines, were examined histologically. Congestion was observed in the lamina propria of the bladder in 13 out of 16 treated animals and in all but one case this was accompanied by oedema and /or haemorrhage. Epithelial hyperplasia was seen in 13/16 treated animals. Bladder tumours were found in 3/13 treated animals: an adenocarcinoma in the lamina propria, an adenocarcinoma in the muscle layer and a transitional cell carcinoma in the lamina propria. All of the animals with bladder tumours and one other treated animal had haematuria. An adenocarcinoma of sebaceous gland was found in one of the treated animals. Six of the treated animals had inflammatory changes in the intestines, 3 had epithelial "proliferative changes" to the epithelium and in addition one had squamous

metaplasia of the ileal epithelium. Proliferation of goblet cells was seen in almost all of the treated animals. Other changes seen in treated animals included severe congestion of the sinusoids and periportal vessels in the liver, mild to severe hepatocellular degeneration, congestion and mild to moderate degenerative changes in the myocardium, areas of emphysema in the lungs, severe congestion of the cortex of the kidneys. It was reported that no "significant histopathological changes were observed" in the control group."

Groups of 4 to 14 female Hartley strain guinea-pigs were fed either a control diet or a diet containing 30% (w/w) of dried bracken crosiers that had been collected in central Hokkaido, Japan (Ushijima, *et al*., 1983). These diets were fed for periods of 5-10 days (males and females used in this experiment only), 20-80 days or 11-15 months. The animals fed bracken had haematuria and intense oedema of the urinary bladder from the 5th day of exposure onwards. Panmyelopathy of the bone marrow was seen in those given bracken for 5-10 days. All animals given bracken for 11-15 months developed transitional cell carcinomas of the bladder and 83.3% of these animals also developed intestinal tumours, including carcinomas (53%) and sarcomas.

A group of 13 guinea-pigs (sex not specified) were given a supplement of Welsh bracken fronds *ad lib* to their diet for 77 days, and were then kept on a bracken-free diet for the rest of their life (Evans IA, 1968; Evans IA, *et al*., 1967). The amount of bracken eaten was not reported. A control group of 6 untreated guinea-pigs was kept for comparison. One treated animal died of adenocarcinoma of the jejunum at 23 months. Nine of the 13 treated animals had chronic intermittent haemorrhages from the urinary bladder and epithelial hyperplasia, papillary adenoma and carcinoma of the bladder were seen in several (numbers not reported) of the treated animals. No such changes were seen in the bladders of control animals.

6.2.4.4. Quail:

An unspecified "active" extract of Welsh bracken was administered to Japanese quail (*Coturnix coturnix japonica*) by an unspecified route from hatching until death. A high proportion of the exposed quail were reported to have developed adenocarcinomas of the caecum, colon and, to a lesser extent, the ileum (Evans IA*, et al.,* 1967).

6.2.4.5. Toads:

A group of 98 Egyptian toads (*Bufo regularis*) was fed 10 mg bracken/toad/week for 5 months. Neoplasms developed in 18 toads: 7 developed ileal adenocarcinomas and 16 developed hepatomas, six of which metastasised to the kidneys. No bladder tumours were found. No tumours developed in the ileum, liver, kidney or bladder of 100 control toads. (El-Mofty, *et al*., 1980)

6.2.5. Animal studies of the carcinogenicity of inhaled bracken:

Inhalation exposure of rats to high levels of bracken spores caused severe pathological effects on the respiratory system and caused carcinomas in the respiratory tract (Caulton, 1999).

6.2.6. Anticarcinogenic properties of bracken

Kim, *et al*. (1993) showed that an extract from bracken induced the anticarcinogenic marker enzyme, quinone reductase, *in vitro* in hepa1c1c7 cells. There was also a slight induction of arylhydrocarbon hydroxylase activity. The bracken had been bought on a food market in Seoul, Korea. The extract had been prepared by freeze-drying the bracken, dissolving in 80% methanol, filtering, evaporating and freeze-drying to provide a powder that was re-dissolved in 80% methanol prior to testing.

Some of the chemicals in bracken, including quercetin and caffeic acid, have been shown to have anticarcinogenic properties (see Sections 6.5.5 and 6.5.9 for more details).

6.3. **Characterisation of the carcinogenic agent in bracken**

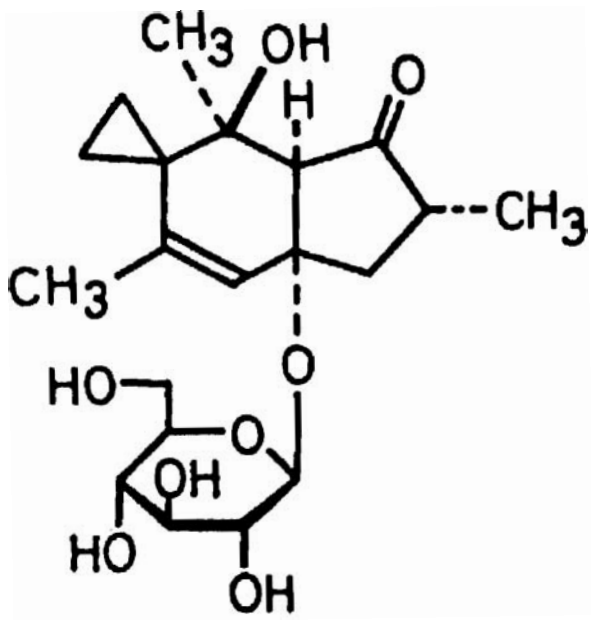
In an attempt to isolate the carcinogenic substance in bracken, a series of experiments were performed on various fractions of the aqueous extract of bracken (Niwa, *et al.,* 1983; Hirono, 1986). Bracken was collected in Hokkaido in Northern Japan and was freeze dried for the experiment. Aqueous extracts of dried bracken powder were fractionated using a series of methods, including solvent partitions (water/butanol), adsorption onto neutral resins (Amberlite XAD-2 and Toyopearl HW-40), thin-layer chromatography and HPLC. The mutagenicity of the resultant fractions was tested in *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of S9 from the livers of PCB-treated rats (Fukuoka, et al., 1978). The carcinogenicity of the fractions was assessed in a series of bioassays (Hirono, *et al.,* 1984a) in which freeze-died samples were mixed with rat basal diet at concentrations to achieve "levels of 3-7.5 times as much as the bracken content of the usual bracken diet in which the proportion by weight was 1 part bracken to 2 parts of basal diet". The experimental diets were fed to groups of either 7 female Sprague–Dawley rats (Charles River CD) or 5 female ACI rats (Hoshido Animal Farm) for periods of 164 to 218 days. Initial fractions that caused tumours in the intestines, mammary glands and urinary bladder of most of the test rats were further purified and tested again. The final most purified fraction was analysed by NMR spectroscopy to determine the chemical structure of the principle compound present, which was taken to be responsible for the carcinogenicity. The compound identified was referred to as "ptaquiloside". The structural formula of ptaquiloside is shown in Figure 1.

In the same year (1983), van der Hoeven, *et al*., reported the isolation and gross structure of a mutagenic compound that they named "aquilide A", which had a chemical structure that was identical to that of ptaquiloside. Freeze-dried bracken (*Pteridium aquilinum* L. Kuhn) was extracted in a Soxhlet apparatus with light petroleum and methanol. The

methanol extract was shown to be mutagenic in a *Salmonella typhimurium* strains TA98 and TA100 in the absence of an external metabolic activation system (positive in both strains). An acetone extract from bracken also gave positive results in the Ames test in the presence of metabolic activation, but not in its absence (White, *et al*., 1983).
The methanol extract was fractionated by liquid chromatography and the fractions were tested for mutagenicity using the *Salmonella* assay. The most mutagenic fraction was concentrated using a "Seppak" mini-column and further separated by HPLC. A single HPLC peak was found to contain all of the mutagenic activity. By further HPLC and mass- and NMR-spectroscopy the mutagenic component was characterised as ptaquiloside (which the authors referred to as aquilide A). In addition to ptaquiloside, mutagenic activity in the *Salmonella* assay was detected in another liquid chromatography fraction from bracken. The authors suspected the mutagenicity might be due to the aglycone of ptaquiloside, which they named aquiline A. The mutagenicity of both ptaquiloside and the other mutagen was dependent upon the pH of the medium, with the mutagenicity being greater at pH12 than at pH7.4 and it was proposed that, in the case of ptaquiloside, this was due to the substance being converted into a highly reactive direct-acting mutagen by the removal of the OH-group from the molecule. Further mutagenicity tests on ptaquiloside that had been treated with alkali showed it to be positive in tests for sister chromatid exchanges and gene mutations (HGPRT) in V79 cells and for unscheduled DNA synthesis in human fibroblasts (HAN-cells) and to cause transformation of C3H 10T1/2 cells (van der Hoeven, *et al*., 1983 and van der Hoeven, 1986).

The carcinogenic agent that was isolated from bracken is called ptaquiloside. This substance is not unique to bracken. Ptaquiloside or analogues of ptaquiloside have been detected in 19 out of 31 species of ferns that were tested (Potter and Baird, 2000).

Figure 1: The structural formula of ptaquiloside (aka. aquilide **A.** aka. braxin C)



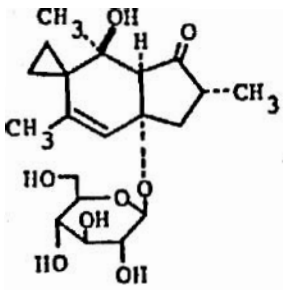
Ptaquiloside

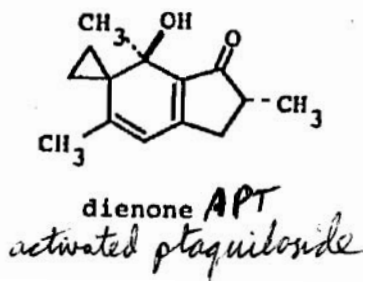
6.4 **Toxic substances found in bracken**

The whole of the bracken plant contains toxins, at least some of which remain after cutting and drying (Cooper and Johnson, 1998; Cooper, *et al*, 2003). Bracken contains several substances that have the potential to cause illness in animals and humans including thiamine deficiency and cancer. The potentially harmful substances identified in bracken include ptaquiloside (also known as aquilide A and braxin C) and other illudanes and protoilludanes, indanones (including various pterosins and pterosides), quercetin and its glycoside rutin, kaempferol, shikimate, thiaminases, prunasin, and various ecdysteroids (Duffus and Duffus, 1991; MAFF, 1996; Campo, 1997; Alonso-Amelot and Avendaño, 2002; Rasmussen, *et al.,* 2003a). Other substances that have been identified in bracken include the dihydrocinnamic acids, tannins, phloretic acid, braxins, astragalin, isoquercetin, tiliroside (kaempferol-3-*p*-coumaroylglucoside), *p*-hydroxystyrene glycocides, dihydroferulic acid, 2,3-butanediol, 3-methylbutan-2-ol, monomethylsuccinate, 2-hydroxymethyl ester of propanoic acid, 4-methoxymethyl ester of butanoic acid, monomethyl ester of butanedioic acid, methyl-5-oxoproline, 2(3H)-dihydrofuranone, *t*-2-methylcyclohexanol and other low molecular weight materials (Wang, *et al*., 1973; IARC, 1983; Hirono, 1986; Saito T, *et al*., 1986 & 19909; Evans, *et al*., 1984; Méndez, 2005;). The structural formulae of some of the substances detected in bracken are shown in Figure 2.

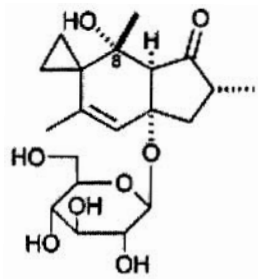
In addition to these substances, several unstable analogues of ptaquiloside have been identified in a neotropical variety of bracken, *Pteridium aquilinum* var. *caudatum* (also known as *Pteridium caudatum* L. Maxon)) that is not found in the UK. The analogues of ptaquiloside that were identified were caudatoside, iso-ptaquiloside, ptaquiloside A, ptaquiloside Z and protoilludalane (Castillo, *et al*., 1997 and 1998; Alonso-Amelot and Avendaño, 2002). Other substances found in *Pteridium aquilinum* var. *caudatum* included ptaquiloside and pterosins A, B, K and Z (Castillo, *et al*., 1997). In acid conditions, ptaquiloside Z broke down to form two indanone compounds, pterosin Z and pterosin I (Castillo, *et al*., 1998).

Figure 2: The structural formulae of some of the chemicals found in bracken

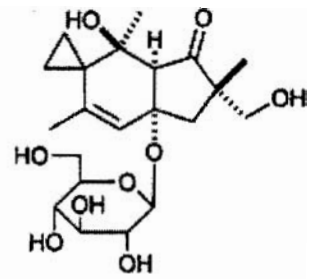




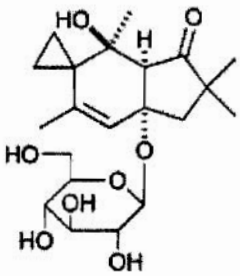
ptaquiloside



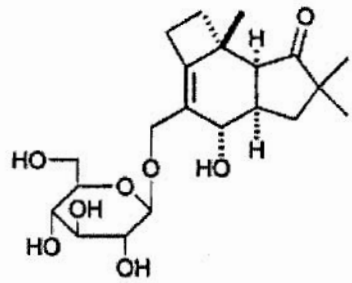
isoptaquiloside



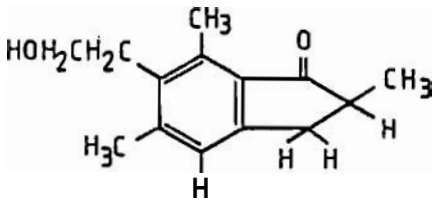
caudatoside



ptaquiloside Z

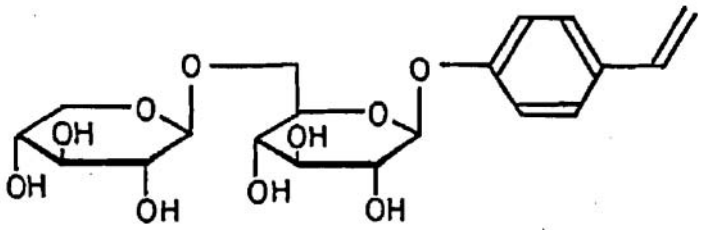


pteridanoside



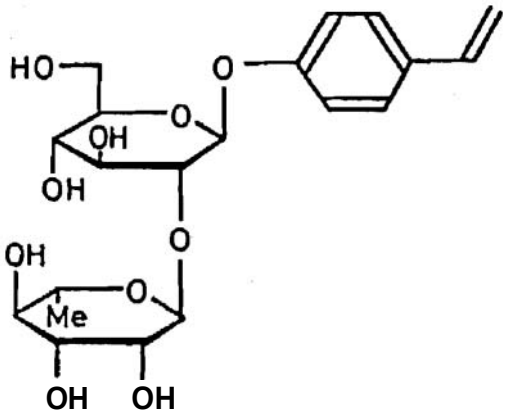
Pterosin B, a 1- Indanone derivative

Figure 2 (part 2): The structural formulae of some of the chemicals found in bracken



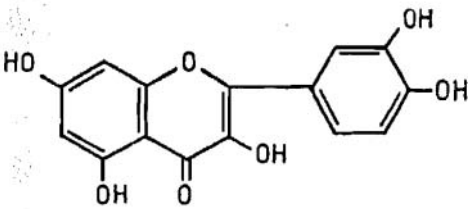
ptelatoside-A

ρ-β-primeverosyloxystyrene

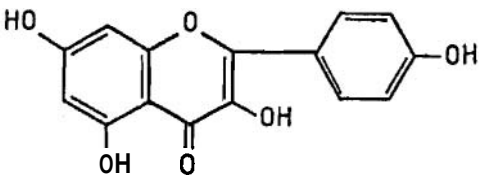


ptelatoside-B

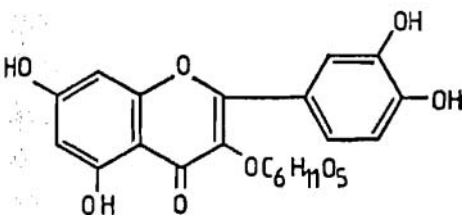
ρ-β-neohesperidosyloxystyrene



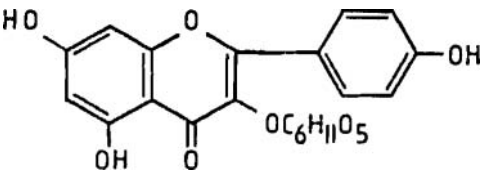
Quercetin



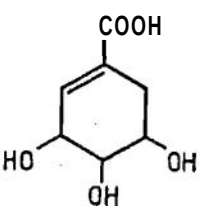
Kaempferol



iso-Quercitrin



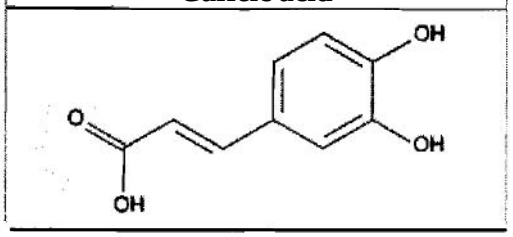
Astragalin



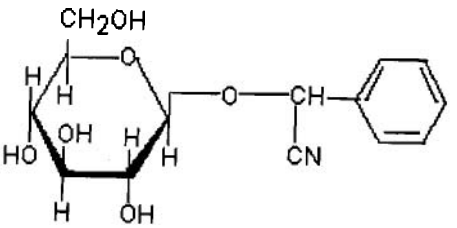
Shikimic Acid

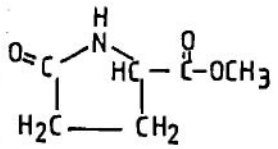
Figure **2** (part 3): The structural formulae of some of the chemicals found in bracken

Caffeic acid

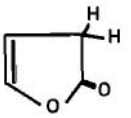


Prunasin (CAS No. 99-18-3)

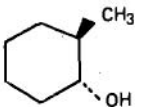




. Proline, 5-oxo, methylester



2(3H)-furanone-dihydro



Cyclohexanol, 2-methyl-, trans

Compounds found in rhizome and **crozier** samples

Sample	Number	Compound name	Compound formula	Scan number	Fit	%
Rhizome	1	Ethanedioic acid	$C_2H_2O_4$	3	944	0.64
	2	2.3-butanediol	$C_4H_{10}O_2$	441	958	594
	3	3-methylbutan-2-ol	$C_5H_{12}O$	839	923	6·02
	4	Butanedioic acid, monomethylester	$C_5H_8O_4$	1035	997	1.28
	5	Proline, 5-oxo-methylester	$C_6H_9O_3N$	1206	989	1·68
	6	Methanamine, N-methyl comp D with borane (1:1)	$C_2H_{10}NB$	1412	781	3.31
Crozier	1	Propanoic acid, 2-hydroxy-methylester	$C_4H_8O_3$	351	922	11·69
	2	2,3-butanediol	$C_4H_{10}O_2$	567	961	7·60
	3	2(3H)-furanone-dihydro	$C_4H_4O_2$	622	958	0·86
	4	Acetic acid, methoxy-ethylester	$C_5H_{10}O_3$	822	842	1·05
	5	Ethanol, 2,2'-/1,2-ethanediylbis(oxy)/bis-	$C_6H_{14}O_4$	829	729	1·05
	6	3-methylbutan-2-ol	$C_5H_{12}O$	950	925	5·89
	7	Butanoic acid, 4-methoxy-, methylester	$C_6H_{12}O_3$	1035	779	44·75
	8	Butanedioic acid, monomethylester	$C_5H_8O_4$	1145	996	7·80
	9	Proline, 5-oxo-methylester	$C_6H_9O_3N$	1315	990	5·63
	10	Cyclohexanol, 2-methyl-, trans	$C_7H_{14}O$	1734	721	1·24

6.5. Hazards from substances in bracken

6.5.1. Ptaquiloside and APT

6.5.1.1. Identity and amounts of ptaquiloside:

Ptaquiloside (aquilide A; braxin C; CAS number 87625-62-5) is a water-soluble (log K_{ow} = -0.63 ± 0.01) highly electrophilic norsesquiterpene glycoside of the illudane type that was independently isolated from bracken *Pteridium aquilinum* var. *latisculum* by workers in Japan (Niwa et al, 1983) and from *Pteridium aquilinum* (L.) Kuhn (variety not reported) by workers in The Netherlands (van der Hoeven et al, 1983). Its structure was elucidated in 1987, and this is shown in Figure 1 (Ojika, *et al*., 1987). Its molecular weight is 398.45. Ptaquiloside has been found in bracken at concentrations of 210-2400 mg/kg (on a dry weight basis) (van der Hoeven, *et al*., 1983; Niwa, *et al*., 1983).

Isolated ptaquiloside is unstable in alkaline conditions, with 80% of the substance decomposing within 10 mins at pH 11.5 and room temperature (Alonsono-Amelot, 2002). It is also unstable to acid, heat and sunlight. Degradation of ptaquiloside followed second-order kinetics, with a half-life of 7 days at pH 4, while at pH 5 and pH 7 respectively 60% and 90% of the original concentration remained after 7 days at 37°C (Saito K, *et al.,* 1989). Ayala-Luis, *et al*. (2006) studied the breakdown of ptaquiloside at a variety of pHs and temperatures. In contrast to earlier findings, they reported that the kinetics of ptaquiloside hydrolysis follows first-order kinetics with respect to ptaquiloside at all pHs and temperatures. At 22ºC, reactions at pH 4.43 or less had a rate constant of 25.7 h^{-1}; at pHs of 6.39 or greater the rate constant was 4.83 x 10^{4} h^{-1}; and at neutral pH the rate constant was 9.49 – 10^{-4} h^{-1}. Ptaquiloside had the lowest rate of hydrolysis at low temperatures and slightly acidic conditions. This implies that ptaquiloside is most likely to leach deep into soils and enter aquifers in areas with cold climates and slightly acidic soils.

In contrast with the instability of the isolated substance, ptaquiloside is stable when *in situ* within the live plant. The ptaquiloside in cut fronds of bracken is also fairly stable within the plant and when exposed to sunlight, with more than 25% of the original concentration in cut fronds remaining after 6 weeks (Saito K, *et al*., 1989).

Early attempts to quantify the concentrations of ptaquiloside in bracken were hampered by the reactivity of the compound, but this difficulty has been overcome by converting ptaquiloside to the stable pterosin B during sample preparation and then analysing using an HPLC method with UV detection (Agnew and Lauren, 1991; Alonso-Amelot, *et al*., 1998; Rasmussen, *et al*., 2003). A GC-MS method of analysis that can directly detect ptaquiloside has also been described (Bonadies, *et al*., 2004).

Different species and varieties of bracken contain different amounts of ptaquiloside (Smith, *et al*., 1994; Villalobos-Salazar, *et al.,* 2000). Ptaquiloside has also been detected in certain other ferns, including those in the genera *Cheilanthes, Cibotium, Dennstaedtia, Histiopteris, Hypolepis, Microlepia, Onychium, Pityrogramma* and *Pteris* (Agnew and

Lauren, 1991; Alonso-Amelot and Avendaño, 2002). Different parts of the bracken plant contain different amounts, with particularly high concentrations being found in young crosiers. The concentration of ptaquiloside in bracken can vary at different times of the year, with the content normally being highest at the start of the growing season, when young crosiers emerge from the ground (Alonso-Amelot, *et al*., 1992a; Rasmussen, *et al.,* 2003a). Smith, *et al*. (1994) measured ptaquiloside in samples of bracken taken from different sites worldwide. They found concentrations of up to 12.9 mg/g in young fronds (crosiers of *Pteridium aquilinum* var. *esculentum* from Australia), up to 7.4 mg/g in rhizomes and up to 9.8 mg/g in spores, but the levels in different varieties varied widely, with some containing no detectable ptaquiloside (< 0.009 mg/g). Samples of *Pteridium aquilinum* var. *aquilinum* from the UK contained between 0.447 and 1.211 mg/g, with a mean of 0.919 mg/g. Analysis of fronds and rhizomes taken from common bracken (*Pteridium aquilinum* var. *aquilinum*) taken from four different sites in Denmark showed that concentrations of ptaquiloside ranged between 213 and 2145 mg/kg in fronds and 11 to 902 mg/kg in rhizomes, and the soils from around the plants contained 0.22 to 8.49 mg/kg with 0.2-8.5 mg/L in the aqueous phase (Rasmussen, *et al*. 2003b).

Laboratory tests showed that ptaquiloside could leach from fresh mature bracken fronds that were exposed to water aerosols for 1.5 hours inside plastic bags. The concentration of ptaquiloside in the leachate water was 0.099 to 0.734 mg/L, which was equal to 0.18 to 0.24% of the amount of ptaquiloside in the fronds (Rasmussen, *et al*. 2003b). Recent measurements (Engel, *et al*., 2007) found 2.1 ± 0.5 mg/g in fronds of *Pteridium aquilinum* var. *aquilinum* sampled in Denmark and 37.0 ± 8.7 mg/g in fronds of *Pteridium aquilinum* var. *esculentum* sampled in New Zealand.

Studies of the behaviour of ptaquiloside in different soils (Rasmussen, *et al.,* 2005) showed three different first-order degradation patterns:

- Rapid degradation in acid (pH<4) sandy soils with half-lives of 8-30 h, decreasing with the amount of organic matter in the soil;
- Slow degradation in less acid (pH>4) sandy soils with half-lives of several days; and
- Fast initial degradation with a concurrent solid phase-water partitioning reaction observed for non-acid clayey soils.

Degradation at 4ºC was 800 times slower than at 25ºC. There was negligible sorption of the ptaquiloside to the soils. The authors concluded that leaching of ptaquiloside to the aqueous environment and to aquifers would be most extensive on sandy soils of pH>4 that are poor in organic matter and are exposed to high precipitation rates during cold weather.

Villalobos Salazar, *et al*. (2000) measured the amounts of ptaquiloside in two sub-species of bracken, *Pteridium aquilinum arachnoideum* and *Pteridium aquilinum caudatum*, that were collected at various altitudes in Costa Rica. The results are summarised in Table 4. The concentrations of ptaquiloside decreased with the age of the fronds (statistically significant trend, $p < 0.0001$). At each growth stage, the concentration in the *arachnoideum* sub-species was about two times higher than in *caudatum*. Concentrations

were higher in the samples collected at higher altitudes (statistically significant trend, $p < 0.005$).

Table 4: Concentrations of ptaquiloside in two sub-species of bracken

Altitude (m)	Mean concentration (mg/kg) ± standard deviation		
	Young fronds	Intermediate fronds	Mature fronds
Arachnoideum			
1800	15104 ± 1725	2127 ± 271	585 ± 77
1400	7961 ± 3610	1271 ± 377	396 ± 38
1000	5770 ± 1296	890 ± 260	300 ± 60
Caudatum			
1800	6838 ± 965	1030 ± 117	278 ± 29
1400	3726 ± 743	695 ± 108	190 ± 20
1000	5135 ± 1003	313 ± 121	81 ± 44

Potter and Pitman (2006) reported the results of analyses for ptaquiloside and pterosins in fresh, dead and composted bracken fronds that had been harvested in the New Forest, England, in summer (sporulating) or autumn (non-sporulating) 1992. Fresh summer-cut bracken contained ptaquiloside and pterosin B, whereas fresh autumn-cut bracken contained pterosins B and L, but only a trace of ptaquiloside. Summer-cut bracken that had been composted for 3 weeks still contained pterosin B, but contained no ptaquiloside. Bracken that had been composted for 12 weeks or longer and dead bracken that had been collected contained no ptaquiloside or pterosins.

6.5.1.2. **Consumer exposure to ptaquiloside**:

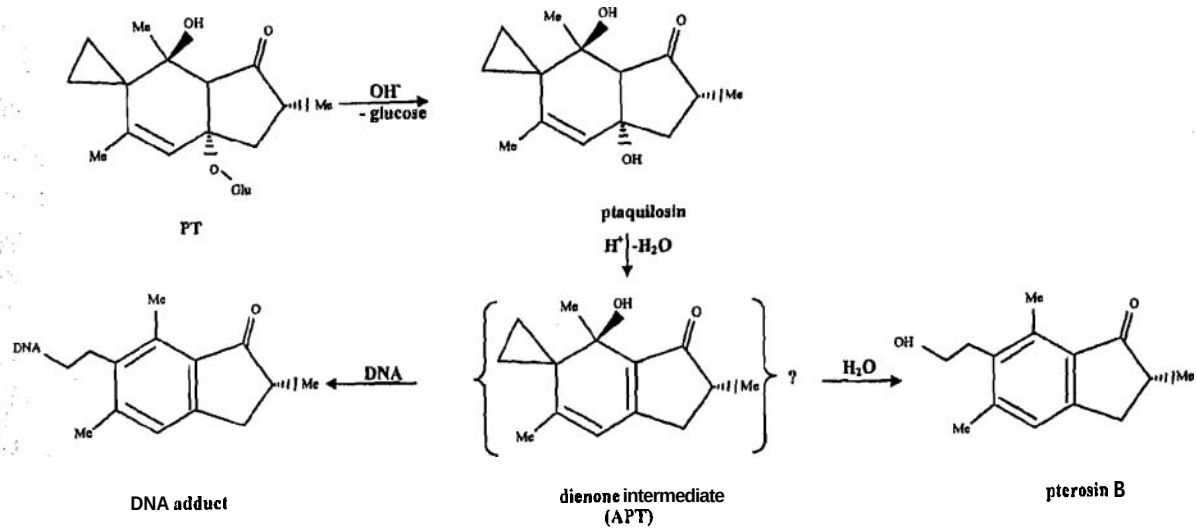
Consumers can be exposed to ptaquiloside by eating bracken or by eating food or drinking water that is contaminated with ptaquiloside. Ingestion and/or inhalation of spores are other possible routes of direct human exposure. Residues may occur in foods derived from animals that ate bracken. Contamination of drinking water may also occur. As ptaquiloside is very water soluble and does not bind with soil, run-off of water from bracken infested land can potentially contaminate drinking water. However, degradation of ptaquiloside to the relatively non-toxic pterosin B can occur in acidic conditions (and activation to the more reactive activated ptaquiloside (APT) can occur in alkaline conditions) – see Figure 3. Microorganisms in the soil can also degrade ptaquiloside (Engel, et al., 2007) and this is more rapid than the chemical degradation in acidic soils. The highest amount of contamination of aquifers with ptaquiloside is likely to occur in bracken infested areas with well-drained slightly acidic soils and cold climates (Ayala-Luis, *et al*., 2006).

The half-life of ptaquiloside is about 8 weeks if stored in aqueous extracts at 37ºC (Burkhalter, *et al*., 1996). Villalobos-Salazar, *et al*. (2000) fed 8kg of fresh bracken (not clear whether this was *Pteridium aquilinum arachnoideum* or *Pteridium aquilinum caudatum*), to 2 Holstein cows and milked them prior to feeding and at 30, 54, 78 and 115 hrs after feeding. The mean concentrations of ptaquiloside detected in the milk at

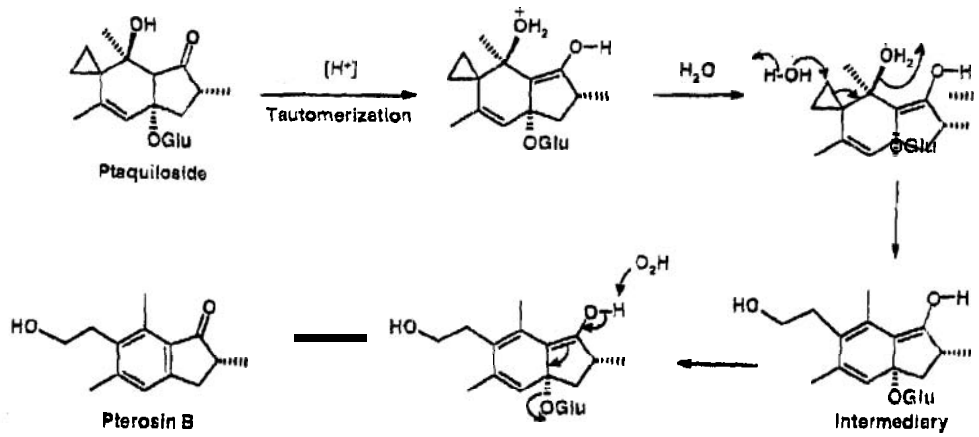
these times were, respectively 0, 0, 80 (87 & 73), 180 (188 & 172) and 79 (86 & 72) μg/L. This shows that ptaquiloside takes longer than 115 hrs after feeding to clear from cows' milk.

In another experiment, Villalobos-Salazar, *et al*. (2000) fed 16kg of fresh bracken (*Pteridium aquilinum arachnoideum* and/or *Pteridium aquilinum caudatum*), to 2 Holstein cows and milked them at 78 hrs after feeding. Samples of the milk were pasteurised, boiled or left unprocessed and the ptaquiloside contents were measured. Boiling caused a 74% reduction in the ptaquiloside concentration and pasteurisation caused a 48% reduction.

Figure 3: Ptaquiloside reaction pathwav (Shahin, et al., 1999)



Proposed scheme of ptaquiloside reaction pathway.



Proposed mechanism for the acid hydrolysis *of* ptaquiloside.

Ptaquiloside has been detected in milk from cows (Alonso-Amelot, *et al.* , 1992b,1993, 1996 and 1998; Alonso-Amelot, 1997; Villalobos-Salazar, *et al*., 2000). A concentration of ptaquiloside of 0.11 mg/L was found in milk from a 3-year-old Holstein cow that had been fed bracken (*Pteridium aquilinum*) for 7 days in 6 kg/day doses of freshly-cut fronds mixed with grass and molasses that provided a dose of ptaquiloside of 216 mg/day (Alonso-Amelot, *et al.,* 1992b and 1993). The bracken contained 0.25 ± 0.05 mg of ptaquiloside per gram of bracken frond. The total amount of ptaquiloside excreted in milk was equal to 1.2% of the total oral intake of ptaquiloside. The cow showed no clinical signs of illness and there was no treatment-related reduction in the amount of milk produced.

Alonso-Amelot, *et al.* (1993) kept 4 Holstein cows in a field that was heavily infested with bracken. After 24 months, three of the cows had developed enzootic haematuria, however no ptaquiloside or pterosin B was detected in any of three weekly samples of milk taken from each of the cows.

With an improved analytical technique, the same group was able to show that as much as 8.6 ± 1.2% of the ptaquiloside eaten by six two-year-old cows was excreted in the milk (Alonso-Amelot, *et al.*, 1998). Six two-year-old Jersey-Holstein cows were fed 6 kg/cow/day of fresh bracken fronds (neotropical ssp. *caudatum* var *caudatum*). The bracken fronds had been sorted according to their content of ptaquiloside and different batches containing were fed for a series of 5-day runs. The average intakes of the cows over the 5-day runs were 2400, 4500, 8100 and 10000 mg ptaquiloside/cow/day. The cows given the highest dose (10000 mg/cow/day) refused to eat the bracken on the fifth day so their treatment was for only 4 days. The average milk production of the cows was 20 ± 2 L/cow/day. No ptaquiloside was found in the first samples of milk, which were taken 14 hours after the start of feeding of bracken. Ptaquiloside was first detected at 38 hours and the amount of ptaquiloside in the milk increased for the first 86 hours of ingestion of bracken. The amount of ptaquiloside in milk levelled off at peak concentrations of approximately 10, 27, 38 and 55 mg ptaquiloside/L of milk for the respective doses 2400, 4500, 8100 and 10000 mg ptaquiloside/cow/day. When feeding of bracken stopped, the amount of ptaquiloside decreased until none was detectable at 86 hours after feeding with bracken was stopped.

The authors estimated (Alonso-Amelot, *et al.*, 1998) that a person consuming about 0.5 L of milk per day from a cow producing 20 L of milk per day and which had eaten a subtoxic dose of 5000 mg ptaquiloside/cow/day (contained in 6-7 kg of fern) for a few days would ingest between 1.75 and 13.4 mg ptaquiloside/person/day, depending on the time between the cow eating bracken and the time it was milked. Note that the estimate of consumer intake of milk is quite low compared with the JECFA food basket value (1.5 L/person/day) and with UK figures for chronic consumption of milk (97.5^{th} percentile chronic consumption = 569-1054 g/person/day for various subgroups of the UK population).

UK data on chronic consumption of milk are shown in Table 5. The highest chronic intake of milk by any UK consumer in the surveys was 3105 g/person/day for adults.

Extrapolating from the data of Alonso-Amelot, *et al*. (1998), UK consumption data suggest exposure of this extreme consumer would be 10.9 to 83.2 mg ptaquiloside/person/day (equal to 0.14 – 1.09 mg/kg bw/day) if the milk was from cows ingesting a subclinical dose of 5000 mg ptaquiloside/cow/day. The highest individual intake of milk by an infant was 1459 g/person/day. This would give this individual an estimated intake of ptaquiloside of 5.1 to 39.1 mg/person/day (or 0.59 to 4.49 mg/kg bw/day).

A similar calculation was applied using the 97.5th percentile intakes of UK sub-populations of milk consumers. The highest chronic the 97.5th percentile intake of milk was seen in infants aged 6 to 12 months, with a level of 1054 g milk/person/day for total milk intake including infant formulae and breast milk. It was considered very unlikely that UK nursing mothers would be exposed to high levels of dietary ptaquiloside and it was considered likely that the processing of cows milk for the production of infant formula would probably destroy or remove much of any ptaquiloside present. Therefore intakes of milk excluding breast milk (1010 g milk/person/day) and excluding breast milk and formulae (850 g milk/person/day) were also considered. The estimates of the intakes of ptaquiloside by these groups of infants and other UK populations are shown in Table 6. The intake of ptaquiloside in milk by infants could be up to 28.2 mg/person/day (3.24 mg/kg bw/day), on the assumption that infant formula and breast milk contain as much ptaquiloside as untreated cows' milk. However, this estimate drops to 27.1 mg/person/day (3.11 mg/kg bw/day if we exclude breast milk. It drops further to 22.8 mg/person/day (2.62 mg/kg bw/day if we also exclude infant formulae from our consideration.

The data on milk intakes by UK infants are derived from a survey that was conducted in 1992-1993. Since this date the FSA has advised mothers not to feed infants of 1 year or less on cows' milk. The reason for this was that cows' milk alone does not contain all of the nutrients needed by infants. Assuming that this advice has been observed, the intake of milk by infants is expected to be now lower than the estimates given above. The subpopulation, other than infants, that is most at risk is toddlers, which an estimated intake of ptaquiloside in milk of up to 21.6 mg/person/day (1.49 mg/kg bw/day). Institutional elderly have a higher intake per person than toddlers (up to 22.1 mg/person/day) but in comparison with bodyweight the level of intake is less (up to 0.36 mg/kg bw/day).

No information was available on the amount of ptaquiloside or its metabolites in meat or offal from food-producing animals that had eaten bracken.

In conclusion, human consumption of milk from cows receiving subclinical doses of ptaquiloside from eating bracken could potentially be as high as 4.49 mg/kg bw/day (using data from the infant with the highest individual chronic consumption of milk). The age groups most at risk from contaminants in milk are infants and toddlers, due to their relatively high consumption of milk in comparison with their body weight. Following the FSA advice for infants not to be given cows' milk to drink, it might be more realistic to regard the intake of the 97.5th percentile toddler consumer of cows milk as the highest

intake likely to occur as a result of consuming milk from cows eating bracken but not showing signs of toxicity. In practice most people will be exposed to less than this, due to drinking less milk, dilution of any ptaquiloside in the milk by bulking with milk from unexposed cows, loss of ptaquiloside during pasteurisation or other processing.

Intakes of ptaquiloside from consuming milk from bracken poisoned cows are likely to be higher than from milk from asymptomatic cows.

Ptaquiloside might or might not leave residues in meat and offal from animals exposed to bracken. No data are available.

Table 5: Chronic intakes of milk by UK consumers (g/person/day)

Population	Percent of population who consume milk	Mean consumption	Median consumption	Maximum consumption	97.5th percentile consumption	99th percentile consumption
Adults	99.67	234	202	3105	639	789
Infants aged 6-12 months (excluding infant formula and breast milk)	99.39	329	645	1459	851	1039
Infants aged 6-12 months (including infant formula but not breast milk)	99.80	483	989	1459	1011	1234
Infants aged 6-12 months (including infant formula and breast milk)	100	541	532	1459	1054	1239
Toddlers aged 1½-4½ years	99.82	301	264	1361	808	968
Children aged 4-18	100	219	188	1014	594	697
Children aged 4-6	100	253	229	890	619	724
Children aged 7-10	100	222	193	956	584	624
Children aged 11-14	100	209	179	1014	569	729
Children aged 15-18	100	199	165	859	601	700
Institutional elderly	100	219	386	1973	825	978
Free-living elderly	99.78	274	254	1384	635	796
Vegetarians	99.26	219	374	1143	661	777

The information in Table 5 was obtained using data on individual consumption that are compiled in the "Intake 2" programme. The data include intakes of cows', sheep's and goats' milk, milk in chocolate and milk used in recipes. Chocolate was assumed to be 25% milk.
The "Intake 2" programme used data from the following food intake surveys: Infants 86, Toddlers' Survey, Young Persons '98 Survey, Vegetarian 1994-95 Survey, Adults 2001 National Diet and Nutrition Survey and Free-Living and Institutional Elderly Surveys (Mills and Tyler, 1992; Gregory, *et al*., 1995; Gregory, *et al*., 2000; MAFF, 1996; Henderson, *et al*., 2002 and Finch, *et al*., 1998).

Table 6: Estimated intakes of ptaquiloside by populations in the UK

Population	**97.5th percentile consumption of milk (g/person/day)**	**Estimated intake of ptaquiloside (mg/person/day)**	**Mean bodyweight in UK survey (kg)**	**Estimated intake of ptaquiloside (mg/kg bw/day)**
Adults	639	2.3 – 17.1	76.5	0.003 – 0.22
Infants aged 6-12 months (excluding infant formula and breast milk)	851	3.0 – 22.8	8.7	0.34 – 2.62
Infants aged 6-12 months (including infant formula but not breast milk)	1011	3.6 – 27.1	8.7	0.41 – 3.11
Infants aged 6-12 months (including infant formula and breast milk)	1054	3.7 – 28.2	8.7	0.43 – 3.24
Toddlers aged 1½-4½ years	808	2.8 – 21.6	14.5	0.19 – 1.49
Children aged 4-18	594	2.1 – 15.9	41.5	0.051 – 0.38
Children aged 4-6	619	2.2 – 16.6	20.5	0.11 – 0.81
Children aged 7-10	584	2.1 – 15.6	30.9	0.068 – 0.49
Children aged 11-14	569	2.0 – 15.2	48.0	0.041 – 0.32
Children aged 15-18	601	2.1 – 16.1	63.8	0.033 – 0.25
Institutional elderly	825	2.9 – 22.1	61.6	0.047 – 0.36
Free-living elderly	635	2.2 – 17.0	70.8	0.031 – 0.24

6.5.1.3. **Toxicity of ptaquiloside**:

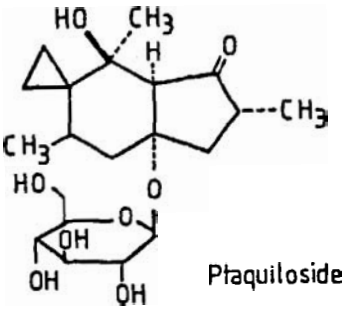
It has been suggested that ptaquiloside is involved in various animal diseases, including acute haemorrhagic syndrome in cattle (sometimes simply called "bracken poisoning"), enzootic haematuria in cattle (as a result of chronic exposure to bracken) and progressive retinal degeneration (PRD or "bright blindness") in sheep (another disease of chronic exposure) (Cooper and Johnson, 1998). Hirono, *et al.* (1995) produced PRD in 2 young male Suffolk Down lambs by intravenous administration of 80-130 mg/animal by catheter to one lamb and 46-400 mg/animal by injection to the other lamb of ptaquiloside every other day for 6 months. A third lamb was kept as a control. The first signs of PRD were seen in the ptaquiloside-treated lambs after 230 and 168 days into the treatment regime.

Ptaquiloside was of low cytotoxicity when tested *in vitro* in Chinese hamster ovary (CHO) cells, 3T3 mouse fibroblasts and normal rat kidney cells, with the concentrations inhibiting cell growth by 50% (IC_{50}) after 48 h of incubation being respectively 0.4×10^{-2}, 0.2×10^{-7} and 0.2×10^{-2} M (Ngomuo and Jones, 1996a). The authors suggested that these results indicated that ptaquiloside was unlikely to be directly responsible for acute bracken poisoning.

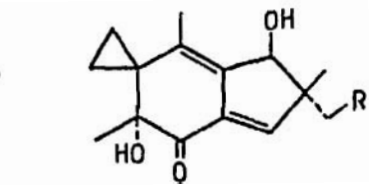
Ptaquiloside and APT are chemically similar to illudin S and illudin M (see Figure 4), which are responsible for the poisonous properties of the Jack O'Lantern mushroom (*Omphalotus olearus*). The acute oral toxicity of ptaquiloside to mice was about 150 times less than that of illudin S, but the acute effects of the two substances were quantitatively and qualitatively broadly similar to oneanother in rats and in calves (Evans IA, 1987). A single dose of illudin S or ptaquiloside caused very high leucocytosis with no indications of haemorrhage or pyrexia in calves.

Single sub-cutaneous (sc) injections of ptaquiloside were administered to mice, rats and guinea-pigs (Yoshida and Saito T, 1994a). Doses of up to 100 mg/kg bw caused no adverse effects on clinical signs, bodyweight gain, or histopathology in 9 male ddY mice and in 7 male Sprague-Dawley rats. However, adverse effects were seen in Hartley guinea-pigs that received ptaquiloside. Groups of 5 male guinea-pigs (only 3 in the top dose group) were given single sc-injections of 0, 1, 5 or 10 mg/kg bw. No clinical signs were seen in those given 0 or 1 mg/kg bw. However, in those given 5 or 10 mg/kg bw, there was haematuria within 14 h and at 3 days there was a bodyweight loss, piloerection, anorexia, anuresis and a loss of vigour. The guinea-pigs were killed for autopsy at 3 days after dosing. No pathology was seen in the animals given 0 or 1 mg/kg bw. In those given 5 or 10 mg/kg bw, there was oedema in the urinary bladder and haemorrhage in the urinary bladder perirenal adipose tissue, the cords of the bladder and the abdominal wall. Histology showed loss of the epithelial cell layer in the mucosa of the bladders of severely affected guinea-pigs. Neutrophilic infiltration of the lamina propria of the bladder was seen in all animals given 5 or 10 mg/kg bw, and severely affected animals also had infiltration in the kidneys and ureters.

Figure 4: Comparison of ptaquiloside and illudins



Ptaquiloside (Aquilide A)



Illudin S (R=OH)

Illudin M (R=H)

Another study (Yoshida and Saito, 1994b) was performed using sc doses of 0, 1 or 10 mg/kg bw of ptaquiloside given to groups of 4 Hartley guinea-pigs with or without their ureters cannulated to divert the urine away from the bladder. Doses of 0 or 1 mg/kg bw had no adverse effect, but a sc dose of 10 mg/kg bw caused haemorrhagic cystitis irrespectively of whether or not the ureters were cannulated. It was concluded that the cystitis was caused by toxic agents carried by the blood, rather than by direct contact with material excreted into the urine.

The microcirculatory effects of ptaquiloside were investigated in rabbits (Asano, *et al.,* 1989). A single dose of either 50 or 100 mg/kg of ptaquiloside in 10 ml of saline was injected intravenously into the ear of one of two rabbits. The subsequent effects on the cutaneous microcirculation of the ear were observed microscopically using a transparent chamber fitted to the ear. There was marked persistent vasoconstriction at arteriolar level at both dose levels. Leucocytes became much more adherent and appeared to increase in numbers. In postcapillary beds the adherences of leucocytes acted like thrombi, causing aggregation of erythrocytes, plasma skimming and stasis in the microvascular net. This persisted throughout the 4 days observation period following ptaquiloside administration. Haematuria was seen from about 20 h after the injection and packed cell volumes were lowered. The rabbit given the highest dose died at 4 days post-injection and the other rabbit survived for more than 6 months. Post-mortem of the rabbit that died showed hyperaemia and oedema of the serous surfaces of the kidneys, small intestines and bladder, hyperaemia of the lungs, haemorrhages within the kidneys. The mucosa of the bladder was hyperaemic and swollen in large blister-like formations all over the interior surface.

Ptaquiloside was isolated from a boiling water extract of bracken and was administered by oral drench to a single female Holstein-Friesian calf. Doses of 400 mg of ptaquiloside were given six days per week for 24 days, then 800 mg/day was given for 14 days, and finally 1600 mg/day was given for 4 days. Blood samples showed granolocytopaenia from day 50 and thrombocytopaenia from day 35 of treatment. The calf was killed for autopsy 86 days after the start of administration. The autopsy showed no haemorrhaging, but femoral and sternal bone marrows were replaced almost entirely by fat with only small foci of erythropoietic cells and few megakaryocytes remaining. (Hirono, *et al*., 1984)

6.5.1.4. **Carcinogenicity of ptaquiloside and of APT**:

Ptaquiloside is thought to be the major carcinogenic substance in bracken. It was shown to be a major carcinogenic component of bracken in series of experiments that were performed independently by workers in Japan (Niwa, *et al*., 1983; Hiono, *et al*., 1984a & 1984b) and in The Netherlands (van der Hoeven, *et al*., 1983). It has been estimated that ptaquiloside accounts for more than 50% of the carcinogenic potency of bracken (van der Hoeven, *et al*., 1983).

In an initial experiment (Hirono et al, 1984a), two groups of 12 female Sprague-Dawley rats were given ptaquiloside that had been isolated from bracken and a control group of 15 females was kept for comparison. Two different dosing regimes were used: rats in Group 1were each given an intra-gastric dose of 780 mg/kg bw of ptaquiloside when they were 25 days-old followed by weekly doses of 100-200 mg/kg bw for the next 8 weeks; Group 2 were given twice-weekly doses of 100-150 mg/kg

bw for 8½ weeks. The experiment was terminated at 300 days after the initial administration. All rats were autopsied. All rats in Group 1 were reported to have loss of bodyweight, urinary incontinence and haematuria following the initial administration of 780 mg/kg bw, and 5 rats in this group died within the first 83 days of the experiment. All of the 7 animals in Group 1 that survived more than 190 days developed mammary tumours (adenocarinomas, papillary carcinomas and anaplastic carcinomas), with an average of 4.8 tumours per rat. Seven of the rats in Group 1 also developed multiple ileal carcinomas and most of the rats in this group had hyperplasia or metaplasia of the urinary bladder mucosa. In Group 2, 10 out of the 11 rats that survived at least 165 days developed mammary tumours (3.9 tumours per rat on average) of similar types to those seen in Group 1 and multiple ileal adenocarcinomas. Transitional cell hyperplasia was seen in the bladders of 7 out of the 11 Group 2 survivors and a urinary bladder papilloma was found in one of them. No tumours were found in any of the control rats.

A group of 15 female ACI rats was fed a diet containing 0.027-0.08% ptaquiloside for 210 days: 0.04% for the first 15 days, then 0.027% for 40 days, 0.04% for 52 days, 0.08% for 40 days and then 0.04% for 60 days (Hirono, *et al*., 1987). The amount of ptaquiloside in the diet was adjusted throughout the study according to bodyweight gain and clinical signs. A control group consisted of 20 female ACI rats. All of the bracken-treated rats developed tumours in the ileum (adenomas, adenocarcinomas and malignant fibrous histiocytomas) and bladder (transitional cell carcinomas, keratinising squamous cell carcinomas and sarcomas). Four of the control rats developed bladder papillomas.

The illudane dienone metabolite of ptaquiloside (also known as activated ptaquiloside or APT) was tested for toxicity in groups of 5 female Sprague-Dawley rats (Shahin, *et al*., 1998d). One group was given 10 weekly doses of 3 mg/rat (20.7 mg/kg bw) of APT and other groups were given weekly oral doses of 3 or 6 mg/rat (20.7 or 41.4 mg/kg bw) for up to 10 weeks. Animals receiving 6 mg/rat had to be euthanased after 3 weeks, due to anorexia, weakness and haemorrhages; the other animals were killed 30 weeks after the final dose of test material. The rats that had received 6 mg/rat for 3 weeks had apoptotic bodies in their liver, coagulation necrosis in the kidneys and bone marrow hypoplasia. The rats that had received APT (i.v. or orally) at 3 mg/rat for 10 weeks had monocytosis with elevated levels of the tumour necrosis factor TNFα in the plasma and also renal tubular necrosis. The i.v.-dosed animals also had type II pneumocyte proliferation. Mammary tumours (adenocarcinoma) were found in 2 of the 5 animals dosed by the i.v. route.

In a slightly larger study, Shahin, *et al*. (1998a & 1998c) gave weekly intravenous (i.v.) doses of either 3 mg/rat of ptaquiloside or 3 mg/rat of APT for 10 weeks to groups of 10 female Sprague-Dawley rats of 145 g bodyweight. The doses of 3 mg/rat were equivalent to 20.7 mg/kg bw. Control rats were kept for comparison. Both groups of treated rats and the controls were kept for 30 weeks after the end of the dosing period, before being killed. An additional group of 10 rats was given i.v. doses of 3mg/rat of APT for 10 weeks, but was killed immediately after dosing to assess the amount of DNA adduct formation before there had been time for DNA-repair to have occurred. Monocytosis was evident in the rats given ptaquiloside and in those given APT and there were also elevated levels of the tumour necrosis factor TNFα in the plasma, which remained high even at 30 weeks after the end of dosing. Focal renal

tubular necrosis was seen in rats given ptaquiloside or APT. Focal type II pneumonocyte necrosis was seen in the rats given APT. Four out of 10 rats that were given APT developed mammary tumours (adenocarcinomas or papillary carcinomas) and one rat had an ileal adenocarcinoma, but no tumours were seen in the ptaquiloside-dosed rats. ^{32}P-Postlabelling showed the presence of DNA-adducts in the ileum of all of the APT-treated rats that were killed at the end of the dosing period. The position of the spot on the TLC for the APT-DNA-adduct was identical that found with *in vitro* studies of DNA-adduct formation with ptaquiloside.

In a follow-up study, Shahin, *et al*. (1998b) administered groups of 10 female Sprague-Dawley rats with weekly doses of APT at 3 mg/rat (20.7 mg/kg bw) by i.v. injection for 10 weeks, 6 mg/rat (41.4 mg/kg bw) by oral gavage for 3 weeks, or 3 mg/rat by oral gavage for 10 weeks. The treatments caused monocytosis along with elevation of levels of the tumour necrosis factor TNFα in the plasma. One group of i.v.-dosed rats was killed at the end of the dosing period, whereas another group that received a similar regime of i.v. doses and the groups that received oral doses were killed when their monocyte counts had returned to normal. The i.v.-dosed rats were killed at 30 weeks after the last dose and the orally-dosed rats were killed at 20 weeks. All groups developed ischemic tubular necrosis of the kidneys. Mammary adenocarcinomas were found in 4 out of 10 of the rats that were given i.v. doses and left for 30 weeks. In contrast, the orally-dosed group did not develop tumours but showed necrosis of blood cell precursors in the bone marrow and had apoptotic bodies in the liver. No mutations of the H-*ras* or *p53* genes were found in the mammary glands of the rats that were killed after 30 weeks in either of the groups that were given oral doses or in the mammary tumours or normal mammary tissue of i.v.-dosed rats that developed tumours. However, in 6 out of 10 of the rats that had received i.v doses but had been killed immediately after dosing, there were double mutations at codons 58 (G to T or G to A) and 59 (A to C or A to G) of the H-*ras* from mammary tissue. No mutations were seen at *p53* in mammary tissue from the rats that were killed immediately after dosing.

When DNA was treated *in vitro* with APT and recovered by butanol extraction or P1 treatment, only one adduct was detected by ^{32}P-postlabelling (Freitas, *et al*., 1999a and 2001). The DNA-adduct was different to any of the adducts that had previously been detected by the same workers in mice that had been fed bracken (Povey, *et al*., 1996) but its chromatographic mobility when examined by thin-layer chromatography was similar to that of an adduct that had been reported in the ileum of bracken-fed calves (Prakash, et al., 1996). The results of Povey, *et al*. (1996) are discussed further in Section 6.2.3.4 and those of Prakash, et al. (1996) in 6.5.1.6..

Alonso-Amelot (2003) suggested that the combined activity of APT and quercetin in bracken might have greater genotoxic/carcinogenic potency than either substance on its own as a result of their combined effects on H-*ras* and p53 genes.

6.5.1.5. **Mutagenicity of ptaquiloside**:

Ptaquiloside was tested for bacterial mutagenicity in strains TA98 and TA100 of *Salmonella typhimurium* at different pHs (Nagao, *et al*., 2007; Matoba, *et al*., 1989). It was not mutagenic in either strain when tested at pH 7.4 in the absence of metabolic activation, but, when the ptaquiloside was preincubated at pH 8.5, it was mutagenic in

both strains. Burkhalter, *et al*. (1996) also showed that ptaquiloside was mutagenic to TA98 when tested in the absence of metabolic activation at pH 8.4.

Ptaquiloside was tested *in vitro* for clastogenicity at different pHs in a Chinese hamster lung fibroblast cell line (CHL) in the presence and absence of S9 from the livers of PCB-treated male F344 rats (Matsuoka, *et al*., 1989). Metaphase analyses were performed after 24 and 48 h of treatment. Ptaquiloside caused chromatid exchange type aberrations under all experimental conditions, but it was less potent at low pH, with the concentrations needed to cause aberrations being 400, 4.5 and 4.5 µg/ml at pH 5.3, 7.4 and 8.3, respectively. It also produced DNA-adducts *in vitro* (Shahin, *et al*., 1995) and caused *in vitro* unscheduled DNA repair at pH 7.2 in a primary culture of rat hepatocytes (Mori, *et al*., 1985).

6.5.1.6. **Mode of action of carcinogenicity of ptaquiloside**:

In alkaline conditions ptaquiloside is transformed by first splitting off the glucose moiety from the molecule to form ptaquilosin and then splitting off a hydroxyl group to form an illudane-dienone compound (APT) (Niwa, *et al*., 1983; van der Hoeven *et al*., 1983; Shahin, *et al*., 1999a). APT is electrophilic and has a greater capacity to alkylate DNA than ptaquiloside (Alonso-Amelot and Avendaño, 2002). APT is stable in mildly alkaline conditions (Alonso-Amelot, 2002), but is immediately converted to pterosin B under weakly acidic conditions (Matsuoka, *et al*., 1989). This observation is reflected in the results of mutagenicity studies in *Salmonella typhimurium* strains TA98 and TA100 that found that ptaquiloside was not mutagenic in either the presence or absence of S9 when preincubated at the usual pH 7.4 but was mutagenic when the preincubation was at pH 8.5 in the absence of metabolic activation (not tested with S9) (Matoba, *et al.,* 1987). Similarly Matsuoka, *et al*. (1989) found that ptaquiloside was less potent at causing chromosomal aberrations when tested at pH 5.3 than at pH 7.4 or pH 8.0, needing concentrations about 90 times greater to cause a significant effect when tested without metabolic activation.

A series of further mutagenicity tests using various chemical analogues and derivatives of ptaquiloside gave results that were consistent with APT being the substance responsible for the mutagenicity in *Salmonella typhimurium* strains TA98, TA100 (Nagao, *et al.,* 1989) and TA1535/pSK1002 (a strain containing an inserted plasmid containing a fused umuC"lacZ gene) (Schmidt, *et al*., 2005). Clastogenicity was produced in CHL cells (Matsuoka, *et al*., 1989). Compounds that were chemically similar to APT, but lacked an activated cyclopropane moiety, were not mutagenic to *Salmonella typhimurium* TA98 or TA100 strains.

APT is considered to be the ultimate carcinogen of bracken, with ptaquiloside being its pro-carcinogen.

APT has been shown to bind covalently to purine bases on salmon DNA, forming adducts with the N-7 of guanine and N-3 of adenine, with the opening of the cyclopropyl ring (Ojika, *et al*., 1989). It also caused cleavage of the double strand supercoiled pBR 322 DNA. As the cleavage occurred under ambient conditions without metal complexation or photoirradiation, the authors concluded that the cleavage mechanism was neither radical nor photochemical mediated. An experiment using a 3'-^{32}P end-labelled DNA restriction fragment indicated that base-specific

DNA cleavage occurred at the adenine residues. Alkylation of the N-3 of the adenine residue caused DNA cleavage while alkylation at N-7 of the guanine residue did not result in DNA strand cleavage. Further experiments (Kushida, et al., 1994) showed that, under physiological conditions, spontaneous cleavage of the N-glycosidic linkage occurred primarily at the modified adenines to produce abasic sites. The abasic sites were unstable and breakage subsequently occurred in the phosphodiesterpentose backbone of the DNA molecule via a β-elimination reaction. The rates of strand cleavage at adenosine residues were affected by both the 5'- and 3'- flanking nucleotides, with the most favourable sequence being 5'-AA***A***T, where ***A*** is the site of cleavage.

Ptaquiloside-DNA adducts were detected in the ileums of two Friesian male calves that had been fed dried bracken (*Pteridium esculentum*) in their diet (Prakash AS, *et al*., 1996). Mutations to the H-*ras* proto-oncogene were also seen in the ileums of these calves. The mutations corresponded to pyrimidine transversions at codon 61 of H-*ras*. *In vitro* experiments (Prakash AS, *et al*., 1996), showed that the APT-alkyated H-*ras* primarily at the adenines and the rate of rate of depurination was sequence-dependent. Investigation of DNA template activity using a plasmid DNA showed that DNA synthesis by T7-DNA-polymerase was terminated by the presence of all alkyated bases, but certain apurinic sites allowed DNA-synthesis to continue. The authors claimed that these results suggested that initial alkylation by ptaquiloside in codon 61, followed by depurination and error in DNA-synthesis, led to activation of the H-*ras* proto-oncogen.

Shahin, *et al*. (1998a and 1999b) found DNA-adducts in the ileum of rats that been treated with weekly i.v. doses of 3 mg/rat of APT for 10 weeks. Shahin, *et al*. (1998b) found double mutations at codons 58 (G to T or G to A) and 59 (A to C or A to G) of the H-*ras* from the mammary tissue of 6 out of 10 rats that had been given weekly doses of APT at 3 mg/rat by intravenous (i.v.) injection for 10 weeks. (These studies are described in a little more detail in the section on "Carcinogenicity of ptaquiloside".) Note that ileal and bladder tumours from rats fed bracken or water extract of bracken did not have any mutations at exons 5-9 of the p53 gene or exons 1 and 2 of the K-ras and H-ras genes (Freitas, *et al*., 2002) – see Section 6.2.4.2.

6.5.2. **Other illudanes and protoilludanes**

Four unstable illudane-type sesquiterpene glycosides (pteroside A2, iso-ptaquiloside, caudatoside and ptaquiloside Z) and a protoilludane sesquiterpene glycoside (pteridanoside) have been detected in young fronds of the neotropical bracken *Pteridium aquilinum* var. *caudatum* in the Venezuela Andes (Castillo, et al., 1999 & 2003). The young fronds contained 1% ptaquiloside, 0.4% isoptaquiloside, 0.6% caudatoside and 0.2% ptaquiloside Z. Ptaquiloside Z was acutely toxic to the brine shrimp *Artemia salina*, with a LC_{50} of 7.8 µg/ml after 24 h exposure, which was the same LC_{50} as obtained for ptaquiloside itself.

Pteridanoside was found at a concentration of 3.2 mg/kg in fronds. It was moderately toxic to brine shrimp *Artemia salina*, with LC_{50}s of 250 and 62.5 µg/ml after 24 and 48 h exposure (Alonso-Amelot, 2002).

No conventional toxicological data were available for any of these compounds.

6.5.3. **Indanones**

A large number of terpenic indanones have been isolated for bracken (see Table 7). These include sesquiterpenyl indalones called pterosins and their glycosides, pterosides. Pterosins are metabolites and breakdown products of ptaquiloside and other illudanes present in bracken. Pterosins A, A2, B, K and Z have been isolated after extraction from *Pteridium aquilinum* var. *caudatum* followed by pH adjustment (Castillo, et al., 1997 and 2003).

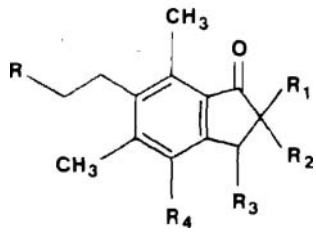
The highest concentrations of pterosins are found in young crosiers and the levels in fronds decrease as they mature. In temperate regions, the highest concentrations of total pterosins occur in May to June, reaching about 24 µg/kg (w/w) in fresh fronds and waning to 4 µg/kg (w/w) by August(Alonso-Amelot, 2002). Dried bracken, collected in July 2003 from the Gantsevich region of Brest district of Belarus, was found to contain concentrations of 0.245 mg/kg of pterosin A and 0.360 mg/kg of pterosin B (Kovganko, *et al.*, 2004).

The breakdown of ptaquiloside can result in the formation of certain pterosins. It was reported by Niwa, *et al*. (1983) and van der Hoeven *et al*. (1983) that, in acid conditions, ptaquiloside breaks down, via its illudane dienone (APT), to form pterosin B. Alonso-Amelot and Avendaño (2002) reported that ptaquiloside forms pterosin F when exposed to chloroform at room temperature.

It has been claimed that pterosin B is much less electrophilic than ptaquiloside (Bonadies*, et al*., 2004) and that indanones do not act as alkylating agents (Alonso-Amelot, 2002). Fukuoka, et al. (1978) tested various pterosin sesquiterpenoids (pterosins A, B, C, D, E, F, G, K, L, N and Z; acetyl pterosin C; benzoylpterosin B; palmitylpterosins A and B) for mutagenicity at pH 7.4 in *Salmonella typhimurium* strains TA98 or TA100 in the presence and absence of S9 from the livers of PCB-treated rats. All of the pterosins gave negative results in this test. Pterosins B and Z did not cause mutations in *Salmonella typhimurium* strains TA98 or TA100 in the absence of metabolic activation (Nagao, *et al.,* 1989; Matoba, *et al*., 1987). Pterosins A, B, C, F and L and pterosides A, B and C were not clastogenic when tested *in vitro* in CHL cells in the absence of metabolic activation (Ishidate, *et al*., 1988).

Pterosin-containing extracts from dried frond or rhizome of bracken (*Pteridium aquilinum* var. *latisculum*) collected from Nayoro, Hokkaido, Japan, did not cause poisoning in 2 male Welsh Black calves when given at a daily dose equivalent to 1 kg/day of fresh bracken for 30 days (Evans, *et al.,* 1983). Leucocyte and thrombocyte counts remained normal The extract from fronds contained pterosins A, B, C and others at concentrations of 0.04, 0.19, 0.04 and 0.02 g/kg; the extract from rhizomes contained pterosins A, B and others at 0.02, 0.02 and 0.02 g/kg and pterosides A, B, C and others at 0.32, 0.57, 0.37 and 0.09 g/kg.

Table 7: Pterosins and pterosides isolated from bracken (Fenwick, 1988)



		R	R_1	R_2	R_3	R_4
Pterosin	A	$-CH_2OH$	CH_3	CH_2OH	H	H
	B	CH_2OH	CH_3	H	H	H
	C	CH_2OH	CH_3	H	OH	H
	D	CH_2OH	CH_3	CH_3	OH	H
	E	CO_2H	CH_3	H	H	H
	F	CH_2Cl	CH_3	H	H	H
	G	OH	CH_2OH	H	H	H
	J	CH_2Cl	CH_3	H	OH	H
	K	CH_2Cl	CH_3	CH_2OH	H	H
	L	CH_2OH	CH_3	CH_2OH	OH	H
	N	CH_2OH	CH_3	OH	H	H
	O	CH_2OCH_3	CH_3	H	H	H
	V	CH_2OCH_3	CH_3	CH_2OH	H	H
	Z	CH_2OH	CH_3	CH_3	H	H
Pteroside	A	$-CH_2O$ glu	CH_2OH	CH_3	H	H
	B	CH_2O glu	CH_3	H	H	H
	C	CH_2O glu	CH_3	H	OH	H
	M	CH_2O glu	CH_3	H	H	OH

6.5.4. *p*-Hydroxystyrene glycosides

Apart from ptaquiloside, the carcinogenic fraction isolated from aqueous extracts of bracken also contained two new *p*-hydrostyrene glycosides, that were named ptelatoside A (ρ-β-primeverosyloxystyrene) and ptelatoside B (ρ-β-neohesperidosyloxystyrene). Ptelatoside A was synthesised and tested in Sprague-Dawley rats. A total of 23 rats of both sexes were fed a diet containing 0.13% ptelatiside A for 109 days (for males) or 125 days (for females). The rats were killed at 520 days after the start of feeding the test material. The animals were examined for tumours and for hyperplastic liver nodules. There was no significant difference between the test group and the control group for tumours or nodules. The authors suggested that the negative result for carcinogenicity in this study might be due to the small dose of ptelatoside A that was administered. (Hirono, 1986)

6.5.5. Quercetin and rutin

Quercetin (CAS No 117-39-5) is a flavinoid that is commonly found in many fruits and vegetables (often as a sugar condensation product, such as rutin, the 3-rhamnoglycoside of quercetin), with particularly high concentrations of 25000 to 65000 mg/kg being found in coloured onions (IARC, 1983). Tea can contain concentrations of more than 10000 mg/kg of quercetin and kaempferol, combined (IARC, 1983). Bracken from Turkey has been found to contain 570 mg/kg (dry weight) of quercetin (Pamukcu, *et al.,* 1980b); and was found to contain 860 mg/kg when examined on a different date (Hatcher, *et al.,* 1981).

The average UK intake of flavinoids in foods has been estimated to be 30 mg/person/day, of which two-thirds is quercetin, ie. 20 mg quercetin/person/day (MAFF/DH, 1995).

Quercetin has a molecular weight of 302.24.

Quercetin is not very well absorbed from the gut lumen. The majority of orally administered quercetin is either metabolised to phenolic acids by the gut flora or voided unchanged in the faeces (Stavric, 1994).

Quercetin was of low cytotoxicity when tested *in vitro* in Chinese hamster ovary (CHO) cells, 3T3 mouse fibroblasts and normal rat kidney cells, with the concentrations inhibiting cell growth by 50% (IC_{50}) after 48 h of incubation being respectively 0.8×10^{-4}, 1.2×10^{-2} and 0.7×10^{-4} M (Ngomuo and Jones, 1996a).

Quercetin is suspected to be carcinogenic (Cooper and Johnson, 1998), but the evidence for this from a series of animal studies is not strong. The International Agency for Research on Cancer (IARC, 1983) concluded in its evaluation of quercetin: “Results from one experiment in rats provide limited evidence for the carcinogenicity of quercetin in experimental animals. In the absence of epidemiological data, no evaluation of the carcinogenicity of quercetin to humans could be made.” A 2-year study in F344 rats given 1000, 10000 or 40000 ppm quercetin in their diets (equivalent to (40, 400 and 1900 mg/kg bw/day) showed increased incidences of hyperplasia and adenomas in the renal tubules of male rats at all doses and increased incidences of renal tubule adenomas and of adenomas and

adenocarcinomas combined in the top dose males, but no effect on females (NTP, 1992). A dietary concentration of 0.1% quercetin for 58 weeks caused multiple ileal tumours (adenomas, fibroadenomas and adenocarcinomas) and transitional cell carcinomas of the urinary bladder in Norwegian albino rats (Pamukcu, *et al*., 1980b). In another study (Dunnick and Hailey, 1992), 40000 ppm (1900 mg/kg bw/day) of quercetin in the diet of F344/N rats caused benign renal tumours in males but not females, but no other tumours were found at any site at doses of 100 or 10000 ppm. Other long-term studies have not confirmed the observations of a carcinogenic effect of quercetin in rats (Hirono, *et al*., 1981; Takanashi, *et al*., 1983; Ito, *et al.,* 1989), in mice (Saito D, *et al*., 1980) or in hamsters (Morino, et al., 1982). In groups of 8 to 20 ACI rats of each sex that were given 1 or 5% of quercetin or rutin in the diet for 540 days or 10% quercetin or rutin for 850 days, there was no significant increase in the incidence of any type of tumour (Hirono, *et al*., 1981). In Fischer 344 rats given diet containing 1% quercetin for 540 days, there was no significant effect on tumours incidence at any site in a treated group of 15 males and 15 females as compared with 16 male and 16 female controls, although one jejunal adenocarcoma was seen in the treated group (Takanashi, et al., 1983). Ito, *et al*. (1989) found no carcinogenic effect in groups of 50 F344/DuCrj rats of each sex that were fed quercetin at 1.25 or 5% in their diet for 104 weeks. There was no evidence of carcinogenicity in ddY mice upon lifetime feeding of diet containing 2% quercetin (Saito D, *et al*., 1980) or in golden hamsters fed 10% quercetin or 10% rutin for 735 days, or 4% quercetin for 351 days followed by 1% croton oil for 350 days (Morino, et al., 1982).

In cattle, oral doses as high as 20 g/calf/day for several months had no effect on incidences of BEH or papilloma-induced lesions of the urinary bladder (Campo, *et al*., 1992), but quercetin has been linked to other bovine cancers of the upper alimentary tract (Alonso-Amelot, 2002).

Quercetin acted as an initiator in an *in vitro* two-stage transformation assay (Campo, 1997). It also can activate or inhibit various protein kinases and phosphatises, thus potentially interfering with critical steps in signal transduction (Campo, 1997). Quercetin arrested primary bovine fetal palate cells (PalF) in cell cycle stages G1 and G2/M, in correlation with activation of p53 and elevation of the cycin-dependent kinase inhibitor $p27^{Kip1}$, but the expression of bovine papillomavirus type 4 (BPV-4) oncogene E7 overcame this arrest and led to the development of tumourigenic cell lines (Bensiston, *et al*., 1999 and 2001). In human cells, it arrested the cell cycle at G1 in both primary cultures and in human papilloma virus HPV-16 transformed (expressing E6 and E7 oncogenes) human keratocytes, with concomitant elevation of $p27^{Kip1}$ (Beniston and Campo, 2003), but HPV-18 transformed (E6/E7) cells did not arrest after quercetin treatment (Beniston and Campo, 2005). The transcriptional up-regulation of the long control region (LCR) of HPV-18 was mediated by a "quercetin responsive element" homologous to the one identified in BPV-4.

There is some evidence that quercetin might have effects that could be anticarcinogenic: inhibition and induction of different phase I and phase II metabolism enzymes, antioxidant effects, induction of apoptosis and down regulation of oncogenes (Musonda and Chipman, 1999). Orally administered quercetin in the rat reduced the ability of bezo(a)pyrene metabolites to bind to DNA, and *in vitro* experiments inhibited the growth of cells from various human cancers (Stavric, 1994).

Quercetin can bind to DNA and can cause single-strand breaks (Fazal, *et al*., 1990). Complexes are formed between quercetin, DNA and metal ions (eg. Cu(II)). The quercetin reduces oxygen to superoxide and, in the presence of metal ions, the hydroxyl radical is formed. The hydroxyl radicals cause the formation of deoxyribose radicals within the DNA and strand breakage subsequently occurs. The mutagenicity of quercetin was tested in bacteriophage λ*vir* using indication bacterial strains of *Escherichia coli* that had genetic defects in their corrigendase complex (*uvrA*), SOS repair system (*recA*) or their repair polymerase (*polA*). The genotoxic potencies of quercetin in the *uvrA* and *recA* indicator strains were similar to that in the wild type *E. coli*, but the genotoxicity was greatly enhanced in the *recA* indicator strain. This is the result that would be expected for a substance that causes mutagenicity as a result of single-strand breakage of DNA.

Quercetin has given positive results in several mutagenicity tests. In the *Salmonella*/microsome assay it gave positive results in strains TA1537, TA1538, TA98 and TA100, but not in TA1535, in the absence of S9 and with increased mutagenic activity in the presence of S9 (Sugimura, *et al*., 1977; MacGregor and Jurd, 1978; Brown and Dietrich, 1979; NTP, 1992). It gave positive results in a forward mutation assay, the multi-gene sporulation test, in *Bacillus subtilis* strain 168, with and without S9, and strain hrc-9, with S9 (MacGregor, 1979). It was positive in the *Escherichia coli* pol A^-/A^+ assay in the presence of S9, but was negative in the *Bacillus subtilis rec* assay when tested in the absence of S9. In mammalian cell systems, quercetin caused gene mutations to 8-azoguanine resistance in V79 Chinese hamster cells in the presence and absence of S9 from livers of Sprague-Dawley rats that had been pretreated with PCBs (Kanechlor 400) (Maruta, et al., 1979), at the tk-locus in Chinese hamster ovary CHO-AT3-2 cells in the absence of S9 (Carver, *et al*., 1983) and in several studies caused mutation at the TK locus of L5178Y mouse lymphoma cells (IARC, 1983). It had no mutagenic effect however at the HGPRT locus of L5178Y mouse lymphoma cells (IARC, 1983) or at the HGPRT, APRT or Na^+/K^+-ATPase loci of Chinese hamster ovary CHO-AT3-2 cells (Carver, *et al*., 1983). It was clastogenic in several cytogenetics assays in various cell systems, including Chinese hamster CHL fibroblasts (Ishidate, *et al*., 1988), Chinese hamster ovary cells (Carver, *et al*., 1983; NTP, 1992), PaIF bovine fetal cells that had been transformed by E7 protein of bovine papillomavirus type 4 (Leal, *et al*., 2003), human fibroblasts and human lymphocytes (IARC, 1983). It caused sister chromatid exchanges (SCE) in Chinese hamster fibroblasts, human fibroblasts and human lymphocytes. One group of experimenters (NTP, 1992) found that it induced sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells with and without S9, but other workers found that it did not cause SCE in CHO cells (Carver, *et al*., 1983) or V79 cells (IARC, 1983). Morphological cell transformation was caused in SHE cells and in Balb/c 3T3 cells. Quercetin caused sex-linked recessive lethal mutations in male *Drosophila melanogaster* (Watson, 1982).

Quercetin was tested by several different workers for *in vivo* mutagenicity in the mouse bone marrow micronucleus assay. Sahu, *et al*. (1981) gave quercetin or rutin at intraperitoneal doses of 200 and 400 mg/kg bw to Swiss mice and found significant dose-related increases in the numbers of micronucleated bone marrow erythrocytes for quercetin, whereas rutin was negative in this test. MacGregor (1979 & 1980) and Aeschbacher (1982) reported that administration of up to 1000 mg/kg bw of quercetin by the oral or intraperitoneal (ip) route gave negative results in the mouse bone

marrow micronucleus test. Urine from rats treated with oral or ip doses of 1000 mg/kg bw of quercetin was mutagenic to strains TA98 and TA100 of *Salmonella typhimurium* (MacGregor, 1979).

Quercetin gave negative results for *in vivo* mutagenicity in a mouse bone marrow micronucleus test in which mice were given 3 daily intraperitoneal (i.p.) injections of 200 mg/kg bw (Ngomuo and Jones, 1996b). Shikimate also did not cause unscheduled DNA synthesis in the gastric mucosal cells of Wistar rats that had been given oral doses of 50-800 mg/kg bw (Ngomuo and Jones, 1996b). Plasma concentrations of quercetin after oral or i.p. doses of 200 mg/kg bw were less than 0.1 μg/ml and the authors suggested that the negative results in these tests might have been due to limited bioavailability of quercetin.

Urine from rats treated with quercetin or rutin (and various fractions derived by silica gel column chromatography from solvent extracts from the urine) was not mutagenic when tested in *Salmonella typhimurium* strain TA98 and TA100 in the absence of metabolic activation (Hatcher, *et al.,* 1981).

In addition to having mutagenic characteristics itself, quercetin can enhance the mutagenic potency of other substances (Musonda and Chipman, 1999). It caused a 7.87-fold enhancement of the mutagenic potency of 2-acetylaminofluorene in *Salmonella typhimurium* strain TA98 in the presence of S9 from the livers of PCB-treated Sprague-Dawley rats (Ogawa, *et al*., 1987). In a host-mediated mutagenicity assay, the feeding of dietary concentrations of 14 g/kg quercetin or 25 g/kg rutin to female BALB/c mice increased the mutagenic potency of the heterocyclic amines MeIQ and Trp-P-2 in *Salmonella typhimurium* strain TA98 (Alldrick, et al., 1989), possibly as a result of its inhibiting or stimulating effects on various cytochrome P450 enzymes in the liver (Musonda and Chipman, 1999).

Quercetin acted synergistically with bovine papilloma virus type 4 (BPV-4) in the *in vitro* transformation of PalFs cells, primary bovine cells from a fetal palate (Campo, *et al*., 1999). Whereas quercetin caused normal cells to arrest in G1, cells that had been pre-treated with BFP-4 were not arrested in G1 and continued to proliferate. On its own, BFP-4 was not capable of fully transforming PalFs cells, but caused morphological transformation of the cells whilst they remained anchorage dependent and did not induce tumours in nude mice. In the presence of 20mM quercetin, BFP-4 pre-treated PalFs cells were transformed so that they were anchorage independent, immortal and oncogenic in nude mice. The transcriptional activity of the long control region (LCR) of BPV-4 was increased four-fold in the presence of quercetin. A similar effect on transcription of the LCR (a 3- to 4-fold increase in transcriptional activity) was seen when human papilloma virus type 16 (HPV-16) was treated with 50mM quercetin. The authors suggested that the presence of quercetin in bracken could cause cancers in cattle and in humans as a result of synergism with papillomaviruses.

6.5.6. **Kaempferol**

Kaempferol (CAS No 520-18-3) is a flavinoid that is chemically similar to quercetin. It has a molecular weight of 286.24. It is commonly found in many plant species. Tea can contain concentrations of more than 10000 mg/kg of quercetin and kaempferol, combined (IARC, 1983). Bracken from Turkey has been found to contain 1100 mg/kg (dry weight) of kaempferol (Pamukcu, *et al.,* 1980b); and was found to contain 2550 mg/kg when examined on a different date (Hatcher, *et al*., 1981).

Kaempferol occurs in bracken in the glycoside complexes astragalin and tylerside. These substances have been reported to cause bladder cancer in rats (Duffus and Duffus, 1991). 78.8 g of dried bracken, collected in July 2003 from the Gantsevich region of Brest district of Belarus, was found to contain 16.7 mg of astragalin – ie. 0.21 mg/kg (Kovganko, *et al.*, 2004).

Kaempferol can be metabolised into quercetin by microsomal enzymes (Brown and Dietrich, 1979).

Takanashi *et al*. (1983) tested kaempferol for carcinogenicity in ACI rats. Groups of 6 males and 6 females were fed a diet containing a concentration of 400 mg/kg of kaempferol (purity 99%) for 540 days. A control group consisted of 30 males and 22 females. There was no statistically significant ($p>0.05$) effect on the incidence of tumours.

Kaempferol was negative for DNA damage in the *rec* assay in *Bacillus subtilis* in the presence and absence of S9 (Brown, 1980). However, it was mutagenic to *Salmonella typhimurium* strains TA98 and TA100 at pH 7.4 in the presence and absence of S9 from the livers of PCB-treated rats (Fukuoka, *et al*., 1978). Further tests showed mutagenicity in *Salmonella typhimurium* strains TA1537, TA98 and TA100, but not to TA1535 or TA1538 (Sugimura, *et al*., 1977, Hardigree and Epler, 1978; MacGregor and Jurd, 1978, Brown and Dietrich, 1979). Kaempferol induced mutations to 8-azoguanine resistance in V79 Chinese hamster cells, with increased mutagenic activity in the presence of S9 from livers of Sprague-Dawley rats that had been pretreated with PCBs (Kanechlor 400) (Maruta, *et al.,* 1979) and caused gene mutations at the tk-locus in Chinese hamster ovary CHO-AT3-2 cells in the presence and absence of S9 (Carver, *et al*., 1983). It had no mutagenic effect however at the HGPRT, APRT or Na^+/K^+-ATPase loci of CHO-AT3-2 cells (Carver, *et al*., 1983). It caused chromosome breaks in CHO-AT3-2 cells in the presence and absence of S9, however it did not cause sister chromatid exchanges in these cells (Carver, *et al*., 1983). It did not cause any morphological transformation of Syrian hamster embryo (SHE) cells *in vitro* (Umezawa, *et al*., 1977). It caused sex-linked recessive lethal mutations in male *Drosophila melanogaster* (Watson, 1982). *In vivo*, there was an increase in the number of micronuclei in polychromatic erythrocytes in the bone marrow of male mice that had been given intraperitoneal injections of 200 or 400 mg/kg bw of kaempferol (Sahu, *et al*., 1981).

Kaempferol caused a 5.1-fold enhancement of the mutagenic potency of 2-acetylaminofluorene in *Salmonella typhimurium* strain TA98 in the presence of S9 from the livers of PCB-treated Sprague-Dawley rats (Ogawa, *et al*., 1987).

The International Agency for Research on Cancer (IARC, 1983) concluded in its evaluation of kaempferol: "The available data were inadequate to evaluate the carcinogenicity of kaempferol to experimental animals. In the absence of epidemiological data, no evaluation of the carcinogenicity of kaempferol to humans could be made."

6.5.7. **Shikimic acid**

Shikimic acid (CAS No 138-59-0) has a molecular weight of 174.15. It is very water-soluble. It is found in a variety of different plant materials, including star anise, green tea and soybeans
. Bracken collected in North Wales has been found to contain 1440 mg/kg (dry weight) of shikimic acid (Hirono, *et al.,* 1977).

Shikamate was of low cytotoxicity when tested *in vitro* in Chinese hamster ovary (CHO) cells, 3T3 mouse fibroblasts and normal rat kidney cells, with the concentrations inhibiting cell growth by 50% (IC_{50}) after 48 h of incubation being respectively 0.8×10^{-3}, 0.7×10^{-3} and 1.0×10^{-3} M (Ngomuo and Jones, 1996a).

Intraperitoneal injections of 10 mg on shikimic acid caused death in mice within a few hours of dosing, with haemorrhaging and "denudation" of the intestinal mucosa (Leach, *et al*., 1971).

Shikimic acid and 9 of its bacterial or mammalian metabolites were tested for mutagenicity in a plate test using *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 in the presence and absence of S9; for mutagenicity in a fluctuation test using *Salmonella typhimurium* strains TA98 and TA100; and for cell transformation in BHK-21/C1-13 cells (fibroblast-like cells derived from Syrian hamster kidney) (Jones, *et al*., 1983). All 10 of the substances tested gave negative results for mutagenicity in both *Salmonella* tests and all 9 metabolites were negative in the transformation test, but shikimic acid itself gave a positive result for cell transformation.

Shikimate gave negative results for *in vivo* mutagenicity in a mouse bone marrow micronucleus test in which mice were given 3 daily intraperitoneal (i.p.) injections of 100-1000 mg/kg bw (Ngomuo and Jones, 1996b). Shikimate also did not cause unscheduled DNA synthesis in the gastric mucosal cells of Wistar rats that had been given oral doses of 250-1000 mg/kg bw (Ngomuo and Jones, 1996b). A dominant lethal assay was conducted using groups of 5 male TF1 mice given either 25 mg of shikimic acid by intraperitoneal injection or 80 mg by stomach tube (Evans IA and Osman, 1974). Increased numbers of dominant lethal mutations were caused by both treatments with shikimic acid.

Shikimic acid was carcinogenic in mice (Evans IA and Osman, 1974). A group of 10 male and 3 female TF1 mice received a single intraperitoneal (ip) injection of 1-30 mg shikimic acid and was observed for up to 70 weeks. One male was given 100 mg of shikimic acid by stomach tube. A control group of 57 mice was used. Six of the treated mice developed tumours in the glandular stomach, 3 developed leukaemias: a reticulum cell A leukaemia, a reticulum cell B leukaemia, and a lymphocytic leukaemia. Five of the ip-treated mice (given 10-30 mg) developed no tumours. The

orally-treated mouse developed a tumour of the glandular stomach and a reticulum cell B leukaemia. No neoplasms were seen in the controls.

Hirono, *et al.*, (1977) fed shikimic acid to a group of 6 male and 6 female ACI rats at a dietary concentration of 0.1% (1000 mg/kg) for 142 days, followed by an observation period of 70 days. This dietary level of shikimic acid was equivalent to the amount of shikimic acid that would be provided by a diet containing about 70% (dry weight) of bracken containing 1440 mg/kg of shikimic acid. A control group of 9 males and 10 females was given normal diet containing no shikimic acid. One male and 2 females from the treated group died of pneumonia before the termination of the experiment. No tumours were found in any of the treated or control animals. The authors commented that, by comparison with the results of feeding studies with bracken, they would have expected this dietary dose of shikimic acid to have produced tumours in the treated animals if shikimic acid were the sole carcinogenic agent in bracken.

Leach, *et al*. (1971) reported that shikimic acid was destroyed by alkaline treatment. As bracken had increased mutagenicity and carcinogenicity in alkaline conditions, it is unlikely that shikimic acid was the main substance responsible for the carcinogenicity of bracken.

The developmental toxicity of shikimic acid was tested in groups of 7-14 pregnant CF-1 mice that were given 0, 0.25 or 1.0 g shikimic acid/kg bw by daily gavage doses from day 1 to day 17 of gestation (O'Donovan, et al., 1977). Fetuses were examined for any possible external, visceral or skeletal abnormalities. The numbers of implants in treated groups were slightly but significantly less than in controls (92, 77 and 69 implantations in groups given 0, 0.25 and 1 g/kg bw/day per day, respectively), and the number of live fetuses per litter was slightly but not significantly reduced as compared with controls (mean number of fetuses per dam = 12, 10 and 9 at 0, 0.25 and 1 g/kg bw/day per day, respectively). None of the control dams showed less than 80% implantation but 50% of those given 0.25 g/kg bw/day and 56% of those given 1 g/kg bw/day had less than 80% implantation. No significant increases in the numbers of any fetal abnormalities were seen in the groups treated with shikimic acid.

6.5.8. **Tannins**

The tannins in bracken are mainly condensed tannins derived from procyanidin and prodelphinidin. Condensed tannins are stored chiefly in the vacuoles of the parenchyma and cuticle of the bracken pinnulettes. Here they accumulate, giving higher concentrations in the older parts of the plant. Concentrations of up to 120 µg/kg have been found in the fronds of tropical bracken (Alonso-Amelot, 2002).

Tannic acid and tannins were evaluated by IARC (1976). It reported that tannic acid is carcinogenic in rats following subcutaneous (sc) injection, producing liver tumours. In mice, sc injection of hydrolysable tannins produced liver tumours and sc injection of condensed tannins produced local sarcomas and liver tumours. No oral studies were available. No human epidemiology studies were available. IARC (1987) classified tannic acid and tannins in Group 3: *The agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans.*

Repeated sub-cutaneous injections of 0.1 mg/kg bw/day of tannins extracted from bracken for 38 days caused fibrous histiocytomas with some malignant features in F344 rats that were killed at 47 weeks after the start of treatment (Pamukcu, *et al*., 1980a). However, bracken tannins added to the diet at concentrations of up to 4000 mg/kg for up to 72 weeks did not induce cancer in F344 or Sprague-Dawley rats (Pamukcu, *et al*., 1980a). The urine from tannin-fed rats was mutagenic to strain TA100 but not TA98 of *Salmonella typhimurium*, whereas the tannins themselves were not mutagenic in either strain when tested in the absence of metabolic activation (Pamukcu, *et al*., 1980a).

6.5.9. **Thiaminases**

Thiaminases are anti-thiamine enzymes that have yet to be chemically characterised. It is fairly heat-stable but is destroyed by autoclaving. As a component of bracken, it has caused thiamine deficiency in non-ruminant animals, including horses, pigs and humans. Thiaminase activity is highest in the rhizomes and very young fronds of the bracken plant, with the concentration in the rhizome being 10-30 times greater than in mature fronds (Cooper and Johnson, 1998; Alonso-Amelot, 2002; Cooper, *et al*, 2003).

Bracken contains a least two thiaminases. Bracken collected in November in Pretoria district, South Africa had mean activities of thiaminase types 1 and 2 of 3.1 and 3.5 µg thiamine destroyed/g plant material/hour, respectively, and a thiamine content of 0.04 µg thiamine/g plant material (Meyer, 1989). An additional heat-stable anti-thiamine factor, possibly caffeic acid, has been shown to be involved in bracken-induced thiamine deficiency in horses (Cooper and Johnson, 1998). [Caffeic acid is a phenolic substance with antioxidant properties that is found in many plants. It has caused stomach papillomas in rats and has decreased the growth of colon tumours in the same rats (Hirose, *et al*., 1998).]

When bracken containing active thiaminase was fed to rats, they developed a disease that simulated the typical nervous lesions of antivitaminosis B_1 and could be cured by vitamin B_1 (thiamine) therapy (Hirono, 1986). Similar effects have been seen in other monogastric animals, including horses and pigs, fed on bracken.

In order to test if the anti-thiamine activity of bracken contributed to the carcinogenicity of bracken, Pamukcu, *et al*. (1970) performed a 52 week feeding study in groups of rats fed either control diet (10 males 7 12 females), a diet containing bracken (12 males and 14 females) or a diet containing bracken supplemented with a sc injection of 2 mg/rat/week of thiamine (38 males and 52 females). No tumours were found in controls. All survivors in the other two groups developed multiple intestinal tumours. Bladder tumours were found in 1/9 (11%) males and 1/13 (7%) females in the group given bracken alone and in 19/36 (53%) males and 35/51 (67%) females in the group given bracken plus thiamine. It was noted that thiamine supplements did not reduce the incidence of tumours, so it seemed unlikely that thiaminase contributed to the carcinogenicity of bracken.

6.5.10. **Prunasin**

Prunasin (molecular weight = 280) is a cyanogenic glycoside that is present in some varieties of bracken. It is usually present in bracken in quantities that do not harm animals that eat the fronds. Nevertheless, sudden death, thought to be due to hydrocyanic acid has been recorded in animals fed on young fronds of bracken. Cyanogenic glycosides are toxic because they form hydrocyanic acid (HCN) when hydrolysed by β-glycosidase enzymes that are released when tissues are damaged. β-Glycosidase excises a glucose moiety from the bracken glycoside, producing the unstable α-cyanhydrin mandelonitrile. This is split by mandelonitrile β-elimination catalysed by oxynitrilase to form HCN and benzaldehyde. Crushing or chewing the plant is sufficient to cause the release of HCN. A polymorphism occurs in bracken: some plants are not cyanogenic because they lack either the enzyme or prunacin. When non-cyanogenic plants dominate, bracken is heavily grazed by farm animals. The cyanogenic form of bracken is not normally eaten by animals when an alternative forage is available, but they will readily graze it under some circumstances (Cooper and Johnson, 1998; Alonso-Amelot, 2002).

Croziers of *Pteridium aquilinum* var. *arachnoideum* from Venezuela were found to contain 10 to 61 mg prunasin/g fresh plant tissue (Alonso-Amelot and Oliveros, 2000). HCN was produced from crushed crosiers at a rate of approximately 0.005 μmol/g min. One gram of prunacin has the potential to release up to 96 μg of HCN. Therefore, 1 gram of Venezuelan bracken crozier has the potential to release about 1 to 6 μg of hydrogen cyanide. Further work on the neotropical bracken species *Pteridium arachnoideum* (Alonso-Amelot and Oliveros-Bastidas, 2005) showed that crosiers contained 1.84 to 107.7 mg/kg of prunacin, which in crushed crosiers decomposed into HCN in less than 1200 mins yielding 0.51 to 47.86 μg HCN/min/g dry weight. There was a pulse of HCN production soon after injury of the plant tissue, followed by a slower rate of formation of HCN. The β-glucosidase enzyme was not rate limiting, occurring in excess in the natural system.

The toxicity of hydrogen cyanide has been reviewed (IPCS, 2004). For HCN, the minimum lethal oral dose in humans is widely quoted to be in the region of 0.5 to 3.5 mg/kg bw. The Council of Europe (2000) identified a TDI of 0.02 mg/kg bw, expressed as cyanide. JMPR (1965) identified an ADI of 0.05 mg/kg bw. More recently, the WHO Guidelines for Drinking-water Quality (WHO, 2003) derived a TDI of 0.012 mg/kg bw.

JECFA (1993) concluded that because of lack of quantitative toxicological and epidemiological information, a safe level of intake of cyanogenic glycosides could not be estimated.

No quantitative information was found on the prunasin content of UK varieties of bracken, but the highest concentrations of prunacin are reported to be found in early to mid spring and the lowest in early autumn. In tropical varieties there is no season variation, but the highest levels are found in young crosiers. (Alonso-Amelot, 2002)

6.5.11. **Braxins**

Saito T, *et al.* (1989) isolated four toxic glycosides from rhizomes of bracken (*Pteridium aquilinum* (L.) Kuhn) and named them braxin A1, braxin A2, braxin B and braxin C. Braxins A1 and A2 were not detected in the fronds of bracken (Saito T and Mochizuki, 1986), but up to 600 mg/kg was detected in rhizomes (Saito T and Mochizuki, 1986). Chemical analysis showed braxin C to be chemically identical to ptaquilaside (Yoshida and Saito, 1994a), so studies on this substance have been reported in this document under the heading "Ptaquiloside and related substances". Braxins A1 and A2 are β-glucopyranosides with an aromatic structure (Saito T and Mochizuki, 1986), but their precise chemical structure has not been reported. They are chemically unstable and braxin A2 appears to be a breakdown product of braxin A1. No information was found on the chemical identity of braxin B.

It was demonstrated that braxins A1, A2, B and C (ptaquiloside) could each induce haemorrhagic cystitis in guinea-pigs within 24 to 48 h of the start of dosing (Saito T, *et al*., 1990; Tjatur Rasa, *et al*., 1998). Braxins A1 and A2 caused a dose-related release of histamine from rat peritoneal mast cells *in vitro*, and caused the cells to swell greatly (Saito T and Mochizuki, 1986; Saito T, *et al*., 1990). The histamine-releasing activities of the glycosides in bracken rhizomes (including braxins A1 and A2) are higher than those of the glycosides that occur in bracken fronds (braxins A1 and A2 are absent), with respective ED_{50} values of approximately 10 mg/ml and 100 mg/ml (Saito T and Mochizuki, 1986). It was suggested that the rashes that bracken causes in some people might in part be due to the effects of braxins on mast cells.

6.5.12. **Ecdysteroids**

There are several ecdysteroids present in bracken, including α-ecdysone, ecdysterone, pterosterone, ponasterone A and ponasteride. They act on insects, causing premature moulting and preventing them developing into adults. (Alonso-Amelot, 2002)

Although, most of the ecdysteroids are regarded as non-toxic to vertebrates, α-ecdysone induced neoplastic lesions in Egyptian toads (Campo, 1997).

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Secretariat
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TOX/2008/12 – Annex B

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

BRACKEN: LITERATURE SEARCH

Articles were identified and selected for review as follows.

Computerised literature search

Several computerised searches of the scientific literature published over the last 10-38 years were conducted by the Information Centre of the Food Standard Agency. Databases searched were Toxicology Bibliographic Information (TOXLINE), MEDLINE, FoodlineWeb, Ingenta.com, Food Science and Technology Abstracts (FSTA), British Library Inside, Current Contents, Campden and Chorleywood Food Research Association (CCFRA) site, Barbour Index, DART Developmental Toxicology Literature, UK Publications.

Keywords searched individually and in various combinations included bracken, bracken fern, Pteridium aquilinum, Pteridium. ptaquiloside, bracken toxin, sesquiterpene glycoside, pterosin, pterosin B, isoptaquiloside, caudatoside thiaminase, quercetin, prunacin, toxicology, toxicological, cancer, carcinogenic, mutagenic, genotoxic, epidemiology.

Printouts of details of articles including abstracts were read and articles that looked like they would be relevant to the consumer safety of bracken were ordered.

Secondary search of the literature

Review articles on bracken were read and from these further key articles were identified. This was particularly helpful for finding older articles.

The relevant articles were read and summarised for this COT paper. During this process, several further important articles that had been missed earlier were identified and ordered.

Selection of articles for review

A large number of articles on the safety of bracken and on related issues were obtained. As a result of time restraints, not all of the articles were read in detail. Some selection had to be made. It was quickly apparent that certain articles were too general or dealt with the issues only peripherally. Such articles were left aside.

Sometimes several articles referred to the same piece of work. Wherever possible the primary source of information was the source that was cited. In some instances,

information was pieced together from several sources and it was necessary to cite several articles for a single piece of information.

Criteria for selection of articles

Articles were selected for inclusion in the review on the basis that they dealt with some aspect of the safety of bracken and its constituent chemicals to human consumers. This could be a direct reference to human safety by dealing with exposure, toxicological or epidemiological aspects or less direct by looking at effects in exposed animals or of exposure by routes other than the diet. Related issues of interest included the taxonomy and worldwide distribution of different varieties of bracken ferns.

Some articles were excluded as they covered areas of work already dealt with in other articles. This was particularly the case concerning the large number of studies from the 1980s that reported the testing various fractions of bracken in standard short-term assays for mutagenicity or carcinogenicity in order to isolate the carcinogenic component of bracken.

There were numerous review articles available that added no new information. Most of these have not been cited.

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