Ptaquiloside

an environmental hazard?

Rasmussen, L. H.

Publication date:
2003

Document version
Publisher's PDF, also known as Version of record

PTAQUILOSIDE – AN ENVIRONMENTAL HAZARD?

Occurrence and fate of a Bracken (*Pteridium* sp.) toxin in terrestrial environments

Ph.D.-thesis by Lars Holm Rasmussen

Chemistry Department, The Royal Veterinary and Agricultural University
DK-1871 Frederiksberg C, Denmark

July 2003

PTAQUILOSIDE – AN ENVIRONMENTAL HAZARD?

Occurrence and fate of a Bracken (*Pteridium* sp.) toxin
in terrestrial environments

PTAQUILOSIDE – AN ENVIRONMENTAL HAZARD?

Occurrence and fate of a Bracken (*Pteridium* sp.) toxin
in terrestrial environments

Ph.D.-thesis by
Lars Holm Rasmussen
Chemistry Department
The Royal Veterinary and Agricultural University
Thorvaldsensvej 40
DK-1871 Frederiksberg C
Denmark
Email: LHR@KVL.DK (work) or PTERIDIUM@OFIR.DK (private)

1st edition.

Chemistry Department, KVL
Thorvaldsensvej 40
DK-1871 Frederiksberg C
Denmark

Thesis conducted under the Ph.D.-programme for
Natural Sciences at The Royal Veterinary and Agricultural University.

Supervisor:

Professor Hans Christian B. Hansen
Chemistry Department
The Royal Veterinary and Agricultural University
Thorvaldsensvej 40
DK-1871 Frederiksberg C
Denmark

Examination committee:

Professor Ole K. Borggaard (Chairman)
Chemistry Department
The Royal Veterinary and Agricultural University
Thorvaldsensvej 40
DK-1871 Frederiksberg C
Denmark

Senior scientist Inge Fomsgaard
Danish Institute of Agricultural Sciences, Department of Crop Protection
Research Centre Flakkebjerg
DK-4200 Slagelse
Denmark

Professor Miguel Enrique Alonso-Amelot
Grupo de Química Ecológica
Departamento de Química
Fracultad de Ciencias
Universidad de Los Andes
Mérida
Venezuela

Examination date:

October 3, 2003

CONTENTS

RESUMÉ

Ørnebregnen (*Pteridium aquilinum* (L.) Kuhn) er en af de hyppigst forekommende planter i Verden. Bregnen findes på alle kontinenter undtagen Antarktis. Ørnebregnen er et almindeligt ukrudt på landbrugsjord, i den primære bush eller i lysbrønde inde i skove. Ørnebregnen indeholder en lang række sekundære planteindholdsstoffer, hvoraf nogle er giftige overfor mennesker og andre levende organismer. Det mest fremtrædende af disse stoffer er det norsesquiterpene glykosid ptaquilosid.

Ptaquilosid forårsager en lang række sygdomme hos dyr strækkende sig fra induceret thiamin-mangel til kræft. Specielt kræft i urinblæren hos køer, kendt som *Bovin Enzootisk Hæmaturi*, forekommer hyppigt i nogle områder af Verden. Ptaquilosid er også mistænkt for at forårsage mave- og spiserørskræft hos mennesker, idet stoffet nemt overføres til mælk hos bregnespisende køer, og idet ptaquilosid muligvis kan udvaskes fra Ørnebregnebevoksninger til grund- og drikkevand. Formålet med denne afhandling har været at undersøge forekomsten af ptaquilosid i terrestriske økosystemer, samt at undersøge ptaquilosids stabilitet og mobilitet i jord for at svare på spørgsmålet: *Udgør ptaquilosid en miljømæssig fare?*

Ptaquilosid findes i koncentrationer op til 15,100 μg g^{-1} i alle bregnedele af alle Ørnebregne-varieteter. Indholdet er normalt størst i de unge bregneblade. Indholdet falder som væksten skrider frem. Indholdet i rhizomer er nogenlunde inverst relateret til indholdet i bladene. Det maksimale indhold på op til 7,050 μg g^{-1} forekommer i rhizomerne når væksten af bladene stopper. Indholdet af ptaquilosid bregne-bevoksninger imellem er meget variabelt. Overordnet set skyldes ptaquilosid-variationen genetiske forhold, mens indvirkning af vækst-forhold kan ses på lokalt niveau (eksempelvist topografi, næringsstofforsyning, lysintensitet o. lign.). Økologisk stress, såsom fysisk påvirkning og skygge, kan forårsage øget indhold af ptaquilosid i blade, mens kvælstof-

mangel kan øge indholdet i rhizomerne. Forskellige studier af variationen i ptaquilosid-indhold har dog leveret modstridende resultater, og yderligere undersøgelser er nødvendige før der kan opstilles en generel model for forekomsten af ptaquilosid i Ørnebregne-bevoksninger.

Nedbør kan udvaske ptaquilosid fra Ørnebregne-bevoksninger til jordbunden. Ptaquilosid er fundet i litter og overjordshorisonter i mængder op til 8.5 μg g^{-1}. Stabiliteten og mobiliteten af ptaquilosid i jord afhænger af jordbundens egenskaber og de klimatiske forhold, idet at: 1) Ptaquilosid er relativt stabilt ved pH 4 til 8; 2) Ptaquilosid kan være beskyttet mod nedbrydning ved svag sorption til uorganiske partikler samt organisk materiale i jorden; og 3) Ptaquilosid nedbrydes langsommere ved lav temperatur i jorden. Under nogle forhold kan ptaquilosid derfor være meget mobilt (som nitrat) og stabilt, hvorved ptaquilosid kan nå grund- og drikkevand. Koncentrationen af ptaquilosid i jordvand blev ikke bestemt direkte i overjordshorisonter, men ligevægtskoncentrationer på op til 400 μg L^{-1} blev estimeret udfra ptaquilosids sorptionsegenskaber. Disse koncentrationer er i overensstemmelse med koncentrationer på op til 7 μg L^{-1} målt i jordvæske ekstraheret fra 90 cm dybde. Ptaquilosid i koncentrationer op til 45 μg L^{-1} er fundet i drikkevand ved to lejligheder! Sammenlignet med den *tolerable koncentration* af ptaquilosid i drikkevand på 0.002 μg L^{-1}, er der tale om meget høje koncentrationer.

Selvom ptaquilosid ser ud til at udgøre en miljømæssig fare, så er den overordnede risiko for mennesker formentlig relativt lille, da ptaquilosidforurening kun vil berøre mennesker der lever og arbejder i områder med mange Ørnebregner. Hvor Ørnebregnen forekommer udbredt, er det dog vigtigt at udvise agtpågivenhed for at minimere risikoen for ptaquilosidforurening af vandressourcer såvel som af mælk.

ABSTRACT

Bracken fern (*Pteridium aquilinum* (L.) Kuhn) is one of the most common plant species on Earth. The fern is found on all continents except from Antarctica where it occurs as a common weed on agricultural lands, as part of the primary bush or below canopy openings inside forests. Bracken contains a wide range of secondary metabolites of which some are toxic towards humans and other living organisms. The most prominent of these compounds is the norsesquiterpene glucoside ptaquiloside.

Ptaquiloside causes a wide range of diseases in animals ranging from thiamine deficiency to cancer. Especially bovine urinary bladder cancer known as *Bovine Enzootic Haematuria* occurs widespread in some parts of the world. Ptaquiloside is also suspected of causing human gastric and oesophageal cancer as the carcinogen readily passes into bovine milk of cows browsing on Bracken and may leach from Bracken stands into drinking water supplies. The aim of this thesis has been to monitor the occurrence of ptaquiloside in terrestrial ecosystems and to investigate ptaquiloside stability and mobility in soils, hereby seeking answers to the question: *Is ptaquiloside an environmental hazard?*

Ptaquiloside is found in all fern compartments of all varieties of Bracken in amounts up to 15,100 μg g^{-1}. The content is usually highest in the young fronds. The content decreases as growth proceeds. The content in rhizomes is almost inversely correlated with the content in the fronds. Maximum contents of up to 7,050 μg g^{-1} occur in rhizomes when frond growth ceases. The content of ptaquiloside in Bracken stands is quite variable. On a large-scale, the content seems mainly to be caused by genetic variation, while influence of local growth conditions can be seen on a smaller scale (e.g. altitude, nutrient availability, light intensity etc.). Ecological stress, such as physical disturbance and shade may induce increased contents of ptaquiloside in fronds, while nitrogen-depletion of soils may raise the content found in rhizomes. However, many studies on the effect of growth conditions have been

contradictory and further investigations are needed before a general model for the ptaquiloside occurrence in Bracken stands can be established.

Ptaquiloside can be leached by rainwater from Bracken stands to the soil. Ptaquiloside is found in topsoil's and litter in amounts up to 8.5 μg g^{-1}. The stability and mobility of ptaquiloside in soils depends on the soil properties and climatic conditions, as: 1) ptaquiloside is rather stable when pH is in the range 4 to 8; 2) ptaquiloside may be protected from degradation by weak sorption to inorganic soil particles and soil organic matter; and 3) ptaquiloside degrades slower at low soil temperatures. Hence, under certain soil conditions, ptaquiloside can be highly mobile (comparable to nitrate) and stable. Ptaquiloside may therefore reach ground- and drinking water. Soil solution concentrations were not measured directly in topsoil's, but equilibrium concentrations up to 400 μg L^{-1} was estimated from the sorptive properties of ptaquiloside, which is in accordance with measured amounts of up to 7 μg L^{-1} in soil water extracted from 90 cm depth. On two occasions, ptaquiloside has been found in drinking water in concentrations of up to 45 μg L^{-1}! Compared to the *tolerable concentration* of ptaquiloside in drinking water of 0.002 μg L^{-1}, these concentrations are very high.

However, even though Bracken may seem like an environmental hazard, the overall risk to humans is likely rather low, as ptaquiloside will only affect people living or working in areas with many Bracken ferns. Where Bracken does occur, care must be taken to minimize the risk of ptaquiloside contamination of water sources as well as of bovine milk!

PREFACE

The thesis is composed of two parts: a general part thoroughly reviewing existing literature covering Bracken in general and ptaquiloside in particular. The review includes the work carried out by the author. The second part of the thesis comprises the following papers and submitted manuscripts:

- Rasmussen, L.H., Donnelly, E., Strobel, B.W. Holm, P.E. and Hansen, H.C.B. (2003) Ptaquiloside in Bracken (*Pteridium aquilinum* ssp. *aquilinum*) in Scotland. Contents in fronds, rhizomes, and litter. *Submitted to Journal of Chemical Ecology.*

- Rasmussen, L.H. and Hansen, H.C.B. (2003) Growth of Bracken in Denmark and the content of ptaquiloside in fronds, rhizomes and roots. *In T. Acamovic, C.S. Stewart, and T.W. Pennycott (Eds.). Poisonous Plants and Related Toxins. CAB International, Wallingford. In press.*

- Rasmussen, L.H., Jensen, L.S. and Hansen, H.C.B. (2003) Distribution of the carcinogenic terpene ptaquiloside in Bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *Journal of Chemical Ecology* 29:3:771-778.

- Rasmussen, L.H., Kroghsbo, S., Frisvad, J.C. and Hansen, H.C.B. (2003) Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere* 51:117-127.

- Rasmussen, L.H., Lauren, D.R. and Hansen, H.C.B. (2003) Sorption, degradation and mobility of ptaquiloside, a carcinogenic Bracken (*Pteridium* sp.) constituent, in the soil environment. *Submitted to Chemosphere*.

- Rasmussen, L.H., Lauren, D.R., Smith, B.L. and Hansen, H.C.B. (2003) Variation in the ptaquiloside content in Bracken from New Zealand. *Submitted to Journal of Chemical Ecology*.

- Rasmussen, L.H., Schmidt, B., Lauren, D.R., Olsen, C.E., Engelsen, S., and Hansen, H.C.B. (2003) A refined method for isolation of pure ptaquiloside from Bracken (*Pteridium aquilinum* (L.) Kuhn.). *Will be submitted to Journal of Agricultural and Food Chemistry in August 2003*.

Behind these seven papers are many hours of work spent in the laboratory, in the field, and numerous hours behind the computer. The work has been hard, but also inspiring and challenging.

Chemistry Department at the Royal Veterinary and Agricultural University (KVL) has been the base for my work, and I would like to express my sincere gratitude to all the people (faculty, staff and students) affiliated with the department, but especially to my friend and colleague Anne Louise Gimsing.

Almost 6 months passed as visiting scientist at HortResearch in Hamilton, New Zealand. The visit was challenging and invaluable to me, since my New Zealand supervisor Dr. Denis R. Lauren taught me how to prepare ptaquiloside in a much easier way than previously reported. Many other people assisted me in New Zealand, but the list would be too long if I should mention all.

A special thanks goes to my colleague Dr. Carlos Pinto, Servica de Desenvolvimento Agrario de Sao Miguel, Ponta Delgada, the Azores, who turned up to be an excellent guide to the beautiful nature at the Azores as well as a competent and enthusiastic scientist working with the toxicological and veterinary aspects of Bracken and ptaquiloside in dairy and cattle production.

Lots of other people have assisted me in the last four years of Bracken work – ranging from students at the KVL, farmers and foresters in Denmark, Scotland, the Azores, and

New Zealand to scientists at other departments and institutions than the ones mentioned above. All are thanked for their invaluable assistance.

Finally my deepest gratitude and thanks to Solveig Warnecke for her assistance during field work and travels, her patience during my long working hours, and her understanding and tolerance of my love to this challenging fern, the Bracken.

Frederiksberg, July 2003

Lars Holm Rasmussen

This thesis is the 1st edition. A 2nd edition will be published soon after the examination based on the comments of the examination committee.

Chapter 1

INTRODUCTION

For many years focus in environmental organic chemistry has been on anthropogenic substances such as pesticides, dioxins, and endocrine disruptors and their occurrences in the environment - especially in ground- and drinking water. However, toxic substances are produced by Nature as well. In fact, some authors estimate that more than 99% of the toxic substances humans are subjected to in total are of natural origin (Ames and Gold, 1990; Ames et al. 1990). These natural toxins are mainly of plant or fungal origin, being a part of their present or past chemical interaction with herbivores as well as other plants or fungi (Ames et al. 1990; Teuscher and Lindequist, 1994; Kaufman et al. 1999).

Humans on all continents have utilized vast numbers of plants and fungi in traditional medicine. This usage can often find its roots in the content of bioactive compounds in the species used, and many species are today screened for their content of bioactives by the pharmaceutical industry. A typical example of this usage is Foxglove (*Digitalis purpurea* L.), which contains a series of glycosides used as heart medicine (Teuscher and Lindequist, 1994). Another widespread usage in North America and Europe of bioactive compounds is aromatherapy based on plant extracts and herbal teas (Anonymous, 2002b). A well-known example of this usage is the Australian *Tea Tree Oil* (made of leaf extracts from the *Melaleuca alternifolia* Cheel) having antiseptic properties. A not so well known example is the extract made from the needles of Western Red Cedar (*Thuja plicata* J. Donn ex D. Don) also having antiseptic properties due to its content of thujone (Teuscher and Lindequist, 1994). The effects of these drugs is caused by their content of bioactive compounds, and are generally considered safe for use due to their natural origin. But the risk of overmedication or intoxication may also be caused by such drugs or the plants/fungi themselves. For example, in Denmark and Britain several people are intoxicated and some are killed every year as a consequence of eating toxic fungi – e.g. Destroying Angel (*Amanita virosa* (Fr.) Bert.) which can be taken for a mushroom when in the young stage

(Cooper and Johnson, 1998). Thus, bioactive compounds may be lethal when consumed in too high amounts.

Due to their potent effect, some bioactive compounds have been examined thoroughly and are monitored regularly by the food safety authorities as some of these compounds occur regularly in human food, traditional/natural medicine, or animal fodder (e.g. Gry et al., 2000). For example, aflatoxins are produced by the fungi *Aspergillus flavus* Link. Aflatoxins are some of the most toxic natural compounds known, and are intensively monitored by the Danish food safety authorities as they occur regularly in certain tropical and subtropical crops, such as groundnuts.

The socio-economic impact of natural toxins can be immense, e.g. in Southern Brazil, cattle intoxication by plants is estimated to make up 17% of all diseases of cattle (resulting in a cattle mortality of 5%). Bracken fern (*Pteridium aquilinum* (Scop.) Gled) is responsible for approx. 40% of these plant intoxications, and in the Santa Catarina State, this sums up to an annual loss of $2,100,000 (Gava et al. 2002)! The impact is usually socially biased since poor and/or uneducated farmers may not be able to eradicate the fern from pastures and hence improve their living standards. The economic loss is likely higher due to lost productivity of the cattle caused by sub-lethal doses of Bracken toxins (Smith and Beatson, 1970). Cases of Bracken-poisoning is also known from the industrialised part of the world, e.g. Germany and United Kingdom (Caldow and Burns, 2000; Schrader et al. 2001).

How does toxic bioactive compounds behave in Nature seen from an environmental point of view? It is a question yet to be answered. Almost no environmental scientists have addressed this problem so far – most have probably not given it any thoughts, and some scientists generally believe that natural substances are easily biodegraded, only found in small amounts and in general not being as toxic and harmful as anthropogenic toxins. Not much research has therefore been carried out on potential adverse effects of the natural toxins on the environment. Recently, North American and Danish research groups have although looked more carefully at a few toxins, for example: aflatoxins from the fungi *Aspergillus flavus* at Maize (*Zea mays* L.); Bt-toxins from genetically modified Maize – so-called Bt-Maize; and thujone from Western Red Cedar needles. The results show that:

- Aflatoxins from infected Maize may leach through the topsoil and reach drain channels (Madden and Stahr, 1993).

- Bt-toxins are found in the rhizosphere of Bt-Maize. The toxin has also proved to be stable in the soil (Saxena et al. 1999).

- Thujone are found in high concentrations in plant debris (litter) from Western Red Cedar, and in soil layers down to 90 cm depth below such stands (Strobel et al. 2003).

Taken together, these results shows the potential for natural toxins to be transferred from plants/fungi to the soil environment, where they are stable enough to reach deeper soil layers and hence may reach groundwater or other recipients.

Before a natural toxin exhibits a threat to soil or water resources, a series of prerequisites must be fulfilled. The compound must be:

- Produced in vast amounts by plants or fungi.
- Found in commonly occurring plants or fungi.
- Found in plants or fungi with a high biomass-production.
- Relatively stable in soil and soil solutions.
- Highly water-soluble and mobile in the soil environment.
- Highly toxic or carcinogenic.

Ptaquiloside found in Bracken and some other ferns might represent such a substance, since (van der Hoeven et al. 1983; Niwa et al. 1983; Niwa et al. 1983a; Saito et al. 1989; Smith et al. 1994; Taylor, 1999; Rasmussen et al. 2003c):

- ✓ Bracken is one of the most common plants on Earth.
- ✓ Ptaquiloside is found in high amounts in the biomass.
- ✓ Dry matter production of Bracken is high, 1,400 g m^{-2} per year or more.
- ✓ The degradation rate of ptaquiloside may be rather slow.
- ✓ Ptaquiloside is readily water soluble and hence theoretically quite mobile in the soil.
- ✓ Ptaquiloside is genotoxic and carcinogenic.

The aim of this thesis is to investigate the potential hazardous nature of ptaquiloside seen from an environmental point of view by seeking answers to the following questions:

1. Can ptaquiloside be found in soil materials?

2. Is ptaquiloside stable in contact with soil materials?
3. What is the mobility of ptaquiloside in soil?

To be able to answer these questions, a few other questions regarding the occurrence of ptaquiloside in different fern components has to be answered:

4. When is ptaquiloside present?
5. What is the natural variation in the ptaquiloside content of Bracken?
6. How much ptaquiloside can be produced under natural conditions?

The research carried out by the author has focused on the Bracken varieties vars. *aquilinum* and *esculentum* (12 varieties exist, see Chapter 2), but reference will also be made to the other Bracken varieties. There is no generally accepted taxonomy of Bracken, as well as many different common names has been used for the different varieties of Bracken. Therefore, Latin names following the taxonomy of Tryon (1941) as referred by Burge and Kirkwood (1992) and Smith et al. (1994) are used instead of common names throughout the text. However, newer taxonomy is used in some of the articles in the second part of the thesis.

Chapter 2

BRACKEN

2.1 Morphology, taxonomy and distribution.

Bracken (*Pteridium aquilinum* (Scop.) Gled, Dennstaedtiaceae, Figure 2.1) is a large deciduous rhizomatous perennial fern, having fronds with a maximum height of 2-4 m originating from a well-developed underground system of rhizomes (Øllgaard and Tind, 1993).

Bracken is an old English word used for all large ferns, but applied to this species in particular. The name *Bracken* may originate from the German *Brache* or *Brach-feld* which means uncultivated land or land open to tillage – showing old recognition of Bracken as a colonizer of abandoned arable land (Rymer, 1976). Other common names for the different varieties of Bracken include: Brake, Brake Fern, Eagle Fern, Female Fern, Fiddlehead, Hog Brake, Pasture Brake, Western Brackenfern, Devil's Foot (Scotland), Fern of God (Ireland), Grande fougère (France), Fougère d'aigle (France), Warabi (Japan), Örnbräken (Sweden), Einstape (Norway), Ørnebregne (Denmark), Sananjalka (Finland), Adlerfarn (Germany), Kilpjalg (Estonia), Warabi (Japan), Broto de Samambaia (Brazil), and Rahurahu (Maori, New Zealand) (Santos et al. 1987; Brownsey and Smith-Dodsworth, 1989; Rook, 2002; Grieve, 2002). There is some disagreement on the taxonomy of Bracken (see below), and several Latin synonyms have been used: *Allosorus aquilinus*, *Asplenium aquilinum*, *Filix aquilina*, *Filix-foemina aquilina*, *Ornithopteris aquilina*, *Pteris aquilina*, and *Pteris latiuscula* (Rook, 2002).

Morphology and growth.

The fronds of *Pteridium aquilinum* are soft herbaceous to leathery (depending on light

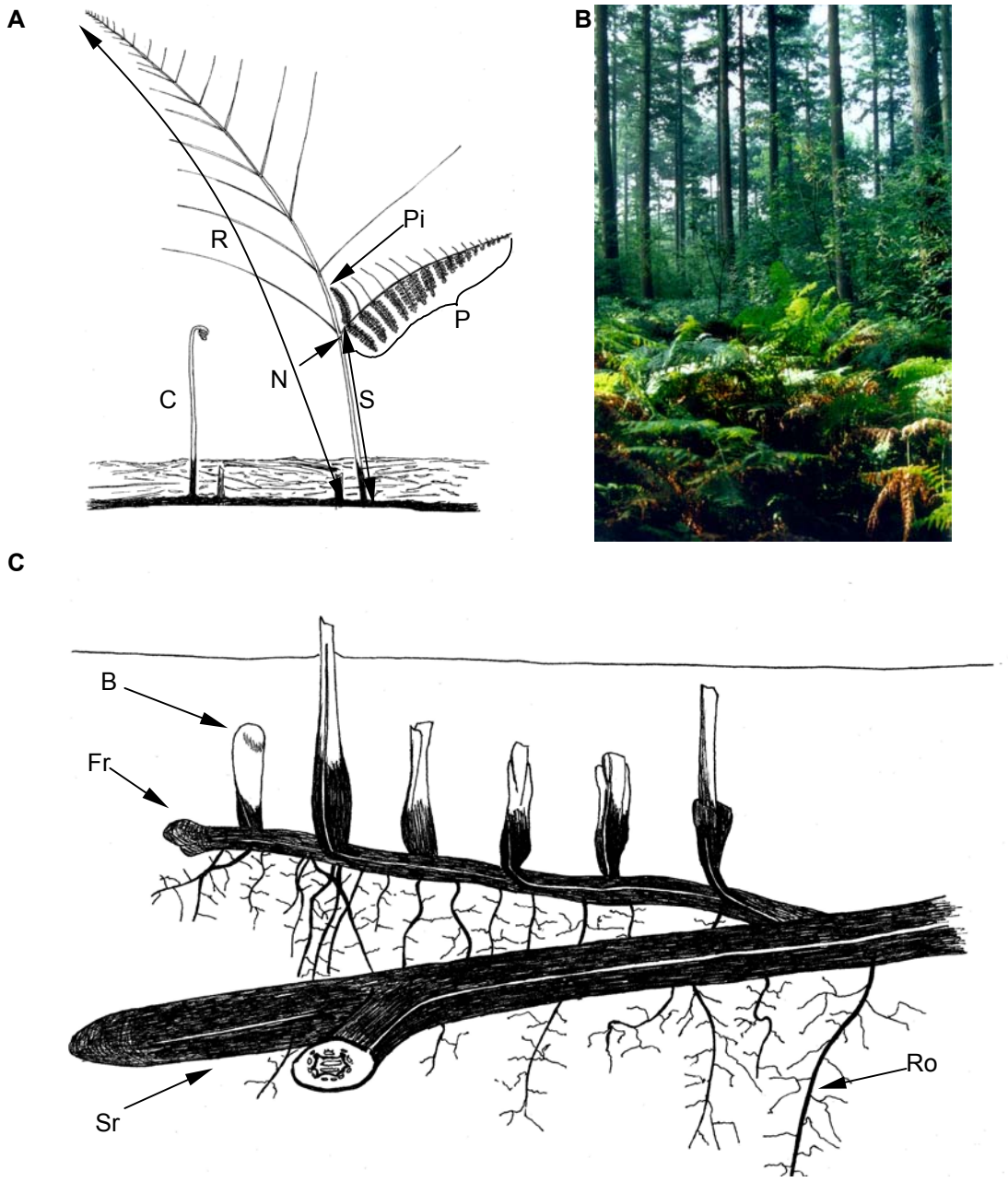


FIGURE 2.1. Bracken (*Pteridium aquilinum* (Scop.) Gled, Dennstaedtiaceae). A: Bracken frond and crosier. B: Mature Bracken stand (var. *aquilinum*), Salten Langsø, Denmark. C: Bracken rhizome system. Abbreviations: B = Bud; C = Crosier; Fr = Frondbearing rhizome; N = Nectaries; P = Pinnae; Pi = Pinnulet; R = Rachis; Ro = Root; S = Stipe; Sr = Storage rhizome. Drawings and photograph by L.H. Rasmussen.

exposition), triangular to triangular-ovate in outline, and bipinnate-pinnatisect to tripinnate. The pinnae are or are nearly opposite (except at apex), the lower ones stalked while the upper are sessile. The pinnae are triangular to triangular-ovate in outline. The outline of the pinnules is linear-lanceolate. The ultimate segments are closely set running together at the base, entire and obtuse, glabrous above and hairy to variable degrees below. The venation

is free, except from fertile segments having continuous marginal, sorus-bearing vein connecting the outer ends of the veinlets (Brownsey and Smith-Dodsworth, 1989; Øllgaard and Tind, 1993). Paired nectaries are found at the base of pinnae. The nectaries are only active in the early development stages of the individual pinnae. The nectaries may be an adaptation to attract ants that will protect the young fronds from herbivores (Øllgaard and Tind, 1993; Rumpf et al. 1994).

The dark rhizomes are subsurface (down to 0.5 m), although some may be found in the litter layer (Watt, 1976). Two types of rhizomes exist: frondbearing rhizomes (blackish short shots from which the fronds emerge), and storage rhizomes (brownish long shots forming the main stem of the fern; used for storage of nutrients). A third type of rhizome may be delineated, the transitional shoot, which is transitional between the two other types (Øllgaard and Tind, 1993; Whitehead and Digby, 1995). The frondbearing rhizomes are usually found above the storage rhizomes (Watt, 1976). The thin (1-3 mm thick) brittle blackish roots are attached to both types of rhizomes, and may extend 150 cm into the soil (Watt, 1976; Rook, 2002). The highest density of roots is found at the frondbearing rhizomes (author, personal observation). The roots may penetrate the mineral soil surface and extend into the litter. Up to 7,900 cm of roots per L of soil can be found in this region (Watt, 1976)! Large numbers of buds are found at rhizomes. Between 400 and 600 per m^2 has been reported for var. *aquilinum*, but only approx. 20% was active at the same time. Dormant buds may be up to 10 years old (var. *latiusculum*). Cutting, burning, and application of nitrogen and phosphorous nutrients increase bud activity. The large number of dormant buds is likely partly responsible for the invasive character of Bracken (Figure 2.2) (Shorina, 1990; Tolhurst, 1990; Whitehead and Digby, 1995; Whitehead and Digby, 1997a). The development from bud to sporulating frond takes 3 year (Christiansen and Jahns, 1981). It takes 3 years for the fern to develop from spore to fully mature frond (Figure 2.3) (Bell, 1976).

Bracken reproduction.

Bracken is genetically diploid and reproduces by spores, but is able to spread vigorously by the use of rhizomes too. The distal part of the rhizomes can reach an age of 35-75 years (Watt, 1940). The expected life span of a clone is uncertain, but estimates of several hundred years or more have been reported (Alonso-Amelot et al. 2001). However, the clone is often killed before reaching this age due to natural succession and ecosystem change (Page, 1976; Rook, 2002). The maximum extent of a single clone has been genetically determined to 390 m (Sheffield et al. 1989).

FIGURE 2.2. Mature Bracken stand at Præstø Fed, Denmark (var. *aquilinum*). The stand is situated on a former moor dominated by Heather (*Calluna vulgaris* L.). Photograph by L.H. Rasmussen.

Bracken is highly variable at the genetic level, which is interesting since Bracken often reproduces only by extension of the rhizome system. The high genetic variation is likely due to high gene flow rates between populations and to homoeologous pairing and cross over of chromosomes at meiosis resulting in a genetically variable population even when originating from a single spore (Jones, 1983; Wolf et al. 1988; Wolf et al. 1988a; Wolf et al. 1990). Bracken stands may originate from a single spore, but usually several genotypes are present resulting in a heterogeneous stand (Sheffield et al. 1989). At high latitudes, Bracken does not produce spores every year, and gene transfer might therefore be of less significance in such regions. Sporulation is related to the energy balance of the fern at the time of spore-production initiation (midsummer), where it is favoured by increased light and temperature. Sporulation is also affected by genotype. Sporulation takes place in early autumn (Øllgaard and Tind, 1993; Kendall et al. 1995; Simán et al. 1999; Ershova, 2000; Wynn et al. 2000). Vegetative formation seems to be the dominant way of stand propagation inside established stands, while establishment of new stands takes place from spores (Watt, 1976; Øllgaard and Tind, 1993).

Bracken taxonomy.

Bracken is well defined at the generic level, but uncertainties are found regarding the nomenclature and taxonomy within the genus (e.g. Thomson (2000) and Thomson and Alonso-Amelot (2002)). Bracken (*Pteridium aquilinum*) belongs to the family Dennstaedtiaceae, and is divided into two subspecies: ssp. *aquilinum* and ssp. *caudatum* (Figure 2.4 and 2.5, Tryon (1941)).

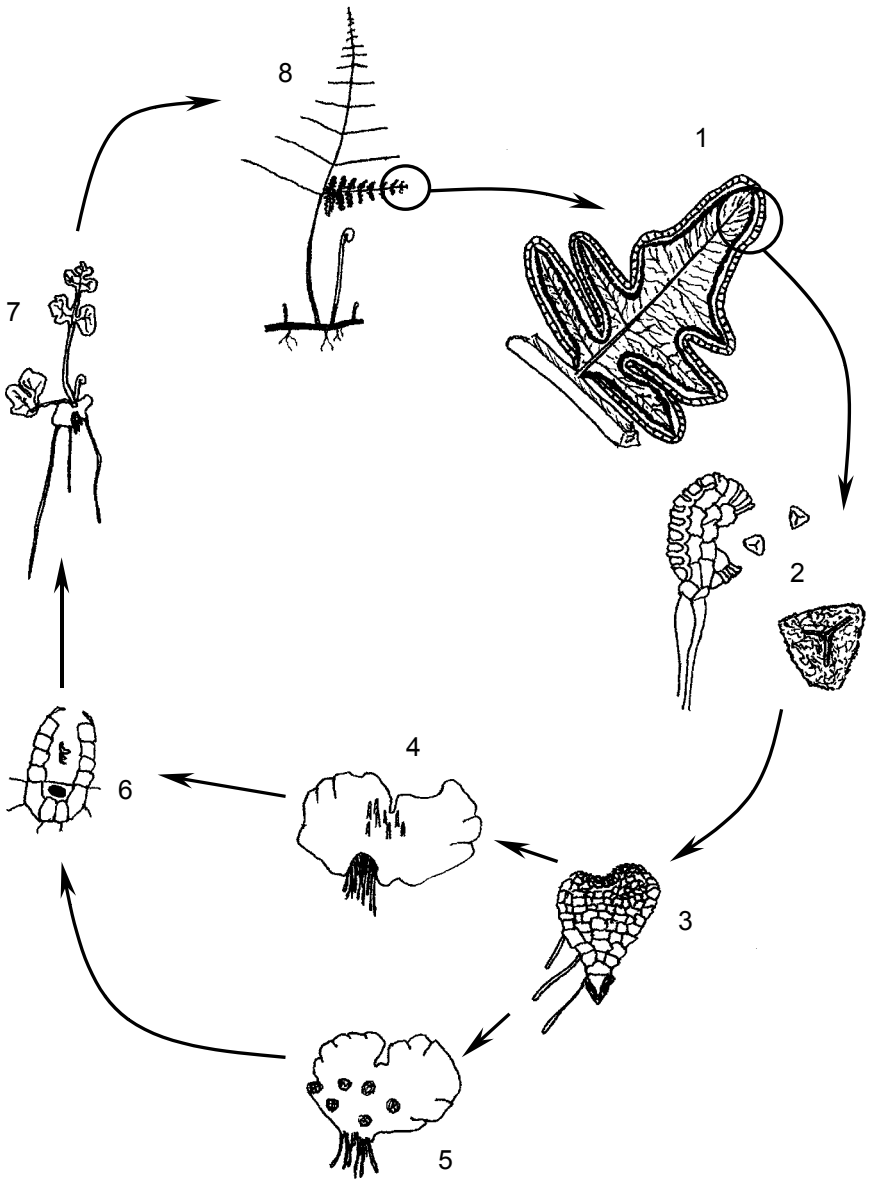


FIGURE 2.3. Bracken life cycle. 1: Lower surface of pinnulet. Sporangia situated along the marginal sorus. 2: Sporangium and spores. 3: Young prothallus formed from spores. 4: Old prothallus with archegonia. 5: Old prothallus with antheridia. 6: Spermatozoids (from antheridium) migrates to the archegonium where they enter the egg cell to form an gametophyte. 7: Young sporophyte still attached to the gametophyte. 8: Adult sporophyte. Drawing by L.H. Rasmussen. Modified from (Brownsey and Smith-Dodsworth, 1989; Burge and Kirkwood, 1992; Øllgaard and Tind, 1993).

These subspecies are furthermore divided into 8 and 4 geographical varieties, respectively. The 12 geographical varieties may be further divided into a series of chemotypes and/or phenotypes according to their content of different natural compounds/secondary

metabolites and/or morphology. In England, Australia, and New Zealand cyanogenic genotypes have been identified within vars. *aquilinum* and *esculentum* (Cooper-Driver and Swain, 1976; Low and Thomson, 1990), while a ptaquiloside chemotype has been suggested within var. *esculentum* (Rasmussen et al. 2003d). Recently, genetic evidence has proven differences between some varieties at generic level, and the following varieties are now proposed full species status by Thomson (2000) and Thomson & Alonso-Amelot (2002): *africanum, aquilinum, arachnoideum, decompositum, esculentum, latiusculum,* and *revolutum*. Varieties *pseudocaudatum* and *pubescens* should be treated as varieties within *latiusculum* while var. *yarrabense* is an *esculentum* x *revolutum* hybrid (Thomson, 2000). Var. *aquilinum* should be perceived as a complex and heterogeneous species comprising several phenotypes and hybrids with other bordering Bracken varieties (Cooper-Driver and Swain, 1976; Sheffield et al. 1989; Wolf et al. 1990; Wolf et al. 1995; Thomson, 2000; Speer, 2000; Thomson and Alonso-Amelot, 2002). It must be emphasized that Bracken is an actively evolving species, and further work on its taxonomy is needed before the taxonomy is fully understood (Page, 1990).

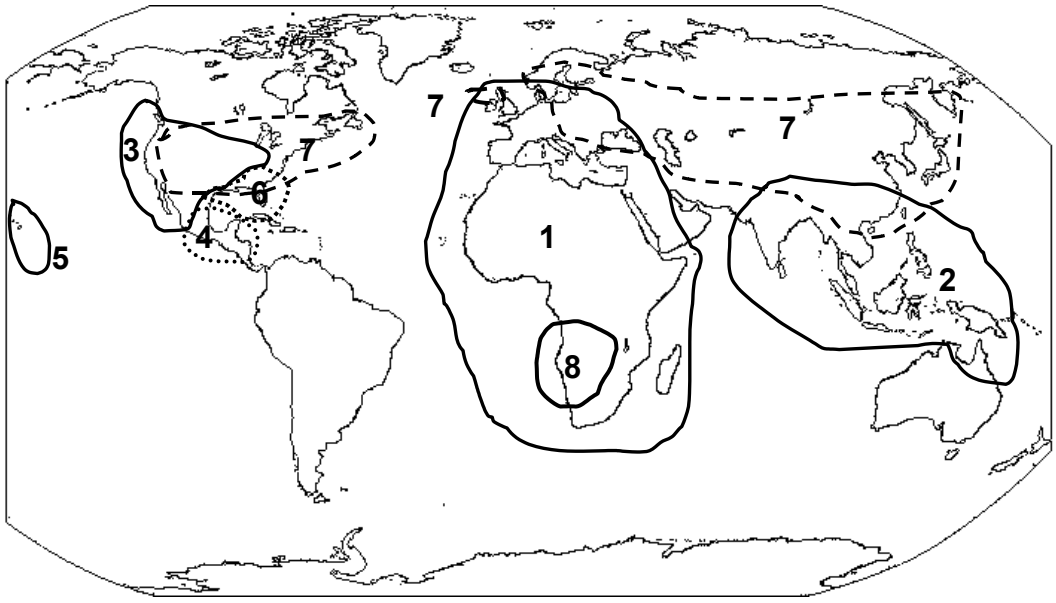


FIGURE 2.4. Geographical distribution of the Bracken subspecies *aquilinum*. 1: var. *aquilinum*. 2: var. *revolutum* (syn. *wightianum*). 3: var. *pubescens*. 4: var. *feei*. 5: var. *decompositum*. 6: var. *pseudocaudatum*. 7: var. *latiusculum*. 8: var. *africanum*. Taxonomy as defined by Tryon (1941). Map adapted from Burge and Kirkwood (1992), Mosberg et al. (1994) and Page (1989).

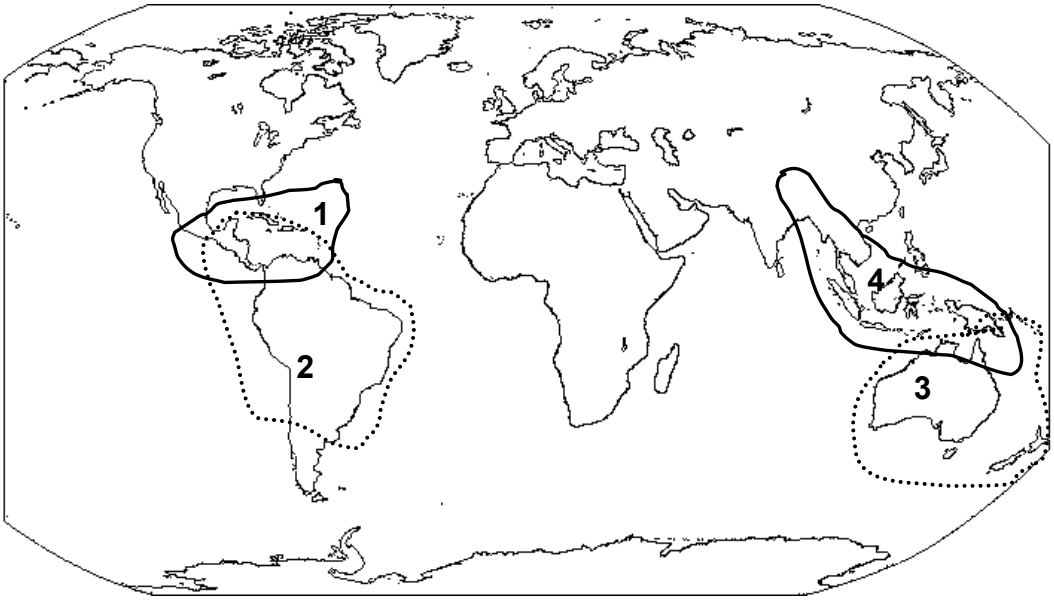


FIGURE 2.5. Geographical distribution of the Bracken subspecies *caudatum*. 1: var. *caudatum*. 2: var. *arachnoideum*. 3: var. *esculentum*. 4: var. *yarrabense*. Taxonomy as defined by Tryon (1941). Map adapted from Burge and Kirkwood (1992).

Bracken distribution and ecology.

Bracken is found on all continents except Antarctica. It is an invasive and opportunistic species well suited for dominating the forest floor below canopy openings. Hence, Bracken is often found in glades, on clear-cut or newly burned areas, and along tracks in forested areas, on the edge of forests and in open woodland and primary bush. Bracken may also dominate more open landscapes such as moors. Bracken is found in semiarid areas, as well as in tropical and temperate forests, moors and heath lands on both hemispheres (Tryon, 1941; Nicholson and Paterson, 1976; Page, 1976; Fenwick, 1988; Brownsey and Smith-Dodsworth, 1989; Henning and Dickmann, 1996).

There are two ways in which Bracken influences succession in Northern Europe (var. *aquilinum* – see Box 2.1): Through invasion into early-successional plant communities, and through control of invasion of later-successional species (Marrs et al. 2000).

The earliest fossil records of Bracken are from Oligocene (Hungary) and Miocene (England) (Rymer, 1976). Spores have been found in sediments throughout the Quaternary, but it is only during the last 5,000 years Bracken has reached its worldwide distribution and abundance. This increase of abundance is thought related to the advance of Neolithic Man causing widespread forest clearings as agriculture was introduced and domestication

of large herbivores took place (Rymer, 1976). Today, Bracken is one of the five most common plants on Earth. In the industrialized countries, the extensive character of agriculture on marginal land - such as mountains, moors and heather favours the still continuing advance of Bracken (Fenwick, 1988; Taylor, 1999). Overgrazing or grazing by species not willingly eating Bracken such as sheep may also contribute (Smith, 2000).

Box 2.1. The five natural reasons for the success of Bracken.

The success of Bracken is due to (Atkinson, 1989; Marrs et al. 2000):

1. Large rhizome system with huge potential for storage of nutrients and carbohydrates.
2. Large number of buds capable of producing new fronds.
3. High productivity resulting in a dense frond canopy that casts deep shade (removes up to 94% of full sunlight (Humphrey and Swaine, 1997)).
4. Large accumulation of litter.
5. A wide range of toxic compounds within living tissues and dead plant material.

Besides from the above mentioned reasons, Bracken also consumes large amounts of water, which adds to the competiveness of Bracken (Tolhurst and Burgman, 1994; Pakeman and Hay, 2001).

Increased atmospheric deposition of pollutants such as NO_x and heavy metals favours Bracken in Northern Europe compared to species Bracken usually compete with, e.g. Heather (*Calluna vulgaris* L.) (Smith, 2000). Bracken is not likely directly affected by higher CO_2-concentrations in the atmosphere, but increased temperature may widen the climatic zones where Bracken can grow (Caporn et al. 1999). Drainage of low-lying soils and peat bog's can also partially explain the Bracken advance (Smith, 2000). In the United Kingdom, Bracken advance has been reported to 1 to 3% per annum, resulting in a total Bracken cover in Scotland and Wales of more than 6% in some areas - in some counties even as much as 40% of the land is covered by Bracken (Rymer, 1976; Fenwick, 1988; Taylor, 1999). Recent counts of Bracken spores in pollen records from the United Kingdom do although indicate that the abundance of Bracken today is less than or only equivalent to maximum historical levels (Pakeman et al. 2000). However, this study is based on few sites only, and includes the questionable assumption that sporulation is constant over time and therefore usable for indication of Bracken cover.

Bracken soils and elemental composition of Bracken.

Bracken is usually found on well-drained acid soils (from pH 2.6) such as Spodosols (Figure 2.6), Inceptisols, and Entisols, but may at times be found at nutrientrich and even calcareous soils such as Alfisols and Mollisols (up to pH 7.5) (Watt, 1976; Frankland, 1976; Jarvis and Duncan, 1976; Skeffington, 1983; Ader, 1989; Hetherington and Anderson, 1998; Rasmussen et al. 2003b). This distribution in relation to soil type is a result of the edaphic demands of the fern as well as a result of human clearance of Bracken

from more fertile soils and arable lands (Thompson et al. 1986; Ershova, 2000; Smith, 2000; Wind, 2002).

The elemental composition of Bracken fronds is not related to the nutrient status of the soil (Hunter, 1953), but some variation in the pH of Bracken litter is typically found (Rasmussen et al. 2003b). Bracken growth may result in formation of a thick (up to 86 cm) and dense litter layer composed of frond remnants. In some areas, litter continues to accumulate until breakdown of the stand. The litter is apparently not digested by earthworms, and a strict horizontal layering of the litter can be observed (Watt, 1976; den Ouden and Vogels, 1997). In the first 20-40 cm of the topsoil, large amounts of dead and living rhizomes can be observed, imposing large impact on soil structure and carbon content (Rasmussen et al. 2003). Due to the large production of nutrient rich biomass, high water consumption, increased pH and salt content in frond leachates and internal nutrient recycling within the fern, Bracken can improve the quality of very poor soils such as Spodosols (Table 2.1) (Williams et al. 1987; Smith, 2000). Bracken may also reverse leaching processes at moors compared to vegetation dominated by Heather (Mackney, 1961; Jarvis and Duncan, 1976). On nutrient-rich soils, Bracken (vars. *aquilinum* and *latiusculum*) can have the opposite effect, as the acidity of Bracken litter and Bracken leachates enables buffering of soil solution pH around pH 5-5.5 which is 1-1.3 pH-units lower than in soil solutions from adjacent non-Bracken soils.

TABLE 2.1. Elemental composition of mature var. *aquilinum* fronds (12 sites) and rhizomes (1 site) in Scotland (Hunter, 1953).

Element:	**Content in fronds:** (% in dry matter)	**Content in rhizomes:** (% in dry matter)
Ash content	5.20 - 6.45[¶]	5.71
Nitrogen	1.31 - 2.12	1.05
Phosphorous	0.09 - 0.22	0.05
Potassium	1.02 - 2.95	1.24
Calcium	0.15 - 0.37	0.18
Magnesium	0.11 - 0.27	0.18
Sodium	0.07 - 0.24	0.26

[¶] Only two sites.

Increased soil solution concentrations of dissolved organic matter (DOM) are also found below Bracken. Up to 4 times the content found in soils with no Brackens have been measured (up to 50 mg L^{-1}) (Johnson-Maynard et al. 1998). The acidifying effect and the increased content of DOM in Bracken soils, can change the soil mineralogy by increasing the rate of weathering and the formation of organo-mineral complexes (e.g. aluminium-

iron-humus complexes). The high annual input of organic matter to the Bracken soil can also change the classification of a soil. For example, Haplocryands has been reported to change to Fulvicryands in Bracken dominated areas (Johnson-Maynard et al. 1997; Johnson-Maynard et al. 1998; Pitman and Webber, 1998; Cwen et al. 1999; Soil Survey Staff, 1999). Changes in mineralogical composition may also alter classification (Johnson-Maynard et al. 1997).

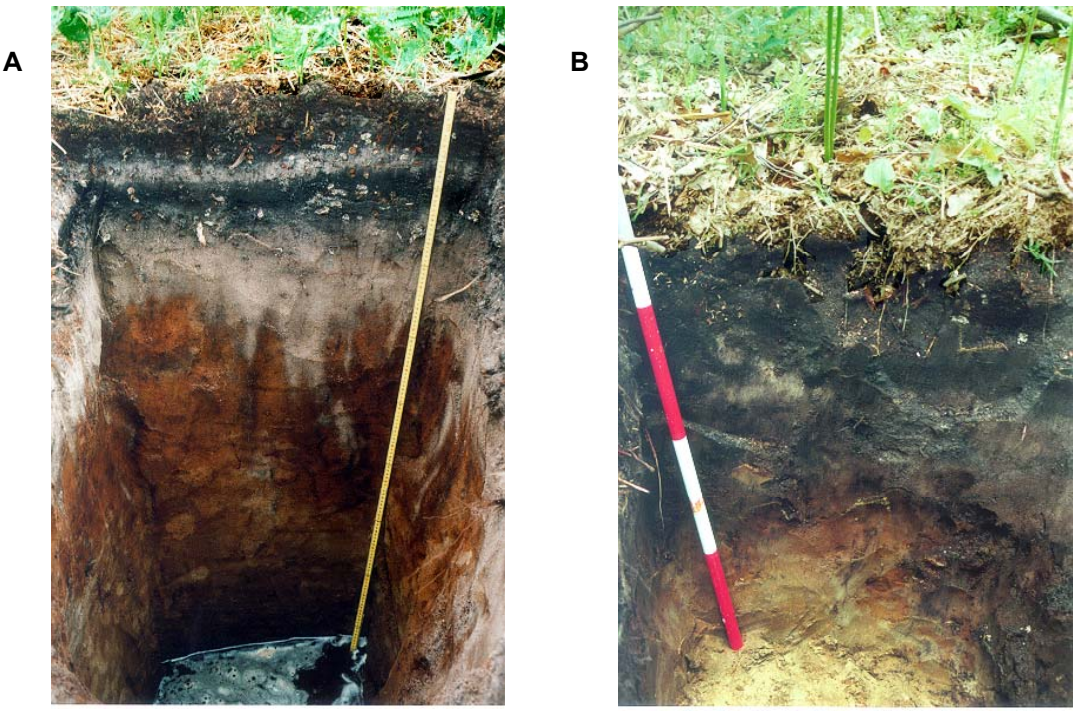


FIGURE 2.6. Two soils with Bracken cover in Denmark (var. *aquilinum*). A: Oxyaquic Quartzipsamment at Præstø Fed (June 2000; ptaquiloside load (fronds in September) = 20 mg m^{-2}). B: Histic Endoaquod at Sorø (May 2000; ptaquiloside load (fronds in September) = 260 mg m^{-2}) (Soil Survey Staff, 1999; Rasmussen et al. 2003b).

2.2 Growth of Bracken.

Bracken grows rapidly, and the fronds reach maximum length in a few months (Figure 2.7). The average growth can be several centimetres per day in the initial growth phase (Alonso-Amelot and Rodulfo-Baechler, 1996; Alonso-Amelot et al. 2000; Ershova, 2000; Rasmussen and Hansen, 2003). Frond maturation and spore production in fertile fronds takes place 100-130 days after emergence for var. *aquilinum* in Denmark, while vars. *caudatum* and *arachnoideum* only use 40-70 days in Venezuela (Evans and Galpin, 1990; Alonso-Amelot et al. 1992; Alonso-Amelot et al. 1995; Alonso-Amelot and Rodulfo-Baechler, 1996; Whitehead and Digby, 1997; Alonso-Amelot et al. 2000; Rasmussen and

Hansen, 2003). When fronds mature, translocation of nutrients (carbohydrates and minerals) and secondary metabolites takes place from the fronds to the rhizomes (Hunter, 1953; Watt, 1976; Williams and Foley, 1976; Rasmussen and Hansen, 2003). The precise growth pattern is highly dependent on the physical growth conditions as well as on the variety of Bracken in question. Frond emergence of var. *aquilinum* in Scotland was found to be dependent on soil temperature as bud growth initiates at 5.5°C (Ader, 1989; Birnie et al. 2000). In areas with no frost, individual Bracken fronds may reach an age of more than 12 months. For example, var. *esculentum* fronds in Australia can be up to 36 months old (Tolhurst, 1990).

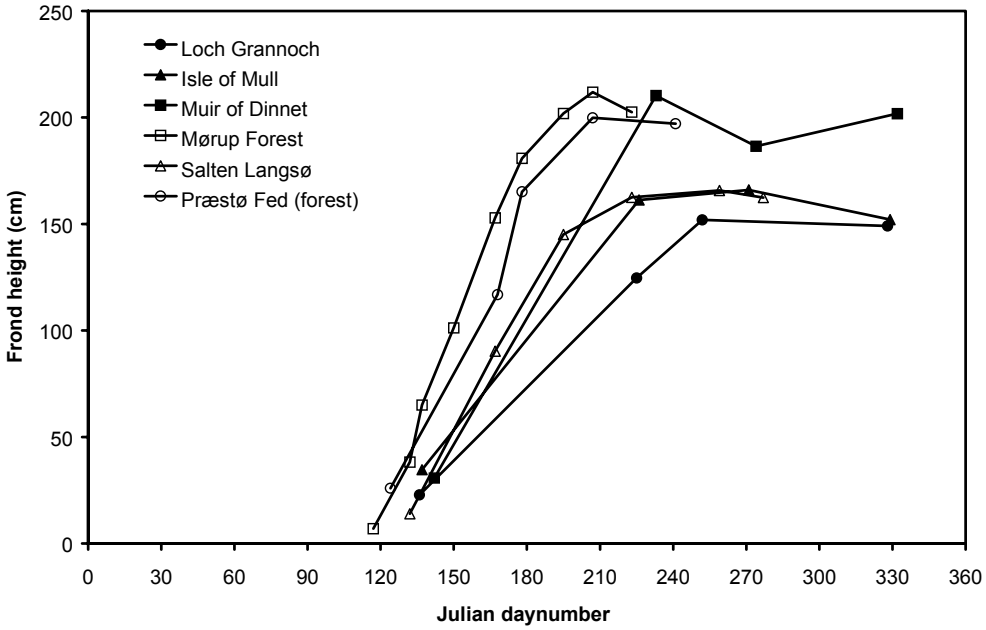


FIGURE 2.7. Growth of var. *aquilinum* in Denmark (white marks) and Scotland (black marks). Data from Rasmussen and Hansen (2003) and Rasmussen et al. (2003).

Five annual growth stages of Bracken can be delineated (partly after Rasmussen et al. (2003b), Tolhurst (1990) and Williams and Foley (1976)):

1. Crosier stage: Fronds has not opened 1st pair of pinnules on 1st pair of pinnae. Carbohydrates are transferred from rhizomes to fronds.
2. Young stage: Fronds has opened 1st pair of pinnules on 1st pair of pinnae, but are not fully expanded. Carbohydrates are transferred from rhizomes to fronds. By the end of this stage, carbohydrate replenishment of the rhizomes begins, especially to storage rhizomes.
3. Mature stage: Fronds are fully expanded. Sporulation may take place on fertile fronds. 1st and 2nd pair of pinnae may exhibit some signs of deterioration in

shaded and dense Bracken stands. Carbohydrate replenishment of both rhizome systems is taking place.

4. Senescent stage: Old fronds show strong signs of deterioration. The colour may change to yellow, orange or brown. Carbohydrate replenishment of both rhizomes systems and translocation of carbohydrates from frondbearing to storage rhizomes.
5. Dormancy: Fronds are dead. No aboveground activity. Slow decrease in reserve carbohydrates and slow increase in the proportion of mobile carbohydrates.

In temperate and subtropical climatic zones, Bracken stands exhibits an annual growth pattern where the crosiers emerge from the soil in the spring and the fronds die back in the autumn (often killed by frost; growth stage 1 to 5) or the following year (growth stage 1 to 4) (Watt, 1976; Øllgaard and Tind, 1993). In the tropics, Bracken stabds has a 12-month growing season (growth stage 1 to 4, but only at individual fronds, as new fronds continue to emerge all year round) (Alonso-Amelot et al. 1992).

Bracken (var. *aquilinum*) stand development in forested and open habitats in Denmark, the United Kingdom and New Zealand (var. *esculentum*) can be divided into four separate phases (Watt, 1976; Rasmussen et al. 2003; Rasmussen et al. 2003b; Rasmussen et al. 2003d):

1. Pioneer phase (new community): Low frond density. No Bracken litter. Few rhizomes. No dead rhizomes.
2. Building phase (young community): Medium frond density. Low amounts of Bracken litter. Many rhizomes. No dead rhizomes.
3. Mature phase (mature community): Maximum frond density. Deep layer of Bracken litter. Multiple rhizomes. Dead rhizomes can be found.
4. Degenerate phase (old community): Low-medium frond density. Medium-deep layer of Bracken litter. Multiple rhizomes, high proportion of dead rhizomes. Patches of other herbs (not usually associated with Bracken) can be found inside the stand.

The frond density can be quite high, resulting in an enormous pressure on competing species. Frond (var. *aquilinum*) densities in Denmark and United Kingdom are usually in the range 5-55 fronds per m^2. The density is affected by natural variations in growth conditions, nutrient availability, and control measures such as mowing (cutting increases the frond density) (Nicholson and Paterson, 1976; Atkinson, 1989; Whitehead and Digby,

1997; Whitehead and Digby, 1997a; Buttenschøn, 2000; Rasmussen et al. 2003; Rasmussen et al. 2003b; Rasmussen and Hansen, 2003). Densities of var. *latiusculum* up to 60 fronds per m^2 has been reported for the former USSR (Shorina, 1990). In Australian forests, var. *esculentum* has densities up to 4 fronds per m^2 (Tolhurst, 1990). In Venezuela, densities between 2 and 67 fronds per m^2 are found for var. *caudatum* and 5-15 fronds per m^2 for *arachnoideum* (Alonso-Amelot et al. 1995; Alonso-Amelot and Rodulfo-Baechler, 1996; Alonso-Amelot et al. 2000). In the United Kingdom, new fronds continue to emerge during most of the growth season. However, in Scotland maximum frond density seems to be reached during the first 30-40 days of the growth season (Ader, 1989; Whitehead and Digby, 1995; Whitehead and Digby, 1997; Rasmussen et al. 2003). This is not the case in Denmark, where allmost all fronds have emerged from the ground within 1-2 weeks (author, personal observation).

Several frond biomass production functions exist, e.g. Paterson et al. (1997). Alonso-Amelot et al. (2000), Alonso-Amelot and Rodulfo-Baechler (1996), and Rasmussen and Hansen (2003) have presented simple models for vars. *aquilinum*, *arachnoideum* and *caudatum* based on the length of the frond (Table 2.2). Models like those and direct measurements have been used to quantify the annual frond dry matter production, which reach maximum at frond maturity in late summer/early autumn before the fern begins retrieval of nutrients to rhizomes (Watt, 1976; Williams and Foley, 1976).

TABLE 2.2. Bracken biomass production functions.

Bracken:	**Equation:** Biomass (g frond^{-1})	**Country:**	**Reference:**
Unmanaged var. *caudatum*	$0.29 \times \exp^{(0.044 \times Lf)}$	Venezuela	Alonso-Amelot et al. (2000)
Unmanaged var. *caudatum*	$-19.33 + 0.512 \times Lf$	Venezuela	Alonso-Amelot and Rodulfo-Baechler (1996)
Unmanaged var. *arachnoideum*	$-10.80 + 0.458 \times Lf$	Venezuela	Alonso-Amelot and Rodulfo-Baechler (1996)
Unmanaged var. *aquilinum*	$0.72 \times \exp^{(0.018 \times Lf)}$	Denmark	Partly Rasmussen and Hansen (2003)
Managed var. *aquilinum*	$0.13 \times \exp^{(0.074 \times Lf)}$	Denmark	Unpublished

Lf: frond length (cm)

Var. *aquilinum* has an annual frond dry matter production up to 1,400 g m^{-2} in United Kingdom while the production in Denmark is up to 550 g m^{-2} (Watt, 1976; Buttenschøn, 2000; Rasmussen and Hansen, 2003; Rasmussen et al. 2003b). Vars. *caudatum* and *arachnoideum* in Venezuela have a whole year growth cycle resulting in a standing aboveground biomass between 25 and 1,440 g m^{-2} with *caudatum* having the highest

production (dry matter) (Watt, 1976; Alonso-Amelot et al. 1995; Alonso-Amelot et al. 2000). The belowground biomass is mainly composed of rhizomes. For var. *aquilinum* this can make up to 660 g dry matter per m^{-2}, while vars. *caudatum* and *arachnoideum* have up to 140 and 570 g m^{-2} respectively (Galpin and Smith, 1986; Alonso-Amelot et al. 1995; Alonso-Amelot and Rodulfo-Baechler, 1996). Rhizomes may make up around 80% of the total biomass of Bracken populations, with storage rhizomes counting for 60-80% of the rhizome biomass (Whitehead and Digby, 1995; Whitehead and Digby, 1997a; Alonso-Amelot et al. 2001). The dry matter content in the rhizomes is lowest during late spring and early summer while the fronds are growing (var. *aquilinum*). From midsummer till mid autumn, the carbohydrate-content of the rhizomes is replenished before the fern goes into dormancy (Williams and Foley, 1976).

2.3 Ethnobotannical history and recent usage of Bracken.

Bracken has been widely utilized by Man in all parts of the world. In Europe, Bracken was mainly used as food at times of scarcity – a sort of flour has been made out of the rhizomes. The fronds have also been used as bedding for animals and Man (e.g. by Roman soldiers, Vikings, and peasants in Denmark and Scotland), floor cover, fuel, as ornamental and ritual plant, as bleaching and dying agent (tartans and tweed), for dykes, roofing (thatch), baskets, mulch, compost and as source of potash for the glass and soap industry. Bracken fronds have also been used for packaging of fruit and breakable materials (Rymer, 1976; Brøndegaard, 1978; Fenwick, 1988; Taylor and Smith, 1995; Project Runeberg, 1997; Bento et al. 2000; Anonymous, 2002a).

The Forest Authority in United Kingdom recently investigated the potential of using Bracken compost as a peat alternative for making suitable potting media for calcifuge plants (in combination with peat) or alone as a mulching material (Pitman and Webber, 1998). Bracken litter may also be used as a remedy for soil pH modification in the restoration of former moors used as farmland. Here, litter has been showed to reduce soil pH to pH 4-4.5 (Owen et al. 1999). An evaluation of var. *esculentum* for use as mulch in the glasshouse industry showed promising results (Taylor and Thomson, 1998). Bracken may also have a future as fertilizer or mulch in organic farming (Eric Donnelly, University of Aberdeen, personal communication).

In New Zealand, Australia, and North America, the pre-European settlers widely used Bracken rhizomes as everyday food or during travel (Newsome, 1987; Leach, 2000; Turner, 2002). In Japan, Korea and some parts of South America (e.g. Brazil) Bracken is

still used for food (many recipes can be found at the internet: search word *Bracken* or *warabi*). In Tokyo alone, more than 300 tonnes of young fronds were consumed every year in the 1970's, and the annual Bracken import to Japan in the 1980's and 1990's were 6,800-15,500 tonnes (commercial price in Japan (1997): 1.3 US$ kg^{-1}) (Rymer, 1976; Hirono, 1986; Nguyen, 2000). The fern is usually collected from natural habitats, but Bracken cultivation do take place some places in Japan and elsewhere (Fujio Morrow, 2000). Bracken collection does also take place among Japanese and Korean people as a recreational, social and outdoor activity. Immigrants from Japan and Korea tend to continue this activity in a cultural context (Anderson et al. 2000). The crosiers or young fronds are usually steeped or boiled in water sometimes added sodium bicarbonate or ordinary wood ash (to preserve the fresh colour of the crosier and to reduce the toxicity and bitterness of the fern – however, not all toxicity is removed by this treatment) (Hirono et al. 1972; Ortega, 1994; Silva et al. 2000; Fujio Morrow, 2000). However, a study of the ptaquiloside content in some commercial Bracken products from Siberia and Japan showed that the content of ptaquiloside was below the limit of detection (100 μg per g-fresh-weight) (Saito et al. 1989).

From time to time Bracken is advocated as food in Europe and North America (Galpin and Smith, 1986). In Denmark, the crosiers have been used as food too; either cooked or pickled (Brøndegaard, 1978). In Denmark, Bracken is not alowed as human food or as traditional medicine due to its toxic constituents, although the Danish army still advocates Bracken rhizomes as food in time of war (Inspektøren for Hæren, 1986; Gry et al. 2000).

Bracken has and is still some places used for beer brewing (replaces hops), as tea, traditional medicine (e.g. in Denmark and in the United Kingdom for killing intestinal worms, to heal wounds, as an abortificant and for alleviating constipation), and in modern herbal medicine (e.g. Digestodoron®, WELEDA AG, Switzerland - used for regulation of the digestive system) (Rymer, 1976; Øllgaard and Tind, 1993; Ortega, 1994; Burkhalter et al. 1996; Wind, 2002; Anonymous, 2002a).

Besides from local use and cultivation of Bracken in Japan, Brazil and Korea, Bracken is now generally only perceived as a weed in agriculture and forestry. Bracken can be controlled in several ways (Cooper-Driver and Swain, 1976; Martin, 1976; Lowday, 1986; Bassett et al. 1990; Tolhurst, 1990; Taylor, 1990; McElwee et al. 1990; Auld, 1990; Lawton, 1990; Burge and Kirkwood, 1992; Breach, 1999; Backshall, 1999; Anonymous, 1999a; Anonymous, 1999b; Le Duc et al. 2000; Buttenschøn, 2000):

1. Chemical control: RoundUp® and Asulam® can be used for management. The success is highly dependent on timing of the herbicide application, and use of non-ionic surfactants in the pesticide formulations. Genetic polymorphism in sensitivity towards herbicides may make spraying difficult. Spraying may also damage other species than the fern, hereby making spraying less useful in conservation areas.
2. Biological control: Several mycoherbicides has been tested and some seems promising. Anthropogenic spread of Bracken-feeding insects has also been proposed, but is not likely to take place.
3. Mechanical control: Bracken breakers, ploughing, rotavating, and crushing by rolling are useful tools. The control measures have to be done 2-3 times per year for several years to eradicate the fern.
4. Manual control: Bracken cutting with scythe, fern hooks and similar tools. The control measure has to be done 2-3 times per year for several years to eradicate the fern.
5. Burning: Not advisable, since Bracken seems to become favoured by burning.
6. Grazing: Cows, goats, and sheep may browse Bracken, and tramping by the animals may break and kill young fronds. Generally not advisable due to the content of toxic principles in the fern that may kill or injure the animals (see Chapter 3 and 4). Grazing may be possible in areas with low contents of toxins in the ferns. Cows and goats generally avoid Bracken, and will often only eat the fern when forced to do so. However, calves breed without presence of adult animals and cattle not recognizing Bracken as toxic due to their upbringing in Bracken-free areas may eat the fern more willingly (Carlos Pinto, Servica de Desenvolvimento Agrario de Sao Miguel, and Eric Donnelly, University of Aberdeen, personal communication). In Denmark, poisonous fodder is not allowed, and attempts to use animals for Bracken control is therefore illegal (Anonymous, 2000). Such attempts should also be considered unethical from an animal welfare point-of-view.

Chapter 3

BRACKEN TOXICITY

3.1 Bracken induced diseases in higher animals and Man.

Bracken causes a wide range of human and animal diseases, including cancer. Sublethal intoxication may also lead to immunosuppression, hence making animals and humans more susceptible for attack from pathogenic bacteria and virus such as the bovine papilloma virus (BPV-4) or MCF-virus (malignant catarrhal fever) (Saveria Campo, 2002; Twomey et al. 2002). Animals do not usually eat large amounts of Bracken, but may do so when: 1) Paddocks are overgrazed; 2) When the young ferns are more palatable than associated forage; 3) When in need of coarse fibres later in the growth season; or 4) When the animals are not familiar with the toxic properties of Bracken (Ralphs (2002); Carlos Pinto, Servica de Desenvolvimento Agrario de Sao Miguel, personal communication). Some animals may even get addicted to Bracken – a phenomena known from many toxic plant species (Figure 3.1) (Watson et al. 1972; Allsup and Griffiths, 1978; Cooper and Johnson, 1998).

In this section, a review will be given of the chemical interaction between Bracken and animals. Emphasis will be on the interaction between Bracken and farm animals, especially on the effect of ptaquiloside. The interaction between Bracken and lower animals, plants and fungi is described in Section 3.2. A selection of the secondary metabolites identified in Bracken will be described and their abundance given in Section 3.3. In the rest of the thesis, all weights are given per unit of air-dry sample, unless otherwise stated.

Thiamine (vitamin B_1) deficiency.

Bracken contains thiaminase enzyme (type 1), especially in the rhizomes and in the crosiers. Bracken consumption may therefore lead to thiamine deficiency (*beri beri*) in monogastric (non-ruminant) vertebrates (e.g. horse, mule, and Man) (Nakabayashi, 1955;

Somogyi, 1971; Evans, 1976; Fukuoka, 1982; Evans, 1986; Munro, 1996). The thiaminase enzyme requires a co-substrate to proceed. Caffeic acid, astragalin, isoquercitrin and 5-*o*-caffeoylshikimic acid has been proposed as co-substrates (Nakabayashi, 1955; Somogyi, 1971; Fukuoka, 1982). The enzyme may also act as part of the ferns insect defence (Jones, 1983).

FIGURE 3.1. Holstein cow eating Bracken (var. *aquilinum*) growing at stonewall, Sao Miguel Island, Azores. Photograph by L.H. Rasmussen.

Potential for cyanogenesis.

Some Brackens contain prunasin, which is a bitter tasting cyanogenic glycoside deterring herbivores (Cooper-Driver and Swain, 1976). Prunasin could cause cyanogenesis, but no deaths of higher animals has been reported as result of cyanogenesis caused by Bracken (Cooper-Driver and Swain, 1976; Hadfield and Dyer, 1986; Munro, 1996; Smith, 1997; Alonso-Amelot et al. 1998; Smith et al. 2000; Alonso-Amelot et al. 2001). However, insecticidal effects have been observed (Schreiner et al. 1984). Cyanogenic chemotypes have been identified and cyanogenic polymorphism within individual Bracken stands has been found among vars. *aquilinum* and *esculentum*. The content of prunasin in Bracken fronds seems to be correlated with ecosystem type, as higher levels of prunasin are found in shaded woodland habitats compared to open land. The higher content in ferns growing in shaded areas might have been evolved as response to increased grazing pressure in such areas (Cooper-Driver and Swain, 1976; Cooper-Driver et al. 1977; Blair et al. 1983; Hadfield and Dyer, 1986). The HCN release from shaded Brackens seems also correlated with rainfall and temperature (Hadfield and Dyer, 1986).

Haemorrhagic syndromes – internal bleedings in sheep and cattle.

Bracken intake causes acute internal bleedings in sheep and cattle as well as necrosis of tissue in the gut epithelium. The disease is cumulative, and the animal has to eat approx. 1 kg of Bracken dry matter each day for 2-4 weeks to produce the syndrome (depending on the toxicity of the fern) (Evans, 1986). Internal bleedings in all parts of the body occurs as the bone marrow ceases to produce blood platelets. The production of granulocytes and lymphocytes are also affected (Smith, 1997). Death is often the result of this poisoning known as *the [Acute] Bracken [Fern] Poisoning* (Caldow and Burns, 2000). The haemorrhagic syndromes are likely caused by ptaquiloside or ptaquiloside-like substances (Hirono et al. 1984a; Yoshida and Saito, 1994; Yoshida and Saito, 1994a; Castillo et al. 2000). Other Bracken compounds may be co-responsible for the formation of this disease when acting as immunosuppressants (Evans, 1986).

Progressive retinal degeneration in sheep.

Degeneration of the retina neuroepithelium in the eyes of sheep is found among animals browsing Bracken for 2-3 years. The syndrome is called *Bright Blindness* due to the increased reflectance of the eyes. The disease is found in the United Kingdom, usually in hill farms. Bright Blindness is caused by ptaquiloside and/or ptaquiloside-like substances (Watson et al. 1972; Allsup and Griffiths, 1978; Hirono et al. 1993; Smith, 1997; Cooper and Johnson, 1998).

Enzootic haematuria – tumours in the urinary bladder in sheep and cattle.

Bovine Enzootic Haematuria (BEH) is found among sheep and cattle feeding on Bracken all over the world, and is characterised by the presence of tumours in the urinary bladder causing blood in the urine (Figures 3.2 and 3.3) (Price and Pamukcu, 1968; Smith and Beatson, 1970). The bladder wall may also become thickened (Hoque et al. 2002).

BEH occurs widespread in some areas, e.g. up to 11.5% of the cattle are affected in some regions of Northern India and Nepal, in the Mérida district of Venezuela 18% of the cattle displayed symptoms of BEH, in Bolivia 11% of the cattle in Bracken affected areas had active BEH while carcinomas were present in 100% of the cattle investigated (206 bovines), 20 cases from 340 farms each year in a district in Wales, and at the Azores, up to 4% of the cows slaughtered at the Ponta Delgada abattoir between 1994 and 1997 had urinary bladder tumours while up to 7% had neoplasias (Hopkins, 1987; Lawson, 1989; Pinto et al. 1997; Dawra et al. 2000; Marrero et al. 2001; Alonso-Amelot and Avendaño, 2001; Dawra et al. 2002).

FIGURE 3.2. Symptoms of *Bovine Enzootic Haematuria* at anaemic Holstein cow, Sao Miguel Island, Azores. Note the yellowish colour of the udder. Photograph by C. Pinto.

In India, the occurrence of BEH seems somewhat correlated with the content of ptaquiloside in Bracken (Dawra et al. 2002). In China, BEH is common in the mountainous southwestern part and seems to be associated with the occurrence of var. *revolutum*. BEH occurred in 1-16% of the cattle, while the incidence among water buffalos was between 1 and 5% (Xu, 1992). In Costa Rica, Venezuela, and Columbia higher levels of BEH-occurrence is found at higher altitudes in areas of intensive Bracken cover compared to areas of lower altitude. The occurrence of BEH is thought to be caused by higher ptaquiloside contents in Brackens from mountainous areas (Villalobos-Salazar et al. 2000). However, the influence of altitude on the ptaquiloside content is disputed (see Chapter 5). The abundance of BEH may exhibit seasonal variation, e.g. in New Zealand BEH became evident in the early spring and disappeared in late summer (Smith and Beatson, 1970).

Ptaquiloside is likely responsible for the formation of these tumours in the urinary bladder, as administration of pure ptaquiloside to cattle has reproduced the clinical syndromes. Presence of cattle with BEH in Australia is correlated with the occurrence of Brackens having a ptaquiloside content >100 μg g^{-1} (var. *esculentum*). Similar correlations has been observed in New Zealand and at the Azores too. BEH usually develops after 2-3 years of Bracken exposure, and shorter periods of Bracken ingestion will not cause BEH (Smith et al. 1993; Smith et al. 1994; Smith, 1997; Pinto et al. 1999; Rasmussen et al. 2003d). Ptaquiloside-like substances such as *caudatoside*, *iso-ptaquiloside* and *ptaquiloside Z* may

also cause BEH (Castillo et al. 2000). Bovine papilloma virus (BPV-1 and 2) may take part in the formation of bladder cancer (Campo, 1997; Saveria Campo, 2002).

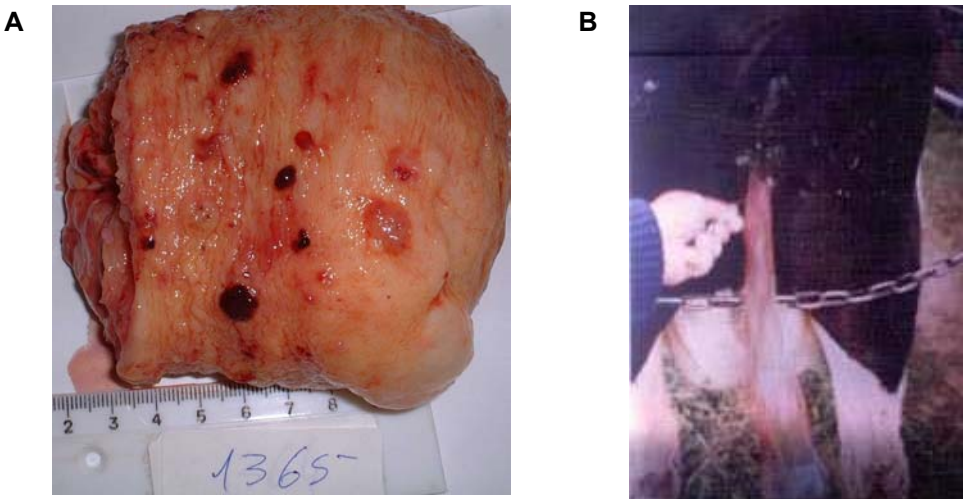


FIGURE 3.3. Clinical syndromes of *Bovine Enzootic Haematuria* in cows at the Azores. A: Tumours in urinary bladder. B. Blood in urine. Photographs by NN (photographer could not be traced) (A) and C. Pinto (B).

Alimentary cancer – tumours in the upper alimentary channel in cattle.

In United Kingdom, Italy, Kenya, and Brazil epithelial tumours have been found in the upper alimentary channel in cattle from Bracken-infected areas. This disease is likely caused by latent bovine papilloma virus (*BPV-4*) infections in cattle (Figure 3.4).

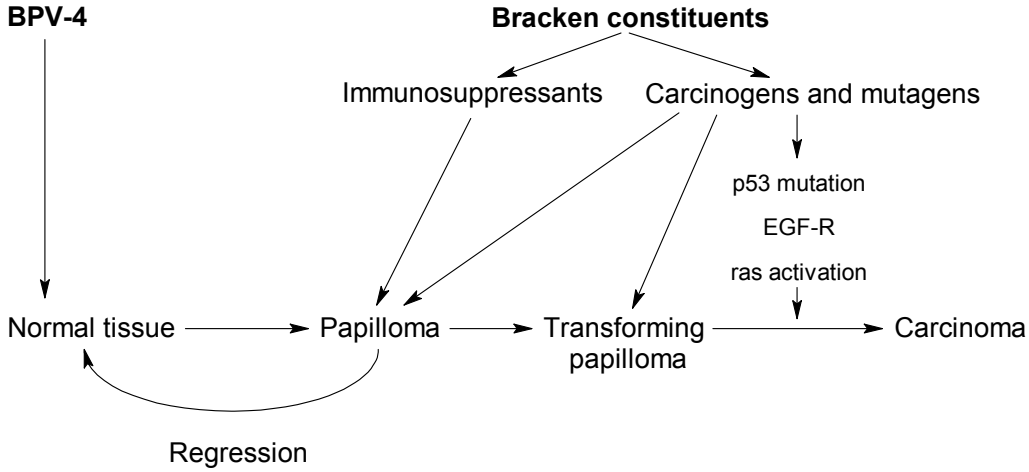


FIGURE 3.4. Interaction between Bracken constituents (e.g. quercetin or ptaquiloside) and Bovine Papilloma Virus 4 (BPV-4) in the development of upper gastrointestinal tract carcinoma. Modified from Saveria Campo (2002).

In Italy, slaughterhouse monitoring revealed oesophageal lesions caused by papillomas in 13% of the cattle. BPV-4 was identified in more than 60% of these papillomas (Borzacchiello, 2003).

Investigations point at quercetin and/or ptaquiloside as the active mutagens and carcinogens, but the aetiology might be very complex, and the general immunosuppressive effects of Bracken may just be the trigger of this viral cancer (Bjeldanes and Chang, 1977; Moura et al. 1988; Campo et al. 1994; Smith, 1997; Stocco dos Santos et al. 1998; Connolly et al. 1998; Campo et al. 2000; Beniston et al. 2000; Saveria Campo, 2002).

Symptoms found in laboratory animals, bacteria and cell cultures.

Numerous investigations have been carried out on laboratory rodents to elucidate the toxic effects of Bracken. The animals have been served a diet containing a certain amount of dried Bracken (crosiers, fronds, spores, or rhizomes) or pure ptaquiloside. The result of such feeding experiments has been rapid development of tumours, especially of the ileum, and formation of ptaquiloside DNA-adducts. Tumours are also found in the mammary (mammary gland carcinomas), colon, urinary bladder, and other tissues (Evans and Mason, 1965; Evans and Mason, 1965; Hirono et al. 1972; Hirono et al. 1973; Saito et al. 1975; Yoshihira et al. 1978; El-Mofty et al. 1980; Hirono et al. 1984; Hirono et al. 1984b; Evans, 1986; Hirono et al. 1987; Santos et al. 1987; Smith et al. 1988; Villalobos-Salazar et al. 1995; Shahin et al. 1998; Shahin et al. 1998a; Freitas et al. 2000; Simán et al. 2000a; Silva et al. 2000; Freitas et al. 2001).

Lymphocytic leukaemia has also been reported in rats given oral or intraperitoneal doses of fresh crosier washings as well as in mice fed with spores (Evans et al. 1984; Evans, 1986; Evans and Galpin, 1990; Povey et al. 1995).

Very often, a large proportion of the laboratory animals dies during the experiments as consequence of fast developing tumours or other Bracken induced diseases (Evans and Mason, 1965; Hirono et al. 1972; Hirono et al. 1984). A single study on the effect of sun dried Bracken crosiers from Brazil (unknown variety) showed no carcinogenic effect on cancer in the urinary bladder (de Oliveira et al. 1995). This may be due to Bracken with no or low content of ptaquiloside/ptaquiloside-like substances (see Chapter 5).

Clinical symptoms have also been found in other animals than rodents used for toxicological testing, such as the Egyptian Toad, Japanese Quail, rabbits and farm animals. In fern fed rabbits, mild to moderate vascular changes were observed (Gounalan et al.

1999). Further examples of syndromes in other types of laboratory animals can be found in several papers, including review papers (El-Mofty et al. 1980; Cooper, 1980; Ushijima et al. 1983; Hirono and Yamada, 1987; Hirono, 1989; Yoshida and Saito, 1994; Yoshida and Saito, 1994a; Shahin et al. 1999).

Ptaquiloside induces chromosomal aberrations in cultured hamster lung cell lines as well as in modified Ames test on *Salmonella typhimurium* strains TA98 and TA100. The clastogenic and mutagenic effect is pH-dependent and effects can be observed after 24 hours at 4.5 mg L^{-1} at pH 7.4/8.0 at and at 400 mg L^{-1} at pH 5.3 (van der Hoeven et al. 1983; Matoba et al. 1987; Matsuoka et al. 1989; Nagao et al. 1989; Burkhalter et al. 1996). The low concentrations of ptaquiloside causing mutations at high pH compared to the high concentration at low pH are due to activation of ptaquiloside at alkaline pH (see Section 4.1). The cytotoxic effect of ptaquiloside on 3T3-cells (mouse fibroblast) and normal rat kidney cells (NRK) has been measured to 24 g L^{-1} (inhibitory concentration, 50% inhibition (IC50), 48 hours). This high concentration indicates no effect of ptaquiloside in acute diseases (e.g. Acute Bracken Poisoning). However, the result may be erroneous due to lack of activation of ptaquiloside in the test experiments (see above and Chapter 4) (Ngomuo and Jones, 1996). Ptaquiloside has a much lower IC50 concentration of 2.9 mg L^{-1} towards HL60-cells (McMorris et al. 1992). Acute toxic properties of ptaquiloside has been demonstrated in the Brine Shrimp assay (LC50 (24 hours): 15.3-62.5 mg L^{-1}; LC50 (48 hours): 3.9-7.8 mg L^{-1}) (Castillo et al. 1998; Castillo et al. 1999). For comparison, strychnine sulphate has a LC50 (48 hours) of 77 mg L^{-1} (Meyer et al. 1982). A multistage model for Bracken carcinogenesis has been proposed by Shahin et al. (1999) and is shown in Figure 3.5.

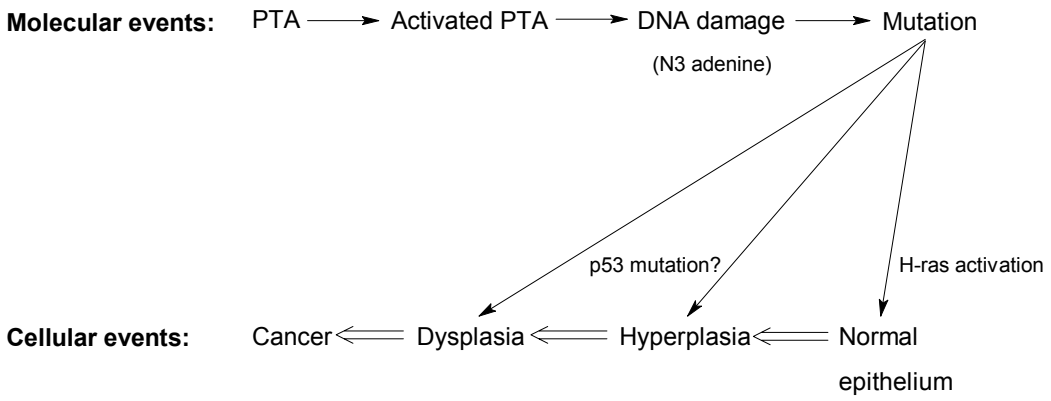


FIGURE 3.5. Multistage cancer model for Bracken carcinogenesis. PTA = ptaquiloside. Modified after Shahin et al. (1999).

Aqueous Bracken spore extracts have proved capable of damaging human cell line DNA. However, human liver enzymes are capable of detoxifying the spore extracts (Simán et al. 2000a).

Oesophageal and gastric cancer in Man.

Bracken is used for food in some parts of the world (Section 2.3) (Rymer, 1976; Haenszel et al. 1976; Hirono and Yamada, 1987; Hirono and Yamada, 1987; Fenwick, 1988; Ortega, 1994; Silva et al. 2000). In Japan and Brazil, the habit is correlated with the occurrence of oesophageal and gastric cancer (Hirayama, 1979; Marlière et al. 2000; Marlière et al. 2000a; Marlière et al. 2002). In fact, the occurrence of gastric and oesophageal cancer are between 2.1 and 8.1 times higher among people including Bracken in their daily intake *as well as* for people just living in Bracken infested areas compared when to people not having the risk behavior (Hirayama, 1979; Villalobos-Salazar, 1987; Galpin et al. 1990; Alonso-Amelot and Avendaño, 2001)!

Correlation between Bracken exposure and cancer has been demonstrated in Wales, Costa Rica, and Venezuela – areas where Bracken is not used as food (Galpin and Smith, 1986; Villalobos-Salazar, 1987; Galpin et al. 1990; Villalobos-Salazar et al. 1990; Alonso-Amelot and Avendaño, 2002). It is not finally proved that Bracken - or ptaquiloside - cause this human cancer. Contamination of water sources with Bracken leachates including ptaquiloside has been proposed as the cause. This theory might apply where shallow groundwater or surface water sources are used in the household, as the maximum *tolerable concentration* of ptaquiloside in drinking water is very low (Box 3.1 and Chapter 6) (Galpin and Smith, 1986; Galpin et al. 1990; Rasmussen et al. 2003c). Potentially carcinogenic water soluble compounds have been detected in young fronds and rhizomes (Evans et al. 1984), and recently, Rasmussen et al. (2003c) showed that ptaquiloside can be leached by rain from the fronds of var. *aquilinum*, and is found in soils below Bracken stands (see Chapter 6). Ptaquiloside is generally believed to be responsible for the carcinogenic activity of Bracken towards humans, but ptaquiloside-like substances may also contribute to the carcinogenicity of Bracken towards humans (Castillo et al. 2000).

Besides from leaching from the fern, ptaquiloside may also be transferred to the soil with urine or droppings from animals browsing on Bracken, as the urine from Bracken fed bovines is carcinogenic and therefore may contain ptaquiloside (Pamukcu et al. 1966; Smith, 1997). Another connection between humans and Bracken carcinogens is bovine milk. It has been found that up to 9% of ptaquiloside found in Bracken used to fed cows, will be excreted in their milk (Villalobos-Salazar et al. 1990; Alonso-Amelot et al. 1993;

Alonso-Amelot et al. 1996; Alonso-Amelot, 1997; Alonso-Amelot et al. 1998; Alonso-Amelot et al. 2000a; Alonso-Amelot and Avendaño, 2001): people drinking 0.5 L milk per day from cows eating subtoxic amounts of Bracken will ingest up to 13 mg of ptaquiloside per day – an amount exceeding the tolerable intake of 0.2-3.2 ng per day (Box 3.1)! Pasteurisation or boiling of milk only inactivates approx. 50-75% of the ptaquiloside (Villalobos-Salazar et al. 2000). Milk contaminated with ptaquiloside is thought to be part of the explanation for the abundance of gastric cancer in some parts of South America as high levels of ptaquiloside was found in Brackens from these areas (Villalobos-Salazar et al. 2000). The abundance of bovines with BEH and other Bracken correlated diseaces in some areas of South America and Europe may indicate potential for large-scale milk-contamination (e.g. Borzacchiello et al. (2003) - see also Section 3.1). Galpin et al. (1990) demonstrated that consumption of buttermilk by infants was associated with a increased risk of obtaining gastric cancer later in life. Ptaquiloside may also be present in meat from cows browsing on Bracken (Smith, 1997).

Box 3.1. Tolerable concentration of ptaquiloside in Danish drinking water.

Following the guidelines from the Danish Environmental Protection Agency, a tolerable concentration of a carcinogenic compound in drinking water for adults can be calculated from results obtained in carcinogenicity tests with laboratory rodents (Miljøstyrelsen, 1992). Using existing laboratory investigations, a tolerable concentration of ptaquiloside in drinking water can be calculated to (see Appendix A):

Animal:	**Mode of intake:**	**Tolerable concentration of ptaquiloside in drinking water:** ($ng\ L^{-1}$)	**Reference:**
Sprague-Dawley rats	Dried and powdered Bracken frond mixed with fodder	1.4 - 1.6	Smith et al. (1988)
ACI rats	Pure ptaquiloside mixed with fodder	0.1	Hirono et al. (1987)
Sprague-Dawley rats	Pure ptaquiloside fed intragastric	0.1 - 0.3	Hirono et al. (1984)

Burkhalter et al. (1996) proposed a human maximum intake of 700 ng per day (adult) corresponding to a safe drinking water concentration of 350 ng L^{-1} based on Hirono et al. (1984). The reason for the discrepancy between the results from Burkhalter et al. (1996) and the tolerable concentrations listed in the table is likely due to different security assessment.

Bracken spores in breathing air have also been considered a potential environmental hazard. Up to 800 spores per L of air can be encountered in sporulating Bracken stands, and up to 10 g of spores can be produced per frond (var. *arachnoideum*). Bracken spores has proven genotoxic towards human cell lines, but only minute amounts of ptaquiloside

have been found in Bracken spores (author; E. Sheffield, the University of Manchester, Manchester, England; and B. Smith, the Royal Veterinary and Agricultural University, Frederiksberg, Denmark unpublished results) (Evans and Galpin, 1990; Simán et al. 1999; Simán et al. 2000; Alonso-Amelot et al. 2001).

The development of oesophageal cancer in Man may be caused by human papilloma virus (HPV-16 and/or 18) interacting with Bracken carcinogens (ptaquiloside and/or quercetin, Figure 3.4) (Chang et al. 1990; Togawa et al. 1994; Suzuk et al. 1996; Campo et al. 2000; Saveria Campo, 2002).

Even though many indices exist for Bracken and/or ptaquiloside induced cancer among humans, the strength of the evidence must be questioned. The results of the animal tests are quite conclusive showing carcinogenicity of Bracken in general and of ptaquiloside in particular. The evidence of human cancer is less conclusive. Epidemiological studies indicate higher abundance of cancer in areas with many Brackens and/or high concentrations of ptaquiloside in the fronds, as well as case-control studies for example indicate higher cancer rates among people living in Bracken infested areas as child compared to those who did not. These retrospective studies should be treated with caution as they include very small populations and are - retrospective (Wilson et al. 1998; Brown et al. 1999). However, the work of Marlière et al. (2002) is very convincing showing a significant higher occurrence of oesophageal cancer among people eating Bracken than people not ingesting Bracken. No raised level of gastric cancer could be observed in this study.

Bracken in itself is classified as possible carcinogenic (Group 2B) by theInternational Agency for Research on Cancer (IARC - WHO), while ptaquiloside occurs on the Dutch list of cancer promoting substances (Anonymous, 2000a). The British Department of Health evaluated the carcinogenic and epidemiological evidence in 1993, and concluded that the risk to the population was very low (Anonymous, 1998).

3.2 Chemical interaction with plants, fungi, and insects.

Several studies have been performed on the allopathic potential of Bracken compounds and leachates, but the results have been quite contradictory (e.g. den Ouden (1995) and Dolling (1996)). The reason is likely different methods applied and differences in susceptibility of target species for the allelopathic substances. For example, seedlings of Scots Pine (*Pinus sylvestris* L.) and Norway Spruce (*Picea abies* (L.) Karst.) respond quite differently to

Bracken litter leachates (var. *aquilinum* and/or *latiusculum*) (Dolling, 1996), while var. *esculentum* used as mulch appears to have no phytotoxic effect at all (Taylor and Thomson, 1998). A test of the inhibitory effects of volatile Bracken constituents, fresh fronds and pinnule extracts (vars. *aquilinum* and/or *latiusculum*), showed inhibitory effects on Scots Pine and Aspen (*Populus tremula* L.). The most pronounced effects were observed in spring and autumn (Dolling et al. 1994).

The allelopathic strategy of Bracken is related to the climatic conditions of the fern (Gliessman, 1976; Gliessman and Muller, 1978; den Ouden, 1995):

- Humid areas: Allelopathic substances are released throughout the year, primarily from the fronds.
- Semiarid areas: Allelopathic substances are released from old or dead fronds at the start of the wet season.
- Temperate areas: Allelopathic substances are released from the litter, rhizomes and roots in the spring (after snow melting starts).

As mentioned earlier, the allelopathic substances may also be released from the fronds in semiarid and temperate areas in spring and autumn.

The organisation of Bracken litter - horizontal layering – can constrain root development (den Ouden and Vogels, 1997), and smothering by dying fronds and litter can kill young seedlings (Humphrey and Swaine, 1997).

Bracken is also capable of changing soil properties and soil solution chemistry hereby changing the growth conditions of other plants and of the fern it selves. Aluminium toxicity due to increased levels of aluminium in the soil solution caused by the acidifying effect of Bracken leachates has been proposed as an integral part of the ferns chemical interactions with competing plants (Johnson-Maynard et al. 1997; Johnson-Maynard et al. 1998). However, the aluminium in soil solution below Bracken stands is likely complexed by dissolved organic matter found in the leachates (Johnson-Maynard et al. 1998) (see Section 2.1).

At least 100 species of fungi have been found on dead Bracken fronds and in Bracken derived litter (e.g. *Cladosporium* sp., *Trichoderma* sp., and *Penicillium* sp. – see Frankland (1976) for further information). Only few species are pathogenic to living fronds or prothalli (Figure 2.3) (Hutchinson, 1976; Frankland, 1976).

Few insects are generally observed feeding on living or dead Bracken (e.g. Frankland (1976) and authors own experience). Toxic substances in Bracken or the occurrence of constituents with deterrent activity might be the cause. The deterrent activity of var. *aquilinum* seems to have three annual peaks (Cooper-Driver et al. 1977; Jones and Firn, 1979; Jones, 1983; Schreiner et al. 1984):

1. Early spring: Likely due to prunasin (cyanogenesis), ptaquiloside and pterosin F.
2. Autumn: Likely due to tannins.
3. Winter: Likely due to tannins and lignin.

The highly complex and variable chemical composition of Bracken might be one of the keys explaining the success of Bracken, as it hinders simple adaptation of insects to the chemical composition of the fern (see Section 3.3) (Jones, 1983). However, some authors like Jones (1983) question the general statement of few insects eating Bracken. For example, in Britain 40 species have been found feeding on var. *aquilinum*.

Most studies on Bracken allelopathy have been performed on individual compounds, but it may be necessary to investigate the potential synergetic effects of the different Bracken compounds to fully understand the allelopathic and deterrent activity of Bracken (Jones, 1983).

3.3 Toxic and allelopathic compounds in Bracken

The variation in the chemical composition of Bracken is immense - more than 100 secondary metabolites have been identified in Bracken so far (Fenwick, 1988). The complicated chemical composition may explain the worldwide distribution and success of Brackens in a wide range of ecosystems. A collection of these secondary metabolites is presented below - especially those suspected of having toxic effects towards animals.

Illudanes and related compounds.

Several compounds having an illudane skeleton are found in Bracken. The most investigated of these is the norsesquiterpene glucoside ptaquiloside (syn.: aquilide A, braxin C - Figure 3.6) (Niwa et al. 1983; Niwa et al. 1983a; van der Hoeven et al. 1983; Hirono et al. 1984). Other so-called *ptaquiloside-like compounds* in Bracken comprises: *iso*-ptaquiloside, caudatoside, and ptaquiloside Z (Figure 3.6) (Saito et al. 1990; Koyama et al. 1991; Potter and Pitman, 1994; Castillo et al. 1997; Castillo et al. 1998; Castillo et al.

2000; Potter, 2000). Ptaquiloside is the most predominant of these compounds (Alonso-Amelot and Avendaño, 2002). Ptaquiloside-like compounds are also found in other ferns from the Dennsteadtiaceae (e.g. hypacrone and hypoloside A, B and C from *Hypolepis punctata* Mett. and dennstoside A from *Dennstaedtia hirsta* Mett.) (Hayashi et al. 1977; Matsuoka et al. 1989; Saito et al. 1989; Saito et al. 1990; Koyama et al. 1991; Potter, 2000).

Ptaquiloside is acute toxic, clastogenic, mutagenic, carcinogenic and cytotoxic (van der Hoeven et al. 1983; Matsuoka et al. 1989). However, a few authors disagree (see Section 3.1: *Symptoms found in laboratory animals, bacteria and cell cultures*). Ptaquiloside will be described in detail in Chapter 4. Ptaquiloside is unstable in aqueous solution and will transform to give stable indan-1-one derivatives, such as pterosin B, the most common pterosin (at least 29 different pterosins exist in Bracken, Figure 3.6) (Saito et al. 1975; Fukuoka et al. 1978; Jones, 1983; Saito et al. 1989; Castillo et al. 1997). The high number of pterosins indicates the presence of other ptaquiloside-like compounds than those mentioned above, as pterosins are likely to have formed from ptaquiloside-like compounds. The different pterosins are present in fronds and rhizomes, most notably pterosin B. The content ranges between 20 and 2,100 μg g^{-1} in fronds and between 10 and 100 μg g^{-1} in rhizomes (var. *latiusculum*). In fronds from vars. *caudatum* and *arachnoideum* pterosin B contents between 6 and 520 μg g^{-1} and 0.1 and 100 μg g^{-1} are found respectively (Saito et al. 1989).

Several pterosins (especially pterosin F) exhibit cytotoxic effects and antibacterial activity, but these compounds are not suspected of causing cancer (Evans et al. 1983; Finkielsztein et al. 1999). Pterosin F has been found in the range 1-27 μg g^{-1} (fresh-weight, var. *aquilinum* frond, highest concentrations found in spring) (Jones and Firn, 1979). Pterosin C has an IC50 concentration of 2.7 μg mL^{-1} towards HL60-cells (McMorris et al. 1992). Pterosin B does not show any mutagenic activity, but some cytotoxic effects towards HeLa cells and toxicity towards the following soil and plant borne bacteria and fungi has been observed (minimum inhibitory concentration: 100 μg mL^{-1}): *Bacillus subtilis*, *Alternaria tenuis*, *Ceratocystis fimbriata*, *Endothia parasitica*, *Guignaria laricina*, *Rhizoctonia solani*, and *Trametes sangunea* (Kobayashi et al. 1975; Saito et al. 1975; Yoshihira et al. 1978; van der Hoeven et al. 1983; Niwa et al. 1983; Nagao et al. 1989).

Pteridanoside is a stable protoilludane sesquiterpene glycoside found in var. *caudatum*. It is acute toxic (LC50 (48 hours, Brine Shrimp assay): 63 mg L^{-1}) (Castillo et al. 1999; Castillo et al. 2000).

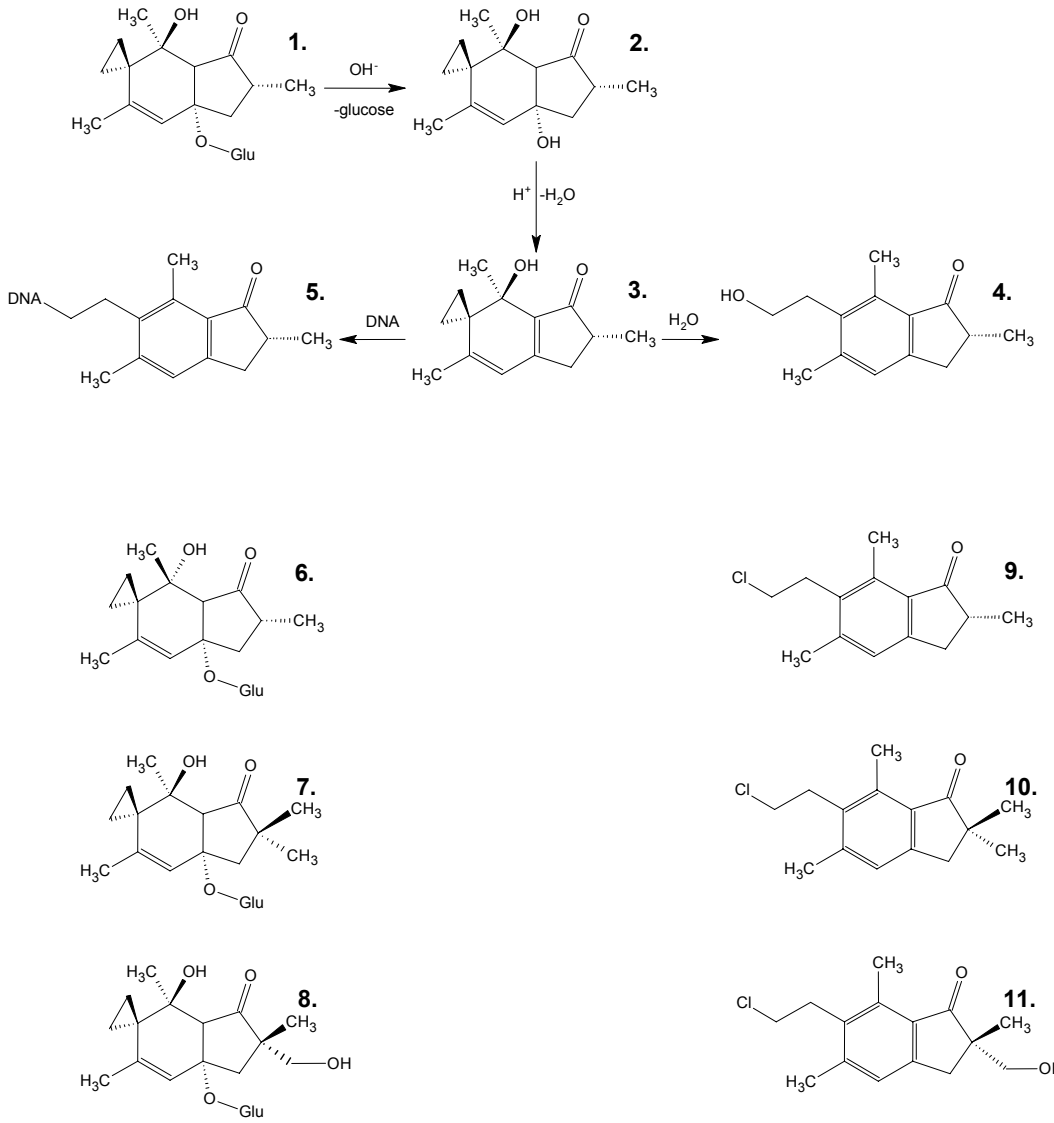


FIGURE 3.6. Ptaquiloside, ptaquiloside reactions, some chlorinated pterosins, and ptaquiloside-like compounds from Bracken. 1: Ptaquiloside. 2: Ptaquilosin. 3: Activated ptaquiloside (dienone intermediate). 4: Pterosin B (acid hydrolysis of ptaquiloside yields pterosin B as well). 5: DNA-adduct. 6: *Iso*-ptaquiloside. 7: Ptaquiloside Z (syn: methylptaquiloside). 8: Caudatoside. 9: Pterosin F. 10: Pterosin H. 11: Pterosin K. Reaction scheme partly after Shahin et al. (1999).

Pterosides are glycosides of pterosins. Four compounds have been identified in Bracken (e.g. pteroside B and braxin A2) (Fenwick, 1988; Alonso-Amelot et al. 1992). The content is in the same range as the ptaquiloside-like substances (60-1,000 µg g^{-1} in fronds, 20-80 µg g^{-1} in spores and 400-9,500 µg g^{-1} in rhizomes) (Saito et al. 1975; Fukuoka et al. 1978; Saito and Mochizuki, 1986).

Cyanogenic glycosides and potential for cyanogenesis.

Prunasin is the only cyanogenic glycoside identified in Bracken (Figure 3.7). Up to 1,600 μg g^{-1} are found in var. *aquilinum* (fronds, fresh weight) (Kofod and Eyjolfsson, 1966; Cooper-Driver and Swain, 1976). Enzymatic hydrolysis of prunasin yields HCN - up to 400 μg g-fresh-$weight^{-1}$ has been observed (pinnae tips, var. *aquilinum*). Hydrolysis of the glucose bond is necessary for the toxic expression of prunasin and the release of HCN (Majak, 2001). Not all Brackens contain the β-glycosidase enzyme necessary for release of HCN, but many rumen bacteria posses this activity making ruminants vulnerable anyway (Cooper-Driver et al. 1977; Majak, 2001). Cyanogenic genotypes have been identified in vars. *aquilinum* and *esculentum* (Cooper-Driver and Swain, 1976; Hadfield and Dyer, 1988; Low and Thomson, 1990). Prunasin may be extruded from Bracken as HCN can be found in organic soil materials inside Bracken stands at contents up to 12.2 nmol g^{-1}) (Dartnall and Burns, 1987). Prunasin may act as deterrent towards herbivores due to the potential for cyanogenesis and/or due to its bitter taste. The highest amounts of prunasin and potential for cyanogenesis are found in the spring (var. *aquilinum*) (Cooper-Driver et al. 1977; Schreiner et al. 1984).

Flavonol and other glycosides.

Several glycosides (at least 17 compounds) of quercetin, quercitrin, rhamnetin and kaempferol have been isolated from Bracken (Figure 3.7). Maximum contents are reported in the range 500-1,100 μg g^{-1} (Nakabayashi, 1955; Cooper-Driver and Swain, 1976; Bjeldanes and Chang, 1977; Fukuoka et al. 1978; Fukuoka, 1982; Tanaka et al. 1993; Imperato, 1995; Imperato, 1996; Imperato and Minutiello, 1997; Imperato, 1997; Imperato, 1998; Imperato, 1998a). The role of the flavonols is complex as the individual compounds exhibit different properties towards different insects, ranging from feeding stimulation to deterrence and growth inhibition (Jones, 1983).

Four *p*-hydroxystyrene glycosides have been identified in Bracken: *p*-hydroxystyrene β-vicianoside and ptelatosides A, B and C (Figure 3.7) (Ojika et al. 1985; Ojika et al. 1987a; Tanaka et al. 1993).

The ecdysteroid glycoside ponasteroside A (warabisterone) and (5S,6S,9S,10S)-15-hydroxycadina-3,11-dien-2-one has been identified in var. *latiusculum* (Takemoto et al. 1968; Tanaka et al. 1993).

Braxin A1 is present in rhizomes at concentrations up to 600 μg g^{-1} (Saito and Mochizuki, 1986; Saito et al. 1987; Saito et al. 1990a).

Besides from the glycosides, a range of sugars comprising: glucose, galactose, mannose, glycuronic acid, galacturonic acid, rhamnose, fucose, xylose, arabinose, and sulphoquinovose are present in Bracken (Duncan and Jarvis, 1976).

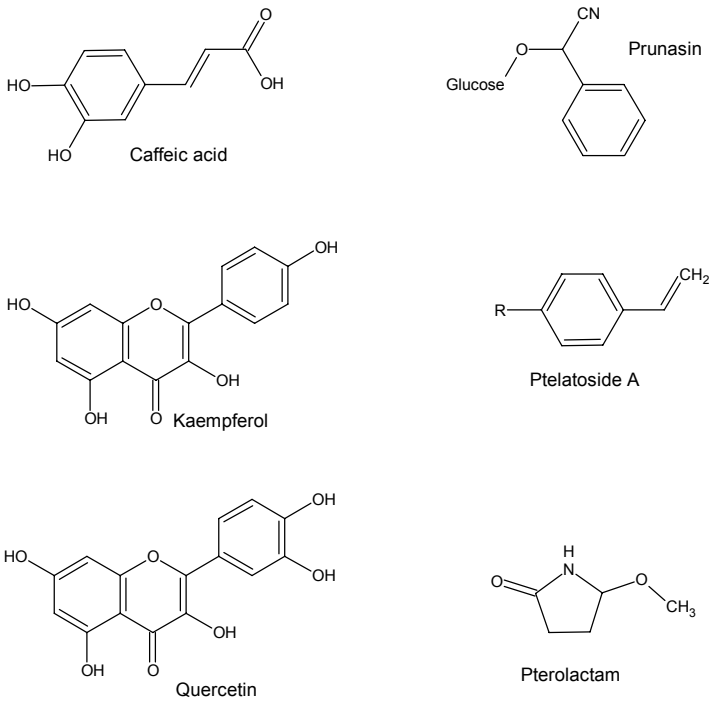


FIGURE 3.7. Overview of some secondary metabolites other than illudanes and illudane-like compounds found in Bracken. R = β-D-xylopyranosyl-β-D-glucopyranosyl.

Flavonoids.

The flavonoids kaempferol, apigenin, and quercetin are pigments producing red and yellow colours (Figure 3.7). Kaempferol and quercetin are aglycons of the corresponding flavonol glycosides. They occur in the range 10,000-25,000 μg g^{-1} – the highest contents observed in spring and early autumn. More flavonoids are produced in open land in contrast to shaded habitats (Cooper-Driver et al. 1977; Smith and Seawright, 1995; Imperato, 1997; Musonda and Chipman, 2000). The flavonoids do not have any deterrent effects towards herbivores, but may have an antimicrobial role (Jones, 1983). Quercetin has been shown to have mutagenic activity towards *Salmonella typhimurium*, carcinogenic activity towards Rats, and might be involved in the papilloma virus carcinogenesis (Figure 3.4) (Cooper-Driver et al. 1977; Pamukcu et al. 1980; Ngomuo and Jones, 1996).

Simple phenolic compounds.

The following cinnamic acid and benzoic acid derivatives has been isolated from Bracken: Benzoic acid, caffeic acid, *o*-coumaric acid, *p*-coumaric acid (*p*-hydroxycinnamic acid), ferulic acid, *p*-hydroxybenzoic acid, protocatechuric acid, vanillic acid, shikimic acid, 5-*o*-caffeolylshikimic acid, chlorogenic acid, dicaffeoyl-tartaric acid, salicylic acid, succinic acid, catecholamines, and *p*-hydroxy benzaldehyde (Figure 3.7) (Bohm and Tryon, 1967; Glass and Bohm, 1969; Somogyi, 1971; Evans, 1976; Whitehead et al. 1982; Fukuoka, 1982; Jones, 1983; Fenwick, 1988; Tanaka et al. 1993; Smith and Seawright, 1995; Alonso-Amelot et al. 1995; Anonymous, 1999; Alonso-Amelot et al. 2001). Some variation in the content of phenolic acids between varieties has been observed (e.g. between vars. *latiusculum* and *caudatum*) (Bohm and Tryon, 1967). The amounts are variable and heritage may influence the abundance of the individual compounds. Contents up to 100 μg g-C^{-1} of *p*-hydroxy benzaldehyde, *p*-coumaric, *p*-hydroxy benzoic, and vanillic acid have been found in aqueous rhizome extracts (Whitehead et al. 1982). Cinnamic acid derivatives seem to have higher deterrent activity than benzoic acid derivatives (Jones, 1983).

Other compounds.

The epicuticular wax of fronds (vars. *aquilinum* and *esculentum*) consists of 92% C_{40}-C_{50} alkyl esters, 2% C_{24}-C_{32} n-alkanols, and 2% C_{27}-C_{31} hydrocarbons (Baker and Gaskin, 1987).

At least four different phytoecdysteroids (polyhydroxy sterols - analogues of insect moulting hormones) are found in Bracken fronds and rhizomes: α-ecdysone, ecdysterone (20-hydroxyecdysone), ponasterone A, and pterosterone (Kaplanis et al. 1967; Takemoto et al. 1968; Takemoto et al. 1968a; Jones, 1983; El-Mofty et al. 1987; Svatos and Macek, 1994). The frond contents are low and peaks in late autumn (var. *aquilinum*: up to 53 μg kg^{-1} fresh-weight). Frond levels are likely too low to affect insects (Alonso-Amelot et al. 2001). The levels in rhizomes are reported to be much higher, and may occur in sufficiently high concentrations to act as deterrents towards soil-insects or nematodes (Jones, 1983).

Tannins (complex phenolic polymers with quite variable structure) are found in large amounts up to 16% (dry mass)). The total concentration of condensed tannins continues to be build up in the fronds during the growth season and may influence their digestibility. More tannins seems to be produced by Bracken growing in open land compared to shaded stands (Cooper-Driver et al. 1977; Jones, 1983; Alonso-Amelot et al. 2001). Tannins may

act as deterrents towards insects and mammalian herbivores due to their astringent taste. However, interpretations of previous investigations have been problematic (see discussion in Jones (1983)).

Pterolactam has been found in var. *latiusculum* (Figure 3.7) (Takatori et al. 1972). Coumarin occurs in the fronds of var. *caudatum* (Alonso-Amelot et al. 1995), while a range of simple aliphatic carboxylic acids and esters has been identified in frond and rhizome leachates: Propanoic acid, 2-hydroxy-methylester; 2,3-butanediol; 2(3H)-furanone-dihydro; acetic acid, methoxy-ethylester; ethanol, 2,2'-/1,2ethanediylbis(oxy)/bis-3; 3-methylbutan-2-ol; butanoic acid, 4-methoxy-methylester; butanedioic acid, monomethylester; proline, 5-oxo-methylester; cyclohexanol, 2-methyl-, trans; ethanedioic acid; 2,3-butanediol; 3-methylbutan-2-ol; butanedioic acid, monomethylester; proline, 5-oxo-methylester; and methanamine (Evans et al. 1984).

Chapter 4

PTAQUILOSIDE – A CARCINOGENIC TERPENE

4.1 Chemical and physical properties.

Ptaquiloside is the most intriguing secondary metabolite found in Bracken seen from a toxicological point of view, as ptaquiloside cause more than 50% of the carcinogenic activity of Bracken (van der Hoeven et al. 1983). Besides from Bracken, ptaquiloside is also found in the following ferns: *Cheilanthes farinosa* (Forsk.) Kaulf., *Cheilanthes sieberi* Kunze, *Dryopteris juxtaposita* Christ., *Histiopteris incisa* (Thunb.) J. Sm., *Pteris cretica* L., and *Onychium contiguum* Hope (Smith et al. 1989; Saito et al. 1989; Saito et al. 1990; Agnew and Lauren, 1991; Gounalan et al. 1999; Aswani Kumar et al. 2001). Ptaquiloside and ptaquiloside-like substances has not been found in other organisms.

The chemical name for ptaquiloside is (SciFinder Scholar, 2002):

> *Spiro[cyclopropane-1,5'-[5H]inden]-3'(2'H)-one,7'a-(β-D-glucopyranosyloxy)-1',3'a,4',7'a-tetrahydro-4'-hydroxy-2',4',6'-trimethyl-, (2'R,3'aR,4'S,7'aR)*

Ptaquiloside is a norsesquiterpene glucoside with an illudane skeleton (Figure 3.6). The biosynthetic pathway for formation of ptaquiloside is not known, but a possible route following humulene cyclization is shown in Figure 4.1 (Castillo et al. 2000). The exact location of ptaquiloside formation inside the fern is not known at present, but biosynthesis of sesquiterpenes is known to take place inside cells following either the acetat/mevalonat path or the glyceraldehyd 3-phosphate/pyruvat path (Chappell, 1995; Newman and Chappell, 1999).

The physical and chemical properties of ptaquiloside are presented in Table 4.1. Ptaquiloside is unstable in aqueous solution, as it decomposes under acid as well as

alkaline conditions due to hydrolysis. Under alkaline conditions, the reaction products are first ptaquilosin and then an unstable dienone formed after liberation of D-(+)-glucose (van der Hoeven et al. 1983; Niwa et al. 1983). Under acid conditions aromatization takes place and the reaction product is mainly pterosin B. Ptaquiloside is rather stable at neutral pH.

The speed of hydrolysis, i.e. disappearence of ptaquiloside from aqueous solutions, is temperature dependent and light sensitive, as the reaction is faster at higher temperature/exposed to light (Figure 3.6 and 4.2) (Ojika et al. 1987; Saito et al. 1989). The reaction is reported to be of second order with respect to ptaquiloside concentration and pH (Saito et al. 1989; Burkhalter et al. 1996). With respect to the stability of ptaquiloside, there is a discrepancy between the former authors (Saito et al. (1989) and Burkhalter et al. (1996) on one side and Ojika et al. (1987) on the other (Table 4.1). Saito et al. (1989) and Burkhalter et al. (1996) finds longer half-life's in their chemical assays than Ojika et al. (1987), although the reaction was running at higher temperature. This may be due to different initial concentrations as the rate of degradation of a second order reaction is dependent on concentration. However, Saito et al. (1989) and Burkhalter et al. (1996) does not report the initial concentrations used in their tests and this discrepency can therefore not be explained at the moment. The rate of reaction may also depend on how pH-adjustment is accomplished as Saito et al. (1989) used prefabricated commercial buffers while Ojika et al. (1987) used simple sulphuric acid or sodium bicarbonate-buffer.

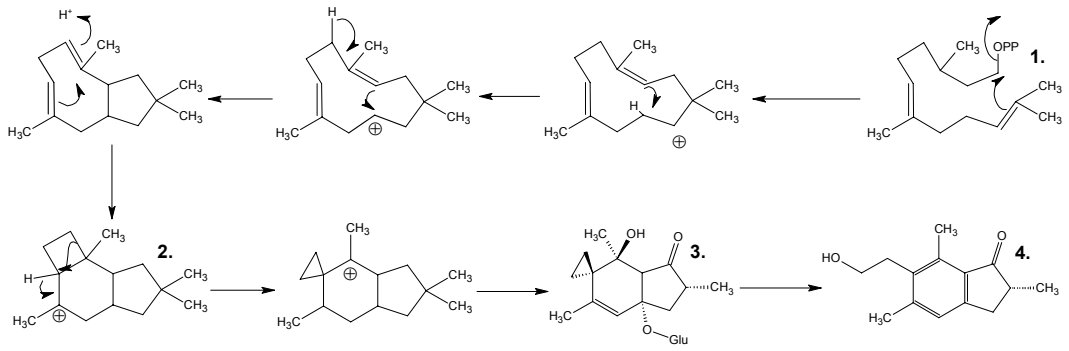


FIGURE 4.1. Proposed biosynthetic pathway for the formation of ptaquiloside in Bracken (var. *caudatum*). 1. Humulene. 2: Protoilludyl cation. 3: Ptaquiloside. 4: Pterosin B (formed after degradation of ptaquiloside). OPP: Pyrophosphate. Modified from Castillo et al. (2000).

A preliminary energy of activation of 65 kJ mol^{-1} was estimated by Burkhalter et al. (1996) for the degradation of ptaquiloside in Bracken extracts.

TABLE 4.1. Physical and chemical properties of ptaquiloside.

Property:		Reference:
Beilstein registry number	3,632,862	Anonymous (1997)
CAS registry numbers	87,625-62-5; 88,825-03-0	Anonymous (1997)
Molecular formula	$C_{20}H_{30}O_8$	
Molecular weight	398.45 g mol^{-1}	
Melting point	85-89°C (hexane, acetone)	Saito et al. (1990)
Optical rotatory power, α (*CH_3OH*-solution, at 589 nm)	-188 deg	Saito et al. (1990)
UV absorption maxima, λ nm (*Na_2CO_3*-solution)	212; 325	Saito et al. (1989)
IR absorption maxima, ν cm^{-1} (*KBr*-pellet and ATR-crystal)	3,440/3,400; 3,020; 2,970; 2,932; 2,874; 1,720/1,724; 1,643/1,640; 1,455/1,448; 1,395; 1,383; 1,279; 1,265; 1,196; 1,165/1,160; 1,080/1,074; 1,040; 933; 889	Saito et al. (1990) Ojika et al. (1987) Rasmussen et al. (2003e).
Raman scattering, ν cm^{-1} (direct measurement)	3,086; 2,975; 2,932; 2,874; 1,717; 1,642; 1,454; 1,374; 1,275; 601.	Rasmussen et al. (2003e)
Half-life, $t_{½}$ (25°C)	2.9 hours (pH 2.0, H_2SO_4) 40 min (pH 9.0) 6.3 min (pH 10.0) 69 sec (pH 11.0)	Ojika et al. (1987) All alkaline measurements were performed in a Na_2CO_3-buffer.
Half-life, $t_{½}$ (25°C)	40-50 days (near neutral pH)	Burkhalter et al. (1996)
Half-life, $t_{½}$ (37°C)	Approx. 12 min. (pH 11.5, buffer) Approx. 25 min. (pH 10,0, buffer) Approx. 8 hours (pH 8.5, buffer) Months or years (pH 7, buffer) Approx. 10 days (pH 5.5, buffer) Approx. 5 days (pH 4.0, buffer)	Saito et al. (1989)
Half-life, $t_{½}$ (in sunlight)	10 days (solid phase)	Saito et al. (1989)
Half-life, $t_{½}$ (at 60°C)	7 days (solid phase)	Saito et al. (1989)
Half-life, $t_{½}$ (75:25 water:methanol)	70-90 days (near neutral pH)	Burkhalter et al. (1996)
Rate constant, k (degradation, aqueous frond extract)	1.56 10^{-2} s^{-1} M^{-1}	Burkhalter et al. (1996)
Activation energy (ptaquiloside degradation)	65 kJ mol^{-1}	Burkhalter et al. (1996)
Partition coefficient, *log*K_{ow} (octanol-water)	-0.63	Rasmussen et al. (2003c)

[1]H and [13]C NMR spectral data can be obtained from several sources (Niwa et al. 1983; Ojika et al. 1987; Oelrichs et al. 1995; Castillo et al. 1997). Note that the half-life's mentioned in Table 4.1 are half-life's of the initial concentrations (half-life of second order reactions are concentration dependent).

The partition coefficient for the octanol-water system for ptaquiloside is -0.63 (*log*-value), indicating high solubility and strong affinity for the aqueous phase (Rasmussen et al. 2003c).

Pterosin B is stable under both acid and alkaline conditions, while ptaquilosin and the dienone only are stable under alkaline conditions (van der Hoeven et al. 1983; Niwa et al. 1983; Kigoshi et al. 1989). The partition coefficient (*log*K_{ow}) for pterosin B is 3.33 while for ptaquilosin it is 1.94 (*log*K_{ow}) (Rasmussen et al. 2003c).

4.2 Reactivity of ptaquiloside and derivatives.

Ptaquiloside is not toxic *per se,* as it has to be activated (i.e. transformed to the unstable dienone, which is believed to be the ultimate carcinogen). Activation takes place at alkaline conditions, which help explain the localization of tumours in bovines (pH(saliva): 8.1-8.2; pH(urine): 7.5-8.5) (Fenwick, 1988).

It is the electrophilic properties of the cyclopropane ring system that cause the alkylating properties of activated ptaquiloside (the dienone) (Prakash et al. 1996). The dienone reacts willingly with nucleophiles such as water, alcohols, amines, thiol and sulphide groups in amino acids, DNA etc. Some reactions are shown in Figure 4.2-4.4 (Ojika et al. 1987; Kushida et al. 1994).

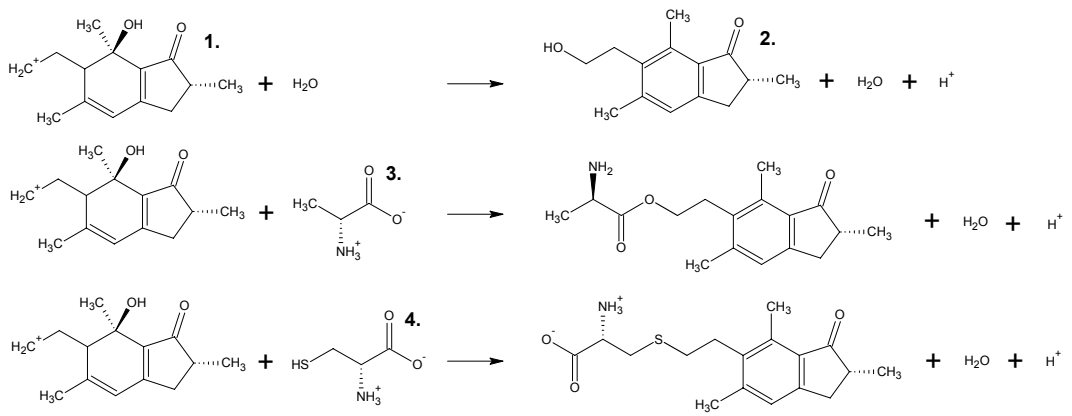


FIGURE 4.2. Examples of reactions between activated ptaquiloside (1) water, and the amino acids alanine (3) and cysteine (4). The reaction product in aqueous solutions is mainly pterosin B (2) (Ojika et al. 1987).

The dienone is capable of cleaving DNA by forming adducts through N-3 in adenine and N-7 in guanine Figure 4.4) (Ojika et al. 1987; Ojika et al. 1989; Kushida et al. 1994; Prakash et al. 1996). The DNA-cleaving/alkylating pot0ential combined with potential high biological uptake and mobility due to the glucose moiety are likely responsible for the carcinogenicity and toxicity of ptaquiloside (Prakash et al. 1996). Since water is the usual solvent in chemical analysis and in Nature, pterosin B is likely a major product of all dienone reactions (Ojika et al. 1987; Ojika et al. 1989). Ptaquilosin is reported to have the same reactivity as the dienone and ptaquilosin mutagenicity has been demonstrated (van der Hoeven et al. 1983; Kigoshi et al. 1989; Nagao et al. 1989).

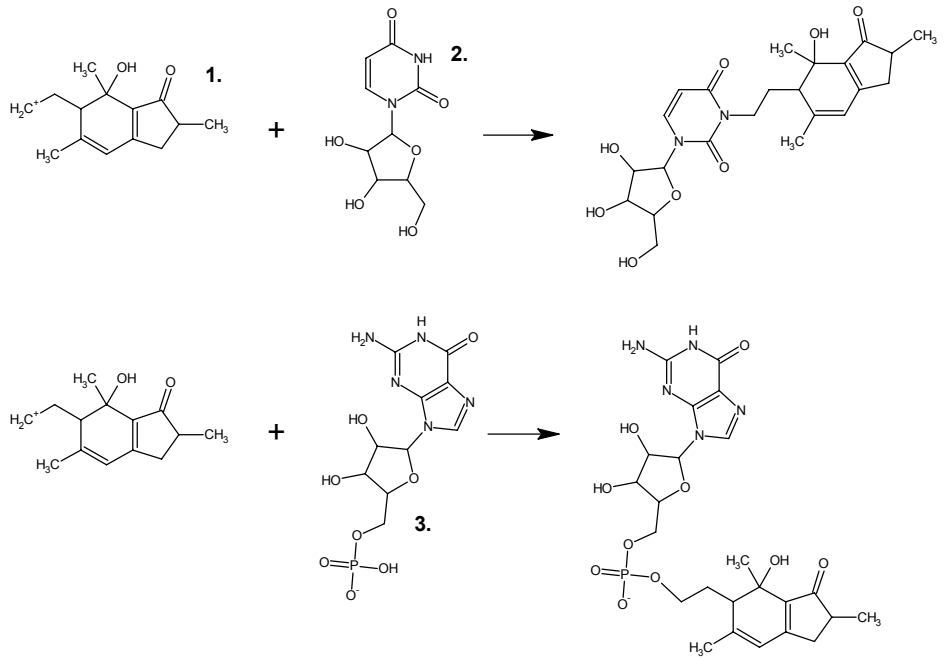


FIGURE 4.3. Selected reactions between activated ptaquiloside (1), the nucleoside uridine (2) and the nucleotide 5'-guanylic acid (Ojika et al. 1987).

4.3 Purification and determination of ptaquiloside.

No method for the synthesis of ptaquiloside has been published yet, although synthesis of ptaquilosin has been accomplished (Padwa et al. 1994; Cossy et al. 1995; Finkielsztein et al. 1999). Existing methods for purification of ptaquiloside from Bracken fern have all been quite laborious, resulting in low yields and have included use of noxious organic solvents such as chloroform (Table 4.2) (van der Hoeven et al. 1983; Niwa et al. 1983; Oelrichs et al. 1995; Burkhalter et al. 1996). However, recently Rasmussen et al. (2003e) developed a fast method with less use of organic solvents. Purification has so far been made using low-pressure chromatography on different resins, partitioning between hydrophilic-hydrophobic solvents and most often a final step of preperative HPLC (high-performance liquid chromatography, sometimes repeated 2 or 3 times with different combinations of eluents; Table 4.2).

The yield and performance of the different methods described has been quite variable, most likely as result of the varying content of ptaquiloside and other secondary metabolites in Bracken (Cooper-Driver et al. 1977; Alonso-Amelot et al. 1995; Rasmussen and Hansen, 2003; Rasmussen et al. 2003). Some other methods than those reported in Table 4.2 exist, but are mainly modifications of the methods reported here.

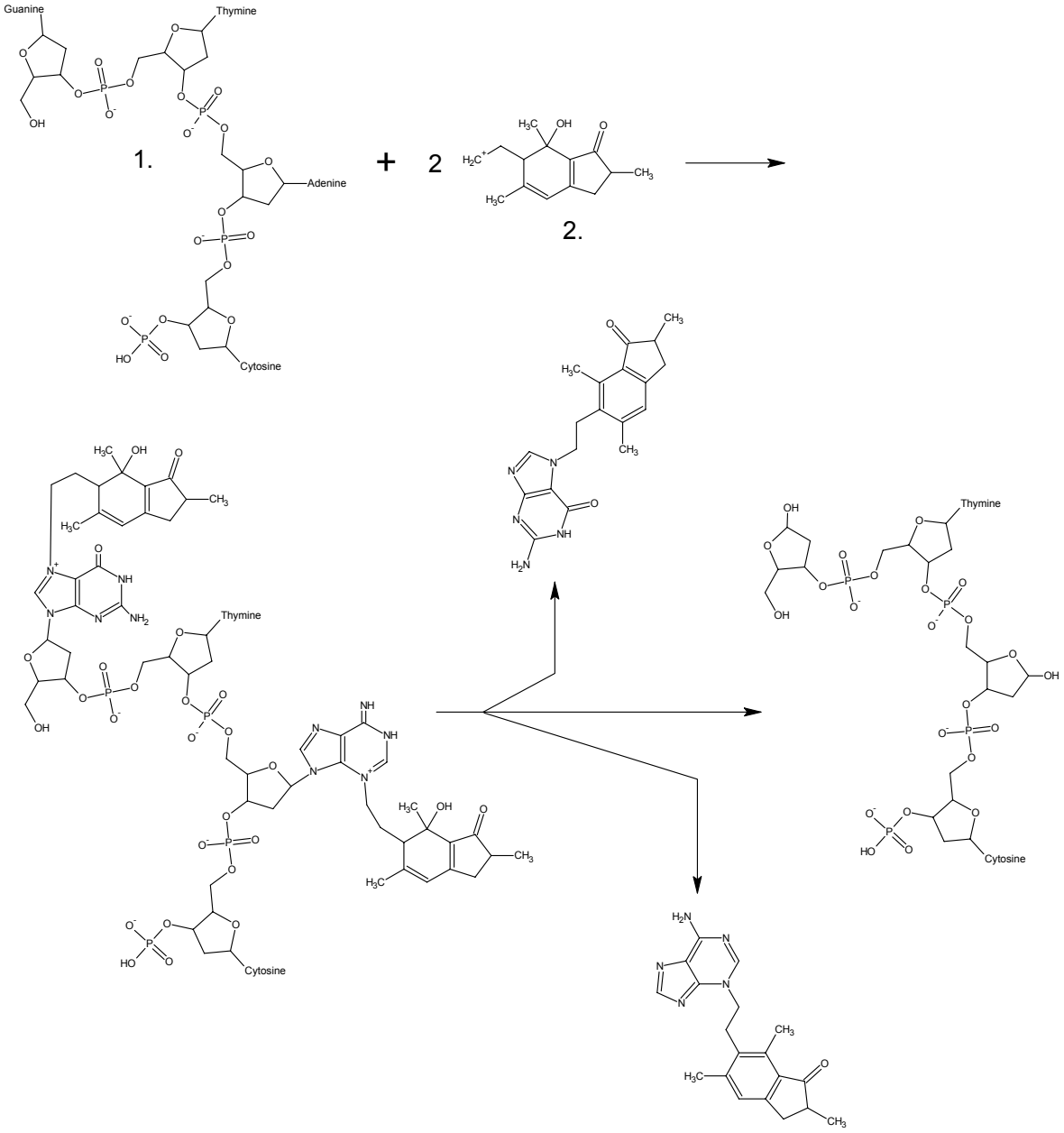


FIGURE 4.4. Reactions between deoxytetranucleotide d(GTAC) (1) and activated ptaquiloside (2). After intitial alkylation by activated ptaquiloside, cleavage of the nucleotide chain takes place (Kushida et al. 1994).

Three main methods exist for extraction and determination of ptaquiloside. The methods have been developed for determination of ptaquiloside extracted from Bracken but have since been modified for determination of ptaquiloside in other matrices such as milk and soil (Table 4.3).

TABLE 4.2. Some examples of methods for purification of ptaquiloside from Bracken fern[¶].

Reference:		Method:
van der Hoeven et al. (1983)	Sample pretreatment:	Freeze-drying.
	Extraction from fern:	Soxhlet (light petroleum and methanol).
	Purification:	Polyamide 6 S resin (eluted with water).
	Concentration:	RP18 Seppak minicolumn (released with methanol).
	Purification:	Preperative HPLC (methanol-water eluent).
Niwa et al. (1983)	Sample pretreatment:	Drying and powdering.
	Extraction from fern:	Boiling water.
	Recovery from water:	XAD-2 resin (released with methanol).
	Concentration:	Partitioning with butanol-water.
	Purification:	Silica gel (eluted with chloroform-methanol).
	Purification:	Preperative HPLC (unknown eluent).
Ojika et al. (1987)	Sample pretreatment:	Drying and powdering.
	Extraction from fern:	Water (30ºC).
	Recovery from water:	XAD-2 resin (released with methanol-water).
	Concentration:	Freexe-drying.
	Purification:	Partitioning with butanol-water.
	Purification:	Toyopearl HW-40 resin (eluted with water).
	Purification:	Silica gel (eluted with chloroform-methanol).
	Purification:	Develosil ODS 30/60 (eluted with methanol-water).
Saito et al. (1989)	Sample pretreatment:	Drying and powdering.
	Extraction from fern:	Cold water.
	Concentration:	Evaporation.
	Purification:	Silica gel (eluted with chloroform-methanol).
	Purification:	Preperative HPLC (3 times: ethyl acetate-methanol, methanol-water, and dichloro methan-methanol).
Oelrichs et al. (1995)	Sample pretreatment:	Freexe-drying and powdering.
	Extraction from fern:	Soxhlet (chloroform and methanol).
	Concentration:	Evaporation.
	Purification:	Partitioning with diethyl ether-water.
	Purification:	Polyamide 6 S resin (eluted with water).
	Recovery from water:	XAD-2 resin (released with methanol.
	Purification:	Silica gel (eluted with ethyl acetate-methanol).
	Purification:	LH-20 (eluted with methanol).
	Purification:	Preperative HPLC (dichloro methan-methanol).
Burkhalter et al. (1996)	Sample pretreatment:	Frozen ferns (liquid N_2) were grinded.
	Extraction from fern:	Cold water.
	Concentration:	Evaporation.
	Purification:	Bakerbond SPE C18 (eluted with water-methanol).
	Concentration:	Evaporation.
	Purification:	Preperative HPLC (water-methanol).
Rasmussen et al. (2003)	Sample pretreatment:	Drying and powdering.
	Extraction from fern:	Cold water.
	Purification:	Polyamide 6 resin (eluted with water).
	Recovery from water:	XAD-2 resin (released with methanol).
	Concentration:	Evaporation.
	Purification:	Polyamide 6 resin (eluted with water).
	Recovery from water:	Liquid-liquid partitioning (aqueous phase sorbed onto prepacked ChemElut diatomaceous soil columns, ptaquiloside retrieved with ethyl acetate).
	Concentration:	Evaporation.
	Purification:	Preperative HPLC (water-methanol).

[¶] Full description of materials (manufacturers etc.) can be found in the original articles.

TABLE 4.3. Some examples of methods for purification of ptaquiloside from Bracken fern[¶].

Method:	Description:	References:
Organic solvent	Water extraction of fresh fronds for 2 hours. Removal of interfering substances with acetone-dichloromethane. Prepurification on a silica gel microcolumn. Quantification of ptaquiloside after conversion to pterosin B by HPLC. The method has been modified to allow determination of ptaquiloside in milk. Recovery of ptaquiloside (standards): 94%.	Alonso-Amelot et al. (1992) Alonso-Amelot et al. (1992a) Alonso-Amelot et al. (1993)
Polyamide resin	Water extraction of dried fronds for 1 hour. Removal of interfering substances with polyamide 6 S resin. Quantification of ptaquiloside by HPLC directly or after conversion to pterosin B. The method has been modified to allow determination of ptaquiloside in soil and in fresh fronds. Recovery of ptaquiloside (standards): 95%.	Agnew and Lauren (1991) Rasmussen et al. (2003d) Rasmussen et al. (2003e)
TLC	Water extraction of freeze-dried fronds for 1.5 hour. Lyophilization of aqueous extract. Quantification of ptaquiloside by two-dimensional thin layer chromatography. Recovery of ptaquiloside: ?.	Matoba et al. (1987) Saito et al. (1989) Oelrichs et al. (1995)

[¶] Full description of materials (manufacturers etc.) can be found in the original articles.

Determination of ptaquiloside in Bracken fern involves three main steps:

1. Extraction of ptaquiloside from the fern. Due to the high hydrophilicity of ptaquiloside, water-extraction is most commonly used. The fern material can be fresh or dried. Care must be taken when drying the ferns as ptaquiloside may decompose at high temperatures (see below).

2. Removal of interfering substances from the extract. Lots of other compounds than ptaquiloside is usually found in Bracken extracts. To secure full resolution of ptaquiloside in the final step of quantification, these componds must be removed. Especially presence of pterosin B in the aqueous extract can be problematic, as ptaquiloside under some analytical conditions is converted to pterosin B before quantification. Removal of pterosin B and other compounds can be performed by partitioning the aqueous extract with a hydrophobic organic solvent as in the methods of Alonso-Amelot et al. As an alternative, pterosin B can be removed with an organic polymer resin like Polyamide 6 (Fluka, Steinheim, Switzerland).

3. Quantification of ptaquiloside. Quantification is most commonly carried out on HPLC with use of external standards. Under some circumstances, ptaquiloside is converted to pterosin B (Figure 3.6) before analysis, as pterosin B is more stable than ptaquiloside and has stronger absorption. Alternatively 2-dimensional thin layer chromatography can be applied.

No comparative study has been carried out between the different methods. When looking at the recovery of standards, there seems not to be any differences between the resin and organic solvent methods. However, extraction time and sample pre-treatment may be essential for the performance of the methods. Agnew and Lauren (1991) and Burkhalter et al. (1996) found that the amount of ptaquiloside extracted decresed as time of extraction increased. Hence, extraction time should not exceed 1 hour. Drying may also decrease the amount of ptaquiloside extracted, as the compound is heat-sensitive (Saito et al. 1989).

4.4 The role of ptaquiloside in the fern.

The role of ptaquiloside in Bracken is not quite clear, but ptaquiloside might act as deterrent towards recent or past herbivores (Alonso-Amelot et al. 1992). This hypothesis is supported by the observation that only few insects feed on Bracken, and that no herbivores eat large quantities of Bracken when enough food is available. Bracken crosiers are also very delicate, fragile and sensitive to damage and attacks, and the high ptaquiloside content often observed in young fronds might be an adaptation to prevent attacks from herbivores in this sensitive stage (see Section 5.1) (Alonso-Amelot et al. 1992). However, the *protection* of the fern could just as well be carried out by other secondary metabolites than ptaquiloside. The timespan from ingestion of ptaquiloside by larger herbivores until they become sick is also rather long (days to years). As ptaquiloside does not show any immediate effect, this compound alone will probably not deter such animals. Prunasin or tannins are likely much more efficient in deterring larger herbivores (Cooper-Driver et al. 1977). Ptaquiloside and ptaquiloside-like compounds might instead serve as mobile precursors for the less hydrophilic compounds: pterosin B and F, hereby enabling the fern to allocate these compounds to points of attack. These pterosins are both reported of having some deterrent and/or antimicrobial effect (see Section 3.3) (Alonso-Amelot et al. 1992; Alonso-Amelot et al. 1995).

Another explanation for the occurrence of ptaquiloside that has been proposed states, that it is not Bracken itself that produces ptaquiloside, but a symbiotic endomychorrhizal fungus instead. Due to the close association between fungi and fern in mycorrhizal networks, the

fungi could introduce ptaquiloside into the vascular system of the fern (Schoental, 1984). This hypothesis is partly supported by the observation that some compounds very similar in structure to ptaquiloside as well as some pterosins have been identified in basidiomycetes (e.g. illudin M and S from *Omphalotus illudens*/*olearis* and pterosins in *Fomes annosus* and *Cyathus bulleri*) (McCloud et al. 1996; Potter, 2000; Rasmussen et al. 2003d). However, no solid evidence for the fungal infection theory exists at present. Hence, the origin and exact role of ptaquiloside in Bracken remains still to be explained.

Chapter 5

PTAQUILOSIDE IN BRACKEN

5.1 Ptaquiloside content in the frond

The content of ptaquiloside in the fronds of different varieties of Bracken ranges between 0 and 15,100 μg g^{-1} (Table 5.1). There seems to be no significant differences between the varieties (Smith et al. 1994). However, genetic heritage may influence the ptaquiloside content as different proveniences still have different ptaquiloside contents when grown under the same conditions (Smith et al. 1988; Smith et al. 1992; Smith et al. 1994; Rasmussen et al. 2003d). For example, Smith et al. (1984) found that var. *esculentum* from the southern part of Australia had higher ptaquiloside contents than ferns from the northern part, even when grown under uniform conditions. Significant differences were also observed between var. *esculentum* from Australia and New Zealand in that study. Some proveniences from vars. *esculentum* and *revolutum* do not contain ptaquiloside at all, indicating the existence of ptaquiloside chemotypes (Smith et al. 1994; Rasmussen et al. 2003d). The existence of ptaquiloside chemotypes in other Bracken varieties is yet to be unveiled. This variation is discussed further in Sections 5.3-6.

The ptaquiloside content in Bracken fronds from proveniences with ptaquiloside is quite variable. The content in fronds with ptaquiloside is usually at maximum just after the crosiers emerge from the ground. The content then decreases gradually during the growth season - but never reach zero. For example, vars. *caudatum* and *arachnoideum* both have maximum ptaquiloside contents in the crosier-stage, whereafter the content decreases rapidly to 10% of the maximum content 12-20 days after crosier emergence. The distribution of ptaquiloside seems to be constant between different pinnae in these varieties when the fronds are 42-45 days old (85% mature) (Alonso-Amelot et al. 1992; Alonso-Amelot et al. 1995). The annual variation in vars. *aquilinum* and *esculentum* from New Zealand, Scotland and Denmark do also follow this pattern, although not as marked

TABLE 5.1. Ptaquiloside contents in different Bracken varieties.

Bracken variety:		Ptaquiloside content: (µg g-drymatter^{-1})		Reference:
Pteridium aquilinum ssp. *aquilinum*	var. *aquilinum*	210-3,140	C[¶]	Burkhalter et al. (1996)
		60-9,780	Y	Ghergariu et al. (2000)
		110-4,450	M	Pinto et al. (1999)
				Rasmussen and Hansen (2003)
				Rasmussen et al. 2003)
				Rasmussen et al. 2003a)
				Rasmussen et al. 2003b)
				Smith et al. (1994)
	var. *decompositum*	960	Y	Smith et al. (1994)
	var. *latiusculum*	40-1,830	C/Y	Gounalan et al. (1999)
		5-1,200	M	Saito et al. (1989)
				Smith et al. (1994)
	var. *pseudocaudatum*	50	Y	Smith et al. (1994)
	var. *revolutum*	0-1,340	Y	Smith et al. (1994)
Pteridium aquilinum ssp. *caudatum*				
	var. *arachnoideum*	30-15,100	C	Alonso-Amelot et al. (1995)
		0[§]-2,130	Y	Smith et al. (1994)
		0[§]-590	M	Villalobos-Salazar et al. (2000)
	var. *caudatum*	1,880-6,820	C	Alonso-Amelot et al. (1992)
		10-1,850	Y	Alonso-Amelot et al. (1992a)
		110-1,960	M	Alonso-Amelot et al. (1995)
				Alonso-Amelot et al. (2000)
				Smith et al. (1994)
				Villalobos-Salazar et al. (2000)
	var. *esculentum*	700-7,000	C	Smith et al. (1988)
		0-13,260	Y	Smith et al. (1990)
		0-8,580	M	Smith et al. (1993)
				Smith et al. (1994)
				Rasmussen et al. (2003d)
	var. *yarrabense*	50-1,330	Y	Smith et al. (1994)

[¶] C: Crosiers; Y: Young developing fronds; M: Mature fronds. [§] Trace amounts (Alonso-Amelot et al. 1995).

(Figure 5.1 and 5.2) (Smith et al. 1990; Rasmussen and Hansen, 2003; Rasmussen et al. 2003). A Japanese survey of var. *latiusculum* fronds showed no marked annual variation, except from a single stand with a shorter summer season compared to the other sites (Saito et al. 1989).

Ptaquiloside is quite stable inside dead fronds exposed to sunlight, but protected from rain. An initial half-life of 3 weeks was observed for var. *latiusculum* fronds kept that way, but the degradation rate seemed to decline with time (Saito et al. 1989).

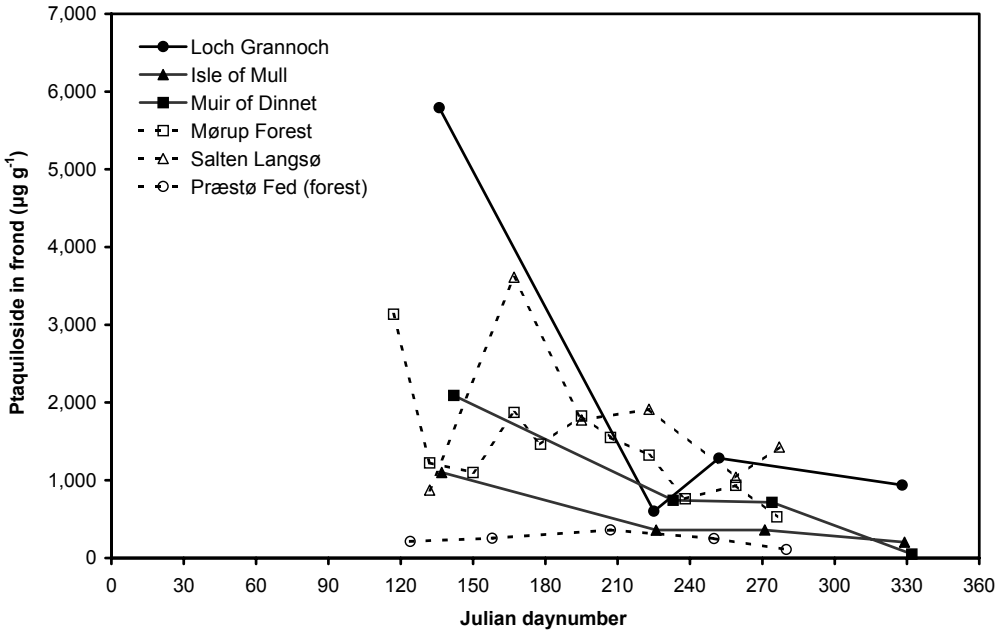


FIGURE 5.1. Ptaquiloside contents in var. *aquilinum* fronds in Denmark (open symbols) and Scotland (filled symbols). Data from Rasmussen and Hansen (2003) and Rasmussen et al. (2003).

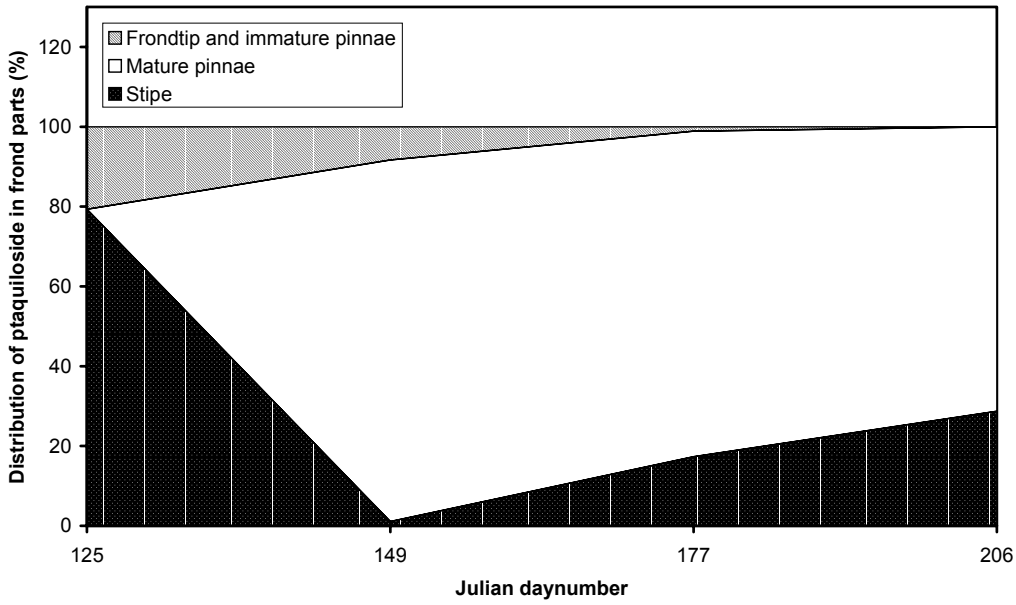


FIGURE 5.2. Ptaquiloside contents in different frond parts of var. *aquilinum* in Denmark. The different frond parts from Denmark were collected at Mørup Forest and Salten Langsø in 2000. The material was analysed as described in Rasmussen and Hansen (2003). Author, unpublished results.

Ptaquiloside load in the standing biomass.

The ptaquiloside load is the total amount found in the fronds per m^2. This is the maximum amount of ptaquiloside that can be transferred to the litter-layer when the fronds die naturally or after mowing. It is also the maximum amount that can be ingested by grazing animals or become leached from fronds by rainwater. The annual variation in the ptaquiloside load is shown in Figure 5.3 for 3 Danish sites. The maximum ptaquiloside load is found when the fronds mature in late summer. The load then declines gradually – maybe as a function of rhizome replenishment with carbohydrates and nutrients – an increase in the content of ptaquiloside in the rhizomes has been observed in the autumn (Rasmussen and Hansen, 2003). When compared to Figure 5.1, the differences in ptaquiloside contents between the Danish sites have diminished and the time of maximum content has changed somewhat from spring to late summer. This indicates more constant amounts of ptaquiloside found inside mature and senescent Bracken stands in contrast to what could be believed based on measurements of the ptaquiloside contents alone. This observation indicates rather constant ptaquiloside levels per m^2 and that the amount of ptaquiloside is *diluted* into the biomass. A survey of 20 Danish Bracken sites in autumn showed that the ptaquiloside load ranged from 10 to 300 mg m^{-2} (Figure 5.3 and Rasmussen et al. 2003b).

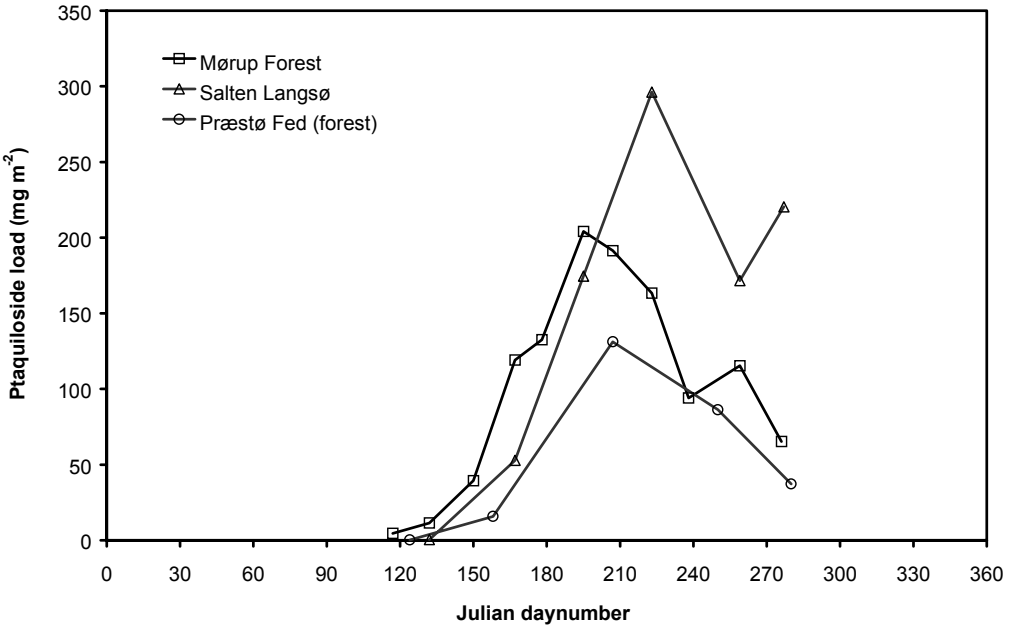


FIGURE 5.3. Ptaquiloside load in the standing biomass (var. *aquilinum*, Denmark). The figure is drawn based on data from Rasmussen and Hansen (2003) and Rasmussen et al. (2003b).

5.2 Ptaquiloside content in rhizomes, roots and spores.

Until recently, rhizome studies have only been carried out during the growth season on vars. *aquilinum*, *latiusculum*, *caudatum* and *arachnoideum*. The results indicated relatively low contents in the rhizomes (<1,200 μg g^{-1}) with the highest concentrations in vars. *aquilinum* and *latiusculum* (Saito et al. 1989; Alonso-Amelot et al. 1992; Alonso-Amelot et al. 1992a; Ghergariu et al. 2000; Rasmussen et al. 2003)). Recently, Rasmussen and Hansen (2003) and Ramussen et al. (2003) found drastic annual variations in the ptaquiloside content in the storage and the frond bearing rhizomes of var. *aquilinum* (2-7,050 μg g^{-1}). The lowest contents were measured during the frond growth season, and the highest concentrations just after fronds had reached maturity (Figure 5.4). Raised levels were also found in late winter/early spring. Storage rhizomes had in general a higher content of ptaquiloside than frond bearing rhizomes. The annual variation in the rhizome content were correlated with the phase of carbohydrate regeneration of the rhizome-system in autumn, and the time of rhizome- and belowground shoot-elongation as well as bud-growth in early spring (Williams and Foley, 1976; Ershova, 2000; Rasmussen and Hansen, 2003).

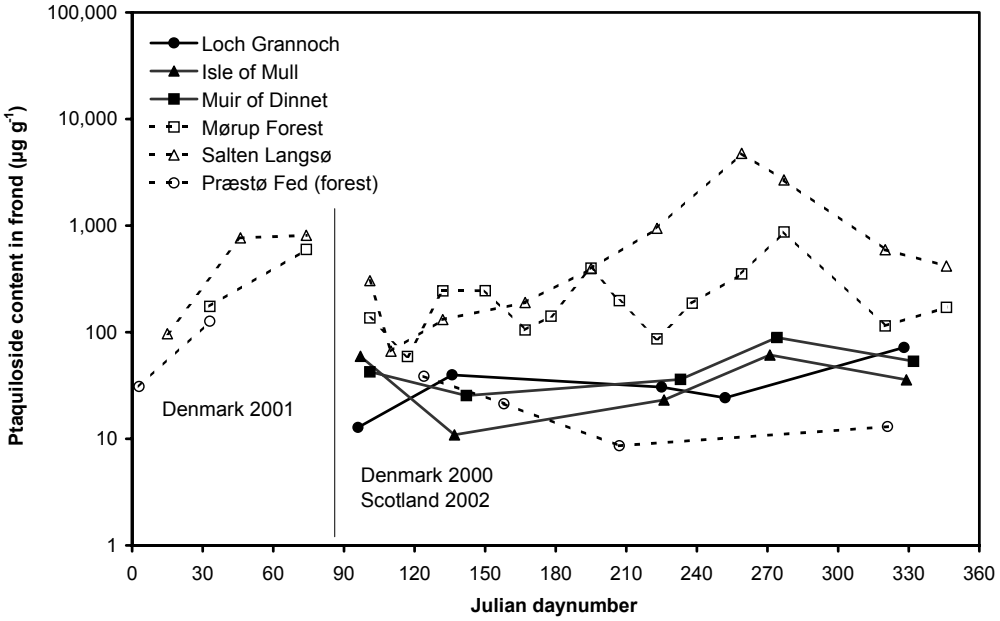


FIGURE 5.4. Ptaquiloside content in rhizomes, var. *aquilinum*, in Denmark (open symbols) and in Scotland (filled symbols). The figure is made from data in Rasmussen and Hansen (2003) and Rasmussen et al. (2003). Note logarithmic scale on abscissa.

The content of ptaquiloside in var. *aquilinum* rhizomes collected in the autumn in Scotland has been found to correlate with longitude, topography and the content of soil nitrogen. Especially nitrogen had strong negative influence on the ptaquiloside content in the rhizomes, indicating higher levels of ptaquiloside in rhizomes from Bracken growing nitrogen-depleted soils (Rasmussen et al. 2003). For further discussion of ptaquiloside variation, see Section 5.6.

Only a single study of the ptaquiloside content in roots has been carried out (var. *aquilinum*) (Rasmussen and Hansen, 2003). The content was very low compared to other parts of the fern, and was found to be highest in the growth season (5-230 μg g^{-1}, Figure 5.5).

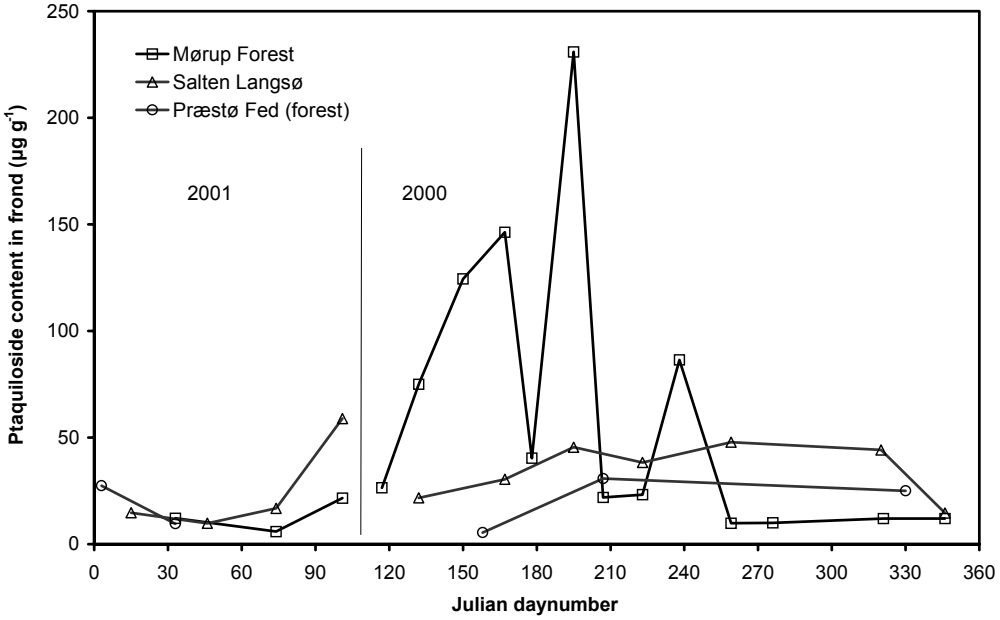


FIGURE 5.5. Ptaquiloside content in roots, var. *aquilinum* (Denmark). The content was only measured in duplicate in 48% of the samples due to limited sample amount. Median coefficient of variation = 10.5%. Analytical method from Rasmussen and Hansen (2003). Author, unpublished results.

The annual variation of the ptaquiloside content in rhizome buds is shown in Figure 5.6. The content was in the range 25 to 1,080 μg g^{-1}. The content seems to increase during the growth season. Rhizome growth takes place in late summer and autumn, and the increase in the ptaquiloside content might be a mirror of that.

Large amounts of ptaquiloside are not found in Bracken spores in contrast to what has been expected due to their genotoxic/carcinogenic effects (Matoba et al. 1987; Saito et al. 1989; Evans and Galpin, 1990; Freitas et al. 2001). Recent anayses of ptaquiloside in spores (var.

aquilinum) showed that the content in spores was less than 20 µg g^{-1} (author; E. Sheffield, the University of Manchester, Manchester, England; and B. Smith, the Royal Veterinary and Agricultural University, Frederiksberg, Denmark unpublished results).

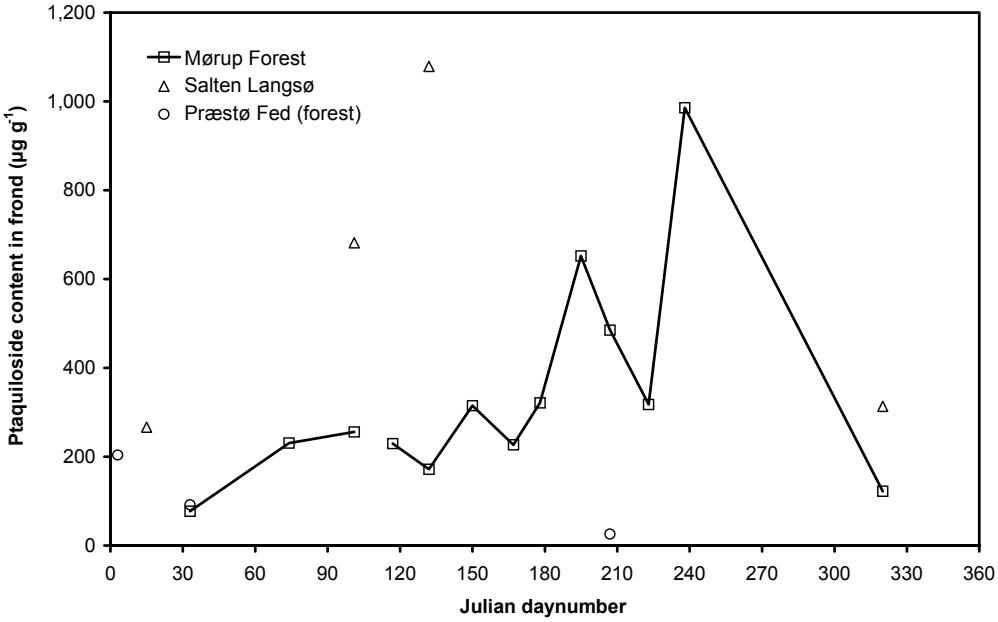


FIGURE 5.6. Ptaquiloside content in rhizome buds, var. *aquilinum* (Denmark). The content was only measured in duplicate in 50% of the samples due to limited sample amount. Median coefficient of variation = 9.0%. Analytical method from Rasmussen and Hansen (2003). Author, unpublished results.

5.3 Daily variation in the ptaquiloside content in fronds.

The content of secondary metabolites is known to be closely linked to the metabolic activity of plants (Kaufman et al. 1999). The daily variation of the ptaquiloside content in Bracken fronds was therefore investigated by Johansen (2003) (Figure 5.7). No significant differences were found between the different times of sampling. At the first time of sampling (Figure 5.7A, average ptaquiloside content: 2,900±800 µg g^{-1}), no increasing or decreasing trend could be observed in the ptaquiloside content during 24 hours, while the ptaquiloside content increased during the second sampling period (Figure 5.7B, average ptaquiloside content: 1,900±730 µg g^{-1}). Weather conditions were similar at the two times of sampling (night temperature: 10-12ºC; day temperature: 20-22ºC; no precipitation). This indicates no influence of the metabolic activity of the fern on the ptaquiloside content in the fronds. A decrease in the ptaquiloside content was found between the two sampling

times in accordance with previously observed ptaquiloside variation in Danish Bracken fronds with respect to age of the fronds (Rasmussen and Hansen, 2003).

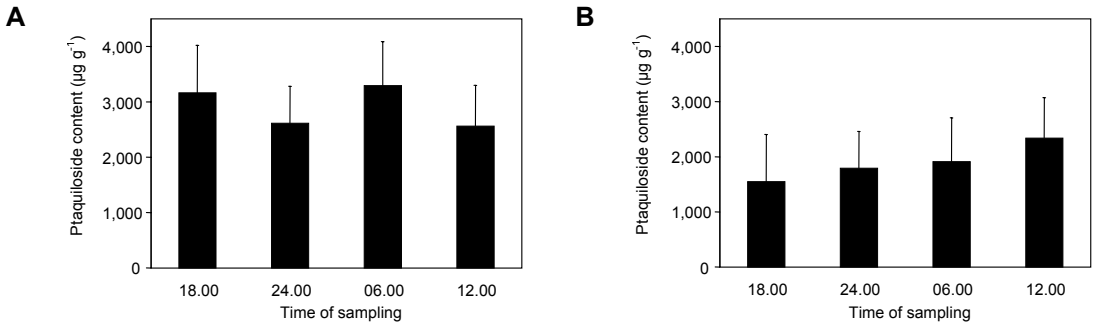


FIGURE 5.7. Daily variation in the ptaquiloside content of almost mature Bracken fronds (var. *aquilinum*). A: 5-6 July 2002. B: 20-21 July 2002. 20-25 randomly sampled replicates at each time of sampling, standard deviations shown. The stand was situated at Præstø Fed, Denmark below a stand of Oak (*Quercus robur* L.). Analytical conditions as in Rasmussen et al. (2003). Unpublished data by Johansen (2003).

5.4 Variation in the ptaquiloside content between fronds inside a stand.

In Figure 5.8, the measurements presented in Figure 5.7 are mapped in the grid used for sampling. No correction has been made for the lower level of ptaquiloside in the fronds collected the second time of sampling. The content ranges from 440 to 5,080 µg g^{-1} with most fronds having contents between 2,000 and 3,999 µg g^{-1}. A few fronds had very high or low ptaquiloside contents (<1,000 and >3,999 µg g^{-1}). These extreme contents were apparently not linked to easily recognizable factors such as height, health status or growth stage. According to Figure 5.8 there seems not to be any pattern in the distribution of ptaquiloside in fronds inside the investigated Bracken stand, i.e. the content of ptaquiloside inside a stand seems to be more or less randomly distributed within a certain concentration range. However, some grouping of samples with extreme ptaquiloside contents could be observed.

The observed pattern of variation in ptaquiloside contents is rather similar to previous observations of cyanogenic polymorphism in Bracken stands (var. *aquilinum*) (Cooper-Driver and Swain, 1976). This polymorphism may be caused by either the possible formation of genetically different sporophytes from the same spore or that several genotypes are present in the stand originating from different spores (Jones, 1983; Wolf et al. 1988; Wolf et al. 1988a; Sheffield et al. 1989; Wolf et al. 1990).

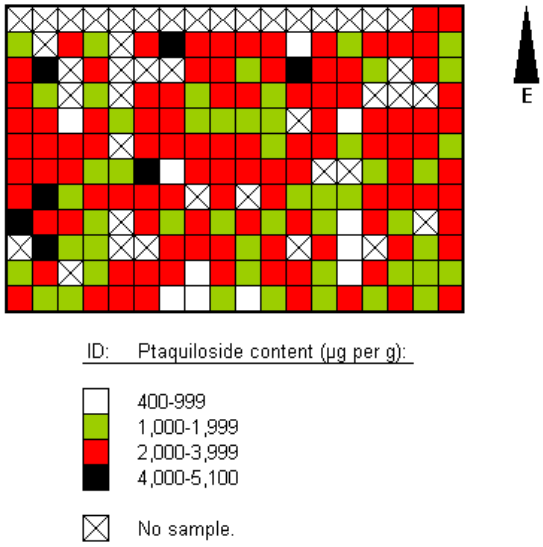


FIGURE 5.8. Variation in the ptaquiloside content between individual Bracken fronds (5-21 July 2003, var. *aquilinum*). One frond was sampled from the central part of each square (2x2 m). Details as in Figure 5.7 (Johansen, 2003).

5.5 Small-scale geographical variation in the ptaquiloside content of Bracken.

Several investigations of the local variation have been carried out on vars. *aquilinum*, *esculentum*, *caudatum*, and *arachnoideum* (Alonso-Amelot et al. 1992; Alonso-Amelot et al. 1995; Rasmussen et al. 2003a; Rasmussen et al. 2003d). The content seems to be quite variable. In a survey of mature var. *aquilinum* fronds, Rasmussen et al. (2003a) found contents between 210 and 2,150 µg g^{-1} in 12 stands located at 4 geographically distinct locations. Significant differences were found between the locations. A detailed survey of the ptaquiloside content in the fronds of young var. *aquilinum* at Præstø Fed in Denmark did also exhibit quite large variation (Figure 5.8). The same kind of variation was also observed between young var. *esculentum* fronds at a farm in New Zealand (Rasmussen et al. 2003d). In the latter study, stands without any ptaquiloside at all were also found! The variation in the ptaquiloside content in the fronds with ptaquiloside (var. *esculentum*) was positively correlated with the frond development stage (number of pinnae) and the altitude of the stand. However, the model could explain only 76% of the data. The geographical variation of the ptaquiloside content in fronds will be discussed further in Section 5.6) (Cooper-Driver and Swain, 1976; Cooper-Driver et al. 1977; Jones, 1983).

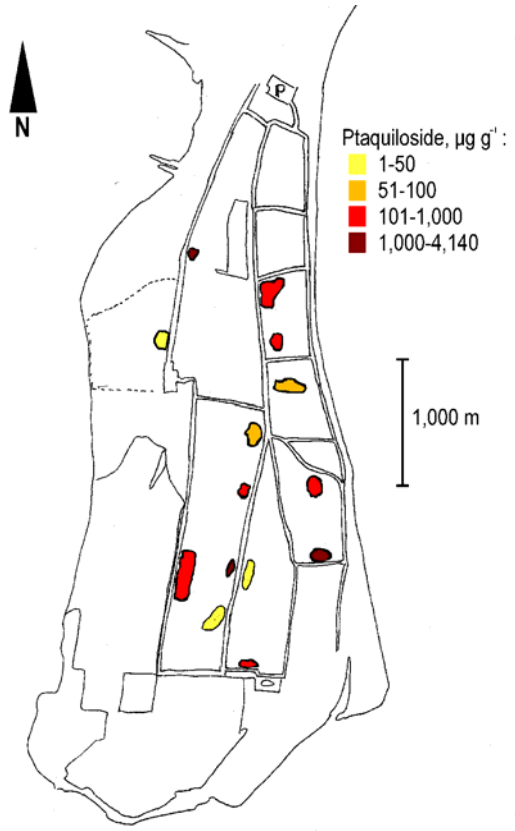


FIGURE 5.9. Local variation in the ptaquiloside content in Bracken fronds (var. *aquilinum*), Præstø Fed, Denmark. Analytical method as in Rasmussen et al. (2003). Author, unpublished results.

5.6 Large-scale geographical variation in the ptaquiloside content in Bracken fronds.

Large-scale variation in the concentration of terpenoic constituents of higher plants is generally believed to occur in response to variations in genetic, biochemical and ecological conditions. For example, genetic mutations can affect biosynthesis of secondary metabolites; mixtures of terpenes may result of the ability of some plant enzymes to produce different compounds; and the complex composition of the mixture of secondary metabolites may have evolved in response to herbivore pressure (Langenheim, 1994). Besides from these biotic causes of natural variation in the content of secondary metabolites, abiotic factors may influence the levels found as well. According to Gershenzon and Croteau (1991, cited from Langenheim (1994)), the content of terpenes generally increases with increased light intensity and decreases with increased abundance of nitrogen, phosphate and potassium. However, the abundance of secondary metabolites is

quite complex due to the interaction between abiotic and biotic factors (Langenheim, 1995; Kaufman et al. 1999).

Several studies have been carried out to monitor the large-scale geographical variation in the ptaquiloside content of Bracken fronds. In New Zealand and Australia, large geographical surveys comprising hundreds of var. *esculentum* stands have been carried out while geographically less extensive studies have been performed in Costa Rica and Venezuela (vars. *arachnoideum* and *caudatum*) (Smith et al. 1994; Alonso-Amelot et al. 1995; Alonso-Amelot et al. 2000; Villalobos-Salazar et al. 2000; Rasmussen et al. 2003d).

The ptaquiloside content seems to be correlated with altitude for stands in South America (vars. *arachnoideum* and *caudatum*) (Alonso-Amelot et al. 1995; Alonso-Amelot et al. 2000; Villalobos-Salazar et al. 2000; Alonso-Amelot et al. 2001). However, the correlation is complex since Villalobos-Salazar et al. (2000) found a positive correlation between altitude and ptaquiloside content for vars. *arachnoideum* and *caudatum*, while Alonso-Amelot et al. (1995) found a negative correlation for var. *caudatum*. The South American studies did only include few Bracken stands and the observed variation may therefore not be statistically significant. Similar confusing results were obtained in New Zealand (var. *esculentum*), where the ptaquiloside content was positively correlated with altitude in a confined region. However, negative effect of altitude was found in a larger study comprising Bracken stands from all over New Zealand (Rasmussen et al. 2003d). Alonso-Amelot et al. (2000) suggested that the observed decrease in ptaquiloside content with height may be due to lower temperatures at higher altitudes imposing restrictions in the metabolic rate and production of secondary metabolites. As evidence for this hypothesis, Alonso-Amelot et al. (2001) found increased levels of ptaquiloside in mountain-rhizomes moved from high altitudes to lower altitudes. The ptaquiloside production by these rhizomes rose to levels comparable to surrounding low-altitude Brackens. Other edaphic variables such as soil drainage, soil composition and precipitation may also explain this variation, as they are known to vary as result of altitude (Jenny, 1980). Further studies are needed to fully explain the effect of altitude on the ptaquiloside content in fronds.

In Australia and in New Zealand, var. *esculentum* from southern latitudes had higher ptaquiloside contents than ferns originating from northern latitudes for Brackens having more or less the same growth stage (Smith et al. 1994; Rasmussen et al. 2003d). The effect of latitude may be due to differences in climate on a North-South gradient, but latitude may also be a proxy variable for some other hidden variable, e.g. parent material and hence soil properties such as content of available nutrients. A similar effect of longitude was also

observed in Scotland (var. aquilinum) (Rasmussen et al. 2003). In this study, correlation with aspect was also observed, showing higher ptaquiloside contents in stands with a southwestern aspect.

Looking more into the direct acting edaphic conditions, then the following factors have been found to correlate positively with the ptaquiloside content in var. *aquilinum* fronds from Denmark: Shade, lack of water holding capacity of the soil and low amounts of available nutrients in the soil (Smith et al. 1994; Villalobos-Salazar et al. 2000; Rasmussen et al. 2003b). All three variables indicate that edaphic stress may directly trigger ptaquiloside production. However, Alonso-Amelot et al. (2001) found a 100-fold increse in the ptaquiloside content of var. *caudatum* fronds following a fire that destroyed the surface vegetation - a situation usually associated with increased availability of nutrients. The raised ptaquiloside-level could although also be caused by higher ptaquiloside contents in second growth fronds (see below). A more subtle indirect effect of the edaphic conditions cannot be ruled out, as climatic stress is known to increase toxin concentrations in many plants (Ralphs, 2002). The effect of shading has also been observed in var. *esculentum* growing under controlled circumstances (Smith et al. 1990). Smith et al (1990) investigated the effect of soil substrate, and found that Brackens grown artificially in soil originating from Bracken stands had lower ptaquiloside contents than Brackens grown in sterilized potting soil. The authors did not conclude on this, but the stress hypothesis might be used to explain the observations, as the sterilized soil might lack the soil micro flora necessary for Bracken vigour and establishment of mycorrhizal systems. Hence, Brackens grown in sterilized soil may not receive the necessary nutrients resulting in increased ptaquiloside contents in the fronds.

Another stress factor known to influence the amount of ptaquiloside found in Bracken stands is physiological stress caused by mowing or grazing. Higher contents have been found in fronds of vars. *esculentum* and *aquilinum* from stands subjected to mowing or grazing compared to undamaged stands (Figure 5.10) (Smith et al. 1994; Rasmussen et al. 2003). This indicates that higher ptaquiloside contents occur in second growth fronds compared to first growth and/or that higher contents are present in damaged fronds. The high contents in ferns collected from stands subjected to browsing or mowing may also be due to the lower growth stage of the actually collected Brackens representing regrowth of new fronds after frond damage (Rasmussen et al. 2003). The physiological response to damage inside a frond needs further investigation.

Genetic variation may also contribute significantly to the observed variations in the ptaquiloside content. Smith et al. (1990 and 1994) found that the primary reason for the differing content of ptaquiloside in fronds was *origin*, i.e. provenience. Bracken from different locations all over the world were grown under controlled environments, and the observed variation in the ptaquiloside content could be tracked to the origin of the Bracken material. However, the study of Smith et al. (1990) was problematic since marked morphological differences were observed between the different proveniences.

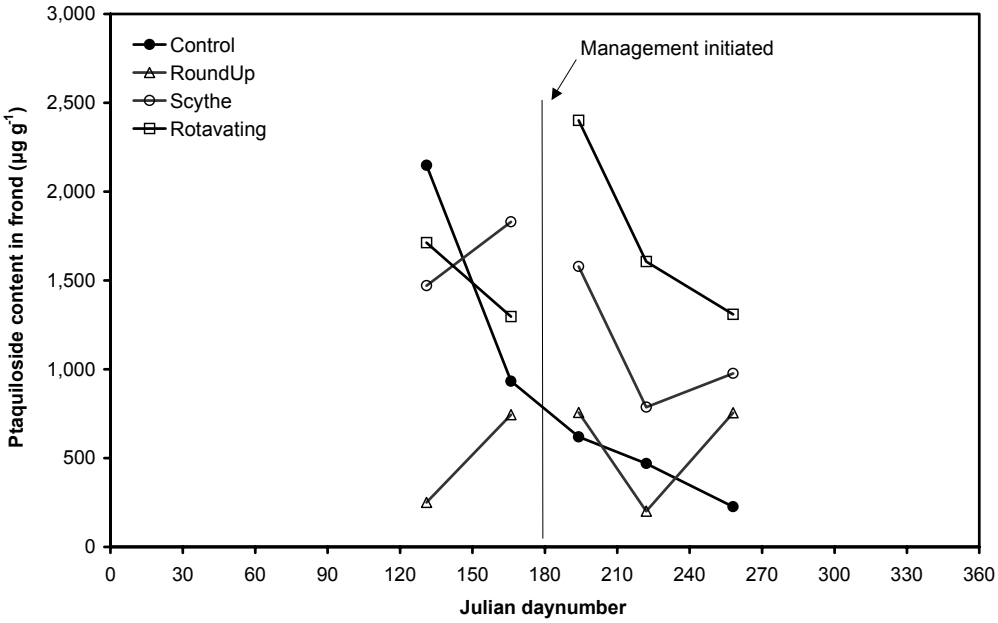


FIGURE 5.10. Ptaquiloside contents in managed Bracken fronds (var. *aquilinum*). Management trial in a Christmas tree plantation (Noble Fir, *Abies nordmanniana* (Steven) Spach) at Hvalsø, Denmark. The experimental set-up comprised a weed management trial having three treatments and a control: 1) Spraying with RoundUp®; 2) Manual management with scythe; and 3) Mechanical management by rotavating. Analytical details as in Rasmussen and Hansen (2003). Author, unpublished results.

The ptaquiloside content in fronds might also be affected by precipitation – especially heavy showers. Rasmussen et al. (2003a) found a decreased content in Danish var. *aquilinum* stands sampled just after heavy showers. The same pattern was observed in Venezuela with var. *caudatum* by Alonso-Amelot et al. (1995). One plausible reason for this is that ptaquiloside is leached from the fronds by the rain. Rasmussen et al. (2003a) found that ptaquiloside can leach from var. *aquilinum* fronds, although only in minute amounts. Crosier and rhizome leaching tests (var. *aquilinum*) have also previously demonstrated presence of many compounds in leachates (Evans et al. 1984). Leaching of ptaquiloside from fronds was tested by Alonso-Amelot et al. (1995) that submerged a var. *caudatum* frond in water. However, this research group observed no ptaquiloside in the water used for the test. This might have been due to too much water used or that no

leaching actually occurs from these fronds. Physiological and morphological differences between the two Bracken varieties exist, but the effect of these differences on ptaquiloside leaching needs further investigation.

5.7 Summary.

Ptaquiloside can be found in all varieties of Bracken, and in all fern compartments. The frond content is usually highest in young fronds and decreases during the growth season. The content in rhizomes is almost inversely correlated with the frond content. The recorded distribution is likely caused by differences in biological activity of the different compartments during the growth season and/or transfer of ptaquiloside between the compartments. The content of ptaquiloside in different Bracken stands is quite variable. On a large scale, the content of ptaquiloside in Bracken fronds seems mainly to be caused by genetic variation, while influence of local growth conditions can be seen on a smaller scale. However, recorded effects of local growth conditions are conflicting between different studies. There are some indications that ecological stress, such as physical disturbance, shade, and climate, may induce increased ptaquiloside contents in fronds, while nitrogen-depletion of soils may raise the level of ptaquiloside in rhizomes. These indications need further investigation before a general model for the ptaquiloside content in Bracken fronds can be established. Apparently, there is no variation in the frond content during the day, but the content varies significantly between individual fronds inside a single Bracken stand indicating ptaquiloside polymorphism at local scale. Non-ptaquiloside Brackens are found in two varieties of Bracken, indicating the existence of ptaquiloside chemotypes.

Chapter 6

PTAQUILOSIDE IN SOIL

6.1 Ptaquiloside in soil materials.

The most obvious way for ptaquiloside to enter the soil environment is by leaching from fern material, either as dead or living biomass. Williams et al. (1987) found significantly increased pH (from 4.2 to 5.5) and salt contents in frond leachates compared to plain rain water (through fall and stem flow of var. *aquilinum*). The highest pH values and salt contents werefound in the leachates from senescent Bracken stands (Williams et al. 1987), hereby indicating the highest potential of leaching from old Bracken fronds.

Ptaquiloside may be transferred from the fern to the soil environment in the following ways (Figure 6.1):

- Leaching of living fronds by rain (Rasmussen et al. 2003a).
- Physical transfer of ptaquiloside inside fronds to the litter layer when fronds die at the end of the growth season (or when fronds have been managed).
- Leaching of dead fronds in the litter layer by rain or melting snow.
- Leaching of living and dead roots and rhizomes by the soil solution.
- Transfer of ptaquiloside inside dead roots and rhizomes to soil.
- Excretion of ptaquiloside from all fern compartments (fronds, rhizomes and roots).
- Transfer to soil of ptaquiloside dissolved in urine or in droppings of animals eating Bracken.

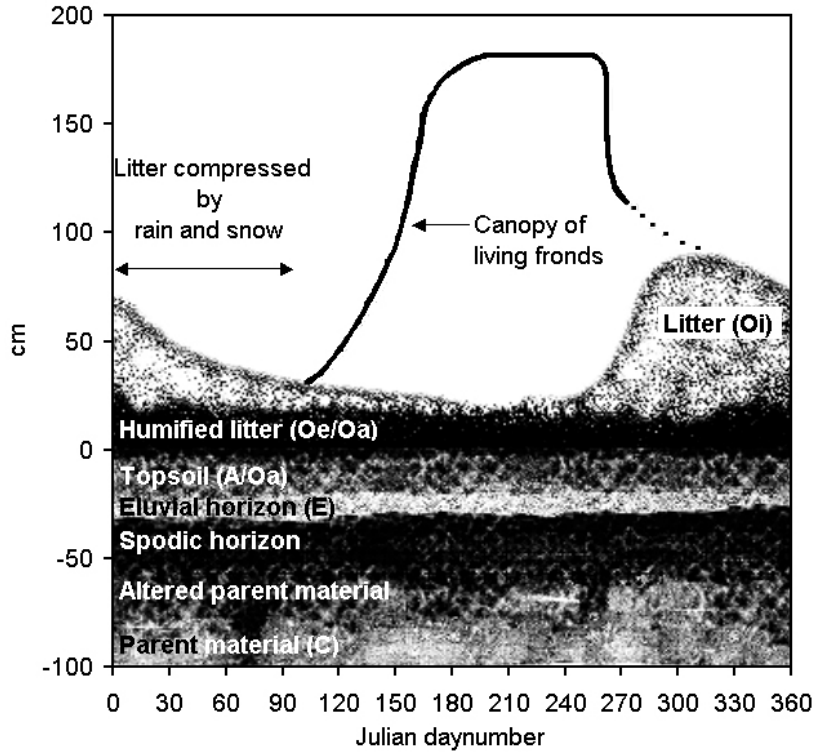


FIGURE 6.1. Annual variation in the physical appearance of a Bracken stand (var. *aquilinum*) growing on a Spodosol. Bracken rhizomes are usually situated in the topsoil (A/Oa-horizons) and in the lower part of the humified litter layer (Oe/Oa-horizons). The litter layer can be very thick, but with very low density. Modified after Nicholson and Paterson (1976).

The content of ptaquiloside ranges between 0.09 and 8.49 μg g^{-1} in litter below stands of var. *aquilinum* in Denmark and Scotland (Oi and/or Oe horizons), while Oa or A-horizons (topsoil) had ptaquiloside contents between 0.01 and 0.09 μg g^{-1} (Denmark only) (Rasmussen et al. 2003; Rasmussen et al. 2003a; Rasmussen et al. 2003b). The variation in the ptaquiloside content of Oi-horizons inside individual stands seems rather high (Rasmussen et al. 2003a). At first sight, the soil contents are quite low compared to frond and rhizome contents. But due to the high density of soil compared to fern materials, contents between 0.3 and 160 mg m^{-2} are found in Oi and/or Oe-horizons while contents between 0.9 and 57 mg m^{-2} are found inside Oa or A-horizons. The corresponding frond contents (the ptaquiloside load) were 15-500 mg m^{-2} (see Section 5.1) (Rasmussen et al. 2003b)!

The fact that ptaquiloside can be leached from fronds and that ptaquiloside occurs in soil strongly indicates that ptaquiloside can be transferred to the soil by rainwater. However, ptaquiloside in Oi- and Oe-horizons may also be relict ptaquiloside found inside remnants of Bracken (Frankland, 1976; Kawai et al. 1981; Saito et al. 1989). The content of

ptaquiloside in Bracken litter from var. *aquilinum* stands in Scotland is correlated with the content of ptaquiloside in the fronds (Rasmussen et al. 2003). Dead Bracken fronds are rather persistent although large amounts of easily soluble compounds are leached within a few months after frond death. Initial half-life's of 3-6 years has been reported for the turnover of var. *aquilinum* litter in Britain (Frankland, 1976). No correlation has been found between the content of ptaquiloside in fronds and soil materials, but a strong correlation has been found between ptaquiloside in Oa or A-horizons (topsoil's) and precipitation showing low ptaquiloside contents in areas with high precipitation in contrast to high ptaquiloside contents in soils from areas with low amounts of precipitation (Rasmussen et al. 2003b). This finding is supported by Rasmussen et al. (2003a) finding higher concentrations of ptaquiloside in soil materials below stands just exposed to heavy showers, reflecting the high solubility and mobility of ptaquiloside in the soil and that ptaquiloside may be added to the soil as discrete additions following the precipitation pattern. Continuous precipitation might instead leach ptaquiloside out of the upper soil layers. No straightforward explanation has been found for the ptaquiloside content in Oi- and Oe-horizons (Rasmussen et al. 2003b).

An example of the annual variation in the ptaquiloside concentration in soil solutions below a Bracken stand is shown in Box 6.1. The concentration is generally low, but peaks in November. The peak is situated in time approx. 1 month after the dieback of the Bracken stand, but also 1 month after the maximum concentrations in the rhizome system. This may indicate leaching of ptaquiloside from dying and degrading fronds or from the rhizome system. This is in line with the findings of Williams et al. (1987) for pH and salts.

Box 6.1. Ptaquiloside concentrations in soil solutions.

Ptaquiloside was measured after cleanup and conversion to pterosin B in soil solutions sampled by ceramic suction cells at 90 cm depth below four sub sites inside a Bracken stand (var. *aquilinum*) in a Christmas tree plantation (Noble Fir, *Abies nordmanniana* (Steven) Spach) at Hvalsø, Denmark (see Figure 5.10). Management took place in June. The experimental set-up was a weed management trial having three treatments and a control: 1) Spraying with RoundUp®; 2) Rotavating; and 3) Scythe. Analytical method as in Rasmussen et al. (2003b). No replicate measurements.

Subsite:	June ($ng\ L^{-1}$)	July ($ng\ L^{-1}$)	August ($ng\ L^{-1}$)	September ($ng\ L^{-1}$)	October ($ng\ L^{-1}$)	November ($ng\ L^{-1}$)	December ($ng\ L^{-1}$)
Control:	ND	ND	ND	ND	0.0	5.6	0.3
RoundUp®:	6.5	4.8	3.8	3.2	ND	ND	ND
Rotavating:	3.2	0.0	1.9	3.6	ND	1.6	ND
Scythe:	1.3	4.7	1.5	1.3	0.9	7.2	2.4

ND: Not determined.

Ptaquiloside has been found two times in drinking water from small wells using shallow groundwater and situated below Bracken stands (var. *aquilinum*) in Denmark (4-6 µg L^{-1}) and southern part of Sweden (45 µg L^{-1}) (author, unpublished results). Both soil water and well water concentrations are very high compared to the tolerable concentration of ptaquiloside in drinkingwater of 0.002 µg L^{-1} (Box 3.1).

6.2 Degradation of ptaquiloside in soil.

Only one study has been carried out to investigate the stability of ptaquiloside in contact with soil materials (Rasmussen et al. 2003c). This study shows that ptaquiloside may be stable in soils for relative long time (Figure 6.2).

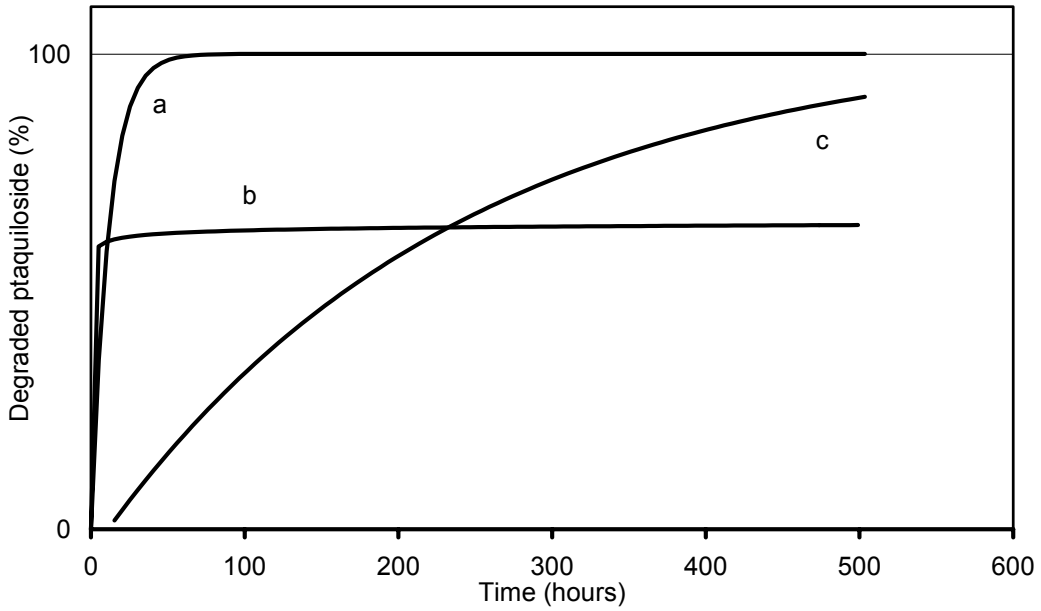


FIGURE 6.2. Stability of ptaquiloside in contact with soil at 25°C. Three degradation patterns exist: a) Fast degradation (within 240 hours, most degraded in 140 hours); b) Fast initial degradation (40-60% within 24 hours) followed by slow degradation; and c) Slow degradation. $[ptaquiloside]_{START}$ = 22-30 µg mL^{-1}, soil:solution ratio = 1:1 (weight:volume). The figure is modified after Rasmussen et al. (2003c).

Based on Rasmussen et al. (2003c), three degradation patterns can be delineated for Danish soils at 25°C (Figure 6.2):

a. Fast and full degradation described as a first order degradation reaction $PTA_{SOLUTION} \xrightarrow{k_1} PTA_{DEGRADED}$. The soils resulting in this degradation

pattern have low clay contents (0-8%), low to high carbon-contents (1-40%), and low pH (less than 4).

b. Fast initial degradation followed by slow degradation. The overall reaction can be fitted as the sum of a first order degradation reaction and a chemical equilibrium between ptaquiloside in solution and in the solid phase. The soils causing this pattern are weak acid to neutral (pH 4.8-7.9), with low carbon-contents (0.06-1.7%), and have high clay contents (14-23%).
c. Slow degradation. The reaction can be described as for group-a. The soils resulting in this pattern are weakly acidic (pH 4.0-4.6), with low carbon-contents (0.2-0.8%), and with low clay contents (1-2%).

In this context, *degradation* covers any process resulting in reduction in the water-extractable amount of ptaquiloside. The pattern for group-a is likely due to acid hydrolysis combined with irreversible reactions between ptaquiloside and humic substances/clay. Microbial degradation may also take part in the degradation. The group-b pattern might be due to fast irreversible sorption to clay minerals followed by a period where ptaquiloside is somewhat protected from degradation, e.g. due to weak interlayer sorption in clay minerals. Such sorption has been observed previously with other glycosides and sugars (Greenland, 1956; Greenland, 1956a). The degradation pattern in group-c is likely due to hydrolysis, just at a lower pace than in group-a (due to higher pH than in group-a) (Rasmussen and Hansen, 2003).

Rasmussen et al. (2003c) repeated their studies of ptaquiloside degradation at 4°C over a period of 1,728 hours. The overall pattern found was the same as at 25°C, but with two notable exceptions:

1) Fast group-a like degradation did not take place.
2) Slow group-c like degradation ceased after the first days.

In two of the four soils investigated degradation ceased within 144 hours, while in the two other soils degradation continued at a lower pace. A sudden unexplained increase in degradation was although observed by Rasmussen et al. (2003c) in one soil after 350 hours. Interestingly, ptaquiloside was still found in all the soil suspensions when the investigation ceased after 1,728 hours. The effect of the soil microbiology, chemistry and mineralogy needs further investigation before a thorough understanding of ptaquiloside degradation and reactions between ptaquiloside and soil can be achieved.

6.3 Soil sorption studies.

Rasmussen et al. (2003c) did also investigate the sorption of ptaquiloside to soil materials. Due to degradation of ptaquiloside caused by hydrolysis and irreversible sorption it was not possible for the authors to determine the sorbed amount of ptaquiloside *sensu strictu*. A further problem faced by the authors was the high solubility of ptaquiloside reflected in the low *log*K_{ow}-value for ptaquiloside (Table 4.1) resulting in a very low – almost non-detectable – amount of sorbed ptaquiloside. Hence, the authors calculated distribution coefficients based on sorption including degradation and irreversible sorption for 24 hours (Equation 6.1, Figure 6.3).

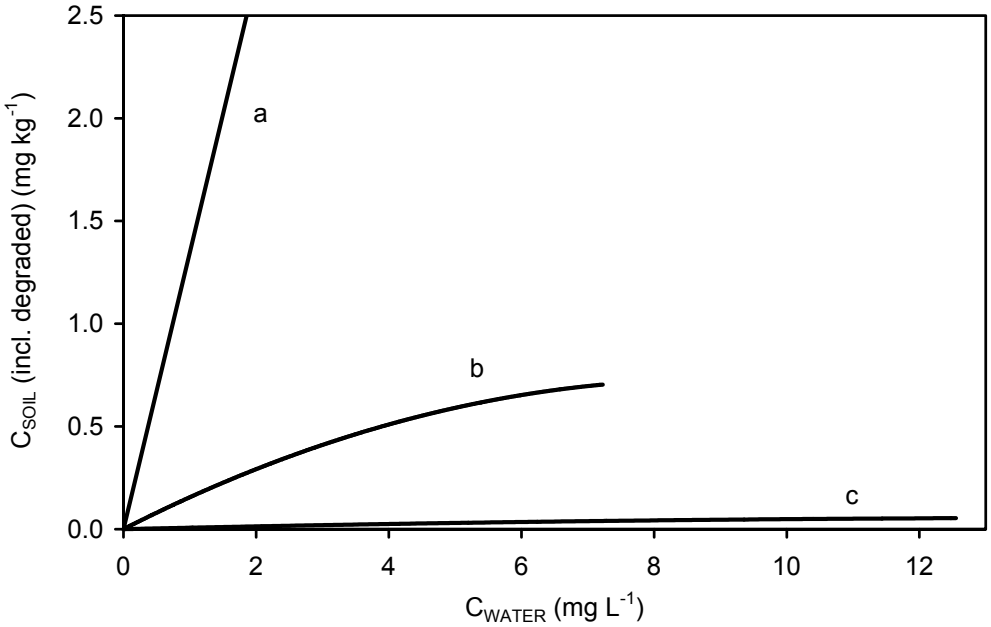


FIGURE 6.3. Freundlich sorption isotherms for ptaquiloside in contact with Danish soils. A range of isotherms were observed: a) Organic soil (Oa-horizon), $K_{FREUNDLICH}$ = 1.23 L kg^{-1}, n = 1.00); b) Mineral soil (clayey A-horizon), $K_{FREUNDLICH}$ = 0.07 L kg^{-1}, n = 0.75); and c) Mineral soil (sandy C-horizon), $K_{FREUNDLICH}$ = 0.01 L kg^{-1}, n = 0.73). Partly after Rasmussen et al. (2003b). $[ptaquiloside]_{START}$ = 0-14 mg L^{-1}. C_w: Concentration in water. C_s: Content on soil.

The sorption of ptaquiloside could be fitted with Freundlich isotherms (Eq. 6.1):

$$C_s = K_{FREUNDLICH} \cdot C_w^n \qquad \text{Eq. 6.1}$$

where C_S = equilibrium concentration of ptaquiloside on soil (mg kg^{-1}), $K_{FREUNDLICH}$ = Freundlich-type distribution coefficient (the Freundlich constant), C_W = equilibrium concentration of ptaquiloside in water (mg L^{-1}), and n = measure of the non-linearity

involved in adsorption. The Freundlich constants ranged from 0.01 to 0.22 with a single extreme constant of 1.23 found in very acid soil materials (probably caused by acid hydrolysis). The Freundlich constant was positively correlated with the carbon-content and the clay content of the soils indicating that sorption and degradation of ptaquiloside is correlated with the clay- and carbon-contents of the soils and that only little sorption of ptaquiloside to soil organic matter takes place (P (level of significance) = 0.03; Eq. 6.2) (Rasmussen et al. 2003c). However, the correlation coefficient between the Freundlich constant and the carbon/clay content of the soils investigated was not very good indicating that ptaquiloside bonding in soil depends on other soil parameters than the clay and carbon-content of the soil ($R^2 = 0.75$).

$$K_{FREUNDLICH} = 0.000 \pm 0.036 + 0.082 \pm 0.026 \cdot C(\%) + 0.006 \pm 0.002 \cdot clay(\%) \qquad \text{Eq. 6.2}$$

The non-linearity parameter n ranged between 0.64 and 1.00, and was correlated with the carbon content and pH ($P = 0.03$; $R^2 = 0.83$; Eq. 6.3).

$$n = 1.074 \pm 0.108 - 0.057 \pm 0.018 \cdot pH + 0.162 \pm 0.046 \cdot C(\%) \qquad \text{Eq. 6.3}$$

Equations 6.2 and 6.3 could only predict the sorption parameters on 8 respectively 7 out of 10 possible sets of data, hereby-indicating influence of other parameters than carbon, pH and clay on the sorption of ptaquiloside to soil materials. However, the models do indicate that sorption of ptaquiloside could be considered as a function of: 1) the acidity, governing the hydrolysis of ptaquiloside; 2) irreversible sorption of ptaquiloside to soil organic matter and clay minerals; and 3) a weak reversible sorption to soil organic matter and clay minerals (Rasmussen et al. 2003c).

The equilibrium concentrations for ptaquiloside in soil solutions are estimated for 6 common Danish topsoil's from the Bracken stands investigated by Rasmussen et al. (2003b) in Table 6.1. The ptaquiloside concentrations are based on the $K_{FREUNDLICH}$ equations (Eq. 6.2-3). These estimated concentrations are in some range with the measured ptaquiloside concentrations in the soil water from Hvalsø (Box 6.1). The concentrations are also well above the maximum tolerable concentration for ptaquiloside in drinking water of 0.1-1.6 ng L^{-1} (Box 3.1).

TABLE 6.1. Estimated equilibrium concentrations in soil solutions based on the full porosity of the soil horizon (Oa/A-horizons, 0-20 cm), and 100 viz. 30% saturation. Data from Rasmussen et al. (2003b). Details in Appendix B.

Location:	Ptaquiloside content:	Soil carbon	Clay	pH	Equilibrium concentration	Percentage of total in soil water
(saturation in %)	(µg kg^{-1})	(%)	(%)		(µg L^{-1})	(%)
Organic soils:						
Hundshage (100)	30	32.5	0.0	2.8	20	30
Præstø I (100)	20	25.7	0.0	3.0	20	30
Præstø II (100)	230	31.3	0.0	3.0	170	30
Ulfshale (100)	710	43.6	0.0	2.8	410	20
Mineral soils:						
Løvenholm (100)	20	2.5	1.8	4.1	30	60
Østbirk (100)	10	2.5	4.3	3.7	20	60
Low saturation:						
Præstø II (30)	230	31.3	0.0	3.0	200	10
Løvenholm (30)	20	2.5	1.8	4.1	60	40

6.4 Soil column studies.

Rasmussen et al. (2003c) investigated the mobility of ptaquiloside in soil materials by leaching experiments in packed soil columns (Spodosol materials).

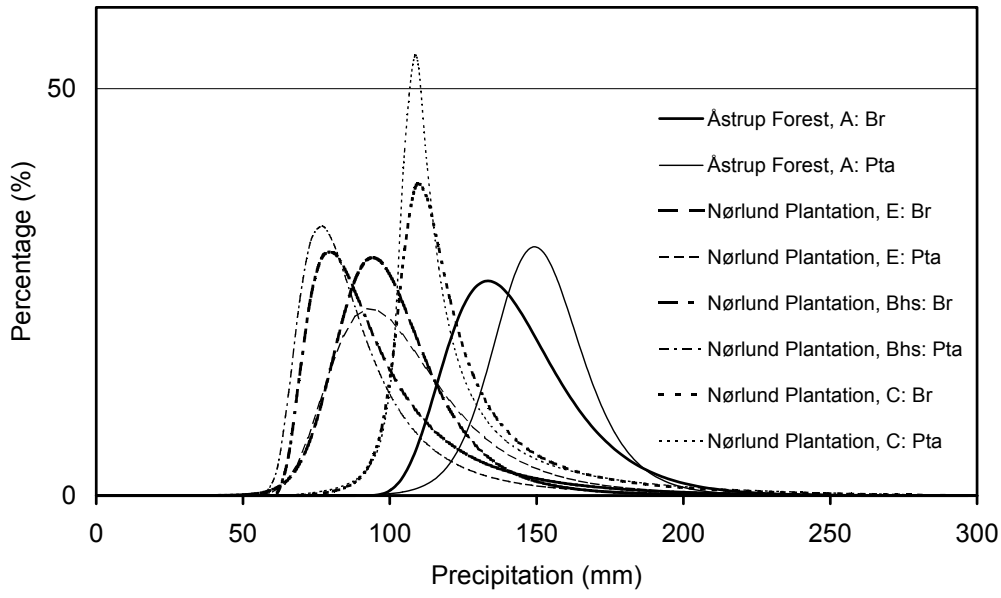


FIGURE 6.4. Breakthrough curves for ptaquiloside and bromide in soil columns comprising soil materials from Danish Spodosols. 80% ptaquiloside recovery from Åstrup Forest, A; 100% ptaquiloside recovery from Nørlund Plantation, E, Bhs, and C (Rasmussen et al. 2003c).

The authors found very weak sorption of ptaquiloside to the soil material and very high mobility (Figure 6.4). Distribution coefficients and retardation factors were calculated from the retardation of ptaquiloside compared to bromide and ranged from 0.00 to 0.05 L kg^{-1}, which is in the same range as the Freundlich distribution coefficients (Rasmussen et al. 2003c).

6.5 Summary.

Ptaquiloside can be leached from Bracken fronds to the soil by rain, but may also be transferred to the soil from dead Bracken material (roots, rhizomes, and fronds). Whatever reason, ptaquiloside is found in topsoil's and litter in amounts up to 8.5 μg g^{-1}. Soil solution concentrations were not measured directly in topsoil's, but equilibrium concentrations up to 400 μg L^{-1} in the soil solution can be estimated from the sorptive properties of ptaquiloside. This range is in accordance with measured ptaquiloside concentrations of up to 7 μg L^{-1} in soil solutions extracted 90 cm below the soil surface. These concentrations are much higher than the tolerable ptaquiloside concentration in drinking water with respect to the carcinogenicity of ptaquiloside.

Ptaquiloside is highly mobile in soils due to weak sorption and may therefore reach groundwater or other recipients. The stability of ptaquiloside in soil depends on the geochemical properties of the soils:

1) Ptaquiloside degrades rapidly when pH is below 4 or higher than 8.
2) Ptaquiloside may sorb irreversibly to clay minerals and soil organic matter.
3) Ptaquiloside may be sorbed reversibly to clay minerals and soil organic matter
4) Ptaquiloside degradation is temperature dependent (faster at higher temperatures).

Besides from the geochemical properties of the soil, soil microbiology may also influence the stability of ptaquiloside in soil.

Chapter 7

PTAQUILOSIDE – AN ENVIRONMENTAL HAZARD?

7.1 Conclusion.

In Chapter 1, six fundamental questions were raised that this thesis should seek answers to:

1. Can ptaquiloside be found in soil materials?

Ptaquiloside can indeed be found in soil materials! Contents ranging between 0.09 and 8.49 μg g^{-1} were found in litter while topsoil material just below the litter had contents between 0.01 and 0.09 μg g^{-1}. The contents seem low, but correspond to 0.3-160 mg m^{-2} in the litter and 0.9-57 mg m^{-2} for the topsoil's.

2. Is ptaquiloside stable in contact with soil materials?

The stability of ptaquiloside in contact with soil materials depends on the physical composition and chemical properties of the soil. Ptaquiloside degrades rapidly due to hydrolysis when pH is less than 4, especially in acid sandy soils where half-lifes in the range of 8 - 30 hours were observed at 25ºC. Degradation is probably quite rapid too in soils having pH higher than 8. Ptaquiloside degrades more slowly in slightly acid to neutral soils due to decreased rate of hydrolysis. In clayey soils, the degradation rate is further reduced probably due to a concurrent solid-phase partitioning reaction. The results indicate, that with longer reaction times, clay silicates may sorb ptaquiloside into the interlayer space reducing the amount of ptaquiloside available for degradation in solution.

Ptaquiloside were found to have very low affinity for sorption to soil organic matter. However, irreversible bonding might take place between the alkylating group of ptaquiloside and soil organic matter in line with what has been observed with DNA. Irreversible bonding may also take place between ptaquiloside and soil minerals. However, this topic needs further investigation before final conclusions can be made.

Ptaquiloside degradation in soil is strongly temperature dependent, and at 4ºC overall degradation is much reduced compared with 25ºC. This raises the question if laboratory studies performed at 20-25ºC are capable of mimicking near natural conditions with respect to the fate of ptaquiloside in the terrestrial ecosystem in much of the temperate zone. An unresolved question is also the effect of microbial activity causing degradation of ptaquiloside.

3. What is the mobility of ptaquiloside in soil?

Soil sorption studies revealed very low distribution coefficients between ptaquiloside found in the soil solution and ptaquiloside bound to soil particles, hence demonstrating a high mobility of ptaquiloside in the soil system. This is especially due to the very hydrophilic character of the glucose moity of ptaquiloside. Even though sorption to soil organic matter is low, ptaquiloside sorption to soil materials seems correlated with the amounts of carbon and clay in the soils as well as to soil pH. However, the correlation is not very good indicating influence of other parameters than those mentioned.

Studies of ptaquiloside mobility in packed soil columns confirmed the results of the soil sorption studies and reveiled mobility of ptaquiloside comparable to an inert tracer, e.g. nitrate. The mobility of ptaquiloside was to some degree confirmed by finding of ptaquiloside in soil solutions extracted from 90 cm below the soil surface.

4. When is ptaquiloside present in Bracken?

Ptaquiloside is found in all varieties of Bracken. However, some proveniances of vars. *esculentum* and *revolutum* does not contain ptaquiloside (see below). Ptaquiloside seems to be present in all fern compartments (fronds, rhizomes, roots and spores) at all times, but in very variable amount. The frond content (maximum content approx. 13,000 μg g^{-1}) is usually highest when the fronds are at the crosier stage and then gradually declines during the growth season. The content in rhizomes (maximum content approx. 7,000 μg g^{-1}) seems to be highest by the end of the growing season. Only few studies have investigated the content in spores and roots. The content in these parts of the fern seems to be rather low, showing maximum contents of approx. 200 and 20 μg g^{-1} respectively. The highest ptaquiloside contents are apparently found in fern compartments with high biological/metabolic activity. Further studies are needed to fully understand the dynamics and biological role of ptaquiloside in the Bracken fern.

5. What is the natural variation in the ptaquiloside content of Bracken?

Apparently no significant differences exist between Bracken varieties regarding their content of ptaquiloside. However, genetic heritage is likely to have some impact on the content of ptaquiloside as Bracken proveniences from the same variety have different ptaquiloside contents when grown under the same conditions. Ptaquiloside chemotypes may also exist as some Bracken varieties include proveniences not containing ptaquiloside.

The variation of the ptaquiloside content in fronds is quite large. As described above, annual variation takes place. Besides from that, large variation in the ptaquiloside content is found within Bracken stands; frond contents varying between 400 and 5,000 μg g^{-1} were found in an area of 24m x 36m. However, significantly different frond contents exist between different Bracken stands. The level of ptaquiloside in fronds of different Bracken stands is likely affected by external growth factors such as climate, ecosystem competition and soil properties. The exact cause of the variation is still to be established.

The level of ptaquiloside in fronds is affected by physiological stress as raised levels of ptaquiloside are found in ferns subjected to mowing or browsing by animals. Physiological or edaphical stress may also be the explanation for the effects of the external growth factors on the ptaquiloside content in fronds. However, this stress hypothesis needs further investigation to be prooved.

6. How much ptaquiloside can be produced under natural conditions?

To make a full risk assessment of Bracken stands it is essential to know the amount of ptaquiloside that can be produced in a Bracken stand. Due to the large annual variation of the ptaquiloside content in the different parts of Bracken, this is a very complicated task. Until now, a detailed model has only been acheived for the content of ptaquiloside in fronds. This model indicated that Bracken fronds could produce at least 500 mg ptaquiloside per m^2. This model is only a first approximation of the ptaquiloside production potential of Bracken stands as it only takes aboveground production into account and does not consider ptaquiloside flux between plant compartments during the growth season or leaching of ptaquiloside from the fern.

7.2 General discussion.

It is still an open question whether ptaquiloside contamination of milk and/or drinking water or the presence of Bracken spores in breathing air/surface waters forms the link between human cancer and the Bracken toxins (Evans, 1979; Galpin and Smith, 1986;

Galpin et al. 1990; Alonso-Amelot and Avendaño, 2002). In areas where milk consumption is dependent on cows browsing on Bracken, then milk is likely the main vector (Alonso-Amelot and Avendaño, 2001). In areas where only few cows browse on the fern, then water contamination might be important as well, especially in rural areas depending on local water sources like surface wells or shallow aquifers. The findings presented in this review may therefore be a part of the explanation of the high cancer rates observed in these areas. However, actual findings of ptaquiloside in drinking water from these areas are still to be made.

The large variations of ptaquiloside stability in contact with soil materials are due to differences in soil composition. Such variations coupled with the natural variation in the ptaquiloside content in Bracken may explain the lack of correlation between Bracken cover, cancer and water supplies as reported by Galpin and Smith (1986) in the Gwynedd-study (Rasmussen et al. 2003; Rasmussen et al. 2003b; Rasmussen et al. 2003c).

The amount of ptaquiloside found in Bracken stands capable of being transferred to the soil, seems quite high when compared to the amounts of pesticides and other potentially harmful compounds added to soils by humans. The question must therefore be raised if Bracken management in forestry and agriculture should be targeted from an environmental point of view besides from a traditional crop science point of view.

When considering the possible harmful properties of Bracken, distinguishing between the concepts of hazard and risk is useful. A hazard is a set of circumstances that may have harmful consequenses; risk is the probability of harmful consequenses occurring from a hazard (Wilson et al. 1998). Therefore, even though Bracken represents an environmental hazard, it must be remembered that the fern most likely only affects people living and working in areas with large densities of Bracken (Smith, 2000). For people only being in sporadic contact with Bracken and/or ptaquiloside the risk of being affected is low, e.g. people having summer residence homes in Bracken infested areas or scouts tramping through Bracken stands. Further studies are needed to unveil the full environmental impact of Bracken.

7.3 Perspectives and further studies.

Seen from a world-perspective, Bracken has generally changed its role from being a useful or used part of Nature to being perceived as a pest in agriculture and forestry. Most research on Bracken is therefore now conducted on the problematic effects of the fern, in

particular how to eradicate or control it. Much progress in the understanding of Bracken ecology and biochemistry has taken place in this light during the last 10-15 years. However, much is still to be done, such as:

- Development of integrated control systems for organic farmers in industrialized countries.
- Development of low-cost control systems/pesticides for use in the developing world.

A thorough understanding of the connection between the fern and its toxins and their implications on human health and the environmental is also strongly needed. This thesis has sought answers to some central questions regarding the presence of ptaquiloside in terrestrial ecosystems. However, several new questions rose such as:

- Can ptaquiloside be leached from roots and rhizomes by the soil water?
- What is the effect of ptaquiloside on the soil microflora?
- Can soil microorganisms mineralise ptaquiloside and how fast?
- Which interactions exist between ptaquiloside and soil minerals?
- To which degree is ptaquiloside transferred from a Bracken stand to the soil?
- What is the total production of ptaquiloside in a Bracken stand (comprising all parts of the fern)?

Until now, most research has been carried out in highly specialized research groups, although some general Bracken conferences have taken place. To secure future advances in this field of science, international cooperation between scientists from different research areas must take place.

REFERENCES

Ader, K.G. (1989) The effect of climate and soils on Bracken vigour and distribution in Scotland. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 141-151. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Agnew, M.P. and Lauren, D.R. (1991) Determination of ptaquiloside in Bracken fern (*Pteridium esculentum*). *Journal of Chromatography* **538**, 462-468.

Allsup, T.N. and Griffiths, W.R. (1978) Progressive retinal degeneration in sheep in South Wales. *The Veterinary Record* **103**, 268-268.

Alonso-Amelot, M.E. (1997) The link between Bracken fern and stomach cancer: milk. *Nutrition* **13**, 694-696.

Alonso-Amelot, M.E. and Avendaño, M. (2001) Possible association between gastric cancer and Bracken fern in Venezuela: An epidemiologic study. *International Journal of Cancer* **91** , 252-259.

Alonso-Amelot, M.E. and Avendaño, M. (2002) Human carcinogenesis and Bracken fern: A review of the evidence. *Current Medicinal Chemistry* **9**, 675-686.

Alonso-Amelot, M.E., Calcagno, M.P. and Pérez-Inojosa, M. (2000) Growth and selective defensive potential in relation to altitude in neotropical *Pteridium aquilinum* var. *caudatum*. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 43-47. Aberystwyth: International Bracken Group. United Kingdom.

Alonso-Amelot, M.E., Castillo, U.F., Avendaño, M., Smith, B.L. and Lauren, D.R. (2000a) Milk as a vehicle for the transfer of ptaquiloside: a Bracken carcinogen. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 86-90. Aberystwyth: International Bracken Group. United Kingdom.

Alonso-Amelot, M.E., Castillo, U.F. and de Jongh, F. (1993) Passage of the Bracken-fern carcinogen ptaquiloside into bovine milk. *Lait* **73**, 332

Alonso-Amelot, M.E., Castillo, U.F., Smith, B.L. and Lauren, D.R. (1996) Bracken Ptaquiloside in milk. *Nature* **382**, 587

Alonso-Amelot, M.E., Castillo, U.F., Smith, B.L. and Lauren, D.R. (1998) Excretion through milk, of ptaquiloside in Bracken-fed cows. A quantitative assessment. *Lait* **78**, 413-423.

Alonso-Amelot, M.E., Oliveros, A., Calcagno, M.P., and Arellano, E. (2001) Bracken adaptation mechanisms and xenobiotic chemistry. *Pure and Applied Chemistry* **73**, 549-553.

Alonso-Amelot, M.E., Pérez-Mena, M., Calcagno, M.P. and Jaimes-Espinoza, R. (1992) Quantification of pterosins A and B, ptaquiloside, the main carcinogen of *Pteridium aquilinum* (L. Kuhn), by high pressure liquid chromatography. *Phytochemical Analysis* **3**, 160-164.

Alonso-Amelot, M.E. and Rodulfo-Baechler, S. (1996) Comparative spatial distribution, size, biomass and growth rate of two varieties of Bracken fern (*Pteridium aquilinum* L. Kuhn) in a neotropical montane habitat. *Vegetatio* **125**, 137-147.

Alonso-Amelot, M.E., Rodulfo-Baechler, S. and Jaimes-Espinosa, R. (1995) Comparative dynamics of ptaquiloside and pterosin B in the two varieties (*caudatum* and *arachnoideum*) of neotropical Bracken fern (*Pteridium aquilinum* L. Kuhn). *Biochemical Systematics and Ecology* **23**, 709-716.

Ames, B.N. and Gold, L.S. (1990) Dietary carcinogens, environmental pollution, and cancer: some misconceptions. *Medical Oncology and TumorPharmacotherapy* **7**, 69-85.

Ames, B.N., Profet, M. and Gold, L.S. (1990) Nature's chemicals and synthetic chemicals: comparative toxicology. Proceedings of the National Academy of Science, USA. Electronic citation: <http://socrates.berkeley.edu/mutagen/ames.PNASIII.html>.

Anderson, J.A., Blahna, D.J. and Chavez, D.J. (2000) Fern gathering on the San Bernardino National Forest: cultural versus commercial values among Korean and Japanese participants. *Society and Natural Resources* **13**, 747-762.

Anonymous (1997) *Beilstein Institut für Literatur der organischen Chemie.* Licenced to Beilstein Chemie Daten und Software GmbH and Beilstein Informationssysteme GmbH.

Anonymous (1998) *1996-Report of the Committees on Toxicity, Mutagenicity, Carcinogenicity of Chemicals in Food, Consumer Products and the Environment*. Department of Health. United Kingdom.

Anonymous (1999) Alleochemical and attractants. Electronic citation: <http://www.wmdc.edu/HTMLpages/Academics/Biology/botanyweb/plantbiochemicals/alleochemicals&attractants.html>.

Anonymous (1999a) ASULOX®. Rhône-Poulenc Inc. (New Zealand). Electronic citation: <http://www.hortnet.co.nz/hortdirectory/rhone_poulenc/product/asutech.htm>.

Anonymous (1999b) Controlling pasture weeds - AgFACT. AgResearch. Electronic citation: <http://www.agresearch.co.nz>

Anonymous (2000) Bekendtgørelse om mindstekrav til beskyttelse af landbrugsdyr, BEK nr 707 af 18/07/2000 (in English: Announcement on minimum standards for the protection of agricultural animals, BEK no. 707 dated 18 July 2000).

Anonymous (2000a) Kankerverwekkende stoffenlijst RDMG uitgeverij. Electronic citation: <http://www.rdmg.nl/>.

Anonymous (2002) Bregner (ferns). Electronic citation: <http://www.vesong.gs.ah.no/sporepl1.htm>.

Anonymous (2002a) Einstape - *Pteridium aquilinum*. Electronic citation: <http://fuv.hivolds.no/ prosjekt/espelra/keinstape.htm>.

Anonymous (2002b) *Politikens bog om det alternative. En Håndbog om Mystik og Videnskab (in English: Politikens Book on Alternative Science. A Handbook on Mysticism and Science)*. København: Politikens Forlag. Denmark.

Aswani Kumar, K., Kataria, M., Somvanshi, R., Kumar, S. and Saini, M. (2001) Characterization of toxin from *Cheilanthes* fern and its effect on lymphocyte proliferation and DNA fragmentation. *Indian Journal of Experimental Biology* **39**, 1065-1067.

Atkinson, T.P. (1989) Seasonal and altitudinal variation in *Pteridium aquilinum* (L.) Kuhn: frond and stand types. *New Phytologist* **113**, 359-365.

Bassett, C., Whitehouse, L.J. and Zabkiewicz, J.A., (Eds.) (1990) Alternatives to the chemical control of weeds. Forest Research Bulletin **155**. Rotorua: Ministry of Forestry. New Zealand.

Backshall, J. (1999) Views of English nature on Bracken control. Papers given at a meating held at the University of Lancaster, U.K. on 10th September 1998. In: Taylor, J., (Ed.) *Bracken Perceptions and Bracken Control in the British Uplands*, pp. 18-29. Aberystwyth, International Bracken Group. United Kingdom.

Baker, E.A. and Gaskin, R.E. (1987) Composition of leaf epicuticular waxes of *Pteridium* subspecies. *Phytochemistry* **26**, 2847-2848.

Bell, P.R. (1976) Gametogenesis and fertilization in *Pteridium*. *Botanical Journal of the Linnean Society* **73**, 47-78.

Beniston, R.G., Morgan, I.M., O'Brien, V. and Campo, M.S. (2000) Synergi between papillomavirus and quercetin: Significance of quercetin-induced G1 arrest for cellular transformation. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 123-127. Aberystwyth: International Bracken Group. United Kingdom.

Bento, M.C., Clemente, M.L., Costa, M.M., Gouveia, M.P. and Spínola, T.M. (2000) *Bovine Enzootic Haematuria* in Madeira Island Portugal. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 141-143. Aberystwyth: International Bracken Group. United Kingdom.

Birnie, R.V., Miller, D.R., Horne, P.L., Leadbeater, S. and MacDonald, A. (2000) The potential distribution and impact of Bracken in upland Scotland: an assessment using a GIS-based niche model. *Annals of Botany* **85**, 53-56.

Bjeldanes, L.F. and Chang, G.W. (1977) Mutagenic activity of quercetin and related compounds. *Science* **197**, 577-578.

Blair, R.M., Alcaniz, R. and Harrell, A. (1983) Shade intensity influences the nutrient quality and digestability of Southern Deer browse leaves. *Journal of Range Management* **36**, 257-264.

Bohm, B.A. and Tryon, R.M. (1967) Phenolic compounds in ferns: 1. A survey of some ferns for cinnamic acid and benzoic acid derivatives. *Canadian Journal of Botany* **45**, 585-593.

Borzacchiello, G., Ambrosio, V., Roperto, S., Poggiali, F., Tsirimonakis, E., Venuti, A., Campo, M.S. and Roperto, F. (2003) Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. *Journal of Comparative Pathology* **128**, 203-206.

Brown, L.M., Zahm, S.H., Hoover, R.N. and Fraumeni, J.F. (1999) Bracken fern consumption and human bladder cancer. *Journal of Epidemiology and Community Health* **53**, 653-653.

Brownsey, P.J. and Smith-Dodsworth, J.C. (1989) *New Zealand Ferns and Allied Plants.* Auckland: David Bateman. New Zealand.

Brøndegaard, V.J. (1978) *Folk og Flora. 1. Dansk etnobotanik (in English: People and Flora. 1. Danish Ethnobotany)*. Copenhagen: Rosenkilde og Bagger. Denmark

Burge, M.N. and Kirkwood, R.C. (1992) The control of Bracken. *Critical Reviews in Biotechnology* **12**, 299-333.

Burkhalter, P.W., Groux, P.M.J., Candrian, U., Hübner, P. and Lüthy, J. (1996) Isolation, determination and degradation of ptaquiloside - a Bracken fern (*Pteridium aquilinum*) carcinogen. *Journal of Natural Toxins* **5**, 141-159.

Buttenschøn, R.M. (2000) Ørnebregne bekæmpes med flere slåninger årligt (in English: Bracken are to mowed several times a year). *Skovbrug Videnblad* **4**, 10-12.

Caldow, G. and Burns, F. (2000) Bracken poisoning in farm animals in Great Britain: a brief outline in relation to disease surveillance data. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 197-200. Aberystwyth: International Bracken Group. United Kingdom.

Campo, M.S. (1997) Bovine papillimavirus and cancer. *The Veterinary Journal* **154**, 175-188.

Campo, M.S., Beniston, R.G., Connolly, J.A. and Grindlay, G.J. (2000) Synergism between papillomavirus and Bracken fern in carcinogenesis of the upper gastrointestinal tract in cattle and humans: quercetin and cell transformation. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology*

and Control. Proceedings of the International Bracken Group Conference Manchester 1999, pp. 116-122. Aberystwyth: International Bracken Group. United Kingdom.

Campo, M.S., O'Neil, B.W., Barron, R.J. and Jarrett, W.F. (1994) Experimental reproduction of the papilloma-carcinoma complex of the alimentary canal in cattle. *Carcinogenesis* **15**, 1597-1601.

Caporn, S.J.M., Brooks, A.L., Press, M.C. and Lee, J.A. (1999) Effects of long-term exposure to elevated CO_2 and increased nutrient supply on Bracken (*Pteridium aquilinum*). *Functional Ecology* **13**, 107-115.

Castillo, U.F., Ojika, M., Alonso-Amelot, M.E. and Sakagami, Y. (1998) Ptaquiloside Z, a new toxic unstable sesquiterpene glucoside from the neotropical Bracken fern *Pteridium aquilinum* var. *caudatum*. *Bioorganic and Medical Chemistry* **6** , 2229-2233.

Castillo, U.F., Ojika, M., Sakagami, Y., Wilkins, A.L., Lauren, D.R., Alonso-Amelot, M.E. and Smith, B.L. (2000) Isolation and structural determination of three new toxic illudane-type sesquiterpene glucosides and a new protoilludane sesquiterpene glucoside from *Pteridium aquilinum* var. *caudatum*. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester*, pp. 55-59. Aberystwyth: International Bracken Group. United Kingdom.

Castillo, U.F., Sakagami, Y., Alonso-Amelot, M.E. and Ojika, M. (1999) Pteridanoside, the first protoilludane sesquiterpene glucoside as a toxic component of the neotropical Bracken fern *Pteridium aquilinum* var. *caudatum*. *Tetrahedron* **55**, 12295-12300.

Castillo, U.F., Wilkins, A.L., Lauren, D.R., Smith, B.L., Towers, N.R., Alonso-Amelot, M.E. and Jaimez-Espinoza, R. (1997) *Iso*-ptaquiloside and caudatoside, illudane-type sesquiterpene glucosides from *Pteridium aquilinum* var. *caudatum*. *Phytochemistry* **44**, 901-906.

Chang, F., Syrjänen, S., Shen, Q., Ji, H. and Syrjänen, K. (1990) Human papillomavirus (HPV) DNA in esophageal precancer lesions and squamous cell carcinomas from China. *International Journal of Cancer* **45**, 21-25.

Chappell, J. (1995) Biochemistry and molecular-biology of the isoprenoid biosynthetic pathway in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 521-547.

Christiansen, M.S. and Jahns, H.M. (1981) *Bregner - Mosser - Laver i Mellem-, Nord- og Vesteuropa (in English: Ferns - Mosses - Lichens in Central, North and Western Europe).* Copenhagen: G.E.C. Gads Forlag. Denmark.

Connolly, J.A., Morgan, I.M., Jackson, M.E. and Campo, M.S. (1998) The BPV-4 co-carcinogen quercetin induces cell cycle arrest and up-regulates transcription from the LCR of BPV-4. *Oncogene* **16**, 2739-2746.

Cooper-Driver, G.A., Finch, S. and Swain, T. (1977) Seasonal variation in secondary plant compounds in relation to the palatability of *Pteridium aquilinum*. *Biochemical Systematics and Ecology* **5**, 177-183.

Cooper-Driver, G.A. and Swain, T. (1976) Cyanogenic polymorphism in Bracken in relation to herbivore predation. *Nature* **260**, 604-604.

Cooper, M.R. and Johnson, A.W. (1998) *Poisonous Plants and Fungi in Britain - Animal and Human Poisoning*, 2nd edn. London: The Stationery Office. United Kingdom.

Cooper, P. (1980) More chapters in the Bracken saga. *Food and Cosmetics Toxicology* **18**, 311-312.

Cossy, J., Ibhi, S., Kahn, P.H. and Tacchini, L. (1995) A formal synthesis of ptaquilosin. the aglycon of a potent Bracken carcinogen ptaquiloside. *Tetrahedron Letters* **36**, 7877-7880.

Cwen, K.M., Marrs, R.H., Snow, C.S.R. and Evans, C.E. (1999) Soil acidification: the use of sulphur and acid plant materials to acidify arable soils for the recreation of heathland and acidic grassland at Minsmere, UK. *Biological Conservation* **87**, 105-121.

Dartnall, A.M. and Burns, R.G. (1987) A sensitive method for measuring cyanide and cyanogenic glucosides in sand culture and soil. *Biology and Fertility of Soils* **5**, 141-147.

Dawra, R.K., Kurade, N.P. and Sharma, O.P. (2002) Carcinogenicity of the fern *Pteridium aquilinum* collected from enzootic bovine haematuria-free hilly area in India. *Research Communications* **83**, 1005-1009.

Dawra, R.K., Sharma, O.P. and Somvanshi, R. (2000) Experience with enzootic bovine haematuria in India. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 150-154. Aberystwyth: International Bracken Group. United Kingdom.

de Oliveira, M.L., Rocha, N.S., Fukushima, S. and de Camargo, J.L. (1995) Absence of promoting potential of Bracken fern (*Pteridium aquilinum*) in rat urinary bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine and uracil. *Teratogenesis, Carcinogenesis, and Mutagenesis* **15**, 191-199.

den Ouden, J. (1995) Allelopathy in Bracken in the Netherlands. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 43-46. Aberystwyth: International Bracken Group. United Kingdom.

den Ouden, J. and Vogels, D. (1997) Mechanical resistance by an ectorganic soil layer on root development of seedling *Pinus sylvestris*. *Plant and Soil* **197**, 209-217.

Dolling, A., Zackrisson, O. and Nilsson, M.C. (1994) Seasonal variation in phytotoxicity of Bracken (*Pteridium aquilinum* L. Kuhn). *Journal of Chemical Ecology* **20**, 3163-3172.

Dolling, A.H.U. (1996) Interference of Bracken (*Pteridium aquilinum* K. Kuhn) with Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies* L. Karst.) seedling establishment. *Forest Ecology and Management* **88**, 227-235.

Duncan, H.J. and Jarvis, M.C. (1976) Nucleotides and related compounds in Bracken. *Botanical Journal of the Linnean Society* **73**, 79-85.

El-Mofty, M., Sadek, I. and Bayoumi, S. (1980) Improvement in detecting the carcinogenicity of Bracken fern using an Egyptian Toad. *Oncology* **37**, 424-425.

El-Mofty, M., Sadek, I., Soliman, A., Mohamed, A. and Sakre, S. (1987) α-Ecdysone, a new Bracken fern factor responsible for neoplasm induction in the Egyptian Toad (*Bufo regularis*). *Nutrition and Cancer* **9**, 103-107.

Ershova, E. (2000) Bracken (*Pteridium aquilinum*) in Siberia. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 52-53. Aberystwyth: IBG. United Kingdom.

Evans, I.A. (1976) Relationship between Bracken and cancer. *Botanical Journal of the Linnean Society* **73**, 105-112.

Evans, I.A. (1979) Bracken carcinogenicity. *Research in Veterinary Science* **26**, 339-348.

Evans, I.A. (1986) The carcinogenic, mutagenic and teratogenic toxicity of Bracken. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology. The proceedings of the International Conference - Bracken '85*, pp. 139-146. Carnforth: Parthenon Publishing. United Kingdom.

Evans, I.A., Al-Samarrai, A.M.H. and Smith, R.M.M. (1984) Bracken toxicology: identification of some water soluble compounds from crozier and rhizome. *Research in Veterinary Science* **37**, 261-265.

Evans, I.A. and Galpin, O.P. (1990) Bracken and leukaemia. *The Lancet* **335**, 231-231.

Evans, I.A. and Mason, J. (1965) Carcinogenic activity of Bracken. *Nature* **208**, 913-914.

Evans, W.C. (1976) Bracken thiaminase-mediated neurotoxic syndromes. *Botanical Journal of the Linnean Society* **73**, 113-131.

Evans, W.C. (1986) The acute diseases caused by Bracken in animals. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology,* pp. 121-132. Carnforth: Parthenon Publishing. United Kingdom.

Evans, W.C., Korn, T., Natori, S., Yoshihira, K. and Fukuoka, M. (1983) Chemical and toxicological studies on Bracken fern, *Pteridium aquilinum* var. *latiusculum*. VIII. The inability of Bracken extracts containing pterosins to cause cattle Bracken poisoning. *Journal of Pharmacobiodynamics* **6**, 938-940.

Fenwick, G.R. (1988) Bracken (*Pteridium aquilinum*) - toxic effects and toxic constituents. *Journal of the Science of Food and Agriculture* **46**, 147-173.

Finkielsztein, L.M., Alesso, E.N., Lantaño, B., Aguirre, J.M. and Iglesias, G.Y.M. (1999) A simple synthetic route to pterosin F and other pterosins. *Journal of Chemical Research (S)* **1999**, 406-406.

Frankland, J.C. (1976) Decomposition of Bracken litter. *Botanical Journal of the Linnean Society* **73**, 133-143.

Freitas, R.N., O'Connor, P.J., Prakash, A.S., Shahin, M. and Povey, A.C. (2001) Bracken (*Pteridium aquilinum*)-induced DNA adducts in mouse tissues are different from the adduct induced by the activated form of the Bracken carcinogen ptaquiloside. *Biochemical and Biophysical Research Communications* **281**, 589-594.

Freitas, R.N., Prakash, A.S., Shahin, M. and Povey, A.C. (2000) Differences between DNA adducts in BDF1 mice treated with Bracken fern extract or spores and the principal DNA adduct formed *in vitro* by ptaquiloside. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 96-98. Aberystwyth: International Bracken Group. United Kingdom.

Fujio Morrow, M. (2000) Bracken as food: a Japanese perspective. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 204-205. Aberystwyth: International Bracken Group. United Kingdom.

Fukuoka, M. (1982) Chemical and toxicological studies on Bracken fern, *Pteridium aquilinum* var. *latiusculum*. VI. Isolation of 5-o-caffeoylshikimic acid as an antithiamine factor. *Chemical and Pharmaceutical Bulletin* **30**, 3219-3224.

Fukuoka, M., Kuroyanagi, M., Yoshihira, K. and Natori, S. (1978) Chemical and toxicological studies on Bracken fern, *Pteridium aquilinum* var. *latiusculum*. II. Structures of pterosins, sesquiterpenes having 1-indanone skeleton. *Chemical and Pharmaceutical Bulletin* **26**, 2365-2385.

Fukuoka, M., Kuroyanagi, M., Yoshihira, K., Natori, S., Nagao, M., Takahashi, Y. and Sugimara, T. (1978) Chemical and toxicological studies on Bracken fern, *Pteridium aquilinum* var. *latiusculum*. IV. Surveys on Bracken constituents by mutagen tests. *Journal of Pharmacol Dyn* **1**, 324-331.

Galpin, O.P. and Smith, R.M.M. (1986) Bracken, stomach cancer and water supplies: Is there a link? In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology - The Proceedings of the International Conference - Bracken '85*, pp. 147-159. Aberystwyth: Parthenon Publishing. United Kingdom.

Galpin, O.P., Whitaker, C.J., Whitaker, R. and Kassab, J.Y. (1990) Gastric cancer in Gwynedd. Possible links with Bracken. *British Journal of Cancer* **61**, 737-740.

Gava, A., da Silva Neves, D., Gava, D., de Moura Saliba, T., Schild, A.L. and Riet-Correa, F. (2002) Bracken fern (*Pteridium aquilinum*) poisoning in cattle in Southern Brazil. *Veterinary and Human Toxicology* **44**, 362-365.

Gershenzon, J. and Croteau, R. (1991) Terpenoids. In: Rosenthal, G.A. and Berenbaum, M.R. (Eds.) *Herbivores, their Interaction with Secondary Metabolites, Vol. 1 The Chemical Participants*, pp. 165-219. New York: Academic Press. USA.

Ghergariu, S., Lauren, D.R. and Riond, J.L. (2000) Ptaquiloside measurements in samples from an area of *Bovine Enzootic Haematuria*. Preliminary data. (Personal communication J.L. Riond, University of Zürich, Zürich, Switzerland).

Glass, A.D.M. and Bohm, B.A. (1969) A further survey of ferns for cinnamic and benzoic acids. *Phytochemistry* **8**, 629-632.

Gliessman, S.R. (1976) Allelopathy in a broad spectrum of environments as illustrated by Bracken. *Botanical Journal of the Linnean Society* **73**, 95-104.

Gliessman, S.R. and Muller, C.H. (1978) The allelopathic mechanisms of dominance in Bracken (*Pteridium aquilinum*) in Southern California. *Journal of Chemical Ecology* **4**, 337-362.

Gounalan, S., Somvanshi, R., Kataria, M., Bisht, G.S., Smith, B.L. and Lauren, D.R. (1999) Effect of Bracken (*Pteridium aquilinum*) and Dryopteris (*Dryopteris juxtaposita*) fern toxicity in laboratory rabbits. *Indian Journal of Experimental Biology* **37**, 980-985.

Greenland, D.J. (1956) The adsorption of sugars by montmorillonite. I. X-ray studies. *Journal of Soil Science* **7**, 323-328.

Greenland, D.J. (1956a) The adsorption of sugars by montmorillonite. II.Chemical studies. *Journal of Soil Science* **7**, 329-335.

Grieve, M. (2002) Botanical.com - A modern herbal. Electronic citation: < http://botanical.com>.

Gry, J., Hallas-Møller, T., Pedersen, E., Pilegaard, K. and Strube, M. (2000) *Drogelisten - Vurdering af Planter, Svampe og dele heraf anvendt i Kosttilskud og Urtete (in English: The List of Herbal remedies - Assessment of Plants, Fungi and Parts Hereof used as Supplementary Food and in Herbal Teas)*. Copenhagen: Fødevaredirektoratet, Ministeriet for Fødevarer, Landbrug og Fiskeri. Denmark.

Hadfield, P.R. and Dyer, A.F. (1986) Polymorphism of cyanogenesis in British populations of Bracken (*Pteridium aquilinum* L. Kuhn). In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology. The Proceedings of the International Conference - Bracken '85*, pp. 293-300. Carnforth: Parthenon Publishing. United Kingdom.

Hadfield, P.R. and Dyer, A.F. (1988) Cyanogenesis in gametophytes and young sporophytes of Bracken. *Biochemical Systematics and Ecology* **16**, 9-13.

Haenszel, W., Kurihara, M., Locke, F.B., Shimuzu, K. and Segi, M. (1976) Stomach cancer in Japan. *Journal of the National Cancer Institute* **56**, 265-274.

Hayashi, Y., Nishizawa, M. and Sakan, T. (1977) Studies on the sesquiterpenoids of *Hypolepis punctata* Mett. - I. *Tetrahedron* **33**, 2509-2511.

Henning, S.J. and Dickmann, D.I. (1996) Vegetative responses to prescribed burning in a mature Red Pine stand. *Northern Journal of Applied Forestry* **13**, 140-146.

Hetherington, S.L. and Anderson, J.M. (1998) Lignin signatures show the effects of changes in Heather and Bracken cover on the composition of organic matter in a moorland soil profile. *Oecologia* **117**, 194-200.

Hirayama, T. (1979) Diet and cancer. *Nutrition and Cancer* **1**, 67-81.

Hirono, I. (1986) Human carcinogenic risk in the use of Bracken fern. In: Hayashi, Y., Nagao, M., Sugimua, T., Takayama, S., Tomatis, L., Wattenberg, L.W. and Wogan, G.N., (Eds.) *Diet, Nutrition and Cancer. Proceedings of the 16th International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, 1985*, pp. 139-145. Tokyo: Japan Scientific Societies Press. Japan.

Hirono, I. (1989) Carcinogenic Bracken glycosides. In: Cheeke, P.R., (Ed.) *Toxicants of Plant Origin - Volume II Glycosides*, pp. 239-251. Boca Raton: CRC Press, Inc. USA.

Hirono, I., Aiso, S., Yamaji, T., Mori, H., Yamada, K., Niwa, H., Ojika, M., Wakamatsu, K., Kigoshi, H., Niiyama, K. and Uosaki, Y. (1984) Carcinogenicity in rats of ptaquiloside isolated from Bracken. *Gann* **75**, 833-836.

Hirono, I., Fushimi, K., Mori, H., Miwa, T. and Haga, M. (1973) Comparative study of carcinogenic activity in each part of Bracken. *Journal of the National Cancer Institute* **50**, 1367-1371.

Hirono, I., Ito, M., Yagyu, S., Haga, M., Wakamatsu, K., Kishikawa, T., Nishikawa, O., Yamada, K., Ojika, M. and Kigoshi, H. (1993) Reproduction of progressive retinal degeneration (*Bright Blindness*) in sheep by administration of ptaquiloside contained in Bracken. *Journal of Veterinary and Medical Science* **1993**, 6-979.

Hirono, I., Kono, Y., Takahashi, K., Yamada, K., Niwa, H., Ojika, M., Kigoshi, H., Niiyama, K. and Uosaki, Y. (1984a) Reproduction of acute Bracken poisoning in a calf with ptaquiloside, a Bracken constituent. *The Veterinary Record* **115**, 375-378.

Hirono, I., Ogino, H., Fujimoto, M., Yamada, K., Yoshida, Y., Ikagawa, M. and Okumura, M. (1987) Induction of tumors in ACI rats given a diet containing ptaquiloside, a Bracken carcinogen. *Journal of the National Cancer Institute* **79**, 1143-1149.

Hirono, I., Shibuya, C., Shimisu, M. and Fushimi, K. (1972) Carcinogenic activity of processed Bracken used as human food. *Journal of the National Cancer Institute* **48**, 1245-1249.

Hirono, I. and Yamada, K. (1987) Bracken fern. In: Hirono, I., (Ed.) *Naturally Occuring Carcinogens of Plant Origin*, pp. 87-120. Tokyo - Amsterdam: Kodansha & Elsevier. Japan.

Hirono, I., Yamada, K., Niwa, H., Shizuri, Y., Ojika, M., Hosaka, S., Yamaji, T., Wakamatsu, K., Kigoshi, H., Niiyama, K. and Uosaki, Y. (1984b) Separation of carcinogenic fraction of Bracken fern. *Cancer Letters* **21**, 239-246.

Hopkins, N.C. (1987) Enzootic haematuria in Nepal. *Tropical Animal Health and Production* **19**, 159-164.

Hoque, M., Somvanshi, R., Singh, G.R. and Mogha, I.V. (2002) Ultrasonographic evaluation of urinary bladder in normal, fern fed and enzootic bovine haematuria-affected cattle. *Journal of Veterinary Medicine, A* **49**, 403-407.

Humphrey, J.W. and Swaine, M.D. (1997) Factors affecting the natural regeneration of *Quercus* in Scottish oakwoods. I. Competition from *Pteridium aquilinum*. *Journal of Applied Ecology* **34**, 577-584.

Hunter, J.G. (1953) The composition of Bracken: some major- and trace-element constituents. *Journal of the Science of Food and Agriculture* **4**, 10-20.

Hutchinson, S.A. (1976) The effects of fungi on Bracken. *Botanical Journal of the Linnean Society* **73**, 145-150.

Imperato, F. (1995) Flavonol glycosides from *Pteridium aquilinum*. *Phytochemistry* **40**, 1801-1802.

Imperato, F. (1996) Kaempferol 3-*o*-(5"-feruloyalapioside) from *Pteridium aquilinum*. *Phytochemistry* **43**, 1421-1423.

Imperato, F. (1997) Rhamnetin 3-*o*-laminaribiocide from *Pteridium aquilinum*. *Phytochemistry* **45**, 1729-1730.

Imperato, F. (1998) Kaempferol 7-*o*-rhamnoside-4'-*o*-glucoside from *Pteridium aquilinum*. *Phytochemistry* **47**, 911-913.

Imperato, F. (1998a) Two new flavonol glycosides from the fern *Pteridium aquilinum*. ECSOC-2. Electronic citation: < http://ecsoc2.hcc.ru/posters/DP_TOP2/dp074/dp074.htm>.

Imperato, F. and Minutiello, P. (1997) Kaempferol 3-*o*-(6"-caffeoylglucoside) from *Pteridium aquilinum*. *Phytochemistry* **45**, 199-200.

Inspektøren for Hæren (1986) *Feltkundskab - Førstehjælp - Sanitetstjeneste (in English: Service duties - First aid - Field hygiene)*. 2nd edn. Inspektøren for Hæren. Denmark.

International Agency for Research on Cancer (IARC) - Summaries & Evaluations. Bracken fern (*Pteridium aquilinum*) and some of its constituents (1998). Electronic citation: <http://www.iarc.fr>.

Jarvis, M.C. and Duncan, H.J. (1976) Profile distribution of organic carbon, iron, aluminium and manganese in soils under Bracken and Heather. *Plant and Soil* **44**, 129-140.

Jenny, H. (1980) *The Soil Recource*. New York: Springer-Verlag Inc. USA.

Johansen, M.S. (2003) Variation in the ptaquiloside content in a Bracken stand at Præstø Fed, Denmark. **Unpublished work**. Chemistry Department, the Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

Johnson-Maynard, J.L., McDaniel, P.A., Ferguson, D.E. and Falen, A.L. (1997) Chemical and mineralogical conversion of Andisols following invasion by Bracken fern. *Soil Science Society of America Journal* **61**, 549-555.

Johnson-Maynard, J.L., McDaniel, P.A., Ferguson, D.E. and Falen, A.L. (1998) Changes in soil solution chemistry of Andisols following invasion by Bracken fern. *Soil Science* **163**, 814-821.

Jones, C.G. (1983) Phytochemical variation, colonization, and insect communities: the case of Bracken fern. In: Denno, R.F. and McClure, M.S., (Eds.) *Variable Plants and Herbivores in Natural and Managed Systems*, pp. 513-558. New York: Academic Press, Inc. USA.

Jones, C.G. and Firn, R.D. (1979) Resistance of *Pteridium aquilinum* to attack by non-adapted phytophagous insects. *Biochemical Systematics and Ecology* **7**, 95-101.

Kaplanis, J.N., Thompson, M.J., Robbins, W.E. and Bryce, B.M. (1967) Ecdysone. *Science* **157**, 1436-1436.

Kaufman, P.B., Cseke, L.J., Warber, S., Duke, J.A. and Brielmann, H.L. (1999) *Natural Products from Plants*. Boca Raton: CRC Press. USA

Kawai, T., Takanashi, H., Nakayama, M., Mori, H. and Hirono, I. (1981) Effect of storage on carcinogenic activity of Bracken fern. *Cancer Letters* **12**, 29-35.

Kendall, A., Page, C.N. and Taylor, J.A. (1995) Linkages between Bracken sporulation rates and weather and climate in Britain. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 77-81. Aberystwyth: International Bracken Group. USA.

Kigoshi, H., Sawada, A., Imamura, Y., Niwa, H. and Yamada, K. (1989) Ptaquilosin, the aglycone of a Bracken carcinogen ptaquiloside: chemical derivation from ptaquiloside and the reactivity. *Tetrahedron* **45**, 2551-1556.

Kobayashi, A., Egawa, H., Koshimizu, K. and Mitsui, T. (1975) Antimicrobial constituents in *Pteris inequalis* Bak. *Agricultural and Biological Chemistry* **39**, 1851-1856.

Kofod, H. and Eyjolfsson, R. (1966) The isolation of the cyanogenic glycoside prunasin from *Pteridium aquilinum* (L.) Kühn. *Tetrahedron Letters* **12**, 1289-1291.

Koyama, K., Takatsuki, S. and Natori, S. (1991) Dennstoside A, an analogue of ptaquiloside, from *Dennstaedtia scabra*. *Phytochemistry* **30**, 2080-2082.

Kushida, T., Uesugi, M., Sugiura, Y., Kigoshi, H., Tanaka, H., Hirokawa, J., Ojika, M. and Yamada, K. (1994) DNA damage by ptaquiloside. A potent bracken carcinogen: detection of selective strand

breaks and identification of DNA cleavage products. *Journal of the American Chemical Society* **116**, 479-486.

Langenheim, J.H. (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. *Journal of Chemical Ecology* **20**, 1223-1280

Lawson, B. (1989) A veterinary practice in South Gwynedd. In: Taylor, J.A., (Ed.) *Bracken Toxicity and Carcinogenicity as Related to Animal and Human Health*, pp. 36-39. Aberystwyth: International Bracken Group. United Kingdom.

Le Duc, M.G., Pakeman, R.J., Putwain, P.D. and Marrs, R.H. (2000) The variable responses of Bracken fronds to control treatments in Great Britain. *Annals of Botany* **85**, 17-29.

Leach, H.M. (2000) European perceptions of the roles of Bracken rhizomes (*Pteridium esculentum* (Forst. F.) Cockayne) in traditional Maori diet. *New Zealand Journal of Archaelogy* **22**, 31-43.

Low, V.H.K. and Thomson, J.A. (1990) Cyanogenesis in Australian Bracken (*Pteridium esculentum*): distribution of cyanogenic phenotypes and factors influencing activity of the cyanogenic glucosidase. In: Thomson, J.A. and Smith, R.T., (Eds.) *Bracken Biology and Management*, pp. 105-111. Wahroonga: The Australian Institute of Agricultural Science. Australia.

Lowday, J.E. (1986) A comparison of the effects of cutting with those of the herbicide asulam on the control of bracken. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology - The Proceedings of the International Conference - Bracken '85*, pp. 359-367. Aberystwyth: Parthenon Publishing. United Kingdom.

Mackney, D. (1961) A podzol development sequence in oakwoods and heath in Central England. *Journal of Soil Science* **12**, 23-40.

Madden, U.A. and Stahr, H.M. (1993) Preliminary determination of mycotoxin binding to soil when leaching through soil with water. *International Biodeterioration and Biodegradation* **31**, 265-275.

Majak, W. (2001) Review of toxic glycosides in rangeland and pasture forages. *Journal of Range Management* **54**, 494-498.

Marlière, C.A., Santos, R.C., Galvao, M.A.M., Soares, J.F., Evangelista, C.L.M. and Gomes, R.F.Q. (2000) Ingestao de broto de samambaia e risco de cancer de esofago e estomago na regiao de Ouro Preto, MG. *Revista Brasileira de Cancerologia* **44**, 225-229.

Marlière, C.A., Wathern, P., Freitas, R.N., Castro, M.C.F.M. and Galvao, M.A.M. (2000a) Bracken fern (*Pteridium aquilinum*) consumption and oesophaegal and stomach cancer in the Ouro Preto region, Minas Gerais, Brazil. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 144-149. Aberystwyth: International Bracken Group. United Kingdom.

Marlière, C.A., Wathern, P., Castro, M.C.F.M., O'Connor, P., and Galvao, M.A. (2002) Bracken fern (*Pteridium aquilinum*) ingestion and oesophageal and stomach cancer. *IARC Scientific Publications* **156**, 379-380.

Marrero, E., Bulnes, C., Sánchez, L.M. and Palenzuela, I. (2001) *Pteridium aquilinum* (Bracken fern) toxicity in cattle in the humid Chaco of Tarija, Bolivia. *Veterinary and Human Toxicology* **43**, 156-158.

Marrs, R.H., Le Duc, M.G., Mitchell, R.J., Goddard, D., Paterson, S. and Pakeman, R.J. (2000) The ecology of Bracken: its role in succession and implications for control. *Annals of Botany* **85**, 3-15.

Martin, D.J. (1976) Control of Bracken. *Botanical Journal of the Linnean Society* **73**, 241-246.

Matoba, M., Saito, E., Saito, K., Koyama, K., Natori, S., Matsushima, T. and Takimoto, M. (1987) Assay of ptaquiloside, the carcinogenic principle of Bracken, *Pteridium aquilinum*, by mutagenicity testing in *Salmonella typhimurium*. *Mutagenesis* **2**, 419-423.

Matsuoka, A., Hirosawa, A., Natori, S., Iwasaki, S., Sofuni, T. and Ishidate, M. (1989) Mutagenicity of ptaquiloside, the carcinogen in Bracken, and its related illudane-type sesquiterpenes. II. Chromosomal aberration tests with cultured mammalian cells. *Mutation Research* **215**, 179-185.

McCloud, T. G., Klueh, P. A., Pearl, K. C., Cartner, L. K., Muschik, G. M., and Poole, K. K. Isolation of illudin S from the mature basidiocarp of *Omphalotus illudens*, and isolation of illudin S and M from the fermentation broth of *Omphalotus olearis*. Electronic citation: <http://npsg.ncifcrf.gov/illudin.htm>.

McMorris, T.C., Kelner, M.J., Wang, W., Estes, L.A., Montoya, M.A. and Taetle, R. (1992) Structure-activity relationships of illudins: analogs with improved therapeutic index. *The Journal of Organic Chemistry* **57**, 6876-6883.

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. and McLaughlin (1982) Brine Shrimp: a convenient general bioassay for active plant constituents. *Journal of Medicinal Plant Research* **45**, 31-34.

Miljøstyrelsen (1992) *Sundhedsmæssig Vurdering af Kemiske Stoffer i Drikkevand (in English: Health Safety Assessment of Chemical Compounds in Drinkingwater).* Copenhagen: Miljøstyrelsen. Denmark.

Moura, J.W., Stocco dos Santos, R.C., Dagli, M.L., D'Angelino, J.L., Birgel, E.H., and Beçak, W. (1988) Chromosome aberrations in cattle raised on Bracken fern pasture. *Experientia*, **44**, 785-788.

Munro, D. (1996) *Pteridium aquilinum* poisoning. Electronic citation: <http://res.agr.ca/cgi-bin/brd/poisonpl/ddplant5?plant=Pteridium+aquilinium&info=all&name=sci>.

Musonda, C.A. and Chipman, J.K. (2000) Carcinogenic and anti-carcinogenic potential of quercetin. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 106-115. Aberystwyth: International Bracken Group. United Kingdom.

Nagao, T., Saito, K., Hirayama, E., Uchikoshi, K., Koyama, K., Natori, S., Morisaki, N., Iwasaki, S. and Matsushima, T. (1989) Mutagenicity of ptaquiloside, the carcinogen in Bracken, and its related illudane-type sesquiterpenes. I. Mutagenicity in *Salmonella typhimurium*. *Mutation Research* **215**, 173-175.

Nakabayashi, T. (1955) Isolation of astragalin and isoquercitrin from Bracken, *Pteridium aquilinum*. *Bulletin of the Agricultural and Chemical Society* **19**, 104-109.

Newman, J.D. and Chappell, J. (1999) Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway. *Critical Reviews in Biochemistry and Molecular Biology* **34**, 95-106.

Newsome, P.F.J. (1987) *The Vegetative Cover of New Zealand*. Wellington: National Water and Soil Conservation Authority. New Zealand.

Ngomuo, A.J. and Jones, R.S. (1996) Cytotoxicity studies of quercetin, shikimate, cyclohexanecarboxylate and ptaquiloside. *Veterinary and Human Toxicology* **38**, 14-18.

Nguyen, Q.V. (2000) *Pickled and Dried Asian Vegetables*. Barton: Rural Industries Research & Development Corporation. Australia.

Nicholson, I.A. and Paterson, I.S. (1976) The ecological implications of Bracken control to plant/animal systems. *Botanical Journal of the Linnean Society* **73**, 269-283.

Niwa, H., Ojika, M., Wakamatsu, K., Yamada, K., Hirono, I. and Matsushita, K. (1983) Ptaquiloside, a novel norsesquiterpene glucoside from Bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Letters* **24**, 4117-4120.

Niwa, H., Ojika, M., Wakamatsu, K., Yamada, K., Ohba, S., Saito, Y., Hirono, I. and Matsushita, K. (1983a) Stereochemistry of ptaquiloside, a novel norsesquiterpene glucoside from Bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Letters* **24**, 5371-5372.

Oelrichs, P.B., Ng, J.C. and Bartley, J. (1995) Purification of ptaquiloside, a carcinogen from *Pteridium aquilinum*. *Phytochemistry* **40**, 53-56.

Ojika, M., Kigoshi, H., Kuyama, H., Niwa, H. and Yamada, K. (1985) Studies on *Pteridium aquilinum* var. *latiusculum*, IV. Isolation of three *p*-hydroxystyrene glycosides and an efficient method for the isolation of ptaquiloside, an unstable Bracken carcinogen. *Journal of Natural Products* **48**, 634-637.

Ojika, M., Sugimoto, K., Okazaki, T. and Yamada, K. (1989) Modification and cleavage of DNA by ptaquiloside. A new potent carcinogen isolated from Bracken Fern. *Journal of the Chemical Society,Chemical Communications* **22**, 1775-1777.

Ojika, M., Wakamatsu, K., Niwa, H. and Yamada, K. (1987) Ptaquiloside, a potent carcinogen isolated from Bracken fern *Pteridium aquilinum* var. *latiusculum*: structure elucidation based on chemical and spectral evidence, and reactions with amino acids, nucleosides, and nucleotides. *Tetrahedron* **43**, 5261-5274.

Ojika, M., Wakamatsu, K., Niwa, H. and Yamada, K. (1987a) Structures of new p-hydroxystyrene glycosides, ptelatoside-A and ptelatoside-B isolated from Bracken fern, *Pteridium aquilinum* var. *latiusculum*, and synthesis of ptelatoside-A. *Tetrahedron* **43**, 5275-5280.

Ortega, M. (1994) Ethnobotanical notes on the Bracken fern (*Pteridium aquilinum* (L.) Kuhn) in the Amazon region and possible carcinogenic risks. *Biollania* **10**, 7-12.

Owen, K.M., Marrs, R., Snow, C.S.R. and Evans, C.E. (1999) Soil acidification - the use of sulphur and acidic plant materials to acidify arable soils for the recreation of heathland and acidic grassland at Minsmere, UK. *Biological Conservation* **87**, 105-121.

Padwa, A., Sandanayaka, V.P. and Curtis, E.A. (1994) Synthetic studies toward illudins and ptaquilosin. A highly convergent approach via the dipolar cycloaddition of carbonyl ylides. *Journal of the American Chemical Society* **116**, 2667-2668.

Page, C.N. (1976) The taxonomy and phytogeography of Bracken - a review. *Botanical Journal of the Linnean Society* **73**, 1-34.

Page, C.N. (1990) Taxonomic evaluation of the fern genus *Pteridium* and its active evolutionary state. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 23-34. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Pakeman, R.J. and Hay, E. (2001) Heathland seedbanks under Bracken *Pteridium aquilinum* (L.) Kuhn and their importance for re-vegetation after Bracken control. *Journal of Environmental Management* **47**, 329-339.

Pakeman, R.J., Le Duc, M.G. and Marrs, R.H. (2000) Bracken distribution in Great Britain: strategies for its control and the sustainable management of marginal land. *Annals of Botany* **85**, 37-46.

Pamukcu, A.M., Olson, C. and Price, J.M. (1966) *Cancer Research* **26**, 1745.

Pamukcu, A.M., Yalçiner, S., Hatcher, J.F. and Bryan, G.T. (1980) Quercetin, a rat intestinal and bladder carcinogen present in Bracken fern (*Pteridium aquilinum*). *Cancer Research* **40**, 3468-3472.

Paterson, S., Pakeman, R.J. and Marrs, R. (1997) Evaluation of a Bracken (*Pteridium aquilinum* (L.) Kuhn) growth model in predicting the effects of control strategies across a range of climatic zones in Great Britain. *Annals of Applied Biology* **130**, 305-318.

Pinto, C., Geraldes, M., Medeiros, F., Pacheco, P. and Louza, A.C. (1997) Slaughterhouse monitoring of cattle neoplasias on São Miguel Island, Azores. *Epidémiol santé animale*, p. 04.A.40.

Pinto, C., Lima, R., Louza, A.C., Almeida, V., Melo, M., Vaz, Y., Fonseca, I.N., Lauren, D.R. and Smith, B.L. (1999) Bracken fern-induced *Bovine Enzootic Haematuria* in Sao Miguel Island, Azores. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control - Proceedings of the International Bracken Group Conference, Manchester 1999*, pp. 136-140. Leeds: The International Bracken Group. United Kingdom.

Pitman, R. and Webber, J. (1998) Bracken as a peat alternative. Edinburgh: The Forestry Authority. United Kingdom.

Potter, D.M. (2000) The *Pteridaceae* as a source of compounds with pharmaceutical activity. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester*, pp. 60-67. Aberystwyth: International Bracken Group. United Kingdom.

Potter, D.M. and Pitman, R.M. (1994) The extraction and characterization of carcinogens from Bracken and the effect of composting. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 110-115. Aberystwyth: International Bracken Group. United Kingdom.

Povey, A.C., Evans, I.A., Taylor, J.A. and O'Connor, P.J. (1995) Detection of DNA adducts by ^{32}P-postlabelling in Mice treated with Bracken extract and Bracken spores. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 95-98. Aberystwyth: International Bracken Group. United Kingdom.

Prakash, A.S., Pereira, T.N., Smith, B.L., Shaw, G. and Seawright, A.A. (1996) Mechanism of Bracken fern carcinogenesis: evidence for H-ras activation via initial adenine alkylation by ptaquiloside. *Natural Toxins* **4**, 221-227.

Price, J.M. and Pamukcu, A.M. (1968) The induction of neoplasms of the urinary bladder of the Cow and the small intestine of the Rat by feeding Bracken fern (*Pteris aquilina*). *Cancer Research* 2251.

Project Runeberg (1997) Bilder ur Nordens Flora (in English: Pictures of the Nordic Flora). Electronic citation: <http://lysator.liu.se>.

Ralphs, M.H. (2002) Ecological relationships between poisonous plants and rangeland condition: a review. *Journal of Range Management* **55**, 285-290.

Rasmussen, L.H., Donnelly, E., Hansen, H.C.B., Strobel, B.W. and Holm, P.E. (2003) Ptaquiloside in Bracken (*Pteridium aquilinum* ssp. *aquilinum*) in Scotland. Contents in fronds, rhizomes, and litter. *Journal of Chemical Ecology*. **Submitted**.

Rasmussen, L.H. and Hansen, H.C.B. (2003) Growth of Bracken in Denmark and the content of ptaquiloside in fronds, rhizomes and roots. In: Acamovic, T., Stewart, C.S. and Pennycott, T.W., (Eds.) *Poisonous Plants and Related Toxins. **(In press)***. Wallingford: CAB International. United Kingdom.

Rasmussen, L.H., Jensen, L.S. and Hansen, H.C.B. (2003a) Distribution of the carcinogenic terpene ptaquiloside in Bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *Journal of Chemical Ecology* **29**, 771-778.

Rasmussen, L.H., Kroghsbo, S., Frisvad, J.C. and Hansen, H.C.B. (2003b) Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere* **51**, 117-127.

Rasmussen, L.H., Lauren, D.R. and Hansen, H.C.B. (2003c) Sorption, degradation and mobility of ptaquiloside, a carcinogenic Bracken (*Pteridium* sp.) constituent, in the soil environment. *Chemosphere.* **Submitted**.

Rasmussen, L.H., Lauren, D.R., Smith, B.L. and Hansen, H.C.B. (2003d) Variation in the ptaquiloside content in Bracken from New Zealand. *Journal of Chemical Ecology*. **Submitted**.

Rasmussen, L.H., Schmidt, B., Lauren, D.R., Olsen, C.E., Engelsen, S.B., and Hansen, H.C.B. (2003e) A refined method for isolation of pure ptaquiloside from Bracken (*Pteridium aquilinum* (L.) Kuhn.). *Journal of Agricultural and Food Chemistry.* **Submitted**.

Rook, E. J. S. (2002) *Pteridium aquilinum* Bracken fern. Electronic citation: <http://www.rook.org /earl/bwca/nature/ferns/pteridiumaqui.html>.

Rumpf, S., Cromey, M. and Webb, C.J. (1994) Ultrastructure and function of the nectaries of New Zealand Bracken (*Pteridium esculentum* (Forst. f.) Cockayne). *New Zealand Journal of Botany* **32**, 487-496.

Rymer, L. (1976) The history and ethnobotany of Bracken. *Botanical Journal of the Linnean Society* **73**, 151-176.

Saito, K., Nagao, T., Matoba, M., Koyama, K., Natori, S., Murakami, T. and Saiki, Y. (1989) Chemical assay of ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the Pteridaceae. *Phytochemistry* **28**, 1605-1611.

Saito, K., Nagao, T., Takatsuki, S., Koyama, K. and Natori, S. (1990) The sesquiterpenoid carcinogen of Bracken fern, and some analogues, from the Pteridaceae. *Phytochemistry* **29**, 1475-1479.

Saito, M., Umeda, M., Enomoto, M., Hatanaka, Y., Natori, S., Yoshihira, K., Fukuoka, M. and Kuroyanagi, M. (1975) Cytotoxicity and carcinogenicity of pterosins and pterosides, 1-indanone derivatives from Bracken (*Pteridium aquilinum*). *Experientia* **31**, 829-831.

Saito, T., Kurata, Y. and Takeno, K. (1990a) Characteristics of histamine release from Rat mast cells induced by a Bracken toxin, braxin A1. *Japanese Journal of Pharmacology* **53**, 165-173.

Saito, T. and Mochizuki, D. (1986) Isolation of two active glucosides, braxins A1 and A2 from rhizomes of Bracken fern. *Journal of Toxicological Science* **11**, 15-27.

Saito, T., Takeno, K., Nakamura, S. and Uehara, M. (1987) Acute poisoning with braxin A1, a Bracken glucoside, in Guinea Pigs. *Japanese Journal of Veterinary Science* **49**, 181-183.

Santos, R.C., Brasileiro-Filho, G. and Hojo, E.S. (1987) Induction of tumors in rats by Bracken fern (*Pteridium aquilinum*) from Ouro Preto (Minas Gerais, Brazil). *Brazilian Journal of Medical and Biological Research* **20**, 73-77.

Saveria Campo, M. (2002) Animal models of papillomavirus pathogenesis. *Virus Research* **89**, 249-261.

Saxena, D., Flores, S. and Stotzky, G. (1999) Insecticidal toxin in root exudates from *Bt*-corn. *Nature* **402**, 480-480.

Schoental, R. (1984) Bracken toxicity and soil mycotoxins. *The Veterinary Record* **115**, 500-500.

Schrader, A., Schultz, O., Völker, H. and Puls, H. (2001) Actual plant intoxications in ruminants of North-Eastern Germany. *Berliner Münchener Tierärztlicher Wochenschrift* **114**, 218-221.

Schreiner, I., Nafus, D. and Pimentel, D. (1984) Effects of cyanogenesis in Bracken fern (*Pteridium aquilinum*) on associated insects. *Ecological Entomology* **9**, 69-79.

SciFinder Scholar (2002). 87625-62-5 Ptaquiloside.

Shahin, M., Moore, M.R., Worrall, S., Smith, B.L., Seawright, A.A. and Prakash, A.S. (1998) H-ras activation is an early event in the ptaquiloside-induced cacinogenesis: comparison of acute and chronic toxicity in rats. *Biochemical and Biophysical Research Communications* **250**, 491-497.

Shahin, M., Smith, B.L. and Prakash, A.S. (1999) Bracken carcinogens in the human diet. *Mutation Research* **443**, 69-79.

Shahin, M., Smith, B.L., Worral, S., Moore, M.R., Seawright, A.A. and Prakash, A.S. (1998a) Bracken fern carcinogenesis: multiple intravenous doses of activated ptaquiloside induce DNA adducts, monocytosis, increased TNF alpha levels, and mammary glad carcinoma in rats. *Biochemical and Biophysical Research Communications* **244**, 192-197.

Sheffield, E., Wolf, P.G. and Haufler, C.H. (1989) How big is a Bracken plant? *Weed Research* **29**, 455-460.

Shorina, N.I. (1990) The structure of Bracken fern stands in relation to morphology. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 52. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Silva, M.E., Brasileiro-Filho, G., Santos, R.C. and Freitas, R.N. (2000) The effect of pretreatment with vitamin E on Bracken tumorigenicity in rats. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 128-131. Aberystwyth: International Bracken Group. United Kingdom.

Simán, S.E., Povey, A.C., O'Connor, P.J., Ward, T.H., Margison, G.P. and Sheffield, E. (2000) The genotoxicity of fern spores. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 99-105. Aberystwyth: International Bracken Group. United Kingdom.

Simán, S.E., Povey, A.C. and Sheffield, E. (1999) Human health risks from fern spores? - A review. *Fern Gazette* **15**, 275-287.

Simán, S.E., Povey, A.C., Ward, T.H., Margison, G.P. and Sheffield, E. (2000a) Fern spore extracts can danage DNA. *British Journal of Cancer* **83**, 69-73.

Skeffington, R.A. (1983) Soil properties under three species of tree in southern England in relation to acid deposition in throughfall. In: Ulrich, B. and Pankrath, J., (Eds.) *Effects of Accumulation of Air Pollutants in Forest Ecosystems*, pp. 219-231. Dordrecht: D. Reidel Publishing Company. Holland

Smith, B.L. (1997) The toxicity of Bracken fern (genus *Pteridium*) to animals and its relevance to Man. In: Felix D'Mello, J.P., (Ed.) *Handbook of Plant and Fungal Toxicants*, pp. 63-76. New York: CRC Press. USA:

Smith, B.L. and Beatson, N.S. (1970) *Bovine Enzootic Haematuria* in New Zealand. *New Zealand Veterinary Journal* **18**, 115-120.

Smith, B.L., Embling, P.P., Agnew, M.P., Lauren, D.R. and Holland, P.T. (1988) Carcinogenicity of Bracken Fern (*Pteridium esculentum*) in New Zealand. *New Zealand Veterinary Journal* **36**, 56-58.

Smith, B.L., Embling, P.P., Lauren, D.R. and Agnew, M.P. (1989) Carcinogen in Rock Fern (*Cheilanthes sieberi*) from New Zealand and Australia. *Australian Veterinary Journal* **66**, 154-155.

Smith, B.L., Embling, P.P., Lauren, D.R. and Agnew, M.P. (1992) Carcinogenicity of *Pteridium esculentum* and *Cheilanthes sieberi* in Australia and New Zealand. In: James, L.F., Keeler, R.F., Bailey, E.M., Cheeke, P.R. and Hegarty, M.P., (Eds.) *Poisonous plants. Proceedings of the Third International Symposium*, pp. 448-448. Iowa State University Press. USA.

Smith, B.L., Lauren, D.R., Embling, P.P. and Agnew, M.P. (1990) Ptaquiloside in Australian and New Zealand ferns as a cause of Neoplasia. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 241-246. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Smith, B.L., Lauren, D.R., Embling, P.P. and Agnew, M.P. (1993) Carcinogenicity of Bracken fern (*Pteridium* spp.) in relationship to grazing ruminants. In: Broughan, J., (Ed.) *Proceedings of the 17th International Grasslands Congress*, pp. 1396-1398. New Zealand Grasslands Association. New Zealand.

Smith, B.L., Lauren, D.R. and Prakash, A.S. (2000) Bracken fern (*Pteridium*): toxicity in animal and human health. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 76-85. Aberystwyth: International Bracken Group. United Kingdom.

Smith, B.L. and Seawright, A.A. (1995) Bracken fern (*Pteridium* spp.) carcinogenicity and human health - a brief review. *Natural Toxins* **3**, 1-5.

Smith, B.L., Seawright, A.A., Ng, J.C., Hertle, A.T., Thomson, J.A. and Bostock, P.D. (1994) Concentration of ptaquiloside, a major carcinogen in Bracken fern (*Pteridium* spp.), from eastern Australia and from a cultivated worldwide collection held in Sydney, Australia. *Natural Toxins* **2**, 347-353.

Smith, R.T. (2000) The spread of Bracken: an end-of-century assessment of factors, risks and land use realities. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control.*

Proceedings of the International Bracken Group Conference Manchester 1999, pp. 2-8. Aberystwyth: International Bracken Group. United Kingdom.

Soil Survey Staff (1999) *Keys to Soil Taxonomy,* 8th edn. Blacksburg, Virginia: Pocahontas Press. USA.

Somogyi, J.C. (1971) On antithiamine factors of fern. *The Journal of Vitaminology* **17**, 165-174.

Speer, W.D. (2000) A systematic assessment of British and North American *Pteridium* using cpDNA gene sequences. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester*, pp. 37-42. Aberystwyth: International Bracken Group. United Kingdom.

Stocco dos Santos, R.C., Lindsey, C.J., Ferraz, O.P., Pinto, J.R., Mirandola, R.S., Benesi, F.J., Birgel, E.H., Pereira, C.A. and Becak, W. (1998) Bovine papillomavirus transmission and chromosomal aberrations: An experimental model. *Journal of Genetics and Virology* **79**, 2127-2135.

Strobel, B.W., Jensen, P.H., Rasmussen, L.H. and Hansen, H.C.B. (2003) Thujone in soil under Western Red Cedar (*Thuja plicata*). *Chemosphere.* **Submitted**.

Suzuk, L., Noffsinger, A.E., Hui, A.E. and Fenoglio-Preiser, C.M. (1996) Detection of human papillomavirus in esophageal squamous cell carcinoma. *Cancer***78**, 704-710.

Svatos, A. and Macek, T. (1994) The rate of ecdysteroid production in suspension cultured cells of the fern *Pteridium aquilinum*. *Phytochemistry* **35**, 652-654.

Takatori, K., Nakano, S., Nagata, S., Okumura, K., Hirono, I. and Shimizo, M. (1972) Pterolactam, a new compound isolated from Bracken. *Chemical and Pharmaceutical Bulletin* **20**, 1087-1087.

Takemoto, T., Arihara, S. and Hikino, H. (1968) Structure of ponasteroside A, a novel glycoside of insect-moulting substance from *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Letters* **39**, 4199-4202.

Takemoto, T., Arihara, S., Hikino, Y. and Hikino, H. (1968a) Isolation of insect moulting substances from *Pteridium aquilinum* var. *latiusculum*. *Chemical and Pharmaceutical Bulletin* **16**, 762.

Tanaka, N., Yuhara, H., Wada, H., Murakami, T., Cambie, R.C. and Braggins, J.E. (1993) Phenolic constituents of *Pteridium esculentum*. *Phytochemistry* **32**, 1037-1039.

Taylor, J. (1999) Perceptions of Bracken. In: Taylor, J., (Ed.) *Bracken Perceptions and Bracken Control in the British Uplands - Papers given at a meeting held at the University of Lancaster, U.K. on 10th September 1998*, pp. 1-7. Ceredigion: International Bracken Group. United Kingdom.

Taylor, J.A. and Smith, R.T. (1995) Bracken 2000 and beyond. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 214-224. Aberystwyth: International Bracken Group. United Kingdom.

Taylor, J.E. and Thomson, J.A. (1998) Bracken litter as mulch: glasshouse evaluation of phytotoxicity. *Australian Journal of Experimental Agriculture* **38**, 161-169.

Teuscher, E. and Lindequist, U. (1994) *Biogene Gifte*. 2nd edn. Stuttgart: Gustav Fischer. Germany.

Thompson, T.R.E., Rudeforth, C.C., Hartnup, R., Lea, J.W. and Wright, P.S. (1986) Soil and slope conditions under Bracken in Wales. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken - Ecology, Land Use and Control Technology. The Proceedings of the International Conference - Bracken '85*, pp. 101-107. Carnforth: Parthenon Publishing. United Kingdom.

Thomson, J.A. (2000) Morphological and genomic diversity in the genus *Pteridium* (Dennstaedtiaceae). *Annals of Botany* **85**, 77-99.

Thomson, J.A. and Alonso-Amelot, M.E. (2002) Clarification of the taxonomic status and relationships of *Pteridium caudatum* (Dennstaedtiaceae) in Central and South America. *Botanical Journal of the Linnean Society* **140**, 237-248.

Togawa, K., Jaskiewicz, K., Takahashi, H., Meltzer, S.J. and Rustgi, A.K. (1994) Human papillomavirus DNA sequences in esophagus squamous cell carcinoma. *Gastroenterology* **107**, 128-136.

Tolhurst, K.G. (1990) Response of Bracken to low intensity prescribed fire in open Eucalypt forest in West-central Victoria. In: Thomson, J.A. and Smith, R.T. (Eds.). Bracken Biology and Management, pp. 53-62. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Tolhurst, K.G. and Burgman, M. (1994) Simulation of Bracken cover in forested areas in Victoria in response to season, overstory and fire conditions. *Australian Journal of Ecology* **19**, 306-318.

Tryon, R.M. (1941) A revision of the genus *Pteridium*. *Journal of the National English Botanical Club* **43**.

Turner, S. (2002) *Pteridium aquilinum*. Electronic citation: <http://www.wsdot.wa.gov/eesc/environmental/programs/culres/ethbot/m-p/Pteridium.htm>.

Twomey, D.F., Holt, G.J. and Reid, H.W. (2002) Malignant catarrhal fever in cattle with suspected Bracken poisoning. *The Veterinary Record* **October 19 2002**, 486-487.

Ushijima, J., Matsukawa, K., Yuasa, A. and Okada, M. (1983) Toxicities of Bracken fern in Guinea Pigs. *Japanese Journal of Veterinary Science* **45**, 593-602.

van der Hoeven, J.C.M., Lagerweij, W.J., Posthumus, M.A., van Veldhuizen, A. and Holterman, H.A.J. (1983) Aquilide A, a new mutagenic compound isolated from Bracken fern (*Pteridium aquilinum* (L.) Kuhn). *Carcinogenesis* **4**, 1587-1590.

Villalobos-Salazar, J. (1987) *Pteridium aquilinum* and gastric cancer. In: *Abstracts XXIII World Veterinary Congress - August 16-21, 1987, Montréal, Québec, Canada*, p. 90. Montreal: World Veterinary Association & Canadian Veterinary Medical Association. Canada.

Villalobos-Salazar, J., Hernandez, H., Meneses, A. and Salazar, G. (2000) Factors, which may affect ptaquiloside levels in milk: effects of altitude, Bracken fern growth stage, and milk processing. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, pp. 68-74. Aberystwyth: International Bracken Group. United Kingdom.

Villalobos-Salazar, J., Meneses, A. and Salas, J. (1990) Carcinogenic effects in mice of milk from cows fed on Bracken fern Pteridium aquilinum. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 247-251. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Villalobos-Salazar, J., Mora, J., Meneses, A. and Pashov, B. (1995) The carcinogenic effects of Bracken spores. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 102-103. Abeerystwyth: International Bracken Group. United Kingdom.

Watson, W.A., Terlecki, S., Patterson, D.S.P., Sweasey, D., Hebert, C.N. and Done, J.T. (1972) Experimentally produced retinal degeneration (*Bright Blindness*) in sheep. *British Veterinary Journal* **128**, 457-469.

Watt, A.S. (1940) Contributions to the ecology of Bracken (*Pteridium aquilinum*). I. The rhizome. *New Phytologist* **39**, 401-422.

Watt, A.S. (1976) The ecological status of Bracken. *Botanical Journal of the Linnean Society* **73**, 217-239.

Whitehead, D.C., Dibb, H. and Hartley, R.D. (1982) Phenolic compounds in soil as influenced by the growth of different plant species. *Journal of Applied Ecology* **19**, 579-588.

Whitehead, S.J. and Digby, J. (1995) Morphological and physiological behaviour of Bracken at advancing fronts: Implications for control. In: Smith, R.T. and Taylor, J.A., (Eds.) *Bracken: An Environmental Issue. Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 33-37. Aberystwyth: International Bracken Group. United Kingdom.

Whitehead, S.J. and Digby, J. (1997) The Morphology of Bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors - a Comparison of the Mature Stand and the Interface with Heather (*Calluna vulgaris* (L.) Hull) 1. The Fronds. *Annals of Applied Biology* **131**, 103-116.

Whitehead, S.J. and Digby, J. (1997a) The morphology of Bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors - a comparison of the mature stand and the interface with Heather (*Calluna vulgaris* (L.) Hull) 2. The rhizome. *Annals of Applied Biology* **131**, 117-131.

Williams, A.G., Kent, M. and Ternan, J.L. (1987) Quantity and quality of Bracken throughfall, stemflow and litterflow in a Dartmoor catchment. *Journal of Applied Ecology* **24**, 217-230.

Williams, G.H. and Foley, A. (1976) Seasonal variations in the carbohydrate content of Bracken. *Botanical Journal of the Linnean Society* **73**, 87-93.

Wilson, D., Donaldson, L.J. and Sepai, O. (1998) Should we be frightened of Bracken? A review of the evidence. *Journal of Epidemiology and Community Health* **52**, 812-817.

Wind, P. (2002) Månedens fokus: Ørnebregne (in English: Focus of the month: Bracken). *Naturens Verden* **11/12**, 14-17.

Wolf, P.G., Haufler, C.H. and Sheffield, E. (1988) Electrophoretic variation and mating systems of the clonal weed *Pteridium aquilinum* (L. Kuhn) (Bracken). *Evolution* **42**, 1350-1355.

Wolf, P.G., Haufler, C.H. and Sheffield, E. (1988a) Maintenance of genetic variation in the clonal weed *Pteridium aquilinum* (Bracken). *SAAS Bulletin of Biochemistry and Biotechnology* **1**, 46-50.

Wolf, P.G., Sheffield, E. and Haufler, C.H. (1990) Genetic attributes of Bracken as revealed by enzyme electrophoresis. In: Thomson, J.A. and Smith, R.T. (Eds.). *Bracken Biology and Management*, pp. 71-78. Occasional Publication No. 40, Australian Institute of Agricultural Science: Hawthorn. Australia.

Wolf, P.G., Sheffield, E., Thomson, J.A. and Sinclair, R.B. (1995) Bracken taxa in Britain: a molecular analysis. In: Smith, R.T. and Taylor, J., (Eds.) *Bracken: An Environmental Issue*, *Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994*, pp. 43-46. Aberystwyth: International Bracken Group. United Kingdom.

Wynn, J.M., Small, J.L., Pakeman, R.J. and Sheffield, E. (2000) An assessment of genetic and environmental effects on sporangial development in Bracken [*Pteridium aquilinum* (L.) Kuhn] using a novel quantitative method. *Annals of Botany* **85**, 113-115.

Xu, L.R. (1992) Bracken poisoning and enzootic haematuria in cattle in China. *Research in Veterinary Science* **53**, 116-121.

Yoshida, M. and Saito, T. (1994) Acute toxicity of braxin C, a Bracken toxin, in guinea pigs. *Journal of Toxicological Science* **1919**, 17-23.

Yoshida, M. and Saito, T. (1994a) Non-urotoxic induction of hemorrhagic cystitis by braxin C, a Bracken toxin, in Guinea Pigs. *The Journal of Toxicological Sciences* **19**, 55-59.

Yoshihira, K., Fukuoka, M., Kuroyanagi, M., Natori, S., Umeda, M., Morohoshi, T., Enomoto, M. and Saito, M. (1978) Chemical and toxicological studies on Bracken fern, *Pteridium aquilinum* var. *latiusculum*. I. Introduction, extraction and fractionation of constituents, and toxicological studies including carcinogenicity tests. *Chemical and Pharmaceutical Bulletin* **26**, 2346-2364.

Øllgaard, B. and Tind, K. (1993) *Scandinavian Ferns*. Copenhagen: Rhodos. Denmark.

APPENDICES

A **Calculation of tolerable concentration of ptaquiloside in drinking water.**

B **Calculation of equilibrium concentrations in soil solutions.**

Appendix A

CALCULATION OF TOLERABLE CONCENTRATION OF PTAQUILOSIDE IN DRINKING WATER

The Danish Environmental Protection Agency recommends the *One-hit* model for estimation of a *tolerable daily intake* of genotoxic compounds through drinking water (Miljøstyrelsen, 1992):

$$\textit{One hit}\ \text{model}: C = \frac{W_h \times I_t \times \left(\frac{L_e}{L}\right)^3 \times \frac{D \times le}{L_e}}{-\ln\left(\frac{1-P_t}{1-P_c}\right) \times \sqrt[3]{\frac{W_h}{W_a}} \times V_h}$$

where C (tolerable concentration of a compound in drinking water (mg L^{-1})); W_h (human body mass in kg (70 kg)); I_t (tolerable life-time risk (10^{-6})); L_e (actual age of laboratory test animals (number of days)); L (theoretical average life-time of laboratory test animals (days)); D (daily exposure of the carcinogen to the laboratory test animals (mg ($kg_{BODYWEIGHT}$·day)$^{-1}$)); le (exposure time (days)); P_t (tumour incidents among exposed laboratory test animals (0-1)); P_e (spontaneous tumour incidents among control laboratory test animals (0-1)); W_a (average weight of laboratory test animals (kg)); and Vh (daily intake of drinking water (adults: 2 L)).

The estimates of a tolerable concentration of ptaquiloside in Box 3.1 are based on the toxicological studies on rats by Hirono et al. (1984), Hirono et al. (1987) and Smith et al. (1988). These studies are made for estimation of Bracken/ptaquiloside carcinogenicity on laboratory animals, and it must be emphasized that the experiments are not optimised for estimation of a tolerable concentration of ptaquiloside in drinking water. For example, the dose of ptaquiloside is likely somewhat too large resulting in too high mortality of the test animals. The experiments are also somewhat short compared to what usually is performed

in toxicology testing. The estimates were checked by Karl-Heinz Cohr (M.Sc. Biochem) from the Danish Toxicology Centre (Kogle Alle 2, DK-2970 Hørsholm, Denmark).

Example: Smith et al. (1988).

Data obtained from Rat exposure to Taumarunui ferns and information's obtained from the authors.

Calculation of daily dose:	Period 1:	Period 2:	Average:
Average intake of food (g day^{-1}):	25	30	
Ptaquiloside in food (mg g^{-1}):	0.135	0.355	
Weight of laboratory animal (kg):	0.26	0.3	
Daily dose (mg ($kg_{BODYWEIGHT}$*day)$^{-1}$):	13.0	35.5	27.2

Variables

Wh	Human body mass (kg):	70
It	Tolerable life-time risk:	0.000001
Le	Actual age of laboratory test animals (days):	400
L	Theoretical average life-time of laboratory test animals (days):	1095
D	Daily exposure of the carcinogen (mg ($kg_{BODYWEIGHT}$*day)$^{-1}$):	27.2
le	Exposure time (day):	162
Pt	Tumour incidents among exposed laboratory test animals (0-1):	0.89
Pe	Tumour incidents among control laboratory test animals (0-1):	0.06
Wa	Average weight of laboratory test animals (kg):	0.3
Vh	Daily intake of drinking water (L):	2
C	Tolerable concentration of ptaquiloside in drinking water (ng/L):	1.4

Appendix B

CALCULATION OF EQUILIBRIUM CONCENTRATIONS IN SOIL SOLUTIONS.

The equilibrium concentrations in Table 6.1 are based on the measured concentrations reported in Rasmussen et al. (2003a) and:

1. Mineral soils: The correlation between $K_{FREUNDLICH}$, soil carbon and clay content (Eq. 6.2), and n = 1.
2. Organic soils: The $K_{FREUNDLICH}$ determined for Præstø soils normalized for carbon content ($C_{SOIL} = 0.032 \cdot f_{OC} \cdot C_{WATER}$) (Rasmussen et al. 2003).

Example 1: Præstø II, 100% saturation.

$$\text{Equilibrium}: C_{SOIL} = f_{OC} \cdot K_{OC} \cdot C_{WATER}^{n},\ f_{OC} = 31.3\%,\ K_{OC} = 0.032,\ \text{due to low concentration in soil water},\ n = 1.$$

$$C_{SOIL} = 31.3 \cdot 0.032 \cdot C_{WATER}^{1} \Leftrightarrow C_{WATER} = \frac{1}{1.002} \cdot C_{SOIL} \Leftrightarrow C_{WATER} = \frac{1}{1.002} \cdot C_{SOIL}$$

$$\text{Porosity}: 1 - \frac{\text{soil density}}{\text{density of soil particles}} = 1 - \frac{0.8\ \text{kg dm}^{-3}}{1.1\ \text{kg dm}^{-3}} = 27\%, \text{ corresponding to 800 g soil per dm}^3 \text{ and 270 mL soil water per dm}^3.$$

$$\text{Total amount of ptaquiloside per dm}^3 : \text{Amount of soil per dm}^3 \cdot C_{TOTALSOIL} = 800\ \text{g} \cdot 225\ \text{ng g}^{-1} = \underline{180{,}000\ \text{ng}}.$$

$$180{,}000\ \text{ng} = C_{SOIL} \cdot 800\ \text{g} + C_{WATER} \cdot 270\ \text{mL} \Leftrightarrow C_{SOIL} = 225\ \text{ng} - \frac{270\ \text{mL}}{800\ \text{g}} \cdot C_{WATER} \Leftrightarrow C_{SOIL} = 225\ \text{ng} - 0.3375 \cdot C_{WATER}$$

$$C_{WATER} = \frac{1}{1.002} \cdot C_{SOIL} = \frac{1}{1.002} \cdot (225\ \text{ng} - 0.3375 \cdot C_{WATER}) = \underline{\underline{168\ \mu\text{g L}^{-1}}}$$

$$\text{Percentage in soil water}: \frac{C_{WATER} \cdot \text{Volume of soil water}}{\text{Total amount of ptaquiloside}} = \frac{168\ \text{ng mL}^{-1} \cdot 270\ \text{mL}}{180{,}000\ \text{ng}} = \underline{\underline{25\%}}$$

Example 2: Løvenholm, 30% saturation,

$$\text{Equilibrium}: C_{SOIL} = K_{FREUNDLICH} \cdot C_{WATER}^{n}$$

$$K_{FREUNDLICH} = 0.0819 \cdot C(\%) + 0.0056 \cdot \text{clay}(\%) \Leftrightarrow K_{FREUNDLICH} = 0.0819 \cdot 2.5 + 0.0056 \cdot 1.8 = 0.2148$$

$$\text{Due to the low concentrations, } n = 1 \Rightarrow C_{WATER}^{1} = \frac{1}{0.2148} \cdot C_{SOIL} \Leftrightarrow C_{WATER} = 4.6555 \cdot C_{SOIL}$$

$$\text{Porosity}: 1 - \frac{\text{soil density}}{\text{density of soil particles}} = 1 - \frac{1.3\ \text{kg dm}^{-3}}{2.65\ \text{kg dm}^{-3}} = 50\%$$

$$\Rightarrow \text{corresponding to } 1{,}300\ \text{g soil per dm}^3 \text{ and } 30\% \cdot 500\ \text{mL} = 150\text{mL soil water per dm}^3.$$

$$\text{Total amount of ptaquiloside per dm}^3 : \text{Amount of soil per dm}^3 \cdot\ C_{TOTAL\ SOIL} = 1{,}300\ \text{g} \cdot 20\ \text{ng g}^{-1} = \underline{26{,}000\ \text{ng}}.$$

$$26{,}000\ \text{ng} = C_{SOIL} \cdot 1{,}300\ \text{g} + C_{WATER} \cdot 150\ \text{mL} \Leftrightarrow C_{SOIL} = 20\ \text{ng} - \frac{150\ \text{mL}}{1{,}300\ \text{g}} \cdot C_{WATER} \Leftrightarrow C_{SOIL} = 20\ \text{ng} - 0.1154 \cdot C_{WATER}$$

$$C_{WATER} = 4.6555 \cdot C_{SOIL} = 4.6555 \cdot (20\ \text{ng} - 0.1154 \cdot C_{WATER}) = \underline{\underline{61\ \mu\text{g L}^{-1}}}$$

$$\text{Percentage in soil water}: \frac{C_{WATER} \cdot \text{Volume of soil water}}{\text{Total amount of ptaquiloside}} = \frac{61\ \text{ng mL}^{-1} \cdot 150\ \text{mL}}{26{,}000\ \text{ng}} = \underline{\underline{35\%}}$$

ARTICLES AND MANUSCRIPTS

Due to restrictions from the publishers of the journals in which two of the papers in this thesis have been published, these papers are not present in this PDF. The papers can be found in:

Rasmussen, L. H., Jensen, L. S., & Hansen, H. C. B. (2003). Distribution of the Carcinogenic Terpene Ptaquiloside in Bracken Fronds, Rhizomes (Pteridium aquilinum), and Litter in Denmark. *Journal of Chemical Ecology, 29*, 771-778.
DOI: 10.1023/A:1022885006742

Rasmussen, L. H., Kroghsbo, S., Frisvad, J. C., & Hansen, H. C. (2003). Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere, 51*, 117-127.
DOI: 10.1016/S0045-6535(02)00694-X

Ptaquiloside in Bracken (*Pteridium aquilinum* ssp. *aquilinum*) in Scotland: Contents in fronds, rhizomes, and litter.

Lars Holm Rasmussen[*], Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Eric Donnelly, Department of Plant and Soil Science, School of Biological Sciences, University of Aberdeen, Scotland, United Kingdom.

Bjarne W. Strobel, Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Peter E. Holm, Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Hans Christian Bruun Hansen, Chemistry Department, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

[*] To whom correspondence should be addressed. E-mail: LHR@KVL.DK

Abstract—Compounds found in bracken (*Pteridium aquilinum* (L.) Kuhn) are suspected of causing cancer in humans and animals. Ingestion of bracken, or food and drinking water contaminated with bracken carcinogens may cause the cancer. The main carcinogenic principle in bracken is the water-soluble indanone-type compound ptaquiloside. The aim of the study was to investigate the annual and regional variation in the content of ptaquiloside in Scottish bracken and associated litter, hereby trying to identify environmental conditions influencing these contents. The ptaquiloside content in fronds, rhizomes and litter (Oi-horizons) was monitored during the growth season at 3 sites, and at 17 additional sites all over Scotland by the end of the growth season. Ptaquiloside contents in the fronds ranged between 50 and 5,800 μg/g, with highest concentrations recorded in May and June. Ptaquiloside contents in the rhizomes ranged between 10 and 660 μg/g, with the highest contents recorded by the end of the growth season. Ptaquiloside contents in bracken litter ranged between 0.1 and 5.8 μg/g, which tended to increase in the autumn. This is the first report of the annual variation in ptaquiloside concentrations in bracken litter. The latitude and altitude of the stand, the frond development stage and content correlated with the amount of ptaquiloside found in the litter, while the content in rhizomes were correlated with the longitude, topography, and nitrogen content in the soil. This is the first report correlating the content of ptaquiloside in rhizomes with external growth factors. The content in the fronds were partly correlated with the longitude and aspect of the stand, the frond-height and the degree of damage to the fronds. Ptaquiloside contents increased in fronds that emerged following harvest compared to uncut fronds of the same development stage. Bracken management should therefore be undertaken with great care if animals are to graze on paddocks having managed brackens.

Keywords—Ptaquiloside, bracken, *Pteridium aquilinum*, Scotland, frond, rhizome, litter.

INTRODUCTION

In Scotland, bracken (*Pteridium aquilinum* (L.) Kuhn) is found as an invading weed all over the country, mainly on acid soils in forests, in paddocks, on moors and on less-intensively managed hilly areas (Frankland, 1976; Page, 1997). Bracken is well defined at the generic level, but uncertainties are found regarding the nomenclature and taxonomy within the genus, especially in recent literature (Thomson, 2000; Thomson and Alonso-Amelot, 2002). To allow comparison with older literature, the taxonomy introduced by Tryon (1941) is adapted in this paper: Bracken belongs to the family Dennstaedtiaceae, and the species *P. aquilinum* is divided into two subspecies: ssp. *caudatum* and *aquilinum*, of which only ssp. *aquilinum* is found in Europe. Two varieties of ssp. *aquilinum* grow in Europe: Common Bracken (var. *aquilinum* - Western and Southern Europe) and Pinewood Bracken (var. *latiusculum* - Northern and Eastern Europe). Common and Pinewood Bracken are both found in Scotland as separate species and as hybrids. Common Bracken is although the most widespread variety in Scotland while Pinewood Bracken is mainly found at the westcoast (Page, 1989).

Ptaquiloside is a nor-sesquiterpene glycoside found in all bracken varieties (Figure 1). Ptaquiloside is a compound of environmental and veterinary concern due to its carcinogenic effects to animals and Man. Bracken-fern consumption by browsing animals, especially by cattle and sheep, may lead to severe diseases such as the *Acute Bracken Fern Poisoning* and *Bovine Enzootic Haematuria* (BEH) (Smith, 1997), while bracken consumption by Man may lead to gastric or oesophaegal cancer (Haenszel et al., 1976; Marlière et al., 2000). Ptaquiloside has prooved to be carcinogenic, mutagenic and genotoxic (van der Hoeven et al., 1983; Ojika et al., 1987; Ojika et al., 1989; Kushida et al., 1994; Shahin et al., 1998). Recently, Marliére et al. (2002) published a study demonstrating a 8.1-fold greater chance of developing oesophageal cancer among people including bracken in their daily intake. Human intake of ptaquiloside with milk contaminated with ptaquiloside may occur in some

areas, as up to 9% of ptaquiloside in bracken digested by cows is transferred to the milk. Water contamination may also occur as at least 0.2% of the total ptaquiloside present in pinnae can be leached by a single rain-event resulting in a frond-drip concentration of up to 730µg/liter (Galpin and Smith, 1986; Alonso-Amelot et al., 1998; Rasmussen et al., 2003a; Alonso-Amelot et al., 2000b)

Ptaquiloside is stable at neutral pH but degrades rapidly at alkaline conditions or when pH less than 4. In the pH-range of 4-6.5 ptaquiloside can exist for months - even in contact with soil. Ptaquiloside is highly water-soluble and is very poorly sorbed to soil organic matter. However, irreversible sorption to soil components may take place under specific conditions (Rasmussen et al., 2003c).

In the temperate parts of Northern Europe, bracken fronds emerge above the soil surface from rhizomes in mid-May in the form of tightly curled crosiers, with exapansion triggered by increasing soil temperatures (Ader, 1990; Page, 1997). The fronds reach maturity from late July to early September, which is followed by senescence. In the United Kingdom frond heights from 60 to 110 cm are typical in open ecosystems, while the fronds are higher in forests (150 cm or more) (Watt, 1976; Whitehead and Digby, 1997). Typically, the frond density is between 16 and 60 fronds m^{-2} in the United Kingdom (Nicholson and Paterson, 1976; Watt, 1976; Callaghan et al., 1984; Whitehead and Digby, 1997).

The ptaquiloside content in fronds decreases gradually during the growth season but never reaches zero. Maximum is found in the newly emerged crosiers, e.g. vars. *caudatum* and *arachnoideum* in Venezuela both show maximum ptaquiloside contents when in the crosier-stage, decreasing rapidly to 10% of maximum after a few weeks. In 7-8 weeks old and older fronds, the ptaquiloside contents remains consistently low (Saito et al., 1989; Alonso-Amelot et al., 1992; Alonso-Amelot et al., 1995). The annual variation in

ptaquiloside content in var. *aquilinum* fronds from Denmark follows this pattern, although not as marked (Rasmussen and Hansen, 2003). The ptaquiloside contents in bracken fronds of all varieties ranges between 0-15,000 µg/g, from 100-9,800 µg/g in var. *aquilinum* and 40-1,800 µg/g in var. *latiusculum* (Smith et al., 1994). There are no significant differences between varieties, but genetic heritage may influence the content and explain the occurrence of fronds free of ptaquiloside (Smith et al., 1994). Smith et al. (1994) reported that fronds from var. *esculentum* sampled in South Australia contained higher ptaquiloside contents compared with those from the North Australia. The pattern was consistent when the ferns were grown at similar conditions for several years.

Rasmussen and Hansen (2003) investigated the annual variation in the ptaquiloside content in the rhizomes of var. *aquilinum* growing in Denmark, and reported contents between 2 and 7,046 µg/g. Generally, rhizome contents fell from those in spring, increasing to maximum after fronds had reached maturity.

Ptaquiloside contents ranging from 0.09 to 8.49 µg/g have been recorded in bracken litter in Denmark. In associated topsoil, levels between 0.01 and 0.09 µg/g were found (Rasmussen et al. 2003a; Rasmussen et al. 2003b).

Various studies have investigated the environmental/edaphic causes for variations in ptaquiloside contents in fronds. The content in fronds was correlated with altitude in South America (vars. *arachnoidum* and *caudatum*; Alonso-Amelot et al., 1995; Alonso-Amelot et al., 2000a; Villalobos-Salazar et al., 2000). However, the correlation is complex, as Villalobos-Salazar *et al*. (2000) found positive correlation between altitude and ptaquiloside content for vars. *arachnoideum* and *caudatum*, while Alonso-Amelot *et al*. (1995) found negative correlation for var. *caudatum*. Similar confusing results were obtained in New Zealand for var. *esculentum* (Rasmussen et al. 2003d). Alonso-Amelot et

al. (2000a) suggests that the decrease in ptaquiloside content with altitude may be due to lower temperatures at higher altitudes imposing restrictions in the metabolic rate and production of secondary metabolites. Climatic stress (drought, temperature and frost) is known to increase toxin concentrations in many plants (Ralphs, 2002).

An investigation of frond ptaquiloside contents in var. *aquilinum* in Denmark indicated that ptaquiloside might be produced in response to environmental stress as growth conditions characterized by shading from trees, low amounts of plant available water in the soil and scarcity of nutrients increased the level of ptaquiloside in fronds (Rasmussen et al., 2003b). The effect of shading has also been demonstrated for var. *esculentum* growing under controlled circumstances (Smith et al., 1990). The stress-hypothesis is supported by Smith et al. (1994) finding higher levels of ptaquiloside in fronds of var. *esculentum* subjected to mowing or grazing compared to non-disturbed fronds. However, the higher contents in damaged ferns may have been due to the fronds being at an earlier growth stage (regrowth after damage) than the undamaged fronds included in the study.

Bracken has been reported to respond to edaphic growth conditions in its production of other secondary metabolites than ptaquiloside. For example higher prurasin contents are found in fronds from shaded woodland habitats compared to open land and the release of HCN from brackens seems to be correlated with rainfall and temperature (Cooper-Driver and Swain, 1976; Cooper-Driver et al., 1977; Hadfield and Dyer, 1986).

A general need exists for understanding the dynamics in ptaquiloside variation inside bracken stands as well as of the geographical variation in the ptaquiloside content of different bracken stands. The aims of this study were therefore to: 1) Monitor the annual and regional variation in the level of ptaquiloside encountered in fronds, rhizomes and litter from Scottish bracken; 2) Unveil any edaphic influence on the observed ptaquiloside

contents, and 3) Examine the effect of bracken cutting on the ptaquiloside level in fronds, rhizomes and litter.

MATERIALS AND METHODS

Field sites, study of annual variation:

Three locations in Scotland with bracken-dominated ecosystems were chosen for the investigation (Loch Grannoch, Muir of Dinnet, Isle of Mull; Figure 2). The locations were situated on open land exept from Muir of Dinnet, which was situated in a glade. The stands were growing on different kinds of soil materials (Table 1-4). Sampling was performed on all sites in April, May, August, September, and November 2002.

Field sites, study of regional variation:

Twenty bracken stands situated across Scotland were chosen for the investigation (Figure 2). The stands were situated in forests or on moors. On all sites the bracken coverage was high or dominant (Table 1-4). Sampling was performed one time at every site in the period September 16 to October 2, 2002, i.e. by the end of the growth season.

Field site, cutting trial:

A single location (Craibstone) was chosen for the cutting trial (Figure 2, Table 1-4). The site was located on open land. Sampling was performed monthly in the first week of June, July, August, September, October, and November 2002. Fronds, which emerged in response to sampling in June and July plots, were resampled 30 days later.

Field work and sample pre-treatment:

Before sampling, the stand was divided into 5 homogeneous sub-sites, from which one square metre (the sampling-area) was delineated in the central part of each sub-site. At each sampling-area the frond-density was counted, and 2 whole fronds were harvested for analysis, while approx. 0.5 m of rhizomes (both frondbearing and storage rhizomes) were collected from a small trench dug at the southern part of the sampling-area. The diameter

and abundance of rhizomes was measured and counted in the trench. All fronds were separated from the rhizome at the soil surface, their frond- and stipe length was measured, and the number of unfurling pinnae counted, enabling determination of the bracken growth stage. Each trench was dug to the depth of the R-horizon, and this depth recorded. Detailed site-descriptions were performed according to the FAO-Unesco system (FAO, 1990). The FAO-Unesco site-descriptions were modified to include: 1) Size of bracken stand (<50m^2, 50-200m^2, 200-500m^2, and >500m^2); 2) Bracken ground cover (low [15%], moderate [15-40%], high [40-80%], and dominant [80-100%]); 3) Light conditions (shaded [below light tree species], glade [stand encircled by trees {>3m tall}], shrub [fronds growing between small trees {<3m tall}, open land]); 4) Bracken status (suppressed [other species dominate the herbal part of the ecosystem], subdominant [bracken is outnumbered by another herb], dominant [bracken is the most prominent herb]); 5) Bracken community growth stage (new [only new plants {max. 1 year}], young [no old fronds present, no litter], mature [old fronds and litter present], and old [bracken community is degenerating] (Watt, 1976)); and 6) Bracken disturbance (none, bitten [by animals], managed [cut, sprayed etc.]). All collected samples from the 5 sub-sites were pooled to give one sample from each site. Growth, density, soil- and stand descriptions were reported as averages of the 5 sub-sites. All samples were brought back to the laboratory as soon as possible and dried at 45°C for 5 days before milling to a particle size less than 2 mm and storage at –20°C.

Ptaquiloside determination:

The ptaquiloside-content was determined in aqueous bracken extracts after cleaning of the sample with Polyamide 6 resin (Fluka, Steinheim, Switzerland) (Agnew and Lauren, 1991). Ptaquiloside from dried samples of fronds and rhizomes were extraced with deionized water for 60 min. in the ratio 1:50 (weight:volume). Ptaquiloside from litter were extracted the same way, but in the ratio 1:7.5 (weight:volume). The suspension was centrifuged at 11,200 g for 5 min. before an aliquot was passed through a 1.0 x 10 cm I.D. glass Econo-

Column® (BIO-RAD, New York, USA) drypacked with Polyamide resin in the ratio 8:1 (volume:weight). Ptaquiloside was converted to pterosin B by base-acid treatment following the method of Agnew and Lauren (1991), and pterosin B determined by UV-absorption at 214 nm at 35°C using a Merck Hitachi HPLC system (L-4200 UV-VIS Detector; 655A-40 Auto-Sampler; D-6000A Interface; L-6200 Intelligent Pump) equipped with Merck LiChroCART® column (125 x 4 mm) packed with LiChrospher® 100 RP-8, 4 µm. The separations were performed at isocratic mode with a water-acetonitril eluent (74:26 volume:volume) using a flow of 1.0 ml/min. The column was flushed after each analysis, and the total time of analysis was 30 min, with a retention time for pterosin of approx. 9 min. Sample injection-volumes of 20-100 µl were used for analysis, and all samples were measured in duplicate. Pterosin B was quantified by using external standards. The limits of detection were 0.1 0.1 µg/g for litter and 0.7 µg/g for fronds and rhizomes. Standards of pterosin B was made from pure ptaquiloside obtained from Professor Ojika (Nagoya University, Japan).

Auxiliary analysis:

Soil pH was measured in a 1:2.5 or 1:10 soil:0.01 M $CaCl_2$ suspension in mineral soil or organic soil samples respectively (Rowell, 1994). The content of organic carbon was determined in all samples (soil, litter, rhizomes and fronds) by dry combustion using an Eltra CS 500 Carbon Sulphur Determinator (Rowell, 1994). Soil, litter, frond and rhizome nitrogen was determined by the Kjeldahl method (Rowell, 1994) in air-dried samples. All analyses were made as dup- or triplicates. The results are given as weight percentages on air-dry basis.

Statistical analysis:

Multiple linear regression (MLR) were carried out on all ptaquiloside- site- and stand data shown in Tables 1 and 2 to unveil correlations between environmental/edaphic parameters

and the ptaquiloside-content in fronds, rhizomes and litter. The significance of individual variables and of the resulting model was tested in analysis of variance. All statistical calculations were performed in the software package *The Unscrambler® ver. 7.6 SR1* (Camo ASA, Oslo, Norway). Geographical coordinates in latitude and longitude were transformed to 10-digit numbers before modelling.

RESULTS AND DISCUSSION

Bracken growth at Loch Grannoch, Muir of Dinnet, and Isle of Mull:

Aboveground frond growth began in the first part of May, and the fronds matured in August (Figure 3). The maximum frond lengths were between 150 and 200 cm, which are in accordance with previous investigations of var. *aquilinum* in the United Kingdom. The frond densities were in the range 24 to 42 fronds m^{-2}, which is relative high compared to investigations of var. *aquilinum* in Denmark, but in accordance with previously reports from the United Kingdom (Nicholson and Paterson, 1976; Whitehead and Digby, 1997; Rasmussen and Hansen, 2003; Rasmussen et al. 2003a; Rasmussen et al. 2003b). The longest fronds were found at Muir of Dinnet, the site with the lowest density, while the smallest fronds were encountered at Loch Grannoch, which had the highest density of the three sites. However, this inverse relationship between frond height and density does not exist at regional scale (Table 3). Frond density increased from May to August, in accordance with earlier reports from the United Kingdom, but in contrast with the growth pattern of var. *aquilinum* observed in forested ecosystems in Denmark (Rasmussen et al. 2003b).

Annual variation in the ptaquiloside content in fronds, rhizomes and litter at Loch Grannoch, Muir of Dinnet, and Isle of Mull:

Ptaquiloside was found in all frond, rhizome and litter samples analysed (Figure 4A-C). Ptaquiloside contents in the fronds ranged between 50 and 5,790 µg/g, with maximum contents at all sites recorded in May. Concentrations decreased as the fronds matured, similar to previous observations of ptaquiloside variations in var. *aquilinum* fronds. However, the ptaquiloside content was higher when compared to var. *latiusculum*. Ptaquiloside contents in the rhizomes ranged between 11 and 90 µg/g. Contents at Isle of Mull and Muir of Dinnet were both low between June and August, with maximum contents

around 1st of October. Rasmussen and Hansen (2003) noted a similar trend in rhizome contents in Denmark, although with considerably higher contents in the autumn of up to 7,000 µg/g. Ptaquiloside contents in the rhizomes at Loch Grannoch were lowest in May and August/September, with maximum contents recorded in November. The difference between Loch Grannoch and the other sites is likely due to fronds reaching maturity later at this site due to higher altitude (Atkinson, 1989). Ptaquiloside contents in bracken litter ranged betwen 0.1 and 3.5 µg/g, similar to that observed for var. *aquilinum* in Denmark (Rasmussen et al., 2003a; Rasmussen et al., 2003b). At Loch Grannoch and Muir of Dinnet, the contents increased towards autumn. Ptaquiloside contents at Isle of Mull displayed no marked variation. Higher contents in the litter were expected in the autumn due to higher amounts of precipitation in this season that might leach ptaquiloside from living, dead or dying bracken fronds. Also, the litter at this time would contain fronds that had died after being shaded out, releasing ptaquiloside. Why this was not the case at Isle of Mull cannot be explained from the observations performed in this study, although it might have been due to the higher rate of precipitation in West Scotland compared to the eastern part.

Regional variation in ptaquiloside contents in Scotland:

Ptaquiloside was found in all samples analysed (Table 4). Contents in the fronds ranged between 87 and 2,450 µg/g with a median content of 787 µg/g. This is in accordance with earlier findings for vars. *aquilinum* and *latiusculum*. MLR analysis resulted in a rather poor cross-validated model for the impact of the environmental/edaphic parameters on the ptaquiloside content in fronds, although showing significant impact of longitude (western longitude), orientation (aspect), disturbance (mowing or grazing damage), and frond height (Figure 5A; Equation 1, standard deviations shown; units as in Table 1-4).

$$\log\text{Pta} = 3.085(\pm 0.310) - 0.103(\pm 0.046)\bullet\text{Longitude} \qquad \text{Eq. 1}$$

+ 0.004(±0.001)•Orientation - 0.591(±0.196)•Disturbance

- 0.004(±0.002)•Frond height

1

2 However, the calibration correlation coefficient was acceptable indicating high leverage of
3 individual sites on the cross-validated model. The model indicates that fronds with high
4 ptaquiloside contents are found in the eastern part of Scotland, in stands pointing to the
5 south and west, which are not being disturbed and which are small. The model might reflect
6 genetic variation across Scotland like previously observed in Australia for var. *esculentum*
7 or it merely reflects the distribution of the two Scottish bracken varieties and hybrids. The
8 negative effect of longitude might also be due to the more senescent stage of brackens
9 sampled in the western part of Scotland compared to the eastern part. A climatic gradient
10 with respect to precipitation is also found going east-west in Scotland, with lower amounts
11 of precipitation found in the eastern part. However, it is warmer in the western part of
12 Scotland, due to the Gulf Stream, leading to a higher number of frost-free days. The
13 negative effect of disturbance is surprising, but might be due to the fact that only two stands
14 showed signs of physical disturbance and the effect could therefore be random. The
15 negative effect of frond height is in accordance with the findings of Rasmussen et al.
16 (2003b).

17

18 Ptaquiloside contents in rhizomes ranged between 17 and 657 μg/g, with a median content
19 of 118 μg/g (Table 4). MLR-analysis showed correlation between ptaquiloside in the
20 rhizomes and longitude (western longitude), topography (slope), and content of soil
21 nitrogen (Figure 5B; Equation 2, standard deviations shown; units as in Table 1-4).

22

*log*Pta = 2.532(±0.286) + 0.124(±0.057)•Longitude Eq. 2

- 0.040(±0.009)•Topography - 1.844(±0.636)•Soil N

23

The model indicates that high levels of ptaquiloside are found in rhizomes originating from stands growing at flat terrain in the western part of Scotland on soils with low amounts of nitrogen. The effect of longitude might be due to the same reasons as indicated for the fronds. The effect of topography is not easily explainable, and might just be a proxy variable for some other cause. This is the first attempt to correlate the ptaquiloside content in the rhizome-system with external growth factors.

Ptaquiloside contents in bracken litter ranged between 0.13 and 5.77 µg/g with a median content of 0.62 µg/g (Table 4). The content was well correlated with the latitude (northern latitude), altitude, frond development stage (number of pinnae) and the content of ptaquiloside in the frond (Figure 5C; Equation 3, standard deviations shown; units as in Table 1-4).

$$log(\text{Pta}+1) = -6.202(\pm 1.548) + 0.095(\pm 0.027)\bullet\text{N} - 0.001(\pm 0.000)\bullet\text{Altitude} + 0.047(\pm 0.010)\bullet\text{Development stage} + 0.185(\pm 0.079)\bullet log\text{Pta(frond)} \quad \text{Eq. 3}$$

High levels of ptaquiloside in litter was found in the northern part of Scotland, inside stands situated at low altitudes, below well-developed fronds having high ptaquiloside contents. The effect of altitude and latitude is not readily explainable. It is colder in the northern part of Scotland and as ptaquiloside degradation is strongly temperature dependent, colder climate should preserve ptaquiloside longer (Rasmussen et al., 2003c). The same effect should be seen with respect to altitude, as temperature tends to decrease with this parameter. Instead, the effect of altitude is negative. However, the negative effect could be caused by increased precipitation at altitude. The effect of altitude needs further investigtion. The positive effect of frond content, may have three reasons: 1) Ptaquiloside is present in the litter from dead fronds originating from earlier growth seasons; 2) Ptaquiloside is leaching from the fronds to the soil, or 3) Ptaquiloside originates from parts

of damaged fronds from the actual growth season (broken pinnae etc.). The effect of frond development stage is unclear, since it was expected that all fronds would have reached the same and maximum number of pinnae and pinnules by the end of September. However, stands at altitude have shorter growing season, so they would not necessarily reach the same stage before growth ceased. Compared to the explanation of the ptaquiloside content in litter given by Rasmussen et al. (2003b), the result obtained in the present investigation is more straightforward, but still indicates the complexity of the origin of ptaquiloside in litter.

When compared to Danish investigations of the regional variation in the ptaquiloside content, the result is somewhat complex. Broadly speaking, ptaquiloside in bracken may be a function of the ferns genetic heritage and its growth conditions. The inability to obtain results similar to the Danish findings may therefore indicate: 1) Relatively large genetic variation in Scottish bracken compared to Danish bracken partly attributed to the presence of var. *latiusculum* and *latiusculum-aquilinum* hybrids in Scotland, and/or 2) More complex or different interactions between environmental/edaphic variables and ptaquiloside contents than in Denmark; 3) Greater range of habitats sampled.

Cutting trial:

The effect of the harvesting of fronds on the ptaquiloside content in fronds, rhizomes, and litter is shown in Figure 6A-B. The ptaquiloside content in the fronds and rhizomes follow a declining pattern as shown for Loch Grannoch, Isle of Mull, and Muir of Dinnet. However, in October a large increase in the frond content was observed. The content in the recut fronds originating from rhizomes, which have had their fronds cut 30 days earlier, is significantly higher than in the uncut fronds. There is no effect on the ptaquiloside content in rhizomes. The content in the recut fronds is also much higher than in uncut fronds at the same growth stage (i.e. compare July-Recut with June). It is possible that some ptaquiloside

in the re-cut fronds is translocated from storage rhizomes, as frond-bearing rhizome carbohydrate stores are likely to be depleted due to harvesting. Storage rhizomes contain higher contents of ptaquiloside (Rasmussen and Hansen, 2003). When comparing the litter at the cut area with the uncut area, a lower ptaquiloside content in the recut area is seen in July and August. The lower content is likely due to the more exposed nature of the recut litter lying on bare ground compared to litter below protecting bracken fronds. Less ptaquiloside leaching from fronds into the litter may also explain this, as the fronds were cut.

CONCLUSION

Ptaquiloside contents in Scottish bracken fronds, rhizomes and litter are in accordance with previous findings for vars. *aquilinum* and *latiusculum*. Also, the annual variation in contents is consistent with earlier reports for var. *aquilinum* in Denmark. The latitude and altitude of the stand, the frond development stage and the ptaquiloside-content was well correlated with the ptaquiloside content in the litter, while the content in rhizomes were correlated with the longitude, topography and development stage of the stand. The ptaquiloside levels in the fronds were partly correlated with longitude and aspect of the stand, frond-height and presence of frond damage. Fronds that emerged following harvesting, contained higher amounts of ptaquiloside compared to uncut fronds of the same development stage. However, decreased levels of ptaquiloside in the litter were found on harvested areas. Compared to similar studies of the ptaquiloside occurrence in bracken from Denmark, the genetic heritage and other growth factors than the recorded may influence the ptaquiloside content in bracken.

REFERENCES

ADER, K. G. (1990) The effect of climate and soils on bracken vigour and distribution in Scotland, pp 141-151 *in* J.A. Thomson and R.T. Smith (eds.). Bracken Biology and Management. Occasional Publication No. 40, Australian Institute of Agricultural Science, Hawthorn.

AGNEW, M.P. and LAUREN, D.R. 1991. Determination of ptaquiloside in bracken fern (*Pteridium esculentum*). *J. Chromatogr.* 538:462-468.

ALONSO-AMELOT, M.E. and AVENDAÑO, M. 2001. Possible association between gastric cancer and bracken fern in Venezuela: An epidemiologic study. *Int. J. Cancer* 91:252-259.

ALONSO-AMELOT, M.E., CALCAGNO, M.P. and PÉREZ-INOJOSA, M. 2000a. Growth and selective defensive potential in relation to altitude in neotropical *Pteridium aquilinum* var. *caudatum*, pp. 43-47 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. IBG. Aberystwyth.

ALONSO-AMELOT, M.E., CASTILLO, U.F., AVENDANO, M., SMITH, B.L. and LAUREN 2000b. Milk as a vehicle for the transfer of ptaquiloside: A bracken carcinogen, pp. 86-90 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. IBG. Aberystwyth.

ALONSO-AMELOT, M.E., CASTILLO, U.F., SMITH, B.L. and LAUREN, D.R. 1998. Excretion through milk, of ptaquiloside in bracken-fed cows. A quantitative assessment. *Lait* 78:413-423.

ALONSO-AMELOT, M.E., PÉREZ-MENA, M., CALCAGNO, M.P., JAIMES-ESPINOSA, R. and CASTILLO, U.F. 1992. Ontogenic variation of biologically active metabolites of *Pteridium aquilinum* (L. Kuhn), pterosins A and B, and

ptaquiloside in a bracken population of the tropical Andes. *J. Chem. Ecol.* 18:1405-1420.

ALONSO-AMELOT, M.E., RUDOLFO-BAECHLER, S. and JAIMES-ESPINOSA, R. 1995. Comparative dynamics of ptaquiloside and pterosin B in the two varieties (*caudatum* and *arachnoideum*) of neotropical bracken fern (*Pteridium aquilinum* L. Kuhn). *Biochem. Syst. Ecol.* 23:709-716.

ATKINSON, T. P. (1989) Seasonal and altitudinal variation in *Pteridium aquilinum* (L.) Kuhn: frond and stand types. *New Phytologist*, 113: 359-365.

BLAIR, R.M., ALCANIZ, R. and HARRELl, A. 1983. Shade intensity influences the nutrient quality and digestability of Southern Deer browse leaves. *J. Range Management* 36:257-264.

CALLAGHAN, T. V., SCOTT, R., LAWSON, G. J. and MAINWARING, A. M. (1984) An experimental assessment of native and naturalised species of plants as renewable energy in Great Britain. IV Energy Crop Nutrition. Institute of terrestrial Ecology (Natural Environmental Research Council), ITE Project 674.

COOPER-DRIVER, G.A., FINCH, S. and SWAIN, T. 1977. Seasonal variation in secondary plant compounds in relation to the palatability of *Pteridium aquilinum*. *Biochem. Syst. Ecol.* 5:177-183.

COOPER-DRIVER, G.A. and SWAIN, T. 1976. Cyanogenic polymorphism in bracken in relation to herbivore predation. *Nature* 260:604-604.

FAO. 1990. Guidelines for Soil Description. 3rd edn. Food and Agriculture Organization of the United Nations. Rome.

FRANKLAND, J.C. 1976. Decomposition of bracken litter. *Bot. J. Linnean Soc.* 73:133-143.

GALPIN, O.P. and SMITH, R.M.M. 1986. Bracken, stomach cancer and water supplies: Is there a link? pp. 147-159 *in* R.T. Smith. and J.A. Taylor (eds.). Bracken - Ecology,

land use and control technology - The proceedings of the International Conference - Bracken '85. Parthenon Publishing. Aberystwyth.

HADFIELD, P.R. and DYER, A.F. 1986. Polymorphism of cyanogenesis in British populations of bracken (*Pteridium aquilinum* L. Kuhn). pp. 293-300 *in* R.T. Smith, and J.A. Taylor (eds.). Bracken - Ecology, land use and control technology. The proceedings of the International Conference - Bracken '85. Parthenon Publishing. Aberystwyth.

HAENSZEL, W., KURIHARA, M., LOCKE, F.B., SHIMUZU, K. and SEGI, M. 1976. Stomach cancer in Japan. *J. Natl. Cancer Inst.* 56:265-274.

KUSHIDA, T., UESUGI, M., SUGIURA, Y., KIGOSHI, H., TANAKA, H., HIROKAWA, J., OJIKA, M. and YAMADA, K. 1994. DNA damage by ptaquiloside. a potent bracken carcinogen: Detection of selective strand breaks and identification of DNA cleavage products. *J. Amr. Chem. Soc.* 116:479-486.

MARLIÈRE, C.A., WATHERN, P., FREITAS, R.N., CASTRO, M.C.F.M. and GALVAO, M.A.M. 2000. Bracken fern (*Pteridium aquilinum*) consumption and oesophaegal and stomach cancer in the Ouro Preto region, Minas Gerais, Brazil. pp. 144-149 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. International Bracken Group. Aberystwyth.

MARLIÈRE C.A., WATHERN, P., CASTRO, M.C.F.M., O'CONNOR, P., and GALVAO, M.A. (2002) Bracken fern (*Pteridium aquilinum*) ingestion and oesophageal and stomach cancer. *IARC Scientific Publications* 156:379-380.

NICHOLSON, I.A. and PATERSON, I.S. 1976. The ecological implications of bracken control to plant/animal systems. *Bot. J. Linnean Soc.* 73:269-283.

OJIKA, M., SUGIMOTO, K., OKAZAKI, T. and YAMADA, K. 1989. Modification and cleavage of DNA by ptaquiloside. A new potent carcinogen isolated from bracken fern. *J. Chem. Soc., Chem. Com.* 22:1775-1777.

OJIKA, M., WAKAMATSU, K., NIWA, H. and YAMADA, K. 1987. Ptaquiloside, a potent carcinogen isolated from Bracken Fern *Pteridium aquilinum* var. *latiusculum*: Structure elucidation based on chemical and spectral evidence, and reactions with amino acids, nucleosides, and nucleotides. *Tetrahedron* 43:5261-5274.

PAGE, C.N. 1989. Three subspecies of bracken, *Pteridium aquilinum* (L.) Kuhn, in Britain. *Watsonia* 17:429-434.

PAGE, C. N. 1997. The Ferns of Britain and Ireland. Cambridge University Press, Cambridge.

RALPHS, M.H. 2002. Ecological relationships between poisonous plants and rangeland condition: A review. *J. Range Management* 55:285-290.

RASMUSSEN, L.H. and HANSEN, H.C.B. 2003. Growth of bracken in Denmark and the content of ptaquiloside in fronds, rhizomes and roots, *in* T. Acamovic, C.S. Stewart, and T.W. Pennycott (eds.). Poisonous Plants and Related Toxins. CAB International, Wallingford. In press.

RASMUSSEN, L.H., JENSEN, L.S. and HANSEN, H.C.B. 2003a. Distribution of the carcinogenic terpene ptaquiloside in bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *J. Chem. Ecol.* 29:3:771-778.

RASMUSSEN, L.H., KROGHSBO, S., FRISVAD, J.C. and HANSEN, H.C.B. 2003b. Occurrence of the carcinogenic bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere* 51:117-127.

RASMUSSEN, L.H., LAUREN, D.R., HANSEN, H.C.B. 2003c. Sorption, degradation and mobility of ptaquiloside, a carcinogenic bracken (*Pteridium* sp.) constituent, in the soil environment. *Chemosphere* Submitted.

RASMUSSEN, L.H., LAUREN, D.R., SMITH, B.L., HANSEN, H.C.B. 2003d. Variation in the ptaquiloside content in bracken from New Zealand. *J. Chem. Ecol.* Submitted.

ROWELL, D.L. 1994. Soil Science - Methods & Applications. Prentice Hall. Edinburgh.

SAITO, K., NAGAO, T., MATOBA, M., KOYAMA, K., NATORI, S., MURAKAMI, T. and SAIKI, Y. 1989. Chemical assay of ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the Pteridaceae. *Phytochem.* 28:1605-1611.

SHAHIN, M., SMITH, B.L., WORRALL S., MOORE, M.R., SEAWRIGHT, A.A. and PRAKASH, A.S. 1998. Bracken fern carcinogenesis: Multiple intravenous doses of activated ptaquiloside induce DNA adducts, monocytosis, increased TNF alpha levels, and mammary glad carcinoma in rats. *Biochem. Biophys. Res. Comm.* 244: 192-197.

SMITH, B.L. 1997. The toxicity of bracken fern (genus *Pteridium*) to animals and its relevance to Man. pp. 63-76 *in* J.P. Felix D'Mello (ed.). Handbook of Plant and Fungal Toxicants. CRC Press. New York.

SMITH, B.L., LAUREN, D.R., EMBLING, P.P. and AGNEW, M.P. 1990. Ptaquiloside in Australian and New Zealand ferns as a cause of Neoplasia, pp. 241-246 *in* J.A. Thomson and R.T. Smith (eds.). Bracken Biology and Management. Occasional Publication No. 40, Australian Institute of Agricultural Science, Hawthorn.

SMITH, B.L., SEAWRIGHT, A.A., NG, J.C., HERTLE, A.T., THOMSON, J.A. and BOSTOCK, P.D. 1994. Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium* spp.), from eastern Australia and from a cultivated worldwide collection held in Sydney, Australia. *Nat. Toxins* 2:347-353.

THOMSON, J.A. 2000. Morphological and genomic diversity in the genus *Pteridium* (Dennstaedtiaceae). *Annals Botany* 85:77-99.

THOMSON, J.A. and ALONSO-AMELOT, M.E. 2002. Clarification of the taxonomic status and relationships of *Pteridium caudatum* (Dennstaedtiaceae) in Central and South America. *Bot. J. Linnean Soc.* 140:237-248.

TRYON, R.M. 1941. A revision of the genus *Pteridium*. *J. Natl. English Bot. Club* 43.

VAN DER HOEVEN, J.C.M., LAGERWEIJ, W.J., POATHUMUS, M.A., VAN VELDHUIZEN, A. and HOLTERMAN, H.A.J. 1983. Aquilide A, a new mutagenic compound isolated from bracken fern (*Pteridium aquilinum* (L.) Kuhn). *Carcinogenesis*. 4:1587-1590.

VILLALOBOS-SALAZAR, J., HERNANDEZ, H., MENESES, A. and SALAZAR, G. 2000. Factors which may affect ptaquiloside levels in milk: Effects of altitude, bracken fern growth stage, and milk processing. pp. 68-74 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. International Bracken Group. Aberystwyth.

WATT, A.S. 1976. The ecological status of bracken. *Bot. J. Linnean Soc.* 73:217-239.

WHITEHEAD, S.J. and DIGBY, J. 1997. The morphology of bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors - a comparison of the mature stand and the interface with heather (*Calluna vulgaris* (L.) Hull) 1. The fronds. *Ann. Appl. Biol.* 131:103-116.

FIGURE CAPTIONS

Table no:	Text:
1	TABLE 1 SITE DESCRIPTION I: LOCATION AND SITE CHARACTERS.
2	TABLE 2 SITE DESCRIPTION II: SOIL PROPERTIES. SD[a] SHOWN.
3	TABLE 3 STAND DESCRIPTIONS (SEPTEMBER 2002) I: PHYSICAL DESCRIPTION. SD[a] SHOWN.
4	TABLE 4 STAND DESCRIPTIONS (AUTUMN 2002) II: PTAQUILOSIDE CONTENTS ETC. SD[a] SHOWN.
Figure no:	**Text:**
1	FIG. 1. Ptaquiloside.
2	FIG. 2. Map of Scotland (minus Shetland and Orkney Islands). Study sites marked. All sites were used for the regional survey. Craibstone (white star) also used for cutting trial. Loch Grannoch, Isle of Mull, and Muir of Dinnet (grey stars) also used for monitoring the annual variation in the ptaquiloside content.
3	FIG. 3. Bracken growth pattern at Loch Grannoch, Isle of Mull, and Muir of Dinnet. Standard deviations marked.
4	FIG. 4. Annual variations in the ptaquiloside content in: A) Fronds; B) Rhizomes; C) Litter. Sites: Loch Grannoch, Isle of Mull, and Muir of Dinnet. Standard deviations shown.
5	FIG. 5. Multiple linear regression models for the ptaquiloside content in: A) Fronds ($P = 0.0035$); B) Rhizomes ($P = 0.0005$); C) Litter ($P = 0.0001$). All variables have $P < 0.05$. R^2 = Calibration correlation coefficient. Q^2 = Cross-validated correlation coefficient.

6	FIG. 6. Craibstone cutting trial: A) Ptaquiloside content in fronds (filled bars) and rhizomes (open bars); B) Ptaquiloside content in litter (September is missing due to contamination of sample). Standard deviations shown.

TABLE 1 SITE DESCRIPTION I: LOCATION AND SITE CHARACTERS.

Site	N	W	Altitude (m)	Orientation (deg)	Topography (%)	Landform	Land element	Human influence	Land use	Vegetation	Light conditions
Loch Grannoch	54:59:29	4:16:49	200	276	15	upland	slope	none	conservation	forest-scrub	open land
Isle of Mull	56:28:52	6:07:18	50	210	20	upland	slope	none	semi-extensive sheep	grassland	open land
Muir of Dinnet	57:05:16	2:54:39	170	198	5	upland	slope	none	conservation	forest	glade
Craibstone	57:11:20	2:13:00	90	115	2	hill	depression	none	conservation	grassland-scrub	open land
Balloch Wood	54:54:45	4:33:51	40	210	0.5	valley	floodplain	none	forest	oak, alder, hazel	glade
Ben Nevis	56:46:47	4:59:46	210	175	27	mountain	slope	none	conservation	grassland	open land
Elrick Hill High	57:10:54	2:15:01	190	155	20	hill	slope	none	conservation	grassland-scrub	open land
Elrick Hill Low	57:10:53	2:14:51	150	260	5	valley	valley floor	none	conservation	grassland-forest	glade
Glencoe	56:39:43	4:57:31	280	20	15	mountain	slope	none	conservation	grassland	open land
Grey Mare's Tail	55:25:18	3:16:58	320	185	15	mountain	slope	none	semi-extensive sheep	grassland	open land
Kirkhill	57:11:37	2:14:49	150	100	3	upland	terrace	none	exotic timber production	forest	glade
Loch Trool	55:05:36	4:29:09	150	170	15	upland	slope	burning	conservation	grassland-scrub	open land
Muir of Dinnet cut	57:05:21	2:54:56	170	200	2	upland	slope	clearing	conservation	grassland-forest	glade
Newton Stewart	54:58:46	4:33:51	100	160	5	upland	plateau	none	exotic timber forestry	forest-scrub	glade
Rockcliffe	54:52:14	3:48:25	30	140	2	hill	slope	none	conservation	grassland-forest	open land
Schiehallion	56:40:32	4:02:25	350	75	5	hill	slope	none	semi-extensive sheep	grassland	open land
Skye	57:12:53	5:57:05	30	240	2	upland	valley floor	none	semi-extensive sheep	grassland	open land
Stronord	54:57:26	4:25:11	70	130	2	hill	terrace	none	exotic timber production	forest	glade
Torridon Beach	57:32:23	5:30:39	5	260	2	coastal plain	floodplain	none	conservation	misc.	open land
Torridon Hill	57:32:50	5:30:36	40	160	7	upland	slope	none	conservation	grassland-scrub	open land

TABLE 2 SITE DESCRIPTION II: SOIL PROPERTIES. SD[a] SHOWN.

Site	Thickness of litter[b] (cm)	Effective soil depth (cm)	Litter C[bc] (%)	Litter N[bc] (%)	Soil pH[cd]	Soil C[cd] (%)	Soil N[cd] (%)
Loch Grannoch	9±5	36±12	48.7	1.12	3.46	8.90	0.49
Isle of Mull	9±3	56±35	46.8	1.23	4.40	8.94	0.47
Muir of Dinnet	8±2	44±20	47.8	1.06	4.03	5.17	0.32
Craibstone	6±3	54±25	50.1	1.37	3.93	3.76	0.28
Balloch Wood	0±0	79±7	49.2	0.81	3.85	2.43	0.14
Ben Nevis	5±5	32±18	47.9	0.60	4.11	4.01	0.25
Elrick Hill High	13±2	56±4	49.3	1.01	3.31	4.77	0.20
Elrick Hill Low	6±3	66±18	45.3	1.27	3.51	9.08	0.48
Glencoe	3±2	41±20	48.2	0.62	4.09	4.42	0.31
Grey Mare's Tail	4±2	24±6	48.2	1.37	3.72	6.68	0.51
Kirkhill	7±2	43±10	48.4	1.00	3.68	4.95	0.17
Loch Trool	3±1	31±15	48.4	0.73	4.19	5.35	0.36
Muir of Dinnet cut	4±1	44±34	45.4	1.51	4.02	3.48	0.24
Newton Stewart	13±5	44±17	49.7	1.29	3.60	9.69	0.42
Rockcliffe	14±3	84±8	49.6	1.26	3.86	4.44	0.32
Schiehallion	1±0	76±21	46.8	0.56	4.34	3.98	0.28
Skye	2±1	71±23	48.3	0.47	4.22	4.24	0.36
Stronord	12±4	43±12	48.9	1.46	3.99	6.42	0.43
Torridon Beach	4±2	71±17	46.3	0.86	4.00	6.15	0.44
Torridon Hill	9±2	44±20	46.7	0.60	3.28	2.86	0.21

[a] Standard deviation.
[b] Litter comprises Oi, Oe and Oa-horizons.
[c] Average coefficient of variation: 3.0% (litter C); 2.1% (litter N); 0.2% (soil pH); 0.7% (soil C); 0.2% (soil N).
[d] Soil refers to the 20 cm below the litter layer (A or A/Oa horizons).

TABLE 3 STAND DESCRIPTIONS (SEPTEMBER 2002) I: PHYSICAL DESCRIPTION. SD[a] SHOWN.

Site	Community development stage	Disturbance	Bracken status	Bracken coverage	Stand size (m^2)	Frond density (#/m^2)	Frond height (cm)	Bracken development stage (# pinnae)	Mineralization of litter	Rhizomes (#/m)	Rhizomes[b] (FBR:SR)
Loch Grannoch	developing	none	dominant	high	50-200	42.6±7.2	152±35	17.0±2.0	strong	25.0±2.0	69:31
Isle of Mull	mature	none	dominant	dominant	200-500	29.6±4.4	166±14	15.0±0.0	moderate	37.8±11.9	73:27
Muir of Dinnet	mature	none	dominant	dominant	>500	24.2±4.3	187±23	20.6±1.7	strong	23.2±3.6	68:32
Craibstone	mature	none	dominant	dominant	50-200	34.4±7.8	183±18	ND[c]	moderate	32.2±10.8	72:29
Balloch Wood	mature	none	subdominant	high	<50	17.0±4.3	176±19	12.3±5.9	strong	13.6±5.9	59:40
Ben Nevis	mature	none	subdominant	high	200-500	44.8±8.2	79±11	16.0±2.2	strong	19.6±14.4	73:27
Elrick Hill High	mature	none	dominant	dominant	>500	28.4±6.2	105±16	ND	slight	26.0±3.7	62:38
Elrick Hill Low	mature	none	subdominant	dominant	50-200	33.0±8.5	153±24	10.0±2.8	moderate	23.0±5.6	61:39
Glencoe	mature	none	subdominant	high	50-200	36.6±11.1	88±10	16.1±1.6	strong	14.8±6.5	76:24
Grey Mare's Tail	mature	none	subdominant	dominant	>500	40.4±13.0	97±14	14.3±2.9	strong	30.8±7.5	68:32
Kirkhill	mature	none	subdominant	high	50-200	21.0±4.8	102±18	14.4±2.7	moderate	23.4±11.2	67:33
Loch Trool	mature	none	dominant	dominant	>500	32.8±6.5	113±9	13.6±2.3	moderate	37.2±10.1	71:29
Muir of Dinnet cut	mature	managed	subdominant	dominant	50-200	20.4±5.1	129±25	15.3±3.9	strong	24.2±18.3	59:41
Newton Stewart	developing	none	dominant	high	50-200	27.0±6.9	133±17	12.2±3.2	moderate	26.8±8.0	70:30
Rockcliffe	mature	none	dominant	dominant	>500	27.2±9.3	172±15	16.8±5.2	slight	38.8±10.6	65:35
Schiehallion	mature	none	dominant	dominant	200-500	47.4±7.5	97±11	14.6±2.7	strong	36.0±8.3	66:34
Skye	developing	grazing damage	suppressed	high	200-500	50.0±4.0	57±12	12.1±2.7	strong	29.0±4.7	55:45
Stronord	mature	none	dominant	dominant	50-200	38.2±2.4	117±10	12.7±4.8	slight	38.6±12.2	79:21
Torridon Beach	mature	none	dominant	dominant	50-200	48.6±4.0	126±25	ND	moderate	27.4±9.7	75:25
Torridon Hill	mature	none	subdominant	high	>500	47.8±4.4	94±12	13.7±3.5	slight	26.4±3.0	75:25

[a] Standard deviation.
[b] FBR: Frond bearing rhizomes; SR: Storage rhizomes.
[c] ND: Not determined.

TABLE 4 STAND DESCRIPTIONS (AUTUMN 2002) II: PTAQUILOSIDE CONTENTS ETC. SD[a] SHOWN.

Site	Frond C[b] (%)	Frond N[b] (%)	Rhizome C[b] (%)	Rhizome N[b] (%)	Frond-PTA[c] (µg/g)	Rhizome-PTA[c] (µg/g)	Litter-PTA[c] (µg/g)
Loch Grannoch	45.9	1.71	ND	ND	1,280±30	24±0	0.13±0.02
Isle of Mull	45.9	1.72	ND	ND	360±15	61±2	0.40±0.03
Muir of Dinnet	46.0	1.25	ND	ND	720±30	89±5	3.52±0.42
Craibstone	46.9	1.32	43.5	1.40	650±20	125±5	ND[d]
Balloch Wood	48.1	1.22	45.1	0.90	510±15	271±1	0.22±0.04
Ben Nevis	44.9	0.72	45.1	0.42	980±20	18±1	1.29±0.09
Elrick Hill High	46.3	0.81	43.7	0.74	1,280±10	50±2	3.85±0.66
Elrick Hill Low	47.1	1.23	44.1	1.18	1,490±50	38±1	0.43±0.15
Glencoe	43.8	1.03	44.2	0.67	90±10	290±3	0.43±0.13
Grey Mare's Tail	46.3	1.72	46.3	1.11	2,450±280	37±5	0.63±0.05
Kirkhill	45.2	1.03	44.4	0.88	490±60	282±1	1.37±0.10
Loch Trool	45.6	1.32	46.1	0.69	1,150±180	42±1	0.44±0.25
Muir of Dinnet cut	46.3	1.10	46.4	1.34	240±30	256±18	0.62±0.05
Newton Stewart	46.3	1.45	46.2	1.20	1,115±40	60±6	0.84±0.73
Rockcliffe	47.6	1.52	45.9	0.91	820±20	510±10	0.96±0.51
Schiehallion	45.3	1.22	42.4	1.11	860±10	657±18	0.41±0.01
Skye	44.6	1.13	44.7	0.43	760±30	155±6	0.35±0.17
Stronord	45.6	1.86	44.7	1.31	260±5	112±7	0.24±0.04
Torridon Beach	45.3	1.24	43.5	1.33	1,780±10	421±2	5.77±0.71
Torridon Hill	44.3	1.08	43.2	0.41	565±5	519±15	2.52±0.39

[a] Standard deviation.
[b] Average coefficient of variation: 1.8% (frond C); 1.3% (frond N); 2.3% (rhizome C); 2.0% (rhizome N).
[c] PTA: Ptaquiloside-equivalents.
[d] ND: Not determined.

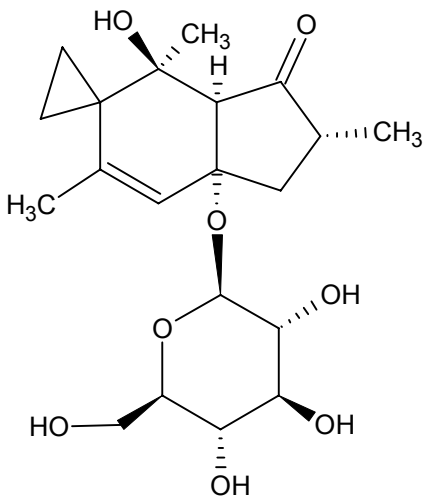


FIG. 1. Ptaquiloside.

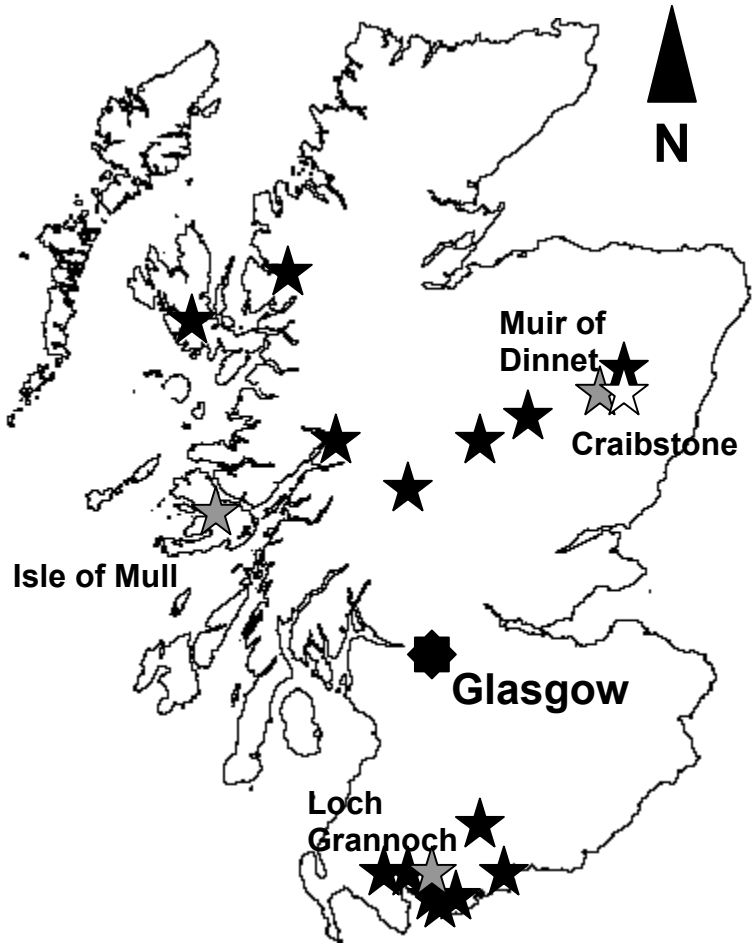


FIG. 2. Map of Scotland (minus Shetland and Orkney Islands). Study sites marked. All sites were used for the regional survey. Craibstone (white star) also used for cutting trial. Loch Grannoch, Isle of Mull, and Muir of Dinnet (grey stars) also used for monitoring the annual variation in the ptaquiloside content.

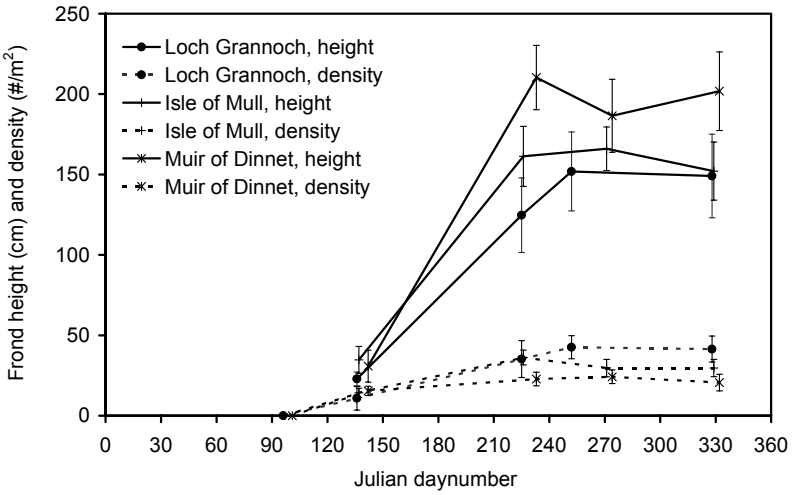


FIG. 3. Bracken growth pattern at Loch Grannoch, Isle of Mull, and Muir of Dinnet. Standard deviations marked.

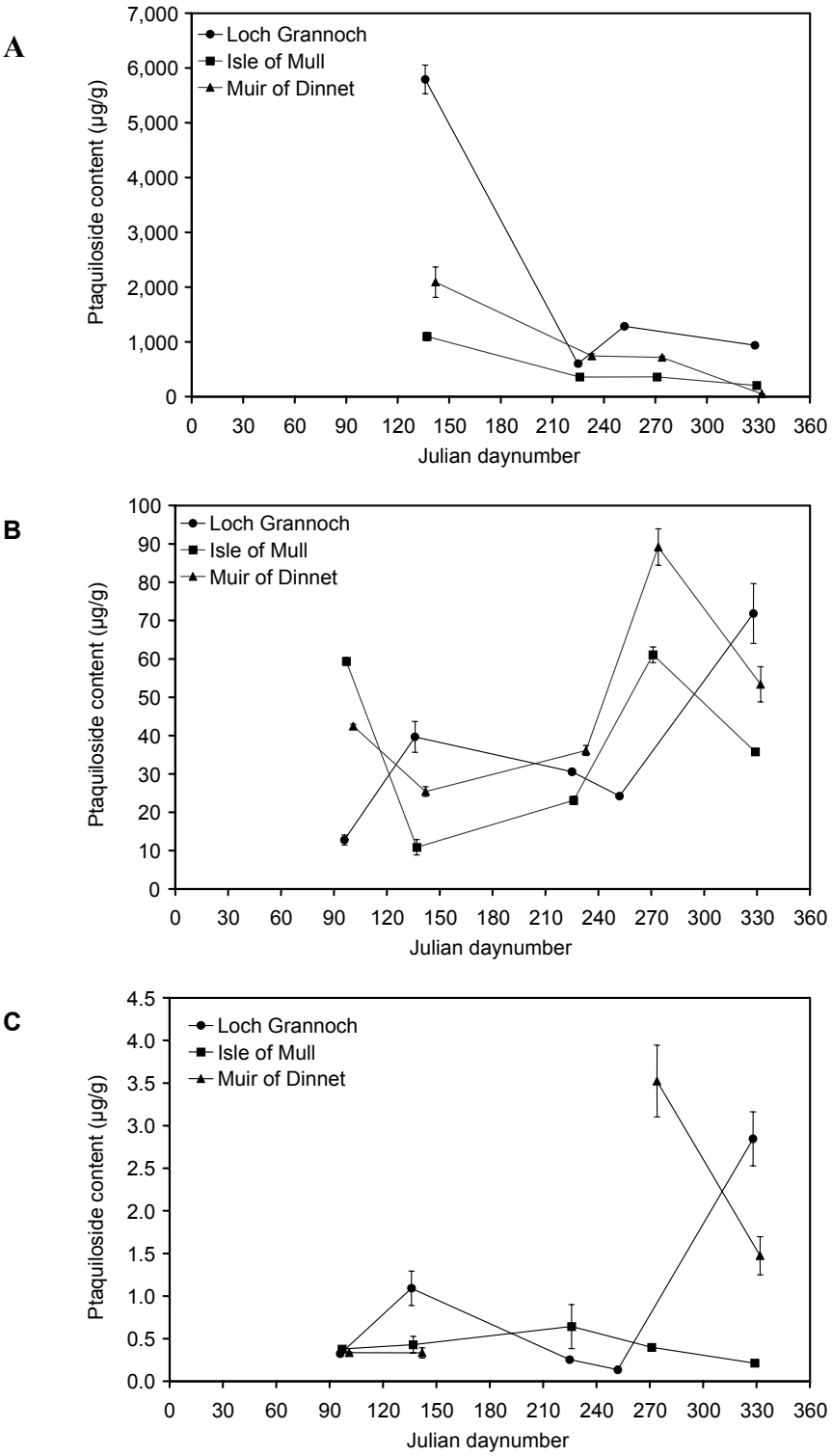


FIG. 4. Annual variations in the ptaquiloside content in: A) Fronds; B) Rhizomes; C) Litter. Sites: Loch Grannoch, Isle of Mull, and Muir of Dinnet. Standard deviations shown.

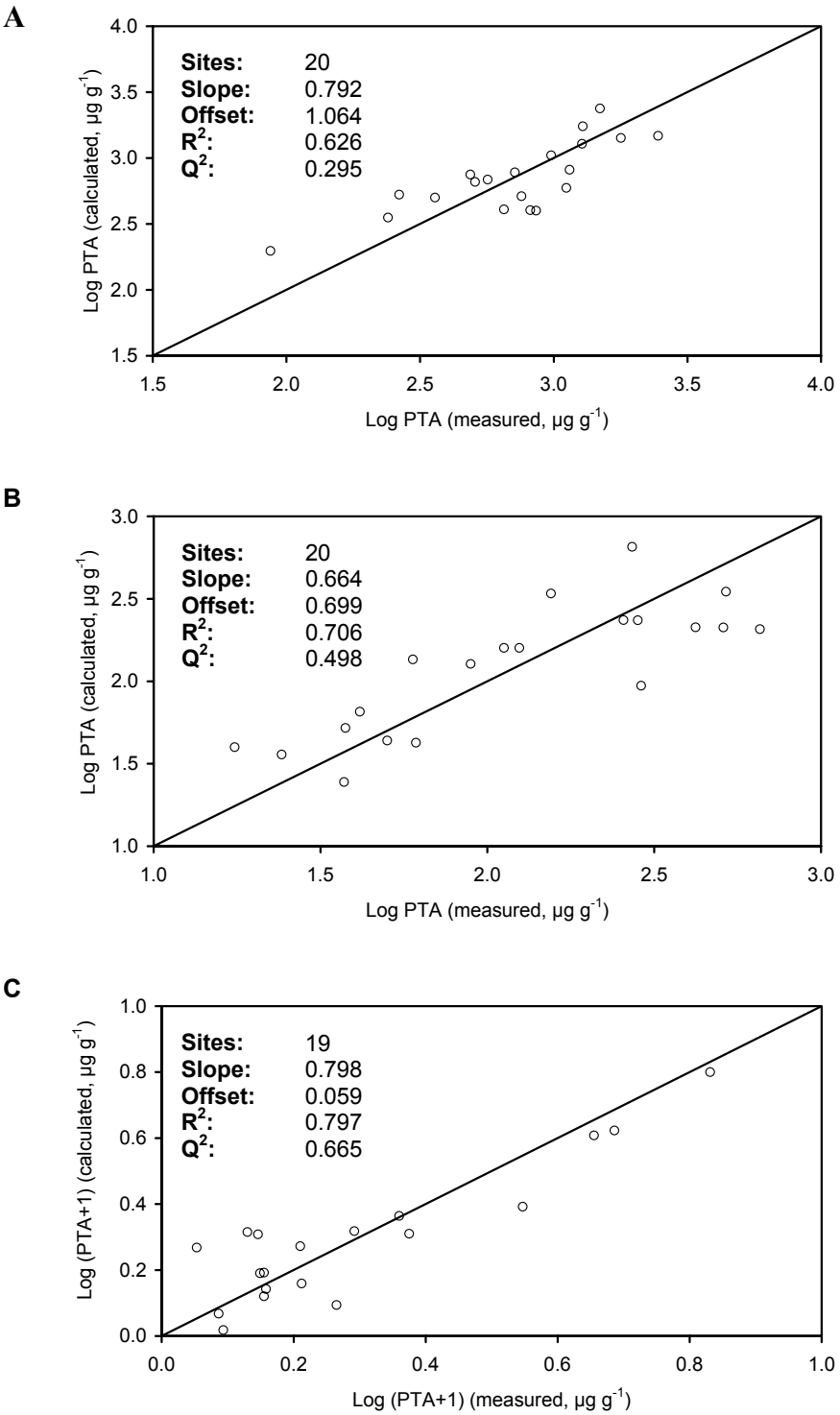


FIG. 5. Multiple linear regression models for the ptaquiloside content in: A) Fronds (P = 0.0035); B) Rhizomes (P = 0.0005); C) Litter (P = 0.0001). All variables have $P < 0.05$. R^2 = Calibration correlation coefficient. Q^2 = Cross-validated correlation coefficient.

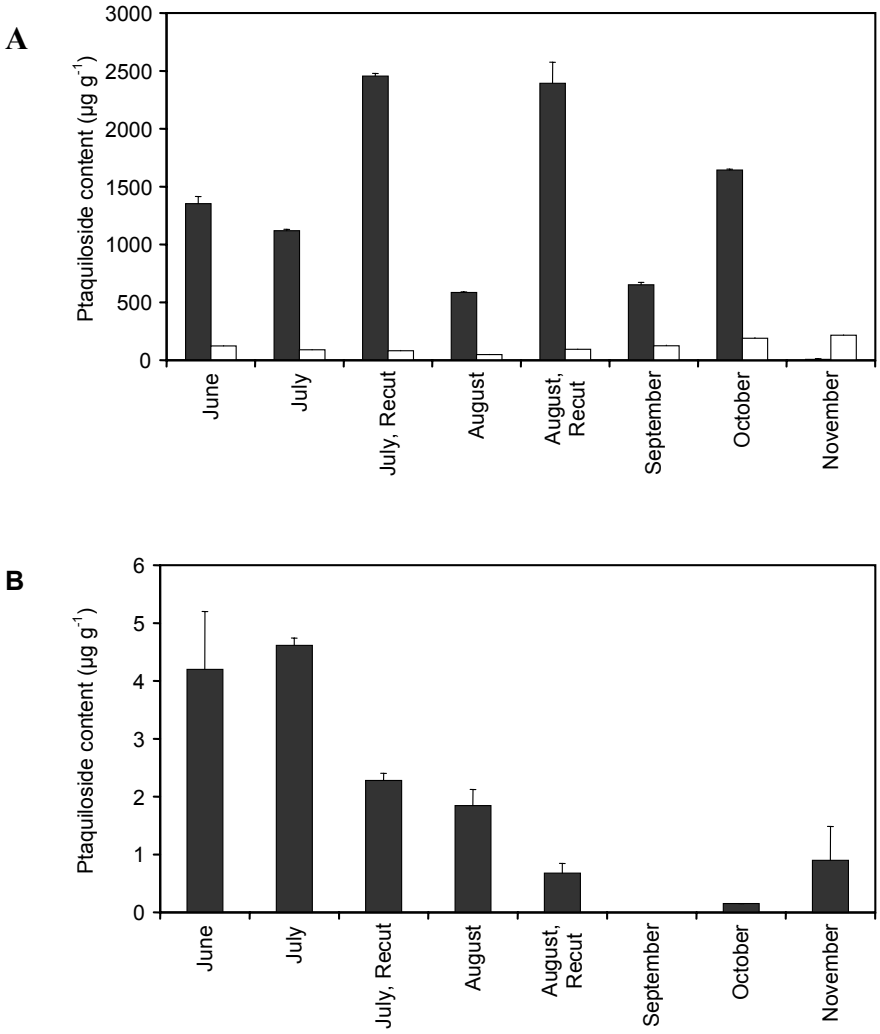


FIG. 6. Craibstone cutting trial: A) Ptaquiloside content in fronds (filled bars) and rhizomes (open bars); B) Ptaquiloside content in litter (September is missing due to contamination of sample). Standard deviations shown.

Growth of Bracken in Denmark and the Content of Ptaquiloside in Fronds, Rhizomes and Roots

L.H. Rasmussen and H.C.B. Hansen.
The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Introduction

In Denmark, the Bracken variety Common Bracken (*Pteridium aquilinum* ssp. *aquilinum* var. *aquilinum* (L.) Kuhn) is encountered as a weed on acid soils in plantation forestry and on recreational areas. As a part of an investigation of soil- and groundwater-contamination with the carcinogenic compound ptaquiloside, three typical Common Bracken populations situated in plantations were investigated from April 2000 until April 2001 to quantify the above ground biomass-production as well as in ptaquiloside-production in different plant-compartments (fronds, frondbearing rhizomes, storage rhizomes, and roots).

Frond growth commences around May 1 in Denmark. The fronds reach maturity in late summer (July and August), and finally die back from late September. In case of early autumn night frost, the growth season may stop at an earlier time (Hansen, 1991; Øllgaard and Tind, 1993; Watt, 1976). The fronds reach a height of 100-250 cm. The highest fronds are typically found within forests and plantations, where the ferns are protected against wind and frost. In the United Kingdom where Common Bracken also are found widespread in forests, on moors, and on heath lands, frond heights from 60 to 110 cm are typical on open terrain, while the fronds generally are higher in protecting ecosystems like forests (150 cm or more) (Watt, 1976; Whitehead and Digby, 1997; Nicholson and Paterson, 1976). No investigations have been published regarding the frond density of Common Bracken in Denmark. In the United Kingdom, densities between 16 and 51 fronds m^{-2} was measured (Whitehead and Digby, 1997; Nicholson and Paterson, 1976).

Several frond biomass production functions have been established over the years (Paterson *et al.* 1997; Alonso-Amelot *et al.* 2000). Recently Alonso-Amelot *et al.* (2000) presented a simple exponential model for the Neotropical Bracken variety *caudatum* based on rachis length. Models like this and direct measurements have been used to estimate the annual frond dry matter production. The dry matter content in Common Bracken fronds reach a maximum at frond maturity in late summer/early autumn before the fern begins retrieval of nutrients to rhizomes (Watt, 1976; Williams and Foley, 1976). Common Bracken has been reported to have an annual frond dry matter production up to 1400 g m^{-2}, while Neotropical Brackens (var. *caudatum* and

arachnoidum) with a whole year growth cycle have a standing biomass in the fronds between 25 and 1439 g m^{-2} (Alonso-Amelot *et al.* 1995; Watt, 1976; Alonso-Amelot *et al.* 2000). A few investigations have been performed regarding the belowground biomass found in the rhizomes. The dry matter content of Common Bracken rhizomes is lowest during late spring and early summer while the fronds are growing. From July to October the Common Brackens in England replenish the carbohydrate-content of the rhizomes before going into dormancy by the end of October (Williams and Foley, 1976). The belowground biomass for Common Bracken can be very large (660 g-dry-matter m^{-2}, 8630 g-fresh-matter m^{-2}), and it is estimated that the rhizomes makes up around 80% of the total biomass of Bracken populations. Of the rhizomes, the storage rhizomes make up around 60% of the rhizome biomass (Whitehead and Digby, 1997; Baker *et al.* 1997).

The ptaquiloside-content in fronds is usually highest just after the crosiers emerge from the ground. The content typically decreases during the growing season. In Neotropical Brackens less than 5% of the maximum content is found in mature fronds (Alonso-Amelot *et al.* 1995; Alonso-Amelot *et al.* 1992; Saito *et al.* 1989). In Common Bracken contents between 60 and 9800 μg g^{-1} were encountered in a world-wide collection held in Sydney (Smith *et al.* 1994).

The ptaquiloside-content in rhizomes lies between 5 and 1200 μg g^{-1}. Only a few studies have been performed on rhizomes, and they were performed in the growth season on Common Bracken and Neotropical Bracken. Highest contents were found in Common Bracken rhizomes (Alonso-Amelot *et al.* 1992; Saito *et al.* 1989; Rasmussen *et al.* 2001).

Materials and Methods

Three Bracken-dominated ecosystems situated all-over Denmark on different kinds of soil materials were chosen for the investigation (Figure 1). Description of the sites can be found in Table 1. Frond-heights (30 fronds per site) and dry matter content of fronds (3 fronds per site) were measured once (Præstø Fed and Salten Langsø) or twice (Mørup Skov) a month from the time crosiers emerged in April 2000 until the fronds reached maturity in September 2000. The dry matter content was measured after drying for 5 days at 110°C. In addition frond-densities were measured at the end of the growing season (3 times per site (1 m^2). Several fronds, rhizomes, and roots were sampled from each site for ptaquiloside analysis. A distinction was made between frondbearing (short shots) and storage rhizomes (long shots). Roots from both kinds of rhizomes were pooled to obtain a suitable sample size. Plant material were brought to the laboratory, cut into small pieces, and dried at 50°C for 5 days, milled (resulting in diameter less than 2 mm), and finally stored at 4°C until further analysis. Before drying of rhizomes and roots, they were gently washed free from soil. The ptaquiloside-content was measured in duplicate once or twice a month during the period of investigation following the water-extraction method of Agnew and Lauren (1991). Ptaquiloside were measured as ptaquiloside-

Fig. 1. Map of Denmark. Study sites shown on map.

equivalents after conversion to pterosin B at 214 nm on a Perkin Elmer Series 10 liquid chromatographer, equipped with a Shimadzu SPD-10A UV Detector, a Perkin Elmer LCI-100 Laboratory Computing Integrator, and a Merck LiChroCART 250-4, Lichrospher 100 RP-8 as analytical column. Water-methanol (45:55 vol-%) was used as eluent with a flow rate of 1.50 ml min^{-1}. A 50 µL sample loop was used for all samples. Standards in the range of 0.0112-22.5000 µg ml^{-1} ptaquiloside-equivalents were used for quantification, resulting in measurement of both ptaquiloside and *iso*-ptaquiloside (Agnew and Lauren, 1991; Castillo *et al.* 1997). A standard of ptaquiloside was obtained from Professor Ojika, Nagoya University.

Table 1. Site description according to FAO (1990) and Soil Survey Staff (1999).

Site:	Parent material	Soil order	Drainage class	Land use	Herbal dominants
Mørup Skov	Glaciofluvial deposits	Inceptisol	Well drained	Pedunculate Oak (*Quercus robur* L.)	B,C,D,E,F G,H
Salten Langsø	Fine sandy till	Inceptisol	Well drained	Douglas Fir (*Pseudotsuga menziesii* Franko.)	B,D,I,J,C K
Præstø Fed	Beach ridges	Spodosol	Somewhat excessively drained	Silver Birch (*Betula pendula* Roth)	B,I

B=Bracken (*Pteridium aquilinum* (L.) Kuhn), C=Shield fern (*Drypteris dilatata* (Hoffm.) A.Gray), D=Honeysuckle (*Lonicera periclymenum* L.), E=Wood anemone (*Anemona nemorosa* L.),F=Dutch rush (*Equisetum hyemale* L.), G=Raspberry (*Rubus idaeus* L.), H=Nettle (*Urtica dioica* L.), I=Wavy Hair-grass (*Deschampsia flexuosa* (L.) Trin.), J=Blackberry (*Rubus fruticosus* L.), K= Wood sorrel (*Oxalis acetosella* L.).

Results and Discussion

The fronds reached maturity 97-134 days after emergence from the ground (Fig. 2). The maximum frond lengths were 166-212 cm, which is in accordance with previous investigations of Common Bracken in Denmark. The frond densities were 9-14 fronds m^{-2}, which is rather low compared to investigations of

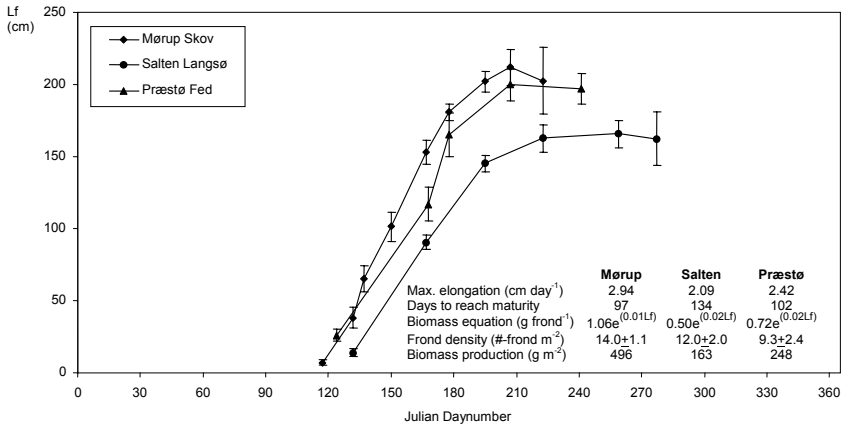


Fig. 2. Bracken growth. Lf = Length of frond (cm). The population at Mørup Skov tilted after heavy rain at day number 210.

Common Bracken in the United Kingdom (Whitehead and Digby, 1997; Nicholson and Paterson, 1976). Exponential biomass equations based on frond length and dry matter content were established and yielded correlation coefficients between 0.9209 and 0.9277 (Fig. 2). Using these functions and the frond density, a total aboveground biomass production between 163 and 496 g dry-matter m^{-2} were estimated at the end of the growth season, which is somewhat low compared to other investigations (Watt, 1976).

Ptaquiloside were found in all plant-compartments all year round (Fig. 3-4). The fronds had ptaquiloside-contents between 360 and 3612 µg g^{-1}. Salten Langsø and Præstø Fed showed maximum ptaquiloside-content in June and July, while Mørup Skov showed maximum in April.

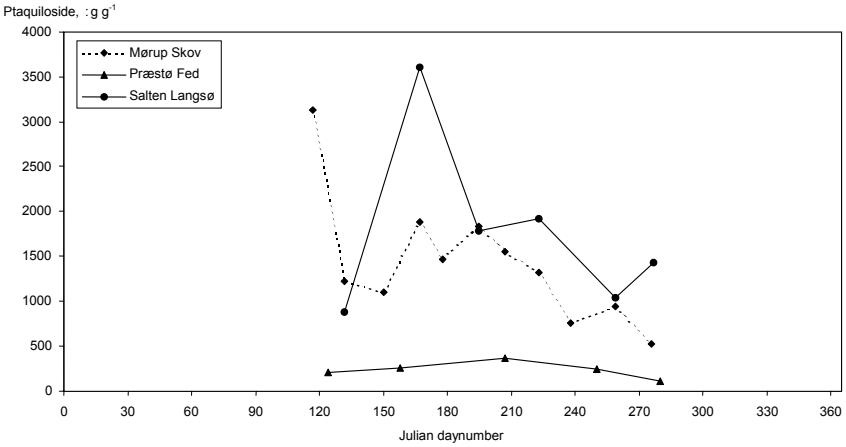


Fig. 3. Ptaquiloside-content in Bracken fronds. SDev<10%.

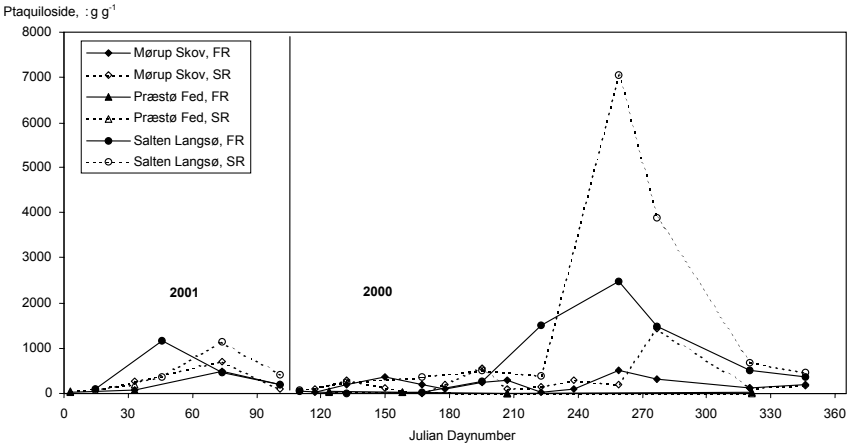


Fig. 4. Ptaquiloside in rhizomes. FR=Frondbearing rhizome. SR=Storage Rhizome. SDev<10%.

Both Mørup Skov and Salten Langsø exhibited a tendency for the ptaquiloside-content to decrease during the growth-season in accordance with other findings, while on Præstø Fed no marked difference in the ptaquiloside-content was observed. The different kinds of rhizomes had ptaquiloside-contents between 2 and 7046 μg g^{-1}, with the lowest contents just after the fronds emerged from ground and during the frond growth season (Fig. 4). The largest contents were encountered after fronds had reached maturity as fully enrolled fronds, in late summer and autumn (after day number 210). Raised ptaquiloside-content was also measured in late winter/early spring before the crosiers emerged from the ground. The storage rhizomes had in general a higher content of ptaquiloside than the frondbearing rhizomes, especially in the autumn. The content seem to be rather high compared to other investigations of rhizomes (Alonso-Amelot *et al.* 1992; Saito *et al.* 1989). The rhizome-contents tend to be positively correlated with carbohydrate regeneration of rhizomes in the autumn, rhizome- and belowground shoot-elongation, and negatively correlated with frondgrowth during summer (Williams and Foley, 1976).

The ptaquiloside-content in roots was generally low compared to the other fern-compartments (5-230 μg g^{-1}). This is the first time ptaquiloside is measured in Bracken roots. The content seems not to be correlated with the ptaquiloside-content in fronds and rhizomes.

Conclusion

Compared to other investigations, Danish populations of Common Bracken seem to have relatively low growth rates and frond densities resulting in a low biomass production. The content of ptaquiloside in the fronds is in accordance with other observations but do not follow the marked decline after frond

emergence as reported elsewhere. The ptaquiloside contents found in roots and rhizomes are higher than previously reported. The high ptaquiloside-contents found in the rhizomes in the autumn are probably caused by replenishment of the rhizomes with carbohydrates.

References

Agnew, M.P. and Lauren, D.R. (1991) Determination of Ptaquiloside in Bracken Fern (*Pteridium esculentum*). *Journal of Chromatography* 538, 462-468.

Alonso-Amelot, M.E., Calcagno, M.P. and Pérez-Inojosa, M. (2000) Growth and Selective Defensive Potential in relation to Altitude in Neotropical *Pteridium aquilinum* var. *caudatum*. In: Taylor, J.A. and Smith, R.T., (Eds.) *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999.* IBG, Aberystwyth, pp. 43-47.

Alonso-Amelot, M.E., Pérez-Mena, M., Calcagno, M.P., Jaimes-Espinosa, R. and Castillo, U.F. (1992) Ontogenic Variation of Biologically Active Metabolites of *Pteridium aquilinum* (L. Kuhn), Pterosins A anD B, Ptaquiloside in a Bracken Population of the Tropical Andes. *Journal of Chemical Ecology* 18, 1405-1420.

Alonso-Amelot, M.E., Rodulfo-Baechler, S. and Jaimes-Espinosa, R. (1995) Comparative Dynamics of Ptaquiloside and Pterosin B in the Two varieties (*caudatum* and *arachnoideum*) of Neotropical Bracken Fern (*Pteridium aquilinum* L. Kuhn). *Biochemical Systematics and Ecology* 23, 709-716.

Castillo, U.F., Wilkins, A.L., Lauren, D.R., Smith, B.L., Towers, N.R., Alonso-Amelot, M.E. and Jaimez-Espinoza, R. (1997) Isoptaquiloside and Caudatoside, Illudane-type Sesquiterpene Glucosides from Pteridium aquilinum var. caudatum. *Phytochemistry* 44, 901-906.

Hansen, K. (1991) *Dansk Feltflora* (in Danish). Nordisk Forlag A.S., Copenhagen, 759pp.

Nicholson, I.A. and Paterson, I.S. (1976) The Ecological Implications of Bracken Control to Plant/animal Systems. *Botanical Journal of the Linnean Society* 73, 269-283.

Paterson, S., Pakeman, R.J. and Marrs, R. (1997) Evaluation of a Bracken (*Pteridium aquilinum* (L.) Kuhn) Growth Model in Predicting the Effects of Control Strategies across a range of Climatic Zones in Great Britain. *Annals of Applied Biology* 130, 305-318.

Rasmussen, L.H., Jensen, L.S. and Hansen, H.C.B. (2001) The Variation of the Ptaquiloside-content Inside and Between Four Danish Bracken Populations (Fronds, Rhizomes, and O-horizons). *Submitted to Natural Toxins*

Saito, K., Nagao, T., Matoba, M., Koyama, K., Natori, S., Murakami, T. and Saiki, Y. (1989) Chemical assay of Ptaquiloside, the Carcinogen of *Pteridium aquilinum*, and the Distribution of Related Compounds in the Pteridaceae. *Phytochemistry* 28, 1605-1611.

Smith, B.L., Seawright, A.A., Ng, J.C., Hertle, A.T., Thomson, J.A. and Bostock, P.D. (1994) Concentration of Ptaquiloside, a Major Carcinogen in Bracken Fern (*Pteridium* spp.), From Eastern Australia and From a Cultivated Worldwide Collection held in Sydney, Australia. *Natural Toxins* 2, 347-353.

Watt, A.S. (1976) The Ecological Status of Bracken. *Botanical Journal of the Linnean Society* 73, 217-239.

Whitehead, S.J. and Digby, J. (1997) The Morphology of Bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors - a Comparison of the Mature Stand and the Interface with Heather (*Calluna vulgaris* (L.) Hull) 1. The Fronds. *Annals of Applied Biology* 131, 103-116.

Whitehead, S.J. and Digby, J. (1997) The Morphology of Bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors - a Comparison of the Mature Stand and the Interface with Heather (*Calluna vulgaris* (L.) Hull) 2. The Rhizome. *Annals of Applied Biology* 131, 117-131.

Williams, G.H. and Foley, A. (1976) Seasonal variations in the carbohydrate content of bracken. *Botanical Journal of the Linnean Society* 73, 87-93.

Øllgaard, B. and Tind, K. (1993) *Scandinavian Ferns*. Rhodos, Copenhagen, 317pp.

Sorption, degradation and mobility of ptaquiloside, a carcinogenic Bracken (*Pteridium* sp.) constituent, in the soil environment

Lars Holm Rasmussen[*a], Hans Christian Bruun Hansen[a] and Denis Lauren[b]

[a]*The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.* [b]*The Horticulture and Food Research Institute Ltd., Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand.*

[*] Corresponding author: Phone (+45) 35282828; Fax (+45) 35282398; E-mail LHR@KVL.DK

Keywords: *Natural toxin, cancer, soil contamination, soil sorption, K_{ow}.*

Abstract

Ptaquiloside (PTA) is a carcinogenic norsesquiterpene glucoside produced by Bracken in amounts up to at least 500 mg m^{-2}. The toxin is transferred from Bracken to the underlying soil from where it may leach to surface and groundwater's impairing the quality of drinking water. The objectives of the present study were to characterize the solubility, degradation and retention of PTA in soils in order to evaluate the risk for groundwater contamination. PTA was isolated from Bracken. The logarithmic octanol-water and ethyl acetate-water partitioning coefficients for PTA were -0.63 and -0.88, respectively, in agreement with the high water solubility of the compound. PTA hydrolysed rapidly in aqueous solution at pH 4 or lower, but was stable above pH 4. Incubation of PTA with 10 different soils at 25ºC showed three different first-order degradation patterns; i) rapid degradation observed for acid sandy soils with half life's ranging between 8 and 30 hours decreasing with the soil content of organic matter, ii) slow degradation in less acid sandy soils with half lives of several days, and iii) fast initial degradation with a concurrent solid phase-water partitioning reaction observed for non-acid, mostly clayey soils. The presence of clay silicates appears to retard the degradation of PTA, possibly through sorption. Degradation at 4^{o}C was generally of type (iii) and degradation rates were up to 800 times lower than at 25^{o}C. Sorption isotherms for the same set of soils were almost linear and generally showed very low sorption affinity with distribution coefficients in the range 0.01 to 0.22 L kg^{-1} at a solution concentration of 1 mg L^{-1} except for the most acid soil; Freundlich affinity coefficients increased linearly with clay and organic matter contents. Negligible sorption was also observed in column studies where PTA and a non-sorbing tracer showed almost coincident break-through. Based on these results leaching of PTA to the aqueous environment will be most extensive on sandy soils, pH > 4 and poor in organic matter exposed to high precipitation rates during cold seasons.

1. Introduction

Plants and fungi produce vast amounts of natural substances as part of their chemical interaction with other species. Just like pesticides of human origin, such substances can be quite toxic towards intended or accidental target organisms (Kaufman et al., 1999; Teuscher and Lindequist, 1994). Such a toxic natural compound is the norsesquiterpene glycoside ptaquiloside (PTA, Table 1) encountered in several ferns, but most notably in the Bracken ferns, which are classified as carcinogenic by the International Agency of Research on Cancer (IARC, 1998).

Bracken fern (*Pteridium aquilinum* (L.) Kuhn., Dennsteadtiaceae) is a globally widespread species comprising 2 subspecies and 12 geographic varieties. It is found in a wide range of ecosystems, mainly on acid sandy soils (Mosberg et al., 1994; Page, 1976). PTA cause acute poisoning, blindness and cancer in animals browsing on Bracken (Fenwick, 1988; Smith, 1997; Hirono, 1989). PTA can be transferred to Man when Bracken is utilized as food. In Japan and Brazil occurrence of oesophageal cancer is observed among people eating Bracken (Hirono et al., 1972; Haenszel et al., 1976; Marlière et al., 2000). Correlation between Bracken exposure and cancer has also been demonstrated in rural parts of South America where Bracken is not used for food. Milk from cows eating Bracken are thought to be the vector for the transfer of PTA to humans in such areas, as approx. 9% of the PTA content in fronds eaten by cows are excreted into the milk (Alonso-Amelot et al., 1998; Alonso-Amelot, 1997; Alonso-Amelot et al., 1996; Alonso-Amelot, 1999). Contamination of drinking water with Bracken leachates including PTA has also been proposed. No direct or indirect evidence has although been found for contamination of drinking water (Galpin and Smith, 1986; Galpin et al., 1990). Recently, Danish investigations have demonstrated the occurrence of PTA in

soil materials, and the drinking water hypothesis was repostulated (Rasmussen et al., 2003a; Rasmussen et al., 2003b).

PTA is found in all varieties of Bracken, but in rather variable amounts - between 0 and 13,000 μg g^{-1} in the fronds (Smith et al., 1994; Alonso-Amelot et al., 1995; Rasmussen and Hansen, 2003; Rasmussen et al., 2003a; Rasmussen et al., 2003b). The content is usually highest at the beginning of the growth season, and is determined by the genetic heritage of the ferns as well as external growth factors like climate and soil properties (Smith et al., 1994; Smith et al., 1988; Alonso-Amelot et al., 1992; Rasmussen and Hansen, 2003; Rasmussen et al., 2003b). The content in the belowground fern rhizomes lies between 5 and 7,050 μg g^{-1}, exhibiting peak concentrations in the autumn, while the PTA-content in roots is in the range 5-230 μg g^{-1} (Alonso-Amelot et al., 1992; Rasmussen and Hansen, 2003; Saito et al., 1989).

PTA might be transferred to the soil when dead or living Bracken materials are leached by rain (Ojika et al., 1987; Rasmussen et al., 2003a). PTA-contents in the range of 0.09 to 8.49 μg g^{-1} has been found in Oi/Oe-horizons from Bracken stands in Denmark, while corresponding Oa and A-horizons had contents between 0.01 and 0.09 μg g^{-1} (Rasmussen et al., 2003a; Rasmussen et al., 2003b). These levels equal 0.3-160 mg m^{-2} (Oi/Oe-horizons) and 0.9-57 mg m^{-2} (Oa or A-horizons). The corresponding content in the fronds were 15-500 mg m^{-2} (Rasmussen et al., 2003b). Saito et al. (1989) found that more than 25 % of the initial PTA-content were still present after 6 weeks in dead fronds exposed to sunlight in open air protected from rain. However, it is until now an unresolved question whether PTA is stable enough and has sufficient mobility to reach streams, lakes or aquifers.

Another question is whether the potential concentrations in drinking water could be high enough to cause cancer. Based on carcinogenicity-tests with rats (Smith et al., 1988; Hirono et al., 1987; Hirono et al., 1984), it is possible to estimate a maximum tolerable concentration of PTA in drinking water by using the *One-hit model* and a tolerable increase in mortality of 1

per million (Danish standards and methodology (Miljøstyrelsen, 1992)). This gives a maximum tolerable concentration of PTA in drinking water of 0.5-16 ng L^{-1}. Rasmussen et al. (2003a) found PTA-concentrations in artificial frond run-offs of 100,000-730,000 ng L^{-1}, hereby indicating a large potential for dangerous soil and water contamination.

The degradation of PTA by hydrolysis (Fig. 1) is a second order reaction in aqueous systems, with approx. 50% degradation after 7 days at pH 4.0 (37°C), while at pH 5.5 and 7.0 approx. 60 and 90% of the initial concentration were still to be found after 7 days (Saito et al., 1989). The estimated activation energy of hydrolysis of 65 kJ mol^{-1} is high, showing that the reaction is strongly temperature dependent (Burkhalter et al., 1996). Topsoil's with Bracken-cover usually exhibits pH-values between 3 and 5 (Nagao et al., 1989; Johnson-Maynard et al., 1998; Johnson-Maynard et al., 1997; Hetherington and Anderson, 1998; Mackney, 1961; Rasmussen et al., 2003a; Rasmussen et al., 2003b). Such pH-values should result in a relative fast degradation of PTA due to hydrolysis. As well as simple hydrolysis, PTA, or more correctly, the unstable dienone (Fig. 1: [3]) is known to be involved in a series of reactions due to the electrophile nature of the cyclopropane ring system. The cyclopropane ring has a high affinity for thiol and sulphide groups in amino acids, and is capable of cleaving DNA by forming DNA-adducts (Kushida et al., 1994; Ojika et al., 1989; Shahin et al., 1998). An unresolved question is whether microbial or enzymatic degradation of PTA occurs in soil.

The overall aim of this study is to characterize the stability and retention of PTA in soil. The specific aims comprise: 1) Determination of the octanol-water distribution coefficient for PTA and its reaction products; 2) Investigation of the stability of PTA in different soils and fern extracts at near natural temperatures; 3) Determination of the distribution coefficient for PTA in soil materials, and 4) Determination of the distribution coefficient for PTA in different soil horizons in a typical Bracken soil as well as the leaching pattern for PTA. As an

integrated part of the study, methods for PTA analysis in soil samples were developed as well as a new fast and simple method for PTA recovery and purification from ferns.

2. Materials and methods

Figure 2 provides an overview of the different experiments performed. All studies were performed with non-sterile soil materials hereby mimicking near-natural conditions. The development of the soil sorption and stability studies was performed according to the guidelines in OECD-method 106 (OECD, 2000). Sterilization of soil samples by $HgCl_2$-addition was tested, but was found unusable due to changes in the soil sorption properties caused by this treatment. Sterilization with sodium azide was not possible in the experimental set-up due to acid-treatment of the soils involved in PTA-determination (see below), as this would cause liberation of toxic HN_3-gasses and nucleophiles which could interfer with quantitative conversion of PTA to pterosin B for analysis (see Section 2.1). All laboratory equipment was washed thoroughly with ordinary cleaning detergent, thoroughly rinsed with deionised water and left to dry after being washed with methanol or ethanol (technical grade). Due to its high hydrophilicity, PTA did not bind to laboratory glassware or plastic used in the experiments.

2.1. PTA Analysis. The PTA-content in all samples was determined after clean-up of the sample with Polyamide 6 resin (Fluka, Steinheim, Switzerland) (Agnew and Lauren, 1991): 4 mL aquous extract was passed through 0.50 g resin (dry packed) on a 1.0 x 10 cm I.D. glass Econo-Column® (BIO-RAD, New York, USA) fitted with a 28 μm polymer filter. In all soil extracts, the PTA was converted to pterosin B following the method of Agnew and Lauren (1991). Pterosin B was determined at 214 nm at 35°C (Merck Hitachi L-4200 UV-VIS Detector, Merck Hitachi oven) using a Merck Hitachi 655A-40 Auto-Sampler, Merck Hitachi D-6000A Interface and a Merck Hitachi L-6200 Intelligent Pump equipped with a Merck LiChroCART® column (125 x 4 mm) packed with LiChrospher® 100 RP-8, 4 μm. The

measurements were performed with a double deionised-H_2O:acetonitrile (HPLC grade) eluent (83:17 V:V) at a flow rate of 1.00 mL min^{-1}. 20-100 µl sample injection volumes were used for analysis resulting in a detection limit of 0.1 µg mL^{-1}. Standards of pterosin B was made from analytically pure PTA obtained from Professor Ojika (Nagoya University, Japan). PTA for extraction studies was determined at 220 nm (Shimadzu SPD-6AV UV-VIS Spectrophotometric Detector) on a Shimadzu LC-6A Liquid Chromatograph (Shimadzu SCL-6A System Controller) equipped with similar columns as used for pterosin B. The measurements were performed at 35°C (Shimadzu CTO-6A Column Oven) with an MillQ-H_2O:acetonitrile (HPLC grade) eluent (92:8 V:V) at a flow rate of 1.00 mL min^{-1}. A 20 µl sample injection-volume was used for PTA analysis resulting in a detection limit of 5 µg mL^{-1}. Standards of PTA were made from pure PTA obtained from Professor Ojika (Nagoya University, Japan). The stability of PTA as well as of pterosin B standards was evaluated each day of analysis. PTA standards were generally only stable for a single working day while standards of pterosin B were stable for at least 5 days (likely well over that). New working standards were prepared each week.

2.2. Extraction and Purification of PTA. Semi-pure PTA for the soil sorption, degradation, and partitioning studies, was obtained by extraction of PTA from freeze-dried and milled Bracken with MillQ-water (40 g Bracken in 400 mL, shaken for 60 min). 100 mL fractions of the crude extract were passed through dry packed Polyamide 6 resin (5.00 g in a 1.0 x 20 cm I.D. glass Econo-Column®) under gravity. The first 50 mL of eluate was used directly in the further clean-up, while the last 40 mL eluate was cleaned a second time with Polyamide 6 (2.00 g in a 1.0 x 20 cm I.D. glass Econo-Column®). The combined eluates (approx. 200 mL) were then added to a XAD2-column and passed through at a rate of approx. 2 drops per second (20 mL Amberlite® XAD® 2 resin (Supelco, Bellefonte, PA, USA) in a 2.5

x 20 cm I.D. glass Econo-Column®, wetpacked). The column was washed with 3 x 15 mL of MillQ-water. All eluates to this point were discarded. The PTA was extracted with 3 x 15 mL methanol (HPLC grade) into a round-bottomed flask. This extract was evaporated to dryness under reduced pressure at 45°C using a rotary-evaporator. The residue was dissolved in 4 mL MillQ-water and the solution added to a Polyamide 6 column (1.00 g Polyamide, 1.0 x 10 cm I.D. glass Econo-Column®, drypacked). The first 0.8 mL eluate was discarded, then a Chem Elut 1020 column (Varian, USA) was then mounted below the Polyamide column and the rest of the eluate passed on to the Chem Elut column. The round-bottomed flask was washed with 4 mL of MillQ-water, and was also added to the Polyamide column. The wash was repeated twice with 2 mL of MillQ-water. Once all the aqueous eluates had adsorbed into the Chem Elut column, it was flushed with 10 x 20 mL ethyl acetate (HPLC grade), and the eluates collected in a round-bottomed flask and evaporated until dry at low pressure (rotary-evaporator, 45ºC). The resulting amorphous yellowish gum of raw-PTA was stored in a desiccator in vials under N_2-atmosphere at -20ºC. The PTA yield through this process was 90-100%, and the resulting raw-PTA had a purity of 20-80% (Fig. 3). The purity of the raw-PTA was determined against a standard of pure PTA. The resulting purity depends on the PTA-content in the ferns used for extraction as well as the content of interfering substances. Only raw-PTA with a purity of 70-80% was used in subsequent work.

2.3. Determination of Octanol-Water Partitioning Coefficient (K_{ow}) for PTA. Analytical pure PTA was dissolved in MillQ-water resulting in a stock solution with [PTA] = 88 μg mL^{-1}. The PTA was partitioned between water and n-octanol (analytical grade) by careful shaking 1 part of the PTA stock solution with 6 parts of n-octanol for 5 min in a 10 mL glass centrifuge tube at 25°C before separation of the phases by centrifugation at 3,600 g (Hansch

and Leo, 1995). The PTA-content in the aqueous fraction was measured directly. The experiment was repeated 8 times.

2.4. Determination of Octanol-Water Partitioning Coefficient (K_{ow}) for Pterosin B. Pterosin B was made from analytical pure PTA following the method of Agnew and Lauren (1991) and dissolved in MillQ-water resulting in a stock solution with [pterosin B] = 31 μg mL^{-1}. The pterosin B was partitioned between water and n-octanol (analytical grade) by careful shaking 500 parts of pterosin B stock solution with 1 part n-octanol for 5 min in a 10 mL glass centrifuge tube at 25°C before separation of the phases by centrifugation at 3,600 g (Hansch and Leo, 1995). The pterosin B-content in the aqueous fraction was measured directly. The experiment was repeated 10 times.

2.5. Determination of Ethyl Acetate-Water Partitioning Coefficient ($K_{EtOAc/H2O}$) for PTA. A stock solution of analytical pure PTA was made in MillQ-water with [PTA] = 72 μg mL^{-1}. The PTA was partitioned between water and ethyl acetate (HPLC grade) by careful shaking 1 part PTA stock solution with 2 parts of ethyl acetate for 5 min in a 10 mL glass centrifuge tube at 25°C before separation of the fases by centrifugation at 3,600 G (Hansch and Leo, 1995). The PTA-content in the aqueous fraction was measured directly. The experiment was repeated 9 times.

2.6. Estimation of Octanol-Water Partitioning Coefficient for Ptaquilosin and the Unstable Dienone. The distribution coefficients were estimated by using the method of structural groups contribution by the ACD log*P*-algorithm (ACD, 1995).

2.7. Stability of PTA in Aqueous Solutions. Raw-PTA was dissolved in double deionised-water or in 0.01 M $CaCl_2$ with preset pH in the range 2-7 resulting in $[PTA]_{START}$ = 13 μg mL^{-1}. 0.001 M acetic acid – acetate buffer systems were used as well as pH adjustment with 0.001 M HCl or 0.001 M NaOH. The stability was investigated during 24 hours at 25°C to enable comparison with soil sorption studies. PTA-concentrations were measured after Polyamide 6 treatment and conversion to pterosin B.

2.8. Stability of PTA in Aqueous Bracken Extracts. 16.00 g Bracken (dried and milled) was extracted with 800 mL MillQ-water for 60 min. The suspension was centrifuged and filtered (Whatman No. 3). Two aliquots were stored in 500 mL Schott®-bottles at 25ºC ($[PTA]_{START}$ = 113 μg mL^{-1}), while two similar samples were kept protected from light at 4ºC in a refrigerator ($[PTA]_{START}$ = 105 μg mL^{-1}). The PTA-contents were measured after clean-up (Polyamide 6) and conversion to pterosin B daily for the first 5 days, and at day 7, 10 and 25. In addition, pH was measured in the extracts. The extracts were aerated for 5-10 min. when samples were collected untill day 10, whereafter aeration was carried out three times a week.

2.9. Soil Materials and Extraction of PTA from Soil. 10 soil types representing typical Danish natural and cultivated soils with properties similar to soils where Bracken could be found were chosen for the sorption and degradation studies. The criteria given in OECD method 106 were used as a guideline to ensure a wide range in soil properties with respect to content of organic carbon, pH, and texture (Table 2, OECD, 2000).

In the sorption and degradation studies, the content of PTA in contact with soil was determined after extraction of 10 g soil with 10 mL 0.01 M $CaCl_2$ for 60 min (flatbed shaker, approx. 40 rpm.). The soil was then separated from the solution by centrifuging for 5 min at

11,200 G and 4 mL of the clear extract added to a Polyamide 6 column (0.50 g Polyamide, 1.0 x 10 cm I.D. glass Econo-Column®, drypacked). For each sample, the extraction was repeated 4 times with 4 mL 0.01 M $CaCl_2$ added to the soil and shaking by hand for 5 min before centrifuging. The PTA-content was determined in the different fractions after conversion to pterosin B. Extracts estimated of having PTA-content well above the detection limit were pooled before measurement. No further PTA could be extracted after 5 extractions. The recovery for spiked samples was generally below 100% since some PTA reacts irreversibly with the soil materials (see: *3. Results and Discussion*).

2.10. Stability of PTA in Contact with Soil Materials. 10.00 g of dry soil was equilibrated with 9 mL 0.01 M $CaCl_2$ over night. 1 mL raw-PTA dissolved in 0.01 M $CaCl_2$ was then added to the suspension resulting in a total of 10 mL aqueous solution in contact with the soil material. The $[PTA]_{START}$ in the aqueous phase ranged between 22 and 30 μg mL^{-1}. The suspensions were gently shaken on a flatbed shaker (approx. 40 rpm) protected from light in a black box kept at 25°C for 1 to 21 days. The samples were aerated 3 times a week. PTA was extracted from the soil according to the method described above. All analyses were made in duplicate. The experiment was repeated at 4ºC for 65 days without shaking with 4 soils using a soil:solution ratio of 10:3 (W:V), $[PTA]_{START}$ in the aqueous phase was 94 μg mL^{-1}.

2.11. Sorption of PTA to Soil Materials. For each soil material 10.00 g of dry soil was equilibrated with 9 mL 0.01 M $CaCl_2$ over night. 0-1 mL raw-PTA dissolved in 0.01 M $CaCl_2$ and 1-0 mL 0.01 M $CaCl_2$ was then added to the suspension resulting in a total of 11 samples containing 10 mL aqueous solution with $[PTA]_{START}$ ranging between 0 and 14 μg mL^{-1}. The suspensions were shaken on an orbital shaker (30-40 rpm) protected from light at 25°C for 24 hours (wrapped in aluminium foil). The aqueous phase was then separated from the soil solids

by centrifugation for 5 min at 11,200 G, and the PTA-content analysed after conversion to pterosin B and clean-up on Polyamide 6.

2.12. Soil Column Studies. Soil columns (30 x 5 cm I.D.) with densities of 1.1-1.6 g cm^{-3} were prepared in acid proof stainless steel columns according to Danish standard (Kjølholt, 1998). The columns were equilibrated with 0.01 M $CaCl_2$ at approx. 65 mm $hour^{-1}$. The influent was added with a peristaltic pump (Alitea C-6Y, Ventur Alitea AB, Sweden). Raw-PTA was dissolved in 0.01 M $CaCl_2$, and 1 mL of the PTA-solution was then mixed with 4 mL of 0.01 M KBr (used as inert tracer). Initial PTA-concentrations were in the range 177-680 μg mL^{-1}. The PTA/bromide solution was added to the column by pipette and the breakthrough curve was obtained by analysing 23 mL fractions each equalling 11.7 mm of precipitation/influent added. PTA in the fractions was measured after conversion to pterosin B, while bromide was measured directly with a Br-sensitive crystal-membrane electrode (Metrohm, Denmark). The pH of the eluates was measured after 35, 130, 220, and 310 mm of influent had passed the column.

2.13. Soil Chemical and Physical Analysis. Soil pH was measured in a 1:2.5 or 1:10 soil:0.01 M $CaCl_2$ suspension in mineral soil or organic soil samples, respectively. Organic carbon was determined by dry combustion using an Eltra CS 500 Carbon Sulfur Determinator (Nelson and Sommers, 1982). The content of calcite was measured gas-volumetrically (Collins, 1906). The soil samples were fractionated into the following particle-size classes after treatment with 0.1 M sodium pyrophosphate: Sand (20-2,000 μm), silt (2-20 μm), and clay (<2 μm). All soil chemical and physical analysis except from soil textures were performed as dup- or triplicates.

2.14. Statistical and computational methods. Statistical analyses were performed in the *Unscrambler ver. 7.6 SR-1* software package (Camo ASA software, Oslo, Norway) and in *Microsoft® Excel 2000* (Microsoft Corporation, USA). Multiple Linear Regression (MLR) analysis was conducted on soil properties (pH, texture, and carbon-content) and soil sorption data obtained for PTA in the soil sorption and degradation studies to unveil correlations between soil properties and PTA-behaviour in soil. The MLR-models were validated and evaluated by full cross validation and one-way analysis of variance. Distribution coefficients for PTA in soil materials were determined by fitting data from soil sorption studies with Freundlich-isotherms in the software package *TableCurve™ 2D* (Jandel Scientific, AISN Software, USA).

3. Results and discussion

3.1. Partitioning Coefficients in Non-soil Systems. The logarithm of the partitioning coefficient for PTA between water and ethyl acetate was -0.88 (Table 3), which is in accordance with previous findings (Ojika et al., 1987). The experimental logarithmic partitioning coefficients between octanol and water for PTA, pterosin B, and the estimated coefficients for ptaquilosin and the dienone intermediate were –0.63, 3.33, 1.03 and 1.94 respectively (Fig. 1, Table 3). The low value for PTA indicate a low sorption and hence a high mobility of the compound in the soil environment due to weak sorption to soil organic matter, all other things being equal (Schwarzenbach et al., 1993). However, formation of covalent bonds by reaction of the cyclopropane moiety of ptaquiloside as well as of ptaquilosin and the unstable dienone with functional groups of humic substances or soil minerals may reduce mobility. Due to the high reactivity of the cyclopropane moiety of of ptaquilosin and the unstable dienone, formation of covalent bonds is likely the main reaction of these compounds.

3.2. Stability of PTA in Aqueous Solutions. PTA is stable in the range pH 4-7 for at least 24 hours, but hydrolyses rapidly at pH-values below 4 (Fig. 4), which is in accordance with other findings (Burkhalter et al., 1996; Saito et al., 1989).

3.3. Stability of PTA in Bracken Extracts. The time course of PTA degradation and the resulting variation in pH of aqueous Bracken extracts stored at 4°C and 25°C are shown in Figure 5. The incubation temperature was the only parameter which was varied between the two experiments. Initial pH is 6.1 in all extracts for the first 24 hours. Hereafter pH drops to 5 followed by an increase to 7.5 in the extracts stored at 25°C. The pH-variation is likely due to fermentation, as production of different carboxylic acids will lower the pH followed by an

increase in pH resulting from the liberation of proteins from the decaying Bracken compounds. Extracts stored at 4°C do not exhibit the same variations in pH. At ambient temperature (25ºC), PTA seems to be stable for 192 hours after which degradation of PTA starts. Thereafter, the concentration of PTA decreases with approx. 5.25 % per day. This pattern is likely due to growth of bacteria capable of utilizing PTA as energy source, increased concentration of β-glucosidase-enzyme in the suspension combined with increased hydrolysis of PTA when pH increases after 192 hours (Saito et al. 1989). For the experiment run at 4ºC, the ptaquiloside degradation is much slower with no degradation taking place during the first 260 hours. These investigations clearly demonstrate that PTA can exist outside the fern in aqueous extracts for prolonged periods of time, especially at low temperatures.

3.4. Stability of PTA in Contact with Soil Materials. The stability of PTA in contact with different soil materials at 4 and 25ºC is plotted against time in Figure 6. At 25°C (Fig. 6A), three degradation patterns can be distinguished:

1) Fast degradation (full degradation within 240 hours, half life between 8 and 30 hours, most degraded within 140 hours). The reaction can be described as a first order degradation reaction: $PTA_{SOLUTION} \xrightarrow{k_1} PTA_{DEGRADED}$. The rate constants ($k_1$) are listed in Table 4. This group (group 1) comprises: Jyndevad Farm A; Åstrup Forest A; Nørlund Plantation E, and Præstø Bracken Stand Oa.
2) Fast initial degradation (degradation of 40-60% within 24 hours) followed by slow or almost no degradation. The overall reaction can be fitted as the sum of a first order reaction degradation reaction (rate constant k_{21}, Table 4) and an equilibrium step characterized by the forward and reverse constants k_{22} and k_{23} (Fig. 7, Table 4). This group (group 2) comprises: Tåstrup Organic Farm A; Christianssæde Ap; Flakkebjerg Farm Btg2, and Flakkebjerg Farm 2Cg1.

3) Slow degradation throughout the whole timespan (half life between 150 and 180 hours). The reaction can be described as degradation pattern 1, and first order rate constants are found in Table 4. This group (group 3) consists of Nørlund Plantation Bhs and Nørlund Plantation C.

In this context, *degraded* is defined as the amount of PTA, which cannot be extracted by water, comprising irreversible bound PTA, hydrolysed PTA, and PTA otherwise degraded.

The soils from group 1 are characterized by having low clay contents (0-8%), low to high carbon-contents (1-40%), and low pH (pH < 4 - with Jyndevad Farm A as an notable exception). The soils making up group 2 are slightly acid to neutral (pH 4.8-7.9), with low carbon-contents (0.06-1.7%), and higher clay contents (14-23%). The two soils in group 3 are slightly acid (pH 4.0-4.6), having low carbon-contents (0.17-0.78%), and low clay contents (1-2%). It is interesting to note that PTA in several of the investigated soils is still present in the soil suspensions after incubation for more than 500 hours at 25°C. The results are in accordance with the recent detection of PTA in the soil environment, but in contrast with the general perception of PTA as being highly unstable (Castillo et al., 1998; Rasmussen et al., 2003a; Rasmussen et al., 2003b).

The degradation pattern observed in group 1 is likely due to a combination of hydrolysis (Burkhalter et al., 1996; Saito et al., 1989), irreversible reactions between PTA and humic substances/clay and possibly microbial degradation in the case of the non-acid Jyndevad Farm A soil. The hypothesis was supported by MLR performed on the reaction constant and soil pH, carbon and clay content showing strong correlation between the first order rate constant and the carbon content (Eq. 1; 95% confidence limits shown, r^2 (calibration) = 0.87, q^2 (cross validation) = 0.80)).

$$k_1 = 0.0150 \pm 0.0058 + 0.0019 \pm 0.0004 \cdot C(\%) \qquad \text{Eq. 1}$$

However, it should be emphasized that the distribution of data with respect to content of organic matter was somewhat skewed due to Præstø Bracken Stand Oa being an organic soil horizon, in contrast to the other horizons being mineral soils (Table 2).

The pattern for group 2 is likely due to fast irreversible sorption of PTA to organic matter and clay minerals combined with hydrolysis followed by a period where PTA is somewhat protected from degradation by reversible sorption to clay minerals (Fig. 7). The protection of PTA by sorption to clay minerals might be due to weak interlayer sorption in clay minerals, a phenomena previously observed for other glycosides (Greenland, 1956a; Greenland, 1956b). The same sorption reaction has been reproduced by using pure smectites, and is affected negatively by application of surfactants (phthalates) to the soil solution (Authors, unpublished results). One exception from the general group 2 pattern is Taastrup Organic Farm A, where a sudden degradation of PTA takes place from 140 to 240 hours, which could be due to microbial degradation. The degradation-pattern observed for group 3 is similar to group 1, but with degradation reactions running at a lower rate. No correlations were found between the reaction constants for group 2 and soil pH, carbon- or clay-content.

The degradation of PTA was repeated at 4°C without shaking for 1,728 hours (Fig. 6B). The overall pattern found at 25°C was observed at 4°C too, but with three exceptions:

1) Fast group 1-like degradation did not take place (see Åstrup Forest A).
2) Slow group 3-like degradation ceased after the first days (see Nørlund Plantation C).
3) All degradation patterns could be modelled as the sum of a first order degradation reaction and an equilibrium step governed by the constants k_{22} and k_{23} (Table 4), hereby indicating temperature influence on the degradation pattern and speed of hydrolysis.

At Nørlund Plantation C, and Flakkebjerg Farm Btg2, the degradation ceased after 24-144 hours, while at Åstrup Forest A, and Taastrup Organic Farm A, it continued, but at a lower rate than in the first few days. A sudden increase in degradation took place at the Flakkebjerg Farm Btg2-soil after 350 hours. These degradation patterns clearly demonstrate the ability for PTA to persist in contact with soil for prolonged time. Since the experimental conditions for the degradation experiment performed at 4°C are more similar to natural conditions in temperate climates than the experiments performed at 25°C, this study indicates that PTA can exist in soil environments for prolonged periods, and supports the findings of Rasmussen et al. (2003a,b).

3.5. Sorption of PTA to Soil Materials. Freundlich-modelling of the sorption data were chosen due to the lack of obvious adsorption maximum in the isotherms:

$$C_{SOIL} = K_{FREUNDLICH} \cdot C_{WATER}^{n} \qquad \text{Eq. 2}$$

where C_{SOIL} = Content of PTA sorbed on soil ($\mu g\ kg^{-1}$), $K_{FREUNDLICH}$ = Freundlich coefficient, C_{WATER} = Concentration of PTA in water ($\mu g\ L^{-1}$), and n = measure of non-linearity. The amount of PTA sorbed to the soils (C_{SOIL}) was calculated as the difference of the amount added and the amount remaining in the soil solution after 24 hours (C_{WATER}). Due to degradation of PTA caused by hydrolysis in some of the soils (mainly group 1) and/or as a result of irreversible sorption (mainly group 2) C_{SOIL} is the sum of sorbed PTA and PTA degraded during 24 hours. It was attempted to extract the actual amount of PTA sorbed to the soil, but due to extremely low sorption to some soils, this was not experimentally possible (Authors, unpublished results). Three soils had pH less than 4.0: Præstø Bracken stand Oa (pH 2.7); Åstrup Forest A (pH 3.3); and Nørlund Plantation E (pH 3.3). According to the rates of hydrolysis shown in Figure 4, this would result in degradation of approx. 55, 30, and 30% of the added PTA within 24 hours when in pure aqueous solution. In the less acid soils, the

amount of PTA which had degraded due to hydrolysis during the 24 hours of sorption is likely close to zero. The results of the soil sorption studies are plotted in Figure 8. Two types of isotherms exist:

1) A linear type with n = 1 (Præstø Bracken Stand Oa; Nørlund Plantation E and Bhs).
2) A convex type with n < 1 (all other soils).

The sorption parameters and the regression coefficients of the sorption isotherms are listed in Table 5. The Freundlich-constants ($K_{FREUNDLICH}$) were found to be in the range 0.0070 to 0.2165 with a single extreme constant of 1.2311 (Præstø Bracken Stand, Oa), clearly demonstrating very low sorption of PTA to soil materials. The n-values were between 0.65 and 1.00. The sites with n = 1.00 did actually have n-values slightly above 1 (1.04 – 1.09), but could be fitted with linear-isotherms as indicated by the r^2-values (Table 5). MLR was performed using the soil characteristics from Table 2 to estimate any correlation to $K_{FREUNDLICH}$ and n (Fig. 9). Præstø Bracken Stand Oa and Åstrup Forest A, were removed from the $K_{FREUNDLICH}$ analysis due to high leverage on the model, while Christianssæde Forest A in addition was removed from the n analysis. The leverage of Præstø Bracken Stand Oa and Åstrup Forest A might be due to a large proportion of degraded PTA caused by hydrolysis. $K_{FREUNDLICH}$ was positively correlated with the carbon-content and the clay-content (Eq. 3; 95%-confidence limits shown, r^2 (calibration) = 0.75, q^2 (cross validation) = 0.42).

$$K_{FREUNDLICH} = 0.0819 \pm 0.0264 \cdot C(\%) + 0.0056 \pm 0.0021 \cdot clay(\%) \quad \text{Eq. 3}$$

The positive regression coefficients clearly indicate that *sorption* of PTA is correlated with the clay- and carbon-contents of the soils.

The non-linearity parameter n was correlated with the carbon-content and pH (Eq. 4, 95%-confidence limits shown, r^2 (calibration) = 0.83, q^2 (cross validation) = 0.57).

$$n = 1.074 \pm 0.108 - 0.0567 \pm 0.0181 \cdot pH + 0.162 \pm 0.046 \cdot C(\%) \quad \text{Eq. 4}$$

The positive correlation with the carbon-content is likely a result of PTA-sorption to organic matter, while the negative correlation with pH could indicate that the sorption capacity of the soil is pH-dependent, i.e. high pH results in a convex adsorption isotherm likely as a result of surface charge changes of the organic matter.

The calibration correlations obtained for $K_{FREUNDLICH}$ and n are rather good when looking at the calibration correlation coefficients only. It must although be emphasized that samples were removed from the analysis, and the cross-validated correlation coefficients are rather low, indicating high leverage of individual soils.

3.6. Soil Column Studies. Breakthrough curves for PTA and bromide are presented in Figure 10. The pH-values measured in the eluates from Nørlund Plantation E and C differed slightly from the reported soil pH values in Table 2, as they were 0.6 and 0.5 units lower respectively. No decreasing or increasing trend in pH development was observed in eluates during the experiment. All added PTA was recovered in the eluates from Nørlund Plantation soils, while 80% was recovered from Åstrup Forest A. Satisfactory modelling of all the resulting breakthrough curves for PTA and bromide could not be performed by the use of convection-dispersion equation due to tailing likely caused by preferential flow (Hansen, 1997). Instead, curves were fitted as Pearson peaks allowing more tailing (Fig. 10). Calculation of distribution coefficients (K_D) based on the retardation of PTA compared to bromide at maximum breakthrough was only possible for Åstrup Forest A, since PTA moved slightly faster than bromide in Nørlund Plantation Bhs and Nørlund Plantation C, and no significant difference between PTA and bromide was observed in Nørlund Plantation E (Table 6). The retardation factor ranged from 0.96 to 1.12. The retardation factors less than 1 is likely due to experimental conditions resulting in insufficient accuracy of the breakthrough curve, but indicates retardation very close to 1 resulting in PTA distribution coefficients close

to 0. The corresponding distribution coefficient therefore ranges from 0 to 0.05 hence showing PTA mobility comparable to that of bromide and demonstrating that PTA does not sorb significantly to soil materials, which is in agreement with the results of the sorption studies, where the $K_{FREUNDLICH}$-coefficients for ranged from 0.0089 to 0.0661. Better resolution of the peaks is likely obtained if longer soil columns are used.

3.7. General Discussion. This investigation shows that PTA is quite mobile in many soil types and may persist for long time when present in sandy, non-acid and cold subsoils - such as many aquifers - or in clayey soil horizons. Based on the results reported above the following statements can be made:

1) PTA degrades rapidly due to hydrolysis when pH is below 4 (i.e. soils in group 1 and 3, Fig. 6) or higher than 8 (Burkhalter et al., 1996; Saito et al., 1989). Consequently, half-lives of PTA in acid sandy soils are short, in the range of 8 - 30 hours at 25 ^{o}C (Fig. 6, Table 4). Half-lives in calcareous soil materials have not been quantified, but probably are quite short due to pH > 8.
2) PTA degrades more slowly in slightly acid to neutral soils where observed half-lifes are usually in the range of days. For clayey soils, the degradation rate at longer reaction times is further reduced probably due to a concurrent solid-phase partitioning reaction (i.e. group 2 soils, Fig. 6, Table 4). The results indicate, that with longer reaction times, clay silicates may sorb PTA in the interlayer space reducing the amount of PTA that is available for degradation in solution.
3) PTA shows low affinity for sorption to organic matter in agreement with its low octanol-water partitioning coefficient (Fig. 8, Table 3). For group 1 soils degradation rates increases with increasing soil organic matter contents which may be due to

irreversible bonding between the alkylating group of PTA and organic matter, or due to increasing microbial activity with increasing content of organic matter (Eq. 1).

4) PTA degradation is strongly temperature-dependent; at 4 ^{o}C overall degradation is much reduced compared with 25 ^{o}C, either due to a decrease in the rate of the first-order degradation reaction, strong retardation due to the solid phase-partioning process, or both (i.e. Fig. 6A vs. 6B).

These sorption and degradation properties of PTA might help explain the high occurrences of cancer in some Bracken dominated areas, as PTA can leach from Bracken stands and enter aquifers or other recipients such as streams and lakes. The apparent lack of correlation between Bracken cover and human cancer on a small scale in some investigations might be partly due to variations in soil properties and/or microbial activity as mirrored in the degradation patterns observed for PTA. It is although still an open question whether PTA-contaminated milk, water or Bracken spores form the links between humans and Bracken. In areas where milk-consumption is dependent on cows browsing on Bracken, then milk is likely the main vector. In areas with only few cows browsing on Bracken or where people obtains milk from areas without Bracken-browsing, then water-contamination might be important as well. This may be the case in areas with many surface wells or shallow aquifers situated below slightly acid clay soils. However, the PTA content in drinking waters still needs to be measured. A preliminary study carried out by the authors did show PTA-contents between 0 and 7 μg L^{-1} in 15 samples of soil solutions sampled at 90 cm depth in a Bracken stand, while two water samples from surface wells encircled by Bracken yielded PTA-contents of 30 and 45 μg L^{-1}. The transfer of PTA from the fern to the soil environment is not yet fully understood; especially leaching from living and dead Brackens needs further examination. PTA can be leached from living fronds by rain (Rasmussen et al., 2003a). This, combined with the fact that PTA can be quite mobile in the soil, indicates that PTA may occur in the soil

water in very variable concentrations correlated with the precipitation pattern. Leaching studies must therefore be very detailed with respect to sampling of soil water. Currently, further studies are being carried out to unveil the incidence of PTA in drinking water.

4. Conclusion

PTA is capable of existing in the soil environment for prolonged time as a highly mobile compound. The octanol-water partitioning coefficient for PTA was extremely low ($log K_{ow}$ = -0.63), while the *log* values of the coefficients for ptaquilosin, the dienone and pterosin B were between 1.03 and 3.33. The stability of PTA in contact with soil or fern extracts was highly temperature dependent as ptaquiloside degraded faster at high temperatures. The observed patterns for degradation of PTA in contact with soil could be grouped into three patterns: 1) Fast degradation (half life between 8 and 30 hours, all degraded within 240 hours); 2) Fast initial degradation (40-60% degraded within 24 hours) followed by poor or almost no degradation; and 3) Slow degradation (half life between 150 and 180 hours). In several soils, PTA was still present after 72 days. The degradation patterns observed was due to differences in acidity, clay content, carbon-content, and probably microbial activity. Sorption isotherms could be fitted by the Freundlich equation with $K_{FREUNDLICH}$–constants between 0.01 and 0.22 except from one soil with a constant of 1.23. n vaulues were between 0.64 and 1.00. The shape of the isotherms was correlated with pH, carbon- and clay-content. Soil column studies performed in a sandy media resulted in breakthrough curves very similar to the inert tracer bromide, hence demonstrating high mobility of PTA in all soils examined.

5. Safety

Use respiration protection-gear while working with powdered Bracken.

Acknowledgement

The authors are grateful to Professor Ojika (Nagoya University, Japan), for the gift of PTA, which made this study possible.

References

ACD 1995. ACD/logP. v. 1.0. Advanced Chemistry Development Inc. Canada.

Agnew, M.P., Lauren, D.R. 1991. Determination of ptaquiloside in Bracken fern (*Pteridium esculentum*). J. Chrom. 538, 462-468.

Alonso-Amelot, M.E. 1997. The link between Bracken fern and stomach cancer: Milk. Nutrition 13, 694-696.

Alonso-Amelot, M.E. 1999. Helecho macho, salud animal y salud humana (Bracken fern, animal and human health). Revista de la Facultad de Agronomia, Universidad del Zulia, Maracaibo, Venezuela 16, 528-541.

Alonso-Amelot, M.E., Castillo, U.F., Smith, B.L., Lauren, D.R. 1996. Bracken ptaquiloside in milk. Nature 382, 587.

Alonso-Amelot, M.E., Castillo, U.F., Smith, B.L., Lauren, D.R. 1998. Excretion through milk, of ptaquiloside in Bracken-fed cows. A quantitative assessment. Lait 78, 413-423.

Alonso-Amelot, M.E., Pérez-Mena, M., Calcagno, M.P., Jaimes-Espinosa, R., Castillo, U.F. 1992. Ontogenic variation of biologically active metabolites of *Pteridium aquilinum* (L. Kuhn), pterosins A ans B, ptaquiloside in a Bracken population of the tropical Andes. J. Chem. Ecol. 18, 1405-1420.

Alonso-Amelot, M.E., Rodulfo-Baechler, S., Jaimes-Espinosa, R. 1995. Comparative dynamics of ptaquiloside and pterosin B in the two varieties (*caudatum* and *arachnoideum*) of neotropical Bracken fern (*Pteridium aquilinum* L. Kuhn). Biochem. Syst. Ecol. 23, 709-716.

Burkhalter, P.W., Groux, P.M.J., Candrian, U., Hübner, P., Lüthy, J. 1996. Isolation, determination and degradation of ptaquiloside - a Bracken fern (*Pteridium aquilinum*) carcinogen. J. Nat. Toxins 5, 141-159.

Castillo, U.F., Ojika, M., Alonso-Amelot, M.E., Sakagami, Y. 1998. Ptaquiloside Z, a new toxic unstable sesquiterpene glucoside from the neotropical Bracken fern *Pteridium aquilinum var. caudatum*. Bioorg. Med. Chem. 6, 2229-2233.

Collins, S.H. 1906. Scheibler's apparatus for the determination of carbonic acid in carbonates: An improved construction and use for accurate analysis. J. Soc. Chem. Industry 25, 518-522.

Fenwick, G.R. 1988. Bracken (*Pteridium aquilinum*) - Toxic effects and toxic constituents. J. Sci. Food Agr. 46, 147-173.

Galpin, O.P., Smith, R.M.M. 1986. Bracken, stomach cancer and water supplies: Is there a link? In *Bracken - Ecology, land use and control technology - The proceedings of the International Conference - Bracken '85* (eds. R.T. Smith and J.A. Taylor), pp. 147-159. Parthenon Publishing, Aberystwyth.

Galpin, O.P., Whitaker, C.J., Whitaker, R., Kassab, J.Y. 1990. Gastric cancer in Gwynedd. Possible links with Bracken. Brit. J. Cancer 61, 737-740.

Greenland, D.J. 1956a. The adsorption of sugars by montmorillonite. I. X-ray studies. J. Soil Sci. 7, 323-328.

Greenland, D.J. 1956b. The adsorption of sugars by montmorillonite. II. Chemical studies. J. Soil Sci. 7, 329-335.

Haenszel, W., Kurihara, M., Locke, F.B., Shimuzu, K., Segi, M. 1976. Stomach Cancer in Japan. J. Natl. Cancer I. 56, 265-274.

Hansch, C., Leo, A. 1995. *Exploring QSAR - Fundamentals and Applications in Chemistry and Biology.* The American Chemical Society, Washington.

Hansen, S. 1997. Water and solute transport in soil. In *Environmental Soil Chemistry* (ed. H.C.B. Hansen), pp. 395-440.

Hetherington, S.L., Anderson, J.M. 1998. Lignin signatures show the effects of changes in heather and Bracken cover on the composition of organic matter in a moorland soil profile. Oecologia 117, 194-200.

Hirono, I. 1989. Carcinogenic Bracken glycosides. In *Toxicants of Plant Origin - Volume II Glycosides* (ed. P.R. Cheeke), pp. 239-251. CRC Press, Inc., Boca Raton.

Hirono, I., Aiso, S., Yamaji, T., Mori, H., Yamada, K., Niwa, H., Ojika, M., Wakamatsu, K., Kigoshi, H., Niiyama, K. and Uosaki, Y. 1984. Carcinogenicity in rats of ptaquiloside isolated from Bracken. Gann 75, 833-836.

Hirono, I., Ogino, H., Fujimoto, M., Yamada, K., Yoshida, Y., Ikagawa, M., Okumura, M. 1987. Induction of tumors in ACI rats given a diet containing ptaquiloside, a Bracken carcinogen. J. Natl. Cancer I. 79, 1143-1149.

Hirono, I., Shibuya, C., Shimisu, M., Fushimi, K. 1972. Carcinogenic activity of processed Bracken used as human food. J. Natl. Cancer I. 48, 1245-1249.

IARC 1998. International Agency for Research on Cancer (IARC) - Summaries & Evaluations. Bracken fern (*Pteridium aquilinum*) and some of its constituents. Homepage: http://www.inchem.org/documents/iarc/iarc848.htm. Access date: 14-8-2002.

Johnson-Maynard, J.L., McDaniel, P.A., Ferguson, D.E., Falen, A.L. 1997. Chemical and mineralogical conversion of Andisols following invasion by Bracken fern. Soil Sci. Soc. Amr. J. 61, 549-555.

Johnson-Maynard, J.L., McDaniel, P.A., Ferguson, D.E., Falen, A.L. 1998. Changes in soil solution chemistry of Andisols following invasion by Bracken fern. Soil Sci. 163, 814-821.

Kaufman, P.B., Cseke, L.J., Warber, S., Duke, J.A., Brielmann, H.L. 1999. *Natural products from plants.* CRC Press, Boca Raton - Boston - London - New York - Washington, D.C.

Kjølholt, J. 1998. *Critical Variables in Soil Column Leaching Tests*. Pesticides Research No. 38. Ministry of Environment and Energy, Denmark and Danish Environmental Protection Agency. Copenhagen.

Kushida, T., Uesugi, M., Sugiura, Y., Kigoshi, H., Tanaka, H., Hirokawa, J., Ojika, M., Yamada, K. 1994. DNA damage by ptaquiloside, a potent bracken carcinogen: Detection of selective strand breaks and identification of DNA cleavage products. J. Am. Chem. Soc. 116, 476-486.

Mackney, D. 1961.. A Podzol development sequence in oakwoods and heather in Central England. J. Soil Sci. 12, 23-40.

Marlière, C.A., Wathern, P., Freitas, R.N., Castro, M.C.F.M., Galvao, M.A.M. 2000. Bracken fern (*Pteridium aquilinum*) consumption and oesophaegal and stomach cancer in the Ouro Preto region, Minas Gerais, Brazil. In *Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999*, (Eds. J.A. Taylor, J.A. and R.T. Smith, R.T.), pp. 144-149. IBG, Aberystwyth.

Miljøstyrelsen 1992. *Sundhedsmæssig vurdering af kemiske stoffer i drikkevand*. Miljøstyrelsen, Copenhagen.

Mosberg, B., Stenberg, L., Ericsson, S. 1994. *Den store nordiske flora*. GEC Gads Forlag, Copenhagen.

Nagao, T., Saito, K., Hirayama, E., Uchikoshi, K., Koyama, K., Natori, S., Morisaki, N., Iwasaki, S., Matsushima, T. 1989. Mutagenicity of ptaquiloside, the carcinogen in Bracken, and its related illudane-type sesquiterpenes. I. Mutagenicity in *Salmonella typhimurium*. Mutat. Res. 215, 173-175.

1 Nelson, D.W., Sommers, L.E. 1982. Total carbon, organic carbon, and organic matter. In
2 *Methods of Soil Analysis. Part 2.*, 2 edn. (eds. A.L. Page, R.H. Miller and D.R.
3 Keeney), pp. 539-579. American Society of Agronomy and Soil Science Society of
4 America, Madison, Wisconsin.
5 OECD 2000. Adsorption-desorption using a batch equilibrium method, method 106. OECD
6 Online Bookshop.
7 Ojika, M., Sugimoto, K., Okazaki, T., Yamada, K. 1989. Modification and cleavage of DNA
8 by ptaquiloside. A new potent carcinogen isolated from Bracken Fern. Journal of the
9 Chemical Society, Chem. Com. 22, 1775-1777.
10 Ojika, M., Wakamatsu, K., Niwa, H., Yamada, K. 1987. Ptaquiloside, a potent carcinogen
11 isolated from Bracken fern *Pteridium aquilinum* var. *latiusculum*: Structure
12 elucidation based on chemical and spectral evidence, and reactions with amino acids,
13 nucleosides, and nucleotides. Tetrahedron 43, 5261-5274.
14 Page, C.N. 1976. The taxonomy and phytogeography of Bracken - a review. Bot. J. Linn. Soc.
15 73, 1-34.
16 Rasmussen, L.H., Hansen, H.C.B. 2003. Growth of Bracken in Denmark and the content of
17 ptaquiloside in fronds, rhizomes and roots. In *Poisonous Plants and Related Toxins*
18 (eds. Acamovic, T. Stewart, C.S. and Pennycott, T.W.). CAB International,
19 Wallingford.
20 Rasmussen, L.H., Jensen, L.S., Hansen, H.C.B. 2003a. Distribution of the carcinogenic
21 terpene ptaquiloside in Bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in
22 Denmark. J. Chem. Ecol. 29, 771-778.
23 Rasmussen, L.H., Kroghsbo, S., Frisvad, J.C., Hansen, H.C.B. 2003b. Occurrence of the
24 carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil
25 layers in Denmark. Chemosphere 51, 117-127.

Saito, K., Nagao, T., Matoba, M., Koyama, K., Natori, S., Murakami, T., Saiki, Y. 1989. Chemical assay of ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the Pteridaceae. Phytochemistry 28, 1605-1611.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M. 1993. *Environmental Organic Chemistry*. John Wiley & Sons, Inc., New York.

Shahin, M., Smith, B.L., Worral, S., Moore, M., Seawright, A.A., Prakash, A.S. 1998. Bracken fern carcinogenesis: Multiple intravenous doses of activated ptaquiloside induce DNA adducts, monocytosis, increased TNF levels, and mammary gland carcinoma in Rats. Biochem. Bioph. Res. Co. 244, 192-197.

Smith, B.L. 1997. The toxicity of Bracken fern (genus *Pteridium*) to animals and its relevance to Man. In *Handbook of Plant and Fungal Toxicants* (ed. J.P. Felix D'Mello), pp. 63-76. CRC Press, New York.

Smith, B.L., Embling, P.P., Agnew, M.P., Lauren, D.R., Holland, P.T. 1988. Carcinogenicity of Bracken fern (*Pteridium esculentum*) in New Zealand. NZ Vet. J. 36, 56-58.

Smith, B.L., Seawright, A.A., Ng, J.C., Hertle, A.T., Thomson, J.A., Bostock, P.D. 1994. Concentration of ptaquiloside, a major carcinogen in Bracken Fern (*Pteridium* spp.), From Eastern Australia and from a cultivated worldwide collection held in Sydney, Australia. Nat. Toxins 2, 347-353.

Teuscher, E., Lindequist, U. 1994. *Biogene Gifte*. 2 edn. Gustav Fischer, Stuttgart - Jena - New York.

FIGURE CAPTIONS

Table no:	**Text:**
1	Table 1. Properties of PTA.
2	Table 2. Properties of soils used in the investigation.
3	Table 3. Distribution coefficients for PTA and derivatives in n-octanol-water and ethyl acetate-water systems (95% confidence limits shown). The coefficients for ptaquilosin and the dienone are estimated by the ACD*logP*-algorithm.
4	Table 4. First order rate constants for PTA degradation in the soil-water systems (see Figures 6 and 7).
5	Table 5. Fitted Freundlich parameters for sorption of ptaquiloside to the soils investigated.
6	Table 6. Retardation factors and distribution coefficients for the soil column studies.
Figure no:	**Text:**
1	Fig. 1. PTA [1] transformation reactions. During alkaline conditions, first ptaquilosin [2] and then the unstable dienone [3] are formed. Both [1] and [3] yields pterosin B [4] under acid conditions. The unstable dienone may alkylate DNA [5] and is likely to react with numerous other substances including humic substances.
2	Fig. 2. Overview of main analyses.
3	Fig. 3. HPLC chromatograms of raw-ptaquiloside. A) Raw-ptaquiloside (purity: 80%). Substances causing peaks eluting later than 15 min was removed from the raw-ptaquiloside by Polyamide treatment before use; B)

	The same sample as A, but after conversion to pterosin B (late eluting peaks not removed).
4	Fig. 4. PTA stability in aqueous solutions at 25°C after shaking for 24 hours: ○) pH adjusted with HCl or NaOH; ◊) acetic acid-acetate buffer. $[PTA]_{START}$ = 13 μg mL^{-1}. Dotted line represents the range where PTA is unstable (pH < 4), while the solid line is the stable range (pH 4-7).
5	Fig. 5. PTA stability in aqueous Bracken extracts (25 days): A) PTA stability; B) Variation of pH; ○) At 4°C ($[PTA]_{START}$ = 105 μg mL^{-1}); □) At 25°C ($[PTA]_{START}$ = 113 μg mL^{-1}).
6	Fig. 6. PTA stability in soil suspensions: A) At 25°C, $[PTA]_{START}$ ranges between 22 and 30 μg mL^{-1}, thick lines represent first order degradation reactions, dotted lines represent the sum of a first order degradation reactions and a concurrent solid phase-water equilibrium; B) At 4°C, $[PTA]_{START}$ = 94 μg mL^{-1}.
7	Fig. 7. Proposed PTA degradation reaction scheme in clayey soils comprising a first order degradation reaction (rate constant k_{21}) and a concurrent solid phase-water partitioning equilibrium characterized by the forward (k_{22}) and reverse (k_{23}) rate constants.
8	Fig. 8. Sorption isotherms for PTA bonding to soil materials (0.01 M $CaCl_2$, 24 hours, $[PTA]_{START}$ in the range 0 to 14 μg mL^{-1}). Solid lines represent Freundlich-fits. Fitting parameters are listed in Table 5. Only the first 5 data points are shown for Præstø Bracken Stand, Oa.
9	Fig. 9. Multiple linear regressions of sorption parameters in Freundlich models versus soil properties: A) $K_{FREUNDLICH}$; B) n. Two soils removed from both regressions: Præstø Bracken Stand Oa and Åstrup Forest A.

	Christiansæde Forest A removed from B. ○ = Calibration. ● = Validation .
10	Fig. 10. Breakthrough curves for ptaquiloside and bromide in packed soil columns composed of soil materials from Danish Spodosols: A) Nørlund Plantation, E-curves fitted with the convection-dispersion equation (dispersion coefficients: 2.1 cm^2 $hour^{-1}$ (bromide) and 17.0 cm^2 $hour^{-1}$ (ptaquiloside)); B) Curves fitted with Pearson curves, R^2 (calibration curves, bromide/ptaquiloside): Åstrup Forest, A = 0.8300/0.9283; Nørlund Plantation, E = 0.9934/0.9582; Nørlund Plantation, Bhs = 0.9982/0.9995; Nørlund Plantation, C = 0.9841/0.9868. 80% ptaquiloside recovery from Åstrup Forest, A; 100% ptaquiloside recovery from Nørlund Plantation, E, Bhs, and C.

Table 1
Properties of PTA

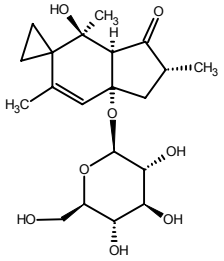
CAS numbers	87625-62-5 88825-03-0
Chemical name	[2'R-(2'α,3'aα,4'β,7'aα)]- 7'a-(β-D-glucopyranosyloxy)-1',3',a,4',7'a-tetrahydro-4'-hydroxy-2',4',6'-trimethyl-spiro[cyclopropane-1-5'-[5H]inden]-3'(2'H)-one
Chemical structure	
Molecular formula	$C_{20}H_{30}O_8$
Molecular weight	398.45 g mol^{-1}

Table 2
Properties of soils used in the investigation

Site and soil horizon	pH (0.01 M $CaCl_2$)	Organic C (%)	Clay content (%)
Præstø Bracken stand, Oa	2.7	38.3	ND¶
Jyndevad Farm, A	6.1	1.8	4
Åstrup Forest, A	3.3	5.2	8
Tåstrup Organic Farm, A	7.2	1.3	14
Christanssæde Forest, Ap	4.8	1.7	17
Nørlund Plantation, E	3.3	0.29	1
Flakkebjerg Farm, Btg2	6.6	0.1	23
Nørlund Plantation, Bhs	4.0	0.78	1
Nørlund Plantation, C	4.6	0.17	2
Flakkebjerg Farm, 2Cg1	7.9	0.06	19

¶ Not determined, but probably close to 0.

Table 3
Distribution coefficients for PTA and derivatives in n-octanol-water and ethyl acetate-water systems (95% confidence limits shown). The coefficients for ptaquilosin and the dienone are estimated by the ACD*logP*-algorithm

PTA	
$log K_{EtOAc/H2O}$	-0.88±0.06
$log K_{ow}$	-0.63±0.01
Pterosin B	
$log K_{ow}$	3.33±0.01
Ptaquilosin	
$log K_{ow}$	1.03±0.48
Dienone	
$log K_{ow}$	1.94±0.41

Table 4
First order rate constants for PTA degradation in the soil-water systems (see Figures 6 and 7)

Temperature	Site and soil horizon	k_1¶	r_1^2	k_{21}	k_{22}	k_{23}	r_2^2
		$hours^{-1}$		$hours^{-1}$	$hours^{-1}$	$hours^{-1}$	
25ºC	Præstø Bracken stand, Oa	0.0864	0.9998	¥			
	Jyndevad Farm, A	0.0239	0.9994				
	Åstrup Forest, A	0.0246	0.9959				
	Tåstrup Organic Farm, A			0.0379	0.0575	0.0943	0.9859
	Christanssæde Forest, Ap			0.0024	0.0038	0.1082	0.9948
	Nørlund Plantation, E	0.0331	0.9941				
	Flakkebjerg Farm, Btg2			0.0009	0.0012	0.0766	0.9791
	Nørlund Plantation, Bhs	0.0045	0.9794				
	Nørlund Plantation, C	0.0043	0.9814				
	Flakkebjerg Farm, 2Cg1			0.0007	0.0008	0.2907	0.9924
4ºC	Åstrup Forest, A			$5.40\ 10^{-5}$	0.0011	0.0174	0.9880
	Tåstrup Organic Farm, A			$4.81\ 10^{-5}$	0.0005	0.0189	0.9829
	Flakkebjerg Farm, Btg2			0.0029	0.0033	0.0546	0.9128
	Nørlund Plantation, C			0.0043	0.0053	0.3525	0.7268

¶ k-values: Rate constants, k_1 = 1. order rate constant; k_{21} = 1. order degradation rate constant; k_{22} = forward rate equilibrium constant (sorption); k_{23} = reverse rate equilibrium constant (desorption), ¥ Empty cells: Not appropriate.

Table 5
Fitted Freundlich parameters for sorption of ptaquiloside to the soils investigated

Site and soil horizon	$K_{FREUNDLICH}$¶	n	r^2
Præstø Bracken stand, Oa	1.23	1.00	0.99
Jyndevad Farm, A	0.22	0.96	0.97
Åstrup Forest, A	0.07	0.75	0.99
Tåstrup Organic Farm, A	0.19	0.94	0.99
Christanssæde Forest, Ap	0.20	0.78	0.99
Nørlund Plantation, E	0.08	1.00	0.99
Flakkebjerg Farm, Btg2	0.18	0.72	0.97
Nørlund Plantation, Bhs	0.01	1.00	0.98
Nørlund Plantation, C	0.01	0.73	0.80
Flakkebjerg Farm, 2Cg1	0.08	0.64	0.87

¶ C_{SOIL} measured in mg kg^{-1}, C_{WATER} measured in mg L^{-1}.

Table 6
Retardation factors and distribution coefficients for the soil column studies

Site and soil horizon	Soil density ($kg\ L^{-1}$)	Porosity (%)	Effluent at max. bromide concentration (mm)	Effluent at max. PTA concentration (mm)	Retardation¶	K_D ($L\ kg^{-1}$)	K_{OC} ($L\ f_{OC}^{-1}\ kg^{-1}$)§
Åstrup Forest, A	1.3	51	133.4±4.8	149.3±2.1	1.12	0.05	0.91
Nørlund Plantation, E	1.4	47	94.2±0.6	93.3±2.0	0.99	NA¥	NA
Nørlund Plantation, Bhs	1.5	43	79.4±1.7	76.4±0.3	0.96	NA	NA
Nørlund Plantation, C	1.6	40	110.0±0.7	108.0±0.3	0.98	NA	NA

¶: Retardation of PTA max. concentration compared to bromide max. concentration. §: f_{OC}: fraction of organic matter. ¥ NA: Not available.

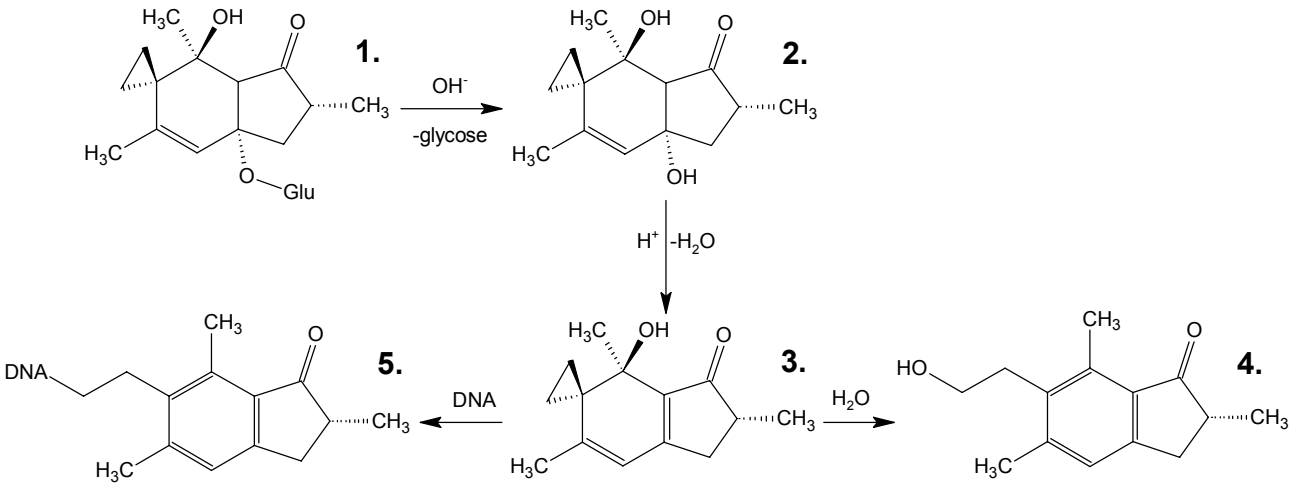


Fig. 1. PTA [1] transformation reactions. During alkaline conditions, first ptaquilosin [2] and then the unstable dienone [3] are formed. Both [1] and [3] yields pterosin B [4] under acid conditions. The unstable dienone may alkylate DNA [5] and is likely to react with numerous other substances including humic substances.

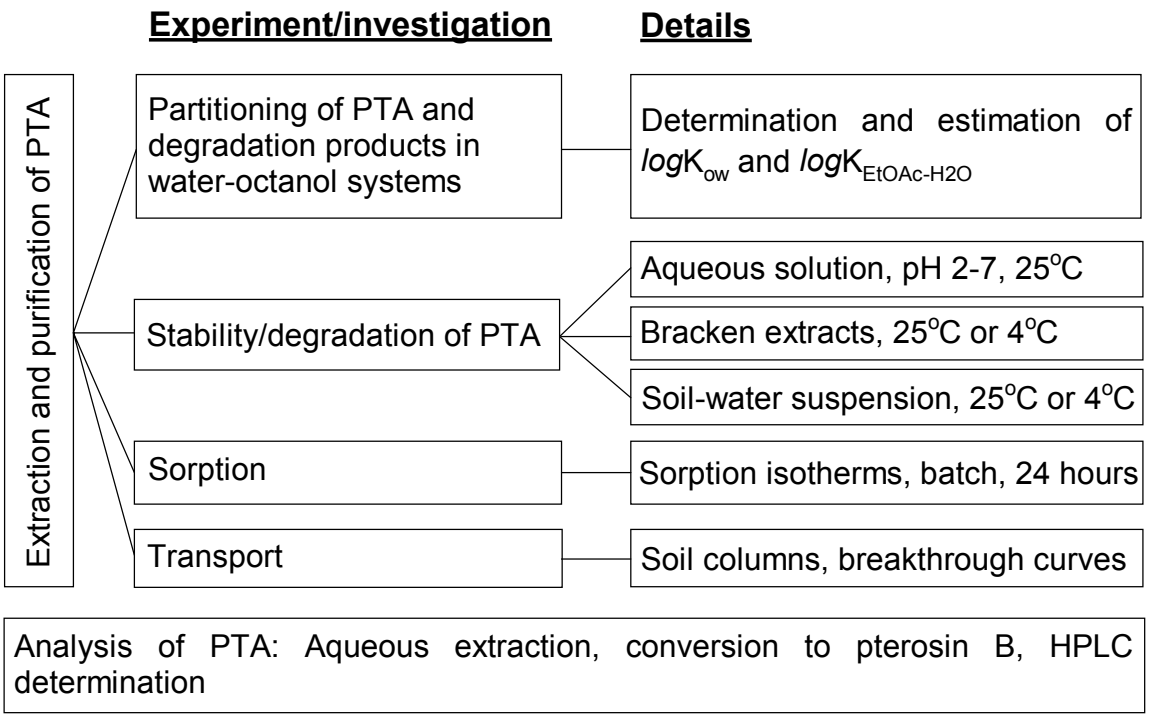


Fig. 2. Overview of main analyses.

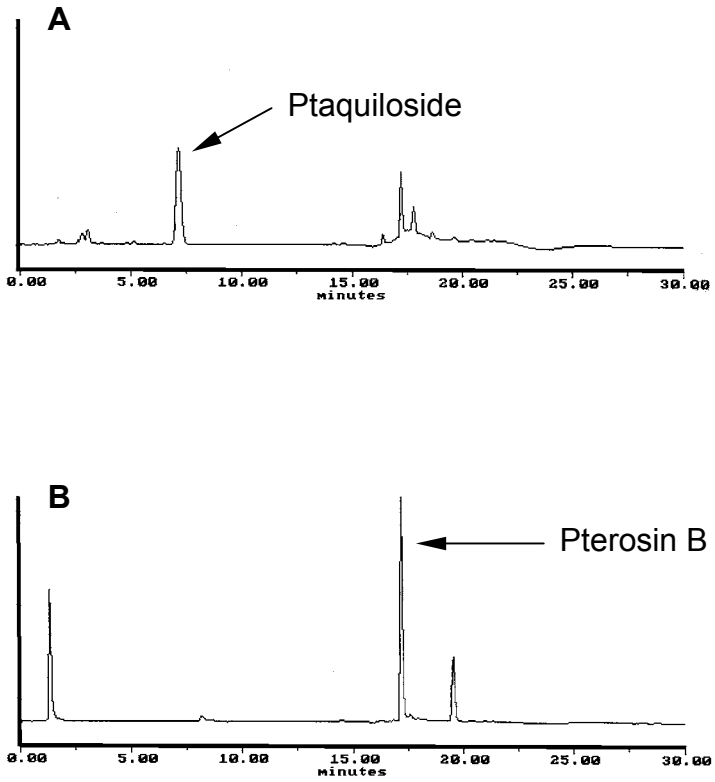


Fig. 3. HPLC chromatograms of raw-ptaquiloside. A) Raw-ptaquiloside (purity: 80%). Substances causing peaks eluting later than 15 min was removed from the raw-ptaquiloside by Polyamide treatment before use; B) The same sample as A, but after conversion to pterosin B (late eluting peaks not removed).

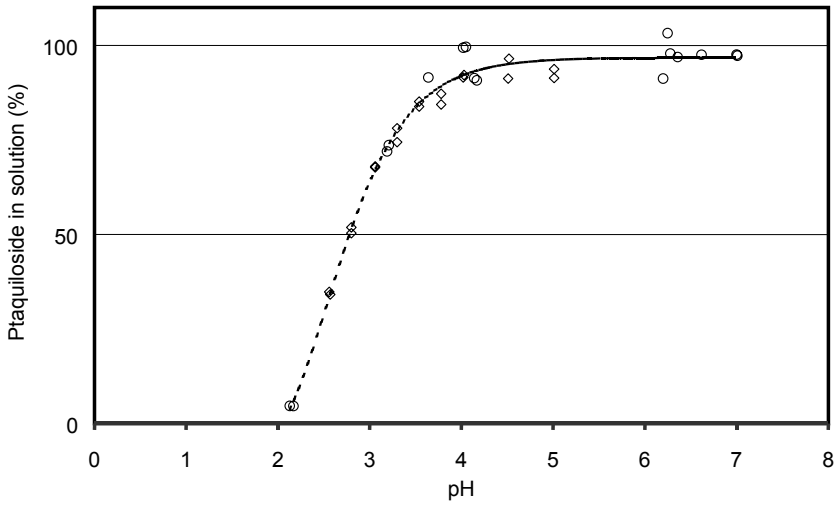


Fig. 4. PTA stability in aqueous solutions at 25°C after shaking for 24 hours: ○) pH adjusted with HCl or NaOH; ◊) acetic acid-acetate buffer. $[PTA]_{START}$ = 13 μg mL^{-1}. Dotted line represents the range where PTA is unstable (pH < 4), while the solid line is the stable range (pH 4-7).

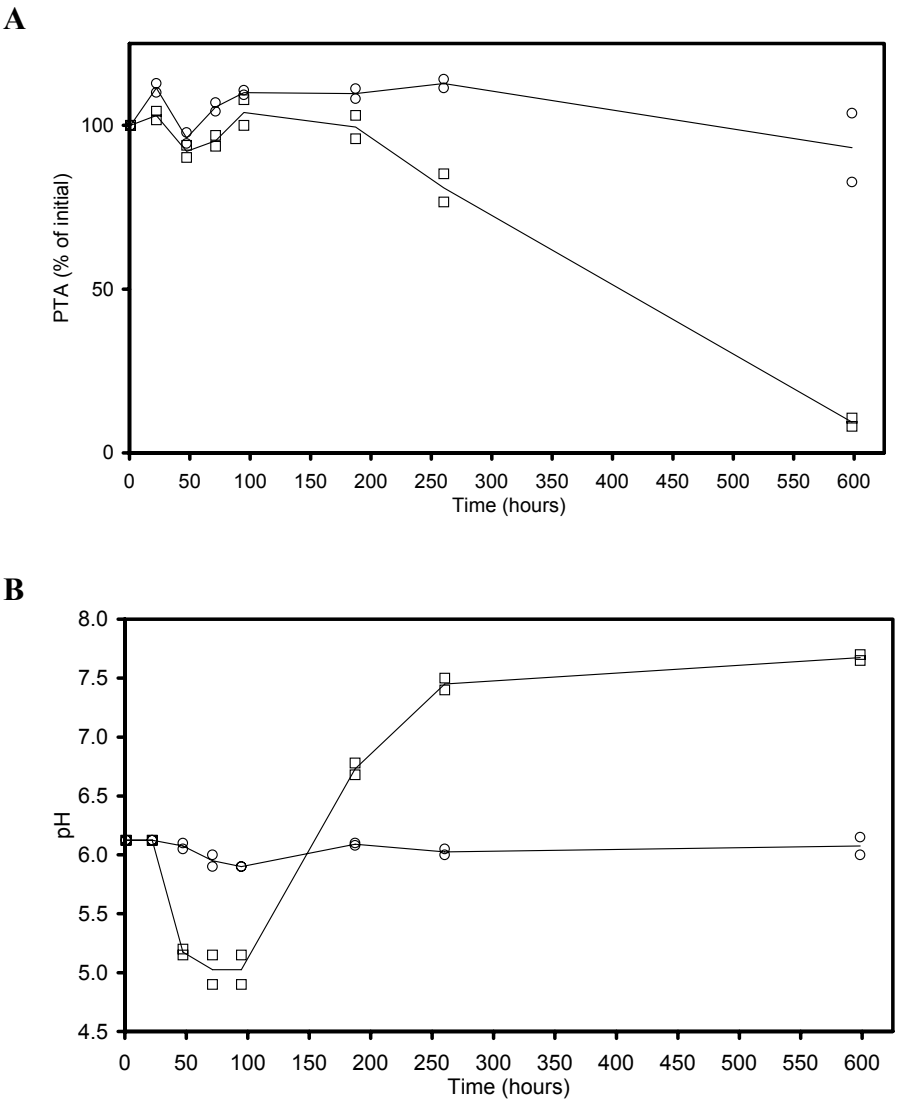


Fig. 5. PTA stability in aqueous Bracken extracts (25 days): A) PTA stability; B) Variation of pH; ○) At 4°C ($[PTA]_{START}$ = 105 µg mL^{-1}); □) At 25°C ($[PTA]_{START}$ = 113 µg mL^{-1}).

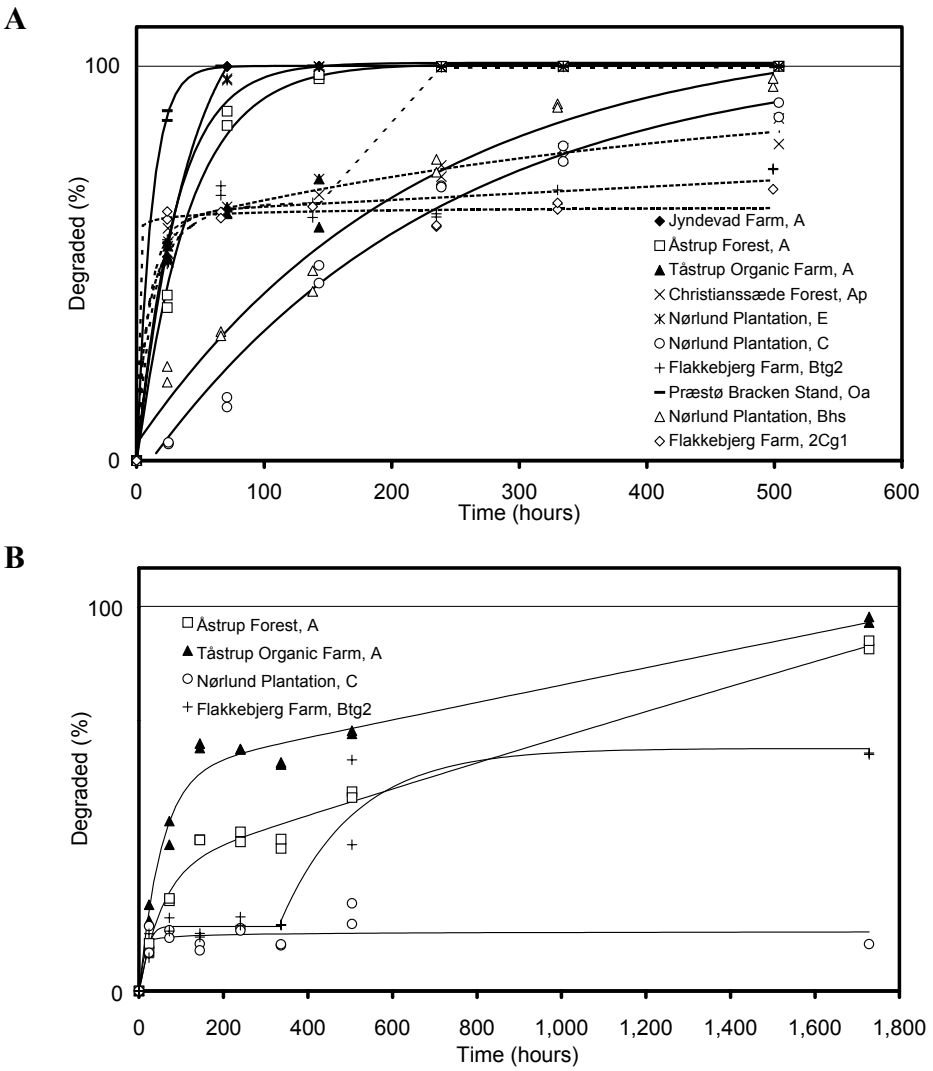


Fig. 6. PTA stability in soil suspensions: A) At 25°C, $[PTA]_{START}$ ranges between 22 and 30 µg mL^{-1}, thick lines represent first order degradation reactions, thin lines represent the sum of a first order degradation reactions and a concurrent solid phase-water equilibrium; B) At 4°C, $[PTA]_{START}$ = 94 µg mL^{-1}.

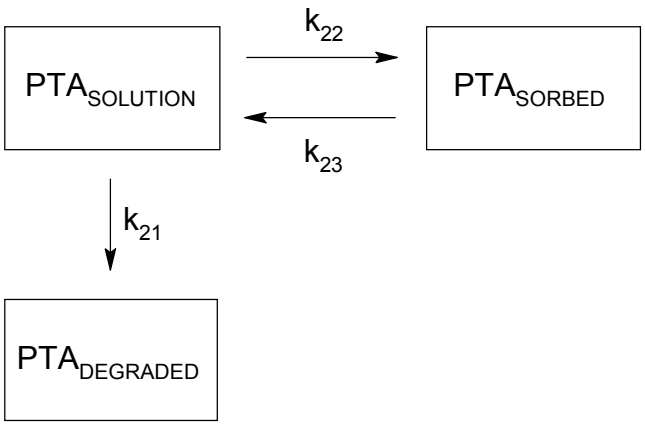


Fig. 7. Proposed PTA degradation reaction scheme in clayey soils comprising a first order degradation reaction (rate constant k_{21}) and a concurrent solid phase-water partitioning equilibrium characterized by the forward (k_{22}) and reverse (k_{23}) rate constants.

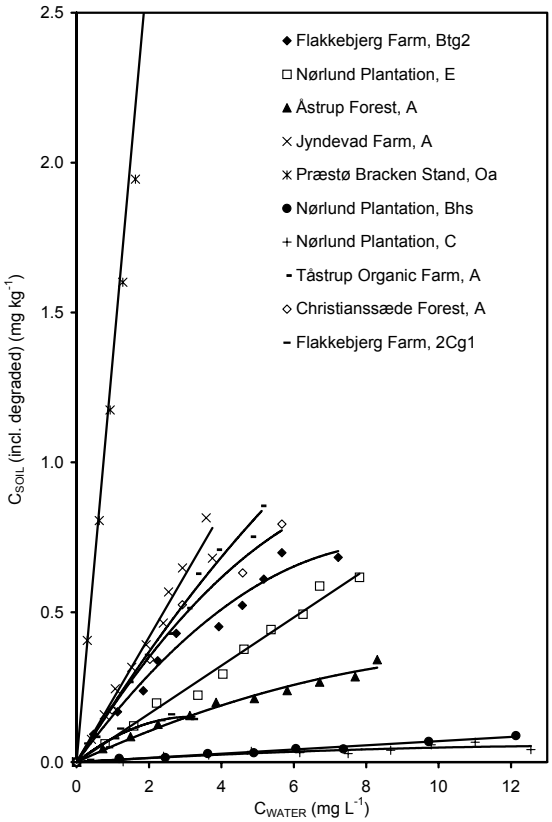


Fig. 8. Sorption isotherms for PTA bonding to soil materials (0.01 M $CaCl_2$, 24 hours, $[PTA]_{START}$ in the range 0 to 14 µg mL^{-1}). Solid lines represent Freundlich-fits. Fitting parameters are listed in Table 5. Only the first 5 data points are shown for Præstø Bracken Stand, Oa.

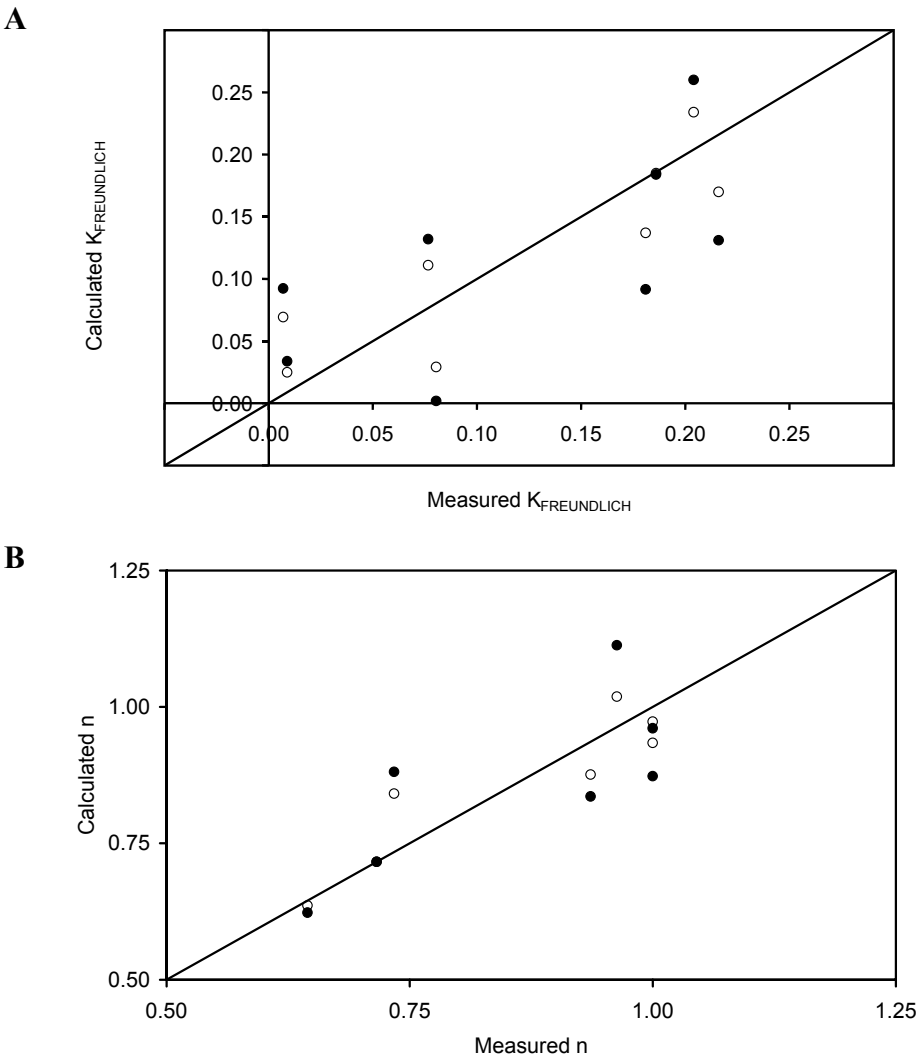


Fig. 9. Multiple linear regressions of sorption parameters in Freundlich models versus soil properties: A) $K_{FREUNDLICH}$; B) n. Two soils removed from both regressions: Præstø Bracken Stand Oa and Åstrup Forest A. Christiansæde Forest A removed from B. ○ = Calibration. ● = Validation .

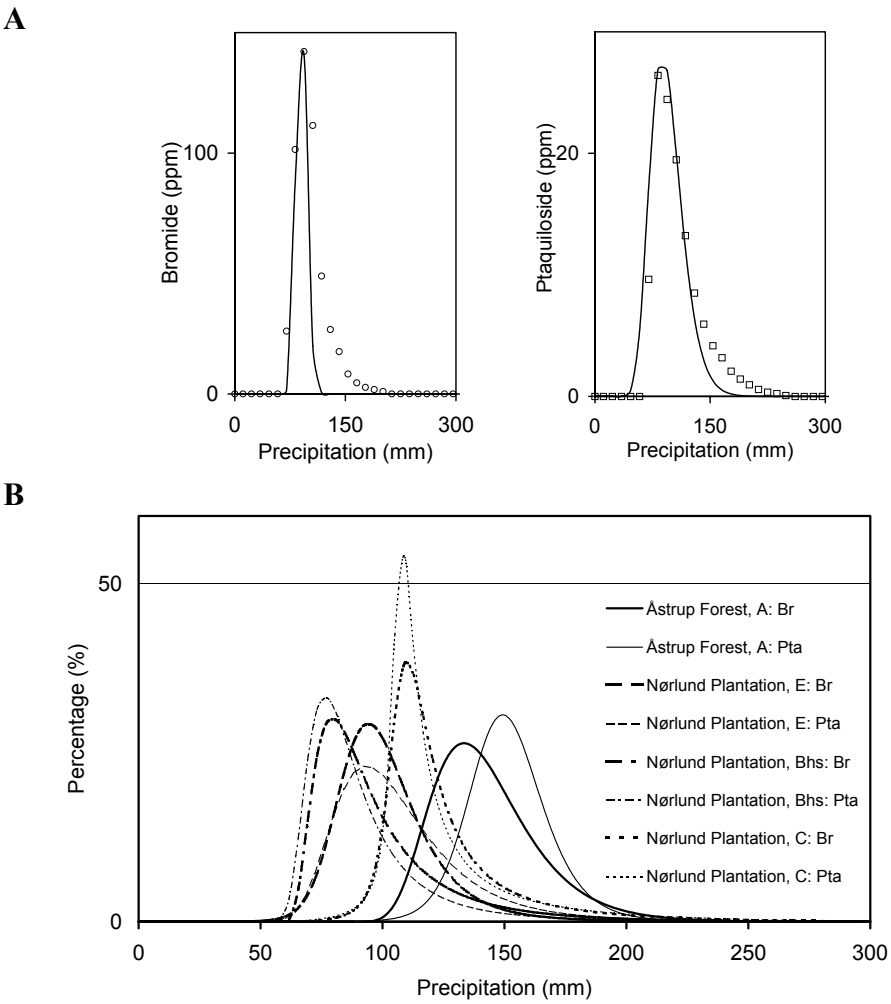


Fig. 10. Breakthrough curves for ptaquiloside and bromide in packed soil columns composed of soil materials from Danish Spodosols: A) Nørlund Plantation, E-curves fitted with the convection-dispersion equation (dispersion coefficients: 2.1 cm^2 $hour^{-1}$ (bromide) and 17.0 cm^2 $hour^{-1}$ (ptaquiloside)); B) Curves fitted with Pearson curves, R^2 (calibration curves, bromide/ptaquiloside): Åstrup Forest, A = 0.8300/0.9283; Nørlund Plantation, E = 0.9934/0.9582; Nørlund Plantation, Bhs = 0.9982/0.9995; Nørlund Plantation, C = 0.9841/0.9868. 80% ptaquiloside recovery from Åstrup Forest, A; 100% ptaquiloside recovery from Nørlund Plantation, E, Bhs, and C.

Variation in the Ptaquiloside content in Bracken from New Zealand

Lars H. Rasmussen[*1], Denis R. Lauren[2], Barry L. Smith[3], and Hans Christian Bruun Hansen[1]

[1] The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark; [2]HortResearch, Ruakura Research Centre, Hamilton, New Zealand; [3]AgResearch, Ruakura Research Centre, Hamilton, New Zealand

Corresponding author: The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark. Phone (+45) 35282828; e-mail LHR@KVL.DK.

Abstract—Bracken (*Pteridium esculentum* (Forst. f) Cockayne) is a common fern encountered all over New Zealand and in many parts of the surrounding pacific islands and in Australia. Bracken causes tumours in the urinary bladder of cattle browsing on the fern. This disease, known as *Bovine Enzootic Haematuria* (BEH), is caused by the norsesquiterpene glycoside ptaquiloside, which can be found in appreciable amounts in the fern. The purpose of the study was to investigate the variation in the ptaquiloside content in Bracken from New Zealand, and to compare the ptaquiloside content with the occurrence of BEH. A further purpose of the study was to identify the correlation between geographic and edaphic parameters and the ptaquiloside content in the fronds. The frond samples collected in the survey had ptaquiloside content between 0 and 13,259 μg/g (on dry matter basis). A significant proportion (87%) of the investigated bracken stands did not contain ptaquiloside, but brackens in 6 out of 11 areas with reported cases of BEH did contain ptaquiloside. The correlation between ptaquiloside content and geographic and edaphic parameters was modelled by partial least squares regression. At national level, the content in fronds having ptaquiloside correlated positively with latitude and negatively with the covarying variables altitude and degree of disturbance (signs of mowing or browsing damage). However, these relationships could not be reproduced at local level. The possible existence of ptaquiloside chemotypes as cause of the observed ptaquiloside distribution is discussed.

Key Words—Bracken, New Zealand, ptaquiloside, Bovine Enzootic Haematuria, *Pteridium*, chemotaxonomy

INTRODUCTION

Bracken ferns (*Pteridium sp.* (Scop.) Gled, Dennstadiaceae) are some of the most common plants on Earth, found on all continents except Antarctica (Page, 1976). The genus comprises at least 12 species and geographic varieties. The species *Pteridium esculentum* (Forst. f) Cockayne occurs on the Pacific islands, New Zealand and in Australia. In New Zealand, this Bracken is found as a common invasive species throughout the country, in open places, along paddocks and roads, in disturbed habitats, in forest glades and clearings, on the edge of wooded areas and as part of the native bush. It normally reaches a height of 2 m, although taller fronds (3-4 m) appear in warm lowland environments (Brownsey and Smith-Dodsworth, 1989). Bracken is usually considered a weed in New Zealand, but the pre-European settlers, the Maoris, have used the fern rhizome as food, especially during travel (Leach, 2001).

Bracken ferns contain several toxins, which can cause a range of diseases in animals and possible man, ranging from induced thiamine deficiency to cancer, e.g. gastric cancer is found among humans who utilize the fern as food, and *Bovine Enzootic Haematuria* (BEH) occurs in cattle browsing on bracken (Hirayama, 1979; Smith et al., 1988; Ojika et al., 1989; Kushida et al., 1994; Marlière et al., 2000; Potter and Baird, 2000; Alonso-Amelot and Avendaño, 2002; Gava et al., 2002). BEH is a chronic neoplastic disease among cattle older than 3 years characterized by intermittent haematuria and numerous tumours in the urinary bladder (Xu, 1992; Hopkins, 1995; Smith, 1997; Pinto et al., 1999; Caldow and Burns, 2000; Dawra et al., 2000). The norsesquiterpene glycoside ptaquiloside has been shown to be responsible for the formation of these tumours in laboratory animals and administration of pure ptaquiloside to cattle has reproduced similar haematological changes (Hirono et al., 1984). Presence of cattle with BEH is also correlated with the ptaquiloside-content (>100 μg/g) in Australia (Smith et al., 1993; Smith et al., 1994). Ptaquiloside-like substances in bracken such as caudatoside, isoptaquiloside and ptaquiloside Z may also

cause BEH (Castillo et al., 1997; Castillo et al., 1998). Bovine papillomavirus (BPV 1 and 2) may also take part in the formation this bladder cancer (Saveria Campo, 2002).

Ptaquiloside (Table 1) is regarded as the major and most significant carcinogenic compound in bracken although other compounds with mutagenic and carcinogenic properties like have been described (e.g. caudatoside). Ptaquiloside is found in the fronds of all bracken varieties, although in quite variable amounts; surveys have shown contents between 0 and 13,000 μg/g (Smith et al., 1994; Alonso-Amelot et al., 1995; Rasmussen et al., 2003b). The frond content is usually highest at the start of the growing season when the young crosiers emerge from the ground. The content then declines as growth proceeds, but never reaches zero (Alonso-Amelot et al., 1992; Alonso-Amelot et al., 1995; Rasmussen and Hansen, 2003). Spring is the time of year when cattle are most susceptible to poisoning by bracken, since the young fronds are appealing to the cattle, while at the same time being in their most toxic state. Surveys of bracken populations in South America (*P. aquilinum* vars. *arachnoidum* and *caudatum* (Alonso-Amelot et al., 1992; Alonso-Amelot et al., 1995)) and Australia (*P. esculentum* (Smith et al., 1994)) showed that altitude and latitude might affect the ptaquiloside content in fronds. In Australia, the brackens growing at southern latitudes had higher ptaquiloside content than ferns coming from northern latitudes, while in Venezuela the fern on mountain slopes at higher altitudes had lower ptaquiloside content than those growing at lower altitudes. However, a Costa Rican study indicated the opposite pattern (Villalobos-Salazar et al., 2000). In a recent Danish investigation of the ptaquiloside contents in *P. aquilinum* var. *aquilinum*, it was demonstrated that the ptaquiloside content in fronds was correlated with the edaphic conditions of light, water and available nutrients (Rasmussen et al., 2003b). Together, these investigations indicate possible existence of ptaquiloside variation caused by recognizable geographic and edaphic parameters. Smith et al. (1994) examined also the ptaquiloside content in fronds from a collection of brackens from all-over the World grown at the same conditions in Sydney, Australia, and found large variations in the ptaquiloside content

inside different bracken species and varieties. However, no significant differences could be found between different varieties and species. In addition, some samples did not contain ptaquiloside at all, indicating existence of ptaquiloside chemotypes within several species and varieties. These observations, although based on small numbers of samples, suggest that the level of ptaquiloside encountered in a particular bracken frond is partly due to genetic heritage as well as edaphic parameters.

The aims of the present study were to: 1) Investigate the range of ptaquiloside contents in young bracken fronds from different sites in New Zealand; 2) Investigate the correlation between the ptaquiloside content in bracken fronds and the occurrence of BEH in New Zealand, and 3) Investigate possible correlations between ptaquiloside in bracken fronds and edaphic parameters.

MATERIALS AND METHODS

Organization of the Study. The study were carried out as 3 separate surveys:

1) Regional Survey: Investigation of the variation in the ptaquiloside content in bracken fronds on a regional basis. This survey was carried out in the Waikato-region and on the Coromandel Peninsula (North Island) on several sampling-trips in September and October 2001. Both old and young fronds were sampled. A total number of 62 Bracken stands were investigated (Fig. 1A). Bracken samples were taken along roadsides, near fences, and in paddocks, covering a wide geographical range of locations in the Waikato-region and on the Coromandel Peninsula.
2) Farm Survey: Investigation of the local variation in the ptaquiloside content in bracken fronds encountered on a sheep and cattle farm situated 30 km west of Taumarunui, North Island. BEH has previously been reported from this farm, and ptaquiloside was found in fronds from this site in the Regional Survey. The survey was carried out November 5-9, 2001 by sampling young fronds from 27 bracken stands within the farm. Samples were taken over a steep hilly section of the farm on grazed paddocks (Fig. 1B).
3) National Survey: Investigation of the variation in the ptaquiloside content in bracken fronds on a national level. This survey was carried out November 21-23 and 27-29 (North Island), and December 15-29, 2001 (South Island). In total, 186 bracken stands were investigated over both main islands in the National survey (Fig. 1C). The survey included 9 areas where BEH was known to occur. Bracken stands sampled were located 30-40 km apart (2-3 km in BEH-areas) in different ecosystems, hereby trying to include as many bracken ecosystems as possible

(roadsides, fences, forests, bush, and paddocks grazed by deer, sheep, and cattle).

All surveys taken together, 275 different bracken stands all over New Zealand were investigated as a part of the study.

Fieldwork and Sample Preparation. At each bracken stand in the Farm and Regional Surveys, 3-20 whole fronds were harvested for analysis, while a number of 6 young fronds were used in the National Survey. The fronds were collected among average sized undamaged fronds from the central part of the bracken stands. In the National Survey only the unfurling frond tips were collected resulting in sampling of the outermost 20-30 cm of frond. All fronds were separated from the rhizome at the soil surface, their length was measured, and the number of unfurling pinnae counted, in order to determine the Bracken growth stage. In the National Survey, three mature fronds from the previous growth season were also harvested for measurement of height and maximum development stage. In the Farm and the National Surveys, detailed site descriptions were performed according to the FAO-Unesco system (FAO, 1990), although adapted to New Zealand (NZ) conditions based on the experiences obtained during the Regional Survey, where a simplified FAO-Unesco site description system was used (FAO, 1990). The FAO-Unesco site descriptions used in the Farm and National Surveys included; NZ landuse classes, NZ vegetation classes, size of bracken stand (<50m^2, 50-200m^2, 200-500m^2, and >500m^2), degree of bracken ground cover (low [15%], moderate [15-40%], high [40-80%], and dominant [80-100%]), light conditions (shaded [below light tree species], glade [stand encircled by trees {>3m tall}], scrub [fronds growing between small trees {<3m tall}, open land]), bracken status (suppressed [other plant species dominate the ecosystem], subdominant [bracken is outnumbered by another plant], dominant [bracken is the most prominent plant]), bracken community growth stage (new [only new plants {max. 1 year}], young [no old fronds present, no litter], mature [old fronds and litter present], and old [bracken community is

degenerating] (Watt, 1976)), and bracken disturbance (none, bitten [by animals], managed [mowed, sprayed etc.]).

All samples from the Farm and Regional Surveys were frozen within 4 hours of sampling, while samples from the National Survey were dried in an Ezidry FD 1000 Food Dehydrator at 45°C (until crisp dry - 12 hours) before freezing.

Ptaquiloside Analysis. Ptaquiloside was extracted from thawed and milled or macerated fronds using MilliQ-water at room temperature for 60 min in BlueCab® bottles at a flat-bed orbital shaker (350 rpm) (1:10 w/v plant-water ratio, Farm and Regional Survey; 1:25 w/v plant-water ratio, National Survey). The ptaquiloside content in the fronds was measured in the aqueous extracts, after treating the extracts with resin (Polyamide 6, Fluka, Steinheim, Switzerland) to remove interfering substances and conversion to pterosin B following the method of Agnew and Lauren (1991). The conversion results in the determination of the sum of ptaquiloside and of any isoptaquiloside present. Both compounds are carcinogenic, therefore this conversion should be a better measure than determination of ptaquiloside alone when comparing the occurrence of bracken related diseases with the content of ptaquiloside in bracken (Castillo et al., 1997). The pterosin B was determined at 220 nm (Shimadzu SPD-6AV UV-VIS Spectrophotometric Detector) on a Shimadzu LC-6A Liquid Chromatograph (Shimadzu SCL-6A System Controller) equipped with Merck LiChroCART® column (125×4 mm) packed with LiChrospher® 100 RP-8, 5 μm. The measurements were performed at 35°C (Shimadzu CTO-6A Column Oven) using a water-acetonitrile (83:17 v/v) eluent and a flow rate of 1.00 ml/min. A 20 μl sample injection-volume was used for all analyses. The limit of detection of the method was below 5 μg/g when expressed as ptaquiloside in dried plant (Agnew & Lauren, 1991). The ptaquiloside content of all samples in the Farm and Regional Surveys was determined in duplicate. The average coefficient of variance (CV_{AVG}) in the samples containing ptaquiloside was 19%. This relatively high CV was due to a few samples which gave markedly different results for

the duplicates, but further analysis was not practical due to the potential degradation of ptaquiloside in the thawed and chopped samples The median CV (CV_{MED}) was 9%. For the National Survey 70% of the samples were analysed in duplicate, and the samples with ptaquiloside gave a CV_{AVG} of 6%. The water-content in the samples from the Farm and the Regional Surveys were measured after drying subsamples of the thawed and chopped samples at 50°C. In the National Survey, the dry weight at 45°C was used for quantification. All results are expressed on a dry weight basis.

Statistical Methods. Statistical analyses were performed in the *Unscrambler ver. 7.6 SR-1* software package (Camo ASA software, Oslo, Norway) and in *Microsoft® Excel 2000* (Microsoft Corporation, USA). Partial Least Squares Regression (PLSR) was carried out on data from the Farm and National Surveys, using all qualitative and quantitative data, to investigate potential correlations between ptaquiloside contents and ecological parameters. PLSR models were developed using jack-knifing techniques and Martens' uncertainty-test. Removal of outliers and non-significant variables were permitted. The models were validated and evaluated by full cross validation (Bro, 1996; Esbensen, 2000).

RESULTS AND DISCUSSION

The investigated Bracken stands are located all-over New Zealand in a multitude of ecosystems ranging from roadsides to undisturbed forests (Fig. 1, Tables 2-6).

Regional Survey. The content of ptaquiloside in the young unfurling fronds as well as in the old fronds from the previous growth season sampled in the Regional survey was zero or below the detection limit in all samples except from 4 samples collected at a cattle and sheep farm situated 30 km west of Taumarunui and in 2 samples from bracken stands nearby this farm (Fig. 1A, Table 2). The content in these fronds ranged from 5 to 1,200 µg/g. The highest levels were found in stands from a hill ridge at the farm, and in a small stand growing at the roadside just outside the farm. BEH has previously been reported at the farm. The low number of stands with ptaquiloside and the occurrence of stands with and without ptaquiloside close to each other at the Taumarunui farm within an area of a few square kilometres prompted a more detailed survey at this site.

Farm Survey. 27 Bracken stands were described and sampled (Tables 3 and 4). The median development stage was 3.1 pinnae resulting in a median height of 45.3 cm. Most stands were situated on sloping land or on hill ridges. The majority had a southern or western aspect, which was due to the topography of the farm. All areas investigated were used as sheep and cattle paddocks. The vegetation class of the paddocks were mainly mixed as many shrubs, small patches of three-ferns and other native as well as exotic species were scattered all-over the farm growing mixed with bracken. In most stands, no signs of disturbance were observed.

The content of ptaquiloside in the fronds ranged from 0 to 13,300 µg/g with a median content of 2,151 µg/g among 17 out of 27 sites (62%) that had measurable ptaquiloside contents (Fig. 1B, Table 4). The median development stage of all stands was 3.1 pinnae, while the development stage among stands having ptaquiloside was only 2.6 pinnae. Compared to stands containing no ptaquiloside, the ptaquiloside containing stands were in

general mature stands placed in grasslands with higher bracken cover. The content was within the range observed previously in bracken fronds in New Zealand and Australia (Smith et al., 1988; Smith et al., 1994). The ptaquiloside content in the stands having ptaquiloside was generally high, and high enough to cause BEH, which is in accordance with the disease history of the farm (Smith et al., 1994). The observed pattern with nearby stands having ptaquiloside concentrations ranging from zero to over 10,000 μg/g resembles previously observed patterns of variation among different stands and even individual fronds of *P. aquilinum* var. *aquilinum* in England regarding the content of the cyanogenic glycoside prunasin (cyanogenic polymorphism) (Cooper-Driver and Swain, 1976; Cooper-Driver et al., 1977).

PLSR was carried out to unveil correlations between the ptaquiloside content and the edaphic parameters (Fig. 2). The analysis was restricted to sites having ptaquiloside in the fronds. By using a logarithmic model based on the number of pinnae (development stage) and the altitude of the stand it was possible to predict the ptaquiloside content on 13 out of 17 sites (76%). Both variables were positively correlated with the ptaquiloside content. The model could not describe 4 sites out of 17, which subsequently were removed from the modelling. Those where outlying stands having either (Table 4): 1) Extreme ptaquiloside contents (F15 and F32); 2) Extremely long stipes (F14); and 3) Extreme variation in the number of pinnae (F19). In addition, two (F14 and F15) of the outliers showed very high variation among duplicates in the ptaquiloside determination. The PLSR analysis indicate that the ptaquiloside content in the fronds could be partly caused by ecological stress since higher contents were found in fronds growing in more stressed environments (hill-ridges; harsher micro climate and lower soil quality compared to down-slope soils). It should be noticed that the development stage is positively correlated with the ptaquiloside content, which is in contradiction to the usual finding that the ptaquiloside content decreases with the age and hence, the development stage of the frond. This might be due to the time span of the sampling: The Farm Survey was carried out over a relatively short time, while the

ptaquiloside content usually decreases during the entire growth season, i.e. over a period of several months.

National Survey. 186 Bracken stands were investigated in the national survey (Table 5 and 6). The median development stage of the young fronds from 2001 was 3.3 pinnae resulting in a median height of 68.3 cm, while the old fronds from the previous year (2000) had reached a median height of 138 cm and a development stage of 17.3 pinnae for mature fronds. 36 % of the stands were situated along roads on strongly disturbed soils, while the rest of the stands were situated on flat terrain, valley floors or on sloping land on more natural soils. Most stands had a northern aspect - oriented towards the sun - and were situated on unused areas or in forests. The vegetation class was therefore mainly grassland and grassland mixed with scrub. The stands were generally situated in open land or among scrub, as small dominant mature communities. Approximately 25% of the stands showed signs of disturbance.

Among the 24 sites (13%) that contained detectable ptaquiloside in the fronds, the concentration ranged from 5 to 2,603 μg/g with a median content of 290 μg/g (Fig. 1C, Table 6). The median development stage among stands having ptaquiloside was 3.2 pinnae, close to the development stage of fronds without ptaquiloside. The ptaquiloside contents were lower than the concentrations found at the Taumarunui Farm, and within the range observed elsewhere for bracken (Smith et al., 1988; Smith et al., 1994). The contents were generally moderate, and only a few sites had ptaquiloside contents considered high enough to cause BEH. There were no marked morphological or edaphic differences between Bracken stands having ptaquiloside and those without.

PLSR was carried out on ptaquiloside containing stands to investigate any correlations between ptaquiloside content and edaphic parameters (Fig. 3). It was possible to predict the ptaquiloside content in the fronds on 21 out of 24 sites (88%) using a logarithmic model based on the altitude of the stand, the latitude (geographical coordinate: Southern latitude), and the degree of bracken disturbance (signs of animals browsing). The altitude and

bracken disturbance were negatively correlated, while latitude was positively correlated with the content of ptaquiloside. In addition, altitude and bracken disturbance were covariate. The significance of altitude is subordinate to the significance of latitude and disturbance. The model was not able to describe three stands, of which two were stands having extremely low ptaquiloside contents (Table 6: N457 and N467) while stand N228 did not have any measurement of altitude, and hence could not be modelled. Those sites were therefore removed from the final model. The PLSR analysis indicates that the ptaquiloside content in the fronds is generally higher in fronds growing in the southern part of New Zealand, which is in line with the findings from Australia (Smith et al., 1994). The altitude of the stand is negatively correlated with the ptaquiloside content, which is in contrast with the results of the Farm Survey. This confusing but intriguing influence of altitude on ptaquiloside in fronds is in line with previous reports from South America and needs further investigation.

Correlation between ptaquiloside content and the occurrence of BEH. Among the 24 ptaquiloside containing stands from the National Survey, 13 (54%) were found in areas where BEH had been observed (Table 7). Among the 10 BEH areas investigated in the whole study, 6 areas had bracken containing ptaquiloside. The number of BEH areas with ptaquiloside was expected to be higher, due to previous field and laboratory-findings indicating the connection between ptaquiloside, bracken, and BEH (Hirono et al., 1984; Smith et al., 1988). Furthermore, bracken from some of the BEH-areas did not show any presence of ptaquiloside. The explanation for these observations may be due to the limited number of bracken stands investigated inside the BEH areas, i.e. the high degree of ptaquiloside polymorphism results in high proportion of no-ptaquiloside stands and therefore a high probability of not finding brackens with ptaquiloside even if they are present in the investigated area.

GENERAL DISCUSSION

Ptaquiloside was found in the range 0-13,259 μg/g in young unfurling fronds of bracken in the Farm Survey, while the levels were 0-2,603 μg/g in the National Survey, which is within the range reported earlier for bracken in Australia and in New Zealand. A high proportion (84%) of the stands did not contain ptaquiloside at all. This predominant lack of ptaquiloside producing bracken for large areas of the country has not been reported until now, although consistently large differences between two locations have been reported earlier in New Zealand (Smith et al., 1988). The finding is similar to the work of Smith et al., (1994) who found large variations in the ptaquiloside content as well as some ferns containing no ptaquiloside at all. Bracken chemotypes have been found with respect to the occurrence of the cyanogenic glycoside prunasin (Cooper-Driver, 1976; Cooper-Driver and Swain, 1976; Low and Thomson, 1990). The lack of ptaquiloside in some stands in the present study might therefore be explained by the existence of ptaquiloside chemotypes of bracken. An attempt was made to model the occurrence of zero-ptaquiloside stands by multiple linear regression, PLSR and principal component analysis in line with the analysis carried out on the fronds containing ptaquiloside, but no significant correlations could be identified. Another plausible explanation of the lack of ptaquiloside in some brackens could be the existence of more than one bracken variety in New Zealand, or that the fern itself does not produce ptaquiloside. Instead, a symbiotic endomycorrhizal fungus could produce the compound. Due to the close association between fungi and fern in mycorrhizal networks, the fungi might introduce ptaquiloside or a biosynthetic precursor into the ferns vascular system (Schoental, 1984). Ptaquiloside-like substances and their products of hydrolysis, the pterosins, have been identified in some basidiomycetes (e.g. illudin M and S from *Omphalotus illudens* and *O. olearis* and pterosins in *Fomes annosus* and *Cyathus bulleri)* (McCloud et al., 1996, Potter, 2000). Ptaquiloside is, however, found in ferns grown in sterilized potting soil. Those had even higher contents than similar ferns grown

artificially in natural bracken soil (Smith et al., 1990). The origin and exact role of ptaquiloside in bracken remains still to be explained. If the fungal theory is correct, then the presence of ptaquiloside might just be an effect of fungal infection, and the observed pattern for the ptaquiloside content is a mirror of the distribution of a ptaquiloside producing fungi.

Detailed studies of the ptaquiloside content in different species and varieties of bracken from Australia, South America and Europe have demonstrated correlations between ptaquiloside content, growth stage, and edaphic/geographic variables, such as altitude, latitude, light exposure and nutritional status. The present study supports these results to some degree, although the Farm and National Surveys exhibited contradictory results regarding the effect of altitude. Based on the present study, it is not possible to explain the variations in the frond ptaquiloside content. It is likely that the variations may be caused by unexplained variations in other edaphic parameters, such as plant available nutrients, soil texture, precipitation, fungal infection, genetic heritage, a combination of those, or by other factors entirely. Further studies are needed to uncover the complex correlations between edaphic parameters, the occurrence of ptaquiloside chemotypes and contents before general applicable conclusions can be made.

There is a reasonable association of ptaquiloside containing stands of bracken to areas where BEH has been observed. However, the proportion of stands containing ptaquiloside was low. In addition, the ptaquiloside contents were in general low compared to the contents, which have been found to cause BEH. These findings may simply reflect the very localised nature of high-ptaquiloside bracken as shown in the Farm Survey. The overall low ptaquiloside contents observed are in agreement with the declining occurrence of BEH in New Zealand. The declining BEH-occurrence is probably due to improved farming practises, namely, the recognition of bracken as a signicant weed, the use of high stock numbers and effective herbicides to remove bracken from grazed areas.

Great care must be taken in future investigations of the geographical variation in the ptaquiloside content in bracken. Such surveys must be very detailed to ensure accurate and reliable results. Interpretations of studies based on low sample numbers must therefore be made with great care.

CONCLUSION

This study shows that ptaquiloside is found in the range 0-13,259 μg/g in young unfurling bracken fronds in New Zealand, although a high proportion of the investigated bracken stands did not contain ptaquiloside at all. The contents of ptaquiloside are within the range previously reported for bracken in Australia and in New Zealand. The content in fronds with ptaquiloside was modelled by partial least squares regression and was found to be correlated with the following edaphic and geographic variables: Altitude, development stage, latitude, and degree of disturbance. At the local level (the Farm Survey), more ptaquiloside was found in older fronds growing at higher altitudes compared to younger fronds at lower altitudes. At national level, more ptaquiloside was found in fronds from southern compared to northern latitudes, while altitude and the degree of disturbance decreased the ptaquiloside content in a covarying complex manner. Models developed at local and national levels were convincing, but due to opposing correlation coefficients between the two separate studies, a general model for the ptaquiloside content could not be obtained. Possible causes for the high variation in ptaquiloside concentrations and occurrence could be the existence of ptaquiloside chemotypes of bracken, the existence of endomycorrhizal fungi, which produces, or triggers the production of ptaquiloside in the fern, or the fern responces to some edaphic growth factors not investigated.

Compared to previous reports on the correlation between ptaquiloside and BEH, the number of stands found to contain ptaquiloside in BEH areas compared to the total number of stands investigated in those areas ptaquiloside was low, as was the concentrations of ptaquiloside found. However, overall, 60% of the stands found nationally to contain ptaquiloside were found in areas with a known record of BEH, supporting the association between BEH and ptaquiloside.

Acknowledgements—This study was made possible by generous grants from Eides Legat, Studiefonden for Danmarks Jordbrugsvidenskabelige Ph.D.-forening, Skovrider Mogens Andersens og Hustrus Legat, Knud Højgaards Fond and OHF og AJ-E Heilmanns Fond. Dawei Deng, Dwayne Jensen, Heather McBridie, Bruce Willoughby, and Solveig Warnecke are all thanked for their valuable assistance during the bracken sampling trips.

REFERENCES

Agnew, M. P. and Lauren, D. R. 1991. Determination of ptaquiloside in bracken fern (*Pteridium esculentum*). *J. Chromatogr.* 538:462-468.

Alonso-Amelot, M.E. and Avendaño, M. 2002. Human carcinogenesis and bracken fern: A review of the evidence. *Curr. Med. Chem.* 9:675-686.

Alonso-Amelot, M.E., Pérez-Mena, M., Calcagno, M.P., Jaimes-Espinosa, R. and Castillo, U.F. 1992. Ontogenic variation of biologically active metabolites of *Pteridium aquilinum* (L. Kuhn), pterosins A and B, ptaquiloside in a bracken population of the tropical Andes. *J. Chem. Ecol.* 18:1405-1420.

Alonso-Amelot, M.E., Rodulfo-Baechler, S. and Jaimes-Espinosa, R. 1995. Comparative dynamics of ptaquiloside and pterosin B in the two varieties (*caudatum* and *arachnoideum*) of neotropical bracken fern (*Pteridium aquilinum* L. Kuhn). *Biochem. Syst. Ecol.* 23:709-716.

Bro, R. 1996. Håndbog i Multivariabel Kalibrering (in English: Handbook of Multivariate Calibration). MLI, the Royal Veterinary and Agricultural University, Frederiksberg.

Brownsey, P.J. and Smith-Dodsworth, J.C. 1989. New Zealand Ferns and Allied Plants. David Bateman, Auckland.

Caldow, G. and Burns, F. 2000. Bracken poisoning in farm animals in Great Britain: A brief outline in relation to disease surveillance data, pp. 197-200 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. International Bracken Group, Aberystwyth.

Castillo, U.F., Ojika, M., Alonso-Amelot, M.E. and Sakagami, Y. 1998. Ptaquiloside Z, a new toxic unstable sesquiterpene glucoside from the neotropical bracken fern *Pteridium aquilinum* var. *caudatum*. *Bioorg. Med. Chem.* 6:2229-2233.

Castillo, U.F., Wilkins, A.L., Lauren, D.R., Smith, B.L., Towers, N.R., Alonso-Amelot, M.E. and Jaimez-Espinoza, R. 1997. Isoptaquiloside and caudatoside, illudane-type sesquiterpene glucosides from *Pteridium aquilinum* var. *caudatum*. *Phytochemistry* 44:901-906.

Cooper-Driver, G.A. 1976. Chemotaxonomy and phytochemical ecology of bracken. *Bot. J. Linn. Soc.* 73:35-46.

Cooper-Driver, G.A., Finch, S. and Swain, T. 1977. Seasonal variation in secondary plant compounds in relation to the palatability of *Pteridium aquilinum*. *Biochem. Syst. Ecol.* 5:177-183.

Cooper-Driver, G.A. and Swain, T. 1976. Cyanogenic polymorphism in bracken in relation to herbivore predation. *Nature* 260:604-604.

Dawra, R.K., Sharma, O.P. and Somvanshi, R. 2000. Experience with enzootic bovine haematuria in India, pp. 150-154 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999, International Bracken Group, Aberystwyth.

Esbensen, K.H. 2000. Multivariate Data Analysis - In Practice. 4 edn. Camo ASA, Oslo.

FAO 1990. Guidelines for Soil Description. 3 edn. Food and Agriculture Organization of the United Nations, Rome.

Gava, A., da Silva Neves, D., Gava, D., de Moura Saliba, T., Schild, A.L. and Riet-Correa, F. 2002. Bracken fern (*Pteridium aquilinum*) poisoning in cattle in Southern Brazil. *Vet. Hum. Toxicol.* 44:362-365.

Hirayama, T. 1979. Diet and Cancer. *Nutr. Cancer* 1:67-81.

Hirono, I., Kono, Y., Takahashi, K., Yamada, K., Niwa, H., Ojika, M., Kigoshi, H., Niiyama, K. and Uosaki, Y. 1984. Reproduction of acute bracken poisoning in a calf with ptaquiloside, a bracken constituent. *Vet. Record* 115:375-378.

Hopkins, A. 1995. Factors influencing cattle bracken-poisoning in Great Britain, pp. 120-123 *in* R.T. Smith and J.A. Taylor (eds.). Bracken: An Environmental Issue.

Contributions to an International Conference. Bracken 94. University of Wales, Aberystwyth, 18-21 July 1994, International Bracken Group, Aberystwyth.

Kushida, T., Uesugi, M., Sugiura, Y., Kigoshi, H., Tanaka, H., Hirokawa, J., Ojika, M. and Yamada, K. 1994. DNA damage by ptaquiloside. a potent bracken carcinogen: Detection of selective strand breaks and identification of DNA cleavage products. *J. Am. Chem. Soc.* 116:479-486.

Leach, H. M. 2001. European perceptions of the roles of bracken rhizomes (*Pteridium esculentum* (Forst. F.) Cockayne) in traditional Maori diet, *N. Z. J. Archaeol.* 22:31-43.

Low, V.H.K. and Thomson, J.A. 1990. Cyanogenesis in Australian bracken (*Pteridium esculentum*): Distribution of cyanogenic phenotypes and factors influencing activity of the cyanogenic glucosidase, pp. 105-111 *in* J.A. Thomson and R.T. Smith (eds.). Bracken Biology and Management. The Australian Institute of Agricultural Science, Wahroonga.

Marlière, C.A., Santos, R.C., Galvao, M.A.M., Soares, J.F., Evangelista, C.L.M. and Gomes, R.F.Q. 2000. Ingestao de broto de samambaia e risco de cancer de esofago e estomago na regiao de Ouro Preto, MG. *Revista Brasileira de Cancerologia* 44:225-229.

McCloud, T. G., Klueh, P. A., Pearl, K. C., Cartner, L. K., Muschik, G. M., and Poole, K. K. Isolation of illudin S from the mature basidiocarp of *Omphalotus illudens*, and isolation of illudin S and M from the fermentation broth of *Omphalotus olearis*. <http://npsg.ncifcrf.gov/illudin.htm>.

Ojika, M., Sugimoto, K., Okazaki, T. and Yamada, K. 1989. Modification and cleavage of DNA by ptaquiloside. A new potent carcinogen isolated from bracken fern. *J. Chem. Soc., Chem. Comm.* 22:1775-1777.

Page, C.N. 1976. The taxonomy and phytogeography of bracken - a review. *Bot. J. Linn. Soc.* 73:1-34.

Pinto, C., Lima, R., Louza, A.C., Almeida, V., Melo, M., Vaz, Y., Fonseca, I.N., Lauren, D.R. and Smith, B.L. 1999. Bracken Fern-induced bovine enzootic haematuria in Sao Miguel Island, Azores, pp. 136-140 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control - Proceedings of the International Bracken Group Conference, Manchester 1999. The International Bracken Group, Leeds.

Potter, D.M. 2000. The *Pteridaceae* as a source of compounds with pharmaceutical activity, pp. 60-67 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester. International Bracken Group, Aberystwyth.

Potter, D.M. and Baird, M.S. 2000. Carcinogenic effects of ptaquiloside in bracken fern and related compounds. *Brit. J. Cancer* 83:914-920.

Rasmussen, L.H. and Hansen, H.C.B. 2003. Growth of bracken in Denmark and the content of ptaquiloside in fronds, rhizomes and roots, *in* T. Acamovic, C.S. Stewart, and T.W. Pennycott (eds.). Poisonous Plants and Related Toxins. CAB International, Wallingford. In press.

Rasmussen, L.H., Jensen, L.S. and Hansen, H.C.B. 2003a. Distribution of the carcinogenic terpene ptaquiloside in bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *J. Chem. Ecol.* 29:771-778.

Rasmussen, L.H., Kroghsbo, S., Frisvad, J.C. and Hansen, H.C.B. 2003b. Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere* 51:117-127.

Saveria Campo, M. 2002. Animal models of papillomavirus pathogenesis. *Virus Res.* 89:249-261.

Schoental, R. 1984. Bracken toxicity and soil mycotoxins. *Vet. Record* 115:500-500.

Smith, B.L. 1997. The toxicity of bracken fern (genus *Pteridium*) to animals and its relevance to Man, pp. 63-76 *in* J.P Felix D'Mello (edt.). Handbook of Plant and Fungal Toxicants. CRC Press, New York.

Smith, B.L., Embling, P.P., Agnew, M.P., Lauren, D.R. and Holland, P.T. 1988. Carcinogenicity of bracken fern (*Pteridium esculentum*) in New Zealand. *N. Z. Vet. J.* 36:56-58.

Smith, B.L., Lauren, D.R., Embling, P.P. and Agnew, M.P. 1990. Ptaquiloside in Australian and New Zealand ferns as a cause of Neoplasia, pp. 241-246 *in* J.A. Thomson and R.T. Smith (eds.). Bracken Biology and Management. Occasional Publication No. 40, Australian Institute of Agricultural Science, Hawthorn.

Smith, B.L., Lauren, D.R., Embling, P.P. and Agnew, M.P. 1993. Carcinogenicity of bracken fern (*Pteridium* spp.) in relationship to grazing ruminants, pp. 1396-1398 *in* J. Broughan (edt.). Proceedings of the 17th International Grasslands Congress, New Zealand Grasslands Association, Palmerston North.

Smith, B.L., Seawright, A.A., Ng, J.C., Hertle, A.T., Thomson, J.A. and Bostock, P.D. 1994. Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium* spp.), from Eastern Australia and from a cultivated worldwide collection held in Sydney, Australia. *Nat. Toxins* 2:347-353.

Villalobos-Salazar, J., Hernandez, H., Meneses, A. and Salazar, G. 2000. Factors which may affect ptaquiloside levels in milk: Effects of altitude, bracken fern growth stage, and milk processing, pp. 68-74 *in* J.A. Taylor and R.T. Smith (eds.). Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. International Bracken Group, Aberystwyth.

Watt, A.S. 1976. The ecological status of bracken. *Bot. J. Linn. Soc.* 73:217-239.

Xu, L.R. 1992. Bracken poisoning and enzootic haematuria in cattle in China. *Res. Vet. Sci.* 53:116-121.

FIGURE CAPTIONS

Table no:	Text:
1	TABLE 1. Properties of ptaquiloside.
2	TABLE 2. Stand descriptions and ptaquiloside contents, Regional survey.
3	TABLE 3. Comparison of ecological variables between ptaquiloside (Pta) and non-ptaquiloside (Non-Pta) containing bracken stands, Farm Survey.
4	TABLE 4. Descriptions of ptaquiloside containing stands, Farm Survey.
5	TABLE 5. Comparison of ecological variables between ptaquiloside (Pta) and non-ptaquiloside (Non-Pta) containing bracken stands, National Survey.
6	TABLE 6. Description of ptaquiloside containing stands, National Survey.
7	TABLE 7. Correlation between the occurrence of BEH and ptaquiloside.
Figure no:	**Text:**
1	FIG. 1. A: Map of the North Island, Waikato and Coromandel Regions. Investigated sites in the Regional Survey are marked on the map (●) as well as the Taumarunui farm area (⬤). B: Map of the investigated area at the farm at Taumarunui, North Island, showing the location of the investigated sites (UTM 60H) and the ptaquiloside levels encountered (µg/g): ○ = 0; ✖ = 1-999; ✚ = 1,000-9,999; ⬤ = >10,000. C: Map of New Zealand. Investigated sites in the National Survey are marked on the map (●) as well as BEH-sites included in the survey (◉). P indicates bracken stands with ptaquiloside.
2	FIG. 2. PLSR-model of the ptaquiloside content in young fronds, Farm Survey (77% explained calibration Y-variance, 70% explained validation Y-variance, 1 PC (principal component)). ○: Samples used for modelling. ×: Outlying samples not included in the model. Thick line: LogPTA (measured) = LogPTA

	(calculated). Thin line: Model trend line (offset and slope shown). R^2 = the variance in LogPTA (calculated) according to the model. Q^2 = the variance in LogPTA (calculated) according to cross validation. RMSEP = root mean square error of prediction.
3	FIG. 3. PLSR-model of the ptaquiloside content in young fronds, National Survey (82% explained calibration Y-variance, 78% explained validation Y-variance, 2 PC's). ○ Samples used for modelling. ×: Outlying samples not included in the model. Solid line: LogPTA (measured) = LogPTA (calculated). Thin line: Model trend line. R^2 = the variance in LogPTA (calculated) according to the model. Q^2 = the variance in LogPTA (calculated) according to cross validation. RMSEP = root mean square error of prediction.

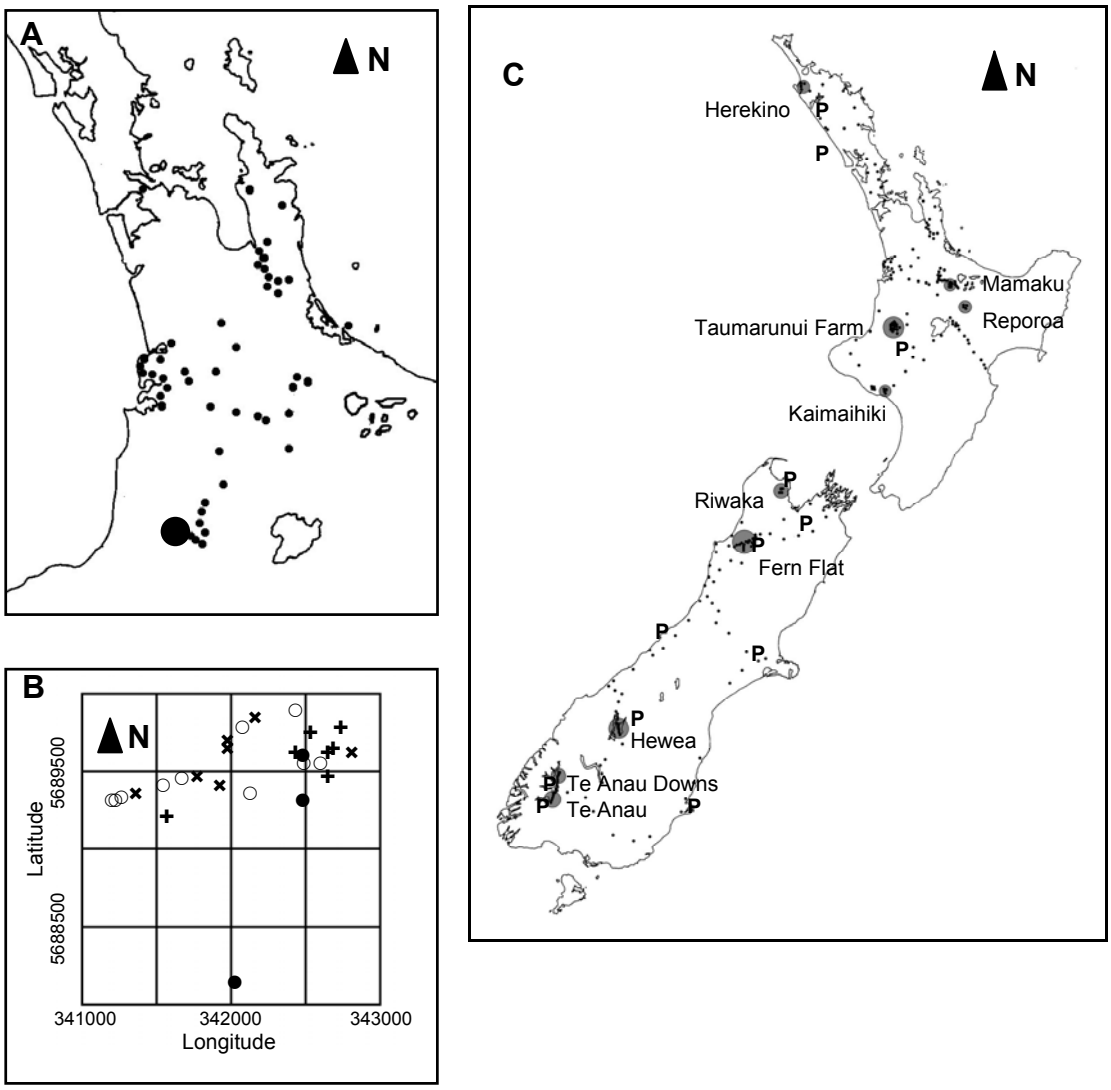


FIG. 1. A: Map of the North Island, Waikato and Coromandel Regions. Investigated sites in the Regional Survey are marked on the map (●) as well as the Taumarunui farm area (⬤). B: Map of the investigated area at the farm at Taumarunui, North Island, showing the location of the investigated sites (UTM 60H) and the ptaquiloside levels encountered (µg/g): ○ = 0; ✖ = 1-999; ✚ = 1,000-9,999; ⬤ = >10,000. C: Map of New Zealand. Investigated sites in the National Survey are marked on the map (●) as well as BEH-sites included in the survey (◉). P indicates bracken stands with ptaquiloside.

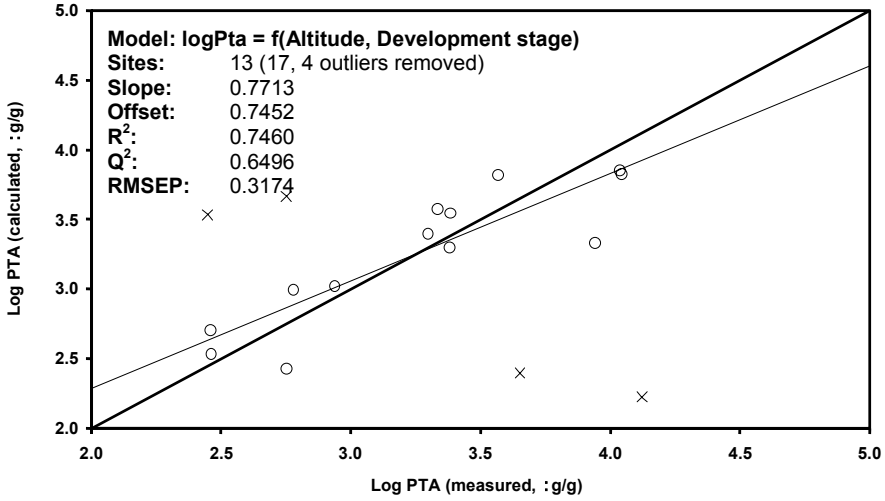


FIG. 2. PLSR-model of the ptaquiloside content in young fronds, Farm Survey (77% explained calibration Y-variance, 70% explained validation Y-variance, 1 PC (principal component)). ○: Samples used for modelling. ×: Outlying samples not included in the model. Thick line: LogPTA (measured) = LogPTA (calculated). Thin line: Model trend line (offset and slope shown). R^2 = the variance in LogPTA (calculated) according to the model. Q^2 = the variance in LogPTA (calculated) according to cross validation. RMSEP = root mean square error of prediction.

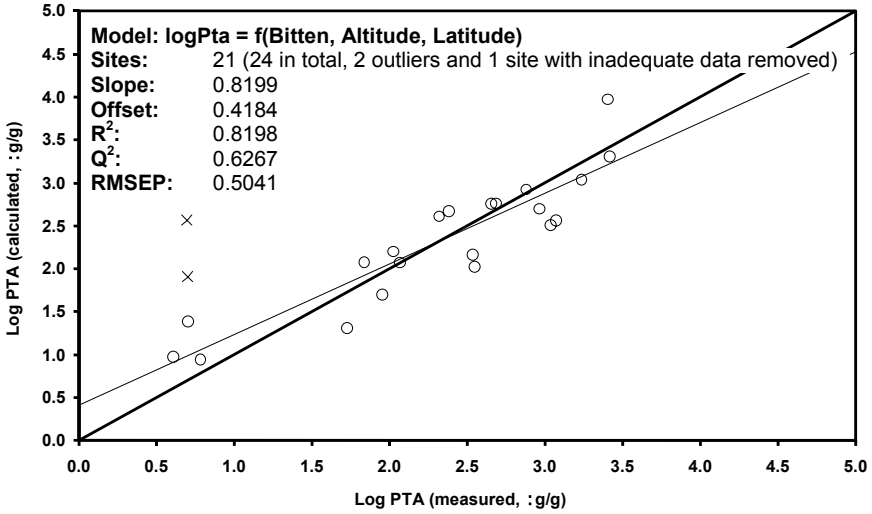


FIG. 3. PLSR-model of the ptaquiloside content in young fronds, National Survey (82% explained calibration Y-variance, 78% explained validation Y-variance, 2 PC's). ○ Samples used for modelling. ×: Outlying samples not included in the model. Solid line: LogPTA (measured) = LogPTA (calculated). Thin line: Model trend line. R^2 = the variance in LogPTA (calculated) according to the model. Q^2 = the variance in LogPTA (calculated) according to cross validation. RMSEP = root mean square error of prediction.

TABLE 1. Properties of ptaquiloside.

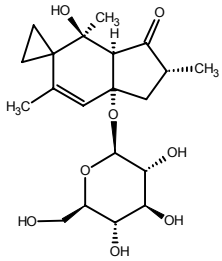
CAS numbers:	87625-62-5 88825-03-0
Chemical name:	Ptaquiloside
Chemical structure:	
Molecular formula:	$C_{20}H_{30}O_8$
Molecular weight:	398.45 g mol^{-1}

TABLE 2. Stand descriptions and ptaquiloside contents, Regional survey.

Stand	Elevation m	Frond length cm	Growth stage pinnae	Ptaquiloside content µg/g	Site description						
					Land element	Aspect	Vegetation	Stand size m^2	Bracken cover %	Light conditions	Growth stage
R1	N.D.	200	N.D.	0	Road side	N.D.	Grass-Scrub	50 - 200	80 - 100	Open land	Old
R2	N.D.	150	N.D.	0	Road side	N.D.	Grassland	50 - 200	40 - 80	Open land	Mature
R3	N.D.	150	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R4	N.D.	50	N.D.	0	Slope	East	Scrub	< 50	< 15	Shaded	Mature
R5	N.D.	15	N.D.	40±0	Hill ridge	None	Grassland	N.D.	N.D.	Open land	Mature
R6	N.D.	150	N.D.	30±10	Hill ridge	None	Grassland	> 500	80 - 100	Open land	Mature
R7	N.D.	150	N.D.	0	Valley	N.D.	Scrub	> 500	80 - 100	Open land	Mature
R8	N.D.	150	N.D.	0	Valley	N.D.	Scrub	> 500	80 - 100	Open land	Mature
R9	N.D.	200	N.D.	6±1	Valley	South	Scrub	> 500	80 - 100	Open land	Mature
R10	N.D.	200	N.D.	139±36	Valley	N.D.	Scrub	> 500	N.D.	Open land	Mature
R11	N.D.	200	N.D.	0	Valley floor	N.D.	Scrub	< 50	N.D.	Open land	Mature
R12	N.D.	200	N.D.	2±0	Valley floor	N.D.	Forest	< 50	< 15	Shaded	Old
R13	N.D.	200	N.D.	0	Valley floor	East	Forest	< 50	80 - 100	Open land	Mature
R14	N.D.	150	N.D.	1198±57	Road side	South	Scrub	< 50	80 - 100	Open land	Mature
R15	N.D.	150	N.D.	0	Slope	N.D.	Scrub	< 50	< 15	Shaded	Old
R16	N.D.	150	N.D.	0	Slope	N.D.	Grass-Scrub	50 - 200	N.D.	Open land	Mature
R17	N.D.	200	N.D.	0	Road side	N.D.	Grassland	200 - 500	80 - 100	Open land	Mature
R18	N.D.	150	N.D.	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
R19	N.D.	150	N.D.	0	Road side	N.D.	Grassland	< 50	40 - 80	Open land	Mature
R20	N.D.	N.D.	N.D.	0	Road side	N.D.	Grassland	< 50	40 - 80	Open land	Mature
R21	N.D.	200	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R22	N.D.	200	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R23	N.D.	200	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R24	N.D.	150	N.D.	0	Road side	N.D.	Grassland	< 50	40 - 80	Open land	Mature
R25	N.D.	150	N.D.	0	Slope	N.D.	Scrub	200 - 500	< 15	Scrubs	Old
R26	N.D.	200	N.D.	0	Road side	N.D.	Grass-Scrub	50 - 200	80 - 100	Open land	Mature
R27	N.D.	200	N.D.	0	Slope	N.D.	Forest	< 50	< 15	Scrubs	Old
R28	N.D.	100	N.D.	0	Ridge	N.D.	Grass-Scrub	200 - 500	< 15	Open land	Old
R29	217	150	N.D.	0	Slope	N.D.	Scrub	> 500	< 15	Scrubs	Old
R30	200	150	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R31	9	125	N.D.	0	Road side	North	Grassland	< 50	80 - 100	Open land	Mature
R32	40	150	N.D.	0	Road side	N.D.	Grassland	< 50	< 15	Open land	Mature
R33	40	125	N.D.	0	Road side	N.D.	Scrub	< 50	< 15	Open land	Mature

R34	42	N.D.	N.D.	0	Slope	N.D.	Scrub	> 500	15 - 40	Scrubs	Mature
R35	44	150	N.D.	0	Road side	None	Grassland	< 50	80 - 100	Open land	Mature
R36	15	100	N.D.	0	Valley	N.D.	Grass-Scrub	> 500	40 - 80	Scrubs	Mature
R37	60	80	N.D.	0	Road side	N.D.	Grassland	N.D.	40 - 80	Open land	Mature
R38	11	110	N.D.	0	Road side	South	Grassland	< 50	40 - 80	Open land	Mature
R39	8	225	N.D.	0	Slope	South	Scrub	> 500	80 - 100	Glade	Mature
R40	35	150	N.D.	0	Slope	None	Scrub	< 50	< 15	Glade	Old
R41	22	125	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R42	20	125	N.D.	0	Road side	South	Grassland	< 50	80 - 100	Open land	Mature
R43	182	175	N.D.	0	Slope	None	Grass-Scrub	> 500	40 - 80	Scrubs	Old
R44	317	175	N.D.	0	Road side	South	Grass-Scrub	< 50	40 - 80	Scrubs	Mature
R45	237	175	N.D.	0	Road side	West	Grassland	< 50	40 - 80	Open land	Mature
R46	N.D.	150	N.D.	0	Road side	N.D.	Grassland	50 - 200	80 - 100	Open land	Mature
R47	125	125	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R48	209	175	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R49	113	125	N.D.	0	Road side	South	Grassland	50 - 200	80 - 100	Open land	Mature
R50	207	225	N.D.	0	Road side	East	Scrub	< 50	80 - 100	Scrubs	Old
R51	195	90	N.D.	0	Road side	North	Grass-Scrub	< 50	80 - 100	Scrubs	Mature
R52	N.D.	200	N.D.	0	Road side	N.D.	Grass-Scrub	< 50	80 - 100	Open land	Mature
R53	N.D.	150	N.D.	0	Road side	N.D.	Grass-Scrub	< 50	40 - 80	Scrubs	Young
R54	N.D.	150	N.D.	0	Road side	N.D.	Grass-Scrub	< 50	< 15	Scrubs	Young
R55	N.D.	100	N.D.	0	Road side	N.D.	Grass-Scrub	< 50	< 15	Scrubs	Young
R56	N.D.	100	N.D.	0	Road side	N.D.	Grass-Scrub	50 - 200	80 - 100	Shaded	Mature
R57	N.D.	150	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R58	N.D.	125	N.D.	0	Road side	N.D.	Grass-Scrub	200 - 500	80 - 100	Shaded	Mature
R59	N.D.	200	N.D.	0	Road side	N.D.	Grassland	< 50	80 - 100	Open land	Mature
R60	N.D.	N.D.	N.D.	0	Road side	N.D.	Forest	< 50	40 - 80	Glade	Mature
R61	N.D.	N.D.	N.D.	0	Road side	N.D.	Grass-Scrub	< 50	80 - 100	Shaded	Mature
R62	N.D.	150	N.D.	0	Road side	N.D.	Grassland	50 - 200	80-100	Open land	Mature

N.D. Not described.

TABLE 3. Comparison of ecological variables between ptaquiloside (Pta) and non-ptaquiloside (Non-Pta) containing bracken stands, Farm Survey.

	Altitude	2001 growth			2000 growth			Land element					Aspect				
		Frond length	Stipe length	Develop. stage	Frond length	Stipe length	Develop. stage	%					%				
	m	cm	cm	pinnae	cm	cm	pinnae	Ridge	Slope	Valley	Road	Other	North	East	South	West	None
Non-Pta	221	45.1	N.D.¶	3.7	N.D.	N.D.	N.D.	20	80	0	0	0	30	20	20	30	0
Pta	346	45.3	N.D.	2.6	N.D.	N.D.	N.D.	29	53	12	6	0	12	18	41	24	6
All	257	45.3	N.D.	3.1	N.D.	N.D.	N.D.	26	63	7	4	0	19	19	33	26	4

	Landuse					Vegetation						Bracken cover			
	%					%						%			
	Sheep	Cows	Forestry	Unused	Other	Grassland scrub	Scrub	Grassland forest	Forest scrub	Forest	Grassland	Low	Moderate	High	Dominant
Non-Pta	100	0	0	0	0	80	0	10	10	0	0	70	30	0	0
Pta	100	0	0	0	0	41	6	18	6	0	29	29	41	18	12
All	100	0	0	0	0	55	4	15	7	0	19	45	37	11	7

	Light conditions				Bracken status			Community development stage			Bracken disturbance			Size of Bracken area			
	%				%			%			%			%			
	Open land	Scrubs	Glade	Shaded	Sup-pressed	Sub-dominant	Dominant	Young	Mature	Old	None	Bitten	Managed	< 50 m^2	50-200 m^2	200-500 m^2	> 500 m^2
Non-Pta	20	60	20	0	0	0	100	80	10	10	100	0	0	20	10	70	0
Pta	35	35	12	18	0	0	100	35	59	6	94	6	0	25	25	50	0
All	30	44	15	11	0	0	100	55	41	4	96	4	0	23	58	19	0

¶ N.D. = Not determined.

TABLE 4. Descriptions of ptaquiloside containing stands, Farm Survey.

Stand	Altitude m	Frond length cm	Growth stage pinnae	Ptaquiloside content µg/g¶
F7	205	37.7±5.5	3.6±1.8	862±240
F11	204	72.1±19.1	2.0±1.1	288±19
F12	216	62.8±25.7	1.5±0.8	562±17
F14	192	113.3±14.6	1.7±0.6	4,491±2,375
F15	164	57.9±33.6	1.5±1.1	13,259±6,918
F19	231	45.3±17.5	5.4±2.9	564±117
F20	235	59.0±11.6	3.1±1.5	598±98
F22	273	45.3±18.4	1.7±1.2	286±9
F24	430	18.1±5.6	2.5±1.2	2,151±1,521
F25	386	32.0±5.8	3.9±1.7	3,684±0
F26	378	33.1±10.8	4.0±1.3	11,052±797
F29	365	49.6±14.5	3.3±1.2	2,416±111
F30	368	61.8±15.3	2.5±0.5	8,721±364
F31	379	35.8±8.5	2.3±1.3	2,395±100
F32	380	29.6±4.8	3.0±2.1	279±38
F33	379	34.1±12.9	2.4±1.0	1,978±47
F34	346	40.8±15.8	4.5±2.4	10,872±1,185

¶ CV_{AVG} = 19%, CV_{MED} = 9%.

TABLE 5. Comparison of ecological variables between ptaquiloside (Pta) and non-ptaquiloside (Non-Pta) containing bracken stands, National Survey.

	Altitude	2001 growth			2000 growth			Land element					Aspect				
								%					%				
	m	Frond length cm	Stipe length cm	Develop. stage pinnae	Frond length cm	Stipe length cm	Develop. stage pinnae	Ridge	Slope	Valley	Road	Other	North	East	South	West	None
Non-Pta	196	119.0	68.3	3.3	138.0	61.0	17.3	16	28	13	37	6	33	17	4	17	29
Pta	167	121.1	67.4	3.2	135.0	67.3	17.0	14	19	22	36	6	25	11	13	21	30
All	183	119.3	68.3	3.3	138.0	61.5	17.3	16	27	14	36	7	26	12	12	21	30

	Landuse					Vegetation						Bracken cover			
	%					%						%			
	Sheep	Cows	Forestry	Unused	Other	Grassland scrub	Scrub	Grassland forest	Forest scrub	Forest	Grassland	Low	Moderate	High	Dominant
Non-Pta	13	10	19	57	1	25	14	8	13	2	38	19	27	31	23
Pta	4	4	25	63	4	13	17	17	4	0	49	13	29	37	21
All	12	9	20	57	2	24	15	10	12	2	37	18	27	32	23

	Light conditions				Bracken status			Community development stage			Bracken disturbance			Size of Bracken area			
	%				%			%			%			%			
	Open land	Scrubs	Glade	Shaded	Sup-pressed	Sub-dominant	Dominant	Young	Mature	Old	None	Bitten	Managed	< 50 m^2	50-200 m^2	200-500 m^2	> 500 m^2
Non-Pta	48	39	6	7	8	23	69	11	86	3	73	12	15	48	27	12	13
Pta	60	16	8	16	8	25	67	8	88	4	76	12	12	42	25	29	4
All	50	36	6	8	8	23	69	10	87	3	74	12	14	46	27	15	12

TABLE 6. Description of ptaquiloside containing stands, National Survey.

Stand	Altitude m	Frond length cm	Growth stage pinnae	Ptaquiloside content¶ μg/g
N227	19	83.8±22.2	4.7±2.0	53±2
N228	N.D.	94.7±43.5	1.5±1.8	455±10
N238	106	97.2±13.1	3.0±1.4	4±0
N401	278	120.0±18.7	6.0±2.1	5N.D.
N411	241	101.7±26.3	3.4±1.4	6N.D.
N414	167	214.7±31.2	2.3±1.4	68N.D.
N416	146	122.2±31.9	3.8±1.9	105N.D.
N417	153	127.8±18.3	1.0±0.6	341N.D.
N418	179	181.2±26.3	2.7±1.5	351N.D.
N419	172	122.5±17.3	2.2±1.0	115±8
N423	86	59.8±15.6	3.9±1.9	239N.D.
N428	65	106.2±18.5	3.7±1.2	1,709N.D.
N429	125	140.0±21.9	3.0±1.5	482N.D.
N442	362	110.2±38.9	1.7±0.8	89±2
N455	221	152.5±19.8	4.2±2.1	450±35
N456	246	155.0±17.7	3.0±0.6	206N.D.
N457	253	125.3±27.1	0.2±0.4	5N.D.
N458	248	69.5±9.3	3.0±0.6	1,080N.D.
N459	239	161.0±14.3	3.5±1.0	1,173N.D.
N467	241	103.0±4.8	4.0±1.6	5±1
N471	25	56.6±22.9	1.6±1.4	2,603N.D.
N472	19	106.3±5.7	3.8±1.0	2,530N.D.
N480	98	148.8±12.7	5.8±1.3	748±51
N505	22	164.8±34.0	5.7±2.4	913N.D.

N.D. Not determined. ¶ VC_{AVG} = 6.

TABLE 7. Correlation between the occurrence of BEH and ptaquiloside.

BEH area	Number of stands	Stands with Pta	Median Pta-content μg/g¶
Taumarunui Farm	27	17	2,151
Herekino	4	0	0
Mamaku	6	0	0
Reporoa	4	0	0
Kaimaihiki	5	0	0
Fern Flat	10	6	115
Hewea	5	1	89
Te Anau	5	3	206
Te Anau Downs	5	2	1,080
Riwaka	4	1	913

¶ Median values only for ptaquiloside-containing stands.

A Refined Method for Isolation of pure Ptaquiloside from Bracken (*Pteridium aquilinum* (L.) Kuhn.)

Lars Holm Rasmussen[*1], Bjørn Schmidt[1], Denis Lauren[2], Søren Balling Engelsen[3], Carl Erik Olsen[1] and Hans Christian Bruun Hansen[1]

[1]The Royal Veterinary and Agricultural University, Chemistry Department, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark. [2]The Horticulture and Food Research Institute Ltd., Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand. [3]The Royal Veterinary and Agricultural University, Department of Dairy and Food Science, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

[*] Corresponding author: Phone (+45) 3528 2828; Fax (+45) 3528 2398; E-mail LHR@KVL.DK

ABSTRACT

A simplified and refined method for isolation of the carcinogen ptaquiloside from processed Bracken fern was developed. Crude ptaquiloside was obtained by extraction with water followed by stepwise purification by low-pressure liquid chromatography using various resins including Fluka Polyamide 6, Amberlite XAD-2 and the Varian Chem Elut 1020 columns. Further purification of the crude ptaquiloside by preparative HPLC afforded ptaquiloside as a whitish amorphous powder with a purity of close to 100% and the gross formula $C_{20}H_{30}O_8 \cdot \frac{2}{3}H_2O$. The purity was confirmed by ^{1}H- and ^{13}C-NMR and mass spectrometry. UV/VIS, FTIR and FT-Raman spectra are presented. This method enables purification of large amounts of ptaquiloside in a timesaving manner, which can be used for analytical purposes as well as for toxicological testing.

Keywords: *Cancer, HPLC, low-pressure chromatography, Raman.*

INTRODUCTION

Bracken (*Pteridium aquilinum* (L.) Kuhn., Dennsteadtiaceae) is a globally widespread fern encountered on all continents except Antarctica (*1,2*). Bracken consumption by cattle and sheep can result in acute intoxication, blindness and cancer among the animals, and the fern is consequently classified as carcinogenic by the International Agency of Research on Cancer (*1,3-5*). Ptaquiloside (Figure 1) is a carcinogenic and mutagenic nor-sesquiterpene glucoside found in high concentrations in Bracken (*6-10*). Ptaquiloside is likely responsible for these clinical syndromes, as administration of pure ptaquiloside to laboratory rodents, sheep and calves have reproduced the syndromes (*11-13*). Bracken and ptaquiloside are also suspected of causing cancer among humans as intake of young Bracken fronds in Japan and Brazil is associated with the occurrence of human gastric and oesophageal cancer (*14,15*). Ptaquiloside may also be consumed by humans via cow milk (*16-18*), or, as recently postulated, through drinking water (*19-21*). However, an unresolved question is if ptaquiloside is stable enough to exist in the soil environment for prolonged periods of time enabling penetration of the carcinogen into deeper soil layers from where contamination of ground and drinking water may occur. Toxicological, ecotoxicological and chemical investigations of the fate of ptaquiloside in the soil environment have been limited due to lack of larger amounts of pure, analytical grade ptaquiloside.

Ptaquiloside is relatively stable in neutral solution, however, in alkaline solution ptaquiloside forms first ptaquilosin and then an unstable dienone (Figure 1). Under acid conditions both ptaquiloside and the unstable dienone react to give pterosin B. Other compounds than pterosin B may also be formed from ptaquiloside under acid conditions (*7,10*).

No method for the synthesis of ptaquiloside has been published so far. Existing methods for purification of ptaquiloside from Bracken fern have all been quite laborious, resulting in low yields and have included use of toxic organic solvents (e.g. chloroform) (*7,9,10,22-24*).

Here, we report a simple, refined and time-efficient method for the isolation and purification of ptaquiloside from Bracken fern based on aqueous extraction, low-pressure liquid chromatography and preparative HPLC.

EXPERIMENTAL PROCEDURES

General Experimental Procedures. Figure 2 provides an overview of the different experimental procedures used for ptaquiloside purification. Analytical HPLC was performed as gradient elution with a water:acetonitrile eluent at 35°C to quantify both ptaquiloside and pterosin B (*25*) by using a Merck Hitachi (Tokyo, Japan) HPLC system (T-6300 Column Thermostat, L-4200 UV-VIS Detector; 655A-40 Auto-Sampler; D-6000A Interface; L-6200 Intelligent Pump) and 125 x 4 mm I.D. Merck LiChroCART® (LiChrospher® 100 RP-8, 5 μm) columns equipped with 4 x 4 mm I.D. Merck LiChroCART® (LiChrospher® 100 RP-18e, 5 μm) guard columns. Quantification was by UV at 220 nm (ptaquiloside) or 214 nm (pterosin B). Preparative HPLC was performed in isocratic mode with a water:methanol (65:35 V:V) eluent at 35ºC to purify ptaquiloside with a Perkin Elmer Series 10 liquid chromatograph (Connecticut, USA) equipped with a Shimadzu SPD-10A UV-VIS Detector (Kyoto, Japan) and a Perkin Elmer LCI-100 Laboratory Computing Integrator (Connecticut, USA). The preparative column was a 250 x 10 mm I.D. Merck LiChroCART® (LiChrospher® 100 RP-8e, 10 μm) column equipped with a 10 x 10 mm I.D. Merck LiChroCART® (LiChrospher® 100 RP-8, 10 μm) precolumn. Detection was by UV at 220 nm. The UV-VIS spectrum was obtained using a Hewlett Packard – 8453 UV-VIS spectrophotometer with a 2 mm QX absorption cell, and methanol as the solvent. NMR spectra were recorded on a Bruker Avance 400 instrument in methanol-*d4*. The ^{1}H and ^{13}C NMR spectra was obtained at 400,1 and 100,6 MHz, respectively. The chemical shifts are relative to internal TMS. LC-MS with ESI ionisation was performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) using an Xterra C18 column (Waters, Milford, USA) and a NaCl modified acetonitrile:water gradient. FTIR spectra were obtained using a Perkin Elmer

System 2000 Fourier Transform infrared spectrometer with a KBr beam splitter. Spectra were obtained directly using an ATR-crystal. FT-Raman spectra were obtained using a Perkin Elmer System 2000 Fourier Transform infrared spectrometer equipped with a Raman module attachment (quartz beam splitter). Excitations of the Raman spectra were made with an Nd^{3+}/YAG laser operating at 1064 nm with a source power of 400 mW.

Reagents, Materials and Standard Solutions. HPLC grade acetonitrile and methanol were supplied by Sigma-Aldrich (Seelze, Germany), ethyl acetate from Fischer Scientific (Loughborough, U.K), and analytical grade sodium hydroxide and hydrochloric acid from Mallinckrodt Baker B.V. (Deventer, Holland). Polyamide 6 resin was supplied by Fluka (Steinheim, Switzerland), and Amberlite supplied XAD-2 resin (Supelco, Bellefonte, PA, USA). Prepacked Chem Elut 1020 columns were obtained from Varian, Inc. (Palo Alto, CA, USA). A standard of pure ptaquiloside was donated by Professor Ojika (Nagoya University, Japan), while standards of pterosin B were made from pure ptaquiloside (*25*). Glass-columns equipped with 28 μm polymer filters (1.0 and 2.5 cm I.D. Econo-Columns, length: 10 and 20 cm) were purchased from BIO-RAD (Hercules, CA, USA). Absorption cells used for obtaining UV-VIS spectrum was obtained from Hellma GmbH & Co KG (Müllheim, Germany). Methanol-*d4* used for NMR was supplied by Cambridge Isotope Laboratories, Inc. (Andover, USA).

Quantification of Ptaquiloside and Pterosin B. The ptaquiloside content in crude Bracken extracts was quantified by analytical HPLC after removal of interfering substances with Polyamide 6 resin following the method of Agnew and Lauren (*25*). The extract (4 mL) was passed through 0.50 g Polyamide 6 resin (dry packed in a 1.0 cm I.D. glass column). Ptaquiloside in the crude samples was then converted to pterosin B by treatment of a 1 mL aliquout of the purified extract with 0.075 mL 1 M NaOH, heating at 45°C in sealed autosampler vials for 60 min., followed by addition of 0.075 mL 5 M HCl

(25). This method ensures full conversion of ptaquiloside to pterosin B. The stability of ptaquiloside and pterosin B standards was evaluated each working day. Ptaquiloside standards were stable for a single working day while standards of pterosin B were stable for at least 5 days.

The purity of extracted and purified ptaquiloside (crude ptaquiloside and pure ptaquiloside) was quantified by analytical HPLC both directly and after conversion to pterosin B. These quantifications were performed without prior Polyamide treatment.

Harvest and Sample Pretreatment. Pinnae (approx. 10 kg) were harvested 12 August 2002 from mature Bracken (*Pteridium aquilinum* var. *aquilinum*) growing at Præstø Fed approx. 60 km south of Copenhagen, Denmark, at 2-4 m above sea level (20). The pinnae were dried at room temperature for 7 days before being milled into a powder (particle diameter less than 2 mm). The resulting Bracken powder had a water-extractable ptaquiloside content of approx. 2,150 $\mu g\ g^{-1}$ and was stored at –20°C.

Extraction of Bracken Materials and Isolation of Crude Ptaquiloside by Low-Pressure Liquid Chromatography. Aqueous extraction of ptaquiloside from Bracken powder was carried out by shaking 60 g Bracken powder with 900 mL triple deionised water for 60 min on a flat bed shaker (approx. 200 shakes per min.). The plant material was then separated from the aqueous solution by centrifuging at 11.200 g for 15 min. Aqueous extract retained by the fern materials fern materials was expelled by squeezing the material. This liquid was combined with the supernatant resulting in a total volume of approx. 600 mL crude extract. Aliquots of 50 mL crude extract were then passed through twelve dry packed Polyamide 6 columns (5.00 g resin in 1.0 cm I.D. glass columns). The combined eluates (approx. 550 mL) were loaded onto 100 mL of pre-conditioned (methanol washed) and wet packed (3-times deionised water) XAD-2 resin (2.5 cm I.D. glass column) at a rate of 360 mL $hour^{-1}$. After loading of the column, the XAD-2 resin

was washed three times with 50 of mL 3-times deionised water before elution of the column by addition of three times 50 mL methanol. The column was not allowed to run dry at any moment. The methanol extract was evaporated until dryness under vacuum using a rotary evaporator (45°C). The resulting gum was dissolved in 10 mL 3-times deionised water and passed through a dry packed Polyamide 6 column (2.00 g resin in a 1.0 cm I.D. glass column) directly onto a dry Chem Elut CE1020 column. The polyamide column was rinsed with a further 2 x 3 mL 3-times deionised water, collecting the total eluate in the Chem Elut column. The Chem Elut column was finally eluted with 2 x 100 mL of ethyl acetate and this eluate was evaporated to dryness under vacuum with a rotary evaporator (45°C). The resulting gum was dissolved in small amounts of ethyl acetate and transferred to glass storage vials with PTFE-caps before being evaporated till dryness by flushing with N_2. The resulting crude ptaquiloside (Figure 3A) was stored at –20°C in sealed vials in a desiccator over silica gel. The entire preparation of crude ptaquiloside lasted one working day. Up to 120 mg of crude ptaquiloside could be produced in this time from the Bracken material obtained in this study.

Purification of Ptaquiloside from Crude Ptaquiloside by Preparative HPLC. One batch of crude ptaquiloside produced as described above was dissolved in 6 mL of 3-times deionised water. Preparative HPLC separation was performed with 1 mL injections. Ptaquiloside containing fractions from each separation run was combined to give approx. 60 mL water-methanolic solution of ptaquiloside (65:35 V:V). Aliquots of 20 mL were then added to each of three Chem Elut CE1020 columns. Ptaquiloside was recovered from the Chem Elut columns with two times 100 mL ethyl acetate. The eluates were combined and evaporated to dryness under vacuum using a rotary evaporator at 45°C. The resulting gum was dissolved in ethyl acetate, transferred to a glass sample vial and evaporated till dry with a stream of N_2 giving ptaquiloside as a gum. When ptaquiloside powder was

needed, the gum was dissolved in 5 mL 3-times deionised water and frozen immediately before being freeze-dried. Ptaquiloside gum and powder (Figure 3B) were stored in sealed glass sample vials with PTFE caps in N_2-atmosphere at –20°C. The purification of ptaquiloside from crude ptaquiloside lasted one working day.

Confirmation of Ptaquiloside Structure by NMR. Pure ptaquiloside was dissolved in methanol-*d4*, and the identity confirmed by ^{1}H and ^{13}C NMR spectra (Table 1 and 2). The spectra proved similar to previously published NMR spectra (*7,8,10,22*). No signs of impurities could be detected in the ^{13}C spectra.

LC-MS of Purified Ptaquiloside. Pure ptaquiloside was dissolved in 3 times deionized water and the mass spectrum obtained by electro spray ionization. Mass spectrum (ESI) of ptaquiloside, *m/z*: 421 [$M+Na^+$]. The spectrum confirmed the identity of the compound.

Determination of UV-VIS, Infrared, and Raman Spectra of Purified Ptaquiloside.

Pure ptaquiloside was dissolved in methanol resulting in a concentration of ptaquiloside of 3.65 mg mL^{-1} and the UV-VIS absorption spectrum obtained in the range 190-1100 nm.

FTIR-spectra were obtained from pure ptaquiloside directly using an ATR-crystal. 256 scans were obtained for the spectrum at 4 cm^{-1} spectral resolution from 4000-550 cm^{-1}.

FT-Raman spectra were obtained directly from pure ptaquiloside at 4 cm^{-1} spectral resolution from 3600-200 cm^{-1} (64 scans in total).

Determination of Stability for Purified Ptaquiloside stored as a Gum.

A stock solution of pure ptaquiloside dissolved in ethyl acetate was produced to give a ptaquiloside-concentration of 65.9±5.2 mg L^{-1}, then 32 autosampler vials, each containing 40 µl of stock solution, were prepared and evaporated until dry by a stream of N_2. The vials were kept at -18º C in a desiccator. On each day of analysis (Figure 6), the content of ptaquiloside was determined by dissolving the content of four vials in 1 mL of 3-times deionised water. Ptaquiloside was converted to pterosin B in two of the vials, and the two

remaining vials left unaltered to give a measurement of the background conversion of ptaquiloside to pterosin B. All four vials were quantified using analytical HPLC with an external standard of pterosin B. This procedure was repeated seven times during a time span of 116 days and the stability of purified ptaquiloside determined.

RESULTS

Purification Technique. Crude ptaquiloside in the form of a yellowish transparent gum was prepared successfully by low-pressure liquid chromatography. The yield was 90-100% of the ptaquiloside content in the crude Bracken extract when quantified by HPLC by use of an external standard. The purity was approx. 70% as determined by HPLC and quantified on a weight basis (Figure 3A). Preparation of crude ptaquiloside from other specimens of Bracken than those used in this study revealed that the purity of crude ptaquiloside produced by this method depends on the initial ptaquiloside content in the ferns harvested as well as the content of interfering substances with solubility properties similar to ptaquiloside. Crude ptaquiloside purities between 20 and 80% have been obtained by this method from different batches of Bracken collected at different sites and times of the year.

Pure ptaquiloside was obtained by preparative HPLC in combination with low-pressure liquid chromatography (Figure 3B). The yield was 15-20% of the ptaquiloside content in the dried and milled bracken fern. Ptaquiloside was isolated either as a transparent gum after evaporation of ptaquiloside dissolved in ethyl acetate or as a whitish transparent amorphous powder after freeze-drying an aqueous solution of ptaquiloside. The purity was 100% (107±5). Clarification of the purity and identity of ptaquiloside by elemental analysis, LC-MS, ^{1}H and ^{13}C NMR (Tables 1 and 2) revealed that water was bound to ptaquiloside resulting in a gross formula for ptaquiloside of $C_{20}H_{30}O_8 \cdot \frac{2}{3}H_2O$.

The UV-VIS spectrum of pure ptaquiloside dissolved in methanol had 3 absorbance maxima, λ_{max} *nm*: 222 (ε 1810); 259 (ε 1060); and 303 (ε 250). The spectrum was in accordance with previously published spectra. However, the absorbance at 303 nm was weaker than reported earlier (24).

The FTIR spectrum (Figure 4) was characterized by the following absorbance maxima, cm^{-1}: 3,394 (broad); 2,970; 2,932; 2,874; 1,720; 1,643; 1,448; 1,383; 1,279; 1,196; 1,160; 1,074 (very strong); 1,040 (very strong); 933; 889. The spectrum was rather similar to previously reported spectra. However, new assignments were made in the range 3,000-2,800 cm^{-1}, likely originating from sp^3 C-H stretch (*7,9,26,27*).

The FT-Raman spectrum (Figure 5) was characterized by the following transmittance maxima, cm^{-1}: 3,086; 2,975; 2,932 (very strong); 2,874 (very strong); 1,717; 1,642; 1,454; 1,374; 1,275; 601. This is the first published FT-Raman spectrum of ptaquiloside.

Ptaquiloside as a gum was stored in sealed vials at –20°C in N_2-atmosphere. Ptaquiloside was stable for the first 50 days (Figure 6). Signs of degradation were observed after 68 days (2.6%). After 116 days 17.6% of the initial amount had degraded.

DISCUSSION

Several methods for recovery of ptaquiloside from Bracken already exist. However, these methods are all rather laborious, include the use of highly toxic chemicals or have low yield. No methods have so far been published for the synthesis of ptaquiloside. The method described in this paper yields high purity ptaquiloside by a combination of low-pressure liquid chromatography and preparative HPLC in a timesaving manner with limited use of organic solvents. If a lower quality of ptaquiloside is acceptable for analytical work or experimental work, the crude ptaquiloside can be used directly. This quality can be produced in large quantities very quickly.

The purity of ptaquiloside produced by this method is very high and comparable to existing methods as demonstrated by LC-MS and NMR-spectroscopy (Figure 4, Tables 1 and 2). The mass spectrum matched previously reported spectra, and the ^{1}H and ^{13}C NMR chemical shifts for ptaquiloside were similar to existing data.

Spectral analysis of ptaquiloside by UV-VIS and FTIR methods confirmed previously published spectra, although new peaks were observed in the range 3,000-2,800 cm^{-1}. The FT-Raman spectrum was characterized by distinct peaks between 3,086 and 2,874.

CONCLUSION

In summary, a rapid method of producing a high yield of approx. 70% pure ptaquiloside has been developed. This has then led to the production of pure production as a gum or as an amorphous powder using a refined and simplified method. Ptaquiloside can be rapidly produced in sufficient amounts for use in soil chemical, toxicological and ecotoxicological studies. Compared to previously published methods for ptaquiloside production, this refined method facilitates production of large amounts of high-quality ptaquiloside in a time-efficient manner with less use of harmful chemicals. Full FTIR- and FT-Raman spectra were recorded. Ptaquiloside produced in this manner was stable for 50 days when stored at -18ºC.

ABBREVIATIONS USED

^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon nuclear magnetic resonance; ATR, attenuated total reflection; COSY, correlated spectroscopy; DMF, dimethylformamide; TMS, tetra methyl silane; I.D., inner diameter; FT, Fourier transform; FTIR, Fourier transform infrared spectrometry; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization; *m/z*, mass-to-charge ratio; PTFE, poly(tetrafluoroethylene); UV-VIS, ultraviolet-visible range; V, volumetric percentage.

Safety-Because of the presence of carcinogens in Bracken, respiration protection-gear must be used while working with powdered Bracken. Plant materials and extracts should be handled wearing gloves.

Acknowledgement-Professor Ojika (Nagoya University, Japan) is gratefully thanked for his donation of ptaquiloside making this study possible.

LITERATURE CITED

(1) Fenwick, G.R. Bracken (*Pteridium aquilinum*) - Toxic effects and toxic constituens. *J. Sci. Food Agr.* **1988**, *46*, 147-173.

(2) Mosberg, B.; Stenberg, L.; Ericsson, S. Den Store Nordiske Flora. Copenhagen. GEC Gads Forlag. **1994**.

(3) International Agency for Research on Cancer (IARC) - Summaries & Evaluations. Bracken fern (*Pteridium aquilinum*) and some of its constituents. Homepage: <http://www.iarc.fr/>. **1998**. Access date: 14-8-2002.

(4) Smith, B.L. The toxicity of Bracken Fern (genus *Pteridium*) to animals and its relevance to Man; in Felix D'Mello JP (ed): Handbook of Plant and Fungal Toxicants. New York. CRC Press. **1997**. pp 63-76.

(5) Hirono, I. Carcinogenic Bracken glycosides; in Cheeke PR (ed): Toxicants of Plant Origin - Volume II Glycosides. Boca Raton. CRC Press, Inc. **1989**, pp 239-251.

(6) Smith, B.L.; Seawright, A.A.; Ng, J.C. Hertle, A.T.; Thomson, J.A.; Bostock, P.D. Concentration of ptaquiloside, a major carcinogen in Bracken Fern (*Pteridium* spp.), from eastern Australia and from a cultivated worldwide collection held in Sydney, Australia. *Nat. Toxins.* **1994**. *2*, 347-353.

(7) Niwa, H.; Ojika, M.; Wakamatsu, K.; Yamada, K.; Hirono, I.; Matsushita, K. Ptaquiloside, a novel norsesquiterpene glucoside from Bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Lett*. **1983**, *24*, 4117-4120.

(8) Niwa, H.; Ojika, M.; Wakamatsu, K.; Yamada, K.; Ohba, S.; Saito, Y.; Hirono, I.; Matsushita, K.: Stereochemistry of ptaquiloside, a novel norsesquiterpene glucoside from Bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron Lett*. **1983**, *24*, 5371-5372.

(9) Ojika, M.; Wakamatsu, K.; Niwa, H.; Yamada, K. Ptaquiloside, a potent carcinogen isolated from Bracken Fern *Pteridium aquilinum* var. *latiusculum*: Structure elucidation based on chemical and spectral evidence, and reactions with amino acids, nucleosides, and nucleotides. *Tetrahedron* **1987**, *43*, 5261-5274.

(10) van der Hoeven, J.C.M.; Lagerweij, W.J.; Posthumus, M.A.; van Veldhuizen, A.; Holterman, H.A.J. Aquilide A, a new mutagenic compound isolated from Bracken Fern (*Pteridium aquilinum* (L.) Kuhn). *Carcinogenesis*. **1983**, *4*, 1587-1590.

(11) Hirono, I.; Ito, M.; Yagyu, S.; Haga, M.; Wakamatsu, K.; Kishikawa, T.; Nishikawa, O.; Yamada, K.; Ojika, M.; Kigoshi, H. Reproduction of progressive retinal degeneration (bright blindness) in sheep by administration of ptaquiloside contained in Bracken. *J. Vet. Med. Sci*. **1993**, *6*, 979.

(12) Hirono. I.; Kono, Y.; Takahashi, K.; Yamada, K.; Niwa, H.; Ojika, M.; Kigoshi, H.; Niiyama, K.; Uosaki, Y. Reproduction of acute Bracken poisoning in a calf with ptaquiloside, a Bracken constituent. *Vet. Rec.* **1984**, *115*, 375-378.

(13) Hirono, I.; Aiso, S.; Yamaji, T.; Mori, H.; Yamada, K.; Niwa, H.; Ojika, M.; Wakamatsu, K.; Kigoshi, H.; Niiyama, K.; Uosaki, Y. Carcinogenicity in rats of ptaquiloside isolated from Bracken. *Gann* **1984**, *75*, 833-836.

(14) Marliére, C.A.; Wathern, P.; Castro, M.C.F.M.; O'Connor, P.; Galvao, M.A.M. Bracken Fern (*Pteridium aquilinum*) ingestion and oesophageal and stomach cancer. *IARC Scientific Publications.* **2002**, *156*, 379-380.

(15) Haenszel, W.; Kurihara, M.; Locke, F.B.; Shimuzu, K.; Segi, M. Stomach cancer in Japan. *J. Natl. Cancer I.* **1976**, *56*, 265-274.

(16) Alonso-Amelot, M.E. The link between Bracken Fern and stomach cancer: Milk. *Nutrition.* **1997**, *13*, 694-696.

(17) Alonso-Amelot, M.E.; Avendaño. M. Human carcinogenesis and Bracken Fern: A review of the evidence. *Curr. Med. Chem.* **2002**, *9*, 675-686.

(18) Alonso-Amelot, M.E.; Castillo, U.F.; Avendaño, M.; Smith, B.L.; Lauren, D.R. Milk as a vehicle for the transfer of ptaquiloside: A Bracken carcinogen; in Taylor JA, Smith RT (eds): Bracken Fern: Toxicity, Biology and Control. Proceedings of the International Bracken Group Conference Manchester 1999. Aberystwyth. International Bracken Group. **2000**. pp 86-90.

(19) Rasmussen, L.H.; Lauren, D.L.; Hansen, H.C.B. Sorption, degradation and mobility of ptaquiloside, a carcinogenic Bracken (*Pteridium* sp.) constituent, in the soil environment. **2003**. Submitted to *Chemosphere*.

(20) Rasmussen, L.H.; Jensen, L.S.; Hansen, H.C.B. Distribution of the carcinogenic terpene ptaquiloside in Bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *J. Chem. Ecol.* **2003**, *29*, 771-778.

(21) Rasmussen, L.H.; Kroghsbo, S.; Frisvad, J.C.; Hansen, H.C.B. Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere* **2003**, *51*, 117-127.

(22) Oelrichs, P.B.; Ng, J.C.; Bartley, J. Purification of ptaquiloside, a carcinogen from *Pteridium aquilinum*. *Phytochemistry* **1995**, *40*, 53-56.

(23) Burkhalter, P.W.; Groux, P.M.J.; Candrian, U.; Hübner, P.; Lüthy, J. Isolation, determination and degradation of ptaquiloside - a Bracken Fern (*Pteridium aquilinum*) carcinogen. *J. Nat. Toxins.* **1996**, *5*, 141-159.

(24) Saito, K.; Nagao, T.; Matoba, M.; Koyama, K.; Natori, S.; Murakami, T.; Saiki, Y. Chemical assay of ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the Pteridaceae. *Phytochemistry* **1989**, *28*, 1605-1611.

(25) Agnew, M.P.; Lauren, D.R. Determination of ptaquiloside in Bracken Fern (*Pteridium esculentum*). *J. Chromatogr.* **1991**, *538*, 462-468.

(26) Saito, K.; Nagao, T.; Takatsuki, S.; Koyama, K.; Natori, S.: The sesquiterpenoid carcinogen of Bracken Fern, and some analogues, from the Pteridaceae. *Phytochemistry* **1990**, *29*, 1475-1479.

(27) Pavia, D.L.; Lampman, G.M.; Kriz, G.S. Introduction to Spectroscopy. Fort Worth, USA. Harcourt College Publishers **2001**.

FIGURE CAPTIONS

Table no:	Text:
1	**Table 1.** ^{1}H NMR Chemical Shifts (δ in ppm relative to TMS, MeOD) and Coupling Constants (Hz) of Ptaquiloside.
2	**Table 2.** ^{13}C NMR Chemical Shifts (δ in ppm relative to TMS, MeOD) of Ptaquiloside.
Figure no:	**Text:**
1	**Figure 1.** Ptaquiloside [1] reactions. During alkaline conditions, first ptaquilosin [2] and then the unstable dienone [3] are formed. Both [1] and [3] yield pterosin B [4] under acid conditions. Other compounds than pterosin B may also be formed from ptaquiloside under acid conditions. The unstable dienone may alkylate DNA [5], and hence be responsible for the mutagenicity and carcinogenicity associated with ptaquiloside.
2	**Figure 2.** Overview of ptaquiloside purification.
3	**Figure 3.** HPLC chromatograms of (A) crude ptaquiloside (purity: 40%) and (B) pure ptaquiloside (purity: 105±11%) using UV 220 nm. Chromatogram A obtained at ambient temperature while B was obtained at 35ºC.
4	**Figure 4.** FTIR spectrum of pure ptaquiloside (purity 105±11%).
5	**Figure 5.** Raman spectrum of pure ptaquiloside (purity 105±11%).
6	**Figure 6.** Stability of ptaquiloside stored at -18ºC (initial concentration: 65.9±5.2 mg L^{-1}). Standard error bars shown.

Table 1. ^{1}H NMR Chemical Shifts (δ in ppm relative to TMS, MeOD) and Coupling Constants (Hz) of Ptaquiloside.

signal	chemical shift[a]	coupling constants
H-2	2.17-2.28 (m)	
H-3a	1.93 (t)	12.7
H-3b	2.50 (dd)	8.2; 12.4
H-5	5.76 (q)	1.3
H-9	2.64 (d)	1.3
H-10	1.07 (d)	6.9
H-11	1.53 (d)	1.2
H-12a -H-13b	0.45-0.90 (m)	
H-14	1.28 (s)	
H-1'	4.60 (d)	7.7
H-2'	3.20 (dd)	7.7; 9.1
H-3' - H-5'	3.26-3.38 (m)	
H-6'a	3.65 (dd)	5.7; 11.8
H-6'b	3.89 (dd)	1.9; 12.0

m: multiplet; s: singlet; d: doublet; t: triplet; q: quintet; dd: doublet-doublet

Table 2. ^{13}C NMR Chemical Shifts (δ in ppm relative to TMS, MeOD) of Ptaquiloside.

signal	chemical shift[a]
C-1	224.1 (s)
C-2	45.3 (d)
C-3	45.3 (t)
C-4	82.1 (s)
C-5	123.2 (d)
C-6	144.5 (s)
C-7	30.1 (s)
C-8	71.9 (s)
C-9	62.5 (d)
C-10	13.6 (q)
C-11	19.5 (q)
C-12	5.9 (t)
C-13	10.6 (t)
C-14	27.0 (q)
C-1'	99.4 (d)
C-2'	75.3 (d)
C-3'	77.8 (d)
C-4'	71.9 (d)
C-5'	78.2 (d)
C-6'	63.0 (t)

[a] Nomenclature: C (s), CH (d), CH_2 (t), or CH_3 (q) given in brackets.

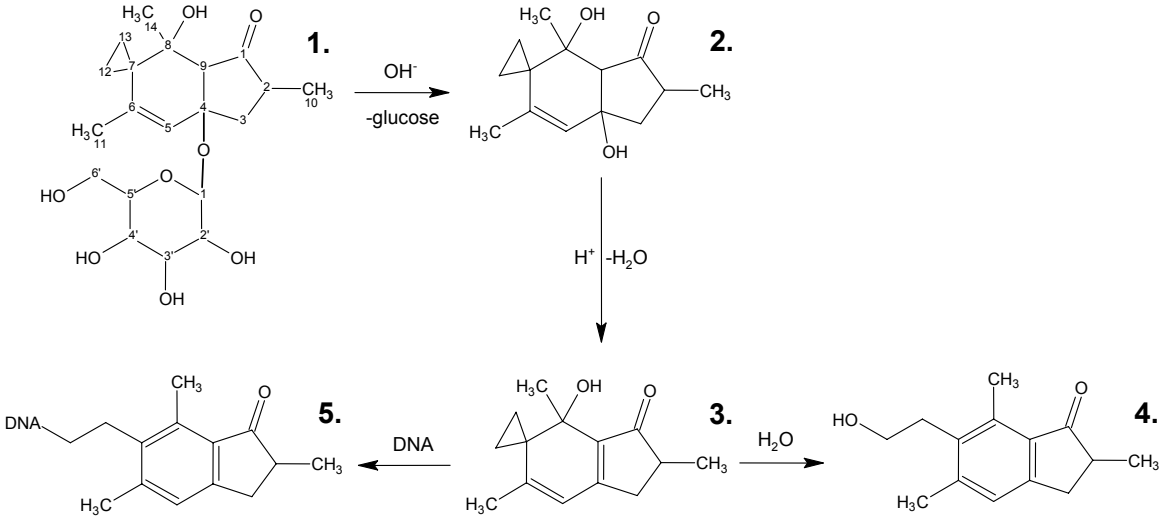


Figure 1. Ptaquiloside [1] reactions. During alkaline conditions, first ptaquilosin [2] and then the unstable dienone [3] are formed. Both [1] and [3] yield pterosin B [4] under acid conditions. Other compounds than pterosin B may also be formed from ptaquiloside under acid conditions. The unstable dienone may alkylate DNA [5], and hence be responsible for the mutagenicity and carcinogenicity associated with ptaquiloside.

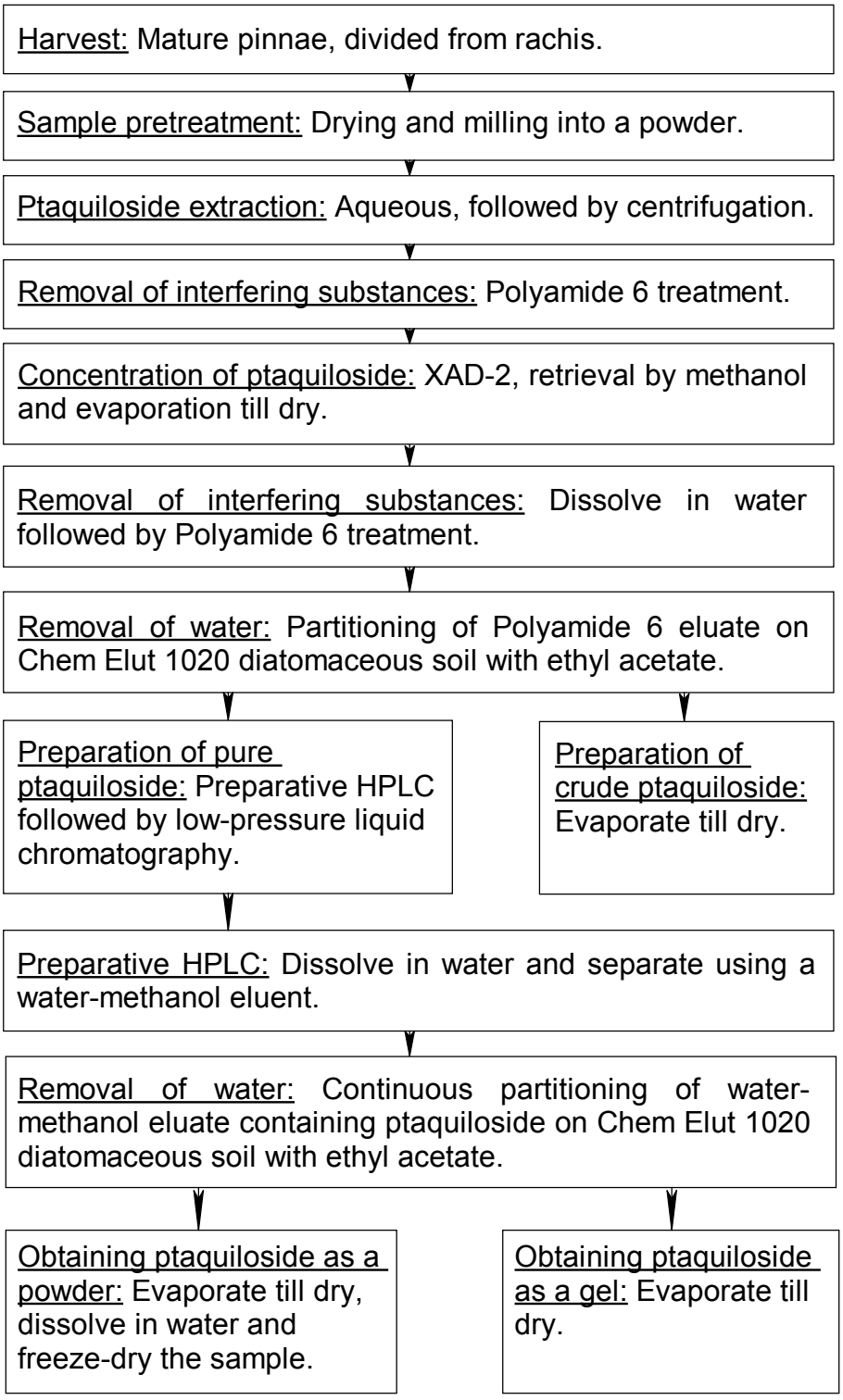


Figure 2. Overview of ptaquiloside purification.

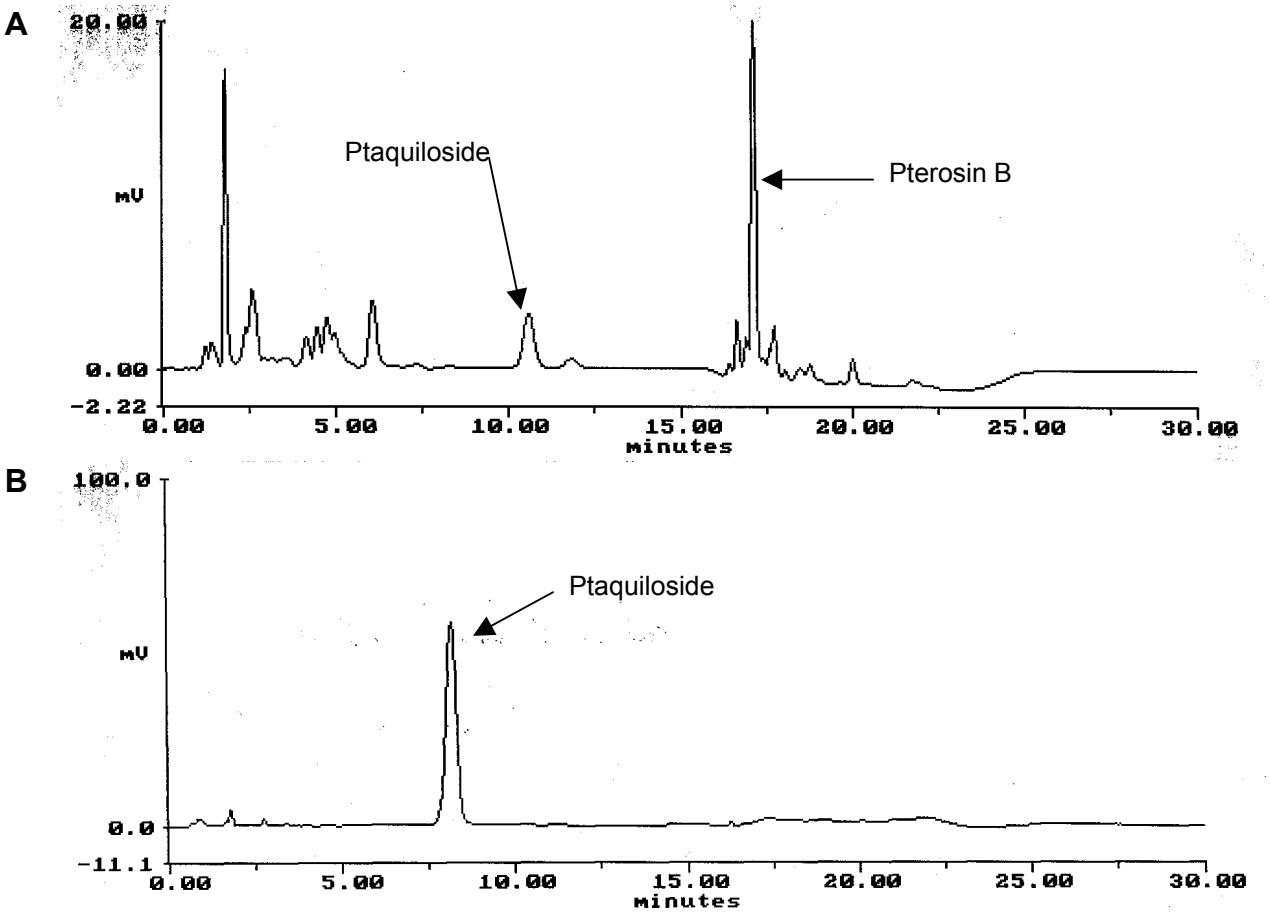


Figure 3. HPLC chromatograms of (A) crude ptaquiloside (purity: 40%) and (B) pure ptaquiloside (purity: 105±11%) using UV 220 nm. Chromatogram A obtained at ambient temperature while B was obtained at 35ºC.

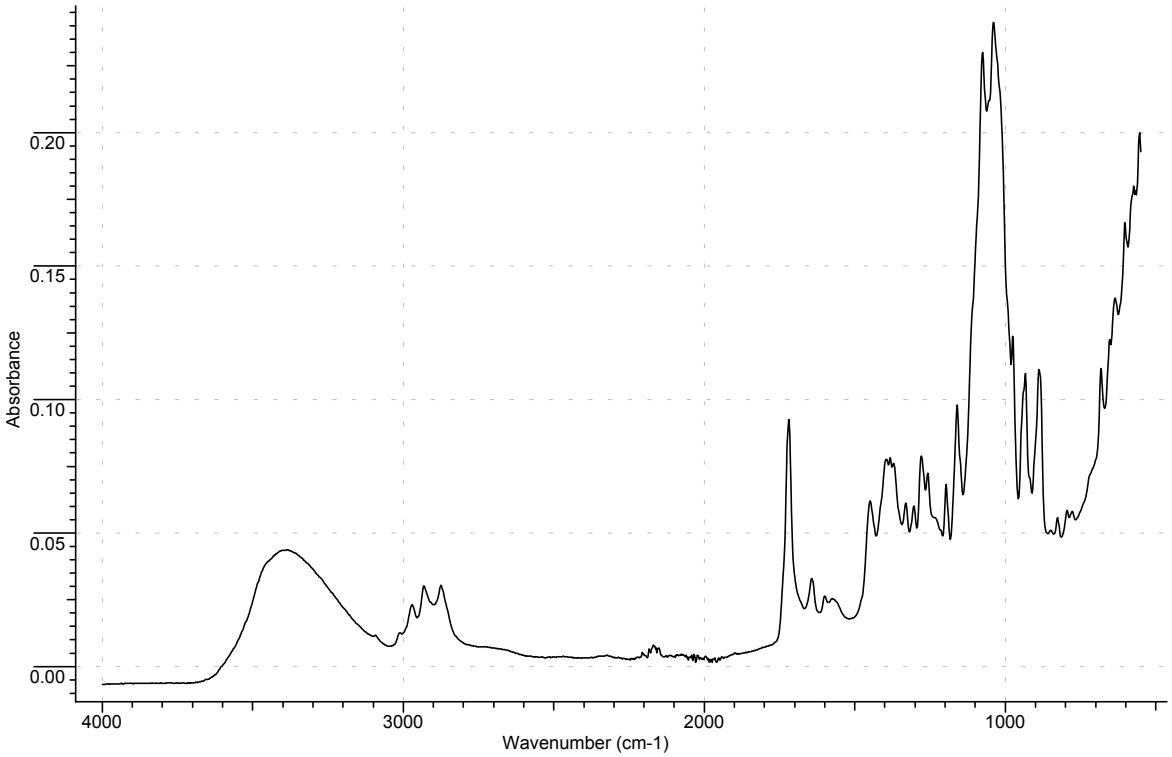


Figure 4. FTIR spectrum of pure ptaquiloside (purity 105±11%).

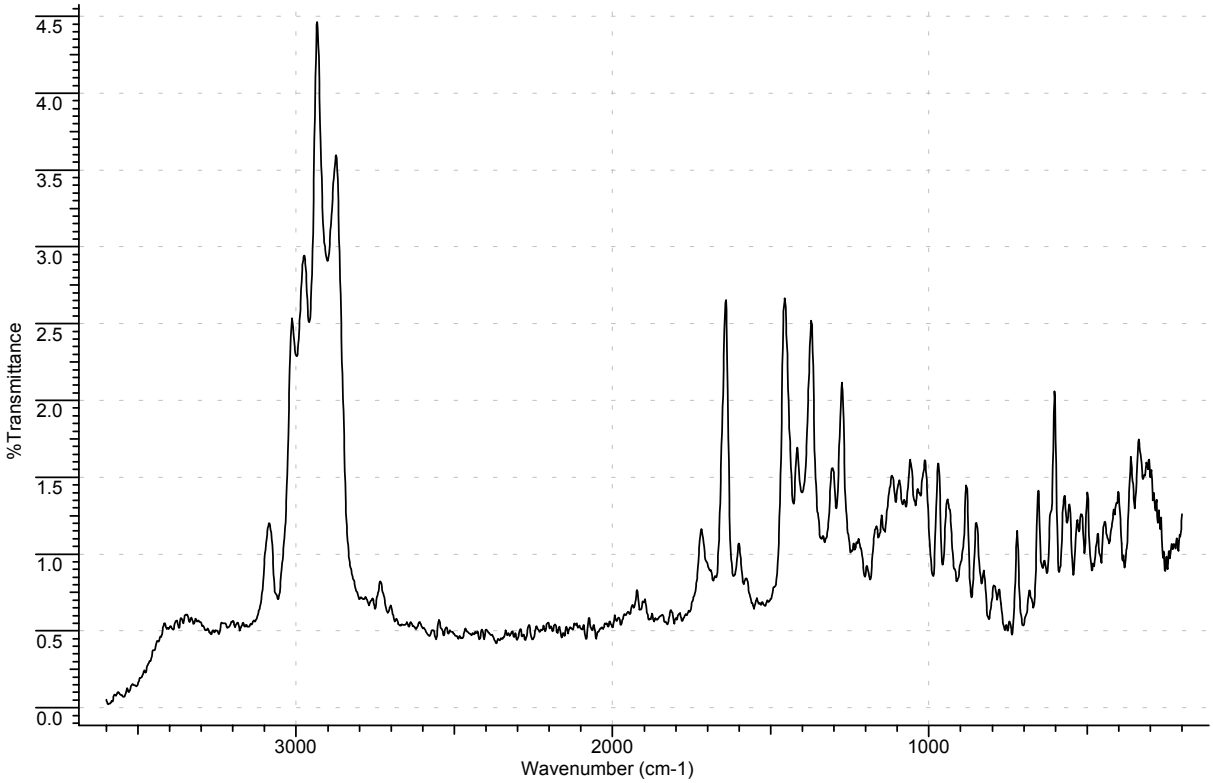


Figure 5. Raman spectrum of pure ptaquiloside (purity 105±11%).

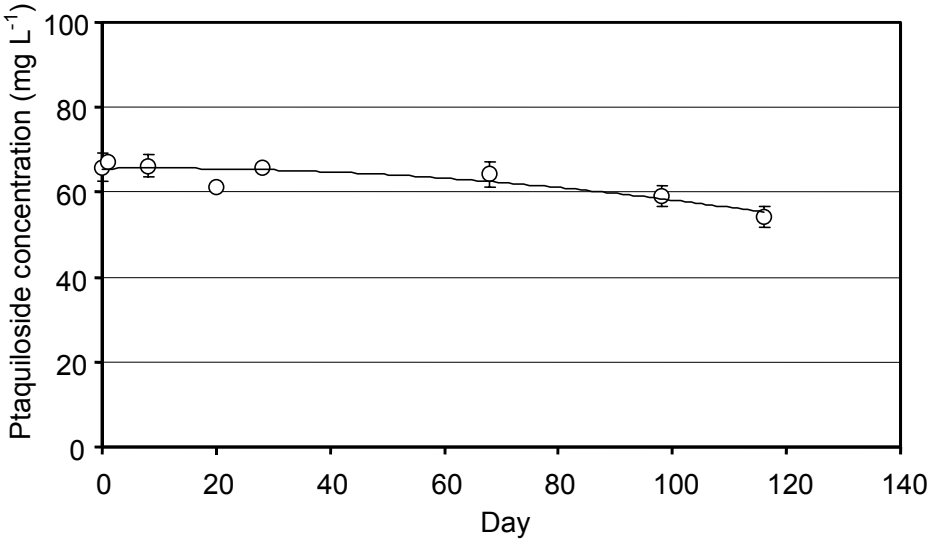


Figure 6. Stability of ptaquiloside stored at -18ºC (initial concentration: 65.9±5.2 mg L^{-1}). Standard error bars shown.