

Passage of the bracken fern carcinogen ptaquiloside into bovine milk

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Summary — Ptaquiloside has been discovered for the first time in milk samples of a bovine fed a dietary complement (6 kg fresh weight/day) of freshly-cut bracken fronds. This was equivalent to ca 216 mg/day pure ptaquiloside. The compound was found in a quantity of no less than 0.11 mg/l in milk. This was determined by the appearance of pteroin B – the decomposition product of ptaquiloside – in prepurified milk samples treated with mild alkali (pH 8.0 and 11.5). The lack of base treatment led to the absence of pteroin B. A milk prepurification protocol based on methanol-acetonitrile precipitations in succession, hexane and dichloromethane extractions, alkaline treatment, reextraction and silica gel microcolumn chromatography followed by reverse-phase HPLC was developed for the detection of ptaquiloside.

bracken / *Pteridium aquilinum* / milk / ptaquiloside / human cancer / HPLC

Résumé — Passage du carcinogène ptaquiloside de la fougère Bracken dans le lait de vache. Le ptaquiloside fut découvert pour la première fois dans le lait de bovins dont l'alimentation avait été supplémentée avec des frondes de fougère fraîchement coupées (6 kg de matériel frais par jour). Cette quantité équivaut à 216 mg/jour de ptaquiloside pur. Pas moins de 0,11 mg/l de ce composé a été détecté dans le lait. Cette détermination a été réalisée en mesurant la ptérosine B produite par la décomposition du ptaquiloside après traitement du lait prépurifié en milieu légèrement basique (pH 8,0 et 11,5). En l'absence de traitement alcalin, aucune production de ptérosine B n'a été observée. Afin de détecter le ptaquiloside, nous avons développé un protocole de prépurification du lait basé sur des précipitations successives au méthanol-acétonitrile, extractions à l'hexane et au dichlorométhane, traitement alcalin, réextraction et chromatographie sur microcolonne de gel de silice, le tout suivi par l'analyse par HPLC en phase inverse.

fougère / *Pteridium aquilinum* / lait / ptaquiloside / cancer humain / HPLC

INTRODUCTION

Bracken fern *Pteridium aquilinum* is considered 1 of the 5 most successful organisms of the plant kingdom (Page, 1976, 1982; Harper, 1977; Fletcher and Kirkwood, 1979). It grows on every continent and at every latitude except for the polar ice-caps (Tryon, 1941), with an extensive coverage of agricultural land (Fenwick, 1988). The widespread clearance of forests in the tropics by ranchers and mine prospectors is rapidly expanding the encroachment of bracken on hillsides, mountain grasslands and cool Andean valleys of the formerly bracken-free pastureland (Gliessman, 1978; Ortega, 1990).

In addition, the range of chemicals with biological impact in this fern is of major economic importance. In addition to the cyanogenic glycosides and tannins (Cooper-Driver and Swain, 1976) and the cytotoxin 1-indanone derivatives - pterosins- (fig 1) (Saito *et al*, 1975), there is the pro-histaminic activity and mutagenicity of braxin 1A (Saito *et al*, 1990), and the thiamine-destroying compounds dactylifric acid (Fukuoka, 1982) and thiaminase (Evans, 1976; McCleary and Chick, 1977) which cause coordination disorders and death in horses and mules (Diniz *et al*, 1984). Still other compounds appear associated with damage to the bone-marrow tissue (Evans, 1986), severe leucopenia, thrombocytopenia (Philp and Gowdey, 1967), excess oral mucus breathing difficulty and death in cattle (Evans, 1976), bright blindness in sheep (Barnett and Watson, 1970), heart failure in pigs (Evans *et al*, 1972; Harding, 1972), and bovine enzootic hematuria (Rosenberger, 1971), a chronic condition of the urinary tract that invariably leads to death. The combination of its wide-spread growth and aggressive chemistry makes bracken a most severe problem for the animal farming industry.

Of major concern is the possible link between bracken infestation and the appearance of esophageal and stomach cancer in individuals living in these areas. This situation was first proposed by Evans *et al* (1971), then reported in Costa Rica (Villalobos-Salazar, 1985, 1987; Villalobos-Salazar *et al*, 1989a), and suggested for some areas of Wales (Galpin and Smith, 1986) and the Andean states of western Venezuela (Alonso-Amelot and Castillo, unpublished observations). The actual mechanism of this connection has never been established since bracken is not considered, apart from rare occasions, as being suitable for human consumption (Hodge, 1973). However, there is sufficient evidence to believe that milk may provide that link. On the one hand, bracken contains ptaquiloside, a glucoside norsesquiterpene (Van Der Hoeven *et al*, 1983; Niwa *et al*, 1983) (see fig 1) of strong carcinogenic potential (Hirono *et al*, 1987). Once separated from the glucose, the ptaquiloside aglycone becomes a potent electrophilic cyclopropane dienone that reacts strongly towards various weak nucleophiles including water, amines and other very mild bases (Matoba *et al*, 1987). Also ptaquiloside must be capable of passing through the cell and nucleus membranes, as it brings about chromosomal aberration (Matsuoka *et al*, 1989) via irreversible damage to uncoiled DNA (Ojika *et al*, 1989).

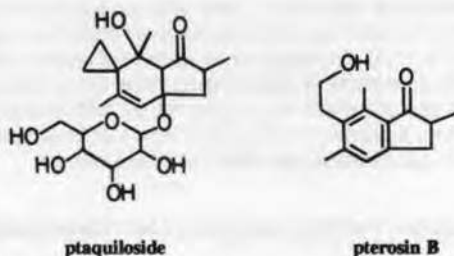


Fig 1. Structure of ptaquiloside and pterosin B. Structure du ptaquiloside et de la pterosine B.

On the other hand, various cancerous tumors have been induced in rats and mice given oral doses of milk obtained from cows fed with dietary complements of bracken fern (Evans *et al.*, 1972; Pamukcu *et al.*, 1978; Villalobos-Salazar *et al.*, 1989b). Earlier results have also indicated that calves experienced lowered bone marrow activity when allowed to suck their bracken-fed mother cow's milk (Evans, 1986).

All this evidence points toward the presence of deleterious bracken metabolites in bovine milk. Some toxic compounds in animal feed such as aflatoxins and some pesticides have been known for some time either directly or after derivatization by body enzymes to pass into the milk in bovines. Bracken metabolites may be no exception. However, in spite of these advances, so far the chemical analysis of milk has been poor in providing direct evidence of secondary bracken compounds. It has been considered that to formally demonstrate the existence of a link between bracken and human cancer, the detection of the bracken carcinogen 1 in milk is required. It is the purpose of this paper to report on the first detection of ptaquiloside in milk from cattle exposed to bracken fern.

MATERIALS AND METHODS

Milk extraction procedure

The entire sequence was carried out at 36 °C to increase the rate of protein and lipid precipitation. A milk sample (2 l) was warmed to 36 °C and methanol (4 l) was added with stirring for 30 min (5 h at 36°C). The suspension was filtered through cheesecloth, then filter paper and finally centrifuged (3 000 rpm, $r = 38$ cm). The supernatant was evaporated *in vacuo* at 36 °C to 300 ml and acetonitrile (1 l) was added in portions while the deposited solids were gradually removed to prevent the formation of a sticky

mass. The suspension was centrifuged, the volume of the liquid phase corrected to 300 ml by evaporation at 36 °C, and 20 g sodium chloride was added. This solution was extracted successively with hexane (3 x 50 ml) and dichloromethane (6 x 25 ml) (extract A). To the aqueous layer was added 15 ml of an aqueous sodium hydroxide (0.003 N) solution and allowed to stand at 36 °C for 2 h with stirring. The mixture was then extracted with dichloromethane (6 x 25 ml) (extract B) to draw out the pteroin B derived from ptaquiloside in the milk sample.

Microcolumn prepurification

The modified procedure of Alonso-Amelot *et al.* (1992a) was used. Extracts A and B were submitted to the same protocol: the dichloromethane solutions were evaporated over silica gel (100 mg) at room temperature *in vacuo*, and the solid residue was transferred to a microcolumn prepared from a Pasteur pipette filled with 300 mg silica gel 70–230 mesh. The column was eluted with 1) 2.5 ml hexane–ethyl acetate 9:1 and 2) 3.0 ml hexane–ethyl acetate 1:4. Only the second fraction contained pteroin B. This eluent was evaporated in a current of dry nitrogen gas, and redissolved in HPLC grade methanol (2.0 ml).

HPLC analysis

A LDC Milton Roy HPLC apparatus composed of a Constametric II pump, a Rheodyne 20- μ l loop injector and a Waters 490E multichannel UV-VIS detector tuned at 260 nm connected to a radial 0.5 x 10 cm C-18 column was used. The optimum separation conditions were as follows: solvent: methanol–water 65:35 in the isocratic mode, flow: 1.0 ml/min, at room temperature. The pteroin B peak appeared at $R_t = 10'10''$.

Cow conditioning and milk sampling

A 3-year old 100% Holstein female in its 4th milking month was selected from a herd and maintained on a Kikuyu (*Pennisetum clandestinum*) plot (altitude 1 900 m) and commercial animal feed concentrate at a rate of 2 kg/day. The

positive health condition of the animal was monitored for 4 months before the bracken-feeding experiment was started. Freshly-cut bracken fronds (6 kg) were then administered orally in an admixture with fresh Kikuyu grass and molasses every morning for 7 consecutive d. After the morning milking was collected (24 l daily average), 2 l samples were separated and immediately taken to the laboratory for analysis as described above.

RESULTS AND DISCUSSION

Ptaquiloside is a very unstable compound. Once isolated from the plant it decomposes quickly into pterosin B under mild acid, base or heat (Saito *et al*, 1989). Any clean-up procedure used to detect this compound in milk must avoid these conditions in order to preserve its molecular integrity. In particular, commonly-used reagents for milk analysis such as acetic, sulfuric and hydrochloric acids must not be utilised. The direct analysis of ptaquiloside in a complex matrix such as milk is therefore not practical. Nevertheless, a milk pre-purification sequence was designed to defat and deproteinise milk samples under neutral conditions. It was based on the precipitation of insoluble material *via* methanol and acetonitrile, followed by extraction with hexane and dichloromethane as described in figure 2 (see also the *Materials and Methods* section). The remaining aqueous phase was then analyzed by HPLC after silica gel microcolumn prepurification (Alonso-Amelot *et al*, 1992a). This strategy ensured that any pterosin B that was to be detected at this point would not come from ptaquiloside decomposition, but from the ingested bracken that contained pterosin B as a natural metabolite. Indeed, a ptaquiloside-enriched sample obtained from the purification of a water extract of bracken resisted the indicated milk precipitation and microcolumn prepurification scheme without apparent change.

Conversely, any pterosin B appearing in the liquid chromatograms of milk extracts treated with mild base after precipitation and solvent extraction should come exclusively from the decomposition of ptaquiloside. Another potential pterosin B precursor present in bracken is the pterosin glucoside pteroside B. When an authentic sample of this compound was added to a milk sample which was then subjected to the purification sequence including the base treatment step at a higher temperature (50 °C), it remained totally unchanged and no detectable pterosin B was produced. This analytical strategy is depicted in figure 3.

The bovine specimens for milking were distributed into 2 groups. In the first group a herd of 4 Holstein cows were kept in a 2-ha plot that was heavily infested with bracken growth. After 24 months 3 of the animals had developed enzootic hematuria. At their medium lactation period, milk samples were taken from the animals once a wk for 3 wk and analyzed for the contents of pterosin B naturally occurring in the fern, and for ptaquiloside *via* its base transformation into pterosin B following the analytical sequence described above. None of these samples showed signs of natural or induced pterosin B in the HPLC chromatograms down to a detection limit of 0.4 ng per injection.

In the second group, one healthy 3-year-old Holstein cow in her 4th month of lactation was given oral complements in the order of 6 kg fresh weight (850 g DM) of freshly-cut bracken fronds of intermediate growth (*ca* 40–50 days from emergence) every 24 h in addition to its normal Kikuyu grass diet and animal-feed concentrate. The ptaquiloside content of the fronds was estimated by HPLC (Alonso-Amelot *et al*, 1992b) to be 0.250 ± 0.050 mg per g vegetation biomass. This gave an average of 216 mg/carcinogen/d added to the diet. This treatment was extended

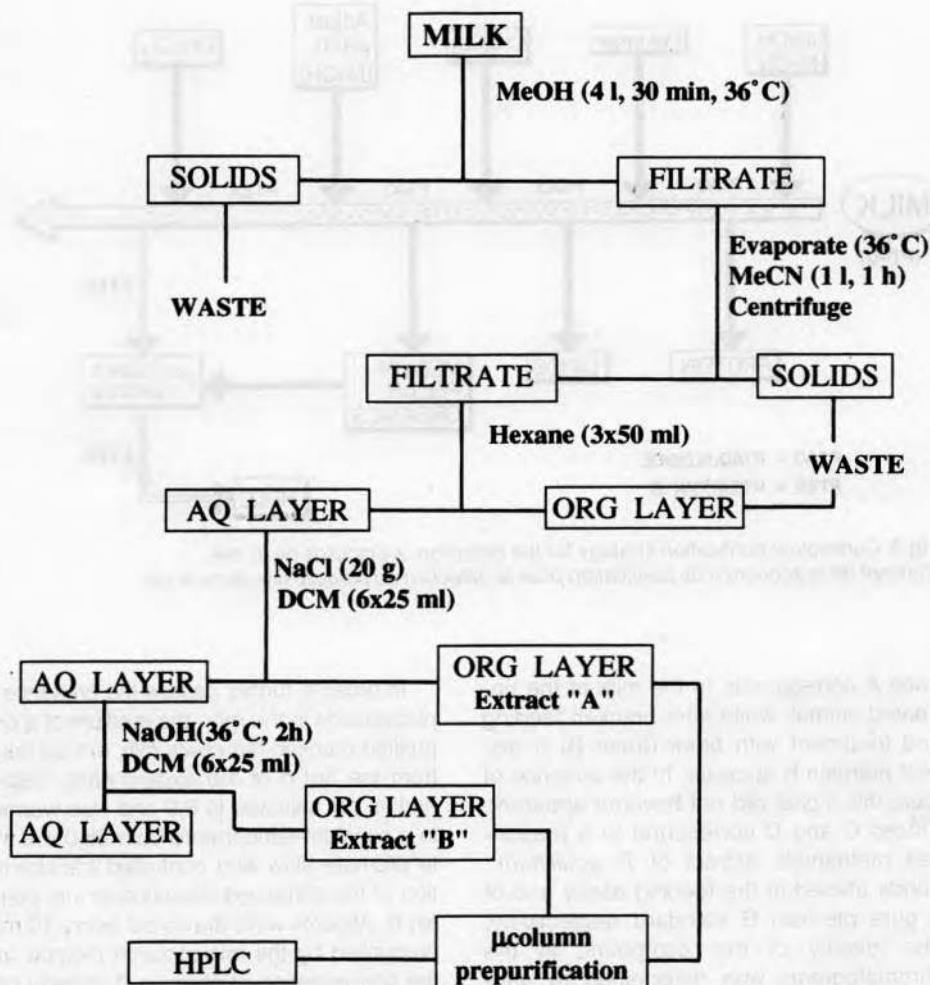


Fig 2. Milk purification sequence for the detection of ptaquiloside, the bracken fern carcinogen.
Séquence de purification pour la détection du ptaquiloside, le carcinogène de la fougère.

for 7 consecutive d and then discontinued. Milk samples were taken daily in the early morning and 7 d after the bracken treatment had been ceased. During the treatment there was no net reduction of the average volume of milk produced (24 l/d), nor was there any indication of sickness or disturbance in the animal. Hence the amounts of bracken metabolites administered were

under the toxicity level (as yet undetermined) of the cow in question.

All the milk samples from this animal that were taken after the first 48 h of bracken feeding did not contain any detectable natural pteroin, but the extracts treated with mild base at pH 11.5 showed a distinct pteroin B peak in the liquid chromatograms. This is shown in figure 4 where

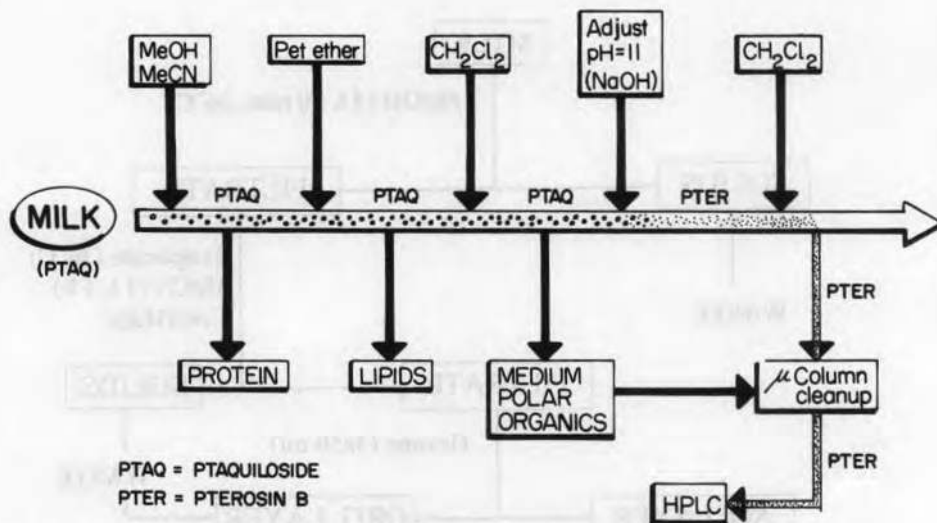


Fig 3. Conceptual purification strategy for the detection of ptaquiloside in milk.
Concept de la séquence de purification pour la détection du ptaquiloside dans le lait.

trace A corresponds to the milk of the untreated animal, while after bracken feeding and treatment with base (trace B) a distinct pterosin B appears. In the absence of base this signal did not become apparent. Traces C and D correspond to a prepurified methanolic extract of *P. aquilinum* fronds utilised in the feeding assay and of a pure pterosin B standard respectively. The identity of this compound in the chromatograms was determined by various methods, including comparison of retention times, co-injection and peak enrichment with pterosin B standards. In addition, the UV spectrum of this peak obtained with the aid of the multichannel spectrophotometric detector employed was identical to that of an authentic sample, as illustrated in figure 5. It is clear that the only way for pterosin B to appear after the base treatment of the milk extract must have been the base-induced decomposition of ptaquiloside originally present in the milk.

In order to further assess the presence of ptaquiloside in the milk, the medium of a prepurified bracken-fed cow's milk extract taken from the 3rd d of the experimental feeding had its pH adjusted to 8.0 and was warmed in a constant-temperature bath $38.0 \pm 0.5^\circ\text{C}$ to promote slow and controlled transformation of the contained ptaquiloside into pterosin B. Aliquots were drawn out every 10 min, prepurified by the microcolumn method and the concentration of pterosin B thereby produced determined by HPLC. The ptaquiloside-pterosin conversion was assessed by following the height of the pterosin B peak. It was found to increase smoothly and asymptotically to completion during the first 60 min of warming. A parallel experiment using a ptaquiloside-enriched milk sample under the same pH and temperature conditions, displayed a similar behaviour was observed, as shown by the data in figure 6. Hence the hypothesis that ptaquiloside was present in the milk of the bracken-fed cow was fully demonstrated.

That the herd of 4 cows exposed to voluntary feeding of bracken fern did not show signs of ptaquiloside in the milk can be explained on 2 grounds. First, although it was clear that the animals did in fact eat limited amounts of fern since they developed enzootic hematuria only attributable to fern intoxication, their feeding on this plant may have been only casual or under stress when other grasses became scarce. Therefore, the concentration of ptaquiloside

excreted through their milk may have been minimal, possibly under the detection limit of the present method. Second, in the voluntary feeding situation there was no precise record of the last time that the cows had actually eaten bracken. It could well be that, ptaquiloside being an unstable chemical, its mean residence time in the rumen could be short, particularly under the rather high temperatures of the rumen mass (40–42 °C). In fact, a milk sample from the bracken-force-fed cow obtained 7 d after the last meal including bracken did not show any detectable ptaquiloside-derived pterosin B in the extracts. Hence, animals exposed to bracken

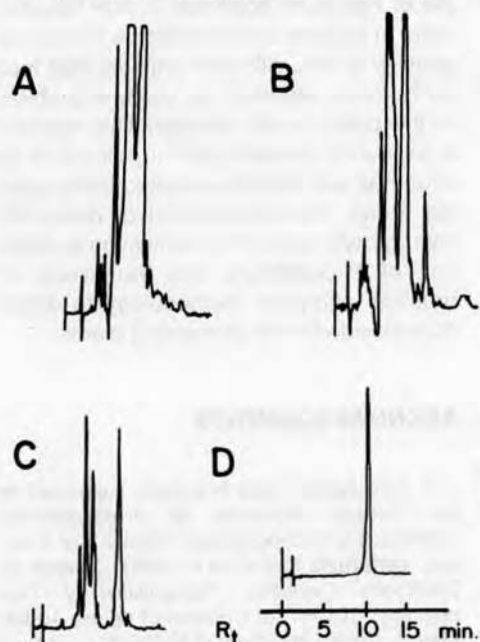


Fig 4. HPLC chromatograms of milk and bracken frond fractions: **A)** milk extract **A.** **B)** Milk fraction treated with dilute base (extract B) showing a distinct pterosin B peak. **C)** Water-dichloromethane extract of bracken fronds used for the feeding experiment. **D)** Pterosin B standard. For specific HPLC conditions see text.

Chromatogrammes de HPLC des fractions de lait et frondes de fougère. A) Extrait A du lait. B) Fraction du lait traité avec une base diluée (extrait B) qui montre clairement un pic de ptérosine B. C) Extrait d'eau-dichlorométhane des frondes de fougère utilisées dans l'expérience de l'alimentation. D) Standard de ptérosine B. Pour les conditions spécifiques de HPLC, voir le texte.

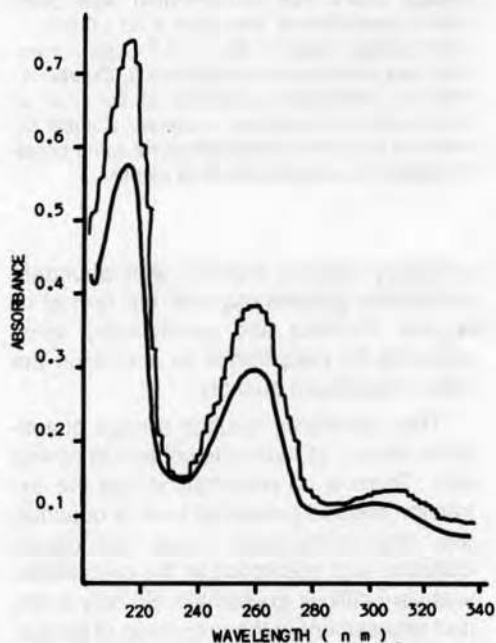


Fig 5. Comparison of the UV spectra of pterosin B peak obtained in the HPLC trace after base treatment. Solid line: spectrum of pterosin B standard. Broken line: spectrum of HPLC peak attributed to pterosin B.

Comparaison des spectres UV du pic de ptérosine B obtenu par HPLC après traitement alcalin. Trait continu : spectre de ptérosine standard. Trait pointillé : spectre du pic de HPLC attribué à la ptérosine B.

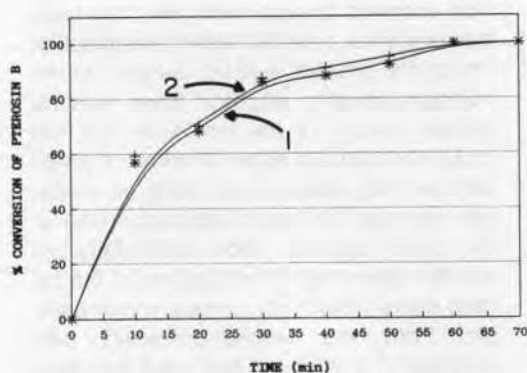


Fig 6. Relative % conversion of ptaquiloside into pterosin B. Curve (1): in a prepurified milk sample of a bracken-fed bovine. Curve (2) : in a healthy cow's milk contaminated with plant-derived ptaquiloside. Both runs at $38 \pm 0.5^\circ\text{C}$. *Pourcentage relatif à $38,0 \pm 0,5^\circ\text{C}$ de conversion de ptaquiloside en ptérosine B. Courbe A : dans un échantillon prépurifié de lait chez un bovin alimenté avec des fougères. Courbe B : dans un lait provenant d'une vache saine contaminé avec le ptaquiloside de la plante.*

voluntary feeding together with abundant alternative grasses may eat the fern at irregular intervals and insufficiently small amounts for ptaquiloside to appear in the milk in significant quantity.

This hypothesis required a more quantitative view of ptaquiloside content in bovine milk. There is no presumption that the extraction method presented here is quantitative, since ptaquiloside losses from coprecipitation and adsorption in the precipitates was very difficult to estimate. So only a limited assessment of the proportion of ptaquiloside relative to the ingested material can be advanced at this time. The concentrations of observed pterosin B were calculated against a calibration curve by standard procedures. When these values were translated in terms of ptaquiloside, it was found to be no less than 0.11 mg/l fresh milk. In terms of the 24 l/d produced by the test animal, this becomes 2.60 mg/d ptaquiloside

excreted into the milk, which represents $\approx 1.2\%$ of the total ingested carcinogen.

CONCLUSIONS

The exposure of dairy cattle to bracken fern is not only of economic concern owing to disease and death of livestock, but the discovery of the potent carcinogenic principle of *Pteridium aquilinum* in milk reported here, in addition to the evidence of carcinogenicity of the milk from animals that feed on bracken, extends the bracken problem to the public health domain. This requires a review of bracken control policies in all countries with bracken-infested cattle grazing areas, the development of more efficient growth control or eradication technology of *P. aquilinum*, and the design of practical analytical methodology to detect ptaquiloside in milk processing plants.

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