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Excretion, through milk, of ptaquiloside in bracken-fed cows. A quantitative assessment

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Abstract — The content of ptaquiloside (ptaq), a major *Pteridium aquilinum* carcinogen found in milk from cows fed bracken in Venezuela, has been estimated quantitatively in its passage through bovines into milk. Six cows were given 6 kg/animal/d of fresh bracken fronds whose ptaq content was determined. Ptaq first appeared in milk 38 h after initially feeding this plant to the cows and continued to be excreted at a relatively constant level, after an induction period, for 62–70 h after the bracken diet had ceased. The average value of total ptaq excreted ($[ptaq]_e$) from milk amounted to 8.60 ± 1.16 % of the total ptaq ingested ($[ptaq]_i$) during the entire feeding period. $[ptaq]_e$ was linearly dose dependent in the 2 400 to 10 000 mg/animal/d range of ptaq given to the test animals and the ratio $[ptaq]e/[ptaq]_i$ remained relatively constant at various ptaq dosages. In a two-pulse feeding sequence with a 72-h interlude when no bracken was given, a bimodal ptaq excretion curve was obtained. The implications to human health are discussed. © Inra/Elsevier, Paris

ptaquiloside / bracken fern / cow's milk

Résumé — Excrétion du ptaquiloside dans le lait de vaches alimentées avec de la fougère Bracken. Évaluation quantitative. La quantité de ptaquiloside (ptaq), le principal carcinogène de *Pteridium aquilinum*, retrouvé dans le lait de vaches alimentées avec la fougère bracken au Venezuela, a été estimée quantitativement. On a donné à six vaches 6 kg/j de frondes fraîches de fougère, dont le contenu en ptaq a été determiné. Le ptaq est apparu d'abord dans le lait 38 heures après la première prise et a continué à être excrété à une concentration plus ou moins constante après une période d'induction, jusqu'à 62–70 heures après que l'alimentation en fougère a cessé. Ensuite, le ptaq a graduellement diminué jusqu'à ce qu'il ait disparu du lait. La valeur moyenne de ptaq excrétée dans le

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lait ($[ptaq]_e$) était de 8,60 ± 1,16 % du ptaq total ingeré ($[ptaq]_i$) pendant toute la période d'alimentation. $[ptaq]_e$ était linéairement dépendant de la quantité de ptaq ingéré par l'animal entre 2 400 et 10 000 mg/animal/j, alors que le rapport ($[ptaq]_e$) /($[ptaq]_i$) est demeuré relativement constant à plusieurs doses de ptaq. Dans une séquence de deux administrations de fougère avec un repos de 72 heures, on a obtenu une courbe d'excrétion bimodale. Les implications pour la santé humaine font l'objet d'une discussion. © Inra/Elsevier, Paris

ptaquiloside / fougère Bracken / lait de vache

1. INTRODUCTION

Milk has been recognized as a potential source of toxic natural products for many years. In addition to mycotoxins acquired by the animal by way of molds present in formulated feed, various other plant secondary metabolites of xenobiotic character have been detected in milk from animals exposed to some range plants. For example, to 5-acetylbenzofuran derivatives in *Eupatorium rugosum* and *Isocoma wrightii* was attributed the notorious 'milk sickness' that affected many early pioneers in Indiana, Illinois, and Ohio in the USA [8, 16]. Glucosinolates from the Cruciferae [55], alkaloids [9, 30, 32, 36] and sesquiterpene γ-lactones from the Compositae [24] add to the list of natural toxins found in milk in proportions as high as 0.4 $mg \cdot L^{-1}$ and higher [13]. Recently we discovered another plant xenobiotic in milk, namely ptaquiloside, from cows exposed to dietary complements of bracken *Pteridium aquilinum* [4, 5].

Ptaquiloside (ptaq), a norsesquiterpene glucoside of bracken fern (genus *Pteridium*) and some other related ferns, with an illudane skeleton [40, 51] is one of the most potent known carcinogens of natural origin [26]. It has been shown in vitro to bind covalently to nucleotides hence splitting the uncoiled DNA chain [33, 41]. Ptaq's potential for causing tumors in the urinary bladder, mammary glands, intestine and other organs has been recognized in laboratory rodents and livestock [28, 53]. In addition, bracken or ptaq ingestion leads to retinal degeneration in sheep [7, 29] and may cause rapid death of cattle when the high levels of ptaq found naturally in some bracken fronds are ingested [17, 18, 20, 21, 27, 47, 56].

Before ptaq was discovered, it was shown that milk from cows fed on a diet containing bracken fronds was carcinogenic and mutagenic to laboratory mice and rats [19, 46, 53]. Ptaq was later isolated and identified as the principal bracken carcinogen and the cause of the retinal degeneration [29], clastogenicity [35], and mutagenicity [38, 39, 51]. It was hypothesized that ptaq was transferred through milk to calves and possibly humans from bracken-fed lactating cows [53, 54]. The development of a method for detecting ptaq in milk by its quantitative transformation into pterosin B followed by HPLC separation [4], allowed for the first time the identification of moderate amounts of ptaq in milk from cows that had eaten bracken; however, these quantities could not justify the dramatic effects reported earlier.

In conjunction with our recent finding [5] that a greater proportion of ptaq is contained in bracken-fed cow's milk, a quantitative approach to this problem was undertaken to estimate the relationship between the amount of ptaq ingested by these animals and the amount excreted through milk, as well as the dependency of ptaq excretion with time. This would allow the assessment of the risk to farmers and other local consumers, as well as to calves, of consumption of raw milk with ptaq. Here, we report that ptaq is effectively excreted through milk in about 8.60 ± 1.16 % of the amount inges-

ted by the cow, and is linearly dose dependent. If one considers the status of bracken as one of the five most common plants on Earth [23], its prevalence in pasture lands in all five continents [43], its increasing spread [44] and the high concentration of ptaq found in most bracken varieties [49], then the occurrence of ptaq in milk should be of great concern to human health and small farm economic progress.

2. MATERIALS AND METHODS

Milk from six two-years old Jersey-Holstein half breed cows were used for testing approximately two months after calving. The animals were maintained in an open kikuyu grass (*Pennisetum clandestinum*) field at 1 900 m above sea level in an agricultural research station in Mérida, Venezuela. The cows were herded twice a day for mechanical milking at 6 AM and 4 PM. In the afternoon shift of each day for five consecutive days, the cows were given 2 kg/animal/d commercial concentrate feed supplement mixed with a previously weighed sample (6.0 kg) of freshly collected 20–25 days old bracken fronds (neotropical ssp. *caudatum* var. *caudatum*) cut in small pieces, kikuyu grass (6 kg), and molasses (1 kg) to induce the animals to eat the entire diet. A bracken lot containing a known amount of ptaq was offered to the animals in each five-day long run. Dosages of ptaq were increased in three similar consecutive experiments by using bracken lots known to contain larger concentrations of ptaq. Samples from each fern frond lot were analysed for contents of ptaq using the HPLC method reported earlier [2] in order to know the amount of ptaq ingested. This enabled the calculation of dose rates of ptaq within the 6 kg of fern daily intake in the range of 2 400 to 10 000 mg of ptaq/animal/d. Only the morning milking was used for chemical analysis. The average milk production of test animals was 20 ± 2 L·day^{-1}.

In a second trial, the same six cows were given four daily doses of 6 kg/animal/d of bracken fronds containing the lowest ptaq concentration (2 400 mg) by the same means for four days, the treatment was discontinued for three days (72 h) and the diet was again resumed for two additional days. Milk analyses were continued until ptaq dropped below detection limits. None of the cows showed signs of distress or intoxication during treatments, performed normally and did not vary their milk production during the test period. Only the cows that were given the highest dose (approx. 10 000 mg of ptaq/animal/d) rejected the diet after the fourth day but were not affected otherwise. None of the test animals developed any disease symptoms attributable to bracken poisoning, including bovine enzootic haematuria (BEH) during 18 months after treatment. Therefore, bracken regimes in this experiment were all subtoxic and inconsequential to animal health in the mid term.

Milk samples were frozen (–10 °C) immediately after milking. After thawing, proteins were precipitated from 1.0 L milk samples using methanol and acetonitrile and fat extraction was accomplished using hexane and dichloromethane. The remaining aqueous fraction was reduced to 1/3 of its volume by evaporation under vacuum at 36 °C, the pH was adjusted to 11 with dilute sodium hydroxide, then the solution was warmed at 36 °C for 2 h. After this time, the solution was extracted exhaustively with dichloromethane and the organic layer reduced in volume. The passage of the evaporated organic phase through a microcolumn of silica gel secured the quantitative conversion of unstable ptaq and the dienone derived therefrom into pterosin B [2]. HPLC of this material with detection at 260 nm allowed the more sensitive quantitation of pterosin B and therefore, of ptaq present in each milk sample by conversion of the molecular weight, as described earlier [4]. In no case was pterosin B detected as such in milk without previous treatment with alkali. This compound appeared only after conversion of ptaq with dilute sodium hydroxide, and thus represents only its precursor (ptaq). Pteroside B, a pterosin B glycoside also present in bracken, was not converted to pterosin B to any detectable extent under the conditions of this analysis, and thus did not interfere with the results. The recently discovered isoptaquiloside [10], which may also yield pterosin B by alcali-acid treatment, was absent in second stage blades given as feed to the test animals.

The amount of ptaq excreted {$[ptaq]_e$ (mg/animal/d)} in the entire daily milk yield was calculated using the following equation:

$$[ptaq]_e = \frac{[Pte\ (ppm)]}{Yr} \times Df \times \frac{PM(Ptaq)}{PM(Pte)} \times V\ (L)$$

where Pte is the amount of pterosin B indicated by the HPLC detector against a calibration curve drawn with pure pterosin B isolated from bracken fern; Yr is the recovery yield of ptaq from a stan-

dard milk sample (five replicates, Yr = 0.91), Df is the analytical dilution factor, PM (Ptaq) and PM (Pte) are the molecular weights of ptaq and pterosin B, respectively, and V is the volume of milk produced by the test cow in a 24-h period. The reliable limit of detection for quantitation using the present technique was in the order of 0.5 ppm of pterosin B.

3. RESULTS

The first milk sample drawn 14 h after the first bracken feeding did not contain detectable amounts of ptaq. This compound appeared only several hours later, since it was first detected in the 38-h milking. The amount of ptaq excreted daily increased gradually until the fourth day when it reached a plateau (*figure 1*). At this point, the average ptaq being excreted through milk (411 ± 199 mg in 20 L) (± SD) represented 9.6 ± 1.6 % of ptaq ingested 38 h before, for an intake of 4 422 ± 79 mg/animal/d. If the diet was maintained for five days, ptaq continued to be excreted at approx. the same rate as during the dosing period for 48 h after the last treatment. Then it dropped down rapidly to levels below the detection limit 86 h after the end of ingestion. The calculated average of total ptaq excreted $[ptaq]_e$ in milk was 8.60 ± 1.16 % of the total ptaq ingested $[ptaq]_i$ over the whole feeding period of several days.

Later, the cows were fed five daily doses of 6 kg of fresh bracken fronds containing in succession approx. 2 400, 4 500, 8 100 and 10 000 mg of ptaq per 6-kg lot. For each daily dose, $[ptaq]_e$ was compared with $[ptaq]_i$ during the five days and their daily ratio was found to be similar for all ptaq doses (*table I*). The results in this table showed that the ratio of $[ptaq]_e$ over $[ptaq]_i$ for the previous feeding period 38 h before was also independent of the dose ingested. Only those animals exposed to the highest dose began to excrete larger amounts of ptaq by the third day, although there was no statistical significance with the other values. The small SD obtained for each point underscore-

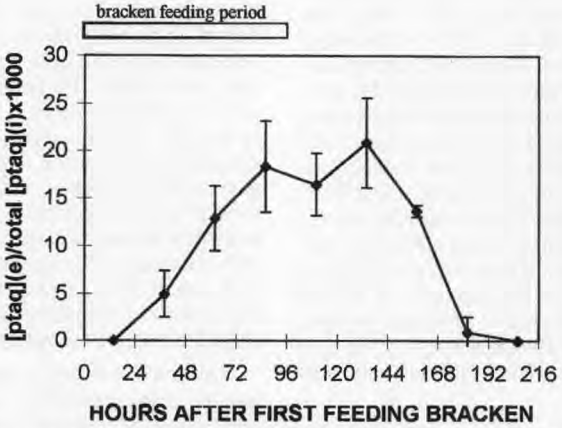

Figure 1. Ratio between ptaquiloside excreted through milk $[ptaq]_e$ daily and total amount ingested $[ptaq]_i$ by cows (N = 6) during the entire feeding period. Five days of treatment with 6.0 kg per day of fresh young bracken fronds containing an average of 4 422 ± 79 mg of ptaquiloside. Error bars in all charts represent SD.

Figure 1. Quotient entre le ptaquiloside excrété par jour dans lait $[ptaq]_e$ et la quantité totale ingérée $[ptaq]_i$ par les vaches (n = 6) pendant toute la période d'alimentation. Cinq jours de traitement, avec 6,0 kg par jour de frondes jeunes et fraîches de fougères qui contiennent une moyenne de 4 422 ± 79 mg de ptaquiloside. Les barres d'erreurs dans tous les graphiques représentent l'écart type.

Table I. Ratio between $[ptaq]_e$ and the daily intake $[ptaq]_i \times 10^2$ taken 48 h before. This timing was taken as reference under the presumption that it takes between 38 and 48 h for ptaquiloside to first appear in milk and thus depends on the dosage of braken eaten by the cow in the previous days.

Tableau I. Quotient entre $[ptaq]_e$ et la quantité journalière $[ptaq]_i$ ($\times 10^2$) ingérée 48 heures auparavant. On a pris cette période de temps comme référence, en supposant qu'il faut entre 38 et 48 heures pour que le ptaquiloside apparaisse dans le lait et qu'il dépend donc de la dose de fougère ingérée par la vache les jours précédents.

Ingested	$[ptaq]_e/[ptaq]_i \times 10^2$ (SD)			
daily dose →	2 400 mg	4 500 mg	8 100 mg	10 000 mg
day 1	0	0	0	0
day 2	3.2 (3.0)	1.4 (1.5)	3.4 (2.5)	2.5 (1.5)
day 3	5.5 (2.5)	4.4 (3.5)	6.1 (3.1)	10.0 (4.0)
day 4	7.6 (4.0)	9.2 (3.2)	9.8 (3.5)	11.1 (4.0)
day 5	9.2 (4.5)	10.7 (3.8)	9.7 (3.0)	7.4 (2.5)

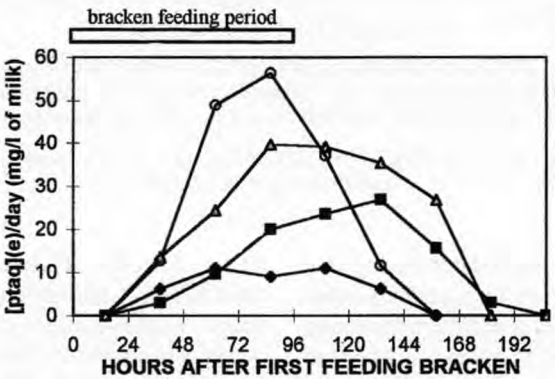

Figure 2. Variation of $[ptaq]_e$ daily with time in relation to ptaquiloside dosage ingested: (◆) 2 400; (■) 4 500; (△) 8 100; and (○) 10 000 mg/animal/d.

Figure 2. Variation quotidienne $[ptaq]_e$, en fonction du temps par rapport à la dose de ptaquiloside ingérée : (◆) 2 400 ; (■) 4 500 ; (△) 8 100 et (○) 10 000 mg/animal/j.

red the fact that the ratio was independent of the dosage. The last bracken feeding occurred 96 h after the beginning of the experiment. Ptaq continued to appear in milk at high doses for 48–62 additional hours as before and then dropped rapidly to disappear after a further 24–48 h period.

The excretion curves at various dosages of ptaq showed similar bell shapes but had different maxima (*figure 2*). At the minimum $[ptaq]_i$ employed, $[ptaq]_e$ decayed shortly after the last dosage (96 h) whereas larger ingested amounts required a longer period of time to be completely excreted from the system. The highest dosed animals (approx. 10 000 mg/animal/d) showed a more rapid decline in excretion (*figure 2*) as the animals rejected the feed after the third day, possibly on account of the effect of prunasin contained in the young fronds. There was also a linear dependency of $[ptaq]_e$ with the dose given (*figure 3*) within

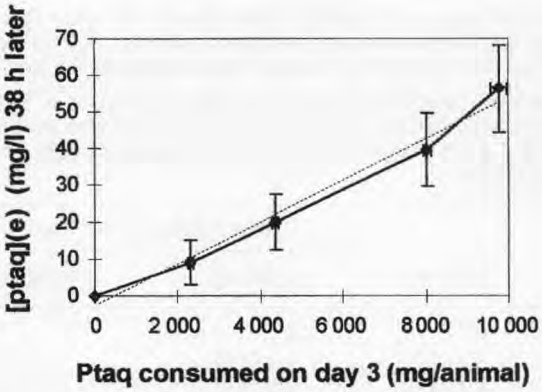

Figure 3. Variation of $[ptaq]_e$ by hour 38 in relation to the total dose of $[ptaq]_i$ within the range 2 400–10 000 mg/animal/d, at a constant bracken frond intake of 6.0 kg/animal/d. Data for 5 animals showing the linear regression line (·····). Regression equation:

$$[ptaq]_e \text{ (mg)} = -2.62(\pm 2.62) + 0.00562(\pm 4.3 \times 10^{-4}) \times [ptaq]_i \text{ (mg)}$$

$$r^2 = 0.9829, F = 171.8, P = 0.001$$

Figure 3. Variation de $[ptaq]_e$ pour l'heure 38 par rapport à la dose totale de $[ptaq]_i$ comprise dans le décalage 2 400-10 000 mg/animal/j, avec une ingestion continuelle de 6,0 kg/animal/j de frondes de fougère. Données pour cinq animaux, représentée par la droite de régression (·····). Équation de régression :

$$[ptaq]_e \text{ (mg)} = -2,62(\pm 2,62) + 0,00562(\pm 4,3 \times 10^{-4}) \times [ptaq]_i \text{ (mg)}$$

$$r^2 = 0,9829, F = 171,8, p = 0,001$$

the 2 400 to 10 000 mg/d range during five consecutive days. Therefore, animals exposed to higher doses of ptaq which are found in many samples of bracken are likely to excrete even more ptaq through their milk.

When bracken feeding covered two consecutive periods of four and two days with a 72-h pause, the bimodal excretion curve of *figure 4* was obtained. The shorter second pulse of $[ptaq]_i$ resulted, not unexpectedly, in a reduced ptaq excretion period comparable to that of the unimodal trial of the lowest ptaq content (2 400 mg/animal/d).

4. DISCUSSION

The neotropical bracken variety used in this series of experiments (*caudatum*) was found to contain from 0.4 to 1.70 mg of ptaq per gram of fresh frond depending on the site of collection. These values were calculated to give 1.18–5.00 $mg \cdot g^{-1}$ of blade biomass – based on an average 66 % moisture contents measured for this stage of growth in our *caudatum* plants. These fall within the ptaq concentration observed in 10 to 20 % of the world bracken samples [49], so our bracken population represented the ptaq contents found in many other places. The fronds collected for feeding the cows were in the second phenological stage, that is blades with the first pinnae unfurled while the rest of the blade still possessed the typical crozier shape. It is known that ptaq concentration decreases rapidly from the crozier to adult stage ([3, 48] inter alia). Ideally therefore, the crozier which contains the highest ptaq concentration should have been used for these experiments. Nonetheless, the croziers of this bracken variety also contain rather high amounts of the cyanogenic glycoside prunasin (Alonso-Amelot and Oliveros, unpublished data), that is known to deter

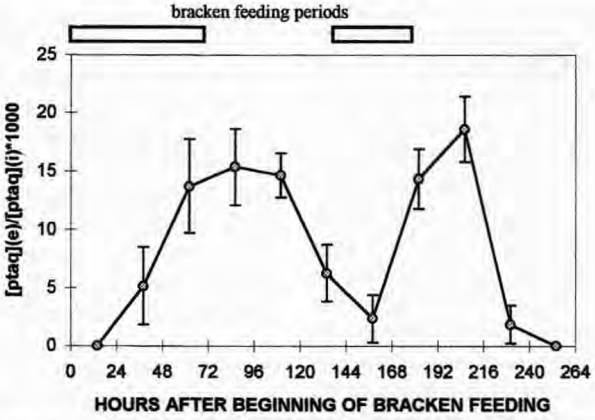

Figure 4. Ratio of $[ptaq]_e$ / $[ptaq]_i$ total over the entire feeding period in a two-pulse sequence. Cows (N = 3) were fed with 6 kg/animal/d of fresh bracken fronds. The last bracken feeding of the first pulse was given at hour 72 and was resumed at hour 144 for 48 additional hours. See bars at the top of the plot.

Figure 4. Quotient $[ptaq]_e$ /$[ptaq]_i$ total sur toute la période d'alimentation dans une séquence de deux pulsations. Les vaches (n = 3) ont été alimentées avec 6,0 kg/animal/j de frondes jeunes et fraîches de fougère. La dernière dose de la première pulsation a été donnée à l'heure 72 et a été recommencée à l'heure 144 pour 48 heures supplémentaires. Voir les barres dans la partie supérieure du graphique.

cattle and sheep herbivory [11, 12, 37], although without any known deleterious effect on animal health at the concentration level found. Cyanogenesis also decreases sharply with frond age [34] along with other secondary metabolites including ptaq [12, 15, 31] and blades become more palatable to bovines from the second stage onwards. A trade-off between these conflicting chemical compositions and acceptability by the test animals was achieved in the second phenological stage of bracken fronds, in which prunasin concentration had decreased to low levels while ptaq content was still significant [3].

There was a delay in the appearance of ptaq in milk (*figure 1*, *table I*), probably resulting from the time required for partial digestion of ruminal material, absorption of ptaquiloside and transport to the milk fluid. After that, ptaq excretion increased progressively to amounts that ranged from 7 to 11 % of the ingested material. However, the ratio $[ptaq]_e$ over $[ptaq]_i$ was independent of the dose offered to the animals, indicating that their system could process still larger quantities of ptaq before showing any detectable physiological stress related to fodder digestibility. This was confirmed by the excellent regression of the dose/excretion correlation of *figure 3* and by the lack of acute intoxication symptoms or BEH-type sequels in any of the test animals after several months. This means that the LD_{50} of ptaq in cows or the dose required to induce acute toxicity by this compound must be considerably larger than 20 ppm which was the largest concentration given to our test animals (10 000 mg to an approx. 500 kg bovid). After bracken feeding was suspended, ptaq continued to be excreted for a period of up to 70 hours. Probably, amassed bracken material in the rumen could potentially act as a slow-release reservoir for ptaq, and thus maintain enough compound in the gut available for absorption.

The bimodal feeding experiment paralleled the observations of the first trial at the lowest ptaq dose in the first feeding pulse – as predicted – with the results shown in

figure 4. The appearance of a second wave of excreted ptaq suggested that cattle exposed irregularly to bracken feeding as would be expected in bracken-infested grazing lands, will yield pulses of ptaq in milk a few hours following the first feeding on the fern and then for a variable period of many hours after the animal ceases eating bracken. The presence of ptaq in milk over such a long period increases the chances of ingestion of ptaq by calves and humans. Although cattle does not favor bracken as feed, they do eat it either during periods of drought when bracken remains green [47], when natural grasses are overgrazed as is frequently the case in developing countries, or when animals stray into dense bracken thickets that exclude other vegetation [45] as it occurs in lands repeatedly affected by fire [1]. These results suggest that the risk to farmers and their families of ingesting milk from areas of bracken dominance is rather high.

For a dietary compound to appear in milk, it has to be incorporated first into the bloodstream and then transferred across the mammary cell membranes into the milk. The glucose portion of ptaq makes it partially water-soluble so it would be expected to be excreted through urine. However, ptaq is also endowed with selective lipophilicity as a consequence of the sesquiterpene portion in its chemical structure. Its teratogenic and mutagenic potential may be derived in part from this attribute since for this compound to act chemically upon nuclear DNA, it must be accrued in the cell yet cross at least three lipophilic barriers: cellular and nuclear membranes, and the likely obstacle raised by chromosomal histone. Therefore, ptaq must be capable of being transported into the mammary cells by a similar expedient.

5. CONCLUSIONS

According to our data, a person drinking about 0.5 L of milk daily from a cow producing 20 L·day^{-1} and which had eaten a subtoxic ptaq dose of 5 000 mg/animal/d (contained in 6–7 kg of fern) for a few days, would ingest between 1.75 and 13.4 mg of ptaq depending on the time between milking and the date when the cow ate bracken. Although no data exists on the toxicology of ptaq in people, the carcinogenic effects of ptaq-contaminated milk, well demonstrated over relatively short periods in laboratory animals [6, 25, 33, 41, 50], allows for a reserved prognosis of its effect on humans. The substantial amounts of ptaq found in milk in the present work should be a cause of concern, especially for farmers consuming milk from herds in bracken infested areas. A much lesser impact would be expected in urban areas where raw milk possibly laden with ptaq is first pooled with ptaq-free milk with the consequential dilution effect.

These results make us believe that the postulated connection between bracken infestation and cancer of the upper gastrointestinal tract in farming populations inhabiting cattle range areas in Costa Rica [52] and other territories where bracken growth is dense such as Central America, northern South America [42], Brazil [14] and elsewhere, is likely to be through the consumption of locally produced milk. Similarly, ptaq-laden milk may have been the link between bracken thickets and gastric cancer believed to occur by other mechanisms such as well water and spore dispersal in some parts of England [22]. The ecological implications of ptaq acting through the multitrophic interaction fern/adult bovine/calf by way of milk are also intriguing. It may be a mechanism of chemical defence evolved in bracken to reduce herbivory pressure by large mammals.

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