To the Editor-in-Chief

Sir,

Mass spectrometric analysis of ptaquiloside, the toxic sesquiterpene from bracken fern

Ptaquiloside (Pta, I) is a potent carcinogen, first isolated from a variety of bracken fern (Pteridium aquilinum, var. latiusculum); its structure was elucidated in 1987.1 The same substance and several analogues have been identified in other Pteridacae.2 Bracken fern is currently consumed by farm animals, and this causes a number of well-known syndromes in domestic animals.3 In large ruminants, chronic enzootic haematuria, the clinical expression of multiple neoplasia of the urinary bladder, occurs. The occurrence of this pathology has been associated with the geographic distribution of bracken fern, which is a plant widely distributed all over the world, and with its content of Pta.5 Pta has been shown in laboratory experiments to be carcinogenic,6 and it is co-responsible for tumors of urinary bladder in cow; it seems to act synergistically with bovine papilloma virus type 2 (BPV-2), that is a well-known virus associated with neoplastic pathology of the bovine urinary bladder.7 The carcinogenic properties of Pta have been related to its electrophilic nature, and its capability to react with (alkylate)8 DNA.

The growing public awareness of the risk implied in continuous exposure to carcinogenic substances present in foods has prompted some researchers to evaluate the possibility that Pta may represent a pollutant of foods derived from cattle.9 This work has also demonstrated a significant increase in gastric cancers (x2.34) in humans who spend their childhood in bracken-infested areas,10 and milk has been proposed as the carrier. Indeed, Pta has been recently detected in milk of cows growing in infested areas.11 Pta (I), a glucoside, is unstable in both acidic and basic conditions; in basic conditions, Pta undergoes a \( \beta \)-elimination, affording the dienone II, which is converted by acids into pterosine B (Ptb, III).12 Analyses of Pta are difficult because of this instability. This difficulty has been overcome by converting Pta into the stable Ptb during sample preparation. Detection and quantitative determinations of this product (an aromatic ketone) are currently achieved using high-performance liquid chromatography (HPLC)13 with UV detection. To our knowledge, no methods that directly detect Pta have been published.

Analytical problem by using mass spectrometry; results of a preliminary study are the subject of the present paper.

Gas chromatographic (GC) analyses were performed using an HP 5890 A instrument (Agilent) equipped with a DB1 column (15 m). Injector and detector temperatures were 250°C. All analyses used a temperature program from 100–250°C, at 15°C/min. GC/MS analyses were performed using an HP 5970 quadrupole mass spectrometer (Agilent) coupled with an HP5890 gas chromatograph; an HP5 MS column (30 m) was used with a temperature program from 120–290°C at 10°C/min. Injector and ion source temperatures were 250°C. EI spectra (70 eV) were acquired for the range \( m/z \) 40–550 at 1.2 scan/s using unit-mass resolution.

Extraction of Pteridium aquilinum was achieved as follows: 200 g of fresh plant were cut into small pieces (discarding the branches) and milled in a blender with 400 mL of water. The mixture was centrifuged at 2500 rpm for 10 min and the supernatant decanted. The aqueous extract of the plant (pH 7–7.1) can be lyophilized and the residue stored at \(-30 \)°C for several months. From 200 g of fresh plant the yield of dry extract was 6 g. This lyophilized extract was used for further experiments.

Pterosine B (Ptb, III) was prepared as follows: to the aqueous solution (400–500 mL) obtained from 200 g of fresh plant, 1 M NaOH (ca. 4.5 mL) was added (pH > 11). The solution was heated at 38–40°C for 1 h, then acidified with 5 M HCl to pH < 2. After

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10 min, the solution was extracted with diethyl ether three times. The usual work-up of the extract afforded 180 mg of residue which was chromatographed on silica gel (20 g). The column was eluted with a gradient of hexane/ethyl acetate, and collection and evaporation of fractions eluting at a hexane/ethyl acetate ratio of 7:3 yielded a substance which, on purification by thin-layer chromatography (TLC), yielded 40 mg of a product (Ptb) which crystallized on standing.

Bromopterosine (VI) was prepared as follows: to 1 mL of a solution containing 100 mg of the lyophilized extract, 600 mg of NaBr were added. The solution was basified with 75 µL of 1 M NaOH and heated at 37–40°C for 1 h. The solution was then acidified with 5 N sulfuric acid and extracted with diethyl ether. The residue was analyzed by GC and GC/MS.

Methoxypterosine (VII) was prepared as follows: to 1 mL of a solution containing 100 mg of lyophilized extract, 2.5 mL of methanol were added. The solution was basified with NaOH (1 M in water/methanol solution), heated at 38–40°C for 1 h, acidified with 5 N sulfuric acid (water/methanol solution), and extracted with diethyl ether. The residue was analyzed by GC and GC/MS.

In order to have a reference sample we prepared Ptb (III) directly from a natural source. Separation and purification of Pta are no simple tasks, as shown by the several procedures proposed to prepare this compound.14 Plants (bracken fern, Pteridium aquilinum, var. latiusculum) were collected in Campania, in areas where related diseases of cattle are currently observed. After some preliminary experiments a very fast procedure of extraction was found, suitable for small amounts of sample (see above). The isolated Ptb showed an NMR spectrum identical to that described previously.1

We then repeated the method described for the HPLC analysis of plants.15 The mixture obtained was extracted with diethyl ether, the residue derivatized with TFA, and analyzed by GC/MS. The spectrum of Ptb trifluoroacetate (IV) is shown in Fig. 1; it includes significant peaks at m/z 217 (\(\text{M} - \text{CF}_3\text{CO}_2\text{H}\)) arising from benzylic cleavage of the side chain). This spectrum was also compared with that obtained from an authentic sample of Ptb, converted into the trifluoroacetate ester. Surprisingly, the resulting mixture contained another related product (V; Fig. 2, abundant ions at m/z 201 and 187), containing chlorine. There is little doubt that the HCl used to acidify the mixture does activate the expected ring opening, but the chloride ion also acts as nucleophile to give the chloro derivative in a significant yield (ratio of peak areas are roughly 3:1).

We thought it might be possible to take advantage of the property of the dienone to react with different species at different rates depending on the nucleophilicity of the reagent. Thus we performed several experiments to convert Pta into ring-opening products with different reactant media; the aqueous extract of the plants was used for these experiments. With sodium bromide, when its concentration in the aqueous solution was higher than 35% (w/w), the bromo derivative VI was obtained as the main product instead of Ptb; GC analysis showed an area peak ratio larger than 6 in favor of VI. HCl was no longer used and sulfuric acid was used instead. The mass spectrum of compound VI was obtained by GC/MS (Fig. 3).

In the same way, when the medium of the reaction was a water/methanol mixture (1:2 v/v), the methoxy derivative VII was obtained as the main ring-opening product, as shown by GC (ratio between peak areas 5:1). The mass spectrum of VII, recorded using GC/MS, is reported in Fig. 4.

It is generally accepted that the most sensitive and general analytical

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Figure 1. Mass spectrum for Ptb trifluoroacetate (IV) obtained by GC/MS.

Figure 2. Mass spectrum, obtained by GC/MS, of the chlorinated compound V found in the reaction mixture of Ptb with HCl.
method to detect organic compounds in complex mixtures are those based on mass spectrometry. HPLC with UV detection is useful for reliable quantitative determination of known analytes present in simple matrices. However, when analytes are present as trace components in complex matrices, and the main objective is to detect the presence and obtain an estimate of the amount, methods like GC/MS or LC/MS are the methods of choice. Therefore, it is surprising that, even though there is a large interest in Pta, to the best of our knowledge no MS methods have been described. Moreover, analysis of Pta via a transformation product (Ptb) is not entirely satisfactory, as discussed below.

EI spectra of pterosine B and related substances exhibit abundant ions at high m/z and their use in single-ion monitoring (SIM) for purposes of detection appears advantageous. The GC/MS data are summarized in Table 1. Benzylic cleavage of the side chain is an important process that gives rise to the ion at m/z 187 which is always abundant in all spectra of related compounds. The loss of the substituent carried by the side chain affords the ion at m/z 201. This ion is present and abundant only if the substituent can be released as a low-energy (stabilized) radical. In this respect the comparison between the abundances of ions at m/z 201 and 187 in the spectra of the chloro and bromo derivatives is significant. When this fragmentation channel is unfavorable, the loss of the methyl group becomes important, as in the cases of Ptb or VII. For purposes of comparison, we obtained also an ESI-MS/MS spectrum of Ptb in a Q-TOF instrument, in positive ion mode. Here the most intense peak is due to the loss of water from the MH⁺ ion; ions at m/z 187 or 188 are not present.

The high toxicity of Pta has been reasonably related to its electrophilic properties, which are no longer present in Ptb, and this same property has been exploited to design the analytical method to detect Ptb as representative of Pta. However, since the transformation of Pta into Ptb may also occur in body fluids and/or in living organisms, the identification of the latter in foods does not guarantee, at least in principle, the presence of Pta in the matrix in question. It is possible, however, to object that the analytical procedures are based on an initial cleanup or extraction of lipophilic substances including the Ptb. The validity of this objection is limited, especially in the case that traces (ppb or less) are to be detected; actually the possibility that the Ptb is the lone or the dominant pollutant present is significant.

The preparation of the described compounds from Pta overcomes the difficulty outlined above. Since the ring-opening occurs only for Pta or the dienone II, products V and VI are much more representative of Pta than is Ptb, taking into consideration also that the adverse properties of Pta are related to its electrophilic character, i.e. the presence of a three-membered ring. It is also worth mentioning that the chemistry of both the dienone II and ptaquiloside, particularly the possibility to convert them into ring-opened products differing from Ptb, is well known and has been studied also for

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**Table 1. Summary of GC/MS data for compounds III–VII**

<table>
<thead>
<tr>
<th>Compound</th>
<th>GC (min)</th>
<th>GC/MS (min)</th>
<th>m/z (abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pterosine B (III)</td>
<td>7.89</td>
<td>12.66</td>
<td>218(42); 203(85); 187(100)</td>
</tr>
<tr>
<td>Pterosine B-TFA (IV)</td>
<td>—</td>
<td>11.68</td>
<td>314(54); 201(100); 69(48)</td>
</tr>
<tr>
<td>Chloropterosine (V)</td>
<td>7.66</td>
<td>12.21</td>
<td>236(33); 201(100); 187(66)</td>
</tr>
<tr>
<td>Bromopterosine (VI)</td>
<td>8.94</td>
<td>13.18</td>
<td>280-2(25); 201(100); 187(32)</td>
</tr>
<tr>
<td>Methoxypterosine (VII)</td>
<td>8.95</td>
<td>11.69</td>
<td>232 (31); 217(47); 187(100)</td>
</tr>
</tbody>
</table>
very recently described analogues of Pta;\(^1\) however, this property has never to our knowledge been exploited to design analytical methods.

The transformation reactions of Pta described here open several new analytical possibilities to detect this pollutant. Products VI and VII are easily analyzed by GC, and sensitivity in GC/MS operating in SIM mode may be at the ppb level or better. In particular, in the bromo derivative VI, the \(m/z\) values of the important ions are shifted to high values and this leads to a reduction of the 'chemical noise' associated with complex matrices. Moreover, there is also the possibility to detect this compound with NICI (negative ion chemical ionization) and, since the production of bromo derivatives under the conditions described here is highly selective, a further increase in the signal-to-noise ratio can be anticipated.

It is also worth noting that even simple GC methods with ECD (electron capture detector) could give good results with the bromo derivative.

Other structurally related substances (for instance paquituloside Z)\(^1\) could be easily detected in plants by the same approach since they presumably have similar chemical properties, i.e. they can be converted into bromo derivatives. Analytical methods based on the approaches presented here are now under study, and will be described in due course. Experiments designed with the objective of detection of the Pta are in progress.

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REFERENCES


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