JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Norsesquiterpene Glycosides in Bracken Ferns (*Pteridium esculentum* and *Pteridium aquilinum* subsp. *wightianum*) from Eastern Australia: Reassessed Poisoning Risk to Animals

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ABSTRACT: Austral bracken *Pteridium esculentum* contains three unstable norsesquiterpene glycosides: ptaquiloside, ptesculentoside, and caudatoside, in variable proportions. The concentration of each of the glycosides was determined in this study as their respective degradation products, pterosin B, pterosin G and pterosin A, by HPLC-UV analysis. Samples of *P. esculentum* collected from six sites in eastern Australia contained up to 17 mg of total glycoside/g DW, with both ptaquiloside and ptesculentoside present as major components accompanied by smaller amounts of caudatoside. Ratios of ptaquiloside to ptesculentoside varied from 1:3 to 4:3, but in all Australian samples ptesculentoside was a significant component. This profile differed substantially from that of *P. esculentum* from New Zealand, which contained only small amounts of both ptesculentoside and caudatoside, with ptaquiloside as the dominant component. A similar profile with ptaquiloside as the dominant glycoside was obtained for *Pteridium aquilinum* subsp. *wightianum* (previously *P. revolutum*) from northern Queensland and also *P. aquilinum* from European sources. Ptesculentoside has chemical reactivity similar to that of ptaquiloside and presumably biological activity similar to that of this potent carcinogen. The presence of this additional reactive glycoside in Australian *P. esculentum* implies greater toxicity for consuming animals than previously estimated from ptaquiloside content alone.

KEYWORDS: ptaquiloside, ptesculentoside, caudatoside, bracken, *Pteridium esculentum*, *Pteridium aquilinum*,

INTRODUCTION

Austral bracken, *Pteridium esculentum* (G. Forst.) Cockayne, occurs in Australia, New Zealand, and New Caledonia and forms part of the Austral/South American clade of the ubiquitous genus *Pteridium* (bracken ferns).[1] In Australia this species occurs widely in nonarid regions, across diverse habitats from coastal sand dunes to elevated inland regions,[2] and has been associated with livestock poisoning causing bone marrow damage leading to a fatal hemorrhagic disease of cattle ("bracken" poisoning), urinary bladder neoplasia of cattle (bovine enzootic hemeaturia), and "bracken staggers" of horses (an effect of thiaminase).[3,4] As with other members of the genus *Pteridium*, these cattle poisonings have been attributed to the presence of ptaquiloside, **1a** (Figure 1), a reactive norsesquiterpenoid glucoside, which readily undergoes glucose elimination to form an unstable conjugated dieneone intermediate capable of alkylating amino acids and DNA.[5]

We recently reported the isolation of the previously unknown hydroxyptaquiloside analogue ptesculentoside, **1b**, from Australian *P. esculentum*, together with similar amounts of ptaquiloside, **1a**, and lesser amounts of caudatoside, **1c**.[6] Before this phytochemical investigation, chemical studies of *P. esculentum* from both Australia and New Zealand were limited to surveys of ptaquiloside, **1a**, content by HPLC-UV analysis either directly or as the more stable decomposition product pterosin B, **2a**.[7−11] Interestingly, comparison of published HPLC-UV traces from New Zealand *P. esculentum*[8] with HPLC-UV traces generated from our Australian *P. esculentum* demonstrated an apparent absence of ptesculentoside, **1b**, and its elimination product pterosin G, **2b**, in the New Zealand bracken extracts.

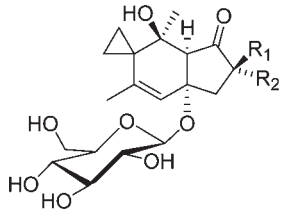
In the present investigation we developed an analytical method to determine the ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, content of *P. esculentum* from various locations in eastern Australia and, for comparison, that of imported New Zealand samples of the same species.

MATERIALS AND METHODS

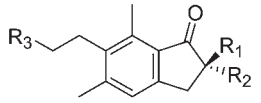
Plant Material for Analysis. Recently emerged furled fronds of *P. esculentum* were collected at two sites in northeastern New South Wales (NE NSW) and four sites in southeastern Queensland (SE QLD), eastern Australia, and from each collection site a separate pressed mature plant sample was submitted to Queensland Herbarium for species identification and retention as vouchers. Recently emerged furled fronds of *Pteridium aquilinum* subsp. *wightianum* were similarly collected at two sites near Malanda in NE QLD. Crozier samples for analysis were generally a composite of 10−15 plants collected at a single site and were dried after collection (48 h at 60 °C or similar) and then milled (1 mm screen) to give a fluffy green powder. *P. esculentum* from New Zealand was kindly provided by Denis Lauren (Plant and Food Research, Hamilton, New Zealand) and Lars Rasmussen (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark). These samples had been stored frozen at Plant and Food Research (New Zealand) for approximately 4 years and were heat treated at 85 °C for 8 h by Australian Quarantine and Inspection Service (AQIS) during importation into Australia (AQIS QM05001140).

Plant Extraction. Dried and milled plant material (1 g) was extracted with reverse osmosis (RO) water (25 mL) (Millipore, 18.2 MΩ) by mechanical shaking for 1 h at room temperature. The extract

Received: November 3, 2010
Revised: April 1, 2011
Accepted: April 4, 2011
Published: April 04, 2011



1a: R_1 = H; R_2 = CH_3
1b: R_1 = H; R_2 = CH_2OH
1c: R_1 = CH_3; R_2 = CH_2OH



2a: R_1 = H; R_2 = CH_3; R_3 = OH
2b: R_1 = H; R_2 = CH_2OH; R_3 = OH
2c: R_1 = CH_3; R_2 = CH_2OH; R_3 = OH

3a: R_1 = H; R_2 = CH_3; R_3 = Cl
3b: R_1 = H; R_2 = CH_2OH; R_3 = Cl
3c: R_1 = CH_3; R_2 = CH_2OH; R_3 = Cl

Figure 1. Structures of norsesquiterpenoid glycosides and pterosin elimination products.

was filtered, and a portion of the filtrate (4 mL) was applied to a Fluka polyamide 6 column (0.45 g) (Sigma-Aldrich, Sydney, Australia) loosely packed into a glass wool stoppered Pasteur pipet. The eluent could be analyzed directly for ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, by HPLC-UV, but quantitation was performed by conversion to the more stable pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c**.

Pterosin Conversion. A portion of the polyamide 6 column eluent (1 mL) was treated with 1 M NaOH (75 μL), vortexed, and incubated at 40 °C for 1 h. After cooling to room temperature, the solution was treated with 5 N H_2SO_4 (75 μL), vortexed, and filtered for HPLC analysis of pterosins, **2a**−**c**. This solution could also be extracted into dichloromethane (1 mL) for GC-MS analysis.

A similar procedure using 5 M HCl (75 μL)[8] was initially trialed, but found to produce unwanted chloro-analogues, **3a**−**c**.

***P. aquilinum* Extracts.** Extacts of *P. aquilinum* from the Isle of Mull (Scotland), Østbirk Sø (Denmark), and San Miguel Island (Azores) that had undergone similar treatment and pterosin conversion (with HCl[8]) were provided by Lars Rasmussen (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark).

HPLC-UV Analysis. HPLC separations were performed using a 250 mm × 4.6 mm i.d, 5 μm, Luna C18 column (Phenomenex, Sydney, Australia) at 35 °C with a flow rate of 1 mL/min and an injection volume of 10 μL. The mobile phase was a mixture of (A) 40:60 CH_3CN in water (v/v) and (B) CH_3CN with a gradient as follows: 0−9 min, held at 100% A; 9−11 min, 100% A−100% B; 11−16 min, held at 100% B; 16−16.5 min, 100% B−100% A; 16.5−25 min, held at 100% A. Detection of individual components was by photodiode array or UV−vis at 220 nm with the following retention times: ptesculentoside, **1b**, 2.8 min; caudatoside, **1c**, 2.9 min; ptaquiloside, **1a**, 3.2 min; and pterosin conversion products pterosin G, **2b**, 3.7 min; pterosin A, **2c**, 4.8 min; and pterosin B, **2a**, 11.1 min. Quantitation was by peak area of pterosins. Chloro-analogues eluted in similar order after 14 min and were not quantitated: pterosin G chloroanalogue, **3b**, pterosin K, **3c**, and pterosin F, **3a**.

Reference Standards. Ptaquiloside and pterosin were provided by Lars Rasmussen (The Royal Veterinary and Agricultural University, Frederiksberg, Denmark) and also isolated from *P. esculentum*[6] (approximately 95% pure by NMR). Standard solutions for HPLC analysis were prepared by conversion of a 0.03 mg/mL solution of ptaquiloside, **1a**, to

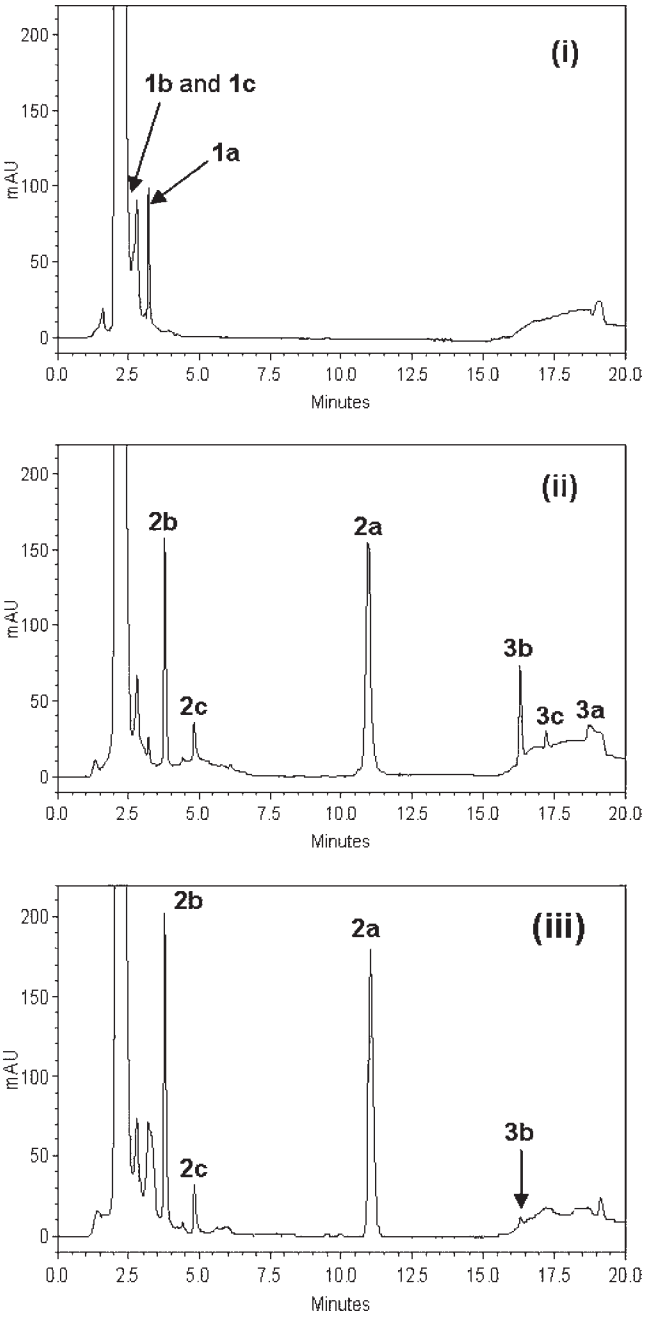


Figure 2. HPLC-UV chromatograms of a *Pteridium esculentum* extract after (i) polyamide cleanup, (ii) pterosin conversion with NaOH−HCl, and (iii) pterosin conversion with NaOH−H_2SO_4.

pterosin, **2a**, as described above. The concentration was calculated on the basis of 100% conversion of ptaquiloside, **1a**, to pterosin B, **2a**, and expressed as ptaquiloside equivalents.

Ptesculentoside, **1b**, isolated from *P. esculentum*[6] (>95% pure by NMR) was dissolved in water (0.06 mg/mL) and converted to pterosin G, **2b**, as described above. The resultant solution was analyzed by HPLC-UV analysis and quantitated in comparison with equivalent ptaquiloside-derived pterosin B solution. The relative absorbance (in glycoside equivalents) was calculated on the basis of 100% conversion of ptesculentoside, **1b**, to pterosin G, **2b**.

Quantitation of ptaquiloside, **1a**, in sample extracts was by direct comparison of pterosin B product absorbance with this standard.

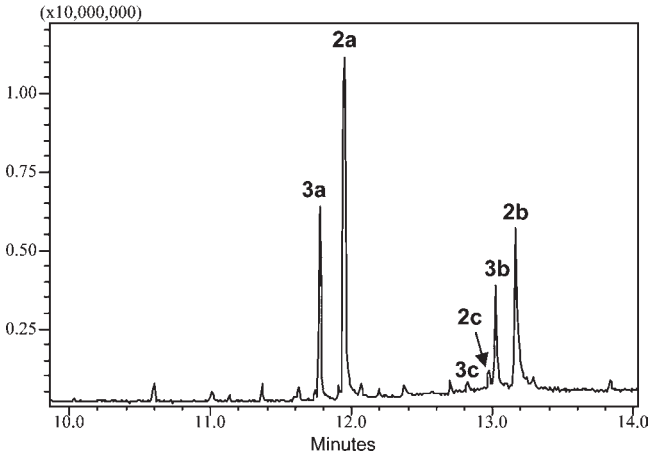


Figure 3. GC-MS chromatogram of a dichloromethane extract of pterosins from *Pteridium esculentum* after pterosin conversion with NaOH−HCl.

Quantitation of ptesculentoside, **1b**, and caudatoside, **1c**, in sample extracts was performed by comparison of measured absorbance of pterosin G, **2b**, and pterosin A, **2c**, with ptaquiloside-derived pterosin B standard and allowing for relative absorbance factors of 0.73 and 0.87 (in glycoside equivalents), respectively, for these pterosins with respect to pterosin B, **2a**.

GC-MS Analysis. GC-MS analysis was carried out on a Shimadzu GC-17A and QP5050 MS instrument equipped with a 30 m × 0.25 mm i.d. DB-5ms column (J&W Agilent Technologies, Melbourne, Australia). Analysis conditions were as follows: injector, 300 °C; temperature program, from 50 °C (2 min) at 20 °C/min to 300 °C; carrier gas, He; column flow, 1.5 mL/min; interface, 300 °C; and electron impact ionization, 70 eV.

RESULTS AND DISCUSSION

Bracken Analysis Method. A simple method to determine ptaquiloside, **1a**, in bracken fern has previously been described by Agnew and Lauren,[8] and this method was readily adapted to determine ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, as their respective degradation products pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c**. The more polar glycosides ptesculentoside, **1b**, and caudatoside, **1c**, eluted with HPLC retention times similar to those of other bracken extract components (Figure 2) and were not completely resolved even when eluted under less polar conditions. Direct HPLC quantitation of all glycosides in *P. esculentum* is thus difficult, and glycosides **1a**−**c** have been quantitated as pterosin products in this study.

The glycosides **1a**−**c** were extracted from bracken samples by aqueous extraction followed by cleanup through a polyamide column to remove native pterosins. Agnew and Lauren[8] employed a NaOH−HCl conversion of ptaquiloside, **1a**, to pterosin B, **2a**, but we found that treatment of our extract of glycosides **1a**−**c** by this method produced pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c**, together with substantial amounts of the chloro-analogues **3a**−**c**, detectable by HPLC-UV analysis (Figure 2). GC-MS analysis of dichloromethane extracts confirmed the presence of all six pterosins **2a**−**c** and **3a**−**c** (Figure 3). The identity of pterosin products was confirmed by comparison with literature mass spectra for pterosin B, **2a**,[12] and pterosin A, **2c**,[13,14] and for chlorides pterosin F, **3a**,[12] pterosin G chloro-analogue, **3b**,[15] and pterosin K, **3c**.[13,14] No literature mass spectrum was available for pterosin G, **2b**, but the mass spectroscopy data reported here for **2b** closely parallel those of the related pterosins with major ions at m/z 234, 219, 201, 185, and 173 consistently 14 Da lower than comparable ions in pterosin A, **2c** (m/z 248, 233, 215, 199, and 187), which contains an additional methyl group at C-2. Literature mass spectroscopy data for the pterosins **2a**, **2c**, and **3a**−**c** are largely incomplete, and mass spectra for all six pterosins are reported here in retention order (Table 1).

To overcome the formation of unwanted chloride byproduct, HCl in the literature procedure[8] was replaced by H_2SO_4, and conversion of *P. esculentum* extracts with NaOH−H_2SO_4 provided pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c**, with only small amounts of the corresponding chlorides **3a**−**c** (Figure 2). These trace chloro-analogues were presumably derived from chloride ions present naturally in the plant sample.

Concentrations of individual glycosides **1a**−**c** were determined by comparison of HPLC-UV absorbance of pterosin products **2a**−**c** at λ_{max} 220 nm with the absorbance of pterosin B, **2a**, derived from standard solutions of ptaquiloside, **1a**. Concentrations of ptaquiloside were calculated on the basis of 100% conversion of ptaquiloside, **1a**, to pterosin, **2a**, and expressed as ptaquiloside equivalents.[8] Pterosins **2a**−**c** all contain similar UV chromophores and in the absence of appropriate standards ptesculentoside, **2b**, and caudatoside, **2c**, were initially quantitated as ptaquiloside equivalents by comparison with ptaquiloside-derived pterosin B standard.

Pterosins **2a**−**c** all have very similar and distinctive UV spectra with λ_{max} at approximately 220, 260, and 305 nm. The log molar absorptivities (log ε) of pterosins **2a**−**c** have previously been determined,[16] and these values were used to determine mass attenuation coefficients relative to each glycoside parent concentration (based on 100% conversion of glycoside to pterosin) (Table 2). In this manner, relative absorbance factors of 0.70 and 0.87 (in glycoside equivalents) were determined for these pterosins, **2b** and **2c**, with respect to pterosin B, **2a** (as ptaquiloside equivalents).

Standard ptesculentoside, **1b**, was also available,[6] and the relative absorbance of the pterosin product **2b** derived from a 0.06 mg/mL solution of **1b** was determined by HPLC-UV comparison with standard ptaquiloside, **1a** (0.05 mg/mL), converted to pterosin B, **2a**. This experimental comparison provided a relative absorbance factor for pterosin G, **2b**, of 0.73 (in glycoside equivalents) with respect to pterosin B, **2a** (as ptaquiloside equivalents), in good agreement with the 0.70 value derived in Table 2. Insufficient pure caudatoside, **1c**, was available to perform a comparable analysis and calculation, and it was necessary to employ the relative absorbance factor derived in Table 2 for this compound. Thus, absorbance factors of 0.73 and 0.87 relative to pterosin B were used to convert measured absorbances for pterosins **2b** and **2c** to ptesculentoside, **1b**, and caudatoside, **1c**, equivalent concentrations (assuming 100% conversion of glycoside to pterosin).

Glycoside Content of Bracken Specimens. HPLC-UV analysis showed the presence of glycosides **1a**−**c** in all samples of *P. esculentum* and *P. aquilinum* subsp. *wightianum* sourced from eastern Australia, and the glycoside composition of each sample determined by quantitation of pterosin products is presented in Table 3. Samples of *P. esculentum* collected from NE NSW and SE QLD all contained ptaquiloside, **1a**, and ptesculentoside, **1b**, as major components with lesser amounts of caudatoside, **1c**. There was considerable variation in both relative glycoside composition and in total glycoside content, with the greatest

Table 1. GC-MS Data for Pterosins 2a−c and 3a−c with Rentention Indices (RI) on a DB-5 ms Column

compound	t_R (min)	RI	*m/z*
pterosin F (**3a**)	11.8	1989	238/236 (10/35, M^+), 202 (16), 201 (100), 188 (10), 187 (68), 159 (14), 141 (9), 129 (21), 128 (24), 115 (18), 91 (13), 77 (10)
pterosin B (**2a**)	12.0	2024	218 (32, M^+), 204 (11), 203 (76), 188 (17), 187 (100), 175 (44), 159 (17), 144 (15), 143 (11), 141 (11), 131 (11), 129 (32), 128 (30), 115 (24), 91 (18), 77 (11)
pterosin K (**3c**)	12.8	2210	268/266 (13/44, M^+), 237 (40), 236 (40), 235 (100), 213 (65), 199 (39), 186 (22), 185 (42), 173 (56), 171 (27), 157 (22), 143 (22), 141 (35), 129 (33), 128 (39), 115 (49), 91 (37)
pterosin A (**2c**)	13.0	2244	248 (63, M^+), 233 (52), 218 (49), 217 (100), 215 (51), 199 (51), 189 (40), 187 (64), 185 (38), 173 (63), 171 (62), 159 (50), 157 (38), 143 (38), 142 (33), 130 (34), 129 (54), 128 (89), 115 (59), 81 (41), 67 (44)
pterosin G chloro-analogue (**3b**)	13.0	2254	254/252 (13/39, M^+), 234 (20), 221 (25), 217 (16), 200 (15), 199 (100), 185 (43), 171 (22), 159 (22), 157 (21), 142 (27), 141 (31), 129 (32), 128 (39), 115 (32), 91 (19)
pterosin G (**2b**)	13.2	2288	234 (45, M^+), 219 (79), 203 (27), 201 (80), 191 (41), 186 (28), 185 (100), 173 (99), 170 (29), 157 (44), 145 (29), 143 (26), 142 (58), 141 (55), 129 (55), 128 (65), 127 (25), 115 (62), 91 (38), 77 (25), 55 (25)

Table 2. Molar Absorptivity of Pterosins 2a−c in Ethanol at λ_{max} 217 nm and Calculation of Relative Absorbance (in Glycoside Equivalents)

	log ε^{16}	molar extinction coefficient (ε) (abs/mol/L)	MW parent glycoside	mass attenuation coefficient (abs/g/L) (glycoside equiv)	relative absorbance (glycoside equiv)
2a	4.57	37154	398	93.4	1
2b	4.43	26915	414	65.0	0.70
2c	4.54	34674	428	81.0	0.87

Table 3. Norsesquiterpenoid Glycoside Content of Uncurling Heads of Australian *Pteridium* Taxa Collected in this Study

Queensland herbarium voucher no.	source location	species	ptaquiloside, **1a** (mg/g DW)	ptesculentoside, **1b** (mg/g DW)	caudatoside, **1c** (mg/g DW)	total glycoside (mg/g DW)
AQ610813	Bribie Island, SE QLD	*P. esculentum*	2.88	5.77	1.49	10.13
AQ840152	Cabarita, NE NSW	*P. esculentum*	4.68	7.83	1.09	13.61
AQ744800	Casino, NE NSW	*P. esculentum*	5.42	10.62	1.03	17.06
AQ840154	(Oxley) Brisbane, SE QLD	*P. esculentum*	4.12	3.47	0.54	8.12
AQ840299	(Eight Mile Plains) Brisbane, SE QLD	*P. esculentum*	1.00	0.79	0.10	1.88
AQ744801	Conondale, SE QLD, sample A	*P. esculentum*	1.97	2.40	0.42	4.79
AQ744801	Conondale, SE QLD, sample B	*P. esculentum*	1.78	2.09	0.31	4.17
AQ744801	Conondale, SE QLD, sample C	*P. esculentum*	2.50	1.86	0.34	4.70
AQ744801	Conondale, SE QLD, sample D	*P. esculentum*	1.44	4.40	0.47	6.31
AQ744801	Conondale, SE QLD, sample E	*P. esculentum*	1.61	3.02	0.33	4.96
AQ773335	Malanda, NE QLD (site 1)	*P. aquilinum* subsp. *wightianum*	12.45	0.35	1.45	14.24
AQ840298	Malanda, NE QLD (site 2)	*P. aquilinum* subsp. *wightianum*	4.49	0.74	0.38	5.61

glycoside content (17.06 mg of glycoside/g DW) being determined in a sample of very young croziers from a roadside location near Casino (NE NSW).

There was considerable variation in the proportion of ptaquiloside (**1a**)/ptesculentoside (**1b**) present in individual samples, and this variation was not clearly linked to plant tissue maturity alone. For example, analysis of five individual croziers collected from patches of bracken over about 1 ha, with all samples located <200 m apart at the same Conondale location (samples A−E), had similar total glycoside contents (4.17−6.31 mg of glycoside/g DW), but with the ptaquiloside (**1a**)/ptesculentoside (**1b**) content ratio varying from 1:3 to 4:3. These samples were collected within a relatively small area with extensive (although patchy) bracken coverage and would be expected to represent a single clone of the plant. The observed variation in the ptaquiloside (**1a**)/ptesculentoside (**1b**) ratio might be affected by stage of growth, localized growing conditions (shade, moisture, soil pH, nutrients, etc.), predation by herbivores, or even competition for resources with other plants.

Samples of *P. esculentum* from Taumaranui, New Zealand, were obtained for comparison, and HPLC-UV analysis of pterosin products demonstrated considerably smaller ptesculentoside, **1b**, content with ptaquiloside (**1a**)/ptesculentoside (**1b**) ratios greater than 38:1 in all samples (Table 4). HPLC-UV traces for these New

Table 4. Norsesquiterpenoid Glycoside Content of *Pteridium* Species from International Locations

source location	species	ptaquiloside, **1a** (mg/g DW)	ptesculentoside, **1b** (mg/g DW)	caudatoside, **1c** (mg/g DW)
Isle of Mull, Scotland	*P. aquilinum*	0.48	<LOD	0.04
Østbirk Sø, Denmark	*P. aquilinum*	2.88	0.30	0.30
Sao Miguel Island, Azores	*P. aquilinum*	1.03	<LOD	0.21
Taumaranui, New Zealand (sample II)[a]	*P. esculentum*	1.19	0.03	0.03
Taumaranui, New Zealand (sample IV)[a]	*P. esculentum*	0.54	<LOD	0.02
Taumaranui, New Zealand (sample VII)[a]	*P. esculentum*	0.61	0.02	0.03

[a] Concentrations of ptaquiloside in these samples were less than originally reported, apparently affected by storage and heat treatment.

Zealand samples were consistent with those previously published by Agnew and Lauren.[8] *P. esculentum* in New Zealand is considered to be morphologically different from the same species in Australia (personal communication, John Thomson), with New Zealand fronds reported to achieve heights of 3−4 m or, exceptionally, 9 m,[17] far in excess of that seen in Australia. Our chemical analysis results suggest that more than one chemotype exists within the species *P. esculentum*, and more extensive phytochemical surveys are required to establish the distribution of these chemotypes across the entire species range[1] and determine whether these chemotypes can be morphologically differentiated.

A second bracken taxon, *Pteridium aquilinum* subsp. *wightianum* (previously *P. revolutum*), has localized distribution in NE QLD,[1,2] and samples of this species were also analyzed for ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, by HPLC-UV analysis of pterosin products (Table 3). This taxon has previously been reported to contain high levels of ptaquiloside, **1a**.[9,10,18] In our analysis all three glycosides **1a**−**c** were detected in this taxon with ptaquiloside, **1a**, as the major glycoside accompanied by small amounts of both ptesculentoside, **1b**, and caudatoside, **1c**. Whilst high levels of ptaquiloside, **1a**, are seen in *P. aquilinum* subsp. *wightianum*, the total norsesquiterpenoid glycoside content of *P. aquilinum* subsp. *wightianum* is less than that seen in some samples of *P. esculentum* due to the abundance of ptesculentoside, **1b**, in the latter species (Table 3).

The most common bracken species in the northern hemisphere is *P. aquilinum*, and initial isolations of ptaquiloside, **1a**, relate to this species.[19,20] To determine whether ptesculentoside, **1b**, is also present, extracts of *P. aquilinum* from Scotland, Denmark, and the Azores that had undergone pterosin conversion (with HCl) were provided by Lars Rasmussen (Denmark). In addition to the previously reported **1a** and **1c**, we detected small amounts of ptesculentoside, **1b**, in the *P. aquilinum* extract from Denmark but not in the extracts from Scotland and the Azores by HPLC-UV analysis of pterosin products (Table 4). GC-MS analysis confirmed the presence of pterosin G, **2b** (from ptesculentoside **1b**), in the converted *P. aquilinum* extract from Denmark.

Risk to Animals. It has been demonstrated that ptaquiloside, **1a**, carcinogenicity occurs as a consequence of initial DNA damage through alkylation of adenine mononucleotide via the reactive cyclopropyl ring.[21,22] Ptesculentoside, **1b**, has chemical reactivity similar to that of ptaquiloside, **1a**,[6] and would be expected to alkylate DNA in a similar manner with associated carcinogenicity. Consequently, previous studies of the toxicity of Australian *P. esculentum* that considered only ptaquiloside, **1a**, content may have significantly underestimated the toxicity of this taxon. In this study, we have found concentrations of ptesculentoside, **1b**, up to 3 times that of ptaquiloside, **1a**, in Australian *P. esculentum*, and this bracken would be expected to present a much greater risk to grazing livestock than bracken containing only similar amounts of ptaquiloside (with no ptesculentoside present). The total reactive norsesquiterpenoid glycoside content rather than only the ptaquiloside content should be considered in the assessment of the potential risk of bracken to livestock, and to humans exposed to these toxins, either through direct ingestion of bracken or from secondary sources such as residues in meat and milk.

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Funding Sources

This study was partly funded by Meat and Livestock Australia (Project AHW.017).

ACKNOWLEDGMENT

Plant identifications were determined by staff of Queensland Herbarium (Environmental Protection Agency, QLD). Technical assistance was provided by Madeleine Modina. Barry Robinson, Gary Everingham, and Jason Cole collected plant samples for this study. John Thomson provided invaluable comment on bracken taxonomy and morphology differences.

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