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Ptaquiloside, the Major Carcinogen of Bracken Fern, in the Pooled Raw Milk of Healthy Sheep and Goats: An Underestimated, Global Concern of Food Safety

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ABSTRACT: Bracken fern (*Pteridium aquilinum*) is a worldwide plant containing toxic substances, which represent an important chemical hazard for animals, including humans. Ptaquiloside, **1**, a norsesquiterpenoid glucoside, is the major carcinogen of bracken detected in the food chain, particularly in the milk from farm animals. To date, ptaquiloside has been shown in the milk of cows feeding on a diet containing bracken fern. This is the first study that shows the systematic detection of ptaquiloside, **1**, and reports its direct quantitation in pooled raw milk of healthy sheep and goats grazing on bracken. Ptaquiloside, **1**, was detected by a sensitive method based on the chemical conversion of ptaquiloside, **1**, into bromopterosine, **4**, following gas chromatography−mass spectrometry (GC−MS) analysis. The presence of ptaquiloside, **1**, possibly carcinogenic to humans, in the milk of healthy animals is an unknown potential health risk, thus representing a harmful and potential global concern of food safety.

KEYWORDS: *bracken fern, ptaquiloside, pterosine, milk, sheep, goats*

INTRODUCTION

Bracken fern (*Pteridium aquilinum*) is a ubiquitous fern, one of the five most common plants on the planet.[1] This plant contains a number of toxic components, the consumption of which produces acute and chronic toxic syndromes in different animal species. Acute bracken poisoning is characterized by a thiamine deficiency in horses and pigs and a depression of bone marrow activity leading to severe leucopenia, thrombocytopenia, and acute hemorrhagic crisis in cattle. Chronic bracken poisoning is responsible for a progressive retinal degeneration, resulting in blindness in sheep, and is associated with tumors of the upper alimentary tract in cattle;[2] furthermore, it causes a syndrome known as chronic enzootic hematuria in cattle and in water buffaloes characterized morphologically by urothelial tumors of the urinary bladder and clinically by the presence of blood in the urine.[3,4]

Ptaquiloside, **1**, a norsesquiterpene glucoside of the illudane type, is known to be the major carcinogen of bracken, readily undergoing glucose elimination to form an unstable conjugated dieneone intermediate, named ptaquilodienone, **2**, and pterosine B, **3** (Figure 1).[5] The reactive cyclopropane moiety of ptaquilodienone, **2**, is capable of breaking DNA strands and/or alkylating selected DNA bases. Thus, the biological properties of ptaquiloside, **1**, are characterized by mutagenic, clastogenic, and carcinogenic effects.[6,7]

It has been suggested that bracken fern increases the oncogenic risk also in humans.[8,9] Indeed, ptaquiloside, **1**, is responsible for severe human chromosomal abnormalities[10,11] and is suspected of causing cancer in humans.[7,12−16]

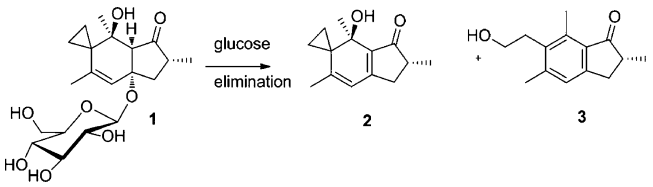

Figure 1. Structure of ptaquiloside, **1**, and of its degradation products ptaquilodienone, **2**, and pterosine B, **3**.

Epidemiological surveys revealed that bracken consumption is positively correlated with esophageal cancer in many geographical areas of the world[17] and with stomach cancer in population of farmers living in some rural regions of Wales and Costa Rica.[17−21]

Several routes may led to human exposure to ptaquiloside, **1**, namely, eating the plant,[22] breathing air containing bracken spores,[23] and consumption of milk and meat of affected animals.[24−26] Drinking water appears to be an additional important route of transmission.[27] Indeed, ptaquiloside, **1**, has been detected in both groundwater below bracken vegetation and surface water.[28]

Ptaquiloside, **1**, is responsible for H-Ras activation via an initial alkylation of adenine of the codon 61, resulting in a

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transition (A to G) or transversion (A to T/C).[6] Any amino acid substitution at this position, except glutamic acid, blocks the guanosine triphosphate (GTP) hydrolysis of the Ras−GTP complex. The deregulation of hydrolysis is believed to result in the aberrant Ras signaling pathway, which causes an abnormal cell growth, proliferation, and survival.[29] Although oncogenic pathways remain to be elucidated, recent molecular studies provided new insights. Indeed, ptaquiloside, **1**, has been shown to induce a deregulation of the expression of a panel of genes related to cell cycle arrest in gastric epithelial cells. Furthermore, ptaquiloside, **1**, has been found to be responsible for glycophenotypic alterations of gastric mucosa and play a synergistic effect with *Helycobacter pylori* infection, leading to the gastric carcinogenesis process.[30,31]

Thus far, ptaquiloside, **1**, has been detected in milk of cows experimentally fed on a diet containing bracken fern.[32−34] It has been shown that the administration of milk from bracken-fed cows to mice was responsible for a high incidence of pulmonary and mammary tumors.[33] No systematic data are available about the naturally occurring presence of ptaquiloside, **1**, in milk of farm animals other than cows. The aim of the present paper is to report the detection and direct quantitation of ptaquiloside, **1**, in the pooled raw milk of healthy sheep and goats grazing on bracken fern-infested pastures, using a gas chromatography−mass spectrometry (GC−MS)-based method.

MATERIALS AND METHODS

Materials, Chemicals, and Reagents. Acetonitrile, ethyl ether, and ethyl acetate were purchased from Sigma-Aldrich (Milan, Italy). All solvents were of analytical grade and used as supplied. Ultrapure water was obtained from a Milli-Q plus system (Millipore, Bedford, MA). Sulfuric acid (Suprapur grade) was purchased from Merck (Darmstadt, Germany). Cartridges (1.2 cm inner diameter) filled with graphitized carbon black (500 mg of Carbograph 4) were prepared and provided by LARA (Rome, Italy). Carbograph 4 has a surface area of 200 m^2/g, and it is commercially also referred to as Carboprep (Resteck, Bellefonte, PA). Polyethylene frits (20 μm) were included. d_4-Bromopterosine, used as an internal standard in the calibration procedure, was synthesized according to a recent protocol.[35] Solutions of deuterated standard were prepared in methanol or ethyl acetate and stored at −20 °C.

Instrumentation. An Agilent Technologies 6850 Series II gas chromatograph, coupled with a 5973 mass selective detector (MSD) (Agilent Technologies, Santa Clara, CA), was used. The carrier gas was helium at 1.7 mL/min of 99.999% purity. The HP-5MS capillary column used was a 30 m × 0.25 mm inner diameter, 0.25 μm film thickness (Agilent, 5% phenyl methyl siloxane). The GC oven temperature was initially held at 100 °C for 1 min, then ramped at 20 °C/min to 280 °C, and held at this temperature for 2 min. Temperatures of injector and transfer line were 280 °C. Analyses were performed in splitless mode, and 0.5 μL was injected. The software MSDchem (Agilent Technologies) has been used to obtain graphics and quantitative data.

Calibration Procedure for Milk Samples. For the quantitative analysis of milk, a five-point calibration curve was obtained by spiking milk, free from ptaquiloside, **1**, with known amounts of internal standard to cover a concentration range of 3−20 ng/mL ptaquiloside, **1**. The amount of ptaquiloside, **1**, was determined in each experiment as previously described,[36] namely, by comparing the sum of the areas of three ions monitored m/z 187, 201, and 280 for the bromopterosine and m/z 189, 205, and 284 for the deuterated reference (d_4-bromopterosine). Each experimental value corresponds to the average of three independent measurements.

The limit of detection (LOD) and limit of quantitation (LOQ), calculated following the directives of the International Union of Pure and Applied Chemistry (IUPAC) and American Chemical Society's Committee on Environmental Analytical Chemistry, were 0.3 and 0.4 ng/mL, respectively. For comparison, the method recently published by Aranha and co-workers[34] declared a LOQ of 5.8 ng/mL.

Sample Preparation. Sample preparation was carried out following a protocol reported elsewhere.[37] The carbograph cartridge was washed with 6 mL of a mixture of acetonitrile/water (7:3, v/v); it was subsequently activated with diluted HCl (pH 2, 20 mL) and, finally, washed with 6 mL of water. A total of 10 mL of milk was passed through the cartridge; the eluate was discarded; and the cartridge was washed with 6 mL of water. The cartridge was then eluted with 7 mL of a mixture of acetonitrile/water (7:3, v/v), and 20 ng of d_4-bromopterosine and 2 g of NaBr were added to this eluate. After evaporation of the upper phase (acetonitrile) in nitrogen stream, the solution was made alkaline (pH 12) and heated at 45 °C for 1 h. The solution was cooled, acidified at pH 2, and extracted 3 times with ethyl ether. The resulting ether solution was evaporated to dryness, and finally, the extract was diluted to 20 μL in a micro (conical) test tube with ethyl acetate.

Collection of Milk Samples. For this study, flocks composed of both goats and sheep that had grazed on pasturelands covered by an abundance of bracken fern in several topographically distinct territories in southern Italy were selected. Milk samples were collected in different periods of the year from healthy sheep and goats sharing the same pastures located within 300−500 m above sea level (asl) (Martirano), within 500−800 m asl (Decollatura), and within 800−1000 m asl (Serrastretta and Carlopoli) altitudinal ranges. In Martirano, the flocks were composed of 117 crossbred sheep and 115 goats that have grazed on lands where bracken fern germinates in March−mid-April. Furthermore, in these lands, borage (*Borago officinalis* L.), Rowan tree (*Sorbus domestica* L.), myrtle (*Myrtus communis* L.), Scotch broom (*Spartium junceum* L.), poppy (*Papaver rhoeas* L.), chicory (*Cichorium intybus* L.), fennel (*Foeniculum vulgare* Mill.), blackberry bush (*Rubus ulmifolius* Schott), clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and sulla clover (*Hedysarum coronarium* L.) were also present. Flocks from Decollatura were composed of 124 Sarda sheep and 46 Maltese and Syria-derived goats that have grazed of lands covered with bracken fern, the fronds of which start to emerge from underground rhizomes in May. Amaranth (*Amaranthus retroflexus* L.), heather (*Erica arborea* L.), clematis (*Clematis vitalba* L.), butcher's broom (*Ruscus aculeatus* L.), ivy (*Hedera helix* L.), and hawthorn (*Crataegus monogyna* Jacq.) are the prevailing herbaceous plants of those lands. Flocks from Serrastretta were composed of 60 crossbred sheep and 67 Nicastrese goats; Carlopoli flocks were composed of 109 Sarda sheep and 100 crossbred, Saanen and Alpine, goats. Pigweed (*Portulaca oleracea* L.), asparagus (*Asparagus acutifolius* L.), aniseed (*Pimpinella anisum* L.), watercress (*Nasturtium officinale* R.), Italian ryegrass (*Lolium multiflorum* L.), tall fescue (*Festuca pratensis* Huds.), oregano (*Origanum heracleoticum* L.), bush (*Rubus canescens* DC.), strawberry (*Fragaria vesca* L.), wild rose (*Rosa canina* L.), horsetail (*Equisetum telmateia* Ehrh.), milk thistle [*Silybum marianum* (L.) Gaertn.], nettle (*Urtica dioica* L.), and wild lettuce [*Reichardia picroides* (L.) Roth.] were herbs prevalently found in the lands of Serrastretta and Carlopoli.

Furthermore, we collected pooled raw ovine and caprine milk samples from flocks composed of 90 crossbred sheep and 86 Nicastrese goats grazing on bracken fern-free lands located near the sea (Sant'Eufemia Gulf of Calabria, southern Italy). In these samples, no ptaquiloside, **1**, has been detected in either ovine or caprine milk.

In each flock, bulk ovine milk was harvested and separately kept from pooled caprine milk. The daily average of milk production for each flock was 60−80 L for ovine milk and 80−100 L for caprine milk. Following milking of all animals, the samples were obtained from tanks containing the pooled raw milk before undergoing any physical and/or other treatments. According to European Union (EU) legislation, "raw milk" is defined as milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40 °C or undergone any treatment that has an equivalent effect [Regulation (EC) No. 853/2004]. All milk samples were investigated within 1 week since harvesting.

Determination of Ptaquiloside, 1, Content. Determination of ptaquiloside, **1**, content in milk samples was obtained with the

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previous method,[37] with small modifications. All of the analyses were made as triplicates. This method takes advantage of the click conversion of ptaquiloside, **1**, into bromopterosine, **4**, in the presence of bromide ions (Figure 2) and the stability and easy detectability of bromopterosine, **4**, by conventional GC−MS techniques.

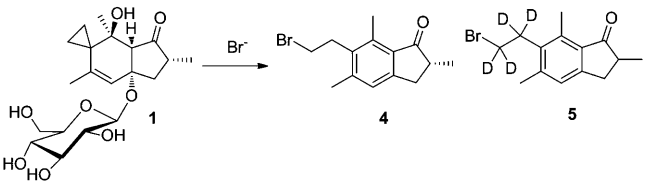

Figure 2. Structure of bromopterosine, **4**, obtained upon treatment of ptaquiloside, **1**, with bromide ions. On the right, the structure (**5**) represents the tetradeuterated standard obtained by total synthesis.

Fresh milk has been filtered over cartridges filled with graphitized carbon black, a matrix with high affinity toward free sugars and glycosylated compounds (such as ptaquiloside, **1**). Freezing and subsequent unfreezing of the milk resulted in a dense sample, likely because of protein precipitation, whose filtration over cartridges gave nonreproducible results, and therefore, this procedure was avoided.

Cartridges were then eluted with a mixture of acetonitrile/water (7:3, v/v), and the reference compound d_4-bromopterosine (**4**) (in place of d_2-bromopterosine used in the previous method) and NaBr were added to the eluate. After evaporation of the acetonitrile phase and workup, the resulting ethyl acetate solution was injected in the GC−MS instrument, using helium as the carrier gas and a HP-5MS capillary column.

RESULTS AND DISCUSSION

The amount of ptaquiloside, **1**, present was determined as a result of the comparison of the sum of the areas of three ions for bromopterosine (m/z 187, 201, and 280) and the corresponding ions of the deuterated reference compound (m/z 189, 205, and 284) (Figure 3). The obtained data, reported in Table 1, show a significant presence of ptaquiloside, **1**, in the milk of both goats and sheep and an absence in the milk from animals as controls. Ptaquiloside, **1**, was detected earlier in milk of goats that have grazed on lowlands (Martirano), which may suggest that the higher the altitude of land where the animals are at pasture, the later bracken fern toxin in the milk can be detected. Furthermore, higher doses have been detected in goat's milk rather than sheep's milk from the same mixed flocks and in the same seasonal period. Very likely, this is because goats more than sheep tend to browse young leaves and nibble the palatable shoots, such as bracken crosiers. It has been shown that ptaquiloside, **1**, occurs in the highest concentrations in the young developing part parts of bracken, such as the crosiers, and the unfolding parts during the spring and early summer.[7] Furthermore, it has been suggested that a few herbaceous plants other than bracken fern contain high levels of ptaquiloside, **1**.[38,39] The variety of herbs of all lands that we examined allowed us to suggest that ptaquiloside, **1**, came from bracken fern only. *Pteridium aquilinum* ssp. *aquilinum* (L.) Kuhn is the only species of bracken fern contaminating all of the lands where the animals, the milk of which we examined, have grazed. Therefore, also edaphic and/or environmental causes might influence the ptaquiloside, **1**, content detected in milk in different seasonal periods.

This is the first study that shows the systematic detection and reports the direct quantitation of ptaquiloside, **1**, in the pooled raw milk of healthy sheep and goats sharing pasturelands covered by bracken fern. The ptaquiloside, **1**, amount was detected by a sensitive method based on the chemical conversion of ptaquiloside, **1**, into bromopterosine, **4**, and following GC−MS analysis,[36,37] easily reproducible in non-specialized laboratories. Because this method has been reported to be able to quantitate up to 80% of ptaquiloside, **1**, present in the sample,[37] it is reasonable to presume that the real ptaquiloside, **1**, values in the pooled raw milk are even higher than we found. We have no data about the amount of bracken fern ingested daily; therefore, experimental procedures are needed to know ptaquiloside, **1**, metabolism in small animals too. It has been experimentally shown that the average value of total ptaquiloside, **1**, excreted in milk of cows is 8.6 ± 1.2% of the amount ingested; furthermore, ptaquiloside, **1**, was detected in milk, for the first time, 38 h after feeding cows with bracken fronds.[33] Direct quantitation of ptaquiloside, **1**, was evaluated in plasma and several tissues (liver, kidney, and skeletal muscle) after feeding two daily cows with a diet containing about 19% of *P. esculentum* for 25 days.[26] New sensitive methods allowing ptaquiloside, **1**, and its decay product, pterosine B, **3**, to be quantified directly and simultaneously in both soil, groundwater, and surface water[27,28] and biological fluids, such as plasma, urine, and milk, have been proposed.[34] All of these methods, including ours, showing low detection limits, have important implications for studies of ptaquiloside, **1**, toxicokinetics in animals and for screening animal tissues suitable for human consumption.

It has been recommended that the priority to reduce the risk to consumers from bracken should be the identification of the amount of ptaquiloside, **1**, that occurs in meat, offal, milk, and dairy products from animals naturally exposed to bracken without any signs of toxicity.[40] Thus far, reports about the presence of ptaquiloside, **1**, in milk and tissues have been from cows in experimental procedures.[26,32,33] No information was available on the amount of ptaquiloside, **1**, or other components of bracken that can occur in milk from animals naturally grazing on bracken fern-infested lands.[40] It is believed that the toxicity of bracken toward domestic animals may affect people too, particularly in the case of ptaquiloside-induced carcinogenesis.[12] Indeed, it has been suggested that ptaquiloside, **1**, can be potentially carcinogenic to humans at all levels of ingestion.[40]

Currently, the International Agency for Research on Cancer (IARC) assigned ptaquiloside, **1**, to Group 2B (possibly carcinogenic to humans) on the basis of epidemiological and experimental evidence. However, the sixth IARC Monographs Advisory Group on priorities for future evaluations made a urgency list of agents and exposures to be considered in the future IARC Monographs Programme. The IARC Group considers ptaquiloside, **1**, and bracken fern high priorities among "naturally occurring substances". Furthermore, the IARC Group recommends that new animal carcinogenicity data should be collected soon and encourages the need to have more defined molecular, epidemiological, and ecological aspects of the impact of bracken fern.[41] In this context, it is worthwhile remembering that urinary bladder tumors caused by ptaquiloside, **1**, intoxication have recently been described, for the first time, in captive fallow deer.[42] Furthermore, a novel syndrome characterized by intestinal adenocarcinomas in adult farmed Sika deer that have grazed on bracken-infested pastures has just been described. Therefore, it has been suggested that ptaquiloside, **1**, plays a crucial role in deer intestinal tumorigenesis too.[43]

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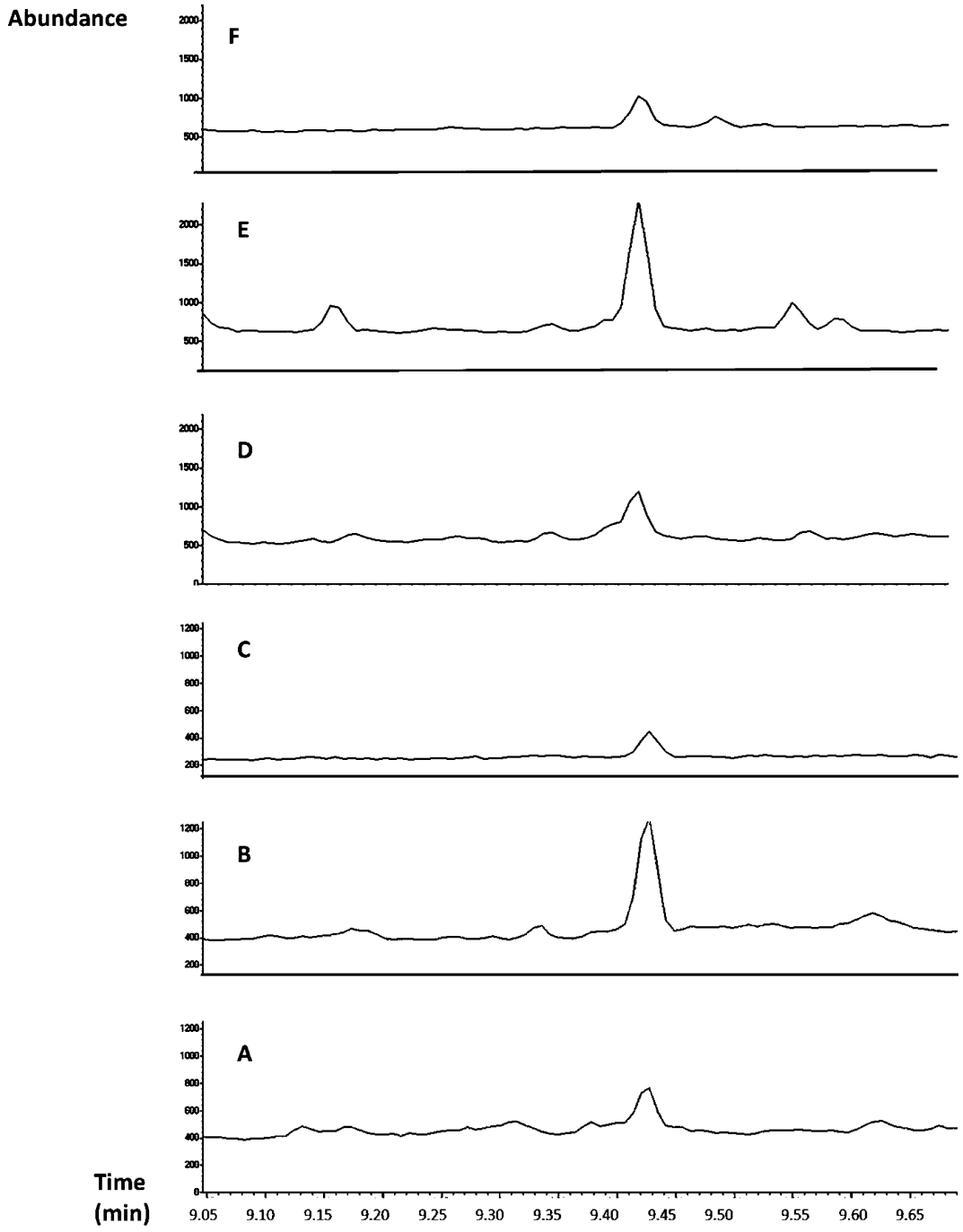

Figure 3. Representative GC traces of the monitored ions. The graphic corresponds to the sample goat/Decollatura/June 2014 in Table 1. Panels A, B, and C refer to the monitoring of ions at m/z 187, 201, and 280. Panels D, E, and F refer to the corresponding deuterated ions at m/z 189, 205, and 284. The retention time of the analyte is 9.42 min.

Finally, it is well-known that thousands of chemicals to which humans are exposed have inadequate data useful to predict their potential for toxicological effects.[44] Risk assessment of ptaquiloside, **1**, presents particular difficulties because the effects of some substances are normally regarded as being without a threshold.[45] Therefore, how long ptaquiloside, **1**, takes to reach toxicological thresholds necessary for its identification and characterization as hazardous, two important steps in the concept of "safe levels of exposure", remains to be elucidated.[46]

Recent epidemiological studies emphasize the potential role of sheep's milk and goats' milk as a risk factor for esophageal squamous cell carcinoma in the high risk area of northern Iran.[47,48] It has been shown that low doses of diterpene ester toxins can be detected in the milk of small ruminants fed by a family of Euphorbiaceae flower plants; therefore, it has been suggested that the high incidence of esophageal cancer observed in certain areas in the Caspian littoral of Iran may be associated with a greater consumption of sheep's milk and goats' milk contaminated by these toxins.[49]

The stability of ptaquiloside, **1**, and its hydrolysis have already been carefully analyzed according to different pH conditions in aqueous solutions.[5] Further interesting aspects to be investigated in the near future are (i) the effect of thermal

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Table 1. Amounts of Ptaquiloside Content Found in Milk Samples

animal	collection and analysis date	place (farm) of collection	bromopterosine content (ng/mL)[a]	ptaquiloside content (ng/mL)[b]
goat	March 2014	Martirano	<LOD[c]	
goat	May 2014	Martirano	0.68 ± 0.14	0.97 ± 0.20
goat	June 2014	Martirano	0.45 ± 0.12	0.64 ± 0.17
goat	April 2014	Decollatura	<LOD	
goat	May 2014	Decollatura	<LOD	
goat	June 2014	Decollatura	1.45 ± 0.25	2.06 ± 0.35
goat	July 2014	Decollatura	1.29 ± 0.18	1.83 ± 0.26
goat	June 2014	Serrastretta	0.40 ± 0.09	0.57 ± 0.13
goat	July 2014	Serrastretta	0.43 ± 0.11	0.61 ± 0.16
goat	September 2014	Serrastretta	0.40 ± 0.08	0.57 ± 0.11
goat	July 2013	Carlopoli	0.77 ± 0.15	1.09 ± 0.21
goat	December 2013	Carlopoli	2.21 ± 0.27	3.14 ± 0.38
sheep	March 2014	Martirano	<LOD	
sheep	June 2014	Martirano	<LOD	
sheep	April 2014	Decollatura	<LOD	
sheep	May 2014	Decollatura	<LOD	
sheep	June 2014	Decollatura	0.60 ± 0.12	0.85 ± 0.17
sheep	July 2014	Decollatura	<LOD	
sheep	June 2014	Serrastretta	<LOD	
sheep	July 2014	Serrastretta	<LOD	
sheep	September 2014	Serrastretta	<LOD	
sheep	July 2013	Carlopoli	0.96 ± 0.18	1.36 ± 0.26
sheep	December 2013	Carlopoli	1.15 ± 0.21	1.63 ± 0.30
goat/C[d]	April 2015	Sant'Eufemia	<LOD	
sheep/C	April 2015	Sant'Eufemia	<LOD	

[a]Each value corresponds to the average of three distinct experiments. [b]Indirectly derived from the bromopterosine content. [c]LOD = limit of detection. [d]C = control.

treatment on ptaquiloside, **1**, dose naturally present in milk of farm animals, (ii) determination of naturally occurring ptaquiloside, **1**, in meat and dairy products for human consumption in animals naturally grazing on pasturelands with bracken abundance, and (iii) functional studies in an attempt to show the relationship, if any, between ptaquiloside, **1**, and intracellular catabolic systems, such autophagy and apoptosis. The design and implementation of the analytical methods useful to reach the above goals are underway in our laboratories.

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Notes

The authors declare no competing financial interest.

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