Faculdade de Engenharia da Universidade do Porto

Ptaquiloside, a Natural Compound with Toxicological Interest: Monitoring and Optimization of Isolation

Joana do Paço Caçador

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Supervisor: Prof. Dr. Margarida Maria da Silva Monteiro Bastos
Co-supervisor: Prof. Dr. Rui Miguel Gil da Costa Oliveira

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“The moment you doubt whether you can fly,
you cease for ever to be able to do it.”

J.M. Barrie, *Peter Pan*

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Abstract

Bracken fern (*Pteridium aquilinum*) is one of the most abundant and widely distributed plant species worldwide and has been associated with a variety of health problems in humans, namely cancer, and animals since the XIX century. Bracken has the ability to endure insect attacks, microorganisms and unfavourable weather. This, coupled with its aptitude to spread, made it necessary to focus considerable efforts on its control and on understanding its toxicity.

About 100 metabolites have been isolated from bracken; of these, ptaquiloside, an unstable sesquiterpene glycoside, is the one responsible for most of the fern's carcinogenicity.

Studies on the toxicological effects that ptaquiloside has on animals and, potentially, on humans are dependent of its isolation from the plant. At LEPABE efforts are being made to develop a faster and more efficient method for the ptaquiloside isolation from *Pteridium aquilinum*.

A key aspect for implementing these methods and assessing their efficiency is the quantification of ptaquiloside present in the plant and the amount obtained by the isolation method, as well as during the stages of the procedure. Thus, it becomes necessary to develop an appropriate method for ptaquiloside quantification.

The objective of this work was to develop, validate and implement a fast, simple and optimized HPLC method to quantify the content of ptaquiloside during the extraction procedure.

By applying a conversion methodology, quantification of ptaquiloside in bracken samples was possible, monitor the concentration/clean-up step of ptaquiloside isolation and identify some unnecessary stages, expediting the isolation process and therefore reducing the loss of ptaquiloside due to hydrolysis.

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Symbols and Abbreviations

BEH	Bovine Enzootic Haematuria
CH_3OH	Methanol
DNA	Deoxyribonucleic Acid
GC-MS	Gas Chromatography-Mass Spectrometry
H_2O	Water
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric acid
HPLC	High Pressure Liquid Chromatography
IARC	International Agency for Research on Cancer
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass spectrometry
N	North
NaOH	Sodium hydroxide
NMR	Nuclear Magnetic Resonance
ODS	Octadecylsilane
P.	*Pteridium*
PA	*Pteridium aquilinum*
Pta	Ptaquiloside
PtB	Pterosin B
RSD	Relative Standard Deviation
SD	Standard Deviation
TLC	Thin Layer Chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
W	West

1. Introduction

Bracken fern (*Pteridium aquilinum* (L.) Kuhn) is one of the most abundant and widely distributed plant species worldwide (Alonso-Amelot et al. 1993; Castillo et al. 1998; Pathania et al. 2012) and has proven itself to be a very resilient plant (Davis et al. 2013). Bracken can be found in most ecosystems (Alonso-Amelot et al. 1993; Dawra et al. 2002; Rasmussen et al. 2005) and has been associated with a variety of health problems in humans and animals since the XIX century (Agnew & Lauren 1991; Castillo et al. 1998). *Pteridium aquilinum* is classified as carcinogenic for animals and possibly carcinogenic for humans (2B group) by the International Agency for Research on Cancer (IARC) (Somvanshi & Ravisankar 2004; Rasmussen et al. 2005; Gil da Costa 2011).

Figure 1 - Mature bracken (*Pteridium aquilinum*) plants at Arcos de Valdevez, Portugal. Removed from (Gil da Costa 2011)

1.1. *Pteridium aquilinum*

Bracken is a perennial fern (Whitehead & Digby 1997) that grows in the mountainous areas of the tropics and in the temperate regions of the world (Alonso-Amelot et al. 1992a; Wilson et al. 1998) in a wide diversity of climates and soil types (Davis et al. 2013). In fact, it grows in all continents (except Antarctica) (Hojo-Souza et al. 2010) and can be found most often at forest margins, in recently deforested areas, abandoned agricultural regions (Smith & Seawright 1995), on clear-cut or newly burned areas and in glades (Rasmussen 2003).

P. aqulinum is normally found on well-drained acid soils (from pH 2.6), but may at times be encountered at nutrient rich and even calcareous soils (up to pH 7.5) (Rasmussen 2003).

The species *Pteridium aquilinum* belongs to the *Pteridium* genus and to the Dennstaedtiaceae family (Cruz & Bracarense 2004; Der & Thomson 2009). Bracken ferns are easily distinguished from other genera in Dennstaedtiaceae, and several taxa within this genus are attributed according to the morphological structure associated with their geographical location (Der & Thomson 2009). In Table 1 the classification of the genus *Pteridium* is described.

Table 1 - Genus *Pteridium* classification; where 2n=diploid number of chromosomes. Adapted from Hojo-Souza et al. 2010

Species	Sub-Species	Occurrence
***Pteridium aquilinum* (L.) Kuhn** Diploid strain (2n=104)	*latiusculum*	North America
	pinetorum	Europe
	japonicum	Asia
	capense	Sub-Saharan Africa
	aquilinum	Europe
	wightianum	India, Southeast Asia and Northern Australia
	decompositum	Hawaii
	centrali-africanum	Sub-Saharan Africa
	pseudocaudatum	Eastern North America
	pubescens	North America
	feei	Central America
***Pteridium esculentum* (G. Forst.) Cockayne** Diploid strain (2n=104)		South Hemisphere (Australia)
***Pteridium arachnoideum* (Kaulf.) Maxon** Diploid strain (2n=104)		South Hemisphere (South America)
***Pteridium caudatum* (L.) Maxon** Allotetraploid strain (4n=208)		Southern and Central America
***Pteridium semihastatum* (Wall. Ex J. Agardh) S. B. Andrews** Allotetraploid strain (4n=208)		South-East Asia and Northern Australia

The genus *Pteridium* can be divided into five species: *P. aquilinum*, *P. esculentum*, *P. arachnoideum*, *P. caudatum* and *P. semihastatum* (Hojo-Souza et al. 2010; Thomson 2004). Along with this classification, these ferns can be grouped according to their morphology and genetic characteristics, which are associated with their geographical location. The diploids species *P. arachnoideum* and *P. esculentum* can be found in the Southern hemisphere and can be genetically and morphologically distinguished from the northern hemisphere bracken, *P. aquilinum*. Additionally, the allotetraploid *P. caudatum* is characteristic of Southern and Central America, whereas the *P. semihastatum* is mostly present in South-East Asia and Australia (Thomson 2004).

1.2. *Pteridium aquilinum* Morphology and Biology

Bracken fern efficiently competes for light, soil nutrients and water with other species (Dolling et al. 1994), due to the plant's rhizome system (horizontal underground stems) that can spread underneath the roots of other vegetation and sprout fronds preventing sunlight from reaching those plants (Davis et al. 2013). Rhizomes function as a storage organ for nutrients and, by advancing through the soil, also benefit colonisation. The plant dies in autumn, growing again from the rhizome in spring as a crozier that slowly straightens as it develops. Bracken also can spread and colonise as result of the release of spores that can travel hundreds of kilometres and colonise other areas (Fenwick 1988; Cruz & Bracarense 2004).

P. aquilinum possesses an erect, rigid stalk with large triangular blades (Fenwick 1988). Mature fronds can grow between 0.6 to 1.8 meters, but can reach 3 meters depending on the environment (Davis et al. 2013) and may reach maximum length in a few months (Rasmussen 2003), also, in colour, the fronds vary from yellowish-green to dark green (Shahin et al. 1999).

This plant can reach the mature stage in only 60 to 70 days (depending on its location), becoming a source of food for herbivores, mainly during the dry season when other plants become scarce (Alonso-Amelot et al. 1992a; Rasmussen 2003), possessing a dry matter production of 1.400 g/m^2 (Rasmussen 2003).

1.3. *Pteridium aquilinum* Toxicity

Bracken has the ability to resist insect attacks, microorganisms and unfavourable weather (Alonso-Amelot & Avendaño 2002) which coupled with its aptitude to spread, makes it necessary to dedicate great efforts on its control and on understanding its toxicity.

Since the end of the XIX century, the ingestion of the plant by cattle was associated with the presence of haematuria and others disorders (Yamada et al. 2007). Nonetheless, only in 1965, Evans and Mason, were able to induce tumours in rats fed with bracken (Evans & Mason 1965), thus proving its carcinogenicity. Since then, bracken has been intensively studied biologically and chemically, to assess animal susceptibility to this plant.

Bracken fern is the only plant known to naturally cause cancer in animals (Smith & Seawright 1995; Rasmussen 2003). This plant is the cause of various diseases in cattle, sheep, horse and other farm and laboratory animals (Alonso-Amelot et al. 1992a). The ingestion of bracken can cause thiamine deficiency (Hojo-Souza et al. 2010), bovine enzootic haematuria (BEH) (Hopkins 1990; Alonso-Amelot et al. 1992a), bladder and intestinal carcinomas in cattle (Oelrichs et al. 1995; Potter & Baird 2000) and liver, kidney, urinary bladder, mammary gland and intestine carcinomas in rats and mice (Alonso-Amelot et al. 1992a; Oelrichs et al. 1995).

In addition to the direct ingestion of bracken, the inhalation of its spores and consumption of milk produced by cattle exposed to bracken may be responsible for stomach and oesophageal cancer in humans (Alonso-Amelot et al. 1992a; Oelrichs et al. 1995; Rasmussen et al. 2013).

The concentration of toxic bracken compounds varies with the age and the plant section. The major amount of carcinogenic substances can be found in bracken croziers (Yamada et al. 2007).

1.4. Ptaquiloside

About 100 metabolites have been isolated from bracken and of these, ptaquiloside (Pta) (Alonso-Amelot et al. 1992a), an unstable norsesquiterpene glycoside of the illudane type (Yamada et al. 1998), is the one responsible for most of the fern's carcinogenicity (Alonso-Amelot et al. 1992a; Oelrichs et al. 1995). Ptaquiloside (Figure 2) was characterised and independently isolated by two groups: one in Japan (Niwa et al. 1983) and other in The Netherlands (Van der Hoeven et al. 1983) in 1983.

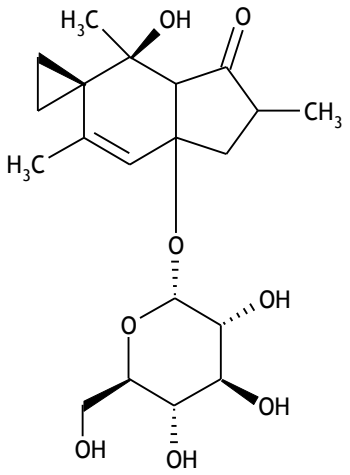

Figure 2 – Ptaquiloside chemical structure.

Ptaquiloside is an amorphous compound, colourless, with the molecular formula $C_{20}H_{30}O_8$ (Niwa et al. 1983) and chemical name [2'R-(2'α,3'aα,4'β,7'aα)]- 7'a-(β-D- glucopyranosyloxy)-1',3',a,4',7'a- tetrahydro-4'-hydroxy-2',4',6'-trimethyl- spiro[cyclopropane-1-5'-[5H]inden]-

3'(2'H)-one (Rasmussen 2003). It is stable at room temperature only for a week but remains stable for six months at low temperatures (from 0°C to -20°C) (Yamada et al. 2007). In aqueous solutions, ptaquiloside is gradually converted into pterosin B (PtB) and D-glucose, at a rate dependent of the solution's pH (Yamada et al. 1998).

Table 2 - Physical and chemical properties of ptaquiloside.

Property		Reference
Molecular Formula	$C_{20}H_{30}O_8$	(Niwa et al. 1983)
Molecular Weight	398.45 g/mol	(Rasmussen 2003)
Melting Point	85-89ºC	(Saito et al. 1990)
UV absorption maxima	214; 220 nm	(Saito et al. 1989)

Ptaquiloside rapidly loses its glucose moiety creating an unstable intermediary - pteridienone, a strong electrophile capable of alkylating amino acids and DNA (Alonso-Amelot et al. 1993; Fletcher et al. 2011; Jensen et al. 2008; Yamada et al. 1998). In Figure 3 are represented the hydrolysis reactions of ptaquiloside, which is unstable in acid and basic conditions (Agnew & Lauren 1991). In alkaline conditions, ptaquilosin is formed and this one leads to the unstable pteridienone; in acidic conditions, both ptaquiloside and the pteridienone form pterosin B (Alonso-Amelot et al. 1995; Rasmussen et al. 2005). Pterosin B is stable under both acid and alkaline conditions, while ptaquilosin and the pteridienone are only stable under alkaline conditions (Van der Hoeven et al. 1983; Niwa et al. 1983).

Ptaquiloside is soluble in water and it has been reported that it is clastogenic (causes structural damages to chromosomes), mutagenic (causes genetic mutations) and teratogenic (leads to modifications in the embryonic structure) (Ayala-Luis et al. 2006; Pathania et al. 2012). Its elevated toxicity is associated with the electrophilic properties of the unstable three carbon ring (cyclopropyl group), which alkylates a wide variety of nucleophiles (biomolecules such as nucleotides) (Alonso-Amelot et al. 1993; Yamada et al. 1998; Bonadies et al. 2004). Nevertheless, anti-tumour activity and cytotoxicity to cancer cells has been described (Zhao 2011).

As stated above, after ptaquiloside loses its glucose moiety, the ptaquiloside aglycone converts to a strongly electrophilic cyclopropane pteridienone that reacts towards various weak nucleophiles like water, amines and other mild bases. Additionally, ptaquiloside is capable of penetrating the cell and nucleus membranes (Alonso-Amelot et al. 1993) since its aglycone fragment allows solubility in chloroform, methylene chloride, acetonitrile and methanol, while the glucose portion enables dilution in water (Alonso-Amelot & Avendaño 2002).

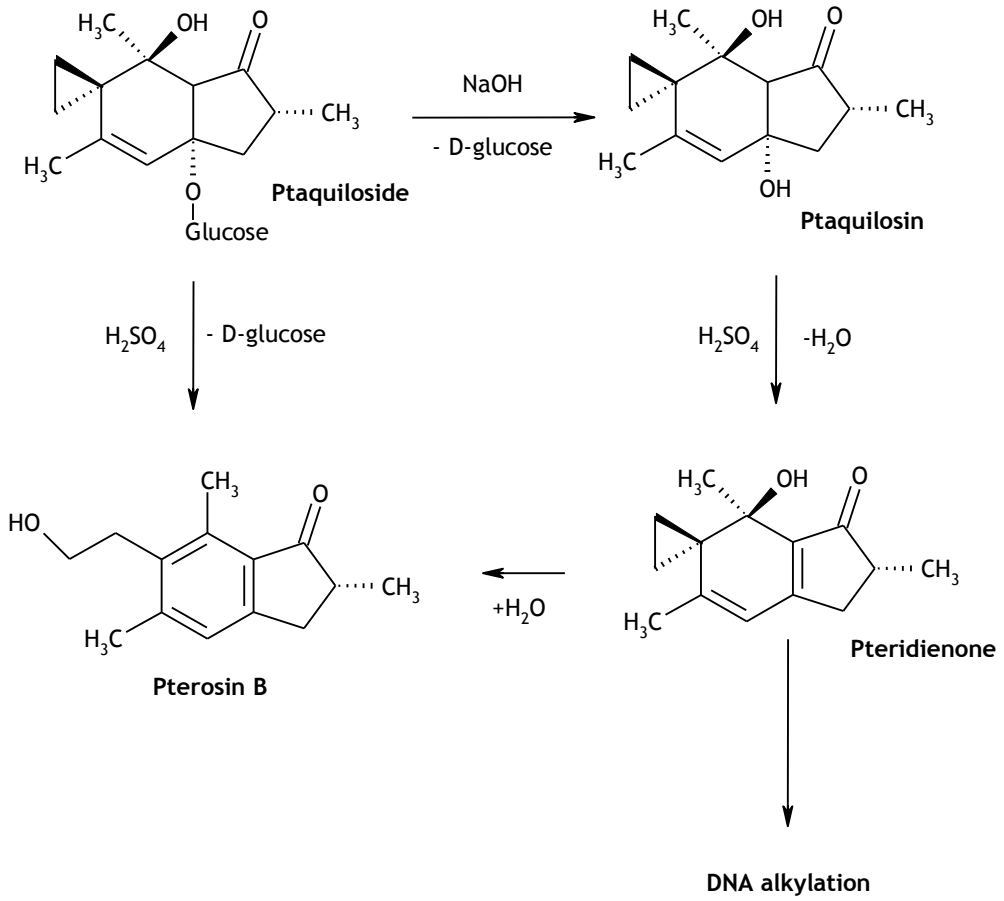

Figure 3 - Reactions of ptaquiloside.

For the activation of ptaquiloside (conversion to pteridienone) there is no need for metabolic activation, since the pteridienone formation is only pH dependent (Yamada et al. 1998). As mentioned before, ptaquiloside loses its glucose moiety under alkaline conditions to originate ptaquilosin and afterwards pteridienone is formed which is transformed into an aromatic compound, pterosin B. The prerequisite of alkaline conditions for the activation of ptaquiloside is reinforced by biological observations, since induced tumours of the bovine oesophagus and bladder are associated with environments of pH 8.1-8.2 and 7.5-8.5, respectively (Fenwick 1988; Saito et al. 1989). In rats, *P. aquilinum* mostly induces tumours of the terminal section of the ileum, a segment with the highest pH in the intestine (Price & Pamukcu 1968; Shahin et al. 1998).

When in contact with physiological nucleophiles, like purine bases in uncoiled DNA, the pteridienone will promote a DNA alkylation that may disrupt gene transcription (Figure 4) (Alonso-Amelot & Avendaño 2002). DNA cleavage occurs through a reaction between DNA and pteridienone to form adducts through the *N*-3 of adenine and/or *N*-7 of guanine (Yamada et al. 2007). Its DNA-cleaving potential combined with potentially high biological uptake and mobility due to the glucose moiety, are likely responsible for the carcinogenicity and toxicity of ptaquiloside (Rasmussen 2003).

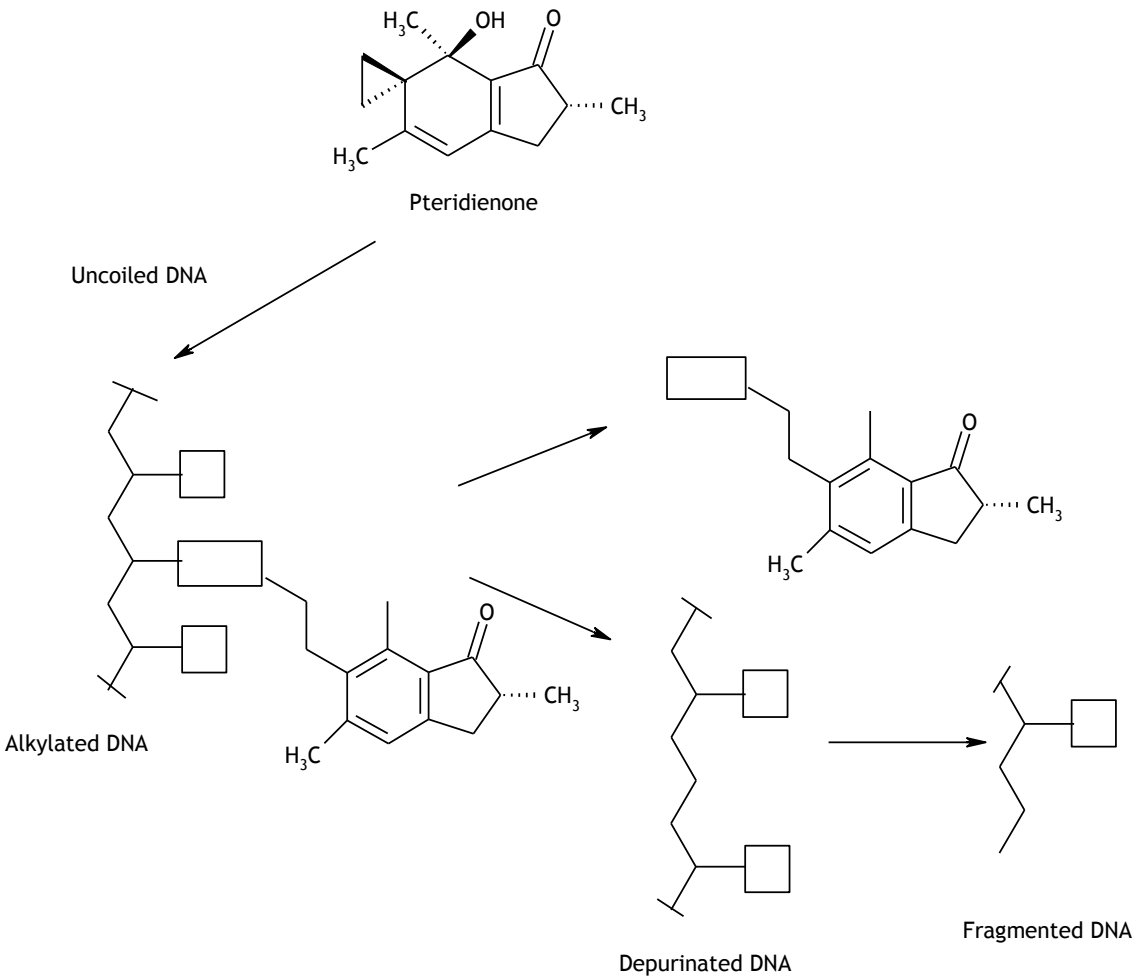

Figure 4 - DNA alkylation via pteridienone; squares represent pyrimidine bases and rectangle represent purine bases. Adapted from (Alonso-Amelot & Avendaño 2002).

The pteridienone causes a permanent change in the genes attacked. These genes may regulate the activation of other genes or have regulatory functions of multiple biochemical and cellular processes (Alonso-Amelot & Avendaño 2002), such as the p53 gene which regulates cell proliferation, DNA repair, apoptosis and tumour suppression. Thus, DNA alkylation by the pteridienone is considered to be the basis of bracken-induced carcinogenesis (Cruz & Bracarense 2004).

The administration of ptaquiloside to animals causes acute poisoning, blindness and tumours (Dawra et al. 2002; Rasmussen et al. 2005; Gil da Costa 2011), depending on the intake dose and on the animal species. Other possibility is the contamination of ground water, since ptaquiloside can be washed by rain and subsequently contaminate the soil and ground waters (Jensen et al. 2008).

In bracken, the concentration of some sesquiterpenoids has been known to vary significantly along the growing season and to be dependent on the plant compartment (Hojo-Souza et al. 2010). Ptaquiloside concentrations of up to 4.450 mg/g in dry weight basis in mature fronds have been reported (Rasmussen 2003). It is known that ptaquiloside appears in higher

concentration in the crozier than in the mature frond (Yamada et al. 2007), which combined with the fern availability increases the risk of animal poisoning (Alonso-Amelot et al. 1992a; Alonso-Amelot et al. 1993).

1.5. Human exposure to *Pteridium aquilinum*

Over the centuries, the use of *Pteridium aquilinum* as human food has been described, mostly in times of food shortage, like during the First World War – a sort of flour was made out of the rhizomes. The fronds have also been used as bedding for animals, floor cover, fuel, as ornamental and ritual plant, as bleaching and dying agent, for dykes, roofing, baskets, mulch and compost (Rasmussen 2003). Nevertheless, in Canada, parts of the United States of America, China and specially, Japan, bracken fern is grown commercially for human use, as food or herbal remedy (Fenwick 1988). The raw fern is usually processed in some manner, *e. g.* with boiling water and soda ash treatment, which reduces the carcinogenic activity (Smith & Seawright 1995; Fenwick 1988).

Indirect contact with ptaquiloside may also occur. Evans et al. and Alonso-Amelot et al. reported the discovery of this carcinogenic principle in milk and meat from animals that feed on bracken (Evans et al. 1972; Alonso-Amelot et al. 1993; Recouso et al. 2003; Fletcher et al. 2011) and in 1988, it was stated that rain might leak carcinogens from the plant and eventually passage them to water supplies (Fenwick 1988). Additionally, the inhalation of bracken spores can expose humans to the toxic effects of bracken fern (Potter & Baird 2000).

The contact with bracken by humans has been associated with a greater occurrence of upper oesophageal carcinoma (Smith & Seawright 1995), stomach cancer (Alonso-Amelot & Avendaño 2001) and respiratory neoplasias (breathing of spores) (Alonso-Amelot & Avendaño 2002) (Figure 5).

The results of the animal tests are quite conclusive, demonstrating the carcinogenicity of bracken in general and of ptaquiloside in particular. The evidence for human cancer is less conclusive, although studies indicate higher abundance of cancer in areas with bracken abundance. These studies should be treated with caution as they include very small populations (Wilson et al. 1998; Brown et al. 1999). Nevertheless, Marliére et al. showed a significant higher occurrence of oesophageal cancer among people eating bracken than people not ingesting bracken (Marliére et al. 2002).

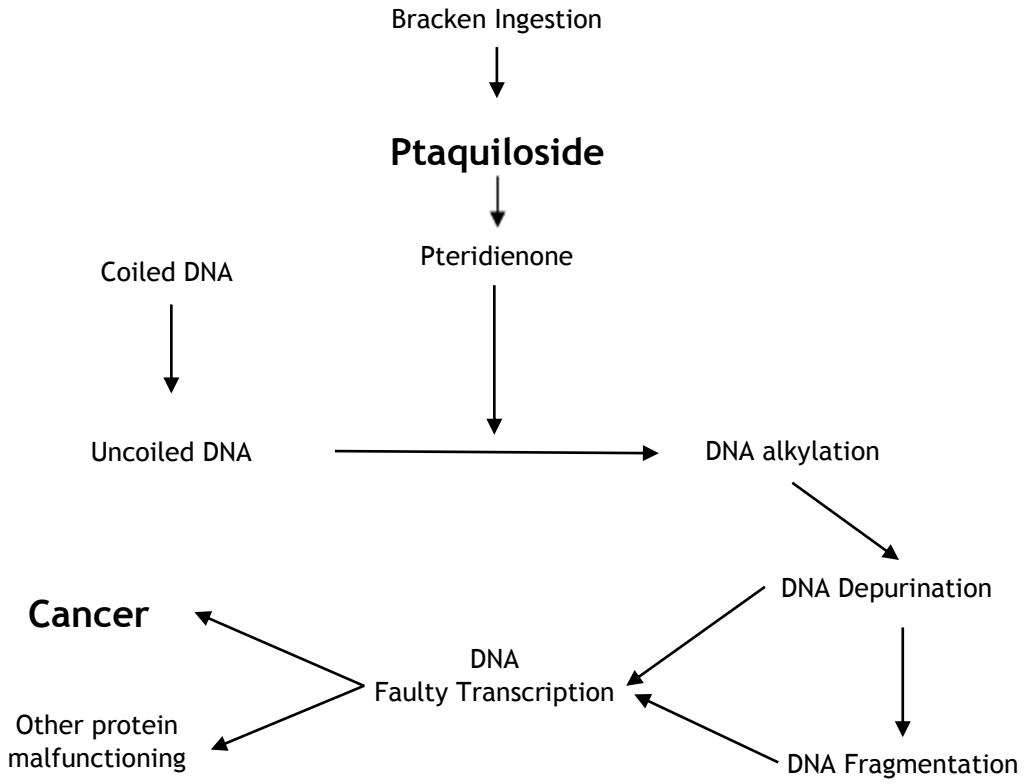

Figure 5 - Consequences of bracken ingestion. Adapted from (Alonso-Amelot & Avendaño 2002).

1.6. Main Objective

Studies of the toxicological effects of ptaquiloside on animals and, potentially, on humans are dependent on ptaquiloside isolation from the fern. However, the available isolation methods are extremely time-consuming and present low efficiency. At LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy) efforts are being made to develop a faster and more efficient method for the isolation of ptaquiloside from *P. aquilinum*.

A key aspect for the implementation of these methods and assess their efficiency is the quantification of ptaquiloside present in the plant and the quantity obtained by the isolation method, as well as during the stages of the procedure. Thus, the development of an appropriate method for ptaquiloside quantification becomes necessary. The present work aimed to develop such a method, in the context of the ongoing research at LEPABE.

2. State of the Art

The global economic importance of *Pteridium aquilinum*, due to its abundance, its toxicity to animals and potential affect to human health through production of carcinogens (McGlone et al. 2005), has resulted in extensive scientific literature, most of which with the objective of isolating ptaquiloside. According to the objectives of this work, the literature review will only focus on the quantification methods of ptaquiloside.

Throughout the years, a variety of methods were developed with the objective of quantifying ptaquiloside, as described in the Appendix. The majority of these methods are based on the one originally developed by Agnew & Lauren (1991).

Due to the extremely unstable nature of the compound, direct analysis is problematic. Also, treatments with acid, base, light or heat should be evaded in the clean-up steps of the analysis (Saito et al. 1989). Additionally, ptaquiloside content in bracken can vary greatly with species, growth stage, altitude and within stands grown in analogous conditions (Ramwell et al. 2010). As a result of ptaquiloside degradation by heat, it is of extreme importance the use of fresh frond material without drying, in order to obtain the best yields of this compound (Alonso-Amelot et al. 1992b).

2.1. Quantification Methods

Firstly, in 1987 a modified mutagenicity test was applied to quantify ptaquiloside in the crude extract of *Pteridium aquilinum* (Matoba et al. 1987). In 1989, a new method of quantification was applied, using thin layer chromatography (TLC) coupled with photodensitometry (Saito et al. 1989). This technique is based on the ptaquiloside instability, since the sample was applied on a TLC plate at 110ºC during 2 hours, allowing ptaquiloside to convert into pterosin B. Nonetheless, TLC was inadequate for ptaquiloside quantification, since the complete transformation of ptaquiloside and the single production of pterosin B were not established (Alonso-Amelot et al. 1992a).

High pressure liquid chromatography (HPLC) offered higher sensitivity over TLC for the quantification of ptaquiloside and pterosin B (Alonso-Amelot et al. 1992b). In this work of 1992, Alonso-Amelot and co-workers successfully applied high pressure chromatography for the quantification of ptaquiloside and pterosin B. They were able to produce a good compound separation and using a mobile phase of CH_3OH:H_2O (65:35, v/v) it was possible to obtain retention times around 10 minutes for pterosin B (Alonso-Amelot et al. 1992b).

In 2004, Bonadies et al. 2004 suggested the advantages of using gas chromatography-mass spectrometry (GC-MS) to analyse trace amounts of ptaquiloside in a complex mixture as a bromo-compound derived from ptaquiloside. The bromo-compound is exclusively derived from ptaquiloside, so the advantage of quantifying this compound instead of pterosin B is that it does not exist in the original sample, so it is not necessary to perform the initial analysis of the sample (Bonadies et al. 2004).

In the Appendix, only quantification methods of ptaquiloside are described. HPLC is the most used method in literature (Ayala-Luis et al. 2006). Although ptaquiloside can be directly quantified (at 220 or 214 nm), a recurring method is to convert ptaquiloside into pterosin B, through a base-acid treatment, following by analysis at 214, 220 or 260 nm (Jensen et al. 2008) due to ptaquiloside's instability (Rasmussen 2003; Yamada et al. 2007). This treatment is also preferred to the heat treatment due to its mildness (pH 11.5, 40 ºC) and completeness within a short period of time (Alonso-Amelot et al. 1992a). Also the UV response is duplicated, lowering the detection limit (Jensen et al. 2008) and allowing the determination of low levels of ptaquiloside (Agnew & Lauren 1991).

Agnew & Lauren (1991) also studied the reaction conditions required to convert ptaquiloside to pterosin B in either acid or basic conditions (Figure 6). In the first case, they discovered that this reaction gave two or more products, while the second only converted ptaquiloside to the pteridienone followed by acidic treatment to yield pterosin B.

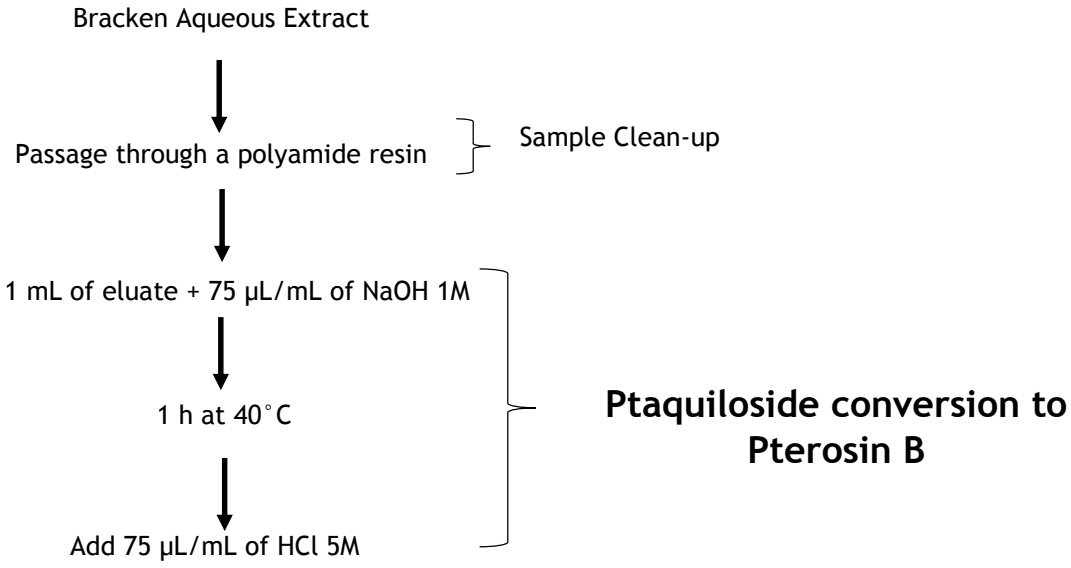

Figure 6 - Representation of Agnew and Lauren (1991) conversion method.

The method chosen by Agnew & Lauren (1991) adds sodium hydroxide (NaOH) to the sample and, after the reaction occurs, adds hydrochloric acid (HCl). However, a modified method is now preferred, because HCl can produce unwanted chloro-analogues, so HCl is substituted by sulphuric acid (H_2SO_4) (Fletcher et al. 2011; Bonadies et al. 2011). Nevertheless, this method only decreases the concentration of chloro-analogues, since the chloride ion is naturally present in the plant and acts as nucleophile to give the chloro-derivative in a significant yield (Bonadies et al. 2004).

The purpose of the clean-up step is to remove all pterosin B present in the sample extract, by passing it through a polyamide resin, in order to only quantify the pterosin B obtained by ptaquiloside conversion (Agnew & Lauren 1991; Jensen et al. 2008; Rasmussen et al. 2013). Alternatively to this clean-up step, an initial analysis of the sample can be performed to assess the quantity of pterosin B present in the plant due to light-induced decomposition of ptaquiloside (Alonso-Amelot et al. 1992a). The difference between pre- and post-conversion values corresponds to pterosin B formed from ptaquiloside during the conversion step.

As described in Table 3, the majority of researchers preferred the quantification by HPLC, although some use different types of detectors, as ultraviolet detector or a combination of ultraviolet with visible light. Several studies used acetonitrile in water or a methanol in water gradient as the mobile phase (Alonso-Amelot et al. 1993; Rasmussen et al. 2013), these being the majority of mobile phases used in ptaquiloside and pterosin B quantification.

In the literature, some reports used mass spectrometry (MS), because substances with a similar retention time and UV-VIS spectrum may be misidentified as the compound in question. As a result, there is a risk for false positive as well as negative analytical results using unspecific detection, such as UV-VIS. Mass spectrometric detection is preferable because it provides the required specificity and a lower limit of detection (Jensen et al. 2008). As previously mentioned, some authors also use GC-MS for ptaquiloside quantification, after conversion to pterosin B (Bonadies et al. 2004).

Throughout the years, researchers have analysed samples of bracken fern from around the world and quantified their ptaquiloside content. In Table 4, the ptaquiloside concentrations are summarized.

Ptaquiloside content in fronds of different varieties of bracken varies between 0 and 15.1 mg/g (Rasmussen 2003). Although there seems to be no significant differences between varieties (Smith et al. 1994), the genetic inheritance can impact the ptaquiloside content once different proveniences still have different ptaquiloside contents when grown under the same conditions (Smith et al. 1994; Rasmussen, Kroghsbo, et al. 2003). Ptaquiloside content is usually at is maximum just after the croziers arise from the ground and the content decreases progressively during growth season (Rasmussen 2003).

Table 3 - Main characteristics for Pta/PtB HPLC quantification.

Reference	Pta/PtB Quantification		
	Detector	**Column**	**Mobile Phase**
Agnew & Lauren 1991	UV	ODS 5 µm or C8 (25 cm x 4.6 mm I. D.), RP-8 guard column	H_2O: CH_3OH (60:40, v/v) for Pta H_2O: CH_3OH (40:60, v/v) for PtB
Alonso-Amelot et al. 1993	UV-VIS	C-18 Radial 0.5 x 10 cm	CH_3OH:H_2O (65:35, v/v) in the isocratic mode
Dawra et al. 2002	UV-VIS	C-18 Reverse-phase (4.6 x 250 mm)	C_2H_3N:H_2O gradient (20:80, v/v for first 20 min followed by increase to 100% acetonitrile in the next 20 min)
Rasmussen 2003	UV-VIS	RP-8, 5 µm (125 x 4 mm) and 4x4 mm I.D. RP-18e, 5 µm safe guard column	H_2O: C_2H_3N gradient
Rasmussen et al. 2005	UV-VIS	100 RP-8, 4 µm (125 x 4 mm)	MillQ-H2O: C_2H_3N (92:8, v/v) for Pta Double deionised H_2O: C_2H_3N (83:17, v/v) for PtB
Ayala-Luis et al. 2006	UV-VIS	100 RP-8, 4 µm (125 x 4 mm), safe guard column 100 RP-18e, 4 x 4 mm	Isocratic mode: C_2H_3N: H_2O (18:82, v/v) Gradient mode: start with the isocratic mode and after 10 min, a gradient was applied until reaching C_2H_3N: H_2O (55:45, v/v)
Latorre 2010	UV	ODS (4 mm x 150 mm, Shimadzu), C18 reverse phase safe guard column	H_2O: CH_3OH: 40% CH_3OH (0-2 min); 40-80% CH_3OH (2-15 min);80-40% CH_3OH (15-16 min);40 CH_3OH (16-25 min)
Fletcher et al. 2011	UV-Vis	C18 column 250 x 4.6 mm, 5 µm	Mixture of (A) 40:60 C_2H_3N in water (v/v) and (B) C_2H_3N 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.
Rasmussen et al. 2013	UV-VIS	100 RP-8, 4 µm (125 x 4 mm)	H_2O: C_2H_3N (69:31, v/v)

Table 4 - Ptaquiloside content in *Pteridium aquilinum*.

Reference	Country	Sample	Pta content
Saito et al., 1989	Japan	Fronds	0.02 – 0.14 % dry weight
		Rhizomes	0.03 – 0.12 % dry weight
Agnew & Lauren 1991	New Zealand	Bracken Fern	0.03 mg/g – 4 mg/g dry weight
Alonso-Amelot et al. 1992b	Venezuela	Large fronds	1.9 mg/g
		Young croziers	
		Smaller or mature fronds	0.07 mg/g
Alonso-Amelot et al. 1992a	Venezuela	Crozier	1.6 mg/g
		Mature Frond	0.113 mg/g
		Rhizomes	0.0046 mg/g
Smith et al. 1994	Australia	*Pteridium* samples from around the world	0 – 9.776 mg/kg
Castillo et al. 1998	Venezuela	Fresh fronds	0.007 mg/g
Rasmussen 2003	Denmark	Mature fronds	0.110 – 4.450 mg/g dry weight
Rasmussen, Kroghsbo, et al. 2003	Denmark	Mature Fronds	0.108 – 3.795 mg/g
Rasmussen, Jensen, et al. 2003	Denmark	Fronds	0.213 – 2.145 mg/g
		Rhizomes	0.011 – 0.902 mg/g
Rasmussen et al. 2013	Denmark	Spores	Maximum of 0.029 mg/g

3. Materials and Methods

3.1. Overall Methodology

The aim of this work was to quantify the ptaquiloside content in bracken fern samples and in samples obtained throughout ptaquiloside isolation process.

As stated before, ptaquiloside analysis is challenging due to the extremely unstable nature of the compound. On the other hand, this feature can be used to develop an indirect quantification method based on the ptaquiloside conversion to pterosin B. Pterosin B is present in the plant due to light-induced decomposition of ptaquiloside and with this method pterosin B concentration is increased, taking advantage of ptaquiloside instability in water, acid and base.

Ptaquiloside quantification will be carried out by calculating the difference between the initial content of pterosin B in the samples and the content present after ptaquiloside conversion to pterosin B.

Thus, the implementation of pterosin B quantification chromatographic conditions and the validation of HPLC quantification of pterosin B was performed. Afterwards, ptaquiloside conversion to pterosin B was implemented, as well as the validation of the ptaquiloside quantification method, employing samples obtained from previous work (Gil da Costa 2011).

Ultimately, this quantification method was used to monitor a new cycle of ptaquiloside isolation, allowing a faster and more efficient processing of bracken samples.

3.2. Bracken Samples

The research carried out in this work focused on the bracken variety *Pteridium aquilinum*. Bracken crozier samples were collected in the mountainsides of Arcos de Valdevez, Portugal, 41º 49’ 12’’ N, 8º 24’ 11’’ W, a location where BEH is known to occur. For the method implementation phase, the samples used were collected in April 2010 (PA - 2010) and for the monitoring stage the samples were collected in April 2014 (PA - 2014). Bracken samples were frozen at -20ºC until use.

3.3. Reagents and standard solutions

Pterosin B was obtained in previous work done by Gil da Costa (2011) by isolation from *Pteridium aquilinum* and possessed a purity superior to 99%, determined by nuclear magnetic resonance (NMR). HPLC grade reagent methanol (CH_3OH) was obtained from VWR International and distilled water was used, filtered with Sartolon® polyamide membrane filters (diameter: 47 mm; pore dimension: 0.45 µm) from Sartorius AG. All other reagents used were of analytic or HPLC grade. Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 15 minutes.

Stock standard pterosin B solutions were prepared in methanol at 200 µg/mL. Working standards in the range of 1 - 80 µg/mL were obtained by dilution with methanol. Stock solutions were stored at -18 °C and standard solutions at 5 °C. Ptaquiloside is stable for at least 6 months in methanol at -5°C (Oelrichs et al. 1995).

3.4. Instrumentation

The HPLC used for the quantification was a JASCO modular system with a pumping system (PU-2080 Plus Intelligent), a ternary gradient unit (LG-2080-02), a degasser (DG-1580-54 4-Line), a multiwavelength detector (MD-2015 Plus), a sampler (AS-2057 Plus Intelligent), a LC-NetII/ADC and a Column Thermostat (CO-2060 Plus Intelligent). The analytical column (Inertsil® ODS-3 5 µm; 250 x 4.6 mm I. D.) was preceded by a C18 4x3.0 I. D. Security Guard™ (Phenomenex®) guard column. The column was maintained at 25 °C in the column oven. Detection was performed at 220 nm, maximum wavelength of pterosin B (Alonso-Amelot et al. 1992a). All the samples were filtered through nylon filters (diameter: 13 mm; pore dimension: 0.22 µm) from Nantong FilterBio Membrane Co., Ltd prior to injection.

Also, for sample processing VirTis benchTop K freeze dryer and Büchi RE 111 Rotavapor with a Büchi 461 Water Bath were used.

3.5. Experimental Procedure to Ptaquiloside Isolation from *Pteridium aquilinum*

The procedure here described was performed according to the one from previous work (Gil da Costa 2011). Sample PA - 2010 was processed in this previous work and sample PA - 2014 is currently being processed, in accordance with the following procedure.

Aqueous Extraction: a water-based method, designed for ptaquiloside isolation from bracken, was initially adopted, and afterwards modified (Oelrichs et al. 1995). Frozen bracken (6 Kg), was blended in distilled water (7.5 L) and stirred at room temperature for 1 hour.

Aqueous Extract Adsorption-Desorption: the aqueous extract (7 L) was filtered through a series of strainers and adsorbed onto a 1 L resin column, Amberlite XAD-2 (Supelco), at an

average flow of 10 mL/min. The adsorbed compounds were eluted with methanol (1.5 L), through a series of adsorption-desorption cycles in order to maximize the removal of all relevant compounds. A final desorption with water was performed.

Extraction with Butanol: the methanol extract was concentrated at low temperature (below 50ºC) under reduced pressure, dissolved in distilled water (400 mL) and extracted with *n*-butanol (5 x 500 mL).

Butanolic Extract Fractioning with Silica Gel Column Chromatography: the *i*-butanol extract was concentrated at low temperature (below 50ºC) under reduced pressure, and chromatographed on silica gel 60 (0.2-0.5 mm, Merck), collecting 500 mL fractions with $CHCl_3$ (5 L), EtOAc (6 L), EtOAc – CH_3OH in different percentages (%, v/v, 3 L): 92:8; 75:25; 50:50; 25:75 and CH_3OH (3 L). All fractions were controlled for the presence of ptaquiloside by ^{1}H and ^{13}C NMR, with a Brucker Avance III 400 spectrometer. Samples dissolved with adequate deuterated solvents.

Purification on ODS-silica gel Column Chromatography: the fractions found to contain ptaquiloside were chromatographed twice on octadecyl-sylane (ODS) silica gel (C18 100-40/75, Fuji-Silysia) with CH_3OH -H_2O (20:80 and 40:60, %, v/v) and CH_3OH.

3.6. Development and Validation of Ptaquiloside Quantification Method

3.6.1. Chromatographic Conditions Studies to Pterosin B Quantification

In order to assay time retention variation of pterosin B with different mobile phase compositions, different compositions were used, from CH_3OH:H_2O (60:40 %, v/v) until CH_3OH:H_2O (80:20 %, v/v), using the same sample of known concentration (25 µg/mL). Analysis time was 30 minutes to all the experiments. A flow rate of 1 mL/min was used and all samples were measured in quadruplicate.

Determination of ***linearity*** was made by calculation of a regression line from the peak area *vs* concentration plot for six standard solutions (1, 5, 10, 25 and 80 µg/mL).

The ***limit of detection*** (LOD) was expressed as (ICH 2005):

$$\mathbf{LOD = 3.3\ sb/a} \qquad (1)$$

where **sb** is the error associated with the intercept and **a** is the slope of the calibration curve. The LOD was studied using samples from the calibration curve.

The ***limit of quantification*** (LOQ) was expressed as (ICH 2005):

$$\mathbf{LOQ = 10\ sb/a} \qquad (2)$$

where **sb** is the error associated with the intercept and **a** is the slope of the calibration curve. The LOQ was studied using samples from the calibration curve, according to ICH guidelines (ICH 2005).

The ***specificity*** of the analytical method was ascertained with samples of pterosin B submitted to conditions of thermal, acidic and alkaline stress. To assess thermal degradation, a known concentration (25 µg/mL) of pterosin B was placed in an oven at 80ºC for 24 h. Then, the samples were filtered and subjected to HPLC analysis. For evaluation of pterosin B degradation in acidic and alkaline conditions, 100 µL of a known concentration (25 µg/mL) of pterosin B was mixed with 100 µL of 2.5 M H_2SO_4 and with 100 µL of 1 M NaOH. Following 36 h at room temperature, the samples were filtered and subjected to HPLC analysis. Control samples (with CH_3OH) were also prepared and assayed.

With the objective of confirming the ability of the method to detect pterosin B in samples, a standard of known concentration (200 µg/mL) was added to a sample. This solution was filtered and injected.

In order to verify the method specificity when using complex samples rather than just standards, a peak enrichments of extracts with known amounts of standards was performed. 0.3 mL of a sample were added to 0.3 mL of a standard (200 µg/mL). The analysis was performed in the conditions stated above.

Accuracy was evaluated using a minimum of nine determinations over a minimum of three concentrations levels (5, 25 and 80 µg/mL) and by calculating the relative standard deviation (RSD).

Repeatability (intra-assay precision) of the chromatographic method was investigated by the analysis on the same day of five pterosin B standard solution (three replicates each). Intermediate precision was studied by the analysis of the same standard solutions on three different days, and described as SD and RSD.

The total analytical method repeatability was determined by executing six replicate samples of the same sample containing pterosin B.

For calculations and statistic determination Microsoft® Excel was used.

Quantification of pterosin B in samples from previous ptaquiloside isolation procedure: in order to validate this method for pterosin B quantification in complex samples, a sample known to possess this compound, previously obtained by column chromatography (PA 2010), was analysed.

The mobile phase was methanol-water (70:30 %, v/v) for pterosin B. The flow rate was 1 mL/min. A 20-µL sample injection volume was used for all analysis. Detection was performed at 220 nm, maximum wavelength of pterosin B. Concentration calculations were based on area measurements relative to standards of concentrations in the range of 1-80 µg/mL. JASCO ChromPass Chromatography Data System software managed chromatographic data.

3.6.2. Studies on Ptaquiloside Conversion to Pterosin B

In order to validate this method for ptaquiloside quantification, samples controlled for the presence of ptaquiloside by ^{13}C NMR, from previous work (Gil da Costa 2011), obtained using column chromatography, were analysed.

Preparation of sample solutions for Pterosin B determination before conversion: samples were collected from each step of the isolation procedure. For the samples in methanol, solvent was evaporated under a current of nitrogen, the sample was weighted and the residue re-dissolved in HPLC-grade methanol. Aqueous samples were freeze dried, weighted and re-dissolved in water. In order to assess the initial content in pterosin B, 100 µL of sample were diluted in 1050 µL of CH_3OH. Samples were filtered and injected.

***Ptaquiloside to Pterosin B Conversion*:** 100 µL of the sample were diluted in 900 µL of water and treated with 75 µL of 1 M NaOH and allowed to react for 1 h at 40 °C. Afterwards, 75 µL of 2.5 M H_2SO_4 were added. The concentration was calculated on the basis of 100% conversion of ptaquiloside to pterosin B.

Chromatographic Analysis: the mobile phase was methanol-water (70:30 %, v/v) for pterosin B. The flow rate was 1 mL/min. A 20 µL sample injection volume was used for all analysis. The mobile phase was selected to result in a retention time of approximately 8-9 min for pterosin B. Detection was performed at 220 nm. Concentration calculations were based on area measurements relative to standards of concentrations in the range of 1-80 µg/mL. JASCO ChromPass Chromatography Data System software managed chromatographic data. In order to validate the calibration curve obtained, a standard (25 µg/mL) was injected daily.

Ptaquiloside Determination: with the initial concentration of pterosin B and the concentration after conversion, it is possible to calculate ptaquiloside concentration in the sample. Knowing the initial weight of the analysed samples the concentration of ptaquiloside is expressed in % (w/w). For expressing the concentration of ptaquiloside in terms of dry weight basis, moisture content of bracken fern was estimated keeping a known quantity of fern in an oven at 120ºC until constant weight. The result was expressed as mg/g.

3.7. Monitoring of Ptaquiloside Isolation Method

After validation of the quantification method, the same methodology described above was applied to the samples collected in April 2014 (PA - 2014), with the objective of optimizing the

number of adsorption-desorption cycles in the isolation process. Samples analysed are described in Table 5.

As stated in Table 6 the number of adsorption-desorption cycles varied with the extraction experiment.

Table 5 - Samples analysed to monitor the extraction process, from PA – 2014 bracken samples.

Extraction	Sample	Description	Weight (mg)
A	A1	Aqueous Extract	26
	A2	1st cycle of methanol adsorption-desorption	15
	A3	2nd cycle of methanol adsorption-desorption	2
	A4	3rd cycle of methanol adsorption-desorption	4
	A5	4th cycle of methanol adsorption-desorption	2
	A6	5th cycle of methanol adsorption-desorption	7
	A7	Final Aqueous Extract	10
B	B1	Aqueous Extract	20
	B2	1st cycle of methanol adsorption-desorption	18
	B3	2nd cycle of methanol adsorption-desorption	20
	B4	3rd cycle of methanol adsorption-desorption	19
	B5	Final Aqueous Extract	19
C	C1	Aqueous Extract	22
	C2	1st cycle of methanol adsorption-desorption	6
	C3	2nd cycle of methanol adsorption-desorption	2
	C4	3rd cycle of methanol adsorption-desorption	1
	C5	Final Aqueous Extract	17

Table 6 - Sample collection and variation of adsorption-desorption cycles.

	Date of Sampling	Extractions	Weight (kg)	Adsorption-desorption cycles
PA - 2010	April 2010	1	11	7
PA - 2014	April 2014	1	6	5
		2	6	3
		3	6	3

4. Results and Discussion

4.1. Development and Validation of Ptaquiloside Quantification Method

4.1.1. Chromatographic Conditions Studies to Pterosin B Quantification

Based on the literature, several proportions of mobile phase CH_3OH: H_2O were studied in order to obtain a satisfactory retention time, varying from CH_3OH: H_2O (60:40, v/v) to CH_3OH:H_2O (80:20, v/v). In this study, the injected standard had a concentration of 25 µg/mL, the analysis time was 30 minutes and all the injections were performed in duplicate.

With mobile phase composition CH_3OH:H_2O (60:40, v/v) a peak around 21 minutes was obtained (Figure 7a). For the quantification of numerous samples this is a long time of analysis. Since with increasing methanol the mobile phase polarity decreases and with decreasing polarity, pterosin B elutes faster (since a reverse phase column was used), a less polar mobile phase was tested.

For CH_3OH:H_2O (70:30, v/v), as it would be expected, a decreasing of the retention time occurs and pterosin B is eluted at approximately 10 minutes (Figure 7b). This is a more adequate time of analysis for compound quantification and with this mobile phase composition there is no other interference peaks in the surroundings. Also, the peak signal suffers an intensification and a narrowing (increased resolution), as anticipated.

This is a mobile phase composition that fulfils all the criteria for compound quantification, with an acceptable retention time and with a good separation factor, that also allows compound separation.

As can be observed in Figure 7c, with an increase in methanol (CH_3OH:H_2O (80:20, v/v)) a further decrease in retention time occurs (around 7 minutes). Although it is a shorter analysis time, the pterosin B peak is too close to the mobile phase peaks, which implies a decrease in the separation factor. This parameter measures the analysis selectivity for two adjacent peaks.

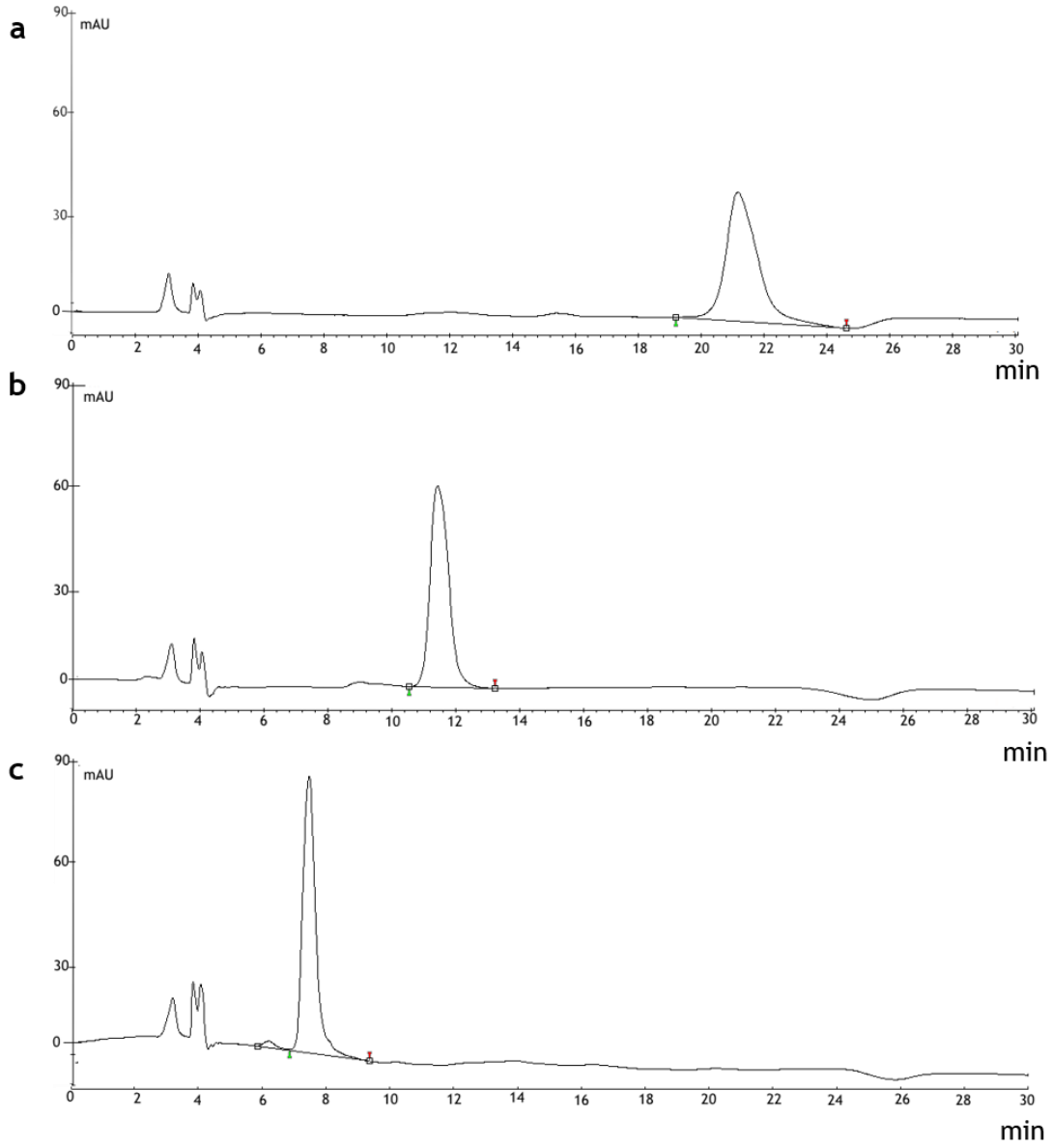

Figure 7 - Mobile phase analysis chromatograms. a: CH_3OH:H_2O (60:40, v/v); b: CH_3OH:H_2O (70:30, v/v); c: CH_3OH:H_2O (80:20, v/v).

In Table 7 the variation of retention time with the variation of the mobile phase composition can be observed.

Table 7 – Pterosin B retention time with mobile phase variation.

Mobile Phase (CH_3OH:H_2O, v/v)	**Retention time (minutes)**
60:40	21
70:30	11
80:20	7

The mobile phase composition that provides the lower retention time is CH_3OH:H_2O (80:20, v/v), however the separation factor obtained in this conditions is lower than the one of CH_3OH:H_2O (70:30, v/v).

Best conditions were achieved with CH_3OH:H_2O (70:30, v/v). A flow rate of 1 mL/min gave an optimal signal to noise ratio and a reasonable separation time. The maximum absorption of pterosin B in the experimental conditions was found at 220 nm (Agnew & Lauren 1991; Ayala-Luis et al. 2006; Somvanshi & Lauren 2006; Jensen et al. 2008; Fletcher et al. 2011), which was the selected wavelength for the analysis. These conditions are adequate to isolate and quantify the compound and they provide a suitable time of analysis (around 15 minutes).

The method has an adequate ***linearity*** over the range of 1-80 µg/mL. The regression equation was: y=0.7009(±0.0195)x-0.3841(±0.7804). The square of correlation coefficient (R^2) was 0.9969 and the correlation coefficient (R) was 0.9985. The calibration curve can be found in the Appendix B.1. The calibration curve obtained also satisfies all the criteria for validation (the slope relative standard deviation lower than 5% and b-sb < 0 < b+sb, in order to guarantee that for null concentration a null response is obtained) (Skoog et al. 1992).

The ***limit of quantification*** was determined to be 11.13 µg/mL and the ***limit of detection*** was 3.67 µg/mL. Other authors were able to achieve smaller LOD, such as 0.5 µg/mL (Alonso-Amelot et al. 1998), 0.0003 µg/mL (Bonadies et al. 2011) and 0.1 µg/mL (Rasmussen et al. 2005). The limit of detection is the concentration corresponding to a signal to noise ratio of 3:1. Therefore, there are only two things that can be done in order to improve the LOD of a HPLC method: increase the signal or decrease the noise. The signal can be increased by operating at the optimal UV wavelength of the analyte. As mentioned before pterosin B has maximum wavelengths at 214, 220 and 260 nm. In this work analysis at 260 nm was tested and at this wavelength the signal intensity was decreased when compared with 220 nm. For future work a calibration curve at 214 nm should be tested to verify if the signal intensity can be increased. To diminish the noise of the analysis other mobile phase solvents should be tested, and comparing with the literature other alternative for methanol (cutoff 205 nm) is acetonitrile (cutoff 190 nm) (Pavia et al. 2001).

The ***specificity*** of the method was evaluated by comparing the chromatograms of standard pterosin B with the chromatograms resulting from the stress conditions (temperature (80ºC), 1 M H_2SO_4 and 1 M NaOH).

When pterosin B standard (25 µg/mL) was submitted to thermal, acidic and alkaline stress conditions the chromatograms remain similar to the chromatogram of the standard solution. For acid and alkaline conditions a peak with the retention time of approximately 2.5 minutes appeared due to the addition of acid and base. Additionally, the absence of any peak in the region of pterosin B elution time indicates the specificity of the developed method, regarding the conversion interferents. In general, the data acquired gives evidence that the method can be considered as specific since no interfering peaks were observed.

The ***accuracy*** of this method was also tested and detailed results for the three concentration levels tested are presented in the Appendix B.2. The mean recovery was found to be 104.93±8.49 %, revealing a solid agreement between theoretical and experimental values.

Repeatability (intra-day precision) and intermediate precision (inter-day precision) of the developed method can be consulted of the Appendix B.3. As can be observed in the Appendix B.3, all of the relative standard variations were inferior to 10%, showing that the method has intra-day and inter-day precision.

The analytical method is effective, fast and meets all criteria for method validation and can be applied to the quantification of pterosin B.

Simple sample preparation procedure and retention time lower than 10 minutes allow the quantification of several samples in a short period of time. The suggested method is free from interferences of other compounds used in the conversion method and no degradation of the compound was found.

The quantification of pterosin B in samples from previous ptaquiloside isolation procedure was performed in order to assess this method validity analysing, one sample derived from the purification with ODS step of the isolation process (PA – 2010).

The chromatogram of the sample with addition of the standard was compared with the one of sample without addition of standard. In Figure 8, a comparison between the two chromatograms is made (black represents the sample and red represents the sample with standard addition). At 7 minutes there is an increase in the peak of pterosin B, which confirms the presence of this compound in the analysed sample. Due to the standard addition, a decrease in the remaining peaks can be observed, once this compounds were diluted while pterosin B was concentrated.

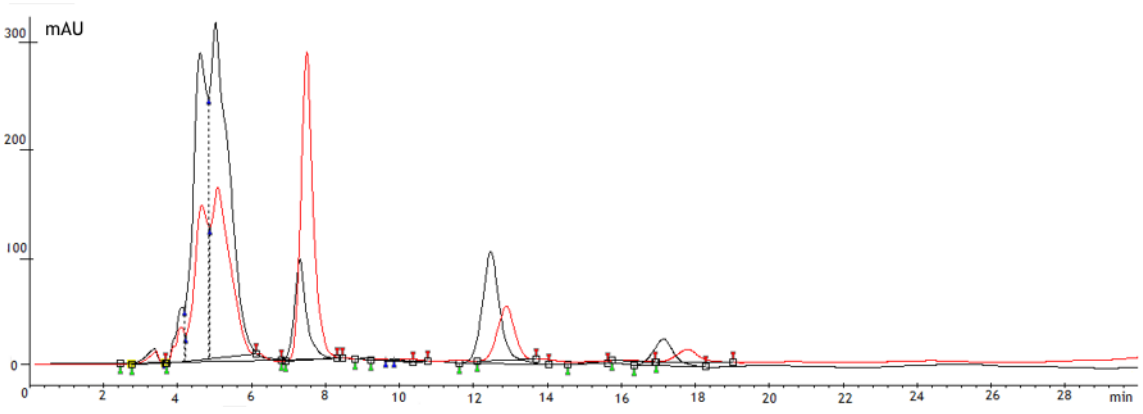

Figure 8 - Comparison of the sample with and without addition of a standard. Black: sample without standard; red: sample with addition of standard.

4.1.2. Studies on Ptaquiloside conversion into Pterosin B

To assess the application of this quantification method, samples of the isolation performed by Gil da Costa (2011) were used for its implementation. Nine samples, representing different stages of the isolation process, were chosen from this previous work (Figure 9). These samples correspond to: aqueous extract (sample A), methanolic eluate from the adsorption-desorption step of the extract from the Amberlite resin (sample B), butanolic extract obtained after Bu-H_2O partition of dried methanolic extract (sample C), two EtOAc/CH_3OH fractions from preparative chromatography (stationary phase –ODS silica gel) (samples D and E) and four fractions derived from sequential purifications in preparative chromatography (stationary phase – ODS silica gel) (samples F to I).

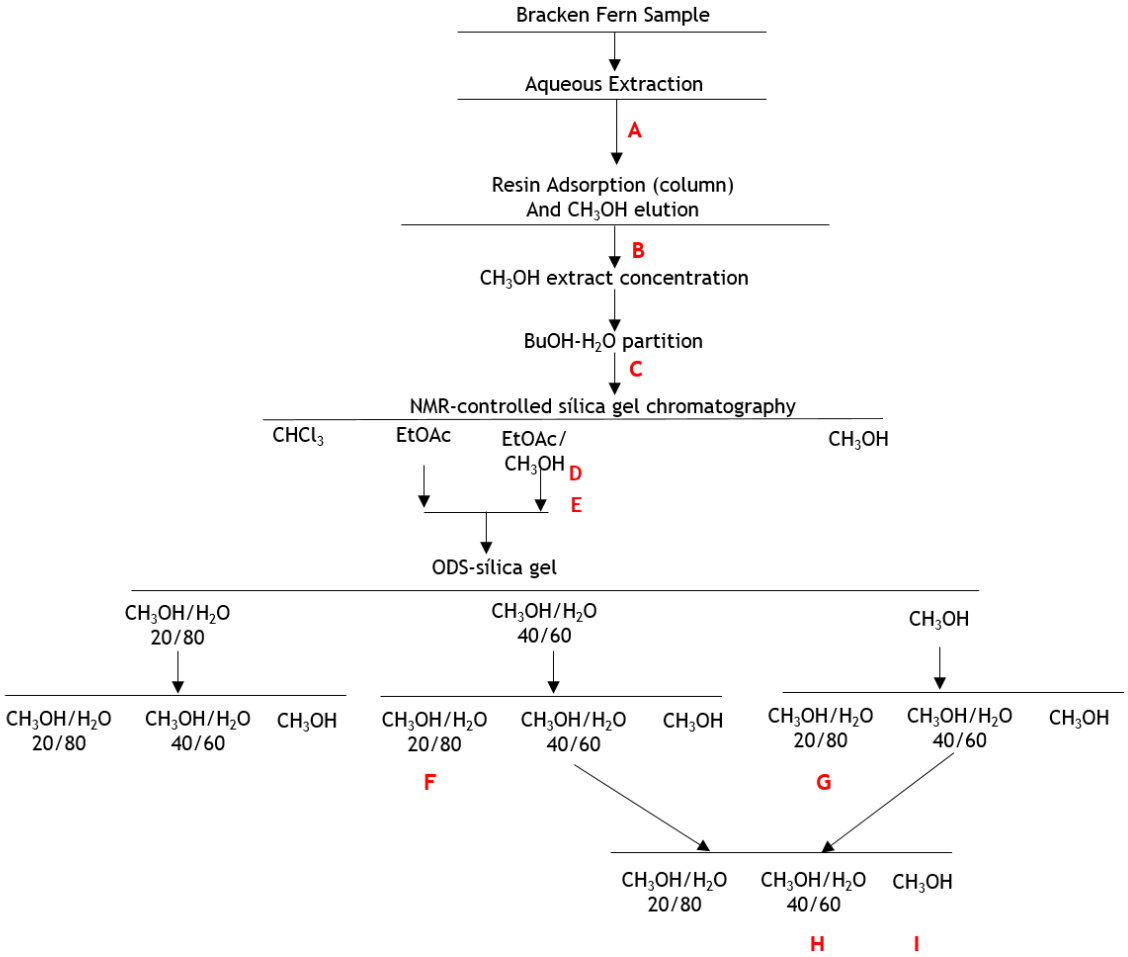

Figure 9- Ptaquiloside isolation method from bracken. Adapted from (Gil da Costa 2011)

All the fractions obtained during the chromatography steps were controlled by ^{13}C NMR, in order to detect ptaquiloside and/or pterosin B. This is possible due to the existence of two signals around δ 6 and 11 ppm, very unusual and characteristic of the ptaquiloside chemical family, corresponding to the carbons C-12 and C-13, respectively. These signals do not appear in the pterosin B spectrum and the corresponding signals appear at δ 32 and 62 ppm. The ^{13}C NMR spectra (attached proton test (APT) technique) of ptaquiloside (a), pterosin B (b) and of

sample G (c) are presented in Figure 10. NMR data related to ptaquiloside and pterosin B is presented in the Appendix C.

As can be observed sample G contains the characteristics signals of ptaquiloside around δ 6 and 11 ppm, confirming the presence of this compound in the sample while in pterosin B those carbons appear at δ 32 and 62 ppm, respectively. Additionally the presence of a signal between δ 200-220 ppm typical of the ketone carbonyl carbon is detected. This type of carbon can be found not only in ptaquiloside but also in pterosin B and corresponds to carbon C-1 and this signal corresponds to the carbon 1 both in ptaquiloside and pterosin B. However, in pterosin B this signal occurs at inferior values (around δ 210 ppm) of ptaquiloside (around δ 220 ppm), due to the conjugation of the carbonyl group with the aromatic ring present in pterosin B. These signals allow an assessment of ptaquiloside degradation in samples during storage/processing.

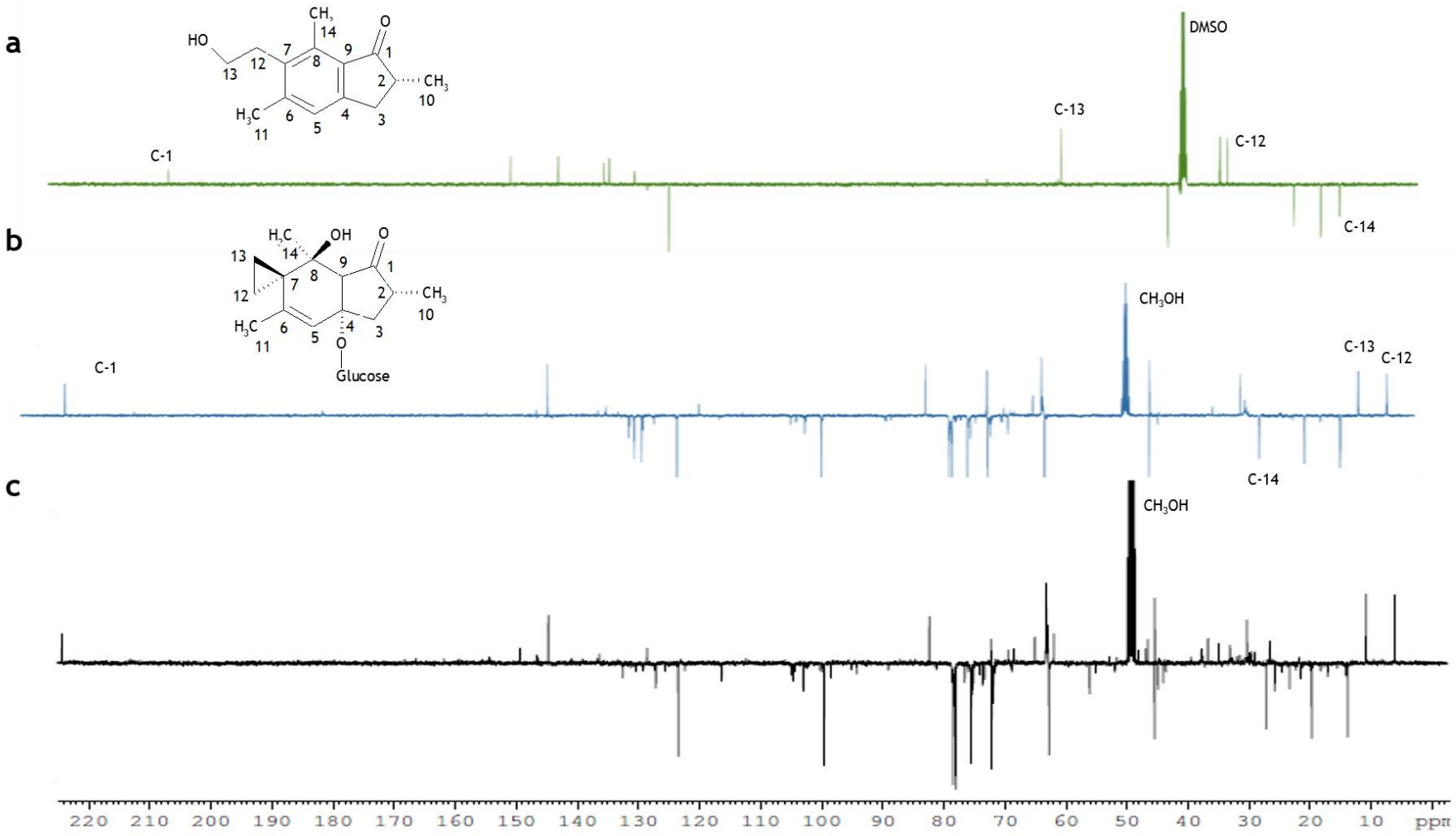

Figure 10 - ^{13}C NMR spectra (apt) for pterosin B (a), ptaquiloside (b) and sample G (c).

Ptaquiloside Determination

In order to assess this method's validity for ptaquiloside quantification, the ptaquiloside content in samples from previous work (Gil da Costa 2011) was determined (PA - 2010). After all samples were analysed, pterosin B content before and after conversion was calculated through the calibration curve previously obtained. Ptaquiloside concentration was obtained subtracting the initial pterosin B content to the conversion one. This calculations assumed a 100% conversion of ptaquiloside to pterosin B.

The results obtained are summarized in Table 8.

Table 8 - Samples analysed from PA - 2010 extraction process.

Sample	$PtB_{initial}$ (µg/mL)	Pta (µg/mL)	w (mg)		% w/w (mg/mg sample)	
			PtB	Pta	PtB	Pta
A	42.97±0.90	0.00	0.09	0.00	0.86	0.00
B	8.80±1.73	0.00	0.02	0.00	0.18	0.00
C	784.22±15.30	267.73	3.14	1.07	5.60	1.91
D	318.27±66.21	559.91	0.32	0.56	0.38	0.67
E	272.74±16.57	130.44	0.27	0.13	0.29	0.14
F	249.36±22.51	873.70	0.25	0.87	0.17	0.60
G	304.14±11.87	2269.77	0.60	4.54	3.03	22.93
H	661.60±34.34	2794.60	0.66	2.79	0.94	3.96
I	4739.68±132.96	630.05	4.74	0.63	6.84	0.91

With this method it is possible to monitor the extraction process and to understand which steps are relevant to the procedures and where the compound is might being lost.

Analysing the results obtained for this extraction process monitoring, a null conversion of ptaquiloside to pterosin B can be observed in sample A. This sample corresponds to the aqueous extract and due to its nature it can be stated that any ptaquiloside or ptaquiloside-alike compound present in the sample was slowly transformed into pterosin B during storage or ptaquiloside was present in the sample in a concentration below the limit of detection.

In sample B, the ptaquiloside content in this sample is residual or non-existent. This sample corresponds to the aqueous extract after passage through the amberlite resin, in which all of ptaquiloside should have been adsorbed, which is confirmed by the monitoring method. Nevertheless, once this sample is also of aqueous nature the absence of ptaquiloside content can also be due to degradation during storage. As stated in the Introduction section of this work, ptaquiloside is only stable for six months at low temperatures (Yamada et al. 2007).

The isolation process described in Figure 9 was performed aiming for ptaquiloside concentration and eliminating the other bracken components present in the aqueous extract. When analysing the remaining samples, an increase of the ptaquiloside content in sample G can

be observed, which is in accordance with the NMR conclusion that this sample possessed ptaquiloside. Of all the analysed samples this was the one with superior ptaquiloside content.

With this ptaquiloside quantification method, it is possible to promptly monitor ptaquiloside isolation, unlike with NMR, which is a slower technique and not so adequate for rapid quantification. NMR requires a more expensive equipment as well as the use of deuterated solvents which results in a costly analysis method. The technique applied for ptaquiloside quantification (^{13}C) is time-consuming and has a lower sensitivity due to the decreased percentage of carbon 13 isotope (1.1%) present in the molecules (Silvestein & Webster 1998). NMR has the advantage of being a non-destructive method.

In the other hand, HPLC is a quicker method for compound quantification (only requires a quick preparation of the initial sample and the conversion of the same, without elimination of solvents) and also allows the identification of other compounds in samples as well as the analysis of several samples in a short period of time, which enables a good monitoring of the isolation process. In this case, the results obtained from both HPLC and NMR are in accordance.

4.2. Monitoring of the Ptaquiloside Isolation Method

A new extraction process was initiated in April of 2014 and samples (P. A. - 2014) were taken from the resin adsorption and methanol elution steps (Figure 11), as described in Table 5. This extraction process was divided into three phases (A, B and C) due to the amount of bracken samples collected (18 kg) and corresponds to the clean-up and concentration of the aqueous extract. In this process the aqueous extract is adsorbed to the resin and then the compound of interest is desorbed with methanol (CH_3OH elution).

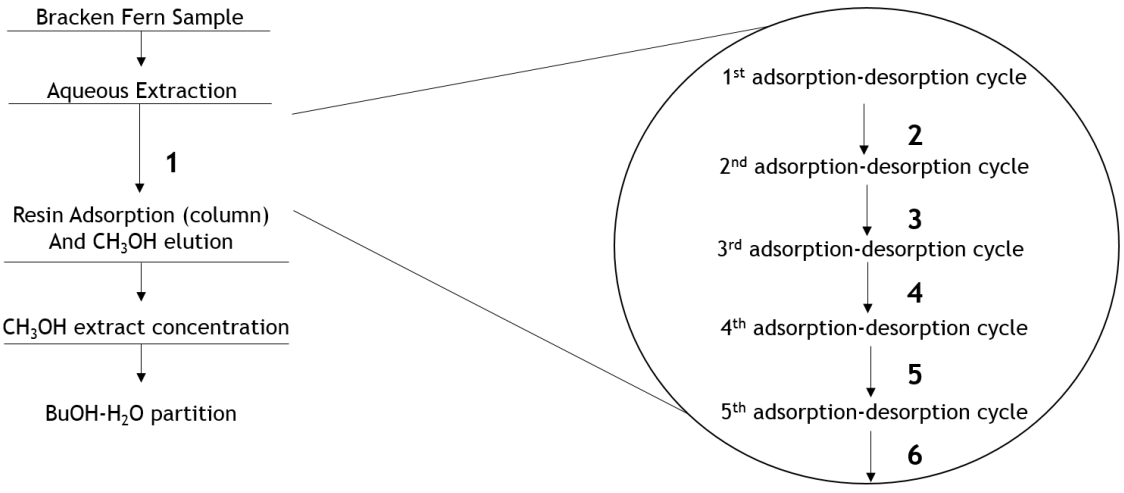

Figure 11 - Sampling for the monitoring of ptaquiloside extraction.

For extraction A an unknown volume of the aqueous extract was freeze dried, whereas for extractions B and C the volume lyophilized was 2 mL. Afterwards the aqueous extracts were

weighted and re-dissolved in distilled water. An initial analysis of the aqueous extract was performed in order to assess the initial content in pterosin B (Figure 12a).

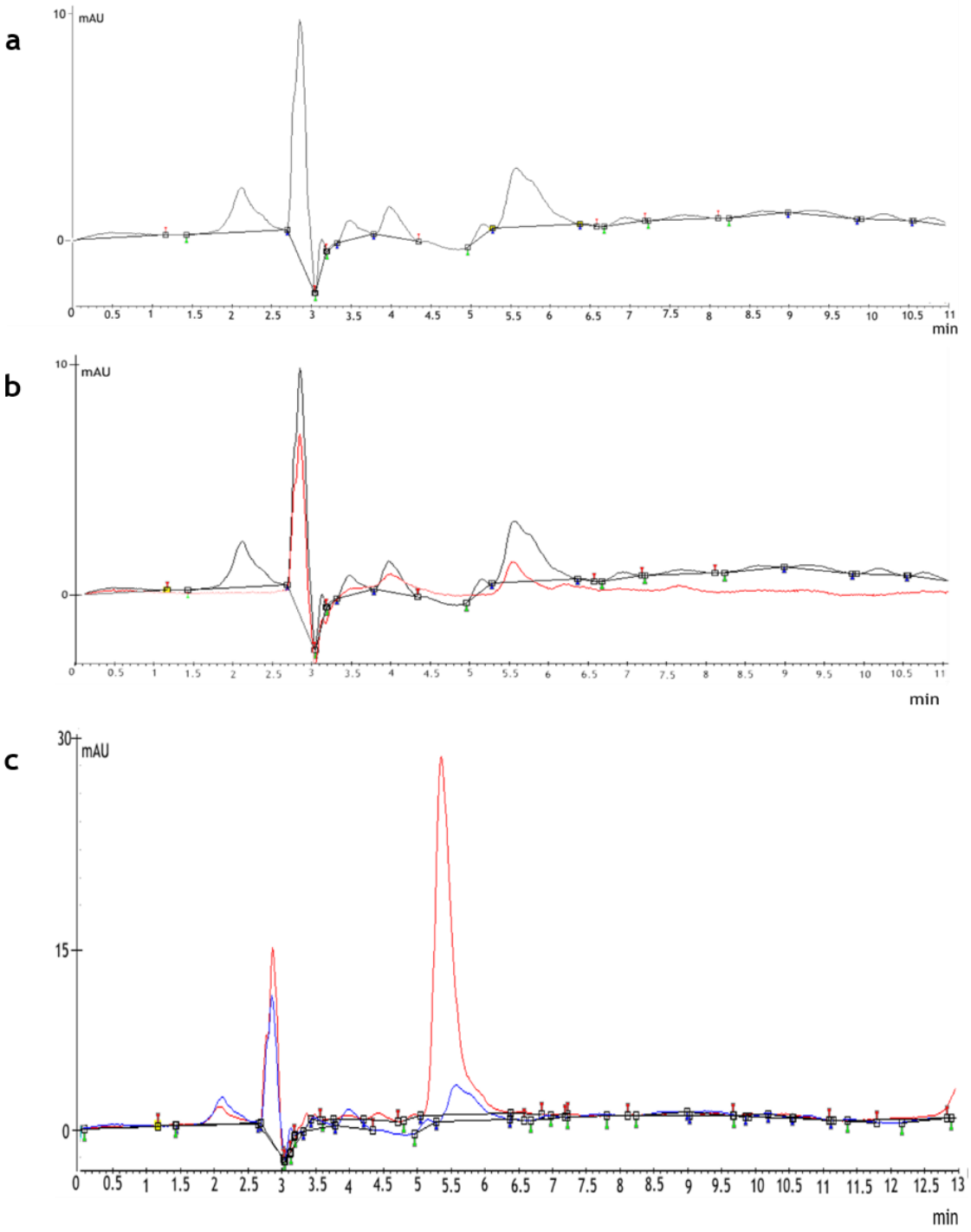

Figure 12 - Chromatogram obtained for initial analysis of Sample A1. a: at 220 nm; b: at 220 nm (black) and 260 nm (red); c: with (blue) and without (red) addition of a PtB standard.

As can be observed, there are 3 peaks between 6 and 8 minutes. Although the pterosin B peak usually occurs at approximately 8 minutes, there was some doubt about the retention time of this compound. With the objective of ascertaining which peak corresponded to the compound, an analysis at 260 nm (other of pterosin B maximum wavelength), due to the aromatic nature of pterosin B, was also performed (Figure 12b).

At 260 nm, a peak can be observed at around 6 minutes of retention time. This supported the hypothesis that the retention time of pterosin B had changed. Nevertheless, another confirmation analysis was performed, an injection of Sample A1 with addition of a standard of pterosin B (Figure 12c).

This analysis confirm the hypothesis of a variation in the pterosin B retention time. This change can be due to the usage of the chromatographic column, alterations in sample temperature or due to the matrix effect of analysing real samples. At this point the pterosin B retention time was considered to be around 5-6 minutes.

For the quantification of the converted sample a confirmation of the peak was also performed, resorting to the 260 nm analysis and to the standard addition method (Figure 13).

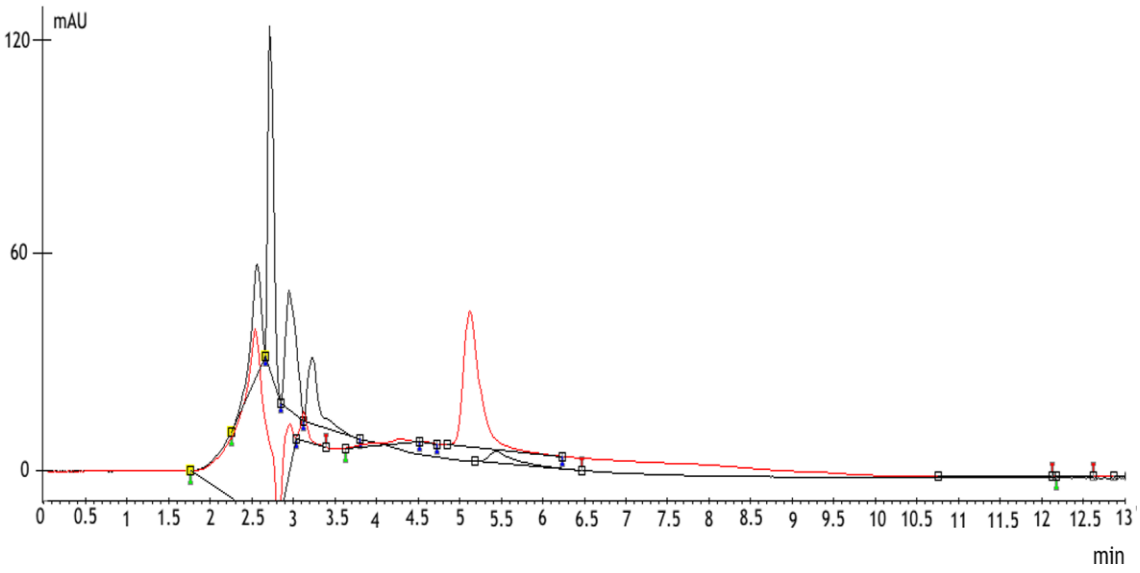

Figure 13 - Chromatogram of Sample A1 converted (black) and with standard addition (red) at 220 nm.

In this case, the pterosin B peak occurs around 5.5 minutes of retention time, in accordance with what was stated above.

In Table 9, the results obtained for the analysed samples of extraction A are summarized.

Table 9 – Ptaquiloside and Pterosin B content for analysed samples of extraction A.

Sample	$PtB_{initial}$ (µg/mL)	Pta (µg/mL)	w (mg)		% w/w (mg/mg sample)	
			PtB	Pta	PtB	Pta
A1	10.08±0.30	2.90	0.30	0.13	1.16	0.49
A2	28.58±2.16	28.59	0.11	0.11	0.76	0.76
A3	977.97±36.38	0.00	3.91	0.00	55.88	0.00
A4	26.57±6.06	0.00	0.11	0.00	5.31	0.00
A5	22.34±3.91	0.00	0.09	0.00	2.23	0.00
A6	34.41±7.51	0.00	0.14	0.00	6.88	0.00
A7	11.88±0.00	0.00	0.05	0.00	0.68	0.00
A8	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

In Sample A1 is important to highlight that the value of initial pterosin B is inferior to the limit of quantification (11.13 µg/mL), nevertheless this value was considered for the ptaquiloside determination due to its proximity to the LOQ.

By evaluating the results described in the table above, it can be observed that, after sample A3, the content in ptaquiloside is null. Accordingly, it can be concluded that all the interest compound was removed from the resin with only one passage of methanol, and, therefore, the following methanol cleanings can be removed from the isolation method.

In sample A8 the value obtained for pterosin B content was inferior to the limit of detection (LOD: 3.67 µg/mL). In this case, a null concentration of ptaquiloside can be considered, according to the results for the previous samples, which had no ptaquiloside content.

Nevertheless, in Sample A3, an increase on the initial content in pterosin B can be observed. This increase in pterosin B can be due to ptaquiloside conversion during the adsorption-desorption, which means that in this step a great amount of ptaquiloside was lost. This proves the importance of celerity in the isolation process in order to reduce the loss of ptaquiloside due to decomposition.

In this extraction process, the volume of the samples collected was unknown, which limited the calculation of the ptaquiloside content in bracken samples. This way, in future sampling all the volumes were registered in order to allow ptaquiloside quantification.

In Table 10 results obtained for the analysed samples of extraction B are summarized.

Table 10 - Ptaquiloside and Pterosin B content for analysed samples of extraction B.

Sample	$PtB_{initial}$ (µg/mL)	Pta (µg/mL)	w (mg)		% w/w (mg/mg sample)	
			PtB	Pta	PtB	Pta
B1	130.55±7.57	0.00	0.26	0.00	1.31	0.00
B2	23.61±0.61	15.71	0.05	0.03	0.026	0.17
B3	16.68±1.65	6.52	0.03	0.01	0.17	0.07
B4	15.08±3.40	3.75	0.03	0.01	0.16	0.04
B5	33.13±1.74	0.00	0.07	0.00	0.35	0.00

In extraction B, and taking into account the results obtained from extraction A, only three adsorption-desorption cycles were performed and the waiting time between cycles was minimized. Sample B1 displays no ptaquiloside content, however since this is the aqueous extract this result cannot be truthful. The freeze drying process that was performed during sample processing before quantification may be the cause for this result. During the lyophilisation of sample B1 a technical problem occurred with the equipment which led to the sample being in aqueous solution at room temperature some time, thus, resulting in ptaquiloside decomposition previous to the conversion step.

Nevertheless, a 0.17 % (w/w) ptaquiloside content can be observed in the results for Sample B2, which confirms that the results for sample B1 are not accurate. From this adsorption-desorption cycle a decrease in the ptaquiloside content is detected until reaching a 0.04 % (w/w) ptaquiloside content.

Sample B5 corresponds to a final aqueous extract and the outcome for ptaquiloside content (0.00 % (w/w)) corroborates the hypothesis that all ptaquiloside was removed from the aqueous extract with three adsorption-desorption cycles.

Concerning extraction C ptaquioside determination, the results are present in Table 11.

Table 11 - Ptaquiloside and Pterosin B content for analysed samples of extraction C.

Sample	$PtB_{initial}$ (µg/mL)	Pta (µg/mL)	w (mg)		% w/w (mg/mg sample)	
			PtB	Pta	PtB	Pta
C1	64.11±2.46	25.95	0.13	0.03	0.58	0.24
C2	19.92±10.30	35.60	0.04	0.07	0.66	1.19
C3	121.03±7.37	0.00	0.24	0.00	12.10	0.00
C4	21.52±10.45	0.00	0.04	0.00	4.30	0.00
C5	27.80±3.55	0.00	0.06	0.00	0.33	0.00

By analysing the results from extraction C, a decrease in ptaquiloside content can be perceived through the adsorption-desorption cycles. In this extraction, all the ptaquiloside content was removed with the first resin adsorption-methanol elution cycle and after that ptaquiloside was not possible to detect. Nonetheless, in sample C3 and increase in pterosin B content can be noticed, which can be related with loss of ptaquiloside during the extraction process or during sample storage.

One of the major concerns with this quantification method is the loss of ptaquiloside due to pour handling of the samples. It is vital to highlight the importance of celerity not only in the isolation process, but also in the quantification method, in order to avoid inadequate ptaquiloside quantifications.

Furthermore, the ptaquiloside content in bracken may be estimated through the ptaquiloside concentration in the initial aqueous extract. Assuming a 100% conversion of ptaquiloside to pterosin B, the total content of ptaquiloside can be calculated in terms of dry weight basis. Once Sample C1 represents the aqueous extract and considering a full extraction of ptaquiloside, it can be extrapolated that the ptaquiloside content in bracken fern is 0.30 mg/g in dry weight basis. This value is similar to the ones found in literature, as the maximum concentration was 4.450 mg/g measured by (Rasmussen, Kroghsbo, et al. 2003) when analysing samples from the whole plant.

This is the first estimate of ptaquiloside content in *Pteridium aquilinum* made in continental Portugal and regarding the reported values for bracken croziers in dry weight basis, this value can only be compared with studies of Alonso-Amelot (Alonso-Amelot et al. 1992a; Alonso-Amelot et al. 1992b),. In 1992, this group reported a ptaquiloside concentration in croziers of 1.6 mg/g and 1.9 mg/g. This value is a superior to the one obtained in this work, which, as stated before, is correlated to the plant growth region, as well as the climacteric conditions during growing season.

5. Conclusions

Bracken fern is an ancient plant that grows abundantly worldwide and is toxic to many animals' species.

P. aquilinum possesses a unique ability to spread (through the rhizome system and/or spores proliferation), which combined to its aptitude to resist insect attacks, microorganisms and unfavourable weather, contributed to the efforts that has been dedicated to its control and to understanding its harmfulness.

About 100 metabolites have been isolated from bracken. Of these, ptaquiloside, an unstable norsesquiterpene glycoside of the illudane type, is the one responsible for most of the fern's carcinogenicity.

Ptaquiloside is soluble in water and it has been reported that it is clastogenic, mutagenic and teratogenic. Its elevated toxicity is associated with the electrophilic properties of the unstable three carbon ring (cyclopropyl group), that alkylates a wide variety of nucleophiles (biomolecules such as nucleotides).

In bracken, the concentration of some sesquiterpenoids have been known to vary significantly along the growing season and is dependent of the plant compartment, even so, ptaquiloside concentrations up to 37 mg/g dry weight in mature fronds have been reported. It is known that ptaquiloside appears in higher concentration in the crozier than in the mature frond. In the present work, ptaquiloside has been quantified in bracken croziers, for the first time in samples from continental Portugal, representing 0.30 mg/g (dry weight).

Nowadays, high pressure liquid chromatography is the most used method for ptaquiloside quantification. In this work, an HPLC quantification was applied, recurring to a conversion of ptaquiloside to pterosin B, through a base-heat-acid treatment, followed by analysis at 220 nm and using as mobile phase methanol:water 70:30 (v/v).

After monitoring the first extraction it was possible to conclude that there was no need for five adsorption-desorption cycles, and for the next extraction the number of cycles was reduced to three, while the waiting time between cycles was minimized.

Using this ptaquiloside quantification method, it is possible to promptly monitor ptaquiloside isolation, unlike with NMR, which is a slower technique and not so adequate for

rapid quantification. The HPLC quantification only requires a quick preparation of the initial sample and the conversion of the same.

The application of this method throughout all the isolation process is of extreme importance in order to ascertain how this process can be accelerated, since ptaquiloside losses due the process's duration is one of the major causes of its low yield.

References

Agnew, M.P. & Lauren, D.R., 1991. Determination of ptaquiloside in bracken fern (*Pteridium esculentum*). *Journal of Chromatography*, 538, pp.462–468.

Alonso-Amelot, M.E. et al., 1998. Excretion, through milk, of ptaquiloside in bracken-fed cows. A quantitative assessment. *Le Lait*, 78, pp.413–423.

Alonso-Amelot, M.E. et al., 1992a. Ontogenic variation of biologically active metabolites of *Pteridium aquilinum* (L. Kuhn) pterosins A and B, and ptaquiloside in a bracken population of the tropical andes. *Journal of Chemical Ecology*, 18(8), pp.1405–1420.

Alonso-Amelot, M.E. et al., 1992b. Quantitation of pterosins A and B, and ptaquiloside, the main carcinogen of *Pteridium aquilinum* (L. Kuhn), by high pressure liquid chromatography. *Phytochemical Analysis*, 3, pp.160–164.

Alonso-Amelot, M.E. & Avendaño, M., 2002. Human carcinogenesis and bracken fern: a review of the evidence. *Current Medicinal Chemistry*, 9(6), pp.675–86.

Alonso-Amelot, M.E. & Avendaño, M., 2001. Possible association between gastric cancer and bracken fern in Venezuela: an epidemiologic study. *International Journal of Cancer*, 91(2), pp.252–9.

Alonso-Amelot, M.E., Castillo, U. & Jongh, F. De, 1993. Passage of the bracken fern carcinogen ptaquiloside into bovine milk. *Lait*, 73, pp.323–332.

Alonso-Amelot, M.E., Rodulfo-Baechler, S. & Jaimes-espinoza, R., 1995. Comparative dynamics of ptaquiloside and pterosin B in the two varieties (*caudatum* and *arachnoideum*) of neotropical bracken fern (*Pteridium aquilinum* L . Kuhn). *Biochemical Systematics and Ecology*, 23(7/8), pp.709–716.

Ayala-Luis, K.B. et al., 2006. Kinetics of ptaquiloside hydrolysis in aqueous solution. *Environmental Toxicology and Chemistry*, 25(10), pp.2623–9.

Bonadies, F. et al., 2011. A new, very sensitive method of assessment of ptaquiloside, the major bracken carcinogen in the milk of farm animals. *Food Chemistry*, 124(2), pp.660–665.

Bonadies, F. et al., 2004. Mass spectrometric analysis of ptaquiloside, the toxic sesquiterpene from bracken fern. *Rapid communications in mass spectrometry*, 18(7), pp.825–8.

Brown, L.M. et al., 1999. Bracken fern consumption and human bladder cancer. *Journal of Epidemiology and Community Health*, 53(10), p.653.

Castillo, U.F. et al., 1998. Ptaquiloside Z, a new toxic unstable sesquiterpene glucoside from the neotropical bracken fern *Pteridium aquilinum* var. *caudatum*. *Bioorganic & Medicinal Chemistry*, 6(11), pp.2229–33.

Cruz, G.D. & Bracarense, A.P.F.R.L., 2004. Role of bracken fern (*Pteridium aquilinum* (L.) Kuhn) in animal and human heath. *Ciências Agrárias*, 25(3), pp.249–258.

Davis, Z. et al., 2013. *Growth of* Pteridium Aquilinum *in Varying Habitats*,

Dawra, R.K., Kurade, N.P. & Sharma, O.P., 2002. Carcinogenicity of the fern *Pteridium aquilinum* collected from enzootic bovine haematuria-free hilly area in India. *Current Science*, 83(8), pp.1005–1009.

Der, J.P. & Thomson, J.A., 2009. Global chloroplast phylogeny and biogeography of bracken (*Pteridium*; Dennstaedtiaceae). *American Journal of Botany*, 96(5), pp.1041–1049.

Dolling, A., Zackrisson, O. & Nilsson, M.C., 1994. Seasonal variation in phytotoxicity of Bracken (*Pteridium aquilinum* L. Kuhn). *Journal of Chemical Ecology*, 20(12), pp.3163–3172.

Evans, I.A., Jones, R.S. & Mainwaring-Burton, R., 1972. Passage of bracken fern toxicity into milk. *Nature*, 237, pp.107–108.

Evans, I.A. & Mason, J., 1965. Carcinogenic activity of bracken. *Nature*, 208(5013).

Fenwick, G.R., 1988. Bracken (*Pteridium aquilinum*) - toxic effects and toxic constituents. *Journal of the Science of Food and Agriculture*, 46, pp.147–173.

Fletcher, M.T. et al., 2011. Norsesquiterpene glycosides in bracken ferns (*Pteridium esculentum* and *Pteridium aquilinum* subsp. *wightianum*) from Eastern Australia: reassessed poisoning risk to animals. *Journal of agricultural and food chemistry*, 59(9), pp.5133–8.

Gil da Costa, R.M., 2011. *Studies on the biological activities of* Pteridium aquilinum *(L.) Kuhn*. Universidade do Porto.

Van der Hoeven, J.C.M. et al., 1983. Aquilide A, a new mutagenic compound isolated from bracken fern (*Pteridium aquilinum* (L.) Kuhn). *Carcinogenesis*, 4, pp.1587– 1590.

Hojo-Souza, N.S., Carneiro, C.M. & Santos, R.C. dos, 2010. *Pteridium aquilinum*: what we know and what is yet to be learnt. *Bioscience Journal*, pp.798–808.

Hopkins, A., 1990. Bracken (*Pteridium aquilinum*): Its distribution and animal health implications. *British Veterinary Journal*, 146(4), pp.316–326.

ICH, 2005. Guidelines Q2(R1) Validation of Analytical Procedures: Text and Methodology. *International Conference on Harmonisation*, pp.1–15.

Jensen, P.H. et al., 2008. Quantification of ptaquiloside and pterosin B in soil and groundwater using liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Journal of Agricultural and Food Chemistry*, 56(21), pp.9848–54.

Marliére, C.A. et al., 2002. Bracken fern (*Pteridium aquilinum*) ingestion and oesophageal and stomach cancer. *IARC Scientific Publications*, 156, pp.379–380.

Matoba, M. et al., 1987. Assay of ptaquiloside, the carcinogenic principle of bracken, *Pteridium aquilinum*, by mutagenicity testing in salmonella typhimurium. *Mutagenesis*, 2(6), pp.419–423.

McGlone, M.S., Wilmshurst, J.M. & Leach, H.M., 2005. An ecological and historical review of bracken (*Pteridium esculentum*) in New Zealand, and its cultural significance. *New Zealand Journal of Ecology*, 29(2), pp.165–184.

Niwa, H. et al., 1983. Stereochemistry of ptaquiloside, a novel norsesquiterpene glucoside from bracken, *Pteridium aquilinum* var. *latiusculum*. *Tetrahedron letters*, (38), pp.4117–4120.

Oelrichs, P.B., Ng, J.C. & Bartley, J., 1995. Purification of ptaquiloside, a carcinogen from *Pteridium aquilinum*. *Phytochemistry*, 40(1), pp.53–56.

Pathania, S. et al., 2012. Detection of ptaquiloside and quercetin in certain Indian ferns. *Current Science*, 102(12), pp.1683–1691.

Pavia, D.L., Lampman, G.M. & Kriz, G.S., 2001. *Introduction to spectroscopy: a guide for students of organic chemistry* 3rd Edition, Brooks/Cole.

Potter, D.M. & Baird, M.S., 2000. Carcinogenic effects of ptaquiloside in bracken fern and related compounds. *British Journal of Cancer*, 83(7), pp.914–920.

Price, J.M. & Pamukcu, A.M., 1968. The induction of neoplasms of the urinary bladder of the cow and the small intestine of the rat by feeding bracken fern (*Pteris aquilina*). *Cancer research*, 28, pp.2247–2251.

Ramwell, C.T. et al., 2010. *Ptaquiloside & other bracken toxins : A preliminary risk assessment*,

Rasmussen, L.H., Kroghsbo, S., et al., 2003. Occurrence of the carcinogenic Bracken constituent ptaquiloside in fronds, topsoils and organic soil layers in Denmark. *Chemosphere*, 51(2), pp.117–27.

Rasmussen, L.H., 2003. *Ptaquiloside: an environmental hazard?* The Royal Veterinary and Agricultural University. Frederiksberg.

Rasmussen, L.H., Hansen, H.C.B. & Lauren, D., 2005. Sorption, degradation and mobility of ptaquiloside, a carcinogenic Bracken (*Pteridium* sp.) constituent, in the soil environment. *Chemosphere*, 58(6), pp.823–35.

Rasmussen, L.H., Jensen, L.S. & Hansen, H.C.B., 2003. Distribution of the carcinogenic terpene ptaquiloside in bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. *Journal of chemical ecology*, 29(3), pp.771–8.

Rasmussen, L.H., Schmidt, B. & Sheffield, E., 2013. Ptaquiloside in bracken spores from Britain. *Chemosphere*, 90, pp.2539–2541.

Recouso, R.C. et al., 2003. Clastogenic effect of bracken fern (*Pteridium aquilinum* v. *arachnoideum*) diet in peripheral lymphocytes of human consumers: preliminary data. *Veterinary and Comparative Oncology*, 1(1), pp.22–29.

Saito, K. et al., 1989. Chemical assay of ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the pteridaceae. *Phytochemistry*, 28(6), pp.1605–1611.

Saito, K. et al., 1990. The sesquiterpenoid carcinogen of bracken fern, and some analogues, from the Pteridaceae. *Phytochemistry*, 29, pp.1475–1479.

Shahin, M. et al., 1998. Bracken fern carcinogenesis: multiple intravenous doses of activated ptaquiloside induce DNA adducts, monocytosis, increased TNF alpha levels, and mammary gland carcinoma in rats. *Biochemical and Biophysical Research Communications*, 244(1), pp.192–7.

Shahin, M., Smith, B.L. & Prakash, A.S., 1999. Bracken carcinogens in the human diet. *Mutation research*, 443(1-2), pp.69–79.

Silvestein, R.M. & Webster, F.X., 1998. *Spectrometric Identification of Organic Compounds* 6th Edition, New York: John Wiley & Sons, Inc.

Skoog, D.A., West, D.M. & Holler, F.J., 1992. *Fundamentals of Analytical Chemistry* 6th Edition, Saunders College Publishing.

Smith, B.L. et al., 1994. Concentration of ptaquiloside, a major carcinogen in bracken fern (*Pteridium aquilinum* spp.) from Eastern Australia and from a cultivated worldwide collection held in Sidney, Australia. *Natural Toxins 2*, pp.347–353.

Smith, B.L. & Seawright, A.A., 1995. Bracken fern (*Pteridium* spp.) carcinogenicity and human health—a brief review. *Natural toxins*, 3, pp.1–5.

Somvanshi, R. & Lauren, D.R., 2006. Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. *Current Science*, 91(11), pp.1547–1552.

Somvanshi, R. & Ravisankar, R., 2004. Recent advances in bracken fern toxin research. *Natural Product Radiance*, 3(4), pp.1–5.

Thomson, J.A., 2004. Towards a taxonomic revision of *Pteridium* (Dennstaedtiaceae). *Telopea*, 10(4), pp.793–803.

Whitehead, S.J. & Digby, J., 1997. Morphology of bracken (*Pteridium aquilinum* (L.) Kuhn) in the North York Moors—a comparison of the mature stand and the interface with heather (*Calluna vulgaris* (L.) Hull) 2. The rhizome. *Annals of Applied Biology*, 131(1), pp.117–131.

Wilson, D., Donaldson, L.J. & Sepai, O., 1998. Should we be frightened of bracken? A review of the evidence. *Journal of Epidemiology and Community Health*, 52(12), pp.812–7.

Yamada, K., Ojika, M. & Kigoshi, H., 1998. Isolation, chemistry, and biochemistry of ptaquiloside, a bracken carcinogen. *Angewandte Chemie International Edition*, 37(13-14), pp.1818–1826.

Yamada, K., Ojika, M. & Kigoshi, H., 2007. Ptaquiloside, the major toxin of bracken, and related terpene glycosides: chemistry, biology and ecology. *Natural Product Reports*, 24, pp.798–813.

Zhao, J., 2011. *The extraction of high value chemicals from heather (*Calluna vulgaris*) and bracken (*Pteridium aquilinum*)*. The University of York Chemistry.

Appendix

A. Survey from the literature

Table 12 - Review of the Ptaquiloside/Pterosin B quantification methods developed so far.

Reference	Sample Cleaning	Conversion of Pta to PtB	Pta/PtB Quantification						
			Equipment	Column	Mobile Phase	Flow-rate (mL/min)	Retention Time	Sample Injected	Conditions
Saito et al. 1991	------	Thin Layer Chromatography	Silica Gel TLC plate (20x10 cm)	------	------	------	------	------	TLC-densitometry at 250 nm
Agnew & Lauren 1991	20 mL of filtrate were added to a glass column (25 cm x 12 mm) dry packed with polyamide 6S resin (2.5 g)	1 mL of eluate + 75 µL/mL of NaOH 1 M ↓ 1 h at 40 °C ↓ Add 75 µL/mL of HCl 5 M	HPLC system Spectra-Physics 740B (binary pumping system with a Rheodyne 7120 manual injector or a Micrometrics 725 autosampler; a 50 µL sample loop; a Micrometrics 731 column oven; a Shimadzu SPD-2A UV Detector; a Spectra-Physics SP4170 integrator)	Zorbax ODS 5 µm or Chrompak CPSpher C_8 (25 cm x 4.6 mm I. D.) with a MPLC RP-8 guard column	H_2O: CH_3OH (60:40, v/v) for Pta and H_2O: CH_3OH (40:60, v/v) for PtB	1	10-12 min for Pta and PtB	50 µL	35 ºC 220 nm

Reference	Sample Cleaning	Conversion of Pta to PtB	Pta/PtB Quantification						
			Equipment	Column	Mobile Phase	Flow-rate (mL/min)	Retention Time	Sample Injected	Conditions
Alonso-Amelot et al. 1992	---	---	HPLC system LDC Milton Roy (Constametric II pump; Rheodyne 20 µL loop injector; Waters 490E multichannel UV-VIS detector)	Radial 0.5 x 10 cm C-18	H_2O: CH_3OH (35:65, v/v)in isocratic mode	1	10 min for Ptb	20 µL	Room temperature 260 nm for PtB
Dawra et al. 2002	---	---	Waters HPLC system (510 and 515 pump; Rheodyne injector; 490E multichannel detector)	C-18 Reverse-phase (4.6 x 250 mm)	C_2H_3N:H_2O gradient (20:80, v/v for first 20 min followed by increase to 100% acetonitrile in the next 20 min)	1	---	---	---
Rasmussen 2003	Agnew and Lauren method	1 mL of eluate + 75 µL/mL of 1 M NaOH ↓ 1 h at 45 °C ↓ Add 75 µL/mL of 5 M HCl	HPLC system Merck Hitachi (T-6300 column thermostat; L4200 UV-Vis detector; 655A-40 Auto sampler; D-6000A Interface; L-6200 Intelligent pump)	Merck LiChroCART® (LiChrospher® 100 RP-8, 5 µm) 125 x 4 mm, 4x4 mm I.D. Merck LiChroCART® (LiChrospher® 100 RP-18e, 5 µm) safe guard column	H_2O: C_2H_3N gradient	---	---	---	35 ºC 220 nm Pta 214 nm PtB B
Bonadies et al. 2004	-----	400-500 mL of aqueous solution + 75 µL/mL of 1 M NaOH ↓ 1 h at 45 °C ↓ Add 75 µL/mL of HCl 5 M	GC/MS HP5890 gas chromatograph	HP5 MS column (30 m)	-----	-----	-----	-----	-----

Reference	Sample Cleaning	Conversion of Pta to PtB	Pta/PtB Quantification						
			Equipment	Column	Mobile Phase	Flow-rate (mL/min)	Retention Time	Sample Injected	Conditions
Rasmussen et al. 2005	Polyamide 6 resin (Fluka): 4 mL aqueous extract was passed through 0.50 g resin (dry packed) on a 1.0 x 10 cm I.D. glass Econo-Column® (BIO-RAD) fitted with a 28 µm polymer filter	Agnew and Lauren Method	Merck HPLC system (L-4200 UV-VIS detector; 655A-40 auto-sampler, D-6000A Interface and L-6200 Intelligent pump)	Merck LiChroCART® (125 x 4 mm) packed with LiChrospher® 100 RP-8, 4 µm	Pterosin B: double deionised H_2O: C_2H_3N (83:17, v/v) Ptaquiloside: MillQ-H_2O: C_2H_3N (92:8, v/v)	1	---	20 µL	35 ºC 220 for Pta 214 nm for PtB
Ayala-Luis et al. 2006	---	---	Merck Hitashi HPLC system (T-6300 column thermostat; L-4000 UV-VIS detector; 655A-40 auto-sampler; D-6000A Interface, L-6200 intelligent pump)	Merck LiChroCART® (125 x 4 mm) packed with LiChrospher® 100 RP-8, 4 µm Merck LiChroCART guard column (Lichrospher 100 RP-18e, 4 x 4 mm)	Isocratic mode: C_2H_3N: H_2O (18:82, v/v) Gradient mode: started with the isocratic mode and after 10 min, a gradient was applied until reaching C_2H_3N: H_2O (55:45, v/v)	1	Pta: 8 min PtB: 12 min	---	35 ºC 220 nm
Somvanshi & Lauren 2006	0.75 mL of supernatant was added to 0.250 g of methanol-washed polyamide powder	1 mL of supernant + 75 µL of 1 N NaOH ↓ 1 h at 40°C ↓ Add 0.76 µL of 5N HCl	Reversed-phase HPLC (Waters HPLC system) 510 and 515 binary pumping system, rheodyne injector, 490 E multichannel detector	C_{18} reverse phase column (4.6 x 250 nm)	Acetonitrile-water gradient: 20:80 for the first 20 min followed by increase to 100% acetonitrile for the next 20 min.	----	Pta and PtB at 29.50 min	20 µL	220 nm 260 nm
Jensen et al. 2008	Agnew and Lauren method	-----	LC-MS 2690 LC Waters System LC-UV	50 x 2.0 mm inner diameter, 3 µm, Gemini C6-hexyl	40& CH_3OH and 3 mM ammonium acetate	0.2 mL/min	3.0 min for Pta and 10.0	----	220 nm for PtB 214 nm for Pta

Reference	Sample Cleaning	Conversion of Pta to PtB	Pta/PtB Quantification						
			Equipment	Column	Mobile Phase	Flow-rate (mL/min)	Retention Time	Sample Injected	Conditions
			Series 1100/1200 LC system with a diode array detector (DAD)	Phenomenex® column			min for Ptb		
Latorre 2010	---	25 mL filtrate + 1 mL NaOH 1 M ↓ 2 h at 45 – 50 °C ↓ 3 x 10 mL of dichloromethane	---	Shim-pack ODS (4 mm x 150 mm, Shimadzu) with safe guard column reverse phase C18	H_2O: CH_3OH 40% CH_3OH (0-2 min) 40-80% CH_3OH (2-15 min) 80-40% CH_3OH (15-16 min) 40 CH_3OH (16-25 min)	1	---	---	Pterosin B 260 nm
Fletcher et al. 2011	4 mL of filtrate was applied to a Fluka polyamide 6 column (0.45 g) (Sigma-Aldrich)	1 mL of eluent + 75 µL/mL of NaOH 1 M ↓ 1 h at 40 °C ↓ Add 75 µL/mL of H_2SO_4 2,5 M	---	Luna C18 column 250 x 4.6 mm, 5 µm	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: 09 min, held at 100% A; 911 min, 100% A100% B; 1116 min, held at 100% B; 1616.5 min, 100% B100% A; 16.525 min, held at 100% A.	1	3.2 min for Pta and 11.1 min for Ptb	10 µL	35 ºC 220 nm
Rasmussen et al. 2013	4 mL aqueous extract was passed through 0.50 g resin (dry packed) on a 1.0 x 10 cm I.D. glass Econo-Column® (BIO-RAD) fitted with a 28 µm polymer filter	Base-acid treatment	Agilent 1100 Series HPLC system (G1314A VWD detector; G1312A Binary Pump; G1328A Manual Injector)	Merck LiChroCART® column (125 x 4 mm) packed with LiChrospher 100 RP-8, 4 lm	H_2O: C_2H_3N (69:31, v/v)	1	10.8 min Ptb	500 µL	Room temperature 214 nm

B. Method Validation

In this Appendix data related with the pterosin B quantification method validation is presented.

B.1. Linearity Study

Table 13 - Summary of linearity study.

Theoretical Concentration (µg/mL))	Average peak area (mAU.min)	Standard Deviation	Relative Standard Deviation (%RSD)
1	1.15	0.25	21.43
5	3.35	0.28	8.49
10	7.30	0.22	3.02
25	15.53	0.28	1.82
50	33.28	0.60	1.81
80	59.94	0.80	1.40
Y intercept		-0.3841	
Slope		0.7009	
Correlation coefficient (R)		0.9985	
Coefficient of Determination (R^2)		0.9969	
Sa/a		2.78 %	
b-Sb		-1.16	
b+Sb		0.40	

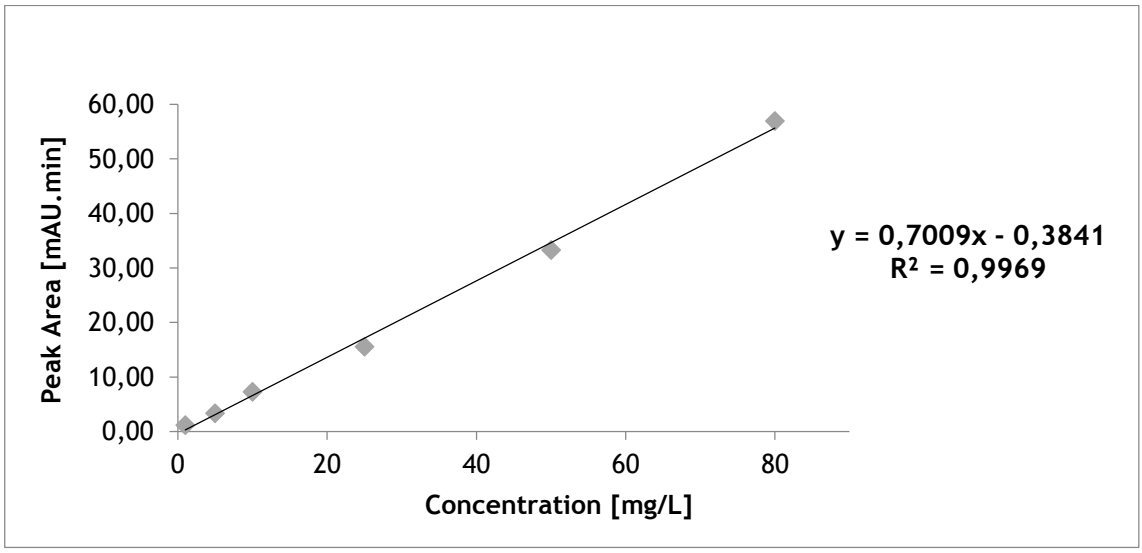

Figure 14 - Calibration Curve for Pterosin B (HPLC UV-Vis) at 220 nm.

B.2. Accuracy

Table 14 - Accuracy results for different concentrations of Pterosin B.

Theoretical Concentration (µg/mL)	**Mean Experimental Concentration (µg/mL) (n=3)**	**Recovery (%)**
5.00	5.74	114.74
25.00	24.99	99.97
80.00	80.07	100.08
Mean Recovery (%)		104.93
% RSD		8.09

B.3. Precision

Table 15 - Results of precision determinations.

Repeatability (Intra-day Precision)		
Concentration (µg/mL)	SD	%RSD
1.00	0.04	7.53
5.00	0.09	1.08
10.00	0.42	4.70
25.00	0.45	2.63
50.00	0.95	1.80
Intermediate Precision (Inter-day precision)		
1.00	0.03	5.57
5.00	0.94	7.65
10.00	0.45	4.84
25.00	0.37	2.19
50.00	1.04	1.96

C. NMR Data

In this Appendix data regarding ptaquiloside and pterosin B NMR spectra are presented.

Table 16 - ^{13}C NMR data for pterosin B (DMSO) and for ptaquiloside (CH_3OH).

Position	δ ^{13}C	
	Pterosin B	**Ptaquiloside**
1	209.44	209.44
2	41.85	41.85
3	33.08	33.08
4	152.07	152.07
5	125.51	125.51
6	144.05	144.05
7	135.52	135.52
8	136.44	136.44
9	131.24	131.24
10	16.18	16.18
11	20.69	20.69
12	31.86	31.86
13	59.75	59.75
14	13.04	13.04