

Occurrence of Carcinogenic illudane Glycosides in Drinking Water Wells

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Occurrence of carcinogenic illudane glycosides in drinking water wells

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Abstract

Background

Ptaquiloside (PTA), caudatoside (CAU) and ptesculentoside (PTE) are carcinogenic illudane glycosides found in bracken ferns (*Pteridium* spp.) world-wide. The environmentally mobile PTA entails both acute and chronic toxicity and comparable risk might be associated with the structurally similar CAU and PTE. It is of great concern if these compounds are present in drinking water wells in bracken dominated regions, since they might pose a threat to human health. This study investigates the presence of PTA, CAU, PTE, and their corresponding hydrolysis products pterosins B (PtB), A (PtA) and G (PtG) in water wells in Denmark, Sweden and Spain. In total, 77 water samples from deep groundwater wells (40 – 100 m) and shallow water wells (8 – 40 m) were collected and preserved in the field, pre-concentrated in the laboratory and analysed by liquid chromatography-mass spectrometry (LC-MS).

Results

Deep groundwater wells contained neither illudane glycosides nor their pterosins. However, seven private shallow wells contained at least one of the illudane glycosides and/or pterosins at concentrations up to 0.27 $\mu\text{g L}^{-1}$ (PTA), 0.75 $\mu\text{g L}^{-1}$ (CAU), 0.05 $\mu\text{g L}^{-1}$ (PtB), 0.03 $\mu\text{g L}^{-1}$ (PtA) and 0.28 $\mu\text{g L}^{-1}$ (PtG).

Conclusions

Detected concentrations of illudane glycosides in some of investigated wells exceeded the suggested maximum tolerable concentrations of PTA, although they were used for drinking water purpose. Contaminated wells were characterized by shallow depth, lower pH and electrical conductivity compared to deep groundwater wells where no illudane glycosides or pterosins were found.

Key words: Caudatoside; Emerging contaminants; Ptaquiloside; Ptesculentoside; Pterosins; Water quality

1. Background

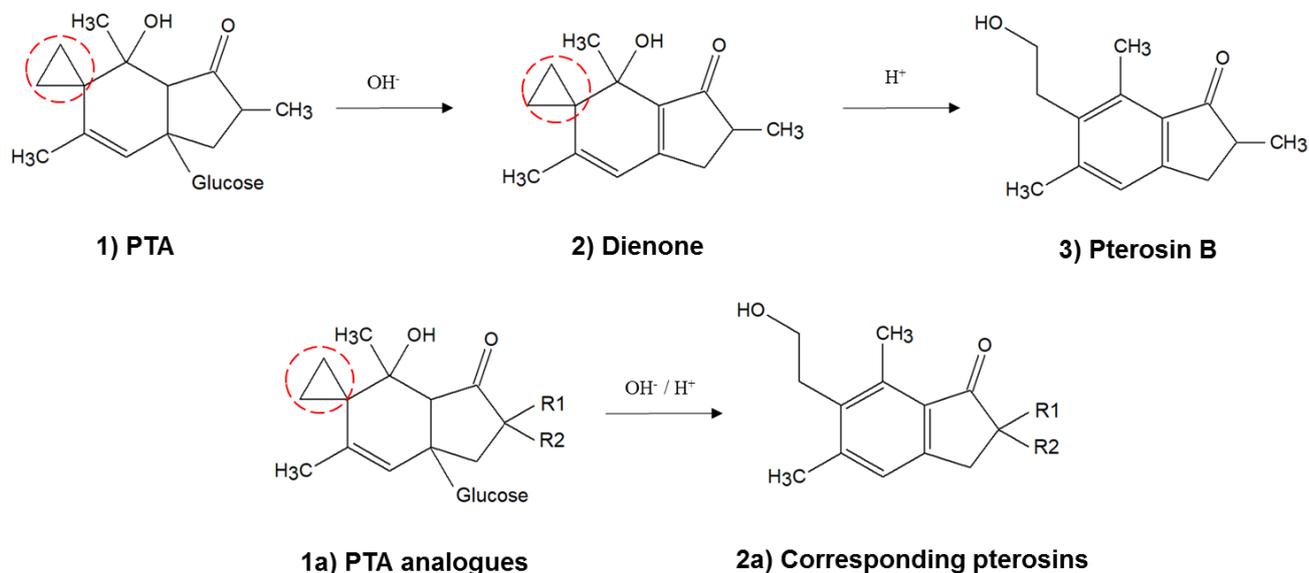
Anthropogenic chemicals, such as pesticides and pharmaceuticals, are of high concern as water contaminants because of their persistence and toxicity [1][2]. However, living organisms can also produce hazardous substances, which are both mobile, persistent and toxic [3]. Plants synthesize a broad variety of toxic chemical compounds e.g. as a mean of protection against pathogens and herbivores [4]. Such plant produced compounds are typically not harmful to the plants themselves but can adversely affect human or animal health through exposure via air, soil, water or food. By their physicochemical properties, many of them can be compared to the persistent and mobile organic compounds (PMOCs), which are often not easily removed from water due to their high water solubility [5]. Plant produced toxins have a potential to pose a higher risk to water quality than anthropogenic chemicals because of their continuous production and comparatively high concentration in plants [6]. Nonetheless, they are still an unexplored group of emerging contaminants and we currently lack the tools to monitor, regulate and remove them.

One of the best studied phytotoxins with PMOC characteristics is the carcinogen ptaquiloside (PTA) from bracken fern [6]. Bracken (*Pteridium* spp.) is globally widespread and the fifth most abundant plant genus in the world [7][8]. It has an extremely high rate of reproduction and re-growth due to its extensive rhizomes, and can rapidly adapt to new ecological conditions [9]. Bracken is acutely toxic and classified as possibly carcinogenic towards humans by the International Agency of Research on Cancer/WHO [10][11]. Intake of toxins takes place via bracken based food in some countries like Korea, China and Japan. In addition, contamination of drinking water is proposed as a vector for human exposure [12].

Ptaquiloside is considered the principle compound responsible for both acute and chronic bracken toxicity [13]. Ptaquiloside is an illudane glycoside with carcinogenic, mutagenic and clastogenic properties and the most studied compound found in bracken [14][15]. Under weak alkaline conditions, PTA (1) is converted into an unstable compound, a dienone (2) which subsequently leads to the formation of non-carcinogenic pterosin B (3) (Figure 1). The dienone (2) is assumed to be the ultimate agent responsible for carcinogenicity with a cyclopropyl group being responsible for reaction with amino acids and DNA, and causing mutations [9][16][17]. Other compounds with structural similarity to PTA have also been found in bracken (PTA analogues) with caudatoside (CAU) and ptesculentoside (PTE) being prevalent [18][19] (Figure 1). Although there is only limited knowledge about the toxic properties of these compounds, they share the reactive cyclopropyl group and are expected to show similar reactivity and toxic effects as PTA [20].

Ptaquiloside is a highly polar compound ($\log K_{ow} = -0.63$) and can be easily transferred to the soil by release from living or dead bracken [21][22]. Because of the high water solubility of PTA and its low sorption to soil, it is prone to leaching from topsoil to surface and groundwater [21]-[23]. Hydrolysis rates of PTA are highly pH dependent, with the lowest degradation rates at neutral to slightly acidic conditions (pH 4.5 – 6.5) and at low temperatures [13][21][24]. PTA is prone to microbial degradation [26], which makes PTA hydrolysis slow in environments with low microbiological activity (e.g. in groundwater). Hence, PTA presence in groundwater should be governed by pH and temperature and PTA can sustain for months in cold water under neutral to slightly acid conditions [24][25]. Ptaquiloside has already been detected in different water bodies. Concentrations of $0.6 \mu\text{g L}^{-1}$ have been reported in spring water wells in Ireland [27], up to $0.09 \mu\text{g L}^{-1}$ in shallow groundwater (0.1 – 2.5 m) in Denmark [28], and up to $2.2 \mu\text{g L}^{-1}$ in stream water in

United Kingdom [29]. In contrast to PTA, occurrence of CAU and PTE in the aqueous environment has been much less investigated. A recent study reported the first occurrence of CAU and PTE in surface waters up to 0.07 and 5.3 $\mu\text{g L}^{-1}$, respectively [30]. This finding suggests that assessments of water toxicity based on PTA only were underestimated. CAU and PTE are slightly more polar compounds and as stable as PTA in surface waters [30].



	R1	R2	Pterosins	Molecular formula	Estimated LogP [31]
1) PTA	H	CH ₃	B	C ₂₀ H ₃₀ O ₈	-1.3
1a) CAU	CH ₃	CH ₂ OH	A	C ₂₁ H ₃₂ O ₉	-1.4
1a) PTE	H	CH ₂ OH	G	C ₂₀ H ₃₀ O ₉	-2.0

Figure 1. Hydrolysis of PTA (1) to PtB (3) and PTA analogues CAU and PTE (1a) to the corresponding pterosins (2a). The reactive cyclopropyl group is marked with a circle [20][32].

Recent attempt of modelling PTA fate in plant-soil matrix indicates that intense precipitation events during cold periods and with a fully developed canopy induce high probability of significant PTA leaching [33]. Furthermore, predicted high concentration of PTA in groundwater is also attributed to macropore transport [33]. Macropores have proven to be important for fast leaching of organic contaminants such as pesticides, bypassing biologically active layers, and thus resulting in low extent of biodegradation [34]. Leaching of pesticides is pronounced in fractured clayey till soils compared to sandy soils due to rapid well-connected macropores [35]. Hence, transport of organic contaminants from upper soil layers into deep groundwater is frequently observed for a variety of pesticides and herbicides [35]-[37]. It has never previously been investigated whether illudane glycosides are mobile and stable enough, to reach deep aquifers used for drinking water supply.

In Denmark, groundwater-based drinking water utilities mainly apply deep groundwater abstraction wells, and subsequently water is treated at waterworks before distribution to the consumers. In contrast to this, in some areas the consumers rely on private water wells, typically more shallow and the raw water might not be treated prior to consumption. While deep groundwater wells usually

abstract the water from confined aquifers, shallow water wells typically abstract water from unconfined aquifers and may be affected by seepage water. Several studies suggest that shallow wells are more prone to contamination by organic contaminants [38][39]. Pesticides are found in higher concentration in Danish shallow water wells as compared to deep groundwater wells [39]. Therefore, shallow water wells might also be more vulnerable to contamination by illudane glucosides.

It is currently unknown whether the bracken illudane glycosides PTA, CAU and PTE pose a threat to groundwater-based drinking water supplies. Thus, it is important to: 1) Investigate if the illudane glycosides are found in wells used for drinking water production, and subsequently 2) Identify the characteristics of vulnerable wells. To do so, we studied the presence of PTA, CAU and PTE and their corresponding pterosins PtB, PtA and PtG in a variety of water wells in bracken dominated areas in Denmark, Sweden and Spain, as function of well depth and water chemistry (pH and electric conductivity).

2. Materials and Methods

Solvents, chemicals and resins

Acids, bases and buffers (glacial acetic acid, formic and hydrochloric acids, sodium hydroxide, ammonium acetate) were all of analytical grade from Sigma-Aldrich (Germany). LC-MS grade acetonitrile was obtained from Merck Millipore (LiChrosolv hypergrade for LC-MS, Germany) and LC-MS grade methanol was purchased from Honeywell (LC-MS Chromasolv, Germany). MilliQ water (electrical resistivity 18.2 M Ω cm, TOC less than 2 μ g/L) was produced by Sartorius Ultrapure water system (Sartorius Stedim Biotech GmbH, Germany). Polyamide for column chromatography was from Fluka Analytical, Sigma-Aldrich Co (Germany). Oasis MAX (20 cc, 60 mg Sorbent, 30 mm particle size) was purchased from Waters (Milford, USA).

Preparation of analytical standards

No certified reference materials of PTA, CAU and PTE exist and therefore analytical standards were produced in-house. They were prepared from bracken plant material by the method described by Kisielius *et al.* [40].

Analytical procedure

Prior to LC-MS analyses, all water samples were pre-concentrated by solid-phase extraction (SPE) by a factor 250. The SPE method used in this study was optimized for PTA and PtB and validated for various groundwater samples [41]. A SPE protocol with more details is provided in Figure S2 of the supplementary material (SM). Together with each set of SPE samples, positive (for PTA and PtB spiked at concentration of 0.5 μ g L⁻¹) and negative controls (DI water) were assessed at the same time. In order to determine the efficiency of the SPE method for CAU, PTE, PtA and PtG, 50 mL of MilliQ water was spiked to a concentration of 0.5 μ g L⁻¹ for each compound. Following the SPE

protocol, samples were processed in separate SPE cartridges and analysed by LC-MS (n = 2). The recovery results for each compound are presented in Table 1.

Table 1. Recovery of illudane glycosides and corresponding pterosins in MilliQ spiked samples. OASIS MAX column (60 mg). The “±” represents standard deviation (n = 2).

Compounds	Recovery (%)
CAU	70 ± 3
PtA	95 ± 8
PTE	56 ± 5
PtG	99 ± 6
PTA	85 ± 2
PtB	91 ± 3

Note: The recovery values for PTA and PtB are from the recent study [41].

The samples were analysed using Agilent 1260 Infinity HPLC System equipped with Agilent 6130 Single Quadrupole mass spectrometer by the method described by Kisielius *et al.* [40]. Chromatographic separation was performed using Agilent Poroshell 120 EC-C18 column and the eluent comprised water (eluent A) and acetonitrile (eluent B), both with 0.1% v/v formic acid at gradient elution mode. The linear range of quantification was 20-500 µg L⁻¹ for PTA and PtB, and 10-250 for the remaining compounds (r > 0.999). The instrumental limits of detection (LOD) and quantification (LOQ) were: 0.22-0.68 µg L⁻¹ (PTA), 0.26-0.78 µg L⁻¹ (CAU), 0.08-0.25 µg L⁻¹ (PTE) respectively, and for pterosins, 0.03-0.09 µg L⁻¹ (PtB), 0.02-0.05 µg L⁻¹ (PtA) and 0.01-0.03 µg L⁻¹ (PtG). The LOD of the entire method including pre-concentration and instrumental LOD was ≤ 0.001 µg L⁻¹ for all illudane glycosides and pterosins.

For all samples with reported presence of illudane glycosides or pterosins, a qualitative confirmation protocol was used. Three levels of confirmation were applied: a) retention time of the compounds (always compared with retention time of the standards); b) spiking test for suspected positive samples (spiked with corresponding compound that was detected, which resulted in appearance of only one larger peak in the chromatogram); c) presence of confirmation ions extracted from the total ion chromatogram, e.g. [M-glucose-H₂O + H]⁺, [M-glucose + H]⁺, [M + Na]⁺, [M + K]⁺ reported by Kisielius *et al.* [40]. All samples with reported presence of illudane glycosides or pterosins were confirmed with spiking test and accurate retention times of the analytes. Furthermore, at least one of the confirmation ions was detected. More details on spiking test are provided in Figure S1. The concentrations of the compounds are reported as average concentrations measured in duplicates. In case the compound was detected in only one of the sample pairs the concentration was reported as a *trace*.

Identity of illudane glycosides and pterosins in bracken

Bracken plant material was collected from 11 locations during the water sampling campaign in order to quantify the content of illudane glycosides. From each location, 40 cm tip of a fully matured frond was collected in duplicates. Plant materials were dried in an ordinary plant press and grinded to powder prior to extraction according to Kisielius *et al.* [40]. Aqueous extracts were passed through a filter vial with 0.2 µm pore size membrane (Syringeless filter device Mini-Uniprep, GE Healthcare Life Sciences UK) prior to LC-MS analyses by which the contents of PTA, CAU and PTE and the pterosins were quantified using in-house produced standards [40].

Origin of water samples

Water samples from 77 wells in Denmark, Sweden and Spain were collected for analyses of PTA, CAU, PTE and the corresponding pterosaurs between June and October 2019. Water chemistry (pH and EC), well depth and more details on each investigated location is provided in Table S1. The majority of investigated wells belong to HOFOR utility (44 locations), the largest drinking water utility in Denmark, that is providing drinking water to approximately one million customers in the Greater Copenhagen area. The remaining investigated locations were selected based on presence of dense bracken ferns in their vicinity, diverse depth of water wells, geographical area and water type. In order to identify water wells with these features, an advertising campaign was performed through different public media prior to monitoring. Raw water (with no treatment) was abstracted from single wells. In case of HOFOR wells, mixed groundwater was collected (raw water mixed from several single wells). Only one location (number 9) does not represent a water well, but is seepage water collected from a creek (3 – 4 m deep) just after a rain event. Wells 68 and 69 were sampled on two occasions, summer and fall 2019.

Most of the monitored wells serve for drinking water purpose. The 44 groundwater wells operated by HOFOR utility included in the study were deep groundwater wells (40 – 100 meters). The remaining 33 wells were less than 40 meters deep. In the following text, HOFOR wells are classified as deep wells (44) and the others as shallow wells (33).

Water sample collection

Water samples were collected and preserved in the field according to the method of Skrbic *et al.* [41]. The preservation protocol was originally developed for PTA and PtB, but was further validated for CAU and PTE and their pterosaurs in this study. 50 mL groundwater was collected in amber glass bottles, buffered in the field by adding between 0.05 and 1 mL of 0.5 M ammonium acetate adjusted to pH 5 with glacial acid for stabilizing the PTA against hydrolysis. Samples were transported to the lab without ice for transportation up to 2 hours (otherwise, placed on ice and transported within 4 hours) and stored at 4 °C. SPE was performed in the lab within 72 hours after sampling. Final samples were stored in vials at -18 °C until LC-MS analyses. Filtration with sterile syringe through 0.45 µm pore size filters was performed only on two occasions, when very shallow/murky water was collected (locations 9 and 62). The samples were collected in duplicates, except for the HOFOR samples that were collected as single samples. The field blanks (DI water) were collected and treated as field samples during transport, preparation and analysis. The samples from Spain were packed with dry ice and delivered to Denmark by express mail (within 2 days).

3. Results

Identity of illudane glycosides and pterosins in bracken

The most commonly found illudane glycoside in bracken plant material was PTA, which was detected at all eleven sampled locations, while at eight locations CAU could also be detected (Table 2). The highest PTA concentration was measured at location 12 (3.23 mg g⁻¹ dry weight) and the highest CAU content was detected at location 66 (0.42 mg g⁻¹ dry weight). Neither PTE nor its degradation product PtG was detected in any of the analysed plant samples.

Table 2. Measured content of illudane glycosides and corresponding pterosins in bracken ferns. The “-“ represents no detection (< LOD) and “±“ represents standard deviation (n = 2). Average coefficient of variation equals 33%, 37%, 21% and 44% for PTA, PtB, CAU and PtA, respectively. Location numbers correspond to the map in Figure 2.

Location no.	Country of origin	Sampling date	mg g ⁻¹ dry weight					
			PTA	PtB	CAU	PtA	PTE	PtG
1	Denmark	08.08.19	0.05±0.01	0.01±0.01	-	-	-	-
4	Denmark	07.08.19	1.10±0.97	0.07±0.05	0.12±0.05	0.02±0.02	-	-
6	Denmark	07.08.19	0.13±0.01	0.02±0.00	-	0.01±0.00	-	-
7	Denmark	07.08.19	0.74±0.23	0.11±0.01	0.18±0.02	0.11±0.01	-	-
8	Denmark	07.08.19	0.10±0.06	0.03±0.02	0.04±0.01	0.01±0.01	-	-
12	Denmark	21.08.19	3.23±1.50	0.18±0.25	0.37±0.19	0.08±0.06	-	-
13	Denmark	21.08.19	0.63±0.07	0.07±0.01	0.07±0.01	0.01±0.01	-	-
17	Denmark	05.08.19	0.08±0.03	0.01±0.00	-	-	-	-
66	Sweden	13.10.19	0.24±0.12	-	0.42±0.07	0.01±0.00	-	-
68	Sweden	01.08.19	3.19±0.18	0.18±0.03	0.10±0.02	0.01±0.01	-	-
69	Sweden	01.08.19	3.02±0.19	0.45±0.05	0.12±0.01	0.11±0.03	-	-

Leaching of illudane glycosides after a rain event

The presence of illudane glycosides and the corresponding pterosins was determined in seepage water collected just after a rain event in a bracken dominated region (location 9). At this location, both PTA (1.27 µg L⁻¹) and CAU (0.28 µg L⁻¹) and their hydrolysis products were detected (Table 3).

Water samples

We sampled 77 water wells in Denmark (64), Sweden (5) and Spain (8) representing different geological settings. Neither bracken illudane glycosides (PTA, CAU and PTE) nor their hydrolysis products (PtB, PtA and PtG) were detected in 70 of the investigated wells (91%). However, in seven wells (9%) at least one of the compounds was detected (Figure 2).

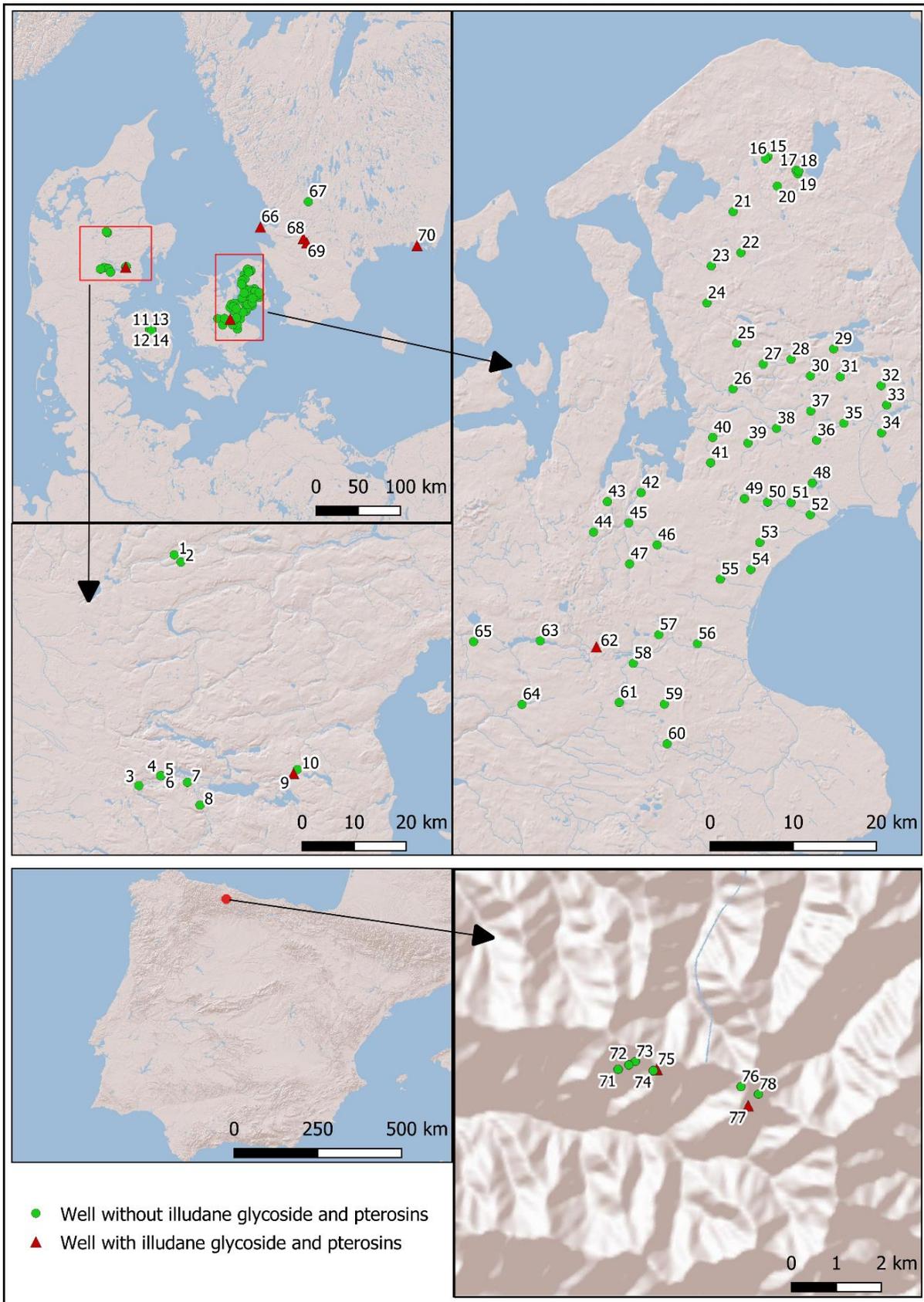


Figure 2. The investigated water wells in Denmark, Sweden and Spain. All sampling locations were associated with adjacently growing bracken ferns. Map is produced by Qgis 3.4 Madeira and background map is ESRI Shaded Relif.

The deep groundwater wells did not contain illudane glycoside or pterosins. All seven wells in which at least one of the compounds was detected were shallow wells (Figure 3). In terms of geography, one of the shallow wells with presence of illudane glycosides and/or pterosins was located in Denmark, four in Sweden and two in Spain (Figure 2). Apart from well 62, all these wells serve for drinking water purpose for humans and/or livestock (Table 3).

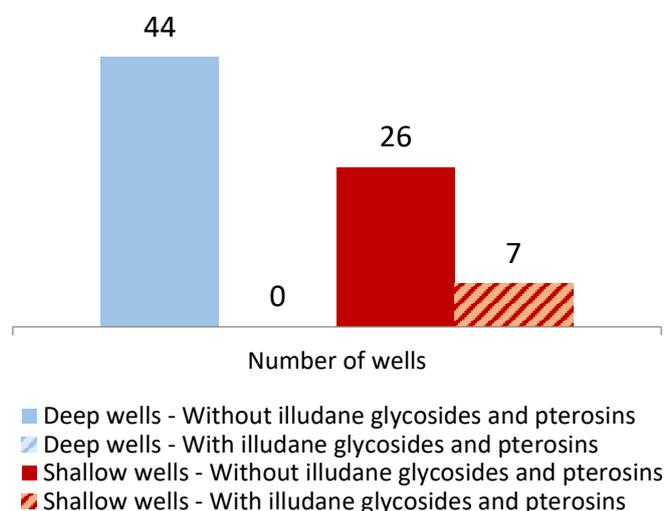


Figure 3. Number of deep and shallow wells with and without illudane glycosides and/or pterosins.

Three shallow wells contained CAU in concentrations up to $0.75 \mu\text{g L}^{-1}$ (Table 3). However, PtA, the degradation product of CAU, occurred in six out of the seven wells, indicating even more frequent presence of CAU. PTA was present in three wells up to $0.27 \mu\text{g L}^{-1}$, while two wells contained PtB. PTE was not detected in any of the wells, while its degradation product PtG was detected in three of them.

Table 3. Water wells with presence of illudane glycosides and/or corresponding pterosins. Empty cells represent no detection ($< \text{LOD}$) and “ \pm ” represents standard deviation ($n = 2$). Average coefficient of variation equals 8%, 24%, 6%, 15% and 42% for PTA, PtB, CAU, PtA and PtG, respectively. Only location 9 does not represent a water well.

Site number	9	62	66	68a	68b	69a	69b	70	75	77
Country	DK	DK	SE	SE	SE	SE	SE	SE	ES	ES
Sampling date	07.08	05.07	13.10	01.08	13.10	01.08	13.10	15.08	01.10	01.10
Distance to Bracken (m)	4	5	1	1	1	3	3	3	2	2
Well depth (m)	-	8	n.a.	40	40	10	10	20	n.a.	n.a.
Water pH	5.9	7	7.1	n.a.	7.2	n.a.	6.4	6.8	7.1	7.3
EC ($\mu\text{S/cm}$)	171	320	467	n.a.	310	n.a.	121	487	812	737
Rain event (day b.s.)	1	1	1	1	2	1	2	n.a.	n.a.	n.a.
Soil type [42]	CS	SC	BR	BR	BR	n.a.	n.a.	n.a.	SS	SS
Groundwater purpose	Tech	Tech	Drink	Drink	Drink	Drink	Drink	Drink	Drink	Drink
PTA ($\mu\text{g L}^{-1}$)	1.27 ± 0.08	-	0.27 ± 0.02	-	-	-	-	-	trace	trace
PtB ($\mu\text{g L}^{-1}$)	0.08 ± 0.03	-	-	-	-	0.05 ± 0.00	-	-	-	0.003 \pm 0.00
CAU ($\mu\text{g L}^{-1}$)	0.28 ± 0.01	trace	-	0.59 ± 0.05	-	-	-	0.75 ± 0.04	-	-
PtA ($\mu\text{g L}^{-1}$)	0.01 ± 0.01	0.03 ± 0.00	0.01 ± 0.05	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.003 ± 0.00	0.01 ± 0.00	-
PTE ($\mu\text{g L}^{-1}$)	-	-	-	-	-	-	-	-	-	-
PtG ($\mu\text{g L}^{-1}$)	-	-	0.28 ± 0.04	trace	-	-	0.02 ± 0.02	-	-	-

Notes: DK=Denmark, SE=Sweden, ES=Spain; a=summer, b=autumn; n.a.=not available; CS=clayey sand soil; SC=sandy clay soil; BR=bedrock; SS=sandstone; Drink=drinking water, Tech= technical water (groundwater not used for drinking water purpose), EC=electrical conductivity, day b.s.=before sampling. All dates relate to year 2019.

In wells 62, 68 and 70 we detected both CAU and PtA. Similarly, PTA and PtB were detected in well 77. Detection of glycosides and corresponding pterosin in the same well validates the presence of glycosides. Well 62 was very shallow and not used for drinking purpose, but contained CAU and PtA at concentration up to $0.03 \mu\text{g L}^{-1}$. Presence of PTA has previously been reported for this location [41]. Wells 72 and 77 showed presence of PtA, PtB and trace levels of PTA. The depth of these wells is unknown.

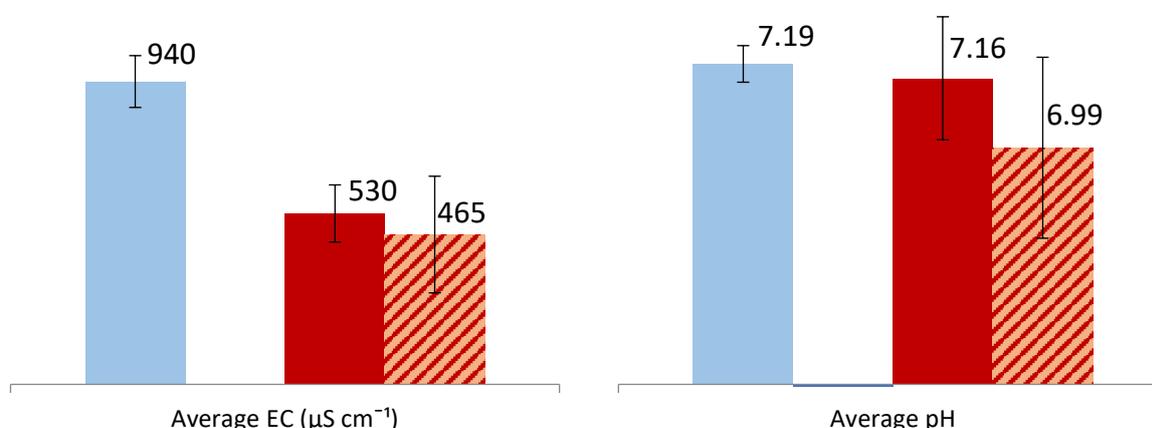
To include seasonal variations, we sampled well 68 and 69 both during summer and fall. In the summer, location 68 contained both CAU and PtA, while only PtA was found in the fall. At location 69, PtB and PtA were detected in the summer, but again only PtA appeared in the fall.

Characteristics of the water wells in relation to presence of illudane glycosides

To investigate determining factors for the presence of illudane glycosides in water wells, we analysed three characteristics of the sampled wells: depth, electrical conductivity (EC) and pH. First, for the subset of wells with known depths (59 out of 77 wells), the shallow wells with presence of illudane glycosides and/or pterosins were on average 19 meters deep (based on only three wells with known depths). Shallow wells without detected compounds were on average 30 meters deep, while deep wells were 40+ meters.

Secondly, EC in the deep wells were $940 \pm 80 \mu\text{S cm}^{-1}$, while conductivity in the shallow wells were $516 \pm 79 \mu\text{S cm}^{-1}$, showing different water chemistry in the deep versus the shallow wells (p -value $\ll 0.001$) (Figure 4). Additionally, shallow wells containing illudane glycosides and/or pterosins had lower average EC of $465 \pm 181 \mu\text{S cm}^{-1}$ when compared to the other shallow wells which had an average EC of $530 \pm 89 \mu\text{S cm}^{-1}$ but the difference was not statistically significant.

Finally, the wells with presence of illudane glycosides and/or pterosins had lower average pH (6.99 ± 0.22) when compared to the other shallow and deep wells ($\text{pH } 7.16 \pm 0.15$ and 7.19 ± 0.05 , respectively) (Figure 4). However, this difference was also not significant.



- Deep wells - Without illudane glycosides and pterosins
- Deep wells - With illudane glycosides and pterosins
- Shallow wells - Without illudane glycosides and pterosins
- Shallow wells - With illudane glycosides and pterosins

Figure 4. Deep and shallow wells with and without illudane glycoside and/or pterosins in relation to water pH and EC. The error bars represent 95% confidence interval.

4. Discussion

Presence of illudane glycosides in drinking water wells and bracken

In this study, we detected illudane glycosides and their corresponding hydrolysis products in drinking water wells for the first time. Interestingly, CAU occurred more frequently than PTA, even though PTA is considered the most abundant carcinogen in bracken [43][44]. Based on estimated LogP values and order of chromatographic elution, CAU is observed to be more polar than PTA (Figure 1) [40][31], and is thus more prone to be mobile in the environment, and potentially end up in water recipients. In a recent study, concentrations of CAU and PTE in surface water were also found to be similar or higher than PTA [30]. Additionally, the same study showed that content of CAU in bracken was higher in Sweden than in Denmark [30], which could explain higher CAU occurrence in wells in Sweden in this study.

In the present study, we did not detect PTE and PtG in any of the collected bracken plant samples, and in accordance with this none of the water samples contained PTE. In Northern Europe PTE has only been detected rarely in bracken and in very low concentrations compared to PTA and CAU [30]. Nevertheless, we detected PTE's degradation product PtG in three wells, indicating prior presence of PTE (at locations where no PTE or PtG were found in the bracken plant material). The content of illudane glycosides in bracken is decreasing from June to November [27][30], thus their hydrolysis products pterosins might still be present in water sampled in autumn while the glycoside (mother compound) is not present anymore, or the content is below LOD. The content of PTE in bracken populations is highly variable with random distribution [30]. Hence, bracken plant material collected for this study might not be the representative for PTE occurrence in the study area.

Maximum measured illudane glycoside concentrations in drinking water wells in this study were $0.27 \mu\text{g L}^{-1}$ for PTA and $0.75 \mu\text{g L}^{-1}$ for CAU. The maximum estimated tolerable concentration of PTA in drinking water is $0.002 \mu\text{g L}^{-1}$ (based on a model with one cancer incidence per million) [45], and the PTA analogue CAU, is presumably equally toxic [20]. The tolerable concentration were thus exceeded with more than a factor of 100 in these specific drinking water samples, indicating a health threat to humans or animals who are supplied with this drinking water.

Leaching of illudane glycosides after a rain event - pulse effects

Both PTA ($1.27 \mu\text{g L}^{-1}$) and CAU ($0.28 \mu\text{g L}^{-1}$) as well as their hydrolysis products were detected in seepage water coming from clayey sand soil in a small creek (location 9). The sampling was performed just after a rain event during summer when concentration of illudane glycosides in bracken

is expected to be the highest [27][30]. Even though the water sample at this location was not collected from a water well, this demonstrates potential of bracken toxins to leach quickly from plant material through the top soil layers.

A rainfall event had occurred prior to sampling from a drinking water well in Sweden (well 66), when PTA was detected ($0.27 \mu\text{g L}^{-1}$). Similar situation was observed for locations 68 and 69 that were sampled on two occasions. During summer, CAU, PtA and PtG were detected after a rain event, while only PtA was found after a rain event in the autumn (location 68). Precipitation related pulses of higher PTA concentrations have already been observed for surface water and shallow groundwater [27][29][41]. The presence of fissured bedrock in the sediment profiles could facilitate fast transport of CAU to deeper layers and explain CAU presence in a 40 m deep well (location 66 and 68). The conditions leading to an extreme toxin wash out event (pulse effect) are when intense precipitation takes place over the fully developed bracken canopy with maximum PTA content, moist soils and continuous macropores/fracture systems stretching from the soil surface to groundwater [33]. Such an event is expected to be more likely for shallow wells due to better macropore connectivity over shorter than long distances.

This study was neither designed to cover temporal/spatial presence of illudane glycosides nor precipitation related pulses. Furthermore, by sampling the raw groundwater mixed from different wells in this study, a potential contamination could be diluted to below detection limit by water from non-contaminated wells.

Characteristics of the water wells in relation to presence of illudane glycosides

Water wells with presence of illudane glycosides and/or pterosins in this study shared several features. They were mostly privately owned, shallow and with low pH and EC. Electrical conductivity is typically higher in deep groundwater [46] as confirmed by this study. Opposed to that, in the shallow wells including wells with presence of illudane glycosides, we observed significantly lower EC that indicates a likely connection with rain and surface water (i.e. presence of seepage water). In addition, water in shallow wells (0 – 40 meters) that contain illudane glycosides and/or pterosins had slightly lower average pH in comparison to deep groundwater wells (40 – 100 meters). Lower pH (pH 4.5 – 6.5) is known to have stabilizing effect on PTA hydrolysis [24]. Furthermore, a very short distance to high bracken biomass (< 10 m) and rain event prior to sampling supported illudane glycoside occurrence.

The majority of wells with presence of illudane glycosides (6 out of 7) serve for drinking water supply. In Denmark there are 55,000 similar private single-abstraction drinking water wells [47] and it is estimated that that 1 in 10 citizens of the EU receives drinking water from small and local systems, including private wells [48]. These systems are more vulnerable to illudane glycoside contamination and might not have any sort of water treatment.

Illudane glycosides and their hydrolysis products were not detected in deep groundwater wells investigated in this study (40 – 100 m). This complies with the fact that PTA's half life in alkaline water is short, ranging from seconds to hours at $\text{pH} > 7$ [24]. Thus, in 80 days the PTA concentration will be reduced by 99.9% at pH 7 (or much shorter at higher pH) [24], indicating PTA is not sufficiently stable to reach deep groundwater aquifers as travel times from soil surface to deep

aquifers take 25 to 100 years [46] when macropore transport does not intervene. In contrast, shallow wells investigated in this study showed presence of illudane glycosides and/or pterosins. Shallow wells are easily affected by younger seepage water that is more vulnerable to illudane glycosides contamination. The presence of cracks or macropores in soil profiles of shallow wells could support fast transport of illudane glycosides in relation to pulses [35][49]. Similar observations were noticed when leaching of pesticides was investigated in clayey tills with up to 5-6 meters deep macropores [50]. In that study, leaching of pesticides into shallow groundwater was noticed and the transport occurred in macropores and was driven by precipitation events [50]. Hence, leaching of illudane glycosides to shallow drinking water wells is possible, it is site specific and probably occurs in pulses.

5. Conclusions

In this study, we detected at least one illudane glycoside or pterosin from bracken fern in seven of the 77 studied wells. Thereby we demonstrated that leaching of illudane glycosides to drinking water wells is possible and could pose a threat to human health. Deep groundwater wells did not contain bracken illudane glycosides, probably due to the long travelling time to these aquifers. However, we detected bracken illudane glycosides in shallow wells, which could be due to intrusion of seepage water. A dense bracken biomass aboveground is a risk factor, especially in relation to rain events.

Six out of seven wells with presence of illudane glycoside were used for drinking water supply. The concentrations in these drinking water wells were $0.27 - 0.75 \mu\text{g L}^{-1}$, which violate the maximum estimated tolerable PTA concentration in drinking water by approximately 100 – 300 fold. In this study, CAU was more frequently detected in drinking water wells than PTA. Hence risk assessments are likely to underestimate water toxicity if they are based on PTA only and should also include CAU and PTE. In particular, these results are of great importance for consumers supplied by shallow wells in bracken dominated regions.

List of abbreviations

PTA: Ptaquiloside

CAU: Caudatoside

PTE: Ptesculentoside

PtB: Pterosin B

PtA: Pterosin A

PtG: Pterosin G

PMOC: Persistent and mobile organic compounds

WHO: World Health Organization

DNA: Deoxyribonucleic acid

Kow: Octanol-water partition coefficient

LC-MS: Liquid chromatography–mass spectrometry

SPE: Solid Phase Extraction

LOD: Limit of detection

LOQ: Limit of quantification

dw: Dry weight

EC: Electrical conductivity

DK: Denmark

SE: Sweden

ES: Spain

n.a: Not available

CS: Clayey sand soil

SC: Sandy clay soil

BR: Bedrock

SS: Sandstone

Drink: Drinking water

Tech: Technical water

day b.s.: Before sampling

EU: European Union

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: N.S., A.-K.P., S.C.B.C., M.J.H., H.C.B.H. and L.H.R. Investigations: N.S., V.K. and L.H.R. Data curation: N.S. and L.H.R. Visualization: N.S. Funding acquisition: A.-K.P., H.C.B.H. and L.H.R. Supervision: A.-K.P., S.C.B.C., M.J.H., H.C.B.H. and L.H.R. Writing-original draft: N.S. Writing-Review and editing: N.S., A.-K.P., S.C.B.C., M.J.H., V.K., H.C.B.H. and L.H.R. All authors read and approved the final manuscript.

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Conflict of interest

There are no conflict of interest to declare.

Supplementary material

Occurrence of carcinogenic illudane glycosides in drinking water wells

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Table S1. More details on all locations included in the study (77 wells and seepage water sample - location 9).

Operating by	Well no. (map)	Country	Coordinates (WGS 84)	Date of sampling	Distance to Bracken (m)	Well Depth (m)	Ground water pH	Ground water EC ($\mu\text{S}/\text{cm}$)	Well purpose
Private well	1	Denmark	56.4464534 9.5991463	08.08. 2019	20	n.a.	7.5	399	Drinking
Private well	2	Denmark	56.4338899, 9.6198298	08.08. 2019	15	n.a.	7.3	572	Drinking
Private well	3	Denmark	56.0452958, 9.4834311	08.08. 2019	15	n.a.	7.4	346	Drinking
Private well	4	Denmark	56.0623034, 9.5512950	07.08. 2019	12	n.a.	6.1	194	Drinking
Private well	5	Denmark	56.0623034, 9.5512950	07.08. 2019	10	n.a.	6.6	183	Drinking
Private well	6	Denmark	56.0625166; 9.5532426	07.08. 2019	20	n.a.	7.6	305	Drinking
Private well	7	Denmark	56.0503431, 9.6337951	07.08. 2019	30	21	7.4	330	Drinking
Private well	8	Denmark	56.0104950, 9.6720990	07.08. 2019	15	n.a.	7.3	572	Drinking
-	9	Denmark	56.0639; 9.5617647	07.08. 2019	5	-	5.9	171	Technical
Private well	10	Denmark	56.0639 9.9617647	07.08. 2019	20	18	6.9	358	Drinking
VCS	11	Denmark	58.5421, 61.38983	21.08. 2019	~ 1000	24	7.1	820	Drinking
VCS	12	Denmark	58.4643, 61.39	21.08. 2019	~ 1000	40	7.3	691	Drinking
VCS	13	Denmark	58.5003, 61.38873	21.08. 2019	~ 1000	20	7.2	782	Drinking
VCS	14	Denmark	55.3932, 10.37301	21.08. 2019	~ 1500	n.a.	7.1	750	Drinking
Kagerup	15	Denmark	56.000368; 12.278585	05.08. 2019	~ 50	25	7.5	586	Drinking
Kagerup	16	Denmark	55.998108; 12.273644	05.08. 2019	~ 50	22	7.4	600	Drinking
Nøddebo	17	Denmark	55.983958; 12.331381	05.08. 2019	5	40	7.4	568	Drinking
Nøddebo	18	Denmark	55.983013; 12.336524	05.08. 2019	4	51	7.4	533	Drinking
Nøddebo	19	Denmark	55.98006; 12.33447	05.08. 2019	10	34	7.6	446	Drinking
Gadevang	20	Denmark	55.967861; 12.293716	05.08. 2019	50	36	7.4	577	Drinking
HOFOR	21	Denmark	55.942212; 12.2063971	12.06. 2019	n.a.	40 - 100	7.5	843	Drinking
HOFOR	22	Denmark	55.897089; 12.2176525	12.06. 2019	n.a.	40 - 100	7.3	628	Drinking
HOFOR	23	Denmark	55.885135; 12.1722189	12.06. 2019	n.a.	40 - 100	7.2	816	Drinking
HOFOR	24	Denmark	55.844043; 12.1478820	12.06. 2019	n.a.	40 - 100	7.1	954	Drinking
HOFOR	25	Denmark	55.798904; 12.2014806	11.06. 2019	n.a.	40 - 100	7.2	733	Drinking
HOFOR	26	Denmark	55.749306; 12.1901284	19.06. 2019	n.a.	40 - 100	7.2	918	Drinking
HOFOR	27	Denmark	55.774720; 12.2503511	11.06. 2019	n.a.	40 - 100	7.1	736	Drinking
HOFOR	28	Denmark	55.778493; 12.3040772	11.06. 2019	n.a.	40 - 100	7.1	1000	Drinking
HOFOR	29	Denmark	55.774959; 12.3620990	13.06. 2019	n.a.	40 - 100	7.1	814	Drinking

HOFOR	30	Denmark	55.769753; 12.3556521	13.06. 2019	n.a.	40 - 100	7.1	834	Drinking
HOFOR	31	Denmark	55.765310; 12.3757434	13.06. 2019	n.a.	40 - 100	7.0	929	Drinking
HOFOR	32	Denmark	55.734447; 12.4497087	19.06. 2019	n.a.	40 - 100	7.1	914	Drinking
HOFOR	33	Denmark	55.720583; 12.4575461	20.06. 2019	n.a.	40 - 100	6.9	1072	Drinking
HOFOR	34	Denmark	55.705495; 12.4551038	20.06. 2019	n.a.	40 - 100	7.0	956	Drinking
HOFOR	35	Denmark	55.706182; 12.3993642	17.06. 2019	n.a.	40 - 100	7.0	956	Drinking
HOFOR	36	Denmark	55.701389; 12.3824231	17.06. 2019	n.a.	40 - 100	6.9	839	Drinking
HOFOR	37	Denmark	55.721150; 12.3372063	20.06. 2019	n.a.	40 - 100	7.0	884	Drinking
HOFOR	38	Denmark	55.704145; 12.2700968	19.06. 2019	n.a.	40 - 100	7.1	745	Drinking
HOFOR	39	Denmark	55.701225; 12.2404061	19.06. 2019	n.a.	40 - 100	7.4	886	Drinking
HOFOR	40	Denmark	55.697330; 12.1471551	19.06. 2019	n.a.	40 - 100	7.1	783	Drinking
HOFOR	41	Denmark	55.673482; 12.1412499	19.06. 2019	n.a.	40 - 100	7.1	703	Drinking
HOFOR	42	Denmark	55.636130; 11.9965528	12.06. 2019	n.a.	40 - 100	7.4	969	Drinking
HOFOR	43	Denmark	55.629909; 11.9685397	12.06. 2019	n.a.	40 - 100	7.1	1664	Drinking
HOFOR	44	Denmark	55.597285; 11.9396549	12.06. 2019	n.a.	40 - 100	7.1	806	Drinking
HOFOR	45	Denmark	55.608339; 11.9792592	12.06. 2019	n.a.	40 - 100	7.4	1619	Drinking
HOFOR	46	Denmark	55.583068; 12.0315054	24.06. 2019	n.a.	40 - 100	7.3	1016	Drinking
HOFOR	47	Denmark	55.579841; 11.9770266	24.06. 2019	n.a.	40 - 100	7.5	963	Drinking
HOFOR	48	Denmark	55.643366; 12.3099030	20.06. 2019	n.a.	40 - 100	7.3	1076	Drinking
HOFOR	49	Denmark	55.629608; 12.2209762	20.06. 2019	n.a.	40 - 100	7.5	864	Drinking
HOFOR	50	Denmark	55.624738; 12.2646894	19.06. 2019	n.a.	40 - 100	7.1	851	Drinking
HOFOR	51	Denmark	55.623169; 12.2674585	19.06. 2019	n.a.	40 - 100	7.1	851	Drinking
HOFOR	52	Denmark	55.616804; 12.2954776	20.06. 2019	n.a.	40 - 100	7.2	1050	Drinking
HOFOR	53	Denmark	55.580792; 12.2278963	20.06. 2019	n.a.	40 - 100	7.3	791	Drinking
HOFOR	54	Denmark	55.575190; 12.2218387	17.06. 2019	n.a.	40 - 100	7.2	751	Drinking
HOFOR	55	Denmark	55.542768; 12.1492360	17.06. 2019	n.a.	40 - 100	7.3	795	Drinking
HOFOR	56	Denmark	55.474569; 12.0616637	25.06. 2019	n.a.	40 - 100	7.2	910	Drinking
HOFOR	57	Denmark	55.486894; 12.0004963	25.06. 2019	n.a.	40 - 100	7.3	667	Drinking
HOFOR	58	Denmark	55.476192; 11.9761353	25.06. 2019	n.a.	40 - 100	7.3	1239	Drinking
HOFOR	59	Denmark	55.466542; 12.0063029	24.06. 2019	n.a.	40 - 100	7.3	1012	Drinking
HOFOR	60	Denmark	55.442168; 12.0114650	25.06. 2019	n.a.	40 - 100	7.3	695	Drinking
HOFOR	61	Denmark	55.468914; 11.9473276	25.06. 2019	n.a.	40 - 100	7.0	1047	Drinking

Private well	62	Denmark	55.475063,11.907543	05.07.2019	5	8	7.0	320	Technical
HOFOR	63	Denmark	55.478000; 11.8918956	19.06.2019	n.a.	40 - 100	7.2	1016	Drinking
HOFOR	64	Denmark	55.468702; 11.8277530	24.06.2019	n.a.	40 - 100	7.3	662	Drinking
HOFOR	65	Denmark	55.491024; 11.8291847	26.06.2019	n.a.	40 - 100	7.3	2090	Drinking
Private	66	Sweden	56.446492; 12.5622580	13.10.2019	1	n.a.	7.1	467	Drinking
Private	67	Sweden	56.40420; 13.29530	10.10.2019	5	n.a.	7.1	543	Drinking
Private	68	Sweden	56.29849; 13.370333	13.10.2019	1	40	7.2	310	Drinking
Private	69	Sweden	56.2983257; 13.3703952	01.08.2019	3	10	6.4	121	Drinking
Private	70	Sweden	56.12597; 15.52270	15.08.2019	3	n.a.	6.8	487	Drinking
Private	71	Spain	43.13347; 5.41273	01.10.2019	~ 2	na	7.0	837	Drinking
Private	72	Spain	43.13392; 5.41177	01.10.2019	~ 2	n.a.	7.1	812	Drinking
Private	73	Spain	43.13383; 5.41120	01.10.2019	~ 2	n.a.	6.5	615	Drinking
Private	74	Spain	43.13425; 5.41120	01.10.2019	~ 2	n.a.	7.0	1000	Drinking
Private	75	Spain	43.13397; 5.40498	01.10.2019	~ 2	n.a.	6.5	483	Drinking
Private	76	Spain	43.13380; 5.39253	01.10.2019	~ 2	n.a.	7.2	74	Drinking
Private	77	Spain	43.13252; 5.39150	01.10.2019	~ 2	n.a.	7.3	737	Drinking
Private	78	Spain	43.13252; 5.39150	01.10.2019	~ 2	n.a.	7.2	842	Drinking

Note: VCS – VandCenterSyd, Kagrup, Nøddebo and Gadevang are drinking water utilities in Denmark from which water samples were collected. Technical water is water not used for drinking water supply.

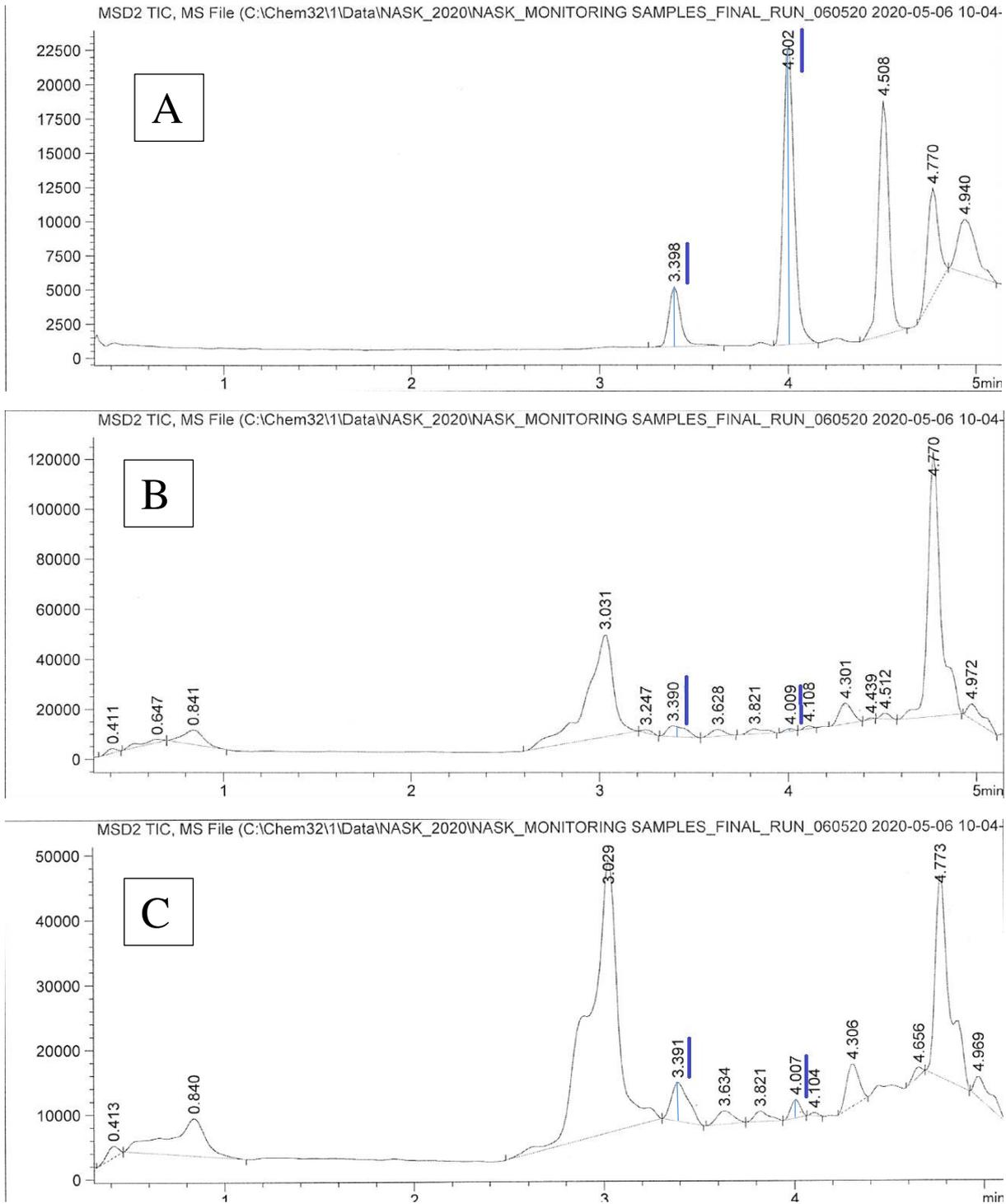


Figure S1. Confirmation spiking test. A – chromatogram of CAU (retention time 3.39) and PtA (retention time 4.00) in the standard, B – chromatogram with detected CAU and PtA in water sample (location 70), C - chromatogram with detected CAU and PtA in water sample (location 70), after the spiking test was performed

Solid-phase extraction (SPE) protocol

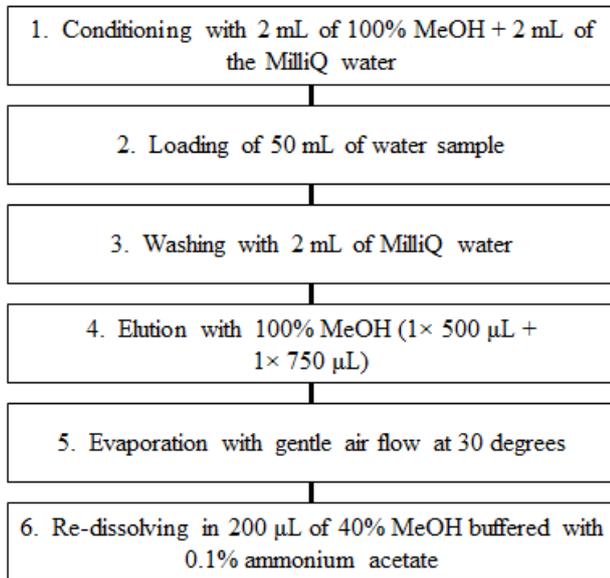


Figure S2. SPE protocol [41].

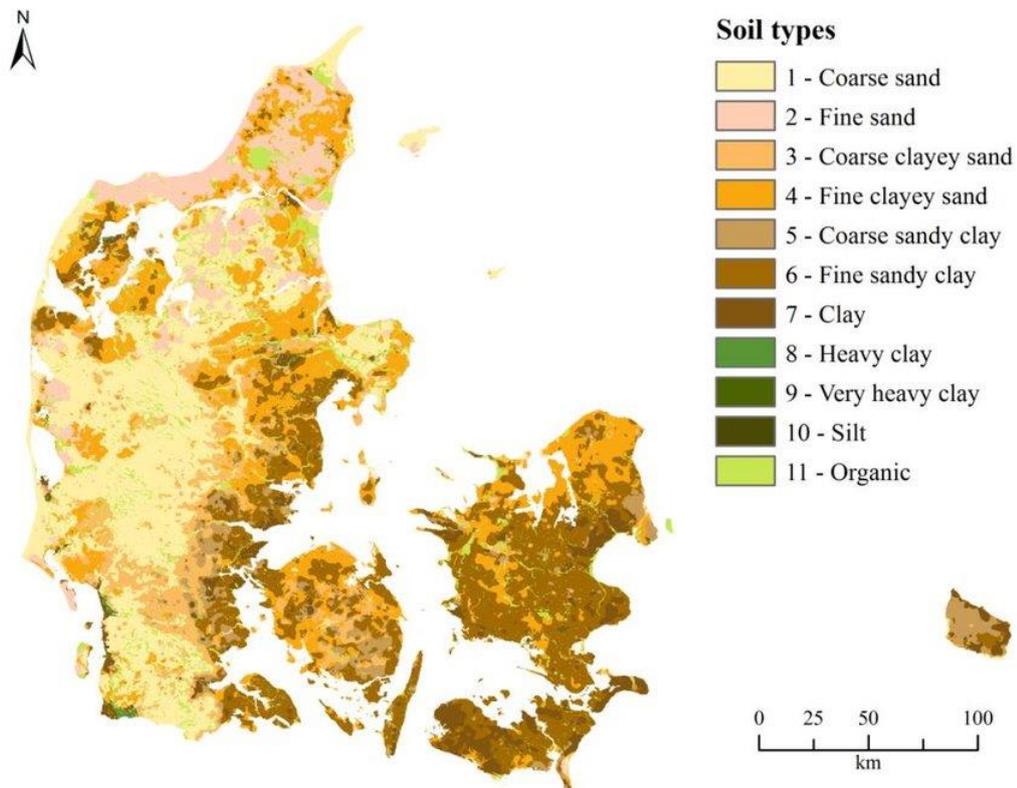


Figure S3. Soil map of Denmark according to the Danish Soil Classification [42].

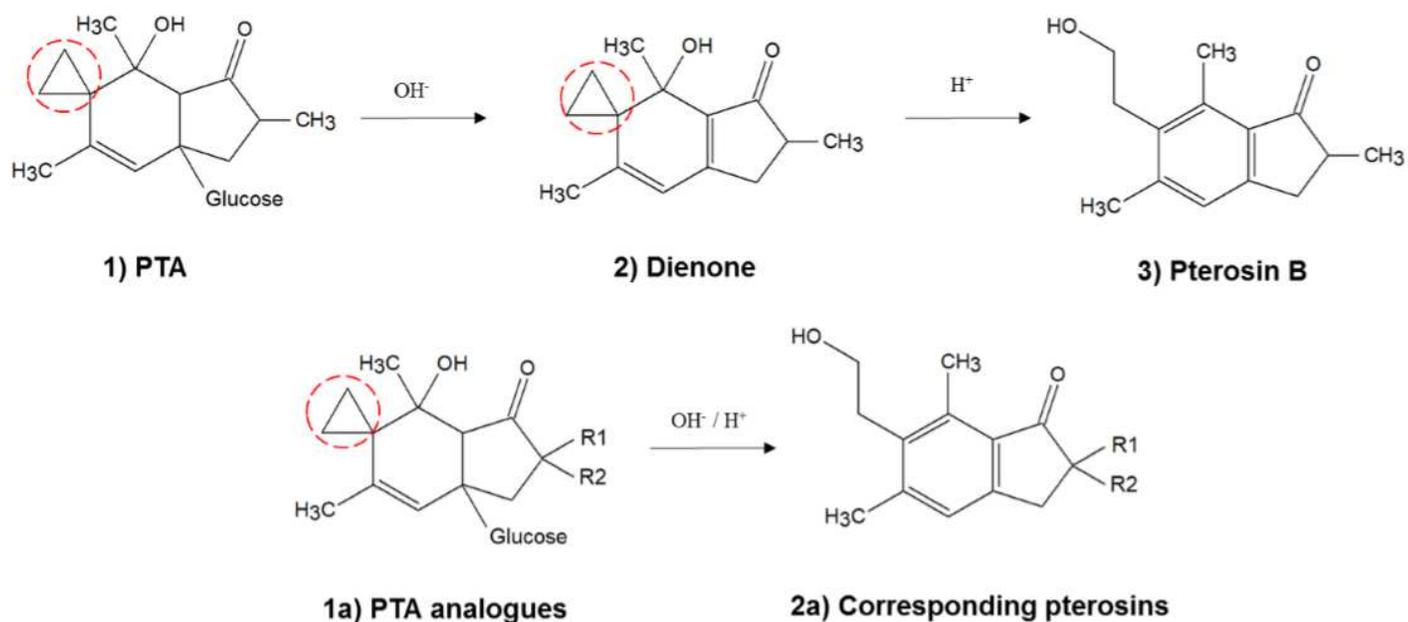
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Figures



	R1	R2	Pterosins	Molecular formula	Estimated LogP [31]
1) PTA	H	CH ₃	B	C ₂₀ H ₃₀ O ₈	-1.3
1a) CAU	CH ₃	CH ₂ OH	A	C ₂₁ H ₃₂ O ₉	-1.4
1a) PTE	H	CH ₂ OH	G	C ₂₀ H ₃₀ O ₉	-2.0

Figure 1

Hydrolysis of PTA (1) to PtB (3) and PTA analogues CAU and PTE (1a) to the corresponding pterosins (2a). The reactive cyclopropyl group is marked with a circle [20][32].

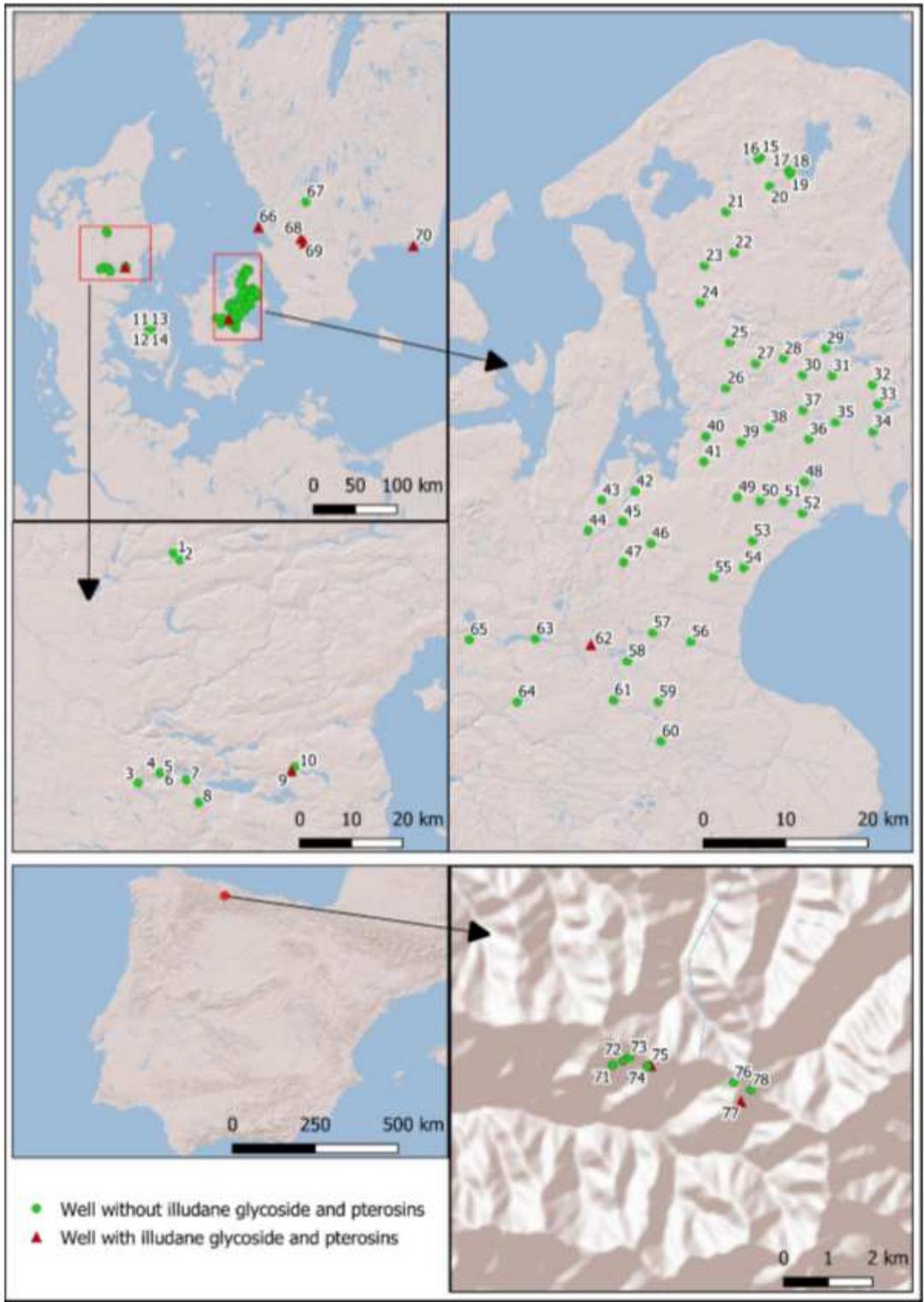


Figure 2

The investigated water wells in Denmark, Sweden and Spain. All sampling locations were associated with adjacently growing bracken ferns. Map is produced by Qgis 3.4 Madeira and background map is ESRI Shaded Relif.

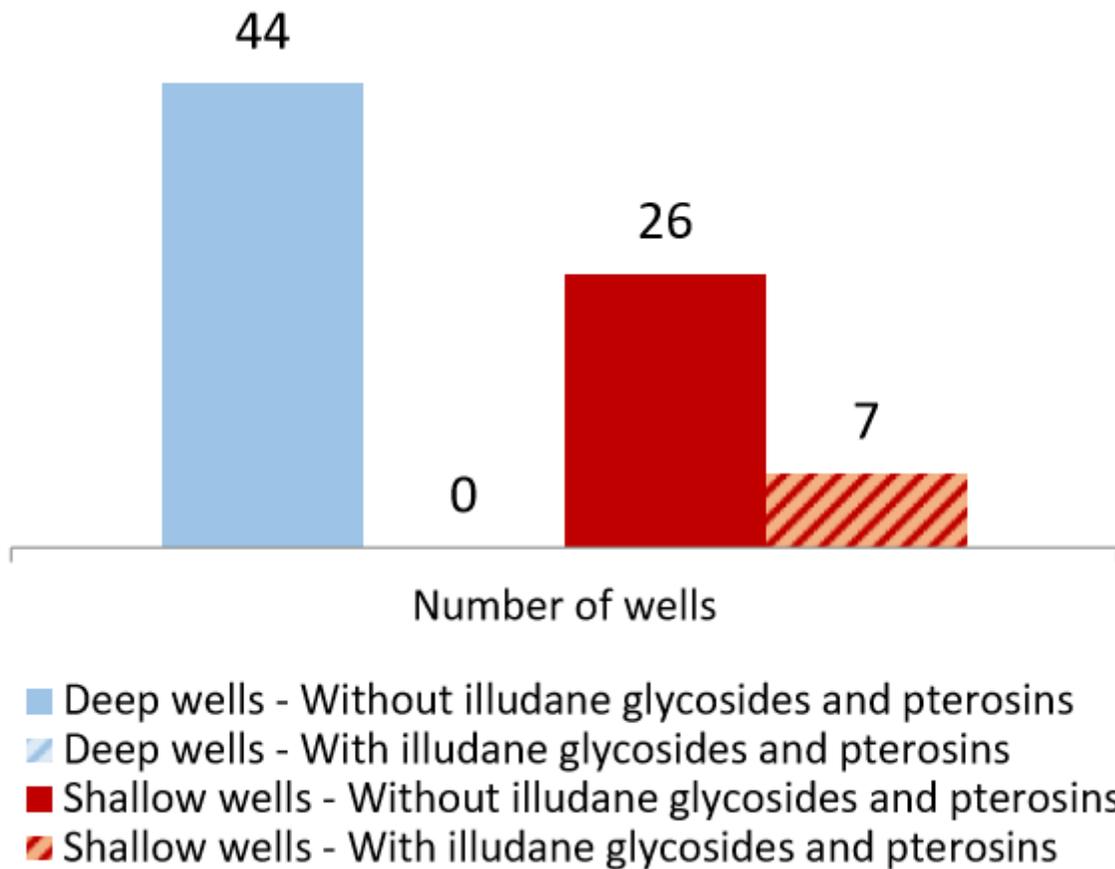


Figure 3

Number of deep and shallow wells with and without illudane glycosides and/or pterosins.

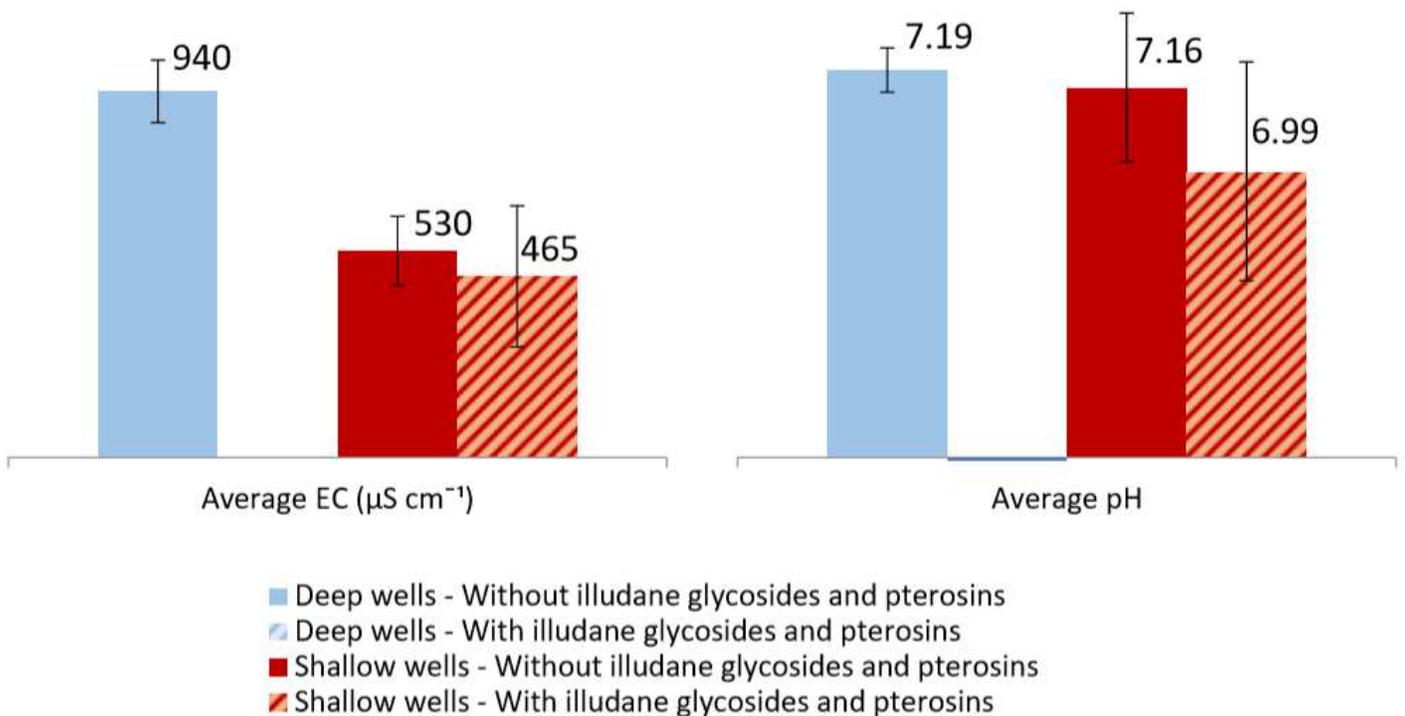


Figure 4

Deep and shallow wells with and without illudane glycoside and/or pterosins in relation to water pH and EC. The error bars represent 95% confidence interval. 4