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Hydrophilic interaction liquid chromatography with tandem mass spectrometric detection applied for analysis of pteridines in two *Graphosoma* species (Insecta: Heteroptera)

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ABSTRACT

A new separation method involving hydrophilic interaction chromatography with tandem mass spectrometric detection has been developed for the analysis of pteridines, namely biopterin, isoxanthopterin, leucopterin, neopterin, xanthopterin and erythropterin in the cuticle of heteropteran insect species. Two columns, Atlantis HILIC Silica and ZIC®-HILIC were tested for the separation of these pteridines. The effect of organic modifier content, buffer type, concentration and pH in mobile phase on retention and separation behavior of the selected pteridines was studied and the separation mechanism was also investigated. The optimized conditions for the separation of pteridines consisted of ZIC®-HILIC column, mobile phase composed of acetonitrile/5 mM ammonium acetate, pH 6.80, 85/15 (v/v), flow rate 0.5 mL/min and column temperature 30 °C. Detection was performed by tandem mass spectrometry operating in electrospray ionization with Agilent Jet Stream technology using the selected reaction monitoring mode. The optimized method provided a linearity range from 0.3 to 5000 ng/mL ($r > 0.9975$) and repeatability with relative standard deviation < 8.09% for all the studied pteridines. The method was applied to the analysis of pteridines in the cuticle of larvae and three adult color forms of *Graphosoma lineatum* and one form of *Graphosoma semipunctatum* (Insecta: Hemiptera: Heteroptera: Pentatomidae). The analysis shows that different forms of *Graphosoma* species can be characterized by different distribution of individual pteridines, which affects the coloration of various forms. Only isoxanthopterin was found in all the five forms tested.

1. Introduction

Pteridines belong to a group of compounds widely distributed in organisms, being present in Prokaryota as well as in Eukaryota (protists, plants, fungi and animals) [1]. Pteridines and their derivatives have several important biological functions. They are important compounds in the process of cell metabolism, such as hydroxylation reactions, conversion of tyrosine into 3,4-dihydroxyphenylalanine, precursor of melanin and play a role in cellular electron transport etc. [1–5]. The increased urinary excretion of these compounds has been found to indicate several disorders, for example viral infections [6] or different types of cancer [7–10]. Pteridines are also one of the families of pigmentary colors of insect cuticle, and some of them are also important eye pigments [11]. They have various colors (structure based), ranging from white (leucopterin), or yellow (xanthopterin) over red (erythropterin) to fluorescent blue under ultraviolet light (biopterin) [12]. Pigmentation caused by pteridines often serves as protective coloration which may be roughly divided into aposematic (warning) and cryptic (part of camouflage) colorations [13–15].

In this paper, pigmentary colors caused by pteridines in two species of heteropteran insects (true bugs) were studied. The pigments of both the species studied, *Graphosoma lineatum* and *Graphosoma semipunctatum* (Insecta: Hemiptera: Heteroptera: Pentatomidae), are formed in the epithelial part of the integument and deposited in the cuticle. The color patterns on the body of the *Graphosoma* species are formed by pigmentose pteridine ground-coloration (ranging from yellow to beige, red and brown) with overlaid longitudinal black stripes and spots formed by black melanin. It has been known for years that the hue of the ground

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color of West Palaearctic *Graphosoma* species varies. However, only recently has the seasonality of one of the northernmost population of *G. lineatum* (Sweden: Stockholm area) been investigated: the larvae are brown, metamorphosed pre-hibernating adults are pale (beige), over winter and emerge in spring as vividly red post-hibernating individuals. Similar, not so regular and less contrasting changes also occur in its Central European populations while the situation in the Mediterranean populations and in the closely related *G. semipunctatum* is more complex and possibly more area-dependent [15]. To complete the color picture and the pteridine array we have additionally included in our study yellow *G. lineatum* from Sardinia (post-hibernating population) and orange post-hibernating individuals of *G. semipunctatum* from the island of Zakynthos (Greece). Henceforth, all five samples examined will be called "forms" for simplicity.

Analysis of pteridines is a difficult task because of their physicochemical characteristics, mainly sensitivity to light resulting in their degradation, the possibility of diverse oxidation products, low solubility and very low concentrations in biological materials [16]. The most widely applied technique for their analysis is high performance liquid chromatography (HPLC) usually coupled with fluorescence detection [7,10,17–22]. HPLC methods typically use reversed phase (RP) mode often employing almost 100% aqueous mobile phases. These mobile phases can generate non-reproducible retention times and reduce the lifetime of analytical columns [23–25]. Due to of solving these difficulties in the separating of pteridines hydrophilic interaction liquid chromatography (HILIC) was tested. However, only two papers on HILIC analysis of pteridines have been published to date [23,26]. Retention properties of ultra-HPLC hybrid stationary phases have been studied for the group of polar basic pteridines (neopterin, biopterin, dihydroneopterin and dihydrobiopterin) in HILIC with fluorescence and mass spectrometic detections [23]. HILIC coupled with fluorescence detection has been applied to the analysis of neopterin, biopterin and isoxanthopterin in urine samples [26]. Another method employed for the determination of pteridines was capillary electrophoresis (CE) [27,28,8,29,30]. Most of these cited papers are focused on the analysis of pteridines as cancer markers in humans. The reported analyses of pteridines in insect cuticles have been performed mainly by paper chromatography and/or by thin layer chromatography [1,12,31–37] and especially by HPLC [38]. According to Nemec et al. [12] the pigment pattern in the studied insect (locust), particulary with respect to pteridine-like molecules, is much more complex than it has been reported in previous publications and the identification of pteridine-like pigments would require more sophisticated methods, namely HPLC coupled with mass spectrometry [12].

As mentioned above, HILIC, which is profitable for the analysis of polar compounds especially in proteomics, glycomics and clinical analysis [39–41], could be a good alternative to RP HPLC for the analysis of pteridines. In HILIC mode the analytes interact with the hydrophilic stationary phase and the elution is generated by hydrophobic binary mobile phase containing water as a strong eluting solvent. The suggested mechanism in HILIC involves partitioning the hydrophobic mobile phase and a layer of mobile phase enriched with the aqueous part being partially immobilized on the surface of stationary phase [42]. However, the retention mechanism in HILIC does not seem to be completely clear. Hydrogen bonding is supposed to play an important role in this process [43]. It may also include ionic, dispersion and hydrophilic interactions [43–52].

The aim of this work was to develop a new LC–MS/MS method applicable for identification and quantitation of selected pteridines (biopterin, isoxanthopterin, leucopterin, neopterin, xanthopterin and erythropterin, for their structures, see Fig. 1), which can be present in integuments of *G. lineatum* and *G. semipunctatum*. The analysis of integuments from individual morphological forms should provide a relative distribution of pteridines, which is assumed to be responsible for the color variation of heteropteran insects. Due to the polar character of pteridines HILIC combined with high sensitivity tandem mass spectrometric detection was used. The method development process also contributed to a better understanding of HILIC separation. To the best of our knowledge (according to the literature) the application of HILIC with MS/MS detection for analysis of pteridines in insects has not been used yet.

2. Experimental

2.1. Chemicals, reagents and real samples

Acetonitrile (ACN, gradient grade), ammonium acetate (purity ≥ 99%), acetic acid (purity > 99.8%), formic acid (purity > 98%) and dimethyl sulfoxide (purity > 99.9%) were supplied by Sigma–Aldrich (St. Louis, USA). Ammonium hydroxide (solution 25%) was obtained from Lachner (Neratovice, Czech Republic). The deionized water used was purified with a Milli-Q water purification system from Millipore (Bedford, USA). Standards of pteridines, namely biopterin, isoxanthopterin, leucopterin, neopterin and xanthopterin, were purchased from Sigma–Aldrich (St. Louis, USA), and erythropterin was provided by Serva Feinbiochemica (Heidelberg, Germany).

Stock solutions of the individual standards were prepared at a concentration of 0.1 mg/mL by dissolving the compounds in dimethyl sulfoxide. Stock solutions were kept in the dark at 4 °C. The stock solutions were further diluted by acetonitrile to attain the required concentrations.

Real samples consisted of 5th (last) instar larvae (Stockholm, Sweden), pale pre-hibernating adults (Stockholm, Sweden), red post-hibernating adults (Stockholm, Sweden), yellow post-hibernating adults (Sardinia, Italy) of *G. lineatum*, and orange post-hibernating adults of *G. semipunctatum* (Zakynthos Is., Greece).

2.2. Instrumentation

All chromatographic measurements were carried out on a HPLC system Agilent HPLC series 1200 coupled with a Triple Quad 6460 tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) consisting of an automated injector, a column oven, a degasser and a quarternary pump. For data acquisition, the Mass Hunter Workstation software was used. Two columns were tested: Atlantis HILIC Silica (4.6 mm × 150 mm, 3 μm), based on silica gel, from Waters (Milford, USA) and ZIC®-HILIC (4.6 mm × 150 mm, 3.5 μm), based on zwitterionic sulfobetaine groups, from Merck (Darmstadt, Germany).

The temperature of the columns was kept at 30 °C and samples were termostated at 20 °C. The injection volume was 5 μL and the flow rate of mobile phase was 0.5 mL/min. The MS/MS measurements were performed in the selected reaction monitoring mode (positive and negative mode were switched every 30 ms in one run) using electrospray ionization with Agilent Jet Stream technology (AJS). AJS with thermal gradient technology uses super-heated nitrogen as a sheath gas to improve ion production and desolvation. Nitrogen was used as the collision, nebulizing and desolvating gas.

2.3. Chromatographic conditions

Ammonium acetate or ammonium formate buffers were prepared by dissolving the appropriate amounts of ammonium acetate or ammonium formate in deionized water and adjusted with acetic acid or formic acid or ammonium hydroxide to the required pH value.