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Komplexní analýza výstražných a obranných látek ploštic vysokoúčinnými separačními metodami

Comprehensive analysis of warning and defense compounds of true bugs by high-performance separation methods

Disertační práce

Vedoucí disertační práce: Doc. RNDr. Zuzana Bosáková, CSc.

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Prohlašuji, že se Mgr. Jan Krajíček podílel na níže uvedených publikací měrou uvedenou v závorkách:

[1] Krajíček, J.; Kozlík, P.; Exnerová, A.; Štys, P.; Bursová, M.; Čabala, R.; Bosáková, Z.: Capillary electrophoresis of pterin derivatives responsible for the warning coloration of Heteroptera. *Journal of Chromatography A* **1336**, 94-100 (2014).

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Abstrakt

V průběhu evoluce se vyvinulo mnoho strategií obrany hmyzu před jeho predátory. Evolučně nejstarší a nejrozšířenější je chemická obrana následovaná akustickou nebo optickou obranou. Spousta druhů hmyzu však používá najednou více typů varovných signálů, které působí na různé smyslové receptory jejich predátora. Takovýto komplexní způsob varovné signalizace se nazývá multimodální. Může vznikat například současným podílem chemických a optických signálů nebo kombinací akustické a optické signalizace. Právě kombinace chemicko-optické signalizace kořisti vůči predátorovi je pravděpodobně nejčastější formou multimodální signalizace. Předkládaná práce se zabývá analýzou biologicky aktivních látek, které participují při obranných mechanismech jednoho z rozšířených druhů hmyzu – ploštic.

Deriváty pterinu představují rozsáhlou skupinu přírodních látek odvozených od bicyklického heterocyklu pteridinu a vyskytují se prakticky ve všech živých organismech od bakterií k obratlovcům. U hmyzu primárně slouží jako pigmenty a například kutikulám ploštic propůjčují jejich nápadné zabarvení. První část disertační práce byla zaměřena na identifikaci a kvantifikaci derivátů pterinu v kutikulách vybraných druhů ploštic. Pro separaci a kvantifikaci deseti derivátů pterinu (L-sepiapterin, 7,8-dihydroxanthopterin, 6-biopterin, pterin, D-neopterin, isoxanthopterin, leukopterin, xanthopterin, erythropterin, pterin-6-karboxylová kyselina) byla vyvinuta metoda kapilární zónové elektroforézy s UV detekcí a hydrofilní interakční chromatografie ve spojení s tandemovou hmotnostní detekcí. Obě metody byly validovány z hlediska běžných validačních parametrů – linearita, správnost, přesnost, limit detekce a kvantifikace aj. Metody byly použity pro identifikaci a zjištění distribuce derivátů pterinu ve vybraných druzích ploštic.

Ploštice disponují velmi dobře vyvinutými pachovými žlázami, které při podráždění produkují velké množství silně zapáchajících látek, fungujících jako chemická ochrana proti jejich predátorům. Druhá část této práce se věnovala vhodným strategiím odběru těkavých sekretů ploštic. Byly navrženy nové neinvazivní metody odběrů těkavých sekretů ploštic s jejich následnou separací pomocí plynové chromatografie s hmotnostní detekcí. Prekoncentrace vzorků sekretů byla provedena sorpcí na vlákně SPME. Z důvodu velké komplexnosti vzorků sekretů byly testovány tři

typy SPME vláken: nepolární polydimethylsiloxan (PDMS), polární polyakrylát (PA) a bipolární divinylbenzen/carboxen/polydimethylsiloxan (DVB/CAR/PDMS). Celá optimalizační procedura byla prováděna pomocí faktorového plánu – RSM (response surface methodology).

Pro GC separaci byla použita moderní kapilární fluorovaná fáze Rtx-200 poskytující dobrou selektivitu pro látky z široké škály polarity. Vypracovaná metodika byla aplikována pro analýzu obranných sekretů samic a samců u vybraných druhů ploštic.

Abstract

Insects have developed many strategies of defence against predators in the course of evolution. The evolutionarily oldest and most widely used type of defence is chemical defence, followed by acoustic or optical defence. However, many species of insects use simultaneously multiple types of warning signals, which affect different sensory receptors of the given predator. Such a complex method of warning signals is called multimodal method. It may consist of a combination of simultaneous chemical and optical signals, or a combination of acoustic and optical signalling. The combination of chemical and optical signalling used against a predator is probably the most common form of multimodal signalling. The presented work deals with the analysis of biologically active substances, which participate in the defence mechanisms of a widespread species of insects – true bugs (Heteroptera).

Pterin derivatives represent a large group of natural compounds derived from pteridin, bicyclic heterocycle, and they are found in virtually all living organisms from bacteria to vertebrates. In insects, they primarily serve as pigments, resulting for example in striking coloration of cuticles of Heteroptera. The first part of the dissertation was focused on identification and quantification of pterin derivatives in cuticles of selected species of true bugs. The method of capillary zone electrophoresis with UV detection and hydrophilic interaction chromatography in combination with tandem mass spectrometry was developed for the purpose of separation and quantification of ten pterin derivatives (L-sepiapterin, 7,8-dihydroxanthopterin, 6-biopterin, pterin, D-neopterin, isoxanthopterin, leukopterin, xanthopterine, erythropterin, pterin-6-carboxylic acid). Both methods were validated in terms of conventional validation parameters - linearity, accuracy, precision, limit of detection and quantification etc. The methods were used to identify and detect distribution of pterin derivatives in selected species of true bugs.

True bugs have very well developed scent glands, which –when irritated - produce large amounts of highly odorous substances functioning as chemical protection against predators. The second part of this work dealt with appropriate strategies of collection of volatile secretions of true bugs. New, non-invasive methods of collection of volatile secretions of true bugs have been designed, with their subsequent separation

by means of gas chromatography with mass spectrometry. Pre-concentration of secretion samples was performed by adsorption on SPME fibre. Three types of SPME fibre were tested for the reason of high complexity of the secretion samples: non-polar polydimethylsiloxane (PDMS), polar polyacrylate (PA) and bipolar divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). The entire optimization procedure was performed using a factorial design - RSM (response surface methodology).

Modern capillary fluorinated phase Rtx-200 was used for GC separation providing good selectivity for substances from a wide range of polarities. The elaborated methodology was applied to analyse defensive secretions of males and females in selected species of true bugs.

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1 Seznam zkratek a symbolů

ACN	acetonitril
CZE	kapilární zónová elektroforéza
DVB	divinylbenzen
ELISA	Enzyme-Linked Immunosorbent Assay
GC	plynová chromatografie
HILIC	hydrofilní interakční chromatografie
HPLC	vysokoúčinná kapalinová chromatografie
LOD	limit detekce
LOQ	limit kvantifikace
PA	polyakrylát
PDMS	polydimethylsiloxan
RE	relativní chyba
RSD	relativní směrodatná odchylka
RSM	response surface methodology
SERS	povrchem zesílený Ramanův rozptyl
SPME	mikroextrakce tuhou fází (solid-phase microextraction)
SRM	selected reaction monitoring

R^2	linearita
t	doba analýzy

2 Cíle práce

Předložená disertační práce si klade za cíl přispět k poznání obranných mechanismů jednoho z významných zástupců hmyzu – ploštic (Insecta: Hemiptera: Heteroptera). Přítomnost či absence jednotlivých látek, které jsou odpovědné za výstražnou a obrannou signalizaci ploštic, může pomoci při fylogenetickém hodnocení jednotlivých druhů, která může být v čistě biologickém přístupu značně obtížná. Na základě dostupné literatury nebyla doposud žádná práce věnována komplexní analýze výstražného zabarvení a těkavých obranných látek u stejného druhu. V tomto směru může takovýto chemicko-biologický přístup přispět k poznání evoluce bimodální obrany ploštic. Hlavní cíle disertační práce tvoří:

1) Vývoj, optimalizace a validace metodiky kapilární elektroforézy a vysokoúčinné kapalinové chromatografie pro stanovení derivátů pterinu v kutikulách vybraných druhů ploštic.

2) Porovnání obou vyvinutých metod z hlediska účinnosti separace, citlivosti detekce, doby analýzy a běžných validačních parametrů.

3) Identifikace a stanovení obsahu derivátů pterinu zodpovědných za charakteristické zabarvení kutikul u vybraných druhů ploštic.

4) Navržení neinvazivních způsobů odběrů těkavých složek obranných sekretů ploštic.

5) Provedení optimalizace navržených způsobů odběrů s využitím RSM.

6) Aplikace nově vyvinuté metodiky na analýzu těkavých sekretů samců a samic vybraných druhů ploštic.

Výstražné zabarvení ploštic

3 Pterin a jeho deriváty

3.1 Aposematismus

Antipredační strategie živočicha, která pomocí výstražných signálů informuje případné predátory o nevýhodnosti (nepoživatelnosti) živočicha se nazývá aposematismus [1].

Nejnápadnější složkou aposematické strategie jsou vizuální signály. Optické signály (barva, vzor, kontrast vůči podkladu, symetrie aj.) bývají často spojovány s chemickými a/nebo akustickými signály. Takový komplexní způsob výstražné signalizace se označuje jako multimodální [2]. Predátoři mohou vnímat tyto výstražné signály kořisti různými smyslovými receptory a spojit si je tak s její nepoživatelností. Smyslem multimodální signalizace je, aby predátoři aposematickou kořist snadněji rozeznali, dlouhodoběji si zapamatovali a vyvarovali se opakovanému útoku [2-4]. V současné době se ale neví, jak přesně multimodální signalizace kořisti funguje, zda (a) urychluje detekci takové kořisti predátorem [5] nebo (b) chemické a/nebo akustické signály působí jako zesilovače optických (výstražných) signálů [5-10].

Deriváty pterinu propůjčují hmyzu typické zabarvení jejich kutikul počínaje od bílé barvy (leukopterin), přes žlutou (xanthopterin), červenou (erythropterin) až po fluorescenční modrou (biopterin) [11]. Červená, žlutá, oranžová a bílá barva mnoha druhů hmyzu je často kombinována s tmavými (obvykle černými) vzory a tím vytváří nápadné aposematické (varovné) zbarvení.

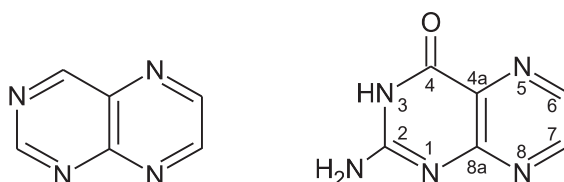
Ploštice jsou modelovým taxonem pro studium aposematismu, mimiker a antipredační ochrany. Jejich zabarvení je často kombinováno s černými skvrnami nebo pruhy. Barvy kutikul se mohou měnit v závislosti na stupni vývoje jedince, ročním období nebo také na geografickém výskytu daného druhu [12, 13]. Odstín zabarvení kutikul ploštic je pravděpodobně výsledkem přítomnosti určitých derivátů pterinu a jejich vzájemným poměrem.

3.2 Rozdělení a vlastnosti derivátů pterinu

Prvním izolovaným derivátem pterinu byl v roce 1889 žlutý xanthopterin , který obsahovala křídla motýlů z čeledi Pieridae [14]. Skupině těchto pigmentů se začalo později říkat pteridiny z řeckého slova *φτερό* = křídlo [15].

Tyto látky jsou hojně zastoupeny prakticky ve všech biologických systémech. Mají důležité biologické funkce jako inhibitory [16], senzitizéry [17, 18], enzymy [19], koenzymy [20], senzory [21] a dále fungují jako pigmenty [22-26] nebo toxiny [27]. Relativně vysoké koncentrace derivátů pterinu se nacházejí ve formě pigmentů u hmyzu, plazů, obojživelníků, krabů, mořského planktonu nebo v lidských tělních tekutinách [28].

Základním skeletem těchto heterocyklických sloučenin je pteridin, který se skládá z pyrimidinu a pyrazinu (obr. 3.1).



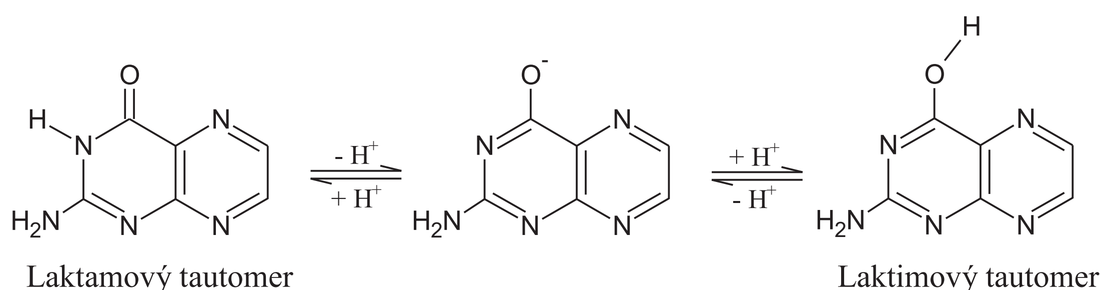
Obr. 3.1: Strukturní vzorce pteridinu (vlevo) a oxidovaného pterinu (vpravo)

Deriváty pterinu lze rozdělit do dvou velkých skupin na základě velikosti postranního řetězce. Nekonjugované mají na pteridinovém cyklu navázány malé substituenty. Konjugované obsahují velké substituenty s p-aminobenzoovou kyselinou jako je např. listová kyselina nebo riboflavin [29]. V přírodě se rovněž vyskytují isopteriny, např. xanthopterin a isoxanthopterin, lišící se polohou oxo skupiny v pozici 6 a 7. Pteriny se mohou vyskytovat ve třech oxidačních stavech, a to 7,8-dihydro-, 5,6,7,8-tetrahydro- a plně oxidované (aromatické) formě. Redukované formy pterinů působí jako silné lapače reaktivních forem kyslíku a jejich antioxidační účinky lze zvýšit interakcí s určitými kationty kovů např. Zn^{2+}, Cd^{2+} nebo Hg^{2+}. Oxidované formy pterinů vykazují silnou fluorescenci, která se může lišit v různých oblastech pH. Xanthopterin vykazuje v kyselé oblasti červenou fluorescenci, zatímco v alkalické oblasti modrou fluorescenci [15, 30-32].

Deriváty pterinu se na světle rozkládají, jsou fotosenzitivními sloučeninami. Redukované formy pterinu podléhají oxidaci vzdušným kyslíkem. Jejich oxidace je podpořena zvláště přítomností iontů některých kovů. Z mateřské sloučeniny nevzniká oxidací pouze plně oxidovaná forma, ale v důsledku různých přesmyků, které probíhají během oxidace, vznikají i různé deriváty pterinu [15, 30].

Díky přítomnosti amino- a oxo skupin, které jsou schopny tvořit intramolekulární vodíkové vazby, jsou substituované pteriny ve vodě prakticky nerozpustné. Rozpustnost ve vodě lze zvýšit derivatizací, která zabrání tvorbě těchto vazeb, např. alkylací aminové či amidové skupiny [27, 33].

Ve vodných roztocích se pteriny chovají jako slabé kyseliny a většina pterinů může existovat ve více acidobazických formách. V prostředí při pH 4 – 12 se ustavuje rovnováha mezi dvěma formami, kyselou formou s amidovou skupinou nebo zásaditou formou s fenolátovou skupinou, přičemž pK_a této rovnováhy je přibližně 8. Při pH okolo 5 se deriváty pterinu nacházejí z více než 99 % v kyselé formě, zatímco okolo 10 v zásadité formě [34, 35]. Pteriny existují v několika tautomerních strukturách, ale pouze dvě struktury se považují za významné – laktamová a laktimová (obr. 3.2).



Obr. 3.2: Významné tautomerní formy pterinů [36].

Laktamový tautomer má nejnižší energii a je proto nejstabilnější. Tautomerní rovnováha je posunuta ve prospěch této struktury a z tohoto důvodu se pteriny ve vodných roztocích chovají jako slabé kyseliny [36-38].

Vzhledem k fyzikálně-chemickým vlastnostem derivátů pterinu (citlivost na světlo, nízká rozpustnost a nízká koncentrace např. v biologických tekutinách) je analýza derivátů obtížná.

3.3 Studované analyty

Struktury studovaných derivátů pterinu jsou uvedeny na obr. 3.3.

Xanthopterin je žlutá krystalická látka, která se vyskytuje hlavně v křídlech motýlů a v moči savců. Dříve se předpokládalo, že xanthopterin je močová kyselina. Tato látka poskytuje murexidovou reakci, a proto vykazuje podobnost s kyselinou

močovou. Ovlivňuje růst a diferenciaci buněk, např. inhibuje růst lymfocytů stimulovaných konkanavalinem (látka rostlinného původu patřící k lektinům [39]). Některé mikroorganismy jsou schopny převést xanthopterin na listovou kyselinu (konjugovaný pterin a vitamin ze skupiny B) [40, 41].

Isoxanthopterin spolu s xanthopterinem patří mezi isopteriny. Tato látka je oproti xanthopterinu bezbarvá. U těchto isopterinu byla prokázána silná aktivita proti karcinomu mléčné žlázy u myší [15, 42].

Leukopterin je také bezbarvou krystalickou látkou a je hlavně obsažen v křídlech bělásků. Vyznačuje se vysokým bodem tání a je společným oxidačním produktem xanthopterinu a isoxanthopterinu [32, 43]. Protože také poskytuje murexidovou reakci, se i u něj dříve předpokládalo, že se jedná o močovou kyselinu [14].

6-Biopterin je vůbec nejrozšířenějším derivátem pterinu a poprvé byl objeven v moči savců. Jeho redukovaná forma, tetrahydrobiopterin, je kofaktorem některých monooxygenas aromatických aminokyselin [15, 44]. Porucha metabolismu biopterinu je spojena s řadou onemocnění, např. hyperfenylalaninemií nebo schizofrenií [45-47].

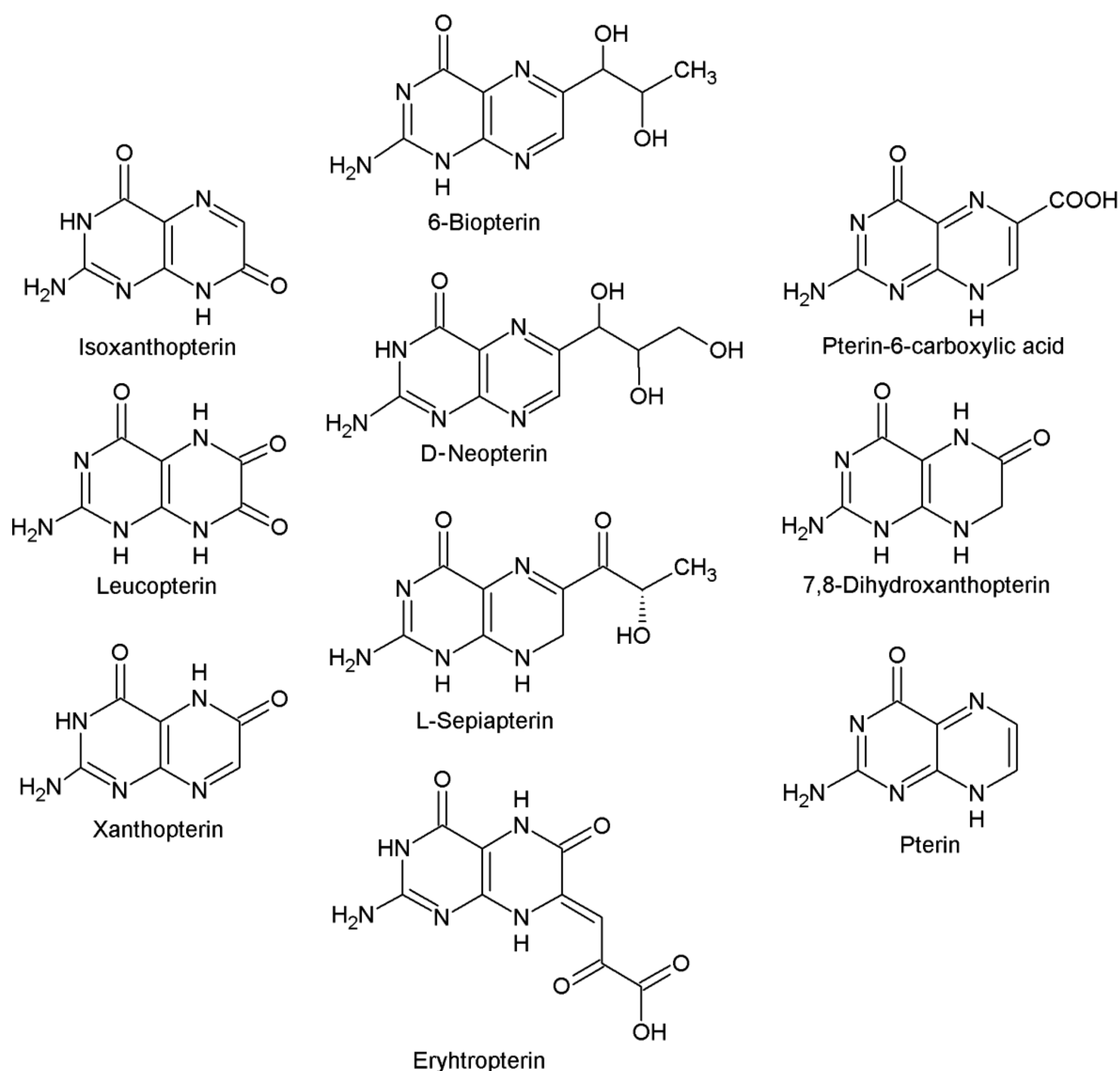
D-Neopterin spolu s 6-biopterinem jsou nejvýznamnějšími deriváty pterinu v lidském organismu. Poprvé byl izolován v roce 1963 z larev včel, včelích dělnic a mateří kašičky [48]. Jeho zvýšená koncentrace doprovází řadu onemocnění a souvisí s aktivací imunitního systému. U pacientů trpících maligními a virovými onemocněními byla koncentrace D-neopterinu poprvé stanovena metodou HPLC a později metodou radioimunoanalýzy [49-53].

Erythropterin je červenooranžové barvivo, které se nejčastěji vyskytuje v pigmentových granulích epidermálních buněk u hmyzu [54].

L-Sepiapterin je žlutá krystalická látka. Slouží jako substrát pro biosyntézu tetrahydrobiopterinu [55-57]. Spolu s 6-biopterinem slouží jako biomarker pohybových poruch a/nebo vývojového opoždění u dětí [58].

7,8-dihydroxanthopterin byl identifikován v moči pacientů trpícími fenylketonurií a hyperfenylalaninemií [59, 60]. Spolu s D-neopterin vznikají ze stejné výchozí látky - 7,8-dihydroneopterinu. Zda-li vznikne D-neopterin nebo 7,8-dihydroxanthopterin záleží na způsobu oxidace [61].

Pterin-6-karboxylová kyselina se v současné době studuje jako potencionální biomarker rakoviny močového měchýře [62, 63]. Vzniká například fotolytickým rozpadem listové kyseliny [64].



Obr. 3.3: Struktury studovaných derivátů pterinu.

3.4 Metody stanovení derivátů pterinu

Deriváty pterinu se nejčastěji stanovují v moči, plazmě, séru nebo mozkomíšním moku [15], kde se jejich koncentrace pohybuje v rozmezí 10^{-6} – 10^{-9} mol.dm^{-3}. V současné době se pro stanovení derivátů pterinu nejvíce využívají chromatografické metody, a to papírová chromatografie [25], reverzní [24, 65-72], iontově-párová [73], hydrofilní interakční [74, 75], iontoměničová [76, 77] a chirální [78] vysokoúčinná

kapalinová chromatografie. Kapilární elektroforéza byla poprvé použita pro analýzu derivátů pterinu v moči savců [79].

Díky přirozené fluorescenci derivátů pterinu se také používá fluorescenční detekce. Tyto analýzy se často provádí za přítomnosti pufru a některé anionty pufru (fosfátové, acetátové) mohou za určitých experimentálních podmínek (pH, koncentrace aniontu) významně snížit emisní záření derivátů pterinu ve vodných roztocích, což může vést k chybné interpretaci a závěrům [80].

K separaci derivátů pterinu v různých oxidačních stavech je vhodné použít iontově-párovou nebo iontoměničovou chromatografii, zatímco pro plně oxidované formy pterinu je výhodnější použít chromatografii na reverzních fázích. Pro přímé stanovení oxidovaných i redukovaných forem pterinu v biologických vzorcích se využívá elektrochemická detekce [15].

Pro analýzu derivátů pterinu byly aplikovány i spektrometrické metody, např. povrchem zesílený Ramanův rozptyl – metoda SERS [81, 82] nebo synchronní fluorescenční spektrometrie [83, 84]. Biopterin a D-neopterin lze stanovit radioimunoanalýzou nebo metodou ELISA a v současné době jsou již dostupné i komerční sety [15].

Pro analýzu derivátů pterinu v kutikulách hmyzu byla využita hlavně papírová chromatografie a/nebo tenkovrstvá chromatografie [11, 23, 25, 85-90], kapilární elektroforéza [91, 92], vysokoúčinná kapalinová chromatografie v reverzním [24] čihydrofilním interakčním módu [90, 93]. Na základě dostupné literatury lze konstatovat, že analýzám derivátů pterinu ve hmyzu se v současné době věnuje velmi málo vědeckých skupin a veškerý výzkum je orientován na klinické využití těchto látek.

Na následujících stránkách jsou uvedeny výsledky týkající se analýz derivátů pterinu v kutikulách ploštic metodami kapilární zónové elektroforézy (Publikace I a II) a vysokoúčinné kapalinové chromatografie (Publikace III). Porovnání obou vyvinutých metod je uvedeno v kapitole 3.5.

3.5 Kapilární elektroforéza a vysokoúčinná kapalinová chromatografie – publikace I – III

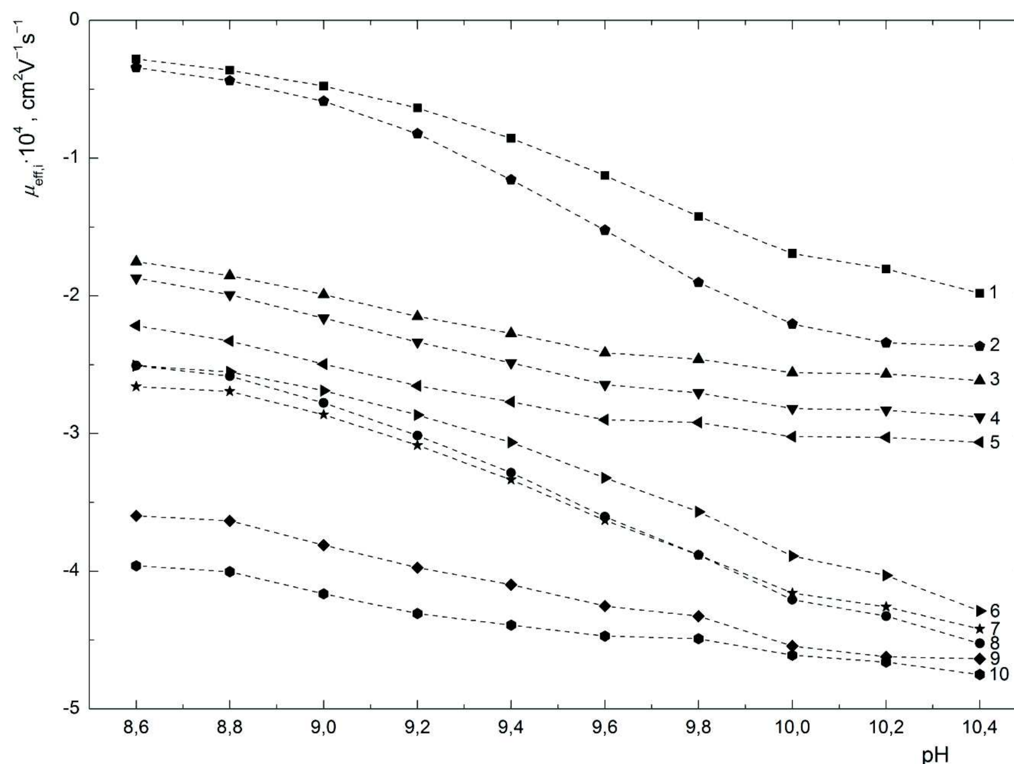
Deriváty pterinu se ve vodných roztocích chovají jako slabé kyseliny. Kapilární elektroforéza je vhodnou alternativou k poměrně komplikovaným a finančně náročným separačním a detekčním systémům používaných pro analýzu derivátů pterinu v HPLC. Na základě dostupné literatury nebyla doposud kapilární elektroforéza pro analýzu derivátů pterinu v kutikulách hmyzu použita.

Pro identifikaci a kvantifikaci deseti derivátů pterinu - L-sepiapterinu, 7,8-dihydroxanthopterinu, 6-biopterinu, D-neopterinu, isoxanthopterinu, leukopterinu, xanthopterinu, erythropterinu, pterin-6-karboxylové kyseliny byla vyvinuta nová metoda kapilární elektroforézy.

V rámci optimalizační procedury byl studován vliv pH pufru, koncentrace jednotlivých složek základního elektrolytu, velikosti separačního napětí a vlnové délky UV detekce na elektromigrační chování a citlivost detekce. Největší vliv na elektromigrační chování studovaných analytů měla hodnota pH (viz. obr. 3.4).

Z obr. 3.4 je patrné, že s rostoucí hodnotou pH separačního elektrolytu dochází k nárůstu záporných hodnot efektivních elektroforetických pohyblivostí všech sledovaných derivátů, což je ve shodě s předpokladem jejich větší disociovanosti. Z obr. 3.4 je také zřejmé, že separace derivátů pterinu je velmi citlivá na hodnotu pH základního elektrolytu. Oddělení všech studovaných derivátů pterinu lze získat pouze ve velmi úzkém rozmezí pH a zejména isoxanthopterin, leucopterin a xanthopterin, jež jsou si strukturně velmi podobné látky a jsou obecně hojně zastoupene v hmyzu, migrují ve velmi podobných časech.

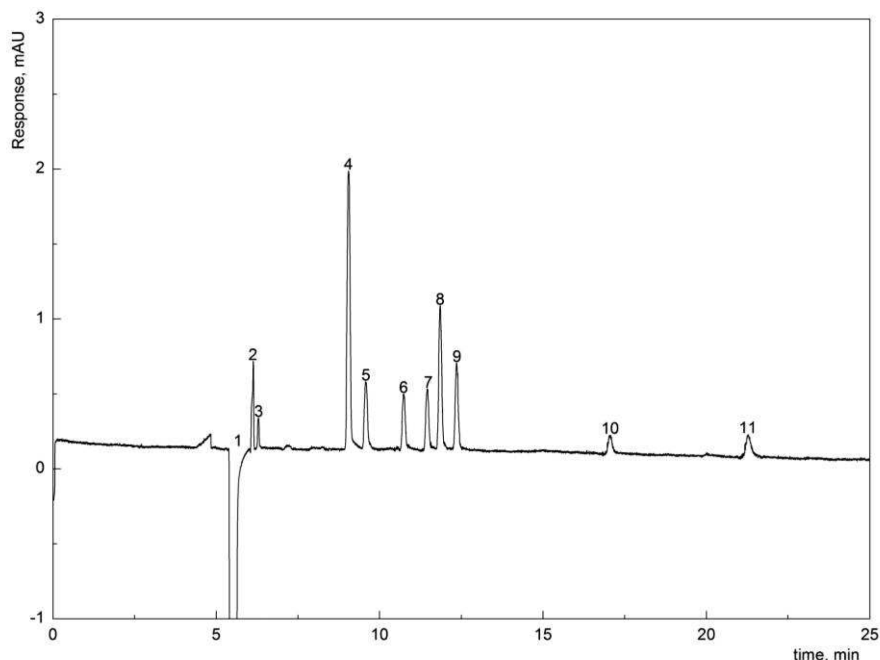
Zatímco při pH $\leq$ 8,8 komigruje isoxanthopterin s leukopterinem při pH $\geq$ 9,6 komigruje leukopterin s xanthopterinem a při pH $\geq$ 10,0 dochází k záměně migračního pořadí leukopterinu a xanthopterinu. Celkového oddělení těchto tří derivátů lze dosáhnout pouze při pH 9,0 a 10,2.



Obr. 3.4: Závislosti efektivních elektroforetických pohyblivostí $\mu_{eff,i}$ jednotlivých derivátů pterinu na pH v separačním elektrolytu skládajícího se ze 100 mmol·L^{-1} kyseliny borité, 2 mmol·L^{-1} Na_2EDTA a 100 mmol·L^{-1} TRIS, (1) L-sepiapterin, (2) 7,8-dihydroxanthopterin, (3) 6-biopterin, (4) D-neopterin, (5) pterin, (6) isoxanthopterin, (7) xanthopterin, (8) leukopterin, (9) erythropterin a (10) pterin-6-karboxylová kyselina.

Optimalizované podmínky separace byly: základní elektrolyt o složení 2 mmol·L^{-1} Na_2EDTA, 100 mmol·L^{-1} TRIS a 100 mmol·L^{-1} kyseliny borité, pH 9,0; vložené napětí 20 kV a UV detekce při 250 nm.

Za těchto podmínek došlo k separaci všech 10 studovaných derivátů pterinu až na základní linii do 22 min (obr. 3.5).



Obr. 3.5 Elektroferogram separace 10 derivátů pterinu v základním elektrolytu skládajícího se ze 2 mmol·L^{-1} Na_2EDTA, 100 mmol·L^{-1} TRIS a 100 mmol·L^{-1} kyseliny borité, pH 9,0. Elektrokinetické dávkování 10 s při 20 kV, separace při 20 kV, teplota 30 °C, procházející proud 19.4 μA a UV detekce při 250 nm. Koncentrace derivátů pterinu ve směsi 2.5 μg·mL^{-1}. Pík (1) dimethylsulfoxid (2) L-sepiapterin, (3) 7,8-dihydroxanthopterin, (4) 6-biopterin, (5) D-neopterin, (6) pterin, (7) isoxanthopterin, (8) leukopterin, (9) xanthopterin, (10) erythropterin a (11) pterin-6-karboxylová kyselina.

Vysokoúčinná kapalinová chromatografie je založena na jiném separačním mechanismu než kapilární elektroforéza a je proto vhodnou srovnávací metodou pro potvrzení identifikace derivátů pterinu přítomných v kutikulách ploštic.

Vzhledem k vysoké polaritě derivátů pterinu byla zvolena HPLC v hydrofilním interakčním módu s tandemovou hmotnostní spektrometrií. Pro analýzu byly vybrány dvě kolony: Atlantis HILIC Silica (150 × 4,6 mm, 3 μm, Waters, Milford, USA) a ZIC-HILIC (150 × 4,6 mm, 3,5 μm, Merck, Darmstadt, Německo).

V rámci optimalizační procedury byl studován vliv obsahu organického modifikátoru, typu pufru, jeho koncentrace a hodnota pH v mobilní fázi na retenční a separační chování. Nejvyšší vliv na retenční chování derivátů pterinu měl poměr organické a vodné složky v mobilní fázi.

Na základě výsledků byla zvolena kolona ZIC-HILIC s následujícími separačními podmínkami: mobilní fáze 85/15 (*v/v*) ACN/5 mM octan amonný, pH = 6,80, průtoková rychlost 0,5 ml·min^{-1}, 30 ºC.

PUBLIKACE I

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Capillary electrophoresis of pterin derivatives responsible for the warning coloration of Heteroptera

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ABSTRACT

A new capillary electrophoretic (CE) method has been developed for analysis of 10 selected derivatives of pterin that can occur in the integument (cuticle) of true bugs (Insecta: Hemiptera: Heteroptera), specifically L-sepiapterin, 7,8-dihydroxanthopterin, 6-biopterin, D-neopterin, pterin, isoxanthopterin, leucopterin, xanthopterin, erythropterin and pterin-6-carboxylic acid. Pterin derivatives are responsible for the characteristic warning coloration of some Heteroptera and other insects, signaling noxiousness or unpalatability and are used to discourage potential predators from attacking. Regression analysis defining the parameters significantly affecting CE separation was used to optimize the system (the background electrolyte (BGE) composition, pH value and applied voltage). The optimized separation conditions were as follows: BGE with composition 2 mmol L^{-1} the disodium salt of ethylendiamintetraacetic acid, 100 mmol L^{-1} tris(hydroxymethyl)aminomethane and 100 mmol L^{-1} boric acid, pH 9.0, applied voltage 20 kV and UV detection at 250 nm. Under these conditions, all the 10 studied derivatives of pterin were baseline separated within 22 min. The optimized method was validated from the viewpoint of linearity ($R^2 \geq 0.9980$), accuracy (relative error $\leq$7.90%), precision (for repeatability RSD $\leq$ 6.65%), detection limit (LOD in the range 0.04–0.99 μg mL^{-1}) and limit of quantitation (LOQ in the range 0.13–3.30 μg mL^{-1}). The developed method was used for identification and determination of the contents of pterin derivatives in adults of four species of Heteroptera (*Eurydema ornata* cream color morph, *Scantius aegyptius, Pyrrhocoris apterus* and *Corizus hyoscyami*) and their distribution in the individual species was determined

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1. Introduction

Pterin and its derivatives belong in a group of heterocyclic compounds that are frequently found in biological systems. They have important biological functions as inhibitors [1], sensitizers [2,3], enzymes [4], coenzymes [5] and sensors [6] and also act as pigments [7–11] or toxins [12]. Relatively high concentrations of pterin derivatives are found in the form of pigments in insects, reptiles, amphibians, crabs, marine plankton and human body fluids [13].

Pterin derivatives give insects their typical cuticle coloration, from white (leucopterin) through yellow (xanthopterin and isoxanthopterin) and red (erythropterin) to fluorescent blue (biopterin) [14]. Red, yellow and white colors of many insects are combined with dark (usually black) pattern forming a conspicuous aposematic (warning) coloration. Aposematic signals are used by prey animals to advertise their noxiousness or unprofitability to their potential predators [15]. They may be visual, acoustic, or chemical, and are often combined in a multimodal signaling systems [16]. True bugs (Heteroptera) are a model taxon for studies of antipredatory defenses, aposematism and mimicry. Many heteropteran species are aposematic, and their coloration ranges from white across yellow and orange to red, combined with black melanic spots and stripes. The coloration of cuticle of a heteropteran species changes during the development of the individual, sometimes also according to the season and sometimes also varies geographically [17,18]. The shade of the coloration is probably the result of the presence of certain pterin derivatives and their mutual ratios.

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The analysis of pterin derivatives in the form of insect cuticle pigments is an extremely complicated problem because of the poor solubility of the pigments in water and common organic solvents, high sensitivity to light and low concentrations in biological samples [19].

Pterin derivatives are most often determined in urine, plasma, serum or cerebrospinal fluid [20]. They are mostly analyzed by chromatographic methods, particularly paper chromatography [10], reverse [9,21,22], ion-pair [23], hydrophilic interaction [24], ion-exchange [25] and chiral [26] high performance liquid chromatography. Spectrophotometric methods have also been used to analyze pterin derivatives, e.g. surface-enhanced Raman scattering method [27] or synchronous fluorescence spectroscopy [28]. Biopterin and D-neopterin can be determined by radioimmune analysis or the ELISA method and commercial sets are currently available [20]. The capillary electrophoretic method was developed for analysis of pterin derivatives in urine with detection based on laser induced fluorescence [29-31]. Separation of six derivatives was performed using a three-component background electrolyte (BGE) consisting of an aqueous solution of tris(hydroxymethyl)aminomethane (TRIS), the disodium salt of ethylendiamintetraacetic acid (Na_2EDTA) and boric acid and the separation concentrated on identifying selected pterins in urine as biomarkers for certain tumor diseases [29].

Pterin derivatives have been analyzed in insect cuticle mainly by paper chromatography and/or thin-layer chromatography [8,10,14,32–37] and HPLC in the reverse [9] or hydrophilic interaction mode [38]. Only one paper has been focused on mechanisms of color production in a highly variable shield-back stinkbug where capillary electrophoresis was used [39].

Pterin derivatives exist in several tautomeric equilibrium forms. However, there are only two important structures in biological systems [40,41], lactim and preferentially lactam structures, thanks to which pterin derivatives act as weak acids in aqueous solutions. Consequently, capillary electrophoresis can be a suitable alternative to relatively complicated, instrumentally demanding and expensive separation and detection systems used for pterin analysis in HPLC.

This work was performed to develop an effective CE method for the separation and quantitation of 10 pterin derivatives (L-sepiapterin, 7,8-dihydroxanthopterin, 6-biopterin, D-neopterin, pterin, isoxanthopterin, leucopterin, xanthopterin, erythropterin and pterin-6-carboxylic acid, structures Fig. 1) that would enable simple screening of the relative contents of the individual pterin derivatives in the cuticle of Hemiptera: Heteroptera and would be applicable in biological practice. Cuticles from selected species of Heteroptera were used as real samples: *Eurydema ornata* cream color morph (Pentatomidae), *Scantius aegyptius* (Pyrrhocoridae), *Pyrrhocoris apterus* (Pyrrhocoridae) and *Corizus hyoscyami* (Rhopalidae). Comparative analysis of the content of individual pterins in the cuticle of various species of Heteroptera is important for studying the evolution of aposematism and mimicry in this group and for understanding the biochemical basis of color polymorphism found in some species.

2. Material and methods

2.1. Pterin derivatives

6-Biopterin (purity ≥ 98%), D-neopterin (purity ≥ 97.5%), leucopterin (purity ~ 95%), pterin (purity ≥ 97.8%), leucopterin (purity ~ 95%) and pterin-6-carboxylic acid (purity ≥ 98%) were supplied by Sigma Aldrich (Munich, Germany). Isoxanthopterin (purity ≥ 97.5%) and xanthopterin (purity ≥ 97.5%) were obtained from Fluka (Buchs, Switzerland). L-Sepiapterin (purity ≥ 99%) was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA). 7,8-Dihydroxanthopterin and erythropterin were kindly provided by Scott Fabricant and Ron Rutowski, respectively.

2.2. Chemicals and Heteroptera samples

The following chemicals were employed to prepare the BGE: $Na_2EDTA \cdot 2\ H_2O$ (purity ≥ 99%, Lachema, Brno, Czech Republic), boric acid (purity ≥ 99.5%, Penta, Prague, Czech Republic) and TRIS (purity > 98%, Lach-Ner, Neratovice, Czech Republic). The required pH value of the BGE was adjusted using sodium hydroxide (purity > 98%, Lach-Ner, Neratovice, Czech Republic). Dimethyl sulfoxide (purity > 99%, Lachema, Brno, Czech Republic) was used to prepare the stock solutions and for the extractions. The deionized water used was purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Real samples consisted of the cuticle from dorsal body parts (pronotum, mesoscutellum and forewings) of dry-mounted specimens of four species of Heteroptera. We used 6.2 mg of cuticle from cream color morph of *E. ornata* (Pentatomidae), 3.5 mg from *S. aegyptius* (Pyrrhocoridae), 4.7 mg from *P. apterus* (Pyrrhocoridae) and 7.2 mg from *C. hyoscyami* (Rhopalidae). The localities and dates of individuals studied are, as follows (all collected by P. Štys): *E. o*: Romania, Agigea, 7/13/1957; *S. a.*: Bulgaria, Sozopol, 6/21/1964; *P. a.*: Czech Republic, Prague, 5/15/2013; *C. h.*: Czech Republic, Pavlovské Hills, Děvín, 5/14/1964.

2.3. Preparation of the BGE

The BGEs consisted of a mixture of TRIS (50–300 mmol L^{-1}), Na_2EDTA (0–3 mmol L^{-1}) and boric acid (50–250 mmol L^{-1}) with various concentrations of the individual components. The required pH of the separation electrolyte was adjusted by adding 1.5 mol L^{-1} NaOH. Then the buffer was filtered through a nylon filter with pore size 0.20 μm (Sigma Aldrich, Munich, Germany) and placed in ultrasound for 15 min prior to use.

2.4. Preparation of stock solutions of pterin derivatives and extracts of Heteroptera

Stock solutions of each of the 10 pterin derivatives were prepared by dissolving 1 mg of the relevant standard in 10 mL dimethyl sulfoxide with a final concentration of 0.1 mg mL^{-1}. The solutions were stored in dark vials at a temperature of 5 °C. The required concentrations were prepared by diluting the stock solutions with the BGE.

The cuticle from dorsal body parts (pronotum, mesoscutellum and forewings) of Heteroptera specimens were extracted with dimethyl sulfoxide. Relevant amount of cuticle was crushed and 0.5 mL of dimethyl sulfoxide was added. The samples were then placed in ultrasound for 2 h with simultaneous heating to 50 °C and then were placed in a shaker at a rotation speed of 1250 RPM for 1 h. The vials were then wrapped in aluminum foil and stored for 11 days at a temperature of 5 °C to be sure that the equilibrium concentrations of pterin derivatives present in the cuticle were reached. The extracts were then centrifuged for 10 min at 10,000 RPM. The supernatant was then poured off and stored at a temperature of 5 °C. Prior to the analysis, 10 μL of the supernatant was diluted with 30 μL of the appropriate BGE.

2.5. Instrumentation, conditioning and separation conditions

All the electrophoretic measurements were performed on an $HP^{3D}CE$ instrument system with a built-in diode-array detector (Agilent Technologies, Waldbronn, Germany). The data were collected and evaluated using the programs Agilent ChemStation

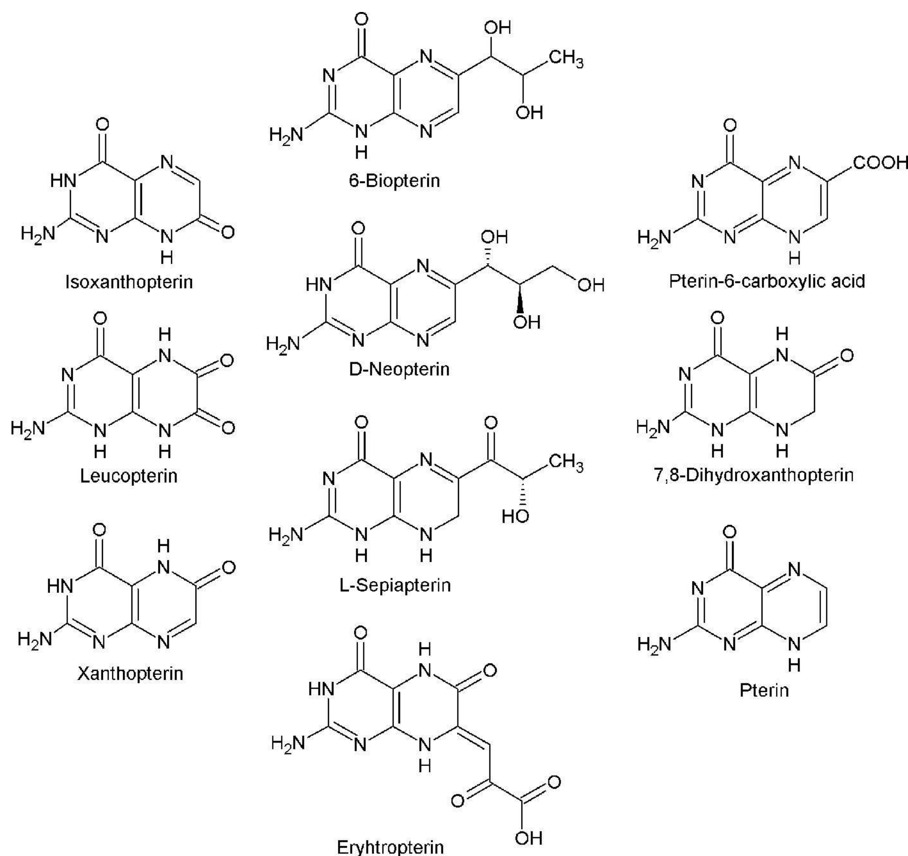

Fig. 1. Structures of the studied pterins.

(Agilent Technologies, Waldbronn, Germany), Origin 8 (OriginLab Corporation, Northampton, MA, USA), Microsoft Excel 2003 (Microsoft Corporation, Redmond, DC, USA) and Minitab 16 (Minitab Inc., State College, PA, USA.).

Separation was performed in an uncoated fused-silica capillary (CACO, Bratislava, Slovakia), total length 70 cm, 55 cm to the detector, inner diameter 50 μm which was thermostated at 30 °C during all the separations. Separation voltage was set at 20–30 kV with cathode at the capillary outlet.

At the beginning of the daily measurement, the capillary was conditioned in the following manner: rinsing with deionized water (2 min), 1 mol L^{-1} NaOH (4 min), again deionized water (2 min) and the electrolyte (6 min) and a voltage of 20 kV was then applied to the electrode (10 min). After each analysis the capillary was washed with deionized water (2 min), 0.1 mol L^{-1} NaOH (1 min) and electrolyte (2 min). After the last measurement of the day, the capillary was rinsed with distilled water (2 min), 1 mol L^{-1} NaOH (3 min), again deionized water (6 min) and air (6 min). A pressure of 1000 mbar was used for all the rinsing.

The standards of the pterin derivatives and real samples were injected electrokinetically for a period of 10 s at a voltage of 20 kV. Dimethyl sulfoxide, in which the analyte and real samples were dissolved, was used as a label for electroosmotic flow.

3. Results and discussion

3.1. Optimization of separation of pterin derivatives

Pterin derivatives act as weak acids and thus carry negative charge in alkaline medium; this can be utilized for their analysis by capillary electrophoresis. The BGE whose composition was based on the previously reported work [29] was selected for the separation of the 10 studied derivatives.

The three-component electrolyte was based on TRIS acting as a buffering component, with the addition of boric acid and Na_2EDTA as a complexing agent. The following parameters were monitored to optimize the composition of the BGE or separation system: concentrations of the individual BGE components in the range TRIS 50–300 mmol L^{-1}, boric acid 50–250 mmol L^{-1} and Na_2EDTA 0–3 mmol L^{-1}, pH of the BGE in the range 8.6–10.4 and applied voltage value between 20 and 30 kV.

Because of the multicomponent character of the separation system, the method of regression analysis (Minitab 16 statistical program) was used for optimization of the procedure; on the basis of the examined responses, application of this method enables determination of statistically significant parameters (at the confidence level of 95%). The investigated responses were: the effective electrophoretic mobility, separation efficiency expressed as the number of theoretical plates and chromatographic response function (CRF). CRF is based on the simultaneous optimization of the resolution and analysis time and was calculated according to the Derringer's function ($S^{p,p+1}$ = 0.95 for $R^{p,p+1}$ = 3.00, and $S^{p,p+1}$ = 0.10 for $R^{p,p+1}$ = 1.50, and g = 0.9 for t = 10 min, and g = 0.1 for t = 20 min); for exact calculation procedure see cit [42]. The experimental results were treated by the regression analysis method and, on this basis, it was found that the above-listed responses are statistically affected by the voltage, concentrations of TRIS and boric acid and especially the pH value of the BGE. The concentration of Na_2EDTA was found not to be a statistically significant parameter.

The dependences of the effective electrophoretic mobility $\mu_{\text{eff},i}$ of the individual pterin derivatives on the pH in the range 8.6–10.4 are given in Fig. 2.

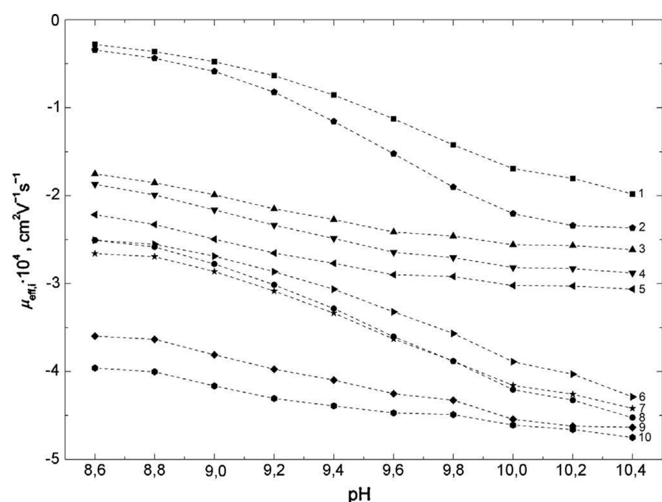

Fig. 2. Dependence of the effective electrophoretic mobilities $\mu_{eff,i}$ of the individual pterin derivatives on the pH in the separation electrolyte consisting of 100 mmol L^{-1} boric acid, 2 mmol L^{-1} Na_2EDTA and 100 mmol L^{-1} TRIS, (1) L-sepiapterin, (2) 7,8-dihydroxanthopterin, (3) 6-biopterin, (4) D-neopterin, (5) pterin, (6) isoxanthopterin, (7) xanthopterin, (8) leucopterin, (9) erythropterin and (10) pterin-6-carboxylic acid.

It can be seen from Fig. 2 that, as the pH of the separation electrolyte increases, the negative values of the effective electrophoretic mobility of all the studied derivatives increase, in agreement with the assumption of their greater dissociation. It is apparent from Fig. 2 that the separation of the pterin derivatives is very sensitive to the pH value of the BGE. All the studied pterin derivatives can be separated only in a very narrow pH range and especially isoxanthopterin, leucopterin and xanthopterin, which are structurally very similar and are generally very common in insects, migrate with very similar times, which is depicted in detail in Fig. 3.

It can be seen in Fig. 3 that, while isoxanthopterin and leucopterin comigrate at pH ≤ 8.8, leucopterin and xanthopterin comigrate at pH ≥ 9.6 and the migration order of leucopterin and xanthopterin is even reversed at pH ≥ 10.0. These three derivatives can be baseline separated at pH 9.0 and 10.2.

A change in the concentrations of boric acid and TRIS has a much smaller effect on the migration behavior of all the studied analytes than the pH value of the BGE. An increase in the concentrations of the individual components results in only a slight increase in the effective electrophoretic mobility of all the derivatives for TRIS and vice versa for boric acid.

The separation voltage was another studied parameter. It was found that the analysis can be performed in the entire tested voltage range; however, because the presence of other substances can be anticipated in Heteroptera extracts, it is preferable to perform the separation at a voltage of 20 kV, which provides greater resolution; this is important especially for isoxanthopterin, leucopterin and xanthopterin, however at the expense of longer analysis times. The wavelength was tested in the range 200–400 nm and a wavelength of 250 nm was selected as a compromise on the basis of comparison of the spectra of all the derivatives.

The optimized conditions were as follows: the BGE with composition 2 mmol L^{-1} Na_2EDTA, 100 mmol L^{-1} TRIS, 100 mmol L^{-1} boric acid; the pH of the BGE was 9.0 (or 10.2 for better separating leucopterin from other unknown compounds present in real samples), voltage 20 kV and wavelength of UV detection 250 nm.

Fig. 4 depicts the electropherogram of a standard mixture of all 10 studied pterin derivatives obtained under the optimized conditions, from which it can be seen that good separation of all 10 monitored derivatives was achieved at pH 9.0 within 22 min. It

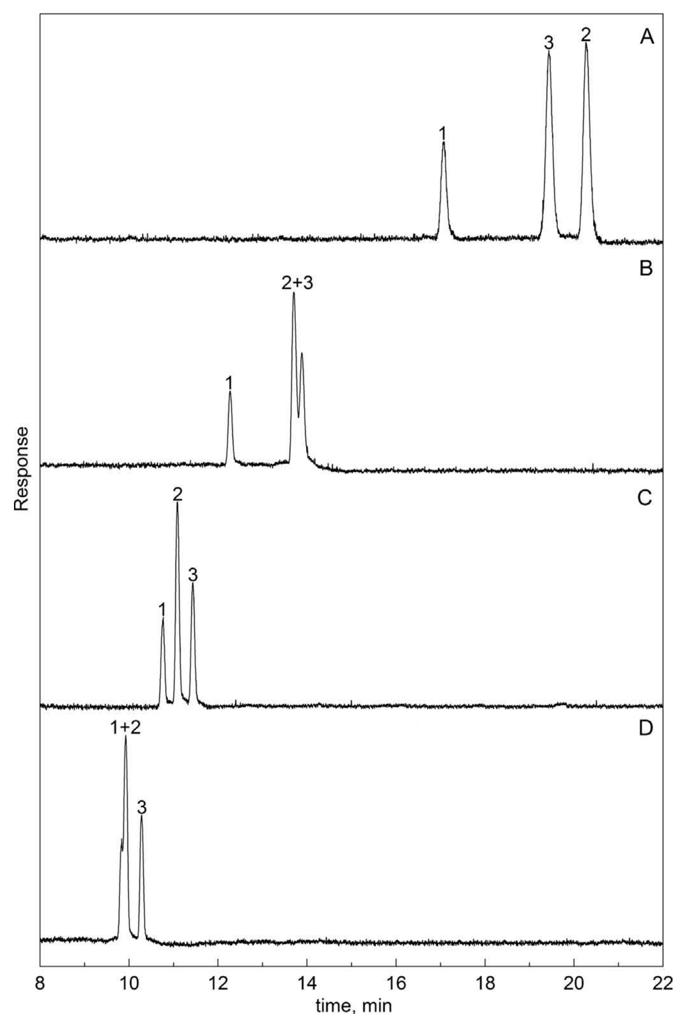

Fig. 3. Separation of standards of pterin derivatives at various pH values. (A) pH 10.2, (B) pH 9.6, (C) pH 9.0 and (D) pH 8.8. Peak (1) isoxanthopterin, (2) leucopterin and (3) xanthopterin.

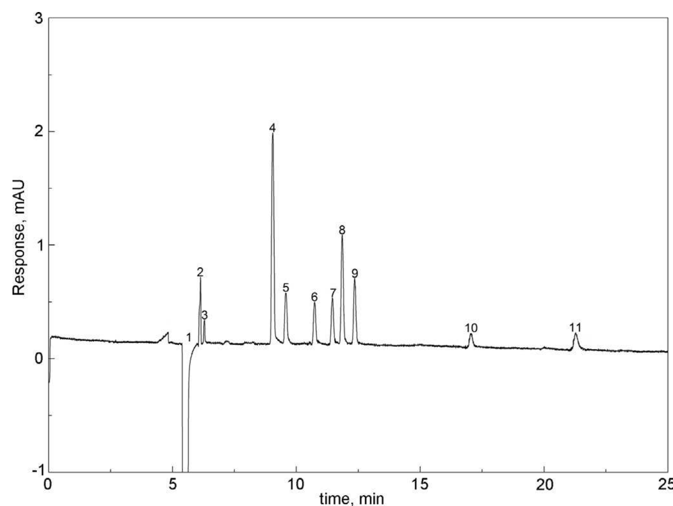

Fig. 4. Electropherogram of the separation of 10 pterin derivatives in the BGE consisting of 2 mmol L^{-1} Na_2EDTA, 100 mmol L^{-1} TRIS and 100 mmol L^{-1} boric acid, pH 9.0. Electrokinetic injection for 10 s at 20 kV, separation at 20 kV, temperature 30 °C, current 19.4 μA and UV detection at 250 nm. Pterin derivative concentration in the mixture 2.5 μg mL^{-1}. The peaks of (1) dimethyl sulfoxide (2) L-sepiapterin, (3) 7,8-dihydroxanthopterin, (4) 6-biopterin, (5) D-neopterin, (6) pterin, (7) isoxanthopterin, (8) leucopterin, (9) xanthopterin, (10) erythropterin and (11) pterin-6-carboxylic acid.

Table 1
Basic measured parameters of the separation of pterin derivatives under optimized conditions ($n = 3$). Effective electroforetic mobility $\mu_{eff,i}$, standard deviation SD, relative standard deviation RSD, retention factor k_i, separation factor $\alpha_{i,i+1}$, resolution of neighboring peaks $R_{i,i+1}$ and separation efficiency expressed as the number of theoretical plates N.

	$\mu_{eff,i} \times 10^4$ (average ± SD) $cm^2 V^{-1} s^{-1}$	RSD (%)	k_i	$\alpha_{i,i+1}$	$R_{i,i+1}$	$N \times 10^{-3}$
L-Sepiapterin	−0.48 ± 0.01	0.38	0.09	1.33	1.99	167
7,8-Dihydroxanthopterin	−0.60 ± 0.03	0.95	0.12	4.58	18.20	91
6-Biopterin	−2.00 ± 0.02	0.53	0.55	1.13	2.27	36
D-Neopterin	−2.18 ± 0.01	0.39	0.62	1.27	5.48	35
Pterin	−2.51 ± 0.01	0.32	0.79	1.15	4.09	56
Isoxanthopterin	−2.71 ± 0.01	0.38	0.91	1.07	2.02	62
Leucopterin	−2.79 ± 0.01	0.34	0.97	1.34	2.14	72
Xanthopterin	−2.88 ± 0.01	0.38	1.03	1.62	31.93	72
Erythropterin	−3.83 ± 0.01	0.33	2.06	1.33	24.01	116
Pterin-6-carboxylic acid	−4.19 ± 0.01	0.34	2.79			169

Table 2
Equation of the linear regression of the calibration curves of pterin derivatives (dependence of the peak area on the concentration of the pterin derivative). Standard deviations in parentheses, coefficient of determination R^2, limit of detection LOD and limit of quantitation LOQ.

Pterin derivatives	Slope (mV s mL μg^{-1})	Intercept (mV s)	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
L-Sepiapterin	0.369 (0.009)	−0.090 (0.056)	0.9981	0.10	0.33
7,8-Dihydroxanthopterin	0.070 (0.002)	−0.180 (0.038)	0.9991	0.99	3.30
6-Biopterin	4.353 (0.087)	1.724 (0.424)	0.9980	0.04	0.13
D-neopterin	1.406 (0.019)	0.612 (0.128)	0.9996	0.14	0.47
Pterin	1.788 (0.017)	2.634 (0.586)	0.9996	0.10	0.32
Isoxanthopterin	0.826 (0.009)	0.418 (0.058)	0.9996	0.15	0.50
Leucopterin*	3.498 (0.093)	0.389 (0.538)	0.9986	0.11	0.36
Xanthopterin	0.570 (0.003)	0.269 (0.021)	0.9999	0.29	0.96
Erythropterin	0.526 (0.009)	0.027 (0.059)	0.9997	0.28	0.92
Pterin-6-carboxylic acid	1.311 (0.027)	0.187 (0.156)	0.9987	0.27	0.90

* Measured in BGE with pH 10.2.

Table 3
Intra- and inter-day of accuracy and precision of the capillary electrophoretic method for determining pterin derivatives ($n = 3$).

Pterin derivatives	Concentration (μg mL^{-1})	Intra-day			Inter-day		
		(average ± SD) (μg mL^{-1})	RSD (%)	RE (%)	(average ± SD) (μg mL^{-1})	RSD (%)	RE (%)
L-Sepiapterin	10	10.15 ± 0.08	0.84	1.50	9.86 ± 0.13	1.30	−1.40
	5	4.83 ± 0.22	4.52	−3.40	5.02 ± 0.12	2.42	0.40
	1	0.98 ± 0.03	2.79	−2.00	0.99 ± 0.01	1.29	−1.00
7,8-Dihydroxanthopterin	50	49.94 ± 2.21	4.42	−0.12	50.70 ± 1.36	2.69	1.40
	10	10.79 ± 0.72	6.65	7.90	10.45 ± 0.59	5.65	4.50
	5	5.04 ± 0.06	1.14	0.80	5.01 ± 0.12	2.34	0.20
6-Biopterin	10	10.02 ± 0.60	6.02	0.20	9.79 ± 0.29	2.93	2.10
	5	5.19 ± 0.05	0.92	3.80	5.22 ± 0.02	0.36	4.40
	1	1.03 ± 0.02	1.82	3.00	1.01 ± 0.04	3.73	1.00
D-Neopterin	10	9.94 ± 0.10	1.01	−0.60	10.05 ± 0.16	1.60	0.50
	5	5.06 ± 0.03	0.67	1.20	5.11 ± 0.06	1.17	2.20
	1	0.94 ± 0.02	1.63	−6.00	0.95 ± 0.03	3.53	−5.00
Pterin	50	50.92 ± 0.70	1.37	1.84	49.98 ± 1.37	2.74	−0.04
	10	10.27 ± 0.46	4.46	2.70	9.71 ± 0.46	4.70	−2.90
	5	5.27 ± 0.32	6.03	5.40	5.12 ± 0.21	4.04	2.40
Isoxanthopterin	10	10.07 ± 0.28	2.77	0.70	9.94 ± 0.13	1.27	−0.60
	5	5.07 ± 0.09	1.84	1.40	4.98 ± 0.04	0.76	−0.40
	1	0.94 ± 0.01	0.48	−6.00	1.01 ± 0.01	0.98	1.00
Leucopterin*	10	9.92 ± 0.03	0.30	−0.80	9.62 ± 0.01	0.15	−3.80
	5	5.12 ± 0.02	0.04	2.40	4.91 ± 0.01	0.29	−1.80
	1	0.94 ± 0.01	0.11	−6.00	1.01 ± 0.01	0.57	1.00
Xanthopterin	10	9.98 ± 0.33	3.32	−0.20	10.02 ± 0.37	3.69	0.20
	5	4.95 ± 0.05	1.10	−1.00	5.01 ± 0.15	2.91	0.20
	1	0.99 ± 0.03	2.89	−1.00	1.01 ± 0.05	5.32	1.00
Erythropterin	10	10.04 ± 0.13	1.32	0.40	9.99 ± 0.14	1.39	−0.10
	5	4.91 ± 0.08	1.61	−1.80	4.94 ± 0.05	0.99	−1.20
	1	1.05 ± 0.04	3.44	5.00	1.06 ± 0.03	2.51	6.00
Pterin-6-carboxylic acid	10	10.06 ± 0.10	0.95	0.60	9.98 ± 0.13	1.30	−0.20
	5	4.83 ± 0.27	5.54	−3.40	4.95 ± 0.17	3.48	−1.00
	1	1.03 ± 0.05	4.78	3.00	1.02 ± 0.06	5.51	2.00

Standard deviation SD, relative standard deviation RSD and relative error RE.

* Measured in BGE with pH 10.2.

can be seen from Table 1 summarizing the basic measured parameters that effective baseline separation of all the derivatives is achieved under the optimized conditions and that the repeatability of the effective electrophoretic mobilities is very good (the relative standard deviation is of the order of tenths of one percent).

3.2. Validation of the capillary electrophoretic method

The newly developed method was validated under the optimized separation conditions from the viewpoints of linearity, limits of detection (LOD) and quantitation (LOQ), precision and accuracy.

Linearity was determined by gradual dilution of standard solutions of the individual derivatives with the BGE in the concentration range 5–50 μg mL^{-1} for pterin and 7,8-dihydroxanthopterin and 1–10 μg mL^{-1} for the other derivatives. The results of linear regression of the dependence of the peak areas on the concentrations are given in Table 2 together with the coefficients of determination (R^2 in the interval 0.9980–0.9999). The obtained results indicate that the linearity is very good in the tested range.

The limits of detection (LOD) and quantitation (LOQ) were obtained by linear regression of the dependence of the peak height on the concentration of the pterin derivative as 3.3 times (LOD) and 10 times (LOQ) the noise level (see Table 2). It can be seen from Table 2 that, with the exception of 7,8-dihydroxanthopterin, the LOQ values vary in the range of tenths of μg mL^{-1}.

The intra- and inter-day precision and accuracy of the proposed method were determined for the individual derivatives by measuring three concentration levels in three repeated

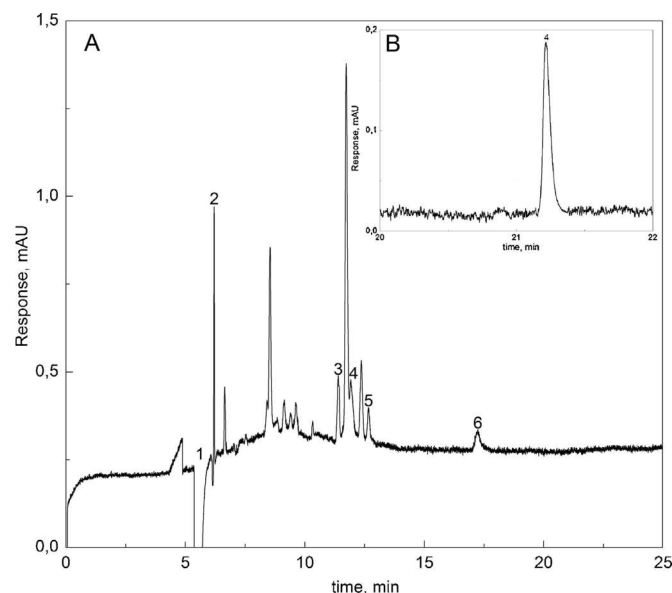

Fig. 5. Electropherogram of the separation of an extract of the cuticle of *Scantius aegyptius* (Pyrrhocoridae) in the BGE consisting of 2 mmol L^{-1} Na_2EDTA, 100 mmol L^{-1} TRIS and 100 mmol L^{-1} boric acid. Electrokinetic injection for 10 s at 20 kV, separation at 20 kV, temperature 30 °C and UV detection at 250 nm. The BGE (A) pH 9.0, current 19.0 μA and (B) pH 10.2, current 45.4 μA. Peak (1) dimethyl sulfoxide (2) L-sepiapterin, (3) isoxanthopterin, (4) leucopterin, (5) xanthopterin and (6) erythropterin.

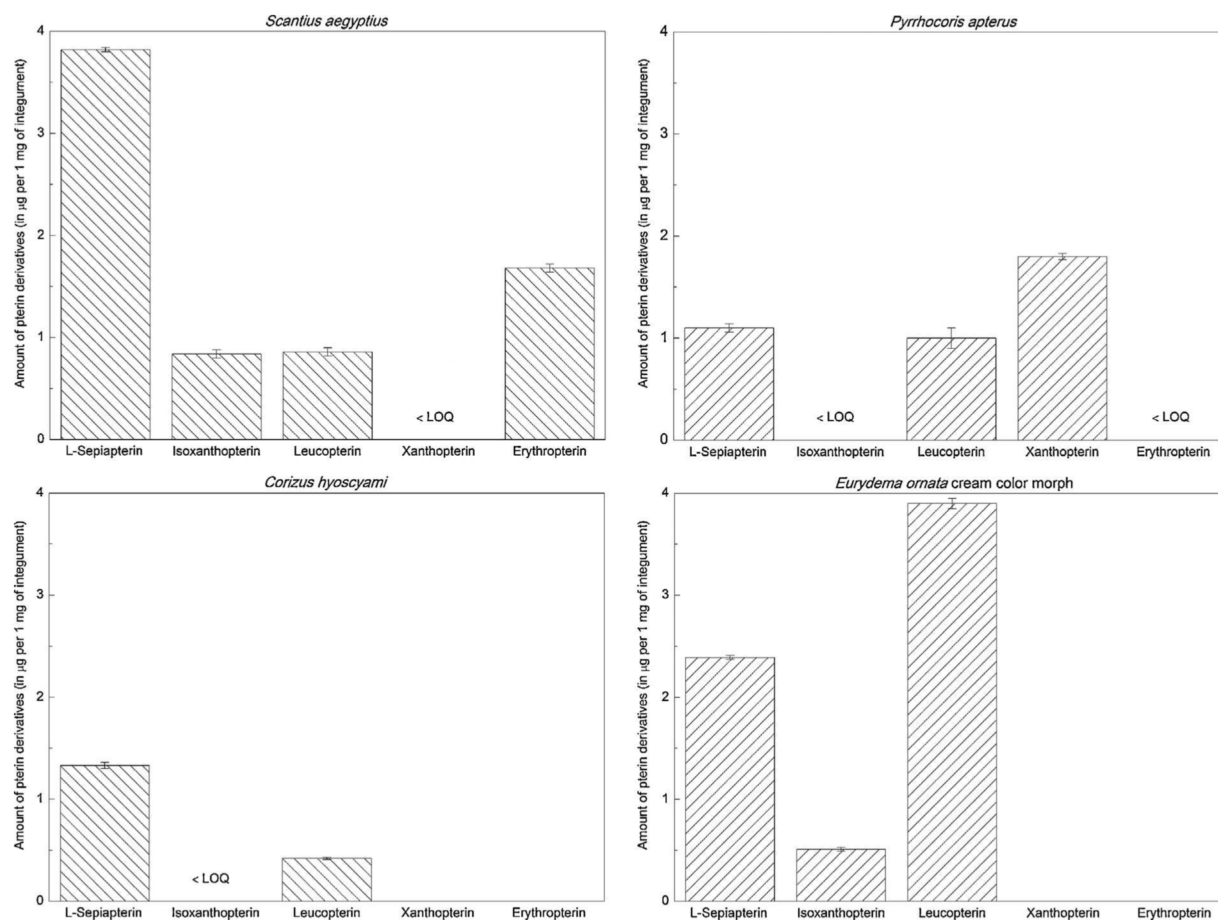

Fig. 6. Comparison of the amounts of pterin derivatives found in the cuticle of the individual species of Heteroptera. Designation < LOQ means only the presence of the relevant derivative.

measurements; the results are summarized in Table 3. The precision was expressed as the relative standard deviation (RSD%) and the accuracy as the relative error (RE%). The precision (RSD) varied in the range 0.04–6.65% and the accuracy (RE) ≤7.90%. This indicates that the newly developed method has acceptable accuracy and precision for quantitation of pterin derivatives.

3.3. Application of capillary electrophoresis for analysis of extracts of Heteroptera cuticle

An optimized capillary electrophoretic method was employed for identification and quantitation of pterin derivatives in extracts from four species of Heteroptera, *E. ornata* (cream colored morph), *S. aegyptius*, *P. apterus* and *C. hyoscyami*. The extracts were prepared by the procedure described in Section 2.4.

Identification of the individual pterin derivatives in the extracts was performed on the basis of comparison of the migration times (effective electrophoretic mobilities) of the peaks of the pterin derivatives in standard mixtures and the peaks for the extracts of the individual species of Heteroptera. The confirmation was done by the method of standard additions. Fig. 5 gives an example of a recording of the entire separation of extract of the cuticle of *S. aegyptius* at pH 9.0. It can be seen that two unknown substances migrate among the similarly migrating three pterin derivatives (isoxanthopterin, leucopterin, xanthopterin). Subsequent quantitation of leucopterin in an electrolyte with pH 9.0 cannot be performed because leucopterin cannot be baseline separated from the unknown substances. The BGE with pH 10.2 must be used for quantitation of this derivative. Leucopterin is more dissociated in this electrolyte and its migration time is prolonged by 10 min, as can be seen in the insert in the upper right-hand corner of Fig. 5. Consequently, all the quantitations of leucopterin in Heteroptera extracts were performed in the BGE with pH 10.2. The other pterin derivatives were quantified in the BGE with pH 9.0.

The individual extracts were measured in triplicate and the concentrations of the identified pterins were calculated from the relevant regression equations, which are given in Table 2. The results of the quantitation for the individual species of Heteroptera are given in Fig. 6. Yellow L-sepiapterin and white leucopterin were determined in the cuticle of all the Heteroptera, but in different mutual ratios. Isoxanthopterin was determined in extracts of *S. aegyptius* and in *E. ornata* (cream color form) and only identified in *P. apterus* and *C. hyoscyami*. Two more colored derivatives – xanthopterin and erythropterin – were either determined or only identified in *S. aegyptius* and *P. apterus*. The highest leucopterin concentration was found in the cuticle of *E. ornata* (cream colored morph). 7,8-Dihydroxanthopterin, 6-biopterin, D-neopterin, pterin and pterin-6-carboxylic acid were not identified in any of the studied samples. Either these derivatives were not present at all or were below their LODs.

4. Conclusions

This work demonstrates for the first time that pterin derivatives present in Heteroptera cuticle can be identified and quantified by the capillary electrophoresis. Multiparameter optimization was performed using the Minitab 16 statistical program, whose results indicated that the pH value has the greatest effect on the separation of analytes in the three-component BGE (TRIS, boric acid, Na_2EDTA) and, of other parameters, the TRIS and boric acid concentrations and the applied voltage. All 10 studied derivatives can be separated under the optimized conditions within 22 min and the proposed system is suitable for quantitation of all the analytes.

Application of the fast and instrumentally simple method for analysis of the organic extracts from the cuticle of Heteroptera demonstrated that the newly developed method provides information on the relative distribution of the individual pterin derivatives in various Heteropteran species. The method can become an important analytical tool for comparative biological studies considering the coloration of insects in precise chemical terms and enable to assess its development and evolution. It is an important topic in the studies of warning coloration and mimicry.

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PUBLIKACE II

Název	*Mechanisms of color production in a highly variable shield-back stinkbug, Tectocoris diopthalmus (Heteroptera: Scutelleridae), and why it matters*
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Mechanisms of Color Production in a Highly Variable Shield-Back Stinkbug, *Tectocoris diopthalmus* (Heteroptera: Scutelleridae), and Why It Matters

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Abstract

Theory suggests that aposematism, specifically the learned avoidance of unprofitable prey via memorable color patterns, should result in selection for pattern uniformity. However, many examples to the contrary are seen in nature. Conversely, honest sexual signals are likely to exhibit greater variation because they reflect underlying variation in mate quality. Here we aim to characterize and quantify the mechanistic causes of color in *Tectocoris diopthalmus* to shed light on the costs of color production, and thus the potential information content of its color signals. We use *Tectocoris diopthalmus* because it is a weakly-defended stinkbug, and presents elements that have classically been studied in the context of aposematism (red coloring), and sexual selection (sexual dichromatism and iridescent coloring). Pigment analysis reveals that variation in orange coloration is due to the amount of erythropterin pigment, stored in intracellular granules. This pigment is common in Heteroptera, and as an endogenously produced excretory byproduct is unlikely to reflect mate quality or variation in unprofitability of the bug. Electron microscopy reveals the iridescent patches are caused by an epicuticular multilayer reflector, and the hue and patch size are directly related to the layer widths and extent of coverage of this layering. Furthermore, we identified melanin as an essential component of the multilayer reflector system; therefore, the quality of the iridescent patches may be affected by aspects of rearing environment and immunocompetence. We posit that *T. diopthalmus* has co-opted the melanic patches of a 'typical' red and black aposematic signal, transforming it into a complex and variable iridescent signal that may enhance its capacity to display individual quality.

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Introduction

Aposematism is the phenomenon wherein defended prey advertise unprofitability through conspicuous signals. Often these take the form of bright 'warning colors', as it is theorized that conspicuous coloration aids predator recognition and memory [1]. Generally, the 'typical' aposematic patterns of insects are red or yellow coloration with black markings [2] [3].

Theory suggests that aposematic patterns should be under strong frequency-dependent selection by predators to be monomorphic [4] [5], and/or strong directional selection for conspicuousness [6] [7]. However, examples of species with variable patterns or other deviations from theoretical predictions are common [3]. Recent theoretical treatments have addressed possible causes of intermediate aposematism and intraspecies variation in pattern, via such mechanisms as predator community structure [8], or variation in prey defense levels [9]. Notably, Blount's et al. [10] model of intraspecific variation explicitly explores color production mechanisms as drivers of variation, through the vehicle of pigments acting as antioxidants to defend against autotoxicity. Recent empirical studies have also demonstrated possible trade-offs between warning signal quality and physiological traits such as toxin excretion costs [11] and pathogen resistance [12], It is hence possible than the mechanisms of aposematic signals themselves, and related physiological traits, are contributing to variation.

Another potential factor contributing to intraspecific variation in aposematic signals is the interaction between selection by predation and sexual selection [13]. Honest sexual signals are likely to exhibit variation because they reflect underlying variation in mate quality [14], and sexual selection may act in opposition to predator selection for uniformity [15]. Identifying the proximate causes of color production can reveal potential costs and constraints, and suggest aspects of the information content of signals. For example, structural coloration is produced by submicron-level organization creating constructive interference with light [16]. Quality and regularity of the ultrastructure can affect its visual qualities [17]. Structural colors can be sensitive to perturbations during development, so poor-quality patches may indicate developmental stress [18], or poor genetic regulation [19]. Pigments are light-absorbing molecules that do not necessarily require fine-scale organization, but can also be sensitive to

developmental stress [20], and may reveal different information to structural color [21]. Two well-studied examples are carotenoids, which produce red or yellow coloration and are involved in antioxidant defense [22], and melanin, which produces brown and black coloration and is an integral component of the insect encapsulation response to parasites [23]. Some pigments can only be sequestered from diet, while others may be costly to manufacture *de novo*, so different types of pigment can relate to different aspects of mate quality [24]. Variation in color patterns may therefore be informative to mate choice and may be under strong sexual selection and/or physiological constraints, counter-acting predator selection for uniformity of aposematic signals.

In addition to selection by predators, sexual selection, and physiological trade-offs, elements of color patterns may be sensitive to environmental factors. Examples include temperature-induced melanization [25] [26], and effects of food limitation on carotenoid intake [22] or nitrogen intake necessary for pteridine synthesis [27] [28]. Such 'direct' costs may induce variation irrespective of the 'indirect' costs of sexual or predator selection. Therefore, we suggest that a 'bottom-up' approach of investigating color production mechanisms is a productive avenue of research for identifying the potential selective pressures, trade-offs, and constraints that may be shaping color patterns.

In this study, we characterize and quantify the color production mechanisms of *Tectocoris diopthalmus* (Heteroptera: Scutelleridae), the Hibiscus Harlequin Bug. This large, charismatic stinkbug is widely distributed along the eastern and northern coasts of Australia and nearby Pacific islands [29]. Its defensive secretions have been identified [30], and these chemicals are known to be aversive to some predators, including birds [31] and praying mantids [32]. Rather than employing the more 'typical' aposematic color scheme of red or yellow with black markings, *T. diopthalmus* display a matte red-orange background with bright metallic blue-green iridescent patches. Because of their iridescence (the phenomenon of observed hue changing with viewing angle), these patches are likely to be produced by structural coloration. Both pattern elements are highly variable; the base color varies from a saturated red to very pale orange, while the iridescent patches range in hue from violet to green, and range in size from almost covering the dorsal surface to being entirely absent (figure 1). The species is sexually dichromatic, with males more likely to have large iridescent patches and deeper red coloration [33]. There are broad latitudinal [34] and seasonal [33] patterns in variation, as well as variation between individuals in one population at a given time. The use of iridescence in aposematic patterns is somewhat surprising, because the hue shifts with viewing angle introduce even further variability to the pattern (but see [35] and [36] for other potential cases of iridescent aposematic signals). The prominent use of both putatively structural and pigmentary color make *T. diopthalmus* an ideal candidate for detailed investigation. Furthermore, *T. diopthalmus* is sympatric over part of its range with *Cantao parentum*, a similar-sized scutellerid with similar life-history that displays a more 'conventional' red with black spotted pattern [29], which raises the question of why *T. diopthalmus* in particular features iridescent patches.

Our aim is to use histological and chemical methods to examine the color production mechanisms of *T. diopthalmus*, and thus the proximate causes of color variation in a putatively aposematic, sexually dimorphic bug. Characterizing the mechanisms will also facilitate identifying potential physiological costs and help elucidate information content of the various signal components. Furthermore, identification of color mechanisms will highlight specific avenues for future research into factors maintaining color variation, and is an important step for exploring the paradox of intraspecific variation in aposematism.

Materials and Methods

Adult male and female Hibiscus Harlequin Bugs (*Tectocoris diopthalmus*) were collected off Norfolk Island Hibiscus (*Lagunaria patersonia*) trees planted on streets near the beaches of Narrabeen and Dee Why, New South Wales, Australia. No specific permissions were required to collect insects from local council-planted trees, and this species is not considered threatened or otherwise protected under law. Bugs were maintained in the lab on potted Beach Hibiscus (*Hibiscus tileaceus*) plants supplemented with seed pod cuttings from Norfolk Island Hibiscus and Native Rosella (*Hibiscus heterophyllus*) plants. Individuals were killed with ethyl acetate fumes, as freezing appeared to alter the appearance and likely the ultrastructure of the iridescent patches.

To quantify the color of the iridescent and orange patches, reflectance spectra of bug integument (12 males, 10 females) were obtained using an Ocean Optics USB 4000 Spectrophotometer with a fiber optic probe positioned at a 45° angle to the incident light source. The light source was an Ocean Optics PX-2 Pulsed Xenon lamp with an optic fiber positioned above the specimen. Insects were pinned to a three-axis freely rotating stage and rotated to positions of maximum brightness reflected. Samples were measured between 300 and 700 nm. Polytetraethylene (Teflon^{TM}) tape was used as the white standard, which provides over 97% reflectivity over the spectral range of interest [37]. To test for linearly polarized reflectance, specimens were viewed through a Hoya linear polarizer while rotating it; to test for circularly polarized light, specimens were viewed through a Hoya linear polarizer rotated behind a quarter-wave plate [38].

Electron microscopy was used to image the cuticular ultrastructure responsible for creating the iridescent patches. One representative male and one female were imaged by Scanning Electron Microscopy (SEM). Pieces of the scutellum were fixed in a 3% glutaraldehye solution in phosphate buffer pH 7.2 overnight, dehydrated in a graded ethanol series (50%, 70%, 90%, 100%, 100%; 15 minutes each step), and dried with an Emitech K850 critical point dryer. The pieces were then mounted on aluminium stubs and sputter coated with gold (approximately 20 nm thick). Specimens were viewed with a Jeol JSM 6480 scanning electron microscope.

Two males, one female, and one juvenile bug were imaged for Transmission Electron Microscopy (TEM). Pieces of scutellum were fixed in a 3% glutaraldehyde solution in phosphate buffer pH 7.2 overnight, and post-fixed in a 1% osmium tetroxide solution for 2 hours, followed by a 2% uranyl acetate solution for 30 minutes. The samples were dehydrated using a graded ethanol series (50%, 70%, 80%, 90%, 95%, 100%, 100%; 30 minutes each step), and infiltrated in LR White resin using a resin-ethanol series (1:3, 1:2, 1:1, 2:1, 3:1, 100% ×2, 1.5 hours each). The final immersion was held under vacuum for 5 hours, before being loaded into molds and polymerized at 70°C. Semi-thin (0.7 μm) and ultra-thin sections (~70 nm) were cut perpendicular to the dorsal surface using a Reicherts Ultracut S microtome with a diamond knife. Semi-thin sections were viewed with an Olympus BH-2 microscope with Scion CFW-1310C color digital camera. Ultra-thin sections were mounted on copper TEM grids coated with 0.3% pioloform, and stained with 7.7% uranyl acetate for 30 minutes, followed by Reynolds lead citrate [39] for 5 minutes. Specimens had a sectioned transversely across the color interface. Sections were imaged with a Philips CM10 transmission electron microscope with Olympus SIS Megaview G2 digital camera.

Figure 1. Examples of variation among individual of *T. diopthalmus*. Pictured are six individual *T. diopthalmus*; the top row is females, the bottom row is males, as examples of the variation between individuals in *T. diopthalmus*. This is not the extent of possible variation, as both males and females are capable of displaying patterns between complete absence and near total coverage of iridescence, and between rich red and virtually white pale orange.
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Measurements of layer width were made at six random points along the transverse section using Olympus iTEM 5.1 software.

For preliminary pigment analysis, carotenoid presence was tested using the acidified pyridine method [40]. To distinguish between other candidate pigment classes (melanins, pterins, flavonoids, and ommochromes), a discriminatory extraction test, modified from Lindstedt et al. [41], was used. Pterins and flavonoids are soluble in strong acids and bases, but flavonoids are also soluble in neutral organic solvents such as methanol, while ommochromes are soluble in acidified alcohols [28] [42]. Both intact and crushed-whole bugs were placed in vials of 0.1M sodium hydroxide, 90% methanol, or 1:10 hydrochloric acid/ methanol solution, and incubated at room temperature for 24 hours. Insect parts were removed and the extracts were centrifuged at 14,000 RPM for 5 minutes. The supernatants were then measured using a Shimadzu UVmini 1240 UV-Vis Spectrophotometer using quartz cuvettes across a wavelength range of 200 to 800 nm. Absorbance spectra were compared against published spectra [43]. Both intact bugs and extracted supernatants were observed under short-wave (254 nm) and long-wave (366 nm) ultraviolet light to visually inspect for the presence of fluorescence, as pterins and flavonoids fluoresce under UV light while carotenoids, ommochromes, and melanins do not [28] [42].

Based on the preliminary results of pigment analysis, we focused our identification efforts on pterin pigments. For the separation and identification of pterins potentially present in the integuments of *T. diopthalmus*, a capillary electrophoretic method (CE) was developed. The separation system was modified from Han et al. [44]. The CE measurements with UV detection were carried out on an Agilent Technologies HP^{3D}CE system with built-in diode array detector operated at 250 nm. CE analyses were conducted in an uncoated fused-silica capillary (CACO) total length 70 cm, 55 cm to the detector, inner diameter 50 μm, thermostated at 30°C. Background electrolyte (BGE) contained a mixture of 100 mmol/L boric acid, 100 mmol/L tris(hydroxymethyl)amino-methane (TRIS), pH 9,0, and 2 mmol/L ethylenediaminetetra-acetic acid disodium salt dihydrate (Na_2EDTA). Samples were injected electrokinetically at 20 kV for 10 seconds, and the applied separation voltage was 20 kV. Pterin standards included biopterin, isoxanthopterin, leucopterin, neopterin, xanthopterin, and erythopterin (all from Sigma Aldrich except erythropterin provided by R. Rutowski). Stock solutions of the individual standards were prepared by dissolving the compounds in dimethyl sulfoxide at a concentration of 0.1 mg/mL and were kept in dark at 4°C. Working standard solutions were prepared by diluting the stock solutions with BGE to a concentration of 0.025 mg/ml. Identi-

fication was carried out by spiking samples with pure standard solutions.

Dried integuments were used in the CE analysis. Three bugs of each color morph ('red' vs 'orange', as judged visually by experimenter) were used in each extraction, to minimize the effects of individual variation. Integuments were weighed (~6 mg per red extract and ~8 mg per orange extract) and put in a vial with 0.5 mL dimethyl sulfoxide to be incubated in the dark at room temperature for 98 hours. The extract was centrifuged at 13,000 RPM for 10 minutes. The supernatant was then diluted 4× with BGE before being used for CE analysis. Three extracts of red form and orange form were prepared, for a total of nine individuals sampled per morph.

To test whether the otherwise-insoluble iridescence is dependent on melanin for its optical properties, photographs and spectra of 5 sample bugs were taken before being immersed in 20% hydrogen peroxide for 24 hours, after which the bugs were washed with water, photographed and measured spectrally (see above) again. Hydrogen peroxide breaks down melanin [45], and degrade melanin-containing layers in the ultrastructure in situ, decreasing both peak reflectance wavelength and brightness [46].

Results

Selected example spectra for the iridescent and orange patches can be seen in Figure 2. The iridescent patches show a sharp peak, which, in the individuals sampled, can range between 480 and 570 nanometers, while the orange base shows a monotonic increase beginning at between 512 and 590 nanometers, leveling off by 700 nanometers. In this study male scutellum iridescent patches are on average more blue-shifted (average peak of 520±24 nm versus 542±17 nm for females), while the male's orange patches are more red-shifted, reflecting on average light of 580±14 nm or greater (versus 538±24 nm or greater for females), creating greater chromatic contrast. No polarized reflectance, either linear or circular, was detected at normal light incidence and viewing angle in either sex.

In the differential solubility test, an orange-colored pigment was soluble in 0.1M NaOH and acidified methanol, but not neutral methanol (Table 1). The absorbance peak in acidified methanol was at 430 nm, with a trough at 380 nm, closely approximating published spectra for erythropterin [43]. Extracts could not be obtained from intact bugs, but only from ground specimens. Unstained semi-thin sections of cuticle reveal red granules inside epidermal cells underneath unpigmented cuticle (Figure 3). Both pigment extracts and the venters of intact bugs fluoresce under ultraviolet light (Table 1).

For confirmation and specification of pterin derivative(s) responsible for the coloration, organic extracts of integuments from orange and red morphs of *T. diopthalmus* were analyzed by capillary electrophoresis (CE). The results obtained from one extraction of 'red' and 'orange' forms are shown in Figure 4. Four peaks were identified in both morphs. Peak 1, the negative peak, is due to dimethyl sulfoxide in the injected sample. Peaks 2 and 3 correspond to isoxanthopterin and leucopterin respectively, which are colorless in visible wavelengths but absorb ultraviolet light. Peak 4 is erythropterin, a red-reflecting pigment which absorbs shorter wavelengths. Both morphs also contain small amounts of colorless biopterin [unlabeled]. Qualitatively, electrophoreograms obtained for a given color morph were nearly identical. The key color difference identified between red and orange morphs is the greater amount of erythropterin present in the red morph (mean peak area 4.23±0.59 milliabsorption units*seconds, from 6 mg of integument) versus the orange morph (mean peak area 2.87±0.15 milliabsorption units*seconds, from 8 mg of integument). Xanthopterin, a common reddish-orange pigment, was not found in any tested samples.

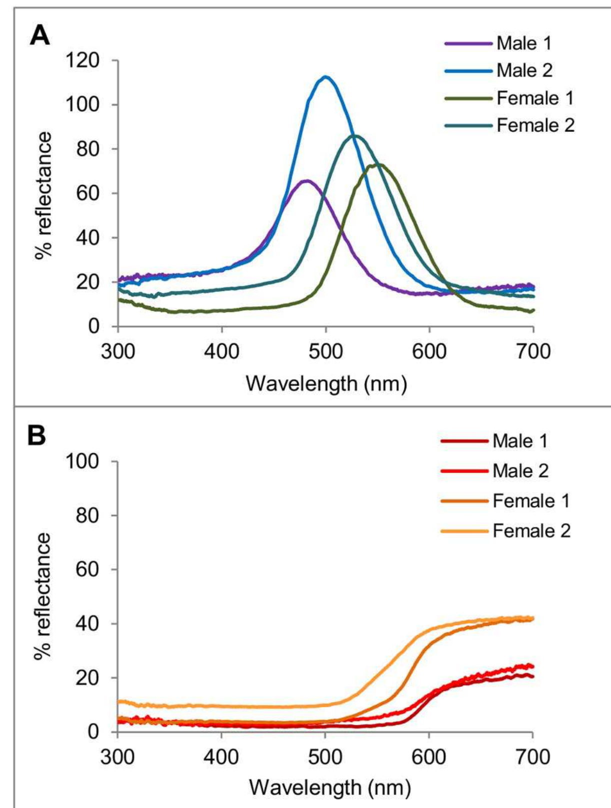

Figure 2. Example reflectance spectra of *T. diopthalmus* color patches. The spectral reflectance curves of the color patches of four individuals (two males, two females) are shown here. Curves from iridescent dorsal patches are shown in A, while the curves from the dorsal orange patches of the same individual are shown in B. Precent reflectance is against a Teflon white standard.
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Scanning electron microscopy revealed that the dorsal surface is covered by microtubules of uneven size but roughly even spacing. These microtubules can be found uniformly over the scutellum of both males and females regardless of underlying color (Figure 5). Transmission electron microscopy revealed no microstructure in the cuticle of the orange patches.

TEM imaging did however reveal ultrastructure to the iridescent patches compromising a multilayer system in the epicuticle (Figure 6). The structure consists of alternating layers of 'dark' (electron-dense) and 'light' (electron-lucent) material, bordered on the outside by a thinner semi-lucent layer, and underlaid by a thick layer of pigmented exocuticle (Figure 7). There are between 5 and 9 layers, on average 7, of each layer type. The average width of the layers is between 60 and 90 nanometers, depending on the individual measured (Table 2). At the base of the multilayer system, and at the interface between the iridescence patches and the black border regions (not shown), the 'light' layers degenerate, and the 'dark' layers seamlessly merge with the underlying pigmented layer. The underlying layer appears as a thick brown line under light microscopy. For a comparison of measured and predicted peak wavelengths of the iridescent patches in the individuals examined, see Table 2.

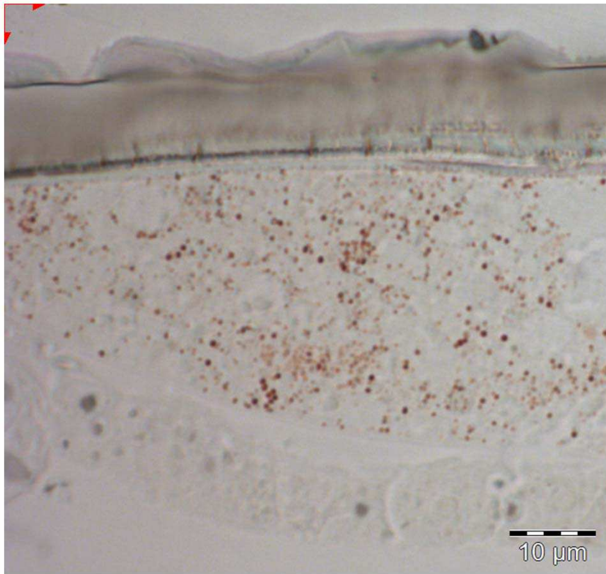

Figure 3. Light microscopy image of epidermal cell in *T. diopthalmus*. Unstained thin section of an epidermal cell underneath cuticle in the ventral surface of *T. diopthalmus*. The small red granules near the distal end are believed to be pigment granules containing pterins.
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For the bugs soaked in hydrogen peroxide, the peak reflectance of the iridescent spots was shifted to a shorter wavelength, the peak brightness was dramatically reduced, and the broadband reflectance was increased, with a monotonic rise at longer wavelengths (Figure 8). This is indicative of both a marked reduction in the optical thickness of the layers and leeching of the underlying pigment.

Discussion

Identification of color production mechanisms

We identified two major color production mechanisms in *Tectocoris diopthalmus*, a red-orange pigment and a multilayer reflector structure. The structure exists in the outermost exoskeletal layer, while the pigment is contained in intracellular granules in the epidermis. We identified the pigment as a pterin-based compound due to its fluorescence characteristics and solubility in acidified or basic aqueous or organic solvent (Table 1). The color

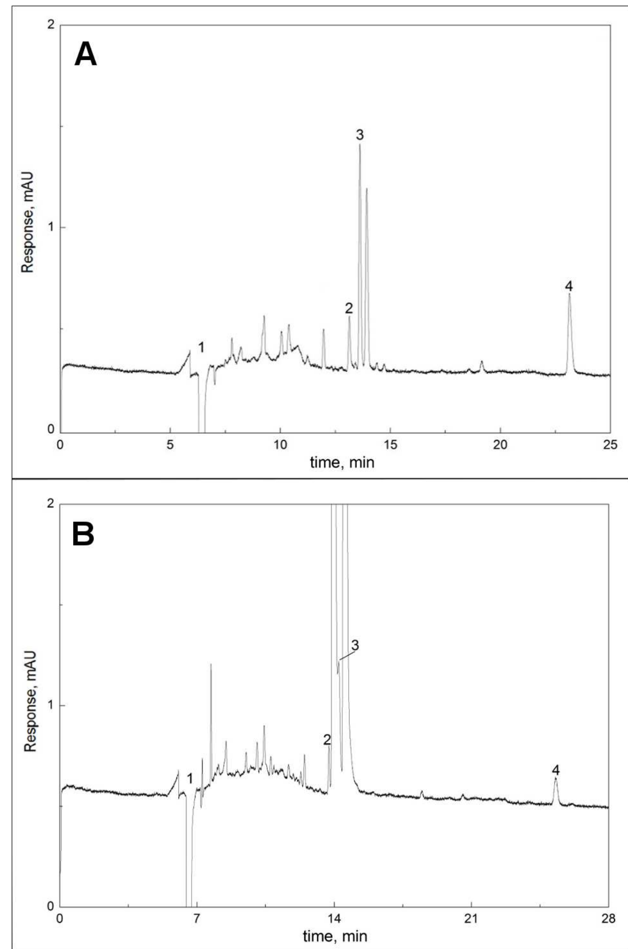

Figure 4. Analysis of pterin-based pigments of *T. diopthalmus* by Capillary Electrophoresis (CE). Capillary Electrophoresis (CE) analysis of red (A) and orange (B) forms of integuments of *Tectocoris diopthalmus* at 250 nm. Peaks: 1- dimethyl sulfoxide,2- isoxanthopterin, 3-leucopterin, 4-erythropterin. Note the larger erythropterin peak in the red form.
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causing pigment has been specifically identified as erythropterin by capillary electrophoresis, and differences in the hue of the red-orange coloration can be largely explained by differences in the amount of erythropterin pigment. This result agrees with previous

Table 1. Discriminatory identification of pigment class.

	Dissolves in solvent				Fluoresces in UV
Pigment Class	**Acidified Pyridine**	**0.1M NaOH**	**90% Methanol**	**10:1 Methanol/HCl**	
Carotenoids	X				
Pterins		X		X	X
Flavinoids		X	X	X	X
Ommochromes				X	
T. diopthalmus extract		X		X	X

X indicates that the pigment named in the row is readily soluble in the solvent named in the column, or fluoresces under ultraviolet light. Lack of X indicates no visible extraction or fluorescence.
doi:10.1371/journal.pone.0064082.t001

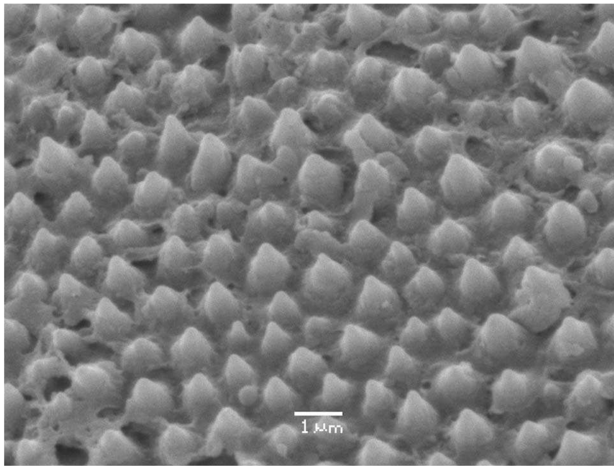

Figure 5. Scanning Electron Microscopy (SEM) image of scutellum surface. SEM imaging shows conical tubercles of varying dimensions covering the surface of the scutellum. These protrusions can be found on both males and females and overlying both iridescent and orange patches.
doi:10.1371/journal.pone.0064082.g005

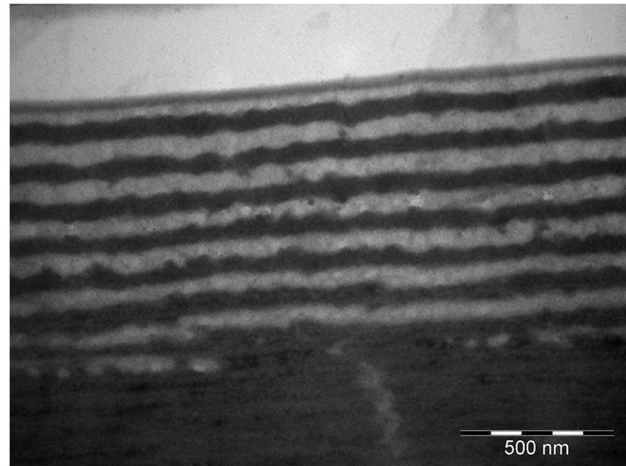

Figure 6. Transmission Electron Micrograph (TEM) of the multilayer reflector. TEM image of the multilayer reflector in *T. diopthalmus* (from sample 2 in table 2). Note the bottommost thin 'dark' layer appears contiguous with the underlying thick dark layer where the separating 'light' layer is disrupted.
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studies that have identified erythopterin as a major red or orange pigment in heteropterans [47] [48] [49] [50].

The iridescent blue-green coloration is produced by a multilayer reflector in the epicuticle, identifiable in transmission electron micrographs (TEM). Both the structure and results of hydrogen peroxide bleaching closely match the results of Schultz and Rankin [46], thus the Harlequin Bug and *Cicindela* tiger beetles have likely evolved a convergent system. We therefore posit that the multilayer structure in *T. diopthalmus* is also is composed of alternating layers of melanin-laced and melanin-free protein matrix, overlaying a melanized exocuticle base [46]. These iridescent patches could be an elaboration evolved directly from melanic patches in a more 'typically aposematic' red and black ancestor, perhaps in shared ancestry with *Cantao*, but this hypothesis requires phylogenetic analysis for further investigation.

The ultrastructure of the epicuticular reflector appears similar to those found in other non-aposematically colored scutellerids [51] [52], as well as cicindelid tiger beetles [53], buprestid jewel beetles [54], and calopterygid damselflies [55]. This is structurally distinct from the rotated helicoidal rotated exocuticle found in scarab reflectors [16], as well as the epicuticular reflector in wasps [56]. The function of iridescence in these animals is often unknown, though it is believed to aid crypsis in *Cincindela oregona* tiger beetles [57] while functioning as a sexual trait, as an indicator of male condition, in male *Calopteryx maculata* damselflies [55].

The predicted peak reflectance of the multilayer system can be calculated using Snell's Law, $\lambda_{\max} = 2(n_1 d_1 + n_2 d_2)$ wherein the peak reflectance (λ_{max}) is dependent on the refractive index (n) and thickness (d) of each layer type. Based on measurements of peak reflectance and layer widths, the average refractive index is estimated to be 1.78. Durrer and Villiger [58] estimated the average refractive index of a melanin-based epicuticular reflector to be 1.75, with the melanic layers having a refractive index of 2.0 and non-melanic layers having an index of 1.5. This estimate has been used successfully by Schultz and Rankin [46] and Fitzstephens and Getty [55] to predict the peak reflectance of multilayer systems. More recent studies by Noyes et al. [59] and Stavenga et al. [54] place the estimate for melanic layers lower, at between 1.6 and 1.7. Using these lower estimates results in a predicted peak wavelength far lower than the value we measured. We therefore utilize the higher value of 2.0 as the refractive index of our electron-dense layer, and consequently 1.56 for our electron-lucent layer. However, we acknowledge the limitations of our analysis and do not intend to make broad claims as to the optical nature of ultrastructural components. Furthermore, diverse claims of the refractive index of ultrastructure components have been made in other scutellerids, including chitin and water (1.58 and 1.33) in *Poecilocoris lewisi* [51], and chitin and chitin-air grating (1.56 and 1.4) in *Calidea panaethiopica* [52]. Therefore there may be great evolutionary lability and diversity in ultrastructural components.

The outer surface of the scutellum in both sexes is uniformly covered by small bumps visible in scanning electron micrographs. These microtubercles appear superficially similar to structures

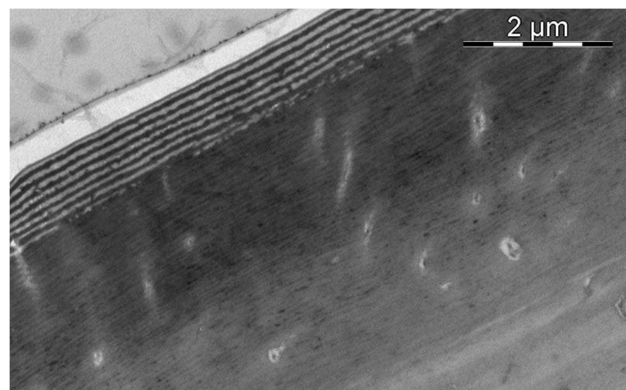

Figure 7. Transmission Electron Micrograph (TEM) of Epicuticle and Exocuticle. Zoomed out version of Figure 6. Three regions of cuticle can be seen. The thin banding in the top-left region is the epicuticle, the outermost layer of cuticle, containing the multilayer reflector. Below that is the exocuticle, with fine helicoidal layering. The upper region of the exocuticle contains an electron-dense pigment which appears brown in unstained thin sections for light microscopy (not pictured). The smoother region in the bottom-right corner is the upper edge of the endocuticle. The apparent thick white layer in the upper-right corner of the micrograph is an artefact due to resin separation from the sample.
doi:10.1371/journal.pone.0064082.g007

Table 2. Multilayer reflector layer measurements and predicted peak reflectance.

Sample	Age/Sex	Measured peak reflectance (nm)	Number of 'dark' layers	Width of 'dark' layers (nm)	Number of 'light' layers	Width of 'light' layers (nm)	Predicted peak reflectance (nm)
1	Male	462	7.2±1.2	59.2±5.9	7.3±0.8	70.3±4.5	456±21
2	Male	532	6.8±1.5	73.1±5.9	6.5±1.3	79.4±6.3	540±21
3	Female	538	6.2±1.3	68.6±9.8	5.8±0.4	90.6±5.9	557±34
4	Juvenile	463	4.8±0.4	60.9±4.6	5.2±0.4	64.3±5.7	444±19

Layer widths (and resulting predicted peak reflectance) given are the mean and standard deviation of the average layer width at six points along the section; therefore, standard deviations reflect horizontal regularity rather than vertical. For examples of 'dark' and 'light' layers, see Figure 6.
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found in another scutellerid, *Poecilocoris lewisi*, which produce a diffuse blue reflectance via Mie scattering [51]. Curiously, we see no evidence for a similar effect in reflectance spectra of *T. diopthalmus* (Figure 2), and do not believe the microtubercles function in color production. It may be that these protrusions act as an anti-reflector structure to reduce specular reflectance, but their dimensions are too large to function effectively in this capacity [60]. Alternatively, they may not have any visual function, and act as an anti-wetting structure [60]; further studies are required to elucidate their biological function.

Exploring the implications of identified mechanisms

The red-orange color is created primarily by erythropterin pigment. Pterins are endogenously produced, though they are some of the most nitrogen-heavy pigments [28] [61]. It has been proposed that pterins are used as a nitrogenous waste product in insects, suggesting the pigment is 'cheap' [62]. However, plant herbivory often results in nitrogen limitation as an important factor in life history trade-offs [27]. Variation in pterin pigmentation between the sexes and between individuals could reflect differences in the ability to acquire dietary nitrogen, but variation in color can be found between individuals reared on the same host plant (personal observation). While the role of pterins as antioxidants in insects has not to our knowledge been studied, certain pterins are known to have antioxidant and immune modulating functions in vertebrates [63]. Given the prevalence of pterins as heteropteran pigments [47], we suggest that the use of erythropterin is as a phylogenetically conserved 'cheap' pigment for aposematic function, but whether it has protective function in Heteroptera requires further study. Furthermore, the average shift in reflectance between males and females suggests that the red-orange pigment may also be playing a role in the creation of a sexual signal in addition to any aposematic function. Selective forces such as mate choice and predators may be selecting on the color pattern as a whole, so it is unsurprising that an element of the color pattern is likely contributing to multiple functions.

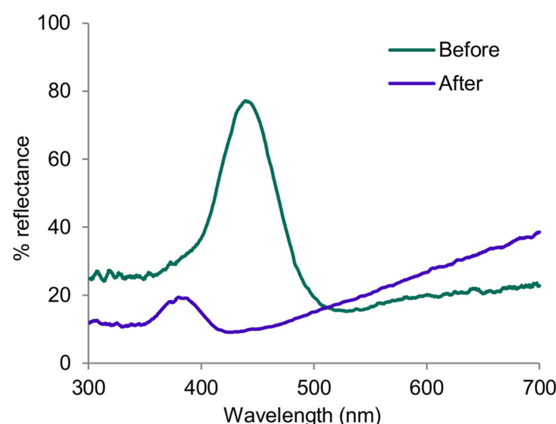

Figure 8. 'Before' and 'After' reflectance spectra after soaking bug in 30% hydrogen peroxide. 'Before' is the reflectance spectrum of the bug before soaking 24 h in 30% H_2O_2, 'After' is the spectra taken after drying. Percent reflectance is against a Teflon white standard. Note the peak shifting to shorter wavelengths, reflective of the thinning of the layers in the multilayer system, and the monotonic increase at longer wavelengths.
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As a potentially 'derived' element of the color pattern, we are particularly interested in how the multilayer reflector system in the iridescent patches may act as an informative, honest sexual trait. It is more structurally complex than the pigmentary component of the signal. In addition to patch size and symmetry, signal quality depends on the regularity of both the dark and light layers, in both the vertical and horizontal dimensions. The precision required for this structure may be disturbed by developmental instability, making it a sensitive trait to display genetic quality and resistance to perturbation [19]. Pigmentary colors can also be sensitive to rearing conditions, and the reliance on melanin pigment as a major component may make the patches even more sensitive to environmental disturbance. For example, cuticular melanization can be upset by high temperatures in many taxa including Lepidoptera [64], *Drosophila* flies [26], and other Heteroptera [25]. This can help explain population differences, with high ambient temperatures suppressing the patches in tropical populations [34], and depending on the window of susceptibility, can also be contributing to intrapopulation variation, via seasonal or daily fluctuations in temperature [33].

Melanin-based signals may also be informative through trade-offs with physiological uses for melanin and its precursors, in ways unique to invertebrates [65]. Tyrosine limitation may result in trade-offs with production of neurotransmitter dopamine or cuticle hardening agent sclerotin [23] [66]. Moreover, melanin and its precursors are utilized in the encapsulation response to endoparasites, a vital component of insect innate immunity, and cuticular coloration could trade off with immunocompetence [67] [68]. Conversely, enhanced melanic ornamentation could also be a result of greater systemic levels of melanin precursors or enzymes. In this way, the iridescent patches could act as an honest indicator of condition or immunocompetence [69]. Juvenile immune challenge can also influence adult expression of melanin coloration, rendering it an informative artefact of juvenile infection history [70].

Variation and its effects on aposematism

Despite the inherent variability in the iridescent patches, there may be aposematic benefit in iridescence. The 'complimentary' nature of green/blue and red/orange (e.g. little overlap in reflected wavelengths) enhances conspicuousness compared to a red/black pattern of comparable brightness [71]. The patches are also highly reflective and saturated, and the juxtaposition of bright and chromatic patches with a low brightness and chroma background (e.g. the red-orange base color) also enhances signal conspicuousness [71]. This brightness contrast may be especially important in aposematism against invertebrate predators such as praying mantids, whose hunting tactics may be more reliant on luminance contrast [72]. The downside of having iridescence is its variability, as there is increased predation risk for individuals that do not match the common morph [4] [73]. However, Ihalainen et al. [74] found evidence that being variable may in fact be beneficial to avoidance learning of moderately defended prey. *Tectocoris diopthalmus*' defensive capacity is moderate compared to other heteropterans [30] [75], and thus may benefit from this variation.

Conversely, variability in hue of orange pigmentation may have little influence on aposematic defense. Exnerová et al. [76] demonstrated that four bird species showed no difference in avoidance learning between red and orange morphs of the firebug *Pyrrhocori apterus*. Great Tits (*Parus major*) trained to avoid red artificial prey will generalize to avoid orange prey as well [77]. Lindstedt et al. [78] showed that while birds in the lab can discriminate between red and orange morphs and will preferentially attack orange, 'survival' of models in the field was equal between morphs. With bugs of varying red and orange hues coexisting, birds may quickly learn to generalize. Variation in the size of iridescent patches has the potential for consequences on aposematism, as larger pattern elements increase avoidance in naïve chicks [79].

In conclusion, investigation into the mechanisms of color production has opened up a wealth of possible physiological and environmental interactions, and generated specific hypotheses to fuel future research directions. We have only used one species as a case study, but the commonplace chemical and microscopic techniques used here are easily applicable to many species. Variation in aposematism is a longstanding question in behavioral ecology, and a bottom-up approach of studying prey physiology is one way to help answer it.

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Author Contributions

Conceived and designed the experiments: SAF DJK MEH JK ZB. Performed the experiments: SAF JK ZB. Analyzed the data: SAF JK ZB. Contributed reagents/materials/analysis tools: DJK MEH ZB. Wrote the paper: SAF ZB.

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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.

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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.

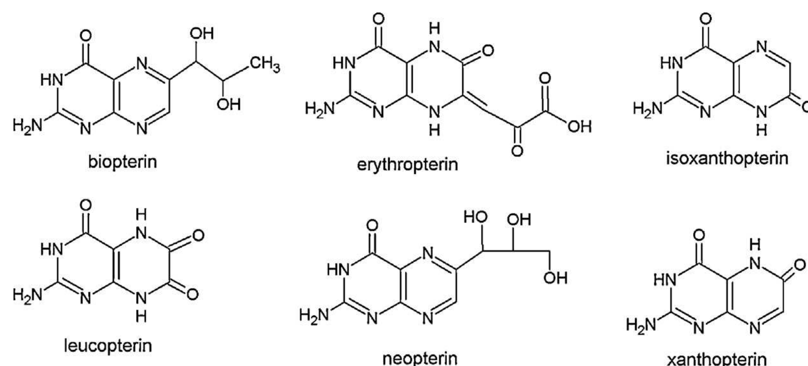

Fig. 1. Chemical structures of the pteridiens studied.

The effect of the ratio of organic solvent to water content in the mobile phase and the influence of additive type (acetic acid, formic acid, ammonium acetate, ammonium formate), its pH and concentration on retention of pteridines were investigated. The effect of pH was tested in the range of 4–6 pH units for Atlantis HILIC Silica column and within 4–8 pH units for ZIC®-HILIC column (pH was varied in steps of 0.5 unit for both columns). The effect of buffer concentrations was tested in the range of 1–25 mM for both columns (buffer concentrations were varied in steps of 5 mM).

The retention times of all analytes were measured in triplicate. The retention was expressed as retention factor (k).

2.4. Sample preparation of G. lineatum and G. semipunctatum

The insect integuments were extracted by dimethyl sulfoxide. The extraction time was optimized to achieve the equilibrium concentration of pteridines by monitoring the pteridine extract during one week. An appropriate amount of dry insect integuments ranging from 2.5 to 6.5 mg was crushed, homogenized and extracted in 2 mL of dimethyl sulfoxide. Then the sample was centrifuged for 10 minutes at 11,337 × g, the supernatant was collected and diluted 1:100 with acetonitrile. Four forms of *G. lineatum* (larvae, pale, red and yellow adults) and one form of *G. semipunctatum* (orange adults) were analyzed.

2.5. Validation of the method

The method was validated for linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, precision, selectivity, carry-over effect and matrix effects. Linearity was established using standard solutions of pteridines serially diluted by acetonitrile within the range of 0.1–2500 ng/mL for biopterin (the calibration concentrations were 0.1, 1, 5, 25, 125, 625, 1250, 2500 ng/mL) and 1–5000 ng/mL for other pteridines (the calibration concentrations were 1, 5, 25, 125, 625, 1250, 2500, 5000 ng/mL), which reflected the expected concentrations of analytes in the real samples. The accuracy of the method was evaluated by analysis of quality control (QC) samples at low (10 ng/mL for biopterin and 20 ng/mL for others pteridines), medium (1 μg/mL for biopterin and 2.5 μg/mL for others pteridines) and high (2.5 μg/mL for biopterin and 5 μg/mL for others pteridines) concentration levels. The agreement between the theoretical and measured values was confronted. The precision was established by replicate analysis of QC samples at the same concentrations as for accuracy measurements.

Method selectivity was monitored by the injection of the sample of *G. lineatum* (mass spectrometer was set in scan mode) and the carry-over effect was checked by the injection of a blank sample (consisting of 1/99 (v/v) dimethyl sulfoxide/acetonitrile). Matrix effects were determined using two types of quantitation methods, namely the calibration curve method and the standard addition method. The calibration curves were constructed by plotting the peak area against the concentration of pteridine standards. The calibration equations were calculated using linear regression analysis. Each concentration was measured in triplicate. For the standard addition method 25 μL of the supernatant was put to each of the five 10 mL volumetric flasks. Then the standard solutions at concentration of 100 μg/mL for erythropterin and leucopterin, 10 μg/mL for isoxanthopterin and 1 μg/mL for biopterin, neopterin and xanthopterin were added in amounts of 0, 25, 50, 75 and 100 μL. Finally, each flask was made up to the mark with acetonitrile and mixed well. After measuring the response for a series of standard additions, a plot of analytical signals versus concentrations of added analytes was plotted. The concentration of the analytes was determined from the point, at which the extrapolated line crossed the concentration axis at zero signal.

3. Results and discussion

3.1. Optimization of mass spectrometric detection

Selected reaction monitoring conditions were established for each analyte by an infusion of standards (1 μg/mL) into the ion source by a syringe pump. The precursor ions were optimize-scanned with the second quadrupole for the best fragmentor voltage. The mass spectra of the molecules were recorded by the employment of fragmentor potentials between 50 and 200 V. After choosing the most intense fragmentor voltage for the found precursor ions, the collision energies of the ion transitions were optimized between 0 and 100 V using a product ion scan. The resultant values are listed in Table 1. To maximize the signals in the SRM mode the ion source parameters were tuned. The ion source was finally set as follows: gas temperature: 350 °C, gas flow: 10 L/min, sheath gas

Table 1
SRM conditions used for LC-MS/MS determination of the pteridines (ESI, positive mode (+), negative mode (−)). The first transition is used for quantitation and the second one for confirmation.

Compound	Molecular weight	Precursor ion	Product ion	Fragmentor (V)	Collision energy (V)
Biopterin	237.1	238.1 (+)	178.1	115	17
		236.1 (−)	192.1	140	3
Erythropterin	265.1	266.1 (+)	220.1	110	8
		264.1 (−)	192.1	95	6
Isoxanthopterin	179.1	180.1 (+)	135.1	125	20
		180.1 (+)	108.1	125	25
Leucopterin	195.1	196.1 (+)	140.1	120	16
		194.1 (−)	166.1	130	8
Neopterin	253.2	254.2 (+)	206.2	115	14
		254.2 (+)	236.2	115	8
Xanthopterin	179.1	180.1 (+)	135.1	125	20
		180.1 (+)	108.1	125	25

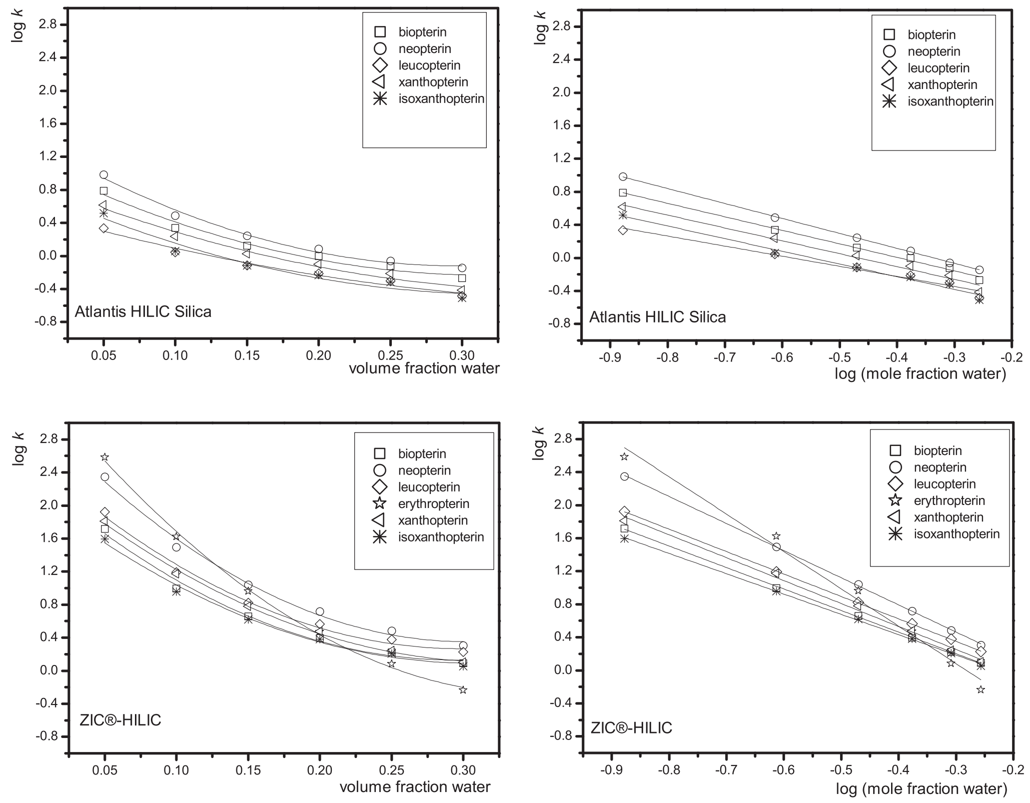

Fig. 2. Dependences of log *k* of studied pteridines on the volume fraction of water (second order polynomial fit) and on the logarithm of the mole fraction of water (linear fit) obtained for the both tested columns (for more details see Eqs. (1) and (2) in Section 3.2). The aqueous part of the mobile phase: 1 mM ammonium acetate buffer, pH 4.00.

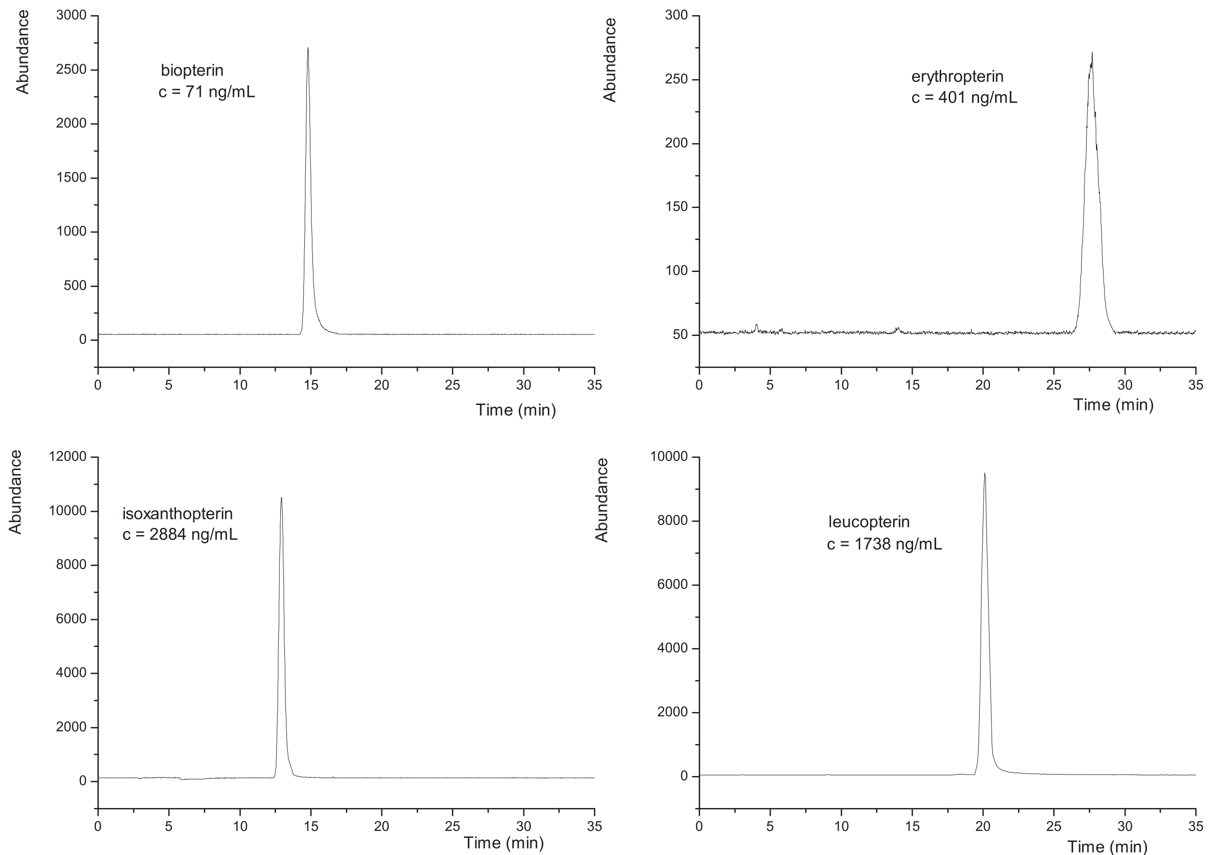

Fig. 3. SRM chromatograms of the orange form of *Graphosoma semipunctatum* under optimized LC–MS/MS conditions; for details see the Section 2 and Table 1.

Table 2
Parameters of the calibration curves (standard deviations in parentheses), limit of detection (LOD) and limit of quantitation (LOQ) obtained for the optimized separation and mass spectrometric detection conditions.

Compound	Slope (mL/ng a.u.)	Intercept (a.u.)	Correlation coefficient	LOD (ng/mL)	LOQ (ng/mL)
Biopterin	1845.9 (65.4)	−1025.2 (89.9)	0.9991	0.1	0.3
Erythropterin	98.0 (5.6)	−49.3 (3.2)	0.9998	6.2	19.3
Isoxanthopterin	122.0 (7.6)	−245.5 (44.2)	0.9982	4.3	12.1
Leucopterin	400.7 (21.8)	−180.5 (32.8)	0.9975	1.4	3.8
Neopterin	304.5 (9.2)	−245.5 (45.6)	0.9986	0.4	1.3
Xanthopterin	448.2 (11.3)	−49.3 (3.3)	0.9977	1.2	3.7

temperature: 350 °C, sheath gas flow: 10 L/min, nebulizer pressure: 310 kPa, capillary voltage: 4000 V for positive mode and 3500 V for negative mode, nozzle voltage: 500 V for positive mode and 600 V for negative mode.

3.2. Separation of pteridines

All studied pteridines are highly hydrophilic molecules, which can be separated in HILIC. The significant effect on retention and thereby separation of pteridines on both columns was observed by changing the ACN/aqueous buffer ratio in the mobile phase. On Atlantis HILIC Silica column erythropterin exhibited a very broadened chromatographic zone and therefore, these data are not included in the results.

The series of measurements performed during the optimization of the mobile phase composition were also used for the characterization of the interaction mechanism involved in the separation system. When the logarithm of the retention factor of an analyte is plotted against the volume fraction of the aqueous part of the mobile phase the empirical Eq. (1) proposed by Schoenmakers et al. [53] should provide a description of the partitioning mechanism:

$$\log\ k = A\varphi^2 + B\varphi + C \tag{1}$$

where φ is the volume fraction of the water in the mobile phase.

A linear plot of log k vs. log (mole fraction water) can indicate the adsorption process, according to the Snyder–Soczewinski expression:

$$\log\ k = \log\ kw - \frac{A_s}{n_w} \log N_w \tag{2}$$

where k_w is the hypothetical retention factor when the mobile phase is purely aqueous, A_S and n_w are the cross-sectional areas occupied by the solute molecule and the water molecules on the surface, respectively, and N_w is the mole fraction of water in the eluent [54].

Fig. 2 shows the dependences of log k of pteridines plotted against the volume fraction of water and against the logarithm of the mole fraction of the water for the both columns. Fitting dependences of log k on the volume fraction of water according to Eq. (1) provide correlation coefficients over 0.9947 for both columns for all the studied pteridines. Dependences of log k on the logarithm of the mole fraction of water fit very well with the linear regression curve of Eq. (2), with correlation coefficients ranged from 0.9905 to 0.9992 for Atlantis HILIC Silica column and from 0.9960 to 0.9991 for ZIC®-HILIC column for all the studied analytes. Based on these observations both partitioning and adsorption seem to contribute to the retention mechanism on both columns.

For mass spectrometric detection, substantially lower concentrations of additives have to be used in order to prevent signal suppression. The effect of buffer concentration on the retention of pteridines was tested in the mobile phase, consisting of

Table 3
Accuracy and precision of the method for LC–MS/MS determination of the pteridines (n = 5).

Concentration (ng/mL)	Intra-day (n = 5) Measured concentrations (ng/mL) (mean ± SD)	RSD (%)	RE (%)	Inter-day (n = 5) Measured concentrations (ng/mL) (mean ± SD)	RSD (%)	RE (%)
Biopterin						
10	9.53 ± 0.42	4.40	−4.73	9.61 ± 0.37	3.85	−3.93
1000	961.70 ± 10.38	1.07	−3.83	956.80 ± 9.28	0.97	−4.32
2500	2346.50 ± 91.28	3.90	−6.14	2317.00 ± 76.92	3.32	−7.32
Erythropterin						
20	20.75 ± 1.31	6.31	3.73	20.84 ± 1.25	5.99	4.21
2500	2570.75 ± 108.74	4.23	2.83	2572.00 ± 95.94	3.73	2.88
5000	5446.50 ± 431.36	7.92	8.93	5481.50 ± 443.45	8.09	9.63
Isoxanthopterin						
20	20.97 ± 0.91	4.34	4.83	20.87 ± 0.89	4.26	4.33
2500	2598.00 ± 76.38	2.94	3.92	2609.50 ± 37.05	1.42	4.38
5000	5368.50 ± 339.31	6.32	7.37	5344.50 ± 311.05	5.82	6.89
Leucopterin						
20	20.27 ± 0.65	3.20	3.66	20.48 ± 0.87	4.25	2.38
2500	2425.25 ± 51.90	2.14	−2.99	2598.25 ± 50.93	1.96	3.93
5000	4708.50 ± 232.60	4.94	−5.83	4809.00 ± 275.56	5.73	−3.82
Neopterin						
20	19.63 ± 0.24	1.22	−1.85	20.62 ± 0.27	1.31	3.10
2500	2594.25 ± 20.49	0.79	3.77	2607.25 ± 26.33	1.01	4.29
5000	5219.00 ± 167.53	3.21	4.38	4808.50 ± 163.97	3.41	−3.83
Xanthopterin						
20	21.34 ± 1.26	5.90	6.72	20.76 ± 0.89	4.29	3.81
2500	2649.75 ± 91.15	3.44	5.99	2624.50 ± 137.26	5.23	4.98
5000	5453.00 ± 397.52	7.29	9.06	5354.50 ± 332.51	6.21	7.09

SD, standard deviation; RSD, relative standard deviation; RE, relative error.

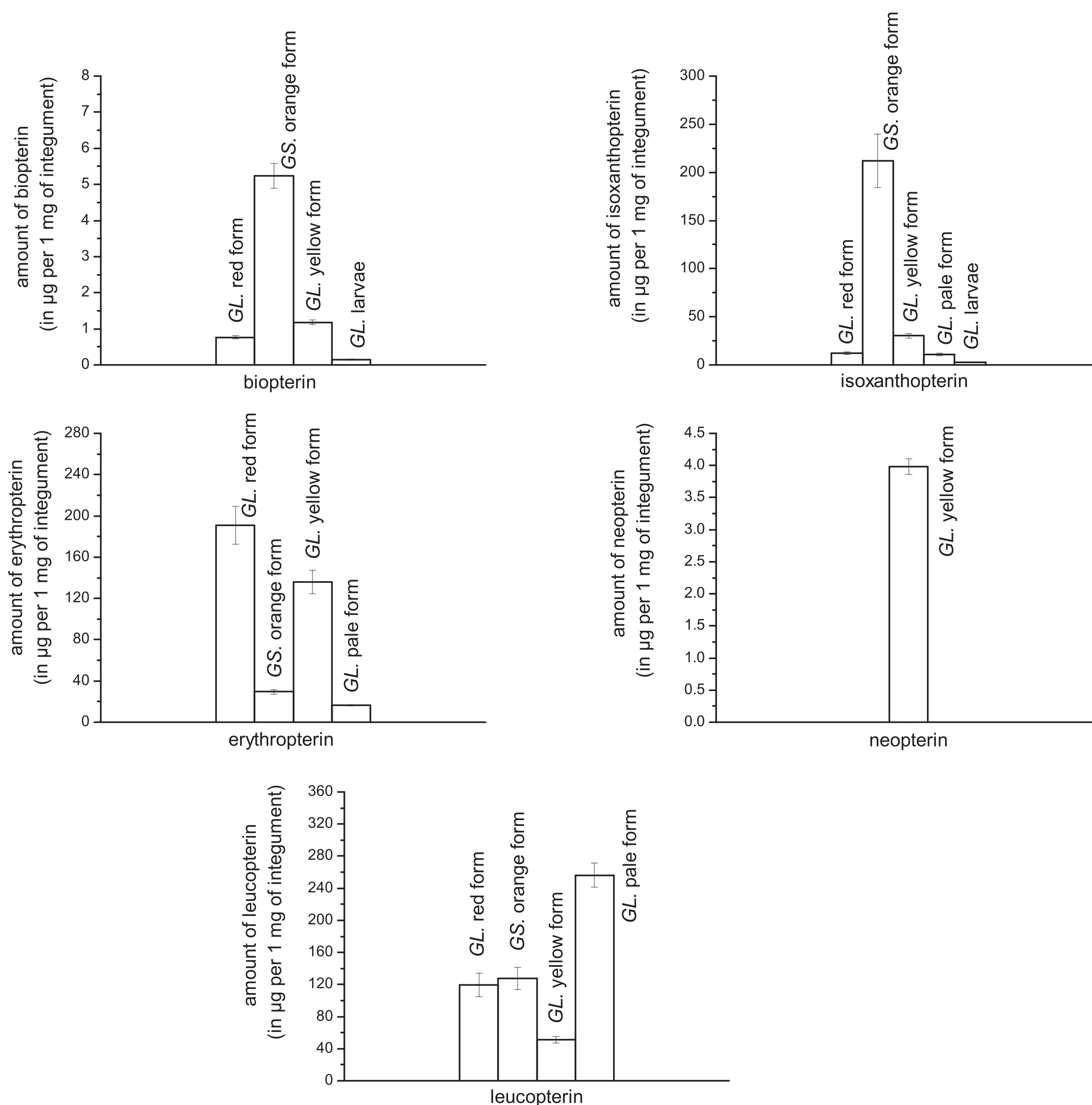

Fig. 4. Comparison of the amounts of pteridines found in different forms of *Graphosoma lineatum* (GL) and *Graphosoma semipunctatum* (GS).

ACN/ammonium acetate, pH 6.8 85/15 (v/v). With increasing buffer concentration the retention of pteridines slightly increased, which can be related to hydrophilic partitioning [55]. Presumably, high concentrations of organic solvent in the mobile phase cause salt partition preferentially into the water-rich layer on the stationary phase. The presence of solvated ions in this water-rich layer would increase its volume, potentially leading to stronger retention of polar solutes [55]. The ZIC®-HILIC column demonstrated a great deal of salt concentration on retention of erythropterin, where an increasing salt concentration from 1 to 25 mM increased its retention from $k = 2.85$ to $k = 23.72$. It can also be related to the suppression of repulsive electrostatic interactions between the sulfonate groups of sulfobetaine ligands and carboxyl group of erythropterin.

The effect of buffer pH on the retention and separation of pteridines was tested in the mobile phase composed of ACN/5 mM ammonium acetate 85/15 (v/v) in the range of 4–6 and 4–8 for Atlantis HILIC Silica column and ZIC®-HILIC column, respectively. On Atlantis HILIC Silica column only slightly increasing retention of pteridines was observed with increasing buffer pH, probably due to attraction of cationic solutes to gradually dissociating silanol groups at higher pH. No influence of pH on pteridine retention has been observed on ZIC®-HILIC column containing permanent zwitterionic sulfobetaine ligands. Replacing ammonium acetate buffer with ammonium formate buffer caused a marked deterioration of peak shapes. Additions of acetic acid or formic acid as an aqeuous part of the mobile phase within the range of 0.001–0.1% caused a peak tailing of the pteridines on the Atlantis HILIC Silica column.

Based on these results, the ZIC®-HILIC column was chosen for the analysis of pteridines. The optimized composition of the mobile phase, with respect to yielding sufficient retention of polar analytes (retention factor > 1), acceptable resolution of pteridines ($R_S > 1$), satisfactory peak shapes ($0.9 < A_S < 1.2$) and a sufficient detection sensitivity for all the analytes, was found to be ACN/5 mM ammonium acetate, pH 6.80, 85/15 (v/v). Under these conditions pteridines eluted with the following retention factors: isoxanthopterin $k = 4.18$, biopterin $k = 4.57$, xanthopterin $k = 5.99$, leucopterin $k = 6.62$, erythropterin $k = 9.16$ and neopterin $k = 11.03$.

3.3. Quantitation

Two calibration approaches were used for determining pteridines in the red form of *G. lineatum*, namely calibration curve method and standard addition method. Results gained from both methods were statistically compared and the matrix effect was evaluated. Calibration curve method is most convenient when a large number of similar samples can be analyzed. The results of the weighted linear regression of dependences of peak area on concentrations are listed in Table 2. The high correlation coefficients indicate an excellent linearity. Testing at a significance level of $1-\alpha=0.95$ demonstrates that the computed intercepts are significantly different from zero. The peak height vs. concentration dependences were treated by linear regression, to determine the limits of detection and quantitation, as the three- and ten-times the noise level, respectively.

Standard addition method is useful when the analyte is present in a complex matrix and matrix-matched calibration samples are not available. This method was tested on the sample of the red form of *G. lineatum*.

Lord's test was used for testing the conformity of the two sets of analytical results. No statistically significant difference was observed among the results obtained by the calibration curve method and the standard addition method. The results showed that ion suppression or enhancement from sample matrix was negligible under the applied conditions. Therefore, only the calibration curve method was used for the determination of pteridines in real samples.

3.4. Selectivity and carry over effect

Selectivity was verified by inspecting chromatograms of real samples in MS scan mode. These chromatograms did not contain any interfering compound at the retention times of the studied pteridines. Sample carry-over was verified by visual inspection of the chromatograms of the injected blank. No carry-over effect was observed at concentrations of pteridines <1000 ng/mL. For standards of pteridines at concentration of 5000 ng/mL the carry-over was found at 5% but it was eliminated during the following blank injection. Therefore, a blank injection was inserted between each unknown sample in order to prevent the carry-over effect.

3.5. Precision and accuracy

Intra-day and inter-day accuracy and the precision of the developed method were assessed by determining standards at three concentration levels in five replicates. The accuracy was expressed as the relative error (RE) and the precision expressed by repeatability as the relative standard deviation (RSD). The data determined for the studied pteridines are summarized in Table 3. For the three concentrations of pteridines, the inter- and intra-day precisions (RSD%) ranged from 0.79 to 8.09%, and accuracy (RE%) was within ±9.63%. The results indicate that the newly developed method has an acceptable accuracy and precision for the analysis of pteridines.

3.6. Analysis of pteridines in G. lineatum and G. semipunctatum

The optimized LC–MS/MS method was used for determining the selected pteridines into four forms of *G. lineatum*, namely larvae, pale, red, and yellow adult forms; and orange adult form of *G. semipunctatum*. Dimethyl sulfoxide was used for the extraction of pteridines from the insect integuments. The extraction time was 96 h (for sample preparation see the Section 2). For illustration, the SRM chromatograms of the orange form of *G. semipunctatum* are given in Fig. 3. Obtained results of the analysis of pteridines in different forms of *G. lineatum* and *G. semipunctatum* are summarized in Fig. 4. It is evident that xanthopterin was not found in any studied form. Neopterin was found only in the yellow form at a concentration of 4 μg per 1 mg of integument. Isoxanthopterin was the only pteridine present in all forms of *G. lineatum* and *G. semipunctatum*. Based on the results, it is obvious that larvae of *G. lineatum* contains only a small amount of biopterin and isoxanthopterin, whereas the pale adult form contains mainly leucopterin and isoxanthopterin and erythropterin are minor. Red and yellow forms of *G. lineatum* as well as the orange form of *G. semipunctatum* contain different proportions of pteridines resulting in the characteristic coloration. The results show big differences in the distribution of individual pteridines and their relative amounts among the individual forms.

4. Conclusion

This work expands the application of HILIC separation combined with MS detection. A new, simple and sensitive method employing hydrophilic interaction liquid chromatography with tandem mass spectrometry was developed and validated for the identification and determination of six pteridines which can be present in insect integuments.

The study also contributed to a better understanding of HILIC separations because the retention of pteridines was investigated in detail on two HILIC columns and the effects of the mobile phase composition, acetonitrile/buffer ratio, buffer type, concentration and pH value were examined. The retention of pteridines was affected mainly by the acetonitrile content in the mobile phase. The obtained results indicated a multimodal retention mechanism on both tested columns. The ZIC®-HILIC column provided stronger retention of pteridines and higher selectivity and efficiency of separation than Atlantis HILIC silica.

Under optimized LC–MS/MS conditions, four forms of *G. lineatum* and one form of *G. semipunctatum* were analyzed. The results showed that different forms of *Graphosoma* species can be characterized by different distributions of individual pteridines. This fact affects the coloration of various forms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.05.004.

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3.6 Porovnání kapilární zónové elektroforézy a vysokoúčinné kapalinové chromatografie v analýze derivátů pterinu v kutikulách ploštic

Byly navrženy metody kapilární zónové elektroforézy [92] a vysokoúčinné kapalinové chromatografie pro analýzu derivátů pterinu v kutikulách ploštic. Optimalizované podmínky pro obě metody jsou uvedeny v sekci 3.5. Metody byly validovány a validační parametry jsou uvedeny v tabulce 1.

Tabulka 1 Validační parametry navržených metod (CZE-UV, HPLC-MS/MS) pro analýzu derivátů pterinu v kutikulách ploštic. Kde R^2 je koeficient determinace, RE relativní chyba [%], RSD relativní směrodatná odchylka [%], LOD limit detekce [$\mu g \cdot mL^{-1}$], LOQ limit kvantifikace [$\mu g \cdot mL^{-1}$] a *t* doba analýzy [min].

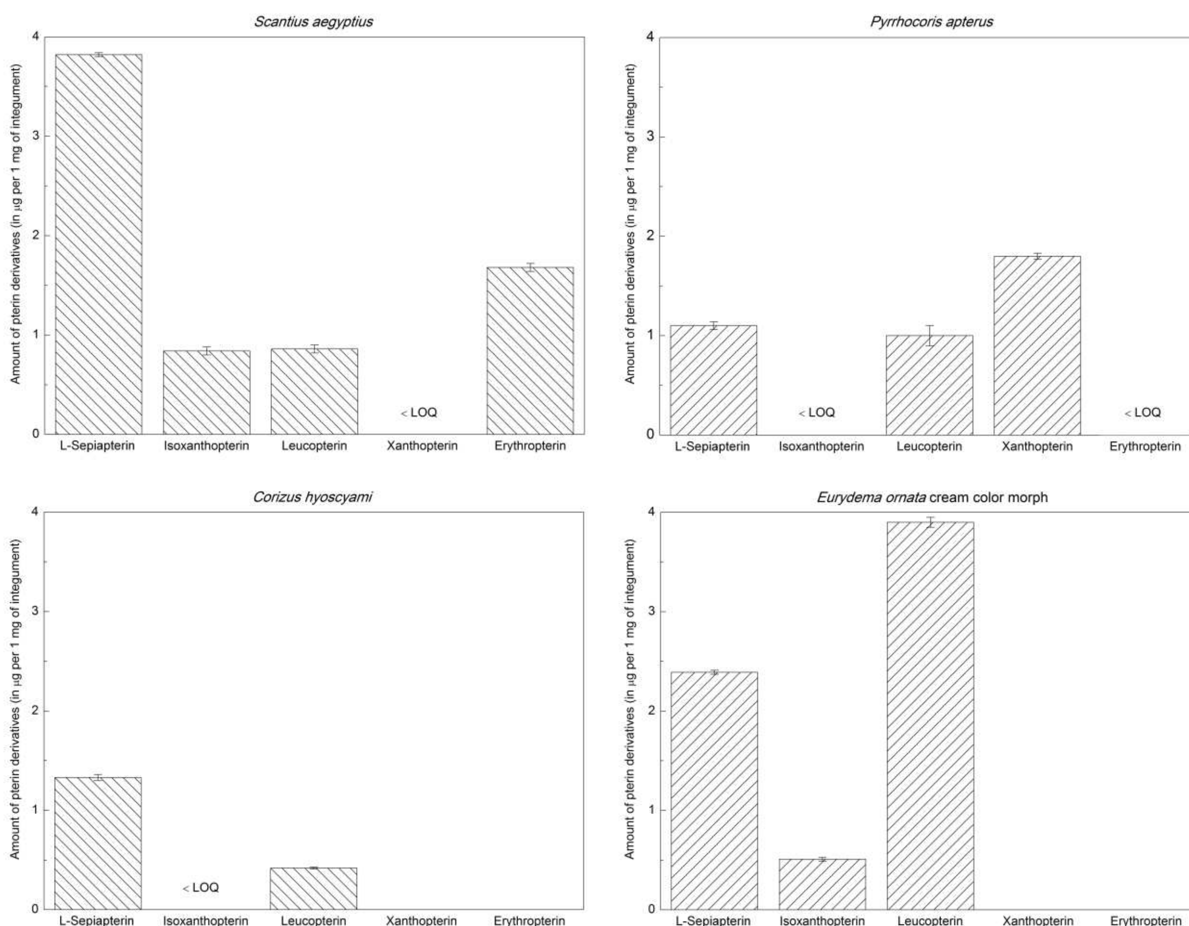
Validační parametr	CZE-UV	HPLC-MS/MS
R^2	≥ 0,9980	≥ 0,9975
správnost, RE	± 7,90	± 9,63
přesnost, RSD	≤ 6,65	≤ 8,09
LOD	0,04 – 0,99	$4 - 62 \cdot 10^{-4}$
LOQ	0,13 – 3,30	$3 - 193 \cdot 10^{-4}$
t	22	30

Z výše uvedeného lze konstatovat, že metoda CE-UV může kromě limitů detekce a kvantifikace poskytovat obdobné charakteristiky jako mnohem komplikovanější systém HPLC-MS/MS. Obě metody se však vzájemně doplňují, protože jsou založené na různém separačním mechanismu, a to je v případě identifikací výhodné.

Obě metody byly použity pro identifikaci a stanovení obsahu derivátů pterinu ve čtyřech druzích ploštic - *Eurydema Oleracea, Scantius Aegyptius, Pyrrhocoris Apterus* a *Corizus Hyoscyami*. Výsledky jsou uvedeny na obr. 3.6 a 3.7. V tabulce 2 jsou uvedeny retenční časy a SRM podmínky pro stanovení jednotlivých derivátů pterinu. Tato tabulka doplňuje publikaci III, protože HPLC-MS/MS byla následně rozšířena o separaci a kvantifikaci dalších 3 derivátů - L-sepiapterinu, pterinu a pterin-6-karboxylové kyseliny, které nebyly v uvedené publikaci sledovány.

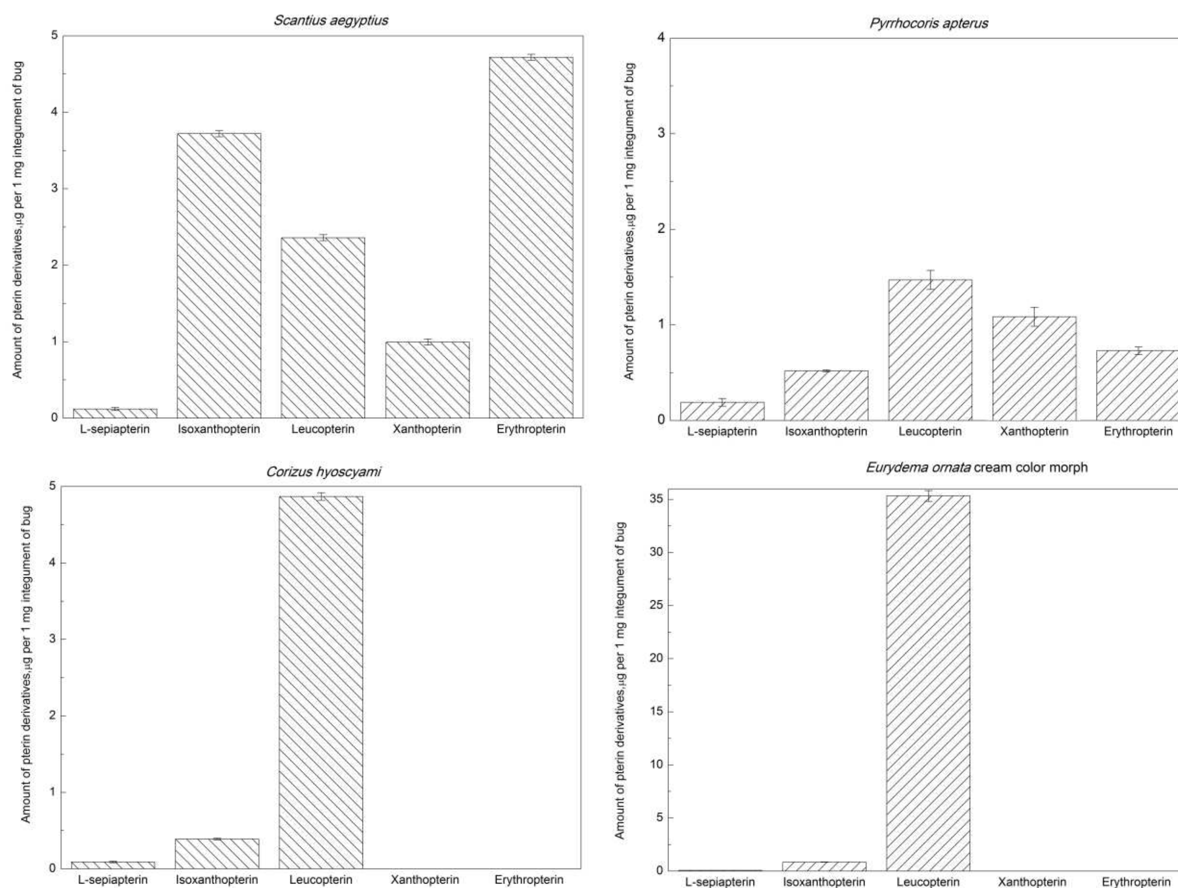
Tabulka 2 Retenční časy a SRM podmínky použité pro analýzu derivátů pterinu metodou LC-MS/MS (ESI, pozitivní mód). První přechod byl použit pro kvantifikaci a druhý pro identifikaci. Ostatní podmínky viz. publikace III.

Derivát	Retenční čas	Prekurzorový iont	Produktový iont	Fragmentor	Kolizní energie
	(min)			(V)	(V)
L-sepiapterin	7,14	238,1	220,1	120	15
Pterin	9,61	164,0	143,1	120	35
Isoxanthopterin	12,25	180,1	135,1	125	20
6-biopterin	12,44	238,1	178,1	115	17
Xanthopterin	14,45	180,1	135,1	125	20
Leukopterin	18,27	196,1	140,1	120	16
Erythropterin	23,60	266,1	220,1	110	8
D-neopterin	29,78	254,2	206,2	115	14
Pterin-6-karboxylová kys.	29,81	208,0	190,0	120	15



Obr. 3.6 Množství derivátu pterinů nalezených ve štítcích jednotlivých ploštic metodou kapilární zónové elektroforézy. Označení < LOQ znamená pouze přítomnost daného derivátu, jehož koncentrace je pod LOQ. Měřeno za optimalizovaných podmínek, uvedených v publikaci I.

Z obr. 3.6 je patrné, že ve štítcích všech ploštic byl identifikován/stanoven žlutý L-sepiapterin, isoxanthopterin a bílý leukopterin, avšak v různých vzájemných poměrech. Další dva barevné deriváty - xanthopterin a erythropterin - byly buď stanoveny, nebo pouze identifikovány v *Scantius aegyptius* a v *Pyrrhocoris apterus.* Stejné výsledky byly získány metodou vysokoúčinné kapalinové chromatografie s tandemovou hmotnostní detekcí (obr. 3.7).

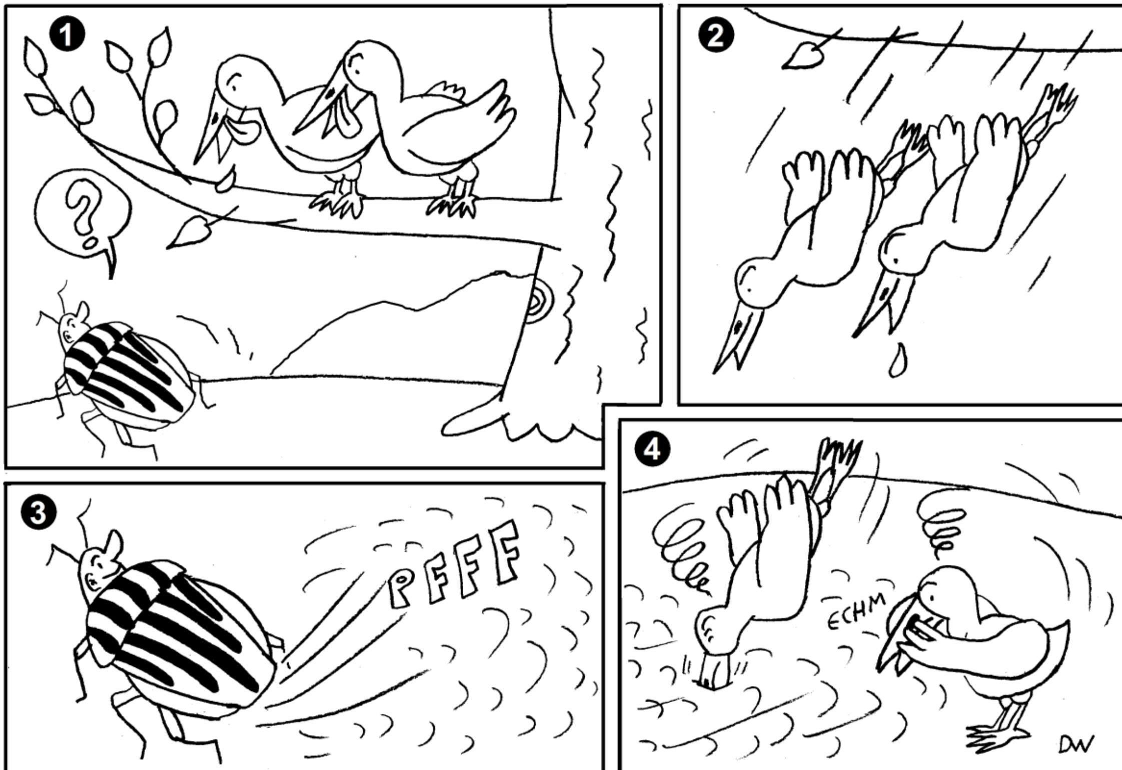


Obr. 3.7 Množství derivátů pterinů nalezených ve štítcích jednotlivých ploštic metodou vysokoúčinné kapalinové chromatografie s tandemovou hmotnostní detekcí. Měřeno za optimalizovaných podmínek, uvedených v publikaci III a sekci 3.6.

Oběma metodami se podařilo identifikovat v daných druzích stejné analyty. Z obou obrázků jsou patrné pouze kvantifikační rozdíly, které jsou však na úrovni jednotek µg daného pterinu, vztaženého na 1 mg kutikuly. Výjimku tvoří pouze leukopterin, který byl nalezen v černo-krémové kutikule ploštice *Eurydema ornata* cream color morph v řádech desítek µg pterinu, vztaženého na 1 mg kutikuly.

Za barevnou rozdílnost kutikul jednotlivých druhů pravděpodobně může rozdílný poměr derivátů pterinu. V současnosti se však ukazuje, že také antrachinony a karotenoidy mohou přispívat k zabarvení kutikul. Tyto látky a jejich potencionální přítomnost v kutikulách budou předmětem dalšího studia v navazujícím grantovém projektu GA UK č. 760216, Využití multidimenzionálních separačních technik pro studium obranného mechanismu ploštic.

Obranné sekrety ploštic



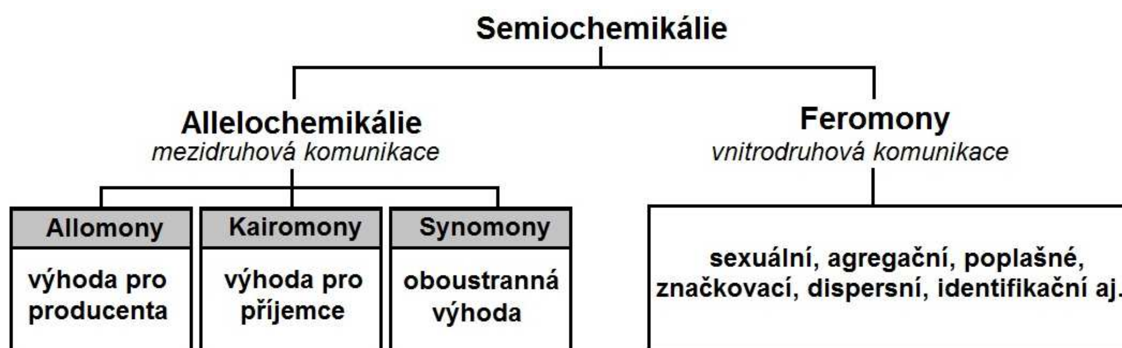
4 Těkavé obranné látky ploštic

4.1 Chemická komunikace

Chemická komunikace u hmyzu je evolučně nejstarší a nejrozšířenější způsob komunikace a je zásadní pro většinu živočichů. Chemická signalizace je výhodná díky své všestrannosti a velké citlivosti. Signál setrvává delší dobu v prostředí a může beze změny překonávat prostorové bariéry. Cenou za časté používání může být „rozšifrování“ signálu predátorem a jeho využití k nalákání kořisti.

Chování živočichů je do značné míry ovlivňováno nebo řízeno stopovým množstvím nízkomolekulárních organických látek a tyto sloučeniny se mohou podílet jak v regulaci vnitrodruhového, tak i mezidruhového chování.

Látky používané živočichy k chemickému přenosu informací se nazývají semiochemikálie (*semion* = signál). Dělení semiochemikálií je uvedeno na obr. 4.1.



Obr. 4.1 Dělení semiochemikálií.

Semiochemikálie lze rozdělit na allelochemikálie a feromony. Allelochemikálie lze dále rozdělit na několik podskupin. *Allomony* produkují chemický signál výhodný pro producenta zprávy. Příkladem může být pavouk *Mastophora cornigera*, který produkuje směsi aldehydů, které slouží jako sexuální feromony u cca 15 druhů můr, které tímto láká a loví. *Kairomony*, u nichž je chemický signál výhodný pro příjemce zprávy. Například brouk pestrokrovečník je přilákán ke své kořisti (kůrovcům) jejími feromony. Poslední skupinou jsou *Synomony*, látky jejichž produkce poskytuje oboustrannou výhodu; např. soužití jedovatých dravých mořských sasanek s korálovými rybami [94].

4.2 Chemická komunikace u ploštic

Ploštice (Hemiptera: Heteroptera) jsou velkou, rozšířenou a kosmopolitní skupinou savého hmyzu. V současné době je více než 50 000 popsaných druhů, které jsou rozděleny do 95 rodin [10, 95]. Ploštice lze nalézt na celém světě a na různorodých stanovištích od suchozemských po druhy vyskytující se v mořích [95]. Spousta druhů se živí vysáváním rostlinných šťáv a z celé hmyzí říše se řadí na čtvrté místo nejvíce devastujících druhů zemědělských plodin [96]. Některé druhy jsou masožravé, krmící se členovci, šneky nebo i malými rybami. Několik druhů funguje jako biologické regulátory škůdců. Pár druhů se živí krví obratlovců a mohou být přenašeči lidských nemocí [95].

Ploštice lze charakterizovat jako druh s velmi dobře vyvinutými pachovými žlázami. Antipredační chemické látky ploštic jsou většinou těkavé a silně pro člověka zapáchající. Ovšem existují druhy, které voní např. po třešních, vanilce, skořici nebo růžích [97, 98].

Sekrety jsou produkovány z rozdílných typů žláz závisících na vývojovém stupni jedince. Tyto látky slouží k odrazení suchozemských obratlovců od útoku a mohou být toxické i pro samotné ploštice. Antipredační infochemikálie jsou obvykle produkty sekundárního metabolismu a u fytofágních druhů (býložravé druhy) mohou být převzaty z hostitelských rostlin [91, 99-104].

4.3 Složení sekretů

Larvy typicky produkují směsi nenasycených 4-oxo-(*E*)-2-alkenů, (tyto látky byly zatím nalezeny pouze u ploštic) a (*E*)-2-alkenů (C_6 –C_{10}). Naproti tomu obranné sekrety dospělců obsahují směsi např. alkoholů a aldehydů (C_4 – C_{10}), nerozvětvených alkanů (zejména tridekan), ketonů, kyselin s krátkými řetězci (rozvětvenými nebo nenasycenými) a jejich esterů. Dospělci některých druhů také produkují 4-oxo-(*E*)-2-alkeny. Dalšími složkami mohou být terpenoidy jako např. α- a β-pinen, limonen nebo linalool, aromatické sloučeniny jako např. benzylalkohol, benzaldehyd, *p*-hydroxybenzaldehyd, fenylethanol, guajakol aj. [95, 100-103, 105-123]. Složení sekretů může záviset na fyzickém stavu jedince, potravě, vývojovém stádiu, ročním období nebo také na rostlině, kterou se živí v případě fytofágních druhů [124, 125].

4.4 Metody analýzy

Pro odběr těkavých sekretů hmyzu bylo navrženo několik postupů, z nichž většina je založena na usmrcení jedinců. Jedním z nich je jejich oplach dichlormethanem, který vede ke smrti jedince v řádu sekund [126]. Nevýhodou této metody je, že dochází k velkému naředění vzorku a do rozpouštědla se mohou extrahovat i látky, nepocházející ze sekretu, ale např. z povrchu těla hmyzu. Další možností je uspání jedince, nejčastěji pomocí CO_2,ethylacetátem nebo usmrcení mrazem s následným vypreparováním žlázy extrahované methyl-*tert*-butyletherem, hexanem nebo dichlormethanem [100, 109, 110, 127-132], dále taképropíchnutím žlázy skleněnou kapilárou a odsáním jejího obsahu [133]. Hlavní nevýhodou těchto postupů je velká pracnost a časová náročnost, protože je třeba vypreparovat velké množství žláz (Farine a kol. používali až 2000 jedinců [126]). Neletální způsoby odběru se prováděly foukáním vzduchu přes nádobu s plošticemi a následným zachycením látek na různých typech adsorbentů, např. Super Q, aktivní uhlí nebo Porapaq Q [112, 134-136], sorpcí na vlákně (SPME) [137-139] nebo vložením papírového ubrousku mezi ploštice a jeho následnou extrakcí dichlormethanem [140]. Tyto postupy však nezahrnují prakticky žádné dráždění či obranný prvek. Relativně nejnovějším způsobem je teplotně-desorpční systém, který byl využit pro analýzu feromonů [141, 142].

Pro analýzu obranných sekretů byla využita plynová chromatografie ve spojení s plamenově ionizačním, hmotnostně spektrometrickým nebo elektroantenografickým detektorem [110, 113, 125, 128, 139, 143-150]. Protože sekrety obsahují velké množství látek, může být před plynovou chromatografii předřazena vysokoúčinná kapalinová chromatografie pro předběžnou separaci [151], ale v poslední době se začíná využívat i komprehenzivní dvoudimenzionální plynová chromatografie (GCxGC) [129, 152]. Pro identifikaci látek se kromě porovnávání s databází hmotnostních spekter také využívá nukleární magnetická rezonance [118, 135, 153].

4.5 Faktorové plány – RSM

Klasické pojetí optimalizace (one factor at a time, OFAT nebo one variable at a time, OVAT) využívá jen jednu veličinu jako proměnný parametr a ostatní parametry se při měření nemění. V případě, kdy je potřeba prozkoumat několik optimalizovaných parametrů se stává tento přístup velice zdlouhavým a nákladným procesem.

Proto se začaly používat nové přístupy označované jako RSM (response surface methodology), které jsou založeny na faktorových plánech. Ty minimalizují tyto náklady snížením množství pokusů na takovou míru, aby bylo možné dosáhnout nebo se alespoň přiblížit optimálnímu bodu (nastavení) bez nutnosti hledání a proměřování všech variant zkoumaných parametrů. Pomocí statistických programů je možné zjistit polynomickou rovnici, která popisuje chování souboru experimentálních dat [154-156].

Proceduru RSM lze rozdělit do čtyř hlavních kroků: (a) výběr experimentálních parametrů a definici odezvy systému (hledaná odpověď), (b) screening pro nalezení parametrů (např. teplota, objem, rychlost), které mají statisticky významný vliv na studovanou odpověď (např. plocha píku), (c) modelování a nalezení matematického popisu systému pomocí rovnice 1 jako funkce (většinou polynom) statisticky významného ukazatele, (d) optimalizace a určení takové kombinace faktorů, která poskytuje optimum [154, 155].

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \qquad (1)$$

Kde y je odpověď, β_0 konstanta, β_i lineární koeficient, β_ii kvadratický koeficient, β_ij interakční koeficient, x_i a x_j úrovně jednotlivých faktorů [154, 155].

4.6 Analýza těkavých obranných sekretů – publikace IV

Pro analýzu těkavých obranných sekretů ploštic byla využita plynová chromatografie spojená s hmotnostně spektrometrickým detektorem. Pro odběr těkavých obranných sekretů byly navrženy a optimalizovány tři způsoby mechanického dráždění ploštic – pomocí ultrazvuku, třepačky a stlačování ploštic pístem injekční stříkačky. Nakoncentrování sekretů bylo prováděno pomocí sorpce na vlákně SPME a v rámci optimalizační procedury byla studována tři vlákna s různou polaritou – polyakrylát (PA), polydimethylsiloxan (PDMS) a kompozitní vlákno skládající se z carboxenu (CAR), polydimethylsiloxanu (PDMS) a divinylbenzenu (DVB); (CAR/PDMS/DVB). Výhodou posledního vlákna je, že dovoluje sorbovat látky z širokého rozmezí polarity. Vlákno obsahuje tři fáze: CAR pro adsorpci polárních látek a velmi těkavých látek, PDMS pro adsorpci nepolárních látek a DVB pro sorpci středně polárních a aromatických sloučenin. Pro analýzy byla využita moderní kapilární GC

kolona obsahující trifluoropropylmethyl polysiloxanovou stacionární fázi, která taktéž dovoluje separovat látky z velmi širokého rozmezí polarity.

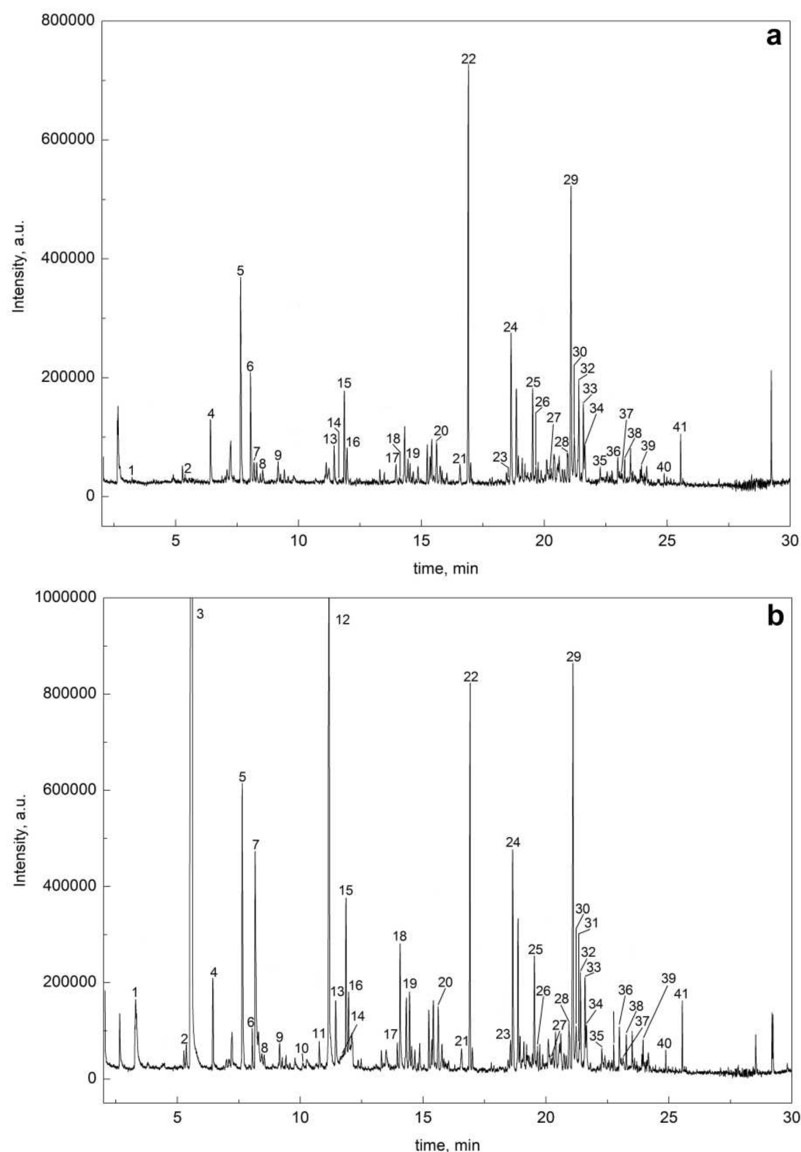
Pro navržené způsoby odběrů těkavých sekretů ploštic byly studovány různé parametry např. typ vlákna, teplota dráždění, teplota při sorpci na vlákně SPME, čas sorpce na vlákno SPME aj. Z důvodu většího množství zkoumaných parametrů byla provedena multiparametrická optimalizace s využitím programu Minitab 16.

Pro optimalizaci navržených metod byla vybrána ploštice *Pyrrrhocoris apterus* (Hemiptera: Heteroptera: Pyrrhocoridae). Tato ploštice je běžným euroasijským druhem, jednoduše dostupná ve velkém množství a její sekrece je velmi dobře prostudována. Tento druh je také charakteristický nízkým počtem obranných látek s jejich nízkou koncentrací a obtížným uvolněním. Z tohoto důvodu se tato ploštice považuje za relativně náročnou k analýzám její obranné sekrece.

V rámci optimalizace byly sledovány dva parametry, a to celkový počet píků a jejich celková suma ploch. Požadavkem na navrženou metodu bylo, aby generovala co největší množství píků a sumu jejich ploch. Na základě této podmínky byla pro další studium vybrána metoda založená na stlačování ploštic pístem injekční stříkačky.

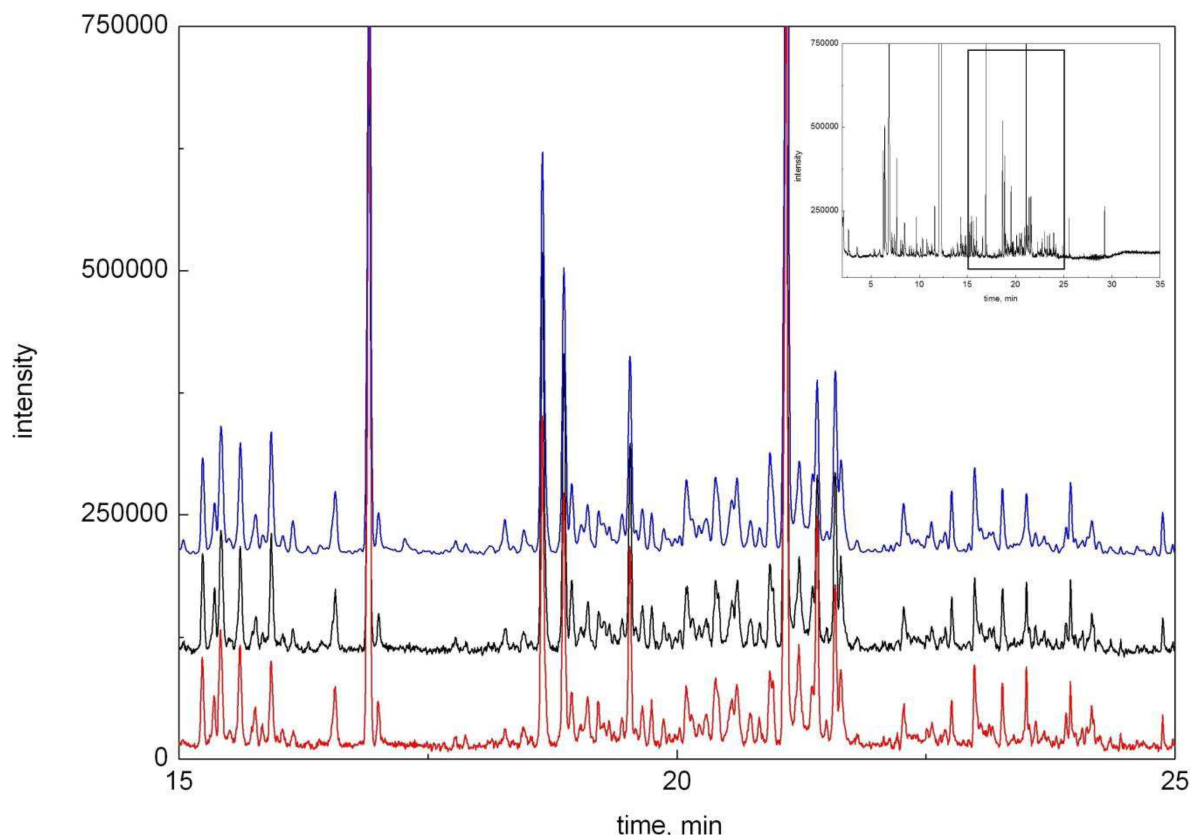
Optimalizované podmínky odběrů sekretů byly: kompozitní vlákno CAR/PDMS/DVB, temperace tří ploštic v injekční stříkačce při 40 °C po dobu 1 minuty, teplota při sorpci na vlákně SPME 40 °C, doba sorpce na vlákně SPME 90 minut.

Nově navržená metoda SPME-GC-MS byla využita pro analýzu sekretů samců a samic dvou evropských druhů – *Pyrrhocoris apterus* a *Scantius aegyptius* a jednoho čínského druhu – *Pyrrhocoris tibialis*. Příklady chromatogramů získaných analýzou sekrece samců a samic produkovaných plošticí *Pyrrhocoris tibialis* jsou uvedeny v obr. 4.2. Obr. 4.3 dokumentuje velmi dobrou opakovatelnost analýz získaných nově vyvinutou metodou SPME-GC-MS.



Obr. 4.2 SPME-GC-MS analýza *Pyrrhocoris tibialis*. Sekrece (**a**) samců a (**b**) samic ploštice *Pyrrhocoris tibialis*. Odběr vzorku sekretů metodou založenou na stlačování ploštic pístem injekční stříkačky; SPME sorpce na vlákně CAR/PDMS/DVB. Pík [1] oktan-1,3-dien; [2] hexenal; [3] (*E*)-2-hexen-1-ol; [4] 3-methylbutanová kyselina; [5] limonen; [6] 1-decyn; [7] (*E*)-2-hexenal; [8] *p*-cymene; [9] undekan; [10] (*E,E*)-2,4-hexadienal; [11] (*E*)-2-hexen-1-ol acetát; [12] (*E*)-2-okten-1-ol; [13] 2,6-dimethyl-7-okten-2-ol; [14] 6-dodeken; [15] dodekan; [16] 1-dodeken; [17] nonanal; [18] (*E*)-2-oktenal; [19] tridekan; [20] 2-isopropyl-5-methyl-1-heptanol; [21] dekanal; [22] tetradekan; [23] 2-ethylcyclohexanon; [24] 4-*tert*-butylcyclohexyl acetát; [25] pentadekan; [26] 4-*tert*-butylcyclohexyl acetát; [27] 2-methyl-1-undekanol; [28] allyl

cyclohexanepropionát; [29] 1-dodekanol; [30] 3,5-Bis(1,1-dimethylethyl)fenol; [31] tetradekanal; [32] hexadekan; [33] indan-1,3-diol monoacetát; [34] verdyl acetát; [35] 2-ethyl-1-dodekanol; [36] 1-tridekanol; [37] 1-(4-*tert*-butylfenyl)propan-2-on; [38] 1-ethoxynaftalen; [39] dodecyl acetát; [40] hexyl salicylát a [41] *α*-hexylcinnamaldehyd.



Obr. 4.3 Opakovatelnost analýzy sekrece samců *Scantius aegyptius*. Každá analýza byla provedena s třemi novými jedinci za optimalizovaných podmínek popsaných v publikaci IV a sekci 4.6.

Identifikace byla provedena na základě porovnání získaných hmotnostních spekter s knihovnou hmotnostních spekter NIST 2008 a na základě porovnání retenčních časů příslušných standardů.

V sekreci *Pyrrhocoris apterus* bylo identifikováno 25 sloučenin: 6 uhlovodíků (hlavně dodekan a tridekan), 3 alkoholy, 7 aldehydů (zvláště (*E*)-2-hexenal, (*E,E*)-2,4-hexadienal, nonanal a (*E*)-2-oktenal), 5 esterů (hlavně methyl 2-hydroxy-3-methyl pentanoát) a 3 organické kyseliny.

Sekrece *Pyrrhocoris tibialis* obsahovala 11 uhlovodíků, 9 alkoholů (hlavně 1-dodekanol, (*E*)-2-hexen-1-ol a (*E*)-2-okten-1-ol), 8 aldehydů (zvláště (*E*)-2-oktenal a (*E*)-2-hexenal), 2 ketony a 8 esterů karboxylových kyselin. Sekrece také obsahovala limonen a malé množství 1-ethoxynaftalenu.

Složení sekrece *Scantius aegyptius* bylo podobné: 10 uhlovodíků, 10 alkoholů (hlavně 2-cyklohexen-1-ol, cyklopentanemethanol a 1-dodekanol), 3 aldehydy, 2 ketony a 11 esterů karboxylových kyselin. Sekrece také obsahovala limonen a 1-ethoxynaftalen. Sekrece *S. aegyptius* a *P. tibialis* je stejná ve 24 sloučeninách, zatímco sekrece *P. apterus* a *S. aegyptius* v 9 sloučeninách a *P. tibialis* a *P. apterus* ve 14 sloučeninách.

Výhodou navržené metody je, že se jedná o neinvazivní postup. Pro jednu analýzu stačí pouze 3 jedinci - Farin a kol. používali až 2000 jedinců, kteří byli pro získání sekretů usmrceni [126]. Lze analyzovat i vzácné druhy např. *Pyrrhocoris tibialis*, které nejsou běžně dostupné a je nutné pro další výzkum udržovat jejich chovy.

PUBLIKACE IV

Název	*Comparative analysis of volatile defensive secretions of three species of Pyrrhocoridae (Insecta: Heteroptera) by gas chromatography-mass spectrometric method*
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Comparative analysis of volatile defensive secretions of three species of Pyrrhocoridae (Insecta: Heteroptera) by gas chromatography-mass spectrometric method

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1 **Comparative analysis of volatile defensive secretions of three species of Pyrrhocoridae**
2 **(Insecta: Heteroptera) by gas chromatography-mass spectrometric method**
3
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Abstract

The true bugs (Hemiptera: Heteroptera) have evolved a system of well-developed scent glands that produce diverse and partly strongly smelling compounds that act mainly as chemical protection against predators. A new method of non-lethal sampling with subsequent separation using gas chromatography with mass spectrometric detection was proposed for analysis of these volatile defensive secretions. Separation was performed on Rtx-200 column containing fluorinated polysiloxane stationary phase. Various mechanical irritation methods (ultrasonics, shaking, pressing bugs with plunger of syringe) were tested for secretion sampling with a special focus on a non-invasive irritation. The preconcentration step was performed by sorption on solid phase microextraction (SPME) fibers with different polarity. For optimization of sampling procedure, *Pyrrhocoris apterus* was selected. The entire multi-parameter optimization procedure of secretion sampling was performed using response surface methodology. The irritation of bugs by pressing them with a plunger of syringe was shown to be the most suitable. The developed method was applied to analysis of secretions produced by adult males and females of *Pyrrhocoris apterus, Pyrrhocoris tibialis* and *Scantius aegyptius* (all Heteroptera: Pyrrhocoridae). The chemical composition of secretion, particularly that of alcohols, aldehydes and esters, is species-specific in all three pyrrhocorid species studied. The sexual dimorphism in occurrence of particular compounds is largely limited to alcohols in *P. tibialis* and suggests their epigamic intraspecific function. The similarities in composition of secretion rather reflect the relationship of species (*Pyrrhocoris apterus* and *P. tibialis*) than similarities in antipredatory color pattern (quasi-Batesian mimesi of *P. apterus* by *Scantius aegyptius*). The phenetic overall similarity of secretions shared by the bugs is linked with their antipredatory properties. The proposed method requires only a few individuals, and they remain alive. Thus secretions of a number of species including even the rare ones can be analyzed and broadly conceived comparative studies can be carried out.

51 # 1. Introduction

52 Chemical intraspecific and interspecific communication is essential for most animals, and the
53 low-molecular organic compounds participate in regulation of behavior, synchronization of
54 physiological and developmental activities, and act as secondary defenses against predators,
55 parasitoids and parasites. The organic compounds functioning in interorganismal
56 communication are called infochemicals [1]. These compounds also take part in aposematic
57 signaling and advertise the prey noxiousness to the potential predators by means of olfactory
58 or gustatory cues [2, 3]. These chemical warning signals are often combined with visual and
59 vibrational (acoustic) signals or cues in a multimodal antipredatory signaling system [4].

60 The true bugs (Hemiptera: Heteroptera) is a large, diversified and cosmopolitan group
61 of sucking insects including 95 families and over 50,000 described species [5]. They inhabit
62 all the major biomes, from terrestrial to fresh-water and marine, and include such well known
63 families as water scorpions, backswimmers, water striders, assassin bugs, plant bugs, bed
64 bugs and shield bugs. Many species are phytophagous and true bugs are ranked as the fourth
65 major insect group of agricultural pests [6]. Some species are carnivorous feeding on
66 arthropods, gastropods and even small aquatic vertebrates. The predatory true bugs may
67 function in regulation of arthropod pests of cultivated plants while the hematophagous species
68 may be vectors of human and animal diseases [7].

69 Nearly all true bugs (Insecta: Hemiptera: Heteroptera) are chemically defended against
70 predators and produce–defense infochemicals which are either autogenous (synthesized by
71 bugs themselves from precursors contained in food; [6]) or sequestered (received in food non-
72 metabolized and stored in special reservoirs; [7]). The glands producing defense secretion and
73 their reservoirs differ between the larvae (nymphs) and adults [8]. The larvae produce defense
74 secretion from larval dorso abdominal glands (DAGs). The adult Heteroptera have lost DAGs
75 or retained and transformed them to produce pheromones and allelochemicals, but not

76 infochemicals with defensive function. Nearly all adult Heteroptera produce defense secretion
77 from methathoracic glands (MTGs, scent glands). In some true bug taxa, there are additional
78 specialized prothoracic and mainly abdominal glands producing a variety of infochemicals,
79 some of purely defensive function [9-14]. The identity of the antipredatory chemicals and the
80 composition of secretion may be taxon-specific and sex-dependent [1]. The antipredatory
81 infochemicals of true bugs are mostly volatile and strongly odorous, often repellent for
82 vertebrate predators [15], and may be toxic for predatory arthropods and even for the
83 secreting bug itself [16]. Short-chain alcohols, aldehydes, oxo-aldehydes, ketones, esters,
84 alkanes, organic acids, monoterpenes and aromatic alcohol/aldehydes are typical compounds,
85 and the composition of the secretion may depend on the physiological, nutritional and
86 developmental state of the individual and the season [17].
87 Most adults terrestrial Heteroptera release their defensive secretions readily upon an
88 attack. However, some true bugs (e.g. the firebugs of the family Pyrrhocoridae) are reluctant
89 to release their secretion because of behavioral constraints, reduced metathoracic glands
90 (MTGs) or anatomical architecture of their secretion releasing MTGs system [18]. Even when
91 not released, the secretion may still be effective by making a predator sick after the bug
92 consumption and inducing thus the learned aversion [15, 19].
93 Several procedures have been proposed for sampling volatile secretions, most of them
94 require killing the insects. One of these procedures is rinsing the bug with dichloromethane,
95 which kills it in a few seconds [20]. This method has the disadvantage that the sample is
96 greatly diluted and substances that are not derived from the secretion but, e.g., from the
97 surface of the body, can also be extracted. Another method is anaesthetizing the bugs using
98 CO_2 or ethylacetate or killing them by freezing with subsequent dissection of the glands and
99 extraction of their content with methyl-*tert*-butylether, hexane or dichlormethane [11, 21-28],
100 or puncturing the gland with a glass capillary [29]. Such procedures are appropriate for

101 studies of large species (e.g. shield bugs, Pentatomidae) in which only a few individuals are
102 needed for dissection and extraction of sufficient quantities of secretion [14, 25, 30]. On the
103 other hand, the method is overly time-consuming in smaller species in which it is necessary
104 to dissect a large number of individuals (Farine et al. used up to 2000 specimens of
105 *Pyrrhocoris apterus* [16]). Non-lethal sampling methods include blowing air across a vessel
106 containing the bugs with subsequent collection of the substances on various types of
107 adsorbents, such as Super Q, active carbon or Porapaq Q [31-34], sorption on a fiber (SPME)
108 [35-37] or placing a paper napkin between the bugs and subsequent extraction of the napkin
109 with dichloromethane [38]. The relatively newest procedure involves a temperature-
110 desorption system that was used for analyzing pheromones [39, 40]. However, these
111 procedures do not include any irritation of the insects that would simulate a predator attack,
112 and assure the discharging of the defensive secretion.

113 Gas chromatography (GC) combined with flame ionization, mass spectrometric or
114 electro-antennographic detection was used for analysis of the secretions of true bugs [23, 25,
115 37, 41-48]. Mainly non-polar DB- or HB-5 capillary columns or parallel connected non-polar
116 DB-1 and polar DB-WAX capillary columns have been used [20, 28, 36, 39, 41]. Because the
117 secretions contain many different substances, preliminary separation can be carried out using
118 high performance liquid chromatography prior to gas chromatography [49], but recently
119 comprehensive two-dimensional gas chromatography (GCxGC) has begun to be used [24,
120 50]. Substances are identified by comparison with a mass spectrometric database and by
121 nuclear magnetic resonance [33].

122 Because of the great complexity of the secretion samples and the number of
123 parameters that can affect the sampling process, it can be beneficious to employ the process
124 optimization procedures that combine all the tested parameters together at the same time and
125 substantially reduce the number of analyses required. Response surface methodology (RSM)

126 is based on the fit of a polynomial equation to the multidimensional (multiparametric)
127 experimental data, which must describe the behavior of the data set with the objective of
128 making statistical prediction. RSM is a general approach for designing experiments; it reduces
129 efficiently the development time and costs of the method. The main advantage of RSM as
130 compared with the currently most widely used approach to optimization, namely one-factor-
131 at-a-time OFAT (one parameter is changed while the others are fixed during optimization) is
132 that only the most important experimental parameters are selected and optimized and that
133 more than one parameter are changed simultaneously in one experiment according to the
134 computer-designed plan of experiments [51-53].

135 In this study three species of the family Pyrrhocoridae, namely the Palaearctic
136 *Pyrrhocoris apterus* (L.), Oriental *P. tibialis* (Stål), and Mediterranean *Scantius aegyptius* (L.)
137 were chosen to analyze and compare their secretions for the following reasons. *Pyrrhocoris*
138 *apterus* and *P. tibialis* are congeneric, closely related but largely allopatric species [54]. They
139 have similar hostplants (various Malvaceae incl. *Tilia* spp.; in case of *P. apterus* also an
140 introduced locust tree, *Robinia pseudacacia*) but a different life-style, and both are reluctant
141 to discharge their defensive secretion upon an attack. *P. apterus* is gregarious, mostly
142 brachypterous, flight-less species, and possesses a warning coloration of conspicuous red-and-
143 black color pattern. *P. tibialis* is non-gregarious, readily flying, and rather cryptically colored
144 in shades of gray and brown. *Scantius aegyptius* is a macropterous (but flight-less), epigeic,
145 Mediterranean species occupying the southwestern part of the range of *P. apterus* and sharing
146 with the latter not only similar red-and-black warning coloration, but also the same habitat
147 and major hostplant (*Malva neglecta*; Malvaceae). Like the both *Pyrrhocoris* species, it is also
148 reluctant to discharge its defensive secretion.

149 This study was carried out to i) develop a non-invasive (non-lethal) method for
150 sampling the defensive secretion of true bugs using various kinds of mechanical irritation that

151 would reliably simulate the conditions of when the bug is attacked by a predator, and find a
152 method suitable also for the species that are reluctant to release their secretion; ii) select the
153 most advantageous sampling of the secretion using SPME fibers with various polarities for
154 subsequent GC-MS analysis; iii) optimize the sampling using the response surface
155 methodology; iv) employ the developed method for analysis and comparison of infochemicals
156 secreted by adults of both sexes in three species of Pyrrhocoridae - *P. apterus, P. tibialis* and
157 *S. aegyptius* - differing in their biology and antipredatory strategies. MTG secretion of *P.*
158 *apterus* has already been analyzed by Farine [20, 55] using dissected MTGs and their
159 reservoirs. Except for *P. apterus, Dysdercus intermedius* Distant, *D. superstitiosus* (F.), *D.*
160 *fasciatus* Signoret, and *D. cingulatus* (F.) defensive secretion of no other pyrrhocorid species
161 have been analyzed [20], and to our best knowledge such comparisons of secretion in closely
162 related species of Heteroptera are surprisingly rare.
163

164 # *2.* Material and Methods

165 ## *2.1. Chemicals*

166 A mixture of *n*-alkanes (C_8 – C_{20}) dissolved in hexane for retention index
167 determination was purchased from Fluka (Buchs, Switzerland). Reference compounds (+)-β-
168 citronellene (≥ 98.5 %), (*E*)-2-hexen-1-ol (> 96 %), (*E*)-2-octen-1-ol (> 97 %), 1-decyne (> 98
169 %), 1-dodecene (≥ 99 %), 1-tridecene (> 96 %), (*E*,*E*)-2,4-decadienal (≥ 97 %), (*E*,*E*)-2,4-
170 dodecadienal (> 90 %), (*E*,*E*)-2,4-hexadienal (≥ 95 %), (*E*,*E*)-2,4-nonadienal (> 89 %), (*E*,*E*)-
171 2,4-octadienal (≥ 95 %), 2-cyclohexen-1-ol (> 95 %), 2-decanol (> 98 %), (*E*)-2-decenal
172 (≥ 92 %), 2-heptanol (≥ 97 %), 2-heptanone (≥ 98 %), (*E*)-2-hexen-1-ol (≥ 95 %), (*E*)-2-
173 hexenal (≥ 99 %), 2-hexyl-1-decanol (> 97 %), (*E*)-2-nonenal (≥ 95 %), (*E*)-2-octenal
174 (≥ 95 %), (*E*)-2-undecenal (≥ 95 %), (*Z*)-3-hexenyl acetate (≥ 98 %), 4-*tert*-butylcyclohexyl
175 acetate (mixture of *E* and *Z*, > 98 %), acetoin (≥ 96 %), allyl cyclohexanepropionate (≥ 98 %),

176 benzaldehyde (≥ 99 %), citronellol (≥ 95 %), cyclohexyl acetate (≥ 98 %),
177 cyclopentanecarboxylic acid (≥ 99 %), cyclopentanemethanol (≥ 98 %), cyclopentanol
178 (≥ 99 %), cyclopentanone (≥ 99 %), decanal (≥ 98%), 1-dodecanol (≥ 98 %), dodecyl acetate
179 (> 97%), 1-hexadecanol (≥ 99 %), hexyl acetate (≥ 99 %), hexyl salicylate (≥ 99 %), isoamyl
180 acetate (≥ 97 %), limonene (> 97%), methyl decanoate (≥ 99 %), nonanal (≥ 97 %), octyl
181 acetate (≥ 99 %), *p*-cymene (> 99%), phenyl acetate (≥ 99 %), prenyl acetate (≥ 98 %), 1-
182 tetradecanol (≥ 97 %), 1-tridecanol (≥ 99 %), 1-undecanol (≥ 99 %), and α-
183 hexylcinnamaldehyde (≥ 95 %) were supplied by Sigma Aldrich (Munich, Germany).
184

185 *2.2. Sample preparation and bug samples*

186 The defense secretions of adults of three closely related true bug species (Hemiptera:
187 Heteroptera: Pyrrhocoridae, the firebugs), were studied: (1) *Pyrrhocoris apterus* (L., 1758)
188 from Prague, Czech Republic, a common Eurasian warningly colored species introduced to
189 other continents as well, and frequently used as a model in experimental studies [56], (2) *P.*
190 *tibialis* (Stål, 1874), from Tianjin, China, an East Palaearctic species, and (3) *Scantius*
191 *aegyptius* (L., 1758) from Greece (Kos Island and Crete), a Mediterranean species. The adults
192 were sexed, fed by seeds of their original hostplants (*Tilia cordata* in *P. apterus*, *Alcea rosea*
193 in *S. aegyptius*, and *Hibiscus rosa-sinensis* in *P. tibialis*), and maintained separately in plastic
194 containers at 25 ± 2 °C and photoperiod L16: D8. Three males and three females of both
195 species have been used for each sampling.

196 Three individuals were placed in 4 mL glass vial closed with a polypropylene stopper
197 (Supelco, Bellefonte, PA, USA) with a teflon septum (Supelco, Bellefonte, PA, USA), and
198 the vial was then placed in an ultrasonic bath (Elma, Singen, Germany) or shaker (Heidolph,
199 Schwabach, Germany). In the experiment employing irritation with a plunger, three
200 individuals were placed in a syringe with a volume of barrel 12.5 mL (Eppendorf, Hamburg,

201 Germany), which was placed in an incubated shaker for tempering the specimens (bioSan,
202 Riga, Lithuania). Following a certain tempering time at the given temperature, the bugs were
203 carefully compressed with the plunger of a syringe so that they could not move and the
204 syringe tip was then closed with a rubber stopper. The bugs were compressed with the plunger
205 until a thin liquid film appeared on their body. Then the rubber stopper was removed from the
206 syringe tip, 5 mL of air was drawn in and an SPME (solid phase microextraction) fiber was
207 immediately inserted into the tip of the barrel.

208 The following experimental conditions were studied for each sampling method: i)
209 irritation with ultrasonics: type of fiber, irritation temperature, intensity of the ultrasonics,
210 irritation time, SPME sampling time and temperature of SPME sorption; ii) for irritation in a
211 shaker: type of fiber, irritation temperature, rate of shaking, irritation time, SPME sampling
212 time and temperature of SPME sorption and iii) for irritation using compression in the barrel
213 of a syringe: type of fiber, temperature prior to compression, tempering time prior to
214 compression, time and temperature of SPME sorption.

215 The volatile compounds were extracted from the syringe barrel using a manual SPME
216 sampler with a selected fiber assembly (all Supelco, Bellefonte, PA, USA) coated with: i)
217 7 µm polydimethylsiloxane (PDMS), ii) 85 µm polyacrylate (PA) and iii) triple phase
218 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Each SPME
219 fiber was thoroughly conditioned for 15 min before and after each analysis in an external
220 syringe oven at 250 °C under vacuum. Before each analysis of defensive secretion, a control
221 analysis of SPME sampler itself and SPME sampling of to be used syringe (blank) was
222 performed. The insects were tempered during the sorption on SPME fiber for the increase
223 vapor pressure.

224

225 *2.3. Instrumentation, separation conditions and identification of the*
226 *volatile components*

227 The analyses were performed using a GCMS-QP2010 Plus instrument (Shimadzu,
228 Japan), equipped with 20 m × 0.15 mm i.d., 0.15 μm film thickness Rtx-200 column
229 (trifluoropropylmethyl polysiloxane stationary phase, Restek, USA). This stationary phase
230 was selected as a general purpose one with respect to its unique ability to separate substances
231 in a relatively broad polarity range. Helium (99.999%, Linde, Czech Republic) was used as
232 the carrier gas at a constant linear flow rate of 35 cm s^{-1}. Splitless-mode injection with SPME
233 liner at 250 °C was employed (split valve closed for 1 min). The oven temperature was
234 maintained at 35 °C for 3 min, ramped at 5 °C min^{-1} to 130 °C, then ramped at 20 °C min^{-1} to
235 300 °C and then maintained for 5 min (total run time, 35.50 min). The mass spectrometer was
236 operated in the scan mode (m/z 35 – 500). The ion source and interface temperatures were
237 200 and 250 °C, respectively.

238 The data were collected and evaluated using the GCMS solution (Shimadzu, Japan),
239 Origin 8 (Origin Lab corporation, Northampton, MA, USA), Microsoft Excel 2003 (Microsoft
240 Corporation, Redmond, DC, USA) and Minitab 16 (Minitab Inc., State College, PA, USA)
241 programs. Identification of the secretion components was made by comparing the obtained
242 spectra with those in the NIST 2008 Mass Spectra Library. Series of *n*-alkanes (C_8 – C_{20}) and
243 reference compounds (see Section 2.1) were analyzed under the same experimental conditions
244 as those used for the samples to either establish the retention indices or confirm the identity of
245 the analytes. The identity confirmation of the separated compounds was performed by
246 comparing the experimental retention times, retention indices and mass spectra of the
247 compounds with those of contemporaneously analyzed reference standards.

248

249 # 3. Results and Discussion

250 ## 3.1. *Selection of parameters and response definition*

251 *Pyrrhocoris apterus* was selected for the study of sampling procedure, because it is
252 easily available in large numbers [19] and its secretion has already been studied [20].
253 Moreover, *P. apterus* is characterized by a moderate chemical defense with a delayed effect
254 on predator and only releases its defense secretion when it is strongly irritated. [15, 19] This
255 species was used for optimization experiments focused on finding the most efficient approach
256 for extracting and analyzing defense secretion in the Pyrrhocoridae.

257 The studied parameters were selected with respect to several factors. One of the main
258 requirements on sampling of volatile secretions was that the bugs would not be killed and
259 destroyed, so the method would be suitable for a limited material of rare species. We found
260 that the critical lethal sampling temperature is 40 ^{o}C, because higher temperature is lethal for
261 the bugs. For SPME, the choice of the polarity of the sampling fiber is the most critical
262 condition for reliable and reproducible analysis of a real sample. As the detailed composition
263 of the secretions of the studied species of Pyrrhocoridae is unknown and can depend on many
264 factors (described in the introduction), three different SPME fibers with different polarities
265 were studied – polar PA, nonpolar PDMS fibers and composite bipolar DVB/CAR/PDMS
266 fiber covering a wide range of volatilities and polarities. This bipolar fiber consists of three
267 phases, graphitized carbon (Carboxen) for adsorption of the most volatile and polar
268 components, divinylbenzene-styrene copolymer (DVB) for sorption of medium-polarity and
269 aromatic compounds and finally polydimethylsiloxane (PDMS) for sorption of nonpolar
270 compounds. The type of fiber, irritation temperature and time, and the temperature of SPME
271 sorption constituted the basic set of parameters for each of the proposed irritation methods.

272 Ultrasonics was supplemented by the parameter of the ultrasonics intensity and the shaker by
273 the shaking rate parameter.

274 Selected parameters together with their tested levels (low, middle and high) are
275 summarized in Table 1. The individual parameters for sampling using a syringe are listed on
276 Table 2. For this sampling method the parameter of the type of fiber is omitted, because
277 optimization was performed for each fiber separately. Sets of measurements, combining the
278 factors and their levels are given in the Supporting Information (S1 – S3 Tables).

279

280 **Table 1. Selected Parameters and Their Tested Levels for Sampling Secretion Using**
281 **Ultrasonics and Shaker.**

Parameter	Level		
	Low	Middle	High
irritation time (min)	1	3	5
irritation temperature (°C)	25	32.5	40
intensity of the ultrasonics (%)	30	65	100
rate of shaking (rpm)	300	750	1200
temperature of SPME sorption (°C)	25	32.5	40
SPME sampling time (min)	30	60	90

282

283 **Table 2. Selected Parameters and Their Tested Levels for Sampling Secretion Using**
284 **Compression in the Plunger of a Syringe.**

Parameter	Level		
	Low	Middle	High
temperature prior to compression (°C)	25	32.5	40
tempering time prior to compression (min)	1	3	5
temperature of SPME sorption (°C)	25	32.5	40
time of SPME sorption (min)	30	60	90

285

286 In addition to developing a non-lethal method of sampling volatile secretions, we also
287 attempted to obtain the largest possible amount of secretions. The following responses were

288 chosen: the number of peaks with an area of at least 20,000 arbitrary units (a.u.) and the sum

289 of their areas.

290

291 ## 3.2. *Optimization procedure*

292 The secretion sampling method was optimized by a Minitab 16, and Tables 3 and 4

293 provide the obtained optimum parameters.

294

295 **Table 3. Optimum Conditions for the Method of Sampling Secretions Using Ultrasonics**

296 **and a Shaker.**

Parameter	Ultrasonics	Shaker
type of fiber	DVB/CAR/PDMS	DVB/CAR/PDMS
irritation time (min)	1	5
irritation temperature (°C)	25	25
temperature of SPME sorption (°C)	40	40
SPME sampling time (min)	90	30
intensity of the ultrasonics (%)	100	–
rate of shaking (rpm)	–	300
number of peaks	23	15
sum of absolute peaks areas	49 660 000	13 770 000

297

298 **Table 4. Optimum Conditions for the Method of Sampling Secretions Using**

299 **Compression in the Plunger of a Syringe.**

type of fiber	PDMS	PA	**DVB/CAR/PDMS**
temperature prior to compression (°C)	25	40	**40**
tempering time prior to compression (min)	1	5	**1**
temperature of SPME sorption (°C)	25	40	**40**
time of SPME sorption (min)	30	90	**90**
number of peaks	10	8	**59**
sum of absolute peaks areas	1 092 000	7 079 000	**35 620 000**

300

301 It can be seen from Table 3 that, on the basis of the required responses (maximum

302 number of peaks, maximum sum of the areas of all the peaks), the DVB/CAR/PDMS fiber

303 seemed to be the best of the fibres studied. Irritation by ultrasonics was more effective than
304 irritation in a shaker, because it generated a greater number of peaks and maximum sum of the
305 areas of all the peaks. When the secretion was sampled using compression of the bugs by the
306 plunger of a syringe (see Tab. 4), polar (PA) and nonpolar (PDMS) fibers exhibited much
307 lower responses and again unambiguously the best results were obtained with
308 DVB/CAR/PDMS fibers. The advantageousness of irritation by ultrasonics and compression
309 by the plunger can be seen from comparison of the required responses obtained in all the
310 tested systems. Irritation by ultrasonics generates a smaller number of peaks than compression
311 with a plunger, but the overall response is somewhat higher than when using a plunger. The
312 sampling method employing compression with the plunger of a syringe combined with
313 DVB/CAR/PDMS was chosen as the most advantageous, generating the greatest number of
314 peaks and probably simulating best the real danger to the bug when being seized by a large
315 insectivorous predator, and when releasing its full set of antipredatory chemicals.
316 For the responses "maximum number of peaks" and "maximum sum of their areas"
317 the coefficients of determination (R^2) were 0.9710 and 0.8720, respectively. These results
318 indicate very good agreement between the experimental data and the built up model.
319 Examples of two response surfaces of plots for this sampling method are given in
320 Fig. 1, depicting the interrelationships of selected parameters with the response. It can be seen
321 from the two graphs that increasing time of SPME sorption are accompanied by an increase in
322 the areas of all the peaks and also in their number. The increasing temperature of SPME
323 sorption generates decreasing sum of peaks areas but increasing number of peaks which is
324 more important for qualitative analysis.
325
326
327

Fig 1. The response surface plot.

The plot depicts the dependence of the sum of all the peaks (upper) (a.u., arbitrary units) and the number of peaks (bottom) on the temperature of SPME sorption and time of SPME sorption; irritation by compressing the true bugs with the plunger of a syringe; SPME sorption on a DVB/CAR/PDMS fiber; tempering time prior to compression 1 min; temperature prior to compression 40 °C.

3.3. Analysis of defensive secretions in adults of Pyrrhocoris apterus, Pyrrhocoris tibialis and Scantius aegyptius by GC-MS

Under the optimized conditions defensive secretions of all the three studied species were analyzed. Sampling was performed under the conditions listed in Table 4 (bold column) by sorption on a DVB/CAR/PDMS fiber after compressing the bugs with the plunger of a syringe. The actual GC analysis was performed under the conditions described in Section 2.3. The bugs were sexed and the identical experiments were carried out separately for males and females. Identifications of the individual peaks obtained for males and females of all three studied species are summarized in Table 5, and only peaks with response (A) greater than 20,000 a.u. and similarity (match factor in NIST 08 data base) higher than 85 % were selected for the identification.

 Table 5. Compounds Identified in the Defensive Secretions of Males and Females of *P. apterus, P. tibialis* and *S. aegyptius*.

		Pyrrhocoris apterus		*Pyrrhocoris tibialis*		*Scantius aegyptius*		
Compound	t_{ret} (min)	Male	Female	Male	Female	Male	Female	Identification
Hydrocarbons								
octan-1,3-diene	3.31	×	×	✓(0.03)	✓(2.80)	×	×	B
3,7-dimethyl-1,6-octadiene	5.38	×	×	×	×	✓(0.05)	✓(0.13)	B
1-decyne	8.05	×	×	✓(3.39)	✓(0.48)	✓(0.11)	✓(0.29)	A, B
p-cymene	8.54	×	×	✓(0.41)	✓(0.24)	✓(0.08)	✓(0.13)	A, B
undecane	9.16	✓(0.06)	✓(0.03)	✓(0.76)	✓(0.37)	✓(0.06)	✓(0.03)	A, B
6-dodecene	11.79	×	×	✓(0.13)	✓(0.18)	×	×	B
dodecane	11.86	✓(1.70)	✓(0.65)	✓(2.56)	✓(2.80)	✓(2.13)	✓(1.21)	A, B
1-dodecene	11.97	×	×	✓(1.60)	✓(1.13)	✓(0.96)	✓(0.47)	A, B
tridecane	14.45	✓(8.70)	✓(4.13)	✓(0.73)	✓(0.98)	✓(0.12)	✓(0.44)	A, B
tetradecane	16.91	✓(0.63)	✓(0.27)	✓(11.85)	✓(4.52)	✓(1.23)	✓(3.71)	A, B
pentadecane	19.52	✓(0.66)	✓(0.71)	✓(2.62)	✓(1.36)	✓(0.36)	✓(1.21)	A, B
hexadecane	21.41	✓(0.90)	✓(0.85)	✓(3.73)	✓(1.25)	✓(0.32)	✓(1.21)	A, B
Alcohols								
cyclopentanol	3.55	×	×	×	×	✓(0.06)	✓(0.15)	A, B
2,3-butanediol	5.02	✓(0.64)	✓(1.79)	×	×	×	×	B
(*E*)-2-hexen-1-ol	5.59	×	×	×	✓(25.46)	×	×	A, B
cyclopentanemethanol	6.42	×	×	×	×	✓(2.54)	✓(2.19)	A, B
2-cyclohexen-1-ol	6.88	×	×	×	×	✓(2.91)	✓(6.20)	A, B
2-propyl-1-pentanol	9.70	×	✓(0.79)	×	×	×	×	B
(*E*)-2-octen-1-ol	11.16	×	×	×	✓(8.29)	×	×	A, B
2,6-dimethyl-7-octen-2-ol	11.60	×	×	✓(1.17)	✓(0.89)	✓(0.31)	✓(0.16)	B
2-methoxyphenol	13.52	×	×	×	×	×	✓(0.25)	B
Isotridecanol	15.42	×	×	×	×	✓(0.27)	✓(0.20)	B
2-isopropyl-5-methyl-1-heptanol	15.61	×	×	✓(1.46)	✓(0.86)	×	×	B
2-methyl-1-undecanol	20.22	×	×	✓(0.22)	✓(0.16)	×	×	B
3,7,11-trimethyl-1-dodecanol	20.74	×	×	×	×	✓(0.08)	✓(0.06)	B
1-dodecanol	21.10	✓(0.11)	✓(0.07)	✓(9.32)	✓(5.99)	✓(1.86)	✓(1.10)	A, B
3,5-Bis(1,1-dimethylethyl)phenol	21.22	×	×	✓(2.12)	✓(0.96)	✓(0.28)	✓(0.22)	B
2-ethyl-1-dodecanol	22.27	×	×	✓(0.32)	✓(0.30)	×	×	B
1-tridecanol	22.99	×	×	✓(0.75)	✓(0.56)	✓(0.16)	✓(0.07)	A, B

Aldehydes								
(*Z*)-3-hexenal	5.23	✓(0.16)	✓(0.55)	✗	✗	✗	✗	B
hexanal	5.56	✓(0.06)	✓(0.08)	✓(0.08)	✓(0.27)	✗	✗	B
(*E*)-2-hexenal	8.17	✓(24.36)	✓(9.36)	✓(0.72)	✓(3.86)	✗	✗	A, B
(*E*,*E*)-2,4-hexadienal	10.11	✓(1.25)	✓(0.43)	✗	✓(0.13)	✗	✗	A, B
nonanal	13.96	✓(1.69)	✓(0.37)	✓(0.58)	✓(0.29)	✓(0.07)	✓(0.08)	A, B
(*E*)-2-octenal	14.06	✓(1.83)	✓(0.47)	✓(0.77)	✓(1.80)	✗	✗	A, B
decanal	16.57	✓(0.57)	✓(0.18)	✓(0.59)	✓(0.34)	✓(0.13)	✓(0.09)	A, B
tetradecanal	21.36	✗	✗	✗	✓(0.45)	✗	✗	B
α-hexylcinnamaldehyde	25.55	✗	✗	✓(0.71)	✓(0.44)	✓(0.13)	✓(0.05)	A, B
Ketones								
cyclopentanone	6.27	✗	✗	✗	✗	✓(0.80)	✓(0.03)	A, B
2-methyl-2-cyclopenten-1-one	8.48	✗	✗	✗	✗	✓(0.14)	✓(0.38)	B
2-ethylcyclohexanone	18.55	✗	✗	✓(0.65)	✓(0.42)	✗	✗	B
1-(4-*tert*-butylphenyl)propan-2-one	23.13	✗	✗	✓(0.59)	✓(0.17)	✗	✗	B
Esters								
1-butanol-3-methyl acetate	7.18	✗	✗	✗	✗	✓(0.06)	✓(0.04)	B
2-buten-1-ol-3-methyl acetate	8.19	✗	✗	✗	✗	✓(0.03)	✓(0.03)	B
hexyl acetate	10.12	✗	✗	✗	✗	✓(0.13)	✓(0.18)	A, B
methyl 2-hydroxy-3-methyl pentanoate	10.25	✓(6.29)	✓(2.67)	✗	✗	✗	✗	B
(*E*)-2-hexen-1-ol acetate	10.79	✗	✗	✗	✓(0.30)	✓(0.14)	✓(0.12)	B
acetic acid, undec-2-enyl ester	15.92	✗	✗	✗	✗	✓(0.23)	✓(0.12)	B
octyl acetate	16.14	✗	✗	✗	✗	✓(0.05)	✓(0.06)	A, B
4-*tert*-butylcyclohexyl acetate [a]	18.64	✗	✗	✓(4.97)	✓(3.10)	✓(0.78)	✓(0.43)	A, B
4-*tert*-butylcyclohexyl acetate [a]	19.65	✗	✗	✓(0.66)	✓(0.30)	✓(0.09)	✓(0.05)	A, B
allyl cyclohexane propionate	20.93	✗	✗	✓(1.38)	✓(0.94)	✓(0.26)	✓(0.24)	A, B
indan-1,3-diol monoacetate	21.59	✗	✗	✓(2.35)	✓(1.28)	✗	✗	B
verdyl acetate	21.64	✗	✗	✓(1.29)	✓(0.72)	✗	✗	B
phenylethyl isovalerate	22.84	✓(0.28)	✓(0.06)	✗	✗	✗	✗	B
isopropyl laurate	23.89	✓(0.10)	✓(0.06)	✗	✗	✗	✗	B
dodecyl acetate	23.95	✗	✗	✓(0.36)	✓(0.28)	✓(0.09)	✓(0.05)	A, B
hexyl salicylate	24.87	✗	✗	✓(0.16)	✓(0.25)	✓(0.06)	✓(0.02)	A, B
methyl dihydrojasmonate	25.59	✓(0.08)	✓(0.44)	✗	✗	✗	✗	B
2-ethylhexyl salicylate	25.68	✓(0.07)	✓(0.54)	✗	✗	✗	✗	B

Others								
3-methylbutanoic acid	6.40	✓(0.24)	✓(0.15)	✓(2.10)	✓(2.97)	×	×	B
limonene	7.65	×	×	✓(6.24)	✓(3.46)	✓(0.73)	✓(0.15)	A, B
(*E*)-2-hexenoic acid	11.62	✓(0.12)	✓(0.15)	×	×	×	×	B
2-ethylhexanoic acid	13.28	✓(0.10)	×	×	×	×	×	B
1-ethoxynaphtalene	23.27	×	×	✓(0.66)	✓(0.34)	✓(0.09)	✓(0.05)	B
1-methoxyoctane	25.30	✓(0.98)	✓(2.27)	×	×	×	×	B

347 [a] *E* or *Z* isomer
348 × not found
349
350 t_{ret} is the retention time of the relevant substance, % areas of the relevant peaks are listed in the parenthesis. The methods used for the identification: A – retention time of the
351 relevant substance was compared with the reference compound; B – the mass spectrum of the relevant substance was compared with NIST 2008 mass spectra library

352 The chemical composition of secretions is unique in all the species studied. We can
353 scrutinize the similarities and differences from the viewpoints of two major hypotheses: (a)
354 *Pyrrhocoris apterus* and *P. tibialis* should be more similar to each other (at least in critical
355 traits) than any of them to *Scantius aegyptius* since they are more closely related as indicated
356 by their generic classification (*Pyrrhocoris* x *Scantius*) (b) *P. apterus* and *S. aegyptius* should
357 be more similar since they are Müllerian (or quasi-Batesian [57]) mimics sharing the same
358 conspicuous aposematic coloration and occurring together at the same localities, habitats and
359 hostplants. We may presume that also their other antipredatory mechanisms would be more
360 similar than those shared with *P. tibialis*.

361 It is important to remember that shared presence of compounds suggests functional
362 similarity while shared presence and shared absence may jointly indicate the number of
363 shared evolutionary steps and thus approach the cladogenetic relationship. However, we are
364 still within the realm of phenetic similarity. Our assessment is limited by absence of
365 phylogenetic hypothesis on cladogenesis (relationships) of the genera of Pyrrhocoridae and
366 species of *Pyrrhocoris* and *Scantius*, and on anagenesis of their character states (character
367 evolution). Consequently, we cannot polarize with certainty the characters and estimate
368 whether the presence or absence of a particular compound is plesiomorphic (ancestral) or
369 apomorphic (derived).

370 It can be seen in Table 5 that 25 compounds were identified in the defensive secretions
371 of *Pyrrhocoris apterus*, namely 6 hydrocarbons (mainly dodecane and tridecane), 3 alcohols,
372 7 aldehydes (especially (*E*)-2-hexenal, (*E*,*E*)-2,4-hexadienal, nonanal and (*E*)-2-octenal), 5
373 esters (mainly methyl 2-hydroxy-3-methyl pentanoate) and 3 organic acids. The most of
374 compounds were found in the both sexes, whereas higher relative peak areas were mostly
375 present in the males. In comparison with the results obtained by Farine et al. [20] the profiles
376 of the main compounds were similar and 10 from 35 compounds found by Farine matched

377 (especially (*E*)-2-octenal, (E)-2-hexenal, tridecane and methyl 2-hydroxy-3-methyl

378 pentanoate). The prevalent less polar or non-polar compounds identified in the secretions by

379 Farine may correspond to using non-polar solvent (pentane) for gland extraction. Moreover,

380 the samples extracted with pentane were concentrated under nitrogen flow and therefore,

381 some polar compounds could be lost.

382 Table 5 shows that the defensive secretions of *Pyrrhocoris tibialis* were found to

383 contain 11 hydrocarbons, 9 alcohols (mainly 1-dodecanol, (*E*)-2-hexen-1-ol and (*E*)-2-octen-

384 1-ol), 8 aldehydes (especially (*E*)-2-octenal and (*E*)-2-hexenal), 2 ketones and 8 esters of

385 carboxylic acids. The secretions also contained limonene, probably derived from the seeds

386 with which the insects were fed, and also 3-methylbutanoic acid and smaller amounts of 1-

387 ethoxynaphthalene.

388 Composition of defensive secretions of *Scantius aegyptius* contained 10 hydrocarbons,

389 10 alcohols (mainly 2-cyclohexen-1-ol, cyclopentanemethanol and 1-dodecanol), 3 aldehydes

390 (nonanal, decenal and α-hexylcinnamaldehyde), 2 ketones and 11 esters of carboxylic acid.

391 The secretions also contained limonene and smaller amounts of 1-ethoxynaphtalene.

392 The secretions of all the studied species also contained a large number of minority

393 branched hydrocarbons (especially methylated) which were not included in Table 5 because

394 the determination of methyl group position is difficult without reference standards. Fig. 2

395 demonstrates very good reproducibility of the analyses obtained by the newly developed

396 SPME-GC-MS method. For all measurements, relative standard deviations of retention times

397 and peak areas did not exceed 1.0 and 6.5 %, respectively.

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401 **Fig 2. Reproducibility of the analysis demonstrated on the part of chromatograms**
402 **recorded for the male secretions of *Scantius aegyptius*.** Each analysis was performed with
403 three new individuals under the optimized experimental conditions listed in Table 4 and
404 described in the Section 2.3. Insert: Whole chromatogram with tagging of displayed region.
405
406 Defensive secretions of *S. aegyptius* and *P. tibialis* match in 24 compounds, whereas
407 defensive secretions of *P. apterus* and *S. aegyptius* match in 9, and *P. apterus* and *P. tibialis*
408 in 14 compounds. Most of the compounds found in the secretions are highly volatile
409 substances with low molecular weights and we may assume that most of them have either
410 defensive function or act as intraspecific chemical signals. Saturated short-chain aldehydes
411 are very effective chemical irritants, hydrocarbons can facilitate penetration of reactive
412 aldehydes through the cuticle of arthropod predators and some esters can also assist in wetting
413 the cuticle of arthropod predators [20].
414 Because of the number of compounds produced by scent glands of Heteroptera and
415 because most of these compounds apparently functionally belong to infochemicals, we can
416 assume that the composition of secretion (a) is probably species-specific, (b) may reflect the
417 relationship of the individual species, and, consequently, (c) may be used in assessment of
418 these relationships (in addition to structural characters, and sequencing of nucleotides)
419 provided we know enough about the origins of compounds and about the biosynthetic
420 pathways to understand what are the feasible ways of their transformation and what ways are
421 forbidden.
422 In the present samples we find several patterns of shared similarity of chemicals.
423 Those present in all the three species are undoubtedly conservative plesimorphic characters of
424 the clade *Pyrrhocori*s & *Scantius*; they cannot show any relationship. Their number is high in
425 hydrocarbons (6 compounds out of 12), but very low in alcohols (1 out of 17), aldehydes (2

426 out of 9) and nil in esters (0 out of 18). This suggest that occurrence of the individual
427 compound of alcohols, aldehydes and esters is mostly species-specific, while the few common
428 ones of these groups (1-dodecanol; nonanal and decanal) are probably essential for
429 functioning of the whole system.

430 The shared presence of two compounds in two species is complementary to absence
431 of this particular compound in third species (or its replacement or transformation) in the third
432 species. This happened in 3 hydrocarbons, 3 alcohols and 6 esters shared by *P. tibialis* and *S.*
433 *aegyptius*, and 4 aldehydes shared by *P. apterus* and *P. tibialis*. Strangely, no compounds are
434 shared exclusively by *P. apterus* and *P. tibialis*. Of the complementary absences, there are 3
435 hydrocarbons, 3 alcohols and 6 esters missing in *P. apterus*, 5 aldehydes in *S. aegyptius*, but
436 no unique absence was found in *P. tibialis*.

437 The secretion of the two *Pyrrhocoris* species contained greater number of aldehydes
438 than the secretion of *Scantius aegyptius* (Tab. 5). Since the aldehydes are known to have
439 antipredatory defensive function [6, 58, 59], this difference corresponds with the difference in
440 palatability of *P*. *apterus* and *S*. *aegyptius* for avian predators, where *S*. *aegyptius* appears to
441 be considerably less well defended of the two species and may be a quasi-Batesian mimic of
442 *P*. *apterus*. Moreover, most of the aldehydes are shared by both *Pyrrhocoris* species, with (*E*)-
443 2-hexenal being dominant. Thus the composition of aldehydes appears rather to follow the
444 phylogenetic relationships between the species than be a result of mimetic convergence.

445 The composition of hydrocarbons was mostly similar in all the three species, with only
446 some of them missing in *P*. *apterus*, which is likely to represent a derived situation. In all the
447 three species, one of the dominant hydrocarbons is tridecane, known for its antipredatory
448 function against arthropod predators, where it facilitates penetration of aldehydes through the
449 cuticle [60].

450 Interspecific differences in the composition of alcohols and esters are difficult to
451 interpret, because of their less known and possibly multiple and context dependent functions
452 [6, 59, 61]. High proportion of both alcohols and esters is species-specific, which indicates
453 their interspecific signalling function [59, 61-63]. The strikingly smaller number of different
454 alcohols and esters in *P. apterus* may be connected with its gregarious lifestyle with smaller
455 need for long-distance chemical communication.

456 Limonene, which is like other terpenoids obtained by the bugs from their hostplants,
457 was present only in *S. aegyptius* and *P. tibialis*. Its absence in *P. apterus* may indicate its
458 unavailability in linden seeds in contrast to those of *Alcea* and *Hibiscus*. Along with other
459 terpenoids, limonene plays role in a chemical defense of the bugs, as it is serves as an ant
460 alarm pheromone [64].

461 Sexual dimorphism was found in the presence of 8 (out of 66 - 12.5%) chemicals. It
462 concerns 2 compounds in *P. apterus*, 5 in *P. tibialis*, and 1 in *S. aegyptius*. In most cases it
463 were females that possessed an additional compounds. Sample chromatograms obtained for
464 the secretions produced by the males and females of *Pyrrhocoris tibialis* are given in Figs. 3a
465 and 3b, respectively.

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475 **Fig 3. SPME-GC-MS analysis of *Pyrrhocoris tibialis*.**

476 The secretions of (**a**) males and (**b**) females of *Pyrrhocoris tibialis*. Sampling the secretion by
477 compression with the plunger of a syringe; SPME sorption on a DVB/CAR/PDMS fiber;
478 details of the sampling conditions given in Table 4. Peak [1] octan-1,3-diene; [2] hexenal; [3]
479 (*E*)-2-hexen-1-ol; [4] 3-methylbutanoic acid; [5] limonene; [6] 1-decyne; [7] (*E*)-2-hexenal;
480 [8] *p*-cymene; [9] undecane; [10] (*E,E*)-2,4-hexadienal; [11] (*E*)-2-hexen-1-ol acetate; [12]
481 (*E*)-2-octen-1-ol; [13] 2,6-dimethyl-7-octen-2-ol; [14] 6-dodecene; [15] dodecane; [16] 1-
482 dodecene; [17] nonanal; [18] (*E*)-2-octenal; [19] tridecane; [20] 2-isopropyl-5-methyl-1-
483 heptanol; [21] decanal; [22] tetradecane; [23] 2-ethylcyclohexanone; [24] 4-*tert*-
484 butylcyclohexyl acetate; [25] pentadecane; [26] 4-*tert*-butylcyclohexyl acetate; [27] 2-methyl-
485 1-undecanol; [28] allyl cyclohexanepropionate; [29] 1-dodecanol; [30] 3,5-Bis(1,1-
486 dimethylethyl)phenol; [31] tetradecanal; [32] hexadecane; [33] indan-1,3-diol monoacetate;
487 [34] verdyl acetate; [35] 2-ethyl-1-dodecanol; [36] 1-tridecanol; [37] 1-(4-*tert*-
488 butylphenyl)propan-2-one; [38] 1-ethoxynaphtalene; [39] dodecyl acetate; [40] hexyl
489 salicylate and [41] *α*-hexylcinnamaldehyde.

490
491 The individual chromatograms slightly differ, the female spectrum is enriched by
492 additional 5 compounds, especially (*E*)-2-hexen-1-ol and (*E*)-2-octen-1-ol. Generally, the
493 secretions of females of all the three species contained larger number of alcohols than the
494 secretion of conspecific males (Tab. 5). This may suggest the epigamic pheromonal function
495 of the particular alcohols [61]. On the other hand, there was minimum of sexual dimorphism
496 in composition of aldehydes, supporting their interspecific function in an antipredatory
497 defense [6, 58, 59], and in composition of esters, which were reported as non-epigamic
498 intraspecific atractants and alarm pheromones [59, 62, 63].

499 In no true bug species the exact function of numerous infochemicals of the secretion is
500 known – in antipredatory context it can range from signaling to irritation up to being lethally

501 toxic [65]. In addition to metathoracic glands representing the major source of defense
502 infochemicals in adults, also other specialized glands and sequestered noxious plant
503 compounds may play role in defense [7, 58]. It should be born in mind that also non-defensive
504 infochemicals may be detected in the adults of true bugs (e.g. pheromones produced in
505 dorsoabdominal glands persisting in adults from larval stage though with an altered function
506 and no more secreting defense substances). We should emphasize that a similar extraction "in
507 vivo" as having been used presently is also applicable to other arthropods (e.g., spiders,
508 millipedes, a variety of insects in addition to true bugs), and may be used for all kinds of a
509 rich array of exocrinous, supra-integumentally released, and often extremely complex
510 defensive secretions.
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526 # 4. Conclusions

527 A set of non-lethal methods has been developed for SPME sampling of the volatile
528 secretions of three species of true bugs (Insecta: Hemiptera: Heteroptera: Pyrrhocoridae) as a
529 part of their defensive mechanisms. Particular attention was paid to optimization of the input
530 parameters, including the means of irritation, and the optimization was performed using
531 multi-parameter response surface methodology. On the basis of the required information
532 (maximum number of peaks, maximum sum of all the peaks), the best method seemed to be
533 irritation of the bugs by compression with the plunger of a syringe and use of a composite
534 DVB/CAR/PDMS fiber for SPME sampling. This method also simulates best the natural
535 attack by a vertebrate predator. GC separation was performed using a column Rtx-200 with
536 fluorinated stationary phase providing good selectivity even for substances with very different
537 polarity scales. The developed method was used for sampling of volatile defensive secretions
538 of adult males and females of *Pyrrhocoris apterus*, *P. tibialis* and *Scantius aegyptius* (all
539 Hemiptera: Heteroptera: Pyrrhocoridae). The method applied has the advantage that only
540 three individuals of each sex are sufficient for the analysis and thus even rare and not easily
541 available species can be analyzed. Moreover, the method is non-lethal for the insects, which
542 may be later used for conducting bioassays. The outlined methods can be widely used in
543 similar studies on other insects although the optimization procedure and fiber evaluation
544 would be necessary to perform for any new species studied owing to large variation of
545 chemicals potentially involved. The chemical composition of secretion, particularly that of
546 alcohols, aldehydes and esters, is species-specific in all the three pyrrhocorid species studied.
547 The similarities in composition of secretion rather reflect the relationship of species
548 (*Pyrrhocoris apterus* and *P. tibialis*) than similarities in antipredatory color pattern (quasi-
549 Batesian mimesi of *P. apterus* by *Scantius aegyptius*). The phenetic overall similarity of
550 secretions shared by the bugs is linked with their antipredatory properties, e. g. small number

551 of alcohols and esters in *Pyrrhocoris apterus* is probably associated with its high
552 gregariousness while low numbers of aldehydes in *Scantius aegyptious* reflects its higher
553 palatability. The sexual dimorphism in occurrence of particular compounds is largely limited
554 to alcohols in *P. tibialis* and suggests their epigamic intraspecific function. We are planning to
555 analyze the obtained data more deeply for example by principle component analysis (PCA) to
556 get more insight into the relations between the individual species.

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764 # 6. Supporting Informations

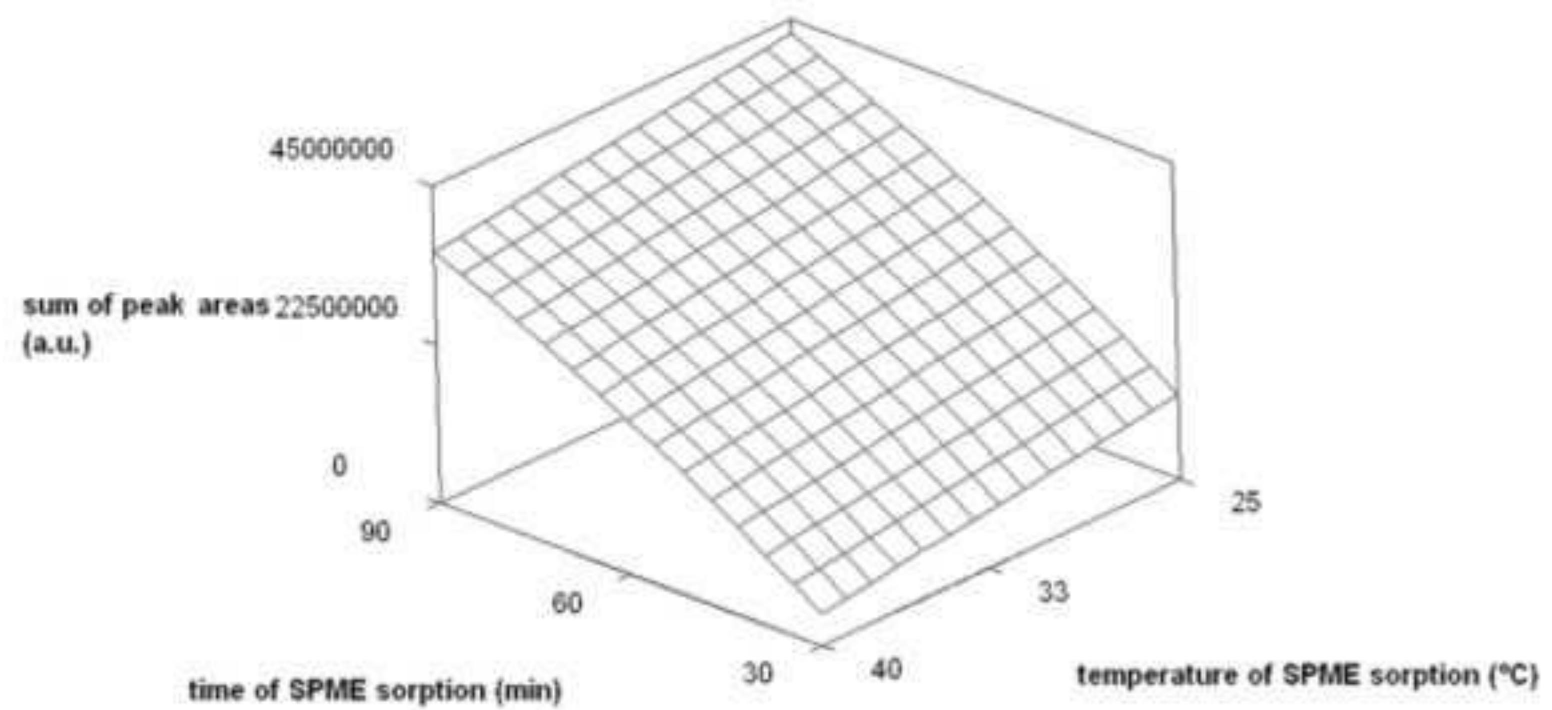
765 **Table S1. Ultrasonics.** Experimental parameters, their levels and modeling experimental plan
766 of the face centered central composite design for sampling secretion using ultrasonics.
767

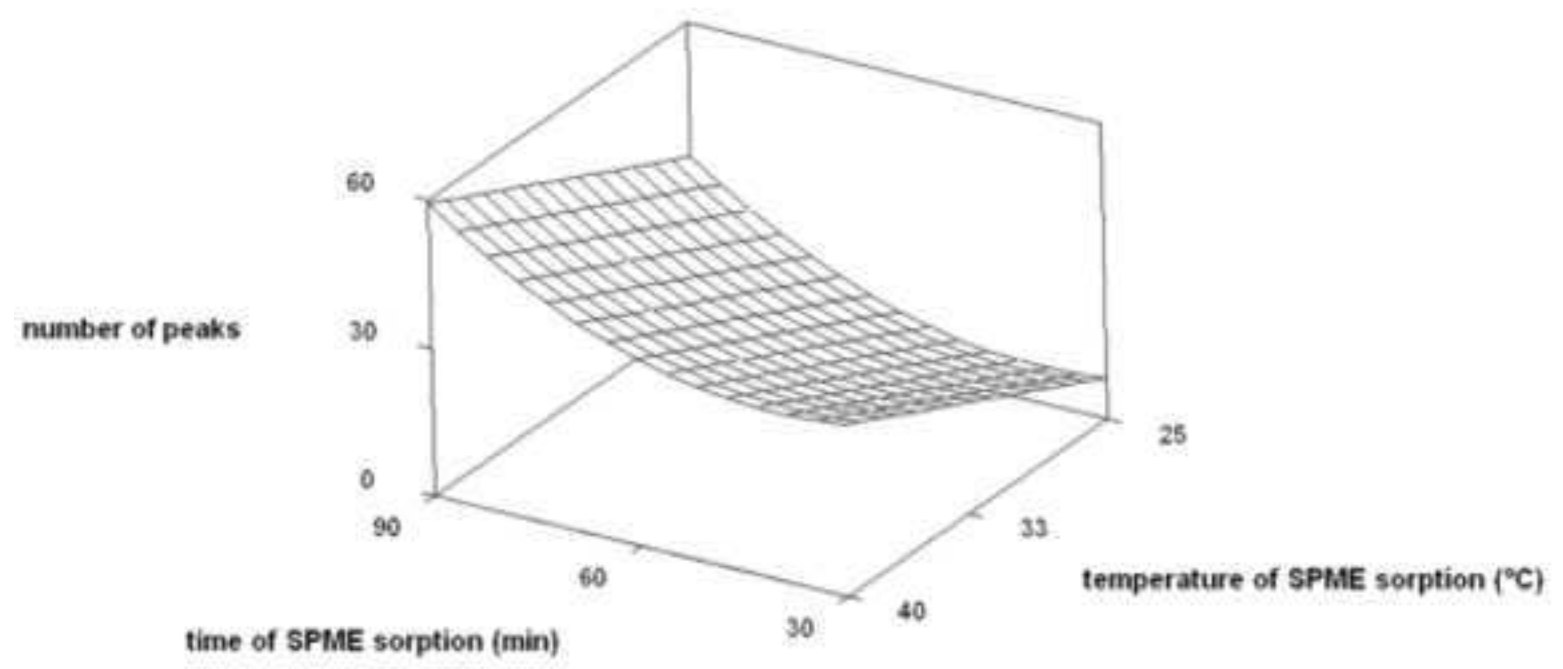
768 **Table S2. Shaker.** Experimental parameters, their levels and modeling experimental plan of
769 the face centered central composite design for sampling secretion using shaker.
770

771 **Table S3. Compression in the Plunger of a Syringe.** Experimental parameters, their levels
772 and modeling experimental plan of the face centered central composite design for sampling
773 secretion using compression with the plunger of a syringe.

Figure

Click here to download Figure Fig 1.tif





Figure

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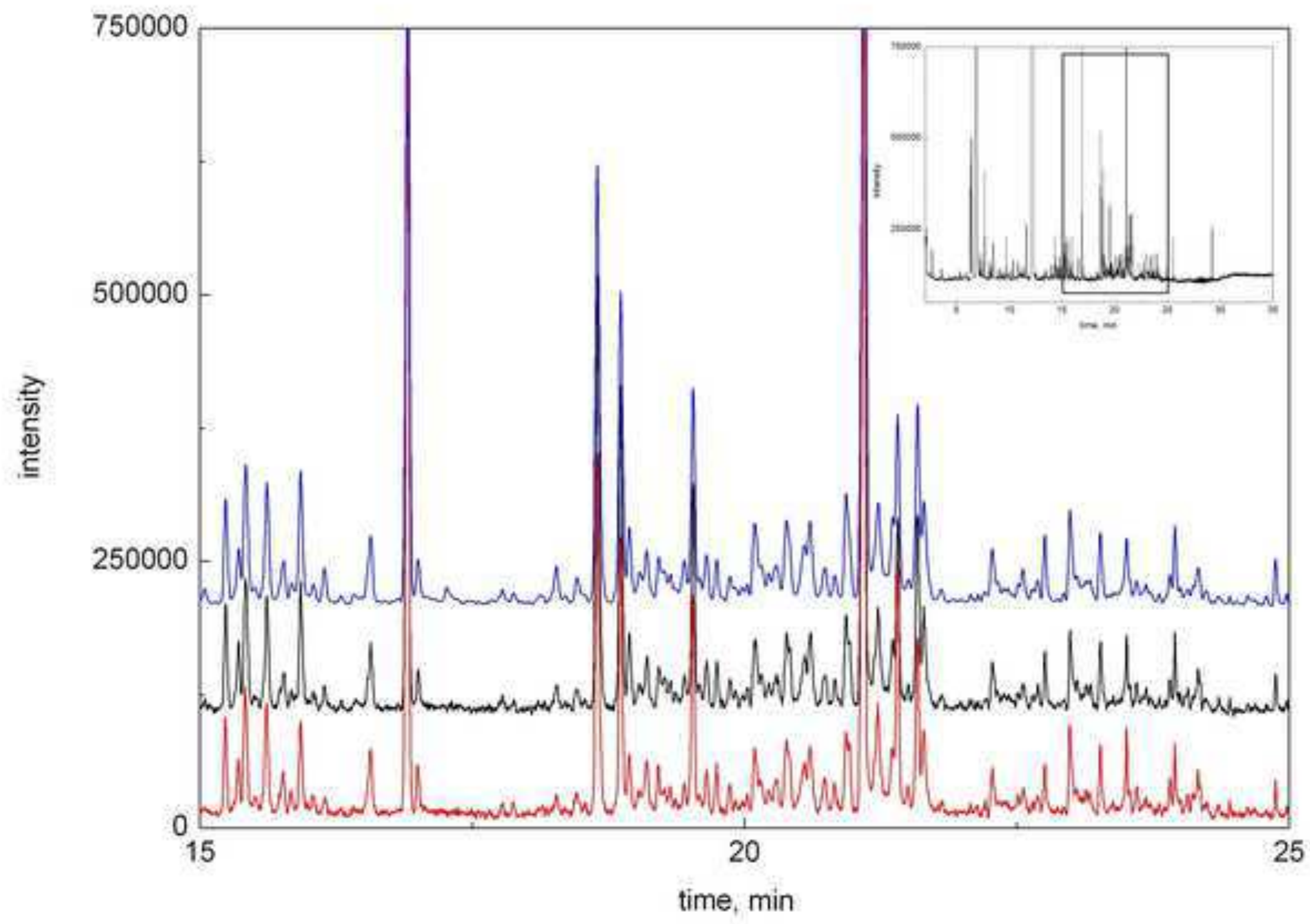

Figure

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4.7 Aplikovatelnost navržené metody

Nově vypracována metoda SPME-GC-MS byla použita pro analýzu sekretů u dvou evropských druhů – *Pyrrhocoris apterus* a *Scantius aegyptius* a jednoho čínského druhu – *Pyrrhocoris tibialis*. Navržená metoda byla optimalizována pro ploštice z rodiny Pyrrhocoridae. Tyto druhy se vyznačují nižší koncentrací látek přítomných v sekretech.

V pilotních studiích bylo zjištěno, že některé další druhy například ploštice *Graphosoma lineatum* z rodiny Pentatomidae, uvolňuje velké množství látek o vysokých koncentracích. Z výše uvedeného důvodu není následná analýza pomocí 1D-GC dostatečně účinná, protože dochází ke koelucím.

Pro rozšíření aplikovatelnosti navržené metody odběru těkavých sekretů i na druhy s vyššími koncentracemi látek přítomných v sekretech budou podmínky odběrů následně upraveny s ohledem k výše uvedeným skutečnostem, například snížením doby sorpce na SPME vlákně nebo snížením počtu jedinců při odběru jejich těkavých sekretů. V následujícím studiu bude také využita metoda komprehenzivní dvoudimenzionální plynové chromatografie (GCxGC). Problematice se bude věnovat navazující projekt grantové agentury GA UK č. 760216 Využití multidimenzionálních separačních technik pro studium obranného mechanismu ploštic.

5 Závěr

Dizertační práce se zabývá komplexní analýzou obranných látek jednoho z důležitých zástupců hmyzu – ploštic. První část dizertační práce popisuje analýzu derivátů pterinu, které jsou obsaženy v kutikulách ploštic, propůjčující jim jejich charakteristické výstražné zabarvení. Druhá část se věnuje novým způsobům odběru těkavých obranných sekretů a jejich následným analýzám plynovou chromatografií. Výsledky dizertační práce jsou:

1) Byla vyvinuta, optimalizována a validována metoda kapilární elektroforézy a vysokoúčinné kapalinové chromatografie s tandemovou hmotnostní detekcí pro stanovení derivátů pterinu v kutikulách vybraných druhů ploštic.

2) Ve vybraných druzích ploštic byly identifikovány a stanoveny obsahy derivátů pterinu zodpovědných za charakteristické zabarvení kutikul ploštic.

3) Byly navrženy nové neinvazivní způsoby odběrů těkavých složek obranných sekretů ploštic – ultrazvuk, třepačka, píst injekční stříkačky. Byly otestovány tři typy SPME vláken, a to nepolární polydimethylsiloxan (PDMS), polární polyakrylát (PA) a bipolární divinylbenzen/carboxen/polydimethylsiloxan (DVB/CAR/PDMS). Celá optimalizace odběru těkavých sekretů ploštic byla provedena pomocí faktorového plánu RSM. Vypracovaná metodika byla aplikována pro analýzu těkavých sekretů samců a samic ploštic *Pyrrhocoris tibialis*, *Pyrrhocoris apterus* a *Scantius aegyptius* nově vyvinutou metodou SPME-GC-MS.

6 Seznam publikací a konferenčních příspěvků

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