Measuring juvenile hormone and ecdysteroid titers in insect haemolymph simultaneously by LC-MS: The basis for determining the effectiveness of plant-derived alkaloids as insect growth regulators

Dissertation

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Dem Andenken meiner Oma

Schade niemandem; sondern hilf allen, so gut du kannst.

(Arthur Schopenhauer)

Abbreviations

λ_{max}	wavelength at maximum absorption
δ	chemical shift (ppm)
20HE	20-hydroxyecdysone
ACN	acetonitrile
CA	corpora allata
$CDCl_3$	deuterochloroform
COSY	correlated spectroscopy
CV	coefficient of variation
DDT	dichlorodiphenyltrichloroethane
DMSO	dimethyl sulfoxide
E	ecdysone
ECD	electron-capture detector
EI	electron ionization
ESI	electrospray ionization
eV	electron-volt
GC	gas chromatography
h	hour
HMBC	heteronuclear multiple-bond correlation
HMQC	heteronuclear multiple-quantum correlation
HPLC	high performance liquid chromatography
JH	juvenile hormone
JHBP	juvenile hormone binding protein
LC	liquid chromatography
LD_{50}	dose estimated to kill 50 % of the test animal population (mg of toxin/kg of body weight)
LOD	limit of detection
LOQ	limit of quantitation
min	minute/s
MeOH	methanol
MS	mass spectrometry
m/z	mass-to-charge ratio
NMR	nuclear magnetic resonance spectroscopy

OCC	open-column chromatography
ppm	parts-per-million
RP	reversed phase
r.t.	room temperature
s	second/s
S.D.	standard deviation
S.E.M.	standard error of the mean
SIM	selective ion monitoring
S/N	signal-to-noise ratio
THF	tetrahydrofuran
TLC	thin-layer chromatography
UV	ultraviolet/ absorption detector
V	voltage

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1. Introduction

Although insects are primarily thought of as pests by many, only 1 % of all insects are considered pests, and only 3,500 species fall into the "serious pest" category. Insects play a key role in maintaining the delicate ecosystem of our planet and therefore necessitate a deeper understanding of their biology and life processes. (Pedigo, 1996) and cannot simply be eliminated indiscriminately.

With the discovery of DDT as an insecticide and the ecological damage brought about by the uncontrolled and exclusive use of organophosphates, carbamates, chlorinated hydrocarbons (such as DDT), and pyrethroids between 1939 and 1962, a more conscientious approach to pest management was envisioned (Carson, 1962). This was implemented by the incorporation of environmentally safer insect control practices with ecological and economical considerations, known as integrated pest management (Steiner, 1968). Techniques include the use of biological control using predators and parasitoids, plant-derived insect deterrents/attractants, utilizing plant resistance, microbial insecticides, sterile-insect techniques, and/or pheromones (Schmidt, 1986).

In the search for more specific pesticides, the predominant differences between the physiology of vertebrates and invertebrates are examined, namely the hormonal control of growth, development and reproduction. Juvenile hormones (JH), a class of sesquiterpenoids, and the steroidal molting hormones, ecdysteroids, found in arthropods and some plants, play a crucial role in insect development and reproduction. During preimaginal development, JHs and ecdysteroids regulate larval and pupal molting. In adult insects, they are involved in egg maturation, yolk deposition and accessory gland development. JHs are also known to be involved in female sexual behavior, division of labor in social insects, and flight and migration (Hagedorn, 1985; Nijhout, 1994; Wyatt and Davey, 1996). Disrupting these processes in agricultural and medicinal pests (Bowers, 1982), but not in non-target insects, and with minimal impact on the environment are the goals of third-generation insecticides, also called insect growth regulators (IGRs) (Williams, 1967; Palli and Retnakaran 2001).

Some IGRs are chitin synthesis inhibitors, others JH mimics, anti-JH hormones, or ecdysteroid agonists, while some have insect regulatory properties in addition to other mode of actions. Benzoylphenyl urea compounds are representatives of chitin synthesis inhibitors, resulting in abnormal development of the endocuticle (for review

see Ishaaya and Horowitz, 1998). Only recently, new chitin synthesis inhibitors, triazines, were discovered with lepidoptericidic activity (Eberle et al., 2003) The juvenoid methoprene developed by Henrick et al. (1973) has been partly successful in controlling California floodwater mosquito *Ochlerotatus* (=*Aedes*) *nigromaculis* (Diptera, Culicidae) and various flies found in stables, by interfering with metamorphosis (Staal, 1975). However, cases of methoprene tolerances in *Ochlerotatus nigromaculis* (Ludlow) (Diptera, Culicidae) have been reported in the U.S.A. (Cornel et al., 2000; Cornel et al., 2002). Mimics of the molting hormone 20-hydroxyecdysone, the bisacylhydrazines tebufenozide and RH-2485, which appear to be mainly selective towards lepidopteran species are ecdysteroid agonists preventing sclerotization of the cuticle and ecdysis (Tarlochan et al., 1998). Examples of anti-juvenile hormone analogs are precocene I, II and III, of which I and II were first isolated from the floss flower *Ageratum houstonianum* (Asteraceae) (Bowers and Cleere, 1976), having anti-allatotropic effects in Heteroptera and some Orthoptera by inducing precocious metamorphosis in larvae and causing sterility in adults (Staal, 1982). Another plant-derived compound, the steroid-like tetranortriterpenoid Azadirachtin from the seeds of the Indian neem tree *Azadirachta indica* (Meliaceae) is unlike other botanical insecticides, such as nicotine, in exhibiting low mammalian toxicity. Besides acting as an IGR (Schmutterer, 1990), Azadirachtin also displays repellent and anti-feedant properties, covering a broad spectrum of pest species (for review see Mordue and Blackwell, 1993).

Testing potential insecticides in the laboratory is usually accomplished by topical application, injection, inhalation or feeding of the test substance and determining LD_{50}, fecundity and/or fertility (Schmidt, 1986). However, to gain a better understanding of the mode of action of IGRs, close monitoring of endocrinologically significant constituents (JHs, Ecdysteroids, and their degradation products) are necessary to comprehend the complexity of insect endocrinology. Various methods have been developed to measure rates of JH-biosynthesis *in vitro* utilizing radiochemical assays (Pratt and Tobe, 1974; Feyereisen and Tobe, 1981) and determine JH and ecdysone titers in insects by immunological methods (Borst and O'Connor, 1972; Hirn and Delaage, 1980; Strambi et al., 1981; Delaage et al., 1982; Baehr et al., 1987; Porcheron et al., 1989; Goodman et al., 1990). However, radiochemical and immunological methods are suitable only for specific applications and lack universal applicability, due to the nonspecific response of the antiserum to the different JH

homologs or degradation products. Physicochemical methods such as GC-MS (Bergot et al., 1981a; Rembold and Lackner, 1985) have been considered the most accurate (approx. 20 pg) in detecting and identifying the six JH homologs currently known to exist. Unfortunately, extensive sample preparation and organic solvent usage has been a major disadvantage when considering GC-MS for routine analysis of JHs, followed by the inability to monitor JH degradation products. In addition to the previously mentioned drawbacks of GC-MS analysis of JHs, more difficulties arise when using GC-MS for ecdysteroid analysis, because of derivatization required of the non-volatile and thermally labile steroids (Ikekawa et al., 1972; Morgan and Poole, 1976a, 1976b; Bielby et al., 1980; Lafont et al., 1980, Bielby and Morgan, 1986; Evershed et al., 1987) and its suitability only to free ecdysteroids. Some HPLC-UV methods exist for the detection of JHs, ecdysteroids, and degradation products (Morgan and Wilson, 1980; Wilson et al., 1980; Lafont et al., 1982; Sasagawa, 1988), but are of limited applicability due to sensitivity problems associated with the detector. By utilizing LC-MS, an advantage over the before hand mentioned methods is the simultaneous detection of JH, JH degradation products, ecdysteroids, and possibly conjugated ecdysteroids; sufficient separation by HPLC has already been demonstrated (i.e. Isaac et al., 1982; Scalia and Morgan, 1982; Scalia and Morgan, 1985). The same degree of accuracy and precision can be afforded as with GC-MS, but with less time-consuming sample preparation and little organic solvent usage.

The aim of this thesis was to develop a LC-MS method, requiring little sample preparation, to monitor developmentally important hormones and their metabolites simultaneously. For future studies involving the identification of novel insect growth regulators (IGRs), such as plant-derived alkaloids, the method should be able to assist in discovering more about the mode of action of IGRs. Some alkaloids have been isolated and identified from the Pink Lime *Glycosmis pentaphylla* (Rutaceae), which is used in India for medicinal and insecticidal purposes (Ambasta, 1986) and might serve as a potential source of IGRs.

2. Overview

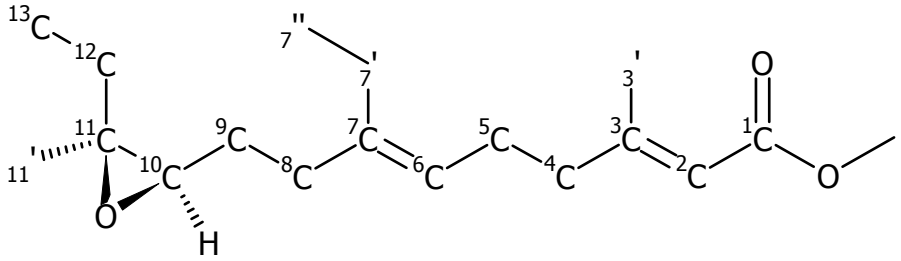
2.1. Juvenile Hormones

2.1.1. Chemistry and Structures

JHs are comprised of a long carbon chain with two double bonds at C_2 and C_6, and an epoxide at C_{10}, which makes them hydrophobic and quite labile. They bind non-specifically to surfaces (Kramer and Law, 1980), which makes handling them extremely difficult, especially at low concentrations.

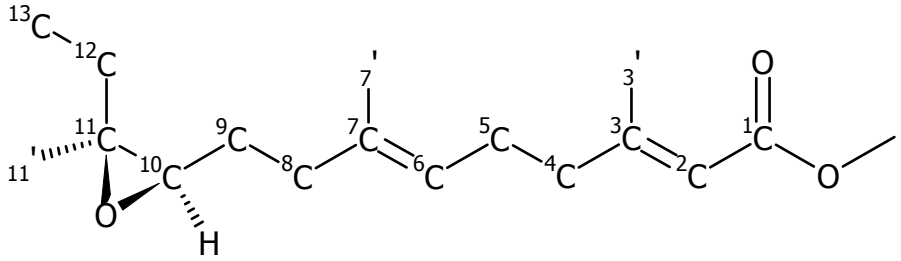
Since the discovery of JHs by Wigglesworth in 1934/36, six JH homologs have been isolated and identified: **JH O** (Methyl-(2*E*,6*E*)-(10R,11S)-10,11-epoxy-3,7-dieethyl-11-methyl-2,6-tridecadienoate) (Bergot et al., 1980), **JH I** (Fig. 1) (Roeller et al., 1967), **4-Me JH I** (Methyl-(2*E*,6*E*)-(4S,10R,11S)-10,11-epoxy-7-ethyl-3,4,11-trimethyl-2,6-tridecadienoate) (Bergot et al., 1981b), **JH II** (Fig. 1) (Meyer et al., 1968), **JH III** (Fig. 1) (Judy et al., 1973) and the bisepoxide **JHB3** (Methyl-(2*E*)-(10R;6S,7S)-6,7;10,11-bisepoxy-3,7,11-trimethyl-dodecadienoate) (Ajami and Riddiford, 1973; Richard et al., 1989).

JH I



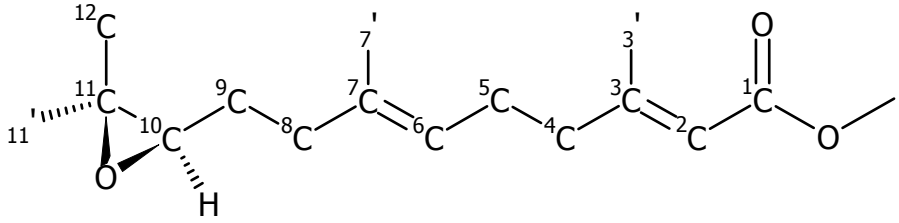
Methyl-(2*E*,6*E*)-(10R,11S)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate

JH II



Methyl-(2*E*,6*E*)-(10R,11S)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate

JH III



Methyl-(2*E*,6*E*)-(10R)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate

Fig. 1 Structures and numbering of JH I, JH II, and JH III

2.1.2. Mode of Action, Biosynthesis, Degradation, and Transport

JHs have a wide range of morphogenetic and gonadotropic effects in insects. Besides their morphogenic effect during larval development, they play an important part in caste determination in social insects, phase polymorphism, and vitellogenin production in adult females, for example. JHs are also involved in mediating diapause, migration, and active flight (for review see Kumaran, 1990).

The primary site of JH biosynthesis in most insects is the CA, where synthesis is regulated by allatostatic and allatotropic neuropeptides (Gäde et al., 1997; Hoffmann, 2003). JH III is produced from acetyl-CoA up to farnesol pyrophosphate according to the biosynthetic pathway of acyclic sesquiterpenoids. The branched side chain homologs, arising from various combinations of acetyl-CoA and propionyl-CoA, follow a similar biosynthetic path as JH III (Schooley et al., 1973; Schooley and Baker, 1985).
Farnesol pyrophosphate is converted to JH III by pyrophosphate ester hydrolysis of farnesol pyrophosphate resulting in farnesol, oxidation yielding farnesal, *O*-methyl transfer producing farnesoic acid and epoxidation providing JH III. In lepidopteran species, however, the CA first converts farnesoic acid to JH III acid through epoxidation (Goodman, 1990) and then to JH III by *O*-methyl transfer, which can occur either in the CA or at different sites. In the Cecropia silkmoth *Hyalophora cecropia* (Lepidoptera, Saturniidae) JH acid is synthesized to JH in the accessory sex glands (Dahm et al., 1976), whereas in the tobacco hornworm *Manduca sexta* (Lepidoptera, Sphingidae) transformation occurs in the imaginal disks (Sparagana et al., 1985; Bhaskaran et al., 1986).

Several JH degradation pathways have been proposed, such as oxidation, conjugation, epoxide hydration and ester hydrolysis, but only the latter two being thoroughly investigated so far. Epoxide hydration is carried out by the epoxide ether hydrolase (microsomal), leading to the formation of JH diols. JH esterase on the other hand, which is present in the haemolymph, hydrolyzes the ester to yield JH acids. JH diols and JH acids can be further converted to JH diol-acids by the same enzymes or conjugated to sulfates (for review see Hammock, 1985; Roe and Vekatesh, 1990).

To ensure solubilization in the haemolymph, JHs are bound to high-affinity and low-affinity carrier proteins (Goodman, 1985; Trowell, 1992). These carrier proteins also function as transport and sequestration agents. They also enhance the stability of JHs by preventing non-specific JH degradation by enzymes (Hammock, 1985).

Since JH I, JH II and JH III are the predominant species in insects and commercially available, these JH homologs were used during the method development in this thesis. JH diols and JH acids of JH I, JH II, and JH III served as representatives of degradation products during method development.

2.2. Ecdysteroids

2.2.1. Chemistry and Structures

Ecdysteroids are polyhydroxylated steroids and contain a *cis* ring connection between rings A and B, a keto-group at C_6, a double bond at C_7 and have an α-configuration at C_{14} (Horn and Bergamasco, 1985). The most widely occurring ecdysteroids in insects are ecdysone (2β,3β,14α,20R,25-pentahydroxy-7-cholesten-6-one) (Fig. 2), which was the first ecdysteroid isolated by Karlson in 1953 from *Bombyx mori* (Lepidoptera, Bombycidae), and 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-7-cholesten-6-one) (Fig. 2), a metabolite of ecdysone. Over 60 ecdysteroids have been isolated and identified *in vivo* and *in vitro* from invertebrates so far (Rees, 1989).

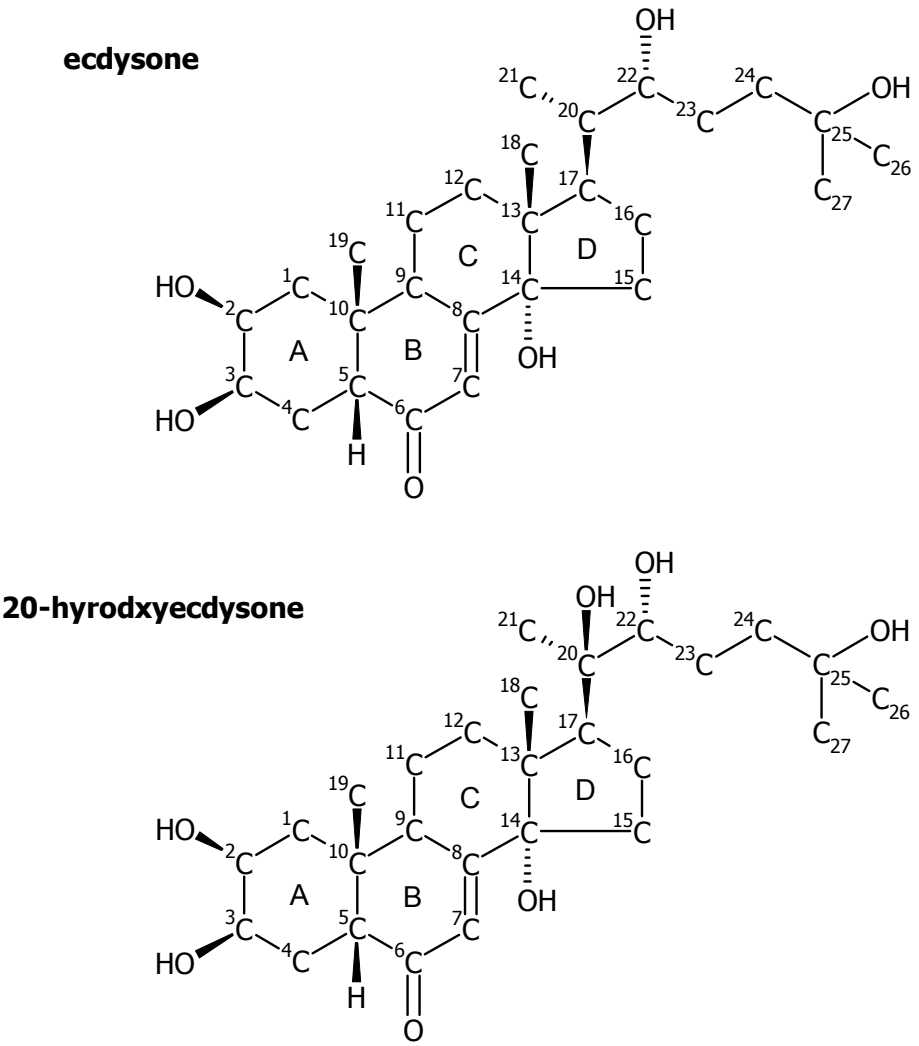


Fig. 2 Structures and numbering of ecdysone and 20-hydroxyecdysone.

2.2.2. Mode of Action, Biosynthesis, Degradation, and Transport

Ecdysteroids are polytropic hormones, having morphogenetic effects, inducing behavioral changes and regulating spermatogenesis, vitellogenesis and oocyte maturation. During insect larval development, 20-hydroxyecdysone and JH regulate onset of molting at high JH titers and metamorphosis at low JH titers. The major site of ecdysone biosynthesis in most insects is the prothoracic gland (ring glands or ventral glands in other insect orders; Koolman et al., 1994; Gäde et al., 1997), whereas in last-instars prior to ecdysis, the testes may also produce ecdysone (Loeb et al., 1982). Other biosynthesis sites in adults, besides the gonads (Rees, 1985), are the abdominal integument and the adjacent fat body (Hoffmann et al., 1992).

The phytosterols campesterol, stigmasterol or β-sitosterol, which are taken in by phytophagous insects through their diet, are converted to cholesterol in the gut. Carnivorous insects obtain cholesterol directly from their diet. Cholesterol is changed to 7-dehydrocholesterol and through numerous steps, which are still being investigated (Lafont, 1996), a 5β-ketodiol is formed. A sequence of hydroxylations of the 5β-ketodiol results in ecdysone (for review see Rees, 1985; Rees, 1995). In Lepidoptera, ecdysone biosynthesis is regulated by the prothoracicotropic hormone, which is released from the brain-corpora cardiaca complex (Rees, 1985). Ecdysone, however, must be first converted to 20-hydroxyecdysone through hydroxylation at the C_{20} position by a cytochrome P_{450} monooxygenase to obtain its biological activity (Smith, 1985); the most active locations being the fat body, the midgut and the Malpighian tubules (Koolman and Karlson, 1985).

Ecdysone can be further metabolized by hydroxylation at the C_{26} position, oxidation at C_3 and C_{26}, reduction at C_3 or side-chain cleavage between C_{20} and C_{22}. Further metabolic pathways include phosphorylation, acetylation, glucosylation or acylation with long-chain fatty acids and hydrolysis of the conjugates at positions C_2, C_3, C_{22}, and C_{26} (Thompson et al., 1990). Conjugated ecdysteroids can either serve as a storage or are eliminated through the Malpighian tubules or the gut (Koolman and Karlson, 1985).

Transport of ecdysteroids in haemolymph by a highly specific protein has been only reported so far by Feyereisen (1980), who was able to confirm the existence of an ecdysteroid binding protein.

Due to the abundance of ecdysteroids and conjugates, the two major ecdysteroids, ecdysone and 20-hydroxyecdysone were chosen for LC-MS method development.

2.3. RP-HPLC and ESI-MS

Separation on a RP column depends on the interaction of the analyte with the solid and the mobile phase. When choosing the solid phase, the structure, physical and chemical properties of the analytes have to be taken into account to obtain adequate separation. The elution strength of the mobile phase has to be high enough to displace the analyte from the solid phase (Young and Weigand, 2002). When coupling HPLC to MS, the flow rate, the solvent types and additives also play an important part in method development. Flow rates for an ESI source can be anywhere from 10 µL/min up to 1 mL/min, depending on the application. Non-volatile solvents and non-volatile electrolyte additives lead to a decrease in sensitivity, due to build up in the MS (Petritis et al., 2002) and should be avoided. Although a high ion concentration can lead to signal suppression, some ions are necessary for the ionization process (Constantopoulos et al., 1999).

ESI is a "soft ionization" technique with none to little fragmentation of the analyte. During ionization in the positive ion mode, some of the ions formed are $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$, whereas in the negative ion mode $[M-H]^-$ is one possible species. Selection of the positive ion or the negative ion mode depends on the chemical make up of the analyte. Chemical ionization takes place at atmospheric pressure, whereas the MS operates at a high vacuum ($<10^{-3}$ Pa) (Fig. 3). The sample is introduced by HPLC through the highly charged electrospray capillary and is dispersed into a fine electrically charged mist by N_2 (g), the nebulizer gas. The charged droplets further disintegrate through solvent evaporation into highly charged minute droplets until ions in the gas phase are produced (Fig. 4). The ions are electrostatically attracted

by a heated capillary and further transmitted through the skimmer and octapole into the mass analyzer (Fig. 3), where ions of different *m/z* are isolated.

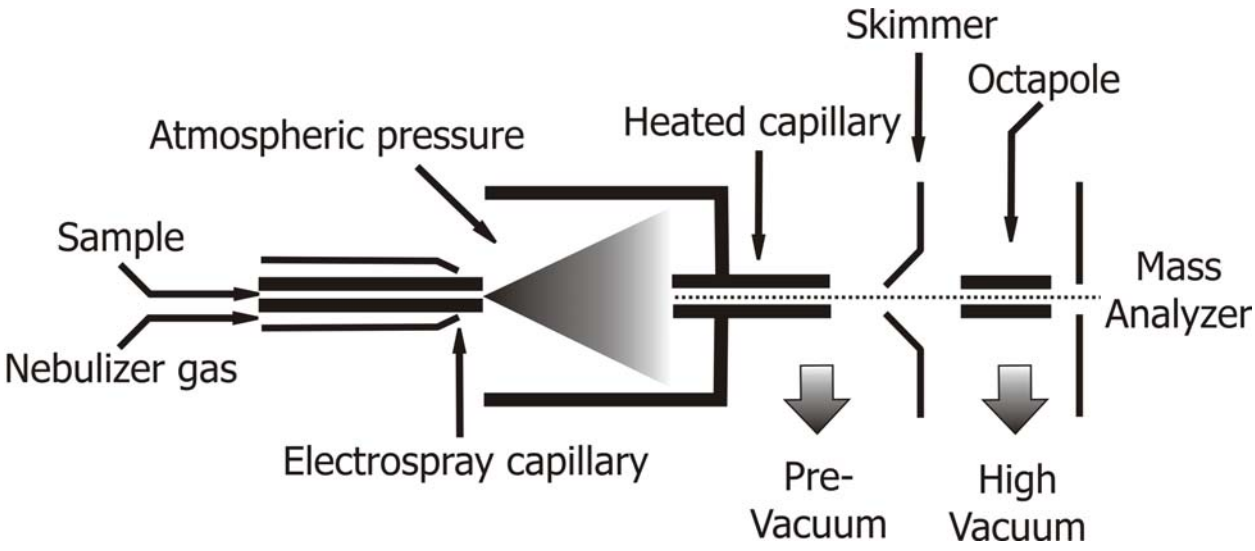


Fig. 3 Schematic of the ESI source

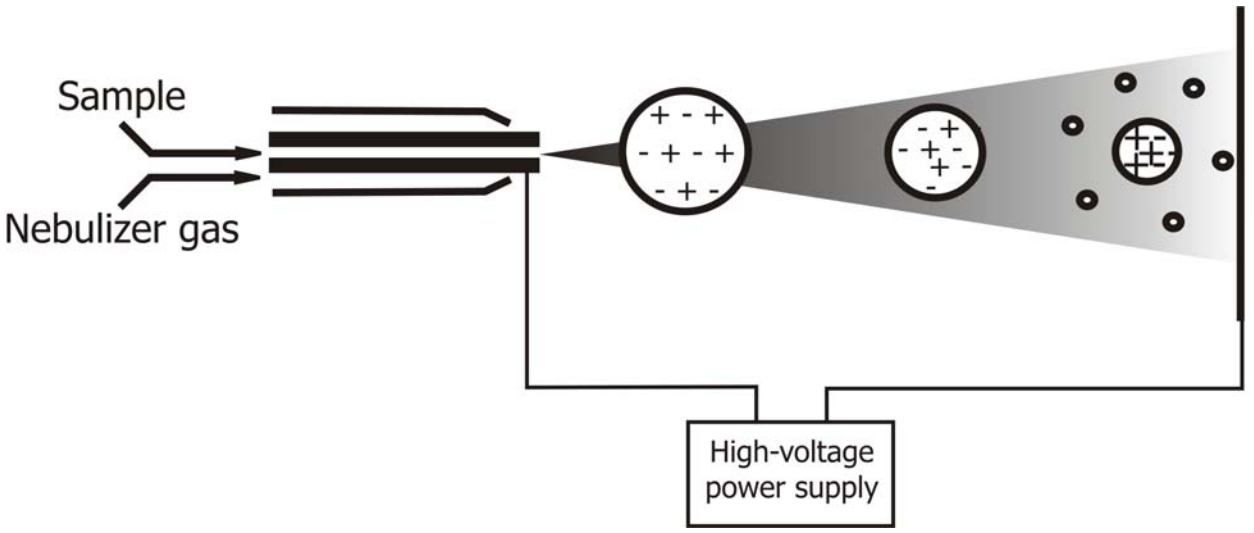


Fig. 4 The desolvation and gas-phase ion process

3. Materials and Methods

3.1. LC-ESI-MS Method Development and Titer Determinations

3.1.1. Materials

3.1.1.1. JH Diol and JH Acid Synthesis

All solvents and chemicals were provided by Prof. K. Seifert (Department of Organic Chemistry I/2, University of Bayreuth, Bayreuth, Germany).

3.1.1.2. JHBP Assay and Haemolymph Sample Preparation

JH I (methyl-(2*E*,6*E*)-10,11-epoxy-7-ethyl-3,11-diemethyl-2,6-tridecadienoate, 78 %) was purchased from SciTech (Prague, Czech Republic) and JH III (methyl-(2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate, 75 %) from Fluka (Taufkirchen, Germany). Triton X-100 was bought from Sigma (Steinheim, Germany), Tris-HCl and KCl from Merck (Darmstadt, Germany) and were of p.a. grade. The solvents used for protein precipitation and haemolymph sample preparation were from Merck (Darmstadt, Germany) and of HPLC grade, except for acetone, which was of p.a. grade. Haemolymph was collected with disposable micropipettes with ring mark (Blaubrand® intra Mark, Merck, Darmstadt, Germany). 0.5 mL reaction tubes (Eppendorf, Hamburg, Germany) and disposable culture tubes (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used for the assay and sample preparation.

3.1.1.3. HPLC and LC-ESI-MS

JH II (methyl-(2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate, 78 %) was purchased from SciTech (Praha, Czech Republic) and methoprene (isopropyl (2*E*,4*E*)- 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, 95.6 %) from Fluka (Taufkirchen, Germany). For JH I and JH III see JHBP assay and haemolymph sample preparation (3.1.1.2). 20-hydroxyecdysone (2β,3β,14α,20β,22,25-hexahydroxy-7-cholesten-6-one, 95 %) and ecdysone (2β,3β,14α,20R,25-pentahydroxy-7-cholesten-6-

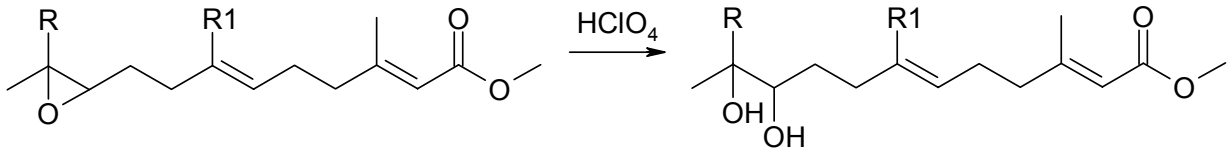
one, 90 %) were obtained from Sigma-Aldrich (Taufkirchen, Germany). All solvents were the same as used for JHBP assay and haemolymph sample preparation (see 3.1.1.2.), except for the LC-MS grade solvent ACN, MeOH and water, which were bought from Riedel-de Haën (Seelze, Germany). All other chemicals were of p.a. grade. The autosampler vials (with silicon-teflon septum) were purchased from Roth (Karlsruhe, Germany). The formic acid was kindly provided by Prof. C. Unverzagt (Department of Bioorganic Chemistry, University of Bayreuth, Bayreuth, Germany).

3.1.2. Methods

3.1.2.1 Synthesis of JH Diols

JH diols were prepared from JHs without double bond isomerization as outlined by Scheme I (McCormick and Schafer, 1977).

Scheme I



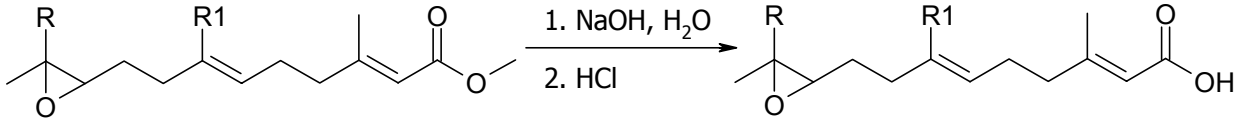
JH I R= Et R1= Et
JH II R= Et R1= Me
JH III R= Me R1= Me

JH was mixed with THF and H_2O (1:1, v/v). 10 % $HClO_4$ was added at r.t. under rapid stirring and left stirring for 23 h. Following saturation of the mixture with NaCl, the aqueous layer was extracted with diethyl ether three times. The organic phases were combined, washed twice with aqueous NaCl, and filtered over Na_2SO_4. The solvents were evaporated under a stream of N_2 (g) and resuspended in hexane and kept at 4 °C.

3.1.2.2. Synthesis of JH Acids

JH acids were prepared by base-induced ester hydrolysis as shown in Scheme II (Goodman and Adams, 1984).

Scheme II



JH I R= Et R1= Et
JH II R= Et R1= Me
JH III R= Me R1= Me

JH was mixed with MeOH and 1 M NaOH (1:1, v/v) in a foil-wrapped vial and incubated in a 40 °C water bath while shaking. After 4 h, the mixture was adjusted to pH 5.0 with HCl and extracted with $CHCl_3$:toluene (9:1, v/v) five times. The solvents were evaporated under a stream of N_2 (g) and resuspended in isooctane and kept at 4 °C. The procedure was performed in semidarkness.

3.1.2.3. HPLC Optimisation

Using a Micro-HPLC-UV (Eldex MicroPro, SunChrom, Friedrichsdorf) different solvent systems were tested (Table 1) at a flow rate of 0.2 mL/min on a 250 x 2.1 mm C_{18} column (Luna C18(2), 5 µm; Phenomenex, Aschaffenburg, Germany or Reprosil-Pur ODS-3, 5 µm, Dr. Maisch-GmbH, Ammerbuch, Germany) protected by a guard column (C_{18} cartridge, Phenomenex) or at a flow rate of 0.1 mL/min on a 250 x 1.5 mm C_{18} column (Reprosil-Pur 120 C18-AQ, 5 µm, Dr. Maisch-GmbH) at 37 °C (column thermostat with forced air circulation, Spark, Emmen, The Netherlands). The column was equilibrated at starting conditions until the pressure was steady for at least 5 min. The UV wavelength was set at the 219 nm, which is λ_{max} for JHs.

time (min)	% solvent	time (min)	% solvent
0-15	60-100 % ACN	0-3	60-80 % MeOH
15-20	100-60 % ACN	3-6	80 % MeOH
		6-9	80-100 % MeOH
		9-15	100 % MeOH

Table 1 ACN and MeOH-gradients used for HPLC

3.1.2.4. JHBP Assay

JHBP assay was conducted with JH I or JH III and partially purified *Gryllus bimaculatus* JHBP (kindly provided by Dr. Amer Tawfik, Assiut University, Egypt and University of Bayreuth, Germany). The release of JH was monitored by HPLC-UV using a MeOH-gradient (Table 1). JH I or JH III and JHBP were incubated at 4 °C for 1 h under shaking in Tris/KCl-buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl) for maximum binding (personal communication Dr. Tawfik). Initially, JH I or JH III (in isooctane) was suspended in buffer: JH I or JH III was added to triton X-100, the isooctane evaporated under N_2 (g) and Tris/KCl-Buffer added. JHBP was then mixed in. Later, JH I or JH III was added directly to the buffer without the addition of triton X-100. Isooctane was evaporated under N_2 (g) and then added to another vial containing JHBP. In order to precipitate the JHBP and disrupt binding, the incubation mixture containing the JHBP-JH III complex was either subjected to sonication and/or MeOH, ACN, acetone, acetone-MeOH (1:1, v/v), or isooctane-MeOH (1:1, v/v), vortexed for 20 s and centrifuged at 10,000 *g* for 30 min. The supernatant was drawn into a syringe and injected onto the column. During the JHBP assay the affinity of JH to polypropylene vials was also tested.

3.1.2.5. Haemolymph Sample Preparation

20 µL of insect haemolymph was collected with a glass capillary and blown into a glass vial containing either MeOH, ACN, or isooctane-MeOH, including methoprene as the internal standard. The haemolymph-solvent ratio was 1:10 (v/v). The haemolymph-solvent mix was vortexed vigorously for 20 s, and allowed to stand at room temperature for 30 min. Samples were centrifuged at 8,500 *g* for 15 min. For isooctane-MeOH, the isooctane phase was transferred to a new glass vial, then the methanol phase was vortexed and centrifuged at 10,000 *g* for 30 min and combined with the isooctane phase. For isooctane-MeOH, additional steps were necessary due to the protein precipitate deposit between the isooctane and MeOH interface. The samples were stored at – 20 °C or concentrated down to 20-50 µL and transferred to an autosampler vial for immediate analysis.

3.1.2.6. LC-ESI-MS Optimisation

3.1.2.6.1. HPLC

Separations were carried out on either the Luna C18(2) or the Reprosil-Pur ODS-3, protected by a guard column (C_{18} cartridge), at a flow rate of 0.2 mL/min utilizing an Agilent HP 1100 HPLC system (coupled to the Micromass LCT) or an Eldex MicroPro HPLC (coupled to the Shimadzu LCMS-2010A) with autosampler. The MeOH- and the ACN-gradients mentioned under HPLC optimization (3.1.2.3.; Table 1) were investigated for MS compatibility.

3.1.2.6.2. ESI-MS

JHs, JH diols, JH acids, ecdysone and 20-hydroxecdysone were analyzed using electrospray ionization (ESI) in the positive mode on a Micromass LCT (1) or a Shimadzu LCMS-2010A (2), which were operated under the following conditions: (1) the electrospray capillary was set at 3.2 kV. The sample cone voltage was tested at 20 V and 60 V. The desolvation and ion source temperatures were set at 150 °C and 120 °C, respectively. The nitrogen flow rates were 2 L/hr for the cone and 420 L/hr for desolvation. (2) Probe high voltage was set at 4.50 kV, CDL voltage at -5.0 V and temperature at 250 °C, the heat block at 200 °C, Q-array voltage was in the scan mode. The nitrogen flow rate was 3.76 L/min. The ionization potential of JH was tested in MeOH and ACN with and without formic acid as an additive by direct injection.

LC-ESI-MS method development was carried out on the Micromass LCT (kindly provided by Prof. C. Unverzagt, Bioorganic Chemistry, University of Bayreuth, Germany). When the LC-ESI-MS method was validated, the department of Animal Ecology I acquired the Shimadzu LCMS-2010A. Therefore, method validation is required for both instruments due to possible differences in sensitivity and precision.

3.1.2.7. Calibration Curves and Method Validation

Calibration curves were compiled by the standard addition method at four different concentrations. Haemolymph samples from adult Mediterranean field crickets *Gryllus bimaculatus* were used to determine reproducibility and repeatability of the LC-MS method. Haemolymph samples of the same individuals were analyzed in triplicate to ensure repeatability. Additional validation was accomplished by spiking haemolymph samples with standards at differing concentrations. For reproducibility studies, processed haemolymph of one individual was analyzed immediately and again one week later after being stored at – 20 °C. Additionally, spiked haemolymph samples were freshly prepared for each analysis, and each analysis was performed on different days. Validation was confirmed when the coefficient of variation did not exceed 15 - 20 %.

3.2. GC-EI-MS

3.2.1. Materials

Hexane GC^2, ethyl acetate GC^2, acetone UV and diethyl ether were from Burdick and Jackson (Baxter, Stone Mountain, GA, U.S.A.). Al_2O_3 (activated, neutral), MeOH-D_3 (99.8 atom % D) and TFA (99 %) were purchased from Aldrich (Aldrich Chem. Company, Inc., Milwaukee, WI, U.S.A.). The internal standard JH III ethyl ester was kindly provided by Prof. S. Ramaswamy (Department of Entomology, Kansas State University, Manhattan, KS, U.S.A.).

3.2.2. GC-EI-MS Method

3.2.2.1. Haemolymph Sample Preparation

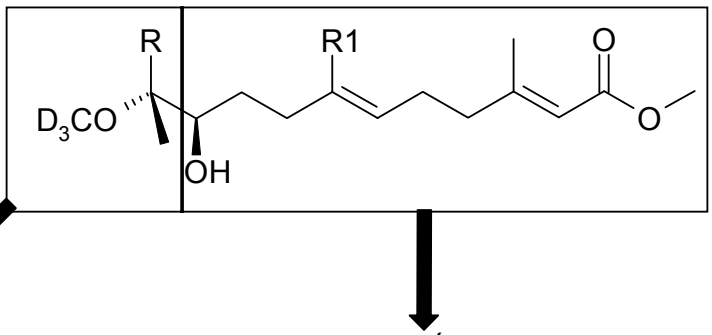
20 µL of insect haemolymph was collected with a glass capillary and blown into ACN:H_2O (1 % NaCl) (1:1, v/v) including the internal standard JH III ethyl ester. The sample was extracted with hexane three times and the hexane phase loaded onto a neutral Al_2O_3 column (6 % H_2O). The columns were assembled by plugging Pasteur pipettes with glass wool and filling them with Al_2O_3 containing 6 % HPLC water (w/w),

having a void volume of 900 µL. The column was rinsed with hexane twice, diethyl ether:hexane (1:9, v/v) twice, and diethyl ether:hexane (3:7, v/v) once, discarding the eluent. Two column volumes of diethyl ether:hexane (3:7, v/v) were used to elute JH from the column, the fraction collected and the solvents evaporated. JH was derivatized by adding MeOH-D_3 (5 % TFA) to the dry sample and incubating at 60 °C for 20 min, obtaining the JH-methoxyhydrin. After incubation, hexane was added and loaded onto newly prepared column again. The column was rinsed with hexane once, diethyl ether:hexane (3:7, v/v) twice, and ethyl acetate:hexane (1:1, v/v) once, disposing of the eluent. The methoxyhydrin was eluted from the column with two column volumes of ethyl acetate:hexane (1:1, v/v). The eluent was evaporated and the residue resuspended in hexane and subjected to analysis.

3.2.2.2. GC-EI-MS Analysis

The JH-methoxyhydrins were separated on a HP 5890 series II Plus GC provided with a 40 m x 0.25 mm Carbowax Econo-Cap column (Alltech, Deerfield, IL, U.S.A.), using helium as carrier gas at a flowrate of 1.0 µL/min and the following temperature gradient: 60 °C for 1 min, increased to 240 °C at 40 °C/min and held at 240 °C for 30 min. The injection port and transfer line were heated to 250 °C. Analysis was carried out on a HP 5972 mass selective detector in the SIM mode, monitoring the fragments resulting from the cleavage at the C_{10} and C_{11} position (see Scheme III).

Scheme III



m/z
JH I = 90
JH II = 90
JH III = 76

m/z
JH I = 239
JH II = 225
JH III = 225

JH I R= Et R1= Et
JH II R= Et R1= Me
JH III R= Me R1= Me

3.3. Insect Rearing Conditions

3.3.1. *Gryllus bimaculatus* de Geer

Mediterranean field crickets *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae) were kept at 27 °C and relative humidity of 30-40 % under a long lighting conditions (16 h light: 8 h dark) rhythm in large plastic containers (30 x 40 x 60 cm, height x width x depth) containing egg cartons for shelter. They were fed a diet consisting of one part commercial rabbit food (2021), one part rat/mouse food (1311) and one part cat food (5031) (Altromin GmbH, Lage, Germany) and supplied with drinking water in watering containers for birds. To prevent drowning of the cricket, the opening was filled with tissue or coarse quartz sand.

In the morning crickets were collected which had just molted and were still white. The age was determined by the days passed since molting. Up to ten crickets were kept in plastic containers under similar conditions, but with moist sand-filled cups for egg laying. Developmental progress in female adult crickets was recorded by determining whole body weights of 0-day to 5-day old *Gryllus bimaculatus* mated females daily in the morning. Ovaries and oviducts were removed and weighed by Mrs. M. Preiß of the same individuals.

3.3.2. *Spodoptera frugiperda*

Eggs of the fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera, Noctuidae) and the artificial diet were provided by Bayer CropScience AG (Leverkusen, Germany). Upon arrival, the eggs were transferred to petri dishes (9.4 cm Ø) and the larvae allowed to emerge. 20 - 30 L1-larvae were transferred using a soft brush to a petri dish containing approximately 35 g of artificial diet (r.t.) and kept at 25 °C under long photoperiod (16 h light:8 h dark). To prevent cannibalism, the larvae were transferred again at L2/3 to an assortment box with 40 compartments (49 x 32 x 36 mm per compartment; Licefa, Bad Salzuflen, Germany) holding each a cube (2.5 - 3 cm; r.t.) of artificial food. For age determination, the larvae were weighed daily, in addition to measuring head capsule width.

3.4. Alkaloids

3.4.1. Materials

Methanol (distilled), diethyl ether, ethyl acetate (distilled), chloroform (distilled), hexane (distilled), water (distilled), Silica Gel 60 (40 - 63 µm) for OCC, Dragendorff's reagent, and diemethyl sulfoxide-D_6 were purchased from Merck (Darmstadt, Germany). The solvents used for extraction were of technical grade and subsequently distilled. Silica Gel 60 aluminum TLC-plates (2 - 24 µm), and chloroform-D_1 were bought from Sigma-Aldrich (Taufkirchen, Germany). Methanol crude extract (12.24 g) of *Glycosmis pentaphylla* leaves was provided by Prof. J. Muthukrishnan (School of Biological Sciences, Madurai Kamaraj University, Madurai, India).

3.4.2. Extraction

The methanol crude extract was acidified and further extracted with diethyl ether, removing lipids, alkanes, and other lipophilic substances. The aqueous layer was adjusted to a pH of 7-8 and again extracted with ethyl acetate (Fig. 1). The diethyl ether (0.931 g) and ethyl acetate fractions (0.320 g) were checked by TLC for nitrogen-containing compounds and fractionated by OCC on silica gel using hexane:ethyl acetate (6:4, v/v), chloroform, and chloroform:methanol (9:1, v/v) successively.

3.4.3. Identification

^{1}H, ^{13}C, H-H COSY, HMBC, and HMQC spectra were recorded on an AC 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz, Bruker) and DRX 500 (^{1}H: 500.13 MHz, ^{13}C: 125.13 MHz, Bruker) spectrometer. Spectra were referenced to chloroform-d_1 (^{1}H: 7.24 ppm, ^{13}C: 77.0 ppm) and diemethyl sulfoxide-D_6 (^{1}H: 2.49 ppm, ^{13}C: 39.5 ppm). The masses were determined either by ESI-MS (Micromass LCT/Shimadzu LCMS-2010A) or EI-MS (Finnigan MAT 312, 70eV) direct injection in methanol or acetonitrile.

4. Results

4.1. LC-ESI-MS Method Development for JH and Ecdysteroid Titer Determinations

4.1.1. Verification of JH diols

The identity of the synthesized JH III diol was confirmed by EI-MS (70 eV) and ^{1}H-, ^{13}C-NMR (in $CDCl_3$, Table 2). EI-MS *m/z*: 284 $[M]^+$. At position 10 the ^{1}H-signal shifts from the JH III signal of 2.70 ppm to 2.64 ppm in the JH III diol. A shift is observed as well for the ^{13}C-signal from 64.10 ppm in JH III to 77.99 ppm in the JH III diol. The ^{13}C-signal at position 11 also changes: from 58.30 ppm in JH III to 73.02 ppm in the JH III diol.

The molecular weights of JH II diol and JH I diol were confirmed by ESI-MS *m/z*: 321 $[M+Na]^+$ and 335 $[M+Na]^+$, respectively.

Position	*^{1}H (δ)*	*^{13}C (δ)*	*Position*	*^{1}H (δ)*	*^{13}C (δ)*
1		159.96	7′	1.60	15.86
2	5.65	115.41	8	2.18	36.60
3		135.90	9	1.61	33.24
3′	1.60	18.74	10	2.64	77.99
4	1.39	40.73	11		73.02
5	2.14	29.45	11′	1.18	26.41
6	5.13	123.69	12	1.14	25.73
7		124.240	OCH_3	3.66	50.86

Table 2 ^{1}H- and ^{13}C-NMR data for JH III diol

4.1.2. Verification of JH acids

The molecular weights of synthesized JH III acid, JH II acid, and JH I acid were confirmed by ESI-MS *m/z*: 275 $[M+Na]^+$, 289 $[M+Na]^+$, and 303 $[M+Na]^+$.

4.1.3. HPLC Optimisation

The ACN gradient (Table 1) gave good separation of the JH diols and the JH homologes within 15 min. Throughout the gradient a steady baseline was observed (Fig. 5). Good separation was also achieved with the MeOH-gradient (Table 1), with a reduction in separation time. The baseline, however, was irregular and absorbance increased with an increase in the MeOH concentration (Fig. 6). The peak width also increased: JH I diol peak width at ½-peak height was 6.42 s for the ACN-gradient, but 10.4 s for the MeOH-gradient. Further modification of the MeOH-gradient by decreasing the steepness of the gradient (0-15 min 80-100 % MeOH) improved the baseline (Fig. 7).

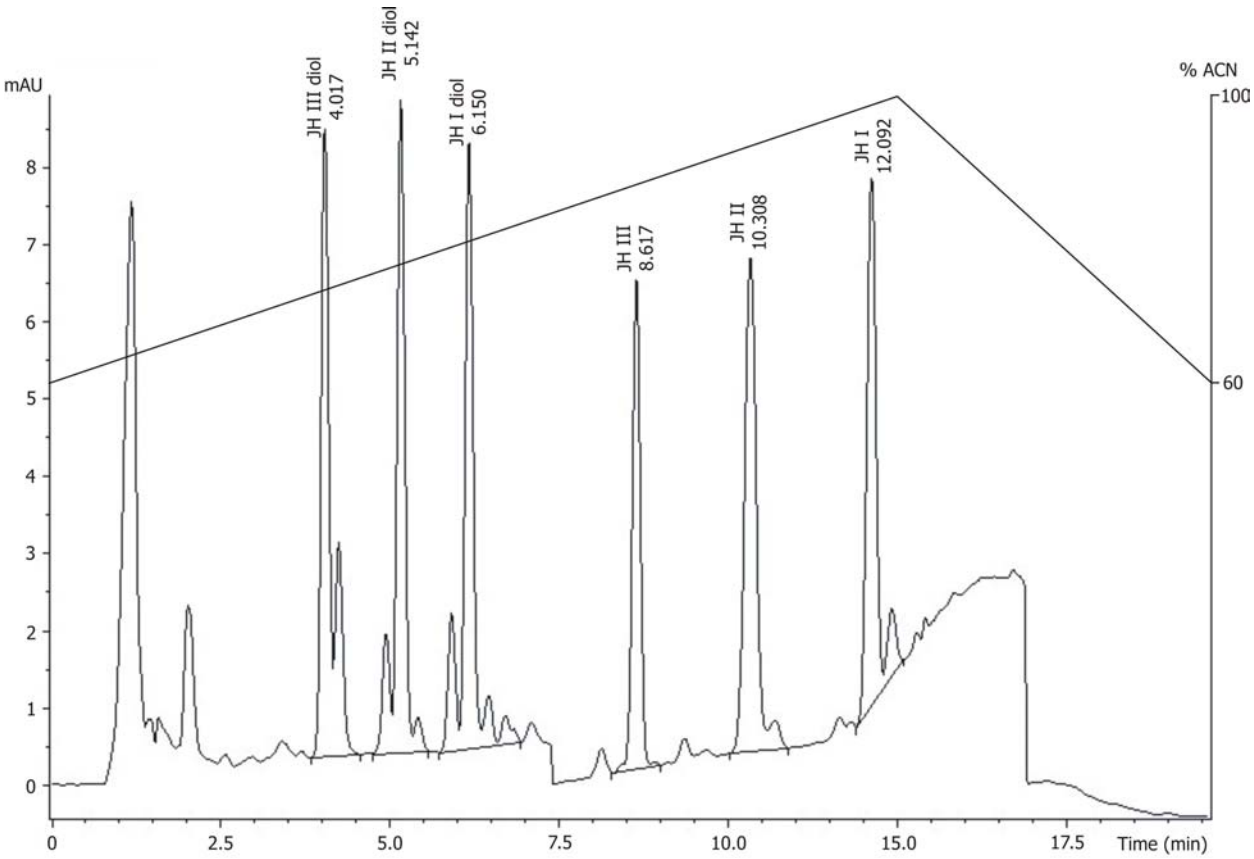


Fig. 5 ACN gradient of a 10 ng mixture of JHs and JH diols

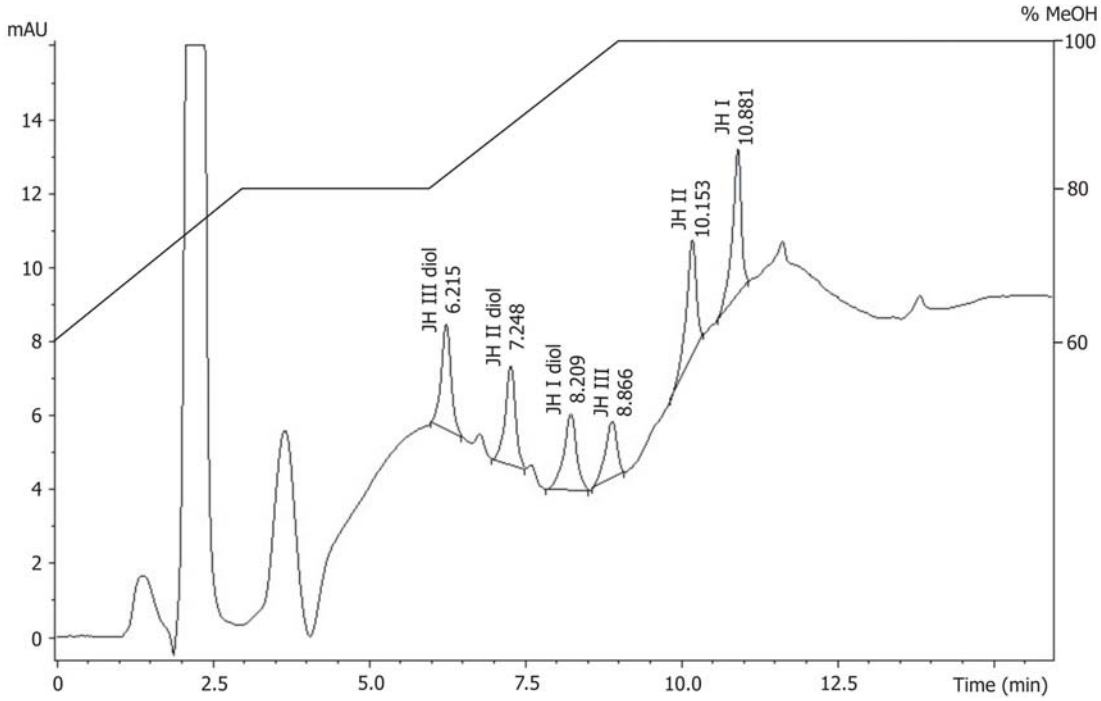


Fig. 6 MeOH gradient of a 5 ng mixture of JHs and JH diols

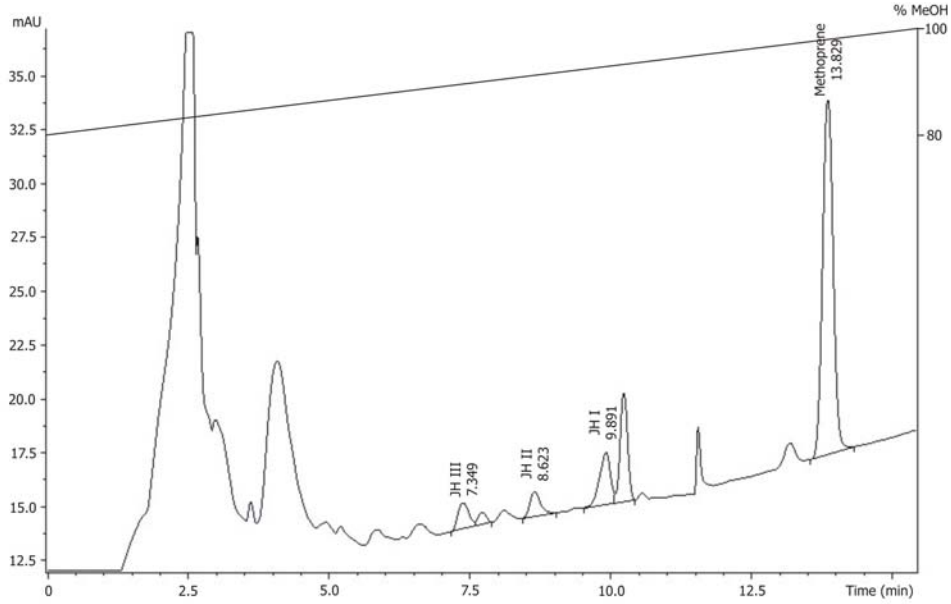


Fig. 7 MeOH gradient of a 4 ng mixture of JHs and methoprene

4.1.4. JHBP Assay

JHBP is a lipoprotein assumed to carry JH in a hydrophobic pocket (Peterson et al., 1977; Goodman et al., 1978; Prestwich et al., 1987). Therefore a JHBP assay was carried out to determine the effectiveness of MeOH, ACN, diethyl ether-MeOH, which are used for delipidation of plasma lipoproteins (Osborne, 1986), acetone and acetone-MeOH, which delipidate and precipitate proteins (Penefsky and Tzagoloff, 1971), and isooctane-MeOH, as a representative of a hydrophilic-hydrophobic system, in disrupting JH binding to the JHBP and ensuring sufficient uptake of JH by the solvent. Besides organic solvents, mechanical treatment is known to be effective in precipitation of proteins and disrupting ligand-protein binding, therefore the effectiveness of sonication was also assessed.

4.1.4.1. Stability of JHs and JH Diols during Sonication

Sonication of JHs for 10 min in Tris/KCl-buffer (pH 7.5) substantially reduced the JH I concentration by 43 %, JH II by 38 %, and JH III by 61 % (Fig. 8). An increase of JH I diol, JH II diol, and JH III diol by 10 %, 12 %, and 14 %, respectively, was observed.

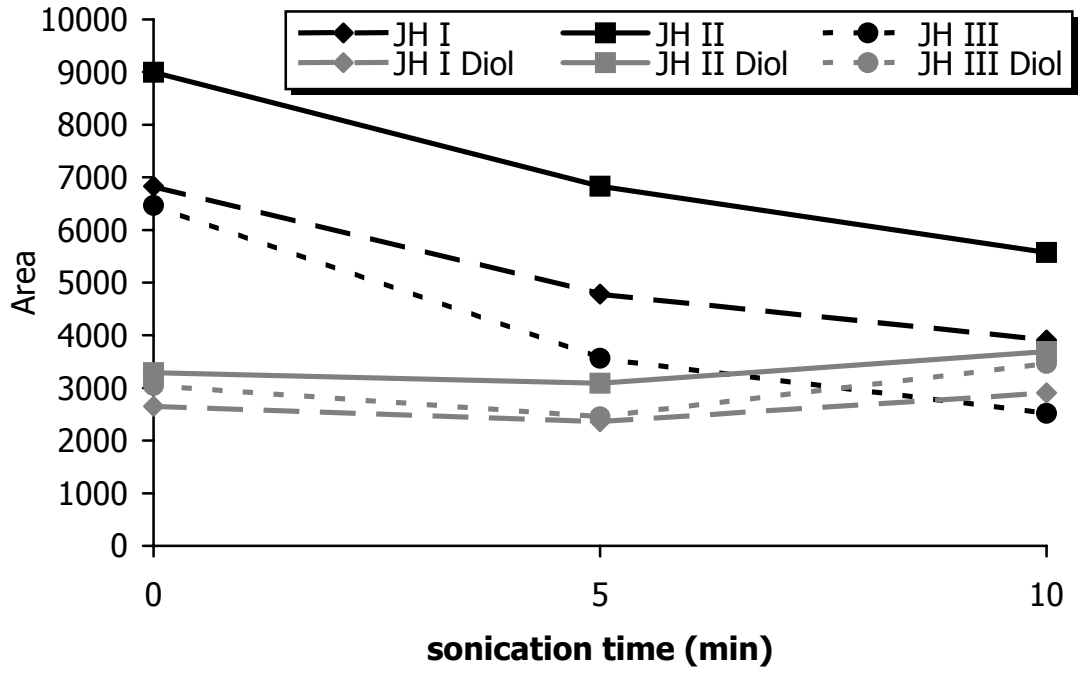


Fig. 8 Stability of JHs and JH diols during sonication

4.1.4.2. Efficiency of MeOH, ACN, Diethyl Ether-MeOH, Acetone, Acetone-MeOH, and Isooctane-MeOH in JHBP denaturation

Initially, triton X-100 was used as a carrier to keep JH in aqueous buffer for the duration of the JHBP assay. HPLC, though, was insufficient in separating Triton X-100 and JH on the column. Triton X-100 eluted at a similar retention time window as JH. Since JH is soluble in aqueous solutions to a certain extent, the assay was carried out without the use of triton X-100. At first, the JHBP was added directly to the buffer containing JH, but JHBP acted as a "sponge" by removing JH from the glass surface.

Finally, JH was allowed to equilibrate at r.t. with the buffer and then an aliquot of the suspension was added to the JHBP, which was in a new vial. The different solvents tested for their efficiency in removing JH from the JHBP were similarly effective in JH removal, except for diethyl ether and acetone. They were incompatible as carrier solvents for the HPLC, resulting in fronting peaks. Evaporation of diethyl ether or acetone under vacuum centrifugation and uptake by MeOH or ACN was not efficient, since MeOH or ACN was not able to remove JH from the glass surface. Further investigation showed that by simply injecting the JH-JHBP complex onto the column, the HPLC solvents were sufficient in releasing JH from the JHBP.

Studies on the affinity of JH to polypropylene vials demonstrated, that JH is highly adsorbed to polypropylene vials, since neither ACN nor MeOH was able to extract approximately 1 ng of JH.

4.1.5. Haemolymph Sample Preparation

Darkening of the haemolymph is due to the eumelanin production by phenol oxidases (Nappi and Sugumaran, 1993). The absence of discoloration in haemolymph served as a visual indicator of enzyme inactivation.

The most effective solvent system for haemolymph preparation was isooctane-MeOH. The haemolymph did not coagulate or show any discoloration at a solvent-haemolymph ratio of 10. Using ACN, the haemolymph did not emulsify well, even after being vortexed vigorously for 1 min. MeOH was not efficient in halting enzyme activity, discoloration of the haemolymph was still observed. Cooling the sample was also considered in order to halt enzyme activity, but proved unsuitable due to incomplete protein precipitation, which caused obstruction of the autosampler needle and HPLC capillary.

JH I, JH II, and JH III were most effectively retained in solution in the presence of haemolymph extracts, when compared to only MeOH (Fig. 9).

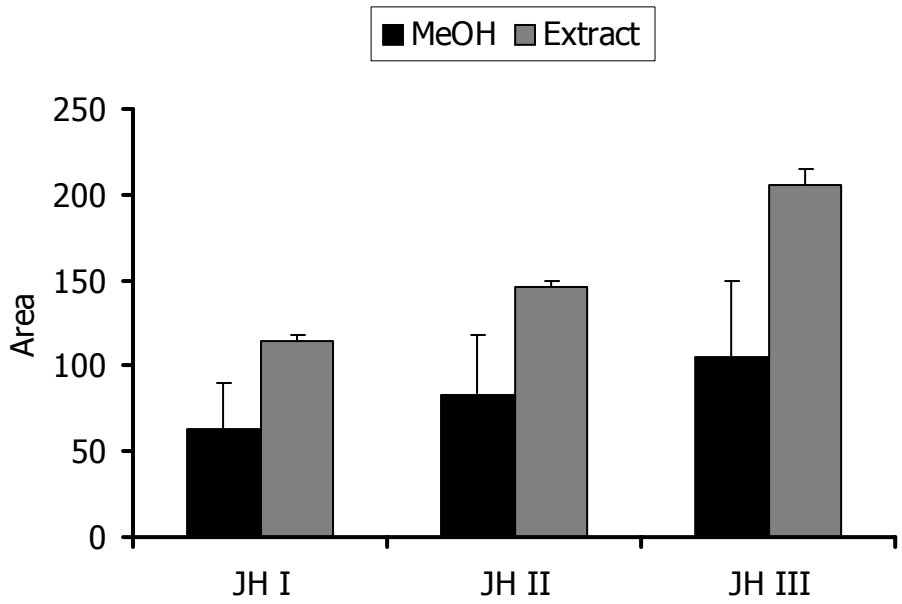


Fig. 9 JH affinity to glass in different media (n = 4; mean ± S.D.)

4.1.6. LC-ESI-MS Optimisation (Micromass LCT)

The ionization of JHs is highest in MeOH at a cone voltage of 20 V in the positive ion mode (100 %). The addition of 0.1 % formic acid to MeOH decreased ionization by 10 %, when compared to MeOH only. A decrease in sensitivity by 37 % was observed when using ACN with 0.1 % formic acid and a 62 % reduction in only ACN. At a higher voltage (60 V), JHs fragment and are no longer detectable. JH I diol, JH II diol, and JH III diol experienced 20 %, 29 %, and 23 % fragmentation, respectively, at 60 V.

In MeOH, the predominant ions for the JH diols and JHs are $[M+Na]^+$, $[M+H]^+$, $[M-OH]^+$, and for JHs also $[M-CH_3O]^+$ (Table 3; Fig. 10). In haemolymph $[M+Na]^+$ is mainly formed, whereas at higher concentrations, the $[M+K]^+$ can also be observed (Fig. 11).

	$[M+Na]^+$	*$[M+H]^+$*	*$[M-OH]^+$*	*$[M-CH_2O]^+$*
JH III Diol	307	285	267	-
JH II Diol	321	299	281	-
JH I Diol	335	313	295	-
JH III	289	267	249	235
JH II	303	281	263	249
JH I	317	295	277	263

Table 3 Ionization of JH diols and JHs at a cone voltage of 20 V in MeOH (Micromass LCT)

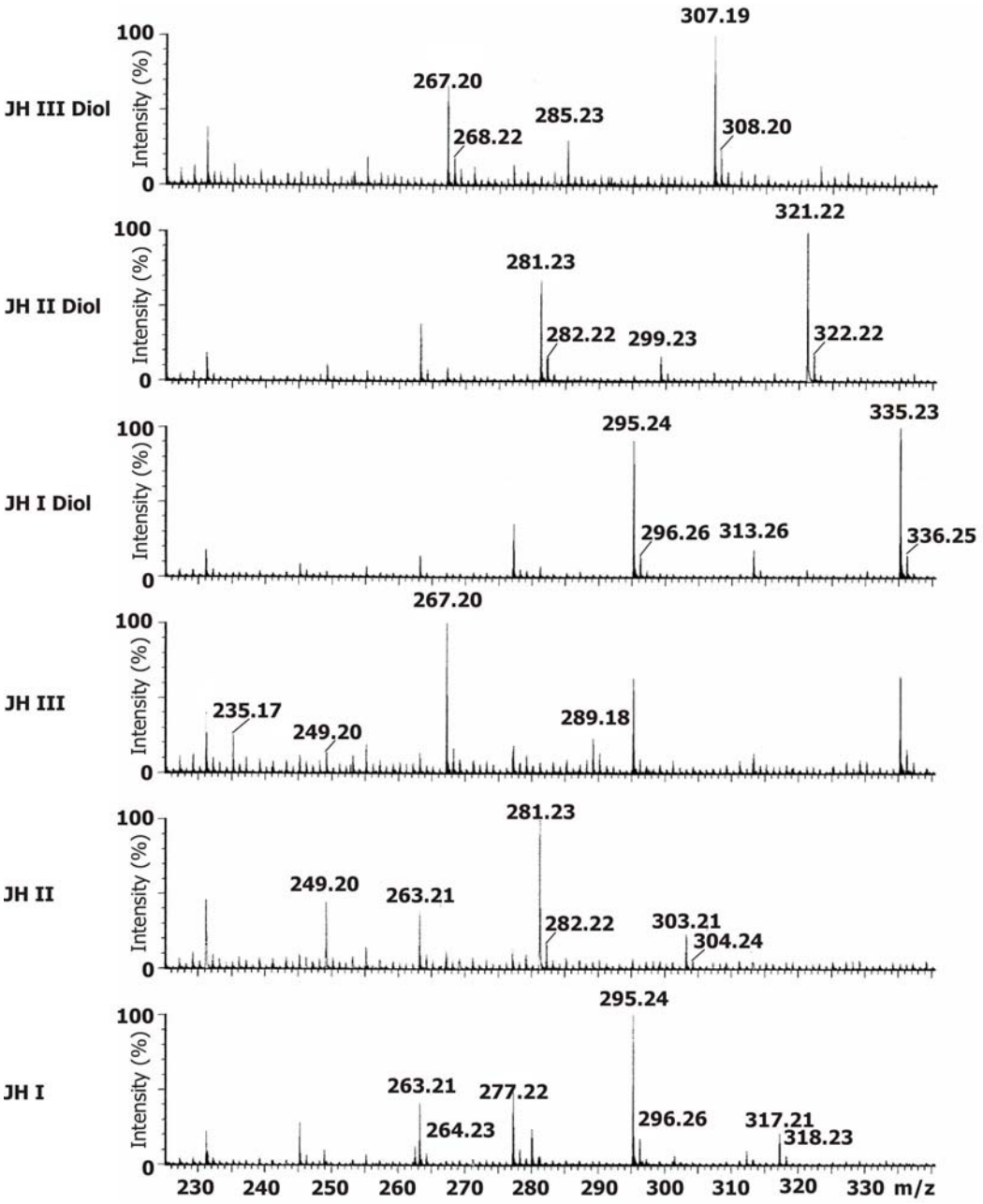


Fig. 10 Mass spectra of JHs and JH diols in MeOH (Micromass LCT)

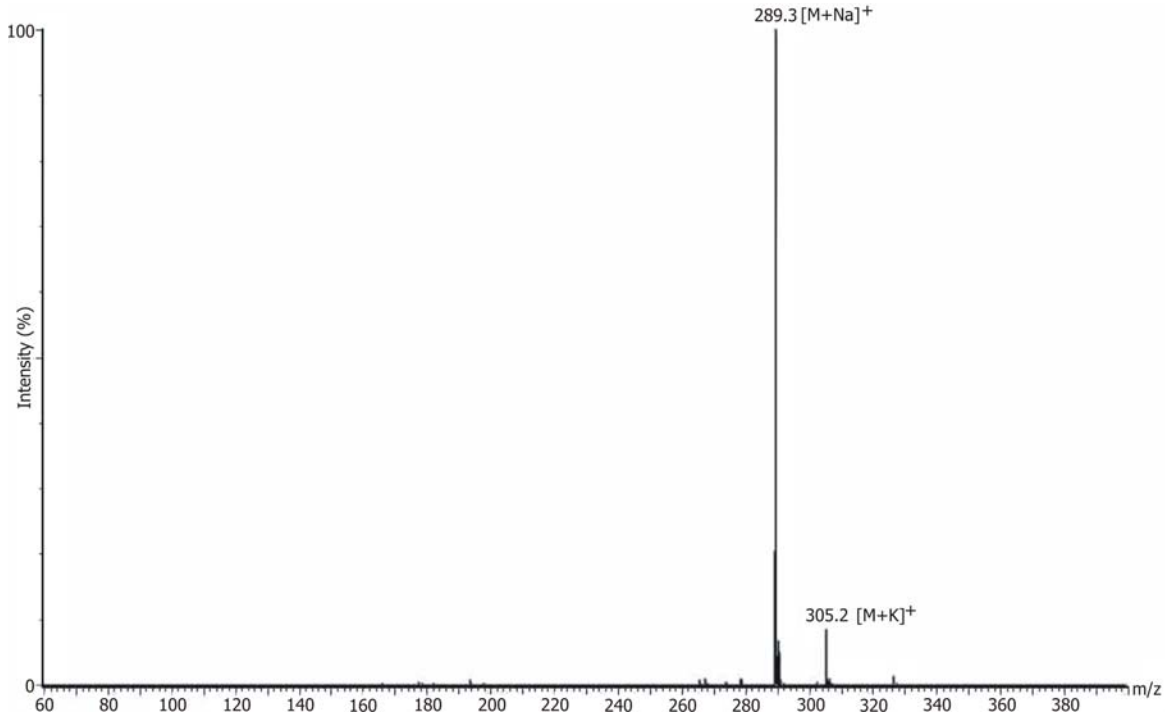


Fig. 11 Mass spectrum of JH III ($[M+Na]^+$ / $[M+K]^+$) in insect haemolymph (Micromass LCT)

Due to the instability of the JH acid standards in solution, quantitative analysis could not be conducted. Qualitative analysis was possible to a certain extent. JH acids ionize to form the $[M+Na]^+$ and the $[M+K]^+$ and elute between 2 and 3 min prior to the corresponding JH.

The MeOH-gradient 80-100 % MeOH in 15 min (3.1.2.3., Table 1) was modified to include an equilibration step at the end of the HPLC run for automation: 0-5 min 80 % MeOH, 5-7 min 80-100 % MeOH, 7-15 min 100 % MeOH, 15-17 min 100-80 % MeOH, and 17-20 min 80 % MeOH.

4.1.7. Quantitation and Method Validation (Micromass LCT)

4.1.7.1. Calibration Curves

The compiled calibration curves for JHs and JH diols were linear and had correlation coefficients greater than 0.98 (Figs. 12-17).

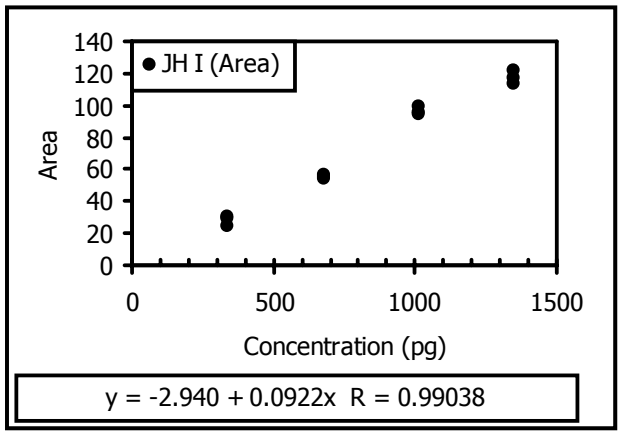


Fig. 12 JH I calibration curve (Micromass LCT)

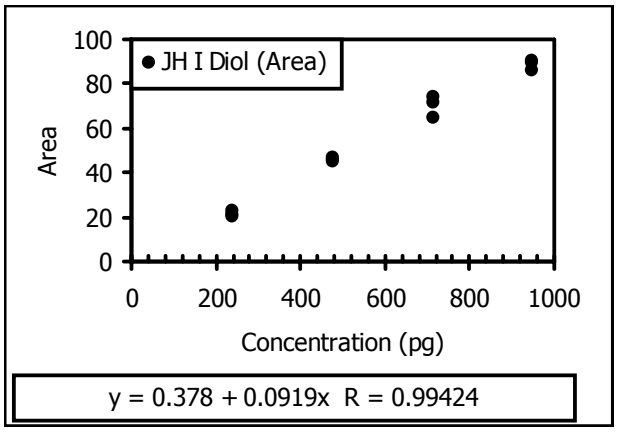


Fig. 13 JH I diol calibration curve (Micromass LCT)

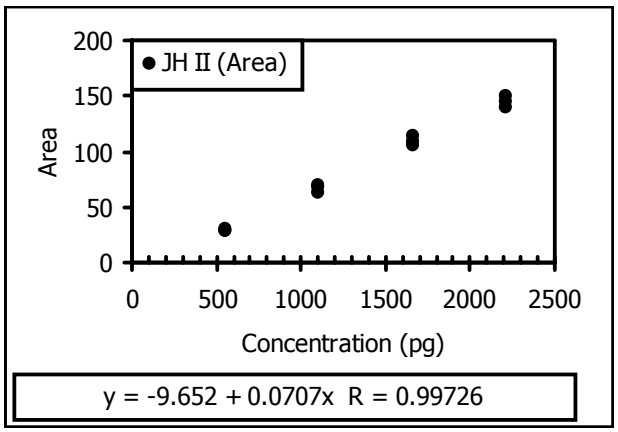


Fig. 14 JH II calibration curve (Micromass LCT)

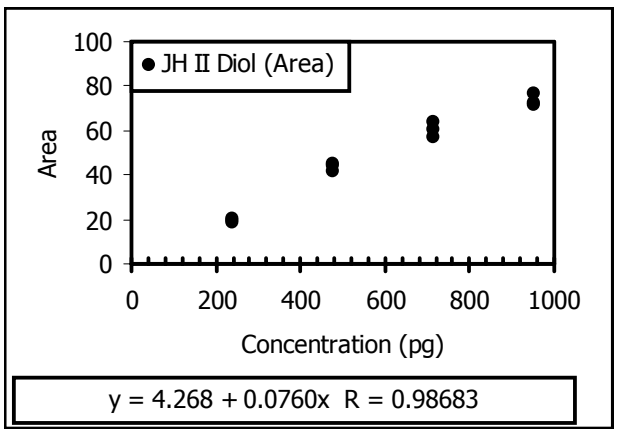


Fig. 15 JH II diol calibration curve (Micromass LCT)

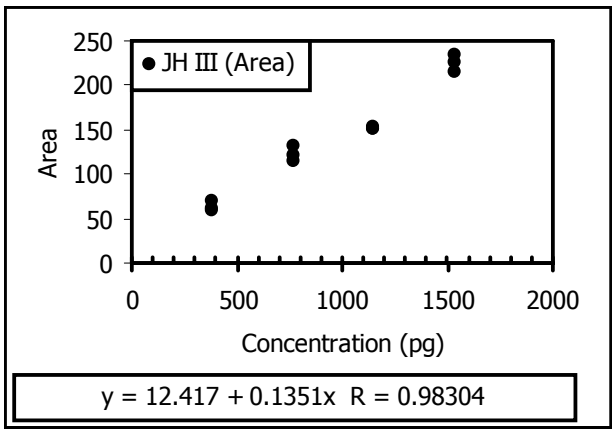


Fig. 16 JH III calibration curve (Micromass LCT)

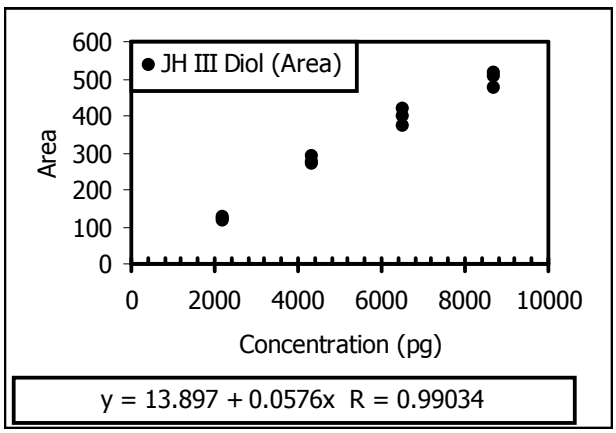


Fig. 17 JH III diol calibration curve (Micromass LCT)

4.1.7.2. LOD and LOQ

The LODs for JHs and JH diols were 8 pg and 6 pg, and the LOQs were 20 pg and 25 pg, respectively. The LODs and LOQs were calculated from the equations given in Fig. 18 for JHs and Fig. 19 for JH diols using a S/N of 3 and 10 for the LOD and the LOQ, correspondingly.

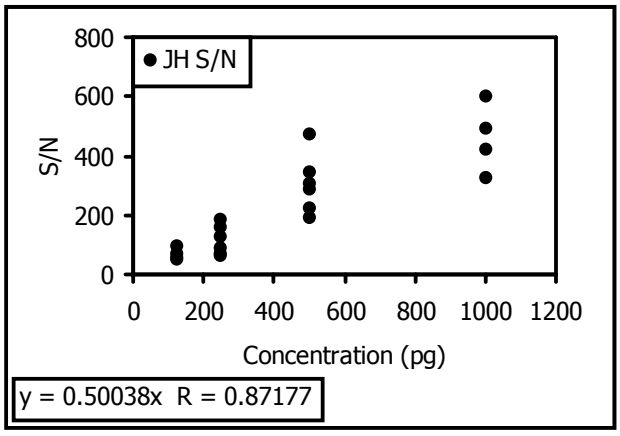


Fig. 18 S/N for JHs (Micromass LCT)

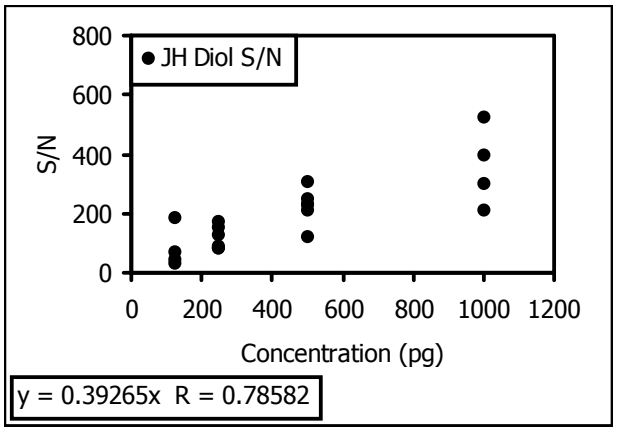


Fig. 19 S/N for JH Diols (Micromass LCT)

4.1.7.3. Method Validation

Repeatability and reproducibility assess precision obtained during repeated analysis of the same sample under varying conditions (see 3.1.2.7. for more detail). A measure of precision is the coefficient of variation, also called the relative standard deviation [(S.D. *100)/mean], which should not exceed 20 % at the lowest concentration and 15 % otherwise (U.S. Food and Drug Administration, 2001). The coefficients of variation show that good reproducibility and repeatability was achieved for unspiked (Table 4) and spiked haemolymph samples (Table 5), except for reproducibility studies of JH I diol where the coefficient of variation is 23.7. The retention time window for all JHs and JH diols was less than ± 0.3 %.

Sample	*Coefficient of variation*		
	N	Repeatability	Reproducibility
6-day old female	3	1.78	
8-day old female	3	2.46	
3-day old female	2		7.09

Table 4 Repeatability and reproducibility studies of unspiked *Gryllus bimaculatus* haemolymph samples (Micromass LCT)

Standard (ng)	*Coefficient of variation*						
	Repeatability						
	N	JH I	JH II	JH III	JH I diol	JH II diol	JH III diol
0.5	3	11.64	5.05	9.72	6.28	4.41	3.56
1.0	3	2.10	5.13	7.01	1.63	3.56	3.91
1.5	3	2.62	3.38	0.72	6.80	5.56	6.04
2.0	3	3.17	3.04	4.15	2.31	3.67	4.05
	Reproducibility						
2.0	3	13.24	6.94	11.22	23.75	14.22	10.66

Table 5 Repeatability and reproducibility studies of spiked *Gryllus bimaculatus* haemolymph samples (Micromass LCT)

4.1.8. LC-ESI-MS Optimisation (Shimadzu LCMS-2010A)

In MeOH and haemolymph, similar JH and JH diol ionization patterns (see 4.1.6.) were observed on the Shimadzu LCMS-2010A. The ecdysteroids exhibit a slightly different ionization pattern in haemolymph, when compared to JHs and JH diols, producing $[M+Na]^+$ and $[M+K]^+$ in approximately a 1:0.5 ratio (Fig. 20 and 21). Analysis was conducted in the positive ion mode, screening only the $[M+Na]^+$ ions listed in Table 3 and Table 6.

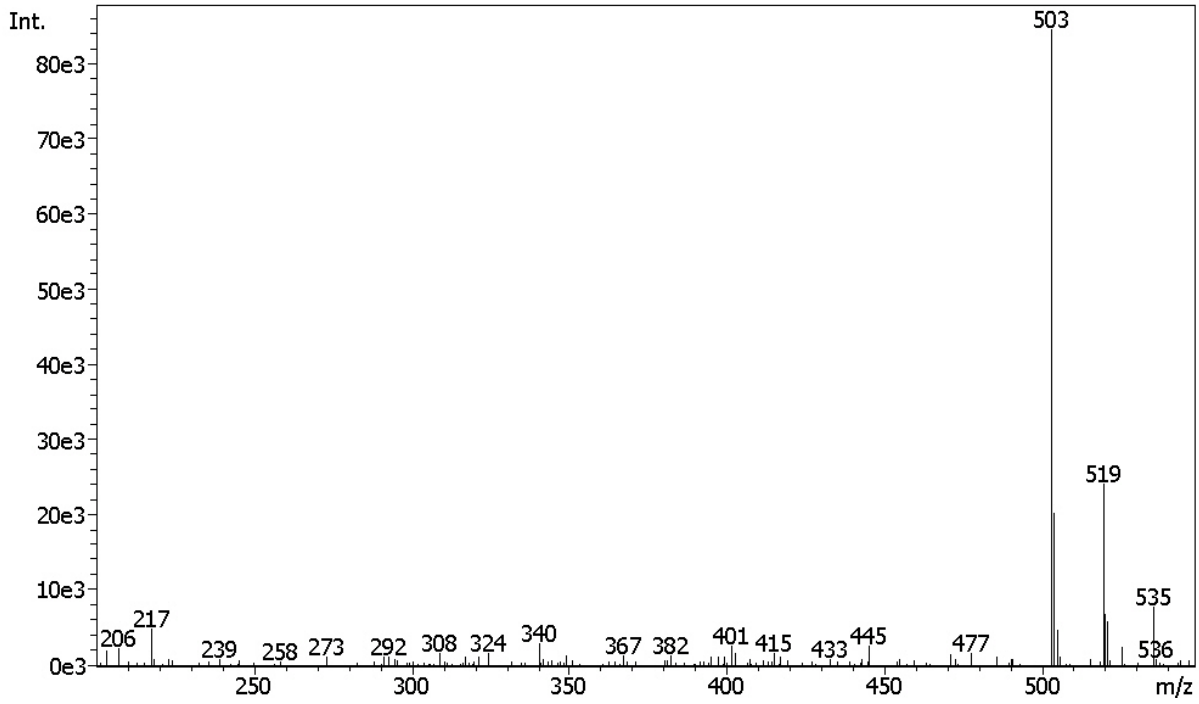


Fig. 20 Ionization pattern of 20-hydroxyecdysone in haemolymph (Shimdazu LCMS-2010A)

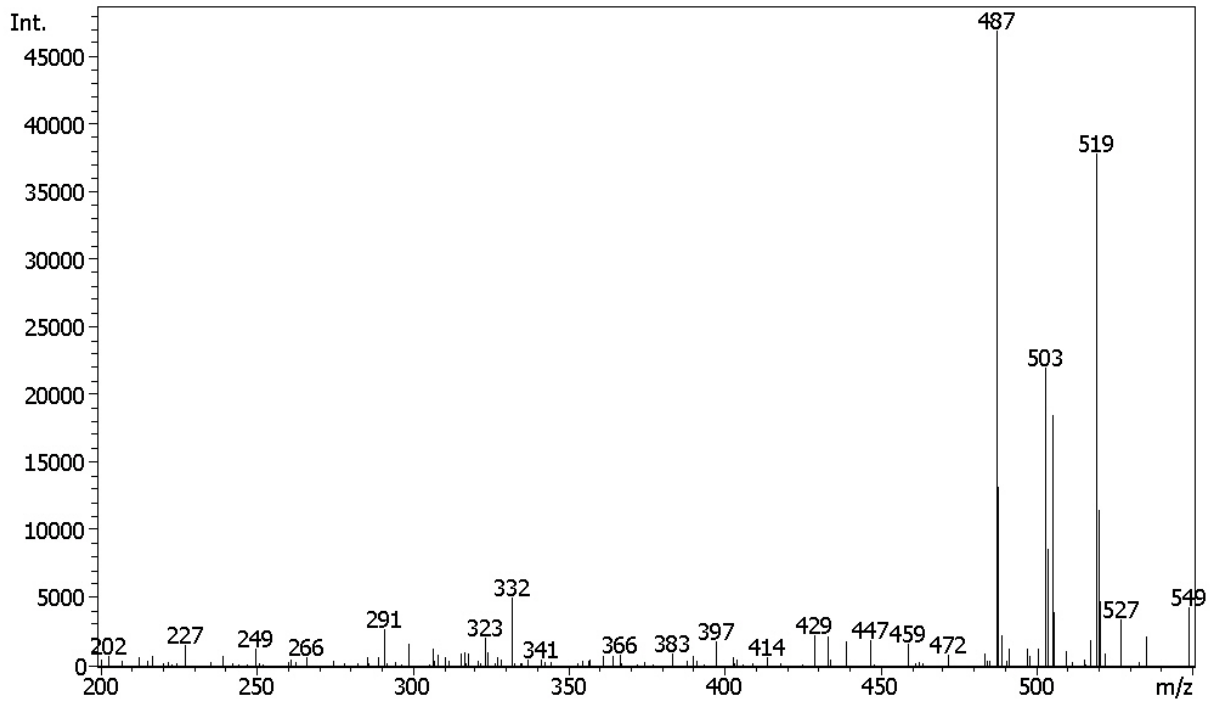


Fig. 21 Ionization pattern of ecdysone in haemolymph (Shimdazu LCMS-2010A)

	20-Hydroxyecdysone	*Ecdysone*
m/z	503/519	487/503

Table 6 Ecdysteroid ions monitored in haemolymph ($[M+Na]^+$ / $[M+K]^+$)

4.1.9. Quantitation and Method Validation (Shimadzu LCMS-2010A)

4.1.9.1. Calibration Curves

The correlation coefficients for the ecdysteroids, JHs and JH diols were greater than 0.98 (Figs. 22 - 29). Linearity was observed throughout all calibration curves.

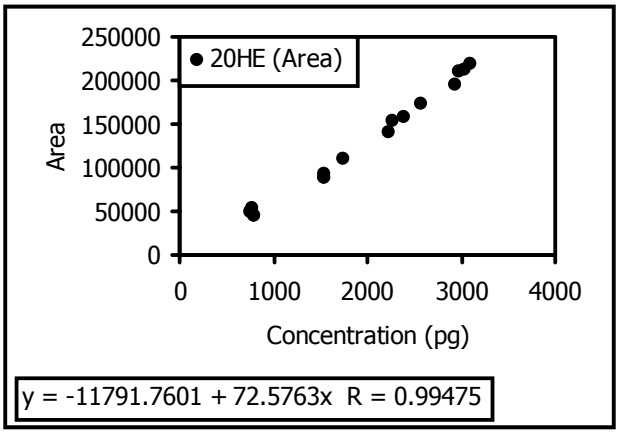


Fig. 22 20-Hydroxyecdysone calibration curve (Shimadzu LCMS-2010A)

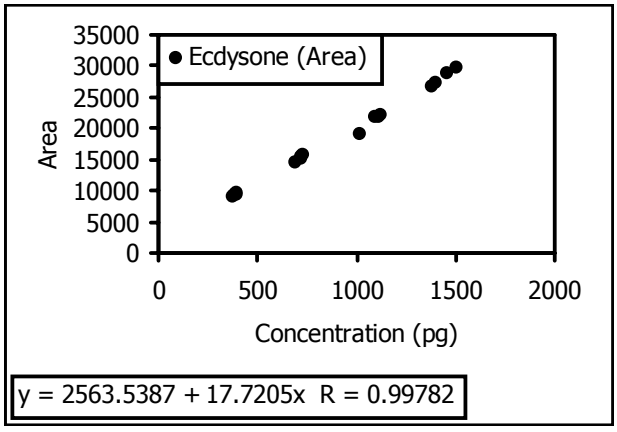


Fig. 23 Ecdysone calibration curve (Shimadzu LCMS-2010A)

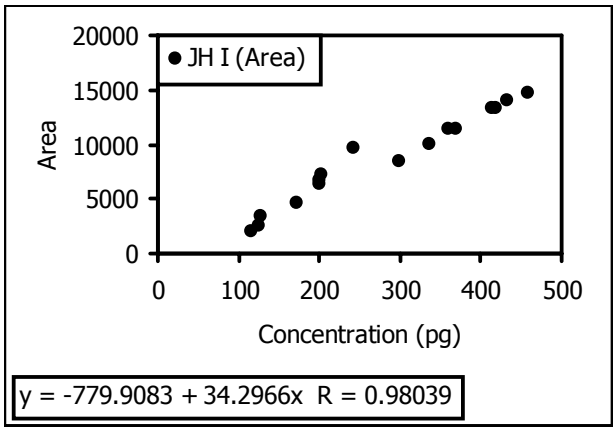


Fig. 24 JH I calibration curve (Shimadzu LCMS-2010A)

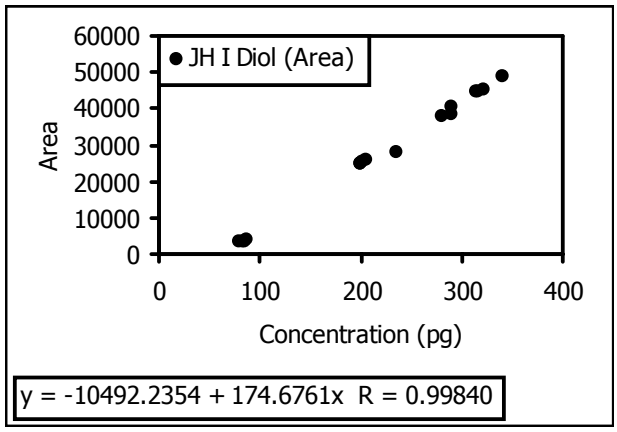


Fig. 25 JH I diol calibration curve (Shimadzu LCMS-2010A)

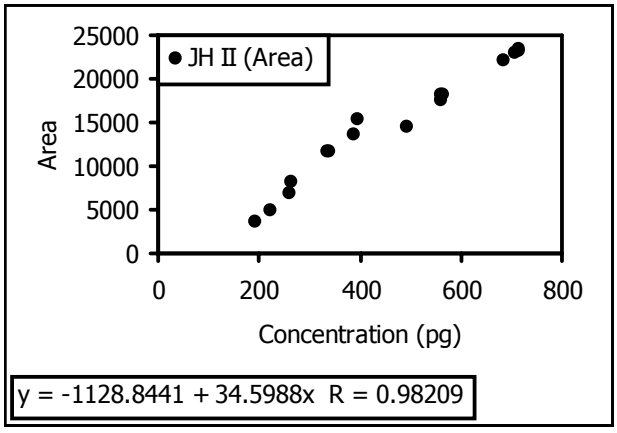


Fig. 26 JH II calibration curve (Shimadzu LCMS-2010A)

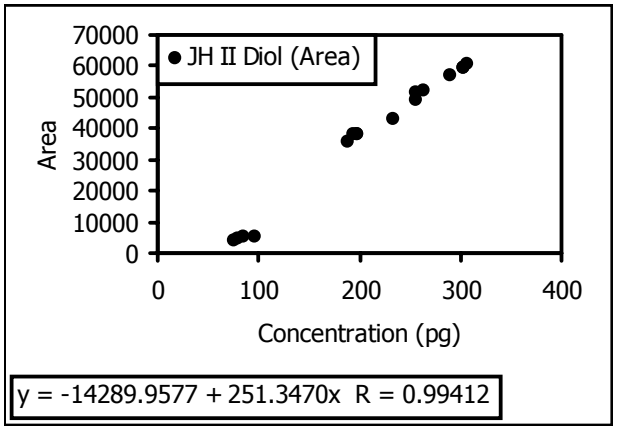


Fig. 27 JH II diol calibration curve (Shimadzu LCMS-2010A)

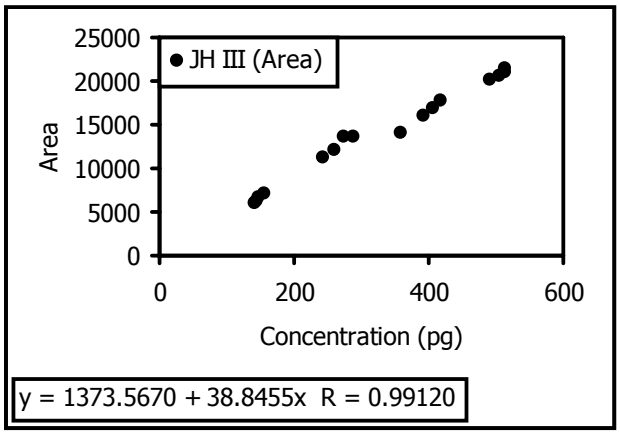


Fig. 28 JH III calibration curve (Shimadzu LCMS-2010A)

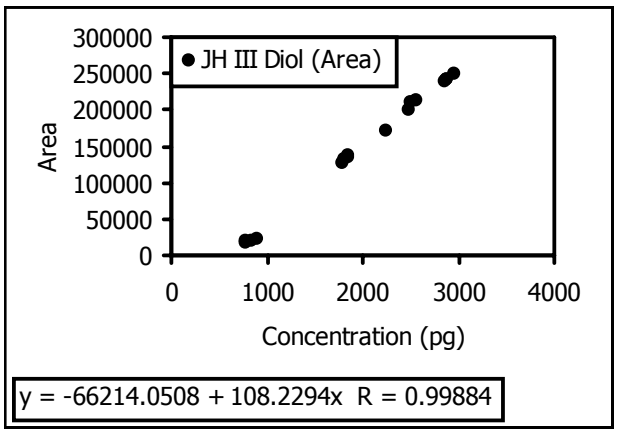


Fig. 29 JH III diol calibration curve (Shimadzu LCMS-2010A)

4.1.9.2. LOD and LOQ

The calculated LOD and LOQ for ecdysteroids were 93 pg and 310 pg, respectively. For JHs and JH diols calculations resulted in the following values for the LOD: JHs 12 pg and JH diols 10 pg. The LOQ values were: JHs 40 pg and JH diols 34 pg. The LODs and LOQs were calculated from the equations given in Figs. 30-32 using a S/N of 3 and 10 as the LOD and the LOQ, correspondingly.

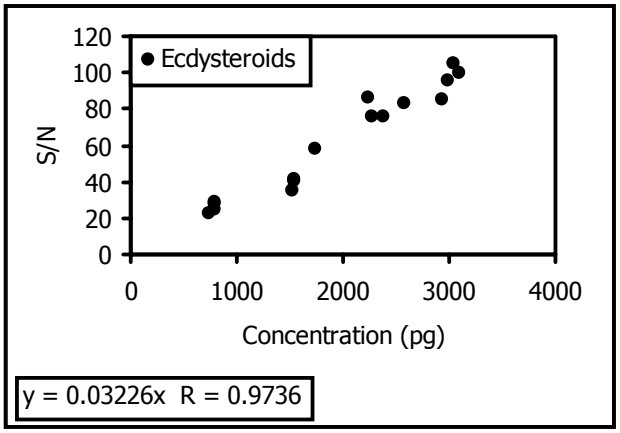


Fig. 30 S/N for ecdysteroids (Shimadzu LCMS-2010A)

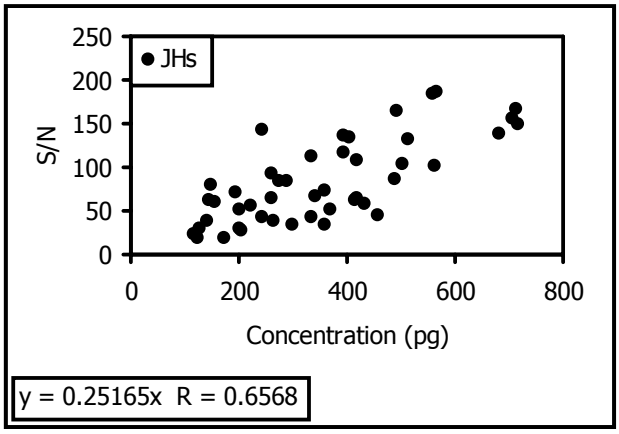


Fig. 31 S/N for JHs
(Shimadzu LCMS-2010A)

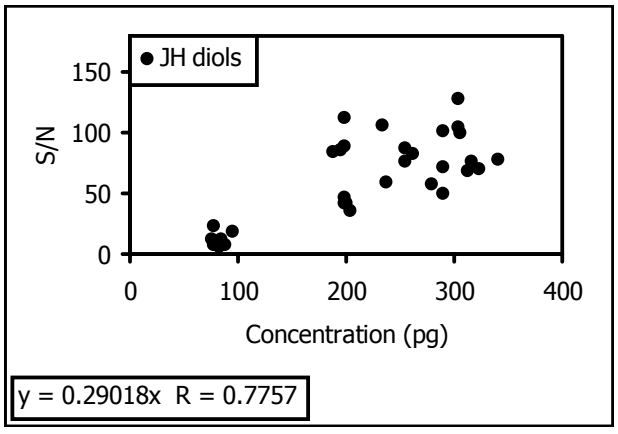


Fig. 32 S/N for JH diols
(Shimadzu LCMS-2010A)

4.1.9.3. Method Validation

Good repeatability was achieved for spiked haemolymph samples (Table 7) with a retention time window of less than ± 0.4 %. The coefficient of variation for ecdysone was slightly above 20 % at a low concentration. The concentrations used are listed in Table 8.

Standard	*Coefficient of variation*								
	Repeatability								
	N	20HE	E	JH I	JH II	JH III	JH I diol	JH II diol	JH III diol
A	4	3.01	21.45	18.84	14.55	4.04	4.48	10.30	7.24
B	4	6.39	10.00	9.81	8.51	7.08	1.32	2.46	1.54
C	4	6.53	8.53	8.95	6.33	6.56	9.12	5.10	5.60
D	4	2.28	4.45	4.52	2.19	2.22	3.76	2.39	1.52

Table 7 Repeatability studies of spiked *Gryllus bimaculatus* haemolymph samples (Shimadzu LCMS-2010A)

	Concentration (pg)							
Standard	20HE	E	JH I	JH II	JH III	JH I diol	JH II diol	JH III diol
A	777	388	115	192	142	87	84	782
B	1554	776	230	384	284	174	168	1564
C	2331	1164	345	576	426	261	252	2346
D	3108	1552	460	768	568	348	336	3128

Table 8 Concentrations used for repeatability studies on spiked *Gryllus bimaculatus* haemolymph samples (Shimadzu LCMS-2010A)

4.2. Determining JH and Ecdysteroid Titers by LC-ESI-MS

4.2.1. *Gryllus bimaculatus*

4.2.1.1. JH Titers

The JH titer determinations are mean values ± S.E.M, unless noted otherwise. The number of individuals are listed in Table 9 (LL = last instar larvae, A = Adult).

Stage / Day	*females*	*males*	*Stage / Day*	*females*	*males*
LL / 2	14	10	A / 3	20	5
LL / 3	8	8	A / 4	10	5
LL / 4	9	8	A / 5	15	5
LL / 5	8	7	A / 6	12	11
LL / 6	8	7	A / 7	16	9
LL / 7	7	3	A / 8	12	5
LL / 8	8	4	A / 9	6	2
A / 0	15	5	A / 10	9	2
A / 1	13	5	A / 11	9	7
A / 2	21	5	A / 12	7	5

Table 9 Number of individuals used for JH titer determinations in *Gryllus bimaculatus* haemolymph at different developmental stages

In last instar larvae and adult *Gryllus bimaculatus* females and males, JH III was found throughout development (Figs. 33 and 34). Last instar larvae of both sexes exhibit a rather steady JH III titer (female: 27 ± 5 pg/µL; male: 27 ± 6 pg/µL) throughout larval development. A maximum JH III titer was observed in adult females on day 4 (94 ± 35 pg/µL) and in adult males on day 3 (111 ± 20 pg/µL).

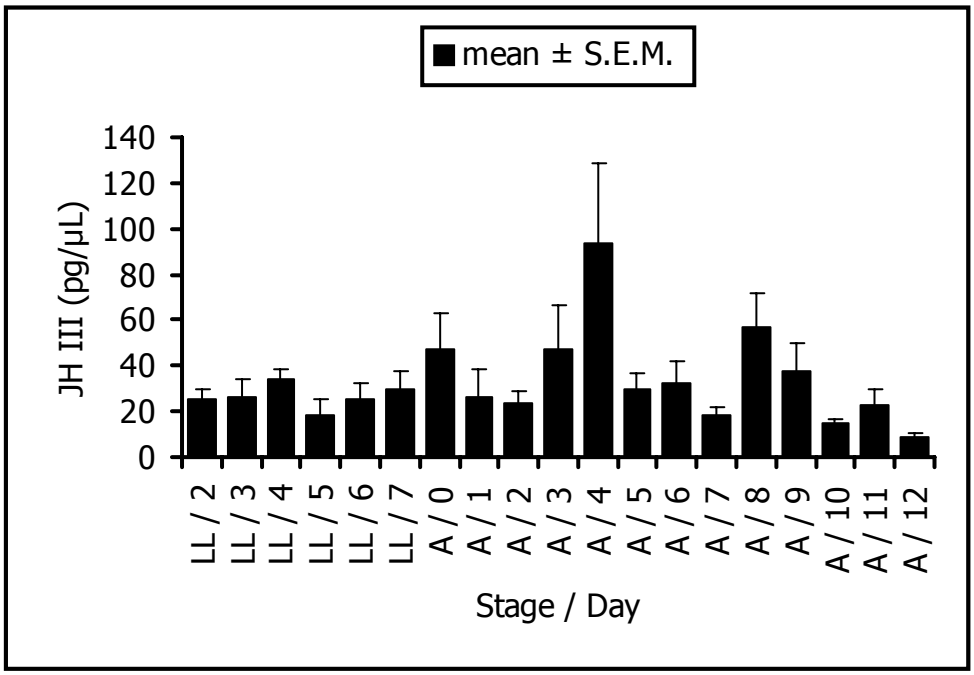


Fig. 33 JH III titers in the haemolymph of last instar larvae (LL) and mated adult (A) *Gryllus bimaculatus* females

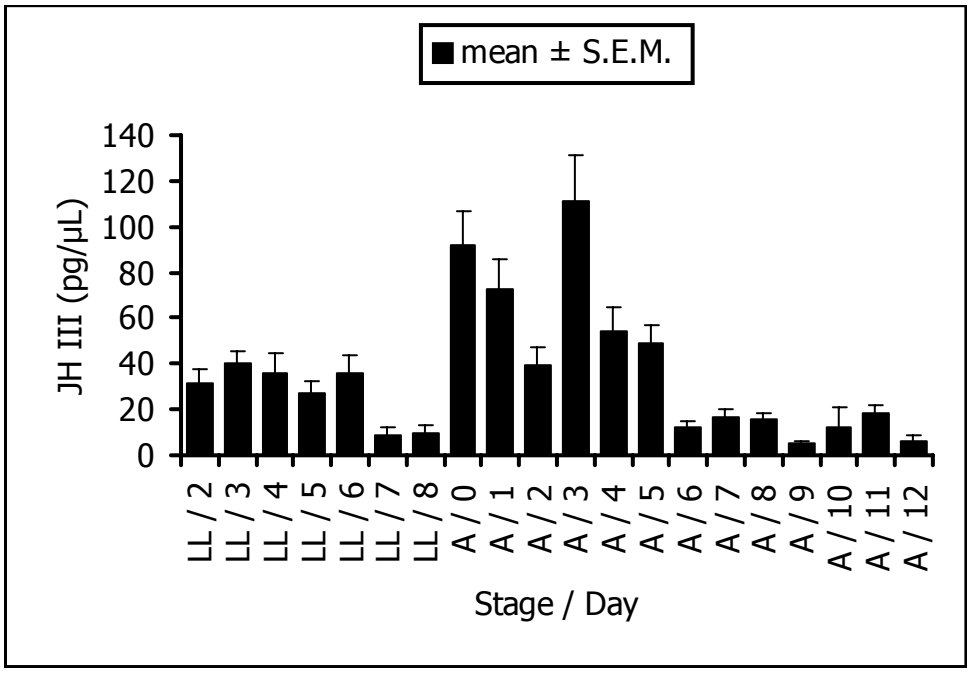


Fig. 34 JH III titers in the haemolymph of last instar larvae (LL) and mated adult (A) *Gryllus bimaculatus* males

Sporadically JH I was also detected in 5-day (11 ± 4 pg/µL, n=2), 6-day (1 pg/µL), 7-day (6 ± 2 pg/µL, n=5), 8-day (4 pg/µL) old female adult crickets and 6-day (1 pg/µL) and 7-day (4 pg/µL) old male adult crickets. The identity was confirmed by the retention time (Fig. 35) and the mass spectrum of JH I (Fig. 36). Neither JH diols nor JH acids could be identified at any of the stages investigated.

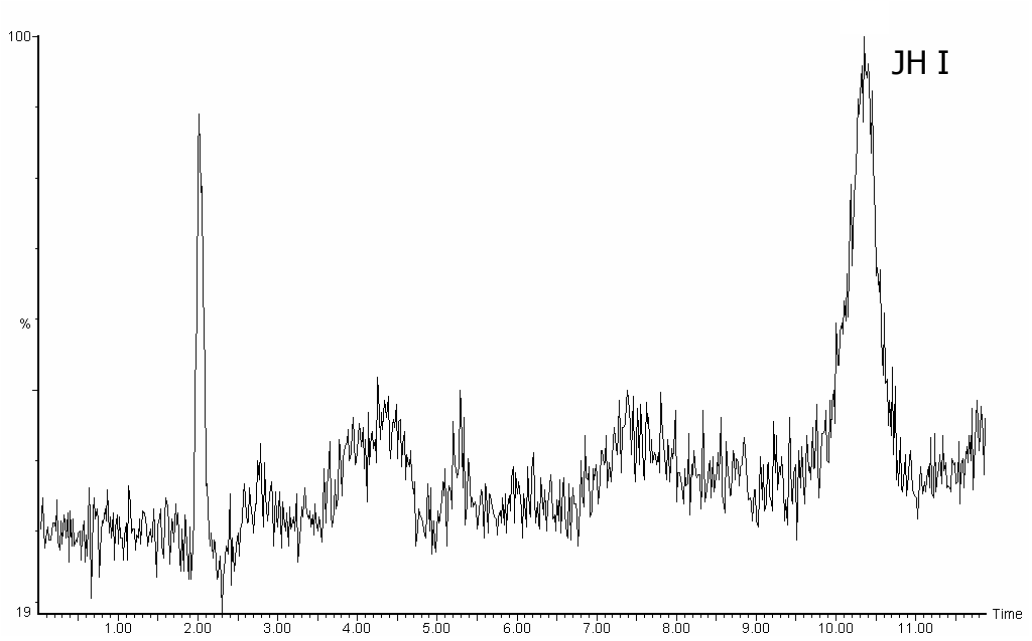


Fig. 35 Chromatogram of JH I found in adult *Gryllus bimaculatus* female

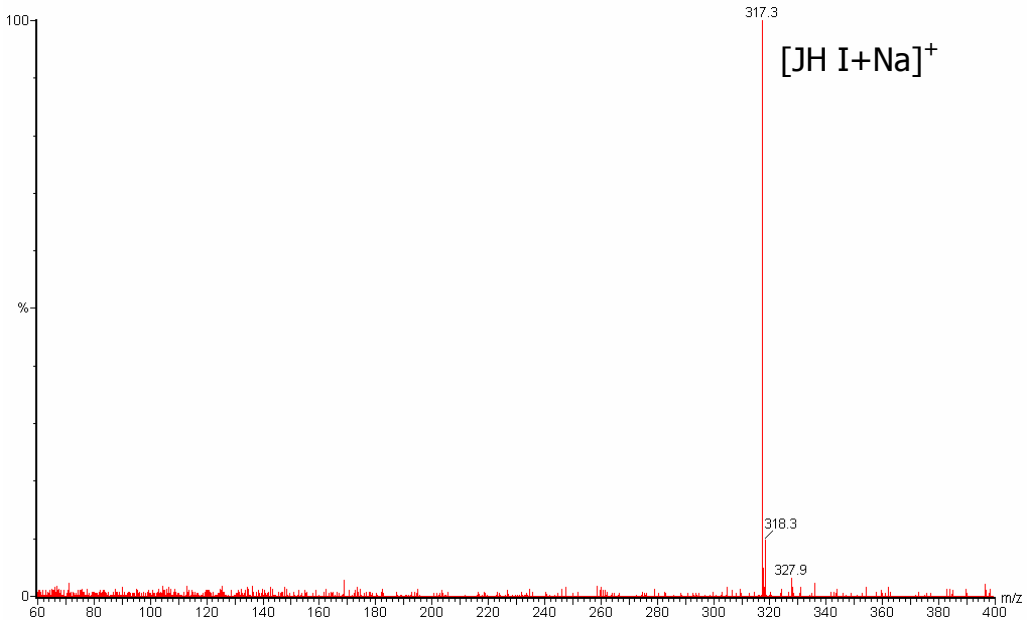


Fig. 36 Mass spectrum of JH I found in adult *Gryllus bimaculatus* female

4.2.1.2. Determining JH Titers of Unmated *Gryllus bimaculatus* Females by GC-EI-MS and LC-ESI-MS

JH III was found in 3-day and 4-day old unmated *Gryllus bimaculatus* females, which were analyzed by GC-MS and LC-MS, respectively. Sample clean-up and analysis by GC-MS of 10 samples took 2 days, whereas LC-MS sample preparation and analysis time of ten samples was accomplished within 4 hours. JH III titers in 3-day and 4-day female crickets by LC-MS were ten-fold higher than by GC-MS (Fig. 37). JH III titers on day 3 and day 4 by GC-MS were 0.5 ± 0.1 pg/µL and 0.3 ±0 pg/µL, respectively. On day 3 and day 4, the JH III titers obtained by LC-MS were 6.2 ± 1.5 pg/µL and 4.7 ± 0.9 pg/µL, respectively. Also by LC-ESI-MS, the values for unmated adult females were much lower than for mated females (see Fig. 33).

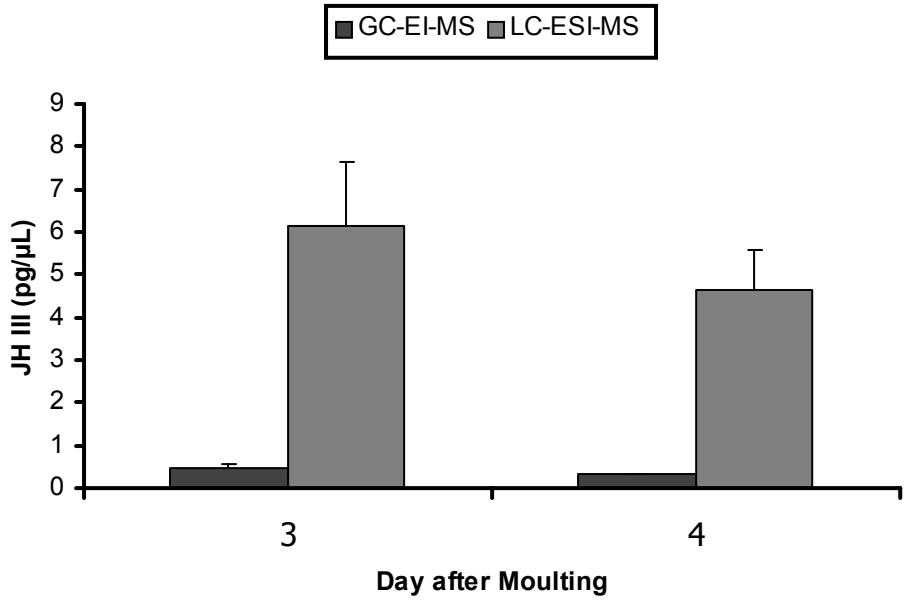


Fig. 37 JH III titers of unmated *Gryllus bimaculatus* females analyzed by GC-EI-MS (n = 5) and LC-ESI-MS (n = 4)

4.2.1.3. Ecdysteroid Titers

Mean values ± S.E.M were calculated for ecdysteroid titer determinations. Table 10 (LL = last instar larvae, A = Adult) gives a list of the number of individuals used.

Stage / Day	*females*	*males*	*Stage / Day*	*females*	*males*
LL / 2	9	10	A / 3	5	5
LL / 3	8	8	A / 4	5	5
LL / 4	9	8	A / 5	5	5
LL / 5	8	7	A / 6	5	11
LL / 6	8	7	A / 7	5	7
LL / 7	7	3	A / 8	4	5
LL / 8	8	4	A / 9	6	2
A / 0	5	5	A / 10	9	2
A / 1	5	5	A / 11	9	7
A / 2	5	5	A / 12	7	5

Table 10 Number of individuals used for ecdysteroid titer determinations in *Gryllus bimaculatus* haemolymph at different developmental stages

Ecdysone was present in last instar larvae of both sexes, as well as in mated adult *Gryllus bimaculatus* females (Fig. 38) and males (Fig. 39), whereas 20-hydroxyecdysone was only found in last instar female larvae and last instar male larvae (Figs. 40 and 41). Prior to a maximum 20-hydroxyecdysone titer in last instar female larvae (day 5; 789 ± 321 pg/µL) and last instar male larvae (days 5 and 6; 371 ± 301 pg/µL and 377 ± 153 pg/µL, respectively), ecdysone titers were high in last instar female larvae and last instar male larvae on day 3 (456 ± 77 pg/µL and 492 ± 85 pg/µL, respectively). In mated adult females and paired adult males, ecdysone titers reached a plateau maximum from day 3 through day 5. In paired adult males, the ecdysone titers averaged 76 ± 6 pg/µL compared to mated adult females, which experienced a 3-fold higher ecdysone titer than paired adult males (235 ± 18 pg/µL).

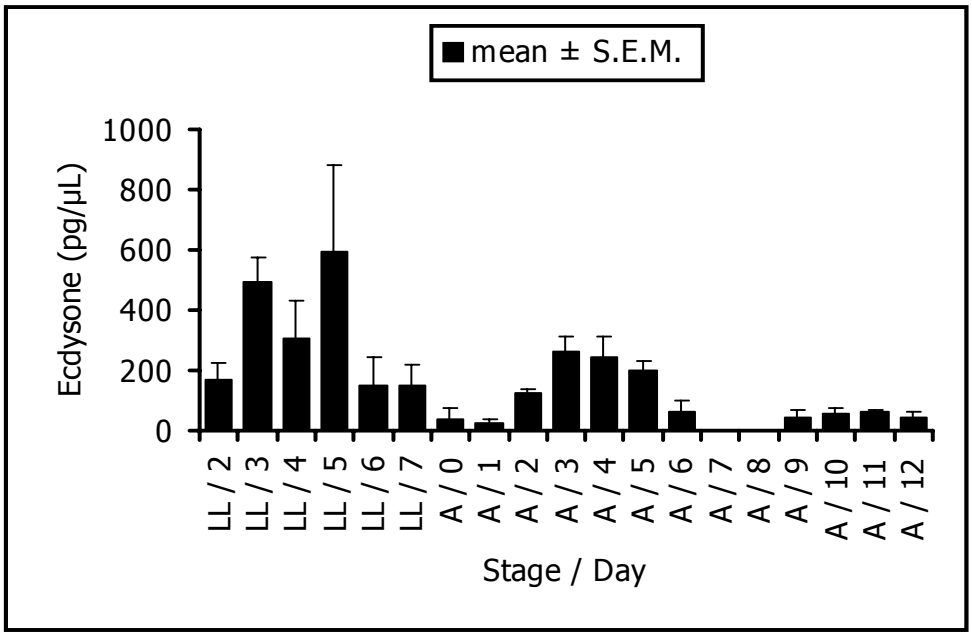


Fig. 38 Ecdysone titers in the haemolymph of last instar larvae (LL) and mated adult (A) *Gryllus bimaculatus* females

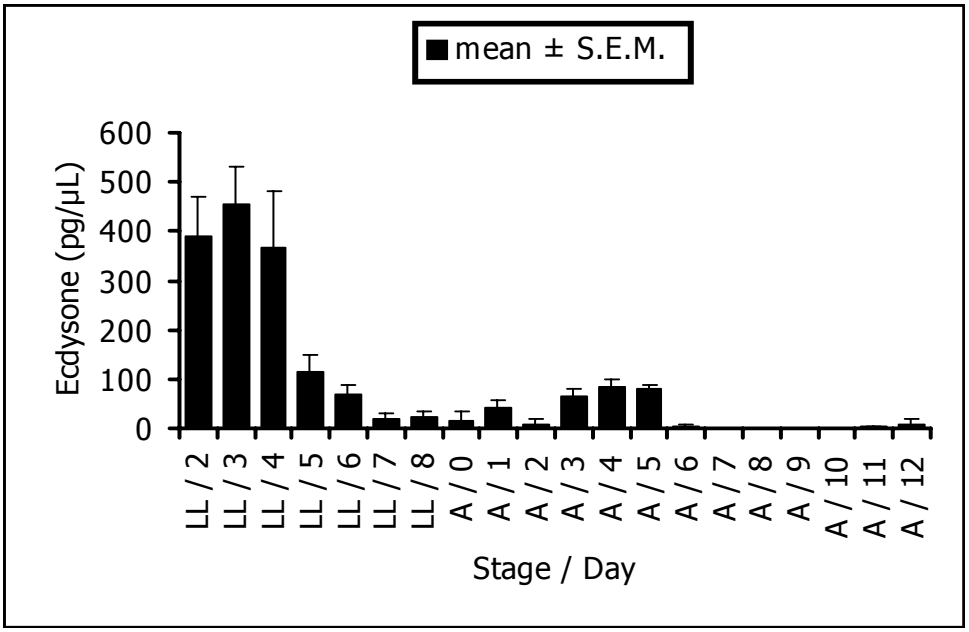


Fig. 39 Ecdysone titers in the haemolymph of last instar larvae (LL) and paired adult (A) *Gryllus bimaculatus* males

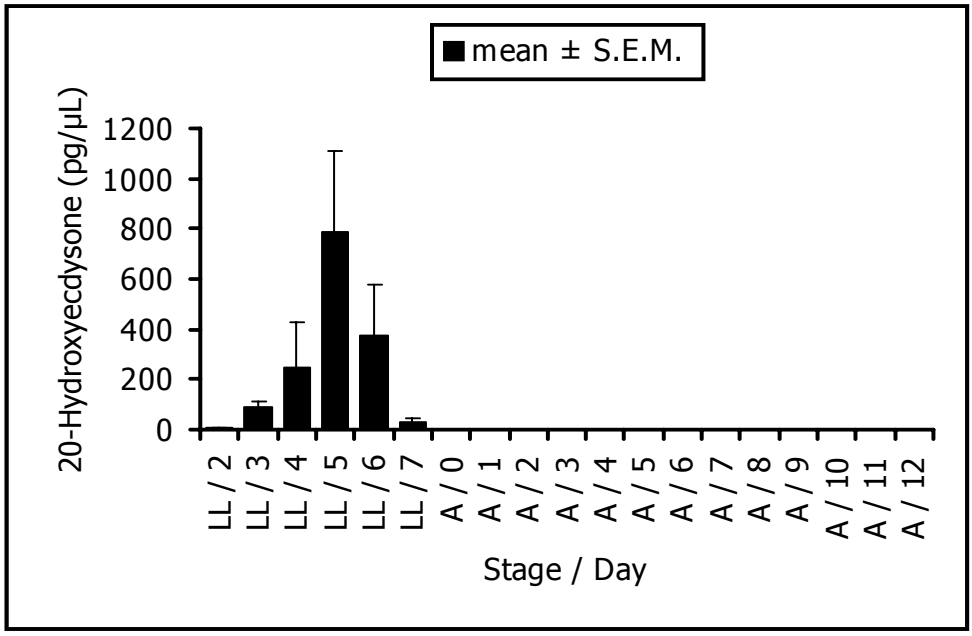


Fig. 40 20-Hydroxyecdysone titers in the haemolymph of last instar larvae (LL) and mated adult (A) *Gryllus bimaculatus* females

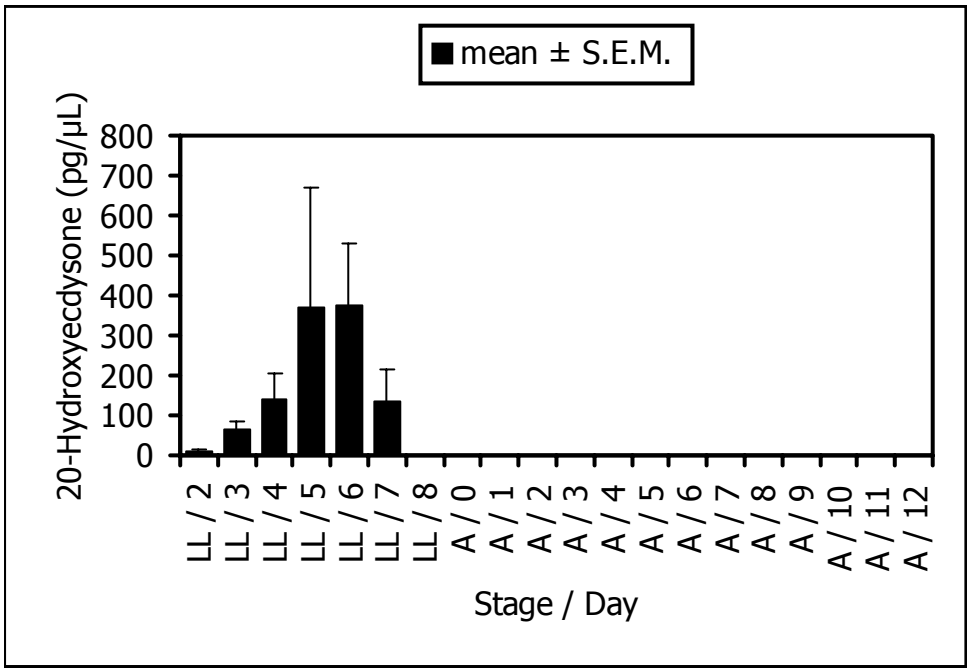


Fig. 41 20-Hydroxyecdysone titers in the haemolymph of last instar larvae (LL) and paired adult (A) *Gryllus bimaculatus* males

4.2.1.4. Ovary Weights and Hormone Titers in Adult *Gryllus bimaculatus* Females

Ovary weights were compared to ecdysone and JH titers to establish a relevant parameter which coincides with ovary development in mated adult female crickets during days 0 through 5 (n = 5 per day; individuals were given codes of identification: 4.1, i.e. would be 4-day old female number 1). Comparison of the titers and ovary weights of single crickets, illustrates high individual variations within "day"-groups (Fig. 42 and 43). Day 4 especially shows low overall titers and low ovary weights for 4.2 and 4.4 (☆), but high overall titers and high ovary weights for 4.1, 4.3, and 4.5 (🡻). Evaluating the mean values of JH III titers, ovary weights, and ecdysone titers (Fig. 44), reveals a steady ovary weight increase until day 4, and a decline at day 5: crickets start laying eggs on approximately day 4. JH III and ecdysone titers increase in synchrony with increasing ovary weights.

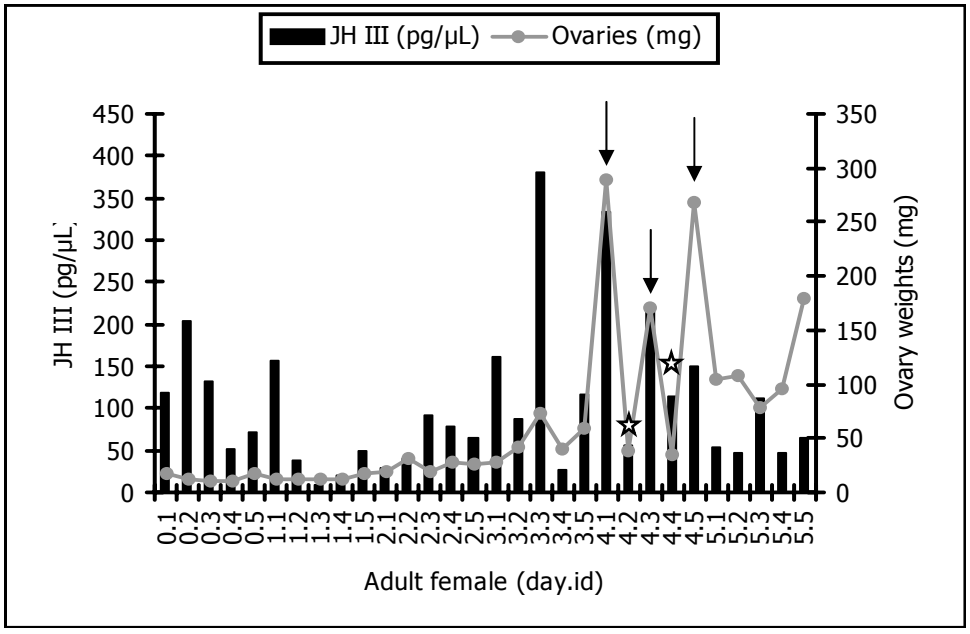


Fig. 42 Comparison of individual ovary weights to individual JH III titers in mated adult *Gryllus bimaculatus* females

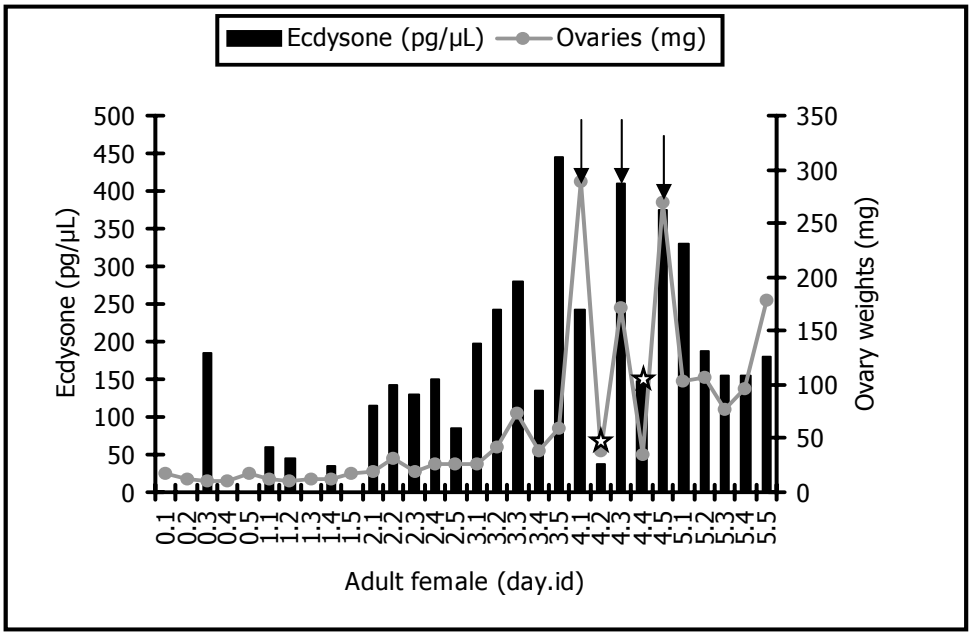


Fig. 43 Comparison of individual ovary weights to individual ecdysone titers in mated adult *Gryllus bimaculatus* females

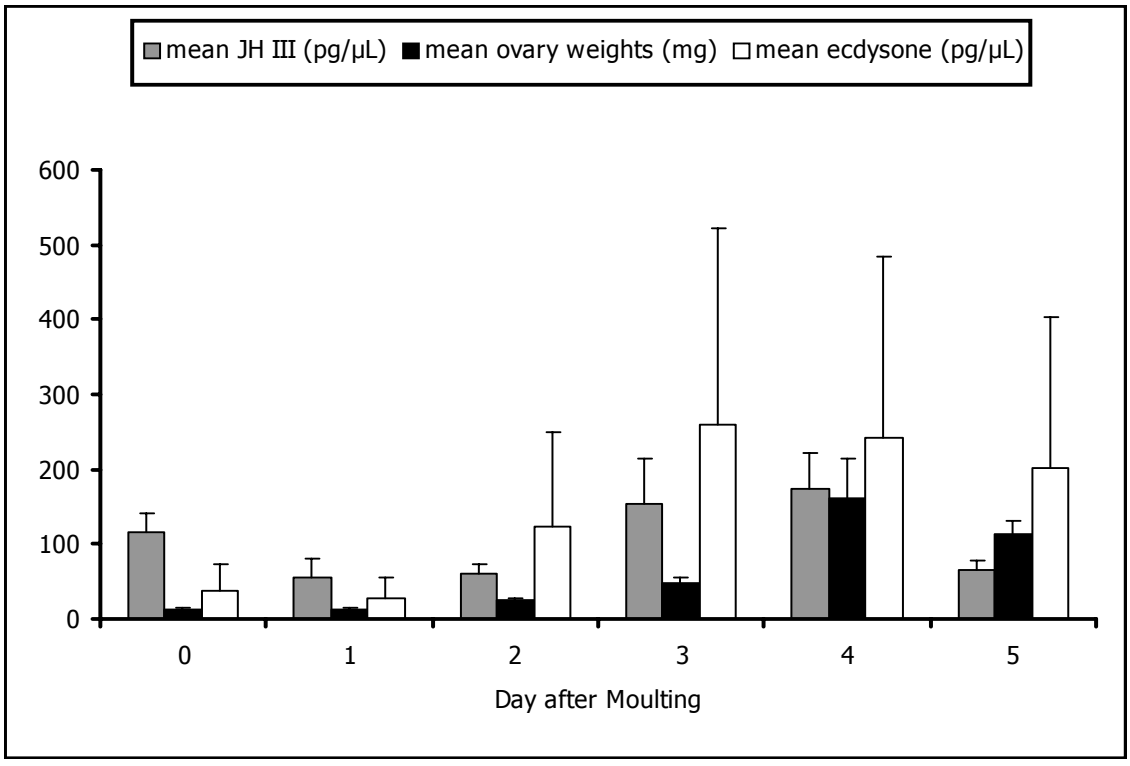


Fig. 44 Comparison of mean JH III titers, mean ovary weights and mean ecdysone titers in mated adult *Gryllus bimaculatus* females

4.2.2. *Spodoptera frugiperda*

Unless noted otherwise, titer determinations are mean values ± S.E.M. The number of individuals are listed in Table 11 (L5 = 5th instar larvae, L6 = 6th instar larvae, PP = pre-pupae).

Stage / Day	*n*	*Stage / Day*	*n*
L5 / 1	22	L6 / 2	23
L5 / 2	26	L6 / 3	17
L5 / 3	14	PP / 1	14
L6 / 1	21	PP / 2	10

Table 11 Number of individuals used for JH and ecdysteroid titer determinations in *Spodoptera frugiperda* haemolymph at different developmental stages

The stage and day were determined by measuring the head capsule width and weighing the larvae daily. Fig. 45 shows the typical weight gain of 5 larvae from day 2 of 4th instar larvae until prepupae on day 2. The head capsule width of 5th and 6th instar larvae were 2.09 and 2.77 mm, respectively (provided by Dr. R. Nauen, Bayer CropScience AG, Leverkusen). Weight curves served as an indicator as to when the larvae had molted and to designate the day of the instar (Fig. 45).

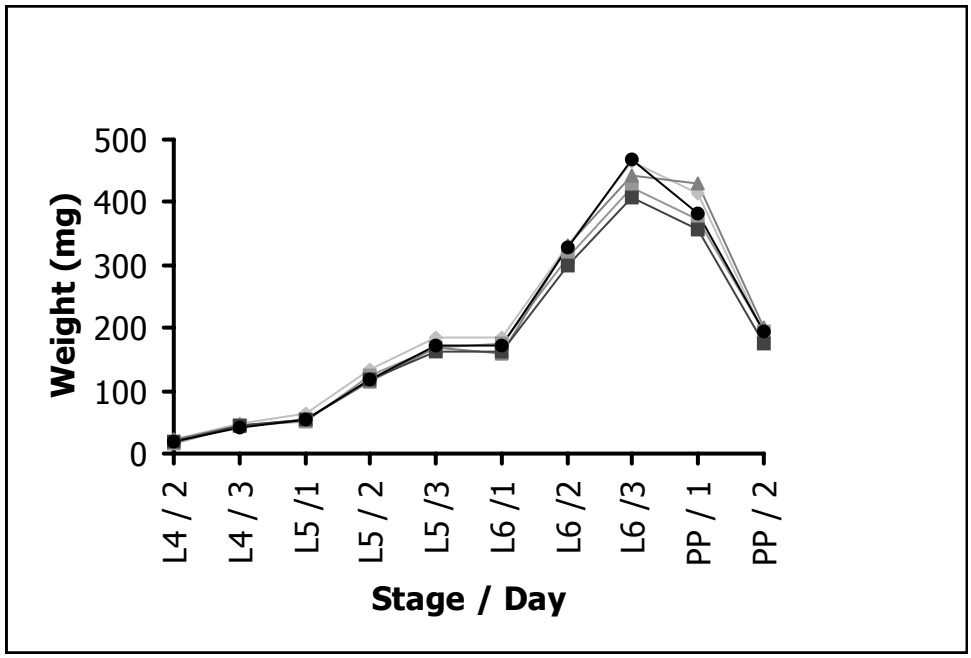


Fig. 45 Weights of 5th instar larvae, 6th instar larvae, and prepupae of *Spodoptera frugiperda*

4.2.2.1. JH Titers

In the haemolymph of *Spodoptera frugiperda* 5th instar larvae JH I, JH II, and JH III were found on day 1 and 2 (Fig. 46). At the end of the 5th instar, through the 6th instar and the prepupal stage, JH I was no longer detectable. JH II was completely absent throughout the 6th instar, but re-emerged on the final day of the prepupal stage. The total JH titer was highest in 5th instar 1-day old larvae, reaching 81 ± 9 pg/µL.

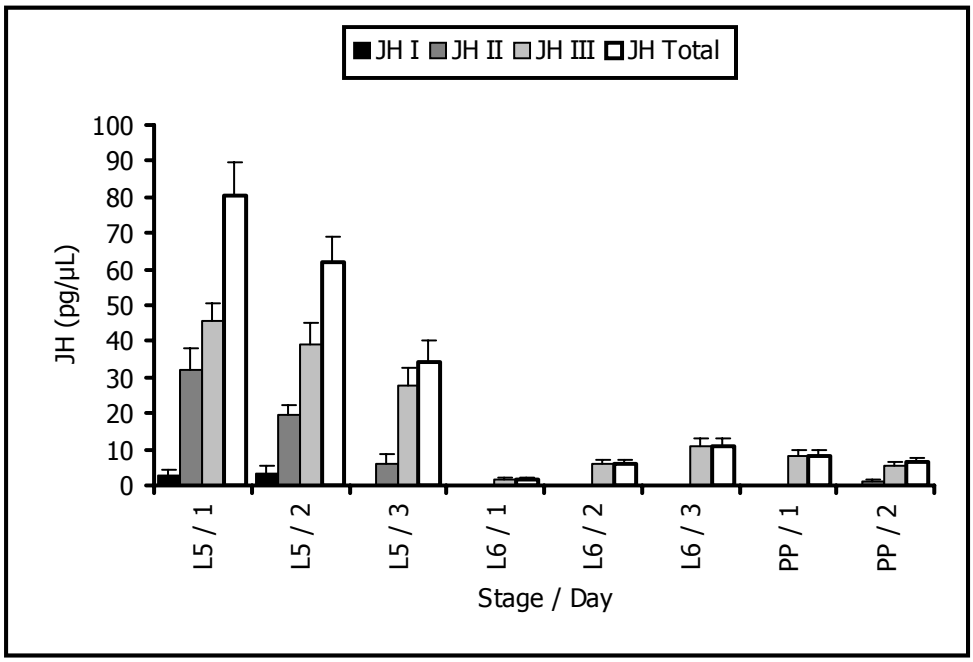


Fig. 46 JH I, JH II and JH III titers in the haemolymph of *Spodoptera frugiperda* 5th instar larvae, 6th instar larvae and the prepupae.

4.2.2.2. Ecdysteroid Titers

Throughout 5th instar larvae, ecdysone was detected at a steady concentration of about 3 pg/µL (Fig. 47). During the last two days of 6th instar larval development, ecdysone titers rose from 1 pg/µL (day 2) to 15 pg/µL (day 3) and increased substantially in prepupal larvae on day 1 (27 ± 8 pg/µL) and day 2 (44 ± 8 pg/µL). 20-Hydroxyecdysone was found only once throughout the larval stages, namely on day 3 of 5th instar larvae (25 ± 20 pg/µL). 20-hydroxyecdysone was present again at the onset of the prepupal stage and reached its maximum on day 2 (47 ± 13 pg/µL).

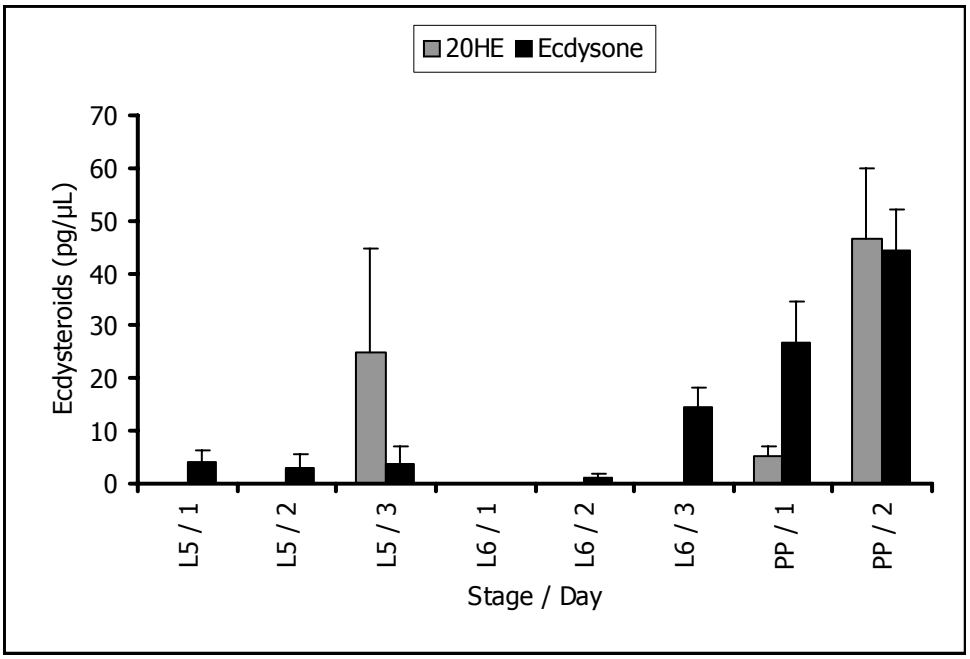


Fig. 47 20-Hydroxyecdysone and ecdysone titers in the haemolymph of *Spodoptera frugiperda* 5th instar larvae, 6th instar larvae and the prepupae.

4.2.3. Method Revalidation and JH Titers in Other Insects

Haemolymph from the ant species *Myrmicaria eumenoides* (Hymenoptera, Formicidae) was kindly provided by Mr. F. Lengyel (Animalphysiology, University of Bayreuth, Bayreuth, Germany). Mrs. G. Kunert (Institute of Ecology, University of Jena, Jena, Germany) supplied haemolymph samples from the pea aphid *Acyrthosiphon pisum* (Homoptera, Aphididae) most generously.

4.2.3.1. Evaluation of Matrix Effects of *Myrmicaria eumenoides* Haemolymph

In order to investigate possible matrix effects when analyzing haemolymph of other insects, haemolymph samples of *Myrmicaria eumenoides* were spiked with standards and analyzed. The results were compared to the values obtained from spiked *Gryllus bimaculatus* haemolymph, and the coefficients of variation calculated (Table 12). The coefficients of variation were less than 20 %, indicating that a matrix effect much different from the one seen in the haemolymph of *Gryllus bimaculatus* is not likely.

Standard (ng)	*Coefficient of variation*						
	Repeatability						
	N	JH I	JH II	JH III	JH I diol	JH II diol	JH III diol
1.0	2	19.85	18.45	12.95	16.11	13.95	11.85
1.5	2	5.56	2.13	11.57	4.59	3.63	7.49

Table 12 Analysis of matrix effects in spiked *Myrmicaria eumenoides* haemolymph samples

4.2.3.2. JH III titers in *Acyrthosiphon pisum* and *Myrmicaria eumenoides* Haemolymph

JH titers were measured in the haemolymph of the pea aphid *Acyrthosiphon pisum* and the ant species *Myrmicaria eumenoides* to demonstrate that the LC-ESI-MS method is applicable to various insect orders without any modification during analysis or sample preparation. In the haemolymph of the pea aphid *Acyrthosiphon pisum* and the ant species *Myrmicaria eumenoides*, JH III was found at concentrations of 640 pg/µL (Fig. 48) and 25 pg/µL (Fig. 49), respectively.

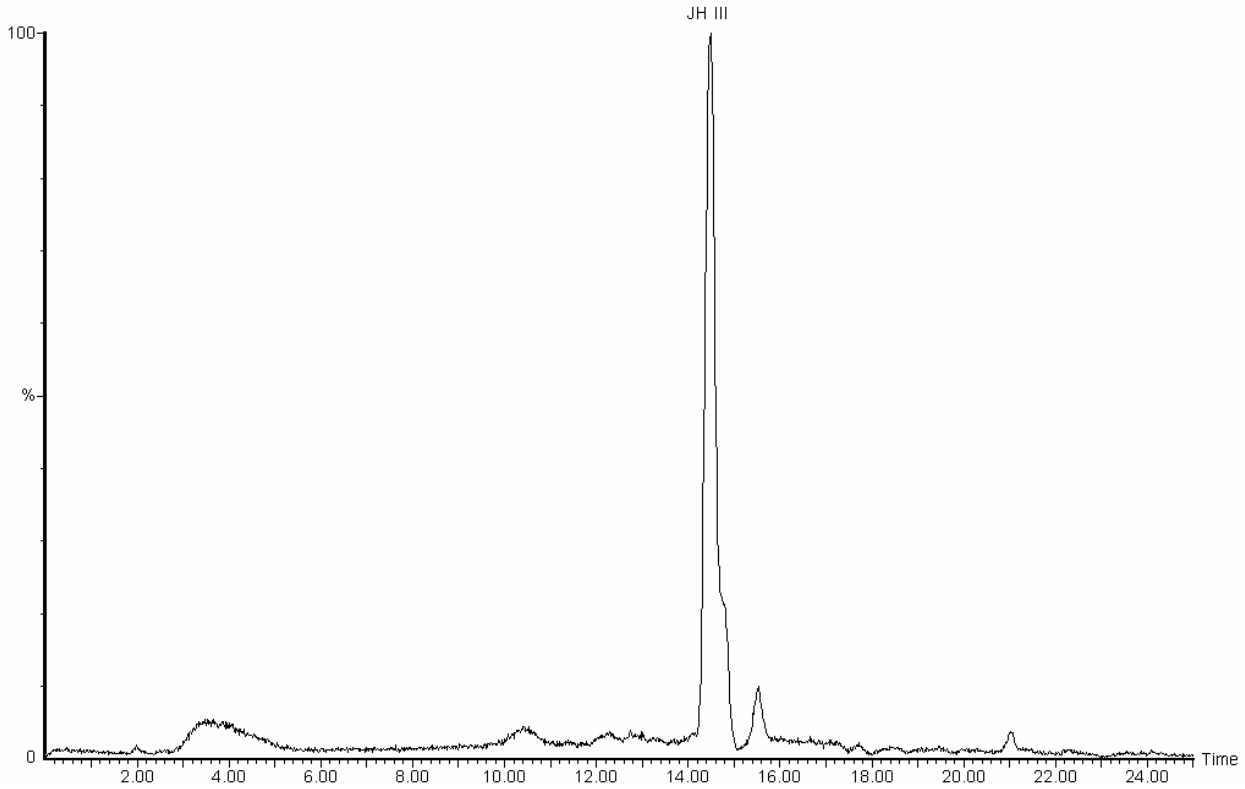


Fig. 48 JH III found in the haemolymph of the pea aphid *Acyrthosiphon pisum* (HPLC gradient: 0-1 min 60 % MeOH, 1-4 min 60-80 % MeOH, 4-6 min 80 % MeOH, 6-9 min 80-90 % MeOH, 9-18 min 90-100 % MeOH)

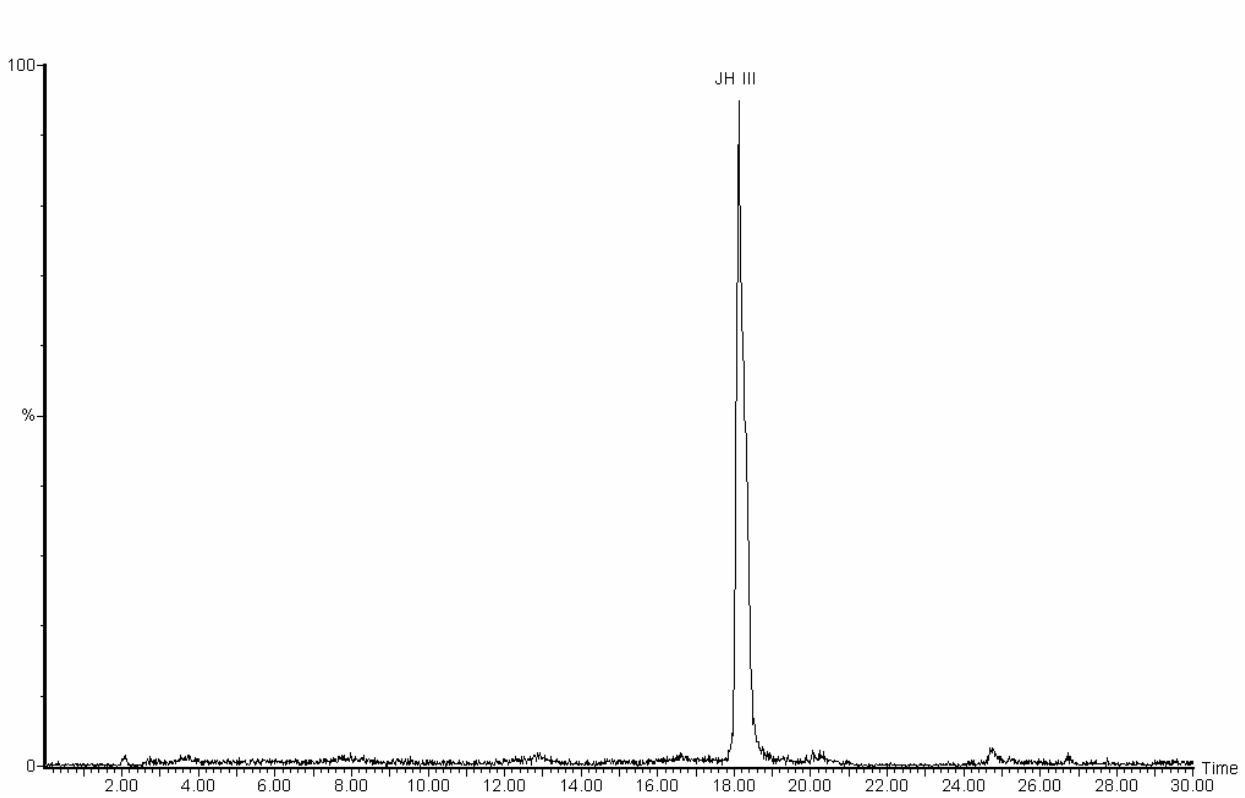


Fig. 49 JH III found in the haemolymph of the ant species *Myrmicaria eumenoides* (HPLC gradient: 0-5 min 60 % MeOH, 5-8 min 60-80 % MeOH, 8-10 min 80 % MeOH, 10-13 min 80-90 % MeOH, 13-22 min 90-100 % MeOH)

4.3. Isolated and Identified Alkaloids of the Pink Lime *Glycosmis pentaphylla* (Rutaceae)

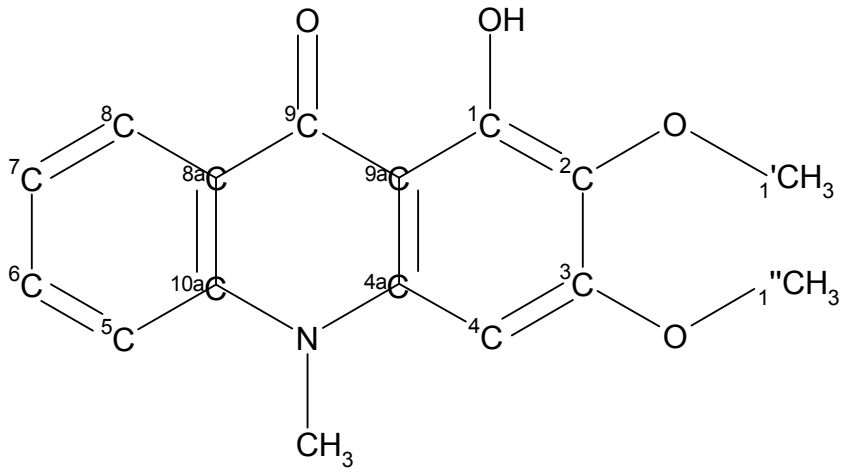
The alkaloids arborinine, 4-methoxy-1-methyl-2(1*H*)-quinolinone, and arborine were isolated from leaves of *Glycosmis pentaphylla* and identified by MS and NMR. Arborinine and aborine are known to be abundant in leaves of *Glycosmis pentaphylla* and have been isolated previously (Banerjee et al., 1961; Chakravarti et al., 1961). 4-Methoxy-1-methyl-2(1*H*)-quinolinone has been found in other members of the Rutaceae, such as in *Ruta chalepensis* L. (El Sayed et al., 2000) and *Ruta montana* (Touati et al., 2000), but not in *Glycosmis pentaphylla*.

4.3.1. Arborinine

The diethyl ether (931 mg) and the ethyl acetate (320 mg) fraction of the MeOH crude extract obtained from leaves primarily contained 4 mg of the yellow acridine alkaloid Arborinine (1-hydroxy-2,3-dimethoxy-10-methyl-9(10*H*)-acridinone) (Fig. 50). Arborinine was identified by EI-MS (70 eV) (*m/z*; %): 285 $[M]^+$ (70), 270 $[M\text{-}CH_3]^+$ (100), 242 $[M\text{-}C_2H_30]^+$ (45), 199 $[M\text{-}2\ C_2H_30]^+$ (30), and ^{1}H- and ^{13}C-NMR in DMSO (Table 13). H-H COSY, HMBC, and HMQC data confirm ^{1}H- and ^{13}C-NMR signal assignments and are in good agreement with literature data (Bergenthal et al. 1979; Spatafora and Tringali, 1997), except for the ^{1}H-NMR signals which differ slightly due to measurements being carried out in DMSO, instead of $CDCl_3$.

Position	*^{1}H (δ)*	*^{13}C (δ)*	*Position*	*^{1}H (δ)*	*^{13}C (δ)*
1	-	154.94	6	7.87	134.31
1′	3.73	59.90	7	7.36	121.63
1″	4.01	56.25	8	8.30	125.30
2	-	129.37	8a	-	119.61
3	-	159.36	9	-	179.85
4	6.64	88.34	9a		104.76
4a	-	140.30	10a		141.83
5	7.83	117.28	N-CH_3	3.92	34.16

Table 13 ^{1}H- and ^{13}C-NMR data of Arborinine



1-hydroxy-2,3-dimethoxy-10-methyl-9(10*H*)-acridinone

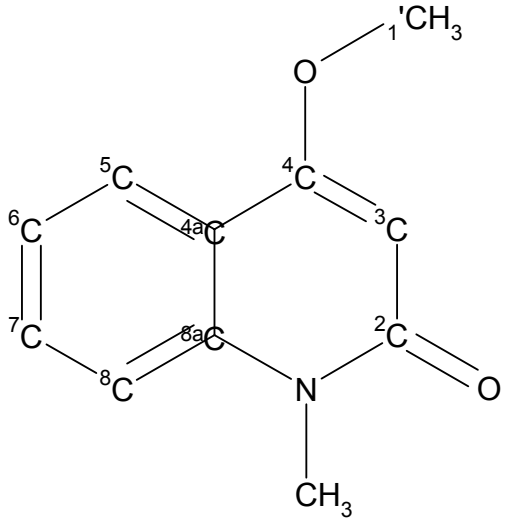
Fig. 50 The structure of the acridine alkaloid Arborinine isolated from *Glycosmis pentaphylla* leaves

4.3.2. 4-Methoxy-1-methyl-2(1*H*)-quinolinone

Further purification of the ethyl acetate (320 mg) fraction of the MeOH crude extract obtained from leaves by OCC with chloroform:MeOH (9:1, v/v) resulted in the isolation of 17 mg of the pale yellow quinoline alkaloid 4-methoxy-1-methyl-2(1*H*)-quinolinone (Fig. 51). 4-methoxy-1-methyl-2(1*H*)-quinolinone was identified by EI-MS (70 eV) (*m/z*; %): 189 $[M]^+$ (100), 174 $[M\text{-}CH_3]^+$ (55), 160 $[M\text{-}CH0]^+$ (15), 146 $[M\text{-}C_2H_30]^+$ (45), and ^{1}H- and ^{13}C-NMR in DMSO (see Table 14), confirming ^{1}H- and ^{13}C-NMR signal assignments by H-H COSY, HMBC, and HMQC. ^{13}C-NMR signals coincide well with published data (Coppola et al., 1981). Literature values of ^{13}C-NMR for position 1′ and ^{1}H-NMR was not available for comparison.

Position	^1H (δ)	^{13}C (δ)
1′	3.93	55.68
2	-	162.49
3	6.04	95.88
4	-	161.90
4a	-	115.56
5	7.88	122.12
6	-	121.10
7	7.65	130.97
8	7.51	114.23
8a	-	139.59
N-CH_3	3.56	29.25

Table 14 ^{1}H- and ^{13}C-NMR data of the quinoline alkaloid 4-methoxy-1-methyl-2(1*H*)-quinolinone



4-methoxy-1-methyl-2(1*H*)-quinolinone

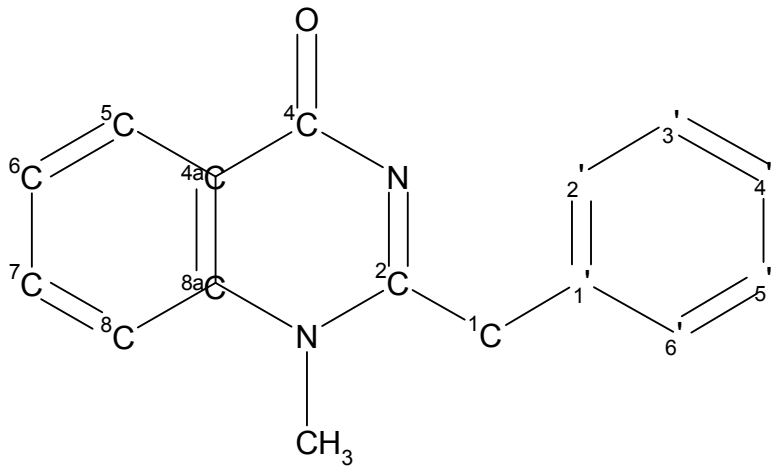
Fig. 51 The structure of the quinoline alkaloid 4-methoxy-1-methyl-2(1*H*)-quinolinone isolated from *Glycosmis pentaphylla* leaves

4.3.3. Arborine

OCC separation of the ethyl acetate fraction (320 mg) of the MeOH crude extract obtained from leaves with chloroform resulted in the isolation of 38 mg of the yellow quinazoline alkaloid Arborine (2-benzyl-1-methyl-4(1*H*)-quinazolinone) (Fig. 52). The EI-MS (70 eV) and ^{1}H-, ^{13}C-NMR(see Table 15), H-H COSY, HMBC, and HMQC in DMSO confirmed the structure of Arborine. EI-MS (*m/z*; %): 250 $[M]^+$ (40), 249 $[M-H]^+$ (100), 235 $[M-CH_3]^+$ (10). The NMR values are in good agreement with ^{1}H- and ^{13}C-NMR data of arborine obtained by Dr. Kamel Shaker (University of Bayreuth, Germany). ^{1}H-NMR assignments published by Bowen et al. (1978) also coincide well with these results.

Position	^{1}H (δ)	^{13}C (δ)
1	4.25	41.78
2	-	162.73
4	-	167.91
4a	-	119.49
5	8.07	127.13
6	7.46	125.64
7	7.75	133.82
8	7.64	116.11
8a	-	141.55
N-CH_3	3.66	34.98
1′	-	135.64
2′	7.31	128.64
3′	7.32	128.76
4′	7.30	126.90
5′	7.32	128.76
6′	7.31	128.64

Table 15 ^{1}H- and ^{13}C-NMR data of Arborine



2-benzyl-1-methyl-4(1*H*)-quinazolinone

Fig. 52 The structure of the quinazoline alkaloid Arborine isolated from *Glycosmis pentaphylla* leaves

5. Discussion

5.1. LC-ESI-MS Method Development for JH and Ecdysteroid Titer Determinations

5.1.1. JH Uptake and JH Degradation Enzyme Activity in Organic Solvents

Since protein removal is essential prior to analysis (Kataoka and Lord, 2002), the uptake of JH by the organic solvent and inactivation of JH esterase had to be taken into consideration when choosing the appropriate organic solvents.

Lipids could possibly serve as JH carriers due to their similar hydrophobic character as JH, preventing JH from adhering to the glass wall. Haemolymph of insects contain high amounts of lipids (Hoffmann, 1995), which remained in solution after protein precipitation. JH adhesion studies to glass in MeOH and haemolymph extracts obtained by using isooctane:MeOH for protein precipitation, clearly showed efficient uptake of JH in haemolymph extracts (Fig. 9), with better repeatability as for in MeOH as indicated by the standard deviation. The concentration of lipids is anywhere from 15 to 55 mg/mL (Hoffmann, 1995), which is at least 150-fold higher than the average concentration of JH. Therefore, the possibility of reaching a point of saturation is unlikely.

As the activity of JH esterase can be increased by organic solvents (Croston et al., 1987) and which could result in the formation of JH acids, JH diols, and JH diol acids (Hoffmann et al., 1994a), haemolymph discoloration was chosen as an indicator of JH esterase deactivation. Darkening of the haemolymph as a result of injury is due to eumelanin production by the phenol oxidase system (Nappi and Sugumaran, 1993). This class of enzymes is known to be stable in 50 % organic solvents (Tyagi and Gupta, 1998) and quite resistant to denaturation by organic solvents. Deactivating phenol oxidase, thereupon inhibiting eumelanin production, serves as an adequate indicator of JH esterase inhibition.

5.1.2. Method Validation (Micromass LCT and Shimadzu LCMS-2010A)

Calibration curves compiled for the Micromass LCT and the Shimadzu LCMS-2010A are in good agreement with each other at correlation coefficients of greater than 0.98. Even though the LODs for JHs were 8 pg for the Micromass LCT and 12 pg for Shimadzu LCMS-2010A, the values were within acceptable levels. The same holds for the JH LOQs, differing by a factor of 2. The discrepancy can be explained by closely examining Figs. 18 and 31. The correlation coefficients are higher for Fig. 18 (0.87177) than for Fig. 31 (0.6568). Implying a better fit and therefore a more accurate calculation of the LODs and LOQs for JH for the Micromass LCT. Similar observation can be made for the JH diols (Fig. 19 and 32).

Analysis of the ecdysteroids on the Shimadzu LCMS-2010A showed good sensitivity and very little noise variation (Fig. 30). Less noise variation may simply be due to less noise observed at higher masses of the ecdysteroids (487/503/519) compared to the JHs (289/303/317) or JH diols (307/321/335). The precision achieved by the Micromass LCT and the Shimadzu LCMS-2010A (Table 5 and 7) did not differ substantially, making both instruments adequate for hormone analysis of haemolymph samples.

5.1.3. JH and Ecdysteroid Analysis by Physicochemical Methods

The first GC-MS method developed for the determination of JH I in the giant silk moth *Hyalophora cecropia* was published in 1972 by Bieber and Sweeley. JH I was extracted from male and female abdomina for 36 h in diethyl ether, then the extract was purified by gel filtration and TLC. The LOD was 200 ng. The GC-MS method by Trautmann and Schuler (1974) was designed to investigate JH I, JH II, and JH III in whole body extracts and obtained a LOD of 200 pg/g whole body weight. But extensive chromatographic clean-up, resulted in only a 67.1 % yield.

A decrease in the LODs and an increase of the recoveries was achieved by Bergot et al. (1981a) and Rembold and Lackner (1985). Bergot et al. (1981a) were able to attain LODs of 10 - 40 pg/g of tissue. JHs were extracted with pentane and cleaned by C_{18} Sep-Pak, alumina filtration and then derivatized to 11-methoxy-d_3-10-hydroxy JH. Finally, the derivatized JHs were further subjected to clean-up by a silica column,

leading to a 70 – 80 % yield. Processing of 15 - 20 samples requires one week. The GC-MS method devised by Rembold and Lackner (1985) included further derivatization of the 11-methoxy-d_3-10-hydroxy JH by dimethyl(nonafluorohexyl)silyl chloride, which resulted in a 67.3 % recovery and a LOD of 3 pg.

The GC-MS results presented in this thesis (see 4.2.1.2) show a high loss of analyte during sample preparation which is not compensated by the internal standard. Since the internal standard is not added to the sample in the form in which it is found in the haemolymph, namely attached to the binding protein, an underestimation of the analyte concentration seems to be a possible explanation.

As with GC-MS methods for JH analysis, sample clean-up was also extensive for ecdysteroid analysis by GC-MS, involving solvent extraction, separation by C_{18} Sep-Pak, alumina column or HPLC. But since ecdysteroids are non-volatile and thermally labile, ecdysteroids also had to be derivatized. Unfortunately, sterically hindered hydroxy groups (i.e. the 14α-hydroxyl group) and the α,β-unsaturated ketone made complete derivatization difficult. Ikekawa et al. (1972) claimed complete silylation of ecdysteroids at 100 °C with trimethylsilylimidazole and good LOD with heptafluorobutyryl derivatives at the nanogram or picogram level for ECD. However, silylation at a higher temperature (140 °C) showed that the 14α-hydroxyl group was originally not silylated at 100 °C (Morgan et al., 1976b). The great diversity of ecdysteroids found in insects, makes the discovery of suitable derivatization conditions challenging (Morgan et al., 1976a, 1976b).

HPLC methods for JHs (Sasagawa, 1988) and ecdysteroids (Isaac et al., 1982; Scalia and Morgan, 1982 and 1985) have been attempted, but the LOD for UV methods is too high (1.6 ng for JHs and 10 ng for ecdysteroid) and proper identification of the analyte can become cumbersome.

Some efforts have been made to combine the advantages of direct analysis by HPLC, without the need to derivatize; and mass identification by MS. Mauchamp et al. (1981) reported a procedure for JH analysis using RP-HPLC coupled with MS, which had a LOD of 10 pg when using a standard mixture and selected ion monitoring. Unfortunately, the LOD for biological samples is not mentioned and sample preparation

included clean-up by alumina column and C_{18} Sep-pak separation, and hexane extraction. Additionally, JHs were stored under dry conditions until analysis and then resuspended in MeOH, which probably resulted in a low recovery, as proven by JH affinity tests to glass (Fig. 9).

Ecdysteroid analysis by MS/MS (Mauchamp et al., 1993) yielded a LOD of 50 ng. Analysis included partly purifying the sample and separating the ecdysteroids by HPLC. The HPLC fractions were collected and pooled and then introduced into the ionization chamber of the MS. The LC-MS method by Evershed et al. (1993) used thermospray ionization and was able to attain a LOD of 5 ng in the selected ion monitoring mode. Wainwright et al. (1997) were able to reduce the LOD to 10 - 100 pg using atmospheric pressure chemical ionization.

Until now, the combination of JH and ecdysteroid analysis by LC-MS has not been mentioned in the literature. The newly developed LC-ESI-MS method can afford the same degree of accuracy and precision as GC-MS. The LODs for JHs and JH diols ranged from 6 to 12 pg, respectively, and 93 pg for the ecdysteroids in biological sample matrix. Sample preparation is less time-consuming, since only proteins are removed by organic solvent precipitation. 72 samples can be processed by a trained person on one day, compared to 10 samples every two days by GC-MS (see 4.2.1.2.). Less organic solvent is used, making the method especially environmentally friendly and economical. Due to stable retention times, the analytes can be identified on the basis of retention times and molecular weights, which obliterates the need of additional ions for identification and suffices in monitoring the $[M+Na]^+$ and at higher JH concentrations or for the ecdysteroids the $[M+K]^+$ as well. The $[M+Na]^+$ and $[M+K]^+$ are formed due to high levels of Na^+ (160 mM) and K^+ (12 mM) in the haemolymph (Hoffmann, 1973). Unlike GC-MS, degradation products, such as JH diols and JH acids, can be monitored by LC-ESI-MS directly. Since no fragmentation is observed by LC-ESI-MS in comparison to GC-MS, maximum sensitivity can be achieved by monitoring not only a fraction of, but the complete signal.

Rapid sample clean-up and short analysis time make the newly developed LC-ESI-MS method suitable for automated routing JH and ecdysteroid analysis of insect haemolymph. Depending on the availability of standards for other JHs, ecdysteroids

(free and conjugated) or degradation product, additional analytes could be incorporated into this method upon validation.

5.2. Ecdysteroid and JH Titers in the Haemolymph of Female and Male *Gryllus bimaculatus* and their Correlation to Developmental Events in Larvae and Adults

5.2.1. Ecdysteroid and JH Titers in Female and Male *Gryllus bimaculatus* Larvae

In hemimetabolous insects, female and male larvae undergo similar developmental processes. As long as JH titers are high, the larva will continue to molt to the next instar when 20-hydroxyecdysone titers increase. However, when JH titers decrease and 20-hydroxyecdysone titers increase, the larva undergoes imaginal ecdysis (Nijhout, 1994).

Using radioimmunoassay, in last instar larvae of *Gryllus bimaculatus* females, the total free ecdysteroid concentration in haemolymph was 2 ng/µL on day 1, 1 ng/µL on day 2, 2 ng/µL on day 3, and 4.5 ng/µL on day 4. A linear increase was observed up to a maximum of 32 ng/µL on day 7. On day 8, the total free ecdysteroid concentrations reached levels below initial concentrations (Gerstenlauer and Hoffmann, 1995), coinciding with ecdysteroid behavior expected in last instar larvae. Similar results were observed from *in vitro* experiments involving ecdysteroid release by the prothoracic gland in female last instar larvae (Oeh et al., 1998). Although the total concentration of 20-hydroxyecdysone and ecdysone obtained by the LC-ESI-MS method was not as high as reported by Gerstenlauer and Hoffmann (1995), the increase in 20-hydroxyecdysone on day 5 and 6 of male and female larvae haemolymph samples, agrees with the ecdysteroid peak required 1 - 2 days prior to imaginal ecdysis (Fig. 40 and 41). Unfortunately, it is difficult to compare the obtained ecdysteroid titers to the radioimmunoassay results, since total ecdysteroid equivalents were measured and 20-hydroxyecdysone and ecdysone cannot be differentiated. The antisera used, also react with other ecdysteroid-like substances, probably explaining the high concentrations observed by Gerstenlauer and Hoffmann (1995).

JH III biosynthesis rates of male last instar larvae showed a similar JH biosynthesis rate pattern as for female last instar larvae (Hoffmann et al., 1995). Starting high on day 0 and 1 (5 - 6 pmol/hr*pair CA) and decreasing to a minimum on day 4 (0.5 pmol/hr*pair CA) and then increasing again after the 9th day. High activity of JH III esterase is observed especially during the last 8 days (Hoffmann et al., 1995). Low biosynthesis rates and high JH III esterase activity keeps JH III titers low, making imaginal ecdysis possible. A low JH III titer was observed in the haemolymph of female and male last instar larvae analyzed in this work. Female and male last instar crickets have relatively close JH III titers until day 6 (Fig. 33 and 34). A decrease in male last instar JH III titers is minimal on day 7 and the JH III titer remains low until imaginal ecdysis.

The obtained results indicate good agreement between the ecdysteroid and JH titers and larval development in female and male *Gryllus bimaculatus* larvae.

5.2.2. Ecdysteroid and JH Titers in Mated *Gryllus bimaculatus* Adult Females

Vitellogenin biosynthesis, oocyte maturation and ovipositioning starting on day 4 for mated *Gryllus bimaculatus* adult females, are controlled by ecdysteroids and JHs (Engelmann, 1979; Engelmann, 1984; Kumaran, 1990). JH stimulates vitellogenin production in the fat body and prepares vitellogenin uptake by the ovaries. Ecdysteroids, which are produced by the follicular cells, are stored in developing eggs (Strambi et al., 1997). An additional site of ecdysteroid synthesis are the abdominal integument and the adjacent fat body, predominantly producing ecdysone (Weidner and Hoffmann, 1992).

Ecdysteroid release *in vitro* by ovaries and the abdominal integument-fat body complex reached a maximum on day 4 and 5, and again on day 7. Another increase in ecdysteroid secretion occurred on day 12. Ecdysteroid titers in the haemolymph of adult females attained by radioimmunoassay were 0.5 ng/µL on day 0, 0.1 ng/µL on day 1, 0.2 ng/µL on day 4, 0.7 ng/µL on day 6 and a maximum on day 8 (0.8 ng/µL). Ecdysteroid titers decreased on day 12 to 0.4 ng/µL (Weidner and Hoffmann, 1992). In the analyzed haemolymph of mated adult females crickets by LC-ESI-MS, a similar pattern was observed, but with a 2 day forward displacement. Ecdysone titers started

increasing on day 2 (124 pg/µL), reaching a maximum on day 3 (260 pg/µL) and staying at 243 pg/µL on day 4. The maximum ecdysone titer coincided with the beginning of ovipositioning (Fig. 38). The release of ecdysteroids *in vitro* revealed a decrease of activity between day 8 and 11. A similar phenomena could be observed for the days 7 and 8 of ecdysteroid titer determinations by LC-ESI-MS, obtaining good agreement between release and titer measurements. The ecdysone titer levels increased slightly by day 9 (44 pg/µL) and held a relatively constant concentration until day 12. Further investigations into the correlation between reproduction and hormone titers, showed a clear relationship between ovary weight increase, JH and ecdysone titers in 0-day to 5-day old mated female crickets (Fig. 44). As the ovary weights increased, the JH III and ecdysone titers also increased until day 4. Then the ovary weights decrease slightly due to ovipositioning (Hoffmann et al., 1996).

20-Hydroxyecdysone, however, could not be detected in the haemolymph of mated *Gryllus bimaculatus* females (Fig. 40), which may raise the possibility that 20-hydroxyecdysone is not the physiologically active hormone or that ecdysone is converted to 20-hydroxyecdysone at the site of action and is not released into the haemolymph.

Since JH III is required in egg maturation and ovipositioning, a high JH III biosynthesis rate and a high JH titer should be observed around day 4, since female crickets start to oviposit at that time. A rapid JH III biosynthesis rate increase was observed between day 1 and 4 (Hoffmann et al., 1994b), agreeing with the maximum JH titer on day 4 (94 pg/µL; Fig. 33). A good correlation between the expected ecdysteroid and JH titers or biosynthesis rates due to vitellogenin biosynthesis, oocyte maturation and ovipositioning and the measured ecdysteroid and JH titers by LC-ESI-MS was reached.

5.2.3. Ecdysteroid and JH Titers in Paired *Gryllus bimaculatus* Adult Males

Little is known about the role of ecdysteroids in *Gryllus bimaculatus* adult males. *In vitro* incubations of various tissues showed that ecdysteroids are released primarily by the abdominal integument. Maximum rates of ecdysteroid release were measured on day 7 of about 0.6 ng ecdysone equivalents/animal*hour (Weidner and Hoffmann,

1992). Unfortunately, this is not in agreement with the ecdysone titers obtained by LC-ESI-MS. Instead, the ecdysone titers of male adult haemolymph samples (Fig. 39) suggest a relationship between the onset of spermatophore maturation and deposition on day 3 and ecdysone titers, since the ecdysone titer increased on day 3 (65 pg/µL) and remained steady until day 5. Just as in mated adult females (see 5.2.2.) 20-hydroxyecdysone was not present in paired *Gryllus bimaculatus* males (Fig. 41) for probably similar reasons.

During the reproductive cycle of adult male crickets, maturation and control of the accessory reproductive glands is regulated by JH (Kumaran, 1990). The spermatophores are first produced 3 days after imaginal molt, which agrees well with JH III biosynthesis data (Klein et al., 1993). The highest JH III biosynthesis rates occurred on day 2 at 44.1 pmol/pair CA * h and rapidly declined on day 4 (8 pmol/pair CA * h), but remained steady until day 12. Analyzed haemolymph JH III titers in adult male crickets (Fig. 34) show a nice relationship to JH III biosynthesis rates *in vitro*. JH III titers are high after imaginal ecdysis (92 pg/µL), but decline to 39 pg/µL on day 2. A maximum is reached on day 3 (111 pg/µL) again, which coincides well with an increase of JH III biosynthesis on day 2. Further reduction of JH III titers up to day 12 is also in good agreement with observed JH III biosynthesis rates and the reproductive cycle of adult male crickets.

5.2.4. JH I in *Gryllus bimaculatus* Adult Females and Males

Some insect endocrinologists presume that JH III is common to all insects and JH I and JH II are exclusively found in Lepidoptera, whereas others claim to have found JH I and/or JH II in other insect orders as well (Schooley and Baker, 1985). Different techniques such as GC-MS, TLC combined with HPLC (Hazarika and Gupta, 2000) or radioimmunoassays were used. None of these techniques, however, were direct measurements of haemolymph contents, such as the developed LC-ESI-MS method, without further clean-up after protein precipitation or derivatization. Therefore it seems feasible to have found JH I in *Gryllus bimaculatus* besides JH III (Fig. 35 and 36). It is interesting that JH I was not found in cricket larvae, but only in 5-day and 6-day old mated female crickets, and in 6-day and 7-day old paired male crickets. Although, a physiological significance is not expected.

5.2.5. Within-Day JH Fluctuations

In the wing-polymorphic cricket *Gryllus firmus ,* JH III titers experience daily cycles in flight-capable morphs towards the evening (Zhao and Zera, 2003). This raises the concern, if this phenomena could also be observed with *Gryllus bimaculatus*, since flight is possible in 4-day old or younger female crickets (Woodring et al., 2002) and could have an impact on JH titers throughout the day. To circumvent this problem, crickets were collected only in the morning between 6 and 9 o'clock. The haemolymph samples were taken between 9 and 12 o'clock.

5.3. Ecdysteroid and JH Titers in *Spodoptera frugiperda* and their Correlation to Developmental Events in 5th and 6th Instar Larvae and Prepupae

Larval molting will prevail as long as JH titers are high at the onset of high ecdysteroid titers. Metamorphosis is initiated when JH titers decrease and ecdysteroid titers rise (Riddiford, 1980). In holometabolous insects, metamorphosis involves many morphological changes (Kumuran, 1990) which are taken place in the absence of JH. Prior to pupation, JH and ecdysteroid titers increase again (Riddiford, 1980). Preceding molting or pupation, larvae cease feeding due to high levels of ecdysteroids (Sehnal, 1989). In *Spodoptera frugiperda* larvae, weight increase was interrupted between molts and during the prepupal stage (Fig. 45). Due to the unavailability of ecdysteroid or JH titers in literature for *Spodoptera frugiperda*, the measured titers were compared to a closely related noctuid, the leaf worm *Spodoptera littoralis* (Lepidoptera, Noctuidae).
The ecdysteroid levels were moderate during 5th and 6th larval instars. Ecdysone titers were below 10 pg/µL during the 5th larval instar and extremely low on day 1 of the 6th larval instar (less than 2 pg/µL). The maximum ecdysone titer peak occurred on day 3 of the 6th larval instar (12 pg/µL). Only one 20-hydroxyecdysone peak was observed on day 3 of the 5th larval instar. The weight curves clearly showed food intake cessation, but the corresponding ecdysteroid peaks were lacking. Further investigation of ecdysteroid titers in *Spodoptera littoralis* indicated that the commitment peak was quite narrow (Steiner et al., 1999); approximately a 12 h window. Since *Spodoptera frugiperda* haemolymph was taken in the mornings at 24 h intervals, the peak might have easily been missed. On day 3 of the 6th larval instar and day 1 and day 2 of the prepupal stage, a steady ecdysone increase was observed (Fig. 47). 20-

Hydroxyecdysone titers also increased substantially from day 1 to day 2 in the prepupal stage (47 pg/µL). Compared to *Spodoptera frugiperda*, the total ecdysteroid titers were also high at the onset of the prepupal stage in *Spodoptera littoralis* (1.75 ng/µL).

The JH titers obtained in 5th and 6th instar larvae of *Spodoptera frugiperda* (Fig. 46) were comparable to JH titers of 5th and 6th instar larvae of *Spodoptera littoralis*. In 5th instar *Spodoptera littoralis* larvae high JH II titers (3 pg/µL) and minimal JH III titers were observed on day 1, which decreased until the next molt. JH II and JH III titers remained low until the end of the 6th larval instar. In prepupae JH II and JH III titers increased again (1 pg/µL) (Steiner et al., 1999). In *Spodoptera frugiperda*, JH I was present only during the first two days of the 5th larval instar. In contrast to *Spodoptera littoralis*, where JH I was detected until the end of the 3rd larval instar. JH II and JH III were the predominant JH homologes in *Spodoptera frugiperda* during the 5th larval instar, with higher titer being recorded for JH III than for JH II. In 6th instar larvae and prepupae (day 1), only JH III prevailed at moderate levels (below 11 pg/µL). JH II appeared again in day 2 prepupae (Fig. 46). Although JH titers were about 10-fold less in *Spodoptera littoralis*, relative increases and decreases were equivalent. The obtained ecdysteroid and JH titer measurements by LC-ESI-MS coincide well with the development of 5th and 6th larval instar and prepupal changes.

5.4. Alkaloids from *Glycosmis pentaphylla* Leaves as Potential Insect Growth Regulators

The Pink Lime *Glycosmis pentaphylla* (Rutaceae), a shrub found in India, South Africa and Australia, has been extensively used in Ayurvedic medicine as a febrifuge (alleviates fever) and a vermifuge (expels worms) (Chakravarti et al., 1961), but also as an insect deterrent. Pesticide studies involving an agricultural pest, the castor semilooper *Achaea janata* Linn. (Lepidoptera, Noctuidae), has lead to the discovery of possible juvenoids in ethyl acetate fractions of *Glycosmis pentaphylla* (Muthukrishnan and Ananthagowri, 1994). The ethyl acetate fraction of leave extracts also inhibits JH III biosynthesis *in vitro* of 3-day old *Gryllus bimaculatus* females and significantly reduces *Culex quinquefasciatus* egg hatchability (Muthukrishnan et al., 1999). In this thesis three alkaloids have been isolated from *Glycosmis pentaphylla* leaves from the

diethyl ether and the ethyl acetate fractions: the acridine alkaloid arborinine, the quinoline alkaloid 4-methoxy-1-methyl-2(1*H*)-quinolinone, and the quinazoline alkaloid arborine.

Since alkaloids from the Rutaceae family are known to be quite effective as insectides, antifeedants and insect growth regulators (Adityachaudhury et al., 1985), the isolated alkaloids offer a promising aspect of acting as insect growth regulators. By utilizing the newly developed LC-ESI-MS method in combination with biotests, the effectiveness of the isolated alkaloids as insect growth regulators can be assessed and perhaps lead to the discovery of novel insecticides.

6. Conclusions and Outlook

A simple, fast and sensitive method was developed for routine determination of JHs, JH acids, JH diols and ecdysteroids in insect haemolymph by LC-ESI-MS. Sample clean-up involves simple protein precipitation by isooctane:MeOH (1:1, v/v), centrifugation and partial evaporation of the organic solvents. The analytes were separated on a C_{18} column by gradient elution with water and MeOH in less than 20 min and analyzed by ESI-MS. Due to the high $[Na^+]$ and $[K^+]$ in insect haemolymph, the $[M+Na]^+$ is primarily formed for the JHs and JH diols, and the $[M+Na]^+$ / $[M+K]^+$ for the ecdysteroids. In haemolymph samples, the LODs for JHs and JH diols were between 6 to 12 pg, respectively. The LOD for ecdysteroids was around 93 pg. The LOD could probably be improved by investigating the use of other additives, other than formic acid, for LC-ESI-MS analysis. Keeping JH and JH diol LODs the same, but improving the LOD for ecdysteroids. Other JHs, ecdysteroids or even other types of hormones could easily be added to the method, thereby improving interpretation of important physiological processes.

The ecdysteroid and JH titers in the haemolymph of female and male *Gryllus bimaculatus* larvae and adults were in good agreement with developmental events. The same holds true for *Spodoptera frugiperda* 5^{th} and 6^{th} larval instar and prepupae. Now, further studies on the effectiveness of alkaloids from *Glycosmis pentaphylla* as potential insect growth regulators can proceed, since endocrinologically important parameters have been established, which could possibly improve interpretations of bioassay results. Additionally, many routes of degradation mechanisms are known for xenobiotics, thereby increasing the possibility to detect and identify degradation products of topically applied, injected, or ingested alkaloids.

7. Summary

- Aim of this thesis was to develop a liquid chromatography-mass spectrometry (LC-MS) method to monitor hormones and their metabolites in the haemolymph of insects simultaneously. Furthermore, some plant-derived alkaloids were structurally elucidated, which may be used as insect growth regulators thus affecting haemolymph hormone titers in putative pest species.

- Juvenile hormones (JHs), JH diols and ecdysteroids were easily separated by high performance-liquid chromatography (HPLC) in less than 20 min using a reversed-phase C_{18} column and a methanol-water gradient.

- Subjecting the JH-JHBP (juvenile hormone binding protein) complex to HPLC was sufficient in releasing JH from the JHBP.

- In order to prevent JH from binding to glass surfaces, it was necessary to include a carrier in the solvent. JHs have a high affinity to polypropylene vials and should therefore be avoided, if no carrier is being used, such as triton X-100.

- The darkening of the haemolymph due to eumelanin production by phenol oxidases served as a visual indicator of general enzyme activity in the haemolymph. Isooctane:MeOH (1:1, v/v) inactivated the phenol oxidase system when used at a solvent-haemolymph ratio of 10.

- An isooctane:MeOH extract of haemolymph was most efficient in keeping JH distributed evenly in solution and prevented JH from adhering to the glass vessel.

- JH concentration in standard solutions was reduced with increasing sonication time.

- Highest ionization of JH was achieved in MeOH for MS compared to ACN or by using formic acid as an additive.

- In the positive ESI (electrospray ionization) mode the most abundant ions formed in haemolymph extract of *Gryllus bimaculatus* was the sodium adduct for JHs, JH diols and JH acids. At higher JH concentrations, the potassium adduct was also observed. The sodium and the potassium adducts were present in ecdysteroid analysis. The same ionization pattern was observed in *Spodoptera frugiperda*, *Myrmicaria eumenoides* and *Acyrthosiphon pisum* haemolymph.

- Method validation of the LC-MS method confirmed reproducibility and repeatability. The LODs for JHs and JH diols were between 6 to 12 pg, and 93 pg for ecdysteroids.

- 72 haemolymph samples can be processed per day by the LC-ESI-MS method using an autosampler.

- JH and ecdysteroid titer measurements showed good agreement between haemolymph titers and developmental events in *Spodoptera frugiperda* and *Gryllus bimaculatus* larvae and *Gryllus bimaculatus* adults.

- In *Gryllus bimaculatus* female and male last instar larvae, the JH titers were low and steady until day 6. The ecdysone peak maximum shifted from day 3 to a 20-hydroxyecdysone maximum peak on day 5, coinciding with the onset of adult ecdysis.

- JH III titers increased on day 3 in paired *Gryllus bimaculatus* males, occurring simultaneously with spermatophore maturation and deposition. A similar response was seen with ecdysone titers.

- Mated female crickets experienced a JH III titer increase on day 4 which coincides with egg deposit on day 4. Ecdysone titers reach a maximum on day 3 and ovary weights on day 4.

- Besides JH III, JH I was found in 5 to 8-day old female adult crickets, and in 6 and 7-day old male adult crickets.

- 20-Hydroxyecdysone was found neither in female nor in male *Gryllus bimaculatus* mated adults.

- The "classical" interplay between JHs and ecdysteroids was observed in 5th instar *Spodoptera frugiperda* larvae. JH titers decreased towards the end of the larval stadium and 20-hydroxyecdysone gave a sharp peak on the last day of the 5th instar. Extremely low levels of JH were measured in 6th instar larvae. 20-Hydroxyecdysone and ecdysone titers increased simultaneously in prepupae.

- The already known alkaloids arborinine and arborine, and the for the first time isolated 4-methoxy-1-methyl-2(1*H*)-quinolinone from *Glycosmis pentaphylla*, were extracted from *Glycosmis pentaphylla* leaves and are discussed as possible insect growth regulators affecting hormone titers in the haemolymph of insect pest species.

8. Zusammenfassung

- Ziel der Untersuchungen war die Entwicklung einer LC-MS (liquid chromatography-mass spectrometry) Methode zur Bestimmung von Hormonen und ihren Metaboliten in der Hämolymphe von Insekten. Darüber hinaus wurden einige Alkaloide aus Pflanzenblättern in ihrer Struktur aufgeklärt, die als Insektenwachstumsregulatoren bei der Bekämpfung von Schädlingen eingesetzt werden könnten.

- Juvenilhormone (JH) und deren Diole (JH-Diole) konnten zusammen mit Ecdysteroiden auf einer HPLC (high performance-liquid chromatography) C_{18}-Säule mühelos in weniger also 20 min aufgetrennt werden, wenn ein Methanol-Wasser-Gradient verwendet wurde.

- Juvenilhormone werden bereits per HPLC Auftrennung von ihrem JH-Bindungsprotein gelöst.

- Ein Trägerstoff ist notwendig, um einen Juvenilhormonverlust durch JH-Bindung an Glas zu vermeiden. Als gute JH-Trägerstoffe haben sich Isooctan:MeOH-Hämolymphextrakte bewährt. Juvenilhormone werden homogener in der Lösung verteilt als in MeOH und bleiben nicht am Glas haften. Beim Umgang mit geringen Juvenilhormonkonzentrationen eignen sich Kunststoffgefäße aufgrund ihrer hohen JH-Affinität nicht.

- Isooctan:MeOH (1:1, v/v) verhinderte die Aktivierung von Phenoloxidasen und eine Verfärbung der Hämolymphe, wenn das 10-fache Lösungsmittelvolumen gegenüber dem Hämolymphvolumen genommen wird. Auf diese Weise werden wohl alle Enzyme in der Hämolymphe inaktiviert.

- Ultraschall-Behandlung vermindert die Juvenilhormonkonzentration in Lösungen.

- Die höchste Ionisierungsausbeute des Juvenilhormons wurde in MeOH erzielt. ACN oder Zusätze (z.B. Ameisensäure) konnten die Empfindlichkeit nicht erhöhen.

- In der Hämolymphe werden im positiven Ionisierungsmodus die Juvenilhormone und deren Diole hauptsächlich zu Natrium-Addukten umgewandelt. Bei hohen JH – Konzentrationen und bei der Ecdysteroidanalyse fanden sich noch zusätzlich Kalium-Addukte. Gleiche Ionisierungsmuster wurden auch in der Hämolymphe von *Spodoptera frugiperda*, *Myrmicaria eumenoides* und *Acyrthosiphon pisum* festgestellt.

- Reproduzierbarkeit und Wiederholbarkeit sind bei der LC-ESI-MS Methode gewährleistet. Die Nachweisgrenzen lagen zwischen 6 und 12 pg für die Juvenilhormone und Diole, und bei 93 pg für die Ecdysteroide.

- Pro Tag können 72 Hämolymphproben mit Hilfe der LC-MS Methode und eines Autoinjektors analysiert werden.

- Die gemessenen Juvenil- und Ecdysteroidtiter stimmen gut mit den jeweiligen Entwicklungsstadien von *Spodoptera frugiperda* und *Gryllus bimaculatus* Larven und *Gryllus bimaculatus* Adulten überein.

- Die JH-Titer in weiblichen und männlichen *Gryllus bimaculatus* Larven im letzten Larvalstadium waren gering, aber konstant bis zum 6. Tag. Ein Anstieg des Ecdysontiters am 3. Tag wurde von einem Anstieg des 20-Hydroxyecdysontiters am 5. Tag begleitet.

- Ein Anstieg des JH III-Titers in verpaarten adulten männlichen Grillen am 3. Tag ist wahrscheinlich mit der sexuellen Reifung verbunden. Zeitgleich steigt auch der Ecdyson-Titer an.

- In den verpaarten Grillenweibchen steigt der JH III-Titer am 3. Tag an und erreicht ein Maximum am 4. Tag. Der Ecdyson-Titer steigt ebenfalls am 3. Tag an. Zu diesem Zeitpunkt sind die Ovarien am schwersten und das Weibchen beginnt mit der Eiablage.

- Neben JH III wurde in 5- bis 8-tägigen weiblichen und in 6- bis 7-tägigen männlichen adulten Grillen JH I nachgewiesen.

- 20-Hydroxyecdyson wurde weder in den adulten Weibchen noch in den adulten Männchen von *Gryllus bimaculatus* nachgewiesen.

- Das „klassisches" Beispiel der Hormonkoordination vor und während der Häutung konnte beim 5. Larvalstadium von *Spodoptera frugiperda* beobachtet werden. Der JH-Titer sinkt zum Ende des Larvalstadiums, während der 20-Hydroxyecdyson-Titer ansteigt. Während des 6. Larvalstadiums sind die JH-Konzentrationen in der Hämolymphe sehr gering. Während des Vorpuppenstadiums ist ein leichter Anstieg des JH-Titers zu beobachten. Ecdyson- und 20-Hydroxyecdyson-Titer steigen rapide im Vorpuppenstadium an.

- Die bereits bekannten Alkaloide Arborinin und Arborin, und das erstmals aus *Glycosmis pentaphylla* extrahierte 4-Methoxy-1-methyl-2(1*H*)-chinolinon, wurden aus *Glycosmis pentaphylla* Blättern isoliert. Ihre Rolle als potentielle Insektenwachstumsregulatoren wird diskutiert.

9. Literature

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Bayreuth, den 21. April 2004

(Stephanie Westerlund)