

Development, validation, and application of liquid chromatography mass spectrometry methods for the determination of juvenile hormone analogues in mealworms

Sjors Rasker, Rita Boerrigter-Eenling, Helmoed Cammin, Ricky Potjes, Lucas Agterberg, Marco H. Blokland, Ane Arrizabalaga-Larrañaga & Saskia S. Sterk

To cite this article: Sjors Rasker, Rita Boerrigter-Eenling, Helmoed Cammin, Ricky Potjes, Lucas Agterberg, Marco H. Blokland, Ane Arrizabalaga-Larrañaga & Saskia S. Sterk (07 Aug 2026): Development, validation, and application of liquid chromatography mass spectrometry methods for the determination of juvenile hormone analogues in mealworms, Food Additives & Contaminants: Part A, DOI: [10.1080/19440049.2026.2710625](https://doi.org/10.1080/19440049.2026.2710625)

To link to this article: <https://doi.org/10.1080/19440049.2026.2710625>



© 2026 The Author(s). Published with license by Taylor & Francis Group, LLC



[View supplementary material](#)



Published online: 07 Aug 2026.



[Submit your article to this journal](#)



Article views: 19



[View related articles](#)



[View Crossmark data](#)



Development, validation, and application of liquid chromatography mass spectrometry methods for the determination of juvenile hormone analogues in mealworms

Sjors Rasker , Rita Boerrigter-Eenling, Helmoed Cammin, Ricky Potjes, Lucas Agterberg, Marco H. Blokland , Ane Arrizabalaga-Larrañaga[†] and Saskia S. Sterk

Wageningen Food Safety Research (WFSR), European Union Reference Laboratory for antivirals, growth promoters and sedatives, Part of Wageningen University and Research, Wageningen, AE, the Netherlands

ABSTRACT

Juvenile hormone analogues (JHAs) are known to affect insect growth and can potentially be used in edible insect rearing. In this study, a reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was developed for the detection of five common juvenile hormone analogues (fenoxycarb, pyriproxyfen, methoprene, kinoprene, and hydroprene) in mealworms. Sample clean-up was performed using reversed-phase solid-phase extraction cartridges. To enhance ionisation, methoprene, kinoprene, and hydroprene are detected as their 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatives. The method was fully validated according to Commission Implementing Regulation (EU) 2021/808 and is capable of both quantitative and qualitative confirmation of fenoxycarb, pyriproxyfen, methoprene, and kinoprene (CC α values of 6.0–29 $\mu\text{g kg}^{-1}$), and qualitative screening of hydroprene (with a CC β of 7.7 $\mu\text{g kg}^{-1}$). Additionally, it was demonstrated that liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) can also be used for the screening of JHA residues at relevant levels. The LC-MS/MS method was used to demonstrate the presence of methoprene in an incurred sample of mealworms. Finally, 14 mealworm samples originating from the Dutch national residue control plan were analysed, and none of the target analytes were found.

ARTICLE HISTORY

Received 27 May 2026
Revised 15 July 2026
Accepted 19 July 2026

KEYWORDS

Juvenile hormone analogue;
mealworm; residue; veterinary
drug; food safety

Introduction

Juvenile hormones (JH) are endogenous compounds found in insects that play a role in growth and metamorphosis (Riddiford 2020). Among the various types, JH III is one of the most prevalent (Dhadialla et al. 2005); see Figure 1. Throughout their life cycle, many insects undergo periodic moulting, during which the exoskeleton is replaced, a requirement for further growth and for metamorphosis from larvae to the next stage. Moulting hormones called ecdysteroids regulate this process by initiating moulting and inducing metamorphosis. JH acts together with ecdysteroids to determine the outcome of each moult as larval, pupal, or adult. The JH concentration is relatively high in the early stages

of insect growth, preventing metamorphosis, and is practically absent at the point when the larvae transform into pupae and then into adults. In short, the presence of JH permits growth but inhibits metamorphosis, maintaining the larval stage (Riddiford 1994; Henrick 2007; Li et al. 2019; Riddiford 2020).

The growth-regulating effects of JH have led to the use of stable, more potent analogues of these substances as insecticides, known as juvenile hormone analogues (JHA), juvenile hormone mimics (JHM), or juvenoids. Like natural JH, JHAs prevent insect larvae from metamorphosing into adults, thereby halting reproduction (Williams 1956; Dhadialla et al. 2005; Henrick 2007). While useful for pest control, studies have demonstrated that this effect can be used to

CONTACT Sjors Rasker sjors.rasker@wur.nl Wageningen Food Safety Research (WFSR), European Union Reference Laboratory for antivirals, growth promoters and sedatives, Part of Wageningen University and Research, Wageningen 6700 AE, the Netherlands

[†]Present address: Department of Applied Chemistry, Faculty of Chemistry, University of the Basque Country (EHU/UPV), 20018, San Sebastian, Basque Country, Spain

Supplemental data for this article can be accessed online at <https://doi.org/10.1080/19440049.2026.2710625>.

© 2026 The Author(s). Published with license by Taylor & Francis Group, LLC

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

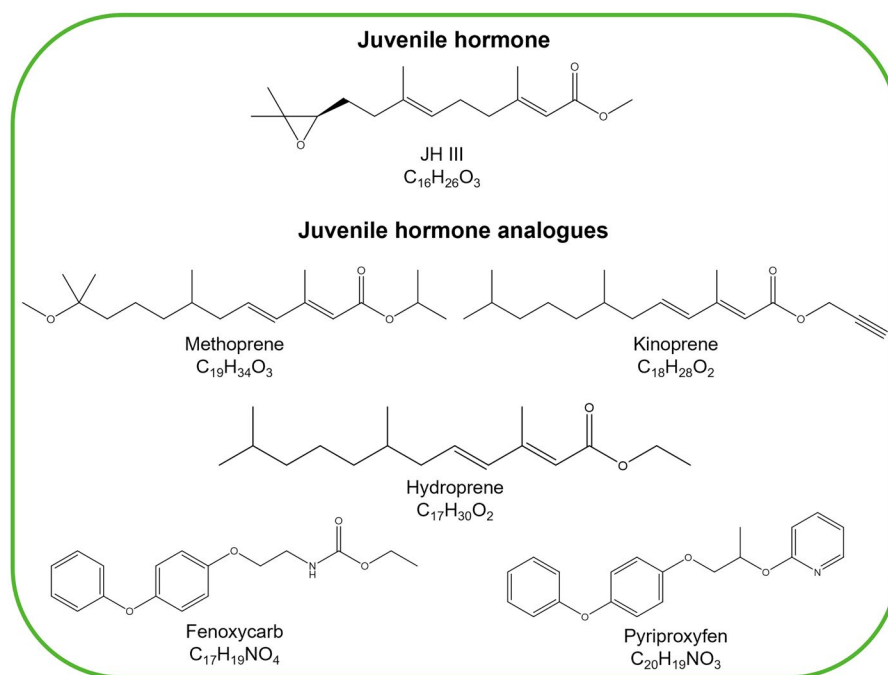


Figure 1. Molecular structures of natural juvenile hormone JH III and the juvenile hormone analogues methoprene, kinoprene, hydroprene, fenoxycarb, and pyriproxyfen investigated in this study.

cause larval growth beyond the point at which they would normally metamorphose, creating abnormally large larvae. For example, the weight of tobacco budworm (*Heliothis virescens*) doubled when fenoxycarb was included in the diet compared to the control group that received no treatment (Mauchamp et al. 1989). Similarly, so-called ‘super larvae’ of the silkworm (*Bombyx mori*) were obtained when methoprene was topically applied at a specific time during larval development, resulting in more than a doubling of their weight compared to the control group (Kadono-Okuda et al. 1986). Indian meal moth larvae (*Plodia interpunctella*) reached three times their normal size when treated with an ecdysteroid analogue and methoprene (Silhacek et al. 1990). Moreover, it has been demonstrated that JHAs can alter the protein and fat composition of yellow mealworms (*Tenebrio molitor*) when included in the diet (Hill et al. 2024). Considering the interest in edible insects as alternative protein sources for food and feed (van Huis 2022) and the well-known effects of JHAs on insect growth, as also reported in popular media (Ken 2017; The Thought Emporium 2025), residue monitoring of these substances in edible insects is becoming increasingly relevant.

Although many compounds with JH-like effects are known (Sláma 1999; Henrick 2007; Jindra and Bittova 2020; Devi et al. 2022; Devi and Awasthi 2022; Tumova et al. 2022), only a few have been widely used as insecticides. The following JHAs were included in this study: fenoxycarb, pyriproxyfen, methoprene, kinoprene, and hydroprene (Henrick 2007; Sláma 2013; Jindra and Bittova 2020; Parthasarathy and Palli 2021; Insecticide Resistance Action Committee 2023). Multiple analytical approaches have been described for the detection of individual or subsets of these analytes in various matrices (Aronov et al. 2005; Payá et al. 2013; He et al. 2020; Wu et al. 2021; Martín-Pozo et al. 2023; Tang et al. 2024). However, to the best of our knowledge, no validated method for the simultaneous analysis of the most relevant JHAs in edible insects has been described and applied for residue monitoring.

This study describes the development of a sample pre-treatment procedure for the analysis of five JHAs in edible mealworms. The extracts are analysed using both liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which can be applied for routine monitoring, and liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS), which can additionally

be applied for research purposes since it provides the added benefit of collecting non-targeted data that can be used for retrospective data analysis (Jongedijk et al. 2023). Furthermore, the proposed LC-MS/MS method has been fully validated as a quantitative, confirmatory method for mealworms in accordance with Commission Implementing Regulation (EU) 2021/808 (European Commission 2021), as the JHAs would be applied as pharmacologically active substances in food-producing animals in the context of this study. The LC-HRMS screening method was further validated through the analysis of samples from a single validation day. Moreover, the proposed LC-MS/MS method was applied to incurred material consisting of mealworms exposed to methoprene to demonstrate its fitness for purpose. Finally, an exploratory survey was conducted on mealworm products originating from the Dutch national residue control plan.

Material and methods

Chemicals and reagents

LC-MS grade water, acetonitrile (ACN), methanol (MeOH), ethyl acetate, formic acid, acetic acid (HAc), and hydrochloric acid (37%) (HCl) were obtained from Actua-All Chemicals (Oss, the Netherlands). Methoprene (99.1%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), and ammonium formate were obtained from Sigma Aldrich (Saint Louis, MO, USA). Kinoprene (91.1%), hydroprene (98.97%), fenoxycarb (99.83%), pyriproxyfen (96%), methoprene-d7, and fenoxycarb-d3 were obtained from LGC standards (Wesel, Germany). Deionised water was used in sample preparation and produced with a Milli-Q Reference A+ system (Merck KGaA, Darmstadt, Germany). Stock solutions were prepared taking purity into account in methanol and stored at -18°C for the duration of the project.

Sample preparation

Mealworm samples were prepared by extracting 0.5 g of material using 5 mL of acetonitrile:water 70/30 v/v% using a Beadruptor (Omni International 24) (Omni International, Kennesaw, GA) set to a speed of 5.65 m s⁻¹, a cycle time of 0.75 min, and a pause dwell time of 0.5 min; two cycles were

used. Next, the samples were centrifuged for 10 min at 3500g. The acetonitrile in the extract was then evaporated at 45°C under a gentle flow of nitrogen until only the aqueous phase remained, after which 1.5 mL of water was added. Cleanup was performed using Strata-X solid-phase extraction cartridges (200 mg) (Phenomenex, Torrance, CA, USA) that were first conditioned using 3 mL of acetonitrile, followed by 2 mL of water. The cartridges were washed with 2 mL of water, then eluted with 2 mL of ethyl acetate, after which the eluate was evaporated at 45°C under a gentle flow of nitrogen. Next, 200 µL of PTAD solution (750 µg mL⁻¹ in acetonitrile) was added, and the extracts were left for 1 h at room temperature to allow derivatisation of methoprene, kinoprene, and hydroprene (Aronov et al. 2005). During method development, Oasis HLB cartridges (200 mg) (Waters Corporation, Milford, MA, USA) and Bond Elute C18 cartridges (500 mg) (Agilent, Santa Clara, CA, USA) were tested.

Instrumental analysis

Targeted analysis was performed in positive ion multiple-reaction-monitoring (MRM) mode using an Acquity Premier UPLC system coupled to a Xevo TQ Absolute Triple Quadrupole Mass Spectrometry equipped with an electrospray ionisation source (Waters Corporation, Milford, MA, USA). See [Supplementary Table S1](#) for MS-related settings. Analysis was performed using an Acquity UPLC BEH C₁₈ column (2.1 mm x 100 mm, 1.7 µm particle size) (Waters Corporation, Milford, MA, USA). Mobile phase A consisted of water and mobile phase B of acetonitrile, both including 0.1 v/v% formic acid. The gradient started at 60% A for 0.5 min, then moved to 100% B at 1.5 min, held until 2.5 min, and then returned to 60% A at 3 min, holding until 4.2 min for re-equilibration, at a flow rate of 0.6 mL min⁻¹. The column temperature was set to 50°C, and an injection volume of 5 µL was used. LC-HRMS analysis was performed using a Vanquish Horizon UHPLC system coupled to an Orbitrap IQ-X Tribrid mass spectrometer equipped with a Heated Electrospray Ionisation source (Thermo Scientific, San Jose, CA, USA) in positive mode. Water was used as mobile phase A and methanol

as mobile phase B, both including 2 mM ammonium formate and 0.1 v/v% formic acid. An Acquity UPLC BEH C₁₈ column (2.1 mm × 100 mm, 1.7 µm particle size) (Waters Corporation, Milford, MA, USA) was used. The gradient consisted of 100% A increasing to 100% B in 15 min, held until 21 min, then returning to 100% A at 22 min, and held until 30 min for re-equilibration at a flow rate of 0.3 mL min⁻¹. The column temperature was kept at 50 °C, and an injection volume of 5 µL was used. Full-scan data were acquired at a resolution of 120,000 full width at half maximum (FWHM) at *m/z* 200 across a scan range of 110–1100 *m/z*, followed by both data-dependent, including dynamic exclusion, and scheduled MS² scan events. The collision energy was set to an absolute value of 20 V for all analytes during scheduled scan events.

Method validation

The proposed method was fully validated as a quantitative confirmatory method for unauthorised substances in mealworms using LC-MS/MS, in accordance with Commission Implementing Regulation (EU) 2021/808 (European Commission 2021). To date, no maximum residue limits (MRLs), minimal method performance requirements (MMPRs), or reference points of action (RPAs) have been established by the European Union for JHA in edible insects in the context of pharmacologically active substances in food-producing animals. MRLs have been set in Commission Regulation 899/2012 for some JHA in the context of pesticide residues in the category 'Other terrestrial animal products' to which edible insects belong, which are 50 µg kg⁻¹ for methoprene and 10 µg kg⁻¹ for both fenoxycarb and pyriproxyfen (European Commission 2012). For the current validation, the lowest calibration level (LCL) was set at 5.0 µg kg⁻¹ (1*LCL), and the validation was performed at three levels: 1*LCL, 2*LCL, and 3*LCL. During the validation study, the identification criteria, decision limit for confirmation (CC_α), trueness, precision (repeatability and within-laboratory reproducibility), selectivity/specificity, stability, and ruggedness were investigated as defined in Table 5 of

Commission Implementing Regulation (EU) 2021/808 (European Commission 2021). The validation study was performed on three separate days by three different technicians using two different LC-MS/MS instruments. Additionally, the samples from validation day 1 were analysed using LC-HRMS. On each validation day, 7 different batches of mealworms were analysed as blanks, and each batch was also fortified at 1, 2, and 3*LCL for a total of 28 validation samples per day. On each validation day, a calibration curve of 6 matrix fortified samples was included for quantification (MFS-line), using a different batch of mealworms each day. Internal standard correction was used for all samples. The identification criteria were assessed by checking if the analytes could be identified in the fortified samples according to the criteria for the relative retention time (within 1% of the relative retention time of the MFS line) and the mass spectrometric identification criteria for LC-MS/MS, meaning that the ratio between the quantifying ion and the qualifying ion falls within 40% of the average ion ratio in the MFS samples. The decision limit for confirmation (CC_α) was calculated at the lowest validation level at which at least 20 of the fortified samples met the identification criteria for unauthorised or prohibited substances, as defined in method 3 of Commission Implementing Regulation (EU) 2021/808 (European Commission 2021). Trueness was assessed at all three levels using the internal standard-corrected MFS lines for quantification of the internal standard-corrected fortified samples and comparing the calculated value to the known fortification value; a criterion of -30 to +20% was used. Repeatability (within-day precision) was expressed as the relative standard deviation (RSD_r, %) of seven replicate extracts, each prepared from different batches of sample, on the same day with the same instruments and technicians; a criterion of 20% was used. Within-laboratory reproducibility (between-day precision) was expressed as the mean repeatability at each LCL obtained across the three validation days with different instruments and technicians (RSD_{rl}, %), and a criterion of 30% was used. To determine the method's selectivity/specificity, the 21 unfortified samples were visually assessed for

signals (signal-to-noise ratio > 3) that could affect data analysis. Stability of the sample extracts was investigated by storing the extracts from validation day 1 at -20°C for 10 days and re-analysing the MFS line and the 7 samples fortified at 1*LCL. Extracts were deemed stable when the criteria for identification, repeatability and trueness were met upon re-analysis. Finally, ruggedness was assessed by including three samples spiked at 2*LCL that were prepared and analysed as normal and by using these samples to assess three potential method deviations, each with three samples prepared and analysed. The final evaporation step was identified as the most likely source of deviations, so it was selected for the ruggedness study. For the first deviation, instead of evaporating until dry after cleanup, evaporation was stopped prematurely. For the second deviation, evaporation was continued for 15 min after the extract was completely dry, and for the third deviation, the evaporation temperature was set to 55°C instead of 45°C . The method was deemed rugged after the modifications, as trueness and repeatability met the criteria. RESVAL version 4.0 (WFSR EURL) was used for LC-MS/MS validation data analysis. The LC-HRMS method was partially validated as a qualitative screening method by assessing selectivity/specificity, and the detection capability for screening ($\text{CC}\beta$) was determined using method 2 (European Commission 2021) with 7 fortified blanks, where the $\text{CC}\beta$ was set at the calibration level at which $\leq 5\%$ false-compliant results were observed.

Incurring mealworms

Methoprene-incurred mealworms were obtained from an insect study on the effects of veterinary drug exposure on mealworms (*Tenebrio molitor*) (Rasker et al. 2026). In short, mealworms were exposed to methoprene for 7 days *via* their substrate, which was fortified with 10 mg kg^{-1} methoprene. After this period, mealworms were removed from the fortified substrate and placed on clean substrate for 48 h to allow the fortified substrate to be excreted from their digestive systems. A sample from this study was analysed using the proposed LC-MS/MS method.

Exploratory survey

The method was used to analyse 14 anonymised monitoring samples from the Dutch national residue plan. These samples consisted of freeze- or oven-dried, frozen, or powdered mealworms taken for official residue monitoring, and they were analysed using a single standard addition approach as described in the EURL Guidance Document on Standard Addition (European Union Reference Laboratories 2022). Two aliquots of each sample were analysed, one as such and one fortified at 1*LCL ($5\mu\text{g kg}^{-1}$) before sample preparation. Results were assessed visually; if the analyte in the fortified sample produced a chromatographic peak that was not visible in the sample as such and the corresponding internal standard was visible in both samples, then the analyte was considered detectable. This approach was used because (freeze)-dried and powdered mealworms were not included in the validation study.

Results and discussion

Method development

Sample pre-treatment

Development of the sample pre-treatment procedure began by investigating multiple extraction solvents, following a generic protocol for subsequent solid-phase extraction with Oasis HLB cartridges, which included ethyl acetate as the elution solvent, as this has been used successfully to elute JHAs in previous work (Aronov et al. 2005). Multiple extraction solvents were assessed across a wide polarity range, with and without the inclusion of acids, see Figure 2A. Optimal performance was observed with water/ACN (3:7, v/v) + 1% (v/v) Hac. This extraction solvent was selected even for the most apolar analyte, hydroprene; see Supplementary Table S2 for analyte characteristics. Next, multiple SPE cartridges were assessed, including Oasis HLB, Strata-X, and C_{18} cartridges, using the same conditioning, washing, and elution procedure (see Figure 2B). Overall, the differences between the cartridges appeared to be minor; methoprene, kinoprene, and hydroprene showed a slightly higher response when Strata-X or C_{18} cartridges were used compared to the

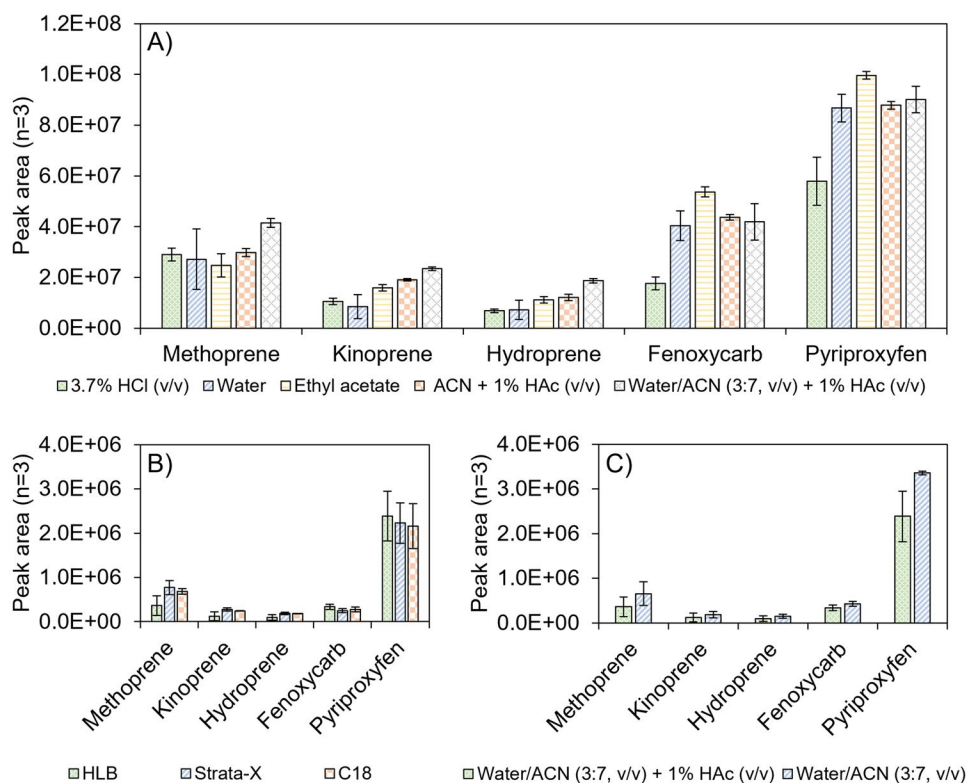


Figure 2. (A) Comparison of different extraction solvents using mealworms fortified with JHAs at 50 $\mu\text{g kg}^{-1}$. (B) Comparison of different SPE cartridges using mealworms fortified with JHAs at 50 $\mu\text{g kg}^{-1}$ extracted using Water/ACN (3:7, v/v) + 1% (v/v) HAc. (C) Effect of addition of acid to the extraction solvent using mealworms fortified with JHAs at 50 $\mu\text{g kg}^{-1}$. Error bars represent standard deviation.

Oasis HLB. It was decided to continue with Strata-X cartridges because of their performance and increased user-friendliness compared to C_{18} cartridges. Finally, the effect of adding acid to the extraction solvent was investigated, and results improved slightly when acid was omitted, allowing for further simplification of the method (see Figure 2C). Except for pyriproxyfen, none of the included analytes exist in an ionised state at low pH, see Supplementary Table S2, so any effects on the response due to the presence of acid can be attributed to the interaction between the acidity of the extraction solvent and matrix components. The optimal initial extraction conditions found for JHAs are similar to those used in previously published multi-methods in the residue analysis field (e.g. Monteiro et al. 2021), suggesting that other compounds can potentially be included in the future.

Instrumental analysis

The non-polar nature of some JHAs, such as methoprene, hydroprene, and kinoprene, results

in a poor ionisation efficiency during electrospray ionisation (ESI) (Aronov et al. 2005). However, since ESI is the most accessible, widely used, and therefore preferred interface between liquid chromatography and mass spectrometry in residue analysis laboratories, it was used in this study. The poor ionisation efficiency of JHAs can be overcome by derivatising the analytes to introduce proton-accepting amide groups into the analyte structure *via* a Diels-Alder cycloaddition using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), as demonstrated previously by Aronov et al. (2005). This derivatisation procedure can be performed at room temperature and does not affect fenoxycarb and pyriproxyfen since these do not contain the diene moiety required for the reaction to occur. The optimal multiple reaction monitoring (MRM) transitions for LC-MS/MS analysis were determined for all (derivatised) analytes and internal standards by infusing standards; see Table 1. To the best of our knowledge, no isotope-labelled internal standards are commercially available for kinoprene and hydroprene,

so methoprene-d7 was used for correction. The exact mass for LC-HRMS analysis was determined based on the molecular formulae, and scheduled MS² scans were included in the method

to ensure detection at low levels, likely required due to co-eluting matrix interferents that increased total ion abundance and reduced analyte sensitivity (Kaufmann 2018). For data analysis, the most

Table 1. Analyte mass spectrometric information.

Compound	Molecular formula	Adduct	Pre-cursor ion (<i>m/z</i>)	Declustering potential (V)	Product ion (<i>m/z</i>)	Collision energy (V)
Fenoxycarb	C ₁₇ H ₁₉ NO ₄	[M + H] ⁺	302	66	88* 116	29
Pyriproxyfen	C ₂₀ H ₁₉ NO ₃	[M + H] ⁺	322	81	96* 78	17 23
Methoprene-PTAD	C ₂₆ H ₃₅ N ₃ O ₄ **	[M + H] ⁺	454	111	242* 328	81 31
Kinoprene-PTAD	C ₂₆ H ₃₃ N ₃ O ₄	[M + H] ⁺	452	86	242* 368	17 27
Hydroprene-PTAD	C ₂₅ H ₃₅ N ₃ O ₄	[M + H] ⁺	442	121	242* 368	19 29
Fenoxycarb-d3	C ₁₇ H ₁₆ D ₃ NO ₄	[M + H] ⁺	305	66	119	21 17
Pyriproxyfen-d4	C ₂₀ H ₁₅ D ₄ NO ₃	[M + H] ⁺	326	81	100	23
Methoprene-d7-PTAD	C ₂₆ H ₂₈ D ₇ N ₃ O ₄ **	[M + H] ⁺	461	111	242	27

*Product ion used for quantification.

**Corresponds to the in-source fragment where CH₄O is lost.

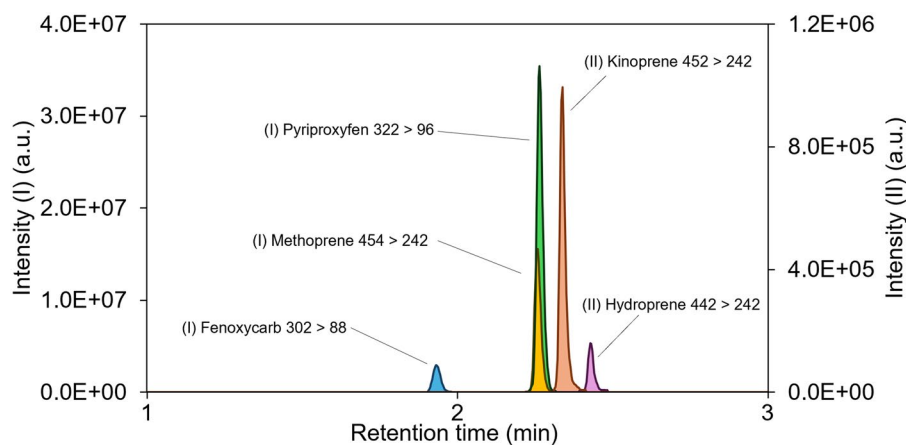


Figure 3. Representative example LC-MS/MS extracted ion chromatograms of mealworm fortified with JHA at 1*LCL (5 µg kg⁻¹), only relevant signal based on retention time range displayed for clarity (a.u. = arbitrary units).

intense production was used; see [Supplementary Table S3](#).

After confirming that all analytes could be detected using both tandem and high-resolution mass spectrometry, the separation was investigated. For LC-MS/MS analysis, a short gradient using a C₁₈ column was sufficient to elute all analytes, and it was decided to prioritise rapid analysis over baseline separation, see [Figure 3](#). For LC-HRMS analysis, a generic, longer

gradient was used to facilitate separation of potential unknowns, see [Supplementary Figure S1](#) for an example LC-HRMS chromatogram. It is worth noting that when the LC-HRMS method is applied for target or suspect screening of compounds other than the JHAs described in this work, the possibility of PTAD-derivatization of residues should be considered, for example, by including the *m/z* of potential PTAD-conjugates in the suspect list.

Table 2. Performance characteristics of the developed LC-MS/MS method per analyte in mealworms ($n=21$).

Compound	Concentration ($\mu\text{g kg}^{-1}$)	Trueness (%)	Repeatability (RSDr, %)	Reproducibility (RSDrl, %)	Confirmed	Classification*	CC α ($\mu\text{g kg}^{-1}$)
Fenoxycarb	5	98	9	9	21/21	Quantitative confirmatory	6.0
	10	97	6	6	21/21		
	15	96	7	7	21/21		
Pyriproxyfen	5	91	23	24	21/21	Qualitative confirmatory	7.5
	10	93	23	23	21/21		
	15	96	21	21	21/21		
Methoprene	5	105	4	11	21/21	Quantitative confirmatory	6.3
	10	102	11	14	21/21		
	15	100	11	12	20/21		
Kinoprene	5	118	32	65	19/21	Qualitative confirmatory	29
	10	120	39	68	21/21		
	15	115	35	63	20/21		
Hydroprene	5	87	25	38	17/21	Qualitative screening	7.7**
	10	88	21	33	21/21		
	15	86	19	35	18/21		

* Analytical method classification according to EU (2021/808) (European Commission 2021).

** CC β detection capability for screening according to EU (2021/808) (European Commission 2021) method 3.

Method validation

Table 2 presents the results of the method validation for the determination of JHA in mealworms. In the case of mealworms, only the yellow mealworm (*Tenebrio molitor*) and the lesser mealworm (*Alphitobius diaperinus*) are currently approved for human consumption within the European Union (European Commission 2022, 2023); however, the latter is often labelled as the buffalo worm (*Alphitobius laevigatus*) (Marien et al. 2022). The samples used for the validation study were obtained from different online vendors, and the labelling often did not include the exact species of insect; therefore, no distinction between species was made for this validation study.

The results for pyriproxyfen from validation days 1 and 2 were corrected using fenoxycarb-d3. Pyriproxyfen-d4 was only included during the final validation day, resulting in an improvement in repeatability (at 1*LCL, day 1 and 2 average of 26% and day 3 12%). However, since the overall repeatability criterion of < 20% was not met at any level for pyriproxyfen, it was classified as a qualitative analyte. The CC α for kinoprene was calculated at the 2*LCL validation level since two fortified samples did not meet the identification criteria at the 1*LCL level. For hydroprene, multiple samples did not meet the identification criteria for ion ratio at multiple levels; therefore, this analyte was classified as a qualitative screening analyte. Visual inspection of the chromatograms revealed signals in all fortified samples. Although the identification criterion for ion ratio

was not always met, this performance is sufficient for screening, after which confirmatory analysis can be conducted using multi-level standard addition (European Union Reference Laboratories 2022). Therefore, the CC β detection capability for screening was calculated, setting the 1*LCL validation level as the screening target concentration. For selectivity, all blank chromatograms were inspected visually, revealing no signals that could interfere with analyte detection. The robustness study revealed that methoprene, kinoprene, and hydroprene are sensitive to residual water if the extracts are not completely evaporated before starting the derivatisation procedure, highlighting this as a critical step. None of the other investigated deviations had significant effects. The stability study showed that derivatized methoprene, kinoprene, and hydroprene in the final extracts were unstable when stored in the freezer for 10 days. Therefore, sample extracts should be analysed immediately after derivatisation.

The partial validation of the LC-HRMS method as a qualitative screening method showed that all compounds could be reliably detected using scheduled MS² scans at the lowest calibration level of 5 $\mu\text{g kg}^{-1}$ with a false compliant rate of $\leq 5\%$, thus the CC β detection capability for screening was set at this level according to method 2 in (EU) 2021/808 (European Commission 2021).

Incurred mealworm analysis

The validated LC-MS/MS method was used to analyse an incurred sample from a trial in which

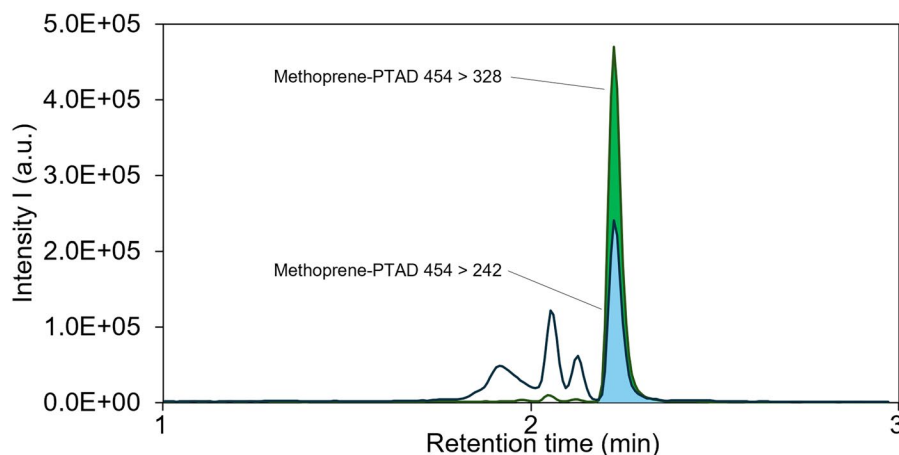


Figure 4. LC-MS/MS chromatogram overlay of methoprene incurred mealworms (a.u.= arbitrary units).

mealworms were exposed to methoprene *via* their substrate (10 mg kg^{-1}) (Rasker et al. 2026); see Figure 4. Analysis of this incurred material demonstrates that the proposed method can readily detect methoprene in mealworms; a level of $26 \mu\text{g kg}^{-1}$ was found in this sample, exceeding the CC α of $6.3 \mu\text{g kg}^{-1}$. In addition to the validation study, the successful detection of methoprene in an incurred sample provides additional assurance that the method can be used for residue detection in real samples, since incurred samples capture variations not captured by fortified samples (McCracken et al. 2000; Lehotay and Cook 2015).

Exploratory survey

To investigate the occurrence of JHA residues in edible insects, 14 anonymised samples from the Dutch national residue control plan were analysed using the proposed method. These samples included mealworm products such as freeze-dried or oven-dried and powdered mealworms and were therefore analysed in two aliquots, one as such and one fortified at $1 \times \text{LCL}$. None of the JHA included in the study were detected in these samples. In 43% of the samples, the fortified sample revealed that hydroprone and kinoprene were not detectable, while methoprene and its corresponding internal standard were not detectable in 14% of the samples. Fenoxycarb, pyriproxyfen and their corresponding internal standards were detectable in all samples. These results show that matrix suppression or matrix-mediated

effects on the recovery differ between non-processed and processed mealworms and that further method optimisation is required reliably to detect JHA in processed insect products. For example, the procedure could potentially benefit from a hydration step before extraction for (freeze)-dried mealworms as is commonly employed in pesticide residue analysis in dry plant-based matrices (e.g. Pszczolińska and Kociołek 2022). Additionally, the difference in detectability for the PTAD derivatives can be explained by differences in ionisation efficiency gain between methoprene (factor 1310), kinoprene (factor 102) and hydroprone (factor 91) as demonstrated by Aronov et al. (2005).

Conclusions

Juvenile hormone analogues (JHAs) can be used in insect rearing to enhance growth and are therefore relevant to food-safety residue monitoring. In this work, an LC-MS/MS method is presented that can detect the five most common JHAs at relevant levels in mealworms. Additionally, it was demonstrated that LC-HRMS, including targeted scans, can detect JHAs at relevant levels. The fitness for purpose of the LC-MS/MS and LC-HRMS methods was demonstrated in accordance with the validation criteria described in Commission Implementing Regulation (EU) 2021/808, and the LC-MS/MS method was applied to an incurred mealworm sample. Finally, mealworm samples from the Dutch national residue control plan were analysed, and no JHA residues

were detected. The proposed LC-MS/MS method can be used for routine monitoring, including screening and confirmation, while the LC-HRMS method can be used to additionally collect data for retrospective analysis for research purposes. Continued regulatory monitoring is recommended, given the widespread knowledge of JHAs' effects on insects.

Author contributions

CRedit: **Sjors Rasker**: Conceptualization, Formal analysis, Methodology, Validation, Visualization, Writing – original draft; **Rita Boerrigter-Eenling**: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing; **Helmoed Cammin**: Investigation, Writing – review & editing; **Ricky Potjes**: Validation, Writing – review & editing; **Lucas Agterberg**: Validation, Writing – review & editing; **Marco H. Blokland**: Conceptualization, Methodology, Writing – review & editing; **Ane Arrizabalaga-Larrañaga**: Conceptualization, Methodology, Writing – review & editing; **Saskia S. Sterk**: Funding acquisition, Project administration, Writing – review & editing.

Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Funding

This project was financially supported by the European Commission DG Health and Food Safety (EURL).

ORCID

Sjors Rasker  <http://orcid.org/0009-0005-9177-6369>
 Marco H. Blokland  <http://orcid.org/0000-0002-3751-6065>
 Ane Arrizabalaga-Larrañaga  <http://orcid.org/0000-0002-9379-5775>
 Saskia S. Sterk  <http://orcid.org/0000-0001-5225-1862>

Data availability statement

Data will be made available on request.

References

- Aronov PA, Dettmer K, Christiansen JA, Cornel AJ, Hammock BD. 2005. Development of a HPLC/tandem-MS method for the analysis of the larvicides methoprene, hydroprene, and kinoprene at trace levels using Diels-Alder derivatization. *J Agric Food Chem.* 53(9):3306–3312. <https://doi.org/10.1021/jf0485842>
- Devi V, Awasthi P. 2022. Synthesis, structural, DFT and bio-assay of novel ethyl 3-(2-(4-chlorophenoxy)acetamido) propanoate on *Galleria mellonella* (wax moth) – a juvenile hormone mimic as potential insect growth regulator (IGR). *J Mol Struct.* 1248:131464. <https://doi.org/10.1016/j.molstruc.2021.131464>
- Devi V, Kumari N, Awasthi P. 2022. Synthesis, characterization and insect growth regulating study of beta-alanine substituted sulfonamide derivatives as juvenile hormone mimics. *Phosphorus Sulfur Silicon Relat Elem.* 197(10):1080–1090. <https://doi.org/10.1080/10426507.2022.2061970>
- Dhadialla TS, Retnakaran A, Smagghe G. 2005. 63 Insect Growth- and Development-Disrupting Insecticides. In L. I. Gilbert (Ed.), *Comprehensive Molecular Insect Science* (p. 55–115. Elsevier. <https://doi.org/10.1016/B0-44-451924-6/00076-4>
- European Commission. 2012. Commission Regulation (EU) No 899/2012 of 21 September 2012 amending Annexes II and III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for acephate, alachlor, anilazine, azocyclotin, benfuracarb, butylate, captafol, carbaryl, carbofuran, carbosulfan, chlorfenapyr, chlorthal-dimethyl, chlorthiamid, cyhexatin, diazinon, dichlobenil, dicofol, dimethipin, diniconazole, disulfoton, fenitrothion, flufenzin, furathiocarb, hexaconazole, lactofen, mepronil, methamidophos, methoprene, monocrotophos, monuron, oxycarboxin, oxydemeton-methyl, parathion-methyl, phorate, phosalone, procymidone, profenofos, propachlor, quinclozac, quintozone, tolylfluanid, trichlorfon, tridemorph and trifluralin in or on certain products and amending that Regulation by establishing Annex V listing default values. *Official Journal of the European Union.* 273/(1).
- European Commission. 2021. Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. *Official Journal of the European Union.* 184/(84).
- European Commission. 2022. Commission Implementing Regulation (EU) 2022/169 of 8 February 2022 authorising the placing on the market of frozen, dried and powder forms of yellow mealworm (*Tenebrio molitor* larva) as a novel food under Regulation (EU) 2015/2283 of the European Parliament and of the Council, and amending Commission Implementing Regulation (EU) 2017/2470. *Official Journal of the European Union.* 28/(10).
- European Commission. 2023. Commission Implementing Regulation (EU) 2023/58 of 5 January 2023 authorising the placing on the market of the frozen, paste, dried and powder forms of *Alphitobius diaperinus* larvae (lesser mealworm) as a novel food and amending Implementing
- Aronov PA, Dettmer K, Christiansen JA, Cornel AJ, Hammock BD. 2005. Development of a HPLC/tandem-MS method for the analysis of the larvicides methoprene, hydroprene, and kinoprene at trace levels using

- Regulation (EU) 2017/2470. Official Journal of the European Union. 5/(10).
- European Union Reference Laboratories. 2022. *EURL Guidance Document on standard addition in the field of the analysis of residues of pharmacologically active substances version 1.1*. https://sitesv2.anses.fr/en/system/files/Guidance-SAM_v1_1_221129.pdf
- He Z, Zhao L, Liu X, Xu Y. 2020. The application of in-source fragmentation in ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry for pesticide residue analysis. *J Chromatogr A*. 1633:461637. <https://doi.org/10.1016/j.chroma.2020.461637>
- Henrick CA. 2007. Methoprene. *J Am Mosq Control Assoc*. 23(2 Suppl):225–239. [https://doi.org/10.2987/8756-971X\(2007\)23\[225:m\]2.0.CO;2](https://doi.org/10.2987/8756-971X(2007)23[225:m]2.0.CO;2)
- Hill V, Lopez-Viso C, Brameld J, Salter A, Parr T. 2024. The juvenile hormone analogue, pyriproxifen, alters protein and fat composition of *Tenebrio molitor* larvae. *J Insects Food Feed*. 10(9):1633–1644. <https://doi.org/10.1163/23524588-00001043>
- Insecticide Resistance Action Committee. 2023. Retrieved from <https://irac-online.org/mode-of-action/classification-online/>
- Jindra M, Bittova L. 2020. The juvenile hormone receptor as a target of juvenoid ‘insect growth regulators. *Arch Insect Biochem Physiol*. 103(3):e21615. <https://doi.org/10.1002/arch.21615>
- Jongedijk E et al. 2023. Use of high-resolution mass spectrometry for veterinary drug multi-residue analysis. *Food Control*. 145:109488. <https://doi.org/10.1016/j.foodcont.2022.109488>
- Kadono-Okuda K, Kajiura Z, Yamashita O. 1986. Induction of perfect superlarvae by the application of juvenile hormone analogue to starved larvae of the silkworm, *Bombyx mori*. *J Insect Physiol*. 32(12):1065–1073. [https://doi.org/10.1016/0022-1910\(86\)90126-5](https://doi.org/10.1016/0022-1910(86)90126-5)
- Kaufmann A. 2018. Analytical performance of the various acquisition modes in Orbitrap MS and MS/MS. *J Mass Spectrom*. 53(8):725–738. <https://doi.org/10.1002/jms.4195>
- Ken C. 2017. *Giant mealworms: the trick professional worm farms use to make huge mealworms*. Retrieved 15 December 2025 from <https://www.youtube.com/watch?v=mgPOkBIY4H0>
- Lehotay SJ, Cook JM. 2015. Sampling and sample processing in pesticide residue analysis. *J Agric Food Chem*. 63(18):4395–4404. <https://doi.org/10.1021/jf5056985>
- Li K, Jia QQ, Li S. 2019. Juvenile hormone signaling - a mini review. *Insect Sci*. 26(4):600–606. <https://doi.org/10.1111/1744-7917.12614>
- Marien A et al. 2022. Detection of alphetobius diaperinus by real-time polymerase chain reaction with a single-copy gene target. *Front Vet Sci*. 9:718806. <https://doi.org/10.3389/fvets.2022.718806>
- Martín-Pozo L, Arena K, Cacciola F, Dugo P, Mondello L. 2023. Development and validation of a multi-class analysis of pesticides in corn products by comprehensive two-dimensional liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 1701:464064. <https://doi.org/10.1016/j.chroma.2023.464064>
- Mauchamp B, Malosse C, Saroglia P. 1989. Biological effects and metabolism of fenoxycarb after treatment of the fourth and the fifth instars of the tobacco budworm, *Heliothis virescens* F. *Pestic Sci*. 26(3):283–301. <https://doi.org/10.1002/ps.2780260308>
- McCracken RJ, Spence DE, Kennedy DG. 2000. Comparison of extraction techniques for the recovery of veterinary drug residues from animal tissues. *Food Addit Contam*. 17(11):907–914. <https://doi.org/10.1080/026520300750038081>
- Monteiro SH, Lehotay SJ, Sapozhnikova Y, Ninga E, Lightfield AR. 2021. High-throughput mega-method for the analysis of pesticides, veterinary drugs, and environmental contaminants by ultra-high-performance liquid chromatography–tandem mass spectrometry and robotic mini-solid-phase extraction cleanup+low-pressure gas chromatography–tandem mass spectrometry, part 1: beef. *J Agric Food Chem*. 69(4):1159–1168. <https://doi.org/10.1021/acs.jafc.0c00710>
- Parthasarathy R, Palli SR. 2021. Stage-specific action of juvenile hormone analogs. *J Pestic Sci*. 46(1):16–22. <https://doi.org/10.1584/jpestics.D20-084>
- Payá P, Mulero J, Oliva J, Cámara MA, Barba A. 2013. Influence of the matrix in bioavailability of flufenoxuron, lufenuron, pyriproxifen and fenoxycarb residues in grapes and wine. *Food Chem Toxicol*. 60:419–423. <https://doi.org/10.1016/j.fct.2013.08.013>
- Pszczolińska K, Kociolek B. 2022. The pesticide residue analysis in commodities with high content of chlorophyll based on the quick, easy, cheap, effective, rugged, and safe method: a review. *J Sep Sci*. 45(1):149–165. <https://doi.org/10.1002/jssc.202100304>
- Rasker S et al. 2026. Effects and fate of chloramphenicol, methoprene, and narasin in yellow mealworms (*Tenebrio molitor*). *J Food Compos Anal*. 153:109139. <https://doi.org/10.1016/j.jfca.2026.109139>
- Riddiford LM. 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. In P. D. Evans (Ed.), *Advances in Insect Physiology* (Vol. 24, p. 213–274. Academic Press. [https://doi.org/10.1016/S0065-2806\(08\)60084-3](https://doi.org/10.1016/S0065-2806(08)60084-3)
- Riddiford LM. 2020. Rhodnius, golden oil, and met: a history of juvenile hormone research. *Front Cell Dev Biol*. 8:679. <https://doi.org/10.3389/fcell.2020.00679>
- Silhacek DL, Oberlander H, Porcheron P. 1990. Action of RH 5849, a non-steroidal ecdysteroid mimic, on *Plodia interpunctella* (Hübner) in vivo and in vitro. *Arch Insect Biochem Physiol*. 15(4):201–212. <https://doi.org/10.1002/arch.940150402>
- Sláma K. 1999. The history and current status of juvenoids. In: *Proceedings of the 3rd International Conference on Urban Pests*. Grafické závody. <https://www.icup.org.uk/media/iq2ji400/icup421.pdf>
- Sláma K. 2013. Insect hormones: more than 50-years after the discovery of insect juvenile hormone analogues (JHA,

- juvenoids). *Terr Arthropod Rev.* 6(4):257–333. <https://doi.org/10.1163/18749836-06041073>
- Tang X et al. 2024. Reduced matrix effects and simultaneous determination of three juvenile hormone analogs in tea by disperse solid-phase extraction (DSPE) and gas chromatography – tandem mass spectrometry (GC-MS/MS). *Anal Lett.* 57(18):3037–3049. <https://doi.org/10.1080/00032719.2024.2308078>
- The Thought Emporium. 2025. *Making giant insects to spite evolution*. Retrieved 15-12-2025 from <https://www.youtube.com/watch?v=D0PjLvlBsWw>
- Tumova S et al. 2022. Unique peptidic agonists of a juvenile hormone receptor with species-specific effects on insect development and reproduction. *Proc Natl Acad Sci U S A.* 119(48):e2215541119. <https://doi.org/10.1073/pnas.2215541119>
- van Huis A. 2022. Edible insects: challenges and prospects. *Entomol Res.* 52(4):161–177. <https://doi.org/10.1111/1748-5967.12582>
- Williams CM. 1956. The juvenile hormone of insects. *Nature.* 178(4526):212–213. <https://doi.org/10.1038/178212b0>
- Wu C et al. 2021. Quantitative determination of pyriproxyfen and its metabolite residues in bee products of China using a modified QuEChERS approach with UPLC-MS/MS. *Ecotoxicol Environ Saf.* 220:112388. <https://doi.org/10.1016/j.ecoenv.2021.112388>