Mass Spectrometry-Based Proteomic Strategies for the Characterization, Identification, and Quantification of Insect Proteins in Novel Foods

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List of Abbreviations

ACHDO	*Acheta domesticus*
ALPDA	*Alphitobius diaperinus*
CV	coefficient of variation
DDA	data-dependent acquisition
DIA	data-independent acquisition
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESI	electrospray ionization
EU	European Union
FDA	U.S. Food and Drug Administration
FDR	false discovery rate
GB	gigabyte
GMO	gene modified organism
GRYSI	*Gryllodes sigillatus*
HCD	higher energy collision induced dissociation
HERIL	*Hermetia illucens*
HPD	heterogeneous phase digestion
HPLC	high performance liquid chromatography
HR	high resolution
IA	immunoaffinity
IA-LC-MS/MS	immunoaffinity tandem mass spectrometry
IS	stable isotope labeled internal standard peptide
LC	liquid chromatography
LLOD	lower limit of detection
LLOQ	lower limit of quantification
LOCMI	*Locusta migratoria*
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight mass spectrometry
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCE	normalized collision energy
pAB	polyclonal antibody
PAP	processed animal protein
PCR	polymerase chain reaction
ppm	parts per million
PRM	parallel reaction monitoring
PSM	peptide-spectrum match
QC	quality control

QQQ	triple quadrupole mass spectrometer
RT	room temperature
SD	standard deviation
SISCAPA	stable isotope standard and capture by anti-peptide antibodies
TENMO	*Tenebrio molitor*
ULOQ	upper limit of quantification
UniParc	UniProt Archive
UniProt	Universal Protein Resource
UniProtKB	UniProt Knowledgebase
UniRef	UniProt Reference Clusters
v/v	volume fraction (volume per volume)
w/w	mass fraction (weight per weight)

Amino acid and nucleotide abbreviations according to IUPAC-IUB Joint Commission on Biochemical Nomenclature.

Abstract

The growing global demand for sustainable and alternative protein sources has increased interest in edible insects. Their regulatory approval in the European Union as novel food and feed ingredients necessitates reliable analytical methods to ensure food safety, authenticity, and allergen risk assessment. This thesis addresses these challenges by developing and validating mass spectrometry-based proteomic workflows for insect protein characterization, identification, and quantification.

A novel homology-based nano-HPLC-HR-MS approach was established to overcome the major limitation posed by the lack of comprehensive, species-specific protein databases. Up to 1893 proteins were identified across six insect species *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Gryllodes sigillatus*, and *Hermetia illucens*, representing up to a 24-fold increase in available proteomic data. Approximately 90 % of tested peptides were further confirmed by parallel reaction monitoring, demonstrating the reliability of the workflow. The allergenic potential was assessed and conserved allergens such as tropomyosin were detected across all species, while species-specific allergens indicated further unique allergenic risks.

Species-specific marker peptides and a novel, conserved pan-insect tropomyosin marker were identified and used to develop a targeted immunoaffinity LC-MS/MS assay for authentication and quantification of five insect species, as well as for general insect protein detection. The immunoenrichment strategy enabled efficient sample cleanup following direct in-suspension proteolysis and supported rapid chromatographic separations with a total cycle time of six minutes. The assays' basic analytical parameters were validated, demonstrating high accuracy, precision, selectivity, and sensitivity. It enabled reliable detection of insect proteins in both commercial products and model food matrices at concentrations relevant for allergen monitoring.

In conclusion, this thesis develops and applies novel analytical tools for the comprehensive proteomic characterization of non-sequenced insect species and delivers methodologies for both qualitative and quantitative detection of insect proteins in food, supporting food safety, traceability, and regulatory compliance.

Zusammenfassung

Die weltweit steigende Nachfrage nach nachhaltigen und alternativen Proteinquellen hat das Interesse an essbaren Insekten erheblich gesteigert. Ihre Zulassung als neuartige Lebensmittel und als Futtermittelzutaten in der Europäischen Union erfordert zuverlässige Analysemethoden, um die Lebensmittelsicherheit, -authentizität und Allergenrisikobewertung zu gewährleisten. Diese Arbeit addressiert diese Herausforderungen, indem sie massenspektrometriebasierte proteomische Methoden für die Charakterisierung, Identifizierung und Quantifizierung von Insektenproteinen entwickelt und validiert.

Um die größte Einschränkung durch das Fehlen umfassender, artspezifischer Proteindatenbanken zu überwinden, wurde ein neuartiger homologiebasierter Nano-HPLC-HR-MS-Ansatz entwickelt. Bis zu 1893 Proteine aus den sechs Insektenarten *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Gryllodes sigillatus* und *Hermetia illucens* konnten identifiziert werden, was einer bis zu 24-fachen Steigerung der verfügbaren Proteomdaten entspricht. Etwa 90 % der getesteten Peptide wurden durch Parallel Reaction Monitoring weiter bestätigt, was die Zuverlässigkeit der Methode demonstriert. Das allergene Potenzial wurde bewertet und konservierte Allergene, wie Tropomyosin, wurden bei allen Arten nachgewiesen, während artspezifische Allergene auf weitere einzigartige allergene Risiken hinwiesen.

Artspezifische Markerpeptide und ein neuartiger, konservierter Pan-Insekten Marker aus Tropomyosin wurden identifiziert und zur Entwicklung eines gezielten Immunoaffinitäts-LC-MS/MS-basierten Testverfahrens zur Authentifizierung und Quantifizierung von fünf Insektenarten sowie zum allgemeinen Nachweis von Insektenproteinen verwendet. Die Immunoaffinitätsstrategie ermöglichte eine effiziente Probenreinigung nach direkter Proteolyse in Suspension und unterstützte schnelle chromatographische Trennungen mit einer Gesamtzykluszeit von sechs Minuten. Die grundlegenden analytischen Parameter des Testverfahrens wurden validiert und zeigten eine hohe Genauigkeit, Präzision, Selektivität und Sensitivität. Dies ermöglichte einen zuverlässigen Nachweis von Insektenproteinen sowohl in kommerziellen Produkten als auch in Modellnahrungsmatrizen in Konzentrationen, die für die Allergenüberwachung relevant sind.

Zusammenfassend entwickelt und wendet diese Arbeit neue analytische Werkzeuge zur umfassenden proteomischen Charakterisierung nicht sequenzierter Insektenarten an und stellt validierte Methoden für den qualitativen und quantitativen Nachweis von Insektenproteinen in Lebensmitteln bereit. Dies trägt zur Gewährleistung der Lebensmittelsicherheit, Rückverfolgbarkeit und regulatorischen Konformität bei.

List of Publications

Accepted Manuscripts

1. **Meisinger, T.**, Planatscher, H., Braeuning, A., Ladenburger, E.-M., Stoll, D., Garino, C., Broll, H., Poetz, O. (2025). Proteomic insights into novel food insects: Homology-based proteome characterization and allergenicity considerations for EU-regulated insect species. Food Control, 177, 111441. https://doi.org/10.1016/j.foodcont.2025.111441

2. **Meisinger, T.**, Planatscher, H., Garino, C., Stoll, D., Ladenburger, E.-M., Braeuning, A., Broll, H., Poetz, O. (2026). From cricket to mealworm: 8-Plex mass spectrometry immunoassay for edible insect detection in novel foods. Microchemical Journal, 222, 117078. https://doi.org/10.1016/j.microc.2026.117078

Accepted Reviews

3. **Meisinger, T.**, Vogt, A., Kretz, R., Hammer, H. S., Planatscher, H., Poetz, O. (2025). Mass spectrometry-based ligand binding assays in biomedical research. Expert Rev Proteomics, 22(3), 123-140. https://doi.org/10.1080/14789450.2025.2467263

Contribution

Accepted Manuscripts

Table 2: Author contribution to the manuscripts presented in this dissertation.

No.	Accepted for publication	Number of authors	Position of candidate in the list of authors	Scientific ideas by candidate (%)	Data generation (%)	Analysis and interpretation by candidate (%)	Paper writing by candidate (%)
1	Yes	8	1	90	100	90	90
2	Yes	8	1	90	100	90	90

The doctoral candidate conceived the studies, developed and optimized the analytical strategies, performed all experimental work, curated, analyzed, and interpreted the data, and prepared the original manuscripts, including all figures, tables, and visualizations. This comprised selection of reference databases, development of the bioinformatic protein discovery pipeline, implementation of allergen detection methodologies, development, optimization, and partial validation of immunoaffinity LC-MS/MS-based assays, discovery and evaluation of peptide markers, assessment of analytical performance, and analysis of model foods and commercial products.

Hannes Planatscher contributed to conceptual discussions, provided scientific supervision, and reviewed all manuscript versions. Oliver Pötz supported the work through conceptual input, supervision, project administration, acquisition of funding, and provision of laboratory infrastructure and resources. Albert Braeuning contributed scientific supervision, methodological input for allergen evaluation, provision of sample material, and manuscript review. Dieter Stoll provided conceptual input, resources, sample material, and manuscript review. Cristiano Garino contributed through project administration, provision of sample material, and manuscript review. Eva-Maria Ladenburger provided sample material and manuscript review. Hermann Broll was responsible for project administration, funding acquisition, manuscript review and provided regulatory information.

Accepted Reviews

Table 3: Author contribution to the reviews presented in this dissertation.

No.	Accepted for publication	Number of authors	Position of candidate in the list of authors	Scientific ideas by candidate (%)	Data generation (%)	Analysis and interpretation by candidate (%)	Paper writing by candidate (%)
3	Yes	6	1	50	n/a	n/a	50

The doctoral candidate co-conceived the scope and structure of the review, defined the thematic focus, and coordinated the overall organization of the manuscript. The doctoral candidate authored substantial sections of the review, integrated and harmonized contributions from co-authors, and performed the final editing and revision of the complete manuscript to ensure conceptual coherence, technical accuracy, and consistency of style. Amelie Vogt and Robin Kretz contributed by authoring individual sections of the review. Oliver Pötz co-defined the scope and content of the review, contributed to manuscript organization, and co-authored sections of the text. Helen S. Hammer (now Helen S. Wagner) and Hannes Planatscher reviewed the manuscript and contributed to scientific discussion and refinement of the content.

1 Introduction

1.1 Global Protein Demand and Food Security

Dietary protein is an essential macronutrient for human health, serving critical roles in growth, tissue repair, immune function, and overall metabolic processes. While all macronutrients serve as energy sources, proteins are unique for supplying amino acids, carriers of nitrogen, sulfur and hydrocarbon skeletons (Prentice, 2005; Wu, 2016). The global demand for dietary protein is increasing, mainly driven by population growth and shifting dietary patterns. It has been projected, that the world's population could grow to 9.7 billion in 2050 and 10.9 billion in 2100 (Nations, 2024). A recent meta-analysis of 57 global food security projections concluded that the global food demand is likely to increase by 35 % to 56 % by 2050 compared to 2010 (van Dijk et al., 2021). Additionally, the composition of this demand is not distributed equally across all macronutrients. Known as Bennett's law, rising incomes especially in developing countries often lead to high meat consumption, which is characteristic for Western food culture. This will increase the global meat demand significantly (Bennett, 1941; Godfray et al., 2018). Projections for China alone expect an increase of approximately 50 % by 2030 compared to 2000, which in turn will raise the demand for protein-rich feeds for the animal agriculture industry (Msangi & Rosegrant, 2012). Estimates of food and crop prices in 2050 vary widely due to differences in modeling approaches and scenarios. Nonetheless, several major studies forecast rising crop prices (Hertel et al., 2016; Nelson et al., 2010; Rosegrant et al., 2014), while only a minority project price declines (Baldos & Hertel, 2016), as described in a recent literature review (Falcon et al., 2022). Higher crop prices can disproportionately affect low-income populations by reducing access to affordable food, thereby increasing the risk of undernutrition. Furthermore, climate change is expected to reduce the total area of arable land through shifting climatic zones, which will alter precipitation patterns including the frequency of extreme weather events, further exacerbating pressure on global food systems and the animal agriculture industry (Fanzo et al., 2022). Beyond the economic and environmental factors associated with increasing global protein demand, the expansion of the meat in-

dustry presents its own set of challenges. Intensive livestock production is a known driver of public health risks due to the emergence and transmission of zoonotic diseases such as influenza A, which are facilitated by close human-animal contact and high animal densities in production and processing environments (Graham et al., 2008; Jones et al., 2013). Livestock production is responsible for a substantial share of global greenhouse gas emissions, particularly methane from ruminants, while also driving extensive land conversion, biodiversity loss, and significant water use (Aleksandrowicz et al., 2016; Tilman & Clark, 2014).

In conclusion, increasing meat production is unlikely to be a sustainable or sufficient strategy to meet future protein demand, given the associated public health risks, environmental impacts, and resource constraints that threaten food security and system resilience. To address these challenges, alternative protein sources have attracted substantial interest, with edible insects, plant-based proteins, and cultured meat emerging as viable options. Their production is rapidly scaling up due to advantages such as reduced environmental impact, efficient resource use, and growing consumer acceptance, positioning them as key components in the transition toward sustainable food systems (Grossmann & Weiss, 2021; Quintieri et al., 2023; Sobczak et al., 2023).

1.2 Edible Insects as Novel Food

1.2.1 Regulatory Status in the European Union

Entomophagy, the consumption of insects as food, has been practiced in various societies since prehistoric times, as it provides an accessible and nutrient-rich food source (Ramos-Elorduy, 2009; Raubenheimer & Rothman, 2013). The cultural and geographical distribution of entomophagy is broad and particularly established in regions of Africa, Asia, Latin America and Oceania (Figure 1.1). In the last century, approximately 20 % of produced animal protein of the Democratic Republic Congo was derived from insects (Gomez et al., 1961). In Thailand, more than 190 species are consumed and entomophagy is an culturally ingrained concept (Krongdang et al., 2023). In contrast, Western countries have only recently begun to adopt edible insects (Yen, 2009), mainly through novelty items,

protein bars, or powdered supplements (Collins et al., 2019). Globally, more than two billion people incorporate insects into their diets (FAO, 2013), with 1,600 to 2,200 edible species identified (Jongema, 2017; Omuse et al., 2024; Van Itterbeeck & Pelozuelo, 2022). Ranked by number of known edible species, the most commonly consumed insects belong to the orders Coleoptera (beetles), Hymenoptera (wasps, bees, ants), Lepidoptera (caterpillars), Orthoptera (grasshoppers, locusts, crickets), and Hemiptera (true bugs) (Omuse et al., 2024).

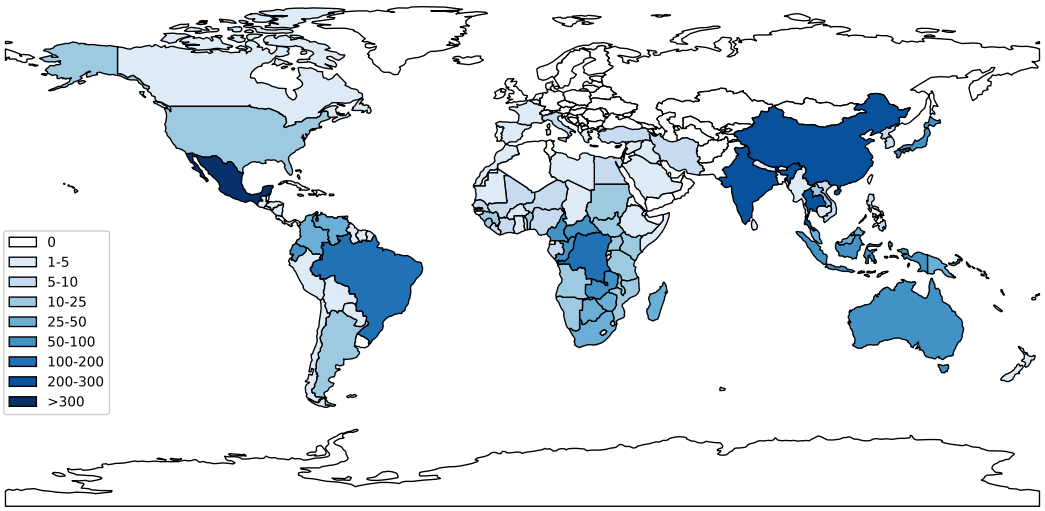

Figure 1.1: Recorded number of edible insect species, by country. Adapted from Ron van Lammeren, Centre for Geo Information, Wageningen University, based on data compiled by Yde Jongema (Jongema, 2017).

In the European Union (EU) insects were not commonly consumed prior to 1997, when the first Novel Food Regulation came into effect and thus, were legally classified as novel food in the EU (European Parliament, 2015; Finardi & Derrien, 2016). Therefore, insect or insect-based products require an authorization after scientific assessment by the European Food Safety Authority (EFSA), before they can be offered on the European market (de Boer & Bast, 2018; European Parliament, 2015). To date, certain products from four insect species, *Tenebrio molitor* (larvae of the yellow mealworm) (European Parliament, 2021b), *Locusta migratoria* (locust) (European Parliament, 2021a), *Acheta domesticus* (house cricket) (European Parliament, 2022), and *Alphitobius diaperinus* (larvae of the darkling beetle, also known as lesser mealworm) (European Parliament, 2023) have been authorized under the Novel Food Regulation 2015/2283.

Table 1.1: Authorized and pending applications for insect-based products under the Novel Food Regulation in the European Union. Data retrieved from OpenEFSA (https://open.efsa.europa.eu), last accessed 01 September 2025.

Insect order	Species name	Common name	Status
Coleoptera	*Tenebrio molitor*	Yellow mealworm	Authorized
Coleoptera	*Alphitobius diaperinus*	Lesser mealworm	Authorized
Diptera	*Hermetia illucens*	Black soldier fly	Pending
Hymenoptera	*Apis mellifera*	Western honey bee	Pending
Orthoptera	*Acheta domesticus*	Domestic cricket	Authorized
Orthoptera	*Locusta migratoria*	Locust	Authorized
Orthoptera	*Gryllodes sigillatus*	Tropical house cricket	Withdrawn*

*Applicant withdrew application without publicly stating reasons.

Further species are currently under evaluation (Table 1.1) and the additional species *Musca domestica* (common housefly), *Gryllodes sigillatus* (tropical house cricket), *Bombyx mori* (silkworm), and *Gryllus assimilis* (field cricket) are listed as safe to be reared in the EU for feed use (European Parliament, 2017, 2021c). Given that these species are already approved for feed use and that industrial production systems are either established or under development for this purpose, it is likely that applications for their authorization as food will follow, as the transition from feed to food markets represents a logical extension for the sector.

1.2.2 Nutritional Profile

Insects compare favorably to conventional protein sources such as pork or beef. While their nutritional value varies based on species, diet, and developmental stage, insects are generally considered highly nutritious, providing substantial amounts of protein, fat, vitamins, minerals, and fiber (Lange & Nakamura, 2021). On a dry matter basis, average protein contents have been reported at approximately 40 % for the orders Coleoptera, 60 % for Orthoptera, and 50 % for Diptera (Rumpold & Schluter, 2013), which are comparable to values for beef (50 %) and pork (40 %) (Parodi et al., 2018). Most edible insect species provide a complete protein profile, meeting recommended levels of phenylalanine, tyrosine, tryptophan, threonine, and lysine, with digestibility comparable to eggs and overall amino acid quality equivalent to that of milk and beef (Lange & Nakamura, 2021; Sobczak et al., 2023).

Fat is another important macronutrient, that is readily available in edible insects. The average fat content varies per insect species ranging from 13 % in Orthoptera to 33 % in Coleoptera and is generally higher in larval stages compared to adult stages (Rumpold & Schluter, 2013). Up to 75 % of the total fatty acids are monounsaturated and especially polyunsaturated, giving edible insects a profile that, while comparable to poultry, fish and vegetable oils, is distinguished by the predominance of these unsaturated forms (Lange & Nakamura, 2021; Tzompa-Sosa et al., 2014; Zielińska et al., 2015).

In addition to their macronutrient composition, edible insects are notable for their rich micronutrient profile, which includes essential vitamins and minerals in concentrations that are often comparable to, or exceed, those found in conventional animal protein sources (Christensen et al., 2006; Payne et al., 2016). Although the micronutrient composition varies by species, many insect species are particularly high in iron, zinc, and magnesium (Sobczak et al., 2023), with bioavailability that can surpass that of plant-derived sources due to the absence of certain antinutritional factors (Dobermann et al., 2017; Oibiokpa, 2017). For example, crickets and mealworms have been reported to contain iron levels comparable to beef, while also providing significant amounts of zinc, an essential micronutrient for immune function, wound healing and cellular metabolism and metalloproteases (Herrera et al., 2018; Mwangi et al., 2018). Furthermore, insects can be a valuable source of vitamin D (Oonincx et al., 2018), B vitamins, including biotin, pantothenic acid, and riboflavin, as well as vitamin B12 (DeFoliart, 1992; Lokeshwari & Shantibala, 2010), which is typically absent from plant-based foods and is critical for neurological function and red blood cell formation.

1.2.3 Environmental Impact

Insect rearing offers several environmental advantages over conventional livestock systems. Insects, such as crickets, possess a high feed conversion efficiency and require only about half the feed needed by chickens and between for and twelve times less feed than pigs or cattle to produce equivalent amounts of protein (Alexander et al., 2017; Collavo et al., 2005; Lange & Nakamura, 2021; Nakagaki & Defoliart, 1991). Their efficiency is attributed to their cold-blooded physiology, which enables reliance on external conditions for metabolic regulation and lowers energy requirements for thermoregulation (Dobermann

et al., 2017; Oonincx et al., 2015). Many insect species can thrive on low-value organic matter, including agricultural by-products and food waste, thereby potentially contributing to waste reduction and supporting circular economy models (Broeckx et al., 2021; Pomari Fernandes et al., 2025). This capacity not only diverts organic material from landfills but also reduces reliance on resource-intensive feed crops such as soy.

In terms of resource use, insect farming demands less land than traditional livestock production (Alexander et al., 2017; Dobermann et al., 2017). While cattle require extensive grazing areas and large volumes of water, insects can be reared in vertically stacked systems that minimize spatial requirements and reduce habitat conversion pressures (Cortes Ortiz et al., 2016). Regarding water use, some studies report that the water footprint per kilogram of mealworm protein is lower than that of beef and pork but higher than that of chicken and fish (Smetana et al., 2023; van Huis & Oonincx, 2017). Greenhouse gas emissions are also substantially lower, as insects do not produce methane and generate less nitrous oxide than ruminants (Oonincx et al., 2010; Smetana et al., 2023). Additionally, the rapid growth cycles and high reproductive rates of many insect species enable efficient protein production with shorter turnover times (Alexander et al., 2017).

Despite these advantages, insect farming at scale remains a developing industry in the European Union, with several unresolved challenges (Dobermann et al., 2017). Maintaining optimal temperature and humidity typically necessitates energy-intensive climate control, which can undermine sustainability benefits if renewable energy is not employed (Smetana et al., 2019). High-density rearing conditions increase the risk of disease transmission within the insect population (Maciel-Vergara et al., 2021), while accidental release of non-native species poses potential ecological risks (Fortuna et al., 2022). Moreover, life cycle assessments indicate that environmental outcomes vary considerably across insect species, substrates, and production systems, suggesting that the benefits are context-dependent rather than universally assured (Salomone et al., 2017; Smetana et al., 2019; van Zanten et al., 2015).

1.2.4 Safety and Allergenicity Concerns

The incorporation of insects into the human diet raises safety considerations, however, current knowledge remains limited (Schlüter et al., 2016). Microbiological hazards include

pathogenic bacteria, fungi, and parasites, while chemical risks involve potential bioaccumulation of heavy metals, pesticides, and other contaminants. Several microorganisms have been reported in edible insects, including spore-forming bacteria and *Salmonella* in mealworm and cricket guts (Klunder et al., 2012; Rumpold et al., 2014) or yeast and mold on fresh insect samples (Caparros Megido et al., 2017). However, effective decontamination techniques have been developed to mitigate these risks (Grabowski & Klein, 2017; Rumpold et al., 2014) and it is assumed, that insects reared and processed under the same sanitary conditions as meat products are microbiologically safe for consumption (Gałęcki et al., 2023). Unlike poultry facilities, which are annual hotspots for influenza outbreaks (Graham et al., 2008), or cattle slaughterhouses, which were significant sites of coronavirus transmission during the pandemic (Brozek & Falkenberg, 2021), insects are additionally considered less likely to transmit zoonoses because of their greater evolutionary distance from humans (Doi et al., 2021). Regarding chemical risks, wild-harvested insects are especially susceptible, as they may consume crops or vegetation treated with chemical agents; however, insects produced under controlled farming conditions can be kept free from such contamination (Lange & Nakamura, 2021).

Of great interest for the safety evaluation of edible insects is their allergenic potential and associated risks for consumers. However, the number of reports is limited and often only available from Asian and African countries (Lange & Nakamura, 2021). Additionally, most clinical studies were conducted with 20 participants or less, thus limiting their informative value (Dobermann et al., 2017). Insects exhibit allergenic potential due to both proteinaceous and non-proteinaceous components (Murefu et al., 2019), including glycans and pigments (Do et al., 2017; Le Coz et al., 2002). Insect proteins and glycoproteins are considered the principal allergenic factors and several insect proteins share homology with established allergens in crustaceans and other arthropods (Lamberti et al., 2021; Leni, Tedeschi, et al., 2020). The allergen.org database lists 239 arthropod allergens, nearly half originating from insects (Schlüter et al., 2016). Allergic reactions to insects may result from primary sensitization or IgE cross-reactivity with known allergens (van Broekhoven et al., 2016). Cross-reactivity is particularly relevant in crustacean-allergic individuals, as tropomyosin and arginine kinase, well characterized and major shrimp allergens, are also present in insects (Barre et al., 2019; De Marchi, Mainente, et al., 2021; Verhoeckx et al.,

2014). Clinical manifestations resemble those of other food allergies, ranging from mild oral symptoms to anaphylaxis (Beaumont et al., 2019; Ji et al., 2009; Ribeiro et al., 2017). Individuals with seafood allergies are advised to avoid insect-derived foods due to high risk of cross-reactivity, emphasizing the need for clear product labeling.

Processing methods to reduce allergenicity have been explored. Heat treatment, though widely used, has been reported to be largely ineffective due to the thermal stability of many insect allergens (Broekman et al., 2015; Schlüter et al., 2016). Consequently, heating alone is insufficient to mitigate allergenic potential. Alternative methods, including enzymatic hydrolysis and fermentation, have shown more promise in reducing IgE binding and allergenic activity (J. Pan et al., 2022). Moreover, incorporation of insects into food matrices adds complexity to allergen risk assessment, as interactions with other food components may further modulate immune responses (De Marchi, Wangorsch, & Zoccatelli, 2021). The identification of allergenic proteins, the verification of species composition, and the detection of contaminants require highly sensitive and specific methods capable of operating in complex food matrices.

1.2.5 Current Authentication Methods

Food authentication is the process of verifying the identity, composition, and origin of food products to ensure compliance with labeling, regulations, and consumer expectations. Fraudulent practices can compromise consumer trust and pose both economic and health-related risks. Common examples include species substitution, where high-value ingredients are replaced with cheaper alternatives, and adulteration through partial substitution or dilution. Fraud also involves misrepresenting origin or falsely claiming production methods such as organic or sustainable practices (Danezis et al., 2016; Schieber, 2018).

At the time of writing, no official reference methods have been approved for authentication of edible insects in food (Marien et al., 2025). However, several promising methods have been published, most of them based on polymerase chain reaction (PCR) protocols (Garino et al., 2021, 2022; Marien et al., 2025; Tramuta et al., 2018). PCR protocols such as quantitative real-time PCR, endpoint PCR or digital PCR are suitable as reference methods due to being widely adopted in food and feed authentication and relatively low

cost (European Parliament, 2013; Holzhauser, 2018). Nevertheless, DNA-based methods present certain limitations. Food processing can impair DNA integrity, reducing assay sensitivity and reliability (Gryson, 2010). DNA does not provide direct evidence of proteins present in the sample, but rather infer their presence from genetic material. Protein-based methods can address these challenges, as proteins provide direct evidence of the presence of specific biological material and and mass spectrometry-based assays allow highly sensitive and specific detection of target proteins or peptides (S. Pan et al., 2008). This allows both qualitative and quantitative assessment of food authenticity. The transition toward proteomic approaches offers a complementary and, in some cases, superior alternative to DNA-based testing, especially for complex or processed food matrices (Ortea et al., 2016).

1.3 Mass Spectrometry-Based Protein Analysis

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the cornerstone of modern proteomics, making the separation, ionization, and high-resolution detection of complex peptide mixtures possible (Cox & Mann, 2011). Following chromatographic separation, peptides are ionized by electrospray ionization (ESI) and thus transferred into the gas phase. ESI remains the dominant ionization technique due to its compatibility with aqueous chromatographic eluents and its ability to generate multiple charged peptide ions (Aebersold & Mann, 2003). The peptide ions are then analyzed in the mass spectrometer based on their mass-to-charge ratio (m/z), which forms the basis for subsequent qualitative and quantitative proteomic strategies. Mass analyzers used in proteomics vary in performance characteristics, with Orbitraps and triple quadrupoles (QQQ) being the most used (Martins et al., 2016). Orbitraps provide high resolution, mass accuracy, and large dynamic range, enabling discovery-oriented approaches. Here, ions are trapped in an electrostatic field and their oscillation frequencies are converted into m/z values, used for peptide identification and modification analysis (Eliuk & Makarov, 2015). In contrast, QQQs, consisting of Q1 for precursor selection, Q2 as collision cell, and Q3 for fragment selection, are optimized for high sensitivity and targeted quantitation of ions (Yost & Enke, 1979).

Proteomic workflows can follow a bottom-up or top-down strategy. In bottom-up proteomics, proteins are enzymatically fragmented into peptides prior to LC-MS analysis (Zhang et al., 2013). This approach is most widely applied due to its robustness, depth of coverage, and compatibility with established database search algorithms (Bittremieux et al., 2017; Cox & Mann, 2008, 2011). By contrast, top-down proteomics analyzes intact proteins directly, preserving information on isoforms and post-translational modifications (Kellie et al., 2010; McLafferty et al., 2007). Due to its inherent technical challenges it is less common in routine studies (Melby et al., 2021). Data acquisition strategies further differentiate between targeted and non-targeted proteomics.

1.3.1 Non-Targeted Proteomics

Non-targeted proteomics is preferably applied in discovery workflows (Ong & Mann, 2005). Here, data-dependent acquisition (DDA) selects the most intense precursor ions for fragmentation to generate MS/MS spectra (Figure 1.2) (Zhang et al., 2013). For subsequent peptide and protein identification, recorded peptide mass spectra are matched against *in silico* generated theoretical peptide spectra derived from curated protein sequence databases, such as UniProt (Dupree et al., 2020). DDA is often applied for broad proteome exploration, allergen or biomarker discovery and for hypothesis generation, and despite the rise of data-independent acquisition (DIA) approaches, DDA remains more widely used (Aebersold & Mann, 2016). While it often provides good proteome coverage, it is inherently stochastic, leading to under-sampling of low-abundance peptides and challenges in reproducibility (Tabb et al., 2009).

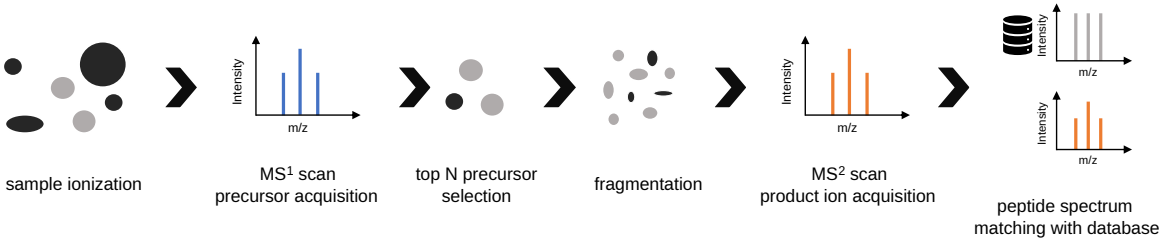

Figure 1.2: Data-dependent acquisition (DDA) workflow in a mass spectrometer. The sample is ionized and a MS1 survey scan is conducted to detect precursor ions. Based on intensity, precursor ions are selected for fragmentation. Resulting product ions are analyzed in an MS2 scan and resulting spectra are compared against a protein sequence database for peptide-spectrum matching (PSM) to enable peptide identification.

The application of non-targeted proteomics to edible insects has been limited by the lack of genomic and proteomic databases used for peptide mass spectra matching (Belghit et al., 2019; Leni, Prandi, et al., 2020; Ulrich et al., 2017). Whereas agriculturally or pharmaceutically important species are generally well characterized, many insect species remain unsequenced (Y. Mei et al., 2022). Consequently, research on proteomic insect authentication has largely focused on approaches that do not depend on sequence databases. Using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), Ulrich *et al.* characterized spectral fingerprints of edible insect species (Ulrich et al., 2017). The idea of this work was later expanded by Belghit and colleagues, who applied direct comparison of tandem mass spectra for species authentication and generated spectral libraries of four insect species relevant for aquaculture (Belghit et al., 2019). A notable exception is the work of Varunjikar and co-authors, who developed a DDA-based workflow, that utilized a small curated protein sequence database derived from the phylum *Arthropoda*. This method was effective for identifying protein homologues in edible insect species but showed a bias toward one species, assumed to be caused by the choice of reference database (Varunjikar et al., 2022). The study by Varunjikar *et al.* marked progress toward database-dependent proteomic authentication of edible insects, but important gaps remain. While databases from related species can facilitate protein identification, both database composition and size strongly influence peptide-spectrum matching outcomes and may introduce biases (Kumar et al., 2017). Comprehensive evaluation of these factors, together with optimized search strategies, is still lacking in the context of edible insect authentication and further research is required to improve reliability.

1.3.2 Targeted Proteomics in Food and Feed Authentication

Targeted methods are commonly utilized to quantify protein biomarkers previously identified in a non-targeted discovery phase. Multiple reaction monitoring (MRM) is performed on triple quadrupole mass spectrometers, where Q1 selects the precursor, Q2 fragments it, and Q3 monitors specific fragment ions. In contrast, parallel reaction monitoring (PRM) on high-resolution instruments such as Orbitraps isolates a predefined set of precursor ions in Q1 and detects all resulting fragment ions simultaneously (Figure 1.3) (Peterson et al., 2012). Both approaches offer high specificity and sensitivity relative to DDA, reduce

interference from co-eluting peptides, and allow accurate quantification across multiple biological samples if known amounts of isotope-labeled peptides are spiked into the sample as internal standard prior to the analysis (Gerber et al., 2003; Rauniyar, 2015). Peptide concentrations are then derived from the ratio of analyte signal to standard signal (Anderson et al., 2009).

Figure 1.3: Predefined precursor ions are isolated from the sample and fragmented. The resulting fragment ions are measured in a high-resolution MS2 scan, generating comprehensive spectra for each targeted precursor. Specific precursor-fragment ion transitions are extracted to generate chromatographic traces. Quantification is performed by comparing endogenous peptide signals to isotopically labeled internal standard peptides.

Carrera *et al.* describes a PRM-based assay for the authentication of the fish-borne parasite *Anisakis* in fish samples. Following a discovery phase, peptide biomarker were identified and applied for detection (Carrera et al., 2016). Applying a similar strategy, Gu *et al.* identified protein biomarker for the differentiation of Atlantic salmon from its common substitution, the rainbow trout. They validated a targeted MRM method for quantification of 1 % to 80 % of rainbow trout in Atlantic salmon meat (Gu et al., 2020).

Targeted proteomics usually detects tryptic peptides (Bereman et al., 2012). This is of advantage for food authentication, where complex food matrices are often heavily processed (Gryson, 2010). Trypsin is able to proteolyze even denatured proteins (Vandermarliere et al., 2013), thus making them available for detection. Nevertheless, matrix complexity remains a major challenge for accurate detection in both PCR and proteomic approaches. Immunoaffinity LC-MS/MS (IA-LC-MS/MS) offers an effective solution to this gap. Antibody-based enrichment selectively captures target proteins or peptides, which reduces background signals and enhances both sensitivity and quantitative accuracy (Sugimoto et al., 2018). A specific implementation of this approach is the stable isotope standards and capture by anti-peptide antibodies (SISCAPA) workflow (Anderson et al., 2009). SISCAPA increases peptide enrichment by two to four orders of magnitude compared to

conventional targeted MS analysis (Anderson et al., 2009; Whiteaker et al., 2012). Unlike other immunoassays, SISCAPA allows the combination of multiple antibodies in multiplex assays, as MS detection ensures absolute specificity and is unaffected by antibody cross-reactivities (Whiteaker et al., 2011, 2012). While well established in biomedical research for pharmacokinetics and biomarker analysis (Ackermann & Berna, 2007; Meisinger, Vogt, et al., 2025), IA-LC-MS/MS is still underexplored in food authentication and especially for edible insects. Recent studies on the detection of processed animal proteins (PAP) in feed, however, emphasize its potential for broader application in this field (Lecrenier et al., 2021; Steinhilber et al., 2019; Steinhilber et al., 2018a, 2018b).

1.4 Assay Validation

Analytical method validation is a critical step in assay development and is recommended by both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (EMA, 2022; FDA, 2022). Key validation parameters include linearity, the proportionality of response to analyte concentration; parallelism, the consistency between test and reference samples; sensitivity, the lowest detectable analyte concentration; accuracy, the closeness of measurements to the true value; precision, the repeatability of measurements; and reproducibility, the consistency across different conditions or laboratories (Andreasson et al., 2015). The lower limits of detection (LOD) and quantification (LOQ) define the assay's dynamic range. Accuracy and intra- and inter-assay precision are assessed through repeated analysis of quality control samples at multiple concentrations. Matrix and carryover effects are evaluated to prevent analysis influences by the matrix or by the chromatographic setup (FDA, 2022). Validation ensures that LC-MS assays provide high specificity, reproducibility and quantitative accuracy suitable for routine use (Nakayasu et al., 2021). Hybrid assays such as immunoaffinity LC-MS/MS assays are not covered by existing guidelines and often acceptance criteria of immunoassays are applied, taking into account the influences of immunoaffinity capture (Jenkins et al., 2014).

2 Objective of the Thesis

In the EU, insects are not considered traditional food sources and thus not widely consumed today. Due to beneficial nutritional profiles and environmentally friendly rearing methods, the use of certain insect species in food products has been authorized by the European Commission in the Novel Foods Regulation (2015/2283). Growing industrial interest for these alternative protein sources has led to further regulatory advancements and to the approval of additional insects and insect-based products.

Despite this momentum, the development of robust analytical tools for safety assessment and species authentication remains challenging and no official methods have been approved. A key limitation is the insufficient genomic and proteomic characterization of the authorized insect species. The absence of comprehensive reference proteomes restricts the development of targeted protein- or peptide-based analytical methods for species differentiation, food authentication, and allergenicity assessment.

To overcome this gap, we established a combined *in silico* and laboratory procedure that allowed the identification of proteins in non-sequenced insect species. This homology-based, non-targeted, proteomic LC-MS/MS-based workflow was applied to the insect species *A. diaperinus*, *A. domesticus*, *G. sigillatus*, *H. illucens*, *L. migratoria*, and *T. molitor*, which are either already authorized or under evaluation for the use in food or feed applications. Together with the comprehensive investigation of their proteomes, we analyzed their allergenic potential and identified numerous potential allergens.

In a subsequent step, we addressed the methodological gap for insect authentication. We identified suitable species-specific peptide marker candidates from the proteomic data. These markers were used to optimize sample preparation protocols and to establish a PRM-based immunoaffinity LC-MS/MS assay for the identification and quantification of selected insect species in food products. The method was partially validated with respect to key performance parameters and subsequently applied to both commercially available food products and model food matrices to demonstrate its practical applicability.

3 Results & Discussion I: Proteomic Insights into Novel Food Insects

The content of this chapter is based on:

Meisinger, T., Planatscher, H., Braeuning, A., Ladenburger, E.-M., Stoll, D., Garino, C., Broll, H., Poetz, O. (2025). Proteomic insights into novel food insects: Homology-based proteome characterization and allergenicity considerations for EU-regulated insect species. Food Control, 177, 111441. https://doi.org/10.1016/j.foodcont.2025.111441

3.1 Development of a Homology-Based Peptide and Protein Identification Procedure

Insects are increasingly recognized as an alternative protein source, with the FAO and EU supporting their use in food and feed (European Parliament, 2015; FAO, 2013). As of 2025, seven insect-based products have been authorized for human consumption in the EU, and eight species have been approved for animal feed (European Parliament, 2013, 2015, 2017, 2021a, 2021b, 2021c, 2022, 2023). Despite their benefits, insects present allergenic risks, especially due to their evolutionary closeness to crustaceans and mites (Kamemura et al., 2019; Pali-Scholl et al., 2019). Comprehensive assessment is challenged by the lack of genomic and proteomic databases, limiting protein and allergen characterization studies (Leni, Prandi, et al., 2020).

At the start of the study in 2021, only one species of interest, *H. illucens* had considerable proteomic information publicly available and to our knowledge, no comprehensive proteomic approaches were published for characterization of the six species *T. molitor*, *A. diaperinus*, *A. domesticus*, *G. sigillatus* and *L. migratoria*. A draft proteome has since been published for *T. molitor*, but as of June 11, 2024, the UniProt knowledge base contained fewer than 50 protein sequences for *A. diaperinus* and *G. sigillatus*, fewer than 200 for *A. domesticus* and fewer than 1600 for *L. migratoria*, even when including both

reviewed and unreviewed sequence entries. These numbers are insufficient for either proteome exploration or identification of species-specific marker peptides.

To maximize proteome coverage in this exploratory study, we developed a novel homology-based search strategy using a comprehensive set of reviewed and unreviewed arthropod protein sequences to identify peptide sequences derived from standard bottom-up proteomics experiments. Homology-based searches rely on sequence similarity. Incorporation of a broad range of arthropod proteins increases the likelihood of identifying true homologues in insects with limited or no sequence information (Liska & Shevchenko, 2003). While this approach carries a higher risk of false positives, its efficiency was later evaluated by us to ensure reliability. In contrast, previous studies, such as Varunjikar et al. relied exclusively on reviewed sequences for peptide-spectrum matching (PSM). Although their method reduced database complexity and increased confidence in protein identifications, it showed a pronounced bias toward *H. illucens*, which is closely phylogenetically related to the well sequenced and reviewed fruit fly (Varunjikar et al., 2022).

Our protein database was constructed by filtering the complete UniRef protein sequence database for the phylum Arthropoda (taxonomy ID 6656) to obtain all known protein sequences closely evolutionary related to insects and insect-related allergens (Li et al., 2018; H. T. Wang et al., 2020). In a second step, this filtered database was deduplicated by clustering of identical sequences, which reduced the total database size without removing relevant protein sequences. Sequence clusters based on single UniParc derived entries were excluded, as these are per definition considered obsolete. The remaining 7.1 million sequence entries were used as database for PSM of proteolyzed edible insect species samples.

Given the extensive internal homology and limited species-specific entries in the database, the false discovery rate (FDR) was set at 5 % to balance sensitivity and specificity. Applying the standard 1 % FDR would have significantly reduced detectable proteins due to the high number of potential matches from the search space and derived decoy hits, which in turn imposed a more stringent significance threshold (Capriotti et al., 2013; Muth et al., 2015). Protein identifications were accepted based on at least one unique peptide and selected targets were further validated using parallel reaction monitoring (PRM) to ensure specificity (Subsection 3.1.2).

Our homology-based approach identified between 1079 and 1893 proteins per investigated species (Figure 3.1 and Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 1). Compared to the number of known proteins derived from the UniProtKB, an one to 24-fold increase for four of the six species of interest was achieved. The high effectiveness of this approach is in line with previous research (Cilia et al., 2011).

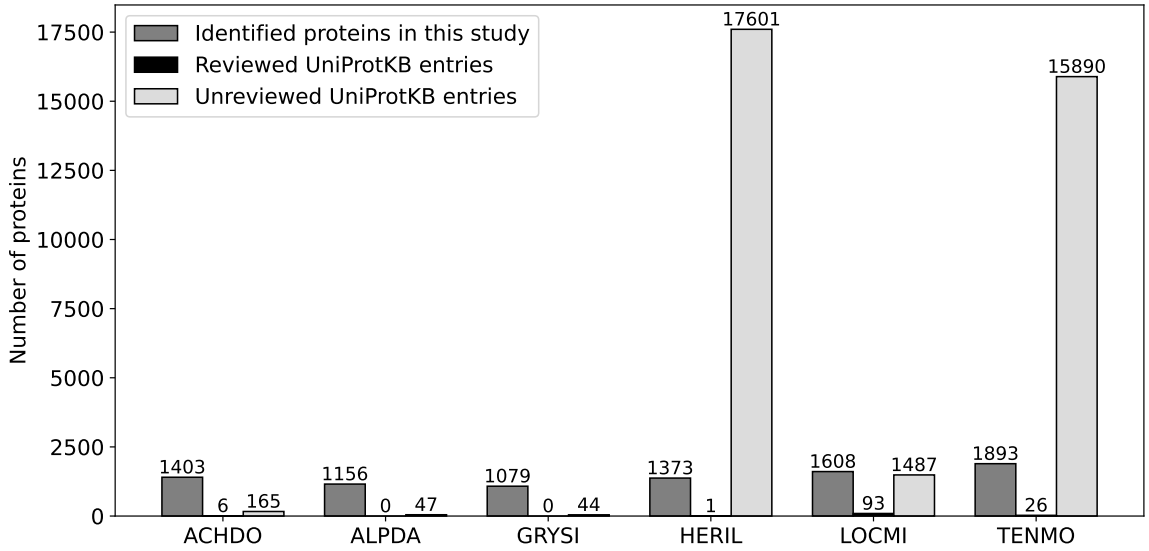

Figure 3.1: Comparison of proteins identified in this study with previously known UniProtKB entries (accessed June 11, 2024) across the examined edible insect species. Using a homology-based approach with a comprehensive arthropod database, large numbers of previously uncharacterized proteins were detected. Species with few UniProtKB entries include *A. domesticus* (ACHDO), *A. diaperinus* (ALPDA), *G. sigillatus* (GRYSI), and *L. migratoria* (LOCMI), while draft proteomes for *H. illucens* (HERIL) and *T. molitor* (TENMO) account for numerous unreviewed entries. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 1.

The number of proteins identified did not correlate with the number of species-specific sequences in the database, as *T. molitor* and *H. illucens* yielded similar protein counts to the four less-characterized species (Figure 3.1). The distribution of identified peptides per protein was similar across all six insect species (Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 2). However three species, *H. illucens*, *T. molitor* and *L. migratoria*, showed single proteins with over 40 unique peptides. The highest number of unique peptides was attributed to the 400 kDa protein apolipophorin, a major component of lipid transporting lipophorins in the hemolymph (Smolenaars et al., 2005). Next, we evaluated protein sequence coverage as indicator for dataset quality. On average over all six species, approximately 12 % coverage was identified, ranging from 0.1 % to 97 %. Per species, the

mean sequence coverage ranged from 11 % in *A. domesticus* to 15 % in *H. illucens*. Exclusion of proteins based on single unique peptides increased these values to 18 % for *A. domesticus* and 27 % for *H. illucens*. Human HEK293 cell line experiments yielded 18 % mean sequence coverage and are thus comparable to our experiments (Thakur et al., 2011). In total, the number of identified peptides, their distribution and protein coverage showed that the arthropod-wide database was sufficiently diverse to capture the protein landscape of the target species, which appear to have comparable proteome complexities.

3.1.1 Investigation of Inter-Species Differences

To assess dataset quality and characterize inter-species proteomic variation, protein identifications were compared across all six insect species (Figure 3.2 A). This comparison revealed that only a small subset of proteins was shared among all species (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 2), primarily conserved proteins like histones, cytoskeletal and structural proteins and EPG5-like autophagy-associated protein, which has previously been described in mice (X. Mei et al., 2023). Species-specific proteins were most abundant in *H. illucens* (1222), *T. molitor* (1588), and *L. migratoria* (1315), reflecting both biological diversity and varying degrees of sequence representation within the arthropod-wide database. These observations are consistent with the reduced efficiency of homology-based peptide identification for evolutionarily distant taxa, as postulated by Renard and colleagues (Renard et al., 2012) and observed by others (Francis et al., 2020; Varunjikar et al., 2022).

Despite the limited availability of species-specific sequences for *A. diaperinus*, *A. domesticus* and *G. sigillatus*, between 494 and 842 unique proteins were identified for each, representing 31 % to 53 % of the numbers obtained for the better characterized species *H. illucens*, *T. molitor* and *L. migratoria* (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 2).

The here generated dataset of species-specific proteins provides a valuable reference for development of assays designed to detect and quantify insect proteins in processed materials. Similar assays for the detection of processed animal proteins (PAP) in feed have been demonstrated recently (Lecrenier et al., 2021; Steinhilber et al., 2019; Steinhilber

et al., 2018a, 2018b). Building on this work, we further confirmed the species specificity of selected unique proteins and established a targeted method for the reliable detection and quantification of insect material in processed food products (Meisinger et al., 2026, Section 4).

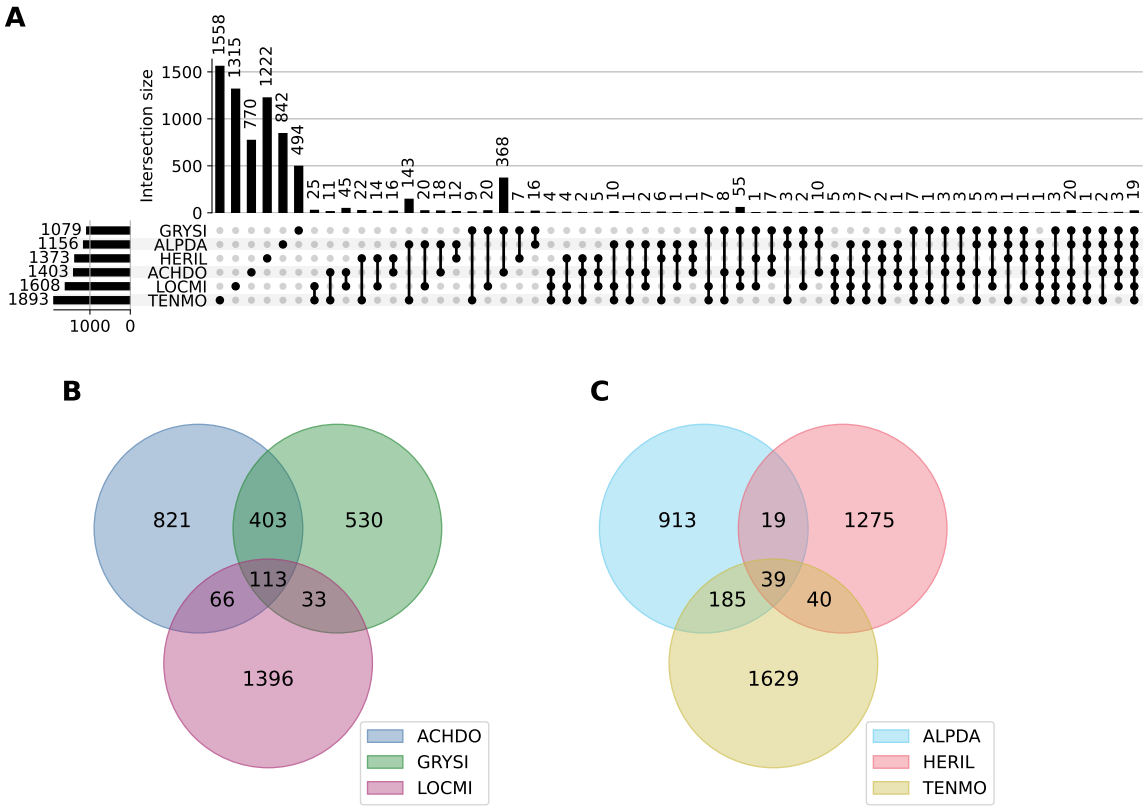

Figure 3.2: Comparison of insect protein profiles in six edible insect species. (A) An UpSet plot visualizing the number of unique and shared proteins among six species. Only a small subset of 19 proteins was common to all six. In contrast, a substantial number of species-specific proteins were identified between the six investigated species. (B) A Venn diagramm visualizing protein identifications across the three Orthoptera species *A. domesticus*, *G. sigillatus* and *L. migratoria*. A high number of 133 common proteins indicates correctly represented phylogenetic relations within our data. (C) Venn diagramm of the remaining three species *H. illucens*, *T. molitor* and *A. diaperinus* showing 39 common proteins between this evolutionary more diverse group. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 3.

To examine finer-scale proteomic relationships, subsequent analyses focused on phylogenetically related taxa. Species from the order Orthoptera, i.e. *A. domesticus*, *G. sigillatus* and *L. migratoria* shared 113 conserved proteins, mainly belonging to actins, myosins, elongation factors, ATPases, and proteasomal families (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 3, and Figure 3.2 B). Pairwise comparisons

showed 33 proteins only shared between *G. sigillatus* and *L. migratoria*, encompassing myosins, ribosomal proteins, twitchins, helicases and clathrins. Further 66 proteins were uniquely shared between *A. domesticus* and *L. migratoria*, mostly metabolic proteins and actin isoforms, receptors and arginine kinases. The two crickets, belonging to the subfamily Gryllinae, shared 403 proteins with diverse functional roles.

A similar comparison was performed for the species from the cohort Endopterygota (Holometabola), i.e. *A. diaperinus*, *T. molitor* and *H. illucens* (Figure 3.2 C). Consistent with their evolutionary distances, both mealworm species exhibited substantial proteomic overlap, whereas only highly conserved proteins were shared with the evolutionary more distant fly *H. illucens* (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 4).

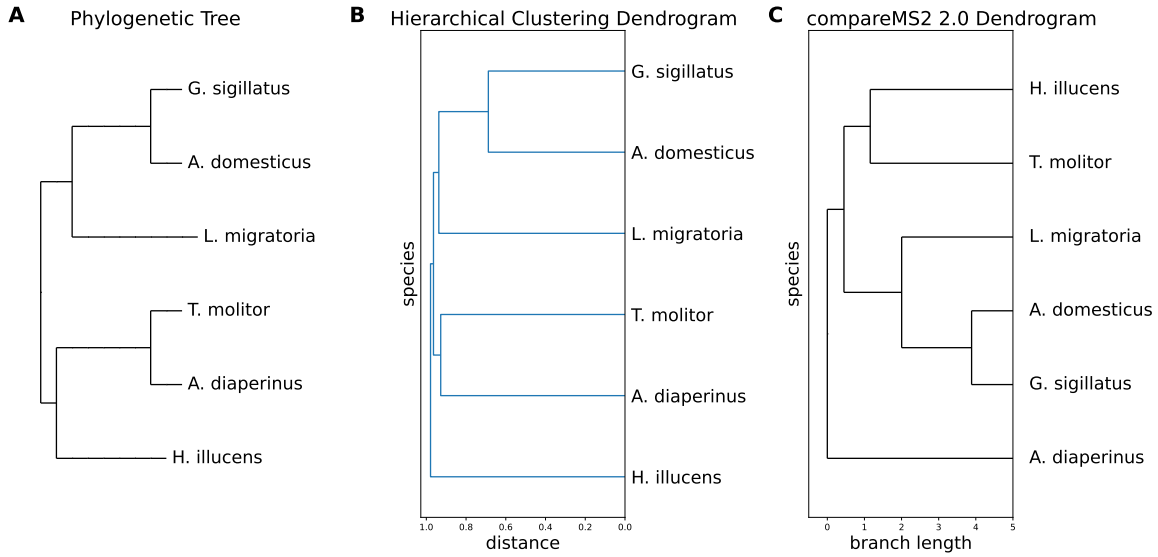

Figure 3.3: Phylogeny and data clustering comparison. (A) Phylogenetic tree of the six investigated species. (B) Using the Jaccard index with a weighted linkage approach resulted in a hierarchical clustering that largely reproduced the phylogeny. (C) Database independent MS/MS spectral similarity clustering confirmed the previous results. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 4.

To confirm the resemblance of the observed patterns to the expected phylogeny, two independent similarity analyses were performed. First, inter-species similarity was quantified using the Jaccard index (Jaccard, 1902) combined with weighted linkage clustering based on peptide presence or absence (Figure 3.3 B). Second, a database-independent comparison was carried out using compareMS2 2.0 (Marissen et al., 2023), which assesses MS/MS spectral similarity without relying on peptide annotation (Figure 3.3 C) (Varunjikar

et al., 2022). Both approaches reproduced the expected phylogenetic relationships among the six species with minor differences between the clustering results (Figure 3.3). These results confirmed the biological validity of the dataset and the reliability of the proteomic pipeline applied to under-characterized insect species.

3.1.2 Experimental Verification of Selected Proteins Applying Targeted Proteomics

The insect proteomes were generated using a combination of data-dependent acquisition (DDA) and peptide identification via MASCOT. Both methods can introduce ambiguities if less conservative search criteria are applied (Chen et al., 2009), hence we performed targeted analysis of 50 randomly selected peptides for each species to estimate the reliability of the selected homology-based identification workflow (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 5). PRM, offering higher reproducibility and accuracy than DDA (Peterson et al., 2012), confirmed on average 90 % of the tested peptides, with minimal interspecies variation (Meisinger, Planatscher, et al., 2025, Appendix A.1, Table 1 and Figure 5). This finding was consistent with recent studies emphasizing the complementary strengths of discovery-based and targeted proteomic approaches (Ahmadi & Winter, 2018; Goetze et al., 2024).

3.1.3 Performance Impact of Error-Tolerant Search Settings

The described approach for peptide and protein identification prioritized identification of evolutionary conserved sequences across all six insect species. To further expand the search space and thus peptide recovery, error-tolerant search settings were applied using a two-step approach: an initial target-decoy search as described previously, followed by a secondary search that tests amino acid substitutions to generate evolutionary plausible peptide variants absent from the database (Creasy & Cottrell, 2002). Between 93 and 1005 additional peptides (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 6), corresponding to 25 (*G. sigillatus*) to 154 (*T. molitor*) proteins (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Figure 1) per species were identified (Figure 3.4).

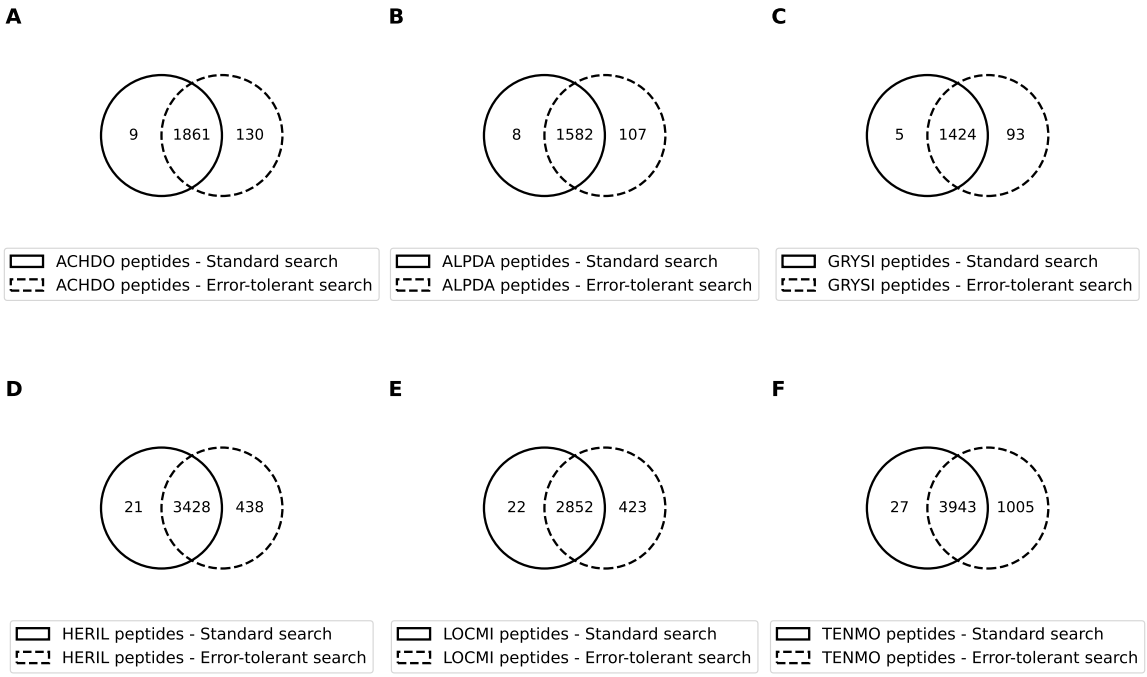

Figure 3.4: Comparison of peptide identifications from standard target-decoy and error-tolerant search settings visualized in Venn diagrams. (A) *A. domesticus* (ACHDO), (B) *A. diaperinus* (ALPDA), (C) *G. sigillatus* (GRYSI), (D) *H. illucens* (HERIL), (E) *L. migratoria* (LOCMI), (F) *T. molitor* (TENMO). Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 6.

In *A. diaperinus*, most inferred proteins were highly conserved (e.g. myosins, histones, ribosomal subunits), whereas the other species showed greater diversity, including enzymes, odorant receptors and cuticle proteins. The number of error-tolerant peptides positively correlated with the abundance of species-specific sequences, with *T. molitor*, *H. illucens* and *L. migratoria* yielding the most new identifications. Error-tolerant searches increased total protein identifications from 2 % to 8 % without affecting protein FDR compared to standard target-decoy searches (0.53 % vs. 0.55 % FDR in *T. molitor*).

The accuracy of peptide identifications was assessed through multiple steps. Most peptide lengths ranged from 7-25 amino acids, consistent with tryptic digestion (Swaney et al., 2010) and manual sequence inspection showed typical composition without obvious artifacts (Figure 3.5). Targeted PRM analysis of an unbiased but length distribution stratified peptide subset (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 7) confirmed 77 % of 110 *H. illucens* peptides and 86 % of 35 *G. sigillatus* peptides. These results supported the reliability of error-tolerant peptide identifications across species with differing database coverage, where *H. illucens* represented the higher end of

database coverage and number of identified error-tolerant peptides, and *G. sigillatus* the lower.

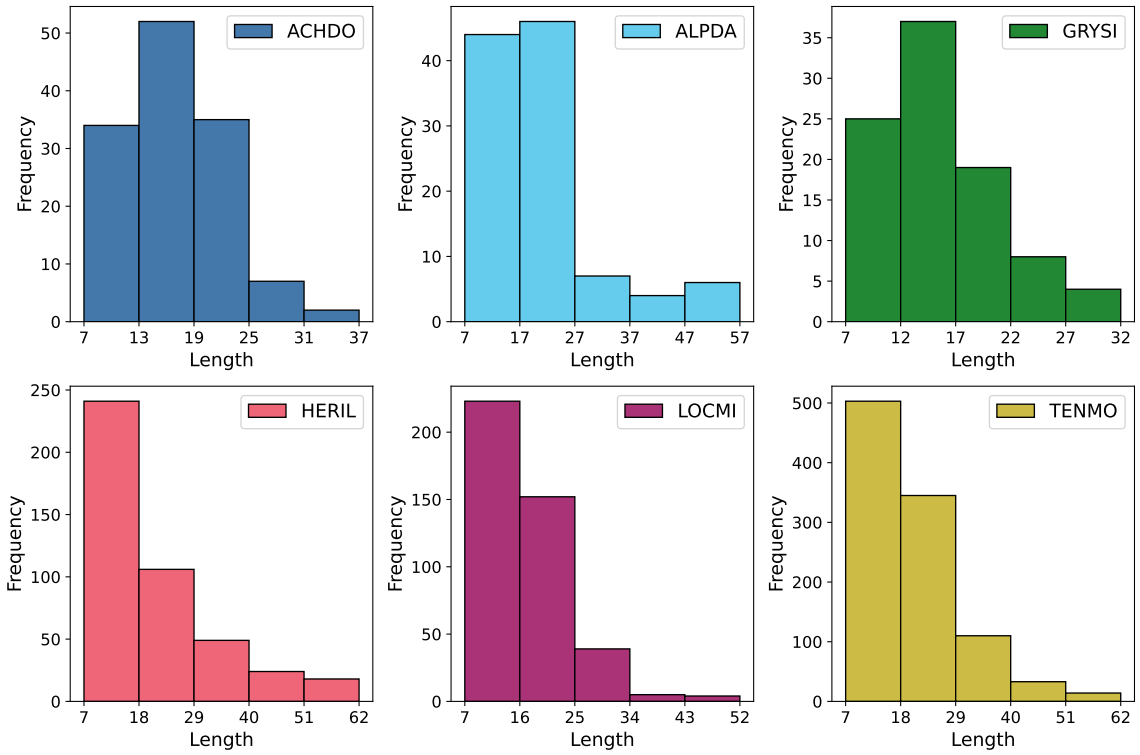

Figure 3.5: Peptide length distribution identified by error-tolerant search settings. Most peptides show a length between 7-25 amino acids, typic for tryptic peptides. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 7.

However, error-tolerant searches substantially increased computational demand by expanding the search space (Renard et al., 2012). For *T. molitor*, standard target-decoy searches required 8 GB memory and 190 h, while error-tolerant searches needed 80 GB and 390 h. Hence, we recommended a two-step approach for similar research questions, i.e. moving to error-tolerant settings only if standard-decoy search results are not sufficient.

3.2 Investigation of Database Composition on Insect Protein Identification

Protein identification accuracy in proteomics depends strongly on the reference database, as variations in sequence coverage, taxonomic specificity or annotation quality affect sen-

sitivity and specificity (Kumar et al., 2017). To assess potential biases, we compared results from different database configurations. We simulated analysis conditions of underexplored species by generation of two additional sequence databases, excluding either *H. illucens* or *T. molitor* sequences prior to sequence identity clustering. Samples of *H. illucens* and *T. molitor* were subsequently analyzed using their respective reduced sequence database and identified peptides were compared to peptides identified from the full sequence database. Using the reduced database for *T. molitor* yielded 47 % fewer peptides (2123 vs. 3970), with 19 % unique to the reduced database (Figure 3.6 A). *H. illucens* showed a 68 % reduction of peptide identifications (1107 vs. 3449), with 18 % unique to the reduced database (Figure 3.6 B).

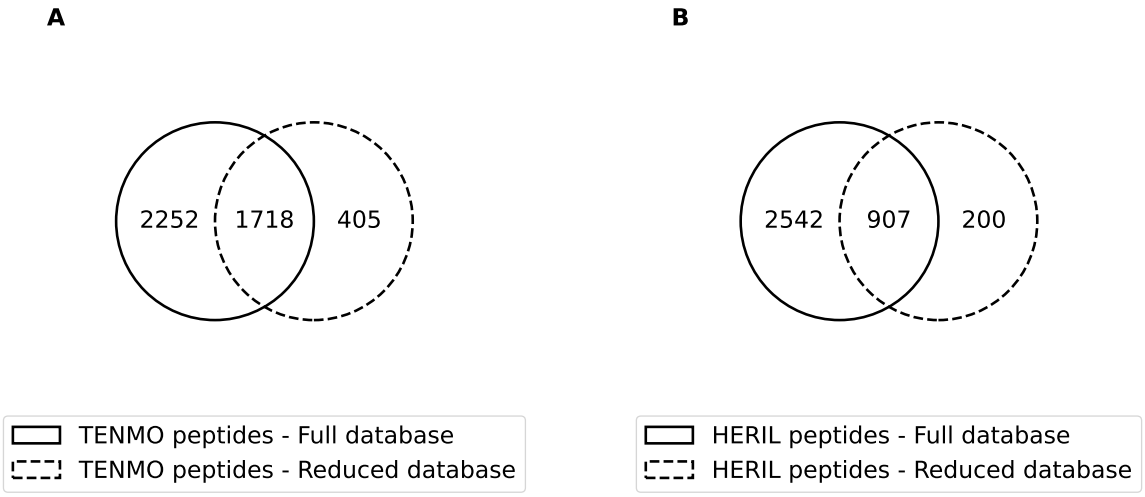

Figure 3.6: Comparison of identified peptides between the full and reduced arthropod databases. The full database was constructed from all available arthropod sequences in UniProtKB, as described previously. The reduced databases were generated by exclusion of *T. molitor* or *H. illucens* specific sequences prior to clustering, thus simulating both species to be underexplored. (A) *T. molitor*, (B) *H. illucens*. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 8.

The overlapping sections in each Venn diagram (Figure 3.6) indicate peptides identifiable without species-specific databases. Accordingly, assuming that *H. illucens* and *T. molitor* represent underexplored species, 43 % of *T. molitor* peptides and 26 % of *H. illucens* peptides were identified using our homology-based approach relative to identifications obtained with the full database. A similar strategy has been reported in human proteomics, where human spectra were searched against a chicken database simulating the absence of species-specific references. A 15 % peptide recovery was achieved (Renard et al.,

2012), which is comparable with our findings considering the large evolutionary distance between humans and chicken. Differences in identification efficiency between *T. molitor* and *H. illucens* reflected the number of homologous sequences in the databases. By exclusion of the respective species from their phylogenetic families, 976 directly homologous sequences for *H. illucens* (Stratiomydiae) and 70,864 for *T. molitor* (Tenebrionidae) remain in UniProt. Belghit and colleages observed similar results and also correlated peptide identification to database size in a previous study (Belghit et al., 2019). Based on this correlation, we roughly estimated the possible peptide recoveries achieved with our homology-based approach for the other investigated insect species, i.e. *A. diaperinus*, *L. migratoria*, *A. domesticus* and *G. sigillatus*. Applying a linear extrapolation yielded estimated percentages of identifiable peptides between 26 % and 47 % (Meisinger, Planatscher, et al., 2025, Appendix A.1, Table 2).

We developed this approach, as direct comparison between species-specific proteomes containing approximately 15,000 entries and the full arthropod database containing approximately 7.1 million entries was impractical due to substantial size differences and the statistical method used for PSM (Kumar et al., 2017). Larger databases expand search space, improving detection of diverse peptides but increasing random matches and FDR, while smaller databases allow more accurate identifications but may miss peptides absent from the reference database (Muth et al., 2015). This illustrates the trade-off between coverage and statistical reliability in database selection.

3.3 Proteomic Profiling of Allergens in Edible Insects

3.3.1 Discovery of Putative Allergens in Edible Insect Species

Insects contain allergenic proteins (van Huis, 2020; Wangorsch et al., 2024; Yang et al., 2024), but ingestion-related risks remain insufficiently assessed due to limited consumption within the EU. Given the scarce protein sequence information for the insect species currently authorized in food products, unbiased proteomic analysis is preferred for initial risk assessment (López-Pedrouso et al., 2020). For this, our previously generated species-specific peptide spectra were searched against the COMPARE database (van Ree et al., 2021), which includes over 2700 peer-reviewed allergens, is not clus-

tered for sequence identity, and was designed for allergy safety assessment by regulatory agencies. We identified between 99 (*A. diaperinus*) and 174 (*L. migratoria*) peptides homologous to known allergens, corresponding to up to 106 putative allergens per species (Meisinger, Planatscher, et al., 2025, Appendix A.1, Table 3 and Appendix A.2, Supplementary Table 8). Most allergens were identified from a single unique peptide, which reflected the large evolutionary distance of the sequence database, missing species-specific sequences (Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 9). Proteins supported by multiple unique peptides were often classified as highly-conserved and abundant proteins, e.g. tropomyosin and α-actinin, and showed good sequence coverage above 25 % (Meisinger, Planatscher, et al., 2025, Appendix A.1, Table 4).

Comparison of identified allergens revealed sixteen conserved proteins shared among all six species (Figure 3.7 A and Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 9) including actin, tropomyosin, glycogen phosphorylase, enolase, ATP synthase β, α-actinin, α-tubulin, filamin C, cytochrome C, GAPDH, and elongation factors. While some (e.g. tropomyosin or enolase) were well established allergens in insects or crustacean (Chuang et al., 2010; De Marchi, Wangorsch, & Zoccatelli, 2021; Wangorsch et al., 2024), others such as GAPDH have so far been only reported in shrimp based on a transcriptomics study (Karnaneedi et al., 2020). This finding reinforced the need for further allergenicity assessments of edible insects, particularly for individuals with shellfish allergies.

We next investigated interspecies differences in allergen profiles. For *H. illucens*, 49 unique putative allergens were identified, including allergens newly reported in insects, such as glutelin, pathogenesis related protein, lipocalin, or nitrile-specifier protein (Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 9). These findings suggested specific allergenic risks associated with *H. illucens*. From the 46 unique allergens of *L. migratoria*, three proteins were particularly noteworthy. Hexamerin, a known locust allergen (Y. Wang, Zhang, et al., 2022), confirmed the reliability of the COMPARE-based approach. Cyclophilin, a pan-allergen associated with pollen sensitization (Fluckiger et al., 2002; Matricardi et al., 2024), has previously been reported in insects only for the American cockroach (L. Wang et al., 2023). The expansin-like protein identified here represents a novel insect allergen previously known only from plants (Grobe et al., 1999).

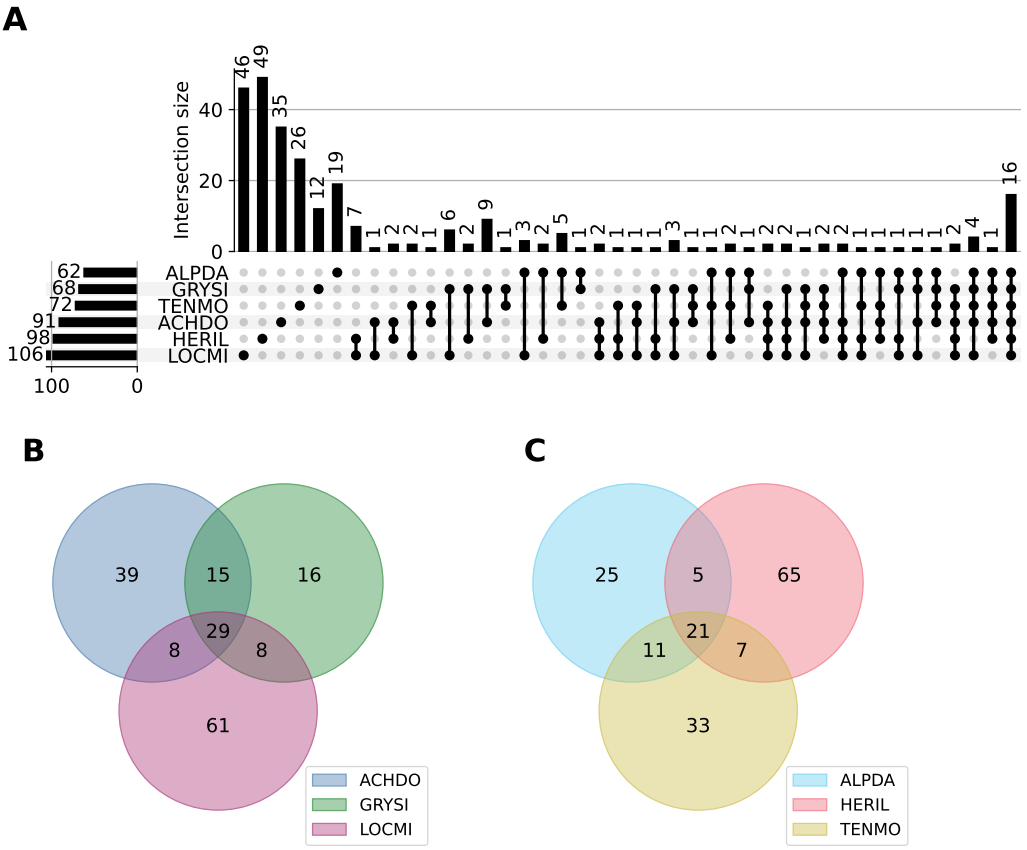

Figure 3.7: Comparison of putative allergen profiles in six edible insect species. (A) Numbers of unique and common allergens identified in the six insect species *A. domesticus* (ACHDO), *A. diaperinus* (ALPDA), *G. sigillatus* (GRYSI), *L. migratoria* (LOCMI), *H. illucens* (HERIL) and *T. molitor* (TENMO), visualized as an UpSet plot. Empty intersections are not shown. (B) In-depth comparison of identified allergens within the Orthoptera order. (C) The species from the Tenebrioninae subfamily were compared with the evolutionary more distant black soldier fly *H. illucens*. Adapted from Meisinger, Planatscher, et al., 2025, Appendix A.1, Figure 8.

Phylogenetic subgroup comparisons largely reflected the evolutionary distances between the species. For the orthopterans, 29 common allergens were identified (Figure 3.7 B and Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 10). In comparison with all six investigated species, three orthopteran-specific allergens, i.e. serine protease, arginine kinase and vicilin, were identified within our dataset. Serine proteases are known insect allergens (Sudha et al., 2008), whereas vicilin, a legume allergen (Burks et al., 1995; Holzhauser et al., 2009), represents a new candidate in edible insects. In the second subgroup, comprising Tenebrioninae and *H. illucens*, 21 allergens were shared, with stronger overlap between Tenebrioninae species (Figure 3.7 C and Meisinger, Planatscher, et al., 2025, Appendix A.2, Supplementary Table 11). This sub-

group contained two unique allergens overall and five unique to Tenebrioninae, including dipeptidyl peptidase (Ves v 3), two tropomyosins, and two arginine kinases, most previously described in insects or mites (Blank et al., 2010; De Marchi, Wangorsch, & Zoccatelli, 2021; Delfino et al., 2024). Furthermore, the particular sequence of an here identified apolipophorin was previously identified in the mite *Sarcoptes scabiei*, underscoring possible cross-reactivity between insects and mites in sensitized individuals.

3.3.2 Refinement and Validation of High-Confidence Allergen Candidates

To refine the initial list of putative allergens and reduce false positives, stricter selection criteria were applied. The Codex Alimentarius recommends a threshold of 35 % identity over at least 80 amino acids to predict cross-reactivity (Codex Alimentarius, 2003; EFSA, 2010). Although this standard has been widely used by EFSA, recent guidance acknowledged that the approach is overly sensitive and prone to false positives when applied without experimental validation (EFSA et al., 2022). Originally designed for GMO risk assessment rather than proteomic screening, this method was adopted here; however, definitive allergen classification requires subsequent validation through targeted analyses. Since tryptic peptides are typically only eight to twelve amino acids long (Swaney et al., 2010), they cannot meet the 80 amino acid alignment window. Thus, only proteins supported by at least two unique peptides within an 80 amino acid region were retained. Using this refined approach, seven to eighteen high-confidence candidate allergens were identified per species, largely confirming the previous findings (Meisinger, Planatscher, et al., 2025, Appendix A.1, Table 4 and Appendix A.2, Supplementary Table 12).

In summary, this study closed a key gap in insect proteomics by establishing a homology-based workflow capable of identifying proteins and allergens, thereby overcoming major limitations caused by the scarcity of species-specific reference sequences. Distinct proteomic and allergenic profiles were revealed among the investigated species. The detection of conserved allergens such as tropomyosin and arginine kinase across multiple species underscores their potential as universal insect allergens. *H. illucens* exhibited a distinct allergen profile, suggesting species-specific allergenic potentials in line with pre-

vious findings (Barre et al., 2021; Palmer et al., 2020). Allergenicity may further be modulated by processing methods such as heating or enzymatic hydrolysis, which can alter protein structure and epitope accessibility (De Marchi, Wangorsch, & Zoccatelli, 2021). Moreover, the comprehensive peptide dataset generated here provides a direct basis for development of targeted PRM assays to validate species-specific proteins and candidate allergens, thereby improving allergen detection, risk assessment, and quality control of insect-based foods. In future studies, experimental confirmation of allergenicity and potential cross-reactivity could be achieved through immunoassays such as ELISA or basophil activation tests, or by using sera from allergic individuals, providing direct evidence of IgE binding and functional responses (De Marchi, Mainente, et al., 2021). Overall, the presented workflow lays the groundwork for improving the safety, authenticity, and traceability of insect-derived foods and can be readily applied to other species with incomplete sequence information.

4 Results & Discussion II: Insect Species Authentication and Quantification

The content of this chapter is based on:

Meisinger, T., Planatscher, H., Garino, C., Stoll, D., Ladenburger, E.-M., Braeuning, A., Broll, H., Poetz, O. (2026). From cricket to mealworm: 8-Plex mass spectrometry immunoassay for edible insect detection in novel foods. Microchemical Journal, 222, 117078. https://doi.org/10.1016/j.microc.2026.117078

4.1 Insect Species Marker Peptide Selection

Several edible insect species have been authorized or are pending authorization by the EU (Quintieri et al., 2023). Although these species contain allergenic proteins, no official method has been established for their authentication in food (Marien et al., 2025). While PCR methods are often the first choice, due to being broadly available and relatively low in cost, they lack the ability to directly detect insect material. In contrast, protein- or peptide-based assays are theoretically capable of direct detection in complex food samples. The development of such assays, including targeted LC-MS/MS methods for species identification and allergen detection in insect-based foods, requires well-defined peptide markers whose uniqueness, robustness and detectability can be assured (Gavage et al., 2020; Meisinger, Vogt, et al., 2025).

Until recently, this process was hindered by the limited availability of annotated protein sequence information for most edible insects, restricting marker selection to approaches based on direct spectral comparison or non-targeted fingerprinting (Belghit et al., 2019; Leni, Prandi, et al., 2020; Tata et al., 2022). The homology-based proteomic workflow established in the preceding study addressed this limitation by expanding the accessible

protein sequence space for six commercially relevant insect species and by providing experimentally verified proteome and allergen profiles (Meisinger, Planatscher, et al., 2025, Appendix A.1).

This dataset and the publicly available draft proteomes of *H. illucens* and *T. molitor* were systematically analyzed for species-specific marker peptides for *T. molitor*, *A. diaperinus*, *A. domesticus*, *G. sigillatus*, *L. migratoria* and *H. illucens*. Candidate peptides were then rigorously filtered to meet the analytical requirements of a targeted assay. Initial evaluation focused on chromatographic behavior and mass spectrometric detectability facilitated by exclusion of peptides outside a specified sequence length window or certain sequence motifs. Candidates were subsequently evaluated for species specificity. Here, a pragmatic approach was adopted since absolute sequence uniqueness, as evaluated in similar assays (Steinhilber et al., 2018b), cannot be confirmed without complete proteomic sequence databases (Pedreschi et al., 2010). Candidate peptides were assessed with BLAST against the non-redundant database. Only peptides with a single or a small number of identified 100 % sequence identity hits were considered for further testing. These hits needed to belong to either taxonomically distant species or economically insignificant species to reduce the potential risk of false positive results of the assay if applied to food samples. The resulting candidates were further filtered for expected abundance. Using a sensitive, targeted PRM-based analysis approach (Peterson et al., 2012), markers were experimentally verified to be of high intensity and unique within the investigated panel of insects. The final list differed from previously reported species-specific peptide markers by Leni and co-workers for *A. diaperinus* and *H. illucens* (Leni, Prandi, et al., 2020), hence we subjected a random selection of their candidates to our stringent BLAST filtering. Most lacked uniqueness or matched bacterial sequences, making them unsuitable for reliable species discrimination.

For five out of six species, at least one marker was confirmed from our candidate list (Table 4.1). Despite the difficulty posed by high sequence similarity, differentiation between *A. domesticus* and *G. sigillatus* was achieved. Although no specific marker for *A. domesticus* was identified, both identified markers for *G. sigillatus* were absent in the evolutionary closely related *A. domesticus* (Yang et al., 2021). The marker selected for *L. migratoria* was initially considered species-specific based on an early BLAST search. A subsequent

search produced a match to *Escherichia coli*; however, since the *E. coli* genome has long been fully sequenced, this hit was likely an artifact rather than a true match, highlighting the need for careful evaluation of database results over time (Martí Jose et al., 2025).

Table 4.1: Final list of marker peptides for the development of an 8-plex IA-LC-MS/MS assay. Peptides were selected after stringent filtering of a homology-based proteomic dataset for analytical properties, species specificity and PRM performance. Specificity was assessed via BLAST against the non-redundant sequence database. Modified from Meisinger et al., 2026, Appendix B.1, Table 1 and Appendix B.2, Supplementary Table 1.

Target species	Peptide sequence	Protein name	BLAST-derived uniqueness
A. diaperinus	DGDVVHGSY-SLTDPDGTR	Larval cuticle protein A3A (LCPA3A)	one 100 % hit in *Tribolium madens*
A. diaperinus	ISIPPFGEILELER	Hemocyanin C (HCC)	two 100 % hits in *Zophobas morio* and *Asbolus verrucocsus*
T. molitor	SLYGGYGSGLGIAR	Larval cuticle protein F1 (LCPF1)	unique
H. illucens	GSYSYNDGFFK	Cuticle protein (HICP)	unique
L. migratoria	DVSPTELEYFEK	Vitellogenin A (VTGA)	one 100 % hit in *Escherichia coli*
G. sigillatus	VSSTLSGLSAELK	Arginine kinase (AK)	one 100 % hit in *Stigmatomma sp. TH05*
G. sigillatus	ASDVADTVLGATGSK	Spermatophylax protein 1C (SP1C)	unique
Insecta	LAFVEDELEVAEDR	Tropomyosin (TPM)	excluding insects: one 100 % hit in *Shewanella electrica*

Besides species-specific markers, a tropomyosin peptide was identified as a class-level identifier for insects. BLAST assessment of this peptide revealed near exclusive occurrence in insects. Full-length tropomyosin is a recognized pan-arthropod allergen (Hall & Liceaga, 2021; Klueber et al., 2020; Ribeiro et al., 2021; Y. Wang, Li, et al., 2022; Wangorsch et al., 2024), and its high degree of sequence conservation and abundance as an actin-binding protein make it a suitable marker for taxonomic identification (Barre et al., 2018; Hitchcock-DeGregori & Barua, 2017; James & Nanda, 2020). To our knowledge, this tropomyosin peptide represents the first reported pan-insect peptide marker. Although some of the selected peptide markers, including tropomyosin and arginine kinase, are derived from known allergens, the peptides itself are not considered allergenic

and thus serve as proxies for species-specific protein material rather than indicators of intact allergens.

In preparation for assay development, isotopically labeled and non-labeled peptides were synthesized (Intavis, Tübingen, Germany) and for each marker peptide, polyclonal antibodies were generated (Hoeppe et al., 2011). Chromatographic and mass spectrometer parameters of each peptide were optimized to yield the highest possible signal intensities (Meisinger et al., 2026, Appendix B.2, Supplementary Figure 1). A chromatographic gradient was identified that enabled near baseline peak separation with a short cycle time of only six minutes (Figure 4.1).

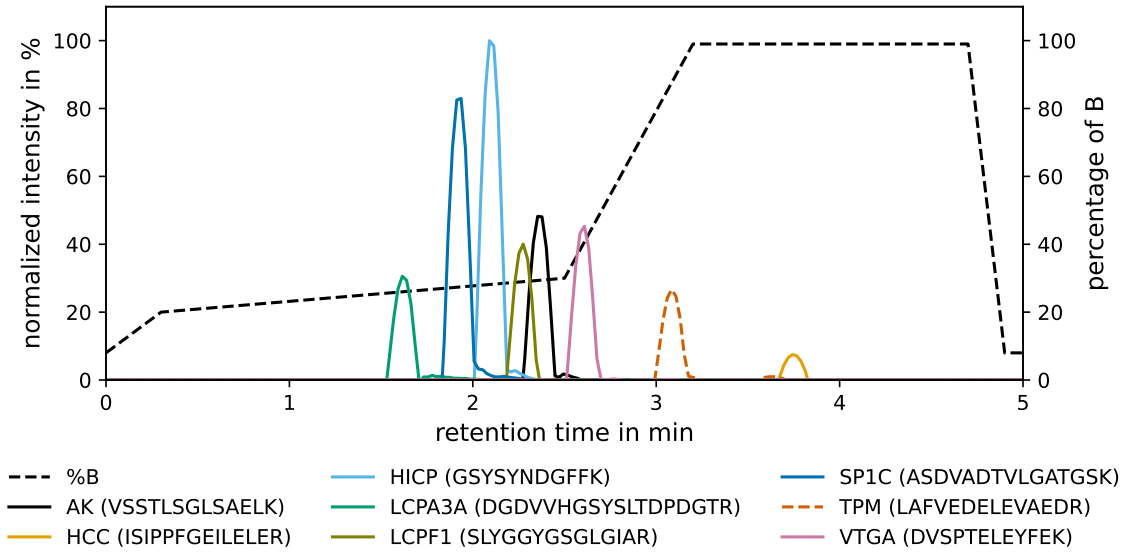

Figure 4.1: Chromatographic gradient with overlaid extracted ion chromatograms for all eight peptide markers. Signal intensities were normalized to the maximum. Data was recorded on an analytical nanoEase M/Z BEH column from Waters, with an inner diameter of 0.75 mm, a length of 150 mm, and a particle size of 3.5 µm. Solvent A consisted of 0.1 % formic acid in LC-MS grade water; solvent B of 80 % acetonitrile and 0.1 % formic acid in LC-MS grade water. The flow was set to 1.5 µL min^{-1} at 55 °C. Adapted from Meisinger et al., 2026, Appendix B.2, Supplementary Figure 2.

4.2 Assay Development and Validation

Assay development and validation are essential steps in establishing a reliable analytical method. The previously identified and selected peptide markers together with their optimized chromatographic gradient and mass spectrometer instrument parameters were used to develop and partially validate a novel peptide-based insect detection and quantification assay.

4.2.1 Sample Preparation

Sample preparation was based on a previously published protocol that combines protein extraction and proteolysis in a single step. This heterogeneous phase digest (HPD) has been shown to be an efficient sample preparation method for milled feed samples, reducing loss of peptide due to its small number of processing steps (Steinhilber et al., 2018a). Key parameters of the HPD have been investigated and optimized for maximal peptide release from pure insect samples, as determined by PRM measurement after semi-automated immunoenrichment.

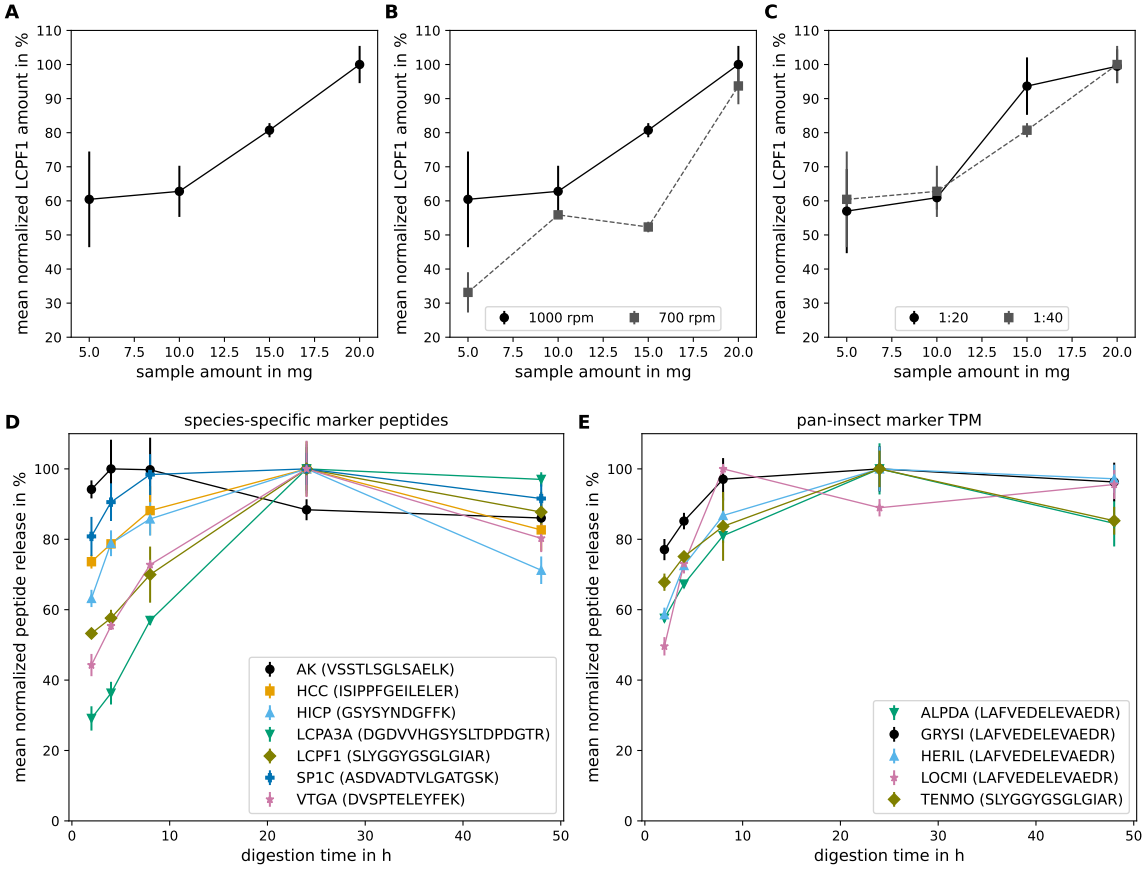

Figure 4.2: Optimization of sample preparation parameters (A) sample amount, (B) shaking speed during incubation steps and (C) trypsin to protein ratios. A time-series analysis was conducted to determine the optimal proteolysis incubation time for the (D) species-specific marker peptides and (E) the pan-insect marker tropomyosin. Error bars represent standard deviation (n = 3). Adapted from Meisinger et al., 2026, Appendix B.1, Figure 1.

Peptide release positively correlated with sample amount and shaking speed but not with tested trypsin to protein ratios (Figure 4.2 A to C). For optimization of the enzymatic fragmentation process, a time dependent analysis was conducted (Figure 4.2 D to E). Incuba-

tion time is a critical factor for proteolysis (Proc et al., 2010; Weiss et al., 2018) and up to 48 h were tested. All markers followed a sigmoid curve and most markers showed highest peptide release after 24 h. The only exceptions being arginine kinase from *G. sigillatus* and tropomyosin of *L. migratoria* peaking at 4 h and 8 h, respectively. Since all eight markers have been analyzed in a multiplexed fashion, a 16 h incubation period has been chosen, thus compromising on the highest possible peptide release for all analytes. This decision is in line with similar assays (Hoeppe et al., 2011; Naboulsi et al., 2024; Vlasakova et al., 2023).

4.2.2 Calibration Curve Performance

The calibration curve determines the analytical range of an assay and was prepared as dilution series of synthetic non-labeled peptides in proteolyzed cookie surrogate matrix. Several dilution series were tested, which led to the selection of an analytical range between 0.23 fmol and 1500 fmol for validation experiments. There, accuracy between 80 % and 120 %, with a CV below 20 % was confirmed for two to four orders of magnitude of concentration (Meisinger et al., 2026, Appendix B.1, Figure 2). While the experimentally determined lower limits of quantifications (LLOQ) varied by analyte between 0.69 fmol (SP1C, HICP) and 6.17 fmol (LCPA3A, HCC), the upper limits of quantification (ULOQ) were constant at 1500 fmol (Meisinger et al., 2026, Appendix B.1, Table 1). Comparison of results obtained from dilution in PBSC or surrogate matrix revealed no matrix effect on peptide recovery (Meisinger et al., 2026, Appendix B.2, Supplementary Figure 3 A). Chromatographic carryover effects were detected for five analytes starting at varying concentrations: SP1C and HCC at 1500 fmol, LCPA3A at 500 fmol, HICP at 166 fmol, and TPM at 55 fmol. However, the amount of each carryover was quantified to be below 1.5 fmol and was effectively mitigated by an additional wash injection after analysis of samples surpassing the determined concentration thresholds.

Pure insect samples were analyzed to generate reference values that enabled translation of the analytical range from analyte concentration to insect percentages in complex food samples (Meisinger et al., 2026, Appendix B.1, Table 1). This assumes homogeneous distribution of insect material and comparable processing-related losses between reference and test matrices. Except LCPF1 and AK with LLOQs that translated to 2.27 % and 0.39 %

insect content respectively, all markers achieved a sensitivity in the lower parts-per-million (ppm) range. This assay thus demonstrated comparable or lower limits of detection (LOD) than previously reported PCR-based assays (Filipa-Silva et al., 2025; Garino et al., 2021; Tramuta et al., 2018). The low sensitivities of LCPF1 and AK in terms of detectable insect percentage deviated from the high technical sensitivities determined by standard curve analysis. This discrepancy suggests that the endogenous abundance of LCPF1 and AK in their respective species may be lower than inferred from initial PRM measurements, despite the high signal intensities observed during initial analyte selection. Alternatively, incomplete solubilization or proteolytic release of these proteins during HPD could limit their extractability from the matrix. Replacing LCPF1 and AK was impeded by limited protein sequence data for both species during the course of the study but offers opportunity for future refinement of the assay.

4.2.3 Intra- and Inter-Assay Accuracy and Precision

Inter- and intra-day accuracy and precision describe the short- and mid-term performance of an analytical method. Intra-day evaluations assess how reliably the assay measures replicate samples within a single analytical run, while inter-day evaluations test its robustness across multiple days, including routine variations in instrumentation, reagents, and operating conditions (FDA, 2022). Both parameters were tested using quality control (QC) samples prepared by spiking non-labeled peptides at defined levels into a proteolyzed cookie surrogate, with nominal values established beforehand from independent measurements. All peptides passed acceptance criteria of lower than 20 % CV and 80 % to 120 % accuracy to the nominal values (Meisinger et al., 2026, Appendix B.2, Supplementary Table 2) and results are comparable to similar assays (Steinhilber et al., 2018b). Artificial QC samples were used because no available food product contained all five target species, and a cumulative increase of the pan-insect tropomyosin signal would have occurred in such a matrix. The use of peptide-spiked surrogate materials is well established in quantitative LC-MS method validation and provides a controlled and reproducible basis for assessing assay performance (Bowen & Volchok, 1980; FDA, 2022).

4.2.4 Assay Specificity

The peptides for this assay were selected from a dataset generated by a homology-based proteomic method based on intrinsic sequence properties and degree of uniqueness, as determined by a comprehensive BLASTp search. Due to the limited available sequence space of insects and arthropods in general, this approach can only approximate true specificity. Hence, the assay setup was rigorously tested experimentally for species specificity. In a first step, applying PRM analysis to species pure insect samples, species specificity for the selected peptides was confirmed within our species subset (Meisinger et al., 2026, Appendix B.2, Supplementary Figure 1 A). Subsequently, antibody specificity within the selected peptide panel was confirmed, showing exclusive enrichment of the respective target peptide (Figure 4.3 A).

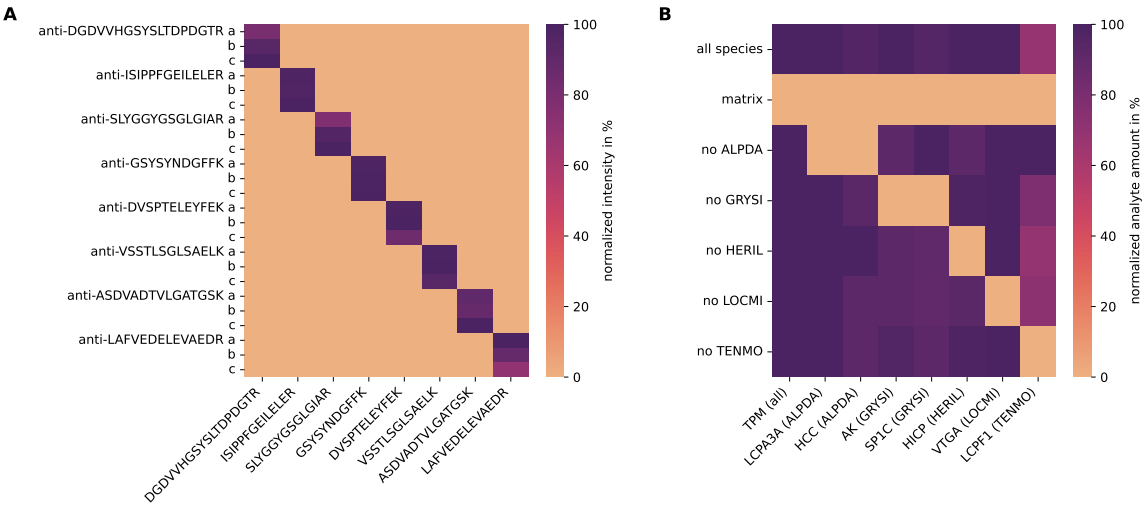

Figure 4.3: Species specificity of the assay was evaluated on the antibody level and with a mixture of all targeted insect species. (A) Each antibodies was incubated with a mixture of all eight isotopically-labeled synthetic peptides (n = 3). Mass signal intensities of the eluted peptides were recorded using PRM and normalized to the maximum signal for each peptide. (B) Sample mixtures containing all insects except one insect each were used for conduct of the assay. Peptide signal intensities from this leave-one-out experiment (n = 3) were normalized to the maximum of each marker. Tropomyosin was observed in all insect containing samples while no marker was present in the matrix control. Markers from omitted insect species were absent in the respective samples. Adapted from Meisinger et al., 2026, Appendix B.1, Figure 3 A and Figure 3 B.

In a combined approach, a leave-one-out experiment was conducted. Mixed samples containing all but one insect species of interest were generated and subjected to immunoenrichment and PRM analysis. Tropomyosin was identified to be present in all tested

insect species, while the tested species-specific marker peptides were not observable in mixtures omitting their respective target insect species (Figure 4.3 B). This highly sensitive approach confirmed the initial PRM results and demonstrated that the peptide marker selection is truly species-specific within the set of target insects.

Comprehensive tests outside of the set of target insects were not feasible due to the high number of potential species. Thus, a more limited strategy was chosen for further tests. Shrimps belong to the subphylum crustacea (Hurzaid et al., 2020), which itself belongs to the arthropods (Schmidt-Rhaesa et al., 1998). Shrimps are therefore the most suitable candidates for additional testing, as their widespread consumption (Jamal, 2022) makes their occurrence in complex foods plausible and their close evolutionary relationship to insects increases the likelihood of shared peptides that could challenge marker specificity. *Litopenaeus vannamei* was selected as representative crustacean (Asmild et al., 2024; Cuzon et al., 2004) due to its economic relevance. After drying and milling, whole and degutted shrimp were analyzed, and no insect-specific marker peptides were detected. Notably, tropomyosin was observed only in whole shrimp, indicating ingested material as its source. The corresponding signal was only marginally above the lower limit of detection (LLOD), implying that in complex food samples, where shrimp typically constitute a small proportion of the matrix, it would be substantially diluted and thus not detectable. In summary, the results demonstrated species-specificity for the insect-species marker, insect specificity of tropomyosin under the tested conditions and that shrimp does not compromise the assay specificity.

4.2.5 Parallelism

Parallelism assesses whether assay responses for diluted samples run in parallel to the calibration curve, indicating that the matrix does not alter analyte quantification (FDA, 2022). This parameter verifies that measured concentrations remain proportional across dilution levels and was tested by serial dilution of proteolyzed samples in proteolyzed cookie surrogate matrix. All analytes demonstrated a strong linear correlation between nominal and measured concentrations (Figure 4.4) and quantification accuracies fell within 75 % to 125 % for all included dilution levels for VTGA, HICP and LCPF1 and for most levels of the other analytes (Meisinger et al., 2026, Appendix B.1, Figure 4 B). For several

analytes, the undiluted samples were excluded due to being above the ULOQ, as well as the tenfold dilution of VTGA and LCPA3A due to observed internal standard suppression, indicating oversaturation of the capture antibodies. In summary, parallelism and thus absence of matrix influence was demonstrated for all analytes over a dilution range spanning three to four orders of magnitude.

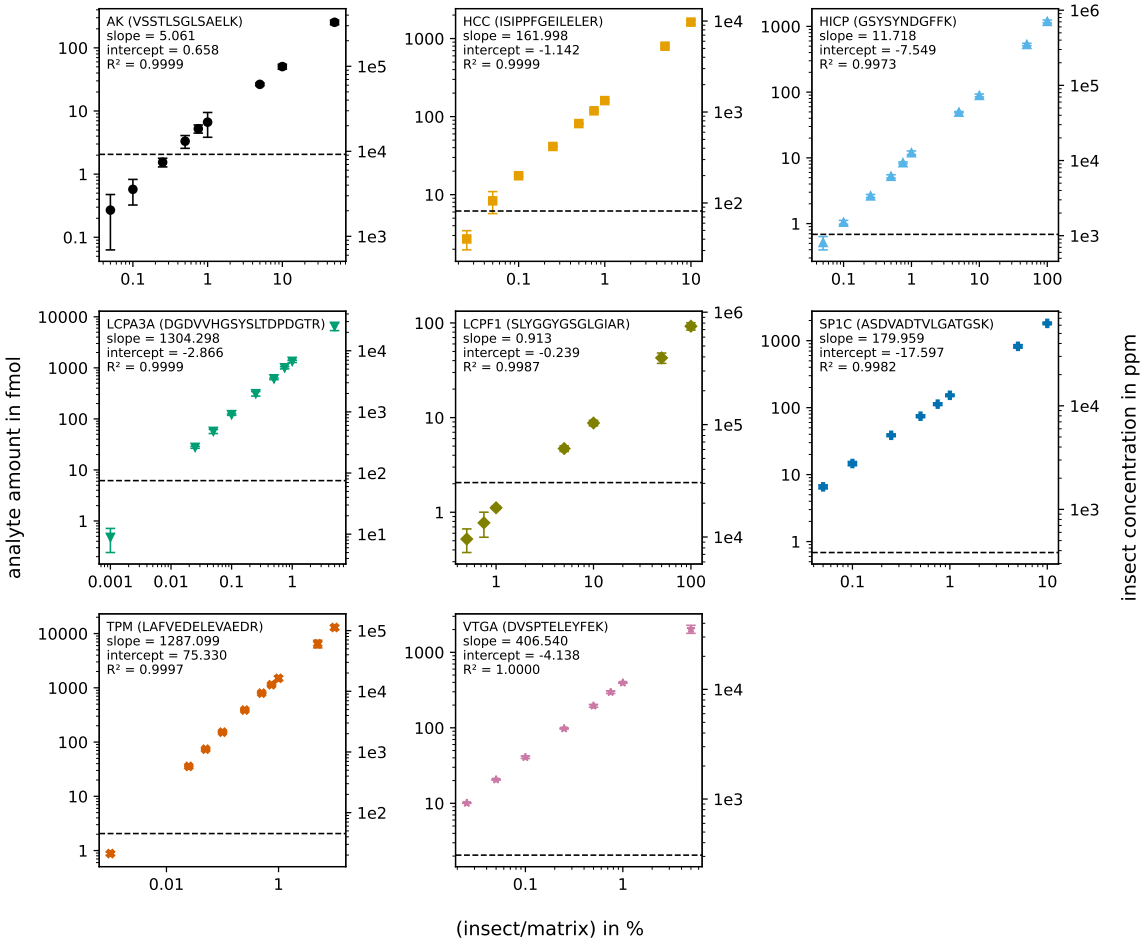

Figure 4.4: Determination of parallelism for all analytes. Proteolyzed insect meal samples were serially diluted into proteolyzed cookie surrogate matrix. A linear regression analysis was performed for each analyte and measured analyte amounts, insect content percentage, slope, intercept and R^2 are given for each plot. A dashed horizontal line indicates the lower limit of quantification and error bars represent standard deviation (n = 3). Adapted from Meisinger et al., 2026, Appendix B.1, Figure 4 A.

4.3 Application in Food Matrices

Following assay development, its applicability was evaluated using real food samples. Food matrices represent a particular analytical challenge due to their complex and hetero-

geneous composition. Interactions between proteins, lipids, carbohydrates, and additives, as well as effects introduced by thermal processing, fermentation, or drying, can alter protein extractability and detectability (Cifuentes, 2012; Pedreschi et al., 2010). These factors may affect marker stability and signal intensity, thereby influencing analytical performance and interpretation of results. In this study, two sample types were investigated: commercially available products containing high proportions of insect material, and model foods spiked with insects at allergen-relevant levels. The former were relevant for food authenticity and label verification, while the latter assessed the suitability of the method for sensitive allergen detection.

4.3.1 Analysis of Commercial Insect Novel Foods

A diverse set of commercially available foods containing insects as single-species ingredients at moderate inclusion levels (5 % to 20 %), complemented by a fried model burger with a high proportion (50 %) of *T. molitor* to extend matrix diversity was analyzed with the 8-plex IA-LC-MS/MS assay (Meisinger et al., 2026, Appendix B.1, Table 2). Across all products, the multiplex assay consistently confirmed the presence of insect-derived proteins, indicating robust qualitative performance irrespective of matrix composition. However, quantitative results were variable (Meisinger et al., 2026, Appendix B.1, Table 2) and depended on the interaction between the selected analyte, the insect species, and the physicochemical properties of the food matrix (Liu et al., 2022; Pedreschi et al., 2010).

For *T. molitor*, quantification was accurate in crackers, a simple, low-fat matrix, where TPM and LCPF1 closely matched the expected levels. In contrast, performance deteriorated markedly in the fried burger patty, likely due to high fat content and intense heat treatment, which reduced protein extractability and promoted heat-induced modifications such as Maillard reactions, particularly in strongly heated regions of the product (Arnold et al., 2015; Liu et al., 2022; Rahaman et al., 2016). In foods containing *A. diaperinus*, LCPA3A showed the most consistent performance, providing accurate or moderately overestimated results in both pasta and cereal bars. Other markers were more affected by matrix composition: tropomyosin performed poorly in carbohydrate-rich pasta, while HCC and TPM underestimated insect content in sugar- and fat-rich cereal bars. These differences indicate marker-specific sensitivity to matrix components and processing-induced

protein modifications (Acquistucci, 2000; Arnold et al., 2015; Tata et al., 2022; Topbas et al., 2018). For *A. domesticus*, quantification was based solely on the pan-insect marker TPM, as no species-specific peptide was available. In the plant-based mince, this resulted in moderate overestimation, attributed to sample heterogeneity (Grant & Pelton, 1973).

Taken together, the results demonstrated that quantitative performance was influenced by both matrix complexity and processing history. Among the tested analytes, LCPA3A and LCPF1 showed the greatest robustness, whereas TPM proved susceptible to matrix-related interference. While the assay is well suited for qualitative screening across a wide range of foods, accurate quantification currently appears reliable only for matrices comparable to simple, low-fat products such as crackers. Broader quantitative application will require further validation across additional food matrices.

4.3.2 Analysis of Allergenic Model Foods

To assess the assay performance at low insect concentrations relevant for allergen control, plain cookies were fortified with a defined mixture of six edible insect species at total levels ranging from zero to 600 ppm. Based on the established quantification ranges, only a subset of analytes was expected to yield quantifiable signals at the upper spiking levels. Experimentally, signals above the lower limit of quantification were observed for several markers, including LCPA3A, VTGA, SP1C, and TPM (Figure 4.5). However, the measured concentrations deviated substantially from the expected values. The poor accuracy was attributed to the heterogeneous distribution of insect material and the intrinsic difficulty of achieving uniform mixing at trace levels in baked, processed foods (Grant & Pelton, 1973). This effect is increased by subsampling, which was necessary to enhance analytical sensitivity (Grant & Pelton, 1973; Rohde et al., 2015). Similar limitations have been reported for other analytical approaches targeting low-level contaminants, illustrating the inherent trade-off between sensitivity and quantitative robustness (Olsvik et al., 2017; Rohde et al., 2015). Alternative strategies that rely on standardized sample input and normalization may improve reproducibility, but often at the cost of reduced sensitivity (Heick et al., 2011). Further optimization of sampling and preparation protocols will therefore be required to fully exploit the assay's quantitative potential at low concentrations.

Despite these limitations, qualitative detection proved to be robust. Several analytes were consistently detected in the 100 ppm samples, with SP1C remaining detectable at 20 ppm and HCC and HICP at levels as low as 5 ppm per species.

total insect content in ppm	per species insect content in ppm	replicate	AK	HCC	HICP	LCPA3A	LCPF1	SP1C	TPM	VTGA
0	0	A	–	–	–	–	–	–	–	–
0	0	B	–	–	–	–	–	–	–	–
0	0	C	–	–	–	–	–	–	–	–
30	5	A	–	–	+	–	–	–	+	–
30	5	B	–	+	–	–	–	–	+	–
30	5	C	–	+	–	–	–	–	+	–
120	20	A	–	+	+	–	–	+	+	–
120	20	B	–	+	–	–	–	–	+ 50%	–
120	20	C	–	+	–	–	–	–	+	–
600	100	A	–	+	–	+	–	+ 38%	+ 1166%	+ 189%
600	100	B	–	+	–	+ 229%	–	–	+ 17%	+
600	100	C	–	+	+	+ 48%	–	+ 225%	+ 49%	+

Figure 4.5: Model cookies were spiked with up to 100 ppm of *T. molitor*, *A. diaperinus*, *A. domesticus*, *G. sigillatus*, *L. migratoria* and *H. illucens*. For each cookie, three subsamples were analyzed. A bold black edge indicates the assay range as determined during assay development. Absence or presence of analytes are indicated by "+" and "–", while percentages describe quantification accuracy of insect content based on experimentally determined protein abundance in pure insect meals. Adapted from Meisinger et al., 2026, Appendix B.1, Figure 5.

This represents a substantial improvement over earlier methods that required at least 1 % insect contents (Leni, Prandi, et al., 2020). Tropomyosin was detected in all spiked samples, underscoring its suitability as a universal insect marker. Given its role as a major

insect allergen (Ribeiro et al., 2021; Wangorsch et al., 2024), the reliable detection of tropomyosin at the lowest concentrations demonstrates the assay's particular value for allergen surveillance and food safety monitoring in complex food matrices. A direct comparison with a recently published real-time PCR method demonstrated competitive sensitivity of the multiplexed peptide-based assay, as both assays achieved detection of *A. diaperinus* at 5 ppm (Garino et al., 2022).

In summary, a multiplexed IA-LC-MS/MS assay for the authentication and quantification of edible insect species in food was developed and partially validated. The resulting 8-plex assay showed high sensitivity, specificity, and reproducibility, with limits of quantification frequently in the low ppm range, thus closing a key gap in insect-based novel food analysis. Qualitative detection was reliable across diverse food matrices, including trace-level applications relevant for allergen monitoring, while quantitative performance was influenced by matrix composition and processing. Overall, the assay provides a sensitive and specific tool for insect detection in foods and a solid basis for further optimization toward broader quantitative applicability.

5 Conclusion and Outlook

The aims of this study were to systematically characterize the proteomes of edible insect species and to develop targeted, peptide-based analytical assays for the authentication and quantification of insect-derived material in complex food matrices.

Several insect species have been authorized for use as food or feed within the European Union, reflecting their growing relevance for sustainable nutrition and alternative protein sources (Quintieri et al., 2023). Despite this regulatory approval and increasing industrial interest, comprehensive molecular knowledge of these species remains limited. In particular, genomic resources are incomplete for many authorized insects, and proteomic data are often scarce (Leni, Prandi, et al., 2020).

To address these limitations, this work established a robust strategy for the detection and identification of proteins in non-sequenced insect species. A non-targeted, homology-based LC-MS/MS workflow, established by combination of *in silico* and laboratory procedures, was developed and applied to six insect species. This approach increased the number of known proteins up to 24-fold per species. Extensive experimental and computational verification steps were conducted to minimize the number of false positives. Furthermore, the influences of database size and error-tolerant PSM settings were investigated and inter-species proteomic differences were explored. This analysis led to the identification of previously unreported putative allergens and revealed distinct allergenic profiles among the investigated species, providing a basis for future clinical and risk assessment studies. Functional validation of the allergenic profiles could be approached by basophil activation tests or skin prick tests in a controlled clinical context. Future application of the here described workflow to edible insect species pending authorization has the potential to accelerate EU approval procedures by providing proteomic data at an early stage of risk assessment. Furthermore, extending this approach to a broader range of insect taxa would support basic biological and evolutionary research by facilitating comparative proteomic analyses. Although the workflow is expected to be transferable to non-

insect species lacking reference genomes, this assumption requires systematic evaluation to better define its general applicability, limitations, and analytical performance.

Despite the authorization or pending authorization of edible insect species and the established recognition for their allergenic potential, no official analytical method is currently available for authentication (Marien et al., 2025). While real-time PCR is often the first method of choice due to its rapid implementation, broad availability and relatively low cost, it relies on indirect analyte detection.

We addressed this methodological gap by development of a direct, peptide-based analytical method. Putative species-specific and class-level insect marker peptides were identified within our previously generated comprehensive proteomic datasets and newly available draft reference proteomes for two species. Notably, the first insect-specific marker peptide was established, derived from tropomyosin, a protein of particular relevance in the context of food allergy. Species-specific markers were identified for all but one species and subjected to extensive verification. These marker peptides formed the basis for the development of the first multiplexed, peptide-based, targeted IA-LC-MS/MS assay for insects. This eight-plex assay was developed and partially validated with respect to sample preparation, calibration behavior, specificity, selectivity, and parallelism, as well as inter-day and intra-day accuracy and precision. It demonstrated high specificity and an analytical range over two to four orders of magnitude. The method was applied to model foods at allergen-relevant concentration levels and commercial insect food products. The experiments confirmed reliable qualitative detection across diverse matrices and at low inclusion levels, demonstrating performance comparable to real-time PCR methods. However, quantitative accuracy showed matrix-dependency and proved challenging at trace concentrations in heterogeneous food matrices. Several complementary development paths can be envisioned for the multiplexed assay. One priority will be expansion of the validation across a wider range of food matrices and processing conditions. This will help identify particularly amenable or challenging matrix composition for quantitative analysis. A second focus will be the replacement of marker peptides with low endogenous abundance, with the aim of improving sensitivity especially at trace concentrations. A third development path involves the establishment of a complementary targeted assay that omits the immunoenrichment and instead relies on an alternative sample preparation strategy. This has the potential to

generate a simplified, low-cost assay with reduced sensitivity, specifically adapted for food authentication in routine analytical laboratories.

In summary, this thesis substantially expanded the known proteomic landscape of edible insect species through a novel homology-based LC-MS/MS workflow applicable to species lacking reference genomes. It further established the first pan-insect marker peptide, providing a molecular basis for class-level detection with direct relevance to food allergy and authenticity assessment. Building on these findings, the first peptide-based, targeted IA-LC-MS/MS assay for the qualitative and quantitative analysis of insect-containing foods was developed, representing a significant methodological advance for insect authentication in food matrices.

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7 Tools and Resources

Parts of the analyses and visualizations presented in this dissertation were conducted using the Python programming language. Data processing and analysis were performed using the pandas and NumPy libraries, and figures were generated with Matplotlib.
ChatGPT (OpenAI) was used exclusively for language editing and stylistic refinement of parts of the dissertation and did not contribute to data generation, analysis, interpretation, or figure preparation. All scientific content, analyses, and interpretations are the sole responsibility of the author.

A Appendix I

A.1 Accepted Manuscript I

Meisinger, T., Planatscher, H., Braeuning, A., Ladenburger, E.-M., Stoll, D., Garino, C., Broll, H., Poetz, O. (2025). Proteomic insights into novel food insects: Homology-based proteome characterization and allergenicity considerations for EU-regulated insect species. Food Control, 177, 111441. https://doi.org/10.1016/j.foodcont.2025.111441

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Proteomic insights into novel food insects: Homology-based proteome characterization and allergenicity considerations for EU-regulated insect species

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Allergens

ABSTRACT

Insects have emerged as a sustainable alternative protein source and recently gained regulatory approval in the European Union as novel foods and animal feed ingredients. However, the limited availability of species-specific proteomic databases presents a significant challenge for the accurate identification of proteins in insect-based food products, relevant for food authenticity, safety and allergenicity concerns. In this study, we developed a homology-based proteomic workflow to address the lack of comprehensive sequence data for edible insect species and to explore their allergenic potential. This nanoflow tandem mass spectrometry method (nano-HPLC HR-MS) aims to be generally applicable to underexplored species. We identified between 1079 and 1893 proteins for each of the six investigated insect species *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Gryllodes sigillatus* and *Hermetia illucens*, increasing their numbers of known proteins up to 24-fold. Furthermore, we assessed the influence of error-tolerant peptide-spectra matching searches and database composition on protein identification and confirmed on average 90 % of selected peptides using targeted methods. We consistently detected known and putative allergens across all tested species, providing further evidence on their allergenic potential. Data from this study may provide the general basis for the development of targeted parallel reaction monitoring mass spectrometry-based assays for so far unsequenced species authentication and quantification of their proteins e.g. in novel foods. The data have been deposited to the ProteomeXchange with identifier PXD061237.

1. Introduction

In recent years, insects have emerged as a promising alternative protein source globally, with growing interest due to their potential contributions to food security, environmental sustainability, and nutritional diversity. The Food and Agriculture Organization (FAO) has highlighted the value of insect farming as a viable option to meet protein demands of a rapidly growing global population in an official document issued in 2013 (van Huis, 2013).

Following the adoption of the Novel Foods Regulation (EU Regulation 2015/2283), the European Commission has already authorized seven insect-based products from four species, i.e. *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, and *Alphitobius diaperinus*, for human consumption. Products consisting of or containing those insect species need to have a respective label to inform the consumer. Several additional insect species are considered novel food candidates. Complementary, in the feed sector eight insect species are approved in feed for swine, poultry and aquaculture (*A. domesticus, A. diaperinus, Gryllus assimilis, Gryllodes sigillatus, Hermetia illucens, Musca domestica*, *T. molitor* and *Bombyx mori*). As the industry develops further new products, the need for rigorous scientific research on the safety, nutritional quality, and allergenic potential of various insect species has become increasingly critical (Ribeiro et al., 2021; Rumpold & Schluter, 2013).

Due to their phylogenetic proximity, many insect proteins show a

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high degree of homology with those of crustaceans and mites, both known for their allergenic potential in sensitive individuals (Kamemura et al., 2019; Pali-Scholl et al., 2019). Sensitization to insect allergens primarily occurs through skin exposure and inhalation. The role of ingestion in sensitization remains underexplored, with ongoing investigations seeking to clarify its potential impact (Wangorsch et al., 2024). It is advisable for sensitive individuals to avoid consumption of insect products to minimize the risk of allergic reactions (Ribeiro et al., 2018). Identification of specific allergens in edible insect species is a crucial step to protect consumers, as allergens may persist through food processing or cooking (Paschke, 2009).

Unlike traditional livestock, the vast majority of insect species lack comprehensive genomic or proteomic sequence databases, creating significant obstacles for accurate species, protein or allergen characterization (Mei et al., 2022). The diversity of insect species and their rapid evolutionary divergence have resulted in significant differences in genomic sequences and, consequently, unique protein structures (Zhang et al., 2007). Traditional proteomic techniques that rely on comprehensive species-specific databases, such as bottom-up mass spectrometry, are therefore only partially applicable.

For this reason, mass spectrometric techniques that do not depend on proteomic databases have been the main research focus regarding insect species discrimination in the recent years. Ulrich and colleagues (Ulrich et al., 2017) applied matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) to characterize spectral fingerprints for identification of edible insect species. This method used whole insect protein extracts as opposed to single insect body parts, which has been the main protein source in previous works. Belghit and co-authors (Belghit et al., 2019) further enhanced species discrimination by direct comparison of tandem mass spectra employing shotgun proteomics. Additionally, spectral libraries for four insect species of interest for aquaculture were generated. Tata et al. (Tata et al., 2022) presented an alternative to proteomics based species identification by applying real-time high-resolution mass spectrometry (DART-HRMS) to insect powders. Distinct metabolic fingerprints were generated that can be applied to detect complete substitution of insect powders in quality control scenarios. Varunjikar and colleagues (Varunjikar et al., 2022) developed a first-of-its-kind analytical flow (AF) shotgun proteomics approach for the authentication of edible insect species. Their approach utilized a reference database comprising all reviewed arthropod sequences to identify proteins across five edible insect species. Additionally, a small curated list of allergens was applied to detect proteins with putative allergenic properties in their datasets. While effective for protein identification, their method focused on identification of black soldier fly specific proteins, attributed to the choice of reference database.

Considering the challenge of protein and allergen identification in non-model species, we propose two complementary proteomic strategies to investigate insect-derived food components. First, foundational proteomic research is essential to characterize the overall protein composition of edible insect species, enabling development of targeted analytical methods and thus the detection of general insect contamination in food products. Second, allergen-specific analyses based on curated protein sequence databases are needed to identify homologous allergens and assess potential cross-reactivity. Both approaches rely on data-dependent acquisition (DDA) mass spectrometry, which remains a powerful tool even in the absence of species-specific genomic or proteomic information.

To support these objectives, this study aimed to establish a robust homology-based proteomic workflow tailored to non-model insect species. Our method integrates nanoflow DDA proteomics with a custom arthropod-wide protein sequence database, error-tolerant peptide identification, and stringent data filtering. The workflow was applied to the insect species *T. molitor*, *H. illucens*, *L. migratoria*, *A. domesticus*, *G. sigillatus*, and *A. diaperinus*, resulting in the identification of numerous proteins absent from public protein sequence databases. Additionally, we assessed the influence of database composition and search strategies on peptide-spectrum matching, demonstrating the broad applicability of our workflow for both general proteomic characterization and targeted allergen detection in under-characterized species. Finally, by employing an extensive curated reference database of known allergenic proteins, we identified several putative insect allergens through sequence homology.

2. Materials and methods

2.1. Chemicals and sample material

The following chemicals were purchased from Carl Roth GmbH und Co. KG, Karlsruhe, Germany: ethanol (≥99.8 %), n-octyl-β-D-glucopyranosid (NOG, (≥98 %), triethanolamine hydrochloride (TEA, (≥99.5 %), and tris-(2-carboxyethyl)-phosphin hydrochlorid (TCEP, ≥98 %). Trypsin TPCK treated was purchased from CellSystems GmbH, Troisdorf, Germany. Acetic acid (0.1 mol/L), and iodoacetamide (IAA, ≥99 %) were purchased from Merck KGaA, Darmstadt, Germany. Phenylmethylsulfonyl fluoride protease inhibitor (PMSF, ≥99.0 %), and formic acid (FA, ≥99 %), were purchased from Life Technologies GmbH, Darmstadt, Germany. Water (HiPerSolv) and trifluoracetic acid (TFA, ≥99.9 %, HiPerSolv) were purchased from VWR International in Darmstadt, Germany. Acetonitrile (ACN, Chromasolv, ≥99.9 %) and water (Chromasolv) were purchased from WICOM Germany GmbH, Heppenheim, Germany.

Insect sample material was purchased from food manufacturer Catch-Your-Bug (Six-Feet-To-Eat), Neu-Ulm, Germany. Only *A. diaperinus* was provided by Snack-Insects, Witzeeze, Germany. *T. molitor*, *A. diaperinus* and *H. illucens* were provided as larvae, whereas *L. migratoria*, *A. domesticus* and *G. sigillatus* were provided as adult insects. All insects were reared in Europe. For *A. diaperinus*, feeding was discontinued 24 h before termination to allow for emptying of the digestive tract. Following killing by freezing, the larvae were blanched, washed, and subsequently dried. Insect sample material provided by Catch-Your-Bug was reared for four to six weeks before drying.

2.2. Sample preparation

Dried whole insects (5 g) were frozen at approx. −80 °C overnight and subsequently ground to powder with a batch mill (IKA Tube Mill control, IKA-Werke GmbH & Co. KG, Staufen, Germany). Milling was repeated three times (10 s, 25000 rpm) and insect powders were stored at approx. −80 °C until further processing.

2.3. Heterogenous phase digestion

Insect powder (15 mg) was added to TEA digestion buffer (620 μL, 100 mM) containing NOG (0.5 %). The suspension was heated (99 °C, 5 min) and cooled down to room temperature. Reduction reagent TCEP (10 μL, 315 mM, final concentration 5 mM) was added and proteins were reduced (21 °C, 5 min, 1000 rpm) on a shaker (ThermoMixer C, Eppendorf, Hamburg, Germany). Protein alkylation was performed by addition of IAA (10 μL, 10 mM, final concentration 5 mM) and subsequent incubation (21 °C, 20 min, 1000 rpm) on a shaker (ThermoMixer C, Eppendorf, Hamburg, Germany) in the dark. Proteolysis was allowed by addition of trypsin (100 μL, 5 mg/mL, final ratio of 1:40 trypsin to protein) and incubation (37 °C, 2 h, 1000 rpm) under agitation. The reaction was stopped by heat denaturation (99 °C, 5 min) and subsequent addition of trypsin inhibitor PMSF (10 μL, 75 mM, final concentration 1 mM). Remaining solid particles were removed by centrifugation (10 min, 16000 rcf) and supernatants were transferred to new reaction tubes. Sample supernatants were diluted 100-fold with formic acid (1 % v/v) and subjected to chromatographic separation.

2.4. LC separation and mass spectrometry

Peptide chromatography was performed using a nanoflow UHPLC system (Ultimate 3000, Thermo Fisher Scientific). Hundred-fold diluted sample supernatants were loaded (5 μL) onto an Acclaim PepMap100 C18 precolumn (0.3 mm i.d. x 5 mm, 5 μm, Thermo Fisher Scientific) for 2 min at a flow rate of 20 μL/min in LC-MS grade water containing 2 % ACN and 0.05 % trifluoracetic acid (TFA). Separation was performed on an Acclaim PepMap RSLC C18 (75 μm i.d. x 150 mm, 2 μm, Thermo Fisher Scientific). Two chromatographic gradients were used: both gradients ranging from 4 % to 55 % organic phase, a short one in 12 min and a long gradient in 92 min. A column temperature of 55 °C or 40 °C and a flow rate of 0.3 μl/min was set. The organic phase consisted of 80 % ACN, 20 % LC-MS grade water and additional 0.1 % FA. The aqueous phase consisted of 0.1 %FA in LC-MS grade water.

The UHPLC system was coupled to a QExactive Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) which was set to data-dependent acquisition (DDA) mode for an untargeted top 10 full MS/ddMS^2 acquisition. For the long gradient method, the full MS resolution was set to 70,000, the AGC target was 3e6, maximum injection time was 50 ms, and the scan range was set to 350–2000 m/z. Data-dependent MS^2 spectra were acquired with an AGC target of 1e5, a resolution of 17,500, a maximum injection time of 60 ms, a normalized collision energy of 27, an isolation window of precursor ions of 1.6 m/z, and dynamic exclusion of precursors was set to 50 s.

For the short gradient method, the full MS resolution was set to 70,000, the AGC target was 1e6, maximum injection time was 50 ms, and the scan range was set to 300–2000 m/z. Data-dependent MS^2 spectra were acquired with an AGC target of 5e4, a resolution of 17,500, a maximum injection time of 60 ms, a normalized collision energy of 27, an isolation window of precursor ions of 2 m/z, and dynamic exclusion of precursors was set to 5 s.

A selection of peptides was confirmed on the same instrument setup using the short chromatographic gradient and the mass spectrometer set to targeted parallel reaction monitoring (PRM) mode. The general settings were: polarity positive mode, dynamic retention time switched off, default charge state set to 2. The MS^2 settings were: one allowed microscan, resolution of 35,000, AGC target on 5e5 with a maximum injection time of 125 ms, an isolation window of 1.5 m/z, a loop count of 1, a normalized collision energy of 27. Spectra were recorded as profiled data.

2.5. Peptide spectrum matching and protein inference

Mass spectra of at least six independent analytical runs of each species were converted to Mascot generic format (*.mgf) and merged to a single species file using ProteoWizzard msConvert (Chambers et al., 2012). Files were submitted to MASCOT 2.8.3 (Perkins et al., 1999) with Mascot Daemon 2.8.0. Peptide identification was done with MASCOT 2.8.3 search engine against protein databases using the following settings: Fixed modification on Carbamidomethyl (C), variable modifications on Acetyl (N-term), Oxidation (M), monoisotopic, enzyme on trypsin with maximum one missed cleavage, peptide charges 1+, 2+, 3+, peptide mass tolerance 0.6 Da, target peptide-spectrum match (PSM) false discovery rate (FDR) of 5 %, MS/MS tolerance of 0.6 Da, target-decoy mode. For error-tolerant searches, option “error tolerant search” was additionally activated. To reduce search space in error-tolerant searches, only amino acid changes were allowed, while the search for PTMs was deactivated.

2.6. Databases

Several sequence databases were used for this study. An arthropod database was generated from UniProt UniRef based on taxonomy ID 6656 (*Arthropoda*), with 100 % identical sequences clustered (accessed on 11-Jun-2024). Retracted or outdated sequence clusters were excluded by removal of clusters containing exclusively UniParc derived entries (see script “fasta_remove_UniParc.py”). Arthropod sequence databases used to assess the impact of database composition were similarly created based on taxonomy ID 6656, excluding either taxonomy ID 343691 *H. illucens* or taxonomy ID 7067 *T. molitor* prior to sequence identity clustering. Allergen identification was performed based on the COMPARE database (version 02-Feb-2024) (van Ree et al., 2021).

2.7. Data processing

2.7.1. DDA protein results filtering

MASCOT search results were auto-exported as comma separated files (CSV) and processed with an in-house Python 3 script (see script “MASCOT-csv_clean.py”). In short, additional header information and unmatched queries were removed, emPAI values were added to each peptide of a protein, non-unique peptides were removed and remaining peptide entries were deduplicated. This was done for each investigated species individually.

2.7.2. Hierarchical clustering analysis

All unique peptides derived from each investigated species were further analyzed by hierarchical clustering. This was done with an in-house Python 3 script (see script “figure_combined-tree.py”) using the SciPy (version 1.10.1) package. A presence matrix was created based on the presence or absence of a specific peptide sequence in each species. For distance calculation, the Jaccard index was used (Jaccard, 1902). Linkages were calculated with a weighted approach.

Additionally, a hierarchical clustering based on raw spectra comparison was performed using compareMS2 2.0 (Marissen et al., 2023). The merged MGF files used for peptide-spectrum matching in MASCOT served as input, and the software was run with default parameters.

2.7.3. Verification of peptides by PRM-mass spectrometry

Fifty peptides were selected from each species to be subjected to targeted analysis methods for verification of the standard target-decoy DDA workflow. The only inclusion criterium for selection was sequence length between 7 and 22 amino acids. Further 110 peptides for *H. illucens* and 35 peptides for *G. sigillatus* were selected for verification of error-tolerant peptide results. Here, the inclusion criteria were: peptides were only identified in error-tolerant MASCOT search runs, and peptide length below 30 amino acids. Isolation lists were generated with Skyline 24.1.0.199 (Pino et al., 2020). Peptide results were loaded into Skyline, manually reviewed, and classified as confirmed following identification of a peak with at least two fragment ions.

2.7.4. Allergen candidate identification

Using an in-house Python 3 script (see script “allergen_80AA-validation.py”), putative allergens with more than one unique peptide were identified and grouped by species and protein accession number. Each group was screened for an 80 amino acid window containing at least two identified peptides. Only these groups were subjected to further analysis. For each selected group, sequence identity was calculated within the first 80 amino acid window, starting from the first amino acid of the initial peptide in the group. Groups with sequence identities of at least 35 % were considered as candidate allergens following the criteria set by Codex Alimentarius and EFSA (Codex Alimentarius, 2003; EFSA, 2010; EFSA et al., 2022).

3. Results and discussion

3.1. Development of the homology-based peptide and protein identification workflow

The ability of research to provide answers to some of the questions regarding the safety of edible insects is often hindered by the lack of knowledge of the proteome of many of the species utilized by the food

industry (Leni et al., 2020). This includes the search for species-specific peptide markers for food authenticity assays, allergen identification for food safety research or phylogenetic studies. The UniProt knowledge base contains only 171 entries for *A. domesticus*, 47 entries for *A. diaperinus*, 44 entries for *G. sigillatus* and 1580 entries for *L. migratoria*. Only *T. molitor*, with 15916 entries, and *H. illucens*, with 17602 entries, have a recently added draft proteome available (as of June 11, 2024). An overwhelming number of all these sequence entries are unreviewed, further showcasing the need for more data. Genomic sequencing of these species is challenging due to the potentially large size of the genomes, the high degree of polymorphism, and the lack of reference genomes (Richards & Murali, 2015). Therefore, we adopted an unconventional approach to broaden the proteomic knowledge of these species of interest for human consumption.

Instead of relying on underexplored species-specific protein sequence databases, we employed a homology-based approach for peptide identification after standard bottom-up proteomic experiments. Previous studies often relied solely on reviewed protein sequences to ensure annotation quality (Varunjikar et al., 2022; Łuciuk et al., 2025). However, such entries are scarce for many edible insect species. To overcome this, we also included unreviewed sequences, applying several filtering steps to manage database complexity. The complete UniRef database filtered for the phylum Arthropoda (taxonomy ID 6656) was clustered for 100 % identity to reduce database size and to remove ambiguous sequences. This further improves comparability of protein identifications. The phylum Arthropoda was chosen instead of the more restrictive class Insecta, as it includes sequences from Crustacea and Arachnida, two phylogenetic groups known for triggering allergic reactions (Li et al., 2018; Wang et al., 2020). In a second step, all clusters consisting of a single entry were removed if that entry was derived from UniParc and thus considered obsolete. The final clustered and filtered FASTA file consists of approximately 7.1 million entries. This database was subsequently used for peptide MS/MS fragment spectrum to sequence matching, which enabled protein inference in proteolyzed food insect samples. We identified, for each of the six species, between 1079 and 1893 proteins that contained at least one unique peptide (see Supplementary Table 1). Thus, the number of known proteins increased from 1 to 24 times for four of the six species of interest (Fig. 1). This result demonstrates the effectiveness of using homology-based sequence databases for proteomics research in under-explored target species and is in line with previous research (Cilia et al., 2011).

We believe that an even more complete proteome could be captured if all developmental stages, such as egg, larva, pupa, and adult, were comprehensively sampled. Different life stages are likely to express stage-specific proteins involved in development, structural changes, or metabolic adaptation (Chatterjee et al., 2024; Gao et al., 2022). However, in the present study, we focused on the life stages most relevant for food production, namely larval and adult stages, to reflect the material actually entering the food and feed chain (Muñoz-Seijas et al., 2024) and ensuring practical relevance of the dataset.

The number of inferred proteins did not appear to be dependent on the number of species-specific sequences in the database, as *T. molitor* and *H. illucens* yielded similar numbers of identified proteins compared to the four under-explored species. This indicates that our arthropod-wide database was sufficiently diverse to represent the protein landscape of our target species, which seem to have comparable proteome complexities. To maximize proteome coverage in this exploratory study, the false discovery rate (FDR) was set at 5 %. This decision was driven by the nature of the search space: the database encompasses a broad range of arthropod sequences, characterized by extensive internal homology and a limited number of species-specific entries. These features increase the number of potential matches and, consequently, increase the number of decoy hits, which in turn imposes a more stringent significance threshold. Applying a standard 1 % FDR under these conditions would have led to a substantial loss in sensitivity, thereby limiting the number of detectable proteins (Capriotti et al., 2013; Muth et al., 2015). Protein identifications were accepted based on at least one unique peptide, and selected targets were further validated using parallel reaction monitoring (PRM) to confirm specificity. The distribution of identified peptides per protein were similar for all six insect species (Fig. 2).

Notable outliers were the species *L. migratoria*, *T. molitor*, and *H. illucens*, which showed single proteins with over 40 identified unique peptides. For all three species, the protein with the highest number of unique peptides is the protein 'apolipophorin' (accessions Q9U943, A0A8J6LD42 and A0A7R8V9P1, respectively), with a size of approximately 400 kDa. Apolipophorins are the major component of

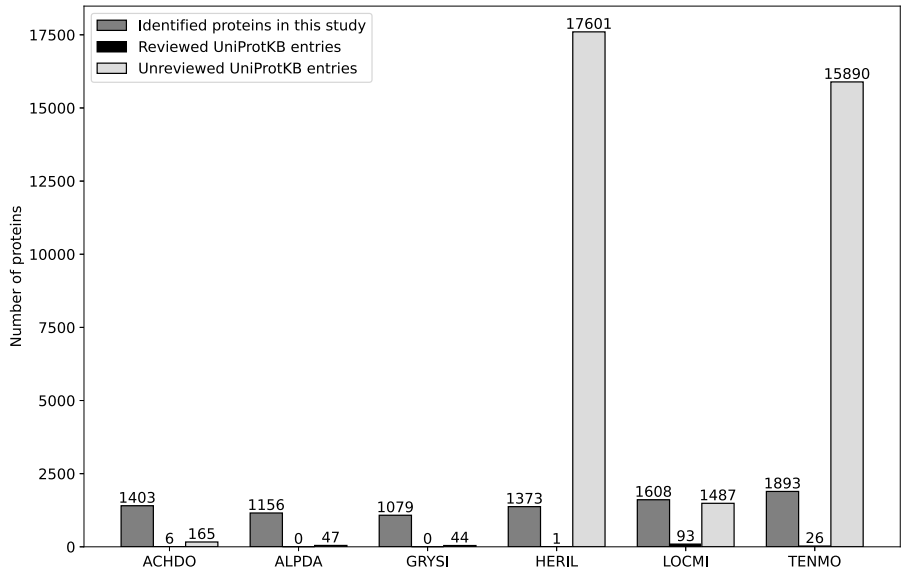

Fig. 1. Comparison of protein numbers identified across the investigated edible insect species with publicly available protein sequence entries in UniProtKB. Proteins in this study were identified using a homology-based approach with a comprehensive arthropod sequence database, revealing large numbers of previously unknown proteins. This contrasts with the limited protein entries available in UniProtKB (accessed June 11, 2024), particularly for species *A. domesticus* (ACHDO), *A. diaperinus* (ALPDA), *G. sigillatus* (GRYSI), and *L. migratoria* (LOCMI), where few entries exist. For *H. illucens* (HERIL) and *T. molitor* (TENMO), draft proteomes are available, resulting in large numbers of unreviewed protein entries in UniProtKB.

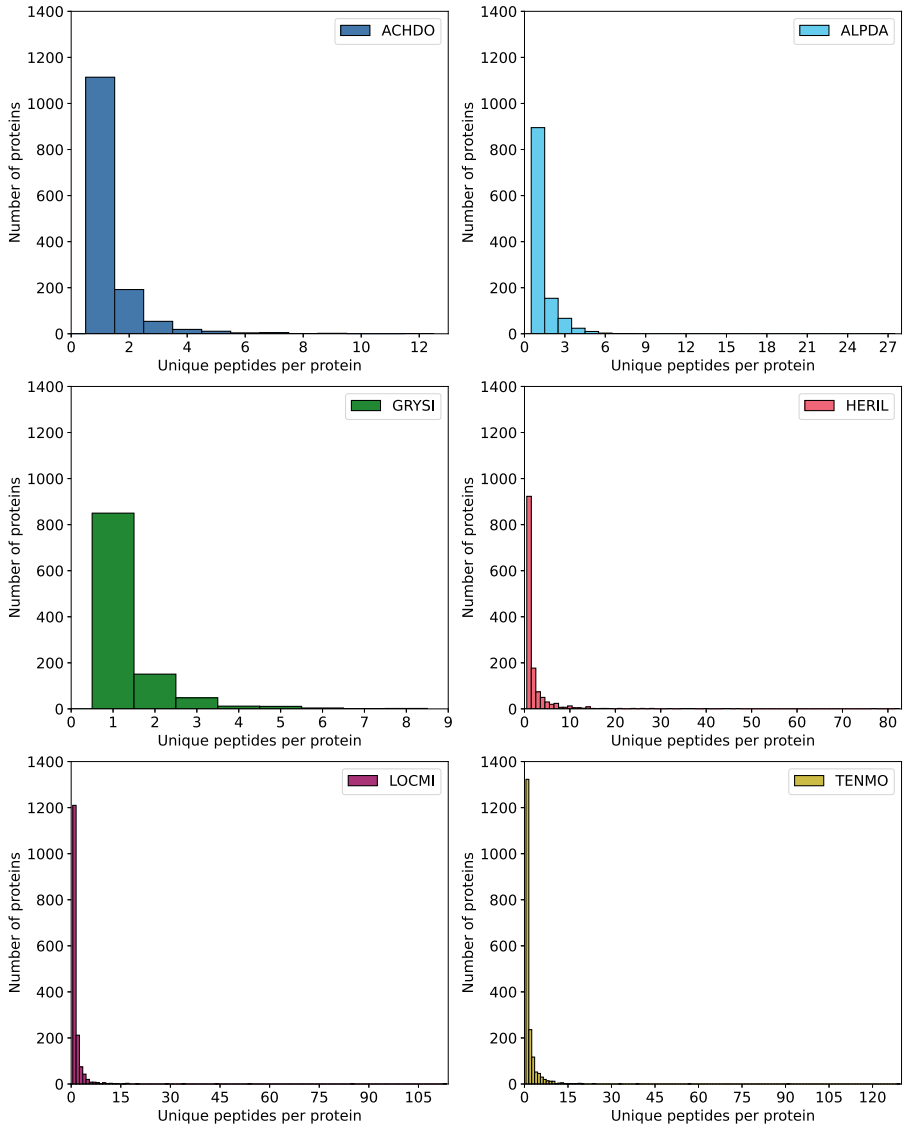

Fig. 2. Distribution of unique peptides per protein for each investigated species. Most of the identifications were based on one unique peptide. In L. *migratoria*, *T. molitor* and *H. illucens* several proteins each with more than 40 unique peptides were identified.

lipophorins, which transport various lipids in the hemolymph (Smolenaars et al., 2005). Protein sequence coverage is heavily dependent on the number of identified peptides and thus showed great variation within each species. On average, approx. 12 % sequence coverage was achieved, with minimum values of 0.1 % and maximum values of 97 % coverage. The highest mean sequence coverage was achieved in *H. illucens* with 15 %, the lowest in *A. domesticus* with 11 %. The exclusion of proteins displaying only one identified unique peptide notably increases these numbers to an average sequence coverage of 18 % for *A. domesticus* and 27 % for *H. illucens*. These values are broadly consistent with those reported in proteomic studies of more extensively annotated organisms. For instance, single-run LC-MS/MS analysis of the human HEK293 cell line yielded average sequence coverage of 18 % (Thakur et al., 2011). Considering the sparse proteome annotation for most insect species and the use of a homology-based arthropod sequence database, the coverage achieved in our study reflects a robust performance.

3.1.1. *Inter-species differences*

We compared proteins identifications for each insect species to further evaluate the quality of the dataset and to obtain detailed information about the distribution of common and different proteins in different insect species. Comparability of identified proteins is given by the design of our approach, as we used a clustered UniRef database with 100 % identity for peptide identification. Thus, a specific peptide will always map to the same protein accession number, regardless of the insect sample.

Comparing all six species, as visualized in Fig. 3A, only a small number of the identified proteins are common to all species (see Supplementary Table 2). This group consists mostly of different isoforms of actin, myosin, tubulin and histones. Notably, we also identified one protein, ectopic P granules protein 5 (EPG5)-like protein, possibly involved in a late step of the autophagy mechanism (Mei et al., 2023). It is reasonable to assume that these well-known proteins are evolutionary conserved in all six species examined. *H. illucens* (1222 unique proteins), *T. molitor* (1588 unique proteins) and *L. migratoria* (1315 unique proteins) showed the highest number of species-specific proteins in our study, namely proteins not identified in the other investigated species. These numbers correlate with the number of species-specific peptide sequences per protein in the database (Fig. 2) and confirms that a homology-based approach for peptide identification and protein inferring is less efficient for evolutionary distant sequences, as postulated before (Renard et al., 2012). A similar effect has been observed

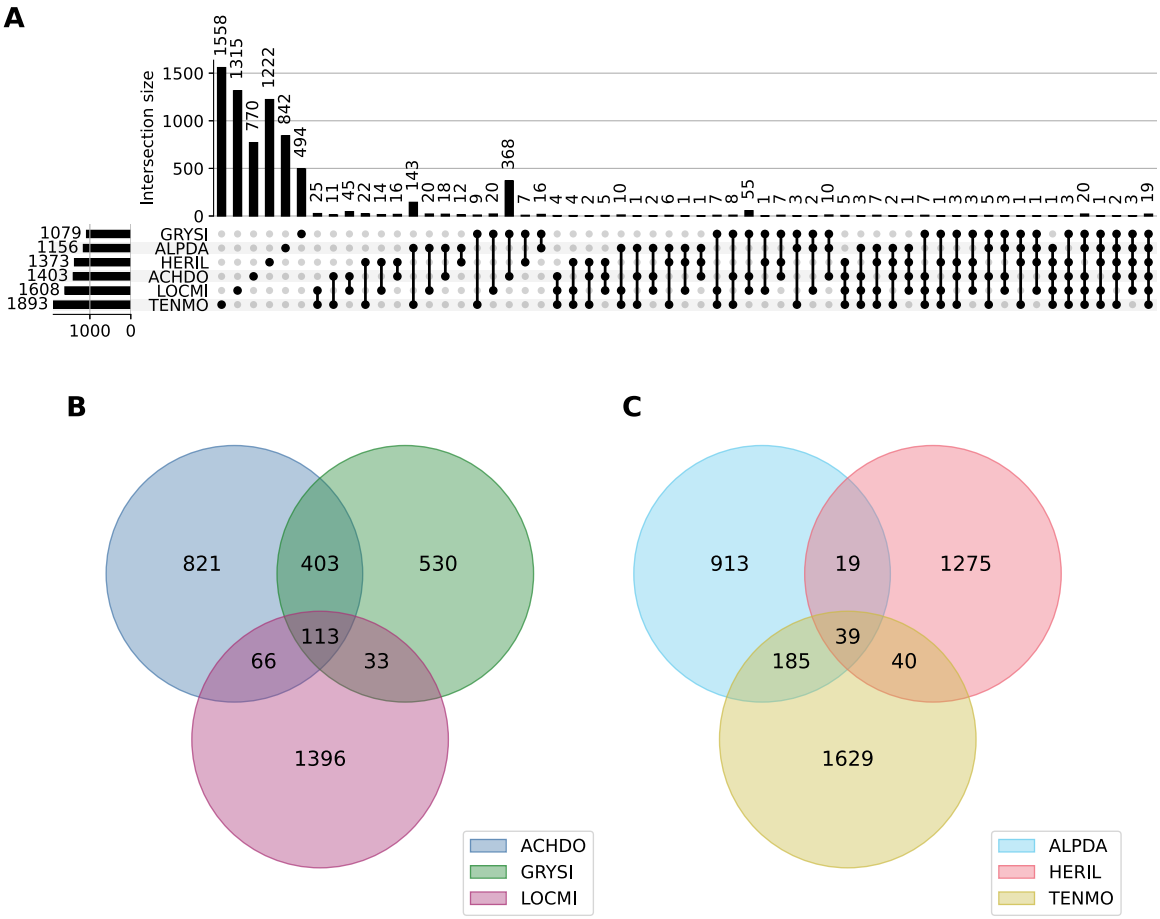

Fig. 3. Comparison of identified proteins across six edible insect species. (A) UpSet plot showing the number of unique and shared proteins among species. Only 19 proteins were common to all six. Each insect species exhibited a substantial number of unique proteins, supporting the development of species-specific PRM-based assays for food and feed samples. (B) Venn diagram comparing protein identifications among the three Orthoptera species, showing 133 shared proteins, indicating that phylogenetic relations are correctly represented in our data. (C) Venn diagram of *T. molitor*, *A. diaperinus* and *H. illucens*, showing 39 proteins shared among these three insect species.

independently in the studies by (Francis et al., 2020; Varunjikar et al., 2022): There, the utilized reference databases contained a great number of *Drosophila melanogaster* sequences, a species that belongs to the same order as *H. illucens*. In both studies, peptide identifications for *H. illucens* were overrepresented. However, in this study, even in the under-explored species *A. domesticus*, *A. diaperinus* and *G. sigillatus*, at least 494 specific proteins were identified. These proteins are valuable candidates for use as species-specific protein and peptide biomarkers for insect proteins in food and feed. Nevertheless, they require further confirmation by targeted LC-MS methods to determine their specificity in such assays (Supplementary Table 2). Therefore, this list offers a key reference for the development of PRM-based assays for the authentication and quantification of insect species, as recently demonstrated for the detection of processed animal proteins (PAP) in feed (Lecrenier et al., 2021; Steinhilber et al., 2018a, 2018b, 2019). Such assays would provide a more accurate and reproducible approach for identifying insect-derived products in various applications, including food quality control, regulatory compliance, and detection of potential adulterations.

Building on the comprehensive comparison of all six species presented in Fig. 3A, we next focused on subsets of phylogenetically related taxa to explore finer-scale patterns in protein composition (Fig. 3B and C). Specifically, we analyzed *A. domestica*, *G. sigillatus* and *L. migratoria* (Fig. 3B), all members of the order Orthoptera, with the two crickets further belonging to the subfamily Gryllinae. This phylogenetic proximity is also reflected in the number of identified proteins among these species (see Supplementary Table 3). The 33 proteins shared only between *G. sigillatus* and *L. migratoria* are mostly myosins, ribosomal proteins, twitchins, helicases, and clathrins. *A. domesticus* and *L. migratoria* share 66 different proteins, mostly arginine kinases, actin isoforms, receptors, and metabolic enzymes. Both crickets share 403 proteins, which vary greatly in function or cellular location. The 113 proteins common to all three species belong again to rather conserved protein families, namely actins, myosins, ATPases, elongation factors, or proteasomal subunits.

A similar analysis was carried out for the remaining three insect species. *A. diaperinus* and *T. molitor* belong to the same subfamily Tenebrioninae, while *H. illucens* is phylogenetically more distant, but all three belong to the same cohort Endopterygota, also known as Holometabola. Unlike members of the Orthoptera order, these insects undergo a complete metamorphosis, going through the life stages egg, larva, pupa, and adult (Rolff et al., 2019). Due to the limited levels of chitin, which is present to a greater extent in the exoskeleton of adults and is poorly digestible, the larval and pupal stages are the only ones used for food and feed purposes (Muñoz-Seijas et al., 2024). The evolutionary distance existing between the three species is directly proportional to the number of overlaps between the identified proteins (Fig. 3C): both mealworms share a great variety of proteins, while only the most conserved proteins are also shared with the black soldier fly (see Supplementary Table 4).

To validate the peptide identification pipeline and arthropod-wide

database, we applied two complementary quality control methods to assess whether proteomic similarity across species aligned with known evolutionary relationships, indirectly confirming identification reliability, database suitability, and data quality.

First, we used the Jaccard index (Jaccard, 1902), in combination with a weighted linkage clustering method, to quantify interspecies similarity based on the presence or absence of identified peptides (Fig. 4B). The Jaccard index is particularly well-suited to binary proteomics data, providing a simple and interpretable measure of peptide overlap between species. To independently validate these findings and control for potential biases introduced by database-dependent identification, we applied compareMS2 2.0 (Marissen et al., 2023), which directly compares MS/MS spectra without relying on peptide annotation. This method, previously applied by Varunjikar and colleagues in a similar study (Varunjikar et al., 2022), assesses similarity based on fragmentation patterns, thus offering an identification-free perspective on sample comparability and technical consistency.

Despite their methodological differences, the two clustering analyses largely reproduced the expected phylogenetic relationships (Fig. 4A) among the six insect species. Minor deviations from the expected pattern were observed between the two dendrograms: in the compareMS2 dendrogram (Fig. 4C), *A. diaperinus* was identified as the most distantly related species, whereas in the Jaccard dendrogram (Fig. 4B), this distinction was given to *H. illucens*. Nonetheless, the general consistency between both methods reinforces the robustness of the peptide identifications and supports the suitability of our analytical workflow for under-characterized insect species.

3.1.2. Experimental verification of selected proteins applying targeted proteomics

We employed DDA to identify a wide range of peptides across six edible insect species. To further verify our dataset, we randomly selected 50 peptides for each species, initially identified in DDA mode, and used PRM mode to verify their presence (Table 1 and Supplementary Table 5). Targeted methods, such as PRM, offer greater reproducibility and accuracy compared to DDA, making them more reliable for verification purposes (Peterson et al., 2012). Peptides were classified as verified, if a peak with at least two fragment ions could be reproducibly measured in two technical replicates. This was demonstrated for an exemplary selection of peptides in Fig. 5. On average, 90 % of the tested peptides were verified by PRM, with only little variation across the species. This indicates robust peptide identification with MASCOT, regardless of sequence background. MASCOT peptide identification based on matching spectra incorporates several numerical and statistical factors. The statistical nature of this method can introduce ambiguities, particularly when using less conservative search criteria (Chen et al., 2009). By verifying the presence of peptides through a less statistically dependent, targeted approach, PRM increases the confidence of the dataset. This finding aligns with recent studies that underscore the complementary strengths of discovery-based and targeted proteomics workflows (Ahmadi & Winter 2019; Goetze et al., 2024).

Table 1
Overview of the number of tested peptides derived from data dependent acquisition (DDA) datasets using parallel reaction monitoring (PRM) and percentage of PRM confirmed peptides.

Species	No. of PRM tested peptides	No. of PRM confirmed peptides	Percentage of confirmed peptides/ %
Acheta domesticus	50	46	92
Alphitobius diaperinus	50	47	94
Gryllodes sigillatus	50	45	90
Hermetia illucens	50	43	86
Locusta migratoria	50	44	88
Tenebrio molitor	50	45	90

3.1.3. Performance of error-tolerant search settings over standard target-decoy approaches

The utilization of an Arthropoda sequence database facilitated the identification of previously unknown peptides across all six species of interests. As designed, this approach prioritizes the identification of evolutionary conserved peptides, as confirmed by the results obtained so far. To expand the search space further, we implemented error-tolerant search settings for peptide identification. This approach employs a two-step search process: initially, a standard target-decoy search is conducted. Subsequently, all unmatched peptide spectra are subjected to a secondary search that iterates through an amino acid substitution matrix. In this way, plausible peptide sequences are *de novo* created,

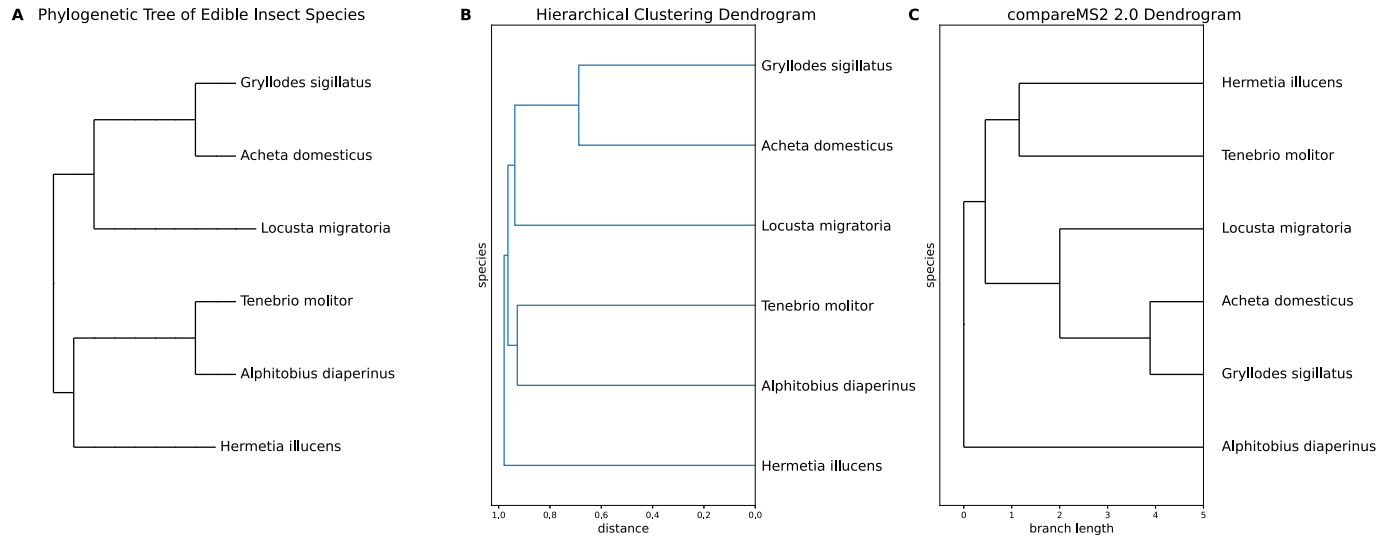

Fig. 4. Comparison of phylogeny and clustering. (A) Phylogenetic tree of the six investigated insect species, retrieved from NCBI Taxonomy (June 11, 2024). (B) Hierarchical clustering of identified peptides using the Jaccard index largely reproduced the expected phylogeny, with minor deviations such as the position of *H. illucens*. (C) Clustering based on MS/MS spectral similarity (compareMS2) confirmed these relationships, supporting data quality and identification reliability.

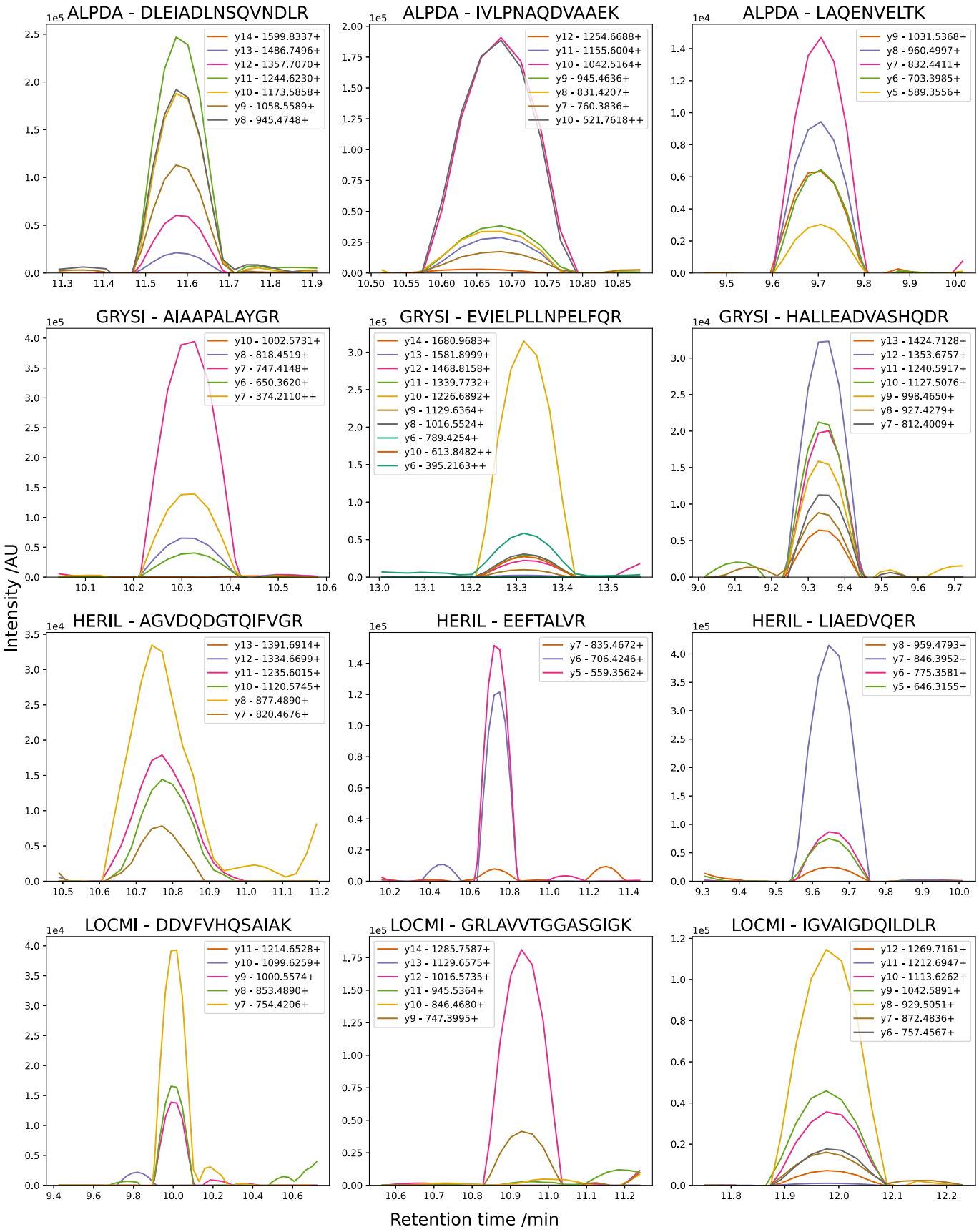

Fig. 5. Selection of PRM verified peptides. An exemplary selection of twelve PRM verified peptides from *A. diaperinus* (ALPDA), *G. sigillatus* (GRYSI), *H. illucens* (HERIL), and *L. migratoria* (LOCMI). Data was visualized using Skyline software. Peptides were classified as verified if a peptide with at least two fragment ions was identified.

mimicking the results of evolutionary processes not present in the database (Creasy & Cottrell, 2002).

With this approach, we identified between 93 and 1005 additional peptides, respectively, for *G. sigillatus* and *T. molitor* (Fig. 6 and Supplementary Table 6). A small subset of peptides unique to the standard search conditions was attributed to numerical instability of the peptide-spectra matching algorithm. The additional peptides correspond to a minimum of 25 proteins, additionally identified for *A. diaperinus*, and a maximum of 154 proteins, additionally identified for *T. molitor* (Supplementary Fig. 1). In case of *A. diaperinus*, the majority of inferred proteins belong to well-conserved protein groups, such as myosins, histones and ribosomal subunits.

However, this pattern was not observed in the data of the other insect species where a more diverse set of proteins, including regulatory factors, enzymes, odorant receptors, heat shock proteins and cuticle proteins, was identified. It appears that the number of identified error-tolerant peptides positively correlates with the total number of species-specific sequences in the database. *T. molitor* and *H. illucens*, both with a draft proteome available, as well as *L. migratoria* with more than 1000 protein sequences available, yielded the largest number of newly identified peptides. Error-tolerant search settings thus increased the total number of identified proteins from 2 % to 8 % without significantly altering false protein discovery rates compared to standard decoy searches. Protein false discovery rates were determined with at least two significant unique peptides per protein at 0.55 % for standard target decoy settings and 0.53 % for error-tolerant settings for *T. molitor*.

To assess the accuracy of peptides identified through the error-tolerant search (Fig. 6), we performed several verification steps. First, we analyzed the peptide length distribution and found that the majority ranged between 7 and 25 amino acids, consistent with typical tryptic peptide lengths (Swaney et al., 2010) (Fig. 7) and indicative of a plausible proteolytic origin. Second, manual inspection of representative sequences confirmed that most exhibited typical sequence composition without artifacts such as extended repeats. Finally, we selected an unbiased subset (Supplementary Table 7) of the error-tolerant peptides from *H. illucens* and *G. sigillatus* for targeted analysis using PRM (Barkovits et al., 2020). Both species represent opposite extremes in database coverage and number of identified error-tolerant peptides within our dataset. For *H. illucens* 110 peptides were tested, of which 77 % could be verified by PRM. We stratified the selection of tested peptides according to peptide length distribution as shown in Fig. 7: 75 peptides belong to the first bin of the histogram, and 35 peptides to the second. In *G. sigillatus,* 30 out of 35 error-tolerant peptides (86 %) were verified, with 10 from each of the first and third bin and 15 from the second. These results support the reliability of the error-tolerant identifications across species with varying database representation.

The increase in peptide and protein identifications comes at a high computational cost: the generation of error-tolerant peptide sequences through amino acid permutation substantially expands the search space (Renard et al., 2012). For *T. molitor*, a standard target-decoy search against the full Arthropoda database required approximately 8 GB of memory and 190 h of total computing time, as determined by htop software (Muhammad, 2004). In contrast, an error-tolerant target-decoy search on the same database required around 80 GB of memory and 390 h of total computing time, representing a tenfold increase in memory usage and a twofold increase in computing time. Given these requirements, the feasibility of this method for larger databases or more species of interest is limited by the significant computational resources required. For this reason, we recommend a two-step approach for similar research questions, starting with a standard target-decoy search and then moving to an error-tolerant setting only if adequate results are not obtained with the initial approach.

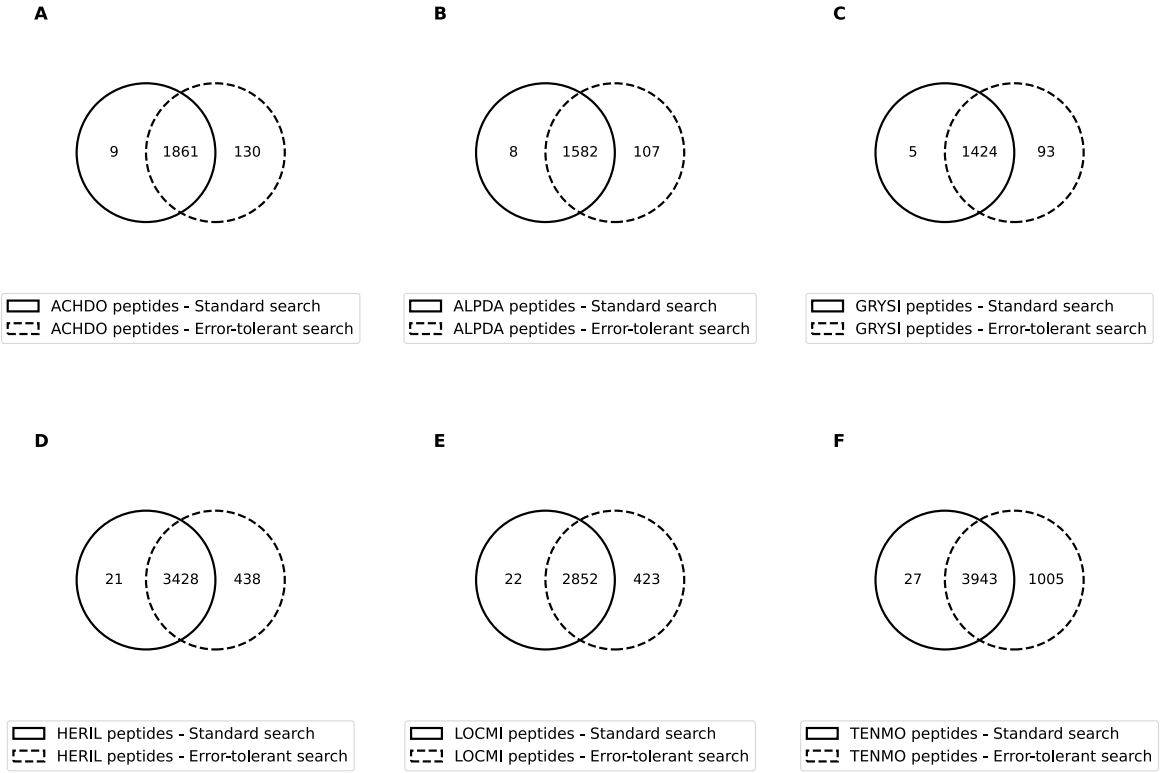

Fig. 6. Comparison of identified peptides between standard target-decoy and error-tolerant search settings. Venn diagrams show the overlap between peptides identified using the full arthropod homology database under standard target-decoy search conditions and those identified using error-tolerant search settings for each species: (A) *A. domesticus* (ACHDO), (B) *A. diaperinus* (ALPDA), (C) *G. sigillatus* (GRYSI), (D) *H. illucens* (HERIL), (E) *L. migratoria* (LOCMI), and (F) *T. molitor* (TENMO).

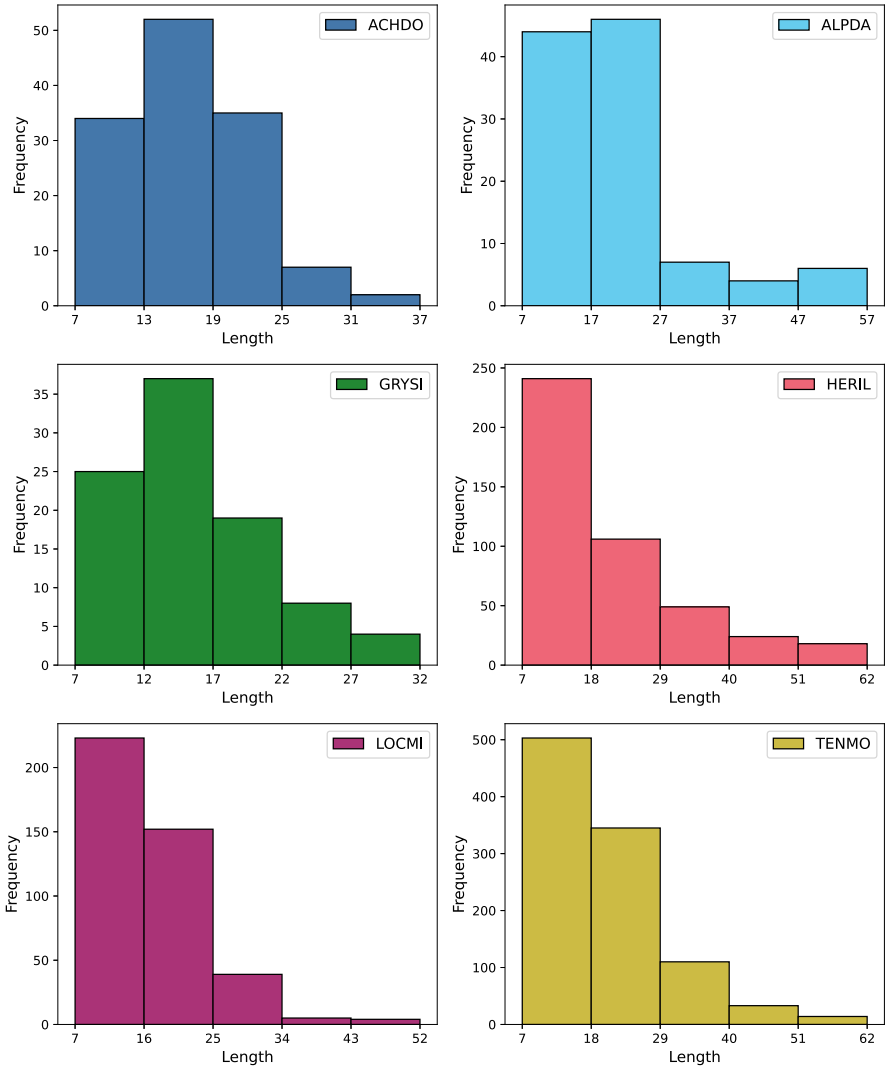

Fig. 7. Peptide length distribution of sequences identified using error-tolerant search settings. The majority of peptides fall within the expected tryptic range of 7–25 amino acids, supporting the plausibility of the identifications.

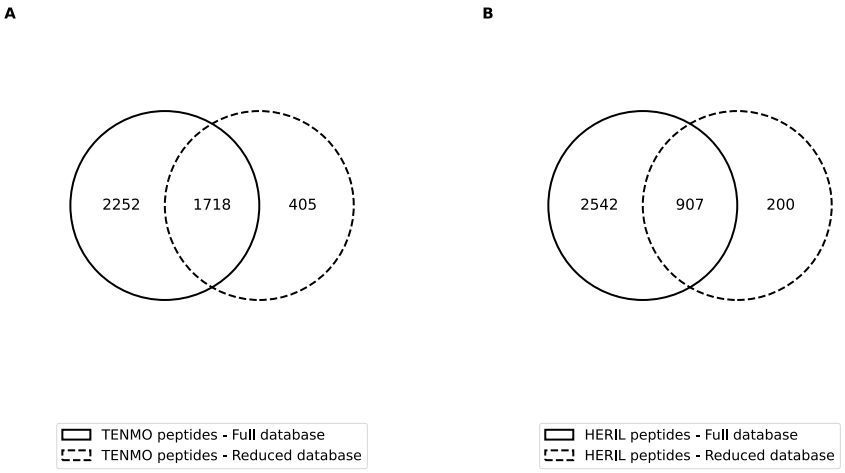

Fig. 8. Impact of database choice. Comparison of identified peptides in (A) *T. molitor* (TENMO) and (B) *H. illucens* (HERIL) using the full or the reduced protein database for MASCOT-based peptide searches. The full database comprises the clustered and filtered homology database constructed from all available arthropod sequences in UniProtKB. In contrast, the reduced database was generated by excluding target-specific protein sequences from UniProtKB prior to clustering and filtering, thus simulating conditions for species without a dedicated proteome.

3.2. Assessing the impact of database composition on protein identification

The accuracy of protein identification in mass spectrometry-based proteomics is highly influenced by the composition of the reference database used for peptide matching (Kumar et al., 2017). Different databases may contain varying levels of sequence coverage, taxonomic specificity, and annotation quality, all of which can impact the sensitivity and specificity of peptide identification. By comparing the results obtained through different database configurations, we aim to highlight potential biases introduced by database selection and their influence on downstream data interpretation.

Out of the six investigated edible insects, only two species, *T. molitor* and *H. illucens*, had publicly available draft proteomes when we did the searches. To assess the applicability of our homology approach on underexplored species, we prepared sequence databases consisting of all available arthropod sequences, excluding *H. illucens* or *T. molitor* sequences prior to identity clustering. This simulates the analysis conditions of species without dedicated proteome available. Using these databases, we reanalyzed our samples of *H. illucens* and *T. molitor* and compared the peptides identified with the two approaches (Fig. 8). A similar approach was previously used in human proteomics, where spectra from human samples were intentionally searched against a chicken database to simulate conditions without species-specific sequence information (Renard et al., 2012).

Applying the reduced database for *T. molitor* resulted in 47 % fewer peptides identified than with the full database (2123 vs. 3970 identified peptides). 19 % of the identified peptides were unique to the reduced database (405 peptides). *H. illucens* showed a less efficient identification, with 68 % fewer peptides identified with the reduced database compared to the full database (1107 vs. 3449 identified peptides). Here 18 % of the identified peptides were unique to the reduced database (200 peptides). The overlapping sections of the peptide identification in Fig. 8A and B represent homologous peptides present in the target species that can be identified by our generalized approach even when no protein sequence background is available. Consequently, we observed that 43 % of peptides, which were identified in *T. molitor* based on the species-specific proteome, were still detected using our homology-based approach. Similarly, for H. illucens, 26 % of the peptides expected from a species-specific analysis were identified through homology search. These findings align with previous work by Renard and colleagues (Renard et al., 2012), who reported approximately 15 % peptide recovery when human spectra were searched against a chicken database, simulating the absence of a species-specific reference despite a large evolutionary distance. This comparison shows that our approach can help to do proteomics identification based on mass spectrometry even if no protein sequence databases are available for the respective analyzed species.

The differences in peptide identification efficiency between the two species can be attributed to the number of available homologous sequences in the arthropod database. *H. illucens* belongs to the family Stratiomyidae (taxonomy ID 34687), which has only 976 sequences available in UniProtKB, that do not belong to this species (as of June 11, 2024). In contrast, the family Tenebrionidae (taxonomy ID 7065) has 70,864 sequences not belonging to *T. molitor*. This results in a larger search space for strongly homologous sequences of this species and consequently to an increase in the number of identified peptides. In a previous study (Belghit et al., 2019), similar results have been presented, further confirming that the rate of peptide identification correlates to the size of the utilized peptide sequence database.

Based on the hypothesis that the success of a homology-based approach correlates to the number of available sequences in a species' family, a corresponding model can be proposed. Applying the findings originally obtained for *H. illucens* and *T. molitor* to the under-explored species *A. diaperinus*, *L. migratoria*, *A. domesticus* and *G. sigillatus* allowed us to roughly estimate – via a linear extrapolation – the theoretical percentage of sequences identifiable using a homology-based approach against a dataset of curated proteomes. This estimation is based on the database of homologous arthropods described here (Table 2).

A direct comparison between a species-specific proteome used as reference database and the full database of homologous Arthropoda is not feasible due to the substantial difference in database sizes (Kumar et al., 2017). The proteomes of *H. illucens* and *T. molitor* contain approximately 15,000 entries, whereas the full Arthropoda database comprises around 7,000,000 entries. This difference in database size impacts peptide-spectrum matching during identification. Larger databases increase the search space, which can improve the chances of matching peptides from diverse species but also increases the likelihood of random matches, potentially raising the FDR. In contrast, smaller databases reduce the search space, allowing for more targeted and accurate identifications, but may miss peptides that are not present in the limited reference set (Muth et al., 2015). These size-related effects highlight the inherent trade-offs in database selection, where a more comprehensive homology-based approach increases coverage but also introduces additional statistical challenges in distinguishing true identifications from random matches.

3.3. Application to putative allergen discovery in edible insects

Insects are known to contain relevant allergenic proteins (Yang et al., 2024). The allergenic risk caused by ingestion is yet to be fully assessed, as wide-spread consumption within the EU is not given. Especially because of the limited knowledge of sequences in most insect species, an unbiased method of analysis such as the one presented in this study is preferred for initial risk assessment. The species-specific peptide spectra files generated for general peptide sequence identification were subjected to a database search against the full COMPARE dataset (van Ree et al., 2021). This database has been designed to meet the requirements of regulatory agencies for allergy safety assessment and contains more than 2700 peer-reviewed allergens. This enables the identification of putative allergens in the species of interest.

Through this approach, numerous peptides homologous to known allergens were identified in all investigated insect species. Peptides were assigned to allergen proteins and summarized in Table 3. A complete list of all identified proteins and peptides can be found in Supplementary Table 8. The number of putative allergens detected varied among the analyzed species, but in a range from 62 proteins for *A. diaperinus* to 106 proteins for *L. migratoria*. Identified peptides covered 0.5 %–100 % of assigned protein sequences. Protein sequence coverage is strongly

Table 2
Overview of the number of protein sequences available for each phylogenetic family that contains one species of interest (accessed June 11, 2024). The theoretical percentage of identifiable peptides using an arthropod homology database was extrapolated based on results from *H. illucens* and *T. molitor*.

Species	Phylogenetic family	Number of protein entries in UniProtKB for this phylogenetic family (excluding target species)	Estimated percentage of identifiable peptides using a homology approach vs. a curated proteome dataset
Acheta domesticus	Gryllidae	2618	26.7
Alphitobius diaperinus	Tenebrionidae	86,733	47.1
Gryllodes sigillatus	Gryllidae	2745	26.7
Locusta migratoria	Acrididae	10,717	28.7
Hermetia illucens	Stratiomyidae	976	26.3
Tenebrio molitor	Tenebrionidae	70,864	43.3

Table 3
Numbers of identified peptides from allergens, allergen proteins and protein sequence coverage information for these allergens in all six edible insect species.

Species	Identified peptides	Identified allergens	Average coverage/%	Coverage range/%
Acheta domesticus	200	91	22.9	1.1–100.0
Alphitobius diaperinus	99	62	30.1	0.5–100.0
Gryllodes sigillatus	139	68	25.2	1.1–100.0
Hermetia illucens	164	98	21.7	1.1–100.0
Locusta migratoria	174	106	28.0	1.1–100.0
Tenebrio molitor	133	72	21.4	0.8–100.0

influenced by the number of tryptic peptides found per protein.

Depending on the research question, different thresholds can be set to exclude low confidence protein hits. The number of unique peptide sequences used for protein identification is one such parameter (Fig. 9).

The majority of allergen proteins were identified based on one unique peptide only. This class of less reliable results can be attributed to the homologous sequence database, which does not include a relevant number of sequences specific to the target species. A selection of proteins with more than one unique peptide is shown in Table 4. The whole group consists overwhelmingly of highly conserved and abundant proteins, such as structural proteins tropomyosin and alpha-actinin. Good coverage of more than 25 % was given for most proteins of this group. Notably some allergenic plant (e.g. wheat, potato) proteins have been identified. Since whole insects were sampled, plant material could be derived from the insects GI tract (Riaz et al., 2023).

Tropomyosins are known food allergens found in crustaceans and insects. Their allergenicity could be lowered but not eliminated by food processing (van Broekhoven et al., 2016) and their diverse patterns of cross-reactivity against IgEs from crustacean sensitive patients has recently been discussed (Palmer et al., 2020). This cross-reactivity linked to the risk of symptom development in allergic patients was the reason why the European Commission imposed mandatory labeling for insects in food as suggested by the EFSA Scientific Committee (EFSA Scientific Committee, 2015).

In-depth comparison of the identified allergens between the species

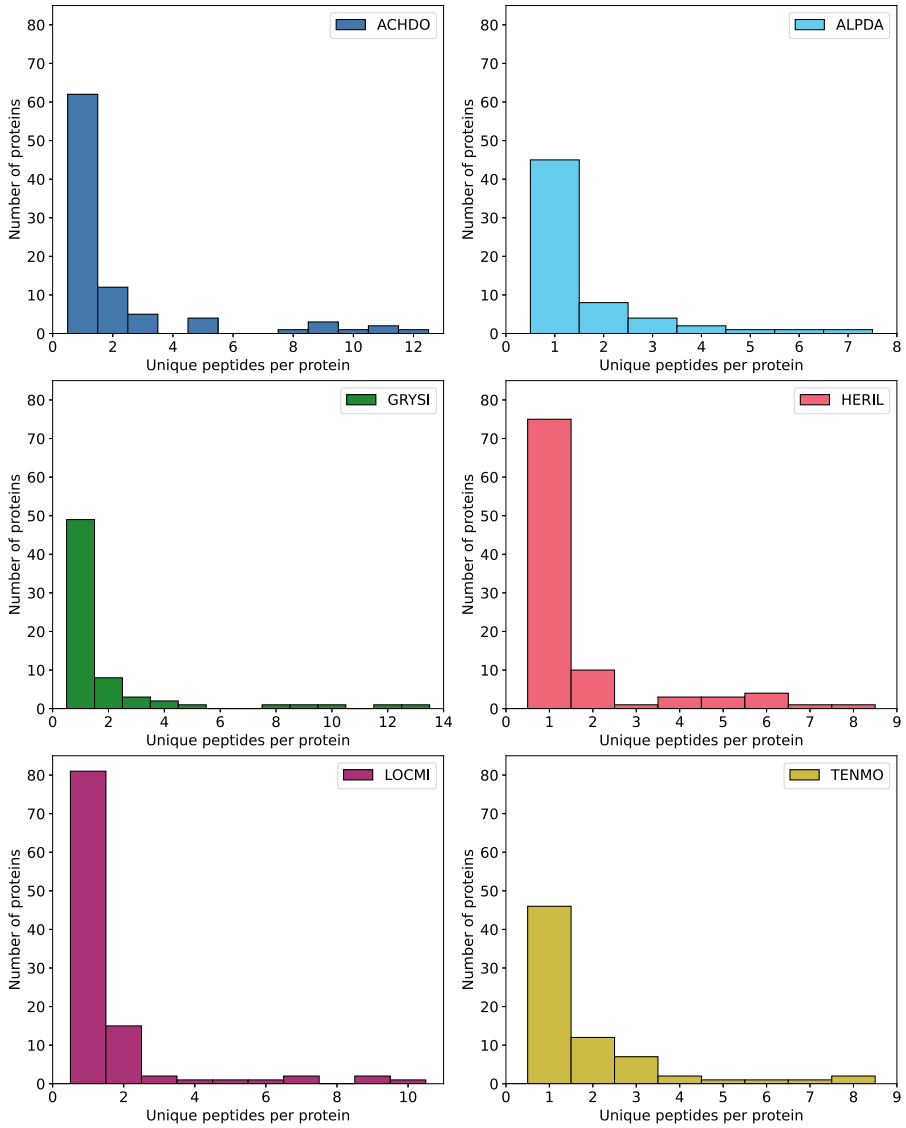

Fig. 9. Distribution of unique peptides per protein for each investigated insect species. Peptides were identified using the COMPARE reference database. A large number of allergen identifications was based on a single unique peptide.

Table 4
Selection of identified putative insect allergens with high numbers of identified unique peptides. High numbers of unique peptides increase the reliability of the allergen detection based on our PRM approach.

Species	Accession number	Protein description	Number of unique peptides
Acheta domesticus	ACM24358.1	Bla g 9; arginine kinase [*Blattella germanica*]	12
	BAA23361.2	Gly m 5; 7S globulin, vicilin, beta-conglycinin [*Glycine max*]	11
	UZC36340	Per a 17; alpha-tubulin [*Periplaneta americana*]	11
	QCI56568.1	tropomyosin [*A. domesticus*]	10
	AAA33966.1	Gly m 6; 11S globulin, glycinin, cupin [*G. max*]	9
	L7UZ85.1	alpha-actinin [*Dermatophagoides farina*]	9
Alphitobius diaperinus	UZC36340	Per a 17; alpha-tubulin [*P. americana*]	7
	COMPARE001	Bla g 9; arginine kinase [*B. germanica*]	6
	L7UZ85.1	alpha-actinin [*D. farina*]	5
	QBP14757.1	elongation factor [*D. farina*]	4
	UZC36337	Per a 14; enolase [*P. americana*]	4
	AVQ67919.1	Per a 13; glyceraldehyde-3-phosphate-dehydrogenase [*P. americana*]	3
Gryllodes sigillatus	ACM24358.1	Bla g 9; arginine kinase [*B. germanica*]	13
	QCI56568.1	tropomyosin [*A. domesticus*]	12
	UZC36340	Per a 17; alpha-tubulin [*P. americana*]	10
	QBP14757.1	elongation factor [*D. farina*]	9
	L7UZ85.1	alpha-actinin [*D. farina*]	8
	QFI57017.1	Scy p 9; filamin C [*Scylla paramamosain*]	5
Hermetia illucens	UZC36340	Per a 17; alpha-tubulin [*P. americana*]	8
	NP_001103782.1	Bomb m 3; tropomyosin [*Bombyx mori*]	7
	AGC56218.1	Der f 28; heat shock protein 70 [*D. farina*]	6
	L7UZ85.1	alpha-actinin [*D. farina*]	6
	QCI56572.1	tropomyosin [*Galleria mellonella*]	6
	URW11955.1	Pen m 14; glycogen phosphorylase [*Penaeus monodon*]	6
Locusta migratoria	L7UZ85.1	alpha-actinin [*D. farina*]	10
	URW11955.1	Pen m 14; glycogen phosphorylase [*P. monodon*]	9
	UZC36340	Per a 17; alpha-tubulin [*P. americana*]	9
	ACM24358.1	Bla g 9; arginine kinase [*B. germanica*]	7
	QBP14757.1	elongation factor [*D. farina*]	7
	QFI57017.1	Scy p 9; filamin C [*S. paramamosain*]	6
Tenebrio molitor	L7UZ85.1	alpha-actinin [*D. farina*]	8
	QBP14757.1	elongation factor [*D. farina*]	8
	UZC36340	Per a 17; alpha-tubulin [*P. americana*]	7
	COMPARE001	Bla g 9; arginine kinase [*B. germanica*]	6
	P20347.3	Sola t 3; cysteine protease inhibitor [*Solanum. tuberosum*]	5
	P16348.1	Sola t 2; aspartic protease inhibitor [*S. tuberosum*]	4

(Supplementary Table 9) showed sixteen proteins found in all six target species (Fig. 10A). The COMPARE database is not clustered for 100 % sequence identity, thus different isoforms of proteins or homolog proteins from different species will count as single proteins. The identified shared proteins were the highly conserved proteins actin, tropomyosin, glycogen phosphorylase, enolase, ATP synthase beta subunit, alpha-actinin, alpha-tubulin, filamin C, cytochrome C, glyceraldehyde-3-phosphate dehydrogenase and elongation factor. While tropomyosin is a well-established allergen in insects and crustaceans (De Marchi, Wangorsch, & Zoccatelli, 2021), glycogen phosphorylase has been reported as shrimp allergen (Wai et al., 2022), and glyceraldehyde-3-phosphate dehydrogenase has been described as unreported allergen in shrimp based on a transcriptomics study published in 2020 (Karnaneedi et al., 2020). This underscores the need for further allergenicity assessments of edible insects, particularly for individuals with pre-existing shellfish allergies. Beta-enolase has been reported as aeroallergen in the German cockroach, while a tubulin homolog has been identified in the storage mite (Chuang et al., 2010; Hindley et al., 2006; Jeong et al., 2005).

While a small subset of allergen homologues was conserved across all six insect species, most identified allergens were species-specific. This is partly due to the COMPARE database containing multiple isoforms of conserved proteins like actin or tropomyosin, which differ slightly in sequence but share the same annotation. However, the distinct presence or absence of allergen classes, even when considering only protein names and disregarding accession numbers, indicates genuine biological variation.

The black soldier fly (HERIL) exhibited 49 unique allergens not identified in the other five species. Multiple of these proteins are homologous to known allergens that were identified repeatedly in this species but only once or not at all in the other analyzed insect species. To our knowledge, these allergens have not been reported as insect allergens before and include glutelin, lipocalin, pathogenesis related protein or nitrile-specifier protein, suggesting unique allergenic hazards associated with this species (Supplementary Table 9).

In a similar fashion, the migratory locust (LOCMI) exhibited 46 unique allergens. Among these, three allergens stand out due to their novelty or their function: hexamerin, cyclophilin and expansin-like protein. Hexamerin was identified multiple times in this species, which validates our use of the COMPARE database, as hexamerin is an already known allergen from the migratory locust (Wang et al., 2022). Cyclophilin (UZC36341) is a pan-allergen commonly showing IgE sensitization in pollen allergic-patients (Fluckiger et al., 2002; Matricardi et al., 2024), that has been identified in insects only in a genome assembly of the American cockroach (Wang et al., 2023). We also identified an expansin-like protein (2103117A) as novel allergen in this species, that was previously identified in several plant types (Grobe et al., 1999), but not in insects.

Following the broader allergen comparison across all six species (Fig. 10A), we next examined patterns within phylogenetically defined subgroups. The orthopterans *A. domesticus*, *G. sigillatus* and *L. migratoria* represent one such closely related subgroup. Their evolutionary proximity is reflected in the number of identified shared allergens within this group, with 29 allergens identified across all three species, 15 shared allergens only between the Gryllidae, and 8 allergens only shared between each Gryllidae and the locust (Fig. 10B–Supplementary Table 10). In comparison with all six target insects, three unique allergens have been identified within the investigated orthopterans: serine protease (AAG44480.1), arginine kinase (ACM24358.1), and vicilin (QFG58557.1) (Fig. 10A–Supplementary Table 9). The COMPARE entry for arginine kinase is derived from the German cockroach and thus is only unique within our dataset and not for this phylogenetic group. Serine proteases have been described as potential allergens in insects before (Sudha et al., 2008), however vicilin has only been described as potential allergen in legumes and their allergenic potential in edible insects is unclear (Burks et al., 1995; Holzhauser et al., 2009).

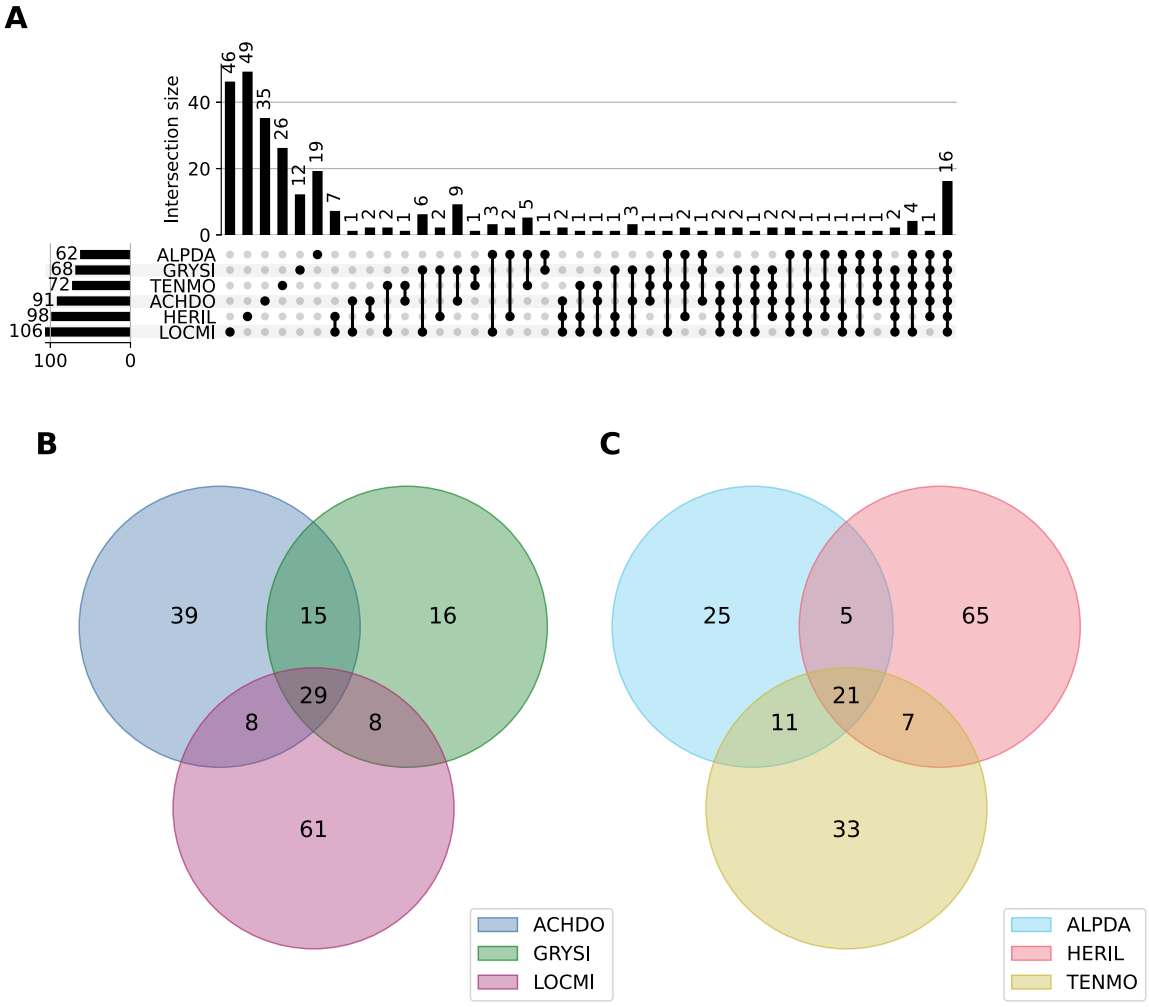

Fig. 10. Comparison of identified allergen proteins across the investigated edible insect species. (A) An UpSet plot showing the number of unique and shared allergens among all six species: *Acheta domesticus* (ACHDO), *Alphitobius diaperinus* (ALPDA), *Gryllodes sigillatus* (GRYSI), *Hermetia illucens* (HERIL), *Locusta migratoria* (LOCMI), and *Tenebrio molitor* (TENMO). (B) Venn diagram comparing identified allergens within the Orthoptera order, reflecting phylogenetic relations. (C) Venn diagram highlighting shared and unique allergens among the Tenebrioninae *Tenebrio molitor* and *Alphitobius diaperinus*, and *Hermetia illucens*.

The second subgroup in our study, consisting of the Tenebrioninae and the black soldier fly, showed similar results. Overall, 21 allergens were commonly shared and a stronger overlap was shown between the Tenebrioninae (ALPDA, TENMO) compared to *H. illucens* (Fig. 10C–Supplementary Table 11). In comparison with all six investigated species, two unique allergens were identified for this subgroup and further five unique allergens for the Tenebrioninae. Arginine kinase (ABB88514.1) and apolipophorin (SHD75397.1) were identified for this second subgroup with arginine kinase being derived from *Bombyx mori* which is thus not unique for this phylogenetic subgroup. Apolipophorin III has been reported as potential allergen in edible insects (Barre et al., 2021), however the here reported sequence is derived from the mite *Sarcoptes scabiei*, thus underscoring the potential of cross-reactivities. For the Tenebrioninae dipeptidyl peptidase (ACA00159.1), two tropomyosins (CAB38044.1, QCI56576.1), and two arginine kinases (COMPARE001, UXW65972.1) have been identified as unique. Most of these allergens have been described before in either insects or mites (Blank et al., 2010; De Marchi, Wangorsch, & Zoccatelli, 2021; Delfino et al., 2024). Noteworthy, the here identified dipeptidyl peptidase sequence is also known as Ves v 3 and typically exposed through injection (Blank et al., 2010).

With our initial approach, we identified putative allergens. To further refine these findings and reduce false positives, we applied more stringent criteria. The Codex Alimentarius recommended a default threshold of 35 % identity over at least 80 amino acids as a minimum requirement to predict cross-reactivity when assessing the risk of allergenicity in genetically modified plants and microorganisms (Codex Alimentarius, 2003; EFSA, 2010). This criterion was originally adopted by the European Food Safety Authority (EFSA) and applied to the allergenicity risk assessment of novel foods. However, in the latest guidance on the scientific requirements for an application for authorization of a novel food in the context of Regulation (EU) 2015/2283, the EFSA Panel on Nutrition, Novel Foods and Food Allergens acknowledged that this *in silico* approach is too sensitive. When it is used in isolation and is not supported by complementary experimental evidence it triggers a high number of false positive hits (EFSA et al., 2022). Importantly, these criteria were designed for regulatory decision-making in the context of risk assessment of GMO, and not for exploratory screening of potential allergens on the proteome level. We recognize that definitive classification of allergens requires further validation and needs to be followed up with more targeted analyses of the potential allergens to asses similarity with known antigens. Tryptic peptides have often average lengths of 8–12 amino acids (Swaney et al., 2010) and are thus too short to fulfill the 80 amino acids window criterion for allergens (Codex Alimentarius, 2003). Therefore, we considered only putative allergens with more than one identified unique peptide per allergen.

These peptides were also grouped according to investigated species and protein accession number. All groups that did not contain at least two peptides in a window of 80 amino acids were excluded from further analysis. For the remaining groups, sequence identity was calculated within an 80 amino acid window starting with the first amino acid of the first peptide of each group. With this approach we were able to identify seven to eighteen candidate allergens per species, most of which are listed in Table 4. A complete list can be found in Supplementary Table 12.

The identification of highly conserved allergens, such as tropomyosin and arginine kinase, across multiple edible insect species underscores their potential role as universal insect allergens. However, the variability in allergen identification and coverage, particularly in *H. illucens*, suggests that not all edible insect species present equal allergenic potential, as already previously suggested (Barre et al., 2021; Palmer et al., 2020). Finally, the potential influence of processing methods on allergen profiles warrants further investigation. Heat treatment, enzymatic hydrolysis, and other food processing techniques may alter protein structures, impacting their allergenicity (De Marchi, Mainente, et al., 2021). Integrating proteomic analyses with processing studies will provide a more complete understanding of allergenic risks in edible insects.

4. Conclusion

This study addresses a critical gap in the proteomic characterization of edible insect species by developing a homology-based workflow that enables the identification of proteins and allergens even in the absence of comprehensive species-specific databases. The proposed approach demonstrates that peptide-spectrum matching using broad arthropod databases can achieve high levels of protein inference across diverse insect species, thereby overcoming most of the limitations posed by incomplete genomic and proteomic data. Our findings reveal significant inter-species differences in proteome composition and identify several species-specific proteins that can serve as markers for food authenticity and allergen detection. Future research should focus on expanding the sequence databases, and validating species-specific peptide markers through targeted proteomic methods. Furthermore, confirmatory studies will elucidate if identified potential allergens are concerningly similar to known allergens. The workflow presented in this study is generally applicable to other incompletely sequenced species and lays a foundation for both the safety and traceability of insect-based food products in the evolving market for sustainable protein alternatives.

CRediT authorship contribution statement

Tobias Meisinger: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hannes Planatscher:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Albert Braeuning:** Writing – review & editing, Supervision, Methodology. **Eva-Maria Ladenburger:** Writing – review & editing, Resources. **Dieter Stoll:** Writing – review & editing, Resources. **Cristiano Garino:** Writing – review & editing, Project administration. **Hermann Broll:** Writing – review & editing, Project administration. **Oliver Poetz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifier PXD061237 and 10.6019/PXD061237.

The Python scripts used in this study are available on GitHub at https://github.com/t-meisinger/InsectProteomics-workflow.

All other data will be made available on request.

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Declaration of competing interest

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 paper.

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Abbreviations

ACHDO – *Acheta domesticus*
ALPDA – *Alphitobius diaperinus*
CSV – comma separated file
DDA – data-dependent acquisition
EFSA – European Food Safety Authority
EU – European Union
FAO – The Food and Agriculture Organization
FDR – false discovery rate
GRYSI – *Gryllodes sigillatus*
HERIL – *Hermetia illucens*
LOCMI – *Locusta migratoria*
PAP – processed animal protein
PRM – parallel reaction monitoring
PSM – peptide-spectrum match
TENMO – *Tenebrio molitor*

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111441.

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A.2 Supplementary Data

Originally published in **Meisinger, T.**, Planatscher, H., Braeuning, A., Ladenburger, E.-M., Stoll, D., Garino, C., Broll, H., Poetz, O. (2025). Proteomic insights into novel food insects: Homology-based proteome characterization and allergenicity considerations for EU-regulated insect species. Food Control, 177, 111441. https://doi.org/10.1016/j.foodcont.2025.111441.

The supplementary tables referenced are not reproduced here due to their large size. They are available from the original publication or upon request.

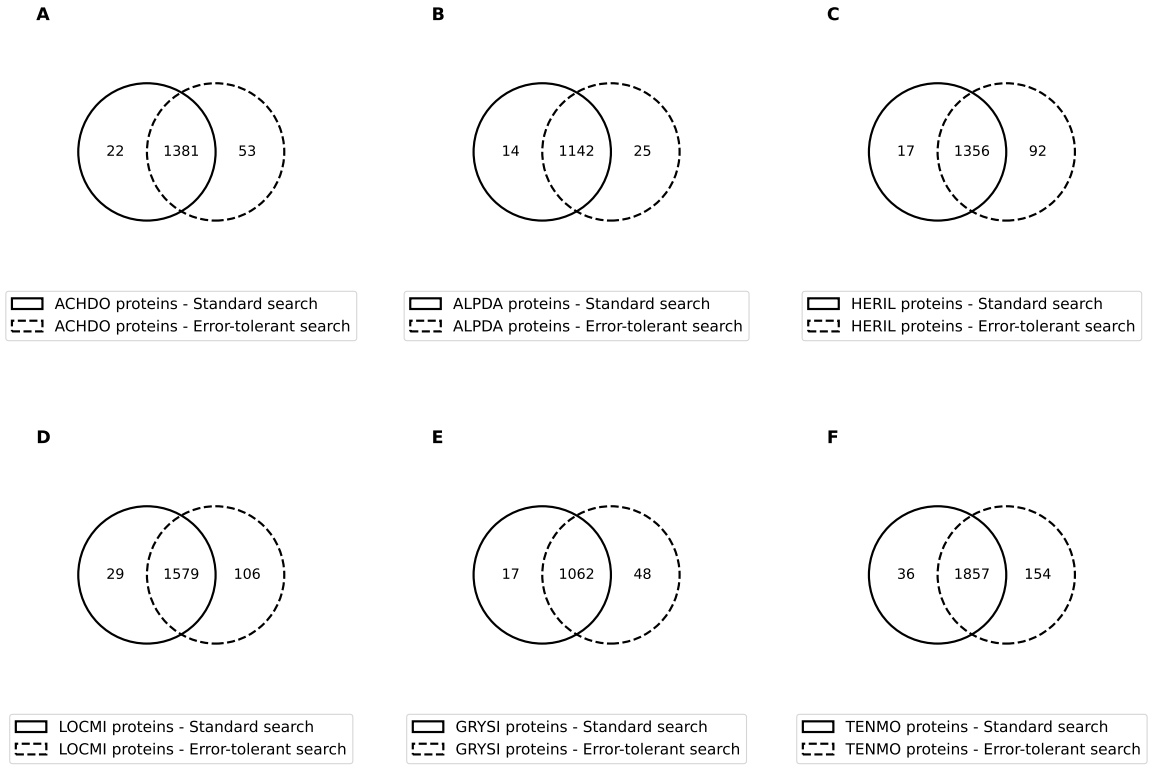

Figure A.1: Comparison of standard target decoy and error-tolerant search settings. Identified proteins using the full arthropod homology database and standard target-decoy search settings versus error-tolerant target-decoy search settings were compared in (A) *A. domesticus* (ACHDO), (B) *A. diaperinus* (ALPDA), (C) *G. sigillatus* (GRYSI), (D) *H. illucens* (HERIL), (E) *L. migratoria* (LOCMI), and (F) *T. molitor* (TENMO).

Supplementary Table 1:

Complete list of all identified unique peptides employing our arthropod reference database and standard target-decoy search settings. Column "sample_taxonomy" contains the species of the analyzed species. Column "prot_acc" contains the protein UniProt accession number. Column "pep_seq" contains the identified peptide sequence. Column "prot_name" contains a cleaned version of the protein description. Column "taxonomy" contains the origin species of the inferred reference protein.

Supplementary Table 2:

Proteins identified using our arthropod reference database and standard target-decoy search settings, grouped by their occurrence across the six investigated insect species. Each column represents a specific intersection in the UpSet plot (see Figure 3A), indicating shared or unique protein identifications.

Supplementary Table 3:

All proteins identified using our arthropod reference database and standard target-decoy search settings were grouped by their occurrence across the three Orthoptera species. Each column represents one intersection of the Venn diagram (see Figure 3B).

Supplementary Table 4:

All proteins identified employing our arthropod reference database and standard target-decoy search settings were grouped by their occurrence within the two Tenebrioninae species and H. illucens. Each column represents one intersection of the Venn diagram (see Figure 3C).

Supplementary Table 5:

Complete list of peptides derived from data dependent acquisition (DDA) datasets tested using parallel reaction monitoring. Column "species" described the occurrence of the peptide within the range of the target species. Column "prm_verification" describes the binary result of the PRM analysis; "0" equals not verified, "1" equals verified.

Supplementary Table 6:

Complete list of all identified unique peptides employing our arthropod reference database and error-tolerant target-decoy search settings. Column "sample_taxonomy" contains the species of the analyzed species. Column "prot_acc" contains the protein UniProt accession number. Column "pep_seq" contains the identified peptide sequence. Column "prot_-

name" contains a cleaned version of the protein description. Column "taxonomy" contains the origin species of the inferred reference protein.

Supplementary Table 7:

Complete list of peptides derived from error-tolerant search settings tested using parallel reaction monitoring. Column "species" described the occurrence of the peptide within the range of the target species. Column "prm_verification" describes the binary result of the PRM analysis; "0" equals not verified, "1" equals verified.

Supplementary Table 8:

Complete list of all identified unique peptides employing the full COMPARE reference database and standard target-decoy search settings. Column "sample_taxonomy" contains the species of the analyzed species. Column "prot_acc" contains the protein UniProt accession number. Column "pep_seq" contains the identified peptide sequence.

Supplementary Table 9:

All proteins identified employing the full COMPARE reference database and standard target-decoy search settings were grouped by their occurrence within all six investigated species. Each column represents one section of a Venn diagram (see Figure 10A).

Supplementary Table 10:

All proteins identified employing the full COMPARE reference database and standard target-decoy search settings were grouped by their occurrence within the three Orthoptera species. Each column represents one section of a Venn diagram (see Figure 10B).

Supplementary Table 11:

All proteins identified employing the full COMPARE reference database and standard target-decoy search settings were grouped by their occurrence within the two Tenebrioninae species and *H. illucens*. Each column represents one section of a Venn diagram (see Figure 10C).

Supplementary Table 12:

Complete list of all proteins identified with at least two unique peptides using the full COMPARE reference database and standard target-decoy search settings. The column "sample_taxonomy" contains the species of the analyzed sample. The column "prot_acc" contains the UniProt accession number of the identified protein. The column "pep_seq" contains the identified peptide sequence. The column "PeptidesTotalSize" indicates the

total number of amino acids covered within an 80-amino-acid window in the corresponding protein. The column "Identity" shows the percentage of sequence identity within this 80-amino-acid window. The column "allergen_status" classifies the protein as either "n/a" if the identity percentage is below 35 % or "putative allergen", and thus as an allergen candidate, if it is 35 % or higher.

B Appendix II

B.1 Accepted Manuscript II

Meisinger, T., Planatscher, H., Garino, C., Stoll, D., Ladenburger, E.-M., Braeuning, A., Broll, H., Poetz, O. (2026). From cricket to mealworm: 8-Plex mass spectrometry immunoassay for edible insect detection in novel foods. Microchemical Journal, 222, 117078. https://doi.org/10.1016/j.microc.2026.117078

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From cricket to mealworm: 8-Plex mass spectrometry immunoassay for edible insect detection in novel foods

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ABSTRACT

After adoption of the Novel Foods Regulation in the European Union, insects are gaining recognition as sustainable protein sources and potential novel allergens. We developed and partially validated a targeted immunoaffinity tandem mass spectrometric assay originally optimized for allergen-focused detection of insect proteins. Its high analytical sensitivity also supports applications in species authentication and quantitative assessment. The assay targets five edible insect species *Alphitobius diaperinus*, *Tenebrio molitor*, *Locusta migratoria*, *Gryllodes sigillatus*, *Hermetia illucens*, and a pan-insect marker based on a conserved tropomyosin peptide. The method demonstrated high accuracy (80% - 120%), precision (coefficient of variation <20%), selectivity, and sensitivity (limits of quantification: 6.17 fmol to 1500 fmol (>30–4500 ppm insect). Matrix and carryover effects observed for some analytes were mitigated by introduction of an additional wash step. Intra- and inter-assay reproducibility and calibration parallelism were confirmed for all analytes. The method was tested on commercial insect-based products and spiked model foods at allergen-relevant levels. Quantitative performance of the assays to determine the insect content in food varied by matrix. The insect allergens were detectable by the pan-insect marker tropomyosin down to 30 ppm and two species-specific markers down to 5 ppm but not consistently across all species-specific analytes. These results demonstrate the capability to detect insects at reasonable levels that might raise allergenic reactions. This assay offers a sensitive, specific approach for detecting and quantifying insect proteins in food.

1. Introduction

The increasing demand for sustainable protein sources has driven interest in the development of alternative protein sources, including those derived from insects. Insects offer a high-quality protein alternative with favorable environmental attributes compared to conventional livestock farming, including efficient feed conversion, low resource use, and reduced greenhouse gas emissions [1]. The Food and Agriculture Organization (FAO) emphasized the potential of edible insects in its 2013 report, highlighting their nutritional value and feasibility for industrial production [2]. This is in line with the goals of the Green Deal in the EU. Consequently, the European Commission, under the Novel Foods Regulation (EU Regulation 2015/2283), has so far authorized insect-based products from four species, i.e., *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, and *Alphitobius diaperinus*, for human consumption. Moreover, approval for two additional applications of products based on the insect species *Hermetia illucens* is pending. Regulatory progress has also been made in the animal feed sector, where eight species, i.e., *A. domesticus, A. diaperinus, Gryllus assimilis, Gryllodes sigillatus, H. illucens, Musca domestica*, *T. molitor,* and *Bombyx mori* have been approved in feeds for swine, poultry, and aquaculture. Despite regulatory advancements, comprehensive safety data and allergenicity

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risk assessments for edible insects remain limited, posing concerns due to their phylogenetic similarity to allergenic crustaceans and house dust mites. Several studies have reported allergenic risks associated with various insect species [3,4] and insect proteins homologous to allergens have been identified in proteomic studies [5,6]. However, the clinical relevance of insect allergens is so far unclear [7–9] as we do not know how often the IgE binding to highly-conserved pan-allergens like tropomyosin or arginine kinase might trigger cross-reactive allergic reactions in susceptible individuals, e.g. with crustacean allergy [3].

To ensure consumer safety and regulatory compliance, the development of reliable qualitative and quantitative methods is essential for accurately detecting and measuring insect-derived components in food and feed products and thereby indirectly assessing insect allergens. These efforts have been negatively impacted by the lack of reliable genomic or proteomic databases for the general majority of insect species, including species already authorized or pending [10]. Most available assays are based on polymerase chain reaction (PCR). For example, Koeppel and collaborators developed and validated the AllInsect tetraplex RT-PCR assay for unspecific detection off all insects and specific detection of *T. molitor*, *L. migratoria* and *A. domestica* down to 0.1% (*w/w*) in food samples [11]. As a proof of concept, Tramuta and colleagues developed a set of multiplex PCRs for detection of nine edible insect species in food using the 16S rRNA gene of mitochondrial DNA, achieving a limit of detection (LOD) of 0.01 ng/μL [12]. Garino and co-authors developed and validated a real-time PCR protocol for the detection of *A. domesticus* in aquaculture feed samples capable of detecting down to 0.1% (w/w) of house cricket in model feeds [13]. Similarly, the group developed and validated a real-time PCR assay for the detection of *A. diaperinus* shortly after its authorization in the EU. Here, they achieved a LOD in food ranging from 1 ppm to 20 ppm [14]. Despite the usefulness of DNA-based methods, they present notable limitations. Highly processed food and feed samples often contain degraded DNA, which can compromise the accuracy and reliability of these techniques [15]. Furthermore, DNA testing provides indirect information about the actual presence of proteins, and quantification is quite limited due to the potential unknown composition of the product to be analyzed and the lack of reference to be used for the quantitative determination [16].

To address these challenges, there is growing interest in protein-based assays as a complementary or alternative approach to DNA-based methods. Here, a mass spectrometer can be used for readout, since peptides enzymatically released from the protein of interest are generally more stable under harsh processing conditions than intact proteins or DNA. Thus, peptide-based quantification is offering improved robustness in detecting insect-derived ingredients in complex food and feed matrices.

In an earlier study, Belghit and colleagues showed that qualitative species discrimination of insect meals in aquafeed is possible. Due to lack of comprehensive proteomic sequence databases, they relied on direct comparison of tandem mass spectra [17]. This approach was improved by Leni and co-authors by using de novo sequencing, identifying specific marker peptides for *H. illucens* and *A. diaperinus* [18]. The known sequence space of edible insect species has been significantly enhanced by proteomic approaches in the recent years [5,6]. However, to our knowledge, no targeted quantitative peptide-based assay using multiple reaction monitoring (MRM) or parallel reaction monitoring (PRM) mass spectrometry has been developed to date to quantify insect proteins in food or feed, as has been established for other food allergens [19,20].

Immunoaffinity-liquid chromatography tandem mass spectrometry (IA-LC-MS/MS) assays represent an analytical strategy to improve PRM or MRM assays, particularly due to their high specificity and sensitivity in complex matrices [21]. These assays combine selective peptide enrichment with targeted quantification, making them well-suited for processed food and feed products. A relevant example is the work by Steinhilber and colleagues, who developed and validated IA-LC-MS/MS methods to quantify species-specific peptides from processed animal proteins (PAPs) in feed, demonstrating the applicability of this approach for regulatory monitoring and species authentication in heterogeneous sample matrices [22–25].

Here we describe the development of a multiplexed targeted IA-LC-MS/MS assay designed for species-specific detection and quantification of the five edible insect species *A. diaperinus*, *T. molitor*, *L. migratoria*, *G. sigillatus*, and *H. illucens* in food and feed samples. The assay includes a conserved peptide from tropomyosin as a pan-insect marker, enabling the detection of non-authorized insect species. Two of the target species are represented by two distinct species-specific analytes each. This 8-plex assay utilizes PRM MS analysis and stable isotope-labeled internal standard peptides for absolute quantification. The immunoenrichment step was incorporated to reduce matrix complexity and to increase assay specificity and sensitivity to enable species-detection and quantification at allergen-relevant concentrations. By selectively capturing target peptides, immunoenrichment minimizes co-eluting matrix components that could interfere with chromatographic separation or mass spectrometric detection, thereby improving signal-to-noise ratios and extending analytical column lifetime [21,26,27].

The method demonstrates good specificity and high sensitivity for several analytes, particularly the pan-insect marker while recognizing that the sensitivity required for comprehensive allergen monitoring is not consistently achieved across all species-specific targets. In application to spiked baked goods, the assay enabled qualitative detection of insects down to 5 ppm per species, demonstrating its potential for allergen monitoring at trace levels, although quantitative performance differed by analyte and matrix complexity. Analysis of commercial insect-containing foods further confirmed robust qualitative performance across diverse matrices, while highlighting the need for additional optimization of certain markers to ensure uniform performance.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and used without further purification. Ethanol, n-octyl-β-D-glucopyranosid (NOG), triethanolamine hydrochloride (TEA), 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and tris-(2-carboxyethyl)-phosphin hydrochlorid (TCEP) were purchased from Carl Roth GmbH und Co. KG (Karlsruhe, Germany). TPCK treated trypsin was obtained from CellSystems GmbH (Troisdorf, Germany). Acetic acid (0.1 mol/L), ammonium bicarbonate, and iodoacetamide (IAA) were obtained from Merck KGaA (Darmstadt, Germany). Phosphate-buffered saline was purchased from Fisher BioReagents (Schwerte, Germany). Formic acid (FA) and phenylmethylsulfonyl fluoride protease inhibitor (PMSF) were obtained from Life Technologies GmbH (Darmstadt, Germany). Deionized water and trifluoracetic acid (TFA) were purchased from VWR International (Darmstadt, Germany), Acetonitrile (ACN) and chromatography-grade water were supplied by WICOM (Heppenheim, Germany).

2.2. Sample preparation

Insect samples *T. molitor* and *H. illucens* were provided as larvae, while *L. migratoria*, and *G. sigillatus* were supplied as adult insects by the food manufacturer Catch-Your-Bug (Six-Feet-To-Eat, Neu-Ulm, Germany). *A. diaperinus* was provided as larvae by Snack Insects (Witzeeze, Germany). All insects were reared in Europe and supplied in dried form. Whole insects (5 g) were frozen at approx. -80 °C overnight and pulverized using a batch mill (IKA Tube Mill control, IKA-Werke GmbH & Co. KG, Staufen, Germany). The milling process was conducted in three cycles of 10 s each at 25000 rpm. The resulting insect powders were stored at approx. -80 °C until further use.

Litopenaeus vannamei was purchased frozen from a supermarket. The shrimp originated from Venezuelan pond cultivation and was used for assay specificity assessment. The intestinal tract of the shrimps was removed from one subgroup of the samples and both groups were lyophilized overnight. Dried shrimps were manually pulverized with a mortar and pestle.

Cookies were prepared as model food as part of the activities by the national project ALLERGEN-PRO (BLE 281A304A18) as described elsewhere [14]. In short, non-insect control cookies were utilized in the preparation of calibration curve matrix and quality control sample preparation. Incurred cookies with total insect contents of 30 ppm, 120 ppm, and 600 ppm were used for allergen level sensitivity tests. Cookie samples were manually pulverized with a mortar and pestle.

Quality control samples were prepared as mixture of proteolyzed non-insect control cookie (cookie surrogate matrix) and non-labeled synthetic peptides at three different concentration levels.

Commercial insect food samples, i.e., pasta ("Insekten-Pasta", 10% *A. diaperinus*), minced meat alternative ("Snack-Insects Hack", 20% *A. domesticus*), and snack bars ("Bug-Break", 10% *A. diaperinus*), were purchased from Snack Insects (Witzeeze, Germany). Crackers ("Protein crackers Tomato and Basil", 5% *T. molitor*) were obtained from Sustainable Food Products LDA (Perafita, Portugal). Commercial food samples were frozen at approx. -80 °C overnight, transferred to cryovials and homogenized by a ball mill (Sartorius, Goettingen, Germany) using 7 mm steel balls at 2000 rpm for 2 min.

Insect burger patties (50% *T. molitor*) were prepared as model food as part of a project of the German Federal Institute for Risk Assessment (BfR). In short, frozen *T. molitor* larvae were pre-minced and mixed with soy slices, methylcellulose, rapeseed oil, breadcrumbs, tomato paste, water, salt, and pepper in a pre-cooled bowl cutter. The mixture was shock-frozen, formed into 150 g patties, and frozen again at −40 °C. Patties were vacuum-sealed, stored at −18 °C, and prepared by pan-frying followed by brief convection oven finishing. Samples were stored at −80 °C until analysis.

2.3. Heterogenous phase digestion (HPD)

TEA digestion buffer (620 µL, 100 mM) containing NOG (0.5%) was added to the sample powder (20 mg). The suspension was denatured (99 °C, 5 min) and allowed to cool down to room temperature. Proteins were reduced under agitation (21 °C, 5 min, 1000 rpm, ThermoMixer C, Eppendorf, Hamburg, Germany) with TCEP (10 µL, 315 mM, final concentration 5 mM). For cysteine alkylation, IAA (10 µL, 10 mM, final concentration 5 mM) was added and the suspension was incubated with shaking (21 °C, 20 min, 1000 rpm). Proteolysis was carried out using TPCK-treated trypsin (100 µL, 5 mg/mL) at a final trypsin-to-protein ratio of 1:40, calculated based on the initial sample weight, under continuous agitation (37 °C, 16 h, 1000 rpm). The reaction was stopped by heat denaturation (99 °C, 5 min) followed by addition of the trypsin inhibitor PMSF (10 µL, 75 mM, final concentration 1 mM). The suspension was pelleted by centrifugation (10 min, 16000 rcf) and supernatants were transferred to new reaction tubes and frozen at −20 °C until further use.

2.4. Target peptide selection

For target peptide selection of *T. molitor*, *A. diaperinus*, *G. sigillatus*, and L. *migratoria*, the dataset previously reported by us [5], deposited at ProteomeXchange with identifier PXD061237, was mined for peptides unique to each species and for peptides common to all five species. For *H. illucens*, a draft proteome was available at the start of this study, thus this was used for identification of peptide candidates instead. Peptide selection was limited to sequences between 8 and 25 amino acids in length, excluding those containing methionine or cysteine due to analytical constraints. Candidate peptides were subjected to homology searches using NCBI BLASTp [28] against the non-redundant protein sequence database to evaluate their uniqueness. Only peptides that were either species-specific or near-specific, i.e., present in few, economically irrelevant species, were retained as species-specific markers. Conversely, peptides conserved across a broad range of insect species, including all five target species, and absent from non-insect taxa (outside NCBI taxonomy ID 50557), were considered for inclusion as pan-insect markers. The candidate list was further refined by excluding peptides derived from proteins not expected to be constitutively expressed.

All remaining candidate peptides were verified by mass spectrometry in targeted parallel reaction monitoring (PRM) data acquisition mode. The final selection of the target peptides was based on maximum signal intensities.

2.5. Immunoenrichment

An aliquot of the enzymatically fragmented sample (50 µL), equivalent to 1.55 mg of the original material, was incubated with antibody mixture (8 µg) for analyte enrichment. This mixture contained 1 µg each of polyclonal antibodies targeting proteotypic peptides derived from *A. diaperinus* larval cuticle protein A3A (LCPA3A) and hemocyanin C (HCC), *T. molitor* larval cuticle protein F1 (LCPF1), *H. illucens* cuticle protein (HICP), *L. migratoria* vitellogenin A (VTGA), *G. sigillatus* arginine kinase (AK) and spermatophylax protein 1C (SP1C), as well as a pan-insect tropomyosin (TPM), respectively. Antibodies were generated and tested as previously described by us [29]. In short, antibody binding properties were tested in peptide-binding experiments, rejecting low affinity binders. Immunoenrichment was conducted using a semi-automated workflow in 96-well format on a computerized plate washer (KingFisher Flex, Thermo Fisher Scientific). Stable isotope-labeled standard peptides ($^{13}C/^{15}N$-labeled, Intavis AG, Tuebingen, Germany) were added at a constant amount (25 fmol), and samples were incubated (1 h, room temperature) allowing formation of peptide-antibody complexes. These complexes were precipitated using protein G-coated magnetic microspheres (16 µL, Thermo Fisher Scientific), and washed twice with phosphate-buffered saline (100 µL, 0.03% CHAPS) and thrice with ammonium bicarbonate (100 µL, 50 mM, 0.03% CHAPS). Peptides were eluted using formic acid (20 µL, 1%).

2.6. Chromatography setup

Peptide separation was done on a nanoflow UHPLC system (Ultimate 3000, Thermo Fisher Scientific). The eluate (5 µL) was loaded on an Acclaim PepMap 100 NEO C18 precolumn (0.3 mm i.d. x 5 mm, 5 µm, Thermo Fisher Scientific) for 0.3 min at a flow rate of 150 µL/min of loading solution (2% ACN, 0.05% TFA in LC-MS grade deionized water). Peptides were then separated on a nano-C18 column (nanoEase *M/Z* BEH, 0.75 mm i.d. x 150 mm, 3.5 µm, Waters) using a two-step gradient: 20% to 30% solvent B (80% ACN, 0.1% FA in LC-MS grade deionized water) in 2.2 min, then 30% to 99% solvent B in 0.7 min at 1.5 µL/min and 55 °C, followed by a washing and equilibration step for 1.8 min. The aqueous solvent A consisted of 0.1% FA in LC-MS grade deionized water.

2.7. Mass spectrometry setup

Peptide detection was conducted using a QExactive Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ion source (EASY-Spray, Thermo Fisher Scientific). The instrument operated in PRM mode for targeted data acquisition with a resolution of 35,000, an AGC target of 2e5, a maximum injection time of 60 ms, an isolation window of 1.5 *m/z*, and a loop count of 1. Normalized collision energies (NCE) were optimized between 15 and 25 for all peptides and supplied with precursor m/z values by an inclusion list. Spectral multiplexing was set to 2 to allow simultaneous acquisition of light and heavy peptide pairs.

2.8. Data processing

Peptide isolation lists were generated with Skyline 24.1 [30]. Raw mass spectrometric data were imported into Skyline, manually reviewed, and exported for quantification using a custom Excel file. Depending on the characteristics of the calibration data, peptide quantification was performed using either a linear or a logistic fit model implemented with Excel's Solver module.

Unit conversion of analysis results in parts per million (ppm) was based on the estimated molecular weight of the target proteins. When full-length protein sequences were unavailable for a given species, molecular weights were approximated using homologous proteins from closely related species or by calculating the mean mass of several such proteins (Supplementary Table 1).

Insect content, expressed in % or ppm, was calculated by referencing analyte levels in pure insect material, which served as the 100% benchmark (Table 1).

3. Results and discussion

3.1. Insect species marker selection

Selection of appropriate marker species is a critical step in the development of targeted LC-MS/MS assays. Marker peptides should meet several key criteria, including being unique to the target species, proteotypic, and stable under processing conditions [10,21,31]. However, this selection process has been significantly constrained by the limited availability of annotated genomic and proteomic data for most edible insect species. As a result, prior studies have largely relied on direct spectral comparison, and proteomic or metabolic fingerprinting approaches for species identification instead [17,18,32].

In this study, we systematically analyzed the proteomic dataset published by Meisinger and co-workers [5], along with the publicly available draft proteome of *H. illucens*, to identify species-specific marker peptides for the species *A. diaperinus*, *A. domesticus*, *G. sigillatus*, *H. illucens*, *L. migratoria*, and *T. molitor*. We narrowed down the list of candidates by applying a list of analytical requirements, such as peptide length or amino acid composition. To assess species specificity, each marker candidate was analyzed using BLAST against the non-redundant database. Due to the limited genomic and proteomic data available for most insect species, absolute sequence uniqueness is currently unlikely to be verifiable [18]. Moreover, our dataset was generated using a homology-based approach, meaning the identified sequences are already known in at least one species. Unlike in well-characterized food-relevant species such as ruminants, where exhaustive proteomic data allow for true uniqueness assessments [23], the limited sequence databases for insects necessitate a more pragmatic approach. Therefore, marker candidates with only a few BLAST hits (100% sequence identity and 100% coverage), even when those hits corresponded to economically insignificant or taxonomically distant species, were still considered acceptable for targeted assay development. Candidates associated with low-abundance proteins were excluded to enhance detection reliability. Final marker peptides were verified using targeted PRM LC-MS/MS [33], confirming that each peptide generated a high-intensity signal that was specific for the respective species (see Supplemental Fig. 1A and Table 1). A particularly noteworthy result was achieved for *G. sigillatus* and *A. domesticus*, two species that are evolutionarily very closely related [34]. Differentiating between them based on peptide markers is especially difficult due to their high sequence similarity. Nevertheless, one *G. sigillatus*-specific peptide absent in *A. domesticus* was identified, although no *A. domesticus*-specific marker could be confirmed. The selected marker for L. *migratoria* was initially determined to be unique to this species based on a BLAST search conducted at the start of the assay development. However, a subsequent BLAST search revealed a new hit with *Escherichia coli*, which was not present in the earlier database version, highlighting the dynamic nature of public sequence repositories and the importance of revalidating markers periodically [35].

In addition to species-specific markers, we identified a conserved tropomyosin-derived peptide present in all six insect species analyzed. BLAST analysis indicated that this peptide is found nearly exclusively within the class Insecta. Hence, we included this peptide to the assay panel as a class-level identifier for insect-based products. Tropomyosin has been widely discussed in the literature as pan-allergen among arthropods, including insects [7,36–39]. Its relevance in allergenicity evaluations, the high degree of sequence conservation across insect taxa

Table 1
Final list of marker peptides selected for targeted LC-MS/MS analysis. All peptides were selected based on analytical properties, species specificity, and detection performance in PRM analysis. Specificity was evaluated using BLAST. Limits of quantification for all proteins, based on multiple accuracy and precision analyses ($n = 12$) and evaluation of the mean ratio of non-labeled to labeled peptides. Mean analyte amounts for each species ($n = 6$) were determined from pure insect samples, and the assay range was calculated in insect ppm according to the previously established analytical range of each analyte.

Target species	Peptide	Protein	BLAST-derived uniqueness	Analytical range in fmol peptide (n = 12)	Mean amount of analyte protein in pure insect in fmol (n = 6)	Theoretical analytical range in ppm insect
A. diaperinus	DGDVVHGSYSLTDPDGTR	Larval cuticle protein A3A (LCPA3A)	one 100% hit in *Tribolium madens*	6.17–1500	147,000	$42.0–1.02 \cdot 10^4$
A. diaperinus	ISIPPFGEILELER	Hemocyanin C (HCC)	two 100% hits in *Zophobas morio* and *Asbolus verrucocsus*	6.17–1500	119,000	$52.0–1.27 \cdot 10^4$
T. molitor	SLYGGYGSGLGIAR	Larval cuticle protein F1 (LCPF1)	unique for *T. molitor*	2.06–1500	90.7	$2.27 \cdot 10^4–1.65 \cdot 10^7$
H. illucens	GSYSYNDGFFK	Cuticle protein (HICP)	unique for *H. illucens*	0.69–1500	1130	$609–1.32 \cdot 10^6$
L. migratoria	DVSPTELEYFEK	Vitellogenin A (VTGA)	one 100% hit in *E. coli*	2.06–1500	41,600	$49.5–3.60 \cdot 10^4$
G. sigillatus	VSSTLSGLSAELK	Arginine kinase (AK)	one 100% hit in *Stigmatomma* sp. *TH05*	2.06–1500	532	$3.88 \cdot 10^3–2.81 \cdot 10^6$
G. sigillatus	ASDVADTVLGATGSK	Spermatophylax protein 1C (SP1C)	unique for *G. sigillatus*	0.69–1500	22,100	$31.2–6.78 \cdot 10^4$
Insecta	LAFVEDELEVAEDR	Tropomyosin (TPM)	excluding insects: one 100% hit in *Shewanella electrica*	2.06–1500	–	–
A. diaperinus	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	68,400	$30.1–2.19 \cdot 10^4$
T. molitor	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	43,700	$47.2–3.44 \cdot 10^4$
H. illucens	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	12,500	$165–1.20 \cdot 10^5$
L. migratoria	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	38,200	$54.0–3.93 \cdot 10^4$
A. domesticus	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	48,500	$42.5–3.09 \cdot 10^4$
G. sigillatus	LAFVEDELEVAEDR	Tropomyosin (TPM)	–	2.06–1500	53,300	$38.7–2.81 \cdot 10^4$

and its structural and functional importance as an actin-binding protein [3,40,41] make tropomyosin a robust molecular marker for taxonomic discrimination.

The here identified species-specific marker peptides differ from those previously reported in literature, as our selection was based on a comprehensive proteomic dataset [5]. In addition, several peptides previously proposed by Leni and colleagues for *H. illucens* and *A. diaperinus* were re-evaluated using our strict BLASTp verification workflow [18]. However, most of the tested candidates were not unique to their respective species or showed numerous matches to bacterial sequences, rendering them unsuitable for robust species discrimination within the framework of our assay. We attributed this lack of specificity largely to the limited sequence information available at the time those studies were conducted, which prevented verification against the broader sequence diversity now present. Our selected peptides act as proxies for species-specific protein material rather than as direct indicators of intact allergen proteins. While some identified markers originate from known allergens, to our knowledge, many do not contain recognized allergenic domains and are not allergenic themselves.

3.2. Optimization of analytical settings

To achieve highly sensitive quantification of the marker peptides, the analytical instrument settings were optimized using both isotope-labeled and unlabeled synthetic peptides. Collision energies were optimized to maximize signal intensity for each analyte (Supplemental Fig. 1B and Supplemental Table 1). In addition, multiple chromatographic gradients were evaluated during method development, resulting in near baseline peak separation for all analytes while maintaining a short cycle time of six minutes (Supplemental Fig. 2).

3.3. Sample preparation optimization

Insect meal and food samples were homogenized using either a mortar and pestle or a ball mill. Protein extraction and proteolysis were carried out in a single step, following a previously published protocol [24]. This one-step method reduced complexity, hands-on time and cost in comparison to other published protocols by omitting defatting, dehydration and protein extraction steps [42–44]. Sample clean-up is facilitated by subsequent semi-automated immunoenrichment. Several parameters of the heterogeneous phase digestion (HPD) were optimized

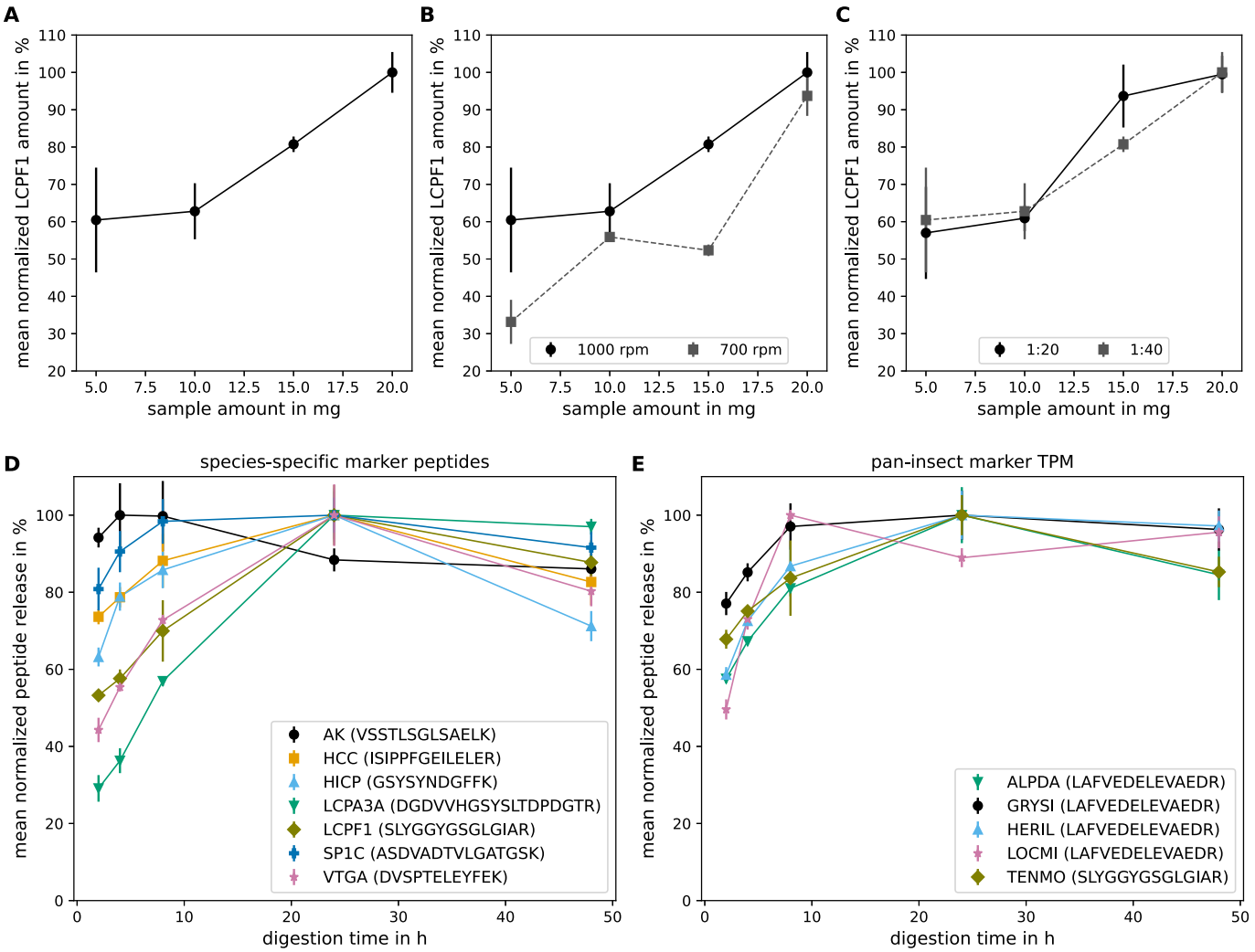

Fig. 1. Sample preparation optimization (n = 3) and time-course analysis (n = 3) during heterogeneous phase digestion (HPD). Error bars represent standard deviation in percent. Data were normalized to the maximum. Sample preparation optimization of peptide release was performed with LCPF1 as representative species marker. The effects of sample amount (A), shaking speed during all incubation steps (B), and the trypsin-to-protein ratio (C) on peptide release were tested. Increased peptide yield was observed with higher sample amounts and shaking speeds, while no significant difference was noted between trypsin-to-protein ratios of 1:20 and 1:40. (E) Release of seven species-specific marker peptides across different proteolysis times. Most peptides reached maximal release after 24 h. (D) Release profile of the pan-insect marker tropomyosin (TPM) across five insect species. Peptide release peaked at 24 h for all species except L. *migratoria*.

for application to insect-derived samples. Specifically, we evaluated the effects of sample amount, shaking speed during incubation, and the trypsin-to-protein ratio exemplary on LCPF1 release ($n = 3$). Analyte release was measured using PRM after immunoenrichment and increased with higher sample amounts, although processing was limited to a maximum of 20 mg due to practical handling constraints (Fig. 1A). From this, a sample volume corresponding to 1.3 mg original sample material could be used for analysis, due to immunoenrichment-dependent matrix depletion [21]. Higher shaking speeds during incubation correlated with increased analyte release (Fig. 1B). In contrast, no significant difference in analyte release was observed between trypsin-to-protein ratios of 1:20 and 1:40 (Fig. 1C).

Tryptic proteolysis is highly influenced by digestion time [45,46], which we evaluated by a time dependent analysis of peptide concentrations in insect samples (Fig. 1, n = 3). Six of the seven species-specific marker peptides followed a sigmoid curve, with maximum peptide release observed after 24 h. The most pronounced increase in peptide release occurred within the first ten hours of incubation. An exception was observed for arginine kinase, the species-specific marker for *G. sigillatus*, which showed peak peptide release at 4 h, followed by a decline beginning after eight hours (Fig. 1E). The pan-insect marker tropomyosin was also analyzed across all five species. In all cases except L. *migratoria*, peptide release peaked at 24 h. For L. *migratoria*, the maximum was reached after eight hours of proteolysis (Fig. 1D). To ensure efficient peptide release for all multiplexed analytes, a compromise needed to be made, hence an incubation time of 16 h was selected. This ensured near optimal peptide release for all analytes that peaked at 24 h, while retaining most of L. *migratoria* and AK peptide intensities.

This time-course analysis also served to assess the robustness of the HPD protocol across different insect sample types, and no protocol-related issues were observed. Based on these results, an incubation time of 16 h, a shaking speed of 1000 rpm, a sample amount of 20 mg, and a trypsin-to-protein ratio of 1:40 were selected as standard conditions, ensuring efficient peptide release for all eight peptides in the multiplex assay. The immunoenrichment workflow was semi-automated by usage of a computerized plate washer and adapted to 96-well format, ensuring high sample throughput, minimal hands-on time and lowering the total cost of the method. A total method time of only one and a half days was achieved.

3.4. Calibration curve performance and analytical range

The calibration curve performance and the analytical range of the assay were evaluated by analysis of a peptide dilution series in 50 µL digested cookie surrogate matrix. Following immunoenrichment, a dilution series of light peptides (0.23 fmol – 1500 fmol) with a constant amount (50 fmol) of isotope-labeled standard peptide ($n = 12$) was analyzed. The calibration curve demonstrated accuracy between 80% and 120%, and CV below 20% across a concentration range spanning two to four orders of magnitude (Fig. 2).

No matrix effect on peptide recovery was observed in the respective analytical range of each peptide when compared with results of the same peptide dilution series in PBSC (Supplemental Fig. 3).

Carryover effects were evaluated by analysis of blank samples after each calibrator level. Carryover was detected for SP1C and HCC at 1500 fmol (6.8% *G. sigillatus* and 1.3% *A. diaperinus*), for LCPA3A at 500 fmol (0.3% *A. diaperinus*), for HICP at 166 fmol (14.6% *H. illucens*), and for TPM at 55 fmol (between 0.1% and 0.4% insect). In all cases, the carryover signal was below 1.5 fmol and was effectively eliminated by introducing an additional washing step following samples containing analyte amounts within the affected concentration range. The lower limit of quantification (LLOQ) was defined as the lowest concentration meeting the following criteria: (1) accuracy and precision within the specified acceptance range (80% to 120%), and (2) a mean ratio of non-labeled to labeled peptides exceeding the blank ratio plus six times the standard deviation. For all analytes, a LLOQ of below 6.17 fmol, and an ULOQ of 1500 fmol was determined. Therefore, an analytical range from 6.17 fmol – 1500 fmol of analyte peptide could be defined (Table 1).

While the analytical range of the assay was established through conventional calibration curve analysis, the LLOQ for the insect-specific protein is of limited practical relevance for food control applications. Instead, the corresponding amount or percentage of insect material detectable in food products is of interest. To enable this, the endogenous analyte concentrations were determined in species-pure insect meal samples to establish a reference value, allowing calculation of the quantifiable range of insect-derived content in complex food samples (Table 1). Six out of eight analytes demonstrated high sensitivity, with quantification achievable in the low parts-per-million (ppm) range of insect material. Exceptions included LCPF1, which enabled quantification of insect content down to only 2.27%, and AK, with a lower limit of 0.39% insect content. Since all peptides showed comparable LLOQs

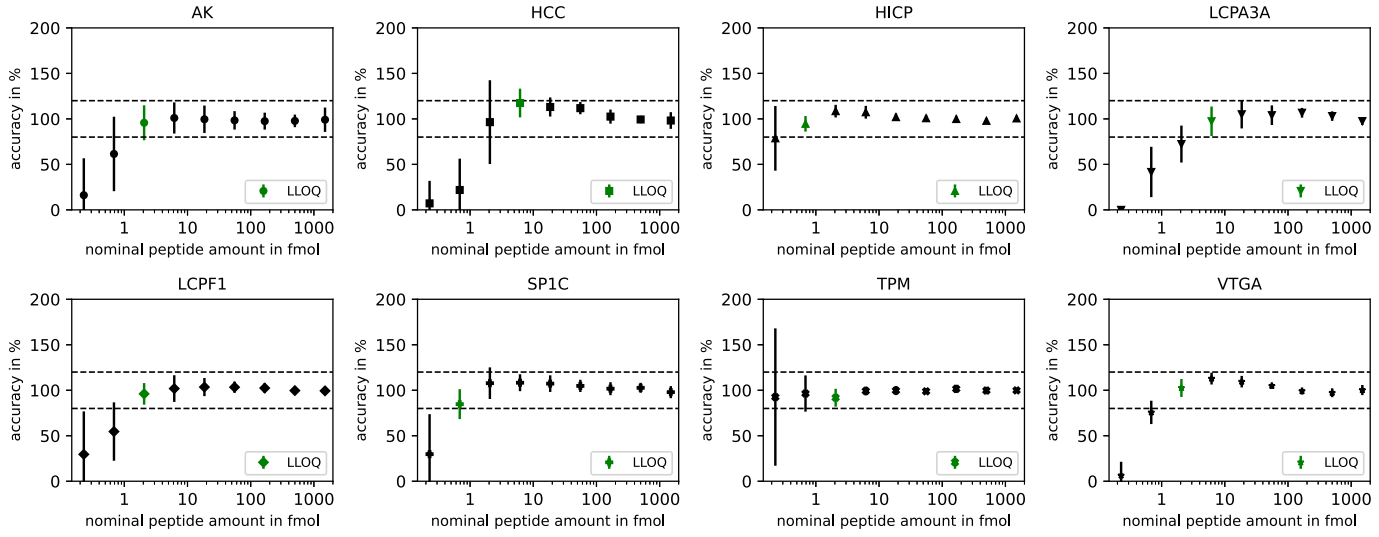

Fig. 2. Calibration curve performance and analytical range of the assay for eight peptide markers. Each plot shows calibrator accuracy (%) versus nominal peptide amount (fmol) in a digested cookie surrogate matrix ($n = 12$; $n = 6$ independent experiments, with $n = 2$ technical replicates each). Horizontal dashed lines indicate the acceptance range of 80% to 120% accuracy. Error bars represent standard deviation. The lower limit of quantification (LLOQ) is indicated in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during calibration curve analysis and high intensities in preliminary targeted LC-MS analysis, the lower sensitivities expressed as insect percentage for AK and LCPF1 were attributed to either low protein expression in the target insects or reduced availability of the respective proteins during the heterogenous phase digest. Besides *T. molitor*, the assay achieved LLOQ values comparable or lower to previously reported limits of detection (LOD) for various PCR-based methods [12,13,47]. Identification and testing of alternative peptides for *T. molitor* and *G. sigillatus* to replace LCPF1 and AK, respectively, has been hindered by the lack of comprehensive genomic and proteomic information during the course of the study, and allows for future improvements.

3.5. Intra- and inter-assay accuracy and precision

Intra- and inter-assay accuracy and precision were assessed using three quality control samples, prepared by spiking non-labeled peptides at three concentration levels into a proteolyzed cookie surrogate matrix. Nominal values of each QC sample were determined beforehand with six independent analytical runs on two different days. The use of spiked QC samples was necessary due to the absence of food matrices containing all

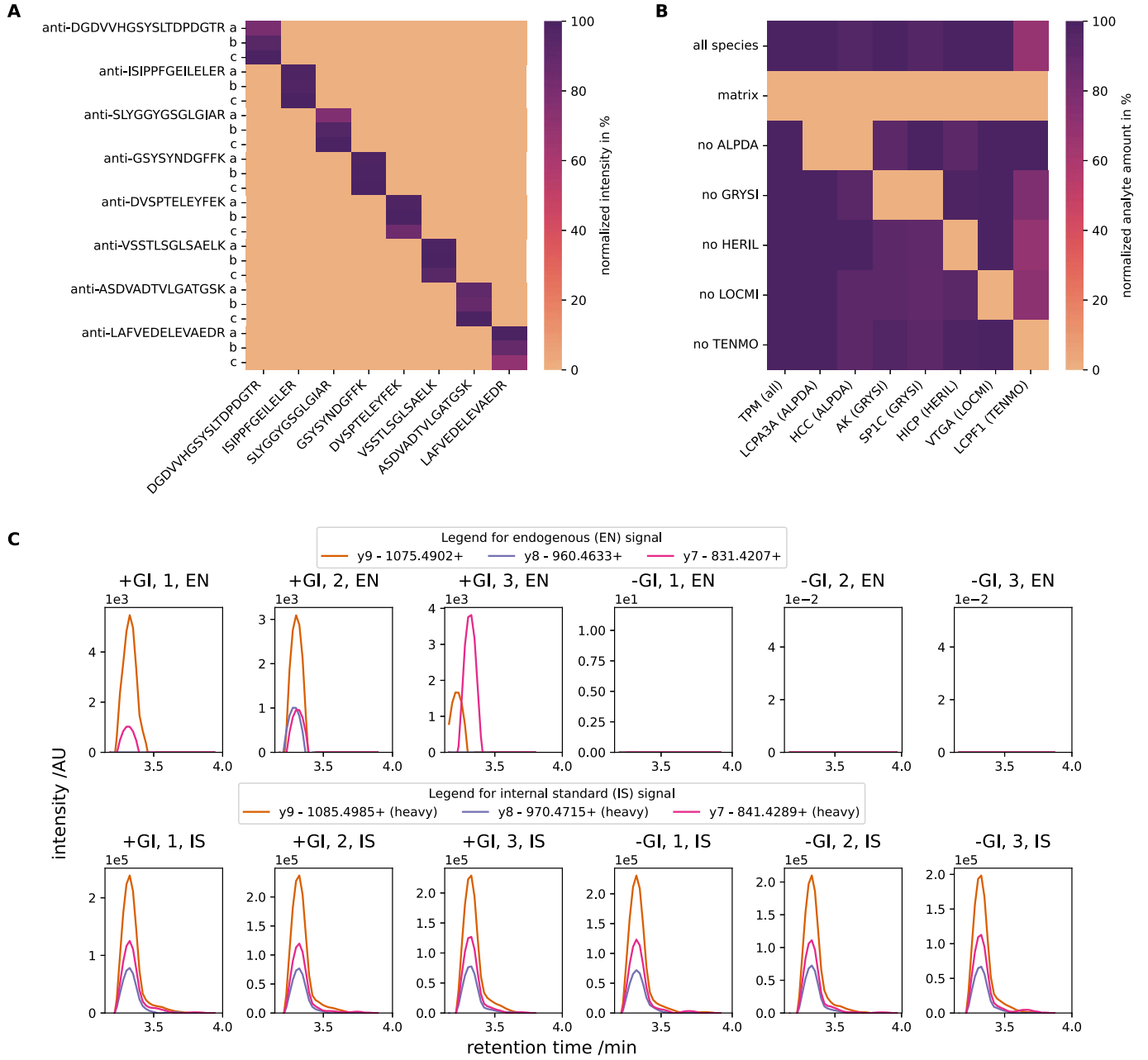

Fig. 3. (A) Antibody specificity was tested in a mixture of eight synthetic isotope-labeled peptides ($n = 3$). Each antibody exclusively enriched its designated target peptide as assessed by parallel reaction monitoring of the eluted peptide from each antibody. (B) Leave-one-out specificity experiment to assess species-specificity of each marker (n = 3). Analyte levels were normalized to the respective maximum value for each marker. Strong signals were observed in PRM following immunoenrichment for all markers in the full mixture, while signals corresponding to the intentionally omitted species were absent in the respective mixes. The pure matrix showed no peptide signal in PRM. (C) Evaluation of potential cross-reactivity of the pan-insect marker tropomyosin (TPM) in L. *vannamei* shrimp samples. Endogenous (EN) and isotope-labeled standard (IS) signals from PRM analysis across three replicates of whole shrimp (with gastrointestinal tract, +GI) and three replicates of shrimp with the gastrointestinal tract removed (-GI) are shown. A barely detectable endogenous TPM signal (maximum intensity of $5{\cdot}10^3$, three fragment ions were detected in only one replicate) was observed only in whole shrimp samples, suggesting the signal originated from intestinal content rather than shrimp tissue.

five insect species simultaneously. Moreover, because the assay includes a pan-insect marker that responds cumulatively to the presence of multiple insect species, combining insect materials would result in additive signals and artificially elevated values for this marker. To ensure consistency and avoid the need for separate QC sets for each species-specific marker and the pan-insect marker, we opted for a standardized approach using spiked non-labeled peptides in a controlled surrogate matrix [48].

Inter- and intra-assay performance were determined on the immunoenrichment level. Intra-assay performance was evaluated by analyzing six replicates of each sample within a single day, while inter-assay performance was determined by analyzing the samples across two independent days. The MS-based immunoassay demonstrated consistent performance, with all peptides measured with a coefficient of variation (CV) below 20% and accuracy within the accepted range of 80% to 120% (Supplemental Table 2).

3.6. Assay specificity

New analytical methods for the quantification and authentication of insects in novel foods need to be highly specific, especially considering the limited knowledge regarding insect proteomes. Thus, we performed rigorous specificity testing of all assay stages. Antibody specificity was tested in a mixture of all eight synthetic isotope-labeled peptides. Each antibody exclusively enriched its designated target, confirming target-specific binding and absence of cross-reactivity within the selected peptide panel (Fig. 3A).

Although PRM was previously used to verify that each peptide marker is exclusively detectable in its expected species, a confirmatory leave-one-out experiment was conducted for enhanced sensitivity through immunoenrichment prior to detection. Here, a mixture of all five species and cookie surrogate matrix was prepared on the proteolysis level, with one species intentionally omitted. Immunoprecipitation was performed using the full antibody panel. The absence of signal corresponding to the omitted species confirmed that the enriched analytes were specific to their respective species. The pan-insect marker tropomyosin was found in all insect containing samples and not found in a proteolyzed cookie surrogate matrix blank sample (Fig. 3B).

Due to the limited genomic and proteomic annotation of insects and other arthropods, additional verification of the pan-insect marker tropomyosin was necessary, as its selection was based on BLAST homology searches only. To confirm that this marker does not occur in other arthropods, and guided by the frequent inclusion of shrimp in food products, the crustacean *L. vannamei* was selected as a representative potentially interfering species [49,50].

Both whole shrimp meal and meal derived from shrimp with removed intestines were analyzed using the insect-specific assay. No insect species-specific marker signals were detected in either sample. However, a barely detectable tropomyosin signal was observed in whole shrimp, which was absent in samples without intestines (Fig. 3C). These results suggest that the detected tropomyosin originates from ingested material rather than shrimp tissue. Importantly, even in undiluted whole shrimp samples, the tropomyosin signal remained only marginally above the lower limit of detection (LOD), indicating that any potential contribution from shrimp intestines is negligible. In typical food matrices, where shrimp would represent only a portion of the total composition, the intestinal-derived tropomyosin signal would be further diluted and fall below detectable levels, ensuring it does not interfere with assay performance.

These findings support the conclusion that tropomyosin is specific to insects under the tested conditions. Despite their phylogenetic proximity to insects, shrimp does not compromise the specificity of the assay.

3.7. Assay parallelism and matrix effects

To assess assay parallelism and potential matrix effects, dilution linearity experiments were conducted. Species-pure insect meal samples were proteolyzed under HPD conditions and serially diluted into a digested cookie surrogate matrix to simulate realistic sample conditions. Measured analyte concentrations were compared to theoretical values calculated from the applied dilution factor and the most concentrated quantifiable sample ($n = 3$). A strong linear correlation between measured and expected concentrations was observed for all analytes across the tested dilution range (Fig. 4A).

For the analytes AK, HCC, LCPA3A, SP1C, TPM, and VTGA, the undiluted samples exceeded the upper limit of quantification and were thus excluded from evaluation. Instead, the most concentrated sample within the quantifiable range was used as the reference for assessing dilution accuracy. Parallelism was considered acceptable if quantification accuracy fell within 75% to 125% of the expected value (Fig. 4B). This criterion was fully met for VTGA, HICP, and LCPF1 across the entire dilution series. LCPA3A, SP1C, TPM, and HCC met the acceptance range at all dilution levels except the highest tested dilution. AK showed consistent accuracy at all dilutions except for a single outlier at dilution factor 133, which slightly deviated below the acceptance threshold. Internal standard suppression was observed for VTGA and LCPA3A at a tenfold dilution. Therefore, these high concentration levels of both analytes were excluded from parallelism evaluation. Given the immunoenrichment format of the assay, this suppression may result from oversaturation of the capture antibodies by high analyte concentrations, leading to reduced binding efficiency and signal distortion [51].

3.8. Analysis of food samples

Following the development of the multiplex assay, it was subsequently applied to the analysis of complex food samples to evaluate its performance under practical conditions. Two categories of samples were evaluated: commercially available food samples with high insect content, and plain cookies spiked with a mixture of six insect species meals at allergen-relevant concentrations.

3.8.1. Analysis of commercially available complex food samples

Commercially available food samples were bought from European vendors and included crackers, pasta, plant-based mince, and cereal bars. Each product contained a single insect species at concentrations ranging from 5% to 20% by weight. To further increase the diversity of tested food matrices, a fried burger patty containing 50% *T. molitor*, prepared in an experimental kitchen as model food, was included in the analysis.

The multiplex assay demonstrated robust qualitative performance across all tested food samples, successfully detecting the presence of insect-derived proteins in every case. However, quantification accuracy varied substantially depending on the analyte, insect species, and matrix composition, reflecting the challenges of complex food matrices (Table 2) [52]. For *T. molitor* in crackers, a matrix with moderate carbohydrate (44 g/100 g) and a low fat content (11 g/100 g), both TPM and LCPF1 yielded high quantitative accuracy (97% and 91%, respectively). Performance declined in the complex burger patty matrix, characterized by high fat content and thermal processing, with accuracies dropping to 34% and 11%. This indicates that matrix effects and processing conditions significantly impair detection efficiency, likely due to reduced protein extractability, epitope degradation or epitope modification by Maillard reactions [52–54]. The burger patties were sampled from the edge of the patty, which exhibited severe heat damage; therefore, strong modifications due to Maillard reactions and other heat-induced chemical processes were expected [55].

For *A. diaperinus*, LCPA3A consistently performed well, achieving 100% accuracy in pasta and 128% in cereal bars. In the pasta sample, HCC performed equally well (89% accuracy) but TPM failed to quantify accurately in this high-carbohydrate (64 g/100 g), low-fat (4 g/100 g) matrix, yielding only 12% accuracy. In the high-sugar (38.5 g/100 g), high-fat (31 g/100 g) cereal bar matrix, HCC and TPM underestimated

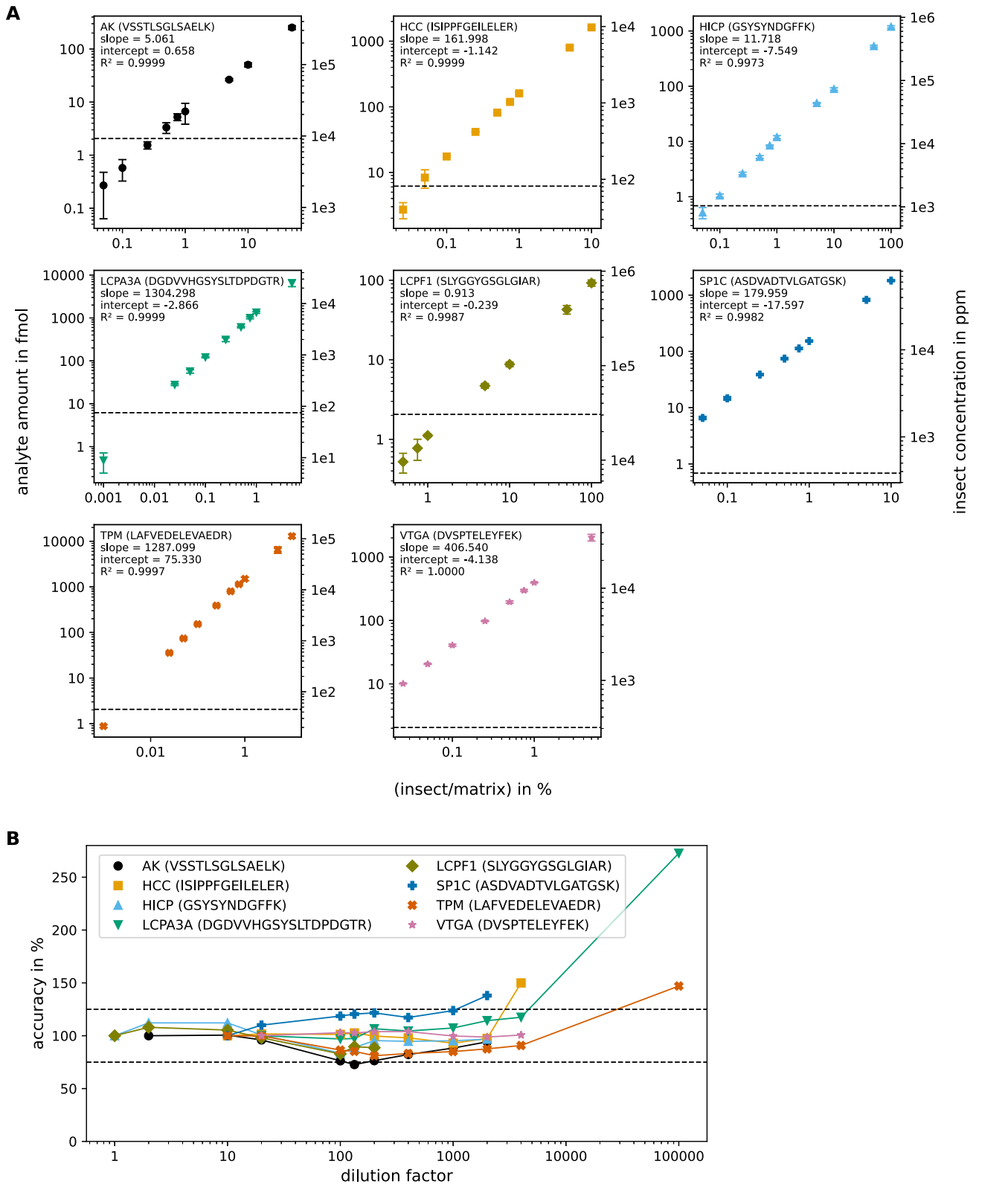

Fig. 4. (A) Assessment of assay parallelism and matrix effects for all analytes. Species-pure insect meal samples were proteolyzed and serially diluted into a digested cookie surrogate matrix to evaluate dilution linearity under realistic sample conditions. Analyte amounts in fmol were determined after immunoenrichment (n = 3). Each subplot displays the linear regression of measured analyte amounts versus insect content (%), including slope, intercept, and R^2. Error bars represent standard deviation. The lower limit of quantification (LLOQ) for each analyte is indicated by a dashed horizontal line. All analytes demonstrated strong linearity across the tested dilution range. (B) Assay parallelism accuracy assessment for all analytes. Accuracy (%) was calculated by comparing measured analyte concentrations at each dilution level of proteolyzed species-pure insect samples in digested cookie surrogate matrix to the concentration of the most concentrated quantifiable sample, which served as the reference (n = 3). Signals of internal standard peptides were suppressed at high analyte concentrations (dilution level 1 and 10) for LCPA3A and VTGA. Therefore, data for these analytes and dilution levels were excluded from the graph. Accuracy limits at 75% and 125% are shown as dashed lines.

Table 2
Analysis of complex food samples ($n = 3$ proteolysis replicates with n = 3 immunoenrichment replicates each). Insect content was determined using analyte amounts in pure insect samples as reference. Determined accuracies are dependent on the analyte and matrix type.

sample type	species	nominal insect content in %	analyte	mean analyte amount in fmol	mean determined insect content in %	SD	accuracy in %
cracker	*T. molitor*	5	LCPF1	4.13	4.55	1.5	91
cracker	*T. molitor*	5	TPM	2110	4.84	0.22	97
pasta	*A. diaperinus*	10	LCPA3A	14,800	10.0	0.65	100
pasta	*A. diaperinus*	10	HCC	1650	8.85	0.36	89
pasta	*A. diaperinus*	10	TPM	840	1.23	0.032	12
plant-based mince	*A. domesticus*	20	TPM	14,600	30.1	1.8	150
cereal bar	*A. diaperinus*	10	LCPA3A	18,700	12.7	1.8	127
cereal bar	*A. diaperinus*	10	HCC	1010	5.45	0.76	54
cereal bar	*A. diaperinus*	10	TPM	4510	6.59	0.68	66
burger patty	*T. molitor*	50	LCPF1	3.94	5.66	1.7	11
burger patty	*T. molitor*	50	TPM	7500	17.2	6.3	34

by 46% and 34%, respectively. The performance differences among analytes suggest that matrix components such as carbohydrates, sugars, and lipids may interfere with specific protein targets to varying degrees [56,57]. Furthermore, it has been shown for pasta that drying and extrusion methods directly affect the extent of Maillard reactions [58]. It is reasonable to assume that other heat- and processing-induced alterations also occur under these conditions, which may explain the limited accuracy of the tropomyosin quantification in the pasta samples.

total insect content in ppm	per species insect content in ppm	replicate	AK	HCC	HICP	LCPA3A	LCPF1	SP1C	TPM	VTGA
0	0	A	–	–	–	–	–	–	–	–
0	0	B	–	–	–	–	–	–	–	–
0	0	C	–	–	–	–	–	–	–	–
30	5	A	–	–	+	–	–	–	+	–
30	5	B	–	+	–	–	–	–	+	–
30	5	C	–	+	–	–	–	–	+	–
120	20	A	–	+	+	–	–	+	+	–
120	20	B	–	+	–	–	–	–	+ 50%	–
120	20	C	–	+	–	–	–	–	+	–
600	100	A	–	+	–	+	–	+ 38%	+ 1166%	+ 189%
600	100	B	–	+	–	+ 229%	–	–	+ 17%	+
600	100	C	–	+	+	+ 48%	–	+ 225%	+ 49%	+

Fig. 5. Analysis results of cookie samples containing trace amounts of insects (n = 3 technical replicates and n = 3 biological replicates). Each cookie contained 0 ppm to 100 ppm of six individual insect species. A "+" indicates detection without quantification, a "–" indicates that the analyte was not detected, a bold black edge indicates that the sample lies within the dynamic range of the respective assay. Quantification results are presented in percent accuracy of the supplemented insect calculated based on the experimentally determined protein abundance of AK, HCC, HICP, LCP3A, LCPF1, SP1C, TPM, and VTGA in a pure insect meal.

For *A. domesticus*, no species-specific marker was available, and quantification relied on the pan-insect marker TPM. In the plant-based mince, TPM led to substantial overestimation (150% accuracy), indicating possible sample heterogeneity or signal modulation influenced by the specific composition of the matrix [59,60]. Alternatively, the discrepancy may indicate that the actual insect content in the commercial product exceeds the amount stated on the label.

Overall, the data underscore that the performance of individual analytes is highly dependent on both the insect species and the sample matrix. LCPA3A demonstrated the highest consistency, while TPM exhibited limited reliability for quantification due to matrix sensitivity. Matrix complexity and processing appear to introduce significant variability in quantification [53], thus further validation work for other matrices is required prior to quantitative sample analysis. While qualitative analysis can be applied to all tested matrices, only matrices similar to crackers yielded accurate quantification results.

3.8.2. Evaluation of assay performance at concentrations relevant for allergen detection

To further evaluate the assay's sensitivity and quantification capabilities at trace concentrations relevant for allergen detection, a second set of experiments was conducted using plain cookies, spiked with a defined insect meal mixture at total insect contents of 0 ppm, 30 ppm, 120 ppm, and 600 ppm, corresponding to 0 ppm, 5 ppm, 20 ppm, and 100 ppm per insect species, respectively. The mixture contained the six edible insect species: *A. domesticus*, *A. diaperinus*, *G. sigillatus*, *H. illucens*, *L. migratoria*, and *T. molitor*.

Based on the assay's quantification range, only the analytes LCPA3A, HCC, VTGA, SP1C, and TPM were theoretically sensitive enough to quantify insect protein in the 100 ppm cookie sample. TPM, as a pan-insect marker, was expected to be quantifiable in the 600 ppm (total) and 120 ppm samples. In practice (Fig. 5), quantifiable signals above LLOQ were obtained in the expected samples only for LCPA3A, VTGA, SP1C, and TPM. However, quantitative accuracy in these samples was poor, with values deviating significantly from expected concentrations. This lack of quantitative reliability is likely due to sample heterogeneity and the challenges of evenly distributing low levels of insect proteins in a complex, processed food matrix, such as baked goods [60]. These effects are assumed to become more pronounced as the percentage of insect protein decreases. In this study, only subsamples of the cookies were processed rather than the entire cookie, to maximize analytical sensitivity and due to practical limitations. However, subsampling can introduce variability, particularly at low analyte concentrations, where heterogeneous distribution can lead to over- or under-representation of target proteins. Similar effects have been seen in other studies, including the official PCR method for detection of ruminant PAP in feed [61,62]. This reflects a fundamental tradeoff between sensitivity and accuracy. Subsampling improves sensitivity for trace detection but compromises quantitative reliability. In contrast, protocols such as described by Heick and colleagues [63] aim to improve reproducibility through standardized sample input and protein normalization, albeit with a potential loss in sensitivity due to dilution. In its current state, the assay shows low quantitative accuracy in processed complex food matrices with low to very low concentrations and further testing implementing other sampling strategies are required to explore its full potential.

Although quantification was only partially possible, qualitative detection was consistently observed for several analytes (Fig. 5), indicating significantly lower LODs compared to the methods validated LLOQs. Qualitative detection was defined as a quantifier ion signal that was high enough to give a concentration value greater than zero when the quantifier ion signal was converted using the calibrator curve. LCPA3A and VTGA were detectable in the 100 ppm sample, while SP1C was found in the 100 ppm and 20 ppm samples. HCC and HICP showed the highest qualitative sensitivity, being detected in samples down to 5 ppm per insect species demonstrating a substantial improvement over a previously reported method with a limit of detection of 1% insect content [18]. TPM was detected in all spiked samples, confirming its suitability as a pan-insect presence marker. Beyond its utility for species-independent detection, TPM holds particular relevance due to its role as a major insect allergen [7,39]. Its consistent detectability across all concentration levels, including the lowest spiking level, highlights the assay's potential for monitoring allergenic risk in food products. Thus, TPM not only enables the verification of insect presence but also contributes directly to improved food allergen surveillance and consumer safety by allowing sensitive detection of an allergenic protein in complex matrices. The qualitative sensitivity achieved for *A. diaperinus* and *H. illucens* in this multiplex assay is comparable to that of a real-time PCR method developed for the detection of *A. diaperinus* in food samples, which also detected the species at concentrations as low as 5 ppm in the same cookie samples [14].

4. Conclusions

A targeted immunoaffinity LC-MS/MS assay was developed and partially validated for the absolute quantification of insect proteins in complex food matrices. Marker peptides for five insect species and a pan-insect tropomyosin peptide were selected, and analytical parameters including chromatography gradient and normalized collision energy were optimized. The assay demonstrated high specificity, broad analytical range over two to four orders of magnitude, and robust performance in terms of calibration linearity, intra- and inter-assay accuracy and precision, as well as parallelism. Application to commercial insect-containing foods and spiked model foods confirmed the method's qualitative reliability, even at allergen-relevant levels, though quantification at low levels remains challenging in heterogeneous matrices. Notably, the assay's qualitative sensitivity is comparable to a previously reported real-time PCR method was applied to the same cookie samples. The validation results showed that the sensitivity required for comprehensive allergen monitoring is not consistently achieved across all species-specific analytes. In particular, the *T. molitor* marker LCPF1 and the *G. sigillatus* marker AK exhibited lower expression levels than anticipated, limiting their performance at trace concentrations. Future work will therefore focus on identifying alternative marker peptides for these species, refining protease selection to reduce impacts of Maillard-related modifications, and improving sample preparation to enhance accuracy at low analyte levels. Additionally, we are considering the development of a complementary assay without immunoenrichment, optimized specifically for species authentication in foods with higher insect content, while continuing to optimize the current immunoenrichment-based assay to achieve robust allergen detection across all markers. Expanding validation to additional food matrices will help assess the assay's robustness under diverse processing conditions. Overall, the assay provides a sensitive and highly specific tool for insect protein detection and quantification, while acknowledging that further optimization is required for uniform achievement of allergen-relevant sensitivity across all markers.

CRediT authorship contribution statement

Tobias Meisinger: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hannes Planatscher:** Writing – review & editing, Supervision, Conceptualization. **Cristiano Garino:** Writing – review & editing, Resources, Project administration. **Dieter Stoll:** Writing – review & editing, Resources, Conceptualization. **Eva-Maria Ladenburger:** Resources, Writing – review & editing. **Albert Braeuning:** Writing – review & editing, Supervision, Resources. **Hermann Broll:** Writing – review & editing, Project administration, Funding acquisition. **Oliver Poetz:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-4o in order to improve readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare the following competing financial interest(s): HP and OP are shareholders of SIGNATOPE GmbH. SIGNATOPE offers assay development and service using MS-based immunoassay technology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2026.117078.

Data availability

Data will be made available on request.

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B.2 Supplementary Data

Originally published in **Meisinger, T.**, Planatscher, H., Garino, C., Stoll, D., Ladenburger, E.-M., Braeuning, A., Broll, H., Poetz, O. (2026). From cricket to mealworm: 8-Plex mass spectrometry immunoassay for edible insect detection in novel foods. Microchemical Journal, 222, 117078. https://doi.org/10.1016/j.microc.2026.117078

The supplementary table B.1 was adapted for print. The original table was not reproduced due to their large size. It is available from the original publication or upon request.

Table B.1: Properties and origin of selected peptide marker. Protein mass origin describes the source of the utilized protein mass in case the selected peptide marker is not unique to its respective species.

Target species	Peptide	Protein name (long)	Protein name (short)	Protein mass in Da	Protein mass origin
A. diaperinus	DGDVVHGSY-SLTDPDGTR	Larval cuticle protein A3A	LCPA3A	15413.42	Tribolium castaneum, D6W8Q9
A. diaperinus	ISIPPFGEI-LELER	Hemocyanin C	HCC	79298.34	mean of A0A482VPX6, L7UVJ1
T. molitor	SLYGGYGSG-LGIAR	Larval cuticle protein F1	LCPF1	14919.13	Q9TXD9
H. illucens	GSYSYN-DGFFK	Cuticle protein	HICP	16451.02	A0A7R8UWC1
L. migratoria	DVSPTEL-EYFEK	Vitellogenin A	VTGA	150811.04	A0A1L5LBJ0
G. sigillatus	VSSTLSGLS-AELK	Arginine kinase	AK	45426.05	Gryllus bimaculatus, GLG96688.1
G. sigillatus	ASDVADTVL-GATGSK	Spermatophylax protein 1C	SP1C	9223.72	A0A0N7HK19
Insecta	LAFVEDELE-VAEDR	Tropomyosin	TPM	40527.2	mean of KAF6210625.1, GLH05633.1, ABK30920.1

Target species	Precursor mass (m/z)	Precursor charge	Quantifier ion	Qualifier ions	NCE
A. diaperinus	945.926851	2+	p++ > y5+	p++ > y11+, y10+, y9+	25
A. diaperinus	806.950881	2+	p++ > y10+	p++ > y11+, y9+, y8+, y7+	25
T. molitor	685.856783	2+	p++ > y8+	p++ > y10+, y9+	20
H. illucens	642.78021	2+	p++ > y8+	p++ > y7+, y6+	20
L. migratoria	728.845747	2+	p++ > y9+	p++ > y8+, y7+, y6+	15
G. sigillatus	646.358824	2+	p++ > y8+	p++ > y7+, y6+	20
G. sigillatus	696.35427	2+	p++ > y10+	p++ > y9+, y8+	20
Insecta	817.899042	2+	p++ > y9+	p++ > y8+, y7+	20

Table B.2: Intra- (n = 6) and inter-assay accuracy and precision (n = 12), evaluated by three different QC samples with all spiked-in analyte peptides in proteolyzed food matrix (cookie) at three concentration levels.

			Intra-day			Inter-day		
analyte	sample	nominal value (fmol)	mean amount (fmol)	accuracy (%)	%CV	mean amount (fmol)	accuracy (%)	%CV
LCPA3A	QC1	24.8	24.2	98	9	24.4	98	7
	QC2	124	117	94	11	123	99	6
	QC3	1260	1200	95	7	1250	99	6
HCC	QC1	27.4	25.9	95	8	26.9	98	9
	QC2	127	124	98	3	124	98	5
	QC3	1270	1260	99	9	1240	98	10
LCPF1	QC1	18	16.4	91	4	18.3	102	11
	QC2	92.3	82.6	89	3	91.7	99	5
	QC3	999	807	81	10	995	100	8
HICP	QC1	20.6	20.5	100	2	20.7	100	8
	QC2	104	97.5	94	3	102	98	7
	QC3	1110	949	85	5	1110	100	6
VTGA	QC1	26.4	26.2	99	2	26.2	99	9
	QC2	127	124	98	3	127	100	8
	QC3	1330	1120	84	4	1340	101	9
AK	QC1	24.2	22.9	95	4	24.4	101	10
	QC2	124	117	94	3	125	101	7
	QC3	1310	1420	108	17	1340	102	12
SP1C	QC1	27.6	25.8	93	2	26.7	97	12
	QC2	132	132	100	5	132	100	7
	QC3	1370	1360	99	5	1420	104	6
TPM	QC1	23.4	23.3	100	5	23.7	101	13
	QC2	123	121	98	3	122	99	8
	QC3	1280	1270	99	4	1340	105	10

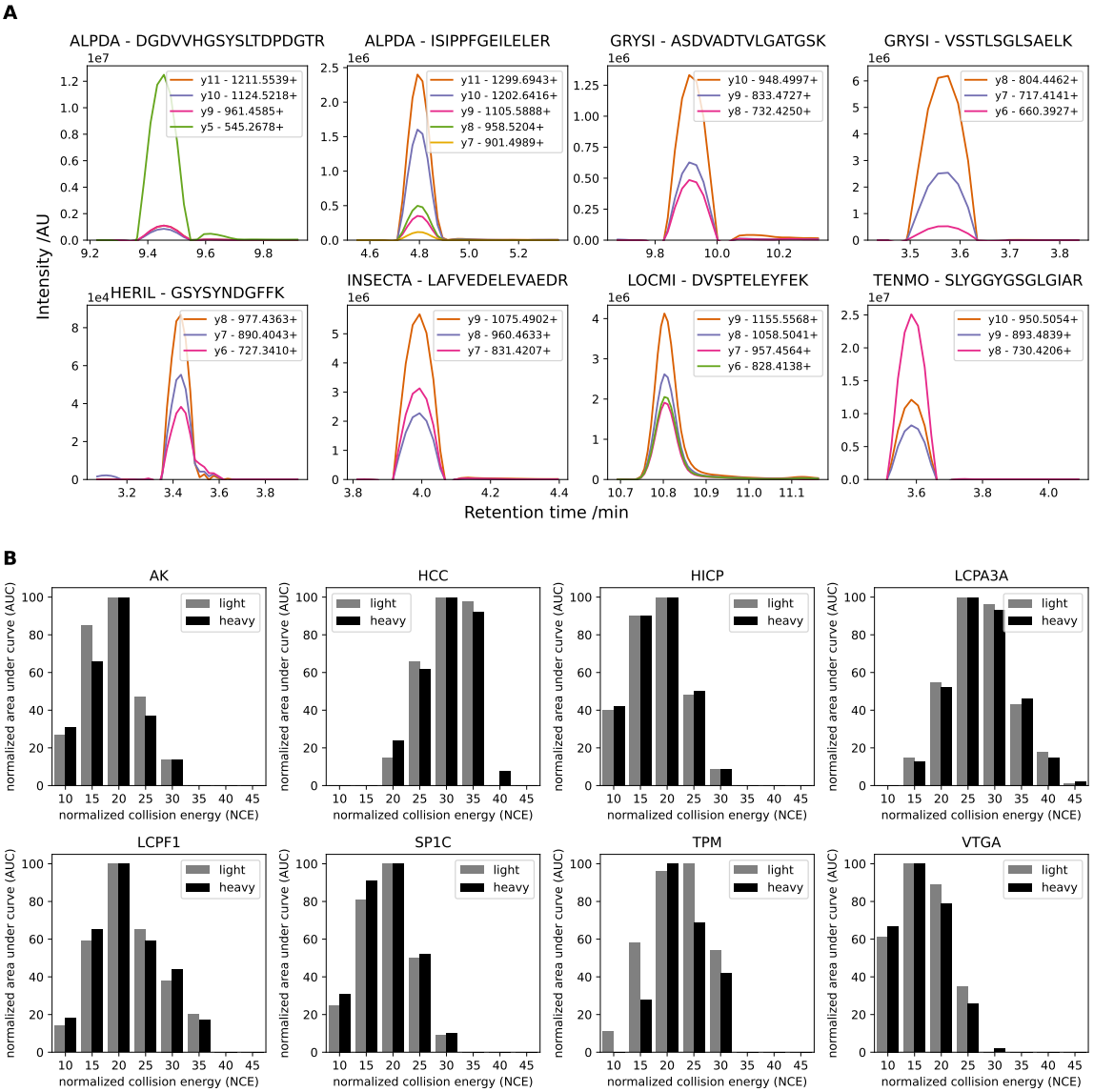

Figure B.1: (A) Marker peptides were verified using parallel reaction monitoring (PRM) LC-MS/MS methods. Each peptide generated a high intensity species-specific signal. At least three peptide fragments per parent ion were detected by PRM. The maximum signal was used for quantification, the other signals were used as specificity control based on the signal ratios. An exception was ALPDA – ISIPPFGEILELER, for which the second highest signal was used for quantification, as it showed greater reproducibility across measurements. (B) Normalized collision energy (NCE) optimization for all analytes. Synthetic isotope-labeled and non-labeled peptides were analyzed using parallel reaction monitoring (PRM) and a range of NCEs. Area under the curve values were normalized relative to the highest value of each analyte.

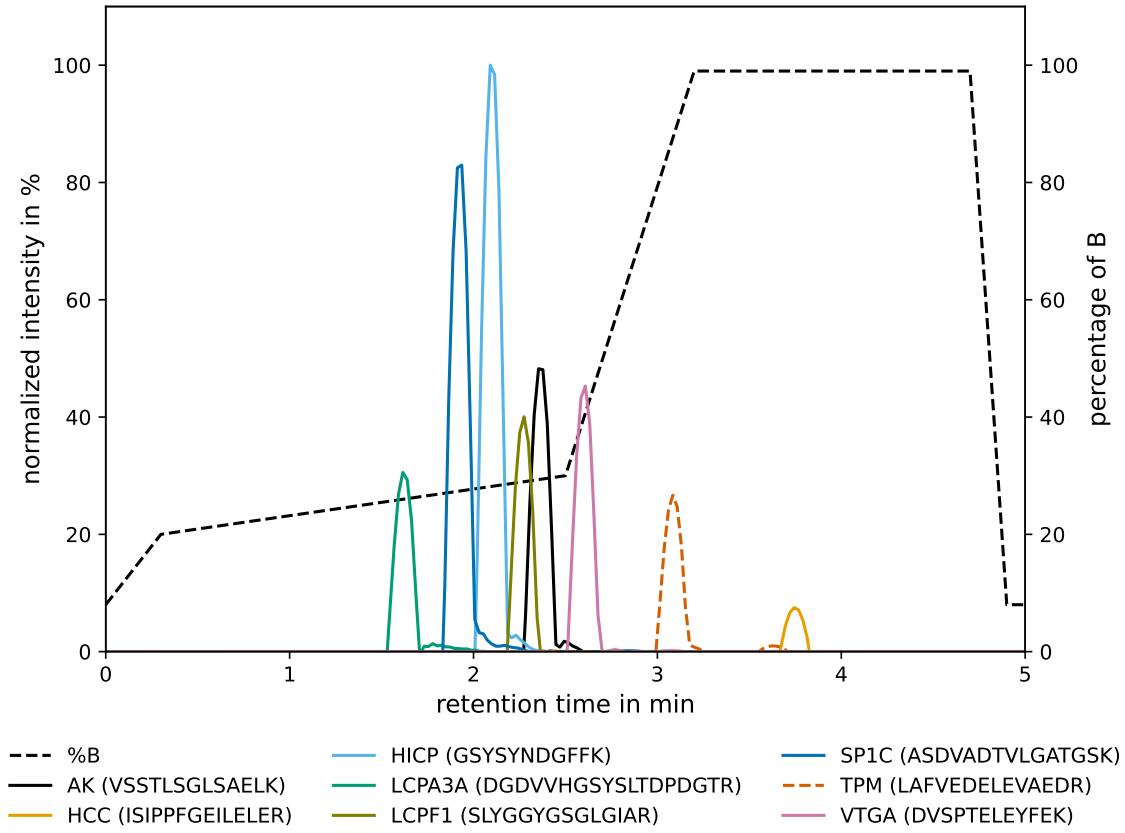

Figure B.2: Final chromatographic gradient with overlaid extracted ion chromatograms of all peptide markers. Intensities were normalized to the highest peak. The total cycle time was 6 minutes, demonstrating retention time stability and separation efficiency across analytes. (column: nanoEase M/Z BEH, 0.75 mm i.d. x 150 mm, 3.5 µm (Waters), Solvent A: 0.1 % FA in LC-MS grade water. Solvent B: 80 % ACN, 0.1 % FA in LC-MS grade water. Flow: 1.5 µL min^{-1} at 55 °C).

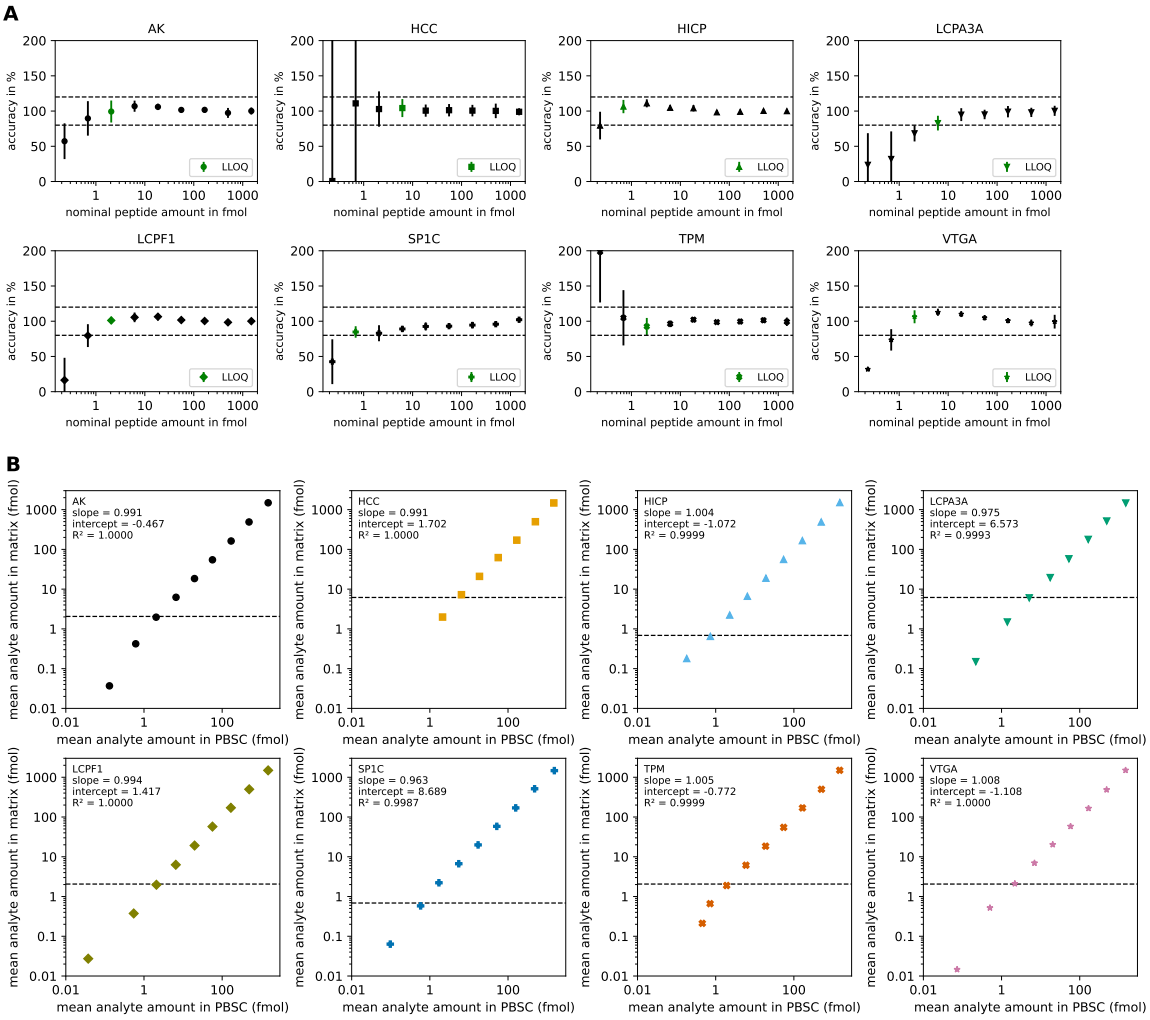

Figure B.3: (A) Calibration curve performance and analytical range of the assay for eight peptide markers. Each plot shows calibrator accuracy (%) versus nominal peptide amount (fmol) in PBSC (n = 12; n = 6 independent experiments, with n = 2 technical replicates each). Horizontal dashed lines indicate the acceptance range of 80 % to 120 % accuracy. Error bars represent standard deviation. (B) Correlation between calibration curves analyzed in PBSC and in proteolyzed cookie surrogate matrix. Each plot shows mean analyte amounts in fmol of one analyte (nn = 12; n = 6 independent experiments, with n = 2 technical replicates each). Linear regression parameters including slope, intercept, and R^2 were added to each plot. The LLOQ is visualized as dashed horizontal line. All calibration curves demonstrate strong correlation between PBSC and matrix.

C Appendix III

C.1 Accepted Review I

Meisinger, T., Vogt, A., Kretz, R., Hammer, H. S., Planatscher, H., Poetz, O. (2025). Mass spectrometry-based ligand binding assays in biomedical research. Expert Review of Proteomics, 22(3), 123-140. https://doi.org/10.1080/14789450.2025.2467263

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