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# Development of protein-based assay methods for authentication of insects in food and feed

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## ABSTRACT

Rising global protein demand for animal and human nutrition requires new, safe sources of protein that support healthy, nutrient-rich diets and sustainable food chains. Insects meet these requirements in terms of their high protein content, polyunsaturated fatty acids, vitamins and ability to convert organic side streams into high value protein products, thereby reducing carbon dioxide emissions. With the recent approval of first insect products under the EU Novel Food Regulation 2015/2283, food authenticity and safety concerns including allergic risks need to be assessed.

Existing methods for authentication of food products are based on genomic DNA using polymerase chain reaction (PCR). While suitable as a qualitative approach, this method is lacking in terms of quantification and is therefore not able to investigate legal limits of insect contents in food. Moreover, food and feed processing can impede DNA-based detection methods. The development of protein-based immunoassays for species identification and quantification remains challenging due to high degree of processing and lacking genomic and proteomic data for most insect species of current interest.

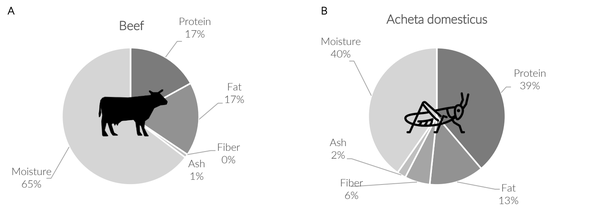


Figure 1: Nutrient contents of (A) beef and (B) an edible cricket species. Williams, J.P. (2016). doi:10.1016/B978-0-12-802856-8.00003-X

## METHOD - IMMUNOAFFINITY-LC-MS/MS

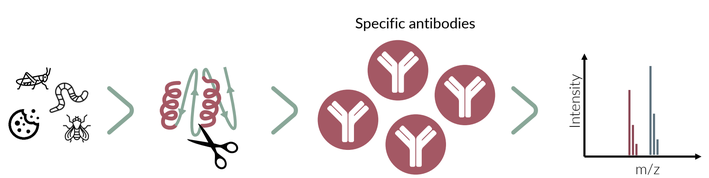


Figure 2: Workflow of immunoaffinity-MS. Insect proteins are tryptically digested and isotopically labeled standard peptides are added. Sequence-specific antibodies targeting proteotypic sequence motifs are employed in an automated immunoprecipitation workflow to enrich peptides derived from proteins of interest and standards. Finally, unique peptides derived from the protein biomarkers are quantified via reference peptides and targeted nLC-mass spectrometry.

## ACKNOWLEDGMENT

This work was funded by Programm zur Innovationsförderung from BMEL project AllergenPro (FKZ 281A304B18).

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## WORKFLOW FOR TARGET PEPTIDE IDENTIFICATION

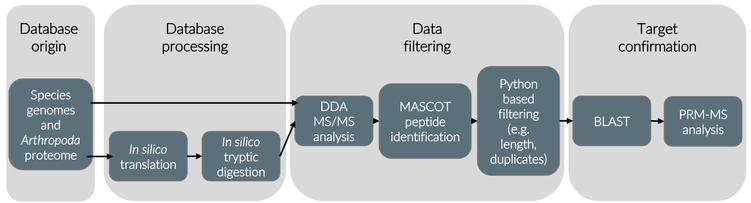
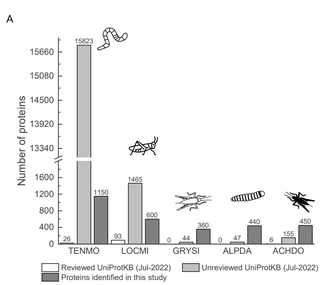



Figure 3: Workflow for target peptide identification for poorly sequenced species. DDA MS/MS data is required to enhance sequence databases. Through a series of identification and filtering steps, species-specific marker peptides can be identified.

Utilizing a combination of wet-lab and in silico techniques, species-specific peptide marker and a pan-*Insecta* peptide marker were identified. To be considered for assay development, marker candidates had to fulfill several selection criteria, such as: length below 25 amino acids, derived from highly abundant protein, presence in sample after sample processing, unique within subset of target species, non-existent in livestock species and arthropods. From 45,000 unique peptides, 173 were tested with PRM analysis, from which 28 potential targets were identified and 9 species marker were selected.

## GAIN OF INSECT PROTEOME DATA

Whole insects meals were directly fragmented by trypsin applying heterogeneous phase digestion (Steinhilber, 2019). Peptide samples were diluted in formic acid and subjected to nLC-mass spectrometry in DDA mode. Peptide spectra were analyzed using an *Arthropoda* proteome database in MASCOT 2.8. High numbers of proteins previously unknown in the target species were identified (Figure 4). Additionally, data filtering was employed to identify species marker candidate peptides in this data set. Species-specific biomarkers for five out of six target species and a pan-*Insecta* biomarker were identified within the acquired insect proteome data.



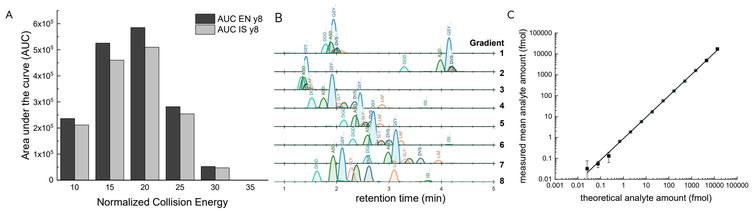

B

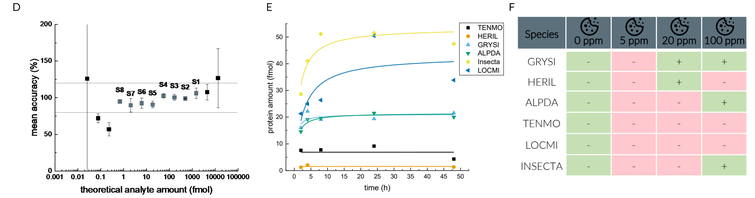
| Species | Marker protein name |
|---|---|
| Tenebrio molitor (TENMO) | Larval cuticle protein F1 |
| Locusta migratoria (LOCMI) | Vitellogenin A |
| Gryllodes sigillatus (GRYSI) | Arginine kinase, Spermatophylax protein C |
| Alphitobius diaperinus (ALPDA) | Larval cuticle protein A3A, Hemocyanin C |
| Hermetia illucens (HERIL) | Cuticle protein, Superoxide dismutase |
| Insecta | Tropomyosin |

Figure 4: (A) Comparison of known protein entries from UniProtKB (Jul-2022) to identified proteins from DDA MS/MS experiments, utilizing a part of the here developed identification workflow. (B) Identified species-specific marker proteins for 5 out of 6 target species. Additionally, a pan-*Insecta* marker was identified.

## ASSAY DEVELOPMENT

We developed an immunoassay with mass spectrometric readout using the identified insect markers. Sequence-specific antibodies and isotopically labeled standard peptides were generated. Antibody specificity was tested; normalized collision energies (NCEs), analytical LC-gradients, number and concentration of calibrators were optimized and a tryptic digestion kinetics study was performed. We demonstrate applicability of this assay in pure insect samples and food samples containing trace amounts of insect meals.




F

| Species | 0 ppm | 5 ppm | 20 ppm | 100 ppm |
|---|---|---|---|---|
| GRYSI | - | - | + | + |
| HERIL | - | - | + | - |
| ALPDA | - | - | - | + |
| TENMO | - | - | - | - |
| LOCMI | - | - | - | - |
| INSECTA | - | - | - | + |

Figure 5: (A) For NCE optimization measurements, peptides were measured in PRM mode using a range of NCE values. (B) Eight analytical LC-gradients were tested for optimal analyte separation, peak form and method time. (C) Standard curve is prepared by dilution of synthetic peptide in constant isotopically labeled peptide concentration (area under the curve (AUC) ratio) ranging from 0.03 fmol – 40500 fmol. A linear fit was used for back calculation of measured analyte amounts. (D) Eight calibrator levels were chosen based on acceptance criteria of 80% to 120% accuracy. (E) Optimal tryptic digestion time was tested between 2 h to 48 h incubation time. Analytes were quantified with the assay and a standard incubation time of 16 h was chosen. (F) Cookies spiked with 6 insect species meals ranging from 0 ppm to 100 ppm per species were processed and analyzed with the assay. Four out of six species were detectable at levels relevant for allergen analysis.

## SUMMARY AND OUTLOOK

nLC-MS enabled the expansion of the known proteome of several edible insect species by factor 2 to 15. Using a new bioinformatic workflow adapted to poorly sequenced species, 46 target species-unique marker peptides were identified. A selection of nine promising targets was optimized for analytical LC-gradient, normalized collision energies, calibrator range and number of calibrator levels and tryptic digestion parameters to provide high sensitivity and robust identification. Antibodies were generated and standard curve experiments show good sensitivities in the high attomole range. A proof of concept experiment in insect and novel food samples was successful. Further development of the quantitative immunoassays to determine insect content in food and feed is in progress.

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