Application Note

Food

# Authenticity Evaluation of Insect Protein-Containing Food Products

## Using the Agilent 1290 Infinity III Bio LC and Agilent 6545XT AdvanceBio LC/Q-TOF


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## Abstract

This application note presents an LC/MS workflow for the taxonomic authentication of insect-derived ingredients in commercial cricket-based protein bars. Food samples were cryogenically ground and delipidated ahead of protein extraction. Proteins were subsequently enzymatically digested prior to LC/MS and MS/MS analysis in data-dependent acquisition mode, thereby allowing comprehensive protein profiling across samples. Protein identification was performed via database searches, followed by peptide-centric taxonomic mapping to achieve species-level resolution. The approach enabled verification of animal- and plant-derived ingredients, supporting label compliance. Notably, unique peptides from *Acheta domesticus* were consistently detected in cricket-containing bars, confirming species-specific identification. The absence of insect peptides in the negative control sample further validated the specificity of the approach.

## Introduction

The increasing demand for sustainable and alternative protein sources has led to the rise of novel foods, of which insect-based products are gaining traction.[1] Among edible insects, *Acheta domesticus* (house cricket) stands out due to its favorable nutritional profile, ease of farming, and regulatory acceptance.[2,3] Cricket-derived ingredients are now incorporated into various consumer products, such as protein bars, offering high protein content with a lower environmental footprint compared to traditional animal proteins.[2,4]

However, incorporating insect proteins into the food chain raises questions related to species authentication, product labeling (both when insect proteins are present and when they are absent), compliance with evolving regulatory frameworks, and the management of potential allergenic risks. To address these challenges, advanced analytical techniques such as liquid chromatography/mass spectrometry (LC/MS) are required. This approach enables comprehensive protein profiling and the identification of species-specific peptide markers, even in highly processed food matrices.

In the current application note, the Agilent 1290 Infinity III Bio LC and Agilent 6545XT AdvanceBio LC/Q-TOF were used to support the identification of insect-derived proteins in processed food matrices.

## Materials and methods

### Materials

Hexane, water, acetonitrile, and formic acid were purchased from Biosolve (Valkenswaard, The Netherlands). Urea, thiourea, dithiothreitol (DTT), 2-iodoacetamide (IAA), and Bradford reagent kit were bought from Merck (Darmstadt, Germany). Tris-HCl pH 8 (1M solution) and bovine serum albumin (BSA) were sourced from Thermo Fisher Scientific (Waltham, MA, USA). Porcine sequencing-grade modified trypsin was acquired from Promega (Madison, WI, USA). A selection of commercially available protein bars (referred as C-BAR01 to C-BAR05) containing insect-derived ingredients (*A. domesticus*) was obtained through local retail stores. Additionally, one protein bar without insect content was included as a reference sample (negative control, referred to as N-BAR). Table 1 gives an overview of the main ingredients present in the protein bars. Indicative content of cricket is provided, according to packaging information.

**Table 1.** Overview of main ingredients present in protein bars, based on package labeling.

| Main Ingredients | N-BAR | C-BAR01 | C-BAR02 | C-BAR03 | C-BAR04 | C-BAR05 |
|---|---|---|---|---|---|---|
| Soy Protein | x | x | x | x | x | x |
| Cricket Powder (%) | – (N/A) | x (4.6%) | x (4.7%) | x (6.3%) | x (13%) | x (13%) |
| Rice Protein | x | x | – | – | x | x |
| Whey Proteins Concentrate | x | – | – | – | – | – |
| Dark Chocolate (Cocoa Powder, Cocoa Butter) | x | – | – | x | x | x |
| Rapeseed Oil | – | x | x | x | – | – |
| Grated Coconut | – | x | – | x | x | – |
| Milk | x | x | x | x | x | x |
| Peanuts And Nuts | x | x | x | x | x | x |
| Chicory Root Fiber | – | – | – | – | x | x |
| Freeze-Dried Blackcurrants | – | – | – | – | – | x |
| Oat | – | x | x | – | – | – |
| Flaxseed | – | x | x | – | – | – |
| Pumpkin Seeds | – | x | x | – | – | – |
| Buckwheat | – | – | x | – | – | – |
| Hazelnuts | – | – | x | – | – | – |

### Protein extraction

Commercial protein bars were ground in liquid nitrogen using a tissue homogenizer (3 × 30 seconds at 30 Hz). 100 mg of each powder was delipidated using hexane. After sonication and centrifugation, the supernatant was discarded. Pellets were subjected to two additional rounds of delipidation under the same conditions and subsequently air dried prior to protein extraction. Proteins were extracted using a buffer containing 7 M urea, 2 M thiourea, 50 mM DTT, and 100 mM Tris-HCl pH 8.0. Samples were sonicated for five minutes, vortexed to facilitate solubilization, and centrifuged for 10 minutes at 21,000 × g. Supernatants were collected and protein concentration was assessed using the colorimetric Bradford assay with BSA as calibrant. Samples were prepared in triplicate.

## In-solution digestion

A volume corresponding to 1 mg of total protein was diluted to a final volume of 120 µL in 100 mM Tris-HCl, pH 8.0. Samples were subsequently reduced at 60 °C for 30 minutes with 10 mM DTT (final concentration), followed by alkylation at 37 °C for 1 hour using 30 mM IAA (final concentration). The sample volume was then adjusted with 100 mM Tris-HCl, pH 8.0 to 800 µL, prior to digestion. Proteolytic digestion was performed overnight at 37 °C using trypsin at an enzyme-to-substrate ratio of 1:50 (w/w). Digestion was quenched by adding formic acid to a final concentration of 1%. Solid-phase extraction of the resulting digests was performed and purified digests were subsequently dried and reconstituted in 160 µL 2% acetonitrile and 0.1% formic acid prior to LC/MS analysis.

## LC/MS acquisition

Tables 2 and 3 respectively summarize the LC/MS instrumentation and acquisition parameters used.

**Table 2.** Details of instrument configuration.

| Module | Details |
|---|---|
| Pump | Agilent 1290 Infinity III Bio High-Speed Pump (G7132A) |
| Autosampler | Agilent 1290 Infinity III Bio Multisampler (G7137A) with Integrated Sample Thermostat |
| Column Compartment | Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with Agilent Quick Connect Bio Heat Exchanger Std. (G7116-60071) |
| Detector | Agilent 6545XT AdvanceBio LC/Q-TOF (6549AA) with Dual Jet Stream ESI Source |

## Protein identification

Raw data files were processed and peptide spectra were matched against the UniProtKB reference proteome (485,423 protein entries), the *A. domesticus* proteome (171 protein entries), and a custom contaminant database containing commonly observed contaminants. Search parameters included trypsin as proteolytic enzyme allowing up to two missed cleavages, precursor mass tolerance of 20 ppm, fragment mass tolerance of 0.05 Da, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine.

The open-source web application Unipept[5] (https://unipept.ugent.be/) was used to identify the unique peptides, i.e. sequences found exclusively in a single taxon (e.g., species, genus, or family), providing strong evidence for the taxonomic origin of the sample. Unipept also flags nonspecific peptides that are shared across multiple taxa, helping assess the specificity and reliability of taxonomic assignments.

**Table 3.** LC/MS data acquisition parameters.

| | |
|---|---|
| **LC** | |
| Column | Agilent AdvanceBio Peptide Mapping C18, 2.1 × 150 mm, 2.7 µm |
| Mobile Phase | A) 0.1% formic acid in water<br>B) 0.1% formic acid in acetonitrile |
| Flow Rate | 0.3 mL/min |
| Injection Volume | 40 µL (250 µg of peptide material) |
| Autosampler Temperature | 8 °C |
| Gradient | Time (min) %B<br>0 to 2 3<br>2 to 92 3 to 30<br>92 to 102 30 to 70<br>102.1 to 112 90<br>112.1 to 125 3 |
| Column Temperature | 60 °C |
| Needle Wash | Acetonitrile using flush port, 3 sec |
| Seal Wash | 10% isopropanol |
| **MS** | |
| **Source** | |
| Ionization | Positive ESI |
| Drying Gas Temperature | 320 °C |
| Drying Gas Flow | 8 L/min |
| Sheath Gas Temperature | 350 °C |
| Sheath Gas Flow | 11 L/min |
| Nebulizer Pressure | 35 psig |
| Capillary Voltage | 3,500 V |
| Nozzle Voltage | 1,000 V |
| Fragmentor Voltage | 175 V |
| **Acquisition MS** | |
| Acquisition Mode | Extended dynamic range |
| Mass Range | *m/z* 300–3,200 |
| Data Acquisition Rate | 2 spectra/s |
| **Acquisition MS/MS** | |
| Acquisition Mode | Auto MS/MS |
| Mass Range | *m/z* 50–3,200 |
| Data Acquisition Rate | 8 spectra/s |
| Isolation Width | Medium (*m/z* 4) |
| Collision Energy | Formula for all charge states: (4 × (*m/z*))/100 – 4.8 V |
| Max Precursors Per Cycle | 8 |
| Precursor Selection | – Precursor threshold: 1,000 counts/0.01%<br>– Isotope model: Peptides<br>– Charge state: 2, 3, > 3, unknown<br>– Sort precursor by abundance only<br>– Active exclusion after 2 spectra (released after 1 min) |

## Results and discussion

LC/MS was employed for the taxonomic authentication of insect-derived ingredients in a selection of commercially available cricket-based protein bars. Prior to protein extraction, samples were cryogenically ground and delipidated using hexane to remove interfering lipids and improve extraction efficiency. Isolated proteins were subsequently reduced, alkylated, and digested with trypsin before LC/MS analysis. Peptides were separated on an octadecyl reversed-phase column over a 90-minute acetonitrile gradient, and MS/MS data were collected in data-dependent acquisition mode. Total ion chromatograms (TICs) of the tryptic digests are displayed in Figure 1.

Chromatographic profiles varied substantially across the different protein bars. The resulting MS/MS data were subsequently subjected to protein database searching. On average, over 78,600 MS/MS spectra (n = 3) were submitted, and over 8750 MS/MS spectra could be confidently assigned to peptides. This corresponds to an average identification rate of approximately 11%, which is typical for high-complexity food matrices such as protein bars (Table 4).

**Table 4.** Summary of identification metrics obtained from database searches for each sample and replicates (n = 3), including total MS/MS spectra submitted, peptide matches, uniquely identified peptides, and overall identification percentage.

| Sample Name (n = 3) | MS/MS Spectra Submitted to Database | Peptide Matches | Uniquely Identified Peptides | % ID |
|---|---|---|---|---|
| NBAR | 74,716 | 7,730 | 946 | 10.3 |
| C-BAR01 | 78,801 | 7,169 | 931 | 9.1 |
| C-BAR02 | 78,806 | 7,703 | 926 | 9.8 |
| C-BAR03 | 81,106 | 7,546 | 803 | 9.3 |
| C-BAR04 | 79,033 | 10,638 | 1,359 | 13.5 |
| C-BAR05 | 79,636 | 11,731 | 1,536 | 14.7 |
| **Average** | **78,683** | **8,753** | **1,084** | **11.1** |

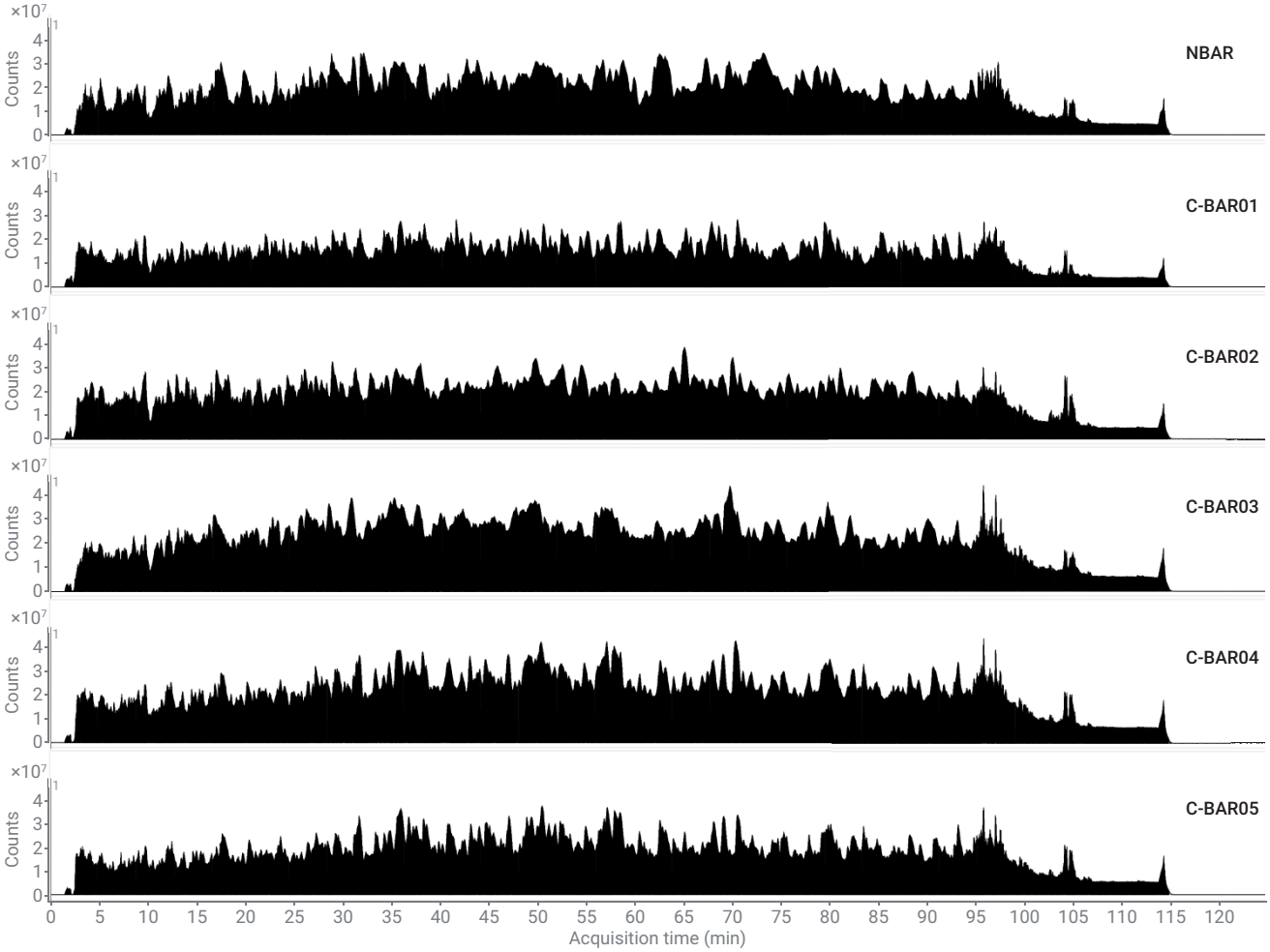


**Figure 1.** Comparison of LC/MS total ion chromatogram profiles of tryptic digests from protein bars.

To further assess peptide uniqueness and support species-level identification, the open-source web application Unipept was used. It maps tryptic peptides to taxa using the UniProtKB database, enabling species-level or higher-level identification. This peptide-centric approach allows for taxonomic authentication of complex mixtures, helping assess the specificity and reliability of taxonomic assignments.

Table 5 summarizes the unique peptides identified and classified according to their taxonomic origin. For example, in sample N-BAR, a total of 378 peptides were assigned to the plant order *Fabales*, which includes legumes. Notably,

**Table 5.** Taxonomic distribution of unique peptides identified by Unipept across protein bar samples (n = 3), based on protein identification via database search.

| | Sample Names | | | | | |
|---|---|---|---|---|---|---|
| | **N-BAR** | **C-BAR01** | **C-BAR02** | **C-BAR03** | **C-BAR04** | **C-BAR05** |
| **Unique Peptides** | **946** | **931** | **926** | **803** | **1359** | **1536** |
| **Metazoa (kingdom)** | **206** | **45** | **49** | **58** | **59** | **79** |
| *Ruminantia (suborder)* | 139 | 9 | 2 | 23 | 19 | 20 |
| Bovinae (subfamily) | 61 | 5 | 0 | 11 | 9 | 10 |
| Bos (genus) | 17 | 0 | 0 | 2 | 1 | 1 |
| ***Anthropoda (phylum)*** | **0** | **28** | **33** | **25** | **31** | **41** |
| **Gryllinae (subfamily)** | **0** | **10** | **12** | **12** | **12** | **12** |
| **Acheta domesticus (Cricket)** | **0** | **4** | **5** | **5** | **5** | **5** |
| **Viridiplantae (kingdom)** | **531** | **549** | **598** | **474** | **834** | **887** |
| *Magnoliopsida (Class)* | 495 | 518 | 571 | 434 | 775 | 819 |
| Malvales (order) | 12 | 0 | 4 | 20 | 25 | 26 |
| Theobroma (genus) | 11 | 0 | 3 | 17 | 20 | 22 |
| Theobroma cacao (species) | 8 | 0 | 3 | 14 | 17 | 18 |
| Caryophyllales (order) | 0 | 0 | 31 | 0 | 0 | 0 |
| Fagopyrum (genus) | 0 | 0 | 31 | 0 | 0 | 0 |
| Fagopyrum esculentum (species) | 0 | 0 | 25 | 0 | 0 | 0 |
| Sapindales (order) | 0 | 20 | 0 | 0 | 0 | 0 |
| Anacardium (genus) | 0 | 20 | 0 | 0 | 0 | 0 |
| Anacardium occidentale (species) | 0 | 20 | 0 | 0 | 0 | 0 |
| Malpighiales (order) | 0 | 2 | 0 | 0 | 0 | 0 |
| Linum (genus) | 0 | 2 | 0 | 0 | 0 | 0 |
| Linum usitatissimum (species) | 0 | 2 | 0 | 0 | 0 | 0 |
| Rosales (order) | 0 | 2 | 58 | 1 | 2 | 1 |
| Prunus (genus) | 0 | 2 | 57 | 1 | 2 | 1 |
| Prunus dulcis (species) | 0 | 0 | 9 | 0 | 0 | 0 |
| Fabales (order) | 378 | 373 | 379 | 343 | 404 | 376 |
| Glycine subgen. Soja (subgenus) | 283 | 249 | 249 | 258 | 275 | 283 |
| Glycine max (species) | 12 | 12 | 12 | 12 | 12 | 11 |
| Arachis (genus) | 0 | 43 | 42 | 0 | 37 | 0 |
| Arachis hypogaea (species) | 0 | 7 | 5 | 0 | 5 | 0 |
| Poales (order) | 47 | 33 | 36 | 1 | 242 | 288 |
| Oryza (genus) | 36 | 3 | 1 | 0 | 154 | 174 |
| Oryza sativa (species) | 2 | 0 | 0 | 0 | 4 | 4 |
| Avena (genus) | 0 | 20 | 23 | 0 | 0 | 0 |
| Avena Sativa (species) | 0 | 14 | 17 | 0 | 0 | 0 |
| Arecales (order) | 0 | 12 | 0 | 9 | 0 | 0 |
| Cocos (genus) | 0 | 5 | 0 | 3 | 0 | 0 |
| Cocos nucifera (species) | 0 | 5 | 0 | 3 | 0 | 0 |

this analysis enables differentiation between soybean species, as soybeans can originate from either the wild East Asian species (*Glycine soja*) or the cultivated soybean (*Glycine max*).[6] Although these two species are closely related, the current approach successfully identified 12 peptides uniquely mapped to *Glycine max*, confirming the presence of the cultivated type in the protein bars and supporting the authenticity of the declared ingredients. Interestingly, the main ingredients declared for C-BAR02—such as almond, peanut, oat, and buckwheat (Table 1) are reflected in the taxonomy of the identified unique peptides, corresponding to *Prunus dulcis*, *Arachis hypogaea*, *Avena sativa*, and *Fagopyrum esculentum*, respectively.

Focusing on the presence of insect-derived peptides, samples C-BAR01 to C-BAR05 revealed between 25 and 41 peptides assigned to the *Arthropoda* phylum, most likely originating from house crickets, as indicated in the ingredient list (Table 1). Among these, between 10 and 12 peptides specifically originate from the *Gryllinae* subfamily. It is important to mention that not all identified peptides are present in each sample, meaning that the sequences detected may differ from one sample to another. More interestingly, five distinct peptides were found to be unique to the species *A. domesticus*, supporting the taxonomic identification at the species level. It could be suggested that these peptides be used as indicator peptides for label compliance. In contrast, the noninsect control sample (N-BAR) yielded no peptides assignable to insect-related proteins, confirming its suitability as a negative control for taxonomic authentication.

These five peptides correspond to three proteins: Apolipophorin-III (19.8 kDa, UniProt: Q16989), Tropomyosin 1 (23.0 kDa, UniProt: A0A4P8D324) and Tropomyosin 2 (23.0 kDa, UniProt: A0A4V1DVH3). Other insect-specific peptides support these protein identifications. Their sequences, taxonomy, retention times, charge states, and *m/z* are provided in Tables 6 to 8. The corresponding summed extracted ion chromatograms of the unique peptides (EICs, 20 ppm mass accuracy) for sample N-BAR and C-BAR04 are shown in Figure 2. Additionally, MS/MS spectra of these specific peptides are reported in Figures 3A to 7A. Fragmentation patterns, including b- and y-series ions, confirm the peptide sequences with high confidence. EIC areas collected in the different samples (n = 3) are presented in Figures 3B to 7B. These were used

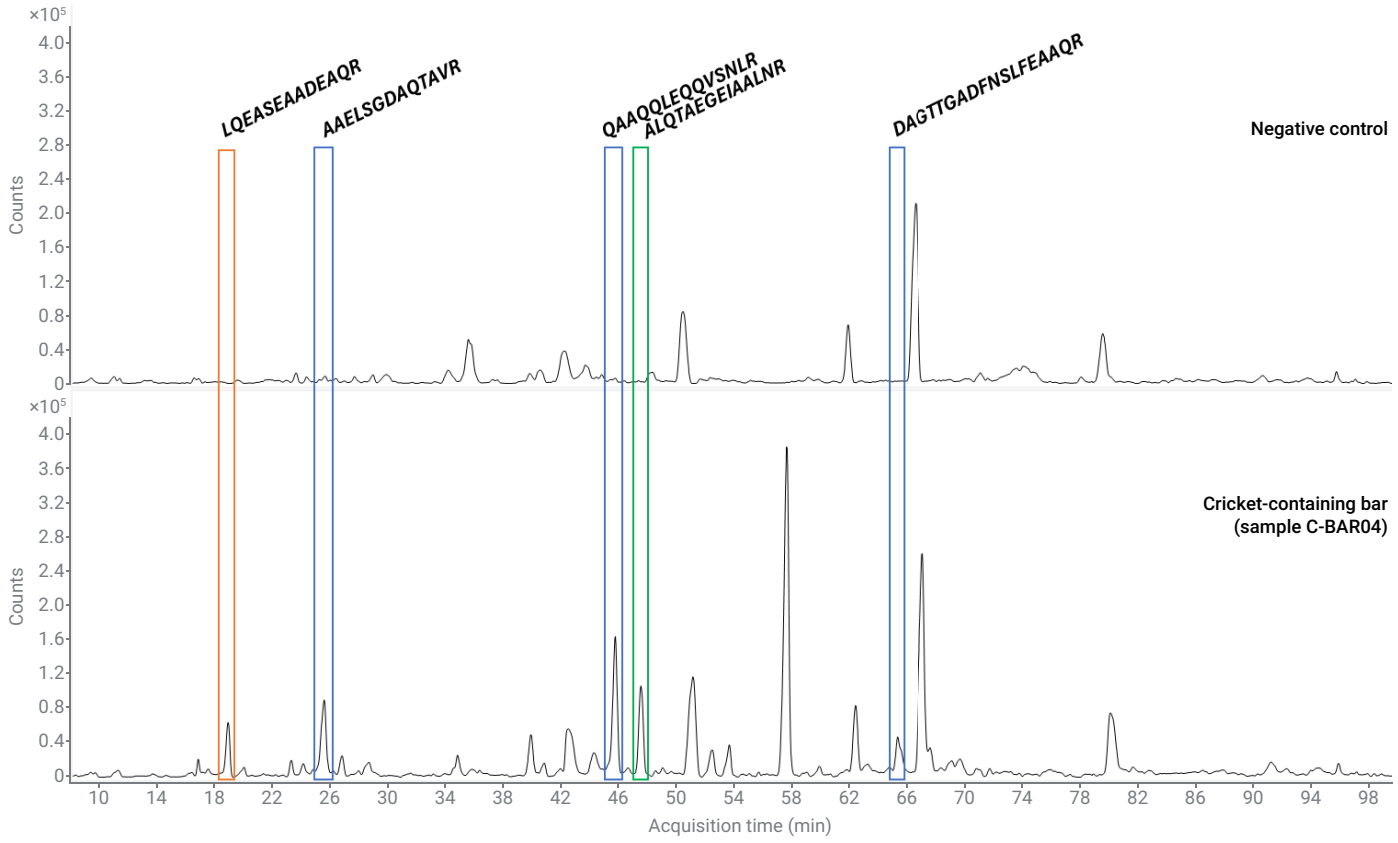


**Figure 2.** Summed EICs of *A. domesticus* specific peptides observed in C-BAR04 and in negative control. (blue = Apolipophorin-III, orange = Tropomyosin 1, green = Tropomyosin 2).

to assess the presence of the *A. domesticus* taxon-specific peptides across the different samples. It can be noticed that they were consistently detected in each insect-containing protein bar, while they were absent in the negative control sample (N-BAR). Interestingly, the intensity of the detected signals (EIC peak area), illustrated with error bars, appears to correlate with the relative amount of cricket-derived material present in the different bars, suggesting a potential semiquantitative application of the method.

Importantly, Apolipophorin-III and Tropomyosins are known to exhibit allergenic potential in edible insects.[7] Tropomyosins are well-characterized pan-allergens in invertebrates, with high thermal stability and conserved IgE-binding epitopes, contributing to cross-reactivity with shellfish allergens.[8,9] Apolipophorin-III, while primarily involved in lipid transport and immune response, has also been identified as a potential allergen in insect allergen profiling studies.[10,11] The presence of these proteins in cricket-based bars not only supports species-level identification but also raises considerations for allergen monitoring. Notably, these five peptides may serve as a species-specific biomarker for *A. domesticus* with relevance for allergen detection.

Overall, this study demonstrates the value of LC/MS for ingredient authentication in complex food matrices, using curated databases and taxonomic tools to ensure confident species-level assignment and support product transparency.

**Table 6.** Peptides assigned to Apolipophorin-III. Peptides in bold are specific to *A. domesticus*; others are assigned to the *Gryllinae* subfamily.

| Protein | Sequence | Taxonomy | RT (min) | z | *m/z* |
|---|---|---|---|---|---|
| Apolipophorin-III | **AAELSGDAQTAVR** | ***Acheta domesticus*** | **25.4** | **2** | **644.8282** |
| | **DAGTTGADFNSLFEAAQR** | ***Acheta domesticus*** | **65.2** | **2** | **935.9319** |
| | **QAAQQLEQQVSNLR** | ***Acheta domesticus*** | **45.6** | **2** | **806.9237** |
| | QQFPDGAQAADK | *Gryllinae* (subfamily) | 22.4 | 2 | 638.3018 |
| | RVQEAVQPHADAVAESLK | *Gryllinae* (subfamily) | 34 | 2 | 974.5160 |
| | | | | 3 | 650.0131 |
| | TAVEQATVLTNQVQQSVQQAANAH | *Gryllinae* (subfamily) | 64.8 | 2 | 1,268.6411 |
| | | | | 3 | 846.0965 |
| | TQLQTHAQTFANNLQAAATQFNEK | *Gryllinae* (subfamily) | 59.7 | 2 | 1,338.1622 |
| | | | | 3 | 892.4439 |
| | VQEAVQPHADAVAESLK | *Gryllinae* (subfamily) | 37.2 | 2 | 896.4654 |

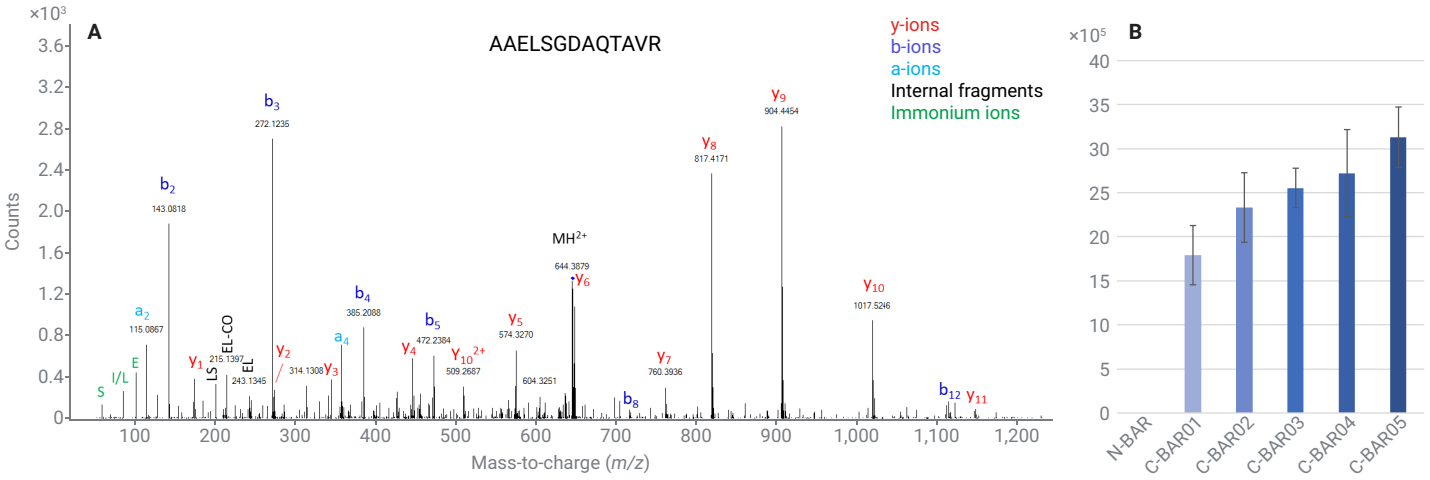


**Figure 3.** (A) Annotated MS/MS spectrum of the peptide AAELSGDAQTAVR (precursor ion: *m/z* 644.8282, charge state z = 2), identified as taxon-specific to *A. domesticus*. (B) EIC area of the peptide AAELSGDAQTAVR across multiple samples (n = 3).

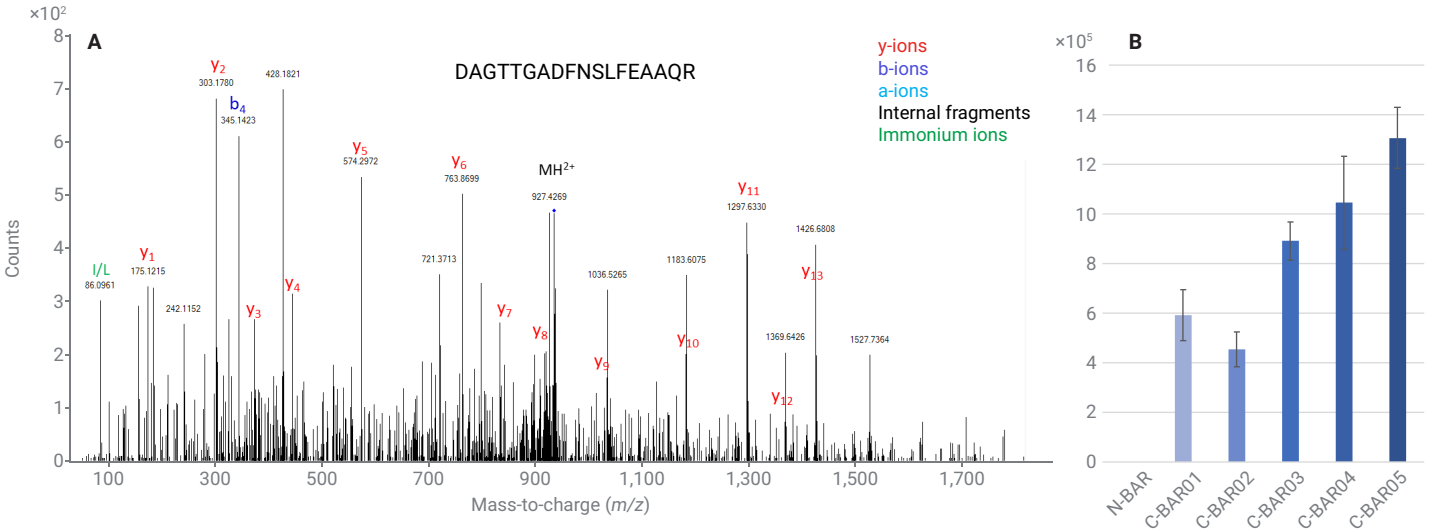


**Figure 4.** (A) Annotated MS/MS spectrum of the peptide DAGTTGADFNSLFEAAQR (precursor ion: *m/z* 935.9319, charge state z = 2), identified as taxon-specific to *A. domesticus*. (B) EIC area of the peptide DAGTTGADFNSLFEAAQR across multiple samples (n = 3).

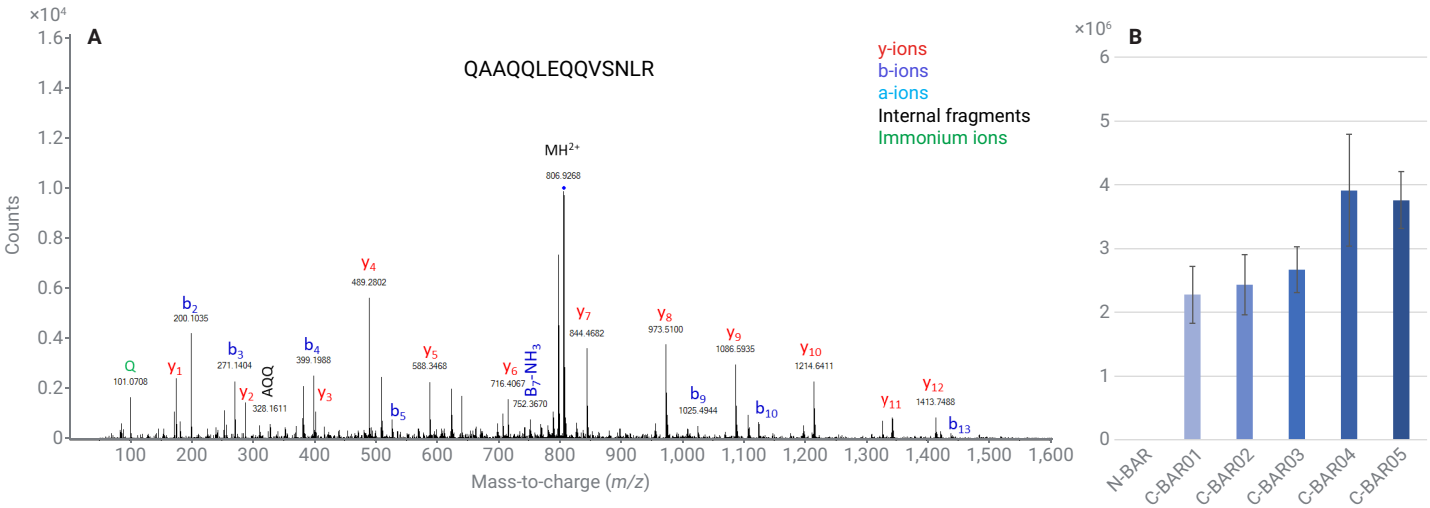


**Figure 5.** (A) Annotated MS/MS spectrum of the peptide QAAQQLEQQVSNLR (precursor ion: *m/z* 806.9237, charge state z = 2), identified as taxon-specific to *A. domesticus*. (B) EIC area of the peptide QAAQQLEQQVSNLR across multiple samples (n = 3).

**Table 7.** Insect peptides assigned to Tropomyosin 1. Peptide in bold is specific to *A. domesticus*; others are assigned to the *Gryllinae* subfamily.

| Protein | Sequence | Taxonomy | RT (min) | z | *m/z* |
|---|---|---|---|---|---|
| Tropomyosin 1 | ANLEQANKDLEDKEK | *Gryllinae* (subfamily) | 21.9 | 3 | 582.2952 |
| | DNAMDKADTCEGQAK | *Pterygota* (subclass) | 13.5 | 2 | 672.8788 |
| | INEDVQELTK | *Gryllinae* (subfamily) | 33.2 | 2 | 865.9372 |
| | KLAQVENDLITTK | *Gryllinae* (subfamily) | 39.9 | 2 | 736.9196 |
| | LAQVENDLITTK | *Gryllinae* (subfamily) | 43.5 | 2 | 672.8721 |
| | **LQEASEAADEAQR** | ***Acheta domesticus*** | **18.8** | **2** | **709.3313** |

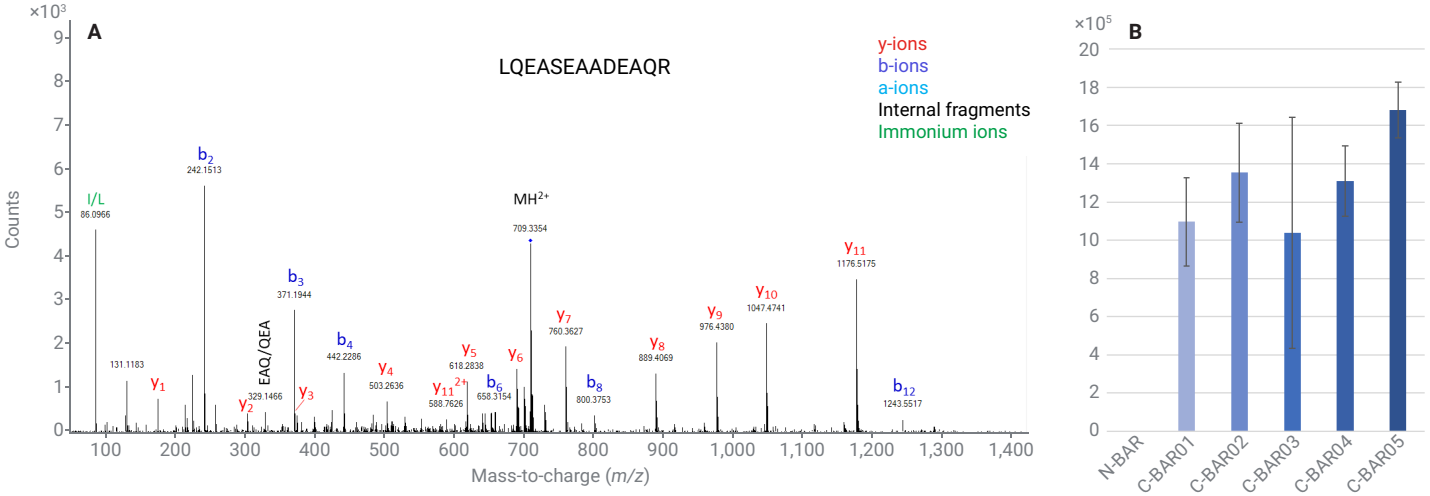


**Figure 6.** (A) Annotated MS/MS spectrum of the peptide LQEASEAADEAQKR (precursor ion: *m/z* 709.3313, charge state z = 2), identified as taxon-specific to *A. domesticus*. (B) EIC area of the peptide LQEASEAADEAQKR across multiple samples (n = 3).

**Table 8.** Insect peptides assigned to Trpomyosin 2. Peptide in bold is specific to *A. domesticus*; others are assigned to the *Gryllinae* subfamily.

| Protein | Sequence | Taxonomy | RT (min) | z | *m/z* |
|---|---|---|---|---|---|
| Tropomyosin 2 | ALLCEQQAR | *Pterygota* (subclass) | 24.0 | 2 | 544.7795 |
| | **ALQTAEGEIAALNR** | ***Acheta domesticus*** | **47.6** | **2** | **728.8914** |
| | IQTIENELDQTQEQLMQVNAK | Insecta (class) | 68.3 | 2 | 1,237.1156 |
| | | | | 3 | 825.0795 |
| | KIQTIENELDQTQEQLMQVNAK | Insecta (class) | 64.2 | 3 | 867.7778 |

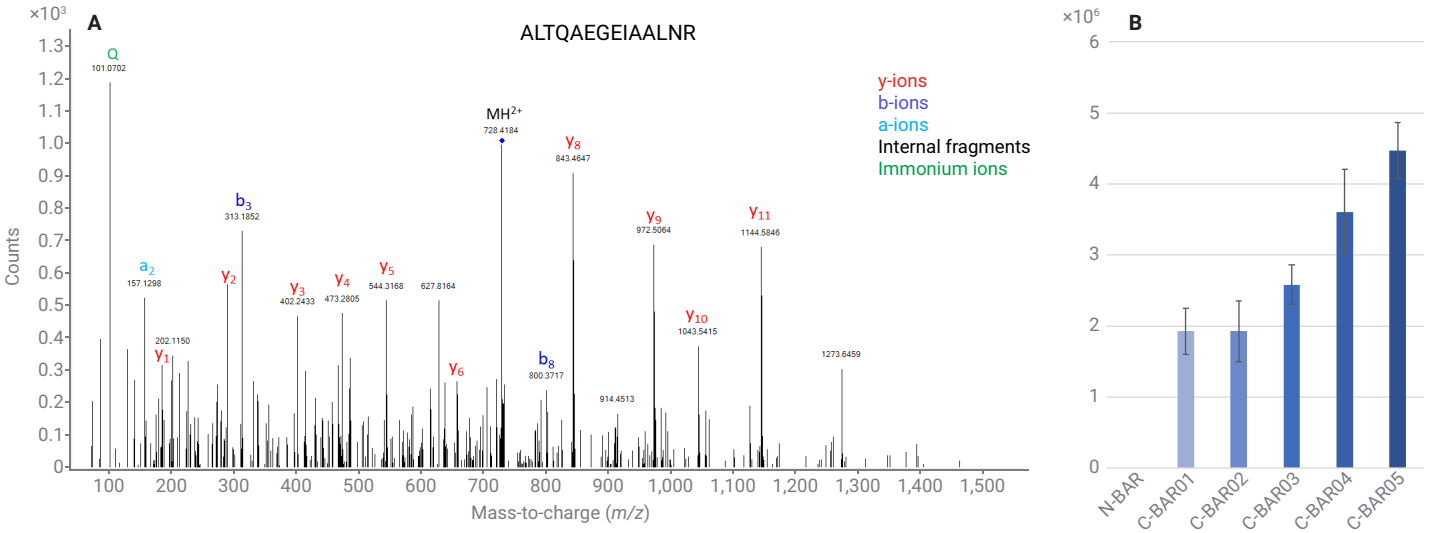


**Figure 7.** (A) Annotated MS/MS spectrum of the peptide ALQTAEGEIAALNR (precursor ion: *m/z* 728.8914, charge state z = 2), identified as taxon-specific to *A. domesticus*. (B) EIC area of the peptide ALQTAEGEIAALNR across multiple samples (n = 3).

## Conclusion

This application note highlights the power of LC/MS for authenticating *A. domesticus* in commercial protein bars. The combination of the Agilent 1290 Infinity III Bio LC and Agilent 6545XT AdvanceBio LC/Q-TOF offers a robust, precise, and biocompatible front-end with a high-resolving, accurate, and sensitive backend incorporating smart data-dependent acquisition. Species-specific peptides from Apolipophorin-III and Tropomyosins were confidently identified using curated databases and taxonomic tools. The consistent detection of five peptides supports their role as robust markers for *A. domesticus*, with particular attention to peptides LQEASEAADEAQKR and ALQTAEGEIAALNR, which may be relevant for allergen monitoring. Beyond species authentication, the workflow also enables verification of major ingredients, supporting label compliance and demonstrating the method's value for food transparency and safety in complex matrices.

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