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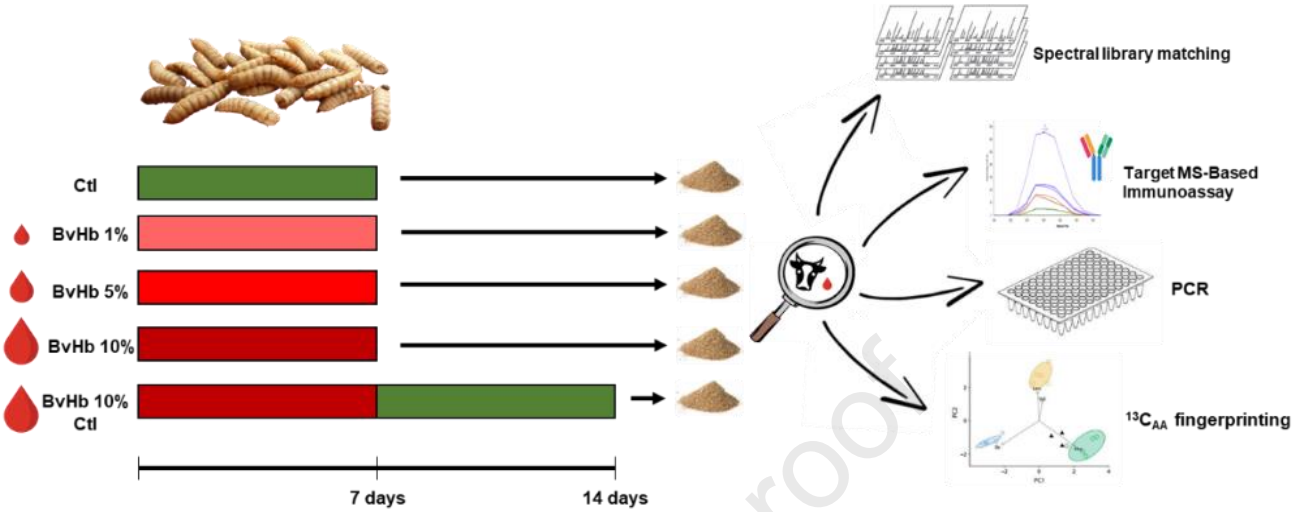
On the behalf of all the co-author's, we affiliate the work performed in the manuscript "Future feed control – Tracing banned bovine material in insect meal" with the different institutions (IMR; CRA-W, Signatope, BfR, Max Planck Institue, University of Namur, WUR University, NMI Natural and Medical Sciences Institute at the University of Tuebingen and Leiden University), we have no conflicts of interest, and declare that we have contributed to the acquisition, analysis and interpretation of data.

**Graphical abstract**



# Future feed control – Tracing banned bovine material in insect meal

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**Abstract**

In the present study, we assessed if different legacy and novel molecular analyses approaches can detect and trace prohibited bovine material in insects reared to produce processed animal protein (PAP). Newly hatched black soldier fly (BSF) larvae were fed one of the four diets for seven days; a control feeding medium (Ctl), control feed spiked with bovine hemoglobin powder (BvHb) at 1% (wet weight, w/w) (BvHb 1%, w/w), 5% (BvHb 5%, w/w) and 10% (BvHb 10%, w/w). Another dietary group of BSF larvae, namely *BvHb 10%, were first grown on BvHb 10% (w/w), and after seven days separated from the residual material and placed in another container with control diet for seven additional days. Presence of ruminant material in insect feed and in BSF larvae was assessed in five different laboratories using (*i*) real time-PCR analysis, (*ii*) multi-target ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), (*iii*) protein-centric immunoaffinity-LC-MS/MS, *(iv)* peptide-centric immunoaffinity-LC-MS/MS, (*v*) tandem mass spectral library matching (SLM), and (*vi*) compound specific amino acid analysis (CSIA). All methods investigated detected ruminant DNA or BvHb in specific insect feed media and in BSF larvae, respectively. However, each method assessed, displayed distinct shortcomings, which precluded detection of prohibited material versus non-prohibited ruminant material in some instances. Taken together, these findings indicate that detection of prohibited material in the insect-PAP feed chain requires a tiered combined use of complementary molecular analysis approaches. We therefore advocate the use of a combined multi-tier molecular analysis suite for the detection, differentiation and tracing of prohibited material in insect-PAP based feed chains and endorse ongoing efforts to extend the currently available battery of PAP detection approaches with MS based techniques and possibly $\delta^{13}C_{AA}$ fingerprinting.

**Keywords:** Feed control; BSF larvae; Proteomics; Carbon isotope fingerprinting of amino acids; qPCR; Spectral libraries.

**Abbreviations**

(PAP), Processed Animal Proteins; (BvHb), Bovine Hemoglobin powder; (BSF), Black Soldier Fly; (UHPLC-MS/MS), Multi-target Ultra-High-performance Liquid Chromatography coupled to tandem Mass Spectrometry; (TSE), Transmissible Spongiform Encephalopathies; (BSE), Bovine Spongiform Encephalopathies; (SOP), Standard Operating Procedures; (EURL-AP), European Union Reference Laboratory for Animal Protein; (SLM), Spectral library matching; (ULOQ), Upper limit of quantification; (CSIA), Compound specific stable isotope patterns of amino acids; (AA), Amino acid; (MRM), multiple reaction monitoring; (GC), Gas chromatography; (PCA), Principal component analysis.

## 1. Introduction

Research on the use of insects as feed ingredients for terrestrial and aquatic animals has developed rapidly in the last five years. By 2017, seven different insect species have been authorized for use in feed for farmed fish (EU Regulation 2017/893). Among these species, black soldier fly (BSF) (*Hermetia illucens*) is considered one of the most relevant species for the production of insect ingredients for fish feed (Belghit et al., 2019). The production of BSF larvae yields fish feed ingredients of high nutritive qualities, and offers certain environmental benefits since these production animals have exceptionally fast growth rates, and efficiently convert low-grade organic matter into high-value protein and fat compounds (Ewald et al., 2020; Liland et al., 2017). According to EU regulation 2017/893, insects reared to produce processed animal protein (PAP) are to be considered as farmed animals. Consequently, just like any other farmed animal species in the EU, insects are subject to the same rules established for the prevention of transmissible spongiform encephalopathies (TSE).

In the EU, following an outbreak of bovine spongiform encephalopathies (BSE) in the early 90s, the use of all mammalian-derived proteins in farmed ruminants was banned in 1994. The ban was extended in 2001 to a new regulation, which generally prohibited the use of PAP (except for use in fish meal) and the use of blood products in feed for any farmed animal, respectively (EC, 2001, EC, 2003). In 2013, the EU has set out a progressive working plan for the re-authorization of non-ruminant PAP and blood product in aquafeed (EC, 2011; 2013). This partial re-authorization of PAP gave rise to new regulatory challenges and called for the development and validation of sensitive analytical approaches, which allow for both species and tissue specific differentiation of PAP in feed to differentiate authorized from non-authorized use (Lecrenier et al., 2016; Rasinger et al., 2016).

To guarantee that the use of PAP in feed is in line with current legislation, standard operating procedures (SOP) have been established by the European Union Reference Laboratory for Animal Protein (EURL-AP) for the control of feed stuffs. Optical light microscopy has been the first official method for the detection and characterization of PAP in feed (EC, 2009). However, species-specific identification of PAP is not achievable with microscopy (EC, 2013). This shortcoming led to the development of a second official method, the EURL-AP validated qualitative polymerase chain reaction (qPCR) for ruminant DNA-detection (Fumière et al., 2009; EURL-AP 2013). Even though qPCR is rapid and sensitive, this method is not tissue specific. For example, authorized milk powder cannot be differentiated from prohibited PAP or blood products from the same species (Lecrenier et al., 2020). Therefore, additional approaches have been developed which allow for the determination of both species and tissue specific origin of PAP and blood products in animal feeds (Lecrenier et al., 2018; Marbaix et al., 2016; Rasinger et al., 2016; Steinhilber et al., 2019).

Proteomic-based methods using (tandem) mass spectrometry (MS) were, in a recent scientific opinion by the European Food Safety Authority (EFSA), identified as promising tools to complement current

standard techniques of PAP detection in feed (EFSA, 2018). Different laboratories specialized in feed and food safety analyses have been developing complementary MS-based approaches for identification and quantification of peptide markers as protein surrogates for the detection of prohibited PAP and blood products. Among those, targeted MS-methods have been established for detection of bovine specific PAP and blood products as well as permitted ruminant milk products in feed material (at 0.1%, w/w) (Lecrenier et al., 2018; Marchis et al., 2017). The detection of species-specific blood peptides in feed matrices (between 0.05 and 1%, w/w) has also been shown to be useful by applying antibody-based enrichment approaches prior LC-MS/MS read out (Niedzwiecka et al., 2019; Steinhilber et al., 2019). When genomic information is sparse or unavailable, untargeted MS approaches based on direct spectra comparisons and spectral library matching have been used to identify and quantify species and tissue-specific adulteration in food and feed (Belghit et al., 2019; Ohana et al., 2016; Rasinger et al., 2016; Wulff, Nielsen, Deelder, Jessen, & Palmblad, 2013).

In addition to proteomic-based tools, the detection of stable carbon isotope patterns of amino acids (AA) (hereafter $\delta^{13}C_{AA}$ fingerprinting), has shown great promise for food and feed authentication (Wang, Wan, Krogdahl, Johnson, & Larsen, 2019; Wang et al., 2018). The $\delta^{13}C_{AA}$ fingerprinting method can trace the biosynthetic origins of proteinogenic amino acids via two different routing mechanisms of their carbon skeletons. While there is little or no changes in the $\delta^{13}C$ values of the essential amino acids during trophic transfer, shifts in $\delta^{13}C$ values for the non-essential AAs can be considerable because animals can synthesize them *de novo* from building blocks derived from dietary macromolecules (McMahon, Fogel, Elsdon, & Thorrold, 2010; McMahon, Polito, Abel, McCarthy, & Thorrold, 2015). Since the $\delta^{13}C_{AA}$ fingerprints reflect diets over a time period that depends on the particular metabolic turnover rate of the analysed tissue, the method can in theory detect traces of feed material well after the feed sources have changed. This feature makes it highly complementary to our other tested molecular methods that are suited for detecting the most recent diets only.

The aim of this study was to compare the current official method (qPCR) to MS-based approaches and $\delta^{13}C_{AA}$ fingerprinting for detection of prohibited bovine material in BSF larvae that could be used as feed ingredients for farmed fish. BSF larvae were reared on substrate with or without added bovine hemoglobin powder at three different concentrations. Detection of ruminant material in (*i*) the feed media of BSF larvae and in (*ii*) the BSF larvae reared on the adulterated substrate were performed using (*i*) qPCR, (*ii*) multi-target UHPLC-MS/MS, (*iii*) protein-centric immunoaffinity-LC-MS/MS, *(iv)* peptide-centric immunoaffinity-LC-MS/MS, (*v*) tandem mass spectral library matching (SLM) and (*vi*) $\delta^{13}C_{AA}$ fingerprinting technique.

## 2. Materials and methods

### *2.1. Feed preparation*

The control feeding medium (Ctl) for the BSF larvae consisted of a standard poultry feed (Kasper Faunafood Kuikenopfokmeel 1, Woerden, The Netherlands, 600320), used as a reference feed medium for BSF larvae by the Laboratory of Entomology (Wageningen, The Netherlands). The control feed medium was spiked with bovine hemoglobin powder (BvHb) (92B, 06000-131-17-0705) at three different concentrations, as follows: (*i*) to 1098 g of ground poultry feed in a sampling bag was added 11.1 g of BvHb, to obtain 1% (w/w) spiked control diets (BvHb 1%), (*ii*) to 1054.5 g of ground poultry feed in a sampling bag was added 55.5 g of BvHb, to obtain 5% (w/w) spiked control diets (BvHb 5%), and (*iii*) to 999 g of ground poultry feed in a sampling bag was added 111 g of BvHb, to obtain 10% (w/w) spiked control diets (BvHb 10%). The design of the experiment is described in Table 1.

### *2.2. Rearing of BSF larvae and sample preparation*

The experiment was carried out at the Laboratory of Entomology (Wageningen, The Netherlands) with seven-day old BSF larvae taken from the stock colony of the Laboratory of Entomology. Experimental units were plastic containers (17.8 × 11.4 × 6.5 cm) to which a homogenized mixture of feed consisting of 18 g of the respective feed media (Ctl, BvHb 1%, BvHb 5% and BvHb 10%); 36 mL of water and ~100 BSF larvae were added. The containers were closed with perforated transparent plastic lids to allow for air exchange and were placed in a climate-controlled cabinet (27 ± 1 °C and 80 ± 1% RH). In addition to the four dietary groups (Ctl, BvHb 1%, BvHb 5% and BvHb 10% (w/w)), another dietary group of BSF larvae, namely *BvHb 10%, were first grown on BvHb 10% (w/w) medium, and after seven days separated from the residual material and placed in another container with control diet for seven additional days (decontamination period). At the end of the feeding experiment with a total feeding period of seven days for larvae grown on Ctl, BvHb 1%, BvHb 5%, BvHb 10% (w/w), and a period of 14 days for the decontamination treatment (*BvHb 10% (w/w)), larvae were separated from residual material, rinsed with lukewarm tap water, dried on tissue paper and immediately frozen at – 80 °C. Frozen BSF larvae were ground to a powder using a blender (Braun Multiquick 5 (600 W), Kronberg, Germany) and freeze-dried (freezing for 24 h at -20°C in vacuum (0.2–0.01 mBar) followed by vacuum at 25°C until constant weight was reached. Feed media and freeze-dried BSF larvae were divided into different fractions and distributed to different laboratories (laboratories A-E) for the multi-laboratory analyses: (*i*) qPCR (laboratories A and B), (*ii*) multi-target UHPLC-MS/MS (laboratory A), (*iii*) protein-centric immunoaffinity-LC-MS/MS (laboratory B), *(iv)* peptide-centric immunoaffinity-LC-MS/MS (laboratory C), (*v*) direct comparison of tandem mass spectra (laboratory D) and (*v*) $\delta\ ^{13}C_{AA}$ fingerprinting technique (laboratory E). The five dietary groups of BSF larvae were studied in biological duplicates at the five laboratories (n = 2).

### *2.3. Detection of bovine hemoglobin in the feeding media and in BSF larvae*

#### *2.3.1. Real time-PCR (laboratories A and B)*

Samples were characterized by real time-PCR according to EURL-AP Standard Operating Procedures 'DNA extraction using the "Wizard® Magnetic DNA purification system for Food" kit' and 'Detection of ruminant DNA in feed using real-time PCR' (https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/), as laid down in European Commission (EC) Regulation No 152/2009 (Commission, 2009). At laboratory A, PCR were performed on a LightCycler® 480 (Roche Diagnostics GmbH, Rotkreuz, Switzerland). The Ct values were calculated using the "Abs Quant/2nd Derivative max" analysis type of the LightCycler® 480 Software release 1.5.1.62 (Roche Diagnostics GmbH, Rotkreuz, Switzerland). At laboratory B, PCR was performed on a QuantStudio 6 flex thermocycler (ThermoFisher Scientific, Waltham, MA, USA) with automatic baseline setting and a fixed threshold of 0.04 in all experiments. All analyses were done with universal mastermix DMML-D2-D600 from Diagenode (Liège, Belgium). All samples were analysed in technical duplicates.

#### *2.3.2 Multi-target UHPLC-MS/MS (laboratory A)*

A multi-target UHPLC-MS/MS approach was used for the simultaneous detection of targeted ruminant blood and milk proteins. Protocols for protein extraction, digestion, peptide purification and MS analysis were based on the protocol described by Lecrenier et al. (2018) with minor changes. Before extraction, 1 µg of each heavy-labelled concatemers, used as internal standards, were spiked to 1 g of sample. Proteins were extracted in 10 mL of extraction buffer (200 mM TRIS-HCl, pH 9.2, 2 M urea) for 30 min by shaking at 20 °C followed by sonication for 15 min at 4 °C. Tubes were then centrifuged at 4660 g for 10 min at 4 °C and 5 mL of supernatant was transferred into new tubes. The protein extracts were diluted with 5 mL of 200 mM ammonium bicarbonate and reduced with 500 µL of 200 mM DTT at 20 °C for 45 min prior to alkylation with 500 µL of 400 mM IAA for 45 min in the dark at 20 °C. Subsequently, digestion was performed by adding 500 µL of trypsin (1 mg/mL in 50 mM acetic acid) for 1 h at 37 °C and trypsin action was stopped by the addition of 150 µL of 20% (v/v) formic acid in water. Tubes were then centrifuged at 4660 g at 4 °C for 10 min. Peptides were purified by reversed-phase extraction using Sep-Pak tC18 cartridges (Waters – Milford, Massachusetts, USA). Cartridge pre-conditioning was performed with 18 mL acetonitrile followed by equilibration with 18 mL of 0.1% (v/v) formic acid in water. Digested supernatant (10 mL) was loaded on the column. Next, 9 mL of 0.1% (v/v) formic acid in water was used to flush out impurities. Elution was then performed with 5 mL of acetonitrile/0.1% (v/v) formic acid in water 80/20 (v/v). Before evaporation at 45 °C using Centrivap, 15 µL of DMSO was added to each tube to prevent dryness. Finally, the pellets were resuspended in 375 µL of 0.1% (v/v) formic acid in water/acetonitrile 95/5 (v/v) and centrifuged at 4660 g for 10 min at 4 °C. The supernatants were transferred into a new tube and stored at -20 °C before injection.

Samples were analyzed using a Xevo TQS micro triple quadrupole system with a positive electrospray and multiple reaction monitoring (MRM) mode coupled with an Acquity system (Waters – Milford, Massachusetts, USA). Peptides were separated by reverse-phase liquid chromatography using a C18 Acquity BEH Waters column (2.1×100 mm). A gradient (Mobile phase A = 0.1% (v/v) formic acid in water (ULC/MS grade) and mobile phase B = 0.1% (v/v) formic acid in acetonitrile) of 16 min (at 0.2 mL/min) allowed the separation of the peptide biomarkers. Elution was carried out as follows: 0–2 min: 92% A; 2–10 min: 92–58% A; 10–10.10 min: 15% A; 10.10–12.50 min: 15% A; 12.50–12.60 min: 92% A, 12.60–16 min: 92% A. The acquisition and processing of data were carried out by MassLynx software (v. 4.1, Waters). The peptides described in previous studies were selected to be used as biomarkers for the detection of bovine hemoglobin, casein and beta-lactoglobulin (Lecrenier et al., 2018). All samples were extracted and analyzed in technical triplicates.

*2.3.3 Protein-centric immunoaffinity LC-MS/MS (laboratory B)*

Sample preparation and semiautomatic immunoprecipitation with an antibody raised against bovine hemoglobin for the MS-based immunoassays were previously described by Niedzwiecka et al. (2019) and Steinhilber et al. (2019). For the analysis of insects, some minor changes were made to the protocols. Based on the protocol by Niedzwiecka et al. (2019), a total amount of 1 g was used for sample preparation in 10% trichloroacetic acid and 2% 2-mercaptoethanol in acetone for 2 h at -20 °C. After washing, proteins were extracted using 7 M urea, 2 M thiourea and 12.5 µg/mL α-amylase in water. For semiautomatic immunoprecipitation, the amount of protein extract was changed to 1 mL to increase the maximum amount of hemoglobin available for immunoprecipitation. The samples were then digested with trypsin and analyzed as described in the original publication using a nano-LC-ESI-MS/MS maXis Impact UHR-TOF equipped with a nanoFlow ESI sprayer interface (Bruker, Bremen, Germany) and a 1290 Infinity nano high performance LC (Agilent Technologies, Waldbronn, Germany). LC and MS parameters were used without modifications from the protocol. All samples were extracted and analyzed in technical triplicates.

*2.3.4 Peptide-centric immunoaffinity LC-MS/MS (laboratory C)*

The peptide-centric immunoaffinity LC-MS/MS method was a modified version of the method previously published in Steinhilber et al. (2018). Two of the plasma protein markers (SERPINF2 and HP252) were removed from the assay to keep complement (C9) and α-2-macroglobulin (A2M), and the peptide for hemoglobin α-chain (HBA), myosin-7 (MYH7), matrilin-1 (MATN1) and osteopontin (OPN) were added. The chromatographic method was modified by using a faster trapping method (0.15 min at 150 µL/min) and a shorter separation method (8% to 50% eluent B in 3.0 min followed by a washing and equilibration step for 2.0 min, 1.5 µL/min flowrate). Peptide separation was performed on an Acclaim Pepmap RSLC C18 (75 µm I.D. × 150 mm, 3 µm, Thermo Fisher Scientific). Mass spectrometric detection was performed using a Sciex QTRAP 6500+ triple

quadrupole mass spectrometer operating in MRM mode. All samples were extracted and analyzed in technical triplicates.

*2.3.5 Spectral library matching (laboratory D)*

Protein extraction, quantification and digestion were performed as described in Belghit et al. (2019) and in Rasinger et al. (2016) without any modifications. The protein digest was analyzed by using nano-LC-ESI-MS/MS maXis Impact UHR-TOF (Bruker, Bremen, Germany) coupled with a UPLC Dionex UltiMate 3000 (Thermo). The digests were separated by reverse-phase liquid chromatography using a 1.0 mm X 15 cm reverse phase Thermo column (Acclaim PepMap 100 C18) in an Ultimate 3000 liquid chromatography system. Mobile phase A was 98 % of 0.1 % formic acid in water and 2 % acetonitrile. Mobile phase B was 0.1 % formic acid in acetonitrile. The flow rate was 30 µL/min. Mobile phase A was 95% water, 5% acetonitrile, 0.1% formic acid. Mobile phase B was 20% water, 80% acetonitrile, 0.1% formic acid. The digest (10 µl) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40 % in 75 min and from 40 % B to 95 % B in 10 min. The column effluent was directly connected to the MS. In survey scan, MS spectra were acquired for 0.5 s in the m/z range between 50 and 2200. The 10 most intense peptides ions 2+ or 3+ were sequenced. The collision-induced dissociation (CID) energy was automatically set according to mass to charge (m/z) ratio and charge state of the precursor ion. MaXis and Thermo systems were piloted by Compass HyStar 3.2 (Bruker). Mass spectrometry data generated were converted using DataAnalysis 4.2 (Bruker) and exported as mzML files. Bovine hemoglobin and milk data were searched against the bovine reference proteome obtained from UniProt (UP000009136; accessed on December 2020); insect data was matched against *Hermetia illucens* specific proteins (UniProtKB; accessed on December 2020) using X! Tandem (Craig & Beavis, 2004) as implemented in the Trans-Proteomics Pipeline (TPP) (Deutsch et al., 2015; Ohana et al., 2016). Spectral libraries were created using SpectraST (Version 5.0), as described in Lam, (2011), and all sample spectra were searched against their respective spectral libraries for relative quantification of BvHb (Deutsch et al., 2015). Dot products above 0.8 were considered as valid matches and used for quantification. The data used in this study and spectral libraries created are available on MassIVE (ftp://MSV000087026@massive.ucsd.edu). A graphical overview of the SLM workflow and an example output of matched spectra are shown in Supplementary Figures 1 and 2, respectively.

*2.3.6. Stable isotope analyses (laboratory E)*

The detailed procedure for AA hydrolyses, Gas Chromatogramy (GC) settings, derivatization, carbon correction and data calibration are described in Wang et al. (2018). In short, each sample of about 3 mg was hydrolyzed with 6 N HCl at 110 °C for 20 h before derivatizing the AAs to *N*-acetyl methyl esters following the protocols by Larsen et al. (2013) and Corr, Berstan, & Evershed, (2007). The AA derivatives were injected with an autosampler into a InertCap 35 column (60 m, 0.32mm i.d., 0.50 µm film thickness, GL Sciences) in a GC and then combusted on a Combustion Isotope Ratio Mass

Spectrometer (IRMS, Elementar Isoprime visION System, Langenselbold, Germany) at the Max Planck Institute for the Science of Human History, Jena Germany. Isotope data are expressed in delta ($\delta$) notation in per mil (‰) in per mil (‰): $\delta$ (‰) = [($R_{sample}/R_{standard}$) – 1] ×1000, where $R$ is the ratio of heavy to light isotope. The carbon isotope ratios are expressed relative to the international standards VPDB. Our in-house reference AA-mixture was calibrated against the n-alkane A7 mixture with well-established $\delta^{13}$C values (available from A. Schimmelmann, Biogeochemical Laboratories, Indiana University). All samples were analyzed in technical triplicates. The average standard deviation for the internal reference standard nor-leucine (Nle) was 0.3‰ (n = 3 for each batch) and the in-house amino acid standards ranged from 0.2‰ for Pro to 0.6‰ for Ala (n = 4–7 for each batch). We obtained the well-defined peaks for the following 15 amino acids: NEAA; alanine (Ala), asparagine/aspartic acid (Asx), glutamine/glutamic acid (Glx), glycine (Gly), proline (Pro), tyrosine (Tyr) and serine (Ser), and EAA; histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val). We also determined the bulk $\delta^{13}$C and $\delta^{15}$N values with the latter expressed relative to AIR. Approximately 1 milligram of the dry mass of diets and BSF larvae from each treatment were analyzed in duplicates for bulk carbon and nitrogen isotopes with an EA-IRMS in the Iso Analytical Limited Inc, UK. For quality control, internal lab standards (IA-R068, IA-R038, IA-R069) and a mixture of IAEA-C7 and IA-R-R046) were analyzed in between sample runs. These standards were calibrated against international reference material IAEA-CH-6, IAEA-N-1, IAEA-C-7 for both $\delta^{13}$C and $\delta^{15}$N. Internal standard yielded 1s = 0.03‰ and 0.03‰ for $\delta^{13}$C and $\delta^{15}$N respectively.

## 3. Results and discussion

In the EU, insects are considered farmed animals, and as such, are subject to the same legal standards as other production animals; this includes rules and regulations concerning the prevention and control of TSE. For efficient control and monitoring of compliance with current feed and food safety regulations, fast and sensitive analytical approaches complementary to the current official methods are required. To the best of our knowledge, this is the first study to compare the suitability of different legacy and novel molecular tools for the detection of prohibited blood products in insect feed and insect larvae, respectively. The data generated here, shows that each of the six analytical approaches applied, can detect the presence of BvHb in insect feed media and/or in BSF larvae. We also found that each method suffered from some inherent shortcomings in the detection of prohibited material in insect feed and insects; these can however easily be overcome if the tools discussed below are used in unison in tiered PAP-analysis systems.

### *3.1 Black soldier fly larvae development*

In general, adulteration of the feeding media with BvHb at 1%, 5% and 10% (w/w) prepared for the BSF growth trial supported similar larval development as Ctl-fed diets. Despite differences in non-essential $\delta\,^{13}C_{AA}$ patterns between dietary treatment groups (see Supplementary Table 7), there were no differences in survival (>95%) or growth (mean individual larval body mass *ca.* 180 mg at day 14 of larval development) between BSF larvae fed the control or feed media spiked with BvHb at 1%, 5% and 10% (w/w, data not shown). These results confirm previous findings on the ability of the BSF larvae to grow on adulterated feed media without affecting their survival or growth performance (Bosch, Fels-Klerx, Rijk, & Oonincx, 2017; Camenzuli et al., 2018).

### *3.2 Detection of bovine hemoglobin powder in the feeding media and in BSF larvae*

#### *3.2.1 qPCR*

Tables 2 and 3 provide a summary of qPCR results obtained for the detection of prohibited BvHb in the media used for the rearing of BSF larvae and for BSF larvae grown on these media, respectively. Unexpected results are marked in red (Tables 2 and 3). Detailed analysis outputs are presented in Supplementary Table 1. Feeding media adulterated with BvHb at the 1%, 5% and 10% (w/w) level were all correctly identified as positive for ruminant DNA (Table 2). Control feed media, which consisted of a standard poultry feed without BvHb adulteration, also were found to be positive for ruminant DNA by qPCR (laboratories A and B, Table 2 and Supplementary Table 1). As dictated by EU legislation, standard poultry feed, including feed material used in the present study, must not contain ruminant PAP or blood products. The positive result obtained by qPCR thus could indicate the presence of non-permitted ruminant material in control feed media. On the other hand, the positive finding also could be due to the presence of permitted feed ingredients of bovine origin such as milk.

At the lowest level of adulteration (1% (w/w) BvHb, Table 3, Supplementary Table 1) tested in the current study, qPCR performed by laboratory A confirmed the presence of ruminant DNA in BSF larvae. Real-time PCR, which is based on the detection of DNA, allows for amplification of minute amounts of target sequences specific to a species or group of species and in general displays very high sensitivities with respect to its target analytes (Fumière, Dubois, Baeten, von Holst, & Berben, 2006; Olsvik et al., 2017; Tanabe et al., 2007). Therefore, qPCR can detect less than 0.1% (w/w) in mass fraction of PAP or blood products in feed and in feed ingredients, respectively. However, when applying the same official qPCR assay in another laboratory (B), in the insect larvae fed the BvHb 1% (w/w) diet, ruminant DNA was not detected (Table 3, Supplementary Table 1). In cases of trace levels of ruminant DNA contamination, interlaboratory differences for ruminant PAP detection using the EURL-validated qPCR assay have been described before. For example, Olsvik et al. (2017) reports on qPCR data obtained at three different national reference laboratories, which analyzed 19 non-ruminant PAP and compared these data to results obtained using an immunoassay-based method. Ruminant PAP was detected in five out of 19 samples and in accordance with the findings of the present study, methodological and multi-laboratory differences for qPCR assay results were reported (Olsvik, et al., 2017). The authors speculated that the observed differences in the results obtained might be due to a shift in the normal distribution of Ct-values close to the cut-off of the PCR assay, PCR inhibition or different process during homogenization and grinding step (Olsvik et al., 2017).

*3.2.2 LC-MS/MS-based approaches*

Contrary to current legislation on PAP, qPCR does not distinguish between non-authorized and authorized ruminant products such as bovine milk (EFSA, 2018). When tissue specificity is the goal, proteomics approaches can be applied to complement and refine current methods of PAP detection (Rasinger et al., 2016). In 2014, EURL-AP initiated an international laboratory network to investigate and develop alternative techniques for PAP detection including, MS-based techniques, immunoassays or spectroscopic methods to complement current standard analytic approaches (Lecrenier, et al., 2020; van Raamsdonk, et al., 2019). MS-based proteomic approaches were listed among the most promising methods for complementing current standard techniques of feed PAP and blood products detection in a report published by EFSA (EFSA, 2018). The potential of MS-based methods for resolving current challenges of official regulatory PAP analyses recently was confirmed in an inter-laboratory study performed across five different European laboratories in which different MS-based protocols for detection of prohibited bovine material in feed samples were compared (Lecrenier, et al., 2021). The study concluded that MS-based analyses efficiently identified non-authorized bovine protein in feed sample mixes at an adulteration level of 1% (w/w) (Lecrenier et al., 2021). The finding by Lecrenier et al. (2021) is further corroborated by results obtained in the present work in which four different MS-based analyses protocols were applied to detect BvHb in the insect-PAP feed chain. Two

complementary proteomic approaches were used; (*i*) targeted MS with or without the use of stable isotope-labeled standards (laboratories A, B and C) and (*ii*) SLM (laboratory D).

Targeted MS (laboratories A, B and C) positively identified bovine haemoglobin powder in feeding media spiked with 1%, 5% or 10% (w/w) BvHb (Tables 2 and Supplementary Tables 2-3). When using non-targeted SLM (laboratory D), a linear increase of bovine specific peptides was observed in the feeding media with increasing concentrations of BvHb (Supplementary Tables 4-5). Multi-target UHPLC-MS/MS (laboratory A), SLM (laboratory D) and peptide-centric immunoaffinity LC-MS/MS (laboratory C) (Table 2 and Supplementary Tables 2-5) detected the presence of bovine hemoglobin also in control feeding media. However, determined abundances of BvHb in Ctl media were very low when compared to feeding media spiked with 1%, 5% or 10% (w/w) BvHb (Supplementary Tables 3-5). For example, using quantitative peptide-centric immunoaffinity LC-MS/MS (laboratory C), in control feed, 19.0 ±1,3 fmol of BvHb specific peptide, bovine hemoglobine α chain (HBA), were detected, whereas at the 1% (w/w) level of BvHb adulteration, over 15000 fmol of HBA were measured; at 5% and 10% (w/w) BvHb in feed, levels of HBA were above the upper limit of quantification (Supplementary Table 3). As was discussed above, control feeding media consisted of standard poultry feed, which should be free of ruminant PAP or blood, but ruminant DNA was detected in these samples by qPCR (Table 2 and Supplementary Table 1). Since three of the MS datasets obtained also were indicative of the control feeding media being contaminated with bovine hemoglobin, the positive finding of the qPCR analyses could indeed indicate that the poultry feed used as control diet in the present study was indeed contaminated with trace amounts of ruminant blood products or blood meal. In addition to bovine specific blood proteins, bovine plasma proteins were detected by peptide-centric immunoaffinity LC-MS/MS (laboratory C), presumably being plasma residues of the BvHb preparation. All MS-based methods investigated, also positively identified bovine milk peptides in the standard chicken feed, which was used as control feeding media in the present study (β-lactoglobulin, casein or osteopontin Table 2 and Supplementary Tables 2-6).

In the BSF larvae fed control feed media or feed adulterated with BvHb at 1% (w/w) level, only peptide-centric immunoaffinity LC-MS/MS detected the presence of bovine blood (Figure 1A, Table 3). One reason as to why the remaining MS approaches failed to detect BvHb in the BSF larvae at the 1% (w/w) level might be the lower sensitivity of these methods compared to the immunoaffinity-based approach. Also, the fact that SLM method detected the presence of BvHb in the BSF larvae in a linear manner with increasing concentration of BvHb only at 5% and 10% (w/w) but not at 1% (w/w) (Figure 1B and Supplementary Table 6) points to a lack of sensitivity of these approaches when compared to the immunoaffinity-based approach. When using multi-target UHPLC-MS/MS method (laboratory A), only one of the two replicate samples of BSF larvae fed diets adulterated with 5% was positive for BvHb (Table 3). These results are probably due to differences in homogeneity and particle size distribution between the two replicate samples. As described earlier, the heterogeneity of

the samples can interfere with the correct detection of specific peptide in certain matrices (Marbraix et al., 2016). Taken together, our data indicate that, as with classic PAP, also for detection and differentiation of insect PAP, LC-MS/MS-based proteomics show great potential to resolve current analytical gaps but technical challenges remain to be addressed in the future.

*3.2.3 $\delta^{13}C_{AA}$ fingerprinting method*

In the current study, $\delta^{13}C_{AA}$ fingerprinting (laboratory E) detected BvHb contamination in BSF larvae fed 10% (w/w) for one week, when this was followed by a decontamination period during which larvae were fed control diets for an additional week (*BvHb 10%) (Figure 2A). In addition to $\delta^{13}C_{AA}$ fingerprinting, peptide-centric immunoaffinity LC-MS/MS (laboratory C) successfully detected traces of non-permitted bovine blood residues in BSF after decontamination. However, given that control-media used in the present study was found to contain traces of bovine material, it is not clear if positive MS finding in the *BvHb 10% group is result of the background contamination detected in the control diet or if this method indeed is able to detect traces of non-permitted material in larvae after decontamination. The challenge of detecting non-permitted material using MS-based assays could be due to the removal of easily detectable residual exterior BvHb contamination stemming from direct contact of BSF larvae with the 10% (w/w) BvHb diet and frass when placing larvae in clean containers during the decontamination period. In addition, after seven days feeding on Ctl-media, BvHb-exposed larvae may have effectively cleaned their gut of any internal BvHb residues. Actually, before harvesting insect larvae, the inclusion of a starvation period, also called gut purging, of at least 24 hours has been recommended, since the gut content of insects was found to contribute considerably to overall contaminant levels and the microbial loads detected in harvested larvae (Bosch, et al., 2017; van Huis, 2013). Bosh et al. (2017) showed that feeding yellow mealworm larvae with poultry feed for 2 days after being fed media containing aflatoxin, considerably reduce the content of this mycotoxin in the larvae. In the current study, substitution of adulterated feeding media with clean poultry diets for seven days prior to harvest, thus allowed the larvae to significantly reduce or possibly eliminate any left-over BvHb in the gut.

Despite the hypothesized lack of internal or external BvHb residues present in BSF larvae fed control diets for a week after one-week of BvHb 10% (w/w) exposure, $\delta^{13}C_{AA}$ fingerprints detected differences in non-essential AA composition (Figure 2, Supplementary Table 7). $\delta^{13}C_{AA}$ values for BSF larvae fed control diets (Ctl) or BvHb 10%* (w/w) were the highest for almost all AA (Figure S3). Principal component analysis (PCA) of the most discriminative AAs (Ala, Val, Leu, Glx, Phe, Lys and Tyr) (Figure 2A) display significant correlations ($p<0.05$) in rank regression analysis in relation to increasing concentrations of BvHb in feeding media (Supplementary Table 7). To discern between BSF larvae fed the different feeding media, Ala, Glx, His, Ile, and Ser were identified as the most discriminative AA that explain the clustering variation (Figure 2B). The fact we were able to discern between Ctl and the depurated larvae (*BvHb 10%) shows that AAs originating from BvHb

proteins had not been replaced completely after seven days on the Ctl diet. This time period is considerably longer than the 100 minutes required for ingested feed to pass though the digestive system of BSF larvae (Mumcuoglu et al., 2001). These promising $\delta^{13}C_{AA}$ fingerprinting results warrant further sensitivity tests with depurated larvae.

The data obtained in the present study indicate that $\delta^{13}C_{AA}$ fingerprinting, while less sensitive than LC-MS-based approach discussed above, was able to cluster the BSF larvae fed *BvHb10% together with groups of insects fed BvHb at the 5% and 10% (w/w) level. $\delta^{13}C_{AA}$ fingerprinting has recently been used to address questions of food authenticity in the aquaculture sector, successfully discriminating between wild-caught, organically, and conventionally farmed salmon groups, as well as salmon fed alternative diets such as insects or macroalgae (Wang et al., 2019; Wang et al., 2018). In other words, based on previous studies and the findings presented here, in addition to MS-based approached, $\delta^{13}C_{AA}$ fingerprinting should also be considered for use in a multi-tier molecular analysis toolbox that can efficiently address questions of food authenticity and detect trace amounts of illegal material through the insect-PAP feed chain.

## 4. Conclusions

The aim of this study was to assess the suitability of legacy and novel molecular analysis tools (i.e. qPCR, MS-based approaches and $\delta^{13}C_{AA}$ fingerprinting) for detection of prohibited bovine material in the food chain when including insect PAP. The data generated here, show that each of the analytical approaches investigated is capable of detecting the presence of BvHb in insect feeding media and/or in BSF larvae. It also was found that each method displayed distinct shortcomings, which precluded detection of prohibited material in some instances. We therefore advocate the use of a combined multi-tier molecular analysis suite for the detection, differentiation and tracing of prohibited material in insect-PAP based feed chains. Taken together, the results confirmed earlier reports on the shortcomings of official monitoring methods and endorse ongoing efforts to extend the currently available battery of PAP detection approaches with MS based techniques and possibly $\delta^{13}C_{AA}$ fingerprinting.

## Figure captions

**Figure 1: (A)** Quantification of hemoglobin α chain (HBA, fmol absolute/200µg sample weight, by peptide-centric immunoaffinity LC-MS/MS (laboratory C, Y axis) in the black solider fly larvae fed the control (Ctl) or feed media spiked with BvHb at 1%, 5% and 10% (w/w); BvHb 1%, BvHb 5% and BvHb 10% (w/w), respectively; *BvHb 10%: BvHb 10% for 7 days followed by Ctl diet for 7 additional days (n = 2, X axis). **(B)** Total count of spectra matching against hemoglobin spectral library (laboratory D, Y axis) determined in the black solider fly larvae fed the control (Ctl) or feed media spiked with BvHb at 1%, 5% and 10% (w/w); BvHb 1%, BvHb5% and BvHb10% (w/w), respectively; *BvHb 10%: BvHb 10% for 7 days followed by Ctl diet for 7 additional days (n = 2, X axis).

**Figure 2:** Detection of bovine hemoglobin powder (BvHb) using $\delta^{13}C_{AA}$ fingerprinting. Principal component analysis (PCA) of **(A)** BvHb in feeding media and **(B)** in black soldier fly (BSF) larvae fed the control (Ctl) or feed media spiked with BvHb at 1%, 5% and 10% (w/w); BvHb 1%, BvHb5% and BvHb10% (w/w), respectively; *BvHb 10%: BvHb 10% for 7 days followed by Ctl diet for 7 additional days (n = 2). PCAs are based on $\delta^{13}C_{AA}$ displaying significant correlation ($p<0.05$) in rank regression analysis in relation to concentrations of BvHb in BSF fed adulterated diets. **(A)** The green, turquoise, blue and red dots represent the control (Ctl), or feed media spiked with BvHb at 1%, 5% and 10% (w/w); BvHb 1%, BvHb 5% and BvHb 10% (w/w), respectively. **(B)** The green, turquoise, blue, red and orange dots represent BSF larvae fed on Ctl, BvHb 1%, BvHb 5%, BvHb10% and *BvHb 10% (w/w), respectively. *BvHb 10%: BvHb 10% for 7 days followed by Ctl diet for 7 additional days.

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## Tables

**Table 1. Description of the different feeding media prepared for the black soldier fly larvae growth trial**

| Conditions | Ctl | BvHb 1% | BvHb 5% | BvHb 10% | *BvHb 10% |
|---|---|---|---|---|---|
| BvHb in medium (%, w/w) | 0 | 1 | 5 | 10 | 10 |
| Total feeding period (days) | 7 | 7 | 7 | 7 | 14 |

Ctl = control diet, Kasper Faunafood Opfokmeel 1; BvHb = bovine hemoglobin powder. *BvHb 10% = BvHb 10% for 7 days followed by Ctl diet for 7 additional days.

**Table 2: Detection of ruminant material in the feeding media used for the black soldier fly larvae growth trial**

| | qPCR (labs A, B) | | Targeted MS (labs A, B, C) | | | | | | | | SLM (lab D) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | LC-MS/MS | | IA-LC-MS/MS (protein IP) | IA-LC-MS/MS (peptide IP) | | | | | | |
| | Ruminant DNA | | Hb | MP[1] | Hb | Hb | PP | MP[2] | MY | CP | Hb | MP |
| Ctl | + | + | + | + | - | + | - | - | - | - | + | + |
| BvHb 1% | + | + | + | + | + | + | - | - | - | - | + | + |
| BvHb 5% | + | + | + | + | + | + | + | | | - | + | + |
| BvHb 10% | + | + | + | + | + | + | + | | | - | + | + |

Plus sign (+) indicates a positive result; minus sign a (-) negative result. Red show unexpected results. Workflows: LC-MS/MS (laboratory A, triple quadrupole); immunoaffinity-LC-MS/MS (IA-LC-MS/MS), IA on protein level (laboratory B, Q-TOF); IA-LC-MS/MS, IA on peptide level (laboratory C, triple quadrupole); SLM, spectral library matching (laboratory D, Q-TOF). Bovine proteins identified: Hb, hemoglobin; PP, plasma proteins: α2 macroglobulin and complement component 9; MP, milk protein: [1] Beta-lactoglobulin, casein and [2] osteopontin; MY, muscle protein: myosin 7; CP, cartilage protein: matrilin 1. Detailed analysis outputs are presented in Supplementary Tables 1-6.

**Table 3: Detection of ruminant material in the BSF larvae grown on feeding media containing bovine hemoglobin powder (n=2)**

| | qPCR (labs A, B) | | Targeted MS (labs A, B, C) | | | | | | | | SLM (lab D) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | LC-MS/MS | | IA-LC-MS/MS (protein IP) | IA-LC-MS/MS (peptide IP) | | | | | | |
| | Ruminant DNA | | Hb | MP[1] | Hb | Hb | PP | MP[2] | MY | CP | Hb | MP |
| Ctl | - | - | - | - | - | + | - | - | - | - | - | + |
| | - | - | - | - | - | + | - | - | - | - | - | + |
| BvHb 1% | + | - | - | - | - | + | - | - | - | - | - | + |
| | + | - | - | - | - | + | - | - | - | - | - | + |
| BvHb 5% | + | - | - | - | + | + | - | - | - | - | + | + |
| | + | + | + | - | + | + | - | - | - | - | + | + |
| BvHb 10% | + | + | - | - | + | + | - | - | - | - | + | + |
| | + | + | - | - | + | + | - | - | - | - | + | + |
| *BvHb 10% | - | - | - | - | - | + | - | - | - | - | - | + |
| | - | - | - | - | - | + | - | - | - | - | - | + |

Plus sign (+) indicates a positive result; minus sign a (-) negative result. Red show unexpected results. Workflows: LC-MS/MS (laboratory A, triple quadrupole); immunoaffinity-LC-MS/MS (IA-LC-MS/MS), IA on protein level (laboratory B, Q-TOF); IA-LC-MS/MS, IA on peptide level (laboratory C, triple quadrupole); SLM, spectral library matching (laboratory D, Q-TOF). Bovine proteins identified: Hb, hemoglobin; PP, plasma proteins: α2 macroglobulin and complement component 9; MP, milk protein: [1] Beta-lactoglobulin, casein and [2] osteopontin; MY, muscle protein: myosin 7; CP, cartilage protein: matrilin 1. Detailed analysis outputs are presented in Supplementary Tables 1-6.

**Figure 1**

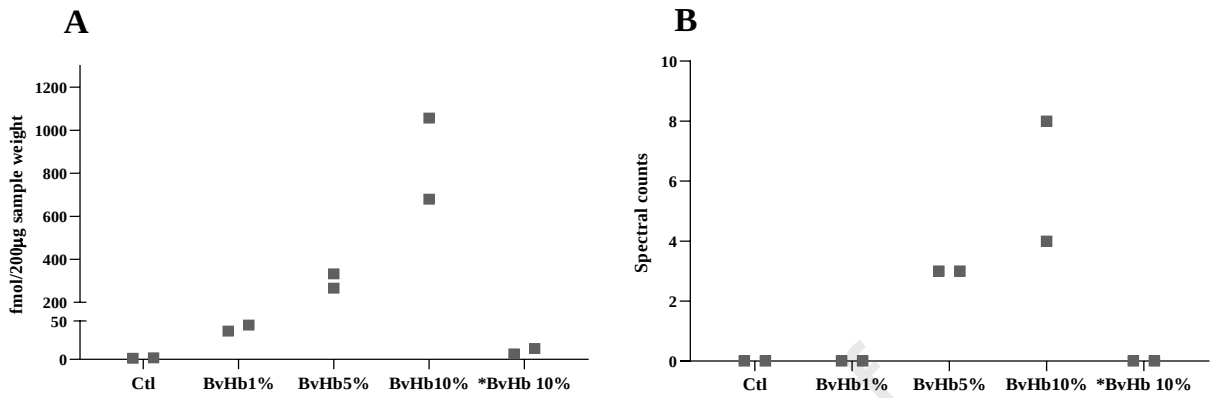
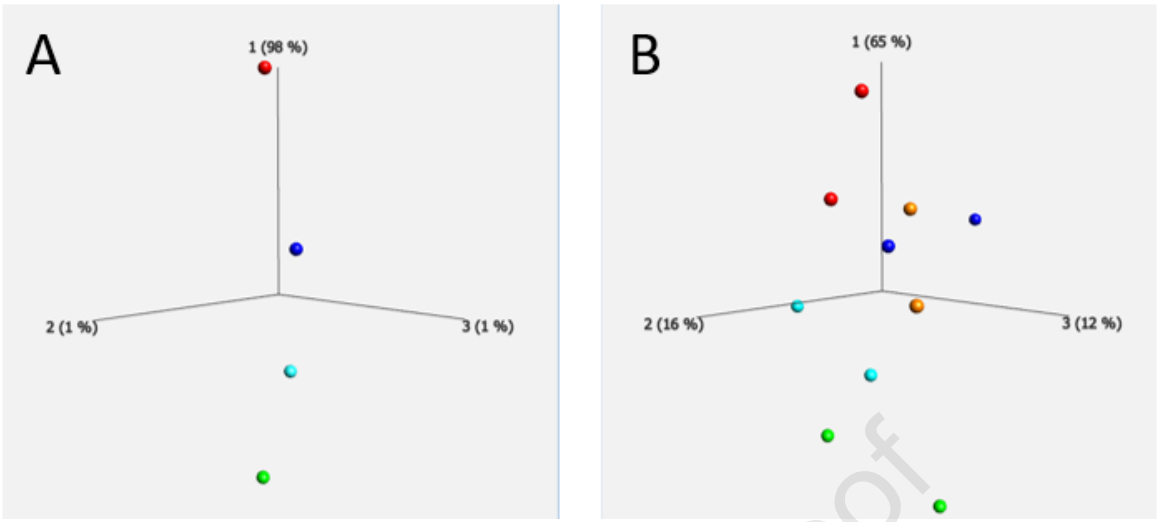


**Figure 2**



**Highlights**

- Detect prohibited bovine material in BSF with qPCR, MS and $^{13}C_{AA}$ fingerprinting
- Compared molecular tools for bovine hemoglobin detection across five laboratories
- Effective insect-PAP feed chain control possible by combining molecular tools
- New perspectives for tracing prohibited bovine material in insect-PAP feed chain

**Conflicts of Interest:** Olivier Poetz (O.P) is shareholder of SIGNATOPE GmbH. SIGNATOPE offers assay development and service using immunoaffinity LC-MS/MS technology.