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Unveiling the potential of proteomics in addressing food and feed safety challenges

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Abstract

The food and feed sector in Europe is rapidly evolving to address contemporary challenges, striving for fairer, safer, greener and more sustainable food systems. This includes the exploration of new protein sources for human consumption and animal feed such as protein derived from insects, algae or novel plant-derived proteins, and the re-evaluation of existing sources like processed animal protein (PAP). To generate reliable data on the diverse array of emerging protein sources for future food and feed safety assessments, a growing demand for the development and implementation of advanced analytical techniques exists. New approach methodologies (NAMs) including, mass spectrometry (MS)-based proteomics methods have been emerging as valuable techniques which potentially can be implemented in regulatory laboratory settings to complement conventional approaches in this realm. These MS-driven strategies have already proven their utility in diverse applications, including the detection of prohibited substances in feed, identification of allergens, differentiation of fish species in complex mixtures for fraud detection and the verification of novel foods and alternative protein sources. This EU-FORA programme was focused on three core objectives namely: (i) the training of the fellow in utilising MS-based proteomics for food and feed safety analyses, (ii) the involvement of the fellow in the development of standardised operating procedures (SOP) for targeted and non-targeted proteomic MS-based workflows for species and tissues specific PAP identification in a national reference laboratory (NRL) and (iii) the transfer and implementation of MS-based approaches and standardised protocols for PAP analysis at the fellow's home institution. Altogether, this programme facilitates the broadening and diversification of use of MS-based proteomic methodologies for reinforcing their significance within the domains of food and feed safety research and regulatory science applications.

Keywords: proteomics, alternative protein sources, processed animal protein, species identification, tissue identification, mass spectrometry

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1. Introduction

With the currently occurring green shift in food and feed domains in Europe and the envisaged focus on value creation from protein derived from novel sources and circular bio-based economies, new data gaps and challenges arise which cannot any longer be addressed with classic analytical tools and regulatory paradigms (Belghit et al., 2021; Delgado et al., 2022). In addition to novel terrestrial feed ingredients, also the authentication of fish species has become is a major concern worldwide (Varunjikar et al., 2022b). In the context of food safety, the development of novel methodologies and analytical tools to authenticate both species and tissue specific composition and origin of proteic material has become key to create and evaluate data which is fit for purpose for future feed safety risk assessments needs. This EU-FORA programme was performed under the auspices of, and contributed to, national and international initiatives aimed at fortifying the capacity of national regulatory laboratories for swift safety interventions and the enhancement of current risk assessment practices. Given the transformative dynamics within the food and feed sectors in relation to the emergence of novel proteic constituents, the training of the fellow in omics-based feed and food authenticity analyses and fraud detection will contribute to a wider implementation and dissemination of the use of new approach methodologies (NAMs) such as proteomics, for addressing emerging and future food and feed safety challenges.

1.1. Applicability of proteomics in food and feed safety sectors

Proteomics, one of the so-called 'omics[1] methods and part of a suite of methods considered as NAMs for use in risk assessment (Marx-Stoelting et al., 2023), is an approach used to study proteins whereby the entire complement of proteins in a given sample (of tissue, cells or a biological fluid such as blood) is analysed simultaneously. Proteomic-based methods using liquid chromatography coupled to mass spectrometry (LC–MS) are increasingly recognised as promising tools to complement current standard techniques for food and feed quality assessments and traceability analysis (Rasinger et al., 2016).

As highlighted in EFSA Scientific Colloquium 24 – 'omics in risk assessment: state of the art and next steps', '*omics technologies are a valuable addition in some aspects of risk assessment of food and feed products and, if the current pitfalls associated with data collection, processing, interpretation and curation were resolved*, i.e. *by application of FAIR (findable, accessible, interoperable and reusable) data management principles, such methods could be routinely used in food and feed safety risk assessment*' (EFSA, 2018).

Originating in the last decades of the 20th century, the advent of MS-based proteomics owes its dawn to the ground-breaking efforts of John B. Fenn and Koichi Tanaka, who ushered in the era of soft desorption ionisation techniques for analysing biological macromolecules via MS (Patterson and Aebersold, 2003). While various forms of these techniques have witnessed extensive adoption across multiple facets of biological research, their integration into the sphere of food and feed safety research remained relatively modest. Nonetheless, several applications of proteomics have surfaced in the context of food and feed safety. These encompass the testing of proteinaceous materials to discern levels of adulteration and ensure traceability, the identification of food-borne pathogens, the detection of genetically modified organisms (GMO) and allergen identification. At the moment, the most foreseeable avenue for incorporating proteomics into routine food and feed testing is in the field of PAP. In 2019, the European Union Reference Laboratory for Animal Proteins in Feedingstuffs (EURL-AP) organised an inter-laboratory study on the detection of bovine PAP in feed by MS-based proteomics. The results of the study indicated that LC–MS was able to successfully identify the presence of various proteins of bovine origin in feed at an adulteration level of 1% (w/w), demonstrating that proteomics has matured enough to become a complementary method for the official control of the use of PAP in feedstuffs (Lecrenier et al., 2021). Furthermore, proteomics can provide a more comprehensive understanding of the molecular mechanisms of toxicity and, hence, be used to identify new biomarkers of exposure and toxicity, which can be used to develop new endpoints for chemical risk assessment (Li et al., 2023). A more exhaustive compilation of examples spotlighting the applicability of MS-based proteomics in the domain of food and feed safety is listed in Table 1, whereas a visual infographic is presented in Figure 1.

[1] As described in *Encyclopedia Britannica*, **omics** are any of several areas of biological study defined by the investigation of the entire complement of a specific type of biomolecule or the totality of a molecular process within an organism. Examples of well-established fields include genomics, transcriptomics, proteomics and metabolomics.

Table 1: Application examples of MS-based proteomics in food and feed safety as well as risk assessment

Subcategory	Application	Reference/–es
Allergens	Detection of known food allergens in food commodities by targeted proteomics	Monaci et al. (2020)
	Application of discovery untargeted proteomics to identify allergens in novel foods	López-Pedrouso et al. (2023), Barre et al. (2021), Bose et al. (2021)
Adulteration and traceability	Discovering protein indicators of freshness of refrigerated fish products via untargeted proteomics	Deng et al. (2019)
	Identification of species and the estimation of their respective relative abundances in a mixed samples for the purpose of authentication analysis of fish and meat	Varunjikar et al. (2022b), Stachniuk et al. (2021)
	Detection and identification of non-authorised proteic material (e.g. prohibited ruminant by-products) in processed animal protein (PAP) by targeted proteomics	Lecrenier et al. (2021)
	Species-specific discrimination of insect meals for aquafeeds via untargeted proteomics	Belghit et al. (2019), Varunjikar et al. (2022a)
	Identification of unique peptide markers for authentication of honey via untargeted proteomics	Bong et al. (2021)
	Detection of powdered milk in fresh cow's milk	Calvano et al. (2013)
Food-borne pathogens and contaminants	High-throughput diagnostic methods detection of parasites, fungi and food-borne bacteria in food and feed via MALDI-MS	Kästner et al. (2021), Pavlovic et al. (2013), Lima and Santos (2017)
	Detection and quantification of protein toxins, e.g. Botulinum toxin	Duracova et al. (2018)
	Identification of undergoing illicit veterinary treatments in livestock	Donna et al. (2009)
	Detection of Maillard reaction induced changes in thermally processed milk	Arena et al. (2017)
Risk assessment	Integration of proteomics in a multi-omics approach within a broader systems biology framework for augmenting current risk assessment practices of GMOs	Benevenuto et al. (2023)
	Applying standalone untargeted proteomics for the detection of transgenic events and evaluating proteome-level equivalence to bolster safety assessments of novel crop varieties destined for use in feed and food	Varunjikar et al. (2023)
	Integration of proteomics in a multi-omics toolbox as new approach methodology (NAM) to define biological responses for chemical safety assessment	Li et al. (2023)
	Application of proteomics along with other omics tools to study host-pathogen interactions for next generation microbiological risk assessment	Haddad et al. (2018)

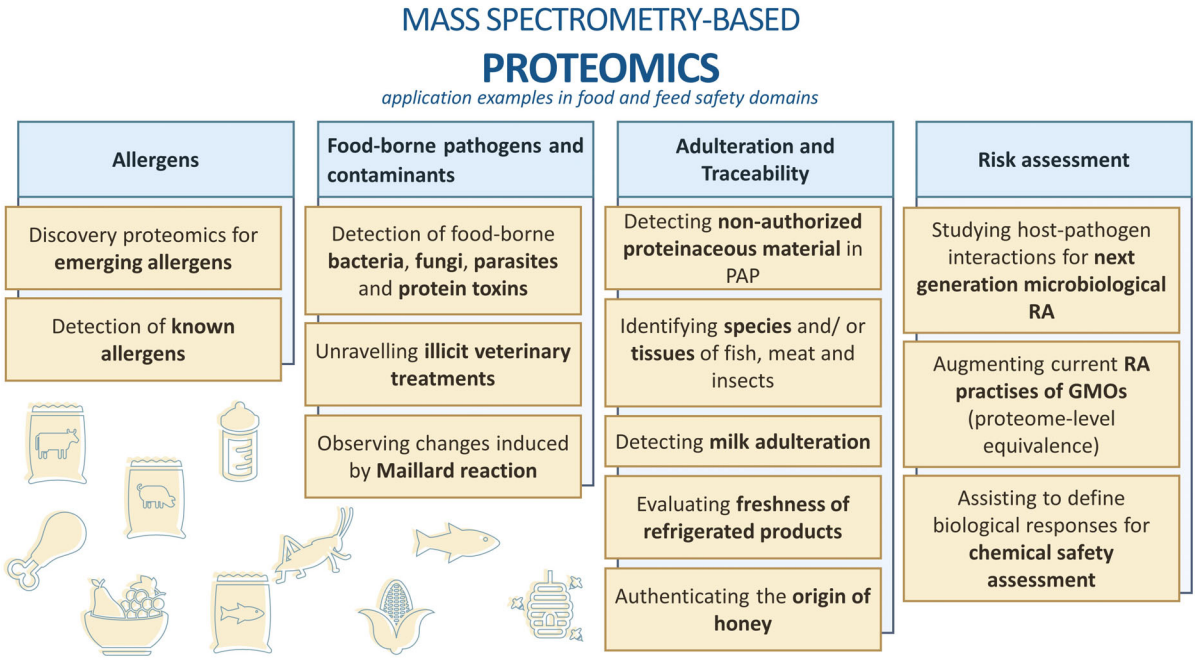

Figure 1: An infographic summarising the use of MS-based proteomics in food and feed safety and risk assessment

2. Description of work programme

The work programme was conducted within the framework of The European Food Risk Assessment (EU-FORA) Fellowship Programme at two institutions: the fellow's home institution, Institute of Food Safety, Animal Health and Environment 'BIOR' (BIOR, Latvia) and the hosting institution, Institute of Marine Research (IMR, Norway). Dr. Josef D. Rasinger, part of IMR's Marine Toxicology group, provided supervision. In essence, the work programme sought to prepare the fellow with practical training and theoretical insights, enabling confident use of MS-based proteomics techniques for use in feed and food safety assessments. Through knowledge sharing, expertise from the hosting site was transferred to the fellow's home institution. Thus, the work programme directly contributed to core EU-FORA objectives: enhancing risk analysis readiness and facilitating knowledge exchange for a unified EU risk assessment strategy.

2.1. Aims

While proteomics is not yet a widely used technique within the multi-omics toolbox applied in risk assessment, it is gaining recognition as one of the NAMs. Being prepared to address future challenges is essential, and proteomics has the potential to enhance risk assessment practices. Therefore, the work programme aimed to provide a thorough training experience, combining hands-on learning with strong theoretical foundations. Beyond technical expertise, the programme intended to acquaint the fellow with diverse application possibilities for proteomics. This strategy fostered versatility beyond a singular context and promoted a holistic view of the technique's possibilities.

2.2. Activities/methods

2.2.1. Laying the theoretical foundations of proteomics within the context of food and feed safety

Given the inaugural hands-on training in January 2023, it was vital to provide the fellow with solid theoretical grounding in MS-based proteomics prior to the arrival. Under IMR's guidance, the fellow conducted literature research, focusing on relevant EU documents regarding proteic materials in feed and novel foods, EURL-AP guidance documents and research papers from the past decade by IMR's Marine Toxicology group. The group has an extensive expertise in this field and has applied proteomics in various contexts; for instance, distinguishing insect meals for aquafeeds, detecting unauthorised proteic

material in PAP, the analysis of GM soy and the detection of mislabelled fish species in mixes (Rasinger et al., 2016; Belghit et al., 2019, 2021; Varunjikar et al., 2022b, 2023). The group also has ample experience in the use of omics for chemical risk assessment (Rasinger et al., 2014, 2017, 2018; Reffatto et al., 2018) and the generation and storage of data in line with FAIR principles (Pineda-Pampliega et al., 2022). Additionally, remote learning was pursued to deepen the fellow's technical knowledge and biological understanding of the field. Therefore, the fellow accessed complementary learning resources from two sources: (i) UC Davis Proteomics Online 2nd Short Course and (ii) the Proteomics Academy, a collaboration between the European Proteomics Association and the European Bioinformatics Community. In addition to that, the fellow also received training on the 'Application of omics in risk assessment', a 1-day online course held by the fellow's supervisor as part of the second training module of the EU-FORA course series. These endeavours, coupled with frequent guidance from the supervisor and other members of the IMR's Marine Toxicology group, ensured the fellow's readiness for practical training at the hosting facility.

2.2.2. Training in FASP and SP3 strategies for sample preparation in proteomics

Sample preparation strategies in contemporary proteomic studies differ considerably from previous gel-based approaches, because protein extraction and digestion prior to MS analysis is required. Commonly employed methods encompass filter-aided sample preparation (FASP), suspension-trapping (S-Trap), single-pot, solid-phase-enhanced sample preparation (SP3) and in-StageTip digestion (iST) (Sielaff et al., 2017; Araújo et al., 2021). Each laboratory typically leans towards a preferred approach, tailored to the specific proteomics application. IMR predominantly adopts the FASP technique, in which the fellow received training. To enrich the work programme's scope, the fellow also participated in SP3 protocol training at the University of Bergen's Proteomics Unit (PROBE), under the supervision of Olav Mjaavatten.

FASP, introduced by Manza et al. (2005), has gained widespread popularity due to its practicality. It effectively eliminates cellular debris, salts, lipids, chaotropes, detergents like sodium dodecyl sulphate (SDS) and other low-molecular weight contaminants through a straightforward centrifugation process using a molecular weight cut-off (MWCO) ultrafiltration device (Manza et al., 2005; Sielaff et al., 2017). This method retains proteins on a filter membrane, making them available for subsequent enzymatic digestion. Generated peptides, small enough to pass the filter, can be collected via centrifugation. After FASP-assisted digestion, roughly 50% of the initial material can be recovered, with no adverse impact on proteome coverage (Sielaff et al., 2017). This quality is particularly beneficial for applications in food and feed safety, where the initial sample amount of proteic material (e.g. insect-based aquafeed or PAP) is comparatively high, in contrast to mass-limited samples such as tissue biopsies from clinical research areas.

An in-depth description of the applied FASP protocol is available in research paper by Belghit et al. (2019). In brief, around 50–100 mg of protein containing sample (e.g. insects, animal tissue, PAP, etc.) is homogenised in lysis buffer (0.1 M Tris–HCl/4% SDS). Protein concentration in the extract is determined spectrophotometry (660 nm Protein Assay). Purification of protein extract is done via FASP, where disposable centrifugal ultrafiltration units allow for detergent depletion, protein digestion and isolation of peptides released by proteases from undigested material. Reduction of disulphide bonds and alkylation is done by dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Protein digestion is performed with trypsin (37°C for 16 h). Additional clean-up of the peptide extract is carried out by dispersed solid phase extraction methodology using C18 spin-columns. The final extract is evaporated and reconstituted in solvent mixture that matches the initial gradient conditions of the LC–MS gradient method (Belghit et al., 2019).

Unlike FASP, SP3 follows a paramagnetic bead approach, where ethanol-driven solvation captures proteins on hydrophilic beads functionalised with carboxylate groups. This mechanism effectively separates a broad range of biomolecules, ensuring unbiased recovery of proteins and peptides for bottom-up proteomics analysis. Additionally, SP3 stands out for its straightforward single-tube protocol, which is less labour-intensive than other methods, including FASP (Hughes et al., 2019). Nevertheless, both SP3 and FASP facilitate the removal of unwanted chemicals and biological components before digestion; the resulting peptide digest can be subjected to a further clean-up if needed. Detailed instructions for the complete SP3 protocol can be found in Hughes et al. (2019). A simplified scheme of the analytical pipeline of both SP3 and FASP protocols is given in Figure 2. During the work programme, the FASP protocol served as the principal sample preparation strategy, while SP3 was exclusively utilised for training purposes.

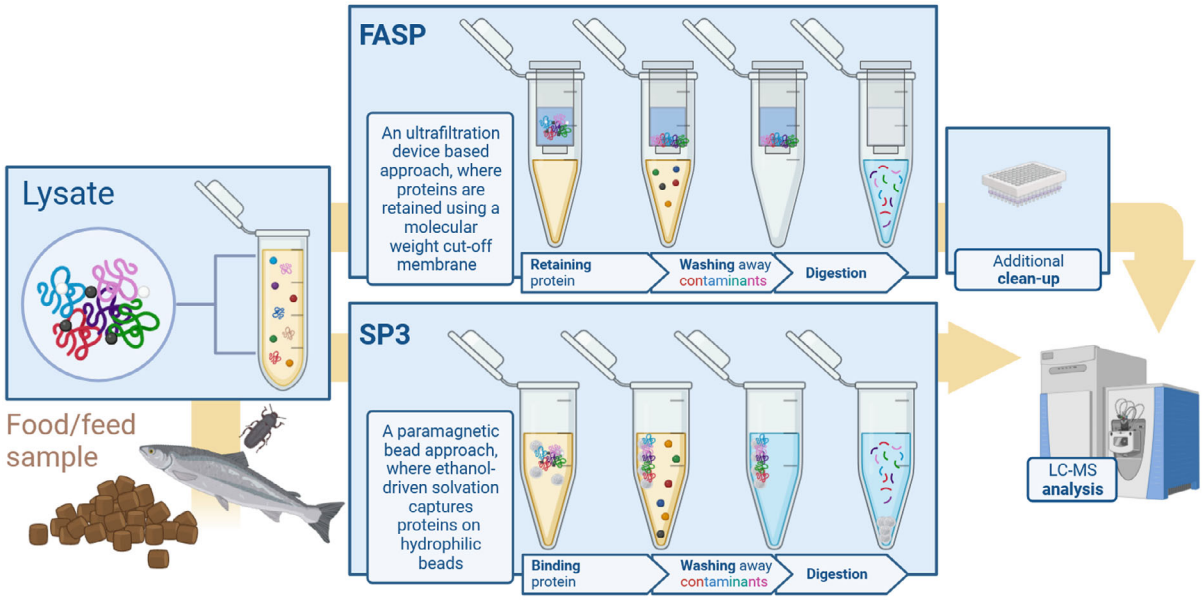

Figure 2: SP3 and FASP protocols: analytical workflow comparison (figure created with BioRender.com with licence number ZE25RV2OJQ)

2.2.3. LC–MS and interpretation of proteomics data utilising different tools

In this work program, a bottom-up proteomics method was employed. The sample extract comprises peptides generated through proteolytic digestion, which are subsequently analysed via LC–MS. Given the known digestion mechanism by a protease (trypsin in this case), the resulting peptide signals can be translated into amino acid sequences, resembling pieces of a puzzle that unveil the sample's proteome. There are different ways to implement instrumental part for bottom-up proteomics. For instance, nanoflow-LC (nano-LC) is used in combination with hybrid high-resolution MS systems, such as time-of-flight MS, Orbitrap-MS or even Fourier transform ion cyclotron resonance MS, to achieve the most out of the proteome analysis in non-target mode. On the other hand, simpler tandem mass spectrometry (MS/MS) systems combined with conventional high-performance liquid chromatography (HPLC) can be effectively used to perform targeted analyses for selected peptide markers (Lecrenier et al., 2021; Stachniuk et al., 2021). In this programme non-targeted approach was utilised. However, instead of using nano-LC, we opted to achieve peptide separation via HPLC with higher flow rates. This decision was rooted in the technical complexity and required expertise associated with nano-LC, despite its industry recognition as the gold standard. As a result, HPLC becomes a more feasible entry point for routine and research labs in the field of food and feed safety. This is particularly relevant for those less familiar with the intricate aspects of implementing proteomics protocols.

Concurrently, mass spectra measurements were executed using a Q Exactive Orbitrap MS system. This decision stemmed from the shared presence of this equipment at both the hosting site (IMR) and the fellow's home institution (BIOR), streamlining the method transfer process in comparison to using distinct setups. The method's tune, acquisition and source parameters were directly adapted from Varunjikar et al. (2022a), where non-target bottom-up proteomics was successfully utilised for authentication, biological analyses and allergen detection in feed and food-grade insect species. These specific parameters can be located in the original research article (Varunjikar et al., 2022a).

The processing of proteomics data and its subsequent bioinformatics analysis are key for the translation of raw MS data into meaningful insights, necessary for delving into a sample's proteome. Amid the vast array of workflows that are routinely employed, a simplified strategy for handling bottom-up non-targeted proteomics data can be boiled down into the following steps (Chen et al., 2020; Deutsch et al., 2022):

1) Raw data pre-processing, e.g. conversion of the raw vendor format to mzML.
2) Peptide identification using peptide sequence search engine (usually from FASTA format), spectral library search engines or *de novo* search engines.

3) Validation of search engine results by assigning a probability (with or without the aid of a decoy database) to each peptide-spectrum match (PSM) to lower the false discovery rate (FDR).
4) Reconstruction of the peptide sequences into their original proteins, a process known as protein inference, where peptide assembly models normally adhere to the Occam's razor approach, which reports the smallest set of proteins that account for the detected peptides.
5) Quantifying the abundance of measured proteins based on the experimental workflow, label-free, isobaric labelling or isotopic labelling approaches.

In the course of this work programme, the participant became familiar with three data processing workflows employed by IMR researchers in the domain of food and feed safety. Each of these workflows is briefly summarised in Table 2.

Table 2: Summary of employed data processing workflows for the analysis of proteomics data in this EU-FORA work programme

Workflow	Summary
Direct spectral comparison using compareMS2 2.0	CompareMS2 2.0 is a versatile tool that enables molecular phylogenetics by aligning and matching tandem mass spectra features of peptides across different input datasets (Palmblad and Deelder, 2012; Marissen et al., 2023). This method has already been used in a range of applications, including food and feed species identification and has been recently updated with an improved user interface un functionalities providing even better applicability potential.
Spectral library building and matching with SpectraST in Trans-Proteomic Pipeline (TPP)	In this workflow spectral library is created from reference samples (Lam, 2011), for example, single-species samples of fish and insects. This library can subsequently be used to scrutinise unknown samples. By implementing a dot product threshold, matching spectra can be pinpointed and used as potential markers for species identification using the untargeted approach (Varunjikar et al., 2022a).
A standard bottom-up proteomics data analysis workflow with sequence search engine Comet in TPP	In this method, a traditional data analysis approach is employed. It involves using UniProt databases (FASTA files) to search for species-specific proteomes via the Comet sequence search engine in the TPP platform (Eng et al., 2013). Subsequently, pepXML files produced in this process are subjected to validation through PeptideProphet and ProteinProphet (Keller et al., 2002; Nesvizhskii et al., 2003), implementing a 1% FDR threshold to ensure accurate identification of markers in line with verified sequences. Despite its reliable nature, the scope of this approach remains somewhat limited beyond model species due to the scarcity of reviewed datasets within the UniProt database for species relevant to the food and feed sector.

Last but not the least, benefitting from work done in an earlier EU-FORA project performed at the hosting site (Pineda-Pampliega et al., 2022), the fellow gained insight into the FAIR (Findability, Accessibility, Interoperability and Reusability) data principles that govern the storage and dissemination of data. For the present project, this entailed familiarity with utilising ProteomeXchange resources, especially by the application of the MassIVE repository. In particular, datasets from two studies by Belghit et al. (2019) and Varunjikar et al. (2022a) were used throughout the programme with massIVE repository IDs MSV000083737 and MSV000088034, respectively.

2.2.4. Transitioning from knowledge to practical implementation of MS-based proteomics for food and feed safety

The introduction has set the stage for the multifaceted applications of MS-based proteomics in food and feed safety research. Across diverse applications intended for authentication and traceability of food and feed, a common objective prevails – capitalising on proteomic variations to distinguish taxa, species and notably tissues. The latter holds pivotal significance within proteomics. For instance, in authenticating PAP in regulatory laboratory settings, species identification predominantly relies on real-time polymerase chain reaction (qPCR), while tissue identification depends on light microscopy. Recent insights from the 2022 EURL-AP proficiency test result reveal that species identification through qPCR is far more accurate compared to light microscopy-based tissue analysis (Fumière et al., 2022). Thus, MS-based proteomics emerges as a potential complement to conventional light microscopy.

A meta-analysis conducted by Sudmant et al. (2015) showed that most transcriptomic studies have observed greater similarity in gene expression between homologous tissues from different vertebrate species than between diverse tissues of the same species (Sudmant et al., 2015). To some extent this holds true even at a level of amino acid profiles, where liver, kidney and brain are very much alike in amino acid composition but differ from stomach and lung in cystine, tryptophane, tyrosine and phenylalanine (Beach et al., 1943). As a result, tissue specification via proteomics emerges as one of the most attainable near-future goals within this domain. Within this context, proof-of-concept experiments were carried out, focusing on muscle tissues extracted from diverse species relevant to the food and feed sector (i.e. fish, pig, ruminant, poultry and crustacean). These were analysed alongside individual single-tissue samples sourced from salmon. This design aimed to gain insight whether non-target proteomics data could discern pronounced differences between tissues, enabling comparisons not only across species but also taxa. Detailed findings beyond the scope of this report have been omitted to prevent potential copyright conflicts, as these results are intended for publication in scientific journals.

With a solid foundation in the theoretical and practical aspects of MS-based proteomics, the fellow also embarked on the development of standard operating procedures (SOPs) at the hosting site. This involved devising SOPs for two distinct workflows: (i) a multi-purpose non-targeted bottom-up proteomics workflow intended for general application in food and feed samples and (ii) a targeted proteomics approach tailored for identifying prohibited materials in PAP. The former was curated by amalgamating methodologies from previous IMR research endeavours, while the latter was adapted from the inter-laboratory study on detecting adulterated PAP via MS-based proteomics, organised in 2019 by EURL AP, which included the participation of IMR among six laboratories (Lecrenier et al., 2021).

The final objective of this fellowship was the task of adapting the complete proteomics protocols implemented at the IMR for use at the fellow's home institution, BIOR. Despite BIOR's resourceful inventory of MS systems, the implementation of MS-based proteomics had not been previously undertaken in house. This transfer of protocols was executed in stages and followed a step-wise implementation described in (Varunjikar et al., 2022a,b).

Initially, the adapted method was evaluated based solely on instrumental capabilities, analysing varying amounts of HeLa digest. Data post-processing was performed against the UniProt human reference proteome (up000005640) using Comet search via TPP. Subsequent refinement of data through PeptideProphet and ProteinProphet was omitted. These results were then compared to analogous HeLa measurements conducted using the IMR's in-house method within the Varunjikar et al., 2022a study (massIVE ID: MSV000088034). Figure 3 (panel A) shows that the adapted instrumental method exhibited reduced sensitivity and required higher amount of HeLa to achieve a similar count of peptide and protein matches.

Moreover, additional experiments compared the instrumental method and the adapted sample preparation protocol through four scenarios. In scenarios 1 and 2, insect samples from various species (e.g. Black soldier fly, Yellow mealworm, Lesser mealworm) underwent FASP protocol at BIOR (scenario 1) and IMR (scenario 2). These extracts were analysed in BIOR and the data were processed by Comet search in TPP with reviewed Arthropoda sequences (accessed July 2023). Significant differences between samples prepared at both facilities were not observed (Figure 3, panel B). However, given that HeLa analysis indicated the BIOR's instrumental method is less sensitive than IMR's in-house method, it was essential to estimate the extent of this shortcoming. Thus, a batch of salmon tissue samples were prepared at the IMR by the fellow and the extracts were analysed on both LC–MS systems (scenario 3: BIOR, scenario 4: IMR). Data were processed in TPP via Comet search against Atlantic salmon proteome (UP000087266). Results confirmed that the LC–MS method at the IMR was superior yielding nearly double data compared to BIOR. Lastly, an independent insect dataset from Varunjikar et al., 2022a (massIVE ID: MSV000088034) was used to assess the overall performance of the transferred method. Similar proteomics post-processing pipeline was applied to the raw data as in scenarios 1 and 2. The number of features obtained from the study's data were around two times higher compared to data from insect samples that were independently prepared and analysed in BIOR by the fellow (Figure 3, panel B). However, the data fall within the same range as in scenario 4 and, hence, it becomes evident that sample preparation is not the key issue for the adapted method and the main shortcomings are related to the instrumental method. This could be partly attributed to the variance in column lengths (150 mm at BIOR vs. 250 mm at IMR). Future improvement of the method would entail fine-tuning the scanning and ionisation parameters of the Q Exactive Orbitrap MS system, and considering the possibility of increasing the protein load per injection.

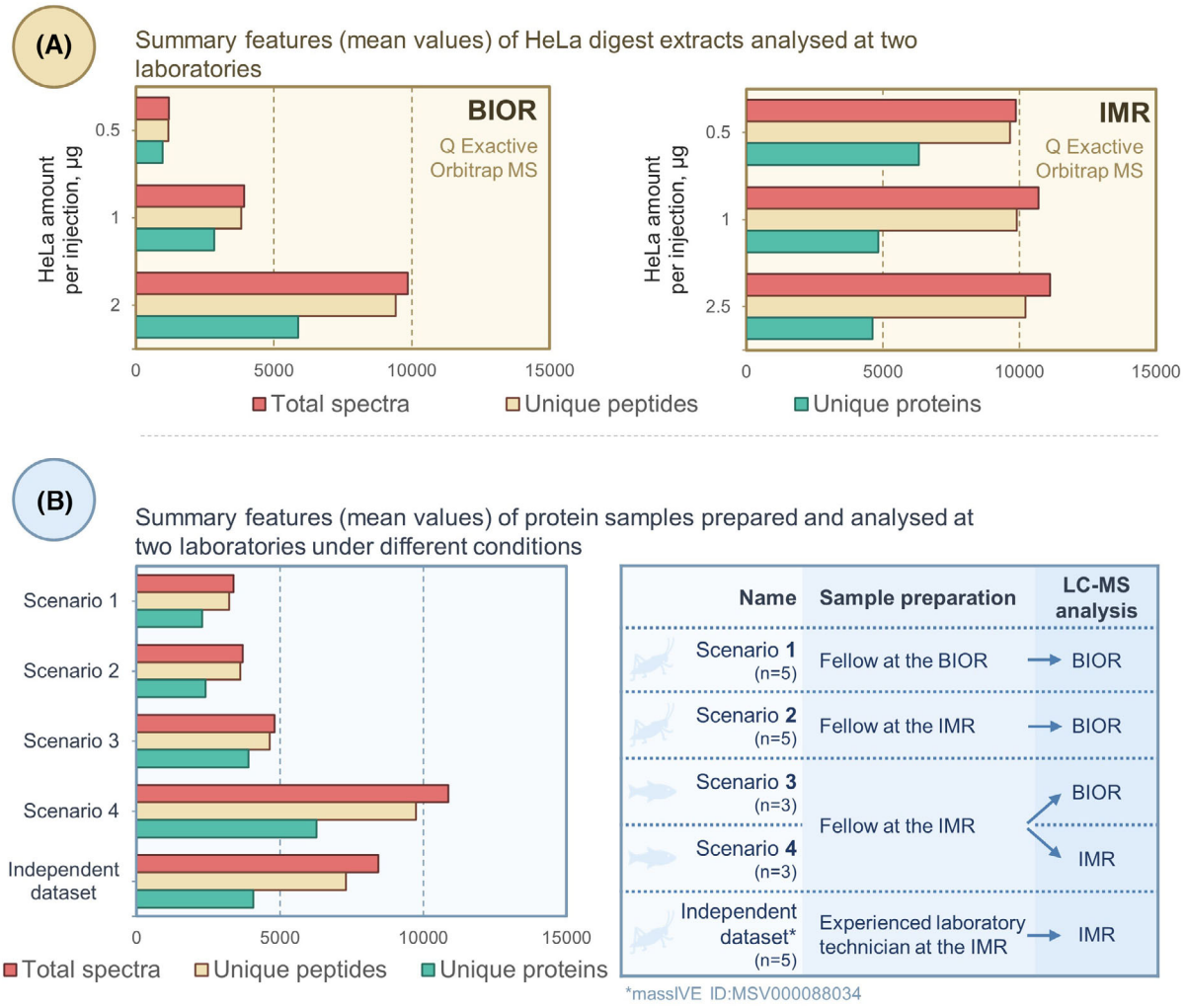

Figure 3: Comparative analysis of adapted instrumental method and sample preparation protocol (panel A – results from HeLa digest analysis, panel B – results from real sample analysis)

2.3. Secondary activities

In addition to the direct implementation of the work programme and participation in the five EU-FORA risk assessment training modules, the fellow engaged in extracurricular activities. On 10 March, 2023, the fellow attended Madhushri Shrikant Varunjikar's thesis defence, titled 'Proteomic Tools for Food and Feed Authentication', at the University of Bergen (Varunjikar, 2023). From 23 to 24 May, 2023, the fellow participated in a remote workshop organised by EURL-AP, which delved into MS-based proteomics approaches for use in regulatory settings for PAP detection. To further enhance practical skills, the fellow assisted in preparation of cod liver samples for proteomics analysis performed under the auspice of a ClimSeaFood research project (Norwegian Research Council; project number: 324374) at the IMR which focuses on the effects of climate change on marine eco-system and seafood safety. On 7 July, 2023, the fellow and the supervisor attended a brainstorming workshop for future joint project applications organised by the Unit Effect-based Analytics and Toxicogenomics of the German Federal Institute of Risk Assessment (BfR); the meeting focused on alternative protein sources, including the application of omics for tracing and risk assessment of novel protein sources.

3. Conclusions

Considering the evolving landscape of the European food and feed sectors, new avenues have emerged, such as the ascent of alternative protein sources as novel foods, shifts in PAP legislation, new aquafeed ingredients, GM ingredients and increased demand for aquaculture products. In all of these instances, proteins play a significant role within the risk assessment framework and, therefore,

the demand for advanced techniques to rigorously assess the safety, authenticity and traceability of protein materials has surged. Furthermore, these approaches must be fit for purpose for future feed safety risk assessments needs. Amidst this context, MS-based proteomics have surfaced as one key NAM and have already demonstrated their utility across various applications including, the detection of prohibited substances in feed, allergen identification and differentiation of species and tissues within intricate mixtures.

Within the framework of this EU-FORA programme, three central objectives were addressed. Firstly, the programme equipped the fellow with the skills to employ MS-based proteomics for food and feed safety analyses. Secondly, it contributed to SOP development for both targeted and non-targeted proteomic workflows that greatly benefitted the implementation of these techniques in both hosting site and home institution. The latter method was successfully transferred to the fellow's home institution, with the intent of evaluating the origin of proteinaceous material in food and feed. This directly aligns with the EU FORA programme's goals – facilitating knowledge exchange for a unified EU risk assessment strategy. Lastly, the programme endeavoured to conceptualise and implement a proof-of-concept strategy, applying MS-based proteomics to discern tissue and species-specific attributes within samples of animal origin. Collectively, this programme expands the realm of MS-based proteomic methodologies, enhancing their relevance and availability in the domains of food and feed safety.

4. Disclaimer

Detailed results obtained from the sample analysis are not included in this report to avoid certain copyright claims, as these results are intended for subsequent publication in peer-reviewed articles.

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Abbreviations

BIOR	Institute of Food Safety, Animal Health and Environment 'BIOR', Latvia
DTT	dithiothreitol
EU-FORA	The European Food Risk Assessment Fellowship Programme
EURL AP	European Reference Laboratory for Animal proteins in feedingstuffs
FAIR	an acronym for data which meet principles of findability, accessibility, interoperability and reusability
FASP	filter-aided sample preparation
FDR	false discovery rate
GMO	genetically modified organism

HPLC	high-performance liquid chromatography
IAA	iodoacetamide
IMR	Institute of Marine Research, Norway
LC–MS	liquid chromatography coupled to mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MWCO	molecular weight cut-off
NAM	new approach methodology
nano-LC	nanoflow liquid chromatography
PAP	processed animal protein
PSM	peptide-spectrum match
qPCR	real-time polymerase chain reaction
RA	risk assessment
SDS	sodium dodecyl sulphate
SOP	standard operating procedure
SP3	single-pot, solid-phase-enhanced sample preparation