Clinics in Oncology

Review Article
Published: 05 Jun, 2018

Mass Spectrometry based Approaches for Analysing Proteins in Processed Food and Feed

Martine Raes[1], Mélanie Planque[1], Hélène Marbaix[1], Nathalie Gillard[2], Marie-Caroline Lecrenier[3], Marc Dieu[1,4], Sergio Mauro[5], Thierry Arnould[1] and Patricia Renard[1,4]*

[1]URBC-NARILIS, University of Namur, Belgium

[2]Health Department, CER Group, Marloie, Belgium

[3]Valorisation of Agricultural Products, CRA-W, Gembloux, Belgium

[4]MaSUN, Mass spectrometry facility, University of Namur, Belgium

[5]Biotechnology Department, CRA-W, Gembloux, Belgium

Introduction

Food and feed traceability and authentication have become major societal issues, requiring adequate analytical approaches. Proteins in food and feed are of paramount importance for providing amino acids to humans and animals. Processing (in particular cooking at high temperature) and rendering, drastically affect proteins [1], leading to denaturation, amino acid modifications, cleavage and aggregation, hampering their extraction. That is why for characterizing food and feed, for their authentication in particular regarding the species origin, DNA-based methods were first developed, as well as alternative methods such as microscopy at least for Processed Animal Proteins (PAPs). Regarding the proteins, ELISAs have been proposed and even commercialised. However these approaches have shown their limitations: PCR-based approaches are highly discriminative for species, but can be problematic in complex matrices, frequent in food and feed and do not provide information about cell/tissue origin, which is a major issue in feed, given the mad cow disease epidemics. In food, in particular for allergenic proteins, which we will focus on, proteins are the target to monitor, and not DNA, and ELISAs at least for some proteins face the problem of cross-reactivity and low sensitivity. The developments in mass-spectrometry, but also in proteomics bioinformatics, computation and data mining over the last years (see for instance [2-4]), have paved the way for expanding peptidomic/proteomic approaches in food and feed characterization and authentication (for a recent review in food, [5]). Table 1 summarizes the possible MS based strategies in proteomics, with their characteristics, pros and cons, when searching for biomarkers. We will illustrate some of these approaches in food and feed proteomics. For the processed food, we will limit ourselves to allergenic proteins, which are a major health issue all over the word, while for feed we will focus on PAPs, in particular as by-products of pig and poultry breeding, since they are a convenient sustainable source of proteins for non-ruminants including farmed fish.

Allergenic Proteins in Food

In order to improve the accuracy of product labelling regarding the presence of allergenic ingredients, some routine laboratories are now considering the use of reliable, sensitive and robust MS-based analytical methods for detecting traces of allergenic foodstuff, but the diversity of food products/matrices and industrial processes makes this quite a challenge. The first step in developing the method consists in selecting marker peptides originating from enzymatic digestion of the proteins. These peptide markers could be identified by either a discovery approach with High-Resolution Mass Spectrometry (HRMS) or an *in silico* approach with triple quadrupole mass spectrometry (for instance [6]). The *in silico* approach consists in retrieving target protein sequences from a database (e.g. Uniprot or NCBI), performing an *in silico* digestion of proteins (e. g. with Skyline), and predicting MS/MS parameters according to a list of criteria such as peptide length (8 to 25 amino acids), digestive enzyme (trypsin), charge states of the peptide (+2, +3), and fragmentation (b, y), which allows generating a theoretical list of peptides and several hundreds of MRM transitions per protein (first column in Table 1). UHPLC-MS/MS for detecting and quantitating several allergens simultaneously in processed food products (requiring preliminary extraction buffer and digestion condition optimizations) has been proposed for the analysis of several allergenic foodstuffs such as: milk, egg, soy, peanut, and tree nuts (almond, hazelnut, walnut, pecan nut, cashew, and pistachio), possibly in several complex matrices taking into account the effects of temperature, acidity, and of

OPEN ACCESS

Correspondence:
Martine Raes, URBC-NARILIS, University of Namur, Belgium, E-mail: martine.raes@unamur.be

Received Date: *23 Mar 2018*
Accepted Date: *20 May 2018*
Published Date: *05 Jun 2018*

Citation:
Raes M, Planque M, Marbaix H, Gillard N, Lecrenier M-C, Dieu M, et al. Mass Spectrometry based Approaches for Analysing Proteins in Processed Food and Feed. Clin Oncol. 2018; 3: 1485.

Table 1: Overview of the main MS strategies for biomarker identification in food and feed.

	Sequence-oriented		Unbiased (sequence-independent)	
Strategy	***In silico analyses: MRM mode***	***Discovery analyses: HRMS mode***	***Global sample peptide profiling***	***Comparison with spectral libraries***
Characteristics	Peptide selection criteria • Protein abundance • Preference for proteins expressed as one single isoform or peptides shared by all the isoforms • Specific protein/peptide • Digestion compatible (K/R occurrence, avoiding sequences prone to miscleavage) • MS friendly (avoiding residue modifications,…) • Robustness to process/matrix effect (unpredictable parameter)		• Unsupervised statistical analysis based on the retention time, m/z ratio and peptide intensity (MS analysis) • Blank and contaminated samples are compared to highlight the MS signals associated with the target contaminated compounds. • MS/MS data can be further used to sequence the peptides specific to the contaminated sample	• Spectral libraries derived from HRMS data are built for each pure target compound, either raw or processed
Pros	• Rapid • Sensitive • Possibility for multitargeting • Ideal for routine analyses	• No need for prior protein selection (sample profiling in discovery mode) • Most known peptide modifications can be integrated (Met oxidation, …) • Adequate to identify and select the markers, but not for routine analyses	• Unbiased towards the peptide sequence --> compatible with yet undescribed modifications due to the process or matrix • Adequate to select and identify the markers, but not for routine analyses	• MS/MS spectra are independent of the retention time --> no need to analyse the different samples concomitantly • Spectral libraries can be built and shared by different laboratories
Cons	• The selection of the protein is based on pre existing knowledge (literature) • Peptide modifications due to process and/or matrix effects cannot be forecast, readily eliminating numerous peptides • Difficulties to take into account species/cultivar variants	• Not as rapid and sensitive as the MRM mode	• Need for reference samples for each matrix and process: a blank reference and a contaminated reference • Any slight modification of the set up will affect the retention time –> the reference and test samples have to be analysed concomitantly	• Detecting a target compound by this approach requires pre-existing spectral libraries as reference
Quantitation purposes	***Quantitation-compatible***		***Not compatible with quantitation***	

tannin and fat content (see for instance [7-9]).

Food laboratories encounter barriers for developing efficient methods. Bottlenecks include the lack of European regulatory thresholds, delays in the emergence of reference materials and guidelines, and the need to detect highly processed allergens. However, the identification of major influences of both food processing and matrix effects in detection of allergens in foodstuffs is possible [10]. The quantitation of allergenic proteins and the sensitivity of the method remain major issues in processed or complex food products, as hundreds of foodstuff varieties and matrices would need to be tested for the presence of antigens. The quantitation of allergens (but also of non-allergenic proteins) can be performed using protein extracts or synthetic peptides as standards, either without ([7,11] or with correction with internal standards such as labelled peptides/proteins [12,13]). Depending on the quantitation strategy, calibration curves can be established for fortified or incurred matrices, protein extracts, synthetic peptides, or labelled peptides. This usually involves matrix matching with the food product. We have shown that the extraction/digestion steps are not fully corrected by the addition of isotope-labelled peptides used as internal standards [10]. An alternative would be to develop new internal standards such as long isotope-labelled peptides digested together with the analyzed sample, and an external calibration curve (preferably unique, whatever the matrix of interest) in order to correct for, at least, the digestion step. While the ultimate approach for quantitation would be the use of labelled proteins that should be treated identically to samples for extraction and digestion - but this expensive strategy is not really compatible with routine analyses -, a promising alternative strategy could be to combine labelled internal standard peptides and standard addition. This strategy consists in adding to the sample increasing amounts of allergens and a fixed amount of labelled internal standard in order to obtain a calibration curve and determine the initial concentration of the target allergen that could be detected. The strategy combining standard addition with labelled peptides should allow a quantitation of allergens in all kinds of foodstuffs, with a good recovery. However, limiting the number of matrices that could be tested is always restrictive. In an approach to test the matrix effects, allergens can be spiked in a variety of matrices enriched, for example, in fat (sauce, mayonnaise), protein (ham), carbohydrates (cookie, chocolate, jam, and compote), tannin (spices and chocolate), or polyphenol (ham and compote) in order to assess allergen detection at the LOQ (Limit of Quantification). The rates of identified false positives and negatives could provide a basis of reflexion and of advice for analytical laboratories regarding the development of routine UHPLC-MS/MS methods for the detection of allergens (Planque et al., Food Chemistry, submitted). Moreover the fit-for-purpose technology should be described as well as the matrices for which the method is functional or not [14]. Eventually, at least two major problems do remain in the field. First, no legal, no universal thresholds have been set up to now. The Voluntary Incidental Trace Allergen Labelling (VITAL) system (developed in Australia and New Zealand, but commonly adopted) sets thresholds based on clinical studies determining no or low observable adverse effect levels for allergens. Norms such as ED_{01} and ED_{05} (eliciting doses) have been determined by VITAL, allowing protection of 99% and 95% of the allergic population, respectively. Secondly, while the basis of these references is useful, data is still expressed using many different units of measure, hampering the comparison of results between studies, which is a major limitation.

PAPs in Feeding Stuffs

Regarding PAPs in feed, different approaches have been used, although the number of publications remains limited compared to the literature about (cooked) meat. The protein extraction step is critical in PAPs, due to the post-mortem modifications of the proteins and to the rendering process. Collaborative work between our groups and the National Institute of Nutrition and Seafood Research (NIFES, Norway), comparing different workflows, with different extraction procedures, concluded that the protein extraction yield has to be optimized, but taking into account the number of reproducibly identified peptides from the extracted proteins [15]. Regarding the proteomic analysis, targeted approaches focused on collagens, were able to identify several species (cow, pig and chicken), based on peptide mass fingerprints [16], while for myosin the same authors used a 2D LC-MS/MS analysis, with peptide sequencing. We were able to identify proteins and peptide biomarkers for the detection for banned processed proteins [17]. We started with a non-targeted approach to detect and identify sets of unknown peptide markers (second column in Table 1), unique to one species (cow, pig and sheep), possibly to some tissues (muscle, cartilage….), with a focus on prohibited tissues (in Europe, blood or muscle from ruminants, muscle from pork for instance). Using a Q-TOF mass spectrometer, the most abundant species - specific peptides were selected (3 for beef and 4 for pork). These peptides were then used for developing a targeted multiple reaction monitoring (MRM) method (first column in Table 1), with a routine triple quadrupole MS, allowing to detect beef and/or pork in a total run of 20 min. Finally, when porcine or vegetal feed was spiked with beef PAPs, the latter were detected when present at 5% w/w, with either the Q-TOF or the triple quadrupole. Based on these developments, the same approach was applied on other feed matrices (feed for fish) for the specific detection of bovine blood meal and blood products. Hemoglobin peptides identified by HRMS were used to develop the MRM method [18]. In this study, milk peptides, previously selected in the context of milk allergens detection, were included in the MRM. The combination of the high sensitive MS/MS method and a sample preparation suitable for a routine use (one-day protocol) allowed us to reach the 0.1% (w/w) limit of detection (LOD) expected for this type of application This multi-target UHPLC-MS/MS method enables the simultaneous detection of multiple by-products (bovine blood-derived products and milk) in animal feed [19]. Complementary to these classical bottom-up proteomic workflows, other strategies have been recently developed, such as for instance PCA (Principle Component Analysis) and the use of spectral libraries (3rd and 4th columns of Table 1). The use of PCA, an unsupervised statistical method, in the analysis of PAPs makes it possible to differentiate the samples no longer on the basis of a few biomarker peptides but by using all of the peptide mass data generated during LC-MS analyses. With the PCA, a global peptide fingerprint of the sample is generated. A (unpublished) study of our laboratory was able to differentiate pork, mutton, beef and poultry PAPs. The advantage of PCA is to use all available mass information without necessarily knowing the sequence of the peptides. The main disadvantage of this method is the unavoidable presence of many peptides of identical masses due to the huge amount of peptide data. Without the MS/MS as a second dimension to characterize these peptides, one relies on the retention time information that allows creating a retention time/mass pair that will be used by the PCA. We are therefore dependent on the LC analysis and the retention time of the peptides, which is dependent on the material used (solvent, column, flow etc.). An alternative to the PCA that allows to get rid of this retention time factor that prevents any inter laboratory analysis, is the use of spectral libraries. The latter are created from experimental MS/MS data, without necessarily requiring identification. They are often used for organisms that remain incompletely sequenced, but for which a spectral library with most spectra identified can be created [20]. According to Ahrné et al [21], the use of a search engine in a spectral library in combination with a sequence search engine in the data banks made it possible to increase identifications by 156% and also increase the speed of data processing. According to Griss [20], this improvement is mainly due to the fact that low quality MS/MS spectra (often synonymous of low-abundance protein) are identifiable with more confidence in a spectral library compared to sequence database research engines. The weakness of this approach is that only peptides present in the used library can be identified, while with a traditional search engine, homology can still be used with other species to identify a peptide. Another drawback is that these databases have to be created. Rasinger et al [15] in their study on PAPs in aqua feeds were also able to discriminate species (porcine, bovine, ovine and poultry) and origin (carcase, meal, blood meal, greaves, muscle, and feather meal) by direct spectral comparison based on the bulk of high quality tandem mass spectra. Of course, even though the extraction procedures might differ, the approaches described here for PAPs in animal feed are also applied to (cooked) meat, for which the discrimination between for instance pork and beef and the detection of traces of pork are critical in several countries (for a recent review [22]). Spectral matching has already been applied to identify meat products in 16 mammalian and 10 bird species [23]. Worth to mention are the recent developments of ambient mass spectrometry, applied by Montowska et al [24] for the authentication of processed meat products.

Conclusion

In conclusion, proteomic/peptidomic MS based approaches have become inescapable in food and feed analysis and different strategies are now available, but have to be selected properly according to the addressed questions. However even though sequence-oriented approaches are considered as quantitation compatible (Table 1), absolute and accurate quantitation of peptide biomarkers in processed food and feed remains, despite continuous progress in the field, a challenge for the next-coming years.

References

1. Mills EN, Sancho AI, Rigby NM, Jenkins JA, Mackie AR. Impact of food processing on the structural and allergenic properties of food allergens. Mol Nutr Food Res. 2009;53:963-9.
2. Karimpour-Fard A, Epperson LE, Hunter LE. A survey of computational tools for downstream analysis of proteomic and other omic datasets. Hum Genomics. 2015;9:28.
3. Oveland E, Muth T, Rapp E, Martens L, Berven FS, Barsnes H. Viewing the proteome: how to visualize proteomics data? Proteomics. 2015;15(8):1341-55.
4. Chen C, Huang H. WCH. Protein bioinformatics databases and resources. Methods Mol Biol. 2017;1558:3-39.
5. Ortea I, O'Connor G, Maquet A. Review on proteomics for food authentication. J Proteomics. 2016;147:212-25.
6. Downs ML, Johnson P. Target selection strategies for LC-MS/MS food allergen methods. J AOAC Int. 2018;101(1):146-51.
7. Planque M, Arnould T, Dieu M, Delahaut P, Renard P, Gillard N. Liquid

chromatography coupled to tandem mass spectrometry for detecting ten allergens in complex and incurred foodstuffs. J Chromatogr A. 2017;1530:138-51.

8. Parker CH, Khuda SE, Pereira M, Ross MM, Fu TJ, Fan X, et al. Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. J Agric Food Chem. 2015;63:10669-80.
9. Heick J, Fischer M, Kerbach S, Tamm U, Popping B. Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. J AOAC Int. 2011;94(4):1060-8.
10. Planque M, Arnould T, Renard P, Delahaut P, Dieu M, Gillard N. Highlight on bottlenecks in food allergen analysis: detection and quantification by mass spectrometry. J AOAC Int. 2017;100(4):1126-30.
11. Montowska M, Fornal E. Label-free quantification of meat proteins for evaluation of species composition of processed meat products. Food Chem. 2017;237:1092-100.
12. Pilolli R, de Angelis E, Monaci L. In house validation of a high resolution mass spectrometry orbitrap-based method for multiple allergen detection in a processed model food. Anal Bioanal Chem. 2018.
13. Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, et al. Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics. 2007;6(12):2139-49.
14. Yeung J, Robert MC. Challenges and path forward on mandatory allergen labeling and voluntary precautionary allergen labeling for a global company. J AOAC Int. 2018;101(1):70-6.
15. Rasinger JD, Marbaix H, Dieu M, Fumière O, Mauro S, Palmblad M, et al. Species and tissues specific differentiation of processed animal proteins in aquafeeds using proteomics tools. J Proteomics. 2016;147:125-31.
16. Reece P, Chassaigne H, Collins M, Buckley M, Bremer M, Grundy H. Proteomic analysis of meat and bone meal and animal feed. In: Jorgensen JS, Baeten V., editors. Detect. Detection, identification and quantification of processed animal proteins in feedingstuffs. Namur, Belgium : Les editions namuroises; 2012.p. 113-23.
17. Marbaix H, Budinger D, Dieu M, Fumière O, Gillard N, Delahaut P, et al. Identification of proteins and peptide biomarkers for detecting banned Processed Animal Proteins (PAPs) in meat and bone meal by mass spectrometry. J Agric Food Chem. 2016;64(11):2405-14.
18. Lecrenier MC, Marbaix H, Dieu M, Veys P, Saegerman C, Raes M, et al. Identification of specific bovine blood biomarkers with a non-targeted approach using HPLC ESI tandem mass spectrometry. Food Chem. 2016;213:417-24.
19. Lecrenier MC, Planque M, Dieu M, Veys P, Saegerman C, Gillard N, et al. A mass spectrometry method for sensitive, specific and simultaneous detection of bovine blood meal, blood products and milk products in compound feed. Food Chem. 2018;245:981-8.
20. Griss J. Spectral library searching in proteomics. Proteomics. 2016;16(5):729-40.
21. Ahrné E, Masselot A, Binz PA, Müller M, Lisacek F. A simple workflow to increase MS2 identification rate by subsequent spectral library search. Proteomics. 2009;9(6):1731-6.
22. Montowska M. Using peptidomics to determine the authenticity of processed meat. In: Colgrave ML, editor. Proteomics in food science - From farm to fork. Elsevier; 2017.p.225–40.
23. Ohana D, Dalebout H, Marissen RJ, Wulff T, Bergquist J, Deelder AM, et al. Identification of meat products by shotgun spectral matching. Food Chem. 2016;203:28-34.
24. Montowska M, Alexander MR, Tucker GA, Barrett DA. Authentication of processed meat products by peptidomic analysis using rapid ambient mass spectrometry. Food Chem. 2015;187:297-304.