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Inter-laboratory study on the detection of bovine processed animal protein in feed by LC-MS/MS-based proteomics

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ABSTRACT

Since the beginning of the feed ban regarding the use of processed animal protein (PAP) in feedstuffs, one of the main challenges has been to develop methods to detect and identify the type and origin of PAP or animal products. By now, in the context of a controlled relaxation of the ban, complementary methods are sought to characterize the product when positive responses are delivered by current official methods, with special attention on feed materials (whether or not authorized) originating from ruminants. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics was identified as the most promising method to meet this challenge. This study presents the first inter-laboratory test constructed to evaluate and compare the LC-MS/MS methods currently developed in six different laboratories from four countries. Nine samples consisting of blank feed matrix or feed adulterated with bovine processed animal proteins and/or milk products were analyzed using in-house protocols and the results obtained were discussed. This study confirms the potential of LC-MS/MS-based proteomics to resolve the current analytical gaps in the detection and differentiation of PAP and highlights the technical challenges that need to be addressed in future.

1. Introduction

PAP is defined by the European Commission (2011) as animal protein derived entirely from Category 3 material, which has been treated to render them suitable for direct use as feed material, organic fertilizers or soil improvers. This definition includes both blood meal and fishmeal but excludes blood products, milk products, colostrum products, gelatin, collagen, egg products, hydrolyzed proteins, dicalcium phosphate and tricalcium phosphate. In practice, PAPs look like meal and are produced by heat treatment under high temperature and pressure in a kind of “carcass soup” made up of, among other things, bone, cartilage, muscle, blood and fat.

Official control of the use of PAP in feedstuffs is based on two analytical methods, light microscopy (LM) and polymerase chain reaction

(PCR), which are combined according to the feed destination (European Commission, 2009). With the relaxing of the feed ban, complementary analytical methods must be developed to support the forthcoming lifting of the feed ban. In a previous publication, Lecrenier et al. (2020) highlighted the analytical gaps that exist, the possible scenarios for future operational schemes, and the new expected challenges that should be associated. Complementary methods need to bridge these gaps by making an accurate distinction between authorized feed materials and non-authorized ones when current official methods deliver positive results. In particular, the joint discrimination of the type of tissues (e.g. dairy products vs PAP) and the species or taxonomic origin (e.g. ruminant vs porcine) is required.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics is a promising approach to fill the analytical gaps

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with respect to animal by-products in feedingstuffs (Lecrenier et al., 2020). MS-based proteomics has the advantage of providing information about both the type of protein and the species through the selection of specific peptide targets. Moreover, the use of a multi-targeted MS strategy, aimed at testing several protein markers, allows the detection and identification of different ingredients in a single analysis. In recent years, interest in determining the origin of PAP using this technology has grown exponentially (Belghit et al., 2019; Lecrenier et al., 2016, 2018; Marbaix et al., 2016; Marchis et al., 2017; Niedzwiecka et al., 2018; Rasinger et al., 2016; Steinhilber et al., 2018a, 2018b, 2019). Consequently, different approaches and protocols varying in terms of sample preparation protocols, choice of MS equipment and analytical strategies, respectively were developed.

The objective of the present inter-laboratory study was to assess the performance of LC-MS/MS methods already developed in different laboratories from both public and private sectors. Each participating laboratory applied their own LC-MS/MS-based proteomic method. The sample set consisted of blank feed or feed adulterated with bovine PAP and/or milk product at various concentration levels.

2. Materials

2.1. Feed and feed material

The feed matrix used was commercial pig feed intended for sow feeding (Table 1). Its labelling indicated that it was composed of wheat middlings, wheat, barley, rice, maize, rapeseed meal, sugar beet pulp, soybean meal, calcium carbonate, lard, salt, premix, dicalcium phosphate and amino acids. PCR and LM analyses confirmed that the feed was free of ruminant DNA and free of terrestrial animal particles, respectively. The protein content was estimated at 14.1% (w/w) based on nitrogen content, determined in duplicate by the Kjeldahl method (ISO 8968-1, 2014), and using the nitrogen-to-protein conversion factor of 6.25 (Jones, Munsey, & Walker, 1942).

Two different PAPs and one milk powder were used as adulterant materials. Bovine PAP-1 was a commercial feed material. PCR and LM analyses showed that it contained ruminant DNA and terrestrial particles (bone and muscle), respectively (Table 1). Its sediment fraction was 62% (w/w) and the protein content was estimated at 49.5% (w/w). Bovine PAP-2 was produced in a pilot plant with about 50% (w/w) bone, 20% (w/w) meat and fat and 10% (w/w) blood. PCR and LM analyses showed that it contained ruminant DNA and terrestrial animal

particles (bone, muscle and blood), respectively. Its sediment was at 53% (w/w) and the protein content was estimated at 35.4% (w/w).

The milk product originated from a commercial calf milk replacer predominantly composed of skimmed milk powder and whey powder. PCR and LM analyses showed that this product contained ruminant DNA, but no bone or muscle particles, respectively (Table 1). The protein content was estimated at 21.8% (w/w).

2.2. Sample preparation

Nine different test materials were prepared for the study (Table 1). Three reference samples (samples #1, #2 and #3) were included in the set and their content was communicated to the participants. Laboratories were free to use these references to develop or optimize their methods and no results were required at this stage. One quality control (QC), sample #4, was included as positive control and its composition was also communicated. Data obtained on this sample had to be listed in the result file but without interpretation as the sample was not blind. Finally, five blind samples (samples #5, #6, #7, #8 and #9) with or without adulteration at levels from 1%, up to 5% (w/w) completed the sample set. Results obtained on these samples were used to evaluate the different MS methods.

Adulterated samples were prepared by stepwise dilutions. To ensure homogeneity, in final step, all samples except sample #2 were ground with an Ultra Centrifugal rotor Mill ZM 200 (Retsch, Haan, Germany) in combination with a sieve of 2 mm mesh size. The blank matrix was prepared and conditioned first in order to avoid any contamination by the other samples. QC and blind samples were characterized by LM and PCR and results were in accordance with the sample composition. Based on the known composition of the samples, the presence or absence of prohibited ruminant by-products was determined for each blind sample (Table 1).

3. Study organization

Each participant was assigned a unique identifier ("Lab 1 to 6") to keep the results anonymous. Each Lab received about 50 g of each reference sample and about 5 g of QC and blind samples. The organizer endorsed the grinding and homogeneity of the samples. Nevertheless, participating laboratories were solely responsible to reach appropriate homogeneity for the sample sub-portions taken for analysis.

A protected Excel file containing a report form and instructions on how to fill it was sent to the participants together with the samples.

Table 1
Sample set composition and results obtained by official methods (LM and PCR).

| Sample # | Composition | LM results | | PCR results | Prohibited ruminant by-products |
|----------|---|-----------------|------|-------------|---------------------------------|
| | | Sediment (bone) | | Ruminant | |
| | | Terrestrial | Fish | | |
| 0 | Bovine PAP-2 | + | - | + | + |
| 1 | Bovine PAP-1 | + | - | + | + |
| 2 | Milk product | - | - | + | - |
| 3 | Pig feed | - | - | - | - |
| 4 | QC (Pig feed + 5% Bovine PAP-1 + 1% Milk product) | + | - | + | + |
| 5 | Pig feed + 1% Bovine PAP-1 | + | - | + | + |
| 6 | Pig feed + 1% Bovine PAP-2 | + | - | + | + |
| 7 | Pig feed + 5% Milk product | - | - | + | - |
| 8 | Pig feed + 1% Bovine PAP-1 + 1% Milk product | + | - | + | + |
| 9 | Pig feed (Blank) | - | - | - | - |

Legend: Flotate, solid phase of the sedimentation made of the floating material in trichloroethylene; LM, Light microscopy; PAP, processed animal protein; PCR, polymerase chain reaction; QC, quality control; +, presence of animal particles, ruminant DNA or prohibited ruminant by-products; -, absence of animal particles, ruminant DNA or prohibited ruminant by-products. Sample #0 was not sent to participant as such but was used to prepare sample #6. Samples sent blind to the participants are in bold. Concentrations are expressed as weight-weight (w/w) percentage (%).

Based on the results obtained for the targeted peptides and individual evaluation criteria, laboratories had to make a conclusion on the presence or absence of prohibited ruminant by-products in the blind samples.

4. Methods

Six laboratories analyzed the samples using their own in-house LC-MS/MS-based proteomic approach. The applied methods used were based on recent publications (Belghit et al., 2019; Lecrenier et al., 2016, 2018; Marchis et al., 2017; Niedzwiecka et al., 2018; Steinhilber et al., 2019). Major differences between methods are summarized in Tables 2–5.

4.1. Sample preparation

Table 2 summarizes the major differences observed in the sample preparation protocols used by the participating laboratories. Test portion size ranged from 15 mg up to 1 g of sample. All the laboratories performed the analyses in technical duplicates, except for Lab 5 that analyzed only one test portion of each sample. Labs 2 and 5 did not pre-treat the samples before extraction, whereas Labs 1 and 3 included an additional grinding step. Lab 1 ground a larger subsample before weighing the test portions in order to ensure a good homogenization. Labs 4 and 6 primarily defatted the samples by using a buffer containing trichloroacetic acid (TCA) and acetone. Each lab used a different extraction buffer, but the reduction and alkylation agents were similar, except for Lab 1 that used another reduction agent. The tryptic diges-

Table 2
Comparison of the sample preparation steps used by the participating labs.

| | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 |
|---------------------|-----------------------------------|---|-------------------------------|---|---------------------------------|---|
| Test portion size | 15 mg | 100 mg | 50 mg | 1 g | 1 g | 50 mg |
| No of replicates | 2 | 2 | 2 | 2 | 1 | 2 |
| Pre-treatment | Grinding | / | Grinding | Defatting step with TCA 10%/2-ME in acetone | / | Precipitation/defatting step with TCA 10%/acetone washes. |
| Extraction buffer | TEA-HCl (50 mM), 0.5% (w/v) nOG | Laemmli Buffer 4x (4% SDS, 20% glycerol, 10% 2-ME, 0.004% bromophenol blue, Tris.HCl (125 mM) | 0.1 M Tris HCl, SDS 4% | Urea (7 M), Thiourea (2 M), 0.03% (w/v) CHAPS, 1.25 mg/L alpha-Amylase | Tris.HCl (200 mM), Urea (2 M) | Urea (7 M), Thiourea (2 M), Tris (30 mM), 4% (w/v) CHAPS |
| Reduction agent | TCEP | DTT | DTT | DTT | DTT | DTT |
| Alkylation agent | IAA | IAA | IAA | IAA | IAA | IAA |
| Digestion enzyme | Trypsin, HPD | Trypsin | Trypsin | Trypsin | Trypsin | Trypsin |
| Digestion time | 2 h | Overnight | 16 h | > 12 h | 1 h | Overnight |
| Purification method | Immuno-precipitation ^b | SDS-page ^a | C18 spin columns ^b | Immuno-affinity enrichment ^a ; C18 spin columns ^b | C18 SPE cartridges ^b | FASP digestion |

Legend: 2-ME, 2-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; IAA, iodoacetamide; FASP, filter aided sample prep; HCl, hydrogen chloride; HPD, heterogeneous phase digestion; No, number; nOG, n-octylglucoside; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; TCA, trichloroacetic acid; TCEP, tris (2-carboxyethyl) phosphine; TEA, triethanolamine.

^a Applied prior to digestion.

^b Applied after digestion.

Table 3
Comparison of the liquid chromatography (LC) and Mass spectrometer (MS) system used by the participating labs and the main parameters.

| | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 |
|---------------------|--|---|--|---------------------------------|--|-------------------------------------|
| LC system | Ultimate TM 3000 nano RSLC (Thermo Fisher Scientific) | ExionLC TM system (SCIEX) | Vanquish TM Horizon binary UHPLC (Thermo Fisher Scientific) | nanoHPLC (Agilent Technologies) | UPLC TM Acquity system (Waters) | NanoElute [®] (Bruker) |
| Gradient time (min) | 10 | 15 | 15 | 74 | 16 | 40 |
| MS system | Q-Exacte TM Plus (Thermo Fisher Scientific) | QTRAP TM 5500 System (SCIEX) | Q-Exacte TM Orbitrap (Thermo Fisher Scientific) | maXis TM (Bruker) | Xevo [®] TQ-S micro (Waters) | Tims TOF TM Pro (Bruker) |
| Acquisition mode | PRM | MRM | PRM | DDA with inclusion list | MRM | DDA |
| Ionization mode | ESI positive | ESI positive | ESI positive | ESI positive | ESI positive | ESI positive |

Legend: DDA, Data-dependent acquisition mode; ESI, electrospray ionization; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; PRM, parallel reaction monitoring.

Table 4
List of the peptide sequences used by the participating labs and the targeted tissue/product.

| Protein | Peptide | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 |
|-------------------------|----------------------|-----------|--------|-------|-------|-------|-------------|
| Alpha-2-Macroglobulin | GSGGTAEHPTVEEFVLPK | Blood | | | | | |
| Alpha-2-Antiplasmin | LPPLSLK | Blood | | | | | |
| Protein HP-25 homolog 2 | FGFDIELFQHAVK | Blood | | | | | |
| Complement Component 9 | YTPVEAIEK | Blood | | | | | |
| Myosin-7 | MLSSLFANYAGFDTPIEK | Muscle | | | | | |
| Osteopontin | YPDVAVTWLKPDPSQK | Bone/Milk | | | | | |
| Matrilin-1 | AGGIELFAIGVGR | Cartilage | | | | | |
| Desmin | TSGGAGGLGALR | | Muscle | | | | |
| Vimentin | TLYTSSPGGVYATR | | Muscle | | | | |
| Myoglobin | YLEFISDAIHVLHAK | | Muscle | | | | |
| Hemoglobin alpha-chain | VGGHAAEYGAEALER | | | Blood | Blood | Blood | Blood |
| Hemoglobin beta-chain | AAVTAFWGK | | | Blood | Blood | Blood | Blood |
| | EFTPVLQADFQK | | | Blood | Blood | Blood | Blood |
| | VVAGVANALHR | | | | Blood | Blood | Blood |
| Collagen alpha-2 (I) | GEPGAVGAVGAVGPR | | | | | | Bone/Tendon |
| Casein alpha-S1 | FFVAPFPEVFGK | | | Milk | | | Milk |
| | HQGLPQEVLNLLR | | | Milk | | | Milk |
| | YLGYLEQLLR | | | | | | Milk |
| Casein alpha-S2 | NAVPTITLNR | | | Milk | | | Milk |
| Beta-lactoglobulin | LSFNPTQLEEQCHI | | | | | | Milk |
| | VLVLDTDYK | | | Milk | | | Milk |
| | VYVEELKPTPEGDLEILLQK | | | Milk | | | Milk |

Table 5
Comparison of the evaluation criteria applied by the participating labs.

| | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 |
|--|--|--|---|---|---|---|
| To detect and identify the peptide | RT identical to IS (± 1 s) Qualifier ion > LLOQ (CV = $\pm 20\%$, CV = $\pm 25\%$ at LLOQ) Specificity of antibody Ion ratios similar to IS ≥ 3 product ions detected | RT similar to ref sample ^a S/N > 3 for all product ions Ion ratios similar to ref sample ^a | RT similar to IS (± 0.07 min) Product ions < 3 ppm mass error S/N > 3 for all product ions ≥ 3 product ions detected ≥ 1 product ion is a y-ion type | ≥ 1 peptide identified Peptide Mascot score ≥ 39.0 | RRT similar to ref sample ^a S/N > 10 for quantifier ion Ion ratios similar to ref sample ^a | RT and IM closed to QC sample Product ions < 5 ppm error Peptide Mascot score \geq identity score Peptide threshold 1% FDR |
| To conclude on the presence of the targeted by-product | CV of the ratio between quantifier ion and IS intensity $\leq 20\%$ | ≥ 2 process IS identified IS identified ≥ 1 peptide identified | ≥ 1 peptide identified | ≥ 1 peptide identified in both replicates. If results differ, bovine Hb considered to be present, if detected in a 3rd replicate | ≥ 2 peptides identified (including AAVTAFWGK for bovine Hb, and FFVAPFPEVFGK for Milk or LSFNPTQLEEQCHI if casein is not main milk source) | ≥ 1 peptide identified |

Legend: CV, coefficient of variation; FDR, false discovery rate; Hb, hemoglobin; IM, ion mobility; IS, internal standard; LLOQ, lower limit of quantification; ppm, part per million; QC, quality control; ref sample, reference sample; RRT, relative retention time; RT, retention time; S/N, signal to noise ratio.

^a RT or ion ratio tolerances described by European Commission (2002).

tion time varied between the laboratories: Labs 1 and 5 had a rapid digestion step ranging from 1 to 2 h, while the other labs performed the digestion for more than 12 h. A last important distinction among the methods relates to purification and enrichment. Lab 1 used an immuno-affinity enrichment technique (peptide immunoprecipitation) and Labs 3 and 5 used C18 solid phase extraction (SPE) cartridges. Lab 4 combined an immuno-affinity enrichment technique (protein immuno-affinity) and a C18 SPE using spin columns. Lab 2 performed SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Lab 6 carried out the reduction, alkylation, and digestion in ultrafiltration filters by filter-aided

sample preparation (FASP) (Distler, Kuharev, Navarro, & Tenzer, 2016).

4.2. LC-MS/MS

The LC-MS/MS systems applied by the participants are presented in Table 3. All laboratories worked with ultra-high-pressure liquid chromatography (UHPLC), except for Lab 4 that used high-pressure liquid chromatography (HPLC). Labs 2, 3 and 5 used conventional internal diameter columns of 2.1 mm whereas Labs 1, 4 and 6 worked with nano

columns. Labs 1, 2, 3 and 5 applied a short gradient time (≤ 16 min), while Labs 4 and 6 had a longer gradient time ≥ 40 min.

Two classes of MS instruments were used by the laboratories: (i) high-resolution mass spectrometers (HRMS) with quadrupole-orbitrap (Q-Orbitrap) or quadrupole-time-of-flight (Q-TOF) used by Labs 1, 3, 4, and 6 and (ii) low-resolution mass spectrometer (LRMS) with triple quadrupole (triple-Q) used by Labs 2 and 5. Differences can also be observed in the acquisition mode: Parallel reaction monitoring (PRM) for Labs 1 and 3, multiple reaction monitoring (MRM) for Labs 2 and 5 and data-dependent acquisition (DDA) for Labs 4 and 6. In summary, the laboratories can be separated in three groups based on their MS approach: LRMS with MRM mode (Labs 2 and 5), HRMS with PRM mode (Labs 1 and 3) and HRMS with DDA mode (Labs 4 and 6).

Table 4 lists the peptide sequences (and the protein from which they origin) used in this study. The targeted bovine tissues or products (i.e., blood, muscle, bone, cartilage, tendon and milk) were identified for each peptide. Lab 1 designed its set with plasma, muscle, bone and cartilage peptides. The selected bone peptide is also present in milk products. Lab 2 has focused its method on muscle peptides. The four other labs (Labs 3, 4, 5 and 6) have chosen comparable sets of hemoglobin peptides, supplemented by milk peptides for Labs 3, 5 and 6 and a bone/tendon marker for Lab 6.

4.3. Criteria for interpretation of the results

The last major parameter of variation concerns the interpretation of the results. Table 5 summarizes the criteria used by each lab to evaluate the MS data and to deliver a conclusion on the presence or absence of the targeted by-product. Regarding the identification and detection of the peptides, criteria were similar for a same cluster in acquisition mode (MRM/PRM or DDA). However, an important difference was the number of identified peptides requested to conclude on the presence of the related by-product. Some laboratories concluded on the identification of by-products with at least one protein marker whereas Lab 5 sets its criteria to a minimum of two peptides.

5. Results and discussion

Individual results are summarized in Supplementary Table 1–6. Table 6 summarizes each participating laboratory's conclusion on their findings. Erroneous results are marked with an asterisk.

The overall results, expressed in terms of global accuracy confirm the suitability of the mass spectrometric approaches for the detection of bovine PAP. The percentage of total error only accounted for 16.5% of the total responses. There are no false positive results for the detection of prohibited ruminant by-products. However, bovine PAPs, when present in the samples, were not always detected. Labs 1, 5 and 6 had no false negative results. Labs 2 and 3 showed one false negative result (sample #8) and Lab 4 had three false negative results (samples #5, #6

Table 6
Summary of the labs conclusion on the presence of prohibited ruminant by-products in blind samples.

| Sample # | Adulteration | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 |
|----------|--------------|-------|-------|-------|-------|-------|-------|
| 5 | 1% PAP-1 | + | + | + | -* | + | + |
| 6 | 1% PAP-2 | + | + | + | -* | + | + |
| 7 | 5% Milk | - | - | - | - | - | - |
| 8 | 1% PAP-1 | + | -* | -* | -* | + | + |
| | + 1% Milk | | | | | | |
| 9 | Blank | - | - | - | - | - | - |

Legend: PAP, processed animal protein; +, the sample contains prohibited ruminant by-products; -, the performed analyses does not allow a conclusion on the presence of prohibited ruminant by-products. False results are marked with an asterisk. Concentrations are expressed as weight-weight (w/w) percentage (%).

and #8). The first conclusion is that, except for sample #8, five out of six participating laboratories correctly detected bovine PAPs at the level of 1% (w/w).

To explain the results obtained by Lab 4 for samples #5 and #6, the method employed in Lab 4 was compared to those of Labs 3, 5, and 6. Even if the methods used by the laboratories listed above differed in sample preparation protocols, MS instrument and the acquisition mode, all four laboratories targeted hemoglobin chains with a comparable set of peptides. Labs 5 and 6 used the same set of peptides as Lab 4, while Lab 3 used only three out of this set of four. Whereas Lab 4 did not detect any of the target peptides, Lab 3 detected one of them (VGGHAAEYGAELER) in one of the two replicates of samples #5 and #6. Lab 5 detected three hemoglobin peptides (VGGHAAEYGAELER, AAVTAFWGK and VVAGVANALAHR) in sample #5 and four hemoglobin peptides (VGGHAAEYGAELER, AAVTAFWGK, EFTPVQLQADFQK and VVAGVANALAHR) in sample #6. Lab 6 also detected three hemoglobin peptides (VGGHAAEYGAELER, EFTPVQLQADFQK and VVAGVANALAHR) in sample #5 and four hemoglobin peptides (VGGHAAEYGAELER, AAVTAFWGK, EFTPVQLQADFQK and VVAGVANALAHR) in sample #6. Lab 4 further investigated the results obtained by analyzing pure bovine PAP-1 (data not shown). This sample was found to be positive for bovine material based on the detection of two out of the set of four peptides (VGGHAAEYGAELER and VVAGVANALAHR). Repeated analysis of the QC sample (containing PAP-1 at a much lower level) by Lab 4, once again yielded negative results for all targeted peptides. This inter-laboratory comparison confirms the presence of bovine hemoglobin in PAP-1 and PAP-2 and suggests that the false negative results obtained by Lab 4 in the study is probably due to a lack of sensitivity of the method on the PAPs used. After investigation, it appeared that the lack of sensitivity compared to prior tests with the method may result from a lowered LC resolution.

The results obtained by all participants on sample #5 (1% PAP-1) and sample #6 (1% PAP-2) were also compared. Labs 5 and 6 detected 100% (4/4) and 75% (3/4) of their hemoglobin markers in sample #6 and in sample #5, respectively. Similar results were also observed by Lab 1 for their blood marker peptides. Lab 3 detected only one blood peptide (VGGHAAEYGAELER) in both samples (samples #5 and #6). Comparison of the signal intensities or peptide counts for the peptides detected in both samples revealed that signals for the blood peptides were generally higher in sample #6 than in sample #5. Muscle markers (myosin-7 and myoglobin) were detected in both samples by Labs 1 and 2. The signal intensity of myosin-7 (MLSSLFANYAGFDTPIEK) and of myoglobin (YLEFISDAIHVLHAK) was also higher in sample #6 than in sample #5. In contrast, bone markers (osteopontin and collagen alpha-2 (I)) used by Labs 1 and 6 were detected in sample #5 and not in sample #6. In order to see whether these differences originate from the PAP's composition, these observations were compared to the results obtained by LM. Using tetramethylbenzidine-hydrogen peroxide staining (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2013), PAP-2 gave a positive reaction for blood (immediate blue-green coloring and release of O₂ bubbles), while PAP-1 did not react positively to this staining. Therefore, the blood concentration in PAP-1 is probably lower than in PAP-2. The comparison of sediment percentage indicates a higher bone content in PAP-1 than in PAP-2. LM observations thus confirms the higher blood content of PAP-2 and the higher bone content of PAP-1 that were suggested by the MS data.

Sample #8, containing 1% PAP-1 and 1% milk powder, appears to be the most complex sample as three out of six laboratories (Labs 2, 3 and 4) failed to detect PAP in it. Lab 3 detected two product ions of the peptide VGGHAAEYGAELER in sample #8 but declared it as negative because their criteria for peptide identification (≥ 3 product ions detected) were not met. Lab 2 did not detect any product ion in this sample. When the same PAP material (PAP-1) was used at the same concentration in sample #5, Labs 2 and 3 could correctly detect it. Labs 1, 5 and 6 detected the presence of prohibited ruminant by-products but the comparison of the data obtained for sample #8 and sample #5

shows some differences. Peak intensities observed by Lab 5 for the hemoglobin peptides VGGHAAEYGAEALER, AAVTAFWGK and VVAGVANALAHR were lower in sample #8 than in sample #5. Peptide counts detected by Lab 6 for VGGHAAEYGAEALER and VVAGVANALAHR were also lower in sample #8 than in sample #5. EFTPVLQADFQK (hemoglobin) and GEPGPAGAVGPAGAVGPR (collagen) had the same peptide counts. For Lab 1, the comparison was limited to muscle and cartilage peptides as the other ones may appear in both adulterants of sample #8, PAP and milk. In this case, the signal intensities of MLSSLFANYAGFDTPIEK (myosin-7) and AGGIELFAIGVGR (matrilin-1) were higher in sample #8 than in sample #5. These results revealed that the presence of milk can have different impacts on the detection of PAP depending on the method and the peptides used. This phenomenon is probably linked to a cause of disturbance well described in ESI-LC-MS: matrix effects. These effects are often due to the alteration of the ionization efficiency of the target analytes due to the co-elution of other compounds. Matrix effects can affect analytical performance in the form of a decrease (ion suppression) or an increase (ion enhancement) in signal intensity (Zhou, Yang, & Wang, 2017). The fact that the presence of milk in sample #8 has a different impact on the results of Lab 1 vs other labs can therefore be explained: the specific purification (immunoaffinity enrichment) used by Lab 1 minimizes the matrix effect by removing more co-eluting molecules.

Some participants also included the detection of milk powder (Labs 1, 3, 5 and 6) in their results. Correct detection of milk proteins was achieved by four laboratories (more details in the Supplementary Tables 1–6), meaning that no false negative result was reported. However, milk powder induced many false positive results and all laboratories found milk peptides at least in one sample that did not contain milk powder. As PCR analyses cannot distinguish the origin of detected ruminant DNA, QC and blind samples (samples #4, #5, #6, #7, #8 and #9) were analyzed by enzyme-linked immunosorbent assays (ELISAs) in order to evaluate if the unexpected results were not due to an unknown milk contamination during sample preparation. Analyses were performed by the CER Groupe using ELISAs developed for allergen detection in food (Dumont et al., 2010). These methods are very sensitive for the detection of beta-lactoglobulin and casein, with a limit of quantification of 0.25 ppm and 0.5 ppm, respectively. No milk protein was detected in samples #5, #6 and #9 and both beta-lactoglobulin and casein were detected in samples #4, #7 and #8.

Lab 1 erroneously classified sample #5 as positive with regard to milk content. The classification was based on the presence of high osteopontin content in the sample. YPDAVATWLKPDPSQK (osteopontin) is used by Lab 1 both to detect the presence of milk and of bone-containing PAP. The identification of the tissue of origin is based on the protein ratio of osteopontin vs the blood proteins. The high bone content of PAP-1, used for sample #5 adulteration, probably caused this misclassification. This study underlines the difficulty of the approach to identify the tissue origin of a protein based on its ratio to other proteins, especially when analyzing mixtures with variable tissue ratios. Hence, a broader data knowledge of PAP composition ranges will be necessary for more reliable milk detection based on the ratio between osteopontin and other tissue markers. The reasons for false milk detection of Labs 3, 5, and 6 are less obvious though based on the same peptides. Lab 3 found milk peptides in one of the two replicates for sample #5 and sample #6. Lab 5 detected one casein peptide in sample #6 but did not declare the sample as positive for milk according to their evaluation criteria, which requires the detection of two peptides to confirm the presence of the targeted by-product. Lab 6 also detected only one milk marker in sample #6 and declared the sample as positive for milk, according to their acceptance criterion. As described by Ramachandran, Yang, and Downs (2020), some milk peptides are known to be sticky leading to increase carryover effects. This phenomenon may explain the false positive results observed. Carry-over is due to non-specific adsorption of peptides that can occur at every part of the LC-MS

system (Maes, Smolders, Michotte, & Van Eeckhaut, 2014). The potential risk is increased consecutively to the injection of high-concentration samples. In order to overcome this problem, it is essential to understand the origin of carry-over and to develop efficient strategies to reduce it. If, despite everything, it is not possible to manage it, the exclusion of troublesome peptides from the target list is another option.

6. Conclusion

The versatility and discriminative power of LC-MS/MS-based proteomics methods to identify both species and tissue origin allows to efficiently and unambiguously determine an array of prohibited and authorized animal by-products in feed samples. The present study shows that LC-MS/MS is able to successfully identify the presence of various proteins of bovine origin in feed at an adulteration level of 1% (w/w). Both LRMS and HRMS exhibited very similar performances in term of accuracy. The results also revealed that there are still some pitfalls to solve. For example, as similarly observed when using other analytical approaches for PAP detection and differentiation, the heterogeneity of the composition of feed materials can interfere with the correct detection of specific peptide in certain matrices. This inter-laboratory study thus provides important data required to build robust and reproducible LC-MS/MS methods for future routine PAP analyses for the detection of prohibited animal by-products. In addition to the very specific application of MS-based proteomics for PAP detection, the results obtained in the present study also provide evidence that protein markers may be a very useful tool for more general questions of feed and food authentication issues, especially in cases of highly processed products.

CRedit authorship contribution statement

Marie-Caroline Lecrenier: Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Resources. **Aline Marien:** Conceptualization, Investigation, Writing - review & editing, Resources. **Pascal Veys:** Conceptualization, Investigation, Writing - review & editing, Resources. **Ikram Belghit:** Investigation, Writing - review & editing. **Marc Dieu:** Investigation, Writing - review & editing. **Nathalie Gillard:** Investigation, Writing - review & editing. **Jean Henrottin:** Investigation, Writing - review & editing. **Uta M. Herfurth:** Investigation, Writing - review & editing. **Daniela Marchis:** Investigation, Writing - review & editing. **Sara Morello:** Investigation, Writing - review & editing. **Eystein Oveland:** Investigation, Writing - review & editing. **Oliver Poetz:** Investigation, Writing - review & editing. **Josef Daniel Rasinger:** Investigation, Writing - review & editing. **Andreas Steinhilber:** Investigation, Writing - review & editing. **Vincent Baeten:** Conceptualization, Writing - review & editing, Resources. **Gilbert Berben:** Conceptualization, Writing - review & editing, Resources. **Olivier Fumière:** Conceptualization, Writing - review & editing, Resources.

Declaration of competing interest

The authors declare the following competing financial interest(s): O.P. is shareholder of SIGNATOPE GmbH. SIGNATOPE offers assay development and service using MS-based immunoassay technology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2021.107944>.

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