Feasibility study of the transfer of the MS method to external lab

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M.-C. LECRENIER, S. PEDERIVA, A. CORDONNIER, L. PLASMAN, A. ANSELMO, V. BAETEN, P. VEYS, D. MARCHIS & O. FUMIÈRE

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Editor :
Centre wallon de Recherches agronomiques
Service Communication
Rue de Liroux, 9
5030 Gembloux (Belgium)

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Introduction:

In 2019, a mass spectrometry (MS) interlaboratory study was conducted by the EURL-AP (Lecrenier et al., 2021). The aim of the study was to evaluate the MS methods already developed in the network of National Reference Laboratories (NRLs) on common set of samples adulterated with various animal by-products at a level of 1% (w/w). Results obtained by the laboratories have confirmed the potential of MS-based proteomics to resolve current analytical gaps in the detection and discrimination of processed animal proteins (PAPs).

The objective of this feasibility study was to complete the previous study by the evaluation of the transferability of a MS method from a laboratory that had developed the method (Lab A) to another laboratory that had never used this method (Lab B). The selected method was the one developed by the EURL-AP and the collaborating laboratory was the Experimental Zooprophylactic Institute of Piedmont Liguria and Valle d'Aosta (IZSTO). Even if the sample preparation methods to use was strictly the same, different MS instruments were used requiring some optimizations. This study presents and discusses these results.

Material:

The commercial feed matrix used was a **pig feed (PigF)** intended for sow feeding. It was the same that the one used for a previous study (Lecrenier et al., 2021). Its declaration 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. Polymerase Chain Reaction (PCR) and light microscopy analyses proved that it was free of ruminant, porcine and poultry DNA and free of terrestrial animal particles, respectively. The protein content was estimated at 14.1 %. Nitrogen content was determined in duplicate according to the Kjeldhal method with an applied conversion factor (kp) of 6.25. PigF was ground with an Ultra Centrifugal rotor Mill ZM 200 (Retsch) in combination with a sieve of 2 mm mesh size, to ensure the homogeneity.

As bovine adulterant feed materials, two different PAPs, two blood products (haemoglobin powder and plasma powder), one gelatine powder and one milk powder were used:

- **Bovine Paps01 (BvPaps01)** was a commercial feed material. PCR and light microscopy analyses showed that it contained ruminant DNA exclusively (no pig and poultry DNA detected) and terrestrial particles (bones and muscles), respectively. Its sediment was of 62 % and the protein content was estimated at 49.5 %.
- **Bovine Paps02 (BvPaps02)** was produced in a pilot plant. Its bone content was about 50 %, meat and fat content was approximatively 20 % and blood content was about 10 %. PCR and light microscopy analyses showed that it contained ruminant DNA exclusively and terrestrial animal

particles (bones, muscles and blood), respectively. Its sediment rate was 53 % and the protein content was estimated at 35.4 %.

- Bovine Haemoglobin powder (BvHb) was a commercial feed material. PCR analyses confirmed that it contained ruminant DNA exclusively.
- Bovine plasma powder (BvPlm) was also a commercial feed material. PCR analyses showed that it contained ruminant DNA exclusively.
- Bovine gelatine (BvGel) was obtained by a manufacturers association. According to the labelling, it was produced from bovine hides by lime (alkali) processing. PCR analyses showed that it contained ruminant DNA and was free of porcine DNA.
- Milk product was a calf milk replacer. It was predominantly composed of skimmed milk powder and whey powder. PCR analyses showed that it contained only ruminant DNA. The protein content was estimated at 21.8 %.

BvPaps01, BvPaps02 and Milk product were also used in the 2019 interlaboratory study (Lecrenier et al., 2021).

Fifteen different test materials were prepared for the study (Table 1). Details of the sample set are indicated in Table 1. The composition of the sample set was established taking into account the following considerations:

- Six reference samples (sample Ref-01 to Ref-06) were included in the set. Participants were free to use them to optimise or develop their methods. Results obtained on these samples are not discussed in this report.
- Seven samples (sample TS-01 to TS-07) were prepared with PigF without or with adulteration at levels of 0.5 % w/w. Adulterations were proceeded by direct spiking with the adulterant.
- Two more challenging samples were finally included as sample TS-08 and TS-09. TS-08 was prepared by the adulteration of PigF with a protein extract in order to obtain a final concentration of 2.5 ppm BvHb, 100 ppm Milk powder and 200 ppm BvGel. TS-09 was prepared in the same way in order to obtain a final concentration of 500 ppm BvPlm.

Table 1: Composition of the sample set

Sample #	Composition
Ref-01	Bovine haemoglobin powder (BvHb)
Ref-02	Bovine plasma powder (BvPlm)
Ref-03	Bovine gelatine (BvGel)
Ref-04	Bovine Paps01 (BvPaps01)
Ref-05	Bovine Paps02 (BvPaps02)
Ref-06	Milk product
TS-01	Pig feed (PigF)

TS-02	PigF + 0.5 % w/w BvHb
TS-03	PigF + 0.5 % w/w BvPlm
TS-04	PigF + 0.5 % w/w BvGel
TS-05	PigF + 0.5 % w/w BvPaps01
TS-06	PigF + 0.5 % w/w BvPaps02
TS-07	PigF + 0.5 % w/w Milk powder
TS-08	PigF + 2.5ppm BvHb + 100 ppm Milk powder + 500 ppm BvGel
TS-09	PigF + 500 ppm BvPlm

Methods

1. Sample preparation (pre-treatment, extraction, digestion, purification) :

Sample preparations were performed in the two labs following the same protocol. Extraction A was performed by Lab A. Lab B received also PigF and feed materials to be able to perform the sample preparation in their own laboratory (Extractions B & C). Sample preparation was based on previously published protocols (Lecrenier et al., 2018) with minor changes. Extraction was performed in 15 ml tubes containing 100 mg of Ref-samples or 1 g for TS-samples. Heavy-labelled standards were spiked in each tube prior to the addition of 10 ml of extraction buffer (200 mM TRIS-HCl, pH 9.2, 2 M urea). Tubes were shaken at room temperature (RT) for 30 min followed by sonication for 15 min. Tubes were then centrifuged at 4660 g for 10 min and 5 ml of supernatant was transferred into new tubes. The protein extracts were diluted with 5 ml of 200 mM ammonium bicarbonate and reduced with 500 µl of 200 mM dithiothreitol (DTT) at RT for 45 min prior to alkylation with 500 µl of 400 mM iodoacetamide (IAA) for 45 min in the dark at RT. Digestion was then performed by adding 500 µl of trypsin (1 mg/ml in 50 mM acetic acid) for 1 h at 37 °C and trypsin action was stopped by the addition of 150 µl of 20 % (v/v) formic acid in water. Peptides were purified by reverse-phase extraction using Sep-Pak tC18 cartridges (Waters – Milford, Massachusetts, USA). Cartridge pre-conditioning was performed with acetonitrile followed by equilibration with 0.1 % (v/v) formic acid in water. Digested supernatant was loaded on the column. Next, 0.1 % (v/v) formic acid in water was used to flush out impurities. Elution was then performed with acetonitrile/0.1 % (v/v) formic acid in water 80/20 (v/v). Before evaporation at 60 °C using Centrivap, 15 µl of DMSO was added to each tube to prevent dryness. Finally, pellets were resuspended in 375 µl of 0.1 % (v/v) formic acid in water/acetonitrile 95/5 (v/v) and supernatants were stored at – 20 °C before injection.

2. Liquid chromatography and mass spectrometer system (LC-MS)

Information about operational conditions for liquid chromatography (LC) and mass spectrometry (MS) analyses was also shared between the two laboratories. Lab B evaluated different LC and MS conditions following several studies (Studies 1 to 6) in order to optimise the method on its instrument. Table 2 and 3 compare the major parameters.

Table 2. Liquid chromatography (LC) operating conditions used by the different labs

		Lab A		Lab B – study 1		Lab B – study 2 & 5		Lab B – study 3 & 6		Lab B – study 4	
LC system		UHPLC Acquity system (Waters)		Exion LC system (SCIEX)							
Column	**Brand**	Waters		Phenomenex							
	Type	ACQUITY UPLC BEH C18		Kinetex® C18 with a security guard				Aeris® Peptide XB-C18			
	Length (mm)	100		50				150			
	Diameter (mm)	2.1									
	Particle size (µm)	1.7		1.7				2.6			
Column temperature (°C)		50		40							
Flow rate (µl/min)		200		250		200					
Injection volume (µl)		2									
Eluent A composition		Water + 0.1 % formic acid									
Eluent B composition		Acetonitrile + 0.1 % formic acid									
Gradient time (min)		16		15		16				17	
Gradient elution program		**Time (min)**	**Eluent A (%)**	**Time (min)**	**Eluent A (%)**	**Time (min)**	**Eluent A (%)**			**Time (min)**	**Eluent A (%)**
		0	92	0	97	0	92			0	92
		2	92	0.5	97	2	92			2	92
				2.5	75					4	85
				4.5	65						
		10	58	5.5	55	11.9	58			11.5	58
				7.5	45						
		10.1	15	8.0	5	12	15			12.5	15
		12.5	15	12	5	14	15			14.2	15
		12.6	92	13	97	14.2	92			16	92
		16	92	15	97	16	92			17	92

Table 3. Mass spectrometry (MS) operating conditions used by the different labs

	Lab A	Lab B
MS system	Xevo TQ-XS micro (Waters)	QTRAP 5500 System (SCIEX)
Acquisition mode	MRM	MRM
Ionisation mode	ESI positive	ESI positive

MRM = Multiple Reaction Monitoring; ESI = Electrospray ionisation

3. Biomarkers and targets

The UHPLC-MS/MS approach was used for the simultaneous detection of four targeted ruminant proteins or protein clusters: haemoglobin, plasma proteins, milk proteins and collagen. The peptides used as markers were selected according to previous studies (Lecrenier et al., 2018; Lecrenier et al., 2021; Fumière et al., 2022). Due to instrumental limitation in mass range (max 1000 Da), Lab B has used in some cases other product ions. Details about monitored ions are summarised in Annex 1 and differences are highlighted.

4. Criteria for interpretation of the results

In order to avoid false positive identifications, strict acceptance criteria had to be applied to consider a signal as positive. As no legal evaluation criteria already existed for this type of application, criteria employed in a previous study were used (Lecrenier et al., 2018) with minor changes. Permitted tolerances were applied according to the Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed (European Commission, 2017).

In order to report the detection and the identification of one peptide marker, the observation of at least two product ions was required at the estimated retention time (RT) with a tolerance of +/- 0.1 min. Peaks from both product ions had to fully overlap. When the corresponding internal standard was available, the RT of the internal standard was used. When no standard was available, the RT observed in matrix-matched standards was applied.

The second acceptance criterion used was the peak area ratio. This also varied depending on the internal standard availability. The peak area ratio between the most intense and the second most intense product ion shall correspond to that from the standard or that from the matrix-matched standards if no standard is available and should not deviate more than 30 % (relative) from the reference value.

The last acceptance criterion was the signal-to-noise ratio (S/N), calculated peak-to-peak in a range equal to six times the peak width at half height. The threshold was fixed at a S/N of 10 for the most intense product ion.

Finally, in order to conclude on the detection of the related ruminant protein or protein cluster, a minimum threshold of two peptides identified per protein/protein cluster was fixed. Peptides used for each targeted ruminant protein (haemoglobin and collagen) and protein cluster (milk proteins and plasma proteins) are identified in Annex 1.

Study timetable:

The 22nd October 2021, Lab A performed the peptide extraction A (samples Ref-01 to Ref-06 and TS-01 to TS-09). The 9th November 2021, the extracted peptides were sent to Lab B and were received on the 10th November 2021. Extracts were stored at -20°C until analyses and meal samples were stored at 4°C until extraction by Lab B. In order to test other LC and MS conditions on fresh extracts, new sample preparations (extraction B and C) were realised for samples TS-01 to TS-07 by Lab B on 10th January 2022 and 13rd June 2022. Samples TS-08 and TS-09 (extraction A) were also reanalysed during these different studies. Timetable of peptide extraction and MS analyses are summarized in Table 4.

Table 4. Study timetable in Lab A and Lab B for samples TS-01 to TS-07

Laboratory and study		Extraction	Extraction date	Analyses date	Extract storing duration (days)
Lab A		A	22/10/2021	27/10/2021	5
Lab B	1	B	10/01/2022	13/01/2022	83
	2			16/05/2022	45
	3			19/05/2022	48
	4				3
	5	C	13/06/2022	16/06/2022	1
	6				

Results:

Table 5 summarizes the overall results for the detection of targeted ruminant proteins in each sample type (TS-01 to TS-07). The two more challenging samples (TS-08 and TS-09) were not taken into account in this table as analyses were only performed on extraction A during all the studies. Due to the differences in term of extract storing duration, data were not comparable to the other ones. These samples are discussed in the detailed results. Major ruminant proteins or protein clusters, expected to be detected according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

The overall results, expressed in terms of global accuracy (AC) confirm the suitability of the MS approaches for the detection of ruminant proteins. The percentage of total error accounted for 17.8 % (10/56) of the total responses, regardless the studied conditions. Among the 10 deviations, only one was due to a false positive finding of collagen in sample TS-07 containing 0.5 % of milk powder (study 1 using extraction B). No other specificity issue was recorded. Some sensitivity issues were noticed link

to the absence of detection of collagen (1/8) and haemoglobin (8/8) in sample TS-05 (PigF + 0.5 % BvPaps01).

Table 5: Global results expressed as accuracy for the detection of ruminant by-product

		AC			
	n	Haemoglobin	Milk proteins	Plasma proteins	Collagen
TS-01: PigF	8	1.000	1.000	1.000	1.000
TS-02: PigF + 0.5 % BvHb	8	1.000	1.000	1.000	1.000
TS-03: PigF + 0.5 % BvPlm	8	1.000	1.000	1.000	1.000
TS-04: PigF + 0.5 % BvGel	8	1.000	1.000	1.000	1.000
TS-05: PigF + 0.5 % BvPaps01	8	0 (8)	1.000	1.000	0.875 (1)
TS-06: PigF + 0.5 % BvPaps02	8	1.000	1.000	1.000	1.000
TS-07: PigF + 0.5 % Milk powder	8	1.000	1.000	1.000	0.875 (1)

Accuracy (AC) is the fraction of the correct positive and negative results. It is calculated by dividing the number of correct results by the total number of results. The number of FN or FP is given in brackets.
n = number of results. Cells corresponding to major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

Detailed results obtained by each studied condition are summarized in Annex 2. Results obtained on each sample are discussed hereunder:

Sample TS-01 (PigF): Blank pig feed gave negative results for all peptides.

Sample TS-02 (PigF + 0.5 % BvHb): 100 % (4/4) of the peptide markers for haemoglobin detection were identified regardless of the studied conditions. Plasma peptides have been classified as minor peptides as they can also be present in haemoglobin powder but at a low level. All plasma peptides were detected in all studies except in study 1 in which the serotransferrin peptide (ELPDPQESIQR) was not detected. No false positive detection was observed.

Sample TS-03 (PigF + 0.5 % BvPlm): 100 % (3/3) of the peptide markers for plasma proteins detection were identified regardless of the studied conditions. Haemoglobin peptides have been classified as minor peptides, just as it was in the previous sample, since low levels of haemoglobin are expected in plasma powder. Here again, 100 % of the haemoglobin peptides were detected in all studies except in study 1 in which two out of the set of four peptides (VGGHAAEYGAEALER and AAVTAFWGK) were not detected. No false positive detection was observed.

Sample TS-04 (PigF + 0.5 % BvGel): 100 % (3/3) of the peptide markers for collagen detection were identified regardless of the studied conditions. Two false positive detections were observed in study 1 using extraction B: one haemoglobin peptide (EFTPVLQAFQK) and one plasma peptide derived from alpha-2-macroglobulin (SNSFVYLEPLPR). However, as only one peptide was detected per targeted ruminant protein/protein cluster, the sample was declared as negative for haemoglobin and plasma proteins. No other false positive detection was observed in the other conditions studied.

Sample TS-05 (PigF + 0.5 % BvPaps01): Unlike adulterants used in previous samples, PAPs is composed of several tissues (e.g. bone, blood, muscles) and the proportion of these tissues varies from one PAPs to another. This is why it is important to use different targeted proteins to be able to detect PAPs whatever their composition. Haemoglobin and collagen were selected as marker for PAPs detection. All collagen peptides (3/3) were identified by Lab A and Lab B under study 1, study 2, study 5 and study 6 conditions. One peptide was not identified in study 4 (GPpGESGAAGPTGPIGSR) and two (GSTGEIGPAGPpGPpGLR & GPpGESGAAGPTGPIGSR) in study 3. No haemoglobin peptide was detected in any study. Paps01 was already used in previous study (Lecrenier et al., 2021) and was known to contain a high bone content and, at the opposite, a low blood concentration. The haemoglobin concentration seems to be too low to be detected at 0.5 % of Paps01 adulteration. No false positive detection was observed.

Sample TS-06 (PigF + 0.5 % BvPaps02): All collagen peptides (3/3) were identified in all studies except in study 4 in which one collagen peptide (GPpGESGAAGPTGPIGSR) was not detected. All haemoglobin peptides (4/4) were detected in study 5 and study 6. 75 % (3/4) of the haemoglobin peptides were identified by Lab A and Lab B using study 1 (extraction B), study 2 and study 3 conditions. Under study 1 (extraction A) and study 4 conditions, only two peptides (AAVTAFWGK and EFTPVLQAFQK) were identified. No false positive detection was observed.

Sample TS-07 (PigF + 0.5 % Milk powder): All milk peptides (4/4) were identified in all studies. One false positive detection of collagen peptide (GEPGPAGAVGPAGAVGPR) was observed in study 1 (extraction A) given a negative result for the detection of collagen following the threshold of two identified peptides. Two false positive detections of collagen peptides (GEPGPAGAVGPAGAVGPR & GPpGESGAAGPTGPIGSR) were observed in study 1 using extraction B given a positive result for the detection of collagen. No other false positive detection was observed in the other conditions studied.

For sample TS-08 and TS-09, only extraction A performed by Lab A was analysed. Study 5 and study 6 were not performed as the analytical conditions were the same as study 2 and study 3 respectively.

Sample TS-08 (PigF + 2.5ppm BvHb + 100 ppm Milk powder + 500 ppm BvGel): Lab A and Lab B using study 1 and study 2 conditions have detected 100 % of the targeted peptides. Study 3 has identified 100 % of the haemoglobin (4/4) and collagen (3/3) peptides and 75 % of the milk peptides (3/4). Under study 4 conditions, 100 % of the haemoglobin (4/4) and milk (4/4) peptides and 66.6 % of the collagen peptides (2/3) were identified. However, despite these misdetections, sample TS-08 can still be declared as positive for haemoglobin, milk proteins and collagen regardless the studied conditions. No false positive detection was observed.

Sample TS-09 (PigF + 500 ppm BvPlm): Lab A and Lab B using study 1, study 2 and study 4 conditions have detected 100 % of the targeted peptides. Study 3 has identified 66.6 % of the plasma peptides (2/3). However, despite these misdetections, sample TS-09 can be declared as positive for plasma proteins regardless the studied conditions. One false positive detection of collagen peptide (GEPGPAGAVGPAGAVGPR) was observed in study 1. However, as only one peptide was detected per targeted ruminant protein, the sample had declared as negative for collagen with regard to the chosen criteria. No other false positive detection was observed in the other conditions studied.

Discussion and conclusion

This study allowed evaluating the transfer of the sample preparation and MS analyses.

Based on all collected data, it can be concluded that sample preparation does not cause no major problem. Some conservation issues were hypothetically underlined and need further investigation. This is probably the reason for the misdetection of some peptides in more challenging samples (TS-08 and TS-09). The extract aging process combined with the low protein concentration within these samples are probably the cause of these false negative detections (Kraut et al., 2009). The maximum delay between the sample preparation as well as the conservation condition (in solution versus lyophilised, -80 ° C versus of -20 °C) have to be studied and noted in the protocol.

Lab B had the opportunity to evaluate several conditions as regards to column type, gradient, …. Studies 1, 3 and 4 gave the poorest results.

For study 1, regardless of the extract used (A or B), one plasma peptide (ELPDPQESIQR) and two haemoglobin peptides (VGGHAAEYGAEALER and AAVTAFWGK) were not detected in TS-02 and TS-03 respectively. This misdetection was not observed in study 2 and study 5, even if the same column was used. One hypothesis is that it could be linked to the gradient used. Indeed, an efficient peptide separation is critical for maximizing the number of peptide identifications and minimizing ion suppression (Hsieh et al., 2013). Using study 1 parameters, all peptides elute in a short period (between 3 min and 4.4 min); it has probably impacted the detection of co-eluent peptides which can cause a masking effect of peptides having a low concentration in the sample such as plasma peptides in sample adulterated with haemoglobin powder or haemoglobin peptides in sample containing plasma powder. A few false positive detections were also observed, usually in both extracts. Even if it is difficult to explain these false positive detections linked to haemoglobin, plasma, milk or collagen peptides, the results rather suggest carry-over between sample injections due to lack of experience with the method used. As it was also noticed in the inter-laboratory study of 2021 (Lecrenier et al., 2021), some peptides can be really sticky leading to increase carry-over effects.

Errors in study 3 and study 4 are more linked to an absence of detection of collagen peptides in the samples containing PAPs. The same observation was not made in study 6 suggesting that it could be related to the aging of the sample.

In view of the results, the conditions used in study 5 and 6 seem to be the best combination. The results are mostly the same as the results obtained in Lab A with even better results on sample TS-06 containing Paps02 since 100 % of the haemoglobin peptides were detected whereas only 75 % (3/4) of them were detected by Lab A. In conclusion, the transfer of the analytical method was successful. However, TS-05 sample, containing 0.5 % of BvPaps01, showed the current limits of the method, regardless of the conditions tested. None of the analyses revealed the presence of haemoglobin due to its very low concentration. One challenging sample (TS-08) has proven that the method is able to detect 2.5 ppm of haemoglobin, which may suggest that the haemoglobin concentration in TS-05 sample had to be lower than this level. Although collagen was detected, it was not possible, given the current legislation reauthorizing ruminant gelatine in non-ruminant feed, to establish whether the sample contained an unauthorised ingredient. Indeed, the detection of collagen alone is not sufficient since the present study has shown that it was detected in both PAPs and gelatine products. Efforts are now being focused on the improvement of haemoglobin detection.

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Annex 1.

Multiple Reaction Monitoring (MRM) ions (m/z=mass/charge) and optimized MS parameters: Retention time (RT, min), cone voltage (CV, volts) and collision Energy (CE, volts). When different product ions have been monitored ions, these were highlighted in grey. **p** indicates the presence of hydroxyproline.

Targeted Protein/cluster				Lab A				Lab B																							
								Study 1				Study 2				Study 3				Study 4				Study 5				Study 6			
	Protein	Peptide	Parent Ion (m/z)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)	RT (min)	Product Ion (m/z)	CV (V)	CE (V)
Haemoglobin	Haemoglobin alpha-chain	VGGHAAEYGAEALE**p**	510.6	4.8	617.3	35	14	3.0	745.4	90	20	5.3	745.4	90	20	6.9	745.4	90	20	8.2	745.4	90	20	5.3	745.4	90	20	6.9	745.4	90	20
					745.4	35	16		617.3	90	20		617.3	90	20		617.3	90	20		617.3	90	20		617.3	90	20		617.3	90	20
					622.3	35	16		622.3	90	20		622.3	90	20		622.3	90	20		622.3	90	20		622.3	90	20		622.3	90	20
	Haemoglobin beta-chain	AAVTAFWGK	475.8	6.6	709.4	35	14	4.0	709.4	80	22	7.2	709.4	80	22	9.0	709.4	80	22	6.9	709.4	80	22	7.2	709.4	80	22	9.0	709.4	80	22
					608.3	35	15		608.3	80	22		608.3	80	22		608.3	80	22		608.3	80	22		608.3	80	22		608.3	80	22
					537.3	35	11		537.3	80	22		537.3	80	22		537.3	80	22		537.3	80	22		537.3	80	22		537.3	80	22
		EFTPVLQADFQK	711.9	7.0	523.3	35	21	4.1	849.4	80	34	7.7	849.4	80	34	9.4	523.3	80	34	7.5	523.3	80	34	7.7	523.3	80	34	9.4	523.3	80	34
					1045.6	35	21		523.3	80	34		523.3	80	34		736.4	80	31		736.4	80	31		736.4	80	31		736.4	80	31
					849.4	35	24		/	/	/		736.4	80	31		378.2	80	31		378.2	80	31		378.2	80	31		378.2	80	31
		VVAGVANALAHR	393.2	5.2	490.3	35	9	3.5	681.4	80	17	5.5	681.4	80	17	7.3	681.4	80	17	7.2	681.4	80	17	5.5	681.4	80	17	7.3	681.4	80	17
					454.8	35	9		490.3	80	17		490.3	80	17		490.3	80	17		490.3	80	17		490.3	80	17		490.3	80	17
					681.4	35	13		454.8	80	17		454.8	80	17		454.8	80	17		454.8	80	17		454.8	80	17		454.8	80	17

Milk proteins	Casein alpha-S2	HQGLPQEVLNENLLR	587.32	6.9	436.2	35	15	4.1	758.4	90	20	7.6	758.4	90	20	9.3	758.4	90	20	8.5	758.4	90	20	7.6	758.4	90	20	9.3	758.4	90	20
					758.4	35	14		436.2	90	20		436.2	90	20		436.2	90	20		436.2	90	20		436.2	90	20		436.2	90	20
					790.4	35	15		790.4	90	20		790.4	90	20		790.4	90	20		790.4	90	20		790.4	90	20		790.4	90	20
		NAVPITPTLNR	598.34	5.9	285.2	35	14	3.7	911.5	90	25	6.4	911.5	90	25	8.0	911.5	90	25	8.0	911.5	90	25	6.4	911.5	90	25	8.0	911.5	90	25
					911.5	35	15		456.3	90	25		456.3	90	25		456.3	90	25		456.3	90	25		456.3	90	25		456.3	90	25
					456.3	35	16		285.2	90	25		285.2	90	25		285.2	90	25		285.2	90	25		285.2	90	25		285.2	90	25
	Beta-lactoglobulin	LSFNPTQLEEQCHI	858.4	7.2	1254.6	35	28	4.3	928.4	80	41	8.2	928.4	80	41	9.8	928.4	80	41	7.4	928.4	80	41	8.2	928.4	80	41	9.8	928.4	80	41
					928.4	35	29		627.8	80	41		627.8	80	41		627.8	80	41		627.8	80	41		627.8	80	41		627.8	80	41
					627.8	45	29		/	/	/		462.2	80	35		462.2	80	35		462.2	80	35		462.2	80	35		462.2	80	35
		VLVLDTDYK	533.30	6.3	853.4	35	15	3.9	853.4	90	20	6.9	853.4	90	20	8.6	853.4	90	20	8.7	853.4	90	20	6.9	853.4	90	20	8.6	853.4	90	20
					754.4	35	16		754.4	90	20		754.4	90	20		754.4	90	20		754.4	90	20		754.4	90	20		754.4	90	20
					641.3	35	18		641.3	90	20		641.3	90	20		641.3	90	20		641.3	90	20		641.3	90	20		641.3	90	20
Plasma proteins	Serotransferrin	ELPDPQESIQR	656.33	5.4	429.2	35	24	3.5	857.4	80	31	5.8	857.4	80	31	7.5	857.4	80	31	7.9	857.4	80	31	5.8	857.4	80	31	7.5	857.4	80	31
					535.3	35	18		535.3	80	31		535.3	80	31		535.3	80	31		535.3	80	31		535.3	80	31		535.3	80	31
					857.4	35	26		429.2	80	31		429.2	80	31		429.2	80	31		429.2	80	31		429.2	80	31		429.2	80	31

	Alpha-2-macroglobulin	SNSFVYLEPLPR	711.38	7.4	482.3	35	20	4.4	887.5	80	34	8.2	887.5	80	34	9.9	887.5	80	34	7.4	887.5	80	34	8.2	887.5	80	34	9.9	887.5	80	34
					887.5	35	21		724.4	80	34		724.4	80	34		724.4	80	34		724.4	80	34		724.4	80	34		724.4	80	34
					724.4	35	22		482.3	80	34		482.3	80	34		482.3	80	34		482.3	80	34		482.3	80	34		482.3	80	34
	Apolipoprotein A1	VAPLGEEFR	509.27	6	424.2	35	14	3.7	750.4	90	20	6.5	750.4	90	20	8.3	750.4	90	20	9.0	750.4	90	20	6.5	750.4	90	20	8.3	750.4	90	20
					637.3	35	20		637.3	90	20		637.3	90	20		637.3	90	20		637.3	90	20		637.3	90	20		637.3	90	20
					750.4	35	21		424.2	90	20		424.2	90	20		424.2	90	20		424.2	90	20		424.2	90	20		424.2	90	20
Collagen	Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	758.90	5.3	665.9	35	18	4.2	880.5	80	36	8.2	880.5	80	36	9.5	880.5	80	36	9.5	880.5	80	36	8.2	880.5	80	36	9.5	880.5	80	36
					781.4	35	26		781.4	80	36		781.4	80	36		781.4	80	36		781.4	80	36		781.4	80	36		781.4	80	36
					880.5	35	25		665.9	80	36		665.9	80	36		665.9	80	36		665.9	80	36		665.9	80	36		665.9	80	36
		GSTGEIGPAGP**p**GP**p**GLR	824.9	5.4	1047.6	35	24	3.5	879.5	80	39	6.0	879.5	80	39	7.5	879.5	80	39	7.2	879.5	80	39	6.0	879.5	80	39	7.5	879.5	80	39
					822.4	35	23		822.4	80	39		822.4	80	39		822.4	80	39		822.4	80	39		822.4	80	39		822.4	80	39
					879.5	35	25		/	/	/		/	/	/		950.5	80	39		950.5	80	39		950.5	80	39		950.5	80	39
		GP**p**GESGAAGPTGPIGSR	790.9	4.3	841.5	35	23	3.2	912.5	80	38	4.3	912.5	80	38	6.4	912.5	80	38	6.5	912.5	80	38	4.3	912.5	80	38	6.4	912.5	80	38
					1426.7	35	25		841.5	80	38		841.5	80	38		841.5	80	38		841.5	80	38		841.5	80	38		841.5	80	38
					912.5	35	23		/	/	/		/	/	/		983.5	80	38		983.5	80	38		983.5	80	38		983.5	80	38

Annex 2.

TS-01: PigF		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-	-	-	-	-	-	-	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	-	-	-
	EFTPVLQADFQK	-	-	-	-	-	-	-	-
	VVAGVANALAHR	-	-	-	-	-	-	-	-
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	-	-	-
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	-	-	-	-	-	-
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	-	-	-
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	-	-	-	-	-	-	-	-
	GSTGEIGPAGPpGPpGLR	-	-	-	-	-	-	-	-
	GPpGESGAAGPTGPIGSR	-	-	-	-	-	-	-	-

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-02: PigF + 0.5 % w/w BvHb		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	+	+	+	+	+	+	+
Haemoglobin beta-chain	AAVTAFWGK	+	+	+	+	+	+	+	+
	EFTPVLQADFQK	+	+	+	+	+	+	+	+
	VVAGVANALAHR	+	+	+	+	+	+	+	+
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	+	- *	- *	+	+	+	+	+
Alpha-2-macroglobulin	SNSFVYLEPLPR	+	+	+	+	+	+	+	+
Apolipoprotein A1	VAPLGEEFR	+	+	+	+	+	+	+	+
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	-	-	-	-	-	-	-	-
	GSTGEIGPAGPpGPpGLR	-	-	-	-	-	-	-	-
	GPpGESGAAGPTGPIGSR	-	-	-	-	-	-	-	-

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-03: PigF + 0.5 % w/w BvPlm		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	- *	- *	+	+	+	+	+
Haemoglobin beta-chain	AAVTAFWGK	+	- *	- *	+	+	+	+	+
	EFTPVLQADFQK	+	+	+	+	+	+	+	+
	VVAGVANALAHR	+	+	+	+	+	+	+	+
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	+	+	+	+	+	+	+	+
Alpha-2-macroglobulin	SNSFVYLEPLPR	+	+	+	+	+	+	+	+
Apolipoprotein A1	VAPLGEEFR	+	+	+	+	+	+	+	+
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	-	-	-	-	-	-	-	-
	GSTGEIGPAGPpGPpGLR	-	-	-	-	-	-	-	-
	GPpGESGAAGPTGPIGSR	-	-	-	-	-	-	-	-

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-04: PigF + 0.5 % w/w BvGel		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-	-	-	-	-	-	-	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	-	-	-
	EFTPVLQADFQK	-	-	+*	-	-	-	-	-
	VVAGVANALAHR	-	-	-	-	-	-	-	-
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	-	-	-
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	+*	-	-	-	-	-
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	-	-	-
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	+	+	+	+	+	+	+	+
	GSTGEIGPAGPpGPpGLR	+	+	+	+	+	+	+	+
	GPpGESGAAGPTGPIGSR	+	+	+	+	+	+	+	+

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-05: PigF + 0.5 % w/w BvPaps01		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-*	-*	-*	-*	-*	-*	-*	-*
Haemoglobin beta-chain	AAVTAFWGK	-*	-*	-*	-*	-*	-*	-*	-*
	EFTPVLQADFQK	-*	-*	-*	-*	-*	-*	-*	-*
	VVAGVANALAHR	-*	-*	-*	-*	-*	-*	-*	-*
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	-	-	-
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	-	-	-	-	-	-
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	-	-	-
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	+	+	+	+	+	+	+	+
	GSTGEIGPAGPpGPpGLR	+	+	+	+	-*	+	+	+
	GPpGESGAAGPTGPIGSR	+	+	+	+	-*	-*	+ (low)	+

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-06: PigF + 0.5 % w/w BvPaps02		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	-*	+	+	+	-*	+	+
Haemoglobin beta-chain	AAVTAFWGK	+	+	+	+	+	+	+	+
	EFTPVLQADFQK	+	+	+	+	+	+	+	+
	VVAGVANALAHR	-*	-*	-*	-*	-*	-*	+	+
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	-	-	-
	NAVPITPTLNR	-	-	-	-	-	-	-	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	-	-	-
	VLVLDTDYK	-	-	-	-	-	-	-	-
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	-	-	-
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	-	-	-	-	-	-
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	-	-	-
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	+	+	+	+	+	+	+	+
	GSTGEIGPAGPpGPpGLR	+	+	+	+	+	+	+	+
	GPpGESGAAGPTGPIGSR	+	+	+	+	+	-*	+	+

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-07: PigF + 0.5 % w/w Milk powder		Lab A	Lab B						
			Study 1		Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A	Extraction A	Extraction B	Extraction B	Extraction B	Extraction B	Extraction C	Extraction C
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-	-	-	-	-	-	-	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	-	-	-
	EFTPVLQADFQK	-	-	-	-	-	-	-	-
	VVAGVANALAHR	-	-	-	-	-	-	-	-
Casein alpha-S2	HQGLPQEVLNENLLR	+	+	+	+	+	+	+	+
	NAVPITPTLNR	+	+	+	+	+	+	+	+
Beta-lactoglobulin	LSFNPTQLEEQCHI	+	+	+	+	+	+	+	+
	VLVLDTDYK	+	+	+	+	+	+	+	+
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	-	-	-
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	-	-	-	-	-	-
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	-	-	-
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	-	+*	+*	-	-	-	-	-
	GSTGEIGPAGPpGPpGLR	-	-	-	-	-	-	-	-
	GPpGESGAAGPTGPIGSR	-	-	+*	-	-	-	-	-

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-08: PigF + 2.5ppm BvHb + 100 ppm Milk powder + 500 ppm BvGel		Lab A	Lab B					
			Study 1	Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A						
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	+	+	+	+ (low)	/	/
Haemoglobin beta-chain	AAVTAFWGK	+	+	+	+	+	/	/
	EFTPVLQADFQK	+	+	+	+	+	/	/
	VVAGVANALAHR	+	+	+	+	+	/	/
Casein alpha-S2	HQGLPQEVLNENLLR	+	+	+	+	+	/	/
	NAVPITPTLNR	+	+	+	+	+	/	/
Beta-lactoglobulin	LSFNPTQLEEQCHI	+	+	+	+	+	/	/
	VLVLDTDYK	+	+	+	-*	+(low)	/	/
Serotransferrin	ELPDPQESIQR	-	-	-	-	-	/	/
Alpha-2-macroglobulin	SNSFVYLEPLPR	-	-	-	-	-	/	/
Apolipoprotein A1	VAPLGEEFR	-	-	-	-	-	/	/
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	+	+	+	+	+	/	/
	GSTGEIGPAGPpGPpGLR	+	+	+	+	+	/	/
	GPpGESGAAGPTGPIGSR	+	+	+	+	-*	/	/

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.

TS-09: PigF + 500 ppm BvPlm		Lab A	Lab B					
			Study 1	Study 2	Study 3	Study 4	Study 5	Study 6
Protein	Peptide	Extraction A						
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-	-	-	-	-	/	/
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	/	/
	EFTPVLQADFQK	-	-	-	-	-	/	/
	VVAGVANALAHR	-	-	-	-	-	/	/
Casein alpha-S2	HQGLPQEVLNENLLR	-	-	-	-	-	/	/
	NAVPITPTLNR	-	-	-	-	-	/	/
Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-	-	/	/
	VLVLDTDYK	-	-	-	-	-	/	/
Serotransferrin	ELPDPQESIQR	+	+	+	+	+	/	/
Alpha-2-macroglobulin	SNSFVYLEPLPR	+	+	+ (low)	+	+	/	/
Apolipoprotein A1	VAPLGEEFR	+	+	+	-*	+	/	/
Collagen alpha-2(I) chain	GEPGPAGAVGPAGAVGPR	-	+*	-	-	-	/	/
	GSTGEIGPAGPpGPpGLR	-	-	-	-	-	/	/
	GPpGESGAAGPTGPIGSR	-	-	-	-	-	/	/

p indicates the presence of hydroxyproline. + means that the peptide was identified; - means that the peptide was not identified. Erroneous results are marked with an asterisk (*). Major ruminant proteins or protein clusters, expected to be detect according to the sample composition, are highlighted in orange. Minor proteins or protein clusters, potentially present but at a low level, are highlighted in grey.