EURL-AP Interlaboratory MS study 2025

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1. Introduction:

Over the past two years, the EURL-AP has worked with the expert group to harmonize the different mass spectrometry (MS) methods used within the network. To achieve this, it was notably proposed to these laboratories to analyse proficiency test samples by MS. The goal was to identify critical points where a standardized approach was necessary, while allowing flexibility in certain steps so that the method could adapt to the conditions of different laboratories, particularly in terms of equipment.

These studies (Lecrenier et al., 2021; Lecrenier et al., 2022; and evaluation of EURL-AP Proficiency test (PT) samples from 2020 to 2024) enabled a global harmonization regarding the target peptides (haemoglobin, milk, and connective tissue peptides), the MS method (targeted), and the type of instrument used (low-resolution MS). It also highlighted the necessity to set a common threshold for data interpretation. This criterion is crucial to fix before the finalisation of the SOP and requires harmonization, as it significantly affects the results.

To address this, the EURL-AP planned to organize an MS interlaboratory study (ILS-MS). The objective of the present study was to establish the minimal number of peptides identified to confirm the detection of targeted products. The selected threshold should ensure sufficient sensitivity to detect adulteration at around 0.1 % processed animal proteins (PAPs) (w/w), but most importantly—and this is crucial— to avoid any false positive results.

2. Material and methods

2.1. Study organisation

Five laboratories participated to the study by using their own-targeted MS method: IZSTO (Italian NRL-AP), IMR (Norwegian NRL-AP), CER Groupe (private Belgian company), BfR (German NRL-AP) and AGES (Austrian NRL-AP). The method focused solely on closed list of ruminant markers targeting three protein categories: haemoglobin, milk proteins (casein and beta-lactoglobulin) and connective tissue proteins (collagen and prolargin).

The study was based on a set of five samples consisting of blank feed or feed fortified with ruminant-derived proteins at levels close to 0.1 % (w/w). The sample preparation (extraction, digestion, purification…), the MS method and the detection criteria to be used were free.

A common heavy-labelled standard (ISTD) was incorporated into the study to facilitate results comparison across participants and was distributed prior to the study, on 4 February 2025 (as described in 2.2 Material section).

Official announcement of the study was sent by mail on 28 March 2025 to all invited participants. On 1 April 2025, the sample sets were shipped to the participants. On that same day the Excel report form

containing the instructions and the Skyline template to import raw data were also sent by email to all participants. The deadline for the delivery of the results was fixed in the announcement and in the instructions on 1 May 2025.

Three laboratories delivered their results on time to the organiser. Due to instrumental or standard issues, two laboratories requested a delay in delivering their results. Taking into consideration that this was not a proficiency test and that no feedback has yet been given to other participants, the deadline was extended to 15 May, enabling all participants to submit their results.

2.2. Material:

Heavy-labelled standard (ISTD)

This standard has been synthesized as a polypeptide comprising two concatenated peptides with the following sequence: AAVTAFWG**K**HQG**L**PQEVLNENLL**R**. Heavy labelled amino acids are indicated in bold. Each laboratory received 50 µg of ISTD in a lyophilized form with storage instruction.

Description of the sample set

Five different blind test materials were prepared for the study. The composition of the sample set was established considering the following parameters:

- Use of feed intended to ruminant, pig, poultry and fish
- Use of various ruminant PAPs with different tissue contents
- Use of milk powder, frequently responsible of carry-over between sample injections
- Use of at least one blank feed

Each participating lab received a sample set of five vials, each of about 80 g, to which a unique random number was assigned. Details of the sample set are indicated in Table 1.

Table 1: Composition of the sample set and intended results according to the sample composition

		Intended results:		
Samples	**Composition**	**Milk proteins of ruminant origin**	**Haemoglobin of ruminant origin**	**Connective tissues of ruminant origin**
ILS-01	Pig feed + 0.1 % ovine PAP + 0.1 % milk powder	+	+	+
ILS-02	Ruminant feed + 0.1 % bovine PAP I	-	+	+
ILS-03	Aquafeed + 0.1 % bovine PAP II	-	+	+
ILS-04	Poultry feed	-	-	-
ILS-05	Aquafeed + 0.1 % bovine bloodmeal	-	+	-

Legend: "+" = protein expected to be identified according to the sample composition; "–" = protein not expected to be identified according to the sample composition.

For this interlaboratory study, participants were not required to conclude on the presence or absence of the target proteins, since the purpose of the study was to determine the positivity threshold (number of peptides detected per protein). The intended results were therefore based on the composition rather than on the threshold applied by the EURL-AP.

Materials used in the preparation of the samples

Four commercially available feed materials or feed were used as matrices:

- A **pig feed**, used to prepare sample 1, was a feed intended for sow feeding. The protein content indicated on the label was 13.8 %. The sediment fraction obtained after a sedimentation with TCE (tetrachloroethylene) represents 1.3 % w/w of the test portion. PCR and light microscopy (LM) analyses proved that it was free of ruminant DNA and free of terrestrial animal particles, respectively.
- A **ruminant feed**, used in sample 2, was a commercial compound feed for sheep and goat. The protein content indicated on the label was 16.1 %. Its sediment content was about 1.3 %. No animal remains could be detected by LM and no animal DNA (ruminant, pig, poultry or fish DNA) was detected by PCR.
- An **aquafeed**, used for preparing sample 3 and 5, was a complement pelleted feed for Atlantic salmon. The protein content indicated on the label was 37.9 %. Its sediment content was about 1 %. On the exception of fish DNA and particles, no other animal DNA (ruminant, pig or poultry DNA) was detected by PCR and no terrestrial particles was detected by LM.
- A **poultry feed**, used in sample 4 was a complete feed for broiler chickens. The protein content indicated on the label was 17.5 %. Its sediment content was about 2.6 %. No particles or DNA from animal origin was detected by LM and PCR.

Adulterant material used:

- An **ovine PAP** was used to prepare sample 1. Its sediment content was about 29 %. PCR analyses revealed the presence of ruminant, porcine and poultry DNA and LM analyses showed that it contained terrestrial animal particles (bones, muscles and sheep hairs).
- A **milk powder** was also used for sample 1. Its sediment content was about 0.35 %. PCR analyses revealed the presence of ruminant and porcine DNA.
- A **bovine PAP** (bovine PAP I) was used for preparing sample 2. Its sediment content was about 52 %. Only ruminant DNA was detected by PCR and LM analyses showed that it contained terrestrial animal particles (bones, muscles and blood).
- Another bovine PAP (bovine PAP II) was used for preparing sample 3. Its sediment was about 60 %. PCR and LM analyses showed that it contained ruminant DNA exclusively and terrestrial particles (bones and muscles), respectively.
- A **bovine blood meal** was used for sample 5. It had no sediment. Only ruminant DNA was detected by PCR.

The two bovine PAPs used were the same that the ones used for the two previous EURL-AP MS studies (Lecrenier et al., 2021; Lecrenier et al., 2022).

Preparation procedure

Feed were 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 a rule of best practice, all feed matrices were ground and conditioned separately. Only after the whole conditioning of the vials with the feed, adulterations were realised by direct spiking into the vials. Participating laboratories are responsible to ensure adequate homogeneity of the sample sub-portions taken for analysis (as specified in the instructions).

2.3. Homogeneity study

A homogeneity study was performed on five replicates of each sample, obtained by randomly selecting five vials. For each replicate, protein extraction was conducted on three test portions of 1 g of the sample material. In cases where terrestrial particles were detected by LM (ILS-01, ILS-02 & ILS-03), additional analyses were carried out on three sediment test portions per replicate. For this purpose, 30 g of feed material were sedimented with TCE for each replicate, and the resulting sediment was divided into three portions. For each sample, two additional matrix-matched samples, used as quality control, were prepared by adding a pool of protein extracts to the matrix at two different levels. This pool is obtained by the prior extraction of proteins from different feed materials (milk powder, haemoglobin powder & bovine gelatine).

As complementary information, the homogeneity study was completed by LM analyses on 10 g of sample material for each replicate and PCR analysis on two test portions of 100 mg of material for each one. Table 2 summarizes the results and detailed MS results for each peptide are given in Annex 1.

Table 2: Results of the homogeneity study

			MS on feed			MS on sediment			Light microscopy			PCR		
Samples	Composition	Nr	Milk proteins	Ruminant Haemoglobin	Ruminant Connective tissues	Milk proteins	Ruminant Haemoglobin	Ruminant Connective tissues	Terrestrial vertebrate	Terrestrial invertebrate	Fish	Ruminant	Porcine	Poultry
ILS-01	Pig feed + 0.1 % ovine PAP + 0.1 % milk pwd.	5	5/5	0/4	2/4	4/5	1/4	2/4	+	n/a	-	+	-	-
ILS-02	Ruminant feed + 0.1 % bovine PAP I	5	0/5	2/4	4/4*	0/5	4/4	4/4	+	-	-	+	-	-
ILS-03	Aquafeed + 0.1 % bovine PAP II	5	0/5	1/4	4/4	0/5	3/4	4/4	+	n/a	+	+	-	-
ILS-04	Poultry feed	5	0/5	0/4	0/4	n/a			-	n/a	-	-	-	-
ILS-05	Aquafeed + 0.1 % bovine bloodmeal	5	0/5	3/4	0/4	n/a			-	n/a	+	+	-	-

Legend: Nr = number of replicates; MS Results are reported as the ratio of detected marker peptides to the total number of peptides defined in the method for each corresponding protein; * for one of the five replicates, only 3/4 peptides were detected; n/a = not analysed; "+" = detected, "-" = not detected.

ILS-01 (Pig feed + 0.1 % ovine PAP + 0.1 % milk powder): All milk peptides (5/5) were detected in the feed sample and four (4/5) in the sediment. Haemoglobin was not detected in the feed sample but one peptide (VVAGVANALAHR) was detected in all replicates of the sediment. Two collagen peptides (2/5) were detected in both the feed or the sediment.

BLAST analysis against the NCBI database confirmed that the haemoglobin peptide detected in the sediment corresponds to the only targeted haemoglobin marker present in ovine haemoglobin sequence. Since this peptide was included in all participating laboratories' methods, and no minimum requirement regarding the number of peptides was defined in this study, the sample was kept in the study to allow evaluation of the method's performance on other ruminant species. However, the interpretation of the laboratory results concerning haemoglobin detection for this sample was limited to applying a positive threshold of one identified peptide as it is not possible to evaluate two peptides due to the limited number of ovine haemoglobin peptide available.

LM revealed the presence of terrestrial vertebrates in the sample. No traces of fish were detected. Only DNA from ruminant was detected by PCR analyses.

ILS-02 (Ruminant feed + 0.1 % bovine PAP I)**:** No milk proteins (0/5) were detected in either the feed or the sediment. Two haemoglobin peptides (2/4) and all collagen peptides (4/4) were detected in the feed, except in one replicate where only three collagen peptides were detected. The use of sedimentation improved the detection of haemoglobin peptides, allowing the detection of all peptides (4/4) while maintaining the detection of all collagen peptides (4/4).

By LM, the sample was positive for the presence of terrestrial vertebrates. No traces of fish or invertebrates could be found. Only DNA from ruminant was detected by PCR analyses.

ILS-03 (Aquafeed + 0.1 % bovine PAP II) **:** No milk proteins (0/5) were detected in either the feed or the sediment. Only one haemoglobin peptides (1/4) were detected in the feed and all collagen peptides (4/4) were detected. The use of sedimentation improved the detection of haemoglobin peptides, allowing the detection of three peptides (3/4) while maintaining the detection of all collagen peptides (4/4).

Sample was positive for the presence of fish and terrestrial vertebrates. Only DNA from ruminant was detected by PCR analyses.

ILS-04 (Poultry feed): No ruminant peptide markers were detected in the feed. Analyses on sediment were not performed as no terrestrial particles were detected in sediment by LM.

LM analyses confirmed that sample was free from any animal particles. No DNA from ruminant, pig and poultry was detected by PCR analyses.

ILS-05 (Aquafeed + 0.1 % bovine bloodmeal): No milk proteins (0/5) were detected in either the feed or the sediment. Three haemoglobin peptides (3/4) were detected in the feed and no collagen peptides (0/4) were detected. Analyses on sediment were not performed as no terrestrial particles were detected in sediment by LM.

Sample was positive for the presence of fish. No traces of terrestrial vertebrates were detected. Only DNA from ruminants was detected by PCR analyses.

2.4. Methods

Each laboratory contributed to the study by applying its own sample preparation protocol, targeted MS methodology and applying its own acceptance criteria for peptide detection. The peptide list was nevertheless restricted to sequences derived from bovine haemoglobin, collagen, prolargin, casein, and β-lactoglobulin, to ensure comparability and facilitate the evaluation of the results (see Table 5). The internal standard (ISTD) was mandatorily added to each sample prior to any subsequent preparation steps. However, the concentration of the ISTD was determined independently by each participating laboratory before the beginning of the study.

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

Major differences between protocols are summarised in Table 3.

Table 3: Comparison of the sample preparations used by the different labs + EURL-AP

	EURL-AP	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Test portion size	1 g (or 10 g for sedimentation)	0.5 g	1 g	10 g	0.5 g	1 g
Nr of replicate	3	2	2	2	4	1
Pre-treatment	None (or sedimentation)	Defatting (if necessary)	Defatting	Sedimentation	None	None
[ISTD] (µg/g feed)	1 (or 0.01 for sedimentation)	1	0.1	0.001	1	1
Extraction buffer	200 mM Tris, 2 M Urea; pH 9.2	200 mM Tris, 2 M Urea pH 9.2	2M ThioUrea, 7 M Urea, pH: not controled	200 mM Tris, 2 M Urea pH 9.2	100 mM Tris, SDS 4 % pH 7.6	200 mM Tris, 2 M Urea pH 9.2
Reduction agent	DTT					
Alkylation agent	IAA					
Digestion enzyme	Trypsin	Trypsin	Trypsin	Trypsin	Trypsin	Trypsin
Digestion T°	37 °C					
Digestion time	1 h	16 h	~17 h	1 h	12-18 h	1 h
Purification method	tC18 Sep-pack	SPE SDB-XC	RP-SPE	tC18 Sep-pack	Oasis Peptide-96 well plate	tC18 Sep-pack

Legend: No, number; [ISTD], concentration of heavy labelled standard in feed; DTT, dithiothreitol; IAA, iodoacetamide; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction

Liquid chromatography and mass spectrometer system (LC-MS)

LC-MS system and major parameters used are summarised in Table 4.

Table 4: Comparison of the liquid chromatography (LC) and Mass spectrometer (MS) system used by the different labs and the main parameters + EURL-AP

	EURL-AP	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
LC system	UHPLC Acquity I-Class FL (Waters)	UHPLC H-Class (Waters)	UHPLC 1290 II Infinity (Agilent)	HPLC Excion LC (Sciex)	UHPLC Vanquish Horizon (Thermo scientific)	UHPLC Acquity I-Class FTN (Waters)
Column length (mm)	100	150	150	150	100	100
Column diameter (mm)	2.1	1	2	2.1	2	2
Gradient time (min)	16	50	18	16	15	16
MS system	Xevo TQ-XS (Waters)	Xevo G2 XS QTOF (Waters)	QTRAP 6500+ System (Sciex)	QTRAP 5500 System (Sciex)	Q-Exactive Orbitra (Thermo scientific)	Xevo TQ-Sμ (Waters)
Acquisition mode	MRM	PRM	MRM	MRM	PRM	MRM
Ionisation mode	ESI positive					
Software	TargetLynx/ Masslynx V4.2 (Waters)	TargetLynx/ Masslynx V4.2 (Waters)	Analyst 1.7/Sciex OS (Sciex)	Analyst 1.6.3/Multiquan t3.03 (Sciex)	Free style (Thermo scientific)/skyline	TargetLynx/ Masslynx V4.2 (Waters)

Legend: LC, liquid chromatography; MS, mass spectrometry; PMR, parallel reaction monitoring; MRM, multiple reaction monitoring; ESI, electrospray ionisation.

Peptide markers

Table 5 lists the peptides (and their corresponding proteins) used as markers in this study. The proteins are classified in three main categories: ruminant haemoglobin, ruminant connective tissues and milk proteins. Peptides were selected based on results obtained by MS during previous EURL-AP studies (Lecrenier et al., 2021; Lecrenier et al., 2022), and particularly on the participation of laboratories in the analysis of EURL-AP PT samples (2020-2024).

Table 5: Comparison of the peptide markers used by the different labs

Protein category	Protein name	Peptide sequence	Precursor ion m/z^z	EURL-AP	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	510.6^{3+}	X		X	X	X	X
	Haemoglobin beta	AAVTAFWGK	475.8^{2+}	X	X	X	X	X	X
		EFTPVLQADFQK	711.9^{2+}	X	X	X	X	X	X
		VVAGVANALAHR	393.2^{3+}	X	X	X	X	X	X
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	758.9^{2+}	X	X	X			X
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	824.9^{2+}	X	X	X	X		X
		GP**Hyp**GESGAAGPTGPIGSR	790.9^{2+}	X		X	X		X
		IGQPGAVGPAGIR	596.8^{2+}		X	X			
		IGQ**Hyp**GAVGPAGIR	604.8^{2+}	X			X		X
	Prolargin	ISSVPAISSR	508.8^{2+}		X				
		IEAIPSGYFK	562.8^{2+}		X				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	692.9^{2+}	X	X	X		X	X
		HQGLPQEVLNENLLR	587.3^{3+}	X	X	X	X	X	X
		YLGYLEQLLR	634.4^{2+}		X			X	X
	Alpha-s2 casein	NAVPITPTLNR	598.3^{2+}	X		X	X	X	X
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	858.4^{2+}	X		X	X	X	X
		VLVLDTDYK	533.3^{2+}	X		X	X	X	X
		VYVEELKPTPEGDLEILLQK	771.7^{3+}		X			X	X
ISTD	Haemoglobin beta	AAVTAFWG(K)	479.8^{2+}	X	X	X	X	X	X
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	593.0^{3+}	X	X	X	X	X	X

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; X = peptide included in the laboratory method.

3. Results and discussion:

Table 6 summarizes the overall results for the detection of targeted ruminant proteins in each sample type (ILS-01 to ILS-05). Ruminant proteins expected to be detected according to the sample composition are highlighted in grey. Detailed results provided by each laboratory are summarized in Annex 1.

The initial evaluation of the results was based on the detection of the ISTD. Unfortunately, one laboratory (Lab 4) failed to detect this one. Since the use and detection of this standard were mandatory, the corresponding results could not be considered in this study.

For the other laboratories, the overall results, expressed in terms of global accuracy (AC), confirmed the suitability of the MS approaches for detecting ruminant proteins at a 0.1 % (w/w) level of adulteration. The percentage of total errors, regardless of the targeted proteins or the laboratories, accounted for 15 % (9/60) of the total responses when considering the positive threshold of one identified peptide (T1P). The calculation could not be carried out with the two-peptide threshold (T2P), because haemoglobin was not assessable for ILS-01 when applying this threshold. This percentage of total errors is similar and even slightly lower to those observed in the previous EURL-AP Interlaboratory Test MassSpec 2019 (Lecrenier *et al.*, 2021), where it reached 16.5 %. It should be noted that the adulteration level in the earlier study was set at 1 %, which made the present study comparatively more challenging.

Among the deviations, only one was due to a false positive finding of milk in sample ILS-02 containing 0.1 % of bovine PAP I. No other specificity issue was recorded. Some sensitivity issues were noticed, mainly related to the lack of haemoglobin detection. To a lesser extent, connective tissue was also difficult to detect in ILS-01.

All laboratories had at least one false result. Lab 2 had one false positive result (ILS-02) for milk detection, and the applied threshold didn't change the conclusion as two milk peptides were detected. The false results from the other laboratories only involved false negative, and the applied threshold didn't change the conclusion as no peptide was detected in their false-negative results. Lab 3 showed two false negative results (ILS-01 & ILS-05) linked to haemoglobin detection. Lab 1 had two false negative results related to haemoglobin detection (ILS-01 & ILS-02) and one false negative result for connective tissue (ILS-01). Lab 5 had also two false negative results related to haemoglobin detection (ILS-01 & ILS-03) and one false negative result for connective tissue (ILS-01).

Table 6: Overall accuracy for the detection of ruminant proteins by Labs 1, 2, 3 & 5, applying thresholds of a minimum of one (T1P) or two peptides (T2P).

			Milk proteins		Haemoglobin		Connective tissues	
	Composition	n	T1P	T2P	T1P	T2P	T1P	T2P
ILS-01	Pig feed + 0.1 % ovine PAP + 0.1 % milk powder	4	1.00	1.00	0.25 (3)	not assessed	0.50 (2)	0.50 (2)
ILS-02	Ruminant feed + 0.1 % bovine PAP I	4	0.75 (1)	0.75 (1)	0.75 (1)	0.75 (1)	1.00	1.00
ILS-03	Aquafeed + 0.1 % bovine PAP II	4	1.00	1.00	0.75 (1)	0.75 (1)	1.00	1.00
ILS-04	Poultry feed	4	1.00	1.00	1.00	1.00	1.00	1.00
ILS-05	Aquafeed + 0.1 % bovine bloodmeal	4	1.00	1.00	0.75 (1)	0.75 (1)	1.00	1.00

Legend: Accuracy is the fraction of the correct results. It is calculated by dividing the number of correct results by the total number of results. The number of false positive or false negative is given in brackets. n = number of results. Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.

Results obtained on each sample are discussed hereunder. Results obtained by the EURL-AP for the homogeneity study are also included in the discussion.

ILS-01 (Pig feed + 0.1 % ovine PAP + 0.1 % milk powder):

Among all samples, ILS-01 appeared to be the most complex sample of the study.

Milk proteins, even at low inclusion level (0.1 %), were successfully detected by all laboratories, with more than two peptides identified (ranging from two to six detected peptides).

In contrast, the detection of ovine PAP was considerably more challenging.

VVAGVANALAHR was detected by Lab 2. No other laboratories detected it. As explained in the homogeneity study, VVAGVANALAHR is the sole targeted haemoglobin marker present in ovine haemoglobin sequence, making its detection more challenging.

For connective tissues, two collagen peptides (GSTGEIGPAGPHypGPHypGLR & GPHypGESGAAGPTGPIGSR) were detected by Labs 2 and 3. BLAST analysis confirmed these two peptides as the only targeted peptides able to detect ovine collagen. Lab 5 reported their results for these peptides as non-interpretable, while Lab 1 didn't detect GSTGEIGPAGPHypGPHypGLR, GPHypGESGAAGPTGPIGSR not being included in their MS method. Regarding prolargin, only IEAIPSGYFK matched the ovine sequence but was not detected in the sample by Lab 1.

No false positive results were reported.

ILS-02 (Ruminant feed + 0.1 % bovine PAP I):

Expected to be positive for both haemoglobin and connective tissues peptides, ILS-02 gave consistent results for collagen detection, with all laboratories identifying more than two peptides (on average three). Prolargin peptides were not detected by Lab 1, the only participant that included them in its method.

Results for haemoglobin detection were more variable. Labs 2, 3 and 5 detected 4/4, 3/4, and 2/4 peptides, respectively, whereas Lab 1 didn't detect any.

Besides that, false positive detection of two milk peptides (HQGLPQEVLNENLLRP & NAVPITPTLNR) was reported by Lab 2.

ILS-03 (Aquafeed + 0.1 % bovine PAP II)

Results for ILS-03 were broadly comparable to those of ILS-02. Nevertheless, it has to be highlighted that PAP II had previously been characterised (Lecrenier *et al.*, 2021) as containing a higher bone fraction and lower blood content than PAP I, making its detection more challenging.

Collagen peptides were again consistently identified by all laboratories with more than two peptides (average of three), while prolargin peptides were not detected.

Haemoglobin results were again variable. Lab 2 detected 3/4 peptides, Labs 1 and 3 detected 2/4, and Lab 5 detected none.

No false positive results were reported.

ILS-04 (Poultry feed)

Serving as the blank control, ILS-04 was expected to contain no targeted ruminant proteins. All laboratories reported results consistent with expectations, with all analyses returning negative findings.

ILS-05 (Aquafeed + 0.1 % bovine bloodmeal)

Composed solely of blood proteins, ILS-05 showed variable haemoglobin detection. Lab 2 detected all four peptides (4/4), while Labs 1 and 5 detected 3/4. Lab 3, however, failed to detect any.

No false positive results were reported.

4. Conclusions

As a reminder, the objective of the present study was to establish the minimum number of peptides required to confirm the detection of targeted products. The selected threshold should ensure sufficient sensitivity to detect adulteration at around 0.1% (w/w) PAPs, but most importantly—and this is crucial—avoid any false-positive results.

For this purpose, and based on the observations, it was decided that one sample (ILS-01) could not be used to determine this limit. Indeed, the insufficient number of ovine haemoglobin peptides did not allow a reliable evaluation. Nevertheless, the analysis of this sample within the study was particularly interesting and opened new perspectives for further developments, particularly in relation to peptides able to detect small ruminant proteins. This point will be discussed in the perspective point.

For the other samples, this interlaboratory study showed that sensitivity and specificity achieved by the participants were sufficient to reach the 0.1 % PAPs adulteration threshold for at least three out of four participants in each case. When proteins were detected, they were consistently identified by at least two peptides. Connective tissues were detected by all laboratories in every sample containing bovine “PAPs” (with blood meal not included under this term in this context). Haemoglobin detection, however,

remains more challenging, and some false negative results indicate that further improvements are still required.

Regarding the false positive detection of milk, applying the two-peptide threshold did not resolve the issue for sample ILS-02. Milk peptides are known to be highly sticky, which can lead to false positive results through cross-contamination or carry-over. Since milk proteins may occur at relatively high concentrations in feed, taking strict precautions during sample preparation is essential to prevent cross-contamination. Concerning the carry-over, strong washing between analytical series, as well as the injection of a "blank feed extract" (rather than a blank solvent) before each batch of analyses, is strongly recommended to both prevent and monitor such carry-over.

Overall, the interlaboratory evaluation reveals several critical points that must be addressed before establishing a definitive threshold. In particular, the current limitations in haemoglobin detection, as well as the variability introduced by laboratory-specific acceptance criteria for peptide detection, underline the need for further refinement and harmonisation. The harmonisation efforts undertaken in recent years now make it feasible to integrate these parameters into a common and robust threshold setting framework. In the light of these considerations, and to ensure that the final threshold is scientifically sound, operationally realistic, and widely applicable (in the perspective of a validation study), the decision regarding its formal adoption has been postponed.

5. Perspectives

Building on the results of this interlaboratory study, several perspectives emerge for strengthening the reliability and robustness of PAP detection by mass spectrometry.

A first priority concerns the improvement of haemoglobin detection, which remains one of the most challenging aspects of the method. Considering the diversity of analytical approaches currently used across laboratories, several avenues for enhancement can be proposed. These include refining sample preparation strategies and addition of new peptide markers.

The use of sedimentation as complementary sample preparation protocol can be used to improve the detection of haemoglobin peptides, as demonstrated in the homogeneity study. Lab 1 conducted a post-study evaluation of the sedimentation approach on sample ILS-02, for which it had previously obtained a false-negative result for haemoglobin detection. The analysis of the sediment enabled the laboratory to detect all three haemoglobin peptides included in its method, whereas none had been detected in the feed fraction, showing that the use if sedimentation could be a solution for improvement for this lab.

However, the analysis of the sediment should not replace the analysis of the feed. The feed analysis must remain the primary test to perform in order to obtain an overall result, as it also taking into account

the proteins that may be present in the flotate. Sediment analysis should be considered a complementary approach in cases where PAPs containing sedimenting particles are present. The result obtained by the Lab 3 on sample ILS-05 provides clear evidence of this. Lab 3 analysed this sample only on the sediment fraction; however, blood meal does not produce sediment, making it undetectable when only the sediment is tested. This explains the false-negative result obtained by this laboratory.

Other potential solutions were also identified based on the participants' experience. Among them, testing a higher urea concentration in the protein extraction buffer, as implemented by Lab 2, represents an additional approach that could improve the sensitivity of haemoglobin detection. Increasing urea from moderate levels (e.g., 2 M) to a fully denaturing concentration (8 M) can significantly enhance the solubilisation and recovery of haemoglobin. A complete denaturation also improves the accessibility of cleavage sites, thereby promoting more efficient and reproducible peptide digestion.
In addition, Lab 1 and Lab 2 incorporated a preliminary defatting step into its sample preparation protocol, which may contribute to reduce matrix complexity by removing lipids that can otherwise hinder protein extraction and enzymatic digestion. This additional clean-up step may further facilitate the release and detection of haemoglobin peptides.

The second priority concerns the ovine PAPs detection, which are subject to the same regulatory framework as bovine PAPs. The limitation in ovine haemoglobin detection identified in this study must also be considered. To address this, the EURL-AP performed an *in silico* digestion of the ovine haemoglobin alpha sequence (P68240) using Skyline software, which identified one particularly promising peptide (**VAAALTK**) for ovine PAP detection. The details on the peptide are summarized in Table 7.

Table 7: MRM Parameters of the new ovine haemoglobin peptide marker

Protein category	Protein name	Peptide sequence	precursor ion: m/z^z	transitions: m/z^z
Ruminant haemoglobin	Haemoglobin alpha	VAAALTK	337.2 2+	**503.3 +** 574.3 + 248.2 +

Legend: MRM transitions are classified by decreasing peak intensities, and the most intense transition is in bold. m/z = mass/charge; z = ion charge

In addition, BLAST analysis against NCBI Database confirmed its occurrence in bovine and caprine haemoglobin, thereby extending its usefulness to the detection of haemoglobin from other farmed ruminants. The specificity was confirmed experimentally on porcine haemoglobin powder and PAP samples (bovine, ovine, caprine and poultry origin). This peptide, still under evaluation, is likely to be integrated soon into the EURL-AP MS method.

Another peptide, MLSSLFANYAGFDTPIEK, derived from Myosin-7, was suggested by Lab 2. BLAST analysis against UniProt Database confirmed its presence in ovine, caprine and bovine myosin.

However, the specificity and sensitivity of this peptide still need to be experimentally confirmed on ovine and caprine PAP samples.

The EURL-AP encourages laboratories to evaluate these peptides. If appropriate, their incorporation in the peptide list would be considered. Should additional peptides be identified that could enhance ovine and caprine PAP detection, laboratories are requested to communicate them to the EURL-AP for further assessment.

Finally, a table summarising the acceptance criteria applied by the laboratories will be prepared. These criteria will be discussed with the laboratories in relation to the results obtained during the ILS and/or additional results generated on the ILS samples following improvements in sample preparation or use of new peptides. A follow-up report will then be prepared and diffused to the network.

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Annex 1.

ILS-01: Pig feed + 0.1 % ovine PAP + 0.1 % milk powder									
Protein category	**Protein name**	**Peptide sequence**	**Homogeneity study**		**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**
			Feed	**Sediment**					
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	-	-		-	-	-	-
	Haemoglobin beta	AAVTAFWGK	-	-	-	-	-	-	-
		EFTPVLQADFQK	-	-	-	-	-	-	**N/I**
		VVAGVANALAHR	-	+	-	+	-	-	-
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	**N/I**	**N/I**	-	-			**N/I**
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	+	+	-	+	+		**N/I**
		GP**Hyp**GESGAAGPTGPIGSR	+	+		+	+		**N/I**
		IGQPGAVGPAGIR			-	-			
		IGQ**Hyp**GAVGPAGIR	-	-			-		-
	Prolargin	ISSVPAISSR			-				
		IEAIPSGYFK			-				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	+	+	+	+		+	+
		HQGLPQEVLNENLLR	+	+	+	+	+	+	+
		YLGYLEQLLR			+			-	+
	Alpha-s2 casein	NAVPITPTLNR	+	+		+	-	+	+
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	+	-		+	-	-	+
		VLVLDTDYK	+	+		+	+	-	+
		VYVEELKPTPEGDLEILLQK			+			-	+
ISTD	Haemoglobin beta	AAVTAFWG(K)	+	+	+	+	+	-	+
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	+	+	+	+	+	-	+

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; "+" = peptide identified; "-" = peptide not detected and/or not identified or below LOD; N/I = results not interpretable for this peptide; Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.

ILS-02: Ruminant feed + 0.1 % bovine PAP I									
Protein category	**Protein name**	**Peptide sequence**	**Homogeneity study**		**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**
			Feed	**Sediment**					
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	+	+		+	+	-	+
	Haemoglobin beta	AAVTAFWGK	+	+	-	+	+	-	+
		EFTPVLQADFQK	-	+	-	+	+	-	N/I
		VVAGVANALAHR	-	+	-	+	-	-	-
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	+	+	-	-			N/I
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	+	+	+	+	+		+
		GP**Hyp**GESGAAGPTGPIGSR	+	+		+	+		+
		IGQPGAVGPAGIR			+	+			
		IGQ**Hyp**GAVGPAGIR	+	+			+		+
	Prolargin	ISSVPAISSR			-				
		IEAIPSGYFK			-				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	-	-	-	-		+	-
		HQGLPQEVLNENLLR	-	-	-	+	-	-	-
		YLGYLEQLLR			-			-	-
	Alpha-s2 casein	NAVPITPTLNR	-	-		+	-	-	-
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	-	-		-	-	-	-
		VLVLDTDYK	-	-		-	-	-	-
		VYVEELKPTPEGDLEILLQK			-			-	-
ISTD	Haemoglobin beta	AAVTAFWG(K)	+	+	+	+	+	-	+
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	+	+	+	+	+	-	+

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; "+" = peptide identified; "-" = peptide not detected and/or not identified or below LOD; N/I = results not interpretable for this peptide; Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.

ILS-03: Aquafeed + 0.1 % bovine PAP II									
Protein category	**Protein name**	**Peptide sequence**	**Homogeneity study**		**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**
			Feed	**Sediment**					
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	-	+		+	+	-	-
	Haemoglobin beta	AAVTAFWGK	-	+	+	+	+	-	-
		EFTPVLQADFQK	-	-	+	-	-	-	N/I
		VVAGVANALAHR	+	+	-	+	-	-	-
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	+	+	-	-			N/I
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	+	+	+	+	+		+
		GP**Hyp**GESGAAGPTGPIGSR	+	+		+	+		+
		IGQPGAVGPAGIR			+	+			
		IGQ**Hyp**GAVGPAGIR	+	+			+		+
	Prolargin	ISSVPAISSR			-				
		IEAIPSGYFK			-				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	-	-	-	-		-	-
		HQGLPQEVLNENLLR	-	-	-	-	-	-	-
		YLGYLEQLLR			-			-	-
	Alpha-s2 casein	NAVPITPTLNR	-	-		-	-	-	-
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	-	-		-	-	-	-
		VLVLDTDYK	-	-		-	-	-	-
		VYVEELKPTPEGDLEILLQK			-			-	-
ISTD	Haemoglobin beta	AAVTAFWG(K)	+	+	+	+	+	-	+
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	+	+	+	+	+	-	+

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; "+" = peptide identified; "-" = peptide not detected and/or not identified or below LOD; N/I = results not interpretable for this peptide; Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.

ILS-04: Poultry feed									
Protein category	**Protein name**	**Peptide sequence**	**Homogeneity study**		**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**
			Feed	**Sediment**					
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	-			-	-	-	-
	Haemoglobin beta	AAVTAFWGK	-		-	-	-	-	-
		EFTPVLQADFQK	-		-	-	-	-	**N/I**
		VVAGVANALAHR	-		-	-	-	-	-
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-		-	-			**N/I**
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	-		-	-	-		-
		GP**Hyp**GESGAAGPTGPIGSR	-			-	-		-
		IGQPGAVGPAGIR			-	-			
		IGQ**Hyp**GAVGPAGIR	-				-		-
	Prolargin	ISSVPAISSR			-				
		IEAIPSGYFK			-				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	-		-	-		**N/I**	-
		HQGLPQEVLNENLLR	-		-	-	-	-	-
		YLGYLEQLLR			-			-	-
	Alpha-s2 casein	NAVPITPTLNR	-			-	-	-	-
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	-			-	-	-	-
		VLVLDTDYK	-			-	-	-	-
		VYVEELKPTPEGDLEILLQK			-			-	-
ISTD	Haemoglobin beta	AAVTAFWG(K)	+		+	+	+	-	+
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	+		+	+	+	-	+

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; "+" = peptide identified; "-" = peptide not detected and/or not identified or below LOD; N/I = results not interpretable for this peptide; Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.

ILS-05: Aquafeed + 0.1 % bovine bloodmeal									
Protein category	**Protein name**	**Peptide sequence**	**Homogeneity study**		**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**
			Feed	**Sediment**					
Ruminant haemoglobin	Haemoglobin alpha	VGGHAAEYGAEALER	+			+	-	-	+
	Haemoglobin beta	AAVTAFWGK	+		+	+	-	-	+
		EFTPVLQADFQK	-		+	+	-	-	N/I
		VVAGVANALAHR	+		+	+	-	-	+
Ruminant connective tissues	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-		-	-			N/I
		GSTGEIGPAGP**Hyp**GP**Hyp**GLR	-		-	-	-		-
		GP**Hyp**GESGAAGPTGPIGSR	-			-	-		-
		IGQPGAVGPAGIR			-	-			
		IGQ**Hyp**GAVGPAGIR	-				-		-
	Prolargin	ISSVPAISSR			-				
		IEAIPSGYFK			-				
Milk proteins	Alpha-s1 casein	FFVAPFPEVFGK	-		-	-		-	-
		HQGLPQEVLNENLLR	-		-	-	-	-	-
		YLGYLEQLLR			-			-	-
	Alpha-s2 casein	NAVPITPTLNR	-			-	-	-	-
	Beta-lactoglobulin	LSFNPTQLEEQ**C[CAM]**HI	-			-	-	-	-
		VLVLDTDYK	-			-	-	-	-
		VYVEELKPTPEGDLEILLQK			-			-	-
ISTD	Haemoglobin beta	AAVTAFWG(K)	+		+	+	+	-	+
	Alpha-s1 casein	HQG(L)PQEVLNENLL(R)	+		+	+	+	-	+

Legend: mass modifications: **Hyp** = hydroxyproline; **C[CAM]**= Cysteine with carbamidomethylation; ISTD, heavy labelled standard; in parentheses=heavy labelled Aa; "+" = peptide identified; "-" = peptide not detected and/or not identified or below LOD; N/I = results not interpretable for this peptide; Ruminant proteins expected to be detected according to the sample composition, are highlighted in grey.