EURL-AP Interlaboratory Test MassSpec 2019

August 2020

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ISBN 978-2-87286-116-3
Legal Deposit D/2020/1463/5

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Abbreviation list

2-ME	2-mercaptoehtanol
BfR	Federal Institute for Risk Assessment (Germany)
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CRA-W	Walloon agricultural research centre
CV	Coefficient of variation
DDA	Data-dependent acquisition mode
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assays
ESI	Electrospray ionisation
EURL-AP	European Union Reference Laboratory for animal proteins in feedingstuffs
FASP	Filter aided sample prep
FDR	False discovery rate
Hb	Haemoglobin
HPD	Heterogeneous phase digestion
IAA	Iodoacetamide
IM	Ion mobility
IMR	Institute of marine research (Norway)
IS	Internal standard
IZSTO	Istituto Zooprofilattico Sperimentale del Piemonte (Italy)
kp	Conversion factor
LC	Liquid chromatography
LLOQ	Lower limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
No	Number
nOg	N-octylglucoside
PAPs	Processed animal proteins
PCR	Polymerase chain reaction
PMR	Parallel reaction monitoring
ppm	Part per million
Q	Quadrupole
QC	Quality control
ref sample	Reference sample
RRT	Relative retention time
RT	Retention time
S/N	Signal to noise ratio
SDS	Sodium dodecyl sulfate
SPE	Solid-phase extraction
TCA	Trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phosphine
TEA	Triethanolamine
Unamur	University of Namur
w/w	Weight to weight ratio

Introduction

In February 2019, the European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised an expert meeting on mass spectrometry (MS). The question was to identify a complementary method enabling further characterisation of the origin of the processed animal proteins (PAPs) or animal products when positive responses are delivered by current official methods, with special attention on identification of feed materials (authorised or forbidden) originating from ruminants. The experts were selected on basis of applied MS methods on animal by-products detection already in development in their labs. One of the actions decided during the MS expert meeting was the organization of an interlaboratory test on mass spectrometry conducted by the EURL-AP.

The objective of the present EURL-AP Interlaboratory Test MassSpec 2019 was strictly to evaluate the MS methods already developed in the network on a common set of samples.

Six labs participated to the study by using their own-targeted or non-targeted MS method: IZSTO (Italian NRL-AP), Signatope (private German company), IMR (Norwegian NRL-AP), CER Groupe (private Belgian company), BfR (German NRL-AP) and UNamur (University of Namur, Belgium).

All laboratories delivered results. The study was based on a set of nine samples consisting of blank feed or feed adulterated with bovine PAPs and/or milk product. The sample preparation (extraction, digestion, purification,…) and MS method to be used were free.

Material

The commercial feed matrix used was a **pig feed** intended for sow feeding. 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 light microscopy analyses proved that it was free of ruminant 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.

As adulterant materials, two different PAPs and one milk powder were used:

- **Bovine Paps01** was a commercial feed material. PCR and light microscopy analyses showed that it contained ruminant DNA and terrestrial particles (bones and muscles), respectively. Its sediment was of 62 % and the protein content was estimated at 49.5 %.
- **Bovine Paps02** was produced in a pilot plant. Its bone content is of about 50 %, meat and fat content of about 20 % and blood content of about 10 %. PCR and light microscopy analyses showed that it contained ruminant DNA and terrestrial animal particles (bones, muscles and blood), respectively. Its sediment was of 53 % and the protein content was estimated at 35.4 %.
- **Milk product** was a calf milk replacer. It was predominantly composed of skimmed milk powder and whey powder. PCR and light microscopy analyses respectively showed that it contained ruminant DNA and no bones nor muscles. The protein content was estimated at 21.8 %.

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

- Three reference samples (sample #1, #2 and #3) were included in the set. Their labelling was communicated to the labs. Participants were free to use them to optimise or develop their methods. No result had to be sent for these samples.
- One quality control (QC) sample was included as sample #4. Its composition was also communicated to the labs. This sample had to be used as a positive control. Data obtained on this sample had to be indicated in the result file but without interpretation as the sample was not blind.
- Five blind samples (sample #5, #6, #7, #8 and #9) without or with 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. With the exception of sample #2, all other samples were ground, as final step, with an Ultra Centrifugal rotor Mill ZM 200 (Retsch) in combination with a sieve of 2 mm mesh size, to ensure the homogeneity. The blank matrix was prepared and conditioned first in order to avoid any contamination by the other samples.

Each participating lab received about 50 g of each reference samples and about 5 g of QC and blind samples. Each sample was assigned with a unique random number. Details of the sample set are indicated in Table 1.

Table 1: Composition of the sample set

Sample #	Composition	Expected results: Contains prohibited ruminant by-products
1	Bovine Paps01	/
2	Milk product	/
3	Pig feed	/
4	QC (Pig feed + 5 % w/w Bovine Paps01 + 1 % w/w Milk product)	/
5	**Pig feed + 1 % w/w Bovine Paps01**	**+**
6	**Pig feed + 1 % w/w Bovine Paps02**	**+**
7	**Pig feed + 5 % w/w Milk product**	**-**
8	**Pig feed + 1 % w/w Bovine Paps01 + 1 % w/w Milk product**	**+**
9	**Pig feed (Blank)**	**-**

Legend: Samples sent in blind to the participants are in bold

Expected results were internally determined based on the known composition of the samples (presence or absence of prohibited ruminant by-products). For the reference samples and the QC, the interpretation of the result was not done (/) as the participants knew the composition.

Methods

Each lab participated to the study by using its own sample preparation and targeted MS method. In most cases, the method used was based on a recent publication (Lecrenier et al., 2016, Marchis et al., 2017, Lecrenier et al., 2018, Niedzwiecka et al., 2018, Belghit et al., 2019, Steinhilber et al., 2019).

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

Major differences between protocols are summarised in table 2.

Table 2: Comparison of the sample preparations used by the different 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
Replicate No	2	2	2	2	1	2
Pre-treatment	Grinding	/	Grinding	Defatting step with TCA 10 % / 2-ME in aceton	/	Precipitation/ defatting step with TCA 10 % /aceton 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**	SDS-page*	C18 spin columns**	Immuno-affinity enrichment*; C18 spin columns**	C18 SPE Cartridges**	FASP digestion

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

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

LC-MS system and major parameters used are summarised in table 3.

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

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

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

3. Biomarkers and targets

Table 4 lists the peptides (and related protein) used as biomarkers in this study. The targeted bovine products are identified for each lab.

Table 4: Comparison of the peptide biomarkers used by the different labs and the product/tissue targeted

Protein	Peptide	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6
Alpha-2-Macroglobulin	GSGGTAEHPFTVEEFVLPK	Blood					
Alpha-2-Antiplasmin	LPPLSLLK	Blood					
Protein HP-25 homolog 2	FGFDIELFQHAVK	Blood					
Complement Component 9	YTPVEAIEK	Blood					
Myosin-7	MLSSLFANYAGFDTPIEK	Muscle					
Osteopontin	YPDAVATWLKPDPSQK	Bone/Milk					
Matrilin-1	AGGIELFAIGVGR	Cartilage					
Desmin	TSGGAGGLGALR		Muscle				
Vimentin	TLYTSSPGGVYATR		Muscle				
Myoglobin	YLEFISDAIIHVLHAK		Muscle				
Haemoglobin alpha-chain	VGGHAAEYGAEALER			Blood	Blood	Blood	Blood
Haemoglobin beta-chain	AAVTAFWGK			Blood	Blood	Blood	Blood
	EFTPVLQADFQK			Blood	Blood	Blood	Blood
	VVAGVANALAHR				Blood	Blood	Blood
Collagen alpha-2 (I)	GEPGPAGAVGPAGAVGPR						Bone, tendon
Casein alpha-S1	FFVAPFPEVFGK			Milk		Milk	
	HQGLPQEVLNENLLR			Milk		Milk	
	YLGYLEQLLR					Milk	
Casein alpha-S2	NAVPITPTLNR			Milk		Milk	Milk
Beta-lactoglobulin	LSFNPTQLEEQCHI					Milk	
	VLVLDTDYK			Milk		Milk	Milk
	VYVEELKPTPEGDLEILLQK			Milk		Milk	

4. Evaluation criteria

Table 5 summarises the criteria used by the labs to evaluate the MS data and to deliver the conclusion on the presence of the targeted product.

Table 5: Comparison of the evaluation criteria applied by the different 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 = +/- 20 %, CV = +/- 25 % at LLOQ) Specificity of antibody Ion ratios similar to IS ≥ 3 product ions detected	RT similar to ref sample * S/N > 3 for all product ions Ion ratios similar to ref sample*	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 ions is a y-ion	≥ 1 peptide identified Peptide Mascot score ≥ 39.0	RRT similar to ref sample * S/N > 10 for quantifier ion Ion ratios similar to ref sample*	RT and IM closed to QC sample Product ions < 5 ppm error Peptide Mascot score ≥ 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 ≤ 20%	≥ 2 process IS identified IS identified ≥ 1 peptide identified	≥ 1 peptide identified	Bovine Hb present in both extractions. If results differ, bovine Hb considered to be present, if a 3rd extraction detects Hb	≥ 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: RT, retention time; IS, internal standard; LLOQ, lower limit of quantification; CV, coefficient of variation; S/N, signal to noise ratio; ref sample, reference sample; ppm, part per million; RRT, relative retention time; IM, ion mobility; QC, quality control; FDR, false discovery rate; Hb, haemoglobin.
** RT or ion ratio tolerances described by European Commission (2002).*

Study organisation

A protected Excel file containing a report form and instructions on how to fill it was sent on the 22nd November 2019 to the participants together with the samples.

The EURL-AP endorsed the grinding and the homogeneity of the samples. Nevertheless, each laboratory participating was sole responsible to reach appropriate homogeneity for the sample sub-portions taken for analysis.

The deadline for the delivery of the results was fixed at the 17th January 2020.

The 7th February, the correspondence list of samples per lab was sent to each participant (supplementary table 1 in annex 1). This document provided all information available on the samples: sample composition, expected results, light microscopy and PCR results.

Results and discussion

All results were delivered on time by labs to the organiser, except for one laboratory which delivered its results on the 7th February. These results were nevertheless accepted, as the correspondence list for the sample set composition was not yet sent.

Based on the results obtained for biomarkers used (supplementary tables 2.1 – 2.6 in annex 2) and individual evaluation criteria, labs had to conclude about the presence of prohibited ruminant by-products in blind samples. Table 6 summarises the lab conclusion. False results are in red.

Table 6: Summary of the labs conclusion on blind samples

Sample #	Composition	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6
5	1 % Paps01	+	+	+	-	+	+
6	1 % Paps02	+	+	+	-	+	+
7	5 % Milk	-	-	-	-	-	-
8	1 % Paps01 & 1 % Milk	+	-	-	-	+	+
9	Blank	-	-	-	-	-	-

Legend: "+" means that the sample was identified as containing prohibited ruminant by-products;
"-" means that the performed analyses did not allow to conclude about the presence of prohibited ruminant by-products. False results are in red cells.

Globally, the results obtained by the different methods are good except for lab 4.

There is no false positive result for the detection of prohibited ruminant by-products. However, ruminant by-products, when present in the samples, were not always detected. Lab 1, Lab 5 and Lab 6 have no false negative result. Lab 2 and Lab 3 showed one false negative result (sample #8) and Lab 4 had three false negative results (sample #5, #6 and #8).

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

In order to explain the results obtained by Lab 4 for samples #5 and #6, their method was compared to that of Lab 3, Lab 5 and Lab 6. Indeed, the four labs targeted the haemoglobin chains with comparable sets of peptides.

- Lab 5 and Lab 6 used exactly the same set of peptide biomarkers as Lab 4 while Lab 3 used only three out of this set of four biomarkers. Lab 3 only detected one of them (VGGHAAEYGAEALER) in one of the two replicates of samples #5 and #6. Lab 5 detected three haemoglobin peptides (VGGHAAEYGAEALER, AAVTAFWGK and VVAGVANALAHR) in sample #5 and four haemoglobin peptides (VGGHAAEYGAEALER, AAVTAFWGK,

EFTPVLQADFQK and VVAGVANALAHR) in sample #6. Lab 6 also detected three haemoglobin peptides (VGGHAAEYGAEALER, EFTPVLQADFQK and VVAGVANALAHR) in sample #5 and four haemoglobin peptides (VGGHAAEYGAEALER, AAVTAFWGK, EFTPVLQADFQK and VVAGVANALAHR) in sample #6. This confirms the presence of bovine haemoglobin in Paps01 and Paps02.

- The methods of the four labs differ by the sample preparation involved as well as by the mass spectrometer used: Q-Orbitrap for Lab 3, Q-TOF for Lab 4 and Lab 6 and triple-Q for Lab 5. Moreover, the results of Lab 4 and Lab 6 are based on a Data-dependent acquisition mode while for Lab 3 and Lab 5 they are based on a parallel reaction monitoring (PRM) and a multiple reaction monitoring mode (MRM), respectively.
- Lab 4 investigated their results by analysing pure bovine Paps01 (data not shown). This sample was declared as positive by them with the detection of only two out of the set of four biomarkers (VGGHAAEYGAEALER and VVAGVANALAHR). When their method was applied to the QC sample that contained Paps01 at a much lower level, they got negative results for all biomarkers. Therefore, the explanation of the results obtained by Lab 4 in the study is probably a lack of sensitivity on these PAPs (Paps01 and Paps02).

The results obtained by all labs on sample #5 (1 % Paps01) and sample #6 (1 % Paps02) were also compared. Lab 5 and Lab 6 have both detected 100 % (4/4) of their haemoglobin biomarkers in sample #6 and only 75 % (3/4) of their haemoglobin biomarkers in sample #5. Even if Lab 1 doesn't use the same proteins, the same phenomenon was observed for their blood biomarkers with the detection of one more biomarker (4/4) in sample #6 than in sample #5 (3/4). Lab 3 has detected only one blood peptide (the same) in both samples. The 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.

This difference was less evident to see for the muscle biomarkers (myosin-7 and myoglobin) detected in both samples by Lab 1 and Lab 2. Nevertheless, the signal intensity of myosin-7 (MLSSLFANYAGFDTPIEK) observed by Lab 1 was higher in sample #6 than in sample #5. On the contrary, bone biomarkers (osteopontin and collagen alpha-2 (I)) used by Lab 1 and Lab 6 were detected in sample #5 and not in sample #6. In order to see if these differences could be explained by the PAPs composition, these observations were compared to the results obtained by light microscopy on Paps01 and Paps02. Using Tetramethylbenzidine-Hydrogen peroxide staining (European Union Reference Laboratory for Animal Proteins in feedingstuffs, 2013), Paps02 gave a positive reaction for blood: immediate blue-green colouring and release of O_2 bubbles. Paps01 did not react positively to this staining. The blood concentration in Paps01 is therefore probably lower

than in Paps02. Regarding the bone content, the sediment percentage of Paps01 is higher than in Paps02. The differences in MS data are therefore confirmed by the light microscopy observations.

Sample #8, containing 1 % Paps01 and 1 % milk powder (w/w), appears as the most complex one to evaluate within the set of samples provided as three out of six labs (Lab 2, Lab 3 and Lab 4) failed to detect PAPs in it. When the same PAPs material (Paps01) is used at the same concentration in sample #5, then Lab 2 and Lab 3 correctly detect it. Lab 3 has detected two product ions of the peptide VGGHAAEYGAEALER in sample #8 but declared it as negative because their criteria for peptide identification (≥ 3 product ions detected) were not reached. Lab 2 did not detect any product ion in this sample. Lab 1, Lab 5 and Lab 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 haemoglobin 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 (haemoglobin) and GEPGPAGAVGPAGAVGPR (collagen) have the same peptide counts. For Lab 1, the comparison was limited to muscle and cartilage peptides as other peptides are present in the two adulterants of sample #8, PAPs and milk. In this case, the signal intensities of MLSSLFANYAGFDTPIEK (myosin) and AGGIELFAIGVGR (matrilin) 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 PAPs depending of the method and the peptide biomarkers used.

Some labs have also included in their results the detection of milk powder (Lab 1, Lab 3, Lab 5 and Lab 6). Correct detection of milk proteins was achieved by four labs (more details in the supplementary tables of annex 2), meaning that no false negative result was reported. However, milk powder induced many false positive results and all labs 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 analysed by enzyme-linked immunosorbent assays (ELISAs) in order to evaluate if the results obtained by the labs 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 ELISAs are very sensitive in 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 sample #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 the content of milk. The classification was based on the presence of a higher osteopontin abundance in the sample. This biomarker is used by Lab 1 both to detect the presence of milk and of PAPs and the identification of

the tissue of origin is based on the protein ratio. The high bone content of Paps01, used for sample #5 adulteration, is probably the cause of the misclassification. This study underlines the difficulty of this approach and a broader data knowledge of PAPs will be necessary to allow a better specificity in the classification of milk presence based on the ratio formed between osteopontin and the other tissue markers. For the three other labs, it is less clear what led them to a false detection of milk. 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. Lab 6 also detected only one milk biomarker in sample #6 and declared the sample as positive for milk, according to their acceptance criteria, that requires the identification of only one peptide of particular nature. The milk peptides used by the three labs are the same. As described by Ramachandran et al. (2020), some milk peptides are known to be sticky leading to give carryover effect. This particularity can probably explain the false positive results obtained.

Conclusion

Results obtained by the labs prove that mass spectrometry can identify the presence of various proteins of bovine origin in feed at a level of adulteration of 1 % w/w. Moreover, thanks to the discriminative power of the method to identify the tissue/product of origin, it is possible to determine if the feed is containing prohibited and/or authorised animal by-products, even though there are still some pitfalls to solve. Furthermore, the study highlights the heterogeneity of PAPs composition and, as already observed by other analytical approaches, this can interfere with the correct detection process of some markers. Based on these results, one can better grasp the potential of the several proposed methods. This study is an important step towards the building of the MS methods for the detection of prohibited animal by-products.

Acknowledgement

The authors are grateful to the participating laboratories who volunteered for this study, who shared additional data upon request and who participated to the discussion: **Daniela Marchis** and **Sara Morello** from IZSTO, **Oliver Poetz** and **Andreas Steinhilber** from Signatope, **Ikram Belghit** and **Josef Rasinger** from IMR, **Jean Henrottin** and **Nathalie Gillard** from CER Groupe, **Uta Herfurth** from BfR, **Marc Dieu** from UNamur and **Gilles Rousseau** from the Protection, control products and residues Unit of CRA-W.

We would like also to give a special thanks to **Lisa Plasman**, **Alexandra Cordonnier** and **Julien Maljean**, from the Quality and authentication of agricultural products Unit of the CRA-W, for their technical assistance in the sample preparation and analysis.

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ANNEX 1

Supplementary table 1: Sample set composition and results obtained on samples by official methods

Laboratory code: **xxxx**

Contact person e-mail: xxxxxxxxxxx

Material set:

Sample			Expected result:	Light microscopy results *			PCR result**
ID	type	Composition	Contains prohibited ruminant by-products	Sediment		Flotate	Target
				Terrest.	Fish		Ruminant
xxx	1	Bovine Paps01	/	+	-	Muscle	+
xxx	2	Milk product	/	-	-	-	+
xxx	3	Pig feed	/	-	-	-	-
xxx	4	QC (Pig feed + 5% Bovine Paps01 + 1% Milk)	/	+	-	Muscle	+
xxx	5	Pig feed + 1% Bovine Paps01	YES	+	-	Muscle	+
xxx	6	Pig feed + 1% Bovine Paps02	YES	+	-	Muscle	+
xxx	7	Pig feed + 5% Milk	NO	-	-	-	+
xxx	8	Pig feed + 1% Paps01 + 1% Milk	YES	+	-	Muscle	+
xxx	9	Pig feed (Blank)	NO	-	-	-	-

*
- in orange: analyses performed on minimum 2 replicates before the preparation of interlaboratory test.
- in blue: analyses performed on a pooled sample of 2 vials prepared for the interlaboratory test in order to obtain the 10 g of sample need for analysis.

**
- in purple: analyses performed on 3 independent vials prepared for the interlaboratory test.

ANNEX 2

Supplementary table 2.1: Results of Lab 1

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	# 5 1 % Paps01	# 6 1 % Paps02	# 7 5 % Milk	# 8 1 % Paps01 & 1 % Milk	# 9 Blank
Alpha-2-Macroglobulin	GSGGTAEHPFTVEEFVLPK	+	+	+	+	+	-
Alpha-2-Antiplasmin	LPPLSLLK	+	-	+	+	+	-
Protein HP-25 homolog 2	FGFDIELFQHAVK	+	+	+	+	+	-
Complement Component 9	YTPVEAIEK	+	+	+	+	+	-
Myosin-7	MLSSLFANYAGFDTPIEK	+	+	+	-	+	-
Osteopontin	YPDAVATWLKPDPSQK	+	+	-	+	+	-
Matrilin-1	AGGIELFAIGVGR	+	+	+	-	+	-

Supplementary table 2.2: Results of Lab 2

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	# 5 1 % Paps01	# 6 1 % Paps02	# 7 5 % Milk	# 8 1 % Paps01 & 1 % Milk	# 9 Blank
Desmin	TSGGAGGLGALR	-	-	-	-	-	-
Vimentin	TLYTSSPGGVYATR	-	-	-	-	-	-
Myoglobin	YLEFISDAIIHVLHAK	+	+	+	-	-	-

ANNEX 2

Supplementary table 2.3: Results of Lab 3

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	#5 1 % Paps01	#6 1 % Paps02	#7 5 % Milk	#8 1 % Paps01 & 1 % Milk	#9 Blank
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	+ (1/2)	+ (1/2)	-	-	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	-
	EFTPVLQADFQK	-	-	-	-	-	-
Casein alpha-S1	FFVAPFPEVFGK	+	+ (1/2)	+ (1/2)	+	+	-
	HQGLPQEVLNENLLR	+	+ (1/2)	+ (1/2)	+	+	-
Casein alpha-S2	NAVPITPTLNR	+	+ (1/2)	+ (1/2)	+	+	-
Beta-lactoglobulin	VLVLDTDYK	+	-	+ (1/2)	+	+	-
	VYVEELKPTPEGDLEILLQK	+	-	-	+	+	-

Legend: (1/2) means positive in one of the two replicates

Supplementary table 2.4: Results of Lab 4

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	#5 1 % Paps01	#6 1 % Paps02	#7 5 % Milk	#8 1 % Paps01 & 1 % Milk	#9 Blank
Haemoglobin alpha-chain	VGGHAAEYGAEALER	-	-	-	-	-	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	-	-	-	-
	EFTPVLQADFQK	-	-	-	-	-	-
	VVAGVANALAHR	-	-	-	-	-	-

ANNEX 2

Supplementary table 2.5: Results of Lab 5

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	#5 1 % Paps01	#6 1 % Paps02	#7 5 % Milk	#8 1 % Paps01 & 1 % Milk	#9 Blank
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	+	+	-	+	-
Haemoglobin beta-chain	AAVTAFWGK	+	+	+	-	+	-
	EFTPVLQADFQK	+	-	+	-	-	-
	VVAGVANALAHR	+	+	+	-	+	-
Casein alpha-S1	FFVAPFPEVFGK	+	-	+	+	+	-
	HQGLPQEVLNENLLR	+	-	-	+	+	-
	YLGYLEQLLR	+	-	-	+	+	-
Casein alpha-S2	NAVPITPTLNR	+	-	-	+	+	-
Beta-lactoglobulin	LSFNPTQLEEQCHI	+	-	-	+	+	-
	VLVLDTDYK	+	-	-	+	+	-
	VYVEELKPTPEGDLEILLQK	+	-	-	+	+	-

Supplementary table 2.6: Results of Lab 6

Protein	Peptide	QC 5 % Paps01 & 1 % Milk	#5 1 % Paps01	#6 1 % Paps02	#7 5 % Milk	#8 1 % Paps01 & 1 % Milk	#9 Blank
Haemoglobin alpha-chain	VGGHAAEYGAEALER	+	+	+	-	+	-
Haemoglobin beta-chain	AAVTAFWGK	-	-	+	-	-	-
	EFTPVLQADFQK	+	+	+	-	+	-
	VVAGVANALAHR	+	+	+	-	+	-
Collagen alpha-2 (I)	GEPGPAGAVGPAGAVGPR	+	+	-	-	+	-
Casein alpha-S2	NAVPITPTLNR	+	-	+	+	+	-
	VLVLDTDYK	+	-	-	+	+	-