Proficiency test samples by MS

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Context

Objective:

- Evaluation of the applicability of MS on common samples: peptide markers, evaluation criteria, LOD, …

Organisation:

- 5 laboratories have analysed the PT 2023 samples by MS
    - CER Groupe (Belgium)
    - Italian NRL
    - Austrian NRL
    - German NRL
    - Norwegian NRL
- Participation based on personal request
- Sample set designed for µscopy-PCR proficiency

$\Rightarrow$ MS Results received before the sending of the µscopy-PCR tabulation forms (first feedback on your results)

Instructions:

Laboratories were free to choose :

- Sample prep

- LC-MS method

- Peptide markers

- Evaluation criteria

But they have to conclude on presence/not:

- Haemoglobin

- Collagen   of ruminant origin

- Milk proteins

Sample N°	1234	5678	9101
The sample is containing **haemoglobin of ruminant origin**	●	○	○
Performed analyses did not allow to conclude about the presence of **haemoglobin of ruminant origin**	○	●	●
The sample is containing **collagen of ruminant origin**	●	○	○
Performed analyses did not allow to conclude about the presence of **collagen of ruminant origin**	○	●	●
The sample is containing **milk proteins of ruminant origin**	○	○	●
Performed analyses did not allow to conclude about the presence of **milk proteins of ruminant origin**	●	●	○
Free comments			

Sample prep

	EURL-AP	CER	Italy	Austria	Germany	Norway
Test portion	1 g			200 mg	1 g	
No Replicates	1 - 3					
Pre-treatment	/ or Sed	/				
Extraction buffer	Tris.HCl (200 mM), Urea (2 M)				7 M Urea (7 M), Thiourea (2 M)	?
Reduction agent	DTT/DTE					
Alkylation agent	IAA					
Digestion enzyme	Trypsin					
Digestion time	1 h			4 h (55°C) / O/N (37°C)	O/N	
Purification method	C18 SPE			SPE	C18 SPE	

LC-MS system

	EURL-AP	CER	Italy	Austria	Germany	Norway
LC system	UHPLC Acquity (Waters)		Exion LC (SCIEX)	UHPLC Acquity (Waters)	?	
Gradient time (min)	16			57	?	
MS System	Xevo TQ-XS	Xevo TQ-S micro	QTRAP 5500 System	Xevo G2 XS QTOF	?	?
		LRMS		HRMS	?	
Acquisition mode	Targeted: MRM/PRM					
Ionisation mode	ESI positive					

(Ruminant) Peptide markers

			EURL-AP	CER	Italy	Austria	Germany	Norway
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	x	x	x		x	x
	Haemoglobin beta	AAVTAFWGK	x	x	x	x	x	x
		EFTPVLQADFQK	x	x	x	x	x	x
		VVAGVANALAHR	x	x	x		x	
Milk	Alpha-s1 casein	FFVAPFPEVFGK	x	x				x
		HQGLPQEVLNENLLR	x	x	x	x	x	x
		YLGYLEQLLR		x		x		
	Alpha-s1 casein	NAVPITPTLNR	x	x	x		x	x
	Beta-lactoglobulin	LSFNPTQLEEQCHI	x	x	x		x	
		VLVLDTDYK	x	x	x		x	x
		VYVEELKPTPEGDLEILLQK		x		x		x
		TPEVDDEALEK				x		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	x	✗		x	x	✗
		GSTGEIGPAGPpGPpGLR	x		x		x	
		GPpGESGAAGPTGPIGSR	x		x		x	
		IGQPGAVGPAGIR	x			x		
	Prolargin	ISSVPAISSR				x		
		IEAIPSGYFK				x		
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR				x		
		AVDQLNELSSEANLYR				x		
	Alpha-2-macroglobulin	SNSFVYLEPLPR	(x)				x	
	Apolipoprotein A1	VAPLGEEFR	(x)				x	
	Serotransferin	ELPDPQESIQR	(x)				x	

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Evaluation criteria

	EURL-AP	CER	Italy	Austria	Germany	Norway
To detect and identify the peptide	RT & IR similar to ISTD/ref sample, S/N > 10 for quantifier ion		RT similar to ISTD IR similar to ref sample	at least 3 transitions with S/N ≥ 10	2 transitions with S/N >3, RT similar to ISTD	?
To conclude on the presence of the targeted by-product	≥ 2 peptides identified			≥ 1 peptide in both replicates	≥ 1 peptide in both replicates	

Need harmonisation…
=> Different criteria for LRMS & HRMS ?

Homogeneity study

- Results in term of presence/not Haemoglobin, Collagen and/or milk

- To conclude on the detection of the targeted protein: min 2 peptides / protein

- **Tabulation forms** (expected results) prepared based on the homogeneity study

Sample	Material	MS on feed				MS on sediment			
		Nr	Hb	Colla	Milk	Nr	Hb	Colla	Milk
1	Bovine feed + 0.05 % bovine PAP	5	-	+	- *	3	+	+	-
2	Sheep feed I + 1 % *Tenebrio Molitor*	5	-	-	-				
3 & 4	Sheep feed II	5	-	-	-				
5	Sheep feed II + 0.5 % Pork Blood meal + 0.1 % dog hair	5	-	-	-				
6	Sheep feed III + 0.01 % bovine PAP	5	-	-	-	3	- **	+	-

If terrestrial particle found in sediment (µscopy)

Sample 1:
* + for one BLG peptide in 5/5 rep.
(Bovine Feed : slightly + for ruminant DNA)
=> Presence of milk?

Sample 6:
** + for one Hb peptide in 2/3 rep.

Global participants results

Sample	Material	Positive results		
		Hb	**Colla**	**Milk**
1	Bovine feed + 0.05 % bovine PAP	3/5	3/3	1/5 *
2	Sheep feed I + 1 % *Tenebrio Molitor*	1/5	0/3	0/5
3 & 4	Sheep feed II	0/5	0/3	0/5
5	Sheep feed II + 0.5 % Pork Blood meal + 0.1 % dog hair	1/5	0/3	0/5
6	Sheep feed III + 0.01 % bovine PAP	3/5	2/3	0/5

* Bovine Feed : slightly + for ruminant DNA)

=> Presence of milk?

			EURL-AP		CER	Italy ✓	Austria ✓	Germany ✓	Norway
			Feed	Sed					
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	-	+	-	+		-	-
	Haemoglobin beta	AAVTAFWGK	-	+	-	+	+	-	-
		EFTPVLQADFQK	-	-	-	-	-	-	-
		VVAGVANALAHR	-	+	-	-		+	-
Milk	Alpha-s1 casein	FFVAPFPEVFGK	-	-	-				+
		HQGLPQEVLNENLLR	-	-	-	-	-	-	-
		YLGYLEQLLR			-		+		
	Alpha-s1 casein	NAVPITPTLNR	-	-	-	-	-	-	-
	Beta-lactoglobulin	LSFNPTQLEEQCHI	+	-	-	-	-	-	
		VLVLDTDYK	-	-		-		-	-
		VYVEELKPTPEGDLEILLQK			-		-		+
		TPEVDDEALEK					-		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-	-			+	-	
		GSTGEIGPAGPpGPpGLR	+	+		+		+	
		GPpGESGAAGPTGPIGSR	+	+		+		-	
		IGQPGAVGPAGIR	+	+			+		
	Prolargin	ISSVPAISSR					-		
		IEAIPSGYFK					+		
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR					-		
		AVDQLNELSSEANLYR					+		
	Alpha-2-macroglobulin	SNSFVYLEPLPR						-	
	Apolipoprotein A1	VAPLGEEFR						-	
	Serotransferin	ELPDPQESIQR						-	

FN (CER) · FN (Norway) · FP ?? 🤔

Sample 2: Sheep feed I + 1 % Tenebrio Molitor

			EURL-AP Feed	CER ✓	Italy ✓	Austria ✓	Germany ✓	Norway
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	-	-	-		-	-
	Haemoglobin beta	AAVTAFWGK	-	-	-	-	-	**+**
		EFTPVLQADFQK	-	-	-	-	-	-
		VVAGVANALAHR	-	-	-		-	
Milk	Alpha-s1 casein	FFVAPFPEVFGK	-	-				**+**
		HQGLPQEVLNENLLR	-	-	-	-	-	-
		YLGYLEQLLR		-		-		
	Alpha-s1 casein	NAVPITPTLNR	-	-	-		-	-
	Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-		-	
		VLVLDTDYK	-	-	-		-	
		VYVEELKPTPEGDLEILLQK		-		-		-
		TPEVDDEALEK				-		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-			-	-	
		GSTGEIGPAGPpGPpGLR	-		-		-	
		GPpGESGAAGPTGPIGSR	-		-		-	
		IGQPGAVGPAGIR	-			-		
	Prolargin	ISSVPAISSR				-		
		IEAIPSGYFK				**+**		
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR				-		
		AVDQLNELSSEANLYR				**+**		
	Alpha-2-macroglobulin	SNSFVYLEPLPR					-	
	Apolipoprotein A1	VAPLGEEFR					-	
	Serotransferin	ELPDPQESIQR					-	

FP ⚠

			EURL-AP Feed	CER ✓	Italy ✓	Austria ✓	Germany ✓	Norway ✓
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	-	-	-		-	-
	Haemoglobin beta	AAVTAFWGK	-	-	-	-	-	-
		EFTPVLQADFQK	-	-	-	-	-	-
		VVAGVANALAHR	-	-	-		-	
Milk	Alpha-s1 casein	FFVAPFPEVFGK	-	-				+
		HQGLPQEVLNENLLR	-	-	-	-	-	-
		YLGYLEQLLR		-		-		
	Alpha-s1 casein	NAVPITPTLNR	-	-	-		-	-
	Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-		-	
		VLVLDTDYK	-	-	-		-	
		VYVEELKPTPEGDLEILLQK		-		-		-
		TPEVDDEALEK				-		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-			-	-	
		GSTGEIGPAGPpGPpGLR	-		-		-	
		GPpGESGAAGPTGPIGSR	-		-		-	
		IGQPGAVGPAGIR	-			-		
	Prolargin	ISSVPAISSR				-		
		IEAIPSGYFK			-/+			
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR				-		
		AVDQLNELSSEANLYR				-		
	Alpha-2-macroglobulin	SNSFVYLEPLPR					-	
	Apolipoprotein A1	VAPLGEEFR					-	
	Serotransferin	ELPDPQESIQR					-	

			EURL-AP Feed	CER ✓	Italy ✓	Austria ✓	Germany	Norway ✓
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	-	-	-	-	-	-
	Haemoglobin beta	AAVTAFWGK	-	-	-	-	-	-
		EFTPVLQADFQK	-	-	-	-	-	-
		VVAGVANALAHR	-	-	-	-	+	-
Milk	Alpha-s1 casein	FFVAPFPEVFGK	-	-				+
		HQGLPQEVLNENLLR	-	-	-	-	-	-
		YLGYLEQLLR		-	-	+		
	Alpha-s1 casein	NAVPITPTLNR	-	-	-		-	-
	Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-		-	
		VLVLDTDYK	-	-	-		-	
		VYVEELKPTPEGDLEILLQK	-		-	-		
		TPEVDDEALEK				-		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-			-	-	
		GSTGEIGPAGPpGPpGLR	-		-		-	
		GPpGESGAAGPTGPIGSR	-		-		-	
		IGQPGAVGPAGIR	-			-		
	Prolargin	ISSVPAISSR				-		
		IEAIPSGYFK				-		
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR				-		
		AVDQLNELSSEANLYR				-		
	Alpha-2-macroglobulin	SNSFVYLEPLPR					-	
	Apolipoprotein A1	VAPLGEEFR					-	
	Serotransferin	ELPDPQESIQR					-	

FP

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			EURL-AP Feed	EURL-AP Sed	CER ✓	Italy ✓	Austria ✓	Germany	Norway ✓
Blood	Haemoglobin alpha	VGGHAAEYGAEALER	-	-	-	+		-	-
	Haemoglobin beta	AAVTAFWGK	-	-	-	-	-	-	+
		EFTPVLQADFQK	-	-	-	-	-	-	-
		VVAGVANALAHR	-	+	-	-	-	+	-
Milk	Alpha-s1 casein	FFVAPFPEVFGK	-	-	-				+
		HQGLPQEVLNENLLR	-	-		-	-	-	-
		YLGYLEQLLR			-		-		
	Alpha-s1 casein	NAVPITPTLNR	-	-	-	-		-	-
	Beta-lactoglobulin	LSFNPTQLEEQCHI	-	-	-	-		-	
		VLVLDTDYK	-	-	-	-		-	
		VYVEELKPTPEGDLEILLQK			-		-		-
		TPEVDDEALEK					-		
Bone/ Connective tissue	Collagen I alpha-2	GEPGPAGAVGPAGAVGPR	-	-	✗		+	- *(FN)*	✗
		GSTGEIGPAGPpGPpGLR	-	+		+		-	
		GPpGESGAAGPTGPIGSR	-	+		+		-	
		IGQPGAVGPAGIR	+	+			-		
	Prolargin	ISSVPAISSR					-		
		IEAIPSGYFK					+		
Immune/ blood cells (blood/milk)	Cathelicidin	LLELDPPPKDNEDLGTR					-		
		AVDQLNELSSEANLYR					+		
	Alpha-2-macroglobulin	SNSFVYLEPLPR						-	
	Apolipoprotein A1	VAPLGEEFR						-	
	Serotransferin	ELPDPQESIQR						-	

Conclusions

- GOOD results on very challenging samples

- Many peptides already in common (haemoglobin/milk/"connective tissue")

- Milk traces in bovine feed (=> sample 1) ? => difficult to find samples for the ≠ methods

- To be continued..

  - Interpretation criteria (LRMS? HRMS?):

    - ? 1 peptide: increase the risk of FP…

    - ? 2 peptides: increase the risk of FN
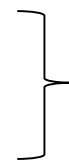
    To be evaluated at 0.1 % PAPs =>
    Proposal:
    MS ILS study (max 5 samples) by the end of 2024 ?

  - Still some problems of carry-over/contamination/ or??

    milk peptide in particular FFVAPFPEVFGK (alpha-casein)

    EURL-AP: used as carry over control, not milk marker (too sensitive)

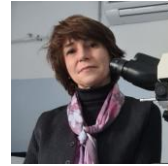 - Interest in keeping plasma peptides and immune cells (informative peptides)

Acknowledgments
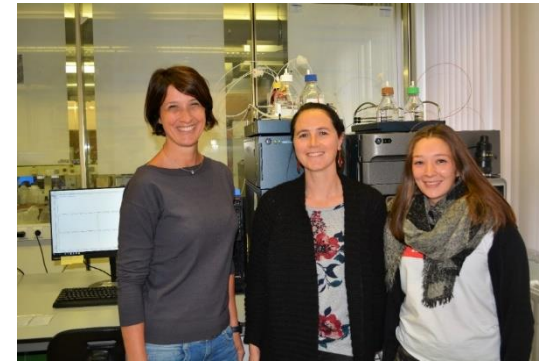
Thanks for your attention