SIGNATOPE

VALIDIERUNG EINES IMMUNOAFFINITATS LC-MS/MS ASSAYS FÜR DIE SPEZIES- UND GEWEBESPEZIFISCHE QUANTIFIZIERUNG VON VERBOTENEN VERARBEITETEN RUMINANTEN PROTEINEN IN FUTTERMITTELN

Bundesinstitut für Risikobewertung
Dr. Oliver Pötz
23-Nov-2023

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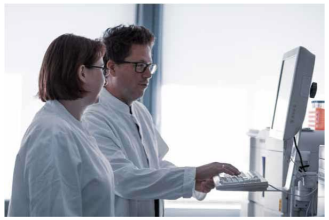
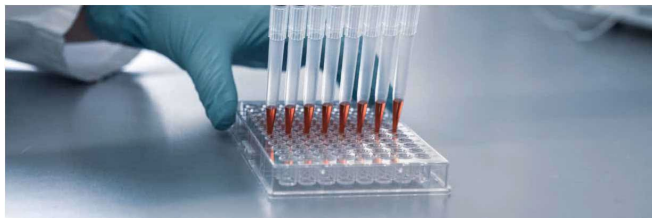
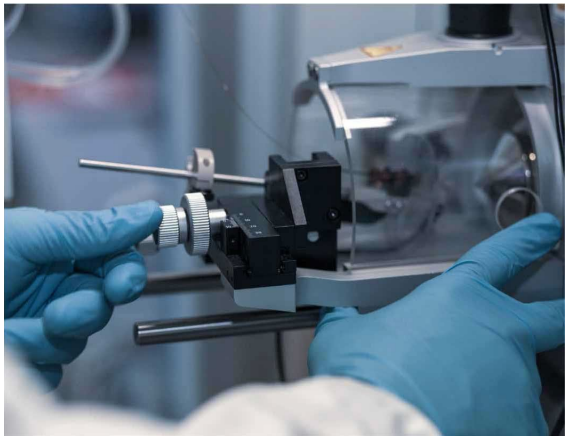
COMPANY

- Founded 2016, fifteen employees, three mass specs
- Novel immunoaffinity mass spectrometry platform for protein quantification
- Industry acceptance: Eight clients from top 20 pharma companies
- Scientific proof: More than 30 peer-reviewed publications
- Critical Path Institute: Predictive Safety Testing Consortium (PSTC) collaboration partner
- Innovation Award Baden Wuerttemberg 2019

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- Challenge – Processed Animal Protein Detection
- Technology – Immunoaffininty LC-MS/MS
- Ring Trial Samples
- Validation
- Summary & Outlook

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CHALLENGE – PROCESSED ANIMAL PROTEIN DETECTION

- Difficult samples

- Species identification

- Tissue differentiation

- Sensitive detection & quantification

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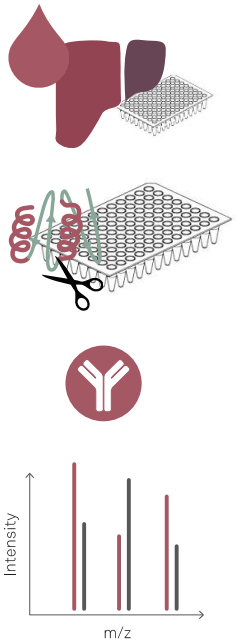
TECHNOLOGY – IMMUNOAFFINITY-LC-MS/MS (IA-LC-MS/MS)



- Sample
- Protein digest using trypsin down to peptides
- Add isotope-labelled peptide standards
- Enrich peptide standards and endogenous peptide derived from protein of interest using antibodies
- Analysis using multiplex targeted nLC-MS/MS

Quantification by ratio of endogenous peptide : internal isotope-labelled standards

Anderson, N. L. et al.. J Proteome Res (2004)
Jiang, J. et al., Proteomics Clin. Appl (2007)

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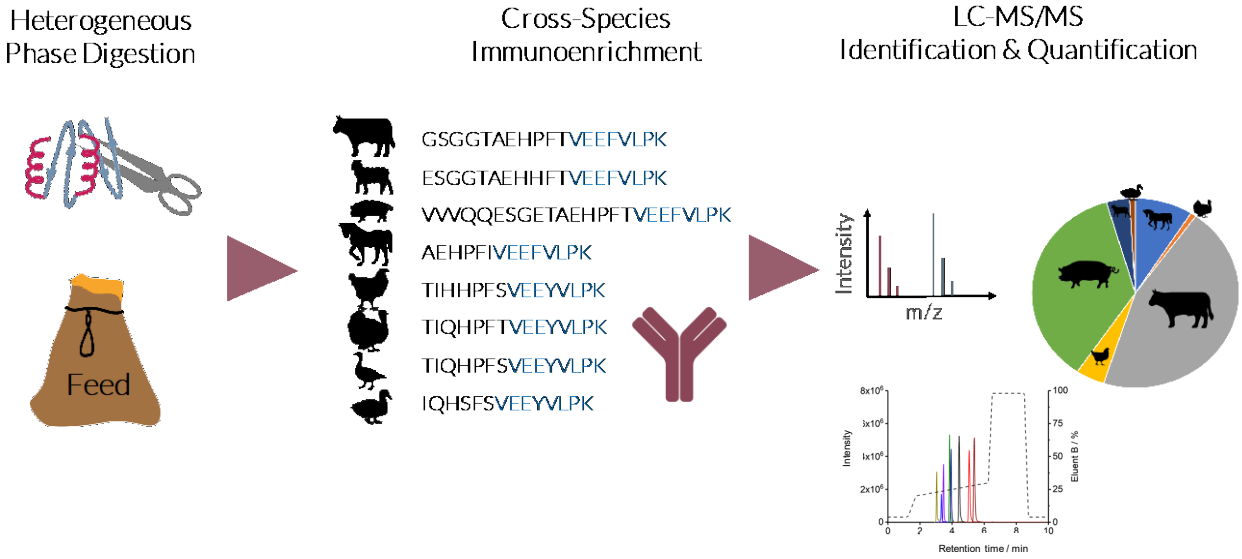
SPECIES IDENTIFICATION ASSAY



Steinhilber, A. E. *et al. J. Agric. Food Chem. 2018, 66, 39, 10327-10335*

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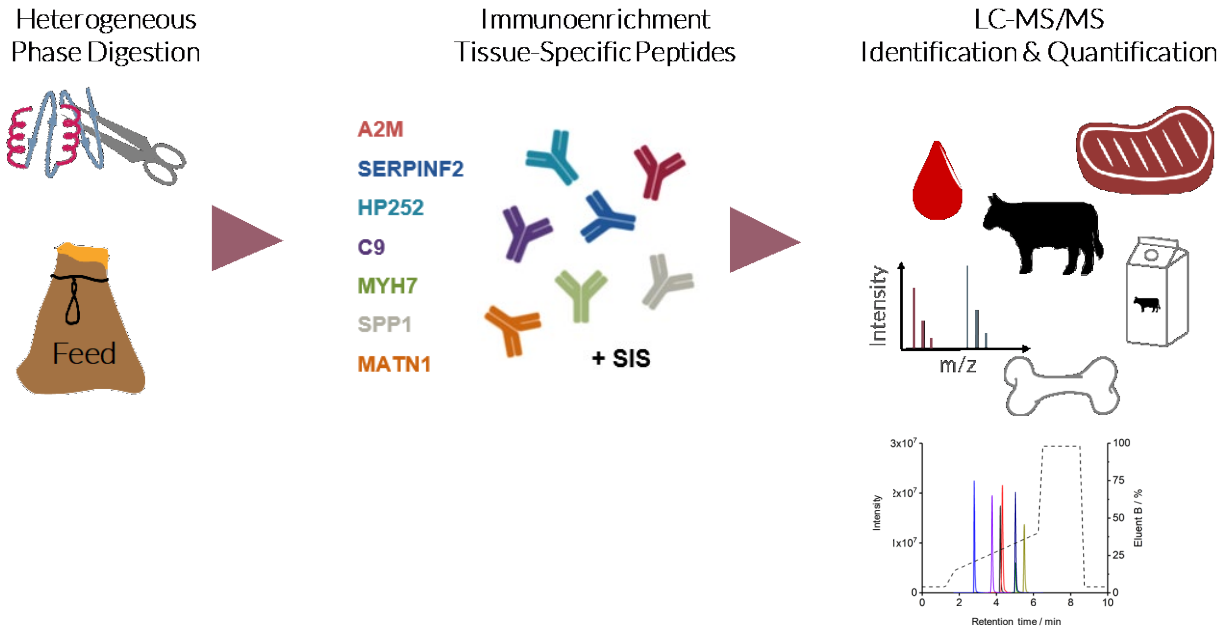
NMI schafft Ergebnisse

RUMINANT TISSUE DIFFERENTIATION ASSAY



Steinhilber, A.E. et al., *Anal Chem* (2019) **91**, 3902-3911 (2019).

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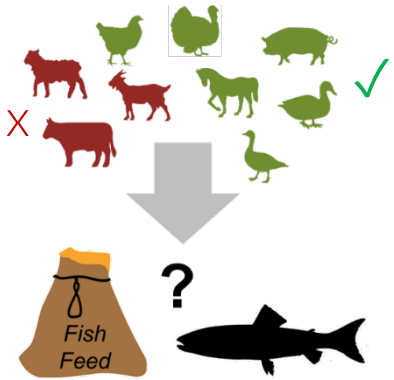
NMI schafft Ergebnisse

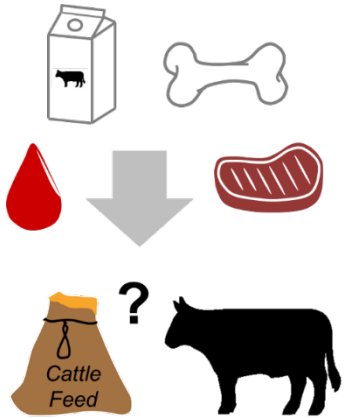
FEED ANALYSIS – A TWO-TIER APPROACH

1. Multi-Species Detection
„which species are present?“



Multiplex 1

2. Tissue-Specific Ruminant Detection
„legal or not?“



Multiplex 2

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RING TRIAL SAMPLE ANALYSIS

Ring trial samples from former proficiency tests provided by the European Reference Laboratory for Animal Proteins (EURL-AP), Gembloux, Belgium

Sample ID	Description	Additional information about the matrix
Feed 1	0.1 % Ruminant PAP	Pig feed
Feed 2	0.1 % Ruminant PAP	Pig feed
Feed 3	1 % Ruminant Blood	Trout feed, compound feed for trout farming
Feed 4	3 % Bovine Plasma	Trout feed, complete feed for fry
Feed 5	5 % Porcine Blood	Salmon feed
Feed 6	Feed containing hemoglobin meal	Fish feed

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SPECIES IDENTIFICATION TIER 1

	Species/ Tissue/ Analyte	Mean peptide amount / fmol						
		Feed 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 6	Control
Species Identification	Cattle 	4.1 ± 0.1	4.4 ± 0.4	88.0 ± 2.9	956.9 ± 32.3	0	0	0
	Sheep/Goat 	0	0	0	0	0	0	0
	Pig 	0	0	834.3 ± 29.7	0	136.8 ± 4.2	537.0 ± 4.7	0
	Horse 	0	0	0	0	0	0	0
	Turkey 	0	0	0	0	0	0	0
	Chicken 	0	0	0	0	0	0	0
	Duck 	0	0	0	0	0	0	0
	Goose 	0	0	0	0	0	0	0

→ Feed 1-4 are positive for bovine A2M and/or porcine A2M
→ Legal or illegal sources?

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TISSUE DIFFERENTIATION TIER 2

	Species/ Tissue/ Analyte	Mean peptide amount / fmol						
		Feed 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 6	Control
Ruminant tissue Differentiation	SERPINF2	0	0	2.3 ± 0.1	156.3 ± 7.0	0	0	0
	C9	0.4 ± 0.1	0.4 ± 0.05	1.2 ± 0.1	50.1 ± 2.5	0	0	0
	HP252	1.2 ± 0.2	1.4 ± 0.1	10.7 ± 0.3	331.5 ± 10.0	0	0	0
	A2M	4.5 ± 0.3	4.7 ± 0.5	94.0 ± 4.3	1065.1 ± 165.9	0	0	0
	SPP1	0	0	0	0	0	0	0
	MYH7	5.6 ± 0.4	6.7 ± 1.0	0	0	0	0	0
	MATN1	7.8 ± 1.5	14.4 ± 4.3	0	0	0	0	0

	Feed 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 6	Control
Samples	Pig Feed + 0.1 % Bovine PAP	Pig Feed + 0.1 % Bovine PAP	Trout Feed + 1 % Bovine Blood + porcine blood (!)	Trout Feed + 3 % Bovine Plasma	Salmon Feed + 5 % Porcine Blood	Fish Feed + containing Hemoglobin Meal	

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NMI schafft Ergebnisse

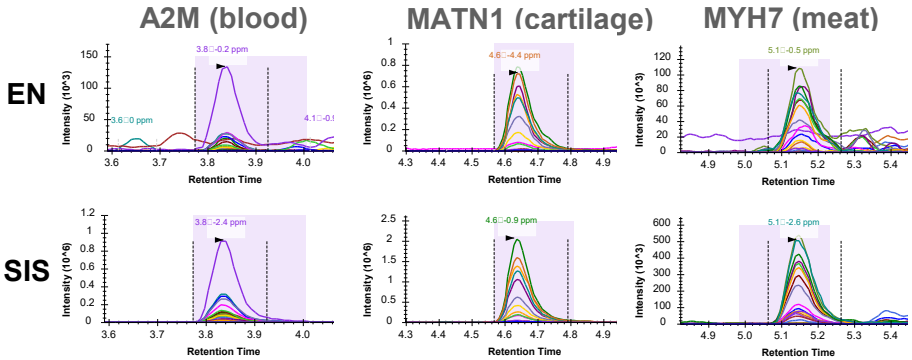
TISSUE DIFFERENTIATION TIER 2

Protein	Sequence	Tissue	Peptide amount / fmol	C.V. / %
A2M	GSGGTAEHPFTVEEFVLPK		4.7	11.4
SERPINF2	LPPLSLLK		0.0	-
C9	YTPVEAIEK		0.4	10.9
HP252	FGFDIELFQHAVK		1.4	4.4
SPP1	YPDAVATWLKPDPSQK		0.0	-
MYH7	MLSSLFANYAGFDTPIEK		6.7	14.2
MATN1	AGGIELFAIGVGR		14.4	29.4

→ Feed 2 contains ruminant blood, meat and bone proteins

→ no SPP1 detected, hence no milk

→ quantified on a level of 0.1%



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VALIDATION – LOWER LIMIT OF QUANTIFICATION

- Accuracy & Precision – calibration curves & 4 quality controls (0.1, 0.5, 1, 5 % animal protein)
- Limit of Quantification – dilution of calibrators in vegetal feed
- Carry over
- Parallelism – dilution of MBM and SDP
- Selectivity – vegetal feed
- Interference – milk powder
- Analyte stability QCs and calibrators – freeze/thaw, long-term (RT), auto sampler

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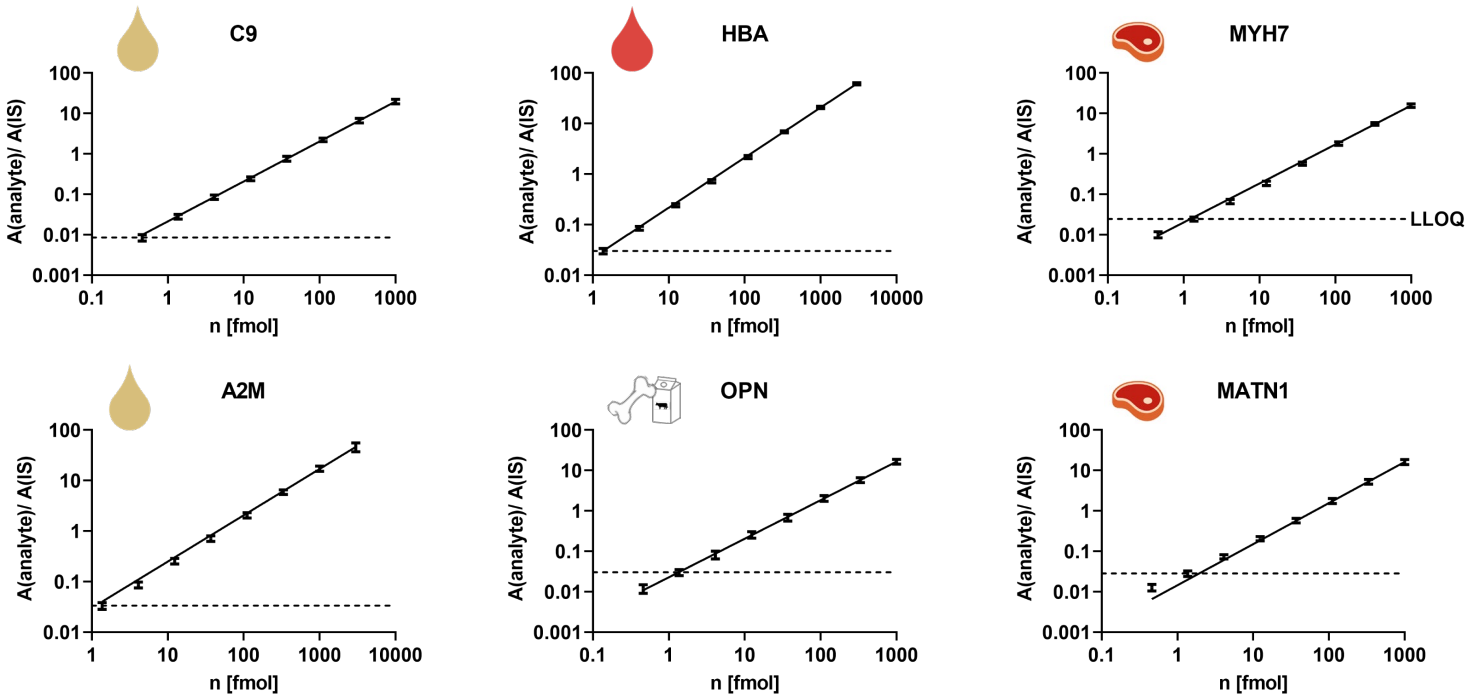
VALIDATION – LOWER LIMIT OF QUANTIFICATION



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VALIDATION – RESULTS

Parameter	Samples	A2M	C9	MATN1	MYH7	OPN	HBA
LLOQ (ng/g)	Calibrators	15.1	1.82	1.56	20.4	2.67	1.38
ULOQ (ng/g)	Calibrators	33000	3980	3420	14900	1950	3010
Inter precision	Calibrators	passed	passed	passed	passed	passed	passed
Inter accuracy	Calibrators	passed	passed	passed	passed	passed	passed
Inter precision	QC2-QC4*	passed	passed	failed	passed	passed	passed
Inter accuracy	QC2-QC4*	passed	passed	passed	passed	passed	passed
Intra precision	QC2-QC4*	passed	passed	failed	passed	passed	passed
Intra accuracy	QC2-QC4*	passed	passed	passed	passed	passed	passed
Reproducibility	contrieved samples (one digest, IP n=2)	passed	passed	passed	passed	passed	passed
	contrieved samples (digest, n=2)	passed	passed	failed	passed	failed	failed

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VALIDATION – RESULTS

Parameter	Samples	A2M	C9	MATN1	MYH7	OPN	HBA
Selectivity	spiked samples / unspiked samples	passed	failed	failed	passed	failed	passed
Parallelism	commercial/artifical samples, serially diluted	passed	passed	failed	passed	passed	passed
Carryover	BS1-BS8	>S2	>S2	>S4	>S3	passed	passed
Interference	spiked samples, spiked milk powder	failed*	failed*	failed*	Up to 60 %	failed*	Up to 10%
Freeze-thaw stability	Proteolyzed sample, 1x-3x	failed	3x	3x	3x	3x	3x
	calibrators, 1x-3x	passed	passed	passed	passed	passed	passed

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VALIDATION – SUMMARY

- Accuracy & Precision: 0.1% animal protein QC1 failed the quantitative acceptance criteria but not the qualitative acceptance criteria.
- Limit of quantification: Method sensitive to detect PAP down to 0.1%.
- Reproducibility: Technical measurement passed acceptance criteria, but 3 out of 6 assays failed most probably due to sample homogeneity.
- Carry over: Samples with content > S3 must be re-run with additional blank run
- Parallelism: Passed, but not MATN assay.
- Selectivity: A2M, MHY7 and HBA assays met criteria.
- Interference: Only MHY7 and HBA assays met criteria, milk powder contains OPN, C9, A2M & MATN.
- Analyte, QCs and calibrators: Long-term stability confirmed for up to 30 months.
- Reagent stability: Confirmed for up to 30 months.

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SUMMARY & OUTLOOK

- Method sensitive to detect PAP down to 0.1% & to quantify down to 0.5%
- Sampling process requires optimization, sample size needs to be determined to achieve statistical robust result
- Interference: MHY7 tolerated up to 60% milk powder

- Reagent stability: Confirmed for up to 30 months

- Ring trial in progress

  Dr. Uta Herfurth German Federal Institute of Risk Assessment, Germany
  Prof. Rene Zahedi, University of Manitoba, Canada
  Dr. Ikram Belgit, Institute of Marine Research, Norway
  Dr. Marie Lecrenier, Wallon Agriculture Research Centre, Belgium
  Prof. Jens Brockmeyer, Universität Stuttgart, Germany

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