Immunoaffinity-Based Mass Spectrometry for the Species Identification and Quantification of Processed Animal Proteins in Feed

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Abbreviations

A280	absorption at a wavelength of 280 nm
Abs	absorption units in 1 cm pathlength equivalents
AGC	automatic gain control
BM	blood meal
BSE	bovine spongiform encephalopathy
C.V.	coefficient of variation
CID	collision induced dissociation
dd	data dependent
DDA	data dependent analysis
ESI	electrospray ionization
FCR	feed conversion ratio
FF	fish feed
FM	fish meal
HCD	higher energy collision induced dissociation
HPD	heterogeneous phase digestion
HRAM	high resolution and accurate mass
IS	stable isotope labeled internal standard peptide
ISD	in-solution digestion
LC	liquid chromatography
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
MALDI	matrix assisted laser desorption ionization
MBM	meat and bone meal
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
msx	spectral multiplexing

n/a	not analyzed
NCE	normalized collision energy
NIR	near infrared
NIRM	near-infrared microscopy
NIRS	near-infrared spectroscopy
PAGE	polyacrylamide gel electrophoresis
PAP	processed animal protein
PCR	polymerase chain reaction
PRM	parallel reaction monitoring
p*X*	porcine *X*
Q	quadrupole
RQ	multiplex assay for ruminant quantification
r*X*	ruminant *X*
S/N	signal to noise ratio
SD	standard deviation
SDHM	spray-dried hemoglobin meal
SDP	spray-dried plasma
SIM	selected ion monitoring
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
SRM	selected reaction monitoring
TOF	time of flight
TSE	transmissible spongiform encephalopathy
TXP	triple-X-proteomics
UHPLC	ultra-high-performance liquid chromatography
UV	ultraviolet
v/v	volume fraction (volume per volume)
vCJD	new variant Kreutzfeldt Jakob Disease
VF	vegetal feed
w/w	mass fraction (weight per weight)
XA2M	cross-species multiplex assay addressing alpha-2-macroglobulin
XIC	extracted ion chromatogram

1 Introduction

1.1 Feed Sustainability

Animal feed sustainability is one of the biggest challenges for the next decades. The world population is constantly growing with an average annual rate of 1-2% and the 10 billion people mark is estimated to be reached until the year 2060 [1]. This growth goes hand in hand with a higher demand for human food and thus animal feed due to an intensification of animal farming. In an era of limited resources, the responsible use of nutrients is crucial to the future food and feed supply.

The expansion of aquaculture, the world's fastest growing food production sector with an average annual growth rate of 8-10% since 1970, was accompanied by a rapid increase in fish feed production [2,3]. The prices for fish oil and fish meal, the most nutritious and digestible ingredients in fish feed, were pushed to historic heights in late 2014 [3]. Also the feed efficiency, expressed by the feed conversion ratio (FCR), was heavily criticized since around 5 kg wild fish are needed to produce 1 kg of carnivorous fish [4]. In this context, cheap and sustainable feed ingredients to substitute expensive fish oil and fish meal gained attention [5]. In the livestock industry, strict regulations concerning the use of animal byproducts as feed additives have also driven the use of alternative, mainly plant-derived, feed ingredients. However, the use of sustainable plant proteins was reported to be limited for several reasons in both livestock [6] and aquaculture industry [7].

Although the bovine spongiform encephalopathy (BSE) crisis has highlighted the risk of their use, animal byproducts can be regarded as valuable nutrient resources. Reutilization of animal byproducts in feed considerably contributes to the goal of keeping nutrients, within the nutrient cycle and dealing responsible with limited resources [6,8].

1.1.1 Animal Byproducts

Every year around 360 million pigs, sheep, goats and cattle as well as more than 6 billion poultry are killed in European slaughterhouses for the purpose of human food production [9]. The meat production sector produced a total of 46.4 million tons of carcass weights in the year 2016 [10]. However, significant amounts of the animal's live weight cannot be used for food production. There are also byproducts of around 25% for chicken, 34% for pigs and even 42% for cattle [9]. As a consequence, more than 20 million tons of animal

byproducts annually emerge from European slaughterhouses, plants producing food for human consumption, dairies and fallen stock from farms [10].

Animal byproducts are not waste, but rather valuable resources for fat, proteins, minerals and even essential vitamins and therefore they can be considered to improve the nutritional value of animal feed [8]. Further fields of utilization are feed additives for fur animals, food for pets, products in oleochemistry, fertilizers and combustibles (Figure 1). It has to be mentioned that the utilization of animal byproducts depends on a risk classification introduced in 2002 by Regulation (EC) No 1774/2002 later amended by Regulation (EC) No. 1069/2009 as a consequence of the BSE crisis [11,12] (see 1.1.3).

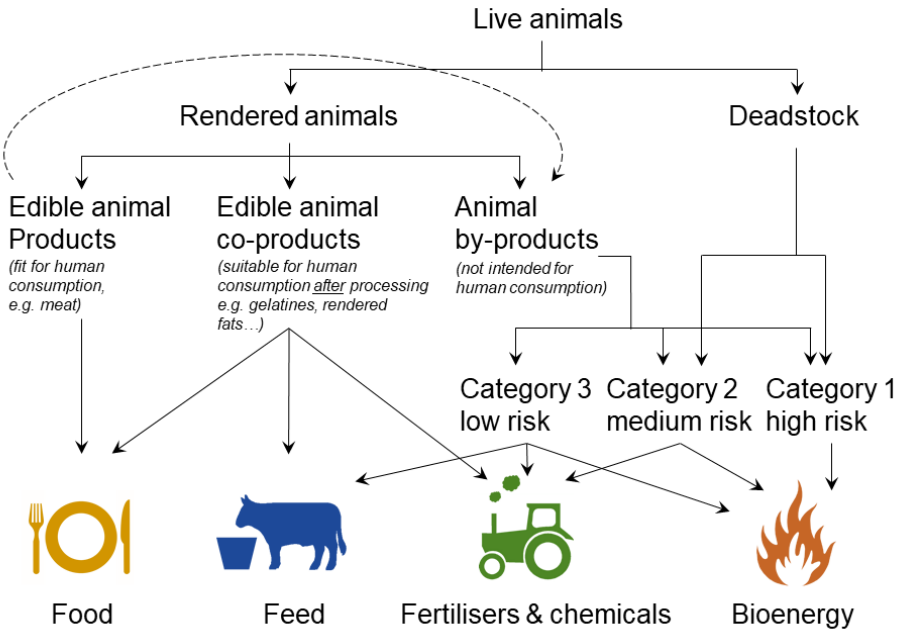

Figure 1. Production flow and fields of utilization of animal products and byproducts modified from Lecrenier [13]. Only animal byproducts of category 3 may be used for feeding purposes. Deadstock and animals with non-classical diseases like transmissible spongiform encephalopathies are not allowed to re-enter the food chain via animal feed (categories 1 and 2).

Category 1 comprises specified risk material linked to non-classical diseases like BSE and scrapie. These products are only allowed to be used as combustibles, which safely disposes the high-risk materials and serves for energy generation. Category 2 comprises medium risk material, for example byproducts from animals that died other than by being slaughtered for human consumption such as fallen stock on farms. Same as category 1, these materials are not allowed to enter the food chain via utilization in animal feed for feed and food safety reasons. Possible fields of utilization are fertilizers or products in the

chemical industry. Category 3 comprises byproducts from animals that are slaughtered for the production of human food, however are not intended for human consumption for commercial reasons. These materials are the ones that are allowed to re-enter the food chain by utilization as protein additives in animal feed. The amount of category 3 material is estimated to be around 12 million tons out of the 20 million tons of animal byproducts annually emerging in the European Union [9].

1.1.2 Processed Animal Proteins

Animal byproducts can contain moisture, fatty tissue, bones, offal, and even entire carcasses of animals from slaughterhouses and animals that died on farm. The process of animal rendering converts them into more stable and usable materials. During this process the animal byproducts are simultaneously dried, crushed into smaller pieces and finally ground to powder. Heat and pressure are applied to sterilize and stabilize the materials. The dry materials are free from harmful microorganisms thus eliminating the risk for diseases and are stable for storage and further reprocessing. The rendering process also separates the fatty part from the bone and protein part yielding two fractions, the rendered animal fats and the so-called processed animal proteins (PAP) [8].

One type of PAP is meat and bone meal (MBM), which typically has a crude protein content of around 48-56% [14,15]. Mammalian MBM improve the nutritional profile of feed for monogastric animals like chicken or pigs [8,16,17]. Poultry MBM effectively substitutes expensive fish meal in aquaculture feed [18,19] or plant proteins [20]. PAPs do not contain anti-nutrients, which are often present in plant-derived products limiting their use as feed supplements [7,21]. Fish meal (FM) produced from water animals excluding mammals is a separately defined type of PAP that is still used in aquaculture and in feed for pig and poultry animals [22].

Blood is another important animal byproduct obtained during slaughter. The global amount of blood emerging from slaughterhouses is roughly estimated with 4.56 billion liters per year [23]. While blood is only poorly used for the production of human food, there is a great interest in blood for animal feed production. Slaughter blood is part of the category 3 materials and is commonly used in their processed form of blood meals (BM). BM is representing a special type of PAP, which is also increasingly used to substitute expensive fish meal in aquaculture feed [8]. The crude protein content of BM reaches 90% surpas-

sing MBM and plant derived protein meals [24]. Not only BM, but also lower processed products such as spray-dried hemoglobin meal (SDHM) and spray-dried plasma (SDP), in the sense of the Regulation (EU) No. 142/2011 [22], are increasingly used since they show a good amino acid balance and a very high digestibility [25,26].

To ensure food safety, PAPs have to be processed under defined conditions before they are allowed to be used as additives in animal feed. The minimum processing conditions for mammalian derived PAPs are 133°C, 3 bar, 20 min [22]. As an exemption, porcine blood meals and PAPs from poultry have to be treated at 80°C for 120 min in order to eliminate pathogenic microorganisms. According to the law, SDHM and SDP are defined as "blood products", a separate category in contrast to PAP. Therefore, they can be treated like porcine BM at 80°C for 120 min.

1.1.3 Feed Ban

The use of PAPs as additives in animal feed was a common procedure for several years until the outbreak of the bovine spongiform encephalopathy (BSE) in 1986 in the United Kingdom (UK). BSE is the bovine variant of the transmissible spongiform encephalopathies (TSE) which affect animals including humans. TSE constitute a group of infectious, transmissible neurodegenerative diseases of the central nervous system caused by misfolded proteins, so-called prions [27]. By protein aggregation, prions cause thread-like depositions and sponge-like tiny holes in the cortex giving the disease its name. After a very long incubation time of several months up to years, the disease progressively destroys the brain, causes mental and motoric disorders and finally always leads to death [28].

The transmission of prions takes place via the consumption of contaminated food. After oral intake, prions enter the enteral nervous system via the intestinal epithelium and subsequently spread into the brain via a neuronal pathway [29,30]. Epidemiological studies came to the conclusion that BSE was spread by infectious cattle feed. In the UK, over 180 000 infected cattle were reported during the crisis and 4.4 million precautionary slaughters took place during the eradication program [31]. The human counterpart to BSE is known as new variant Creutzfeltd-Jakob disease (vCJD) [32]. It was first identified in 1996 in the UK and is was shown to be related to BSE [33]. Until today, 177 people were killed by vCJD in the UK and 52 elsewhere, primarily in Western European countries [34,35]. The European Union took several measures to get the control over the BSE pandemic. The most

important measure was the ban for mammalian derived proteins in animal feed introduced with Commission Regulation (EC) No. 999/2001 and later expanded by amendment No. 1234/2003 [36,37]. The regulation prohibited the feeding of PAPs, such as MBM, to animals that are intended for human consumption. Prion-contaminated cattle MBM, which was supposed as the main vector of disease, was successfully excluded from animal feed which has led to a decreasing number of registered BSE cases nearly down to zero in Europe including the UK, the former hot spot of the BSE pandemic (Figure 2) [31,38]. The few cases still occurring today are most likely spontaneous prion diseases that are not caused by an infection.

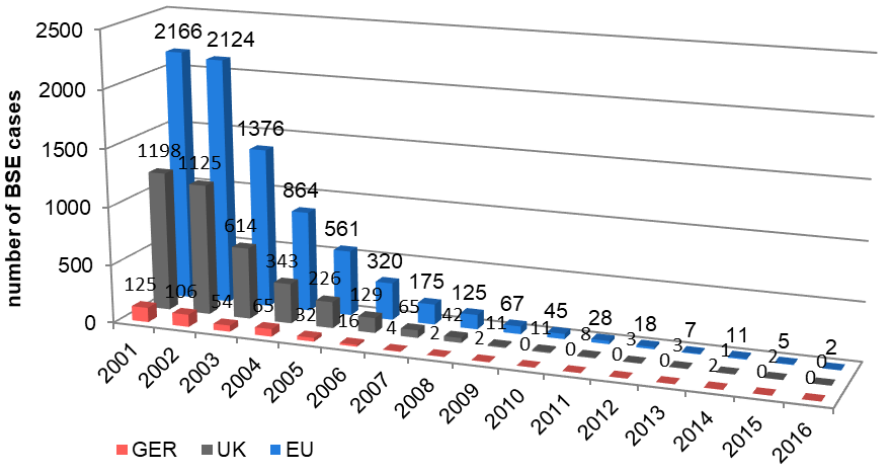

Figure 2. Number of registered cases of bovine spongiform encephalopathy (BSE) in Germany (GER), the United Kingdom (UK) and the European Union (EU) since 2001 reported by the World Organization for Animal Health [31].

1.1.4 Progressive Reauthorization

Today, the pandemic seems to be almost eradicated and a transmission of BSE between non-ruminants is considered unlikely. Nevertheless, the use of animal proteins in feed remains strictly regulated [39]. Except milk and egg products, bovine material is not allowed for feeding purposes, neither in non-ruminant feed nor in aquaculture. The feeding of non-ruminant PAPs to ruminant animals is also prohibited (Table 1). In consideration of a sustainable feed chain the European Commission is currently working on the reintroduction of non-ruminant PAPs in feed without by-passing the ban for intraspecies feeding [11]. The first steps were done with the allowance of fish meal in milk replacer for weaning calves in 2008 [40] and for non-ruminant PAPs in feed for aquaculture in 2013 [41] amended by certain insects in 2017 [42]. Future EU plans comprise legalizing PAPs from pig and poultry origin for use in non-ruminant feed [39].

Table 1. Current legislation concerning the use of PAPs as feed additives. Table modified after an internal strategy paper from the European Commission [39]. A = allowed; P = prohibited.

Product	Ruminant feed	Non-ruminant feed	Fish feed
Ruminant meat and bone meal	P	P	P
Ruminant blood meal	P	P	P
Ruminant blood products	P	P	P
Ruminant gelatin or hydrolyzed proteins	P	P	P
Egg and milk products	A	A	A
Fishmeal	P	A	A
Porcine / poultry meat and bone meal	P	P	A
Porcine / poultry blood meal	P	P	A
Porcine / poultry blood products	P	A	A
Porcine / poultry gelatin or hydrolyzed proteins	A	A	A
Animal proteins other than mentioned above	P	A	A
Insect protein (seven defined species)	P	P	A

The progressive lifting of the feed ban demands for analytical methods with high sensitivity and specificity to prove the origin of PAPs and to ensure the absence of ruminant material. In this regard, the risk assessment as well as the analytical methodology should cope with the real-life situation in the production chain. Minor cross contamination due to allowed animal proteins (e.g., derived from milk powder) or transport between slaughter houses, rendering plants, and feed producers can never be ruled out. Therefore, producers, represented by the European Fat Processors and Renderers Association (EFPRA), but also control laboratories call for quantitative accurate thresholds to replace the pending zero-tolerance-concept. However, any decision on threshold levels of the European Commission will depend on a thorough risk assessment. A risk study of the European Food Safety Authority (EFSA) in the year 2011 concludes that a contamination level of 0.1% of non-ruminant PAP in feed would lead to less than one BSE infected cattle in the EU per year with an upper 95% confidence level [43]. EFSA currently works on an updated risk assessment as well as the implementation of a threshold ("technical zero") for minor ruminant cross contamination on demand of the European Commission [44]. If and at which tolerance level final thresholds for ruminant and non-ruminant PAPs will be implemented is not clear yet, but it can be strongly assumed that quantitative methods will be required with a sensitivity in the range of <1–2% PAP (w/w) [45].

1.2 Feed Authentication

The ban of PAPs in feed introduced in 2001 was one of the main EU measures to control the BSE pandemic. To enforce the ban, analytical methods have been implemented that prove the absence of illegal PAPs. Currently, light microscopy and polymerase chain reaction (PCR) are the official methods for the detection of illegal PAPs in feed. However, the changes in legislation due to the progressive reauthorization, required the development of alternative methods to quantitatively determine the exact origin of animal proteins in feed. The focus in the development of alternative methods is on spectroscopic methods, immunoassays and mass spectrometry. Current developments concerning the official methods and alternative methods are highlighted in the following.

1.2.1 Official Analytical Methods

In 1998 optical light microscopy was implemented as the first official method for PAP detection and characterization in feed [46]. The initial method was implemented to enforce the feed ban and was further developed over the past years [45]. The method relies on the morphological detection of particles such muscle fibers, cartilage, bones, hair or feathers. The technique is very sensitive with a limit of detection (LOD) of <0.1% PAP in a feed compound [47]. However, the method is neither able to differentiate species nor it can differentiate visually not classifiable material such as powders. This led to the adoption of PCR as second official method in 2013 [48]. Based on the analysis of DNA, the PCR method is able to reliably determine the species origin on a level of 0.05% PAP in feed [49], even in visually not classifiable material. Despite the advantages, DNA-based methods suffer from significant drawbacks. Since the genomic information does only differ between species but not between tissues of the same species, a tissue-specific PAP differentiation remains impossible. As an example, the current legislation allows the feeding of bovine milk and egg products; however, the feeding of bovine blood and MBM is illegal. A differentiation of legal and illegal protein additives cannot be accomplished by the current official PCR method [50]. Another drawback is linked to the fact that mammalian-derived PAPs have to be treated at 133°C, 3 bar, 20 min in order to be used as feed additives [22]. At these harsh conditions DNA sequences tend to degrade and therefore the PCR analysis can be affected [51]. It also has to be mentioned that both, the light microscopy and the PCR method are not primarily focused on a quantification but more on a qualitative detection of PAPs.

To summarize, the official methods light microscopy and PCR show a highly sensitive detection of illegal PAPs in feed on a level <0.1% however, they are limited in their species and tissue differentiation as well as quantification capability.

1.2.2 Alternative Methods

The official method light microscopy was further developed and optimized. To become more independent from experienced operators and to increase sensitivity, spectroscopic methods were introduced [45]. Near-infrared spectroscopic methods were used either in combination with the official microscopy method (NIRM) [52] or as standalone method (NIRS) [53]. The NIRM protocol is the same as it is for the official light microscopy but instead of visually evaluating the particles, characteristic NIR spectra of thousands of particles are acquired. The throughput of this method was further increased by the use of a NIR imaging system to parallelly analyze particles [54]. Since NIRM is based on the protocol of the microscopic method, a detection of <0.1% PAP is possible. Standalone NIRS is a non-destructive method that can be used as on-line control directly in feed production plants detecting accidental contaminations within the production chain [55,56]. However, the detection limit of >1% is too high for an analysis in official control laboratories [45,56]. The differentiation of terrestrial and fish PAPs can be accomplished by these methods [55]. Although results indicated that even a differentiation of terrestrial species is possible, there are possible overlaps in the NIR spectra between different species and a safe PAP differentiation is not guaranteed [57].

Immunoassays were recognized as a powerful tool for PAP analysis since certain proteins can be used to trace back the species as well as the tissue origin. Several immunological methods addressing heat-stable PAP fractions were developed [58-62]. In principal, immunoassays are highly sensitive and quantitative. The latter is important since there are plans for the introduction of quantitative accurate thresholds, replacing the pending zero-tolerance-concept of PAPs in feed. However, immunoassays are also affected by the harsh rendering conditions leading to protein denaturation and partial fragmentation reactions [63]. One commercial assay kit (MELISA-TEK), which is usually used for meat analysis, was applied to PAP detection by Bremer and colleagues [64]. In an interlaboratory study this kit showed a detection limit of 0.5% ruminant PAP in non-ruminant PAP. The only immunoassays that was able to detect 0.1% bovine MBM in vegetal cattle feed showed a cross reactivity to porcine material [65]. A developed immunoassay by Kim and colleagues

showed cross reactivities to porcine gelatin, canola and wheat material [60]. A completely different approach that faces the issue of protein fragmentation was published by Huet and colleagues [66]. The group developed competitive immunoassays using antibodies raised against tryptic peptides which are not influenced by high temperature and pressure. One of the immunoassays was able to detect 2% bovine MBM in feed. Although the so far developed immunoassays are promising, immunoassays are always prone to interferences and cross reactivities to either other species or ingredients in the complex feed matrix limiting the sensitivity.

Mass spectrometry is another very powerful tool for the sensitive and specific analysis of proteins. A mass spectrometric analysis can be either performed on the protein's intact level (top down) or on the level of peptides after enzymatic fragmentation (bottom up). In feed analysis, only the bottom up approach is used since protein denaturation and fragmentation prevent an intact protein analysis.

1.3 Protein Analysis by Mass Spectrometry

In the past decade, the mass spectrometric (MS) analysis of tryptic peptides as protein surrogates has emerged as a powerful tool in the field of proteomics [67-69]. This analysis is based on an enzymatic fragmentation of complex biological samples into peptides. These peptide mixtures are subsequently separated by liquid chromatography coupled to a tandem mass spectrometric detection (LC-MS/MS) for the identification of peptides and indirectly proteins. Depending on the type of instrument different experiments can be performed to obtain both qualitative and quantitative information about the proteins present in biological samples. Usually, LC separations are connected via an electrospray ionization (ESI) source to a tandem MS instrument that mostly has a quadrupole (Q) mass-to-charge filter for a first stage MS selection coupled to a mass analyzer, mostly time of flight (Q-TOF), additional quadrupoles (QQQ) or an orbitrap mass analyzer (Q-Orbitrap).

Non-targeted MS detection is commonly used to profile the protein content of a complex biological digest and to qualitatively identify possible markers of diagnostic or therapeutic relevance [70]. The most common non-targeted MS approach is the data dependent acquisition (DDA) which acquires full scan MS spectra in precursor level during chromatographic elution of the peptides and then isolates the most intense peptide precursor mass-to-charge ratios (usually top 10) for a further fragmentation step (Full-MS/ddMS^2). The

characteristic fragment ion spectra produced by collisional induced dissociation (CID) in a collision cell, which is located between mass filter and mass analyzer, can be assigned to peptide sequences by comparison to spectra databases.

Quantitative approaches are targeted MS methods like selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) that allow a highly sensitive and specific detection of selected peptides [71,72]. Tandem MS instruments using quadrupoles as mass-to-charge filters provide a very high sensitivity since background interferences are greatly reduced during analyte isolation. The indirect detection of precursor ions via their fragment ions in the mass analyzer allows for an unambiguous peptide identification by their characteristic mass transitions. A precise and accurate quantification in targeted MS methods can be achieved by the use of stable isotope labeled standard peptides that are spiked into the samples prior LC-MS/MS analysis. The peptides with same sequence and physicochemical properties elute at the same time together with the analyte peptides, however the mass analyzer is able to discriminate the masses. Peptide concentrations can be calculated by referencing the analyte peptide signal to the internal standard signal of known amount [72-74].

1.3.1 Methods for Food and Feed Analysis

Because of their great success in the field of proteomics, MS methods have been quickly adopted in related fields like food chemistry especially in meat science [75-77]. Targeted MS methods for the species identification in both raw [78-81] and cooked [82-84] meat samples were developed. Recently, also a non-targeted MS approach combined with spectral library matching to differentiate species in meat products was reported [85]. In the field of animal feed authentication, MS methods gained attention as well. The BSE crisis has driven the development of MS methods for the species differentiation in processed MBM [86-88]. Non-targeted MS combined with spectral matching was recently adapted to the field of feed authentication by Rasinger and colleagues [89]. The species differentiation in both cooked meat and MBM samples proves the suitability of peptide-centric shotgun MS for the analysis of processed and denatured proteins and pose MS as a superior method compared to immunological methods.

Currently, the focus in the feed sector is on the development of targeted MS methods to detect illegal PAPs in animal feeds that provide species and tissue specificity, high sensitivity as well as the capability for quantification. Heat-stable species- and tissue-specific

marker peptides were identified in non-targeted MS studies of PAPs and blood meals [13,89,90]. First targeted MS methods for the detection of banned ruminant PAPs were established. One targeted assay was developed to simultaneously detect bovine blood and milk proteins, providing a tissue differentiation of legal and illegal feed additives such as blood meals and milk powder [91]. In this assay, blood meal was detected with a sensitivity reaching the 0.1% (w/w) limit, matching the legal requirement for analytical methods imposed by the European Commission. Another targeted assay for the detection of banned ruminant MBM was reported [90]. This assay addresses three bovine peptides derived from the two proteins hemoglobin α and heat shock protein β-1 and allows the detection of banned ruminant MBM in vegetal feed with a limit of detection of 5% (w/w). However, the state of the art MS methods for feed authentication fulfill only some of the requirements imposed by the European Commission. Until now, no method was reported that simultaneously fulfills the following requirements:

- Detection of PAPs like MBM and BM
- Detection of blood products like SDHM and SDP
- Differentiation of PAPs, blood products and milk products
- Detection limit of <0.1% (w/w) for bovine MBM, the most critical type of PAP
- Quantification <1-2% (w/w)

Further developments in targeted MS methods for an improved detection of PAPs are very likely. Isotope labeled standard peptides were already introduced to provide the capability for quantification [91]. A simultaneously detection of different PAP and blood product types could be achieved if appropriate markers were selected. The remaining challenge is to combine the previous achievements with a sensitive detection on a level of 0.1% (w/w) PAP in feed.

1.3.2 Immunoaffinity-Based Targeted Mass Spectrometry

One approach to improve throughput and sensitivity of targeted MS assays are hybrid methods that combine immunoprecipitation with MS detection. Such hybrid methods have been established on both MALDI-MS and ESI-MS platforms [92,93]. Immunoaffinity-based MS assays are used in clinical and pharmaceutical research for the quantification of receptors [94], kinases [95], drug-metabolizing enzymes [96] and plasma proteins [97]. Similar to sandwich immunoassays, the peptides or proteins of interest are captured by antibodies

that are immobilized on a stationary phase. However, instead of using a second antibody for the detection, the analytes are eluted and analyzed by LC-MS/MS. This workflow was termed stable isotope standards and capture by anti-peptide antibodies (SISCAPA) [92]. Applying SISCAPA, a peptide enrichment of at least two and up to four orders of magnitude compared to pure targeted MS analysis was observed [92,98]. An advantage over immunoassays is the capability to combine antibodies in multiplex assays since the MS detection provides an absolute specificity and is not affected by antibody cross reactivities [97,98].
The disadvantage of generating one antibody per analyte was faced by the application of group-specific enrichment strategies. One example for a group-specific peptide enrichment strategy is the concept of triple X proteomics (TXP) [99-102]. This concept uses antibodies that recognize short C-terminal peptide sequences comprising three amino acids plus the terminal arginine or lysine present in tryptic peptides. A group-specific enrichment can be performed for peptides that share a specific TXP motif. The TXP approach greatly reduces the number of antibodies that are necessary to enrich a large number of peptides [103]. The short epitopes are ideally suited to enrich peptides from homologous proteins of different species or protein families [104].

2 Aim of the Thesis

The aim of this thesis is to provide a new analytical method for an improved detection of banned processed animal proteins (PAP) in feed compounds. There are several requirements regarding analytical methods that need to be fulfilled in order to be applied in future feed authentication studies. These requirements are mainly the species and tissue specificity, the sensitivity of ≤0.1% (w/w) PAP or blood product in feed and the ability for quantification on a level of <1-2% (w/w). An overview of the current achievements in state of the art methods is given in Table 2.

The overall objective of this work is the introduction of immunoaffinity-based mass spectrometry to feed analysis in order to face the current limitations in PAP detection. The developed workflow should comprise an improved sample preparation for the release of peptides, an immunoaffinity enrichment of the peptides, and a peptide identification and quantification by LC-MS/MS using stable isotope labeled standards (Figure 3).

The first objective is to improve the peptide release from highly processed protein samples in order to achieve a maximum analyte amount for the following immuno-MS analysis facilitating a detection below the required detection limit of 0.1% (w/w).

The second objective is to apply the improved sample preparation to PAPs and blood products from different species and to identify possible marker peptides by non-targeted mass spectrometric experiments. The identified markers have to be properly selected by help of bioinformatics to achieve species as well as a tissue specificity.

The third objective is to generate marker-specific polyclonal antibodies and to compile them in multiplex immuno-MS assays. The developed assays should be applicable to different species and tissue types in the common feed matrices. One idea is to multiplex peptide-specific antibodies that address bovine tissue-specific marker in order to provide a highly sensitive detection and differentiation of PAPs and blood products from milk powders. Another idea is to apply the concept of a group-specific immunoenrichment using only one cross-species antibody for the detection and differentiation of the main livestock species, cattle, sheep, goat, pig, horse, turkey, chicken, duck and goose. The need for quantification should be fulfilled by the use of stable isotope labeled standard peptides as internal standards in both multiplex assays.

Quantitative multiplex immuno-MS assays should be developed, validated and finally applied to feed compounds for an unambiguous species- and tissue-specific detection of PAPs and blood products on a level of ≤0.1% (w/w).

Table 2. Comparison of state of the art methods and alternative methods for feed authentication.

Method	State of the Art				Aim
	Microscopy	PCR	Immuno-assays	Mass Spectrometry	Immunoaffinity-Based Mass Spectrometry
Detection Limit	0.1%	0.05%	0.5%	5% MBM <0.1% blood	0.1% for all sample types
Quantification	no	yes	yes	yes	yes
Tissue Specificity	no	no	yes	partially	yes
Species Specificity	none	high	medium – high (cross reactivities)	high	high
Time per sample	180 min	300 min	60 min	60 min	15 min

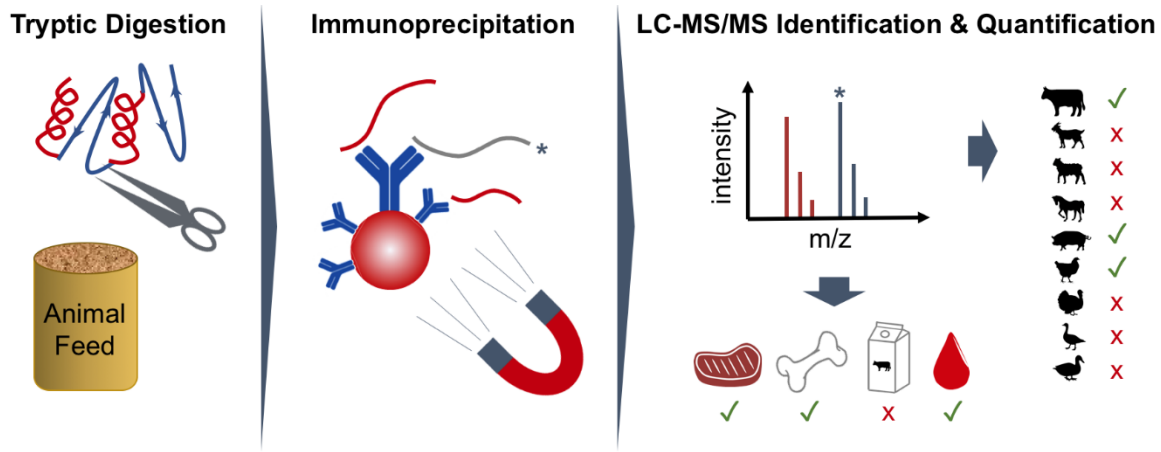

Figure 3. Immunoaffinity-based mass spectrometry for the species and tissue differentiation in feed compounds by the analysis of proteotypic peptides.

3 Materials and Methods

3.1 Materials

3.1.1 Consumables

Table 3. Consumables

Product and specification	Manufacturer (location)
0.2 mL Skirted 96-well robotic plate	Thermo Fisher Scientific (Waltham, USA)
Acclaim PepMap RSLC C18, 75 µm I.D. x 150 mm, 2 µm	Thermo Fisher Scientific (Waltham, USA)
Acclaim PepMap100 C18 µ-precolumn, 0.3 mm I.D. x 5 mm, 5 µm	Thermo Fisher Scientific (Waltham, USA)
Axygen AxySeal	Corning (NY, USA)
Centrifuge tubes, *15 mL conical bottom*	Greiner Bio-One (Frickenhausen, DE)
Centrifuge tubes, *50 mL conical bottom*	Greiner Bio-One (Frickenhausen, DE)
Centrifuge tubes, *50 mL self-standing*	Greiner Bio-One (Frickenhausen, DE)
Dynabeads Protein G	Thermo Fisher Scientific (Waltham, USA)
epT.I.P.S. Standard 0.1-10 µL	Eppendorf (Hamburg, DE)
epT.I.P.S. Standard 100-5000 µL	Eppendorf (Hamburg, DE)
epT.I.P.S. Standard 2-200 µL	VWR (Darmstadt, DE)
epT.I.P.S. Standard 50-1000 µL	Eppendorf (Hamburg, DE)
Filter devices *VIVASPIN 6 30,000 MWCO PES*	Sartorius Stedim Biotech (Göttingen, DE)
KingFisher 96 tip comb	Thermo Fisher Scientific (Waltham, USA)
Micro insert, 0.1 mL, clear glass 15 mm, top	VWR (Darmstadt, DE)
Micro inserts glass, 250 µL, conical	neoLab (Heidelberg, DE)
Microplate, 96 well, PS, F-Bottom, clear	Greiner Bio-One (Frickenhausen, DE)
Nitrile gloves	VWR (Darmstadt, DE)
NuPAGE 4-12% Bis-Tris Gel 12 well	Thermo Fisher Scientific (Waltham, USA)
Pasteur pipettes *Assistant*	Karl Hecht GmbH & Co KG (Sondheim, DE)
pH indicator paper range 1-14	Carl Roth (Karlsruhe, DE)
Pipette tips *SpaceSaver LTS 20 µL*	Mettler Toledo (Columbus, USA)
Pipette tips *SpaceSaver LTS 200 µL*	Mettler Toledo (Columbus, USA)
Pipette tips *SpaceSaver LTS 300 µL*	Mettler Toledo (Columbus, USA)
QUICKRACK Tip Transfer System, 1250 µL	Biozym Scientific (Oldendorf, DE)
Reaction tubes *PCR Tube Strips 0.2 mL*	VWR (Darmstadt, DE)
Reaction tubes *Protein LoBind Tube 1.5 mL*	Eppendorf (Hamburg, DE)
Reaction tubes with screw thread, 1.5 mL, conical	neoLab (Heidelberg, DE)

Reaction tubes with screw thread, 1.5 mL, self-standing	neoLab (Heidelberg, DE)
Reaction tubes with screw thread, 2.0 mL, self-standing	neoLab (Heidelberg, DE)
Reaction tubes, 1.5 mL, PP	Eppendorf (Hamburg, DE)
Reaction Tubes, 4 mL, PP round base	Greiner Bio-One (Frickenhausen, DE)
Reaction tubes, 5 mL, PP	Eppendorf (Hamburg, DE)
Screw caps for microcentrifuge tubes	VWR (Darmstadt, DE)
Screw caps for reaction 1.5 mL and 2 mL tubes	neoLab (Heidelberg, DE)
Screw caps, 9 mm, natural rubber red-orange	VWR (Darmstadt, DE)
Vial short thread, 1.5 mL, amber glass with label	VWR (Darmstadt, DE)

3.1.2 Chemicals, Biochemicals and Reagents

Table 4. Chemicals, Biochemicals and Reagents

Substance (abbreviation/specification)	Manufacturer (location)
1,4-Dithiothreitol (DTT)	Sigma Aldrich (St. Louis, USA)
2-Amino-2-(hydroxymethyl)propan-1,3-diol (TRIS)	Carl Roth (Karlsruhe, DE)
2-propanol, LC-MS grade (IPA)	VWR (Darmstadt, DE)
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Carl Roth (Karlsruhe, DE)
Acetone, ACS grade	VWR (Darmstadt, DE)
Acetonitrile, LC-MS grade (ACN)	Carl Roth (Karlsruhe, DE)
Albumin fraction V, protease-free (BSA)	Carl Roth, Karlsruhe, DE
Ammonia solution 25%, Rotipuran	Carl Roth (Karlsruhe, DE)
Ammonium bicarbonate (ABC)	Sigma Aldrich (St. Louis, USA)
BCA Protein Assay Reagent (bicinchoninic acid)	Thermo Fisher Scientific (Waltham, USA)
Blocking Reagent for ELISA	Roche Diagnostics (Mannheim, DE)
Citric acid, 99%	Sigma Aldrich (St. Louis, USA)
Coomassie Plus Protein Assay Reagent Kit	Thermo Fisher Scientific (Waltham, USA)
Customized polyclonal antibody sera	Pineda GmbH (Berlin, DE)
Customized synthetic standard peptides	Intavis AG (Tübingen, DE)
Dimethylsulfoxide (DMSO)	Sigma Aldrich (St. Louis, USA)
Dionex Cytochrome C Digest, lyophilized	Thermo Fisher Scientific (Waltham, USA)
Ethanol, >99.8%, p.a.	Carl Roth (Karlsruhe, DE)
Formic acid , 99% (FA)	Carl Roth (Karlsruhe, DE)
Hydrochloric Acid, 37% fuming	Carl Roth (Karlsruhe, DE)

InstantBlue Coomassie Based Staining	Expedeon (San Diago, USA)
Iodoacetamide (IAA)	Sigma Aldrich (St. Louis, USA)
Keyhole limpet hemocyanin (KLH)	Thermo Fisher Scientific (Waltham, USA)
L-Cystein hydrochloride monohydrate	Sigma Aldrich (St. Louis, USA)
LTQ Velos ESI Positive Ion Calibration Solution	Thermo Fisher Scientific (Waltham, USA)
m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS)	Thermo Fisher Scientific (Waltham, USA)
Methanol, LC-MS-Grade	Carl Roth (Karlsruhe, DE)
n-Octyl-ß-D-glucopyranoside (NOG)	Carl Roth (Karlsruhe, DE)
NuPAGE Antioxidant	Thermo Fisher Scientific (Waltham, USA)
NuPAGE LDS Sample Buffer (4X)	Thermo Fisher Scientific (Waltham, USA)
NuPAGE MES SDS Running Buffer (20X)	Thermo Fisher Scientific (Waltham, USA)
NuPAGE Sample Reducing Agent	Thermo Fisher Scientific (Waltham, USA)
Ovalbumin *Imject*	Thermo Fisher Scientific (Waltham, USA)
Phenylmethylsulfonyl fluoride (PMSF)	Thermo Fisher Scientific (Waltham, USA)
Phosphate Buffered Saline 10x (PBS)	Thermo Fisher Scientific (Waltham, USA)
Powdered milk, blocking grade	Carl Roth (Karlsruhe, DE)
SeeBlue Plus2 Prestained Standard	Thermo Fisher Scientific (Waltham, USA)
Sodium azide, for synthesis	Merck (Darmstadt, DE)
Sodium hydroxide, ≥99%	Carl Roth (Karlsruhe, DE)
Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclo-hexane-1-carboxylate (sulfo-SMCC)	Thermo Fisher Scientific (Waltham, USA)
Technical buffer solution pH 4.01	Mettler Toledo (Columbus, USA)
Technical buffer solution pH 7.00	Mettler Toledo (Columbus, USA)
Technical buffer solution pH 9.21	Mettler Toledo (Columbus, USA)
Thiourea, p.a.	Thermo Fisher Scientific (Waltham, USA)
Triethanolamine hydrochloride (TEA/TEA-HCl)	Carl Roth (Karlsruhe, DE)
Trifluoroacetic acid, ULC/MS Optigrade (TFA)	LGC Promochem (Wesel, DE)
Tris(2-carboxyethyl)phosphine (TCEP)	Carl Roth (Karlsruhe, DE)
Trypsin from bovine pancreas	Sigma Aldrich (St. Louis, USA)
Trypsin, modified, TPCK treated	Worthington (Lakewood, USA)
Urea, p.a.	Carl Roth (Karlsruhe, DE)
Water, HPLC LC-MS grade	VWR (Darmstadt, DE)

3.1.3 Samples

Table 5. Samples

Sample description	Code AST	Code BFR	Origin
Spray-dried bovine plasma	ANID001	C12129	CCL NutriControl (Veghel, NL)
Spray-dried porcine plasma (1)	ANID002	C12130	CCL NutriControl (Veghel, NL)
Spray-dried porcine blood meal	ANID005	C12131	CCL NutriControl (Veghel, NL)
Blood meal poultry mix	ANID006	C12135	CCL NutriControl (Veghel, NL)
Citrate plasma chicken (1)	ANID007	C14046	Preclincs GmbH (Potsdam, DE)
Citrate plasma turkey (1)	ANID008	C14047	Preclincs GmbH (Potsdam, DE)
Citrate plasma goat (1)	ANID009	C14048	Preclincs GmbH (Potsdam, DE)
Citrate plasma sheep (1)	ANID010	C14049	Preclincs GmbH (Potsdam, DE)
Citrate plasma horse (1)	ANID011	C14050	Preclincs GmbH (Potsdam, DE)
Citrate plasma goose (1)	ANID012	C14057	Preclincs GmbH (Potsdam, DE)
Citrate plasma duck (1)	ANID013	C14058	Preclincs GmbH (Potsdam, DE)
Citrate plasma cattle (1)	ANID014	C14059	Preclincs GmbH (Potsdam, DE)
Citrate plasma pig (1)	ANID015	C14060	Preclincs GmbH (Potsdam, DE)
Citrate plasma cattle (2)	ANID017	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma pig (2)	ANID018	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma sheep (2)	ANID019	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma goat (2)	ANID020	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma horse (2)	ANID021	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma turkey (2)	ANID022	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma duck (2)	ANID023	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma chicken (2)	ANID024	-	Preclincs GmbH (Potsdam, DE)
Citrate plasma goose (2)	ANID025	-	Preclincs GmbH (Potsdam, DE)
Spray-dried plasma, species unknown	ANID026	C15097	T.T. Baits (Erlangen, DE)
Spray-dried porcine plasma (2)	ANID027	C16024	CCL NutriControl (Veghel, NL)
Meat and bone meal, mix from poultry animals	ANID028	C15167	GePro GmbH & Co. KG (Diepholz, DE)
Milk performance feed for cattle (vegetal feed)	ANID029	C08074	H.W. Schaumann GmbH (Pinneberg, DE)
Bovine meat and bone meal (1)	ANID030	C16113	CCL NutriControl (Veghel, NL)
Porcine protein, SP60	ANID031	C16039	LUFA Nord-West (Oldenburg, DE)
Pure porcine meat and bone meal from 2007, first production of a new production line	ANID032	C16042	LUFA Nord-West (Oldenburg, DE)
Porcine meat and bone meal,	ANID033	C16043	LUFA Nord-West (Oldenburg, DE)

Category 3 (PAP)			
Porcine processed animal protein	ANID034	C16044	LUFA Nord-West (Oldenburg, DE)
Milk powder for human consumption	ANID035	-	Nestlé (Vevey, CH)
Milk powder laboratory blocking reagent	ANID036	-	Carl Roth (Karlsruhe, DE)
Fish feed without land living animals	ANID049	C17168	BioMar (Brande, DK)
Fish feed with land living animals	ANID050	C17167	BioMar (Brande, DK)
0.1% ruminant PAP (1)	ANID052	C16001	EURL-AP (Gembloux, Belgium)
0.1% ruminant PAP (2)	ANID053	C16005	EURL-AP (Gembloux, Belgium)
1% ruminant blood	ANID054	C16010	EURL-AP (Gembloux, Belgium)
3% bovine plasma	ANID055	C16171	EURL-AP (Gembloux, Belgium)
5% porcine blood	ANID064	C14011	EURL-AP (Gembloux, Belgium)
Fish feed containing hemoglobin meal	ANID057	C17202	EURL-AP (Gembloux, Belgium)
Bovine meat and bone meal (2)	ANID058	C16151	PerNaturam (Gödenroth, DE)

3.1.4 Laboratory Equipment

Table 6. Laboratory Equipment

Apparatus and type	**Manufacturer**
Analytical balance *CPA225D-0CE*	Sartorius Stedim Biotech (Göttingen, DE)
Analytical balance *Explorer*	OHAUS Waagen (Bad Hersfeld, DE)
Analytical balance *XS205 DualRange*	Mettler Toledo (Columbus, USA)
Ball mill *Micro-Dismembrator U*	Sartorius Stedim Biotech (Göttingen, DE)
Bead-based assay system *FLEXMAP3D*	Luminex (Austin, USA)
Bead-based assay system *FlexMap3D*	Luminex (Austin, USA)
Centrifuge for reaction tubes *5415 D*	Eppendorf (Hamburg, DE)
Centrifuge *Mini Star*	VWR (Darmstadt, DE)
Chromatography System *ÄKTAxpress*	GE Healthcare (Little Chalfont, GB)
Chromatography *UltiMate 3000 RSLC nano*	Thermo Fisher Scientific (Waltham, USA)
DURAN Laboratory bottle with DIN thread, GL 45, 1000 mL	Duran Group GmbH (Wertheim/Mainz, DE)
DURAN Laboratory bottle with DIN thread, GL 45, 500 mL	Duran Group GmbH (Wertheim/Mainz, DE)
DURAN Laboratory bottle with DIN thread, GL 45. 250 mL	Duran Group GmbH (Wertheim/Mainz, DE)
DURAN Measuring cylinder, 100 mL ± 0.5 mL	Duran Group GmbH (Wertheim/Mainz, DE)
DURAN Measuring cylinder, 25 mL ± 0.25 mL	Duran Group GmbH (Wertheim/Mainz, DE)

DURAN Measuring cylinder, 50 mL ± 0.5 mL	Duran Group GmbH (Wertheim/Mainz, DE)
DURAN Measuring cylinder, 500 mL ± 2.5 mL	Duran Group GmbH (Wertheim/Mainz, DE)
Electronic pipette 5 – 100 µL *research pro*	Eppendorf (Hamburg, DE)
Electronic pipette 5 – 300 µL *E4 XLS*	Mettler Toledo (Columbus, USA)
Electrophoresis Power Supply *Power Ease 500*	Thermo Fisher Scientific (Waltham, USA)
Electrophoresis System *XCell SureLock*	Thermo Fisher Scientific (Waltham, USA)
GL 45 Screw Caps	Duran Group GmbH (Wertheim/Mainz, DE)
Ice machine *Scotman AF40*	Frimont S.p.A. (Pogliano Milanese, IT)
Magnet *Dynal MPF -96S*	Thermo Fisher Scientific (Waltham, USA)
Magnet *DynaMag Spin*	Thermo Fisher Scientific (Waltham, USA)
Magnet *KingFisher 96 PCR head*	Thermo Fisher Scientific (Waltham, USA)
Magnet Particel Processor *KingFisher 96*	Thermo Fisher Scientific (Waltham, USA)
Magnet Particel Processor *KingFisher Flex*	Thermo Fisher Scientific (Waltham, USA)
Magnet stirrer *RCT basic*	IKA-Werk (Staufen, DE)
Magnet stirrer *VARIOMAG MONO*	Thermo Fisher Scientific (Waltham, USA)
Mass spectrometer *QExactive Plus*	Thermo Fisher Scientific (Waltham, USA)
Mass spectrometer *QExactive*	Thermo Fisher Scientific (Waltham, USA)
Microplate Reader *FLUOstar Optima*	BMG Labtech (Ortenberg, DE)
Mixer *Vortex Genie 2*	Scientific Industries (Bohemia, USA)
Multichannel pipette 2 – 20 µL *Pipet-Lite XLS*	Mettler Toledo (Columbus, USA)
Multichannel pipette 5 – 50 µL *Pipet-Lite XLS*	Mettler Toledo (Columbus, USA)
Multichannel pipette 50 – 1200 µL *Eppendorf Research Pro*	Eppendorf (Hamburg, DE)
pH-Meter *766 Calimatic*	Knick (Berlin, DE)
Pipette 0.1 – 2.5 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 1 – 10 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 10 – 100 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 100 – 1000 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 2 – 20 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 20 – 200 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Pipette 50 – 5000 µL *Eppendorf Research plus*	Eppendorf (Hamburg, DE)
Sample Mixer *Hulamixer Sample mixer*	Life Technologies (Carlsbad (USA)
Sonication bath *Sonorex*	Bandelin (Berlin, DE)
Sonication bath *Transsonic T780/H*	Elma (Singen, DE)
Spectrophotometer *Lambda Bio +*	Perkin Elmer (Waltham, USA)
Spectrophotometer *NANODROP 2000c*	Thermo Fisher Scientific (Waltham, USA)
SWC Safety Weighing Cabinet	Sartorius Stedim Biotech (Göttingen, DE)
Thermomixer *C*	Eppendorf (Hamburg, DE)

Thermomixer *Comfort*	Eppendorf (Hamburg, DE)
Water purification system *arium 611VF/advance*	Sartorius Stedim Biotech (Göttingen, DE)
Water purification system *Milli Q Plus*	Sartorius Stedim Biotech (Göttingen, DE)

3.1.5 Software and Databases

Table 7. Software and Databases

Software or database and version	Distributor
Chromeleon 6.8	Thermo Fisher Scientific (Waltham, USA)
Endnote X8	Thomson (Philadelphia, USA)
Mascot 2.3.02	Matrix Science (London, GB)
Microsoft Office 2016	Microsoft (Redmond, USA)
OPTIMA 2.20	BMG Labtech (Ortenberg, DE)
Origin 7.5	OriginLab (Northampton, USA)
Pinpoint 1.4	Thermo Fisher Scientific (Waltham, USA)
Proteome Discoverer 2.1	Thermo Fisher Scientific (Waltham, USA)
SEQUEST 28.0.0.0	University of Washington (Seattle, USA)
Skyline 3.7	University of Washington (Seattle, USA)
Tune 2.5	Thermo Fisher Scientific (Waltham, USA)
TXP-Tools	Internal script by Hannes Planatscher
Unicorn 5.11	GE Healtcare (Little Chalfont, GB)
UniProtKB Proteomes (Dec 2016)	UniProt Consortium
XCalibur 3.0	Thermo Fisher Scientific (Waltham, USA)
xPONENT Software Solutions 2.2	Luminex (Austin, USA)

3.2 Identification and Selection of Marker Peptides

3.2.1 *In Silico* Identification of Cross-Species Epitopes

Cross-species epitopes were identified by a bioinformatics workflow (Figure 4). A previously published list of 150 most abundant human plasma proteins served as a basis [105]. The Uniprot accession numbers were translated into their corresponding gene names and the respective protein sequences from all species of interest were collected: cattle *(bos taurus)*, sheep *(ovis aries)*, goat *(capra hircus)*, pig *(sus scrofa)*, horse *(equus caballus)*, turkey *(meleagris gallopavo)*, duck *(anas platyrhynchos)*, goose *(anser anser)* and chicken *(gallus gallus)*. These protein sequences were fragmented *in silico* into tryptic peptides. To minimize analytical issues, the list was filtered for peptide lengths between 8 and 25 amino acids and peptides that do not contain cysteine or methionine. The remaining peptides were grouped based on their C-terminal sequence comprising four amino acids and by their corresponding gene names. The grouped peptide list was further sorted by the species coverage of the cross-species epitope and the maximum number of species-specific peptides for each epitope. The top 5 proteins with the highest species specificity and cross-species coverage were extracted and served as a basis for the final selection of one cross-species epitope.

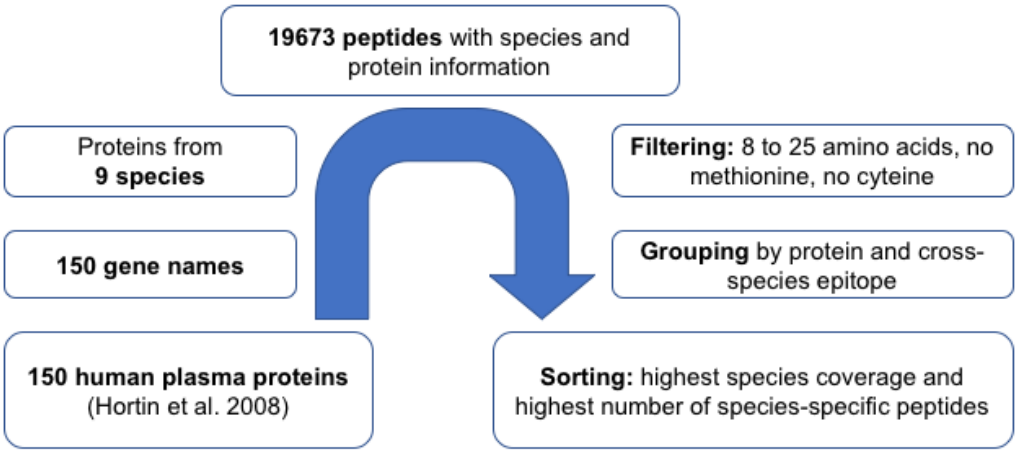

Figure 4. Bioinformatic workflow for the identification of cross-species epitopes. High abundant plasma proteins published by Hortin and colleagues [105] were in silico digested and homologous peptides were analyzed for a cross-species epitopes by a bioinformatic workflow.

3.2.2 Ruminant-Specific Plasma Marker Peptides

Marker peptides were identified by a bioinformatic workflow combined with data obtained from non-targeted mass spectrometric analyses of bovine milk powder and native bovine plasma (see 3.10.1). The proteins identified in the milk powder sample were subtracted from the bovine plasma identifications in order to achieve a specific detection of blood derived proteins. The remaining protein sequences were divided in peptide frames consisting of eight amino acids. The frequency of these sequences in the Uniprot database (December 2016) was calculated using an internal script by Dr. Hannes Planatscher (Figure 5). Sequences of low frequency were manually chosen to minimize sequence similarities to other species. Only peptide lengths between 8 and 25 amino acids, and peptides without cysteine and methionine were considered. Furthermore, only peptides that were already experimentally observed in bovine plasma were considered for a further selection.

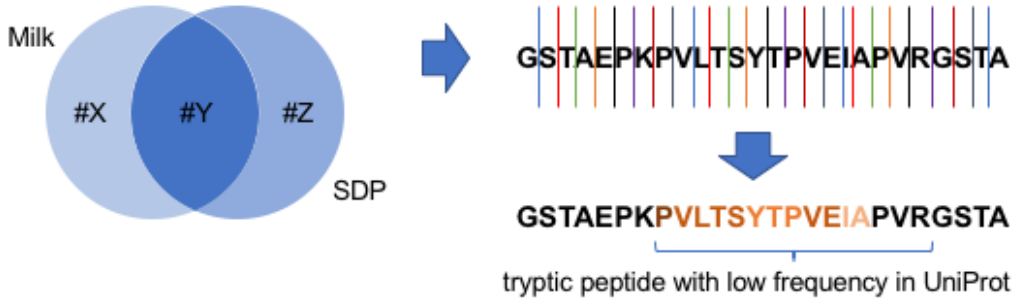

Figure 5. Identification of ruminant sequences with low inter-species similarity on the basis of spray-dried plasma (SDP) data using the tool *Exitope Matcher* developed by Dr. Hannes Planatscher.

3.2.3 Tissue-Specific Ruminant Marker Peptides

To increase tissue specificity, specific marker peptides for bovine meat and bone meal, milk powder and citrate plasma were identified. Therefore, a non-targeted mass spectrometric analysis of bovine milk powder, meat and bone meal and citrate plasma was performed. Using bioinformatics, the results were filtered for peptides that allow a species and a tissue differentiation (Figure 6). The tissue specificity was achieved by filtering the results for unique peptides that were only identified in three different samples, respectively. In this step, all peptides, even methionine and cysteine containing peptides, were

considered. However, peptide lengths below 8 amino acids and longer than 25 amino acids were excluded. The resulting list was filtered for peptides, for which antibodies already were available. To achieve species specificity a sequence alignment of the selected peptides and the sequences from the other species was performed.

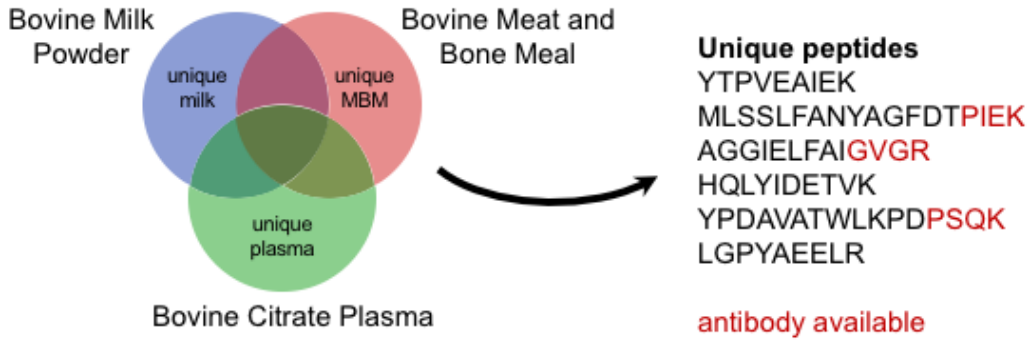

Figure 6. Identification of tissue-specific ruminant marker peptides on the basis of shotgun proteomic data of different tissues with available group-specific polyclonal antibodies addressing four terminal amino acids (triple X proteomics antibodies).

3.3 Polyacrylamide Gel Electrophoresis

The gel electrophoresis was performed under denaturing conditions using the NuPAGE Bis-Tris Mini Gel-System (life technologies). Protein samples (10 µg) were mixed with 2.5 µL 4x lithium dodecylsulfate (LDS) sample buffer and 1 µL reducing agent (500 mmol L^{-1} dithiothreitol) was added to the sample. Afterwards, the samples were diluted with water to reach a total volume of 10 µL. The samples were denatured by heating for 10 min at 70°C and loaded on the 4-12% Bis-Tris gel. The separation was performed in 2-(N-morpholino)ethanesulfonic acid (MES) buffer by applying a constant voltage of 200 V for 35 min. After migration, the gel was washed with water and stained using Coomassie InstantBlue solution for at least one hour. The staining solution was removed, and the gel was washed with water before taking a picture.

3.4 Synthetic Standard Peptides

Standard peptides with different modifications were synthesized by Intavis AG (Tübingen, Germany). Unlabeled peptide standards and stable isotope labeled standards with

a $^{13}C/^{15}N$-labeling of the C-terminal lysine and arginine were synthesized for assay development. Peptide stock solutions for assay development were prepared with a concentration of 1 mmol L^{-1}. Therefore, 1 mg to 2 mg of peptide was weighed. The peptide was dissolved in dimethyl sulfoxide (DMSO) and adjusted with water to achieve a final concentration of 1 mmol L^{-1} in 20% (v/v) DMSO. Peptide stock solutions were stored at this concentration at -20°C and diluted to working concentrations before each experiment.

3.5 Antibody Generation and Purification

Antibodies have been generated as described previously [100]. Antigen conjugation and antibody purification have been performed by Cornelia Sommersdorf. Antibody sera were produced at Pineda GmbH (Berlin, DE).

3.6 Determination of Protein and Peptide Concentrations

3.6.1 Bicinchoninic Acid Assay

The concentration of citrate plasma samples was determined using the Pierce BCA Assay Kit (Thermo Fisher). Plasma samples were diluted to an estimated concentration of <2 mg mL^{-1} assuming a concentration of the undiluted plasma of approximately 60 mg mL^{-1}. Bovine serum albumin was used as a standard for calibration. The BSA stock solution was diluted to concentrations between 25 µg mL^{-1} and 2 mg mL^{-1} using phosphate buffered saline as diluent. BCA reagent A and B were mixed in the ratio 50:1 and 200 µL of the reagent solution was added to 25 µL of sample dilutions in a microplate. The microplate was incubated at 37°C for 30 min. Afterwards, the plate was cooled to room temperature and the absorption at 562 nm was measured. The sample concentrations were back-calculated using a four-parametric calibration function fitted to the BSA standard dilution series.

3.6.2 Bradford Assay

The concentration of protein solutions containing urea and thiourea were determined using the Pierce Coomassie Plus (Bradford) Assay Kit. Sample protein solutions were diluted 1:10 in water. Bovine serum albumin was used as a standard for calibration. The BSA stock solution was diluted to concentrations between 25 µg mL^{-1} and 2 mg mL^{-1} using a

1:10 dilution of urea and thiourea sample buffer as diluent. 150 µL of the Coomassie Plus reagent solution was added to 5 µL of diluted samples in a microplate and mixed for 30 s. The plate was incubated for 10 minutes at room temperature and the absorption at 595 nm was measured. The sample concentrations were back-calculated using a four-parametric calibration function fitted to the BSA standard dilution series.

3.6.3 UV-Absorption Measurement

The total protein and peptide amount of complex protein extracts and tryptic digests was estimated using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Therefore, 2 µL of sample were placed on the sensor and the absorbance at 280 nm was measured. The total protein and peptide amount was calculated on the basis of 1 Abs ≈ 1 mg mL^{-1} [106]. UV absorbance due to added trypsin and hydrogen iodide formed during alkylation with iodoacetamide was subtracted using blank digests. The absorption due to nucleic acids was determined by the A260/A280 ratio (Figure 7). The protein purity of the sample can be calculated on the basis of Formula 1 [107].

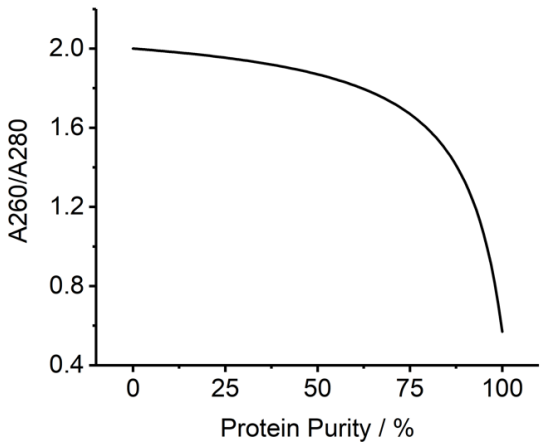

Figure 7. A620/A280 ratio dependency on the protein purity of sample solutions.

$$\frac{A_{260}}{A_{280}} = \frac{(\varepsilon_{260,p} \text{ x } (\%p) + \varepsilon_{260,n} \text{ x } (\%n))}{(\varepsilon_{280,p} \text{ x } (\%p) + \varepsilon_{280,n} \text{ x } (\%n))} \quad (1)$$

ε = extinction coefficient, p = proteins, n = nucleic acids

3.7 Preparation of Validation Samples

3.7.1 Mixtures for Species Differentiation

The mixtures for proving the species differentiation capability of multiplex assay XA2M were prepared with citrate plasma from nine species (Preclinics GmbH, Potsdam, Germany) that were spiked into a fish feed (BioMar A/S, Brande, Denmark). To validate the assay specificity, eight mixtures of digested citrate plasmas in digested fish feed were prepared (10% w/w). In each mixture, one of the nine species was left out. Sheep and goat plasma were mixed in equal amounts and treated as one species since the marker peptide is identical in both species. To validate intra- and interassay variation, mixtures of all species in fish feed on the solid non-digested level were prepared. Native citrate plasmas of nine species were mixed in equal volumes and lyophilized for two days using an alpha I-6 freeze dryer (Christ, Osterode, Germany). The fine multispecies powder was then added to fish feed at three concentrations (1%, 5% and 10% w/w). Consequently, the single species concentrations were 0.1%, 0.6% and 1.1% (w/w), respectively. Before mixing, the coarse fish feed powder was further homogenized into a fine powder using a ball mill (Sartorius Stedim Biotech, Goettingen, Germany). About 80 mg of fish feed was weighed into cryovials, cooled in liquid nitrogen and ground to a fine powder using 7 mm steel balls at 2000 rpm for 2 min. The fine powders were then mixed at the three concentrations as stated above. To obtain a homogenous mixture, glass beads with a diameter of 2 mm were added and mixed properly.

3.7.2 Mixtures for Ruminant Protein Detection

The validation samples for the detection of ruminant proteins were prepared at the BfR in Berlin with two different bovine meat and bone meals obtained from different sources (PerNaturam, Gödenroth, Germany and CCL NutriControl, Veghel, Netherlands) and a bovine spray-dried plasma (CCL NutriControl, Veghel, Netherlands). The bovine samples were spiked in a vegetal cattle feed (H.W. Schaumann GmbH, Pinneberg, Germany). All samples were ground to powder in a Retsch MM 400 ball mill with 25 mm steel balls (Qiagen, Düsseldorf, Germany) prior to the preparation of feed mixtures. Grinding jars, filled with steel balls and sample material, were cooled in liquid nitrogen prior to pulverization at 25 Hz for 1 min. Feed mixtures were prepared in a Turbula Mixer type T2F (Willy A.

Bachofen AG, Basel, Switzerland) for 2 h at room temperature at a speed frequency of 22 min^{-1}. For the determination of intra- and interassay precision at the NMI, about 250 mg of validation samples was cooled in liquid nitrogen and then pulverized for a second time using a ball mill (Sartorius Stedim Biotech, Goettingen, Germany) and 7 mm steel balls at 2000 rpm for 2 min.

3.8 Protein Extraction and Fragmentation

3.8.1 Extraction in Phosphate Buffered Saline

For protein extraction purposes 15 mg of animal protein sample was weighed in a tube and suspended in 750 µL of phosphate buffered saline (PBS). The suspension was shaken over night at 37°C and 1000 rpm. Afterwards, the suspension was centrifuged at 13 000 g for 5 min and the supernatant was collected. The protein concentration was estimated with two methods, the bicinchoninic acid assay (3.6.1) and the measurement of optical density at 280 nm (see 3.6.3). The extracts were stored at -20°C until further use.

3.8.2 TCA Acetone Extraction

The TCA acetone protein extraction was carried out as described by Marbaix and colleagues with slight modifications [90]. 900 µL acetone with 10% trichloroacetic acid (TCA) and 0.3% dithiothreitol (DTT) was added to 100 mg of PAP sample and stored at -20°C overnight. Each sample was centrifuged for 10 min at 16 000 g at 4°C and the supernatants were discarded. The remaining pellets were washed first in 900 µL acetone with 0.3% DTT and second in 900 µL of 90% acetone containing 0.3% DTT with an incubation step of 30 min at -20°C and centrifugation at 16 000 g at 4°C after each washing step. The supernatant after the second washing step was discarded and 500 µL of resuspension buffer (urea 7 M, thiourea 2 mol L^{-1}, Tris 30 mmol L^{-1}, CHAPS 4%) was added. The sample was mixed for 1 h at 650 rpm at 12°C on a thermomixer and centrifuged for 10 min at 16 000 g. The supernatant was transferred to a new tube and stored at -20°C. The protein concentration of the extracts was determined by a Coomassie plus assay (Bradford) as described in section 3.6.2. The tryptic digestion was performed as described in section 3.8.4 with 100 µg protein extract.

3.8.3 Heterogeneous Phase Digestion

620 µL of triethanolamine digestion buffer (50 mmol L^{-1}) containing 0.5% *n*-octylglucoside (NOG) was added to 15 mg of animal protein or vegetal feed sample. The suspension was heated for 5 min at 99°C and cooled down to room temperature. The sample was reduced with tris(2-carboxyethyl)phosphine (TCEP) at a final concentration of 5 mmol L^{-1} for 5 min at room temperature. Iodoacetamide (IAA) was added for a final concentration of 10 mmol L^{-1}, and the samples were alkylated for 30 min at room temperature in the dark. Trypsin (Worthington, Lakewood, USA) was added in a 1:40 ratio (w/w) on the basis of the initial sample weight. The samples were digested at 37°C for 2 h while shaking at 1000 rpm to achieve a stable suspension. The digestion was stopped by adding phenylmethanesulfonyl fluoride (PMSF) for a final concentration of 1 mmol L^{-1}. The suspension was centrifuged for 5 min at 13 000 g. Afterwards, the supernatant was transferred to a new reaction tube. The total protein and peptide content after digestion was estimated by UV absorption measurement at 280 nm as described in section 3.6.3.

3.8.4 In-Solution Digestion

Protein extraction was performed as described in 3.8.3. However, instead of adding trypsin, the sample was incubated under the same conditions without enzyme. The insoluble fraction was removed by centrifugation, and the supernatant was digested by trypsin for 2 h at 37°C while shaking at 1000 rpm. The digestion was stopped by adding PMSF for a final concentration of 1 mmol L^{-1}. The protein estimation was performed via UV spectroscopy as described in section 3.6.3.

3.9 Immunoprecipitation of Peptides

The immunoprecipitation was performed on a *KingFisher* magnetic particle processor (Thermo Fisher Scientific, Waltham, USA). Different amounts of samples were placed in a well of a 96 well PCR plate. Citrate plasma samples were analyzed in amounts ranging from 1 µg to 10 µg. Feed compounds containing processed animal proteins (PAPs) were analyzed in amounts up to 425 µg. The samples were incubated with single antibodies or antibody mixtures. Each antibody was used in an amount of 1 µg. The stable isotope labeled peptide stock solutions were diluted to a working concentration of 5 fmol μL^{-1}. A volume of 10 µL, corresponding to a total amount of 50 fmol was added to the samples.

Phosphate buffered saline (PBS) containing 0.03% (w/v) CHAPS was added for a final volume of 100 µL. The samples were incubated at room temperature for 1 h, followed by the precipitation of the peptide-antibody complexes using protein G-coated magnetic microspheres. The microspheres were used in a ratio of 5 µL per 1 µg of antibody. The microsphere conjugates were washed two times in 100 µL of PBS and three times in 100 µL of 50 mmol L^{-1} ammonium bicarbonate, each containing 0.03% (w/v) CHAPS. The peptides were eluted in 20 µL of 1% formic acid (FA).

3.10 Chromatography and Mass Spectrometry

3.10.1 Non-Targeted Peptide Identification

Chromatography

Marker identification experiments were performed using a non-targeted mass spectrometric workflow. The separation of peptides was performed on a nanoflow UHPLC system (Ultimate 3000 RSLCnano, Thermo Fisher Scientific). 1 µg of sample was loaded on an Acclaim PepMap100 C18 µ-precolumn (0.3 mm I.D. x 5 mm, 5 µm, Thermo Fisher Scientific) for 5 min at a flow rate of 20 µL min^{-1} in LC-MS grade water containing 2% acetonitrile (ACN) and 0.05% trifluoroacetic acid (TFA). The peptides were separated in 180 min by an Acclaim PepMap RSLC C18 (75 µm I.D. x 150 mm, 2 µm, Thermo Fisher Scientific) using a linear gradient from 5% to 55% at 0.3 µL min^{-1} and 40°C. The column was washed and equilibrated for a further 20 min. The aqueous phase consisted of 0.1% FA in LC-MS grade water. The organic phase consisted of 80% ACN and 20% LC-MS grade water containing 0.1% FA.

Mass Spectrometric Detection in Full Scan

The nano UHPLC system was coupled to a QExactive Plus hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific). For a non-targeted data acquisition, a top 10 Full-MS/$ddMS^2$ method was performed. Full MS resolution was set to 70 000 with an AGC target of 3 x 10^6 and a maximum injection time of 100 ms. The scan range was set from 300 to 2000 m/z. Data dependent MS^2 spectra were acquired with a resolution of 17 500 and an AGC target of 5 x 10^5 with a maximum injection time of 50 ms. The isolation window of precursor ions was set to 2.0 m/z. Normalized collision energy was set to 25. Dynamic exclusion of precursors was set to 5.0 s.

3.10.2 Targeted Peptide Quantification

Chromatography

For quantification experiments after immunoprecipitation, a short chromatographic gradient was used. The peptide separation was performed on the same instrument as used in section 3.10.1. Here, 5 µL of the eluate from the immunoprecipitation step (see 3.9) was loaded on the precolumn for 0.25 min at a flow rate of 120 µL min^{-1} in LC-MS grade water containing 2% ACN and 0.05% TFA. The peptides were separated on an Acclaim PepMap RSLC C18 (75 µm I.D. x 150 mm, 2 µm, Thermo Fisher Scientific) using different two-step gradients at 1 µL min^{-1} and 55°C. The two-step gradients are shown in Table 8. The column was washed and equilibrated for further 5 min. Mobile phases were the same as described in section 3.10.1.

Table 8. Linear two-step gradients used for targeted quantification experiments. Ruminant protein quantification (RQ) was performed using RQ1, RQ2 and RQ3. Species identification was performed via cross-species multiplex addressing homologous alpha-2-macroglobulin peptides (XA2M).

Time	Eluent B / %			
/ min	RQ1	RQ2	RQ3	XA2M
0.00	4	4	4	4
0.50	4	4	4	4
5.00	15	15	15	20
5.25	35	45	40	30
7.50	98	98	98	98
7.75	98	98	98	98
10.00	4	4	4	4

Mass Spectrometric Detection using Selected Ion Monitoring

The quantification of marker peptides on the precursor level was performed on the same instrument as in section 3.10.1 using a *selected ion monitoring* (SIM) method with a data dependent acquisition of MS^2 spectra ($ddMS^2$). SIM resolution was set to 35 000. Precursor m/z was supplied by an inclusion list and ions were isolated with a mass window of 3.0 m/z. Spectral multiplexing was set to 2 for light and heavy peptide pairs. For a maximum number of data points per chromatographic peak, the peptide isolation was time scheduled. The AGC target was set to 2 x 10^5 and a maximum injection time of 60 ms used.

The scan range was set from 400 to 1000 m/z. Data dependent MS^2 spectra were acquired with a resolution of 17 500 and an AGC target of 2×10^5 with a maximum injection time of 60 ms. The isolation window of precursor ions was set to 2.0 m/z. Normalized collision energy was set to 25. Dynamic exclusion of precursors was set to 3.0 s. Data analysis was performed using Skyline v3.7.

Mass Spectrometric Detection using Parallel Reaction Monitoring

The quantification of marker peptides on the fragment ion level was performed on the same instrument as in section 3.10.1 using *parallel reaction monitoring* (PRM). PRM resolution was set to 70 000 with an injection time of 120 ms when only 4 peptides were analyzed (RQ1 and RQ2). The resolution was reduced to 35 000 with an injection time of 60 ms when more than 4 peptides were analyzed (XA2M and RQ3). The AGC target was set to 2×10^5 in both cases. Precursor m/z was supplied by an inclusion list and ions were isolated with a mass window of 1.5 m/z. Spectral multiplexing was set to 2 for light and heavy peptide pairs. For a maximum number of data points per chromatographic peak, the peptide isolation was time scheduled. The optimal normalized collision energy (NCE) was found to be at a level of 25 for all selected marker peptides. Data analysis was performed using Skyline v3.7.

3.10.3 Determination of the Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantification (LOQ) was determined by a method evaluated and published by Mani and colleagues [108]. This method was proven to reliably determine LOD and LOQ for mass spectrometry-based peptide assays. The determination of LOD and LOQ in this method is based on the measurement of blank samples as well as low concentrated analyte samples, considering alpha and beta errors (Formula 2). Mani and colleagues did not further specify the low concentration sample. In this thesis, the low concentration sample was chosen as the lowest dilution step of an analyte dilution series showing a signal-to-noise-ratio $S/N \geq 3$. The LOQ was estimated as the threefold of the LOD. To assess the quantification range of assays, the lower limit of quantification (LLOQ) was determined. The LLOQ was chosen as the lowest concentration level showing an accuracy in the range of 80% to 120% with a coefficient of variation ≤20%, according to a guideline for bioanalytical method validation published by the United States Food and Drug Administration [109].

$$\mathrm{LOD} = X_{\mathrm{blank}} + (1-\alpha) \times (\mathrm{SD}_{\mathrm{blank}} + \mathrm{SD}_{\mathrm{low}}) / \sqrt{\mathrm{N}} \quad (2)$$

X_{blank}	*mean of blank measurements*
$(1-\alpha)$	*z value, here alpha = 0.05 with z = 1.645*
SD_{blank}	*standard deviation of blank*
SD_{low}	*standard deviation of low concentration sample (S/N>3)*
$\sqrt{N}$	*square root of number of replicates, here n=3*

4 Results

4.1 Identification of Species- and Tissue-Specific Marker Peptides

4.1.1 Identified Marker Peptides by Non-Targeted Mass Spectrometry

In order to identify species- and tissue-specific marker peptides, non-targeted mass spectrometric (MS) analyses were performed. For the experimental verification of the bioinformatically identified species-specific plasma peptides, non-targeted MS analyses of citrate plasmas from the species cattle, sheep, goat, pig, horse, chicken, turkey, duck and goose were performed. Verifying mass spectra for each species-specific precursor ion are shown in Supplementary Data C. In order to identify tissue-specific marker peptides, non-targeted MS analyses in samples of different tissue origin were performed. Therefore, spray-dried plasmas (SDP) of porcine and bovine origin, a porcine blood meal (BM) and a bovine meat and bone meal (MBM) were analyzed. In order to be able to differentiate legal and illegal protein additives, a bovine milk powder was also analyzed in a non-targeted MS analysis.

Table 9 shows the number of identified proteins and peptides in bovine and porcine citrate plasmas and SDPs, bovine milk powder, porcine BM and bovine MBM. In the non-processed citrate plasmas, 349 proteins were identified in the bovine plasma and 385 proteins in the porcine plasma. The number of identified peptides was highest in the citrate plasmas with 1902 and 1911, respectively. The processed SDPs showed a slightly lower number of identifications with 238 proteins in the bovine SDP and 191 in the porcine SDP and 1297 and 917 identified peptides, respectively. 399 proteins were identified in the processed porcine BM. The number of identified peptides in the porcine BM was 1005 and matched the range of the SDP of porcine and bovine origin, all processed at similar conditions of at least 80°C. The milk powder sample showed 190 protein identifications and 666 peptide identifications. The bovine MBM was prepared using different sample preparation protocols. The comparison is shown in section 4.2.3. The bovine MBM, prepared by heterogeneous phase digestion (HPD) showed 267 protein identifications and 1024 peptide identifications. Therefore, the result was in the same range as it was observed for the SDP and BM samples, although MBM was processed under much higher temperature and pressure (133°C, 3 bar, 20 min). The same MBM prepared by extraction

and in-solution digestion (ISD) showed 86 protein identifications and 193 peptide identifications, and the TCA/acetone-prepared MBM showed 91 protein and 146 peptide identifications. Data from citrate plasma, milk powder and MBM were used for the experimental verification of bioinformatically identified and selected peptides (4.1.2 and 4.1.3).

Table 9. Number of proteins and peptides identified in a non-targeted mass spectrometric analysis of different animal proteins of porcine and bovine origin. Two citrate plasmas, two spray-dried plasmas (SDP), one blood meal (BM), one milk powder and one meat and bone meal (MBM).

Sample type	Sample ID	Preparation	# Runs	Species	#Proteins	#Peptides
Citrate plasma	ANID017	ISD[1]	3	Bovine	349	1902
	ANID018	ISD[1]	3	Porcine	385	1911
SDP	ANID001	HPD[2]	1	Bovine	238	1297
	ANID002	HPD[2]	1	Porcine	191	917
BM	ANID005	HPD[2]	3	Porcine	399	1005
Milk powder	ANID036	HPD[2]	3	Bovine	190	666
MBM	ANID030	TCA[3]	3	Bovine	91	146
	ANID030	HPD[2]	3	Bovine	267	1024
	ANID030	ISD[1]	3	Bovine	86	193

[1] in-solution digestion, [2] heterogeneous phase digestion [3] trichloroacetic acid extraction

4.1.2 Identified Cross-Species Epitopes for Species Differentiation

Five plasma proteins that allow a cross-species enrichment of homologous peptides using one antibody were bioinformatically identified: alpha-2-macroglobulin (A2M), coagulation factor VIII (F8), antithrombin-III (SERPINC1), serum albumin (ALB) and cholinesterase (BCHE). The sequence alignment for these proteins is shown in Table 10. The species coverage describes how many species are covered by the epitope and can be enriched using only one antibody. The highest species coverage was achieved for A2M that covered all 9 species of interest. The other four cross-species epitopes covered 8 of 9 species. The number of species-specific peptides describes how many of the species can be differentiated from the others by a unique peptide sequence. Same peptide sequences of different species were counted as one species-specific peptide as long as they belong to the same taxonomic group, e.g. ruminants or poultry. A2M and F8 showed the highest number of

species-specific peptides with a number of 8. The other proteins showed a lower number of species-specific peptides of 6 for ALB and BCHE and 7 for SERPINC1.

To summarize, A2M showed the highest species coverage in combination with the highest number of species-specific peptides. The peptides' LC-MS/MS properties were considered as suitable, since peptide length, amino acid composition and polarity matched the criteria (see 3.2.1). This epitope was chosen to generate a group-specific polyclonal antibody for the parallel enrichment of 9 species and the differentiation of 8 species. The species sheep and goat cannot be differentiated via the chosen marker peptides. However, both can be differentiated from the bovine-specific peptide.

Table 10. Cross-species epitopes identified in a bioinformatic workflow.

Protein	Peptide sequence	Species coverage	Species-specific peptides	bos taurus	ovis aries	capra hircus	Sus scrofa	equus caballus	gallus gallus	meleagris gallopavo	anser anser	anas platyrhynchos
α-2-Macroglobulin	GSGGTAEHPFTVEEFVLPK	9/9	8	X								
	ESGGTAEHHFTVEEFVLPK				X	X						
	VVVQQESGETAEHPFTVEEFVLPK						X					
	AEHPFIVEEFVLPK							X				
	TIHHPFSVEEYVLPK								X			
	TIQHPFTVEEYVLPK									X		
	TIQHPFSVEEYVLPK										X	
	IQHSFSVEEYVLPK											X
Factor VIII	SWAHHIALR	8/9	8	X								
	IHPQSWVHHIALR				X							
	SWVHHIALR					X						
	IHPTSWAQHIALR						X					
	IHPQSWGHQIALR							X				
	HWHNHIALR								X			
	IHPAWHNHIALR									X		
	QWHNHIALR											X
Antithrombin III	ITDVIPPQAINEFTVLVLVNTIYFK	8/9	7	X								
	ITDVIPPQAIDEFTVLVLVNTIYFK				X	X						
	ITDVIPPEAINELTVLVLVNTIYFK						X					
	ITDVIPHGAINELTVLVLVNTIYFK							X				
	GIDDLTVLVLVNTIYFK								X			
	GIDELTVLVLVNTIYFK									X		
	ITEVIPEGGINDLTVLVLVNTIYFK											X
Serum Albumin	DAFLGSFLYEYSR	8/9	6	X								
	DVFLGSFLYEYSR				X	X						
	HVFLGTFLYEYSR						X					
	DVFLGTFLYEYSR							X				
	SFEAGHDAFMAEFVYEYSR								X			
	SFEAGHDAFMSEFVYEYSR									X		X
Cholinesterase	FSDMGNNAFFYYFEHR	8/9	6	X	X	X						
	FSEMGNNAFFYYFEHR						X					
	FSELGNDAFFYYFEHR							X				
	TIAEVGNNVFFYFFEHR								X			
	IAEIGNNVFFYFFEHR									X		
	FAQLGHNAFFYFFEHR											X

4.1.3 Identified Tissue-Specific Ruminant Marker Peptides

The identification of tissue-specific ruminant marker peptides was performed in two selection processes. First, the identification of ruminant-specific plasma peptides with a very low sequence similarity to other species that allow a comprehensive analysis of MBM, SDP and BM samples (see 3.2.2). Second, the identification of tissue-specific ruminant marker peptides that allow a higher specificity in MBM and blood product detection (see 3.2.3).

Figure 8 shows the number of unique peptides identified in the SDP and the milk sample via non-targeted MS analysis for the selection of ruminant plasma markers. After HPD sample preparation, 238 proteins were identified in bovine SDP and 209 proteins in bovine milk powder with an overlap of 34 proteins. The 204 unique bovine SDP proteins were selected for further candidate selection via bioinformatics. Therefore, these proteins were *in silico* fragmented into overlapping 8 amino acid comprising peptide frames. The occurrence of these peptides in the UniProt database was counted by an internal tool developed by Dr. Hannes Planatscher. The result of the frequency calculations for the most promising marker proteins is shown in Supplementary Data A. The bioinformatic workflow revealed 86 peptide candidates from which 33 were experimentally verified in the performed non-targeted MS analysis of a bovine SDP sample (Table 9). Among these candidates, peptides from the three high-abundant plasma proteins alpha-2-antiplasmin (SERPINF2), complement component 9 (C9) and protein HP-25 homolog 2 (HP252) were selected since they showed very low inter-species similarity. The sequence alignment in Figure 9 shows that the sequences are identical for the ruminant group and different for pig, horse and poultry animals.

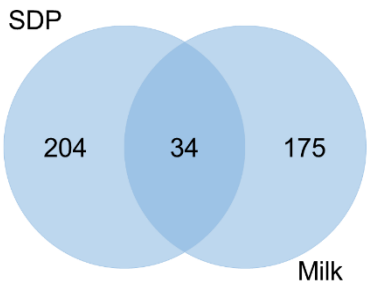

Figure 8. Number of identified proteins in a bovine spray-dried plasma (SDP) and a bovine milk powder.

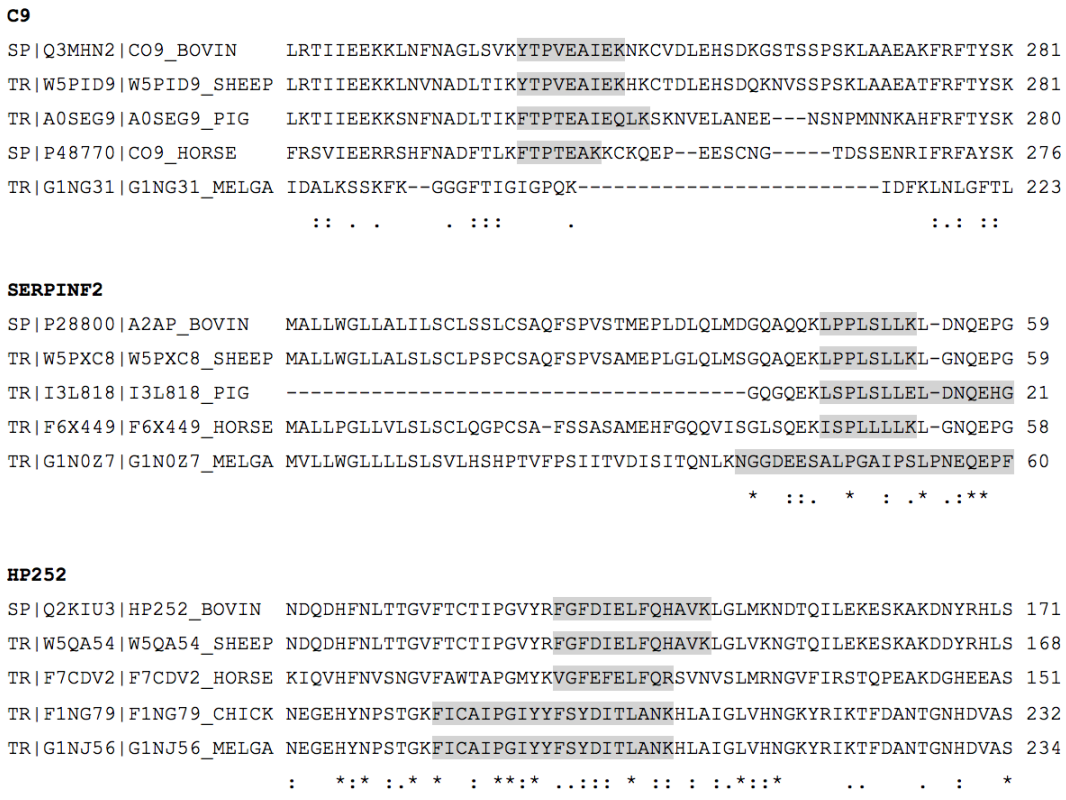

Figure 9. Sequence alignment of ruminant-specific plasma protein sequences identified via bioinformatics.

The second part of the marker peptide identification was performed on the basis of a non-targeted MS analysis of the bovine samples citrate plasma, milk powder and MBM. The focus was set on the identification of tissue-specific markers for the differentiation of ruminant MBM, blood products and milk powder. The result of the non-targeted MS analysis of the three sample types is shown in Figure 10. The non-targeted MS analysis of citrate plasma, milk powder and MBM revealed ruminant-specific targets that are unique for each sample type. By the use of bioinformatics, peptides were filtered that allow a differentiation of ruminants and other species. Out of 913 unique MBM peptide identifications, 138 peptides were suitable for species differentiation and 10 peptides for which antibodies were already available in the working group. Out of these 10 peptides, myosin-7 (MYH7) and matrilin-1 (MATN1) were selected as markers for meat and cartilage tissue. The analysis of milk powder revealed 271 unique peptides, with 160 unique bovine peptides and 20 peptides with already available antibodies. Out of these, osteopontin (SPP1) was chosen as a high-abundant marker for ruminant bone and milk. The sequence alignment in Figure 11 shows that the sequences for the bovine peptides differ from those of

the species pig, horse and poultry animals. An overview over the selected ruminant-specific marker peptides is shown in (Table 11).

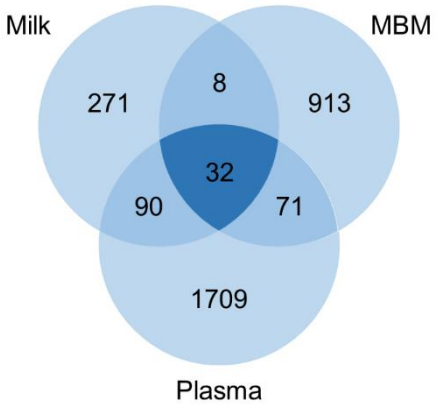

Figure 10. Number of identified peptides in bovine plasma, milk powder and meat and bone meal (MBM).

```
MYH7
SP|Q9BE39|MYH7_BOVIN            LNETVVDLYKKSSLKMLSSLFANYAGFDTPIEK--GKGKAKKGSSFQTVSALHRENLNKL 658
TR|W5QDF5|W5QDF5_SHEEP          LNETVVDLYKKSSLKMLSSLFANYAGFDTPIEK--GKGKAKKGSSFQTVSALHRENLNKL 658
SP|P79293|MYH7_PIG              LNETVVDLYKKSSLKLLSNLFANYAGADTPVEK--GKGKAKKGSSFQTVSALHRENLNKL 658
SP|Q8MJU9|MYH7_HORSE            LNETVVDLYKKSSLKMLSNLFANYLGADAPIEK--GKGKAKKGSSFQTVSALHRENLNKL 658
TR|A0A1D5P600|A0A1D5P600_CHICK  LNETVVGLYQKSALKLLASLFSNYAGADAGGDGGKGKGAKKKGSSFQTVSALHRENLNKL 653
                                ******.**:**:**:*:.**:** * *:  :   ***  ********************

MATN1
TR|E1BMV3|E1BMV3_BOVIN          QFAITKALSDAEGGRPRSPDISKVVIVVTDGRPQDSVRDVSARARAGGIELFAIGVGRVD 185
TR|W5NVN2|W5NVN2_SHEEP          QFAITKALSDAEGGRPRSPDISKVGAHRTDIGGGGSHIHVGREARP--SHIPPLRVGRVD 186
TR|I3L5Q7|I3L5Q7_PIG            HFAITKALSDAEGGRPRSPDISKVVIVVTDGRPQDSVRDVSARARASGIELFAIGVGRVD 184
TR|F6QY08|F6QY08_HORSE          QFAITRAFSEGEGGRARSPDISKVVIVVTDGRPQDSVRDVSARSRASGIELFAIGVGRVD 182
SP|P05099|MATN1_CHICK           QFAISRAFSDTEGARLRSPNINKVAIVVTDGRPQDGVQDVSARARQAGIEIFAIGVGRVD 182
                                :***::*:*: **.* ***:*.**    **    ..  .*. .:*    .:  : *****

SPP1
SP|P31096|OSTP_BOVIN            SSEEKQLNNKY------PDAVATWLKPDPSQKQTFLA--PQNSVSSEETDD-NKQNTLPS 79
SP|Q9XSY9|OSTP_SHEEP            SSEEKQLNNKY------PDAVATWLKPDPSQKQTFLE--PQNSVSSEETDD-NKQNTLPS 79
SP|P14287|OSTP_PIG              SSEEKLLSNKY------TDAVATLLKPDPSQKQTFLA--PQNTISSEETDD-FKQETLPS 79
TR|F7AYC1|F7AYC1_HORSE          SSEEKQLYNKH------SDAVSIWLKPDPSQKQNLLA--PQT-VSSEETDN-LKQETLPS 79
SP|P23498|OSTP_CHICK            SSEEKYDPRSHHTHRYHQDHVDSQSQEHLQQTQNDLASLQQTHYSSEENADVPEQPDFPD 89
                                *****   ..:       * *    : . .* .*. *    *.  ****. :  :*  :*
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Figure 11. Sequence alignment of tissue-specific ruminant meat and bone protein sequences identified via bioinformatics.

Table 11. Selected ruminant- and tissue-specific tryptic marker peptides for the development of immunoaffinity-based mass spectrometric assays.

Protein	Gene	Tissue	Peptide sequence
Alpha-2-macroglobulin	A2M	blood	GSGGTAEHPFTVEEFVLPK
Complement component 9	C9	blood	YTPVEAIEK
Alpha-2-antiplasmin	SERPINF2	blood	LPPLSLLK
Protein HP-25 homolog 2	HP252	blood	FGFDIELFQHAVK
Myosin-7	MYH7	meat	MLSSLFANYAGFDTPIEK
Matrilin-1	MATN1	cartilage	AGGIELFAIGVGR
Osteopontin	SPP1	milk, bone	YPDAVATWLKPDPSQK

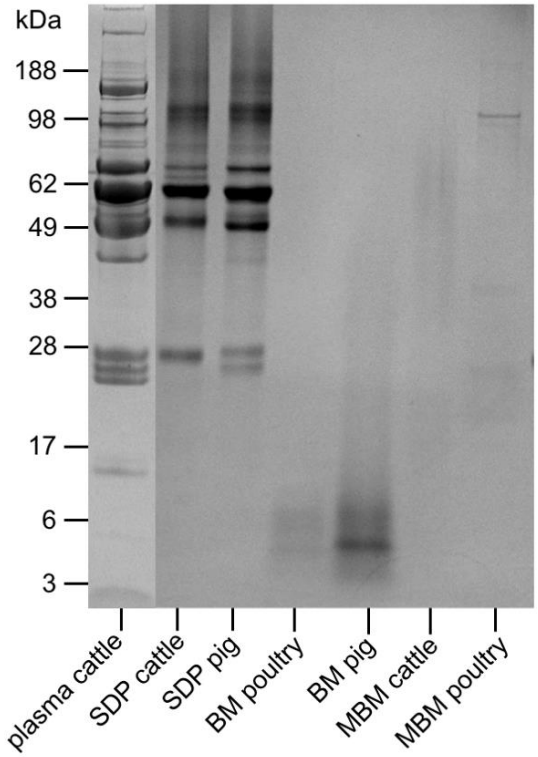
4.2 Sample Preparation of Processed Animal Proteins

4.2.1 Animal Protein Extracts Analyzed by Gel Electrophoresis

The extent of fragmentation in processed animal proteins was analyzed by lithium-dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE). A phosphate buffered saline extraction was performed with SDP, BM and MBM samples of different species origin. The total protein and peptide concentration was estimated by an absorption measurement at 280 nm and equal amounts of the water soluble and extractable fraction were loaded on the gel.

The result of the Coomassie-stained gel is shown in Figure 12. The bovine citrate plasma sample showed a typical band pattern as it was expected. Clear sharp bands of different intensity over the whole molecular weight range were observed. In comparison, the SDP samples still showed high molecular weight proteins, however the bands were blurrier rather than clear, indicating a partial fragmentation of the proteins. In the highly processed PAP samples, BM from porcine and poultry origin and MBM from bovine and poultry origin, little to no protein bands were observed. Since the absorption measurement confirmed the presence of proteins, the extracted protein amount must have been highly fragmented, leading to no visible protein bands within the observed molecular weight range.

A



B

Sample	Protein content / µg µL^{-1}
SDP cattle	4.31 ± 0.02
SDP pig	3.96 ± 0.01
BM poultry	0.66 ± 0.01
BM pig	2.92 ± 0.02
MBM cattle	1.09 ± 0.01
MBM poultry	1.52 ± 0.02

Figure 12. Gel electrophoresis (LDS-PAGE) of different animal protein extracts compared to bovine citrate plasma (A).The result of the A280 protein determination in triplicate measurements is shown in the right table (B). A total amount of 10 µg was loaded on the gel.

4.2.2 A280 Method Evaluation for Complex Sample Analysis

The A280 measurement was considered as a fast and easy way to determine the total peptide content in the supernatant after direct digestion of PAPs. Usually, the method is used for pure proteins with known extinction coefficients [106]. The procedure's suitability for the protein determination in complex mixtures such as blood plasma was evaluated in this thesis. Citrate plasma was measured by bicinchoninic acid assay (BCA) as a reference method and by the A280 method with application of the rule 1 Abs ≈ 1 mg mL^{-1} [106].

The result of the protein determination, the deviation to the reference method and the protein purity of plasmas is shown in Table 12. For most species' citrate plasma, the A280 method achieved a similar result to that of the BCA determination. The maximum deviation was observed in the pig plasma with +13.4% and in the goose plasma with -11.0%. The correlation of the two methods is shown in Figure 13. A correlation coefficient (Pearson) of 0.90 was observed.

Table 12. Protein determination of different species' citrate plasma using the bicinchoninic acid assay (BCA) and the A280 method in triplicate measurements.

Species	BCA assay		A280		Deviation
	mean / mg mL^{-1}	C.V. / %	mean / mg mL^{-1}	C.V. / %	A280/BCA / %
Pig	54.2	3.6	62.6	1.4	+13.4
Cattle	67.6	5.0	67.3	0.1	-0.5
Horse	66.4	4.4	69.2	0.1	+4.1
Turkey	50.1	4.8	54.0	0.2	+7.4
Chicken	41.1	4.8	43.8	0.4	+6.1
Goat	68.1	5.6	62.0	0.2	-9.9
Sheep	68.6	3.9	65.9	0.1	-4.0
Goose	53.3	3.7	48.0	0.6	-11.0
Duck	47.0	3.3	47.8	0.3	+1.7

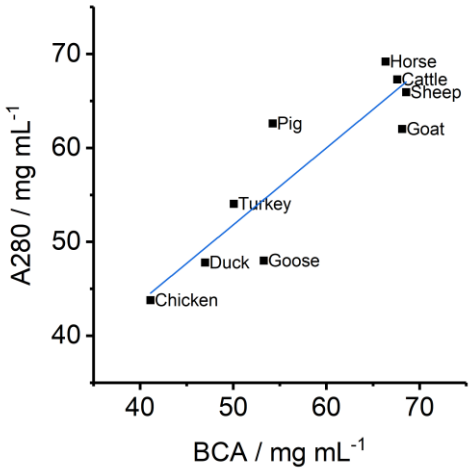


Figure 13. Correlation of bicinchoninic acid assay (BCA) and the A280 method for citrate plasma protein quantification.

Possible interfering substances and reagents at 280 nm were evaluated. Nucleic acids can interfere with the protein determination at 280 nm. The ratio of A260 and A280 absorption was used to calculate the protein purity. In citrate plasma the protein purity was determined with >99% and therefore nucleic acids did not interfere in plasma samples (Table 13). The protein purity determination was repeated with PAPs and feed matrices. The lowest protein purity was observed for HPD-prepared fish feed with 90.1%. Since the protein purities are determined to be >90% a correction was not considered necessary.

Table 13. Protein purity of different sample types determined by A260/A280 ratio.

Sample type	A260/A280	Protein purity / %
Citrate plasma	0.58	99.9
Fish feed	1.32	90.1
Vegetal feed	1.24	92.0
Spray-dried plasma	0.65	99.4
Blood meal	0.82	97.9
Meat and bone meal	1.31	90.4

During enzymatic digestion several reagents were added. Absorption caused by the buffer or the added reagents was evaluated. Neither the digestion buffer nor the used detergent and reducing agent showed an absorption at 280 nm (Figure 14). However, when iodoacetamide was added, the absorption increased. The protease inhibitor PMSF, which was used to stop the enzymatic fragmentation, showed no additional absorption. The UV absorption due to added iodoacetamide and the enzyme should be blanked using a blank digest.

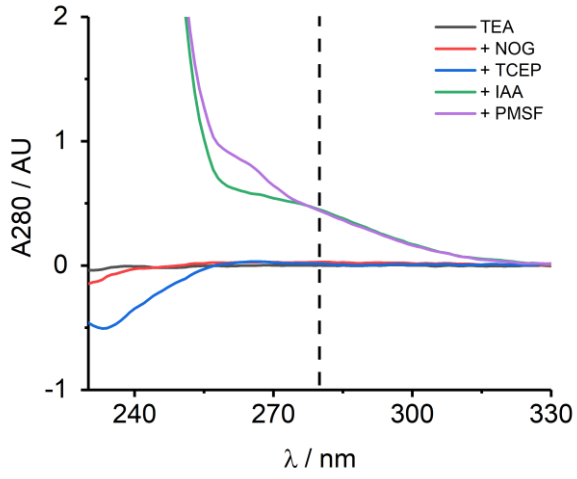


Figure 14. UV absorption spectra of reagents used for tryptic digestion.

4.2.3 Evaluation of Heterogeneous Phase Digestion

Optimization of Enzymatic Fragmentation Parameters

The direct digestion of PAPs in suspension was optimized regarding the sample amount, mixing speed, trypsin type and enzyme ratio to sample (Figure 15 A-D). The parameters were checked for a porcine BM and a porcine SDP in an overnight incubation. The absorption at 280 nm in the supernatants was monitored to evaluate the parameters' effects. Varying the sample amount showed a linear relationship between the initial sample weight and the absorption at 280 nm. The SDP sample showed a slight saturation at 25 mg. The variation of the rotational speed showed higher absorption values with higher rotation speed. The digestion in suspension was supposed to be affected by the degree of mixing and the liquid-solid interface. The trypsin product of two manufacturer had no effect. However, an enzyme-to-sample ratio of 1:40 seemed to be more efficient than a ratio of 1:20. As final settings for HPD, a sample amount of 1 mg per 750 µL corresponding to 20 mg mL^{-1}, 1000 rpm and Worthington trypsin in a ratio of 1:40 were chosen.

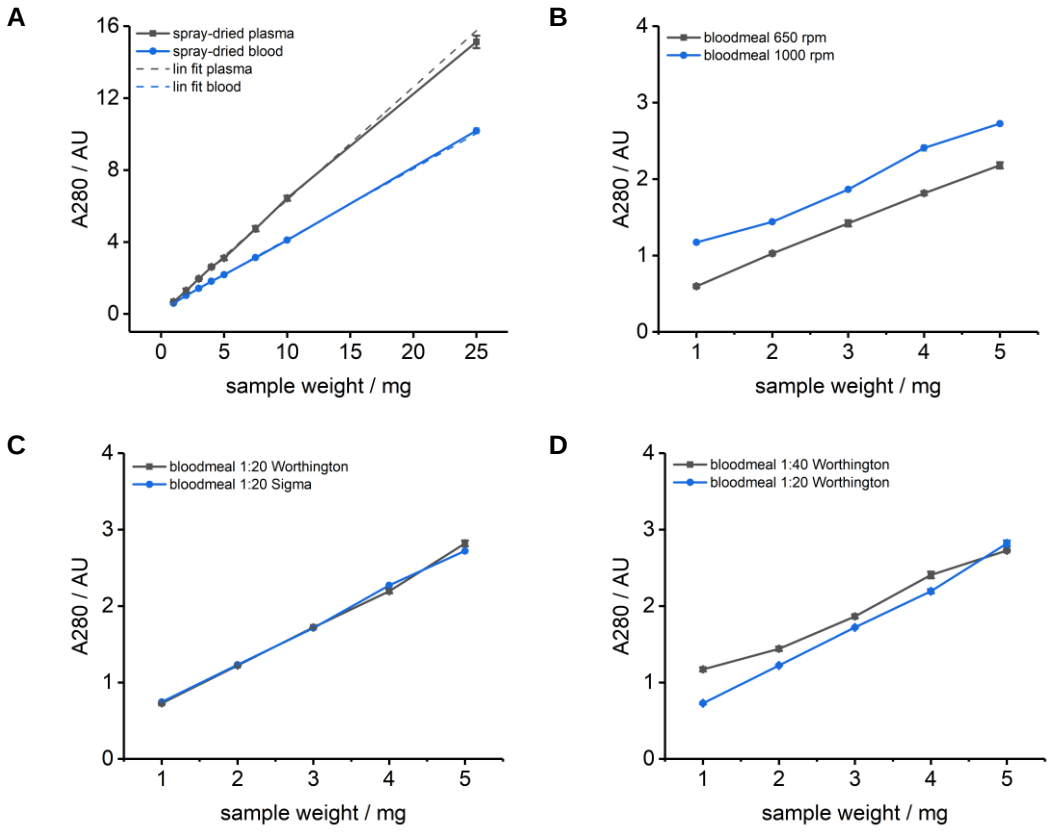


Figure 15. Heterogeneous phase digestion (HPD) parameter optimization in terms of sample amount (A), mixing speed (B), enzyme manufacturer (C) and enzyme ratio to sample (D).

Application of HPD to Different Animal Protein Samples

Using the optimized parameters, HPD was applied to different PAP and blood product types for different treatment times. The peptide release was determined by 280 nm absorption referred to the initial sample weight and its protein content. Typical protein content of the analyzed sample types known from literature are within the range of 67-78% for SDP [110] and 83-93% for BM [110] and 48-56% for MBM [14]. The results were normalized to the mean values of the protein content ranges: 72.5% for SDP, 88% for BM and 52% for MBM.

The total protein release was quite stable for a period from 2 h to 42 h HPD and did not increase with longer digestion times. The highest peptide release with mean of 93% was observed for the group of six SDP samples (Figure 16). SDPs tended to form gels when aqueous buffers were added, however when overnight HPD was applied, the SDPs completely dissolved. Mean releases of 39.9% and 44.5% were observed for BM deriving from pig and poultry, respectively. The MBM samples showed mean releases of 44.5% for poultry and 22.3% for bovine MBM (Table 14).

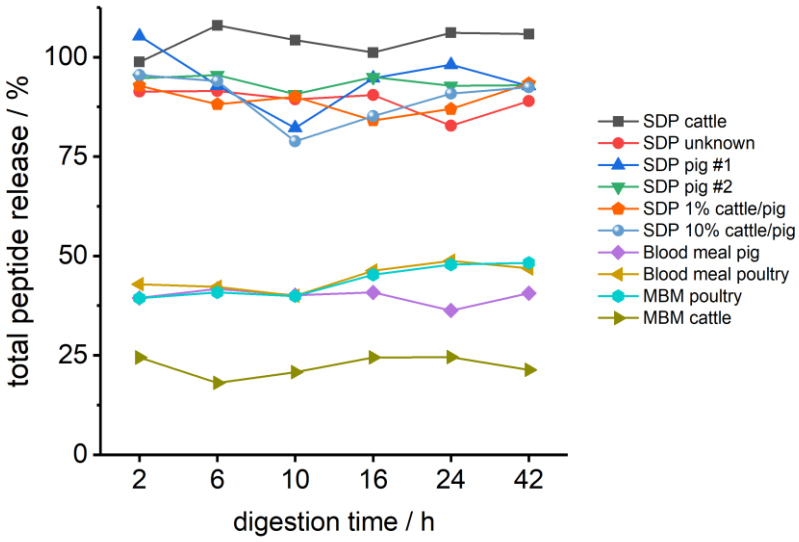


Figure 16. Heterogeneous Phase Digestion applied to different animal protein types for varying treatment times. Total protein release is expressed as percent yield normalized to the initial sample amount and its mean protein content known from literature, 72.5% for spray-dried plasma (SDP), 88% for blood meal (BM) and 52% for meat and bone meal (MBM) [14,110].Single measurements were performed for each digestion time.

Table 14. Mean total peptide release from different animal protein sample types after heterogeneous phase digestion (HPD) in a time frame from 2 h to 42 h. Two spray-dried plasma (SDP), two blood meals (BM) and two meat and bone meals (MBM) of different species origin were analyzed.

Sample	Mean A280 / µg µL^{-1}	Mean protein yield / %
SDP cattle	15.1 ± 0.5	104.1 ± 3.2
SDP pig #1	13.7 ± 1.0	94.4 ± 6.9
BM poultry	7.8 ± 0.5	44.5 ± 3.0
BM pig	7.0 ± 0.3	39.9 ± 1.7
MBM poultry	4.5 ± 0.4	44.5 ± 3.0
MBM cattle	2.3 ± 0.3	22.3 ± 2.4

Comparison of Extraction with In-Solution Digestion to HPD by UV-monitoring

Furthermore, it was assessed if the HPD is not simply a buffer extraction with the digestion of dissolved proteins but more a direct digestion at the liquid-solid interface. Therefore, the buffer extraction with in-solution digestion (ISD) of the supernatant after centrifugation was compared to the direct digestion in suspension (HPD). As indicated by absorption at 280 nm, HPD significantly released more total peptide compared to ISD with a factor of 2 in a bovine MBM and a porcine BM. In the porcine SDP sample, HPD improved the total peptide released by a factor of 5.6 (Figure 17).

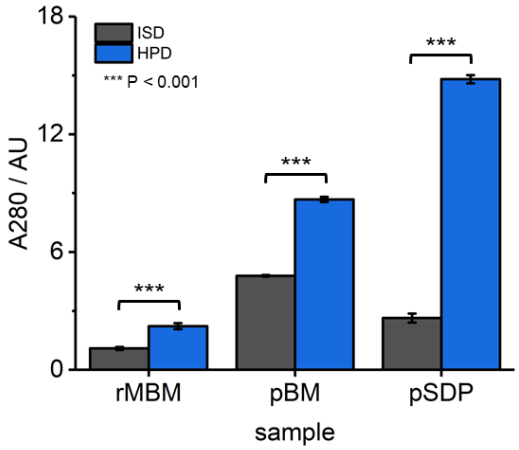


Figure 17. Protein determination via A280 method after application of heterogeneous phase digestion (HPD) and extraction with in-solution digestion (ISD) to the sample types porcine blood meal (pBM), porcine spray-dried plasma (pSDP) and ruminant meat and bone meal (rMBM) in six replicates.

Mass Spectrometric Evaluation of Heterogeneous Phase Digestion

A more detailed comparison of HPD, ISD and the state of the art TCA/acetone (TCA) protocol was performed using a bovine MBM. Here, in addition to the determination by A280 absorption, a detailed non-targeted MS analysis was performed. The non-targeted analysis allowed to draw a conclusion whether HPD only releases more of the same peptides or more different peptides.

The protein content after TCA/acetone extraction was determined with Coomassie Plus (Bradford) assay. 100 µg of extract was then digested according to the ISD protocol, giving the final protein concentration of 0.13 µg μL^{-1} (Table 15). The highest extraction efficiency determined by the protein concentration in the final digest was observed for HPD with 2.22 µg μL^{-1}, followed by the ISD protocol showing half the amount of HPD with a concentration of 1.09 µg μL^{-1}.

Table 15. Protein determination after the application of three different sample preparation protocols to a ruminant meat and bone meal. Three replicates were performed.

Protocol	Mean protein / µg μL^{-1}	SD / µg μL^{-1}	C.V. / %
TCA/acetone	0.13	0.002	1.14
HPD	2.22	0.150	6.84
ISD	1.09	0.080	6.93

A total amount of 1 µg of each digest was subjected to a non-targeted LC-MS/MS analysis. Three runs per sample preparation protocol were performed. The number of identified proteins is shown in Figure 18. The number of identified peptides is given in brackets. The analysis of a bovine MBM using the HPD protocol revealed five times more peptides and three times more proteins compared to ISD. The total number of peptides identified by HPD was 1024 and only 193 by ISD preparation. The numbers of identified proteins are 267 and 86, respectively. A total number of 146 peptides and 91 proteins were identified using the TCA/acetone protocol. The results show that HPD releases not only twice the amount compared to a buffer extraction but also significantly more peptides from a highly processed MBM sample.

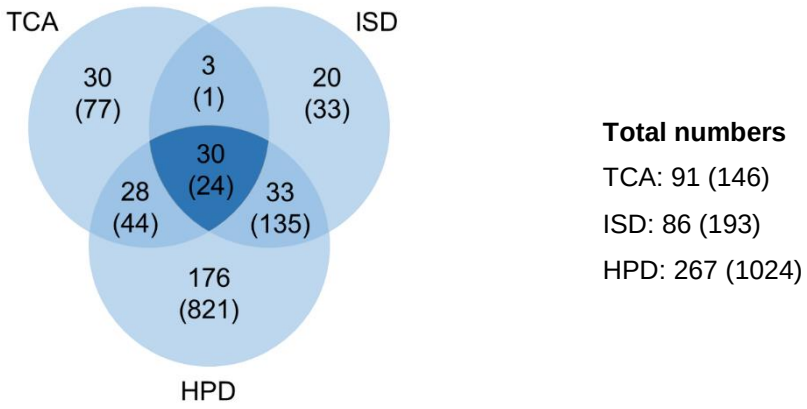


Figure 18. Number of identified proteins and peptides (given in brackets) via non-targeted mass spectrometric analysis of a bovine meat and bone meal after the application of the different sample preparation methods TCA/acetone, extraction with in-solution digestion (ISD) and heterogeneous phase digestion (HPD). Three non-targeted MS runs were performed for each preparation protocol.

4.3 Development of Multiplex Immuno-MS Assays

4.3.1 Multiplex Panel and Chromatographic Separation

The selected marker peptides were compiled in different multiplex assays with different chromatographic gradients. Eight species-specific homologous A2M peptides were compiled in one 8-plex assay to identify the species origin of blood-derived animal protein samples. The three highly ruminant-specific plasma peptides and the bovine A2M peptide were compiled in one 4-plex assay to analyze blood product samples for ruminant protein ingredients. The tissue-specific ruminant peptides from MYH7, MATN1 and SPP1 were compiled together with the plasma-specific target for C9 in another 4-plex assay to analyze MBM for ruminant proteins. The two 4-plex assays, both comprising C9, were finally merged into one 7-plex assay to analyze unknown animal proteins for ruminant contaminations deriving from milk, blood products or MBM. The gradients and the chromatographic separation of the marker peptides is shown in Figure 19. Since the samples were immunoaffinity purified, short 5 min chromatographic gradients with a 5 min column flush and equilibration step could be developed. This allowed a sample cycle time of only 10 min.

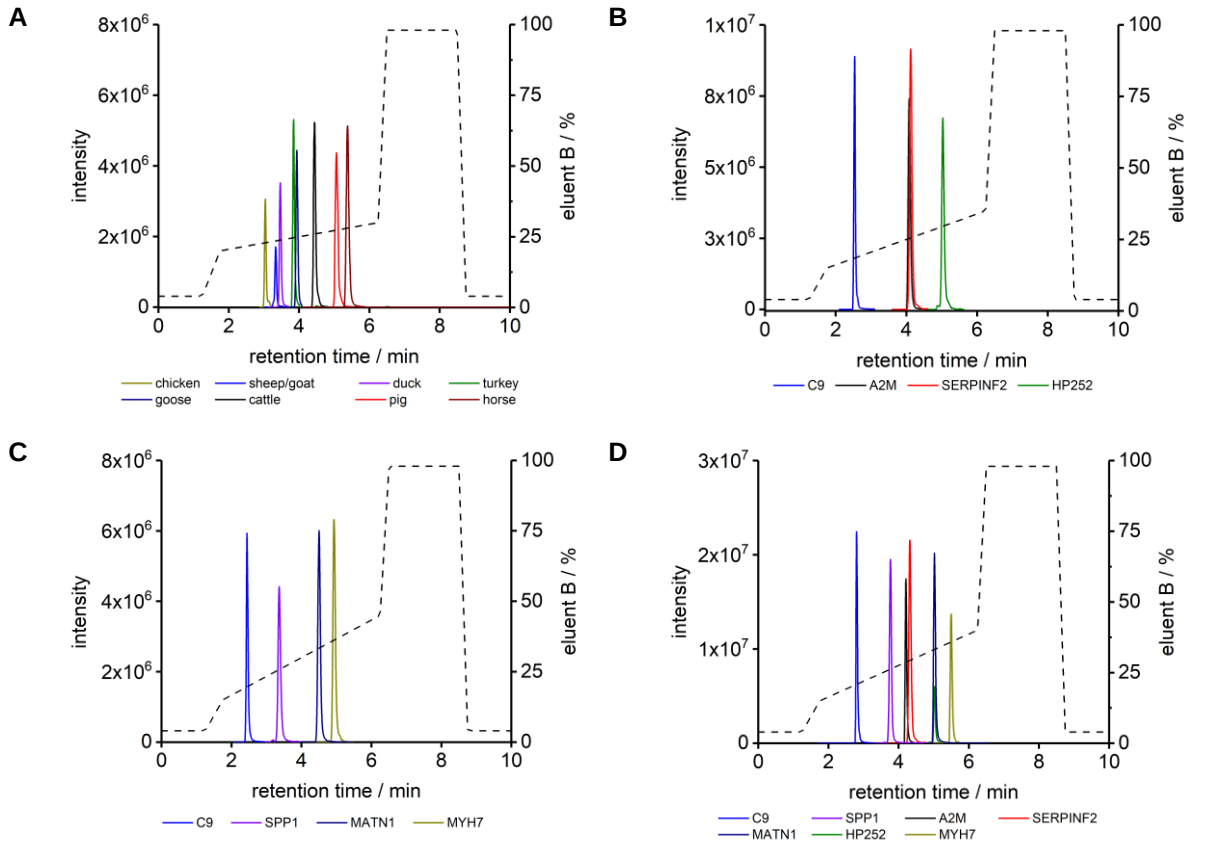


Figure 19. Chromatographic separation of the marker peptides compiled in four multiplex assays: 8-plex assay XA2M targeting homologous peptides from different species (A), 4-plex assay RQ1 targeting ruminant-specific plasma peptides (B), 4-plex assay RQ2 targeting tissue-specific ruminant peptides (C) both combined in 7-plex assay RQ3 (D).

4.3.2 Determination of Peptide Ionization and Fragmentation Properties

The ionization properties of the chromatographically separated peptides were analyzed. A full MS scan of standard peptides was performed and each peptide's charge state with the higher signal intensity was chosen for targeted experiments (Supplementary Data B). In a second targeted experiment the fragmentation properties of the chosen precursor m/z were analyzed. Different normalized collision energies (NCE) settings were checked: 15, 20, 25 and 30. Data analysis was performed for single and double charged fragment ions. To discriminate the analytes from the isotope labeled standards, only y-ions were considered since the isotope labeling was on the peptide C-terminus. The result of the collision energy optimization is shown in Supplementary Data D. Table 16 shows the m/z and charge state of all precursor ions along with the m/z and charge states for the three most intense fragment ions of each peptide analyzed in this work.

Table 16. Selected marker peptides with precursor charge states and its most intense fragment ions.

Protein	Peptide sequence	Precursor m/z	Most intense fragments m/z
C9	YTPVEAIEK	525.2793 p++	785.4403 y7+ 688.3876 y6+ 589.3192 y5+
SERPINF2	LPPLSLLK	440.7969 p++	384.2549 y7++ 670.4498 y6+ 335.7285 y6++
HP252	FGFDIELFQHAVK	517.6049 p+++	971.5309 y8+ 842.4883 y7+ 729.4042 y6+
MYH7	MLSSLFANYAGFDTPIEK	1002.4928 p++	1472.7056 y13+ 1325.6372 y12+ 486.2922 y4+
MATN1	AGGIELFAIGVGR	630.3590 p+++	719.4199 y7+ 961.5465 y9+ 832.5039 y8+
SPP1	YPDAVATWLKPDPSQK	605.9807 p++	459.2562 y4+ 671.3359 y6+ 778.4094 y14++
A2M, Cattle	GSGGTAEHPFTVEEFVLPK	668.0021 p+++	929.4727 y17++ 1305.7089 y11+ 861.4716 y7+
A2M, Sheep/Goat	ESGGTAEHHFTVEEFVLPK	705.3445 p+++	949.4758 y17++ 1061.5877 y9+ 861.4716 y7+
A2M, Pig	VVVQQESGETAEHPFTVEEFVLPK	900.4569 p+++	1137.5499 y20++ 1073.5206 y19++ 1073.5206 y19++
A2M, Horse	AEHPFIVEEFVLPK	552.2995 p+++	861.4716 y7+ 732.4291 y6+ 603.3865 y5+
A2M, Chicken	TIHHPFSVEEYVLPK	599.3174 p+++	877.4666 y7+ 1307.688+ y11+ 748.4240 y6+
A2M, Turkey	TIQHPFTVEEYVLPK	600.9892 p+++	877.4666 y7+ 1321.7038 y11+ 748.4240 y6+
A2M, Goose	TIQHPFSVEEYVLPK	596.3173 p+++	877.4666 y7+ 1307.6882 y11+ 748.4240 y6+
A2M, Duck	IQHSFSVEEYVLPK	559.2945 p+++	877.4666 y7+ 748.4240 y6+ 619.3814 y5+

4.3.3 Antibody Functionality in Feed Matrices

During the antibody generation process, the functionality of the polyclonal antibodies in rabbit serum was evaluated. After purification, the polyclonal antibodies were tested in phosphate buffered saline (Supplementary Data E). However, complex feed matrices can have an effect on the antibody functionality by cross-reactive epitopes. To assess possible matrix effects, 50 fmol of each isotope labeled standard peptide was measured in a background of different known amounts of digested feed matrix.

Figure 20 shows the signal intensities of 8 species-specific A2M peptides, captured by the cross-species antibody at different background levels of digested fish feed. The fish feed matrix had no drastic effect on the measured signal intensity of the standard peptides, indicating that the antibody functionality was not affected. Rather the opposite was observed: The log2-transformed fold changes in Figure 21 show a consistent improvement of the signal intensity for all peptides. Nonetheless, the effects can be considered low as indicated by the mean 95% significance borders shown as dashed horizontal lines.

Figure 22 shows the results for the ruminant standard peptides in vegetal feed matrix. The peptide-antibody-pairs showed very different results in this feed matrix. The log2-transformed fold changes in Figure 23 clearly show a steady decrease in SERPINF2 signal intensity with higher feed matrix amounts. It can be assumed that this antibody was affected by the vegetal feed matrix and the marker peptide was enriched with a lower efficiency. There were also opposite effects: HP252 and A2M peptides were detected with a higher signal intensity up to 10 µg matrix and then, a decrease was observed. Nevertheless, the signals were still higher than in the buffer control with 0 µg feed matrix. However, with the exception of SERPINF2, the effects were also considered low as indicated by the mean 95% significance borders

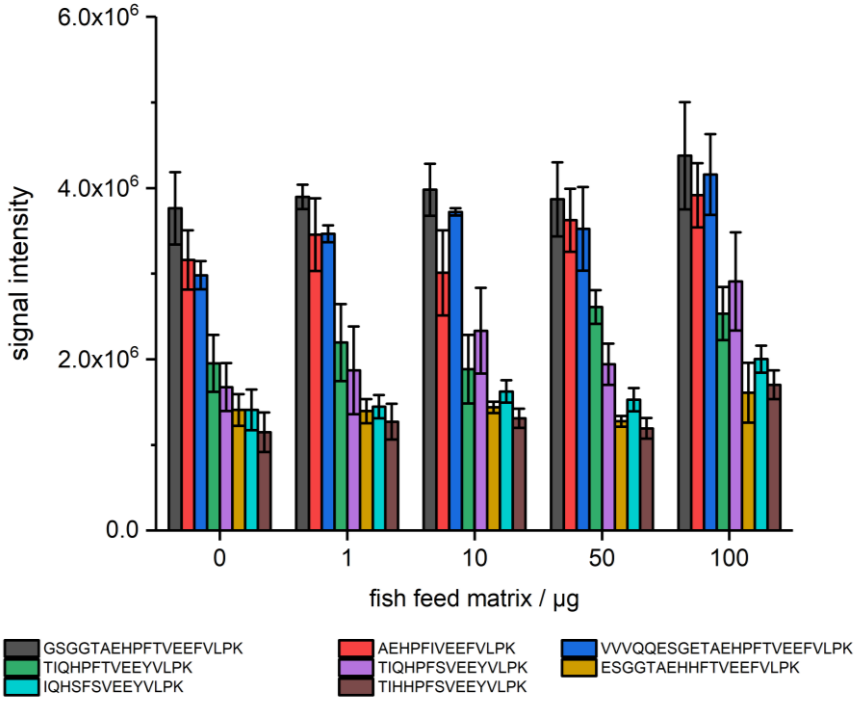


Figure 20. Stable isotope labeled standard signal intensities measured by multiplex XA2M at different amounts of fish feed matrix in triplicates.

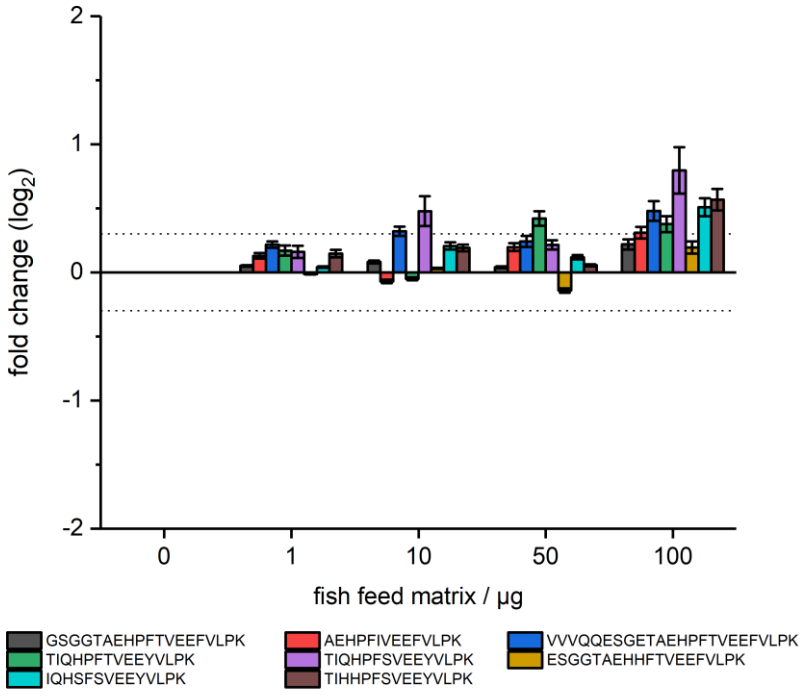


Figure 21. log_2-transformed fold changes in stable isotope labeled standard signal intensities measured by multiplex XA2M at different amounts of fish feed matrix.The mean 95% significance levels are shown as horizontal lines.

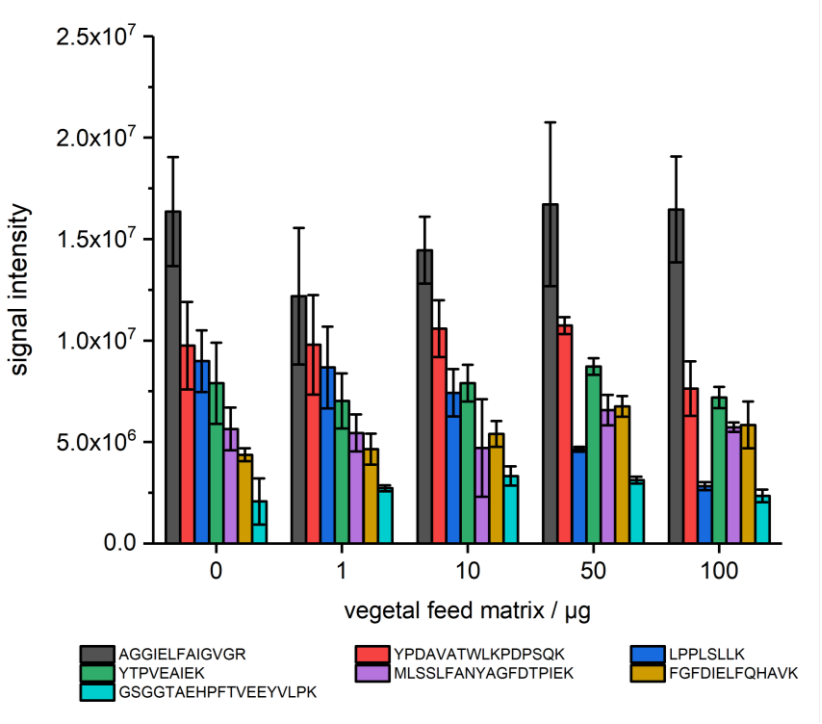


Figure 22. Stable isotope labeled standard signal intensities measured by multiplex RQ3 at different amounts of vegetal cattle feed matrix in triplicates.

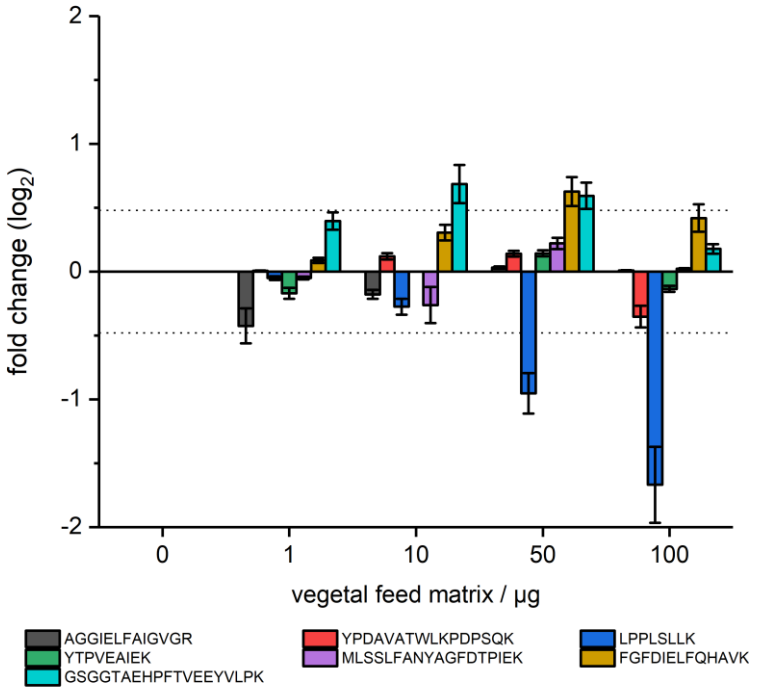


Figure 23. log_2-transformed fold changes in stable isotope labeled standard signal intensities measured by multiplex RQ3 at different amounts of vegetal cattle feed matrix. The mean 95% significance levels are shown as horizontal lines.

4.3.4 Heterogeneous Phase Digestion in Targeted Analyses

In section 4.2.3 it was shown that the HPD protocol is superior to an extraction in the same buffer system. HPD released more proteins which was shown in A280 measurements in the supernatant and in non-targeted MS analyses. However, A280 monitoring and non-targeted MS do not indicate the improve in terms of marker peptide release. Since the release of marker peptides is important for an accurate, precise and sensitive quantification, the efficiency of ISD and HPD in different sample types was determined by an analysis with developed multiplex assays.

The multiplex assay RQ3 was applied to an ISD- and HPD-prepared MBM to analyze the marker peptides for ruminant detection. The multiplex assay XA2M was applied to ISD- and HPD-prepared porcine BM and SDP samples (Figure 24). For the ruminant peptides, very different results were observed. The SPP1 peptide increased by a factor of 1.6, C9 by a factor of 2.6 and SERPINF2 by a factor of 5.7. There were also very drastic increases: The signals for HP252, A2M MYH7 and MATN1 after ISD preparation were in the attomole range and therefore near the limit of detection. After the application of HPD, the peptide amounts reached levels in the range of 19.2 fmol to 737 fmol, resulting in very high improvement factors. The HP252 release increased by a factor of 28, A2M by a factor of 113 and MYH7 and MATN1 by factors of 2344 and 1307, respectively. The release of the porcine A2M peptide increased by a factor of 2.8 in the BM sample and 10.5 in the SDP sample.

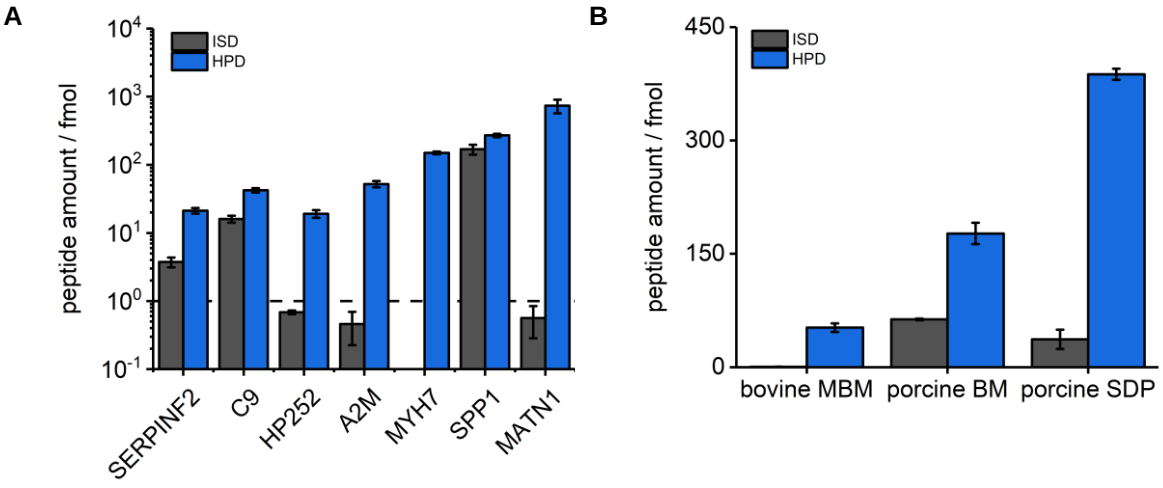


Figure 24. Quantification of seven marker peptides using multiplex RQ3 after application of heterogeneous phase digestion (HPD) and buffer extraction with in-solution digestion (ISD) to a ruminant meat and bone meal (A) and marker peptides for alpha-2-macroglobulin quantified in meat and bone meal (MBM), blood meal (BM) and spray-dried plasma (SDP), respectively (B). Six replicates were prepared for each sample and protocol combination.

4.3.5 Digestion Kinetics

The time dependent release of the marker peptides from different sample types and species was analyzed. In case of the cross-species approach, not for every species a PAP sample was available. Therefore, native citrate plasmas were subjected to an in-solution digestion and the peptide release from the respective proteins was analyzed (Figure 25). Mostly, the peptides showed a relative constant appearance during tryptic digestion up to 42 h. In contrast, the A2M-specific peptides from turkey and goose showed steadily decreasing concentrations after 2 h digestion time. The chicken-specific peptide showed a constant concentration up to 10 h and rapid degradation at overnight digestion (16 h) and longer digestion times. The highest relative mean peptide release was observed to be at 2 h which was therefore chosen as the standard digestion time.

The release from different sample types was determined for the bovine species since bovine plasma, MBM and SDP were available (Figure 25). Citrate plasma and rSDP showed very similar peptide releases. The peptide release from rMBM was slightly different at a digestion time of 16 h, however, the overall trend was the same including the rapid degradation after 24 h to 42 h. The application of HPD to different sample types was considered to be unproblematic.

The peptide release from different ruminant proteins was analyzed by the multiplex assay for ruminant quantification RQ3 (Figure 26). A bovine MBM and a bovine SDP served as samples and were treated by HPD for varying times. Again, some targets showed a constant concentration between 2 h and 42 h of HPD (for example SERPINF2). In contrast, the proteotypic peptides for HP252, MATN1 and MYH7 showed a decreasing concentration over time in the MBM sample. MATN1 and MYH7 were not detected in the SDP sample, however, HP252 showed a decreasing concentration in the SDP sample as well. A digestion time of 2 h was already sufficient to achieve a mean peptide release of 90%, which was the reason to choose this as the standard fragmentation time for HPD in all analyses.

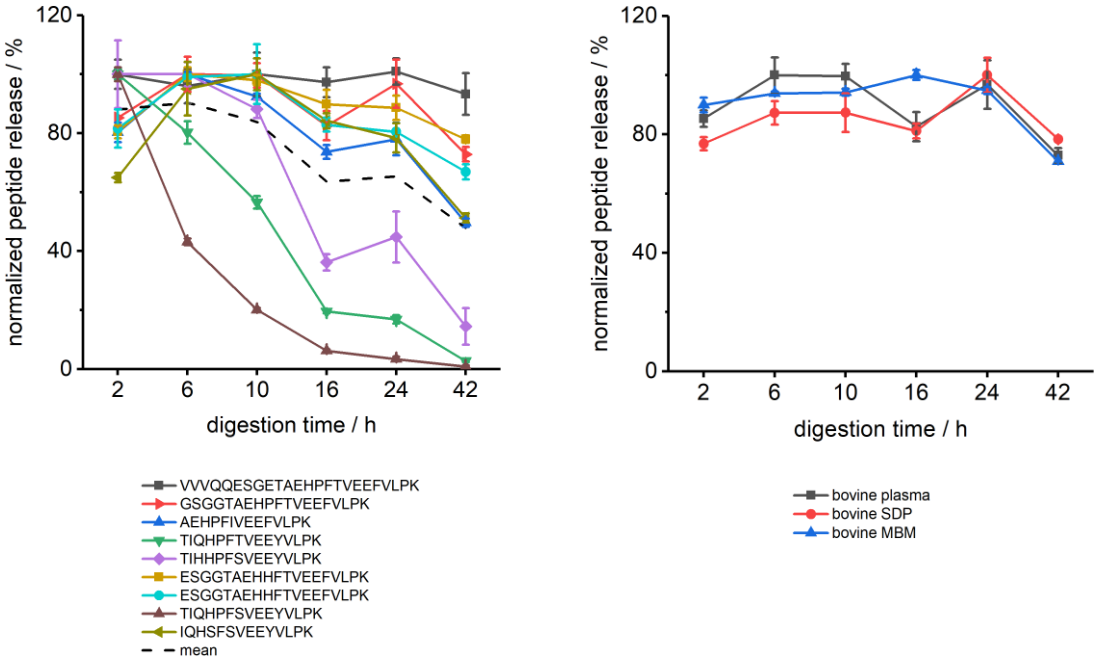


Figure 25. Time dependent marker peptide release from different species' citrate plasma after application of in-solution digestion (ISD) analyzed by multiplex XA2M (left). The bovine peptide released from the different animal protein types citrate plasma, spray-dried plasma and meat and bone meal after application of heterogeneous phase digestion (right). Three replicates were prepared.

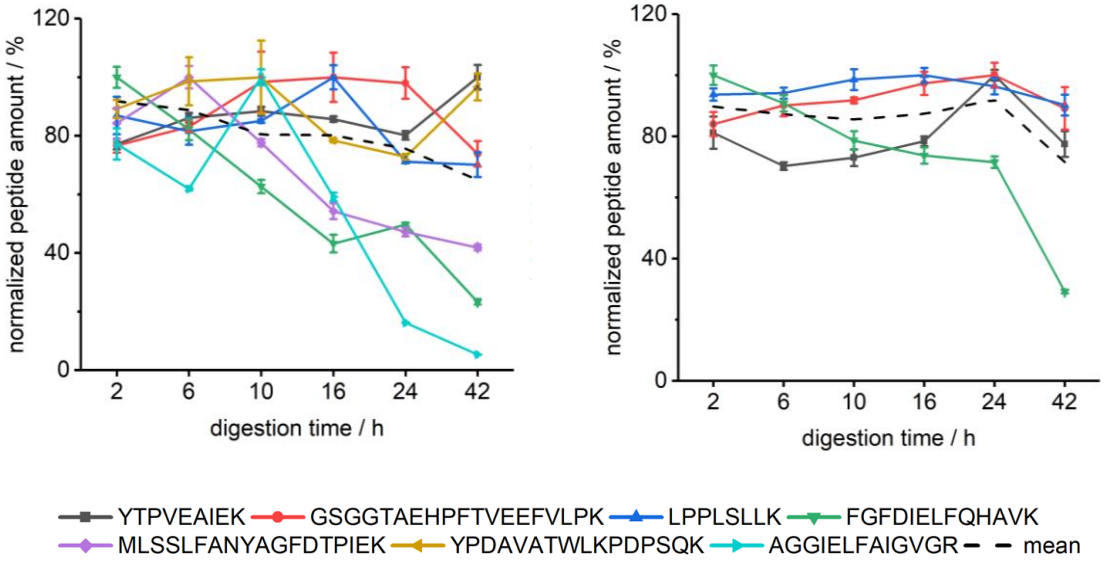


Figure 26. Time dependent marker peptide release after application of heterogeneous phase digestion (HPD) to a bovine meat and bone meal analyzed by multiplex RQ3 (left) and to a bovine spray-dried plasma (right). Three replicates were prepared.

4.3.6 Linearity and Precision in Feed Matrices

Linearity and precision were assessed with a dilution series of the synthetic analyte peptides spiked with a constant amount of isotope labeled standard peptides in digested feed matrices. The prepared samples were measured with the multiplex assays RQ3 and XA2M in both SIM and PRM mode. The signal ratios from light and heavy peptides were plotted against the concentration ratios and a linear regression was performed. Accuracy and precision were calculated using the regression equation and plotted against the total peptide amount in the immunoaffinity step. The limit of detection (LOD) and lower limit of quantification (LLOQ) were determined as described in section 3.10.3. Only PRM data are shown in the following graphs. SIM measurements are shown in Supplementary Data G (RQ3) and H (XA2M).

Multiplex RQ3 showed a linear relationship between the analyte to standard signal ratios and the actual concentration ratios over a concentration range of four to five orders of magnitude in both measuring modes PRM (Figure 27) and SIM (Suppl. Figure 57). The coefficients of determination R^2 ranged from 0.99465 for the bovine MATN1 peptide in SIM to 0.99993 for the bovine A2M peptide in SIM (Table 17). The calculated limits of detection (LOD) ranged from 38 amol for the bovine A2M peptide in PRM to 2.40 fmol for the bovine MYH7 peptide in SIM. For all peptides, the LODs were consistently lower in PRM compared to SIM detection. The lower limits of quantification (LLOQ) were observed in the range from 51 amol for the bovine C9 peptide in SIM (Suppl. Figure 58) to 1.37 fmol for most of the ruminant peptides in PRM (Figure 28).

Multiplex XA2M showed a linear relationship between the analyte to standard signal ratios and the actual concentration ratios over a concentration range of four to five orders of magnitude in both measuring modes PRM (Figure 29) and SIM (Suppl. Figure 63). The coefficients of determination R^2 ranged from 0.98792 for the sheep and goat A2M peptide in SIM to 0.99992 for the turkey A2M peptide in PRM (Table 17). The calculated limits of detection (LOD) ranged from 30 amol for the bovine A2M peptide in PRM to 4.42 fmol for the goose A2M peptide in SIM. With the exception of the porcine A2M peptide, the LODs were consistently lower in PRM compared to SIM detection. The lower limits of quantification (LLOQ) were observed in the range from 152 amol for the chicken A2M peptide in SIM (Suppl. Figure 64) to 37.0 fmol for the sheep and goat A2M peptide in PRM (Figure 30) as well as for the goose A2M peptide in SIM (Suppl. Figure 64).

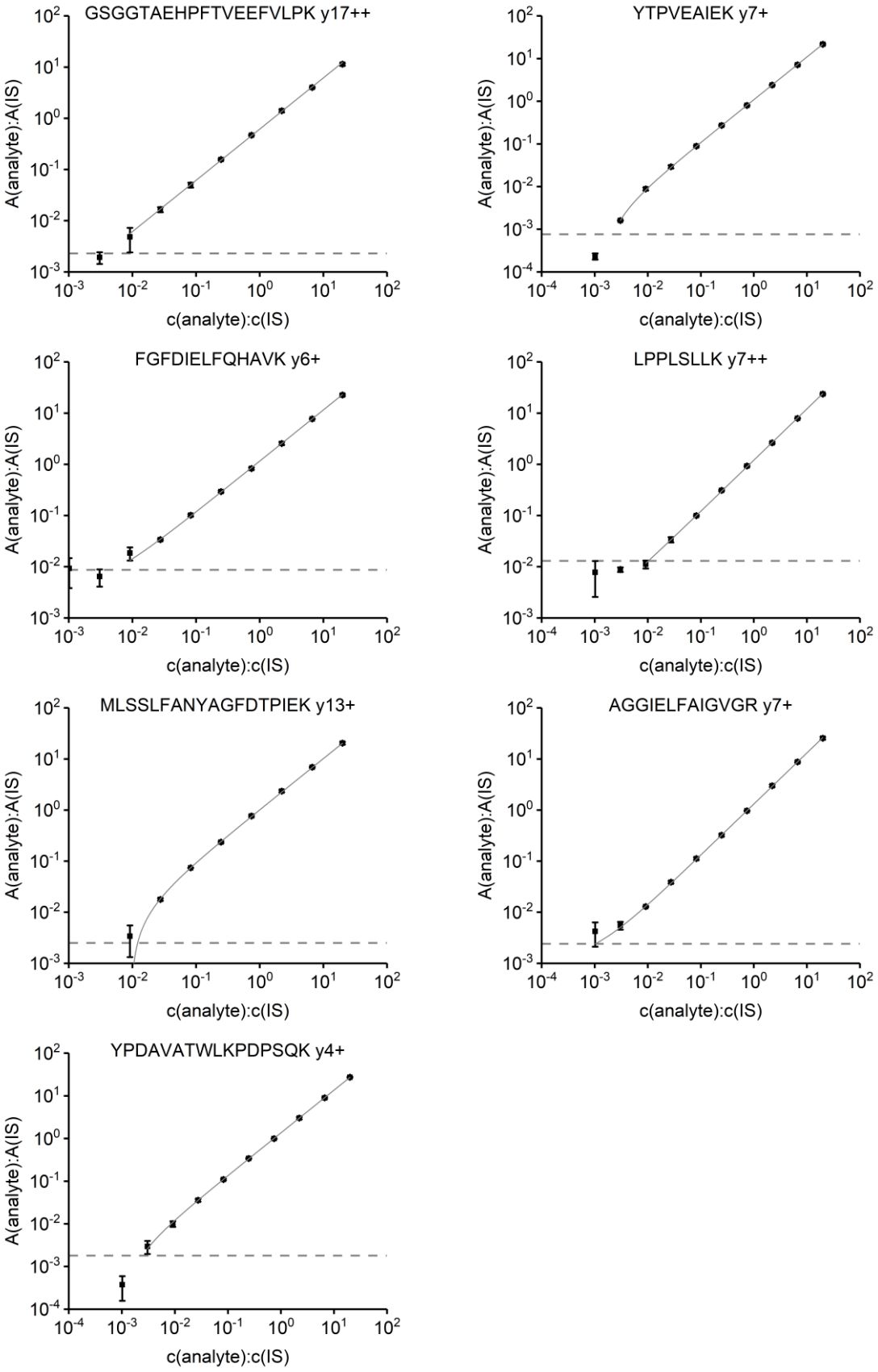


Figure 27. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ3 measured in PRM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

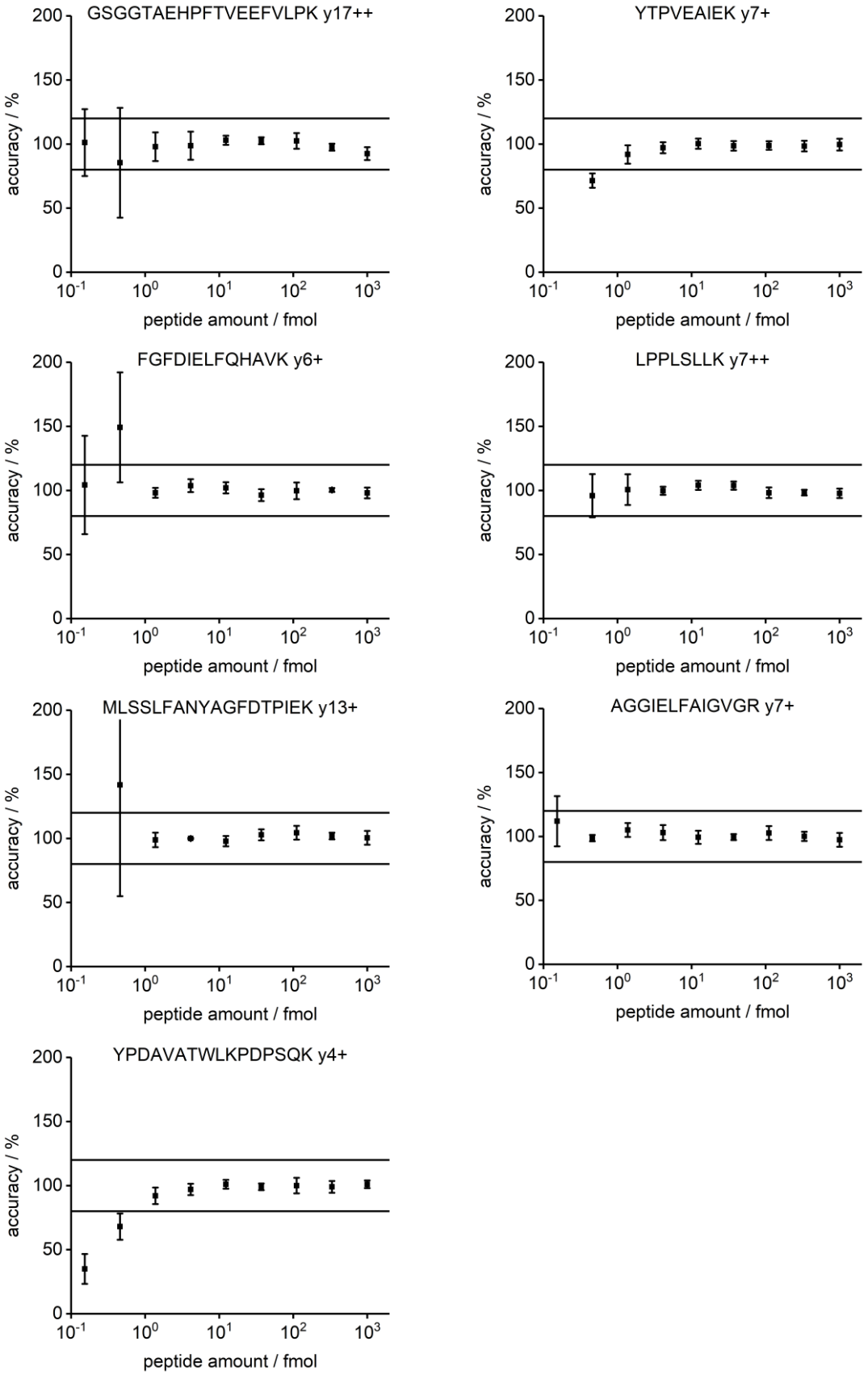


Figure 28. Accuracy and precision of multiplex RQ3 measured in PRM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

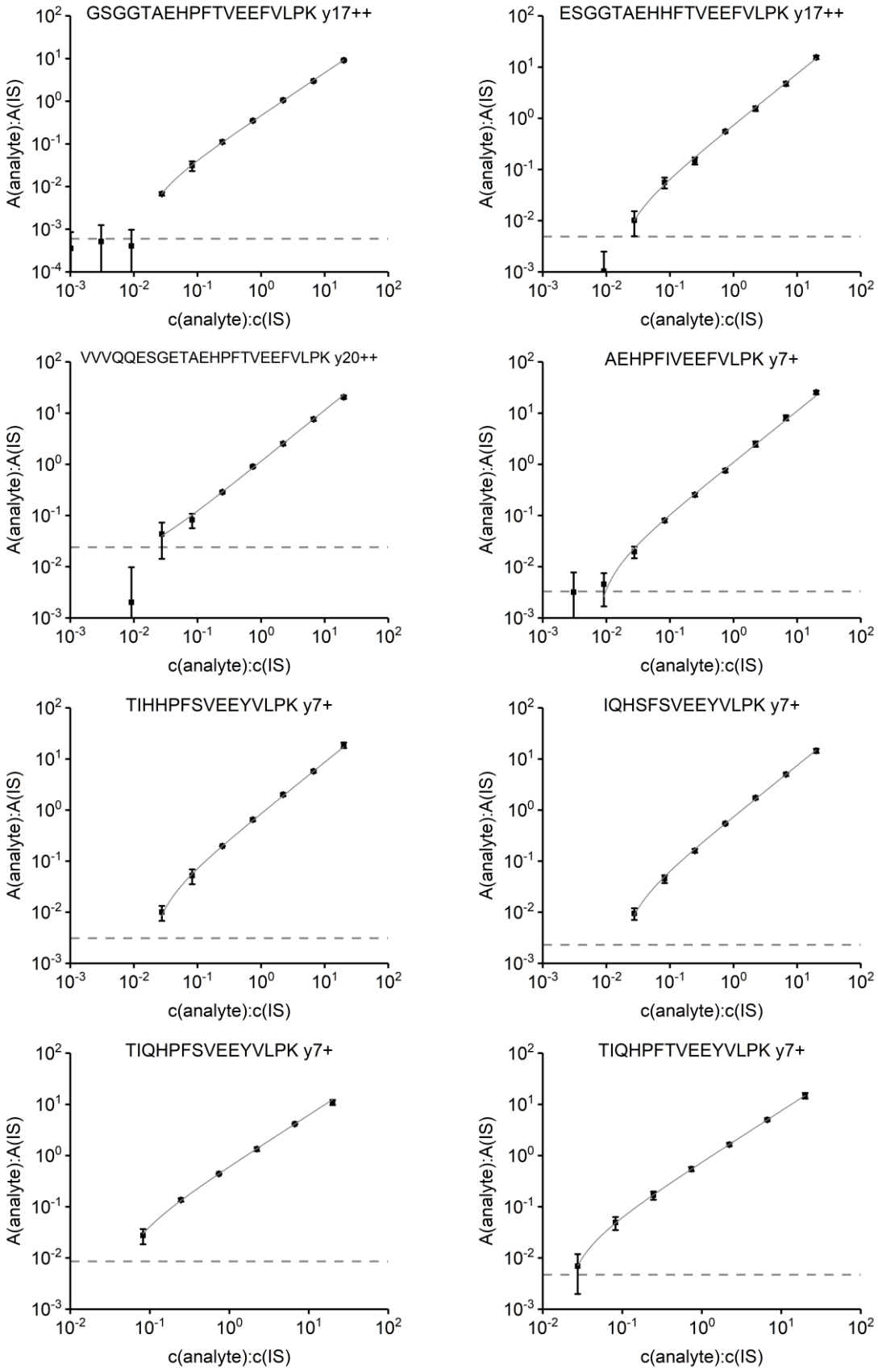


Figure 29. Linearity and limit of detection (shown as dashed horizontal line) of multiplex XA2M measured in PRM mode and fish feed as matrix. Each concentration was prepared as triplicate.

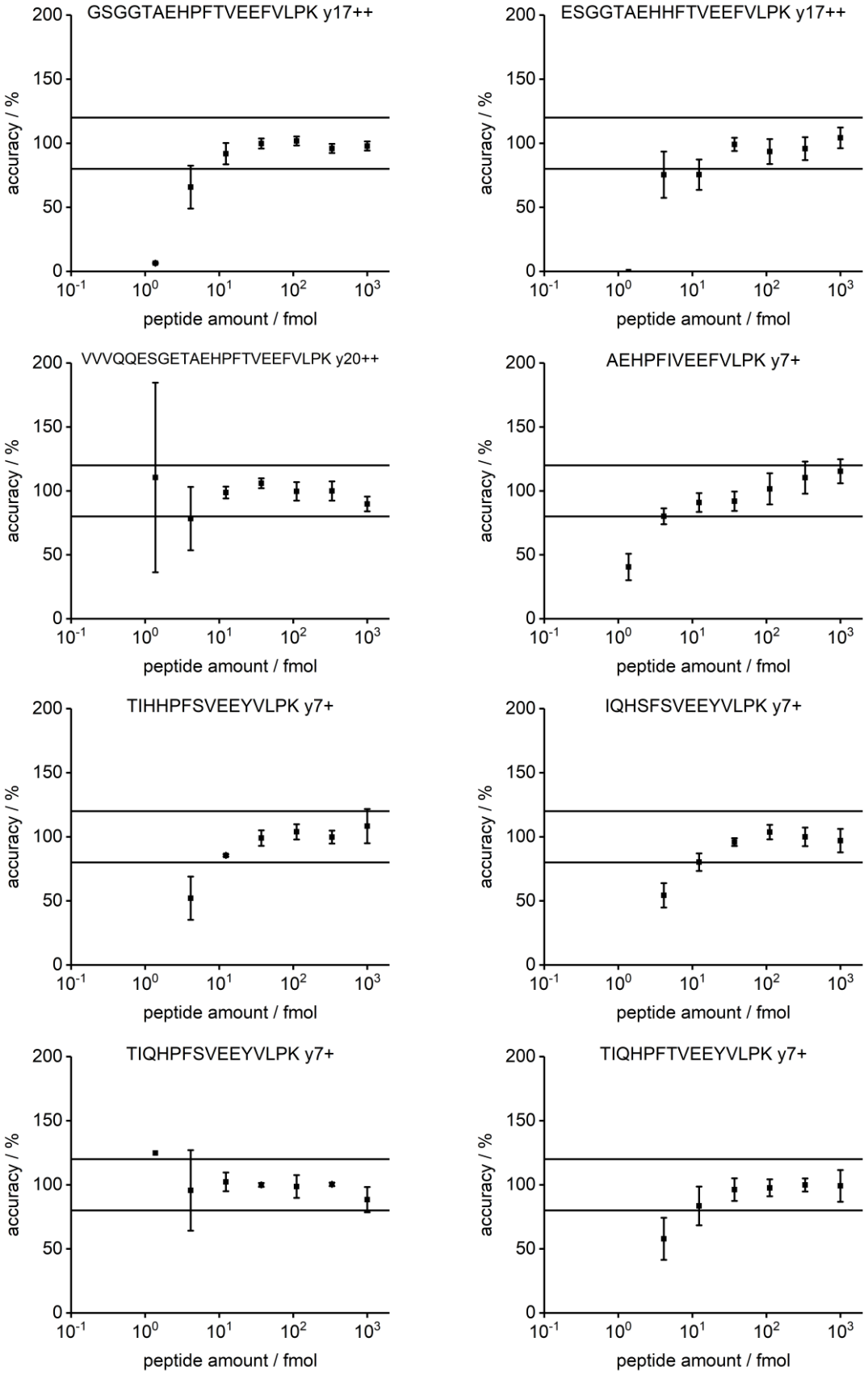


Figure 30. Accuracy and precision of multiplex XA2M measured in PRM mode and fish feed as matrix. Each concentration was prepared as triplicate.

Table 17. Linear regression data and the determined limit of detection (LOD) and lower limit of quantification (LLOQ) of the dilution series prepared in feed matrix and measured by multiplex XA2M and RQ3.

Assay	Peptide sequence	SIM					PRM				
		Slope	Intercept	R^2	LOD / fmol	LLOQ / fmol	Slope	Intercept	R^2	LOD / fmol	LLOQ / fmol
RQ3	YTPVEAIEK	1.02	1.19E-03	0.99786	0.137	0.051	1.09	-1.70E-03	0.99961	0.038	1.372
	LPPLSLLK	1.16	1.68E-02	0.99984	1.239	0.152	1.20	6.35E-04	0.99918	0.645	0.457
	FGFDIELFQHAVK	1.09	1.15E-02	0.99863	0.759	1.372	1.15	2.80E-03	0.99946	0.433	1.372
	MLSSLFANYAGFDTPIEK	1.00	1.20E-02	0.99877	2.397	1.372	1.02	-9.75E-03	0.99904	0.125	1.372
	AGGIELFAIGVGR	1.24	3.29E-03	0.99465	0.390	0.457	1.31	1.06E-03	0.99920	0.121	0.152
	YPDAVATWLKPDPSQK	1.31	2.47E-03	0.99942	0.308	0.152	1.35	-1.53E-03	0.99975	0.088	1.372
	GSGGTAEHPFTVEEFVLPK	0.58	2.49E-03	0.99993	0.212	0.152	0.61	-3.41E-05	0.99853	0.155	1.372
XA2M	GSGGTAEHPFTVEEFVLPK	0.47	2.97E-03	0.99973	0.486	0.457	0.46	-5.94E-03	0.99889	0.030	12.346
	ESGGTAEHHFTVEEFVLPK	0.70	1.55E-03	0.98792	0.336	0.457	0.74	-1.02E-02	0.99623	0.245	37.037
	VVVQQESGETAEHPFTVEEFVLPK	0.96	3.22E-03	0.99967	0.692	4.115	1.14	8.90E-03	0.99274	1.200	12.346
	AEHPFIVEEFVLPK	1.08	4.11E-03	0.99977	1.025	1.372	1.10	-7.45E-03	0.99080	0.167	4.115
	TIHHPFSVEEYVLPK	0.80	1.47E-03	0.99960	0.340	0.152	0.86	-1.51E-02	0.99927	0.155	12.346
	TIQHPFTVEEYVLPK	0.77	5.93E-03	0.99967	0.657	0.457	0.75	-1.35E-02	0.99992	0.235	12.346
	TIQHPFSVEEYVLPK	0.86	6.17E-03	0.99975	4.421	37.037	0.62	-2.13E-02	0.99966	0.431	12.346
	IQHSFSVEEYVLPK	0.91	-6.32E-03	0.99052	0.248	4.115	0.75	-1.18E-02	0.99838	0.116	12.346

4.3.7 Linearity and Precision in Phosphate Buffer

The dilution series were also performed in phosphate buffered saline without feed matrices in order to determine possible matrix effects on the assay performance. Dilution series of the synthetic analyte peptides were prepared in the presence of a constant amount of isotope labeled standard peptides in phosphate buffered saline and measured with the developed multiplex assays RQ3 and XA2M in both SIM and PRM mode. Data analysis was performed in the same way as described in the section 4.3.6. The graphics for SIM and PRM measurements in phosphate buffered saline are shown in Supplementary Data G (RQ3) and H (XA2M). Table 18 provides an overview over the linear regression data and the determined limit of detection (LOD) and lower limit of quantification (LLOQ) for multiplex RQ3 and XA2M.

Multiplex RQ3 showed a linear relationship between the analyte to standard signal ratios and the actual concentration ratios over a concentration range of four to five orders of magnitude in both measuring modes PRM (Figure 53) and SIM (Figure 54). The coefficients of determination R^2 ranged from 0.99445 for the bovine SPP1 peptide in PRM to 0.99976 for the bovine C9 peptide in PRM (Table 18). The calculated LOD ranged from 10 amol for the bovine SPP1 peptide in PRM to 1.66 fmol for the bovine HP252 peptide in SIM. With the exception of the MATN1 peptide, the LODs were consistently lower in PRM compared to SIM detection. The LLOQ were observed in the range from 51 amol for the A2M peptide in SIM (Figure 56) to 4.12 fmol for most of the ruminant peptides in PRM (Figure 55).

Multiplex XA2M showed a linear relationship between the analyte to standard signal ratios and the actual concentration ratios over a concentration range of four orders of magnitude in PRM (Figure 59) and four to five orders of magnitude in SIM mode (Figure 60). The coefficients of determination R^2 ranged from 0.98768 for the turkey A2M peptide in PRM to 0.99997 for the sheep and goat A2M peptide in SIM (Table 18). The calculated LOD ranged from 95 amol for the turkey A2M peptide in PRM to 6.0 fmol for the pig A2M peptide in SIM. For all A2M peptides, the LODs were consistently lower in PRM compared to SIM detection. The LLOQ were observed in the range from 457 amol for the ruminant A2M peptides from cattle, sheep and goat in SIM (Figure 62) to 12.4 fmol for most of the A2M peptides in PRM (Figure 61).

Table 18. Linear regression data and the determined limit of detection (LOD) and lower limit of quantification (LLOQ) of the dilution series prepared in phosphate buffer and measured by multiplex XA2M and RQ3.

Assay	Peptide sequence	SIM					PRM				
		Slope	Intercept	R^2	LOD / fmol	LLOQ / fmol	Slope	Intercept	R^2	LOD / fmol	LLOQ / fmol
RQ3	YTPVEAIEK	1.03	8.34E-04	0.99881	0.101	0.152	1.10	-1.68E-03	0.99976	0.056	1.372
	LPPLSLLK	1.22	4.73E-03	0.99904	0.358	0.152	1.21	-4.72E-04	0.99968	0.086	0.457
	FGFDIELFQHAVK	1.07	2.56E-02	0.99969	1.659	4.115	1.14	-1.38E-03	0.99940	0.277	4.115
	MLSSLFANYAGFDTPIEK	0.99	6.22E-04	0.99845	0.229	0.457	1.02	-6.47E-03	0.99925	0.123	4.115
	AGGIELFAIGVGR	1.29	5.17E-03	0.99950	0.291	0.152	1.26	7.52E-03	0.99448	0.423	4.115
	YPDAVATWLKPDPSQK	1.33	1.38E-03	0.99823	0.157	0.152	1.30	-4.47E-03	0.99445	0.010	4.115
	GSGGTAEHPFTVEEFVLPK	0.60	3.75E-03	0.99513	0.239	0.051	0.61	-3.65E-03	0.99945	0.125	4.115
XA2M	GSGGTAEHPFTVEEFVLPK	0.54	-2.67E-05	0.99990	1.049	0.457	0.53	-7.17E-03	0.99934	0.237	12.346
	ESGGTAEHHFTVEEFVLPK	0.94	3.35E-03	0.99997	1.453	0.457	1.06	-3.79E-02	0.99513	1.341	12.346
	VVVQQESGETAEHPFTVEEFVLPK	1.07	3.40E-04	0.99970	5.999	4.115	1.18	-2.38E-02	0.99053	0.416	4.115
	AEHPFIVEEFVLPK	1.17	3.97E-03	0.99940	4.058	1.372	1.23	-1.66E-02	0.99977	0.518	12.346
	TIHHPFSVEEYVLPK	0.86	-6.50E-04	0.99753	1.401	1.372	0.91	-1.48E-02	0.99929	0.268	12.346
	TIQHPFTVEEYVLPK	0.80	3.50E-04	0.99969	1.829	1.372	0.85	-7.62E-02	0.98768	0.095	12.346
	TIQHPFSVEEYVLPK	0.63	-2.01E-03	0.99924	1.644	1.372	0.63	-2.04E-02	0.99543	0.236	4.115
	IQHSFSVEEYVLPK	0.76	-7.38E-03	0.99994	0.939	4.115	0.78	-3.05E-02	0.99867	0.613	12.346

4.3.8 Specificity of Mass Spectrometric Detection Methods

Dilution experiments revealed that the precursor detection (SIM) is more sensitive, accurate and precise for some marker peptides compared to the fragment ion detection (PRM) (section 4.3.6 and 4.3.7). However, the detection specificity seemed to be dramatically higher using PRM. This was further evaluated in a comparison of SIM and PRM measuring matrix blank samples after immunoaffinity enrichment and analyte measurements in low concentration samples in matrix.

Figure 31 shows mass spectra acquired for three marker peptides in a matrix blank sample (VF). Both PRM and SIM scans were acquired with a mass analyzer resolution of 35 000. The quadrupole isolation window in SIM was set to 3.0 m/z in order to isolate at least three precursor isotopes for a higher analyte specificity. The isolation window in PRM was set to 1.5 m/z since the specificity is already given by several fragment ions of the monoisotopic precursor m/z. The mass spectra acquired on the fragment ion level (PRM) showed a very low noise level and no interfering ions in the mass range of the analytes were observed. Mass spectra acquired on the precursor level (SIM) showed several interfering ions over the whole mass range. Even in the mass range of the analytes, interfering ions were detected in the matrix blank sample.

Figure 32 shows extracted ion chromatograms from mass spectra acquired in the presence of a marker peptide at the lowest detectable concentration of 1.37 fmol. Interfering signals in the chromatogram were observed for precursor detection, which could affect peak integration. In contrast, no interfering ions were observed in the chromatogram of the fragment ions, facilitating peak identification and integration. The higher detection specificity of fragment ion scan was preferred over the higher sensitivity for some markers on the precursor level although the signal intensity was lower on the fragment ion level.

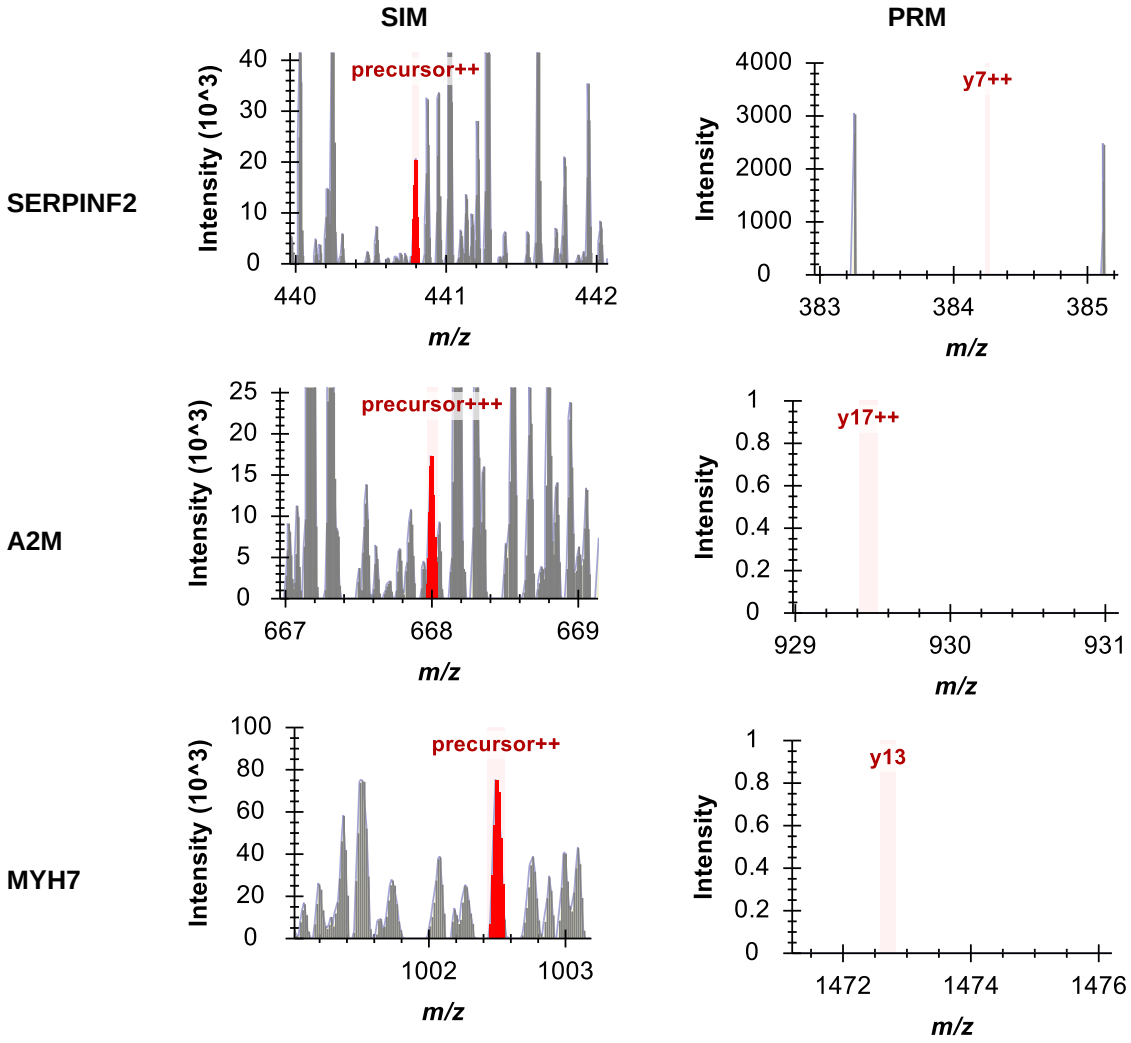


Figure 31 Mass spectra of vegetal feed blank measurements acquired after immunoaffinity enrichment in selected ion monitoring (SIM) and parallel reaction monitoring (PRM) mode for three selected marker peptides.

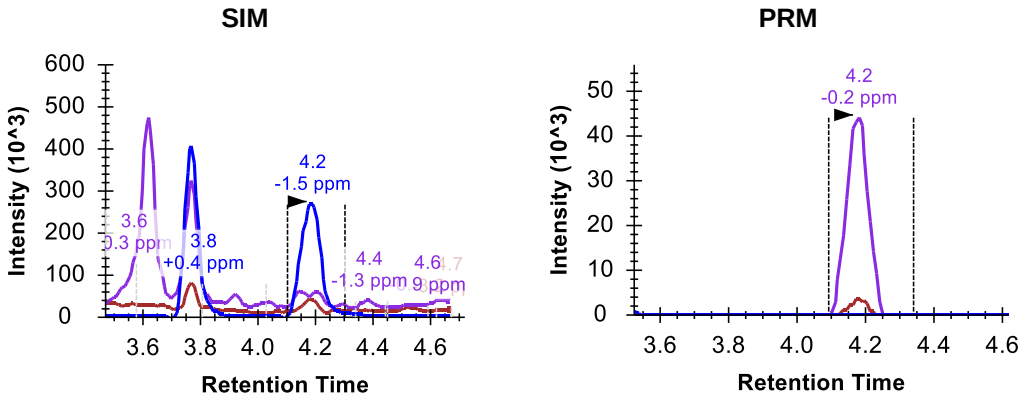


Figure 32. Extracted ion chromatograms (XIC) for SERPINF2 present in a low concentration (1.37 fmol) acquired with selected ion monitoring (SIM) and parallel reaction monitoring (PRM) with a mass analyzer resolution of 30 000. Three ions were analyzed to achieve a specific detection. The three differently colored XICs represent the three most intense precursor isotope ions in SIM, and the three most intense fragment ions of the monoisotopic precursor ion in PRM.

4.3.9 Limit of Detection and Quantification in Spiked Samples

Ruminant Quantification Assay RQ3

Two types of PAPs with different processing degrees and analyte concentrations were spiked into a vegetal cattle feed matrix (VF). Ruminant spray-dried plasma (rSDP) was chosen as high concentration feed additive. The processed ruminant meat and bone meal (rMBM) served as low concentration feed additive. BM was expected to show analyte concentration between these two extrema and was not analyzed in spike-in experiments. Both samples were treated separately according to the HPD protocol, mixed afterwards with the digested VF and then analyzed by targeted MS. Additionally, the rSDP sample was spiked into digested porcine spray-dried plasma (pSDP) in order to determine the detection limit in PAP-PAP admixtures. The spike-in dilution series was used to determine the limit of detection expressed in femtomol as it was done in the standard dilution series (shown as dashed horizontal line). The first spike-in level that exceeded the determined LOD was used as the lowest detectable level expressed in weight percentage. The limit of quantification (LOQ) was determined as the lowest spike-in concentration that was analyzed with a coefficient of variation ≤20% and applying the rule LOQ = 3 x LOD (shown as solid horizontal line). The calculated detection and quantification limits and the regression data are shown in Table 19.

In the rMBM in VF spike-in series, the most sensitive detection was achieved for C9, MATN1 and SPP1, where all spike-in samples starting with 0.05% were detected above the calculated LOD (Figure 33). The 0.1% spike-in sample was positively detected via the marker peptides from MYH7 and A2M. SERPINF2 and HP252 were the least sensitive markers, allowing the detection of 0.50% rMBM in VF. In terms of quantification similar results were observed. SERPINF2 and HP252 showed the highest LOQ of 5.00%. The LOQs for A2M and C9 were 0.75% and 0.25%, respectively. A quantitative detection of rMBM in VF was achieved at 0.05% for MATN1 followed by the markers MYH7 and SPP1 at 0.10%, respectively.

In the rSDP in VF spike-in series all concentrations starting with 0.05% were quantitatively detected via all four plasma targets A2M, C9, SERPINF2 and HP252 (Figure 34). As expected the meat and bone markers MYH7 and MATN1 were not detected above the LOD in the rSDP spike-in sample. Since SPP1 occurs in low levels in plasma, SPP1 was detected

in spike-in levels above 0.75% and quantitatively determined above 5.00%. Plasma protein concentrations were much higher in SDP compared to MBM, indicated by the slope of the regression equations. For this reason, not only the detection limit was lower, but also the upper limit of quantification was achieved. High standard deviations and non-linear signals were observed for A2M, HP252 and SERPINF2. To avoid this, 10% SDP adulterations needed to be diluted.

In the rSDP in pSDP spike-in series the marker A2M was not analyzed since the corresponding antibody enriches in a cross-species manner and therefore also the matrix species. For this reason, the SDP spike-in samples were diluted and measured with a total amount of 20 µg instead of 100 µg. SERPINF2 showed a decreased sensitivity with a detection limit of 5.00% rSDP in pSDP. However, C9 and HP252 were able to detect 0.10% and 0.25% spike-in levels, respectively. A quantification of rSDP in pSDP was possible at 0.25% via C9 and 0.50% via HP252 (Figure 35).

To summarize, developed multiplex RQ3 was suitable for detecting and quantifying a bovine MBM or SDP in a VF background in a tissue-specific way at the regulatory level of 0.1% (w/w) which is the approved limit of detection of the official PCR and microscopic method. Linearity is given up to 100% sample without a need for sample dilution for the rMBM samples. However, to avoid carry-over, the samples with SDP adulterations of 10% or higher should be diluted.

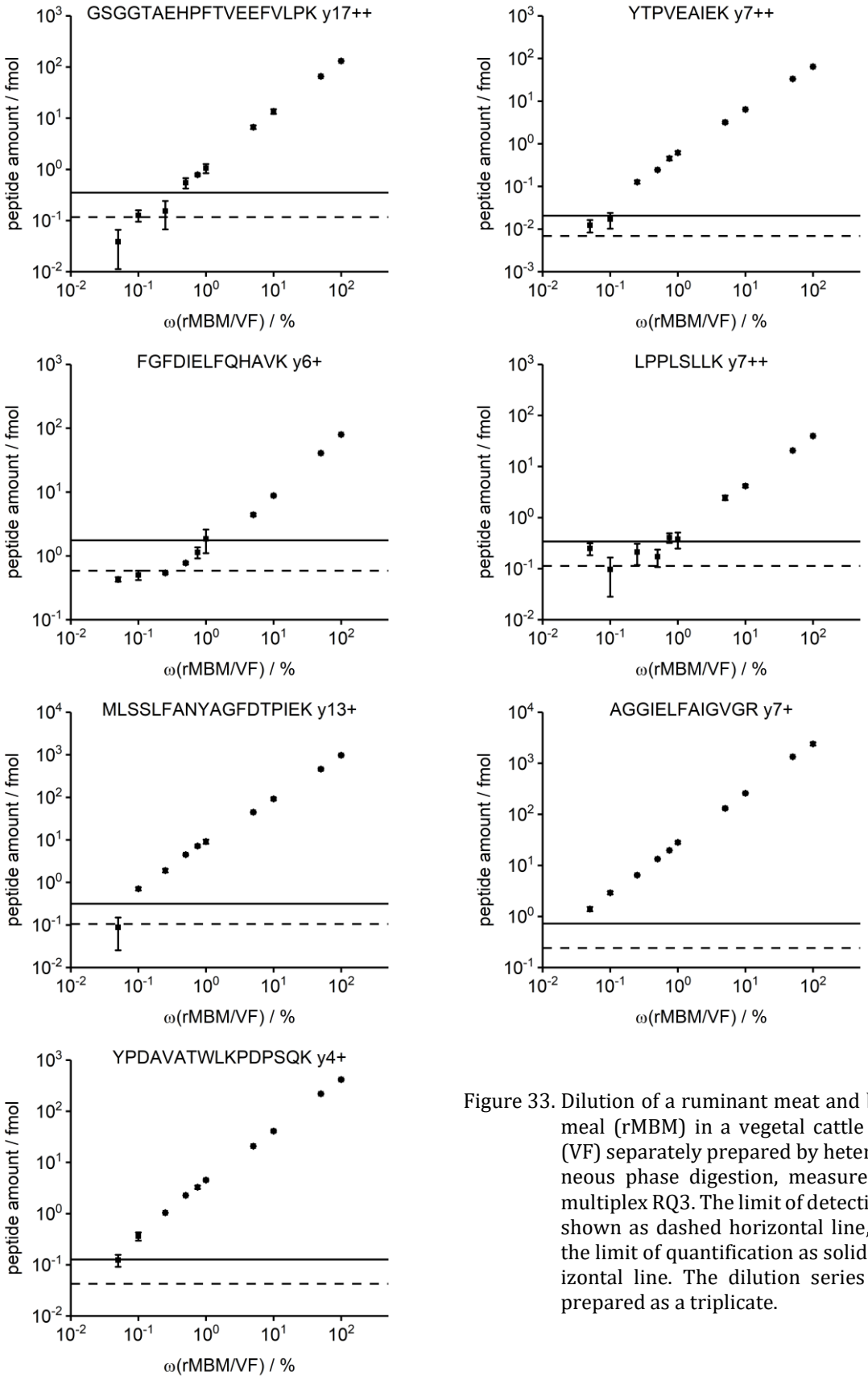


Figure 33. Dilution of a ruminant meat and bone meal (rMBM) in a vegetal cattle feed (VF) separately prepared by heterogeneous phase digestion, measured by multiplex RQ3. The limit of detection is shown as dashed horizontal line, and the limit of quantification as solid horizontal line. The dilution series was prepared as a triplicate.

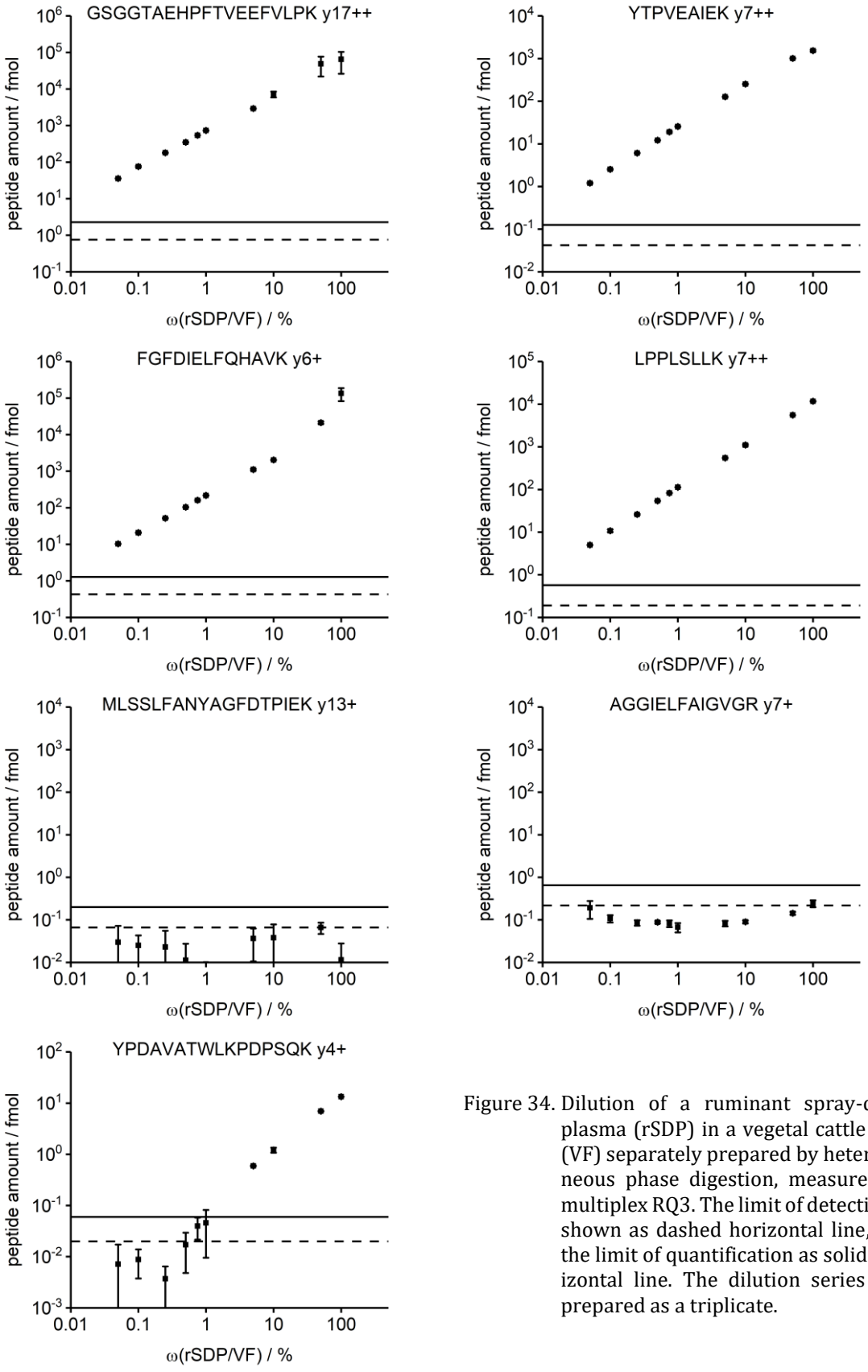
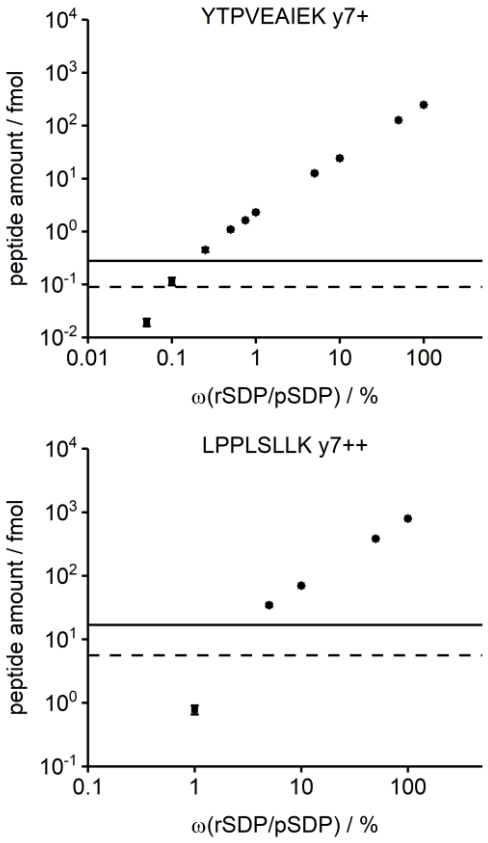


Figure 34. Dilution of a ruminant spray-dried plasma (rSDP) in a vegetal cattle feed (VF) separately prepared by heterogeneous phase digestion, measured by multiplex RQ3. The limit of detection is shown as dashed horizontal line, and the limit of quantification as solid horizontal line. The dilution series was prepared as a triplicate.



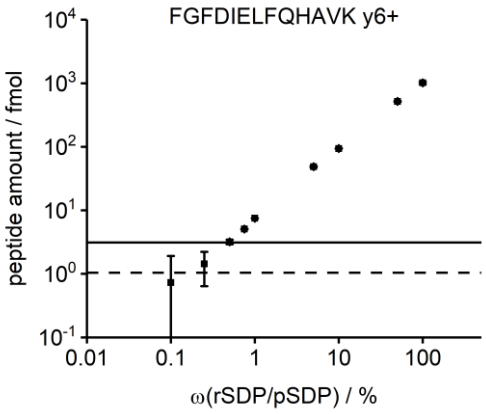


Figure 35. Dilution of a ruminant spray-dried plasma (rSDP) in a porcine spray-dried plasma (pSDP) separately prepared by heterogeneous phase digestion, measured by multiplex RQ3. The limit of detection is shown as dashed horizontal line, and the limit of quantification as solid horizontal line. The dilution series was prepared as a triplicate.

Table 19. Linear regression data and the determined limit of detection (LOD) and limit of quantification (LOQ) of the ruminant meat and bone meal (rMBM) and ruminant spray-dried plasma (rSDP) dilution in a vegetal cattle feed matrix (VF) and a porcine spray-dried plasma matrix (pSDP). In the rSDP in pSDP dilution, A2M was not analyzed (n.a.).

spike-in	protein	slope	intercept	R^2	LOD		LOQ	
					/ fmol	/ %	/ fmol	/ %
rMBM in VF	A2M	1.31	-1.90E-01	0.99995	0.117	0.10	0.351	0.75
	C9	0.64	-5.62E-02	0.99906	0.007	0.05	0.021	0.25
	SERPINF2	0.40	5.62E-02	0.99950	0.113	0.50	0.340	5.00
	HP252	0.80	3.85E-01	0.99967	0.586	0.50	1.757	5.00
	MYH7	4.31	-2.33E-02	0.99565	0.105	0.10	0.316	0.10
	MATN1	9.37	-2.42E-01	0.99811	0.242	0.05	0.726	0.05
	SPP1	26.44	1.14E-02	0.99950	0.043	0.05	0.128	0.10
rSDP in VF	A2M	709.47	8.00E-01	0.99615	0.757	0.05	2.271	0.05
	C9	24.99	-4.52E-02	0.99930	0.042	0.05	0.126	0.05
	SERPINF2	111.26	-5.80E-01	0.99966	0.191	0.05	0.573	0.05
	HP252	208.94	-1.43E-01	0.99897	0.429	0.05	1.286	0.05
	SPP1	0.13	-8.01E-02	0.99968	0.020	0.75	0.060	5.00
	MYH7	0	0	0	0	0	0	0
	MATN1	0	0	0	0	0	0	0
rSDP in pSDP	A2M	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	C9	2.48	-1.99E-01	0.99885	0.050	0.10	0.015	0.25
	SERPINF2	7.87	-7.72E+00	0.99845	0.187	5.00	0.562	5.00
	HP252	9.78	-2.28E+00	0.99827	0.052	0.25	0.156	0.50

Cross-Species Identification Assay (XA2M)

The detection limit of the cross-species multiplex XA2M was determined as described for multiplex RQ3. Due to a lack of PAP samples for all species, the spike-in was performed with three MBMs: ruminant MBM (rMBM), porcine MBM (pMBM) and a poultry-mix-MBM (Figure 36). Table 20 shows the determined LOD and LOQ expressed as peptide amount and as weight percentage. The bovine MBM was detected at 0.1% via the bovine A2M peptide. A quantification was possible at 0.75%. The detection limit for the porcine MBM was 0.25% with quantification limit of 0.75%. The poultry-mix-MBM was analyzed to consist of turkey and chicken proteins in relative amounts of 91.7% chicken and 8.3% turkey, determined in the 100% poultry-mix-MBM. In total, the poultry-mix-MBM was detected by the chicken A2M peptide at 1.00% and a quantification was possible at 5.00%. Taking the relative poultry amounts into account, a pure chicken MBM could be detected at 0.92% and a pure turkey MBM at 0.42%.

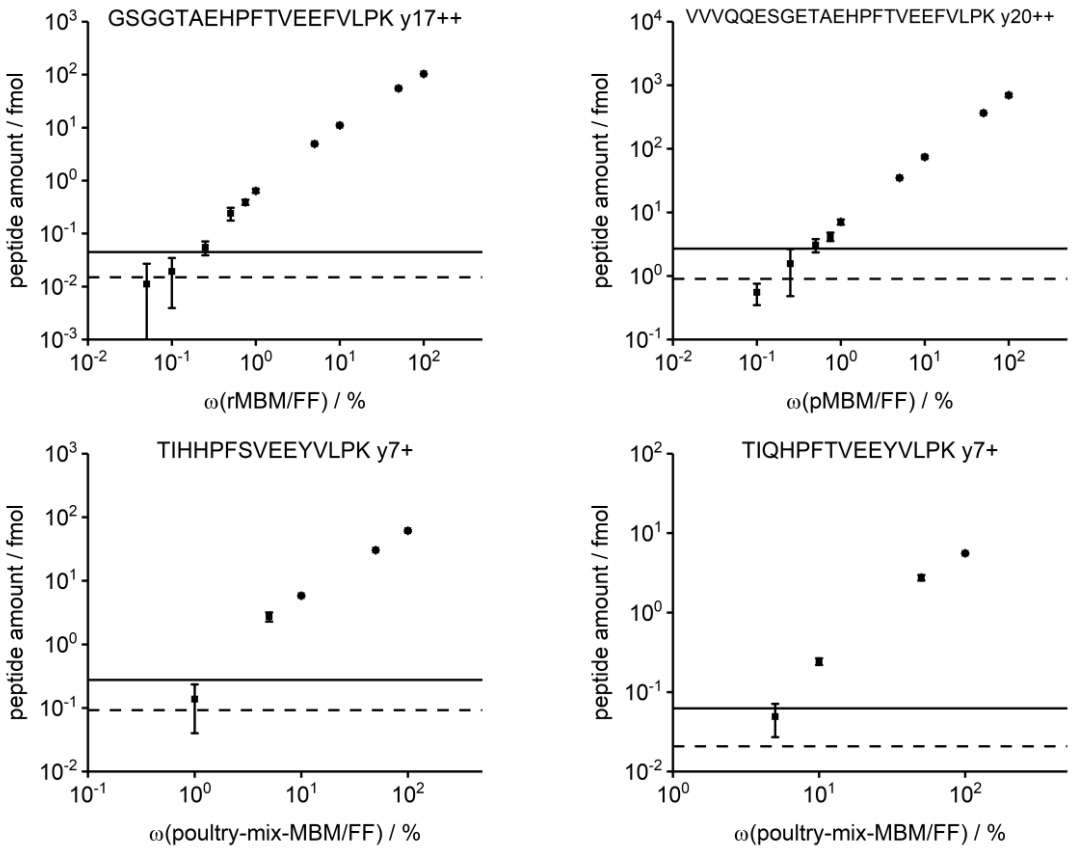


Figure 36. Dilution of a ruminant (rMBM), a porcine (pMBM) and a poultry-mix (poultry-mix-MBM) meat and bone meal in a fish feed (FF) matrix separately prepared by heterogeneous phase digestion, measured by multiplex XA2M. The limit of detection is shown as dashed horizontal line, the limit of quantification as solid horizontal line. The dilution series was prepared as a triplicate.

Table 20. Linear regression data and the determined limit of detection (LOD) and limit of quantification (LOQ) of the bovine, porcine and poultry-mix meat and bone meal dilution in a fish feed matrix.

Species marker	Slope	Intercept	R^2	LOD / fmol	LOD / %	LOQ / fmol	LOQ / %
Bovine	1.03	-0.17	0.99222	0.015	0.10	0.045	0.75
Porcine	7.26	-0.81	0.99938	0.899	0.25	2.697	0.75
Poultry-mix	-	-	-	-	1.00	-	5.00
chicken	0.62	-0.43	0.99998	0.092	0.92	0.277	4.59
turkey	0.06	-0.28	0.99360	0.021	0.42	0.062	0.83

4.3.10 Effect of Sample Homogenization

To assess RQ1 intra- and interassay precision, rMBM in VF mixtures at 0.1%, 1% and 10% were analyzed in five HPD replicates. In a first experiment, no additional grinding step via ball mill was performed and very high coefficients of variation were observed. Only the 10% mixture could be analyzed with an intraassay precision ≤20%. In a second experiment the samples were additionally ground before HPD and LC-MS/MS analysis to improve the sample homogeneity. Thereby, the coefficients of variation improved dramatically and the signal to noise ratios increased for the 1% and 10% samples (Table 21). The 1% and 10% mixtures were analyzed with coefficients of variation of <10%. The low concentration samples (0.1%) still showed high coefficients of variation and slight decreases in signal to noise ratios for three out of four marker peptides. Therefore, the boundary between signal and no signal became clearer. This highlighted the importance of sample homogeneity and representative sample taking for quantitative assays.

However, in terms of a qualitative analysis, the additional grinding step showed a negative effect: The signals were diluted in the matrix and a qualitative detection became challenging for the 0.1% samples (Figure 37). In the non-ground samples, two out of five replicates showed signals with a higher intensity and confidence of detection. In these non-ground samples at 0.1%, the marker peptides were clearly detected above the calculated limit of detection, which is shown as dashed horizontal line (Figure 38). The comparison to the signals of the isotope labeled internal standards proof the fragmentation pattern of the analytes (Figure 37). In the corresponding ground sample, the signal intensity dramatically decreased. Most replicates showed intensities in the range of the calculated LOQ.

Furthermore, the chromatographic peak shape declined and fragment ion detection and therefore the peptide identification was impeded. This experiment revealed that on the one hand, homogeneous samples are indispensable for quantitative determinations, but on the other hand a larger number of random sample taking in non-homogeneous samples allows for a qualitative detection with higher confidence on the level of 0.1%.

Table 21. Effect of an additional grinding step via ball mill on the precision and signal to noise ratio of a ruminant meat and bone meal at three concentration levels (0.1%, 1% and 10%) prepared by heterogeneous phase digestion in five replicates.

Protein	sample concentration / %	non-homogenized mean / fmol	non-homogenized C.V. / %	non-homogenized S/N	homogenized mean / fmol	homogenized C.V. / %	homogenized S/N
SERPINF2	10	19.4	18	584	18.5	7	1452
	1	2.0	88	61	1.1	3	87
	0.1	2.1	133	64	0.4	131	30
HP252	10	33.9	13	169	40.1	6	271
	1	3.3	30	16	2.1	8	14
	0.1	2.4	121	12	1.1	110	8
A2M	10	111.5	15	600	122.8	5	24561
	1	11.0	32	59	6.5	2	1302
	0.1	1.5	115	8	0.2	27	43
C9	10	11.9	16	1775	11.9	2	2385
	1	1.3	37	195	0.7	6	131
	0.1	0.3	145	39	0.1	31	8

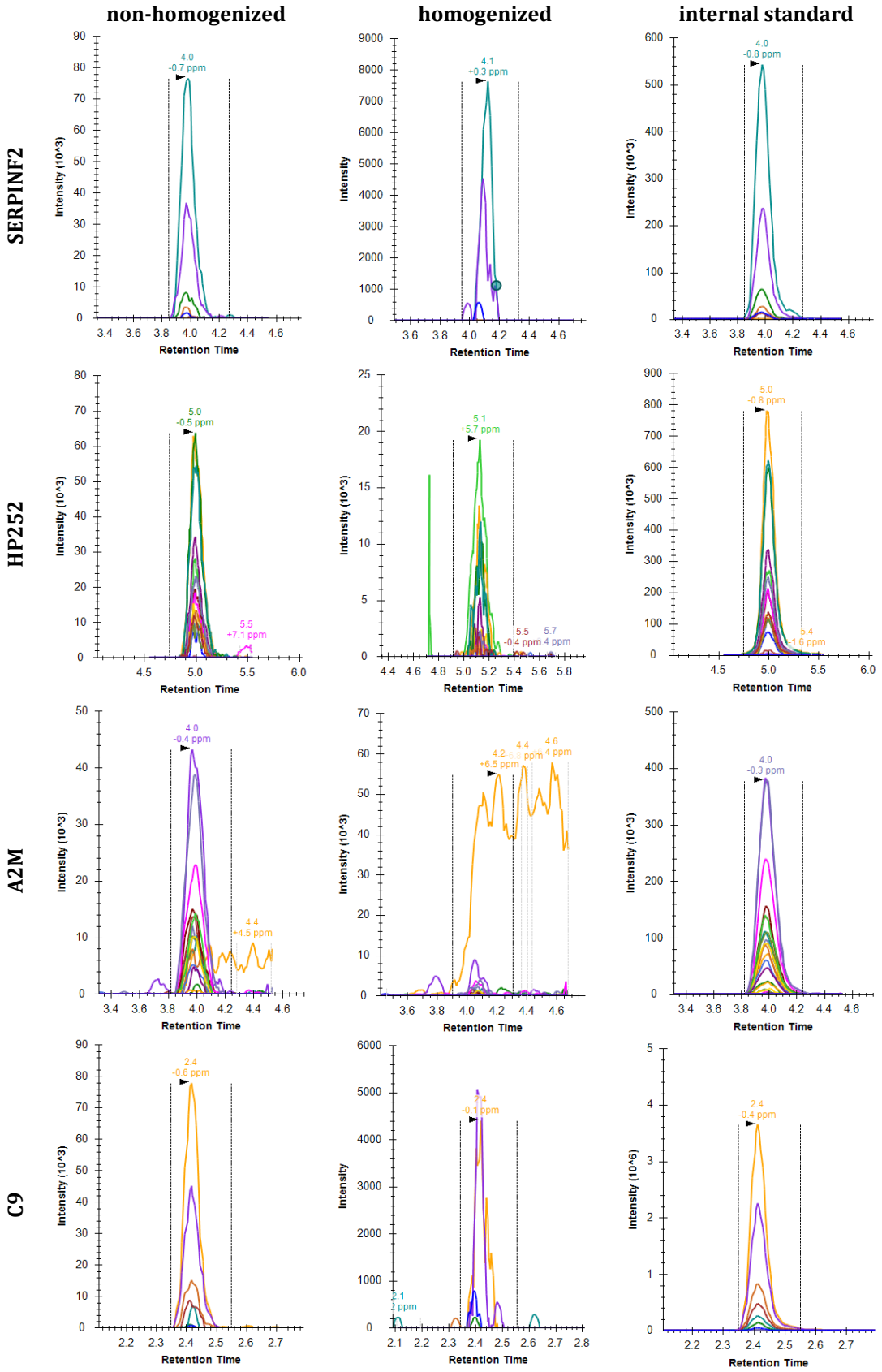


Figure 37. Extracted ion chromatograms for the ruminant SERPINF2, HP252, A2M and C9 marker peptides present in a low concentration sample, with and without additional grinding via ball mill,compared to the internal standard signal at a higher concentration of 50 fmol.

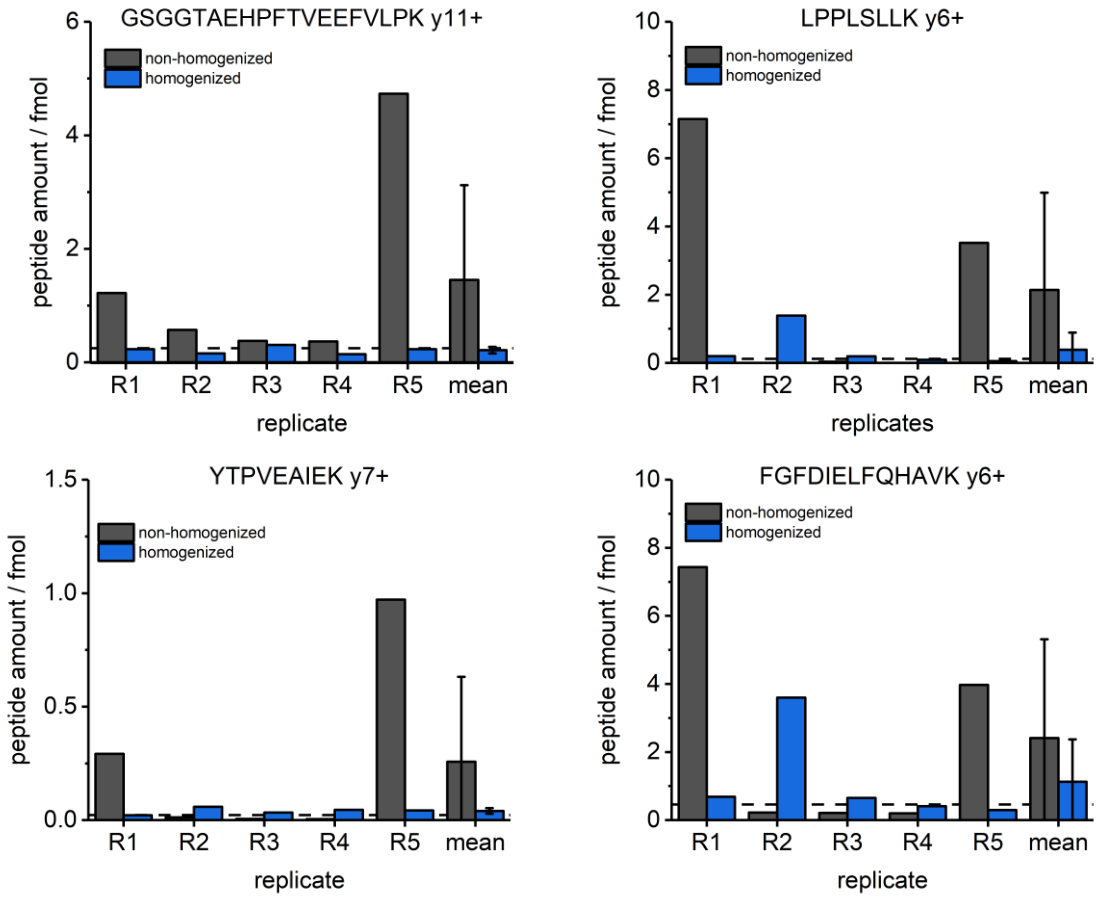


Figure 38. Signal intensity of the marker peptides' most intense fragment ion, present in a low concentration in five replicate runs and the mean, with and without additional grinding via ball mill. The limit of detection is shown as dashed horizontal line.

4.3.11 Tissue Specificity

The developed assays RQ1 addressing plasma proteins and RQ3 addressing plasma, meat, bone and cartilage proteins, were applied to three different animal proteins in order to assess the tissue specificity. Two milk powders, an MBM and two SDP were analyzed (Figure 39).

For the RQ1 multiplex assay, all four plasma proteins were quantitatively detected in each sample. Hence, the presence of the proteins could not be used to differentiate the tissue types. However, the developed assay RQ1 allowed the differentiation of tissues when the relative peptide amounts were compared. The targeted peptides showed characteristic protein ratios in plasma, milk and MBM, respectively. Highest relative A2M amount was observed for SDP samples (65%) with the lowest amount of C9 (3%) with a ratio of 33:1. In the MBM sample, the A2M amount decreased to 42% and C9 increased to 20% (ratio 2:1). In milk, the plasma proteins reached nearly equal relative amounts ranging from

17% (C9) to 28% (HP252). The ratio of A2M and C9 decreased to 1.5:1. These ratios can be compared with unknown samples in order to identify the sample's origin and differentiate between legal and illegal additives.

A higher confidence of tissue differentiation was achieved by the application of the developed RQ3 7-plex assay. The MBM-specific markers MYH7 and MATN1 were detected neither in the milk powders nor in the SDP samples. In contrast, these markers were highest in the MBM sample with relative amounts of 50% for MATN1 and 20% for MYH7, followed by nearly 20% for SPP1 and a sum of 10% plasma proteins. High relative levels of SPP1 of around 99% with less than 1% of the plasma proteins and a lack of MYH7 and MATN1 clearly indicated the presence of milk powder. Expanding the multiplex assay with more tissue-specific markers allowed the differentiation of milk powder, MBM and blood product samples such as SDP.

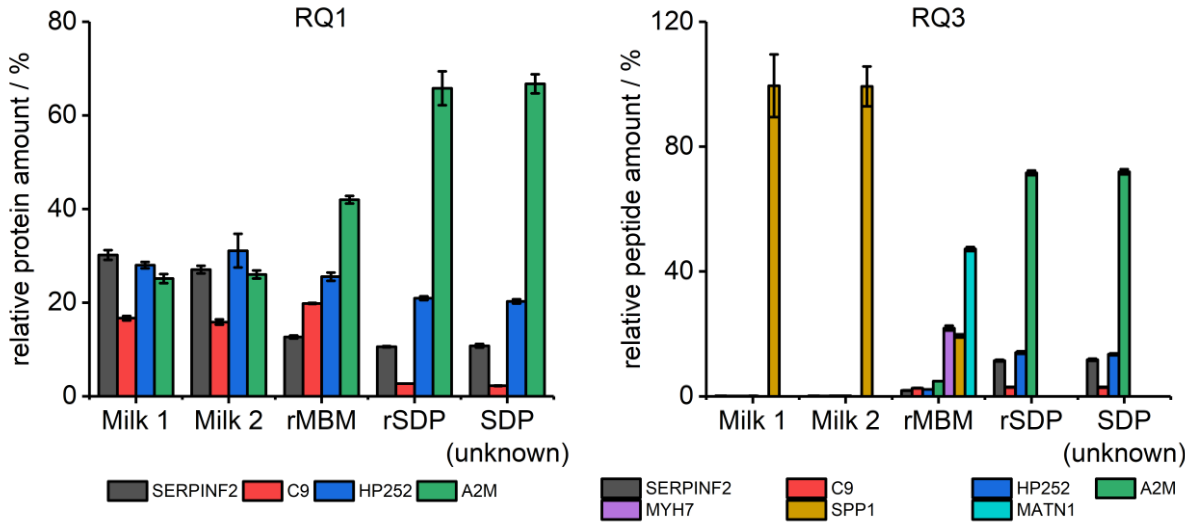


Figure 39. Relative protein amounts determined via marker peptide quantification using multiplex RQ1 and RQ3 in milk powder samples, a ruminant meat and bone meal (rMBM) and two spray-dried plasmas, one of ruminant origin (rSDP) and one of unknown species origin (SDP unknown). Results were normalized to the sum of analytes (set to 100%) in each sample and three replicates were analyzed.

4.4 Validation of Analytical Parameters

4.4.1 Specificity

Species and tissue specificity of the developed multiplex assays were determined with HPD-prepared animal protein samples and ISD-prepared citrate plasmas. A positive detection was defined as detected signals of at least three fragment ions with a signal variation of ≤20% for the most intense fragment ion after referencing to the internal standard. Positive signals are shown in the tables below as mean peptide amounts with their standard deviations.

The specificity of multispecies detection via the XA2M assay was assessed with citrate plasmas as clean reference samples. The plasma mixtures were prepared as described in section 3.7.1. In each mixture one species was left out, while the other species were present in equal volumes. The result of the leave-one-species-out specificity is shown in Table 22. All species were parallelly detected except the species that was left out. One exception was the mixture without porcine plasma, which showed a positive porcine A2M signal. The negative control measured as fish feed matrix without any plasma gave the same result. The negative control measured as phosphate buffer without fish feed and plasma gave no signal for porcine A2M. The signal for the A2M-peptide in the no-porcine mixture was not significantly higher than that of the matrix itself (P=0.67). This indicated a porcine contamination of the fish feed and a porcine-specific detection was assumed.

The specificity for XA2M in processed animal protein samples was also assessed and is shown in Table 23. The expected species origin was confirmed. Again, the fish feed without land living animals showed a slight porcine contamination. The second fish feed supposed to contain land living animals was proven to contain mainly porcine material and a slight amount of chicken material. The unknown MBM and BM samples of poultry mixtures were proven to be pure chicken in case of MBM and a mixture of 80% chicken and 20% turkey material in case of the BM sample. The ruminant MBM and SDP samples were confirmed to be pure samples. Four porcine PAP samples of different origin, a porcine BM and two porcine SDP were confirmed to be pure porcine samples. The SDP sample of unknown species origin, was tested to consist of 80% bovine and 20% porcine material. In combination, the analyzed clean citrate plasmas and the processed animal protein samples showed a highly specific species detection via XA2M.

Table 22. Species specificity of multiplex XA2M assessed with citrate plasma as clean reference samples in triplicates. Only positive detections with C.V. ≤20% are shown, negative signals are shown with a value of zero.

Sample / species	Peptide amount/ nmol g^{-1}							
	cattle	sheep/goat	horse	turkey	goose	duck	chicken	pig
no cattle	0	129 ± 10	265 ± 12	85 ± 2	67 ± 5	61 ± 3	112 ± 4	462 ± 24
no sheep/goat	255 ± 19	0	268 ± 3	82 ± 3	73 ± 5	59 ± 4	119 ± 2	426± 12
no horse	258 ± 10	140 ± 3	0	84 ± 1	72 ± 3	64 ± 2	112 ± 5	405 ± 8
no turkey	231 ± 5	158 ±19	277 ± 6	0	67 ± 2	62 ± 2	119 ± 5	401 ± 9
no goose	259 ± 10	158 ± 16	276 ± 4	81 ± 3	0	62 ± 6	116 ± 2	409 ± 7
no duck	255 ± 5	167 ± 16	276 ± 3	87 ± 5	70 ± 4	0	114 ± 9	436 ± 11
no chicken	261 ± 20	160 ± 9	276 ± 5	85 ± 1	71 ± 1	63 ± 2	0	402 ± 30
no pig	241 ± 15	130 ± 10	248 ± 15	84 ± 5	66 ± 4	56 ± 5	119 ± 7	8 ± 1
all species in FF	246 ± 3	145 ± 11	266 ± 13	86 ± 2	67 ± 3	63 ± 3	112 ± 9	421 ± 6
all species in PBS	238 ± 2	146 ± 17	263 ± 9	86 ± 3	70 ± 4	60 ± 2	104 ± 4	412 ± 5
FF, no plasma	0	0	0	0	0	0	0	8 ± 1
PBS, no plasma	0	0	0	0	0	0	0	0

Table 23. Species specificity of multiplex XA2M assessed with processed animal protein samples and blood products of different species origin in triplicates. Only positive detections with C.V. ≤20% are shown, negative signals are shown with a value of zero.

Sample	Peptide amount / nmol g^{-1}							
	cattle	sheep	pig	horse	turkey	chicken	duck	goose
FF (no mammals)	0	0	0	0	0	0	0	0
FF (with mammals)	0	0	25.8 ± 1.4	0	0	0.5 ± 0.1	0	0
poultry BM	0	0	0	0	2.9 ± 0.1	11.4 ± 0.5	0	0
poultry MBM	0	0	0	0	0	0.5 ± 0.1	0	0
MBM cattle	2.5 ± 0.2	0	0	0	0	0	0	0
porcine meal 1	0	0	6.1 ± 0.2	0	0	0	0	0
porcine meal 2	0	0	5.3 ± 0.3	0	0	0	0	0
porcine meal 3	0	0	6.9 ± 0.5	0	0	0	0	0
porcine meal 4	0	0	9.2 ± 0.7	0	0	0	0	0
porcine BM	0	0	113.1 ± 12.6	0	0	0	0	0
porcine SDP 1	0	0	159.6 ± 5.6	0	0	0	0	0
porcine SDP 2	0	0	294.4 ± 17.9	0	0	0	0	0
bovine SDP	185.0 ± 7.3	0	0	0	0	0	0	0
unknown SDP	151.2 ± 3.8	0	37.1 ± 4.4	0	0	0	0	0

Specificity of multiplex RQ3 was assessed in digested vegetal feed as matrix (10% w/w) and is shown in Table 24. The ruminant marker peptides were not detected in pure vegetal feed and in none of the poultry and porcine animal protein samples. As expected, the ruminant plasma peptides were detected in legal milk powder as it was already observed in section 4.3.11. The SDP samples of bovine and unknown origin showed signals for the plasma derived peptides, as it was expected. The results proved a species-specific detection of ruminant plasma proteins.

In terms of tissue specificity, the results for SPP1, MYH7 and MATN1 have to be considered. These markers also allow a ruminant-specific detection. Neither signals in the porcine and poultry samples, nor in the vegetal feed matrix were observed. High relative levels of SPP1 of around 99% clearly indicated the milk powder samples. A relative amount of 20% SPP1 while also containing 20% MYH7, 50% MATN1 and 10% plasma proteins indicated the MBM. A lack of SPP1, MYH7 and MATN1 signals while showing high levels of A2M and lower levels of the other three plasma targets indicated the presence of BM or SDP in the feed compound. The two meat and cartilage-specific targets MYH7 and MATN1 were only present in bovine MBM and therefore offered an unambiguous species and tissue detection of illegal MBMs in cattle feed.

Table 24. Species and tissue specificity of multiplex RQ3 assessed with processed animal proteins and blood products of different species origin in vegetal cattle feed as matrix (10% w/w). The analysis was performed in triplicate runs. Only positive detections with C.V. ≤20% are shown, negative signals are shown with a value of zero.

Sample	Peptide amount / nmol g^{-1}						
	SERPINF2	C9	HP252	A2M	SPP1	MYH7	MATN1
VF	0	0	0	0	0	0	0
BM poultry	0	0	0	0	0	0	0
MBM poultry	0	0	0	0	0	0	0
BM pig	0	0	0	0	0	0	0
MBM pig	0	0	0	0	0	0	0
Milk powder 1	0.3 ± 0.02	0.2 ± 0.01	0.3 ± 0.03	0.3 ± 0.01	214.3 ± 21.6	0	0
Milk powder 2	0.3 ± 0.02	0.2 ± 0.01	0.4 ±0.02	0.4 ± 0.01	189.1 ± 12.1	0	0
MBM cattle	0.9 ± 0.04	1.3 ± 0.02	1.1 ± 0.01	2.4 ± 0.01	9.4 ± 0.2	10.6 ± 0.4	22.9 ± 0.3
SDP cattle	29.9 ± 0.7	7.6 ± 0.2	36.5 ± 1.1	186.5 ± 1.9	0	0	0
SDP unknown	27.4 ± 0.7	6.8 ± 0.3	31.4 ± 0.7	168.0 ± 1.9	0	0	0

4.4.2 Accuracy and Precision

Assay accuracy and precision for RQ3 and XA2M in feed matrices was already assessed during the method development (4.3.6). The equation of the linear fit was used to calculate the measurements accuracy and precision. The criteria for an accurate and precise measurement in PRM detection were 80% to 120% accuracy with a precision of ≤20%. This was achieved in the concentration range of 152 amol for the bovine MATN1 peptide and 1.37 fmol for most of the other bovine peptides in a vegetal cattle feed matrix. In the fish feed matrix, an accurate measurement was observed in the range of 4.12 fmol for the horse A2M peptide and 37.0 fmol for the sheep and goat A2M peptide (Table 17). The results showed an accurate and precise quantification for at least 3 to 4 orders of magnitude depending on the marker peptide and matrix.

4.4.3 Linearity and Limit of Detection

The assay linearity for RQ3 and XA2M in feed matrices was already assessed during the method development (4.3.6). The PRM detection showed a linear relationship between the measured signal ratios of analyte and internal standard and the concentration ratios of analyte and internal standard over a concentration range of 4 to 5 orders of magnitude (Table 17). The standard dilution was also used to estimate the limits of detection (LOD) for each analyte in PRM detection. The LODs in fish feed matrix ranged between 30 amol for the bovine A2M peptide and 431 amol for the goose A2M peptide (Table 17). The LOD for the porcine A2M peptide was even higher with 1.20 fmol, however the fish feed showed a porcine protein contamination, causing an over-estimation of the LOD. The LOD for this peptide determined in PBSC was only 416 amol. In the vegetal feed matrix, the LODs ranged between 38 amol for the bovine C9 peptide and 645 amol for the bovine SERPINF2 peptide.

4.4.4 Recovery

The recovery of the analytes in complex matrix was determined by measurements in PBSC and spiked in feed matrices, respectively. Since internal standards were used, the signal ratios itself should not be affected by the matrix. However, the absolute signal intensities can be affected by the matrix leading to an increase or decrease in the observed limits of detection. In order to determine the recovery in the quantitative dynamic range, the meas-

urement accuracies in PBSC and in matrix were compared and expressed as relative recovery *R* in percent (Formula 3). An analyte recovery need not to be 100%, however the extent of recovery should be consistent and precise [109].

$$R = \frac{\text{accuracy(matrix)}}{\text{accuracy(PBSC)}} \text{ x } 100\% \qquad (3)$$

The recovery for RQ3 determined by measurements in PBSC and vegetal feed matrix are shown in Table 25. In the quantitative range of 4 fmol to 1000 fmol the recovery did not deviate more than 15% from the nominal value of 100%. The determined recoveries were consistent and precise with a maximum variation coefficient of 6.8%. The recovery for XA2M determined by measurements in PSBC and fish feed matrix are shown in Table 26. In the quantitative range of 37 fmol to 1000 fmol the recovery did not deviate more than 20%. The determined recoveries were consistent and precise with a maximum variation coefficient of 10.8%. The results indicated that the matrices did not have a significant effect on the lower limits of quantification for both multiplex assays.

Table 25. Recovery for multiplex RQ3 determined in phosphate buffered saline and vegetal cattle feed as matrix in triplicate runs.

Peptide amount / fmol	Recovery (matrix:PBSC) / %						
	A2M	SERPINF2	HP252	C9	MYH7	SPP1	MATN1
4	102	102	109	101	115	108	102
12	107	104	104	101	96	101	85
37	101	103	95	99	99	98	94
111	103	98	101	98	96	99	102
333	98	101	97	102	94	97	104
1000	96	100	98	98	95	98	96
mean	**100.8**	**101.1**	**99.2**	**99.6**	**95.9**	**98.5**	**96.2**
SD	**3.7**	**2.2**	**3.1**	**1.4**	**1.7**	**1.3**	**6.6**
C.V.	**3.6**	**2.2**	**3.1**	**1.4**	**1.8**	**1.3**	**6.8**

Table 26. Recovery for the multiplex XA2M determined in phosphate buffered saline and fish feed as matrix in triplicate runs.

Peptide amount / fmol	Recovery (matrix:PBSC) / %							
	cattle	sheep /goat	pig	horse	chicken	turkey	goose	duck
37	105	83	112	92	95	87	91	98
111	105	98	93	101	104	98	83	101
333	96	92	101	114	95	104	103	98
1000	95	88	83	112	93	101	80	95
mean	**100.0**	**90.1**	**97.3**	**104.9**	**96.5**	**97.6**	**89.5**	**97.9**
SD	**4.6**	**5.4**	**10.5**	**8.8**	**4.5**	**6.3**	**8.9**	**2.2**
C.V.	**4.6**	**6.0**	**10.8**	**8.4**	**4.6**	**6.5**	**9.9**	**2.3**

4.4.5 Repeatability

The intra- and interassay repeatability of multiplex XA2M, was assessed at three concentrations of a plasma mixture in fish feed (1%, 5% and 10%, w/w) on the dried and non-digested level applying HPD (n=5). Single species concentrations in the fish feed were 0.1%, 0.6% and 1.1%, respectively. The multiplex XA2M was capable to measure all peptides with coefficients of variation ≤20% for the most intense fragment ion and at least three detected transitions for each marker peptide (Table 27). Precise measurements were achieved in the high, medium and low concentration ranges. The highest concentration precisely determined was 1351 fmol for the sheep and goat A2M peptide. Precise measurements in the medium range were shown for several peptides of different species. The lowest concentration precisely determined was in the range of the limit of quantification with 10 fmol for the duck A2M peptide.

The intra- and interassay repeatability of multiplex RQ3 was assessed by the analysis of three different validation samples (rMBM1, rMBM2, rSDP) at three concentration levels (0.1%, 1% and 10% w/w) in vegetal feed (VF) on the dried and non-digested level applying HPD (n=5). The result is shown in Figure 56. The rSDP in VF mixtures were detected with variation coefficients ≤20% on all three concentration levels for all four plasma targets. As expected, the tissue-specific markers SPP1, MYH7 and MATN1 were not detected in rSDP. In case of the meat and bone meal mixtures with rMBM1, the illegal admixtures were detected at the medium and high concentration levels of 1% and 10% by the presence of all markers with variation coefficients ≤20%. The lowest concentration level of

0.1% was detected by the marker proteins HP252 and MYH7 with coefficients of variation ≤20%. The markers C9, A2M and MATN1 were only qualitatively detected with coefficients of variation >20%. In case of the meat and bone meal mixtures with rMBM2 all marker peptides were precisely quantified on the highest concentration level of 10%. The 1% concentration level was quantitatively detected with coefficients of variation ≤20% for all markers except MATN1, which was only qualitatively detected. The lowest concentration level of 0.1% could not be quantitatively detected for rMBM2, however a qualitative detection was possible for HP252, MYH7 and MATN1 with signals above the calculated LODs but variation coefficients exceeding 20%.

Table 27. Intra- and interassay repeatability of multiplex XA2M, assessed with citrate plasma mixtures on three concentration levels in five replicates, respectively.

Species	Intraassay						Interassay					
	10% w/w (1.1% per species)		5% w/w (0.6% per species)		1% w/w (0.1% per species)		10% w/w (1.1% per species)		5% w/w (0.6% per species)		1% w/w (0.1% per species)	
	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %
cattle	871	6	459	5	107	2	892	4	461	7	107	7
sheep/goat	1423	10	651	7	153	7	1351	12	658	9	161	10
pig	1104	5	571	6	152	7	1110	6	588	3	158	6
horse	500	8	248	8	59	5	526	5	300	7	73	10
chicken	189	7	103	5	22	8	203	6	103	16	24	8
turkey	113	8	54	5	12	12	121	4	66	14	14	8
duck	80	4	40	8	9	11	85	9	48	8	10	19
goose	129	5	68	3	15	7	132	4	68	17	15	14

Table 28. Intra- and interassay repeatability of multiplex RQ3, assessed with two ruminant meat and bone meals (rMBM1 and rMBM2) of different origin and a ruminant spray-dried plasma (rSDP) on three concentration levels with five replicate runs, respectively. Not quantifiable samples are shown with a value of zero. Qualitative detections with coefficients of variations exceeding 20% are marked (0[a]).

	Sample	SERPINF2		C9		HP252		A2M		SPP1		MYH7		MATN1	
		mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %
Intraassay	10% rSDP	4527.2	4.5	1038.4	2.3	8845.1	2.5	34431.8	3.7	0	-	0	-	0	-
	1% rSDP	394.8	4.0	99.8	3.5	760.9	1.7	3215.2	3.0	0	-	0	-	0	-
	0.1% rSDP	36.7	9.9	9.3	3.5	75.5	1.2	298.5	4.6	0	-	0	-	0	-
	10% rMBM1	69.4	5.9	37.3	4.4	162.0	8.9	489.1	7.5	8.1	7.0	2670.9	14.0	248.2	6.7
	1% rMBM1	4.4	16.9	2.5	4.2	10.2	6.0	29.9	6.8	0.7	9.3	212.2	11.5	5.6	7.8
	0.1% rMBM1	0	-	0[a]	-	1.0	11.2	2.6	18.5	0.1	20.0	16.2	16.1	0.4	18.0
	10% rMBM2	12.6	11.7	12.1	12.1	25.5	12.1	71.3	12.3	119.7	8.6	471.5	13.0	136.6	9.0
	1% rMBM2	3.5	16.0	1.9	11.7	7.1	9.4	9.2	8.7	15.6	12.9	73.5	17.3	0[a]	-
	0.1% rMBM2	0	-	0	-	0[a]	-	0	-	0	-	0[a]	-	0[a]	-
Interassay	10% rSDP	4313.4	5.3	1120.5	12.6	9172.9	4.7	34581.5	3.4	0	-	0	-	0	-
	1% rSDP	389.3	5.0	97.8	3.8	742.9	4.4	3038.7	3.8	0	-	0	-	0	-
	0.1% rSDP	36.0	9.1	9.0	4.7	69.8	9.0	282.9	8.9	0	-	0	-	0	-
	10% rMBM1	67.6	6.7	37.7	3.4	155.2	7.2	461.7	5.5	8.7	2.8	2512.9	5.2	203.4	14.6
	1% rMBM1	4.8	10.2	2.9	11.6	11.4	7.9	33.7	11.0	0.7	12.6	255.3	12.6	7.1	17.3
	0.1% rMBM1	0	-	0[a]	-	1.0	13.9	0[a]	-	0	-	19.2	15.0	0[a]	-
	10% rMBM2	14.9	3.7	14.8	12.0	30.4	14.8	83.1	8.9	137.3	8.2	564.1	6.4	185.8	10.7
	1% rMBM2	4.0	18.7	2.1	3.6	7.7	4.3	10.0	5.2	18.4	11.2	91.5	6.2	0[a]	-
	0.1% rMBM2	0	-	0	-	0[a]	-	0	-	0	-	0[a]	-	0[a]	-

4.5 Analysis of Proficiency Test Samples

The developed multiplex assays RQ3 and XA2M were used to analyze official proficiency test samples provided by the German Federal Institute for Risk Assessment, originally obtained in proficiency tests from the European Reference Laboratory for Animal Proteins (EURL-AP, Gembloux, Belgium). An overview over the proficiency test samples, the expected and experimentally determined species and tissues is given in Table 29. An overview about the quantification data is shown in Table 30. A detailed evaluation of the samples with the extracted ion chromatograms of each sample is shown in Supplementary Data I.

In a first step, the cross-species XA2M multiplex assay revealed that all samples were from bovine or porcine origin. Feed 1, feed 2 and feed 4 were proven to contain only bovine material. Feed 3 was analyzed to consist of porcine and bovine material. Feed 5 and feed 6 were proven to consist of only porcine material. In a second step, the ruminant- and tissue-specific RQ3 multiplex assay was applied. As expected, feed 5 and feed 6 did not contain ruminant proteins, which confirmed the findings observed first in the species identification. The highly tissue-specific proteins MYH7 and MATN1 were only detected in feed 1 and feed 2 along with the plasma proteins C9, HP252, A2M. This result indicated that feed 1 and feed 2 were adulterated with a ruminant meat and bone meal. The missing signal for SERPINF2 in low concentrated MBM samples is not surprising, since this target was shown to be less sensitive in comparison to the other plasma targets (4.4.5). The absence of SPP1 in the two feeds proved that the samples did not contain milk powder. In feed 3, missing SPP1, MYH7 and MATN1 signals, but signals for all four plasma targets indicated that this sample consists of blood derived ruminant proteins such as SDP or BM. The same was observed for feed 4, however, the signals were considerably higher compared to feed 3. This can be explained due to fact that the adulteration in feed 4 is pure bovine and not consisting of bovine and porcine material as it was shown for feed 3. To sum up the results, the analysis of proficiency test samples showed the suitability of the two multiplex assays for the species identification and tissue differentiation of PAPs and blood products in animal feed compounds on a level of 0.1%.

Table 29. Overview about the expected and determined species and product types in the analyzed proficiency test feed compounds (n.p. = tissue identification not possible).

Feed	Description	Expected species	Expected product	Determined species	Determined tissue
1	0.1% ruminant PAP in pig feed	Cattle, sheep, goat	BM, MBM	Cattle	Muscle
2	0.1% ruminant PAP in pig feed	Cattle, sheep, goat	BM, MBM	Cattle	Muscle
3	1% ruminant hemoglobin meal in fish feed	Cattle, sheep, goat	BM, SDHM	Cattle in Pig	Blood
4	3% bovine plasma in fish feed	Cattle	SDP	Cattle	Blood
5	5% porcine blood in fish feed	Pig	BM, SDHM	Pig	n.p.
6	Hemoglobin meal in fish feed	no info	BM, SDHM	Pig	n.p.

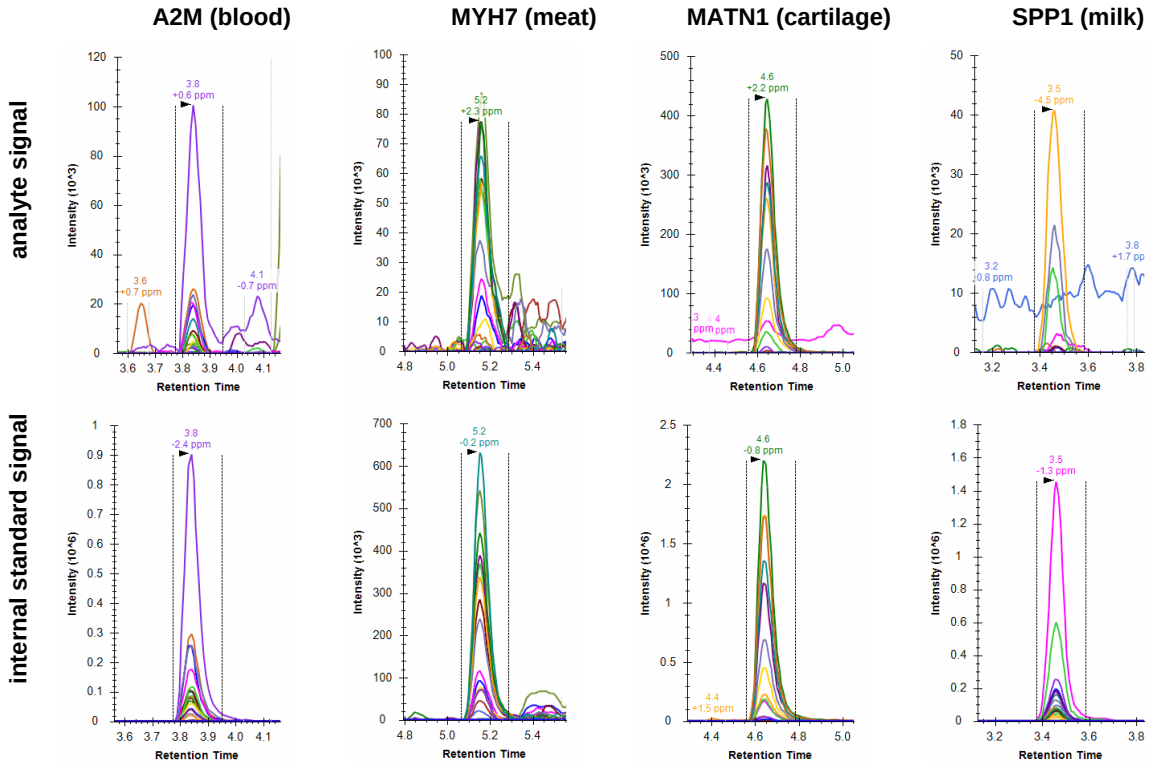


Figure 40. Tissue-specific quantification of 0.1% ruminant PAP in a pig compound feed (Feed 1) determined by multiplex RQ3. The marker for A2M represents the plasma proteins, the other plasma proteins are shown in Supplementary Data I.

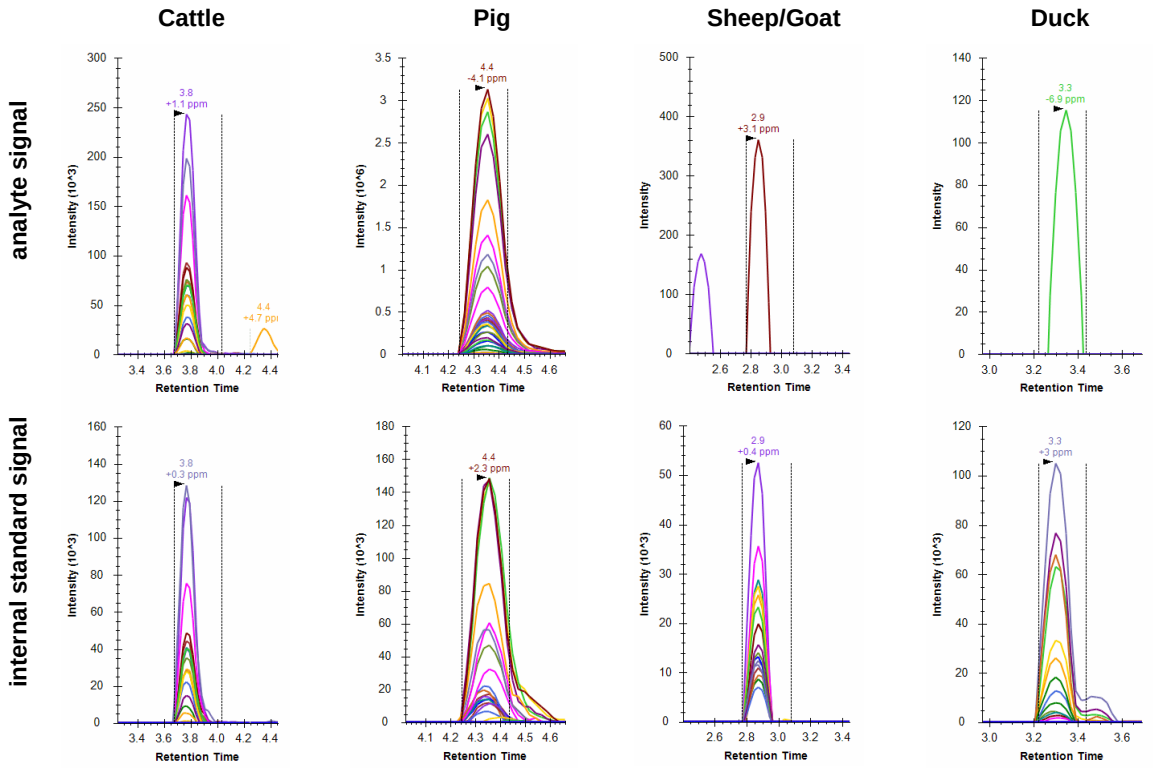


Figure 41. Species differentiation and quantification of 1% ruminant blood in a porcine background (Feed 3) determined by multiplex XA2M. Positive signals for cattle and pig are shown. The species sheep/goat and duck showed negative signals. The other species were also negative and are shown in Supplementary Data I.

Table 30. Species identification and tissue differentiation in official proficiency test feed compounds using the developed multiplex RQ3 and XA2M. Analysis was performed in three replicates for each sample.

Assay	Species	Feed 1		Feed 2		Feed 3		Feed 4		Feed 5		Feed 6		Control	
		mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %	mean / fmol	C.V. / %
Species identification (XA2M)	Cattle	4.1 ± 0.1	2.9	4.4 ± 0.4	8.8	88.0 ± 2.9	3.3	956.9 ± 32.3	3.4	0	-	0	-	0	-
	Sheep/Goat	0	-	0	-	0	-	0	-	0	-	0	-	0	-
	Pig	0	-	0	-	834.3 ± 29.7	3.6	0	-	136.8 ± 4.2	3.0	537.0 ± 4.7	0.9	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	-
Tissue differentiation (RQ3)	SERPINF2	0	-	0	-	2.3 ± 0.1	4.4	156.3 ± 7.0	4.5	0	-	0	-	0	-
	C9	0.4 ± 0.1	14.9	0.4 ± 0.05	10.9	1.2 ± 0.1	8.9	50.1 ± 2.5	5.0	0	-	0	-	0	-
	HP252	1.2 ± 0.2	13.4	1.4 ± 0.1	4.4	10.7 ± 0.3	2.9	331.5 ± 10.0	3.0	0	-	0	-	0	-
	A2M	4.5 ± 0.3	6.6	4.7 ± 0.5	11.4	94.0 ± 4.3	4.6	1065.1 ± 165.9	15.6	0	-	0	-	0	-
	SPP1	0	-	0	-	0	-	0	-	0	-	0	-	0	-
	MYH7	5.6 ± 0.4	6.4	6.7 ± 1.0	14.2	0	-	0	-	0	-	0	-	0	-
	MATN1	7.8 ± 1.5	19.6	14.4 ± 4.3	29.4	0	-	0	-	0	-	0	-	0	-

5 Discussion

5.1 Marker Peptide Identification and Multiplex Panel

Aim of this thesis was the development of a method for the detection of processed animal proteins (PAPs) in feed on the basis of a peptide-centric approach. Basically, a species authentication in unknown PAP samples can be performed without *a priori* knowledge of specific marker peptides. To achieve this, a non-targeted mass spectrometry (MS) experiment can be performed. The obtained mass spectra are then compared with spectral libraries of reference samples [89]. However, for a highly sensitive, accurate and precise quantification, defined peptides have to be identified and a targeted method has to be developed [89-91]. These targeted methods usually address species- and tissue-specific peptides deriving from meat or bone proteins that were previously identified by non-targeted mass spectrometry of meat and bone meal (MBM) samples. An extensive study of possible MBM marker peptides was published by Marbaix and colleagues [90]. However, meat-specific markers do not allow the detection of blood products, such as spray-dried plasma (SDP) or blood meals (BM). In this thesis, the focus was set on the identification of species- and tissue-specific markers, which allow a comprehensive analysis of SDP, BM and MBM products from relevant livestock species.

Non-targeted MS analyses of citrate plasma, SDP, BM, MBM and milk powder of different species served as an experimental basis for the further bioinformatic selection of suitable peptides (Table 9). The highest number of identified proteins was observed in the native citrate plasmas. As expected, the processed SDP, BM and MBM samples showed lower numbers of peptide identifications. Nevertheless, more than 1000 potential marker peptides were identified in these processed samples, when they were prepared using the newly developed sample preparation protocol, named heterogeneous phase digestion (HPD). An in-depth comparison and discussion of HPD and other sample preparation protocols is given in section 5.2.2. These non-targeted MS analyses provided experimentally verified peptides that were detected in differently processed samples and were used for the further bioinformatic marker selection (4.1.2 and 4.1.3).

In a first bioinformatic analysis, possible cross-species epitopes were identified to establish a multispecies multiplex assay using only one antibody. The multispecies assay was

intended to be applied for a multispecies detection of PAPs and blood products in aquaculture feed. Five plasma proteins that allow a cross-species enrichment of homologous peptides using only one antibody were identified: alpha-2-macroglobulin, coagulation factor VIII, antithrombin-III, serum albumin and cholinesterase (Table 10). The cross-species epitope of alpha-2-macroglobulin was chosen to generate a polyclonal antibody since it matched the selection criteria best (3.2).

Cholinesterase was not chosen, because of its lower plasma abundancy compared to the other four targets. Serum albumin was not chosen because of the high risk for cross contaminations in biochemistry laboratories since it is commonly used as blocking reagent and standard protein in immunoassays and protein determination assays. The antithrombin peptides comprise a very long epitope at the C terminus which would allow the generation of a highly specific cross-species antibody. However, the peptides are very large with quite similar sequences and therefore a chromatographic separation was supposed to be challenging. Factor VIII comprises shorter peptides with a higher inter-species sequence variation. The short cross-species epitope of these marker peptides comprises only four amino acids which could increase the risk for antibody cross-reactions to other high abundant sequences.

In comparison, alpha-2-macroglobulin was considered superior to the other target proteins: First, the C-terminus covers 9 species of interest with species-specific peptides for 8 species. A differentiation of sheep and goat via alpha-2-macroglobulin is not possible since the peptide sequences are identical. However, this is not an issue since the legal regulations state ruminants as one group. Second, the N-terminal sequences show a high inter species variability which facilitates a chromatographic separation. Third and most important, the conserved C-terminal sequence offers the possibility of expanding the cross-species epitope to a length of eight amino acids if an immunization with the two sequence variations, containing either phenylalanine (F) or tyrosine (Y) in the X position of the epitope's sequence (*VEE<u>X</u>VLPK)* is performed. This allowed the generation of a cross-species antibody which is able to enrich different species' A2M peptides from PAP and blood products with a high specificity.

However, addressing a single bovine protein like alpha-2-macrogloblin (A2M) was not sufficient for tissue differentiation. A2M was also detectable in milk powder at very low concentrations (Table 24). Furthermore, a cross-species antibody was considered to be

less suitable if only a sensitive detection of ruminants in a background of other species is required. Therefore, a second bioinformatic search was performed in which three further ruminant-specific plasma peptides from alpha-2-antiplasmin (SERPINF2), protein HP-25 homolog 2 (HP252) and complement component 9 (C9) were identified (Figure 9). These markers were chosen because they show only a low sequence similarity to the homologous peptides from other species. In mass spectrometry, a single amino acid exchange that is unique for the species would be sufficient for reliable species identification. However, in immunoaffinity-based mass spectrometry, inter-species sequence similarities could lead to cross reactivity of antibodies in multispecies mixtures. Consequently, the matrix species would block the antibody's binding capacity and probably limit the assay's sensitivity regarding the species of interest. This issue was addressed by the identification of species-specific peptide sequences with low inter-species similarity and the generation of peptide-specific antibodies.

To further improve tissue specificity, additional marker peptides that are unique for each tissue were identified. Non-targeted MS analyses of milk powder, MBM and citrate plasma revealed ruminant-specific marker peptides that are unique for the respective sample types (Figure 10): myosin-7 (MYH7), matrilin-1 (MATN1) and osteopontin (SPP1) were selected as marker peptides for meat, bone and cartilage tissue, respectively. These six additionally selected ruminant-specific peptides offered the possibility for unambiguous species and tissue detection (Table 11).

At first, the 7 bovine-specific marker peptides were compiled in two different 4-plex assays for ruminant quantification (RQ), one addressing the four plasma targets A2M, SERPINF2, C9 and HP252 (RQ1) and another addressing the tissue-specific markers MYH7, MATN1 and SPP1 together with the plasma marker C9 (RQ2). The two multiplexes were intended to address different questions in feed authentication: RQ1 for highly specific blood product detection in feed and SDP-SDP admixtures; RQ2 for tissue-specific MBM detection. While RQ1 performed well, RQ2 was quite unstable and 5 µg of each antibody instead of 1 µg had to be used for immunoprecipitation (Supplementary Data F). Consequently, also the number of magnetic microspheres needed for the precipitation of antibody-peptide-complexes had to be increased. Since the number of magnetic microspheres exceeded the maximum possible volume, the antibody-microsphere-ratio was reduced from 5 to 2.5, limiting the precipitation efficiency. As a consequence of this, new peptide-

specific polyclonal antibodies against the RQ2 targets were generated. The new antibodies performed better and could be used in lower amounts of 1 µg. This also allowed the combination of the two 4-plex assays into one 7-plex assay (RQ3) since the limiting factor of magnetic microsphere amount was not reached. The eight homologous A2M peptides of different species were combined in one cross-species 8-plex assay (XA2M) using the cross-species antibody anti-VEE*X*VLPK. The immunoaffinity enrichment allowed the development of short chromatographic gradients with a cycle time of only 10 mins, thereby increasing the sample throughput.

To sum up, a total number of 14 marker peptides were identified and compiled in two multiplex assays: One that allows a species differentiation of 8 species using one group-specific antibody and another for the differentiation of ruminant tissues using highly ruminant-specific epitopes. The limitations of current analytical methods concerning the species and tissue differentiation of different animal protein types were addressed by the selection of these targets. On this basis, the two quantitative assays XA2M and RQ3 were developed and basic analytical parameters were validated.

5.2 Sample Preparation of Processed Animal Proteins

5.2.1 Optimization of Peptide Release

In the course of marker peptide identification, extracts of SDP, BM and MBM samples were analyzed by gel electrophoresis. Therefore, a detergent-based buffer extraction of the samples' heat stable and water-soluble protein fraction was prepared and compared to a native bovine citrate plasma. The gel electrophoresis revealed a partial fragmentation of proteins in the SDP samples and a high degree of fragmentation in the BM and MBM samples (Figure 12). Missing protein bands in BM and MBM did not indicate an unsuccessful extraction since the protein determination proved the presence of proteins in the extracts (Table 15). Consequently, the extracts loaded on the gel must have been highly fragmented. This result corroborated the hypothesis that protein-centric approaches, such as sandwich immunoassays, are relatively unsuitable for the detection and quantification of PAPs since antibody binding usually requires intact proteins as a prerequisite for detection. Without detailed knowledge of the epitope, the actual protein length and potential modifications of the PAPs, a targeted protein-centric approach remains challenging.

Whereas a peptide-centric approach, which could quantify proteins in PAP samples indirectly through peptides via mass spectrometry, seemed to be the favorable solution. The experiment also revealed that the sample preparation or extraction needed to be optimized since a high protein fraction still remained insoluble.

Commonly used sample preparation protocols for protein extraction from processed animal protein and processed meat samples include urea/thiourea buffers or TCA/acetone followed by clean-up steps because of incompatibility of some reagents with mass spectrometry [82,89,90]. In this thesis, a peptide-centric approach was rated promising. A direct tryptic digestion of PAPs in suspension without prior protein extraction in order to improve the release of peptides from denatured, fragmented and insoluble proteins was applied and evaluated. Since salts, fats and reagents were removed during the immunoaffinity enrichment, a further clean-up before mass spectrometric analysis was not necessary. The direct tryptic digestion required a method to determine the total peptide content in the supernatant after digestion. The A280 method was considered as a fast and easy way to determine the total peptide content in digests and was preferred over a time-consuming amino acid analysis. Usually, the A280 method is used for pure proteins with known extinction coefficients [106]. Applying the rule 1 Abs ≈ 1 mg mL^{-1} citrate plasma samples were measured both with the bicinchoninic acid (BCA) assay and the A280 method (Table 12). The two protein determination methods showed similar results with a correlation coefficient of 0.90 (Figure 13). Therefore, an accurate protein determination via A280 method in complex digests was assumed.

Since the A280 method is not a very specific determination, possible interfering substances were evaluated. The A260/A280 ratio was used to determine the protein purity of the enzymatically fragmented samples and to estimate the impact of potentially extracted nucleic acids. The calculated protein purities of citrate plasmas and SDP were >99%. The porcine BM was calculated with 97.9%. The lowest protein purity was calculated for fish feed with 90.1% (Table 13). A correction of the protein concentration was not considered necessary. In this thesis, the quantification was performed by the use of isotope labeled internal standard peptides added to the samples and then result was referred to the protein concentration determined via A280. Alternatively, the quantification could be referred to A260/A280-corrected concentrations or even directly to the initial sample weight of 15 mg per sample used for HPD. In this case, the A280 determination

would be only used to estimate the protein amount for immunoprecipitation. However, the A280 measurement was proven to be a very important tool to check for reproducibility and efficiency of HPD (Supplementary Data J). The interference of chemical reagents added during HPD was also evaluated. Iodoacetamide (IAA) was shown to interfere with the A280 measurement (Figure 14). Absorption due to IAA and the added trypsin were subtracted using blank digests.

Different parameters of HPD were checked and optimized (Figure 15). In order to achieve a stable PAP suspension during HPD, the sample amount was limited to 15 mg per 750 µL digestion buffer and a mixing speed of 1000 rpm was used. While the enzyme manufacturer had no effect on the total protein release, the enzyme ratio to sample had a slight effect and was best in a ratio of 1:40.

Optimized HPD was applied to different species SDP, BM and MBM for different fragmentation times. The fragmentation time itself had no effect on the peptide release. However, the peptide yields strongly depended on the sample type and its processing conditions (Figure 16). Less processed SDP samples showed the highest peptide yield whereas the highly processed bovine meat and bone meal showed the lowest peptide yield. The different peptide yields were expected to influence the limit of detection of the respective sample type. All HPD-prepared samples were also compared to a buffer extraction in phosphate buffered saline. However, dramatically lower peptide yields were observed for the pure buffer extraction (Figure 12). SDPs tended to form gels by the addition of PBS. In contrast, when HPD was applied, they completely dissolved in an overnight treatment.

In conclusion, it was shown that a peptide-centric workflow was preferable to a protein-centric one. This allowed direct enzymatic fragmentation of PAPs in suspension without prior protein extraction. In combination with immunoprecipitation, time-consuming clean-up steps prior to mass spectrometry could be avoided. However, the suspension stability during HPD had to be considered and a blank digest had to be performed in order to determine the total peptide content of the supernatant using the A280 method. The sensitivity of PAP and blood product detection was supposed to depend on the sample type since different peptide yields were observed. The direct enzymatic fragmentation was also shown to be more efficient than a buffer extraction of proteins. Whether only more total protein or even other proteins became soluble needed further investigation.

5.2.2 Heterogeneous Phase Digestion

It was further investigated if HPD is not only a buffer extraction with tryptic digestion of dissolved proteins in solution, but rather a direct digestion at the PAP's liquid-solid interface. The hypothesis was that HPD not only releases more total peptide due to a higher digestion efficiency but also makes insoluble proteins accessible for analysis by a partial digestion and solubilization of certain protein domains. In course of this, HPD was also compared to the state of the art TCA/acetone protein extraction method. A detailed mass spectrometric analysis of the released peptides was performed.

A side-by-side comparison of an extraction with in-solution digestion (ISD) of the supernatant and a direct digestion (HPD) was performed (Figure 17). Compared to ISD, the A280 measurements after HPD-preparation showed a twofold increase in case of bovine MBM and porcine BM. The protein concentration increased by a factor of 5.8 for porcine SDP. A280 absorption indicated that HPD was significantly more efficient than ISD. The MBM sample was further analyzed in an in-depth comparison of HPD and ISD with a recently published state of the art TCA/acetone protein extraction protocol for PAPs [90]. Therefore, non-targeted mass spectrometric analyses in triplicate runs were performed (Figure 18). Using the TCA/acetone protocol, a comparable number of peptides and proteins to that of the ISD protocol was observed. The analysis of bovine MBM by Marbaix and colleagues revealed a maximum number of 495 peptide identifications after TCA acetone extraction followed by a cleanup step and LC-MS/MS analysis on a Q-TOF mass spectrometer [90]. In direct comparison this is twice the amount of the ISD peptide identifications but half of the HPD result. The different types of mass spectrometers and different MBM sources may contribute to the variations in identified peptides. Hence, the results of the analysis in this thesis and the results from Marbaix and colleagues cannot be directly compared. Nevertheless, the higher number of identified peptides with the application of HPD indicated that trypsin was capable to digest proteins at the liquid-solid-interface releasing peptides from highly processed MBM, which were not accessible via protein extraction in the same buffer system. It was assumed that the additionally released peptides derived from insoluble proteins from which some domains were cleaved and thereby the peptides became soluble.

The comparison of HPD and ISD was also evaluated in terms of marker peptide concentrations. Two bovine samples, rMBM and rSDP, and two porcine samples, pBM and pSDP

were analyzed by the targeted MS assays (Figure 24). The results confirmed that all marker peptides were released in significantly higher amounts using HPD, independent from the sample type that was analyzed (MBM, BM or SDP).

For highly sensitive detection and quantification of proteins, indirectly through the detection of peptides, the digestion step is crucial. It has been reported that tryptic digestion is strongly influenced by digestion time. [96,111]. HPD preparation was optimized in a time dependent analysis of marker peptide concentrations in both rMBM and rSDP samples using multiplex RQ3 (Figure 26) and in citrate plasmas using XA2M (Figure 25). While the total protein release via HPD was proven to be not dependent on fragmentation time (Figure 16), the marker peptide release was strongly time dependent. The experiments revealed that a 2 h HPD-preparation was already enough to achieve mean normalized peptide releases of >90%. Longer digestion times showed negative effects on some marker peptides. Especially the tissue-specific markers MYH7, MATN1 and HP252 of multiplex RQ3 and the turkey, goose and chicken A2M peptides of multiplex XA2M, showed decreasing peptide concentrations over time, probably due to peptide degradation, adsorption or unspecific enzymatic fragmentation. Since different sample types could affect HPD, peptide release for the bovine A2M peptide was analyzed in three different bovine samples: citrate plasma, rMBM and rSDP (Figure 25). Except a slight difference at 16 h for rMBM, the samples showed similar peptide releases over time in different sample types. The application of optimized HPD fragmentation time to different sample types was considered unproblematic.

To summarize, HPD was proven to release both more and different peptides from highly processed animal protein samples compared to ISD or TCA/acetone. The marker peptides were released in significantly higher amounts, beneficial regarding the assays' sensitivities. Unlike the total peptide release, the release of marker peptides was shown to be affected by digestion time. The optimum digestion time was determined and the applicability of HPD to different animal protein types was confirmed.

5.3 Species and Tissue Differentiation

5.3.1 Multispecies Detection

Species differentiation in the feed sector is very difficult due to the strict regulations imposed by the European Union. A safe use of PAPs is only guaranteed if the species origin of proteins in feed can be unambiguously determined. For several reasons, this is an analytical challenge. First, there is a variety of different animal proteins deriving from different slaughter byproducts that have to be detected (e.g. blood, meat, bone). These are processed under different conditions depending on the product type, leading to denaturation and fragmentation reactions. An identification of intact markers that can be measured in all product types is very challenging. Second, identified markers must be suitable for species differentiation of at least the most used livestock species namely cattle, pig and poultry animals. Third, and most critical, all these proteins have to be analyzed in very different feed matrices used for farmed animals.

In this thesis, the issue of different product types of several species in a variety of feed matrices was addressed by a cross-species immunoenrichment of plasma peptides combined with mass spectrometric detection. A group-specific antibody targeting 8 species-specific marker peptides which were present after rendering in PAPs and blood products was generated. This allowed the enrichment of 8 livestock species from MBM, BM and SDP samples in a complex feed background. The immunoprecipitated peptides were then identified via mass spectrometry. The highly specific multiplex detection in a fish feed background was shown for citrate plasma mixtures in Table 22. The specific differentiation of species for several PAP and blood product samples was shown in Table 23.

In conclusion, the multiplex XA2M overcomes current limitations in species differentiation in the field of feed authentication. Multiplex XA2M is able to parallelly quantify the 8 livestock species cattle, sheep/goat, pig, horse, turkey, chicken, goose and duck in the main PAP and blood product types MBM, BM and SDP and works in a complex feed matrix. XA2M is only limited in the differentiation of tissues since only one plasma protein is addressed.

5.3.2 Tissue-Specific Ruminant Detection

The differentiation of legal and illegal proteins is one of the key points in animal feed authentication. Current official methods such as polymerase chain reaction cannot differentiate between allowed milk powder and not allowed BM or MBM ingredients deriving from the same species. Latest developments in PAP detection methods addressed this issue by targeting meat-specific proteins [90] or blood proteins with parallel detection of milk proteins [91]. In the former, meat proteins were targeted which cannot be found neither in milk nor in BM. This assay was able to specifically detect MBM in a cattle feed matrix on a level of 5% (w/w). However, the high detection limit for cattle MBM and the lack for a differentiation of illegal BM and legal milk powders are significant drawbacks of this method. In the latter, blood and milk proteins were targeted simultaneously, allowing the differentiation of the two sample types. A detection limit for BM samples of <0.1% was shown. Probably, the simultaneous detection will work for MBM samples, too. The occurrence of those marker peptides in MBM was observed in this thesis by shotgun proteomic analysis. Nevertheless, the suitability of the assay for MBM was not shown by the authors and it remains unclear, whether a detection limit of 0.1% can be reached for highly processed MBM.

In this thesis, multiplex peptide-centric assays targeting several proteins were used to address the stated issues. Multiplex RQ1 was developed to address four highly ruminant-specific plasma proteins. During the bioinformatic marker peptide identification and selection, proteins identified in milk powder were excluded. However, the developed multiplex RQ1 was able to detect the markers in milk powder samples in very low amounts (Figure 39). On the one hand, this highlighted the advantage of immunoaffinity-based mass spectrometry in terms of a highly sensitive peptide detection. On the other hand, this impeded the differentiation of milk and blood containing samples by measuring A2M as single protein. Nevertheless, RQ1 was able to differentiate MBM and BM from milk powder by calculating the relative protein amounts in the sample types, respectively. The marker peptides in plasma and milk have their own characteristic ratio that can be compared with unknown samples in order to identify the sample's origin and discriminate between legal and illegal. Moreover, the tested samples in this work consisted of 10% milk powder, 10% SDP, or 10% MBM. The concentration of A2M in milk powder was about 500

times lower than in the SDP sample and 10 times lower than in the MBM sample. Therefore, animal feeds with very high amounts of milk powder, namely milk replacers for calves, would pose an analytical worst-case scenario with a milk protein content of up to 50%. Due to monetary reasons, the amount of milk powder used in milk replacers is closer to 20-30% which would result in amounts of A2M comparable to a milk-free feed adulterated with 0.04-0.06% rSDP or 2-3% rMBM, respectively. However, in the case of milk powder, the measured value for HP252 would be comparable to A2M whereas in case of SDP as additive the signal for A2M would be the 2-fold of HP252. Hence, concentration and ratio of the proteins have to be taken into account to judge a sample's PAP content and origin.

Unambiguous species and tissue differentiation are very important criteria for new analytical methods to determine the origin of animal proteins in feed. Although RQ1 was able to differentiate between MBM, SDP and milk, the required tissue specificity was shown to be challenging when only plasma peptides were addressed. For this reason, additional marker peptides that are unique for each tissue type were identified. The additionally selected tissue-specific peptides from SPP1, MYH7 and MATN1 further increased the tissue specificity of detection in multiplex RQ3. While still having information about the absolute and relative plasma protein amounts, additional information about other tissue types were obtained. The marker SPP1 was not detected in SDP samples and in comparable levels to the other proteins in bovine MBM (Table 24). In contrast, the milk powder samples showed a relative SPP1 amount of >99% strongly indicating the presence of a milk adulteration (Figure 39). The markers MYH7 and MATN1 clearly discriminated the bovine MBM from BM, SDP and milk powder.

In conclusion, the results have proven the suitability of the developed multiplex assay RQ3 to unambiguously detect and quantify bovine proteins from MBM, SDP, BM and milk with high precision and confidence. Until now, no method was reported that was able to simultaneously detect and differentiate all tissue types in a feed compound as matrix.

5.4 Detection and Quantification of Processed Animal Proteins

5.4.1 Qualitative Detection

A qualitative detection of 0.1% PAP in feed is the approved level for the official microscopic method evaluated in former ring trials. The detection limit of polymerase chain reaction (PCR) even reaches 0.05% ruminant PAP in plant-based fish feed as demonstrated in recent proficiency tests [49]. Alternative methods are challenged by these qualitative limits since up to now no threshold for PAP in feed is in place.

The qualitative detection capabilities of the developed assays RQ3 and XA2M were evaluated. The absolute limit of the detection (LOD) expressed in peptide amounts was assessed by dilution series of synthetic standard peptides spiked in feed matrices. The application of multiplex assay RQ3 was intended to be in the authentication of cattle feed. For this reason, the corresponding dilution series was spiked into a digested vegetal cattle feed as matrix. Multiplex assay XA2M was intended to be applied for a multispecies detection in aquaculture feed authentication studies. In this case, a digested fish feed was used as matrix. A comparison of different possibilities to determine LOD for targeted mass spectrometric assays was published by Mani and colleagues [108]. It was shown that blank measurements combined with measurements of a low analyte concentration give the most reliable LOD estimation. This method considers alpha and beta errors and is very suitable for highly specific mass spectrometric assays where the signal in blank measurements is often zero or strongly fluctuates, leading to inconsistent LOD calculations.

The used matrix amount and hence the later measured sample amount was assessed prior to the dilution experiment. A maximum sensitivity can be achieved by increasing the absolute sample amount in the immunoaffinity step. Consequently, the matrix amount also increases, possibly leading to more pronounced matrix effects. To assess matrix effects, a constant known amount of stable isotope labeled standard peptides was analyzed at different matrix amounts. Some of the generated antibodies seemed to be affected by the vegetal cattle feed matrix (Figure 23). While the signal intensities increased for A2M and HP252, the signal intensity of SERPINF2 decreased with higher matrix amounts. In case of the cross-species antibody for homologous A2M peptides, the peptide signal intensities slightly increased with higher matrix amounts (Figure 21). It was supposed that contrary effects such as blocking of reagent tubes and cross-reactivity of antibodies with the matrix

determined the actual signal intensity. Therefore, multiplex XA2M was supposed to be not negatively affected by the matrix in higher sample amounts. Consequently, the sensitivity for low level contaminations could be increased if higher matrix amounts were analyzed. The same applied for most of the bovine-specific peptide-antibody pairs. However, the achievable sensitivity for SERPINF2 will most likely decrease when higher matrix amounts are analyzed. It was decided to spike the dilution series in a range of 50 amol to 1000 fmol into a total amount of 100 µg feed matrix in order to assess the absolute amount LOD.

The standard dilution experiments showed that the measurements were linear over a concentration range of 4 to 5 orders of magnitude. The blank measurement and the lowest concentration showing a S/N of ≥ 3 were used to determine LOD. The determined LODs using the method evaluated by Mani and colleagues were between 38 amol and 645 fmol for RQ3 in vegetal cattle feed and between 30 amol and 1.20 fmol for XA2M in fish feed (PRM detection, Table 17).

The mass spectrometric detection after immunoaffinity enrichment and chromatographic separation was performed using a high resolution and accurate mass (HRAM) quadrupole-orbitrap hybrid mass spectrometer. Compared to low resolution triple quadrupole instruments that are common in routine analysis, HRAM instruments allow the measurement of both, precursor and fragment ions with an outstanding accuracy and sensitivity [112,113]. For this reason, the two MS detection modes SIM and PRM were compared regarding sensitivity and specificity in feed matrices. The detection of fragment ions using PRM showed lower LODs for most peptides than the detection of precursor ions using SIM (Table 17). Not only the detection limit improved but also the confidence of detection was higher using PRM. The precursor scans in SIM were shown to be affected by high noise levels in the extracted ion chromatograms even after immunoaffinity enrichment (Figure 31). Peak integration for the three isolated precursor isotopes was shown to be inaccurate (Figure 32). It was decided that for qualitative detections, PRM offers a more confident marker peptide identification.

Since an orbitrap analyzer was used, all fragment ions were detected in parallel, leading to an absolute specificity of peptide detection. However, the number of detected fragments depends on the peptide concentration. The most intense fragment ions exceed the LOD first, followed by other fragment ions according to their relative intensities. In this

thesis, the LOD was determined only for the most intense fragment ion. Further fragment ions were considered only for quantitative analyses (Table 16). Nevertheless, the confidence of illegal product detection while analyzing only one fragment ion per marker is still very high since in multiplex assays, several fragments of different markers are monitored simultaneously.

The detection limits in feed authentication studies are usually expressed as weight percentages of PAP in feed compounds. The weight percentage LOD indirectly depends on the contamination type SDP, BM or MBM and the actual analyte amount present in the respective product type. Highest sensitivity was supposed to be observed for SDP contaminations, since they are less processed and show high relative levels of plasma proteins. In contrast, the lowest sensitivity was expected for highly processed MBMs as contaminants and addressing plasma proteins as markers for MBMs, since their relative abundancy was quite low. Higher concentrated and more tissue-specific targets such as myosin or matrilin should be addressed in that case.

To determine the LOD expressed as weight percentage, feed matrices spiked with HPD-prepared MBM and SDP were analyzed. SDP and MBM were chosen as the two extrema in terms of processing conditions and target analyte concentration. As expected, the detection limit of spiked rSDP was observed to be at the lowest analyzed level of 0.05% (Table 19). An extrapolation of the linear regression indicated even lower detection limits (Figure 34). In case of the highly processed rMBM sample, the lowest level detected in a tissue-specific way was 0.05% via MATN1 and 0.1% via MYH7 as marker peptides. Even a porcine SDP contaminated with 0.1% of a bovine SDP was safely detected via the C9 marker peptide, proving the high specificity of the generated antibody in multispecies mixtures. Although multiplex XA2M was not developed with the intention to be highly sensitive, the assay was able to detect 0.1% rMBM in a fish feed matrix via the bovine-specific A2M peptide (Table 20).

To summarize, the developed multiplex RQ3 qualitatively detected rMBM and rSDP in a VF matrix as well as rSDP in a pSDP matrix on a level of 0.1%. The detection via PRM was shown to provide a higher detection confidence compared to SIM. The VF matrix was observed to affect the functionality of some of the developed polyclonal antibodies.

5.4.2 Quantitative Determination

The European Commission currently works on the introduction of a PAP threshold for the presence in feed compounds [39]. This requires future feed authentication methods to be quantitative. Although there are currently no thresholds in place, it is estimated that alternative methods need to be quantitative in a range of <1-2% [45]. The quantification capabilities of the developed assays were assessed within the same experiments as for the qualitative detection. The linear regression equation was used to calculate the measurement's accuracy and its precision. The lowest concentration level that was measured with an accuracy between 80-120% and a precision of ≤20% was defined as the lower limit of quantification (LLOQ). The LLOQs determined for RQ3 determined in vegetal cattle feed were in the range between 152 amol and 1.5 fmol, and LLOQs for XA2M determined in fish feed were in the range between 4.1 fmol and 37.0 fmol (Table 17).

The quantification range was determined in phosphate buffered saline as well as in feed matrices (Table 18 and Table 17). These data were used to determine the analytical recovery and to answer the question whether the matrices affect the antibodies binding efficiency and therefore the limit of quantification. Slight effects of the matrix on the signal intensities were observed (Figure 21 and Figure 23). However, the quantification was not affected in a range of 4 fmol to 1000 fmol for RQ3 (Table 25) and 37 fmol to 1000 fmol for XA2M (Table 26).

The rSDP- and rMBM-spiked VF samples were used to determine the limit of quantification (LOQ) expressed as weight percentage for multiplex assay RQ3. Here, a quantitative determination was defined as signals above the calculated LOQ with a precision ≤20% for the most intense fragment ion. Additionally, two further qualitatively detected fragment ions, were chosen as criteria for quantification. Applying these rules, the lowest level of rMBM that was quantitatively detected in a tissue-specific way was 0.05% via MATN1 and 0.1% via MYH7 and SPP1 (Table 19). The plasma proteins SERPINF2 and HP252 were least sensitive with a LOQ of 5.0%. In the rSDP spike-in series, all analyzed levels equal or higher than 0.05% were quantitatively determined. Furthermore, the upper limit of quantification was exceeded for A2M, HP252 and C9 as it was shown in Figure 34. Mixtures containing more than 10% SDP as contaminant needed to be diluted in order to achieve a precise and accurate result. The rSDP in pSDP spike-in samples were quantitatively determined on a level of 0.25% via C9, 0.5% via HP252 and 5% via SERPINF2. The group-

specific antibody against A2M peptides was not used in this analysis since it would capture the porcine matrix as well.

For the same reason, no determination of LOQ in SDP-SDP mixtures for multiplex XA2M was performed. However, spike-in samples of different species MBMs in fish feed were investigated. Multiplex XA2M was able to quantitatively determine 0.75% of a porcine and a bovine MBM in fish feed, respectively. The analyzed poultry-mix-MBM was quantitatively determined on a level of 5%. However, single species poultry-MBM would be probably quantified at lower levels.

The RQ1 intraday repeatability was determined with validation samples (section 4.3.10). In this experiment, very high coefficients of variation were observed. It was assumed that these variations were caused by sample inhomogeneities. Indeed, an additional grinding step via ball mill reduced the coefficients of variation and increased the signal-to-noise ratios (Table 21). A detailed analysis of the ground and non-ground samples also revealed that the mean signal intensity decreased when an additional homogenization step was applied (Figure 38). The ground samples' signal intensities for the 0.1% samples were around the detection limit in most cases. In contrast, the non-ground samples showed signals dramatically higher than the limit of quantification for 2 out of 5 replicates. A clear fragmentation pattern in the extracted ion chromatograms for these two samples was observed, proving the peptides' identity (Figure 37). The experiment revealed that a homogeneous sample is indispensable for an accurate and precise quantification. However, when it comes to a qualitative analysis, inhomogeneous samples and a high number of random sampling could increase the chance for detecting the contaminant with a high analytical confidence. The sampling strategy has to be clearly investigated in further projects.

Intra- and interassay repeatability experiments for multiplex RQ3 and XA2M were then performed with additionally ground validation samples (Table 27 and Table 28). The assay repeatability was determined for both of the developed assays at three concentration levels for different sample types. SDP and citrate plasmas were precisely measured with interassay precisions ≤20% on all spiked levels (RQ3 and XA2M). RQ3 assay repeatability was shown for the two different meat and bone meals rMBM1 and rMBM2 on concentration levels of 1% and 10%, respectively. Different results were observed for the two MBMs

on the level of 0.1%. While 0.1% rMBM1 was precisely quantified via HP252 and MYH7, the mixture of rMBM2 was only qualitatively detected.

Cause of this discrepancy could probably be a variation in the relative meat and bone amounts since the samples were obtained from different sources. This can be corroborated by the different total protein contents that were determined in the two MBMs. The plasma, muscle and cartilage protein levels were higher in rMBM1. In contrast, the rMBM2 mixtures showed drastically higher SPP1 levels. This result highlights the importance of the analysis of several protein targets to achieve highest sensitivity in samples with varying protein composition. Furthermore, clear cut-offs for the MBM quantification should be determined on the basis of further analyses with a higher number of different MBMs.

To conclude, in terms of a quantitative determination the admixtures of highly processed rMBMs were quantified with interday variations ≤20% in the range of 0.1-1% (w/w), which was not reported in literature, yet. Measurements of a higher number of both, MBM and SDP samples from different sources are required to confirm these results.

5.5 Ring Trial Samples and Final Conclusion

The developed and partially validated multiplex assays RQ3 and XA2M were applied to proficiency test samples that were provided by the German Federal Institute for Risk Assessment (Berlin) and were originally obtained from the European Reference Laboratory for Animal Proteins (EURL-AP, Gembloux, Belgium) in former ring trials (Table 30). The analyzed feed compounds were two compound feeds for pig, spiked with 0.1% ruminant PAP (Feed 1 and Feed 2); one fish feed, an industrial compound feed for trout farming containing 1% ruminant spray-dried hemoglobin powder (Feed 3); one fish feed, a complete feed for fry containing 3% bovine plasma (Feed 4); one aquafeed (40% complete feed for salmon, 60% fish feed), containing 5% porcine blood meal (Feed 5) and a fish feed with hemoglobin meal, no detailed information available yet (Feed 6).

The application of multiplex XA2M revealed the species origin of the test samples. Porcine- and bovine-specific A2M peptides were identified and quantified with a high analytical confidence. Three pure bovine contaminated feeds (Feed 1, Feed 2, Feed 4), two pure porcine contaminated feeds (Feed 5, Feed 6) and a mix of porcine and bovine contamination in feed were identified (Feed 3). Feed 1 and 2 were discriminated from the other ruminant species sheep and goat by the presence of the bovine-specific marker peptide.

The porcine background in feed 3 did not affect the detection of the bovine material. The intended application of XA2M for species identification in animal feed was proven by this analysis. Furthermore, XA2M successfully identified and quantified the bovine A2M peptides in two pig feeds spiked with ruminant PAP on a level of 0.1%.

Multiplex RQ3 was used to identify the tissue origin (legal or not) of the detected bovine contaminations. At first, two pure porcine contaminated feeds were analyzed with the bovine-specific RQ3 multiplex to prove the absence of ruminant material. Since no ruminant material was detected in these samples, they would be legal for use in aquaculture feed. The four feeds containing bovine material were analyzed to be meat meals (Feed 1 and Feed 2), and blood products (Feed 3 and Feed 4). There were no indications for the presence of milk powder, since osteopontin was not detected in high relative protein levels (>99%). This result proved the illegal source of the bovine material contained in the feed compounds and therefore, their use as pig feed would be illegal.

The analysis of ring trial samples via the two quantitative multiplex assays XA2M and RQ3 showed a reliable species identification and tissue differentiation. The species contained in the feed compounds were identified by XA2M, without being affected by high porcine matrix levels. The tissue origin of the protein source in the bovine contaminated feed compounds was determined by RQ3. The ring trial samples covered common animal protein additives such as PAPs and blood products. These animal proteins can be quantitatively detected on a level of 0.1% in common feed matrices such as fish feed and land living animal feed. A discrimination of illegal animal protein additives and legal milk powder using the developed assays is possible. Figure 42 shows a decision tree to draw a conclusion about the exact species and tissue origin of unknown feed compounds. So far there is no other analytical method reported in literature that offers such a comprehensive quantification and differentiation of animal proteins in feed compounds.

As a final conclusion, the concept of immunoaffinity-based mass spectrometry for the quantitative detection of PAPs in feed was shown to be superior to other current analytical methods. This concept overcomes current limitations and closes the gap in analytical methods for safe PAP detection. The developed assays have great potential to be adopted as official methods in feed authentication studies and are promising candidates for routine feed analysis.

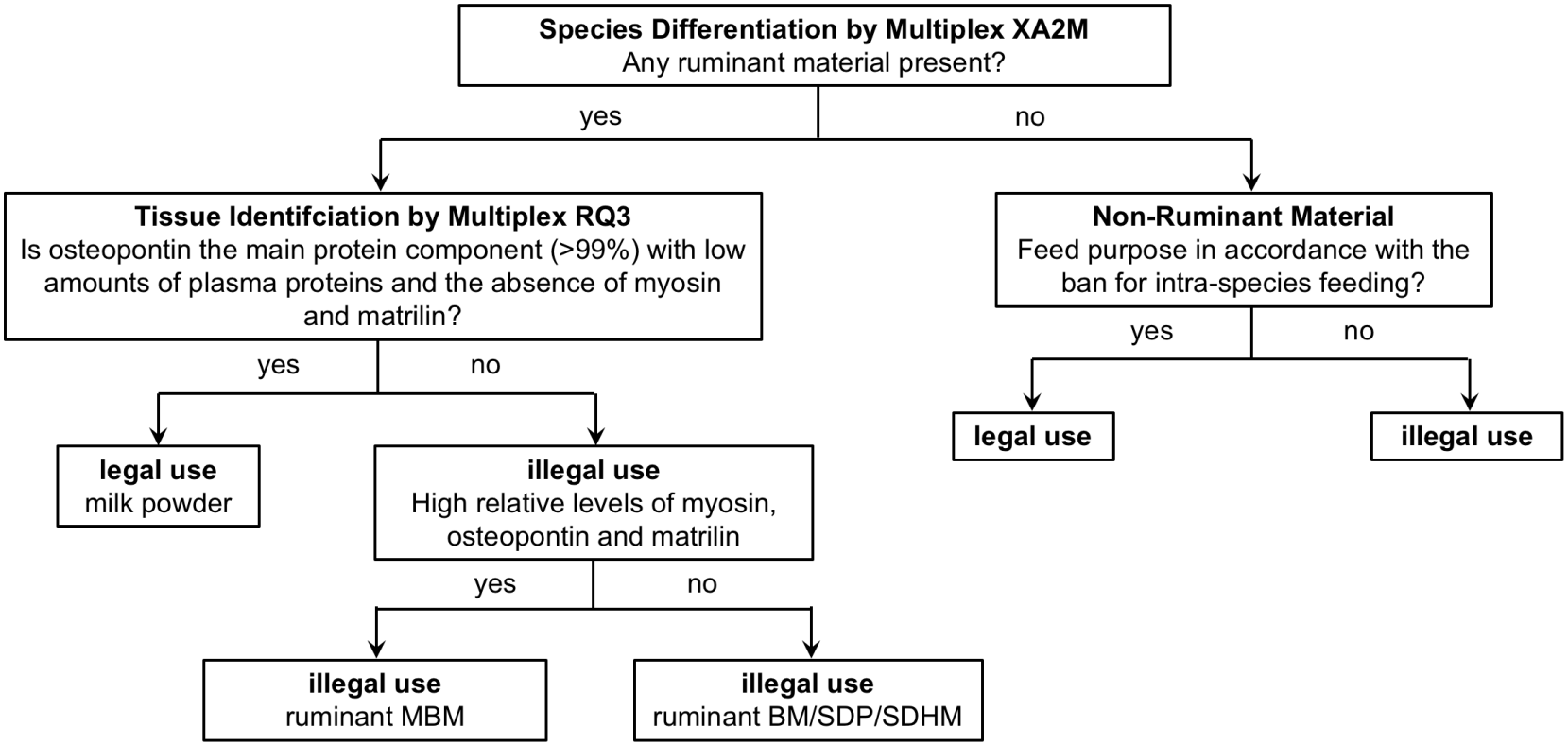


Figure 42. Decision tree for the determination of legal or illegal use of feed compounds analyzed by the two developed multiplex assays for species identification (XA2M) and ruminant tissue differentiation (RQ3).

6 Summary

The present work introduced immunoaffinity-based mass spectrometry to feed analysis and improved the detection of banned processed animal proteins (PAPs) in animal feed. Current analytical methods show deficiencies in either sensitivity, species and tissue specificity or quantification ability. To address this issue, a peptide-centric workflow that comprises a more efficient sample preparation, an immunoaffinity enrichment of species- and tissue-specific peptides, and a LC-MS/MS analysis for identification and quantification using stable isotope labeled standard peptides, was established.

The release of peptides from poorly soluble PAPs and blood products was improved by a direct digestion in suspension. Further time-consuming clean-up steps are not necessary since reagents and salts are removed during the immunoenrichment. The enrichment also allows a fast peptide separation using short gradients with a 10 min cycle time and therefore an increased sample throughput.

The species differentiation of the 8 livestock species cattle, sheep/goat, pig, horse, turkey chicken, duck and goose, was addressed in a multispecies approach. Therefore, a cross-species polyclonal antibody was generated, which is able to enrich 8 homologous peptides from processed meat and bone meal, blood meal and spray-dried plasma, hence allowing a comprehensive analysis of common feed additives. A second multiplex assay was developed to differentiate ruminant tissues by targeting 7 peptides of meat, bone, cartilage, blood and milk proteins. This allows a differentiation of legal and illegal ruminant protein additives. The assays' basic analytical parameters were validated. Both assays showed a detection limit in the picomolar concentration range allowing a qualitative detection over 4 to 5 orders of magnitude and a quantification over 3 to 4 orders of magnitude. Depending on the tissue type, 0.05%-0.75% PAP was specifically and quantitatively determined in an animal feed background.

The multiplex assays were finally applied to official proficiency test samples from the European Reference Laboratory for Animal Proteins (EURL-AP, Gembloux, Belgium). The developed assays showed an unambiguous differentiation and quantification of species and tissues on a contamination level of 0.1% PAP in feed. As a final conclusion, immunoaffinity-based mass spectrometry was shown to overcome the current limitations in PAP detection and meets the requirements for future feed authentication methods.

7 Zusammenfassung

Mit der vorliegenden Arbeit wurde erstmals das Konzept der immunoaffinitätsbasierten Massenspektrometrie im Bereich der Futtermittelanalytik angewendet. Die Detektion von verarbeiteten tierischen Proteinen (VTP) in Futtermitteln wurde damit verbessert. Die derzeitigen Methoden sind aufgrund unzureichender Sensitivität, Spezies- und Gewebsspezifität oder mangelnder Quantifizierung nicht für den zukünftigen Einsatz in der Futtermittelanalytik geeignet. Im Rahmen dieser Arbeit wurde ein Verfahren entwickelt, welches diese Lücke schließen soll. Das peptidzentrische Verfahren umfasst eine verbesserte Probenvorbereitung, eine Immunoaffinitätsanreicherung von tierart- und gewebsspezifischen Peptidsequenzen sowie eine LC-MS/MS Analyse zur Identifikation und Quantifizierung mittels isotopenmarkierten Peptidstandards.

Die Peptidfreisetzung aus schwerlöslichen VTP und Blutprodukten wurde mittels direktem Verdau in Suspension verbessert. Weitere Probenaufarbeitungsschritte zur Entfernung von Salzen und Reagenzien sind nicht notwendig, da diese während der Immunpräzipitation entfernt werden. Außerdem ermöglicht die Anreicherung eine schnelle chromatographische Auftrennung der Peptide mit Zykluszeiten von 10 Minuten und damit einen höheren Probendurchsatz.

Die Tierartdifferenzierung der acht Hauptnutztierarten Rind, Schaf/Ziege, Schwein, Pferd, Pute, Huhn, Ente und Gans wurde in einem Multispeziesansatz verfolgt. Hierfür wurde ein speziesübergreifender polyklonaler Antikörper generiert, um acht homologe Peptide aus Fleischknochenmehlen, Blutmehlen oder sprühgetrockneten Plasmen anzureichern. Eine umfassende Detektion üblicher Proteinadditive in Futtermitteln ist damit gewährleistet. Zusätzlich wurde ein zweiter multiplexer Assay zur gewebsspezifischen Unterscheidung von Rinderproteinen entwickelt. Dieser adressiert sieben gewebsspezifische Peptidsequenzen aus Fleisch, Knochen, Knorpel, Blut und Milchproteinen und ermöglicht eine Unterscheidung von legalen und illegalen Proteinadditiven der Spezies Rind. Es wurden grundlegende analytische Parameter der beiden Assays validiert. Die Assays zeigten eine Nachweisgrenze im pikomolaren Konzentrationsbereich, was eine qualitative Detektion über vier bis fünf Größenordnungen sowie eine Quantifizierung über drei bis vier Größenordnungen erlaubt. Abhängig vom Gewebetyp wurden Verunreinigungen von nur 0,05 % bis 0,75 % VTP in einer tierischen Futtermittelmatrix spezifisch und quantitativ erfasst.

Die entwickelten Tests wurden schließlich auf offizielle Ringversuchsproben des Europäischen Referenzlabors für tierische Proteine (EURL-AP, Gembloux, Belgien) angewendet. Hierbei wurde eine eindeutige Differenzierung und Quantifizierung von Tierarten und Geweben auf einer Konzentrationsstufe von 0,1% VTP in Tierfutter gezeigt. Die immunoaffinitätsbasierte Massenspektrometrie erwies sich damit als eine vielversprechende Methode, um die derzeitige Lücke in der Futtermittelanalytik zu schließen. Die erforderlichen Kriterien an zukünftige offizielle Methoden zur Prüfung der Futtermittelauthentizität sind erfüllt.

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Supplementary Data

Overview:

A. **Exitope Analysis**
B. **Peptide Ionization**
C. **Verification of Species-Specific alpha-2-Macroglobulin Peptides**
D. **Collision Energy Optimization**
E. **Antibody Functionality in Buffer**
F. **Multiplex RQ2 Linearity and Precision**
G. **Multiplex RQ3 Linearity and Precision**
H. **Multiplex XA2M Linearity and Precision**
I. **Proficiency Test Sample Analysis**
J. **A20 Measurements**

A. Exitope Analysis

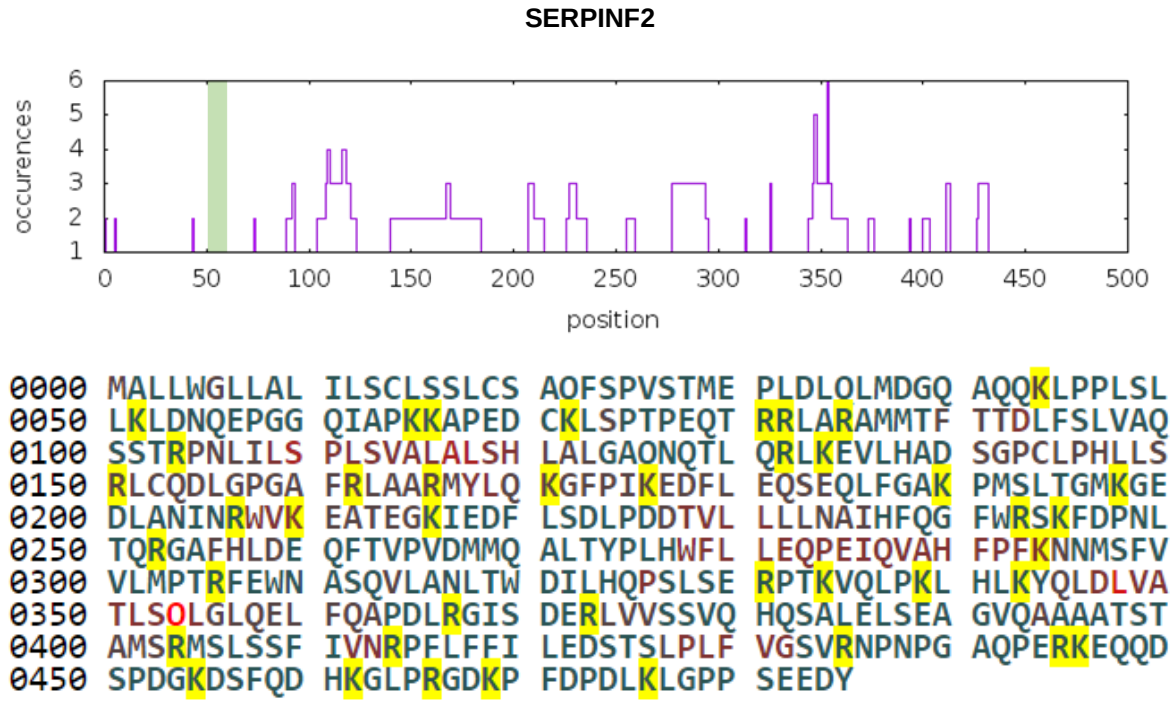

Figure 43. Exitope analysis result for SERPINF2. The occurrence of the peptides in Uniprot is shown in the upper graph. High occurrence sequences are colored red and tryptic cleavage sites are highlighted in yellow.

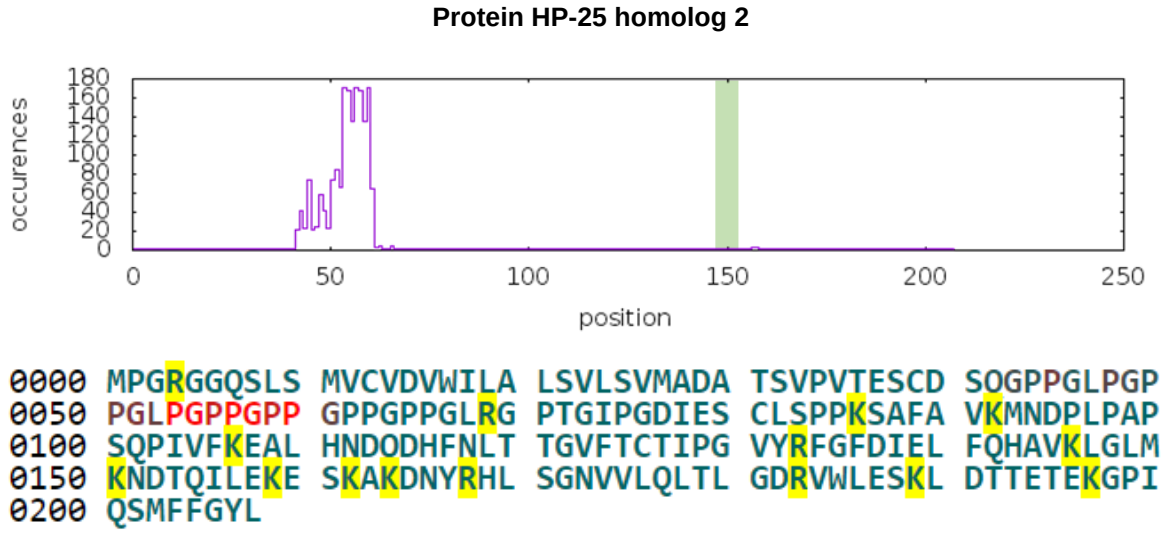

Figure 44. Exitope analysis result for HP252. The occurrence of the peptides in Uniprot is shown in the upper graph. High occurrence sequences are colored red and tryptic cleavage sites are highlighted in yellow.

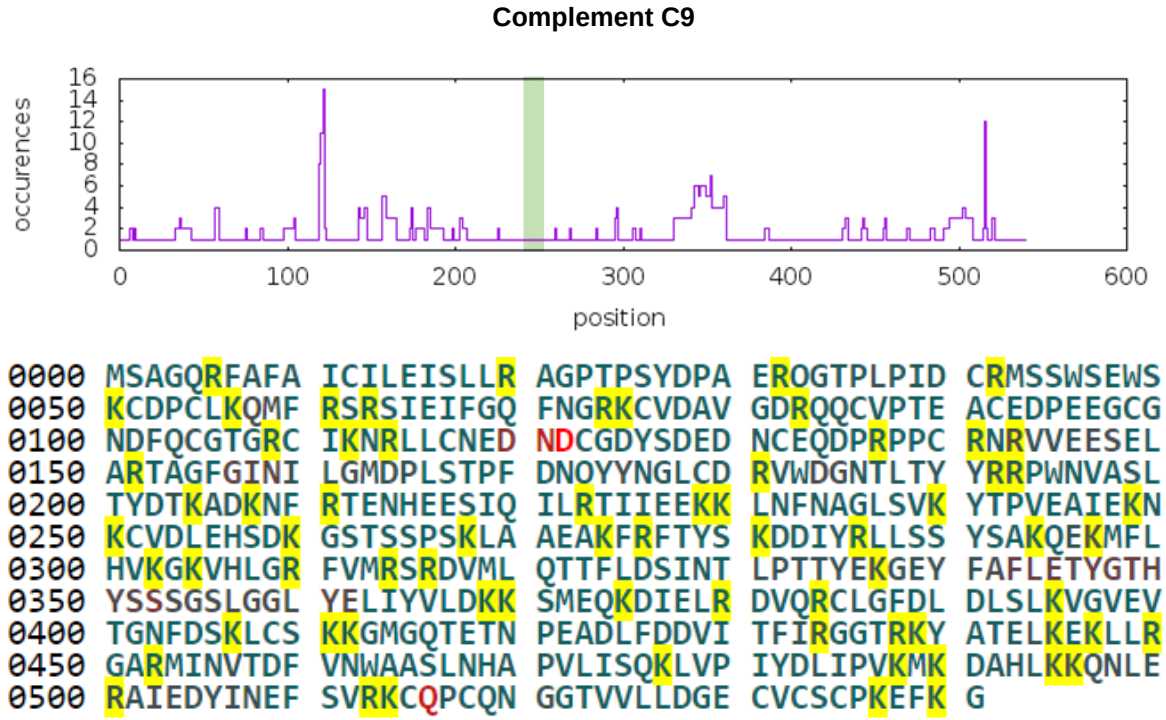

Figure 45. Exitope analysis result for complement C9. The occurrence of the peptides in Uniprot is shown in the upper graph. High occurrence sequences are colored red and tryptic cleavage sites are highlighted in yellow.

B. Peptide Ionization

Table 31. Charge state determination of the selected marker peptides.

Peptide	Intensity p++	Intensity p+++	Most intense Precursor m/z
YTPVEAIEK	6.68E+08	1.79E+05	525.2793
LPPLSLLK	2.82E+09	0.00E+00	440.7969
FGFDIELFQHAVK	1.53E+08	4.56E+08	517.6049
MLSSLFANYAGFDTPIEK	1.06E+08	4.83E+07	1002.4928
AGGIELFAIGVGR	1.68E+09	4.73E+07	630.3590
YPDAVATWLKPDPSQK	1.94E+08	7.44E+08	605.9807
GSGGTAEHPFTVEEFVLPK	3.01E+08	1.64E+09	668.0021
ESGGTAEHHFTVEEFVLPK	1.52E+08	1.21E+09	705.3445
VVVQQESGETAEHPFTVEEFVLPK	6.13E+07	3.16E+09	900.4569
AEHPFIVEEFVLPK	2.12E+09	5.27E+09	552.2995
TIHHPFSVEEYVLPK	4.62E+08	1.44E+09	599.3174
TIQHPFTVEEYVLPK	8.25E+08	2.00E+09	600.9892
TIQHPFSVEEYVLPK	9.02E+08	2.07E+09	596.3173
IQHSFSVEEYVLPK	6.24E+08	1.34E+09	559.2945

C. Verification of Species-Specific alpha-2-Macroglobulin Peptides

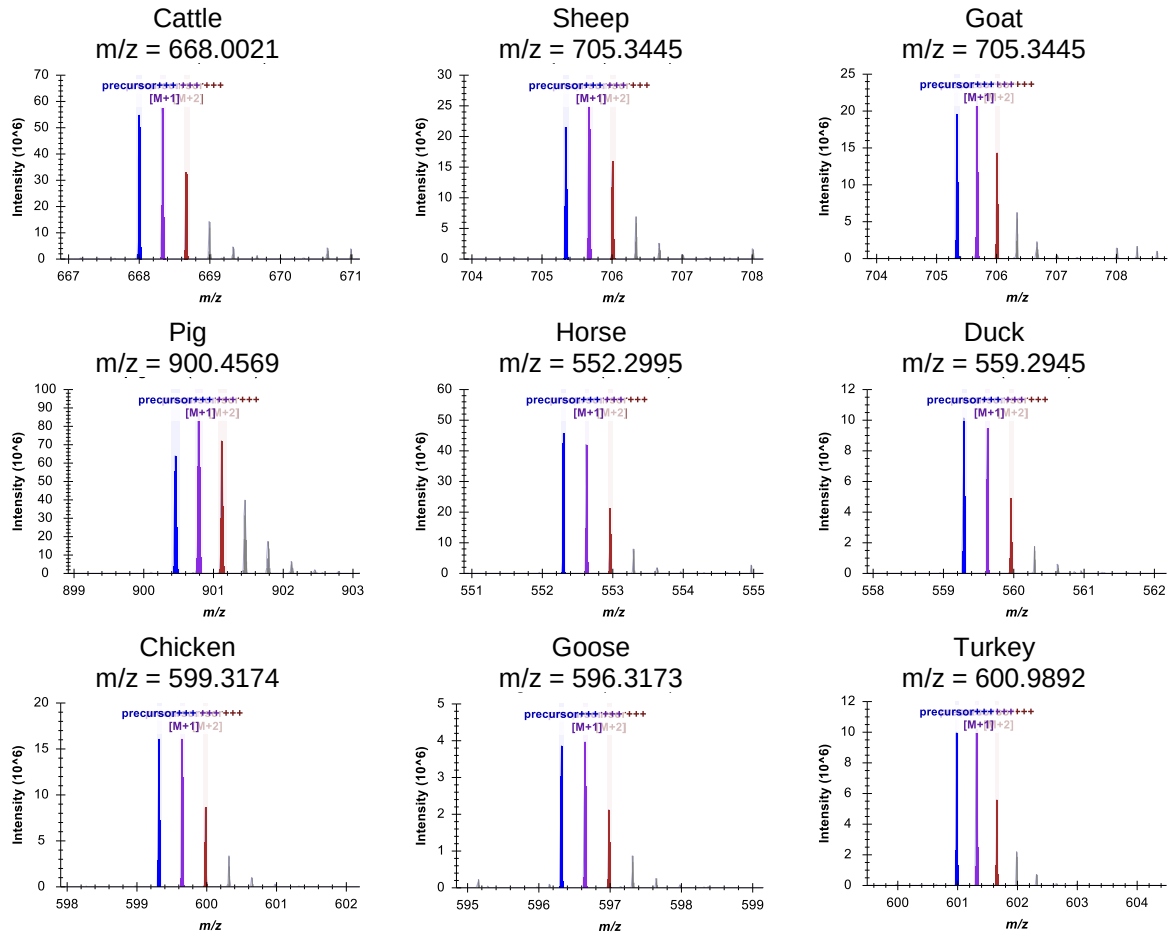

Figure 46. Verification of species-specific alpha-2-macroglobulin peptides in citrate plasmas via non-targeted mass spectrometry. Three most intense isotope signals are highlighted for each precursor.

D. Collision Energy Optimization

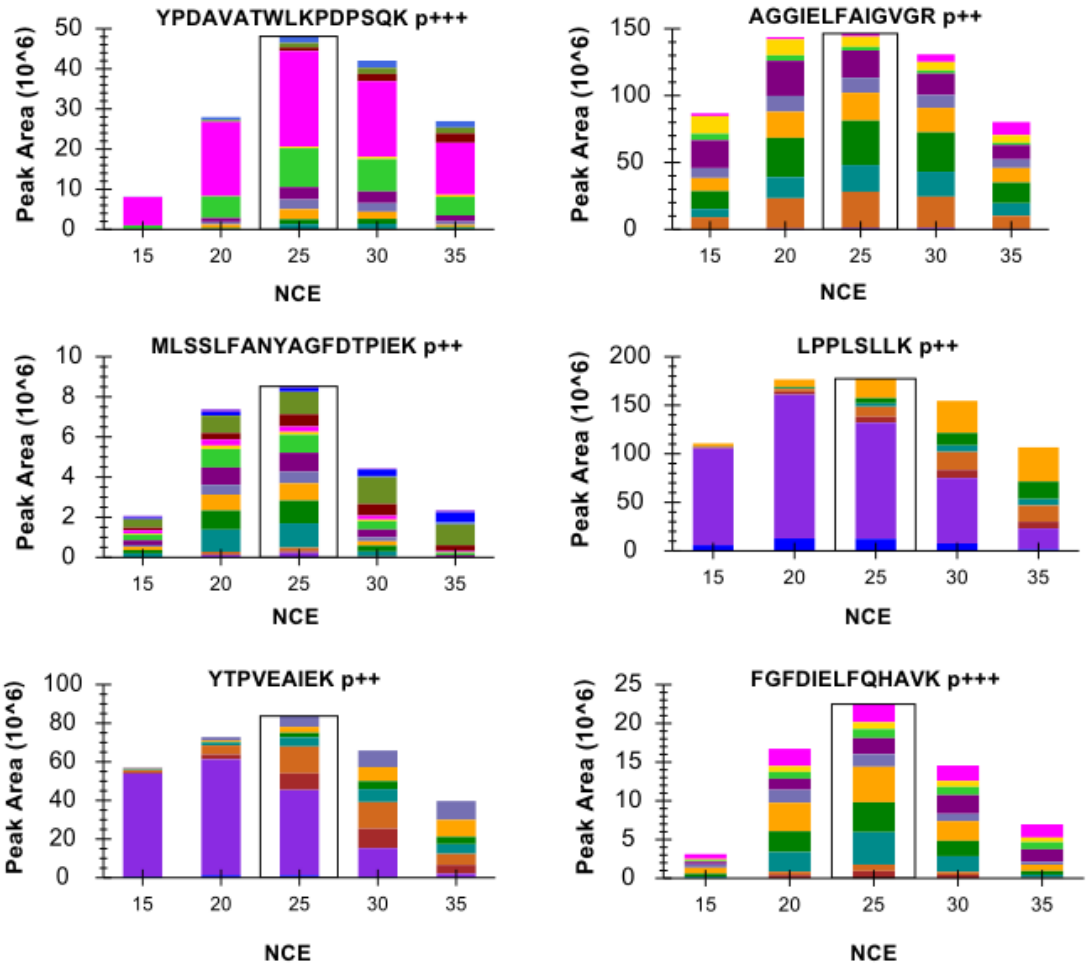

Figure 47. Optimization of collision energy for ruminant peptides. Different fragment ions are indicated by different colors in the stacked bar charts.

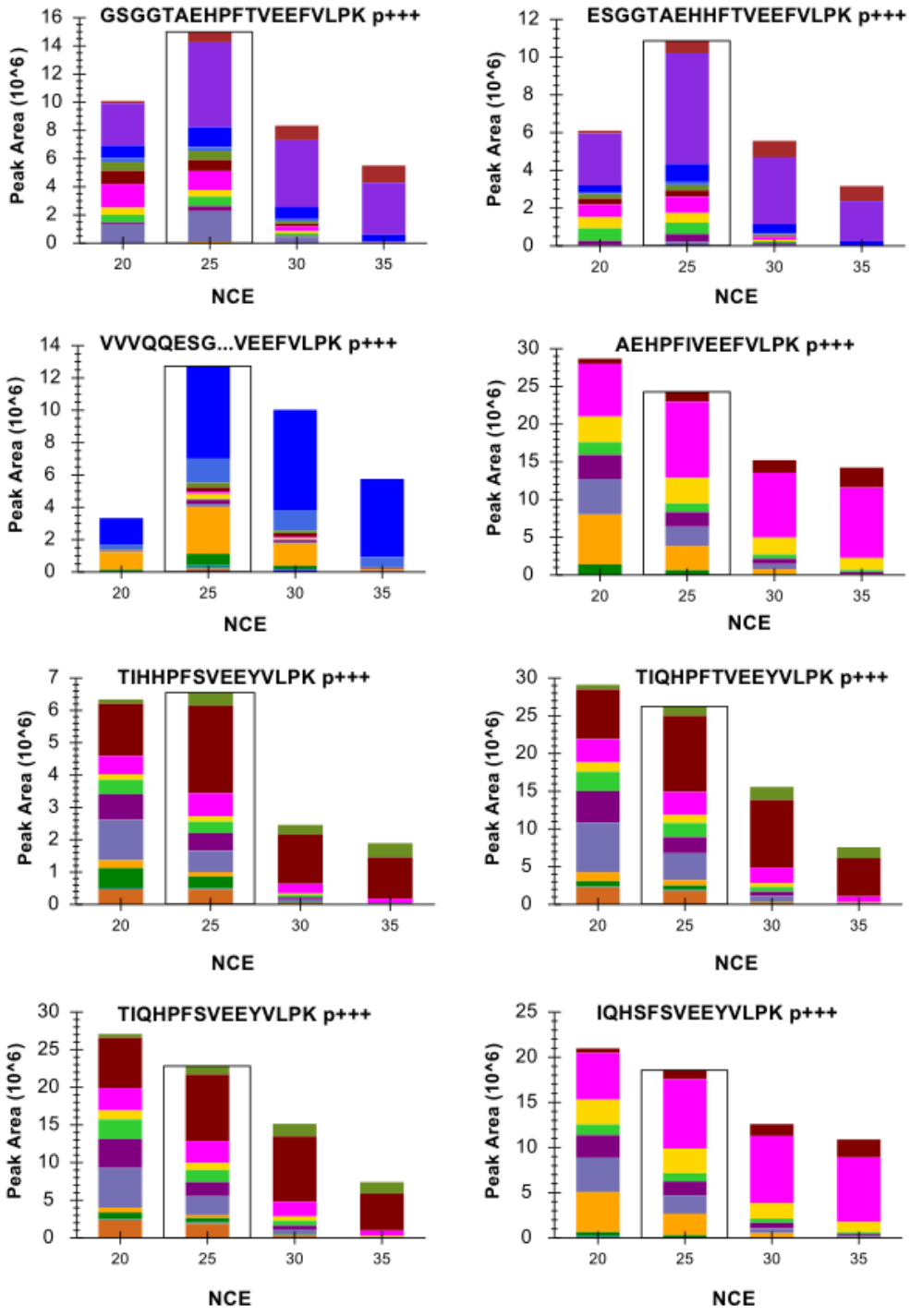

Figure 48. Optimization of collision energy for species-specific alpha-2-macroglobulin peptides. Different fragment ions are indicated by different colors in the stacked bar charts.

E. Antibody Functionality in Buffer

Table 32. Antibody functionality of the two rabbit sera rbt1 and rbt2. determined in PBSC.

Antibody	Epitope	EN signal intensity		IS signal intensity	
		rbt1	rbt1	rbt1	rbt2
Cap_260_261	VEEXVLPK	1.41E+07	3.32E+07	2.23E+07	6.27E+07
Cap33	LPPLSLLK	1.54E+08	5.05E+08	1.45E+08	4.77E+08
Cap34	YTPVEAIEK	5.87E+08	7.06E+08	5.05E+08	6.16E+08
Cap35	FGFDIELFQHAVK	3.05E+08	3.50E+08	1.84E+08	1.90E+08
Cap36	MLSSLFANYAGFDTPIEK	2.85E+06	2.46E+06	2.88E+06	2.58E+06
Cap37	YPDAVATWLKPDPSQK	1.26E+07	8.22E+06	8.93E+06	5.86E+06
Cap38	AGGIELFAIGVGR	1.47E+07	8.95E+06	1.63E+07	1.01E+07

F. Multiplex RQ2 Linearity and Precision

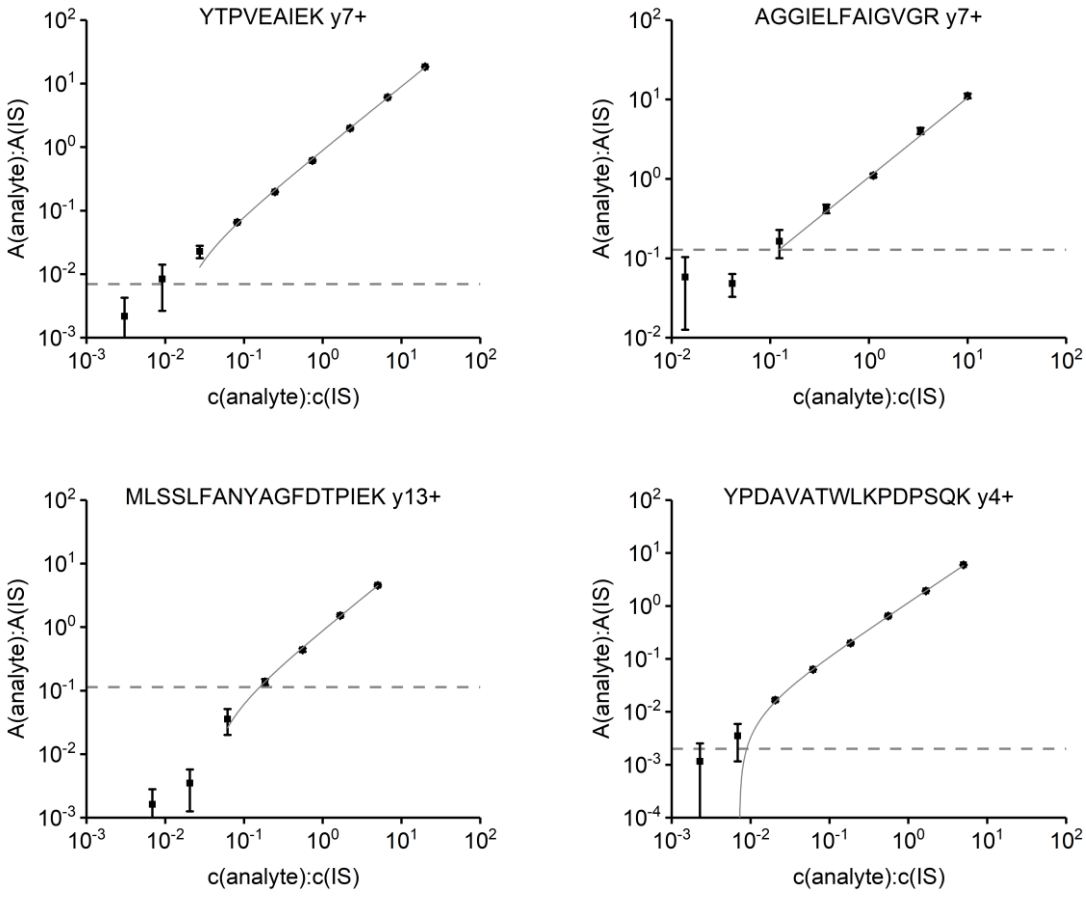

Figure 49. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ2 measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

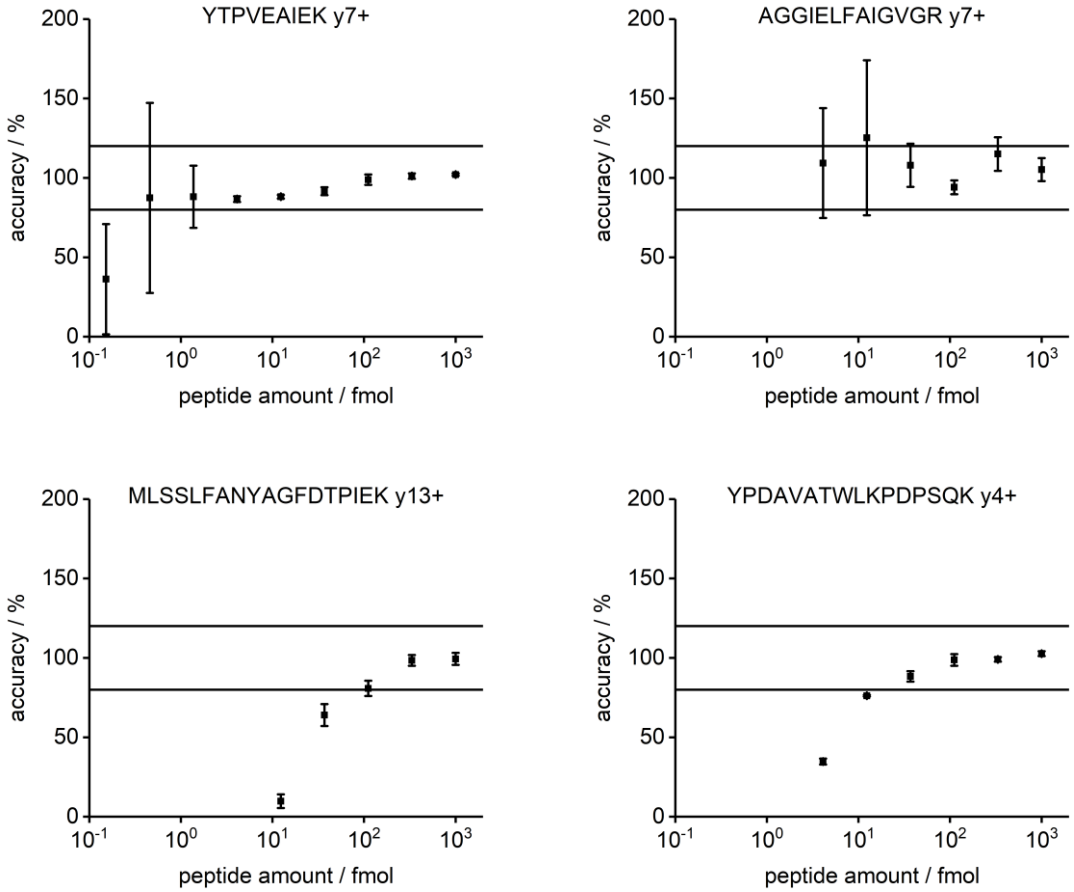

Figure 50. Accuracy and precision of multiplex RQ2 measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

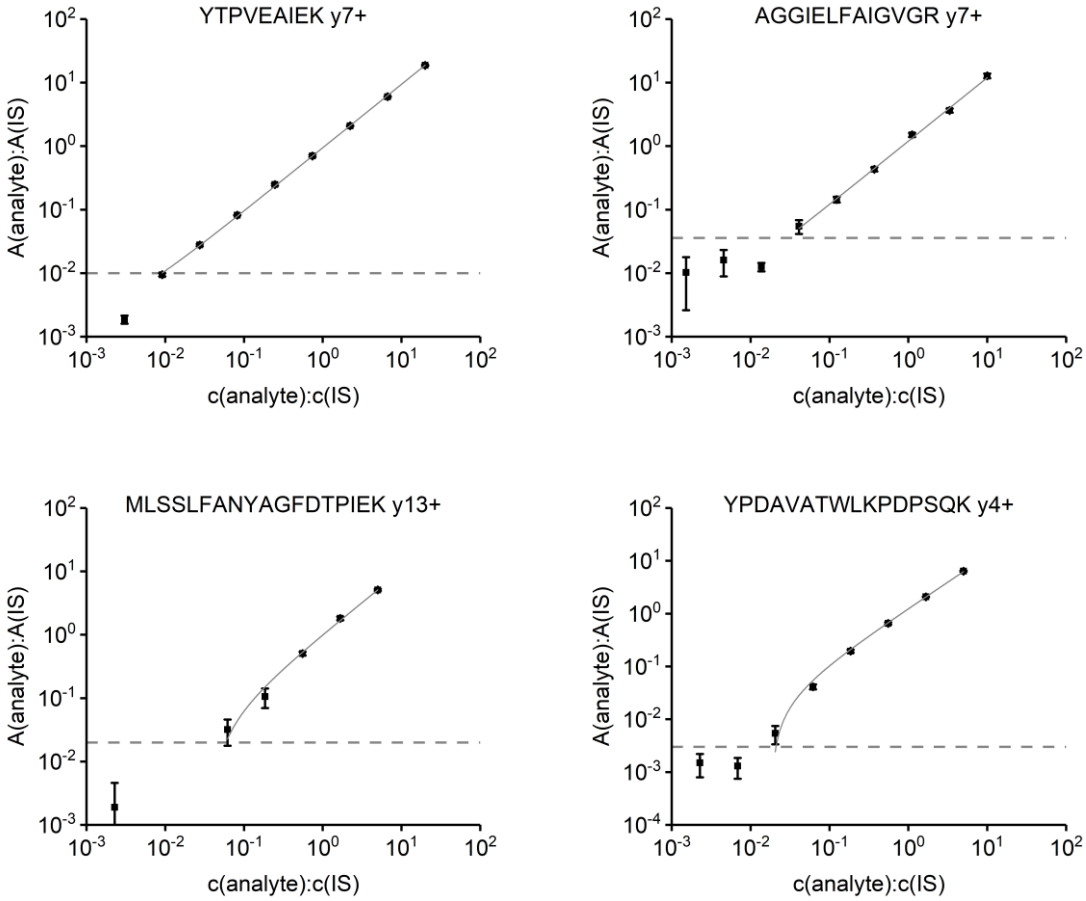

Figure 51. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ2 measured in PRM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

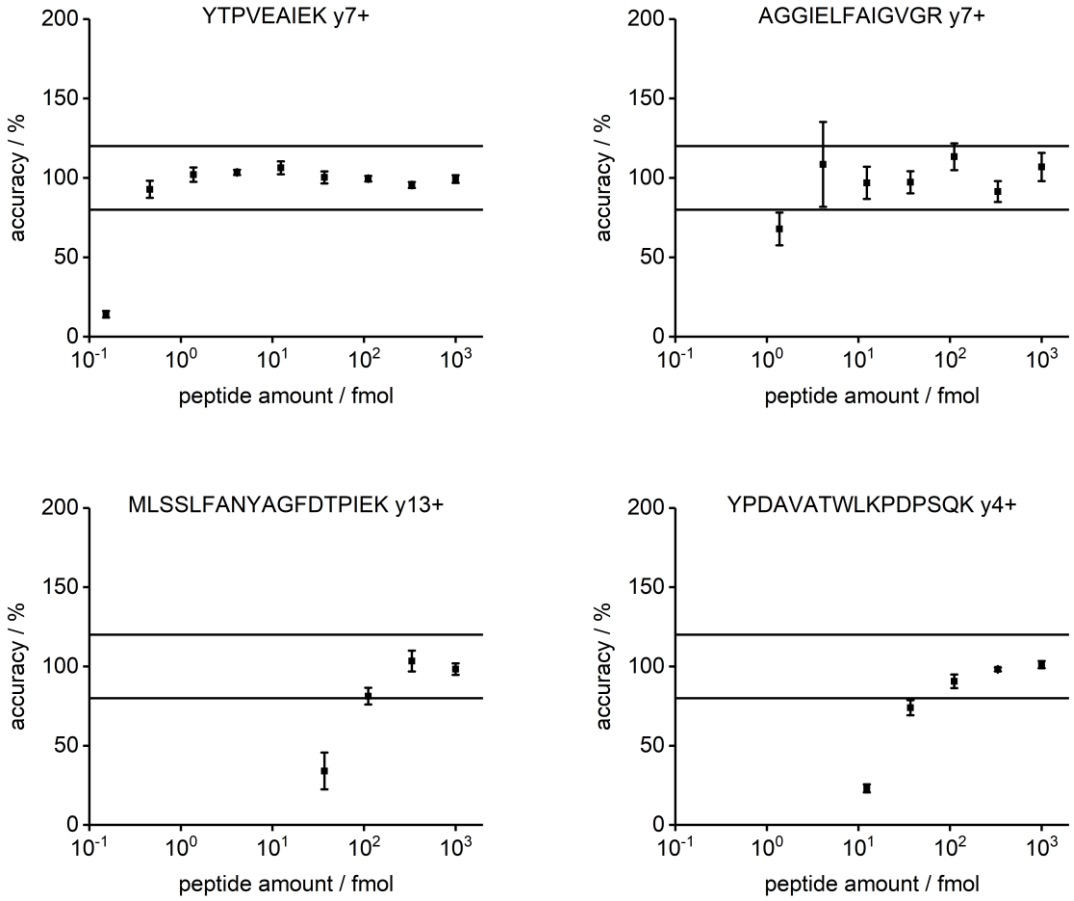

Figure 52. RQ2 Accuracy and precision of multiplex RQ2 measured in PRM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

G. Multiplex RQ3 Linearity and Precision

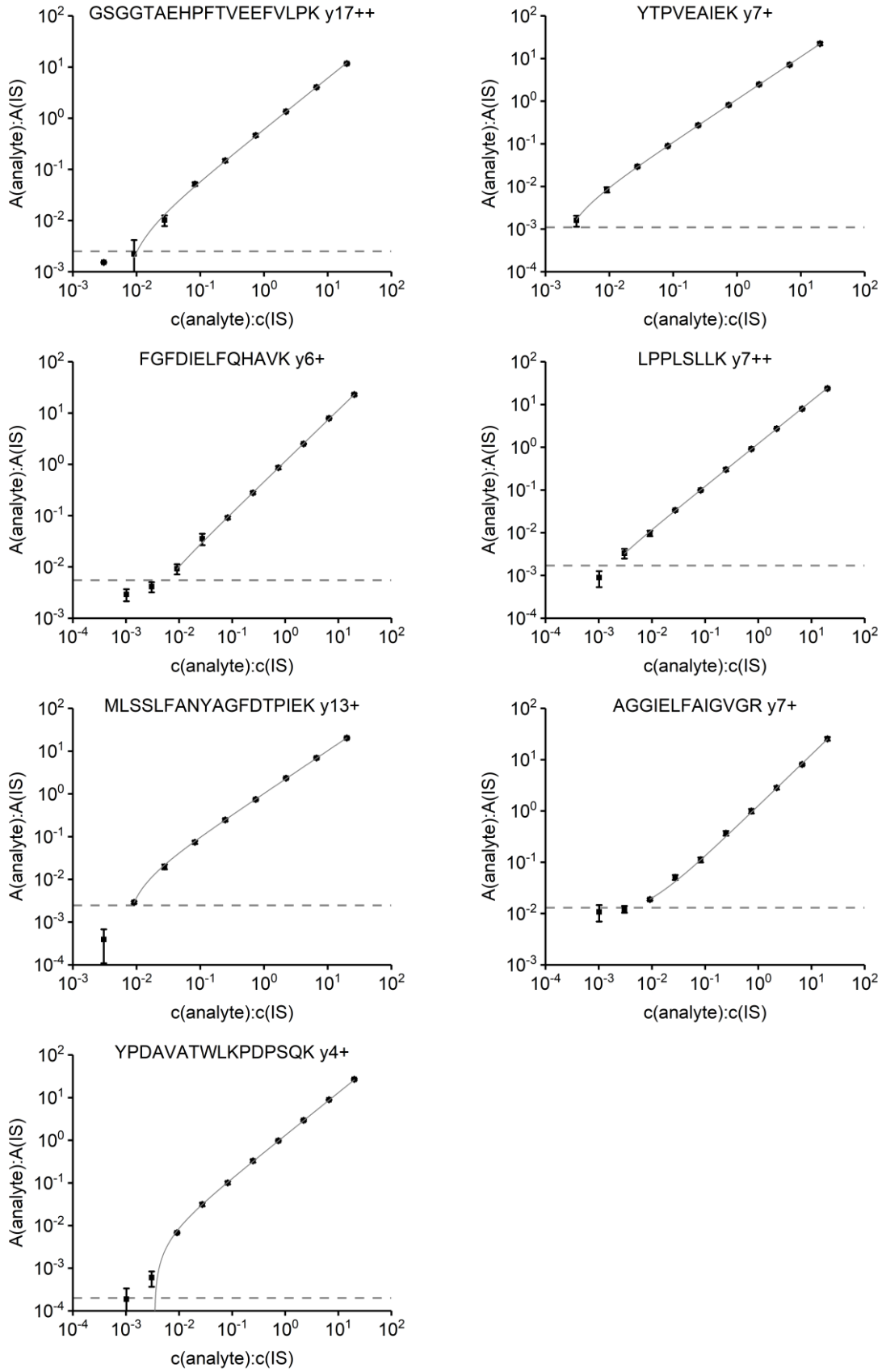

Figure 53. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ3 measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

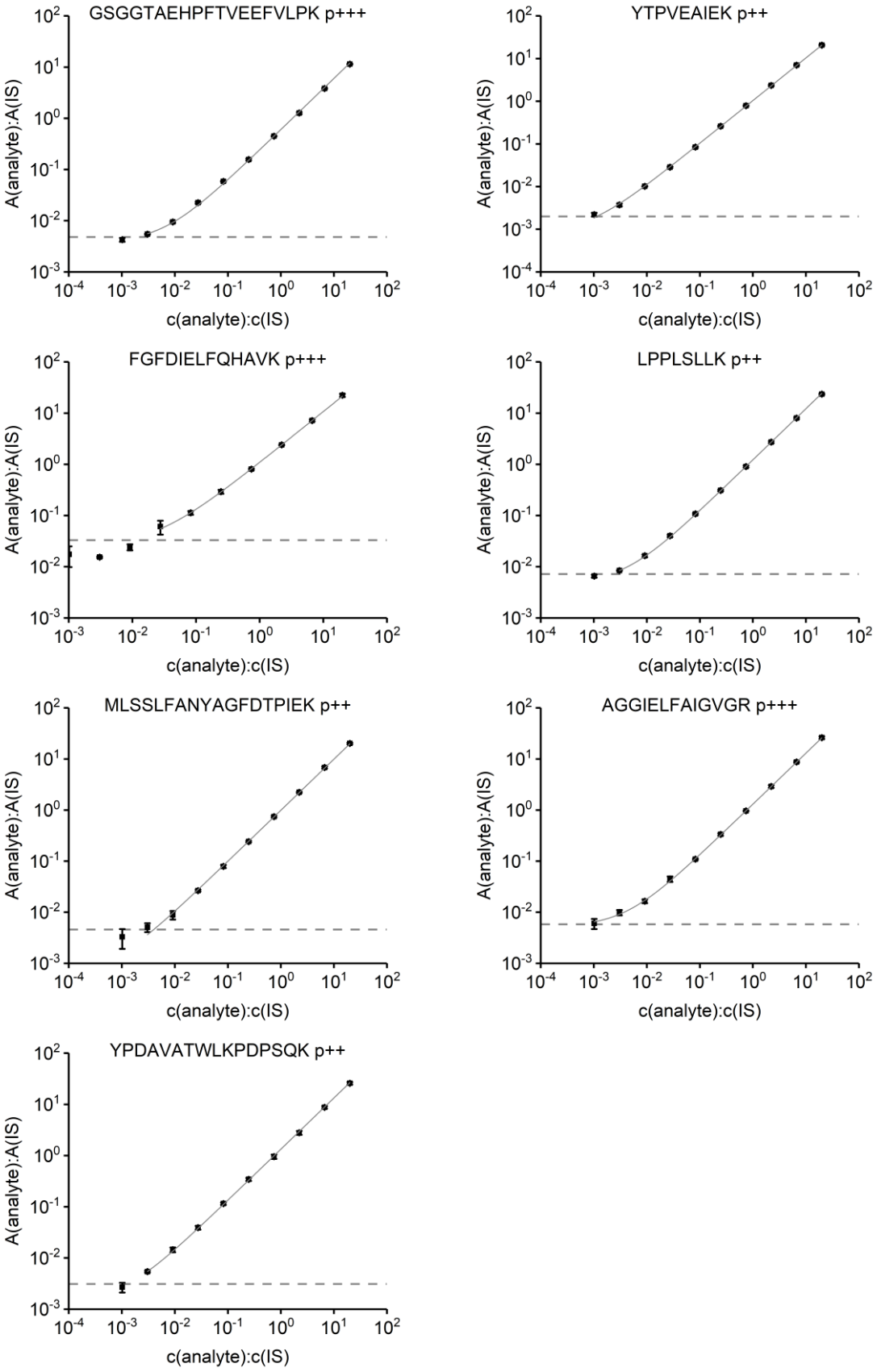

Figure 54. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ3 measured in SIM mode and PBSC as matrix. Each concentration was prepared as triplicate.

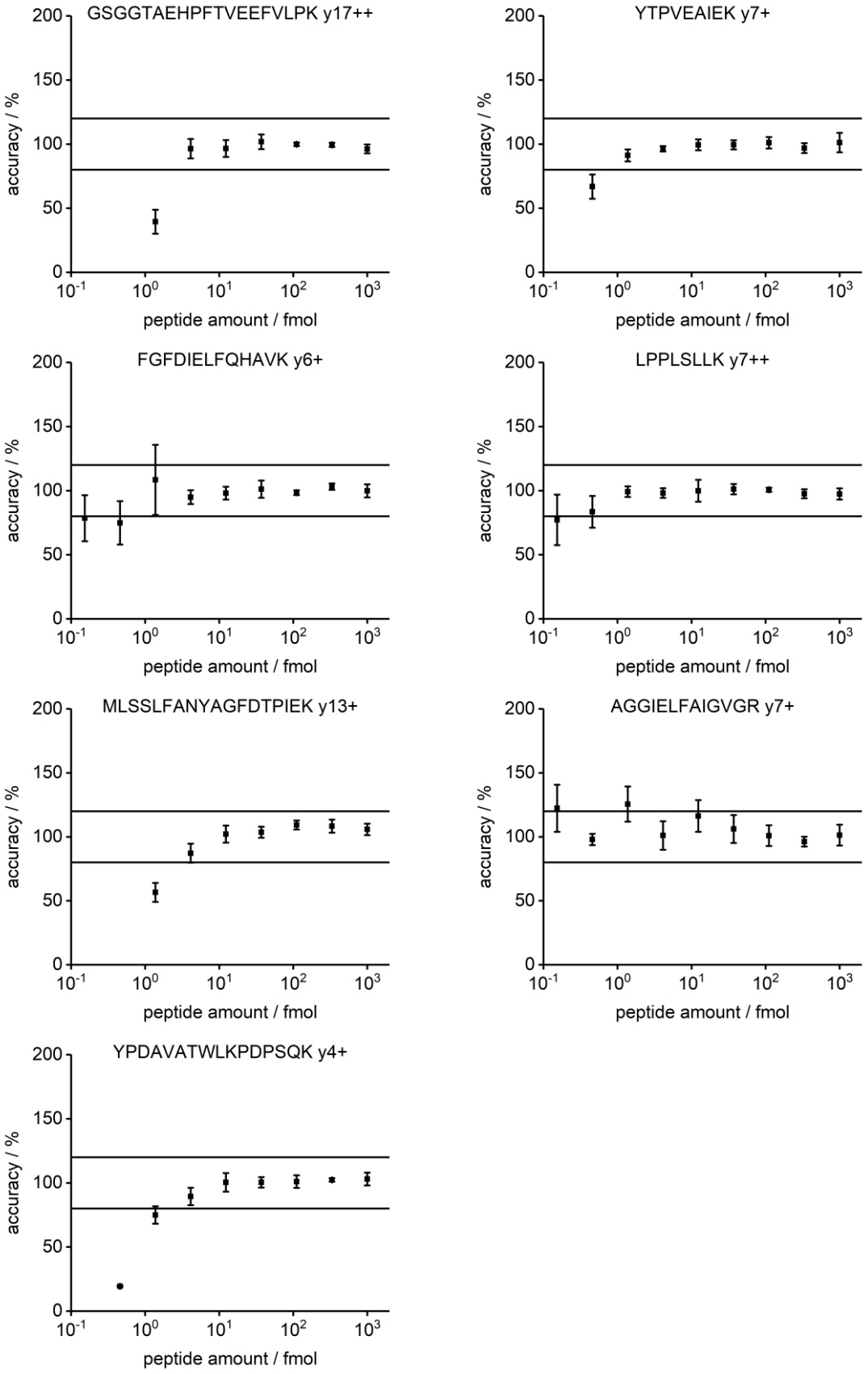

Figure 55. Accuracy and precision of multiplex RQ3 measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

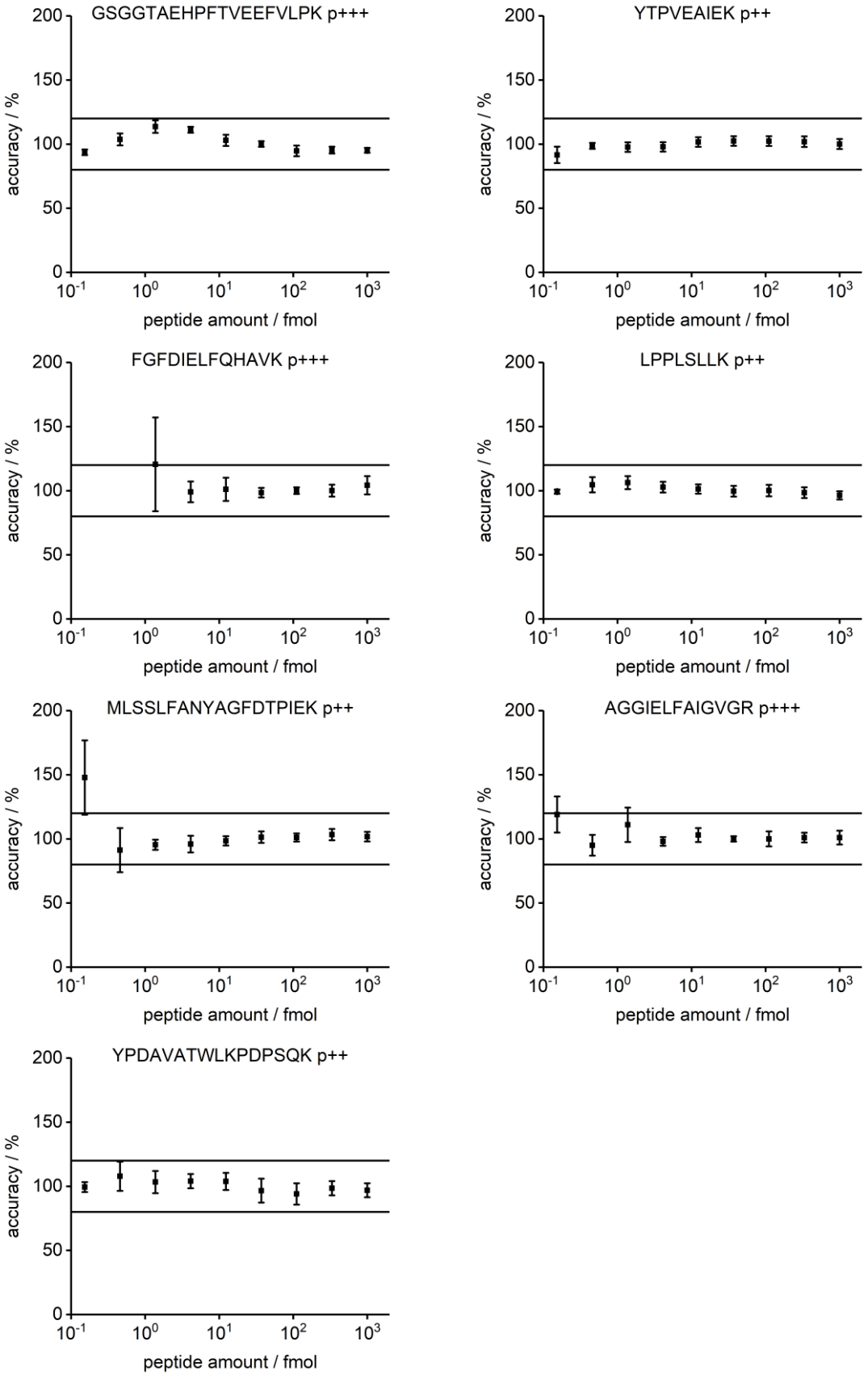

Figure 56. Accuracy and precision of multiplex RQ3 measured in SIM mode and PBSC as matrix. Each concentration was prepared as triplicate.

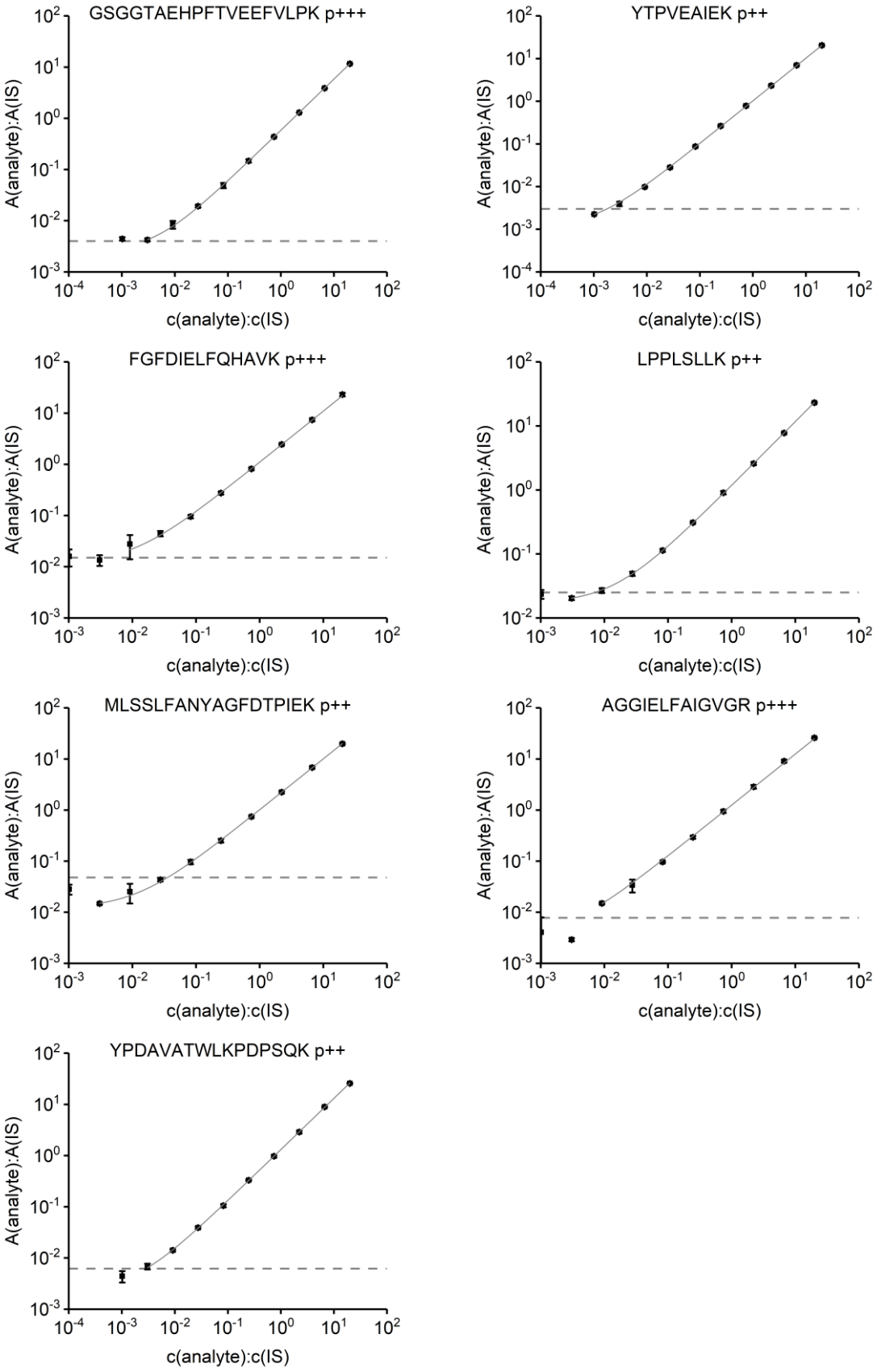

Figure 57. Linearity and limit of detection (shown as dashed horizontal line) of multiplex RQ3 measured in SIM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

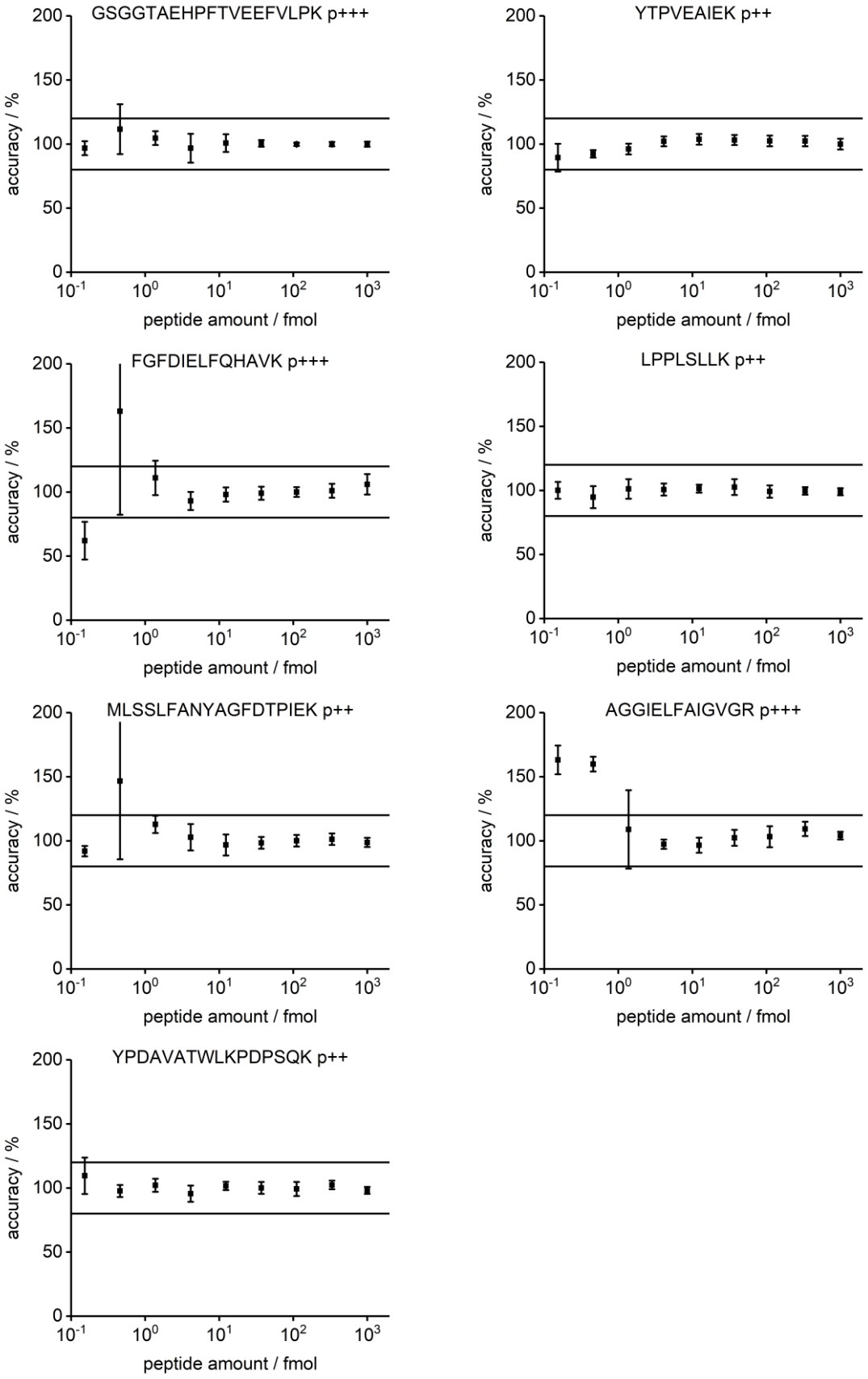

Figure 58. Accuracy and precision of multiplex RQ3 measured in SIM mode and vegetal cattle feed as matrix. Each concentration was prepared as triplicate.

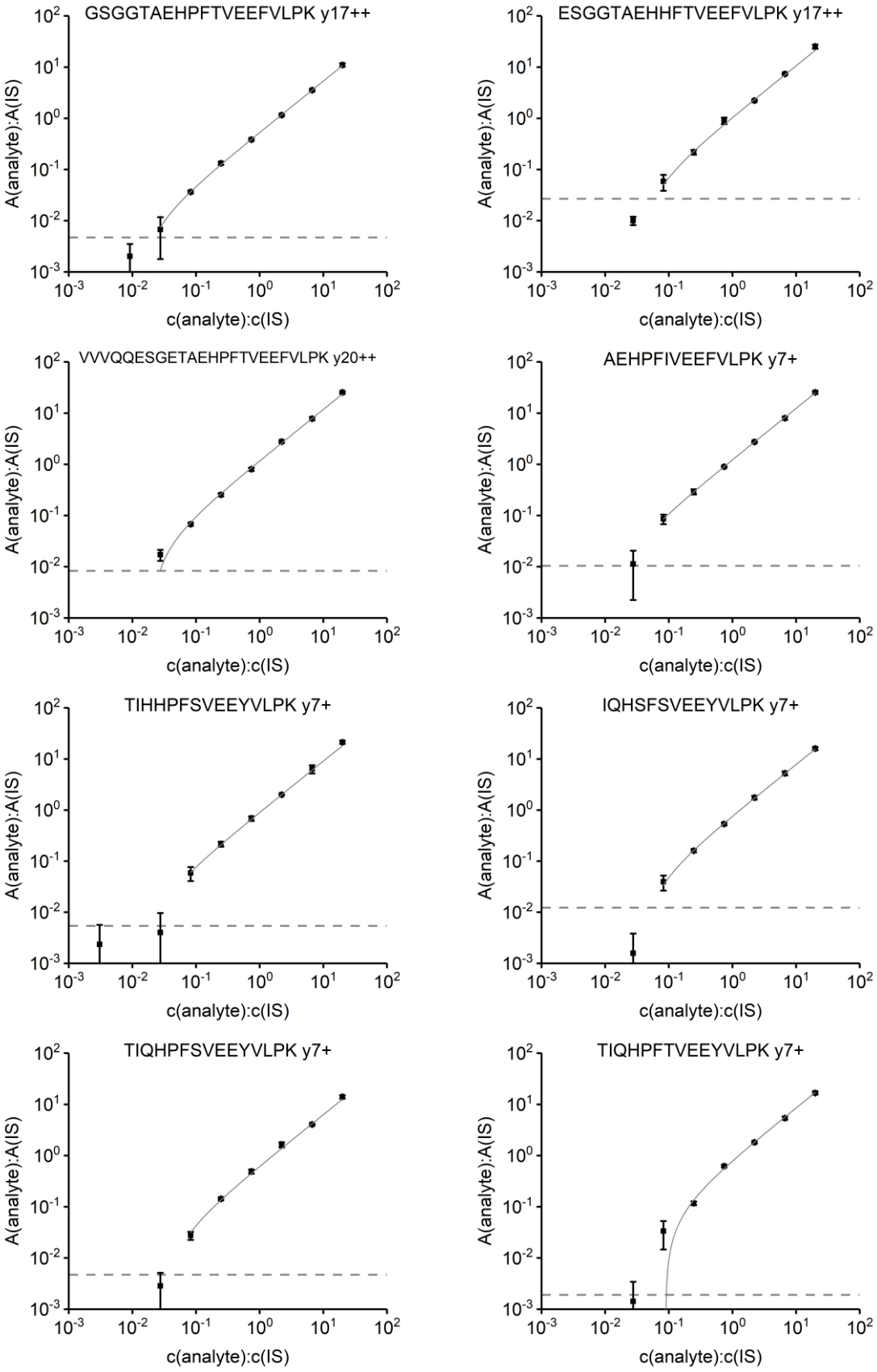

Figure 59. Linearity and limit of detection (shown as dashed horizontal line) of multiplex XA2M measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

H. Multiplex XA2M Linearity and Precision

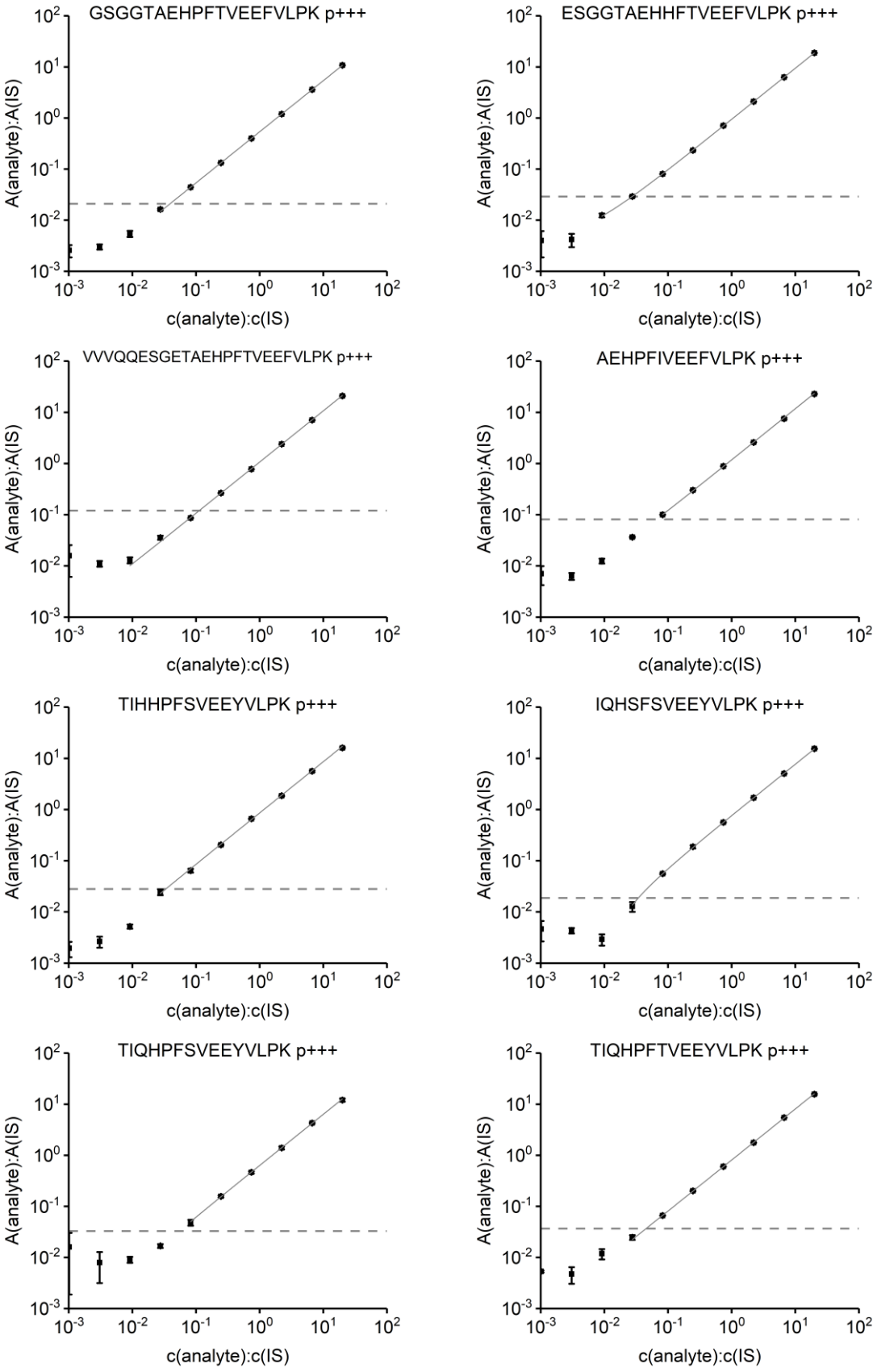

Figure 60. Linearity and limit of detection (shown as dashed horizontal line) of multiplex XA2M measured in SIM mode and PBSC as matrix. Each concentration was prepared as triplicate.

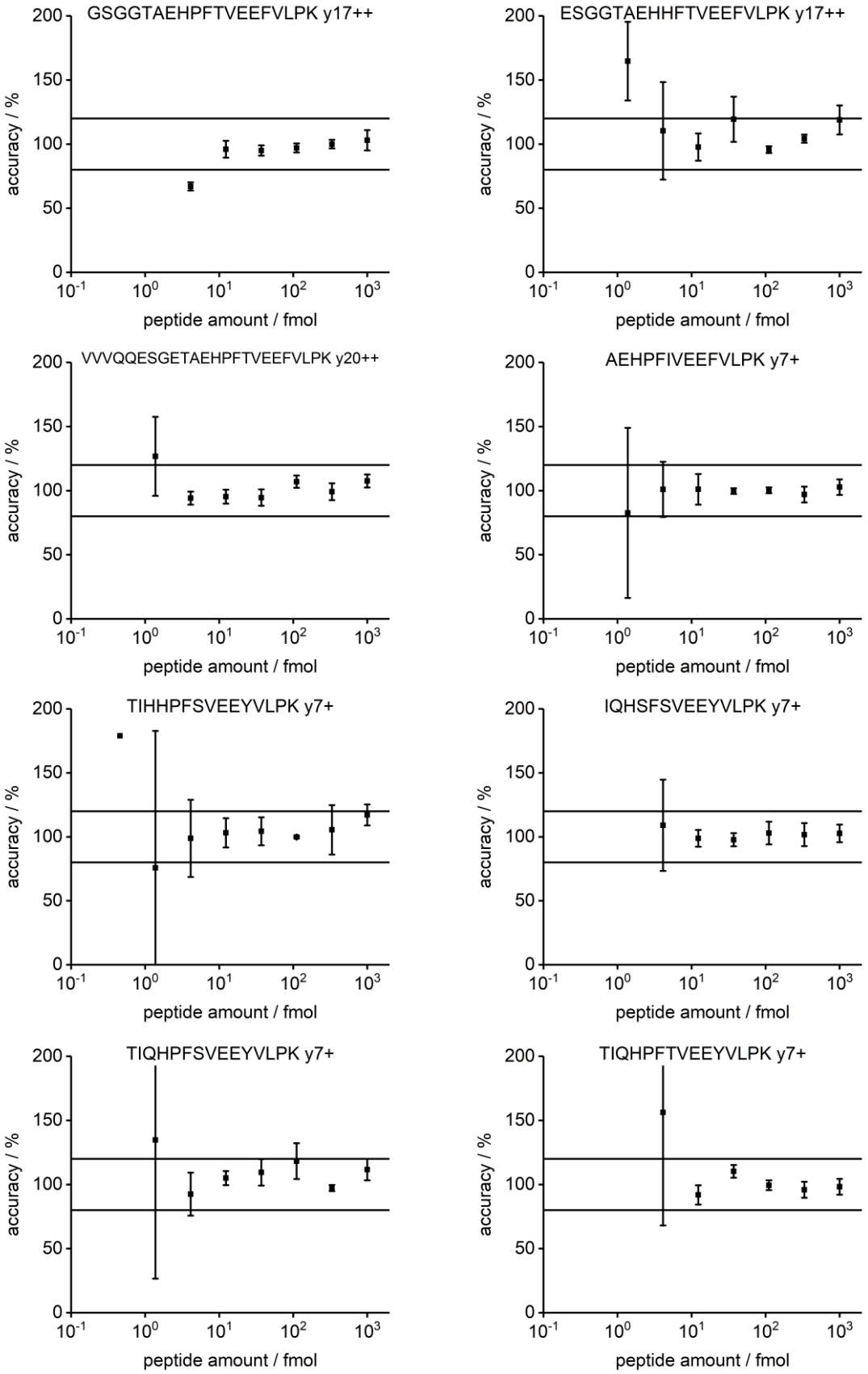

Figure 61. Accuracy and precision of multiplex XA2M measured in PRM mode and PBSC as matrix. Each concentration was prepared as triplicate.

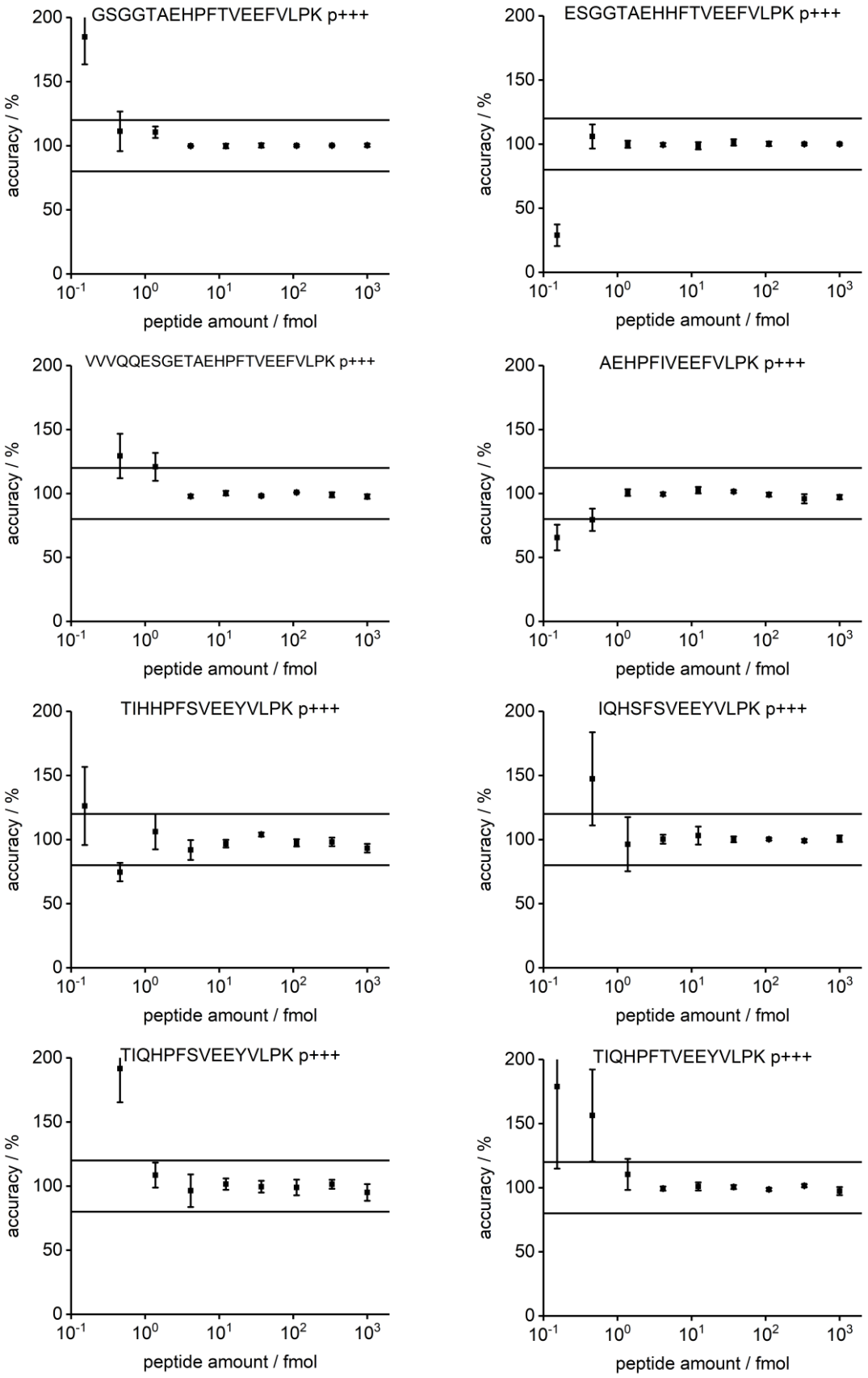

Figure 62. Accuracy and precision of multiplex XA2M measured in SIM mode and PBSC as matrix. Each concentration was prepared as triplicate.

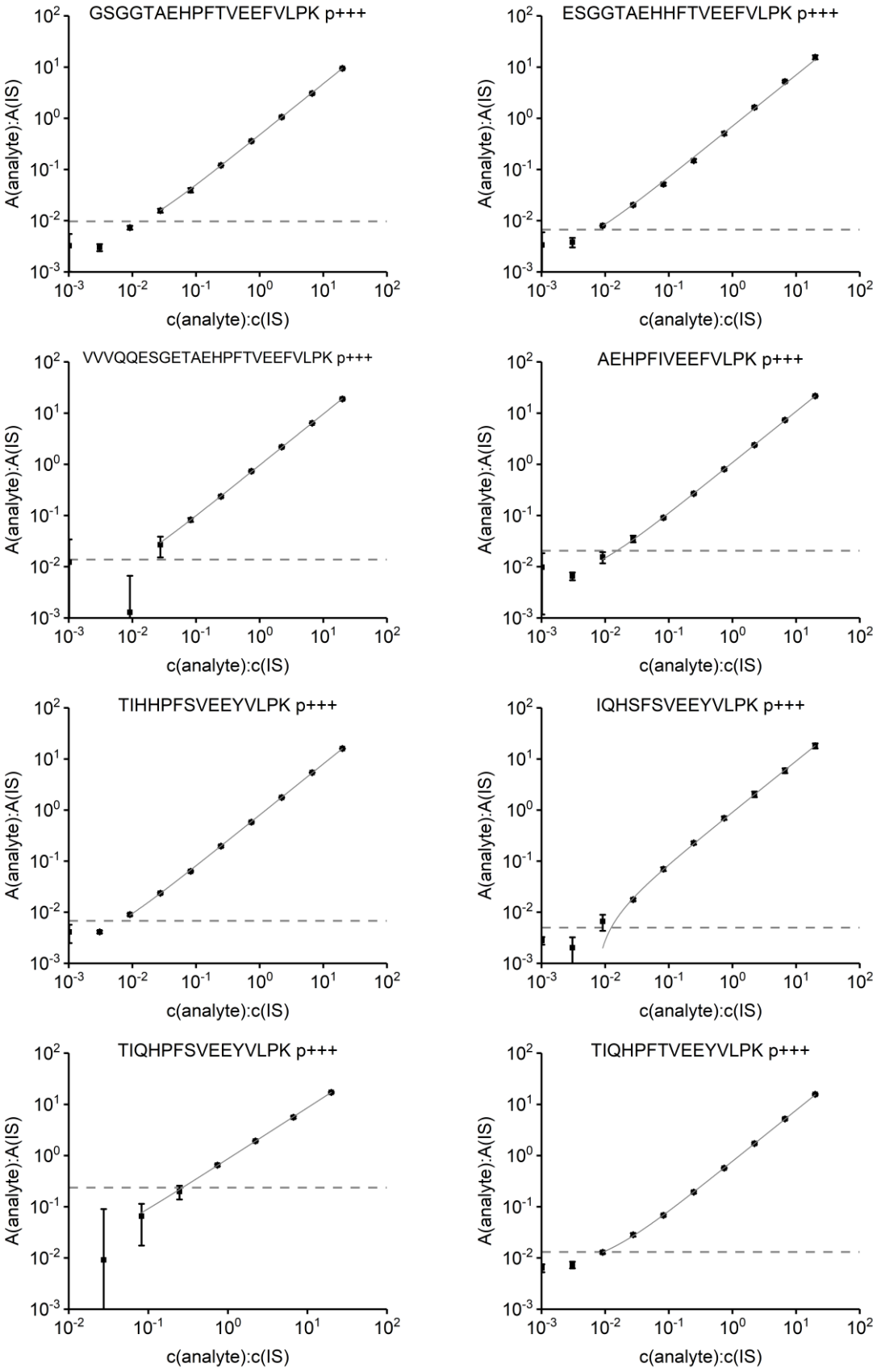

Figure 63. Linearity and limit of detection (shown as dashed horizontal line) of multiplex XA2M measured in SIM mode and fish feed as matrix. Each concentration was prepared as triplicate.

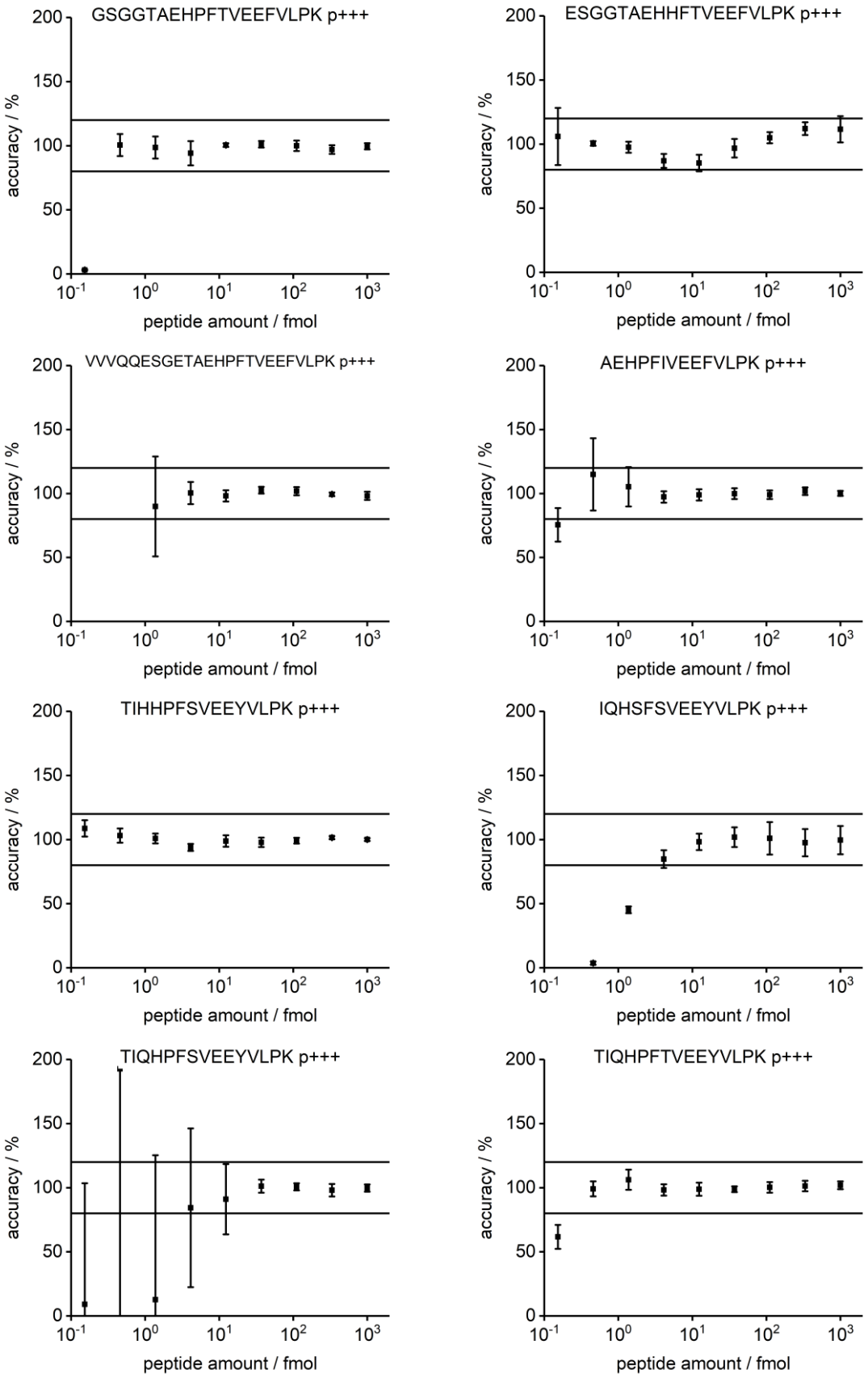

Figure 64. Accuracy and precision of multiplex XA2M measured in SIM mode and fish feed as matrix. Each concentration was prepared as triplicate.

I. Proficiency Test Sample Analysis

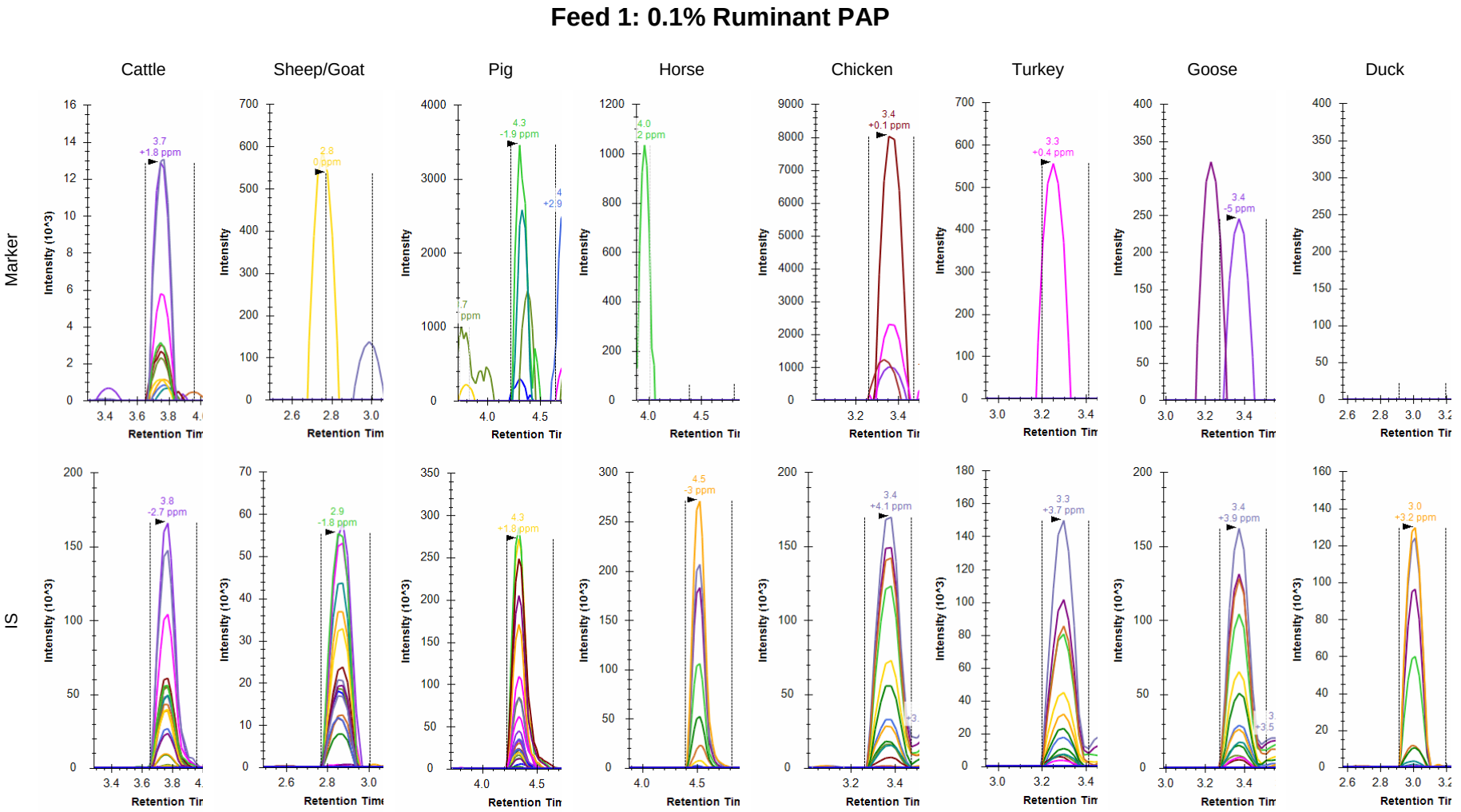

Figure 65. Species identification in proficiency test sample "Feed 1" using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

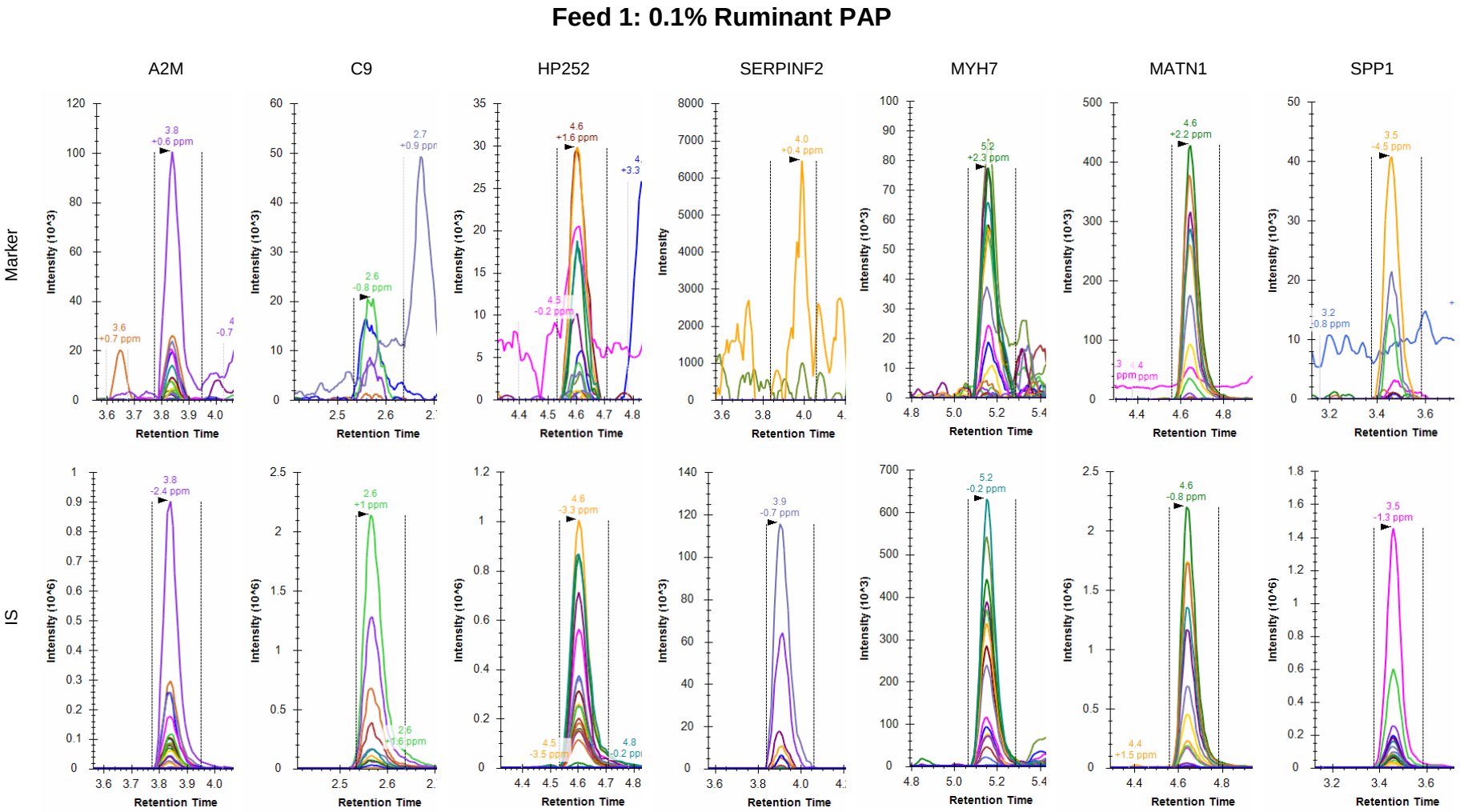

Figure 66. Ruminant tissue identification in proficiency test sample "Feed 1" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

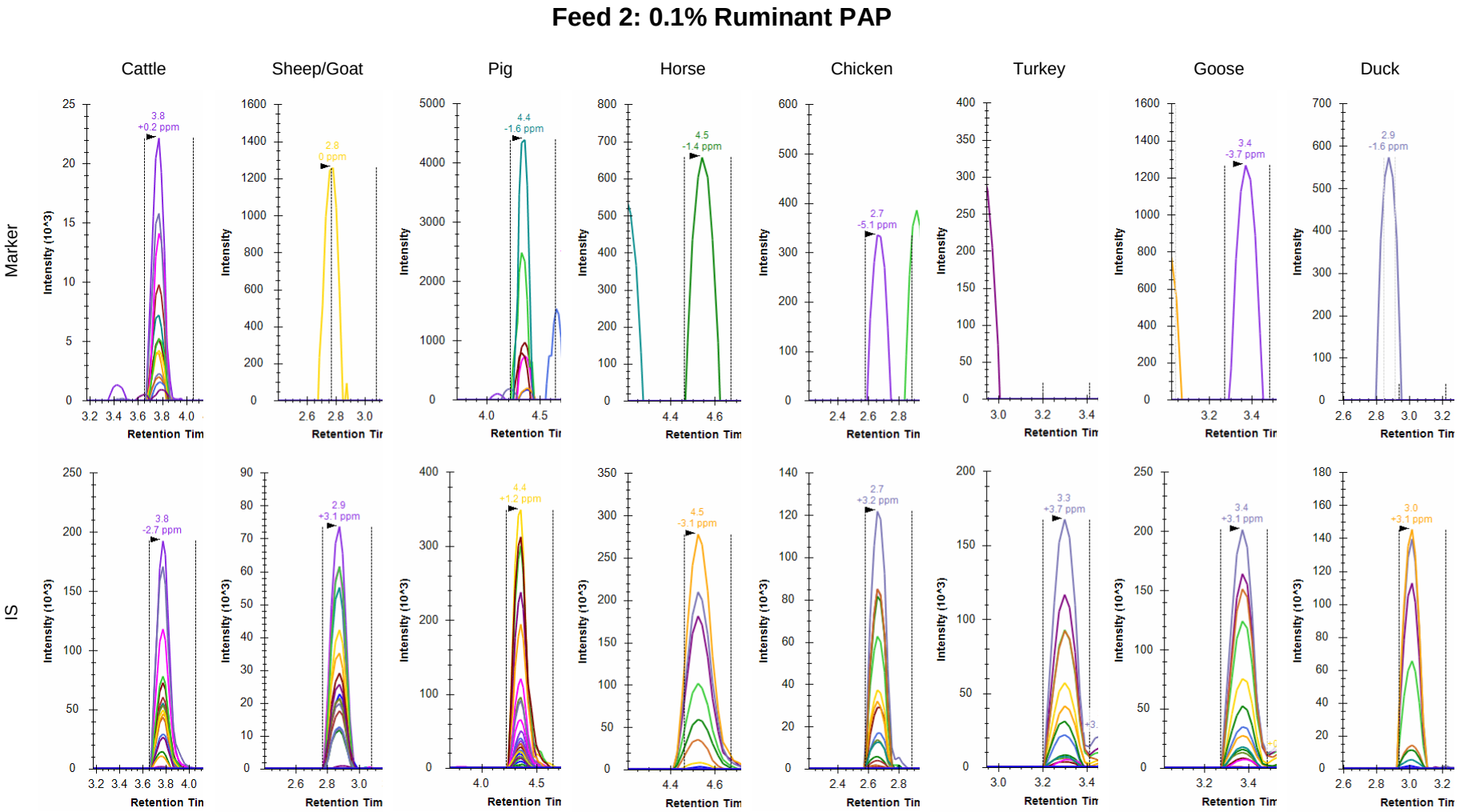

Figure 67. Species identification in proficiency test sample "Feed 2" using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

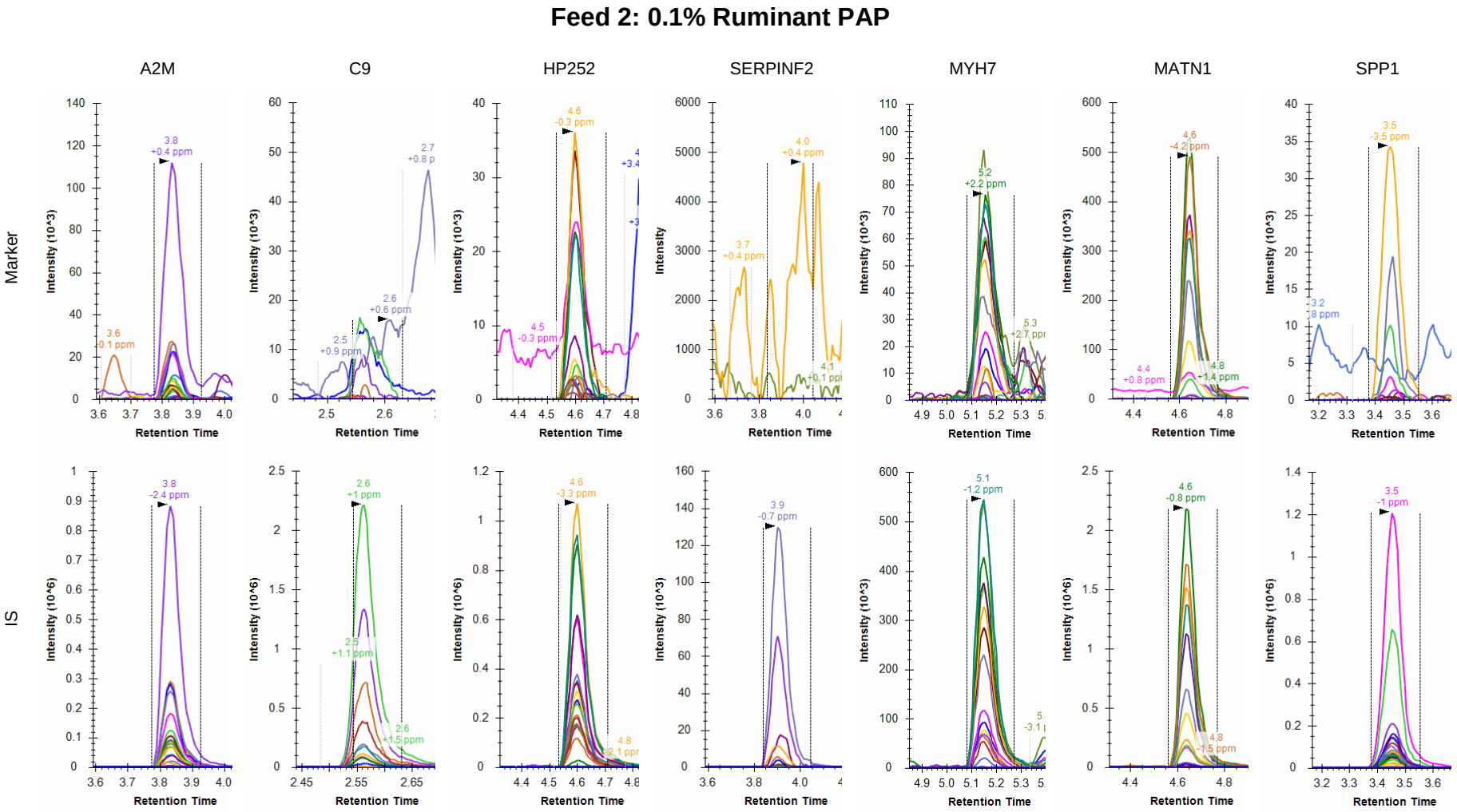

Figure 68. Ruminant tissue identification in proficiency test sample "Feed 2" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

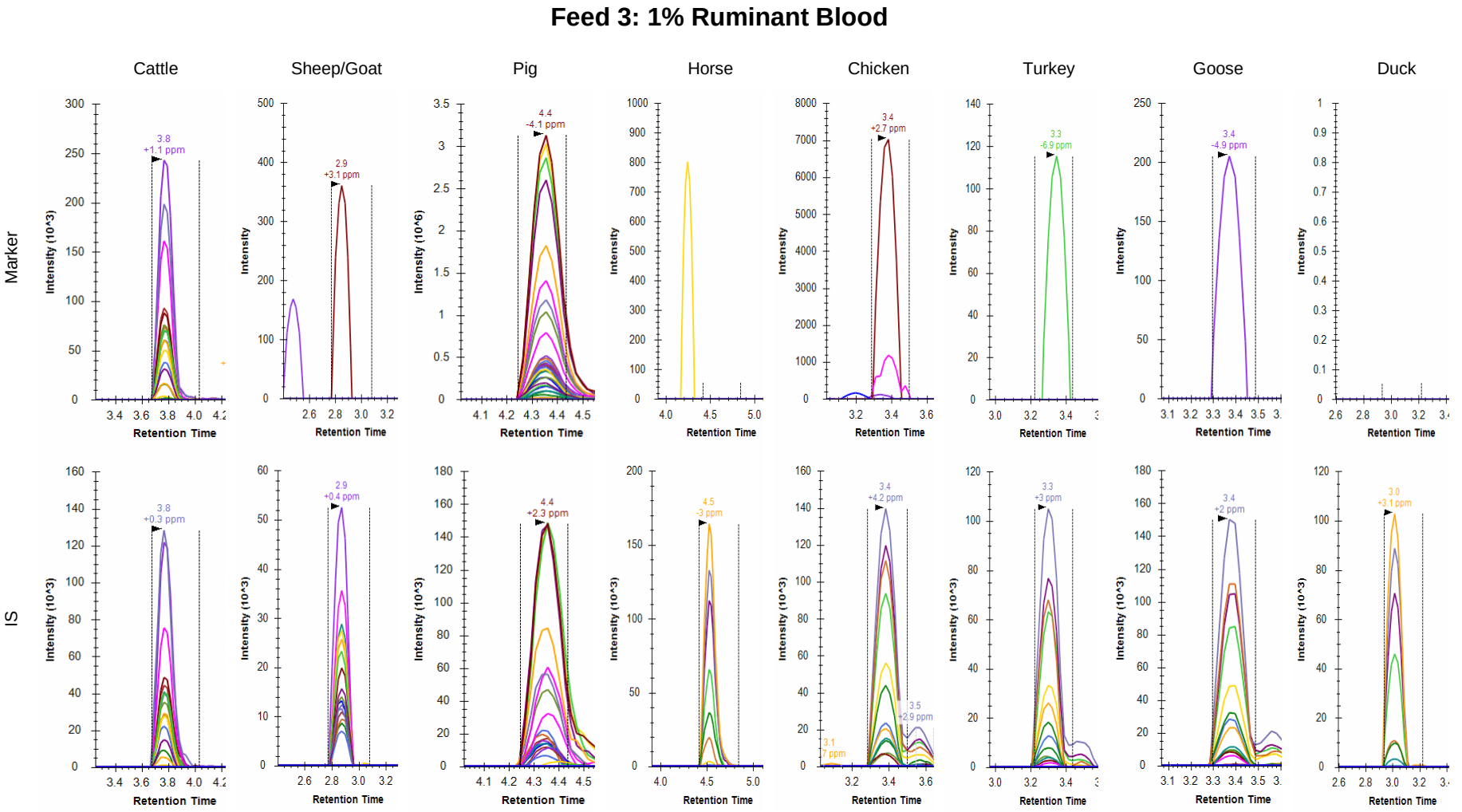

Figure 69. Species identification in proficiency test sample "Feed 3" using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

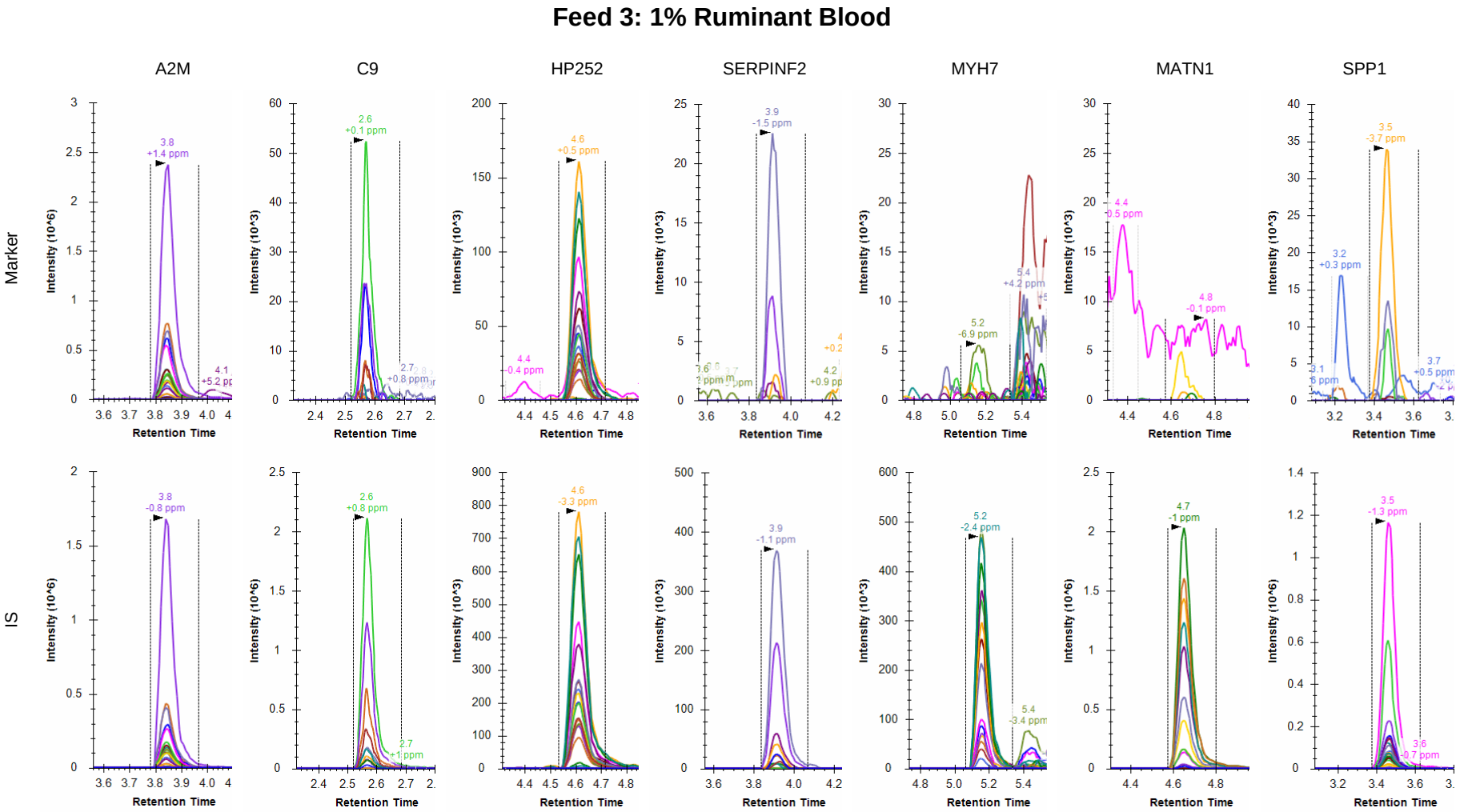

Figure 70. Ruminant tissue identification in proficiency test sample "Feed 3" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

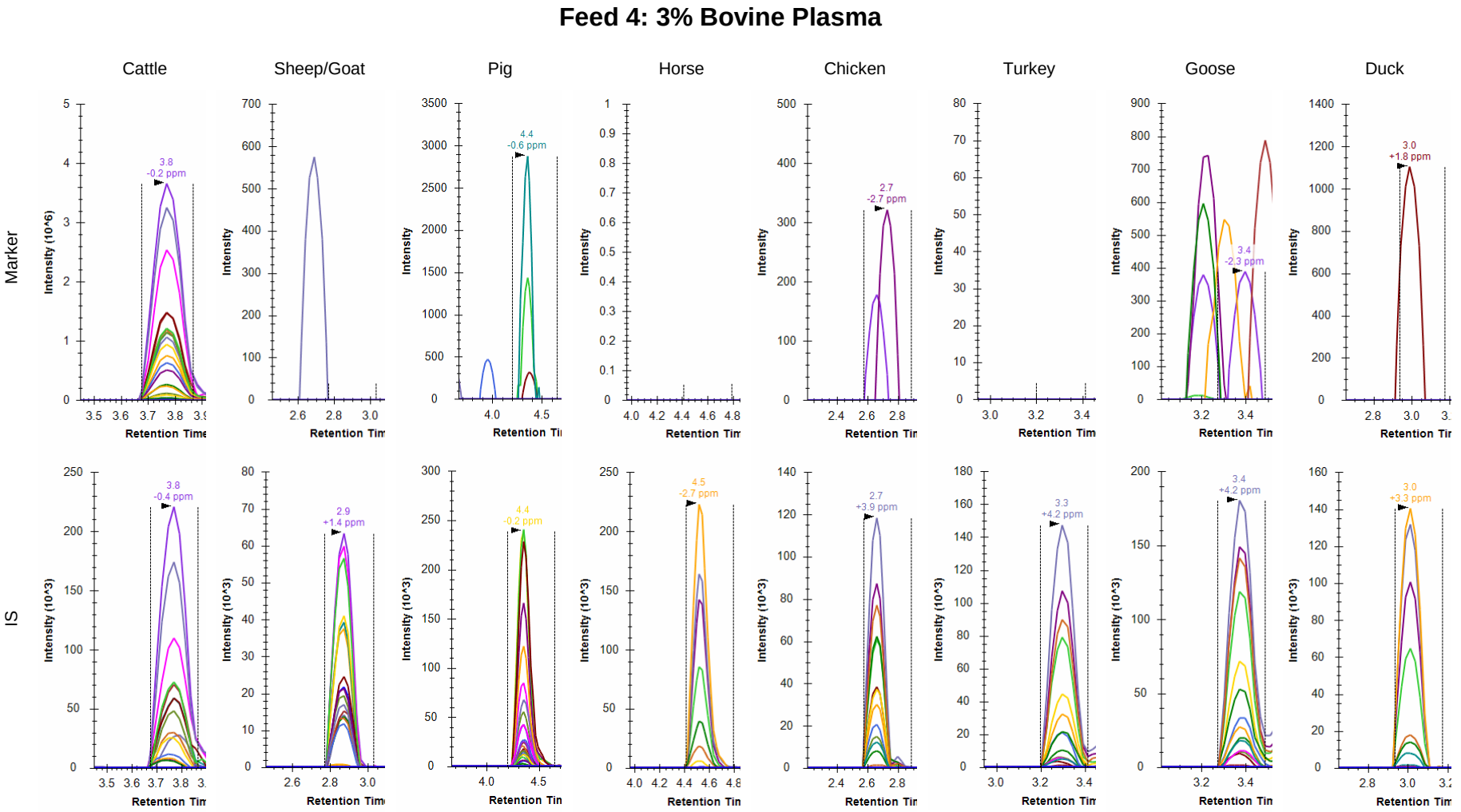

Figure 71. Species identification in proficiency test sample "Feed 4" using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

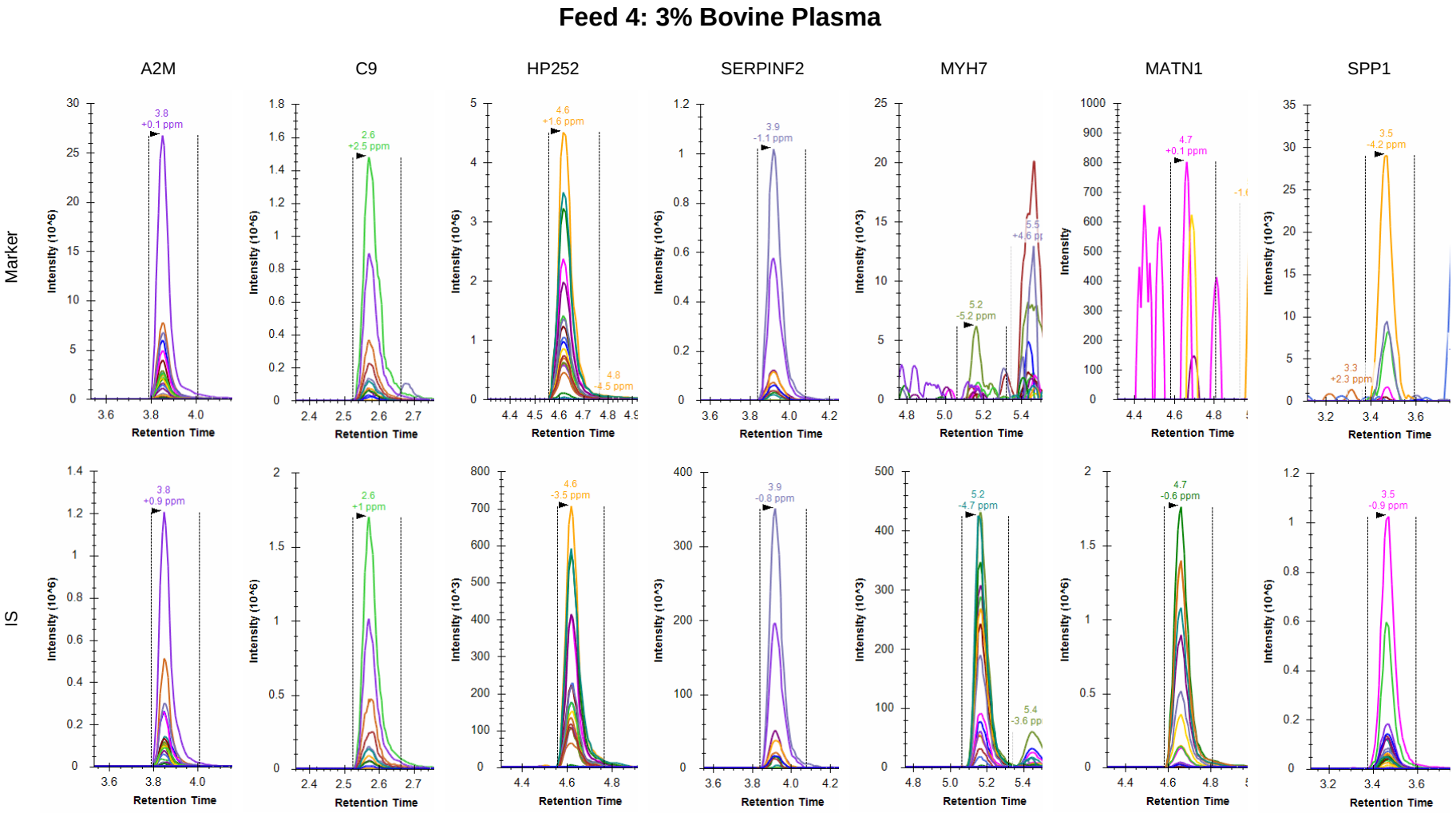

Figure 72. Ruminant tissue identification in proficiency test sample "Feed 4" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

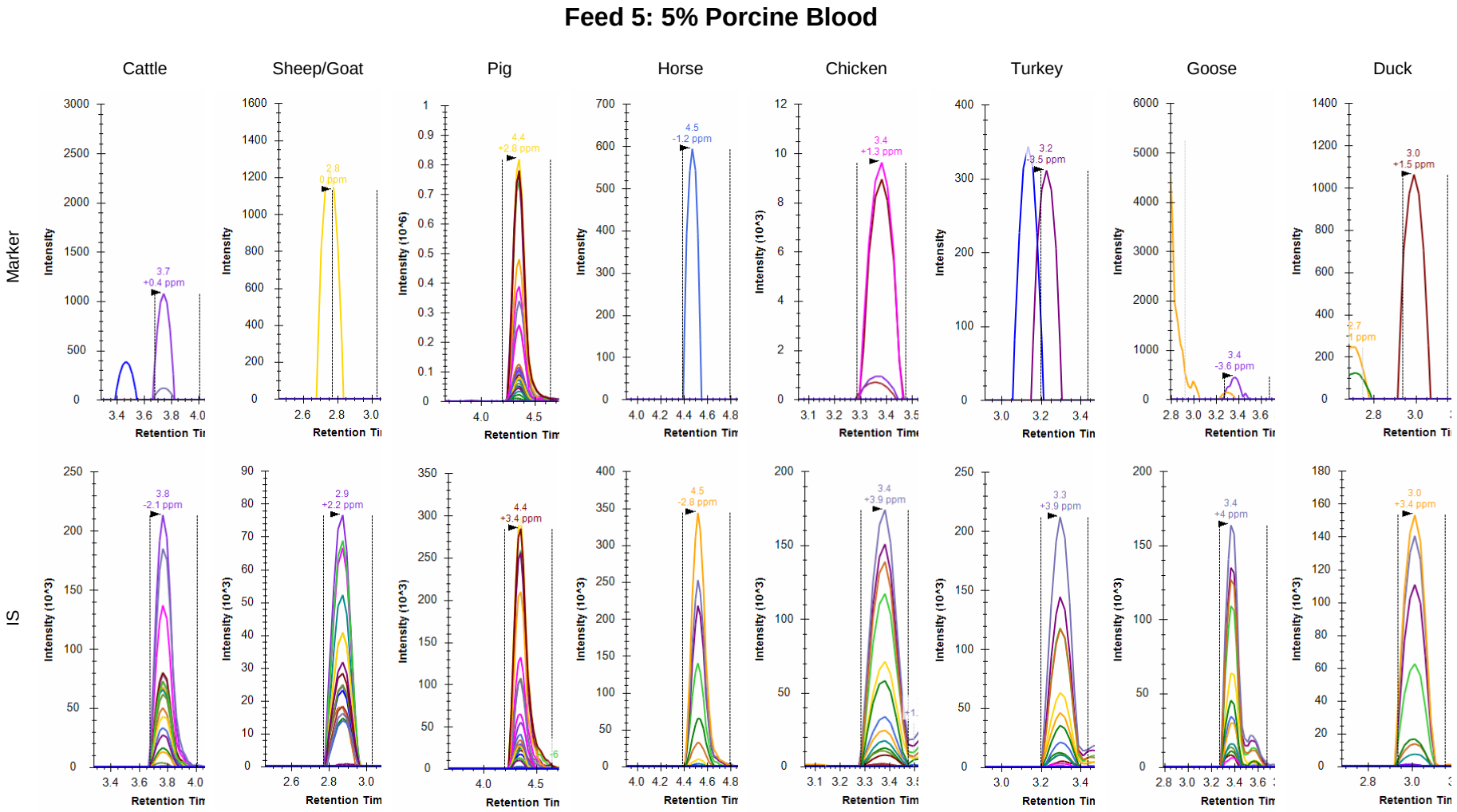

Figure 73. Species identification in proficiency test sample “Feed 5” using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

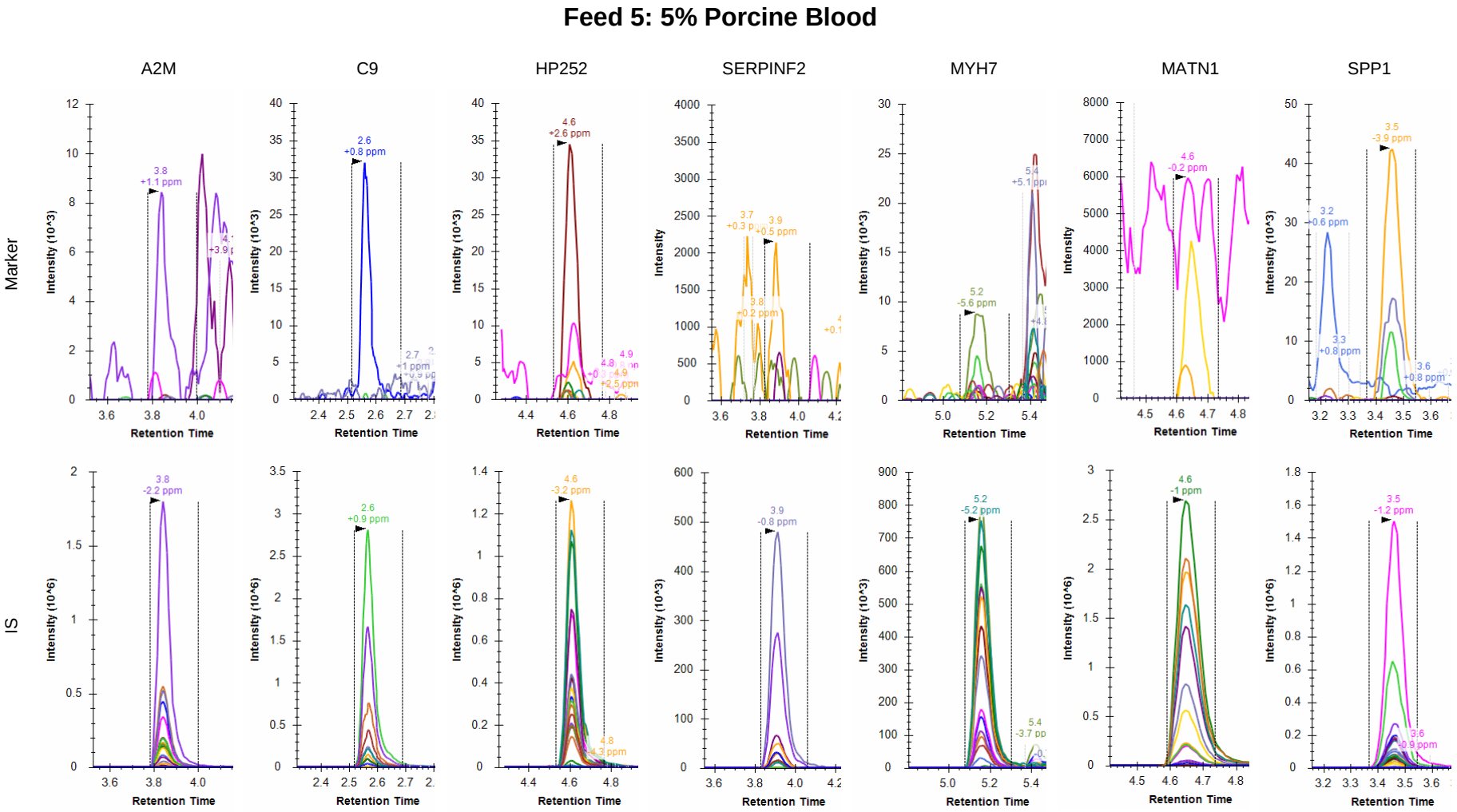

Figure 74. Ruminant tissue identification in proficiency test sample "Feed 5" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

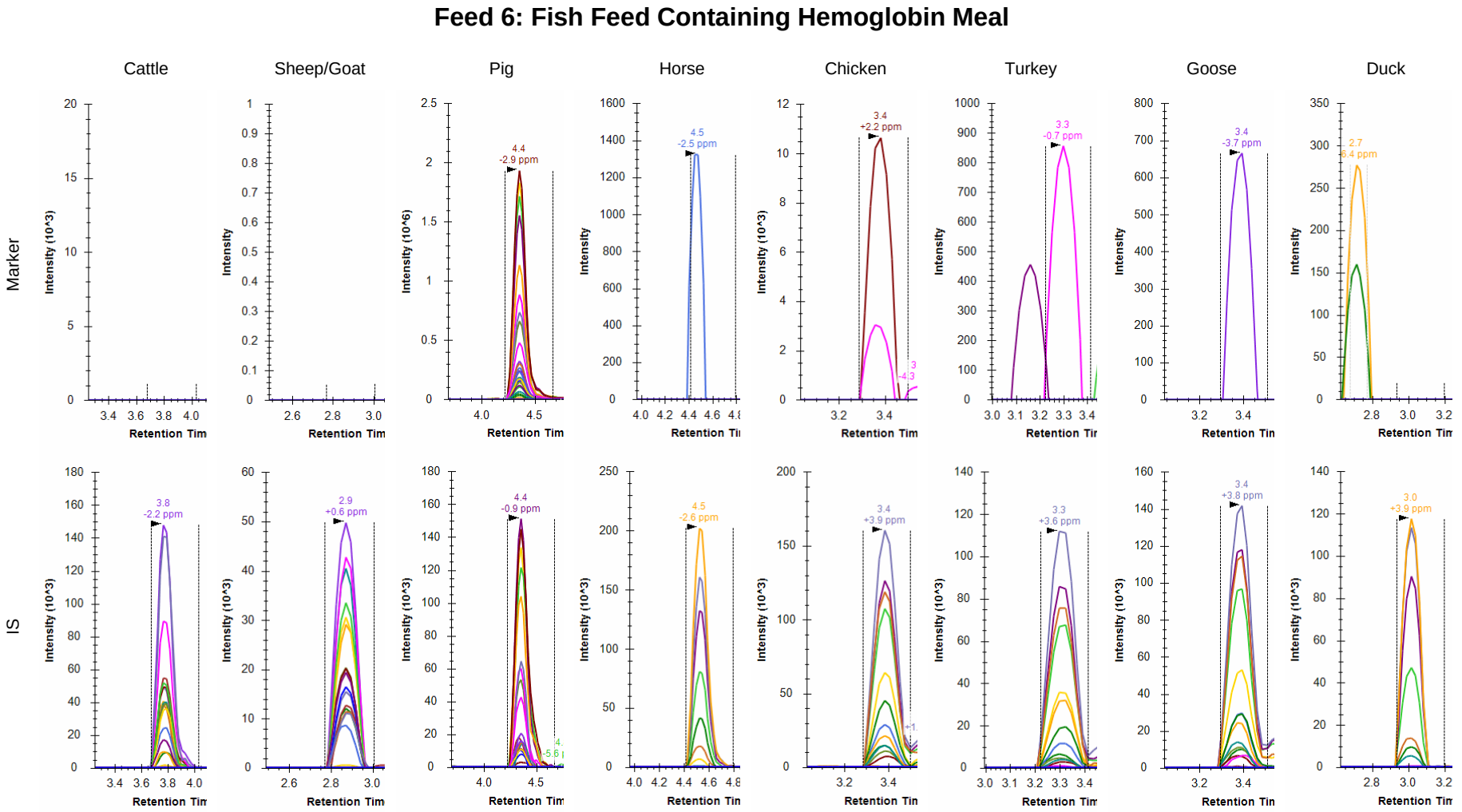

Figure 75. Species identification in proficiency test sample "Feed 6" using multiplex XA2M. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

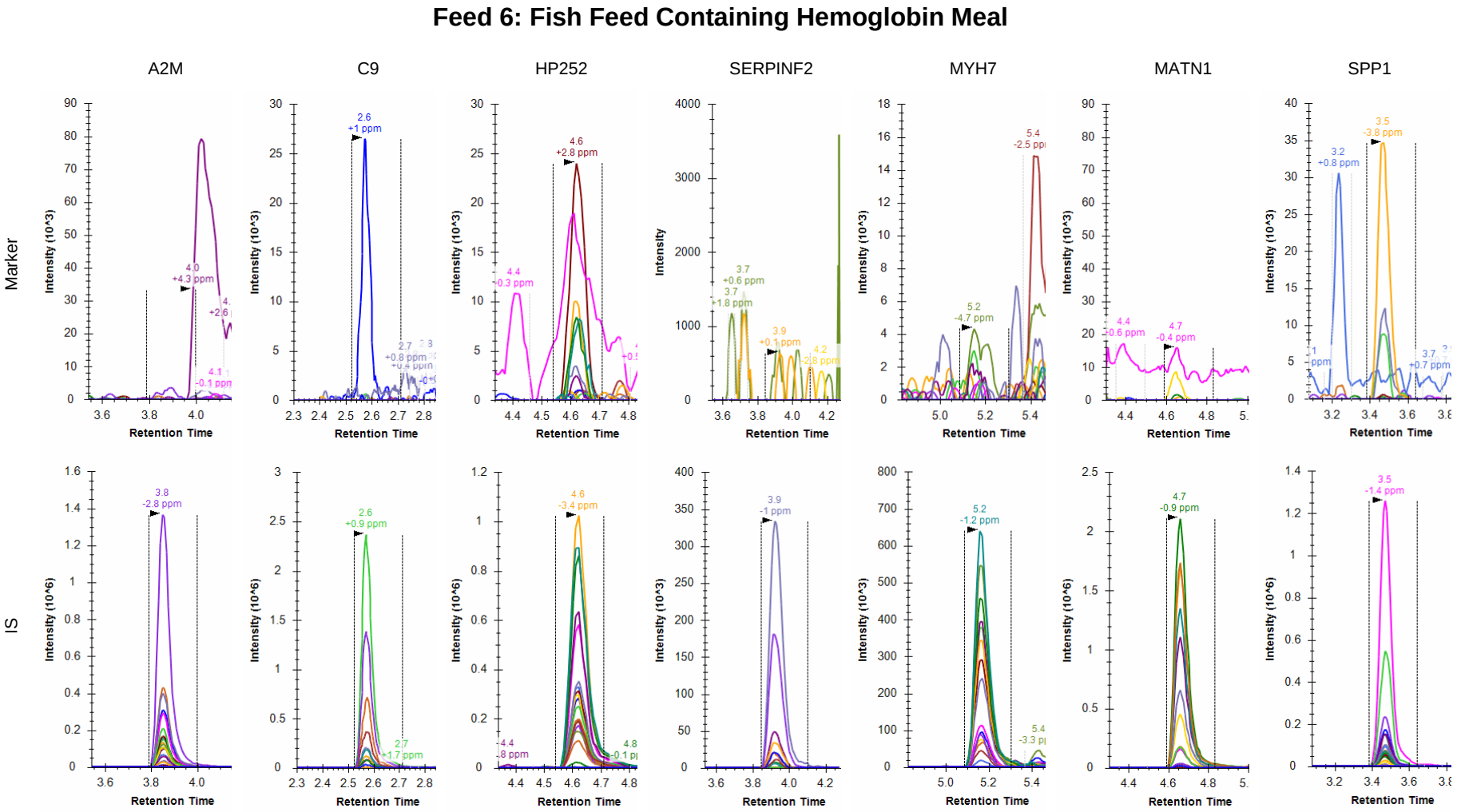

Figure 76. Ruminant tissue identification in proficiency test sample "Feed 6" using multiplex RQ3. Analyte signals are shown in the first row, isotope labeled internal standard signals (IS) are shown in the second row. The different colors indicate the detected fragment ions.

J. A280 Measurements

Table 33. Sample weight and A280 readout of HPD-prepared validation samples.

weight fraction	rMBM1 in VF			rMBM2 in VF			rSDP in VF		
	Replicate #	Sample Weight	A280	Replicate #	Sample Weight	A280	Replicate #	Sample Weight	A280
	1.1	15.3	4.733	4.1	15.2	4.648	7.1	15.2	5.650
	1.2	15.5	4.890	4.2	15.0	4.607	7.2	15.1	5.680
	1.3	15.1	4.665	4.3	15.0	4.535	7.3	15.3	5.722
	1.4	15.1	4.595	4.4	15.1	4.563	7.4	15.3	5.743
10%	1.5	15.2	4.703	4.5	15.0	4.580	7.5	15.5	5.732
	1.6	15.0	4.757	4.6	15.3	4.728	7.6	15.4	5.926
	1.7	15.1	4.985	4.7	15.1	4.764	7.7	15.2	5.996
	1.8	15.0	5.079	4.8	15.2	4.905	7.8	15.2	6.060
	1.9	15.1	5.167	4.9	15.1	4.967	7.9	15.1	6.102
C.V. / %		**1.0**	**3.9**		**0.7**	**3.1**		**0.8**	**2.8**
	2.1	15.0	4.574	5.1	15.3	4.975	8.1	15.1	4.513
	2.2	15.2	4.601	5.2	15.0	4.968	8.2	15.4	4.527
	2.3	15.2	4.686	5.3	15.4	4.889	8.3	15.1	4.919
	2.4	15.2	4.635	5.4	15.1	4.973	8.4	15.5	5.019
1%	2.5	15.0	4.434	5.5	15.4	5.238	8.5	15.0	4.893
	2.6	15.0	4.484	5.6	15.1	5.010	8.6	15.4	5.414
	2.7	15.0	4.562	5.7	15.0	5.175	8.7	15.0	4.680
	2.8	15.0	4.744	5.8	15.4	5.336	8.8	15.2	4.780
	2.9	15.3	4.898	5.9	15.5	5.515	8.9	15.1	4.514
C.V. / %		**0.8**	**2.8**		**1.2**	**3.9**		**1.2**	**5.8**
	3.1	15.4	4.858	6.1	15.2	4.647	9.1	15.4	4.949
	3.2	15.4	5.043	6.2	15.0	4.514	9.2	15.1	4.648
	3.3	15.5	4.834	6.3	15.2	4.505	9.3	15.2	4.821
	3.4	15.1	4.815	6.4	15.3	4.563	9.4	15.4	4.969
0.1%	3.5	15.3	4.816	6.5	15.0	4.559	9.5	15.3	4.910
	3.6	15.5	4.869	6.6	15.2	4.744	9.6	15.4	5.034
	3.7	15.4	4.878	6.7	15.5	4.891	9.7	15.1	5.227
	3.8	15.5	5.012	6.8	15.3	4.805	9.8	15.2	5.244
	3.9	15.0	4.867	6.9	15.0	5.049	9.9	15.4	5.242
C.V. / %		**1.1**	**1.6**		**1.0**	**3.8**		**1.0**	**3.9**

Curriculum Vitae

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03/2015 – 05/2018	PhD thesis at the Natural and Medical Sciences Institute at the University of Tübingen Title: *Immunoaffinity-Based Mass Spectrometry for the Species Identification and Quantification of Processed Animal Proteins in Feed*
08/2014 – 02/2015	Master thesis at the Fraunhofer Institute for Interfacial Engineering and Biotechnology (1.0) Title: *Synthesis and Characterization of Gelatin-. Chondroitin sulfate and Hyaluronic Acid-Based Hydrogels for Cartilage Tissue Engineering*
03/2013 – 07/2014	Study of Applied Chemistry (M.Sc.) at Reutlingen University of Applied Sciences (1.2)
08/2012 – 02/2013	Bachelor thesis at the Fraunhofer Institute for Interfacial Engineering and Biotechnology (1.0) Title: *Development of a HPLC-Based Analytical Method for the Quantitative Determination of Particle-Surface-Coupled Proteins*
03/2010 – 07/2012	Study of Applied Chemistry (B.Sc.) at Reutlingen University of Applied Sciences (1.5)
02/2010 – 07/2009	Military service as medic, Artillerie-Kaserne in Kempten and Graf-Stauffenberg-Kaserne in Sigmaringen
06/2009	Abitur at Quenstedt Gymnasium Mössingen (2.3)