Antibodies

as a versatile tool in detection strategies for proteins:

Recombinant bovine Somatotropin as a model

Nathalie G.E. Smits

Propositions

1. Antibody characterisation is needed, in particular to prepare for a future with non-animal derived biorecognition molecules.
   This thesis

2. For enforcement purposes of forbidden substances abuse, unequivocal identification by mass spectrometry is not always the answer.
   This thesis

3. Distinction between male and female is necessary to come to equal treatment.

4. Mankind's attitude to the climate crisis resembles jumping an amber traffic light, when actually we know it will be red when we cross the line.

5. One asset of marketing is creative interpretation of life cycle assessments.

6. Detection of allergens is not necessary for allergen detection in food.

7. A prerequisite to value science is the presence of a functional moral compass in politics.

Propositions belonging to the thesis entitled:
Antibodies as a versatile tool in detection strategies for proteins: Recombinant bovine Somatotropin as a model.

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Wageningen, 27 September 2022

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'Het is de kunst om in de chaos die ons omringt
een verhaal te vinden dat een tijdje klopt'
Rüdiger Safranski

Contents

Abbreviations

Ab	Antibody
AMR	Antimicrobial Resistance
ASSURED	Affordable, Sensitive, Specific, User Friendly, Rapid, Equipment-free, Deliverable
BSA	Bovine Serum Albumine
CCα	Decision limit
CCβ	Detection capability
CCD	Charge Coupled Divice
CDR	Complementary Determining Region
EC	European Commision
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionisation
eST	Equine Somatotropin
EU	European Union
Fab	Fragment Antigen Binding parts
FAO	Food and Agricultural Organisation
FCIA	Flow Cytometric Immunoassay
FDA	Food & Drug Administration
GAB	Goat Anti Bovine
GAM	Goat Anti Mouse
GAR	Goat Anti Rabbit
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
hGH	Human Growth Hormone
hST	Human Somatotropin
IGF-1	Insulin-like Growth Factor - 1
IGFBP2	Inslulin-like Growth Factor Binding Protein 2
IgG	Immunoglobulin
LC	Liquid Chromatography
LFD	Lateral Flow Device
mAb	Monoclonal Antibody
MIPA	Microsphere Peptide-Based Immunoassay
MRL	Maximum Residue Limit
MS	Mass Spectrometer
pAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween
PE	R-Phycoerythrin
POC	Point of Care

pST	Porcine Somatotropin
rbST	Recombinant Bovine Somatrotropin
rhGH	Recombinant Human Growth Hormone
RIA	Radio Immunoassay
RP	Reversed Phase
scFv	Single Chain Fragment Variable
ST	Somatotropin
UPLC	Ultra Performance Liquid Chromatography
USA	United States of America
VHH	Variable domain on a Heavy chain
VNAR	Variable New Antigen Receptor
WADA	World Anti Doping Agency
WHO	World Health Organisation
XPS	X-ray Photoelectron spectroscopy

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

General Introduction

Introduction

Antibodies are a versatile tool in detection strategies for, for instance protein based forbidden substances. Abuse of forbidden substances in order to increase physiological performance is well known in athletics. The examples of athletes accused of substances abuse after doping control are well known. For this doping control, methods to detect abuse of the forbidden substances need to be available and the criteria to decide whether or not a forbidden substance is abused need to be agreed upon. Parallels can be drawn with food safety. In animal husbandry substances can be used to stimulate growth to increase meat production, or to enhance milk production, in dairy cattle. To enable control of abuse, reliable methods to detect the abuse need to be available and moreover, these methods need to be in accordance with current applicable legislation. This thesis will demonstrate this process of method development with recombinant bovine somatotropin (rbST), a proteohormone stimulating milk production, as a model. Use of rbST is forbidden within the EU [1], but legalized in other regions, such as the United States. Therefore, methods to assess its abuse are required. Unfortunately, revealing rbST abuse by devloped methods is sometimes hampered due to the fact that the criteria set in legislation on control on forbidden substances in animal husbandry cannot always be met. The main reason for this is that these criteria were originally developed for the unequivocal identification of small molecules, e.g. steroid hormones. To enable detection of rbST abuse antibodies have shown to be an indispensable tool. Therefore, this thesis is focused on the importance of antibody use in method development for protein detection, to obtain needed selectivity and sensitivity, resulting in a new generation of highly sophisticated methods. Moreover, attention will be given to strategy development; the process of setting up an analytical strategy for the efficient and reliable food safety control with respect to the detection of undesirable protein hormones.

1 Antibodies as a versatile tool

Antibodies are Y-shaped immunoglobulines (Ig) and are a product of the immune system. Five main isotypes of Ig with their own properties are known, IgA, IgD, IgE, IgG and IgM. They are produced by the immune system to counteract the presence of foreign compounds (antigens) in the blood [2]. When foreign compounds enter the body, the adaptive immune system is responsible for antibody production and their specificity and efficiency. Important actors in this antigen specific immune response are the B-cells, the memory of the immune system which is built by differentiation of these B-cells [3]. In this thesis antibodies are employed in two ways. First, the presence of these specific antibodies can be an indication of exposure to a specific antigen, therefore detection of these specific antibodies can be used as a biomarker, revealing its exposure [4]. Secondly, the immune system can be triggered on purpose with a defined foreign compound, by repeating this trigger, antibody producing B-cells maturate [5]. After maturation the blood containing the antibodies or the antibody producing cells can be carefully collected and after processing the produced antibodies can be used as biorecognition molecules in immunoassays.

1.1 Antibody structure

IgG is the most abundant Ig in serum reaching a concentration up to 10 mg mL^{-1} [6]. The IgG type antibodies are composed of four polypeptide chains: Two identical heavy (H) chains of approximately 50 kDa in size each, and two identical light chain (L) of approximately 25 kDa in size each. The two H chains are connected to each other by disulfide bonds, in the so-called hinge region and each heavy chain is connected to a light chain with a disulfide bond [7] as shown in Figure 1. H and L are composed of constant and variable domains. The constant domains determine the isotype whereas the variable domains of the H and L chain together contribute to the antigen binding site, also known as complementary determining region (CDR) [8]. These antibody binding sites have shown to be a powerful tool in analysis as they can be used to bind and subsequently detect specific analytes (antigens). Antibodies originating from mouse, rabbit, goat and cow (bovine) are similar in structure. However, also differences can be pointed out. Each species has unique constant domains [9,10]. This species-specificity can be used in immunoassay development, for instance in recognizing a constant domain of a target protein antibody, using a secondary antibody from a different species attached to a detection label. In spite of this species-specificity, also heterogeneity is seen within a species, even for different batches of the same monoclonal antibody heterogeneity has been demonstrated [11]. Moreover, also sizes may differ. For antibodies within species, rabbit and mouse antibodies have in average a mass of approximately 150 kDa [12], goat antibodies are a bit smaller with 144 kDa [13]. The bovine antibodies are larger in size due to an unusual long CDR sequence of up to 70 amino acids and therefore able to bind challenging targets, like for instance Human immunodeficiency virus (HIV) [14]. Consequently, bovine antibodies are 160 kDa on average [15].

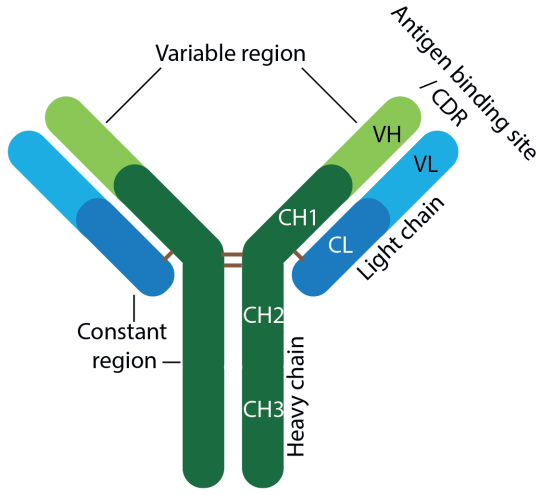

Figure 1. Schematic representation of the antibody structure consisting of two heavy and two light chains which are composed of constant and variable domains. The chains are connected by disulfide bonds (-).

Polyclonal antibodies

Polyclonal antibodies (pAbs) are found in the blood and extracellulair fluids. They are produced to counteract a foreign compound. PAbs are produced by a large number of B-cell clones, resulting in a polyclonal serum containing a composition of antibodies targeting different epitopes of the foreign compound [16]. This usually results in higher sensitivity than for an antibody obtained from one B-cell clone [17]. A downside of pAbs however, is found in their production as full blood of the animal is required. Production of pAbs thereof is limited to the size of the animal and its lifespan [18].

Monoclonal antibodies

Monoclonal antibodies (mAbs) are monospecific, in other words originating from one B-cell clone. The basis for mAb production lies in the immune response to the foreign compound similar to pAb production. To produce mAbs, spleen B-cells from the animal challenged with the foreign compound are collected and fused with myeloma tumor cells. Successful fusions will result in hybridoma cell lines, which can rapidly and indefinitely multiply and are able to produce the antibody of interest [19].

1.2 Antigens

A foreign compound entering the body is called an antigen. To elicit an immune response the antigens need to be at least 2 kDa [20]. Proteins easily exceed this limit as for instance proteins endogenously present in humans range from 1.8 kDa to 515 kDa and therefore are able to provoke an immune response [21]. Proteins have well defined three-dimensional structures as their function arises from their three dimensional conformation [22]. Folded proteins need to be compact to provide stability to the structure. The overall three dimensional structure of a protein is a result of the local folding of the polypeptide chain which leads to α-helices or β-sheets [23]. Proteins used as antigens in this thesis are, IGF-1, IGFBP2, rbST but also the constant domains of the heavy chains of antibodies.

1.3 Antibody-antigen interaction

Antibodies targeting proteins recognize a small part of the protein, also called an epitope, typically 4-12 amino acids long [24]. Epitopes can be categorized in two types, the continuous (linear)- and discontinuous (conformational) epitopes. The first type, the continuous epitopes, are linear peptide fragments of a protein chain recognized by the antibodies. These epitopes are defined in a functional manner, as it is not clear if all aminoacids present in the continous epitope make contact to the antigenic binding site of the antibody. The second type, the discontinuous epitopes, consist of amino acids approximating each other by folding of the polypeptide chain. The native conformation of the protein therefore is key for antigenic reactivity [25,26]. In order for an antigen to be recognized by an antibody, the epitope needs to be accessible for interaction and therefore present on the surface of the antigen [27,28].
Binding affinity of the antibody-antigen interaction is the equilibrium established between association and dissociation of the antibody-antigen complex. Higher association and lower dissociation is characterized by affinity maturation [29]; The first immune response as a reaction on entering foreign compounds results in low affinity antibodies which dissociate easily after binding, however due to an ongoing immune response B-cells maturate, which increases the affinity [30]. Next to affinity, specificity is of high importance for antibody-antigen interaction, *in vivo* to bind only the targeted antigen and *in vitro* to determine only the compound of interest in immunoassay development and application. As monoclonal antibodies target a single epitope, higher specificity is obtained compared to polyclonal antibodies where multiple epitopes are targetted [31].

1.4 Antibody-antigen interactions applied in detection platforms

When antibodies are obtained via serum collection (pAbs) or cell fusions (mAbs), the antibodies can be an essential tool in immunoassay development aiming at detection of their specific (protein) target. Developed assays in this thesis all are build upon antibody-antigen recognition. Basically two detection formats can be distinguished, sandwich and inhibition assay formats as shown in Figure 2.

Assay formats

Sandwich assay format The sandwich immunoassay format traditionally sandwiches the target protein between two antibodies. An antibody is immobilized on a surface to capture the target protein and a second labelled detection antibody, also able to bind the target protein, is added [32,33]. In this thesis two sandwich assay formats are used. However not using 2 antibodies sandwiching the target protein, as the antibody itself is the target protein of interest. Here, the antibody of interest is sandwiched between the target protein or peptide and a secondary species specific antibody. This concerns first, detection of rbST induced bovine antibodies, which are sandwiched between the rbST immobilized on the sensor surface and the secondary labeled anti-bovine antibody in the microsphere based multiplex biomarker immunoassay. And second, detection of the epitope recognized by a selected monoclonal antibody. Here the monoclonal antibody was sandwiched between the peptide epitope and a secondary labeled anti-mouse antibody on a Enzyme Linked Immonosorbent Assay (ELISA) based reaction. For sandwich assay formats the signal increases when the target protein concentration increases.

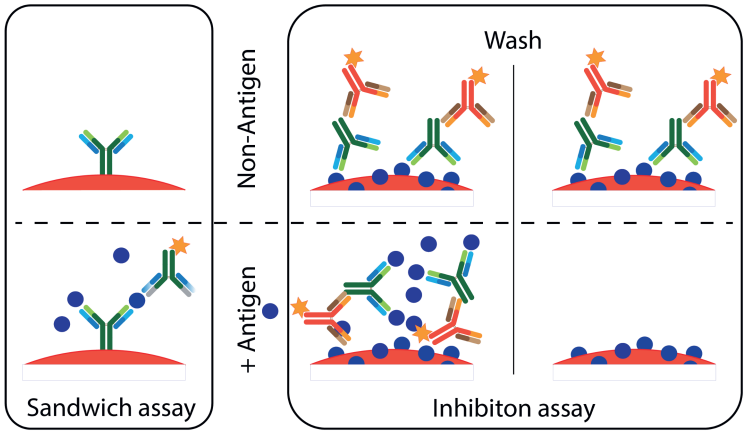

Figure 2. Schematic overview of a sandwich- (left) and inhibition (right) immunoassay. In the sandwich immunoassay the capture antibody immobilized on a surface captures the target protein which will then bind to the detection antibody. In the inhibition assay the target protein or peptide immobilized on the surface and the target protein present in the sample compete for the antibody binding.

Inhibition assay format In the inhibition assay format, the target protein is immobilized on a surface. When the target protein is present in the sample, the target proteins immobilized on the surface and the target proteins present in the sample added will compete for antibody binding. If no target protein is present in the sample, the added antibodies will bind to the surface- immobilized target proteins. Subsequently the labeled

secondary antibody will bind to the first antibody and a high signal is obtained in read-out. On the other hand, when the sample target protein concentration is high, the antibody will bind to the free available target protein instead, which will lead to no antibody binding to the surface immobilized target protein. Consequently no labeled secondary antibody will bind and no signal is obtained in read-out. For inhibition assay formats, decrease in signal is a result of increased concentration of the target protein.

Peptide array immunoassays

For protein targets, defining the epitope recognized by a selected antibody, an array of overlapping peptides corresponding with the protein target is generated. On an amino functionalized polypropylene support, peptides are synthesized with standard Fmoc-chemistry [34]. Both linear and 3D structured (conformational) peptides can be part of the library [35]. To determine the epitope recognized, the generated array is incubated with the selected antibody in an ELISA-based approach. If the epitope present on the array is recognized by the antibody, binding occurs. Subsequently a secondary antibody labeled with peroxidase conjugate confirms this binding [36]. The color change due to the colorimetric enzyme-substrate reaction can be recorded with a CCD camera. From results assaying the total library of overlapping peptides a binding profile is composed and the epitope recognized by the antibody can be deduced, as schematically represented in Figure 3.

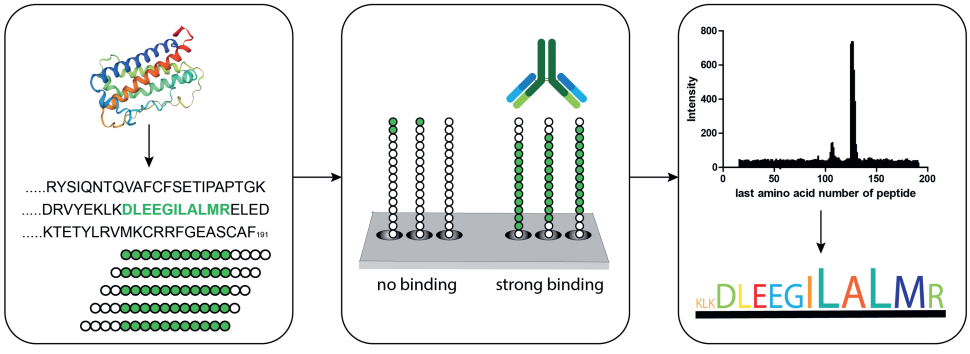

Figure 3. Schematic representation of the process of antibody mapping with generation of overlapping peptides of the selected protein target (left), testing of the antibody with all the produced overlapping peptides for antibody binding (middle) and deduction of the epitope recognized after recording of the binding profile (right).

Microsphere-based immunoassay

For microsphere based immunoassays both flow cytometric and planar imaging platforms can be used. Both platforms use distinct color-coded microspheres which are commercially available. The microspheres are color-coded by an internal dye existing of different ratios of red and infrared fluorophores. The ratio of dyes classifies the microsphere type.

Moreover, for assay read-out both platforms also use fluorophores, like for instance phycoerythrin. Combining multiple regions of color-coded microspheres enables multiplex detection [37]. In the flow cytometric platform, microspheres are transported in a flow and pass two lasers for microsphere type classification (excitation at 638 nm) and assay read out (excitation at 532 nm). Use of the planar imaging platform requires microspheres to be magnetic, as microspheres are scattered over a magnetic planar surface and CCD camera's are used for region classification (excitation 621 nm) and assay read out (excitation 511 nm) [38] as shown in Figure 4.

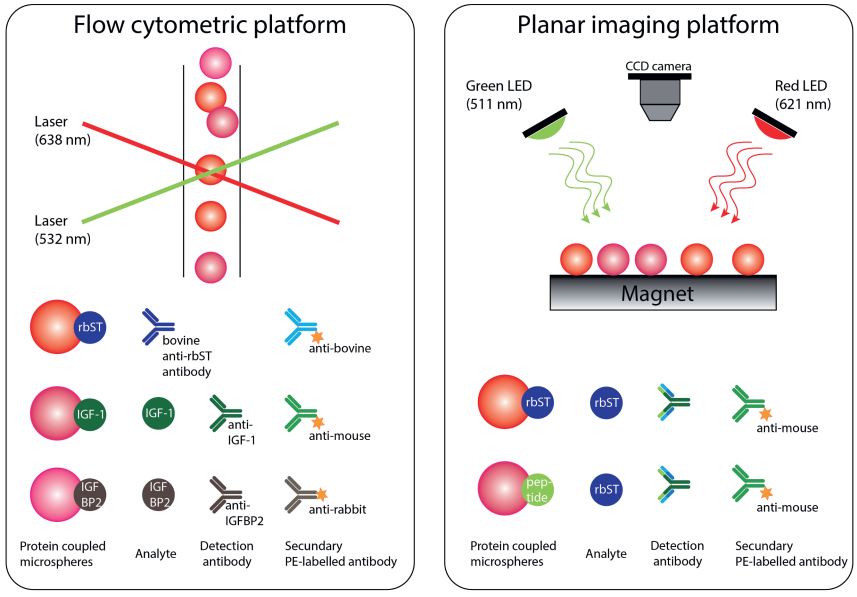

Figure 4. Schematic representation the microsphere based flowcytometric - (left) and planar imaging platform (right). For each platform the executed assay formats are represented; The IGF-1, IGFBP2 on the flowcytometric platform and both rbST assays on the planar imaging platform are inhibition assays. For detection of the rbST induced antibodies in bovine the antibody is sandwiched between the rbST immobilized on the microsphere surface and the anti-bovine secondary antibody.

Microsphere coupling Proteins and peptides are covalently coupled to the carboxylated microspheres. The coupling chemistry for peptides and proteins is build on the same principle, only the coupling buffer is different. A standard two-step carbodiimide reaction employing N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium salt (NHS) is executed [39]. For protein coupling, MES buffer pH 5 is used, a standard buffer prescribed by the manufacturer. Proteins have multiple aminogroups for binding. However, the peptides immobilized in this thesis only

have one aminogroup with a high pKa available for binding. Therefore, peptides are immobilized using a borate buffer pH 8.3 and high salt concentration [40].

Immuno-affinity enrichment LC-MS

Sample pretreatment Antibodies can also be applied to support sample treatment. With immuno-affinity enrichment, a selective purification, high sensitivity for protein detection can be reached [41]. For selective purification, in this thesis, antibodies against rbST are coupled to a micro-column monolith carrier, as monolith carriers show low non-specific binding in comparison with microspheres and enable an intensive contact between the sample and carrier [42]. With these monolith immuno-affinity microcolumns, the low abundant rbST is enriched from the complex protein-rich environment like blood serum. The rbST captured by the antibodies on the monolithic immuno-affinity micro-column is eluted from the column for further treatment (Figure 5). Subsequent tryptic digestion and detection of the enriched peptides improves sensitivity in comparison with detection of the whole protein. For tryptic digestion, the enzyme trypsin is employed to cleave the protein into smaller molecules, peptides. The sizes of these peptides after digestion can be deduced from the protein sequence chain as trypsin specifically cuts the protein chain at the carboxyl side of the amino-acids lysine or arginine at 37 °C in the 7-9 pH range [43].

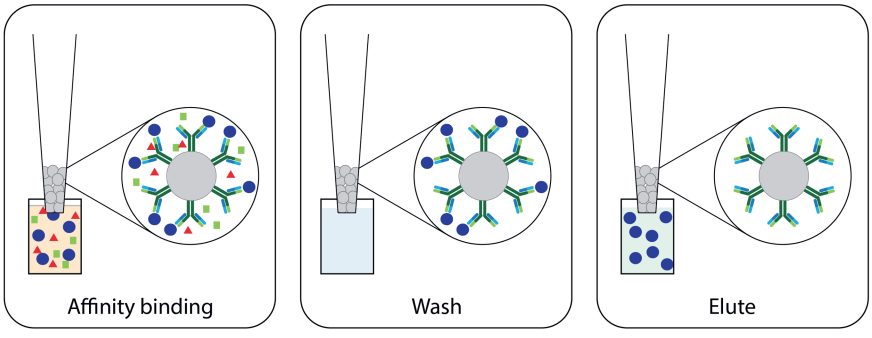

Figure 5. Schematic representation of the immuno-affinity enrichment with monolithic micro columns. The sample containing the target protein is transferred over the column for antibody binding (left); abundant proteins are washed away (middle) and the target protein is eluted from the monolith micro-column (right).

LC-MS analysis Peptides are separated or purified by reversed phase liquid chromatography (RP-LC) in this thesis. RP-LC uses a hydrophobic stationary phase and a mobile phase to separate the peptides based on their polarity. In the mobile phase the percentage organic solvents is increased during a run and depending on the interaction between the stationary and mobile phase peptides will exit the column at different times [44]. To improve the peptide separations in comparison with the conventional high pressure LC,

ultra performance LC (UHPLC) which uses columns packed with smaller silica particles and requires higher operating pressures [45]. When the peptides exit the LC column they are ionized by electrospray ionisation (ESI), and finally are transferred to the high vacuum chamber of the mass analyzer of the system. The mass spectrometer (MS) measures mass to charge ratios (m/z) of the charged ions. For peptide measurement the MS is operated in positive ion ESI-MS mode. With the first quadropole (Q1) analyzer the m/z values of the protonated peptides are selected. If further determination of the peptides is required a triple quadrupole is used. After measurement of the m/z ratios of the peptides, the peptides of interest are selected, fragmented (Q2) and fragments are measured in the third quadrupole (Q3) as shown in Figure 6.

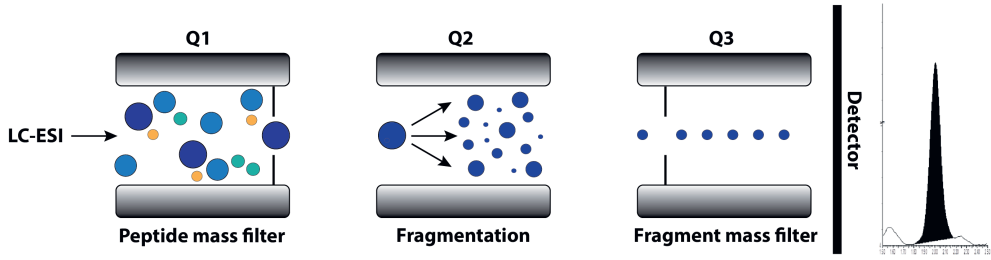

Figure 6. Schematic representation of an MS/MS measurement. The sample is ionized by electrospray ionization (ESI). In Q1 the peptide is mass/charge selected, fragmented (Q2) and fragments are mass/charge analyzed in Q3.

2 Recombinant bovine somatotropin

2.1 Protein structure and function

Endogenous bovine somatotropin (bST), is a single-chain protein hormone of approximately 22 kDa and present in 4 different forms. BST is 190 or 191 amino acids long. In the 190 aminoacid form A_1 is missing, and in both 190 and 191 aminoacid forms either leucine or valine is present on position 126 or 127 respectively as schematically presented in Figure 7. Two internal disulfide bonds contribute to the three dimensional structure of the protein [46,47]

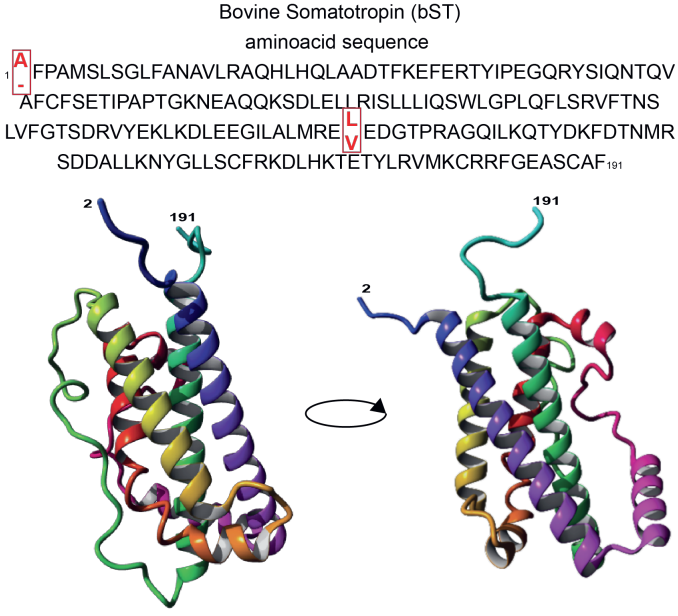

Figure 7. Schematic representation of the aminoacid sequence of the four endogenous bST forms and a model of the 3-dimensional structure.

BST is produced by the anterior pituitary gland and belongs to the same hormonal family as prolactin and placental lactogen [48]. It controls differentiation, growth and metabolism of many cell types of vertebrate species [49] and plays an important role in the control of growth and reproduction. One of its characteristics is stimulation of mammary gland growth and regulation of milk production [50]. Next to stimulation of the mammary gland, rbST treatment influences the growth hormone/insulin like growth factor I (GH/IGF-1) axis, the immune system and cell cycle in the dairy cow [51,52].

2.2 rbST treatment

When bST is extracted from pituitary glands and administered to lactating cows, the milk production is stimulated [53]. To enable unlimited production of bST without being depended on extraction of pituitary glands, a joint cooperation between Monsanto Co. and Genetech Inc. produced recombinant bST (rbST) with biotechnological techniques in the 1980's [54]. Nowadays, two forms of rbST are commercially available: Posilac® and BoostinS®. Posilac® (Met-rbST), developed by Monsanto, is chemically almost identical to the endogenous form, with only a distinct N-terminal end, the alanine is replaced by a methionine. BoostinS® (Hilac, Ala-rbST), is produced by LG Life sciences and is identical to the endogenous form [55]. Currently commercially available rbST vehicles make use of a slow-releasing formula enabling biweekly treatment according to the manufacturers' protocol [56]. For optimizing milk yields gaining approximately 11% [54,57,58], it is advised to start treatment 57-70 days after calving.

2.3 Legislation

The European Union ban of placing rbST on the market and its administration is described in Council Decision EC 1999/879/EC since 2000 [1,59]. Ground for this ban is animal welfare. Adverse health effects determined by meta-analysis showed 25% increased risk for clinical mastitis, 40% increased risk of unsuccessful conception and 55% increased risk of lameness [60]. As Europe, Canada and Australia amongst others banned the use of rbST, its use is permitted in for instance the USA and Southern Africa, and partly by the international food trade this ban will stay subject of discussion. The Codex Alimentarius, a commission established by the Food and Agricultural Organisation (FAO) and the World Health Organisation (WHO) contributing to the safety, quality and fairness of international food trade, reviewed draft maximum residue limits (MRLs) for rbST to be discussed at the 38th session in 2015. At request of the Codex Alimentarius Commission, the Joint Expert Committee on Food Additives (JECFA) evaluated aspects related to the safety of rbST and concluded "there was no evidence to suggest that the use of rbST would result in a higher risk to human health due to the possible increased use of antimicrobials to treat mastitis" [61,62]. Subsequently the European Commission asked the European Food Safety Authority (EFSA) to assess the JECFA conclusion. EFSA concluded the human antimicrobial resistance (AMR)-related concerns are only partially considered by JECFA , and therefore "cannot be considered valid in relation to all AMR-related concerns" [63]. Due to the missing consensus the Codex Alimentarius Commission came to hold draft MRLs for rbST at Step 8, where all 180 member countries decide for or against a policy becoming a universal standard. As a result the draft MRLs for rbST are currently at this step since the 1990s, as there are no time restrictions for this step.

3 Detection strategies for control purposes

Strictly speaking a strategy is a long-term plan for achieving something, or reaching a goal, or the skills of making such plans [64]. To secure the safety of our food, substances like rbST are banned. In practice however, economic benefits and the availability of the product hamper this ban. Its abuse is economically lucrative as profit increases with 26-32% [65,66]. According to EU Regulation 2017/625, it is required to monitor the use of banned substances in food producing animals or their products [67]. Monitoring starts at the farm with rapid identification by inspectors of questionable situations [68]. Next, taking samples at the farm for laboratory measurements is indispensable to pinpoint rbST abuse. Preferably samples are taken by non-invasive methods, but also should be suitable for analysis by the detection method of choice. Consequently, for detection methods criteria were set to assure scientifically sound interpretation of the data obtained. Criteria sets, are layed down in Commission Regulation 2021/808 [69], concerning "the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling". This Commission Regulation distinguishes 2 types of methods, screening- and confirmatory methods, which both have their own set of criteria to abide by. In brief, screening methods are used to detect the presence of a substance having a false compliant rate of $<5\%$ at the level of interest. Screening methods are high sample throughput and able to sift for potential non-compliant results. The confirmatory methods unequivocally identify and if necessary quantify the compound of interest.

3.1 Screening

Detection of growth promotors and peptides is highly complicated due to the similarity with endogenous hormones, low concentrations in blood circulation and short half-life. ELISA methods developed for rbST detection itself, unfortunately do not reach required sensitivity for applicability in real samples [70]. It is known that despite the similarity with endogenous protein hormones and peptides, several growth promoters can trigger an antibody response when administered. In sports doping, erythropoietin has been described to induce antibody formation [71]. Also administration of growth hormone (GH), IGF-1 or growth hormone releasing hormone (GHRH) to human can trigger antibody formation [72-74]. In animals this response is observed as well, and is used in doping and veterinary control to screen for abuse of this class of growth promoters. In horse racing, antibody formation against somatotropin is used to screen for its misuse with surface plasmon resonance (SPR) and ELISA methods [75]. Also in dairy cows rbST administration to enhance milk production can induce antibody formation, detectable in serum and milk by ELISA and flow cytrometric immunoassay (FCIA) [76-78]. For control purposes, these antibodies are proven to be a very powerful screening tool [79]. Moreover, in combination with other biomarkers, like IGF-1, IGFBP-2 and osteocalcin, misuse can be predicted with a high certainty as 95% of the rbST-treated cows can be determined as truly positive [80]. Additional clinical chemistry profiles of blood parameters potentially strengthen screening for rbST abuse in dairy cows [81]. Nowadays, research for rbST abuse detection is also

focussed on gene-expression. This screenings technique however, is not yet compliant with the Commission Regulation 2021/808 [82].

3.2 Confirmation

In doping and food regulatory agencies, confirmation of the presence of the illegally used growth promoter is needed after positive screening results. This, however, is challenging, as the matrices, highly abundant in proteins, can interfere in the LC-MS/MS analysis by suppression of the signals of the low abundant growth promoter(s). Moreover, the ability to detect rbST for the full period between two consecutive administrations requires high sensitivity of the method. Methods without additional enrichment steps lack this sensitivity [83]. In human doping control, promising results are obtained for detection of traces of peptide hormones by immuno-affinity purification prior to liquid chromatography-mass spectrometry [84]. Enrichment using antibody based affinity purification followed by LC-MS/MS confirmation displays high sensitivity and enables the detection of rbST in bovine serum over the full period between two consecutive administrations (when administered according to manufacturers' protocol) compliant with Commission Regulation 2021/808 [85-86].

3.3 rbST detection in practice

Preferably, samples are taken by non-invasive methods. As milk enhancement is the purpose of rbST abuse, milk seems to be the preferable matrix. Using milk as matrix enables to screen for the total farm when analysing tank milk samples. When tank milk samples, which is a mixture of milk of multiple cows, are analysed for induced antibodies to pinpoint rbST abuse over 95% true positive rate can be reached [77]. Unfortunately, confirmatory methods are not yet able to detect rbST in milk samples and plasma (or serum) of dairy cows is still required. When tank milk taken from a farm is screened and found to be non-compliant, serum or plasma samples must be taken from individual cows for confirmation as presented in Figure 8.

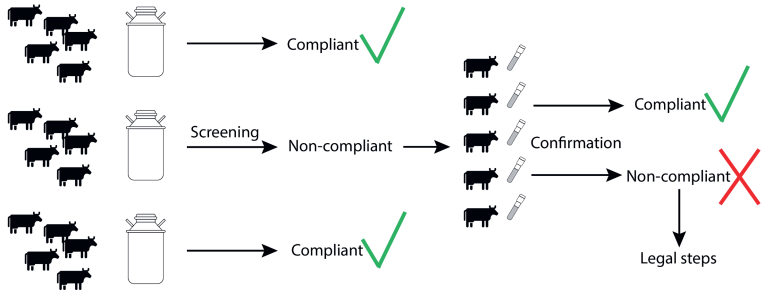

Figure 8. Schematic representation of a control strategy where with screening a sift in samples is made prior to confirmation with rbST as an example.

The number of individual samples needed from a herd to enable confirmation is not yet established. In establishing this number, it should be taken into account that a sample can originate from a cow in her dry period, or shortly after calving prior to treatment. The number of samples, decided upon to be tested on rbST presence must be statistically sound to provide proof of rbST abuse in farms screened for non-compliance. Another challenge in rbST confirmation is the endogenous identical rbST, BoostinS®, as according to legislation the compound of interest must be unequivocally identified. In this case, full identification cannot discriminate between endogenous and recombinant bST. Moreover, it is legally not valid to accept significantly higher bST levels as proof of BoostinS® abuse.

4 Scope and thesis outline

The research presented in this thesis focuses on antibody-based tools for and insights into detection strategies, using rbST as a model. To reach this goal, detection tools to reveal rbST abuse need to be developed and their challenges addressed. Results obtained from these detection tools will lay a foundation to elaborate on applicability of existing legislation for abuse of forbidden protein hormones. To this end, knowledge on detectability of rbST and its biomarkers in dairy cows, when treated according to manufacturers' protocol, is needed. This knowledge is lacking due to i) the absence of representative sample material obtained from Ala- and Met-rbST treated cows and ii) the absence of rbST detection methods that are sensitive and specific enough to be applicable in practice. In this thesis the use of antibody-antigen interactions as a tool for detection of rbST abuse is explored in two ways; on one hand, in **Chapter 2**, presence of rbST-induced antibodies in dairy cows are investigated as they are specific biomarkers, allowing prolongation of the detection window to reveal rbST abuse, compared to detection of rbST itself. On the other hand, in **Chapter 3**, antibodies are used for affinity enrichment of rbST in serum to aim for detection limits applicable in real-life. Antibody-antigen interactions are further characterized in **Chapter 4**. As detailed knowledge on the antigen binding site of rbST-induced antibodies enables to focus on the epitopes responsible for rbST specific antibody-antigen binding, and moreover, reduces dependence on the full rbST protein. To suggest detection strategies for rbST, the developed methods are applied and results are elaborated for real-life applicability in **Chapter 5**. Next to in-depth knowledge on the antigenic binding site of rbST induced antibodies in serum of rbST treated dairy cows, **Chapter 6** contains discussion on the results presented in this thesis. Moreover, suggestions for the rbST detection strategy are made and accompanied with ideas for future research.

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Chapter 2

Multiplex flow cytometric immunoassay for serum biomarker profiling of recombinant bovine somatotropin

Adapted from: Smits, N.G.E., S.K.J. Ludwig, G. Van der Veer, M.G.E.G. Bremer, and M.W.F. Nielen. Multiplex flow cytometric immunoassay for serum biomarker profiling of recombinant bovine somatotropin. *Analyst* **2013**, 138 (1), 111-117.

Abstract

Recombinant bovine somatotropin (rbST) is licensed for enhancing milk production in dairy cows in some countries, for instance the United States, but is banned in Europe. Serum biomarker profiling can be an adequate approach to discriminate between treated and untreated groups. In this study a multiplex screening tool of a small set of biomarkers for pinpointing recombinant bovine somatotropin (rbST) (ab)use was developed and evaluated: insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2) and rbST-induced antibodies were selected as rbST dependent markers and combined in one parallel assay format. For this, the color-encoded microspheres were used in a suspension array, with a dedicated flow cytometer. Serum samples obtained from an animal experiment with rbST-treated and untreated dairy cows were measured with the developed triplex immunoassay and biomarker responses on rbST treatment were evaluated. This resulted in characteristic treatment-dependent responses for all three individual biomarkers. Combining these results with the statistical prediction model k-nearest neighbours (kNN), resulted in good discrimination of treated and untreated animals: an overall sensitivity (true positive rate) of 89.1% and an overall specificity (true negative rate) of 97.7% were reached. Therefore, this is the first multiplex method which can be applied with high confidence for screening of unknown herds of cattle pinpointing at rbST (ab)use.

1 Introduction

Recombinant bovine somatotropin (rbST) can be used to enhance growth and lactating performance in cattle. Within the EU, rbST is banned since 2000 [1], therefore, routine screening methods are urgently needed. A liquid chromatography-mass spectrometry (LC/MS) method for direct rbST detection in blood samples was developed [2], however it showed a small detection window, due to the short half-life of rbST in blood [3]. Moreover, the similarity with the endogenous hormone (bST, also called growth hormone), the low concentrations of bST and rbST in serum, and strong fluctuations of bST hamper the direct detection. Therefore, detection of rbST-dependent biomarkers having a longer half-life offers a promising alternative, as already reported for steroid abuse and in sports doping [4-8]. As rbST strongly influences the growth hormone/insulin like growth factor I (GH/IGF-I) axis, the following biomarkers are considered as indicative for administration of rbST: insulin-like growth factor-1 (IGF-1) and its binding proteins IGFBP2 and 3, rbST-induced antibodies, and several markers of bone and collagen turnover [9-12]. So far, immunoassays detecting single rbST related biomarkers were developed on different platforms like radio immunoassays (RIA) [9, 13, 14], enzyme linked immunosorbent assays (ELISA) [15-19], western blot techniques (WB) [20,21] and flow cytometric immunoassays (FCIA) [22-24]. Looking at single biomarkers, by using the above mentioned techniques, an indication of potential rbST abuse might be obtained. However, a much more powerful screening tool can be designed by combining multiple biomarkers into a multiplex assay format. The advantage of biomarker screening in the serum of dairy cows using flow cytometry in comparison with surface plasmon resonance (SPR) based techniques was demonstrated recently [22]. Using color encoded microspheres in a suspension array format, in theory, 100 different analytes can be detected simultaneously with high throughput in minimal sample volume. In this study we evaluated the suitability of this technique for multiplex detection of rbST related biomarkers: IGF-1, its binding protein IGFBP2 and rbST-induced antibodies. This set was selected from the literature as it includes two biomarkers with a quick response upon rbST treatment (IGF-I and IGFBP2) and one with a long half-life (rbST-induced antibodies), i.e. together offering the possibility of a prolonged detection window. The development of a biomarker-based method for rbST (ab)use required the analysis of a large population of untreated cow samples to determine endogenous background levels, and the biological variation of each biomarker. Decision limits were then established. Next, the applicability of the developed triplex assay was demonstrated with serum samples from rbST-treated and untreated cows. Using the statistical prediction tool k-nearest neighbours (kNN), the origin of serum samples, treated or untreated, was predicted based on single biomarker analysis and combined biomarker analysis. Finally, both biomarker analysis approaches were compared on their capabilities for pinpointing rbST (ab)use in dairy cattle.

2 Experimental

2.1 Materials and instruments

Posilac® 500 mg single-dose syringes and syringes with only the slow-release formula were purchased from Monsanto Company (St Louis, MO). Hydrochloric acid, potassium phosphate, sodium azide, sodium chloride, sodium hydroxide, sodium phosphate, Tween-20 and the ultrasonic cleaner were purchased from VWR International (Amsterdam, The Netherlands) and glycine was from Duchefa (Haarlem, The Netherlands). The N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was supplied by Fluka (Steinheim, Switzerland) and sodium dodecyl sulfate (SDS) by Serva (Heidelberg, Germany). Bovine serum albumin (BSA), insulin-like growth factor-I (IGF-I; human recombinant), 2-(N-morpholino)ethanesulfonic acid (MES hydrate) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Monsanto rbST standard was obtained from the National Hormone & Peptide Program (NHPP) of Dr Parlow (Torrance, CA). Insulin like growth factor binding protein-2 (IGFBP2; bovine recombinant) was purchased from IBT (Reutlingen, Germany). Mouse anti-IGF-1 was supplied by Spring Bioscience (clone SPM406, Fremont, CA) and the rabbit anti-IGFBP2 was from USBiological (Swampscott, MA). R-Phycoerythrin (PE)-labeled goat anti-bovine immunoglobulins (GAB-PE) were from Santa Cruz Biotechnology (Santa Cruz, CA) and R-phycoerythrin (PE)-labeled goat anti-mouse immunoglobulins (GAM-PE) and goat anti-rabbit immunoglobulins (GAR-PE) were purchased from Prozyme (San Leandro, CA). MultiScreen HTS filter plates were purchased from Millipore (Amsterdam, The Netherlands). Protein LoBind Tubes (1.5 mL) and a table centrifuge model 5810R were supplied by Eppendorf (Hamburg, Germany). The Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY platform programmed to analyze a 96-well plate was purchased from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK). SeroMAP microspheres (sets 025, 050 and 078) and sheath fluid were purchased from Luminex (Austin, TX). The Snijder test tube rotator was purchased from Omnilabo International (Breda, The Netherlands). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK).

2.2 Sample materials

Eight 5 year old Holstein dairy cows were divided into two groups. After two weeks adaptation, the treatment given consisted of subcutaneous injections of 500 mg rbST in a slow-release formula for the first group (a–d, referred to as rbST-treated) and the slow-release formula only for the second group (e–h, referred to as untreated). The cows were injected four times at two week intervals and subsequently twice with a one week interval. During the two week adaptation period, blood samples were collected weekly. During the treatment period blood samples were collected a day before, a day after and a week after injection and after the last injection blood samples were collected weekly for four more weeks. Unfortunately, one untreated cow (denoted e) died in the beginning of the animal experiment, due to swollen hocks, which led to general inflammation and

sepsis. Therefore, in this experiment results could be obtained only for 4 treated and 3 untreated cows. The experimental procedure was authorized by the ethical committee of the Faculty of Veterinary Medicine, Ghent University. In addition, blood samples were taken from 20 healthy, lactating cows varying in the age range of two to five years, in different stages of their lactating cycle, to reflect a normal population of untreated dairy cows. Based on the origin of these cows, the assumption of being untreated with rbST was justified.
After blood collection, all blood samples were placed at room temperature for 4 h to coagulate. Then, samples were centrifuged for 10 min at 3000 × *g*, and serum samples were collected and stored at -80 °C until further use.

2.3 Pretreatment of serum samples

For the generic flow cytometric immunoassay (FCIA) sample preparation procedure [22,24], serum samples were pretreated by adding 25 µL glycine solution (27.5 mM glycine pH 0.5 (pH adjusted by addition of HCl)) to 25 µL of serum sample or standard solution in a polypropylene tube under constant vortexing. Samples were then incubated at room temperature for 60 min. After incubation, 50 µL glycine–SDS solution (400 mM glycine, 0.3% (*m/v*) SDS, pH 10 (pH adjusted by addition of NaOH)) was added under constant vortexing. Samples were further diluted with 0.1% BSA in PBST to a final dilution of 80 times. No further sample preparation was needed prior to the FCIA.

2.4 Microsphere preparation for the flow cytometric immunoassay (FCIA)

IGF-1, rbST standard and IGFBP2 were coupled to seroMAP microsphere sets 025, 050 and 078, respectively, according to Bremer et al. [22]. Briefly, for each microsphere set 2.5×10^6 microspheres were coupled with a two-step carbodiimide reaction using 500 µL of a 100 µg mL^{-1} protein solution in MES buffer for IGF-1 and rbST, and 500 µL of a 10 µg mL^{-1} protein solution in MES buffer for IGFBP2. After coupling, the microspheres were stored in a blocking buffer (PBS, 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3) at 2–8 °C in the dark until use. Under these storing conditions, the microspheres were stable for more than one year.

2.5 FCIA procedure

Standards and sera were pretreated as described (Paragraph 2.3). One hundred µL of the pretreated and diluted serum samples or standard solutions were added to a filter bottom microtiter plate. Hereafter, a 10 µL antibody mixture containing 1500 times diluted anti-IGF-1 and 25000 times diluted anti-IGFBP2 antibody was added and incubated for 15 min on a microtiter plate shaker. Then, microspheres (10 µL diluted suspension containing about 1250 microspheres per microsphere set) were added to each well and incubated for 1 h on a microtiter plate shaker. After incubation, the plate was centrifuged (1 min at 130 × *g*) and the microspheres were washed with 200 µL PBST. After washing, a

125 µL PE-labeled antibody mixture containing 625 times diluted GAM-PE, 1000 times diluted GAR-PE and 1000 times diluted GAB-PE was added and incubated for 30 min on a microtiter plate shaker. After this incubation step, the plate was centrifuged and 125 µL of PBST was added per well. Then, the microspheres were detected, according to bead assay type and PE-label in the flow cytometer (1 µL s^{-1} was measured until 100 events per microsphere set were reached with a maximum of 50 µL per well).

2.6 In-house validation study of the developed FCIA

As an in-house validation study the intra- and inter-assay precision levels of the individual biomarkers in the triplex FCIA were assessed. The intra-assay variation was calculated by averaging the percentaged standard deviation of each sample obtained by median fluorescence intensity (MFI) signals of a 10 times repeated triplex FCIA on 8 serum samples obtained from 7 cows. The inter-assay variation was calculated by averaging the percentage standard deviation of each sample obtained by duplicate measurements of the same 8 sera on 9 different days, using B/B_0 results for the IGF-1 and IGFBP2 assay and normalized values as described in the next paragraph for rbST-induced antibodies.

2.7 Signal normalization for rbST induced antibodies

Due to the lack of standard for rbST-induced antibodies, the daily variations in the assay performance and technical performance of the Luminex 100 IS 2.2 system, a normalization step to enable in-between-days comparison of signals from rbST-induced antibodies is needed. As the 8 sera used for the in-house validation study were measured in every experiment, MFI signals of these 8 sera were used for normalization: responses of the 8 sera were averaged and responses of the measured samples were divided by that average.

2.8 Assessment of decision limits

To be able to discriminate between treated and untreated dairy cows, a decision limit is needed for each biomarker. For assessing the decision limits with most accuracy, sera from 27 untreated dairy cows (20 untreated cows and 7 from the animal experiment during their adaption period) were measured with the triplex assay. For IGF-1 concentrations were used for the calculations, for IGFBP2, B/B_0 values were used due to the absence of a pure standard for obtaining a calibration curve, and for rbST-induced antibodies, MFIs were used. Concentrations, B/B_0 values and MFIs were averaged and the standard deviations determined respectively. Decision limits with 95% confidence were calculated. Theoretically, IGF-1 concentrations increase due to rbST treatment [10,12,25], therefore, 2 times the standard deviation was added to the average IGF-1 concentration. IGFBP2 concentrations theoretically decrease due to rbST treatment [12], which results in less inhibition of maximum MFI signals (B_0) and consequently in higher B/B_0 values. Therefore 2 times the standard deviation was added to the average B/B_0 value. RbST-induced antibodies could be formed due to rbST treatment [23,24], therefore 2 times the standard deviation was added to the average MFI signal.

2.9 Statistics

To assess whether the combination of the three analysed biomarkers is already capable to predict rbST abuse, a k-nearest neighbours prediction model in the R environment [26] was used. B/B_0 values for IGF-1 and IGFBP2 as well as MFI signals for rbST-induced antibodies for every sample from the animal experiment were included in the data analysis. First of all, the whole dataset was divided into a training and test set by using a stratified repeated random sub-sampling approach, which means that 70% of the rbST-treated and 70% of the untreated samples were chosen for the training set and the remaining 30% of both groups for the test set. Subsequently, B/B_0 values and MFI signals of the training set were auto-scaled and a kNN model was built using the training dataset. The optimal number of k ($1 \leq k \leq 10$) was chosen based on the bootstrapping approach leaving out 10% of the training data (randomly with replacement), which was repeated 10 times [27] and the resulting model was tested by predicting the remaining auto-scaled test set data. Correctly and falsely predicted results were evaluated carefully. To obtain an average performance of the kNN model, this procedure was repeated 10000 times; each time different randomly chosen training and test sets were applied and an overall sensitivity (true-positive rate), specificity (true-negative rate) and misclassification rate could be calculated for every sample, for every time point and for the whole animal experiment.

3 Results and discussion

3.1 Development of the triplex FCIA

For the development of the triplex screening assay, three single immunoassays were combined: the previously developed IGF-1 assay [22], the assay for rbST-induced antibodies [24] and a newly developed IGFBP2 assay. Despite the fact that IGF-1 and IGFBP2 are indirect competitive assays and the assay for rbST-induced antibodies is an indirect assay, one single straightforward approach in terms of pretreatment and incubation times was feasible. Therefore, the color-encoded microspheres, primary antibodies and secondary antibodies from the individual assays were simply mixed (Figure 1). This approach, however, led to an increase in the MFI signal of approximately 150% for serum samples measured in the multiplex IGF-1 and IGFBP2 assays, compared to the same samples analysed in singleplex format, while no increased signal was observed for the standard solution (B_0). This phenomenon might be caused by serum antibodies that directly bind to the microspheres unspecifically [28], whereas the standard solution only consisted of a 80 mg mL^{-1} BSA in PBS. No influence of multiplexing was found on the detection of rbST-induced antibodies. To further investigate the source of the increased signal, the influence of the individual primary antibodies and the individual secondary antibodies in combination with or without primary antibodies on all three microsphere sets, was tested. This pointed to unspecific binding of PE-conjugated secondary antibodies to all three microsphere sets, in particular GAR-PE and GAB-PE, as the cause of the increased MFI signals. Therefore, to decrease this background, the PE-coupled secondary antibodies were diluted more until MFI signals were just above 1000 MFI for the blank standard. Thus the main modification

of the triplex conditions versus the singleplex assays were secondary antibody dilutions for GAM-PE, GAR-PE and GAB-PE of 625, 1000 and 1000 times, respectively, instead of the former 625, 375 and 100 times dilutions. By doing so, the increase in MFI signals upon multiplexing became less than 2%.

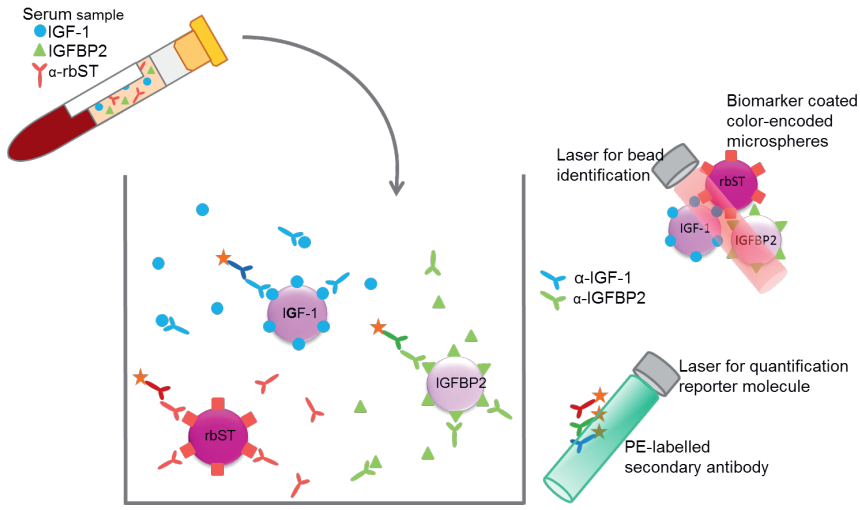

Figure 1. Triplex assay format, the indirect competitive format for IGF-1 and IGFBP2 and indirect format for antibodies formed against rbST, all combined within one well.

3.2 In-house validation study of the developed triplex FCIA

For all three assays, high repeatability in both intra-assay and inter-assay variations were assessed. Average coefficients of variation of 5.4%, 5.4% and 6.5% in the intra-assay variation and 5.3%, 4.3% and 7.5% in the inter-assay variation for the detection of IGF-1, IGFBP2 and rbST-induced antibodies respectively were found. This is in very good agreement with the formerly found variations in the IGF-1 singleplex assay [22].

3.3 Triplex FCIA applicability to real samples

As the ultimate goal is to detect rbST abuse with a biomarker-based method, the applicability of the triplex FCIA was tested.

Establishment of decision limits

For determination of IGF-1 concentrations, a calibration curve was recorded and used to recalculate IGF-1 concentrations in sera of the 27 untreated cows (data not shown). Average IGF-1 concentrations of 94 $\pm$ 21 ng mL^{-1} were found, resulting in an IGF-1 decision limit of 136 ng mL^{-1}. As no recombinant IGFBP2 suitable for obtaining a good

calibration curve was available, a decision limit was determined on MFI signals normalized on the maximum MFI signal (B_0). For IGFBP2, this resulted in a B/B_0 of 0.43 $\pm$ 0.04 resulting in an IGFBP2 decision limit of 0.51. For the decision limit of rbST-induced antibodies, an average MFI signal of 193 $\pm$ 37 was found resulting in a decision limit of 266 MFI. For evaluating the applicability of the assays, sera of the animal experiment (4 rbST-treated and 3 untreated dairy cows) were analysed and results were compared to the decision limits as shown in Figure 2. As expected, for IGF-1 and IGFBP2, a rapid upcoming and decaying response, and for rbST-induced antibodies a response with a long half-life were observed. All three biomarkers showed specific characteristics in their response upon rbST treatment, together offering a wide detection window with great potential.

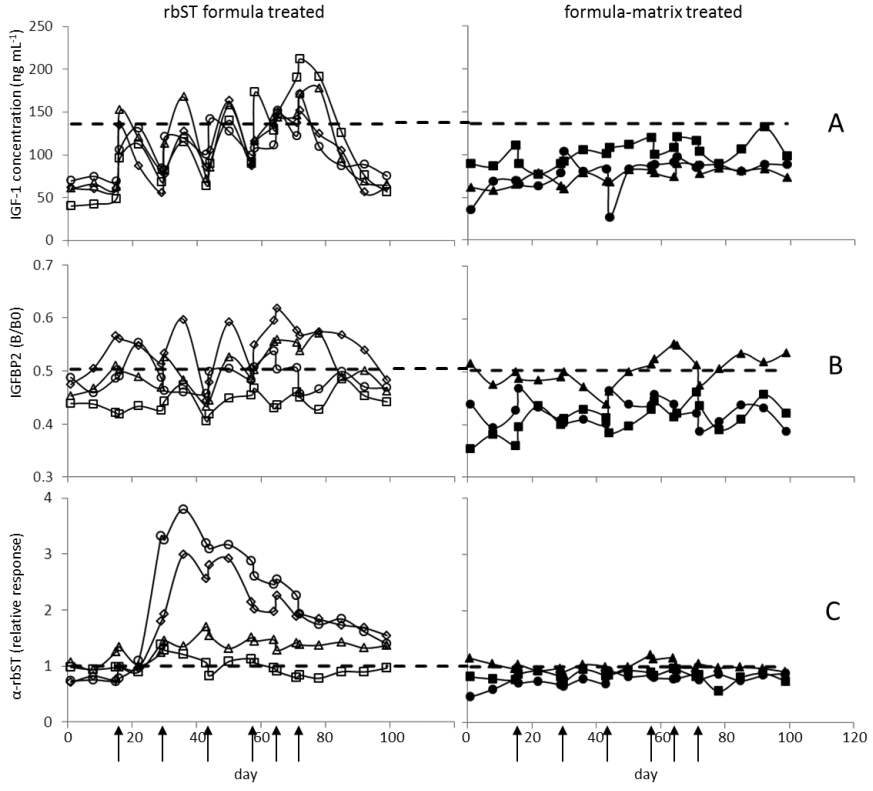

Figure 2. Effect of rbST treatment on serum biomarker levels of dairy cows in time. Time points of treatment (rbST-formula or matrix-formula) are marked by arrows. IGF-1 concentrations (A), IGFBP2 B/B_0 values (B) and normalized signals of rbST-induced antibodies (C) are shown for rbST treated animals a(◇), b(□), c(○) and d (△), and untreated animals f(■), g(▲) and h(●).

Biomarker IGF-1

Following rbST treatment, IGF-1 concentrations were elevated in all four treated cows (Figure 2A). This elevation was, however, only for a short period of time in all treated animals. Serum samples taken on the first day after the first treatment already showed an increase in IGF-1 concentration with the highest IGF-1 concentrations seen in sera taken one week after treatment. Then, concentrations declined towards the initial concentration as it can be seen in sera taken two weeks after treatment. In human serum this increase was also observed, however, only for two days after GH treatment [29], whereas similar responses were determined in lactating cows before [30]. Although the increase in IGF-1 concentration is clearly seen in all 4 cows (a–d) after the last rbST treatment, only a part of the serum samples tested showed IGF-1 concentrations beyond the decision limit. Therefore, 37% of the serum samples were determined as true-positives and subsequently 63% were classified as false-negatives (Table 1). The untreated cows, as expected, showed no increase in IGF-1 concentration, leading to a true negative rate of 100%.

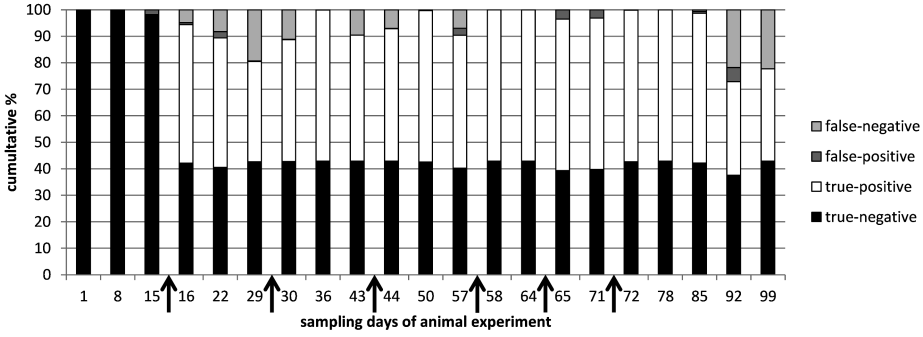

Figure 3. Prediction of correct and incorrect classification, using kNN modelling. Results of all serum samples taken in the animal experiment were categorized by sampling day and used for building the model and predict classification. Time points of treatment (rbST-formula or matrix-formula) are marked by arrows.

Biomarker IGFBP2

The rbST treatment was also reflected by the results from the IGFBP2 assay. As described in the literature, IGFBP2 concentrations decreased due to rbST treatment [9,31]. Consequently, maximum MFI signals (B_0) were inhibited less in sera of rbST-treated animals than in sera of the untreated animals yielding in general an increased B/B_0 response due to the decreased IGFBP2 levels. The first and most pronounced IGFBP2 response upon treatment was found one week after the rbST treatment (Figure 2B), i.e. later in time than for IGF-1. Two weeks after treatment IGFBP2 signals were back to original signals. In the literature, only responses to daily rbST injections were studied, resulting in a return to concentrations within 4 days after rbST treatment cessation [9]. In this study however, responses to the slow release rbST formula were studied. The IGFBP2

assay showed responses upon rbST treatment in three out of the four rbST-treated cows (a, c and d), but only a part of the serum samples taken from treated animals showed a B/B_0 higher than the decision limit. Therefore, 31% of the serum samples were classified true-positive, whereas subsequently 69% were tested as false-negative. The sera from untreated animals f and h, were all true negative; only some serum samples of the untreated animal g showed less inhibition, yielding a total of 90% true-negative samples and 10% false-positives (Table 1).

Table 1. Classification of serum samples from the animal experiment based on the single biomarker results in the triplex FCIA assay

	Untreated		rbST treated	
Biomarker	True negative (%)	False positive (%)	True positive(%)	False negative(%)
IGF-1	100	0	37	63
IGFBP2	90	10	31	69
α-bST	86	14	80	20

Biomarker rbST-induced antibodies

Two weeks after the first rbST treatment, an increase in antibodies specific for rbST was clearly seen (Figure 2C). This increase in rbST-induced antibodies was stable for a longer period of time, i.e. no MFI signal decline to start values in between rbST injections was seen. Even four weeks after the last rbST treatment, MFI signals for rbST-treated animals a, c and d were still beyond the decision limit, the maximum time point being investigated. This resulted in a true-positive rate of 80% and subsequently in a 20% false-negative rate. These results are in agreement with the literature [17,32] where 70–80% of rbST treated cows responded with antibody production. The untreated cows, f and h tested negative and only for cow g some serum samples were found just above the decision limit. This resulted in a true-negative rate of 86% and a false-positive rate of 14% (Table 1).

Table 2. Average classification based on the single biomarker results

Average prediction		
Biomarker	Classified correct (%)	Classified incorrect (%)
IFG-1	64	36
IGFBP2	56	44
α-rbST	83	17

Average classification of serum samples from (un)treated cows based on single and multiple biomarkers

Summarizing, for IGF-1, IGFBP2 and rbST-induced antibodies respectively, on average 64%, 56% and 83% of the tested samples were classified correctly (Table 2).
According to commission decision 2002/657/EC a 95% true positive rate is needed for screening assays. None of the single biomarkers on its own can pinpoint rbST abuse with that confidence. Combining results of the three biomarker assays, however, could increase the confidence rate for pinpointing rbST abuse. A statistical prediction model, the k-nearest neighbours (kNN) algorithm, was used to discriminate between rbST-treated and untreated animals. After building kNN models on all triplex serum sample data, an overall sensitivity (true-positive rate) of 89.1% and specificity (true-negative rate) of 97.7% were obtained (Table 3).

Table 3. Classification of serum samples based on all three biomarker results following kNN statistics

	kNN statistic prediction	
	Classified correct (%)	Classified incorrect (%)
Untreated	97.7	2.3
rbST treated	89.1	10.9

Most of the false-negative results (10.9%) occurred two weeks after the beginning of the rbST treatment and three and four weeks after termination of the treatment. False-negative results at the beginning of the treatment period could be accounted to IGF-1 and IGFBP2 levels, which declined rapidly after injection, and the antibody titers, which did not increase that much after the first rbST treatment. After multiple treatments, as is to be expected in practice, the false-negative rate became lower. Three to four weeks after termination of the rbST treatment, the prediction power of the model diminished for the same reasons (Fig. 3). Overall a correct prediction of 93.6% was observed. Further improvements of this biomarker triplex screening method can be achieved by simply adding additional biomarkers.

4 Conclusion

A unique multiple biomarker FCIA assay has been developed to pinpoint rbST (ab)use in serum samples of dairy cows. Individual immunoassays could be combined into a robust triplex format with only minor modifications. Thus a reproducible and sensitive platform was obtained. The developed triplex FCIA enables pinpointing rbST abuse by combining results of three biomarkers, IGF-1, IGFBP2 and rbST-induced antibodies. The IGF-1 and IGFBP2 biomarkers responded rapidly after the first rbST injection, while responses for rbST-induced antibodies were characterized by a long half-life. For that reason, the

combination of these three biomarkers resulted in a very long detection window. On average the individual biomarkers yielded correct classification of 64% for IGF-1, 56% for IGFBP2 and 83% for rbST-induced antibodies in serum samples. A kNN prediction model built on the combined triplex data enabled even a 93.6% correct prediction rate. Therefore, this triplex FCIA provides a detailed biomarker profile in serum, ultimately pinpointing rbST abuse in cattle with the highest possible confidence.

Acknowledgments

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Chapter 3

Monolith immuno-affinity enrichment liquid chromatography tandem mass spectrometry for quantitative protein analysis of recombinant bovine somatotropin in serum

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Abstract

The use of recombinant bovine somatotropin (rbST) to enhance milk production is approved in several countries, but it is prohibited in the European Union. According to EU legislation, it is necessary to confirm positive screening results prior to enforcement. Although adequate screening assays are available nowadays, development of liquid chromatography tandem mass spectrometry (LC-MS/MS) confirmatory methods to detect low levels of rbST is still a challenge. Here, we present a novel approach using immunoaffinity enrichment on monolithic micro-columns in combination with state-of-the-art ultra-high pressure LC-MS/MS (UHPLC-MS/MS) detection. The developed approach enables detection and confirmation of rbST in serum at a decision limit (CCα) concentration of 0.8 ng mL^{-1}. Furthermore, the method is easy to handle, robust and reproducible. We successfully applied the confirmatory method to serum samples from rbST treated cows that were found suspect after immunoassay-based screening. The use of rbST could be confirmed over 1 week after treatment, and the developed method demonstrated the sensitivity needed for effective control.

1 Introduction

Already in the 1930s, extracts of pituitary glands containing endogenous bST were used to increase milk yield [1,2]. Widespread application, however, was limited; as for one administration, multiple pituitaries of slaughtered bovines were required [1]. This changed in the 1980s when biotechnology offered the opportunity to produce recombinant bovine somatotropin (rbST) [2], enabling unlimited production of the growth hormone and therefore its use on a large scale. Currently, its use for milk production enhancement in dairy cattle is licensed in the USA and other countries, with no restriction to the residue levels. In Europe, there is concern about the effects of rbST on the well-being of the animals and consumers concern related to elevated IGF-1 hormone levels in milk. Because of this, rbST use is banned in Europe [3]. The ban requires methods to detect rbST abuse, and several analytical strategies have been reported [4]. Development of methods to detect rbST abuse is a major challenge due to: (i) the low concentrations in blood, (ii) its sequence similarity with the endogenous hormone bST (only differing in the N-terminal amino acid) and (iii) the strong fluctuations of levels of bST in serum [5,6]. For these reasons, adequate screening methods aiming at rbST itself, at relevant levels, have failed to be developed so far. Recently, a polyclonal antibody highly selective for rbST was produced and used in an ELISA format [7]. This assay, however, still lacks the required sensitivity to detect rbST in serum at relevant levels by factors of 10 to 100. To overcome the problems in detecting rbST directly, indirect screening methods focussed on detection of rbST-related biomarkers instead of rbST itself [8,9]. A multiplex screening approach developed by Ludwig et al. [8] provided a serum biomarker profile which correctly predicted rbST use in 95% of treated dairy cows. The number of true positives met the detection capability (CCβ) requirements of Commission Decision 2002/657 [8,10]. According to this decision, for positive findings (i.e. suspect samples) of a forbidden substance in a screening assay, additional instrumental analysis is needed to confirm its identity. Subsequent confirmation of forbidden substances, like rbST, can be accomplished by targeted LC-MS/MS analysis [11]. So far, only one confirmatory method for rbST in serum of dairy cows has been described in literature [5]. This method was able to detect rbST in cow serum at concentrations from 4 to 10 ng mL^{-1} during a detection window from 4.5 h to 4 days after treatment [5]. However, the method requires a large serum volume of 4 mL and an extensive sample preparation for cleanup [5]. Furthermore, detection of rbST until 4 days after administration is not sufficient to be able to detect the use of slow-releasing rbST formulas applied only biweekly, according to treatment schedules [12]. Several studies showed that overall bST serum levels (i.e. bST and rbST combined) after rbST treatment in dairy cows range from 1.5 to 45 ng mL^{-1} [6, 13-15]. Consequently, to enable detection of rbST during both the (biweekly) treatment period and the period after treatment, detection methods need sensitivity at/or below 1.5 ng mL^{-1}. To achieve this sensitivity, selective purification by use of antibodies against rbST is a promising approach. The use of immuno-affinity purification methods have been shown to be very effective for the detection of clinical important proteins in serum [16]. Therefore, it is expected that application of this approach to rbST will offer the sensitivity needed. In

general, anti-rbST antibodies need to be coupled to a carrier, after which enrichment of the low abundant rbST from the complex, protein-rich, serum background can be accomplished. Monolith micro-columns are a promising carrier for the antibody because of their low nonspecific binding, in comparison with beads and the intensive contact between the analyte and the antibodies, as shown in literature for IGF-1 enrichment [17]. For rbST in serum samples, selective and sensitive enrichment with monolith micro-columns may be promising as well. After enrichment of rbST from the serum samples, tryptic digestion was applied. The main advantage of tryptic digestion is the improved sensitivity of the peptides in comparison with detection of the whole protein. In this study, we present the development of a sensitive confirmatory method for rbST in serum of dairy cattle (Figure 1). Performance characteristics were determined in a preliminary in house validation. Applicability is demonstrated by confirmation of rbST at relevant concentrations in serum samples from rbST treated cows.

2 Experimental

2.1 Materials and instruments

Monsanto rbST standard was obtained from the National Hormone & Peptide Program (NHPP) of Dr. Parlow (Torrance, CA). Elanco rbST was obtained from Elanco (Indianapolis, IN, USA). Lactotropin 500 mg single-dose syringes were purchased from Centro de Tecnologia (Rio de Janeiro, Brazil). Pierce BCA protein assay, the Finnpipette™ Novus i Multichannel Electronic and monolithic micro-columns (MSIA disposable automation research tips (D.A.R.T.), containing approximately 10 mg packed bed Protein A or Protein A/G) were all purchased from Thermo Fisher Scientific (Rockford, Illinois). Ammonium sulphate, hydrochloric acid, potassiumphosphate, sodium hydroxide, sodium phosphate and the ultrasonic cleaner were purchased from VWR International (Amsterdam, The Netherlands). Trypsin, tris(hydroxymethyl)aminomethane, iodoacetamide (IAA), dimethyl sulfoxide (DMSO) and DL-dithiothreitol were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Methanol and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid was purchased from Actu-All chemicals (Oss, The Netherlands). Protein Lobind Tubes (1.5 mL, 2.0 mL) and a table centrifuge model 5810R were obtained from Eppendorf (Hamburg, Germany). The Jouan GR 20-22 ultracentrifuge was obtained from Jouan (Saint-Herblain, France). The Snijder test tube rotator was purchased from Omnilabo International (Breda, The Netherlands). An isotopic-labelled bST peptide AFPAMSLSGLFANAVLR and a synthetic analogue of the rbST peptide MFPAMSLSGLFANAVLR were obtained from Bachem (Bubendorf, Switserland). The LC-column: Kinetex 50×2.10 mm I.D. 1.3 µm C_{18} (100 Å) was purchased from Phenomenex (Utrecht, the Netherlands). Bond Elut Plexa 30 mg solid-phase extraction columns were purchased from Agilent Technologies (Amstelveen, The Netherlands). A Zymark TurboVap was purchased from Biotage (Upsala, Sweden).

2.2 Serum samples

Serum samples from two controlled animal treatment studies were used. In the first animal treatment study, serum samples were obtained from one 3-year-old dairy cow (a) treated twice with subcutaneous injections of 500 mg Lactotropin. This treatment was part of a sequential Lactotropin-steroid treatment schedule existing of three compounds in total. Of each compound, two subcutaneous injections were administered with 1 week interval. After each treatment, an adaptation period of 2 weeks was taken into account. Blood samples were collected daily during the week after each treatment. The second animal treatment study was according to commonly used rbST treatment conditions as recommended by the manufacturer: An adaptation period of 2 weeks was taken into account, and then the cow was treated every second week with 500 mg rbST, according to manufacturers' guidelines. Serum samples were obtained from one 3-year-old dairy cow (b). After blood collection, the blood sample was placed at room temperature for 4 h to coagulate. After coagulation, the samples were centrifuged for 10 min at 3000 x *g*, and serum was collected and stored at -20 °C until further use. The experimental procedure was authorized by the ethical committee of ID-DLO in Lelystad, the Netherlands.

2.3 Preparation of polyclonal antiserum

The preparation of polyclonal antiserum against Elanco rbST was described before by Heutmekers et al. [18]. Briefly, a New Zealand White rabbit (no. 58) was immunized with Elanco rbST at the Centre for Small Laboratory Animals in Wageningen, the Netherlands. Blood was obtained at various moments during the entire treatment period and serum was collected. The sera collected over the total treatment were pooled and stored at -80 °C for further use.

2.4 Ammonium sulphate purification of polyclonal antiserum

To concentrate the antibodies from the antiserum and to remove abundant proteins, first, the combined rabbit antiserum no. 58 was diluted three times with PBS (154 mM NaCl, 5.39 mM Na_2HPO_4, 1.29mMKH_2PO_4, pH7.4). Then, slowly, under constant stirring, an equal amount of saturated ammonium sulphate was added. Next, this solution was left at room temperature for 30 min without stirring. Subsequently, the solution was ultra-centrifuged for 10 min at 10000 x *g*, the supernatant was discarded and the pellet was re-suspended in PBS to restore the starting serum volume. Finally, the resuspended pellet was dialyzed against PBS for 24 h. The protein concentration was 6.6 mg mL^{-1} determined by the BCA protein assay according to the manufacturer's protocol.

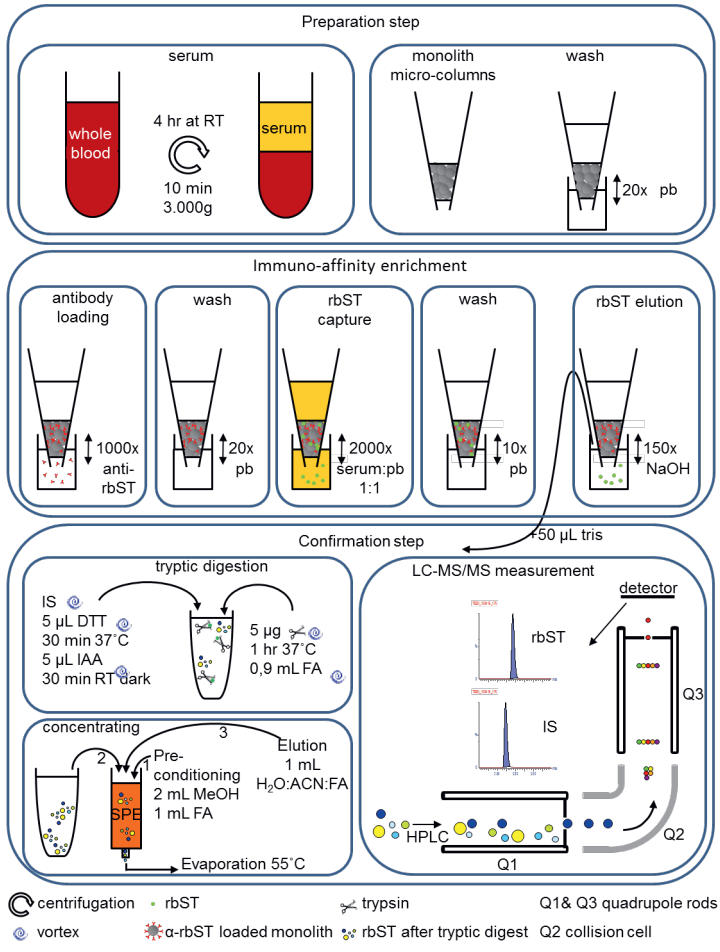


Figure 1. Workflow for serum preparation, enrichment with monolith micro-columns and LC-MS/MS measurement of rbST. SPE solid phase extraction, pb phosphate buffer, RT room temperature, FA formic acid, ACN acetonitrile.

2.5 Immobilization of purified antibodies on potein A and protein A/G monolithic micro-columns followed by rbST enrichment

Polyclonal anti-rbST ammonium-sulphate purified antibodies were immobilized to monolithic micro-columns loaded with, respectively, protein A or protein A/G by affinity binding. The pipet tips containing the protein A and protein A/G monolithic micro-columns were placed on the Finnpipette® Novus i multichannel, which is an automated device having a repetitive cycling function. First, the monolithic micro-columns were washed ten times with 150 µL 20 mM phosphate buffer pH 7. Adherent solution was removed

from the column by air pressure. Next, 75 µL 0.1 mg mL^{-1} of the ammonium-sulphate-precipitated polyclonal anti-rbST antibodies, resuspended in 20 mM phosphate buffer pH 7, was transferred over the column 1000 times. After the last cycle, adherent solution was removed from the column by air pressure. To remove unbound antibodies, the monolithic micro-columns were washed twice by 10 cycles of 150 µL 20mM phosphate buffer pH 7. It took 50 min to obtain these freshly prepared monolith micro-columns immobilized with ammonium-sulphate-precipitated polyclonal anti-rbST antibodies, which were directly used for rbST enrichment. For immuno-enrichment, 1 mL serum sample or spiked serum was diluted with 1 mL 20 mM phosphate buffer pH 7. The sample was transferred 2000 times (300 µL per cycle) through the anti-rbST-immobilized monolithic micro-column. The adherent sample was removed from the column by air pressure. To remove the remaining unbound sample, the monolithic micro-columns were washed ten times with 150 µL portions of 20 mM phosphate buffer. The captured rbST was then eluted from the monolith micro-column by 50 µL 200 mM NaOH (20 µL per cycle, 150 times). The eluate was collected, and 50 µL 50 mM Tris pH 7.9 was added before further use (final pH >10). Eluates were stored at -20 °C until tryptic digestion. The enrichment procedure took 4.5 h.

2.6 Digestion and cleanup of the immuno-affinity-purified extract

For digestion of the proteins with trypsin, the pH of the obtained solution was adjusted to 8-8.5 with 1 M HCl. After addition of 5 µL 45 mM DL-dithiothreitol (DTT), to reduce sulphur bridges, the solution was mixed and incubated for 30 min at 37 °C. The solution was cooled down to room temperature, and 5 µL 0.1 M iodoacetamide was added for methylation of the cysteine residues. The solution was mixed on a vortex and incubated for 30 min at room temperature in the dark. For protein digestion, 5 µg trypsin in 1 mM HCl (pH<3) was added, followed by 20 µL acetonitrile, mixed by vortex and incubated 1 h at 37 °C. Digestion was stopped by the addition of 0.9 mL 5 % formic acid. Then, an internal standard solution containing isotope-labelled bST peptide was added. This peptide is a replicate of the 17 amino acids of the N-terminal end of endogenous bST (AFPAMSLSGLFANAVLR) and only differs in the N-terminal amino acid from the rbST incorporation of alanine-$^{13}C_6{}^{15}N_4$, resulting in a total mass increase of 10 Da. The digest was concentrated on an Agilent Bond Elut Plexa SPE column (60 mg): After conditioning the column with 1 mL methanol and 1 mL 5 % formic acid in water, the sample was applied onto the column. Then, the column was washed with 1 mL 10 % acetonitrile and the sample was eluted with 0.5 mL water/acetonitrile/formic acid (25:70:5, v/v/v). The eluate was collected in 50 µL DMSO and evaporated to approximately 60 µL on a TurboVap at 55 °C under 10 psi N_2. Please note that the exact volume is not critical due to the use of an internal standard. After cooling to room temperature, 25 µL 5 % formic acid was added. The sample was mixed and transferred to an LC injection vial. The digestion and concentration procedure took in total 3 h.

2.7 LC-MS/MS analysis

Analysis was performed using an I-Class UPLC system connected to a Xevo TQS mass spectrometer Waters (Manchester, UK). Thirty microliters from the final extract was analysed by UHPLC-MS/MS in multiple reaction monitoring (MRM) mode. The chromatographic separation was performed on a Kinetex 50×2.10 mm I.D. 1.3 µm C_{18} (100 Å) column. The flow rate was set at 0.5 mL min^{-1}. A gradient was used starting with 75:25 (v/v) water/acetonitrile for the first 30 s, increasing to 70:30 (v/v) water/acetonitrile in the next 3 min. Then, the column was washed for half a minute with 100% acetonitrile. Total run time was 6 min 30 s. The mass spectrometer was operated in the positive ion ESI-MS/MS mode. Ion transitions m/z 913.1 > 774.1 and m/z 913.1 > 1047.6 were measured to detect the rbST specific N-terminal peptide with amino acid sequence, MFPAMSLSGLFANAVLR, after tryptic digestion [19]. To check the retention time of this N-terminal rbST peptide of interest, a synthetic analogue of the rbST peptide was injected at the beginning and the end of each series. For the bST internal standard, the transition m/z 888.1 > 779.13 was followed.

2.8 In house method validation

The decision limit CCα and the detection capability CCβ were determined according to the calibration procedure conform Commission Decision 2002/657/EC. Calculation of the concentration was performed by constructing a linear calibration curve of the response factor (peak area ratio of rbST fragment and internal standard) vs the concentration (expressed as absolute amount rbST protein). For intra-assay variation, four identical rbST spiked serum samples of respectively 2 and 10 ng mL^{-1} rbST in serum were analysed in parallel. Variation was determined and expressed as the percentage of the average. For determination of inter-assay variation, the spiked serum samples of 2 and 10 ng mL^{-1} were prepared, enriched with monolith micro-columns and measured on three different days. Variation was determined and expressed as the percentage of the average. For recovery of the immuno-affinity isolation, rbST-spiked serum samples were analysed and compared to rbST calibration curve in sodium hydroxide, as the latter is compatible with the elution conditions after immune-affinity rbST enrichment.

3 Results and discussion

3.1 Optimization rbST immuno-affinity enrichment

For the enrichment of the low abundant rbST from serum of dairy cattle (Figure 1), two monolith micro-columns were compared: a monolith micro-column prepared with protein A and a monolith micro-column prepared with protein A/G. Both protein A and protein A/G have a high affinity for polyclonal rabbit antibodies [17] and are expected to strongly interact with the rabbit anti-rbST used in this study. After rabbit anti-rbST immobilization on both monolith micro-columns, the protein A monolith micro-column was able to capture 10–20 % more rbST from spiked serum samples compared with the protein A/G

monolith micro-column. Therefore, the protein A monolith micro-column was used for further optimization steps. To obtain the highest recovery of rbST, the number of pipetting cycles, pipetting speed, antibody immobilization concentration and elution conditions were investigated with spiked serum samples. The number of pipetting cycles was determined to be 2000, taking both the efficiency of enrichment and time into consideration. The pipetting speed was found to be of great importance for both immobilization of the antibody on the monolith micro-column and for rbST capture from serum samples. To effectively immobilize the polyclonal anti-rbST antibody onto the monolith micro-column, the solution had to be transferred through the column with slowest pipetting rate as practically possible (approximately 63 µL s^{-1}). Otherwise, immobilization of the antibody was not sufficient and no rbST was captured. The same result was obtained for the transfer of serum samples over the monolith micro-column to enable rbST capture: rbST could only be detected when the serum was transferred slowly through the micro-column (approximately 77 µL s^{-1}). Next, the concentration of anti-rbST antibody used for immobilization to the monolith micro-columns was optimized, aiming for the highest yield of rbST after enrichment. For this, anti-rbST was applied to the column with concentrations of 0.1, 0.07, 0.04 and 0.01 mg mL^{-1} in 20 mM phosphate buffer pH 7. The immobilization concentration of 0.1 mg mL^{-1} showed best rbST capture capacity respectively 2, 3 and 7 times more rbST was captured using 0.1 mg mL^{-1} compared with the other concentrations. Higher concentrations of the antibody were not tested as immobilization with 0.1 mg mL^{-1} antibody was capable to enrich rbST in serum at concentrations in the low nanogram per milliliter range, sensitive enough for incurred serum samples. The binding sites of the micro-columns prepared under these conditions were found to be saturated at a serum rbST concentration of $>$50 ng mL^{-1}, which is at least 50 times higher than the expected levels of rbST in treated cows (Figure 2). For the elution of captured rbST, conditions compatible with the subsequent trypsin digestion are preferred to simplify the workflow. Trypsin digestion compatible buffers are for instance tris and ammonium bicarbonate. These buffers were tested in different molarities for their capacity to elute rbST from the monolith micro-columns. Unfortunately, these conditions are too mild, only eluting 10 % or less rbST from the monolith micro-columns in comparison with harsher elution conditions. A solution of 200 mM NaOH was found to be most effective for elution of rbST from the micro-columns. After elution, a tris solution was added to obtain a trypsin compatible solution. In Figure 1, an overview of the entire workflow developed is given. In short, after enrichment of rbST with monolith micro-columns from serum supernatant, tryptic digestion yields 20 different peptides. The endogenous and recombinant protein differs by one amino acid located at the N-terminal side of the protein. In case of rbST, an alanine is replaced by a methionine. To discriminate between these two forms, the N-terminal peptide is analysed. To be sure that the detected N-terminal peptide is specific, a blast computation was performed at the SIB using the BLAST network service [20]. There were no other peptides found containing the same amino acid sequence as the detected peptide. The addition of the isotope-labelled N-terminal peptide of bST as an internal standard allows to correct for sample cleanup losses after tryptic digestion.

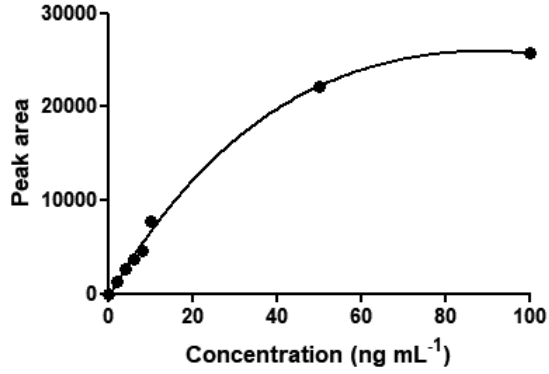
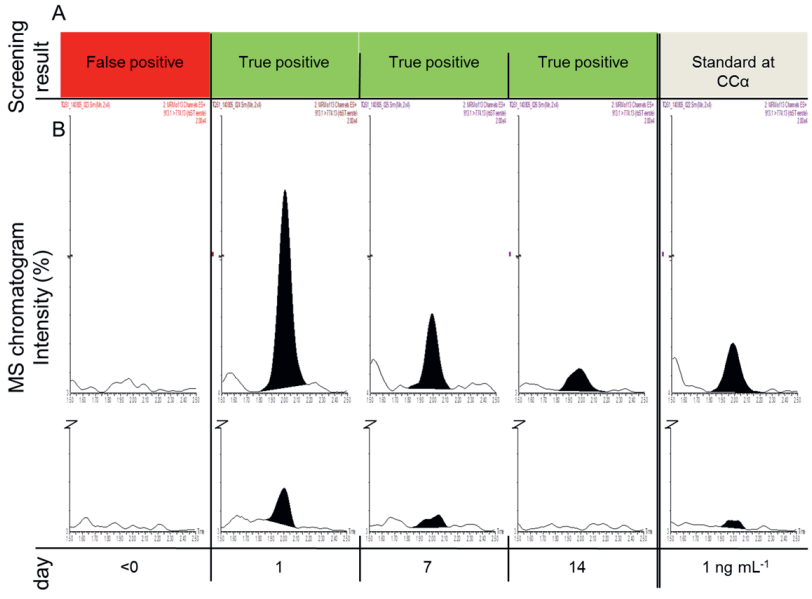


Figure 2. Peak areas obtained with LC-MS/MS transition *m/z* 913.1 > 774.13 after enrichment of rbST in spiked serum samples at: 0, 2, 4, 6, 8, 10, 50 and 100 ng mL^{-1}. The observation of a plateau beyond 50 ng mL^{-1} indicates saturation of the binding sites of the micro-columns.

3.2 Method performance characterization

To characterize the method performance, the developed method was partly validated in-house as a quantitative confirmatory method according to Commission Decision 2002/657/EC [10]. The parameters considered were the decision limit (CCα), the detection capability (CCβ), the intra-assay variation, the inter-assay variation and the recovery. Ion ratio of the measured transition determined for samples from the matrix matched sample (MMS) series and samples from rbST-treated animals were all within the ion ratio limits, as described in the Commission Decision 2002/657/EC [10]. Important parameters for performance characterization are (i) the decision limit (CCα), the limit at and above which it can be concluded, with an error probability of α, that a sample is non-compliant; (ii) the detection capability (CCβ), the smallest content of the substance that may be detected, identified and quantified in a sample with an error probability of β; (iii) the intra-assay variation; (iv) the inter-assay variation; and (v) the recovery. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to truly detect contaminated samples with a statistical certainty of 1-β. Data obtained from the validation study are presented in Table 1. The CCα and CCβ were determined to be 0.8 and 1.6 ng mL^{-1}, respectively. From additional samples spiked with rbST in concentrations of 0.25, 0.5 and 1 ng mL^{-1}, it was concluded that the obtained CCα and CCβ are realistic,as the 0.5 and 1 ng mL^{-1} samples still meet the ion ratio criteria (see also the reconstructed ion chromatogram of the rbST-spiked serum sample at 1 ng mL^{-1} in Figure 3).



Ion ratio MMS (%) (range: 12.0 – 22.3)	-	18.7	20.2	-	14.7
Retention time MMS (min) (range: 1.94 – 2.04)	-	2.00	1.99	1.98	1.98
Confirmation result (Y/N)	N	Y	Y	N	Y

Figure 3. A; Top bar shows the results of the screenings analysis, B; LC-MS/MS confirmation of biomarker screening results. Chromatograms of transition m/z 913.1 > 774.13 and m/z 913.1 > 1047.6 after enrichment of rbST in serum in (from left to right): a serum sample prior to treatment (day 0), a serum sample taken the first day of treatment (determined to contain 9.0 ng mL^{-1} rbST), a serum sample taken 7 days after treatment (determined to contain 1.9 ng mL^{-1} rbST), a serum sample taken 14 days after treatment (determined to contain <1 ng mL^{-1}) and a serum sample spiked with 1 ng mL^{-1} rbST. All y-axes are scaled to the serum sample of the first day of treatment. Below the chromatogram, the compliance with the ion ratio and retention time criteria is indicated (the ion ratio and retention time interval as determined according to the 2002/657/EC are given in brackets).

Moreover, in 75% of blank serum samples spiked at CCα (0.8 ng mL^{-1}, rbST presence was confirmed, which is actually better than the >50% at CCα level as required by 2002/657/EC. Intra- and inter-assay variations were determined for rbST in serum at a concentration of 2 and 10 ng mL^{-1}, in accordance with expected serum concentrations. Variation was expected to be higher at 2 ng mL^{-1} compared with 10 ng mL^{-1}. Although this difference was indeed observed in both intra- and inter-assay variation, intra-assay

variation was found to be only 12% at 2 ng mL^{-1} and 9% at 10 ng mL^{-1} rbST in serum (Table 1). It should be noted that for 10 ng mL^{-1}, one of the four data points was removed as outlier due to the fact that the internal standard was not added correctly. Recovery was determined by comparison of calibration curves prepared in either elution solvent or by fortification of serum. The graphs in Figure 4 shows that not all rbST from the fortified serum samples will be captured and eluted using the monolith micro-columns.

Table 1. In-house validation study to characterize the method performance using monolith micro-columns loaded with protein A, tryptic digestion and LC-MS/MS analyses.

Validation results		
CCα		0.8 ng mL^{-1}
CCβ		1.6 ng mL^{-1}
Intra-assay variation	2 ng mL^{-1}	12 % (n = 4)
	10 ng mL^{-1}	9 % (n = 3)
Inter-assay variation	2 ng mL^{-1}	15 % (n = 3)
	10 ng mL^{-1}	10 % (n = 3)

Comparison of the response factors of the two calibration curves suggests a recovery during immuno-enrichment of approximately 50 % for concentrations up to 10 ng mL^{-1}. For the two highest concentrations, 50 and 100 ng mL^{-1}, the recovery of rbST dropped to 25 and 17%, respectively, due to saturation of the binding sites of the monolith micro-column (Figure 2). Even though not all rbST is recovered, the repeatability and reproducibility data in Table 1 are fit for purpose and the sensitivity required for effective control is reached. To test the stability of the trypsin-digested serum, samples were stored for 2 weeks at 4-8 °C. Comparison of the samples before and after storage showed a decrease in peak area of approximately 50% for all samples (results not shown). The decrease in intensity of the measured N-terminal peptide can be explained by its instability and tendency to adsorb to glassware [21]. Although 50% is a significant loss at 4-8 °C, it did not obstruct measurement and quantification of rbST in the extracts. It is therefore advised to store digested samples and matrix-matched calibrants at 4-8 °C as short as possible prior to analysis. Detection of rbST in bovine serum has already been presented before by Le Breton et al. [5]. In that work, the lowest presented rbST concentration in bovine serum was 3 ng mL^{-1} (data acquired on only one transition). To reach that level, an extensive sample preparation of multiple precipitation steps and overnight digestion was needed. In contrast, the method presented in this study shows high sensitivity (CCα of 0.8 ng mL^{-1}) and the confidence of data acquisition of two ion transitions. Note that using a single-ion transition, rbST in bovine serum with a concentration of 0.25 ng mL^{-1} could even be detected. Moreover,sample preparation is less extensive, less laborious and semi-automated, and tryptic digestion required only 1 h.

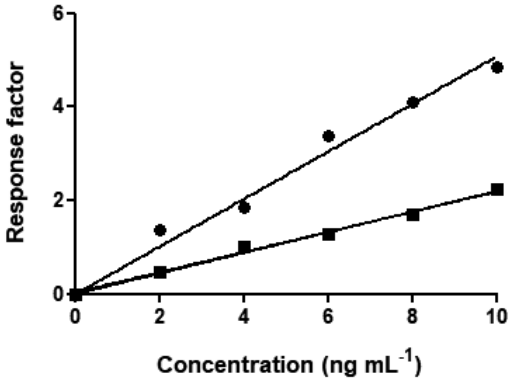


Figure 4. Calibration curves with respectively 0, 2, 4, 6, 8 and 10 ng mL^{-1} of rbST spiked in NaOH (●) to simulate enrichment elution conditions and after immuno-affinity enrichment of fortified serum samples (■).

3.3 rbST analysis in serum of rbST-treated cows

To investigate applicability of the developed method to real-life samples, serum samples from two different animal experiments were analysed. As a proof of principle, from the first animal experiment, 17 serum samples of one treated cow were analysed: two serum samples prior to treatment (t=<0 days), one serum sample for each day after the first and second treatment (t=1-14 days) and one sample 3 weeks after the second treatment (t=29 days). This allowed exploring the detectability of rbST during and after the treatment period. Obtained rbST concentrations, corrected for incomplete recovery, are given in Table 2 and show that rbST can be detected from the first day after the first treatment until 7 days after the second treatment. Only in one serum sample, taken on day 2, no rbST seemed to be present. It is not clear why no rbST was detected in the sample that day. The highest concentration of rbST (21.4 ng mL^{-1}) was detected on the first day after the first treatment. On the first day after the second treatment, an increase in rbST was observed as well, although less apparent. In general, a daily variation in rbST concentration was observed and rbST concentrations decreased only slowly. This suggests the ability of the slow-releasing formulae to release rbST slowly to the blood circulation, with a peak on the first day(s), and maintain a minimum level. Twenty-one days after the last treatment, no rbST could be detected anymore, which is in good agreement with the need of a two-weekly treatment, as advised by the manufacturer [12]. This treatment schedule was applied in the second animal study. Analysis of samples from this study gives insight in the ability of the method to detect rbST use under realistic treatment conditions. Four serum samples were analysed: one serum sample prior to treatment (t=<0 days) and serum samples taken after 1 day (t=1 day), 1 week (t=8 days) and 2 weeks (t=14 days) after the third treatment. In addition, three additional blank serum samples from other animals were analysed. No rbST was detected in any of the five blank serum samples,

and no interferences are observed that could lead to false positive findings. This implies that the method is very specific, though analysis of more non-treated animals is needed to statistically prove these findings. From the treated cow (b), the trend of the determined rbST concentrations is similar to the first animal experiment. The highest concentration was found on the first day after administration, and rbST could be detected until 1 week after administration (see Table 2 and Figure 3).

Table 2. RbST concentrations found in serum samples of two dairy cows, a and b treated with rbST, quantified versus MMS

cow	a	b
day	Concentration rbST (ng mL^{-1})	Concentration rbST (ng mL^{-1})
<0	< CCα	< CCα
1	20.3	9.0
2	< CCα	-
3	4.8	-
4	4.5	-
5	6.7	-
6	2.3	-
7	1.4	-
8	1.8	1.9
9	9.1	-
10	3.0	-
11	2.8	-
12	1.8	-
13	4.0	-
14	2.1	< CCα
29	< CCα	-

The difference in concentration on the first day after treatment between the two cows from the two animal experiments can be explained by natural variation in response, which should be investigated by analysis of more treated animals. For the serum sample taken 2 weeks after rbST administration, just prior to the following administration, a peak is clearly visible at transition m/z 993.1>m/z 774.13 and suggests the presence of rbST (Figure 3). However, the concentration is lower than the CCα of the method, and for confirmation of rbST according to Commission Decision 2002/657/EC, a concentration larger than CCα and a peak at a second transition are necessary. Further enhancement of the sensitivity may be obtained by increasing the sample volume. It is expected that

by this adjustment, rbST presence can not only be detected but also confirmed up to 14 days after rbST administration. The results compare favorably with previous methods [5], in which rbST was only detected until 4 days after treatment.

Confirmation of positive screening results

Control strategies in food and feed safety often include two steps: First, samples are screened in order to obtain a fast indication of the suspect samples, thereby reducing the number of samples, as samples with a negative result will not be investigated further. The second step in the control strategy is the confirmation of positive samples conform Commission Decision 2002/657 with additional (analytical) methods [10]. This two-step strategy was applied to the four samples from the second animal study. The samples were previously screened by Ludwig et al. [8] by a multiple protein biomarker assay, where four different biomarker proteins were measured simultaneously. The serum samples were selected for analysis with the LC-MS/MS method as they were considered suspicious for rbST in the screening assay (Figure 3A). The sample taken prior to treatment (t=<0 days) was screened suspect, which is obviously unlikely and might serve as a false-positive case. Analysis of the serum sample prior to treatment (t=<0 days) with our confirmatory method showed that, indeed, despite the positive screening result, no rbST-specific peptide could be detected and rbST was below our detection limit (Figure 3B). It can therefore be concluded that the screening result of this sample, taken before treatment, is false positive and underlines the necessity of confirmatory methods. The false-positive screening result was most likely due to the apparent presence of rbST-induced antibodies as was observed in less than 5% of the untreated cows and is most likely the result of non-specific interactions of other antibodies or proteins in the screening assay [22]. For the other three serum samples, taken during rbST treatment according to the treatment schedule, screening results were found to be true positive. As shown in Figure 3B by the chromatograms of transition m/z 913.1 > 774.13 and m/z 913.1 > 1047.6, samples taken during the rbST treatment all showed rbST presence. Samples taken after treatment of dairy cows with rbST (t-=>0 days), which were found positive in the screenings assay, were confirmed for the presence of rbST with the developed confirmatory LC-MS/MS method (Figure 3). In addition, the need of a reliable confirmatory method for samples found positive during screening was proven by the example of a false-positive screening result that could only be identified by the LC-MS/MS analysis. Please note that no explicit confirmation criteria have been established yet for protein and/or peptide analysis by targeted MS/MS.

4 Conclusion

In this study, a novel approach to pinpoint rbST abuse has been developed based on rbST enrichment by immunoaffinity on monolith micro-columns. High sensitivity is reached with a CCα of 0.8 ng mL^{-1}. The intra- and interassay variations were determined to be <10% at 10 ng mL^{-1} and <18% at 2 ng mL^{-1}. Applicability of the confirmatory method was demonstrated by analysis of serum samples from a treated animal for which positive

screening results were obtained. With the developed approach, we quantify rbST with a detection window of the total treatment period of 14 days and confirm its presence for over 1 week after treatment. It is therefore that, for the first time, to the best of our knowledge, an approach is presented that successfully proofs rbST abuse under commonly used treatment conditions.

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Chapter 4

Microsphere peptide-based immunoassay for the detection of recombinant bovine somatotropin in injection preparations

Adapted from: Smits, N.G.E., Bovee, T.F.H., Pujari, S.P., van Ginkel, L.A., Nielen, M.W.F and B. Albada. Microsphere Peptide-Based Immunoassay for the Detection of Recombinant Bovine Somatotropin in Injection Preparations. *Biosensors* **2022**, 12, 3: 138.

Abstract

The use of peptides in immunoassays can be favored over the use of the full protein when more cost effective or less toxic approaches are needed, or when access to the full protein is lacking. Due to restricted access to recombinant bovine somatotropin (rbST), a protein enhancing growth and lactating performances of livestock, which use has been banned in the EU, Canada and Australia (amongst others), we developed a peptide-based biorecognition assay on an imaging planar array analyzer. For this, we identified the rbST epitope that is responsible for binding to the rbST-targeting monoclonal antibody 4H12 (MAb 4H12) to be $_{115}$DLEEGILALMR$_{125}$. This linear peptide was synthesized and coupled to microspheres, after which it was tested in a biorecognition competitive inhibition assay format. We observed IC_{50} values of approximately 0.11 µg mL^{-1}, which are lower than observed for the full rbST protein (IC_{50} = 0.20 µg mL^{-1}). Importantly, there was no binding with the scrambled peptide. Preliminary results of directly coupled peptides in a microsphere biorecognition assay for detection of rbST are presented. Real-life applicability for detection of somatotropins (STs) in injection preparations of bovine-, porcine- and equine ST are shown. This newly developed immunoassay strongly supports future developments of peptide-based immunoassays to circumvent the limited access to the full protein.

1 Introduction

When immunoassays detect a full protein, the use of this full protein in assay building is commonplace. However, when access to the full protein is limited as shown in this study, where the regulatory ban in Europe and other countries limits access to recombinant bovine somatotropin (rbST), the use of protein-derived peptides offers advantages. In general, the use of peptides offers advantages in (i) cost effectiveness, (ii) toxicity, (iii) pathogenicity, (iv) specificity, and (v) limited access to the full protein. For instance, in the development of serological tests for COVID-19, the high costs for the S1-protein could be avoided using a 12-mer peptide, while at the same time showing increased specificity, resulting in an assay with high diagnostic performance [1]. In development of neutralizing antibodies for toxin therapy of, e.g., venoms, bacteria toxins, etc., antigenic peptides are used instead of the toxic full protein [2-4]. Antigenic peptides can also be used in peptide-based detection systems, for example, to avoid the use of infectious viruses and toxins as antigen in an immunoassay [5-7]. In diagnosing food allergy, peptide-based assays provide insights on epitope-specific antibody binding [8,9], which is related to pathogenesis, its prognosis and resulting treatment options of the disease [10]. In this study, we show the specific advantage of peptides when access to the full protein rbST is limited. RbST is a 191 amino acid long protein hormone that enhances growth and lactating performance in cattle. When administered to dairy cows according to the manufacturer's protocol, milk production can increase up to 25% [11]. The use of rbST for milk-enhancing properties is approved in several countries, but is banned in the European Union [12]. To control this ban, a strategy to detect rbST misuse in the EU has been described by Smits et al. [13]. The strategy for rbST in serum and milk from dairy cows is based on the digestion of the proteins and detection of rbST-specific peptides formed using an extensive dedicated LC-MS/MS method. To distinguish between farms suspected and not-suspected of rbST misuse, a straightforward immunochemical method measuring rbST induced-antibodies can be performed as an initial screening procedure. To measure the rbST-induced antibody expression, the full rbST protein is attached to a variety of surfaces, e.g., microspheres for suspension arrays [14,15], 96-well plates in ELISA [16,17] and glass chips in microarrays [18], enabling detection by binding to the immobilized rbST [19]. As these assays rely on the banned full rbST protein, they suffer from limited accessibility and applicability. Moreover, promising immunoassays for rbST detection itself, use the temporary available polyclonal antibodies [20,21], and others only demonstrated rbST detection in buffer [22] or show antibodies lacking specificity [23]. To avoid the dependence on the full protein, or an antibody source which can be exhausted, we developed an immunoassay that is built on a specific peptide part to represent the full protein and a monoclonal antibody, which can be produced indefinitely. First, we performed an epitope mapping of rbST using both linear and conformational epitope mimics [24] in an ELISA-based format [25]. After identification of suitable peptide epitopes, we synthesized the relevant peptides, and scrambled versions thereof, and covalently immobilized them to a carboxyl-rich surface of microspheres using an EDC/NHS protocol [26-30]. This enabled us to develop a suspension-based format that uses immobilized peptides for the detection of rbST.

2 Experimental

2.1 Materials and instruments

The rbST protein standard was a gift from the National Hormone & Peptide Program (NHPP) of Dr. Parlow (Torrance, CA, USA) and equine somatotropin (eST) was from Bresagen (Adelaide, Australia). We obtained Posilac® (methionine-rbST) sometribove zinc suspension for injection, and excipient (sesame oil and aluminum monostearate) for injection from Elanco (Indianapolis, IN, USA), and Hilac® (alanine-rbST) was obtained from LG (Seoul, Korea). The hybridoma cell line producing monoclonal antibody (MAb) 4H12 (IgG2b, κ) was developed and produced by Genscript (Leiden, The Netherlands). Chemicals for peptide synthesis were obtained from the following commercial sources: 1-hydroxybenzotriazole hydrate from Acros (Geel, Belgium); PyBOP, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Met-OH, Fmoc Arg(Pbf)-OH, Fmoc Asp(*Ot*Bu)-OH, Fmoc-Leu-OH, Fmoc-Glu(*Ot*Bu)-OH, Fmoc-Gly-OH and Rink Amide MBHA resin HL were purchased from Novabiochem (Zwijndrecht, The Netherlands); HBTU, dichloromethane, N,N-dimethylformamide and 2-propanol from BioSolve (Valkenswaard, The Netherlands). Acetic anhydride, trichloroacetic acid, sodium dihydrogen phosphate monohydrate, sodium chloride, sodium hydroxide, sodium azide, tween-20 and hydrochloric acid were supplied by Merck (Zwijndrecht, The Netherlands). Porcine Somatotropin (pST), human Somatotropin (hST), bovine serum albumin (BSA), 2-(N-morpholino) ethanesulfonic acid (MES hydrate), sodium phosphate dibasic, boric acid, N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and N-(3-methylaminopropyl)-N-ethylcarbodiimide (EDC) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Trifluoracetic acid and N,N-diisopropylethylamine were purchased from Fisher Scientific (Landsmeer, The Netherlands). The analytical LC column ZORBAX Eclipse XDB-C18, the preparative column PreptHT XDB-C18, 21.2 mm I.D. × 250 mm, the Agilent model 1260 Infinity II LC system and the LC/MSD were all supplied by Agilent (Amstelveen, The Netherlands). Triisopropylsilane and ninhydrin were obtained from TCI (Zwijndrecht, Belgium) and Fmoc-ε-Aca-OH and Boc-mini-PEG-OH from Peptides International (Louisville, KY, USA). Drive fluid, paramagnetic color-coded microsphere sets 012, 043, 038, 029, 073 and 064 and the planar microsphere array analyzer (MAGPIX) running on XPONENT software were from Luminex (Austin, TX, USA) and Phycoerythrin (PE)-labeled goat anti-mouse immunoglobulins (GAM-PE) were from Moss (Pasadena, MD, USA). Protein Lobind Tubes were supplied by Eppendorf (Hamburg, Germany) and the Snijder test tube rotator was from Omnilabo International (Breda, The Netherlands). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK) and the magnetic particle concentrator DynaMag™-2 was from Invitrogen Dynal (Oslo, Norway). The ultrasonic cleaner was obtained from VWR International (Amsterdam, The Netherlands) and the centrifuge was from Hermle (Wehingen, Germany). The DS-11 series spectrophotometer was from Denovix (Wilmington, DE, USA).

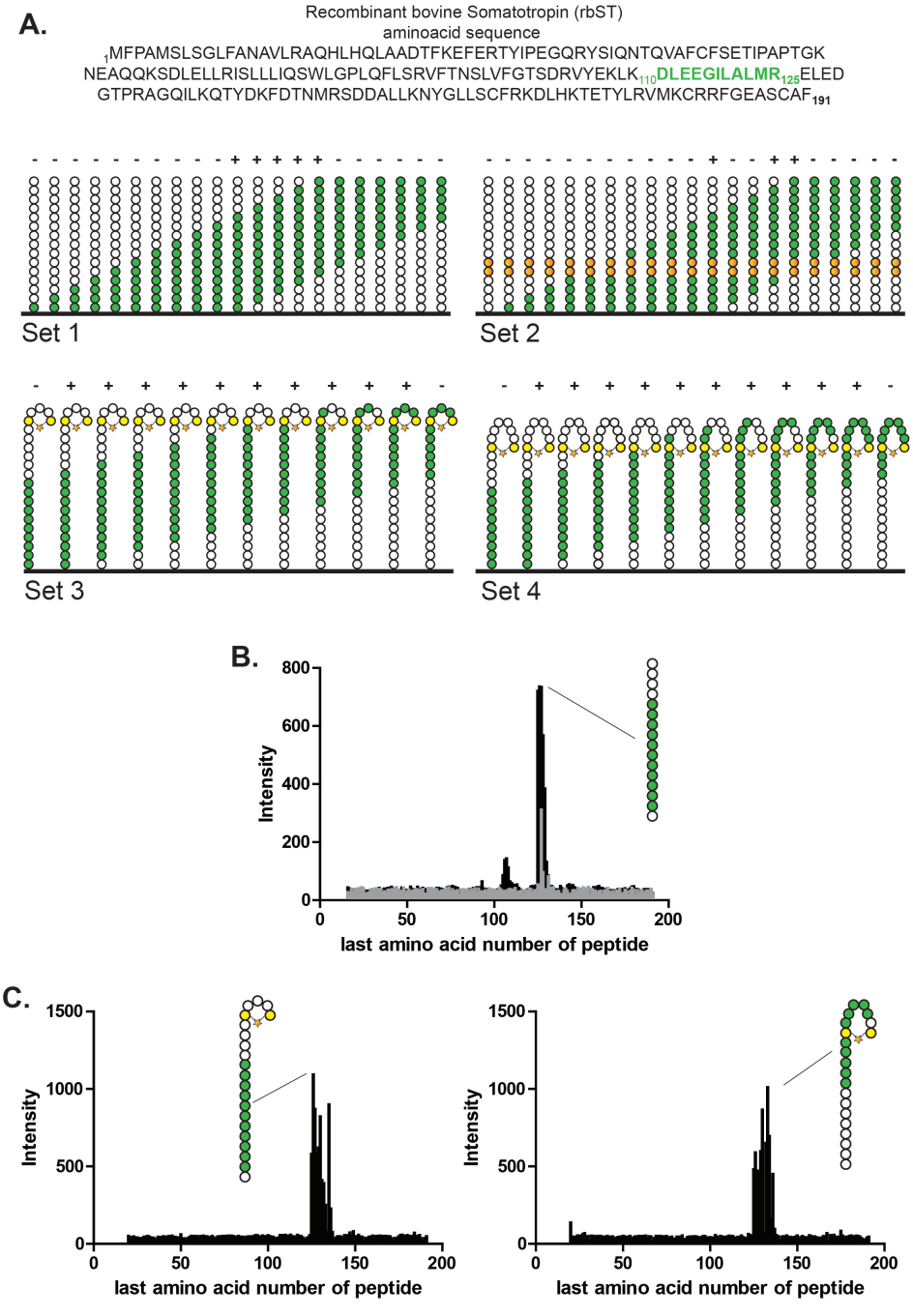
A.
Recombinant bovine Somatotropin (rbST)
aminoacid sequence
1MFPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPEGQRYSIQNTQVAFCFSETIPAPTGK
NEAQQKSDLELLRISLLLIQSWLGPLQFLSRVFTNSLVFGTSDRVYEKLK110DLEEGILALMR125ELED
GTPRAGQILKQTYDKFDTNMRSDDALLKNYGLLSCFRKDLHKTETYLRVMKCRRFGEASCAF191
Set 1
Set 2
Set 3
Set 4
B.
Intensity
800
600
400
200
0
0
50
100
150
200
last amino acid number of peptide
C.
Intensity
1500
1000
500
0
0
50
100
150
200
last amino acid number of peptide
Intensity
1500
1000
500
0
0
50
100
150
200
last amino acid number of peptide

Figure 1. Schematic representation of the four epitope mapping approaches to identify peptide-based mimics of recombinant bovine somatotropin (rbST) that bind to the monoclonal antibody 4H12. (A) The entire rbST amino acid sequence is shown at the top. In the schematic representation of the peptides, only a smaller number of peptides, including the binding epitope, is shown for clarity purposes. Set 1 represents linear peptides of 15 aa with an offset of 1 aa in each subsequent peptide; set 2 represents the same linear peptides as used in set 1 with the 10th and 11th amino acids replaced by alanine or glycine (in case a native alanine is present) (orange beads); set 3 represents peptides of 21 aa with an offset of 1 aa that mimic an textalpha-helical loop with the first and fifth aa replaced by cystine residues (yellow beads), which are connected to an MP2 clip (star); set 4 is similar to set 3, with this exception that the cysteine amino acids are placed on the first and eighth aa. The binding epitope is presented in green in both the sequence of rbST and, schematically, in the four sets. The presence of a potential binding epitope for the antibody is schematically presented by a '+', and absence of binding is presented by a '-'. (B) Intensity results for epitope mapping of monoclonal antibody 4H12 for the rbST protein sequence; binding of antibody 4H12 to 15-mer linear peptides (set 1, black) and peptides subjected to a double-Ala scan (set 2, grey). (C) binding of antibody 4H12 to 21-mer sequences that contain short and extended textalpha-helical turn mimics (set 3, left graph, and set 4, right graph). The highest intensity is schematically represented in the respective graph, the green beads in the schematic depiction represents the binding epitope.

2.2 Epitope mapping

The entire sequence of rbST (Figure 1A) was epitope mapped for antibody 4H12 by Pepscan Presto BV (Lelystad, The Netherlands), as described before [31]. Briefly, the rbST sequence was derived from the endogenous bST sequence by replacing the N-terminal alanine (Ala) with a methionine (Met) residue. With this rbST sequence, four sets of peptides were produced and used to determine the binding epitope of MAb 4H12: (i) linear peptides of 15 amino acids in length with an offset of one amino acid (set 1), (ii) linear peptides similar to set 1, with the amino acids on place 10 and 11 replaced by alanine (Ala); a native Ala present on place 10 and/or 11 was replaced by glycine (Gly) (set 2), (iii) α-helical loop mimics of 21 amino acids in length with an offset of one amino acid and cysteine residues on place 1 and 5 to enable connection with an α-helix-inducing mP2 chemical linkage of peptides onto scaffolds (CLIPS, set 3) and (iv) α-helical loop mimics similar to set 3, with the two Cys residues placed on amino acid numbers 1 and 8, which were also linked with mP2 CLIPS (set 4). Binding of Mab 4H12 to the described peptides is quantified using an ELISA-type read out.

2.3 Peptide synthesis

Three peptides from the epitope mapping were selected and synthesized: DLEEGILALMR (pep), DLEEGILALMRK (pep-K) and MRIEGLADLEL (pep-scr) (Figure 2). For this, 1

mmol of the Fmoc-protected amino acids were weighted separately and placed in the synthesizer. Peptides were built on 0.25 g of Rink Amide resin (loading capacity of 0.6–1 g $mmol^{-1}$, 3:1 amino acids:resin) by standard Fmoc synthesis. To start, the resin was pre-swelled for 3 min in DMF. For Fmoc-removal, the resin was washed two times for 30 s with DMF followed by incubating 1 × 5 min and 1 × 20 min with 20% piperidine in DMF. The piperidine solution was removed by washing for 2 × 30 s with DMF, 1 × 30 s with DCM and 3 × 30 s with DMF again. The next Fmoc-protected amino acid was activated by 2.5 mL 0.4 M HBTU and 2.5 mL 0.8 M DIPEA in DMF for 3 min, which was then added to the resin and incubated for 90 min. This procedure was repeated until the desired peptide sequence was generated. When desired, the peptide was functionalized in the last step before cleavage from the solid support. Specifically, pep-K was acetylated on the N-terminus using 4.7 mL Ac_2O, 230 mg HOBT•H_2O and 2.2 mL DPEA in 100 mL DMF, followed by washing with DMF and DCM (each three times for 2 min, with 2 mL of solvent).

Figure 2. Schematic overview of the magnetic microspheres with their respective microsphere numbers (pink beads). Individual amino acids are presented as a green bead and single letter amino acid code. Abbreviations of the coupled peptide or protein as used in this manuscript are mentioned next to the schematic presentation; linear peptide (pep), linear peptide with extra lysine (pep-K); linear peptide with peg spacer (pep-peg); linear peptide with aca spacer (pep-aca); linear peptide scrambled (pep-scr); total recombinant bovine somatotropin protein (rbST).

For the peptides that contained a spacer, the Fmoc-group was removed and the spacer was coupled to the N-terminus using the coupling procedure mentioned above, but now

with Boc-protected aminocaproic acid (aca) or Boc-protected mini-PEG-COOH (peg). After modification, peptides were disconnected from the resin by incubation with 2 mL 95% TFA, 2.5% triisopropylsilane and 2.5% water (4 h). Each peptide was collected in a tube and precipitated by the addition of diethylether (-20 °C) and centrifuged for 5 min at 3800 x *g*. Then, the supernatant was removed and the pelleted peptide was dried by air at room temperature in the fume hood.

2.4 Peptide purification

Peptide purification was performed using a preparative LC system connected to a single quadrupole mass spectrometer (MS) for mass confirmation of the synthesized peptides. A total of 500 µL from the prepared peptide solution was injected onto the preparative column. The flow rate was set at 20 mL min^{-1} and 0.1% formic acid was added to the mobile phases. A gradient was used starting with 90:10 (*v*/*v*) water/acetonitrile, increasing to 50:50 (*v*/*v*) water/acetonitrile in 14 min. This mobile phase composition was kept constant for 3 min. The next minute the gradient was returned to starting conditions of 90:10 (*v*/*v*) water/acetonitrile and this was kept constant for 7 min. Total run time was 25 min. The MS was operated in positive electrospray ionization mode. Masses of synthesized and modified peptides of interest, purified by HPLC, were verified by MS and fractions containing peptides were collected manually. $[M+2H]^{2+}$ ions at *m/z* 630, *m/z* 715, *m/z* 702.5, *m/z* 686.5 and *m/z* 630 were found for, respectively, pep, pep-K, pep-peg, pep-aca and the scrambled peptide pep-scr, in accordance with the expected theoretical values (Supplementary Materials, Figure S1). After purification and fraction collection, the synthesized peptides were freeze dried for further use.

2.5 Microsphere preparation for microsphere peptide-based immunoassays (MIPA)

Microsphere preparation was executed according to Bremer et al. [32] with the exception that Magplex® microsphere sets instead of seroMAP microspheres were used. During the procedure, microspheres were trapped using a magnet (1 min) and after removal of the supernatant, trapped microspheres were resuspended by vortexing (1 min). The peptides pep, pep-K, pep-peg, pep-aca and rbST standard were coupled to the different internally dyed microsphere sets, i.e., microsphere set numbers 012, 043, 029, 038 and 064, respectively (Figure 2). The internal dye gives the microsphere set a unique spectral signature, and consequently, a unique read-out region. For each microsphere set, 2.5×10^6 microspheres were covalently coupled with a two-step carbodiimide reaction using 500 µL of 100 µg mL^{-1} peptide in boric acid solution (100 mM boric acid, 1M NaCl, adjusted to pH 8.3 with NaOH), and 100 µg mL^{-1} rbST in MES buffer (50 mM MES pH 5.0). After coupling, the microspheres were stable for over 1 year when stored in a blocking buffer (PBS, 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3) at 2-8 °C in the dark until use, with the exception for the microspheres measured with XPS, which were stored in Milli-Q water until use.

2.6 Inhibition MIPA procedure

In this procedure, all dilution and washing steps were executed using PBST containing 0.1% BSA. In total, 100 µL of rbST, eST, pST and hST standard solution or injection preparation extract were added to a low bind 96-well microtiter plate. Next, 10 µL of 20-, 2-, 2-, 1- and 0.7 ng mL^{-1} antibody for microspheres coated with, respectively, pep, pep-peg, pep-aca, k-pep and rbST and microspheres (10 µL diluted suspension containing about 1250 microspheres per microsphere set) were added to each well. The microtiter plate was incubated for 20 min on a microtiter plate shaker to allow competition between the immobilized rbST epitope peptides or protein on the microspheres and the somatotropin (ST) present in the solution for the available antibody binding sites. After incubation, the microspheres were trapped by a magnet and washed twice. After washing, a 125 µL, 625-times diluted PE labeled Goat anti Mouse antibody was added and incubated for 20 min on a microtiter plate shaker. After this incubation step, microspheres were trapped with a magnet, supernatant removed and 125 µL PBST containing 0.1% BSA added. The plate was briefly mixed on the microtiter plate shaker before measuring on the imaging planar array analyzer (MAGPIX). The MIPA procedure for the detection of somatotropins is summarized in Figure 3.

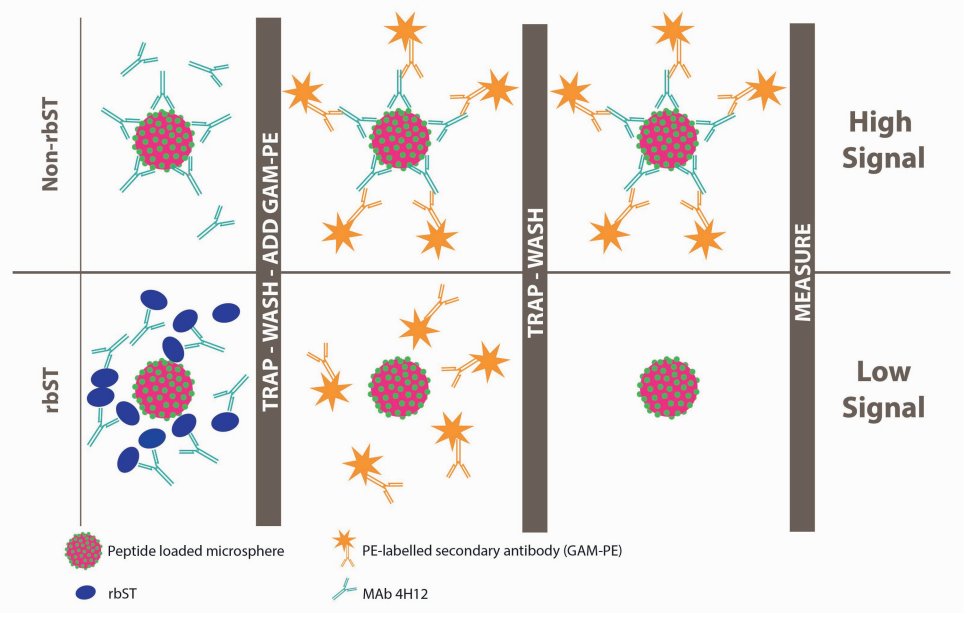

Figure 3. Summarized workflow and assay principle of the indirect competitive assay format for somatotropins. In absence of rbST (upper part), MAb 4H12 binds to the peptide-coated microspheres, which enables binding of the PE coupled secondary antibody. The presence of PE results in a high signal in the imaging planar array analyzer. In presence of rbST (lower part), MAb 4H12 binds to the rbST in solution. This results in no binding

of MAb 4H12 to the peptide-coated microspheres, and therefore, no binding of PE coupled secondary antibody. As no PE is present, this will result in a low signal in the imaging planar array analyzer.

2.7 Extraction of rbSTs of injection preparations

Proteins were extracted from syringes containing methionine-rbST, alanine-rbST and an excipient according to Heutmekers et al. [20]. Briefly, 5 mL CAPS buffer (50 mM CAPS, 100 mM NaCl pH11) was added to 100 mg injection preparation and the mixture was vortexed (1 min), sonicated (10 min) and centrifuged for 10 min at 2000 × *g*. The buffer layer was separated from the white layer on top (slow-release formula) and filtered through a 5 µm filter. Protein concentrations were measured with a spectrophotometer.

2.8 X-ray photoelectron spectroscopy (XPS)

Prior to XPS analysis, modified microspheres (in Milli-Q) were dropcasted on a plasma-cleaned piece of gold and dried in a vacuum oven at 50 °C for at least 2 h. Microspheres and modified gold surfaces were analyzed using a JPS-9200 photoelectron spectrometer (JEOL Ltd., Tokyo, Japan). The spectra were obtained using monochromatic Al KαXray radiation at 12 kV and 20 mA with an analyzer energy pass of 10 eV for narrow scans. The obtained spectra were processed using the CASA XPS peak fit program (Casa Software Ltd., version 2.3.16 PR 1.6).

3 Results and discussion

3.1 Identification of epitopes recognized by MAb 4H12 in rbST

To determine which peptide could mimic the rbST epitope that is recognized by the MAb 4H12 the Pepscan technology for epitope-mapping was used with four different peptide designs (Figure 1). For all four epitope designs, binding between MAb 4H12 and the sequence $_{115}$DLEEGILALMR$_{125}$ was demonstrated (Figure 1B). Especially, in the library containing 15-mer linear peptides of the full rbST protein (set 1), binding to the linear sequence $_{115}$DLEEGILALMR$_{125}$ was found, which was confirmed by a library containing double alanine replacements (set 2), showing the importance of amino acids $_{120}$ILALMR$_{125}$, with the highest negative impact when L_{121} and M_{124} were part of the replacement by alanine with a measured intensity reduction of more than 92% and 83%, respectively. These findings were confirmed by two other libraries (sets 3 and 4), which contained short and extended α-helical loop mimics of the peptide sequences that covered the entire primary sequence of rbST (Figure 1C). Therefore, we synthesized and used the straightforward linear peptide $_{115}$DLEEGILALMR$_{125}$ that was able to bind the MAb 4H12 for our further immunoassay development. We tested whether attachment to the microsphere via either the C- or the N-terminus of the peptide was preferred (the former option was facilitated by a C-terminally positioned Lys residue), and whether the biorecognition would benefit

from the presence of a hydrophilic or hydrophobic spacer between the peptide and the microspheres (Figure 2).

3.2 Performance of the microsphere peptide-based immunoassay (MIPA)

MAb 4H12 showed binding with all microsphere sets, i.e., to full rbST protein and to the mimicking epitope $_{115}$DLEEGILALMR$_{125}$, irrespective of the method by which it was attached to the surface. For example, attachment via the C-terminal additional Lys residue or the backbone amine group of the N-terminus yielded the same results with IC_{50} values of 0.10 and 0.13 µg mL^{-1}, respectively. Similarly, IC_{50} values of 0.10 and 0.12 µg mL^{-1} were obtained for peptides attached to the surface via the hydrophobic aminocaproic acid (aca) or hydrophilic mini-PEG (peg) linker, respectively, which was positioned on the N-terminus, indicating that an additional spacer was not required (Figure 4).

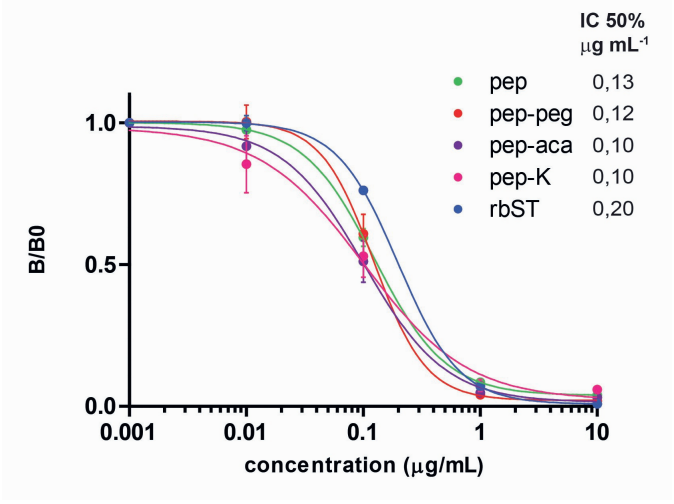

Figure 4. Typical inhibition curves, four-parameter fitted, of rbST in PBST/BSA solution for microspheres coupled with pep (●), pep-K (●), pep-peg (●), pep-aca (●) and the total rbST protein (●) (n = 3). Error bars display the standard deviations on B/B_0 per tested rbST concentration for each individual microsphere set. The half maximal inhibitory concentration (IC_{50}) for each individual microsphere set is shown in the top right corner of the graph. Pep-scr did not show any binding of the MAb; consequently, its inhibition could not be included in this figure.

Although the literature often describes the need for spacers in peptide-based biorecognition assays [33-35], in our assay format based on the MAb 4H12 and the peptide $_{115}$DLEEGILALMR$_{125}$ biorecognition pair, no spacers were required. As expected, no binding occurred of the mAb to the scrambled peptide coupled to a microsphere, showing specificity of MAb 4H12 for the peptide $_{115}$DLEEGILALMR$_{125}$. The presence of peptides

on the microspheres was proven by XPS measurements (Supplementary Materials, Figure S2). In addition, the specificity was compared by initial surface plasmon resonance (SPR) experiments, where the pep-peg peptide was coupled to one microfluidic channel and rbST to another channel of the sensor surface. The sensor channels were incubated with two different rbST-induced monoclonal antibodies, MAb 4H12, which specifically binds the peptide $_{115}$DLEEGILALMR$_{125}$ and MAb 5E2, which recognizes another, yet unknown, epitope. Both MAbs showed binding to the immobilized rbST, as expected, but only MAb 4H12 was able to bind to the immobilized pep-peg, independently confirming its specificity (Supplementary Materials, Figure S3 and Table S1). Next, MAb 4H12 concentrations were optimized for the microsphere peptide-based immunoassay to a dilution factor corresponding to a signal intensity of 1000 MFI for the blank standard (dilution buffer, Supplementary Materials, Table S2). This resulted in MAb 4H12 concentrations of 20, 2, 2, 1 and 0.7 ng mL^{-1} for microspheres coated with pep, pep-peg, pep-aca, pep-K and rbST, respectively. The antibody concentrations in combination with the respective microspheres were used to determine the sensitivity of MAb 4H12 for the determination of full rbST-protein in standard solutions, as shown in Figure 4. For both, rbST-protein and peptide immobilized microspheres, typical dose-response curves were obtained, with IC_{50} values of, respectively, 0.20 µg mL^{-1} and 0.11 µg mL^{-1}. This shows use of peptide-immobilized microspheres give at least comparable sensitivities to rbST immobilized microspheres, although generally, affinity for the full protein is higher than the affinity for the single peptide. Further experiments were executed using microspheres coupled with pep, as all peptides showed similar results and pep is the cheapest and most straight forward peptide to obtain.

3.3 Application of MIPA to somatotropin injection samples

Final protein concentrations in Posilac®, Hilac® and excipient extracts were 5, 3.5 and 17 mg mL^{-1} respectively, in accordance with previously described extraction efficiency [20]. Although Posilac® and Hilac® contain a slightly different form of rbST - as the amino acid sequence of Hilac® is similar to the native bST, whereas in Posilac® the N-terminal amino acid alanine is replaced by a methionine - the MAb 4H12 binding epitope in both rbST forms is intact. Comparison of the injection sample extracts with the rbST protein standard showed 100% cross reactivity in the MIPA and was demonstrated on all peptide microsphere sets, whereas the excipient showed no cross reactivity. To test the applicability of the newly developed assay for ST detection from other species, which would be an advantage for enforcement purposes, Uniprot sequences of other STs were compared for the MAb 4H12 binding epitope and showed only small varieties (Figure 5A). For pST and eST, only one amino acid was different compared to the rbST epitope identified by our epitope mapping study: one of the two amino acids with the highest impact for MAb 4H12 binding, L_{121}, is replaced by Q. For hST, three epitope-related amino acids were different: L_{121}, A_{122} and R_{125} were replaced by Q, T and G, respectively (Figure 5A). Calibration curves of pST, eST and hST showed that due to the replacement of three amino acids in hST, MAb 4H12 was not able to recognize and bind to hST, even at the

highest tested concentration of 100 µg mL^{-1} (Figure 5B). However, cross-reactivity was seen for eST and pST, in which L_{121} is exchanged by Q. As earlier described, a signal reduction of 90% is expected when L_{121} is replaced, which is clearly demonstrated in the calibration curve at 1 µg mL^{-1} when the inhibition of eST and pST is compared to the inhibition of rbST. Here, rbST almost completely inhibits MAb 4H12, whereas inhibition by eST and rbST just started (Figure 5B).

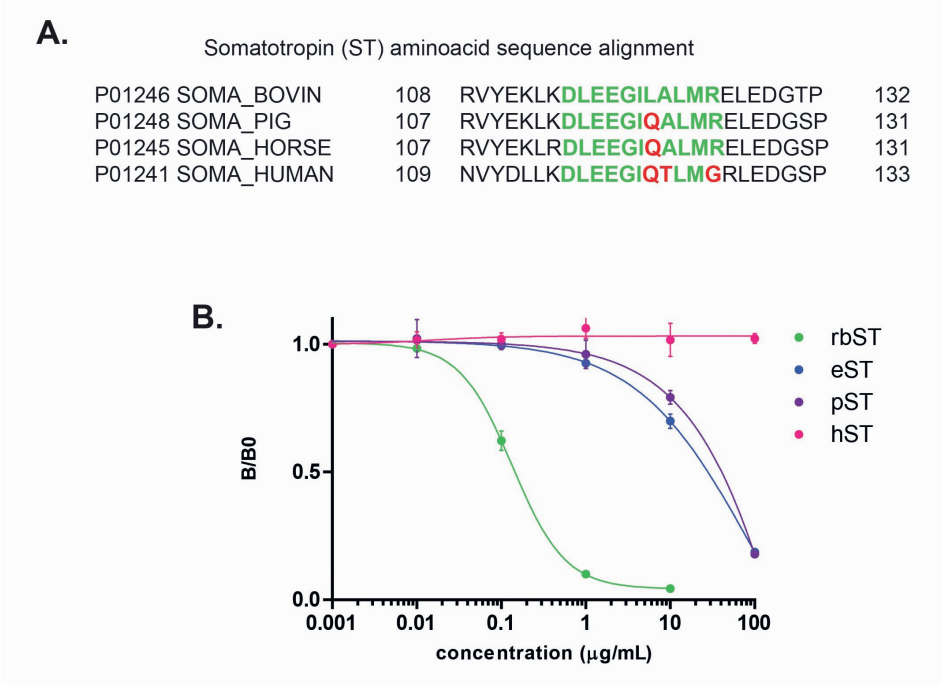

Figure 5. Amino acid sequence alignment for the epitope recognized by Mab 4H12 (green) and its variations (red) for bovine, porcine, horse and human Somatotropin (A). Inhibition curves of rbST (●), porcine somatotropin (pST, ●), equine somatotropin (eST, ●) and human somatotropin (hST, ●) (n = 2) in PBST/BSA solution for microspheres coupled with pep (B).

Full inhibition by eST and pST is observed around 100 µg mL^{-1}. These results show specificity of MAb 4H12 for the peptide and demonstrates the possibility to make an estimation on cross-reactivity, and applicability of the MIPA for other ST at forehand, instead of empirically as done in Heutmeker et al. [20]. Moreover, Heutmekers et al. [29] used polyclonal antibodies in combination with the full rbST protein, of which there is limited access or, for the former, will be exhausted at one point. Both sources of the developed MIPA, MAb 4H12 and peptides, can be produced endlessly, so this MIPA can always be produced and used. Therefore, this MIPA is applicable for screening purposes

of injection preparations containing rbST, pST and eST, and a simple 500-fold dilution of injection preparation extracts should be sufficient to detect these somatotropins, as injection preparations typically contain hundreds of milligrams somatotropins per injection. Furthermore, this approach enables us to 'flag' suspect samples, which would be an advantage for enforcement purposes, provided a confirmatory method is available, which is the case.

4 Conclusion

Compared to the full protein rbST MIPA, the peptide-based MIPA was found to be more sensitive with IC_{50} of 0.20 and 0.11 µg mL^{-1}, respectively. The epitope recognized by MAb 4H12 was determined as DLEEGILALMR, and optimal results were obtained in MIPA when this peptide was attached to the N-terminus without any spacers. A new coupling procedure enabled direct coupling of the peptide to the microspheres and its successful attachment was confirmed by XPS. Real-life applicability was demonstrated for injection preparations of rbST, eST and pST. It is, therefore, important that we present an approach that can circumvent the denied access to the full rbST protein, and opens a new research field for future assay development for the detection of rbST.

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Supplementary information

1 Introduction

To develop a peptide microsphere-based immunoassay, peptides were coupled to the carboxyl-rich surface of the microspheres. In the literature, peptides are often coupled to microspheres using a biotin-streptavidin coupling [1–4], a spacer [5] or BSA conjugate [6,7]. Direct coupling of peptides to the microspheres is most straightforward; however, this is not common practice. Only a few articles describe directly coupled peptides in peptide-based microsphere immunoassays using a standard EDC/NHS protocol. These couplings are executed at pH 5 [8–10] and pH 7 [11,12]. However, when only the N-terminus is available to couple to the carboxylated microspheres, the pH should be adjusted in order to deprotonate the free aminogroup (pKa 9). To the best of our knowledge, peptide coupling at high pH has not been described for microsphere-based immunoassays before; however, optimized protocols for binding of small negatively charged molecules are known from SPR where high salt and pH are used to establish binding [13], and this approach has been executed in our study.

2 Results

2.1 Evidence of peptides on the microspheres

As mentioned in the manuscript, as expected, no binding occurred with the scrambled peptide. To ensure that also the scrambled peptide was correctly coupled to the microspheres and that these microspheres were a true blank, three sets of microspheres were characterized in addition by X-ray photoelectron spectroscopy (XPS). Figure S1 shows an XPS narrow scan of C1s, N1s and S2p for modified and non-modified microspheres. The C1s spectrum of pep-K was fitted with four peaks as hydrocarbon (C-H/C-C) at 285.0 eV, heteroatoms-bound carbon (C-N and C-S) at 286.7 eV, carbonyl groups from amide (HN-C=O) at 288.3 eV and finally carboxylic acid carbonyl carbon (C-C=O) group at 290.6 eV. The ratio carbonyl groups from amide and carboxylic acid carbonyl carbon (HN-C=O)/(C-C=O) in pep-K modified microsphere is 2.03 in accordance with the theoretically (amide carbonyl 11 and acid carbonyl 6) expected ratio of 2.2. In addition, the pep-K modified microsphere shows the characteristic ammonium (402 eV) and amide nitrogen (400 eV) in the N1s narrow scan spectra, and the S2p peak appearing at 164.7 eV, confirming the successful peptide coupling on the microspheres. The C1s spectrum of the pep-scr was fitted with four peaks as hydrocarbon (C-H/C-C) at 285.0 eV, heteroatombound carbon (C-N and C-S) at 286.7 eV, carbonyl groups from amide (HN-C=O) at 288.6 eV and finally carboxylic acid carbonyl carbon (C=O) group at 290.7 eV. The ratio carbonyl groups from amide and carboxylic acid carbonyl carbon (HN-C=O)/(C-C=O) in scr-pep modified microsphere is 2.6, in accordance with the theoretically (amide carbonyl 10 and acid carbonyl 4) expected ratio of 2.5. In addition, the pep-scr-modified microsphere showed the characteristic ammonium (402 eV) and amide nitrogen (400 eV)

in the N1s narrow scan spectra, and S2p peak appeared at 164.4 eV, which is again confirming the successful coupling of pep-scr on the microsphere.

2.2 Quantification of the amount of peptide immobilized on the microspheres

Quantification of the amount of peptide immobilized on the microspheres via the XPS technique is quite reliable. We have estimated the percentage of attachment of peptides by considering 1 N1s atom in unmodified beads, and after modification with pep-K (DLEEGILALMRK), it will be 17 N1s (16 nitrogen from pep-K). Using this, and the fact that the peptide contains a sulfur in methionine, we were able to calculate the N1s:S2p ratio in order to determine the loading of the peptide on the beads. Specifically, the theoretical N1s:S2p ratio for pep-K attached on the microspheres should be 17.0:1.0 and the experimental obtained 17.8:1.0, suggesting that >95% of pep-K attached on the microspheres. In case of scrambled peptide MRIEGLADLEL (pep-scr), the theoretical N1s:S2p ratio on the microspheres should be 15.0:1.0 and the experimental obtained 15.5:1.0. Here, considering 1 N1s groups in unmodified beads and after modification with Pep-scr, it will be 15 N1s (14 nitrogen form pep-scr) and 1 S2p, suggesting that >96% pep-scr attached on the microspheres.

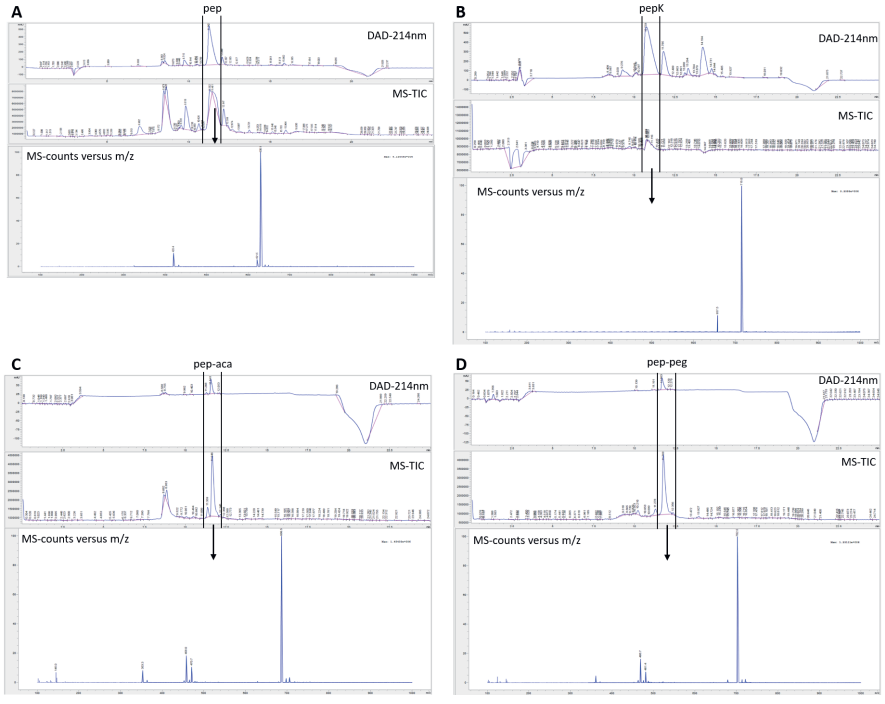

Figure S1. HPLC-DAD (diode array detection) chromatograms (top) and corresponding MS-total ion current (TIC) chromatograms (middle) for A) pep, B) pepK, C) pep-aca and D) pep-peg. Collected fraction is stated between the horizontal lines and the mass charge versus total counts of the fraction is shown (bottom).

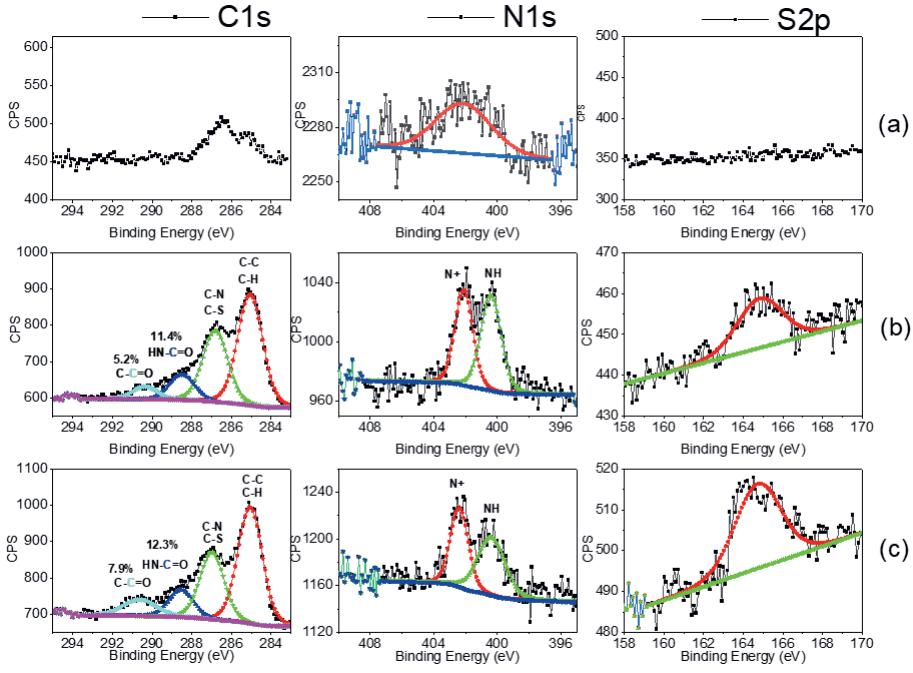

Figure S2. Narrow scan XPS spectra of C1s, N1s and S2p for microspheres: (a) non-modified microsphere-blank, (b) microsphere coupled with peptide DLEEGILALMRK and (c) microsphere coupled with scrambled peptide MRIEGLADLEL.

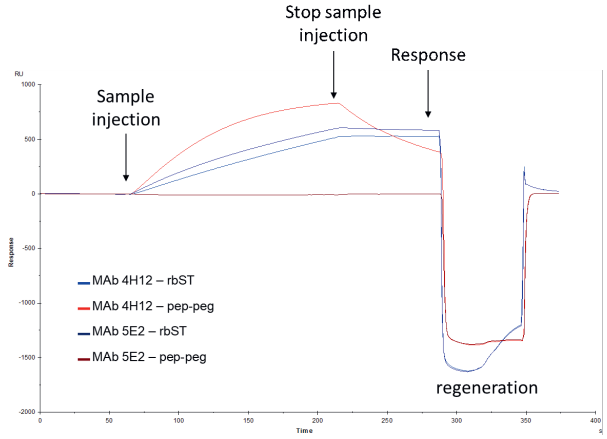

Figure S3. Surface plasmon resonance analysis to show binding of MAb 4H12 and MAb 5E2 on a flow channel (Fc) immobilized with rbST and an Fc immobilized with pep-peg.

Table S1. Response Units (RU) of surface plasmon resonance measurements. Binding of two anti-rbST MAbs were tested (Mab 4H12 and MAb 5E2) for rBST binding and Pep-peg binding. With the latter being the specific epitope for Mab 4H12.

MAb	rbST (RU)	Pep-peg (RU)
4H12	530	408
5E2	584	0

Table S2. Mean fluorescence intensities (MFI) of Mab 4H12 final dilutions (fd) on microspheres coupled with pep, pep-K, pep-aca, pep-peg and rbST.

Mab 4H12 conc. (ng mL^{-1})	Microsphere Pep (MFI)	Pep-K (MFI)	Pep-aca (MFI)	Pep-peg (MFI)	rbST (MFI)
100	5807	14096	14535	20057	23988
50	3953	11471	11777	17707	22331
25	2234	8137	8917	14113	18510
12.5	1277	5629	5581	10443	14056
6.25	711	3413	3146	6949	9384
3.13	323	1805	1630	4171	5645
1.56	170	939	885	2220	3428
0.78	97	466	410	1170	1985

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Chapter 5

Detection of methionine- and alanine-recombinant bovine somatotropins and their induced antibodies in serum and milk of cows suggests blood-milk barrier specificity for these compounds

Adapted from: Smits, N.G.E., M.H. Blokland, K.L. Wubs, T.F.H. Bovee, B. Albada, L.A. van Ginkel and M.W.F. Nielen. Detection of methionine- and alanine-recombinant bovine somatotropins and their induced antibodies in serum and milk of cows suggests blood-milk barrier specificity for these compounds. *Journal of Dairy Science* **2021**, 104, 4, 5069-5078.

Abstract

The elimination of recombinant bovine somatotropin (rbST) and its induced antibodies through milk of 2 formulations is studied to propose a control strategy for its use or abuse. Two dairy cows were treated with alanine-rbST (Ala-rbST), which is identical to endogenous bovine somatotropin, and ten dairy cows were treated with methionine-rbST (Met-rbST), which differs by 1 amino acid from endogenous bovine somatotropin. We developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method able to measure rbST at a decision limit (CCα) of 0.8 ng mL^{-1} and 2.3 ng mL^{-1} for serum and milk, respectively. The results show that the administered Ala-rbST is transferred from blood to milk but that this is not the case for Met-rbST. This suggests a blood-milk barrier-related specificity for these compounds. In addition, rbST-induced antibodies were formed in animals treated with Ala-rbST and those treated with Met-rbST. In both treatments, the rbST-induced antibodies were transferred from blood to milk, showing no blood-milk barrier specificity for these antibodies. These elimination patterns show that, for enforcement purposes, the detection of rbST-induced antibodies in tank milk can serve to screen for rbST administration, and subsequent confirmatory serum analysis by LC-MS/MS is needed to identify whether Ala-rbST or Met-rbST has been used.

1 Introduction

Recombinant bovine somatotropin (rbST) is a 191 amino acid-long protein hormone, commercially available in slow-release formula injection preparations in 2 forms: (1) identical to endogenous bovine somatotropin (bST) with alanine as N-terminal amino acid (Ala-rbST), and (2) 1 amino acid difference from endogenous bST, the N-terminal alanine replaced by methionine (Met-rbST). Recombinant bST is administered to dairy cows to enhance milk production, which can then increase up to 25% [1]. Normally, 500 mg of rbST subcutaneously injected in a slow-release formula allows a low administration frequency of just once every 2 wk. According to the size of rbST (22 kDa), most subcutaneously injected rbST is expected to enter the central lymph system by the lymph absorption pathway. From there, rbST will subsequently migrate into blood circulation [2]. Next to the presence of the rbST in blood circulation, specific antibodies against rbST (anti-rbST) can be formed due to rbST treatment, which will be referred to as "anti-rbST antibody-type biomarker" in the following text, according to its definition by Califf [3]. It is expected that this occurs even when the rbST amino acid sequence is identical to that of endogenous bST, similar to humans treated with human somatotropin [4]. This immune response then is due to rbST aggregate formation during production, shipping, and storage of rbST in formulas. In general, protein aggregates of multiple monomers result in a repetition of monomer parts, which are associated with pathogenic patterns and therefore trigger an immune response [5-7]. The use of rbST is forbidden in the European Union according to Council Decision 1999/879/EC, and control of its misuse is required [8]. For control purposes, the presence of either rbST or anti-rbST antibody-type biomarker in the cow can be used. Because blood sampling is not as easy as taking tank milk samples, and moreover for regulatory simplicity, tank milk is preferred (for example, inspectors in the Netherlands are not allowed to draw blood). Therefore, the present study was undertaken to establish whether both rbST and the anti-rbST antibody-type biomarker transfer from blood to milk. Because the method to determine these compounds in blood [9] did not work for milk, the method first had to be adjusted. After subcutaneous administration, rbST itself and anti-rbST antibodytype biomarker are expected to be present in the bloodstream. However, transfer from blood to milk is a selective process and requires active cellular transport, as the blood-milk barrier is highly impermeable to prevent diffusion of blood and milk components and to protect the calf after birth [10]. Only specific substances are transported from blood to milk, for instance, by transcytosis, 1 route among 5 major known routes [11]. For our proteins of interest, rbST and the anti-rbST antibody-type biomarker, excretion into the milk is expected because endogenous bST is a bioactive compound that regulates the growth of newborn calves via their mother's milk [12]. In addition, newborn calves are agammaglobulinemic, which means they have an immature immune system and no detectable immunoglobulins. They are protected by passive immunity supplied by the colostrum and milk from their mothers; therefore it is also expected that the anti-rbST antibody-type biomarker is transferred from the blood to the colostrum and milk via an Fc receptor-mediated mechanism [13]. Because this mechanism is Fc-based, all IgG found in blood, including rbST-induced antibodies, would thus end up in milk [14, 15]. Four liquid chromatography-tandem mass

spectrometry (LC-MS/MS)-based methods have been described that are able to specifically detect rbST in serum at relevant levels, that is, below 10 ng mL^{-1} [9, 16-18]. Results of rbST measurements in incurred milk samples were presented by [19], but unfortunately the authors did not describe how rbST in the milk was measured. Until now, rbST has only been detected at high levels in spiked blank milk samples, and rbST data in incurred milk samples is lacking [20]. Regarding the measurement of the anti-rbST antibody-type biomarker, many methods on different platforms have been developed for both serum and milk, such as ELISA [21,22], Western blotting [23], and flow cytometric immunoassay (FCIA) [24-26]. In addition, a microarray smartphone readout has been described for the detection of anti-rbST antibody-type biomarkers in milk [27,28]. In the present study, the anti-rbST antibody-type biomarker in milk and serum was measured by using the previously developed FCIA method [24,25]. Eventually, measuring both rbST and the anti-rbST antibody-type biomarker in serum and milk of treated cows enabled us to study the transfer of rbST and its antibodies from serum to milk and to propose a screening strategy for rbST use or abuse based on anti-rbST antibody-type biomarkers in milk.

2 Experimental

2.1 Materials and instruments

The rbST standard was obtained from the National Hormone and Peptide Program (Torrance, CA). We obtained rbST, Posilac sometribove zinc suspension for injection (Met-rbST), and Excipient (sesame oil and aluminum monostearate) for injection from Elanco (Indianapolis, IN). The rbST for injection (Ala-rbST, Hilac) was obtained from LG (Seoul, South Korea). An isotopic-labeled bST peptide AFPAMSLSGLFANAVLR, with incorporation of $^{13}C_6{}^{15}N_4$-alanine, resulting in a total mass increase of 10 Da, and a synthetic analog of the rbST peptide MFPAMSLSGLFANAVLR were obtained from Bachem (Bubendorf, Switzerland). Pierce bicinchoninic acid protein assay and the Finnpipette Novus multichannel electronic and monolithic microcolumns disposable automation research tips protein A were purchased from Thermo Fisher Scientific (Rockford, IL); ammonium sulfate, hydrochloric acid, potassium phosphate, sodium hydroxide, sodium phosphate, and the ultrasonic cleaner from VWR International (Amsterdam, the Netherlands); trypsin, tris(hydroxymethyl)aminomethane, iodoacetamide, dimethyl sulfoxide, and dl-dithiothreitol from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands); methanol and acetonitrile from Biosolve (Valkenswaard, the Netherlands); and formic acid from Actu-All chemicals (Oss, the Netherlands). Protein LoBind Tubes (1.5 and 2.0 mL), Protein LoBind 96 deep-well plates (1.0 and 2.0 mL), and a table centrifuge, model 5810R, were purchased from Eppendorf (Hamburg, Germany); a GR 20-22 ultracentrifuge from Jouan (Saint-Herblain, France); Snijder test tube rotator from Omnilabo International (Breda, the Netherlands); and Zymark TurboVap from Biotage (Upsala, Sweden). Bond Elut Plexa 30-mg solid-phase extraction columns were purchased from Agilent Technologies (Amstelveen, the Netherlands).

2.2 Serum and milk samples

To study rbST elimination kinetics via milk, both serum and milk samples were collected from 2 controlled animal experiments with nonpregnant lactating Holstein Frisians cows (varying in stages of lactation and aged 2–7 yr; information per individual cow is shown in Supplemental Table S1, https://doi.org/10.3168/jds.2020-19209). Cows were housed indoor as a group, to enable sight, noise, and smell contact, but physically separated. Cows were fed roughage and concentrate and had ad libitum access to water. The same treatment schedule was used for both animal experiments: an adaptation period of 2 wk was taken into account before starting the treatment; then cows were treated every second week by subcutaneous injections of rbST according to the manufacturers' guidelines. Milk yields per animal were measured by weighing and registered daily. Blood and milk samples were collected approximately 15 min before the injection, as well as both 1 d and 1 wk after each injection. Additionally, following the last (i.e., the fourth and third) subcutaneous injections for experiments 1 and 2, respectively, blood and milk samples were collected daily for 2 wk; this is schematically presented in Figure 1.

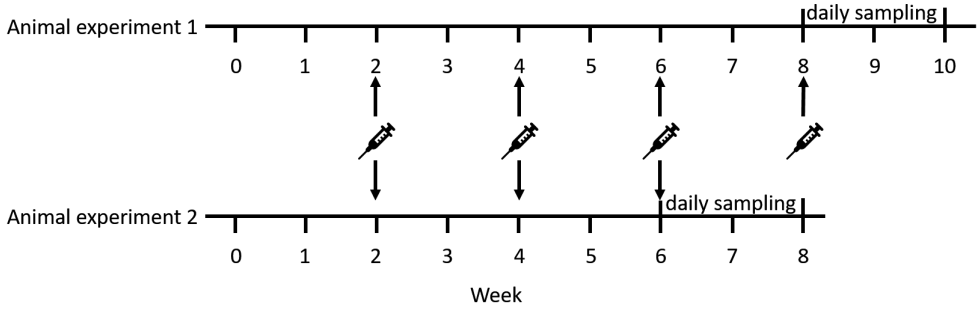

Figure 1. Schematic representation of the treatment schedules of animal experiments 1 and 2. In experiment 1, cows were treated with blank formula (n = 2) and methionine-recombinant bovine somatotropin (Met-rbST) formula (n = 8), and in animal experiment 2 cows were treated with Met-rbST (n = 2) and Ala-rbST (n = 2) formulas. Syringes show the moment of treatment with, respectively, Met-rbST, Ala-rbST, or blank in slow-release formulas.

In the first study, 8 cows were injected with 500 mg of Met-rbST in a slowrelease formula, and 2 cows followed the same treatment schedule, but with a similar slow-release formula without rbST (blank controls). In the second study, 2 cows were treated with a new batch of Met-rbST slowrelease formula, and 2 additional cows were treated with a 500 mg Ala-rbST slow-release formula. Due to the presence of multiple recent blank materials, from an ethical point of view, we chose not to include a blank cow. The Ala-rbST in this formula is identical to the peptide sequence of endogenous bST. Blood samples were stored at room temperature for 4 h to coagulate. After coagulation, the samples were centrifuged for 10 min at 3,000 x *g*, and serum was collected and stored at -20 °C until further analysis. Milk

samples were stored at -20 °C directly until further analysis. Both studies were authorized by the ethical committee of the Animal Sciences Group of Wageningen University and Research Centre, in Lelystad and Wageningen, the Netherlands, respectively.

2.3 Preparation and purification of polyclonal antiserum

Preparation and purification of polyclonal antiserum against rbST was as previously described by Heutmekers et al. [29].

2.4 Pretreatment of serum and milk samples for rbST analysis

Extraction of rbST from blood and milk samples was based on the rbST extraction protocol for serum developed by Smits et al. [9], with further improvements being made for blood serum and small adaptations for milk serum samples. Improvements to the serum extraction method comprised addition of Tween 20 during affinity purification, and, for LC-MS/MS measurements, a column with a smaller internal diameter was used for further improvement of the method. In short, serum samples were thawed, and the rbST in the serum samples was extracted and concentrated by affinity binding using polyclonal anti-rbST ammonium sulfate purified antibodies that were immobilized on monolithic microcolumns loaded with protein A [9]. Protein A-loaded monolithic microcolumns were placed on the Finnpipette, which is an automated device having a repetitive cycling function. First, the monolithic microcolumns were washed 10 times with 150 µL of 10 mM PBS with 0.05% Tween-20 (PBST), pH 7.4. Adherent solution was removed from the column by air pressure. Next, 75 µL of 0.1 mg mL $^{-1}$ of the ammonium sulfate-precipitated polyclonal anti-rbST antibodies, resuspended in 10 mM PBST, pH 7.4, was transferred over the column 1000 times back and forward. After the final cycle, adherent solution was removed from the column by air pressure. To remove unbound antibodies, the monolithic microcolumns were washed twice by 10 cycles of 150 µL of 10 mM PBST, pH 7.4. These freshly prepared monolith microcolumns immobilized with ammonium sulfate-precipitated polyclonal anti-rbST antibodies were then directly used for rbST enrichment. For immunoenrichment, 1 mL of serum sample, including matrix-matched spike controls, were diluted with 1 mL of 10 mM PBST, pH 7.4. The sample was transferred 2000 times (300 µL per cycle) back and forward over the anti-rbST immobilized monolithic microcolumn. Adherent sample was removed from the column by air pressure. To remove remaining unbound sample, the monolithic microcolumns were washed 10 times with 150 µL of 10 mM PBST pH 7.4. Captured rbST was then eluted from the monolith microcolumn by 50 µL of 200 mM NaOH (20 µL per cycle, 150 times). The eluate was collected and stored at -20 °C until tryptic digestion. To extract rbST from milk samples, the same steps were used, except that, after thawing, the milk was first centrifuged at 3000 x *g* for 10 min at 4 °C, and 2 mL from the aqueous layer was used and diluted with 2 mL of 10 mM PBST. For extraction, the sample was divided into 2 portions, and these portions were consecutively transferred 1000 times over a single monolith microcolumn. Thereafter, rbST extraction from the milk samples followed the same procedure as that described for serum.

2.5 Digestion and cleanup of the immunoaffinity-enriched rbST serum and milk extracts

For digestion of proteins with trypsin, 50 µL stored sample extracts were thawed, and 50 µL of 200 mM Tris, pH 8, was added. The pH of the obtained solution was adjusted to between 8 and 8.5 with 5 µL of 5% formic acid. To reduce the formation of sulfur bridges, 5 µL of 45 mM dl-dithiothreitol in 200 mM Tris, pH 8, was added, and the solution was mixed and incubated for 30 min at 37 °C. The solution was cooled to room temperature, and 5 µL of 0.1 M iodoacetamide in 200 mM Tris, pH 8, was added for methylation of the cysteine residues by mixing on a vortex and incubation for 30 min at room temperature in the dark. For protein digestion, 5 µL of 1 µg μL^{-1} freshly prepared trypsin in 200 mM Tris, pH 8, was added, followed by addition of 20 µL of acetonitrile, mixing by vortex, and incubation for 1 h at 37 °C. Digestion was terminated by addition of 0.9 mL of 5% formic acid, and an internal standard of an isotope-labeled bST peptide was added. This labeled peptide is a replicate of the 17 amino acids of the N-terminal end of endogenous bST (AFPAMSLSGLFANAVLR) and differs only in the N-terminal amino acid by incorporation of $^{13}C_6$ $^{15}N_4$-alanine, resulting in a total mass increase of 10 Da. The sample digest was transferred to a Bond Elut solid-phase extraction column previously conditioned with 1 mL of methanol and 1 mL of 5% formic acid in water. Then the column was washed with 1 mL of 15% acetonitrile and eluted with 0.5 mL of water: acetonitrile: formic acid (15:75:10 $v/v/v$). The eluate was collected in a tube already containing 50 µL of dimethyl sulfoxide and evaporated to approximately 100 µL in the TurboVap at 55 °C under 10 psi N_2. After cooling to room temperature and mixing, the sample was ready for LC-MS/MS analysis.

2.6 LC-MS/MS analysis

Analysis was performed on an I-Class ultra-performance liquid chromatography system connected to a Xevo triple quadrupole mass spectrometer (Waters, Manchester, UK), operated in the multiple reaction monitoring mode. Using 20 µL of the final sample extracts, chromatographic separation was performed on an Acquity Peptide CSH C_{18}, 130 Å, 1.7 µm, 1 × 100 mm column (Waters). Mobile phase A consisted of 10% acetonitrile with 0.1% formic acid and 0.01 M ammonium formate, and phase B consisted of 90% acetonitrile with 0.1% formic acid and 0.01 M ammonium formate. The flow rate was set at 0.175 mL min^{-1}, and mobile-phase composition of 80% A and 20% B was used for the first 30 s, increasing to 52% B in the next 4 min. Then the column was washed for 2.5 min with 90% B and re-equilibrated with 80% A and 20% B for 2 min. Total run time was 9 min. The mass spectrometer was operated in the positive ion ESI-MS/MS mode. Ion transitions m/z 883.1 > 774.1, m/z 883.1 > 1,047.6, and m/z 883.1 > 960.6 were measured to detect the bST- and Ala-rbST–specific N-terminal peptide with amino acid sequence AFPAMSLSGLFANAVLR, and ion transitions m/z 913.1 > 774.1, m/z 913.1 > 1,047.6, and m/z 913.1 > 960.6 were measured to detect the Met-rbST specific N-terminal peptide with amino acid sequence MFPAMSLSGLFANAVLR, all formed after tryptic digestion [30]. To check the retention time of the N-terminal rbST peptide of

interest, a synthetic analog of the rbST peptide was injected at the beginning and end of each series. For the $^{13}C_6{}^{15}N_4$-bST internal standard, the transition m/z 888.1 > 779.1 was followed.

2.7 Pretreatment and measurement of serum and milk samples for anti-rbST analysis

Pretreatment of serum and milk samples to detect anti-rbST-induced antibodies was previously described by Ludwig et al. [24] and is schematically presented in Supplemental Figure S1 (https://doi.org/10.3168/jds.2020-19209).

2.8 Initial in-house validation

The decision limit (CCα) and detection capability (CCβ) were determined according to the calibration procedure conforming to Commission Decision 2002/657/EC [31]. Milk samples enriched with rbST at 0, 1, 2.5, 5, 10, and 25 ng mL^{-1} were processed using the described method. In all runs, at least 1 calibration curve was included, leading to a total of 17 calibration curves, which were then used to determine CCα and CCβ. Calculation of the concentration was performed by constructing a linear calibration curve of the response factor (peak area ratio of rbST fragment and internal standard) versus the concentration (expressed as absolute amount of rbST protein). The false negative rate β-error, according to the screening criterion of Commission Decision 2002/657/EC [31], was previously determined and described by Ludwig et al. [24].

3 Results and discussion

3.1 Controlled animal treatment experiment

According to the manufacturer's protocol for Met-rbST, optimum milk yields are obtained when rbST treatment starts 57 to 70 d after calving. Following this protocol, Bauman et al. [32] showed that milk production in dairy cows increased by approximately 10%. Although the treatment in our animal experiment did not start at the prescribed optimal moment, as the study was not set up to determine the effect on the milk production, the effect of the administered rbST hormone on the milk yield was followed. Figure 2 shows that rbST treatment resulted in an increment of the milk yield of, on average, 11%, similar to the findings of Bauman et al. [32] and de Morais et al. [33]. Overall, the standard deviations due to intraindividual differences are rather high, similar to others reported in the literature [34].

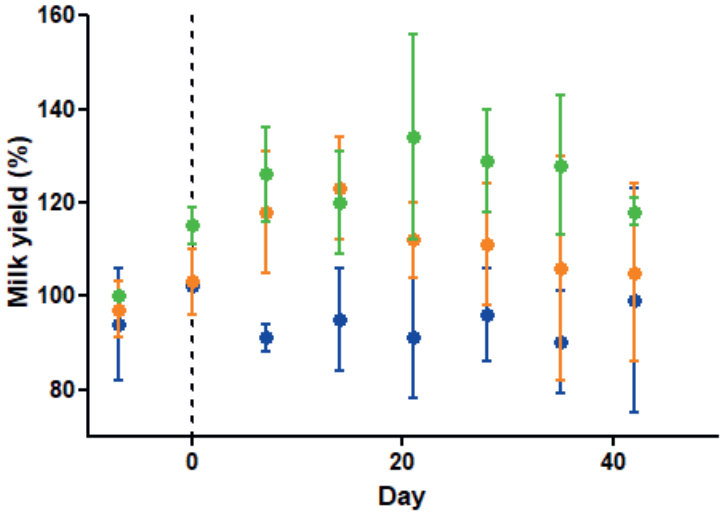

Figure 2. Average milk yield of 10 cows treated with methionine-recombinant bovine somatotropin (Met-rbST, ●), 2 cows treated with alanine-rbST (Ala-rbST, ●), and 2 untreated cows (●). Treatment with Met-rbST and Ala-rbST started at d 0 (- - -). Milk yield is normalized on the average milk yield before treatment (100%), and error bars represent SD.

3.2 Analytical performance of rbST detection in serum and milk

The developed method for the analysis of bST and rbST in milk was based on the previously published method for analysis of rbST in serum [9]. Therefore, the serum method was adjusted, as described in the Materials and Methods section, and an initial in-house validation as a quantitative confirmatory method according to Commission Decision 2002/657/EC [31] was executed.

Table 1. Validation parameters (decision limit, CCα, and detection capability, CCβ) of recombinant bovine somatotropin (rbST) method performance for serum and milk using monolith microcolumns loaded with protein A, tryptic digestion, and liquid chromatography-tandem mass spectrometry analyses

Performance parameter	Serum Met-rbST, (ng mL^{-1})	Milk Met-rbST, (ng mL^{-1})
CCα	0.8	2.3
CCβ	1.6	4.5

The analytical performance of the milk method was compared with the original serum

method: the decision limit (CCα) and detection capability (CCβ) of both methods were compared. As shown in Table 1, the CCα of Met-rbST in serum is 3-fold lower than in milk, which most probably arises from interfering lipids in milk, as milk contains on average 3 to 5% lipids [35], whereas serum contains less than 0.5% lipids [36]. This high fat content of milk is known to block and interfere the antigen-antibody binding during rbST enrichment [37]. However, because of the straightforward sampling compared with blood, a method to detect rbST use or abuse via milk is preferred. To enable Ala-rbST detection, sensitivity of the antibody for Ala-rbST was determined and showed to be similar to that of Met-rbST [29]. Moreover, when a serum sample was spiked with both Met-rbST and Ala-rbST, the binding ratio was, respectively, 6:4, showing antibody affinity for both forms of rbST, even when both forms are present in the sample (data not shown). Figure 3 shows results obtained from a serum sample and its corresponding milk sample taken 1 d after the first Ala-rbST treatment and a milk sample before treatment. One day after treatment, for both milk and serum, a clear peak is present at the expected retention time of the Ala-rbST peptide marker, demonstrated by the comparison with the internal standard. The peak is missing in the sample taken before treatment. This demonstrates that the Ala-rbST was transferred from blood to milk and that the developed milk method was applicable to incurred milk samples.

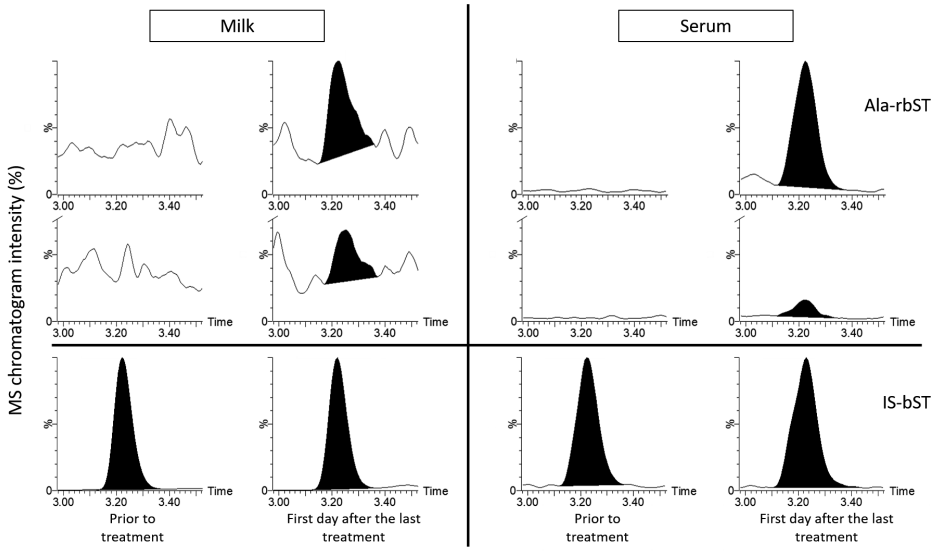

Figure 3. Reconstructed ultra performance liquid chromatography–tandem MS chromatograms [with minor smoothing (2 × 2) using the moving mean (Mn)] of transitions (from top to bottom) m/z 883.1 > 774.1 and m/z 883.1 > 960.6 for the alanine-recombinant bovine somatotropin (Ala-rbST) marker peptide and m/z 888.1 > 779.1 for the methionine-bovine somatotropin (bST) internal standard (IS-bST) of, on the left side, a milk sample taken before treatment and a milk sample taken 1 d after the last Ala-rbST

treatment and, on the right side, the corresponding serum samples. All samples were obtained from the same cow.

3.3 Elimination of rbST into milk

The goal of the present study is to elucidate the elimination of both rbST and anti-rbST from blood to milk. For this, rbST and anti-rbST need to pass the blood-milk barrier. Studying the elimination by measuring both compounds in both serum and milk will yield the required data, enabling development of a control strategy to detect use or abuse of rbST. Both Met- and Ala-rbST were measured in serum and milk samples, starting just before the first administration. It should be noted that serum and milk samples taken before the rbST treatments did not contain detectable levels of endogenous bST and, obviously, no rbST either. Thus, all detected bST and rbST after the treatments is likely to originate from the corresponding Ala-rbST or Met-rbST treatment.

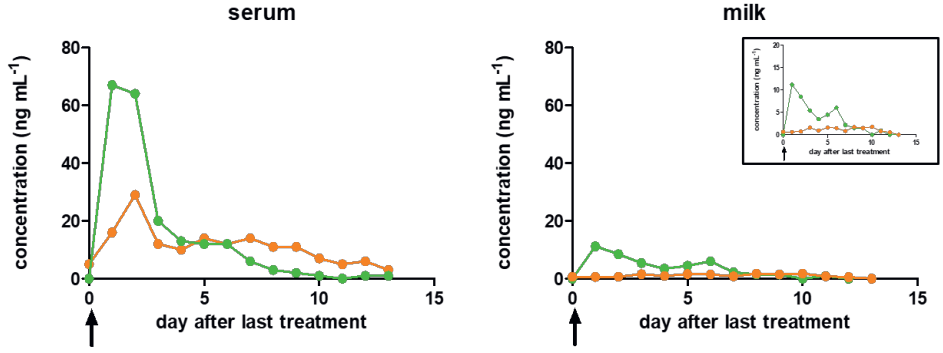

Figure 4. Serum and milk methionine-recombinant bovine somatotropin (Met-rbST, ○●) and alanine-rbST (Ala-rbST, ○●) concentrations, in ng mL^{-1}, as determined by ultra performance liquid chromatography–tandem MS analysis. Arrows mark the moment of the last injection with rbST. The left-hand graph shows average rbST serum concentrations for Met-rbST (n = 10) and Ala-rbST (n = 2); the right-hand graph shows the concentrations in the corresponding milk samples.

Figure 4 shows that for both types of treatment (i.e., with Ala-rbST or Met-rbST), a clear increment in serum rbST was observed already the first day after treatment. This increment was seen after each treatment, from the first treatment onward (Supplemental Figure S2, https://doi.org/10.3168/jds.2020-19209). After the last treatment, samples were taken daily for 14 d to study elimination of rbST via milk. Regarding the Ala-rbST treatment, the highest rbST serum concentrations were measured in the first 2 d after treatment, reaching concentrations of about 70 ng mL^{-1} (n = 2). Two days after the last treatment, the rbST serum concentrations dropped rapidly. But although concentrations became low, approximately 1 ng mL^{-1} of Ala-rbST was still detectable in serum 2 wk

after the last treatment. For cows treated with Met-rbST, serum rbST concentrations increased, reaching a maximum 2 d after the last treatment, although this the level was lower compared with Ala-rbST, approximately 30 ng mL^{-1} (n = 10). Although lower than the maximum reached with Ala-rbST, Met-rbST serum concentrations stayed elevated for a longer period of time, with levels above 10 ng mL^{-1} (n = 10) 10 d after the last treatment and an average concentration of 3 ng mL^{-1} (n = 10) found 2 wk after treatment. The graph on the right-hand side of Figure 4 shows the measured concentrations of rbST in the corresponding milk samples. Ala-rbST was detected in milk, but in contrast and despite the elevated Met-rbST concentrations present in serum during the 2 wk after administration, Met-rbST concentrations in milk stayed below CCα. The time-dependent Ala-rbST concentration curve in milk is similar to that of Ala-rbST in serum, with the distinction that the detected concentrations in milk are lower. The correlation between milk and serum rbST concentration is dependent on the mechanism that enables rbST to pass the bloodmilk barrier. It has been described that endogenous bST (i.e., Ala-bST) is present in the blood and milk, as bST in milk is a growth factor for the calf [12]. Two routes are considered by which bST that is endogenously present in the mother ends up in her milk. First, bST is transported from the blood circulation via the mammary gland to the milk by transcytosis, similar to the well-studied mechanism for prolactin [11], which belongs to the same hormone family and shows homology with bST [38]. Second, it has also been recognized that the mammary gland itself is able to produce bST. However, the exact role and excretion route of mammary bST is not known yet [39,40]. Because endogenous bST is transported from blood to milk, transport of administered Ala-rbST from blood to milk is also expected. Although this has not yet been demonstrated, this theory of rbST passing the blood-milk barrier is supported by our current observations of Ala-rbST in milk samples after Ala-rbST treatment, showing concentration profiles identical to those in serum. However, despite Met-rbST detected in serum, concentrations of Met-rbST in milk after Met-rbST treatment stayed below CCα. The blood-milk barrier transport mechanism was expected to be similar for both forms of rbST, as they differ by only 1 amino acid. It can be speculated how this difference of 1 amino acid can be responsible for the observed effect that Met-rbST is barely found in milk. Methionine cannot be synthesized by the mammary gland, but methionine is an important part of casein, the major protein component in milk [41,42]. It is also known that the mammary gland can use N-terminal-bound methionine of peptides and proteins as a source for its endogenous production of milk proteins, including casein [43]. Although the proteolytic activity within the mammary gland of cows is not yet fully understood, in humans, breast milk has revealed the presence of a set of peptides with sequential removal of amino acids at the N-terminus, indicating that exopeptidase activity in the mammary gland can be responsible for N-terminal methionine cleavage [44]. If, in the cow's mammary gland, N-terminal methionine is cleaved from the 191-AA-long Met-rbST, then future research should also focus on a 190-AA-long rbST protein that has an N-terminal phenylalanine instead of methionine. The targeted LC-MS/MS measurements in milk should then focus on the N-terminal rbST peptide of the Met-rbST without the methionine (i.e., FPAMSLSGLFANAVLR). In addition, assuming that the injection

preparations of Ala-rbST and Met-rbST both contain 500 mg of the recombinant peptide, as declared on the injection preparations, which are legally bound to a strict acceptable deviation of <10% [45], and the analytical performance was similar for both rbST forms, it was expected that we would find similar amounts of rbST in the serum of treated cows. However, more Ala-rbST was found in serum after treatment than Met-rbST. Because exopeptidases are broadly distributed in the living organism, exopeptidases can cleave the N-terminal methionine peptide when present in the blood [46]; therefore, also, presence of FPAMSLSGLFANAVLR in serum should be tested in future research.

3.4 Elimination of rbST antibodies to milk

Antibodies induced by the injection of rbST were detected in serum samples and their corresponding milk samples using a FCIA [25] irrespective of the type of rbST administered. The validation parameter β-error of the FCIA method was described before by Ludwig et al. [24] and is summarized in Table 2.

Table 2. Validation parameter β-error of anti-recombinant bovine somatotropin antibody-type biomarker for serum, milk, and bulk tank milk samples using flow cytometric immunoassay [24].

Performance parameter	Serum	Milk	Tank milk
β-error (%)	6	6	≤ 5

Three samples per cow were tested (sample set), one sample before the rbST treatment, one sample 1 wk after the second rbST administration, and one sample 2 wk after the third rbST administration. In total, sample sets of 14 individual cows were tested. Ten cows were treated with the Met-rbST formula, two cows were treated with the Ala-rbST formula, and two cows did not receive treatment with rbST. Figure 5 clearly shows that both rbST treatments result in increased anti-rbST concentrations in both serum and milk (raw data in Supplemental Table S2, https://doi.org/10.3168/jds.2020-19209). Moreover, comparison of the measured sample responses to the decision level enabled us to discriminate between treated and untreated cows in both serum and milk. The presence of antibodies in milk upon both treatments was expected, as both Ala-rbST and Met-rbST will result in an immune response, and a generic Fc-specific transport mechanism from blood toward milk for immunological protection of newborn calves has been reported [13]. Although Ala-rbST is fully identical to endogenous bST, cows treated with the Ala-rbST formula showed an immune response by anti-rbST production. In humans, such a phenomenon is also observed when subjects are treated with human growth hormone. Recombinant human growth hormone is also identical to the endogenous form, and antibody formation occurred in 50% of children treated with human growth hormone [47]. In both cows treated with Ala-rbST, we found antibody formation (Supplemental

Table S2, https://doi.org/10.3168/jds.2020-19209). For treatment with Met-rbST an antibody response in 67% of the cows was found, as previously described [24]. The immune responses in these cows are likely due to formation of rbST aggregates in the formulas [5,6]. To pinpoint rbST use or abuse for control purposes, rbST-induced antibodies can be detected in both serum and milk from treated cows. Milk samples are preferred, as milk sampling is noninvasive. However, because not all cows will show an immune response upon treatment, for control purposes multiple cows should be sampled, or, even better, samples should be taken from the farmer's milk storage tank, as it has been shown that taking samples from tanks for detection of rbST-induced antibodies is more adequate than sampling from individual cows, resulting in a true positive rate of over 95% for tank milk [24].

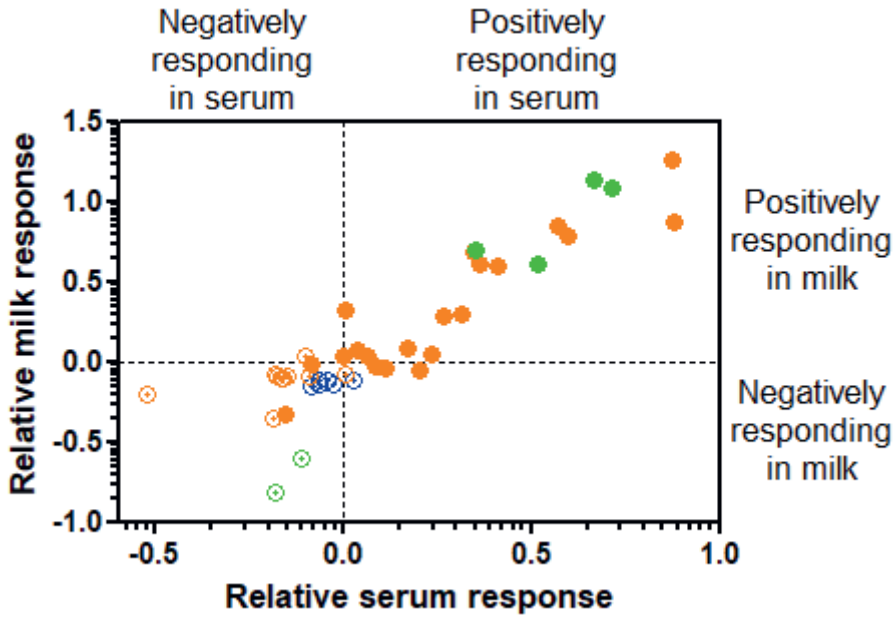

Figure 5. Recombinant bovine somatotropin (rbST) antibody relative responses in corresponding serum and milk samples. Measured flow cytometric immunoassay fluorescence signals are normalized according to Smits et al. [25]. For analysis, samples from each cow at the following time points were used: 1 wk before treatment (○) and 3 and 6 wk after start of treatment (●). Samples are taken from 2 untreated (○●) cows, 10 cows treated with the methionine-rbST formula (○●), and 2 cows treated with the alanine-rbST formula (○●). Dashed lines represent the decision level to discriminate between treated and untreated cows for, respectively, serum and milk.

3.5 Strategy for rbST enforcement purposes

The elimination patterns for rbST and the rbST-induced antibodies, as presented in this study, show that, for enforcement purposes, the detection of rbST-induced antibodies in tank milk can serve as an antibody-type biomarker to screen for rbST administration. Ideally, results from tank milk analysis need to be available within 1 d. When screening results suggest rbST use or abuse, blood samples need to be drawn immediately for subsequent confirmatory serum analysis by LC-MS/MS. These analyses are needed to confirm presence of rbST in the cow and to identify whether Ala-rbST or Met-rbST was used.

4 Conclusion

Investigating the elimination of rbST, the transfer from blood to milk, demonstrated that the blood-milk barrier for rbST is very specific. Both rbST formulations are detectable in serum, but Met-rbST was barely transferred to milk. As expected, Ala-rbST, which is similar to endogenous bST, was transferred to the milk, and the levels were about 5 times lower than those in the serum samples. Thus, a difference of 1 amino acid (methionine) makes an important difference for elimination into milk. Regarding the immune response, both formulations resulted in a clear response and similar levels of anti-rbST in serum. And as expected, these anti-rbST antibodies were transferred to milk. The present study thus demonstrates that, for control purposes, screening for rbST use and abuse can easily be performed by measuring anti-rbST antibodies as an antibody-type biomarker in tank milk. However, subsequent LC-MS/MS analyses of serum samples are needed to confirm the type of rbST used, as Met-rbST is not able to cross the blood-milk barrier.

Acknowledgments

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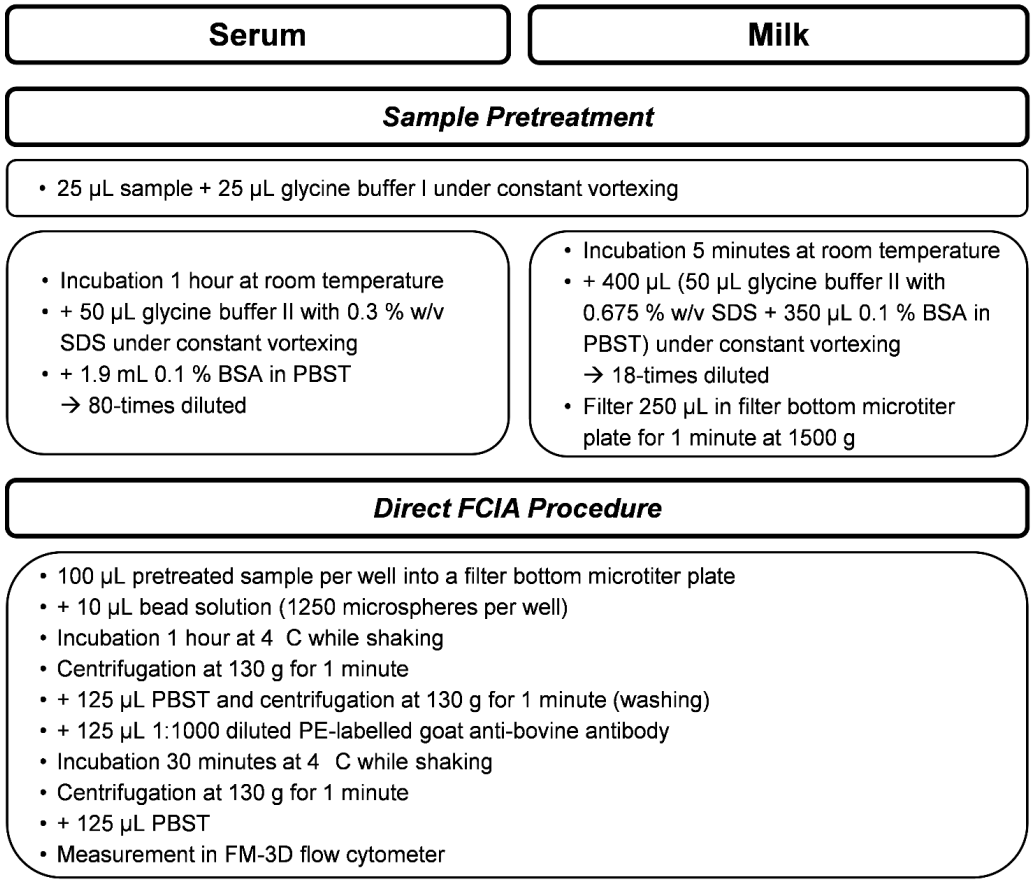
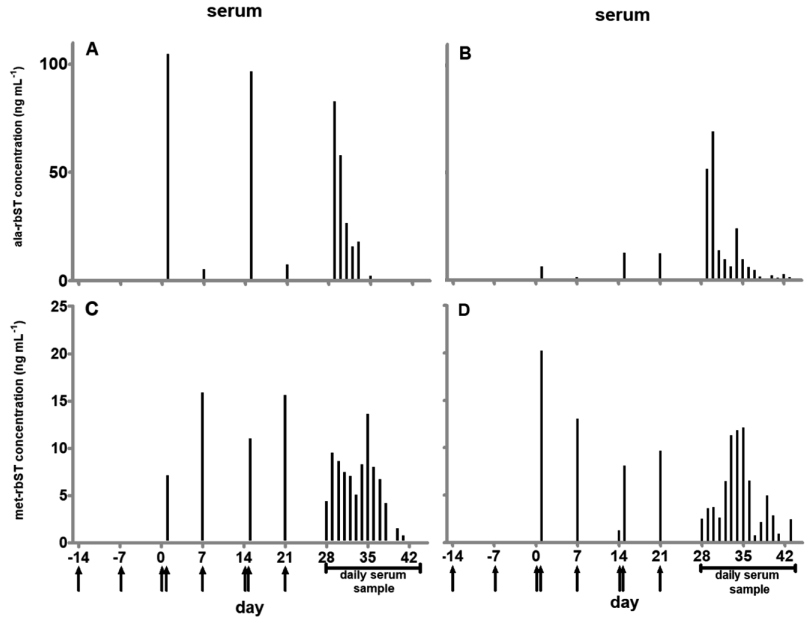
Supplementary information



Supplemental Figure S1. Sample pre-treatment and direct FCIA procedure for anti-rbST detection in serum and milk as presented in Ludwig et al. [24].



Supplemental Figure S2. Detection of rbST concentrations (ng mL^{-1}) in serum samples of Ala-rbST treated cows (A,B) and Met-rbST treated cows (C,D) of animal experiment 2. Samples were collected during the total animal experiment period, also prior to treatment. Days where samples were collected are indicated by arrows (↑) and a horizontal bar during daily sample collection period. Treatment with Ala- and Met-rbST started at day 0. Note the different y-axis in figs. S2A and S2C.

Supplemental Table S1. Information of the individual cows about the treatment they received, age, weight, lactation number, stage of lactation and to which animal experiment they were assigned. *In both animal experiments cows were treated according to the manufacturers guidelines by subcutaneous injection in the tailhead depression, neck or behind the shoulder.

treatment	cow	Animal experiment*	Age (year)	Weight (kg)	Lactation number	Stage of lactation	
						start	end
Met-rbST	J332	1	4	729	3	early	mid
	J350	1	3	680	2	early	mid
	H38	1	7	609	6	mid	late
	H152	1	3	578	2	late	late
	H185	1	2	501	1	mid	late
	Z337	1	7	788	7	late	late
	Z376	1	3	723	2	late	late
	J314	1	4	638	4	early	mid
	8910	2	5	693	3	late	late
	8930	2	5	647	4	mid	late
Ala-rbST	1102	2	3	575	2	late	late
	6137	2	5	677	4	mid	mid
Blanc	J302	1	4	673	3	mid	late
	E160	1	2	687	1	late	late

Supplemental Table S2. Mean FCIA fluorescence signals (MFI), proportional to the levels of anti-rbST in serum and corresponding milk samples from Met-rbST (■), Ala-rbST (■) and untreated (■) dairy cows. Samples are collected prior to treatment, 3 and 6 weeks after the first treatment.

			Prior to treatment		3 weeks after first treatment		6 weeks after first treatment	
treatment	Experiment No.	cow	serum (MFI)	milk (MFI)	serum (MFI)	milk (MFI)	serum (MFI)	milk (MFI)
Met-rbST	1	J332	153	164	423	385	229	218
	1	J350	157	159	368	180	296	183
	1	H38	152	170	905	1211	589	786
	1	H152	159	157	251	235	189	195
	1	H185	163	163	531	816	473	396
	1	Z337	234	171	1735	1482	510	971
	1	Z376	183	220	1713	3713	853	1437
	1	J314	187	166	281	187	268	220
	2	8910	104	55	280	183	242	62
	2	8930	58	79	115	91	166	42
Ala-rbST	2	1102	127	31	856	1367	763	690
	2	6137	108	23	367	570	543	205
Blanc	1	J302	199	147	190	142	244	155
	1	E160	217	147	209	154	199	153

Chapter 6

General discussion and future perspectives

General discussion

Antibodies are a powerful versatile tool in detection strategies for proteins. Detection of low abundant proteins in complex matrices however stays challenging and therefore, improvement of existing methods and development of new methods is an ongoing process. This thesis focuses on tools to reveal (ab)use of the low abundant protein rbST. To enable this antibodies are used either as 1) biomarker or 2) biorecognition element. The methods developed to determine rbST (ab)use, however, are in practice not always compatible with enforcement purposes. The main aim of this thesis was to explore antibody use as a versatile tool for the detection of rbST (ab)use, and how results obtained with the developed methods contribute to strategies to prove this (ab)use. In this chapter the potentials and limitations of the developed antibody based assays will be discussed, next to the challenge of using the incompatible aspects of obtained results within current legislation. Recommendations on future research will be given, as well as an outlook on possible future strategies and possible adaptions to legislation.

1 Antibodies as a versatile tool

Antibodies showed to be an indispensable and versatile tool for immuno-affinity enrichment (**Chapter 3, 5**), to detect target proteins (**Chapter 2, 4 and 5**) and as biomarker (**Chapter 2 and 5**). The methods developed to detect rbST and its abuse are depending on the availability of these antibodies. For instance, the immuno enrichment of rbST from bovine serum samples as described in **Chapter 3 and 5** a polyclonal antibody serum developed in the 1980's is chosen. Polyclonal antibody serum was available for all experiments executed in this thesis, and although some is left for future experiments, this stock is limited. Future research should focus on the use of antibodies or biorecognition molecules which can be produced indefinitely, as we did with the monoclonal antibodies presented in **Chapter 4**. If in the future polyclonal antibodies are replaced by other biorecognition molecules however, behavior and characteristics of these biorecognition molecules will be different from the polyclonal antibodies. Newly developed methods then need to be optimized and validated again. It should be taken into account that this probably leads to other method circumstances and different method performances of parameters such as decision limit (CCα) and detection capability (CCβ). As described before, availability of the existing or newly developed antibodies, determination of their characteristics, is of importance to improve existing or develop new assays.
The rbST-induced antibodies used for biomarker detection as described in **Chapter 2** and **Chapter 5** are discriminative between rbST treated and non-treated dairy cows [1]. These antibodies could be further characterized, for instance by MS, to possibly distinct between specific and a-specific antibodies. Characterization of antibodies by MS is already shown for monoclonal antibodies used for immunotherapy [2,3]. Monoclonal antibodies, however, are one type of protein, present in high concentrations, in a more or less purified form. The rbST antibodies in the dairy cows are polyclonal antibodies, which display high variability and are often present in low concentrations. Therefore, characterization

of polyclonal antibodies by mass spectrometry is extremely challenging and therefore still in its infancy [4,5].

1.1 Future outlook on antibody use

Current and and future use of antibodies is not self-evident. At the moment classic antibody generation faces strict restrictions in accordance with European Union policy on animal use [6]. In legislation the use of animals for antibody production is forbidden, unless it is proven that no other method is available [7]. EURL-EVCAM published a paper on non-animal derived antibodies, including different types of synthetic antibodies, and how to use them in immunoassay applications [8]. This EURL-EVCAM report will reform the production of antibodies within the EU. The transfer from animal derived antibodies to non-animal-derived antibodies, by restricting or banning animal experiments, will be similar as in a previous casus, the cosmetic industry [6]. In the cosmetic industry animal testing for cosmetic purposes was successfully banned by strict EU regulation, moreover this ban also applied for import of cosmetics. Future research based on antibody-antigen interactions, therefore will be in need for non-animal derived biorecognition molecules. Research is focused on development of a variety of biorecognition molecules, for instance recombinant antibodies. Recombinant antibodies can be developed using genetic material of cells producing classical antibodies [9,10], but nowadays also naive phage antibody libraries can be used [11,12]. In production of recombinant antibodies, focus is often on the antigen binding parts/CDRs of the antibody as shown in Figure 1. Compared to conventional antibodies, recombinant antibodies can be limited to the production of specific parts of the antibody, the fragment antigen binding parts (Fab) or the single chain variable parts (scFv). Although developments in the field of recombinant antibodies are promising, affinity of these recombinant antibody fragments for their targets is generally lower than of the original antibody [13]. For camelid and shark antibodies the recombinant form is based on the original antibody, but limited to the part containing the antigen binding site, called VHH (Variable domain on a Heavy chain) and VNAR (Variable New Antigen Receptor) respectively [14,15]. The former mentioned recombinant antibodies use classic generated antibodies as scaffolds. There are, however, also non-antibody based biorecognition molecules under development; for instance aptamers, DARPins (Designed Ankyrin Repeat Proteins), affimers and knottins. They have certain advantages over the antibody scaffolds as they are easier and cheaper to produce and often more stable, but on the downside their current commercial availability is limited [13]. For rbST, non-animal derived biorecognition molecules are not developed yet. However, for the human growth hormone (hGH) the possible affinity between biorecognition molecules and hGH is explored. Developed biorecognition molecules for hGH are aptamers which display a higher affinity for rhGH then for the endogenous hGH [16], peptide ligands for affinity capture [17] and molecular imprinted polymers (MIPS) able to detect hGH in plasma in a 0.1 - 100 ng mL^{-1} range [18]. The knowledge obtained by development of these non-animal derived biorecognition molecules for hGH can serve as a base for future development of biorecognition molecules for (r)bST.

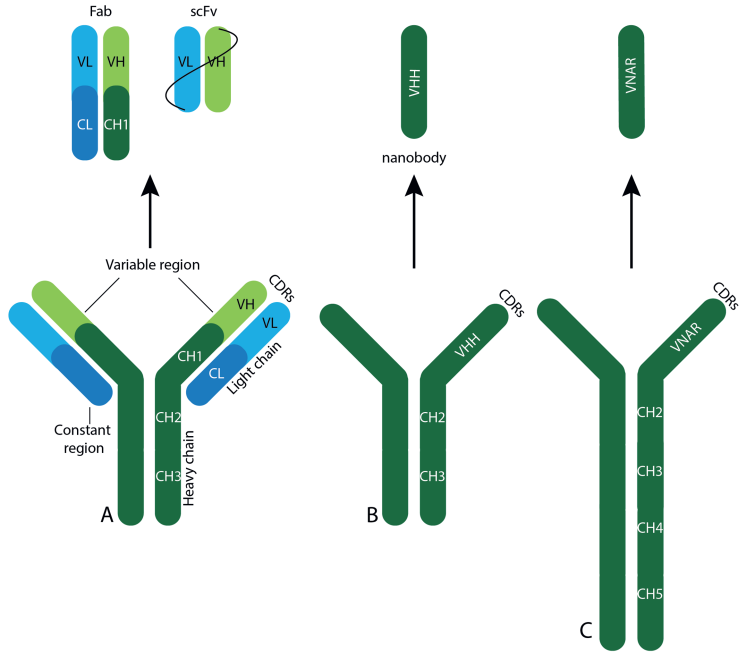


Figure 1. Structure of conventional antibodies (lower) and their recombinant fragments (upper) of human (A); camelid (B); and shark (C). The antigen is recognized by the complementarity determining region (CDR). The recombinant fragment of human (A) exists of the fragment antigen binding (Fab) or the single chain variable fragment (scFv) and for both camelid (B) and shark (C) the antigen binding fragment of the heavy chain is used.

2 Recombinant bovine somatotropin

2.1 Protein structure and function

BST is produced and released by the pituitary gland and is endogenously present in 4 isoforms; first variant is missing the N-terminal alanine (in a 1:1 ratio compared to endogenous bST), and the second variant is the presence of leucine or valine (in a 2:1 ratio) at amino acid position 126 or 127 [19-21]. RbST present in injection preparation was determined as shown in Figure 2. For Met-rbST, the amino acid sequence starts with methionine on the N-terminus and leucine is present at position 127. For Ala-rbST, alanine occupies the N-terminus and also here leucine is present at position 127. Deconvolution of the native mass spectra showed a single peak for Met-rbST and Ala-rbST, with a 60 Da mass difference between the two tested injection preparations. This is in agreement with the mass difference between methionine (149.21 Da) and alanine (89.09 Da), the two aminoacids present on the N-terminal side of respectively Met-rbST and Ala-rbST. When

dairy cows are treated with Met-rbST, it is possible to prove this treatment as Met-rbST is distinct from endogenous bST due to the methionine present on the N-terminal side of the protein as shown in **Chapter 3**.

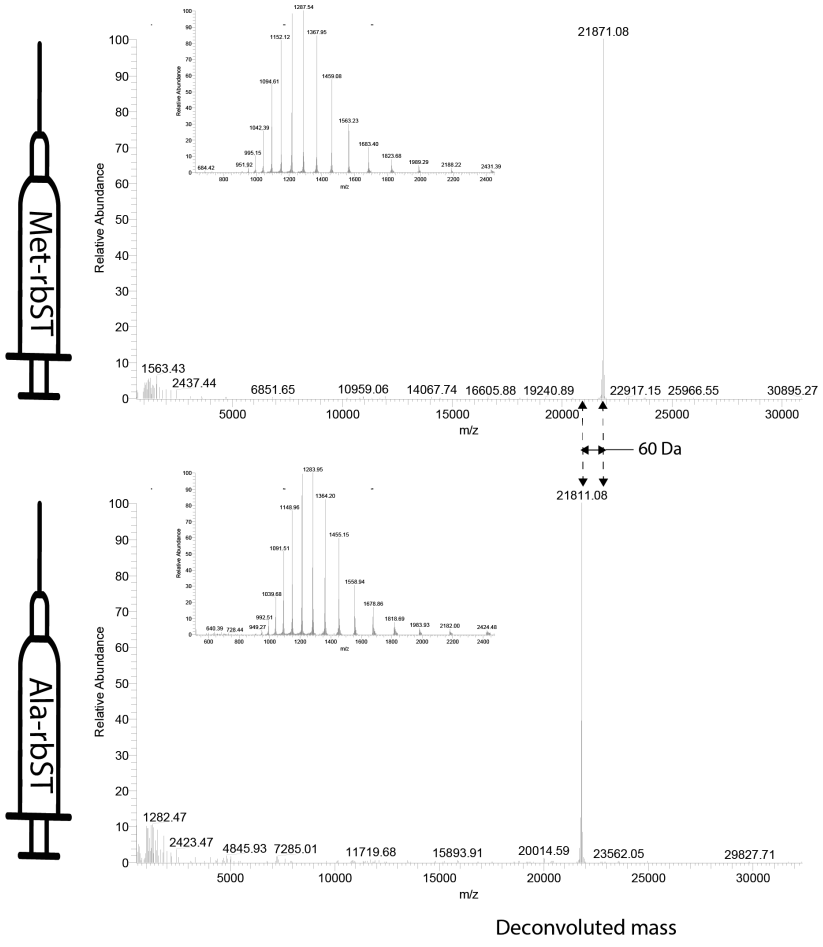


Figure 2. Deconvoluted MS data associated with mass determination of rbST from two different injection preparations and their corresponding mass to charge envelopes (insert); Met-rbST (top) and Ala-rbST (bottom).

For Ala-rbST the distinction from endogenous bST is not possible yet. Different approaches can be explored aiming at this distinction. One approach could be the profiling of the bST forms present, as endogenous bST exists of 4 isoforms in known ratios and injection preparations contain only one form of rbST. Significant distinction from the

natural profile could reveal the (ab)use of Ala-rbST. Unfortunately, our MS experiments where not sensitive enough to detect isoforms of endogenous bST in serum samples. However, technological improvements, might enable this distinction in the future. Another approach for exploration is the $^{12}C^{13}C$ isotope ratios of endogenous- and exogenous bST. Isotope ratios are not known for bST and its recombinant form yet, but when drawing the parallel with hGH, it is hoped that $^{12}C^{13}C$ isotope ratios might enable distinction of the endogenous- from the exogenous bST form. For hGH isotope ratios of injection preparation- and pituitary hGH were compared before and showed a significant difference in $^{12}C^{13}C$ isotope ratio for one of the tested injection preparations [22]. This research, however, dates from 1996 and to the best of the authors knowledge no further research on this topic is published. This is possibly due to the reached sensitivity of the method, as 2-3 µg of hGH was needed for detection, where the physiological range of hGH in human blood is 0.4 - 10 ng mL^{-1} for male and 1 - 14 ng mL^{-1} for female [23].

2.2 rbST treatment

Controlled animal experiments were executed to obtain representable samples of rbST treated dairy cows. These samples were used for method development, to detect rbST and its biomarkers as described in **Chapter, 2, 3 and 5**. Although these samples represent rbST treatment and are of great value for method development, to investigate the influence of treatment on biomarker responses and rbST presence in time, there are still knowledge gaps due to time and size of the controlled animal experiments. Execution of the animal experiments depends on project funding, animal ethical approval and availability of dairy cows. Consequently, rbST treatment periods in the controlled animal experiments are relatively short compared to real practice of rbST treatment on farms where rbST is used for milk enhancement. Therefore, a deviation from real practice is the moment to start the treatment, as best milk enhancement results are established when treatment starts 57-70 days after calving [24]. For practical reasons, this optimal period for starting treatment is not taken into account in the controlled animal experiments. Moreover, due to the relatively short treatment period of the dairy cows, the animal experiment only included a part of one calving cylcle. In real practice, however, rbST treatment prolongs over multiple calving cycles, i.e. calving, starting treatment, dry period, calving, starting the second treatment period, etc. The influence of a prolonged treatment period on presence of the biomarkers is therefore not known. The biomarkers IGF-1 and IGFBP2 respectively increase and decrease after each treatment and return to their initial concentrations prior to the next treatment. It is likely this pattern will also occur when dairy cows are rbST treated for a longer period of time and after a next calving. The formation of rbST induced antibodies, however, increases after the first treatment after which it gradually decreases. How this biomarker will react after a dry period, next calving and restart of the treatment is not clear, and to the best of the authors knowledge, also not described in literature for rbST treatment. Examples of other treatments in human show dual results. For instance, in patients retrieving IFNβ to treat multiple sclerosis, antibodies against IFNβ are formed and although treatment is continuous, antibody production is lost over time [25]. On the other hand, for treatment with

infliximab for inflammatory bowel disease treatment, there are examples of anti-drug antibodies staying present and interfering by diminishing treatment and even exhibit adverse effects in the patient, this then leads to discontinuation of treatment [26]. As the rbST induced antibodies are important biomarkers to point to rbST abuse, further research on prolonged treatment is required.

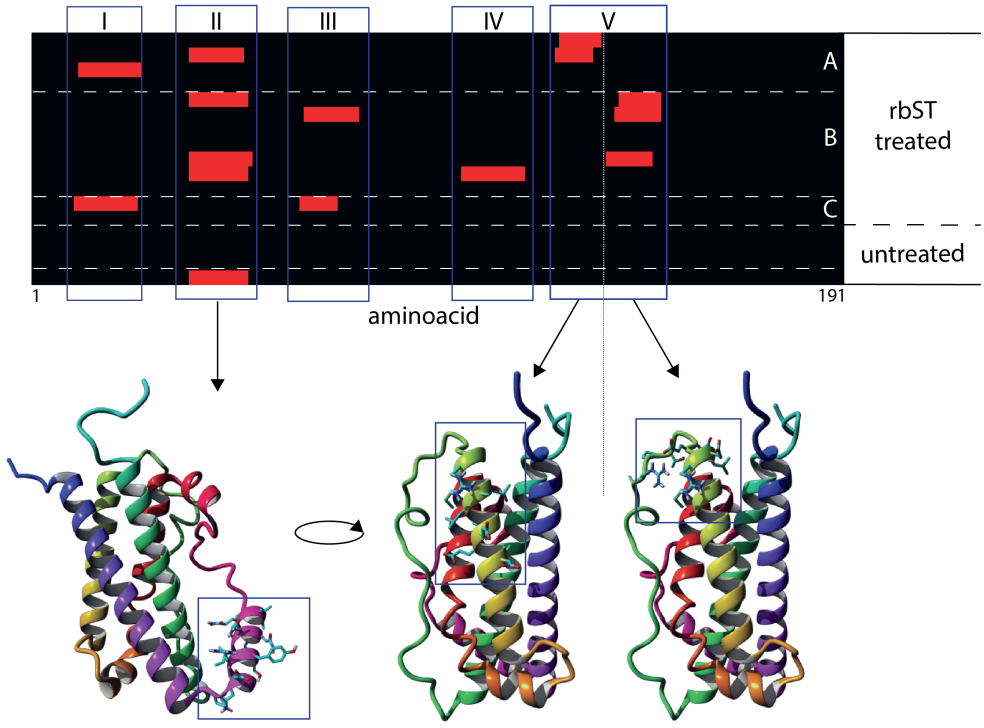
2.3 Legislation

Commission Regulation (EU) 2019/1871 [27] describes that for enforcement purposes, non-compliant results need to be unambiguously confirmed. The similarity between Ala-rbST and the endogenous bST hampers the identification of Ala-rbST. In the previous paragraphs suggestions are proposed for future research to enable unambiguous detection, however, due to the similarity between them it is unknown if this will provide tools for their confirmation. Another approach can be a reference point for action, as can be laid down for substances like bovine somatotropin according to this legislation. For Ala-rbST this is however not provided. Enforcement of abuse of Ala-rbST is therefore currently not possible. New strategies will be suggested, to encourage development of new ideas fit for this legislation, or trigger discussion on the current legislation for this specific compound.

3 Detection strategies for control purposes

3.1 Screening

The rbST induced antibodies have shown to be indicative biomarkers that reveal rbST (ab)use [1]. Consequently, several screening assays aim on the detection of these rbST induced antibodies [1, 28-34,**Chapter 2**]. Screening methods are applied to make a first sift in dairy farms, suspected and non-suspected of rbST abuse. At the moment, these methods are laboratory-based, and consequently time consuming due to the required transport and the type of detection methods used. Focus nowadays is also on testing on location, i.e. point of care (POC), ideally meeting the, by the acronym summarized ASSURED parameters (**A**ffordable, **S**ensitive, **S**pecific, **U**ser friendly, **R**apid and Robust, **E**quipment free, **D**elivered)[23, 35]. In many POC detection methods, antibody-antigen interactions serve as a basis, in Lateral Flow Devices (LFD), fluorescent, magnetic, and electrochemical biosensors amongst others [36,37]. These techniques and methods are employed for a variety of topics e.g. early detection and monitoring of cancer [38], infectious diseases [39] and veterinary diagnostics [40]. Detection of the rbST-induced antibodies at POC, is a promising strategy for rapid detection of rbST abuse. An advantage of testing at POC is that the material needed for confirmatory analysis can be sampled directly of a dairy farm suspected for rbST abuse. Preliminary results for POC testing of rbST induced antibodies are already shown by Ludwig et al. [33,34]. For the former described screening assays the full rbST protein is used as capture protein. This protein, however, is not commercially available and therefore production of these screening assays is hampered.



Region	Amino acid sequence	predicted flexibility
I	$_{11}$FANAVLRAQHLHQLAA$_{26}$	rigid
II	$_{38}$PEGQRYSIQNTQVAF$_{52}$	flexible
III	$_{64}$KNEAQQKSDLELLR$_{77}$	partly rigid
IV	$_{102}$VFGTSDRVYEKLKDL$_{116}$	partly rigid
V	$_{124}$MRELEDGTPRA$_{134}$	intermediate + flexible
	$_{136}$QILKQTYDKFDTN$_{148}$	intermediate + flexible

Figure 3. Binding (red) of serum to 15 aa long linear peptides from rbST treated dairy cows and untreated dairy cows. Sera from rbST treated dairy cows were obtained from 3 different animal experiments which were executed in 2008 (A), 2010 (B), 2014 (C). The blue boxes I-V depict amino acid regions recognized by the sera and their aminoacid sequence and rigidity are described (bottom). For visualisation region II and V are displayed in a 3 dimensional model representing the amino acid side chains by sticks.

As described in **Chapter 4**, the use of full rbST protein can be circumvented by using peptides which represent the epitope recognized by the antibody. For the rbST induced antibodies in the sera of dairy cows epitopes are unknown. Moreover, it is not clear if a single epitope, or a variety of epitopes is recognized by all sera. Therefore, sera from Met-rbST treated cows from our controlled animal experiments were mapped for rbST with linear epitopes. Not all sera showed binding to the linear peptides (Figure 3). Some sera are only able to recognize peptides when locked in a 3D confirmation [41] but also the immune-response can be lacking [28,32, **Chapter 2, 3 and 5**]. Using 15 aminoacid long linear peptides, binding to 5 regions of the rbST protein was observed, as shown in Figure 3. Antibody binding to rigid structures is known to be specific, whereas for more flexible structures, a-specific binding also occurs [42]. Therefore, rigidity of the regions was determined in silico, showing region I to be very rigid, region III and IV partly rigid, region V intermediate rigid and region II to be flexible [43]. Ideally, one rbST peptide from a rigid region is recognized by all sera. This would enable method development using only this specific peptide. Unfortunately, the peptide region recognized by most sera, region II, was also recognized by a serum obtained from an untreated animal. This might be due to the flexibility of this region. Binding to regions I, III and IV was only observed in a sera of a few cows. For region V a pattern was seen, an unexpectedly shift in recognized linear epitope between two different animal experiments. To explain this shift, differences in the two types of injection preparations were investigated. First, the Met-rbST protein amino acid sequences of the two batches injection preparations used in the animal experiments were determined and showed to be similar (data not shown). Second, the slow release formulation was tested on the amount of zinc ion. Zinc is present in the slow release formulations to capsulate and stabilize the growth hormone, forming an insoluble zinc-protein complex [44-46]. It is known that aggregation of proteins used for administration enhances the immune response, even if administered proteins are identical to endogenous proteins [47-49]. The degree of aggregation will be of influence on triggering the immune system. Next to the amount of zinc ion, the manufacturing process can influence the degree of aggregation [49,50]. The Met-rbST formulations used for the animal experiments of 2008 and 2010 contained 2235 mg kg^{-1} and 1159 mg kg^{-1} of zinc ion respectively. Therefore the aggregation degree for both Met-rbST formulations might be different, and might result in a different region recognized by the rbST induced antibodies.

Future perspective on screening

Although we were not able to find one specific peptide recognized by all sera, knowledge on the multiple recognized peptides can provide an alternative approach. For instance, a multiplex immunoassay can be developed for the peptide regions recognized, with exception of region II. This likely enables detection of the rbST induced antibodies without need of the full rbST protein. Moreover, it diminishes aspecific binding due to the absence of region II. The flow cytometric platform as used in **Chapter 2** would be suitable for this approach, and first results of its applicability are also demonstrated in **Chapter 4** where a peptide is used to capture a mouse monoclonal rbST induced antibody. In

other research fields, applicability of a similar approach is shown, for example detection of malaria induced antibodies in human [51] or for determination of the epitopes responsible for an allergic reaction [52].

3.2 Confirmation

According to Commission Regulation 2021/808 [53], confirmatory methods should unequivocally identify and if necessary quantify rbST. The goal was to discriminate bST from Met-rbST [54-57]. Targetted mass was a doubly charged peptide without modifications [58]. For a reliable rbST detection method by LC-MS/MS more peptides should be measured. Commission Regulation 2021/808 [53] prescribes 2 peptides using LC-HRMS/MS and in general, measurement of three to five peptides of a protein is considered to be good practice for quantification. When more peptides are measured, potential truncation or modification of rbST can be detected [59-61] and more information about the detected variants in Ala-rbST and Met-rbST could be obtained in serum samples of rbST treated cows as shown in **Chapter 5**.

Future perspective on confirmation

Due to the low concentrations of rbST present in protein rich matrices, abundant proteins needs to be depleted to enable rbST detection. In this thesis monolith microcolumns are used for the rbST enrichment, but new developments in this field should be tested and compared aiming at an increased rbST yield after enrichment. Moreover, with every pretreatment step more rbST is lost, therefore reducing the amount of pretreatment steps is preferable. By measurement of the intact protein instead of the tryptic digest, pretreatment steps can be reduced. Unfortunately, intact protein measurements still lacks sensitivity to measure concentrations of rbST present in serum [61], but further development and improvements of this technology, might lead to sufficient sensitivity in the future.

3.3 rbST detection in practice

For strategy development it is needed to consider that the antibodies induced by rbST also have a biological function. These antibodies reduce the presence of rbST itself [62], similar to anti-drug antibodies formed with other treatments [63-65]. For the confirmation of rbST this should be taken into account as the low concentrations of circulating rbST are likely to decrease in presence of rbST induced antibodies. In the Netherlands, currently, rbST misuse is monitored by screening for rbST induced antibodies in tank milk. In case of non-compliant screening results, however, no follow up by taking serum samples for confirmatory analysis is executed. The reason for this approach is the invasive character of serum sampling. This choice hampers enforcement as Met-rbST detection is currently only possible in serum as described in **Chapter 5**. Ala-rbST on the other hand, is also detectable in milk, but until now, it is not possible to distinguish the endogenous bST

from the exogenous Ala-rbST form. For enforcement purposes, unambiguous confirmation of forbidden substances identical to endogenous compounds is challenging and often not possible yet. Therefore, the unambiguous confirmation of these identical substances should be discussed, as revealing abuse requires another approach than the standard. To suggest other approaches for confirmation of use of forbidden substances identical to endogenous forms, a point of action can be set by legislation. The variety of the endogenous bST present in blood and milk complicates setting of a reliable point of action. Moreover, also learnings can be drawn from the field of sports where authorities face challenges that are similar to the ones faced in cattle. Forbidden substances have short detection windows and consequently the timing of sample collection is of importance [66]. In the field of sports therefore, natural variations in biomarker profiles are investigated and show to be relatively small. This is probably due to the strong human homeostasis [67]. Therefore, significant variations in biomarker profiles are used to pinpoint misuse of prohibited substance. For this the administration route, gender and training load, amongst others, are taken into account [68-70]. To determine deviated biomarker patterns, a harmonised, scientifically and legally robust programme on profiling of biomarkers during a longer period of time was developed [66]. This longitudinal monitoring of biomarkers is executed via the WADA biological passport [71].

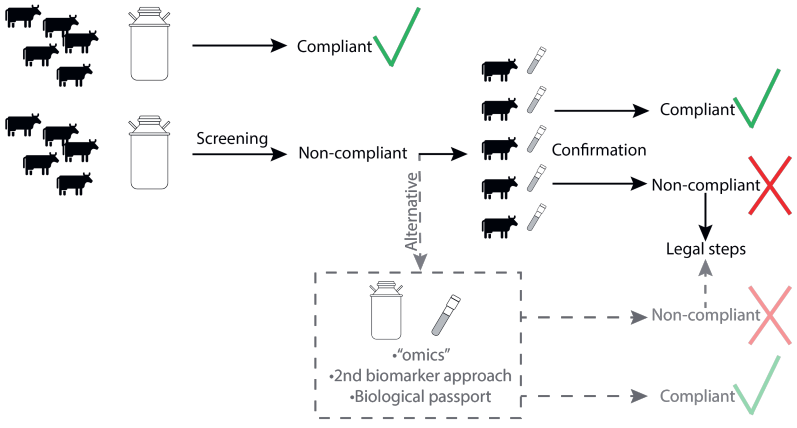


Figure 4. The currently used enforcement approach according to Commission Decission 2021/808 (black arrows), and an alternative route based on the approach used for sports doping in athlete sports by WADA (grey dashed arrows).

For the detection of the human growth hormone (rhGH, the human counterpart of rbST), hGH isoforms are measured and population based decision limits for two hGH biomarkers are set: IGF-I and Procolagen-III-peptide (P-III-NP). In practice atypical results are found in 80% of the cases where low doses of rhGH are used [72]. Moreover, when re-evaluting these results and apply individual longitudinal detection of the hGH biomarkers and hGH isoforms, 88% of the individuals showed responses above their personal upper threshold

[72], pointing to rhGH abuse [73,74]. In the field of sports rhGH abuse is confirmed by 2 biomarker screening assays. Biomarkers are used for the initial testing procedure, and when non-compliant results are found. Confirmation is executed with another screening assay [75]. This is another approach than the unambiguous determination of the compound itself as required for enforcement of prohibited substances in animal farming and can be an approach considered to deal with the similarity between endogenous and exogenous compounds as shown in Figure 4. Moreover, in both athletes doping and use of forbidden substances in animal farming, new developments for detection need to be kept up-to-date and improvements needs to be made. The "omics" to identify more robust individual blood signatures are suggested in both fields [71,76,77].

4 Conclusions

Altogether, this thesis has researched the use of antibodies as a versatile tool in detection strategies for proteins. For this rbST was used as a model, and methods were developed on the interface of multiple research fields. This resulted in a screening method, i.e. a method to indicate rbST abuse, and a method sensitive and specific enough to confirm the abuse of rbST. Moreover, also in-depth knowledge on the antibodies antigenic binding site was obtained, and applied in an peptide based assay format. Subsequently, development of these methods enabled studying the blood-milk barrier specificity for rbST allowing to critically look at legislation for this compound. Finally, gaps and new research motivated by the research chapters were addressed and summarized in Table 1. This includes new developments in the antibody field, new strategies for rbST detection accompanied with unpublished results as discussed, as well as ideas for future detection strategies to enable enforcement for rbST.

Table 1. Summary of knowledge gaps, challenges and directions for future research for each research chapter of the thesis: Antibodies as a versatile tool in detection strategies for proteins: Recombinant bovine somatotropin as a model.

Chapter	Knowledge gaps/ challenges	Directions for future research
2	• No diversity in treated Holstein dairy cows	• Build the animal experiment on a cross section of an average dairy farm population
	• Results not based on treatment according to manufacturers protocol	• Treatment of dairy cows according to manufacturers protocol
	• Lab based method specifically developed for serum matrix	• POC method applicable for matrices of interest
	• Decrease CCβ according to legislation	• Increase prediction rate by for instance adding extra specific biomarkers or increase specificity further
3	• The tested population of rbST treated animals is small	• Increase the sample size of the animal experiment to increase statistical power
	• Reduce the extensive sample preparation prior to measurement	• Investigate the possibility to measure rbST intact and furthermore optimize immuno-affinity enrichment (immunoaffinity formats and bio-recognition molecules)
	• The stock of polyclonal antibodies is finite	• Investigate applicability of lasting bio-recognition molecule
	• Method developed specifically for serum matrix	• Optimize and apply method on matrices of interest
	• Results are depended on in-house available, state of the art machines	• Investigate added value of technical improvements in LC-MS/MS and LC column material and sizes used
	• Results on one targetted peptide	• Target multiple peptides for quantification and to obtain insights on possible truncation
4	• Only results from singleplex assay with linear peptides	• Investigate possibility to multiplex linear and confirmational peptide based assays ultimately aiming for epitope profiling of for instance the rbST induced antibodies of dairy cows
	• Current sensitivity is sufficient for injection formulations however lacking for bST determination in serum of dairy cows	• Increase sensitivity, to enable profiling (see former)
5	Methods developed in chapter 2 & 3 are applied on an extended animal experiment closer to a cross section of an average dairy farm population. Therefore, knowledgegaps, challenges and future directions for the technical aspects of the methods are similar in this chapter	
	• Information on the influence of rbST induced antibodies on the circulating rbST *in vivo*	• The influence of the triggered immune response on the presence of rbST should be investigated to explore a possible correlation
	• Met-rbST is not detectable in milk	• Research presence of rbST fragments in serum and milk aiming at a physiological explanation of its absence in milk
	• No unequivocally identification of Ala-rbST possible	• Research possibilities to discriminate between endogenous bST and Ala-rbST and/or the possibility to set a threshold for Ala-rbST

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Summary

Antibodies are Y-shaped immunoglobulines (Ig) produced by the immune system to counteract the presence of foreign compounds (antigens). This characteristic is due to the specific recognition of the antigens by the antigen binding site. To explore antibodies as a versatile tool in detection strategies, in this thesis recombinant bovine somatotropin (rbST) is used as a model. With rbST as a model, antibody-antigen interactions are explored in two ways; on one hand, rbST-induced antibodies found in dairy cows are used as specific biomarkers indicating rbST abuse. On the other hand, antibodies are developed as assay components, able to detect or via immunoaffinity, enrich the antigen of interest. Chapter 1 describes the general introduction to this thesis and elaborates on antibodies, recombinant bovine somatotropin and detection strategies. The research reported in this thesis takes place on the interface of different research fields; immunochemistry, analytical chemistry and organic chemistry are combined to develop methods sensitive and specific enough to reveal rbST abuse in dairy cows. These tools then form the base for development of detection strategies.

The next three chapters focus on antibodies as versatile tools in detection of rbST and its abuse.

Chapter 2 describes presence of rbST induced antibodies in dairy cows after rbST treatment. These antibodies are specific biomarkers to reveal rbST abuse in dairy cows. In this chapter these rbST induced biomarkers are part of a set of three selected rbST dependent markers and combined in one parallel assay format. This resulted in characteristic treatment-dependent responses for all three individual biomarkers. Subsequently, serum biomarker profiling is explored as an adequate approach to discriminate between rbST treated and untreated groups. These results were combined and with the statistical prediction model k-nearest neighbours (kNN), resulted in good discrimination of treated and untreated animals: an overall sensitivity (true positive rate) of 89.1% and an overall specificity (true negative rate) of 97.7% were reached.

According to EU legislation, it is necessary to confirm these results prior to enforcement. In Chapter 3 we therefore employed rbST specific antibodies for monolith immuno-affinity enrichment of rbST in serum samples. After enrichment presence of rbST is confirmed with state-of-the-art ultra-high pressure LC-MS/MS (UHPLC-MS/MS) detection. The developed approach enables detection and confirmation of rbST in serum at a decision limit ($CC\alpha$) concentration of 0.8 ng mL^{-1}. We successfully applied the confirmatory method to serum samples from rbST treated cows that were found suspect with the in Chapter 2 described biomarker-based method. The use of rbST could be confirmed over 1 week after treatment, and the developed method demonstrated the sensitivity needed for effective control.

Knowledge on antibody specifics is evident to improve methods even further. Next step therefore, is antibody characterization. In Chapter 4 the rbST epitope compatible with the antigenic binding site of a selected rbST-induced antibody is explored. As there is restricted access to the full rbST protein, the use of peptides in rbST immunoassays can be favored. In Chapter 4 we identified the rbST epitope that is responsible for binding to the rbST-targeting monoclonal antibody 4H12 (mAb 4H12) to be $_{115}$DLEEGILALMR$_{125}$. This linear peptide was synthesized and coupled to microspheres, after which it was tested in a biorecognition competitive inhibition assay format. We observed IC_{50} values of ap-

proximately 0.11 µg mL^{-1}, which are lower than observed for the full rbST protein (IC_{50} = 0.20 µg mL^{-1}). Importantly, there was no binding with the scrambled peptide. Real-life applicability for detection of somatotropins (STs) in injection preparations of bovine-, porcine- and equine ST are shown. This newly developed immunoassay strongly supports future developments of peptide-based immunoassays to circumvent the limited access to the full protein.

The developed methods described in the former three chapters are the base to elaborate on detection strategies and propose a control strategy. For this, knowledge on elimination of recombinant bovine somatotropin (rbST) and its induced antibodies through milk is needed and described in Chapter 5. Two formulations are studied alanine-rbST (Ala-rbST), which is identical to endogenous bovine somatotropin, and methionine-rbST (Met-rbST), which differs by 1 amino acid from endogenous bovine somatotropin. The results show that the administered Ala-rbST is transferred from blood to milk but that this is not the case for Met-rbST. This suggests a blood-milk barrier-related specificity for these compounds. In addition, rbST-induced antibodies were formed in both, Ala-rbST and Met-rbST treated animals. In both treatments, the rbST-induced antibodies were transferred from blood to milk, showing no blood-milk barrier specificity for these antibodies. These elimination patterns show that, for enforcement purposes, the detection of rbST-induced antibodies in tank milk can serve to screen for rbST administration, and subsequent confirmatory serum analysis by LC-MS/MS is needed to identify whether Ala-rbST or Met-rbST has been used.

In Chapter 6 in-depth knowledge on the antigenic binding site of rbST induced antibodies in treated dairy cows, discussion points on results of this thesis and suggestions on control strategies are discussed. This discussion will be accompanied with ideas for future research.

About the Author

Curriculum Vitae

Nathalie Gabriëlle Esther Smits was born on the 3rd of April 1975 in Apeldoorn. After graduating from secondary school in 1992 (HAVO, Nederrijn college, Arnhem), she started her education in Clinical chemistry at the "Hogeschool van Arnhem en Nijmegen". In her last study year she learned about the daily routines in a hospitals clinical chemistry- and hematology laboratory (Rijnstate ziekenhuis, Arnhem) and she did research on development of a lateral flow device for detection of sulfadimidines at RIKILT (Wageningen). This working experience triggered her interest and curiosity for working with antibodies, which still is her working field. In 1997 she moved to Maastricht for her first job on tissue typing for transplant immunology at the academical hospital of Maastricht. After three years, her former colleagues from RIKILT offered her a technician position which she accepted, and started working at WFSR, which still is her current employer. During this period, she kept on studying, attending her first module at the Open Universiteit Nederland in 2006, and finishing a full Master in Environmental Sciences in 2013. After finishing her Master she started her PhD in 2013 at WFSR and ORC. This was partly possible on projects focused on method development for growth promotors, but for other parts of the PhD a part-time approach was needeed. During her PhD, Nathalie also kept on developing in her job, where she currently is projectleader of different projects with a current focus on allergens in food.

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Training activities

Discipline specific activities	Organizer	Year
Biomolecular MS and proteomics course	Hecklab	2014
Hormone and veterinary drug residue analysis	VDRA	2014
Euroresidue	ER	2016
Advanced food analysis	VLAG	2017
NVMS conference	NVMS	2015
MS of biomolecules/ applications in biopharmaceutical industry	BSMS	2014
Multiplex detection in the agro-food domain	XSamples	2015
Next-generation antibodies and protein analysis	VIB	2015
The analytical challenge conference	DTL	2015
Ligand-binding Theory and Practice	FEBS	2016
FABIAN symposium	Synthon	2018
FABIAN symposium	Tryskelion	2014

General courses	Organizer	Year
Scientific Writing	WGS	2014
Presenting with Impact	WGS	2015
Project and Time Management	WGS	2015
Writing Grant Proposals	WGS	2016
Guide to Scientific Artwork	WGS	2015

Other activities	Organizer	Year
Preparation of PhD research proposal		2013
Biosensor weekly group meetings	WFSR	2013-2022
Organic chemistry colloqia	ORC	2013-2021
Guiding WUR Molecular Life Science BSc students during their excursion as part of BIC-10807	WFSR	2013-2017

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