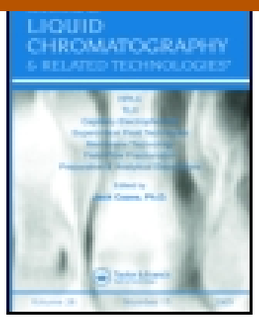

Journal of Liquid Chromatography & Related Technologies

ISSN: 1082-6076 (Print) 1520-572X (Online) Journal homepage: http://www.tandfonline.com/loi/ljlc20

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To cite this article: Patricia Regal, Alexandre Lamas, Cristina A. Fente, Carlos M. Franco & Alberto Cepeda (2017): Tracing (r)bST in cattle: Liquid-based options for extraction and separation, Journal of Liquid Chromatography & Related Technologies, DOI: 10.1080/10826076.2017.1331453

To link to this article: http://dx.doi.org/10.1080/10826076.2017.1331453

Accepted author version posted online: 19 May 2017.

Tracing (r)bST in cattle: liquid-based options for extraction and separation

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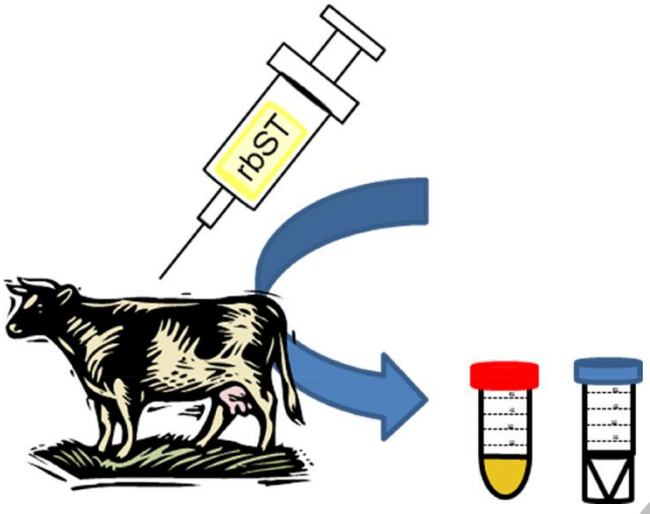
Abstract

Growth hormone (GH) or somatotropin (ST) is a species-specific polypeptide hormone produced in the pituitary gland of vertebrates. When administered exogenously to dairy cattle it has galactopoietic effects and is capable of increasing the milk yield. The beginning of the commercial production of recombinant variants of bovine somatotropin (rbST), by the end of the twentieth century, had a major economic impact in the dairy industry. Recombinant bST enabled large scale applications in farms, enhancing significantly milk production. While it is banned in the European Union (EU), several countries permit the trade and use of recombinant somatotropins in animal husbandry. Also, rbST-free dairy products can be frequently found in the market of those countries, even though these labels are not actually verified in a laboratory. In this context, effective analytical methods are needed for residue control to avoid an illegal use of rbST but also to prevent fraudulent labelling in some cases. The present review includes studies published in the last five years (from 2012 to 2017) to monitor rbST in bovine animals, using liquid-based applications. It is then intended to serve as a practical guide to help

those laboratories interested in developing analytical methods to detect rbST use and abuse.

GRAPHICAL ABSTRACT



KEYWORDS: somatotropin; bovine; bST; rbST; method; chromatography

1. INTRODUCTION TO (R) BST HORMONE

Growth hormone (GH) or somatotropin (ST) belongs to the group of peptide and protein hormones. This single-chain polypeptide is secreted by the anterior pituitary in all vertebrates and exerts a wide range of biological activities including growth, energy metabolism, sexual maturation and immunity. It has two highly conserved disulfide bonds in its molecule, which are the key for its biological activity in animals. GH action is initiated by binding to membrane-bound receptors that are located at various tissues, including liver, bone and mammary gland, amongst others [1].

Early research at the beginning of the 20th century in Russia and England found that milk production in cattle could be enhanced using pituitary extracts containing bST thanks to the ability of this hormone to coordinate and control bovine metabolism and nutrient partitioning. It was in the 1980s when it became economically and technically feasible to produce large quantities of pure hormone using recombinant DNA biotechnology, resulting in what is known as recombinant bST or rbST [2,3]. The galactopoietic potency of different molecular variants of pituitary and recombinant bST were evaluated by Monsanto Agricultural Company in 1992 with a group of fifty four lactating Holstein cows [4]. The animals receiving intramuscular injections of different forms of ST showed a greater response to recombinant variants than to pituitary molecules. In this context, rbST was approved for the first time for its use in the US by the Food and Drug Administration (FDA Agency) in 1993. This new product was released with the name of Posilac®, and it has played a significant role in the industrialization of US dairy production. FDA determined that the drug is safe for the treated animals and the animal products are safe for humans, hence the product is effective [5]. Monsanto's company licensed Eli Lilly to market its product in countries other than the US. The other two companies that developed recombinant versions of bST, Upjohn and American Cyanamid, no longer exist due to mergers and/or acquisitions by other companies.

The administration of exogenous somatotropin was first meant to help to support the growing world population and increasing demands of food, reducing also environmental impact of food production [2]. However, it soon became a tool to increase profit as it happened to many other veterinary drugs and/or growth promoters, and in many cases it

has been used without considering animal welfare [6-8]. Recombinant bovine somatotropin (rbST) can be used to enhance milk production in cows and in other dairy ruminants as sheep, goats and buffaloes [9]. Additionally, its potential as growth promoter has been also demonstrated in cattle and even in aquaculture [10,11]. The administration of rbST is permitted in several countries including the US, Brazil and Mexico, among others, while it has been banned in others, for instance Canada, Australia and New Zealand. In 1999, the European Union (EU) decided to definitively ban possible use of recombinant bovine somatotropin (rbST) in the European Members invoking animal welfare reasons, but also the impact on the European milk policy and consumer fears [12]. Besides, an increase of antimicrobial resistances in humans following to the use of rBST in dairy cattle is plausible [13].

Each time a new piece of legislation is adopted to ban or regulate a specific drug or veterinary treatment, a new analytical need is born to monitor its (ab)use. Additionally, the control of veterinary drug residues in food of animal origin is essential to protecting the consumers and, where necessary, verifying its compliance with the established maximum residue limits. A good example is a relatively recent scandal that took place in Spain in 2013, when a hundred of people were involved in a crime against public health and animal abuse due to illegal somatotropin treatments. According to the authorities, many farmers were administering rbST to cows as a common practice, using commercial injections from Mexico that were introduced illegally in the country. Clearly, recombinant bovine growth hormone is included among the current challenges of European residue control plans [14]. On the other hand, in countries where the rbST use is

permitted it is easy to find in the market products labelled as rbST-free, especially in dairy. In these cases, it might be necessary to check the truth of this indication on the labelling concerned. In this sense, novel analytical platforms based in liquid preparations and in liquid chromatography represent up-to-date technologies which allow very low detection limits as well as excellent precision and accuracy in the analysis [15].

The present review focuses on the existing liquid-based methodology to monitor (r)bST (extraction, separation, detection,…), providing a selection of the most relevant works published in the last 5 years (2012 – 2017). The study is meant to be a useful guide for future analysts and/or laboratories willing to monitor this hormone. The physical-chemical properties of bST and different recombinant versions are also summarized.

2. OPTIONS IN LIQUID EXTRACTION FOR (R)BST ANALYSIS

Liquid extractions and preparative steps using different solvents (precipitation, pH adjustment, dilutions, washings, etc.) are broadly and worldwide applied in analytical laboratories. Also, some chemical reactions as digestions, molecule cleavages and derivatizations, require a liquid medium to be performed. With this regard, the physical-chemical properties of both the solvent and the target analyte are very important factors and must be evaluated carefully to achieve the desired goal of the analytical method.

2.1. Physical-Chemical Properties Of Natural And Recombinant Bst

Pituitary growth hormone is a protein hormone with a molecular weight of about 22 kDa and substantial species-specific variations [16,17]. For instance, bovine growth hormone

differs very substantially from human or porcine hormones (from at least 10% to 40% of all residues), but is very similar to the ovine version [18]. Bovine GH (bGH or bST) is a single-chain polypeptide of 191 amino acids, with two disulphide bridges between the amino acids cysteine at positions 53 and 164, and positions 181 and 189 of its primary structure. It shows two transcriptional variants in its natural form where valine and leucine are found in an approximate ratio 1:2 at residue 127 due to an allelic polymorphism [18]. The frequencies of leucine$_{127}$ and valine$_{127}$ bST gene alleles are not a constant and differ between major dairy breeds but leucine can be considered the most abundant [19]. When purified from pituitary extracts, bGH shows a complex heterogeneity due to these naturally occurring transcriptional variations, but also because of translational and post-translational modifications [17,18]. The co-translational modifications result in two possible aminoacyl N-terminal residues, alanine (191 amino acids) and phenylalanine (190 amino acids), in approximately equimolar quantities (about 0.5 mole of each of them per mole of protein). This means that half of the polypeptide chains lack the N-terminal alanine residue. A third possibility with N-terminal methionine, present with less frequency, has been also described but data concerning its natural occurrence is scarce [18].

The genetically engineered versions, the so-called recombinant bovine somatotropins, have been produced with different N-terminal variants by different companies, all with full biological activity *in vivo*. Up to date, four analogues of bovine somatotropins, i.e. somagrebove, sometribove, somavubove, and somidobove, have been produced by recombinant-DNA techniques (see details in Table 1). Boostin® or Hilac® from LG

LifeSciences (South Korea) and Lactatropin® (Lactotropina® in Latin America) or Posilac® from Elanco (USA) are the two most famous and widespread products containing recombinant bST for use in dairy cattle. The difference between the most abundant endogenous form and the recombinant bST contained in Posilac® and/or Lactatropin® doses (sometribove) is only one extra amino acid at the amino-terminal end, methionine. In contrast, Boostin® (or Hilac®) contains a recombinant analogue (somavubove, developed by The Upjohn Company, USA) with a sequence virtually identical to the natural pituitary protein with terminal alanine. The structural formula (amino acid sequence) of the naturally occurring hormone and the different existing (r)bSTs are shown in Figure 1. Somagrebove and somidobove have been rarely used and mainly for research purposes in the 1990s [4,20,21].

As regard rbST solubility, this is extremely dependent on pH, being the minimum solubility between 7 and 8. Similarly to its natural counterpart, it is soluble in weak acidic or alkaline buffers, insoluble in water, alcohol, acetone, benzene, and chloroform [1]. Temperature has been noted to have very little effect on this solubility, ranging from 8.1 mg/mL at 5 ºC to 11.7 mg/mL at 37 ºC in pH 7.4 [22]. Also, ionic strength has a profound effect on the pH-solubility profile. High-pH buffer solutions, between pH 9 and 12, have been used to maintain solutions since the isoelectric point of bST is around 7.4. Hence, a common used solution consists of 0.025 M $NaHCO_3$ and 0.025 M Na_2CO_3 at pH 9.4 [23]. Conformational changes may occur under acidic conditions, resulting in significant precipitation, as a partial unfolding of rbST molecule can be induced by acid pH. The hydrophobic aggregate formed from precipitation of rbST has an extremely low

solubility, and hence it is very important to avoid this phenomenon [22]. It is also important to consider the occurrence of cross-linking and hydrolysis reactions produced on lyophilized rbST, both sensitive to the pH of the solution before lyophilization. Typical of many proteins, this hormone is susceptible to undergo significant precipitation induced by agitation [22]. Also, it should be noted that lyophilized bovine somatotropin is sensitive to photo-induced degradation of the disulfide bonds [24].

2.2. Liquid-Based Extractions And Preparative Solutions

The determination of recombinant somatotropin in animals has been mainly carried out through biomarker-based approaches, and only a relatively low number of methods to directly detect the hormonal compound have been developed so far [25]. The main challenge during method development for rbST analysis has been sample preparation, due to both the complexity of the biological samples and the very low levels of this protein in the treated animal. Preparative steps to determine rbST usually include different buffers to adjust sample pH and/or provoke precipitation of the hormone. Later, the detection method marks the steps to be followed, including for instance solid phase concentration and tryptic digestion of the target protein.

In Table 2, a summary of the liquid-based methods developed to extract bovine somatotropins from different matrices is presented, including works published in the last five years (2012 – 2017). Protein purification procedures usually take advantage of the changes in solubility of the target protein, altering the solvent conditions to force its precipitation [26]. As it can be observed in this table, the adjustment of sample pH with

phosphate buffer at pH~7 is a prerequisite to precipitate proteins with ammonium sulfate ($(NH_4)_2SO_4$). Firstly, somatotropins in aqueous solution (serum, milk) are lead to aggregation and precipitation by approaching the pH to their isoelectric point, where they are less soluble as they have zero net charge. This tendency to interact and aggregate in the solution is promoted by adding high salt concentrations. To this end, $(NH_4)_2SO_4$ is used as the salt of choice, since it is very soluble and relatively cheap. This salt is also very stabilizing to protein structure and it preserves its activity [26]. This "isoelectric tandem salting-out" precipitation technique was successfully applied for the determination of (r)bST in trout and buffalo serum, in the first case using very little amount of serum (only 50 µL), followed by a SPE clean-up with C4 cartridges and LC-MS determination [27,28]. The misuse of recombinant growth hormones in equine sports is also possible, especially due to the existence of possible cross-species applications to horses (GHs from other species have shown being biologically active in horses). In 2016, Wong et al reported a multiplexed mass spectrometric method for the simultaneous detection of three recombinant hormones in equine plasma: recombinant equine GH (reGH), recombinant human GH (rhGH) and recombinant porcine GH (rpGH) [29]. They used polyethylene glycol (PEG) 6000 and ammonium acetate at pH 5 (instead of phosphate buffer), in combination with ammonium sulfate precipitation. Due to the lower isoelectric point of recombinant human GH in in comparison to the other somatotropins included in this method, buffer and $(NH_4)_2SO_4$ were used at a lower pH than in other works for precipitation.

Regardless of the first precipitations step, a second solid phase extraction (SPE) is usually performed, with very few exceptions, before tryptic digestion and LC-MS determinations [27-29]. In this sense, some authors have reported that the best SPE performance is achieved with a C4 matrix active group, with particle size of 20 μm and 300 Å pore size [28]. They reported recoveries for somavubove and sometribove in buffalo samples around 30%, including the recombinant porcine ST used as internal standard. In some cases, the obtained SPE eluate may be further cleaned up by chloroform/methanol precipitation before digestion procedures [29]. In contrast to the rest, Smits et al developed in 2015 an interesting method of immune-affinity enrichment on monolithic micro-columns to extract rbST from serum of treated animals [30]. A polyclonal antiserum against rbST (sometribove) was obtained and *in-house* purified, to be applied to the monolithic micro-columns. With the monoliths contained inside pipet tips, and these tips placed on a multichannel automated pipette, the authors achieved a very effective extraction in a process that last 4.5 hours but works practically alone. The reported recovery of this immuno-enrichment was of approximately 50 % at concentrations up to 10 ng mL^{-1}, dropping to a half or less for the highest concentrations due to a saturation of the monolithic tips. In this work, after tryptic digestion and not before, the protein fragments were concentrated using non-polar polymeric SPE catridges and analyzed then with UHPLC-MS/MS.

Similarly to animals, synthetic human growth hormone (rhGH) is commercially produced for pharmacological treatments of growth-related diseases in humans [31]. However, it is also suspected of being misused in sports for performance improvements in athletes and

as such it is prosecuted by anti-doping agencies (World Anti-Doping Agency website). Once more, the similitude between this drug and its natural version complicates the detection of administration in sportspeople. In this context, the second most abundant variant of GH, containing fewer amino acids than the most abundant one (176 amino acid residues versus 191 amino acids) and existing at a constant ratio with respect to the other, can be used to demonstrate abuse. In 2015, Such-Sanmartín et al reported an interesting alternative method to purify and detect both human GH variants in plasma that could be transferred to the animal field [32]. With the Mass Spectrometry Immuno-Assay (MSIA) that the authors propose, no protein precipitation is required; instead, GH is purified from plasma using an antibody-based approach providing maximum sensitivity. Nevertheless, the precipitation based method remains as a promising tool, and it also can be scaled up for quantification of additional low-mass proteins that are non-specifically extracted in the precipitated solution, such as GH-related biomarkers [32].

2.2.1. Enzymatic digestions of (r)bST

Tryptic digestion of rbSt is sometimes required for identification purposes, as in LC-MS methods or for amino acid sequence elucidations. Liquid chromatography demands a preliminary enzymatic digestion of the hormone because this simplifies the chromatography and allows focusing the subsequent detection on only one small sequence of the protein. Common digestion reagents include NH_4HCO_3 or $(NH_4)_2CO_3$ buffers, EDTA, acetonitrile and trypsin. Smits et al reported the necessity of a first pH adjustment to 8-8.5 with hydrochloric acid, and addition of DL-dithiothreitol (DTT) to

reduce sulphur bridges. After incubation in this solution for 30min at 37°C, iodoacetamide is added for methylation of the free cysteine residues [30].

The digestion of somatotropin with trypsin is usually performed at 37ºC overnight or 16 hours [27,28], even though some authors have reported that 1 hour is enough [30]. The reaction must be then stopped by acidification with a quenching solution of for instance formic acid and water before instrumental analysis. Trypsin has been the enzyme of choice for digestion of bovine somatotropin so far. However, Such-Sanmartín et al tried both porcine trypsin and *S. aureus* V8-protease (endoproteinase Glu-C) for digestion of human plasma samples concluding that the latter is more adequate to generate individual and specific peptides of human growth hormone variants [32]. If this is also true for animal somatotropins is a fact that remains to be seen.

2.2.2. Extraction of (r)bST from commercial formulations

The different formulations developed for rbST administration in animal husbandry have been aimed mainly at achieving long-releasing (and consequently, long-acting) options [23]. As discussed previously (section 2.1), like many other proteins somatotropin shows pH-dependent solubility and stability, tendency to aggregate, heat and enzyme lability and rapid *in vivo* clearance. These properties make formulation design difficult. The administration of this hormone has to be done by injection, since it would be digested if added to feed, like any other protein. To avoid daily injections, and make its commercial use feasible in large farms, a long-release formula was firstly developed by Monsanto, consisting of the zinc salt of sometribove, suspended in food-grade vegetable oil (sesame

oil aluminum monostearate). This formula is injected under the skin, subcutaneously, and it would protect rbST from tissue proteases, preventing rapid degradation and allowing a prolonged release of the drug. Also, this kind of formula (emulsion instead of solution) protects somatotropin from moisture, thus helping assure chemical and physical stability [23]. Apart from rbST, Boostin® contains vitamin E and lecithin. Lactatropin® only declares zinc sometribove in its composition (equivalent to 500 mg of bovine somatotropin). Posilac®, registered trademark of Monsanto Company, contains a sterile sometribove zinc suspension. All current commercial products are prolonged-release injectable formulations in single-dose syringes, each containing 250 or 500 mg of rbST, capable of maintaining a sustained and "active-effective" level of hormone in the animal for about 14 days.

It may not be easy to obtain pure standard of rbST for research purposes, especially in Europe, as manufacturers are usually reluctant to sell their standards of recombinant variants due to the European ban of rbST use. Also because doubts may arise in relation to the recombinant version used in a specific formulation, it seems a good option to obtain the reference standard from the actual commercial dose. In this context, it is useful to have a reference method to extract the hormone from commercial formulations. Chang et al described a non-denaturing method in 1997 to extract rbST from oleaginous vehicles [21]. The protocol consisted on a simple extraction with a buffer solution of borate-ethylenediaminetetraacetate (EDTA) at pH 9.5 in a VanKel dissolution system, at 40°C for 3 hours. The recovery of rbST, in their case somidobove, was on average more than 90%. Recently, Castigliego et al described a very simple and straightforward method to

isolate rbST (somavubove) from slow-release preparations, i.e. Boostin® [28,33]. The reported method consists of two successive carbonate buffer (pH 9.6) additions, vortexing and cool centrifugation, in order to separate the grease layer from the protein solution. The protein solution concentration can be measured by spectrophotometry (280 nm) and further evaluated by SDS-PAGE.

3. SEPARATION OF (R)BST USING LIQUID CHROMATOGRAPHY

As in the case of extraction methods, physical-chemical properties also have to be taken into account when developing a separation step. The separation (r)bST using liquid chromatography demands a preliminary enzymatic digestion of the hormone using trypsin, which has been discussed in the previous subsection 2.2. This step simplifies the chromatography and allows focusing the detection on the N-terminal part of the protein. Actually, the N-terminal peptide is sufficient (and necessary) to discriminate between natural somatotropin and its recombinant counterpart. Besides, mass spectrometry is also more sensitive and simple than using the whole protein for the analysis.

Table 3 shows the most recently published works (2012-2017) using liquid chromatography to separate rbST (peptides after tryptic digestion) in biological samples. As it can be observed in this table, the preferred column packing sorbents for reversed phase chromatography are C18 and C8. The UHPLC separation of N-terminal rbST peptides from trout serum performed by Rochereau-Roulet et al was based on a gradient elution in a C18 column, using a binary mobile phase composed of acetonitrile and water with 0.1% formic acid [27]. Smits et al developed a method for bovine serum using also

UHPLC with a C18 column, acidified water and acetonitrile, and in this case with a slightly smaller particle size [30]. Similarly, Wong et al successfully separated the proteotypic peptides of four recombinant GH (recombinant equine, porcine, human and chicken growth hormones) and natural equine GH (eGH) using a nano-UPLC system equipped with a C18 column designed specifically for reversed-phase separation of peptides and proteins [29]. Once more, the selected liquid phase was a combination of acidified water and acetonitrile. Peptides were detected by high resolution mass spectrometry in positive ESI mode. For human growth hormones, this same combination of C18 particles and binary mobile phase has been reported [32]. In a method to determine (r)bST peptides present in buffalo serum, Castigliego et al combined a C8 column with a UHPLC binary system and a HPLC quaternary pump, in order to discard both head and tail of the chromatographic runs with the aid of a divert valve [28]. The HPLC pump worked with an isocratic combination of pure methanol and water, and UHPLC worked in gradient mode with acidified acetonitrile/methanol (80/20, v/v) and acidified water.

3.1. Considerations When Using Mass Spectrometry (LC-MS)

The unequivocal detection N-terminal peptides of (r)bST is usually carried out with triple quadrupole mass spectrometers working in multiple reaction monitoring (MRM) mode or high resolution mass spectrometers (HRMS), with positive electrospray ionization [27,28,30,34,35]. In these cases, the determination of only a part of the whole protein of somatotropin requires a digestion step prior its analysis, using the procedures already reported in section 2.2.1. The obtained N-terminal fragments are expected to differ

between natural bovine somatotropin and its recombinant versions (see Figure 1 for details) and are used for unequivocal identification.

As it was described in section 2.1, bovine somatotropin shows natural heterogeneity in its amino acid sequence due to transcriptional, translational and post-translational variations [18]. The main heterogeneity has been detected at two positions: the N-terminus and residue 127. As it is shown in Figure 1, these modifications include two possible residues at position 127 and two N-terminal residues, phenylalanine (NH_2-Phe_1-Pro_2…) and alanine (NH_2-Ala_1-Phe_2-Pro_3…). Clearly, for somidobove and somagrebove, marked differences exist between their NH_2 terminus and those of natural somatotropin variants. These differences simplify the development of analytical methods to monitor its use in bovine animals. However, for the two main rbST forms available on the market, sometribove and somavubove, the situation is not as simple because their structural differences diminish or even disappear. Sometribove, also known as methyonil-rbST, has an extra methionine compared to the phenylalanine pituitary variant, or a substitution if compared to the alanine one. The peptide fragments can be distinguished using mass spectrometry, and for this reason the analytical methods developed so far have targeted this recombinant version. However, the other form, somavubove, which is also widespread for dairy cattle, is identical to one of the variants of pituitary bovine somatotropin: bST with N-terminal alanine. For this reason, no methods for somavubove detection in bovine animals have been developed so far. It is technically impossible to distinguish somavubove, commercially known as Boostin® or Hilac®, from pituitary bST in bovine serum and/or milk. The only LC-MS method found in the literature has

been recently developed by Castigliego et al, not for cows but for buffalos [28]. In this context, it seems pertinent to develop effect-based analytical methods including biomarkers, quantitative ratios, profiling and other indirect techniques that could result in good alternatives. Moreover, the practicability of C^{12}/C^{13} measurements for the detection of somatotropin has not been reported yet.

4. OTHER LIQUID APPLICATIONS AND INDIRECT DETERMINATIONS OF RBST

Flow cytometry is a biophysical technique employed routinely in many scientific fields and for different purposes, including cell counting, immunoassays and protein isolations. The operation of flow cytometers relies on an isotonic liquid or fluid called sheath fluid. This liquid flows in a laminar way with cells or particles in suspension, carrying and aligning them so that they can pass individually through a measuring point [36]. These liquid-based systems have demonstrated its applicability also to monitor somatotropin abuse in cattle. A multiplex flow cytometric immunoassay capable of discriminating between milk from rbST-treated and untreated animals was developed by Ludwig et al in 2012 [37]. The method was based on the detection of anti-rbST antibodies in raw milk. The authors stated that pooled tank milk samples should be preferred to minimize the problem of non-responding cows in the farm. A similar approach was reported a year later by the same team, using a flow cytometric immunoassay to profile various biomarkers of rbST in bovine serum [38].

Other liquid-based platforms include lab-on-a-chip devices, which integrate microfluidic systems which allow solvent-saving, a reduced time of assay and lower cost of equipment. These analytical methods have also been used as an alternative to more expensive platforms in rbST abuse monitoring [39-41]. A microfluidics system coupled to a smartphone enabled to build a portable immunoassay platform capable of detecting rbST biomarkers in milk, including different antibodies and also positive and negative controls [42]. This smartphone-based protein microarray requires different buffers and solutions to function. The analyst is able to analyze the images instantly on the phone thanks to Quantum dots, built-in UV LEDs and a custom-developed Android application.

Enzyme-linked immunosorbent assays (ELISA) are very simple routine procedures based on the binding antigen-antibody in a liquid medium. This procedure has been used to measure the responsiveness of the animal to rbST treatments, using preferably milk and serum as target matrices. A new ELISA based on an acid-stripping procedure was developed very recently to measure buffalo serum and milk responsiveness towards rbST [33]. Antibody production for these assays is a complicated task due to the high degree of homology of rbST, particularly sometribove and somavubove, and bST. A novel strategy for the production of specific polyclonal antibodies for methyonil-rbST based on the use of the full-length protein, a N-terminus fragment and a multiple antigen peptide [43]. The polyclonal antibodies exhibited a great binding ability towards rbST in a competitive antigen-coated ELISA format.

5. CONCLUSIONS

Despite being banned in several countries as the European Union members, Australia or New Zealand, among others, the use of recombinant bovine somatotropin in cattle is quite likely, considering the significant worldwide livestock industry. Also, the potential existence of fraudulent labelling of rbST-free dairy products is not monitored in those countries where its use is permitted, presumably due to the absence of analytical options. It is curious that only one confirmatory method for somatotropin administration in bovine animals has been published in the last five years. The present review can serve as a practical guide to promote the development of analytical methods to detect rbST use and abuse. Liquid-based strategies constitute versatile and straightforward options for this task, including protein precipitations, liquid extractions and hormone digestions, coupled to liquid chromatography and mass spectrometry determination.

ACKNOWLEDGEMENTS

Authors acknowledge the financial support of the Spanish innovation program *"Programa Estatal de Investigación, Desarrollo e Innovación Orientada a los Retos de la Sociedad"* (project AGL2014-58881-R).

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Table 1. Summary of the existing pituitary and recombinant bovine somatotropins, including differential characteristics of their amino acid sequences.

Analyte	Product	Producer	Amino acids	NH_2 - terminus*	Residue-127	M.W.†
bST	natural growth hormone	pituitary gland	191 (or 190)	NH_2 – Ala_1 – Phe_2 – (or NH_2 – Phe_1 -)	Leu_{127} (or Val_{127}) (2:1)	21811.9437 (or 21722.8497)
rbST	Lactatropin® and Posilac® (sometribove)	Monsanto (USA) ELANCO, Eli Lilly (USA)	191	NH_2 – Met_1 – Phe_2 -	Leu	21872.0633
rbST	Boostin® or Hilac® (somavubove)	LG Life Science, Ltd. (Seoul, Korea)	191	NH_2 – Ala_1 – Phe_2 -	Leu	21811.9437

rb ST	Somidobove	ELANCO, Eli Lilly (USA)	199	NH_2 – Met_1 – Phe_2 – Pro_3 – Leu_4 – Asp_5 – Asp_6 – Asp_7 – Asp_8 – Lys_9 – Phe_{10} -	Leu	228 18.0 384
rb ST	Somagrebov e	American Cyanamid (USA)	193	NH_2 – Met_1 – Asp_2 – Gln_3 – Phe_4 -	Leu	221 15.2 810

*NH_2 – $residue_1$ – $residue_2$– (amino acids nomenclature, Ala: alanine, Phe: phenylalanine, Met: methionine, Pro: proline, Leu: leucine, Asp: aspartate, Lys: lysine, Gln: glutamine, Leu: leucine, Val: valine).

†Source: U.S. National Library of Medicine (ChemID*plus*); molecular weight of natural bST is calculated only for 127-L-Leu variants.

Table 2. Options for liquid extraction and preparative steps for (r)bST analysis in animal matrices reported in the last five years (2012-2017).

Analyte	Matrix	Liquid-based extraction	Complementary steps	Recovery and others	Reference
rbGH (Lactatropin®)	trout serum (50 µL)	0.1 M phosphate buffer pH 6.9 + precipitation 15h at 4°C with 45% (NH4)2SO4 + dissolve pellet in 0.1 M phosphate buffer at pH 6	SPE C4 cartridges + concentration + ice-cold MeOH precipitation 1.5h Overnight tryptic digestion in 50 mM NH4HCO3, 10 mM EDTA and 1 µM pepstatin pH 7.9, acetonitrile 16%	Miniaturization capabilities Long precipitation step Long step related to nitrogen drying after elution	[27]
rbGH (Lactatropin®)	bovine serum (1 mL)	immunoaffinity cycling enrichment on *in-house* monolithic micro-columns (4.5	Tryptic digestion: adjust pH 8-8.5 with HCl + DL-dithiothreitol (30 min, 37ºC) + iodoacetamide	Long procedure: enrichment 4.5 h + digestion and concentration 3 h, but semi-	[30]

		hours): *1)* load sample diluted 1:1 with phosphate buffer at pH 7 *2)* wash columns with buffer 3) elute with 200 mM NaOH Add Tris until pH > 10	(30 min, dark) + trypsin + acetonitrile (1h, 37ºC) SPE concentration of digest	automation capabilities ~ 50 % recovery at 10 ng mL^{-1}	
rbST (Boostin®) and mrbST (sometribove)	buffalo serum (1.2 mL)	phosphate buffer 0.1M pH 6.9 + add saturated (NH4)2SO4 under stirring until 45% + 4 ºC overnight + centrifuge + resuspend pellet in	SPE C4 cartridges Ice-cold MeOH + C2H7NO2 1M + centrifuge + resuspend pellet in 50 mM (NH4)2CO3, 5 mM EDTA, pH 8 + trypsin overnight 37 ºC	~ 30 % recovery	[28]

		phosphate buffer 0.1M pH 6	+ dry and reconstitute		
rbST (Boostin®)	oil-based slow-release formula (500 µL)	*1)* eliminate grease layer (40 mM NaHCO3, pH 9.6 + vortex + centrifuge) *2)* 1 mL protein solution (repeat previous step) *3)* collect 2 mL of protein solution	Measure protein concentration in spectrophotometer (280 nm absorbance, extinction coefficient 13610 $M^{-1}cm^{-1}$) Evaluate contaminants by SDS-PAGE		[28]

Table 3. Separation of (r)bST present in animal matrices using liquid chromatography, including methods reported in the last five years (2012-2017).

Analyte	Matrix	LC column	Separative performance	Additional details	Reference
N-terminal rbGH	trout serum	BEH C_{18}	good peak shape; elution < 3 minutes	LOD 0.5 and LOQ 1 μg mL^{-1} rbGH	[27]
N-terminal rbGH	bovine serum	Kinetex C_{18}	elution < 2 min	CCα 0.8 and CCβ 1.6 ng mL^{-1}	[30]
N-terminal rbGH	buffalo serum	ACE Excel 3 C_8	head and tail of HPLC runs are discarded	HPLC quaternary pump + UHPLC binary pump LLOD 0.25 ng/ml and LLOQ 1 ng/ml	[28]

Figure 1. Primary structure of pituitary and recombinant bovine somatotropins.