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Detection of recombinant bovine somatotropin in milk and effect of industrial processes on its stability

Marie-Hélène Le Breton[a,b,*], Andrea Beck-Henzelin[a], Janique Richoz-Payot[a], Sandrine Rochereau-Roulet[b], Gaud Pinel[b], Thierry Delatour[a], Bruno Le Bizec[b]

[a] Nestlé Research Center, Nestec Ltd., P.O. Box 44, CH-1000 Lausanne 26, Switzerland
[b] Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), USC INRA 2013, Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique (ONIRIS), Site de la Chantrerie, BP 50707, F-44307 Nantes Cx 3, France

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ABSTRACT

A LC–MS/MS method has been developed for the direct detection of recombinant bovine somatotropin (rbST) in milk and dairy products. The sample preparation protocol is based on a solid phase extraction step followed by precipitation with cold methanol and enzymatic digestion. The analysis is focused on the tryptic N-terminal peptide, specific of the recombinant form of the hormone and the detection is performed by LC–ESI(+)-MS/MS. This method has been validated according to the European Union criteria described in the Directive 2002/657/EC. Acceptable performances, with a decision limit (CCα) of 1.24 ng mL^{-1} and detection capability (CCβ) of 1.92 ng mL^{-1} were obtained. Calculation of repeatability and intermediate reproducibility of the signal at 100 ng mL^{-1} lead to relative standard deviations lower than 20%, showing the robustness of the method. Samples subjected to various industrial processes namely, heating, freezing, defatting, pasteurization and spray-drying were then analysed in order to determine the consequences of these treatments on the stability of the hormone. Results showed that temperature related processes, such as pasteurization and spray-drying induce a loss of the hormone up to 95%.

1. Introduction

Bovine growth hormone, also called somatotropin is frequently used in animal production, either as a growth promoter or to increase milk yield in dairy farming. It is responsible for various physiological effects, that have been discovered with the injection of pituitary extract to lactating animal [1]. Its ability to enhance milk production is mainly due to an action on the partitioning of absorbed nutrients. It involves both direct effects of the hormone on tissues and indirect effects mediated by somatotropin-dependant factors such as insulin-like growth factor 1 (IGF-1) [2–4]. This increase of milk production has been largely described in the literature and can vary from 5% to 40% [5–9].

Treatment of animals with somatotropin have became possible with the commercialisation of a recombinant form of the hormone (recombinant bovine somatotropin or rbST). Its use in dairy farming is authorized in some countries such as the United States since 1994 [10], but many countries like those from the European Union have prohibited its application [11,12]. All these statements have increased the need for an analytical method able to determine the presence of rbST in biological matrices.

Even if the use of this hormone has been regulated for 15 years now, the detection and quantification of recombinant bovine somatotropin in milk is still an issue, due to a crucial lack of analytical tools in order to comply with regulation. Indeed, no method is available to detect specifically rbST in milk or dairy products.

Bovine somatotropin is a protein hormone with a molecular mass of 22 kDa [13]. The recombinant form has been developed and first commercialised by Monsanto. It is now mainly produced and distributed by Elanco (Eli Lilly). The difference between the endogenous and the recombinant form is limited to one amino acid located at the N-terminal of the sequence. An alanine in the case of the endogenous form is replaced by a methionine in the rbST [14]. This slight difference prevented for a long time the development of an analytical method able to detect and discriminate both forms.

The previous published methods to detect bovine somatotropin were based on immunoassays with no possibility to differentiate recombinant and endogenous form of the hormone [15–18]. Moreover, the quantification of proteins, especially in matrix rich in proteins such as milk and at very low level of concentration has always been a problem. Only recently a mass spectrometry based

* Corresponding author at: Nestlé Research Center, Nestec Ltd., P.O. Box 44, CH-1000 Lausanne 26, Switzerland. Tel.: +41 21 785 80 96; fax: +41 21 785 85 53.
E-mail address: marie-helene.le-breton@rdls.nestle.com (M.-H. Le Breton).

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method has been developed for the specific detection of recombinant somatotropin. It was first developed for control of doping in horse racing [19] and then adapted to the bovine and caprine field [20–22]. But these various methods were dedicated to serum and plasma matrices and a specific development was needed for their adaptation to milk and dairy products.

The aim of this study was to develop a confirmatory method to detect specifically and quantify rbST in milk. This method has been validated in compliance with the requirements set by the EU Commission Decision 2002/657 [23]. This protocol was then applied to different types of milk products in order to study the stability of rbST consequently to various industrial processes.

2. Materials and methods

2.1. Samples and process parameters

The method has been developed and validated in commercial UHT partially skimmed milk samples collected from the local market. Trials have also been performed on raw milk, condensed milk and milk powder. The samples were reconstituted or diluted with water to obtain the corresponding proportion of milk proteins. The analytical procedure was also applied to samples subjected to various industrial processes, namely heating, defatting, pasteurization, freezing and spray-drying. Heating was performed in a water bath at 50 °C for 2.5 h. Defatting consisted in a centrifugation of raw milk at 2200 × *g* for 15 min and removal of the upper fat layer. Freezing was achieved by storing the samples at −20 °C for one night. Pasteurization was performed in a process lab, by heating the milk at 72 °C for 30 min. Spray-drying was carried out in a NIRO tower in which 15 L of milk were processed to obtain around 600 g milk powder.

2.2. Reagents and chemicals

All reagents and solvents were of analytical or HPLC grade quality. The standards of hormone recombinant bovine somatotropin (rbST) and recombinant equine somatotropin (reST) were purchased from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, USA) and Bresagen Limited (Thebarton, Australia), respectively. They were dissolved in ammonium bicarbonate 50 mM, at a concentration of 1 mg mL^{-1} and 10 μg mL^{-1}. The N-terminal tryptic peptides rbST (MFPAMSLSGLFANAVLR), reST (MFPAMPLSSLFANAVLR) and rbST labelled with ^{13}C (MFP(A^{13C})MS(L^{13C})SG(L^{13C})F(A^{13C})N(A^{13C})V(L^{13C})R) were synthesized by Millegen (Labege, France). Sodium carbonate, formic acid and sodium hydroxide were from Merck (Darmstadt, Germany). Trifluoroacetic acid and ammonium bicarbonate were from Fluka (Buchs, Switzerland). Sequence grade modified trypsin was purchased from Promega (Madison, USA). HPLC grade acetonitrile and methanol were from J.T. Baker (Peypin, France). SPE C_4 cartridges (500 mg/6 mL) were obtained from Interchim (Montluçon, France).

2.3. Sample preparation

Ten millilitres of milk or processed milk were spiked with 100 ng mL^{-1} of reST as internal standard and applied onto a SPE C_4 previously conditioned with 10 mL methanol and 10 mL water. The SPE cartridge was then washed with 5 mL water + 0.1% TFA, followed by 5 mL water/acetonitrile 70:30 (v/v) with 0.1% TFA. The cartridges were eluted with 7 mL of a water/acetonitrile mixture 20:80 (v/v) containing 0.1% TFA. The eluate was evaporated under nitrogen stream at 50 °C to reduce the volume approximately to 1 mL. The proteins of interest were then precipitated with addition of 5 mL of cold methanol and stored for at least 1 h at −20 °C. After centrifugation for 10 min at 1500 × *g* and removal of the upper layer, the precipitate was dried under nitrogen stream at 50 °C. Proteins were digested with 2 μg of trypsin in 100 μL of ammonium bicarbonate 50 mM and 20 μL acetonitrile overnight at 37 °C. The extracts were then evaporated and reconstituted with 40 μL of a solution containing 1 μg mL^{-1} N-terminal tryptic rbST labelled ^{13}C peptide in water/acetonitrile 80:20 (v/v) with 0.1% formic acid used as external standard. The samples were subsequently analysed by LC–MS/MS.

2.4. LC–MS/MS measurement

The HPLC system consisted in an Agilent 1200 (Agilent Technologies, Santa Clara, USA) with a C_{18} Interchrom QS Uptisphere 3HDO 150 mm × 2 mm, 3 μm, 100 Å column (Interchim, Montluçon, France). Peptides were separated using a mobile phase composed with acetonitrile + 0.1% formic acid (A) and water + 0.1% formic acid (B). The elution gradient started with 10% A increasing to 55% in 5 min and then to 65% in the following 5 min. The gradient reached 100% A after 15 min and decreased to initial conditions at 17 min and remained at 10% A for 3 more minutes. The total run time was 20 min and a divert valve was used to let the sample pass into the instrument from 5 to 11 min. The solvent flow rate was 300 μL min^{-1} and injection volume 20 μL. The MS instrument was a API 5000 (Applied Biosystems, USA), fitted with an electrospray ion source in positive mode. Mass spectrometric acquisition was performed using the following working parameters: capillary voltage was set at 5000 V, nebulizer at 55 psi, gas flow at 13 L min^{-1} and gas temperature at 300 °C. The triple quadrupole was used on SRM mode and data were collected and analysed with the Analyst 1.5 software (Analyst, USA).

2.5. Method validation

The method has been validated in compliance with the requirements described in the European Commission Decision 2002/657 [23,24] with the determination of the specificity, linearity, decision limit (CCα) and detection capability (CCβ), precision and accuracy. Confirmation criteria as defined in the EU Commission Decision 2002/657 [23] were applied to confirm the presence of rbST within the matrix: at least four identification points, peak area ratios for the different transition reactions recorded within the required limits of acceptance, retention time within 2.5% of the retention time of a standard injected on that day. The selectivity of the method was assessed by analysing 20 different blank milk samples to check the absence of any interfering signal in the zone where the analyte's elution is expected.

The linearity of the analytical method was proved by assessing the linearity using a matrix-matched calibration curve. This calibration curve was performed in the matrix, i.e. milk samples, fortified with 20, 40, 60, 80 and 100 ng mL^{-1} rbST. On each validation day the calibration curves were constructed by means of plotting the detection response of the matrix-matched standard solutions versus the concentrations by means of regression analysis. From these data the regression coefficient of the calibration curve was calculated and the required criteria for correct linearity was $R^2 > 0.99$. The decision limit (CCα) and detection capability (CCβ) were calculated by analysing 20 different blank samples and the same samples spiked with rbST at 100 ng mL^{-1}. Precision represents the variability of independent results obtained under stipulated conditions (repeatability and intermediate reproducibility). Only intermediate reproducibility was determined because all the experiments were performed in the same laboratory. Precision and accuracy (within- and between-day) were calculated from the analysis of six aliquots from a blank matrix, fortified with rbST at 100 ng mL^{-1} and performed in duplicates by three different operators on 6 sepa-