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Identification of Cows Treated with Recombinant Bovine Somatotropin

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A method for the specific detection and quantification of recombinant bovine somatotropin (rbST) in bovine blood has been validated according to criteria described in the EU Commission Decision 2002/657/EC. The method is based on a thorough purification procedure followed with the detection by LC−ESI-MS/MS of the tryptic N-terminal peptide specific of the rbST. The recombinant equine somatotropin (reST) is used as internal standard. Performance of the method was assessed based on specificity, linearity, trueness and repeatability. Decision limit (CCα) and detection capability (CCβ) were found to be 2.5 ng mL^{-1} and 6.8 ng mL^{-1}, respectively. This method was subsequently applied to the analysis of serum and plasma collected from two different animals treated with 500 mg of rbST. No significant variations were observed when analyzing either serum or plasma, but an important difference between animals was encountered. In all cases, recombinant bovine somatotropin was still detected two weeks after administration.

KEYWORDS: Growth hormone; somatotropin; mass spectrometry; dairy cows; validation; kinetic; serum; plasma

INTRODUCTION

In dairy farming, recombinant bovine somatotropin (rbST), also called growth hormone, is used to treat cows in order to increase milk yield. The efficiency of this hormone has been demonstrated in many studies (*1*−*4*), and even if rbST is banned in most of the countries (*5*, *6*), it is a common practice in a few of them. Particularly, it is commonly used in the United States since its authorization by FDA in 1994 (*7*).

The hormone, a 191 amino acid long protein, had always shown some difficulties to be analyzed. So far, methods were limited to immunoassays (*8*, *9*) with the problem that native and recombinant forms were not differentiated (*10*, *11*). A few attempts have been published, but they all remained unsuccessful to specifically detect rbST at physiological levels (*12*, *13*). Only recently, methods based on mass spectrometry have been developed for the specific analysis of recombinant equine and bovine somatotropin in plasma and serum (*14*−*16*) and the kinetics of elimination of rbST in the serum of a treated animal has been described (*16*). The level of rbST never exceeded 10 ng mL^{-1}, and rbST was still detectable 4 days after treatment. This study showed for the very first time the unambiguous detection of rbST in samples collected from an animal subjected to treatment with Lactatropin, a prolonged-release formulation of rbST. However, this work was limited to one animal and the analysis of serum only.

The objective of the present study was first to validate the method according to the criteria described in the EU Decision 2002/657 (*17*) in order to assess performance of the developed protocol. This procedure was then used to analyze samples collected from two animals treated with rbST. It was the opportunity to extend the number of samples analyzed and therefore to ensure the robustness of the protocol. It also allowed detailed description of the kinetics of elimination of rbST in the organism of treated animals. In addition, both serum and plasma have been analyzed and the contents of rbST in the different fractions of blood were compared. The difference of concentration of rbST within two different animals was also examined.

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MATERIALS AND METHODS

Animal Study. The experimental procedure was performed at ENSAIA (Vandoeuvre les-Nancy, France) in agreement with animal welfare rules. Two cows in lactation identified by number 1112 and 1142 were treated with Lactatropin (Elanco Animal Health, Sandton, South Africa). They both received two injections of 500 mg of rbST, one at the beginning of the study and the second injection two days later as described in **Figure 1**. Blood samples were collected on a daily basis during the treatment in two different tubes. The first part was kept in dry tubes, incubated one night at 4 °C and then centrifuged to collect serum. The second tube contained an anticoagulant, heparin. Samples were centrifuged and the supernatant was collected to obtain plasma. Samples used for the validation were collected from various animals never treated with rbST. All the samples were stored at −20 °C until analysis.

Reagents and Chemicals. A standard of recombinant bovine somatotropin (rbST) was obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA). Recombinant equine somatotropin, reST (EquiGen-5) was purchased from Bresagen Limited (Thebarton, Australia). The synthetic peptides with the amino acid sequence MFPAMSLSGLFANAVLR (N-terminal tryptic rbST)

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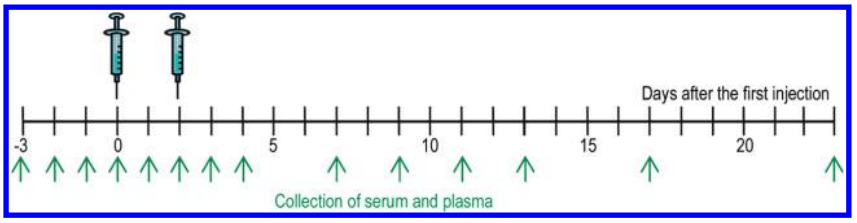

Figure 1. Outline of the animal study.

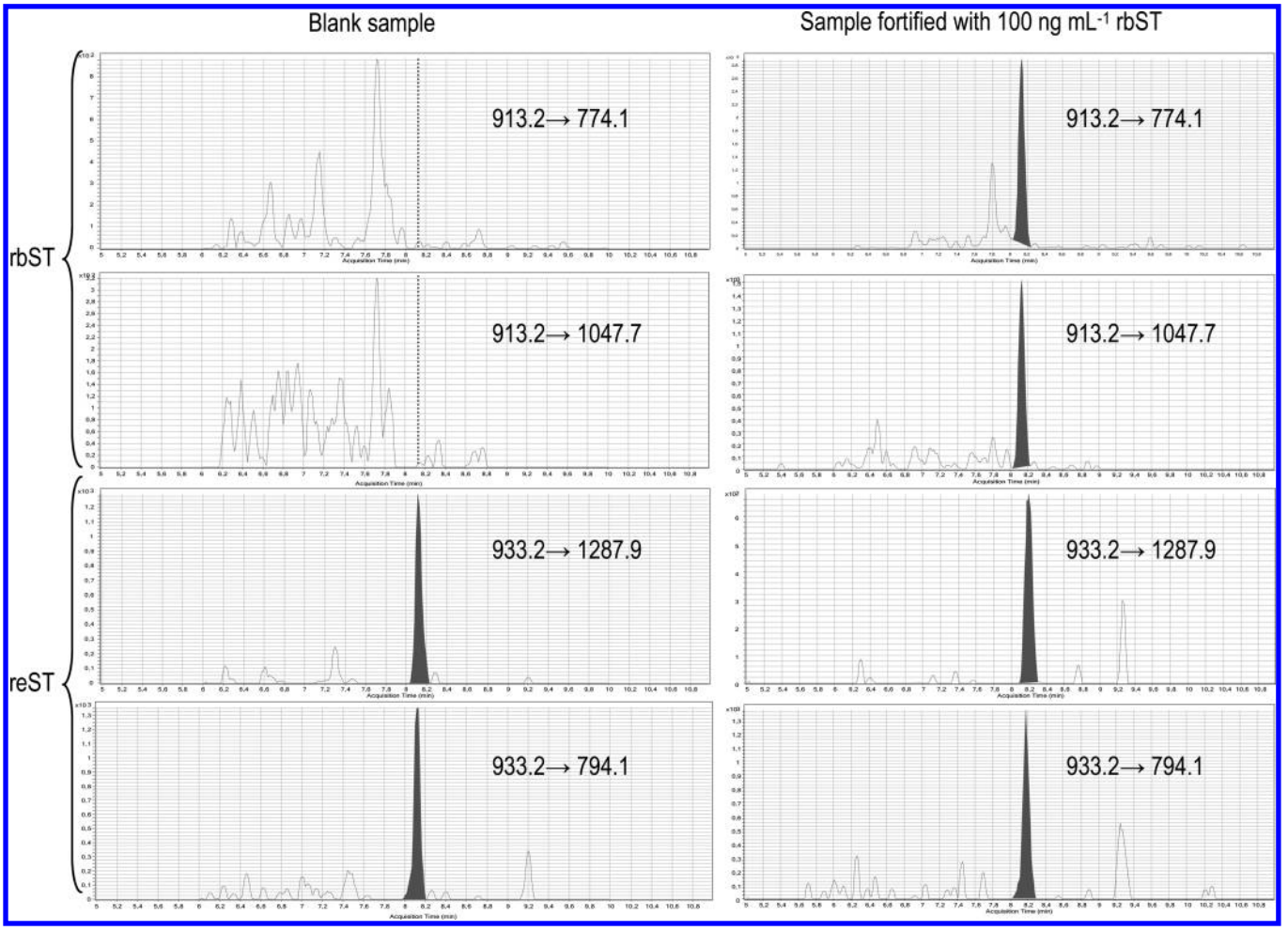

Figure 2. Selected reaction monitoring (SRM) ion chromatograms of a blank serum (left) and a sample fortified with 100 ng mL^{-1} rbST (right). Both samples were fortified with 100 ng mL^{-1} reST as internal standard.

and MFPAMPLSSLFANAVLR (N-terminal tryptic reST) were obtained from Millegen (Labege, France). Pepstatin, EDTA, acetic acid, ammonium bicarbonate, sodium phosphate monobasic monohydrate, disodium hydrogen phosphate and trifluoroacetic acid (TFA) were from Sigma−Aldrich Chemical Co. (St. Louis, MO). Sequence grade modified trypsin was purchased from Promega (Madison, WI). HPLC grade acetonitrile, methanol, ammonium sulfate and formic acid were from SDS (Peypin, France). SPE C_4 cartridges (500 mg/6 mL) were from Interchim (Montluçon, France).

Sample Treatment. The sample preparation was performed as described in a previous study (*16*). In summary, 4 mL of serum or plasma was fortified with 100 ng mL^{-1} of reST as internal standard and proteins were precipitated with 45% ammonium sulfate. The molecules of interest were then extracted on a SPE C_4 followed by a precipitation with cold methanol. The precipitated proteins were digested by trypsin to obtain the N-terminal peptide specific of the recombinant form of the hormone. The analyses were performed by LC−ESI-MS/MS on a triple quadrupole instrument.

LC−MS/MS Measurement. The chromatographic separation of the peptides generated by the tryptic digestion was achieved on a C_{18} Interchrom QS Uptisphere 3HDO 150 mm × 2 mm, 100 Å column (Interchim, Montluçon, France). The HPLC system was an Agilent 1200 (Agilent Technologies, Santa Clara, CA). The solvent flow rate was set at 300 μL min^{-1}. 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 8 more minutes. The total run time was 25 min, and a divert valve was used to let the sample pass into the instrument from 5 to 11 min. The typical expected retention times for the rbST and reST N-terminal peptides were 8.0 and 8.1 min, respectively. The MS instrument was a triple quadrupole 6410 (Agilent Technologies, Santa Clara, CA), fitted with an electrospray ion source. The ESI interface was operated in positive ion mode. A sample volume of 20 μL was loaded onto the column using the autosampler. 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 with monitoring of the transitions 933.2 → 1287.9 and 933.2 → 794.1 with a collision energy set

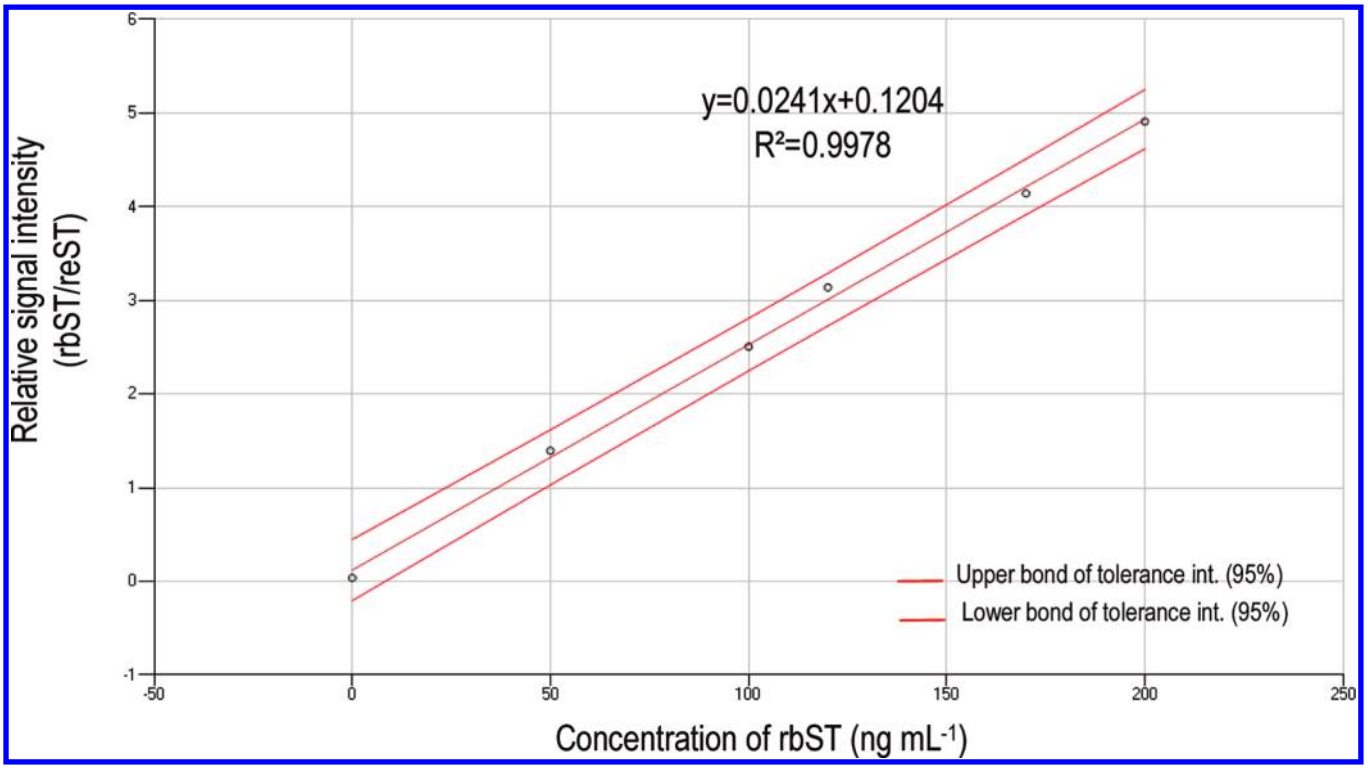

Figure 3. Calibration curve obtained on the pool of the 20 analyzed blank serum samples spiked with recombinant bovine somatotropin (rbST) at 5 fortification levels.

at 30 and 20 V respectively for the internal standard and 913.2 → 1047.7 and 913.2 → 774.1 with a collision energy set at 30 and 20 V respectively for rbST. Data were collected and analyzed with the MassHunterB01 software (Agilent Technologies, Santa Clara, CA).

Confirmation Criteria. In accordance with Commission Decision 2002/657/EC (*17*), recombinant bovine somatotropin was considered as unambiguously detected in samples when the following criteria were met: (i) the ratio of the chromatographic retention time of the analyte to that of the internal standard reST (i.e., the relative retention time) corresponds to that of rbST in standard solution within ±2.5% tolerance, (ii) the presence of a signal at each of the two SRMs monitored for rbST, and (iii) the peak area ratio from the different transition reactions within the tolerances fixed in the decision 2002/657/EC (*17*, *18*).

Method Validation. The method has been validated according to the requirements of the European Decision 2002/657/EC (*17*, *18*). The specificity of the method has been assessed by analyzing 20 different blank samples. These samples were serums collected from various animals never treated with rbST.

The response linearity was evaluated by a calibration curve in the matrix constructed by plotting the ratio of rbST/reST peak area versus analyte concentration. The samples used for the calibration curve were obtained from a pool of the 20 previously analyzed blank samples. There is no regulatory limit (MRL) set for rbST so the working range was chosen in accordance with expected circulating levels after administration. Five levels of fortification were considered, at 50, 100, 120, 170, and 200 ng mL^{-1} of rbST. In all samples, the internal standard reST was added at a concentration of 100 ng mL^{-1}. The trueness and repeatability of the method were determined by analyzing the same 20 serum samples fortified with 100 ng mL^{-1} rbST and with 100 ng mL^{-1} of internal standard reST.

The decision limit and the detection capability were calculated from the calibration curve, blank and spiked samples. The decision limit (CCα) is defined as the limit at and above which it can be concluded, with an error probability α of 1%, that a sample is noncompliant. Recombinant bovine somatotropin is a forbidden compound therefore the CCα can be calculated from the standard deviation of the noise amplitude. The detection capability CCβ is the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of 5%. It corresponds to the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of measured content. It was determined from the analysis of 20 blank samples fortified with 100 ng mL^{-1}.

RESULTS AND DISCUSSION

Method Performance Characteristics. Twenty different blank samples were analyzed to assess the specificity of the method. The ion chromatograms corresponding to one of these samples with two transitions for rbST (913.3 → 774.1 and 913.2 → 1047.7) and two for reST (933.2 → 1287.9 and 933.2 → 794.1) are displayed in **Figure 2**. The absence of any interfering compounds on the ion chromatograms in the expected retention time of rbST (8.1 min) shows a good specificity of the method for both transitions. This is achieved due to MS/MS specificity as well as an efficient purification procedure. The response linearity of the procedure was found to be very satisfactory with a $R^2 > 0.99$ as shown in **Figure 3**. Moreover, the 95% confidence interval for the intercept calculated considering its standard deviation includes the zero value, indicating the absence of systematical instrumental bias. Concerning the 20 samples fortified at 100 ng mL^{-1}, rbST was unambiguously detected, with all the required identification criteria successfully tested. The coefficient of variation of the retention time was equal to 0.1%, and the variation of the ion ratio corresponded to 13%. An example of chromatogram obtained is displayed in **Figure 2**. The variation of the most intense signal was 30%, and, concerning the trueness, a deviation of 58% has been obtained, indicating a rather high variation, nevertheless acceptable for a banned compound. The decision limit (CCα) and detection capability (CCβ) were calculated following the calibration curve procedure as described in the EU guidelines (*17*) and were found at 2.5 ng mL^{-1} for CCα and 6.8 ng mL^{-1} for CCβ. These values appeared very satisfying and coherent with the observation of the ion chromatograms in term of signal-to-noise ratio as shown in **Figure 2**. The overall performance data calculated with this procedure at a fortification level of 100 ng mL^{-1} are summarized in **Table 1**.

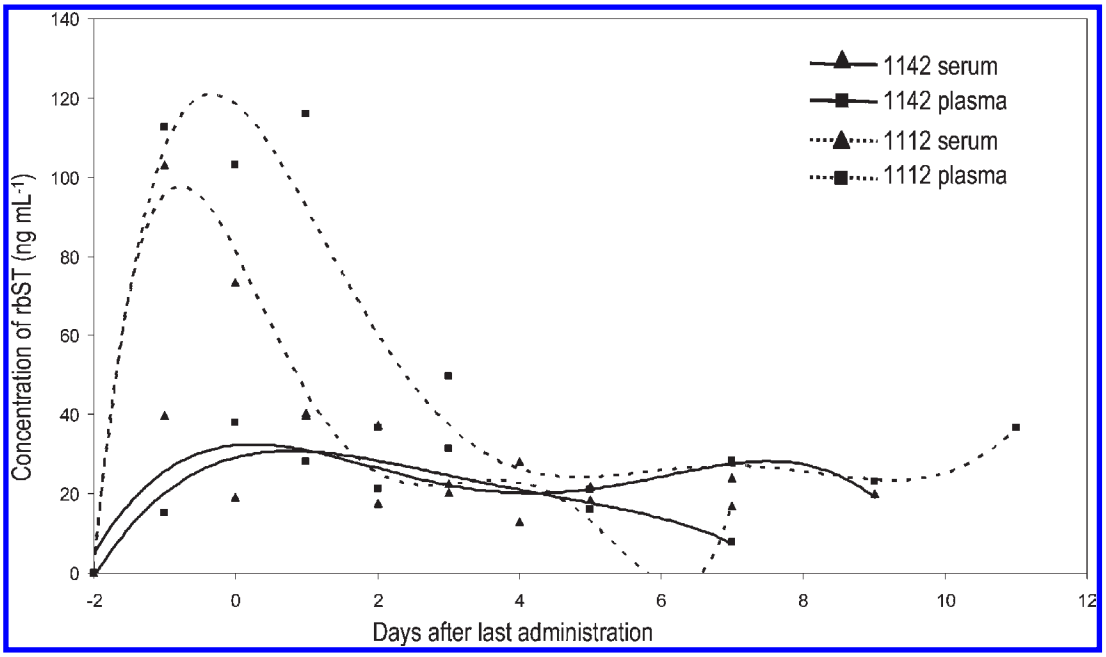

Figure 4. Elimination kinetics of recombinant bovine somatotropin consecutive to the treatment with Lactatropin of two animals.

Table 1. Parameter Characteristics of the Method Obtained for Serum Samples Fortified with 100 ng mL^{-1} rbST

specificity	linearity	repeatability	trueness	limits (ng mL^{-1})
no interfering compound	R^2 = 0.9964 slope = 0.0241 intercept = 0.1204	signal: CV = 30% RT: CV = 0.1% ratio: CV = 13%	deviation 58%	CCα = 2.5 CCβ = 6.8

Kinetics in Serum and Plasma. This method has been successfully used to analyze serum and plasma collected from two cows treated with rbST. As described previously, two cows were treated with two injections of rbST, one at the beginning of the study and the second two days later. The results of the kinetics are displayed in **Figure 4**. As shown in the graph, the amount of rbST in the blood both for serum and plasma of the animal 1112 increases in very high proportion in the first few days after administration to reach a maximum of 120 ng mL^{-1}. Then it drops and stabilizes around 30 ng mL^{-1} for the next 10 days. Concerning animal 1142, the values obtained are much lower, with a maximum of 40 ng mL^{-1} during the first few days but the same range of concentrations than the one measured in animal 1112 were obtained for the next 10 days. This relatively high difference is observed between the two animals, while subjected to the same treatment in the same conditions. This disparity can also be compared to our previously published study (*16*) where very low amounts of rbST have been measured (maximum concentration around 10 ng mL^{-1}). This suggests a great variability between animals. Concerning the comparison of the levels of rbST in serum or plasma, it seems from the analysis of both matrices collected on the two animals that there is no significant difference between the amount of rbST in plasma or serum. The method can therefore be used for the analysis of both without any distinction.

In this study, we analyzed blood samples collected up to 13 days after injection. Even at the end of this period, the recombinant bovine somatotropin was still unambiguously detected and identified in samples, and it is probably still detectable afterward. This is due to the specificity of Lactatropin, commercialized in syringes containing 500 mg of rbST in prolonged-release formulation. The molecule is gradually liberated in vivo for a long-term effect. In consequence, the time window available for the control of rbST in blood is rather important. Even if the protocol of this study did not correspond exactly to the dose normally prescribed in dairy farming, considering that recombinant bovine somatotropin is used in prolonged-release formulation with injection every two weeks, these results show the potentiality of this method to be used for the control of (mis)use of recombinant bovine somatotropin in cattle.

Conclusion. The first part of this work consisted of the full validation of a LC−MS/MS method dedicated to the detection of recombinant bovine somatotropin in blood. Robustness and accuracy of the procedure were assessed with an important number of samples and the compliance with requirements described in the 2002/657/EC. The sensitivity of the method has been found in accordance with physiological levels present in animals treated with the hormone. This method has been accredited and is currently applied at the national level to control the possible misuse of this substance within the European Union.

In addition to the validation, the analysis of samples collected from two different animals treated with Lactatropin showed the high variability between individuals regarding circulating levels of rbST in the organism. The comparison of concentrations measured in serum and plasma demonstrated that both sample pretreatment procedures could be apply without distinction, simplifying the organization of controls and sampling procedure.

The results obtained in this study suggest a trend in terms of level of concentration, nevertheless the number of animals is still very limited. The next step would be to organize an animal study involving a higher number of subjects to ensure that the trend observed in this study is significant. The next objective would also be to apply the method to milk

matrices in order to determine the physiological levels in this matrix.

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