fore, the Ce(IV) methodology is an alternative to present methods of determination of DE for starch hydrolysates, especially where required for process control.

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Registry No. Glucose, 50-99-7; maltose, 69-79-4; isomaltose, 499-40-1; maltotriose, 1109-28-0; maltotetraose, 34612-38-9; maltopentaose, 34620-76-3; maltohexaose, 34620-77-4; maltoheptaose, 34620-78-5.

Rapid and Sensitive ELISA Method for the Determination of Bovine Somatotropin in Blood and Milk

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An avidin/biotin ELISA assay for bovine somatotropin (bST) is described. The method uses affinity-purified polyclonal antisera raised in rabbits to immobilize bST from blood or milk samples on the wells of microtiter plates. Bound bST is quantitated by adding biotinylated anti-bST antibody during the sample incubation step, followed by incubations with horseradish peroxidase labeled avidin D and ABTS substrate. Because high-affinity anti-bST antibody is used, and the biotinylated antibody is added directly to the sample, the assay can be performed in less than 4 h while sensitivities of 0.2 and 2 ng/mL in milk and blood, respectively, are maintained. The advantages of time, sensitivity, and simplicity of methodology have made the assay a valuable tool for defining bST concentrations in the blood and milk of dairy cows treated with somidobove, a recombinant form of bST.

Somatotropin is a 22-kDa polypeptide growth hormone that is normally secreted by the pituitary gland. There is substantial evidence to indicate that, among the various direct and indirect biological activities attributed to the hormone, in ruminants somatotropin also mediates a galactogenic effect (Cotes et al., 1949; Brumby and Hancock, 1955; Machlin, 1972). It has been known for many years that milk production in dairy cows can be increased by as much as 20% or more when circulating levels of somatotropin are increased through the administration of exogenous hormone (Hutton, 1957; Peel et al., 1981, 1982, 1983; Bauman et al., 1985; Hart et al., 1985; Mohammed and Johnson, 1985). The potential application of this finding to the management of milk production in dairy herds had, however, been hampered by the cost of obtaining limited amounts of somatotropin available through extraction of pituitary glands. Recently, the application of recombinant DNA techniques to clone and express the gene for bovine somatotropin (bST) has made it possible to obtain an unlimited amount of hormone and provides an opportunity to investigate benefits that may be derived from controlling milk production through supplemental administration of bST on a commercial scale (Spencer, 1987).

Central to studies conducted to investigate the safety and efficacy of recombinant-derived bovine somatotropin (r-bST) treatment was the need for a sensitive analytical method that could be used to estimate bST levels in various biological fluids. An avidin–biotin enzyme-linked immunosorbent assay (ELISA) is described which is sensitive to 0.2 ng/mL in whole milk and 2 ng/mL in blood and can be completed in less than 4 h.

MATERIALS AND METHODS

Materials. Highly purified somidobove (Griffiths, 1988) was obtained from Dr. R. E. Chance, Eli Lilly and Co., Indianapolis, IN, and was used to raise anti-r-bST antibodies in rabbits. Reference Standard somidobove, lots RS0010, RS0041, and RS0071, was obtained from the Analytical Development Division, Eli Lilly. Reference standards had potencies of 1.4 IU/mg, as determined by the rat body weight gain assay. Total bST protein was based on amino acid analysis and nondiscriminatory peptide assays. For purposes of comparison in the ELISA assay, two pituitary-derived bovine somatotropin (p-bST) preparations were used: (1) USDA-bGH-B-1 (AFP-5200) was obtained

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from the Animal Science Institute, U.S. Department of Agriculture, Beltsville, MD; (2) p-bST, lot 35, was obtained from Miles Laboratories, Elkhart, IN. The USDA material had a potency of 1.9 IU/mg on the basis of the rat tibia epiphyseal width assay, and the Miles material contained 60% p-bST protein by weight as determined by HPLC.

Bovine prolactin (bPL), iodination grade with a purity of 85%, and bovine luteinizing hormone (bLH), iodination grade with <0.1% bST, were obtained from Chemicon, El Segundo, CA. These hormones were used to assess the cross-reactivity of anti-somidobove antibody to non-bST proteins of pituitary origin.

Strain H37 Ra complete adjuvant and incomplete Freund's adjuvant were purchased from Difco Laboratories, Detroit, MI.

Immunoassay plates were NUNC Certified Immunoplate I obtained from Vangard International, Neptune, NJ. *N*-Hydroxysuccinimide–biotin (BNHS) and Type IV porcine skin gelatin, 60 bloom, were from Sigma Chemical Co., St. Louis, MO. Horseradish peroxidase–avidin D (avidin–HRP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS solution, were obtained from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD. All other reagents were certified ACS grade and were obtained from Fisher Scientific, Fairlawn, NJ, or Sigma.

Production of Affinity-Purified Anti-r-bST Antibodies. Lyophilized somidobove was reconstituted in normal saline to concentrations of either 200 or 400 μg/mL and mixed 1:1 with adjuvant. An equal volume of 2% Tween 80/saline was added, and the mixture was emulsified in a Mini-Beadbeater, Biospec Products, Bartlesville, OK.

For primary immunization, strain H37 Ra complete adjuvant was used. Eleven rabbits were injected subcutaneously in each ankle with a total dose of 50 μg of somidobove in a volume of 0.5 mL. Secondary immunizations were performed weekly for 4 weeks with 100 μg of somidobove in incomplete Freund's adjuvant/Tween 80, followed by biweekly injections for 6 weeks. All secondary immunizations were intramuscular, 50 μg into each thigh.

Ten days after the last immunization, rabbits were anesthetized and bled by cardiac puncture. Serum from each animal was titrated for antibody activity against r-bST in an ELISA assay for rabbit IgG, using plates coated with somidobove. All but one rabbit produced high titer antiserum to r-bST. In the responding animals, a 1/4000 dilution of serum gave absorbance readings greater than 2.0 compared to preimmune sera, which gave absorbance readings less than 0.03. Dilutions of antisera yielding absorbances of 0.05 ranged between 1/51 200 and 1/204 800 in individual rabbits.

Antisera were pooled, and an IgG fraction was prepared by using standard techniques of salt precipitation with 18% and then 14% Na_2SO_4, followed by chromatography on DEAE-cellulose (Fahey and Terry, 1978). Affinity-purified anti-r-bST antibody was prepared by chromatography of the purified IgG fraction on a 16 × 180 mm r-bST–Sepharose 4B affinity column prepared by using the cyanogen bromide method (Cuatrecasas, 1970; Parikh et al., 1974). After extensive washing with 0.1 ionic strength borate/saline buffer, pH 8.4, to remove unbound IgG, the anti-r-bST antibody bound to the column was eluted with 0.1 M glycine/saline, pH 2.0. The fractions containing protein (determined by absorbance at 280 nm) were neutralized and pooled. The antibody solution was dialyzed against potassium phosphate buffered saline (KPBS), pH 7.2, containing 0.02% thimerosal, and concentrated in a ProDiCon apparatus using PA-15 membranes (Bio-Molecular Dynamics, Beaverton, OR). Approximately one-third of the antibody was biotinylated, and the remainder, which was used to coat assay plates, was adjusted to a concentration of 4 mg/mL and mixed 1:1 with glycerol. Aliquots were stored in polypropylene storage vials at 2–8 °C.

Biotinylation of Anti-r-bST. Affinity-purified anti-r-bST was dialyzed against 0.1 M sodium bicarbonate buffer, pH 8.0, and biotinylated as described by Subba Rao et al. (1983). Briefly, the protein concentration was adjusted to 1 mg/mL in fresh bicarbonate buffer, and 1 mL was placed into each of a series of 13 × 100 mm test tubes. To each tube, 200 μL of 1 mg/mL BNHS in dimethyl sulfoxide was added; the tubes were sealed and incubated at room temperature for 6 h. After incubation, 6 mL of cold KPBS, pH 7.2, containing 0.01% thimerosal, was added to each tube to dilute the reaction. The contents of each tube were pooled, dialyzed, and concentrated against KPBS/thimerosal. The protein concentration was determined at 280 nm absorbance and was adjusted to 1.5 mg/mL. Glycerol was added in a 1:1 volume, and the solution was aliquoted and stored at 2–8 °C.

Preparation of Milk Serum Fraction. Whole milk was centrifuged at 100000*g* for 1 h at 5 °C. The middle, clear yellow fraction, which constituted the milk serum fraction (Hart et al., 1985) was carefully removed with a pipet and stored at −20 °C.

ELISA Assay for bST. Assay plates were coated by adding 100 μL/well of affinity-purified anti-r-bST, diluted to 0.5 μg/mL in 0.1 M sodium bicarbonate buffer, pH 9.6. Plates were sealed with acetate plate sealers (Linbro/Titertek, Flow Laboratories, McLean, VA) and were incubated on a damp paper towel at 37 °C for 1 h. After the incubation period, the plates were rinsed three times for 30 s with 300 μL/well of 0.02 M KPBS containing 0.01% gelatin. Standards and samples were added immediately after rinsing.

To measure increases in bST levels in milk and blood collected from r-bST-treated animals, standards were prepared by adding Reference Standard somidobove to a pool of samples collected from control animals. The starting concentration of standards was generally 50 ng/mL. The 50 ng/mL standard was serially diluted 1:1 directly in the plate with the pooled control sample as diluent. For most studies, the ending concentration was 0.2 ng/mL. Blood samples were assayed at a 1/10 dilution to reduce background absorbance to less than 0.1 OD unit, whereas milk serum and whole milk samples were assayed undiluted. Fifty microliters of standard or sample was added to each well.

Immediately after the addition of standards and samples, 50 μL of 0.2 μg/mL of biotinylated anti-r-bST, diluted in 0.02 M KPBS containing 0.1% gelatin, was added to each well. The plates were shaken briefly on a Mini-Orbital shaker (Bellco Glass, Inc., Vineland, NJ) and then sealed and incubated at 37 °C for 1 h.

After the plates were rinsed as described previously, 100 μL of 1 μg/mL avidin–HRP was added to each well. Plates were sealed and incubated at 37 °C for 15 min. Plates were rinsed five times for 60 s with 300 μL/well of the rinse buffer, and 100 μL of ABTS substrate solution was added to each well. The OD (410–490 nm) was monitored on an MR600 microplate reader (Dynatech Instruments, Inc., Torrance, CA). When the absorbance of wells containing the 50 ng/mL standard reached a value close to 1.9, the plate was read and absorbance readings were recorded.

Standard curves were constructed by using a four-parameter logistic curve-fitting algorithm (TiterCalc 2.1, HP Genenchem, Palo Alto, CA), and sample bST concentrations were interpolated from the standard curve generated on the same plate. A VAX program, RIASYS (Smith and Suckstorff, 1986) was used to determine the minimum detectable increase (MDI) in bST concentration and provided additional assay performance statistics. The MDI was calculated as described by Rodbard (1978).

RESULTS

In the first experiments, the range of sensitivity of the assay for r-bST and p-bST was investigated. These experiments were performed by spiking KPBS buffer containing 0.1% porcine gelatin with increasing concentrations of bST. As shown in Figure 1, each source of bST was detected in the concentration range 0.3–70 microunits/mL, corresponding to 0.2–50 ng/mL of somidobove. In the buffer system used, the slope of the response curve was greater for the r-bST than for the USDA p-bST material, suggesting the assay may be 2-fold more sensitive for somidobove than for p-bST. Similar results were obtained with the p-bST obtained from Miles Laboratories (data not shown).

Once the approximate range of sensitivity of the assay was established, the extent of cross-reactivity of the rab-

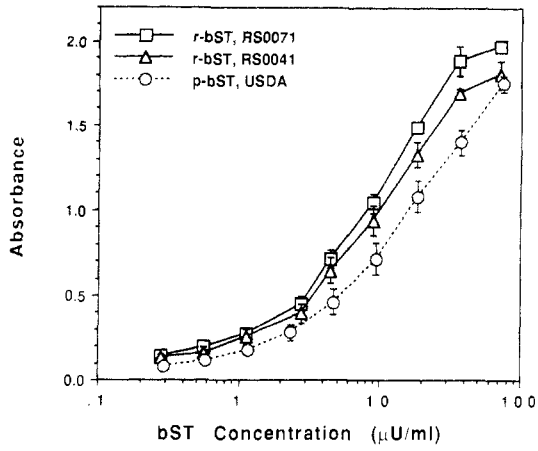

Figure 1. Comparison of response curves of USDA pituitary bST and somidobove. Data are mean responses of assays run on three separate days.

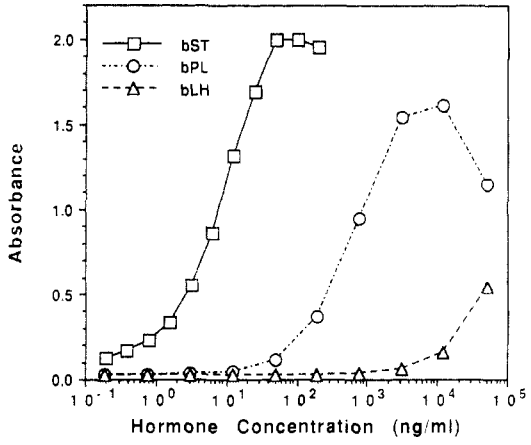

Figure 2. Cross-reactivity of the bST ELISA with bovine prolactin (bPL) and luteinizing hormone (bLH) derived from pituitary.

bit anti-somidobove antibody with two other pituitary hormones, bLH and bPL, was determined (Figure 2). Under conditions used for the determination of bST, the antibody was 20 000-fold less reactive with bLH than with bST. This demonstrated that physiological concentrations of bLH, which range from a basal concentration of 1 ng/mL to a proestrus peak of 40 ng/mL (Catchpole, 1977), are not detected in the assay. The cross-reactivity of the antibody toward bPL was 150-fold less than its reactivity with bST. The normal basal concentrations of prolactin in cow blood range from 28 to 50 ng/mL (Kaprowski et al., 1972; Catchpole, 1977), but may rise to as high as 320 ng/mL at 20 h prepartum. Considering that within 30 h postpartum prolactin levels fall back to base line (Catchpole, 1977), it is only during the short interval immediately prior to and after parturition that the concentration of prolactin is high enough to cause potential interference with the determination of blood bST concentrations at 2 ng/mL and lower.

To determine the optimum conditions for estimating r-bST concentrations in blood, somidobove standards were prepared in 1/10 diluted cow plasma or serum. The results, presented in Figure 3, show that the assay maintained the full range of sensitivity seen in the earlier experiments performed in the KPBS/gelatin buffer. The same results were obtained whether plasma or serum was used. In a further experiment using cow plasma, recoveries of somidobove ranged from 87% to 115% over the concentration range from 0 to 500 ng/mL (Table I). To establish the detection limit for r-bST in blood, the minimum

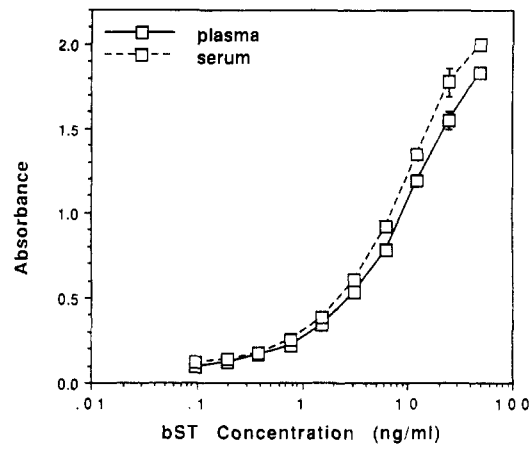

Figure 3. Sensitivity of the bST ELISA compared in 1/10 diluted bovine blood plasma and serum matrices.

Table I. Recovery of Somidobove in Blood Plasma

calcd concn (ng/mL)	measd concn (ng/mL)	% recovery
500	475	95.0
250	259	104
125	129	103
62.5	61.3	98.1
31.3	30.1	96.2
15.6	14.6	93.6
7.81	6.80	87.1
3.91	3.61	92.3
1.95	1.92	98.5
0.997	1.15	115
0.000	0.006	

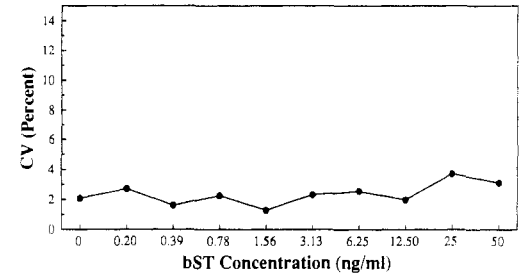

Figure 4. Intraassay precision of the ELISA at various bST concentrations in a 1/10 diluted blood serum matrix. Data are taken from 48 assays.

detectable increase (MDI) in r-bST concentration was determined by analyzing standard curves from 48 plates. Standards consisted of 1/10 diluted cow serum spiked with somidobove ranging in concentration from 0 to 50 ng/mL. The MDI in somidobove concentration ranged from 0.017 to 0.089 ng/mL at the $P < 0.01$ level. Data from the standard curves were also used to construct a precision profile for estimating intraassay coefficients of variation (CVs) at each concentration. Mean CVs were uniformly less than 4% at all concentrations comprising the standard curve (Figure 4). On the basis of these data, and accounting for the 1/10 sample dilution and level of cross-reactivity with prolactin, the detection limit for r-bST was estimated to be at least 2 ng/mL, approximately twice the highest MDI observed. In addition to using cow serum or plasma, the ELISA was found to work equally well in rat plasma and dog serum (data not shown).

In the final set of experiments, the method was evaluated for its ability to quantitate r-bST in milk. Somidobove was spiked into either whole milk or the milk serum fraction, and the resulting response curves were compared. The curves, shown in Figure 5, were superimposable, demonstrating that r-bST could be assayed directly in whole milk. Further, since there was essen-

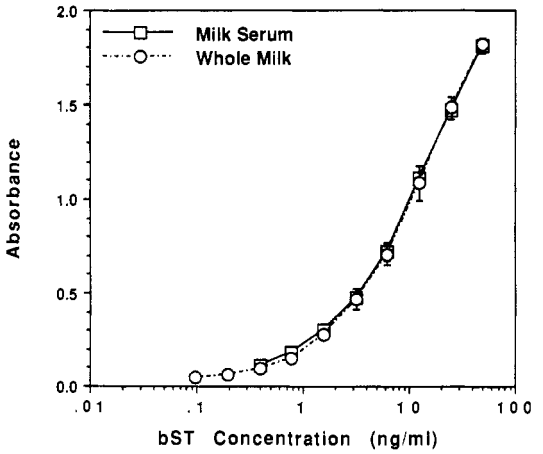

Figure 5. Sensitivity of the bST ELISA compared in undiluted whole milk and undiluted milk serum matrices.

tially no assay interference caused by other milk constituents as evidenced by the very low background response of unspiked milk, whole milk samples could be assayed undiluted. The sensitivity for bST in whole milk was estimated to be 0.2 ng/mL, and as shown in Table II, recoveries in whole milk ranged from 94.9% to 113.6% in the concentration range 0.098–50 ng/mL.

DISCUSSION

The application of recombinant DNA techniques has made possible the large-scale production of proteins that may be used to modulate biological effects on target animals. Bovine somatotropin (bST) is a protein which, due to its ability to promote milk production in dairy cows, has been selected for development by several companies. Central to studies conducted to determine the safety and efficacy of recombinant bST (r-bST) was the need for a rapid and sensitive method to quantitate r-bST levels in several types of biological fluids.

In this paper, the development and characterization of an avidin/biotin ELISA for estimating bST concentrations in blood and milk have been described. The assay offers advantages of speed and sensitivity compared to a similar ELISA method reported by Secchi et al. (1988) and has been extended to the determination of bST in milk. By eliminating the blocking step and combining the biotin-antibody step with sample addition, the number of steps required to complete the assay was reduced from six to four. Coupled with the use of high-affinity anti-r-bST antibody, the time required to complete an assay was reduced from 48 to 4 h, and at the same time, the sensitivity for r-bST in blood was increased to 2 ng/mL.

The assay appeared to be approximately 2-fold less sensitive for pituitary-derived bST (p-bST) than for somidobove. This difference may reflect differences in methods used to determine the bST content of each preparation. The USDA and somidobove reference standards were compared by using biopotency information obtained from different biological endpoints. Inherent differences in these assays, as well as the greater amount of variability generally associated with bioassay methods, may have contributed to differences seen in the ELISA comparison. This does not explain a similar difference seen between somidobove and the Miles p-bST preparation, however, since they were compared by using analytical chemical data as the basis for comparison. Since the antisera were raised against somidobove, it is possible that there is a subpopulation of antibodies that reacts more strongly with portions of the somidobove molecule or with one or more epitopes not present on p-bST.

Table II. Recovery of Somidobove in Whole Milk

calcd concn (ng/mL)	measd concn (ng/mL)	% recovery
50.0	54.2	108
25.0	27.1	108
12.5	12.0	96.0
6.25	6.02	96.3
3.13	3.18	102
1.56	1.57	101
0.781	0.745	95.4
0.391	0.370	94.6
0.195	0.205	105
0.098	0.111	113

The anti-somidobove reagents were assessed for extent of cross-reactivity with other pituitary hormones. It was demonstrated that, under conditions used to determine bST concentrations, physiologically relevant concentrations of prolactin and luteinizing hormone did not interfere with the assay. It is noteworthy that the antibody reagents used in the assay were prepared by immunizing rabbits with highly purified r-bST that did not contain trace amounts of other pituitary hormones as would be expected to be present in pituitary-derived preparations, yet some cross-reactivity with prolactin was observed. The cross-reactivity may be due, in part, to trace contamination of the prolactin with bST, but also probably reflects the amount of structural homology between bST and prolactin. The results underscore the need to determine the extent of relevant cross-reactivities, particularly when the concentration of the analyte is significantly lower than that of the cross-reacting substances in the sample.

The ELISA assay was found to be equally sensitive when whole milk or milk serum fractions were assayed. This is particularly advantageous, since it has been observed by Hart et al. (1985) that a substance in whole milk interfered with the binding of the ^{125}I-labeled bST tracer to antibody in an RIA assay. This may explain the relatively high basal bST concentrations reported by Mohammed and Johnson (1985). The ability to assay whole milk in the ELISA eliminates the need to prepare milk serum fractions and significantly reduces analysis time.

To ensure parallelism of standard curves with unknowns, standards were prepared either in blood or in milk, rather than in buffer. For milk, this was further necessitated by our observation that the background absorbance response of unspiked milk was lower than the background of buffer alone. Although this permitted quantitation of differences in bST concentrations between samples from bST-treated cows and untreated controls, it precluded the direct estimation of basal levels of bST in milk and blood, both of which are at the detection limits of bST assays reported (Purchas et al., 1970; Trenkle, 1970; Machlin, 1972; Gorewit, 1981; Hart et al., 1985; Mohammed and Johnson, 1985; Wheaton et al., 1986; Secchi et al., 1988). However, if basal concentrations are of interest, the maximum basal bST concentration may be estimated indirectly by subtracting the absorbance of the unspiked standard from the absorbance of each spiked standard and using this "corrected" standard curve for interpolation of unknowns (Figure 6). By use of this method, the basal level may be assumed to be included in the range of concentrations defined by interpolation from the uncorrected and the corrected standard curves, which represent the minimum and maximum bST concentrations in the sample, respectively.

CONCLUSIONS

The paper describes an avidin/biotin ELISA that can be used to define increases in bST levels in the blood

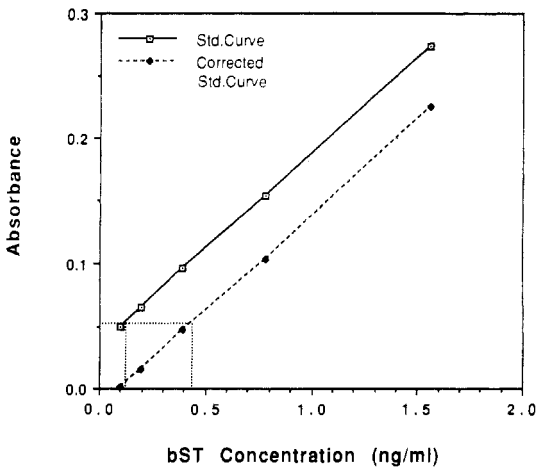

Figure 6. Estimation of the bST concentration range expected to include the basal concentration. Interpolation using the uncorrected standard curve estimates the minimum concentration, whereas interpolation from the corrected standard curve, generated by subtracting the absorbance of the unspiked milk matrix from each point on the standard curve, estimates the maximum bST concentration in the sample. For example, the basal bST level in a milk sample yielding an absorbance of 0.053 would be included in the range 0.1–0.45 ng/mL, on the basis of data presented in the figure.

and milk of cows treated with supplemental bST. The assay is sensitive to 0.2 ng/mL in milk and 2 ng/mL in blood and can be completed in less than 4 h. When compared to RIA, an additional advantage is that whole milk samples can be assayed, eliminating the need to prepare milk serum fractions prior to analysis. Although basal levels of bST cannot be determined directly, estimates of the minimum and maximum basal bST concentrations can be obtained indirectly by using a corrected standard curve.

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