

## GC-MS/MS versus LC-MS/MS for unambiguous identification of $\alpha$ and $\beta$ -trenbolone in bovine urine

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### Abstract

This work involves the optimization of LC-MS/MS and GC-MS/MS methods for determination of  $\alpha$  and  $\beta$ -trenbolone in bovine urine. The parameters recovery, repeatability and intermediate precision in both methods met the acceptance criteria of the *Codex Alimentarius*: recovery between 83.5-114% and repeatability and intermediate precision with RSD between 5.1-14.5% and 9.8-25.5% respectively, for concentrations higher than  $1 \mu\text{g kg}^{-1}$ . These methods were applied to the analyses of 5 real samples suspected of contamination. The analyses showed that derivatization with MSTFA/I<sub>2</sub> followed by pure MSTFA, was an important tool to prevent false positive results. The use of microwave assisted derivatization reduced significantly the analysis time by GC-MS/MS.

**Keywords:** bovine urine, gas chromatography tandem mass spectrometry, liquid chromatography tandem mass spectrometry, microwave assisted derivatization, trenbolone.

## 1. Introduction

Trenbolone is a synthetic anabolic steroid with androgenic properties and is considered an exogenous growth promoter. Trenbolone is administered to beef cattle to increase the feed efficiency and production profitability in the form of esters: acetate, hexahydrobenzylcarbonate and enanthate (1). In Canada and the USA, the use of trenbolone is approved (2-3). However, in Europe (4) and many other parts of the world, such as Brazil (5), its use is banned and the minimum required performance limit (MRPL) is  $2 \mu\text{g kg}^{-1}$  in bovine urine (6). Trenbolone acetate implants are the most commonly used in beef cattle. After application, trenbolone acetate is hydrolyzed to  $17\beta$ -trenbolone, the most active biological form of trenbolone, and excreted primarily as  $17\alpha$ -trenbolone metabolite (7). Therefore, the determination of  $17\alpha$ -trenbolone and  $17\beta$ -trenbolone in bovine urine has been used to identify the use of trenbolone acetate.

Literature data indicate that liquid chromatography (LC) is the preferred analytical technique for identification and quantification of trenbolone in bovine urine (8-16); however, gas chromatography/mass spectrometry (GC/MS) is also used (17-22). Sample preparation of these methods is based on trenbolone hydrolysis, which is mainly excreted in urine in the form of glucuronide and sulfate, followed by liquid-liquid extraction (LLE) and/or solid phase extraction (SPE). GC methods presented the disadvantage of the additional derivatization step, but showed better chromatographic resolution and high sensitivity, when compared to LC methods.

The derivatization of 4,9,11-triene conjugated compounds, such as trenbolone, still pose difficulties due to the keto function in their structures. The combination of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) with catalysts may be the solution for these difficulties. Several reagents were used for derivatization of trenbolone: methoxylamine hydrochloride/pyridine followed by N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (17); O-methylhydroxylamine hydrochloride/

pyridine followed by MSTFA/ trimethylsilylimidazole (TMSI) (20); TMSI:BSTFA (22) and MSTFA/ $I_2$  (18, 21). MSTFA/ $I_2$  forms derivatives with specific mass spectra for  $\alpha$  and  $\beta$ -trenbolone, at very low concentration levels, making it possible to unambiguously identify the presence of trenbolone in bovine urine (18).

In this work, GC-MS/MS and LC-MS/MS methods were optimized and applied to analysis of  $17\alpha$ -trenbolone and  $17\beta$ -trenbolone in bovine urine samples suspected of contamination. The GC-MS/MS method involved derivatization with MSTFA/ $I_2$ , followed by pure MSTFA, employing for the first time microwave assisted derivatization, which significantly reduced the analysis time.

## 2. Experimental

### 2.1. Reagents, buffer solutions and cartridges

All reagents were of analytical grade. Methanol was acquired from Tedia (Fairfield, USA); glacial acetic acid, enzyme  $\beta$ -glucuronidase from *Helix pomatia* (type-2,  $\geq 100,000$  units/mL), diethyl ether, MSTFA and 2,2,4-trimethylpentane (isooctane) were purchased from Sigma-Aldrich (Saint Louis, USA); n-hexane was acquired from Vetec (Rio de Janeiro, Brazil); acetone was provided by J. T. Baker (Philipsburg, USA); resublimed iodine, hydrochloric acid and 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS) were purchased from Êxodo Científica (São Paulo, Brazil); anhydrous sodium acetate was provided by Neon Comercial (São Paulo, Brazil). Ultrapure water was generated by Milli-Q Millipore system (Billerica, USA).

Sodium acetate buffer  $2 \text{ mol L}^{-1}$  (pH 5.2) was prepared from a mixture of sodium acetate with acetic acid. TRIS solution  $2 \text{ mol L}^{-1}$  (pH 9.5) was prepared from a mixture of TRIS with hydrochloric acid.

HLB Supel cartridges (200 mg/6 mL) were purchased from Sigma-Aldrich (Saint Louis, USA) and aminopropylsilane cartridges Sep-Pak (200 mg/6 mL) were purchased from Waters (São Paulo, Brazil).

## 2.2. Standards and standard solutions

The anabolic standards  $\alpha$ -trenbolone ( $\alpha$ TB) and  $\beta$ -trenbolone ( $\beta$ TB) were acquired from Toronto Research Chemicals (North York, Canada). The internal standard methyltestosterone-D3 (MTT-D3) was acquired from RVIM National Institute for Public Health and the Environment (Bilthoven, The Netherlands).

Individual stock standard solutions were prepared at a concentration of 200  $\mu\text{g mL}^{-1}$  by dissolving the mass of each compound in methanol. The anabolic working standard solution was prepared by diluting the stock standard solution in methanol to a final concentration of 0.10  $\mu\text{g mL}^{-1}$ . All solutions were stored at  $-20^\circ\text{C}$ .

## 2.3. Instrumentation

The samples were incubated (TE-420EI Tecnal incubator, Piracicaba, Brazil), centrifuged (CR4i Thermo Electron Corporation centrifuge Ohio, USA) and shaken in a vortex (Ika Genius 3, Wilmington, USA). Next, the samples were dried in a shaking bath (BT-25 Yamato, Tokyo, Japan), derivatized in microwave oven (Philco PMS25N2, Pennsylvania, USA) and, finally, dried in a sample concentrator (Dri-Block DB-3 Techne, Stone, UK).

## 2.4. LC-MS/MS system

LC-MS/MS system consisted of a Agilent 1200 series liquid chromatography system equipped with an autosampler and a quaternary pump (Agilent Technologies, Santa Clara, USA) coupled to an API 5000 triple quadrupole mass spectrometer with a turbo ion spray interface (Applied Biosystems, Foster City, USA). Both systems and data treatment were controlled by Analyst 1.5.1 software (Applied Biosystems, Foster City, USA). Separation was achieved on a Poroshell 120 EC-C18 column (50 x 3.0 mm, 2.7  $\mu\text{m}$ ) (Agilent Technologies, Santa Clara, USA) using an Acquity UPLC BEH C18 vanguard pre-column (5 x 2.1 mm, 1.7  $\mu\text{m}$ ) (Waters, Milford, USA). Flow rate used was

0.7  $\text{mL min}^{-1}$ , column temperature was set at 40  $^\circ\text{C}$  and injection volume was 10  $\mu\text{L}$ . A gradient elution programmer was used with solvent A (ultrapure water) and solvent B (methanol) as follows: from 0 to 0.50 min, the percentage of B was kept at 60% and linearly increased to 80% up to 2.50 min; from 2.50 to 2.51 min, B linearly increased to 100% and was kept constant up to 3.00 min; from 3.00 to 4.50 min, B linearly decreased to 60%, and was kept up to 6 min. Electrospray ionization was operated in the positive mode, and the source block temperature was set at 600  $^\circ\text{C}$  with a capillary voltage of 5.5 kV. Nitrogen gas was used as desolvation agent and as nebulizer gas at a flow rate of 50 psi, and argon was used as collision gas at a flow rate of 4 psi. Data acquisition was performed in selected reaction monitoring (SRM) and two characteristic SRM transitions were monitored for each analyte. For  $\alpha$ TB and  $\beta$ TB, the precursor ion was 271.2  $m/z$  and product ions were 199.2 and 253.2  $m/z$ , with declustering potential of 101 and 100 V, collision energy of 37 and 39 V and collision cell exist potential of 24 and 14 V, respectively. For MTT-D3, the precursor ion was 306.3  $m/z$  and product ion was 91.0, with declustering potential of 111 V, collision energy of 39 V and collision cell exist potential of 20 V.

## 2.5. GC-MS/MS system

GC-MS/MS system consisted of a gas chromatography 7890B with triple quadrupole mass spectrometer 7000C (Agilent Technologies, Santa Clara, USA). Separation was performed in a column HP-5MS (30 m, 0.25 mm I.D., film thickness 0.25  $\mu\text{m}$ ) from J & W column (Agilent Technologies, Santa Clara, USA), using helium (White Martins, Belo Horizonte, Brazil) as carrier gas. The chromatographic method used was optimized and validated by our group (23). The electronic beam on the mass spectrometer was set at 70 eV in the electron ionization mode and was operated in the SRM mode. The selected precursor ions were 442.0 and 449.0  $m/z$  and product ions were 352.0 and 323.0  $m/z$  for  $\alpha$ TB and  $\beta$ TB, respectively, and collision energy was 25 eV.

## 2.6. Sample preparation

The enzymatic hydrolysis, extraction and clean-up procedures consisted of: [1] 2 mL sodium acetate buffer and 50  $\mu$ L  $\beta$ -glucuronidase from *Helix pomatia* were added to 5 mL of bovine urine for enzymatic hydrolysis, performed in an incubator under gentle stirring at 37.5 °C for 16 h; [2] next, pH was adjusted to 9.5 by adding 4 mL TRIS solution; [3] 5 mL diethyl ether were added for analyte extraction, then, a mixture was vortexed for 30 s and centrifuged at 4000 rpm for 10 min; [4] the organic phase was transferred to a 15 mL flask. This procedure was repeated once more and the organic phases were combined; [5] the extract was evaporated to dryness under nitrogen stream in shaking bath at 50 °C and the residue was re-dissolved with a mixture of 1.5 mL of methanol and 3 mL of ultrapure water; [6] 2 mL of hexane were added to the extract, which was vortexed for 30 s and centrifuged at 4000 rpm for 10 min and, then, the organic phase was discarded. This procedure was repeated once more and the aqueous phases were combined; [7] the extract was transferred to a HLB cartridge (pre-conditioned with 5 mL of methanol and 5 mL of ultrapure water), which was washed with 5 mL of ultrapure water and 5 mL of methanol/water (55:45 v/v) solution; [8] the aminopropylsilane cartridge (pre-conditioned with 5 mL of methanol and 5 mL of acetone) was coupled to the HLB cartridge, and the analytes were eluted with 5 and 3 mL of acetone, consecutively; [9] the eluate was evaporated to dryness under nitrogen stream in shaking bath at 50 °C, and residue was re-dissolved with 500  $\mu$ L of methanol/water (55:45 v/v) solution, vortexed for 30 s and transferred to a vial and injected in the LC-MS/MS system. For GC-MS/MS analysis, this extract was dried under nitrogen stream in a sample concentrator at 60 °C and conducted to derivatization.

For blank bovine urine samples, an aliquot of the working standard solution was added before step [1].

## 2.7. Derivatization for GC/MS/MS system

Derivatization with MSTFA/ $I_2$ , followed by pure MSTFA, consisted of: [1] 15  $\mu$ L of MSTFA/ $I_2$  (0.3%

m/v) were added to the dry extract, vortexed and kept at rest at room temperature for 5 min; [2] the mixture was dried under nitrogen stream in a sample concentrator at 60 °C. Then, 25  $\mu$ L of pure MSTFA were added, vortexed and derivatized using a conventional microwave oven for 2 min at 900 W; [3] the mixture was again evaporated to dryness under nitrogen stream in a sample concentrator at 60 °C and the derivatized residue was re-dissolved in 100  $\mu$ L of isooctane, and was then injected in the GC-MS/MS system.

## 2.8. Experiments to determine linearity, recovery and precision

The calibration curves were prepared at six concentration levels (1.0; 1.5; 2.0; 2.5; 3.0 and 4.0  $\mu$ g kg<sup>-1</sup>) by spiking blank bovine urine samples with working standard solution volumes ranging from 0 to 200  $\mu$ L. For LC-MS/MS analysis, 100  $\mu$ L of internal working standard solution were added to each point, and the ratio of the analyte peak areas to the internal standard peak area was employed in the preparation of the calibration curves. For GC-MS/MS analysis, analyte peak areas were used in the preparation of the calibration curves. Each point of the calibration curve was prepared in triplicate and the analytes were extracted according to the procedure previously described. The F-test was applied at 95% confidence level to evaluate the homogeneity of the response variances and the determination coefficient ( $R^2$ ) for linear fit of the curves (24).

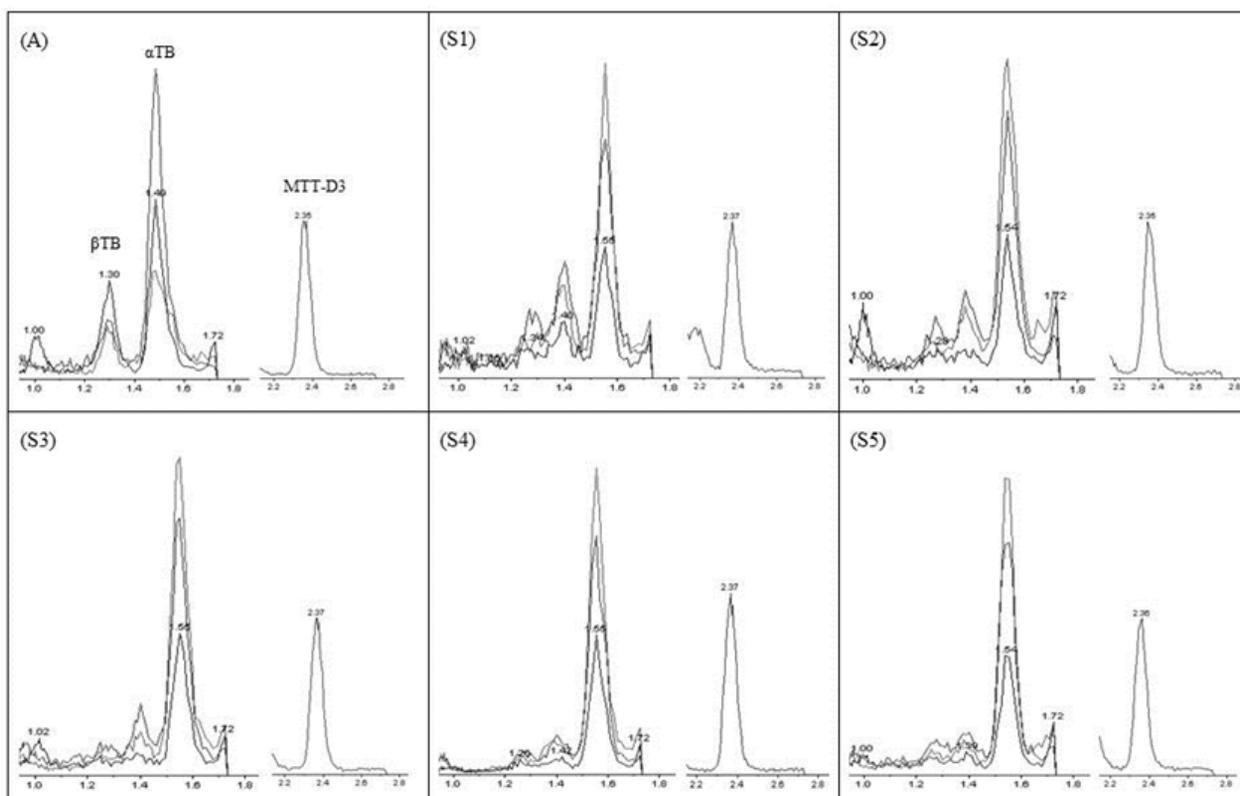
To evaluate precision and recovery, aliquots of 5.0 mL blank bovine urine were spiked at levels 1.0 and 1.5 x MRPL, in six replicates. The experiment was repeated by a second analyst on another day to evaluate intermediate precision. Recovery was calculated by the ratio between estimated concentration and nominal concentration. Precision was estimated from the relative standard deviation (RSD) of the estimated concentrations for six replicates at each level intra-day (repeatability) and for the results inter-day and inter-analyst (intermediate precision).

### 3. Results and Discussion

#### 3.1. LC-MS/MS analysis

In the LC-MS/MS analysis, 5 bovine urine samples presented intense signals for  $\alpha$ TB and minor signals for  $\beta$ TB, for both the quantification transition (271.4 $\rightarrow$ 199.2) and the confirmation transition (271.4 $\rightarrow$ 253.2). However, small shifts in the relative retention times (2.92-3.36%) of the analytes in real samples were observed when compared with the spiked blank sample (Figure 1). These small deviations in the relative retention times, and the

fact that the quantification and identification transitions were identical, led to the suspicion that the samples were contaminated with TB. Taking into account that urine is a complex matrix, composed mainly of water, urea, creatine, ammonia and inorganic salts, other metabolites from the diet and/or medication could be present. Therefore, a substance with the same transitions as TB and similar interaction with the stationary phase of the chromatographic column may be found in the samples, generating analytical signals in the LC-MS/MS chromatogram.



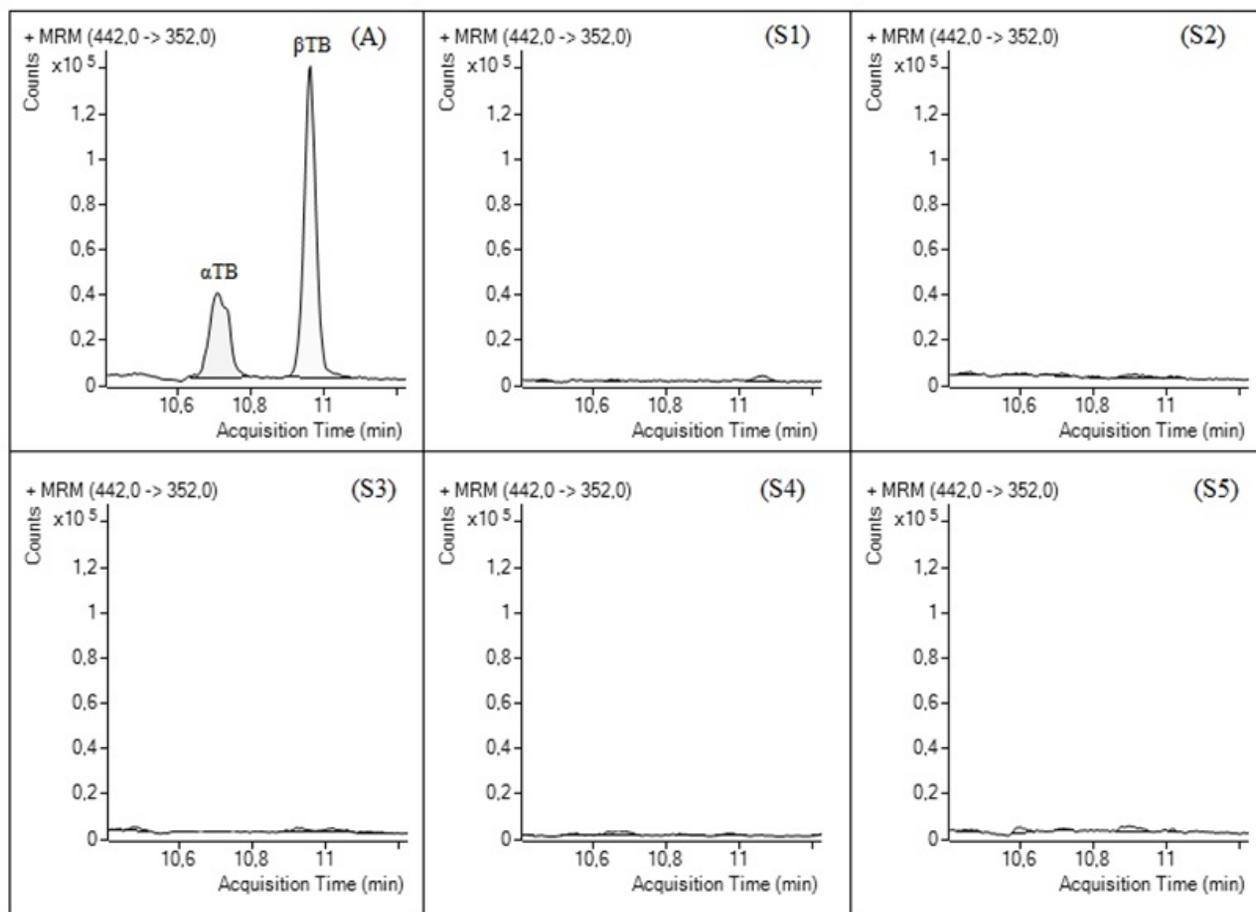
**Figure 1.** LC-MS/MS chromatograms for blank bovine urine spiked with  $1 \mu\text{g kg}^{-1}$  of  $\alpha$ TB and  $\beta$ TB (A) and bovine urine samples (S1, S2, S3, S4 and S5)

#### 3.2. GC-MS/MS analysis

The GC-MS/MS analysis of these samples was performed using a specific derivatization for conjugated 4,9,11-triene compounds, with MSTFA/ $I_2$  followed by pure MSTFA. GC-MS/MS chromatograms did not provide

any signal in the retention times of  $\alpha$ TB and  $\beta$ TB, for the two transitions evaluated for the suspected samples (Figure 2). Thereby, this specific derivatization for conjugated 4,9,11-triene compounds is an important tool to prevent

false positive results for TB, despite being an additional step in sample preparation.

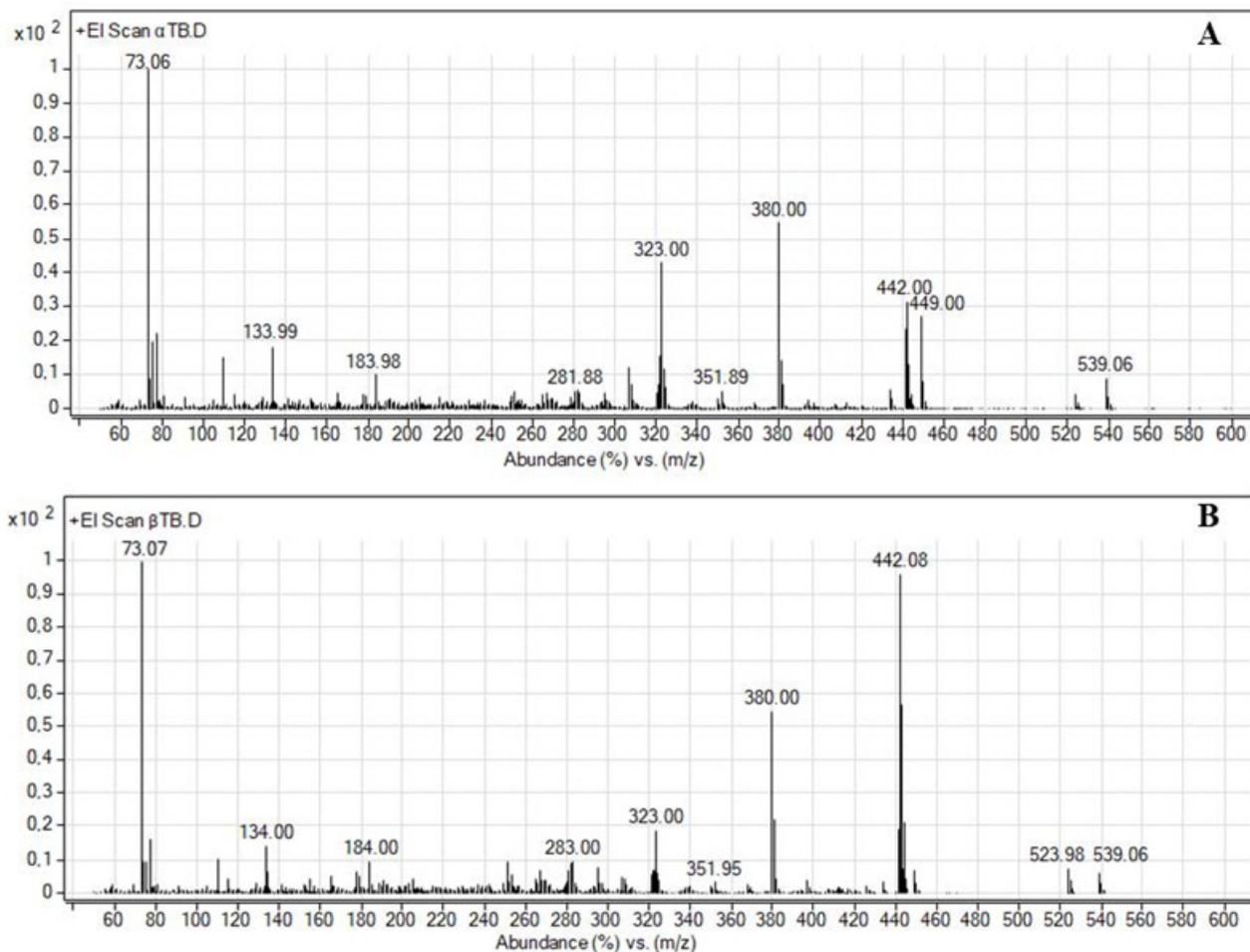


**Figure 2.** GC-MS/MS chromatograms for blank bovine urine spiked with 1  $\mu\text{g kg}^{-1}$  of  $\alpha$ TB and  $\beta$ TB (A) and bovine urine samples (S1, S2, S3, S4 and S5) for quantification transition (442.0 $\rightarrow$ 352.0)

The derivatization reaction used in this work was based on (18, 21), exclusively for conjugated 4,9,11-triene compounds, including  $\alpha$ TB and  $\beta$ TB. In the present work, the second derivatization was conducted in a microwave oven, thus providing a total reaction time (7 min) much shorter when compared to the conventional heating derivatization reaction and the derivatization reaction at room temperature, which require 43 min (21) and 90 min (18), respectively.

Full scan mass spectra for  $\alpha$ TB and  $\beta$ TB in methanol, derivatized with MSTFA/ $\text{I}_2$  followed by pure MSTFA, are shown in Figure 3. The fragmentation pattern was different for each isomer. The ion at  $m/z$  449 was more abundant in the mass spectrum of  $\alpha$ TB while the ion at  $m/z$  442 was more abundant in the  $\beta$ TB mass spectrum. The base peak in the two spectra was the ion at  $m/z$  73, and was attributed to the trimethylsilyl radical cation. The other significant fragments showed similar relative intensities, and the molecular ion in both spectra was measured at  $m/z$

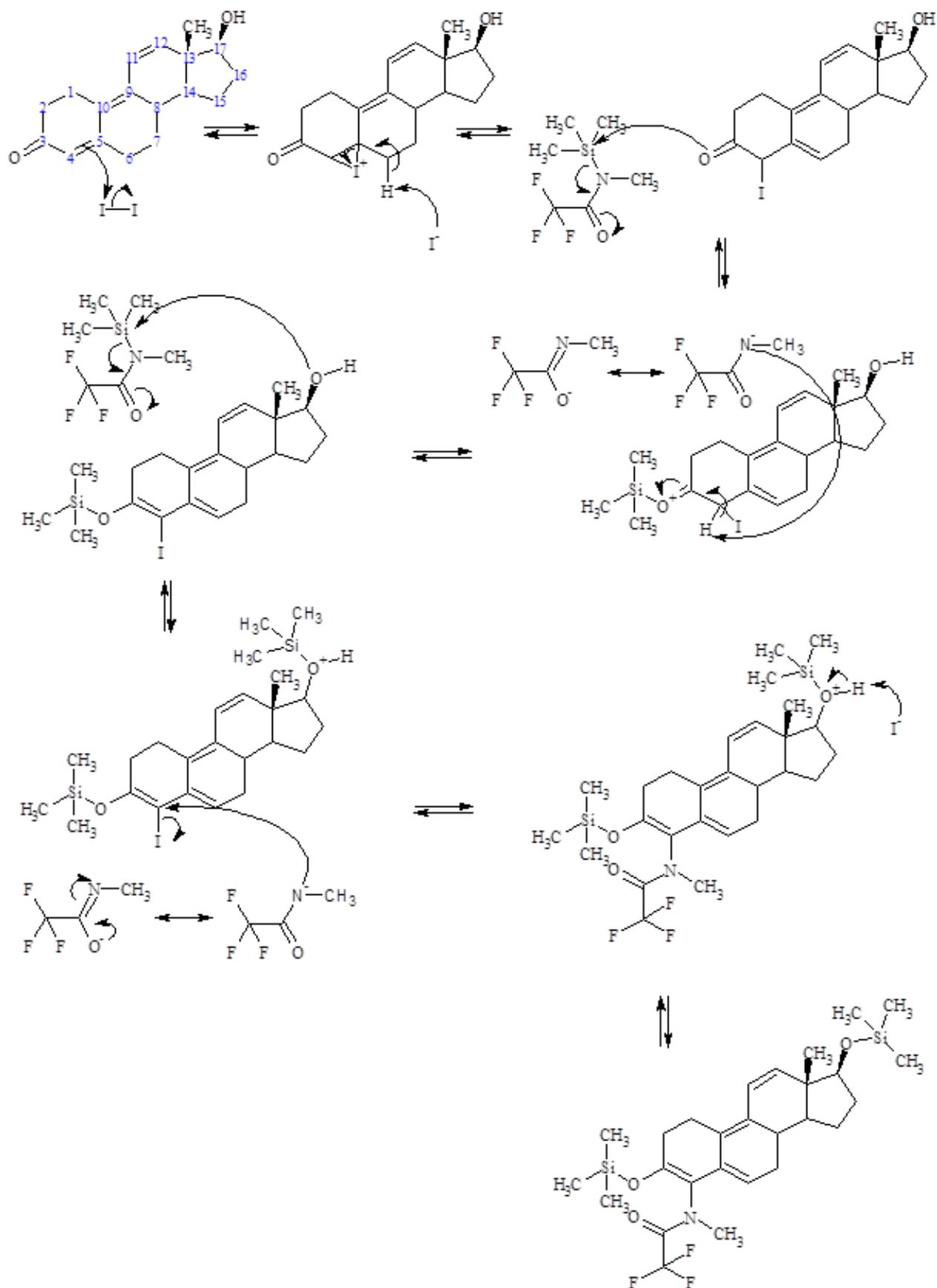
539. These results were in agreement with the works of Maume *et al.* (18) and Wozniak *et al.* (21).



**Figure 3.** Mass spectra of  $\alpha$ TB (A) e  $\beta$ TB (B) after derivatization with MSTFA/I2 followed by pure MSTFA

Based on the full scan mass spectra (Figure 3), a mechanism was proposed (Figure 4). Its main steps are: nucleophilic attack of the carbonyl group, at the C-3 position, on the silicon atom, catalyzed by iodine; nucleophilic attack of the N-methyltrifluoroacetamide group, on the carbon at the C-4 position followed by silylation of the hydroxyl group, at the C-17 position. The present

proposal was in agreement with the works of Maume *et al.* (18) and Wozniak *et al.* (21).



**Figure 4.** Mass Proposed mechanism for derivatization reaction of  $\beta$ TB with MSTFA/I<sub>2</sub> followed by pure MSTFA

### 3.3. Linearity, recovery and precision

In order to evaluate linearity, the F-test was applied and the response variances were considered heterogeneous for both analytes and methods. Thus, the weighted least squares approach was applied, using the inverse of the variance of each concentration level as weighting factor, and  $R^2$ -values were equal to or greater than 0.932 (Table 1).

Recovery, repeatability and intermediate precision met the following acceptance criteria of the *Codex Alimentarius*: recovery between 60-120% and repeatability with RSD lower than 30% for concentrations higher than  $1 \mu\text{g kg}^{-1}$  (25) (Table 1).

**Table 1.** Parameters for evaluation of the linearity, recovery and precision of the optimized methods

Method	Analyte	Linearity			Intra-day condition				Inter-day condition			
		Slope	Intercept	$R^2$	R	RSD	R	RSD	R	RSD	R	RSD
					1.0 MRPL		1.5 MRPL		1.0 MRPL		1.5 MRPL	
LC-MS/ MS	$\alpha$ TB	0.085	-0.017	0.932	83.5	6.8	91.9	12.0	88.5	10.9	87.0	14.1
	$\beta$ TB	0.116	0.054	0.980	101.4	11.6	106.7	6.6	112.5	17.2	112.8	9.8
GC-MS/ MS	$\alpha$ TB	51065	-64257	0.964	94.2	14.2	84.4	9.6	111.0	22.2	86.0	24.8
	$\beta$ TB	36902	-40042	0.940	103.9	14.5	86.0	5.1	114.0	18.5	87.0	25.5

R: Recovery (%); RSD: Relative standard deviation (%)

## 4. Conclusions

The analysis of the real samples allowed the comparison of GC-MS/MS and LC-MS/MS methods for identification and quantification of  $\alpha$ TB and  $\beta$ TB in bovine urine. The GC-MS/MS analysis confirmed the absence of anabolic steroid in these samples, indicating that the LC-MS/MS and GC-MS/MS methods can be complementary. Although the GC-MS/MS analysis is more time taking, it can be employed to investigate suspected results obtained by LC-MS/MS analysis, with more specificity.

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