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Determination of 17α-methyltestosterone in Bovine Serum Using Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

A high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) based method has been developed and validated to confirm the presence of the synthetic growth promoter 17α-methyltestosterone (MT) in bovine serum samples. After a single-step extraction with acetonitrile, the enhanced determination of methyltestosterone was achieved by detecting its oxime derivative. In accordance with the criteria defined in Commission Decision 2002/657/EC and using deuterated testosterone (T-d_3) as an internal standard, the method has been validated in terms of decision limit (0.05 ng/mL), detection capability (0.09 ng/mL), repeatability CV (7.04%), within-laboratory reproducibility CV (11.00%), and accuracy (87.00%).

Key words: methyltestosterone, bovine serum, HPLC-MS/MS, validation

INTRODUCTION

By nature, steroidal hormones are produced by male and female sex organs (i.e. testes and ovaries), adrenal cortex and placenta. Sex hormones are generally applied in veterinary medicine to regulate and improve fertility, as well as to improve growth. In addition to endogenous steroids, many semi-synthetic and synthetic analogues have also been produced and administered to animals[1].

Anabolic steroids have been used as growth promoters in livestock farming for more than 30 years. The analysis of illegal cocktails from the black market shows that the synthetic androgen 17α-methyltestosterone is being used to improve protein deposition, and consequently increase the body weight of animals. The role of natural and synthetic hormones in endocrine disruption is well known. Because of human health risk and carcinogenic potential of their residues[2], hormonal growth promoters are forbidden in the European Union.

Several analytical procedures have been described for the confirmatory analysis of methyltestosterone residues in different control matrixes, using gas (GC) or liquid chromatography (LC) with mass spectrometry (MS) detection, including analysis on hair[3,4], muscle[5,6], urine[7], and milk[4-6,8,9]. GC/MS is a sensitive and suitable technique for the assay of steroids, while HPLC/MS is a good alternative that is increasingly being used. Derivatisation procedures can change the chemical structure of analytes, resulting in higher ionisation efficiency and enhanced detection sensitivity for steroid determination with commonly available mass spectrometer detectors[6,10-12]. One of these derivatisation procedures is oxime formation, which enables the improvement of ketosteroid analysis with ESI/MS-MS[13-15].

In previous work, the analysis of methyltestosterone by LC/MS using atmospheric pressure chemical ionisation (APCI)[5] or electrospray ionisation (ESI)[8] in muscle tissue has played an important role. Recently, Shao *et al*. used electrospray ionisation (ESI) LC/MS for detecting 11 illegal natural and synthetic steroids in meat, liver, kidney, and milk[8]. Sample preparation included overnight hydrolysis and cleanup with Oasis HLB, silica and aminopropyl solid-phase extraction cartridges. Additionally, Xu *et al*. used ESI LC/MS/MS for the determination of 10 steroids in animal tissue. Sample preparation included 3-4 hours hydrolysis, tert-butyl methyl ether (TBME) extraction and cleanup with C18 SPE cartridges.

In order to comply with European Commission Decision 2002/657/EC[16], it is necessary to have validated analytical methods that enable unambiguous detection and confirmation of banned substances. Analysis

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of bovine serum is a suitable tool to control the administration of prohibited drugs in farming livestock[17,18]. No applications have been reported, but the development of sensitive and confirmatory methods in serum can improve methyltestosterone residue control in livestock animals.

The present study concerns development, validation and application of an analytical method for detecting and quantifying 17α-methyltestosterone residue in blood serum from cattle treated with low dosages of this drug. For extraction/purification of the steroid, only acetonitrile was used, and quantification was carried on using HPLC-(ESI) MS/MS. The developed methodology gave satisfactory recoveries and clean final extracts. As a whole, the method proved to be reliable and sensitive (MRPL 2 ppb stated in urine, RIVM-CRL, The Netherlands). Hence, it is a suitable tool for the determination and confirmation of methyltestosterone in serum and can be used for residue control programs and doping control analysis.

MATERIALS AND METHODS

I. *Chemicals*

Experimental materials included 17α-methyltestosterone, obtained from Steraloids Ltd. (Croydon, UK), and testosterone-16, 16, 17-d_3, used as deuterated analogue, which was purchased from CDN Isotopes (Quebec, Canada). Formic acid was from AcrosOrganics (Geel, Belgium). Methyl tert-butylether (MTBE), hexane, HPLC-grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). Hydroxylamine solution (99.999%, 50 wt.% in H_2O) was from Sigma-Aldrich (St Louis, Mo, USA). Milli-Q organic-free water from Millipore (Bedford, MA, USA) was used. All reagents were of analytical grade.

II. *Serum Samples*

Serum samples were obtained from twenty-two Holstein cows (approximately 3 months old, weight 100 ± 5 kg). Two of them were treated with an intramuscular injection (1.5 mL) of an oily methyltestosterone solution containing 5 mg/mL of steroid. The solution was prepared at the laboratory with methyltestosterone in a mixture of arachis oil and benzyl alcohol. Twenty animals were used as untreated controls. Serum samples were collected every three days for one month and kept frozen until analysis.

III. *Standards and Samples Preparation*

A stock solution of 1.0 mg/mL in methanol with 0.1% ascorbic acid was prepared for methyltestosterone and stored at -20°C. Standard solutions were prepared by dilution using methanol with 0.1% ascorbic acid at concentrations of 0.10, 0.15, 0.30, 0.45, 0.50, 0.60, 1 and 1.5 ng/mL. The internal standard testosterone-d_3 was diluted in acetonitrile at 1 ng/mL. Validation calibrators were made using blank bovine serum obtained from control animals.

Standard solutions, serum samples and validation calibrators (300 µL) were placed in 2-mL Eppendorf tubes containing 1000 µL of the internal standard mixture in acetonitrile. Tubes were capped, vortexed for 1 min, sonicated for 30 min and centrifuged at 16100 g for 15 min using an Eppendorf Centrifuge 5415D (Hamburg, Germany). The precipitate was discarded and the acetonitrile layer was evaporated under a nitrogen gas stream at 37°C.

The procedure described previously by other authors was used for steroid derivatisation[14,15]. Residue was redissolved in 300 µL of 1.5 M hydroxylamine solution (pH 10) for 30 min at 90°C. After this derivatisation procedure, 700 µL of water was added and the oxime steroid derivatives were extracted twice with 2 mL of MTBE. After evaporation under a nitrogen gas stream, the residue in 100 µL of methanol-water (50:50, v/v) was used for the chromatographic procedure.

IV. *Apparatus and Conditions*

The high performance liquid chromatography (HPLC) system consisted of a quaternary pump, degasser and autosampler from Agilent Technologies, model 1100 (Minnesota, USA). A Q-Trap 2000 mass spectrometer with Ion Source Turbo Spray from Applied Biosystems MSD Sciex (Toronto, Canada) was used. Nitrogen was produced by a high-purity nitrogen generator (PEAK Scientific Instruments Ltd, Chicago, Ill). Nitrogen was used for curtain, nebulizer and collision gas.

Aliquots (65 µL) from calibrators or sample extracts were separated by HPLC using a Synergi 2.5 µm Fusion-RP 100A (50 × 2 mm) column from Phenomenex (Torrance, CA, USA) with a guard column of the same filling material. The mobile phase was (A) aqueous formic acid solution (0.1%) with (B) methanolic formic acid solution (0.1%) at a flow rate of 200 µL/min. After the first 3 min with the eluent at 90% aqueous mobile phase (A), binary gradient mixing was initiated as follows: (A) 90% to 20% for 4 min, 20% to 50% for 5 min and 50% to 60% for 6 min. For re-equilibrating, the program was set for 5 min at the initial conditions. Multiple reaction monitoring (MRM) in positive mode was used. Unit mass resolution was set for both mass-resolving quadrupoles Q1 and Q3. Table 1 shows the transitions monitored for methyltestosterone and the internal standard testosterone-d_3 as well as ESI source conditions for each analyte. Data were collected using a Dell Optiplex GX400 workstation and processed with the Analyst 1.4.1 software package (MDS SCIEX).

Journal of Food and Drug Analysis, Vol. 18, No. 1, 2010

V. *Method Validation*

Validation was performed according to the European Commission Decision 2002/657/EC, using the ResVal program (version 2.2) from the Community Reference Laboratory CRL for hormones (RIVM, Bilthoven, The Netherlands). A homogeneous mixture of blank bovine serum (35 mL) was divided into 63 sub-samples. Each day (over the course of three days), 21 fortified samples were analyzed: one sample was not spiked, 6 samples were spiked at 0.15 ng/mL, 6 samples at 0.30 ng/mL, 6 samples at 0.45 ng/mL, 1 sample at 0.60 ng/mL and 1 sample at 1.50 ng/mL. Each day a calibration graph was calculated using standard solutions from 0 to 1.50 ng/mL. The concentration of the analyte in the validation and incurred samples were interpolated from calibration curves constructed by calculating the area ratios of analyte peak area / internal standard peak area *versus* analyte concentration. The calibration graph (analyte peak area / internal standard peak area *versus* analyte concentration) can be described by the equation $y = m \cdot x + b$. In addition, 20 blank bovine serum samples were analyzed. Decision limit, detection capability, selectivity and specificity, precision, accuracy (as corrected percentage recovery), repeatability, reproducibility and applicability/ruggedness were evaluated.

RESULTS AND DISCUSSION

I. *Methodology Development*

In previous studies concerning the analysis of steroids in serum, liquid-liquid and/or solid phase extractions were required leading to long and complicated pre-treatments[12,14,19,20]. Deproteinisation and extraction with acetonitrile, without later steps, permitted the HPLC/MSMS analysis of fluoroquinolones[21] or steroids[22] and this procedure has been used in our laboratory for some years. Recently, Guo *et al.* described a similar deproteinisation procedure with acetonitrile for serum preparation, prior to an LC/MS/MS oestrogen measurement[23]. As the present validation demonstrates, the proposed methodology enables efficient measurement of methyltestosterone from serum, with minimal background interference. For the chromatographic method, injection of extracts dissolved in 100 µL of methanol/water (50:50, v/v) within the three first minutes, with a very aqueous mobile phase, holds the steroids at the column head. This produces very sharp peaks and avoids more polar interferents.

Evaluation of mobile phases containing acetonitrile and methanol showed the most efficient ionisation for this last solvent. The Synergy Fusion column provided sufficient retention and separation of the oxime methyltestosterone derivative, assuring a good method performance. Chromatographic separation of a bovine serum sample spiked with the assayed steroid and internal standard at a level of 0.15 ng/mL and a real sample from treated animals are shown in Figure 1. Good efficiency and peak shape were achieved with the proposed gradient conditions. Figure 1 also shows a chromatogram of a blank bovine serum sample.

II. *Mass Spectrometry Conditions*

Oximation with hydroxylamine is considered to be one of the most interesting reactions for derivatisation and offers increasing steroid sensitivity when using ESI[13-15]. In this work, we developed a method for analysing the synthetic androgen 17α-methyltestosterone, which has a reactive keto group. For achieving the maximum signal intensity, mass spectrometric conditions were optimized by directly injecting the oxime derivatives from the steroid and its deuterated analogue (testostenone-d_3) into the mass spectrometer, at a concentration of 1 µg/mL in mobile phase. Addition of formic acid (0.1%) was necessary to improve ionisation. Obtained derivatives were easily ionisable with ESI under positive mode, yielding a protonated oxime ion $[M+H]^+$ (Figure 2). The product ion spectrum of protonated steroid-oxime was evaluated. The compound-dependent parameters were optimised by means of their infusion into the source, leading to declustering potential, entrance potential, collision cell entrance potential, collision cell exit potential, collision cell exit potential

Table 1. MRM transitions and mass spectrometry parameters of derivatized methyltestosterone (m/z 318) and its internal standard T-d_3 (m/z 307)

MRM Transition (Q1,Q3)	Retention Time (min)	Dwell time (ms)	DP (volts)	EP (volts)	CEP	CXP	CE
306.994 / 124.100	13.02	150	111	11	14	4	41
306.994 / 112.000	13.02	150	111	11	14	4	41
318.087 / 124.200	13.33	150	71	9	14	4	45
318.087 / 112.100	13.33	150	71	9	14	4	45

DP: Declustering Potential; EP: Entrance Potential; CEP: Collision Cell Entrance Potential; CXP: Collision Cell Exit Potential; CE: Collision Energy.

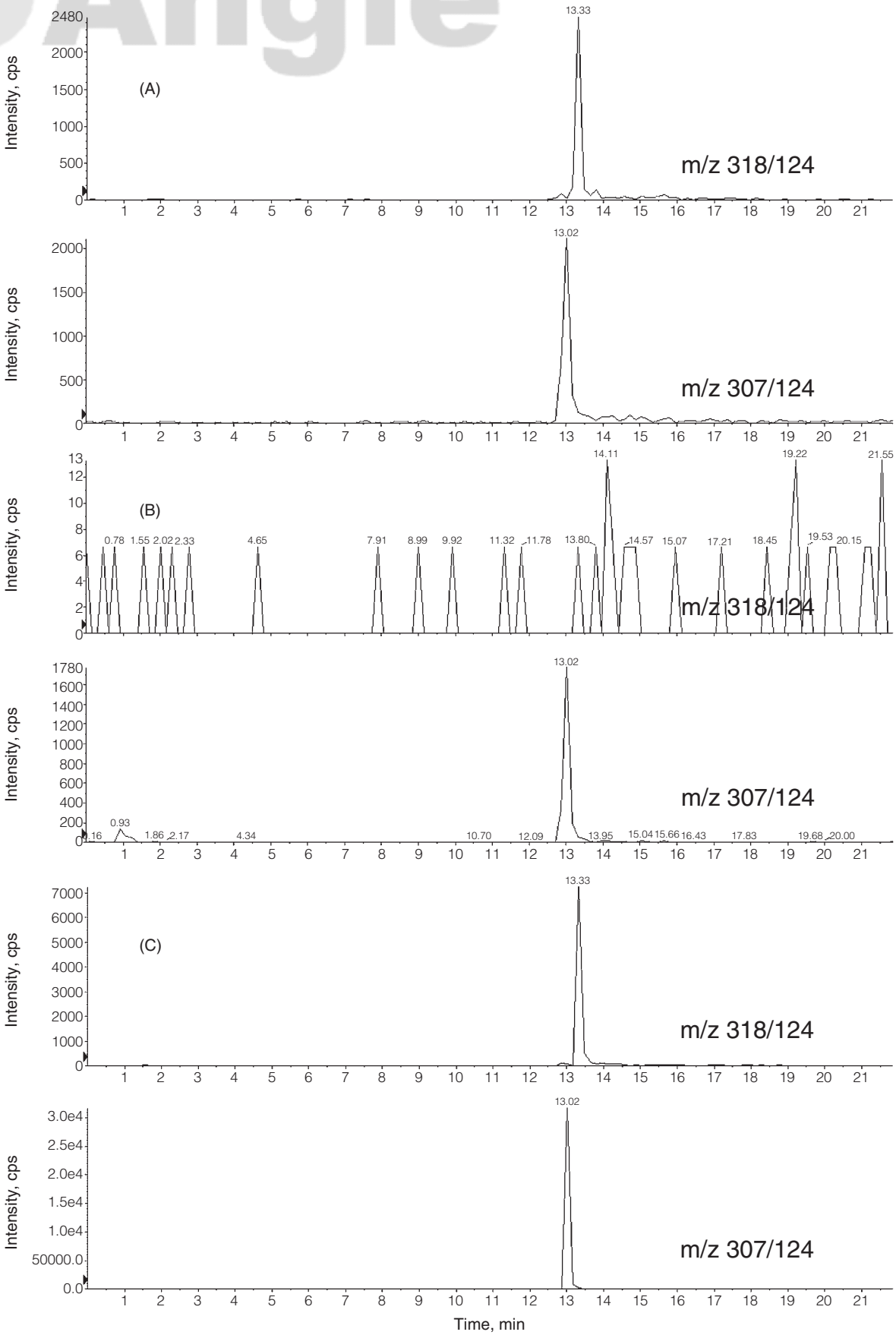
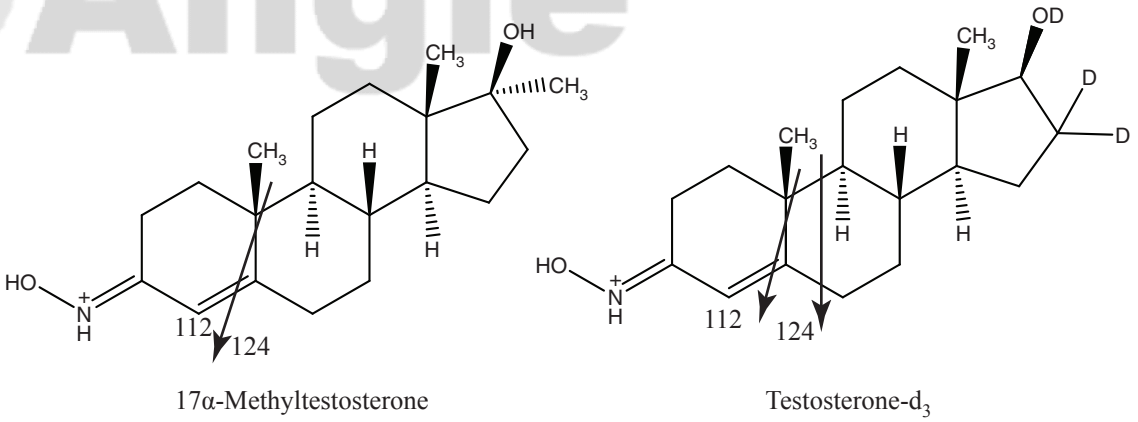


Figure 1. Reconstructed chromatogram of a spiked (0.15 ng/mL) serum sample (A), a blank bovine serum sample (B) from control animals and a real bovine serum sample tested positive (C).



17α-Methyltestosterone Testosterone-d_3

Figure 2. Structure of oxime derivatives of MT and T-d_3 and their possible fragmentation points.

(CXP) and collision energy as shown in Table 1. The ESI source-dependent parameter settings were optimized for intensity under LC conditions using the flow injection mode (FIA) of a methyltestosterone-oxime solution, obtaining the following results: ionspray voltage, 5500 V; heater temperature, 400°C; curtain gas, 30 arbitrary units (AU); collision gas, medium; nebuliser gas, 45 AU and turbo gas, 65 AU. Analytes were quantified in MRM mode. Two MRM transitions (Table 1) were monitored for identifying the analyte (150 ms dwell time/transition). For quantification, the most intense transition was monitored. Evaluation of the ratio of their relative intensities was used to confirm compound identity. Fragmentation produced ions at m/z 112 and 124, which corresponds to fragments of a protonated 3-oxime.

To obtain quantitative results, analyte peak area was divided by internal standard peak area. Deuterated testosterone was used as deuterated analogue of methyltestosterone to carry out the analytical methodology. Similarity of structures, fragmentation patterns and retention times permitted the use of deuterated testosterone for methyltestosterone analytical method validation.

III. *Method Validation*

During validation, blank serum from different animals and 20 blank bovine serum samples from 20 different animals were analysed. Selectivity and specificity of the method were checked by analysing these 20 blank bovine serum samples. No interfering peaks were found in the retention time of the analyte ± 2.5% as shown in the chromatogram of the selected transitions (Figure 1). Calibration curves were constructed by linear fitting using the least squares linear regression calculation covering the entire working range. The calibration curves obtained each day gave a good linear correlation [$y = (0.0237 \pm 0.0037)x + (0.1428 \pm 0.0174)$, $R = 0.9941 \pm 0.0014$). Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant[16]. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. The decision limit (CCα), the concentration level for a confirmatory method in accordance with European Decision[16] was 0.05 ng/mL. The detection capability (CCβ) means the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of β[16]. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability. The detection capability CCβ was found to be 0.09 ng/mL. These concentrations were all well below the MRPL of 2 ppb. The α and β errors should be less than or equal to 1% or 5%, respectively.

There are no reports of analysing methyltestosterone in serum without derivatisation and only a few using derivatisation. Kaklamanos *et al.*[5] developed a highly time-consuming HPLC/MS/MS method to detect methyltestosterone without derivatisation analyzing 5.0 g of muscle and obtaining CCα and CCβ of 0.05 ng/g and 0.09 ng/g respectively. Both values were similar to those reported by this validation but using a larger amount of sample. Shao *et al.*[8] reported a LOD (Limit of Detection) of 3 ng/kg and a LOQ (Limit of Quantification) of 10 ng/kg sampling 10 g of milk, suggesting this method requires more quantity of sample than present methodology (only 300 μL of serum). Recently, Van der Merwe *et al.*[24] reported a GS/MS method to determine methyltestosterone in ostrich's serum using 2 mL of sample, achieving a CCα of 0.3 ng/mL and a CCβ of 0.6 ng/mL, both higher than our values and analyzing larger amount of serum. Malone *et al.*[25] developed a rapid LC/MS/MS method for the analysis of synthetic growth promoters using 5 g of bovine muscle and a similar apparatus, reporting CCα = 0.1 ng/g and CCβ = 0.17 ng/g, both values upper those presented in this work.

Since no certified reference materials were available in our laboratory, Commission Decision 2002/657/EC

offered a means of determining the accuracy of corrected recovery for spiked samples. Each day spiked samples were analyzed at 0.5, 1, 1.5, 2 and 3 times the validation level, following ResVal instructions[26]. The recovery was corrected for losses during sample preparation via the aid of a deuterated internal standard, as indicated above. The analyzed accuracy (87.00%) was in compliance with the criteria to be between 70-110%. The results obtained at the CCα level for repeatibility (CV = 7.04%) and reproducibility (CV = 11.00%) were also in accordance with the Commission Decision 2002/657/EC.

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