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# Liquid chromatography–tandem mass spectrometry methods for determination of stanozolol and 16β-hydroxy-stanozolol in animal urine

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## Abstract

**Introduction:** Because of the activities and effects they induce, hormones are prohibited for use for anabolic purposes in farm animals intended for slaughter, which is regulated in the European Union by relevant legal provisions. Therefore, there is an obligation to monitor residues of hormones in animals and food of animal origin to ensure consumer safety. A hormone banned but used formerly for fattening cattle, stanozolol, and its metabolite 16β-OH-stanozolol are synthetic compounds that belong to a large group of steroid hormones. This study investigates residues of these compounds in animal urine. **Material and Methods:** From 2006–2022, 2,995 livestock urine samples were tested for stanozolol residues in Poland as part of the National Residue Monitoring Programme. A liquid chromatography–tandem mass spectrometry method to determine stanozolol and 16β-OH-stanozolol in animal urine was developed and validated according to the required criteria. Urine sample analysis was based on enzymatic hydrolysis of hormones potentially present in it to the free form, extraction of them from the sample with a mixture of n-hexane and butyl alcohol, purification of an extract on an $NH_2$ amine column and finally, instrumental detection. **Results:** The apparent recovery and precision parameters of the developed method were in line with the established criteria, while its decision limits CCα and detection capabilities CCβ were lower than the recommended concentration for analytical purposes set at 2 μg $L^{-1}$ (valid until December 15, 2022; currently set as 0.5 μg $L^{-1}$). **Conclusion:** All examined samples were compliant with the evaluation criteria.

**Keywords:** urine, stanozolol, hormone residue analysis, LC-MS/MS.

## Introduction

Stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno(3,2-c)-pyrazole) (STAN) is an 17-alpha-methylated synthetic derivative of the male sex hormone testosterone and also of its active metabolite dihydrotestosterone (DHT), which was first synthesised by Clinton *et al.* (5) in 1959. It belongs to the heterocyclic anabolic androgenic steroids group (AAS). It is one of the oldest steroid drugs, in production starting from four years after its synthesis in 1962 in the UK. This compound is the closest structure to the steroid hormone methyltestosterone, but differs from endogenous and most synthetic hormones. Instead of the ketone group at the C3 carbon atom in methyltestosterone, it has a pyrazole ring condensed to the androstane ring system, with favourable structural features and chemical properties, but hindering the isolation of this compound from the biological matrix during determination of residues (29). The product is commercially available under the names "Winstrol" "Strombafort" or "Winobolic". It has been used since the 1980s as a growth stimulant in human athletics and was popular in many sports, from American football through martial arts to bodybuilding and strength sports. However, it has been banned since 1974 by the

International Olympic Committee (IOC). Its action enhances muscle tissue efficiency, accelerates regeneration, and improves the body's quality. Depending on the desired effect, it is combined with other anabolic steroids such as trenbolone, boldenone, nandrolone and testosterone. Stanozolol was also given to racehorses to improve their performance, accounts of which in Australian horseracing are reported in the literature (25). At the end of the 1990s, STAN and drug combinations including it were found in samples of biological material from slaughtered animals taken from the injection sites, even though it has been in the group of anabolic compounds prohibited in fattening animals in the European Union (EU) countries since 1988. Stanozolol is currently in group S1 of Anabolic Agents and subgroup 1 of AAS in the World Anti-Doping Agency (WADA) list of the substances prohibited for athletes at all times during competition (37). Laboratory statistics from WADA show the median annual percentage of positive results for STAN to be 14% in the S1.1 compound class and indicate that for over ten years, it has been one of the most commonly identified steroids among the AAS group of compounds (10, 36). As an orally active steroid, this compound has been used for therapeutic purposes in humans mainly to improve poor protein synthesis by the body and mitigate bone and muscle mass loss, during convalescence after injuries of the musculoskeletal system, and in postmenopausal osteoporosis, hereditary angioedema, anaemia, coagulation disorders and vascular manifestations of Behcet's disease. Used thus, it inflicts no significant damage on internal organs, especially the liver, even in long-term treatment if the dose is low. This synthetic hormone does not directly affect the level of oestrogens or DHT but as a derivative of DHT it can slightly lower progesterone levels. However, there is evidence that it causes lipid metabolism disorders, overproduction of haemoglobin and left ventricular hypotrophy when used in high concentrations, because of the pharmacological action of hepatotoxic effects (23, 27). For this reason, this compound has been superseded by other, more specific drugs with fewer side effects.

Stanozolol is rapidly metabolised after administration, becoming 16β-OH-stanozolol (16β-OH-STAN) as the main urine metabolite of phase I of metabolism in bovines, horses (25, 26) and indisputably also in humans (1, 14, 33). In the group of STAN metabolites in bovine and human urine, 4β-hydroxy-stanozolol, 3′-hydroxy-stanozolol, and 4,16-dihydroxy-stanozolol excreted in the form of conjugates have also been investigated (14, 34). Anabolic steroids are mainly excreted in urine bound with glucuronic or sulfuric acid. According to research by McKinney *et al.* (25) on the urine of horses, stanozolol and its 15C-metabolites in phase II of metabolism bind mainly with glucuronic acid, while its 16α/β-metabolites bind mostly with sulphuric acid in certain epimeric proportions.

The use of hormones in the fattening of animals for consumption has been legally prohibited in EU countries since 1988 following Council Directives 96/22/EC and 2003/74/EC (7, 20). Therefore, hormones should not be present in animal tissues, and maximum residue limits (MRLs) have not been established for them (6, 17). In 2007, the EU Reference Laboratories (EURLs) proposed a recommended concentration (RC) value of 2 μg $L^{-1}$ for analytical purposes and monitoring of STAN residues in urine (32). In connection with the current update of the legislation relating to the use of veterinary drugs in animals and the production of food of animal origin, the analytical limit of the minimum method performance requirement (MMPR) has been lowered for STAN and 16β-OH-STAN in urine to 0.5 μg $L^{-1}$ (12). The possible presence of hormone residues in animal tissues may pose a risk to consumers of food of animal origin. Therefore, all EU countries are obliged to monitor the residues of anabolic hormones in biological samples from animals and of food of animal origin (8, 16, 18, 21). European Union experts have placed STAN and its 16β-OH-STAN metabolite on the list of the minimum recommended number of compounds to screen for in the steroid group (A3) since 2006. The list was covered by the classification of Annex I to Council Directive 96/23/EC (8, 32) until December 15, 2022 and has recently been amended by updated legislation and placed according to Annex I in group A1c (12, 16). In adherence to the EU recommendations, studies of residues of these compounds in animal excreta and food of animal origin were undertaken in Poland in 2006 and introduced to the monitoring programme. Originally, an enzyme-linked immunosorbent assay (ELISA) method was used for this purpose. However, this technique generated many non-compliant results. Union legislation specifies requirements for analytical methods for screening for anabolic hormone residues. In particular the legal framework calls for the use of liquid (LC) or gas chromatography (GC) techniques with mass spectrometry (MS), allowing the detection of compounds at low concentration levels (below the proposed RC values and current MMPR values) and their full identification using certain criteria. In order to meet the residue limit detection requirements and criteria for identification, it was necessary to develop an instrumental confirmatory method suitable both for detecting the compounds mentioned above at low concentration levels and for their identification.

Different instrumental techniques were used for the determination of STAN in urine and other biological matrices like faeces or hair: a gas chromatography–mass spectrometry (GC-MS) technique (2, 3, 4, 22, 28) and most often in recent years, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) technique (1, 9, 13, 14, 25, 28, 33, 34). In light of STAN and OH-STAN being forbidden compounds and the desirability of

obtainment of the lowest possible decision parameters for them, sensitive LC based methods were developed for further implementation in the Polish Residue Monitoring Programme (RMP).

## Material and Methods

**Reagents and chemicals.** The requisite solvents, namely methanol (analytical, HPLC, and residue grade), ethanol, n-hexane and butanol, were purchased from J. T. Baker (Deventer, the Netherlands). Other chemicals were obtained from Merck (Darmstadt, Germany) and these were acetic acid, formic acid, sodium acetate, anhydrous sodium carbonate and β-Glucuronidase (23 U $mL^{-1}$)/Arylsulfatase (68 U $mL^{-1}$) from *Helix pomatia* (AS HP). Solid phase extraction (SPE) $C_{18}$ 500 mg/3 mL columns were obtained from J. T. Baker and type I water with the highest purity was obtained with a Milli-Q apparatus (MilliporeSigma, Burlington, MA, USA). Acetate buffer (2 M, pH 5.2), solutions of sodium carbonate (1 M) and formic acid (0.1%), and mixtures of n-hexane with methanol, methanol with water, and methanol with 0.1% formic acid were prepared in the laboratory.

Standards of stanozolol and 16β-OH-stanozolol (16β-OH-STAN) were purchased from Sigma Aldrich (St. Louis, MO, USA), Dr Ehrenstorfer (Augsburg, Germany) or the National Measurement Institute (North Ryde, Australia). An internal standard (IS) of 16β-OH-stanozolol-D3 (16β-OH-STAN-D3) was provided by the European Union Reference Laboratory (EURL) – Wageningen Food Safety Research (WFSR, Wageningen, the Netherlands). Standards of compounds were kept at room temperature while labelled standards were stored at 2–8°C according to the manufacturer's recommendations. Primary standard stock solutions were prepared in methanol at concentrations of 1 mg $mL^{-1}$, 100 µg $mL^{-1}$ and 10 µg $mL^{-1}$. Working solutions were obtained by tenfold dilution of primary standard solutions to the concentration of 1 µg $mL^{-1}$. The structural formulas of molecules of STAN, 16β-OH-STAN and the analogous IS are presented in Fig.1.

**Sample preparation.** Urine sample processing was based on the analytical procedure developed and shared by the WFSR EURL (30). The samples were stored frozen until the start of the test and were thawed at room temperature prior to testing. The urine was filtered using filters for biological material to remove macroscopic contamination. A 5 mL aliquot of urine was prepared, and 2 mL of acetate buffer pH 5.2 was added to it. Acetic acid or 1M sodium carbonate solution was added to adjust to the required pH. The 16β-OH-STAN-D3 internal standard was added to the sample in an amount of 10 ng, which corresponded to a concentration of 2 µg $L^{-1}$ in the sample. In sequence, 100 µL of AS HP glucuronidase was added and the sample was thoroughly mixed and subjected to enzymatic hydrolysis at 37°C (±2°C) for 16–20 h. After adjusting the pH to 9 with 1 M sodium carbonate, extraction was carried out with 10 mL of a mixture of n-hexane and butanol (80:20, v/v). Then the contents were centrifuged for 3 min at 6,192 × *g*. The organic layer was sequentially transferred to another tube, and the extraction was repeated with 10 mL of a mixture of n-hexane and butanol. After combining the organic phases, the extract was evaporated at 55°C (±2°C) in a nitrogen stream. The urine sample was then purified. For this purpose, the dry evaporation residue was dissolved in 5 mL of a methanol-water mixture (80:20, v/v) and applied to an $NH_2$ column preconditioned previously with 5 mL of the above mixture. After passing through the column, the extract was collected and evaporated at 55°C (±2°C) under a stream of nitrogen. The extract was dissolved in 500 µL of ethanol and re-evaporated under controlled conditions. Finally, the residue was dissolved in 100 µL of a mixture of methanol with 0.1% formic acid (50:50, v/v), mixed thoroughly and dispensed into the specified LC system.

**Liquid chromatography–tandem mass spectrometry analysis.** Two LC-MS/MS systems were used in the instrumental analysis. The first was composed of an 1100 Series HPLC pump system (Agilent Technologies, Waldbronn, Germany) for the LC separation of hormones and a Finnigan $LCQ_{DUO}$ ion trap instrument (Thermo Quest, San José, CA, USA) operating in positive electrospray ionisation mode for spectrometric measurement (LC-$MS^2$ IT).

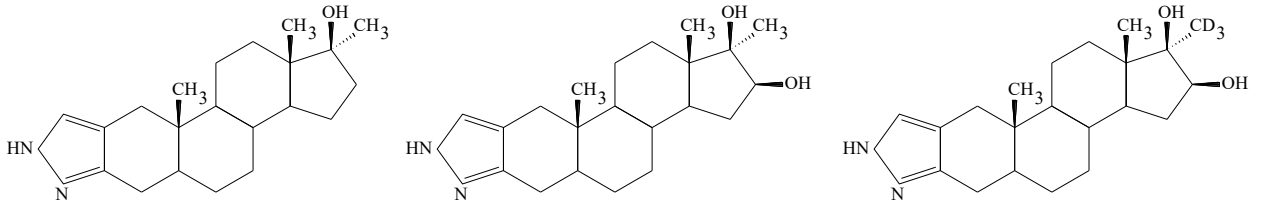


**Fig. 1.** Chemical structures, molecular formulas and weights of molecules of hormones tested

The second set was composed of a 1200 Series binary pump system (Agilent Technologies) for the LC separation coupled with a triple quadrupole mass spectrometer API 4000 with a Turbo Ion source (Sciex, Framingham, MA, USA) (QqQ). For both instruments, nitrogen was used as the nebulisation and desolvation gas. Also in both cases, the same Ultra C18 analytical column (150 mm × 2.1 mm × 5 µm) (Restek, Bellefonte, MA, USA) with an octadecyl guard cartridge (4 mm × 2 mm) (Phenomenex, Torrance, CA, USA) was used. The elution solvents were methanol (A) and an 0.1% aqueous solution of formic acid (B), which were applied in a gradient. The output composition of the mobile phase (A:B (v/v)) was 60:40 and changed to 80:20 from 0 to 20 min, then changed again to 60:40 to 25 min and persisted as 60:40 to 30 min. The mobile phase flow rate was constant and equalled 0.25 mL $min^{-1}$ throughout the analysis. The chromatographic column was kept at a constant temperature of 30°C. The injection volume of the sample was 25 µL. In the case of LC-$MS^2$ (IT), defined diagnostic signals (selected reaction monitoring transitions, SRM) with optimised MS and MS/MS parameters were monitored for analytes and internal standards. The XCalibur software (Thermo Fisher Scientific, Waltham, MA, USA) that controlled the system was used for data registration and processing. However, in the case of LC-MS/MS (QqQ), two or three diagnostic signals (multiple reaction monitoring transitions, MRM) were recorded depending on the compound with optimised ion source and acquisition parameters. The Analyst software (version 1.4.2; Sciex) that controlled the instrument was used for MS/MS analysis, data collection and processing. The parameters used for the identification and confirmation of STAN and 16β-OH-STAN are presented in Table 1.

**Samples.** During the validation study of the method, the reference material used was bovine and porcine urine previously tested in the laboratory for the presence of STAN and 16β-OH-STAN residues, in which these compounds were not found. In further studies, the research material was animal urine samples officially collected in the years 2006–2022 by authorised veterinary sanitary inspectors in Poland under the auspices of the RMP for the presence of prohibited substances and chemical and biological residues of medicinal products in live animals and animal products. Samples were taken on farms and slaughterhouses according to national and European regulations regarding sampling frequency requirements. In sixteen years of research 2,995 official urine samples were tested. Among them 1,332 came from farms and the remaining 1,663 from slaughterhouses. A total of 2,477 samples were taken from cattle, 514 from pigs, 2 from sheep and 2 from horses.

**Table 1.** Liquid chromatography–tandem mass spectrometry ion acquisition parameters used for the identification of stanozolol (STAN) and 16β-OH-stanozolol (16β-OH-STAN) using a 16β-OH-stanozolol-D3 internal standard (16β-OH-STAN-D3)

| LC-MS/MS (QqQ) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Compound | MRM transition (m/z) | Collision energy CE (V) | Declustering potential DP (V) | Entrance potential EP (V) | Collision cell exit potential CXP (V) | Ion ratio average ± SD | Samples fulfilling the confirmation criteria (%)* | |
| | | | | | | | 0.50–5.00 µg $L^{-1}$ | CCα level |
| STAN | 329>121 | 52 | | | | 0.346 ± 0.023 | 98.7 | 100.0 |
| | 329>95 | 54 | 212 | 10 | 10 | 0.395 ± 0.019 | 100.0 | 100.0 |
| | **329>81**[a] | 68 | | | | - | - | - |
| 16β-OH-STAN | 345>95 | 61 | 214 | 10 | 10 | 0.399 ± 0.027 | 100.0 | 100.0 |
| | **345>81** | 70 | | | | - | - | - |
| 16β-OH-STAN-D3 | **348>81** | 72 | 198 | 10 | 5 | - | - | - |
| Additional series of validation | | | | | | Ion ratio average ± SD | Samples fulfilling the confirmation criteria (%)** | |
| | | | | | | | 0.25–5.00 µg $L^{-1}$ | CCα level |
| STAN | 329>121 | | | | | 0.218 ± 0.028 | 100.0 | 100.0 |
| | 329>95 | | as above | | | 0.273 ± 0.009 | 100.0 | 100.0 |
| | **329>81**[a] | | | | | - | - | - |
| 16β-OH-STAN | 345>95 | | as above | | | 0.264 ± 0.039 | 100.0 | 100.0 |
| | **345>81** | | | | | - | - | - |
| LC-$MS^2$ (IT) | | | | | | | | |
| Compound | SRM transition (m/z) | | Collision energy CE (%) | | | Ion ratio average ± SD | Samples fulfilling the confirmation criteria (%)* | |
| | | | | | | | 1.00–10.00 µg $L^{-1}$ | |
| STAN | **329>311**[a] | | | | | - | - | |
| | 329>271 | | 35 | | | 0.228 ± 0.032 | 75.3 | |
| | 329>229 | | | | | 0.363 ± 0.067 | 74.1 | |
| 16β-OH-STAN | 345>327 | | | | | 0.653 ± 0.212 | 51.9 | |
| | **345>309** | | 37 | | | - | - | |
| | 345>227 | | | | | 0.734 ± 0.163 | 55.6 | |
| | 345>159 | | | | | 0.735 ± 0.135 | 58.0 | |
| 16β-OH-STAN-D3 | **348>312** | | 36 | | | - | - | |

LC-MS/MS (QqQ) – liquid chromatography–tandem triple quadrupole mass spectrometry; [a] – transitions shown in bold were used for quantification; SD – standard deviation; CCα – decision limit; *– according to Commission Decision No. 2002/657/EC (6); **– according to Commission Implementing Regulation 2021/808 (17); LC-$MS^2$ (IT) – liquid chromatography–tandem ion trap mass spectrometry

**Validation study.** The developed LC-MS/MS methods were validated following the general guidelines of Commission Decision 2002/657/EC for confirmatory methods (6). The performance parameters of instrumental linearity, specificity, repeatability, reproducibility, apparent recovery, ruggedness, decision limits, and detection capabilities were determined (6). For the analysis of the factor effect, an in-house Microsoft Excel form with data processed by ResVal software, version 2.0 made available by EURL-WFSR was used as a technical tool (31). The instrumental linearity of the methods was assessed based on calibration curves of standard working solutions of stanozolol and 16β-OH-stanozolol drawn at six points each. In standard curves, adjusted to the appropriate range of spiking of urine samples, analyte concentrations corresponded to 0, 0.20, 0.40, 2.00, 4.00, and 10.00 μg $L^{-1}$, while the appropriate amount of IS used corresponded to the content of 2.00 μg $L^{-1}$ in the sample. A validation level (VL) of 2.00 μg $L^{-1}$ corresponding to the set RC value was adopted as suggested by the EURLs in the case of the LC-$MS^2$ (IT) technique, whereas a lower VL of 1 μg $L^{-1}$ was applied to LC-MS/MS (QqQ). Specific spiking levels of urine samples were adapted to the particular detection method, but four experiments of spiked samples were designed for both of them. Three series of samples spiked to concentration levels of 1.00, 2.00, 3.00, 4.00 and 10.00 μg $L^{-1}$ were prepared for LC-$MS^2$ (IT) spectrometric measurement, and three series of samples were also prepared for LC-MS/MS (QqQ) spectrometric measurement; however these were spiked to concentration levels of 0.50, 1.00, 1.50, 2.00 and 5.00 μg $L^{-1}$. In each series, blank reference urine samples were also analysed. An additional series of validation included ten urine samples spiked at the CCα concentration level determined from the previous test series for individual compounds and appropriately adjusted standard curves. Based on these acquisition data, matrix-matched calibration curves were plotted. Regression parameters of both prepared curves were calculated (standard and matrix-matched). Concentration calculations were based on the standard calibration curves prepared with the 16β-OH-STAN-D3 internal standard. Calculations of the detection value CCα (the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant) and detection capability CCβ (the smallest content of the analyte that may be detected and/or quantified in a sample with an error probability of β) were made in the formulae of the Excel spreadsheet (for LC-$MS^2$ (IT)) and automatically by the software (in the case of LC-MS/MS (QqQ)). The α error was the probability of a false non-compliant decision and the β error was the probability of a false compliant decision. The calculation methodology was compliant with the ISO/11843 standard (24). The highest values of these parameters were chosen from the three experiments used (worst-case approach).

According to the ISO/11843 standard, the calculations of CCα and CCβ for prohibited compounds are based on the matrix-matched calibration curve procedure using blank material spiked with analytes below, at and above an adopted validation level. The corresponding concentration at the y-intercept of the calibration line plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit CCα. In comparison, 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 CCβ. Apparent recovery, precision and uncertainty were also assessed using these validation experiments. The validation process also included the step of checking signal specificity. For this purpose, in the case of LC-$MS^2$ (IT), analysis of ten blank urine samples simultaneously with ten samples of urine fortified to 1 μg $L^{-1}$ was carried out, and in the case of LC-MS/MS (QqQ), according to the ResVal software manual, the concentration of compounds in the blank samples was analysed relative to the determined CCα parameters. The expanded uncertainty for STAN and its metabolite was calculated as the sum of variances of reproducibility multiplied by the coverage factor of 2 by the Excel spreadsheet formulae and ResVal software at VLs of 2.00 μg $L^{-1}$ and 1.00 μg $L^{-1}$, respectively. Following the assumptions of the validation, eight robustness study samples were also investigated. Seven different possible factors which could influence the measurement results were selected. The group of selected factors included the pH of extraction with a mixture of n-hexane and butanol, the composition of the mixture of n-hexane and butanol, the species of animal from which the urine sample came, the series of SPE columns, the composition of the mixture of methanol and water used before application of the extract on SPE $NH_2$ column, the temperature of the analytical LC column and the composition of the mobile phase concerning the formic acid content. The method's ruggedness was estimated using the Youden approach under the guidelines of Commission Decision 2002/657/EC, by comparing the calculated standard deviations for the differences between the two levels of each factor with the standard deviations determined under within-laboratory conditions (6).

Concerning the guidelines in Decision 2002/657/EC, confirmation criteria regarding acceptable tolerances of relative ion intensities and compliance with the relative retention time of STAN and 16β-OH-STAN in urine samples spiked for validation purposes were checked (6). In addition, urine samples spiked at the level of estimated CCα values were checked for reliability according to SANCO guidelines (11). Furthermore, it was verified whether, in the case of urine samples spiked at the estimated level of CCα, the confirmation criteria set out in

Decision 2002/657/EC (at least four identification points, IPs) were fulfilled.

In connection with the entry into law of Commission Implementing Regulation (EU) 2021/808, which replaced Decision 2002/657/EC from June 10, 2022, an additional series of validation by LC-MS/MS (QqQ) was analysed. Taking into account the new proposed MMPR analytical limit of 0.5 μg $L^{-1}$ for STAN in urine there was a need to determine CCα limits for confirmation purposes, and in line with EURL recommendations according to the provisions of Regulation (EU) 2021/808 dedicated to prohibited and unauthorised substances (12, 17). The experiment included spiking levels of urine at 0.25, 0.50, 0.75, additionally at 1.00, 2.00, 5.00 μg $L^{-1}$, and ten samples at the calculated CCα. The concentration of 0.25 μg $L^{-1}$ (lowest calibrated level, LCL), as a half of the MMPR was adopted as the lowest level of validation. The CCα value was calculated as the sum of the LCL and the result of multiplication of the k-factor equal to 2.33 (Gaussian distribution) and the standard measurement uncertainty at LCL, expressed as standard deviation. For the analysis of the factor effect, an in-house Microsoft Excel form with data processed by ResVal software, updated version 4.0 made available by EURL-WFSR and adapted to the requirements of (EU) 2021/808 (31). The new criteria for retention time and relative ion ratios required for the confirmatory method were also checked for all spiked samples (17).

Moreover, in line with the current guidelines, the validation was supplemented by an experiment estimating the relative matrix effect (ME) and checking if it affected the signal response. The size of the matrix effect was evaluated by comparing the mass spectrometric response for STAN and 16β-OH-STAN in the urine samples spiked after extraction and in a solvent at the same concentration of 0.5 μg $L^{-1}$ according to the formula MF (standard normalised for IS) = MF(standard) / MF(IS), where MF is the matrix factor, MF(standard) is the peak area of matrix-matched standard / peak area of solution standard and MF(IS) is the peak area of matrix-matched IS / peak area of solution IS) (17). The ME numerical values were evaluated based on the coefficient of variations for the MF (standard normalised for IS).

**Evaluation of method performance in the proficiency tests.** The method for determining STAN and 16β-OH-STAN in the urine of animals was assessed in proficiency tests (PTs) organised by the EURL-WFSR three times, by Fapas PT four times (Fera Science, Sand Hutton, United Kingdom) and Progetto Trieste three times (Test Veritas, Trieste, Italy), once as LC-$MS^2$ (IT) and the remaining occasions as LC-MS/MS (QqQ). In only two PT rounds, one organised by WFSR in 2015 and the other by Fapas in 2022, 16β-OH-STAN and STAN were included in the field of analytes, and the test results were statistically evaluated (15). For the WFSR PT in 2015, bovine urine samples were collected during the experiment from a young heifer treated with STAN (injected four times with 5 mL of ± 10 mg mL STAN solution in the left and right sides of the neck). Coded material for research was dispatched to the participants in the form of three samples: a blank sample (A), a high incurred sample (B) and a low incurred sample (C). In that PT, 16β-OH-STAN was present in two urine samples. The above results were statistically summarised and evaluated with the z-score parameter. In the Fapas PT in 2022, the tested urine sample was obtained from female pigs treated with dienestrol. After collection, it was diluted with hormone-free urine and spiked with STAN, zeranol and nandrolone. These studies were also assessed with the z-score. In addition to external PTs, in order to confirm the competence of the personnel in the laboratory in determination of hormones in urine, quality control sample reference materials purchased from the WFSR were tested. The research material contained residues of STAN and 16β-OH-STAN and was assessed against the declared concentration in an information sheet.

## Results

A summary of the validation results of the confirmatory methods for the analysed steroid hormones is presented in Table 2. Linear regression parameters for the standard and matrix-matched calibration curves were correct for both compounds tested over the entire range of the concentration (1–10 μg $L^{-1}$ for LC-$MS^2$ (IT) and CCα–5 μg $L^{-1}$ for LC-MS/MS (QqQ)). The calculated regression coefficients for the plotted curves were greater than 0.98, as shown in the table. An overview of calibration parameters is summarised in the lower part of Table 2.

Chromatographic analysis of blank urine samples shows no associated peaks in the retention time ranges of compounds in either detection methods used which confirms the specificity of measurement of STAN and 16β-OH-STAN.

Overall apparent recovery of compounds tested from urine for LC-$MS^2$ (IT) at all validation spiking levels ranged from 81.1% to 125.8% for STAN, with relative standard deviation (RSD) not exceeding 30% (11.9–27.7%) and RSD under reproducibility conditions less than 31% (12.3–30.2%). The contrasting much better overall apparent recovery of compounds tested from urine for LC-MS/MS (QqQ) at all validation spiking levels ranged from 64.5% for STAN to 114.6% for 16β-OH-STAN, with RSD not exceeding 16% (4.4–15.3%) and RSD under reproducibility conditions less than 25% (10.5–23.8%). The calculated CCα and CCβ values in Table 2 were under the legislation guidelines of below 2 μg $L^{-1}$ of RC level for both detection techniques and compounds tested. The apparent recoveries of STAN and 16β-OH-STAN from the urine samples spiked to estimated values of the CCα for LC-MS/MS (QqQ) were correct and in the range of 91.9–114.6%.

Additionally, the determined values of expanded uncertainty ranged from 0.31 μg $L^{-1}$ for 16β-OH-STAN to 0.37 μg $L^{-1}$ for STAN, or expressed as a percentage range, 15.5–18.5% for LC-$MS^2$ (IT), while for LC-MS/MS (QqQ) the expanded uncertainty values were higher and ranged from 0.20 μg $L^{-1}$ for STAN to 0.34 μg $L^{-1}$ for 16β-OH-STAN, or 20.0–34.0%.

**Table 2.** Validation parameters of the liquid chromatography–tandem triple quadrupole (LC-MS/MS (QqQ) and –tandem ion-trap (LC-$MS^2$ (IT) mass spectrometry methods for the determination of stanozolol (STAN) and its metabolite 16β-OH-stanozolol (16β-OH-STAN) in bovine urine

| | Number of samples | Spiking level (μg $L^{-1}$) | Compound | | | |
|---|---|---|---|---|---|---|
| | | | STAN* | 16β-OH-STAN* | STAN** | 16β-OH-STAN** |
| Apparent recovery (%) | n = 18*/21** | 1.00*/0.50** | 125.8 | 85.4 | 64.5 | 98.8 |
| | | 2.00/1.00 | 104.7 | 101.7 | 65.9 | 94.0 |
| | | 3.00/1.50 | 88.4 | 90.7 | 66.8 | 88.7 |
| | n = 18*/6** | 4.00/2.00 | 87.4 | 94.3 | 98.8 | 103.2 |
| | | 10.00/5.00 | 81.1 | 88.0 | 96.4 | 97.1 |
| | n = 6** | CCα** | NE | NE | 91.9 | 114.6 |
| Repeatability (RSD, %) | n = 18*/21** | 1.00*/0.50** | 22.4 | 24.0 | 11.2 | 4.4 |
| | | 2.00/1.00 | 22.7 | 19.2 | 6.8 | 6.1 |
| | | 3.00/1.50 | 12.9 | 17.5 | 7.3 | 4.4 |
| | n = 18*/6** | 4.00/2.00 | 20.9 | 11.9 | 6.7 | 13.4 |
| | | 10.00/5.00 | 27.7 | 13.6 | 7.9 | 12.1 |
| | n = 6** | CCα** | NE | NE | 15.3 | 10.7 |
| Within-lab reproducibility (RSD, %) | n = 18*/21** | 2.00*/0.50** | 27.2 | 22.9 | 23.8 | 10.5 |
| | | 3.00/1.00 | 22.0 | 17.6 | 15.2 | 17.7 |
| | | 4.00/1.50 | 30.2 | 12.3 | 10.9 | 10.9 |
| Decision limit (CCα, μg $L^{-1}$) | | | 0.44 | 0.25 | 0.14 | 0.08 |
| Detection capability (CCβ, μg $L^{-1}$) | | | 0.75 | 0.42 | 0.24 | 0.13 |
| Measurement uncertainty at validation level of *2 μg $L^{-1}$/**1 μg $L^{-1}$ (U, k = 2, μg $L^{-1}$/ %) | | | 0.37/18.5 | 0.31/15.5 | 0.20/20.0 | 0.34/34.0 |
| Matrix effect (ME, %) | | | NE | NE | 7.0 | 10.0 |
| | | | Standard calibration curve | | | |
| Slope ± $s_b$ | | | 0.2119 ± 0.0843 | 0.1058 ± 0.2351 | 0.2112 ± 0.2456 | 0.1443 ± 0.0845 |
| y−Intercept ± $s_a$ | | | 0.0812 ± 0.0838 | −0.0027 ± 0.0737 | 0.0570 ± 0.0424 | 0.0125 ± 0.0096 |
| Correlation coefficient | | | 0.9945 | 0.9985 | 0.9866 | 0.9970 |
| Standard error | | | 0.0662 | 0.0127 | 0.0884 | 0.0285 |
| | | | Matrix matched calibration curve | | | |
| Slope ± $s_b$ | | | 0.1781 ± 0.1220 | 0.1228 ± 0.0223 | 0.2516 ± 0.1005 | 0.1564 ± 0.0554 |
| y−Intercept ± $s_a$ | | | 0.1656 ± 0.1199 | −0.0102 ± 0.0088 | −0.0322 ± 0.0291 | −0.0060 ± 0.0103 |
| Correlation coefficient | | | 0.9983 | 0.9995 | 0.0291 | 0.9988 |
| Standard error | | | 0.0426 | 0.0156 | 0.0582 | 0.0148 |

Additional series of validation**

| Compound | Number of samples | Spiking level (μg $L^{-1}$) | STAN | 16β-OH-STAN |
|---|---|---|---|---|
| Apparent recovery (%)// Repeatability (RSD, %) | n = 7 | 0.25 | 89.4//3.1 | 102.7//6.7 |
| | | 0.50 | 70.2//4.4 | 108.3//1.8 |
| | | 0.75 | 79.8//10.2 | 101.7//4.5 |
| | n = 4 | 1.00 | 80.5//5.5 | 100.2//4.7 |
| | | 2.00 | 86.8//15.9 | 96.5//3.1 |
| | | 5.00 | 81.4//9.0 | 94.0//2.9 |
| | n = 10 | CCα | 105.0//4.9 | 101.3//20.0 |
| Decision limit (CCα, μg $L^{-1}$) | | | 0.27 | 0.28 |
| Standard curve: Equation/Correlation coefficient | | | y = 0.6440x−0.0664/0.9998 | y = 0.1570x−0.0168/0.9971 |
| Matrix matched calibration curve: Equation/Correlation coefficient | | | y = 0.5230x−0.0670/0.9997 | y = 0.1453x−0.0033/1.0000 |

RSD – relative standard deviation; * – liquid chromatography–tandem ion trap mass spectrometry; ** – liquid chromatography–tandem triple quadrupole mass spectrometry; NE – not estimated

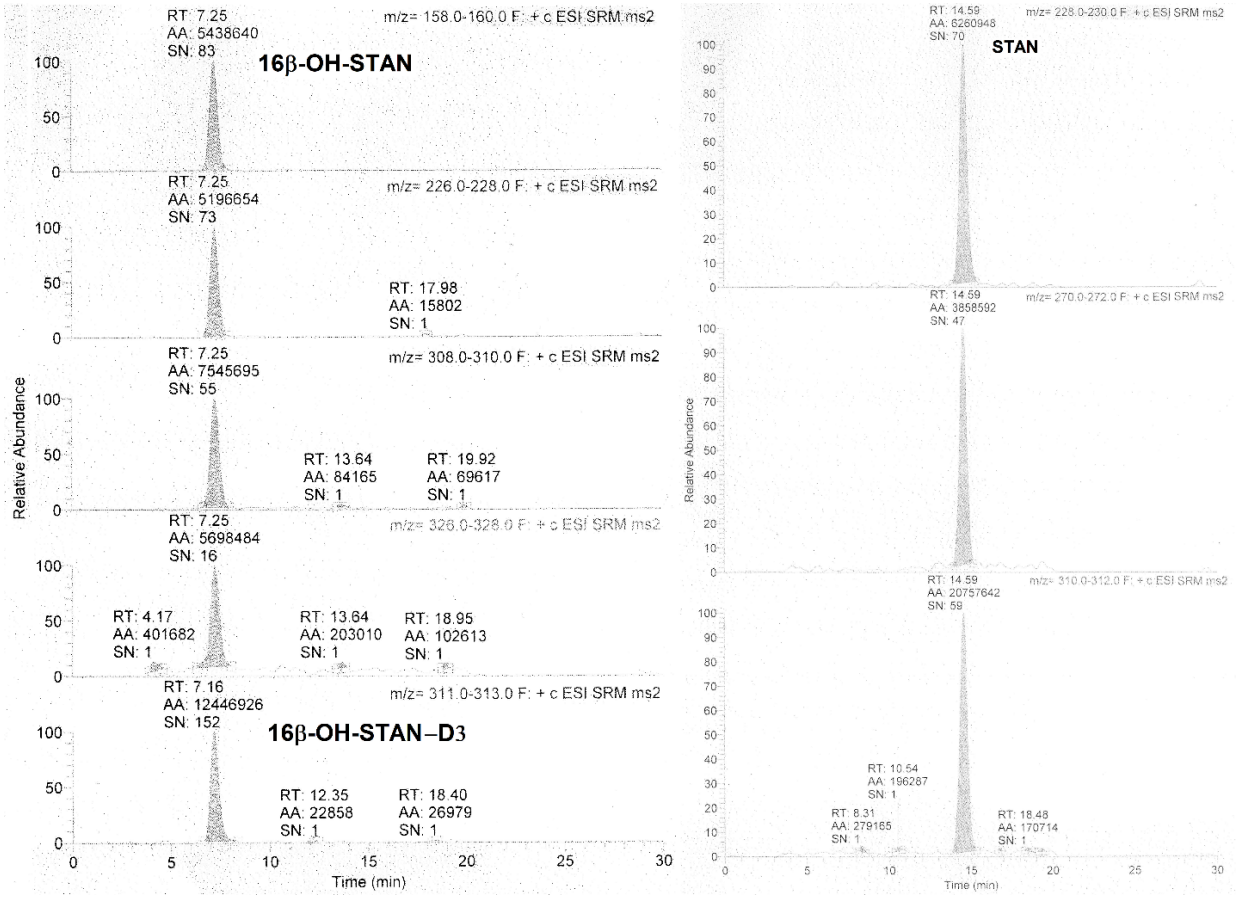


**Fig. 2.** Liquid chromatography–tandem ion trap mass spectrometry selected reaction monitoring (SRM) chromatograms of bovine urine samples spiked with 16β-OH-stanozolol (16β-OH-STAN) and stanozolol (STAN) at 2 µg $L^{-1}$

The numerical values of ME expressed as a percentage indicate matrix enhancement (positive values) for both STAN and 16β-OH-STAN, but their values are less than 20%. In the evaluation of the method ruggedness in terms of possible minor factors that could influence the results, it was shown using the Youden test that the calculated standard deviations of the differences between the two levels of each factor were smaller than the standard deviations under within-laboratory conditions. It was demonstrated that no selected factors crucial for the method affected the analytical performance significantly. Representative LC-$MS^2$ (IT) SRM chromatograms of the urine samples spiked with STAN and 16β-OH-STAN at the validation level of 2.00 µg $L^{-1}$ are presented in Fig. 2.

As to the suitability of the two LC-MS/MS methods as confirmatory, it should be mentioned that the percentage of samples meeting the criteria required for that purpose in the case of LC-$MS^2$ (IT) ranged from 51.9 to 75.3 while in the case of LC-MS/MS (QqQ) the percentage of such samples was markedly higher and ranged from 98.7 to 100 (Table 1).

In an additional validation series aligned with the new proposed analytical limit of 0.5 µg $L^{-1}$, correct values of apparent recoveries were obtained for all spiking levels of urine samples ranging from 70.2 for STAN to 108.3 for 16β-OH-STAN with RSD not exceeding 20% (1.8–15.9%). The regression parameters of the standard and matrix-matched calibration curves were correct as in the full validation. The CCα values determined based on the new recommended protocols were higher than those determined according to previous legislation in the full validation. All samples from the above series also met the criteria for the confirmatory method (Table 1).

Satisfactory z-scores were obtained in the PTs (as defined in the Material and Methods section) assessed in terms of the compounds tested. In the first evaluated test, the WFSR PT 2015, respective z-scores of −0.10 and −0.04 were obtained for the 16β-OH-STAN present in samples B and C. In the second test, Fapas PT 2022 a z-score of 0.0 was achieved for STAN in the tested sample.

In none of the 2,995 urine samples taken as part of the official monitoring studies conducted in 2006–2022 in Poland was the presence of STAN or its metabolite 16β-OH-STAN detected. Nor were they confirmed above the CCα level under the rules for assessing the sample result set out in the legislation (17). Representative LC-MS/MS (QqQ) MRM chromatograms are presented in Fig. 3.

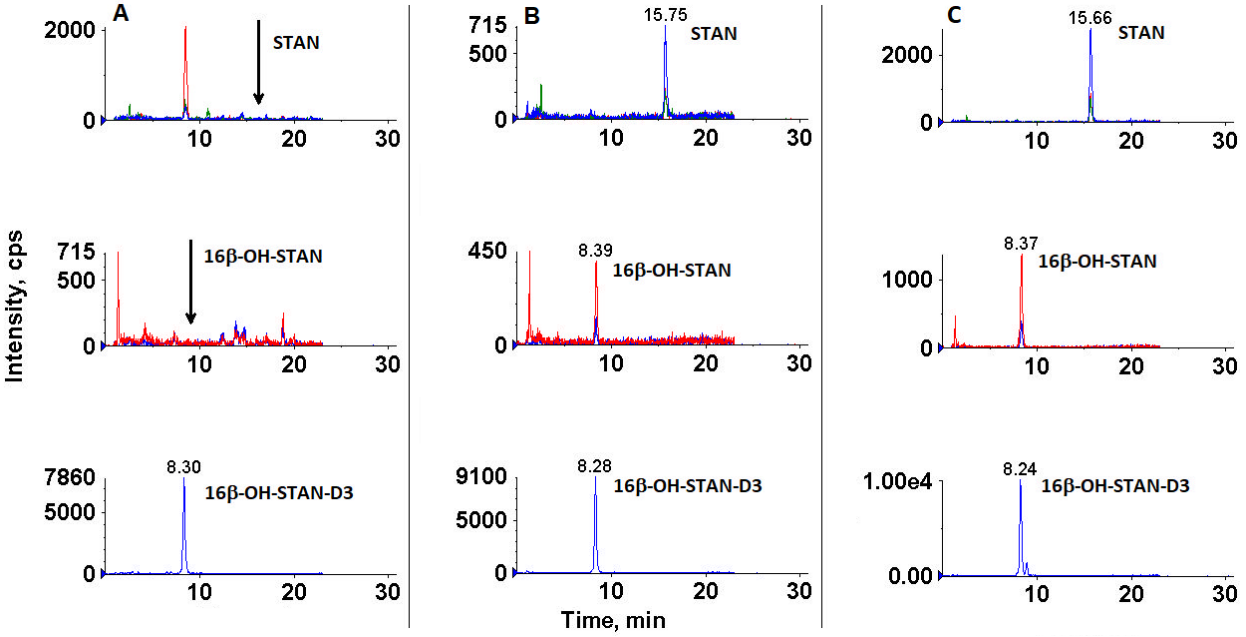


**Fig. 3.** Liquid chromatography–tandem triple quadrupole mass spectrometry multiple reaction monitoring chromatograms of A – a pig blank urine sample; B – a pig urine sample spiked with stanozolol (STAN) and 16β-OH-stanozolol (16β-OH-STAN) at 0.5 µg $L^{-1}$; C – a pig urine sample spiked with STAN and 16β-OH-STAN at 2 µg $L^{-1}$

## Discussion

The condition optimisation for detection of STAN, 16β-OH-STAN and the IS was the initial phase of the analytical procedure development both for LC-$MS^2$ (IT) and LC-MS/MS (QqQ). The conditions of analysis were determined by individually infusing the compound's standards. During the instrument tuning step, positive and negative ionisation were explored, but based on the results obtained and literature reports concerning the LC technique in this area, the positive one was finally selected for further procedures. Positive ionisation was similarly studied and reported on by other authors, who used it for electrospray ion source (ESI) type (25, 33, 34, 35) and atmospheric pressure chemical ionisation source chromatography (1, 14, 35). In the case of the IT analysis, three and four SRM transitions were obtained for STAN and 16β-OH-STAN, respectively, whereas in the QqQ analysis, three and two MRM transitions were obtained, as indicated by the data in Table 1. All the transitions were chosen for the quantitative determination of STAN and 16β-OH-STAN to minimise the interference due to other biological components of the sample. For all transitions, optimal technical parameters of physical quantities have been selected (6, 17). The transitions with the most intensity for particular hormones were used for quantification. The optimal compromise between ionisation, hormone peak geometry and intensity for ESI+ was achieved when methanol and 0.1% formic acid were used as the mobile phase.

For the study of the compounds tested, an Ultra C18 column was applied in which, apart from correct chromatographic separation, good signal intensities and correct peak geometry were also obtained. Other authors also often used reversed-phase columns with identical packing material and film thickness and identical or similar parameters of diameter and length (1, 14, 33, 34, 35).

No optimisation steps were taken in the isolation of compounds or the purification of urine samples, which is a complex matrix because of the countless metabolic products it contains. However, it should be emphasised that STAN is a difficult compound and analytically resistant to separation from the matrix by reason of the structure of the molecule and the presence of a pyrazole ring in it. The symmetry of the cation and its basicity probably facilitate its easy and permanent binding to the elements present in the matrix. Therefore, it was necessary to establish specific conditions enabling the isolation of STAN and its metabolite containing the pyrazole ring in the molecule. The analysis conditions set for the procedure used in National Reference Laboratory were the same as those that have been optimised and recommended by the EURL WFSR. In many publications, the authors declare the use of a different method of urine purification, *e.g.* SPE columns with non-polar, polymeric polystyrene adsorbent PAD-I (33), Strata-XL-A or Strata-XL-C columns, or a combination of Strata-XL or Strata-Si-1 in liquid-liquid extraction with ethyl acetate (1, 34) and Chem-Elut 1010 columns (35).

It was intended to use the developed analytical methods for confirmatory research. Following the guidelines for quantitative confirmatory procedures, the required validation technical parameters were determined (6, 17). The results proved that LC-$MS^2$ (IT) and LC-MS/MS (QqQ) methods have sufficient

selectivity and specificity, which was validated by chromatograms not containing signals of compounds interfering with STAN and 16β-OH-STAN as in Fig. 3A. Correlation coefficients for the standard and matrix-matched calibration curves for both compounds exceeded 0.98, showed good curve fit according to statistical modelling theory and provided a linear regression response in the concentration range tested.

The trueness of both the LC-MS$^2$ (IT) and the LC-MS/MS (QqQ) methods was satisfactory. Almost all apparent recovery values were within the reference range defined in Commission Decision 2002/657/EC as the minimum trueness of quantitative methods and ranged from −50% to +20% under the provisions for concentrations ≤1 μg $L^{-1}$ ($kg^{-1}$), from −30% to +10% for concentrations >1 and <10 μg $L^{-1}$ ($kg^{-1}$), and from −20% to +10% for concentrations ≥ 10 μg $L^{-1}$ ($kg^{-1}$) (6). Only two values of apparent recovery for STAN by both techniques slightly exceeded the range's upper (125.8%) and lower (66.8%) limits. The methods were characterised by good precision (RSD under repeatability) of <30% (LC-MS$^2$ (IT)) and <20% (LC-MS/MS (QqQ)) with within-lab reproducibility of <31% in line with the requirements of the legislation in force. According to established criteria, CV values for concentrations below 100 μg $L^{-1}$ ($kg^{-1}$) are not quantified. The Horwitz equation used to determine them give unacceptably high values; therefore it must be assumed that they are as low as possible (6). The values of parameters of apparent recovery and RSD of repeatability and reproducibility obtained in our validation study are consistent with the values of relevant parameters achieved by other authors (1, 14, 33). Furthermore, apparent recoveries of hormones in urine samples spiked at the estimated CCα in LC-MS/MS (QqQ) were in the required range of 50–120% stipulated by Commission Decision 2002/657/EC for concentrations below 1 μg $L^{-1}$ ($kg^{-1}$) (6). The RSD of repeatability at the CCα level had acceptable values not exceeding 20%. The CCα and the CCβ calculated values for both the LC-MS$^2$ (IT) and LC-MS/MS (QqQ) methods were below the RC level of 2.00 μg $L^{-1}$, which was also in line with the SANCO guide on the implementation of CD 2002/657/EC, assuming that for banned and unauthorised compounds the detection parameters should be as low as reasonably achievable (ALARA principle) (11). The values of the LC-MS/MS (QqQ) method detection parameters expressed as decision limits (CCα) for individual hormones were comparable to the CCα values reported by other researchers using LC-MS/MS instrumental techniques (33, 34).

Also, the uncertainty values in the overall assessment were correct for both compounds tested, even for STAN, despite the lack of an analogous IS for this compound.

The matrix effect phenomenon, so common with LC-based techniques and appearing in the form of enhancement or suppression of the analytical signal, was not an apparent significant influence on the test result in the case of LC-MS/MS (QqQ), for which it was tested. Low numerical values describing ME indicate no interference from the site of endogenous matrix components that could interfere with analytes, influencing the analytical signal. Ignoring the possibility of estimating the numerical size of ME in several ways – based on the principle of spiking after extraction, variation of the calibration curves slopes or comparing the slopes of the matrix-matched calibration curve to the standard calibration curve – until now there has generally been no accepted criterium for assessing the size of the measured phenomenon. According to Regulation 2021/808/EU, it should be assumed that ME does not exist if the estimated coefficient of variation is not greater than ±20% (17). Considering that there is no ME, it is reasonable to perform calculations from the standard calibration curve, which was done during the full validation of the method for both detection techniques.

The investigation of the robustness of the analytical method confirmed that the parameters finally selected as crucial for sample preparation and chromatographic separation are optimal for obtaining satisfactory method performance.

The methods developed were intended for confirmatory purposes; therefore, it was necessary to ascertain whether they met the criteria for confirmatory methods regarding identification points (IPs), relative intensities of ions and relative retention time outlined by 2002/657/EC (6). Four IPs were achieved for one precursor ion and two daughter ions obtained for 16β-OH-STAN by the LC-MS$^2$ (IT) technique. The one precursor and three daughter ions obtained for STAN in the LC-MS$^2$ (IT) and LC-MS/MS (QqQ) techniques gave 5.5 IPs. A better number of 7.5 IPs was achieved for 16β-OH-STAN for the one precursor and four daughter ions obtained. The minimum required number of IPs called for by the 2002/657/EC legislation is four, and consequently the criteria for SRM and MRM relating to the IPs were met. Also, the criteria for compatibility of the relative retention time of STAN and 16β-OH-STAN in spiked samples and standards within the specified 2.5% tolerance range were confirmed as met for both the LC-MS$^2$ (IT) and LC-MS/MS (QqQ) techniques. In terms of compliance of relative ion intensity, only the method based on LC-MS/MS (QqQ) techniques, for which the presence of tested compounds was confirmed in 98.7–100% of samples in the CCα–5.0 μg $L^{-1}$ concentration range, met the requirements for confirmatory purposes. For the LC-MS$^2$ (IT) technique and the concentration range of 1–10, the percentage of samples confirmed as required for the relative ions intensities was 51.9–75.3%, depending on the MRM transition and the analyte. The assumption is that for prohibited compound α-error, the probability that the tested sample is compliant is 1%, which means that the compliance criteria for 99% of samples should be met.

Considering that, it was concluded that the LC-MS$^2$ (IT)–based method is inadequate for confirmatory purposes.

In an additional validation series performed with the LC-MS/MS (QqQ) technique, taking into account the new analytical limit of 0.5 μg $L^{-1}$ for STAN and 16β-OH-STAN in urine, the trueness values were also satisfactory. All apparent recovery values were within the reference range defined in Commission Implementing Regulation (EU) 2021/808 as the minimum trueness of quantitative methods and ranged from −50% to +20% under the provisions for concentrations ≤1 μg $L^{-1}$ ($kg^{-1}$), from −30% to +20% for concentrations >1 and <10 μg $L^{-1}$ ($kg^{-1}$), and from −20% to +20 % for concentrations ≥10 μg $L^{-1}$ ($kg^{-1}$) (17). For all levels of urine spiking with analytes, good precision (RSD under repeatability) not exceeding 20% was obtained. This is in line with the assumptions of the regulation, according to which for concentrations below 10 μg $L^{-1}$ ($kg^{-1}$), it should be as far as possible below the set maximum value of 30%. The CCα calculated values for both STAN and 16β-OH-STAN were below the MMPR level of 0.50 μg $L^{-1}$, which is in line with the legal documents and relevant technical guides and proves the appropriateness of the method for confirmatory purposes. For the reason that the method is only used as confirmatory, the requirements for identifying compounds regarding identification points, relative intensities of ions and relative retention time were also addressed (17). For prohibited compounds, such as hormones, 5 IPs are required, one of which may be related to the type of chromatographic separation. The method used for STAN and one precursor and three daughter ions yielded 6.5 IPs, while the method used for 16β-OH-STAN and one precursor and two daughter ions provided 5 IPs. For all spiked samples tested in this series, the criteria for compatibility of the relative retention time of STAN and 16β-OH-STAN with standards within the specified 1% tolerance range were confirmed. Regarding compliance of relative ion intensity of 100% of samples in the CCα-5.0 μg $L^{-1}$ concentration range, the criteria were met with ±40% RSD.

The LC-MS/MS method has been used in the RMP for over 10 years. As an accredited method, it is regularly verified in PTs to confirm its suitability for the intended purposes. Until now, all samples tested with this method have been qualified as compliant with the specified requirements. Based on the European Food Safety Authority (EFSA)'s published reports summarising the monitoring results in EU Member States, it can be concluded that until now only one non-compliant result for STAN was found in cattle in 2011 in Italy (19). Despite the low percentage of non-compliant results in the EU over the years, ongoing monitoring of residues of banned compounds is justified; therefore, analytical methods in laboratories should be constantly improved to ensure the safety and health of food consumers.

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