ApplicationNOTE

Waters

COMPARING SIR TO MRM FOR THE QUANTITATIVE CONFIRMATION OF STEROID GROWTH PROMOTERS IN BOVINE URINE

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ABSTRACT

As legislation continues to lower the limits of detection (LODs) required for residue quantification and confirmation in food, more specific and sensitive methods of detection are required. This application note describes the use of the Waters® Micromass® Quattro micro™ GC instrument using electron impact (EI+) ionization to quantify and confirm eight trimethyl silyl (TMS) derivatised steroid growth promoters present in bovine urine extracts.

INTRODUCTION

It is suspected that steroid growth promoters are currently used to speed up the rate of growth of muscle tissue in domestic animals grown for public consumption in many countries. However, the use of growth promoters in animals reared for meat is prohibited in the European Union (EU)[1]. EU regulations stipulate that no residual concentration of these compounds should be present at any stage in the production of meat. Therefore, the detection and confirmation of these steroids at any concentration will lead to the condemnation of the produce. In order to effectively monitor the occurrence of these residues the most specific and sensitive methods are required.

Gas chromatography and single quadrupole mass spectrometry, using selected ion recording (SIR), is the favoured method of analysis. Results must satisfy the current EU legislation on confirmation criteria, Commission Decision 2002/657/EC[2].

These steroids require derivatisation to ensure sufficient volatility to chromatograph through a fused silica capillary column. For example, the following derivatisation of 17α-Nortestosterone with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) is required prior to quantitative analysis (Figure 1). The efficiency of the derivatisation step can sometimes be variable or low for these compounds. This variable efficiency is often compensated by the use of stable isotope labelled analogues.

Current legislation stipulates four structure-related ions for each analyte with the correct ion ratio must be monitored when using SIR mode. When the analysis requires the unambiguous identification of these compounds at trace concentrations from highly complex matrices difficulties can be encountered. These are often interferences from co-eluting compounds, which appear on the SIR trace making accurate quantification prone to error.

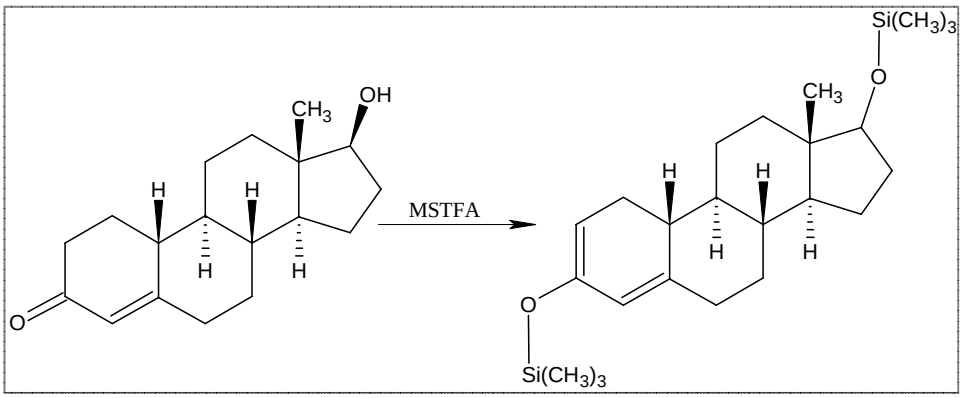

Figure 1. The typical derivatisation process used for the steroid growth promoters.

ApplicationNOTE

Multiple reaction monitoring (MRM) is a tandem mass spectrometric technique that allows the monitoring of specific collision induced dissociation (CID) reactions. The nature of these reactions depends on molecular structure as well as mass and, as a result, significant improvements in analytical selectivity may be achieved using this method. Current legislation stipulates that when using MRM mode, monitoring of two structure-related transitions for each analyte suffices for confirmation of identity.

EXPERIMENTAL

The samples were analysed on a Waters Micromass Quattro micro GC (Figure 2) tandem quadrupole mass spectrometer operated in EI+ mode. Both series of SIR and MRM experiments were performed on the Quattro micro GC.

Extraction

2 mL of standard or sample were enzymatically hydrolysed at 37 °C overnight. Next day, after the addition of sodium acetate buffer (0.25 M, pH 4.8), each extract was purified by passing the sample through a C_{18} and a NH_2 cartridge, respectively. Further cleanup was completed using semi-preparative HPLC. The resulting fractions were derivatised with MSTFA++. 17-α-methyltestosterone-delta 9,11 was added as a derivatisation control standard.

Figure 2. Waters Micromass Quattro micro GC.

Derivatisation

Derivatisation was completed by adding excess MSTFA++ to each sample and heating to 60 °C for 15 min. All extracts were then reduced to dryness by a dry stream of nitrogen. 40 mL of iso-octane/n-decane (4:1 containing 1.0 ng/mL of the PCB 138 internal standard) was added to each sample.

GC Method

The samples were injected by splitless injection (2 µL, 250 °C, purge at 30 mL/min after 2.1 min) into a carrier gas of helium at a constant flow rate of 1.0 mL/min delivered from an Agilent 6890 GC with a 7683 autosampler attached. The GC capillary column employed was a Restek Rtx-CLPesticides, 30 m x 0.25 mm i.d., 0.25µm. The following temperature ramp rate was used: 130 °C (2 min) to 250 °C (3 min) at 12 °C/min, to 300 °C (8.4 min) at 7.5 °C/min. The total run time was 30 min. The temperature of the interface was held at 275 °C during the chromatographic run.

MS Method

In both SIR and MRM modes, the ion source was operated at 180 °C with an electron energy of 70 eV and a trap current of 100 µA.

For the SIR experiments, the four selected ions for each analyte and their associated dwell times are listed in Table 1.

For the MRM experiments, the two transitions for each analyte and their associated dwell times and collision energies are listed in Table 2. The collision gas used was argon at a gas pressure $2.5e^{-3}$ mBar. The product ions in MRM are largely similar to the selected ions in SIR but they still represent higher selectivity due to the way they are generated.

The data were acquired using Waters MassLynx™ software and processed using the TargetLynx™ Application Manager.

Compound	Selected ions (Da)	Dwell time (s)
PCB-138 (I.S.)	289.8, 359.8	0.1
17α-Nortestosterone	182.1, 194.1, 403.2, 418.2	0.05
5β-androstane-17α-methyl 3α,17β-diol (MEAD)	143, 255.2, 270.2, 435.3	0.05
17β-Nortestosterone	182.1, 194.1, 403.2, 418.2	0.05
17α-ethyl-5β-estrane-3α,17β-diol (EED)	157.1, 241.1, 331.2, 421.2	0.1
5α-androstane-17α-methyl 3β,17β-diol (MEAD)	143, 255.2, 270.2, 435.3	0.1
17α-Methyltestosterone	301.2, 341.2, 356.2, 446.3	0.1
Norethandrolone	287.1, 300.2, 356.2, 446.3	0.1
Chloorandrostenedione (CLAD)	429, 449.1, 464.2, 466.1	0.1

Table 1. Selected ions monitored during the SIR experiments.

Compound	Transitions	Dwell time (s)	Collision Energy (eV)
PCB-138 (I.S.)	361.8 > 289.8 359.8 > 324.8	0.1	20 10
17α-Nortestosterone	418.2 > 287.1 418.2 > 194.1	0.05	15 12
5β-androstane-17α-methyl 3α,17β-diol (MEAD)	435.2 > 345.2 270.2 > 255.1	0.05	10 8
17β-Nortestosterone	418.2 > 287.1 418.2 > 194.1	0.05	15 12
17α-ethyl-5β-estrane-3α,17β-diol (EED)	421.2 > 331.2 331.2 > 241.1	0.1	7 10
5α-androstane-17α-methyl 3β,17β-diol (MEAD)	435.3 > 255.1 435.3 > 345.2	0.1	12 8
17α-Methyltestosterone	446.3 > 301.1 301.2 > 169.1	0.1	12 12
Norethandrolone	446.3 > 287.1 446.3 > 356.2	0.1	10 8
Chloorandrostenedione (CLAD)	464.2 > 429.2 464.2 > 449.2	0.1	10 10

Table 2. Transitions monitored during the MRM experiments.

RESULTS AND DISCUSSION

The SIR results for the 100 ng/mL standard, equivalent to 1.0 ng/mL in urine, are illustrated in Figure 3. All four ions of 17α-nortestosterone can be clearly observed and integrated.

It has been observed that as an animal ages the complexity of the urine matrix increases. The urine from a mature bovine (increased complexity) was spiked with a concentration of 1.0 ng/mL producing the SIR results illustrated in Figure 4.

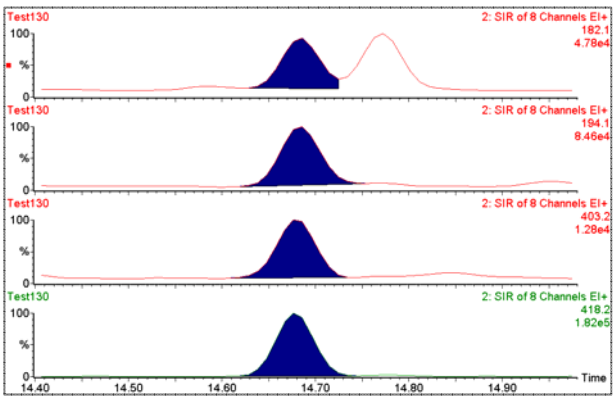

Figure 3. Four SIR ions of 17α-nortestosterone, 100 ng/mL (equivalent to 1.0 ng/mL in bovine urine).

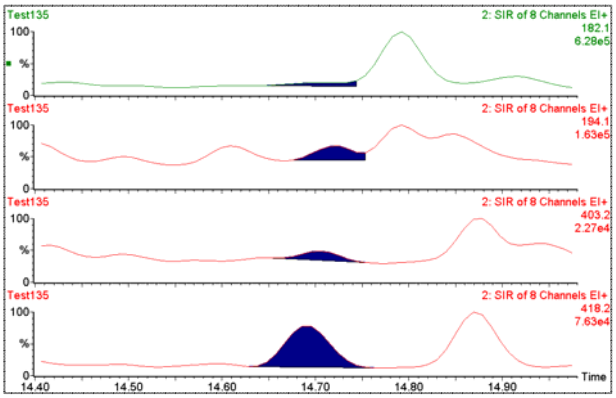

Figure 4. Four SIR ions of 17α-nortestosterone, 1.0 ng/mL in bovine urine.

In this example the molecular ion is still able to be quantified successfully. However, the three fragment ions used for confirmation are poorly resolved from matrix interferences, resulting in a failure of the confirmation criteria.

Selectivity can be gained through the use of the MRM technique. Figure 5 clearly illustrates the advantage of MRM as the analyte at the same concentration and in the same extract is resolved from all matrix interferences and can be easily quantified and confirmed. The product ion from the quantification transition, 418 > 194, is also recorded in SIR mode as one of the confirmation ions (see Figure 3) but the use of MRM results in a much improved signal to noise (S/N) ratio.

The standards and samples were injected and the data were processed using Waters TargetLynx application manager. Correlation coefficients of $r^2 > 0.992$ without weighting were obtained for all eight compounds of interest. A representative calibration curve for 17 α-nortestosterone, with a correlation coefficient of $r^2 = 0.999$, is illustrated in Figure 6.

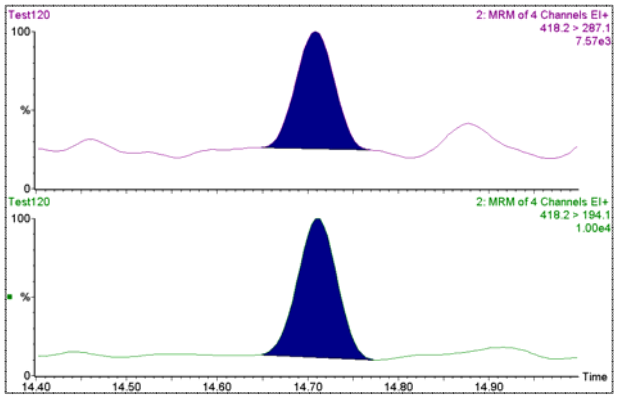

Figure 5. Two MRM transitions of 17α-nortestosterone, 1.0 ng/mL in bovine urine.

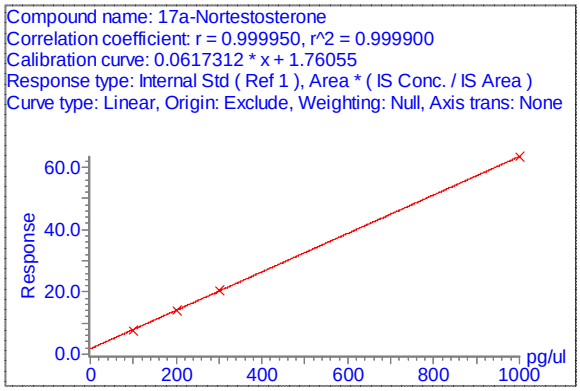

Figure 6. Representative calibration curve for 17α-nortestosterone, 100-1000 ng/mL corresponding to 1-10 ng/mL in urine.

Limits of detection (LOD) were determined for all the compounds in three different urine types, where the degree of interference increases, and are listed in Table 3. The LOD was defined as the concentration in urine injected that gave a signal for the confirmation transition equivalent to three times the baseline noise. From the table it can be seen that, in general, as the age of the bovine increases (clean to complex) the estimated confirmation LOD increases with the complexity of the matrix.

A TargetLynx browser window is illustrated in Figure 7 with a summary window providing quantification information (by compound). The calibration curve for the selected compound and the associated statistics are seen in addition to a manually controlled integration window for rapid screening of the automatic integration routine. In these examples the quantification was completed on the quantification transition with the ion ratio (between this transition and the confirmation transition) criteria as stated in EU Commission Decision 2002/657/EC[2] being obeyed.

The expected ion ratio for each compound was determined from the average of the four solvent standards. Table 4 lists the expected and experimentally determined ion ratios for each steroid in addition to the % relative standard deviation (% RSD) for all sample injections where the compound was detected. The Commission Decision 2002/657/EC[2] criteria, which is dependent on the relative abundance of the confirmation transition to the quantification transition, is also listed in Table 4.

Compound	Estimated confirmation LOD, ng/mL		
	Clean Urine	Medium Urine	Complex Urine
17α-Nortestosterone	0.25	0.30	0.50
5β-androstane-17α-methyl 3α,17β-diol (MEAD)	0.50	1.00	1.00
17β-Nortestosterone	0.20	0.30	0.50
17α-ethyl-5β-estrane-3α,17β-diol (EED)	0.10	0.50	0.50
5α-androstane-17α-methyl 3β,17β-diol (MEAD)	0.50	1.00	1.00
17α-Methyltestosterone	0.07	0.20	0.30
Norethandrolone	0.30	1.00	1.00
Chloorandrostenedione (CLAD)	1.00	2.00	2.00

Table 3. Estimated confirmation LOD for all eight derivatised steroids in urine with various complexities.

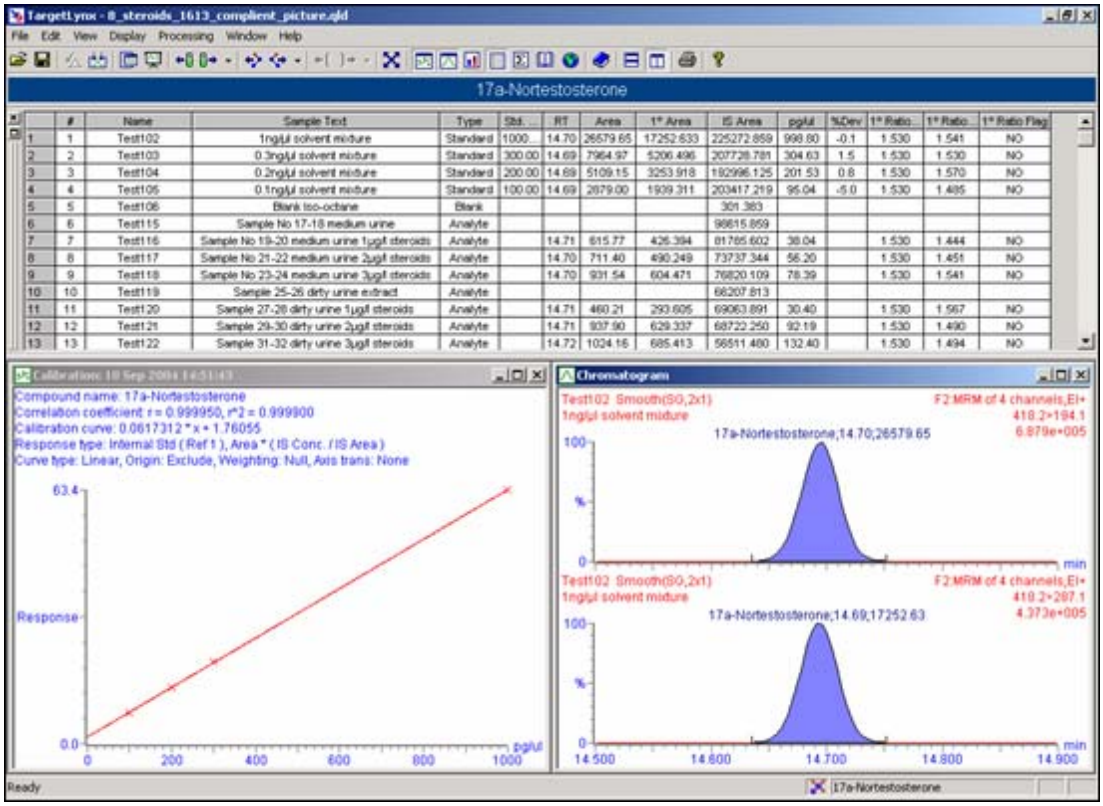

Figure 7. TargetLynx browser containing sample spreadsheet information by compound, a calibration curve and an interactive integration window.

Compound	Expected Ion Ratio	Determined Ion Ratio	% RSD	EC Legislation
17α-Nortestosterone	65.4%	66.2%	2.8	20
5β-androstane-17α-methyl 3α,17β-diol (MEAD)	85.5%	77.5%	12.2	20
17β-Nortestosterone	74.6%	74.1%	6.7	20
17α-ethyl-5β-estrane-3α,17β-diol (EED)	58.8%	53.8%	11.1	20
5α-androstane-17α-methyl 3β,17β-diol (MEAD)	14.3%	15.6%	23.0	30
17α-Methyltestosterone	55.6%	50.0%	7.4	20
Norethandrolone	28.1%	27.0%	14.2	25
Chloorandrostenedione (CLAD)	50.0%	50.0%	1.3	25

Table 4. Ion ratio statistics for the eight derivatised steroids.

CONCLUSIONS

Current EU legislation stipulates that when using selected ion recording (SIR) four ions must be monitored for each analyte to quantify and confirm derivatised steroids in urine extracts. SIR analysis of urine extracts can display difficulties in resolving the analyte from the background matrix and meeting those criteria.

This application note has shown that through the use of tandem quadrupole MS/MS, greater selectivity can be achieved, especially from complex urine extracts (mature bovine). Greater confidence is gained from confirming with two multiple reaction monitoring (MRM) transitions when the relative abundance of those transitions is in agreement with the reference standard. Excellent linearity was achieved for all the analytes. The LODs for all eight analytes (using the confirmation transition) was determined to be equal to or less than 1.0 ng/mL with the exception of CLAD in all types of urine.

All quantitative processing was completed through the use of the TargetLynx application manager. TargetLynx provides advanced quantification with a range of automatic quality control checks including a compliance check in accordance with EU requirements for confirmatory analysis of banned substances.

REFERENCES

1. Le Bizec, B., Marchand, P., Gadé, C., Maume, D., Monteau, F. and André, F. Publication contributed to Euroresidue IV Conference, 8-10th May 2000, Veldhoven, The Netherlands.

2. Commission Decision 2002/657/EC, Official Journal of the European Communities, No. **L221** (2002) 8-36.

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