

Quantitative analysis of anabolic steroids in control samples from food-producing animals using a column-switching LC-HESI-MS/MS assay

ASMS 2014 TP285

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Introduction

The use of natural and synthetic hormones to increase the weight of meat producing animals is prohibited in the European Union (European Council Directive 96/22/EC and amended by Directive 2003/74/EC) to protect consumers from the harmful effects of digesting hormone residues and their metabolites. Compliance with this legislation is monitored by the screening of a large number of animals every year. Urine is collected from animals on farms and liver from animals at abattoirs.

Typically, sample preparation of these urine samples involves time-consuming two-step offline-SPE. Here, we have developed an online-SPE method to considerably shorten the pre-treatment time. In addition to optimisation of the extraction method, chromatographic separation was optimised to decrease ion-suppression and isobaric interference. Compound ionisation was enhanced using a new heated ESI (HESI) source on the LCMS-8050 triple quadrupole mass spectrometer.

Materials and Methods

Sample preparation

Bovine urine samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant (100µL) was spiked at 0, 0.25, 0.5, 1, 1.5, 2, 4 and 6 pg/µL with each target compound. The sample was diluted with 58 µL water (containing internal standards), 40 µL 50mM ammonium

acetate buffer and 2 µL β-glucuronidase solution. This mixture was left at approximately 37°C for 6 hours. This mixture was diluted with 100 µL mobile phase A and centrifuged at 10,000 rpm. The supernatant was filtered through a 0.2 µm filter into the auto-sampler vial.

LC-MS/MS analysis

A Nexera UHPLC system was used for analysis of compounds combined with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto). A 6 port, 2 position switching valve was utilised to accomplish the trapping and back-flushing of the first analytical column. A flow rate of 0.4 mL/min was utilised in pumps A, B and C and the oven temperature was set at 40°C. The

UHPLC configuration is shown in Figure 1. The LC-MS/MS was operated in MRM mode using both ESI positive and negative. Source conditions were optimised: nebulising gas flow, 3 L/min; heating gas flow, 5 L/min; drying gas flow, 10 L/min; interface temperature, 400°C; DL temperature, 150°C, heat block temperature 300°C.

Table 1 – MRM acquisition parameters

Compound	Ion mode	RT	MRM	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
methyltestosterone	ESI+	10.6	303.1 > 97.05, 109.1	-21	-26, -28	-19, -22
β-boldenone	ESI+	9.1	287.1 > 121, 135.1	-20	-23, -16	-13, -26
α-boldenone	ESI+	10.1	287.1 > 121.05, 269.2	-20	-24, -12	-13, -30
α-trenbolone	ESI+	9.2	271.1 > 253.15, 199.1	-19	-20, -24	-18, -21
Progesterone	ESI+	11.9	315.3 > 97.05, 109.05	-22	-23, -26	-19, -21
Testosterone	ESI+	10.0	289.1 > 97.05, 109.1	-20	-25, -26	-18, -21
Hydroxystanozolol	ESI+	10.3	345.3 > 81.1, 95.0	-17	-51, -44	-15, -19
Dienestrol	ESI-	10.6	265.1 > 93.1, 249.2	19	30, 26	16, 26
Hydroxystanozolol d ₃	ESI+	10.2	348.3 > 81.1	-17	-51	-15
β-boldenone d ₃	ESI+	9.1	290.1 > 121.0	-20	-23	-13

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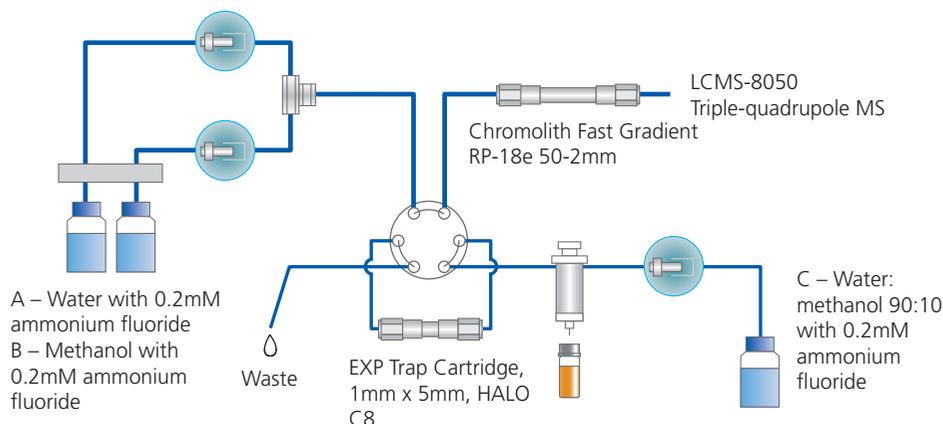


Figure 1 – Online-SPE-LC-MS/MS configuration

Results and discussion

Method development

A heated ESI source was utilised for the effective ionisation of all target compounds using positive and negative mode. Four MRM transitions were optimised for each compound using an automated MRM optimization program within the LabSolutions workstation. Table 1 lists the quantifier and one qualifier transition.

A Chromolith Fast Gradient RP-18e (50 x 2 mm) column was utilised for target compound separation. This column was utilised in order to minimise the system back pressure that can often be high as a result of back-flushing the trapping column. This monolithic column was found to

provide near identical results to a Kinetex C18 (50 x 2.1, 2.6µm), but with a back pressure of only approximately 50 bar, in comparison to the Kinetex of approximately 400 bar.

Various mobile phases were tested for analytical separation. A common mobile phase in steroid analysis is the use of ammonium hydroxide, whereas in this study the response for target compounds was far superior using a mobile phase containing 0.2mM ammonium fluoride. Figure 2 displays chromatograms comparing the two solvent results.

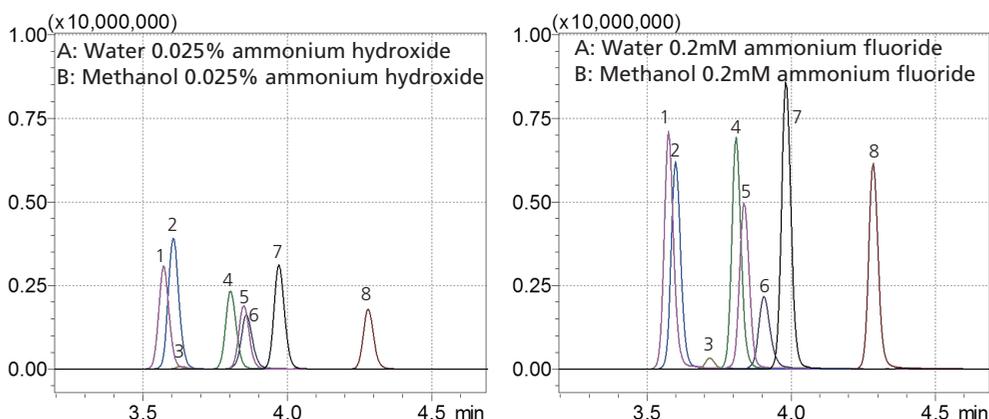


Figure 2 – Comparison of two different mobile phase solvents (pumps A and B) for eight target steroids.

Peak #1 β-boldenone, #2 α-trenbolone, #3 Dienestrol, #4 Testosterone, #5 α-boldenone, #6 Hydroxystanozolol, #7 methyltestosterone, #8 Progesterone

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In order to improve response at low levels and to improve recoveries, a multiple injection scheme was explored, essentially by using the trapping column as pre-concentration device. A bovine urine sample spiked at 10 pg/μL was injected multiple times using injection volumes of (5 μL, 10 μL, 20 μL, 30 μL) to give different total volumes loaded on the trap. Figure 3 displays the

results of this study. It was found that several smaller injections, rather than one larger injection, provided much improved compound response. This difference was particularly evident in the results for β-boldenone. The final method utilised an injection scheme of 3 x 5 μL injections. A total injection volume of 15 μL was enough to acquire the necessary response at the lower calibration levels.

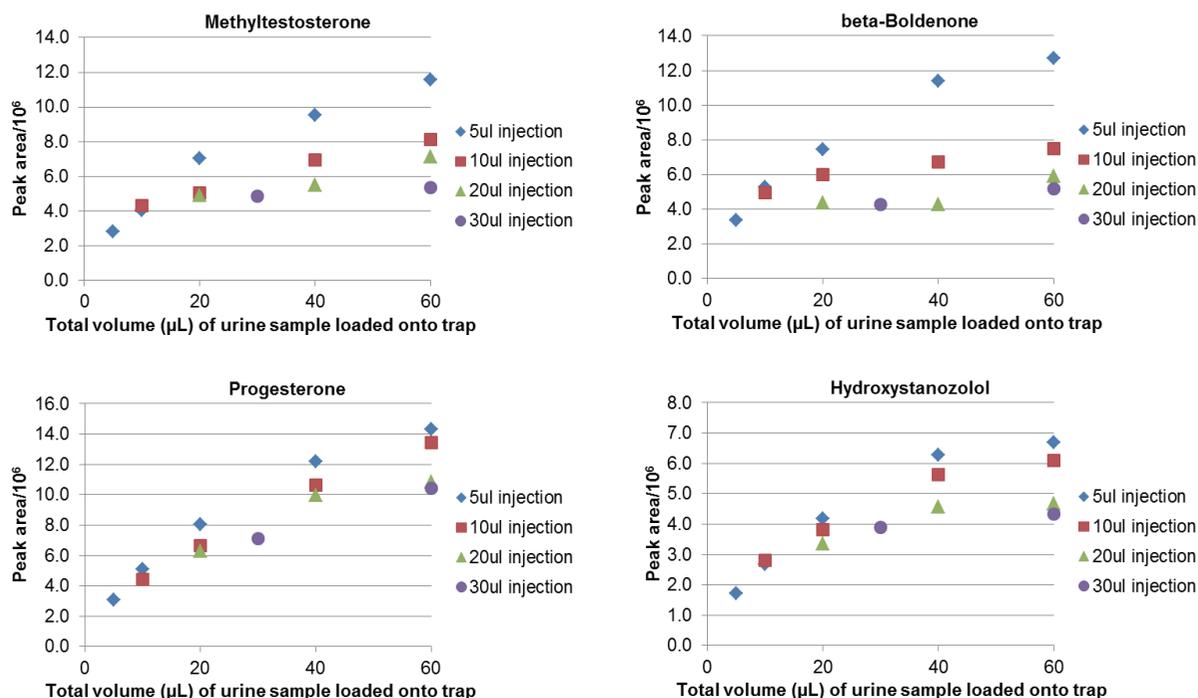


Figure 3 – Optimization of multiple loading injections onto the trapping column with different injection volumes (5 μL, 10 μL, 20 μL, 30 μL)

Final method performance

The final online trapping LC-MS/MS method was developed for eight steroids with LOQs below 0.25 pg/μL and a method cycle time of 20 minutes. This sensitivity permitted the quantification of steroids below the typical screening target concentration (STC) applied by food safety laboratories in bovine urine. Linearity was determined to be excellent for all compounds in the range 0.25 – 6 pg/μL, with $R^2 > 0.998$ for all compounds studied. Peak area repeatability (n=6), assessed on the same day, was determined to be less than 4.6 % RSD for all compounds at 2 pg/μL, and less than 3.6 % RSD at the higher concentration of 6 pg/μL. Matrix effects were assessed at

10 pg/μL for each compound and were found to vary significantly. The highest suppression was observed for dienestrol (90 %) and hydroxystanozolol (68 %), whereas lower suppression was observed for progesterone (14 %) and β-boldenone (29%). Deuterated internal standards were utilised to compensate for recovery and matrix effects. Figure 4 displays the calibration results (linear, 1/x) for each compound and an example chromatogram from a 1pg/ μL bovine urine sample. Calibration levels were analysed in duplicate and either Hydroxystanozolol d₃ or β-boldenone d₃ were used as internal standards for each compound.

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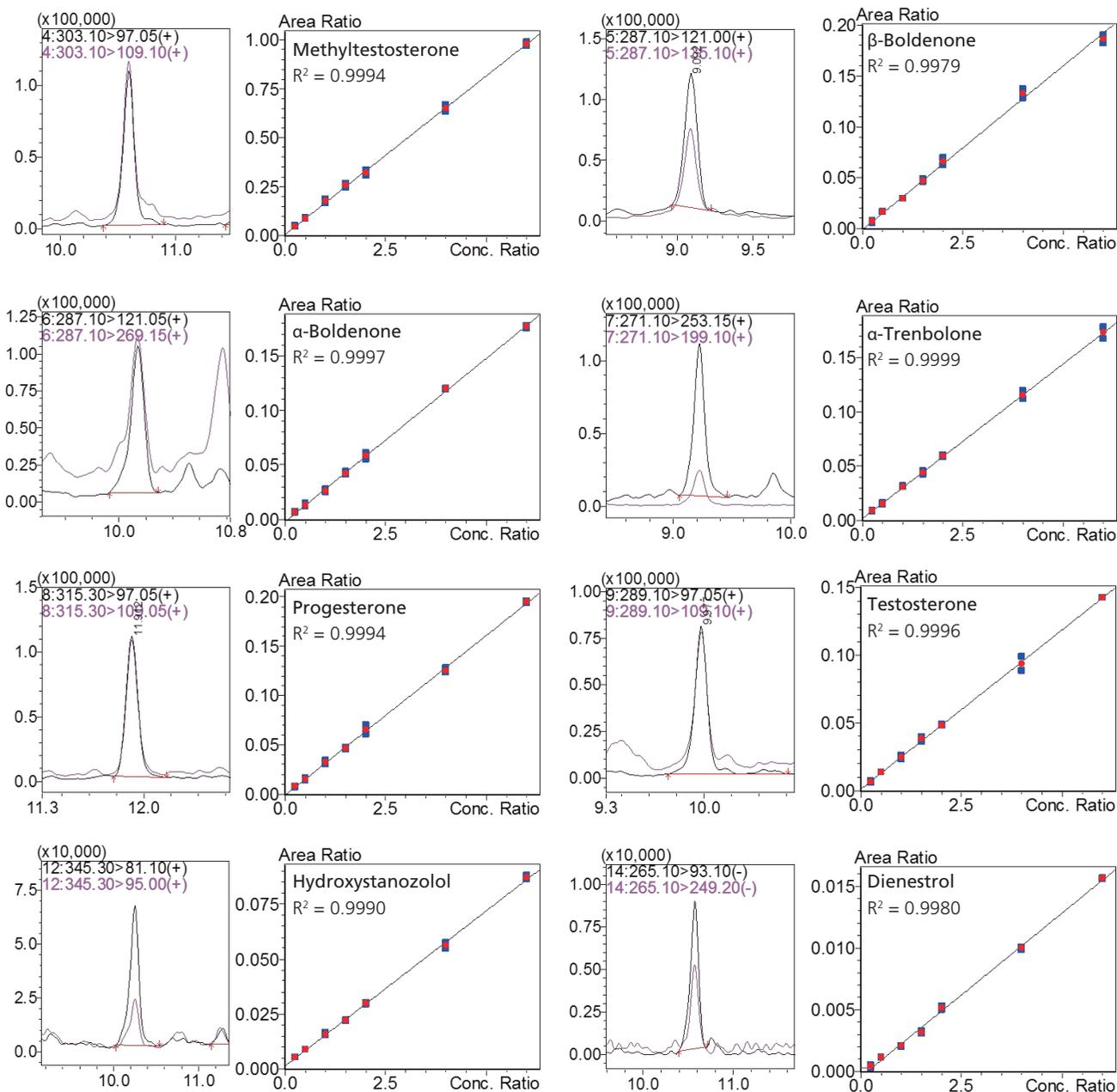


Table 4 – Calibration curves (linear, 1/x) for each compound and example chromatogram in bovine urine spiked at 1 pg/μL

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Conclusion

- An online-SPE-LC-MS/MS method was developed for the quantification of eight steroids at low concentrations required by food safety laboratories.
- The methodology involved simple sample preparation and offers significant time savings in comparison to a two-step offline-SPE procedure that is commonly used for this application.
- Preliminary validation data displayed excellent linearity for all compounds, low intraday %RSD, and good sensitivity at the lowest calibration level of 0.25 pg/ μ L.
- Multiple injections onto the trapping device were explored to improve compound response. It was found that several smaller injections, rather than one larger injection, provided much improved compound detection.
- Matrix effects were determined to be significant for most compounds, therefore deuterated internal standards were utilised to compensate.