

**GERSTEL**

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Fully Automated Analysis of Doping Substances in Equine Urine by Disposable Pipette Extraction (DPX) coupled to GC/QqQ-MS

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KEYWORDS

Doping, Equine Urine, Automated Sample Preparation, Disposable Pipette Extraction, Triple-Quadrupole-MS, Gas Chromatography (GC)

ABSTRACT

This application note describes a fully automated analysis method for selected equine doping compounds in equine urine. A GERSTEL MultiPurpose Sampler (MPS) with Disposable Pipette Extraction (DPX) option is employed for extraction and cleanup. After gas chromatographic separation the analytes are detected by a triple-quadrupole mass spectrometer (QqQ-MS).

The method is rugged, provides an adequate cleanup of the complex sample matrix and shows good limits of detection, from below 0.1 to just under 10 ng/mL for the various analytes that are determined.

INTRODUCTION

The use of performance enhancing substances predates the beginning of ancient Olympics in Greece. However, until the 20th century the word “doping” had not been widespread [1].

Due to the lucrative prizes and/or glory that may be involved in competitive sports, there is a need to ensure

fairness and uphold the integrity of the various sports. Examples of regulatory bodies on doping analysis are World Anti-Doping Agency (WADA), Association of Racing Commissioners International (ARCI), and International Federation of Horseracing Authorities (IFHA) which have laid down strict guidelines regarding the use of certain substances.

Doping agents may be derived from commercially available drugs or natural plant extracts; and they are usually analyzed in either plasma and/or urine. These biological samples are complex matrices, which can hinder analyte detection. Equine urine for example is rich in plant-derived phenolic compounds. Furthermore, due to the advancement of science and technology, some of these prohibited substances may be very potent, requiring only a minute amount to produce a significant effect. Thus, there is a need for a proper cleanup prior to analysis to minimize the degradation impact on the various instruments and consumables, and ensure their detection.

In current literature, solid phase extraction (SPE) followed by conjugate cleavage and derivatization before LC-MS/MS detection is described for the analysis of anabolic steroids from equine urine [2]. The analysis of equine plasma is also quite common. In this context a method including protein precipitation, SPE cleanup (online, by column switching) before LC-MS/MS detection is described [3]. Another method uses MTBE for liquid/liquid extraction of serum in conjunction with LC-MS/MS detection [4]. Automated sample preparation is rather seldomly used.

In this application, Disposable Pipette Extraction (DPX), a dispersive solid phase extraction technique (d-SPE), had been utilized for the extraction of certain prohibited substances in equine urine. DPX was chosen due to the relatively fast extraction, and since the entire process can be automated, thus reducing the risk of human error, improving repeatability, and increasing throughput.

The DPX process is quite similar to solid phase extraction (SPE) on packed cartridges, except that the sorbent material is loosely contained in a pipette tip with a transport adapter on the top to facilitate automated processing with the GERSTEL MultiPurpose Sampler (MPS) (figure 1). One main difference is that the sample is drawn into the tip from the bottom, rather than loading it onto the cartridge through the top as is done in conventional SPE. Thereafter, air is aspirated into the tip to facilitate mixing and exchange between the sample and the sorbent (figure 2). The entire extraction process is depicted in figure 3. As the sample is never

in contact with the autosampler syringe during sample preparation, the potential for carryover is low.



Figure 1. GERSTEL Disposable Pipette Extraction (DPX). Pipette tip with loosely contained sorbent mounted with a transport adapter to facilitate automation.



Figure 2. DPX extraction step. Turbulent mixing of sample and sorbent is achieved by aspirating air.

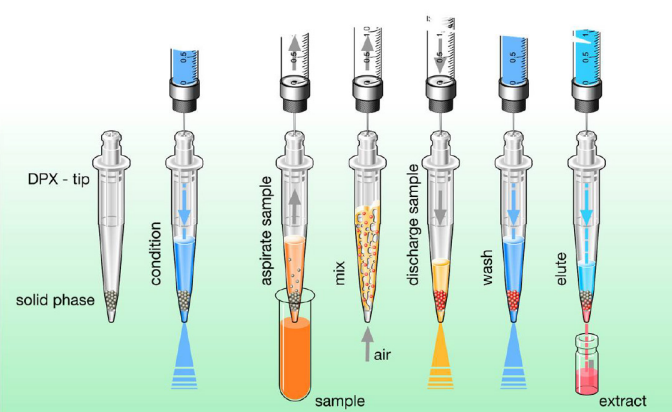


Figure 3. Schematic diagram of the DPX steps.

Model analytes were chosen according to their relevance from lists of the ARCI [5] and the International Federation of Horseracing Authorities (IFHA) [6].

Due to the complexity of the urine matrix, gas chromatography coupled to mass spectrometry (GC/MS) was selected for the separation and analysis of the targeted analytes. Selected ion monitoring (SIM) involving multi-dimensional GC heart cutting (2D GC) of the fractions of interest and selected reaction monitoring modes (SRM) on triple-quadrupole (QqQ) mass spectrometer were utilized to increase the selectivity of the tested analytes.

EXPERIMENTAL

Instrumentation. Analyses were performed using a 7890 gas chromatograph equipped with either a 7000 Triple-Quadrupole MS with extractor source or a 5975 Mass Selective Detector (Agilent Technologies). A Dual Head MultiPurpose Sampler (MPS) with Disposable Pipette Extraction (DPX) was employed for sample preparation and injection into a Cooled Injection System (CIS) PTV-type inlet with Automated Liner EXchange (ALEX), all from GERSTEL (figure 4).



Figure 4. GERSTEL MultiPurpose Sampler (MPS) Dual Head configuration with Disposable Pipette Extraction (DPX) on a 7890 GC/5975 MSD from Agilent Technologies.

Materials. Equine urine samples were provided by the German Sport University, Cologne. The analyzed compounds and internal standards were purchased from Sigma-Aldrich or Lipomed as pure substances or in methanolic solutions of 1 mg/mL. All solvents and salts were of analytical grade from Sigma-Aldrich and LGC.

Preparation of Standards. Pure compounds were dissolved in acetonitrile to give stock solutions of 1 mg/mL of each individual compound. A combined stock solution of 60 ng/ μ L in acetonitrile was prepared from these solutions. This stock solution was diluted to produce a series of working solutions of 10, 5, 1, 0.5 and 0.1 ng/ μ L concentration in acetonitrile.

A working solution containing 100 ng/ μ L of each internal standard (d_{10} -phenanthrene, $^{13}C_3$ -caffeine and d_5 -diazepam) was prepared in methanol.

Sample Preparation. Equine urine samples were prepared with help of the Dual Head MPS. 1.2 mL samples of urine were filled into 1.5 mL vials (093640-046-00) and spiked with 1-8 μ L of analyte working solutions. 3 μ L of internal standard solution was added. The samples were alkalinized by adding 120 μ L of 1N NaOH. After centrifugation, 1.1 mL of the supernatant (equivalent to 1 mL of equine urine) was manually transferred to a culture tube (093640-082-00) and placed onto the DPX sample tray. Since a powerful centrifuge is also available for the MPS, complete automation of these initial sample preparation steps is entirely possible.

Normally, drug metabolites from equine urine samples require cleavage of drug conjugates (e.g. glucuronides, sulfates) before extraction. This step was left out in this study since only spiked urine samples were analyzed.

Sample Extraction. The sample was automatically extracted with a mixed mode weak anion exchange DPX tip (WAX, 017512-119-00). The tip was conditioned with 500 μ L methanol followed by 2000 μ L water, both added from the top. A 740 μ L sample of equine urine was aspirated and extracted following phase wetting with 270 μ L water added from the top. The sample was dispensed back into the vial and the extraction cycle repeated twice. After the extraction steps, the DPX tip was washed with 2000 μ L water and the analytes subsequently eluted with 500 μ L acetone added from above. The eluate was mixed by performing multiple aspiration and dispensing cycles using the injection syringe and an aliquot injected into the CIS. Sample preparation takes approximately 15 minutes and it is completely overlapped with the GC run. This ensures efficient utilization of the GC/MS since the next sample is always ready for injection once a run is completed.

Analysis Conditions

MPS: 8 μ L injection volume
CIS 4: Deactivated glass beads liner
50 mL solvent vent/splitless (3 min)
50°C, 12°C/sec, 300°C (3.4 min);
12°C/sec; 280°C (19.7 min)
Pneumatics: He, constant flow,
mid column backflush
1st column 1.2 mL/min
2nd column 1.4 mL/min
Oven: 100°C (3 min); 15°C/min;
320°C (1 min + 5 min post run)
Columns: 2 x 15 m HP-5ms Ultra Inert (Agilent),
 $d_i = 0.25$ mm $d_f = 0.25$ μ m
QqQ: Selected reaction monitoring (SRM)
Source Temp. 230°C
He Quench Gas 2.25 mL/min
N₂ Collision Gas 1.5 mL/min
full scan, 40-350 amu

RESULTS AND DISCUSSION

Optimization of Extraction and Injection. For optimization of DPX parameters two analytes, caffeine and diazepam, were used. The analyte recovery and cleanup efficiency of different sorbents were evaluated: Reversed phase (RP, 017500-119-00), polar (P, 017514-119-00) and mixed mode weak anion exchange (WAX, 017512-119-00). Although all sorbents showed good recovery of the analytes, the co-extracted sample matrix backgrounds were different. The WAX sorbent eliminated matrix far more efficiently than the RP and P sorbents. Alkalinizing the sample prior to sample extraction largely converted phenolic matrix compounds into anions, which were effectively bound to the anion exchange moiety of the sorbent; while the analytes were extracted by the polymeric backbone of the WAX material. In the elution step, only the neutral analytes were eluted by organic solvent, while the phenolic anions remained on the sorbent, resulting in a relatively clean eluate (figure 5).

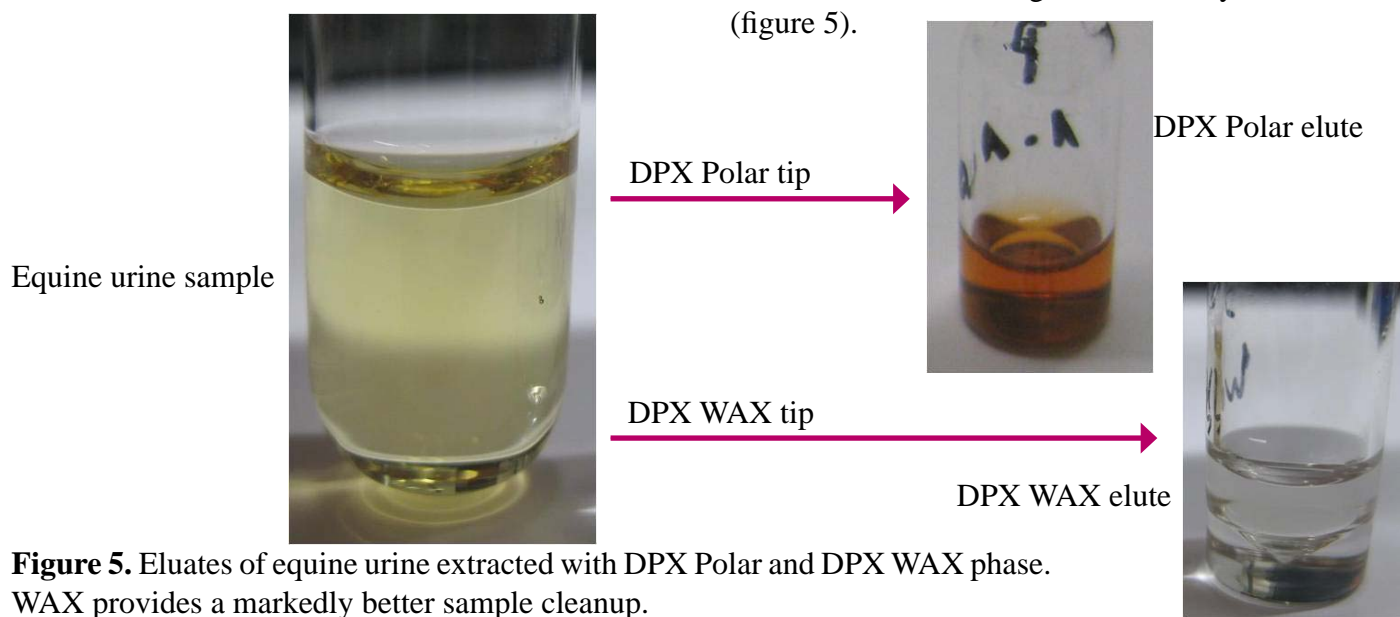


Figure 5. Eluates of equine urine extracted with DPX Polar and DPX WAX phase. WAX provides a markedly better sample cleanup.

Parameters like tip conditioning, extraction, washing and elution were also optimized. Methanol and water were chosen for tip conditioning following a series of experiments with a number of solvents. It was then examined how different water volumes, corresponding to various methanol residues on the sorbent, influenced the extraction of the analytes. Conditioning with 500 μ L methanol followed by 2 mL water was found to produce best recovery of the compounds in question (figure 6).

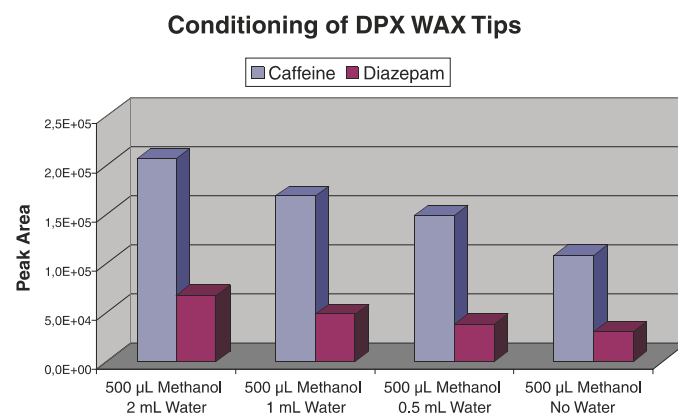


Figure 6. Conditioning of DPX WAX tips with 500 μ L methanol and different volumes of water. Influence on extraction efficiency.

Washing of the DPX tip after sample extraction should remove as much as possible of the interfering matrix, while allowing the analytes to be retained on the sorbent. Different water volumes and mixtures of water and organic solvents were tested. Internal standards were added to the eluate after elution to compensate for matrix effects caused by the different amounts of matrix left after washing. Generally a positive matrix effect enhancing peak areas was visible. Finally, washing with 2 mL of pure water was chosen as the optimum (figure 7).

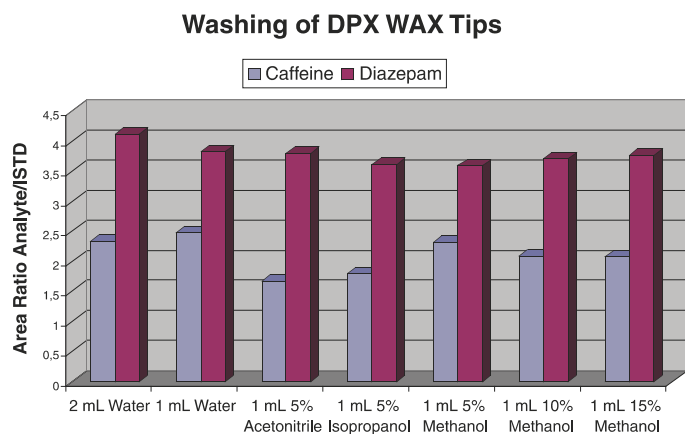


Figure 7. Optimization of the DPX washing step. Internal standards were added after elution in order to compensate for matrix effects caused by different extract cleanup.

Several elution solvents (acetonitrile, ethyl acetate, and acetone) were tested. Acetone was chosen for several reasons: It is more polar than ethyl acetate and more volatile than acetonitrile. Hence, it is able to extract polar analytes more efficiently than ethyl acetate, and is removed more readily than acetonitrile during large volume injection. Furthermore it is water miscible facilitating good sorbent wetting during the elution step. The chosen model analytes were observed to be stable in acetone after elution.

An evaluation of inlet liners was carried out using the Automated Liner EXchange (ALEX). Several inlet liners could easily be tested by running a sequence with automated liner exchange between sample injections. The deactivated liner filled with glass beads (011714-005-00) was found to perform the best and was chosen for further measurements (figure 8).

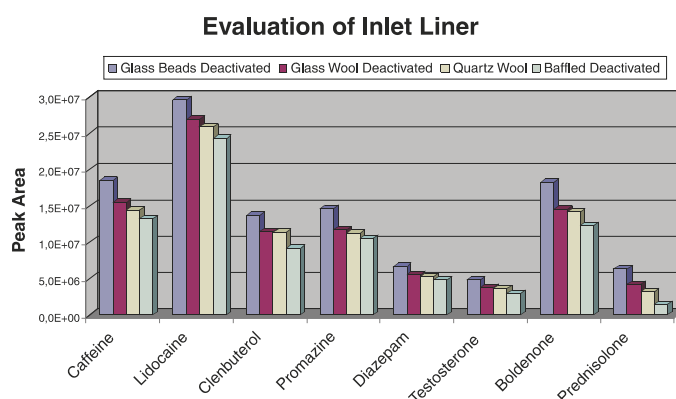


Figure 8. Automated evaluation of inlet liners using GERSTEL Automated Liner EXchange (ALEX).

In order to further improve the limits of detection, large volume injection was evaluated. As the matrix content of the extracts was still relatively high, only a moderate increase of the injection volume up to 8 μL was tested. 10 ng of each analyte was injected contained in different volumes of blank matrix extract respectively. It was expected that peaks for each individual compound yield an equivalent area count. Interestingly, a matrix enhancement effect could be seen when injecting 4 and 8 μL of spiked blank extract resulting in larger peak areas even though the amount of analyte injected was the same. As can be seen in figure 9, the results obtained using 4 and 8 μL injection volume are quite similar. The effect seen here is referred to by Mastovska, Lehotay and Anastassiades as “matrix-induced chromatographic response enhancement” [7], presumably the added matrix covers active sites in the inlet. An injection volume of 8 μL was chosen for the optimized method.

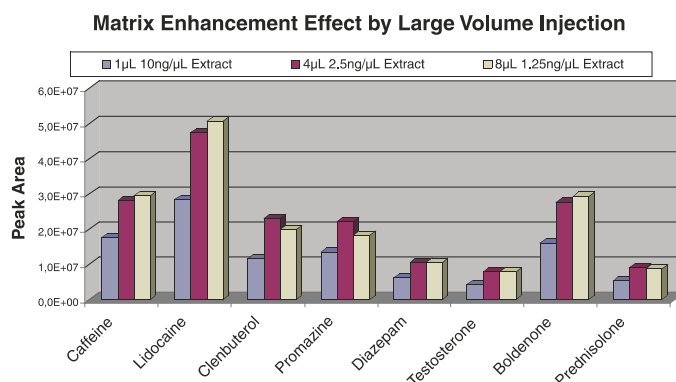


Figure 9. Evaluation of large volume injection. Injection of 10 ng of each analyte in different volumes of blank equine urine extract. Matrix enhancement is visible for 4 and 8 μL injection volume.

Comparison of Detection Techniques. Three different detection techniques were evaluated: Selected ion monitoring (SIM) with a single-quadrupole MS, selected ion monitoring with heart cutting two dimensional GC (2D-GC SIM), and selected reaction monitoring (SRM) with a triple-quadrupole MS. Two different 2D-GC setups were chosen, both employing a non-polar first dimension column (e.g. Rtx-5ms 30 m, $d_i = 0.25$ mm, $d_f = 0.25$ μ m, Restek) and a medium polarity second dimension column with high temperature stability (e.g. Rxi-17Sil MS 30 m, $d_i = 0.25$ mm, $d_f = 0.25$ μ m, Restek) in one GC oven. Fractions were cut from the first dimension column and cryofocused at the beginning of the second dimension column using a Cryo Trap System (CTS 2, GERSTEL). The first dimension column was backflushed after the last analyte was trapped on the CTS 2. After that, the GC oven was cooled down and the trapped fractions were released onto the second dimension column by programmed heating of the CTS 2.

In the first setup, both columns were connected to an open split interface at the inlet to the MS. This setup offers the advantage of monitoring the chromatogram from both the first and second dimension column. However, it has the potential drawback of the matrix contaminating the MS. In the second setup, the first

dimension column is connected to a flame ionization detector (FID), while the second dimension column is connected to the MS. Although monitoring of the heart cutting is more difficult, it minimizes the matrix contamination effect on the MS. Both techniques showed similar performance regarding enhancement of selectivity.

Figure 10 shows the cleanup of the chromatogram by 2D-GC. A large volume injection of 25 μ L equine urine extract detected by full scan MS is compared to the same injection detected by 2D-GC full scan MS. Figure 11 shows the chromatograms obtained from all three detection techniques, enabling a comparison of the signal to noise ratios achieved from equine urine extract spiked with 50 ng/mL diazepam. The triple-quadrupole detection was chosen since it gave the best signal/noise ratios and therefore highest selectivity and sensitivity. Even though the 2D-GC systems used also showed good selectivity and sensitivity, such systems are more suited for determining a limited number (1 - 4) of compounds from complex matrices. In that context, they are an in-expensive alternative to triple-quadrupole MS. They are not suited for analyzing a large number of compounds, as that would lead to a significant amount of the matrix being transferred to the second column, thus diluting the cleanup effect.

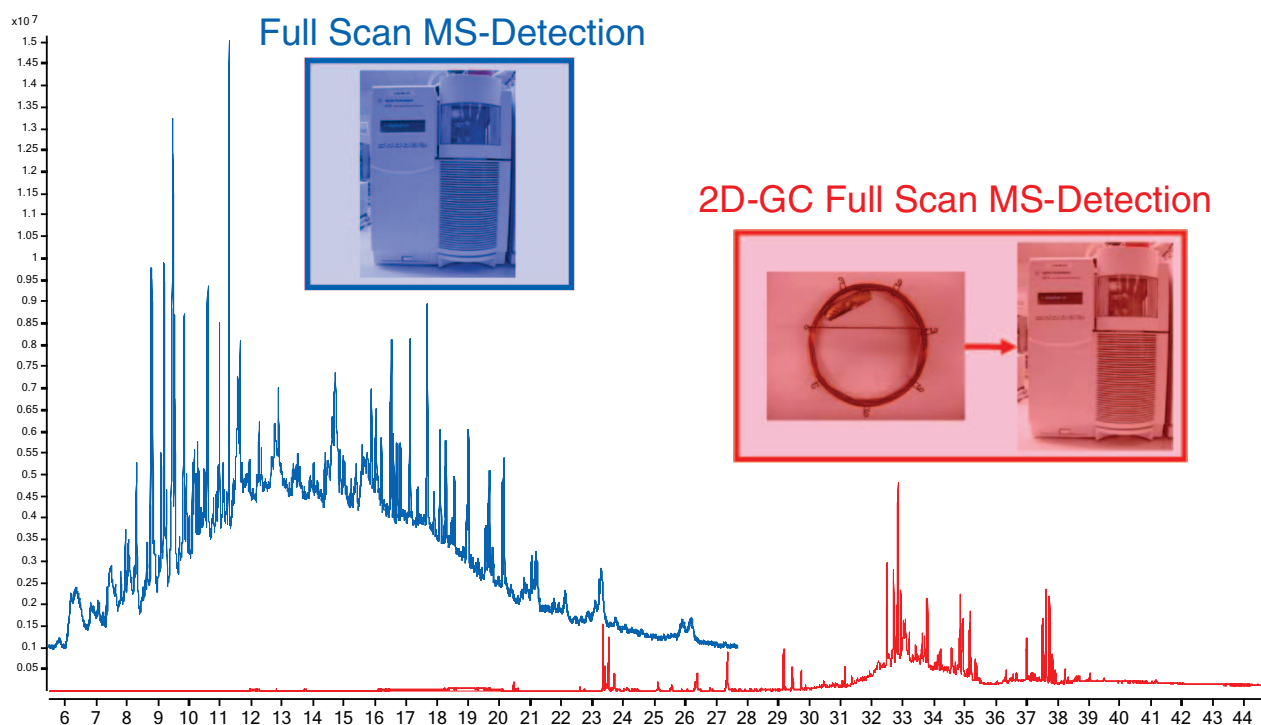


Figure 10. 25 μ L large volume injection of an equine urine extract detected in full scan MS- and 2D-GC full scan MS-mode, showing effective matrix elimination by 2D-GC.

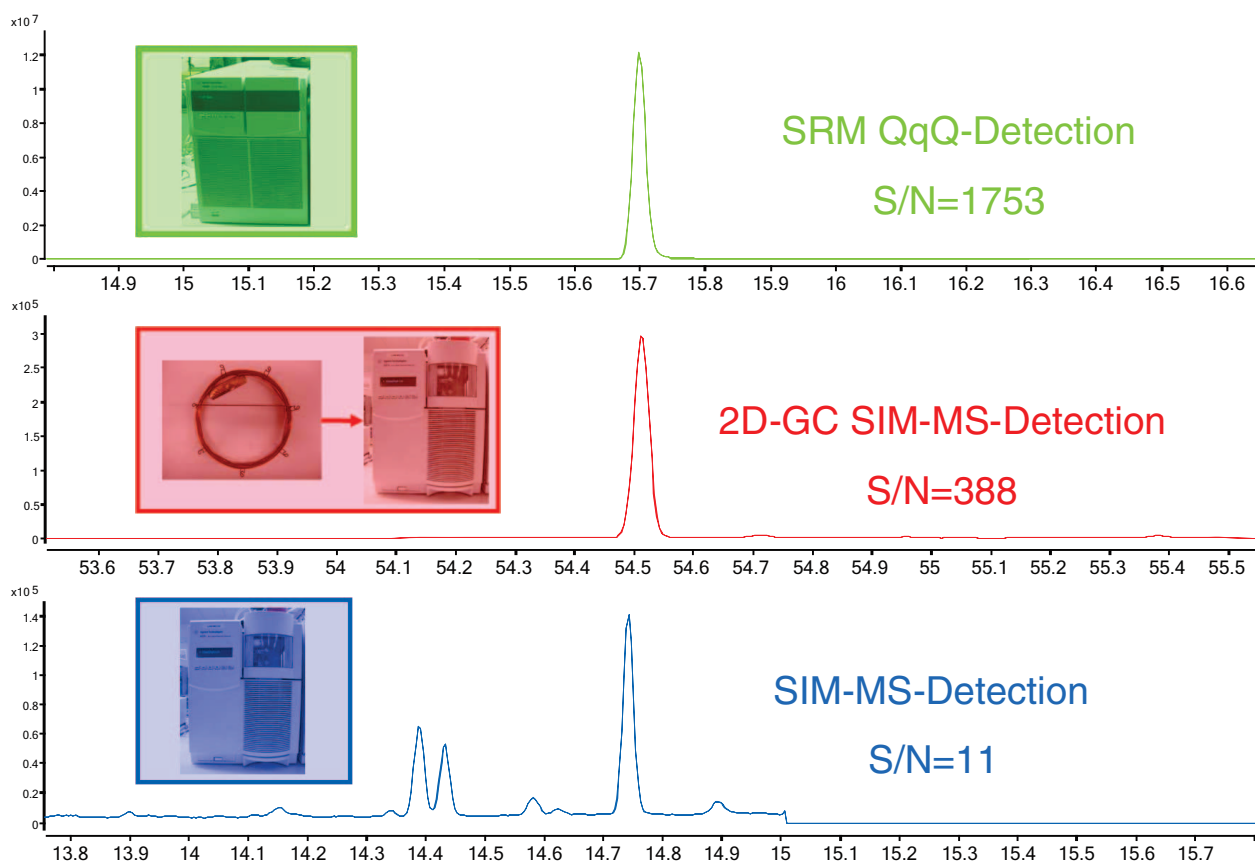


Figure 11. Comparison of different detection techniques. 25 μ L large volume injection of 1 mL extracted equine urine containing 50 ng/mL diazepam.

Optimization of Triple-Quadrupole Detection. Detection in the triple-quadrupole mass spectrometer was optimized with regards to the precursor and daughter ions involving 3 SRM transitions per compound for detection, by varying the collision energy (table 1). A typical chromatogram can be seen in figure 12. Peak areas were corrected by internal standards. The internal standard yielding the most consistent results in calibration measurements was selected for each individual analyte.

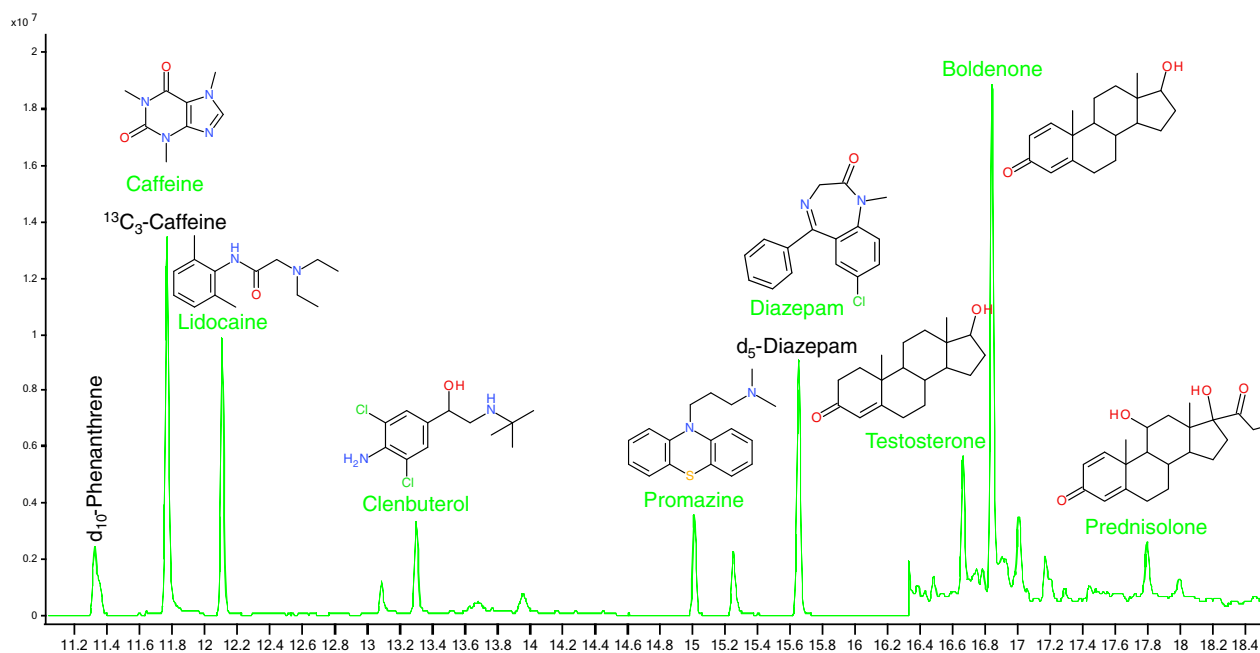


Figure 12. Typical chromatogram of an extracted equine urine spiked at 50 ng/mL measured with triple quadrupole detection.

Table 1. Analytes and internal standards with retention times and SRM conditions.

Compound	Ret. Time [min]	Precursor Ion [amu]	Daughter Ion [amu]	Collision Energy [V]
d ₁₀ -Phenanthrene	11.29	188	160	30
		188	184	35
		188	158	40
Caffeine	11.73	109	55	5
		194	109	10
¹³ C ₃ -Caffeine	11.73	194	165	10
		197	57	30
		111	57	5
Lidocaine	12.08	197	111	10
		86	58	10
		234	86	10
Clenbuterol	13.27	234	205	10
		86	57	10
		190	127	20
Promazine	14.98	127	65	30
		284	86	10
		284	199	10
d ₅ -Diazepam	15.61	199	167	20
		261	226	15
		289	261	10
Diazepam	15.63	289	226	30
		256	221	15
		283	238	20
Testosterone	16.63	283	248	15
		124	109	10
		124	81	20
Boldenone	16.81	124	96	10
		122	107	10
		122	77	40
Prednisolone	17.76	122	79	20
		122	107	10
		121	77	20

Validation Steps and Sample Measurements. Calibration was performed using spiked blank equine urine samples, resulting in good linear correlations being achieved (figure 13). The limits of quantification and linear range were estimated from these measurements. Some analytes, like caffeine, promazine and diazepam, showed very low limits of quantification below 0.1 ng/mL. Other compounds like lidocaine and clenbuterol had quite abundant SRM quantifiers, but weak SRM qualifiers, leading to slightly higher limits of quantification below 2 ng/mL. The selectivity for the steroidal compounds was not as good as for the other compounds since they produce mass fragments with commonly found mass signals such as m/z=122. Therefore, testosterone, boldenone and prednisolone had the highest, but still very good, limits of quantification (figure 14).

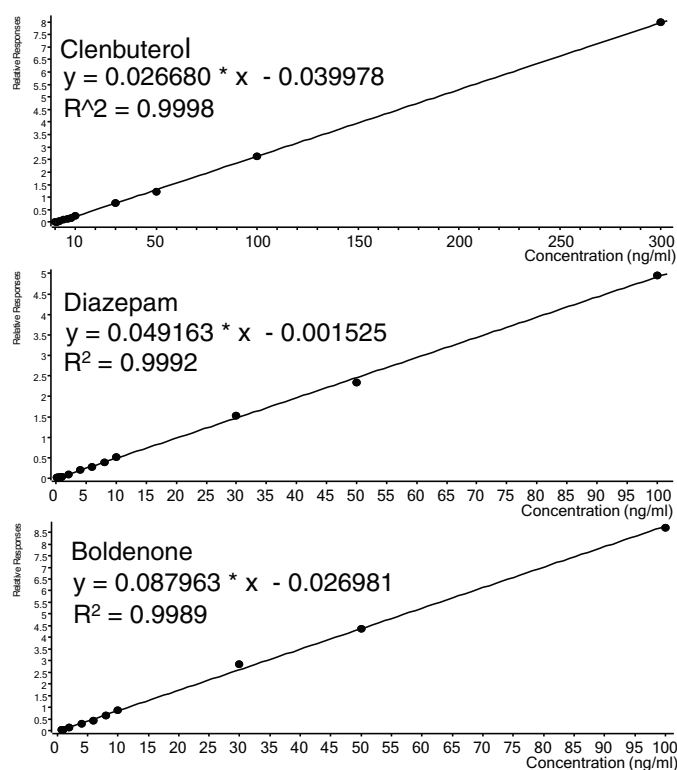


Figure 13. Calibration lines for selected compounds extracted from equine urine.

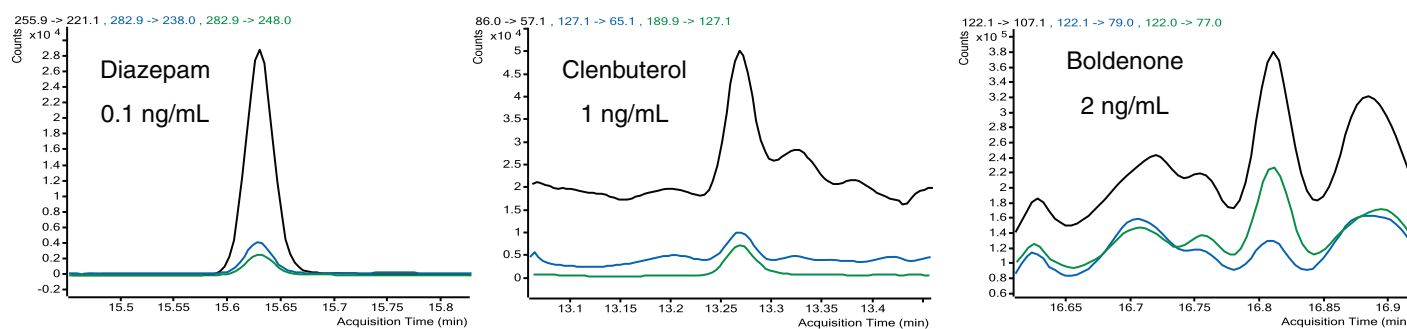


Figure 14. Chromatograms showing quantifier and qualifier SRMs for diazepam at the lowest calibration level and clenbuterol and boldenone at their limit of quantification.

The upper limits of quantification are influenced by the mass spectrometer. A reduction of sample volume or injection in split mode can shift the linear range to higher concentrations, if needed.

Repeatability was determined by 8-fold extraction of equine urine samples spiked at 30 ng/mL resulting in relative standard deviations below 5 % for most compounds. Recoveries were determined at the 30 ng/mL level; for most compounds, the recovery was found to be higher than 85%. Very stable retention times were achieved by employing a mid column backflush, thus keeping the analytical column free from high boiling contaminants. Nowadays, column backflushing is increasingly used in pesticide analysis. Despite its advantages, the technique is not common in doping analysis. An increase of sample throughput and robustness and less need for maintenance are reported in the literature for the analysis of steroids from equine urine using backflush [8].

Table 2. Validation data for equine urine doping analysis.

Analyte	Linear Range [ng/mL]		Correlation coefficient	Recovery [%]	RSD (n=8) [%]	Maximum deviation from mean retention time [min]
Caffeine	<0.1	>300	0.9982	98	4.5	0.027
Lidocaine	<2	>100	0.9981	106	3.2	0.015
Clenbuterol	<1	>300	0.9998	90	5.4	0.026
Promazine	<0.1	>300	0.9979	51	7.7	0.010
Diazepam	<0.1	>100	0.9992	93	1.7	0.010
Testosterone	<2	>300	0.9997	85	4.2	0.018
Boldenone	<2	>100	0.9989	96	3.0	0.008
Prednisolone	<10	>300	0.9993	24	11.9	0.024

Several different equine urine samples were spiked and analyzed using the calibration mentioned above in order to check the accuracy of the method results, which were satisfactory for most samples (table 3). Fields marked with “N/A” indicate that the spiked amount was smaller than the limit of quantification of the respective compound. “High blank!” means that the sample already contained the spiked compound and therefore the listed concentration is too high.

Table 3. Method accuracy evaluated by spiking different equine urine samples.

Analyte	Sample 1 5 ng/mL	Sample 1 10 ng/mL	Sample 1 20 ng/mL	Sample 1 30 ng/mL	Sample 1 40 ng/mL	Sample 1 50 ng/mL	Sample 2 1 ng/mL	Sample 3 5 ng/mL	Sample 4 10 ng/mL	Sample 5 20 ng/mL	Sample 6 30 ng/mL	Sample 7 50 ng/mL
Caffeine	3.0	9.5	22.8	32.9	44.9	62.5	High Blank!	High Blank!	High Blank!	High Blank!	High Blank!	High Blank!
Lidocaine	4.7	9.7	20.0	30.8	40.9	50.3	N/A	6.0	12.8	22.7	29.8	53.0
Clenbuterol	4.7	9.0	17.6	26.4	36.2	51.7	4.0	6.4	11.2	21.6	31.4	50.6
Promazine	3.9	10.5	23.4	30.7	41.3	51.8	1.2	6.9	9.9	High Blank!	High Blank!	High Blank!
Diazepam	4.6	9.5	20.2	31.3	40.9	51.3	1.8	5.1	10.0	20.3	30.8	49.2
Testosterone	5.7	10.0	21.0	29.0	38.3	52.5	N/A	5.4	8.4	18.5	32.8	46.7
Boldenone	4.3	8.8	19.6	29.0	37.2	50.7	N/A	5.1	10.1	21.4	34.3	51.3
Prednisolone	N/A	8.0	16.9	19.1	24.3	39.9	N/A	N/A	8.2	21.9	High Blank!	High Blank!

In addition to the compounds mentioned, procaine and phenylbutazone could be extracted and analyzed. However, they were catabolized in the prepared sample under the chosen conditions. Phenylbutazone was transformed to hydroxy-phenylbutazone. The ester bond in procaine may have been hydrolyzed, catalyzed by the basic conditions.

CONCLUSIONS

An automated analysis method for doping compounds in equine urine was established employing a combination of DPX/LVI/GC/QqQ-MS. The selectivity and sensitivity of the method with triple-quadrupole MS detection was higher than with 2D-GC SIM-MS and SIM-MS detection. For a limited number of analytes, (n~4) 2D-GC MS provides an inexpensive alternative, helping to gain selectivity and sensitivity. In order to determine more polar compounds using GC analysis, derivatization is typically needed; this process can be automated using the described MPS platform.

Coupling the DPX cleanup to an LC/QqQ-MS is also possible and would be beneficial in providing an alternative screening test. DPX elution could be done using acetonitrile for HILIC column analysis or the eluate diluted appropriately for RP column analysis. Generally, more compounds may be included readily in the method. The following achievements were made:

- A completely automated DPX/LVI/GC/QqQ-MS analysis method was developed (from extraction to detection). Sample preparation was performed much faster than methods described in literature [2,3,4].
- Sample preparation was overlapped with the GC/QqQ-MS run ensuring that instrument time is efficiently used.
- Efficient and fast cleanup of equine urine matrix with DPX WAX tips.
- Low limits of quantification from <0.1 to <10 ng/mL, comparable to those found in literature for urine [2]. Limits of quantification in plasma are somewhat lower, for many compounds around 0.1 ng/mL [3,4].
- Good repeatability with RSDs between 1.7 and 11.9 %.
- Column backflush [8] and automated liner exchange (ALEX) were implemented, improving the ruggedness of the analysis method.

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