Analytical Methods

PAPER

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Cite this: *Anal. Methods*, 2016, **8**, 1789

# The potential for complementary targeted/non-targeted screening of novel psychoactive substances in equine urine using liquid chromatography-high resolution accurate mass spectrometry

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Received 18th January 2016
Accepted 22nd January 2016

DOI: 10.1039/c6ay00156d

www.rsc.org/methods

The potential for liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry to identify 'unknown' compounds using non-targeted screening methods provides a potential advantage in the fight against doping in sport. This innovation comes with the requirement for assessment to support its use in the medico-legal context. A method for the LC-HRAM detection of 2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine (NBOMe) compounds in equine urine was validated in order to assess the capabilities of a workflow developed for non-targeted analysis using the SIEVE® differential analysis software platform. Six NBOMe compounds (25B, 25C, 25D, 25E, 25H and 25I) were studied to develop and optimize the proposed non-targeted screening workflow before two additional candidates (25N and 25T2) were used as blind controls for verification. Chromatographic alignment and the integration threshold were found to be the most critical parameters for successful identification of 'unknown' responses. The proposed workflow serves as an example for anti-doping laboratories to implement fit-for-purpose non-targeted screening methods.



## Introduction

Novel psychoactive substances (NPS) are chemical modifications of currently controlled substances that have similar pharmacological effects and chemically designed to circumvent legislation.[1] In 2012, Casale and Hays[2] reported the characterization of eleven 2,5-dimethoxy-*N*-(2-methoxybenzyl)phenethylamine (NBOMe) compounds considered to be more potent serotonin 5-$HT_{2A}$ receptor agonists than their 2,5-dimethoxyphenethylamine ("2C") precursors.[3–5] Since this time there have been multiple reports concerning adverse health effects.[6–13] Misuse of this drug class generally occurs with a single administration of approximately 0.1 g to achieve hallucinations and a varying degree of stimulation.[13] In response to a medico-legal requirement the validation of analytical methods used to detect NBOMe compounds in biological matrices has been reported by forensic toxicology laboratories.[8–10] Furthermore, metabolism studies in human and rat urine have been recently performed.[14]

While there are no reports describing the effect of these compounds in horses, there is concern about the potential for misuse of NPS such as NBOMe compounds in equine athletes where handlers may have made the assumption that these compounds are undetectable by horseracing laboratories. The availability of these compounds presents a serious threat to the integrity of equine sports and to the welfare of the horse. NBOMe compounds are therefore prohibited for use by the International Federation of Horseracing Authorities.[15]

Analytical methods utilizing the sensitivity and specificity of liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry applied to forensic toxicology,[16–18] and more specifically to horseracing laboratories,[19] have been reported in recent years. The potential for LC-HRAM technology to detect 'unknown' compounds using non-targeted screening methods provides a potential advantage in the fight against doping in sport.

Differential analysis is a tool used in studies with large data sets that require the comparison of pre-treatment control samples of biological origin with samples collected following a treatment (such as exposure to particular stimuli) in order to detect and elucidate biomarkers correlating to the treatment response. The control and treatment samples are compared by statistical means resulting in the generation of a list of targets that are independent of the control sample. For the past 10 to 15

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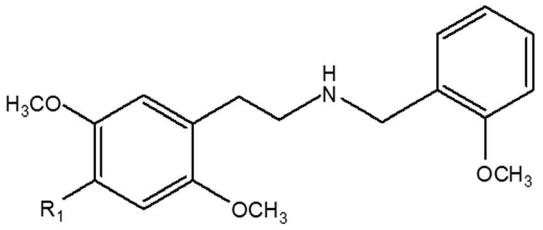



Fig. 1 General NBOMe structure ($R_1$: Br = 25B, Cl = 25C, $CH_3$ = 25D, $C_2H_5$ = 25E, H = 25H, I = 25I).

years, differential analysis techniques have been widely used for the assessment of microarray data.[20,21] More recently following the development of software for MS-based applications, there is considerable potential for differential analysis in analytical chemistry.[22] Depending on the software used standard outputs may include retention time, mass-to-charge ($m/z$) ratio and the statistical probability ($p$-value) of the two samples being statistically different. From this information, a targeted approach can be applied such as tandem mass spectrometry ($MS^2$) to identify unknown compounds. This strategy is preferred over conventional visual assessment of chromatographic data since it can detect compounds that coelute or have low abundances obscured by background noise. In addition, differential analysis applied to full-scan MS provides an advantage over other chemometric approaches for biomarker detection by not excluding raw data. With the rapid proliferation of NPS such strategies have the potential to detect new compounds that have not been previously defined in published targeted methods or databases.

The aim of the study described herein was to develop and validate a method for the detection of six NBOMe compounds (Fig. 1) in equine urine using LC-HRAM spectrometry to support integrity in horseracing. Furthermore, the applicability of using a differential analysis software package such as SIEVE® (Statistical Iterative Exploratory Visualization Environment, Thermo Fisher Scientific)[23] was evaluated for the non-targeted screening and putative identification of two additional NBOMe compounds that were unknown to the analyst under 'blind' testing conditions.

## Materials and methods

### Reference materials, chemicals and reagents

Hydrochloride salts of the NBOMe compounds [2-(4-bromo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25B), [2-(4-chloro-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25C), [2-(4-methyl-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25D), [2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25E), [2-(2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25H), [2-(4-iodo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25I), [2-(4-nitro-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25N) and [2-(4-methylthio-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine] (25T2) manufactured by Lipomed AG (Arlesheim, Switzerland) were purchased as 1 mg $mL^{-1}$ (in methanol) ampoules from PM separations (Capalaba, Queensland, Australia). Desipramine-$d_3$ was purchased from Grace (Deerfield, IL, USA). Trypsin and sodium acetate were obtained from Sigma-Aldrich (St Louis, MO, USA). β-Glucuronidase K12 from *E. coli* was purchased from Roche Diagnostics (Mannheim, Germany). Analytical grade ammonia (aqueous solution 28%) and acetic acid (glacial) together with HPLC grade solvents were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Ultra-pure water was obtained using a Millipore filtration system (Bedford, MA, USA).

### Preparation of standard solutions

Stock solutions for each NBOMe standard were prepared at 100 μg $mL^{-1}$ by quantitatively diluting the purchased 1 mg $mL^{-1}$ solution into 10 mL of methanol in a volumetric flask. For the method validation, a mixed NBOMe intermediate solution (1 μg $mL^{-1}$) containing the six candidates (25B, 25C, 25D, 25E, 25H and 25I) was prepared by diluting 100 μL of each stock into 10 mL of methanol in a volumetric flask. A mixed NBOMe working solution (100 ng $mL^{-1}$) was prepared by diluting 1 mL of the mixed intermediate solution into 10 mL methanol in a volumetric flask. Desipramine-$d_3$ stock solution (1 mg $mL^{-1}$) was prepared from dissolving 5 mg of primary standard, weighed using an analytical balance (Mettler Toledo AT261, Columbus, OH, USA), in methanol (5 mL) using a volumetric flask. The desipramine-$d_3$ working solution (2 μg $mL^{-1}$) was prepared by diluting 20 μL of stock in methanol (10 mL) using a volumetric flask. Methanolic solutions were stored at 4 °C for up to 12 months.

### Preparation of blank equine urine

Authentic blank urine samples were collected from three thoroughbred gelding horses by spontaneous voiding with carrot reward following approval from the Racing NSW Animal Care and Ethics Committee (RP72). These horses were known to have not been administered any pharmaceutical agent for at least two weeks prior to sample collection. Urine samples from the three horses were pooled to provide a profile considered to be representative of the racehorse population for proof-of-concept concerning differential analysis.

### Sample preparation

Equine urine samples (3 mL) were fortified with desipramine-$d_3$ (2 μg $mL^{-1}$, 50 μL) internal standard before addition of pH 5.0 acetate buffer (0.2 M, 4 mL) followed by enzyme hydrolysis with β-glucuronidase K12 from *E. coli* (20 μL) and trypsin solution (625 μg per sample) overnight at 37 °C. The basic organic fraction was isolated by solid phase extraction (SPE) using a mixed-mode C8/strong cation exchange XTRACKT® column (200 mg, 3 cc, UCT, Bristol, PA, USA). The cartridge was conditioned with methanol (2 mL) and water (2 mL) before loading the urine sample and washing with acetic acid (0.1 M, 2 mL) followed by methanol (2 mL). The cartridge was dried using $N_2$ gas under positive pressure before elution with ethyl acetate/ammonia/methanol (100 : 3 : 0.5, 3 mL). HCl/methanol (0.1 M, 20 μL) was added to extracts before evaporation to dryness under nitrogen at 60 °C. Residues were reconstituted in methanol (50 μL) and

pH 4 ammonium acetate (10 mM, 100 μL) for LC-HRAM analysis.

### LC-HRAM analysis

LC-HRAM spectrometry was undertaken using an Ultimate 3000 HPLC coupled to a QExactive benchtop orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed using a Gemini® C18 column (50 × 2.1 mm, 5 μm; Phenomenex, Torrence, CA, USA) operating at 35 °C with a 10 μL injection volume. The mobile phase consisted of A: pH 9 ammonium acetate (10 mM) and B: 0.1% acetic acid/acetonitrile. Gradient elution was performed with a flow rate of 0.5 mL $min^{-1}$ according to the following program: 1% B for 2 min, increased to 80% B linearly during the period between 2 and 8.5 min, before returning to 1% B at 8.6 min and held until 11.2 min. HRAM detection was performed using positive mode heated electrospray ionization (HESI) in full scan at a resolution of 70 000 (full width at half maximum, FWHM) acquiring a mass range of *m*/*z* 50 to 650 at 3 Hz. Mass calibration was performed prior to analysis using Pierce® ESI positive (P/N 88 323) calibration solution (Thermo Fisher Scientific, Bremen, Germany) but no lock mass was used. Source temperature, spray voltage, sheath gas (high purity $N_2$) and auxiliary gas (ultra-high purity $N_2$) were set at 350 °C, +4000 V, 63.74 and 10.30 arbitrary units, respectively. $MS^2$ data was acquired with a normalized collision energy of 25 arbitrary units, automatic gain control target of 2 × $10e^5$ and *m*/*z* isolation window of 0.5 to support confirmation of identity according to criteria prescribed by the Association of Official Racing Chemists (AORC).[24] Instrument control and data processing (±5 ppm) were performed using Xcalibur® software (version 2.2 SP1) from Thermo Fisher Scientific (San Jose, CA, USA).

**Method validation.** Method parameters were assessed according to NATA Technical Note-17 (ref. 25) to support the ILAC-G7:06/2009 document for horse racing laboratories.[26] Quantitative results were obtained from full scan acquisition of $[M + H]^+$ for each analyte provided in Table 1. The limit of detection (LOD) and lower limit of quantification (LLOQ) for the six candidate NBOMe compounds were determined from replicate ($n = 7$) analyses of spiked equine urine samples achieving a signal-to-noise (S/N) ratio of greater than 3 and 10, respectively. Intra- and inter-assay precision (expressed as percentage relative standard deviation, %RSD), together with accuracy, were determined from the ratio of peak areas for the analyte and desipramine-$d_3$ for seven replicates of 1 ng $mL^{-1}$ and 10 ng $mL^{-1}$ each analysed seven days over a period of two weeks. The same spiked sample matrix was used to assess recovery with comparison to urine samples fortified at 1 ng $mL^{-1}$ and 10 ng $mL^{-1}$ post-extraction. In turn, matrix effect was assessed using this set of post-extraction fortified urine samples compared to neat standards reconstituted in LC mobile phase at 1 ng $mL^{-1}$ and 10 ng $mL^{-1}$. Long-term stability trials were performed on additional replicates ($n = 3$) of spiked samples stored at 4 °C, −20 °C and −80 °C for one-, two- and three-month intervals. Short-term stability freeze/thaw trials were conducted on three consecutive days using additional replicates ($n = 3$) of spiked samples stored at −20 °C.

**Non-targeted screening.** Differential analysis of full scan ($MS^1$) data was performed using SIEVE® version 2.0.180. Data files acquired from duplicate sample injections were processed against duplicate matrix blank injections representing the control, the second of which was assigned as the reference file for chromatographic alignment. Recursive-base-peak-framing was used over the entire chromatographic run time (11.2 min) between *m*/*z* 50 and 500 to reflect the retention time and mass range of NPS. The default *Peak Intensity Threshold* of 1.27 × $10^8$ resulted in the generation of 5000 frames while the optimized *Peak Intensity Threshold* of 1 × $10^6$ resulted in the generation of 2000 frames, each with a chromatographic time range of 2.5 min and *m*/*z* width of 10 ppm. Statistical output from SIEVE® provides a *Ratio* value between sample and control with an associated *p-value* for each putative substance.

Analysis of $MS^2$ data to investigate dissociation schemes and apply precursor ion fingerprinting was performed using Mass Frontier™ 7.0 SR1. Each of these software packages were purchased from Thermo Fisher Scientific (San Jose, CA, USA).

## Results and discussion

### Targeted method validation

The limited LC selectivity displayed by the six candidate NBOMe compounds is shown in Table 1 with retention times within 0.8 min, including coelution of 25C and 25D. Nevertheless, specificity was achieved from differences in mass for this group of

Table 1 Specificity for six candidate NBOMe compounds using LC-HRAM

| NBOMe | Retention time (min) | Formula | Experimental $[M + H]^+$ | Theoretical[a] $[M + H]^+$ | Mass error (Δ ppm) |
|---|---|---|---|---|---|
| 25H | 5.72 | $C_{18}H_{23}NO_3$ | 302.1745 | 302.1751 | 2.0 |
| 25B | 6.20 | $C_{18}H_{22}NO_3Br$ | 380.0850 | 380.0856 | 1.6 |
| | | | 382.0829 | 382.0835 | 1.6 |
| 25C | 6.10 | $C_{18}H_{22}NO_3Cl$ | 336.1355 | 336.1361 | 1.8 |
| | | | 338.1325 | 338.1331 | 1.8 |
| 25D | 6.10 | $C_{19}H_{25}NO_3$ | 316.1902 | 316.1907 | 1.6 |
| 25E | 6.48 | $C_{20}H_{27}NO_3$ | 330.2059 | 330.2064 | 1.5 |
| 25I | 6.37 | $C_{18}H_{22}NO_3I$ | 428.0712 | 428.0717 | 1.2 |

[a] Determined by isotope simulation tool in the Xcalibur® operating software.

non-isomeric NBOMe compounds. The mass error ($\Delta$) for the six candidate NBOMe compounds was found to be within 2.0 ppm (Table 1), which is within the accepted value of 5.0 ppm.[24] This included the $[M + H + 2]^+$ isotopes for 25B and 25C due to the presence of bromine and chlorine atoms in their respective structures. Robustness in specificity was demonstrated by stability in mass accuracy without the use of a lock mass. Using caffeine ($[M + H]^+ = m/z$ 195.0877) as the most representative compound of NPS contained in the calibration mix, the intra-assay standard deviation was within 0.05 ppm. For the six-month duration of the study, the inter-assay standard deviation was within 0.1 ppm.

Quantitative validation results are provided in Table 2, which shows the LOD to be 0.1 ng $mL^{-1}$ for 25B, 25E, 25H and 25I, and 0.5 ng $mL^{-1}$ for 25C and 25D. The LLOQ for all six candidate NBOMe compounds was 1 ng $mL^{-1}$. Linearity was achieved between this LLOQ and 200 ng $mL^{-1}$ with $R^2 \geq 0.995$ to establish the calibration range performed in duplicate at the following concentrations: 1, 2, 5, 10, 20, 50, 100 and 200 ng $mL^{-1}$. Intra- and inter-assay precision was assessed at a spiking level of 1 ng $mL^{-1}$ to be within 8.7% (<10%) and 13.2% (<15%), respectively. At 10 ng $mL^{-1}$ these were within 4.0% (<10%) and 10.9% (<15%), respectively. Suitability of the SPE sample preparation method was demonstrated by excellent recoveries (88–97%) from 10 ng $mL^{-1}$ spiked equine urine samples, supporting accuracy between 93 ± 4% and 113 ± 6% (between 80% and 120%). Matrix effects were determined to be negligible from the results that were within 20% of the expected value. Samples were stable after 3 freeze/thaw cycles and stable for up to 3 months at 4 °C, −20 °C and −80 °C.

## Non-targeted analysis using differential analysis

Individual components of the SIEVE® software workflow were assessed; chromatographic alignment, peak detection, statistical analysis and identification.

**Chromatographic alignment.** Since SIEVE® can compare between two or more samples simultaneously, it is important that the sample chromatograms are first aligned for the peak detection process to provide accurate results. The ChromAlign algorithm corrects for inherent chromatographic variability by calculating optimal correlations between full-scan spectra in separate data files.[27] Retention time results for the six candidate NBOMe compounds and desipramine-$d_3$ internal standard ($[M + H]^+ = m/z$ 270.2091) obtained from the precision validation matrix were compared to assess variation that may reduce the robustness of the alignment algorithm used by SIEVE®. The suitability of LC parameters was demonstrated by relative standard deviations of less than 0.1% determined from seven 10 ng $mL^{-1}$ replicates over seven sets of analyses conducted within two weeks. Using the example of 25B spiked at 100 ng $mL^{-1}$, Fig. 2 demonstrates proof-of-concept for SIEVE® to provide visual identification of an abnormal response.

**Peak detection.** Following chromatographic alignment, spectral data is plotted in three-dimensional space with retention time, $m/z$ and relative intensity on the $x$-, $y$- and $z$-axes, respectively. Peaks in the control and treatment sample are detected using an algorithm called recursive-base-peak-framing where spectral data from both samples in the experiment are grouped together by relative intensity. Once the common base peak is known, a frame is established according to user-defined retention time, $m/z$ and intensity tolerances.[28] The *Peak Intensity Threshold* is the lowest intensity for which a spectral data point will be framed and is recommended to be one that is representative of the LOD for the respective class.

The default *Peak Intensity Threshold* setting of $1.27 \times 10^8$ analyzing 5000 frames provided detection of the six candidate NBOMe compounds at concentrations between 20 and 100 ng $mL^{-1}$. While sufficient for proof-of-concept that differential analysis was applicable to the method, these levels were considered too high for routine application. Optimization of the *Peak Intensity Threshold* to $1 \times 10^6$ analyzing 2000 frames resulted in detection levels between 0.5 ng $mL^{-1}$ and 10 ng $mL^{-1}$ that are considered fit-for-purpose.

**Statistical analysis.** At the conclusion of the peak detection process, the mean peak intensity values for peaks in each frame from sample and control replicates are compared using paired $t$-tests for determination of significant differences. The generated frame report contains the associated *p-value* for verification by the user. For simplicity, the frame report also contains a *Ratio* parameter which provides the user with direct comparison of mean peak intensities that can be filtered to identify compounds of interest. By convention, the maximum *Ratio* and minimum *p-value* is 99 999.90 and $1.00 \times 10^{-5}$, which implies the compound in the treatment sample is completely independent to the control sample. In addition to numerical

Table 2 Quantitative validation results for the six candidate NBOMe compounds

| Parameter | 25B | 25C | 25D | 25E | 25H | 25I |
|---|---|---|---|---|---|---|
| LOD (ng $mL^{-1}$) | 0.1 | 0.5 | 0.5 | 0.1 | 0.1 | 0.1 |
| LLOQ (ng $mL^{-1}$) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Linearity (ng $mL^{-1}$, $R^2 \geq 0.995$) | 1–200 | 1–200 | 1–200 | 1–200 | 1–200 | 1–200 |
| Intra-assay at 1 ng $mL^{-1}$ (%RSD) | 6.9 | 8.5 | 7.3 | 6.2 | 8.7 | 5.7 |
| Intra-assay at 10 ng $mL^{-1}$ (%RSD) | 5.2 | 5.4 | 6.1 | 4.0 | 7.3 | 3.5 |
| Inter-assay at 1 ng $mL^{-1}$ (%RSD) | 6.7 | 7.5 | 10.5 | 13.2 | 11.8 | 8.6 |
| Inter-assay at 10 ng $mL^{-1}$ (%RSD) | 5.3 | 7.6 | 5.9 | 5.0 | 10.9 | 6.2 |
| Accuracy (%±SD, at 10 ng $mL^{-1}$) | 93 ± 4 | 109 ± 5 | 108 ± 5 | 106 ± 4 | 107 ± 7 | 113 ± 6 |
| Recovery (%, at 10 ng $mL^{-1}$) | 93 | 97 | 96 | 97 | 91 | 88 |

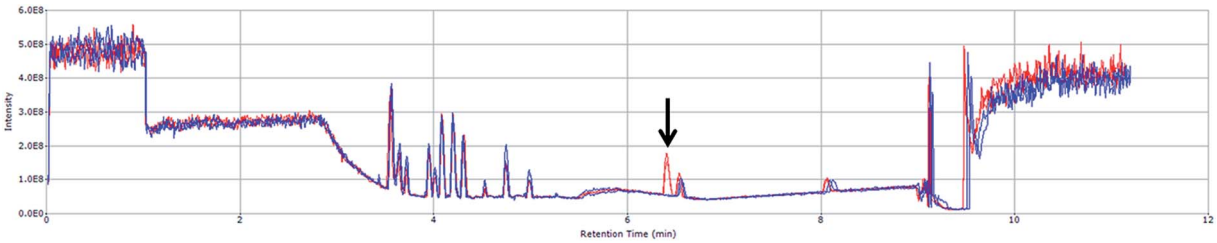



Fig. 2 SIEVE® total ion chromatogram (TIC) alignment showing the presence of 25B (annotated with ↓) spiked at 100 ng $mL^{-1}$ in equine urine by comparison to a blank equine urine sample.

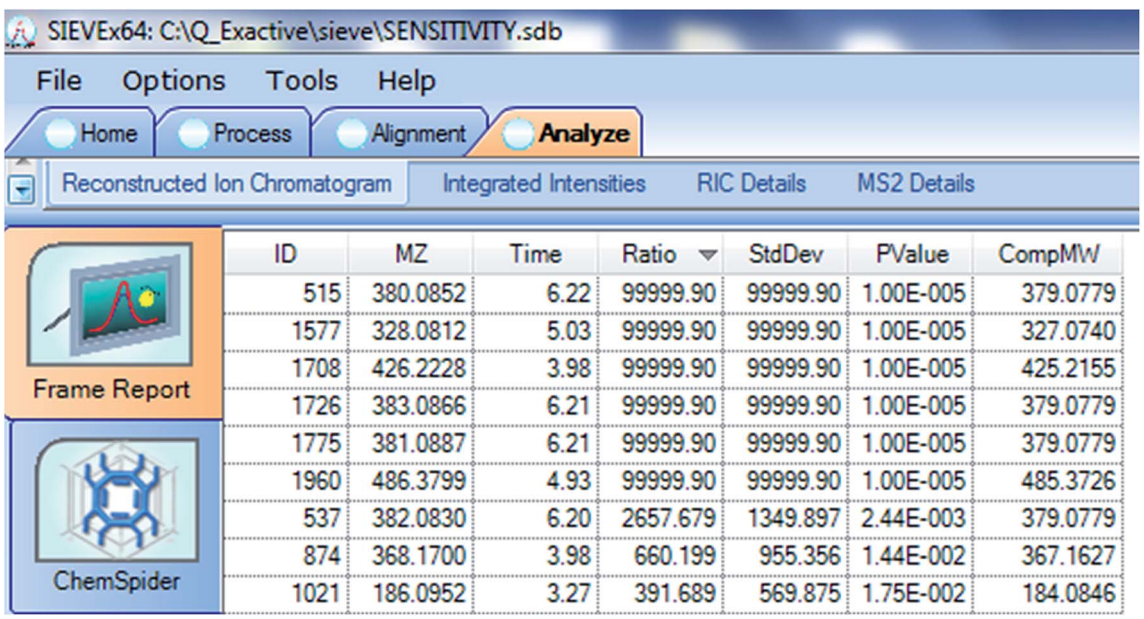


statistical evaluation, the data can be reviewed as volcano plots, scores plots from principal component analysis (PCA) or scatter plots to visualize correlation between *Ratio* and *p-value*.

For this study, compounds exhibiting a *Ratio* > 10 and *p-value* < 0.001 were deemed compounds of interest. Fig. 3 shows the frame report for 25B spiked at 5 ng $mL^{-1}$ with maximum *Ratio* values of 99 999.90 for *m/z* 380.0852 ($[M + H]^+$, $\Delta = 1.1$ ppm) and 2657.679 for *m/z* 382.0830 ($[M + H + 2]^+$, $\Delta = 1.3$ ppm), together with 99 999.90 for their $^{13}C$ isotope responses *m/z* 381.0887 and *m/z* 383.0866, respectively. The *p-value* associated with the maximum possible *Ratio* values was $1.00 \times 10^{-5}$, while that for *m/z* 382.0830 with a lower yet clearly high *Ratio* value was $2.44 \times 10^{-3}$. Interestingly, these *m/z* values only differ by approximately 1 Da yet they are all associated with different frames. For example *m/z* 380.0852, 381.0887, 382.0830 and 383.0866 are associated with frames 515, 1775, 537 and 1726 and respectively.

**Identification.** Comparison of retention times for responses corresponding to an isotope distribution that record high *Ratio* values (>10) and low *p-values* (<$1.00 \times 10^{-3}$) enabled a presumptive finding to be made. Fig. 3 shows the retention time range (6.20 to 6.22 min) for the four responses attributable to 25B at 5 ng $mL^{-1}$ in equine urine. The component molecular weight (*CompMW*) for these responses at 379.0779 Da was input to the elemental composition table of the Xcalibur® operating software for generation of chemical formula using defined parameters. The elements selected were C, H, N, O, F, Cl, Br, I and S with a mass tolerance of ±10 ppm and double-bond equivalents (DBE) range of 4.0 to 10.0. The second of ten results (in order of increasing $\Delta$) was $C_{18}H_{22}NO_3Br$ with $\Delta = 0.4$ ppm and DBE = 8.0. This could be selected as the correct candidate based on two assessments; known DBE for NBOMe compounds of 8.0 due to the presence of two benzene rings and the simulated spectral comparison feature of Xcalibur®. The latter provides a visual comparison of experimental and theoretical isotope patterns for the given chemical formula as shown by Fig. 4 for the example of 25B at 5 ng $mL^{-1}$ in equine urine. Accuracy in the provision of *CompMW* of 25B (379.0779 Da) from SIEVE® was assessed by $\Delta$ to be −0.3 ppm.

With the molecular weight and chemical formula proposed for an 'unknown' compound using SIEVE® and Xcalibur®, respectively, the identification process can proceed using $MS^2$ studies. For this purpose Mass Frontier™ spectral interpretation software was used to interrogate targeted $MS^2$ data




| ID | MZ | Time | Ratio | StdDev | PValue | CompMW |
|---|---|---|---|---|---|---|
| 515 | 380.0852 | 6.22 | 99999.90 | 99999.90 | 1.00E-005 | 379.0779 |
| 1577 | 328.0812 | 5.03 | 99999.90 | 99999.90 | 1.00E-005 | 327.0740 |
| 1708 | 426.2228 | 3.98 | 99999.90 | 99999.90 | 1.00E-005 | 425.2155 |
| 1726 | 383.0866 | 6.21 | 99999.90 | 99999.90 | 1.00E-005 | 379.0779 |
| 1775 | 381.0887 | 6.21 | 99999.90 | 99999.90 | 1.00E-005 | 379.0779 |
| 1960 | 486.3799 | 4.93 | 99999.90 | 99999.90 | 1.00E-005 | 485.3726 |
| 537 | 382.0830 | 6.20 | 2657.679 | 1349.897 | 2.44E-003 | 379.0779 |
| 874 | 368.1700 | 3.98 | 660.199 | 955.356 | 1.44E-002 | 367.1627 |
| 1021 | 186.0952 | 3.27 | 391.689 | 569.875 | 1.75E-002 | 184.0846 |

Fig. 3 SIEVE® frame report for 25B spiked at 5 ng $mL^{-1}$ in equine urine.

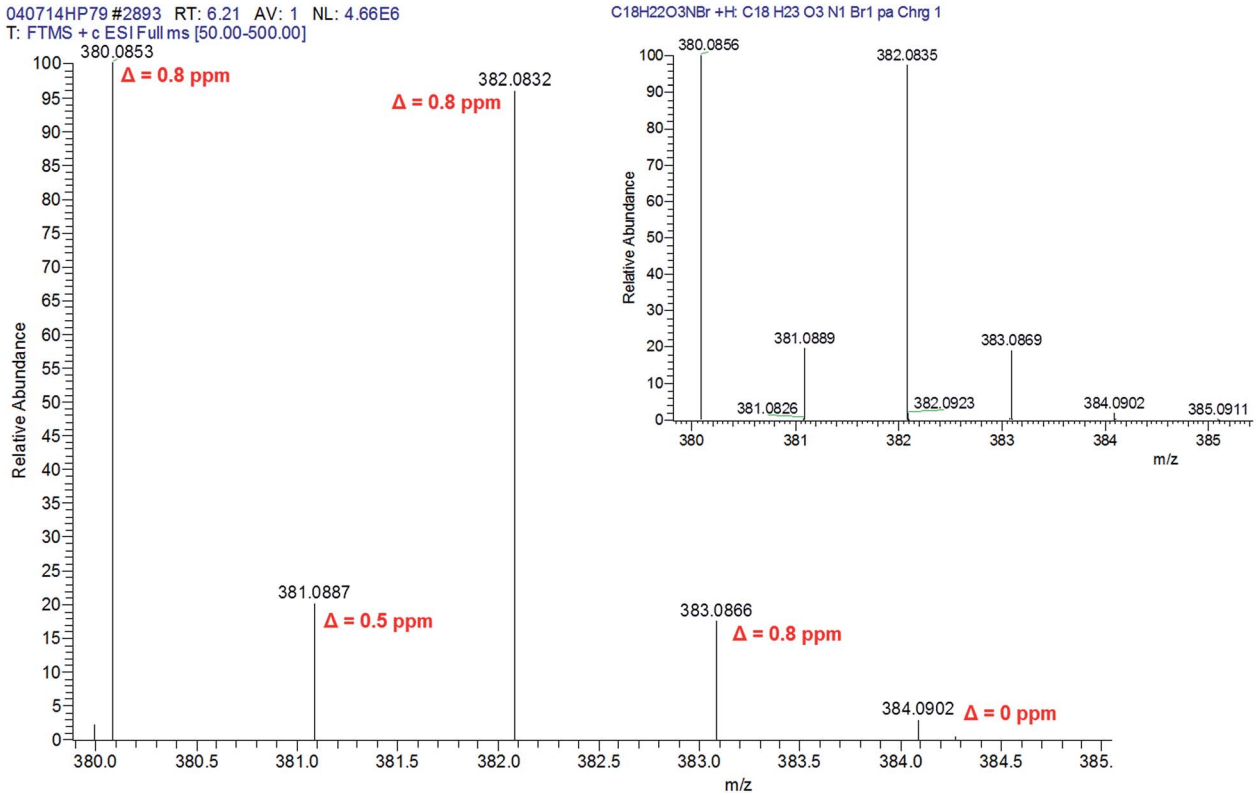



Fig. 4 Xcalibur® simulated isotope pattern for 25B spiked at 5 ng $mL^{-1}$ in equine urine (with annotated $\Delta$) and theoretical pattern for $C_{18}H_{22}O_3NBr$ (right inset).

uploaded from Xcalibur®. Mass Frontier™ software can predict product-ion structures and provide dissociation pathways for user-defined compounds. Precursor ion fingerprinting was investigated as a means for using structurally-related product ions to identify the precursor.[29] $MS^2$ studies of the six candidate NBOMe compounds revealed a common dissociation pathway resulting in the cleavage of benzyl moiety to produce *m/z* 121.0651 (±1.2 ppm) and detection of the tropylium ion (*m/z* 91.0548 (±1.8 ppm)). These diagnostic product ions can be used to confirm the NBOMe class of compounds following investigation of screening abnormalities produced from differential analysis.

**Proposed workflow.** The results for 25B were used to propose a workflow (Fig. 5) that could be implemented by forensic laboratories using differential analysis to perform non-targeted screening. This was successfully applied to the detection of 25C (10 ng $mL^{-1}$), 25D (5 ng $mL^{-1}$), 25E (10 ng $mL^{-1}$), 25H (5 ng $mL^{-1}$) and 25I (0.5 ng $mL^{-1}$) in equine urine. The proposed workflow is composed of four sections that are separated based on the software required; SIEVE®, Xcalibur®, Mass Frontier™ and database searching. At the conclusion of differential analysis by SIEVE®, the frame results can be filtered by *Ratio* (or *p-value*) and components that exhibit a *Ratio* > 10 and *p-value* < 0.001 can be selected for further interrogation. Those that do not meet these criteria can be considered as matrix contributions. Since the majority of NBOMe compounds have one nitrogen atom (*i.e.* an odd number) it would result in an even *m/z* value for the precursor ion due to the addition of a proton from the electrospray ionization process. Components that have an odd *m/z* value can also be included for interrogation since analogues containing an even number of nitrogen atoms are possible, however, odd *m/z* values can imply that the molecule contains no nitrogen atoms and would result in a larger data set for evaluation. Following differential analysis, the molecular formulae for selected components can be generated in Xcalibur® using the elemental composition tool by including C, H, N, O, F, Cl, Br, I, S as elements of interest. If the formula generated for a particular component contains a single nitrogen with three oxygen atoms and a DBE = 8 it can be subjected to $MS^2$ studies. The mass error difference should be within ±10 ppm, however, this value can be modified as required by the user. The $MS^2$ data of selected components can be evaluated for the presence of only two major product ions, specifically *m/z* 121.0648 and 91.0542. The added substituent can be determined by comparing the mass difference of the target analyte and the least substituted analogue 25H–NBOMe. Once the structure has been tentatively identified the likelihood can be assessed by using structural correlation software such as Mass Frontier™ to compare the *in silico* and experimental fragmentation patterns. If they correlate, the data can then be compared to a database and if the database search yields no result the analyte of interest could be a novel NBOMe compound.

It should be considered that while the strategy outlined here is aimed explicitly at users of Thermo Fisher Scientific hardware-software configurations for screening of NBOMe compounds, the general workflow can be adapted by users of

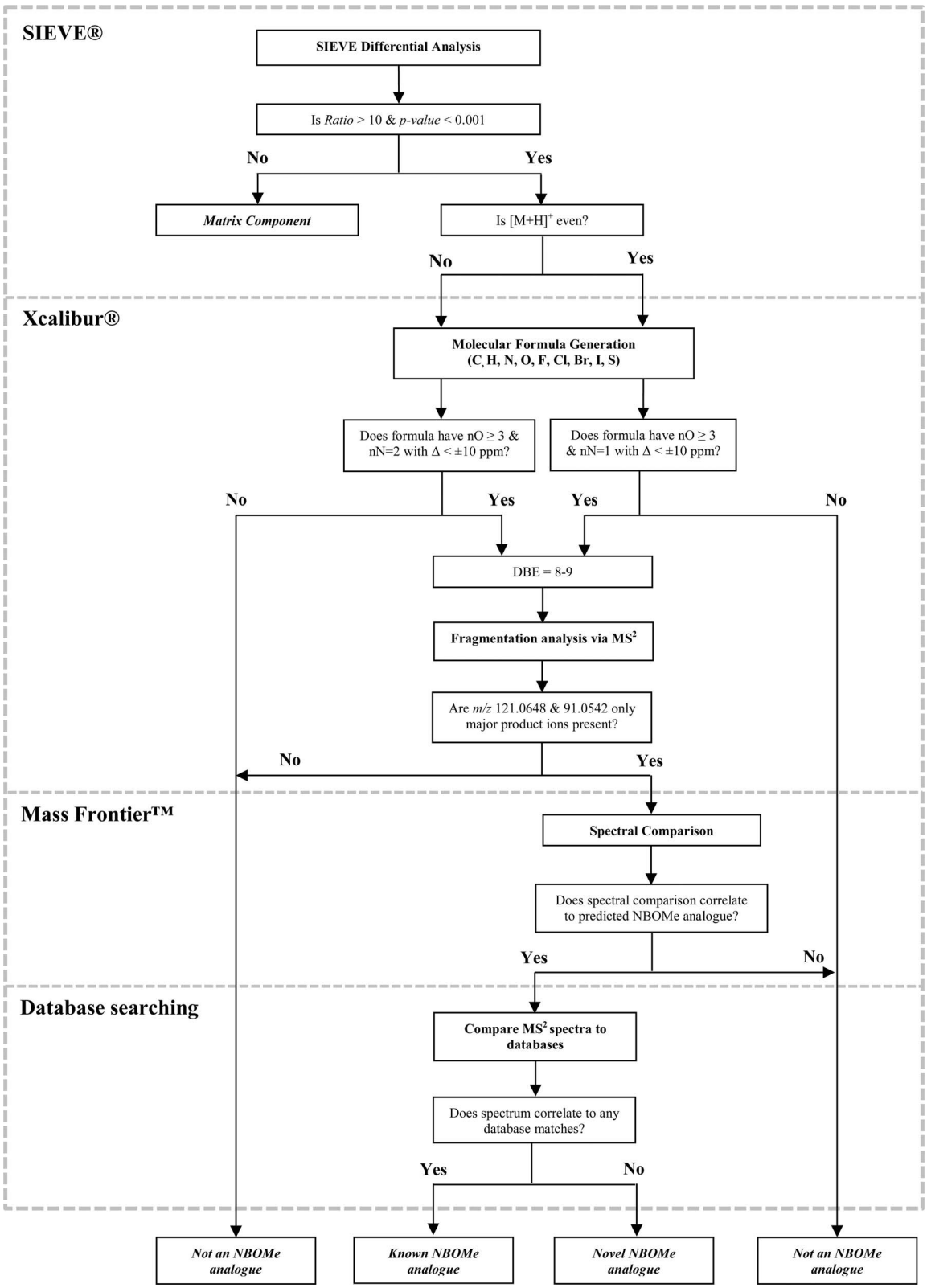



Fig. 5 Proposed workflow for non-targeted screening of NBOMe compounds in equine urine.

instruments manufactured by other vendors. For instance, SIEVE® is not the only differential analysis software package available to LC-HRAM practitioners; Mass Profiler Professional developed by Agilent Technologies (Santa Clara, CA, USA) and MetaboLynx™ developed by Waters Corp. (Milford, MA, USA) are also available.

**Verification.** The proposed workflow was subsequently verified by two blind control tests, each performed by a separate analyst with the knowledge that a NBOMe compound was spiked into equine urine at 100 ng $mL^{-1}$ but not which particular compound it was.

*Test case 1.* In conjunction with the tabulated frame report, a scatter plot of *Ratio versus p-value* is provided by SIEVE® for rapid visual inspection of differential analysis results. Selection of an outlier with a *Ratio* of 416.063 and *p-value* of $1.16 \times 10^{-2}$ had a corresponding *m/z* entry with 347.1596 at a retention time of 6.07 min. In addition, a $^{13}C$ isotopic response with a *Ratio* of 15.252 and *p-value* $1.01 \times 10^{-2}$ was found with *m/z* 348.1631 at the same retention time and *CompMW* of 346.1524 Da. Simulated spectral comparison provided good agreement ($\Delta = 1.4$ ppm and $\Delta = 1.1$ ppm) between the two ions detected by SIEVE® and the theoretical *m/z*, respectively. While the *p-value* for both ions was relatively high (*i.e.* $>1 \times 10^{-3}$), the retention time alignment of these responses with high *Ratio* values was considered sufficient evidence for entry into Xcalibur® to determine the chemical formula as $C_{18}H_{22}N_2O_5$ with $\Delta = 0.2$ ppm and DBE = 9.0. Mass Frontier™ analysis of $MS^2$ data revealed *m/z* 91.0545 and *m/z* 121.0646 belonging to the diagnostic fragments of an NBOMe compound. The above information was used to identify the unknown compound as the nitro-NBOMe compound, 25N with comparison to literature data reported by Casale and Hays.[2] The DBE value found in this case was 9.0 instead of 8.0 found for other NBOMe compounds, thereby providing a characteristic feature of the $NO_2$-analogue.

*Test case 2.* A second analyst selected an outlier with a *Ratio* of 194.980 and *p-value* of $6.38 \times 10^{-3}$ that had a corresponding *m/z* entry with 363.1814 at a retention time of 6.64 min. A second entry at 6.64 min with *m/z* 362.1779 had a *Ratio* of 86.715 and *p-value* of $9.43 \times 10^{-4}$ with the *CompMW* for both entries at 361.1706 Da. Xcalibur® determined the chemical formula to be $C_{20}H_{27}NO_3S$ with $\Delta = -0.04$ ppm and DBE = 8.0. The simulated spectral comparison revealed excellent agreement for *m/z* 362.1781 (100%, $\Delta = 0.8$ ppm), *m/z* 363.1816 (20% rel., $\Delta = 0.6$ ppm), *m/z* 364.1736 (5% rel., $\Delta = 1.6$ ppm) and *m/z* 365.1771 (1% rel., $\Delta = 1.4$ ppm). Precursor ion fingerprinting of $MS^2$ data confirmed the NBOMe class before web searching of the chemical formula provided the putative identification to be 25T2.[30,31]

Illicit administration of NPS such as NBOMe compounds to racehorses is highly possible in response to surveillance of conventional amphetamine-type substances by laboratories. This requires accredited racing laboratories to implement testing methods for substances that become readily available on the black market in order to deter and prosecute licensed persons who are tempted to administer non-approved drugs without knowledge of adverse health effects to an animal. Since 2013, the Australian Racing Forensic Laboratory (ARFL) has no reported findings for NBOMe compounds in equine urine.

The challenge of screening for an ever-increasing number of target compounds, many of which may be 'unknown' to a laboratory or for which no reference material is available, necessitates the use of hardware-software technology combinations that can assist in identifying abnormal responses in a biological sample for further investigation. To this end, the advantages of HRAM instrumentation in racing laboratories can be further enhanced by incorporating differential analysis software such as SIEVE® investigated in this study.

The individual components of the SIEVE® software workflow were assessed to identify critical control points that could be optimized for a proposed non-targeted analysis workflow in forensic laboratories. The importance of mass accuracy for confidence in determining unknowns should not be understated. Following the assessment of this parameter to document specificity in method validation, successful differential analysis was shown by this study to be dependent on robustness of chromatographic alignment and optimization of the *Peak Intensity Threshold* to achieve the required sensitivity. Recursive-base-peak-framing provides retention time, *m/z*, *Ratio*, *p-value* and *CompMW* that can be reviewed to determine an abnormal finding. While the processing time (approximately 1 min) for differential analysis per sample is reasonable, further work is required to enable batch processing of multiple sample files for more efficient translation of this workflow to routine testing. Regardless, the proposed workflow was used by the ARFL in conjunction with routine targeted analysis of 95 equine urine samples over a three-week period during a major racing carnival. No abnormalities requiring further investigation have yet been reported, however work continues to increase the coverage of samples analyzed by this method.

The *CompMW* can be used by the instrument operating software (Xcalibur®) to provide a putative chemical formula within defined parameters for elemental composition, which is critical to ensure sufficient coverage of NPS that may include halogen and sulfur substituents. In addition, DBE was found to be a useful discriminator of NPS classes, with 8 or 9 (in the case of 25N) being characteristic of NBOMe compounds. Subsequent $MS^2$ data was interrogated using Mass Frontier™ to enable precursor ion fingerprinting where diagnostic product ions could identify the NBOMe present. The major limitation of this study was the reliance on conventional literature sources to facilitate the final stage of compound identification from $MS^2$ data. Further work is required to investigate the possibility that *pseudo*-$MS^3$ (using in-source collision-induced dissociation, CID) studies of small molecules such as NBOMe compounds could provide the means for *in silico* structural elucidation. This would allow collaborative approaches such as *mzCloud*,[32] a $MS^n$ spectral database, to be incorporated into the workflow.

An additional limitation is the focus on parent NBOMe compounds without taking into account the likely presence of metabolites following NBOMe administration as recently reported for 25I by Caspar *et al.*[14] Metabolism of NBOMe and other NPS compounds will undoubtedly be important to consider as the research into non-targeted screening strategies continues, however the focus of this study was to use parent NBOMe compounds as model analytes for the development of non-targeted screening strategies.

Consideration must also be given to the presence of isobaric compounds such as structural isomers that are not resolved using HRAM technology. Investigation and subsequent

confirmation of such compounds will require greater LC selectivity than the generic screen presented here.

Notwithstanding the limitations of the method, putative identification of a NBOMe compound using the presented screening strategy can enable an abnormality to be recorded against a sample in order for further investigation to take place. Subsequent confirmation would require the procurement of an authentic reference material but this should not preclude the method from being useful to circumvent the nexus that anti-doping laboratories find themselves in terms of targeted analysis.

## Conclusion

The use of LC-HRAM spectrometry has demonstrated the capability for laboratories to combat the misuse of NPS in horseracing. Specific to this study, the presented method for the detection and quantification of NBOMe compounds in equine urine was validated. In addition, proof-of-concept use of differential analysis with SIEVE® was performed for non-targeted screening to putatively identify two NBOMe compounds, 25N and 25T2 with scope to extend this study to $MS^n$ data for *in silico* elucidation of novel compounds.

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