Application Note

Forensics

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Analysis of Polar and Ionic Drugs in Doping Control by Ion-Exchange Chromatography with the Agilent 1260 Infinity II SFC System

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Abstract

The objective of this application note is to demonstrate the use of the Agilent 1260 Infinity II SFC System for the separation of highly polar and even ionic compounds. The workflow involved using ion-exchange chromatography columns in combination with supercritical fluid chromatography (SFC). Under SFC conditions, unlike HPLC, there is no flowthrough behavior of very polar compounds. The clear separation achieved under SFC conditions allows reliable quantification. Systematic chromatographic method development is described, including stationary phase, modifier, and additive selection. Trends in retention and peak quality were investigated by applying different gradients, column temperatures, and SFC backpressures. As an example of practical relevance, compounds and their metabolites, which are important in doping control analysis, were taken to demonstrate the performance of the developed SFC/MS triple quadrupole method. The precision of peak area and retention time allowed reliable quantification below the required levels, and the limits of quantification and detection were comparable to typical HPLC/MS methods. A basic validation in human urine, showing recoveries, is also included.

Introduction

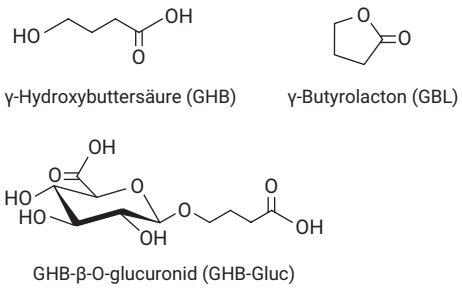
Although UHPLC/MS/MS and GC/MS/MS are the standard trace-analysis techniques applied in doping control, both have their limitations when it comes to the analysis of very polar or even ionic compounds. SFC has a large potential to overcome these problems, and additionally, it is a technique orthogonal to HPLC separations. SFC allows the separation of very polar and ionic compounds by the available variety of solid phases, modifiers, and additives in the trace analysis of residues, doping control, and forensics.[1-3]

Ethyl sulfate (ETS), ethyl-β-D-glucuronide (ETG), γ-butyrolactone (GBL), γ-hydroxybutyric acid (GHB), GHB-β-O-glucuronide (GHB-Gluc), meldonium (Meld), and γ-butyrobetaine (G-BTB) occur in various classes of drugs and metabolites important in doping control and forensics[4] (Figure1).

ETS and ETG are phase-II conjugates from ethanol representing markers of alcohol ingestion. The endogenous metabolite GHB gained public awareness as "liquid ecstasy".[5] GBL is a precursor of GHB and used as an industrial solvent. The phase-II metabolite of GHB, GHB-Gluc, might be a possible biomarker to complement its detection.[6] The zwitterionic Meld is a clinically used cardioprotective drug with reported misuses in sports. The endogenous metabolite G-BTB may give spectral interferences with Meld and, therefore, has been included in this study.

The objective of this application note was the development and optimization of an SFC method for the separation of the mentioned polar and ionic compounds including their determination by means of triple quadrupole MS. The complete study, including supporting material, has already been published in a scientific journal.[7]

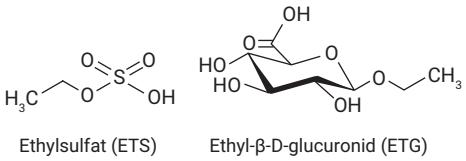
Neurotransmitter ("liquid ecstasy", anabolic effects)



γ-Hydroxybuttersäure (GHB) γ-Butyrolacton (GBL)

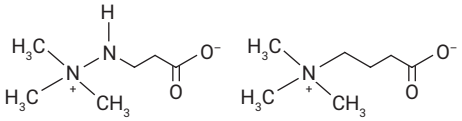
GHB-β-O-glucuronid (GHB-Gluc)

Marker for alcohol consumption



Ethylsulfat (ETS) Ethyl-β-D-glucuronid (ETG)

Metabolic modulator



Meldonium (Meld) γ-Butryobetain (G-BTB)

Figure 1. Structure formulae of the compounds used in this study.

Experimental

Instrumentation

The Agilent 1260 Infinity II SFC System comprised:

- Agilent 1260 Infinity II SFC Control Module (G4301A)
- Agilent 1260 Infinity II SFC Binary Pump (G4782A)
- Agilent 1260 Infinity II SFC Multisampler (G4767A)
- Agilent 1260 Infinity II Diode Array Detector (G7115A) with high-pressure SFC flow cell (G4301-60100)
- Agilent 1260 Infinity II Multicolumn Thermostat (MCT) (G7116A)
- Agilent 6470A Triple Quadrupole LC/MS with Agilent Jet Stream technology

Software

Agilent MassHunter software V. B.07 for data acquisition, qualitative and quantitative data analysis, MRM Optimizer, and source optimizer

Columns

The following stationary phases were tested for method development, typically as a 4.6 × 150 mm, 5 µm column: 2-ethylpyridine, cyano, 1,2-dihydroxypropyl ether, sulfonic acid, aminopropylsilane, silica, zwitterionic HILIC, and amide.

Finally, a silica-based SCX column with sulfonic acid modification mixed with phenyl groups was used (4.6 × 150 mm, 5 µm).

Mobile phases

Initially, nine solvents were used as a modifier for method development. All were based on methanol-containing buffers such as ammonium formate and ammonium acetate in different concentrations combined with different amounts of additional water.

Finally, MeOH/H_2O (95/5, v/v) with additive (ammonium formate (20 mM) + formic acid (15 mM)) was chosen.

Reference standards

All standard stock solutions were prepared in methanol and stored at –20 °C. For chromatographic method development, 10 µg/mL each in methanol was used.

Calibration

Calibrations were created for neat standards and in matrix (urine). The calibration range of 0.5 to 100 ng/mL (1:10 dilution, corresponding to 0.005 to 1.0 µg/mL in undiluted urine) was selected for GBL, ETS, Meld, and G-BTB. For GHB and ETG, a calibration range of 0.05 to 10 µg/mL (1:10 dilution, corresponding to 0.5 to 100 µg/mL in undiluted urine) was selected. GHB-Gluc was calibrated using a calibration range of 0.5 to 50 µg/mL (1:10 dilution, corresponding to 5 to 500 µg/mL in undiluted urine).

Sample preparation

The urine sample preparation included protein precipitation with MeOH at a dilution of 1:2. After centrifugation, the supernatant was diluted with MeOH (1:5, v/v), resulting in a final dilution of 1:10.

Solvents and chemicals

- GHB (D6 sodium, used due to regulatory issues in Germany) and G-BTB were purchased from Sigma-Aldrich GmbH (Munich, Germany).
- ETS and ETG were supplied by Lipomed GmbH (Weil am Rhein, Germany).
- GHB-Gluc was purchased from ResearChem GmbH (Burghof, Switzerland).
- GBL (methanolic solution) was obtained from the Institute for Forensic Medicine, Department for Forensic Toxicology at Wolfgang-Goethe-University (Frankfurt, Germany).
- Meldonium was obtained from Sigma-Aldrich (Taufkirchen, Germany).
- Other chemicals and solvents were of LC/MS grade and purchased from Merck (Darmstadt, Germany).

Final SFC method

Parameter	Value
CO_2-Modifier	MeOH/H_2O (95/5, v/v), additive: ammonium formate (20 mM) + formic acid (15 mM)
Flow Rate	2 mL/min
Gradient	15% modifier for 1 min, 60% modifier in 1 min, with a flow rate change from 2.0 to 2.5 mL/min, hold for 3 min Post-time: 2 min at 15% modifier and 2.0 mL/min
Backpressure Regulator	170 bar, 60 °C
Column Temperature	45 °C
Feed Solvent For Injection	2-Propanol
Feed Speed	400 µL/min
Overfeed Volume	1 µL
Injection volume	2 µL

MS method

For the optimization of MRM transitions, the Agilent MRM Optimizer was used, and for the optimization of the source parameters, the Agilent Source Optimizer software was applied.

Parameter	Value
SFC was splitless coupled to MS; ionization was possible due to the higher amount of modifier and additives	
Polarity	ESI positive and negative
Capillary Voltages	5,500 V (ESI–), 2,500 V (ESI+)
Nozzle Voltages	0 V (ESI–), 2,000 V (ESI +)
Drying Gas Temperature	250 °C
Drying Gas Flow	12 L/min
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Nebulizer Pressure	15 psi
MRM Settings	see Table 1

Table 1. MRM settings (parent ions, fragmentor voltage, fragment ions, collision energy, and polarity) and retention times of analytes used in this study.

Analyte	Parent Ion	RT (min)	Fragmentor (V)	Product Ion Quantifier (CE (V))	Product Ion Qualifier (CE (V))	ESI Polarity
γ-Butyrolactone (GBL)	86.1	1.25	60	42.5 (16)	43.1 (10)	+
γ-Hydroxybutyric acid (GHB)	104.1	1.47	60	61.2 (14)	90.1 (8)	–
Ethyl sulfate (ETS)	126.1	1.85	60	96.9 (16)	79.9 (38)	–
Ethyl-β-D-glucuronide (ETG)	222.2	2.31	80	74.8 (12)	85.1 (16)	–
GHB-β-O-glucuronide (GHB-Gluc)	279.1	2.64	90	113.0 (20)	103.0 (18)	–
Meldonium (Meld)	146.2	3.07	90	42.2 (50)	58.1 (32)	+
γ-Butyrobetaine (G-BTB)	145.2	3.40	90	45.1 (34)	87.1 (14)	+

Results and discussion

Chromatographic method development

To develop the chromatographic method, 12 different stationary phases were screened with nine different modifier/additive combinations by a generic gradient (5 to 50% modifier in 10 minutes). The obtained chromatograms were evaluated in terms of peak shape, retention behavior, and resolution. The most promising stationary phase for the separation was a strong cation exchange material in combination with a methanol/water modifier. With increasing amount of water, the compounds tended to coelute, while less water led to compromised peak shapes. Therefore, the effect of the amount of water and the concentration of additives on retention time and peak shape were evaluated in the next step. The use of more than 5% of water resulted in coelution and peak broadening for G-BTB, Meld, GHB-Gluc, ETS, and ETG. The retention times of G-BTB and Meld shifted from the region between 8 to 10 minutes to the region between 4.5 and 6.5 minutes. The retention time of GHB-Gluc, ETS, and ETG increased from the region between 3 and 4 minutes to 4.5 and 5 minutes. This caused significant coelution of the mentioned compounds with 10% water addition. The increase of the amount of additive ammonium formate resulted in only a minor decrease in retention time for most of the compounds (except GHB). There was nearly no effect on GBL. Due to the proposed ion exchange effect, the peak shapes were evaluated for increasing buffer concentration (Figure 2). Most of the analytes showed a better peak shape with higher additive concentration, especially ETG. Although GHB showed contrary behavior, 5% water in the methanol modifier and 20 mM ammonium formate as additive were kept for further optimization. To improve the ionization in the MS source, 15 mM formic acid was added, too.

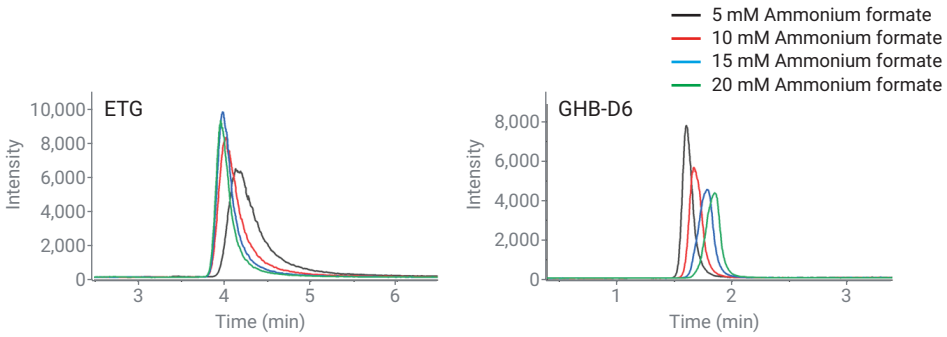


Figure 2. Evaluation of peak shape with increasing amount of additive (ammonium formate). The effects on GHB and ETG are shown as examples.

For optimization of the gradient, the separation started with 15% B followed by a steep increase to 60% B in one minute to elute the compounds earlier, sharpen their peaks, and reduce tailing. To increase the speed of analysis (shorter retention times), the flow rate was changed during the run from 2 to 2.5 mL/min (Figure 3).

Because of their influence on the density of the mobile phase, column temperature and system backpressure also have a significant impact on retention time and selectivity in SFC and therefore were optimized. Column temperatures were tested between 25 and 55 °C; backpressure settings were tested between 90 and 170 bar. The compounds most affected by a backpressure increase were GHB, GBL, and ETS; their retention times decreased from 1.8 to 1.5 minutes, from 1.5 to 1.3 minutes, and from 2.3 to 1.8 minutes, respectively. The increase in temperature moved the retention time of ETS slightly from 2.0 to 2.2 minutes. Finally, a backpressure of 170 bar and a column temperature of 45 °C were chosen for the final method.

Method performance characterization

For the characterization of the performance of the final method, selectivity, linearity, LOD, LOQ, area and retention time precision, carryover, and matrix effects were evaluated.

The final method showed good selectivity for the separation of all seven compounds in spiked urine (Figure 4). No interfering signals were observed for the MRM transitions of GHB, ETG, Meld, and GBL in blank urine. For ETS, there is a transition of *m/z* 125 to 80 (qualifier) near the analyte but the second transition and retention time allow a clear differentiation. For GHB-Gluc, there is a very low matrix signal for transition of *m/z* 279 to 103, which was considered irrelevant. G-BTB was clearly separated from an interference.

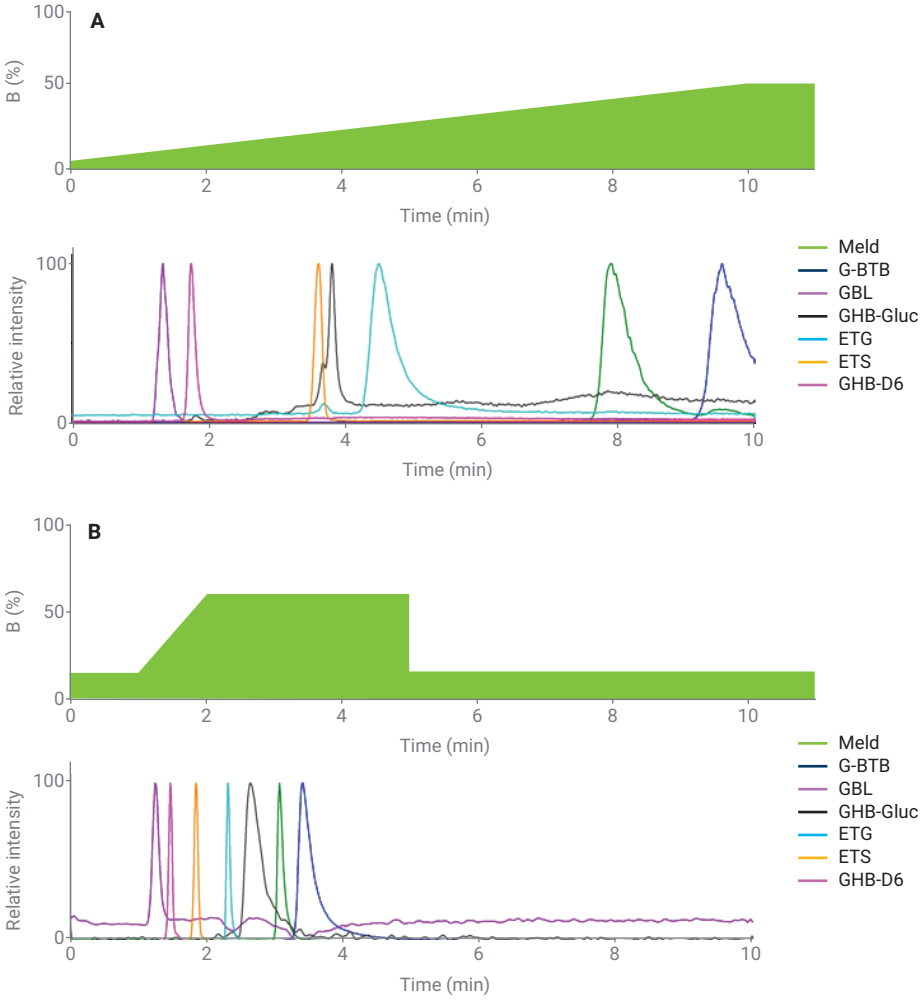


Figure 3. Optimization of gradient. A) Initial gradient from 5% B to 50% B in 10 minutes at a flow rate of 2.0 mL/min. B) Optimized gradient start from 15% B to 60% B in one minute and a change of flow rate from 2.0 to 2.5 mL/min (see Experimental section).

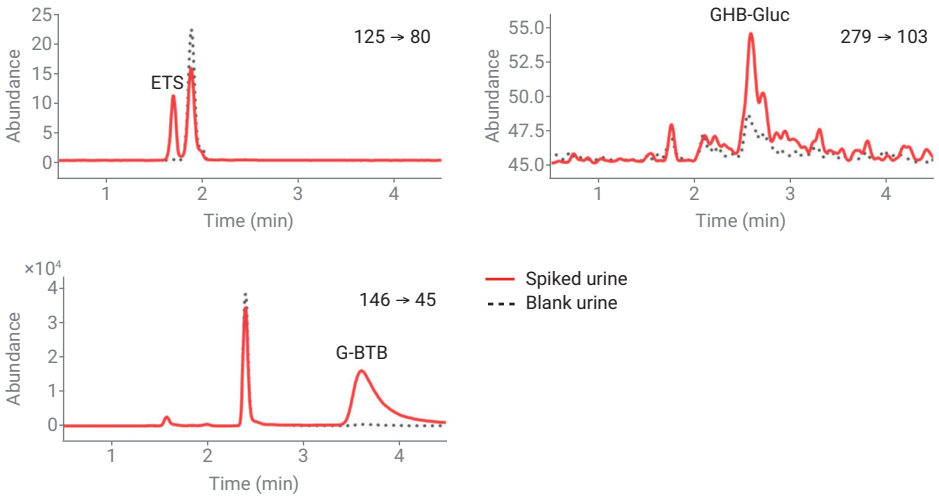


Figure 4. Chromatograms of selected MRM transients with spiked-urine matrix for G-BTB, GHB-Gluc, ETS, and blank matrix.

The limit of detection and limit of quantification were calculated based on signal-to-noise ratios. The lowest levels were achieved for Meld and ETS at LOD = 0.001 mg/L and LOQ = 0.005 mg/L. All other values, together with the achieved linearity, are presented in Table 2. The precision was measured for individual peak parameters. The peak area precisions, CV_{area}%, were between 2.1 and 13.4%. The retention time precision, CV_{RT}%, was below 1% and confirmed the retention time stability.

The matrix effect, which is the influence on ionization of a compound by coeluting matrix compounds, was calculated as matrix factor (MF). This could be either an ion suppression or an enhancement resulting in a quantification error. The MFs were calculated by a comparison of a calibration from neat standard and matrix-matched calibration. Ion enhancement occurred for G-BTB and ETS; ion suppression occurred for GHB-GLUC and ETG.

Proof of concept

For the final evaluation of the method, the obtained data were compared to existing cut-off values and minimum required performance levels (MRPLs). Table 3 shows a comparison of obtained LOD of the developed SFC/MS/MS method with existing HPLC/MS/MS methods.

In case of ETG, there is more than one applied cut-off limit. This depends on the condition of the test person: for instance, in the case of abstinence testing, a urinary cut-off concentration of 0.1 mg/L is used. In doping control, a reporting limit of 5 mg/L for ETG is applied, while 0.5 mg/L for ETS is used.[10] For the differentiation of naturally occurring GHB and an administered drug, 10 mg/L is used as a cut-off limit.[11] According to the achieved LOQ, the SFC/MS/MS method could be used for the measurement of GHB and GBL in urine. The LOQ for Meld[12] is also below the minimum required performance level, and therefore the developed SFC/MS/MS method may be used for screening in doping control.

Table 2. Characterization of the performance of the final method by LOD, LOQ, linearity, precision of area, retention time, and matrix factor (MF).

Compound	LOD (mg/L)	LOQ (mg/L)	Calibration Range (mg/L)	Correlation Coefficient R^2 (n = 5)	Precision Area CV (n = 10)	Precision RT CV (n = 10)	MF
GBL	0.005	0.025	0.005 to 1.0	0.981	2.1	0.89	1.04
GHB	0.5	2.5	0.5 to 100	0.996	13.4	0.85	1.09
ETS	0.001	0.005	0.005 to 1	0.991	5.7	0.33	1.57
ETG	0.1	0.5	0.5 to 100	0.972	9.3	0.52	0.30
GHB-Gluc	0.1	0.5	5 to 500	0.988	12	0.72	0.80
Meld	0.001	0.005	0.005 to 1	0.989	3	0.33	1.01

Table 3. Comparison of LOQs between the developed SFC/MS/MS method and existing HPLC/MS/MS methods.

Compound	Cut-Off Limit (mg/L)	LOQ (mg/L) SFC/MS/MS	LOQ (mg/L) HPLC/MS/MS
GBL	10	0.025	1.0
GHB	10	2.5	0.5
ETS	0.1	0.005	0.005[8]
ETG	0.1/0.5/5.0	0.5	0.01[8]
GHB-Gluc	–	0.5	0.5
Meld	0.1	0.005	0.05[9]

Conclusion

This application note demonstrates the use of the Agilent 1260 Infinity II SFC in combination with a strong cation exchange column for the separation for very polar and ionic compounds like drugs and metabolites by ion exchange with a polar modifier and additives. The advantage of using SFC is, in contrast to HPLC methods, that the polar compounds are retained and clearly separated for reliable quantification. The obtained method showed performance data comparable to already established HPLC methods. The tandem mass-spectrometric detection showed detection limits that are suitable to consider the suggested approach as an alternative separation and detection technique for very polar and ionic compounds in comparison to HPLC/MS/MS techniques.

References

1. Par, M. K. *et al.* SFC/MS as an Orthogonal Technique for Improved Screening of Polar Analytes in Anti-Doping Control. *Anal. Bioanal. Chem.* **2016**, *408*, 6789–6797.
2. Desfontaine, V. *et al.* SFC–MS Versus RPLC-MS for Drug Analysis in Biological Samples. *Bioanalysis* **2015**, *7*, 1193–1195.
3. Fujito, Y. *et al.* Importance of Optimizing Chromatographic Conditions and Mass Spectrometric Parameters for Supercritical Fluid Chromatography/Mass Spectrometry. *J. Chromatogr. A* **2017**, *1508*, 138–147.
4. Novakova, L. *et al.* Ultra High Performance Supercritical Fluid Chromatography Coupled with Tandem Mass Spectrometry for Screening of Doping Agents. II: Analysis of Biological Samples. *Anla. Chim. Acta* **2015**, *853*, 647–659.
5. Abanades, S. *et al.* Gamma-Hydroxybutyrate (GHB) in Humans: Pharmacodynamics and Pharmacokinetics. *Ann. N Y Acad. Sci.* **2006**, *1074*, 559–576.
6. Mehling, L.-M. *et al.* GHB-O-β-Glucuronide in Blood and Urine is not a Suitable Tool for the Extension of Detection Window after GHB Intake. *Forensic Toxicol*. **2017**, *35*, 263–274.
7. Xhaferaj, M. *et al.* Ion Exchange in Supercritical Fluid Chromatography Tandem Mass Spectrometry (SFC-MS/MS): Application of Polar and Ionic Drugs and Metabolites in Forensic and Anti-Doping Control. *J. Chromatogr. A* **2020**, *1614*, 460726.
8. Albertmann, M. *et al.* A High-Performance Liquid Chromatographic-Tandem Mass Spectrometric Method for the Determination of Ethyl Glucuronide and Ethyl Sulfate in Urine Validated According to Forensic Guidelines. *J. Chromatogr. Sci.* **2012**, *50*, 51–56.
9. Kim, Y. *et al.* Method for Screening and Confirming Meldonium in Human Urine by High-Resolution Mass Spectrometry and Identification of Endogenous Interferences for Anti-Doping Testing. *Mass Spectrom. Lett.* **2017**, *8*, 39–43.
10. World Anti -Doping Agency, WADA technical document TD2016EAAS, World Anti-Doping Agency, **2015**.
11. Brailsford, A. D. *et al.* Pharmacokinetic Properties of Gamma-Hydroixybutyrate (GHB) in Whole Blood, Serum and Urine. *J. Anal. Toxicol.* **2012**, *36*, 88–95.
12. Minimum Required Performance Levels for Detection and Identification of Non-Threshold Substances – TD2018MRPL, in: Proceedings of the World Anti Doping Agency, **2018**.

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