

WADA Technical Document – TD2015IDCR

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Written by:	WADA Laboratory Expert Group	Approved by:	WADA Executive Committee
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MINIMUM CRITERIA FOR CHROMATOGRAPHIC-MASS SPECTROMETRIC CONFIRMATION OF THE IDENTITY OF ANALYTES FOR DOPING CONTROL PURPOSES.

The ability of a method to identify an analyte is a function of the entire procedure: sample preparation, chromatographic separation, mass analysis and data assessment. Any description of the method for purposes of documentation should include all parts of the method. The appropriate analytical characteristics shall be documented for the entire identification method and should be sufficiently proven as being fit-for-purpose through proper method validation.

Laboratories shall follow the identification criteria described in this Technical Document in their analytical protocols, including the differentiation between isomers of the same substance (if this is required for the unequivocal identification of a *Prohibited Substance*).

1.0 Chromatographic Criteria

- The retention time (RT) of the analyte's chromatographic peak in the *Sample* shall not differ (ΔRT) by more than one (1) percent (%) or ± 0.1 minutes (whichever is greater¹, but not exceeding the full-width-at-half-maximum, FWHM), from that of the same analyte in a spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.
- Alternatively, the Laboratory may choose to use relative retention time (RRT) as an acceptance criterion, where the RT of the peak of interest is measured relative to a chromatographic reference compound (CRC).
 - If the CRC is not the stable isotope-labeled analyte, the RRT of the analyte in the *Sample* shall not differ by more than $\pm 1\%$ from that of the same analyte in a spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.
 - If the CRC is the stable isotope-labeled analyte, the RRT of the analyte in the *Sample* shall not differ by more than $\pm 0.5\%$ from that of the same analyte in a spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.

¹ The “whichever is greater” criterion may result in a RT difference (ΔRT) unrealistically large for narrow chromatographic peaks (e.g., in UHPLC with FWHM of 1s). Since any ΔRT greater than the FWHM is generally not considered a good match in RT, a maximum ΔRT is set at the FWHM of the reference peak in the spiked sample, Reference Collection sample, or Reference Material.

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2.0 Mass Spectrometric Identification Criteria

Commonly employed strategies for the identification of analytes, using mass spectrometry (MS)-based techniques are often referred to as “top-down” and “bottom-up” approaches.

- The “top-down” approach involves the MS analysis of the intact analyte through the generation of substance-specific ions.
- The “bottom-up” approach involves the measurement of enzymatically or chemically produced fragments of the analyte and the identification of any such fragments.

Although these terms have been particularly used for the analysis of large molecules (*e.g.* proteins), both approaches are valid and applicable to the MS-based identification of any analyte provided the information obtained complies with the criteria defined in this Technical Document.

The specificity of the information obtained to unequivocally identify the analyte shall be established as part of the method validation process (*e.g.* using Basic Local Alignment Search Tool, BLAST, for analysis of a given amino-acid sequence in conjunction with a suitable database such as, for example, UniProtKB) and is not part of this document.

MS criteria for identification by either scanning (*e.g.* Full Scan, Product Ion Scan) or non-scanning (*e.g.* Selected Ion Monitoring, Selected Reaction Monitoring) techniques are based on the presence and relative abundance of a number of ions which are defined by the Laboratory as diagnostic for the analyte. Any data processing (*e.g.* integration, subtraction, averaging, *etc.*) shall be performed consistently across the analytical batch.

The concentration of a *Prohibited Substance*, or its *Metabolite*, or its *Marker* may need to be comparable (*i.e.* signal of the analyte within one order of magnitude) in the *Sample* and the spiked urine, Reference Collection sample, or Reference Material to ensure identification of the target analyte.

The following identification criteria shall be applied:

- Each measured mass used for identification shall be within ± 0.5 Da of the corresponding mass of the same ion acquired from a spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.
- When using single-stage MS, at least three (3) diagnostic ions shall be acquired.
- When using multiple-stage MS (*e.g.* MS/MS), at least two (2) precursor-product ion transitions (*i.e.* two SRM transitions) shall be monitored. The isolation width of the precursor ion shall not be more than m/z 1.3, unless required by its molecular mass and charge state.

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- The abundance of diagnostic ions shall be determined from the peak area or height of integrated selected ion chromatograms. This is also applicable when scan mode only is used for identification.
- The signal-to-noise (S/N) ratio of all diagnostic ions shall be greater than three to one (3:1).
- Relative Abundances shall be calculated by dividing the area or height of the ion trace of each diagnostic ion by the area or height obtained from the ion trace of the most abundant diagnostic ion taken as the base peak ².
- The Relative Abundances of any of the diagnostic ions shall not differ by more than the amount specified in Table 1 from the corresponding Relative Abundances of the same ions acquired from a spiked positive control urine, Reference Collection sample, or Reference Material.
- If three (3) diagnostic ions or two (2) transitions are not available, a second derivative shall be prepared, or a second ionization or dissociation technique³ shall be used. The second ionization technique shall be based on a different physical principle, *i.e.* chemical ionization vs. electron ionization and again should provide different diagnostic ions. It is not acceptable to utilize a technique that changes only the relative abundance of the same ions.
- It is not permissible, without a valid explanation⁴, to collect more than the minimum required number of ions or transitions in SRM or SIM and select those with Relative Abundances within tolerance windows while ignoring other ions or transitions which would not meet the identification criteria.

² The base peak ion shall be the most abundant diagnostic ion acquired from a spiked positive control urine, Reference Collection sample, or Reference Material. This reference base peak ion shall also be applied to the calculation of the Relative Abundances from the *Sample* chromatogram, even if it does not constitute the base peak ion in the *Sample*.

³ A different dissociation technique might be, for example, using MS³ instead of MS² or using Electron Capture Dissociation (ECD) or Electron Transfer Dissociation (ETD) instead of Collision Induced Dissociation (CID).

⁴ A valid explanation might be, for example, clear evidence that one of the primary established ions is being interfered by a partially co-eluting substance.

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Table 1. Maximum Tolerance Windows for Relative Abundances to ensure appropriate confidence in identification

Relative Abundance in the reference specimen ⁵ (% of base peak)	Maximum Tolerance Windows for the Relative Abundance in the <i>Sample</i>	Examples	
		Relative Abundance (% of base peak)	Tolerance Window (% of base peak)
50 - 100	±10 (absolute)	60	50-70
		95	85-105
25 - 50	± 20% (relative)	40	32-48
1 - 25	±5 (absolute) ⁶	10	5-15
		3	>0 ⁶ – 8

⁵ Spiked sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.

⁶ The diagnostic ions must always be detected in the *Sample* (S/N > 3:1).

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3.0 Definitions

Diagnostic ion(s): Molecular ion or fragment ions whose presence and abundance are characteristic of the analyte and thereby may assist in its identification. A second ion belonging to the same isotopic cluster may also be used as diagnostic only when the peculiarity of the atomic composition of the fragment so justifies it (*e.g.* presence of Cl, Br, or other elements with abundant isotopic ions).

Relative Abundance: The abundance of a particular ion relative to the most abundant ion monitored.

Maximum Tolerance Window of Relative Abundances: The maximum permitted difference between the Relative Abundance of a particular ion obtained from the *Sample* and that obtained from the reference specimen. This may be expressed in ABSOLUTE or RELATIVE terms.

Absolute: Determined by adding/subtracting the stated tolerance value to/from the Relative Abundance obtained for the monitored ion in the reference specimen.

Relative: Determined by calculating the stated tolerance percentage of the Relative Abundance obtained for the monitored ion in the reference specimen and then adding/subtracting that value to/from the Relative Abundance.

Scan: Acquisition of ions of a continuous range of m/z values.

Selected Ion Monitoring (SIM): Acquisition of ions of one or more pre-determined discrete m/z values for specified dwell times.

Selected Reaction Monitoring (SRM): Data acquired from specific product ions corresponding to m/z selected precursor ions recorded via two or more stages of mass spectrometry. SRM can be performed as tandem mass spectrometry in time or tandem mass spectrometry in space.

Signal-to-Noise (S/N) Ratio: Magnitude of the instrument response to the analyte (signal) relative to the magnitude of the background (noise).

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