WADA Technical Document – TD2023IDCR

Document number:	TD2023IDCR	Version number:	1.0
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Date:	23 September 2022	Effective date:	01 January 2023

MINIMUM CRITERIA FOR CHROMATOGRAPHIC-MASS SPECTROMETRIC CONFIRMATION OF THE IDENTITY OF ANALYTES FOR DOPING CONTROL PURPOSES

1.0 Introduction

The ability of a Confirmation Procedure (CP) to identify an Analyte is a function of the entire Analytical Method: sample preparation, chromatographic separation, mass analysis and data assessment. Any description of the Analytical Method for purposes of documentation should include all parts of it. 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 [1].

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

The identification of an Analyte using chromatography coupled with mass spectrometry is based upon a comparison of the Retention Time (RT) and Relative Abundances (RAs) of the Diagnostic Ions of the Analyte detected in a *Sample* with those in a Reference Specimen analyzed in the same analytical batch. The most abundant Diagnostic Ion acquired from the Reference Specimen is the Reference Diagnostic Ion, which shall be applied to obtain the RT and to calculate the RAs of the Analyte's Diagnostic Ions.

2.0 Chromatographic Criteria

2.1. Retention Time (RT)

The 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 greater, but not exceeding the full-width-at-half-maximum, FWHM), from that of the same Analyte in a Reference Specimen analyzed in the same analytical batch.

> *Comment:*
> *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 Reference Specimen.*

2.2. Relative Retention Time (RRT)

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 ± 1% from that of the same Analyte in a Reference Specimen 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 ± 0.5% from that of the same Analyte in a Reference Specimen analyzed in the same analytical batch.

3.0 Mass Spectrometric Identification Criteria

3.1. Background

Commonly employed strategies for the identification of Analyte(s) 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 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.

3.2. Requirements

MS criteria for identification by either scanning (*e.g.,* Full Scan, Product Ion Scan) or non-scanning [*e.g.,* Selected Ion Monitoring (SIM), Selected Reaction Monitoring (SRM)] techniques are based on the presence and RA of a number of Diagnostic Ions which has been validated 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.

Depending on the Analytical Method, the concentration of the Analyte may need to be comparable (*i.e.,* signal of the Analyte within one order of magnitude) in the *Sample* and the Reference Specimen to ensure identification of the 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 Diagnostic Ion acquired from the Reference Specimen analyzed in the same analytical batch;
- When using single-stage MS, at least three (3) Diagnostic Ions shall be acquired;

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- When using multiple-stage MS (*e.g.,* MS/MS), at least two (2) Diagnostic Ions [*i.e.,* two precursor-product ion transitions (SRM transitions)] shall be acquired. 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;
- The Signal-to-Noise (S/N) ratio of all Diagnostic Ions shall be greater than (>) three to one (3:1);
- The abundance of Diagnostic Ions shall be determined from the peak area or height in the integrated selected ion chromatograms. This is also applicable when Scan technique only is used for identification;
- The most abundant Diagnostic Ion acquired from a Reference Specimen is the Reference Diagnostic Ion, which shall be applied to the calculation of the RAs. The same ion shall be applied as the Reference Diagnostic Ion from the *Sample* chromatogram, and shall be used to calculate the RAs of the other Diagnostic Ions, even if it is not the most abundant Diagnostic Ion in the *Sample* chromatogram;
- RAs 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 Reference Diagnostic Ion in the same chromatographic peak;
- The RAs of any of the Diagnostic Ions shall not differ by more than (≤) the amount specified in Table 1 from the corresponding RAs of the same ions acquired from the Reference Specimen;
- If the minimum number of Diagnostic Ions is not available (*i.e.,* less than three (3) Diagnostic Ions when using a single-stage MS or two (2) Diagnostic Ions when using multiple-stage MS), a second chemical derivative shall be prepared, or a second ionization or dissociation technique shall be used in addition to the first analysis. 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 RA of the same Diagnostic Ions (*e.g.,* changes of electron energy in electron ionization);

> *Comment:*
> *A different dissociation technique might be, for example, using MS^3 instead of MS^2 or using Electron Capture Dissociation (ECD) or Electron Transfer Dissociation (ETD) instead of Collision Induced Dissociation (CID).*

- It is not permissible to ignore any Diagnostic Ion acquired - without a valid explanation - which would not meet the identification criteria and select only those Diagnostic Ions with RAs within the Maximum Tolerance Windows for Relative Abundances (MTWRAs, see Table 1). It is recommended that more than the minimum required number of Diagnostic Ions (provided they have been validated) are acquired. In any case, all Diagnostic Ions acquired shall be evaluated and shall meet the identification criteria.

> *Comment:*
> *A valid explanation might be, for example, clear evidence that one of the established Diagnostic Ions is being interfered by a partially co-eluting substance in the Sample.*

Table 1. MTWRAs to ensure appropriate confidence in identification

RA in chromatogram of Reference Specimen * (% of the Reference Diagnostic Ion)	**MTWRA in the *Sample* chromatogram**	**Examples (in the *Sample* chromatogram)**	
		RA (% of Reference Diagnostic Ion **)	**Maximum Tolerance Window of RA** (% of Reference Diagnostic Ion)
> 50 - 100	± 10 (absolute)	105 100 60	115 - 95 100 ** (as Reference Diagnostic Ion) 50 – 70
> 25 - 50	± 20 % (relative)	40	32 – 48
1 - 25	± 5 (absolute) ***	10 3	5 – 15 > 0 *** – 8

* Analyzed in the same analytical batch.
** The MTWRA for the Reference Diagnostic Ion shall also be presented.
*** The Diagnostic Ions must always be detected in the *Sample* (S/N > 3:1).

4.0 Definitions

Diagnostic Ion(s): Molecular ion or fragment ion or product ion from a transition whose presence and abundance has been validated as 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 Ion 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).

Reference Diagnostic Ion: The most abundant Diagnostic Ion acquired from the Reference Specimen (spiked positive control, Reference Material or Reference Collection) whose presence is characteristic of the Analyte and thereby may assist in its identification. The Reference Diagnostic Ion is used to calculate the Relative Abundances (RAs) of all Diagnostic Ion(s) of the same chromatographic peak in a chromatogram and it shall also be used to establish the Retention Time (RT) for comparison.

Reference Specimen: A spiked positive control sample, Reference Collection sample, or Reference Material.

Relative Abundance (RA): The abundance of a Diagnostic Ion relative to the Reference Diagnostic Ion.

Maximum Tolerance Window of Relative Abundances (MTWRA): The maximum permitted difference between the Relative Abundance of a Diagnostic 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 RA obtained for the Diagnostic Ion in the Reference Specimen.
- **Relative**: Determined by calculating the stated tolerance percentage of the RA obtained for the Diagnostic Ion in the Reference Specimen and then adding/subtracting that value to/from the RA.

Retention Time (RT): The time that is required for an Analyte to reach the mass spectrometry detector after injecting into a chromatographic column connected to the mass spectrometer.

Relative Retention Time (RRT): Measurement of the Retention Time (RT) of the Analyte of interest relative to a Chromatographic Reference Compound (CRC).

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.

Selected Reaction Monitoring (SRM): Data acquired from specific product ions corresponding to selected precursor ions recorded via two (2) 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).

5.0 References

[1] The World Anti-Doping *Code International Standard* for Laboratories (ISL).

[Current versions of the WADA ISL and Technical Documents may be found at *https://www.wada-ama.org/en/what-we-do/science-medical/laboratories*]