

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

## IDENTIFICATION CRITERIA FOR QUALITATIVE ASSAYS INCORPORATING COLUMN CHROMATOGRAPHY AND MASS SPECTROMETRY

The ability of a method to identify a compound 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 a particular assay. The Laboratory shall establish criteria for identification of a compound.

### 1.0 Sample Preparation

The purpose of the sample preparation and chromatographic separation is to present a relatively pure chemical component from the sample to the mass spectrometer. The sample purification step can significantly change both the performance of the chromatographic system and the mass spectrometer. For example, a change in extraction solvent can selectively remove interferences and matrix components that might otherwise co-elute with the compound of interest. In addition, selective preparation procedures such as immunoaffinity extraction or fractions collected from high performance liquid chromatography separation can provide a solution that is nearly devoid of any other compounds.

### 2.0 Chromatographic Separation

#### 2.1 Gas Chromatography

- For capillary gas chromatography, the retention time (RT) of the analyte shall not differ by more than two (2) percent or  $\pm 0.1$  minutes (whichever is smaller) from that of the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously;
- Alternatively, the laboratory may choose to use relative retention time (RRT) as an acceptance criterion, where the retention time of the peak of interest is measured relative to a chromatographic reference compound (CRC).
  - The RRT shall not differ by more than  $\pm 1\%$  from that of the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously;
  - In general, the CRC is not a stable-isotope-labeled internal standard. If a stable isotope-labeled compound is used as the CRC for the same compound, the agreement of RRT between the *Sample* and the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously should be  $\pm 0.1\%$ .

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

## 2.2 *Liquid chromatography*

- For high performance liquid chromatography, the RT of the analyte shall not differ by more than two (2) percent or  $\pm 0.1$  minutes (whichever is smaller) from that of the same substance in a spiked urine 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 retention time of the peak of interest is measured relative to a chromatographic reference compound (CRC);
  - The RRT shall not differ by more than  $\pm 1\%$  from that of the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously;
  - In general, the CRC is not a stable-isotope-labeled internal standard. If a stable isotope-labeled compound is used as the CRC for the same compound, the agreement of RRT between the *Sample* and the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously should be  $\pm 0.1\%$ .
- If so-called “dilute and shoot” methods are used with LC/MS or LC/MS/MS, the use of a stable-isotope labeled internal standard is strongly advised due to the potential for matrix ion suppression or enhancement;
- When the method relies on chromatographic retention times as part of the identification process, the peak(s) of interest should preferably have retention factors ( $k'$ ) in the range of 3-10 to optimize separation factor ( $\alpha$ ) and detectability;
- The use of liquid introduction without adequate separation, such as introduction of a peak not separated from the solvent front, is not acceptable due to the potential for ion suppression;
- If other purification techniques are used prior to mass spectrometric analysis, the Laboratory shall have method validation documentation demonstrating that the method provides accurate results free of biological matrix interferences.

## 3.0 **Mass Spectrometric Detection and Identification of Molecules with Mass Less than 800 Da**

All Prohibited Substances with a concentration greater than approximately 100 ng/mL in the urine shall have a full or partial scan acquired or shall have an accurate mass determined such that the elemental composition can be defined. Whenever possible, a full scan is the preferred option.

### 3.1 **Full scan mode**

- A full scan generally should begin at an  $m/z$  value of 50 daltons, avoiding the inclusion of ions arising from permanent gases. A partial scan may begin at

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

an m/z value greater than any abundant ion due to the derivatizing agent (e.g., the m/z 73 ion arising from trimethylsilyl derivatives) or chemical ionization reagent;

- When a full or partial scan is acquired in GC/MS, all diagnostic ions with a relative abundance greater than 10% in the reference spectrum obtained from a positive control urine, a Reference Collection sample, or a Reference Material shall be present in the spectrum of the peak to be evaluated;
- For GC/MS<sup>n</sup> and LC/MS<sup>n</sup> techniques in Table 1, all diagnostic ions in the product ion scan with a relative abundance greater than 10% shall be present;
- Ion abundances should be obtained from peak areas or heights from the integration of extracted ion chromatograms;
- The relative abundance of the diagnostic ions may be obtained from a single spectrum at the peak apex or averaged spectra or integration of peak areas of extracted ion profiles;
- The relative abundances of each of the diagnostic ions greater than 10% shall be within the limits specified in Table 1.

### **3.2 Background Subtraction, Averaging Spectra, Peak Deconvolution and Computer-Based Spectral Library Matching**

- Background subtraction should be performed uniformly on all samples analyzed contemporaneously and used to make decisions regarding the presence of a *Prohibited Substance* or *Method*, its *Metabolite*, or *Marker*. Both an un-subtracted and a background-subtracted spectrum should be included in any documentation;
- As a general practice, background subtraction involves averaging (or summing) five (5) or less spectra between the inflection points of the peak. A similar number of spectra from the baseline, either before or after the peak, can be summed and subtracted from the peak spectra. Background subtracted spectra usually provide a more accurate representation of the mass spectrum of pure compounds available in spectral libraries;
- Recent advances in computer-assisted peak resolution using the mass spectral data have been established. One example of such a program is the Automated Mass Spectral Deconvolution and Identification System (AMDIS). The application of these freeware or commercial computer programs is permitted. The use of the program shall be validated as part of the written procedure.

### **3.3 Accurate Mass Measurement**

Accurate mass measurement provides the opportunity to determine the elemental composition of an ion. While accurate mass measurement cannot distinguish isomeric structures, it is often sufficient to determine the number of carbon, oxygen, hydrogen, nitrogen and other atoms in the molecule.

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

Mass accuracy should be reported as parts per million (ppm), as calculated from the equation:

$$\text{Mass accuracy (ppm)} = ((\text{Measured mass} - \text{calculated mass}) / \text{calculated mass}) \times 10^6$$

When using an instrument for exact mass measurement, the method description required under the Technical Document for Laboratory Documentation Packages shall include:

- the mass spectrometer design (e.g., analyzer type and/or geometry);
- resolution;
- lock masses and lock mass reference materials, and;
- mass range.

If all reasonable alternative elemental compositions cannot be excluded on the basis of exact mass, other considerations can be used to eliminate possible compositions. For example, the presence of a chlorine or bromine atom (and other elemental compositions) can be eliminated by examining the isotopomer pattern around the mass of interest. In addition, other types of analyses or derivatization schemes may also be used to eliminate uncertainty of composition.

### 3.4 Selected Ion Monitoring Mode

In cases where the concentration of the suspicious substance is less than approximately 100 ng/mL, it may be necessary to acquire selected ions in order to detect the substance.

- When selected ions are monitored, at least three diagnostic ions shall be acquired. Recent research, in the absence of chromatographic retention time data, has shown that the acquisition of more than three ions increases the probability of correct identification<sup>1</sup>;
- The relative abundance of a diagnostic ion shall preferably be determined from the peak area or height of integrated selected ion chromatograms;
- The integration start and stop points for all of the chromatographic peaks for each of the m/z values of each of the selected ions should be consistent. Ion ratios are then calculated by dividing the area of the each ion trace by the area obtained from the peak corresponding to the m/z of the base peak ion;
- The signal-to-noise ratio of the least intense diagnostic ion shall be greater than three to one (3:1);
- The relative intensities of any of the ions shall not differ by more than the amount in Table 1 from the relative intensities of the same ions acquired from a spiked positive control urine, Reference Collection sample, or Reference Material;

---

<sup>1</sup> Stein, S. E. and D. N. Heller (2006). "On the risk of false positive identification using multiple ion monitoring in qualitative mass spectrometry: Large-scale intercomparisons with a comprehensive mass spectral library." Journal of the American Society for Mass Spectrometry **17**(6): 823-835.

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

- The concentration of *Prohibited Substance*, or its *Metabolite*, or its *Marker* should be comparable in the *Sample* and the spiked urine, Reference Collection sample, or Reference Material.

If the Laboratory protocol requires three ions to be within a tolerance window to identify a substance, it is not permissible to collect additional ions and select those ion ratios that are within tolerance and ignore others that would not result in meeting identification criteria without a valid explanation.

For GC/MS, in order to ensure that a large amount of a co-eluting substance could not give rise to the observed diagnostic ions, a full scan spectrum shall be acquired at the retention time of the peak(s) of interest. The purpose of this scan is **not** identification, but rather to document the lack of presence of other substances that could contribute to the diagnostic ion intensity. The acquisition of a full scan may require analysis an additional aliquot of *Sample* to which the internal standards are not added. This full scan spectrum shall be included in the documentation package.

### **3.5 Computing Ion Ratios from Selected Ion Monitoring Data**

If three diagnostic ions are not available, a second derivative shall be prepared, or a second ionization or fragmentation technique shall be used. The second derivative should yield different diagnostic ions.

The second ionization technique shall be based on a different physical principle, i.e., chemical ionization vs. electronic 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 mass ions. In any case a minimum of two diagnostic ions shall be present in each mass spectrum.

### **3.6 Tandem mass spectrometric (MS<sup>n</sup>) detection and identification**

Tandem mass spectrometry data can be acquired in either the full scan or selected reaction monitoring (SRM) mode. The combination of mass selection of the precursor ion followed by a potentially unique collision-induced dissociation and mass selection or scanning of the product ion gives tandem mass spectrometry increased specificity. In general, two precursor-product ion transitions should be monitored. In some cases, however, the combination of a single precursor-product ion pair may be sufficiently unique to be definitive. If the Laboratory chooses to use one precursor-product ion pair for identification, they shall have acquired validation data documenting the uniqueness of the transition. The mass resolution of the first mass analyzer shall be set to at least unity.

When more than one precursor-product ion pair is monitored, the relative abundance of a diagnostic ion shall preferably be determined from the peak area or height of integrated selected reaction monitoring chromatograms.

- The integration start and stop points for all of the chromatographic peaks for each of the m/z values should be consistent;

## WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

- Ion ratios are then calculated by dividing the area of the each ion trace by the area obtained from the peak corresponding to the m/z of the base peak ion;
- The relative intensities of any of the ions shall not differ by more than the amount in Table 1 from the relative intensities of the same ions acquired from a urine spiked with Reference Material, Reference Collection sample, or Reference Material analyzed contemporaneously;
- The signal-to-noise of the least intense diagnostic ion shall be greater than three-to-one (3:1);
- The relative abundance of a diagnostic ion shall preferably be determined from the peak area or height of integrated selected ion chromatograms.

If unique diagnostic precursor-product ion pair(s) are not available, a second derivative shall be prepared, or a second ionization or fragmentation technique shall be used.

- The second derivative should yield different precursor and/or product ions;
- The second ionization technique may use a different chemical ionization reagent, but should provide different precursor or product ions;
- It is not acceptable to utilize a technique that changes only the relative abundance of the same mass ions.

To ensure that the precursor and product ions are not arising from a co-eluting compound in the chromatogram, a full scan spectrum at the retention time of the peak(s) of interest shall be acquired. The purpose of this scan is **not** identification, but rather to document the lack of presence of other substances that could contribute to the precursor-product ion intensity. This may require analysis of an additional aliquot in which the addition of a stable-labeled internal standard is omitted. The scan shall be included in the documentation package.

**Table 1**  
**Maximum Tolerance Windows for Relative Ion Intensities to**  
**Ensure Appropriate Confidence in Identification**

Relative Abundance (% of base peak)	EI-GC/MS; CI-GC/MS; GC/MS <sup>n</sup> ; LC/MS ; LC/MS <sup>n</sup>
> 50%	±10% (absolute)
25% to 50%	± 20% (relative)
5% to <25%	±5% (absolute)
<5%	± 50% (relative)

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

## 3.7 Estimation of concentration

The concentration may be estimated by any of the above techniques by taking the ratio of the peak height (or peak area) obtained at the retention time for the analyte of interest compared to that obtained from an internal standard.

- An internal standard that contains  $^2\text{H}$  or  $^{13}\text{C}$  in appropriate locations in the molecule is preferred but not required;
- The peak height (or peak area) ratio may then be compared to a reference material appropriately spiked or a positive control urine;
- The use of a single ion at the appropriate mass-to-charge ratio (e.g. m/z 405 for 19-norandrosterone di-TMS derivative) taken from an extracted ion chromatogram or from a selected ion monitoring chromatogram is sufficient for the estimation of concentration. Additional ions shall be used for meeting identification criteria.

## 4.0 Mass Spectrometric Detection and Identification of Molecules with Mass Between 800 and 8000 Da

In the last decade the advances in mass spectrometry of proteins and peptides has been a major contributor to the characterization of complex mixtures of proteins in proteomic research. In contrast, identification of proteins in anti-doping science is limited to specific compounds identified in the relevant sections of the Prohibited List. Thus, selective isolation of target proteins from the biological matrix is an integral part of applications in anti-doping. Numerous articles have been published on the "top down" and "bottom up" identification of proteins using mass spectrometry. The "top down" approach involves the mass measurement of the intact protein or peptide. The bottom up approach involves the sequencing of proteolytic or chemically-produced fragments of the intact protein, and this section applies to any such fragment with a molecular mass less than 8 kDa.

### 4.1 Top Down Approaches

- Determination of molecular mass: Unlike most small molecules, the ionization of proteins produces a number of multiply charged species (e.g.,  $\text{M}+5\text{H}^{5+}$ ,  $\text{M}+6\text{H}^{6+}$ ,  $\text{M}+7\text{H}^{7+}$ , etc.). Deconvolution of these multiply charged ion envelopes allows the calculation of the mass (M) of the intact protein;
  - Accurate mass/High resolution approaches: The mass shall be within 0.5 Da of the mass calculated from the reported amino acid sequence of the protein or peptide;
  - Low resolution mass spectrometric approaches: The mass calculated from the multiply charged ion envelope shall be within 0.5 Da of the mass calculated from the reported amino acid sequence of the protein or peptide.
- Tandem mass spectrometric sequencing: It has been established that the building blocks of proteins, amino acids, cause fragmentation of the protein backbone in predictable ways. Thus, a multiply-charged peptide ion can be

# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

selected and, after low energy collisionally-induced dissociation, a set of multiply charged ions can be analyzed to determine the sequence of amino acids. Since the sequence of amino acids is frequently unique to the protein, this can constitute an identification of the protein. During method validation, amino acid sequences in the target protein should be checked against a sequence database (e.g. BLAST or similar) to ensure that the combination of peptide sequences used for identification are unique to the protein in question or that other steps (e.g., immunoaffinity isolation or HPLC retention time) in the analysis rule out other proteins with identical sequences.

The relative abundances of the sequence ions should be compared to a contemporaneously assayed protein standard. If a stable isotope-labeled protein ( $^{15}\text{N}$  and/or  $^{13}\text{C}$ -labeling of the amino acids) is available, the characteristic sequence ions of the unknown can be compared directly to those derived from the isotopically labeled protein. In either case, the relative abundance of the characteristic ions should agree within the range specified in Table 1 for MS/MS experiments.

During method validation, the sequence of amino acids should be compared to a sequence database (e.g. BLAST database). The uniqueness of the sequence ions should be described in the description of the method included in the documentation package.

## **4.2 Bottom-up approaches**

If the protein has a mass-to-charge ratio that exceeds the range of the instrument, it is necessary to cleave the protein into pieces before mass spectrometric analysis. The protein may be chemically-modified to increase the number of charges on the ions. It is also permissible to consider a single peptide dissociated from a multi-peptide protein (e.g., cleavage of inter-chain disulfide bonds).

## **4.3 Identification of proteolytic or chemically-induced peptides**

A minimum of 10% of the amino acid sequence of the protein or peptide should be verified. During method validation, the sequence of amino acids should be compared to a sequence database (e.g. BLAST database). The uniqueness of the sequence ions should be described in the description of the method included in the documentation package.



# WADA Technical Document – TD2010IDCR

Document Number:	TD2010IDCR	Version Number:	1.0
Written by:	WADA Laboratory Committee	Approved by:	WADA Executive Committee
Approval Date:	08 May, 2010	Effective Date:	01 September, 2010

## 5.0 Definitions

**Accurate Mass Measurement:** This technique usually requires high mass resolution and is frequently associated with the term high resolution mass spectrometry. The mass assignment is generally made to at least two decimal places.

**Diagnostic ion(s):** Molecular ion or fragment ions whose presence and abundance are characteristic of the substance 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 (e.g. presence of Cl, Br, or other elements with abundant isotopic ions).

**High resolution mass spectrometry (HRMS):** For the purposes of the *International Standards for Laboratories*, HRMS is defined as mass spectrometry at a resolving power equal to or greater than 10,000 at full-width half-height maximum.

**Low resolution mass spectrometry (LRMS):** LRMS is defined as mass spectrometry at a resolving power less than 10,000 at full-width half-height maximum.

**Relative abundance (mass spectrometry):** The abundance of a particular ion relative to the most abundant ion monitored expressed as a percentage.

**Maximum difference in relative abundance:** The maximum permitted difference between the relative abundance of a particular ion obtained from the *Sample* and that obtained from the positive control urine. This may be expressed in ABSOLUTE or RELATIVE terms.

**Absolute difference:** Calculated by subtracting the stated percentage from the relative abundance obtained for the studied ion from the positive control urine or Reference Material. For example, if the relative abundance of an ion in the chromatographic peak of interest in the positive control urine or Reference Material is measured as 20%, then the observed relative abundance for the same ion in the peak of interest in the unknown urine sample would be required to be in the range of 15-25% ( $20\% \pm 5\%$ ) for the ion to meet the identification criteria.

**Relative difference:** Calculated by multiplying the stated percentage by the relative abundance obtained for the studied ion from the positive control urine or Reference Material. For example, if the relative abundance of an ion in the chromatographic peak of interest in the positive control urine or Reference Material appears as 30 % and the stated maximum permitted difference is 20 % (relative), then the observed relative abundance for the same ion in the peak of interest in the unknown urine sample would be required to be in the range of 24-36% ( $30\% \pm (30 \times 20 \%)$ ) for the ion to meet the identification criteria.

**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. Selected reaction monitoring can be performed as tandem mass spectrometry in time or tandem mass spectrometry in space.

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