AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry

Preamble

1. This document provides a set of internationally-agreed recommendations for the comparison of chromatographic and mass spectral data during confirmatory analysis consistent with ILAC-G7 Part B "Guide for Establishing the Presence of Prohibited Substances". It is intended as a guideline for analysts and laboratories. Responsibility for ensuring the quality, integrity, defensibility and fitness for purpose of the analytical data lies with the analyst and the laboratory. Laboratories should clearly define and document specific criteria for their own use.

General Analytical Requirements

2. In general, chromatographic separation coupled to mass spectrometric detection is sufficiently specific to be used alone as a confirmatory method.

3. A prohibited substance or a metabolite or artifact of a prohibited substance may be analysed intact or as the end product of a sequence of one or more reproducible chemical and/or enzymatic processes. In the latter case, the end product must be unequivocally attributable to the presence in the test sample of a prohibited substance or a metabolite or artifact of a prohibited substance.

4. The injection sequence for a confirmatory analysis should be consistent with ILAC-G7 Part B Clause 14. An example of a sequence appropriate to a range of analytical circumstances is as follows:

   - Negative control (may also serve as a system blank for non-threshold substances)
   - System blank
   - Test sample
   - Reagent blank or negative control
   - Reference sample (reference material or other positive control)

Chromatography

5. Chromatographic matching is based on a comparison of relative or absolute retention times. In the presence of an internal standard or marker, either approach may be used.

Gas Chromatography and High-Performance Liquid Chromatography

6. When using relative retention time, the relative retention time of the analyte in the test sample should not vary from that in the reference sample by more than ±1%. In the case of peptides and

macromolecules including but not limited to proteins, the relative retention time of the analyte in the test sample should not vary from that in the reference sample by more than ±2%.

7. When using absolute retention time, the absolute retention time in the test sample should not vary from that in the reference sample by more than ±1% or ±6 seconds, whichever is the greater, and should also not vary from that in the reference sample by more than the full width at half maximum of the analyte peak in the reference sample. In the case of peptides and macromolecules including but not limited to proteins, the absolute retention time in the test sample should not vary from that in the reference sample by more than ±2% or ±12 seconds, whichever is the greater, and should also not vary from that in the reference sample by more than the full width at half maximum of the analyte peak in the reference sample.

8. The approach to chromatographic peak smoothing should be consistent across the analytical batch.

Other Techniques

9. Laboratories using chromatographic separation techniques other than gas chromatography or high-performance liquid chromatography should set criteria appropriate for the technique used. In general, the absolute difference in retention time between the test sample and the reference sample should not exceed the full width at half maximum of the peak in the reference sample.

Mass Spectrometry

10. Mass spectral matching is based on a comparison of the relative abundances of selected diagnostic ions. The selected diagnostic ions should be molecular ions, quasi-molecular ions or fragment ions whose presence and abundance are characteristic of the analyte.

11. Mass spectral data may be acquired by either single-stage or multiple-stage mass spectrometry and in either scanning or non-scanning mode. The acquired data may be presented in the form of a mass spectrum and/or as a set of extracted ion chromatograms.

Diagnostic Ions

12. The signal for any diagnostic ion selected for matching must be significantly above any measurable background noise level. Typically, in the presence of measurable noise, the signal-to-noise ratio should be well above 3:1 as determined using extracted ion chromatograms.

13. For single-stage mass spectrometry, the molecular ion or quasi-molecular ion must be included as a diagnostic ion if its relative abundance is greater than 5% in the test sample.

14. For full scan single-stage acquisition presented as a mass spectrum, a minimum of three diagnostic ions are required for matching.

15. An ion transition arising from multiple-stage acquisition is considered more characteristic than a fragment ion in isolation and the number of diagnostic ions required may be reduced by one. For full scan multiple-stage acquisition including at least one precursor isolation step and presented as a full scan mass spectrum, a minimum of two diagnostic ions other than the precursor are required for matching.

16. Full scan mass spectra are data-rich and are generally preferred for confirmatory analysis. However, the use of full scan mass spectra may be problematic when dealing with challenging concentrations or in the presence of severe background interference. In these cases the use of non-scanning acquisition or the presentation of full scan data as extracted ion chromatograms without an accompanying mass spectrum may be more appropriate. To compensate for the loss of data-richness, an additional diagnostic ion is required. For selected ion monitoring or full scan single-stage acquisition presented as extracted ion chromatograms without an accompanying mass spectrum, a minimum of four diagnostic ions are required for matching.

17. The principles in clauses 15 and 16 are additive. For selected reaction monitoring or full scan multiple-stage acquisition including at least one precursor isolation step and presented as a set of extracted ion chromatograms without an accompanying mass spectrum, a minimum of three diagnostic ions other than the precursor are required for matching.

18. If a single technique produces insufficient diagnostic ions suitable for matching, multiple analytical techniques or chemical derivatisations may be used in combination.

Relative Abundance

19. The relative abundance of a diagnostic ion is the abundance of that ion relative to the abundance of a specified diagnostic ion expressed as a percentage. It may be calculated from mass peak heights in a background-subtracted mass spectrum or from integrated chromatographic peak areas in a set of extracted ion chromatograms.

20. The relative abundance for a diagnostic ion in the reference sample is calculated relative to the most intense diagnostic ion selected for matching in the reference sample. The most intense diagnostic ion selected for matching in the reference sample always has a relative abundance of 100%.

21. The relative abundance for a diagnostic ion in the test sample is calculated relative to the diagnostic ion in the test sample corresponding to the most intense diagnostic ion selected for matching in the reference sample. When the most intense diagnostic ion in the test sample differs from that in the reference sample, diagnostic ions in the test sample may have relative abundances greater than 100%.

22. The maximum permitted difference between the relative abundance in the test sample and the relative abundance in the reference sample for each diagnostic ion is 20% of the relative abundance in the reference sample plus 5%.

Example calculations:

Relative abundance in the reference sample	Maximum permitted relative abundance difference	Permissible relative abundance range in the test sample
99%	24.8%	74.2-123.8%
90%	23%	67-113%
80%	21%	59-101%
70%	19%	51-89%
60%	17%	43-77%
50%	15%	35-65%
40%	13%	27-53%
30%	11%	19-41%
20%	9%	11-29%
10%	7%	3-17%
5%	6%	>0-11%
1%	5.2%	>0-6.2%

Full scan mass spectra

23. All ions within the common mass range that can be ascribed to the analyte and which appear in the reference spectrum with a relative abundance greater than 10% must also be present in the test spectrum.

24. All extraneous ions in the test spectrum with *m/z* greater than 100 and abundance greater than 20% of the most intense ion in the spectrum that can be ascribed to the analyte must be demonstrated to be extraneous using extracted ion chromatograms. The abundance for this purpose may be calculated from either the mass spectrum or from extracted ion chromatograms.

25. The approach to background subtraction should be consistent across the analytical batch. The details of the background subtraction applied should be appropriate to the individual sample.

High resolution mass spectrometry

26. In general, when compared to mass spectra obtained using unit mass resolution and nominal mass assignment, the use of high resolution mass spectrometry provides additional confidence in the attribution of a mass peak or ion chromatogram to a confirmatory analyte.

27. The mass error for a diagnostic ion in either the test spectrum or the reference spectrum is calculated by subtracting the measured mass of the diagnostic ion from the exact mass derived from the chemical formula, allowing for charge. The use of mass error is preferred but can only be determined when the formula of the diagnostic ion is known.

28. The mass difference for a diagnostic ion in the test spectrum is calculated by subtracting the measured mass of the diagnostic ion from the measured mass of the corresponding diagnostic ion in the reference sample.

29. The requirements in clauses 10 to 22 and clause 25 shall all apply for high resolution mass spectrometry. The requirements in clauses 23 and 24 shall not apply for high resolution mass spectrometry provided that the mass error or mass difference for each diagnostic ion selected for matching shall fall within the range ±5 ppm or ±2 mDa, whichever is greater.

Macromolecules

Proteins and Their Proteotypic Peptides

30. The amino acid sequence of a target protein or proteotypic peptide must be demonstrated to be unique in the analytical matrix in question. For example, BLAST searches or similar bioinformatic tools should be used to identify proteins or peptides which contain an identical sequence. Where such proteins or peptides are identified, the likelihood of their presence in the analytical matrix and of their generating a species with an identical sequence to the target protein or proteotypic peptide under the analytical conditions employed should be evaluated.

31. The amino acid sequence of a protein may be common to multiple animal species or synthetic analogues. Proteotypic peptides arising from that sequence may therefore have more than one possible origin. Provided that all proteins giving rise to a particular proteotypic peptide should not be naturally present in the analytical matrix, the proteotypic peptide may be taken as evidence of the presence in the analytical matrix of a prohibited substance.

Other Macromolecules

32. Laboratories working with macromolecules other than proteins and their proteotypic peptides should set criteria appropriate for the technique and mass range used.

Reference Materials

33. Reference materials used for chromatographic and mass spectral matching should be consistent with ILAC-G7 Part B Clause 16.

34. Materials of known, certified content are preferred for use as reference materials. Where these are not available, the source of a reference material should be documented and its identity validated.

35. Chromatographic and mass spectral data derived from a reference material provide a definitive reference data set for comparison with the test sample. All data in the reference data set must be attributable to the analyte concerned.

36. The reference material concentration should be such that the reference data set is unaffected by instrument or matrix effects. It is not necessary to match the analyte concentrations of the test sample and reference sample, although it is good practice to consider the potential effects of concentration or matrix on the reference data set.

37. The reference data set may be derived from any of the following:

    - An extracted or non-extracted reference material.
    - An isolate from a matrix-matched sample spiked with a reference material.
    - An isolate from a sample collected after an authenticated administration of an appropriate substance.
    - An isolate from an *in vitro* incubation of an appropriate substance with liver cells, microsomes, plasma or serum.

38. Where a test sample is analysed using chemical and/or enzymatic modification, the reference material used for chromatographic and mass spectral matching may be analysed without modification if it is equivalent to the end product of the modification sequence or it may be derived from the parent substance or any stable intermediate substance formed using equivalent processes to the test sample.

References

ILAC-G7:04/2021, "Accreditation Requirements and Operating Criteria for Horseracing Laboratories" (April 2021; International Laboratory Accreditation Cooperation). Available at https://ilac.org/.

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