Guidelines for Comparison of Mass Spectra

1 Introduction

Many of the analytical procedures used in the Toxicology Subunit rely on mass spectrometry to help establish identification of individual chemical entities within a sample. In order to ensure consistency and reproducibility in compound identification, it is desirable to have guidelines for the comparison of known and unknown mass spectra.

2 Scope

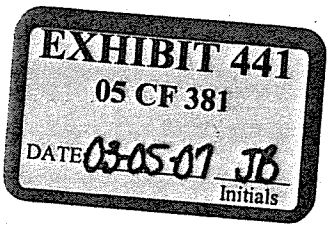
This document provides guidelines to help determine what constitutes a match between known and unknown mass spectra. Various critical characteristics of a mass spectrum are defined, and procedures for using these characteristics to evaluate matching between spectra are laid out. Note that this document provides guidelines for the matching of mass spectra, and does not directly address compound identification. A good quality mass spectral match will normally be only one element in establishing the identity of an unknown substance. These protocols are intended for application to full scan, tandem, and selected ion monitoring (SIM) mass spectra acquired in electron impact (EI), chemical (CI), electrospray (ESI), and atmospheric pressure chemical ionization (APCI) modes. Other mass spectral techniques are beyond the scope of this document.

3 Principle

The spectrum of a given unknown of interest is compared to the known spectrum of a target analyte. The unknown spectrum should contain all the significant ions present in the known spectrum, and should not contain any unexplained significant ions not seen in the known spectrum. The relative intensities (hereafter referred to as "ion ratios") of several selected characteristic ions should match in both spectra, to within defined tolerances. These guidelines draw heavily on technical document 2003IDCR from the World Anti-Doping Agency, the 2003 recommendations of the American Society for Mass Spectrometry's Measurements and Standards Committee, and the 2002 National Committee for Clinical Laboratory Standards Approved Guideline for GC/MS (gas chromatography/mass spectrometry) Confirmation of Drugs.

4 Specimens

Not applicable.



5 Equipment/Materials/Reagents

Not applicable.

6 Standards and Controls

Not applicable.

7 Calibration

The calibration of all mass spectrometers should be verified regularly per the appropriate instrument protocols in the *Instrument Operations and Support Subunit SOP Manual*.

8 Sampling

Not applicable.

9 Procedure

Provided below are procedures for defining and determining critical characteristics of a mass spectrum to be used in establishing whether or not two spectra match. An abbreviated list of the key points is provided in Appendix 1.

9.1 Averaging and Background Subtraction of Mass Spectra

In many real-world samples, it may be necessary to correct mass spectra of interest for the presence of ions resulting from sample background, instrument background, or partially coeluting sample components. The necessary background-subtracted spectrum will usually be generated by averaging not more than five spectra across the peak of interest and then subtracting the average of a number of background spectra equal to not more than twice the number of sample spectra. The background spectra may come before and/or after the sample spectra, and should all be selected from outside the region integrated for determination of ion ratios. This background-subtracted spectrum will be used to establish the list of significant ions and the base peak for that spectrum.

9.2 Determination of "Significant Ions" in a Mass Spectrum

Any ion signal greater than 15% of the most intense ion signal in a background-subtracted mass spectrum will normally be considered a ***significant ion***. An ion that would otherwise be considered significant may be excluded if it can be demonstrated that the ion arises from, or is significantly disturbed by, an uncorrectable chemical interference. Such interferences will

normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

9.3 Determination of "Diagnostic Ions" in a Mass Spectrum

Diagnostic ions are those ions in a mass spectrum that are characteristic of the chemical compound under investigation. Determination of diagnostic ions depends upon knowledge of the chemical structure of a component under investigation, and may therefore only be determined from mass spectra of known standards. The definition of what makes any given ion "characteristic" of a particular chemical structure is somewhat nebulous, and there does not appear to be any universally accepted standard in the field. This means that good and consistent judgment by the examiner is essential. There are, however, recommendations as to what renders some ions nonspecific and not diagnostic, and an examiner should abide by these practices when eliminating ions from consideration as diagnostic.

Adduct ions will normally be excluded, except that one pseudomolecular adduct ion may be considered diagnostic. Isotopomers will be excluded unless they are characteristic of a specific chemical composition. Normally this will be limited to chlorine and bromine isotopomers, but other possibilities may arise. Ions resulting purely from a derivatizing or complexing reagent will normally be excluded from the list of diagnostic ions. For example, the m/z 73 ion of a trimethylsilyl derivative may not be chosen as a diagnostic ion. Normally the (pseudo)molecular ion for a compound will be considered diagnostic, unless the intensity for that ion is less than 5% of the intensity for the base peak in the background-subtracted spectrum of the component in question.

9.4 Determination of the "Base Peak" in a Mass Spectrum

The ***base peak*** for the mass spectrum of a known standard is the most intense signal for a diagnostic ion in the background-subtracted spectrum. For the purpose of determining relative ion intensities the base peak in an unknown mass spectrum will be taken as the base peak of the standard spectrum to which it is being compared, even if a different diagnostic ion shows higher intensity in that spectrum.

In instances where it can be demonstrated that the nominal base peak signal is significantly disturbed by an uncorrectable chemical interference, the second most intense diagnostic ion present in the spectrum may be used as the base peak. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

9.5 Method for Calculating Ion Ratios

Ion ratios will normally be determined by integrating reconstructed ion traces for each diagnostic ion present in a given component. All integrations of reconstructed ion traces from a given

component should have comparable stop and start points. Ion ratios are then calculated by dividing the area for each ion trace by the area for the trace of the base peak ion, and expressing the result as a percentage. In instances where the reconstructed ion traces produce non-integratable data, it is acceptable to substitute ion abundances from the background subtracted spectrum of the compound of interest for the integrated areas from reconstructed ion traces. This will normally happen in situations where multiple sorts of mass spectral data are simultaneously acquired in a single analytical run, resulting in discontinuous data streams for the various individual mass spectral experiments.

10 Instrument Conditions

Not applicable.

11 Decision Criteria

Provided below are guidelines for establishing a match between a known mass spectrum and that of an unknown spectrum. Note that some analytical standard operating procedures (SOP's) include detailed criteria for the evaluation of mass spectra of individual target analytes. Such specific instructions will supercede the guidelines provided below.

In almost all cases, unknown spectra should be matched against known spectra obtained from contemporaneously analyzed reference material. Exceptions are discussed in section 12.5 of these guidelines. When assessing spectra for a targeted analyte from multiple unknown samples in a single analytical run, it is acceptable to compare each unknown spectrum to the known spectrum resulting from a different contemporaneously analyzed reference sample. The mass spectra of many chemical entities are known to vary with analyte load. It is acceptable to dilute and reanalyze a sample containing a high level of a suspected target compound in order to be able to more appropriately match its spectrum to a lower concentration standard, control, or calibrator.

11.1 For Full Scan Mass Spectra

In order to establish a match between known and unknown mass spectra in the full scan mode, both of the following criteria should be met:

a. Every significant ion present in the known spectrum should be present in the unknown spectrum, and vice-versa.

b. All relative intensities for diagnostic ions in the unknown spectrum should match those observed in the known spectrum within the tolerances shown in Table 1 or Table 2. If these limits would produce an acceptable lower bound of less than 1% for a given ion ratio,

the lower limit will be set at 1%. Ion ratios for specific diagnostic ions may be excluded from consideration if they meet any of the following criteria:

1. The ion ratio for that ion in the known spectrum is less than 5% (less than 10% for CI, ESI, or APCI spectra).
2. The signal-to-noise ratio of the reconstructed ion trace for that ion in the unknown spectrum is less than 3.
3. It can be shown that the signal for that ion in either the known or the unknown spectrum is significantly disturbed by an uncorrectable chemical interference. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

If there are more than four diagnostic ions in the known spectrum, then the examiner need only evaluate the ratios for four diagnostic ions (three ratios) in order to establish a scientifically valid match between the spectra. For compounds with a molecular weight less than 80 AMU, only three diagnostic ions (2 ratios) need be evaluated to establish a scientifically valid match. The selected ions will normally include the base peak and the (pseudo)molecular ion, unless those ions meet one of the three exclusion criteria given above. If fewer than three diagnostic ions are available for evaluation, the spectra may still be matched, but the examiner should be aware that the information content derived from such a match is limited, and should be regarded with caution. Examiners should also take care to ensure that the chosen scan range provides adequate "buffer space" around the diagnostic and significant ions of the substance in question.

Table 1: Ion Ratio Matching Tolerances for EI Mass Spectra

If the ion ratio in the known spectrum is:	>50%	≥25% and ≤50%	<25%
Then the ion ratio in the unknown spectrum should be within:	10% absolute	20% relative	5% absolute

Table 2: Ion Ratio Matching Tolerances for CI, ESI, and APCI Mass Spectra

If the ion ratio in the known spectrum is:	>60%	≥40% and ≤60%	<40%
Then the ion ratio in the unknown spectrum should be within:	15% absolute	25% relative	10% absolute

11.2 For SIM Mass Spectra

Selected ion monitoring experiments can allow for the detection of very low levels of analyte in complex sample matrices, at the cost of reducing the information content of that experiment. Examiners should take great care in selecting monitored ions for a SIM experiment. Ions for a SIM experiment must be based upon a known full scan spectrum of the species of interest collected

on the instrument to be used for the SIM experiment. Four diagnostic ions will normally be selected (three ions for compounds with a molecular weight less than 80 AMU; see 12.4 for another exception), and, if possible, all should be significant as well as diagnostic. The base peak will normally be one of the chosen ions, and the (pseudo)molecular ion should be included if it has a relative intensity greater than 5% in the known full scan spectrum. In order to establish a match between a known SIM spectrum and an unknown SIM spectrum, all resulting ion ratios should meet the tolerances specified in Table 1 or Table 2, as appropriate.

11.3 For Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometry can lend a great deal of additional specificity to mass spectral experiments by greatly increasing the confidence that the ions in a given spectrum are all associated with a single substance. Due to the nature of most collision-induced dissociation processes, however, ion ratios in tandem mass spectra tend to be much less stable, and much more dependent on analyte load, than is true for classic electron impact mass spectra.

Tandem mass spectra tend to be much "cleaner" than full scan mass spectra, with fewer extraneous ions. Therefore, the limit for determination of significant ions in a tandem mass spectrum is lowered to 10% (from 15%) of the most intense observed ion in the background subtracted spectrum. The high probability of ion association in tandem mass spectrometry means that nearly all ions of reasonable intensity observed in an MS/MS experiment should be considered diagnostic, with the exception of ions resulting purely from the loss of an adduct.

Due to vagaries of the physical processes involved in the precursor ion isolation and fragmentation events in an ion trap mass spectrometer, tandem mass spectra acquired on such an instrument will occasionally show an "ion-splitting" artifact for a precursor ion returned in a product ion mass spectrum. This is evidenced by the presence of two ions, separated by a fraction of an AMU, at the nominal mass of the precursor ion in the product ion spectrum. In instances where this phenomenon is observed, the response for the affected ion should be taken as the total of the response for both components of the "split" ion signal.

11.3.1 Product Ion Experiments

When conducting product ion experiments, the selection of a precursor ion is critical to obtaining useful and reliable information. In most cases, the (pseudo)molecular ion of the species under consideration should be selected, if available. It is also acceptable to use a diagnostic isotopomer of the (pseudo)molecular ion, if one is available. If the (pseudo)molecular ion is not available, or is not suitable for some reason, then the selected precursor ion should be both significant and diagnostic in the full scan mass spectrum of the substance under consideration. With product ion spectra, it is also important to ensure that the observed fragment spectrum is, in fact, emerging from the selected precursor ion. For this reason, one of the two following criteria should normally be met for a product ion spectrum:

a. The precursor ion should be observed in the product ion spectrum with an ion ratio of at least 5%.
b. If full scan mass spectral data are collected concurrently with the product ion spectra, the full scan spectrum of the component of interest should show no ions within 1.5 AMU of the precursor ion with greater than three times the intensity of the precursor ion.

In order to establish a match between a known product ion spectrum and the product ion spectrum of an unknown, both of the following criteria should be met:

a. Every significant ion present in the known spectrum should be present in the unknown spectrum, and vice-versa.
b. All relative intensities for diagnostic ions in the unknown spectrum should match those observed in the known spectrum to within the tolerances shown in Table 3. If these limits would produce an acceptable lower bound of less than 1% for a given ion ratio, the lower limit will be set at 0.5%. Ion ratios for specific diagnostic ions may be excluded from consideration if they meet any of the following criteria:

    1. The ion ratio for that ion in the known spectrum is less than 5%.
    2. The signal-to-noise ratio of the reconstructed ion trace for that ion in the unknown spectrum is less than 3.
    3. It can be shown that the signal for that ion in either the known or the unknown spectrum is significantly disturbed by an uncorrectable chemical interference. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

If there are more than three diagnostic ions in the known spectrum, then the examiner need only evaluate the ratios for three diagnostic ions (two ratios) in order to establish a scientifically valid match between the spectra. The selected three ions should include the base peak and the precursor ion (if present), unless those ions meet one of the three exclusion criteria given above. If only a single diagnostic ion is observed in the product ion spectrum, spectra may still be matched, but the examiner should be aware that the information content derived from such a match is limited, and should be regarded with caution.

Table 3: Ion Ratio Matching Tolerances for MS/MS Product Ion Spectra

If the ion ratio in the known spectrum is:	>40%	≤40%
Then the ion ratio in the unknown spectrum should be within:	25% relative	10% absolute

11.3.2 Precursor Ion and Neutral Loss Experiments

The practical information content for precursor ion and neutral loss MS/MS experiments is generally low, but circumstances may still arise in which one of these techniques can provide critical additional information about a given substance. For precursor ion experiments, a match between a known and an unknown spectrum may be established if all significant ions present in the known spectrum are present in the unknown spectrum, and vice-versa. For neutral loss experiments, a match between a known and an unknown spectrum may be established if all significant transition pairs present in the known spectrum are present in the unknown spectrum and vice-versa.

11.3.3 Selected Reaction Monitoring (SRM) Experiments

SRM analysis shares many features, advantages, and limitations with SIM analysis, but benefits from the added specificity afforded by tandem mass spectrometry. Three diagnostic ion transitions should be chosen for an SRM experiment. Generally, all three transitions should share a common precursor ion, although it is appropriate to use multiple precursor ions if all are part of a diagnostic isotope cluster in the full scan spectrum of the substance in question. It is desirable that the chosen precursor ion be the (pseudo)molecular ion of the substance in question. If this is not possible, or not practical, then the chosen precursor ion should be both significant and diagnostic in the full scan spectrum of the substance in question. In order to establish a match between a known SRM spectrum and an unknown SRM spectrum, both resulting ion ratios should meet the tolerances specified in Table 3.

11.3.4 Higher Order (MS^n) Tandem Mass Spectrometry

Tandem mass spectra of order higher than 2 are beyond the scope of this document. There is little to no discussion of this subject in the various published technical guidelines, and the technique is rarely practiced in forensic and regulatory settings. When used, higher order tandem mass spectra will be addressed on a case-by-case basis, usually as a part of method validation. Examiners should consider using the criteria for product ion MS/MS in section 12.3.1 as a starting point for such evaluation.

11.4 Exact (Precise) Mass Measurement Techniques

Exact mass measurement can provide a significant additional level of information content in a mass spectrum, giving an examiner more confidence in any conclusions based upon that spectrum. The use of exact mass measurement techniques does not, however, allow an examiner to disregard other aspects of the mass spectrum under consideration. As such, mass spectra obtained using exact mass techniques should still meet all of the matching criteria for the appropriate mass spectral techniques given above, but different standards may be used in selecting diagnostic ions, and more confidence can be placed in matches based upon a limited set of diagnostic ions.

Ions in an unknown spectrum will be considered to be an exact mass match to those in a known spectrum if the measured masses agree to within 0.005 AMU. For a SIM experiment, only three ions, instead of four, need be monitored, and show appropriate ion ratio agreement, if all three ions meet this exact mass match criterion. When determining diagnostic ions, any isotopomer of a (pseudo)molecular ion may be considered diagnostic if it meets this exact mass match criterion. One additional adduct ion, beyond the pseudomolecular ion, may also be considered diagnostic if it meets this exact mass match criterion.

11.5 Matching to Library Spectra

While mass spectral libraries (either commercial compendia or collections generated in-house) can be invaluable tools in helping to direct examinations and suggest possible targets for further investigation, an examiner should remain aware of the limitations of these libraries. Most commercial libraries do not clearly indicate the instrumentation the spectra were acquired on, or at what level of sample loading. In-house library data may have been acquired on the same instrumentation used to obtain a given unknown spectrum, but it is very difficult to ensure that long-term drift in instrument performance has not compromised the reproducibility of those library spectra.

Despite these limitations, there may arise rare instances in which it is necessary to compare an unknown spectrum to a library entry, for example if a standard of the substance in question cannot be readily obtained, or for purposes of screening to direct further investigation. In cases where such matching is attempted, all criteria for the appropriate type of mass spectrometry, given above, should still be observed, with one significant change. In these instances, ion abundances for the determination of ion ratios will be measured as the intensity of the ion in the spectrum, rather than as the integrated area of a reconstructed ion trace. For the unknown spectrum, all criteria regarding averaging and background subtraction from section 10.1 should still be observed.

12 Calculations

$IR_x = (A_x/A_b)x100\%$, where:

IR_x = the ion ratio for ion x
A_x = the integrated area of the reconstructed ion trace for ion x
A_b = the integrated area of the reconstructed ion trace for the base peak ion
(Ion abundances from background subtracted mass spectra may be substituted for integrated areas under certain circumstances detailed in section 9.5.)

13 Uncertainty of Measurement

Not applicable.

14 Limitations

This procedure, while extensive, is not intended to be exhaustive, and situations will arise in which their blind application could lead to inappropriate conclusions. No set of rules can ever replace the good judgment of a trained and experienced examiner. The mere fact that an unknown mass spectrum matches well to the spectrum of a known standard will rarely, *by itself*, be sufficient grounds to claim the presence of that compound in the questioned sample. All of the analytical data for the samples in question should be considered when drawing such final conclusions. Similarly, the fact that an unknown mass spectrum fails to match that of a known standard generally will not, *by itself*, constitute grounds for concluding that the compound is not present in the questioned specimen.

This protocol does not excuse an examiner from exercising care in the acquisition of mass spectral data. It should be remembered that poor practices in the acquisition of mass spectra will yield data that is useless at best, and misleading at worst. Samples that show evidence of severe chromatographic overload should generally be diluted and reinjected in order to obtain reliable mass spectra. Instrument calibration is also critical for obtaining useful mass spectral data, and it is incumbent upon any examiner to run appropriate test samples to demonstrate proper instrument function.

15 Safety

Not applicable.

16 References

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Appendix 1: Mass Spectra Key Points

1. Averaging/Background Subtraction of Mass Spectra: (* = subtracted spectrum)
   a. <6 scans averaged for sample (N)
   b. <2N+1 scans for background
   c. background outside area integrated for ion ratios

2. Significant Ions: Ion signal > 15% (10% for MS/MS) of most intense ion signal (*)

3. Diagnostic Ions (determine from mass spectra of known standard):
   a. Characteristic of chemical compounds chemical structure
   b. Diagnostic criteria (not well defined):
      (Pseudo)molecular ion is diagnostic if intensity > 5% base peak (*)
   c. Non-diagnostic ions (generally exclude):
      Adduct ions except pseudomolecular ion
      Isotopomers except chlorine and bromine isotopomers
      Ions 2^{ndry} to a derivatizing reagent (e.g. m/z 73 TMS deriv)

4. Base Peak (determined from mass spectra of known standard):
   a. Most intense signal for diagnostic ion (*)
   b. Base peak standard is base peak unknown (Exception: can use second most intense diagnostic ion if base peak has an uncorrectable chemical interference)

5. Method for calculating relative ion intensities:
   a. Determine ion abundances = integrate RIC's / EIC's for each diagnostic ion
   b. Integration - comparable stop & start points
   c. Ion ratios (expressed as %) = each ion trace area/base peak ion area
   d. Spectral peak height may be substituted for integrated area if the RIC data in non-integrable.

6. Decision Criteria: Unknown spectra matched against known spectra.

6.1 Full Scan Mass Spectra
   a. Every significant ion in known spectra present in unknown and vice-versa
   b. Relative ion intensities (EI)
      Known Ion ratio > 50% - unknown 10% absolute
      Known Ion ratio 25-50% - unknown 20% relative
      Known Ion ratio < 25% - unknown 5% absolute
   c. Relative ion intensities (CI, ESI, APCI)
      Known Ion ratio > 60% - unknown 15% absolute
      Known Ion ratio 40-60% - unknown 25% relative
      Known Ion ratio < 40% - unknown 10% absolute

6.2 SIM Mass Spectra
- a. Four diagnostic ions (three for compounds <80 AMU), all should be significant
- b. Base peak one of the chosen ions
- c. (Pseudo)molecular ion RI > 5%
- d. Meet relative ion intensities

6.3 Tandem MS (ion ratio dependant on analyte load, less stable)
- a. All ions of reasonable intensity are diagnostic (Exception 2^{ndry} loss of adduct)

6.3.1 Product Ion (Daughter Ion) Experiment
- a. Select precursor ion (parent ion):
  (Pseudo)molecular ion or one of its isotopomers
- b. Product spectra:
  Precursor ion observed with ion ratio >5% OR full scan show no ions within 1.5 AMU of precursor ion with > 3x intensity of the precursor ion
- c. Comparing product spectra (Standard to unknown = std to unk)
  Significant ions present both std and unk
  If > 3 diag ions –need only ratio 3 diag (2 ratios)
  Ion ratios:
    Known > 40% - unknown 25% relative
    Known < 40% - unknown 10% absolute
  Exclude ion ratios if:
    Ion ratio known < 5%
    Signal-to-noise RIC unknown < 3
    Chemical interference in ion

6.3.2 Precursor ion and Neutral loss – must match stds with unknowns

6.3.3 Selected Reaction Monitoring
- a. 3 diagnostic ion transitions
- b. Share common precursor ion, except isotopomer clusters
- c. Ion ratios:
    Known > 40% - unknown 25% relative
    Known < 40% - unknown 10% absolute

6.4 Exact Mass (Accurate Mass)
- a. m/z < 600: Std to Unk measured masses within 3 mAMU
- b. m/z > 600: Std to Unk measured masses within 5ppm
- c. SIM: only 3 ions need monitoring, in correct ratios
- d. If above criteria is met:
    Can use (pseudo)molecular ion if < 5% RI
    Can use any isotopomer of (pseudo)molecular ion

Can use up to one additional adduct ion, beyond pseudomolecular ion

Appendix 2: Glossary of Terms

Adduct Ion – Any ion to which another chemical entity has been attached by a means other than covalent bonding.

Base Peak – The most intense or abundant diagnostic ion in the mass spectrum of a substance.

Diagnostic Ion – Any ion observed in the mass spectrum of a substance that is characteristic of the chemical structure of that substance.

Ion Ratio – The relative abundance or intensity of two ion signals in a mass spectrum.

Isotopomers – Two or more chemical species that differ only in isotopic composition. For example, CH_3OH and CD_3OH are two isotopomers of methanol.

Molecular Ion – A charged intact molecular species, with charge acquired solely through the gain or loss of electrons. Normally denoted as M^+ or M^- for singly charged species.

Precursor Ion – In tandem mass spectrometry, the ion selected for fragmentation. Often referred to as a "parent ion".

Product Ion – In tandem mass spectrometry, an ion resulting from the fragmentation of another ion. Often referred to as a "daughter ion".

Pseudomolecular Ion – A charged molecule in which charge has been acquired through adduction of an ion or through loss of a moiety able to dissociate in solution. Examples include $(M+H)^+$, $(M-H)^-$, $(M+Na)^+$, and $(M+NH_4)^+$.

Reconstructed Ion Trace – A display of the abundance or intensity of a single ion signal as a function of time during an analysis. Often also called an "extracted ion chromatogram" (EIC) or "reconstructed ion chromatogram" (RIC).

Significant Ion – Any ion in the mass spectrum of a substance present above a specified intensity or abundance.