Title: Procedure for Mass Spectral Comparisons (CAS-05)

1. Purpose

Many of the analytical procedures used within the Chemical Analysis Section (CAS) units rely on mass spectrometry to help establish identification of individual chemical entities within evidentiary samples. In order to ensure consistency and reproducibility in compound identification it is a good practice to have guidelines for the comparison of mass spectra between unknown and known analytes. Such practices decrease subjectivity and bias during data evaluation and increase consistent reporting results among analysts.

2. Scope

This document provides guidelines to help determine what constitutes a possible correlation between known and unknown mass spectra. Characteristics of mass spectra are described and procedures for using these characteristics when helping to determine whether there is a possible correlation between known and unknown spectra are listed. This document provides guidelines for the matching of mass spectra between analytes and does not directly address compound identification. Another resource for determining analyte identification is the use of a software algorithm (e.g., quality mass spectral matching within instrument software). These resources will normally provide only one aspect when trying to establish the identity of an unknown analyte. This procedure is intended for applications which involve full scan, tandem, and/or selected ion monitoring (SIM) mass spectra acquired in electron impact ionization (EI), chemical ionization (CI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) modes. Either positive ion (PI) or negative ion (NI) spectra are also applicable to this procedure. Other mass spectral techniques are beyond the scope of this document. Specific mass spectral comparison guidelines in individual analytical standard operating procedures (SOPs) will override any guidelines set forth in this document. This procedure can be used as a tool and final determination whether an analyte is identified or not rests on the analyst who is issuing the result.

The use of this procedure is intented for analyte identification (e.g., drug, metabolite, specific chemicals). When reporting classes of chemicals (e.g., gasolines, distillates, isoparaffins, aromatics) this procedure may not necessarily apply. However, if identification of analytes within a class is a requirement in order to identify a specific class of chemicals, then this procedure will be used. For example, if *m*-ethyltoluene, *p*-ethyltoluene, 1,3,5-trimethylbenzene, *o*-ethyltoluene, and 1,2,4-trimethylbenzene are required to be present in order to report that gasoline was identified in a submitted piece of evidence, then this procuedure will be used to evaluate the mass spectral comparison of those analytes.

3. Responsibilities

This document applies to section personnel who contribute, or assist in contributing, results within a laboratory report according to relevant accreditation guidelines.

4. Specimens

Not applicable.

5. Equipment

Not applicable.

6. Standards and Controls

Not applicable.

7. Calibration

The verification of mass spectrometer calibration should be performed regularly but the description of such verification is beyond the scope of this procedure.

8. Sampling

Not applicable.

9. Procedure

Provided below are procedures for defining mass spectral characteristics which will be used when aiding analysts in determining possible identifications of analytes.

9.1 Averaging and Background Subtraction of Mass Spectra

It is good practice to correct analyte mass spectra in order to minimize ions resulting from sample [matrix] background, instrument background, or partially co-eluting materials. A background-subtracted spectrum is often necessary and can be generated by averaging spectra across a [chromatographic] peak and then subtracting (or taking the ratio of) the average of background spectra. The background spectra may come before and/or after the sample spectra and should be selected from outside any region used for ion ratio evaluations. Background-subtracted spectra will be used to establish a list of significant ions as well as base peaks within spectra.

9.2 Determination of 'Significant Ions' in Mass Spectra

Any ion signal greater than 15% of the base peak (i.e., most intense ion signal) within 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 interferent (e.g., background). Such interferences can be demonstrated by showing that a reconstructed ion trace (i.e., ion chromatogram) for the questionable ion is not consistent with the associated ion trace from an analyte of interest's ion(s).

Note-01: For Tandem MS data this value is 10% instead of 15% (see later section for explanation).

9.3 Determination of 'Diagnostic Ions' in Mass Spectra

Diagnostic ions are those ions within a mass spectrum that are characteristic of a particular analyte. The determination of diagnostic ions depends upon knowledge of an analyte's chemical structure and thus needs to be determined from known [reference standard] mass spectra. There is not a universally accepted standard for determining diagnostic ions, however, the following recommendations should be considered.

- Optimally, diagnostic ions should be significant ions.
- Adduct ions will normally be excluded. However, one pseudo-molecular adduct ion may be considered as a diagnostic ion.
- Isotopomers will be excluded unless they are characteristic of a specific analyte.
- 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.
- One pseudo-molecular ion for an analyte may be considered diagnostic unless the intensity for that ion is less than 15% of the intensity for the base peak in the background-subtracted spectrum of the analyte in question.

Note-02: Pseudo-molecular ion formation is an artifact common to most analyses performed by electrospray ionization mass spectrometry. These species are non-covalent complexes formed between an analyte of interest and any other components (such as mobile phase, additives, and impurities) present in the ionized sample.

Note-03: Isotopomers (or isotopic isomers) are isomers with isotopic atoms having the same number of each isotope of each element but differing in their positions. The result is that the molecules are either constitutional isomers or stereoisomers solely based on isotopic location. Normally this will be limited to chlorine and bromine but other possibilities may arise.

9.4 Determination of the Base Peak in Mass Spectra

The base peak within a known analyte's background-subtracted mass spectrum is a diagnostic ion with the most intense signal. For the purpose of determining ion ratios the base peak will be determined from the known reference analyte's mass spectrum, even if a different ion peak is higher in intensity in the unknown's mass spectrum. If two ions have the same intensity then either one can be used.

In instances where it can be demonstrated that the nominal base peak signal is significantly disturbed by an uncorrectable chemical interference then the second most intense diagnostic ion present in the analyte spectrum may be considered its base peak. Such an interference will normally be demonstrated by showing that a reconstructed ion trace (i.e., ion chromatogram) for the ion in question is not related with the traces for other ions associated with the analyte of interest.

9.5 Calculating Ion Ratios

Ion ratios will normally be determined by integrating reconstructed ion traces for each diagnostic ion present in a given analyte. While using automatic integration is acceptable, all integrations of reconstructed ion chromatograms from a given analyte's ion should have comparable starting and ending points. This can be set up through the display settings of the processing software (e.g., set the display window to only fit the beginning and ending times of the extraction ion's chromatographic peak). Areas of the integrated peaks are obtained and recorded (e.g., using ChemStation's 'Percent Report' feature). Ion ratios can be calculated by dividing each diagnostic ion's integrated peak area by the corresponding base peak ion's integrated peak area and expressing the results as percentages. 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 analyte 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. Sampling

Not applicable.

11. Decision Criteria

Provided below are guidelines for establishing a correlation between a known mass spectrum and that of an unknown spectrum. Unknown spectra should be matched against known spectra obtained from contemporaneously analyzed reference material which have been collected using similar mass spectral parameters. There are exceptions, however, and these will be discussed elsewhere within this procedure. 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 different [contemporaneously analyzed] references. The mass spectra of analytes are known to vary based on concentration and sample injection load into instrumentation. Dilution and/or re-analysis of samples in order to be able to more appropriately match concentration levels and then using subsequent data for mass spectral evaluation is acceptable.

11.1 Full Scan Mass Spectra

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

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

11.1.2 All ion ratios for diagnostic ions for the unknown analyte should correlate to those observed for the known analyte within certain tolerances (see the following tables). If a known analyte's ion ratio is less than 1% for a given diagnostic ion then that ratio may be administratively set at 1%. Specific diagnostic ion ratios may be excluded from consideration if they meet any of the following criteria:

11.1.2.1 A diagnostic ion ratio within a known spectrum is less than 5% (less than 10% for CI, ESI, or APCI spectra).

11.1.2.2 The signal-to-noise ratio of the reconstructed ion trace for an ion for the unknown analyte is less than three (3).

11.1.2.3 It can be shown that the signal for an ion in either the known or the unknown analyte 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 does not behave similar to other diagnostic ions associated with the analyte of interest.

Only a maximum of four (4) diagnostic ions (three (3) ratios) within an known analyte's spectrum need to be used for this evaluation. For analytes with a molecular weight less than 80 AMU, or which possess less than eight (8) atoms, only three (3) diagnostic ions (two (2) ratios) need to be evaluated. The selected ions will normally include the base peak, the pseudo-molecular ion, and fragment ions which make the selection criteria. If fewer than three (3) diagnostic ions are available for evaluation then spectra may still be evaluated – but information derived from such evaluations may be limited. Scan ranges should be chosen to provide an adequate 'buffer space' around the diagnostic and significant ions of analytes. A reasonable variety of diagnostic ions should be used. The final number of diagnostic ions is not a strict number and is based on the characteristics of mass spectra, the technique used, and other factors. Thus, more or fewer than four (4) diagnostic ions can be used in this procedure.

Table-01: Ion Ratio Matching Tolerances for EI 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:	10% Absolute	20% Relative	5% Absolute

Table-02: Ion Ratio Matching Tolerances for CI, ESI, and APCI 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:	15% Absolute	25% Relative	10% Absolute

Note-04: Absolute versus Relative percentages
Relative means the percent relative to the number being evaluated. When calculating the acceptability range this percentage is multiplied by the criteria number and then added/subtracted (Example: Ion ratio of 70% with a criteria of 10% Relative means the acceptable range is 63% to 77%).
Absolute means the range used for the number being evaluated is calculated using an addition/subtraction of the actual value that is listed (Example: Ion ratio of 70% with a criteria of 10% Absolute means the acceptable range is 60% to 80%).

11.2 Selected Ion Monitoring (SIM) 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 for that experiment. Ions for a SIM experiment must be based upon a known full scan spectrum of an analyte which has been collected on the same instrument. Four (4) diagnostic ions will normally be selected (three (3) diagnostic ions for analytes with a molecular weight less than 80 AMU or less than eight (8) atoms), and, if possible, all should be significant as well as diagnostic. The base peak will normally be one of the chosen ions and a pseudo-molecular ion should be included if it has an ion ratio greater than 5% in the known full scan spectrum. In order to establish a correlation between a known SIM

spectrum and an unknown SIM spectrum all resulting ion ratios should meet the tolerances specified in the tables above, as appropriate.

11.3 Tandem Mass Spectra

Tandem mass spectrometry can provide additional specificity because confidence in increased in the assumption that the ions within a given spectrum are all associated with a single analyte. Due to the nature of most collision-induced dissociation (CID) processes, however, analyte ion ratios from tandem mass spectral experiments tend to be much less stable and much more dependent on analyte concentration (or sample load) as compared to classic electron impact (EI) mass spectral ratios. Tandem mass spectra tend to be much 'cleaner' than full scan mass spectra and usually contain fewer extraneous ions. Therefore for data from a tandem MS experiment, any ion signal greater than 10% of the base peak (i.e., most intense ion signal) within a background-subtracted mass spectrum will be considered a significant ion. 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.

Within an ion trap mass spectrometer, due to the physical processes involved in the precursor ion isolation and fragmentation events, tandem mass spectra acquired on such instrumentation will occasionally show 'ion-splitting' artifacts for precursor ions. 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 analyte under consideration. With product ion spectra it is critical to ensure that the observed fragment ions are, 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:

- The precursor ion should be observed in the product ion spectrum with an ion ratio of at least 5%.

- If full scan mass spectral data are collected concurrently with the product ion spectra then the full scan spectra of the component of interest should show no ions within 1.5 AMU of the precursor ion with greater than three times (3x) the intensity of that precursor ion.

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

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

- All ion ratios for diagnostic ions for the unknown analyte should correlate to those observed for the known analyte within certain tolerances (see the following table). If a known analyte's ion ratio is less than 1% for a given diagnostic ion then that ratio may be administratively set at 0.5%. Specific diagnostic ion ratios may be excluded from consideration if they meet any of the following criteria:
  - The ion ratio for that ion in the known spectrum is less than 5%.
  - The signal-to-noise ratio of the reconstructed ion trace for that ion in the unknown spectrum is less than three (3).
  - It can be shown that the signal for an ion in either the known or the unknown analyte 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 does not behave similar to other diagnostic ions associated with the analyte of interest.

For product ion experiment spectra only a maximum of three (3) diagnostic ions (two (2) ratios) within a known analyte's spectrum needs to be used for this evaluation. The three selected ions should include the base peak and the precursor ion (if present) unless those ions met one of the three exclusion criteria given above. If only a single diagnostic ion is observed in the product ion spectrum then spectra may still be evaluated but information will be limited.

Table-03: Ion Ratio Matching Tolerances for MS/MS Spectra

If the ion ratio in the known spectrum is:	> 40%	≥ 40%
Then the ion ratio in the unknown spectrum should be:	25% Relative	10% Absolute

11.3.2 Precursor Ion and Neutral Loss Experiment Spectra

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 analyte. For precursor ion experiments a correlation between a known and an unknown analyte 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 correlation between a known and an unknown analyte spectrum may be established if all significant transition pairs are present in both the known and unknown spectra.

11.3.3 Selected Reaction Monitoring (SRM) Experiment Spectra

Selected Reaction Monitoring experiments share many features, advantages, and limitations with SIM-type experiments, but they benefit by having the added specificity afforded by tandem mass spectrometry. Two or three diagnostic ion transitions may be chosen for an SRM experiment. Generally 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 analyte 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 is not practical, then the chosen precursor ion should be both significant and diagnostic in the full scan analyte spectrum. In order to establish a correlation between a known analyte SRM spectrum and an unknown analyte SRM spectrum, the ion ratio of the unknown should be within ±10% (relative) of the ion ratio of the known when only two (2) transitions are monitored. When three (3) transitions are monitored then both resulting ion ratios should meet the criteria specified in the MS/MS table above.

11.3.4 Higher Order (MS^n) Tandem Mass Spectra

Tandem mass spectra of order higher than two (MS/MS ; MS^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 not frequently used except for special circumstances, such as overcoming chromatographic obstacles. When used, higher order tandem mass spectra will be addressed on a case-by-case basis and will usually be part of a method's validation. The criteria for product ion MS/MS in the previous section may be used as a starting point for such evaluations.

11.4 Exact (Precise ; Accurate) Mass Spectra

Exact mass measurement can provide a significant level of information to analyte identification. The use of exact mass measurement techniques does not, however, allow other aspects of mass spectral evaluation to be disregarded. 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 when selecting diagnostic ions. More confidence can be placed in data evaluations when limited diagnostic ions are available.

Ions in an unknown analyte spectrum can be considered a correlation to those in a known analyte spectrum when the measured masses agree to within 0.005 AMU. When determining diagnostic ions any isotopomer of a pseudo-molecular ion may be considered diagnostic if it meets the exact mass match criterion. One additional adduct ion, beyond the pseudo-molecular ion, may also be considered diagnostic if it meets this exact mass match criterion.

11.5 Using Library Spectra

While mass spectral libraries (either obtained commercially or generated in-house) can be valuable tools when assisting analysts in determining analyte identification, there are limitations to their use. Most commercial libraries do not clearly indicate the instrumentation that spectra were acquired or the level of sample loading. In-house library data may have been acquired on the same instrumentation used to obtain a given unknown analyte spectrum but it is very difficult to ensure that long-term drift in instrument performance has not compromised the reproducibility of in-house generated spectra. Also, algorithms that are used for mass spectral comparisons are not always known and interpretation of library matching percentages can be subjective.

Despite these limitations there may arise a rare instance in which it is necessary to compare an unknown spectrum to a library entry. For example, if an analyte standard cannot be readily obtained or, for purposes of screening, in order to direct further investigation, such library data can be used for comparisons. In cases where such correlations are attempted, all criteria for the appropriate type of mass spectrometry (given above) can be used. Reporting of an analyte as being identified should involve comparison of unknowns to known analyte references. Absent reference samples for comparison, unknown substances may be considered 'consistent with' known analytes if given enough correlation to library (or in-house generated) data. Using such a description within a laboratory report, however, should include a definition as to how it differs from an identification description and why the term was used.

12. Calculations

$IR_d = (A_d/A_b) \times 100$

IR_d = Percent ion ratio for Diagnostic Ion 'd'

A_d = Integrated area of the reconstructed ion trace for each diagnostic ion 'd'

A_b = Integrated area of the reconstructed ion trace for the base peak diagnostic ion (Ion abundances from background subtracted mass spectra may be substituted for integrated areas under certain circumstances detailed in the above 'Calculating Ion Ratios' section)

Example (PI/EI Data):

		Heroin				RT Range
		Diagnostic Ions (m/z)				13.04-13.26
Specimen		**327**	**268**	**310**	**369**	
Pos. Ctrl. Heroin	response	249088	138377	122839	175128	
mpr012914_12.D	ion ratio	100.0	55.6	49.3	70.3	
Item 001-001	response	120567	67966	61484	85784	
mpr012914_04.D	ion ratio	100.0	56.4	51.0	71.2	
	pass / fail	**PASS**	**PASS**	**PASS**	**PASS**	

13. Measurement Uncertainty

Not applicable.

14. Limitations

This procedure, while extensive, is not intended to be exhaustive. Known limitations for specific analytes will be documented in individual analytical procedures. This procedure should be used by analysts when determining how to report an analyte's presence within evidentiary materials. Failure of criteria described within this procedure will not limit or preclude an analyte from being reported if enough data and collaboration between reviewing analysts can support such findings.

15. Safety

Not applicable.

16. References

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Rev. #	History
1	New document.