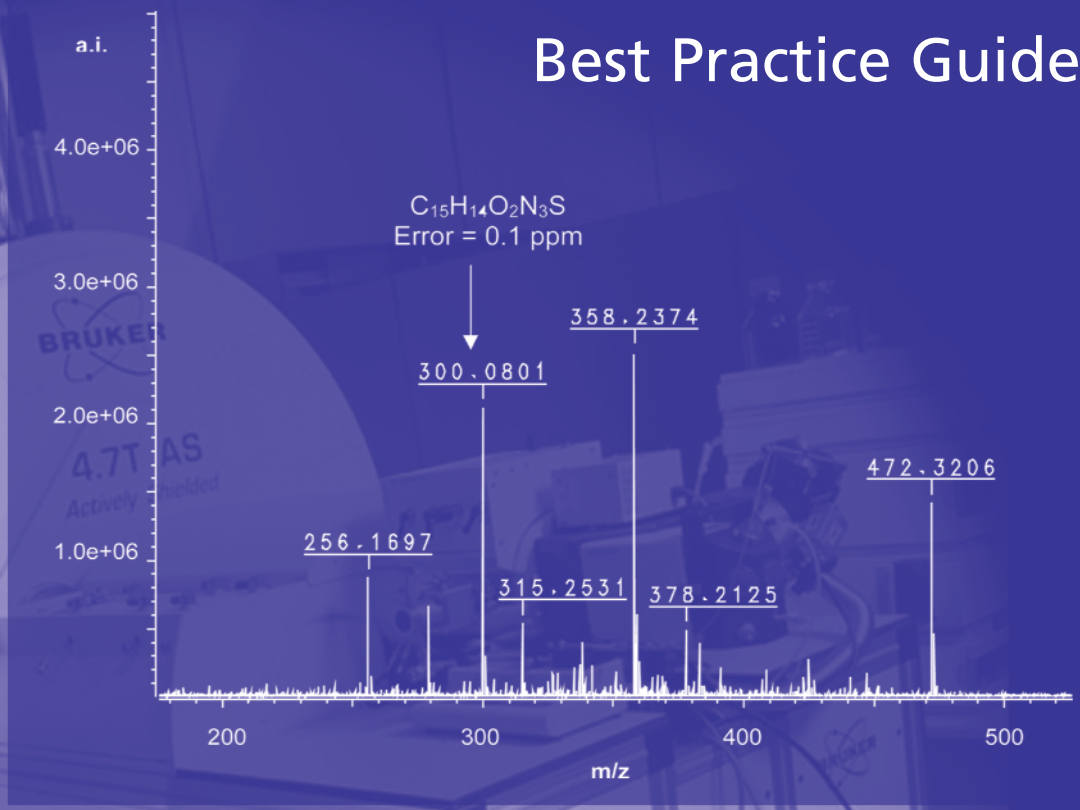
Methodology for Accurate Mass Measurement of Small Molecules

Best Practice Guide



Co-ordinating Editors

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Preface

This Guide was prepared as part of the Department of Trade and Industry's VIMMS Programme, an initiative which formed part of the UK National Measurement System. The Guide was prepared by LGC in collaboration with members of a specially formed Working Group whose assistance is gratefully acknowledged. The members of the Working Group during the course of the work are listed below.

In the past the phrase "accurate mass" was interpreted very broadly and covered a wide variety of mass spectrometry measurements, with varying precision. Today, most instruments used for accurate mass measurements are capable of achieving precisions of 10 ppm or better. This Guide is concerned with application to small to medium size molecules and resulted from a review[1] of accurate mass applications which was undertaken as part of the VIMMS Programme. The review[1] highlighted the recent rapid growth in the use of mass spectrometry for molecular weight determinations of small to medium sized molecules, particularly in the chemical and biochemical industries. This growth has been fuelled by a number of factors, including the rapid pace of instrument development, enabling accurate mass and molecular weight measurements to be made in a more robust and less costly fashion. A number of experts have expressed concern that this rapid growth has resulted in unreliable data being obtained by operators who often have little experience of AccMass applications or even of mass spectrometry.

Discussion of the issues by experts at a forum meeting highlighted the need to prepare guidance on undertaking key aspects of the methodology in order to obtain robust measurements and traceable data. It was emphasised, however, that preparation of such guidance should be supported by an experimental evaluation of the methodology, including the implications of variations between type of spectrometer. Consequently, an inter-laboratory comparison[2] was organised by LGC as part of the VIMMS programme to evaluate variations in accurate mass measurements and to improve understanding of the key experimental factors in obtaining reliable data from each type of mass spectrometer. The lessons learned from this study have played a major role in shaping the advice offered in the Guide.

The main aim of this document is to provide users and suppliers of AccMass instrumentation with a clear summary of the essential steps in obtaining reliable data. In addition, the reader should obtain a better understanding of the limitations of different types of spectrometer and the particular precautions which are necessary in setting up the instruments and making measurements. We hope that the Guide will also facilitate an understanding of the principles of the technique and prove useful for both private study and training courses. A glossary of the terms used in this Guide has been included to assist in its use for training purposes.

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November 2004

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1. Introduction

Accurate mass measurement of small molecules is used to determine elemental formulae. The better the accuracy the less the ambiguity[3]. In general, the interpretation of an accurate mass measurement carried out at a resolution of 10,000 (10% valley definition) is non-ambiguous up to 300 daltons (Da) when only including C, H, N and O due to the low number of potential elemental formulae. With increasing mass to charge ratio (m/z) the number of possible formulae dramatically increases making identification more and more difficult[4]. The increasing variety of instrumentation together with the increasing numbers of new entrants into the field of mass spectrometry has created an urgent need for clear and informative guidance.

FTMS currently offers the highest mass resolution of any mass spectrometer[5], exceeding that of the traditional high resolution magnetic sector instruments. Though not usually considered as tools for accurate mass measurement, lower resolution quadrupole, triple quadrupole, quadrupole ion trap and *TOF* mass spectrometers have also been applied to such measurements. Accuracy of measurement using quadrupole ion trap instruments varies with the mass and the type of ion and is generally no better than 50 ppm. Hence ion trap instruments have not been included in this guidance document.

In order to produce guidance based on sound experimental knowledge, a study to evaluate a wide range of accurate mass measurement techniques was undertaken[1]. This study has enabled the editors to collate experience of the various accurate mass measurement techniques in a single document and to provide comprehensive advice on best practice for accurate mass measurement. This was followed with an inter-laboratory comparison involving a large number of expert users and was co-ordinated by LGC in 2002. It should be noted that instrumentation is always developing and mass measurement accuracy is constantly improving; this is particularly true of TOF instruments. The inter-laboratory comparison data relates only to the instrument capabilities in 2002. The results of the inter-laboratory comparison, which involved the accurate mass measurement of a compound of molecular weight 475 with no interfering ions present, have recently been published[2].

Irrespective of instrument type and the experience of the user, the instrument must be calibrated over an appropriate mass range incorporating the mass of interest by using a suitable reference material. This primary calibration should be established where possible with traceable or authenticated materials and confirmed from time to time by measuring a reference material.

A glossary of the terms used in this guide and a bibliography have been included to assist in the use of this document for training purposes.

2. Background

2.1 General Issues

An accurate and precise mass measurement increases the certainty of identification of an elemental formula. If accuracy and precision (*i.e.* the measurement uncertainty) are known, these values can be used to reduce the number of candidate molecular formulae to be considered. For example, if the mass measurement accuracy of 5 ppm (±2 ppm precision) is routinely achieved, mass measurements to within 7 ppm should be considered in the initial data

evaluation, rather than the top 10 or 20 hits from the results table, which is the more usual approach. This is particularly important for determining the elemental formula of a complete unknown. Each type of analyser will have unique parameters that will contribute to the overall uncertainty of the accurate mass measurement. Very little work has been carried out on this subject but some initial work has been described[6,7]. Although not fully described here, guidance to the general concept and principles of measurement uncertainty is available[8].

It is important to understand the degree of accuracy required relative to the m/z of the ion that is measured. Setting fixed acceptable error limits for exact molecular mass measurement is not recommended[4]. With increasing m/z the number of formulae which will fit a measured molecular mass increases until an unambiguous result becomes impossible to obtain and this is illustrated in Figure 1[9].

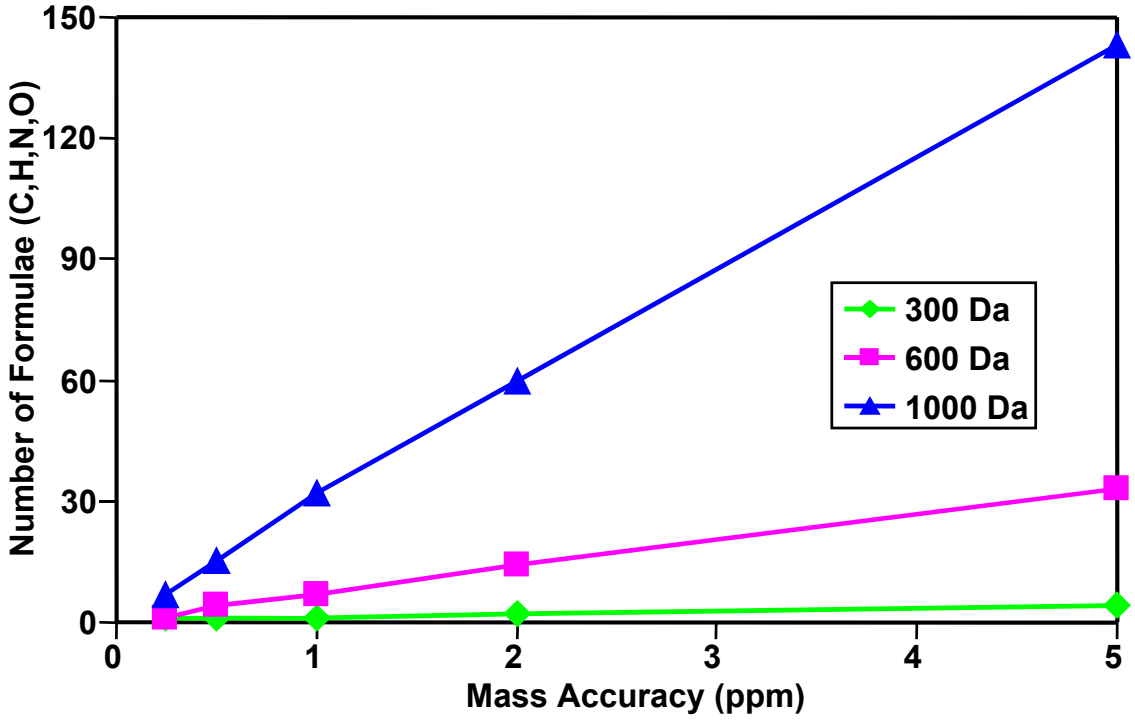

Figure 1: The effect of mass accuracy and molecular weight on the number of potential elemental formulae (source: Quenzer, T. L. *et al.*[9]).

It is also important to distinguish between the integer mass of the nominal mass ion, the calculated exact mass and the average mass. Table 1 below illustrates the different numerical values resulting from these definitions[10] for several molecules.

Table 1: The distinction between common definitions of molecular mass (uncharged species)

	Formula	Molecular Mass (See Glossary for definition of terms)		
		Nominal	**Exact**	**Average**
Sulphamethazine	$C_{12}H_{14}N_4O_2S$	278	278.0837	278.3313
Ketaconazole	$C_{26}H_{28}Cl_2N_4O_4$	530	530.1482	531.4306
Ubiquitin	$C_{378}H_{630}N_{105}O_{118}S$	8556	8560.6254	8565.8730

Sulphamethazine has an average mass of 278.3313, a nominal mass of 278 and a calculated exact monoisotopic mass of 278.0837. Ketaconazole contains two chlorine atoms; the nominal mass ion contains two ^{35}Cl atoms whereas the mass of the average mass ion is calculated using the natural abundance ratio of the ^{35}Cl and ^{37}Cl atoms. At much higher masses the effect of the different definitions can be considerable. Ubiquitin, a protein with a formula $C_{378}H_{630}N_{105}O_{118}S$, has an average mass of 8565.873, a nominal mass of 8556 and a calculated exact monoisotopic mass of 8560.6254. Note that the presence of several hydrogen atoms increases the exact monoisotopic mass above an integral value and when more than some 130 hydrogen atoms are present the exact mass is one or more units higher than the nominal mass. This is because the ^{1}H isotope has an exact mass of 1.0078 Da. The 630 hydrogen atoms in Ubiquitin have a combined exact monoisotopic mass of 634.914, which very significantly increases the calculated exact mass value over the nominal mass (630).

Factors such as temperature, humidity, vibration and local magnetic fields can affect instrumental stability and hence reproducibility of results. It is important to ensure that the temperature is both within the manufacturers' specification range and is also stable; this is of particular importance for *TOF* instruments. A varying temperature will cause an external calibration to drift producing poor mass measurement accuracy. There may be a lesser, but still significant, effect on internal calibration. High humidity can produce condensation on electrical components and high voltage arcing, causing instrument damage. Hence it is advisable to install air conditioning for all accurate mass work. Vibration will arise from lorries, trains, tram lines and lifts. The floor on which the instrument is situated should also be considered, the higher the floor number the worse is the potential for vibration problems. Local magnetic fields such as those caused by other 'magnet scanning' instruments or large machinery such as lifts nearby are important.

2.2 Instrumentation

Magnetic sector instruments offer high mass resolution (up to 150,000 (10% valley, see Section **3.3**)) and have traditionally been used for many years for accurate mass measurement. *FTMS* instruments are capable of very high resolution (up to 1,000,000 (FWHM)), but have only started to become popular during the last few years. Lower resolution instruments such as *TOF*, quadrupole-*TOF* and triple quadrupoles have not been traditionally employed to record accurate mass measurements. However, recent advances in instrument design have made this possible to some extent. Such instruments are compact, generally bench-top, relatively easy to use, rugged and may not require purpose-built accommodation. The cost and ease of use of the different types of instruments vary widely and should be taken into consideration when selecting an instrument. There are a number of different ion sources used to generate ions for

accurate mass measurement. In general the choice of ion source has no significant effect on mass measurement accuracy; however, it is generally accepted that the accuracy using *FAB* or *MALDI* can be lower than with the use of other sources, possibly due to matrix effects.

The inter-laboratory comparison co-ordinated by LGC[2] showed that in many cases an identical protocol and mass spectrometer were used by several participants to make the mass measurements, but an equivalent performance was not reported. This illustrates the need for guidance on the appropriate application of the various protocols employed. The overall observations from the inter-laboratory comparison are summarised below.

In the inter-laboratory comparison the high accuracy achievable when using magnetic sector instruments was readily demonstrated. The results showed that for the three common mass measurement techniques reported when using such instruments, peak matching and dynamic voltage scanning clearly produced the most accurate results, with 88% and 60% of mean mass measurements ≤ 1 ppm, respectively. Only 50% of the laboratories that employed dynamic magnet scanning experiments reported data with mean mass accuracy of ≤ 10 ppm. The results clearly reflect the accepted capability of the three techniques. Further details of the peak matching, dynamic voltage scanning and the dynamic magnet scanning experiments can be found in Section **4.1**.

Using *FTMS*, 83% of the results reported showed mean mass measurement accuracy of ≤ 1 ppm. This result is comparable with that from peak matching and superior to that recorded using dynamic voltage scanning on magnetic sector instruments and meets the expectation of the capability of *FTMS* for accurate mass measurements.

For the lower resolution instruments, quadrupole-*TOF* spectrometers produced the highest accuracy measurements, with 83% of results within 5 ppm compared with 65% from conventional TOF instruments. This may be due as much to the optimisation of current protocols as to any deficiency in instrument capability. *ESI* data was obtained from either orthogonal-*TOF* or orthogonal quadrupole-*TOF* instruments. *MALDI* data was obtained from either axial-*TOF* or orthogonal quadrupole-*TOF* instruments. The orthogonal-*TOF* data showed accuracies with the majority within 10 ppm, whilst the axial-*TOF* data showed accuracies within 25 ppm (see also Figure 4). The capabilities of *TOF* and quadrupole-*TOF* mass spectrometers have only recently been enhanced to allow accurate mass measurements to be recorded.

Only two triple quadrupole instruments were used in the inter-comparison and these produced mean mass measurement accuracy of < 2 ppm. These results illustrate the capability of lower resolution mass spectrometers when a sound experimental protocol is used and there are no unresolved isobaric ions. The way in which the measurement is carried out is critical in achieving high accuracy.

3. Best Practice

In order to achieve high accuracy and good precision a number of key considerations must be understood and optimised where applicable.

1 Tuning and peak shape

2 Ion abundance

3 Resolving power

4 Calibration

5 Sample introduction

6 Data manipulation

7 Validation and Quality Control checks

8 Selection of elemental formulae

These considerations are considered in turn in the following sections.

3.1 Tuning and Peak Shape

As accurate mass measurements are generally carried out with the mass spectrometer operating in peak profile (continuum) mode, the centre of the peak (centroid) on the m/z scale must be accurately assigned. An accurate measurement can only be obtained if there is a homogeneous (mono-isotopic) peak that is symmetrical (an exception here are *TOF* peaks, which are not symmetrical). Regardless of the type of mass spectrometer employed to record accurate mass measurements, poorly defined peaks will result in mass measurement error and poor precision.

The peak shape can be affected by poor instrument tuning, low ion counts, unresolved interferences, mechanical vibration and electronic ripple. Tune the instrument carefully – this should include optimising ionisation conditions, which increases ion counts, and optimising ion transmission from source to analyser and detector which influences peak shape. Consider the use of chromatographic separation techniques to remove unresolved interferences where possible. The effect of poor peak shape on mass accuracy when using *FTMS* is illustrated in Figure 2.

3.2 Ion Abundance

Ion abundance is important in the determination of accurate mass both in terms of being too high or too low. For high intensity signals there is a danger of saturating the detector. The mass measurement of a low intensity signal can result in poor mass accuracy because of poor peak shape and poor ion statistics, which means the centroid is not accurately defined on the m/z scale.

The mass measurement accuracy of the *TOF* or quadrupole-*TOF* mass spectrometer is impaired with high ion counts due to time-to-digital converter (TDC) "dead time", a characteristic of the detectors used in these instruments. This is the time after each ion is recorded when the TDC is not able to record another ion count. The mass accuracy of the *FTMS* can be affected by space charge effects; the best mass accuracy with *FTMS* is achieved when the total number of ions in the analyte experiment is equal to the total number of ions used in calibration[11]. If either phenomenon is encountered a reduction in ion count should be achieved by reducing analyte concentrations or the amount of analyte introduced (injection volume).

When using magnetic sector instruments the three techniques (peak matching, dynamic voltage scanning and dynamic magnet scanning) used for accurate mass measurement are not adversely affected by ions at high abundance unless detector saturation is reached. Low abundance ions can be detrimental to mass accuracy as they may lead to poor peak shape and inaccurate peak centroids.

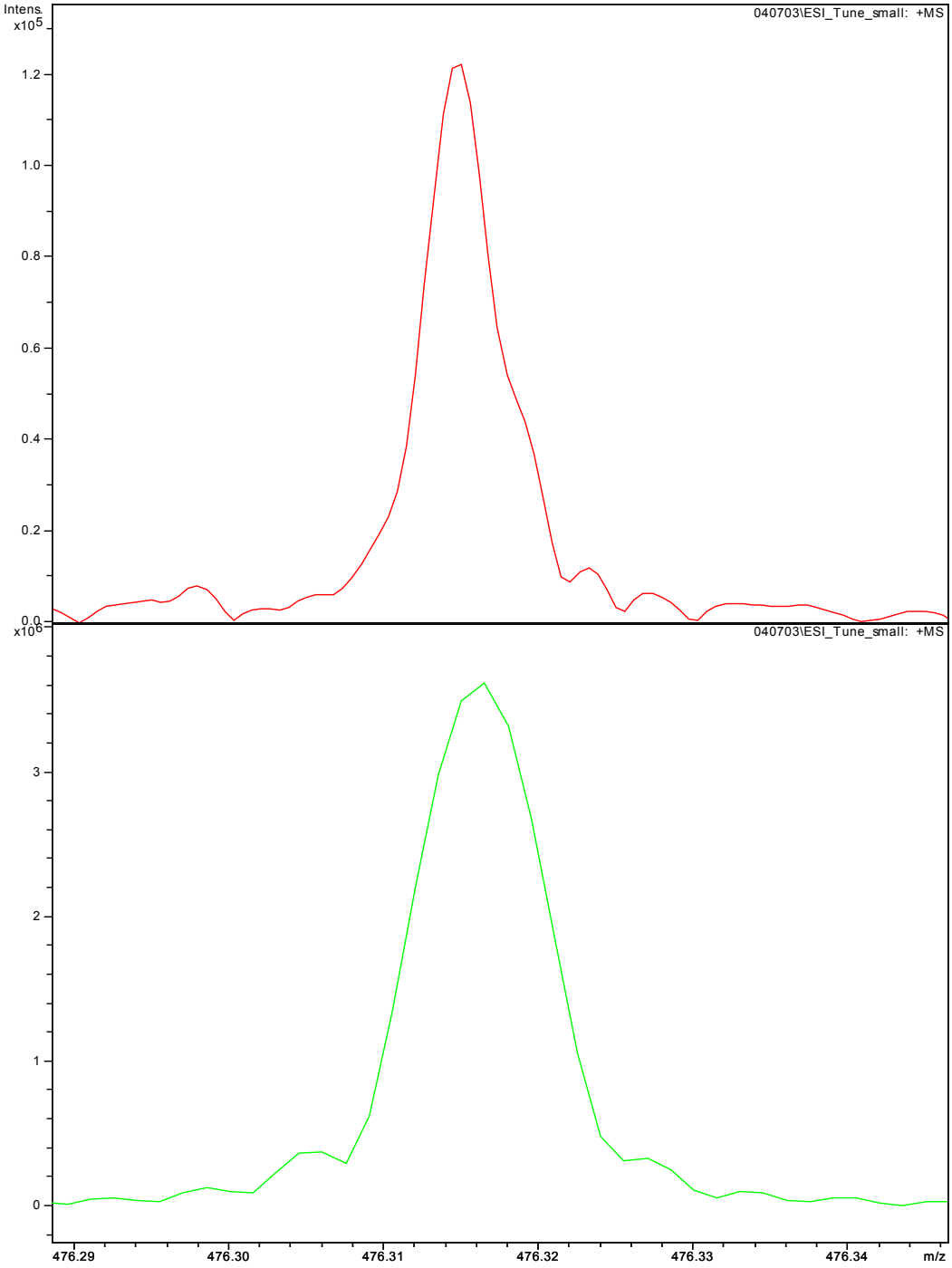

Figure 2: The effect of *FTMS* peak shape on mass accuracy, top = 2.5 ppm, bottom = 0.5 ppm

Ion abundance is also important when calibration is carried out. See Section **3.4.2**.

3.3 Resolving Power

Two definitions are used routinely, depending on the type of instrument employed to make the mass measurement.

10% valley (intensity) definition: this is useful only for instruments giving triangular-shaped peaks (*e.g.* magnetic sector instrument peaks). Two peaks of equal intensity are considered to be resolved when they are separated by a valley which is 10% of the height of each peak (made up from a 5% contribution from each component) (Figure 3.) In practice, by this definition a resolution of 1000 means that m/z 1000 and m/z 1001 have a 10% valley between them. Δm can be measured from the separation of the two peaks or from the width of a single peak at the 5% height (Δm is the difference between m and the value of the next highest m/z value ion that can be separated from m, in m/z units). This definition has been traditionally used with magnetic sector instruments.

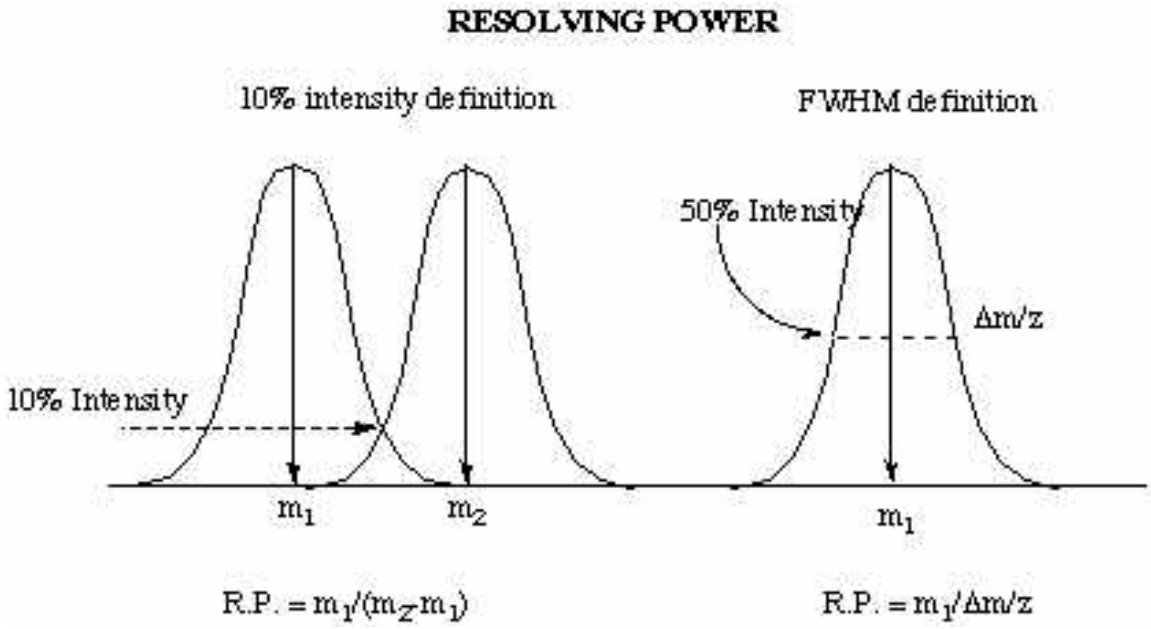

Figure 3: The definitions of resolving power (source: courtesy of Professor Tony Mallett)

50% valley (intensity) definition (full width half maximum, FWHM): the quadrupole, *FTMS*, ion trap and *TOF* definition is based on a peak width measured at 50% peak height, producing a value approximately double that calculated using the 10% valley definition (comparison only valid for instruments giving gaussian-shaped peaks (*e.g.* magnetic sector instrument peaks).

The resolving power required for an accurate mass measurement is determined by the measurement problem (factors here include the m/z values to be resolved, the presence of background interferences) and therefore this determines the instrument that can be used. Increasing the resolving power of the mass spectrometer narrows the peak widths. The result of this is that data systems can centroid the peaks more accurately, reducing the error of mass measurement and decreasing the ambiguity. However, increasing the resolving power decreases the signal strength on some analysers (for example, magnetic sector) and this can affect the precision of measurement. In these cases a balance must be achieved and it is advised that the resolving power used should not exceed that required to resolve background interferences or other m/z values that may need to be resolved. This will limit the selection of

the instrument. For an accurate mass measurement it is essential that interferences are resolved.

3.4 Calibration

Calibration of the m/z scale of the mass spectrometer is achieved using a reference compound yielding ions of known m/z. Appropriate instrument calibration is vital for good mass measurement accuracy. Calibration should cover the complete range of analyte masses; extrapolation of the calibration range will not give good results.

Mass calibration is one of the most critical parameters to consider in the achievement of accuracy. If the calibration is poor then even a high precision mass measurement will be poor in terms of accuracy. Two calibration protocols are used with accurate mass measurement, as described in the following sections. External calibration is generally necessary; the additional use of an internal calibrant can substantially improve accuracy and is necessary on certain instruments.

There are some important considerations that apply to both external and internal calibration:

- Ensure that the reference compound has many reference ions, particularly in the region of the ions to be accurately mass measured
- Ensure that the ions are of a similar nature where possible; for example, the calibrant ion and analyte ions should be in the same charge state.

3.4.1 External Calibration

In this case calibration is carried out prior to analysis. The spectrum of a known reference compound is recorded and the peaks in the spectrum are assigned to their exact masses by the instrument data system to produce the correct calibration. Clearly a good external calibration is required to obtain good mass accuracy, particularly when an internal calibrant (reference mass ion) is not used for subsequent accurate mass measurements. It should be noted that external calibration only works satisfactorily if the instrument is sufficiently stable.

Tips for achieving a good external calibration:

- Carry out the calibration as close in time as possible to the accurate mass measurement to minimise effects of instrument drift
- Maintain temperature control of the instrument environment to minimise drift.

There are many compounds employed as external calibrants for mass spectrometry and their selection is determined by the ionisation technique to be employed and the working m/z range. Details of many are supplied by the instrument manufacturers in the literature provided with the instruments, but a list, though not exhaustive of many of the common calibrants and those used in the inter-laboratory comparison, is shown in Table 2.

Table 2: List of common calibrants

Chemical Name	m/z Range	Uses
Sodium iodide + Caesium iodide mixture	20-4000	ES (+ and -) FAB/LSIMS (+ and -)
Perfluorokerosene (PFK)	31-900	EI (+) and CI (+ and -)
Perfluorotributylamine (PFTBA)	31-671	EI (+) and CI (+ and -)
Polyethylene glycol mixture /(PEG 200+400+600+1000)	80-1000	ES (+) and APCI (+)
Peptide mixtures	ca. 200-3000	ES (+)
Horse heart myoglobin	700-1600	ES (+)
Horse radish peroxidase	1800-2900	ES (+)
Ultramark F-series	69-3000	EI/CI

3.4.2 Internal Calibration

This is generally required for all instruments except *FTMS* for which external calibration can achieve sufficient accuracy. With internal calibration the m/z scale is calibrated using a single ion or a series of ions of known m/z from a reference compound that is introduced into the mass spectrometer ion source at the same time as the analyte. This method is superior to external calibration since the effects of instrumental drift are removed, and should be used whenever possible unless ions from the reference compound interfere with the analyte ions.

The use of a single or double point internal calibration is sometimes referred to as lock mass. Usually the use of two calibrant ions to bracket the analyte ion will achieve better accuracy. To achieve optimum mass accuracy the m/z of a single point calibrant ion should be as close as possible to that of the analyte ion; this is particularly important for magnetic sector mass spectrometers.

The abundance of the internal calibrant ion or ions can have a profound effect on mass accuracy[12,13,14]. For best accuracy these ions should be of the same abundance as the analyte ions. Ion abundance and peak shape are major contributors to the mass accuracy achieved using internal calibrant ions regardless of instrument type.

3.5 Sample Introduction

Where a constant ion abundance for the analyte and reference ion can be maintained (*e.g.* via a gas inlet, syringe pump and in some instances a direct insertion probe), particularly in a range that provides optimum performance, the accuracy of the mass measurement will be improved[14].

It is important to consider whether the ion signal generated by the analyte compound is transient or constant. In the use of chromatography, *MALDI* and in some instances the direct insertion probe, where a transient signal is generated, the abundance of the analyte ion at the point of measurement needs careful consideration.

The point on the transient signal where the measurement is taken can be adjusted to optimise the ion abundance. If ion abundance is too high the detector may be saturated, if too low there

may not be sufficient ions for an accurate measurement. When the transient signal is of high intensity the measurement should be taken near the tail of the peak (the end) to avoid saturating the detector. Where it is of low intensity the measurement should be taken across the top of the peak so as to maximise the number of ions used for measurement. It is suggested that where possible, say, five scans are taken and the results averaged. Too high or too low abundance problems of an internal calibrant ion can be avoided by maintaining the abundance at a constant optimum level. The calibrant can be introduced into the ion source at a constant rate or known background interferences can be used as reference points.

The width of a transient signal is relevant to the scan speed of the mass spectrometer. The residence time of the transient signal must be taken into account when selecting the scan speed. For fast (narrow width) transient signals the scan speed must be sufficiently fast so as to record at least five scans of satisfactory intensity across the signal. This is particularly significant when using capillary gas chromatography columns and sector field instruments in magnet scanning mode.

Multiple measurements should be carried out on the unknown to establish the precision of measurement. This is especially true for hyphenated systems when scans across a transient signal may need to be inspected individually to select scans where the signal intensities for the reference and unknown are roughly equal.

3.6 Data Manipulation

There are a number of ways in which the final mass measurement can be determined and a list of appropriate possible elemental formulae generated. For many mass spectrometers, the calculation of the measured mass and generation of a list of elemental formulae are completely automated within the instrument data system. But it should be noted that the post acquisition software parameters can have an effect on the final result and within the software a number of parameters may be manipulated to obtain the final measurement. For example, the points on a peak used to define the centroid and the peak smoothing can both be adjusted and will have an effect on the final mass measurement. Many data systems employ a series of common procedures including those listed below.

- Record spectrum in profile mode
- Baseline subtract raw data (to remove interferences on or near peak of interest)
- Select centroiding conditions and centroid ion of interest
- Adjust external calibration using internal calibration
- Specify conditions for elemental composition calculator (possible elements, precision, charge state, type of ion $[M+H]^+$, $M^{+\cdot}$, $[M+Na]^+$ etc)
- Produce a list of potential elemental formulae based on the measured accurate mass

It should be noted that with some data systems centroiding and internal calibrant correction are automated processes within the experiment and cannot be modified by user input. When using a peak matching unit to record accurate mass measurements with a magnetic sector mass spectrometer the above processing steps are not necessary and the instrument readout reflects the measured mass. For older instruments the last two steps are carried out manually.

One additional point to consider is the parameters used to calculate an elemental formula. Not all data systems take into account the mass of an electron (0.00055 Da) when calculating the

formula from the specified ion, which can introduce an error at very high accuracy (an error of 5.5 ppm at m/z 100). To avoid this systematic error both the analyte and calibrant ions should take into account the mass of an electron or both should ignore it.

3.7 Validation and Quality Control Checks

The reliability of the automated data manipulation process should be checked by using as an analyte a compound of known accurate mass as a quality control (QC) sample to ensure correct data system operation. It is also important to treat the analyte, QC sample and calibrant in the same manner from a data processing point of view. The QC check should also be used to give an indication of the uncertainty of measurement. The uncertainty information from a QC check should be used to reduce the number of candidate molecular formulae to be considered. See also Section 2.1.

In terms of calibration the analyte should be mass measured relative to a suitable internal reference compound wherever possible. This can be used to adjust any drift in the primary calibration. It is important that the intensity of the signal from the reference and the unknown are roughly equal. At least one reference ion of known mass should be used to verify the accuracy of the primary calibration. If it is not possible to use an internal reference then an external calibration should be carried out immediately before the analyte is measured.

3.8 Selection of Elemental Formulae

Accurate mass measurement is used to determine the elemental formulae of molecule and fragment ions for confirming the presence of known compounds or to assist in identification of an unknown compound. In order to be able to assign elemental formulae and structures there are a number of key strategies that should be employed and these are described below.

3.8.1 Use of Accuracy, Precision or Uncertainty Data

This type of information, obtained from a QC check should be used to reduce the number of candidate molecular formulae to be considered. See also Section **2.1**.

3.8.2 The Nitrogen Rule

Nitrogen has an even atomic mass (14) but is trivalent. Thus any small molecule containing common elements and an odd number of nitrogen atoms will have an odd numbered molecular weight. If the molecule ion is an even number it must have an even number of or zero nitrogen atoms.

3.8.3 Rings Plus Double Bonds (R + DB)

From valency considerations the total number of rings and double bonds in a molecule of formula $C_aH_bN_cO_d$ is equal to:

$$R + DB = a - \tfrac{1}{2}b + \tfrac{1}{2}c + 1$$

Where other elements are present, these are counted as additional atoms of C, H, N or O to which they correspond in valency. For example, the number of silicon atoms should be added to the number of carbon atoms, the number of halogen atoms to the number of hydrogen atoms

and the number of phosphorous atoms to the number of nitrogen atoms. It should be noted that this is based on the lowest valence state of the elements, and does not count double bonds formed to atoms in higher valence states.

For an elemental formula to be theoretically possible 'R + DB' must be greater than –1.5. Moreover, if the ion is odd electron ($M^{+\cdot}$) the 'R + DB' value will be an integer and conversely, a non-integer 'R + DB' value indicates an even-electron ion. So, if the ion type is known, formulae giving an inappropriate 'R + DB' value can be discarded.

3.8.4 Isotope Information

A number of elements have distinctive isotope patterns and if these elements are contained in the analyte of interest then that distinctive pattern can be recognised. Careful scrutiny of the isotope pattern of the ions measured may also allow you to narrow down the number of possibilities. Chlorine has a distinctive isotope pattern (relative abundance of the naturally occurring isotopes ^{35}Cl:^{37}Cl is in the ratio ≈ 3:1), making the presence of chlorine in the sample fairly obvious (*e.g.* add chlorine to the list of possible elements if the isotope pattern indicates it is present). Likewise the ratios for multiple chlorine atoms can be calculated and recognised if present. Other common elements having distinctive isotope patterns include bromine (^{79}Br:^{81}Br ≈ 1:1)and sulphur (^{32}S:^{33}S:^{34}S ≈ 100:1:4).

Observation of the $^{12}C/^{13}C$ ratio can give the number of carbon atoms in the analyte. Naturally occurring carbon contains approximately 1.1% of the ^{13}C isotope. As a result of this an ion containing 10 carbon atoms will have a ^{13}C isotope peak with an abundance ~11% of the ^{12}C peak. Similarly an ion containing 50 carbon atoms will have a ^{13}C isotope peak with an abundance ~55% of the ^{12}C peak. Hence a simple $^{12}C/^{13}C$ ratio measurement will give a good indication of the number of carbon atoms present within the precision of the mass spectrometer.

3.8.5 NMR and other spectroscopic methods

Once an empirical formula has been assigned to an unknown it is often possible to elucidate its structure by combining data from the mass measurement of fragment ions and from other techniques such as NMR spectroscopy and infra-red spectroscopy.

A simple example is now presented to illustrate the selection of elemental formulae[10]. Suppose a molecule ion is determined to have a mass value of 58.0415. Table 3 gives the accurate m/z values for a variety of ion compositions, but which is the most likely elemental composition? Application of the nitrogen rule and 'R + DB' rule eliminate all given formulae except numbers 3, 4, 5, 8 and 11 (even number of N atoms because it has an even molecular mass and integer 'R + DB' because it is a radical cation (odd electron ion)). Of these formulae only C_3H_6O is within 200 ppm of the measured mass and is thus the most likely formula.

See also **Appendix 1** for journal requirements on the use of accurate mass measurement for elemental formulae determination.

Table 3: All possible elemental compositions corresponding to mass value of 58.0415 with element limits C - 4, H - 10, N - 4, O - 2 and mass precision limits of ± 1000 ppm

Fomula Number	Elemental Composition				Calculated Mass	Deviation ppm	R + DB
	C	*H*	*N*	*O*			
1	1		1	2	57.9924	847	2.5
2			3	1	58.0036	654	2.5
3	2	2		2	58.0049	630	2.0
4	1	2	2	1	58.0162	437	2.0
5		2	4		58.0274	243	2.0
6	2	4	1	1	58.0287	220	1.5
7	1	4	3		58.0400	26.3	1.5
8	3	6		1	58.0413	3.2	1.0
9	2	4	1	1	58.0525	-190	1.0
10	3	8	1		58.0651	-407	0.5
11	4	10			58.0777	-623	0.0

4. Instrument Specific Guidance

The following guidance reflects experience from the LGC inter-laboratory comparison[2] and the views of the expert consultation panel. The number of instruments used for each type of spectrometer in the inter-laboratory comparison is shown in Table 4.

Table 4: Instrument types used in the LGC inter-laboratory comparison[2]

Instrument Type	Number Used in Inter-laboratory Comparison
Magnetic sector	21
FTMS	8
Single quadrupole	0
Triple quadrupole	2
TOF (axial and orthogonal)	16
Quadrupole-TOF (orthogonal)	10

4.1 Magnetic Sector

There are a number of different ways in which accurate mass measurement can be carried out using this type of analyser. These are peak matching, dynamic voltage scanning and magnet scanning.

4.1.1 Peak Matching

To record an accurate mass measurement using the peak matching technique, a lock (reference) mass (M_R) is introduced at the same time as the analyte[13,14,15]. With constant magnetic field, the accelerating voltage is adjusted so that M_R and the analyte ion (M_U) are observed to be coincident. The ratio of the two voltages is inversely proportional to the masses so M_U can be calculated. This process can be carried out using a manual peak matching unit and oscilloscope or via the instrument data system and computer monitor.

This method can produce very accurate results, readily to within 1 ppm of the true value. This was observed in the inter-laboratory comparison where 88% of mean mass measurements were ≤ 1 ppm. For best accuracy the reference mass should be as close as possible to the analyte mass (at the same nominal mass is optimum), providing both masses are well resolved.

4.1.2 Dynamic Voltage Scanning

In the dynamic voltage scanning technique (known as V/E scan or accelerating voltage (V) scan) a very narrow mass range (V varied by up to 10%) is scanned by varying the accelerating voltage[16] while the magnetic field is held constant. Initially, the magnet is positioned (or parked) at the starting m/z value just below the mass range of interest. The accelerating voltage (V) is then scanned to permit the m/z range of interest and at least two bridging reference ions to pass through the instrument. The electric sector (formerly known as electrostatic analyser, E) is scanned simultaneously to prevent deterioration in resolution due to kinetic energy spread.

Usually a narrow range internal calibration is used to make the measurements. When using EI, between 1 and 3 ions from PFK or PFTBA (or other appropriate calibrant) are selected. In the inter-laboratory comparison the m/z range for internal calibration was no greater than ±56 Th from the analyte ion. A number of laboratories employed FAB for the inter-laboratory comparison and selected two ions from poly(ethyleneglycol) 600 that bracketed the analyte ion.

The inter-laboratory comparison demonstrated that mass accuracy within 5 ppm could readily be achieved by > 90% of participants using dynamic voltage scanning with 60% of participants reporting mean mass measurement accuracy of ≤ 1 ppm[2].

4.1.3 Magnet Scanning

In this technique the magnetic sector is scanned over a wide m/z range whilst keeping the accelerating voltage constant. The advantage of this method is that all of the ions in the spectrum of interest can be mass measured. Whilst not as accurate as peak matching or dynamic voltage scanning it can provide good quality data provided care is taken. The inter-laboratory comparison showed that the best accuracy that could be achieved was 3 ppm[2], but the variation in the reported mean mass accuracy was significantly greater (3 ppm – 42 ppm) than that observed for both peak matching and voltage scanning. In practice the magnet scan

should be started well above (say 100 Th) the highest mass ion of interest to ensure a smooth and reproducible magnet scan at the masses of interest. The inter-scan time should be sufficient to allow the magnetic field to stabilise before commencing the next scan. The reference compound should be of sufficient concentration to ensure that no reference ions are missed. Best accuracy is achieved using internal calibration; however, if the reference compound is not present when the analyte is run, the use of a single point calibrant (lock mass) is recommended. The magnet scan speed should not be too rapid, 10 sec/decade for directly introduced compounds that can remain resident within the ion source. Where a chromatographic inlet is used a balance must be achieved between scan speed and analyte peak width and scan speeds as fast as 0.1 sec/decade may have to be used, although this will compromise accuracy.

4.2 Fourier-Transform Ion Cyclotron Resonance

FTMS currently offers the highest mass resolution of any mass spectrometer[5]. *FTMS* is routinely used for accurate mass measurement with mass resolution in excess of $1x10^6$ and mass accuracy of 1 ppm[17]. This was clearly demonstrated in the inter-laboratory comparison[2]. For small molecule accurate mass measurement an external calibration is initially carried out covering the mass range of interest. Before measuring the analyte ion, the external calibration should be checked using the calibration compound, and all calibrant ions should ideally be within 1 ppm of their theoretical values. The mass measurement of the analyte ion is then carried out using the external calibration. Further improvement in mass accuracy (≤0.5 ppm) can be achieved using internal calibration. An important parameter to consider when recording accurate mass measurements using *FTMS* is the relative abundance of the external calibrant and analyte ions. Care should be taken to ensure that they are similar[11] and within the range where space charge effects will not occur.

4.3 Single Quadrupole and Triple Quadrupole

These are inherently low resolution instruments that have not been routinely used to record accurate mass measurements. However, accurate mass measurements (<5 ppm) can be obtained from them provided due care is taken, particularly with the calibration procedure, and when no unresolved interferences are present at the masses of interest[18]. This was emphasised in the inter-laboratory comparison as both laboratories that recorded their measurements using triple quadrupole instruments reported mean mass measurement accuracy of < 2ppm[2]. Both laboratories employed an internal calibration protocol using multiple ions (a minimum of 5) in the range ± m/z 74 from the analyte ion. A high sampling rate of 128 points per Th was used.

4.4 Time-of-Flight (TOF) and Quadrupole-TOF

TOF and quadrupole-*TOF*s are inherently low resolution instruments. With current technology the resolutions (FWHM) achievable by *TOF* and quadrupole-*TOF* instruments are up to about 15000. The inter-laboratory comparison showed that accuracies of better than 5 ppm can be achieved provided some care is taken (see Figure 4 for data on orthogonal and axial *TOF* instruments). Quadrupole-*TOF* instruments produced the higher accuracy measurements, with 83% ≤ 5 ppm, compared with 65% from *TOF* instruments. Ideally, external calibration should be carried out just before the mass measurement. In the inter-laboratory comparison, the majority of laboratories that reported data using either a quadrupole-*TOF* or *TOF* instrument employed a lock (reference) mass. In the inter-laboratory comparison some laboratories used a lock mass ion that was as much as 258 m/z units away from the analyte ion. However, such a

large difference in m/z did not adversely effect the mass accuracy, a mean mass measurement accuracy of < 1 ppm was achieved in this case. The key parameter to consider when using a lock mass with *TOF* and quadrupole-*TOF* instruments is the relative and absolute abundance of the analyte and lock (reference) ions. This factor is particularly important with chromatographic introduction and it is recommended that a measurement be taken in the tail of an eluting component peak within a specified range of ion abundance. As ion abundance increase rapidly in the leading edge of an eluting peak, it is more difficult to select the optimum measurement point.

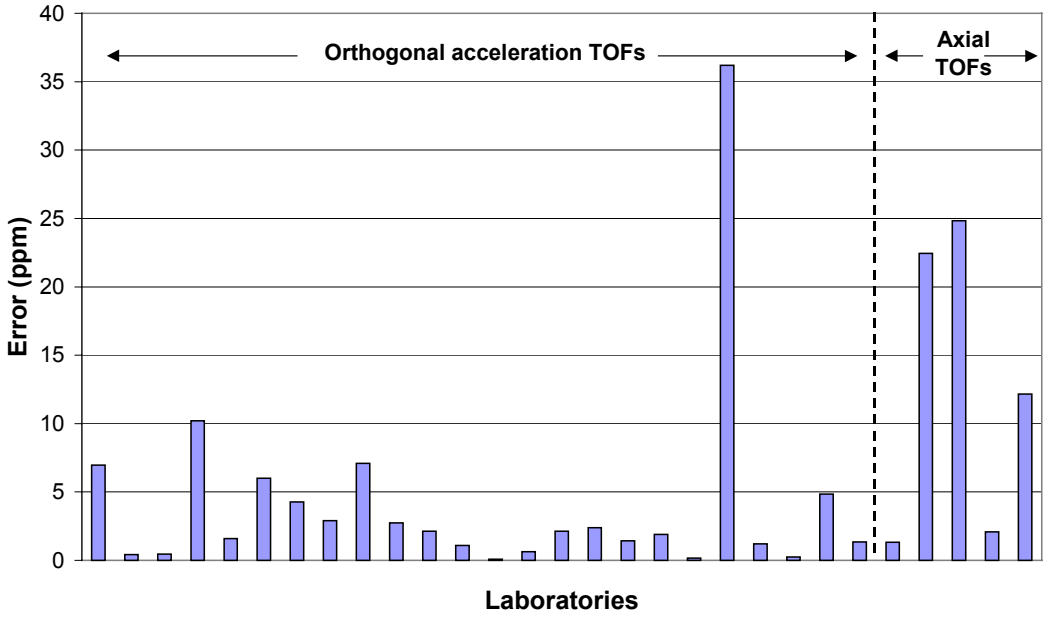

Figure 4: Mass measurement error of orthogonal and axial *TOF* instruments in inter-laboratory comparison

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Glossary of Terms

The terms in this Glossary originate from referenced sources (a-d). Other similar definitions exist in the scientific literature and may be regarded as equally valid. Where possible definitions have been used which most clearly convey the meaning of the term.

Accuracy[a]

A quantity referring to the difference between the mean of a set of results or an individual result and the value that is accepted as the true value for the quantity measured.

AccMass

Colloquial term used for accurate mass or accurate mass measurement.

Accurate mass[b]

Accurate mass is the measured m/z of an ion and, strictly speaking, should be referred to as 'measured accurate mass'. It is used when reporting the mass to some number of decimal places (usually a minimum of three).

Accurate mass measurement[b]

The determination of accurate mass.

Atomic weight[b]

The atomic weight of an element is the weighted average of the naturally occurring stable isotopes of an element.

Average molecular weight/ average molecular mass[b]

The average mass is calculated using all the isotopes of each element along with their natural abundance. It is calculated using the atomic weights of the elements from which the molecule is composed (See ***Atomic weight***).

Calculated exact mass[b]

The mass determined by summing the masses of the individual isotopes that compose a fragment or molecule ion. It is based on a single mass unit being equal to 1/12 the mass of the most abundant naturally occurring stable isotope of carbon.

Centroid[c]

The centroid of a peak is its centre of gravity and is that point on the peak at which the mass is measured. It may be determined in different ways.

Centroiding

The process by which the centroid of a peak profile is determined.

Continuum Spectrum[c]

One displaying the full profile (height and width) of the detected signal (peak) for an ion.

Decade[b]

An order of magnitude change in m/z range (for example m/z 600 – 60 or m/z 100 – 10). Used in description of magnet scan speed (seconds/decade) for magnetic sector instruments where an exponential magnet downscan is used.

ESI[d]

Electrospray ionisation

Exact mass[b]

Equivalent to 'calculated exact mass' of a monoisotopic ion, radical or molecule.

FAB[d]

Fast atom bombardment as a method of ionisation.

FTICRMS[d]

This refers to Fourier Transform ion cyclotron resonance mass spectrometry, also known as Fourier Transform mass spectrometry (FTMS).

FTMS

See ***FTICRMS***.

Isotope Cluster[b]

A group of peaks close to one another that represent ions with the same elemental composition but of a different isotopic composition.

Isotopes[a]

Forms of an element (nuclide) where the numbers of neutrons are different leading to different atomic weights, for example ^{12}C and ^{13}C.

MALDI[d]

Matrix-assisted laser desorption/ionisation

Molecular Mass [b]

The mass of a molecule or molecule ion.

Monoisotopic ion [b]

The ion comprised of the most abundant naturally occurring stable isotope of each of the elements making up the ion.

Most abundant mass [b]

This is represented by the most abundant ion in an isotope cluster.

m/z [b]

Mass-to-charge ratio (where *z* is numerically equivalent to *e*, the mass of the electron, which is 9.109×10^{-28} g)

Nominal mass ion [b]

The integer mass of the ion comprised of the integer masses of the most abundant naturally occurring stable isotopes of the elements making up the ion.

Peak Profile

See ***Continuum Spectrum***

Precision [a]

The closeness of agreement between the independent results obtained when applying the experimental procedure under prescribed conditions.

Resolution [b]

Frequently and incorrectly used interchangeably with 'resolving power'; however, strictly speaking it is the measure of the separation of two mass spectral peaks (Δm at m). Δm is the difference between m and the value of the next highest m/z value ion that can be separated from m, in m/z units. See also Section **3.3**.

Resolving Power [b]

The resolving power of a mass spectrometer is defined in terms of its capacity to separate ions of adjacent m/z. The resolving power necessary to separate masses m and (m+Δm) respectively is given by:

$$R = m/\Delta m$$

See also Section **3.3**.

Thomson[b] **(Th)**

The name for an m/z unit or increment.

TOFMS[c]

Time-of-flight mass spectrometer.

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Appendix 1: Journal Requirements for Accurate Mass Data Used for Formula Confirmation

Very few journals publish guidance to authors on the use of accurate mass data for formula confirmation. Some examples of published guidance are given below, which were correct at the time of writing (March 2004). The most detailed and informative are those of the *Journal of the American Society for Mass Spectrometry*.

Journal of the American Society for Mass Spectrometry

For publication of accurate mass data used to confirm the identities of synthetic and natural products, report the uncertainty in the accurate mass measurement used for formula verification along with the result. The acceptable uncertainty in a measurement by any analytical method must be adequate for the intended use of the data.

Evaluate the uncertainty of accurate mass measurement by any statistically valid method. Determine, for example, the precision and accuracy of replicate measurements or evaluate the performance characteristics of the mass spectrometer. Consider all candidates fitting the experimentally determined value and its reported uncertainty when the result of accurate mass measurement is used for formula confirmation. Do not set fixed acceptable error limits for exact mass measurement. This is illustrated by the discussion below:

> When valence rules and candidate compositions encompassing $C_{0\text{-}100}$, $H_{3\text{-}74}$, $O_{0\text{-}4}$ and $N_{0\text{-}4}$ are considered at nominal parent m/z of 118, there are no candidate formulae within 34 ppm of each other. When the ion is of m/z 750.4 and the formulae are in the range $C_{0\text{-}100}$, $H_{3\text{-}74}$, $O_{0\text{-}4}$ and $N_{0\text{-}4}$, there are 626 candidate formulae that are possible within 5 ppm. Thus, for a measurement at m/z 118, an error of only 34 ppm uniquely defines a particular formula, whereas at m/z 750, an error (and precision) of 0.018 ppm would be required to eliminate all extraneous possibilities.

Journal of Organic Chemistry

Summary information – Compound Characterisation – High resolution mass spectrometry data (HRMS) may be reported to support the molecular formula assignment. The structures of peptides and oligonucleotides may be established by providing evidence about sequence and mass. Typically, a sequence will be accompanied by HRMS data that establishes the molecular weight and formula. HRMS data may be used to support a structure assignment but cannot be used as a criterion of purity. (Note – no criteria for quality are listed.)

American Chemical Society

Characterization Requirements for Routine Organic Substances

(Only mass spectrometry requirements are detailed here)

Molecular Weight. Evidence of molecular weight should be provided, especially if elemental analysis was not performed. Low resolution MS data under conditions that minimize fragmentation are acceptable. If there is a specific need to distinguish alternative formulas with the same molecular mass (within one amu), then HRMS data are necessary.

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