Methodology for Accurate Mass Measurement of Small Molecules

Best Practice Guide

Co-ordinating Editors

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*LGC Limited, Teddington, UK

Setting standards in analytical science

ISBN 0-948926-22-8

Published by LGC Limited

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November 2004

VIMMS/2004/01

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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

Acknowledgement

The work described in this Guide was supported under contract with the Department of Trade and Industry.

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Contents

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

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

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

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.

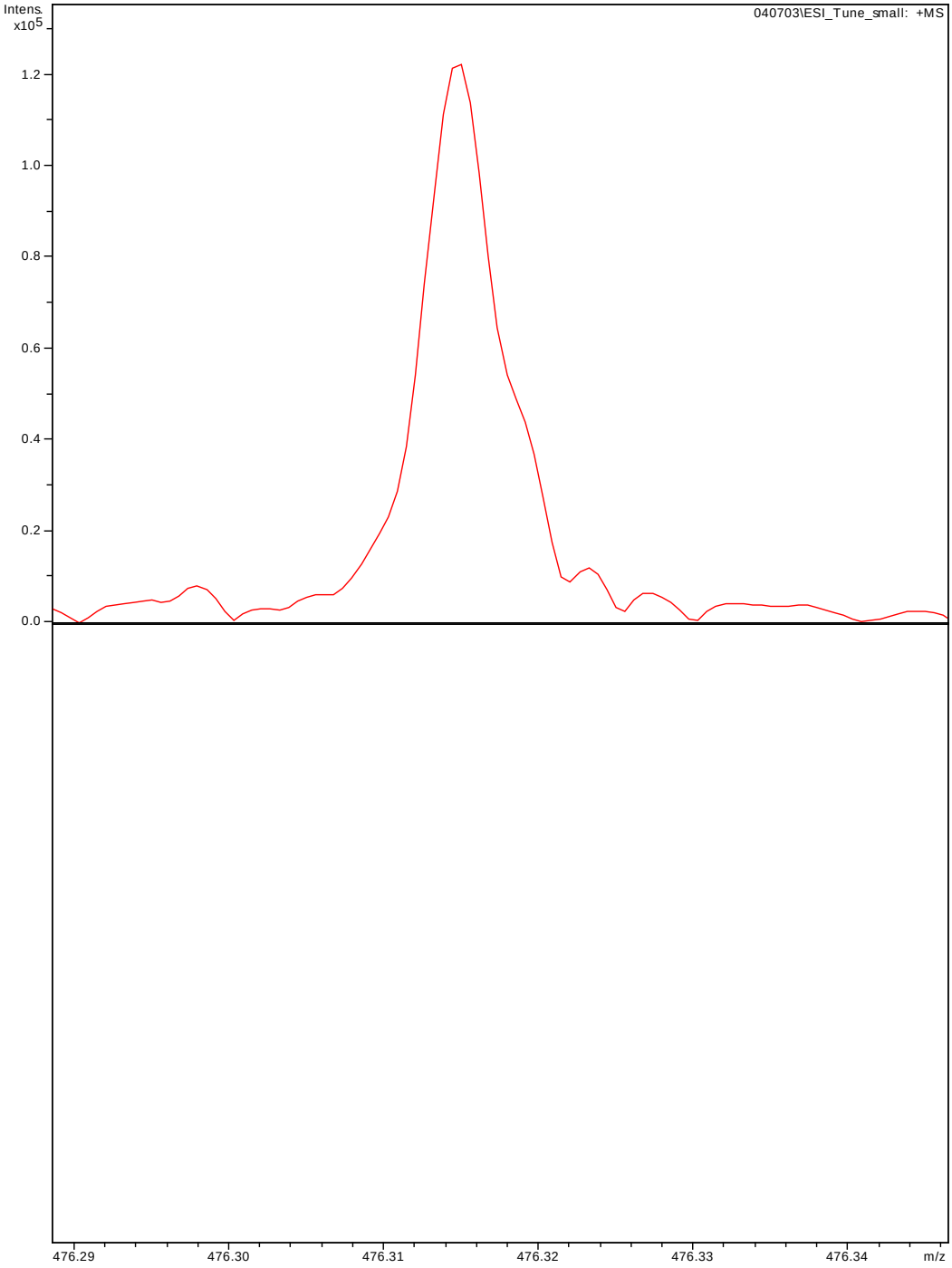
The peak shape can be affected by poor instrument tuning, low ion counts, unresolved interferences, mechanical vibration and electronic ripple. Tune the instrument carefully – this should include optimising ionisation conditions, which increases ion counts, and optimising ion transmission from source 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.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1.0
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0.2
0.0
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476.31
476.32
476.33
476.34
m/z

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 (Δ

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 case007 Tc0.1343 Tw9c0.1343 Tw[(In thi.145hy5 Tw[.T16 BT/TTucal0.0d0.0995re ly4(a04(app

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	EI06.78 6c00 l0-1000

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.

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.

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 dis

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.

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**scan) a v**

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 chao21huog4tis wil nont 5.6(Tc0 TD0.0297 Tc-28.215 -1.1p2Csth)5.6(e .08 a6c-c0038 Tc4 TcTJ11b

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.

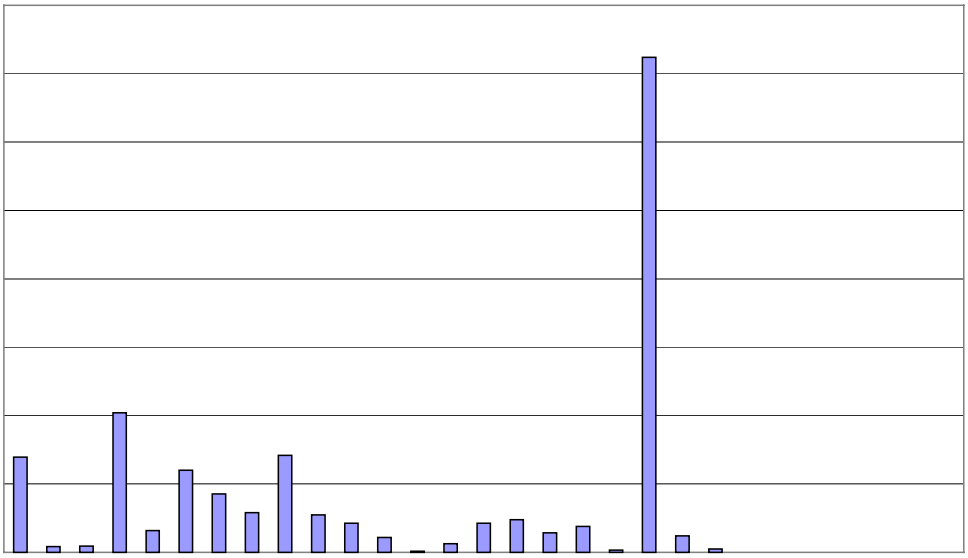

Figure 4: Mass measurement error of orthogonal and axial *TOF* instruments in inter-laboratory comparison

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Glossary of Terms

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 natura

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

ISBN 0-948926-22-8