Signal Response

Mass Spectrum

Chromatogram

**Guide to achieving
reliable quantitative
LC-MS measurements**

LGC

Guide to achieving reliable quantitative LC-MS measurements

RSC Analytical Methods Committee

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Editor

Mike Sargent

Contributors

Ashley Sage

Celine Wolff

Chris Mussell

David Neville

Gwyn Lord

Mansoor Saeed

Rakesh Lad

Ruth Godfrey

Simon Hird

Vicki Barwick

Preface

Mass spectrometry (MS) has the capability to separate organic molecules according to their molecular mass and permits their detection and quantitation with extremely high sensitivity. High performance liquid chromatography (HPLC) facilitates the rapid, quantitative separation of compounds from each other and from the other constituents of complex mixtures or matrices. Used in tandem, the two techniques (usually referred to as LC-MS) provide a unique capability for rapid, cost-effective and quantitative measurements of organic molecules for an enormous variety of applications. Routine use of mass spectrometry began to grow in the 1950s, followed by HPLC in the 1970s. However, development of reliable interfaces to link the two techniques was not easy and it is only during the past twenty or so years that LC-MS has assumed the key role it occupies today. During that time, manufacturers have succeeded in steadily reducing the size and real cost of the instrumentation whilst software and automation have greatly lowered the learning curve for operators. As a result, LC-MS has become ubiquitous as the technique of choice for many quantitative analysis applications. Appearances can, however, be deceptive. Whilst there is no question that modern LC-MS instruments are relatively easy to operate and maintain, there are also many pitfalls awaiting the unwary, especially for accurate quantitative measurement of analytes at trace concentrations or in complex samples. The problem is exacerbated by the apparent ease with which the technique can be applied to ever more demanding applications and the understandable tendency of manufacturers to emphasize the benefits of their products.

In this LC-MS guide we have attempted to bring together *practical* advice which we hope will assist users of the technique to avoid many common problems and to develop reliable, quantitative applications as quickly and cheaply as possible. The guide is not a textbook so readers will find little discussion of theoretical aspects of the techniques. Similarly, we have limited descriptions of the instrumentation to key aspects which we feel are necessary for readers to benefit from the many hints and tips given in the practical advice. Two aspects of the guidance merit further comment. First, achieving reliable quantitation by LC-MS depends not only on correct use of the instrumentation but also on correct development and use of the entire analytical method. We have, therefore, included extensive advice on aspects such as sample preparation and calibration strategies. Secondly, experience shows that many errors in analytical data result not from lack of understanding of the measurement techniques but as a result of simple and avoidable mistakes during their application. Such matters are addressed in many textbooks and guides on quality assurance but we have provided a very brief overview of some common issues. In keeping with the practical approach of this guide, we have also offered a few common sense suggestions to help readers ensure that the time and effort spent on developing a reliable LC-MS method is not wasted by making avoidable errors during its routine use.

Mike Sargent
Chair, AMC Mass Spectrometry Sub-Committee
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1   Introduction

Mass spectrometry is a very sensitive technique and is widely regarded as having good selectivity. However, in many applications it is necessary to isolate the target analyte from what could be a sample containing thousands of other different molecules. Typically mass spectrometry alone is unable to meet this need as it can only differentiate compounds by their mass-to-charge ratio (*m/z*) which is insufficient in most practical applications of the technique. For example, more than 1,500 compounds may have the same molecular mass at around 250 Da. Hence, an additional separation technique is needed before presenting the sample to the mass spectrometer.

Liquid chromatography-mass spectrometry (LC-MS) is the combination of two selective techniques that allows the analyte(s) of interest in highly complex mixtures to be isolated and measured. LC differentiates compounds by their physico-chemical properties and MS differentiates compounds by mass (specifically their mass-to-charge ratio). It is this dual selectivity that makes LC-MS such a powerful analytical tool. The power of the technique is illustrated in Figure 1.1. The mass spectrometer acts not only as the "LC detector" but, at least in principle, it provides the capability to identify the species corresponding to each chromatographic peak through its unique mass spectrum.
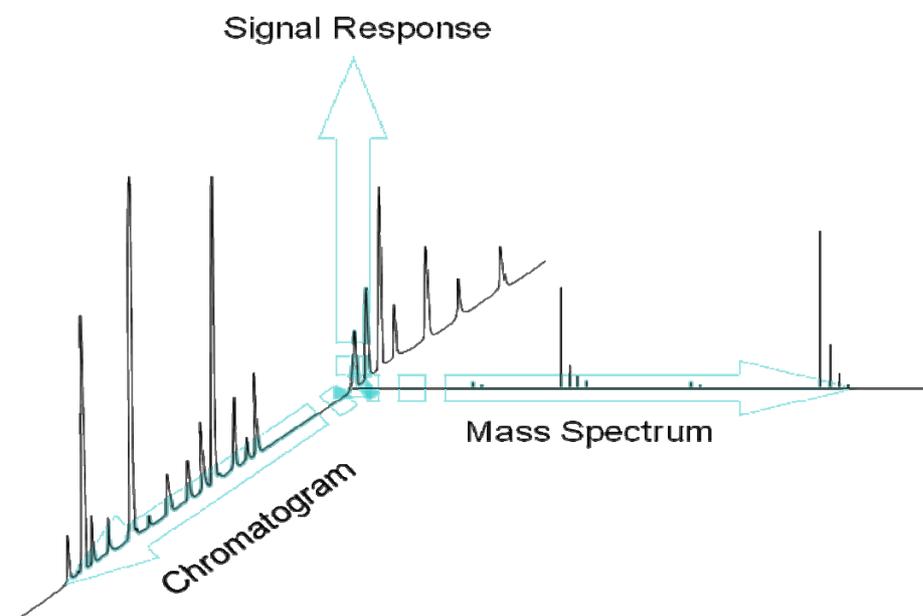
Figure 1.1: The power of the LC-MS technique to separate and identify each component of a complex mixture

1.1   Scope of this guide

The separate topics of liquid chromatography and mass spectrometry cover such a wide and complex field that writing a document to describe them in detail would result in an almost unreadable tome. Instead, this guide aims to bring together the most relevant aspects of the subjects that need to be considered when using LC-MS for accurate and reliable quantitation, *i.e.* measuring precisely and accurately how much of an analyte is present in a sample.

There are many different manufacturers of LC and MS systems, each with their own particular traits and operating instructions, but generally they all follow the same principles. Hence, the guidance in this document should be relevant regardless of the particular instrumentation used in a laboratory.

1.2 Why Use LC-MS for quantitation?

Various features of the technique are summarised in Table 1.1 below.

BENEFITS	
Selectivity	Combining the two separation mechanisms of LC and MS/(MS^n) allows the analysis of complex mixtures. The resulting selectivity allows a particular analyte or analytes to be isolated from the mixture and gives confidence that the correct component is being measured. Since analytes are separated by their mass-to-charge ratio (m/z) the technique allows for the use of isotopically labelled internal standards, which may not separate by LC but can be separated by their mass difference. The use of stable isotopically labelled (SIL) internal standards can help control variability in a quantitative assay.
Speed	Since the MS will distinguish compounds based on mass, the chromatographic method does not have to separate every single component in the sample, so co-elution of non-isobaric analytes is possible. This allows fast LC analysis times and reduced sample preparation, which helps with method development and high throughput sample analysis.
Sensitivity	Mass spectrometry is an inherently sensitive technique. Good selectivity also leads to reduced noise, allowing very low levels (fg mL^{-1}) to be detected.
DISADVANTAGES	
Expense	Mass spectrometers that can couple to LC systems are expensive to buy and run. Regular servicing is also required, adding to the cost. The environmental conditions in the laboratory need to be well controlled to ensure system stability.
Complexity	In their own right, both LC and MS can be difficult to optimise. Combining the two leads to a complex co-dependant synergy. The ionisation mechanism can be especially complicated – often several species are formed in the ionisation source and multiple charging can occur. Care must be taken to choose conditions for optimum sensitivity and reproducibility. Sufficient training is also needed to allow analysts to run the systems effectively.
Limited dynamic range	Compared to other quantitative techniques LC-MS can have a limited range where the response is linear with respect to concentration. Typically, ranges should not exceed 500-fold concentrations.
Excessive selectivity	In quantitative analysis it is usual that the MS is set to only detect specific analytes. This results in a very 'clean' looking chromatogram and it is easy to forget that there can be a lot of components still present, but not seen. These components can cause problems with reproducible quantitation and can be difficult to trace if they are not being looked for (see matrix effects, section 1.3).

Table 1.1: Reasons for using LC-MS and some of the possible limitations of the technique

1.3   Matrix effects (or ion suppression)

Throughout this guide there are numerous references to the concept of a 'matrix effect', which occurs because the ionisation mechanism is prone to disturbance from some components of the sample, especially with biological samples. If these components co-elute with the analyte of interest then data can become biased or have poor precision. Typically the ionisation mechanism is suppressed, meaning that a lower response than expected is observed. There are three approaches to reducing this effect:

1. Chromatographic: physically separate the analyte and interference on column so they elute at different times.
2. Extraction: selectively remove the interference during the sample preparation stage.
3. Compensation: use a stable isotopically labelled (SIL) internal standard or matrix match so that any effect is consistent. The elution time of a SIL internal standard will be virtually identical to that of its unlabelled counterpart and will therefore (in theory) undergo the same amount of matrix effect. Using response ratio to determine concentrations will therefore compensate between different matrices. Matrix matching is the process of ensuring that all standards, quality control (QC) samples and test samples are in an identical matrix so that any ion suppression is constant.

1.4   The key stages of quantitative analysis

1.4.1   Sample collection

For any quantitative analysis it is crucial to ensure that a representative and sufficiently homogeneous sample is taken for analysis. The storage conditions between sampling and analysis must also be controlled to ensure that the samples do not degrade. Stability is usually tested prior to sampling.

1.4.2   Calibration and quality control samples

In order to quantify unknown concentrations of analyte, samples containing known concentrations must be prepared first. For quantitation this normally involves adding a range of concentrations to blank samples, providing a set of calibration solutions which can be used to generate a calibration plot or line. The blank samples must be as close as possible in composition to the samples being analysed (matrix matching). QC samples are typically prepared in bulk and analysed at regular intervals to monitor assay precision and bias. Acceptance of data usually depends on QC samples being successfully quantified within predefined limits. It is becoming routine to reanalyse a proportion of test samples to demonstrate that the precision of the assay is under control (incurred sample reanalysis – ISR). This is because test samples are often subtly different to control samples. For example, biological samples may contain metabolites, where QC samples will not. Predefined criteria must be in place to allow assessment of replicate data sets.

1.4.3   Sample preparation and extraction

Direct analysis of samples using LC-MS is possible but it is usually the case that samples will need to be cleaned up to remove the worst interferences and also to concentrate the sample if the analyte is only present at very low concentrations. Typically an internal standard is added

to control for variations in recovery, matrix effect and ionisation. The end point of extraction will need to be compatible with the chosen LC method.

1.4.4 Analysis

Calibration standards, QCs and samples are injected onto the LC-MS system. It is expected that the assay will have been thoroughly assessed and validated to establish that its performance is fit-for-purpose. The assay should be sensitive enough to detect the lowest sample concentration and selective enough to ensure that interfering components do not compromise quantitation. Before analysing samples it is expected that a system suitability test (SST) is carried out. This typically involves injecting a known solution and comparing its response to previous data. Analysing a blank sample and a sample just containing internal standard is used to demonstrate selectivity.

1.4.5 Data processing

Correct data processing is a fundamental step in generating good quality quantitative data. Most modern software packages contain automated algorithms for integrating peaks and these are preferred over manual integration. Each chromatogram must be inspected to ensure that the baseline is correctly drawn and that the analyte is resolved from any close eluting peaks. The key point here is that integration should be consistent. Typically the analyte:internal standard response ratio is used to create the calibration line and quantify the QC samples and unknowns. Predefined acceptance criteria should be applied for key aspects such as repeatability or calibration linearity.

1.4.6 Reporting

Most modern quantitation software allows direct export of results into word processing packages or spreadsheets. LIMS systems are also designed to integrate with most LC-MS instruments.

1.5 References and further reading

1. Taylor P. J., *Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry*. Clin. Biochem., 2005. 38: p. 328-334.
2. Mei H., Hsieh Y., Nardo C., Xu X., Wang S., Ng K. and Korfmacher W. A., *Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery*. Rapid Commun. Mass Spectrom., 2002. 17: p. 97-103.

2   Instrumentation

In this chapter an overview of the entire LC-MS instrument is provided together with a brief description of the key components: the LC-MS interface/ionisation source and the mass analyser. The aim here is to highlight the reasons for choosing particular types of these components from the many available possibilities in order to obtain an instrument which is best suited to quantitative applications. This guide does not address the application of liquid chromatography in detail but the use and optimisation of the two key components is described in Chapters 5 and 6.

In basic terms, a typical LC-MS system consists of the components shown in Figure 2.1.
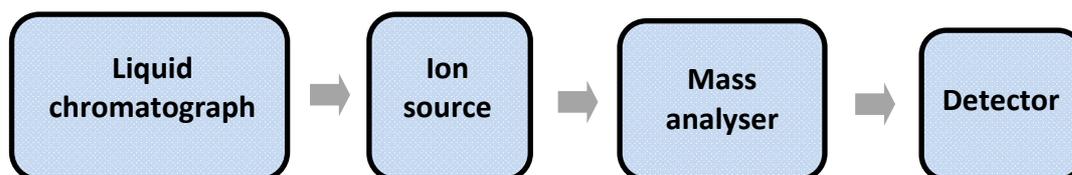
Figure 2.1: Basic components of an LC-MS system

Ion source: used in the vaporisation/ionisation of the target molecules.
Mass analyser: used to separate the gas phase ions by mass-to-charge ratio (*m/z*).
Detector: detection of the mass separated ions and measurement of their relative abundance.

The development of commercial LC-MS systems has led to a wide range of instruments being made available to the end user. Although the choice of ionisation techniques for LC-MS remains somewhat restricted, instruments now utilise many different types of mass analysers. New types or derivatives of the mass analyser have been introduced at regular intervals over recent years. Table 2.1 outlines the main types of LC-MS instrument that can be used for quantitation together with their features/benefits and disadvantages. This guide to achieving reliable quantitation by LC-MS is primarily aimed at the use of the so-called triple quadrupole (QqQ) "tandem" mass analysers as these instruments are considered the "gold standard" for quantitation and remain the most widely used for that purpose. However, much of the advice given in this guide is also relevant to other types of modern LC-MS instrument. The layout of a typical QqQ mass spectrometer is shown in Figure 2.2.
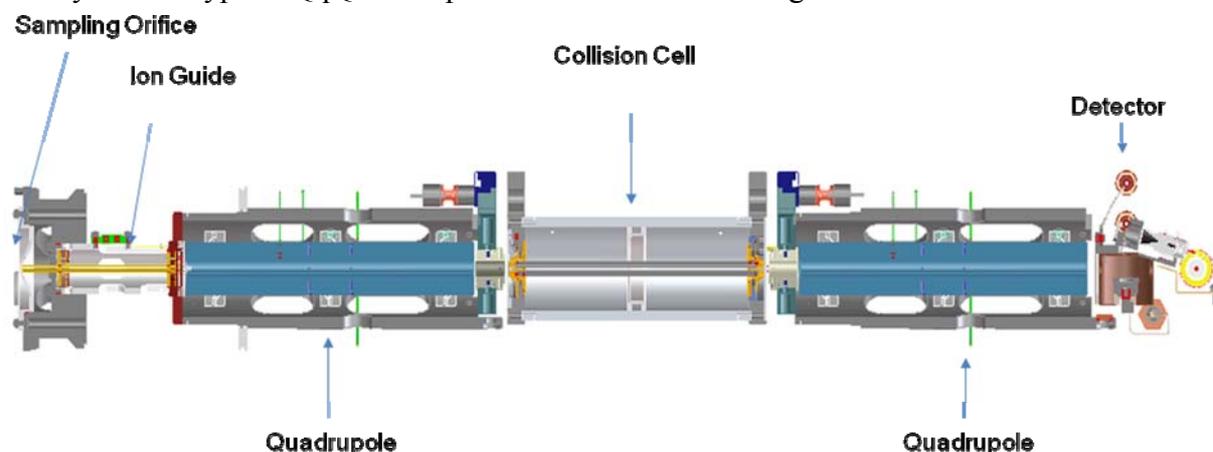
Figure 2.2: Schematic view of a typical triple quadrupole (QqQ) mass spectrometer

Mass spectrometer type	Features and benefits	Disadvantages
Single Quadrupole	Good scan function sensitivity Good selectivity/sensitivity via SIM scanning High duty cycle with SIM Good dynamic range (3-4 orders) Fast positive/negative ionisation	Limited mass range (generally up to 3000 *m/z*) SIM functionality can be prone to matrix interferences thus limit detection limits Low resolution (around 1500 FWHM or 0.7 Da)
Triple Quadrupole	Good scan function sensitivity Good SIM function Excellent selectivity with MRM, even with matrix Excellent duty cycle with MRM Ability to run multiple analytes simultaneously with MRM High dynamic range (4-5orders) Fast pos/neg ionisation Other scan functions available, *e.g.* neutral loss, product and precursor ion	Low resolution generally (1500 FWHM or 0.7 Da) Limited mass range (up to 3000 *m/z* generally)
Ion Trap (low resolution)	Very high full scan sensitivity Full scan MSMS and MS^n capability (ideal for structural identification) Can perform targeted quantitation with 3 orders of dynamic range using SIM scan functions. Some linear ion traps can perform simultaneous full scan and MRM experiments	Can suffer from matrix interferences (particularly 3D traps) Duty cycle is generally slower when compared to a triple quad, especially when doing simultaneous full scan and MRM acquisitions Low resolution generally (1500 FWHM/0.7 Da) but can run at higher resolution (but loses duty cycle)
Ion Trap (high resolution)	High full scan sensitivity in MS, MSMS and MS^n mode Good dynamic range (3 orders) High resolution (>100,000 FWHM) can provide good selectivity using exact mass measurement	Resolution can be affected by scan speed, *i.e.* the faster the acquisition speed, the lower the resolution Orbital trapping devices can have a limited dynamic range and be affected by matrix Limited mass range (up to 4000 *m/z* typically)
TOF (high resolution)	Good scan functionality and sensitivity High resolution (up to 40,000 FWHM) provides high selectivity through exact mass measurement Good dynamic range (with newer ADC based detection systems, typically 3-4 orders) Ability to get quantitation on multiple analytes in a single acquisition Mass range in excess of 20,000 *m/z*	No MS/MS functionality or other scan functions Generally, lower sensitivity when compared to a triple quadrupole running MRM Sensitivity can be affected by scan speed
qTOF (high resolution)	Good full scan sensitivity Good MSMS scan functions High resolution (>40,000 FWHM) providing high degree of selectivity via exact mass measurement Good dynamic range with newer ADC based detection systems (3-4 orders) Ability to get quantitation on multiple analytes during a single run Mass range in excess of 20,000 *m/z* Resolution not affected by increased scan speed	Generally, lower sensitivity when compared to a triple quadrupole running MRM Sensitivity can be affected by scan speed

Table 2.1: The main types of LC-MS instrument used for quantitation

Sample molecules are converted into gas phase ions in the ionisation source before being accelerated into the mass analyser via the sampling orifice and ion guide. Ions are then deflected by electrostatic fields in the quadrupoles according to their mass and their charge within the mass analyser. The detector converts the ion energy into electrical signals, which are then transmitted to a computer for data handling including calculations required for quantification.

Mass spectrometers do not measure the molecular mass directly but rather the mass-to-charge ratio (m/z) of the ions formed from the molecules. For ions with either a +1 or −1 formal charge, as is found with most "small molecules", the mass of the ion is the same as the m/z ratio. Clearly this is not the case when dealing with large molecules such as peptides and proteins. Production of gas-phase ions is necessary for manipulation by the electrostatic field. Electrically neutral particles are not affected by such fields. Mass analysers operate under vacuum as the ions need a free path through the instrument without colliding with molecules in air.

2.1 The ion source

The direct coupling of LC and MS (LC-MS) has developed into one of the most powerful techniques for trace quantitative analysis. The main breakthrough was solving the problem with the incompatibility of introducing the flow of liquid mobile phase from the LC column into the vacuum required in the mass spectrometer by the use of atmospheric pressure ionisation (API) interfaces. Today, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the most common API techniques in routine use for quantitation of small molecules by LC-MS. Schematic diagrams of the two types of source are shown in Figures 2.3 and 2.4. Atmospheric pressure photoionisation (APPI) was developed to increase ionisation efficiencies of non-polar compounds such as polyaromatic hydrocarbons and steroids. The choice of the most appropriate ionisation technique, as well as detection polarity, is based upon analyte polarity and LC operating conditions but many classes of compounds perform well using either technique and sometimes in both ion modes. Interfaces tend to be selected based upon individual preference derived from experience and available techniques as well as the magnitude of any matrix effects.
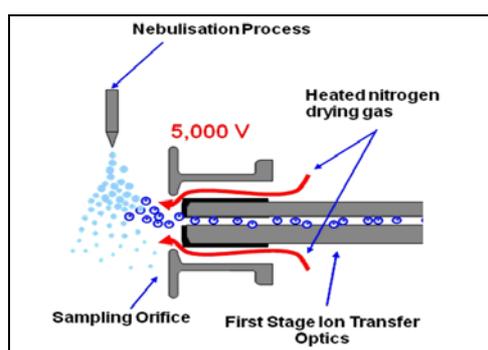
Figure 2.3: Schematic of ESI source   *Figure 2.4: Schematic of APCI source*
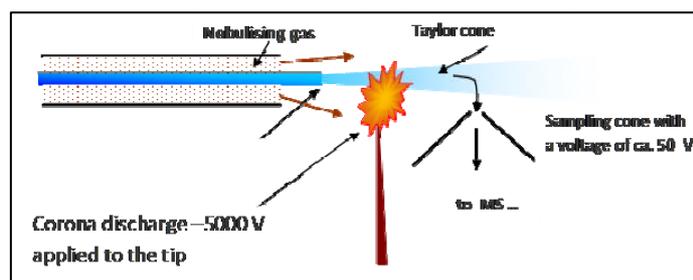
There are many examples of the use of APCI for LC-MS analysis, but more recently the technique appears to have been left in the wake of electrospray ionisation which has achieved overwhelming popularity. This may be related to the increasing use of multi-analyte methods but perhaps also reflects the improvements in source and probe design for electrospray not yet paralleled with APCI. As all the API techniques afford ions having little excess internal energy, the resulting mass spectra are typically dominated by protonated or deprotonated

molecules ([M+H]$^+$ and [M-H]$^-$) or adduct ions (*e.g.* [M+Na]$^+$ and [M+acetate]$^-$), without much fragmentation.

For the purpose of this guide, the API interface and ionisation techniques will be described using reasonably generic terminology. Details of instrument-specific parameters can be found in the manual for each instrument. The API interface is a critical part of the mass spectrometer and needs to produce ions from the molecules of interest whilst coping with typical LC flow rates (0.2-2 mL/min). Each vendor produces instruments with different API interfaces but each design uses a variety of voltages, gases, temperature and sampling orifices/tubes to provide:

- Pressure reduction.
- Evaporative processes called desolvation.
- Ionisation.
- Removal of excess solvent and un-ionised material to waste (exhaust).

Modern API interfaces have been designed to be relatively easy to clean and maintain. Scheduled maintenance of the API interface, as per vendors' instructions, is an important aspect to maintaining good quantitative performance.

2.1.1 Electrospray ionisation (ESI)

There have been considerable efforts directed at understanding the mechanisms involved in ion production for electrospray because understanding how ions are generated from the mobile phase into the gas-phase is invaluable in diagnosing problems such as loss of sensitivity and matrix effects. There are a number of differing theories but ionisation takes place in the liquid phase and involves the following interactive processes:

- The LC eluent flows through a metal capillary contained within the probe.
- Droplets are formed by nebulisation of the LC flow into a spray as it leaves the electrospray capillary.
- A charge is transferred onto the droplets by applying a large (2-5 kV) potential difference between the electrospray capillary and counter electrode.
- The droplet size reduces by evaporating the mobile phase by the use of a heated drying gas.
- This desolvation increases charge density on the surface of the smaller droplets.
- Electric repulsion due to the charge density results in droplet fission.
- When this exceeds the surface tension of the droplet it results in coulombic fission.
- Gas-phase ions are formed as the droplet "explodes" and are sampled typically through some form of orifice.

The main advantage of the use of ESI for quantitative LC-MS is the formation of protonated or de-protonated molecules with little fragmentation, ideal for selection of precursor ions and for maximising sensitivity. The major limitation is ion suppression or enhancement effects due to presence of co-eluting analytes or co-eluting matrix components. This "matrix effect" is recognized as one of the major sources of uncertainty in LC-MS and LC-MS/MS). In addition, response can be non-linear at high concentrations and optimum pH for ESI response can conflict with choices made to control LC selectivity.

2.1.2 Atmospheric pressure chemical ionisation (APCI)

APCI is more suited to the analysis of relatively non-polar molecules still within the scope of LC separations. It is a gas-phase ionisation technique involving the following interactive processes:

- The LC eluent flows through a silica capillary contained within the probe.
- Droplets are produced by nebulisation of the LC flow into a spray.
- Solvent is evaporated by the use of a heater in the probe to produce gas-phase molecules.
- A corona discharge needle placed in the ion source generates electrons and ionises air and gaseous solvent molecules forming reactive species (N_2^+, H_2O^+, O_2^+), which quickly react to form $H_3O^+(H_2O)^n$.
- Proton transfer from/to the ionised solvent results in chemical ionisation of analyte $[M+H]^+$.

Given that APCI is a gas-phase ionisation technique it tends not to suffer from ion suppression as much as ESI. However, considerable care is needed when selecting the type of solvent and additives used for the mobile phase to avoid issues with suppression of the analyte signal from preferential ionisation of solvents or additives with relatively higher proton affinities. APCI also needs greater concentrations of additives in the mobile phase to control the chemical ionisation process taking place in the gas-phase.

2.2 The mass analyser

The traditional view of the mass spectrometer is of a large, floor standing instrument utilising a large electromagnet to provide a high resolution mass spectrum. In practice, this type of instrument has seen only limited application for LC-MS. As mentioned above, most LC-MS instruments use an analyser comprising several components in tandem, with the most popular for quantitative LC-MS being the triple quadrupole (QqQ) spectrometers. These are described briefly below together with some recent advances in instrumentation which combine the advantages of tandem instruments with the high resolution provided by large magnetic sector spectrometers.

2.2.1 Tandem mass spectrometry

Despite the availability of a wide range of mass analysers each with different performance characteristics, QqQ instruments have been the preferred choice for most routine targeted quantitation assays as they offer the best performance characteristics for quantitation. Single quadrupole instruments are rarely used for quantitation as, when combined with API sources, the technique lacks the selectivity required for detection in complex matrices. Single quadrupole MS has been superseded by tandem mass spectrometry (MS/MS). The basic principle of MS/MS is the selection of a precursor ion, fragmentation of this ion, usually by collision-induced dissociation (CID) and measurement of the mass-to-charge ratio (*m/z*) of the product ions formed.

There are two fundamentally different approaches to MS/MS: tandem in space and tandem in time. Tandem in space instruments have separate independent mass analysers in physically different locations of the instrument. A hybrid mass spectrometer is an instrument which combines analysers of different types. Examples of tandem in space mass spectrometers include, but are not limited to:

- Triple/tandem quadrupole (QqQ).
- Quadrupole time-of-flight (QqToF).

- Orbitrap hybrid instruments.

Tandem in time instruments routinely used for quantitation are ion-trapping mass spectrometers, namely:

- 3-D quadrupole ion traps (QIT).
- 2-D or linear ion traps (LIT).

In these instruments, the various stages of mass spectrometry are conducted within the same physical trapping volume but at different times during the experiment. The QIT mass analyser suffers from some significant limitations impacting upon quantitation, including poor dynamic range and a limit on the number of ions that can be simultaneously isolated and fragmented. The future of ion trap technology for quantitative analysis will probably lie with linear ion traps, which can be used either as ion accumulation devices in combination with quadrupole, Orbitrap and ToF devices or as commercially available, stand-alone mass spectrometers with MS^n capabilities.

In addition to the choice of mass analyser, each type of instrument may be operated in different modes. The traditional approach to mass spectrometry involves scanning a wide mass range to obtain a mass spectrum. However, for LC-MS applications QqQ instruments are typically operated in selected reaction monitoring (SRM) mode which is also called multiple reaction monitoring (MRM) by some suppliers. This involves continuously monitoring a small number of selected transitions (*i.e.* mass numbers) for each analyte, typically one precursor ion to a couple of product ions. This approach provides a significant gain in sensitivity compared with acquiring full spectral data. Further gains have been achieved by steady innovation in instrumentation. Modern electronic technology and faster data acquisition interfaces, together with better designs of collision cell to allow faster clearing of ions from the cell, significantly shorten the minimum dwell times that can be used for each precursor/product ion pair monitored, without significantly reducing signal-to-noise ratios (S/N) or introducing cross-talk. The latter is the term used to describe the phenomenon when the fragment ions from one SRM transition are scanned out during another transition. Instrument vendors have also introduced tools for automating the management of the duty cycle resulting in more data points per peak, better reproducibility and higher S/N, even when the number of SRM transitions is doubled. Rapid switching between positive and negative ion detection also facilitates incorporating analysis of a wider range of compounds into one method. Other operating modes using QqQ analysers (*e.g.* product ion, neutral-loss and precursor ion scan) are rarely applied for quantitative analyses.

2.2.2 High resolution mass spectrometry

Over the last decade, new approaches to mass spectrometry such as ToF and orbitrap devices have enabled LC-MS instruments capable of rapidly providing a full range of spectral information with the added bonus of high mass resolving power. The latter provides selectivity and the capability for accurate mass measurement to aid identification. These developments in instrument design have resulted in improved quantitative performance due to extended dynamic range, faster acquisition rates to generate sufficient data points across the chromatographic peaks, and higher sensitivity. The main reason for using high resolution mass spectrometry is often to enable untargeted analysis. This is based upon the retrospective interrogation of the data using databases and libraries for identification of analytes other than those pre-programmed into the acquisition method. However, this approach is of limited value for quantitation as this requires the response from peaks in sample extracts to be

compared to that from standard solutions which are often omitted from the untargeted workflow.

3 Method development and optimisation

Developing a quantitative LC-MS assay is an involved, iterative process that can often be very time consuming if the assay requirements are challenging. Typically the two main demands are achieving adequate sensitivity and selectivity. These two factors are affected by every stage of the assay, as shown in Figure 3.1, and these co-dependencies can be traded off to achieve the required result.
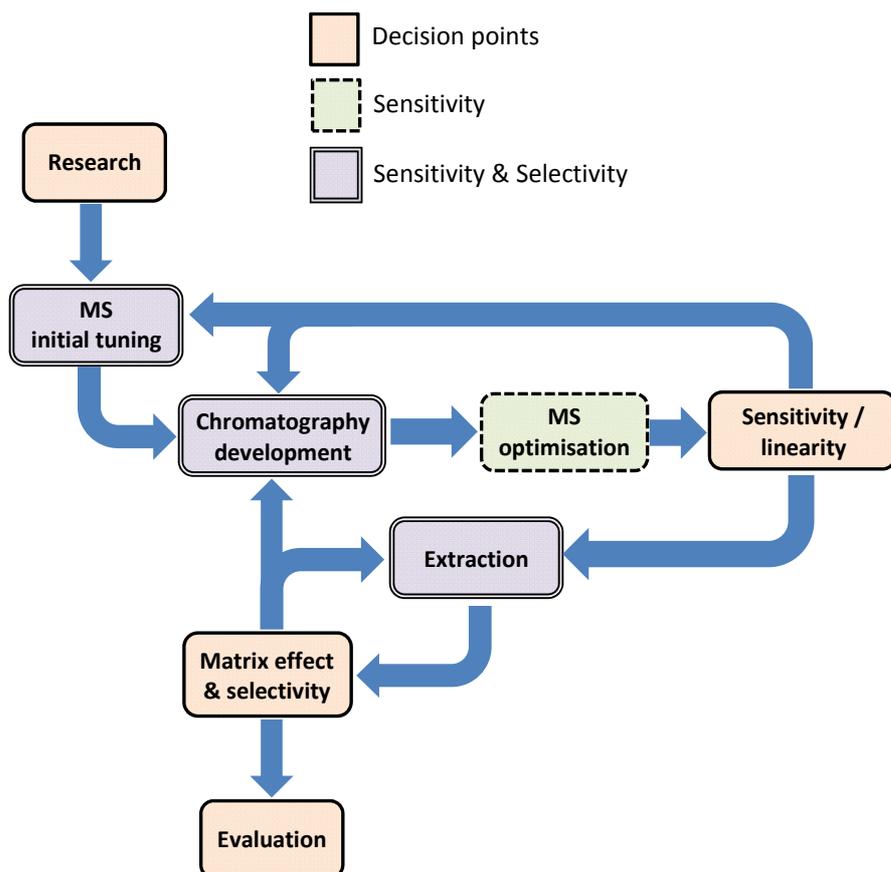
Figure 3.1: Stepwise optimisation of the overall LC-MS method

This complex synergy can easily be broken down into a logical step-wise sequence which avoids some of the cyclical pitfalls of method development. However, it is important to keep in mind that one is optimising a single system within which all the steps need to be compatible.

3.1 Research and planning

This stage is crucial as it will often be the case that a small nugget of information will end up saving days of frustration in the laboratory. The type of information required can be broken down into four categories:

1. Chemical and structural information about the analyte(s) being measured.
2. Information about the type and condition of samples that will ultimately be measured.
3. Information about assays which have been developed in the past.

4. Industry or regulatory guidelines which need to be applied (if any).

Even though the method development process is essentially identical for a wide range of compounds, the finer points within each stage will need planning to ensure that the unique chemical properties of the analytes are taken into account. The planning stage sets the starting point for all subsequent experiments, so time will be wasted if it is poorly considered. It is also worthwhile to ensure that the effort is proportional to the end point. For example, an assay that is measuring clinical data for the pharmaceutical industry will need to be more robust than a screening method used in drug discovery.

3.2 MS initial tuning

The first practical step is determining whether the analyte(s) will ionise in the mass spectrometer. This is usually carried out by introducing a constant stream of analyte into the ion source using a syringe infusion pump. This allows the mass spectrometer parameters, such as gas flow and ionisation voltage, to be 'tuned' to the analyte, creating the optimum conditions for ionisation and therefore sensitivity. It is here that the best ionisation mode (positive/negative) will be determined, adducts will be noted and, if performing MS^n, the most informative fragments chosen.

3.3 Chromatography development

Arguably, developing a good chromatographic method is the most critical factor in a robust assay since it affects selectivity and sensitivity. Some suitable starting conditions should have been identified in the planning stage. In the absence of any previous method information the most sensible approach is to choose a starting point based on the logD of the analyte. There are several software packages available that will calculate logD, including some free on-line services such as ChemIDPlus (http://chem.sis.nlm.nih.gov/chemidplus/). The logD of an analyte is a measure of its lipophilicity at a given pH and is, therefore, usually related to its retention on a reversed-phase column such as C18 (more lipophilic = more retention). If an analyte is determined to be very polar (logD<0), then it may be better to investigate other retention mechanisms such as HILIC or ion-exchange. The standard LC development principles apply but special consideration should be given to flow rate and buffer choice. The flow rate should be within the limits of the mass spectrometer and any pH modifiers should be volatile. Typical additives are formic acid, acetic acid, ammonia solution, ammonium acetate and ammonium formate.

With MS/MS analysis it is often advantageous to monitor two or three MRM transitions for each analyte at first. This gives confidence that the correct analyte is being monitored and the different selectivity of each transition could be useful in the later stages of development when actual sample extracts are analysed. When developing a multi-analyte assay a balance will have to be made between a good chromatographic separation and a useable analysis time. As previously mentioned, co-elution of different components is acceptable as long as the MS can distinguish them and they do not interfere with the ionisation process.

3.4 MS optimisation

Once the LC conditions have been determined it is usually worth retuning the mass spectrometer, since the ion source parameters are dependent on the composition and flow rate of the mobile phase being used to generate the spray. This will ensure that the optimum sensitivity is achieved and can help improve reproducibility.

3.5   Sensitivity assessment

Sensitivity is ultimately determined by a combination of mass spectrometer response, LC conditions and extraction efficiency. Typically the sensitivity is based on a signal-to-noise (S/N) ratio at a particular concentration. A S/N value of 10 is usually acceptable but can be lower depending on the assay requirements. At this stage it is useful to know the concentration of analyte that will achieve the required signal-to-noise value since this will determine whether actual samples will need to be concentrated or diluted. The linear range will also need to be evaluated. A mass spectrometer is only linear over a certain concentration range before saturation occurs either in the ion source or at the detector.

3.6   Sample preparation and extraction

This is probably the most technically demanding stage in development. The aim is to remove as many interferences as possible while maximising recovery of the analyte(s). Where low concentrations are being measured it may also be desirable to introduce a concentration step. Typical extraction techniques include solvent extraction, solid phase extraction (SPE), protein precipitation (for biological matrices) and, if the matrix is simple enough, dilution. As part of this development phase it will be necessary to measure the effect of matrix on the ionisation process (matrix effect) and analyte recovery. If sufficient selectivity cannot be achieved during the extraction it may be necessary to adjust the LC conditions to separate out any interferences.

3.7   Matrix effects

There are two established methods for assessment of matrix effects: the qualitative post-column infusion method [1] and the post-extraction spike method [2] which is able to quantitatively assess matrix effects.

3.7.1   Post-column infusion

In this approach, a constant flow of analyte solution is introduced into the mobile phase post column (see Figure 3.2) resulting in a raised background signal. A blank sample extract is then injected onto the LC-MS system using the LC method under test. The sample components elute from the column and enter the MS source. If any of the eluting components interfere with the ionisation of the analyte the background signal will be disrupted. Using this technique the size and spread (across the chromatogram) of the matrix effect is visually represented which allows one to determine whether adjusting the LC method will be sufficient to avoid the matrix effect or whether a better sample clean-up is required.
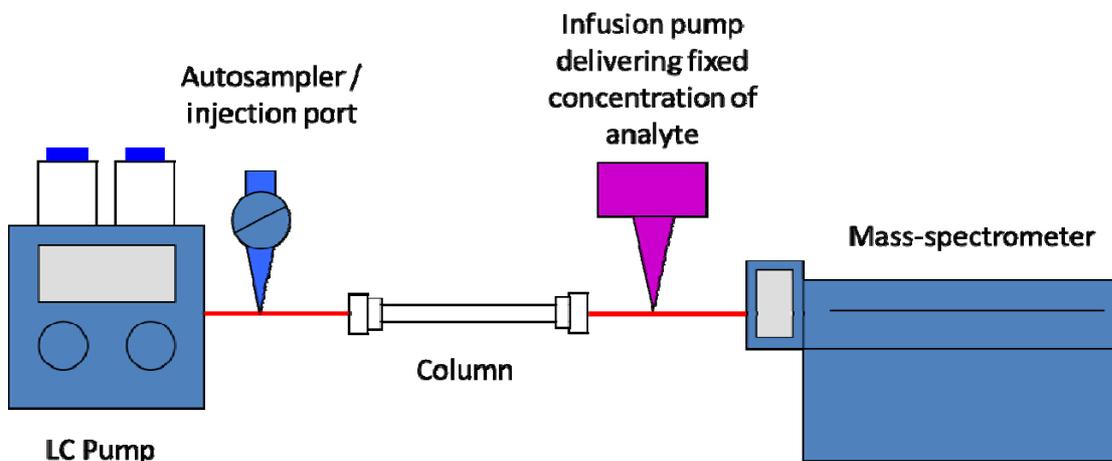
Figure 3.2: Use of post-column infusion for assessment of matrix effects

3.7.2 Post-extraction spike

Here, the MS responses of two samples are compared; one sample is in the matrix under test, *e.g.* plasma, the other in a matrix considered to be clean, *e.g.* water. Using this technique, if there is a matrix effect, the signals measured from the sample and the clean control will be different and can be quantified. It would seem an easy approach to spike analyte into both matrices and then extract them, but it is possible that the recovery of analyte would be different for each, giving a skewed result. The way around this is to extract the samples first and then add the analyte at the final stage. The downside to this technique is that finding a suitable point to add analyte can sometimes be difficult and one cannot visually see how close any matrix effect is to the analyte. Using both techniques together is recommended.

3.8 Extent of development

Knowing when a method is 'good enough' is sometimes difficult to judge. There will always appear to be slight improvements possible at every stage and, whilst striving for the best assay is to be applauded, there will be a point at which any further fine-tuning is a waste of time and effort if the assay fulfils its purpose. The next stage in the development process is evaluation and any deficiencies in the assay will be picked up there.

3.9 Evaluation of the method

Prior to using the method to measure samples, it is usual to assess the overall performance with respect to a number of key parameters. In many applications this assessment will be a formal validation procedure. The criteria against which the parameters are assessed will depend on the intended use.

3.10 References and further reading

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4 Sample preparation

Samples subject to analysis are very rarely in a form ready for acquiring the required data. They are usually a complex mixture where the target compound or analyte is present at relatively low levels in relation to the remaining sample constituents. Although some methods can be used to directly analyse a sample, such as 'dilute and shoot' [1], they rely on the capability of the analytical system to discriminate the target analyte from the rest of the sample. As explained in the introduction, the combination of liquid chromatography (LC) with mass spectrometry (MS) provides an analytical technique with a high capability to discriminate between different molecules. Yet, even with this capability, the vast majority of analyses require greater clean-up or concentration of the sample prior to use of LC-MS in order to achieve the selectivity, sensitivity and robustness required by routine analytical applications.

4.1 General considerations of sample preparation

The entire environment and management of the sample must be considered during the sample preparation procedure for a successful outcome. These considerations include:

- The suitability of sample containers – some analytes are known to adsorb onto plastic, and certain solvent mixtures can dissolve molecular components from the container into the sample thereby increasing its complexity.
- The thermal stability and photostability of the analyte – whether the preparation needs to be carried out at a reduced temperature or in amber glass vials.

Following the selection of appropriate containers and handling conditions for the sample, methods of preparation or extraction can be used in one of two ways:

1. To remove and concentrate the target analyte.
2. To selectively remove other component(s) of the sample that may interfere with the analysis due to their high abundance and/or inability to be distinguished from the target analyte.

The second approach is typically used for qualitative analyses where little is known about the sample, including which prospective target analyte may be present, other than the presence of a highly abundant interference, such as albumin. For most LC-MS quantitation, the target analyte is known and the most pragmatic approach is to selectively isolate it to increase the potential sensitivity of the measurement technique for the analyte.

There are many sample preparation techniques available and they can be classified according to the state of the sample involved, *i.e.* gas, liquid or solid. There are a number of texts [2-5] and reviews [1, 6-8] that comprehensively discuss the theory and practicalities of sample preparation (extraction) in detail. For the purpose of this guide, only techniques which allow the sample to be presented to the LC-MS system in a liquid phase will be discussed as this is normal for the vast majority of LC-MS analyses. Liquid samples can potentially be generated from anything (a surface or free solid) that can dissolve in a polar or non-polar organic solvent. However a key issue is to achieve quantitative dissolution of the target analyte(s) without introducing breakdown or other changes to the composition. The range of

possible samples and analytes is vast, all having different compositions and all posing their own issues for analysis. The following text and reviews give some examples:

- Clinical and forensic applications where samples analysed may be blood [9, 10], serum [9, 10], plasma [9, 10], urine [9], cerebrospinal fluid (CSF) [9], oral fluid [10, 11], dried samples on collection paper/card [9, 12].
- Environmental and industrial applications where samples may be in the form of effluent (*e.g.* waste water) [4, 13], solubilised foodstuffs [14], or particulates and soil samples [4, 13].

Thus, this guide cannot describe and reference every possible approach. What it does attempt is to provide guidance on how a sample preparation procedure may be developed which is 'fit-for-purpose', with the aim of producing a sample extract or solution that is compatible with the LC-MS system (typically consisting of a particulate-free liquid containing concentrated analyte) [4, 7]. This will be dependent on:

- What sample is being analysed.
- What must be extracted from the sample (target analyte or interference).

4.2 Liquid-liquid extraction (LLE)

This is a relatively simple and quick wet chemistry technique based on the solubility of an analyte between two **immiscible** solvents, where the target analyte passes from its solvent of origin into a 'polarity compatible' solvent in which it is (more) soluble. In the case of a highly non-polar analyte, contained in an aqueous biological matrix such as urine, the analyte will partition into the added immiscible non-polar solvent [5].

4.2.1 Partition ratio (K) [15]

The partition ratio is the relative measure of separation at equilibrium conditions (often referred to as 'distribution constant', 'partition coefficient', or *log P)* [3, 5, 15, 16, 17], as shown in Figure 4.1.

$$ K = \frac{[A_2]}{[A_1]} $$
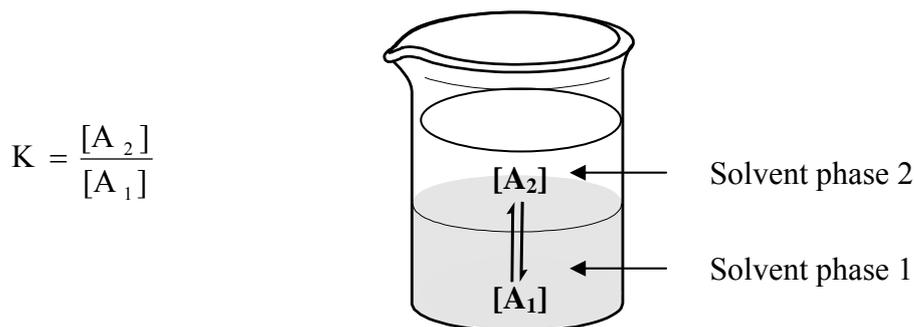
[A$_2$] = molar concentration of the target analyte (A) in solvent phase 2

[A$_1$] = molar concentration of the target analyte (A) in solvent phase 1

Figure 4.1: Derivation of the partition ratio for liquid-liquid extraction

- IUPAC recommends the use of the term partition ratio rather than partition coefficient [15].

- The extraction solvent can be strategically chosen according to its immiscibility with the sample solution and compatibility (*e.g.* polarity or hydrophobicity) with the target analyte [16, 17]. The partition ratio may be used as a gauge of good solubility for an efficient and high recovery extraction [17].
- Ionic (dissociated) analytes typically will remain in the aqueous layer unless neutralised [5].
- As extraction is typically not 100% efficient (unless K is large), some analyte will still remain in the sample liquid phase. Repeated extractions of the sample liquid are often required for high recoveries of the analyte and are more efficient than a single volume of extraction solvent (see the following references for determining extraction efficiency and recovery [3, 4, 5]). For example, if extraction is 80% efficient at recovering the analyte:
  - After 1st solvent extraction, 80% of analyte removed in total.
  - After 2nd solvent extraction, (80/100) x20% = 16% so… 96% of analyte removed in total.
  - After 3rd solvent extraction, (80/100) x4% = 3.2% so... 99.2% analyte removed in total.
  - While a single extraction would have removed only 80% of analyte.

4.2.2 Extraction procedure

Separation can be carried out within a sealed container such as a separating funnel (for larger solvent volumes, typically >5 mL), mixed and possibly centrifuged to improve partitioning. The target solvent layer (containing the analyte) is then removed (*e.g.* using a Pasteur pipette or drained from the separating funnel) and the extraction of the sample liquid repeated to increase the recovery of the analyte. During removal of the target solvent layer, care should be taken to avoid withdrawing solvent too close to the solvent partition as target solvent may become contaminated with sample liquid. The target solvent is then either analysed directly or evaporated ready for reconstitution in a smaller volume of an appropriate solvent [2, 5] (i.e. with consideration for LC-MS analysis [2]).

4.2.3 Practical considerations

- If the organic solvent has lower density than water (*e.g.* diethyl ether, hexane, ethylacetate) – the organic solvent phase is the top layer, above the water [5].
- If the organic solvent has greater density than water (*e.g.* chloroform, dichloromethane) – the organic solvent phase is the bottom layer, below the water [5].
- To concentrate the analyte, or transfer the analyte to a solvent more suited to the subsequent analysis:
  - Organic phase – evaporate (*e.g.* with nitrogen gas) and reconstitute in a smaller volume of suitable organic solvent for analysis (if required) by GC/MS as the solvent is volatile.
  - Aqueous phase – lyophilise (freeze-dry) and reconstitute in a smaller volume of aqueous solvent (if required) for analysis by LC-MS as the solvent is relatively polar.
- Solvents selection:
  - Immiscibility – solvents must be immiscible (check the miscibility table).
    o Typically one solvent is water as it is immiscible with many organic solvents.
  - Cost of solvent and apparatus – typically relatively large solvent volumes are used.
  - Safety – *e.g.* replace benzene with toluene due to the toxicity of the former.
  - Evaporation time and volatility – if the target extraction solvent is organic a more volatile alternative that will evaporate in less time could be an option.
- Drying agents: [16]

- Solvent systems are generally not 100% immiscible and small droplets of aqueous solvent may still be found in the organic layer and vice versa.
- If the target solvent layer is organic it may be dried (removing residual aqueous solvent from target analyte/solvent) using an insoluble inorganic compound (*e.g.* $MgSO_4$, Na_2SO_4, $CaCl_2$), added until no more clumps of solid are stuck to the sides/bottom of the container to ensure drying is successful.
  - As drying agents are insoluble in the target solvent they will remain solid.
  - The solution may be left to settle or centrifuged at low speed – the target organic solvent may then be decanted (and filtered) into an alternative clean container leaving solid drying agent and residual aqueous solvent behind.
- Modifying solubility: [3, 5]
  Acid-base extraction
  - Analyte solubility is dependent on the degree of ionisation (dissociation) in the solvent, *e.g.* neutral non-polar analytes can pass into the organic solvent easily unlike ionic (fully dissociated) polar analytes.
    - pH of solvents can be manipulated to encourage dissociation (or neutralisation) and passage of analyte from one phase to another. For example, decreasing solution pH (2 units below pKa of the weakly acidic anion) by adding an acid can neutralise the anion in an aqueous solution enabling it to transfer into the organic layer more easily, as shown in Figure 4.2.
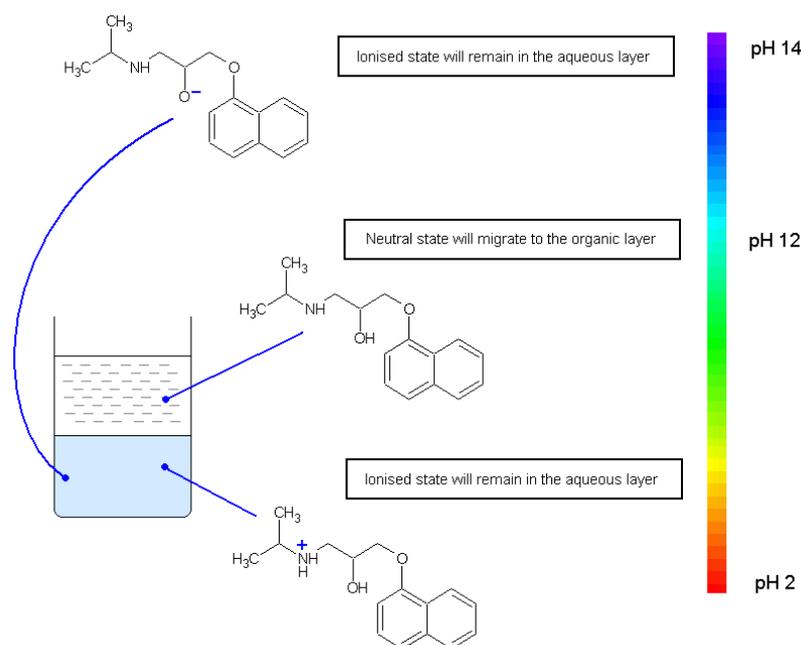
Figure 4.2: Illustration of how solution based chemistry may be manipulated using pH to encourage partition of propranolol (pKa = 9.5)[18] into aqueous or organic liquid phases.

- Agitation of extraction solution:
  - The solution will need to be mixed by shaking/vortexing and then be allowed to settle maximise the efficiency of the transfer of analyte from one solvent to another, and to ensure that immiscible solvent layers do not form an emulsion (suspension of droplets of a solvent in the body of another).

- If an emulsion does form, the sample may be centrifuged to ensure sufficient separation [4, 5].

Metal complexing
- If the target analyte/contaminant is a metal ion, it may be removed into an organic solvent through forming a neutral complex with a metal chelator (*i.e.* an oppositely charged organic ligand such as 8-hydroxyquinoline) [3, 4].

4.3 Protein precipitation

This is one of the more straightforward sample preparation methods used for generating partially clean extracts for LC-MS quantitation from samples with a relatively high abundance of protein. Protocols typically involve [2, 19]:
- Addition of an organic solvent (*e.g.* acetonitrile/methanol/acetone), ammonium sulphate or trichloroacetic acid (TCA) to a biological sample such as blood, serum, or plasma.
- Following precipitation (which may require mixing/vortexing), the sample mixture may be centrifuged to draw the protein precipitate to the bottom of the sample vial, leaving other components in the liquid layer.
- Removal of the 'protein-free liquid' which may be analysed directly, or evaporated to dryness and reconstituted in a (more) suitable solvent (*i.e.* elution strength) prior to analysis by LC-MS [2].

Addition of precipitate-inducing additives which function using different mechanisms, including dehydration (*e.g.* using ammonium sulphate or an organic solvent such as acetone) and denaturing of the proteins through an adverse change in pH (TCA) [19]. Parameters that may be varied within the protocol can include:
- The amount of additive.
- The type and combination of additive (organic solvent/TCA/ammonium sulphate).
- Duration of the precipitation procedure [19].

4.4 Solid phase extraction (SPE)

This preparation technique, which is commonly used in both environmental and clinical applications, uses a solid stationary phase sorbent (normally contained in a cartridge) to clean-up and/or concentrate sample components prior to analysis. There is a wide range of sorbent chemistries available that are typically surface-modified silica supports, providing great selectivity to strategically retain either the target analyte or interferences present in the sample. For trace analysis, SPE is generally used to selectively isolate and concentrate a known target analyte within the sample [2].

4.4.1 SPE protocols

Typical SPE protocols for trace analysis involve these steps (as shown in Figure 4.3) [4, 20]:
1. *Wetting and conditioning* – to solvate and activate the sorbent to retain analytes of interest.
2. *Sample loading/retention* – application of the sample to the cartridge (sorbent) where analytes of interest are retained.
3. *Wash/rinse* – any lesser retained interferences are washed off the sorbent.
4. *Elution* – removal of analytes of interest into a collection tube using a relatively small volume of suitable solvent.

To ensure optimum target analyte retention and, depending on the sorbent chemistry, it may be necessary to pretreat the sample:

- Dilute/adjust the ionic strength/pH of the sample for the target analyte.
- Remove insoluble material (*e.g.* particulates) using filtration [5, 20] or centrifugation prior to sample introduction to the SPE cartridge [20].

The choice of SPE procedure and sorbent characteristics will depend upon a range of issues such as:
- The sample volume [5, 20].
- The sample solvent characteristics and content of the sample matrix (*i.e.* interferences present within the sample) [4, 20, 21].
- The target analyte characteristics (how the analyte is intended to be retrained) [21].
- The amount of target analyte [2, 21] (analyte loading should be <5% of the maximum loading capacity of the sorbent) [2].

i.

CONDITIONING

ii.

RETENTION

iii.

RINSE/WASH

iv.

ELUTION

Key
- Retained undesired matrix component
- Retained target analyte
- Unretained undesired matrix component
- Solvent flow
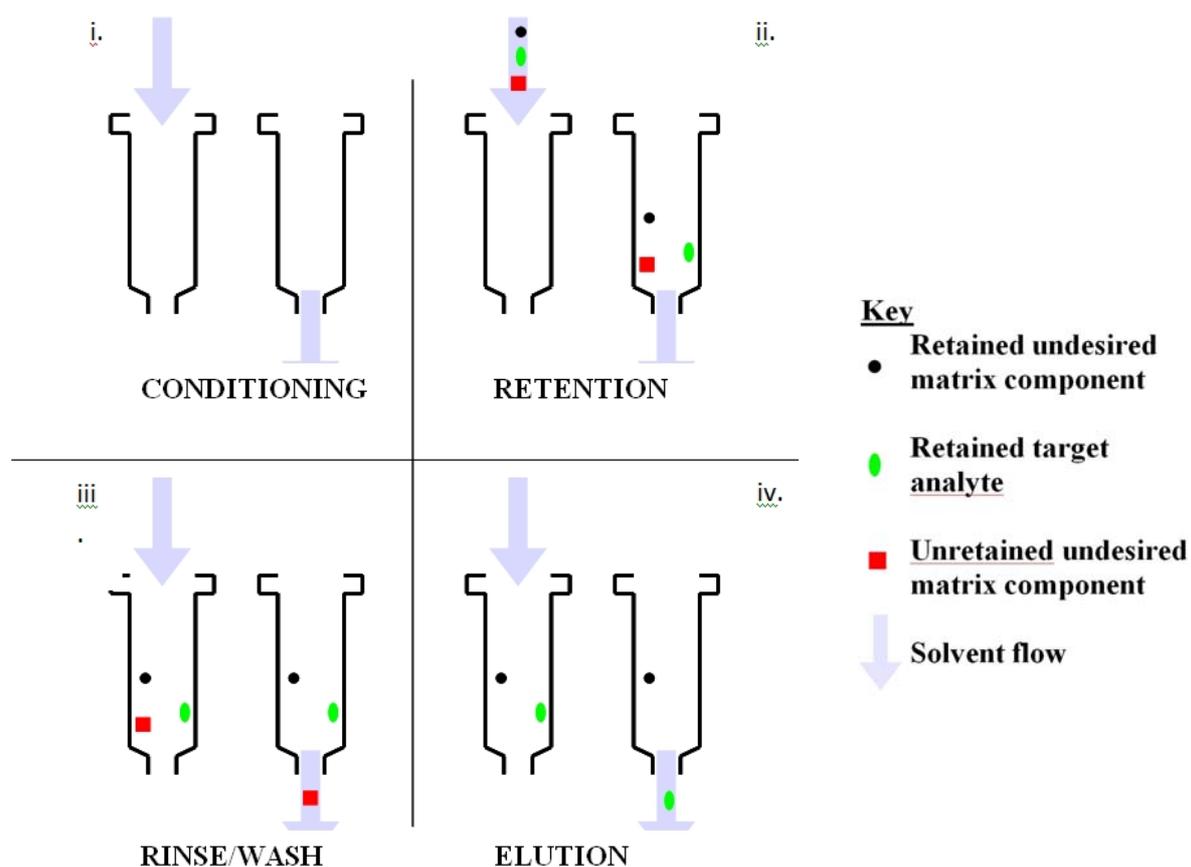
Figure 4.3: Generic experimental workflow for an SPE extraction showing the retention and separation of the target analyte from other components in the sample.

There are a number of helpful texts available for method development that new practitioners of SPE may use. Such texts contain tips and advice that are beyond the scope of this guide [2, 4, 5, 20, 21].

4.4.2 Practical SPE considerations

Sorbent chemistry

- SPE is highly selective with a wide range of sorbent chemistries offering retention by a specific interaction [5, 20] (see Table 4.1). It offers inherent enhanced possibilities for separation compared with liquid-liquid approaches as the solid sorbent is immiscible with extraction solvents.
- Mixed mode sorbents:
  - Capable of a combination of interactions, such as non-polar and cation exchange [5] (see Table 4.1).
  - Enable targeted isolation of analytes according to both polarity and ionic characteristics in a single extraction, with the option of the fractionation of classes of compounds into acids/neutrals and bases if required.
  - Manufacturers of these products provide useful guides regarding the protocols however, this method is summarised quite succinctly in reference 22.
- In addition to these primary interactions, analytes can experience secondary interactions with unreacted silanol groups on the silica surface as the modification process may not provide 100% coverage [4, 20, 21]. To minimize the effect of these interactions, modified sorbents can be further exposed to end-capping reagents such as trimethylchlorosilane (TMCS) to react with any remaining free silanol groups on the silica surface. Practitioners of SPE should, however, be aware that non-endcapped modified sorbents have different extraction properties to those that are endcapped [5, 21].

Solvents and pH

- Depends on the target analyte, other sample constituents, sorbent (see Table 4.1 for further details), and compatibility with the eventual method of detection [2, 21].
- A suitable solvent/sample flow rate should be chosen to ensure the sorbent is properly prepared for analyte retention [4, 20] and elution [20].
- Sorbent conditioning is usually with a solvent having characteristics that would not disrupt the chosen retention mechanism. For example, wetting and conditioning a C8 cartridge may involve using solvents such as methanol or acetonitrile which solvate and 'detangle' the C8 chains, followed by a volume of 100% water if extracting the target analyte from urine [4, 20].

Analyte Retention

- For retention to occur, consideration should be given to the compatibility of the sorbent, the target analyte and the sample matrix [20].
- Biological matrices are generally aqueous solutions and would therefore typically be used with a non-polar or reversed-phase sorbent, such as a C8 or C18, to isolate molecules that have sufficient non-polar character to adsorb [20].
- A solvent wash or rinse may be used to remove any unwanted interferences that are unbound or weakly retained on the sorbent, leaving the target analyte *in situ* [20]. For example, for an aqueous biological matrix and a relatively non-polar target analyte on a reversed-phase sorbent, it is necessary to use a solvent similar to the sample matrix so that it does not disrupt the Van der Waals interaction between the target analyte and the sorbent.

Analyte elution
- Uses a solvent that can disrupt the retention interaction between the target analyte and the sorbent [20]. For example, for an aqueous biological matrix and a relatively non-polar target analyte on a reversed-phase sorbent, this solvent should be of stronger non-polar character than the Van der Waals interaction with the sorbent. See Table 4.1 for further details regarding the alternative sorbent chemistries, retention mechanisms, and solvents.
- Eluants can be analysed directly on a LC-MS system (if compatible) or, more commonly, evaporated to dryness using nitrogen and reconstituted in a smaller volume of solvent compatible with both the LC system and the target analyte, *i.e.* LC mobile phase [2].

Interaction	Class of sorbent	Example sorbent type	Chemical 'R' group	Application	Wash Solvent	Elution Solvent
Polarity	Polar (*i.e.* Normal phase)	Cyanopropyl	$-(H_2C)_3-CN$	Mid to non-polar sample matrix with analytes containing some polar character (retained by hydrophilic interactions)	Non-polar solvent that will maintain polar interaction (*e.g.* hexane)	Solvent stronger than polar interaction (*e.g.* water, methanol)
		Aminopropyl	$-(H_2C)_3-NH_2$			
		Silica				
	Non-polar (*i.e.* Reversed-phase)	C18	$-C_{18}H_{37}$	Polar sample matrix (*e.g.* aqueous biological sample) with analytes containing some non-polar character (retained by van der Waals forces)	Polar solvent that will maintain non-polar interaction (*e.g.* water)	Solvent stronger than non-polar interaction (*e.g.* methanol, hexane)
		Polystyrene-divinylbenzene (PS-DVB)				
		C8	$-C_8H_{17}$			
Ionic	Cation exchange (Weak, WCX)	CBA (Carboxypropyl)	$-(H_2C)_3-COOH$	Basic analytes (sorbent surface is negatively charged so used to retain cations)	Maintain pH at least 2 units below the pKa of the cation (basic analyte) to ensure cation is charged	Increase pH to 2 units above pKa of cation to neutralize and disrupt interaction
	Cation exchange (Strong, SCX)	Benzenesulphonic acid	$-SO_3H$			
		PRS (Propylsulphonic acid)	$-(H_2C)_3-SO_3H$			
	Anion exchange (Weak, WAX)	Aminopropyl	$-(H_2C)_3-NH_2$	Acidic analytes (sorbent surface is positively charged so used to retain anions)	Maintain pH at least 2 units above the pKa of the anion (acidic analyte) to ensure anion is charged	Decrease pH to 2 units below pKa of anion to neutralize and disrupt interaction
		PSA (Primary Secondary Amine)	$-(H_2C)_3-NH-(CH_2)_2-NH_2$			
	Anion exchange (Strong, SAX)	Quaternary amine	$-(H_2C)_3-\overset{+}{N}-(CH_3)_3 \ Cl^-$			
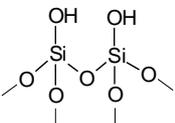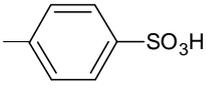
Table 4.1: Summary of common SPE sorbent chemistries and experimental information for practitioners. Note: for ion exchange sorbents the sorbent surface and the analyte must be oppositely charged for retention to occur. This should be taken into account with the control of pH in the SPE protocol [5, 20, 21]

4.5 Sample preparation kits

Unlike application to large biomolecules (DNA, RNA, proteins), the use of sample preparation kits for small molecule quantitation by LC-MS has only recently come to the fore, with the combination of SPE technology and drying agents to improve the recovery of specific classes of analytes. The technique used within these kits is known as 'Quechers' and is a 'QUick, Easy, CHeap, Effective, Rugged and Safe' preparation method designed initially for the targeted extraction of pesticides from foodstuffs [23]. This technique has now been adopted as a product by several sample preparation vendors including Waters (Disque™), Agilent (SampliQ™) and Supelco (dispersive SPE tubes).

'Quechers' methods typically consist of two stages:
- Sample extraction using a water-miscible organic solvent such as acetonitrile, with the addition of a salt (*e.g.* magnesium sulphate) [23-28] and possibly other additives, such as buffer [25-28], to encourage liquid separation and improve recovery of target analytes, respectively.
- Clean-up of the sample extract using dispersive SPE (dSPE) sorbent, typically PSA based, which is mixed with the sample extract [23-28].

Following each stage, the sample is mixed and centrifuged to ensure minimal carryover of interferences in the target solvent layer [23, 26], and the final extracts are analysed by LC-MS after dSPE 'clean-up' [24, 26]. There are a number of experimental parameters that dictate the success of the 'Quechers' extraction [23] including:
- The sample matrix.
- Homogeneity of the sample.
- The ratio weight of sample:solvent volume
- The selection of extraction solvents.
- The type and amount of partitioning salts.
- pH (*e.g.* buffer [25, 27]).
- The agitation/extraction technique (shaking, vortex mixing or blending).

Further adaptations of the 'Quechers' methods have involved variations of these parameters to optimise the method for specific applications.

Pros and cons of 'Quechers' methods
- Benefits
  - Quick [23, 24, 26].
  - Low volumes of solvent required [23, 24].
  - Highly accurate and suitable for quantitation (when used with an internal standard) [23-26].
  - High recovery (for a range of pesticides) [23-26].
  - Not 'resource hungry' with regards to laboratory apparatus and space [23, 24].
  - Low cost [23, 24, 26].
  - Rugged [24, 26].
- Limitations
  - Relatively few validated protocols involving alternative classes of analytes other than pesticides.

Recent investigations of 'Quechers' have involved broadening its use for target analytes of alternative chemistries and for different sample matrices. These include non-polar polycyclic

aromatic hydrocarbons in seafood contaminated with oil following environmental pollution [29] and high throughput pesticide screening within various parts of plants used in Chinese medicines [30], showing 'Quechers' has the potential to be used effectively for a wide range of applications.

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5   Selection and optimisation of the chromatographic system

The most important and widely used LC separation technique for quantitative LC-MS/MS is reversed-phase (RP) separation. This utilises differences in hydrophobicity (termed "non-specific hydrophobic interactions") to achieve partitioning between an apolar stationary phase and a polar mobile phase. Typically, mobile phases use an aqueous blend of water with a miscible polar organic solvent, such as acetonitrile or methanol. This competes effectively with analyte molecules for sites on the apolar stationary phase, displacing analytes causing them to move faster through the column. With control of solvent composition, pH, temperature and flow rate, RP can provide good peak shape and enable separation of many analytes from each other, as well as from isobaric interferences or co-extractives responsible for ion suppression. In cases where there is insufficient retention of polar or ionic compounds, good quantification is still possible using alternatives such as hydrophilic interaction chromatography (HILIC) [1], use of volatile ion pair reagents (*e.g.* heptafluorobutyric acid (HFBA)) [2] and porous graphitic carbon (PGC) [3].

This chapter provides options for establishing and optimising LC conditions. Initially trying some generic LC conditions for RP chromatography is suggested and, depending on the outcome, information on implementing additional changes for further optimisation is provided. This chapter also includes a list of tips to maintain systems for optimum chromatography. A number of books are available that discuss best practice for HPLC [4, 5].

5.1   Key chromatographic parameters impacting on quantification using LC-MS/MS

The separation of any two components can be varied systematically by changing experimental conditions. The key parameter affecting quantification is resolution (R_s), which is expressed in terms of three parameters that are directly related to experimental conditions:

- retention (or capacity) factor (k).
- selectivity (α)
- column efficiency (also known as column plate number) (N).

For many LC-MS/MS applications, ultimate LC selectivity is not required as sufficient selectivity is often provided by the MS/MS. This guide makes reference to the invaluable resource provided by John Dolan via the Separation Science web portal (http://www.sepscience.com). The reader is directed to various relevant tips from the HPLC Solutions series (http://www.sepscience.com/Information/Archive/HPLC-Solutions).

5.1.1   Retention factor (k) [1] [2] [3]

The retention factor (k), which was previously termed capacity factor, is the degree of retention of a sample component in the column. It is defined as the time the solute resides in the stationary phase relative to the time it resides in the mobile phase. When an analyte's elution is too fast, the retention time can vary and there is a greater probability of co-elution of analytes with other components from the matrix. This can result in isobaric interference,

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[1] http://www.sepscience.com/Techniques/LC/Articles/314-/HPLC-Solutions-28-Back-to-Basics-1-Retention-Factor

[2] http://www.sepscience.com/Techniques/LC/Articles/361-/HPLC-Solutions-29-Basics-to-Basics-2-t0

[3] http://www.sepscience.com/Techniques/LC/Articles/170-/HPLC-Solutions-94-How-to-Calculate-k

either completely or partially masking the peak of interest, and/or ion suppression, which reduces the analyte response. Too much retention means that elution takes a very long time and peaks broaden, causing problems with precise and accurate integration.

5.1.2 Selectivity (α)[4]

Selectivity is the measure of relative retention or separation of two sample components. Important variables that affect selectivity are the column packing, the mobile phase composition (mainly pH) and how the properties of the solutes influence retention mechanisms. LC selectivity is more critical with optical detectors than when using LC-MS/MS but good selectivity can avoid isobaric interferences and matrix effects.

5.1.3 Column efficiency (N)[5]

The efficiency of a chromatographic separation is a measure of the dispersion of the analyte band as it travels through the LC system and column. A number of parameters contribute to column efficiency: flow rate, column dimensions, substrate particle size and extra-column effects (including instrumental and plumbing effects). In LC column terminology, particle size refers to the mean diameter of the silica spheres used as the support material to which the stationary phase is bonded. Decreasing particle size has been observed to limit the effect of flow rate on peak efficiency; smaller particles have shorter diffusion path lengths, allowing a solute to travel in and out of the particle faster. Therefore, the analyte spends less time inside the particle where peak diffusion can occur. In addition, column efficiency does not diminish when flow rate increases. The relationship between column flow rate and efficiency is described by the Van Deemter equation governed by three cumulative terms: eddy diffusion, longitudinal diffusion and mass transfer. These terms can be plotted both individually and cumulatively in graphical form to provide a Van Deemter plot of efficiency versus flow rate. Such plots can be used, for example, to illustrate improved efficiency from the use of sub-2 μm particles in specialist ultra-high performance liquid chromatography (UHPLC) systems or by using larger, fused-core/superficially porous silica (SPS) particles (also termed fused core) [6].

5.1.4 Resolution (R$_s$)[6]

The degree to which two compounds are separated is called resolution and is determined by the column efficiency and selectivity. Insufficient chromatographic resolution can lead to co-elution resulting in isobaric interference or ion suppression from the matrix.

5.2 Tips for selection of conditions

The objective of this section is to provide the reader with some information on which to base the selection of LC conditions.

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[4] http://www.sepscience.com/Techniques/LC/Articles/316-/HPLC-Solutions-31-Back-to-Basics-3-Selectivity

[5] http://www.sepscience.com/Techniques/LC/Articles/317-/HPLC-Solutions-32-Back-to-Basics-4-Efficiency

[6] http://www.sepscience.com/Techniques/LC/Articles/359-/HPLC-Solutions-39-Back-to-Basics-6-Resolution

5.2.1 Column

- Silica or alternative support (highly base-deactivated).
- Consider:
  - pH stability.
  - End-capping to reduce tailing.
  - Tolerance to backpressure created both during manufacture and in use.
- Particle size (<2 μm, <3 μm SPS, 3 μm or 5 μm):
  - Increased efficiency vs. increased back-pressure.
  - <2 μm may require specialised UHPLC equipment.
  - It is assumed that high efficiency is mandatory so it is likely that where UHPLC is available short columns (50 mm or less) packed with <2 μm particles will be used, whereas if only conventional HPLC is available columns packed with 3 μm particles or SPS particles (typically 2.5 or 2.7 μm) will be used.
- Internal diameter (2.1, 3.0 or 4.6 mm *i.d.*):
  - Increase in diameter allows higher flow rates, less peak broadening through dispersion and greater speed but it must be ensured that the electrospray source maintains sensitivity at high LC flow rates.
- Length (20-150 mm):
  - Increase in length gives greater peak capacity but higher back pressure.
  - Choice is dependent upon application, typically:
    - Columns for speed: 20-50 mm *i.d.*
    - Columns for increased peak capacity: 100-150 mm *i.d.*
- Phase:
  - Reversed-phase (RP):
    - C8 or C18.
    - Alternative selectivity through phenyl, cyano, perflurorinated, polar-embedded.
    - Modifications for retention of polar compounds with >90% aqueous to avoid dewetting (loss of retention).
  - Alternatives (most likely HILIC).
- Guard column/pre-column filters:
  - Guard column acts as a trap for particulates and strongly retained components arising from samples and LC system:
    - Use same particle size as analytical columns.
    - Installed immediately before the analytical column.
    - If samples have gone through clean-up there may be no need to have a guard column.
  - A pre-column filter protects the column from particulates (from samples, pump seals, and injector valve wear) but not from unwanted co-extractives:
    - Filter must be smaller in size than column frits.
    - Installed immediately before the guard column.
  - Both must be very low dead volume in design to avoid loss in performance.
  - Items must be replaced as soon as performance is observed to have deteriorated or as part of a planned maintenance regime.
- Tubing and fittings:
  - Materials need to be chemically compatible with the sample and the mobile phase, to be able to withstand the operating conditions (pressure and temperature) and be properly prepared.

- Use stainless steel (SS) tubing and fittings in places where once the connection is made there is no need to make further adjustments:
  o Take care to use the correct ferrules for the type of port or nut as manufacturer design varies.
  o Once swaging has taken place only use with that receiving port.
- Use PEEK (poly-ether-ether-ketone) tubing and fingertight fitting for connections that are changed often:
  o As there is no permanently attachment of the fingertight fitting to the wall of the tubing, once the fitting is moved from one receiving port to another, the tubing/fitting can be adjusted to the dimensions of the new port.
- Select the appropriate length, internal and outer diameter of tubing:
  o The most commonly-used tubing size is 1/16" (~1.6 mm) outer diameter but comes in a variety of inner diameters.
  o In some cases specific parts comprising the correct tubing with pre-swaged fittings are available from the vendors.
- The choice and handling of tubing and fittings can impact chromatographic results.[7][8]

5.2.2 Mobile phase for reversed-phase (RP) chromatography

- Organic solvent is the "strong" solvent in RP chromatography:
  - Typically methanol or acetonitrile.
  - Methanol exhibits the weaker elution strength so analytes are eluted from the column in a higher proportion of organic modifier. This results in more effective desolvation thus enhancing the electrospray response.
  - Methanol is more viscous than acetonitrile and will generate higher back pressures.
  - Using acetonitrile will elute analytes earlier and hence reduce run times.
- The buffering of the aqueous mobile phase for chromatographic purposes is often no longer required:[9]
  - The use of base-deactivated silica removes the need to block the ion exchange activity of the silica surface.
  - The pH is no longer adjusted to neutralise the analytes because ionised species can now be retained on RP columns with good peak shape.
  - The presence of low concentration (1-10 mM) of ammonium salt can act as a counter ion to restrict the effect of interactions of basic compounds with the silica surface of some columns.
  - Acceptable additives include acetic acid, formic acid, ammonium hydroxide, ammonium formate (≤10 mM) and ammonium acetate (≤10 mM).
  - Avoid the use of surface-active agents such as surfactants and non-volatile salts (phosphate, borate and citrate) as they suppress the response and deposit in the MS probe and source.
  - Select an appropriate flow rate that gives optimum linear velocity for the column packing selected, whilst maintaining good electrospray sensitivity.
- Injection modes and wash solvents:

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[7] http://www.sepscience.com/Sectors/Miscellaneous/Articles/421-/How-Fittings-Can-Impact-Chromatographic-Results

[8] http://www.sepscience.com/Techniques/LC/Articles/159-/Things-to-Consider-When-Selecting-HPLC-System-Tubing

[9] Buffers are traditionally used in HPLC to adjust the pH of the mobile phase to push ionisable compounds of interest (such as acids or bases) into the non-ionised state for better peak shape and consistent retention times.

- Valve/loop systems (*e.g.* Rheodyne and Valco):
  o Full/filled loop: injects 100% of nominal loop volume:
    - When accuracy and precision are the primary goal.
    - Need to change injection loops to vary volume.
  o Partial loop/fill: injects a portion of nominal loop volume:
    - Check specific instrument recommendation as there are limitations on volumes that can be injected.
- Flow through needle (needle in flow path design):
  o Programmable volume injection.
  o Low carryover.
- Ensure sample submission solvent and injection volume is compatible with initial mobile phase conditions when using the selected injection mode:
  o Sample must also be soluble in and miscible with the sample submission solvent.
- Wash solvents to minimise carry over:
  o Typically two solvents are used sequentially as per instructions in instrument manual (typically "strong" and then "weak").
    - Strong solvent (100% organic solvent):
      o To flush tubing.
      o To remove components injected.
    - Weak wash solvent (excluding buffers):
      o To remove strong solvent.
      o To return system to initial LC conditions.
      o Degassed for good hydraulic properties.

5.3 Suggested initial conditions

The objective of this section is to provide the reader with some generic conditions to act as a starting point.

5.3.1 Column

- HPLC system: C18 3 µm or <3µm SPS, 3.0 mm *i.d.* x 50 or 100 mm.
- UHPLC system: C18 <2 µm, 2.1 or 3.0 mm *i.d.* x 50 mm.
- Fitted with appropriate guard column and/or pre-column filter.

5.3.2 Mobile phase

- Aqueous component: ammonium formate (<10 mM) with or without 0.1 % formic acid (positive ion mode) or 0.1 % ammonia (negative ion mode).
- Organic component: methanol.
- Flow rate 0.3-1.0 mL/min (depending on column dimensions) at 40-60 °C.

5.3.3 Injection mode

- Full/filled loop or flow through needle.

5.3.4 Elution methods

- Isocratic to give k of between 2 and 5:
  - Isocratic conditions may not be appropriate if the requirement is to separate multiple analytes of different physiochemical properties.
- Program a rapid gradient elution (k>2), changing the solvent composition with time.
- Typically start at a high proportion of aqueous (*e.g.* 90 %) and change the composition linearly to high organic (*e.g.* 90 %) over a period of 5 minutes.

- It is prudent to hold the elution at the high organic composition for a fixed period (*e.g.* 2 minutes) to ensure all analytes and co-extractives have eluted off the column before returning to the starting conditions.
- Include an appropriate amount of time for re-equilibration of the column.

5.4 Troubleshooting

The objective of this section is to provide the reader with some options for further optimisation to overcome common issues that impact upon quantification. Ensure that any changes to chromatographic conditions do not have a significant impact on electrospray response, and evaluate changing conditions in the presence of matrix for realistic optimisation.

5.4.1 Retention capacity

- Too much retention – analyte peaks will not elute or will be too broad, reducing sensitivity:
  - Reduce the carbon loading of the column, *e.g.* change from C18 to a C8 phase:
    - Use a stationary phase bonded to the same type of silica to ensure that secondary interactions are kept consistent, although their impact is likely to increase with lower ligand coverage.
  - Alter gradient elution so that the initial proportion of aqueous is reduced and speed up the change to high organic and/or increase the proportion of organic solvent at the end to 100 %.
  - Switch from methanol to acetonitrile which has a greater elution strength.
  - Increase column temperature.
- Lack of retention – analyte peaks elute in the void volume and co-elute with interferences and/or components which suppress the electrospray signal:
  - Change stationary phase to one with multiple retention mechanisms (*e.g.* hydrogen bonding and/or ion exchange with silanols) to aid the retention of the more hydrophilic molecules whilst also allowing the retention of the hydrophobic analytes.
  - Alter the pH of the mobile phase using volatile buffers so that the analyte is in the un-ionised form (*i.e.* for bases increase the pH and for acids decrease the pH):
    - Knowledge of analyte pKa is helpful for selection of pH.
    - Ensure that the LC system and column chosen are stable at the new pH.
  - Change initial conditions to 100 % aqueous and perhaps hold the starting conditions isocratic (*e.g.* 1 minute):
    - Ensure that the chosen column can be used in such conditions and is not susceptible to dewetting, which will lead to complete loss of retention.
  - Use a HILIC column following manufacturers' guidelines to set up isocratic conditions that provides a k between 2 and 5. The sample submission solvent will need adjustment.

5.4.2 Selectivity

- Lack of resolution due to insufficient selectivity – automatic integration of analyte peaks is not possible and subjective manual intervention is required leading to reduced precision:
  - When C18 or C8 columns cannot be optimised to provide a sufficiently rugged separation, columns with more polar stationary phase chemistry should be evaluated:
    - Such phases utilise additional types of interactions (*i.e.* H-bonding and other dipole attractions) available with certain functional groups.

- Polar-embedded phases (*e.g.* amide, urea, ether, and carbamate functionalities) may also show improved peak symmetry for bases over non-polar alkyl phases.
- Adjust pH as described above to offer alternative selectivity for acidic and basic analytes through changes to the mobile phase:
  - In some cases altering the pH of the mobile phase will change the retention of isobaric interferences or components causing ion suppression whilst leaving neutral analytes unaffected.
- Poor peak shape – due to the selectivity from MS/MS, poor peak shape will not always adversely affect quantification but automatic integration is likely to be unreliable and limits of detection and quantification can be reduced:
  - Secondary interaction between positively charged analytes and acidic silanols on the surface of the silica cause peak broadening and peak tailing that compromise resolution and peak shape.
  - Column and associated guard may have become contaminated with co-extractives:
    - Replace guard and analytical column with new.
    - Regenerate analytical columns as per vendor's instructions.
  - Broad peaks or fronting often accompanied by shifts to shorter retention times indicate sample overload:
    - Dilute the sample by a factor of 10 to 20 and re-inject. If sharper peaks are now observed throughout the chromatogram this confirms a sample overloading problem.
  - Check the sample submission/injection solvent strength and sample size:
    - A strong injection solvent can cause the sample to move quickly through the first portion of the column while it re-equilibrates with the mobile phase, thereby broadening or even splitting peaks.
    - Select a weaker solvent as the injection solvent:
      - When using starting conditions with a high aqueous content, this tends to mean dissolving or diluting the sample in water, which can result in problems with a lack of solubility.
    - If there is sufficient sensitivity, inject in such a small volume that it is equilibrated instantaneously with the mobile phase:
      - Make sure that the wash solution is compatible with, and weaker than, the mobile phase.
  - If early eluting peaks are affected more than later peaks, the problem may be related to extra-column effects or injection solvent problems :
    - Extra-column effects might include poor fitting of tubing and ferrules to the injector, filter, guard or column or the improper choice of tubing (*e.g.* too long and/or wide).
    - Keep dead volume to a minimum as it causes severe band broadening with a subsequent decrease in peak heights (detection limits) and tailing making integration inaccurate and imprecise.
  - If the column is found to be functioning properly, the problem may be related to a partially plugged in-line filter, an injector problem, or incorrectly installed tubing.

5.4.3 Carry over and contamination

- The increase in sensitivity of recent LC-MS/MS instruments and the use of wide calibration ranges makes this a significant issue:
  - The amount of carry-over can be estimated from the response of a blank injection after analysis of an appropriate standard solution.

- The primary causes of LC carryover are:
  - Autosampler carryover from the residue of a previously injected sample absorbed on and/or trapped in the autosampler needle, injection port, transfer tube, sample loop, or injector valve resulting in an analyte peak in the chromatogram.
  - Residues adhering to Peek tubing and valves in the flow path.
  - Column carryover from the residue of a previously injected sample on the column resulting in increased background or analyte peak.
- Carryover may be avoided or reduced by:
  - A wash solvent that is strong enough to dissolve any remaining sample from the LC system.
  - Wash duration must be long enough to remove the residue from the LC system.
  - Sufficient hold-time at the final conditions of a gradient (too short can fail to remove all analytes from the LC system).
- The primary cause of elevated background from MS contamination is memory effects (background):
  - Ensure temperatures in the sprayer and source are set high enough for efficient desolvation to avoid condensation of sample in the source region.
  - Do not switch flow to waste at the end of the run without an auxiliary flow preventing the capillary and source to "dry".
- Other contributions to generating background peaks include:
  - Impure isotopically-labelled standards.
  - MS/MS cross-talk (insufficient time to clear ions from collision cell before next transitions monitored).

5.5   References and further reading

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6 Optimisation of the LC-MS interface and mass spectrometer

An overview of the strategy for optimisation is depicted in Figure 6.1.

Formation and selection
of precursor ions

↓

Formation and selection
of product ions

↓

Data acquisition settings

↓

Check using LC-MS/MS
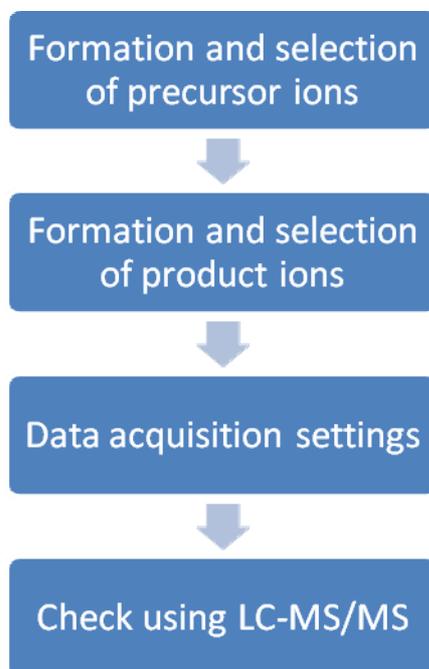
Figure 6.1: Overview of the optimisation strategy

Initially the goal of any optimisation procedure, whether automated or manual, is to select acquisition parameters that provide the best signal for appropriate analyte precursor and product ions. However, it is important to remember that the overall objective is to select transitions that give the best compromise of selectivity and sensitivity (as measured by signal-to-noise ratio). This can only be evaluated with real samples, in the presence of "matrix", when using LC-MS/MS in selected reaction monitoring (SRM) mode. Performance characteristics might include precision, limit of detection and quantification, linearity and selectivity (absence of interferences).

There are a number of parameters that can be adjusted which affect ionisation and desolvation, ion transfer, fragmentation in the collision cell and mass analysis. However, here the focus is only on those that have a significant impact upon quantification. Other parameters should be set as per vendors' instructions. For the purpose of this guide a manual approach is described. The same principles will apply when setting up automated procedures using vendors' software

The optimum values for some parameters depend more on the structure of the compound of interest, whereas others are more dependent on the LC mobile phase composition and flow rate. Some parameters have an impact on more than one process. The advice given here refers to "pneumatically-assisted electrospray ionisation" unless other ionisation modes are cited. Additional key parameters in APCI, which is not covered further here, include corona discharge current and vaporiser temperature.

6.1 Key mass spectrometric parameters impacting on quantification using LC-MS/MS

6.1.1 The nebulisation process

By optimising the parameters that affect the droplet radius and number of charges on the droplet, the production of ions may be maximised and, therefore, the instrument sensitivity enhanced. The formation of a stable nebulisation aerosol is dependent upon:

- The potential difference between the electrospray capillary and a counter electrode.
- Nebuliser gas flow.
- Sprayer position relative to the sampling orifice.
- The eluent composition and flow rate.

6.1.2 The desolvation process

The most effective desolvation is achieved by a combination of processes, the parameters for which are interrelated and should not be optimised in isolation:

- Source temperature.
- Drying/desolvation temperature and gas flow.
- Declustering.

Such parameters are rarely adjustable during the analysis and so should be optimised for the "worst-case scenario".

6.1.3 Mass analyser parameters for selected reaction monitoring (SRM)

There are various voltages associated with the ion optics used to optimise ion transport to the first quadrupole. These parameters should be set as per vendors' instructions. Avoid conditions that result in fragmentation of precursor ions. Other parameters that have an impact on quantification include:

- Mass resolution and calibration.
- Collision cell energy, gas flow and associated voltages.
- Data acquisition settings such as dwell time, number of data points and polarity switching.

6.2 Considerations before starting optimisation

- If low limits of detection are not required, the default parameters recommended by the vendor may be sufficient.
- Try conditions previously determined in other laboratories or published in the literature using the same instrument model.
- The extent to which parameters are optimised may depend on whether a new method is being developed to determine a novel analyte or whether additional analytes are being added to an existing method:
  - Addition of analytes leads to compromise in performance and hence complete optimisation of parameters for determination of the new compounds may not be required.
  - When faced with a novel method it should be possible to provide direction to the optimisation and the solvents used, based upon the molecular structure and any pKa data for the analyte:
    - o If the analyte is basic in nature it suggests that it has a functional group that protonates readily and so will respond well in electrospray using positive ion mode.

- o Optimum response should be obtained by adjusting the solution pH to 2 pH units below the analyte's pKa, as the basic group will spend 99.5 % of its time in the ionised form.
    - o The converse is true for acidic compounds.
  - There may be a number of problems with such a directed approach:
    - o Some compounds can generate either positively or negatively charged molecular species and in some cases better S/N is observed during the analysis of real extracts using the polarity mode that generates lower signal intensity.
    - o The position of the charge on the analyte molecule can direct the fragmentation observed during CID having an impact on the number of product ions observed and the selectivity of the SRM transitions chosen.
    - o The optimum solution pH for maximum sensitivity in electrospray rarely matches the solution pH required for optimum LC retention and separation.
    - o Large changes in solution pH can occur at the electrospray capillary during the electrospray process so the pH of the electrospray droplets may be considerably different from that of the bulk solution.
- Assessment of empirical formulae:
  - Check for the presence of diatomic elements (*e.g.* chlorine), the isotopic abundance of which will be reflected in the mass spectra.
  - Note the likelihood of multiply charged molecular species.
  - Calculate mono-isotopic mass for protonated or de-protonated molecules and any likely adduct ions:
    - o If the analyte is already ionic, calculate mono-isotopic mass for molecular ions.
    - o Ensure that the empirical formula is for the analyte in solution rather than any formulated salt or ester.
    - o Some analytes ionise in an atypical way so check for previous examples and ion assignments in the literature or carry out investigations in-house using mass spectrometers capable of high resolution and accurate mass measurements.
- Assignment of likely structures to product ions:
  - It is advantageous but time-consuming to have some understanding of the fragmentation rather than simply applying an empirical approach.
  - This approach can help avoid the use of non-selective transitions (*e.g.* loss of water).

6.3 Preparation for the optimisation procedure

6.3.1 Choice of solvent

A solution of the analyte(s) at an appropriate concentration (see instrument manual) in an MS compatible solvent is required to optimise conditions. Start with a low concentration of the standard solution and avoid saturating the detector by checking that the isotope patterns in the spectra match the analyte's theoretical isotope pattern.

- The solvent chosen will depend on the solubility of the analytes and upon the means by which the solution is to be introduced.
- If infused directly, then an attempt should be made to try to match the solvent with the likely composition of the mobile phase.
- If using an empirical approach, carry out the optimisation in both positive and negative ion mode in a range of solvents to enable comparison of performance under different conditions:
  - Neutral (*e.g.* 5 mM ammonium acetate).

- Acidic (*e.g.* 0.1 % formic acid).
- Basic (*e.g.* 10 mM ammonium bicarbonate at pH 10.5).
- Methanol.
- Acetonitrile.

6.3.2   Introduction of standard solutions

Three procedures are commonly used to introduce the standard solution containing the analyte into the ionisation source of the mass spectrometer: infusion, infusion combined with LC flow, or flow injection analysis (FIA). Direct infusion is not appropriate for optimisation of source parameters when using APCI.

- Infusion of the standard solution into the ionisation source (*e.g.* at a flow rate of 10-20 µL/min using a syringe pump):
  - Provides a steady signal from analyte ions the response of which is monitored and/or recorded when changes are made to the parameters using the instrument control software.
  - Is relatively straightforward and can be fast if data on only a few analytes are required.
  - Is difficult to automate, limited by the volume of the syringe, and can lead to contamination of the source with the analyte.
  - Results in solvent composition and flow rate that does not match the actual chromatographic elution conditions. Its use should therefore be restricted to optimising compound-dependant parameters such as collision energy.
- The infusion of the standard solution from the syringe pump can be mixed with a second stream of mobile phase from the LC system, delivered at a more relevant flow rate:
  - Optimisation at more relevant flow conditions can include an assessment of source parameters as well as compound-dependant ones.
  - For procedures relying on infusion to be successful, a stable electrospray and analyte response is essential:
    - Sources of instability include faulty syringe pump, leaking syringe and/or tubing, T-piece, faulty LC pump or dirty/faulty/badly positioned electrospray capillary.
- In FIA, multiple injections of a fixed volume of the standard solution are made using the LC system:
  - The instrument parameters are systematically changed after each injection and the parameter giving the best response selected as optimal.
  - Optimisation is carried out in an automated fashion assuming the software has such capability – if not then carrying out this process manually is very time consuming.
  - Optimisation can be achieved in realistic conditions (LC mobile phase and flow rate) with the least contamination of the three options.
  - Optimisation using FIA relies upon transient signals and so stability of response is also essential.

6.3.3   Other considerations

A wide range of contaminants derived from previous analyses, solvents and gases can have an impact upon analyte signal and detection and hence can be detrimental to the optimisation process:
- It is important to use LC-MS grade solvents and reagents, clean the source and flush the LC/fluidics lines out prior to starting optimisation.

6.4 Procedure for optimisation

Modern instruments have automated procedures for optimisation of instrument parameters driven by the operating system software, dealing with delivery of the standard solution for ionisation, setting up the relevant experiments, and for evaluation and reporting of the results. These procedures simply automate the manual procedure given here.

- Record a mass spectrum over a wide mass range:
  - The range will depend on the masses of the required analytes, the mass range of the instrument and its mass calibration.
  - It is important not to acquire data over a limited range but to check for unexpected ions over the full mass range.
  - It is helpful to keep the instrument clean to minimise the background so that the precursor ion can be selected easily.
- Select a suitable precursor ion:
  - Protonated or de-protonated molecules.
  - If the quasi-molecular ion proves unstable, resulting in low abundance, optimise conditions that favour the formation of an ion-source fragment ion to be used as the precursor.
  - Multiple precursor ions may be observed and these may include:
    o Diatomic-containing molecular species, adduct ions, multiply charged ions and dimers.
    o Avoid selecting adducts as these tend to be very stable and difficult to fragment by collision-induced dissociation (CID), *e.g.* sodium in positive ion mode or acetate adducts in negative ion mode.
  - Check that the mass of the precursor ion measured is consistent with the calculated monoisotopic mass of the analyte.
- Select suitable product ions:
  - Change to MS/MS mode, set up a product ion scan with the previously selected precursor ion and select low collision energy to observe the intact precursor ion in the third quadrupole (Q3).
  - Increase the collision energy so the CID fragments are formed.
  - Record product ion mass spectra showing the abundance of the various product ions formed:
    o This may require spectra recorded at different collision energies or an averaged spectrum using a collision energy ramp.
    o Record product ion spectra with a low abundance of the precursor ion still present in the spectra purely for reference.
  - Vary parameters (see below) and then select the value which gives the optimum response for each product ion:
    o The response of un-fragmented precursor ion is now irrelevant.
    o Try not to make a judgement about which ion to select at this stage, but optimise conditions for all that are formed with sufficient abundance.
- If possible avoid selection of product ions associated with transitions that may prove non-selective, for example:
  - Loss of water.
  - Reaction of an adduct to form a protonated or de-protonated molecule.

6.5 Optimisation of the nebulisation process

6.5.1 Potential difference between the electrospray capillary and a counter electrode

- Select an electrospray capillary voltage that produces the highest signal levels under stable and reproducible axial spray conditions:
  - Typically voltages are in the range 1 to 5 kV, depending upon the instrument design and application.
  - Use lower voltages:
    o When using negative ion mode.
    o When using mobile phases with no or low concentrations of buffers (*e.g.* 1 mM ammonium formate).
    o When using lower aqueous content mobile phase eluents, such as those used for HILIC as these solvents have low surface tension and hence a more stable formation of the Taylor cone at the tip of the electrospray capillary.
  - Higher voltages are required to induce ion formation from mobile phases with high water content.
  - Increasing the electrospray capillary voltage further leads to:
    o Unstable spray configuration and changes in the appearance of the resulting mass spectra leading to non-robust quantitative measurements.
    o Electrical arcing through the interface which may cause damage to the instrument electronics.
    o Corona discharge in negative ion mode, since field emissions of electrons from a sharp point occur at relatively low potentials.

6.5.2 The nebuliser gas flow

- The flow rate has a significant impact on the droplet size whereas the potential at the electrospray capillary will govern the charge on the molecule:
  - Some instruments employ a "set it and forget it" approach to nebulising gas flow whereas other models have a fixed flow rate.
  - Where the nebulising gas flow rate can be altered, select the optimum flow rate for the liquid flow employed for the LC separation.

6.5.3 Sprayer position relative to the sampling orifice

- Positioning the sprayer orthogonally to the sampling orifice results in less contamination of the source and ion optics, sampling of fewer charged droplets (resulting in a more stable ion signal) and the capability to tolerate higher flow rates and involatile buffers.
- Care is required with the relative positioning of the electrospray capillary and counter electrode to ensure a stable and symmetrical spray plume:
  - It is usually possible to adjust the sprayer position on three axes.
  - Optimisation of the position of the sprayer and the electrospray capillary voltage are interrelated and neither may be optimised in isolation.
- Adjustment of the probe/sprayer is most effective when operating at low flow rates, but adjustment is more critical when using mobile phases with high aqueous content to avoid formation of large, poorly charged droplets and electrical discharge at lower electrospray capillary potentials and with high LC flow rates.
- Select the optimum sprayer position for the liquid flow and composition employed for the LC separation or for the "worst case scenario" for solvent composition (high aqueous content), as long as the sprayer position selected has no significant detrimental impact on the response of other analytes known to elute in a high proportion of organic solvents.

6.5.4   The eluent composition and flow rate

- It is rarely possible to optimise the LC eluent composition solely for MS performance as the choice of mobile phase solvents is usually dependant on the LC separation required:
  - Use of volatile mobile phases promotes desolvation and droplet fission so the relative percentages of aqueous and organic solvents in a RP mobile phase determine the surface tension of the solvent.
- The electrospray capillary voltage is also dependent upon the conductivity of the eluent solution, which is mostly affected by the concentration and type of buffer used, *i.e.* the relative mobility of the electrolyte species in solution at the electrospray capillary tip:
  - Electrolytes are often added to sample solutions to promote desorption of specific types of ions:
    - o A volatile, weak acid, such as acetic or formic acid, is added to increase conductivity and to facilitate the protonation of the analyte.
    - o Base, such as ammonia, is added to increase the yield of de-protonated analyte.
  - The presence of high quantities of added electrolytes will result in the suppression of analyte signals.
  - The formation of a stable nebulisation aerosol is dependent upon the shape of the electrospray capillary tip being maintained and not consumed by electrochemical processes promoted by excessive electrolyte concentrations.
- Modern source designs exhibit minimal loss of sensitivity at the higher flow rates associated with UHPLC.

6.6   Optimisation of the desolvation process

6.6.1   Source temperature

- Sufficient heat is required in the source to avoid any condensation and clustering in this region.

6.6.2   Drying/desolvation temperature and gas flows

- Heat is required in the source and interface region to ensure the desolvation necessary for the change from liquid to droplet to cluster to ion.
- Enough heat is needed to evaporate the solvent under any LC conditions employed and yet the temperature needs to be low enough to prevent decomposition.
- The source heater is supplemented by the use of an inert, often heated, desolvation gas (usually nitrogen) that is pumped into the interface housing, sometimes via the sprayer assembly:
  - Increase temperature and desolvation gas flow with increasing LC flow rate and proportion of water in the mobile phase.
  - The desolvation gas flow may be switched off at low solvent flows (*e.g.* direct infusions).
  - If temperatures are set too high, labile compounds will thermally degrade.

6.6.3   Declustering

- Parameters that influence declustering should be set as per vendors' instructions or optimised for the LC eluent composition and flow rate in use. They include:
  - Counter current and curtain gas.
  - Heated ion transport tube/capillary.
  - Accelerating voltage (such as fragmentation, cone or declustering potential).

6.7 Optimisation of mass analyser parameters for selected reaction monitoring (SRM)

6.7.1 Mass resolution and calibration

- Ensure the instrument is correctly calibrated.
- When carrying out scanning experiments, the mass analyser should be able to separate masses that differ by one mass unit or less (typically 0.7 Da FWHM).
- When carrying out SRM experiments, mass resolution can be reduced to maximise response if required:
  - The resolution of the first quadrupole (Q1) can be reduced just to the point that the isotope is not seen:
    - o If the mass resolution set in Q1 is too low whilst Q3 remains at unit mass resolution, then additional ions will enter the collision cell and additional product ions for each isotope of the precursor ion will be observed.
  - The resolution of Q3 can also be reduced to give maximum signal intensity.
- In most cases the values of parameters for each resolution setting and their associated mass calibration files will have already been set up leaving the analyst to simply select an appropriate resolution setting for SRM experiments.

6.7.2 Collision energy, gas flow and associated voltages

The degree of fragmentation of each precursor ion in the collision cell after CID is dependent on the amount of collision energy selected:

- Select the optimum collision energy for the response of each product ion.
- If no product ions are observed, optimise collision energy for the transition precursor to precursor ion:
  - Given that the precursor ion of interest is stable, selection of a relatively high value for collision energy might be preferable in order to fragment and hence reduce the contribution from isobaric interference.
- Optimise collision gas flow/pressure if an option.
- On some instruments a potential difference is typically applied to the entrance and exit of the collision cell to transport precursor ions into the cell and product ions out:
  - In some instrument designs, the default recommended values are sufficient whereas in others it is prudent to optimise, especially when faced with relatively low or high masses.

6.7.3 Optimisation of data acquisition settings

- Acquisition of each transition requires provision of an adequate period of time, known as the 'duty cycle':
  - Acquisition (dwell time).
  - Time for the electronics to settle (pause):
    - o Between transitions (inter-channel delay).
    - o Between scans (inter-scan delay).
- Acquisition is constrained by limitations imposed upon cycle time by minimum dwell and pause times:
  - On older designs the longer the dwell time spent acquiring data the greater the sensitivity.
- Consideration also needs to be given to acquiring sufficient data points across a chromatographic peak for the purpose intended:

- Increasing the dwell time for transitions, or the total number of transitions acquired, will lead to a reduction in the number of data points and hence the precision and accuracy of the measurement suffer.
- For example, multiple injections of a fruit extract gave precision for quinoxyfen of 6.6% RSD (relative standard deviation) with 8 data points across a peak which was improved to 2.4 % RSD when the number of data points acquired was increased to 12.
- On modern systems the management of the duty cycle can be automated so that the period of acquisition of each transition is optimised by acquiring only during the time when the peak is likely to be detected.
- Variation in the mass calibration may result in a reduction in sensitivity as the quadrupoles will no longer be "parked" on the apex of each of the ions of interest at the expected *m/z* values:
  - This can be avoided by selecting peak *m/z* values measured under the prevailing conditions but the use of exact masses for each SRM ion combined with good mass calibration is preferred.
- It is usually possible to change polarity within a single acquisition method but the time required for the electronics to switch varies between different instruments.

7   Calibration

Quantitative analysis almost always involves comparing the instrumental response obtained from the analyte in an unknown sample with that from one for which the concentration is known – the calibration standard. As mentioned previously, LC-MS measurements are made by introducing liquids into the instrument so the calibration standards are generally solutions of a known amount of analyte in a suitable solvent. Correct preparation of these solutions and the way in which they are used are key factors in obtaining reliable quantitative LC-MS data. The main issues to consider are:

- Selection of calibration standards.
- Preparation and storage of calibration solutions.
- Use of internal standards.
- The calibration strategy.
- Reduction of calibration data using appropriate statistical or mathematical models.

Most calibration issues are generic rather than specific to the LC-MS technique but practical aspects of calibration are discussed in this guide as they have a major influence on the accuracy of LC-MS data.

7.1   Selection of calibration standards

It is possible to purchase 'pre-prepared' commercially available calibrants for many common analytes and routine applications, either as stock solutions for dilution in the laboratory or at the required working concentrations. This practice is popular with some laboratories in order to save time or remove the risk of calibrant preparation errors in the laboratory. The suitability of such materials should be assessed using the same guidelines as for preparation of in-house calibration solutions.

- The purity of the analyte and its associated measurement uncertainty will impact the quality of the data that are produced and the material selected should be fit for the intended application. Most laboratory grade organic compounds are supplied with only an approximate estimate of purity but this may be adequate if, for example, the sampling variability for the application has a coefficient of variation of 40%. With more critical applications, it may be necessary to use a certified reference standard (*e.g.* with a certified purity of 99.5 ± 0.2 %) but this will be more expensive.
- Where suitable materials are not available commercially, in-house purity and stability checks may be required for solid or liquid/solution standards. For example, water content of a powder material may be determined by Karl Fischer analysis and LC-UV used for quantifying organic impurities. Full scan MS can be used to check for decomposition or conversion products in solution standards. Common problems include hydrolysis, oxidation, dehydration, isomerisation and/or racimerization, decarboxylation and photodegradation.
- Where existing standards are available within the laboratory, it is important to check that they are within any stated expiry date and that they have been stored correctly (following recommendations on the certificate of analysis). For some hygroscopic compounds, once a sealed vessel is opened the rate of water adsorption can be rapid unless they are stored in a desiccator. For example, if a standard absorbs 10% water and the purity is not corrected, all data generated from it will be inaccurate by +10%.

- The accuracy of calibration standards can be checked indirectly through analysis of solutions from alternative sources, ideally a certified reference material but often a previous batch of calibrant.

7.2 Preparation and storage of calibration solutions

The selection of the solvent for preparation of calibration solutions should take into account:

- Solubility, which can be tested using different ratios of solvent to solute and comparing standards against each other (along with visual inspection).
- Stability under storage and use conditions.
- Volatility (high boiling solvents will need to be solvent exchanged prior to LC-MS infusion experiments, volatile solvents can present a problem with evaporation causing changes in concentration).
- Whether the solvent selected (including that used to prepare any internal standards) will be detrimental to the analytical method (*e.g.* cause unwanted protein precipitation or sample breakthrough in a SPE method).

The amounts and procedures used in preparation of calibration solutions are important:

- The quantities of starting material and the most appropriate balance should be chosen in order to minimize measurement uncertainty from weighing. (A best practice guide to weighing and calculating the associated measurement uncertainty is available as a free electronic download from the NPL website, see reference 1.)
- Anti-electrostatic measures may need to be put in place to prevent weighing error and/or enable quantitative transfer of powder standards.
- The volumes used when preparing dilutions will impact on measurement uncertainty. For example, pipetting 10 μL of a stock solution and dissolving in 1 mL of solvent is likely to be less precise than pipetting 1 mL and dissolving in 100 mL of solvent, to achieve the same concentration.

Correct choice of storage vessels and conditions is important, for example:

- Standards should be prepared and stored in silanized glassware, or in plastic vessels if adhesion of the compound to active sites of glassware is an issue.
- If the target analyte is susceptible to degradation by UV light it may be necessary to wrap containers in foil or use amber glassware and work in an environment with non-UV emitting lighting.
- It may be necessary for stock and intermediate standard preparation and storage to be segregated from sample storage, preparation and analysis areas.

7.3 Calibration strategies

In designing the calibration strategy it is necessary to first select the most appropriate calibration approach and then to decide on specific aspects of implementing that approach. The main options for LC-MS calibration are:

- External calibration in which a series of separately prepared calibration solutions is measured during the same LC-MS run as the samples to be analysed. The calibration solutions may be prepared using matrix matched or pure solvent solutions.
- Internal calibration in which aliquots of a known amount of a standard are added directly to the test samples, ensuring that the unknown and known analyte amounts are measured under identical conditions.

Internal calibration may also be referred to as internal standardisation but it is better to avoid this term in order to avoid confusion with the situation where an internal standard is added for purposes other than calibration, for example to allow correction of effects such as instrument drift or variable sample injection volume. An internal standard can be used in this way with any of the calibration strategies described here. Advice on the selection and use of internal standards is given in Section 7.4.

Other aspects of calibration to consider include:
- The number of calibrant levels required, both for method validation and for subsequent batch analysis.
- The appropriate concentration levels for the calibrants bearing in mind the anticipated sample concentration levels.
- The number of replicate measurements for both samples and calibrants.
- The distribution of calibrant solutions within the set of samples, including the number of replicates (sometimes referred to as the degree of bracketing).
- Whether the LC-MS measurements should be made using peak area or peak height, the former generally being preferred for LC based assays.

7.3.1   Matrix matched external calibration
Due to the possibility of matrix suppression and enhancement effects, in general matrix matching is preferred to the use of solvent standards. For many applications the reality is that the blank matrix used to prepare calibrants will not have exactly the same composition as the sample, and the analyst should be aware of this, but it is the best compromise achievable. For example, steroid stripped serum, which has been taken through a carbon filter, will be a cleaner matrix than patient serum. Synthetic urine will be considerably cleaner (and potentially a more stable matrix) than human urine. Use of blank matrix for calibrant preparation can be advantageous in some cases, by reducing the adhesion of target compounds, as active sites have been taken up by the more abundant matrix compounds and/or stabilizing the compounds.

7.3.2   Standard addition internal calibration
In many LC-MS applications, calibration using standard additions may be the best strategy to minimise measurement bias because it ensures almost perfect matrix matching between sample and calibrant. In brief, multiple aliquots (typically >4) of the same volume are taken from the test sample and all but one are spiked with accurately known amounts of calibrant, increasing the amount for each member of the series. The solutions are then diluted to the same volume to generate a set of matrix matched linear calibration standards. Typically the first spiked standard is prepared such that after diluting to volume the analyte concentration is twice that of the anticipated concentration in the sample. This procedure has advantages in terms of taking account of matrix effects, including suppression, but it requires sufficient sample in the first place to take multiple aliquots, significantly more work is needed for each analysis than for other calibration techniques, and the measurement uncertainty is often higher than for other calibration schemes; hence, it is less often employed routinely.

7.3.3   Internal calibration by direct addition
In this approach an aliquot of a standard which differs from the analyte is added to each sample allowing both to be measured in the same solution at the same time by the mass spectrometer. This gives ideal matrix matching, avoids instrumental or other variations between each analysis, and may reduce overall analysis times. However, it is essential to find

such an *internal standard* which behaves like the analyte in almost every respect except that it can be distinguished from the analyte in the mass spectrum.

7.4   Selection and use of internal standards

Addition (often referred to as 'spiking') of a known amount of a carefully chosen compound (different from the analyte) to solutions to be measured is widely used in quantitative LC-MS. As mentioned above, such internal standards may be used for correction of effects, such as instrument drift or variable sample injection volume, or for internal calibration of the measurements. In the case of LC-MS, the latter often exploits a major advantage of using mass spectrometry for quantification, namely the ability to use isotopically labelled internal standards. For this purpose it is necessary to obtain or synthesise an analogue of the analyte with a different isotopic composition from the naturally occurring analyte. Molecules that differ only in their isotopic composition are referred to as *isotopologues* and the calibration approach as *isotope dilution mass spectrometry (IDMS)*. In most LC-MS applications of this technique the isotopologue is based on enrichment with ^{13}C, ^{15}N or ^{2}H (see Section 7.4.2).

7.4.1   General considerations

An important decision is when to add the internal standard. For many applications the internal standard should be added as early on in the analytical procedure as possible, *e.g.* to correct for losses during sample preparation. However, for some applications it may be necessary to add the standard at a later stage. For example, for methods which have a digestion stage, if the chosen internal standard is an analogue of the target compound after digestion, it may be most appropriate to add it after the digestion stage. The following aspects should be considered when selecting a non-isotopically labelled internal standard:

- It should not be present in any of the samples to be analysed. For example, using a prescribed drug as an internal standard for a therapeutic drug monitoring application is not a good idea.
- It should be as similar in physiochemical properties to the target analyte as possible and elute as close to it as possible. Isomers, homologues and structural analogues are often ideal candidates.

7.4.2   Use of isotopically labelled internal standards

If an isotopically labelled version of the target analyte is available it should be spiked at the beginning of the analysis when it can be used to account for a number of method variables, including:

- Recovery during transfer and clean-up stages.
- Variability in extraction efficiency.
- Injection volume variability.
- Matrix suppression.
- Changes in adduct profiles between samples and calibrants.

Care is required when using an isotopically labelled internal standard. If there is a choice of isotope available for an application, ^{13}C and ^{15}N labelled compounds are generally preferred to deuterated compounds for LC-MS applications. This is due to the greater shift in chromatographic retention time from that of the natural compound that occurs with deuterium labelling than for ^{13}C and ^{15}N analogues. Such shifts in retention time are often quite small in LC applications compared with high efficiency GC separations but there is still potential for the natural and labelled compounds to experience slight differences in matrix suppression (and enhancement) which will in turn affect the peak area measured. In addition, the

following points should also be considered when selecting isotopically labelled internal standards:

- The labelling should be on a stable part of the molecule and the degree of labelling should not change due to back exchange occurring during the analysis. Unanticipated exchanges can occur, for example via tautomerization mechanisms, and this aspect should always be checked experimentally.
- There should not be any significant mass spectral overlap between the natural and labelled analogues of the target. For this reason it is preferable for most small molecule applications that the labelled compound has a mono-isotopic mass at least 3 Da higher than that of the natural compound.
- Significant amounts of unlabelled material in the isotopic spike should also be avoided. The overall purity of the material is not critical provided that there is no contribution from any of its impurities to any of the compounds targeted in the analytical method.
- In IDMS methods, complete equilibration of the labelled and natural compounds is essential to ensure accuracy. Equilibration and extraction studies may be required to prove that the target analyte has been completely extracted/released without degradation or that the spike has equilibrated with the sample and is bound to matrix components and/or active surfaces to the same degree as the natural compound.

7.5   Calibration data reduction

A set of standards is prepared over the target concentration range and the response (or ratio of responses when an internal standard is used) is plotted on the *y*-axis against the known concentrations of the calibrants on the *x*-axis. Conventionally, this involved manual preparation of a calibration graph; with modern, computer-orientated instrumentation the graph may no longer be used but the principle remains the same except that the calibration line is fitted using an algorithm rather than by eye. Clearly, the correct choice and use of algorithm is critical. The most commonly used calibration model for quantitative LC-MS/MS is least squares linear regression. Data is processed using a vendor's software or exported to Excel (or another statistical package) and least squares linear regression calculations used to determine the equation of the straight line with the best fit to the data. The equation is then used to convert a measured response for a test sample to the predicted concentration for the sample. In the case of standard additions calibration, the amount of substance in the sample is calculated from the intercept of the least squares linear regression line with the *x*-axis (*i.e.* the value of *x* when *y*=0). The most frequently used parameter to evaluate line fitting is the correlation coefficient *r*, or the related term r^2. The best fit is indicated with a value of *r* close to 1. However, many analysts have been misled by a value presented by the software and which they assume is "close to 1". Best practice during method development is to visually inspect the calibration using the plot of the calibration data. Many statistical guides are available which describe more rigorous procedures to check for any anomalies in the calibration (see, for example, references 2 and 3).

When initially constructing a linear calibration scheme, a minimum of five concentration levels (including a blank) equally spaced across the concentration range is recommended. Ideally, replicates for each concentration should be prepared. As a minimum the set of calibrants should be injected at the beginning and end of the run, and analysed periodically throughout a run. For some applications it may be sufficient to demonstrate the linear range of an assay during initial validation and for future batch analysis a scaled down calibration scheme, even single point calibration, may be all that is required.

Software typically gives an option to set the intercept of the calibration line with the x axis to zero, *i.e.* to force the line through the point (0,0). Generally this option should not be used unless there is evidence to indicate that the true intercept is not significantly different from zero. Typically ESI and APCI instruments can operate over a linear dynamic range of up to 10^5. At the top end of an instrument's dynamic range, a non-linear response may be observed, which is likely to be due to either saturation of the ionization process or overloading of the detector. In some instances a flat topped chromatographic peak will be observed when the detector saturation point has been reached. Providing standards have been prepared such as to cover the concentrations determined in the samples, reduction in sensitivity to achieve linearity may be possible. For example, by modification of instrument parameters such as collision energy voltages, use of an alternative SRM transition, reduced injection volume, or dilution of the sample solutions.

For many applications, the basic least squares linear regression model is sufficient. However if it is clear that the data are poorly fitted using this approach, it may be necessary to apply a non-linear model, such as a quadratic (2nd order polynomial). Use of a quadratic model should be justified and may require additional data points compared to linear calibration in order to define the best-fit line accurately. In some cases, where curvature of the line is observed at higher concentrations, processing of the data using a quadratic equation may give the best estimate of concentration in the sample, which can then be used in order to calculate appropriate calibrant concentrations required for repeat analysis. If a third or fourth degree polynomial equation is required to fit the data, it might be time to rethink the analytical methodology used.

7.6   References and further reading

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4. Barwick V., *Preparation of calibration curves: A guide to best practice*. 2003. LGC/VAM/2003/032 (available from www.nmschembio.org.uk).

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8   Method validation and uncertainty

Development of an LC-MS method is usually undertaken with the intention that it will subsequently be used for routine measurements. Before doing so it is necessary to formally validate the method and to estimate the measurement uncertainty that will be associated with the results it generates. These topics are discussed in detail in many excellent books and guides, some of which are listed at the end of this chapter. A brief overview of the most important aspects is provided here.

8.1   Method validation

Method validation is the formal process of establishing and documenting that a method is capable of producing results that are fit for the intended purpose. This requires experimental evaluation of a range of parameters which may influence the performance of the method. In many cases some or all of the necessary data will have been obtained during method development but additional experiments may need to be planned. However, it is essential that validation evaluates the performance of the 'final' version of the method.

It is important to be aware of the following points when planning a validation study:
- Validation should assess the performance of the entire measurement procedure, including any sample preparation, not just the end measurement by LC-MS.
- The evaluation of method performance should include the analysis of materials that are representative of test samples and materials with known values (such as certified reference materials).
- In order to decide whether a method is 'fit-for-purpose' the level of performance required, such as the limit of quantitation or the acceptable measurement uncertainty, should be agreed in advance with the users of the results.
- A validation study should always include evaluation of the measurement uncertainty, even when there is no formal requirement to report results with a stated uncertainty.
- The validation study itself should be fit-for-purpose, *i.e.* the amount of effort required will depend on the criticality of the results it provides and the extent of prior knowledge, such as use of a published method or the availability of existing data from method development studies or other sources.
- The validation study is only complete when it has been fully documented.

The most common performance parameters studied during method validation, apart from measurement uncertainty (see below), are:
- Selectivity: The ability of the measurement procedure to measure the property of interest (e.g. the amount of analyte present in the sample) without interference from other sample components.
- Precision of replicate analyses:
  - Repeatability provides an estimate of the variability in results when measurements are performed under similar conditions (e.g. same laboratory, same analyst, same equipment, within a short period of time).
  - Intermediate precision provides an estimate of the variability in results when measurements are made in the same laboratory but under more widely varying conditions (e.g. different days, different analysts).

- o Reproducibility provides an estimate of the variability in results when measurements are made in different laboratories (evaluation of reproducibility therefore requires the organisation of an inter-laboratory comparison).
- Bias: An estimate of any systematic deviation of the results from the 'true' value, such as the certified value of a reference material.
- Detection limit: The lowest concentration at which the analyte can be reliably observed. Note that the term 'sensitivity' is frequently used when discussing the detection capabilities of an instrument/method ('good sensitivity' implies the ability to detect low concentrations).
- Quantitation limit: The lowest concentration of the analyte that can be reliably measured.
- Linearity and working range: The range of analyte concentrations over which the method will produce results with an acceptable uncertainty.
- Ruggedness or robustness: Susceptibility of the method to changes in external factors such as environmental conditions, using different analysts on different days, etc.

Most methods are validated in-house by the single laboratory in which they will be applied. However, if the method is intended for use by a group of laboratories, perhaps within a company or an industry sector, it may be appropriate to validate through an inter-laboratory study (also known as a collaborative trial or 'round-robin' exercise). Data from such studies is generally used to assess the reproducibility (but not the intermediate precision) and/or bias of measurement results and for establishing how well a method responds to use in different locations (i.e. the ruggedness of the method).

8.2   Measurement uncertainty

Measurement uncertainty arises from the random and systematic effects that are inherent in all measurement processes, even after correction for known systematic effects. Important points to remember are:
- An uncertainty estimate should combine all the effects that have a significant influence on the result, to give a single value which provides a quantitative indication of the quality of the measurement result.
- When uncertainty is reported it is usually presented in the form (value ± uncertainty) with both expressed in the required units (*e.g.* 22.3 ± 1.9 mg L^{-1}).
- In testing (rather than calibration) laboratories, the measurement uncertainty is not normally calculated for individual test results. Instead, the uncertainty is evaluated for typical test materials and this value is then applied to all results obtained when the method is operated correctly within its specified scope.
- Measurement uncertainty is not about mistakes – the aim is to capture the all the effects that will cause variation in measurement results when the measurement procedure is operating correctly.

Detailed guidance on the evaluation and quantification of measurement uncertainty is given in reference 5. Briefly, each effect contributing to the uncertainty produces an uncertainty in the result which should be expressed as a standard deviation. The individual components may be combined by taking the square of each component, adding them, and taking the square root of the sum. This provides an estimate of the **standard uncertainty (*u*)** of the results.

- The components to be combined are expressed in terms of the target measurand using the same units. For example, if temperature variations significantly affect the result

for an analyte concentration measurement, then the temperature uncertainty component would be expressed as the standard deviation of variations in concentration (*e.g.* in mg L^{-1}) caused by the temperature fluctuations.

- Due to the way uncertainty components are combined, uncertainties that are less than one-fifth of the largest uncertainty component will not make a significant contribution to the combined uncertainty.

- Note that in some cases it is more appropriate to combine the uncertainties as relative values (*i.e.* relative standard deviations).

To give increased confidence that the uncertainty interval includes the true value for the measurand, the standard uncertainty of the measurement result is usually *expanded* by multiplying by a specified factor (known as the **coverage factor, *k***) to give an **expanded uncertainty (*U*)**.

- In most routine testing applications, a coverage factor of 2 is used to give a level of confidence of approximately 95% that the true value lies within the range of the result and its expanded uncertainty.

8.2.1 Evaluating measurement uncertainty

Broadly speaking, two different approaches may be used to evaluate measurement uncertainty: 'bottom-up' and 'top-down' [5]. The former relies on the quantification of every individual uncertainty component which contributes to the overall uncertainty of the measurement. While this approach is feasible for certain types of measurement (particularly in physical metrology) it is often impractical for testing laboratories carrying out chemical analysis. There are simply too many possible sources of uncertainty, many of which are interrelated, to make quantification of individual components feasible. The alternative 'top-down' approach is therefore commonly employed in testing laboratories. This relies on the use of method performance data which encompass the effects of the main sources of uncertainty for the method being evaluated. Typically, data obtained during the method validation study are used but for established methods it is also possible to make use of data from on-going quality control activities.

- Note that it is important to identify and review the likely sources of uncertainty even when using the top down approach. This information should be used to ensure that the data used for the evaluation are obtained under conditions which embrace all the significant uncertainty effects.
- Failure to do this adequately is a common reason for significantly underestimating measurement uncertainty.
- The sources of uncertainty will vary from method to method but the typical areas that need to be considered for LC-MS are summarised in Table 8.1.

In using method performance data, it should be noted that:
- The estimate of precision used should be at least the within-laboratory reproducibility ('intermediate precision') or the reproducibility standard deviation from a collaborative study of the particular measurement method (see reference 11 for information on the use of collaborative study data).
- In principle, it is the uncertainty about bias that is important in uncertainty evaluation rather than the bias itself. In practice, however, uncorrected bias may remain and it is common to make an allowance for uncorrected bias when reporting uncertainty (references 7 and 8 give some details).

- Provided there is assurance that the consensus or assigned value is unbiased, performance in a proficiency testing scheme provides an effective check of the uncertainty estimates provided by laboratories.

Source	Examples
Sampling	(Where sampling forms part of the procedure) – random variations in composition between sub-samples, bias introduced by sampling procedure.
Sample composition	Sample homogeneity and stability. Sample matrix – interferences and influences on analyte recovery.
Sample pretreatment/clean-up	Drying, grinding, blending of sample. Extraction of analyte from sample matrix – extraction conditions, incomplete analyte recovery. Loss of analyte during clean-up steps.
Reagents	Purity of reagents used to prepare calibration solutions. Reagent composition.
Laboratory equipment	Calibration of glassware and analytical balances. Effect of temperature on volume measurements.
LC-MS instrumentation	Instrument calibration. LC and MS conditions.
Laboratory	Environmental conditions.
Computational effects	Effect of calibration model.
Analyst	Small differences in application of the method.
Random effects	Always present for all stages of the method – variation in extraction efficiency during sample clean-up, variation in environmental conditions, variation in instrument response.

Table 8.1: Sources of measurement uncertainty

8.2.2   Use of uncertainty information in developing and improving methods

Evaluating the uncertainty in parallel with method development can help to identify which method parameters are important.

- Even if all components and contributions are not fully characterised, they can be estimated (an "educated guess" can be used in the first instance and then replaced by an appropriate value as more information is gathered).
- Uncertainty estimates can be used to assess which components have the greatest contribution and help to identify where the method needs to be improved to obtain a smaller uncertainty. The information can also be used to confirm whether some contributions are not significant and thus do not need further development or investment in order to achieve the desired result.

- Uncertainty information can be used during method development to help choose the final combination of conditions which can produce the required uncertainty in the measurement result. Examples of options which may be considered include:
  - Using volumetric or gravimetric measurements.
  - The size of sub-sample used (smaller samples may introduce larger uncertainties).
  - The number of dilutions and their sizes (two 1:10 dilutions might give a smaller uncertainty contribution than one 1:100 dilution in some cases).
  - The number of replicates depending on their uncertainty contribution.
  - The appropriate selection of calibration standard/calibrant/reference material, depending on the uncertainties of their values and the contribution they make to the result. It may be possible to save money by demonstrating that a lower level of purity could achieve the desire result, or to justify the purchase of more expensive standards for reduced uncertainty.

8.3   References and further reading

Method validation

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4. Eurachem Guide. *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*. 1998, LGC, Teddington, ISBN: 0 94892 612 0 (www.eurachem.org).

Measurement uncertainty

5. Ellison S. L. R., Williams A. (Eds), *Eurachem/CITAC Guide. Quantifying uncertainty in analytical measurement*, 3rd ed., 2012. Eurachem/Citac (www.eurachem.org).

6. EUROLAB. *Measurement uncertainty in testing*. Technical report No. 1/2002 (www.eurolab.org).

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8. EUROLAB. *Measurement uncertainty revisited: Alternative approaches to uncertainty evaluation*. Technical report No. 1/2007 (www.eurolab.org).

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11. ISO 21748:2010. *Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation.* ISO, Geneva

12. *The expression of uncertainty in testing*, LAB 12, UKAS, 2000 (www.ukas.com)

9 Data quality and reporting

There are many textbooks and guides available that provide detailed information on quality assurance and quality control for analytical laboratories (see the references and further reading at the end of this chapter for examples). It is inappropriate to repeat this information here but this chapter provides a brief overview of some practical issues which are especially relevant to an analyst carrying out routine measurements. If the work is undertaken in a laboratory which is accredited to the ISO/IEC 17025 standard [1], or operates in accordance with it, most of the points discussed would be addressed by the laboratory's documented quality system.

9.1 Common sense precautions

Errors in routine analytical measurements most often arise from random factors such as operator mistakes, malfunction of instruments or other equipment, contamination of samples or calibration standards, calculation or transcription errors (especially using spreadsheets). Hence, no matter how effective the development and validation of the LC-MS method it is always prudent to undertake simple, practical precautions during its routine use. Examples include:

- If it has not already been done during method validation, a clear and detailed description of the validated test method should be prepared to help ensure that the method is applied consistently in the future. This is particularly important when the method may be used by different analysts over a period of time.
- The documented method should be varied only if absolutely necessary, in which case the changes must be authorised appropriately and documented. 'Unofficial' temporary changes (*e.g.* for expedience due to instrument problems) are a particularly common source of errors. Note that if significant changes are made, re-validation of the method will be required.
- Carry out daily instrument checks prior to using an LC-MS (system suitability checks) in accordance with a documented procedure and keep a record of the outcome.
- Before and during a run or batch of samples, inspect chromatograms and spectra visually to check for acceptable separation, peak shape, etc. This is particularly important with automated systems where the operator may be tempted to 'leave things to the computer' and otherwise sees no more than a printed sheet of results.
- When the laboratory develops its own spreadsheets for calculations and/or data recording it is essential that these be properly validated and precautions (*e.g.* password protection) against inadvertent or unauthorised changes included.
- A common issue with techniques such as LC-MS is the enormous amount of raw data generated by the instruments and often available only as temporary (or easily overwritten) files on the instrument computer. It is important to have systems in place that allow the effective archiving and retrieval of **all** the information relating to the analysis of each test sample (*e.g.* raw measurement data, calibration records, quality control data, etc).
- In a busy routine laboratory, large numbers of test samples often generate even greater numbers of sub-samples, prepared solutions, additional dilutions, etc. Use of unlabelled containers, 'private' numbering systems created by the analyst, water soluble marking pens, and so on is to invite problems with wrongly identified samples.

- In many laboratories, particularly those dealing with high sample volumes, sample tracking is addressed through the use of a LIMS (Laboratory Information Management System) but whatever the scale of operations, a reliable system of linking the received sample to the reported result is vital. This should include a means of issuing a unique sample identification number on receipt by the laboratory and recording all subsequent operations or data against that number.
- The format for reporting measurement results to the end users will depend on the intended use of the results and any requirements specified by the customer. Regardless of whether specific requirements must be adhered to, it is important to ensure that measurement reports or certificates issued by the laboratory are correct, unambiguous, and indicate any relevant limitations of the method. A system to cross-check and approve reports before they are released is essential in all but the smallest laboratory.

9.2 Analytical quality control (AQC)

Method validation establishes that a method is fit for a particular purpose. Once the method is put into use, it is important to continue to monitor its performance to ensure the continued reliability of results. Hence, the precautions described above should be underpinned by making the measurements within a system of analytical or statistical quality control (AQC or SQC). This system will not prevent random errors but it may well detect them before erroneous results are reported. Likewise, with effective AQC it should be possible to avoid repeating the same errors in future. Common quality control procedures include:
- Analysis of blank samples to check for contamination/interferences.
- Analysis of quality control (QC) samples to monitor method performance.
- Analysis of test samples in duplicate to check method precision.
- Analysis of CRMs and/or spiked samples to check for method bias.

Important points to note when using QC samples include:
- The QC material should be similar in composition to test samples, stable and sufficiently homogeneous, and available in sufficient quantities to allow analysis over an extended time period.
- The QC material is usually included with every batch of test samples and should receive exactly the same treatment.
- The results obtained from the QC samples should be monitored by plotting them on a control chart. 'Warning' and 'action' limits (based on the mean and standard deviation of QC results when the method is operating correctly) can be used to help identify when problems with a measurement have occurred.
- In particular, a system must be in place to ensure that the control charts are inspected in a timely manner and action taken on any out of limit occurrences. A common source of erroneous data is failure to carry out such an inspection until long after the results were reported.

A common type of QC chart used in analytical laboratories is the Shewhart control chart shown in Figure 9.1. Information on the construction and interpretation of control charts can be found in ISO 8258 [2] and the IUPAC harmonized guidelines for internal quality control [3].
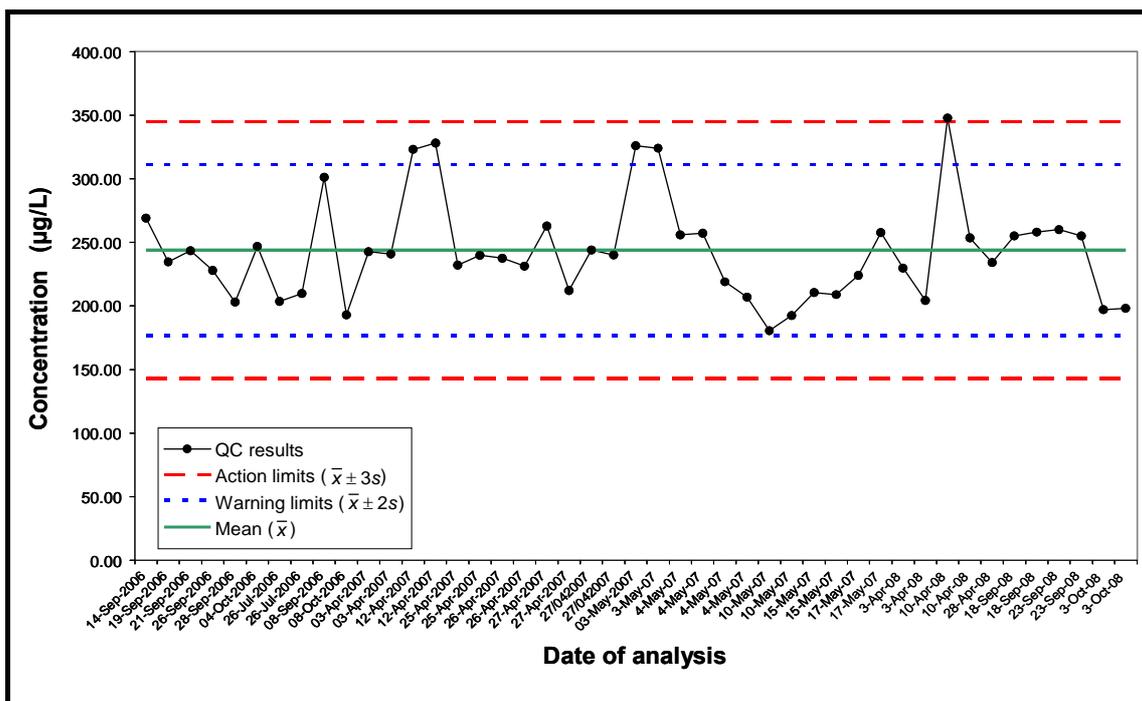
Figure 9.1: Example of a Shewhart control chart

9.3 References and further reading

1. *ISO/IEC 17025, 2005. General requirements for the competence of testing and calibration laboratories.* ISO, Geneva.

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7. Mullins E., *Statistics for the quality control chemistry laboratory*, 2003, RSC, Cambridge.

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10   Glossary of mass spectrometry terms

This glossary provides brief descriptions of some common mass spectrometry terms mentioned in the guide. More comprehensive information is available in an IUPAC compilation.[10]

Accurate mass	Experimentally determined mass of an ion of known charge.
Atmospheric pressure chemical ionisation (APCI)	Chemical ionisation that takes place using a nebulised liquid at and atmospheric pressure corona discharge.
Atmospheric pressure ionisation (API)	Any ionisation process in which ions are formed atoms or molecules at atmospheric pressure.
Atmospheric pressure photoionisation (APPI)	Atmospheric pressure chemical ionisation in which the reactant ions are generated by photionisation
Average mass	Mass of ion/molecule calculated using average atomic mass of each element weighted for its natural isotopic abundance.
Base peak	The peak of greatest intensity within the mass spectrum.
Collision-induced dissociation (CID)	Dissociation of an ion after collisional excitation.
Dalton (da)	See unified atomic mass unit
Electrospray ionisation (ESI)	Spray ionization process in which either cations or anions in solution are transferred to the gas phase via formation and desolvation at atmospheric pressure of a stream of highly charged droplets that result from applying a potential difference between the tip of the electrospray needle containing the solution and a counter electrode.
Exact mass	Calculated mass of ion/molecule containing a single specified isotope of each atom, most frequently the lightest isotope of each element, calculated from masses of these isotopes expressed using an appropriate degree of accuracy.
Fragment ion	Product ion that results from the dissociation of a precursor ion.
Full width half maximum (FWHM)	Measure of peak resolution based on peak width (Δm) measured at 50% peak height
Ion trap	General term for a device for spatially confining ions using electric and magnetic fields alone or in combination.
Isobaric compounds	Atomic or molecular species with the same nominal mass but different exact masses
Isotopes	Atoms of same number of electrons and protons but different number of neutrons, hence with different *m/z*

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[10] Murray K. J K., Boyd R. K., Eberlin M. N., Langley G. J., Li L., Naito Y., *Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013*). Pure Appl. Chem., 2013. 85: p. 1515-1609

	in the mass spectrometer.
Linear ion trap (LIT)	Two-dimensional quadrupole ion trap in which ions are confined in the axial dimension by means of a static electric potential.
Mass spectrometry/mass spectrometry (MS/MS) (also known as tandem mass spectrometry)	Acquisition and study of the spectra of the electrically charged products or precursors of *m/z* selected ion(s).
Mass spectrum	Plot of the relative abundances of ion as a function of their *m/z* values.
Molecular ion	Ion formed by the removal of one or more electrons from a molecule to form a positive ion or the addition of one or more electrons to a molecule to form a negative ion.
Monoisotopic mass	Exact mass of ion/molecule calculated using the mass of the most abundant isotope of each element.
Multiple reaction monitoring (MRM)	Application of selected reaction monitoring to multiple product ions from one or more precursor ions
Multi-stage MS/MS ($\underline{MS^n}$)	Experiment designed to record product ion spectra where n is the number of product ion stages.
Nominal mass	Mass of ion/molecule calculated using mass of most abundant isotope of each element rounded to nearest integer value, *i.e.* sum of the *mass numbers* of all constituent atoms.
Orbitrap	Type of ion trap where ions are orbitally trapped and oscillate harmonically along the trap axis. The oscillation frequency is inversely proportional to the square root of *m/z*.
Precursor ion	Ion that reacts to form particular product ions or undergoes specified neutral losses.
Product ion	Ion formed as the product of a reaction involving a particular precursor ion.
Quadrupole ion trap (QIT)	Ion trapping device that depends on the application of radio frequency potentials between a ring electrode and two end-cap electrodes to confine the ion motion to a cyclic path. The choice of these potentials determines the *m/z* value below which ions are not trapped.
Quadrupole time-of-flight (QTOF)	Hybrid mass spectrometer consisting of a transmission quadrupole mass spectrometer coupled to an orthogonal acceleration time-of-flight mass spectrometer. A collision quadrupole is typically inserted between the two mass spectrometers (*i.e.* a QqTOF configuration).
Relative abundance	Measure of relative amount of ion signal recorded by detector.
Relative atomic mass (a_r)	Ratio of the average mass of the atom to the unified atomic mass unit.
Relative molecular mass (m_r)	Ratio of the mass of a molecule to the unified atomic mass unit; units = Dalton (Da)
Resolving power	Ability of instrument to resolve/separate two closely

	spaced peaks – differs according to type of instrument.
Selected ion monitoring (SIM)	Operation of a mass spectrometer in which the abundances of ions of one or more specific *m/z* values are recorded rather than the entire mass spectrum.
Selected reaction monitoring (SRM)	Data acquired from one or more specific product ions corresponding to *m/z* selected precursor ions recorded *via* two or more stages of mass spectrometry.
Taylor cone	Conical shape of the liquid emanating from a capillary under high potential, e.g. in electrospray ionisation.
Time-of-flight (TOF) mass spectrometer	Mass spectrometer that separates ions of different *m/z* by their time of travel between the ion source and detector, through a filed-free region after acceleration by a constant voltage in the source. The ions will have differing velocities depending on their mass.
Triple quadrupole mass spectrometer (often abbreviated QqQ)	Tandem mass spectrometer comprising two transmission quadrupole mass spectrometers in series, with a (non-selecting) RF-only quadrupole (or other multipole) between them to act as a collision cell
Unified atomic mass unit (amu)[11]	Unit of mass defined as one-twelfth of the mass of a carbon-12 atom and used to express masses of atomic particles. Equivalent to the Dalton unit.

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[11] IUPAC uses the symbol u for 'unified atomic mass unit' and discourages the use of amu (for atomic mass unit) although this abbreviation is still widely found.

National
Measurement
System

The National Measurement System delivers world-class measurement for science and technology through these organisations

amc

National
Measurement
Office

NPL
National Physical Laboratory

LGC

nel
technology for life