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LC-MS INSTRUMENT CALIBRATION

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13.1 INTRODUCTION

As analytical and bioanalytical methods must be validated before using them for routine sample analysis and after changing method parameters (see Chapter 8), instruments such as liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS), which are utilized to perform the analysis, should be calibrated and qualified. In addition, an instrument’s performance should be tested for suitability prior to use on practically a day-to-day basis.

For Good Laboratory Practice (GLP) studies, any equipment (instrument and any computer system used with it) must be tested according to the documented specifications. GLP regulations, which were issued by the U.S. Food and Drug Administration (FDA) in 1976, apply specifically to nonclinical studies used for Investigational New Drug (IND) registration. Shortly after the FDA introduced GLP regulations, the Organization for Economic Cooperation and Development (OECD) published a compilation of Good Laboratory Practices. OECD member countries have since incorporated GLP into their own legislation. In Europe, the Commission of the European Economic Community (EEC) has made efforts to harmonize the European laws. In general, instrument calibration is part of the regulatory compliance for worldwide drug submission. FDA GLP Chapter 21 of the Code of Federal Regulations Part 58 (CFR 21 Part 58) [1] deals with the
maintenance and calibration of equipment, including LC-MS or LC-MS/MS. The following are excerpts:

Equipment shall be adequately inspected, cleaned, and maintained. Equipment used for the generation, measurement, or assessment of data shall be adequately tested, calibrated and/or standardized.

The written Standard Operating Procedures required under §58.81(b)(11) shall set forth in sufficient detail the methods, materials, and schedules to be used in the routine inspection, cleaning, maintenance, testing, calibration, and/or standardization of equipment, and shall specify, when appropriate, remedial action to be taken in the event of failure or malfunction of equipment. The written standard operating procedures shall designate the person responsible for the performance of each operation.

Written records shall be maintained of all inspection, maintenance, testing, calibrating and/or standardizing operations. These records, containing the date of the operation, shall describe whether the maintenance operations were routine and followed the written standard operating procedure.

According to the GLP, SOPs are defined as procedures that contain the details of how specified tasks are to be conducted. The GLP definition of SOP merges the International Organization for Standardization (ISO) definitions of procedure and work instructions where procedure is a general statement of policy that describes how, when, and by whom a task must be performed; and work instructions contain the specific details of how the laboratory or other operation must be conducted in particular cases [2].

In addition to GLP, any instrument used to perform analysis under Good Manufacturing Practices (GMPs), must also be covered by instrument SOPs. If a LC-MS laboratory never performs any nonclinical GLP work for FDA submission, GMP, or bioavailability/bioequivalence testing, that laboratory in theory is not obligated to have LC-MS SOPs [3]. However SOPs are very useful because they provide a measure of consistency in how data are generated, processed, and archived. This consistency has many benefits: for example, facilitating discovery of the cause of any anomalous data that may be produced. For these reasons, many LC-MS laboratories, even though not required to be in GLP compliance, have decided to operate under these regulations and use SOPs at all times. It is important to clarify that the main purpose of the LC-MS SOPs is not to substitute the operator’s manuals but to ensure that a particular instrument is properly maintained and calibrated such that any data generated from it, when operating, can be considered reliable [3].

In this chapter we focus primarily on calibration of LC-MS where the mass spectrometer is operating at unit resolution, resolution that is sufficient to separate two peaks one mass unit apart. This kind of low-resolution mass filter covers almost 90 percent of the instruments commonly used for qualitative and/or quantitative analysis of small molecules. Batch-to-batch qualification testing of the instrument is also described. For the calibration of high-resolution mass spectrometers such as magnetic sector, TOF, or FTICR coupled with liquid chromatography, readers are referred to specific publications.
13.2 PARAMETERS FOR QUALIFICATION

The day-to-day performance of a given LC-MS or LC-MS/MS depends on its calibration, tuning, system suitability test, and final overall validation.

13.2.1 Calibration Parameters

Calibration parameters are instrument parameters whose values do not vary with the type of experiment, such as peak widths, peak shapes, mass assignment, and resolution versus sensitivity.

**Peak Width.** Peak width depends on the mass resolution. A resolution of 1 mass unit is sufficient to distinguish ions in most qualitative/quantitative small molecule applications. A typical definition of unit resolution is when the peak width at half-height is about 0.6 to 0.8 mass unit. The profile scan of ions on a typical benchtop LC-MS has a bandwidth of about 1 mass unit (Figure 13.1).

**Peak Shape and Profile Scan.** In a typical benchtop LC-MS, abundance measurements are collected at 0.10-\( m/\zeta \) increments, as shown in Figure 13.2. When these data are presented in a mass spectrum, a single line can be shown. The height and position are derived from the profile scan.

**Mass Assignment.** It is performed using specific MS calibrants. Calibrants should be well-characterized reference materials. Certification and handling of these

![Figure 13.1. Bandwidth in a typical benchtop LC-MS.](image)

![Figure 13.2. (a) Profile scan and (b) spectral representation in a typical benchtop LC-MS.](image)
Compounds should be documented. LC-MS qualitative analysis always uses reference materials, whereas quantitative analysis uses a combination of reference materials and actual analyte standards as calibrants.

**Resolution versus Sensitivity.** Mass resolution is a compromise between ion intensity and peak width. In general, as the resolution is increased, the ion intensity decreases (see Section 13.3.2).

**Calibration.** Calibration has been defined as “a comparison of a measurement standard or instrument of known accuracy with another standard or instrument to eliminate deviations by adjustment” [4]. The term *calibration* in this chapter is used to indicate a mass-axis calibration. Other calibration (e.g., voltage of power supplies) is outside the scope of this chapter. Calibration of the mass axis requires a series of ions evenly spaced throughout the mass range. When a calibration is completed, it is possible to acquire data over any mass range within the calibrated range. It is therefore sensible to calibrate over a wide mass range. Manual, semiautomatic, and automatic LC-MS calibration require introduction of the solution of the calibrant (calibration solution) into the MS at a steady rate while the procedure is running. The calibration solution is introduced directly (infused) into the MS from a syringe pump or through a loop injector connected to the LC pump. It is recommended that the MS be calibrated at least once every three months and the calibration checked about once a week.

### 13.2.2 Tuning Parameters

Tuning parameters are instrument parameters whose values can vary with the type of experiment. For example, if the experiment requires quantitative data on one or more particular ions, the MS should be tuned to optimize the response for the specific analyte standard. Manual, semiautomatic, and automatic tuning procedures require the introduction of a tuning solution of the analyte of interest into the MS at a steady rate. This can be done in three different ways: (1) by introducing the solution directly from the syringe pump (direct infusion); (2) by introducing the sample from the syringe pump into the effluent of the LC by using a tee union; and (3) by injecting the sample into the effluent of the LC by using a loop injection valve [flow injection analysis (FIA)].

The first method is good for tuning for experiments at a low flow rate involving the syringe pump. The second and third methods are useful for experiments at a higher flow rate involving the LC. In general, the FIA gives a better evaluation of the instrument signal-to-noise ratio (S/N) at the masses of interest. In most cases the tuning parameters obtained from the automatic or semiautomatic tuning procedures are sufficient for many analytical experiments. However, one must ensure that proper parameters are generated. For some applications it is necessary to do manual fine-tuning of several MS parameters.

The optimized parameters, which affect the signal quality, change from instrument model to instrument model and from brand to brand. Examples are: source
temperature, ionization voltages, gases (nebulization, desolvation, and collision), ion path potentials (lens, multipoles, or stacked rings), collision energy, solution, or mobile-phase flow rate. The potentials, RF values, and gas pressure affect the declustering, focusing, fragmentation, and efficiency of ion transmission.

13.2.3 System Suitability Testing

System suitability allows the determination of system performance by analysis of a defined solution prior to running the analytical batch. System suitability should test the entire analytical system, chromatographic performance as well as the sensitivity of the mass spectrometer for the compounds of interest. Some LC-MS SOPs reference analytical methods as the source of operating details for a given analysis. This works particularly well for quantitative analysis, where analytical methods include critical details on instrument parameters and special calibrations that might be required for a particular analyte. Thus, system suitability testing provides the daily checking of the system.

13.2.4 Validation

Validation is the final step to guarantee that a LC-MS system performs as expected. Validation includes instrument calibration, tuning, testing, and checking of the documentation for completeness, correctness, and compliance with SOPs. Validation consists of four separate steps:

1. Validation of the instrument and the computer controlling it (computer system validation or CSV)
2. Validation of the analytical method running on that equipment
3. System suitability testing, to test the equipment and the method together to confirm expected performance
4. QA/QC review of sample analysis data collected on such a system [4]

13.3 CALIBRATION PRACTICES

How often an LC-MS should be calibrated depends on the mass accuracy required. For example, instrument calibration should be verified daily when performing accurate mass measurements of peptides and proteins. However, the quantitative analysis of small molecules requires less frequent calibration.

13.3.1 General Tuning and Calibration Practice for MS

The mass analyzer should be calibrated on a regular basis by infusing a calibration solution. In general, an electrospray ionization source (ESI) is used. The solution should produce ions (with known exact masses) that cover the entire instrument mass range or at least the mass range that will be used for subsequent analyses.
For the most recent LC-MS on the market, an automatic procedure is included in the software package to tune and calibrate in the ESI mode. However, older instruments and/or very specific applications still require manual or semiautomatic procedures to optimize the parameters that affect ion detection. In an LC-MS instrument, the mass spectrometer is tuned and calibrated in three steps: (1) ion source and transmission optimization, (2) MS calibration, and (3) fine tuning (detection maximization of one or more particular ions).

**Ion Source and Transmission Optimization.** In this step, the MS in ESI mode is roughly tuned on one or more specific ions by infusing the calibration solution at a steady rate around 5 µL/min for several minutes. The introduction of the calibration solution compound is best achieved using a large-volume Rheodyne injector loop (50 or 100 µL) or an infusion pump (e.g., a Hamilton syringe pump): When using a large-volume injection loop, the solvent delivery system should be set up to deliver around 5 µL/min of 50:50 acetonitrile/water or 50:50 methanol/water through the injector into the source. An injection of 50 µL of calibration solution will then last for at least 10 min. When using an infusion pump, the syringe should be filled with the calibration solution and then connected to the ESI probe with fused silica or peek tubing.

The ions chosen for the optimization should be in the middle of the calibration range or in a specific region of interest (e.g., m/z = 195 of caffeine or m/z = 906.7 of PPG calibration solution). Before using the automatic tuning procedure to demonstrate that the transmission of ions into the MS is optimum, the ESI source sprayer should be manually adjusted to establish a stable spray of ions into the MS and to ensure that enough ions are detected to calibrate the MS. The degree of adjustment changes from model to model and depends on the source geometry. In general, sources where the spray is off axis with the inlet, such as orthogonal and z-spray, require fewer adjustments. For the on-axis sources, to avoid the contamination of the instrument optics, it is important to check that the source is not spraying directly at the instrument’s orifice. On-axis sources usually have a side window to provide a good view for the spray trajectory. In both cases the sprayer should always be moved in small increments until reaching the optimum position for the highest S/N value of the ions of interest. The source parameters (e.g., nebulizer gas, lens voltages) are optimized automatically, semiautomatically, or manually.

**MS Calibration.** In the second step, the MS in the ESI mode is calibrated using the calibration solution. All mass analyzers must be calibrated. For example, quadrupoles 1 and 3 are both calibrated for the triple-quadrupole mass spectrometer. Here is the calibration process:

1. A mass spectrum of a calibration solution is acquired (calibration file) and matched against a table of the expected masses of the peaks in the calibration solution that are stored in a reference file.
2. Each peak in the reference file is matched to a corresponding peak in the calibration file.
3. The corresponding matched peaks in the calibration file are the calibration points.
4. A calibration curve is fitted through the calibration points.
5. The vertical distance of each calibration point from the curve is calculated. This distance represents the remaining (or residual) mass difference after calibration.
6. The standard deviation of the residuals is also calculated. This number is the best single indication of the accuracy of the calibration [5].

Before starting the automated calibration, a number of the peak match parameters need to be set. These parameters determine the limits within which the acquired data must lie for the software to recognize the calibration masses and result in a successful calibration. Some of these parameters are:

1. **Peak search range.** This is the window used by the calibration software to search for the most intense peak. Increasing this window gives a greater chance of incorrect peak matching. It is important to ensure that the correct peak is located in the peak search range; otherwise, a deviation in the calibration may arise. If an incorrect peak is located, the search peak range should be adjusted to locate only the correct ion.

2. **Peak threshold.** All peaks in the acquired spectrum below the intensity threshold value (measured usually as a percentage of the most intense peak in the spectrum) will not be used in the calibration procedure.

3. **Peak maximum standard deviation or maximum difference between the predicted and actual mass.** During calibration the difference between the measured mass in the acquired calibration file and the true mass in the reference file is taken for each pair of matched peaks. If this value exceeds the set value, the calibration will fail. Reducing the value of the standard deviation gives a more stringent limit, while increasing the standard deviation means that the requirement is easier to meet, but this may allow incorrect peak matching.

4. **Peak width.** This is a measure used to set the resolution, usually specified at 50% of maximum intensity.

If the acquired spectrum looks like the reference spectrum and all of the peaks expected pass the criteria above, the calibration is acceptable. If the instrument has never been calibrated before or the previous calibration file has been misplaced, a more detailed calibration routine must be used. This calibration procedure requires the location of known peaks, starting with the lowest mass ions. When a peak has been located, a digital-to-analog conversion value is assigned to the exact mass of the ion.

Once a full instrument calibration is in place, it is not always necessary to repeat the full calibration procedure when the instrument is next used. Instead of a full calibration, a calibration verification can be performed by infusing the calibration solution and setting all peak matching parameters to the values that were used
for the full calibration. If the difference between the predicted and actual mass is not significant, the original mass calibration curve is still valid. Independent of the instrument model/brand, it is always possible to obtain, at the end of the automated calibration, a final report that displays the predicted exact calibration solution masses, the actual mass of each peak as seen in the mass spectrum, the difference between these two masses (accuracy of the calibration), and in many cases also the intensities and peak widths of all the ions specified in the experiment.

Fine Tuning. In the third step, the detection of one or more particular ions is optimized to tune the MS with the standard of the analyte of interest, if available, in either the ESI or APCI mode. The mass-to-charge ratio of the analyte of interest or, alternatively, when the standard of the analyte is not available, an ion in the calibration solution that is the closest to the mass-to-charge ratio for the ion of interest, can be chosen (see Section 13.2.2).

13.3.2 Quadrupole Mass Filter Calibration

Resolution versus Sensitivity. A quadrupole mass filter can be programmed to move through a series of RF and dc combinations. The Mathieu equation, which is used in higher mathematics, can be used to predict what parameters are necessary for ions to be stable in a quadrupole field. The Mathieu equations are solved for the acceleration of the ions in the X, Y, and Z planes. A selected mass is proportional to \( \frac{\text{dc} \times \text{RF} \times \text{inner radius}}{\text{RF frequency}} \). For a given internal quadrupole radius and radio frequency, a plot can be made of RF and dc values that predict when a given mass will be stable in a quadrupole field. This is called a stability diagram (Figure 13.3). RF and dc combinations follow the value shown

![Stability Diagram](image)

Figure 13.3. Stability diagram of a quadrupole mass filter.
by the line intersecting the stability diagram. The line that reflects the RF/dc is called a *scan line* (Figure 13.4). Modifying the scan line changes the ratio of RF to dc. The gain changes the ratio of RF to dc more rapidly at high values of RF than at low values of RF.

Constant-peak-width profile scans can be achieved by adjusting the RF-to-dc ratio. In this case the points of intersection on the scan line will be at the same distance below the apices (Figure 13.5). To adjust the scan line, the amount of

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**Figure 13.4.** Different scan lines in a quadrupole mass filter.

**Figure 13.5.** Constant peak profile scan in a quadrupole mass filter.
dc is changed for a given RF potential. The scan line can be adjusted in two ways: the slope and y-intercept can be adjusted to achieve high-quality spectra. The term *amu gain* is used to indicate the slope, and *amu offset* is used to indicate the y-intercept of the scan line. The amu offset value is a setting for the mass filter dc voltage adjustment needed to resolve ions and achieve a constant peak width over the entire mass range. The proper scan line produces good resolution and thus good profile scan. This results in optimized mass spectra (Figure 13.6).

**Figure 13.6.** Optimized mass spectra in a quadrupole mass filter.
It is usually desirable to have a consistent peak width over the entire mass range. If the dc is held constant, the peak width varies over the mass range and increases as the mass increases (Figure 13.7). Adjusting the slope of the operating line increases the resolution. The resolution normally obtained is not sufficient to deduce the elemental analysis. Usually, quadrupole mass spectrometers are low-resolution instruments and operate at unit resolution.

The wide peak width at higher masses results in loss of resolution. In Figure 13.8 the heavy ion and its $^{13}$C isotope cannot be distinguished, while

**Figure 13.7.** Increase of peak width when dc is held constant in a quadrupole mass filter.

**Figure 13.8.** Loss of resolution at high masses in a quadrupole mass filter: (a) unit resolution; (b) low resolution.
at low masses (light ion) it is still possible to resolve the isotope peak (Figure 13.9). This is a classic case of poor resolution at high masses. The normal trade-off between high ion transmission and narrow peak widths should be optimized for each application. Most applications require unit mass resolution (i.e., isotope peaks 1 amu apart are clearly defined) which corresponds to peak widths of approximately $0.7 \pm 0.1$ amu at 50% intensity [full width at half maximum (FWHM)].

The entire quadrupole mass range can be divided into several mass regions such that the resolution can be adjusted in each region without affecting the others. This feature allows fine tuning of the instrument resolution for specific applications. The resolution within the individual mass regions is adjusted by increasing or decreasing the offset parameter at the masses that bracket the region. Increasing the offset increases the resolution and decreasing the offset decreases the resolution. Changing offset changes the slope of the scan line. In other words, to obtain unit mass resolution throughout the operating mass range, different scan lines are employed for different $m/z$ ranges. For example, if the resolution of an ion at $m/z$ 500 needs to be increased, the offset at the mass that bracket the region, for example, mass 100 and/or mass 1000, should be increased. Both the mass settings at 100 and 1000 will change the resolution, so either setting may be adjusted. However, if the resolution of an ion at $m/z$ 999 needed to be adjusted, the offset at mass setting 1000 would have a large effect, whereas the mass setting at 100 would have a very small effect on its resolution [6,7].

Resolution is also affected by the actual time that ions spend in the quadrupole. Ions that have higher kinetic energy have shorter residence time and lower resolution. Reducing the kinetic energy typically leads to an improvement in resolution. A dramatic change in resolution may cause a shift in the instrument calibration. After adjusting the resolution, it is necessary to check the calibration (calibration verification), and if it is noted that the difference between the predicted and actual mass values have shifted, the instrument should be recalibrated.
**Calibration Curves.** A quadrupole mass spectrometer can require up to three calibration curves:

1. A *static calibration* is used to accurately “park” the quadrupole mass analyzer on a specific mass of interest. If only a static calibration is performed, the instrument is calibrated for acquisitions where the quadrupoles are held at a single mass as in SIM or SRM.

2. A *scanning calibration* enables peaks acquired in a scanning acquisition to be mass measured accurately (scan mode). If only a scanning calibration is performed, the instrument is calibrated correctly only for scanning acquisitions over the same mass range and at the same scan speed as those used for the calibration. The scan speed recommended for the scanning calibration is 100 amu/s.

3. A *scan speed compensation calibration* compensates for lag time in the system when the instrument is scanned rapidly. If only a scan speed compensation is performed (without a scanning calibration having been performed), the scan speed compensation is treated as a scanning calibration and the instrument is calibrated correctly only for scanning acquisitions over the same mass range and at the same scan speed as those used for the calibration. The scan speed recommended for the scan speed compensation is 1000 amu/s.

For some MS models and brands it is recommended that all three types of calibration are performed so that any mode of data acquisition can be used and mass ranges and scan speeds can be changed while maintaining correct mass assignment [5]. Some other instruments calibrate all together without distinctions [6–8]. When the instrument is fully calibrated, any mass range or scan speed is allowed within the upper and lower limits dictated by the calibrations.

When each peak in the reference spectrum has been matched with a corresponding peak in the spectrum acquired, the mass difference is calculated for each pair of peaks (see Section 3.1.2). These mass differences are plotted as points on a graph; each data point has the mass of the acquired peak as its x coordinate, and the mass difference above as its y coordinate, and a smooth curve is drawn through the points (Figure 13.10) [5]. The polynomial order parameter controls the type of curve that is drawn and can be set to any value between 1 and 5:

![Figure 13.10. Plot of the mass difference between reference spectrum and acquired spectrum. MS1 static calibration, 28 matches of 28 tested references, SD = 0.0465. (Courtesy of Micromass Quattro LC.)](image-url)
**Polynomial order** = 1. A straight line is drawn through the points. This polynomial of order 1 is not used to calibrate quadrupole MS.

**Polynomial order** = 2. A quadratic curve is drawn through the points. An order of 2 is suitable for wide mass ranges at the high end of the mass scale and for calibrating with widely spaced reference peaks. This is the recommended polynomial order for calibration with sodium iodide (see Section 13.3.3), which has widely spaced peaks (150 amu apart) and horse heart myoglobin (see Section 13.3.3), which is used to calibrate higher up the mass scale. In general, for a typical ESI calibration where the mass range calibrated is from 600 amu to greater than 1000 amu, the recommended setting for the polynomial order parameter is 2.

**Polynomial order** = 3. A cubic curve is used.

**Polynomial order** = 4. Used for calibrations that include the lower end of the mass scale, with closely spaced reference peaks. This is suitable for calibrations with polyethylene and polypropylene glycols (see Section 13.3.3) that extend below 300 amu.

**Polynomial order** = 5. Rarely has any benefit over a fourth order fit.

For more information about the calibration procedure for a specific model or brand quadrupole MS, it is suggested that the reader consult the operators manual provided with the instrument.

### 13.3.3 Calibration Solutions

Calibrants are required to calibrate the mass scale of any mass spectrometer, and it is important to find reference compounds that are compatible with a particular ion source. Calibrants commonly used in electron ionization (EI) and chemical ionization (CI), such as perfluorocarbons, are not applicable in the ESI mode. The right calibrants for LC-ESI-MS should (1) not give memory effects; (2) not cause source contamination through the introduction of nonvolatile material; (3) be applicable in both positive- and negative-ion mode. The main calibrants used or still in use to calibrate ESI-MS can be divided into the following categories: polymers, perfluoroalkyl triazines, proteins, alkali metal salt clusters, polyethers, water clusters, and acetate salts.

**Polymers.** Polymers, such as polypropylene glycols (PPGs) and polyethylene glycols (PEGs) are the preferred calibrant for many small molecule applications. PPG calibration solutions produce mostly singly charged ions over the entire instrument mass range in both positive- and negative-ion mode. The PPG ions in general used for calibration in positive mode are: 59.0 (solvent and fragment ion), 175.1 (fragment ion), 616.5, 906.7, 1254.9, 1545.1, 2010.5, and 2242.6 (Figure 13.11) [6,7,10,11,27]. During the calibration procedure, these ions are listed in the reference file, and they should be the most intense peaks within the search range in the calibration file around the predicted mass. It is important to ensure that the correct peaks are located in the search range; otherwise, a
deviation in the calibration may arise. If an incorrect peak is located (typically, a peak with a mass difference greater than 1 amu from the predicted mass), the search range or the experiment scan width should be adjusted to locate only the correct ion. When the calibration is done using PPG, for most applications a difference between the predicted and actual mass of 0.05 amu or less can be considered as not significant. In general, a positive-ion calibration will be sufficient for the negative-ion mode, but occasionally, there might be very small calibration shifts in part of the mass range (e.g., 0.1 amu offset at low mass). The PPG ions used for calibration in negative-ion mode are 45.0 (solvent ion), 585.4, 933.6, 1223.8, 1572.1, 1863.3, 2037.4, and 2211.6 (Figure 13.12) [6,7].

PPG and PEG calibration solutions are used most widely for ESI-MS calibration, although significant source contamination and memory effects may occur [5,9,10,11,27]. Some precautions should be taken when using PEG or PPG as calibrant. Indeed, if a very low threshold and wide peak search range is used, it may be possible to select the wrong peaks and get a “successful wrong” calibration. Therefore, caution should be used when calibrating with PEG or PPG in ESI mode due to the number of peaks that are produced. Although ammonium acetate is added to the calibration solution to produce mainly [M + NH₄]⁺ ions, under some conditions it is quite usual to see [M + H]⁺, [M + Na]⁺, and doubly charged ions.

The spectrum shown in Figure 13.13 demonstrates how a PEG spectrum can be dominated by doubly charged ions ([M + 2 NH₄]²⁺) if the wrong conditions are chosen. In this case the concentration of ammonium acetate in the reference solution was too high (5 mM ammonium acetate is the maximum that should be used) and the declustering potential is too low. Doubly charged ions can be
identified easily because the peaks in a given cluster (e.g., one that for all $n^{12}$C atoms and its neighbor that contains one $^{13}$C and $n-1^{12}$C) are separated by 0.5-$m/z$ units (Thomson) instead of 1.0-$m/z$ units. If the instrument is set to unit mass and data are acquired in continuum mode, the doubly charged peaks
will appear broader, as the isotopes will not be resolved. With PEG the possible calibration range depends on the molecular weight distribution of the PEGs used in the reference solution.

**Perfluoroalkyl Triazines.** Perfluoroalkyl triazines such as Ultramark 1621 have mainly been used as the calibrant for FAB [10–13]. Ultramark 1621 is also used as a calibration solution for ESI-MS calibration [8,10,11,14]. Although effective, this standard is very “sticky” and is very difficult to remove from the ion source. For this reason, Ultramark 1621 calibration solution should not be used at flow rates above 10 µL/min, to avoid system contamination. Ultramark 1621 is in general used together with other calibrants to cover the entire MS range. Calibration solutions containing caffeine, L-methionyl-arginyl-phenylalanylanine acetate H₂O (MRFA) and Ultramark 1621 are commonly employed for ESI-MS calibration [8]. It is possible to observe the following singly charged, positive ions for caffeine, MRFA, and Ultramark 1621 (Figure 13.14): caffeine: m/z 195; MRFA: m/z 524; and Ultramark 1621: m/z 1022, 1122, 1222, 1322, 1422, 1522, 1622, 1722, 1822.

**Proteins.** ESI-MS calibration was initially performed using solutions of gramicidin S, cytochrome c, or myoglobin as calibrant. Proteins produce multiply charged species in ESI. When proteins are used as calibrants for low-resolution LC-MS and LC-MS/MS instruments, it is not possible to resolve individual isotope peaks, and the calibration should be performed by using the average molecular mass for the unresolved isotope clusters. The danger in using proteins as calibrants is illustrated by the fact that equine myoglobin was used as a reference standard by many groups until it was discovered [10,11,15,16] that the amino acid sequence used to calculate the molecular mass of myoglobin was incorrect. The correct molecular mass of equine myoglobin was actually 1 amu higher than the value used as a reference standard.
Alkali Metal Salt Clusters. Alkali metal salt clusters cover a wide m/z range and are used to calibrate mass spectrometers in ESI mode. Cesium iodide solutions produce singly and doubly charged species from m/z 133 up to m/z 3510 or higher [10,11,17]. Unfortunately, cesium iodide calibration solutions are not very commonly used, due to the following drawbacks: (1) sample suppression; (2) persistence in the ESI source; (3) possible cation attachment; and (4) the large spacing, 260 amu, between peaks. A mixture of sodium iodide and rubidium iodide calibration solution is able to cover the instrument’s full mass range from 23 to 3920 (Figure 13.15) [5,10,11,26]. The peak at 23 is sodium, the 85 peak is rubidium, and the others are clusters. A mixture of ammonium acetate, tetrabutylammonium iodide, benzyltriphenylphosphonium chloride, and hexadecylypyridinium chloride was used successfully and described in the literature [18].

Polyethers. Polyethers such as polyethylene oxide (PEO) and polypropylene oxide (PPO) have been used for ESI-MS calibration [10,11,19]. The predominant ions for these calibrants are cation attachments, and sodium attachment is frequently observed, due to traces of sodium in solvents and glassware. The positive-ion ESI mass spectra of PEO and PPO are characterized by abundant \([M + nNa]^n+\) and some \([M + nH]^n+\) species. Macrocyclic polyethers and crown ethers were also used as ESI-MS calibrants [11]. In general, nonderivatized polyethers show the following drawbacks when used as calibrations solutions: (1) they are difficult to flush out of the ion source, (2) they generate complex mass spectra resulting from the presence of several different cation sources, and (3) they have different charge states. Negative-ion ESI-MS show relatively weak \([M - H]\) peaks that can be observed only with difficulty; thus, polyethers are not useful calibration compounds for negative-ion ESI analysis.

![Figure 13.15. Calibration in ESI positive using sodium iodide and rubidium iodide. (Courtesy of Micromass Quattro LC.)](image-url)
Derivatized polyethers such as polyether sulfate have been investigated for both positive- and negative-ion calibration [11]. Although polyether sulfates are not commercially available, they are easily synthesized. Lauryl sulfate ethoxylates were also used as calibrants for negative-ion ESI. Polyether amines and quaternary ammonium salts were used as positive-ion calibration solutions [11]. These commercially available compounds do not exhibit significant sodium or potassium adducts, and they are more easily flushed out of the mass spectrometer ion source than are nonderivatized polyethers. In addition, doubly charged polyether diamines can produce reference peaks at low m/z values.

**Water Cluster.** Numerous groups have used water clusters successfully as calibration solutions [10,11,20–22]. Water clusters do not produce any source contamination in ESI-MS and provide closely spaced reference peaks with a calibration range up to m/z 1000. In positive-ion mode, protonated water clusters with up to 70 water molecules are observed. In negative ESI singly deprotonated water clusters are observed [OH$^-$ · (H$_2$O)$_n$ with n > 20], as well as solvated electrons [(H$_2$O)$_m$$^-$ with m > 11].

**Acetate Salts.** Sodium acetate and sodium trifluoroacetate clusters were used and produce useful reference peaks for both positive and negative ESI [10,11,23]; 0.5% acetic acid in ammonium acetate solutions can be used for calibration in ESI-MS. This calibration solution, which is volatile, produces cluster ions up to m/z 1000. Therefore, it does not produce any source contamination or memory effects. Replacing acetic acid by trifluoroacetic acid (TFA) further enlarges the mass range to m/z 4000, but TFA produces some memory effects and ion suppression, especially in negative-ion mode.

**Other Calibrants.** The use of cesium salts of monobutyl phthalate and several perfluorinated acids to generate cluster ions up to m/z 10,000 has been described in the literature [11,24]. Fluorinated derivatives of glyphosate and aminomethylphosphonic acid were used as reference compounds for negative ESI [11,20]. These calibrants were synthesized as a set of individual compounds that can give singly charge reference ions over the m/z range 140 to 772. Because these are individual compounds rather than a single compound that gives a distribution of oligomers or cluster ions, individual reference masses can be selected to bracket the mass of the analyte [11]. In conclusion, different calibrants are currently used, depending on the instrument manufacturer and/or specific application. The most common are polyethylene or polypropylene glycols, Ultramark 1621, phosphazines, mixtures of caffeine, a small peptide (MRFA) and myoglobin, or other mixtures of peptides and proteins.

### 13.3.4 APCI Source Tuning and Calibration

An atmospheric pressure chemical ionization (APCI) interface is generally considered extremely easy to optimize and operate. This is perhaps best proven by the fact that hardly any optimization of the interface parameters is reported
in the many APCI application papers. The optimization for important interface parameters, such as the liquid flow rate, solvent composition, nebulizer and auxiliary gas flow, probe position, and vaporizer temperature, is less critical than, for instance, in ESI.

Reserpine is the most commonly used calibrant for APCI. The average molecular weight of reserpine is 608.7 and is generally injected using flow injection analysis (FIA) at a concentration of 10 pg/µL in 1% acetic acid in 50:50 methanol/water [6,7]. The use of PEG was described in the literature for calibration of an APCI-MS system in positive-ion mode (Figure 13.16) [5,10,25]. For calibration with PEG, it is best to use a large-volume injection loop (50 µL) with a solvent delivery system set up to deliver 0.2 mL/min of 50:50 acetonitrile/water or methanol/water through the injector and into the APCI source. An injection of 50 µL of PEG calibration solution lasts for approximately 15 s, allowing enough time to perform a slow scanning calibration. Since the PEG1000 has peaks from m/z 63 to 987, it is possible to calibrate over this mass range that is sufficient for the majority of applications with APCI. In many APCI operations, tuning strategies adapted from ESI are commonly used.

13.4 COMMON PROBLEMS AND SOLUTIONS

1. LC-MS SOP and documentation. The importance of having LC-MS SOPs and updated written records for LC-MS calibration and maintenance is particularly
critical during an audit [4]. Answering the following questions should help in verifying if the documentation related to LC-MS instrument is in full compliance with GLP and GMP regulations:

a. Is the LC-MS within specification, and is the documentation to prove this available?
b. If the LC-MS is not within specifications, by how much does it deviate?
c. If the LC-MS is not within specifications, how long has this been the case?
d. If the LC-MS is not within specifications, what action has been taken to overcome the defect?
e. What standard has been used to test and calibrate the LC-MS before sample analysis?
f. What action has been taken to guarantee the reliability of the data produced by the LC-MS used?

2. *Fit the purpose calibration.* It is common sense to check instrument performance each day, and GLP requirements simply formalize the performance and documentation of these checks. On the other hand, it is also important to use the right test (full calibration, verification, system suitability test, or instrument and method validation) to verify the performance and to avoid needlessly lengthy procedures. As already discussed (see Sections 13.2.3 and 13.3.1), it is not always necessary to perform a MS full calibration every day. For example, if a particular MS is used only to record complete full-scan mass spectra, a daily calibration or verification of the calibration of the \( m/z \) ratio scale is required. However, in the case where a MS is coupled with an LC and utilized primarily for the analysis of one or more analytes in the selected ion monitoring (SIM) mode, it does not always require a daily verification of the calibration. In this specific case it is quite common in LC-MS and LC-MS/MS applications to test only the following performance parameters: (a) sensitivity, (b) system precision, (c) linear dynamic range, (d) analytes retention time, and (e) chromatographic peak shape. All these parameters can be checked simultaneously by injections of system suitability solutions. Usually, if the system suitability test fails for nonchromatographic reasons, a possible deterioration of the \( m/z \) axis calibration should be taken into consideration and at least a verification of the calibration should be performed.

3. *Cross-contamination.* During the tuning and calibration procedures a tuning or calibration solution is infused using a syringe pump connected directly to the ion source or going into LC flow using a tee union. In general, the LC-MS is tuned and calibrated before data acquisition in either the ESI or the APCI mode. Due to the fact that many calibration solutions are quite “sticky,” it is suggested to minimize the possibility of cross-contamination by using a different syringe and section of fused silica/peek tubing for the calibration solution and another for the tuning solution containing the analytes of interest. The infusion tuning/calibration procedures put a comparatively large amount of calibrants/analytes into the MS.
Therefore, before performing an analytical run to analyze the analyte, it is suggested that the LC-MS interface be cleaned. These problems are partially avoided if the FIA method (see Section 13.2.2) is used for tuning.

4. Failed calibration. There are number of reasons for a calibration to fail. If an automated calibration method is used, it is possible that the reference peaks are not recognized when the reference file and calibration file are compared. This can be due to the following reasons:

   a. Degradation of the calibration solution.
   b. Serious contamination of the calibration solution.
   c. No flow of solvent into the source.
   d. Multiplier set too low, so that the less intense peaks are not detected.
   e. Reference solution running out, so that the less intense peaks are not detected.
   f. Incorrect ionization mode selected. It should be checked that the data have been acquired with the right ion mode ESI positive or negative;
   g. Intensity threshold set in the peak match parameters (see Section 13.3.1) too high. Peaks are present in the acquired calibration file but are ignored because they are below the threshold level.
   h. Peak search range set in the peak match parameters (see Section 13.3.1) too small. The calibration peaks lie outside the limits set by these parameters;
   i. Wrong reference file selected.

If the correct calibration parameters were used, and good calibration data were acquired, the instrument should be calibrated correctly. However, in some circumstances it is possible to meet the calibration criteria without matching the correct peaks. This situation is unusual, but it is always sensible to examine the on-screen calibration report to check that the correct peaks have been matched. These errors may occur when the following parameters are set:

   a. The intensity threshold is set to 0.
   b. The peak search range is too wide. A more intense peak than the reference could be included in the window.
   c. If a contamination or background peak lies within one of the peak search windows and is more intense than the reference peak in that window, the wrong peak will be selected. Under some conditions this may happen more often when PEG and PPG are used as calibrants.

There are two ways to avoid this problem:

   a. If the reference peak is closer to the center of the peak search range, this range can be narrowed until the contamination peak is excluded. It should be ensured that no other reference peaks are excluded.
b. If the reference peak is not closer to the center of the peak search range, or if by reducing the window other reference peaks are excluded, the calibration should be executed manually.

Before starting an automated calibration, as calibration solution infuses, the value of the ion current signal should always be checked to verify if the signal is present; or the signal is stable, varying by less than about 15% from scan to scan. If these conditions are not verified, the following troubleshooting measures should be tried:

a. If a fused-silica tube is used inside the ESI needle, it should be ensured that the fused-silica sample tube is in the position recommended by the operator’s manual for that model or brand.

b. The interface should be inspected to ensure that the inlet is clean.

c. The ESI probe should be optimized for the flow rate used.

d. The solution entering the probe should be free of air bubbles.

e. The tubing and connectors should be checked for possible leaks.

REFERENCES

1. Code of Federal Regulations, Title 21, Food and Drugs, Office of the Federal Register, National Archives and Records Administration, Washington, DC, 1994, Part 58 (available from New Orders, P.O. Box 371954, Pittsburgh, PA, 15250-7954).


5. User’s Guide Micromass Quattro LC.


