ESSENTIAL QUALITY CONTROL INVESTIGATIONS OF CHIROPTICAL SPECTROSCOPY DATA OF BIOLOGICAL MOLECULES

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ABSTRACT
Circular Dichroism (CD) has become one of the most important techniques for the characterization of biomolecules, determination of absolute configuration and stereochemical analysis. However, literature lacks data related to quality assurance/control of factors affecting the reliability of chiroptical spectroscopic data. Therefore, it is important, prior to the analysis, to establish quality assurance for these factors affecting the reliability of the data, which is especially true when spectra are crucial evidence of the structure and stability of a protein component of a pharmaceutical product, but it is also important when spectra are to be reported for almost any purpose. Nevertheless, to ensure good circular dichroism data that are comparable between instruments and between laboratories, there are several major factors in an optical spectrometer that need to be controlled including calibration (intensity and wavelength calibration), data resolution spectral bandwidth (SBW), time scale of measurements and scan mode. Moreover, signal and noise, post measurement smoothing, stray light, drift, performance characteristics and experimental factors. In this work, we have studied the effect of several factors on the quality of spectral data of proteins (IgG), amino acids (L-phenylalanine) and simulated spectra of nucleic acid. The results showed that increasing the wavelength stepsize distorts the spectrum and can produce a reduced peak height i.e. loss of fine structure. In terms of signal processing, the fewer the steps needed the longer is the time spent averaging the data at a particular wavelength and hence the lower the noise associated with the spectroscopic feature.
KEYWORDS: Calibration; chiroptical spectroscopy; circular dichroism; quality control.

INTRODUCTION
Chiroptical spectroscopic methods of analysis are powerful techniques and find wide areas of applications such as elucidation of protein structure and conformational changes, protein-ligand interactions. The methods have been proven to be sufficiently simple, reliable, and, in many situations, invaluable for rapid determination of molecular structure or binding parameters. Typically, the CD spectra of proteins are recorded in the far-UV region (180–250 nm), the near-UV region (250–320 nm), and in the regions of ligand absorption when studying protein–ligand complexes or proteins with prosthetic groups. The data produced from CD measurements are analysed by several chemometric tools such as partial least squares, principal component regressions, or combinations of several such methods to improve and extend the analyses and to further the applicability of this technique. Quantitative and qualitative information can be obtained, however, to faithfully produce reliable results that reflect the measured sample properties, the assurance of quality of spectroscopic and analytical data should be considered very carefully. The different aspects of quality assurance include demonstrating that a reliable instrument is “fit for the purpose” of the analysis. This implies that the performance characteristics such as sensitivity, selectivity and detection limit must be well demonstrated. Quality assurance studies that can be decisive in obtaining reliable CD data such as absorbance/CD calibration, wavelength calibration, assessment of the effect of wavelength data resolution i.e. stepsize (SS) and spectral bandwidth (SBW) on the quality of spectral data are hardly addressed in the literature. The absorbance scale of a spectrophotometer is readily calibrated with an accredited neutral density filter or defined solutions of potassium dichromate. By contrast, the “intensity” scale of a CD spectrum is not so certain. The accuracy of a polarisation modulation spectrometer depends critically on calibration. The achievement of accuracy and precision in spectroscopic data can be demonstrated by performing measurements that employ standards. These standards are certified reference materials of known characteristics such as wavelength peak maxima position and/or absorbance value at that position, they can be primary, secondary or local laboratory based. CD is defined as the differential absorbance of left (A_L) and right circularly polarised light (A_R), therefore a primary standard implies the measurement of A_L and A_R separately and taking the difference. But values of ΔA are very small and can approach 10^{-5}, making the absolute measurement of ΔA extremely difficult. However, this has only been achieved directly for Tris (ethylenediamine) cobalt (III) chloride.
i.e. (+)-Δ-[Co (en₃)] Cl₃, which has the advantage of having very strong positive CD at 498 nm ($\Delta\varepsilon = 1.89 \text{ M}^{-1}\text{cm}^{-1}$). On the other hand, secondary standards usually offer the advantage of convenience and are based upon samples that have values often accepted simply based upon consensus. Thus, the wavelength accuracy and precision of the modulation polarisation spectrometer are achieved by measuring the spectrum of (+)–10d camphor sulphonate acid, which is used routinely for CD calibration.

Wavelength accuracy affects the quality not only of CD spectra, but also of any other spectra, and, due to this; it is advisable to check the wavelength calibration of the instrument and its general far-UV transmission performance from time to time. Wavelength calibration of an instrument that operates in the UV/vis region can be achieved by using optical filters (holmium or didymium). This is done by measuring the wavelengths of peak maxima and comparison with standard values. The permitted tolerance is ±1nm in the range 200-400 nm and ±3 nm in the range 400-600 nm. Other standards that can be used for wavelength calibration include the sharp emission lines in the UV/vis output of xenon, mercury and deuterium discharge lamps. Also, aromatic compounds such as benzene and acetone in the liquid or vapour form can be used for wavelength calibration as they give sharp absorption features.[8] Moreover, wavelength data resolution i.e. stepsize (SS) and spectral bandwidth (SBW) can significantly affect the quality of spectral data such as the fine structure required for protein structure characterisation. The work here will be focussed mainly on the effect of wavelength data resolution on spectral quality of simulated nucleic acid spectra, and proteins and amino acids using IgG2 and L-phenylalanine as examples. Aspects of quality assurance, artefacts and procedures associated with the measurements of circular dichroism data are also discussed in this work.

**MATERIALS AND METHODS**

**Materials**

L-phenylalanine, Tris buffer and Analar NaOH (0.1M) were purchased from BDH, England, UK, “in– house” distilled water at KCL. Stock solution of IgG2a (11.9 mg/ml), pH 5.5 was supplied by Pfizer Plc, Kent, UK. Sodium acetate trihydrate (CH₃COONa.3H₂O) Analar grade from Hopkin &Williams LTD, England, UK. The cells and the optical filters used in this study were specifically manufactured for circular dichroism measurements from Hellma GmbH & CO.KG, D-79-371, Mullheim, Germany.
Methods
Jasco 720 CD spectrometer from Jasco International Co., Tokyo, Japan and APL CD spectrometer, from Applied Photo Physics, England, UK were used in the study of the L-phenylalanine and the antibody solutions. The instruments was flushed continuously with nitrogen and allowed to warm up for half an hour before measurements were recorded. Absorbance spectra were obtained according to the fundamentals of simultaneous measurements of absorbance and CD as described by the authors.[7] Measurements were made mainly in two regions, near UV (350 – 240 nm) and far UV (240 – 190 nm) using 1.0 cm, 0.2 and 0.05 cm pathlengths as needed. An antibody solution of 0.5 mg/ml was prepared by dilution in 20.0 mM sodium acetate trihydrate buffer pH 5.5 (add titrant in an appropriate manner and mixing with minimal stirring to avoid mechanical aggregation of the IgG). L-phenylalanine was prepared in 50 mM Tris buffer and the pH of the solution was adjusted to pH 7.0 using small amounts of 0.1 M NaOH. The Gram 32/AI, the JASCO standard analysis, Origin 6.0, Excel 97, and Axum 6.0 software packages were used in data processing and analysis. Scanning parameters for L-phenylalanine are shown in table 1.

Table1: The instrumental parameters of the measurements of phenylalanine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan speed</td>
<td>10nm/min</td>
</tr>
<tr>
<td>Step size(SS)</td>
<td>0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 nm.</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>10.0 mdeg.</td>
</tr>
<tr>
<td>Band width(BW)</td>
<td>0.2, 0.5, 1.0, and 2.0 nm.</td>
</tr>
<tr>
<td>Wavelength range</td>
<td>300-240 nm</td>
</tr>
<tr>
<td>Accumulation</td>
<td>1.0</td>
</tr>
<tr>
<td>Response time</td>
<td>4.0 seconds</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSIONS
Wavelength calibration
By their nature, chiral molecules are relatively complex; the simplest example being a molecule having four different groups attached to a carbon atom with one of the groups carrying a chromophore. Accordingly, there are not many chiral molecules with spectral features sharp enough for wavelength calibration certainly at room temperature. Even fewer chiral molecules have vapour pressures high enough for gas phase spectra. These spectral features in CD spectroscopy are generally broad implies that a high precision in wavelength calibration may not be essential. However, there are several methods used and the reader can find them in the literature such as, emission lines from a mercury source.[9] and emission lines from a deuterium source.[10] which are primary methods and should be used when available.
Moreover, other methods using calibrated traceable Holmium & Didymium glasses\(^{11}\) and rare earth oxides in acid solution.\(^{12}\) In general, the wavelength calibration and the CD signal should be tested approximately every 300 hours of instrument use (or once every three months) to insure that the instrument is operating properly, and to check for signals of lamp aging.

**CD Calibration**

The accuracy of a polarisation modulation spectrometer depends critically on calibration. The achievement of accuracy and precision in analytical or spectroscopic data can be demonstrated by performing measurements that employ certified reference materials of known characteristics such as wavelength peak maxima position and/or absorbance value at that position. Standards can be primary, secondary or local laboratory based. CD is defined as the differential absorbance of left (\(A_L\)) and right circularly polarised light (\(A_R\)), therefore a primary standard implies the measurement of \(A_L\) and \(A_R\) separately and taking the difference. Values of \(\Delta A\) are very small and can approach \(10^{-5}\), making the absolute measurement of \(\Delta A\) exceedingly difficult. This has only been achieved directly for cobalt (III) tris-ethylenediamine (Coen) i.e. (+)-\(\Delta[-\text{Co (en}_3]\) Cl\(_3\) which has the advantage of having very strong positive CD at 498nm (\(\Delta c = 1.89\ \text{M}^{-1}\text{cm}^{-1}\)). Secondary standards usually offer the advantage of convenience and are based upon samples that have values often accepted simply based upon consensus. Thus, the wavelength accuracy and precision of the modulation polarisation spectrometer is achieved by measuring the spectrum of (+)–10d camphor sulphonic acid (Figure 1) as this standard is used for routine CD calibration.\(^{13-16}\)

![Figure 1: Absorbance and CD spectra of (+)–10d camphor sulphonic acid](image-url)
DATA RESOLUTION

Effect on CD spectra of IgG molecule

Data resolution (Stepsize, SS) describes the number of points required to define a spectral feature and is controlled by the spectral bandwidth of the spectral feature being measured. Generally, a smaller stepsize will in principle produce more faithful peak shapes.

The CD spectra in the near UV for a solution of 0.5 mg/ml of IgG measured in 1.0 cm cell and in the far UV measured in 0.05 cm cell are presented in Figure 2. The step size (SS) is fixed and the spectral bandwidth (SBW) is varied. Figure 2 a, b and c shows the effect of using 1, 2 and 5 nm SBW with 0.2, 0.5 and 1.0 nm respectively (the spectra were offset along the Y-axis for the purpose of comparison). Table 1 shows the effect of SBW and SS on the signal/noise ratio, the data of SBW at different SS values are plotted against S/N ratio (figure 3d). The SBW is often used to control the light throughput and to improve the S/N ratio, but the instrument resolution is reduced. Examination of Figure 3 clearly shows that the S/N ratio improves by increasing both SBW and SS. The plots in Figure 3d show a linear improvement of S/N with increasing SBW when using 0.2 nm SS. There is a deviation from linearity with increasing SS (0.5 and 1.0 nm). In general, an external drive moves the prisms to select for a given wavelength, starting with longer wavelengths and moving to shorter wavelengths. The step size of the external drive corresponds to a 0.05 nm change in wavelength, and this sets the limit to which wavelengths can be selected. On the other hand, at a given wavelength, the slit width will determine the bandwidth (wavelength distribution of the output) and the intensity. Thus, relatively wide slits give low wavelength resolution and high intensity, and thus, high signal to noise ratio. Conversely, narrow slits give sharp resolution and relatively lesser signal to noise (Table 2). Additionally, signal to noise level is proportional to the square root of the integration time, thus the simplest way to improve spectral quality is increasing the response time. However, in this case the speed of reading needs to be reduced to avoid distortions in the spectrum. For that, the speed of reading multiplied by the response time must be less than 0.33 nm (that is for a speed of 200 nm/min the response time has to be less than 0.1 s and for a speed of 50 nm/min less than 0.4 s).
Table 2 The effect of SBW and SS on signal-to-noise ratio

<table>
<thead>
<tr>
<th>Step size (SS) (nm)</th>
<th>SBW (nm)</th>
<th>Mean CD</th>
<th>STD</th>
<th>(S/N)_exp</th>
<th>(S/N)_rel</th>
<th>(S/N)_theor</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>14.4</td>
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<tr>
<td></td>
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</tr>
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<td></td>
<td>2</td>
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<td>0.16</td>
<td>34.5</td>
<td>1.9</td>
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<td>0.09</td>
<td>64.5</td>
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<td>5</td>
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<td>0.24</td>
<td>22.8</td>
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<td>5</td>
<td>5.2</td>
<td>0.14</td>
<td>37.1</td>
<td>3.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 2: Effect of spectral bandwidth (SBW) and step size (SS) on the CD spectra of IgG (d) Plots showing the effect of SBW and SS on S/N ratio

A theoretical DNA UV absorbance Study

In spectroscopy, most peak shapes can be described by a normal Gaussian curve. The mathematical model of a normal Gaussian curve is given by:

\[
f(x) = H e^{-\left(\frac{x-x_0}{W}\right)^2}(4\ln(2))
\]

Where \(f(x)\) is the observed signal height, \(H\) is the peak height, \(x\) is the wavelength, \(x_0\) is the peak maxima and \(W\) is the full width at half height (FWHH).

The DNA study was undertaken to theoretically determine the minimal number of data points required to faithfully produce a spectral feature. The measured UV spectrum of a DNA solution with \(A_{\text{max}}\) (258 nm) = 1.0 and the full width at half height (FWHH) = 44 nm. A
simulated DNA absorbance spectrum was produced by using equation (1) and setting the stepsize = 0.2 nm i.e. \((x-x_0) = 0.2\) nm. The DNA absorbance spectrum and the fitted Gaussian curve are shown in Figure 3, which shows a good match between the simulated DNA spectrum and the measured Gaussian curve. Reproducing the Gaussian fit with various data SS is presented in Figure 4a. To represent the “worse” case scenario, SS were taken equidistant on either side of the wavelength maximum \(\lambda_{\text{max}}\) at 258 nm. This represents the deterioration in \(A_{\text{max}}\) as a consequence of increasing SS, which is clearly apparent in the magnified part of figure 4a. Figure 4a indicates that a large stepsize deteriorates the Gaussian curve shape. However, setting \((x-x_0) = n/2\) in equation (1) where “n” is the data step size “SS” in nanometres produces the following expression:

\[
H' = H e^{-\frac{n^2}{2w^2}} - 4\ln 2
\]

where \(H'\) is the reduced height due to insufficient data points. The plot of peak height as a function of SS is shown in Figure 4b. It indicates.

Figure 3: Overlay of absorbance spectrum of a DNA and the simulated Gaussian curve.

Figure 4 (a): The effect of step size on the shape of the Gaussian curve (b) The distortion profile of the Gaussian peak integrity.
This plot confirms that a large SS results in the deterioration of peak shape and loss of fine structure. However, to reproduce H faithfully:

\[ e^{-\left(\frac{n}{2w}\right)^2 \cdot (4\ln 2)} = 1.0 \quad (3) \]

Here, a value of \( n \leq 2.0 \) produces a value \( \geq 0.9 \). The graphs shown in Figure 4 suggest that the formula \( SS = \text{FWHH}/20 \) is good enough to faithfully reproduce the spectroscopic data.

Amino acids (L- phenylalanine)

The spectra of L-phenylalanine were recorded to study the effect of SBW and SS on the spectral feature of amino acids. L-phenylalanine is chosen as an example because its CD spectrum has apparent fine structure between 270 and 240 nm, it has signals as a triplet at about 258, 264, and 268 nm which represent different vibrational levels of the excited state,\(^{[17]}\) that will easily signify the effect of using different values of SBW and/or SS. SBW values of 2.0 nm, 1.0 nm, 0.5 nm and 0.2 nm were employed with different SS values (0.1, 0.2, 0.5, 1.0, 2.0, 5.0 nm) in the measurement of L-phenylalanine and the spectra are shown in Figures 5, 6, 7 and 8 respectively. Analysis of the spectra clearly shows a loss of fine structure as evident of increasing SS.

![Figure 5](image)

**Figure 5:** Absorbance and CD spectra of L-phenylalanine in 1.0 cm cell and 2.0 nm SBW with different SS values (a) and (b) show the effect of using SS of 0.1, 0.2 and 0.5 nm on absorbance and CD spectra respectively (c) and (d) show the effect of using SS of 1.0, 2.0 and 5.0 nm on absorbance and CD spectra respectively.
A common feature of the simulated nucleic acid spectra and the measured L-phenylalanine spectra is that increasing the wavelength stepsize distorts the spectrum and can produce a reduced peak height i.e. loss of fine structure. Nevertheless, in terms of signal processing, the fewer the steps needed the longer is the time spent averaging the data at a particular wavelength and hence the lower the noise associated with the spectroscopic feature. A stepsize of 0.2 nm is an appropriate choice to faithfully produce the peak shape of the Gaussian curve.

Moreover, the use of the proper SBW with SS of 0.2 nm is particularly a good choice to avoid spectral distortion and produce the CD fine structure of L-phenylalanine as shown in figure 5b. Considering an analogue signal detection system, a spectroscopic feature has its own natural bandwidth, which in a sense describes its “sharpness”. Two factors are of particular importance; firstly, the instrumental spectral bandwidth must be small enough to avoid a distorted measurement of a spectral feature and yet not so narrow that the light throughput is unnecessarily reduced.

![Figure 6: Similarly, absorbance and CD spectra of L-phenylalanine in 1.0 cm cell and 1.0 nm SBW with different SS values.](image-url)
Figure 7: Similarly, absorbance and CD spectra of L-phenylalanine in 1.0 cm cell and 0.5 nm SBW with different SS values.

Figure 8: Similarly, absorbance and CD spectra of L-phenylalanine in 1.0 cm cell and 0.2 nm SBW with different SS values.

Clearly, smaller bandwidths are noisy, a bandwidth of 2.0 nm seems a good choice as it conserves the spectral features of the L-phenylalanine and provides better signal-to-noise ratio. The entrance and exit slits of a monochromator are normally set to be equal. From the produced spectra the effect of changing the bandwidth from 0.2 to 0.5 nm has little effects on the spectra. A generally accepted figure for the instrumental bandpass is that it should be 1/10 of the natural bandwidth of the measured spectral feature.\[18\] Unlike the natural bandwidth, the spectral bandwidth is set by the operator and is defined as the “spread” of monochromatic light leaving the monochromator or the prism and entering the sample. It is also defined as the band of wavelengths contained in the central half of the entire band passed by the exit slit.
of the monochromator. For example, an SBW = 1.0 nm implies that at 250 nm most of the light is spread between 249.5 nm and 250.5 nm. Secondly, the natural bandwidth is a property of the sample and is defined as the bandwidth at half the height of the sample absorption peak. The natural bandwidths of CD measurements are usually broad; the narrowest likely to be encountered in general work are those associated with the fine structure of L-phenylalanine which can give features with a wavelength width approaching 2.0 nm. For the vast majority of compounds an instrumental bandpass of 1.0 nm is therefore more than sufficient. However, in practice a 2.0 nm SBW giving 4 times more light and hence a two-fold improvement in signal-to-noise ratio is advantageous for many compounds (figure 2 and 5). In general, too narrow bandwidth results in small fraction of the light source to interact with the sample and hence high noise (figure 8). On the other hand, too wide spectral bandwidths are not good for spectroscopic measurements as they contain broad range of wavelengths that are poorly absorbed by the sample resulting in distortion and loss of fine structure as shown in figure 2. Empirical observations showed that the choice of slit width depends on the fine structure in the measured spectrum, the required light level, the optical train and the polarisation modulation constraints. Yet, in relation to the resolution, narrow slits are good for better resolution, but they reduce the signal-to-noise ratio and a compromise should be made.

The APL CD spectrometer measures a single data point every 25 microseconds. The instrument user needs to define the data resolution (stepsize) and decides how many 25 microseconds’ intervals to accumulate and average at every wavelength step. Therefore, the recorded signal is the average of the detector signal sampled every 25 microseconds at every wavelength data interval. The APL CD spectrometer has its own concept of recording data. This was investigated in order to explore its potential and to assess standard conditions. As a result of many measurements varying the data stepsize, the conversion table between the analogue and the digital approach has been drawn up and is represented in Table 3.

Table 3: Comparison of APL and JASCO J720 instrument settings.

<table>
<thead>
<tr>
<th>JASCO instrument settings</th>
<th>APL instrument settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ 10 nm/min, 4s TC× ] x 24 x 10× x SS</td>
<td>SS</td>
</tr>
<tr>
<td>1.0 nm</td>
<td>240 000</td>
</tr>
<tr>
<td>0.5 nm</td>
<td>120 000</td>
</tr>
<tr>
<td>0.2 nm</td>
<td>48 000</td>
</tr>
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<td>[ 20 nm/min, 4s TC ] x 12 x 10× x SS</td>
<td>SS</td>
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<tr>
<td>1.0 nm</td>
<td>120 000</td>
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</table>
CONCLUSION

So far, despite the importance of such parameters, there are no literature dedicated to the study of the effect of these parameters on the quality and accuracy of chiroptical spectroscopic data. The results obtained in this study clearly identify how critical and vitally important to control these parameters i.e. data resolution and spectral bandwidth, in order to obtain reliable CD data particularly in the area of biopharmaceuticals when monitoring therapeutic proteins such as IgGs. When studying biological molecules that have an ambiguous or unknown CD spectra, it is advised to study the effect of these parameters on the CD spectra before commencing experiments to determine the best choice that will produce good quality spectra typical for these molecules and ready for further analysis. Furthermore, it is important to understand the instrument used in the analysis and be able to relate and interpret spectral changes due to instrumental parameters to be able to differentiate between them and changes due to environmental effects e.g. pH and temperature. Also, the type of instruments used whether analogue or digital must be taken into consideration when interpreting the parameters used in spectral analysis. In summary, this article shows the examination of a number of parameters important for producing accurate and reproducible CD spectra. Moreover, methods for improving the accuracy and comparability of CD spectral magnitudes, fine structure and peak positions were presented. Hence, this paper provides practical guidance for "good practice" and quality control of the measurement of CD data.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES


