

Val-CiD Appendix A:
CD spectroscopy: an inter-
laboratory study

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Alex Knight, Stuart Windsor and Chris Jones

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2 Introduction

2.1 Context

This report is a result of part of the work done under the project titled “Development and Validation of Circular Dichroism Methods for the Quality Control of Biopharmaceuticals” (also known as “Val-CiD”) which was formulated in response to the Invitation to Tender (ITT) GBBK/C/019/00009, as part of the DTI’s first “Measurements for Biotechnology” programme. The project was led by the National Institute for Biological Standards and Control and delivered by a broad consortium of interested parties including the National Physical Laboratory, GlaxoSmithKline, Imperial College, Chiralabs, Elliot Scientific and Jasco. A number of linked documents have been produced as a result of this project, and this document has been designated Appendix A.

This report describes a UK-wide intercomparison study of CD measurement, which was performed as part of this project. Full details of the study design and methodology are presented, together with a summary of the results and their analysis. Conclusions drawn from this study have been incorporated into the accompanying good practice document.

2.2 Rationale

One of the important questions addressed in this project is the degree of comparability of CD data between different instruments: whilst it is important to be able to generate internally consistent data from any one CD instrument, it may often be necessary to make use of spectra obtained from different instruments in order to be able to detect differences during biopharmaceutical product development and testing. This may occur because new instruments are purchased to replace older ones, or because products are analysed at multiple sites; indeed, even minor upgrades or servicing of any one instrument can in effect turn it into a 'different' instrument in terms of the spectra it produces for a given sample.

If CD methods are to become pharmacopoeial methods, rather than release tests performed by a single manufacturer, they must be robust enough to be performed on a wide variety of different instruments in different locations. Ideally one would like to be able to make use of such data obtained on more than one CD instrument, without the data containing systematic errors as a result of the 'idiosyncrasies' of each instrument or associated equipment (e.g. sample cells), or indeed operator variation.

In an effort to better understand the extent to which comparable data can be obtained, an inter-laboratory study has been carried out, in which identical samples were sent to multiple laboratories around the UK (and two overseas), to be run on their CD instrument(s). Participants included academic, regulatory, industrial and contract-service laboratories (Table 2.1). Data was analysed to determine the extent of the systematic errors, with a view to determining where these arise and developing methods to minimise them, so as to improve inter-laboratory comparability and confidence in the use of CD spectroscopy in biopharmaceutical product characterisation.

2.3 Approach

2.3.1 Study Participants

Contact; experimental workers	Organisation
Professor George E. Tranter Dr. Alison A. Edwards	Imperial College London
Dr. Andrew Leech	University of York
Dr. Mark S. B. McAlister	AstraZeneca R&D Alderley Park, Macclesfield
Dr. Alison Rodger	University of Warwick
Dr. Stuart Windsor Miss Rachel Yardley Mr. David Butterfield Dr. David Schiffmann	National Physical Laboratory, Teddington
Dr. Chris Jones	National Institute for Biological Standards and Control
Dr. Sharon M. Kelly	University of Glasgow
Dr. Stephen Martin	National Institute for Medical Research, London
Dr. Carl Jone Dr. Anna Hills	GlaxoSmithKline, Beckenham
Professor Bonnie A. Wallace	Birkbeck College, University of London
Professor Jeremy Lakey Helen Ridley	University of Newcastle
Rachel Marrington ¹	Syngenta, Jealott's Hill International Research Centre, Bracknell, Berks.
Dr. Alex F. Drake Dr. Tam Bui	King's College London
Dr. Marcia Holden Dr. James Noble ²	National Institute of Standards and Technology, Gaithersburg, Maryland, USA
Dr. Andrew J. Doig Teuku Iqbalsyah	University of Manchester Institute of Science and Technology (UMIST)
Mr. Kevin Howland	University of Kent
Dr. Robert W. Janes Farah O'Boyle Dr. Alison L. Cuff	Queen Mary, University of London
Dr. Mike Ridout	Institute of Food Research, Norwich
Dr. Yves Dupont	Laboratory of Molecular and Cellular Biophysics, CNRS UMR-5090, CEA Grenoble, France

Table 2.1 Participants in the intercomparison study.

The laboratories that participated in the study are listed in Table 2.1. The contact for each laboratory is listed, followed by the experimental workers, where known. All

¹ University of Warwick

² National Physical Laboratory, Teddington, UK

instruments used were anonymously assigned a reference letter, which is used throughout the report. A few laboratories used more than one instrument.

2.3.2 Proteins

Three proteins were chosen to be used in this study. The criteria were that the proteins were:

- readily commercially available
- not too expensive (since relatively large quantities were to be made up)
- sufficiently different from one another structurally, so as to provide clearly different CD spectra
- not requiring any unusual buffers (so that a common buffer could be used)
- not expected to generate significantly temperature- or pH-dependent CD spectra
- of moderate to high stability, to minimise degradation during the course of the study.
- non-toxic (to facilitate sending them by post).

The proteins selected for this study are shown in Table 2.2 below. Participants were not told the identity of the proteins, which were referred to only by a letter code: A, B, or C.

<i>Code</i>	<i>Name</i>	<i>Origin</i>	<i>Product Code³</i>	<i>Lot</i>	<i>Concentration (mg/ml)</i>	<i>Biological function</i>
A	α (1)-acid glycoprotein (AGP, orosomucoid)	bovine	G3643	051K7440	1.3	Serum protein – levels vary with stress response or certain diseases
B	lysozyme	chicken egg white	L7651	091K7021	1	Enzyme with anti-bacterial action
C	cytochrome C	equine heart	C2506	052K7015	1.3	Necessary for mitochondrial energy production

Table 2.2 Summary of the proteins used for the inter-laboratory CD comparison.

In order to minimise variation due to sample preparation, a single large batch of each of the three proteins was prepared at NPL and immediately divided into aliquots for use at participating laboratories. To prevent growth of microbial contaminants during the course of the study, each batch was prepared and aliquoted aseptically and stored in a refrigerator when not in use.

Each protein solution was prepared in a 30 mM sodium phosphate buffer, pH 7.20, to minimise fluctuations in pH and maintain protein stability. Proteins A and C were prepared at approximately 1.3 mg/ml, and protein B at 1 mg/ml. These concentrations were chosen so as to enable the same aliquots to be used for both far-UV and near-UV/visible CD measurements, thereby simplifying the protocol and minimising the total number of samples for circulation and re-testing. Some compromise was therefore necessary in choosing the concentration for proteins B and C, as it could not

³ All proteins were purchased from Sigma-Aldrich.

simultaneously be optimal for all spectral regions, but this did not pose a major problem. (Protein A was only measured in the far-UV region, see Table 2.3.)

A sample from each of the three proteins was analysed for purity using polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE), which indicated the material was of satisfactory purity. To make baseline measurements, aliquots of water were also sent out with each set of proteins⁴.

For all protein samples where results are shown in section 4.2, samples were re-measured at NPL upon their return from participating laboratories, to verify that no significant changes had occurred to the sample (e.g. large protein concentration changes, degradation or contamination) that could potentially, in and of themselves, explain at least part of any differences seen between instruments.

2.3.3 Calibration

At the outset of this study, it was anticipated that a significant difference between instruments could be due to variations in their calibration state. This could reflect differences in the ammonium-*d*-10-camphorsulfonate (ACS) solutions used to calibrate the instruments (e.g. errors in concentration), drifts in instrument calibration, or other errors in the calibration process. Therefore it was necessary to measure and compare the calibration states of all the instruments used in this study.

To this end, a solution of ACS was prepared at a concentration known only to the organisers, as well as a 10-fold dilution thereof, and water for baseline measurements. These samples are referred to as ACS(A), ACS(B) and ACS(W) respectively. The ACS(A) solution was prepared gravimetrically from ACS powder (Katayama Chemical Co., Japan, via Jasco (UK) Ltd), and filter-sterilised through a 0.22 μm filter. The concentration⁵ was verified by UV absorbance using the literature extinction coefficient for ACS⁶.

Chemical purity of the ACS powder (from three different bottles) was analysed by one-dimensional proton NMR spectroscopy, which indicated it was ~99.4% pure. Using this same powder, another solution of ACS was made, and the extinction coefficient was measured using a reference UV-VIS spectrometer at NPL; this was consistent with the results of [1].

The calibration of the NPL instrument was checked regularly using a set of three 1 cm cylindrical screw-top sealed cells, referred to as 'S', 'T' and 'V', containing ACS(A), ACS(B) and ACS(W) respectively. A number of participating laboratories were sent this or another such set of sealed cells, but in most cases calibration measurements were made using *aliquots* of these samples together with one of a number of cells of 1 cm path length, which participants filled themselves.

2.3.4 Experimental protocol

Aside from the samples to measure, each participating laboratory received a detailed experimental protocol. Scans were carried out at normal room temperature, or at a set

⁴Although this is of course not the same as the sodium phosphate buffer in which the proteins were prepared, it is sufficient for the purpose of obtaining CD baselines for this comparative study.

⁵The concentration used was 0.0670%, slightly above the 0.0600% w/v commonly used for calibration.

⁶ 34.3 M⁻¹ cm⁻¹ at 285.5 nm, (Takakuwa et al., 1985).

temperature of 20 °C, depending on the circumstances. Samples were then returned to NPL for re-measurement. To facilitate inter-instrument comparisons, all participating laboratories were asked to record spectra using the common scan parameters provided below or – where this was not feasible – parameters as close to them as possible. Labs that wished to, in addition, run the samples using their own protocol were encouraged to do so. Users of non-Jasco instruments adapted the parameters with the aim of obtaining scans of broadly similar quality. For example, Aviv instruments were run using: 0.2 nm interval, 0.33 sec. settling time, 1 sec. averaging time, 1 nm bandwidth, and 1 - 3 scans depending on the sample.

Participating laboratories were asked to measure the three proteins in different wavelength ranges (using the appropriate path length of cell), as shown in Table 2.3. The scan parameters recommended for the proteins are given in Table 2.4, as specified for Jasco instruments.

<i>Spectral region</i>		<i>Far UV</i>	<i>Near-UV</i>	<i>Visible</i>
<i>Cell Path Length (mm)</i>		<i>0.1</i>	<i>10</i>	<i>10</i>
<i>Protein sample</i>	A	YES	NO	NO
	B	YES	YES	NO
	C	YES	YES	YES

Table 2.3 Wavelength ranges and cell path lengths for each protein in the inter-lab comparison.

<i>Parameter</i>	<i>ACS</i>		<i>Proteins</i>		
	<i>A</i>	<i>B, W</i>	<i>Far-UV</i>	<i>Near-UV</i>	<i>Visible</i>
<i>Scan speed (nm /min)</i>	50				
<i>Scan range (nm)</i>	200 – 400	180 – 400	180 – 260	240 – 320 ⁷	430 – 600 ⁸
<i>Data pitch (nm)</i>	0.1 ⁹				
<i>Response time (s)</i>	1				
<i>Bandwidth (nm)</i>	1				
<i>No. of accumulations</i>	1		6	6	2

Table 2.4 Parameters specified for use in inter-laboratory study (for Jasco instruments).

2.3.5 Instruments used

As seen in Figure 2.1, the majority of the instruments used in this study were manufactured by Jasco, reflecting the distribution of instruments in current use in the

⁷ Near-UV scans were from 240 to 320 nm, although some were extended to 360 nm, which in the protein B spectra can help one to detect baseline drift. All analysis of the data was performed on the data from 240 to 320 nm.

⁸ Scans were extended to 720 nm, if instrument and time permitted.

⁹ Although the majority of the instruments recorded spectra at a resolution of 0.1 nm, some spectra were recorded at a lower resolution, for a variety of technical reasons. The baseline-corrected protein spectra were converted by linear interpolation to a resolution of 0.1 nm, in order to generate datasets suitable for further mathematical processing, analysis and plotting.

UK. However, instruments from other manufacturers are also represented. We have not attempted to break the analysis down according to manufacturer.

Models of CD instrument used in study

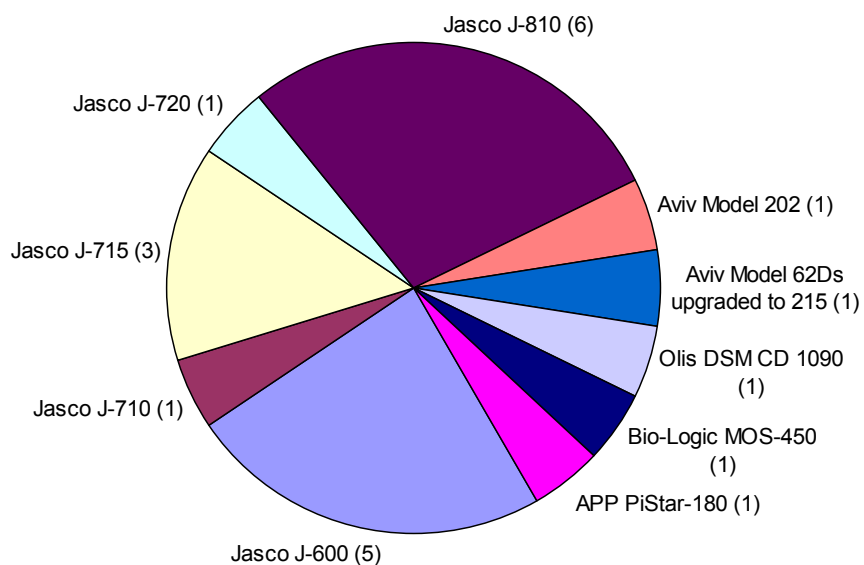


Figure 2.1 The instruments used in this inter-laboratory comparison (figures in brackets indicate the number of each type of instrument).

3 Processing of data from participating laboratories

3.1 Initial processing

Data provided by participating laboratories was imported into Microsoft Excel spreadsheets, where it was processed and plotted. All CD data was kept in units of millidegrees (mdeg). Initial processing steps included averaging over several scans and baseline subtraction, where these had not been performed already.

3.2 ACS spectra

The ACS spectra were analysed to determine the position of the peaks within the spectra, in terms of both wavelength and CD signal intensity (ellipticity). ACS(A) spectra were used to determine the position of the positive peak around 290.5 nm, this being the peak most commonly used to calibrate CD instruments. ACS(B) spectra were used to determine the ratio between this peak and the negative peak around 192.5 nm; this ratio can give insights into the state of the instrument (although there is some disagreement as to what this value should be – see section 4.1.2).

In several cases, it was difficult to reliably assign the peak position of the negative peak around 192.5 nm due to the noise in this part of the spectrum; in these cases, an estimate of the peak position *was* attempted but the measurement was flagged as having a 'poor peak shape', i.e. the ratio determined in these cases should be viewed as having significantly greater error than the others.

The results of this analysis give an indication of the variation in the calibration state of the instruments, and were in turn used to normalize the protein spectra, in the hope of reducing the variation between the spectra recorded on different instruments.

3.3 Normalization of protein spectra for instrument calibration

Part of the differences seen between spectra may reasonably be expected to be due to the calibration state of the instruments. Hence for each instrument used in the study, a common ACS solution (using aliquots, or ready-filled sealed cells) was measured along with the proteins in order to enable spectra from that instrument to be normalized to account for the instrument's calibration state; it was hoped that this would reduce the variation between spectra obtained on different instruments.

In order to compare the calibration state of each instrument, it is important to ensure that, as far as possible, the variation in measured ACS peak values comes from instrumental variation rather than the sample cells or the ACS solution itself.

Variation due to the ACS solution was controlled for by re-measuring the ACS solution on the NPL instrument after its return from participating laboratories. Variation due to sample cell could have come about because not all labs used the same cells for these ACS measurements, and so their path lengths could have varied. This was controlled by measuring the path lengths of these cells¹⁰.

¹⁰ This was expected to be small compared to the differences in ACS peak positions, given the stated manufacturing tolerance for 1 cm cells, which is only ± 0.1 % of the path length.

For this purpose, path lengths were compared by measurement of CD spectra of identical ACS solutions in each cell, and comparison of peak magnitudes. Whilst this method did not give *absolute* path lengths, for the purposes of this inter-laboratory study all that was required was the *relative* path lengths.

These measurements were performed using aliquots of ACS(A) and the NPL CD instrument. Cell 'S' (see section 2.3.3) was chosen as the reference cell for this purpose and therefore was taken to have a relative path length of 1. Repeat measurements of the CD signal for cell 'S' were averaged to give a reference ellipticity, θ_0 . Each cell was then filled with ACS(A) and the CD signal measured (using four accumulations, i.e. four scans); the cell was then emptied and re-filled, to give a total of three spectra; these were averaged to give the measured ellipticity, θ_m . The relative path length is then given by:

$$l_r = \frac{\theta_m}{\theta_0}$$

As mentioned in section 3.2, each participant spectrum of ACS(A) (which gives an indication of each instrument's calibration status) was analysed to determine the magnitude of the positive peak, θ_p . This was then corrected to account for the relative path length of the cell used by that laboratory, and an overall calibration factor, F , was calculated to normalise the participant's protein measurement data:

$$F = \frac{\theta_0}{(\theta_p / l_r)} = \frac{\theta_0 \theta_m}{\theta_p \theta_0} = \frac{\theta_m}{\theta_p}$$

To obtain the ACS-normalised CD signal from the protein data, the participant's 'raw' protein CD signal was multiplied by the participant-specific normalisation factor, F .

4 Results and Analysis

4.1 *Instrument calibration – comparison of ACS CD spectra*

4.1.1 'Positive-peak' analysis

As discussed earlier, an important aim of this project was to compare the state of calibration of CD instruments. The most significant calibrations of CD instruments are for wavelength and CD “intensity” (ellipticity). These calibrations may also be non-linear, in that calibration of ellipticity or wavelength may vary with wavelength, for example [2-4]. However, in order to simplify the work required of each laboratory and make it easily applicable to all instruments, a single, straightforward method of examining calibration was chosen – comparison of the CD spectra of solutions of ACS, the chemical standard most commonly used for setting and assessing calibration of CD spectrometers. By analysing these spectra, it is possible to identify inter-instrument differences in CD intensity calibration and wavelength calibration.

The most direct way to use such data is to determine the wavelength and height of the positive peak located at around 290.5 nm in each spectrum. The height of the peak at 290.5 nm is an indicator of the calibration setting of the instrument, such that – broadly speaking – the higher the ACS peak at 290.5 nm, the greater will be the magnitude (for positive and negative peaks) of any CD spectrum obtained with that instrument¹¹.

To this end, aliquots of the ACS(A) solution were sent to participating laboratories. The baseline-subtracted spectra measured on each instrument (Figure 4.1) were analysed by determining the peak position in each case, the results of which are shown in the right-hand-side of Figure 4.2. The calibration of the NPL instrument was checked frequently throughout the course of study using a set of sealed sample cells ('S', 'T' and 'V' – see section 2.3.3) containing the same ACS solution as that used by the participating laboratories, the results of which are shown in the left-hand part of Figure 4.2.

¹¹ Although, as Miles et al. (2003) showed, the actual factor by which any two instruments' CD spectra vary may not be constant across all wavelength regions.

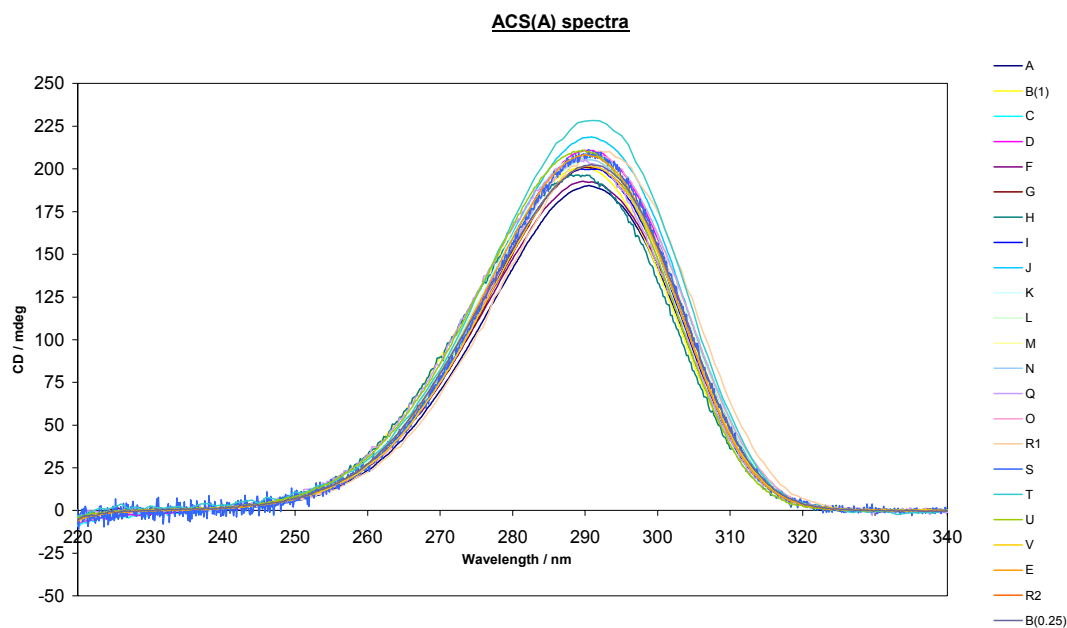


Figure 4.1 The baseline-corrected ACS(A) spectra from participants, together with a representative spectrum from the repeat measurements on the NPL instrument (E)¹².

¹² In this and subsequent plots of participant data, B(1) & B(0.25) refer to spectra obtained using two different scan parameters for instrument B (discussed further in section 3.5.1). R1 and R2 refer to different scan parameters for instrument R: R1 spectra were collected using a scan protocol as near as possible to that specified in the protocol sent to participants; R2 was collected using the scan protocol commonly used for this instrument, which gave less noisy data.

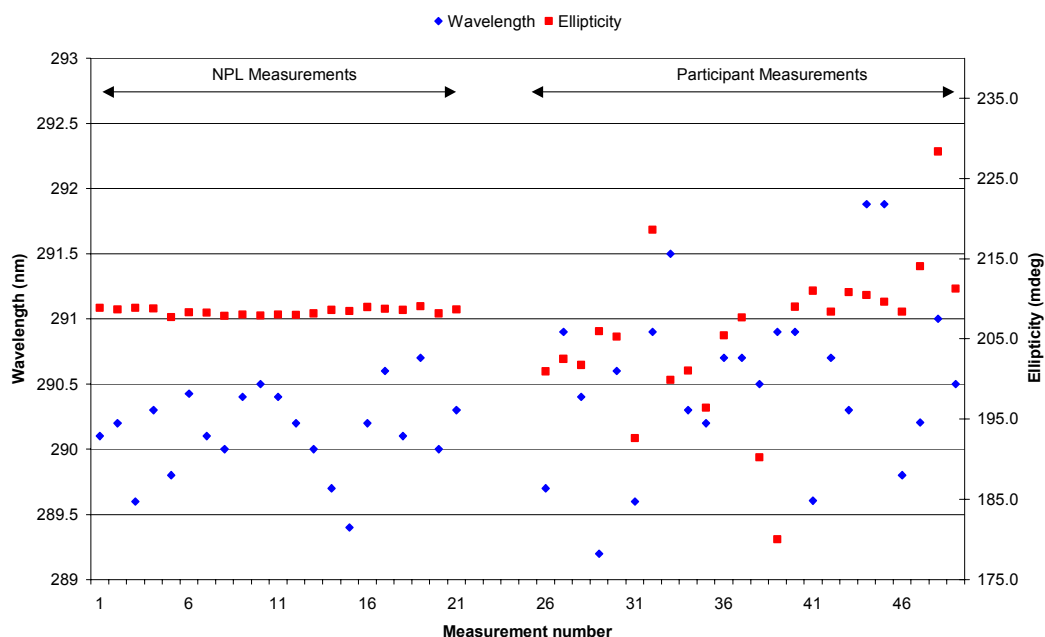


Figure 4.2 Plot of the position (wavelength and ellipticity) of the positive ACS peak for repeat measurements of ACS(A) at NPL, and for participant measurements.

The peak positions shown have not been adjusted for path length differences between the different 10 mm cells used; however, the variation in signal seen here is several times greater than the differences in path length, i.e. it cannot be ascribed simply to differences in path length. If samples had not warmed up sufficiently from the refrigerator, temperature differences could also play a part. There was one slight 'outlier' in the NPL series of measurements, which has not been plotted here.

4.1.2 Peak Ratio Analysis

In addition to ACS(A), aliquots of a 10-fold lower concentration of ACS, referred to as ACS(B), were also measured by participants. This enabled the position of the negative peak around 192.5 nm to be measured, and the ratio between the magnitude of this peak and the positive peak around 290.5 nm to be determined. Measurement of this ratio can help indicate if an instrument is operating correctly, and indeed is often used to check calibration of an instrument in place of the measurement of individual peaks [5]. ACS(B) spectra can be seen in Figure 4.3, and the results of peak analysis are shown in Figure 4.4; Figure 4.5 shows the values of the ACS peak ratios for the different instruments.

These results show a relatively large range in the values of this ratio (even when ignoring those ratios that are unreliable due to noise), suggesting that inter-instrument differences may be more marked than what one may conclude based solely on consideration of peaks round 290.5 nm.

Three ACS(B) spectra are clear outliers, as indicated in Figure 4.3. It is not totally clear why these have occurred. However, re-measurement of ACS(B) on instrument O at a later date using a different cell gave a 'normal' spectrum, suggesting there may have been a defect in the original cell. This is reinforced by the ACS(A) data obtained

using this same cell, which gave a peak magnitude well below what was expected (data not shown¹³). If the original cell used was indeed defective somehow, this may explain, at least in part, why unusual results were seen for the *protein* CD spectra from this instrument in the near-UV and visible regions, where the same cell was used (section 4.2).

Although there is some disagreement about the precise value to be expected, it should be around 2 – a value of 2.1 is generally deemed to be good, whereas a value significantly below or above 2 (e.g. 1.90 or 2.30) might suggest a problem with the instrument (A. Rodger, pers. comm.). Reported literature values range from 1.90 to 2.20 (cited in [5]). However, because it is quite possible for the ratio to be within an acceptable range but the individual peak intensities to be unacceptable, it is important not to rely solely on the peak ratio when calibrating CD instruments [5].

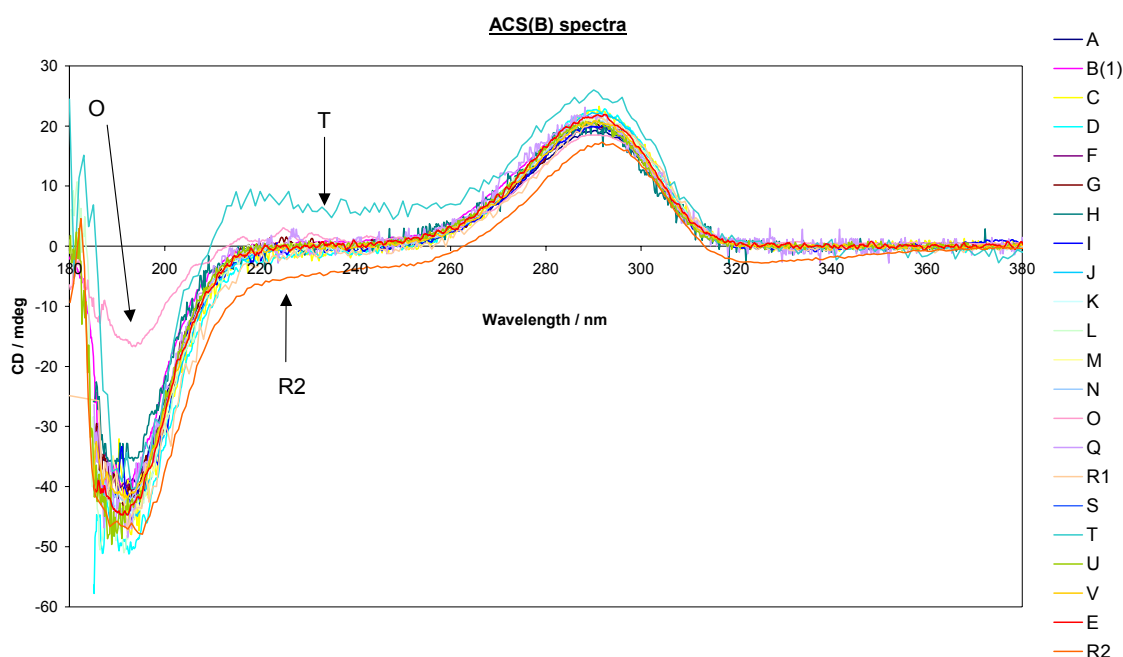


Figure 4.3 The baseline-corrected ACS(B) spectra from participants, together with a representative spectrum from the repeat measurements on the NPL instrument (E).

Most spectra are of similar shape and size, although there is some variation in magnitude; this may in part be due to slight concentration differences between the ACS(B) aliquots used, but this is not of concern, as the primary purpose of this analysis was the determination of the peak ratio. Unusual spectra are marked with arrows. Note that instrument R gave a normal spectrum when the alternative scanning mode was used (R1).

¹³ The ACS(A) spectrum was re-measured using a different cell, which gave more typical data, and it is this which is shown in Figure 4.1; both are plotted in Figure 4.2.

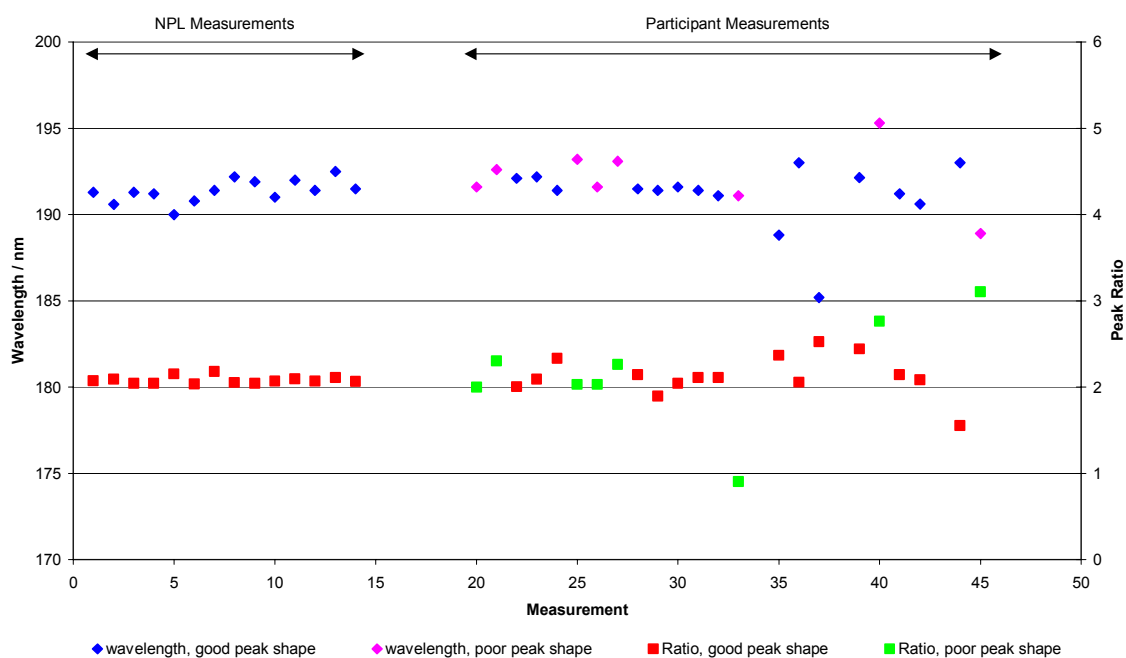


Figure 4.4 Plot showing the wavelength of the negative ACS peak, and the ratio of the magnitudes of the negative and positive CD peaks, from repeat measurements of ACS(B) at NPL, and for participant measurements.

Data points with poor peak shapes are highlighted.

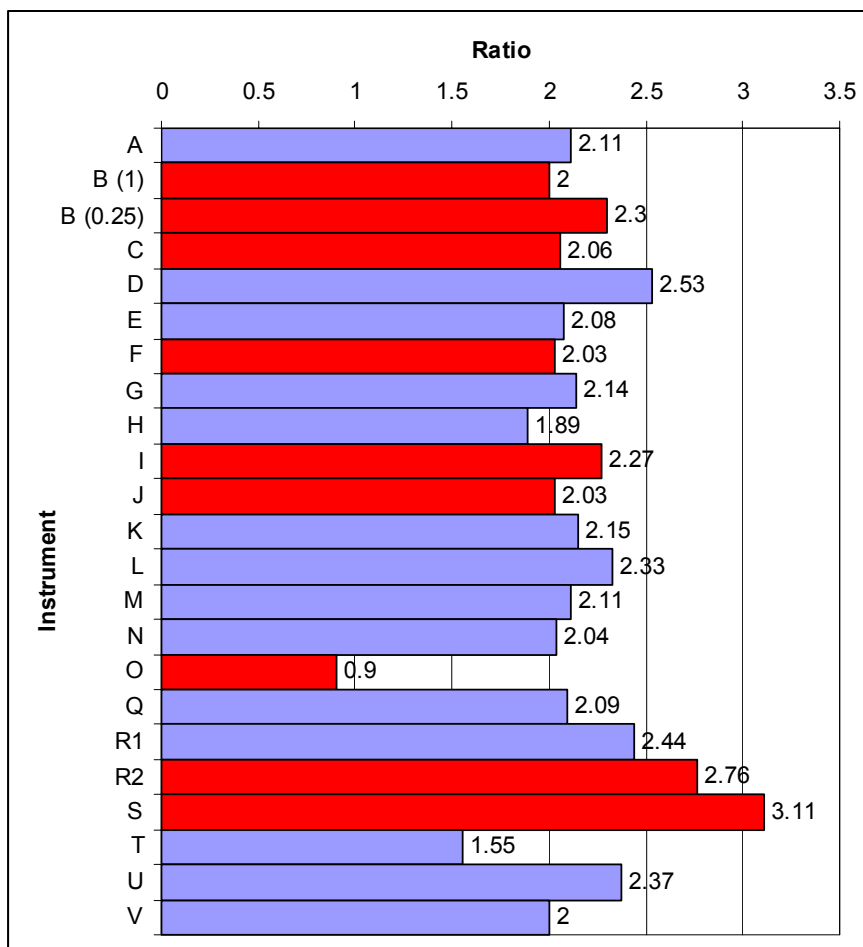


Figure 4.5. Summary of ACS two-peak ratios.

Values in red indicate unreliable measurements due to excessive noise around the negative peak. E is the result of 13 measurements at NPL.

4.1.3 Sample integrity

In all but one case, ACS(A) and (B) samples were re-measured at NPL upon their return from participating laboratories, in order to rule out sample contamination/degradation as a cause of variation. The same scan procedure was followed, and the same cell used, as in the participating laboratories. The sealed cells used by a number of the participating laboratories were also re-measured at NPL. These results for ACS(A) are summarised in Figure 4.6, which shows that all ACS(A) samples measured this way appeared to have maintained their integrity despite having been measured and transported.

Similarly, no unusual results were found for the returned ACS(B) samples (data not shown), indicating they had not degraded, etc., except for the sample returned from instrument M, which showed lower-than-normal positive and negative peak ellipticities; however, as the ACS(B) spectrum obtained from this instrument itself appeared normal, it is likely this was just an 'outlier', and is not of concern.

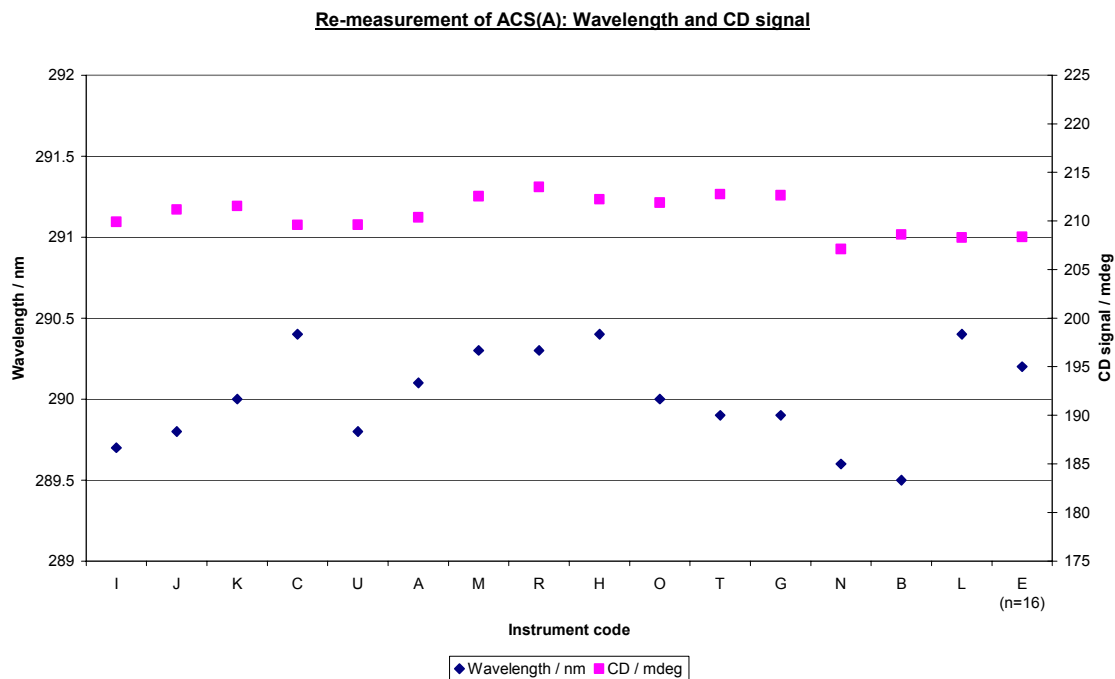


Figure 4.6 Re-measurement at NPL of ACS(A) samples after their measurement by participating laboratories and return to NPL.

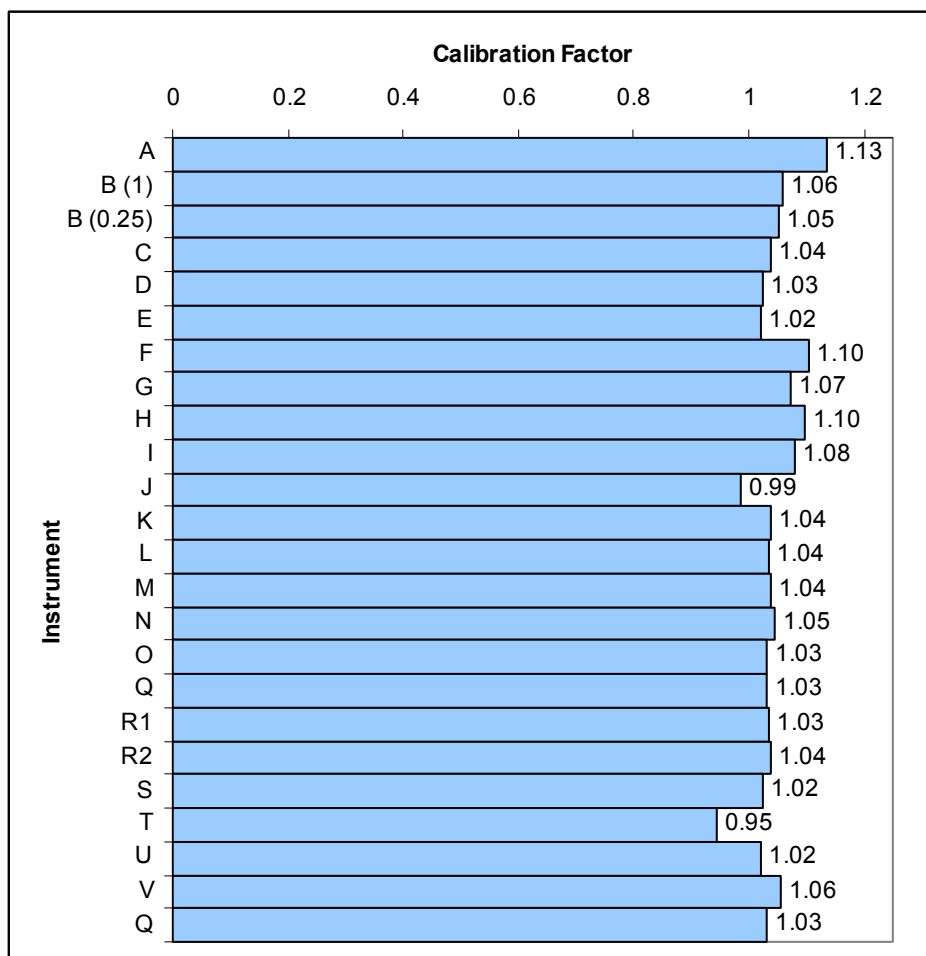


Figure 4.7 Calibration factors derived from ACS and path-length measurements, as described in section 3.3).

Note the relatively narrow range of variation (standard deviation 0.045).

4.1.4 Curve fitting

Finally, an alternative approach to analysing the ACS spectra is to perform curve fitting to the ACS spectra around the peak area. Although this introduces extra steps in the analysis, in principle it could improve the comparison and correction of calibration differences between instruments, because such differences will then be a function of a very large number of data points around the peak, rather than just one point per spectrum, which should reduce the problems of noise and the relative broadness of the peak, i.e. it could increase the 'robustness' of the use of ACS as a measure of calibration. Figure 4.8 shows the results of such an analysis on the NPL repeat measurements. Gaussian functions were fitted to the ACS(A) spectra in the region 280-300 nm and compared with the results obtained from standard peak analysis; this demonstrates that the fitted wavelength values are much less noisy than those obtained by peak position alone.

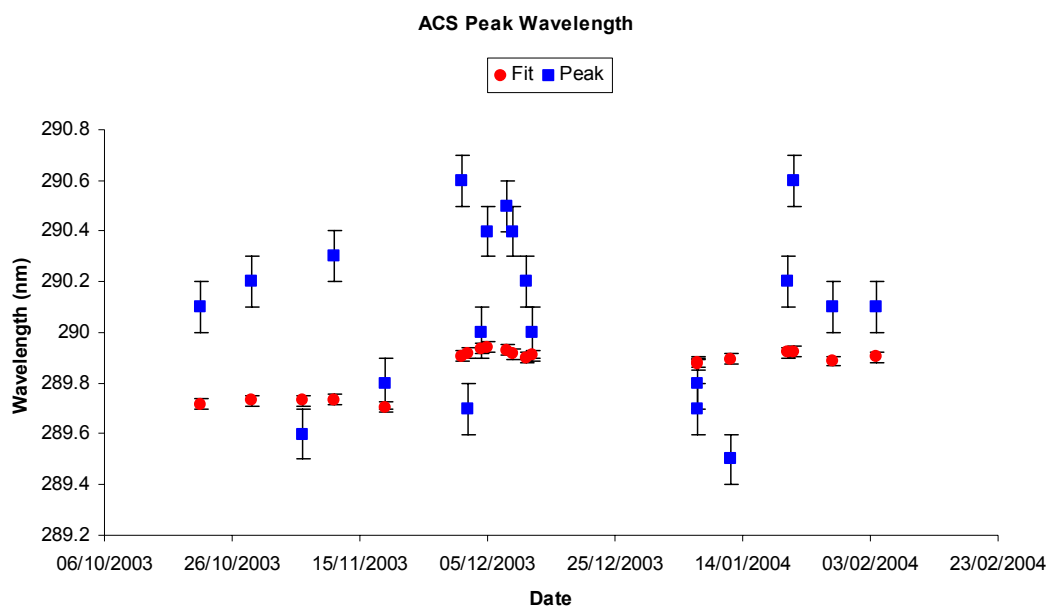


Figure 4.8 Results of applying Gaussian curve fitting to the NPL repeat measurements of ACS(A) in the sealed cells.

Note the improved consistency of these results ('fit', red) compared with those from peak analysis ('peak', blue). A small (0.2 nm) wavelength shift associated with an instrument repair is also visible here. Error bars are 95% confidence limits for the curve fitting; for the peak values they indicate the data interval (± 0.1 nm).

4.1.5 Conclusions

All ACS(A) spectra from participating laboratories were of broadly the same shape and magnitude (considering only the data from ~ 220 nm and upwards). However, there were some small wavelength and scaling differences between them, likely due mostly to differences in instrument calibration. Most ACS(B) spectra were also of broadly similar shape and magnitude, although there was a small number of clear outliers.

The inter-instrument variation in CD intensity calibration setting, as judged by the CD signal at the positive ACS peak around 290.5 nm, was smaller than expected at the outset: the results from the majority of instruments fell into a relatively narrow range (see Figure 4.7). Wavelength calibration, as judged by the wavelength of the ACS positive peak, was very similar for all instruments.

Nonetheless, significant differences between the calibration of instruments *were* seen; these most likely arose due to operator variation or error in the preparation of the ACS calibration solution. This highlights some of the current difficulties of calibrating CD instruments and the need for an approach that does not require chemical calibrants.

4.2 Protein spectra

For each of the spectral regions measured for each protein, three sets of plots are shown in the following sections:

1. An overlay of the unprocessed, baseline-corrected spectra from the participating laboratories, and a representative spectrum measured at NPL.
2. An overlay of the repeat measurements at NPL on the samples returned from the participating laboratories.

3. An example of the results of processing the spectra using an iterative spectral alignment method (ISAM).

For each spectral region, a histogram summarises the results of the ISAM analysis. For more details on this analysis method, please see the accompanying Appendix C.

Together, this data allows one to see the problems that can be encountered in trying to obtain comparable spectra using different instruments.

4.2.1 Far UV Spectra

Spectra in the far UV or “secondary-structure region” (180-260 nm) are often used to obtain *a priori* secondary structure information through spectral deconvolution. Accurate data are important because the results of such a deconvolution depend on the absolute value of the CD, as well as spectral shape. This region is also of interest for biopharmaceutical characterization, as it provides a useful check on the overall structural integrity from batch to batch. Although it is not typically as sensitive to structural variation as near-UV spectra, far-UV spectra have the advantage that they are generally quicker to acquire (as fewer repeat scans are needed for an equivalent signal-to-noise ratio). Furthermore, the short path lengths used may in some cases facilitate measurement of solutions that would otherwise absorb too highly; for example, although it may not be an optimal situation for CD study, one may sometimes have to measure samples at high protein concentration if that is how they are manufactured and stored.

4.2.1.1 Protein A

All three proteins were measured in the far-UV region. Protein A spectra from participant instruments, together with a representative spectrum measured on the NPL instrument, are shown in Figure 4.9. All CD spectra are broadly the same shape and there are no clear outliers, i.e. unusual spectra in terms of shape or position. However, the spectra do differ in their magnitude and, to a certain extent, in their position with respect to the wavelength axis. The noise level of the spectra varies somewhat too.

All samples were re-measured on the NPL CD instrument after their return from participating laboratories, in order to identify possible changes to the sample; CD spectra from these measurements are shown in Figure 4.10. Note the good level of consistency between spectra; with almost no exceptions, spectra are similar from sample to sample across the entire far-UV wavelength range studied. This shows that the principal differences seen in the inter-laboratory measurements are not simply due to sample differences, e.g. protein degradation or concentration differences between samples.

In order to further compare the spectra from the different instruments, they were processed using the iterative spectral alignment method (ISAM) developed as part of this project (discussed Appendix C). An example is shown in Figure 4.11, and results for all spectra are shown in Figure 4.12. In this method, a reference spectrum is chosen (here, the NPL spectrum was used) and the program sequentially attempts to align other of the other 'test' spectra with this reference spectrum so that differences between them are reduced; it achieves this by altering the scaling of each spectrum and its position along the wavelength axis, until the best possible alignment is obtained, in a 'least-squares' sense.

By removing (and quantifying) the 'unwanted' differences in scaling and wavelength offset, it helps one visualise how similar the 'underlying' spectra are in terms of their

overall *shape*. Thus, it demonstrates to what extent one could obtain better comparability between spectra if one could minimise such 'unwanted' differences, which is one of the aims of this project – these differences are likely to be due to factors such as instrument calibration and cell path length. Comparison of Figure 4.9 with Figure 4.12 shows that the spectra are significantly better aligned after processing with ISAM, i.e. there is less difference between spectra after processing (noise levels in any one spectrum are not affected by this method).

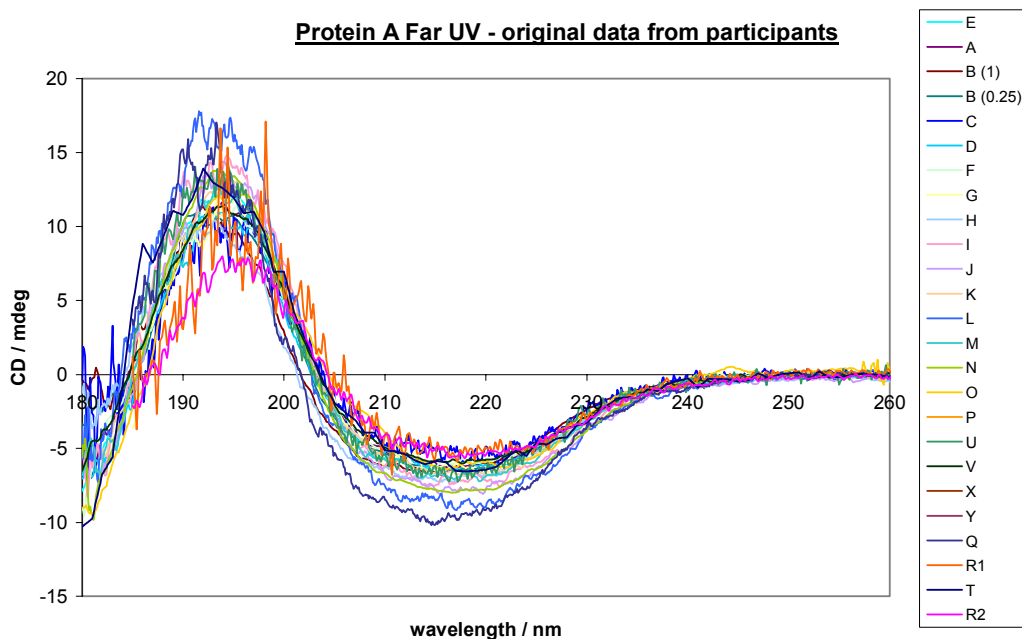


Figure 4.9 Far-UV CD spectra of Protein A.

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL¹⁴.

¹⁴ While most spectra were collected using 0.1 mm cells, for technical reasons far-UV measurements (for all 3 proteins) with instrument G were made using (nominally) 1 mm cells, together with samples that had been diluted by approximately 10-fold. Spectra were scaled appropriately to make them comparable with the others. Similarly, instrument R1/R2 far-UV spectra were recorded using (nominally) 0.2 mm cells, and were then scaled by a factor of 0.5.

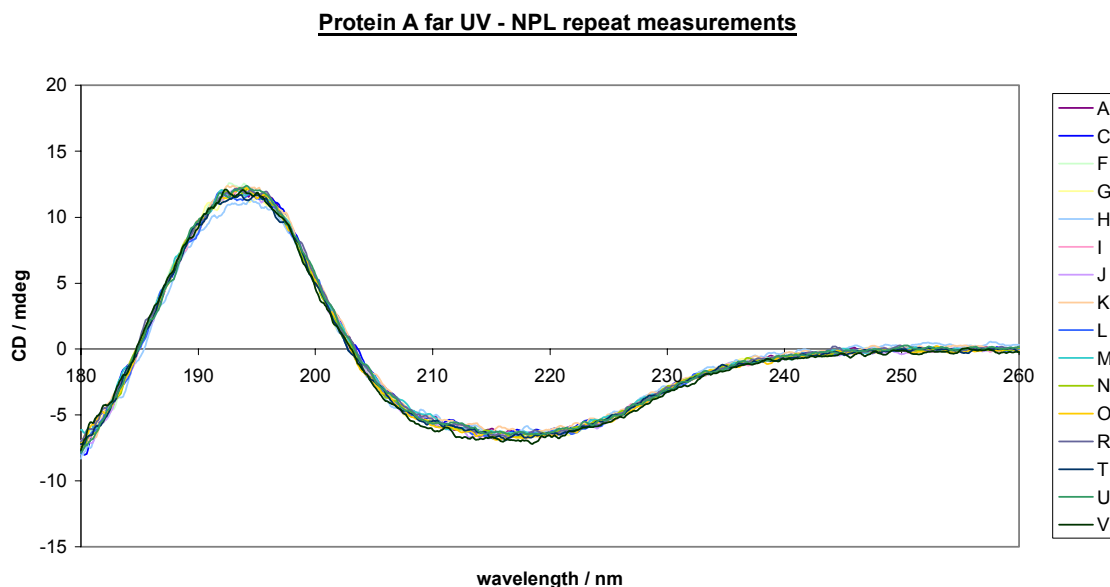


Figure 4.10 Far-UV CD spectra of Protein A.

Repeat NPL measurements of 16 samples returned from participating laboratories. Note the good level of consistency between almost all spectra.

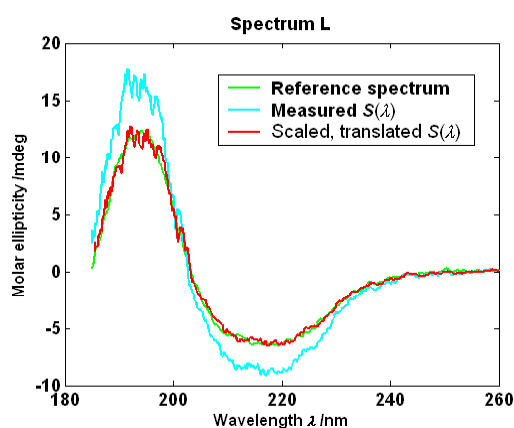


Figure 4.11 An example of the alignment of CD spectra in ISAM, shown here for protein A far-UV spectra.

The NPL spectrum, taken as the reference, is shown in green. The original spectrum obtained from instrument L is shown in cyan; it is clearly distinct from the reference spectrum. By applying scaling and translation factors that give the closest match to the reference, the 'optimally aligned' spectrum is arrived upon, shown in red. The close match now existing between red and green spectra shows the improvement in alignment obtained from applying this technique to this pair of spectra.

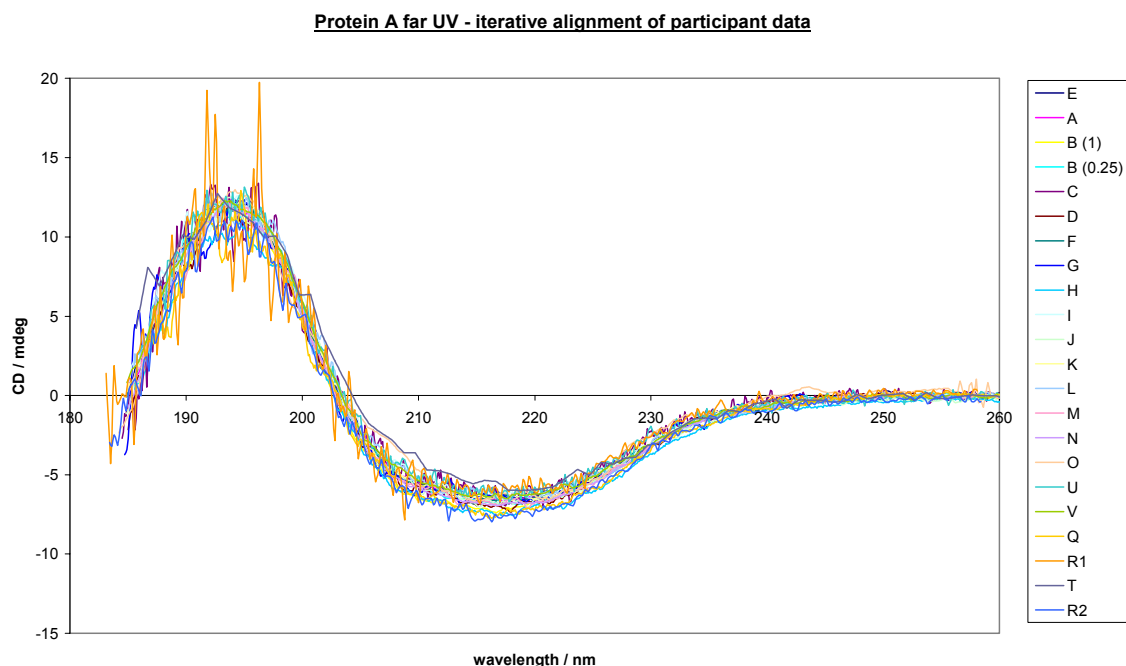


Figure 4.12 Aligned far-UV CD spectra of protein A.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) as the reference. Overall, a clear improvement in alignment can be seen. For the far-UV ISAM results shown here and for proteins B and C, data below 185 nm was removed before processing, due to its high noise levels. Note that the colour coding is different from that in the plot showing the original spectra (Figure 4.9).

4.2.1.2 Protein B

As for protein A above, Figure 4.13 shows protein B far-UV spectra measured on participant instruments and on the NPL instrument. All CD spectra are broadly the same shape and there are no obvious outliers, although one instrument shows unusual results compared to the others in the region ~240-260 nm. As before, inter-instrument differences are seen in wavelength position and especially in scaling, and noise levels also vary. Re-measurement of protein B samples on the NPL CD instrument (Figure 4.14) after their measurement by participants showed that the samples had not degraded or varied in concentration, again indicating that inter-instrument variation was not due to sample differences.

Processing of samples using the ISAM alignment routine (Figure 4.15) showed significant improvement in alignment in most cases, but spectra from two instruments remained clearly distinct from the rest, suggesting the presence of factor(s) not accounted for by the ISAM alignment. From an examination of the spectral plots before and after alignment, it seems that the baseline of these two instruments is offset from the rest, and that this baseline difference becomes accentuated after the others are aligned using ISAM. In addition, a discernible difference in spectral *shape* may also exist for one of these instruments, due perhaps to a differing spectral CD response in this instrument.

The current version of ISAM does not adjust for such baseline differences as part of its optimisation of fitting, but this ability could be added in future versions. However,

to account for spectral response differences, it would be necessary to *measure* calibration at multiple wavelengths, as in [5].

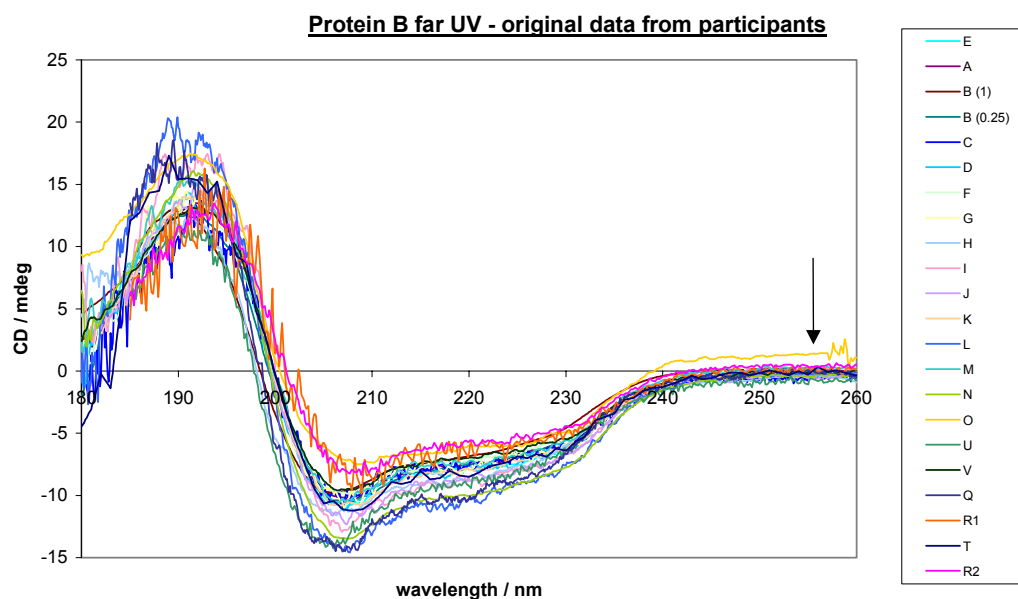


Figure 4.13 Far-UV CD spectra of Protein B.

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL. Note that one instrument (O, arrowed) appears to differ from the others around 240-260 nm.

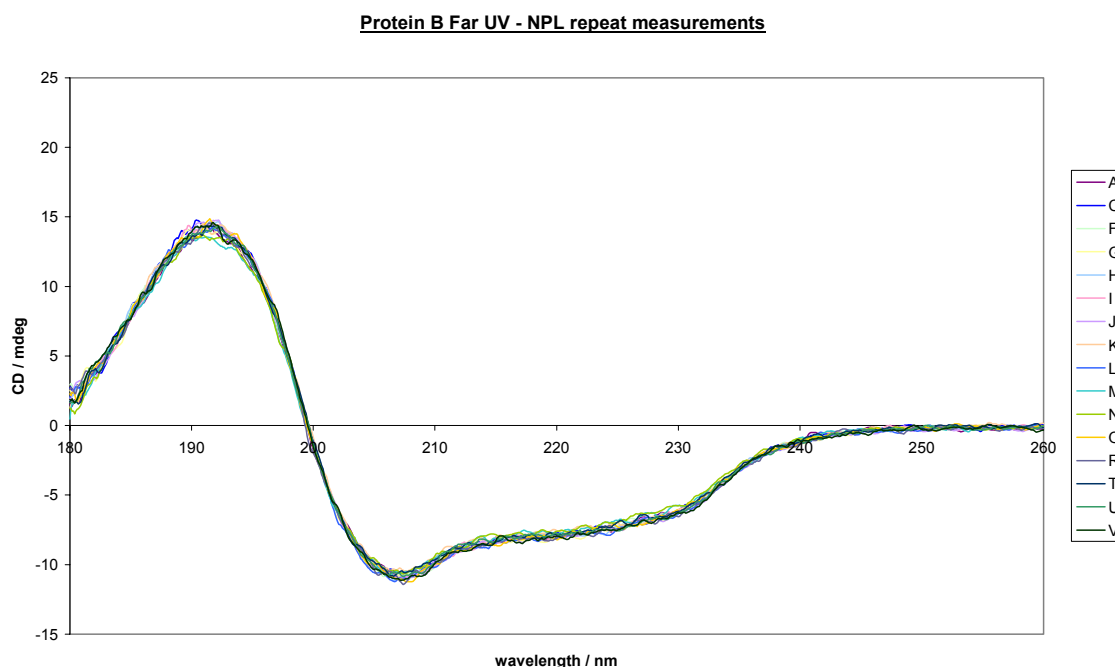


Figure 4.14 Far-UV CD spectra of Protein B.

Repeat NPL measurements of 16 samples returned from participating laboratories.

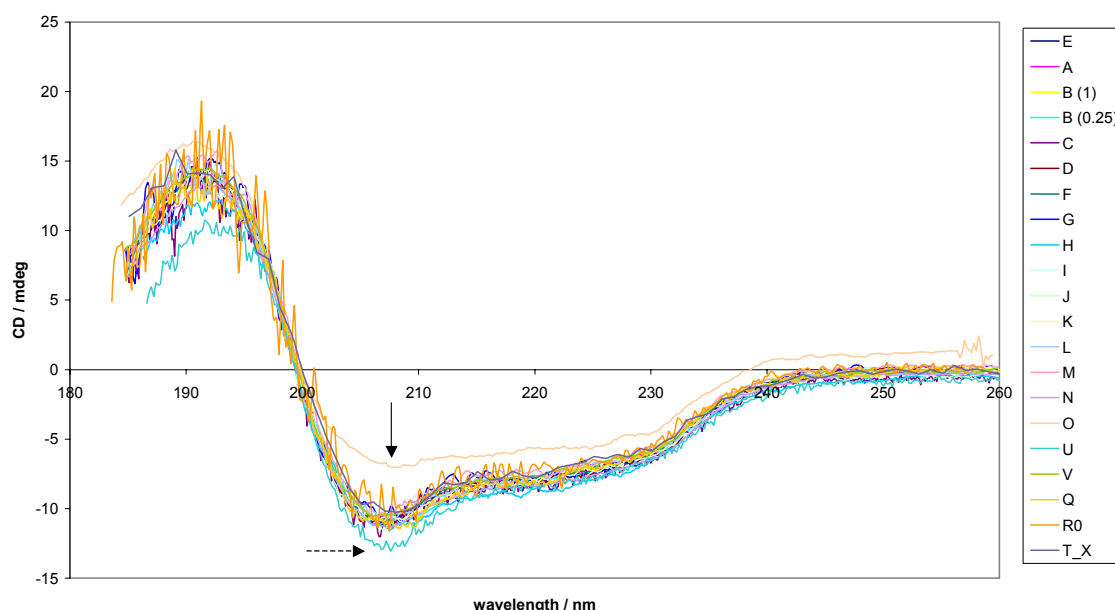
Protein B far UV - iterative alignment of participant data

Figure 4.15 Aligned far-UV CD spectra of protein B.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) as the reference. Good alignment has been obtained except for O (orange, solid arrow) and U (cyan, dashed arrow); baseline differences appear to account for a large part of this, although for instrument O there also appears to be some small difference in spectral response, i.e. shape, compared to the others.

4.2.1.3 Protein C

Protein C spectra from participant instruments can be seen in Figure 4.16; as for proteins A and B, this shows that the spectra have similar shapes but a wide range of scaling differences, and also some difference in position along the wavelength axis. Signal-to-noise ratios also vary. These differences are not simply due to sample degradation or other sample variation, as judged by the results of the repeat NPL measurements of protein C (Figure 4.17). Alignment of these spectra using ISAM (Figure 4.18) shows significantly improved alignment in all but one case. As was seen for protein B, although baseline differences will account for part of this discrepancy, it appears that this instrument's spectral response is also different, as the shape of the spectrum also differs from that of other instruments.

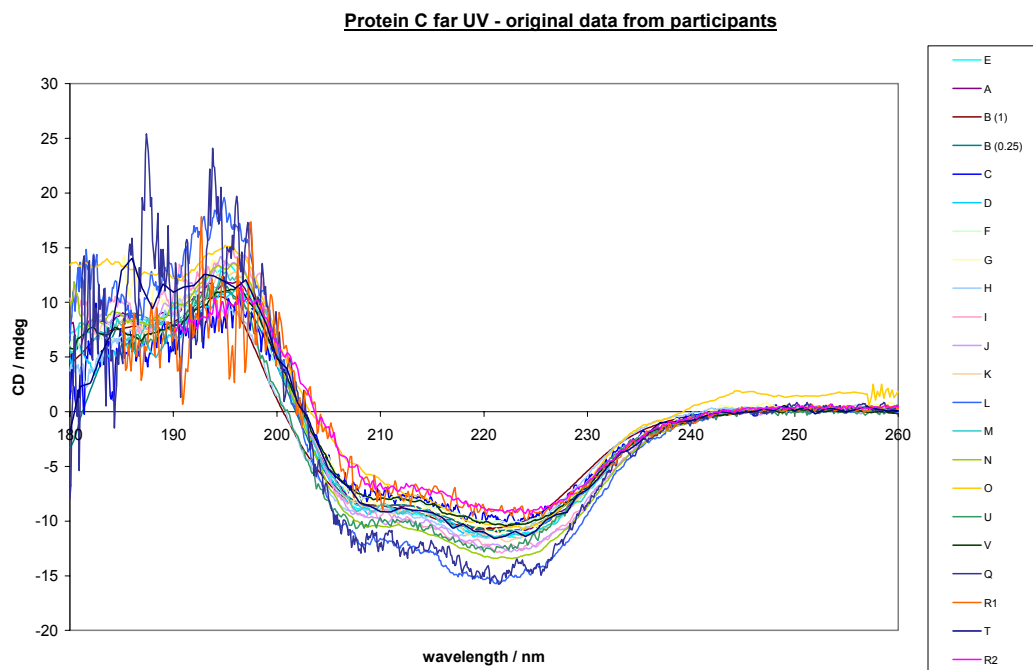


Figure 4.16 Far-UV CD spectra of Protein C.

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL.

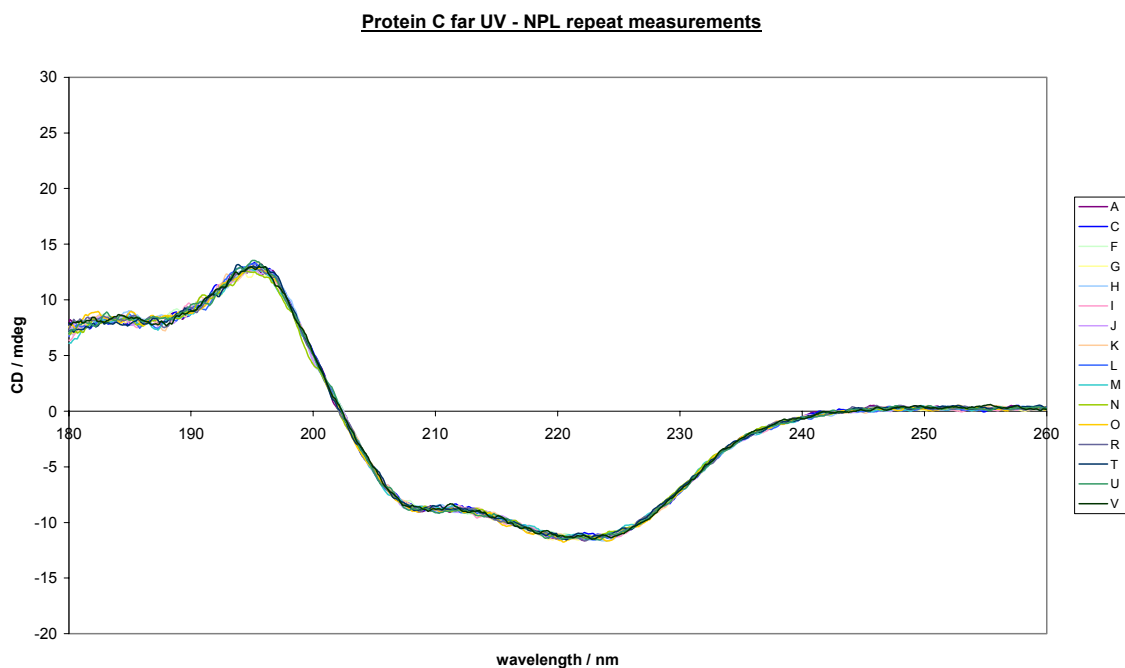


Figure 4.17 Far-UV CD spectra of Protein C.

Repeat NPL measurements of 16 samples returned from participating laboratories.

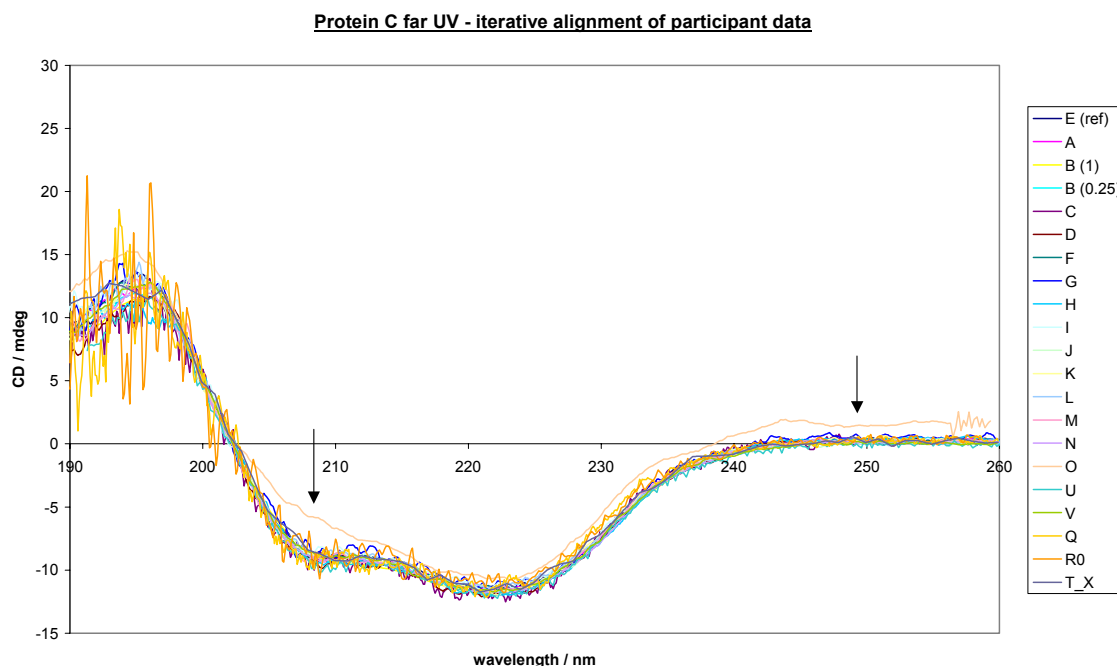


Figure 4.18 Aligned far-UV CD spectra of protein C.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) as the reference. Overall, improved alignment is seen, although one instrument (O, arrowed) remains somewhat different from the majority.

4.2.1.4 Normalizing spectra by ACS-based calibration state

As described in section 3.3, spectra from each instrument were normalized to remove the effects of differing calibration states, using the ACS peak value obtained on that instrument (see Figure 4.7). At the outset of this work, it was expected that this would effect a large improvement in the alignment of spectra. However this was not the case; this was particularly noticeable in the far-UV, where the largest differences in scaling are seen. Figure 4.19 shows the ACS-normalized far-UV CD spectra for protein B; comparing this with Figure 4.13, which shows the spectra without such normalization, it is clear that such normalisation does not greatly improve spectral alignment, i.e. it does not markedly reduce the scaling differences seen.

This is reflected in the relatively small inter-instrument spread in CD intensity calibration settings (Figure 4.2); this means that, for any one protein, the variation seen between the CD spectra obtained on different instruments, especially in the far-UV region, can only to a limited extent be attributed to instrument calibration differences. Pathlength differences can be several-fold larger than such calibration differences, and are the most likely explanation for the spread of data seen here, especially in the far UV.

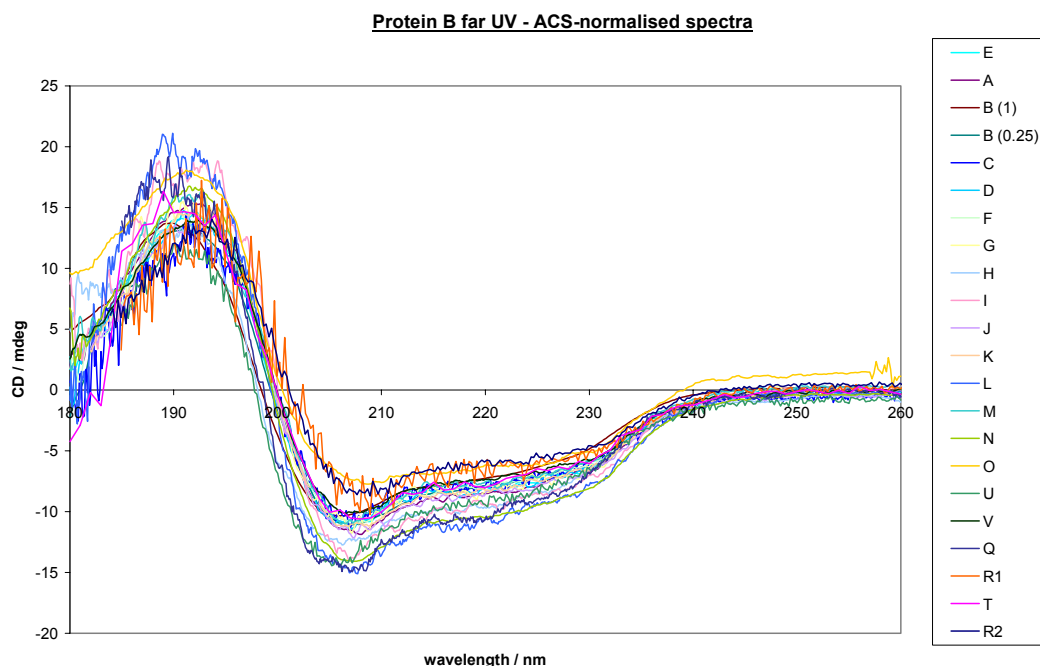


Figure 4.19 Protein B far-UV participant spectra after normalizing by each instrument's calibration state as measured by ACS peak intensity.

Compare with Figure 4.13. Note that no clear improvement has been achieved.

4.2.1.5 Summary of far-UV results

As the above results demonstrate, there are relatively large differences between the far-UV CD spectra obtained on the different instruments, for what are essentially identical samples. Whilst part of this is due to differences in the *shape* of the spectra, a large part is simply due to differences in their magnitude, or scaling, and slight differences in their position along the wavelength axis. There are also differences in noise.

Differences in wavelength position most likely arise because of differences in the calibration of the instruments. These are relatively small in the far-UV region. More dramatic are the scaling effects, which amount to $\sim \pm 50\%$ by eye; these cannot be accounted for solely by instrument calibration, which accounts for only $\sim \pm 8\%$. It is likely that a large proportion of these differences is due to variation in the actual path length of the (nominally) 0.1 mm cells used for these measurements; see also section 4.3, where an example of the correction of spectra using measured path length is discussed.

It is clear that the differences between spectra noted above are in large part instrument dependent, rather than arising from differences between the samples measured. For example, noise levels mostly stay constant across the three proteins, for any one instrument. Similarly, the wavelength offset and scaling (as obtained from the results of the ISAM alignments) are broadly similar for all three proteins, in any one instrument, as shown in Figure 4.20 and Figure 4.21 respectively. That is, across all three proteins, offsets are largely similar in direction and magnitude for any one instrument; this is to be expected since this offset is a property of the instrument and should remain constant across all proteins in the same wavelength range; random errors, however, mean that there are some exceptions to this.

Similarly, on the whole, the scaling factors across all three proteins rise and fall in a similar way across instruments, which is to be expected if – as is thought – most of the differences seen are due to systematic errors in cell path length or instrument calibration, which will affect all three proteins similarly.

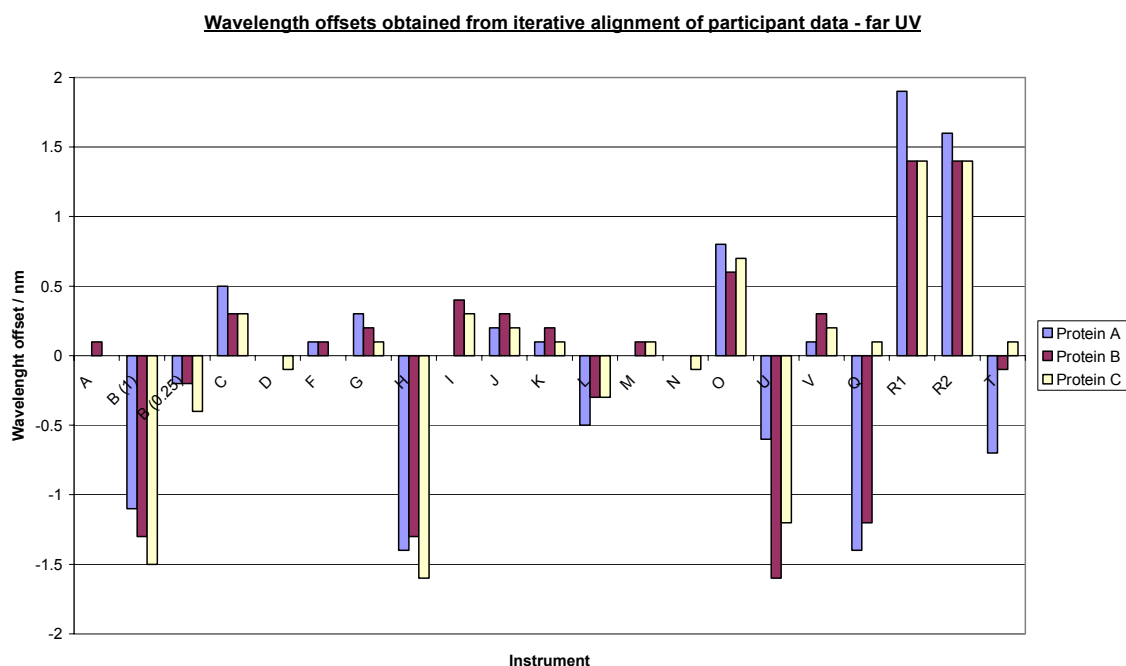


Figure 4.20 Summary of far-UV wavelength offsets for proteins A, B and C on each instrument.

Obtained from the ISAM alignment routine, using a representative spectrum measured on the NPL instrument as reference. Where no bar is plotted for a particular instrument/protein combination, this indicates a zero offset.

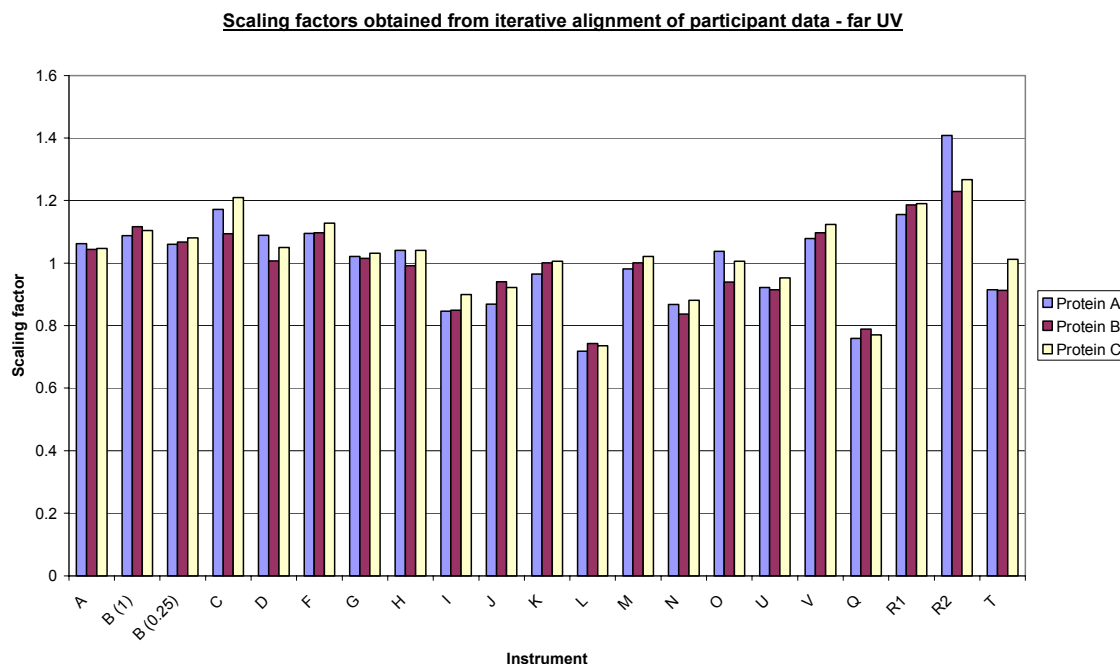


Figure 4.21 Summary of far-UV scaling factors for proteins A, B and C run on each instrument.

Obtained from the ISAM alignment routine using a representative NPL spectrum as reference.

4.2.2 Near UV Spectra

Protein CD spectra in the near UV (or “aromatic”) region chiefly reflect the structural environment of aromatic amino acids in the protein. Whilst one cannot deconvolute these into secondary-structure classes as with far-UV spectra, they are potentially very useful in biopharmaceutical characterisation because they can be sensitive to small structural changes such as may be seen in batch-to-batch variation. Proteins B and C were measured in this spectral region.

4.2.2.1 Protein B

Protein B near-UV spectra from participant instruments, together with a representative spectrum measured on the NPL instrument, are shown in Figure 4.22. Most of the CD spectra are the same shape, and there is certainly significantly less variation in scaling compared with the far-UV spectra. However, there are clearly a number of outliers, for reasons that are unclear. These are not due to problems with the sample, as shown by the results of repeat measurements of these samples using the NPL instrument (Figure 4.23). The variation of noise level appears less than in far-UV spectra.

When these samples were processed through the ISAM alignment method (Figure 4.24), most aligned well, although spectra which appeared as outliers in the original data did not align well and remained as outliers, as their overall *shape* was too different from the majority for the alignment to be of help. The spectrum from instrument C failed to align well, but this was simply due to a deviation in the 240-245 nm region. Figure 4.25 shows more clearly what happens when some of these outlier spectra, as well as a 'normal' spectrum, were processed with ISAM.

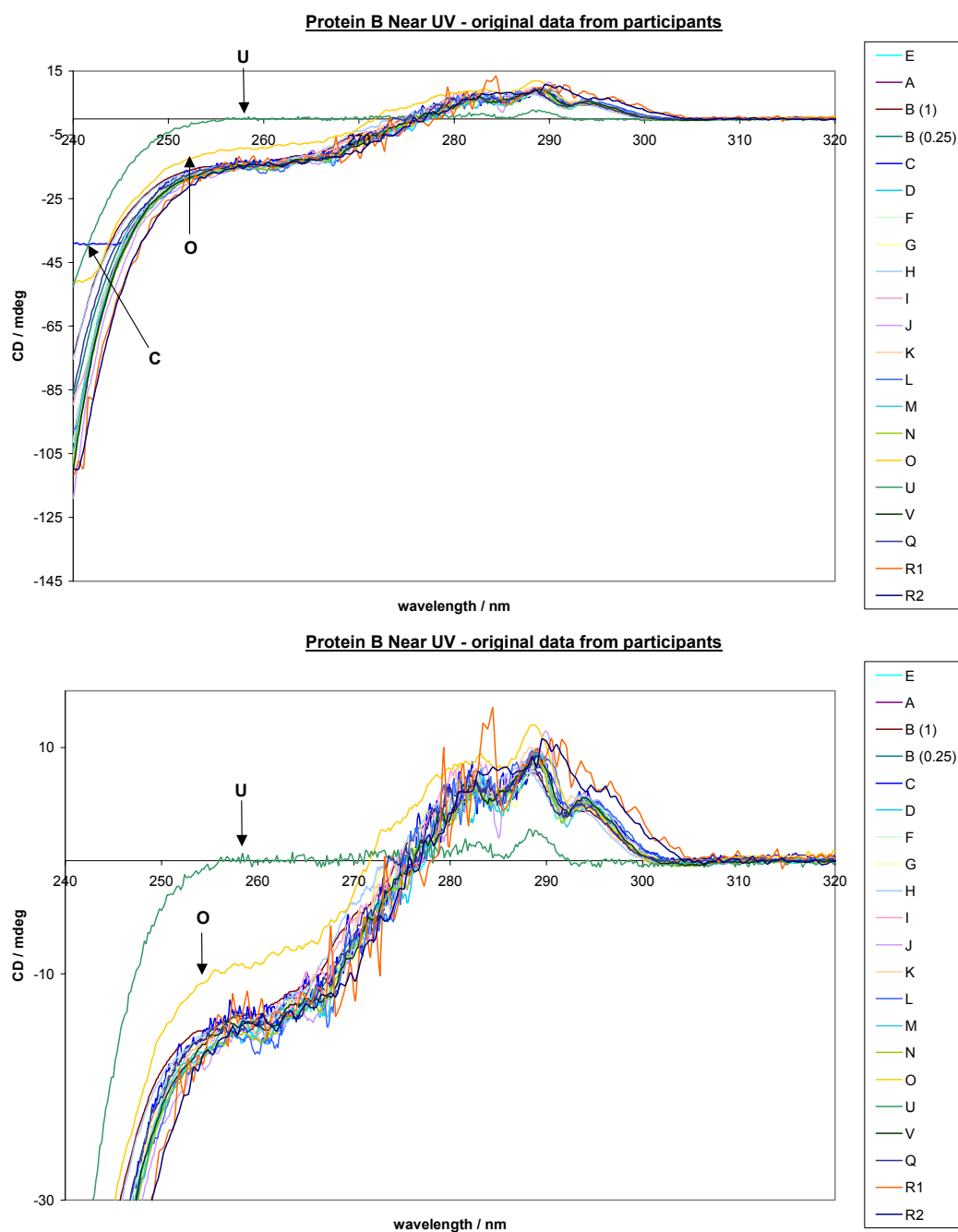


Figure 4.22 Near-UV CD spectra of Protein B. (**lower** panel shows an enlarged view).

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL. Note in particular that data from three instruments are clearly distinct from the majority (O, U and C, arrowed – the deviation of C from the majority is only clear from the upper plot, in the 240-250 nm region). One of these (U) possesses very little CD signal, and may have resulted from a cell that was only partly filled or contained an air bubble in the light path.

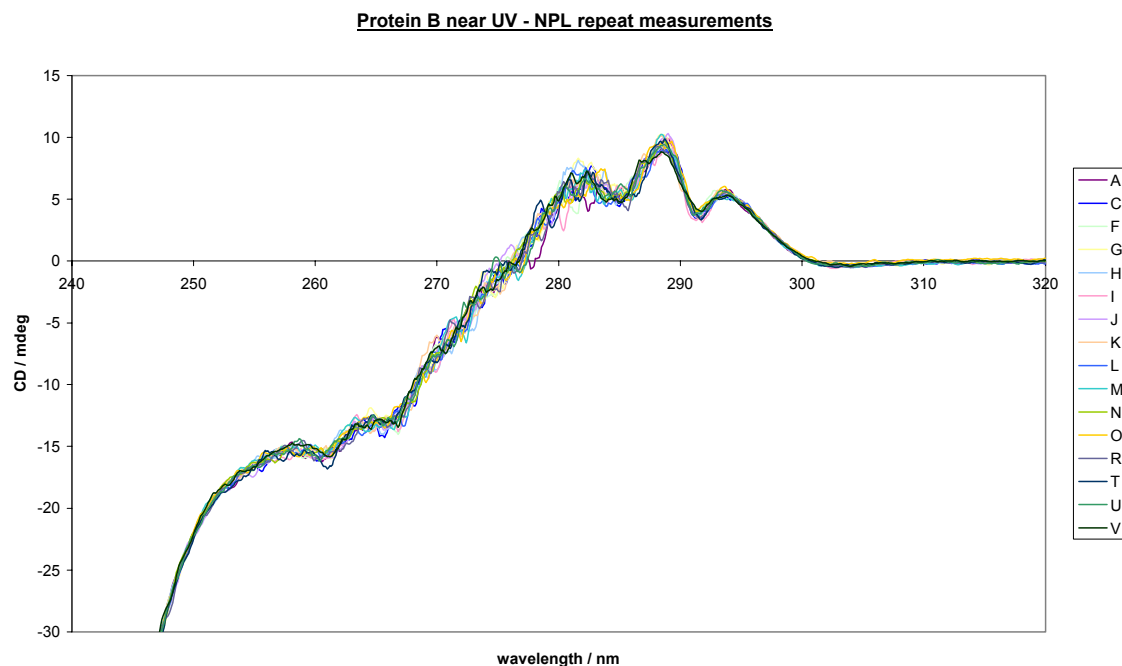


Figure 4.23 Near-UV CD spectra of Protein B.

Repeat NPL measurements of 16 samples returned from participating laboratories. The increased noise around 280 nm as a result of the higher absorbance is clearly visible.

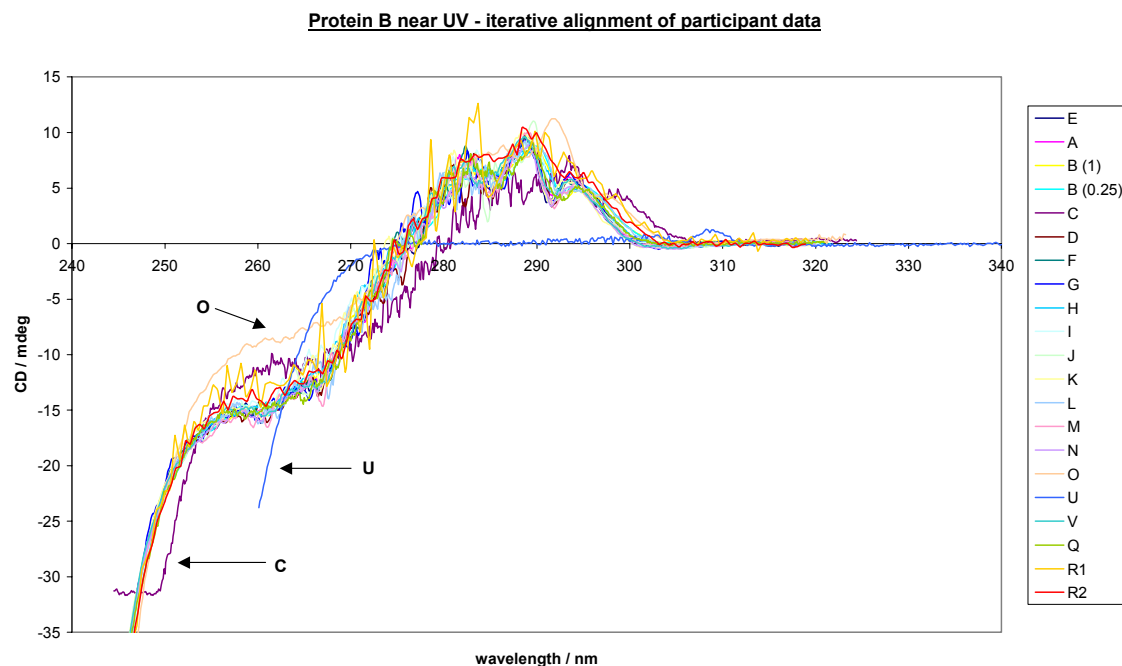


Figure 4.24 Aligned near-UV CD spectra of protein B.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) as the reference. Poor alignment is seen in a number of cases (arrowed).

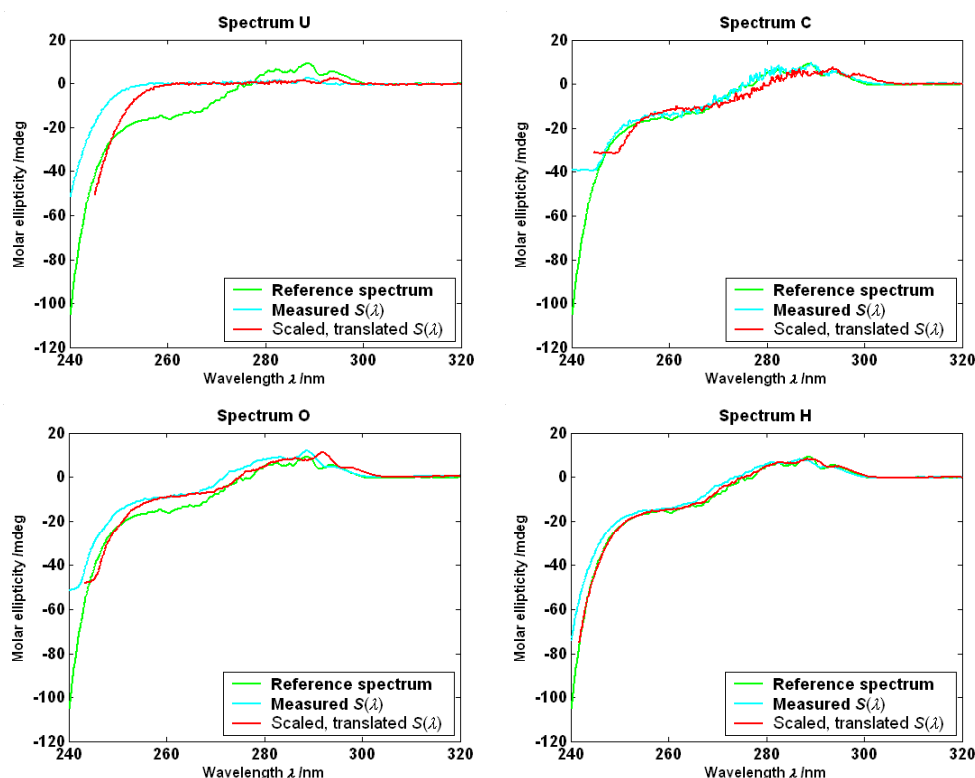


Figure 4.25 Examples of the alignment of protein B near-UV spectra.

Colour scheme is as explained in Figure 4.11. Spectra from instruments U, C and O – which did not align well – are shown, together with a spectrum from instrument H, in which the alignment worked well.

4.2.2.2 Protein C

Figure 4.26 shows the protein C near-UV spectra obtained from participants' instruments, along with a spectrum measured at NPL. There is relatively good alignment in most cases (apart from the region around 240-245 nm, where variation is greater due to high absorbance levels there), and as was the case for protein B near-UV spectra, there is significantly lower variation in scaling compared to the far-UV spectra.

Repeat measurements at NPL (Figure 4.27) show good overlay for all spectra bar one: the spectrum of the sample used with instrument T deviates slightly from the majority of spectra, suggesting some conformational change may have taken place (this is also seen in the visible region – see Figure 4.32 and section 4.2.3.1). However, this is not a cause for concern as instrument T was only used to record *far-UV* measurements; since the protein C far-UV spectrum recorded on that instrument did not appear unusual, any conformational change that occurred does not seem to have affected the far-UV CD region. Indeed, this small change could reflect changes that occurred *after* the sample had been measured by the participating laboratory.

Alignment of spectra using the ISAM routine (Figure 4.28) shows good alignment of all but two spectra, which diverge from the main cluster of spectra from ~265 nm and upwards.

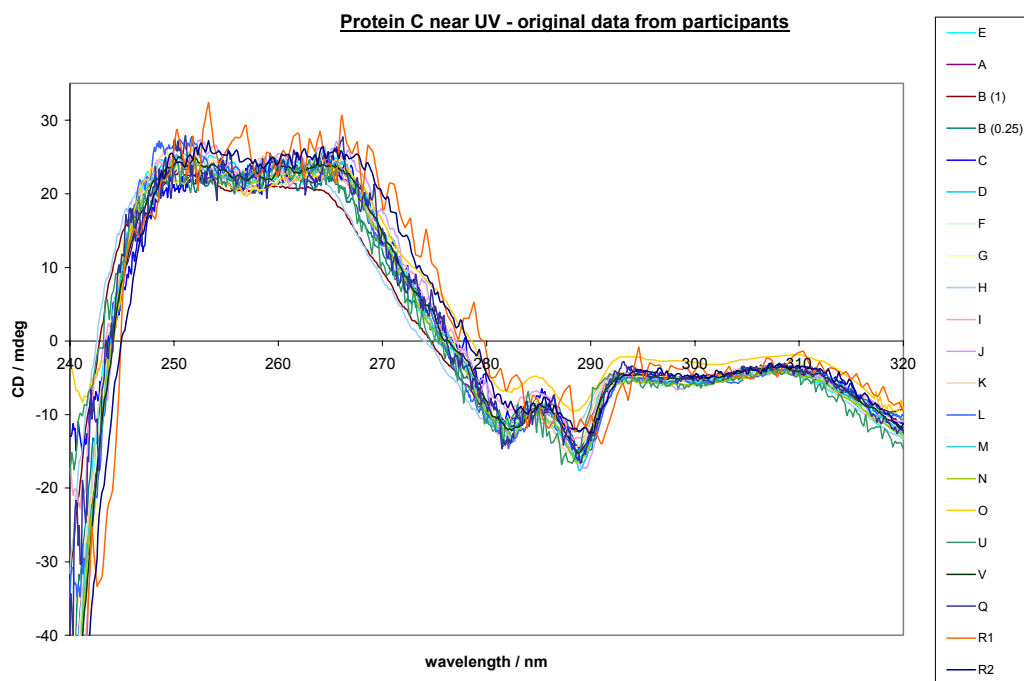


Figure 4.26 Near-UV CD spectra of Protein C.

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL.

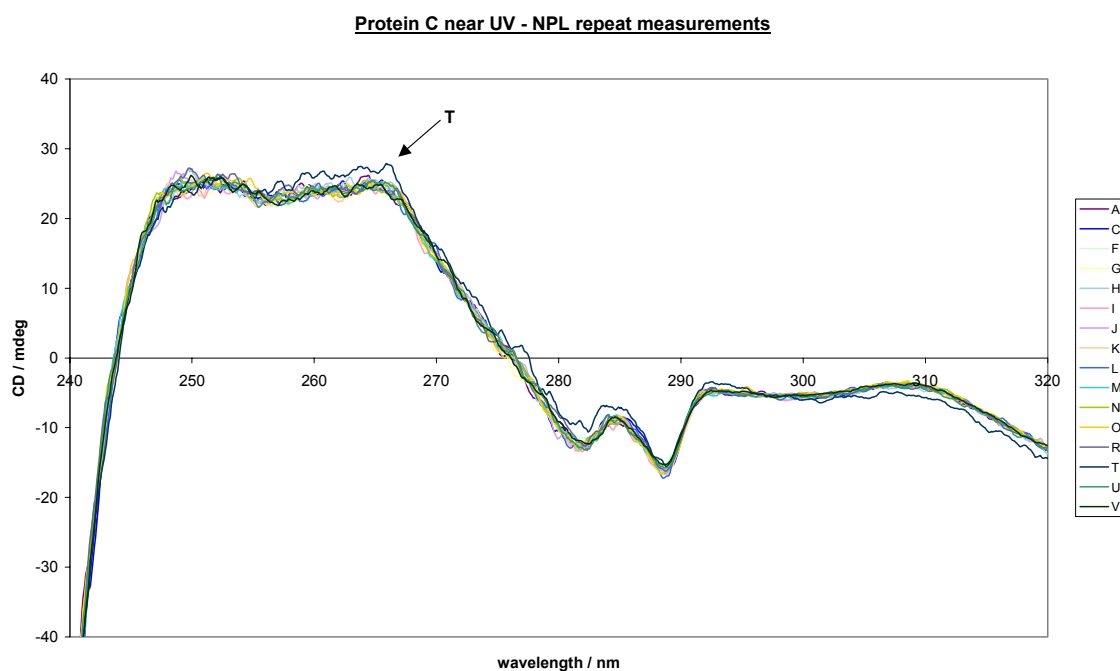


Figure 4.27 Near-UV CD spectra of Protein C.

Repeat NPL measurements of 16 samples returned from participating laboratories. One spectrum (used with instrument T, arrowed) is clearly different from the others, perhaps due to slight degradation in transit.

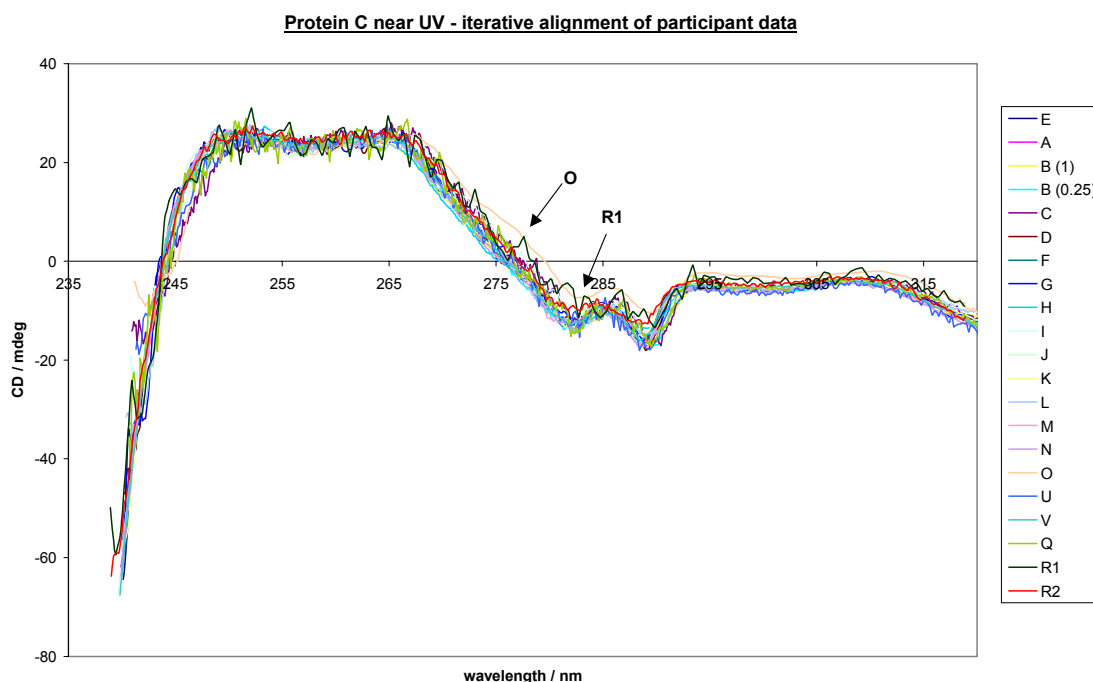


Figure 4.28 Aligned near-UV CD spectra of protein C.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) as the reference. Good alignment is obtained for all spectra except that, from approximately 265 nm upwards, instrument O (orange, arrowed) diverges from the majority; R1 appears to diverge slightly around ~ 277-282 nm (green, arrowed) but this is probably just due to the relatively high level of random noise in this spectrum.

4.2.2.3 Summary of near-UV results

With only a few exceptions, the shape of the near-UV spectra are comparable between instruments; better comparability was seen for protein C than protein B. In terms of inter-instrument variation in scaling, this was lower (~ 30%) amongst the near-UV spectra than was seen for the far-UV spectra. This is most easily explained by the different types of cell used in each case: the near-UV spectra were recorded using cells of nominally 1 cm path length, whereas the far-UV spectra were measured in much shorter cells, mostly of nominally 0.1 mm path. In relative terms, the manufacturing tolerances are better for 1 cm cells than for 0.1 mm cells, i.e. the relative spread of *actual* path lengths is smaller for 1 cm cells, leading to lower variation in the CD spectra measured in those cells. Indeed, that such a large variation is seen in far-UV spectra is further evidence that path length differences are the primary reason for the large variation seen there. Wavelength differences were relatively small, and comparable with those of far-UV spectra.

As was observed for the far-UV spectra, the differences seen are clearly instrument or operator dependent, rather than reflecting sample variation, as the samples gave reproducible signals in the near-UV region too when measured on the NPL instrument.

The near-UV wavelength offsets obtained from the ISAM algorithms are generally similar for both proteins B and C in any one instrument, as shown in Figure 4.29. This is to be expected given that, ignoring random error, wavelength offset is fundamentally a property of the instrument rather than the sample; similar agreement

between proteins was seen in the far-UV data. However, the correlation between the scaling factors obtained for proteins B and C, as shown in Figure 4.30, appears weaker than that seen for proteins A, B and C in the far-UV; this may be because the near-UV spectra showed less scaling variation to begin with, than the far-UV.

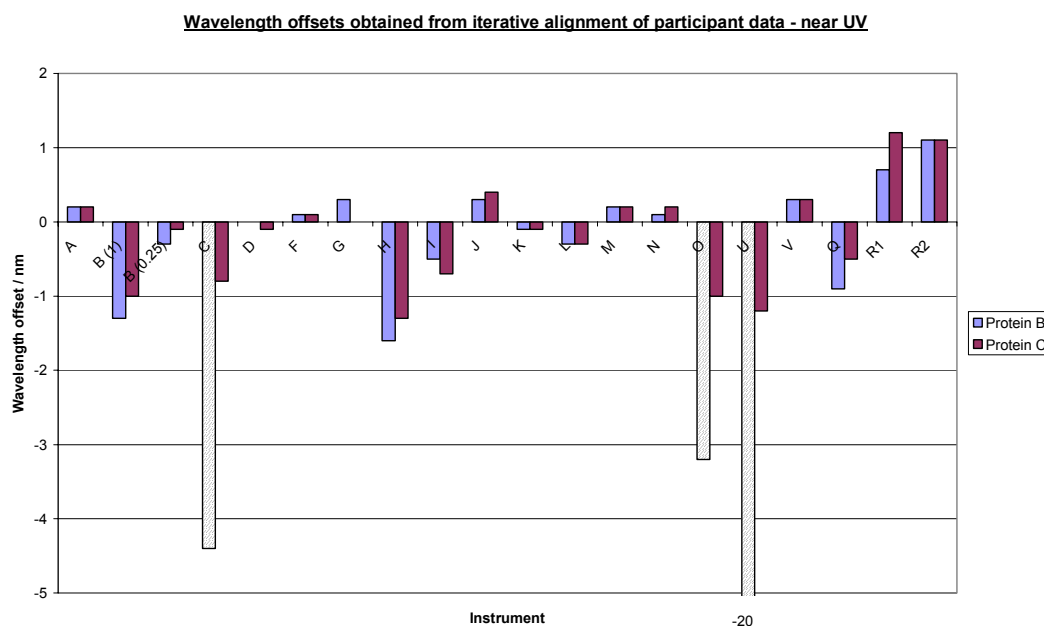


Figure 4.29 Summary of near-UV wavelength offsets for proteins B and C on each instrument.

Obtained from the ISAM alignment routine, using a representative spectrum measured on the NPL instrument as reference. Where no bar is plotted for a particular instrument/protein combination, this indicates a zero offset. The unusually large protein B offset values for O, U and C (shown hatched) are not indicative of true instrument calibration effects and can be ignored, as these spectra were highly atypical.

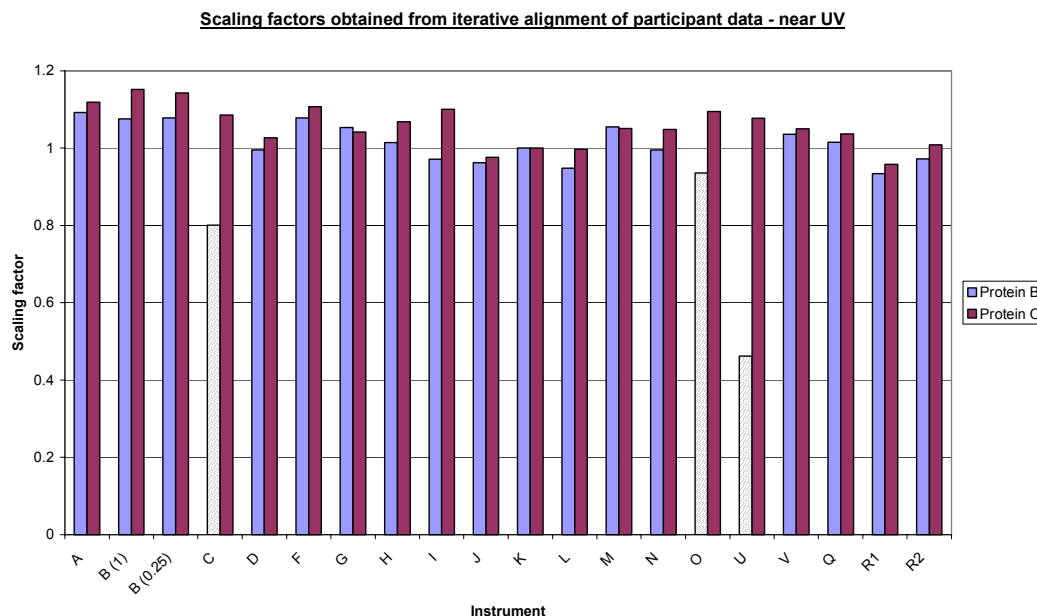


Figure 4.30 Summary of near-UV scaling factors for proteins B and C on each instrument.

Obtained from the ISAM alignment routine using a representative NPL spectrum as reference. The protein B scaling factors for instruments O, U and C (shown hatched) should be ignored as these spectra were highly atypical, such that the scaling factors are misleading, and for these instruments one would not actually expect the protein B and C scaling factors to match.

4.2.3 Visible spectra

CD spectra in the visible region are not generally used for biopharmaceutical characterisation (unless the product contains a visible chromophore). However, such CD spectra can be useful for binding studies, where binding causes an induced CD in the probe. Greater instrument variation was expected in this region, particularly in wavelength, as instruments are not generally optimised for measurement in this region.

4.2.3.1 Protein C

Visible-region CD spectra from participants are shown in Figure 4.31. This shows good comparability between most spectra, but a number of clear outliers. The region around 430 nm is noisy, but this is to be expected due to the high absorbance in that area.

Repeat measurements at NPL are shown in Figure 4.32, again showing good sample stability and instrument repeatability, as for the other wavelength regions. However, the spectrum of sample T shows some divergence from the others. In the near-UV too, sample T of protein C differed from the rest of the samples (Figure 4.27); this reinforces the possibility raised earlier (section 4.2.2.2) that this sample has undergone a change in conformation. Note that nothing unusual was seen in the *far-UV* spectrum of this sample of protein C (Figure 4.17); hence this appears to be an example of a point mentioned in section 4.2.2, namely that the far-UV CD region is less sensitive than the near UV to small structural changes. However, as discussed earlier, this structural change this is not of concern here.

The spread of data was broadly similar to that seen in the near-UV. Alignment of spectra using ISAM (Figure 4.33) showed good alignment for most spectra; baseline drift over the long scan time may have contributed to the divergence of instrument C's spectrum.

The wavelength offsets and scaling factors obtained from the ISAM analysis are shown in Figure 4.34 and Figure 4.35 respectively. Although larger wavelength offsets tend to be found in the visible region compared with the other two regions (clearly apparent in Figure 4.36, section 4.2.4), comparability in this region is generally better than expected, given that instruments are not generally optimised for use in this region.

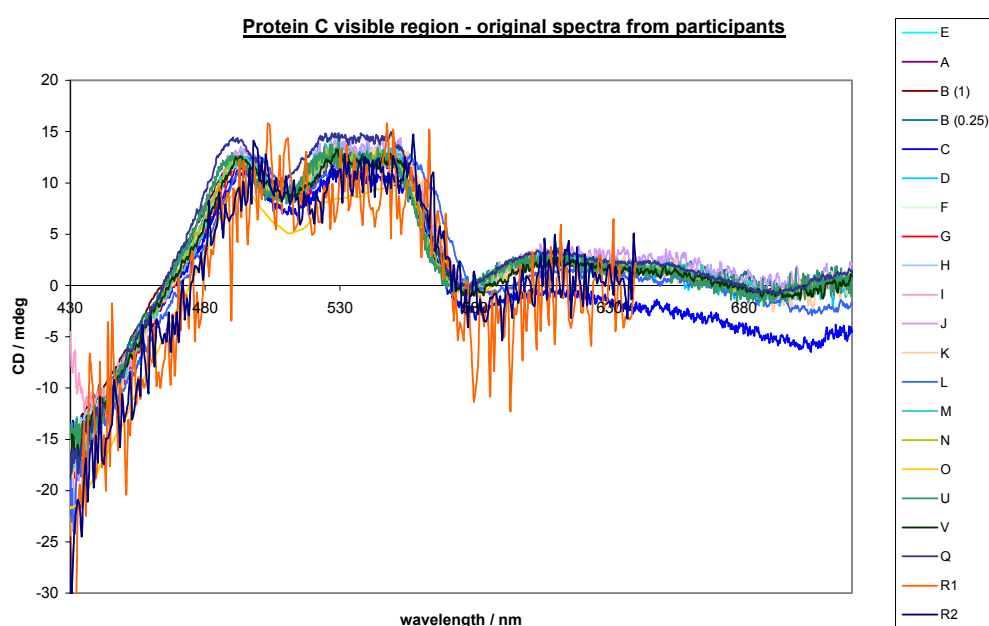


Figure 4.31 Visible-region CD spectra of Protein C.

Data from participating laboratories are shown together with a representative spectrum (E) measured at NPL¹⁵.

¹⁵ For technical reasons, R1/R2 visible-region spectra were recorded in a (nominally) 1 mm cell; these were then scaled 10-fold for comparison with the other spectra.

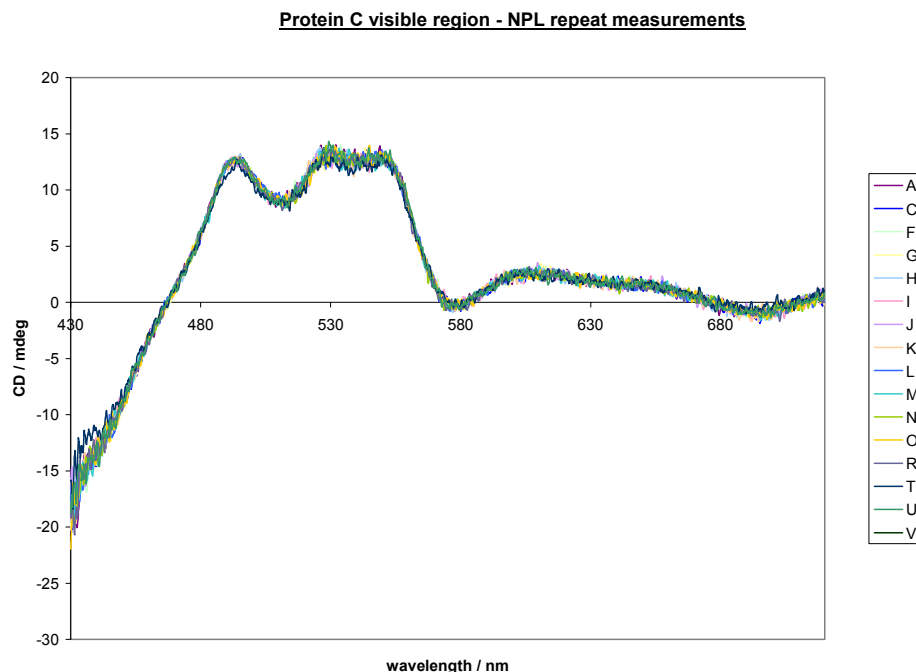


Figure 4.32 Visible-region CD spectra of Protein C.

Repeat NPL measurements of 16 samples returned from participating laboratories. Good agreement is obtained for most spectra, although slight variation is seen for sample T around 430-450 nm and around 490 nm.

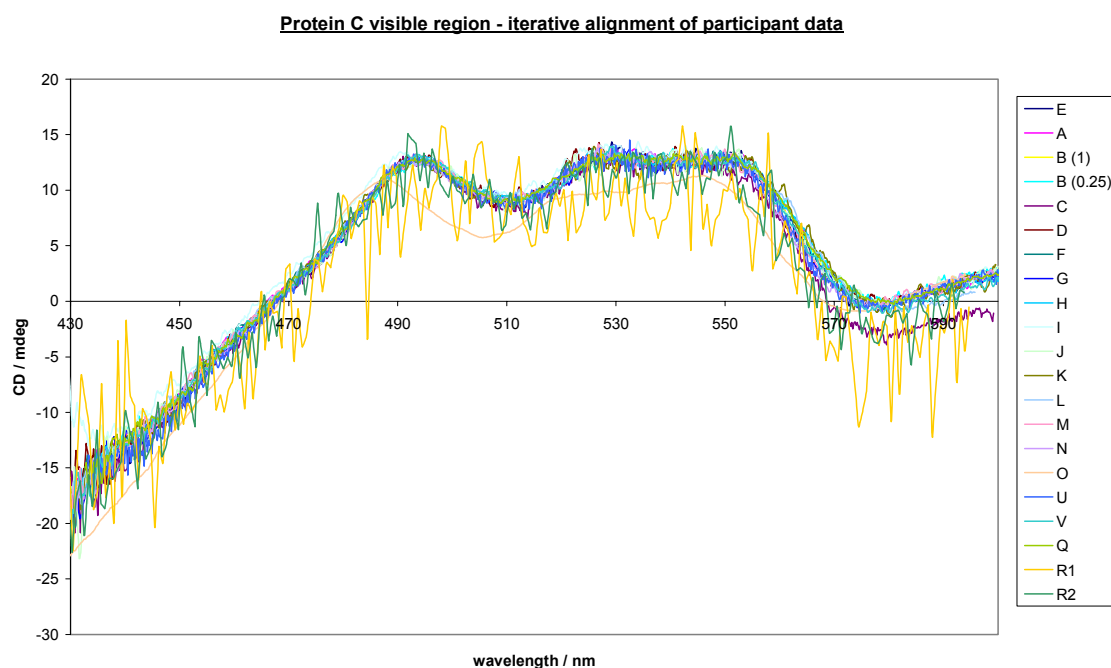


Figure 4.33 Aligned visible-region CD spectra of protein C.

Spectra from participating laboratories were processed with the iterative spectral alignment method (ISAM), using a representative spectrum measured on the NPL instrument (E) used as the reference.

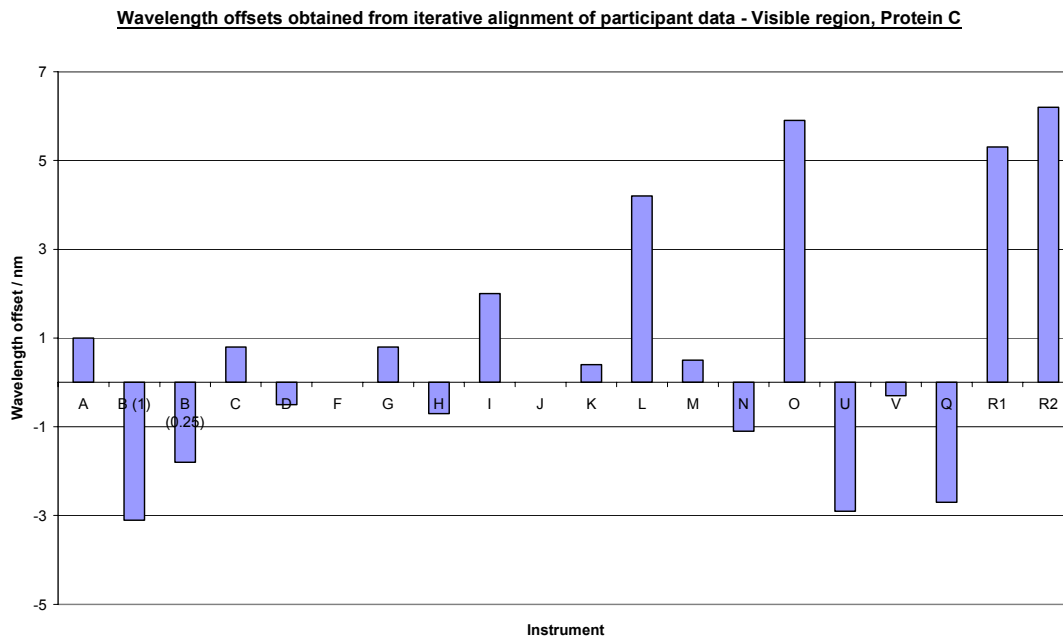


Figure 4.34 Summary of visible-region wavelength offsets for protein C

Obtained from the ISAM alignment routine, using a representative spectrum measured on the NPL instrument as reference.

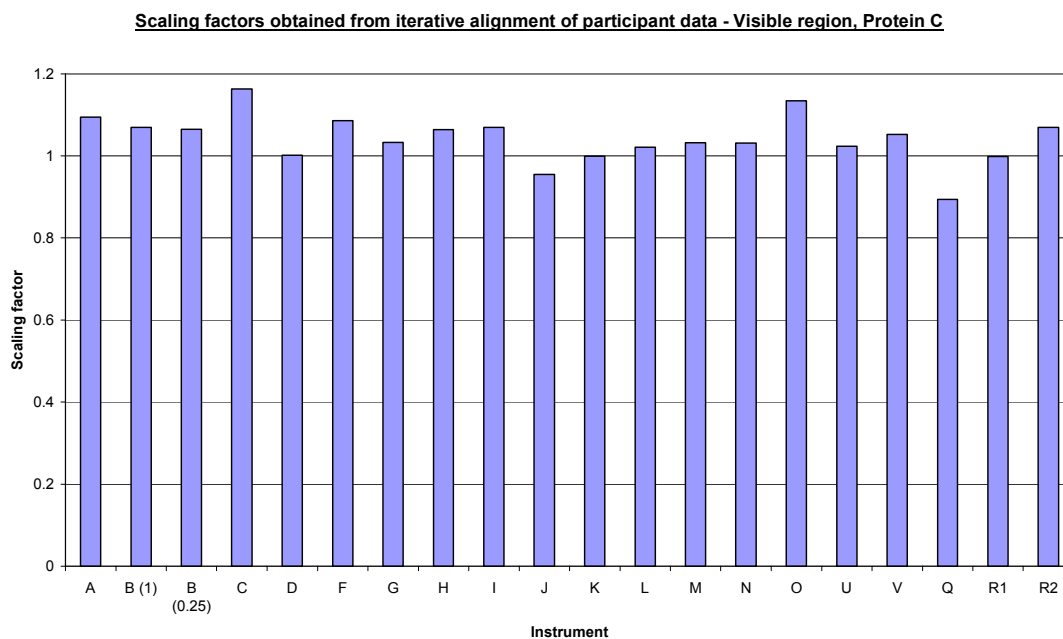


Figure 4.35 Summary of visible-region scaling factors for protein C.

Obtained from the ISAM alignment routine, using a representative NPL spectrum as reference.

4.2.4 Wavelength offsets – a global view

Figure 4.36 shows a summary of the wavelength offsets obtained from alignment of spectra using the ISAM routine, for all proteins across all wavelength ranges studied. This shows that there is no clear, consistent difference in magnitude between the far-

UV and near-UV regions, but that wavelength offsets in the visible region tend to be larger.

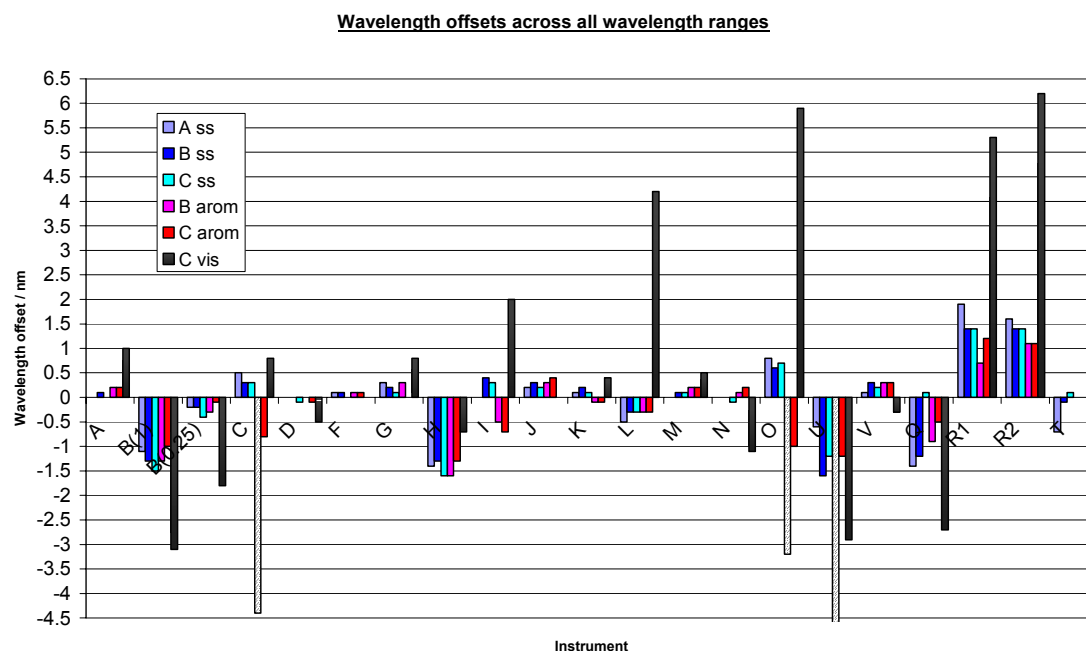


Figure 4.36 Wavelength offsets – a summary across all proteins and spectral ranges.

Key: ss=secondary structure (far UV); arom=aromatic (near UV); vis=visible. Hatched shading indicates values that are unreliable due to highly atypical protein B near-UV CD spectra. Note that the visible region shows the largest shift in most cases.

4.3 Use of cell path length measurement

During the course of this study, participants were asked to supply information about the path length of the cells used for their measurements. In addition to the path length stated by the manufacturer (the 'nominal' path length), they were requested – where this was known – to provide a *measured* path length. In all but a small number of cases, however, only nominal path lengths were reported. Hence it was not possible, in most cases, to adjust the spectra to account for possible path length discrepancies between the nominal and actual path lengths.

However, the use of such path length information can be seen by studying a case where such information *was* available, and where there was a large discrepancy between nominal and actual path length: instrument L was run using a cell whose path length was measured as 0.126 mm, using the ratio of the ACS CD signal in that cell and a 1 cm cell, where path lengths are more accurate. The path length of the cell used at NPL was 0.097 mm, as measured using an interferometric method (similar or identical results were obtained from two other methods). Interestingly, as expected, the ratio between these two cell path lengths ($0.097 / 0.126 = 0.77$) is very close to the scaling factor obtained from the spectral adjustment method, ISAM (a mean of 0.73 over the three proteins studied in this cell).

In other words, a large portion of the difference between spectra measured in instruments L, and those measured at NPL, appears to arise out of *path length* differences, highlighting the potential importance of measuring this path length as part of studies where more than one cell is used, or may be used in the future. Differences

in instrument calibration (as determined by ACS peak magnitudes) will of course also play a role here too. However, as is often the case (unless the instrument is out of calibration by a significant amount), when using short cells, differences due to calibration tend to be less important than differences in path length, and this is the case here too, where the % difference in path length is more than 20-fold greater than the % difference in calibration.

As discussed elsewhere, although such scaling differences can be minimised or removed by an approach such as ISAM or other normalization methods, it can sometimes be preferable to minimise such systematic differences using known measurements as far as possible, before attempting further adjustments. This is especially important if one is interested in detecting differences in protein concentration between samples, since path length and protein concentration have similar, indistinguishable effects on CD spectra, and in such a case it would be beneficial to minimise all other potentially confounding effects first.

However, this correction is of course only as good as the method used to *measure* the path length itself (and indeed, the methods used to compare instrument calibration), and in some cases it will be enough, or indeed better, to remove such scaling differences using ISAM or other normalization methods that do not rely on 'external' *measurements*, rather than attempt to distinguish between concentration, calibration and path length effects. Moreover, if the path length measurement has large errors – or indeed if the method used to compare calibration itself has significant errors – protein concentration differences will not be accurately picked up; indeed, to detect differences in protein concentration, alternative, proven methods for this purpose are often preferable, e.g. a properly validated UV absorption assay.

4.4 The effect of temperature on the protein CD spectra

4.4.1 Introduction

Protein structure can be affected by changes in temperature, and this can be reflected in CD spectra, although proteins vary in their sensitivity to temperature. Hence the possibility existed that differences seen between the repeat measurements at NPL, or between participating laboratories, could in part reflect differences in the temperature at which samples were measured. *A priori*, it was not thought very likely to be a significant factor in this case, but it was important to investigate this further.

In order to study this, we examined how the CD spectra varied with temperature for all three proteins at all the relevant wavelength ranges used in this study. We chose a range of temperatures (15 – 45 °C), over which the proteins were not expected to undergo denaturation; although this range extends beyond the relatively narrow range at which samples were run in this study (around 20 – 25 °C), it was intended that this would help in identifying any real trends likely to exist within the narrow range used for measuring the CD spectra, whether the cell is simply at room temperature or is used with a temperature-controlled unit.

4.4.2 Method

Variations in CD spectra of proteins over a temperature range were measured using a Jasco J-810 CD instrument equipped with a Peltier-type temperature-controlled cell holder, a Peltier temperature-control system (model PTC-423S/L) and Jasco temperature-control software. The Peltier block was supplied with water from a

refrigerated circulator set to 15 °C. The measurement parameters are given in Table 4.1.

<i>Instrument parameter</i>	<i>Value</i>					
Temperatures (°C)	15	20	25	30	35	45
Data pitch (nm)	0.1					
Scanning speed (nm/min)	50					
Response (s)	1					
Bandwidth (nm)	1					
Equilibration time (s)	600 for 1mm cell 1200 for 10mm cell					
Temperature gradient (°C/min)	2					

Table 4.1 Scan parameters used to study the temperature dependence of protein CD spectra.

'Equilibration time' is the amount of time the system waited before starting to record spectra, after reaching each of the six pre-set temperatures.

Spectra were measured over the above 6-point temperature range, for each of the following solutions, at the wavelength ranges shown in Table 4.2.

<i>Solution</i>	<i>Wavelength Range (nm)</i>	<i>Region</i>	<i>Number of accumulations (repeat scans)</i>
Protein A	180-260	Far UV	4
Protein B	180-260	Far UV	4
Protein B	240-360	Near UV	4
Protein C	180-260	Far UV	4
Protein C	240-360	Near UV	4
Protein C	430-720	Visible	2
Water	180-260	Far UV	4
Water	240-360	Near UV	4
Water	430-720	Visible	2

Table 4.2 Samples and scan regions used in the temperature study.

A 10mm cell was used for the near-UV and visible regions, and a 1mm cell (and 10-fold dilution of the proteins) was used for the far-UV spectra. Water baselines were recorded at 15 °C and 45 °C, and their mean spectrum was used as the baseline.

4.4.3 Results

The results can be seen in the following six figures (Figure 4.27-Figure 4.42).

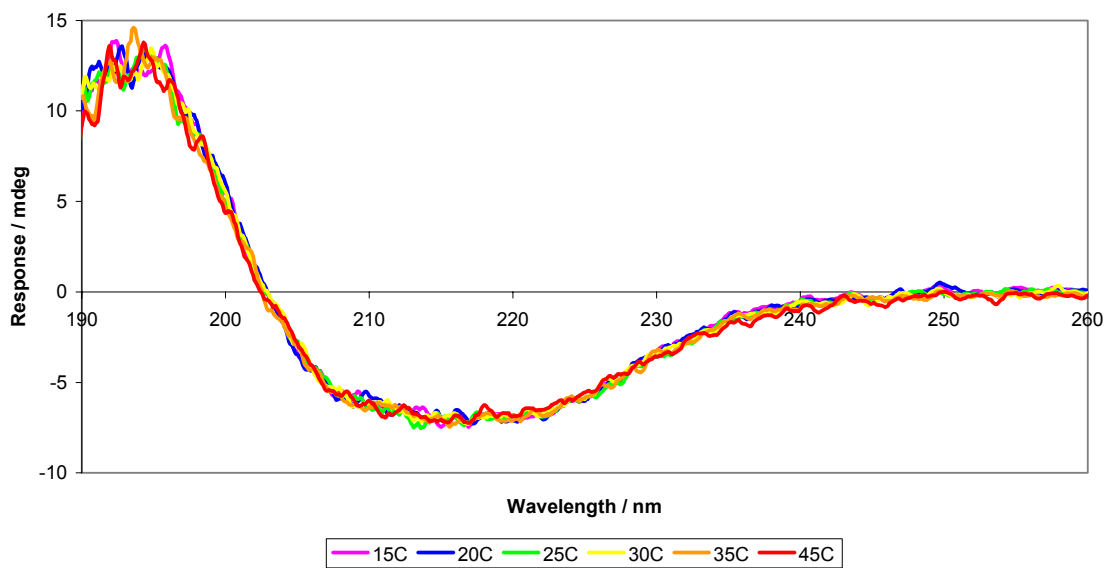
Protein A. Variation of CD spectrum with temperature

Figure 4.37 Variation of CD with temperature for Protein 'A', far UV.

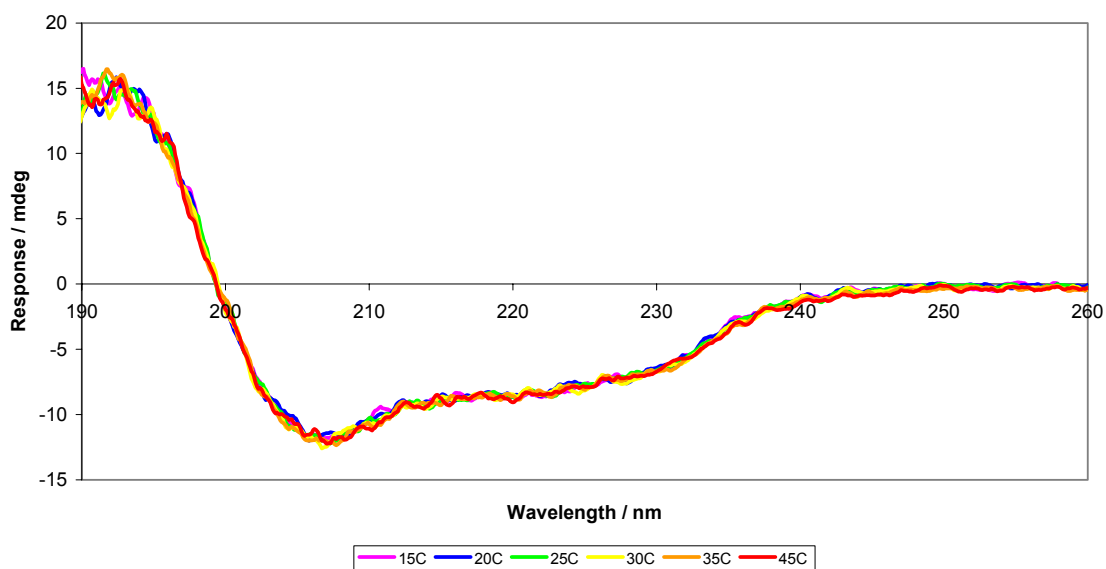
Protein B. Variation of CD spectrum with temperature

Figure 4.38 Variation of CD with temperature. Protein 'B', far UV.

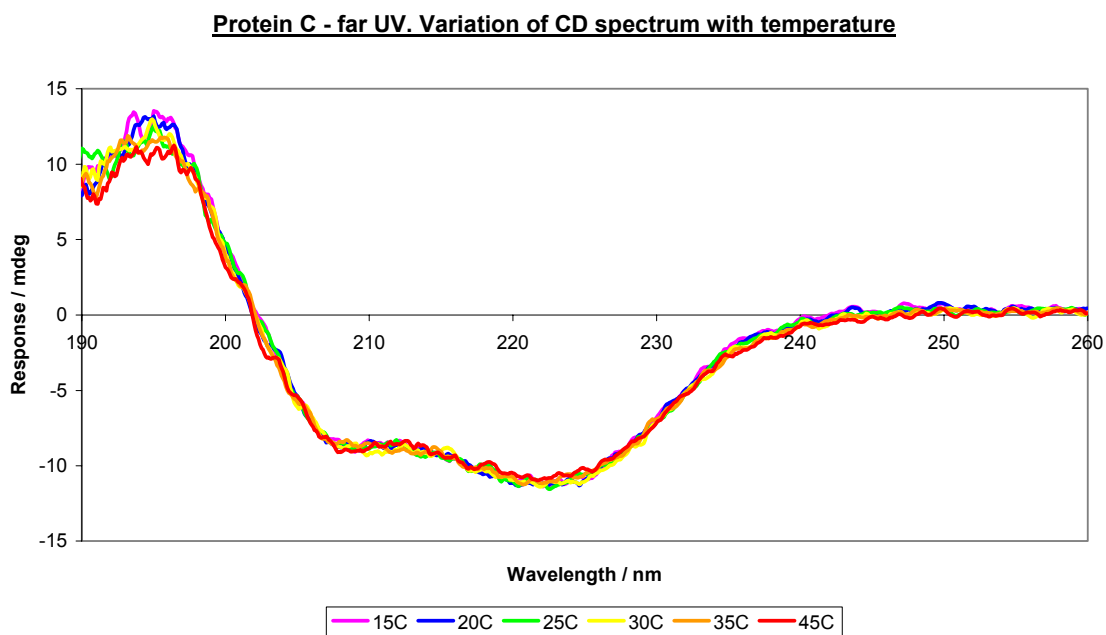


Figure 4.39 Variation of CD with temperature. Protein 'C', far UV.

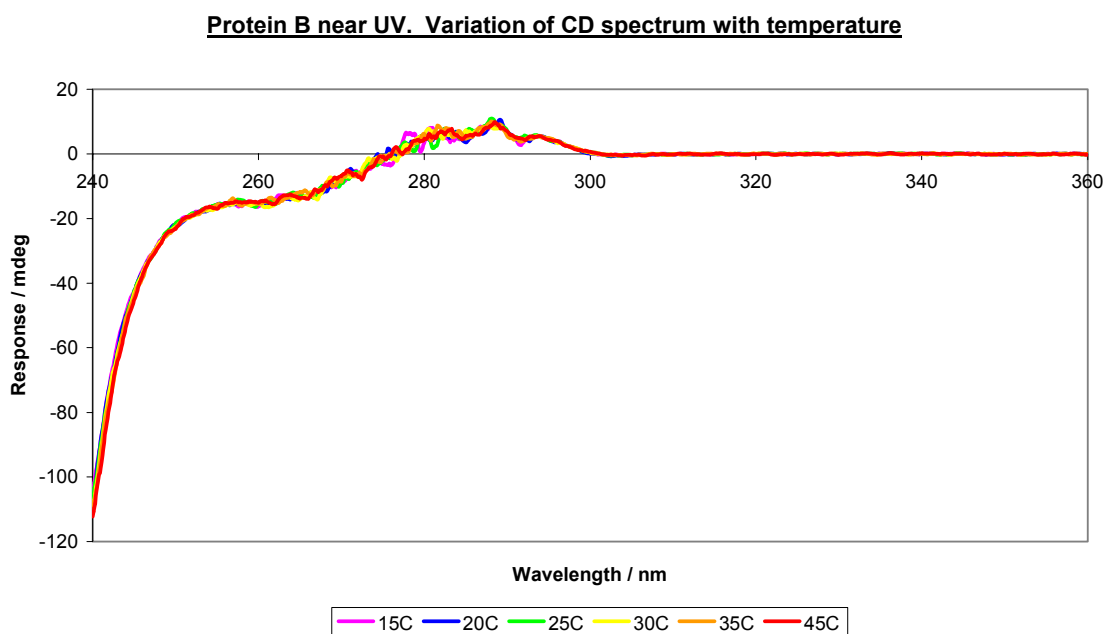


Figure 4.40 Variation of CD with temperature. Protein 'B', near UV.

Protein C - near UV. Variation of CD spectrum with temperature

Figure 4.41 Variation of CD with temperature. Protein 'C', near UV.

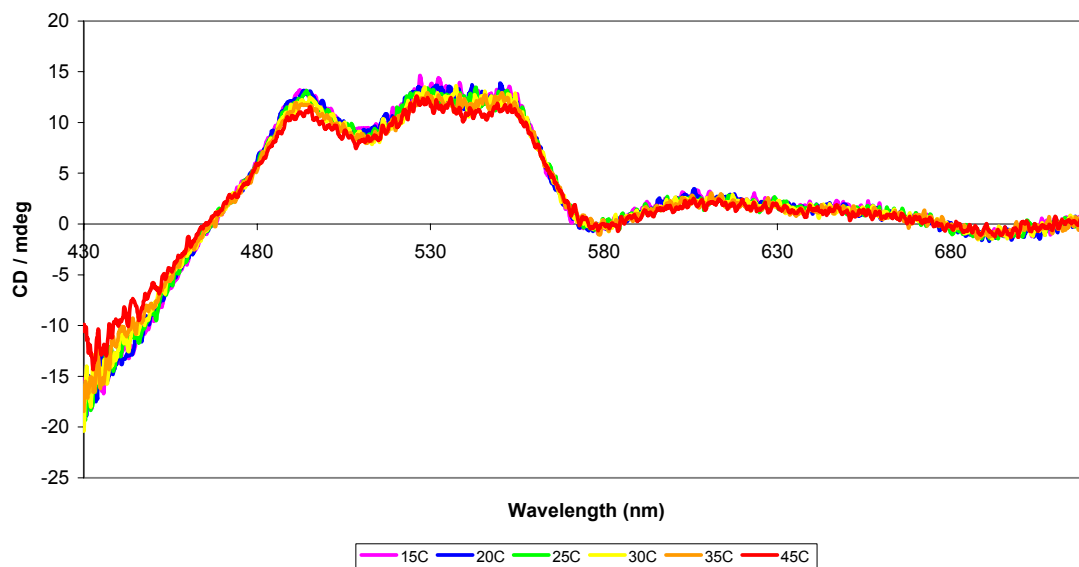
Protein C - visible region. Variation of CD spectrum with temperature

Figure 4.42 Variation of CD with temperature. Protein 'C', visible region.

In the far-UV spectra, small differences can be seen between spectra measured at different temperatures. In the near-UV, small differences can be seen for protein B at the lowest wavelengths, and clearer differences can be seen for protein C in this region, especially in the 240-250 nm range. In the visible region (studied for protein C only) there is a clearer (albeit still relatively small) progressive change with increasing temperature, although, as for the other regions, very little, if any, real difference over the typical room-temperature range (20 – 25 °C).

4.4.4 Conclusions

Although these experiments show some differences apparently due to the temperature of measurement, these do not account for the large differences seen between spectra, especially considering that most samples will have been measured in a more limited temperature range than even the narrow range studied here.

Hence, the principal differences seen between instruments, or between the measurements made on returned samples at NPL, are not likely to be due to differences in the temperature at which the proteins were measured.

4.5 Examples of unusual data

Efforts were made to obtain data that was comparable as possible between instruments. However, certain factors prevented this from being achieved in all cases, and consideration of some of these can be instructive.

4.5.1 Scan parameters

For CD instruments used in a scanning mode (as opposed to collecting data at each wavelength before moving to the next), the spectra obtained should be essentially independent of the particular combination of scan speed and response time chosen, as long as these adhere to certain guidelines (detailed in the accompanying Best Practice Guide). However, in practice this is not always the case, as can be seen with Instrument B. This instrument is relatively old and although its optics are comparable with modern instruments, problems with its electronics mean that it is more sensitive to scan speed and response time, than is usually the case. Hence when measuring at scanning speeds greater than 20 nm/min, a response time of less than 1 sec needs to be used with this instrument, otherwise spectra can be distorted.

To demonstrate this effect, two sets of data were provided from this instrument, referred to here as B(1) and B(0.25). B(1) spectra were obtained using the parameters specified in the instructions sent to participants, which included a 50 nm/min scan speed and 1 sec response time. B(0.25) spectra were obtained by lowering the response time to 0.25 sec. The B(1) spectra are clearly blue-shifted compared to the B(0.25) spectra, as seen in the example shown in Figure 4.43; they are also blue-shifted compared to the majority of other instruments in this study, as seen in Figure 4.36.

A similar result was seen in instrument H. Most spectra on this instrument were collected using the scan parameters recommended in this study; however, a far-UV spectrum of protein C was also collected using the standard parameters used on that instrument (10 nm/min scan speed, 2 sec response time), which are quite different from those suggested in the protocol for this study. This resulted in a blue-shift akin to that seen for instrument B, as shown in Figure 4.44. This is likely to reflect a similar problem to that discussed above for instrument B, as instruments B and H are both the same model.

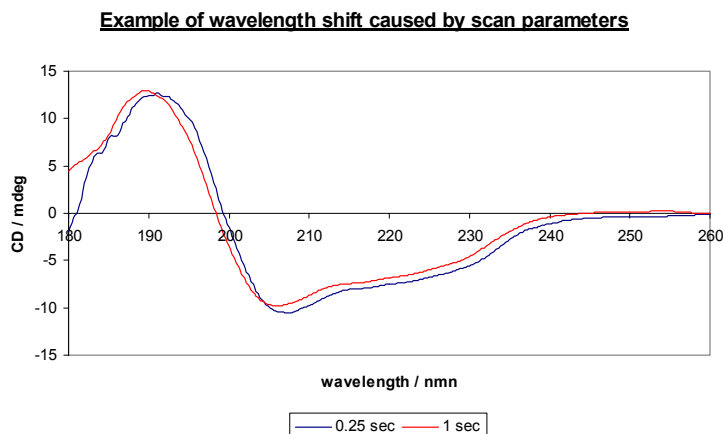


Figure 4.43 An example of the 'blue-shift' effect of scan parameters on instrument B.

Far-UV CD of protein B (baseline subtracted) measured with a response time of 1 sec (as specified in the inter-comparison study) or 0.25 sec. Note that in this spectrum, as well some of the other B(0.25) spectra, smoothing was applied by the participating laboratory after baseline subtraction; this form of the data was used here for plots and analysis, as it has not caused spectral distortion.

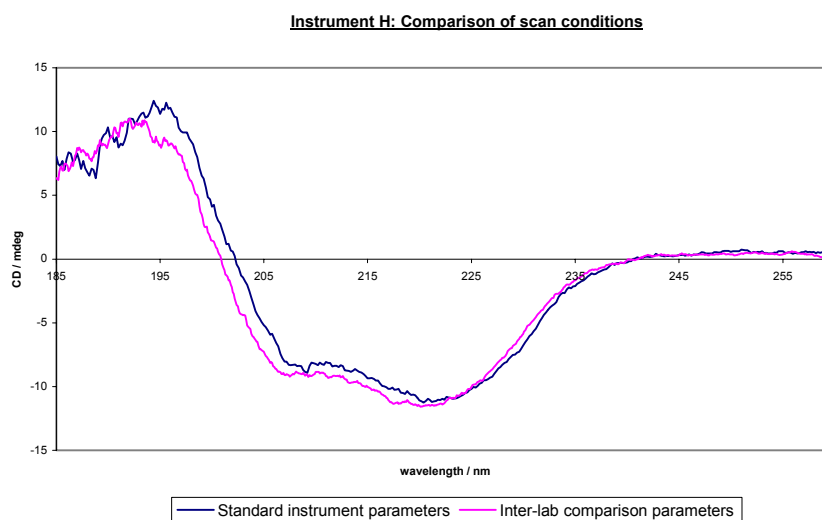


Figure 4.44 Comparison of protein C spectra measured using standard scan parameters and the parameters normally employed for this instrument.

Note how the former spectrum is blue-shifted with respect to the latter. See also Figure 4.43.

4.5.2 Excessive noise levels

Instrument S had lacked proper usage and maintenance. This led to a deterioration in the quality of the data produced by the instrument, and in particular to its producing data with an unusually high noise level in the far-UV region, as shown in Figure 4.45 (only far-UV spectra were measured on this instrument). Due to these problems, protein spectra from this instrument were not included in the overall analysis of the inter-laboratory data; however, ACS(A) spectra were analysed and form part of the ACS data presented in section 4.1, as noise levels for this sample in the near-UV region (where the positive peak of ACS is found) were low, aided by the presence of a high CD signal. This data provides a cautionary example of the importance of proper

running and maintenance of a CD instrument, e.g. of providing an adequate nitrogen supply.

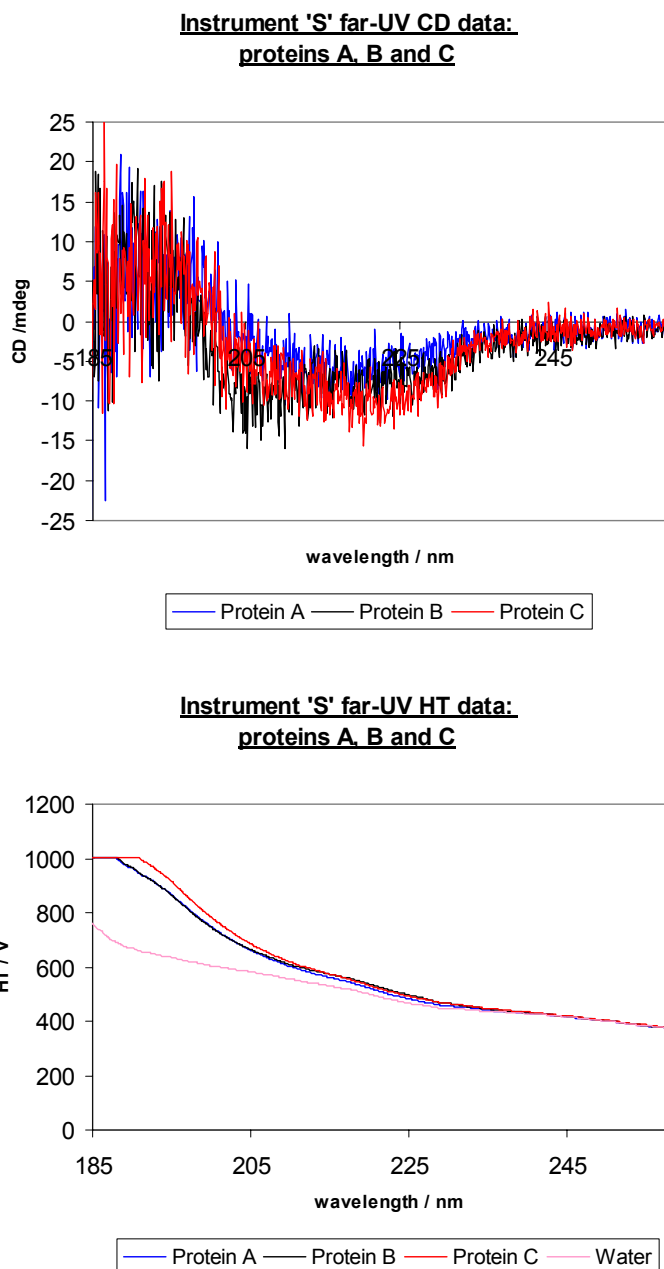


Figure 4.45 Example of the potential results of incorrect usage and lack of proper maintenance of a CD instrument.

Upper: Baseline-corrected far-UV CD spectra for instrument S (average of 6 scans each). Note the very high level of noise. **Lower:** Corresponding HT spectra from four individual scans.

4.6 Conclusions

This study has highlighted some of the current difficulties in obtaining comparable CD spectra from different instruments, and possible solutions to some of these problems.

It is encouraging that, with some exceptions, the many instruments used in this study gave spectra of broadly similar shape, such that most of the variation between spectra obtained on different instruments could be corrected for by a wavelength-independent scaling or shifting of the spectra using ISAM, a software tool developed as part of this project (note that other methods, such as a type of mean centring, are available to normalize for scaling differences (discussed in Appendix C), although these will not account for wavelength shifts.) Furthermore, it is encouraging that instruments were found to be more closely matched than expected in terms of their calibration state.

This study has highlighted the contribution of instrument calibration and, in particular, cell path length, to variation between instruments. Hence to improve comparability, it will be important not only to improve the accuracy of calibration of CD instruments (e.g. through the development and use of non-chemical calibration methods, as discussed elsewhere), but also to measure cell path lengths, especially for short cells used in the far-UV region. This is especially important if one bears in mind that this study was performed under controlled conditions, where sample concentration and batch-to-batch variation was *not* a variable: in a QC application where such variation is possible or indeed likely, it will be particularly important to minimise the above 'non-sample-related' effects.

We have demonstrated that ISAM can be useful for the alignment of real protein spectra, thereby helping to reduce unwanted scaling and offset effects; its operation is discussed in more detail in another Appendix. However, such adjustments did not account for *all* the inter-instrument variation, demonstrating some of the limitations in the current form of this software. For example, it may be necessary to allow it to deal with simple baseline differences and to modify it to be able to make use of multi-wavelength calibration information of the type suggested in [5]; in such a case, it may be important to use both positive and negative calibration peaks to account for differing instrument calibration responses to CD signals of opposite sign.

This study was focussed on UK CD users, where most instruments in current use are from a single manufacturer, and therefore it will be important to expand the study to incorporate more instruments from other manufacturers, in order to better understand to what extent instruments from different manufacturers can give comparable results. An international inter-comparison study is currently being planned, which should enable better cross-manufacturer comparison and help in the production of guidelines and data analysis methods to ensure comparability of CD measurements, which will further facilitate the use of CD spectroscopy as a tool in biopharmaceutical analysis and regulation.

5 Acknowledgements

The authors would like to thank all of the many people at the participating laboratories, without whose efforts this study would have been impossible (see Table 2.1).

6 References

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