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Val-CiD Appendix B: The Use of Chemical Calibrants in Circular Dichroism Spectrometers

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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, University of Warwick, 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 B.

This report describes studies undertaken to identify best practice for the calibration of CD instruments using chemical calibrants. Potential measurement issues are identified, and a candidate for an alternative calibrant is evaluated. The recommendations from this study feed into the accompanying Best Practice Guide.

2.2. Scope of report

In order to obtain accurate, consistent measurements from CD instruments, it is important that they be properly calibrated, and that this calibration is checked regularly. The aim of this work was to develop a method for the calibration of circular dichroism instruments that is appropriate for routine laboratory use. Whilst a number of chemicals may in principle be used for this purpose (see, e.g. [1]), the standard method to calibrate CD spectrometers is by using a chiral compound known as ammonium *d*-10-camphorsulphonate, ACS[1] [2].

Section 3 of this report focuses on some of the important practical issues surrounding the use of ACS as a CD calibrant, in order to obtain a better understanding of the reasons for variability and the steps that should be taken to minimise this. Hence we have attempted to assess the influence of experimental error and instrument variation on the comparability of ACS spectra, in order to understand to what extent one can expect a supposedly stable sample to produce the same spectrum under different circumstances.

A number of alternative compounds exist which may be used for calibration of CD instruments; these may complement the use of ACS in certain applications. For example, they may facilitate measurement of calibration at far-UV wavelength peaks, such as D-pantolactone [1, 3]. It is, in general, preferable to calibrate an instrument at a wavelength close to that at which most of the spectra will subsequently be measured, since calibration accuracy can decrease as one moves away from this point. Hence it is useful to have a choice of calibration chemicals available, each with CD peaks at different wavelengths. In addition, it is useful to be able to determine the state of calibration of a CD instrument over a *range* of wavelengths, because such multi-point calibration has been shown to be important in the standardization and comparability of spectra obtained on multiple instruments [4].

[1] ACS is in fact a secondary standard to the primary standard camphor sulfonic acid, which is extremely hygroscopic and therefore impractical as a routine calibrant.

One such alternative to ACS is the chiral ruthenium complex, $[Ru(phen)_3](ClO_4)_2$ ('phen' = 1,10-phenanthroline), the crystal structure of which has been determined [5]. Section 4 of this report details an evaluation of its properties as a CD calibrant.

3. ACS: The standard CD Calibrant

3.1. Introduction

The principal requirements for a CD calibration chemical standard are that it should: be water-soluble (ideally), be available at high chemical and enantiomeric purity, have simple sample handling requirements (e.g. not hygroscopic), be stable in solution, and possess a large CD signal in the wavelength region of interest, without too high an absorbance. These features are displayed by ammonium dextro-(+)-camphor-10-sulphonate (ACS), so it has traditionally been used for calibration of CD instruments [2]. ACS has a ketone in its structure (Figure 3.1(a)), which absorbs in the UV/Vis region (Figure 3.2). The related molecule, *d*-10-camphorsulphonic acid (CSA, Figure 3.1(b)) has also been used as a standard; however, CSA is hygroscopic and therefore it is very difficult to avoid concentration errors in preparation of a standard solution, and so ACS is generally used instead.

To study ACS and its use for CD calibration, a number of aspects were considered, including choice of instrument parameters, working temperature, sample concentration and long-term stability of the sample, day-to-day variation and cuvette path length. Data sets have been collected on two different instruments to enable inter-instrument comparison. Stability and reproducibility of ACS spectra, and instrument performance, were generally assessed by measurement of ellipticity and wavelengths of signal maxima for ACS in water.

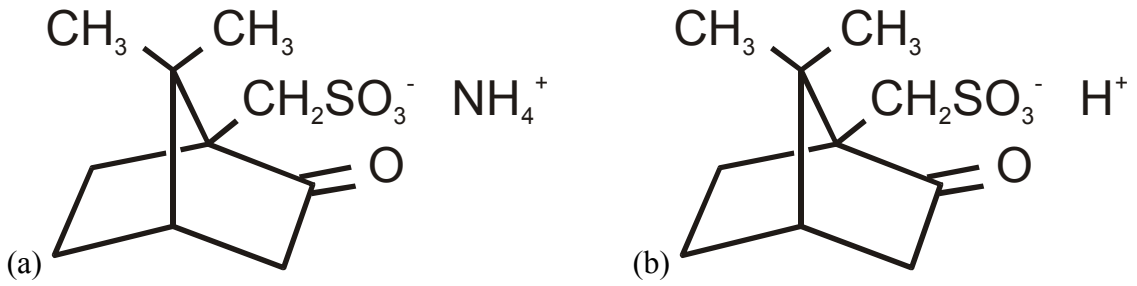


Figure 3.1 (a) Ammonium dextro-(+)-camphor-10-sulphonate (ACS). (b) Dextro-(+)-camphor-10-sulphonic acid (CSA).

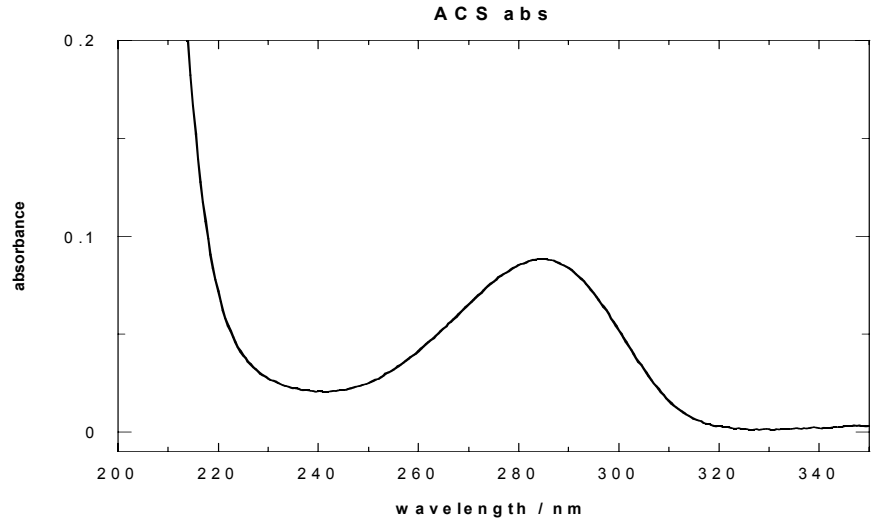


Figure 3.2 UV absorption spectrum of ACS (0.06% w/v), 1 cm path length.

3.2. Methods and Materials

3.2.1. Instruments

The CD spectra reported here were recorded on Jasco spectropolarimeters, models J-600 and J-715. A range of parameters is available on these instruments, including wavelength scan range, spectral bandwidth, response time, scanning speed, step resolution and sensitivity (although in practice 'sensitivity' comes down to a choice of high or low sensitivity as the data collection is unaffected by other choices). One of the goals of this work was to select optimal practical parameters for a laboratory endeavouring to analyse samples as quickly as possible.

As these studies were performed using CD instruments manufactured by Jasco, some of the results will be primarily applicable only to such instruments; for example, the continuous-scanning mode typically used in Jasco CD instruments is not used in all CD instruments, and so optimisation of scan parameters will generally require different considerations for non-Jasco machines. However, the issues highlighted by this study, and the methods to study them, should in large part be readily transferable to non-Jasco instruments. And of course, studies on the stability of ACS itself are independent of instrument manufacturer.

3.2.2. Materials

3.2.2.1. **Ammonium dextro-(+)-camphor-10-sulphonate**

ACS solutions were prepared by accurately weighing 60 mg of ACS and making up to volume with 18.2 MΩ (ultrapure) water in a 100 ml volumetric flask. The solution concentration is then described as 0.0600% (w/v). It appears that such a standard solution in a closed glass vessel can usually be stored for at least eight months, [6] and probably years, in a refrigerator (4^{o}C) (no change in spectral properties were observed for a 4-year-old standard solution compared with a fresh one; results not shown). However, microbial contamination can degrade the solution and prevent it from showing such stability.

3.2.2.2. Potassium chromate (K_2CrO_4)

A solution of potassium chromate was used to assign the path lengths of cuvettes. A 0.2 M potassium chromate solution was prepared by accurately weighing 0.971 g potassium chromate into a 25 ml volumetric flask. Approximately 10 ml of water (18.2 MΩ) and 1 pellet of potassium hydroxide were added, and the sample made up to volume when it had cooled to room temperature. This concentration is appropriate for ~ 0.001 cm path length cuvettes. Serial dilutions into volumetric flasks were undertaken to provide solutions for longer path length cuvettes – see Table 1. The diluted standard is not stable unless additional KOH is added.

3.2.3. Potassium chromate method to determine path length

The path lengths of different cuvettes were determined using a Jasco V-550 UV/Vis spectrometer. This method is based on absorbance measurements of potassium chromate solutions at 372 nm, where the extinction coefficient is 4830 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$. All measurements were performed in rectangular quartz cuvettes with Teflon stoppers. For consistency of measurements, it was essential that the cuvettes be assembled and inserted in the same orientation (vertical and front to back) each time.

3.2.4. UV/Visible absorbance measurement protocol

a) Cuvettes were washed three times with pure water (18.2 MΩ), then three times with a volatile solvent such as acetone, and dried by blowing air or nitrogen into the cuvette.

b) Instrumental parameters were set to be:

wavelength range:	600–275 nm
scanning speed	200 $nm \cdot min^{-1}$
resolution	0.5 nm
bandwidth	1.0 nm
response	fast

c) A baseline was collected every day before the measurements.

d) At least three scans were collected for each cuvette with the appropriate solution, as summarised in Table 1. The path length was then calculated for each scan, and finally the three results were averaged.

	Nominal path length of cuvette	*Sample volume required / ml*	*K_2CrO_4 concentration (mol/dm^3)*	*Number of measurements*
Rectangular quartz cell (with Teflon stopper)	1 cm	2	0.0002	3
	2 mm	0.4	0.0002	3
	1 mm	0.2	0.002	3
	0.5 mm	0.1	0.002	3
	0.1 mm	0.04	0.02	9
	0.01 mm	0.04	0.2	12

Table 1 Method for path length determination.

Nominal path length	*Cuvette number*	*Absorbance*	*Real Path length / cm*
1 cm	1G	0.98 ± 0.04	1.018 ± 0.004
	6Q	0.951 ± 0.002	0.984 ± 0.002
0.5 cm	S	0.464 ± 0.001	0.481 ± 0.001
	R	0.460 ± 0.001	0.476 ± 0.001
2 mm	12Q	1.913 ± 0.003	0.202 ± 0.003
	13Q	1.9106 ± 0.0002	0.198 ± 0.002
1 mm	7C	1.0209 ± 0.0003	0.1057 ± 0.0001
	7Q1	0.992 ± 0.001	0.1026 ± 0.0001
	7Q2	0.947 ± 0.002	0.0981 ± 0.0001
	10	0.9722 ± 0.0004	0.1006 ± 0.0001
0.1 mm	14	1.096 ± 0.005	0.01135 ± 0.00005
0.01 mm	15	0.68 ± 0.05	0.00070 ± 0.00006
0.1 mm	14	1.10155 ± 0.003	0.0114 ± 0.00003
0.01 mm	15	0.74± 0.01	0.00077 ± 0.00009

Table 2 Path lengths of different cuvettes used in this work.

Approximately 20 measurements were undertaken by 2 operators for the demountable cuvettes.

3.2.5. Interference method to determine path length

An alternative method was also used to determine the path lengths of *empty* short path length cuvettes. The cell was assembled taking great care to keep the faces parallel. A UV/Vis scan of the empty cell was taken in transmission mode from 402 to 665 nm. The path length was determined from the following equation:

$$L = \frac{n\lambda_1\lambda_2}{2(\lambda_2 - \lambda_1)}$$

where L is the path length in nm, λ_1 and λ_2 are the start and end wavelengths in nm and n is the number of fringes between λ_1 and λ_2. A typical spectrum is shown in Figure 3.3. Both methods were then compared for 0.1 mm cuvettes and shown to give similar answers. For example, the real path length obtained by the 'empty' cuvette method is 0.119 ± 0.003 nm, which varies by ~4 % from the filled-cuvette average. However, assembling the cells, especially the 0.001 cm one, is operator and measurement to measurement dependent when there is a sample involved.

The results of a further study of path length measurement, involving a larger number of cells and a further method, are presented in the accompanying Best Practice Guide. Together, these show that path lengths can vary significantly from the nominal value, and demonstrate possible experimental means of measuring them. Such measurements can be important for accurate work, especially when working with short (< 1 mm path length) cells, where manufacturing tolerances are relatively poor.

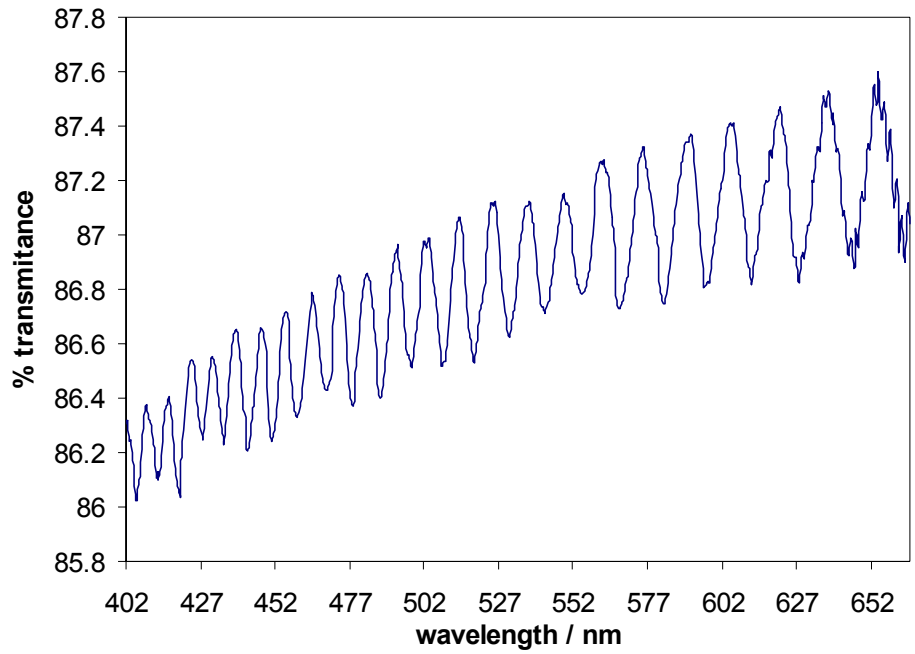


Figure 3.3 UV/Vis spectrum of an empty 0.1 mm cuvette, showing typical interference fringes.

3.2.6. Optimisation of instrument parameters

An important initial requirement for this study was the establishment of an efficient method to obtain good CD spectra of 0.06 % ACS, for which it was necessary to optimise all instrumental parameters. Spectra were therefore collected under several conditions, until the best results were obtained as judged by a reasonable signal-to-noise ratio (S/N) with minimum measurement time. The J-600 instrument was used for most of this work as it will provide the lower bound for instruments used in a range of laboratories, being a somewhat older machine.

1) *Wavelength range (nm)*: The "start" wavelength (for Jasco instruments, this is always the upper end of the wavelength range) in CD needs to be at least 20 nm, and preferably 50 nm, beyond the wavelength where the peak starts to rise. In general the "end" wavelength was determined when the high-tension voltage reached approximately 575 V (see section 3.3.5.2).

2) *Sensitivity (mdeg)*: This depends on the intensity of the larger peak. Note that this is not a necessary parameter to set on most, if not all, non-Jasco instruments.

3) *Scanning speed (nm/min)*: this is one of the most important parameters to obtain a good ratio between signal and noise. It is dependent on response time and bandwidth, and must be chosen carefully so as not to distort the spectrum whilst at the same time minimising run times.

4) *Bandwidth (nm):* The standard bandwidth is 1 nm, but unless peaks are very sharp, we have concluded that measurements with a bandwidth of 2 nm improve the S/N ratio of the spectrum, as more light is incident on the sample.

5) *Response, r (sec):* After defining the other parameters, the response is set so as to optimise the signal of the spectrum without distorting it. S/N is proportional to $\sqrt{r}$.

6) *Accumulations, n*: In the terminology of Jasco instruments, this is the number of repeat scans averaged to give each spectrum. S/N is proportional to √n. However, more scans take more time to measure.

Based on the above considerations, the parameters adopted for obtaining ACS spectra on the J-600/J-715 with an acceptable S/N ratio, in minimum time and without any spectral distortion, are shown in Table 3. Note that scan parameters differ depending on the path length: different information is obtained from each type of experiment.

	Cuvette path length	
Parameters	1 cm	1 or 2 mm
ACS concentration	0.0600% (w/v)	0.0600% (w/v)
Start wavelength / nm	350	350
End wavelength / nm	275	183
Step resolution J-600 / J-715 / nm	0.4 / 0.5	0.4 / 0.5
Speed / nm min^{-1}	100	100
Response / s	1	1
Bandwidth / nm	1.0	2.0
Sensitivity / mdeg	200	100

Table 3 The scan parameters adopted for collecting ACS data on the J-600/J-715.

Typical ACS spectra in 1 cm, 1 mm and 2 mm cuvettes are shown in Figure 3.4. All spectra show a positive maximum at ~290 nm and the shorter path length spectra show a negative minimum at ~191 nm. A 2 nm bandwidth was used for the shorter path length cuvettes as the light throughput at low wavelength was enhanced. It was not necessary for the 1 cm path length wavelength range and so 1 nm was used. For a 1 mm cuvette, a set of parameter conditions equivalent to those of Table 3 would be 200 nm/min, 0.5 s response and 8 scans. However, it takes time for the instrument to reset between scans and so 4 scans was chosen as a reasonable compromise between this loss of time, and the advantage of averaging out any short-term fluctuations.

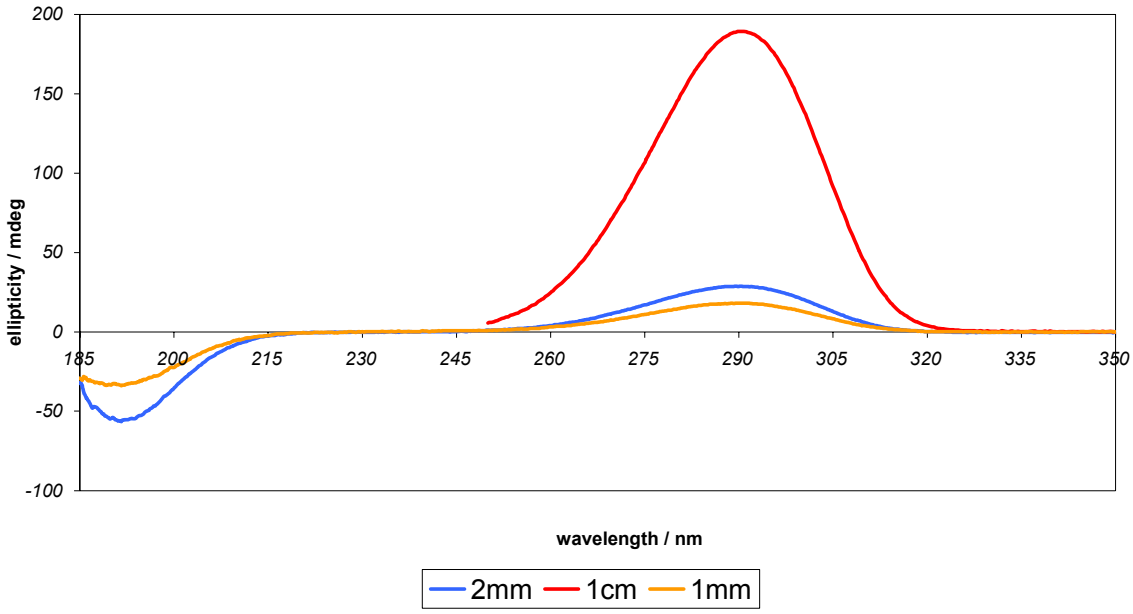


Figure 3.4 CD spectra of ACS in cuvettes of different path length.

Nominal path length is shown in the figure. The spectra were collected using the parameters shown in Table 3.

CD measurement protocol for ACS study

The following protocol was followed to collect spectra for the purpose of the stability studies shown here.

a) Determine the path length of cuvettes as outlined above.

b) Select two cuvettes with equal path lengths, whose CD spectra when filled with water are the same.

c) Fill one of the cuvettes with water and the other with the ACS solution at the required concentration. Seal the stoppers with Teflon tape and keep the cuvettes in the refrigerator between measurement sessions.

d) Measure the spectra of ACS and of water, and subtract that of the water from that of the ACS. Use the parameters summarised in Table 3. If necessary, zero the spectrum in the long-wavelength region of the spectrum.

3.3. *Results and Discussion*

3.3.1. Baseline check

One of the issues to be considered with calibration is how often a baseline spectrum needs to be collected. The variation of the instrument baseline was assessed by running a full baseline (190–350 nm) without any sample, but with the same cell holder in position as is used for CD analysis. A CD baseline spectrum in the J-600 was recorded every hour for 10 days. From this we concluded that the J-600 requires a baseline to be collected every 3 hours for measurements below 210 nm, as there seemed to be a gradual change as well as the run-to-run variation (Figure 3.5). The J-715 was more stable, and the frequency with which baseline measurements are required depends on how closely the operator wishes to interrogate the data.

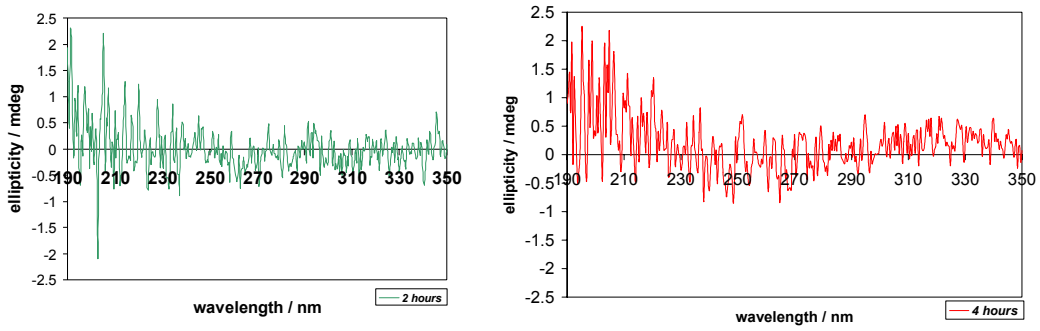


Figure 3.5 Spectra showing single-scan baselines at two-hour intervals.

Scans were collected using the step resolution, scan speed, response and bandwidth parameters of Table 3 (for a 1cm cell).

3.3.2. Cell holder

Another factor considered was the role played by the cell holder. Three cell holders were available for consideration. CD spectra of water-filled cuvettes were collected for each cell holder, with the same instrumental parameters. The difference in the spectra of Figure 3.6 shows that the cell holder does affect the spectrum obtained. In fact, the difference is due to the different cell holders presenting different parts of the cuvette to the light beam, which might be a more general problem: cuvettes must be placed in exactly the same place when measuring both sample(s) and baseline (ideally this requires the same cuvette orientation to be used, although in practice, as light beams are usually central to the holder, this is not imperative[2]). It is also important that the same size of light beam (that is, the same bandwidth setting, and same mask in place) is used for sample and baseline.

[2] However, some cells may exhibit circular birefringence; in this case the orientation of the cell is critical.

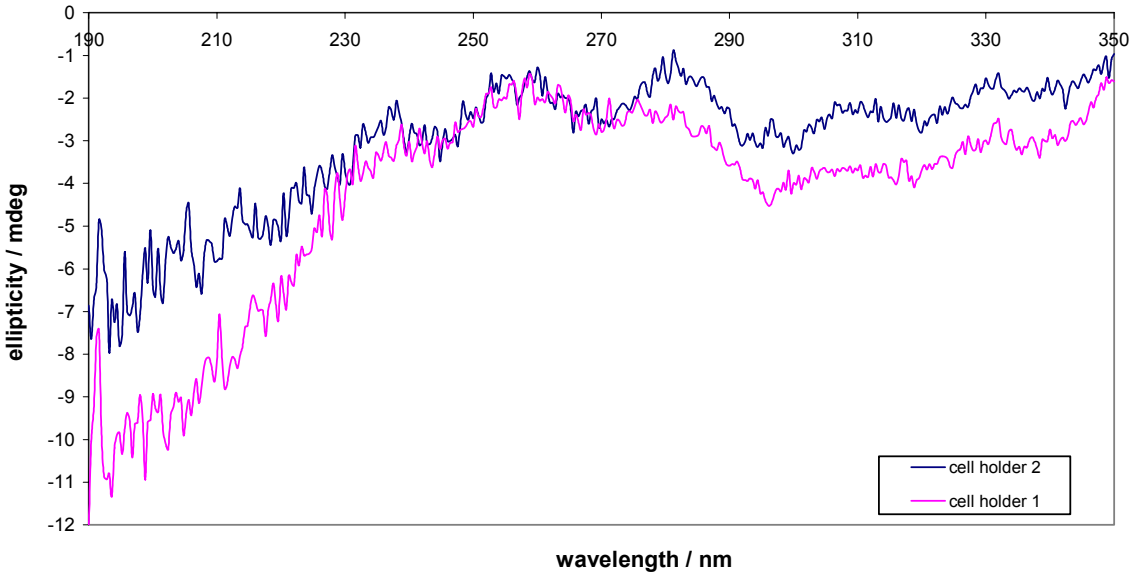


Figure 3.6 Spectra of the same water-filled cuvette in two different cell holders.

3.3.3. Reproducibility of the ACS CD spectrum

As noted above, it is assumed that ACS follows the Beer-Lambert Law, i.e. that the signal is proportional to the sample concentration (and path length). To check this, different concentrations of ACS from 0.075 % to 0.015 % were made by diluting a 0.075 % stock solution using volumetric glass pipettes and volumetric flasks. Three independent samples were prepared. CD spectra were collected in a 1 cm path length cuvette and a water baseline collected using the same cuvette was subtracted. The instrument parameters used in this study are those of Table 3. The data are summarised in Table 4 and Figure 3.7. It is clear from this figure that over this concentration range, the ACS CD signal obeys the Beer-Lambert Law within experimental error, as the CD signal increased linearly with concentration in each of the three experiments.

	ACS % conc. (w/v)	θ / mdeg	ΔA / 10^{-4}
Experiment 1	0.0748	23.1400	7.02
	0.0598	18.5696	5.63
	0.0449	14.0629	4.26
	0.0299	9.3933	2.85
	0.0224	7.3643	2.23
	0.0150	5.4149	1.64
Experiment 2	0.0752	23.3474	7.08
	0.0602	18.7584	5.69
	0.0451	14.0803	4.27
	0.0301	9.7930	2.97
	0.0226	7.3986	2.24
	0.0150	5.1556	1.56
Experiment 3	0.0750	23.2073	7.04
	0.0600	18.5080	5.61
	0.0450	14.2372	4.32
	0.0300	9.4444	2.86
	0.0225	7.5396	2.29
	0.0150	5.2186	1.58

Table 4 Magnitude of ACS CD signals at 290 nm as a function of concentration.

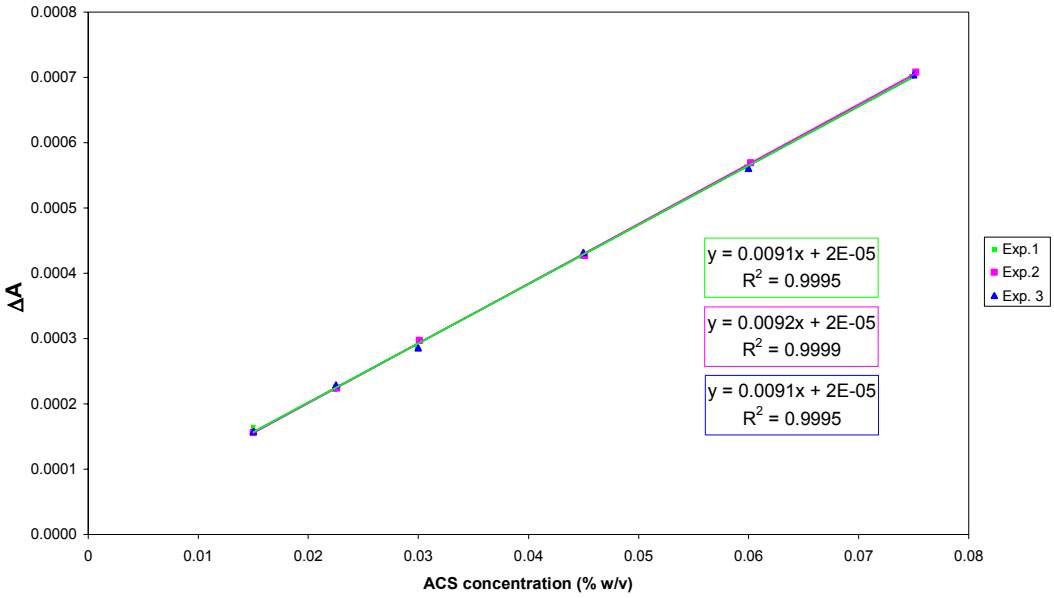


Figure 3.7 Plot of ΔA as a function of concentration.

The 'best-fit' straight lines are shown, together with their equations and the R^2 values: these indicate that the data fits very well to a straight line (R^2 =1 for a perfect straight-line fit), indicating compliance with the Beer-Lambert Law.

3.3.4. Day-to-day variation

The data from the CD instruments were suspected to vary from day to day. For this reason, we assessed the ellipticity of ACS on a daily basis. CD spectra of ACS (0.06 % w/v) were obtained with both the J-600 and the J-715 at room temperature (22°C), using both a 1 cm and a 1 mm path length cuvette and the Table 3 parameters. These were measured by averaging 3 runs per day; all of them were corrected by subtracting the corresponding baseline water spectrum. The data obtained are summarised in Table 5. Note that the % variation in ellipticity increases in the order, 1 cm positive peak→1 mm positive peak→1 mm negative peak. This is as expected, as the 1 cm positive peak is by far the largest signal and is measured in a region where the absorbance is not particularly high, and so should suffer least from noise; the 1 mm positive peak (although smaller in magnitude than the 1 mm negative peak) is measured in a region with significantly lower absorbance than the 1 mm negative peak and so should suffer less from noise than the latter; hence the 1 mm negative peak will tend to have the highest noise levels and therefore the greatest % variation in ellipticity.

CUVETTE PATH LENGTH	*INSTRUMENT*	*WAVELENGTH / nm*	*ELLIPTICITY / mdeg*	*ELLIPTICITY variation / %*
1 cm positive peak	J-600 CD	290.3 ± 0.1	187.7 ± 0.8	0.4 %
	J-715 CD	290.6 ± 0.3	190.8 ± 1.0	0.5 %
1 mm positive peak	J-600 CD	289.6 ± 0.1	17.9 ± 0.1	0.6 %
	J-715 CD	290.6 ± 0.2	19.3 ± 0.3	1.5 %
1 mm negative peak	J-600 CD	191.3 ± 0.2	-32.7 ± 0.6	1.8 %
	J-715 CD	191.9 ± 0.2	-36.7 ± 1.0	2.7 %

Table 5 Day-to-day variations of the CD of ACS (0.06 % w/v).

The errors quoted are the standard deviations from spectra measured for 10 days.

3.3.5. ACS CD calibration and some instrument performance issues

CD calibration is usually undertaken using ACS 0.06 % (w/v) in a 1 cm cuvette, with the required values being: 190.4 ± 1 mdeg at 291 ± 0.8 nm. This is a single-point calibration, and does not consider whether any sample features, or the wavelength range, may cause a problem. Two particular issues are, where is the sample absorbance too high (and few laboratories have absorbance instruments that work below 190 nm), and is the light scattering becoming too much of a problem below 200 nm? The issues to consider here are the ratios of the 290 and 191 nm ACS peaks and the high-tension (HT) voltages.**Signal ratios**

The ACS spectrum of a 1 mm cuvette containing 0.06% w/v ACS on the J-600 has a negative maximum at 191.3 nm and a positive maximum at 289.6 nm. The ratio of these signals is −1.86 ± 0.05. At the time this work was undertaken, the ratio had a value of −1.94± 0.05 on the J-715. After servicing and when operated with very clean cuvettes, the J-715 ratio was ~ −2.05. Subsequent work on the Daresbury synchrotron CD station suggests a value of −2.15 may be correct. Although the value of this ratio is not established, it is clear that values with magnitude below 2.0 are indicative of light-scattering effects, which may be a function of general light loss and poor performance below 200 nm.

3.3.5.2. High-tension voltage

The effect of the high-tension voltage (HT) of the photomultiplier tube on the CD signal was assessed using ACS samples at different concentrations from 0.6000–0.0144 % (w/v) in a

1 mm cuvette, all with the same instrument parameters; data was collected for four independent measurements. The point at which the Beer-Lambert Law broke down was determined — from this one can determine the maximum operating HT value. The J-600 has a maximum HT operating value of 574 ± 5 V, and the J-715 maximum value was 587 ± 5 V. Thus, for these instruments at least, ~600 V may be deemed the maximum 'safe' operating HT voltage. Similar experiments can be carried out for other instruments.

3.3.6. Influence of temperature

As part of a separate stability study on ACS, the effect of temperature on the CD signal intensity of ACS was investigated. A new sample of ACS was prepared (0.06 % w/v); the calibration of the instrument was checked before analysis by measuring the CD signal intensity of the sample in a 1 cm path length cuvette at 290.5 nm at 23 ºC (190.0 mdeg). This is within the calibration limit of 190.4 ± 1 mdeg.

A temperature-ramp experiment was undertaken with the same sample of ACS, starting at 5 ºC and ramping at 1 ºC/min through to 90 ºC; single-wavelength CD measurements were taken every 0.2 ºC (Figure 3.8). Although this is not baseline-corrected data, this is not expected to have a significant effect on these results; furthermore, other experiments (such as that discussed below) involving baseline-corrected full scans showed a clear effect of temperature on measured ACS signal.

It can be seen that at 5 ºC, the CD signal intensity is greater than that allowed for within calibration limits. The signal intensity decreases gradually to 80 ºC, where there is a sudden large drop in intensity. The final CD reading at 90 ºC is 172 mdeg. It is interesting to note that at 23 ºC the CD signal intensity is once again within the calibration limits. After the analysis (once the sample had cooled down from 90 ºC to 23 ºC), a full CD spectrum was collected. This had a CD intensity of 191.6 mdeg at 290.5 nm, which is just out of the calibration limits, suggesting that there may possibly have been some changes to the structure of ACS in solution.

In addition to the above study at a single wavelength, spectral scans were also taken: a full wavelength scan was undertaken every 5 ºC using the usual parameters for the study of ACS in a 1 cm path length cuvette. Figure 3.8 shows the baseline-corrected data, where it can be seen that not only is there a steady decrease in signal intensity but there is also a shift in wavelength maximum of about 1 nm from 5 ºC to 90 ºC. Prior to this analysis being undertaken, a UV spectrum was collected (using a Jasco V-550 spectrometer); this was repeated after the analysis (once the sample had cooled down from 90 ºC to 23 ºC). The results of this are shown in Figure 3.9; there are no obvious differences in the UV data collected.

It was thought that the instability observed throughout the above temperature-stability study could be related to the pH of the solution, and therefore the pH of this ACS solution was measured before and after the analysis. The results are shown in Table 6; a large increase in pH was observed, suggesting a heat-induced chemical change to the ACS.

ACS 0.06 % w/v	*pH at 20 ºC*
Before heating to 90 ºC	6.42
After heating to 90 ºC	8.07

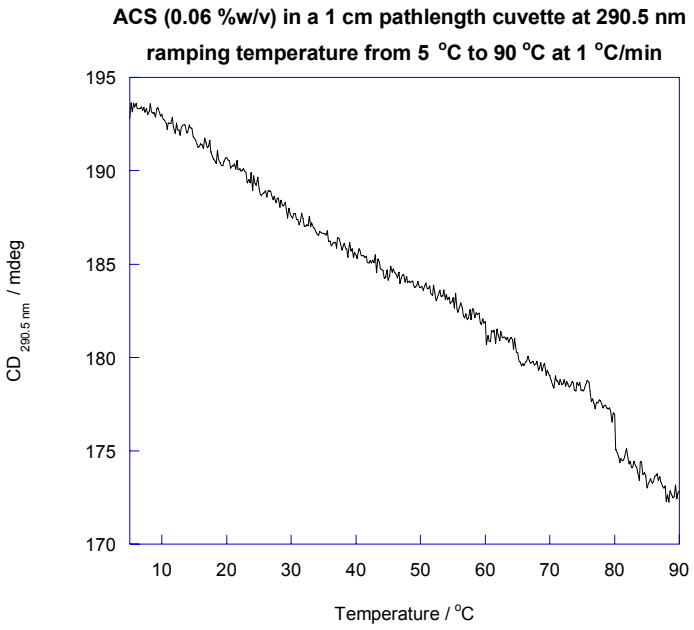
Table 6 The change in pH of a solution of ACS (0.06 % w/v) before and after the temperature-ramp study shown in Figure 3.8.

In a separate experiment focussing on the UV spectrum of ACS, UV spectra were measured using a 0.06% w/v ACS solution. Correct ACS sample preparation was first verified by CD

analysis, which showed a peak at 190.0 mdeg (expected value of 190.4 ± 1 mdeg). Full UV spectra were then measured at 5 °C intervals over the range 5 °C to 90 °C, using a Cary UV spectrometer (Figure 3.10). An extra UV spectrum was also collected at 23 ºC as this is the temperature at which ACS is usually worked with; after the analysis the sample was cooled down to 23 ºC and a further spectrum was collected to evaluate whether any change observed had been permanent or not.

It can be seen from Figure 3.10 that there is a slight shift of up to 2.5 nm at long wavelengths; however, analysis of actual peak positions showed that there is no noticeable shift in the wavelength of the maxima. Comparison of spectra at 23 °C before and after heating showed that the heating had not caused any major change to the ACS solution, as the UV spectra of ACS before and after analysis are quite comparable (data not shown; results were similar to those seen in Figure 3.9). Analysis of peak positions showed that the wavelengths of the maxima are the same before and after heating, although there was a slight increase in UV absorbance. This corresponds well with the observed increase in CD signal intensity, described above.

The temperature-dependence of the ellipticity of a 0.06% solution of ACS, as shown above, may be due to a number of reasons, which includes thermal expansion of glass, thermal expansion of liquids and changes in the solute-solvent interactions which affect the magnitude of the effects which give rise to the CD signal. This effect is large enough that it should not be ignored as part of the calibration process: the difference between the ellipticity measured at 5 °C and at 25 °C is ~ 2%. It is therefore best to always allow ACS samples to reach room temperature before measuring them, or ideally to use a temperature-control system to maintain constant temperature for all CD measurements (i.e. a water-jacketed cell or a Peltier-based system), and to allow the sample adequate time to reach this temperature.



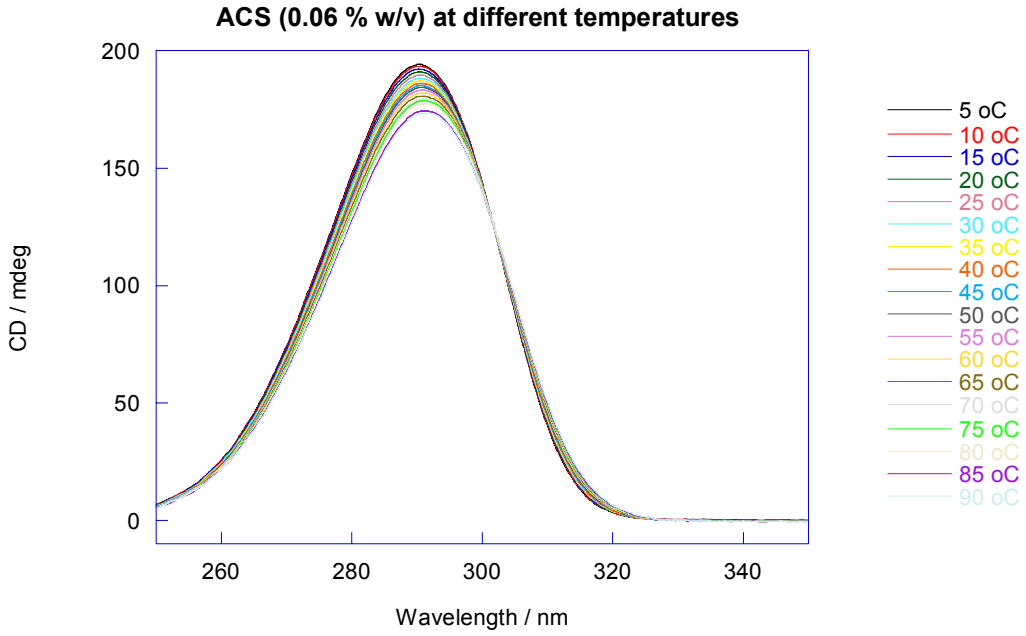


Figure 3.8 The effect of temperature on ACS.

***Upper**: ACS (0.06 % w/v) in a 1 cm path length cuvette at 290.5 nm. The signal intensity decreases gradually to 80 ºC where there is a sudden large drop in intensity.*

***Lower**: ACS (0.06 %w/v) at different temperatures, measured at 5 ºC intervals, showing both a steady decrease in signal intensity and a shift in wavelength maximum of about 1 nm from 5 ºC to 90 ºC.*

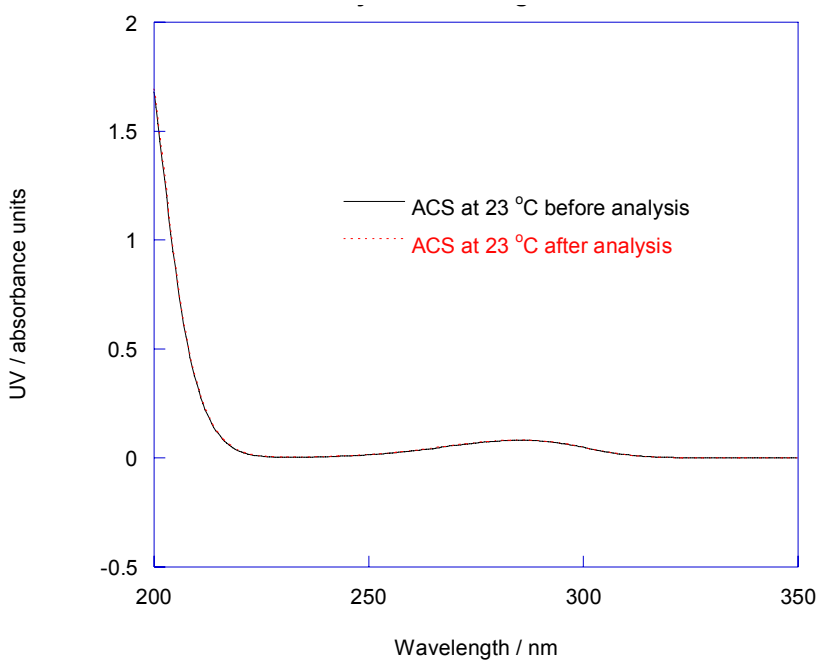


Figure 3.9 Effect of heating on UV absorbance spectrum of ACS.

UV spectra of a solution of ACS (0.06 % w/v) at 23 ºC, before and after heating it from 5 ºC to 90 ºC for the temperature-ramp experiment reported in Figure 3.8. Compare with Figure 3.10.

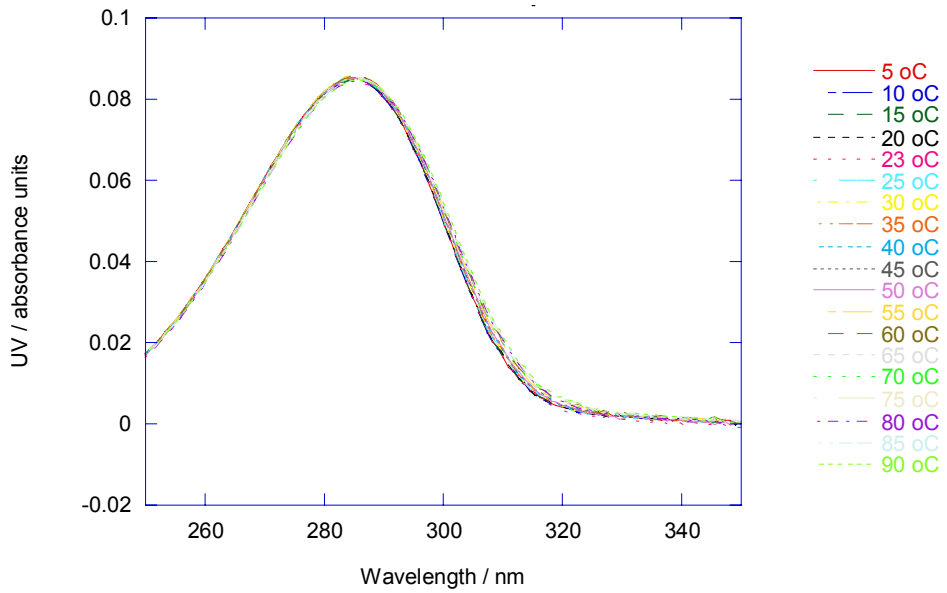


Figure 3.10 UV spectra of ACS at different temperatures.

Absorbance of ACS (0.06 % w/v) was measured in a 1 cm path length cuvette

3.3.7. Stability of ACS under varying storage conditions

In order to study how storage conditions affect ACS, a two-month stability trial was undertaken in which solutions of ACS (0.06 % w/v) were kept under different conditions in sealed 1 cm and 1 mm path length cells, and a range of parameters (including CD signal intensity) was monitored over this time. Time points of analysis were weeks 1, 2, 3, 4, 5 and 9. Storage conditions were: room temperature (in the light); room temperature (kept dark); refrigerator (kept dark); or at 37 °C in an oven (kept dark). A fresh 0.06 % ACS solution was also prepared before each analysis to verify that instrument calibration itself had not drifted (see below).

To ensure that the CD instrument was in calibration before each week's analysis was undertaken, a new ACS sample (25 ml) was prepared and analysed. After use, all of these 'verification' samples were stored in the fridge; at the two-month time point all of these samples were analysed in 1 cm and 1 mm path length cuvettes on both the CD and UV instrument (after first allowing them to warm to room temperature). The scan methods used were the standard ones for the analysis of ACS. pH data was also collected on all samples. The general trend appeared to be that the longer the sample had been left in the fridge, the greater the UV absorbance and pH, suggesting a solution change to the ACS (data not shown). However, the length of storage had no clearly significant affect on the CD peak intensity (above expected random variation).

Figure 3.11 shows that the spectra from the 1 cm cells remained, in most cases, relatively unchanged. However, it is immediately apparent that the sample stored at room temperature in the light exhibits anomalous changes from Week 3 onwards. Fitting of Gaussian curves to the peaks of these spectra allows a more detailed examination. Figure 3.12 shows that all the samples show significant increases in CD by week nine, although in most cases these are quite small – of the order of 2-4 %. However, in the case of the aforementioned sample, there

is a huge increase of the order of 22 % from the baseline value. These results are very hard to explain, particularly as there is not a corresponding increase in absorbance, as one might expect if solvent evaporation was occurring. Note that the sample stored in the fridge in the dark shows the smallest changes. Examination of the peak wavelengths (Figure 3.13) shows that again the changes are mostly small and mostly appear to be due to instrumental drift over time, as they track the behaviour of the reference (fresh) sample. The observed differences are in fact ~ 0.1 nm, which is twice the data pitch, so they probably have no significance.

The spectra obtained in the 1 mm cells (Figure 3.14) show both the positive peak at 290 nm and the negative peak at 190 nm. Much less variation is apparent for all the spectra than is seen in the 1 cm cells. Figure 3.15 shows the changes in the peak CD. Due to the smaller amplitude of the signals, the confidence intervals are relatively larger, and all the sample intensities lie within the confidence limits of the reference sample. The wavelength results are shown in Figure 3.16. Here most of the variation appears to be due to random noise – no consistent patterns emerge.

In summary, there are increases apparent in the 1 cm cell CD for all the samples after 8 weeks. These increases are probably not seen in the 1 mm data because of the lower signal to noise ratio in these spectra. One spectrum shows a 22 % change, however, which would be expected to be picked up in the 1 mm cell also. This may be explained if the result is due, for example, to random bacterial contamination. Further work is needed to investigate this. It is clear, however, that ideally, fresh samples of ACS should be used, or failing this, samples that have been stored for no more then a few weeks at 4°C in the dark.

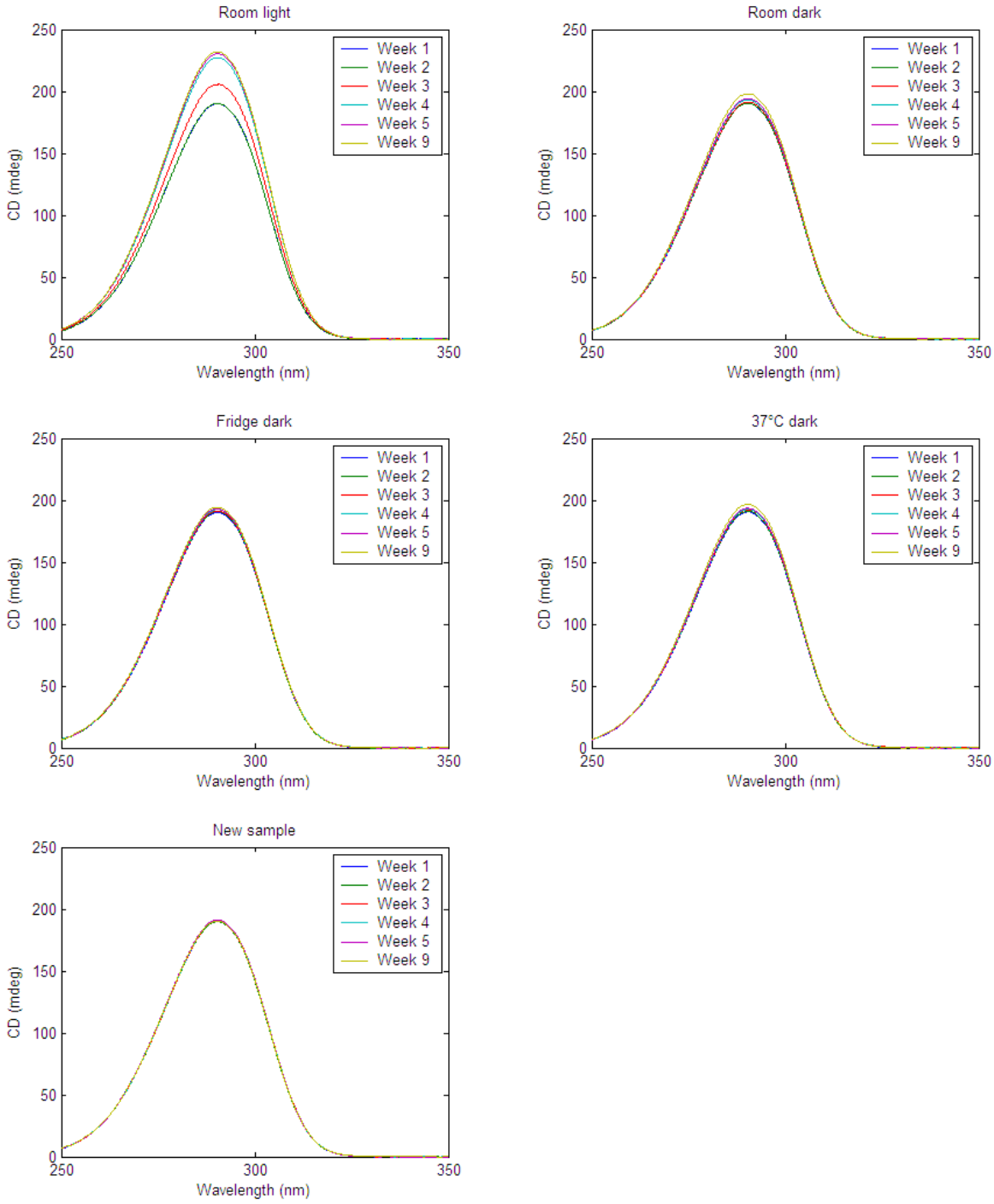


Figure 3.11 Spectra of ACS samples in 1 cm cuvettes.

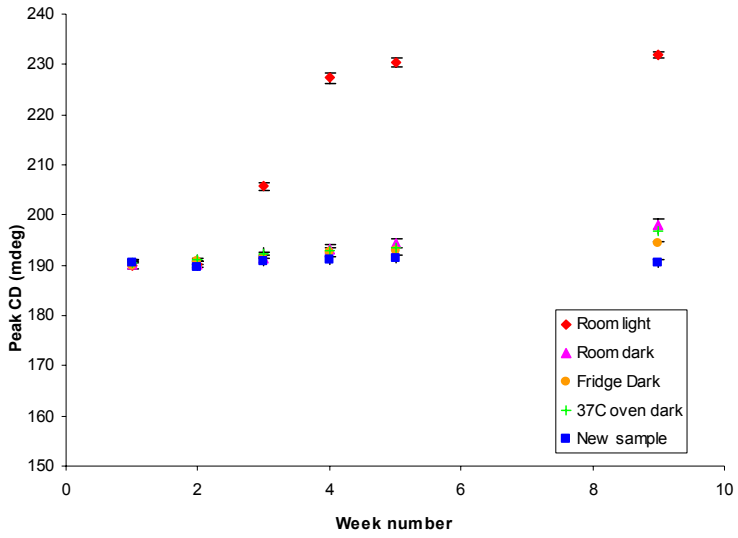


Figure 3.12 Changes in peak CD of samples in 1 cm cuvettes.
Error bars indicate 95 % confidence limits of Gaussian fits to spectra from 285 to 295 nm.

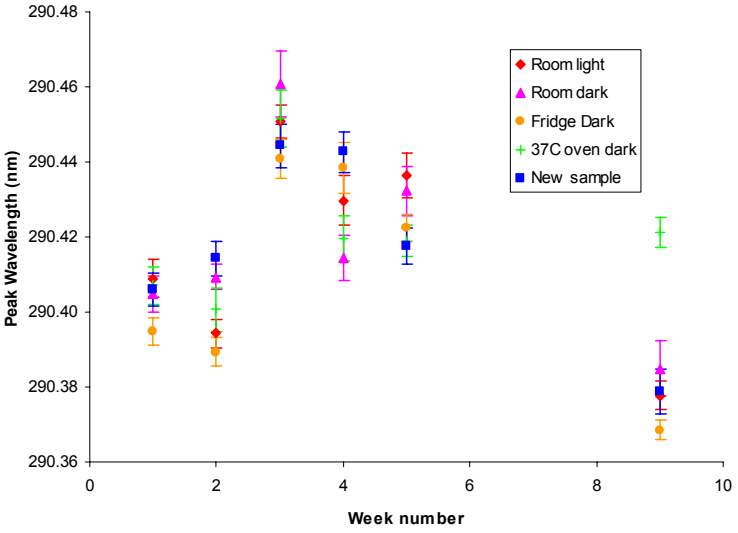


Figure 3.13 Changes in peak wavelength of samples in 1 cm cuvettes.
Error bars indicate 95 % confidence limits of Gaussian fits to spectra from 285 to 295 nm.

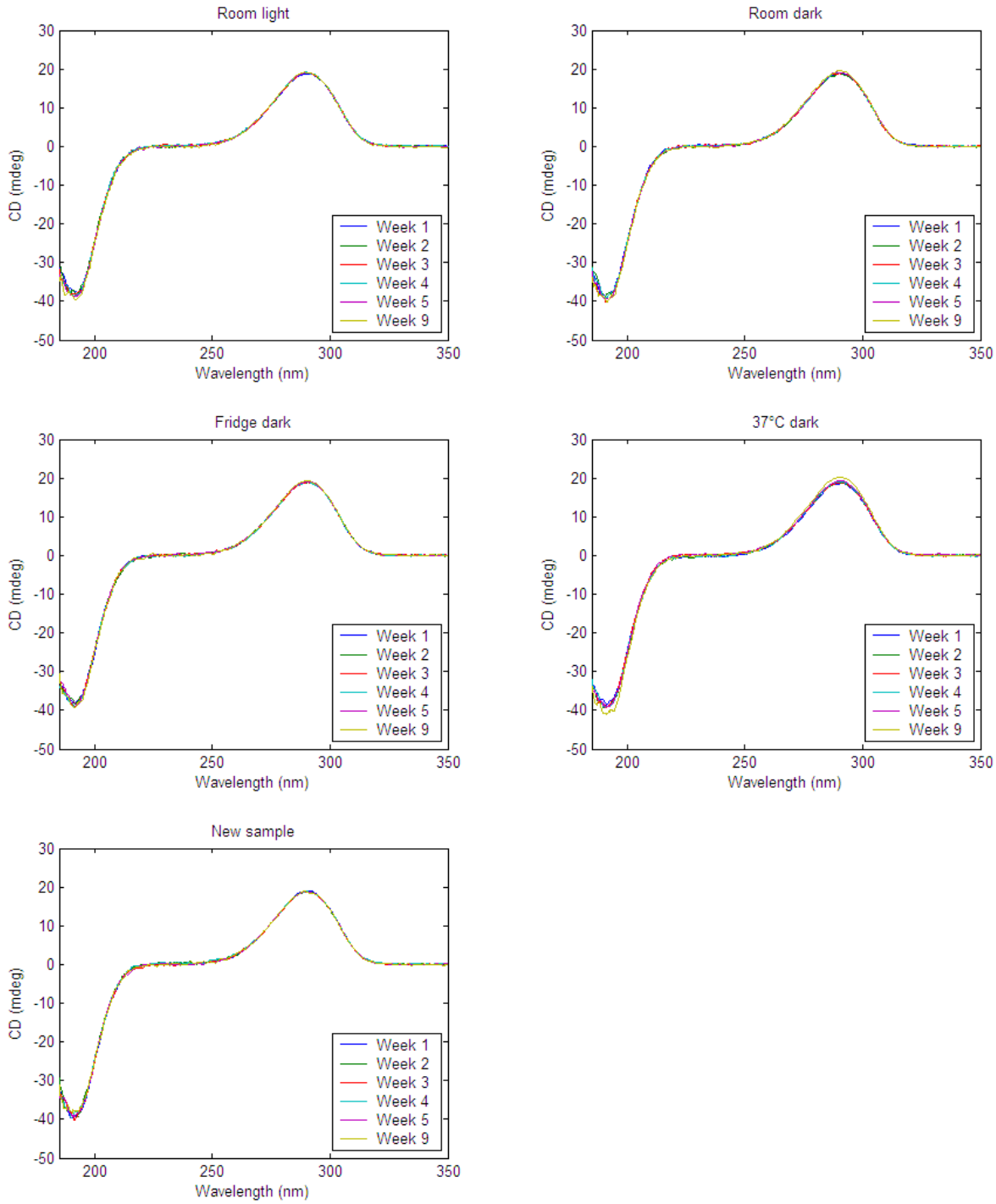


Figure 3.14 Spectra of ACS samples in 1 mm cuvettes.

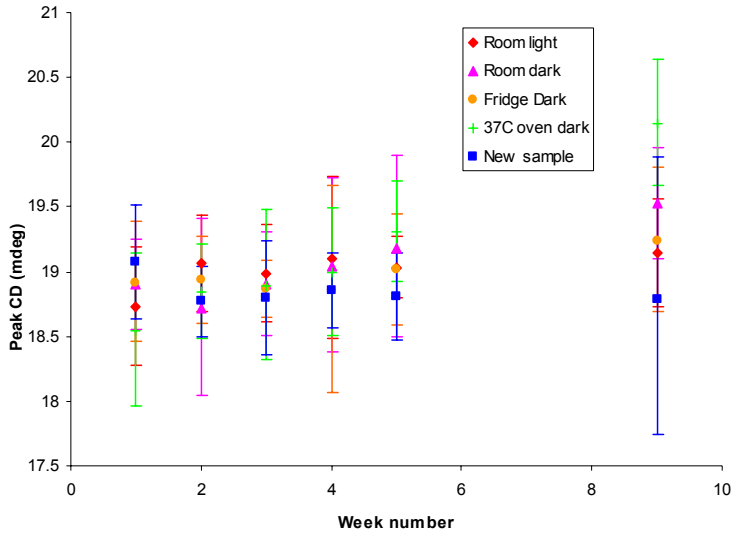


Figure 3.15 Changes in peak CD of samples in 1 mm cuvettes.
Error bars indicate 95 % confidence limits of Gaussian fits to spectra from 285 to 295 nm.

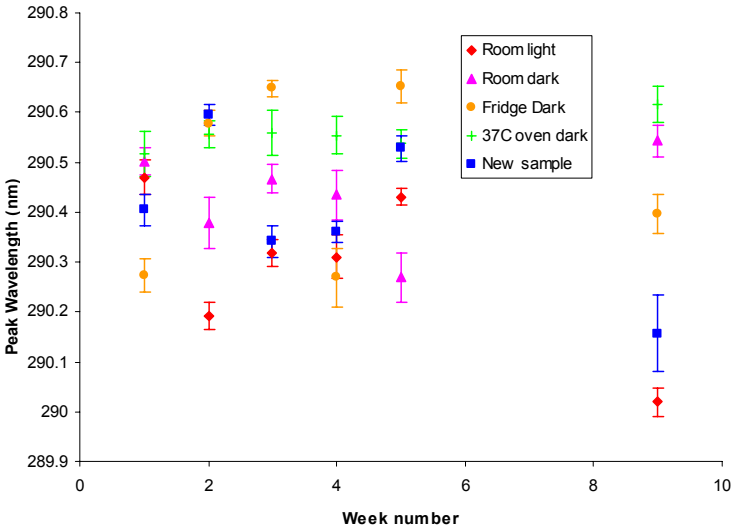


Figure 3.16 Changes in peak wavelength of samples in 1 mm cuvettes.
Error bars indicate 95 % confidence limits of Gaussian fits to spectra from 285 to 295 nm.

4. Ruthenium Complex: an alternative CD calibrant

The suitability of the ruthenium complex mentioned above as a CD calibration standard was studied using D- and L- enantiomers of this complex, provided by Dr. Alison Rodger (University of Warwick). A stability study was undertaken to look for changes in spectral features such as peak CD signals.

4.1. Methods and Materials

Solutions of each of the two enantiomers of the complex, D-[$Ru(phen)_3(ClO_4)_3$] and L-[$Ru(phen)_3(ClO_4)_3$], were prepared and diluted to give a suitable signal; the tubes containing these were covered with aluminium foil and left at room temperature. CD spectra of these solutions were measured with a Jasco J-810 CD instrument, over a period of 4 weeks. Each solution was at ~0.005 mg/ml (the concentrations differed slightly between the two solutions). Each spectrum shown here is the average of two separate fills of the cuvette with the ruthenium complex at each time interval, and has been baseline subtracted. The instrument parameters shown in Table 7 were used for each spectrum.

Instrument parameter	*Value*
Start wavelength (nm)	400
End wavelength (nm)	180
Data pitch (nm)	0.1
Scanning speed (nm/min)	50
Response (s)	1
Bandwidth (nm)	1
Accumulations	1

Table 7 CD instrument scan parameters for ruthenium-complex stability studies.

4.2. Stability study of ruthenium complex solutions

The CD spectra for the D- and L- forms of $Ru(phen)_3(ClO_4)_3$ over the four-week period of the study can be seen in Figure 4.1 and Figure 4.2 respectively; a plot of the peak CD intensities of the positive and negative peaks, for each of the solutions, is shown in Figure 4.3, and a similar plot in Figure 4.4 shows the wavelengths of the peaks. It is clear that the complex is stable over the course of the experiment. Another attraction of these compounds as potential standards is that they show CD signals from the visible down to 180 nm (see absorbance spectrum in Figure 4.5) and with careful choice of concentration of the solution a single sample can be used over the full wavelength range. Since both enantiomers can be produced, the instrument performance, particularly below 200 nm, can be investigated by determining whether the two enantiomers have equal and opposite CD signals.

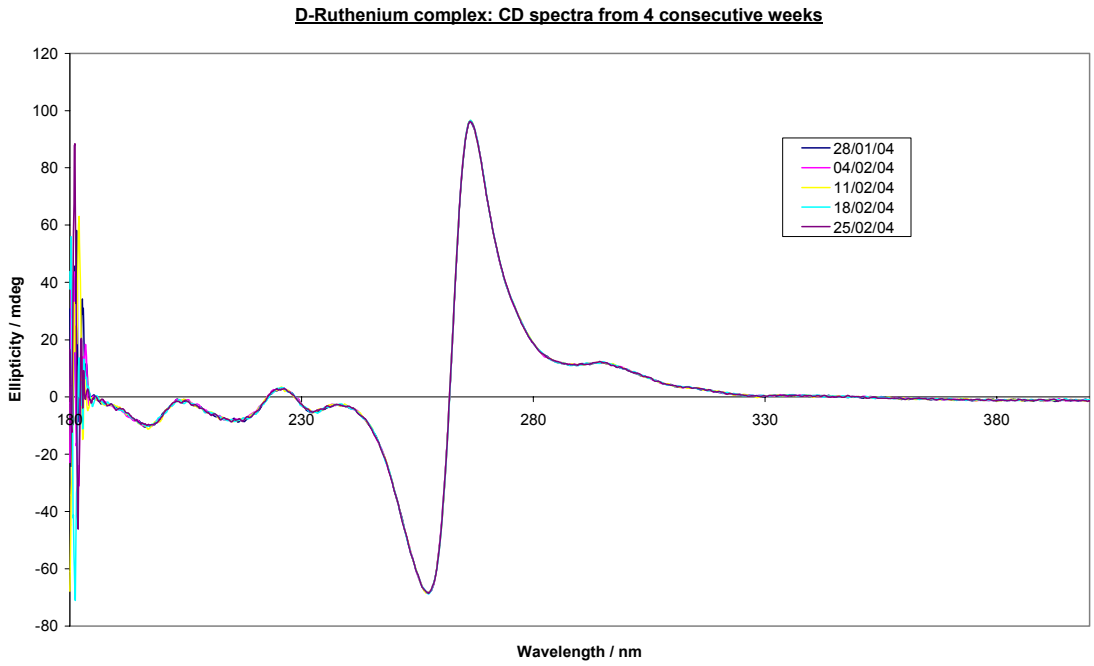


Figure 4.1 CD spectra of D-[Ru(phen)$_3$(ClO$_4$)$_3$] from stability study.

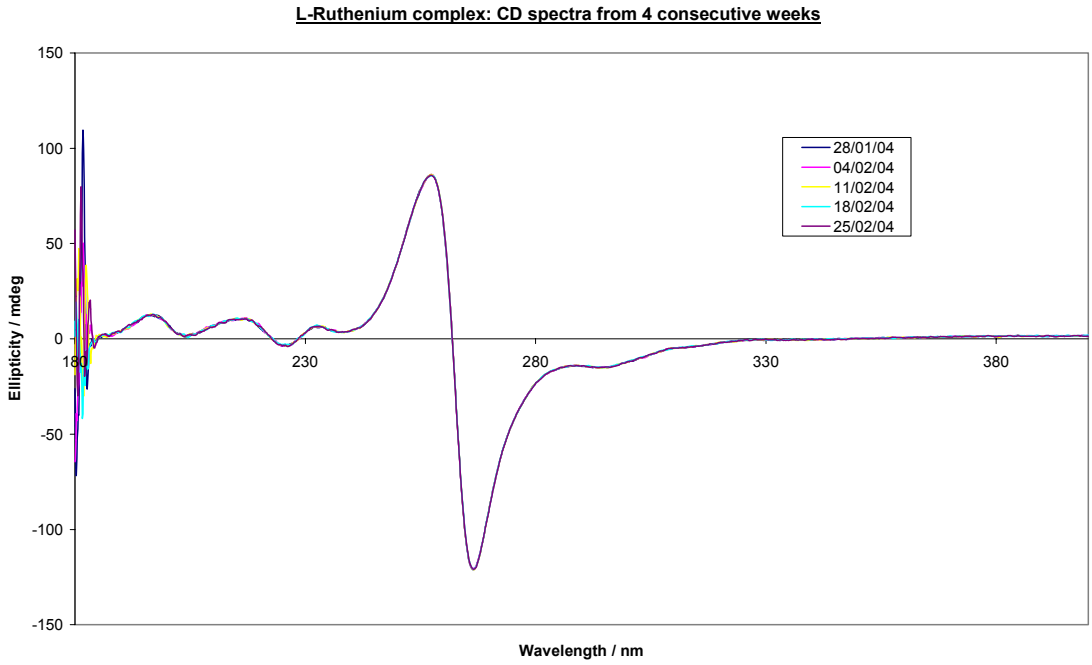


Figure 4.2 CD spectra of L-[Ru(phen)$_3$(ClO$_4$)$_3$] from stability study.

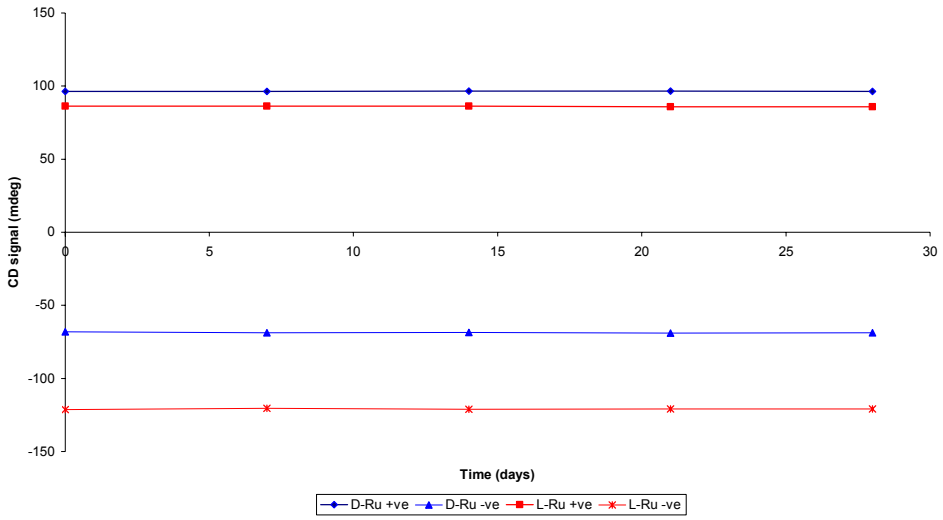


Figure 4.3 Stability of the peak intensities of each of the two calibration solutions.

The peak intensities were determined for the positive (+ve) and negative (-ve) peaks of each solution.

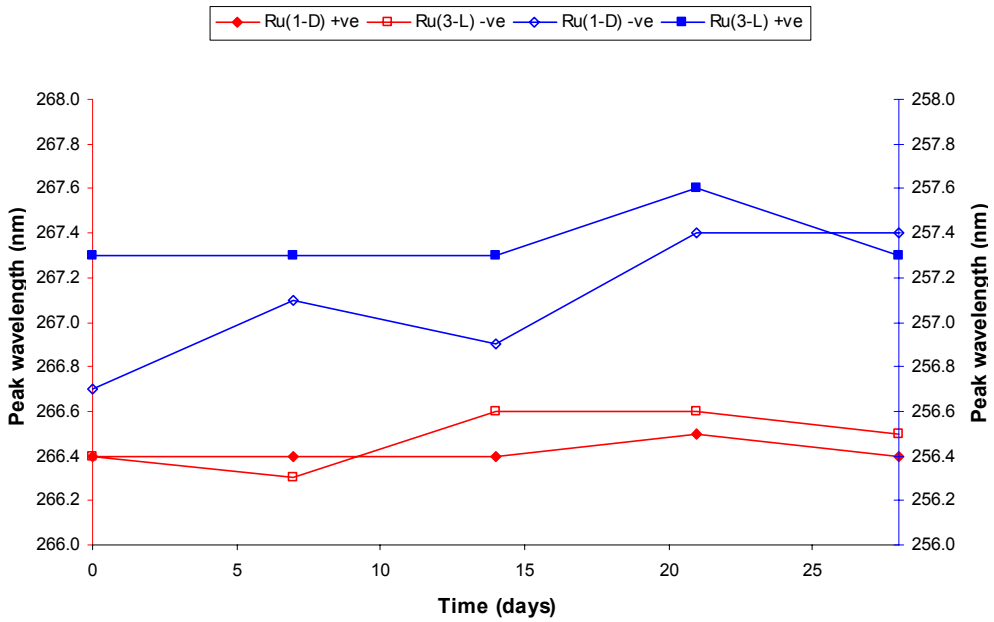


Figure 4.4 Stability of the peak wavelength of each of the two calibration solutions.

The peak wavelengths were determined for the positive (+ve) and negative (-ve) peaks of each solution. For this plot, the peaks at approximately 266 nm are plotted in red and shown on the left-hand axis; the peaks shown in blue are those at approximately 257 nm.

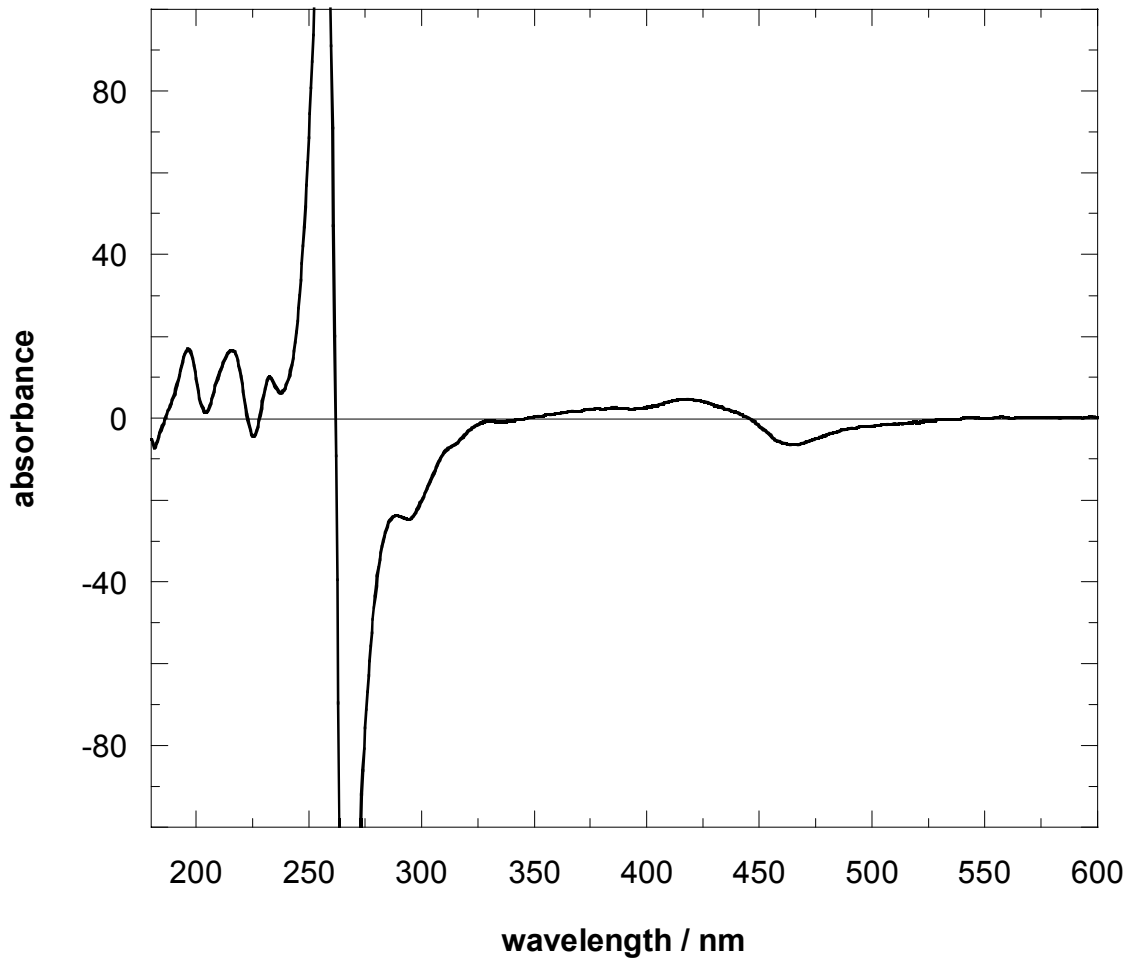


Figure 4.5 Absorbance spectrum of the ruthenium complex.

4.3. Conclusions

Together, these results show that solutions of these ruthenium complexes are stable under the conditions of this study: there was a very good level of reproducibility from week to week (given typical instrument errors) and no indication of sample degradation. This is consistent with earlier work with these samples (Alison Rodger, University of Warwick), which also showed that they are stable at 60 °C for at least 2 weeks.

As mentioned in section 3.1, aside from stability, another characteristic of a good CD calibrant is that it should provide a sufficiently strong CD signal without excessive absorption; these ruthenium complexes meet this requirement.

The ruthenium complex presented here may offer a number of advantages as a CD calibrant:

- Two main calibration peaks
  - at useful wavelengths (often used in protein studies)
  - positive and negative sign
  - similar amplitudes
- Additional spectral features in the far UV
- Single solution for visible, near and far UV regions

- Available in both enantiomers (especially useful for checking performance below 200 nm)
- Appears to be very stable
- Narrow peak shape useful for wavelength calibration

Although these complexes appear to meet the requirements for use as CD calibrants, it is first necessary that they be available commercially in a chemically and enantiomerically pure form, which is currently not the case. Longer term stability studies would also be required.

5. Conclusions

ACS has long been used as a calibrant for CD instruments; this study has highlighted some of the important parameters that need to be considered when using this chemical as a calibrant. Many of these factors are independent of the particular chemical used. These include the importance of regular baseline checks, consistent positioning of cells, ensuring that concentrations used are such that the calibrant operates within the Beer-Lambert Law, taking into account the temperature at which the calibrant is measured, and sample storage.

A stability study of an alternative ruthenium-based calibrant has also been presented, which shows the potential use of this chemical as a CD calibrant, providing it can be made available commercially in chemically and enantiomerically pure forms. The present study on ACS can serve as a model for validation studies of this and other chemicals that may be considered for use as CD calibrants.

6. References

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