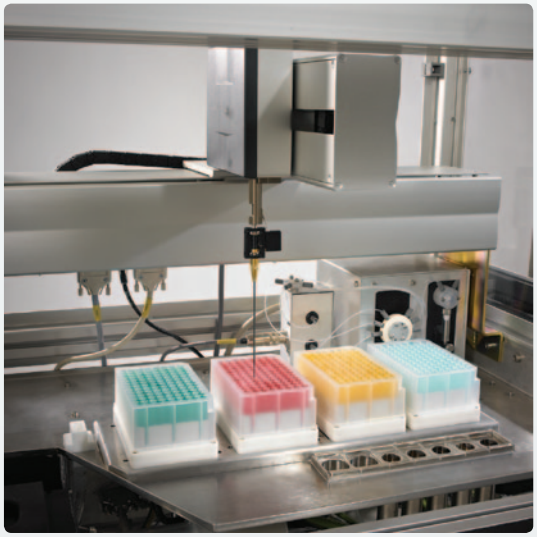
AppliedPhotophysics
Ultrasensitive Spectroscopy for the Life Sciences



CHIRASCAN SERIES APPLICATION NOTE

Strategies to compare far-UV Circular Dichroism spectra of similar proteins using Chirascan™-plus ACD

Abstract *This paper discusses how Chirascan™-plus ACD presents easy-to-obtain, highly reproducible results while achieving high throughput, previously not available for Circular Dichroism. Using advanced robotics combined with the 96 or 384-well plates, Chirascan™-plus ACD can rapidly prepare many replicates and perform multiple measurements for clear determination of standard deviations.*

INTRODUCTION

Circular Dichroism (CD) spectroscopy is an extremely sensitive technique to analyse the conformation of proteins in solution and can be used as a fingerprint of a particular protein structure [Greenfield, 2006]. Due to the very low sample volumes required and the simplicity of the measurement, it is a powerful comparative tool to detect differences in structure between proteins.

Traditionally with manually operated instruments there have been a number of problems in using CD spectroscopy to determine statistically significant results. These include the number of samples needed to be prepared and analysed reproducibly to derive statistically significant results. Also direct comparison of CD spectra usually requires careful preparation of protein samples at accurate concentrations, to allow normalisation of the CD spectra.

An often used approach is to quantitate the concentration of protein using the absorbance at 280 nm. This assumes that any differences between the proteins do not affect the 280 nm extinction coefficient; an assumption that can lead to inaccuracies or misinterpretation of data. It also requires a second experiment to determine the absorbance. The problems described above have limited the wide use of this technique.

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KEYWORDS

▸ Chirascan	▸ Biocomparability
▸ Circular Dichroism	▸ Protein
▸ Automated	▸ Mamalian Serum
▸ Albumin	▸ G Factor

Here we explore two ways of analysing CD spectra taken in the far ultraviolet (UV), i.e. over the wavelength range 180 to 260 nm, that do not require quantitation of the protein concentration. Eight mammalian serum albumins, which are all structurally very similar were used. Additionally, ovalbumin was included as an obvious outlier.

The first method is to normalise the area between 0 and the CD curve so that all spectra can be compared, and then look at statistically significant differences in the normalised spectra.

The second method is to use a property called the G Factor [Baker and Garrell, 2004]. This is the ratio of the CD (as ΔA) to the total absorbance of the sample (A). It retains the conformational information of the CD spectrum, but as a ratio it is dimensionless, with no dependence on the sample concentration or cell pathlength. With earlier generations of CD spectrometers, two experiments were required to determine the G Factor, since the CD spectrum had to be measured separately from the absorbance spectrum.

The ultrasensitive Chirascan™-plus spectrometer from Applied Photophysics provides high performance far-UV CD data and simultaneously collects highly accurate absorbance spectra. This removes the need for running two experiments, dramatically improving time of analysis and providing more robust data.

The other key aspect to this study is to provide measures of statistical significance. The new Chirascan™-plus Automated Circular Dichorism (ACD) autosampler system guarantees easy to obtain highly reproducible results. The advanced robotics allows many replicates to be prepared. Using 96 deep well plates many samples can be prepared and analysed allowing high throughput to be easily achievable.

MATERIALS AND METHODS

All samples of serum albumins were purchased from Sigma-Aldrich Company Ltd., Dorset, U.K. As they were all provided as crystallised or lyophilised powders, the proteins were weighed and dissolved in a pH 7.4, 20 mM sodium phosphate 150 mM sodium fluoride buffer, to a concentration of approximately 1 mg/mL.

A microplate was filled with 100 μL of each sample. A buffer sample was loaded into every fourth well, beginning with well 1, to allow a buffer baseline spectrum to be subtracted from the sample CD and absorbance spectra. Each sample was loaded into 3 separate wells, to allow replicate samplings for the determination of standard deviations.

The samples were sequentially sampled from the microplate and loaded into a 0.1 mm pathlength automated flow-cell by the ACD system attached to the Chirascan™-plus. Spectra were acquired over the wavelength range 180 to 260 nm, with a 1 nm stepsize, at 0.5 seconds per data point. 4 spectra were acquired for each sampling, giving a total of 12 spectra per sample. The whole dataset took less than 7 hours of unattended operation to acquire.

After acquisition the data were processed by automated averaging of the four CD and absorbance spectra for each sampling, followed by the subtraction of the buffer baseline for each averaged spectrum. G Factor spectra were then automatically calculated by division of the baseline subtracted CD value (after conversion from millidegrees to ΔA) by the baseline subtracted absorbance value, for each wavelength.

The CD and G Factor spectra were then averaged and the standard deviation calculated on the 3 repeat samplings, to provide a single mean and standard deviation for each wavelength, which were propagated through all subsequent calculations.

As a positive control, a separate experiment was performed with the same sample of human serum albumin (HSA) under the same conditions as previously, except that the 0.1 mm pathlength cell was replaced by a 0.2 mm pathlength cell, to provide a significant difference in the CD amplitudes.

RESULTS

Intrasample reproducibility was very good. As an example, Figure 1 left shows the four repeat spectra of the first sampling of HSA. It is difficult to observe any significant difference in the spectra. Figure 1 (right) shows the averages of the 4 repeat spectra for each of the 3 samplings of HSA, and differences in the spectra are again difficult to observe.

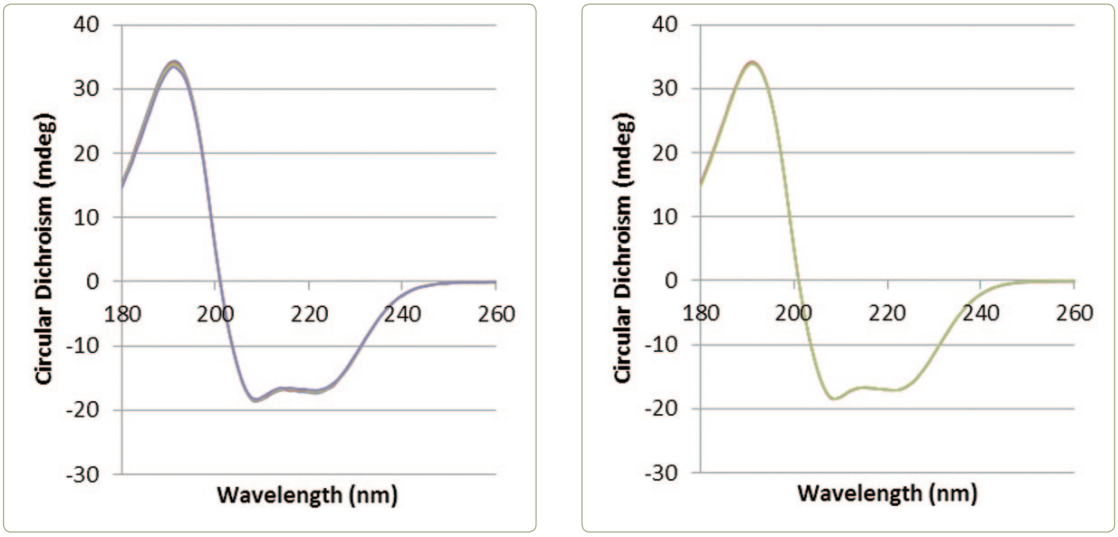

Figure 1. HSA sample repeats. Left are the 4 repeat spectra of the first sampling. Right are the averages of the 4 repeat spectra for each of the 3 samplings.

A plot of the averaged baseline subtracted CD spectrum of each sample (Figure 2), shows a large scatter in CD amplitudes due to differences in final protein concentration or cell pathlength between the samples. Obviously a simple direct comparison of the spectra is not possible at this point.

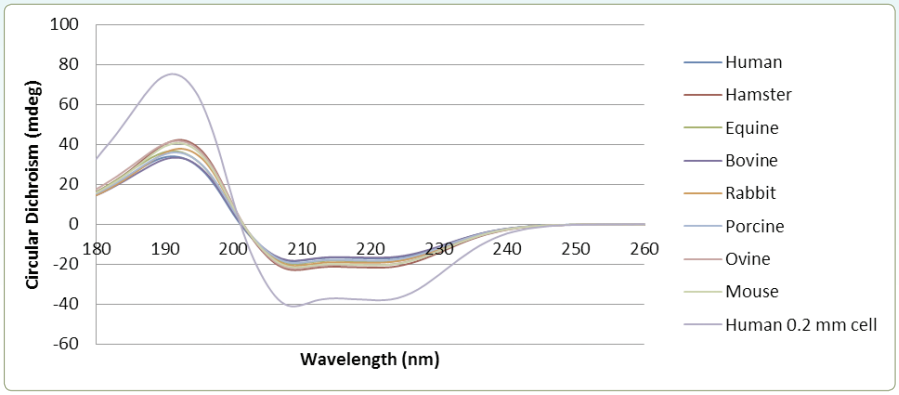

Figure 2. Circular Dichroism spectra of baseline subtracted average of 3 samplings of each serum albumin. Note the large amount of scatter in the amplitudes of the data. The largest spectrum is that of human serum albumin in a 0.2 mm cell.

Without careful normalisation for concentration, it can be quite difficult to obtain useful information from a comparison of the CD spectra. A simple but effective method of normalisation without knowledge of protein concentrations is by the area between 0 and the curve. Figure 3 shows the result of normalisation of the spectra shown in Figure 2 to the area of the 0.1 mm cell HSA spectrum.

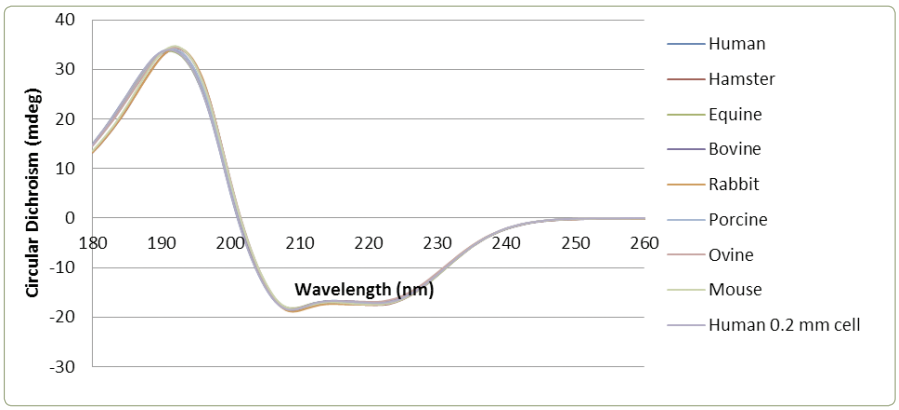

Figure 3. Circular Dichroism after normalising the area between 0 and the curve to the area of HSA in the 0.1 mm cell. Note the small shift in the ~190 nm peak.

Normalisation by area is an effective way of enabling a comparison of the shapes of protein spectra that does not require knowledge of exact or relative protein concentrations. For example in Figure 3 there is evidence of a small red shift at ~ 190 nm in some of the protein samples.

Subtracting one spectrum from another gives us a way of highlighting the spectral differences. In Figure 4, the difference spectra calculated by subtracting the other normalised protein spectra from that of HSA are plotted. The error bars represent 3 x the standard deviation around zero, and provide a way of judging the extent of statistically significant differences between the two protein spectra.

As can be seen, the only spectrum that lies completely within 3 standard deviations of the original HSA spectrum is that of HSA measured in the 0.2 mm pathlength cell. All other spectra show statistically significant differences, to varying extents. What is also interesting is that mouse, rabbit and hamster serum albumin all show very similar difference spectra, which form a distinct group.

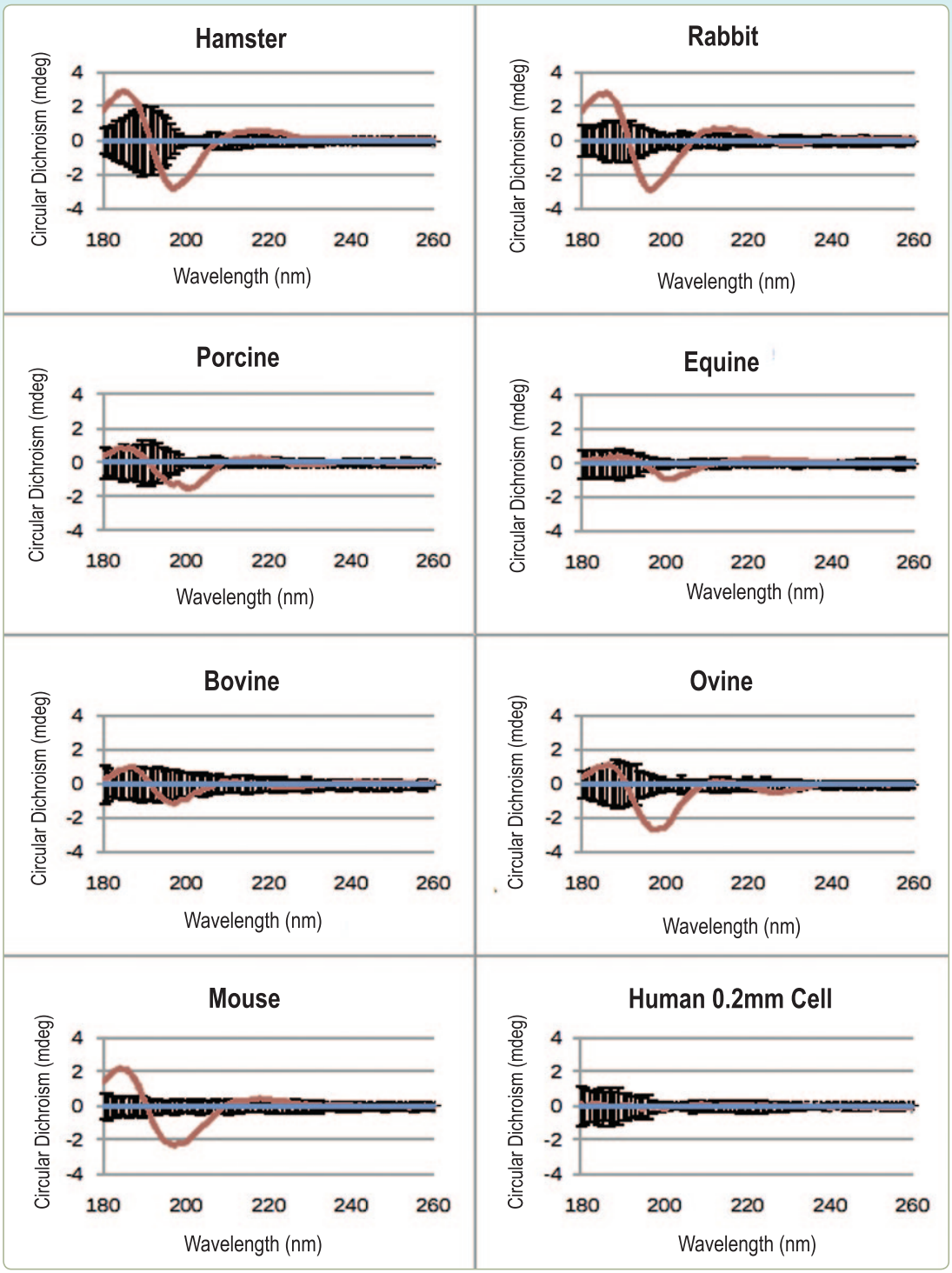

Figure 4. CD difference spectra of the HSA spectrum minus normalised CD spectra from other albumin samples, and a sample of the HSA measured separately in a 0.2 mm cell. The error bars are set at 3 x the standard deviation above and below zero.

Although this is a very useful way of comparing CD spectra, the area normalization method has the potential to artificially decrease the observed spectral changes, because it reveals differences in spectral shapes but not in spectral amplitudes.

This problem with normalisation procedures can be sidestepped completely. The G Factor, i.e. ratio of the CD as ΔA to the absorbance, is very simply calculated by dividing the measured CD signal by the measured absorbance, which on the Chirascan-plus are measured simultaneously. The G Factor is an intrinsic property of a particular chromophore (as the extinction coefficients and quantum yields are for fluorophores). Like the CD, it is sensitive to the conformation of the protein [Baker and Garrell], but it is not dependent on the pathlength of the cell or the concentration of the sample as the CD or absorbance separately are.

Figure 5 shows the overlaid G Factor spectra of all the samples presented in Figure 3. Notice that now between 180 and 200 nm the spectra are similar in amplitude. At longer wavelengths, the CD and absorbance approach zero, and the experimental noise appears to become large. Although this looks disordered, this is not statistically significant.

An experimentally critical aspect is that the buffer and sample have to be carefully matched for concentrations of all absorbing species other than the protein, to provide an accurate baseline absorbance value. A significant problem during manually conducted CD experiments is the introduction of oxygen, which absorbs below 200 nm thereby increasing the variability of the absorbance spectra. The ACD sample chamber is sealed and minimally disturbed during sample loading, which significantly reduces this potential source of experimental error.

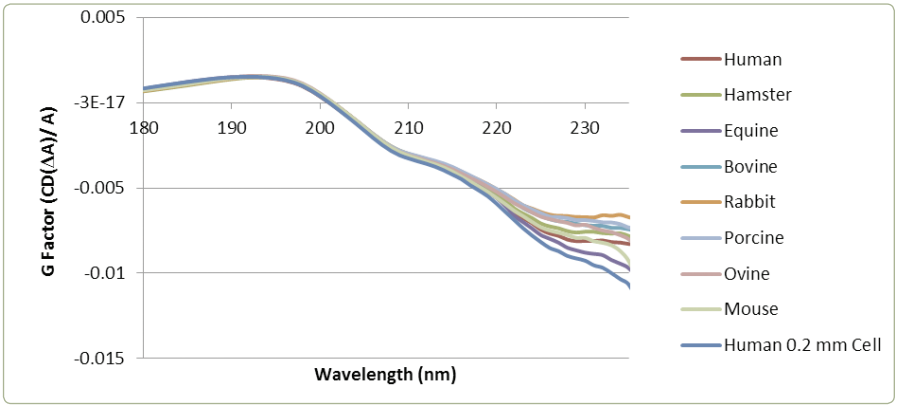

Figure 5. Overlaid G Factor spectra of all serum albumin samples.

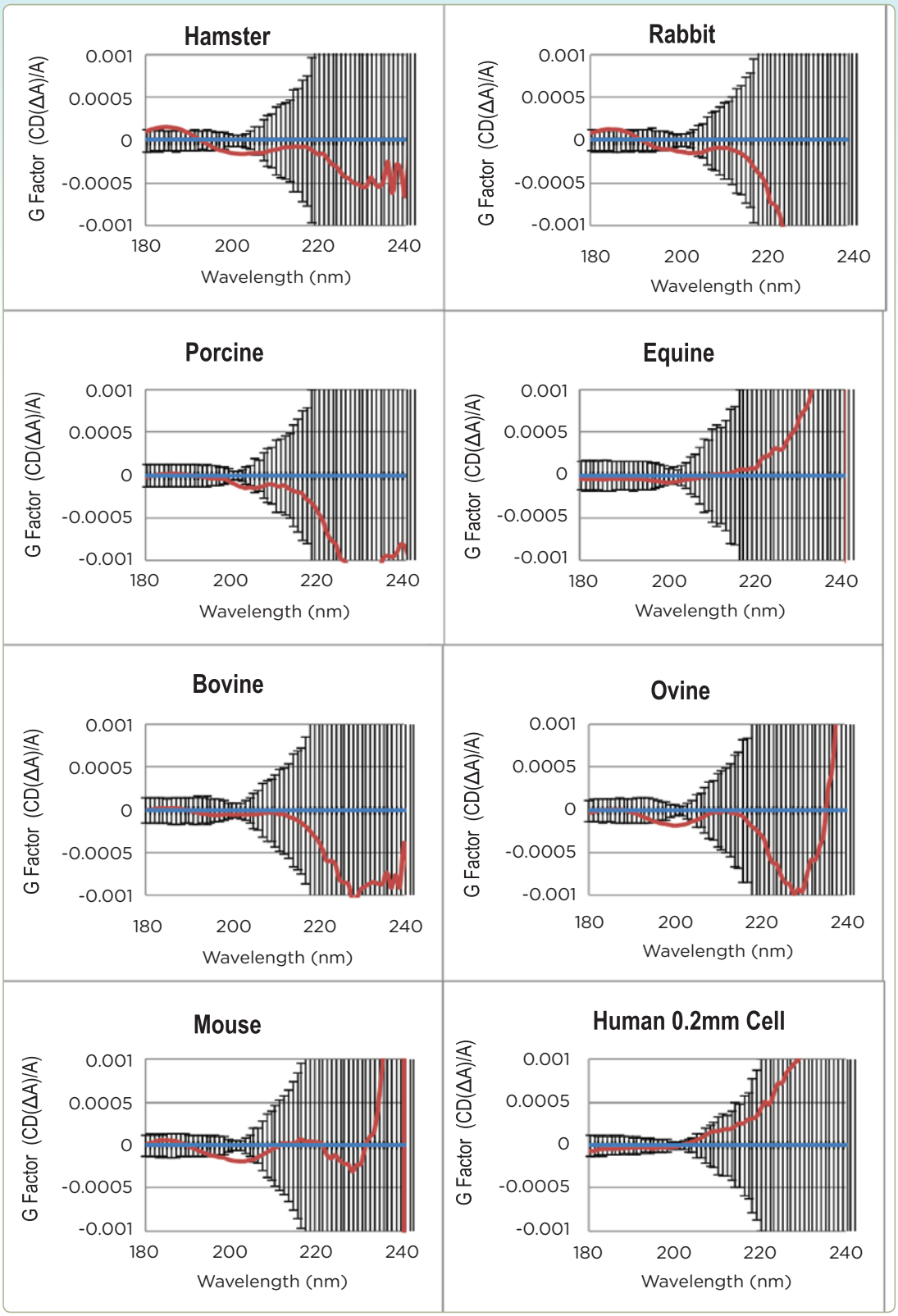

Figure 6. Difference in G Factor spectrum human serum albumin spectrum other serum albumins, or the same HSA in a different pathlength cell. The error bars are set around zero at 3 x the standard deviation.

The spectra can be compared in exactly the same way as the normalised CD spectra shown in Figure 4. In Figure 6 the difference spectra calculated by subtracting the G Factor spectra of the other proteins from that of the HSA are shown. Again there are observable, statistically significant differences in many of the traces, although these are not as distinct as in Figure 5. This is due to the larger error bars arising from the increased error in the absorbance measurements. As before, the mouse, hamster and rabbit serum albumins show the largest differences from HSA, but are very similar to each other. Most importantly, there are no significant differences observed between the HSA spectra from the two different pathlength cells. This demonstrates that similar samples can be successfully identified.

It is interesting that the normalised CD data gave much more significantly different results. This is due to the extra variability in the absorbance measurement adding in extra uncertainty into the G Factor measurement. This also indicates that differences in CD spectra shape is the most significant difference between the spectra of the proteins, not the amplitudes of the CD signal.

COMPARING APPLES AND ORANGES

Because the main differences in the CD spectra of this group of serum albumins are subtle wavelength shifts, the normalised CD spectra produced more statistically significant differences than the G Factor spectra. Are there cases were the G Factor is more sensitive? In Figure 7 we can see the measured and normalised CD spectra of hen egg white ovalbumin compared to those HSA.

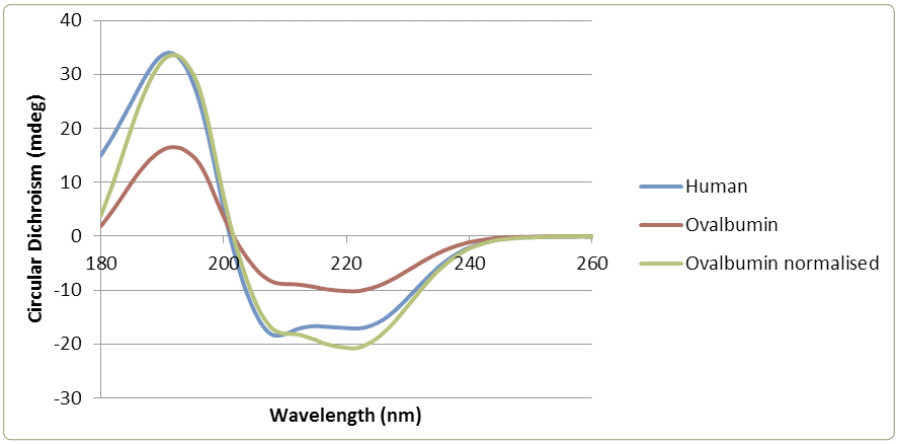

Figure 7. Comparison of directly measured human serum albumin and ovalbumin CD spectra. Also included is ovalbumin normalised to the area of human serum albumin.

When we apply the same techniques as were used to compare the serum albumins, it is the G Factor spectrum that shows the greater significant difference between the spectra over the range 180 and 210 nm, as shown in Figure 8. This is due to the amplitudes of the CD spectra of the ovalbumin and HSA being significantly different, as well as their shapes.

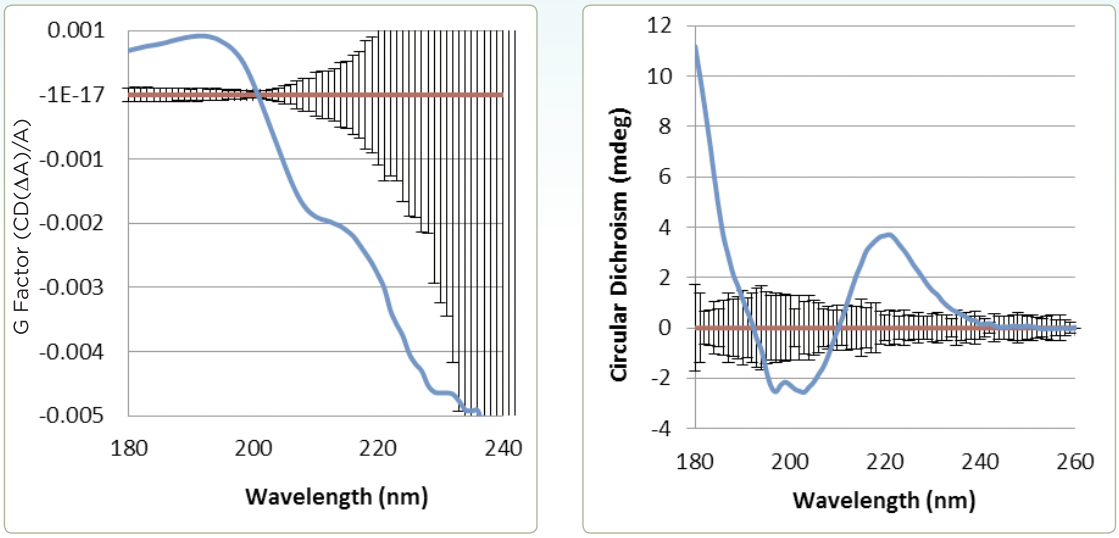

Figure 8. Difference in the spectra of HSA and ovalbumin for the G Factor (left) and CD derived spectra. The error bars are set around zero at 3 x the standard deviation.

Normalisation has the effect of decreasing the apparent differences between the two spectra, which are due to differences in real chiral spectral intensities. In this case the G Factor shows a significantly larger difference in the region from 180 to 210 nm than the normalised CD.

From this it would seem a combined approach may be best, with the normalised CD being very sensitive to differences in spectral shape and peak wavelength shifts, while the G Factor highlights changes in CD spectrum amplitudes that are not due to changes in protein concentration or cell pathlength

CONCLUSIONS

We have examined two approaches of comparing the CD spectra of protein samples without knowledge of the exact protein concentration.

The first method is to normalise all spectra, over the same spectral range, to the same area between 0 and the curve, before carrying out comparisons between them. This provides a very simple and sensitive way of comparing the spectral shapes of CD protein spectra. Also when combined with the high reproducibility and ease of obtaining replicate sample spectra using an Automated Circular Dichroism system, enables statistically significant comparisons to be made.

The second method is to compare the G Factor spectra, which has the advantage of removing dependence on concentration and pathlength, while being sensitive to real amplitude differences between spectra. The key to the technique's successful application is to obtain highly reproducible absorbance spectra across the wavelength range analysed. The unique sample chamber design of the ACD autosampler and the high performance electronics of the Chirascan-plus CD instrument, allow accurate absorbance and CD spectra to be obtained simultaneously.

REFERENCES

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NEW APPLICATIONS FOR CD SPECTROSCOPY

Optimising biotherapeutic formulations

Used as a label free stability-indicating assay, Chirascan™-plus automated circular dichroism (ACD) can identify good formulation candidates earlier for further downstream processing. By culling formulations that are likely to fail early and focusing on those that are more viable for real time and accelerated stability studies, users can make savings in both time and money.

Combining the label-free and information-rich technique of dynamic multimode spectroscopy (DMS) with the productivity of automation gives a whole new approach to establishing conformational stability under different formulation conditions. The conformational integrity of biotherapeutics as a function of more than one stress condition (e.g. Temperature, pH, ionic strength) is readily determined in unattended operation.

Statistical comparison of similar proteins (Biosimilarity)

Research into biosimilar pharmaceutical products has grown exponentially over the last few years turning it into a multi-billion pound business. Structure, biological activity and stability are just a few of the complex studies required and, traditionally, these use multiple techniques which are very time consuming and labour intensive.

Automation lends itself to measuring samples repeatedly and thus to generating statistical comparisons. To answer the question: 'Are these two CD spectra the same?' is no longer a matter of guesswork – a statistical significance can be associated with the measurements and a quantitative judgment about similarity or otherwise can be made.

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